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## Tracking the dynamics of hemp dew retting under controlled environmental conditions

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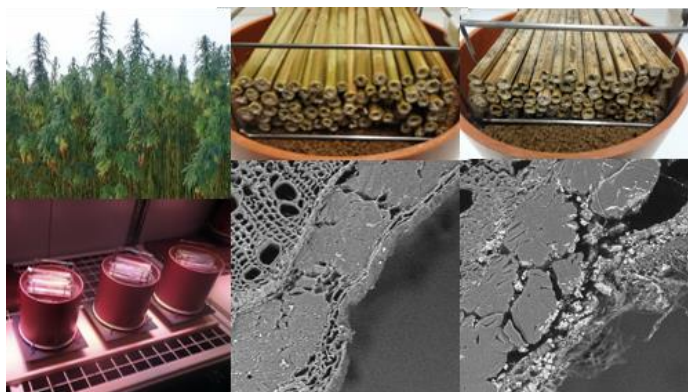
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### Abstract

The use of natural fibers such as hemp (*Cannabis sativa* L.) as substitutes for nonrenewable fibers increases the life cycle performance of composite materials. The management of retting in fields as a natural pretreatment prior to fiber extraction remains challenging due to a lack of knowledge about the relative importance of environmental and biotic factors, which continually interact under field conditions. Here, we studied the dynamics of hemp retting under controlled air temperature and humidity conditions and with simulated rain. We tracked the color and infrared spectral absorbance of the stem surface, the chemical composition and microbial enzyme activities of the bast tissues, and the stem architecture over 42 days at 15 °C. Color changes on the stem surfaces were the first indicators of retting progress, with a significant decrease in the L\*, a\* and b\* values (CIELAB) from day 14 onward. These findings were closely correlated with the surface colonization progress as revealed by scanning electron microscopy, the changes in enzymatic activities and the decohesion of the bast tissues. Additional investigations are needed to study other environmental scenarios to provide an accurate assessment of the retting process over time.

**Keywords.** Colorimetry, Experimental design, Hemp, Infrared, Retting, Scanning electron microscopy

**Abbreviations:** 4-MUB, 4-methylumbelliferone; AHC, ascending hierarchical classification; ANOVA, one-way analysis of variance; ATR-FTIR, attenuated total reflection-Fourier transform infrared; DNS 3, 5-dinitrosalicylic acid; EMC, equilibrium moisture content; PCA, principal component analysis; SEM, scanning electron microscopy.



## 1 Introduction

Improving the life cycle performance of products has become a priority for consumers, and, consequently, for industry (Sauvageon et al., 2017). The use of natural fibers such as hemp (*Cannabis sativa* L.) as a substitute for synthetic or mineral fibers in applications such as composites is part of this trend, and it is broadly under study for industrial applications in Europe. However, natural fibers are still used only in small quantities because, among other reasons, of quality issues related to the heterogeneous properties involved in any biomass production (Fernandez-Tendero et al., 2017; George et al., 2016) (Fernandez-Tendero et al., 2017; George et al., 2016). To facilitate defibrillation and preserve the fiber quality, a pretreatment step known as retting is performed, and it involves the selective degradation of the parenchyma cells surrounding the fiber bundles located in the bast tissues of the stem, leading to their decohesion from the rest of the stem (Müssig, 2010). This interbundle decohesion can continue with intrabundle decohesion involving the selective degradation of the thin primary cell wall and middle lamella around the thick cellulosic secondary cell wall of the cells composing fiber bundles (Liu et al., 2017b). The fiber extraction from the plant is thus easier, making it possible to preserve the fiber integrity and homogenize the fiber properties by reducing the amount of energy needed for the decortication step (Liu et al., 2015a). In the case of hemp, traditional methods of retting, such as dew (or field) retting and water, have been used for centuries and are still the dominant procedures.

Dew retting consists of leaving stems in the field, after the harvest, at the interface between the soil and the atmosphere, facilitating the natural and partial degradation of the bast tissues. Degradation of the pectin-rich middle lamella allows separation of the bast fibres from the stem with partial dissociation of the bundles (Ribeiro et al., 2015). This process occurs under local environmental conditions and is regulated by abiotic and biotic factors such as local weather conditions and soil microbial colonization (Liu et al., 2015a). The process of dew retting is not yet fully characterized and is highly climate-dependent, which makes it difficult to obtain a predefined quality for industrial applications (Bacci et al., 2010; Jankauskienė et al., 2015). Most of the literature published on hemp field retting focuses on comparisons with other retting processes involving only a single field experiment (Jankauskienė et al., 2015; Liu et al., 2017a). With this approach, it is difficult to characterize the factors that drive the retting process and to compare results between experiments. Limited research has been conducted under controlled laboratory conditions, which would allow for the environmental parameters to be set and to be decoupled from one another and thereby avoid confounding effects, e.g., the harvest date, which influences both the hemp composition and the climatic conditions at retting time.

In this study, we developed a new experimental approach performing hemp dew retting under controlled conditions (in relation to the air temperature, relative humidity, simulated rains and lighting cycle). This approach was derived from plant litter decomposition studies (Iqbal et al., 2015; Lee et al., 2014) that were aimed at acquiring a better understanding of the relationships between the properties of plant residue mulches, environmental conditions and decomposition.

The objectives of this work were i) to verify the capacity of the controlled experimental approach to reproduce the hemp dew retting process and ii) to dynamically link the changes in the macroscopic properties of stems with chemical properties and observations of biological processes.

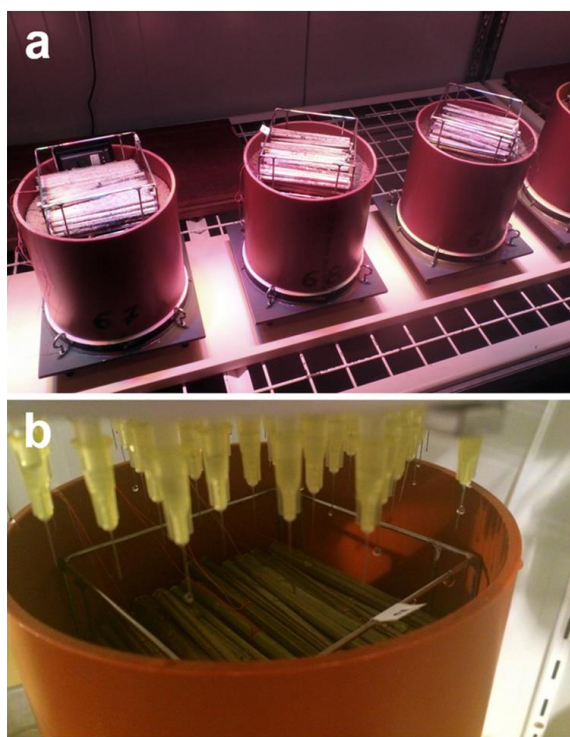
## 2 Material and methods

The experimental approach implies miniaturization and simplifications to ensure homogeneity, standardization and reproducibility for further experiments (i.e., in terms of the soil granulometry, rain, hygrometry, hemp stem segment length, litter layer shape and height).

