Dry-matter content during extension of twigs, buds and leaves reflects hydraulic status related to earlywood vessel development in *Quercus pyrenaica* Willd.

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Abstract

A quantitative method was tested to describe crown phenophases in relation to water content and to secondary growth in ring-porous species, based on the hypothesis that new shoots require hydrated tissues to maintain the necessary turgor for extension, leading to a reduction in dry matter content (DMC).

We collected a three-year-old branch from 11 *Quercus pyrenaica* Willd. trees at 10-day intervals to estimate DMC of newly developing buds, leaves, and twigs, and processed two opposite stem microcores for xylogenesis. Branch phenophases and shoot length were recorded in the field.

The DMC of all organs decreased during crown development, with a minimum in early June, followed by a gradual increase up to initial values in late September. The shoot extension period concurred with the lowest DMC, but also with the beginning of earlywood maturation in the main stem, suggesting a high tissue hydration only when earlywood vessels become functional to fulfil enough water requirements for shoot and leaf extension.

These results confirm the usefulness of DMC to accurately quantify the phenology of primary growth from bud swelling up to full leaf extension, as a complement to qualitative methods. This variation in DMC appears to be linked to secondary growth as a result of earlywood vessel development.

Keywords: crown water status, phenology, ring-porous species, xylem, xylogenesis.

1. Introduction

Earlywood vessels of ring-porous species are considered to transport water only during the year of their formation (Greenidge, 1955, Zasada and Zahner, 1969, Chaney and Kozlowski, 1977, Ellmore and Ewers, 1986, Utsumi *et al.*, 1999, Umebayashi *et al.*, 2008), and therefore water consumption by crown development should depend on the renewal of the earlywood vessel network every spring. Over the last decades, some works focused on the analysis of the relationships between shoot and cambial phenology in ring-porous species, particularly on the association between earlywood vessel development and shoot phenology (Suzuki *et al.*, 1996, Sass-Klaassen et al., 2011, González-González et al., 2013, Takahashi et al., 2015, Pérez-de-Lis et al., 2016, Lavrič et al., 2017, Puchałka et al., 2017).

The conventional approach used to quantify secondary growth (i.e., wood formation) relies on xylogenetic studies involving the extraction of a microcore (Rossi *et al*., 2006) or cambial wounding (Gričar et al., 2005, Gričar and Čufar, 2008), which are in general performed from the main trunk at breast height. Histological methods allow an accurate monitoring the cell division process, and secondary cell wall deposition in order to sequence these events. In conifers, the number of tracheids is counted to establish the dynamics of secondary growth (Rossi et al., 2012), while in angiosperms the ongoing ring width is usually measured for this purpose, (Čufar et al., 2008, Michelot et al., 2012, Prislan et al., 2013, Pérez-de-Lis et al., 2017) although earlywood vessel area has been occasionally considered for ring-porous species (Alla et al., 2011, Sass-Klaassen et al., 2011, Pérez-de-Lis et al., 2016).

Primary growth is more complex to quantify due to the difficult access to the crown of large trees, and to the asynchrony often found among different branches. This problem

77 is usually overcome by estimating phenophases (e.g., swollen buds, bud break,..)
78 following visual observation at the naked eye, binoculars, or photographs (Haggerty and
79 Mazer, 2008; Vilhar et al., 2013). Other authors distinguished between flushing leaves
80 and successive leaves (Takahashi et al., 2015; Takahashi and Takahashi, 2016), even
81 with some attempts to quantify these estimations (González-González et al., 2013).
82 However, visual estimations account for a high degree of subjectivity, and can result in
83 some inaccuracies, especially at defining certain phenophases.

84 Quantitative approaches including bud size, leaf area index, and shoot elongation
85 measurements have been respectively used to monitor bud, leaf, and shoot development
86 (Cuny et al., 2012, Basler and Körner, 2014, Camarero et al., 2016, Lavrič et al., 2017),
87 but all of them fail to account for the whole primary growth phenophases. Palacio et al.
88 (2008) analysed seasonal changes in dry matter content (DMC) at full hydration (the
89 opposite to water content) in different leaf and stem cohorts of several species. Similar
90 high values of DMC were recorded for all species during dormancy, whilst lower values
91 and significant interspecies differences were observed during the vegetative period.
92 Although minimum DMC roughly coincided with the moment of maximum shoot
93 extension, their data were not precise enough to demonstrate the existence of a close
94 relationship between minimum DMC and maximum shoot extension. This relationship
95 can be expected, because plant organs require high amounts of water to maintain the
96 turgor pressure necessary for extension during the growing process (Bradford and
97 Hsiao, 1982). DMC has also been found to be strongly related to dry matter
98 concentration (dry mass per volume of plant organs), but volume estimation is more
99 difficult, particularly in very small organs (Shipley and Vu, 2002).

100 We hypothesize that the measurement of DMC throughout the growing period will
101 allow a quantitative description of shoot development, where low DMC peaks in shoot

parts (leaves and stems) will correspond to the peaks of shoot extension. Such hypothesis relies on the ontogenetic processes related to the onset of shoot extension (Davis and Mooney, 1986). Thus, one of the prerequisites for bud burst is the increase of hydration of bud and shoots (Bradford and Hsiao, 1982; De Fay et al., 2000). Expanding cells adjust their osmotic potential and cell wall elasticity to maintain adequate turgor pressure throughout the growth process (Boyer, 1988; Van Volkenburgh, 1999). Consequently, the mechanisms used by cells to maintain this pressure may increase the capacity of organs to gain water when they are at full turgidity, hence leading to a DMC reduction in leaves and twigs during shoot extension. Once cell division has ceased, dry matter accumulates in cells, causing an increase of DMC associated to a higher volumetric fraction of structure (Roderick et al., 1999). Functional xylem tissues provide water and osmolytes required for bud swelling and bud burst in deciduous species (Bonhomme et al., 2010), but the possible mutual interactions between the timing of xylogenesis and shoots DMC remain unexplored.

In order to test this hypothesis by establishing the relationships between primary and secondary growth, we selected the tree species *Quercus pyrenaica* Willd., which exhibits a short growing period, and a low phenological variation at the individual level in spring. Consequently, the aims of this study were: i) to verify whether the minimum DMC of crown organs in *Q. pyrenaica* is coupled to the maximum rate of shoot extension, and ii) to quantify variations in DMC of crown organs at different phenological phases, and their relationships with earlywood vessel formation in the stem and crown development.

