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1 Assessment of nutrient remobilization through structural changes of palisade and spongy parenchyma  
2 in oilseed rape leaves during senescence

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33 17 **Main conclusion:** Differential palisade and spongy parenchyma structural changes in oilseed rape leaf were  
34 18 demonstrated. These dismantling processes were linked to early senescence events and associated to  
35 19 remobilization processes.  
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20 **ABSTRACT**

21 During leaf senescence, an ordered cell dismantling process allows efficient nutrient remobilization. However, in  
22 *Brassica napus* plants, an important amount of nitrogen (N) in fallen leaves is associated with low N  
23 remobilization efficiency. The leaf is a complex organ mainly constituted of palisade and spongy parenchyma  
24 characterized by different structures and functions concerning water relations and carbon fixation. The aim of the  
25 present study was to demonstrate a specific structural evolution of these parenchyma throughout natural  
26 senescence in *Brassica napus*, probably linked to differential nutrient remobilization processes. The study was  
27 performed on 340 leaves from 32 plants during an 8-week development period under controlled growing  
28 conditions. Water distribution and status at the cellular level were investigated by low field proton Nuclear  
29 Magnetic Resonance (NMR), while light and electron microscopy were used to observe cell and plast structure.  
30 Physiological parameters were determined on all leaves studied and used as indicators of leaf development and  
31 remobilization progress. The results revealed a process of hydration and cell enlargement of leaf tissues  
32 associated with senescence. Wide variations were observed in the palisade parenchyma while spongy cells  
33 changed only very slightly. The major new functional information revealed was the link between the early  
34 senescence events and specific tissue dismantling processes.

35 **Key words:** leaf senescence, microscopy, NMR relaxometry, oilseed rape, palisade

## 36 INTRODUCTION

37 A leaf goes through different phases during its development, the last being senescence. Senescence is a  
38 highly regulated complex process that leads to the death of the tissue. This process has been shown to involve  
39 three phases (Nooden et al. 1997). The first phase is regulated by hormones and is characterized by transduction  
40 of the senescence signal (from environmental or internal factors) that leads to activation of key genes. The  
41 second phase is a degenerative phase corresponding to the disassembly and remobilization of cell components.  
42 Remobilization during that phase allows recycling cell nutrients from senescing tissues to growing organs.  
43 Finally, the terminal phase corresponds to disorganization of the nucleus, degeneration of the tonoplast and  
44 distortion of the cell wall leading to the cell death. Mitochondria remain active until the latest stage of  
45 senescence (Sakamoto 2006). All these events are programmed in order to allow maximal carbon (C) and  
46 nitrogen (N) remobilization in correspondence with sink demand. For crop species, the ability of the plant to  
47 remobilize nutrients highly impacts seed yield, especially in the current context of fertilizer input reduction. For  
48 instance, oilseed rape crop is known to have suboptimal N use efficiency partly due to low leaf organic N  
49 recycling performance and inherent important N residual amount in fallen leaves. As nitrogen fertilization has a  
50 major impact on oilseed rape production costs and environment quality, low N remobilization efficiency (NRE)  
51 has negative economical and agro-ecological consequences. Improving oilseed rape NRE should be a breeding  
52 challenge and would therefore necessitate a better understanding of senescence associated nutrient allocation and  
53 partitioning processes in leaves especially at the cell structure and organization levels where organites and  
54 macromolecules constitute predominant sources for metabolite recycling and sink feeding.

55 A leaf is constituted of the palisade and the spongy parenchyma crossed by vascular tissues and  
56 surrounded by two epidermises. In *Brassica napus*, the palisade parenchyma consists of regular shaped cells  
57 organized in layers whereas the spongy parenchyma, presenting large intercellular spaces, is less well organized  
58 (Castro-Diez et al. 2000). The principal leaf functions are photosynthesis, photorespiration and transpiration.  
59 Even if there is no experimental data supporting that, it is currently accepted that photosynthesis is mainly  
60 performed by palisade tissue (Nardini et al. 2010) whereas the spongy tissue is known to be involved in gas  
61 exchange. Moreover, it has been shown in *Acer hippocastanum* (horse chestnut) with abaxial position of stomata  
62 that the spongy tissue has a major role in the homeostasis of the leaf hydraulic status (Nardini et al. 2010).  
63 Although the leaf has often been studied as a homogeneous organ the structural and functional differences  
64 described above may suggest different patterns of evolution during development and senescence. It might be  
65 expected that palisade and spongy tissues evolve differently in terms of metabolism during senescence possibly  
66 leading to different contributions to remobilization process. The question of differential evolution of the palisade  
67 and spongy parenchyma during leaf development has not been addressed to date in the literature. The only  
68 differential response between leaf parenchyma were revealed by microscopy in plant under water (Bacelar et al.  
69 2006; Martinez et al. 2007) and ozone stresses (Bohler et al. 2013).

70 As already stated structural modifications due to macromolecules dismantling and recycling in  
71 senescing leaves are at the origin of cell compartment disruption, dry matter reallocation and probably water  
72 redistribution between compartments. Low field proton Nuclear Magnetic Resonance (NMR) has been used for  
73 investigation of cell water compartmentalization in various plant organs (Hills and Remigereau 1997; McCain  
74 1995; van der Weerd et al. 2001). The technique allows measurement of relaxation signals that for highly

75 hydrated plant tissues originate mainly from water. In the case of compartmentalized systems characterized by  
1 76 slow diffusion exchange of water molecules between compartments, relaxation times have a multi-exponential  
2 77 character due to differences in physical and chemical properties of water in different compartments. The  
3 78 relaxation signal is therefore used to study changes in water status and distribution. In most studies, transverse  
4 79 relaxation time (T<sub>2</sub>) has been used rather than longitudinal relaxation time (T<sub>1</sub>) as it is more sensitive to  
5 80 variations in water properties occurring in plant tissues. The multi-exponential T<sub>2</sub> has mainly been interpreted at  
6 81 a cell level to assign different signal components to principal cell compartments while assuming homogeneity of  
7 82 cells in the sample being examined (Snaar and Van As 1992b). This model was developed for fleshy fruit (Snaar  
8 83 and Van As 1992a) and vegetable parenchyma (Hills and Remigereau 1997). It has also been used for leaf  
9 84 tissues from different plants (Capitani et al. 2009; Colire et al. 1988). Another approach was used by Qiao *et al.*  
10 85 (2006) who considered only heterogeneity of the sample at the tissue level. Musse *et al.* (2013), investigating  
11 86 leaf senescence of *Brassica napus*, recently drew attention to the fact that both cell compartmentalization and  
12 87 tissue heterogeneity have to be taken into account in the interpretation of the NMR signal. The NMR signal of  
13 88 leaves was shown to be sensitive to the senescence stage and all signal components to be affected by it. The  
14 89 principal signal changes concerned the vacuole-related component that separated into two components in  
15 90 senescing leaves. An interpretation was proposed associating these changes with a process of cell enlargement  
16 91 and hydration, probably linked to remobilization processes.

26 92 The focus of the present study was on changes occurring in vacuole properties, the major place of  
27 93 macromolecules degradation during remobilization, and plast structures that constitute the main N reserve of the  
28 94 leaf, during leaf senescence. NMR measurements and an extended microscopy study were performed to observe  
29 95 changes at both cellular and tissue levels. Light microscopy was used for the quantification of tissue thickness  
30 96 and vacuolar volume, whereas plasts and cell walls were investigated using electronic microscopy. The design of  
31 97 the experiment allowed investigation of natural leaf senescence in a large number of plants grown under  
32 98 controlled conditions through an eight-week kinetics study. This design made it possible to compare different  
33 99 developmental scales commonly used to describe senescence (i.e. leaf rank, leaf age and physiological markers).  
34 100 The output of the study was to demonstrate a differential structural and probably functional evolution linked to  
35 101 nutrient remobilization processes of palisade and spongy parenchyma cells in naturally senescent leaves of  
36 102 *Brassica napus*.

## 46 104 MATERIALS AND METHODS

### 49 105 *Plant material*

51 106 About 10 seeds (homogenous weight) of oilseed rape, *Brassica napus* L., genotype Tenor, were sown in  
52 107 individual containers filled with a growing medium (FALIENOR 9226-6F2) containing 65% light peat, 20%  
53 108 dark peat and 15% perlite. Four sowings (series S-1, S-2, S-3, and S-4) were undertaken at one-week intervals.  
54 109 The 8 most homogenous three-week-old seedlings of each series were individually planted into four-liter pots  
55 110 filled with the same growing medium and grown in a growth cabinet for five (t<sub>1</sub>) to twelve (t<sub>8</sub>) weeks. The 32  
56 111 plants used for this experiment were watered throughout the growing period, with a fertilizing solution

112 (Liquoplant bleu) used at 3‰ and irrigation was adjusted to the evaporation-transpiration rate of the pots. The  
113 growth cabinet conditions were 14hr daylight (at 200  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and 10hr dark (relative humidity:  
114 75/90%; temperature: 20/18°C).

### 115 ***Chlorophyll content***

116 Before sampling, relative chlorophyll content per unit of leaf area was determined using a non-  
117 destructive chlorophyll meter SPAD (Soil Plant Analysis Development; Minolta, model SPAD-502).  
118 Chlorophyll content of each leaf was estimated as an average value of 6 independent measurements.