**2.1 Plant materials and soil sampling.** Hemp (*Cannabis sativa* L. cv. Fedora 17) was grown in the Champagne area (eastern France) and harvested in September 2015 at the physiological stage of seed maturity. The stems were cut a few centimeters above the ground, dried in a forced-air oven at 40 °C for one week and then stored at room temperature. The median parts (between the fourth node from the apex and the third node from the stem base) of the hemp stems were selected to reduce the heterogeneity of the initial chemical composition of the stems, which differs along the stems, as revealed by a previous study (Liu et al., 2015b), and they were hand-cut into 10 cm-long sections. The soil was collected from the 0–10 cm soil layer from the same plot as the plant material and stored at 4 °C. The soil is a silty clay loam with 38.8% clay, 59.2% silt and 2% sand. The initial total carbon and nitrogen concentrations were 15.8 and 1.8 g kg<sup>-1</sup> soil, respectively, and the pH<sub>H2O</sub> was 6.97. Prior to the experiment, the moist sample soil were sieved (< 4 mm), all the plant residues were removed manually, and the moist soil was kept at 4 °C to prevent microbial activities before sample use.

**2.2 Experimental system and retting protocol.** The experimental system consisted of twelve 3-L PVC columns (5 destructive sampling dates x 3 replicates per date) containing a 10-cm layer of soil onto which hemp stems were dropped and lined up to form a 3-cm height pile (**Fig. 1a**). The pile of hemp stems was held in place by a metal frame to maintain its structure and form a hemp litter layer, and in the rest of this article, it will be known as the “litter layer” to distinguish it from the stem segments that are considered individually (**Fig. 1a**). The

columns were set up in a climatic chamber in which the environmental parameters were regulated. The air temperature (15 °C) and relative humidity (60% moisture content) were recorded every 30 min using sensors (174 H, TESTO, Germany) and analyzed with software (Comfort Basic 5.0, TESTO, Germany). A 12-hour daily lighting cycle was provided with a horticultural LED light rail (Rail 36 W, Gemma LED, Sweden) to reproduce the visible spectrum of natural lighting, and regulated with a time switch. Deionized water was applied with rain simulators (Iqbal et al., 2015), allowing for the control of the amount, intensity, and composition of applied water (Fig. 1b). A 6-mm equivalent rain was applied to the columns once per week. These climatic parameters correspond to the mean climatic conditions for hemp retting as performed after harvesting at the seed maturity stage in the area where the hemp and soil were sampled. According to the temperature response function (Brisson et al., 2009), a 42-day duration of retting at 15 °C under our experimental conditions represented (approximately) in the field, for a dew retting duration of 59 days with a mean temperature of 12 °C and 33 days with a mean temperature of 17 °C.

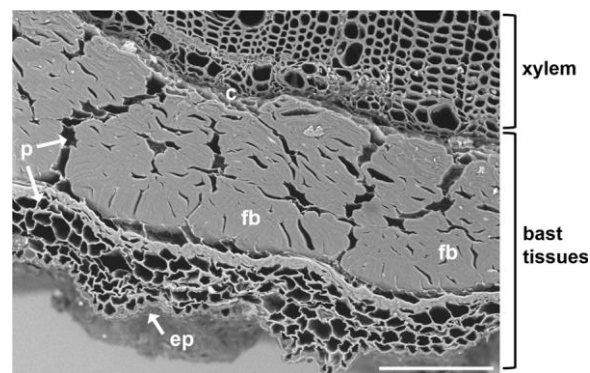


**Fig. 1.** Retting under controlled conditions, experimental system description: (a) experimental columns inside the climatic chamber under the LED light rail (b) rain simulator: © Bleuze/Inra.

The soil water content was adjusted to 20 g of H<sub>2</sub>O 100 g<sup>-1</sup> dry soil with deionized water to ensure that the retting process would not be limited by the soil moisture. The soil was placed in the columns to form a 10-cm-high soil layer at a bulk density of 1.3 g cm<sup>-3</sup> by adding the

corresponding mass (2426 g of moist soil), and the height was adjusted by applying pressure with a manual press (Sauvadet et al., 2016). Before the start of the experiment, the columns containing soil were maintained for 24 h in the climatic chamber at 15 °C. The hemp stems were premoistened between two cotton pieces humidified with deionized water at 4 °C for 24 h to reach an average moisture content of 78%. The excess water (the same free water as that contained in the pith hole) was removed before retting. The litter layer consisted of 50 g of equivalent dry matter in stems per soil column for an average of 70 stem sections and corresponded to approximately 26 t DM ha<sup>-1</sup>. The experiment was run for 42 days. The litter layer was turned over on days 7 and 28 to homogenize the retting process vertically.

**2.3 Stem sampling.** Three randomly selected columns were destructively sampled after 7, 14, 28 and 42 days. This dynamic was selected to monitor the key retting periods in accordance with preliminary experiments that are not reported here. The sampling strategy included three litter layers that were sampled at the start of incubation (day 0). For each sampled litter layer, the analyzed stem samples were taken from the center of the litter layer after removing the stems that were in contact with the soil and those in contact with the atmosphere to minimize the edge effects. The samples were then stored at -20 °C except for 3 stems, which were dried in a forced-air oven at 40 °C for 72 h for dry matter content determination and stem surface analysis. The bast tissues (corresponding to the outer tissues surrounding the xylem core of the stem) (Fig. 2).



**Fig. 2.** SEM micrograph of the outer region of a hemp stem; (c) cambium, (ep) epidermis, (fb) fiber bundle, and (p) parenchyma. Scale bar=100 µm.

**2.4 Stem surface analysis.** The color of the stem surface was measured on a set of 3 stems per sampled layer using a colorimeter (CR-400, Konica Minolta, Japan), with 10 color measurements for each stem (Fig. S1). The results were expressed in the CIE L\*a\*b\* color space system (Fig. S2) for the D65 illuminant with an observation angle of 10° and a 50-mm<sup>2</sup> measuring area. The CIELAB

color space decomposes the color into co-ordinates within a three-dimensional color space ( $L^*$ ,  $a^*$ , and  $b^*$ ) as follows:  $L^*$  is the lightness (black = 0 to white = 100),  $a^*$  indicates redness/greenness, and  $b^*$  indicates yellowness/blueness (McGuire, 1992).

The chemical changes on the stem surface were characterized by infrared spectroscopy. The spectra were acquired by attenuated total reflectance (ATR) using an FTIR spectrometer (CARY 630, Agilent, USA), and measurements were performed in six replicates on 3 stem sections per column (Fig. S1). The spectra were obtained over a spectral range of 4000-650  $\text{cm}^{-1}$  through 64 scans of the sample at a resolution of 8  $\text{cm}^{-1}$  and corrected by baseline subtraction and unit vector normalization to a range of 4000-650  $\text{cm}^{-1}$ .