2 Material and Methods

2. 1 Study site and tree selection

126 The study was carried out in the northwestern Iberian Peninsula (43º 1'43.57'' N, 7º 31'
127 5.19'' W; 500 m a.s.l.), where a dominant woodland of naturally regenerated *Q.*
128 *pyrenaica* mixed with *Q.robur* L. on a siliceous soil forms a transitional forest from
129 Mediterranean vegetation to Atlantic vegetation. Average annual rainfall is ca. 900 mm,
130 mainly falling between October and February, mean annual temperature is 11.8 ºC, with
131 18.3 ºC in the hottest month (July), and 5.7 ºC in the coldest (January) (period 2006-
132 2016; http://www.meteogalicia.es).

133 The selected species *Q. pyrenaica* covers a wide distribution area in the Iberian
134 Peninsula, being fairly common in all inland regions, especially on siliceous soils in the
135 central and western hills between 400 and 1,600 m a.s.l. (Jiménez et al., 1997). The
136 vegetative period mainly extends concomitantly with summer drought, but the strength
137 of the root system allows the maintenance of optimal soil conditions for water retention
138 and protection against erosion (Montserrat, 2008).

139 Wood micro-cores and twigs were collected at each sampling date from March to
140 September 2013 (day of the year 66-266) from 11 isolated trees to prevent competition
141 effects. We followed a 10-day interval in spring during earlywood vessel formation and
142 crown development; and a 20-day interval in summer; in this way, we accounted for the
143 whole dynamics of primary and secondary growth without inducing crown resprouting.
144 The number of trees sampled was twice the minimum sampling size recommended by
145 Cornelissen et al. (2003) for phenological traits, and the heterogeneous size of the
146 selected trees ensured that samples were representative of the study area (Table 1).
147 Trees had a mean diameter of 24.7 ± 4 cm at breast height, with individual values
148 ranging from 19.5 to 32.5 cm, and average height of 8.6 ± 3 m ranging from 6.5 to 16.0
149 m. The lower canopy limit was at 1.6 to 4.8 m above ground, allowing accessibility for
150 crown sampling.

151 **2. 2 Primary and secondary growth measurements**

152 **2.2.1 Dry matter content**

153 We collected one 3-year old branch at each sampling date from each of the 11 selected
154 trees, at sunny orientation below half-crown, always avoiding successive cuttings on the
155 same main branch. No resprouting signals were observed near the cutting wounds.
156 Branches were labelled, bagged, and expeditiously transported to the lab after the end of
157 sampling to be immediately subjected to maximum hydration following Garnier et al.
158 (2001). We photographed all branches upon arrival with a digital camera for the visual
159 documentation of the phenophases. Maximum hydration was achieved by immersing
160 the first 3–4 cm of the stem in distilled water, after having cut off underwater the three
161 proximal centimeters of the stem. During hydration, branches were capped by a wet
162 plastic bag, and kept at 5 °C for 24 h.

163 We measured the full hydration weights from the apical buds, leaves, and twigs of each
164 hydrated branch. When present, apical and top sub-apical buds, leaves, current-year, 1-
165 and 2-year-old cohorts were measured separately. Subsequently, samples were oven-
166 dried at 60 °C for 48 h, and dry weights recorded. All weighing was conducted to the
167 nearest 0.1 mg with a scale (Denver instrument, AA-160, New York, USA). The DMC
168 (mg g^{-1}) of leaves and stems was calculated as the ratio between dry (W_d) and fresh
169 weight (W_f) at full hydration, assuming that the weight difference was due to water:

170 $DMC = W_d/W_f$

171 On average, 6.0 samples of current twigs, 5.0 of one-year-old twigs and 2.5 of two-year-
172 old twigs were collected from each tree and sampling date (Table 2), which is about
173 two-fold the sampling size per tree recommended by Cornelissen et al. (2003).

2. 2. 2 Shoot extension

Current-year twigs were measured before drying in order to assess absolute shoot extension rate (SER). This comprised the distance from the insertion point on the proximal scale scar to the tangent line between the apices of the most apical green leaf. Shoot extension rate was calculated for each sampling date as:

$$SER = (L_n - L_{(n-1)})/T$$

where L_n (mm) is the mean shoot length of twigs per tree from a sampling date n, $L_{(n-1)}$ (mm) the mean shoot length of each individual in the previous date ($n - 1$), and T the days elapsed between dates ($n - 1$) and n.

2. 2. 3 Leaf phenological observations

Leaf phenology of the whole crown was recorded for each individual tree during sampling. Observations were made by screening buds of the main shoots from the top to the bottom of the crown using binoculars (10 × magnification), and were expressed as a particular day of year (DOY).

Six phenophases were considered (Fig. 1), namely a) bud dormancy, b) swollen buds, c) bud burst, d) leaf unfolding, e) appearance of small leaves, and f) full extension. Bud dormancy corresponds to the overwintering stage, while bud swelling was identified by the apparent separation of buds from the stem, together with the exposition of areas of lighter coloured tissue as a result of the initial extension of cataphylls with the separation of scales. Bud burst was characterized by green-coloured expanded buds with no unfolded leaves, and leaf unfolding ended as soon as the leaf blade was clearly visible, but not the petiole. Appearance of small leaves was defined as the moment when at least one leaf was completely out of the bud and current year twigs and petiole

could be visually appreciated; and full extension was recorded when leaves attained at least the 50% of their final size. The period of leaf extension spanned from the presence of turgid small leaves (less than the 50% of their final size) until their full extension.

The proportion of each phenophase in the whole canopy of each selected tree was estimated visually. In order to establish the duration of each phenophase within the population, we averaged its proportion considering the whole population of trees (Fig. 2a).

2. 2. 4 Monitoring of xylogenesis

Two microcores of 2-mm diameter were taken using a Trephor device from the northern and southern sides of each tree at 1.3 m above the ground at each sampling day (Rossi et al., 2006). This allowed us to account for the variation in vessel formation between opposite sides, and also to guarantee the presence of enough vessels to correctly identify each cambial phenophase. We took samples on the main stem because vessel formation progresses basipetally, and consequently the presence of mature vessels at this level assures the maturation of the complete vascular path up to the leaves (Aloni, 2013). After extraction, microcores were placed in Eppendorf microtubes with a 50% ethanol solution and stored at 5 °C until processing.

Wood samples were dehydrated by successive immersions in ethanol and xylene, and embedded in paraffin using a tissue processor (Leica TP1020, Wetzlar, Germany). Thereafter, cross-sections of 8–10 µm thickness were obtained from each paraffin block with a manual rotary microtome (Leica RM2125 RTS, Wetzlar, Germany) and placed on microscope slides. After two immersions in xylene for 5-min each to remove the residual paraffin, cross-sections were rehydrated and stained in two consecutive solutions: safranin for 10 minutes and fast green FCF in ethanol (80%) for 30 s, (Cutler

et al., 2008). Afterwards, they were newly immersed in xylene to remove water residues, and permanently fixed with Eukitt® resin (O. Kindler GmbH, Reiburg, Germany).