### 119 ***Sampling***

120 For all series, one plant was taken each week for 8 weeks (week1 to week 8) and all the leaves for each  
121 plant were numbered according to their rank (L-1 for the first leaf after the cotyledon).

122 Six to ten leaf discs of 8 mm in diameter were cut from the limb tissue for the NMR measurements to  
123 sample similar tissue weights. In order to obtain homogeneous tissues, discs were taken from each side of the  
124 central vein as close as possible to the vein and avoiding lateral nervures. Discs were then placed in NMR tubes  
125 which were closed with a 2-cm long Teflon cap to avoid water loss during measurements. Leaf discs were also  
126 collected for microscopy studies from selected plants and leaf ranks,. The rest of the leaf limb was frozen in  
127 liquid nitrogen and kept at -20°C to be subsequently ground and freeze-dried for starch quantification.

128 For microscopy studies, mesophyll tissues were collected from series 4 (S-4) plants, from three leaves  
129 (leaf ranks 4, 6, and 8) at three times (weeks 3, 4, and 5). Five bands 3-millimeters wide and 1-centimeter long  
130 were taken perpendicularly to the central vein. In order to confirm our vacuolar volume approximation, in the  
131 first plant five bands were also collected alongside the central vein. After sampling, the fixative solution  
132 (phosphate buffer 0.1 mol. L<sup>-1</sup> p H 7.4 (PB), 3% (w/v) glutaraldehyde) was infiltrated into the leaf tissues using  
133 10 cycles of depressurization with a vacuum pump (5 min of low pressure in each cycle). Samples were returned  
134 to the same fixative solution for 24 h at 4°C and then were washed in PB and dehydrated in a graded series of  
135 increasing concentrations of ethanol (50, 70, 90 and 100% v/v). Samples were kept in pure ethanol for 12 h at  
136 4°C.

### 137 138 ***Starch quantification***

139 Starch was extracted from 30 mg dry weight of freeze-dried leaf limb tissue using 1 mL phosphate  
140 buffer (0.2 mol. L<sup>-1</sup>, pH 6.5) for 20 min at 95°C. The supernatant was collected after centrifugation at 15,000 x g  
141 for 5 min at 4°C. The extraction step was repeated twice and the supernatants were pooled. Measurements were  
142 performed three times for each sample (following the manufacturer's recommendations (Sigma, # STA20)) after  
143 hydrolysis by  $\alpha$ -amylase and amyloglucosidase. Starch content was expressed in glucose equivalent after  
144 subtraction of free glucose content.

### 145 ***Water content***

146 Water content (WC) was measured on all leaf discs sampled for NMR relaxometry by weighing before  
147 (fresh weight) and after drying (dry weight) in an oven at 103°C for 16h. WC was expressed as percentage of  
148 fresh weight.

## 149 *NMR Relaxometry*

150 NMR Relaxometry measurements were performed with a 20 MHz spectrometer (Minispec PC-120,  
151 Bruker, Karlsruhe, Germany) equipped with a thermostatted probe. The temperature was set at 18°C. T2 was  
152 measured using the combined FID-CPMG sequence.

153 The FID signal was acquired from 11  $\mu$ s to 70  $\mu$ s at a sampling decay of 0.4  $\mu$ s. For the CPMG  
154 measurements, the 90°-180° pulse spacing was 0.1 ms and the signal of a single point at the echo maximum was  
155 acquired. Data were averaged over 64 acquisitions. The number of successive echoes recorded was adjusted for  
156 each sample according to its T2. The recycle delay for each sample was adjusted after measurement of the T1  
157 with a fast-saturation-recovery sequence. The total time of acquisition of data for T2 (including spectrometer  
158 adjustments and T1 measurement) was about 10 min per sample.

159 Fitting was performed in two steps: first T2 relaxation curves from the CPMG data only were fitted by  
160 Scilab software according to the MEM (Mariette et al., 1996), which provides a continuous distribution of  
161 relaxation components without any assumption concerning their number. In this representation, the peaks of the  
162 distribution are centered at the corresponding most probable T2 values, while peak areas correspond to the  
163 intensity of the T2 components. Then complete T2 relaxation curves obtained by the combined FID-CPMG  
164 sequence were analyzed using the Levenberg-Marquardt algorithm which allows a discrete solution for the  
165 fitting curve according to the equation:

$$166 \quad I(t) = I_1 \exp(-t / T_{21})^2 + \sum_{i=2} I_{0i} \exp(-t / T_{2i}) + offset \quad \text{Eq. 1}$$

167 where  $I_{0i}$  is the intensity of the  $i^{\text{th}}$  exponential at the equilibrium state and  $T_{2i}$  the characteristic transverse  
168 relaxation time for the  $i^{\text{th}}$  exponential. The number of terms that best described the relaxation curve was  
169 determined by examining MEM relaxation curves. Intensity was expressed through the specific leaf water weight  
170 of the  $i^{\text{th}}$  signal component expressed in  $\text{g m}^{-2}$  (LWW). The specific LWW of each component was calculated  
171 according to the equation:

$$172 \quad LWW_i = \frac{I_{R0i} \times m_w}{A} \quad \text{Eq. 2}$$

173 where  $m_w$  is the water mass of the leaf disc use for NMR (in g),  $A$  the leaf disc area (in  $\text{m}^2$ ) and  $I_{R0i}$  the relative  
174 intensity of the  $i^{\text{th}}$  signal component expressed as a percentage of the total CPMG intensity.

## 175 *Electron and light microscopy*

### 176 **Sample preparation and micrograph acquisition**

177 For light microscopy, samples stored in pure ethanol were progressively included in an acrylic resin  
178 (LRWhite) using a graded series of increasing concentrations of resin solubilized in ethanol (20, 40, 60, 80, and

181 100% v/v). Polymerisation was performed at 55°C over 4 days. Thin sections (1 µm) were cut with an  
182 ultramicrotome (LEICA UC7) and stained with toluidine blue before they were observed with a NIKON Eclipse  
183 80i microscope.

184 For transmission electron microscopy analysis (TEM), ethanol in samples was replaced by propylene  
185 oxide by two baths (50 and 100% v/v). Then the propylene oxide was replaced by an epoxy resin (EPON) using  
186 the same method (50 and 100% v/v) and the sample was kept for one night at 4°C in an EPON solution.  
187 Polymerisation was performed over 1 day at 55°C and 60H at 72°C. Ultra-thin sections (60 nm) were then cut  
188 with an ultramicrotome (LEICA RM 2165), and placed on grids post-stained for 60 min with uranyl acetate. The  
189 ultra-thin sections were then examined with a JEOL JEM 1230 transmission electron microscope with an  
190 accelerating voltage of 80 kV

#### 191 **Vacuole volume measurement**

192 The area (A) of each cell from both parenchyma was measured using image J software. The width (W)  
193 of each cell was also measured and the results from samples taken perpendicularly or parallel to the central vein  
194 of the same leaves, were compared. No difference was observed between the two types of sample, allowing us to  
195 estimate the vacuole volume of each cell by multiplying A by W.

### 196 ***Data analyses***

197 All calculations were performed using the software package Rstudio. In order to detect significant  
198 differences ( $P < 0.05$ ) between different leaf ranks, and between different weeks of sampling, one way analysis of  
199 variance test (ANOVA) was applied to all measurements. The multiple range LSD test was used to compare  
200 means of the series. Correlations were revealed through Pearson's rank correlation coefficient analysis.

## 201 **RESULTS**

### 202 ***Physiological characterization of leaf development***

203 Leaf development was characterized through chlorophyll and starch content, water content and dry  
204 weight measurements (Fig. 1). In order to compare leaf pattern of evolution according to the position in the  
205 canopy and to time, two approaches were used to present the results:

206 (i) All leaves were followed during the eight-week measurement period. The eighth rank leaf (LR-8)  
207 was chosen for presentation in Fig. 1 (a, b) because it was present on the plant during the whole measurement  
208 period (from eight weeks to fifteen weeks).

209 (ii) All leaves from one plant were analyzed at one measurement time (b, d). The results presented  
210 correspond to the ten-week-old plant (third week of measurement period).

211 Fig. 1a shows that the chlorophyll content was at its maximum until the sixth week and then decreased  
212 to the end of the measurement period. The amount of starch increased until the fifth week of measurement and  
213 then started to decrease with ageing and destruction of the photosynthetic pigment. The water content was about  
214 85% of fresh weight in young leaves (Fig. 1c) and increased during the last three weeks. Dry weight was  
215 constant until the sixth week and then decreased slightly. The results of fresh weight measurement  
216 (Supplementary data 1) showed that the increase in water content observed in old leaves was due not only to the  
217 decrease in dry weight but also to an increase in the amount of water.



218 Similar patterns were observed while comparing physiological parameters corresponding to different  
219 leaf ranks from the same plant at a given time (Fig. 1b, d) to those from a leaf over eight weeks (Fig. 1a, c). This  
220 demonstrated that analyzing leaves for the parameters measured in the present study throughout the canopy from  
221 old to young leaves can be considered equivalent to studying one leaf during its development.