**2.5 Microscopic imaging of hemp tissues.** Thick cross sections (5 mm) were obtained from the median part of 3 frozen stems per column using a razor blade (Fig. S1). The samples were dried for 2 h at 40 °C prior to scanning electron microscopy (SEM) observation with a tabletop scanning electron microscope (TM-1000, Hitachi, Japan) at an accelerating voltage of 15 kV.

**2.6 Chemical analysis.** Part of the bast tissues was freeze-dried and then hand-cut with scissors before being ground with a ball-crusher (MM 2000, Retsch, Germany) for three min (Fig. S1). The total C and N contents were measured in four replicates using an elemental analyzer (Euro EA, EUROVECTOR, Italy). The cell wall contents of the bast tissues were determined after successive extractions of the soluble fractions using phosphate buffer followed by 95% ethanol. Frozen bast tissues (with the equivalent of a 400 mg dry mass basis) were blended in 100 mL of 50-mM phosphate buffer (pH 7) for 1 min prior to filtration through a GF/A filter. The cell wall residue was obtained after ethanol washing and forced-air oven drying. The cell wall polysaccharides were hydrolyzed into sugar monomers (glucose, mannose, xylose, galactose, rhamnose, arabinose and galacturonic acid) using two-step sulfuric acid hydrolysis. The sugar monomers were separated by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) on a DIONEX CarboPac™ PA1 (4 × 250 mm) analytical column (Thermo Scientific DIONEX, USA). The concentrations of the monosaccharides were quantified in triplicate using 2-deoxy-D-ribose as the internal standard (Lashermes et al., 2016). The lignin contents of the cell wall residues were determined with a spectrophotometric procedure using acetyl bromide and 10 mg of sample as previously described (Iiyama and Wallis, 1990).

**2.7 Enzymatic activities.** All the enzyme assays were performed on a microplate at 25 °C and a pH of 7 with 700  $\mu\text{L}$  of the phosphate buffer-soluble fraction extracted from the bast fibers (Fig. S1). All the enzyme activities were expressed as  $\text{nmol g}^{-1}$  dry bast tissue  $\text{h}^{-1}$ . The

cellobiohydrolase,  $\beta$ -D-glucosidase,  $\beta$ -D-xylosidase,  $\beta$ -D-galactosidase and  $\alpha$ -L-arabinosidase activities were assayed in three replicates with the release of the fluorogenic product 4-methylumbelliferone (4-MUB, Sigma-Aldrich, Germany) according to a method adapted from Bell et al. (2013) as previously described by Sauvadet et al. (2016). The polygalacturonase activity was quantified in duplicate by measuring the reducing end groups formed during the enzymatic degradation of polygalacturonic acid (0.5  $\text{g L}^{-1}$ ) using 3,5-dinitrosalicylic acid (DNS) reagent (Miller, 1959) and a galacturonic acid calibration curve. After 1 h of incubation, the reaction was stopped by adding 500  $\mu\text{L}$  of dinitrosalicylic acid. The solutions were then boiled in water for 15 min. The absorbance of the solution was measured at 510 nm using a microplate spectrophotometer (Versamax, Molecular Devices, USA).

**2.8 Statistical analysis.** A one-way analysis of variance (ANOVA) was performed to assess the effects of the retting duration on the stem surface color, the chemical composition of the bast tissues, and the enzyme activities. ANOVAs were performed using SigmaPlot® software (version 12.0, Systat, USA). The Holm-Sidak method was used to perform pairwise multiple comparison procedures. A statistical analysis of the infrared spectra was carried out with a PCA (principal component analysis) using Unscrambler® X software (CAMO, Germany), and the definition of homogenous groups based on the PCA score values was determined according to an ascending hierarchical classification (AHC) using XLSTAT-Ecology software (version 18.02, Addinsoft, France).

### 3 Results and discussion

#### 3.1 Controlled environmental conditions of retting

Monitoring of the environmental conditions showed that the soil surface temperature and the relative humidity at the hemp litter layer-soil layer interface were maintained throughout the experimental period (Fig. S3). After the rain applications, the soil surface temperature tended to decrease slightly ( $-1^\circ\text{C}$ ) and the air relative humidity increased; subsequently, the temperature and air relative humidity returned to their prerain values except for the rain application on day 21, when the trend was reversed. The moisture content of the stems ( $\text{g H}_2\text{O}$  per 100 g of stem DM), which was measured prior to each rain event, gradually decreased from 78% at day 0 to 30% at day 7, then to 18% at day 14, and finally remained stable until day 42, indicating a progressive drying of the stems over time, despite weekly rains. The loss of water from hemp stems can be explained by the flow of water vapor from the plant tissue to the atmosphere to reach the equilibrium moisture content (EMC) (Nilsson et al., 2005). Similar drying during field retting was observed previously by Gusovius et al. (1998), Coppens et al.

(2006), and Iqbal et al. (2015), who also showed that during the drying of plant residue mulches between rainfall events, the speed and intensity of water losses depended on the physical and chemical characteristics of the litter particles in the mulch. This drying suggests that alterations due to water/drought stress in hemp stem properties occurred throughout the retting experiment, as suggested by Meijer et al. (1995) for flax dew retting.

### 3.2 Changes in the stem surface during the retting process

#### 3.2.1 Colorimetry

A visual examination of the stems indicated that marked changes took place during retting (Fig. 3). The stem surface color, which was bright green before retting (Fig. 3a), became bright yellow after one week (Fig. 3b). Black spots appeared after two weeks (Fig. 3c); the stems then turned pale gray with increased covering in black spots after four weeks (Fig. 3d).



**Fig. 3.** Photographs of the hemp litter layers before and during the retting period: (a) before retting, after (b) 7, (c) 14, (d) 28 and (e) 42 days of retting.

Eventually, after six weeks, the stems became darker gray with widespread black spots (Fig. 3e). The values of the three CIELAB components showed significant variations over the retting period (Table 1). The L\* value decreased gradually by 18.5% and explained the observed evolution of the stem color from bright to pale and eventually dark colors. The a\* and b\* component values declined below the starting values by 32.2 % and 32.4%, respectively, and they tended towards the origin (a\* and b\* values equal to zero), corresponding to the observed transition of the stem color from green to gray (achromatic) (McGuire, 1992). These changes can be explained by the degradation of plant pigments due to abiotic and biotic mechanisms such as photodegradation, biodegradation, and microbial colonization. The appearance of black spots can be attributed to microbial colonization as observed in the hemp dew retting experiment (Akin et al., 2000; Jankauskiene and Gruzdeviene, 2013). The overall color measurements differentiated among three stages, namely, the initial (before retting), intermediate (days 7 to 28) and final stages (day 42), indicating that there were dynamic changes in the stem surface. Although significant change in the stem appearance have been described for hemp dew retting (Akin et al., 2000; Jankauskiene and Gruzdeviene, 2013), to the best of our knowledge, the present study is the first to report quantitative changes in

the stem surface color during the retting period using colorimetry. This rapid and nondestructive method has already been used on fibers (Akin et al., 2000; Martin et al., 2013) and could be applied to monitor the hemp retting process and classify stems according to their retting degree advancement, as described for flax fibers (Pallesen, 1996).

