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Technical note

# Assessment of xylem phenology: a first attempt to verify its accuracy and precision

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**This manuscript aims to evaluate the precision and accuracy of current methodology for estimating xylem phenology and tracheid production in trees. Through a simple approach, sampling at two positions on the stem of co-dominant black spruce trees in two sites of the boreal forest of Quebec, we were able to quantify variability among sites, between trees and within a tree for different variables. We demonstrated that current methodology is accurate for the estimation of the onset of xylogenesis, while the accuracy for the evaluation of the ending of xylogenesis may be improved by sampling at multiple positions on the stem. The pattern of variability in different phenological variables and cell production allowed us to advance a novel hypothesis on the shift in the importance of various drivers of xylogenesis, from factors mainly varying at the level of site (e.g., climate) at the beginning of the growing season to factors varying at the level of individual trees (e.g., possibly genetic variability) at the end of the growing season.**

***Keywords***: **black spruce, methodology, wood formation, xylogenesis.**

## Introduction

In the last 15 years, investigations of xylem phenology have experienced a renewed and increasing interest because of their importance for quantitative wood anatomy, dendroecology and ecophysiology. A number of interconnected research teams have been created worldwide to answer the questions about when and how secondary growth occurs (Mäkinen et al. 2008, Marcati et al. 2008, Lupi et al. 2010, Begum et al. 2013, Fonti et al. 2013, Li et al. 2013). Procedures applying microcoring or pinning (Mäkinen et al. 2008) have been used to detect the intra-annual variations in cell number and to identify the dates when the meristems divide, post-cambial cells differentiate, and xylem is mature and functioning. Analysis allows the climatic variables to be coupled or compared with all phases of xylem phenology to detect the most important drivers of wood formation. Observations of xylem anatomy provide a tree-based assessment of the dynamics of cambial and post-cambial growth. However, several authors point out the high variability in the measurement due to different sampling points. Therefore, to what degree is this estimation representative of the tree?

The aforementioned procedures have been successfully applied on all woody organs, stems, branches or roots, on time scales of a few days to weeks (Thibeault-Martel et al. 2008, Lenz et al. 2012, Lupi et al. 2012). However, they have an important and unavoidable complication: observations have to be performed on different points over time. More precisely, the same cambial zone cannot be sampled or pinned more than once. Thus, sample collection is required to be performed on a sufficiently wide region of the analysed organ according to a random or systematic sampling. This inevitably generates results containing a component that is associated with the within-tree variability. Although well acknowledged, such a component still remains unquantified.

Up to now, the analyses of xylem phenology have been based on the assumption that the differences within trees are negligible

and that the sampling position on the stem has no substantial influence on the final results, at least if samples are taken within a certain distance. In fact, recent findings have demonstrated that this assumption should be reconsidered or verified. Cell differentiation was observed to change along the stem when collecting samples at different tree heights, although growth reactivation was independent of height (Anfodillo et al. 2012). In cross section, the stem is rarely circular but exhibits a more or less pronounced amoeboid shape and heterogeneous tree-ring widths around the circumference. In conifers, different tree-ring widths correspond to different cell productions (Wang et al. 2002), and consequently to different xylem phenologies (Lupi et al. 2010, Rossi et al. 2012). Thus, the variability in tree-ring width could be expected to be the effect of different timings and periods of xylem formation. Procedures for standardizing the variability in cell production have been suggested (Rossi et al. 2003, Seo et al. 2007), but a critical evaluation about the representativeness of the sampling around the stem was missing.

Investigations on xylem phenology are constrained by the number of studied trees, because sample preparation and microscopic observations are time consuming. In some cases, the wide variability among trees, carefully described by Wodzicki and Zajaczkowski (1970), has prevented successful evaluation of the differences in xylem phenology (e.g., Lupi et al. 2012, D'Orangeville et al. 2013). Even if a part of this variability is evidently related to the genetic differences among individuals, tree position, age and soil fertility, the methodological problems associated with the within-tree variability are destined to persist and could raise doubts over the consistency of the results. In this paper, stems of trees from two sites were sampled along two opposite paths at each sampling date to test the variability within and between trees and to quantify the precision and accuracy of the estimations of xylem phenology.