### 222 *NMR characterization of leaf development*

223 Changes in NMR signal were followed throughout leaf development. Fig. 2 presents a continuous  
224 distribution of transverse relaxation times obtained by the maximum entropy method (MEM) calculated from the  
225 separate CPMG (Carr Purcell Meiboom Gill) data from different leaves. The dotted line represents MEM results  
226 for the selected leaf (LR-8) obtained at four different times in the eight-week measurement period. The solid line  
227 shows the results for four leaves (LR-10, LR-7, LR-5, LR-3) from one plant at one measurement time (third  
228 week). The NMR signal for *Brassica napus* leaves is characterized by four or five components, depending on  
229 leaf age (Fig. 2), the first component corresponding to the solid state protons (not represented in Fig.2) and the  
230 others to the liquid fraction (Musse et al. 2013). The longest T2 component attributed to the vacuole (Musse et  
231 al. 2013; Van As 2007) represented more than 75% of the leaf water. The T2 of this component increased with  
232 time and started to split into two components in mature leaves (Fig.2b). For senescent leaves, two separate  
233 vacuolar components were observed (Fig.2c) for which T2 values increased until the end of the measurement  
234 period (Fig.2d). As for the physiological parameters, Fig. 2 shows that the evolution of transverse relaxation time  
235 distributions was the same when monitoring one leaf during several weeks and when analyzing leaves  
236 throughout the plant axis from old to young leaves at one measurement time.

237 The leaf rank for which the longest T2 component observed in young leaves separated into two  
238 components is represented in Fig. 3. The emergence of the new component is from here on referred to as “the  
239 split”. Both our former study (Musse et al. 2013) and the present results showed that this split was linked to the  
240 developmental process of the leaf. Between week 2 and week 7, the leaf rank at which the split occurred  
241 increased by one rank each week (Fig. 3). During the eight-week measurement period, the number of leaves in  
242 which the split occurred represented a constant ratio of 40 - 50% of all the expanded leaves of the plant.

243 The results presented in Figs. 2 and 3 show that it was possible to use the split to target leaves at the  
244 same developmental stage. In this aim, tag zero was assigned to leaves in which the split had occurred, the  
245 subsequent leaf rank was numbered 1, etc. According to this scale, the older the leaf was, the higher its tag, while  
246 negative tags represented young leaves. This NMR split scale made it possible to average values from data  
247 obtained in leaves at different measurement weeks and from different leaf ranks. These results took into account  
248 all 340 leaves from the 32 plants used in this study, giving an overview of leaf development from young to very  
249 senescent leaves. The NMR split scale is used in the subsequent Figures.

250 The discrete solutions for the complete decay curve (FID+CPMG) obtained by the Levenberg-  
251 Marquardt algorithm agreed to a great extent with the MEM results of the separate CPMG curve. In addition, the  
252 FID provided access to a fast-relaxing component linked to the solid phase protons, with T2 of a few tens of  
253 microseconds and 2-5% of total signal intensity. Component 1 was not further analyzed but the correlation  
254 between its intensity and dry weight (Supplementary data 2) may be valuable in future studies to determine leaf  
255 age as the dry weight decreases during senescence (Fig. 1).

256 For the liquid fraction components, Fig. 4 shows the T2 value and relative intensity of the NMR signal  
1 257 (components 2 to 5) expressed in leaf water weight per leaf area during leaf development obtained by the  
2  
3 258 Levenberg-Marquardt algorithm. The T2 of component 2 (Fig. 4a) remained at around 2 ms from the youngest  
4  
5 259 leaves to two ranks before the split occurred (tag -7 to tag -2, according to the split scale). It then decreased  
6  
7 260 progressively until tag +4 and component 2 finally disappeared in older leaves. The T2 of component 3 was  
8  
9 261 relatively stable, at around 20 ms for young leaves (tag -7 to tag -1) and 16 ms for older leaves (tag +1 to tag +7).  
10  
11 262 The T2 of component 4 (Fig. 4b) was around 100ms for the youngest leaves (tag -7) and progressively reached  
12  
13 263 200 ms at tag -1. After the split, the two components (4 and 5) had different pattern of evolutions, component 4  
14  
15 264 increased slightly up to 235 ms (tag +7), while the T2 of component 5 first increased steadily to 400 ms until the  
16  
17 265 tag +5 and then strongly to 800 ms until tag +7.

16 266 Fig. 4c shows the relative signal intensity expressed in leaf water weight per leaf area ( $LWW_i$ ) in order  
17 267 to present the amount of water associated with each component. The  $LWW_2$  remained stable until the  
18  
19 268 disappearance of component 2 (tag +5). The amount of water associated with component 3 increased slightly  
20  
21 269 during leaf development from 40  $g/m^2$  (tag -7) to more than 50  $g/m^2$  in the oldest leaves.  $LWW_4$  was about 220  
22  
23 270  $g/m^2$  before the split. At the first appearance of component 5,  $LWW_5$  was 180  $g/m^2$  whereas  $LWW_4$  was around  
24  
25 271 100  $g/m^2$ . It remained around this value after the split whereas  $LWW_5$  increased markedly to 440  $g/m^2$  from tag 0  
26  
27 272 to tag +6.

27 273 In Fig 5, the physiological parameters are expressed on the NMR split scale. The same patterns for  
28  
29 274 chlorophyll and starch content and can be observed in Fig. 1. By comparing conventional approaches (Fig 1) and  
30  
31 275 the NMR split scale (Fig 5) it was demonstrated that the NMR split scale allowed averaging of more data for  
32  
33 276 each point. Indeed, for the representation according to leaf position or leaf age (Fig. 1), number of data averaged  
34  
35 277 for each point corresponded to the number of repetitions (4 in the present study). In contrast, in the case of the  
36  
37 278 representation using the NMR split scale, all leaves from all plants were considered and each point represents an  
38  
39 279 average of 7 to 21 measurements on individual leaves. The number of averaged data depended on the number of  
40  
41 280 leaves with similar NMR signals, without taking into account the characteristics of the experimental design (leaf  
42  
43 281 rank and measurement week). Moreover, the NMR split scale made it possible to extend the range of the  
44  
45 282 development scale, as can be observed in Fig. 5. The NMR split scale therefore makes it possible to reconstruct  
46  
47 283 the pattern of evolution of parameters throughout leaf development by taking into account all the individual  
48  
49 284 leaves studied

### 285 *Limb structure modifications*

49 286 Changes in leaf internal structure were characterized by electronic and light micrographs. Fig. 6  
50  
51 287 represents examples of leaf light micrographs of young (tag -2), mature (tag +2) and senescing leaves (tag +4).  
52  
53 288 The general structure of the leaf remained constant at all stages of leaf development, with upper epidermis,  
54  
55 289 palisade parenchyma, spongy parenchyma and lower epidermis (from top to bottom). However, the tissue  
56  
57 290 differentiation became trickier in senescing leaves (from tag +4) where the structure tended to be less organized.  
58  
59 291 The most obvious structural modification observed between the leaf stages was the increase in leaf thickness  
60  
61 292 with ageing. This was mostly due to enlargement of the palisade cells. The upper epidermis seemed to  
62  
63 293 experience similar swelling to the palisade parenchyma, and the structure of the lower epidermis, that was less

294 affected by ageing, was likely to change in the same way. Another noticeable structural change that can be  
1 295 observed (Fig. 6) was the disappearance of chloroplasts during leaf development. In addition to changes in  
2 296 numbers of chloroplasts, plast structure was also affected by ageing. This is illustrated in Fig. 7 depicting  
3 297 electronic micrographs of plasts at three developmental stages. Chloroplasts had several starch granules in young  
4 298 and mature leaves, well defined thylakoids and few small plastoglobuli (Fig. 7a). By contrast, in gerontoplasts  
5 299 present in senescing leaves (Fig. 7b), plastoglobuli were larger and numerous. A few thylakoids were observed,  
6 300 but not stacked as in chloroplasts, and no starch granules were visible. Gerontoplasts were found mainly in  
7 301 senescent leaves, although a few of them were observed in mature leaves. In the final stage of development  
8 302 (corresponding to the oldest leaves (Fig. 7c)) plasts were constituted of relatively large lipid droplets surrounded  
9 303 by an envelope. These plasts were not detectable by light microscopy because they were rare and because  
10 304 toluidine blue is known specifically to stain starch in the plast.

### 17 305 *Changes of parenchymal structure*

18 306 The structural modification of leaf tissues seen in Fig. 6 is presented in Figs. 8. The average vacuolar  
19 307 volume of palisade parenchyma cells increased markedly (Fig. 8), while that of spongy parenchyma remained  
20 308 around  $0.3 \text{ mm}^3$ . Leaf thickness increased from around  $250 \text{ }\mu\text{m}$  to  $320 \text{ }\mu\text{m}$ , mainly due to an increase in the  
21 309 palisade parenchyma (from  $100 \text{ }\mu\text{m}$  to  $160 \text{ }\mu\text{m}$ ). The thickness of the spongy parenchyma remained at around  
22 310  $100 \text{ }\mu\text{m}$ . Each epidermis presented a slight increase in thickness.

23 311 Note that, as mentioned earlier in Materials and Methods section, measurements of the area and width  
24 312 of the vacuole on the samples taken perpendicularly and parallel to the central vein confirmed that vacuole depth  
25 313 and width can be considered as identical, validating the model for volume estimation based on micrograph  
26 314 observations (Supplementary data 3).