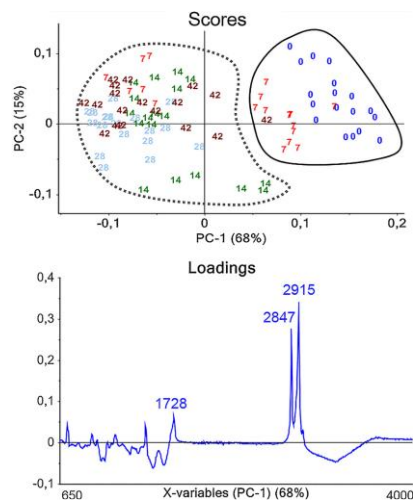
**Table 1.** Changes in the hemp stem surface CIELAB color values according to the L\* (lightness), a\* (redness/greenness) and b\* (yellowness/blueness) during hemp retting under controlled conditions.

Time (days)	CIELAB color values		
	L*	a*	b*
0	53.9 ± 2.1 <sup>a</sup>	3.0 ± 1.0 <sup>b</sup>	23.2 ± 4.8 <sup>a</sup>
7	46.9 ± 2.4 <sup>b</sup>	2.7 ± 1.0 <sup>b</sup>	20.5 ± 2.0 <sup>b</sup>
14	46.2 ± 2.0 <sup>b</sup>	2.5 ± 0.9 <sup>b</sup>	19.0 ± 2.0 <sup>c</sup>
28	46.7 ± 2.5 <sup>b</sup>	3.7 ± 1.0 <sup>a</sup>	20.9 ± 1.7 <sup>b</sup>
42	44.0 ± 3.5 <sup>c</sup>	2.0 ± 1.0 <sup>c</sup>	15.7 ± 2.1 <sup>d</sup>

Values with different letters are significantly different at a level of 5%

#### 3.2.2. Attenuated total reflection-Fourier transform infrared

The colorimetric aspect is greatly influenced by intrinsic factors such as the surface chemical composition. The ATR infrared analysis of the stem surfaces was thus used as a rapid and nondestructive method for evaluating the chemical changes occurring in the stem surface during retting under controlled conditions. The PCA of the spectra indicated that the first two components explained 83% of the variability in the spectra (Fig. 4a). The first component (PC-1) accounted for 68% of the statistical variation between stems, discriminating nonretted stems (day 0) and stems from 2 replicates of litter layers sampled at day 7 from those from the other litter layer samples, i.e., the third 7-day litter layer replicate and stems from litter layers sampled from days 14 to 42.

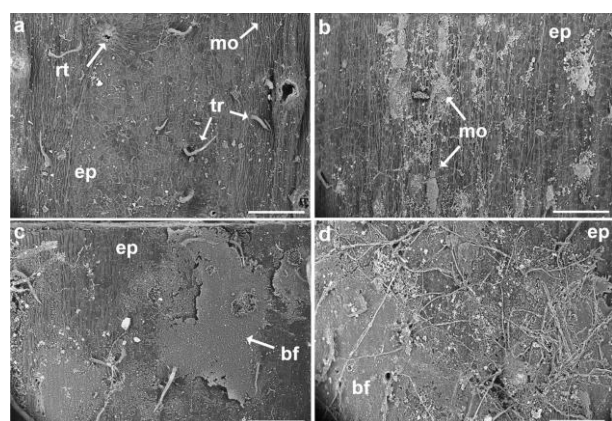


**Fig. 4.** Principal component analysis of attenuated total reflectance infrared spectra for the hemp stem surfaces obtained during retting under the controlled conditions period; (a) the numbers correspond to the sampling date and the circles correspond to the homogenous groups defined by an ascending hierarchical classification; and (b) score loading corresponding to the first component (PC-1).

This result was confirmed by a definition of homogenous groups based on principal component score values from 1 to 4 using an AHC, and it indicated that there were variations in the retting progress depending on the litter layers for the first two weeks. The absorption bands that positively correlated with the first component (PC-1) loading were primarily related to nonretted and 7-day-retted litter layer properties (Fig. 4b). The two primary bands at 2847 and 2915  $\text{cm}^{-1}$  corresponding to ( $-\text{CH}_2$ ) stretching vibrations and a minor band at 1728  $\text{cm}^{-1}$  corresponding to a ( $\text{C}=\text{O}$ ) stretching ester vibration discriminated the samples. These bands can be assigned to the fatty compounds such as the cutin and waxes of the outermost cuticle layer of the stem (Heredia-Guerrero et al., 2016), and they showed decreasing intensity in the retted samples, suggesting that these compounds are degraded during the retting process.

### 3.2.3. Scanning electron microscopy

The stem surface SEM observations taken prior to retting indicated the presence of trichomes, some of which had collapsed (Fig. 5a) thus causing epidermal damage before retting (Fig. S4a). There were very few microorganisms on the surface of the stem before the experiment (Fig. 5a) and even after 7 days (data not shown). After 14 days of retting, significant microbial colonization was observed (Fig. 5b), with fungal hyphae and bacteria on the epidermal surface (Fig. S4b), as previously reported (Jankauskienė et al., 2015). These observations revealed that the trichome remnants constitute the primary spots of colonization by microorganisms, which are then expanded to the rest of the surface (Fig. 5b) and could underlie the bast tissues (Fig. S4b). The microorganism colonization became more pronounced from day 28 with microbial biomass forming a biofilm (Fig. 5c), and the epidermis was almost completely covered on day 42 (Figs. 5d & S4c).



**Fig. 5.** SEM micrographs of the hemp stem surface before and during the retting period; (a) before retting, after (b) 14, (c) 28 and (d) 42 days of retting; (bf) biofilm, (ep) epidermis, (mo) microorganism, (rt) remnant of trichome, and (tr) trichome. Scale bar=200  $\mu\text{m}$ .

Therefore, during retting, the hemp stem surface was colonized by microorganisms, the presence of which might interact with the ATR infrared analysis. Thus, the

decrease in the bands assigned to fatty compounds might also be explained by the masking of the cuticle bands due to the surface colonization by microorganisms (Fig. 5). In fact, the effective path length allowed by the diamond ATR Agilent accessory is below 8  $\mu\text{m}$  in the midinfrared spectrum, which is not sufficient to reach the cuticle with a biofilm thickness of at least 15  $\mu\text{m}$  (Donlan, 2002). Further analysis could confirm this latter hypothesis by identifying the characteristic bands corresponding to the microorganisms that were most frequently isolated during field retting. Taken together, the microscopic observations combined with colorimetry and infrared analysis provided evidence of significant changes on the stem surface after 14 days of retting under controlled conditions, and they clearly indicated microbial colonization.