Ring-width measurements and cambial observations of the microcore cross-sections were performed on images taken with a digital camera (Canon EOS 600D, Tokyo, Japan), coupled to a transmitted light microscope (Olympus BX40, Tokyo, Japan), using a white light polarizing filter (40 × magnification). The width of cell expansion and maturation zones was measured on images along three radial lines. We determined each phenological phase of vascular cambial activity, including: onset of earlywood vessel enlargement, onset of earlywood vessel maturation (Fig. 1c'), cessation of earlywood vessel enlargement (Fig. 1f '), and cessation of cell expansion. The onset of earlywood vessel enlargement was considered when the most recent ring contained at least one enlarging vessel, whereas the onset of earlywood vessel maturation was defined when secondary cell wall deposition in vessels was detected as birefringence under the polarizing light; cessation of earlywood formation occurred when earlywood vessel enlargement was no longer detected, but all expanding cells belonged to latewood; and cessation of cell expansion was defined as no more expanding cells were detected, although lignification could be still ongoing. We attributed the corresponding DOY to each cambial phenophase.

2. 2. 5 Statistical analyses

Raw data of DMC did not follow a normal distribution. Therefore, relationships among the DMC of buds, leaves, and stems of the current and two preceding years were explored using Spearman's correlation coefficient, which were separately performed for each organ, with data from each sampling day and individual tree.

In order to detect the date of significant variation in DMC on the time course evolution of organs, we applied a linear mixed model analysis with time as fixed factor and tree as random factor. The same analysis was used to characterize leaf DMC during leaf phenological phases, with phenological events as fixed factor and tree as random factor. In both cases, residuals followed a normal distribution according to the Shapiro-Wilks test. Analyses were performed with the R statistical software (R Core Team, 2014) using the 'nlme' package (Pinheiro et al., 2016) and "multcomp" package was used to separate least square means.

As previously reported for conifers (Rathgeber et al., 2011) and angiosperms (Pérez-de-Lis et al., 2017), the absence/presence of visually-recorded xylem phenophases were expressed as binary data, and a logistic regression used in order to compute the following four critical dates: beginning of earlywood vessel enlargement (beEW) and beginning of maturation (bmEW), cessation of earlywood vessels enlargement (ceEW), and cessation of ring width expansion (ceRW). The durations of the phases of earlywood with mature vessels (dEW = ceEW - bmEW) and ring in cell extension (deRW = ceRW - beEW) corresponded to the time span between the onset and cessation of these phases. All phases were also compared between northern and southern sides using a Student's *t*-test. A logistic regression between earlywood phenophases and DMC of buds and leaves was computed to test the ontogeny relations removing 'time' as a factor.

The duration of primary growth phenophases in the whole crown was defined as the time elapsed from the first recording of each event in one or several branches to their appraisal in 100 % of the crown. A logistic regression was also chosen to establish the DOY of each crown phenophase being active in 50% of the tree population, using the first recording and appraisal in 100 % of the crown. The standard deviation of each day

expressed as DOY was computed by taking the 2.5% and 97.5% probabilities (Rathgeber et al., 2011).

3 Results

3. 1 Crown phenology

The visual appraisal of crown phenology showed overlapping shoot phases during a crown development period of 90 days, from the swollen bud phenophase in early April to the complete leaf extension at the end of June (Fig. 2a). Bud burst started between the end of April and early May (117 ± 5.7 DOY for first recording and 128 ± 14 DOY for 100%) and was followed by leaf unfolding in the first fortnight of May (130 ± 14.9 DOY for first recording and 133 ± 10.0 DOY for 100%; Table 3a). Leaf extension took place in the second fortnight of May for first recording of the crown (from 137 ± 19.3 to 154 ± 12.1 DOY) and from the beginning of June to three days before the summer solstice for 100% of the crown (from 150 ± 1.9 to 172 ± 1.6 DOY), coinciding with the maximum shoot extension rate (SER) (Fig. 2b). The time elapsed between the first recording and 100% of the crown was 11 days for bud burst, four days for leaf unfolding, and more than 21 days for leaf extension (Table 3a).

3. 2 Relationship between shoot extension rate and DMC of buds, leaves, and twigs

DMC in leaves, buds, and twigs showed a common decreasing pattern, reaching a minimum in the first half of June (150–162 DOY), followed by a gradual increase until the end of September (Fig. 2c). Maximum SER concurring with the lowest organ DMC suggested the hypothesis of high tissue hydration for growth purposes. In addition, time course of DMC variation of individual trees during the sampling period was highly homogeneous (Fig. 3).

SER was negatively correlated with DMC for all organs (Table 4), maximized for current twigs (r=–0.590, $P \leq 0.001$), and minimized for 2-year-old twigs (r = –0.284 $P \leq 0.023$) due to the lower range of variation in DMC (Table 5). The correlation among DMC of organs ranged from 0.924 to 0.516 ($P < 0.001$ in all cases), which confirms that the change in DMC had a similar pattern for all parts of the crown measured.

Mean values of DMC (Table 5) had no clear difference for 2-year twigs among sampling dates (DOY), which is in agreement with the lower proportion of growing tissue; whereas buds and 1-year old twigs showed a significant variation ($P < 0.05$) in hydration, which corresponds to bud swelling and earlywood vessel enlargement. This variation in DMC allows a more accurate definition of the onset of crown development than visual methods. Furthermore, significant ($P < 0.05$) increase in DMC of current year twigs and leaves allows identifying the end of shoot elongation.

The variation in DMC of crown organs through time allowed quantifying the visual perception of crown phenology. Shoot phenophases (Table 6) showed low and similar values of DMC (278.9 to 270.9 ± 13.2 mg g^{-1}) from leaf unfolding to leaf size > 50%. DMC was maximal during bud dormancy (540.1 ± 13.8 mg g^{-1}; $P < 0.05$) and decreased significantly at bud swelling (425.7 ± 13.2 mg g^{-1}; $P < 0.05$) and further at bud burst (346.9 ± 13.8 mg g^{-1}; $P < 0.05$) due to the onset of primordial extension inside buds. In contrast, DMC of leaves increased significantly in early July (DMC 422.3 ± 13.2 mg g^{-1}; $P < 0.05$) dating the appearance of a new critical event inappreciable by binoculars or leaf area index measurements, probably the hardening of leaves associated to biomass accumulation and the increased volumetric fraction of structure (Roderick et al., 1999).

3. 3 Xylogenesis

316 Crucial events of wood formation did not differ significantly ($P > 0.05$) between north
317 and south side when logistic curves were compared (Table 3b). Therefore, we averaged
318 data from both sides, and estimated the duration of ring-width increment as 162 ± 21.8
319 days spanning from early April (94 ± 21.8 DOY) to the end of September (256 ± 3.9
320 DOY). During this period, cambial activity showed a bimodal pattern (Fig. 2e) with two
321 peaks in ring-width increment in spring (early May; 129 DOY) and summer (early July;
322 182 DOY). The first one corresponded to earlywood vessel formation, and was lower
323 than the summer peak of latewood, while the decreasing rate in June (150-160 DOY)
324 pointed out the lower rate before the transition from earlywood to latewood formation.