27 315 Fig. 9 depicts the percentage of the leaf cross section occupied by each tissue according to the NMR  
28 316 split scale. In order to approximate vacuolar water at the tissue level, internal spaces and plasts were removed  
29 317 from the representation. The volume of epidermis cells represented about 15% of the leaf tissues and therefore  
30 318 contributed to the NMR signal. Due to their irregular shape, the volume of epidermis cells could not be estimated  
31 319 from micrographs. However, an increase in both upper and lower epidermis thickness was observed, indicating  
32 320 similar evolution of these tissues to that of palisade parenchyma, i.e. in accordance with the hydraulic designs  
33 321 proposed in leaves by Zwieniecki (Zwieniecki et al. 2007). We therefore supposed that the fifth component of  
34 322 the NMR signal reflected epidermis vacuoles in addition to the palisadic parenchyma vacuoles. In consequence,  
35 323 the volumes occupied by epidermis and palisade cells were pooled for comparison with NMR results (Fig. 9).

36 324 For comparison with the vacuolar water changes measured from micrographs, the percentages of the  
37 325 total intensity of components 4, 5 and 4+5 are represented on the same graph. The results showed an increase in  
38 326 volume of palisade and epidermis tissues whereas the spongy parenchyma remained constant. At the same time,  
39 327 the relative intensity of the fifth component increased, while that of the fourth remained almost constant. The  
40 328 latter indicated that the fourth component might be related to vacuoles of spongy parenchyma cells while the  
41 329 fifth component might correspond to those of the palisade parenchyma cells.

## 58 330 **DISCUSSION**

### 331 *Differential structural changes of leaf parenchyma throughout leaf senescence*

1  
2 332 As described in the introduction, leaf parenchyma have different functions that may lead to different  
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4 333 patterns of evolution during senescence. The major limitations for studying functions of each parenchyma  
5 334 separately are technical; for example, there is no easily available technique that allows differential evaluation of  
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7 335 biochemical traits at the tissue level. In C3 plants, like oilseed rape, only structural differences between  
8 336 parenchyma have been analyzed in detail, due to the fact that they are visible microscopically. Microscopic  
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10 337 studies of leaf tissue structure have mostly focused on the impact of water stress in *Arabidopsis* (Wuyts et al.  
11 338 2012), peas (Martinez et al. 2007), olive trees (Bacelar et al. 2006) and horse chestnut trees (Nardini et al. 2010).  
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13 339 The impact of ozone has also been measured at the tissue level by measuring leaf tissue thickness of different  
14 340 woody plant species, because of the well-known gas exchange role of spongy cells (Günthardt-Goerg et al.  
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16 341 2000). The results reported have shown significant differences between species in response to such stress, in  
17 342 terms of size and structure of parenchyma.

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19 343 The different patterns of evolution of palisade and spongy cell volumes (Fig. 8) reported here could be  
20 344 explained by the specialized functions attributed to each type of parenchyma. In view of their major role in  
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22 345 photosynthesis (Nardini et al. 2010), palisade cells would be more impacted by the nutrient remobilization  
23 346 process associated with plast dismantling, especially in terms of change in solute composition of the vacuole that  
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25 347 is the major site of degradation of different molecules during senescence (Otegui et al. 2005). The increase in  
26 348 palisade cell volume corresponding to leaf hydration (Fig. 1) could be explained by cell wall loosening probably  
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28 349 together with lowering of vacuolar osmotic potential. For instance, it has been shown on *Arabidopsis* that several  
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30 350 cell wall degrading enzymes ( $\beta$ -glucosidase) are enhanced by decline in photosynthesis (Mohapatra et al. 2010),  
31 351 suggesting that cell wall degradation occurs in the leaf cells during senescence. These authors suggested that  
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33 352 polysaccharides bound to the cell wall that remains intact even during the late phase of senescence may be a  
34 353 possible source of sugar. On the other hand, spongy cells, that have a major role in the leaf's hydraulic status  
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36 354 (Nardini et al. 2010), are believed to regulate water status during senescence.

37 355 As a matter of fact water content was shown to increase in oilseed rape leaves during senescence (Fig.  
38 356 1c and d), in accordance with findings previously reported by Musse et al. (2013). This increase is due to loss of  
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40 357 dry weight that materializes remobilization of cell materials (Diaz et al. 2008) and to leaf hydration. This  
41  
42 358 hydraulic characteristic was revealed by both micrographs and the increase in fresh weight observed during  
43 359 senescence progression (Supplementary data 1). The water entry is at the origin of the slight increase of the  
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45 360 intensity of the signal corresponding to the vacuole and probably of the increase in T2. In mature leaves (tag 0),  
46 361 the fifth component appeared as a result of the split in the fourth component. This is supported by the fact that  
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48 362 the sum of the LWW of two longest T2 components in mature and senescing leaves (Fig. 4) represented a  
49 363 continuation of the pattern of LWW of the fourth component in young leaves. After the split, the LWW and the  
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51 364 T2 of the fourth component continue to increase slightly, and both parameters of the fifth component markedly  
52 365 increased. As discussed by Musse *et al.* (Musse et al. 2013), the significant increase in the T2 of the fifth  
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54 366 component is probably due to several phenomena. First, the water influx, at the origin of cell enlargement  
55 367 combined with decreased dry weight, probably induced a dilution of vacuole solutes. This phenomenon was in  
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57 368 part counterbalanced by the increase in solute concentration due to degradation processes occurring during  
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59 369 senescence (Otegui et al. 2005). Secondly, the increase in cell volume had an impact on the T2 value because of  
60 370 the sensitivity of T2 to compartment size (van der Weerd et al. 2001). The trends of components 4 and 5 were

371 linked to the observations from the light microscopy, showing clear tissue differentiation (Figs. 8). Indeed, the  
372 vacuolar volume of the palisade cell increased markedly, whereas such changes did not occur in spongy cells.  
373 The variations in palisade vacuole volume measured in the present study (from 0.4 mm<sup>3</sup> (tag -2) to 1.6 mm<sup>3</sup> (tag  
374 4)) affected the T2 values of the fifth component. A linear relationship between the relaxation rate (1/T2) and the  
375 sum of the inverse of the cell compartment radii has been demonstrated in maize and pearl millet by van der  
376 Weerd et al. (2001), implying constant permeability of the compartment membrane and the same solute  
377 composition of vacuole. However, the linear relationship could not be applied to the results of the present study  
378 because of the changes in the vacuole composition and because there was no evidence that tonoplast  
379 permeability did not vary during leaf development. The sudden increase in T2 of the fifth component at the very  
380 end of senescence (tag +6 and +7) and the high value of standard deviation of the measurements (Fig. 4) may be  
381 linked to active dismantling of the tonoplast of some the palisade cells just before their death. Indeed, the water  
382 enclosed in the vacuoles may then spread over the entire symplast compartment. In spongy cells the volume  
383 measured on micrograph changed very slightly during senescence compared to palisade cells and was in  
384 agreement with changes in signal intensity. The slight increase in the T2 of the fourth component was probably  
385 due to the same mechanisms in young leaves. The relatively small changes in LWW and vacuole volume in this  
386 tissue fits well with the spongy cell supposed role in the homeostasis of the leaf hydraulic status. Comparison of  
387 the relative intensity of the fourth and fifth components with the volume occupied by the two parenchyma (Fig.  
388 9) demonstrated the attribution of the fourth component to the spongy parenchyma vacuoles and of the fifth  
389 component to the palisade parenchyma and epidermis vacuoles.

390 Note that several authors have postulated that leaves lose water during senescence (McIntyre 1987;  
391 Zhang et al. 2012). It was shown by Zhang *et al.* (2012) that detached senescent *Arabidopsis* leaves lose water  
392 faster than mature leaves. However, there is no abscission of the leaf in *Arabidopsis*, unlike *B. napus*, and dead  
393 leaves of *Arabidopsis* remain fastened to the plant while drying. Zhang *et al.* (2012) also showed that the  
394 abscisic acid level is greater in senescent leaves, indicating that stomata are closed. A decrease in stomatal  
395 conductance with aging has already been highlighted in *B. napus* (Albert et al. 2012). Moreover, guard cells live  
396 longer than leaf parenchyma cells (Gotow et al. 1988) and are able to control water flux in leaves until the final  
397 senescence stage.

398 In the present study, the focus was on the T2 components associated with the vacuole. However, certain  
399 results suggested putative attribution of the other components of the NMR signal. The third signal component of  
400 young leaves (tag -3), relaxing at about 20 ms (Fig. 4) and representing about 15% of the water signal, has  
401 previously been linked to the plastids (Musse et al. 2013). Such attribution was supported here by comparison with  
402 the results of quantitative analysis of light micrographs, showing that plastids occupied about 13% of leaf tissue  
403 volume. However, while following senescence to its final stage, as performed here, the results showed that this  
404 component was still detected when plastids had disappeared on light micrographs. Consequently, the third T2  
405 component in senescing leaves probably originates, in addition to the plastidial water, from other proton pool  
406 relaxing at a similar relaxation rate. Several phenomena resulting from plast degradation could be at the origin of  
407 the formation of proton pools. One phenomenon may correspond to the proliferation of small vesicles such as  
408 senescent associated vacuoles (Avice and Etienne 2014; Otegui et al. 2005) and RuBisCo containing bodies  
409 (Hoertensteiner 2006). These lytic compartments have dimensions in the same order of magnitude as plastids. The  
410 second phenomenon may correspond to the proliferation and increase in size of the plastoglobuli (Brehelin et al.