### 3.3. Changes in the bast tissue chemical composition and enzymatic activities

The microbial colonization of the stem surface should induce changes in the stem composition, particularly the outer (bast) tissues, which are rich in cellulosic fibers (Fig. 2). First, the overall chemical composition of the bast tissues did not show significant variation in the total N and C concentrations (0.7 and 44 g per 100  $\text{g}^{-1}$  DM, respectively) during the 42-day retting (Table 2), which is consistent with previous studies about mulch decomposition on the soil surface over a similar duration (Iqbal et al., 2015). However, the cell wall residue content of the bast tissues gradually increased from 63.3% DM at day 0 to 73.6% DM at day 42 (Table 2). This result indicates that the proportion of the soluble fraction decreased, either by leaching or by microbial degradation, as observed during the early stages of litter decomposition (Berg and McClaugherty, 2008). When growing on a substrate, the microorganisms initially consume the readily absorbable compounds present in the soluble fraction, and then they produce extracellular enzymes that degrade structural cell wall compounds (pectins, hemicellulose, cellulose, and lignin) to receive the absorbable forms of carbon and nutrients that match their needs (Lashermes et al., 2016). Although lignin represents a small proportion of the cell walls in bast tissues, the lignin contents of the cell wall residues increased slightly but significantly after two weeks of retting, consistent with the known biological recalcitrance of lignin (Akin, 2008; Liu et al., 2017a; Talbot et al., 2012). Despite the lack of significant variation in the total cell wall polysaccharides (Table 2), a sugar monomer analysis revealed significant changes in the polysaccharide composition during retting (Fig. 6). The typical sugars of the middle lamella/primary cell walls (pectin, xyloglucan) such as arabinose, rhamnose and galacturonic acid, galactose and xylose (Blake et al., 2008; Cr n nier et al., 2005) significantly decreased until 28 days of incubation, with the greatest decrease occurring during the first week (Fig. 6a-b). The glucose content can be primarily attributed to the cellulose of the

secondary cell wall fiber (Crônier et al., 2005), and it increased slightly during the first week and then did not change until the end of the retting (Fig. 6c). Similarly, mannose can be considered an indicator of secondary cell wall hemicelluloses in hemp fiber (Crônier et al., 2005), and its concentration did not vary significantly during the retting period (Fig. 6c).

The dynamics of polygalacturonase showed high pectinase activities at day 0, and the activity did not change significantly during the retting (Fig. 6d), which is consistent with the sharp decrease in galacturonic acid during the first stages of retting. Despite the high activity during the entire retting process, the smaller decrease in pectin monomers at later stages could be explained by the appearance of a recalcitrant pectin structure and/or lower accessibility due to the presence of a lignin-encrusted middle lamella component between the fibers (George et al., 2016). The determination of the other enzymatic activities involved in hemicellulose degradation ( $\alpha$ -L-arabinosidase,  $\beta$ -D-galactosidase, and  $\beta$ -D-xylosidase) and cellulolytic enzymes (glucosidase and cellobiohydrolase) indicated a similar evolution pattern with increasing activities essentially during the first two weeks of incubation (Fig. 6 d–f). These results are consistent with the sharp decrease in cell wall monomers (arabinose, galactose and xylose). However, increasing cellulolytic activities during retting were not related to a decreasing amount of cell wall glucose in the bast tissues. This result is likely attributable to the degradation of the cellulose in the parenchyma cell wall, which represents a very limited quantity when compared to the thick, cellulose-rich cell wall of bast fibers. Taken together, these results suggest that biological activities primarily act on thin-walled cells, which contain mostly pectin, hemicellulose and a certain amount of cellulose, while cellulose-rich fiber secondary walls would be weakly altered as described for dew retting (Müssig, 2010; Tahir et al., 2011). Overall, the dynamic evolution of enzymatic activities during retting under controlled environmental conditions could be explained by the microbial dynamics reported for hemp dew retting (Liu et al., 2017a; Ribeiro et al., 2015). Nevertheless, high variations in the sugar content occurred during the first week, while most enzymes exhibited weak activity, except for polygalacturonase, supporting the hypothesis that abiotic mechanisms such as drying and leaching may be responsible for certain changes in the composition of cell wall sugars in bast tissues.

### 3.4. Effects on the stem tissue architecture and the retting process

SEM was used to reveal the impact of microbial colonization and evolution in the cell wall chemical composition during retting on the tissue scale (Fig. 7). Observations of the stem transverse sections revealed unaltered tissue architecture in the stem prior to retting (Fig. 7a). Xylem is located in the core tissues, and bast tissue includes the parenchyma surrounding the fiber