## Materials and methods

### Study sites and tree selection

This study was conducted in the boreal forest of Quebec, Canada, in two even-aged, mature black spruce (*Picea mariana* (Mill.) BSP) stands. The first site (SIM) is located in the Simoncouche research station (48°13′N, 70°20′W, 350 m above sea level (a.s.l.)), within the Laurentides Wildlife Reserve. The second site (BER) is located at a higher altitude, near the Bernachez lake in the Monts-Valin (48°51′N, 70°20′W, 611 m a.s.l.). Both stands originated from forest fires, which occurred around 1870 and 1922 in BER and SIM, respectively. More detailed description of the study sites is reported in Lupi et al. (2010). The climate is continental with long and cold winters. In 2009, the May–September mean temperatures were 11.3 and 12.8 °C and total precipitations 478.3 and 391.8 mm in BER and SIM, respectively (Lupi et al. 2010).

Three co-dominant trees with similar growth patterns were selected in each site. The similarity in growth rates between trees was assessed with a preliminary sampling, in which, the number of tracheids of the three previous tree rings were counted.

### Data collection and preparation

In 2009, wood microcores were collected weekly from April to October using a Trephor (Rossi et al. 2006*a*). On each sampling date, the opposite sides of the stem were sampled, following a counterclockwise-elevating spiral centred at breast height, resulting in two spiral paths never crossing. The samples usually contained the preceding three to five tree rings and the developing annual layer with the cambial zone and part of the adjacent phloem. The samples were always taken at 5–10 cm intervals to avoid resin ducts forming in response to the disturbance (Deslauriers et al. 2003). The microcores were stored at 5 °C in Eppendorf tubes containing a water : ethanol solution (1 : 1) and processed within a few days. The microcores were dehydrated through successive immersions in ethanol and Histosol™ and embedded in paraffin (Rossi et al. 2006*a*). Transverse sections 6–10 μm in thickness were cut with a rotary microtome, stained with cresyl violet acetate (0.16% in water) and immediately observed at a magnification of 400–500× with visible and polarized light. Cells in the cambial zone and in radial enlargement showed only primary cell wall, which does not shine under polarized light (Gričar et al. 2006). Cambial cells were characterized by a thin cell wall and small radial diameters, while enlarging cells had a radial diameter at least twice that of the cambial cells. Wall thickening cells shone under polarized light and showed a colouration that varied between light and dark violet. Lignification appears as a colour change from violet to blue, starting at the cell corners and middle lamella and spreading centripetally into the secondary walls. When the entire cell presented a blue colouration, lignification was considered complete and tracheids mature (Rossi et al. 2006*b*).

### Xylem phenology and cell production

In spring, xylem formation was considered to have begun when the average number of tracheids in the enlargement phase between the three rows was greater than one. Xylem formation was considered complete when no further tracheids were observed undergoing wall thickening and lignification. Duration of xylogenesis was calculated as the difference between the onset of cell enlargement and the ending of cell wall thickening and lignification.

For each sample, the radial number of cells in the cambial zone, radial enlargement phase, cell wall thickening phase and mature cells were counted along three radial rows. Total number of tracheids was calculated as the sum of the number of cells in radial enlargement, wall thickening and lignification and mature cells. To describe cell production, the total number of tracheids was fitted with a Gompertz function using the

non-linear regression procedure with the Marquart iterative method in SAS (SAS Institute 2010, Cary, NC, USA). The Gompertz function was defined as follows:

$$y = A\exp(-e^{\beta - kt})$$

where $y$ is the number of tracheids, $t$ is the time computed in days of year (DOY), $A$ is the upper asymptote of the total number of xylem tracheids, $\beta$ is the $x$-axis placement parameter and $k$ is the rate of change parameter (Deslauriers et al. 2008). The asymptote represents the number of tracheids produced by a tree during the growing season. The annual growth was estimated as the total number of tracheids produced at the end of the growing season and corresponding to the Gompertz asymptote (Rathgeber et al. 2011).

### *Analysis of variability*

To compute average differences, it was estimated through basic combinatory calculus that 66 combinations of couples of observations were possible with 12 observations (2 sampling path × 3 trees × 2 sites). Indeed, if a group has $n$ elements (in our case 12), the number of $k$ combinations (in our case two, to compute differences) can be calculated using factorials as follows:

$$\frac{n!}{k!(n-k)!}$$

These 66 combinations corresponded to six differences within trees, 24 differences between trees (considering either sampling paths) within the same site and 36 differences between trees (considering either sampling paths) of different sites. For both the phenological variables and wood production, the mean differences were computed as the absolute value of the difference for each type of combinations (i.e., within trees, between trees within the same site and between the sites). Differences were also graphically represented by means of boxplots in Sigmaplot 10.0 (Systat Software, Inc., San Jose, CA, USA, 2006) with the lower and upper limit of the box representing the first and third quartile, the line inside the box representing the median or second quartile.