411 2007) clearly observed on the micrographs (Fig. 3), resulting from the thylakoid membrane remobilization. Note  
1 412 that first and second order vascular tissues were successfully avoided by the sampling procedure; however, a few  
2 413 third and all fourth order vascular tissues were present in leaf samples used for NMR experiments. The  
3 414 contribution of these tissues to the NMR signals, and especially to the third component remains to be evaluated.  
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6 415 The second T2 component, relaxing at about 3 ms and representing about 2% of the water signal, has  
7 416 been attributed to apoplastic water and to a lesser extent to water inside starch granules (Musse et al. 2013). Our  
8 417 microscopy results (Supplementary data 4) are in agreement with the literature (Mohapatra et al. 2010), where it  
9 418 was suggested that an increase in cell wall elasticity due to its thinning can be linked to the decrease in intensity  
10 419 and T2 of this component just before it disappeared. Moreover the disappearance of the second component  
11 420 corresponded to the loss of the plast integrity, and starch granules within them.  
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### 16 421

### 17 422 *Changes in physiological indicators of C and N remobilization throughout leaf senescence*

### 18

19 423 Plasts contain 75% to 80% of total leaf nitrogen (Makino and Osmond 1991) and high amount of lipids  
20 424 (Thompson et al. 1998). They are thus the major source of nutrients remobilized during senescence. Plast  
21 425 dismantling, characterized by chlorophyll breakdown, is generally measured in terms of chlorophyll loss  
22 426 (BuchananWollaston 1997; Otegui et al. 2005). The chlorophyll content was shown to be constant in young and  
23 427 photosynthetically active leaves and to decrease slowly with ageing and plast dismantling (Ghosh et al. 2001).  
24 428 Our results (Fig. 1 A) are in accordance with these results and those of former studies (Inada et al. 1998; Lim et  
25 429 al. 2007; Otegui et al. 2005), demonstrating that the chlorophyll degradation is an early event in senescence.  
26 430 Chlorophyll content, as measured here, was a good indicator of the general plast status but did not make it  
27 431 possible to access information about specific tissues changes. This question was addressed through light  
28 432 microscopy. The light micrographs showed that plast degradation started with a decrease in volume and was  
29 433 followed by a reduction in number, in accordance with results obtained on darkened leaves (Ghosh et al. 2001;  
30 434 Inada et al. 1998; Keech et al. 2007). Despite the higher density of plasts observed in palisade parenchyma and  
31 435 the different functions attributed to leaf tissues, plast disappearance was concomitant in both parenchyma. This  
32 436 could indicate that the degradation mechanisms are similarly regulated.  
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43 437 Electron microscopy was shown to be complementary to light microscopy as it allowed the  
44 438 investigation of plast structure. Electron micrographs (Fig. 7) showed typical young chloroplast presenting well  
45 439 organized thylakoids and starch granules demonstrating plast functionality (Parthier 1988). Plastoglobuli, that  
46 440 are responsible for membrane replacement (Brehelin et al. 2007), were not numerous at this age. Gradual  
47 441 chloroplast shrinkage and transformation into gerontoplasts was clearly visible in our images (Fig. 7b) showing  
48 442 only a few membranes that were stacked and larger and more numerous plastoglobuli. The process described  
49 443 above is characterized by the disintegration and remobilization of thylakoid membranes through plastoglobuli  
50 444 (Thompson et al., 1998, Keech et al. 2007), leading to the accumulation of these plastoglobuli in the plast. The  
51 445 last stage of plast dismantling, corresponding to the oldest leaves studied (Fig. 7c), was characterized by large  
52 446 plastoglobuli without remaining thylakoids, surrounded by the plastidial envelope. As far as we are aware, this  
53 447 ultimate stage of plast dismantling has not been described in the literature to date, probably because plast  
54 448 evolution through senescence has mainly been studied on darkened leaves (Keech et al. 2007; Wada and Ishida  
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449 2009). Dark treatment is known to induce intracellular degradation more aggressively than natural ageing (Wada  
1 450 and Ishida 2009), and it probably enhanced the autophagy remobilization processes. Despite evidence of  
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3 451 autophagy of whole plants during senescence (Guiboileau et al. 2012; Wada and Ishida 2009), the presence of  
4 452 plants at that late stage therefore demonstrated that they were not all dismantled through autophagy. This may be  
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6 453 linked with the suboptimal nutrient remobilization observed in *Brassica napus* leaves during senescence.  
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8 454 Plasts contain an important part of the leaf carbon in the starch granules. Starch content (Diaz et al.  
9 455 2008; Wada and Ishida 2009) is another common parameter used to monitor senescence. Masclaux et al. (2000)  
10 456 proposed that for tobacco leaves studied according to leaf rank, the onset of decrease in starch content represents  
11 457 the source/sink transition. Their results also showed a decrease in dry matter associated with remobilization  
12 458 during senescence. The latter phenomenon was observed in the present study (Fig. 1b and d) and was at the  
13 459 origin of the decrease in the intensity of the first NMR signal component, demonstrating that NMR signal could  
14 460 be used as indicator of remobilization activity.  
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19 461 While the link between tissue-specific structural modifications and efficiency of nutrient remobilization  
20 462 remains unknown, it is clear that the senescence-associated processes of cell enlargement and tissue hydration  
21 463 occurring in palisade layer may have an impact on this efficiency. Even if the cell hydration seems not to have an  
22 464 impact on plast disappearance, with no difference observed in terms of plast density evolution between the two  
23 465 parenchyma layers, one may expect differences in term of cell metabolism between the two layers that remain to  
24 466 be investigated.  
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### 30 468 *Using parenchyma differentiation on NMR signals as a developmental marker*

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32 469 Investigation of leaf senescence requires knowledge of the developmental stage of the leaves studied.  
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34 470 This stage can be determined more or less accurately via parameters such as leaf rank or leaf age, chlorophyll  
35 471 content, gene expression, etc. During plant development, new leaves appear at the top of the plant and leaf  
36 472 senescence progresses from the bottom to the top of the canopy (Masclaux et al. 2000). It should be noted that  
37 473 the progression of senescence is acropetal (Avice and Etienne 2014) in the leaf limb and that this phenomenon  
38 474 was taken into account in the sampling procedure.  
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44 475 In crop plants, such as oilseed rape, wheat, rice and tobacco, leaf rank scale is commonly used to  
45 476 present physiological results (Mae and Ohira 1981; Masclaux et al. 2000). Presentation of the results according  
46 477 to leaf rank and measurement week showed that leaf rank is a good criterion for monitoring natural senescence.  
47 478 Another approach is to tag leaves according to the time of appearance and to follow their development. If the  
48 479 analysis method is destructive, different plants are necessary for such analysis. However, both approaches  
49 480 necessitate averaging values of leaves from different plants, and are susceptible to variability because leaves  
50 481 from different plants of the same leaf rank or time of appearance are not necessarily of the same physiological  
51 482 status, even from plants grown in controlled conditions.  
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57 483 Chlorophyll content is also commonly used to determine physiological status as it represents a simple,  
58 484 non-destructive method (Otegui et al. 2005; Zhang et al. 2012). However, this method cannot be used to  
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485 discriminate between young and mature leaves with the maximum of chlorophyll. Moreover, Gombert et al.  
1 486 (2006) demonstrated that chlorophyll content was not a precise parameter to determine physiological status of a  
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3 487 leaf. For instance, in the case of nitrogen deficiency, the same chlorophyll content was measured in leaves from  
4 488 stressed and control plants of different developmental status. Our results also showed that the method used for  
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6 489 measurement of chlorophyll content was not suitable for determining the senescence stage of very old leaves,  
7 490 characterized by the absence of plastids on micrographs but still with chlorophyll content of around 10% of  
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9 491 maximum (Fig. 5).

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11 492 Similarly, starch content seems to be overvalued in old leaves. Indeed, various authors have detected  
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13 493 small amounts of starch in old leaves where the photosynthesis apparatus has clearly been dismantled (Diaz et al.  
14 494 2008; Masclaux-Daubresse et al. 2008). Our results in old leaves (tag +5 and more) also showed a small amount  
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16 495 of starch whereas no plastids were visible on light microscopy.

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18 496 Another common approach to determine the physiological status of a leaf has been to use the expression  
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20 497 of two genes, *sag12* (*senescence associated gene 12*) that is unregulated during senescence and *cab* (*chlorophyll*  
21 498 *a/b binding protein*) that is downregulated during senescence (Gombert et al. 2006). The cross-over between in  
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23 499 the expression of these genes was proposed as the onset of senescence. While the method is helpful to determine  
24 500 a specific leaf status at the cross-over, it is less clear before and after this cross-over and the method remains  
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26 501 time consuming.

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28 502 Finally, the approach using individually darkened leaves (IDL) allows control of the beginning of  
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30 503 senescence and therefore better monitoring of plastid dismantling occurring in late senescence (Keech et al. 2007).  
31 504 However, it may induce intracellular degradation more aggressively than natural ageing (Wada and Ishida 2009).