325 Earlywood maturation spanned 61 ± 15.7 days (Fig. 2d; Table 3b) for 50% of the trees,
326 with the first mature vessels at the beginning of May (121 ± 15.3 DOY), and earlywood
327 vessel enlargement finished at the end of June (182 ± 3.7 DOY). In this period, DMC
328 values dropped below 410 mg g^{-1} in buds and leaves, corresponding to the earlywood
329 development period (Fig. 3, 4).

330 Significant variation in DMC of buds (Table 5) took place at the end of April (119
331 DOY; $P < 0.05$) pointing out that bud swelling had already taken place, which
332 coincided with the beginning of earlywood maturation (Table 3b; 121 ± 15.3 DOY; $P <$
333 0.05). During leaf extension, the significant variation in DMC of leaves and current
334 twigs was detected at the first day of July (182 DOY; $P < 0.05$), at the same time as
335 cessation of earlywood formation.

336 For all trees, the decline in earlywood enlargement (Fig. 2e), i.e., when earlywood
337 maturation had already started (Fig. 2d,f), coincided with the period of leaf extension
338 (150−170 DOY; Fig. 2a), the lowest DMC of leaves (Fig. 2c), and maximum SER (Fig.
339 2b).

4 Discussion

Our results on *Q. pyrenaica* show significant differences in DMC between dormant and swollen buds, as well as minimum values of DMC from bud burst to full extension of leaves. In addition, shoot extension rate (SER) was found to be negatively related to DMC of leaves, and current and preceding year twigs, while the DMC of buds was positively related to that of 1 and 2-year old twigs. These findings are in agreement with seasonal changes in water content of twigs/leaf tissues reported by Davis and Mooney (1986) and (Tognetti *et al*., 2000) for several Mediterranean woody species, recording increments in water saturated weight/dry weight ratio during spring growth. This was confirmed by Palacio *et al*. (2008), who also established quantitative relationships between SER and DMC of leaves and twigs.

The sequential changes in DMC allowed us to obtain a more objective dating for the timing of primary growth than the visual inspection by binoculars. The timing of reduction in bud DMC facilitated the monitoring of bud swelling, whereas the increase in leaf DMC indicated the hardening of leaves, which is difficult to identify visually in the practice. This explains why most studies record late phenophases, such as bud burst or leaf unfolding, in order to assess the onset of shoot growth. In fact, shoot growth is constituted by two processes, namely the initiation of primordia by the apical meristem, and the extension of these primordia into fully developed organs (Champagnat et al., 1986). The first of them (organogenesis) occurs inside the bud in the vegetative period of the year previous to budburst (Alla et al., 2013). After winter, organogenesis restarts at the onset of budburst, when alteration in cell division at the shoot apical meristem leads to leaf initiation (Sinha, 1999).

363 Changes in DMC of buds in early spring may be associated with the extension of
364 meristems, which has been attributed to an ontogenic process involving increased
365 hydration (Bradford and Hsiao, 1982; De Fay et al., 2000). Although the early phase of
366 bud swelling is often a part of observation protocols, its precise onset is very difficult to
367 ascertain over multiple buds at regular intervals, especially in the field.

368 Expanding cells of crown organs adjust their osmotic potential and cell wall elasticity to
369 maintain adequate turgor pressure throughout the growth process (Bradford and Hsiao,
370 1982; De Fay et al., 2000). The mechanisms used by cells to maintain this pressure may
371 increase the capacity of organs to gain water when set at full turgidity, hence leading to
372 reduced leaf and stem DMC (Palacio et al., 2008). Cessation of this extension, in our
373 case occurs 52 days after bud burst, and is concomitant with dry matter accumulated in
374 leaf cells. This contrasts with the 21-day period of leaf unfolding noted for *Q.*
375 *pubescens* by Lavrič et al., (2017) after monitoring leaf area index. A part of this
376 accumulation may come as of non-structural carbohydrates, following the increase of
377 net photosynthetic rates, once respiratory demands associated with growth decrease, and
378 leaves become net sources of carbon (Palacio et al., 2008). For *Q. robur*, Morecroft et
379 al. (2003) showed that development of full photosynthetic capacity took place between
380 approximately 50-70 days after bud break. This result is also consistent with the
381 carbohydrate accumulation recently recorded in stems of *Q. pyrenaica* in summer
382 (Pérez-de-Lis et al., 2017).

383 On average, the phenophases showing the largest increases in water content (the
384 opposite to DMC) were leaf unfolding and leaf extension, with values up to 72%. Bud
385 swelling and budburst were characterized by a lower hydration (57% and 65%
386 respectively), in contrast to only 46% in dormant buds. These low values of hydration in
387 buds are due to the presence of bud scales, which are only dry protective organs. Such

388 increase in the water content of organs need to be supported by either water stored in
389 living cells, (latewood) vessels and vasicentric tracheids from previous years or
390 earlywood vessels from the current year. Therefore, quantification of the water content
391 in twigs along with monitoring of earlywood formation in the stem may be crucial to
392 characterize the progression of crown development in ring-porous hardwood species.

393 Previous works in deciduous oaks highlighted that some earlywood vessels can be
394 already enlarging at the time of leaf unfolding (Suzuki et al., 1996, Sass-Klaassen et al.,
395 2011, Michelot et al., 2012, Puchałka et al., 2017). According to González-González et
396 al. (2013) and Pérez-de-Lis et al. (2016), earlywood vessel enlargement in the stems of
397 *Q. pyrenaica* takes place before bud burst, while secondary wall deposition is initiated
398 at the moment of bud burst. This may indicate that new vessels are ready for water
399 transport during leaf development, as evidenced the increasing sap flow recorded upon
400 budburst in *Q. pubescens* (Lavrič et al., 2017). On the other hand, functionality of
401 vessels has been defined by observing the presence of perforations in longitudinal thin
402 sections of *Fraxinus excelsior* L. (Atkinson and Denne, 1988), and *Quercus serrata*
403 Murray and *Robinia pseudoacacia* L. (Kudo et al., 2015). These authors found that
404 perforations in the first earlywood vessels were completed along the entire stem when
405 small leaves were visible to the naked eye. Kitin and Funada (2016) concluded that
406 water requirements of early leaves must be fulfilled by latewood vessels formed in
407 previous years. Moreover, Basler and Körner (2014) detected bud swelling and bud
408 burst in cuttings of several species (including *Q. petraea*) collected in late winter, which
409 entails that these phenophases may be attained without the presence of newly formed
410 earlywood vessels.