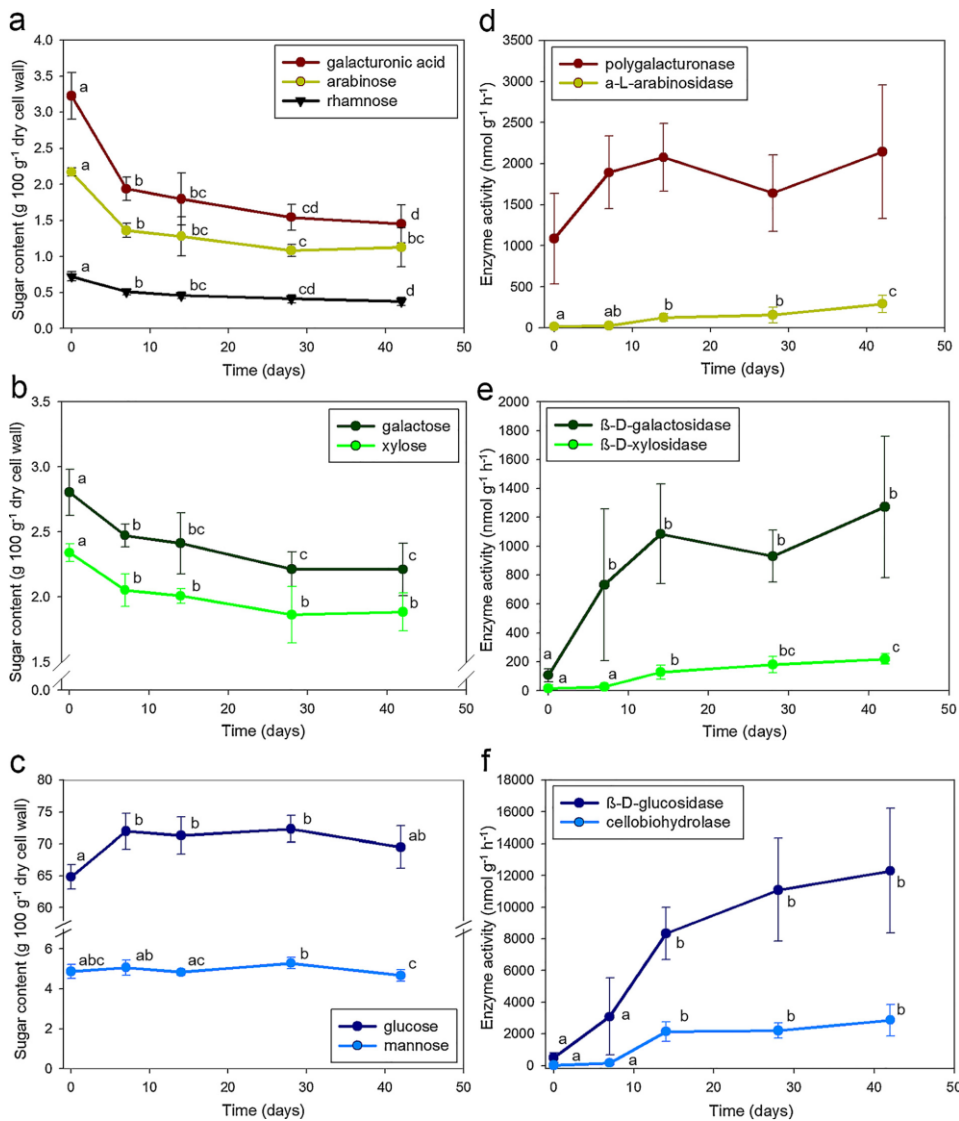
bundles and the epidermis with the cuticle at the interface with the external environment, as previously described (Crônier et al., 2005; Fernandez-Tendero et al., 2017; Liu et al., 2016). The SEM analysis did not reveal any apparent microbial colonization of the stem before retting. After 7 days of retting, almost no change could be observed (data not shown), whereas after 14 days of retting, the stems showed tissue modifications (Fig. 7b). At this stage, fungal hyphae were clearly observed on the epidermis, which is consistent with the stem surface imaging (Fig. 5). The parenchyma located under the epidermis and around the fiber bundles was partially damaged, and fiber bundle decohesion was observed. The observed alterations in the thin cell walls of the bast tissues parenchyma observed at day 14 are consistent with our measurements of a sharp decrease in the primary cell wall polysaccharides, such as pectin and hemicelluloses, and with the high and increasing enzymatic activities, which were also obtained at day 14 (Fig. 6). Although microorganisms were primarily observed on the surface, with only a few inside the bast tissues, one may assume that efficient extracellular enzymes were produced earlier, resulting in a partial dissociation of the fiber bundles. Abiotic factors might also account for bundle dissociation because the drying of the stems from 78% water content at day 0 to 18% at day 14 could induce parenchyma cell-wall disruptions due to severe water loss as proposed by Meijer et al. (1995) for flax dew retting. These phenomena could be even more pronounced if a preferential degradation of soluble components and a loss of parenchyma cells had occurred. On day 28, the colonization had spread below the epidermis on the outer area of the fiber bundles, resulting in a greater extent of decohesion between fiber bundles but no visible alteration in the fiber structures (Fig. 7c). The epidermis seemed to form a continuous layer (Fig. 7c), although surface imaging (Fig. 5c) showed increasing coverage by the microbial biomass. This observation supports our hypothesis that chemical modifications of the stem surface (Fig. 4), as revealed by infrared analysis, are most likely due to the masking of the cuticle by microbial colonization instead of cuticle degradation. At the end of the retting experiment (day 42), the colonization had spread through the bast tissue and reached the cambium area at the interface with the xylem (Fig. 7d). This observation indicates that the colonization during retting originated from the external environment and progressed towards the inner core of the stems. However, the fiber structure remained unchanged after 6 weeks of retting, whereas the micrographs showed fiber bundle decohesion from the rest of the stem. Both changes characterize the desired degree of retting for fiber crops (Akin, 2010). The epidermis was no longer visible and seemed to have been replaced by the biofilm, suggesting possible cuticle degradation..



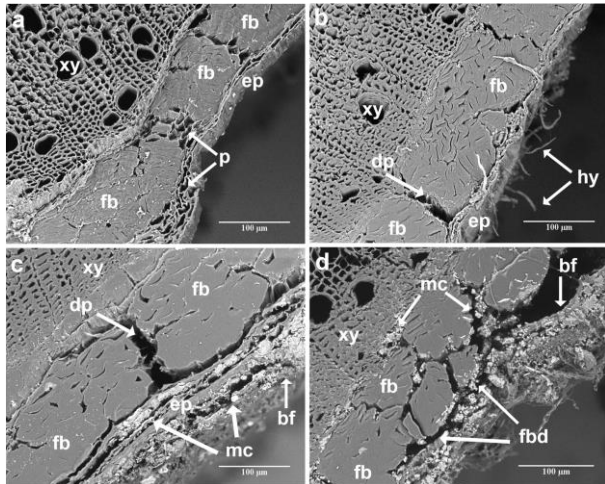
**Table 2.** Chemical changes in hemp bast tissues and cell wall residues during retting under controlled conditions

Time days	Bast tissues			Bast tissue cell wall residues	
	C total	N total	Cell wall content	Sugar content	Lignin content
	g 100 g <sup>-1</sup> dry bast tissues			g 100 g <sup>-1</sup> dry cell wall	
0	43.4 ± 0.4 <sup>a</sup>	0.7 ± 0.0 <sup>a</sup>	63.3 ± 9.2 <sup>a</sup>	81.3 ± 2.7 <sup>a</sup>	4.3 ± 0.3 <sup>a</sup>
7	43.6 ± 0.4 <sup>a</sup>	0.7 ± 0.0 <sup>a</sup>	70.4 ± 3.9 <sup>ab</sup>	85.6 ± 3.4 <sup>a</sup>	4.5 ± 0.2 <sup>a</sup>
14	43.6 ± 0.2 <sup>a</sup>	0.7 ± 0.1 <sup>a</sup>	71.8 ± 1.5 <sup>ab</sup>	84.4 ± 2.5 <sup>a</sup>	5.8 ± 0.2 <sup>b</sup>
28	43.9 ± 0.2 <sup>a</sup>	0.7 ± 0.1 <sup>a</sup>	76.9 ± 2.4 <sup>b</sup>	85.0 ± 2.2 <sup>a</sup>	4.9 ± 0.7 <sup>ab</sup>
42	43.8 ± 0.2 <sup>a</sup>	0.7 ± 0.1 <sup>a</sup>	73.6 ± 4.4 <sup>ab</sup>	81.5 ± 3.6 <sup>a</sup>	4.5 ± 0.1 <sup>a</sup>

Values with different letters are significantly different at a level of 5 %



**Fig. 6.** Changes in the cell wall residues of the hHemp bast tissues cell wall residues content changes in terms of galacturonic acid, neutral sugar content (a-c) and enzyme activities (d-f) during retting under controlled conditions; the period: relative content of (a) arabinose, rhamnose and galacturonic acid, (b) galactose and xylose, (c) glucose and mannose and the enzyme activities of (d) exopolgalacturonase and  $\alpha$ -L-arabinosidase, (e)  $\beta$ -D-xylosidase and  $\beta$ -D-galactosidase, (f)  $\beta$ -D-glucosidase and cellobiohydrolase. Values with different letters are significantly different at a level of 5 %.