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34 505 As described above, all the methods commonly used have specific defects. The main limitations are  
35 506 related to insufficient method accuracy and the limited sensitivity of the parameter selected in specific  
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37 507 developmental ranges. In contrast, NMR signals for oilseed rape leaves were found to be very sensitive to the  
38 508 developmental stage over a very wide period of time. The major modification of the NMR signal is the splitting  
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40 509 of the last T2 component of young leaves, reflecting differences in the pattern of evolution of palisade and  
41 510 spongy tissues. NMR measurement protocol was associated with signal analysis based on two different  
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43 511 approaches (MEM and Levenberg-Marquardt) providing in this way a robust method for split estimation. The  
44 512 split appeared to be an essential stage in leaf development, and its progression in the canopy (Fig. 3) was  
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46 513 constant (about one leaf rank per week). When using the split for tagging, the changes in the NMR signal  
47 514 throughout the entire development process became highly reproducible (Fig. 4). Physiological parameters  
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49 515 occurring according to the split scale (Fig. 5) validated this approach. Moreover, the split scale allowed accurate  
50 516 presentation of the results over a wide range of developmental stages.

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53 517 In view of the high reproducibility of the NMR signal shown in this study, it should be possible to use all the  
54 518 parameters in the NMR signal to identify the age of an individual leaf. As the method described is based on the  
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56 519 differences in the pattern of evolution of palisade and spongy tissues in *Brassica napus* under specific  
57 520 experimental conditions, the general character of the method should be further evaluated. Indeed, this would  
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59 521 require the acquisition of a NMR database for specific experimental conditions and other species, and would be



522 an important step in leaf characterization compared to current methods based on metabolic indicators  
1 523 (chlorophyll, starch content) that require measurement of kinetics.  
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## 4 524 **CONCLUSION**

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7 525 In this study, different patterns of evolution of the palisade and spongy parenchyma of *Brassica napus*  
8 526 leaves during development were revealed, associated with leaf thickening due to cell enlargement and tissue  
9 527 hydration. The NMR signal was shown to be sensitive to tissue modifications, and an original interpretation was  
10 528 therefore proposed taking into account both cellular compartmentalization and heterogeneity at the tissue level.  
11 529 The experimental design of the study made it possible to follow structural and physiological changes in leaf  
12 530 during natural senescence, up to its ultimate stage, where late stage of gerontoplasts was revealed. The link  
13 531 between tissue-specific structural modifications and the efficiency of nutrient remobilization remains unknown;  
14 532 however, the results highlighted the importance of considering the complexity of the tissue structure while  
15 533 studying leaf functioning. Finally, it was also shown that the structural changes can be used instead of long-time  
16 534 measurements of kinetics in order to monitor leaves according to their developmental status. In the current  
17 535 context of fertilization reductions and climatic changes, occurrence of nitrogen depletion and water deficit is  
18 536 expected to increase. One interesting outcome of the present study would therefore be to see how leaf structural  
19 537 modifications and nutrient remobilization during senescence are impacted by these abiotic stresses.  
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## 28 29 30 539 **CONFLIC OF INTEREST**

31  
32 540 The authors declare that they have no conflict of interest.  
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## 48 549 **LITERATURE CITED**

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2 **661 Fig. 1** Changes in chlorophyll and starch content, water content and dry weight during leaf development in  
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4 **662** oilseed rape. **a, c** the eighth rank leaf was followed during the eight-week measurement period. **b, d** all leaves  
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6 **663** from four plants were analyzed at the third week of the measurement period. The values correspond to averages  
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8 **664**  $\pm$  standard deviations of data collected from leaves of four individual plants

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13 **666 Fig. 2** Transverse relaxation time distribution (MEM) calculated from the CPMG signal for Brassica napus  
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15 **667** leaves. The dotted line represents results of the selected leaf (LR-8) obtained at four different times (weeks 1, 4,  
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17 **668** 6 and 8) of the eight-week measurement period. The solid line shows results of four leaves (LR-10, LR-7, LR-5,  
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19 **669** LR-3) from one plant at one measurement time (week 3)

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22 **670 Fig. 3** Leaf rank at which the longest T2 component observed in young leaves split into two components and  
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24 **671** maximum and minimum leaf ranks of the plant studied from each measurement week. Data shown are the  
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26 **672** averages (rounded to the whole number) of the four series

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31 **674 Fig. 4** NMR relaxation parameters of oilseed rape leaves according to the week report to the NMR split scale.  
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33 **675** Transverse relaxation times of the component 2 and 3 (**a**) and 4 and 5 (**b**) in ms and in relation to NMR split  
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35 **676** scale. **c** water distribution in the water-associated components (2-5) expressed as LWW where LWW<sub>i</sub> is specific  
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37 **677** leaf water weight of the i<sup>th</sup> signal component expressed in g m<sup>-2</sup>. Values correspond to average of eight to  
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39 **678** sixteen leaves

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44 **680 Fig. 5** Chlorophyll content and starch content of oilseed rape leaves expressed as a percentage of the maximum  
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46 **681** in relation to the NMR split scale. Values are an average of seven to twenty-four results  $\pm$  standard deviation

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49 **682**

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52 **683 Fig. 6** Light micrographs of cross sections of oilseed rape leaves at three typical developmental stages: young  
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54 **684** (tag -2) (**a**), mature (tag +2) (**b**) and senescent (**c**) (tag +4). UE: upper epidermis; PP: palisade parenchyma; SP:  
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56 **685** spongy parenchyma; LE: lower epidermis

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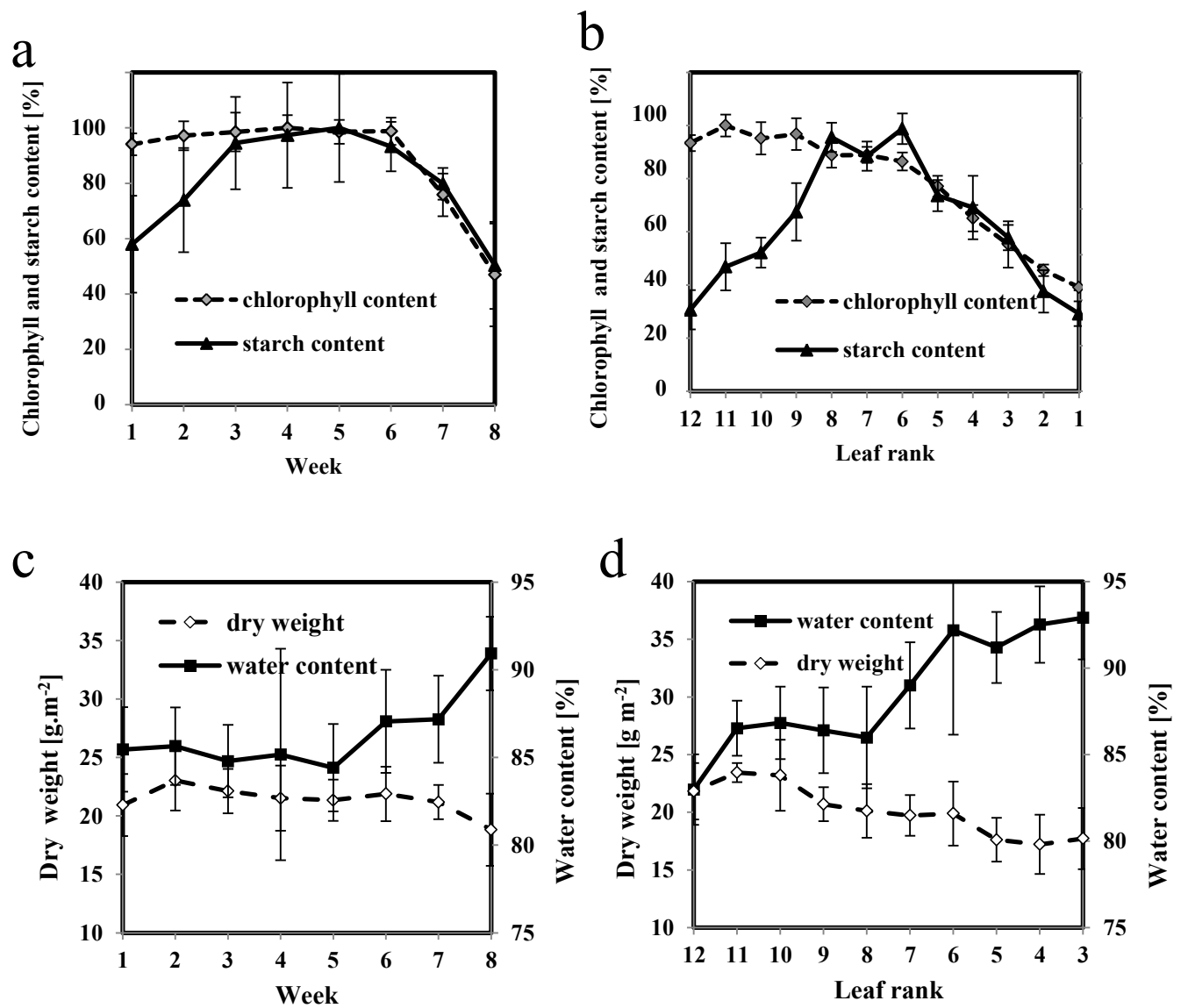
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686 **Fig. 7** Electron micrographs of plastids from oilseed rape leaves: **a** chloroplast from young (tag+2) and mature  
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2 687 leaves; **b** gerontoplast from a senescent leaf (tag+4) and **c** plastids in final developmental stage present only in the  
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4 688 oldest leaves (tag+5)