411 In view of these evidences, our results confirm that new earlywood vessels cannot
412 contribute to bud burst, suggesting that water supply should come from already existing

living cells and previous year latewood vessels and tracheids. Leaf unfolding is closely coupled with significant changes in DMC of one-year-old twigs, probably associated to the increased hydraulic conductance and new tissues formation, which appears to indicate that new vessels are functional within a few days after the onset of secondary wall deposition.

The difficulties found when defining the cessation of full leaf development may be sorted out by DMC measurements as an index of the cessation of water demand for shoot extension. Low values of DMC in the crown last up to the second fortnight of June, c. 40 days after the maturation of the first new vessels, and coincide with the cessation of earlywood formation. In line with previous studies (Lavrič et al., 2017), our results consequently reveal the required functionality of earlywood vessels to attend the high water demand for shoot and leaf extension to complete the development of the crown. In turn, earlywood vessel development would also influence shoot extension in subsequent years, as the number of leaf primordia in dormant buds appears to be positively related to xylem conductance and vessel size (Cochard et al, 2005).

Consequently, our study highlights the sequence of earlywood development in relation to primary growth events as quantified by the water content of branch organs (Fig. 5). Enlargement of earlywood vessels and bud swelling are concomitant events, suggesting that water demanded by developing buds is supplied by latewood vessels, tracheids or living cells, since new vessels did not reach their final size until bud burst. This is followed by leaf unfolding, which coincides with the hydration of 1-year old twigs, probably because the newly formed vessel network is already able to provide enough water, and thus attend the demand for the maximum rate of shoot elongation.

5. Conclusions

To our knowledge, we show for the first time that determining crown organ DMC is a powerful tool to quantify primary growth in trees from bud swelling up to full leaf extension, which are the most difficult phenophases to ascertain with visual inspection by binoculars. This method constitutes a simple tool for understanding the dynamics of tissue hydration during crown development, and allows monitoring the water content of buds, twigs, and leaves in relation to secondary growth under field conditions, providing that the branches are easily accessible. Furthermore, the results show that new earlywood vessels need to be functional to fulfil water requirements for shoot extension, but not for bud burst. We consequently propose an objective classification of bud and leaf phenophases according to their DMC, which avoids the subjectivity of the observer.

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626 **Tables**

627 **Table 1** Characteristics of *Q. pyrenaica* trees selected for the study. Diameter at breast
628 height (*DBH*), total height (*H*), stem height below the crown (*SBC*) and crown diameter
629 (*CD*). Average and standard error are shown at the bottom.

Tree (*n*)	DBH (*cm*)	H (*m*)	SBC (*m*)	CD (*m*)
1	25.8	16	4.8	5.9
2	19.5	6.4	3	6.75
3	22.4	8	4	7.75
4	23	7.2	3	6.25
5	24.2	7.2	3.2	7.05
6	30	12	4	5.85
7	27.2	6.4	3.2	5.85
8	20.3	8	2.4	5.9
9	24.2	5.6	1.6	5.45
10	23.1	6.4	1.6	6
11	32.5	11.2	3.2	6.8
Average ± sd	24.7 ± 4	8.6 ± 3	3 ± 1	6.3 ± 0.7

630

631 **Table 2** Average number of samples collected per *Q. pyrenaica* tree over the 12
632 sampling days. Data provided are the tree identification and number of: current year
633 twigs that were present in the last six dates (*Twigs*), one-year-old twigs (*Twigs - 1*), two-
634 year-old twigs (Twigs - 2), *Buds*, *Small leaves* (those <50% of maximum size; collected
635 during two dates), *Large leaves* (>50% of maximum size; collected in the remaining ten
636 dates), and *cambium* samples. Total sample size is shown at the bottom.

	Sample size						
Tree	Twigs	Twigs - 1	Twigs - 2	Buds	Small leaves	Large leaves	Cambium
1	5.4±1.1	4.8±2.2	2.1± 1.4	42.7±21.9	55±0.0	5.5±0.5	2
2	7.0±1.5	5.5±1.3	2.2± 1.2	37.5±3.4	40±28.3	6.0±1.4	2
3	5.8±2.3	4.9±1.7	2.0± 0.9	31.3±16.1	30±14.1	5.5±1.0	2
4	5.3±1.5	5.0±3.2	2.2± 1.4	32.6±9.2	27.5±10.6	5.5±1.0	2
5	6.0±1.5	4.6±1.7	2.8± 1.5	40.0±22.5	28±2.8	5.75±0.5	2
6	6.3±1.8	5.3±1.3	2.6± 1.1	37.3±25.7	20±0.0	5.5±1.0	2
7	5.2±1.1	4.5±2.4	2.5± 1.4	32.1±16.7	20±0.0	4.5±1.2	2
8	6.0±0.7	5.1±1.9	3.4± 1.3	28.5±13.4	20±0.0	6.5±1.2	2
9	6.3±2.1	4.5±2.6	2.2± 1.0	39.1±14.8	21.5±6.4	6.5±1.7	2
10	7.0±2.4	5.0±2.2	2.5± 1.0	46.1±14.5	56.5±4.9	6.5±0.5	2
11	5.5±2.0	5.2±3.1	2.7± 1.6	27.5±17.4	19.5±4.9	6.0±2.0	2
Total samples	379	658	325	2474	581	255	264

637

638 **Table 3** Critical dates indicated as day of year (DOY) for primary and secondary
639 growth of *Q. pyrenaica* computed by logistic regression. Phases for primary growth are:
640 computed critical days of bud swelling, bud burst, leaf unfolding, less than 50 % and
641 more than 50% of the leaf size for first recording and 100% of crown of each
642 phenophase, and the time elapsed to complete each phase. Phases for secondary growth
643 are: computed critical days for the beginning of earlywood enlargement (beEW) and
644 maturation (bmEW), cessation of earlywood enlargement (ceEW), cessation of ring-
645 width cell expansion (ceRW), duration of earlywood with mature vessels (dEW) and
646 ring-width cell extension (deRW) in the north (N) and south sides (S), as well as the
647 average (S+N).

648 **(a)**

Primary growth phenology

Crown onset	Bud swelling	Bud burst	Leaf unfolding	< 50% leaf size	> 50% leaf size
First recording	80±2.6	117±5.7	130± 14.9	137±19.3	150±1.9
100% of crown	113±1.1	128±14.4	133±10	154±12.1	172±1.6
Time elapsed	33±2.8	11±15.5	4 ±17.9	16±22.8	21±2.5

(b)

Secondary growth phenology

Loc	beEW	bmEW	ceEW	ceRW	dEW	deRW
S	97±18.5	123±16.2	180±3.5	247±24	57±16.6	150±18.8
N	102±21	122±14.6	183±3.7	256±1.4	61±15.1	154±21
S+N	94±21.8	121±15.3	182±3.7	256±3.9	61±15.7	162±21.8

649

650 **Table 4** Spearman's correlation coefficients between shoot extension rate (SER; log
651 (mm d^{-1}) and dry matter content (mg g^{-1}) of different crown organs of *Q. pyrenaica*:
652 One year old twigs (Twigs - 1), two years old twigs (Twigs - 2), current twigs (Twigs),
653 buds and leaves. The corresponding *P* values are also shown at the right side of the
654 table.