The variability in xylem phenology (onset, ending and duration of xylogenesis) and cell production was estimated at different levels using the *glmer* function of the package *lme4* in R (version 3.0.01, R Core Team 2013), fitting a hierarchical model for each response variable, using the restricted maximum likelihood estimation method. The model included site and tree nested within site as a random effect. The variation was attributed to site, tree (within site) and position (residual variance) expressing the ratio between the variance of each random effect and the total variance as a percentage.

Considering onset, duration, ending of xylogenesis and cell production, Euclidean distances were computed using the *dist* function and a multidimensional scaling (MDS) was performed using the *cmdscale* function in R (R Core Team 2013). MDS uses an algorithm that displays the structure of distance-like data as a geometrical picture (Wang 2011).

## Results

### *Cell production and xylem phenology*

The Gompertz function adequately fitted the data producing adjusted $R^2$ ranging between 0.725 and 0.896 (on average 0.83 ± 0.05). The Gompertz functions represented the dynamics of xylem growth by reproducing the rapid increase in tracheid number in spring and estimating the average cell production in autumn (Figures 1 and 2). The differences between trees were markedly higher than those within tree, as shown by the predicted curves that frequently overlapped within each tree (Figure 2).

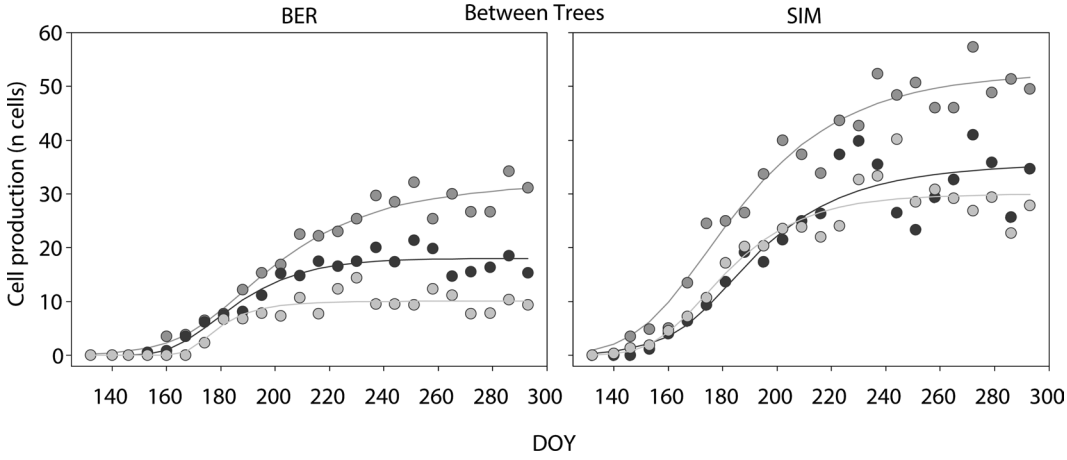


Figure 1. Between-trees variability in the dynamics of cell production fitted by Gompertz functions. Each point corresponds to the average of the two sampling paths. Each colour (black, light and dark grey) refers to a single tree.