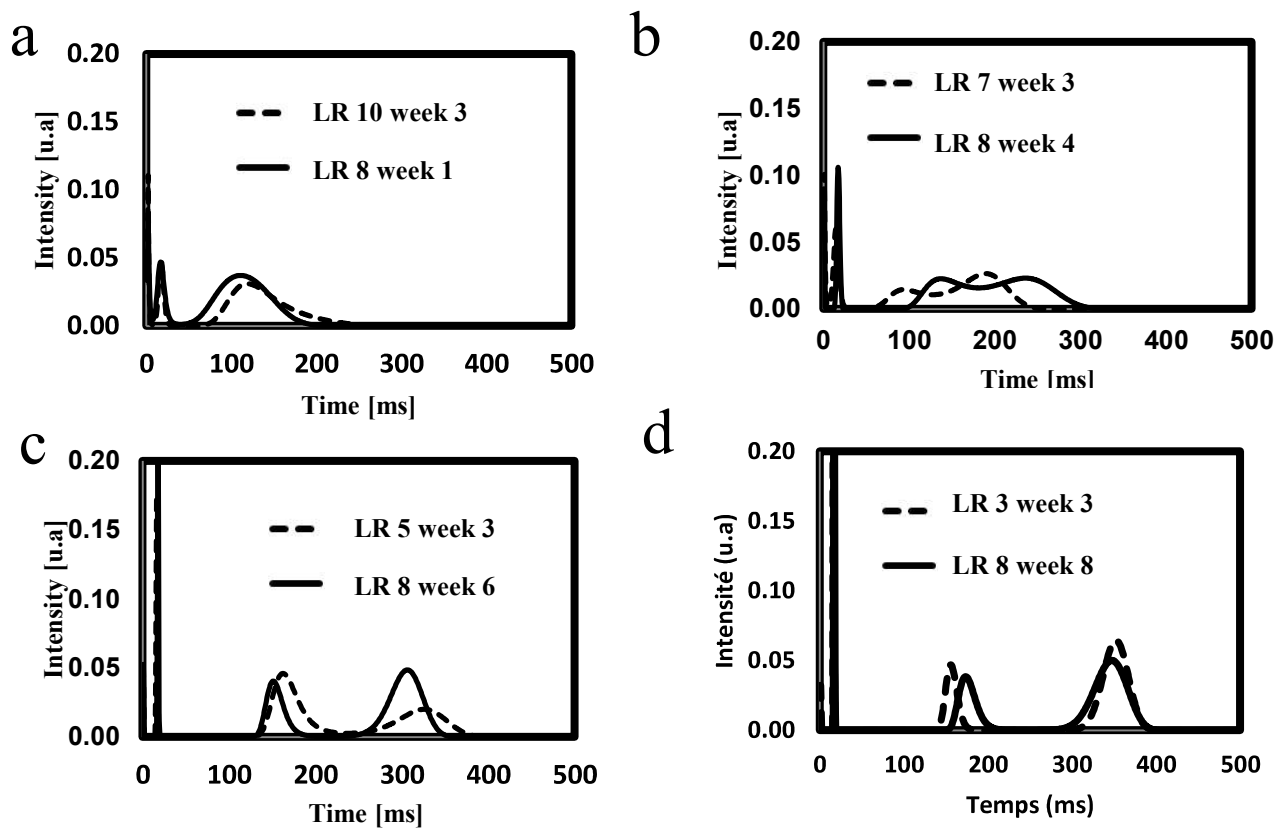
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10 690 **Fig. 8** Vacuole volume of the cells of different parenchyma of the oilseed rape leaf. Each measurement is an  
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12 691 average of at least 40 cells of 4 different images

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17 693 **Fig. 9** NMR signal intensity of the fourth (white triangle) and fifth components (white square) and both  
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19 694 components (white diamond) expressed as percentage of the total NMR signal intensity and in relation to the  
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21 695 split. Relative volume of spongy parenchyma (black triangle) and relative volume of palisade parenchyma and  
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23 696 epidermis (black square) measured on light micrographs of oilseed rape leaf cross-sections and presented in  
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25 697 relation to the split and expressed as percentage of the whole limb volume. Each point represents the average of  
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27 698 measurements from 4 to 10 images (from at least three different leaves)

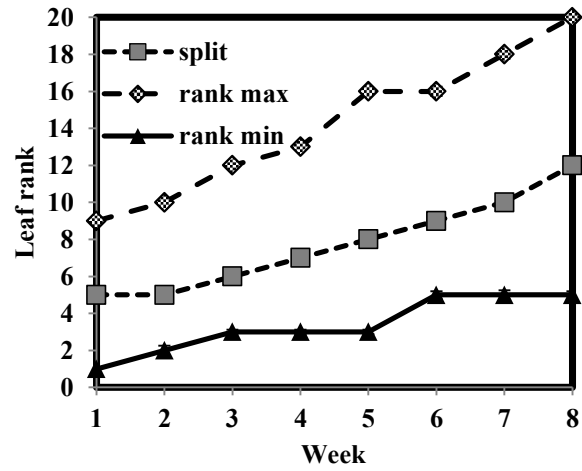
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**Fig. 1** Changes in chlorophyll and starch content, water content and dry weight during leaf development in oilseed rape. **a, c** the eighth rank leaf was followed during the eight-week measurement period. **b, d** all leaves from four plants were analyzed at the third week of the measurement period. The values correspond to averages  $\pm$  standard deviations of data collected from leaves of four individual plants



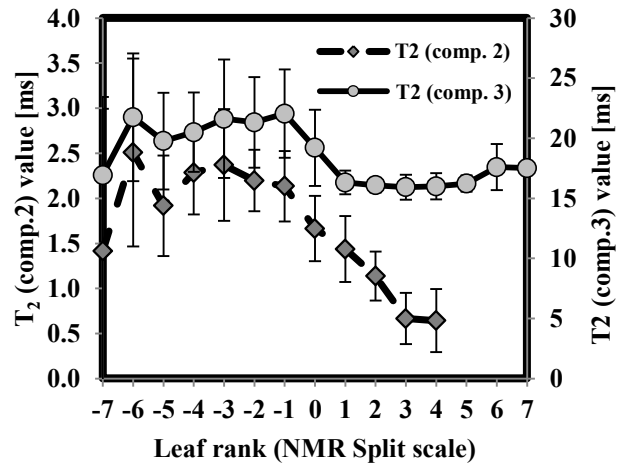
**Fig. 2** Transverse relaxation time distribution (MEM) calculated from the CPMG signal for *Brassica napus* leaves. The dotted line represents results of the selected leaf (LR-8) obtained at four different times (weeks 1, 4, 6 and 8) of the eight-week measurement period. The solid line shows results of four leaves (LR-10, LR-7, LR-5, LR-3) from one plant at one measurement time (week 3)



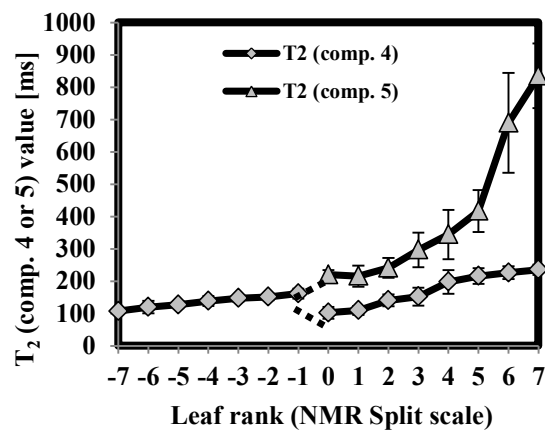
**Fig. 3** Leaf rank at which the longest T2 component observed in young leaves split into two components and maximum and minimum leaf ranks of the plant studied from each measurement week. Data shown are the averages (rounded to the whole number) of the four series