**Fig. 7.** SEM micrographs of the hemp transversal sections before and during retting under controlled conditions; (a) before retting, after (b) 14, (c) 28 and (d) 42 days of retting; (bf) biofilm, (dp) degraded parenchyma, (ep) epidermis, (fb) fiber bundle, (fbd) fiber bundle decohesion, (hy) hyphae, (mc) microbial colonization, (p) parenchyma, and (xy) xylem. Scale bar=100 µm.

Finally, the retting process caused irreversible changes in the bast tissue architectural structure with the degradation of certain tissues such as parenchyma and fiber bundle decohesion. These modifications were triggered by microorganisms with the observed progressive colonization, which is consistent with previous findings (Tahir et al., 2011). However, it may be assumed that initial abiotic mechanisms such as drying can cause damage, facilitating further microbial colonization and degradation

#### 4. Conclusions

This experimental design was found to be adequate for performing hemp retting under controlled conditions, allowing for the quantification of the slight but significant changes occurring during the dew retting process. The microbial colonization of the stem surface and bast tissues revealed by microscopic imaging was linked to the selective degradation of the cell walls of the bast tissues parenchyma, thanks to the enzymatic activity measurements, leading to inter- and intrafiber bundle decohesion. Furthermore, both the colorimetric and infrared measurements of the stem surface also provided accurate assessments of the retting progress over time with results in accordance with the previous methods. It is conceivable that colorimetry and infrared spectroscopy could provide new and rapid monitoring indicators of the stem retting dynamics, thus providing a relevant decision-making tool for evaluating the optimal retting duration.

Thanks to this approach, which decoupled the environmental parameters and prevented possible

confounding effects under field bundle decohesion. Furthermore, both the colorimetric and infrared measurements of the stem surface also provided accurate assessments of the retting progress over time with results in accordance with the previous methods. It is conceivable that colorimetry and infrared spectroscopy could provide new and rapid monitoring indicators of the stem retting dynamics, thus providing a relevant decision-making tool for evaluating the optimal retting duration. These data are crucial for farmers and hemp industries to improve hemp cultivation and dew retting management options for fibers of high, consistent quality.

#### Notes

The authors declare no competing financial interest.

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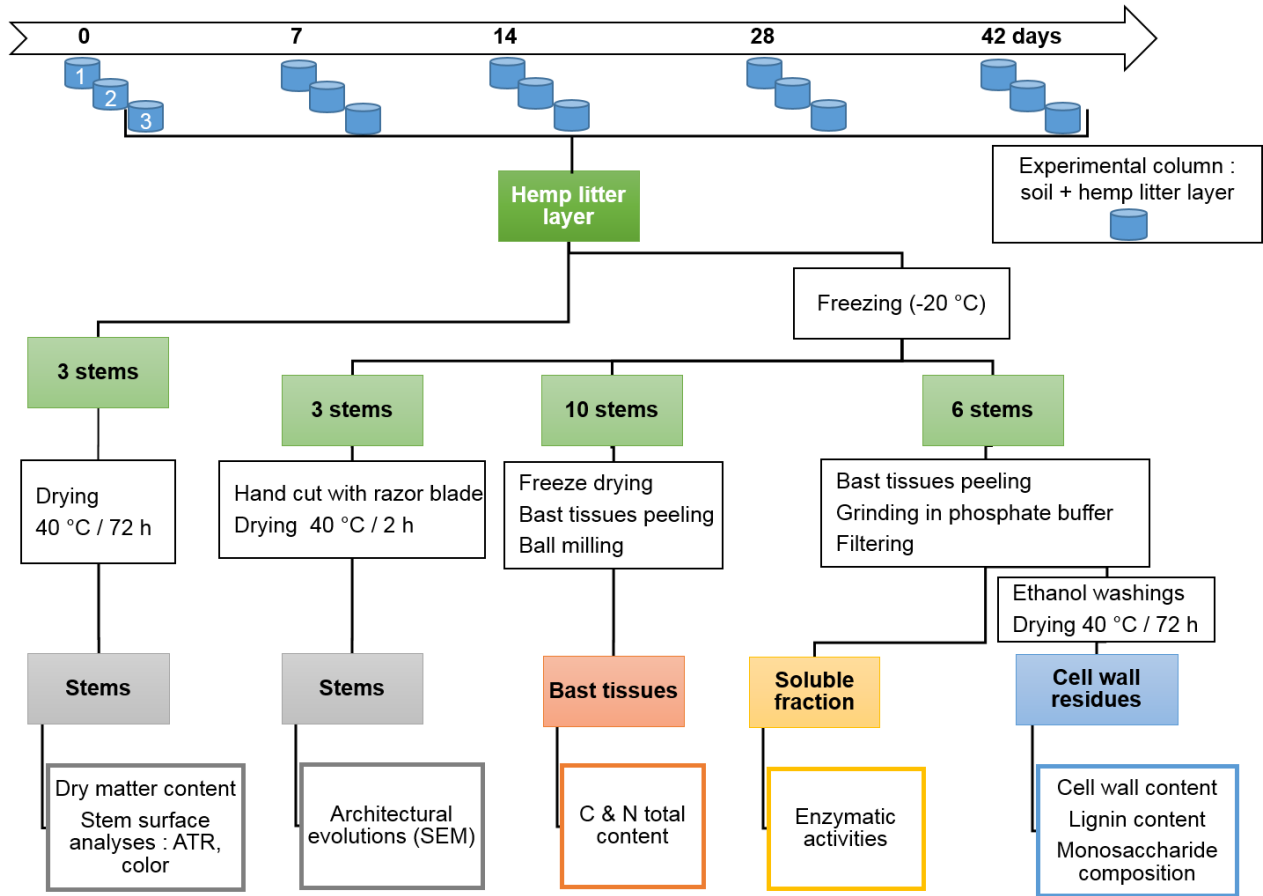
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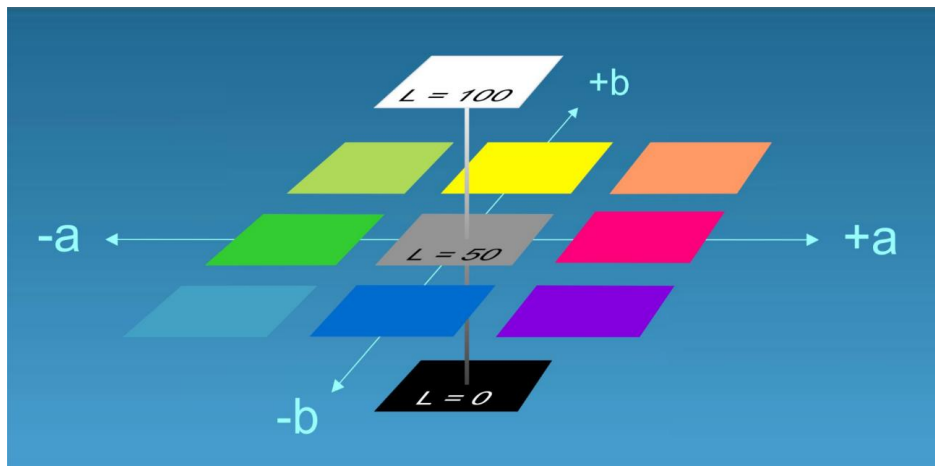
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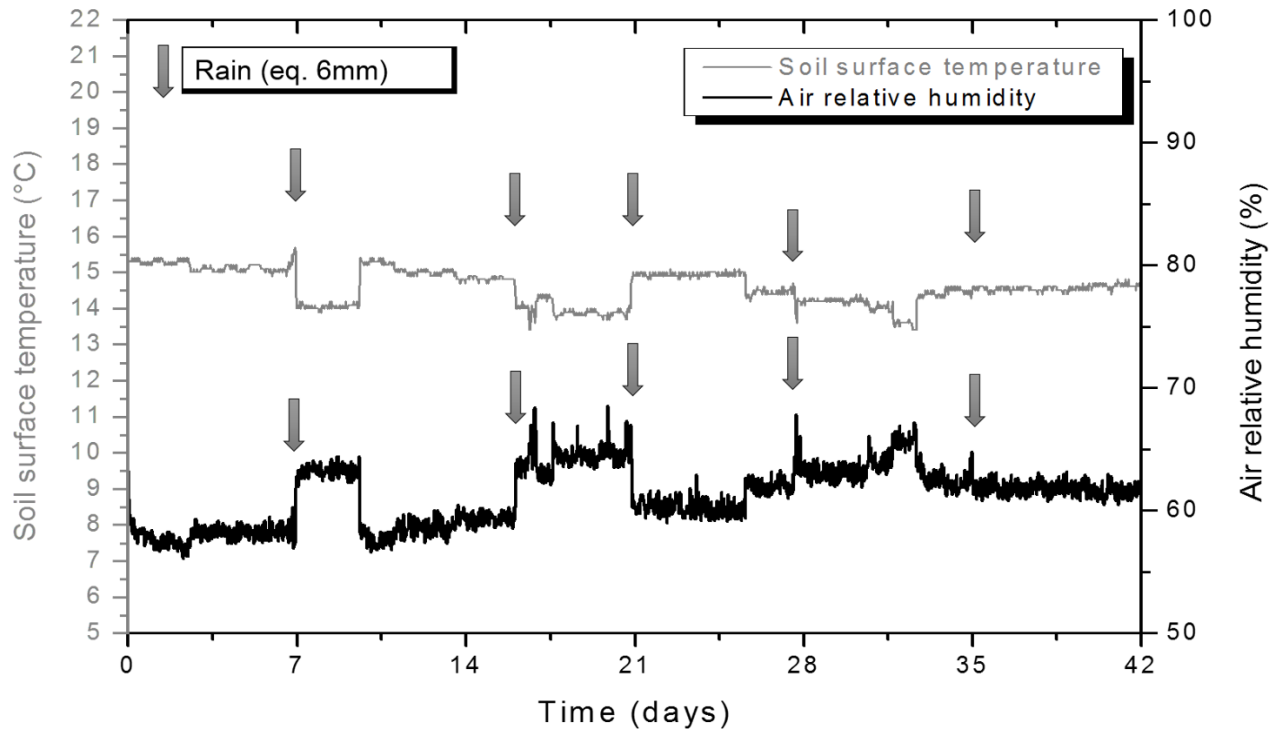
## Supplementary Figures