	Twigs - 1	Twigs - 2	Twigs	Buds	Leaves	SER
Twigs - 1		< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Twigs - 2	0.860		< 0.001	< 0.001	< 0.001	0.023
Twigs	0.785	0.778		-	< 0.001	< 0.001
Buds	0.658	0.516	-		-	-
Leaves	0.645	0.640	0.924	-		< 0.001
SER	-0.425	-0.284	-0.590	-	-0.502	

655
656

657 **Table 5** Time course evolution of DMC (mg g^{-1}) of buds, one, and two years old twigs,
658 leaves and current year twigs of *Q. pyrenaica* during the crown growing season in 2013.
659 Differences in mean values between DOY (P<0.05) are indicated by upper lettering.
660 First values significantly different from previous observations are highlighted in bold.

DOY	Two years old twigs	One year old twigs	Bud	Leaves	Current year twigs
66	510.6±8.5 [a]	482.4±6.6 [a]	543.5±12.6 [a]	-	-
84	510.1±8.5 [a]	487.2±6.6 [a]	529.4±12.1 [a]	-	-
105	508.8±8.5 [a]	490.3±6.6 [a]	506.6±12.1 [a]	-	-
119	508.3±8.5 [a]	481.7±6.6 [a]	**442.9±12.1 [b]**	-	-
129	478.8±8.5 [a, b]	**452.4±6.6 [b, c]**	305.5±12.6 [c]	-	-
140	468.5±8.5 [b, c]	431.9±6.6 [c, d]	285.9±17.0 [c]	276.0±10.2 [c]	-
150	468.3±8.5 [b, c]	421.8±6.6 [d, e]	259.9±18.8 [c]	280.9±7.8 [c]	228.0±7.0.[a]
162	432,7±8.5 [c]	393.2±6.6 [f]	-	259.7±7.2 [c]	207.0±6.3.[a]
182	432.4±8.5 [c]	404.2±6.6 [e, f]	-	**413.9±6.9 [b]**	**319.8±6.5.[d]**
213	478.9±9.3 [a, b]	411.7±6.6 [d, f]	-	435.4±6.9 [b]	411.6±6.3.[b]
246	495.4±8.9 [a, b]	466.3±6.6 [a, b]	-	463.4±6.9 [a]	459.6±6.3.[c]
266	509.9±8.9 [a]	490.9±6.6 a	-	464.1±7.2 [a]	492.0±6.5.[e]

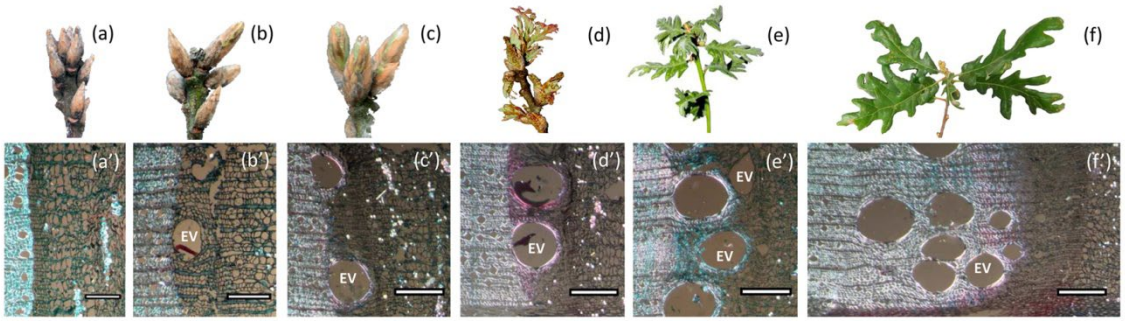
661

662 **Table 6** Characterization of phenological phases of crown by dry matter content (mg g^{-1}) of *Q. pyrenaica*. Differences in dry matter content between branch phenophases (P<0.01) are indicated by upper lettering.

Branch phenophases	Dry matter content
Bud dormancy	540.1±13.8 [a]
Bud swelling	425.7±13.2 [b]
Bud Burst	346.9±13.8 [c]
Leaves unfolding	278.9±13.2 [d]
Leave size < 50%	277.9±17.6 [d]
Leaves size > 50%	270.9±13.2 [d]
Leaf hardening	422.3±13.8 [c]
Full mature leaves	463.4±13.2 [c]