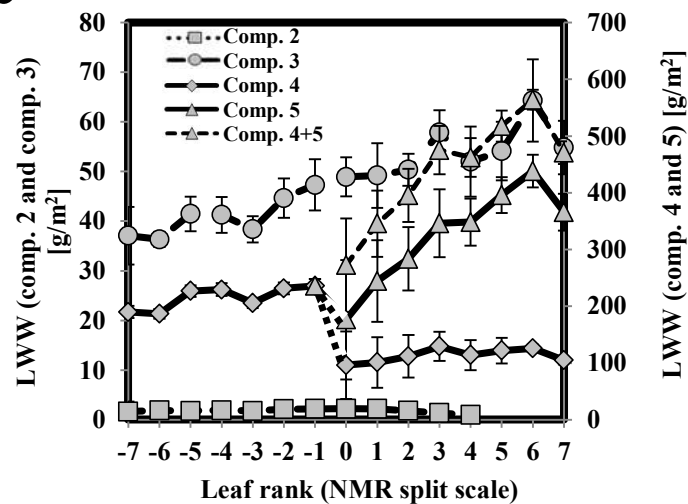
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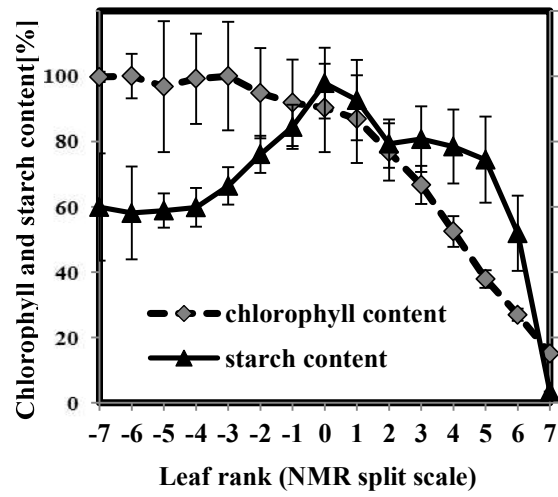
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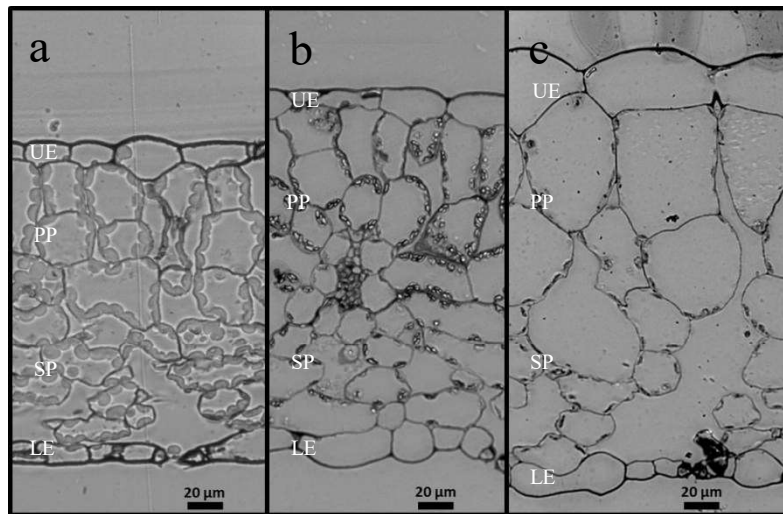
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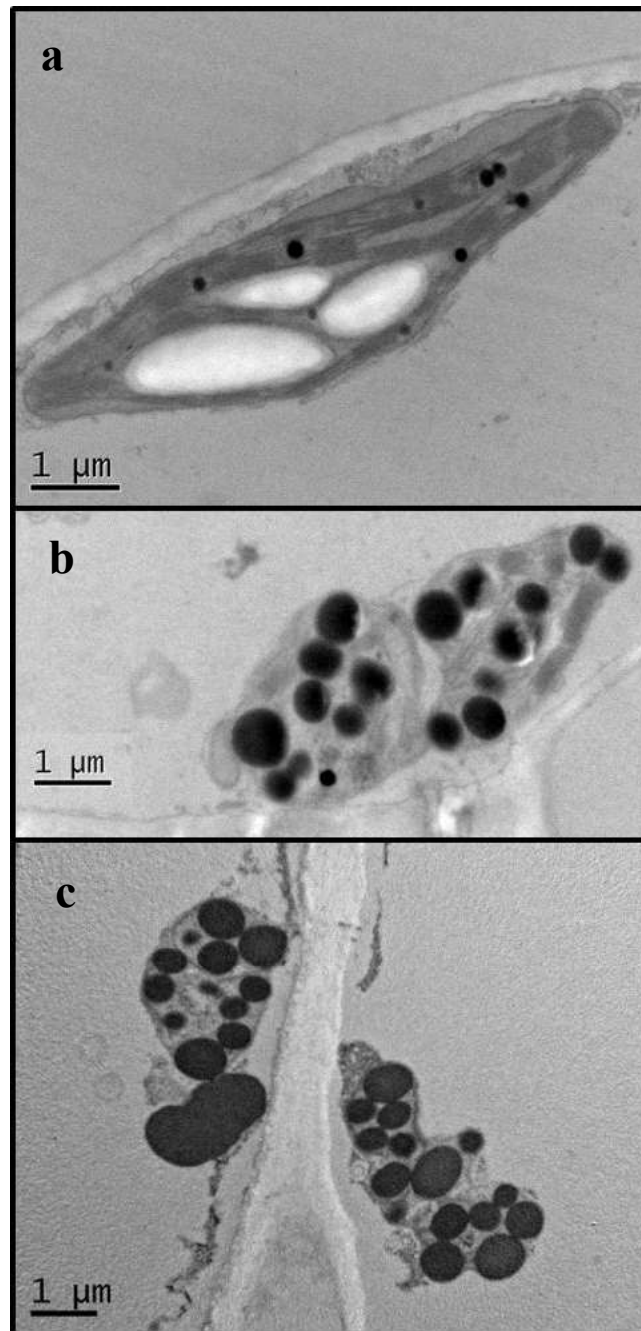
**Fig. 4** NMR relaxation parameters of oilseed rape leaves according to the week report to the NMR split scale. Transverse relaxation times of the component 2 and 3 (a) and 4 and 5 (b) in ms and in relation to NMR split scale. c water distribution in the water-associated components (2-5) expressed as LWW where LWWi is specific leaf water weight of the ith signal component expressed in  $\text{g m}^{-2}$ . Values correspond to average of eight to sixteen leaves



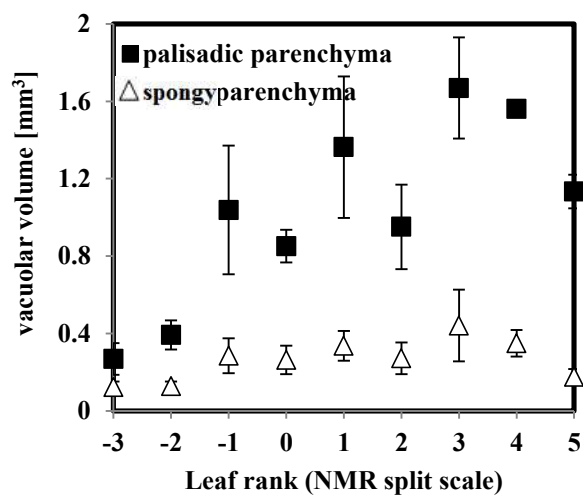
**Fig. 5** Chlorophyll content and starch content of oilseed rape leaves expressed as a percentage of the maximum in relation to the NMR split scale. Values are an average of seven to twenty-four results  $\pm$  standard deviation



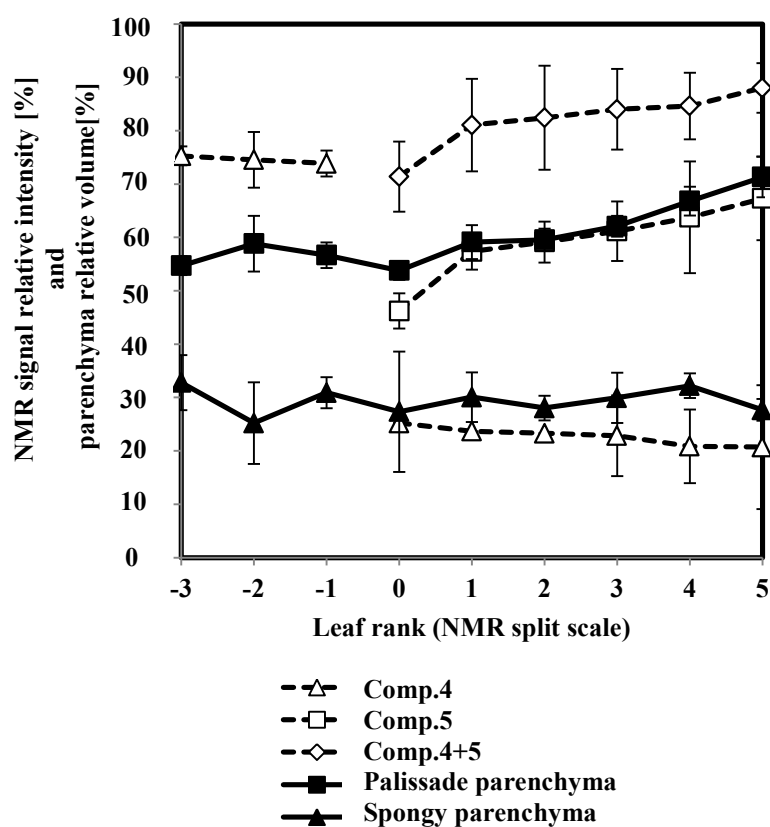
**Fig. 6** Light micrographs of cross sections of oilseed rape leaves at three typical developmental stages: young (tag -2) (**a**), mature (tag +2) (**b**) and senescent (**c**) (tag +4). UE: upper epidermis; PP: palisade parenchyma; SP: spongy parenchyma; LE: lower epidermis



**Fig. 7** Electron micrographs of plastids from oilseed rape leaves: **a** chloroplast from young (tag+2) and mature leaves; **b** gerontoplast from a senescent leaf (tag+4) and **c** plastids in final developmental stage present only in the oldest leaves (tag+5)



**Fig. 8** Vacuole volume of the cells of different parenchyma of the oilseed rape leaf. Each measurement is an average of at least 40 cells of 4 different images



**Fig. 9** NMR signal intensity of the fourth (white triangle) and fifth components (white square) and both components (white diamond) expressed as percentage of the total NMR signal intensity and in relation to the split. Relative volume of spongy parenchyma (black triangle) and relative volume of palisade parenchyma and epidermis (black square) measured on light micrographs of oilseed rape leaf cross-sections and presented in relation to the split and expressed as percentage of the whole limb volume. Each point represents the average of measurements from 4 to 10 images (from at least three different leaves)