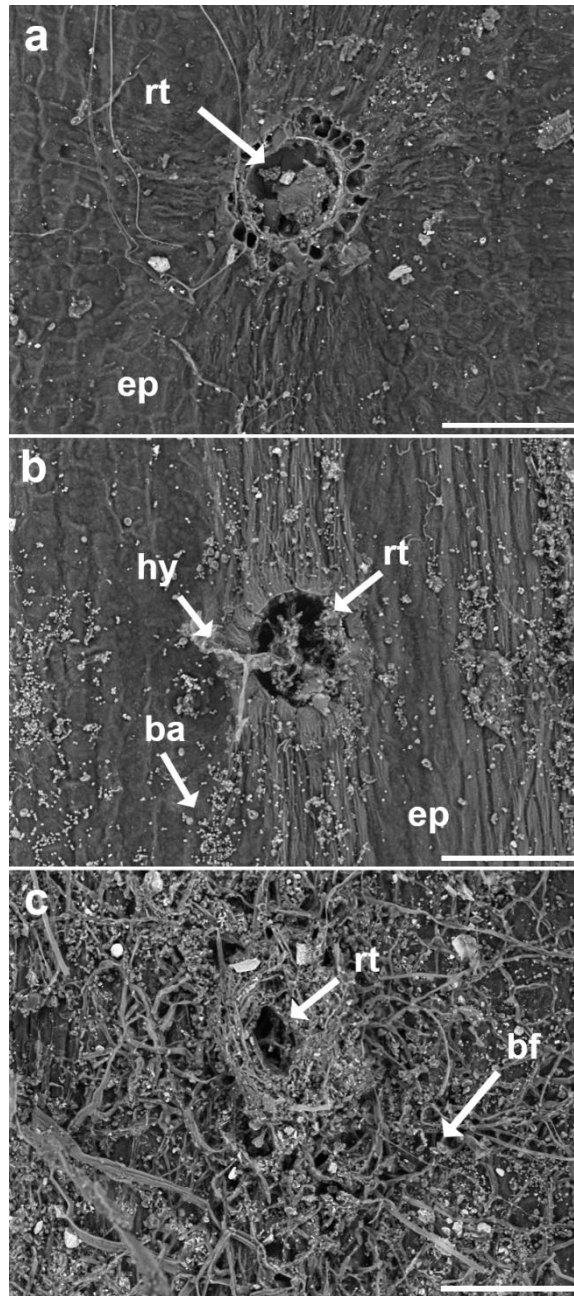
**Fig. S1** Sampling diagram



**Fig. S2** CIELab color space:  $L^*$  (lightness),  $a^*$  (redness/greenness) and  $b^*$  (yellowness/blueness). (zeiss.com)



**Fig. S3** Climatic recording at the soil surface of the experimental columns during retting under controlled conditions period: soil surface temperature (°C) and air relative humidity at soil/litter layer interface (%)



**Fig. S4** Scanning electron micrographs of the hemp stem surface before and during retting period: (a) before retting and after (b) 14 and (c) 42 days of retting; (ba) bacteria, (bf) biofilm, (ep) epidermis, (hy) hyphae, (rt) remnant of trichome. Scale bar = 100  $\mu\text{m}$