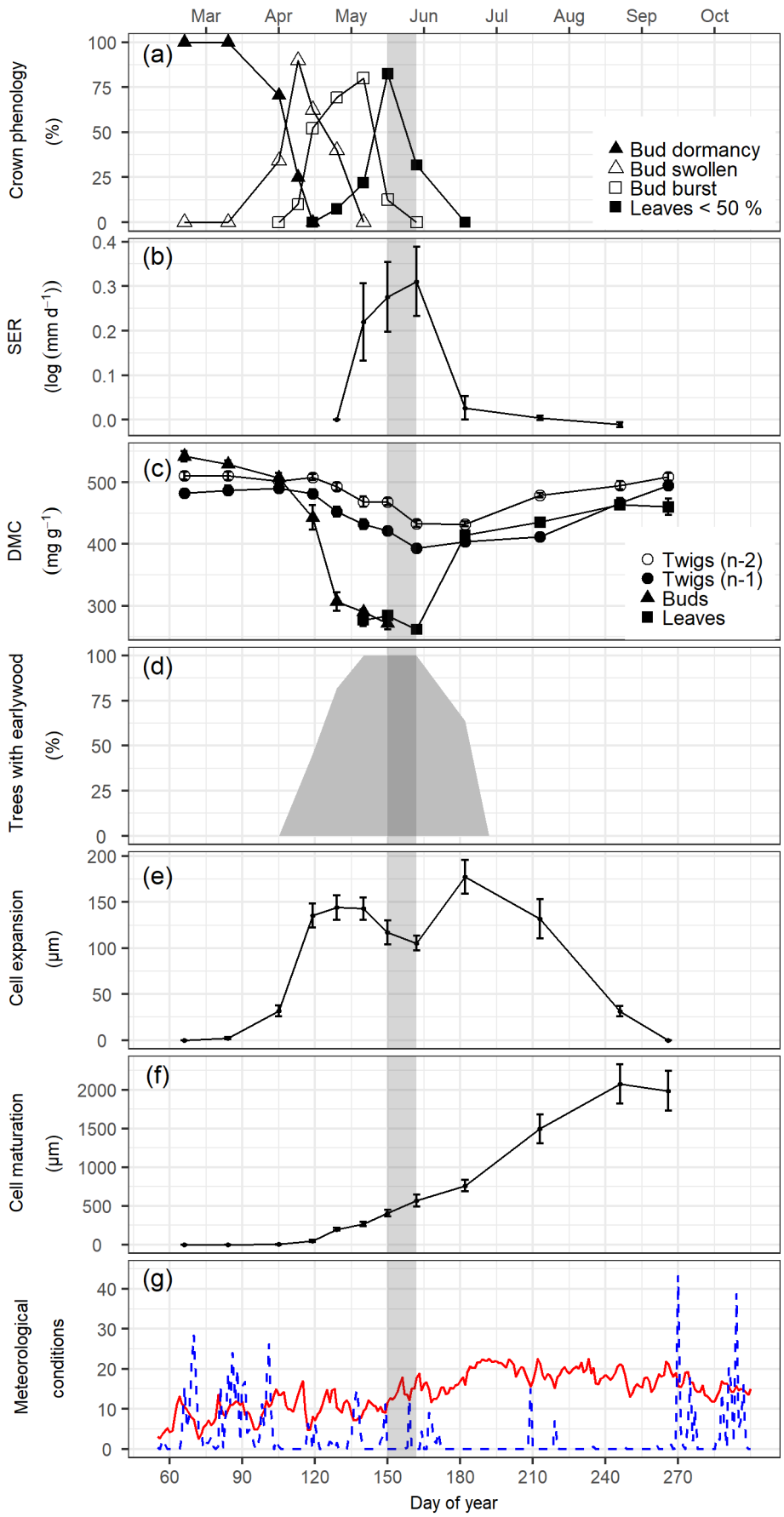
665

666 **Figures**

667 

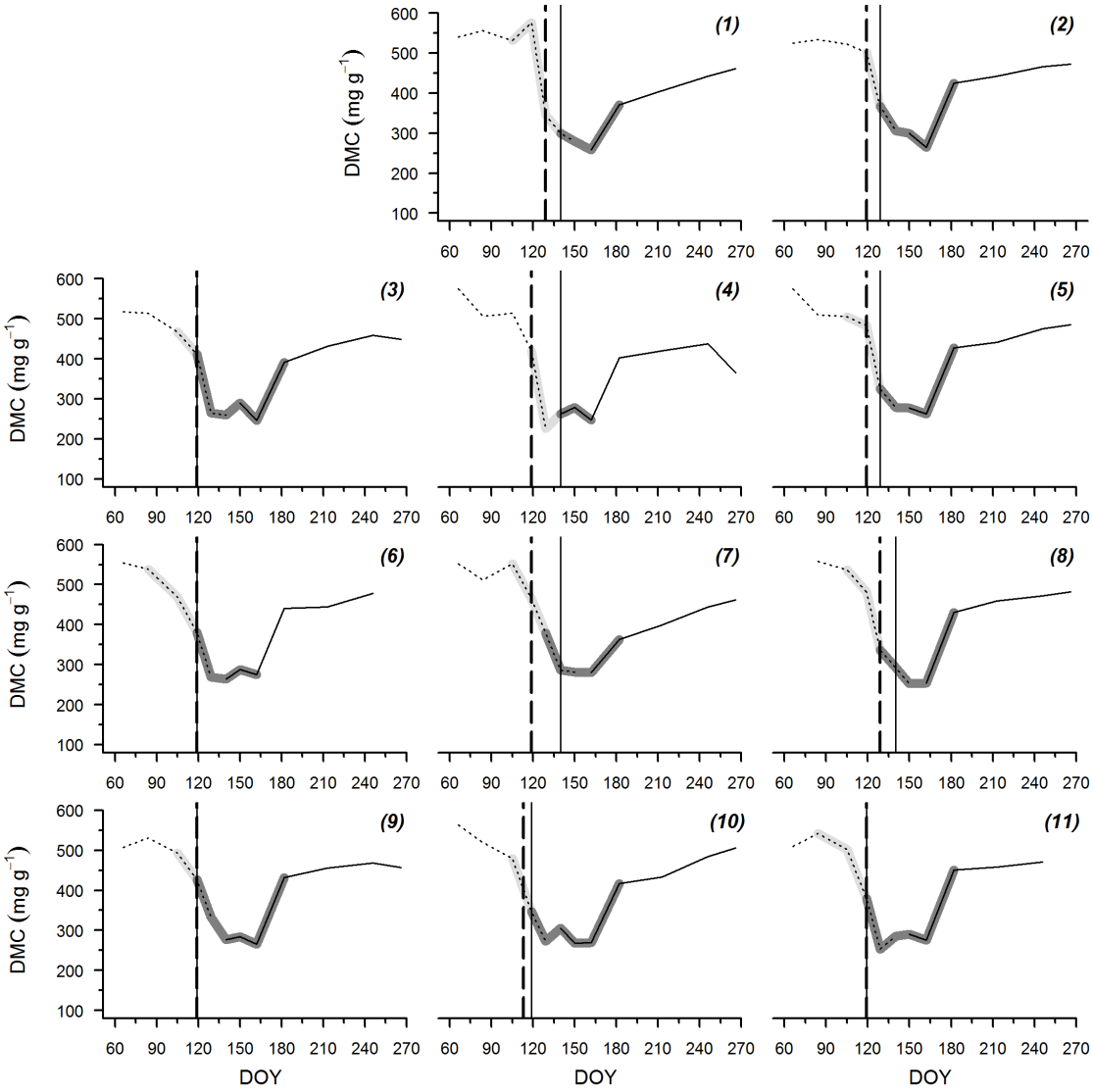
668 **Fig. 1** Leaf growth images of *Q. pyrenaica* and the corresponding transversal thin
669 sections of earlywood development observed under polarized white light, in a time
670 sequence. The upper row shows primary growth phenophases: (a) bud dormancy, (b)
671 swollen buds, (c) bud burst, (d) leaf unfolding, (e) appearance of small leaves and (f)
672 full extension. The lower row shows secondary growth phenophases: (a') dormant
673 cambium, (b') earlywood vessel enlargement, (c') onset of earlywood vessel maturation,
674 (d' and e') earlywood vessel maturation, (f ') cessation of earlywood vessel formation
675 and cell expansion of latewood. Birefringence under the polarizing light indicates
676 secondary cell wall deposition. Scales bars are 150 µm (a', b') and 300 µm (c'- f '). EV,
677 Earlywood vessel

678


Mar
Apr
May
Jun
Jul
Aug
Sep
Oct
(a)
Crown phenology (%)
Bud dormancy
Bud swollen
Bud burst
Leaves < 50 %
(b)
SER (log (mm d⁻¹))
(c)
DMC (mg g⁻¹)
Twigs (n-2)
Twigs (n-1)
Buds
Leaves
(d)
Trees with earlywood (%)
(e)
Cell expansion (µm)
(f)
Cell maturation (µm)
(g)
Meteorological conditions
Day of year

679

680 **Fig. 2** Seasonal phenological trends for *Q. pyrenaica*: Phenological diagrams indicated
681 as (a) Percentage of crown in the phases of: bud dormancy, swollen buds, bud burst,
682 leaves less than 50 % of the leaf size (Leaves < 50 %), (b) Shoot extension rate (SER in
683 log(mm d^{-1}), (c) Dry matter content (DMC in mg g^{-1}) from the buds, leaves, and one-
684 and two-year-old twigs, (n-1) and (n-2) respectively, (d) Percentages of trees with
685 mature earlywood, (e) Ring-width expansion (µm), (f) Wood maturation (µm), (g)
686 Meteorological variables: *continuous red line* is the average temperature (ºC) and
687 *dashed line blue* the precipitation (mm) (data from the nearest meteorological station,
688 Campus Lugo). Grey bar indicates the period of maximum shoot extension rate (SER).
689 Samples taken from day 66 to 266 of year in 2013.



690

691 **Fig. 3** Individual seasonal trends of bud swelling and leaf development of 11 *Q.*

692 *pyrenaica* in 2013 quantified as dry matter content (DMC in mg g^{-1}) of buds during

693 swelling (dotted line) and leaves in development (continuous line); the light gray shade

694 shows the period in which all the earlywood vessels were in expansion, while the dark

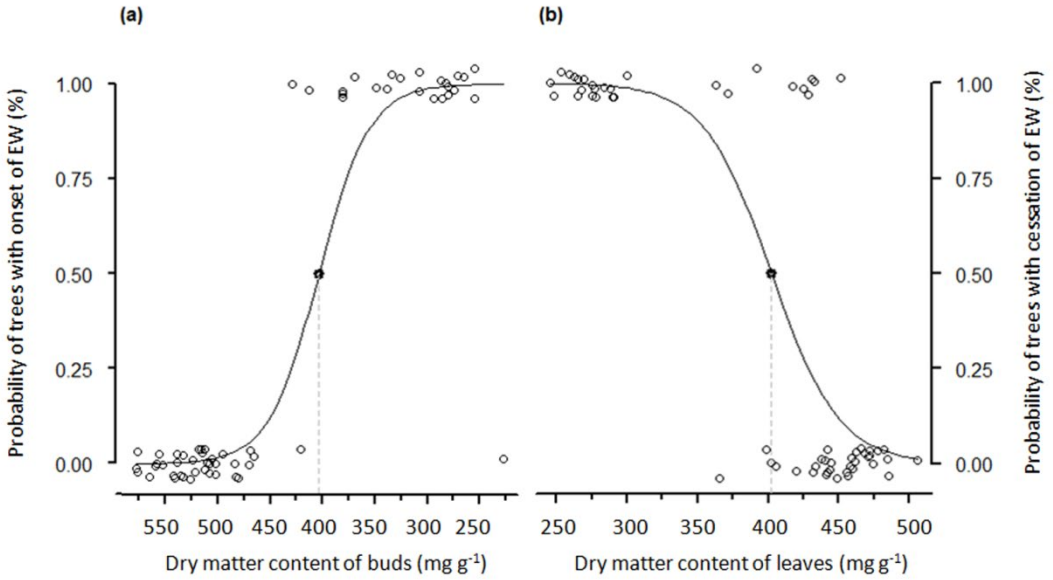
695 gray shadow shows the overlapping period of some expanding vessels with the

696 deposition of secondary cell wall. Vertical lines indicate the time elapsed from the first

697 recording of bud burst (dashed line) in one or several branches to their appraisal in 100

698 % of the crown (solid line). In brackets the number of each tree. Samples taken from

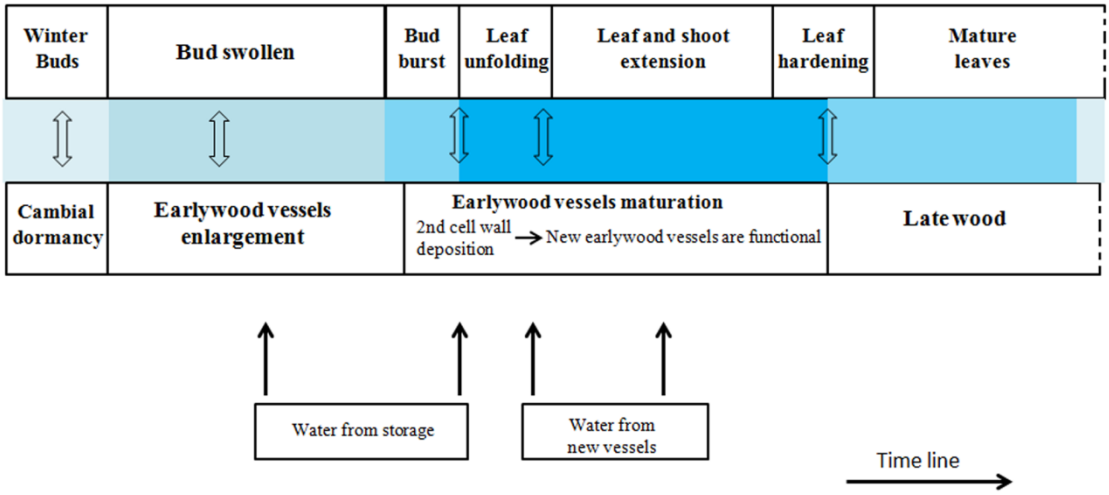
699 day 66 to 266 of year (DOY) in 2013



700

701 **Fig. 4** Probability of trees reaching each phenophase predicted by logistic regression in
702 relation to bud and leaf dry matter content (DMC in mg g^{-1}) in *Q. pyrenaica*. Black point
703 and grey vertical dashed line indicate the DMC when the 50 % of trees reach each
704 earlywood phenophase. a) Dots represent each observation of the onset of earlywood
705 maturation in binary data (0 = no secondary wall deposition in the first earlywood vessel;
706 1 = at least one vessel has started a secondary wall deposition) in relation to bud DMC
707 (mg g^{-1}). b) Dots represent the observation of the cessation of earlywood maturation as
708 binary data (0 = not earlywood enlargement; 1 = at least one early wood vessel in
709 enlargement) in relation to leaf DMC (mg g^{-1})

710



711

712 **Fig. 5** Sequence of events during shoot growth of the canopy in relation with the
713 development of earlywood vessel in the stem of *Q. pyrenaica*. Colour density indicates
714 increased water content