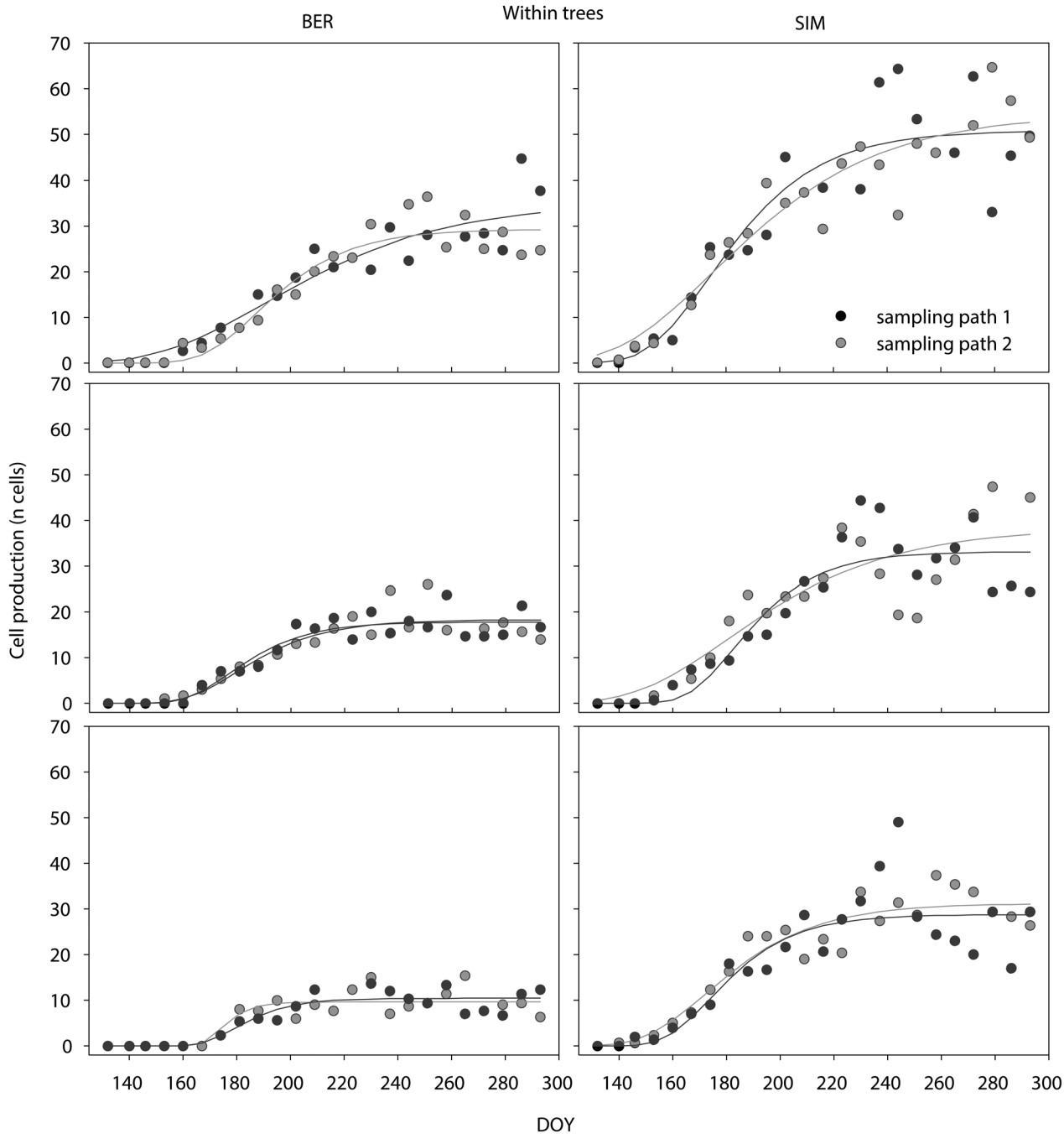


Figure 2. Within-tree variability in the dynamics of cell production fitted by Gompertz functions.

Xylogenesis started ~2 weeks earlier and was 35 days longer in SIM than in BER, which resulted in a greater cell production in SIM (Table 1). A higher variability between trees was observed in BER, especially for the ending and duration of xylogenesis.

### *Within- and between-tree variations*

The differences between trees for the onset of xylogenesis were on average 9.5 and 7.3 days, while the average differences within tree were 4.3 and 2.7, at BER and SIM respectively (Figure 3). The ending of xylogenesis showed a range of more than a month in BER, with an average difference of 3 days between the two sampling paths. In SIM, the differences between trees for the ending of xylogenesis were less pronounced than those within tree (on average, 9.0 vs 11.3 days, respectively). Cell production differed more between trees (14.4–15.2 tracheids), than within tree (2.3–4.9 tracheids) in both study sites.

The random effects model showed that the differences within tree were always smaller than those between trees in

Table 1. Xylem phenology and cell production estimated in the two study sites.

| Variable | BER | SIM |
|---|---|---|
| Onset of xylogenesis (DOY) | 161.8 ± 7.0 | 146.7 ± 5.2 |
| Ending of xylogenesis (DOY) | 260.8 ± 23.1 | 281.0 ± 8.1 |
| Duration of xylogenesis (days) | 99 ± 29.2 | 134.2 ± 7.6 |
| Cell production (n cells) | 20.3 ± 10.5 | 40.1 ± 10.4 |

Values are reported as mean ± standard deviation.

Table 2. Restricted maximum likelihood variance component estimates as a percentage of total variance.

| Source of variation | Onset of xylogenesis (%) | Ending of xylogenesis (%) | Duration of xylogenesis (%) | Cell production (%) |
|---|---|---|---|---|
| Site | 69.0 | 19.6 | 44.2 | 53.1 |
| Between trees | 24.9 | 71.3 | 52.3 | 43.9 |
| Within trees | 6.1 | 9.1 | 3.5 | 3.0 |

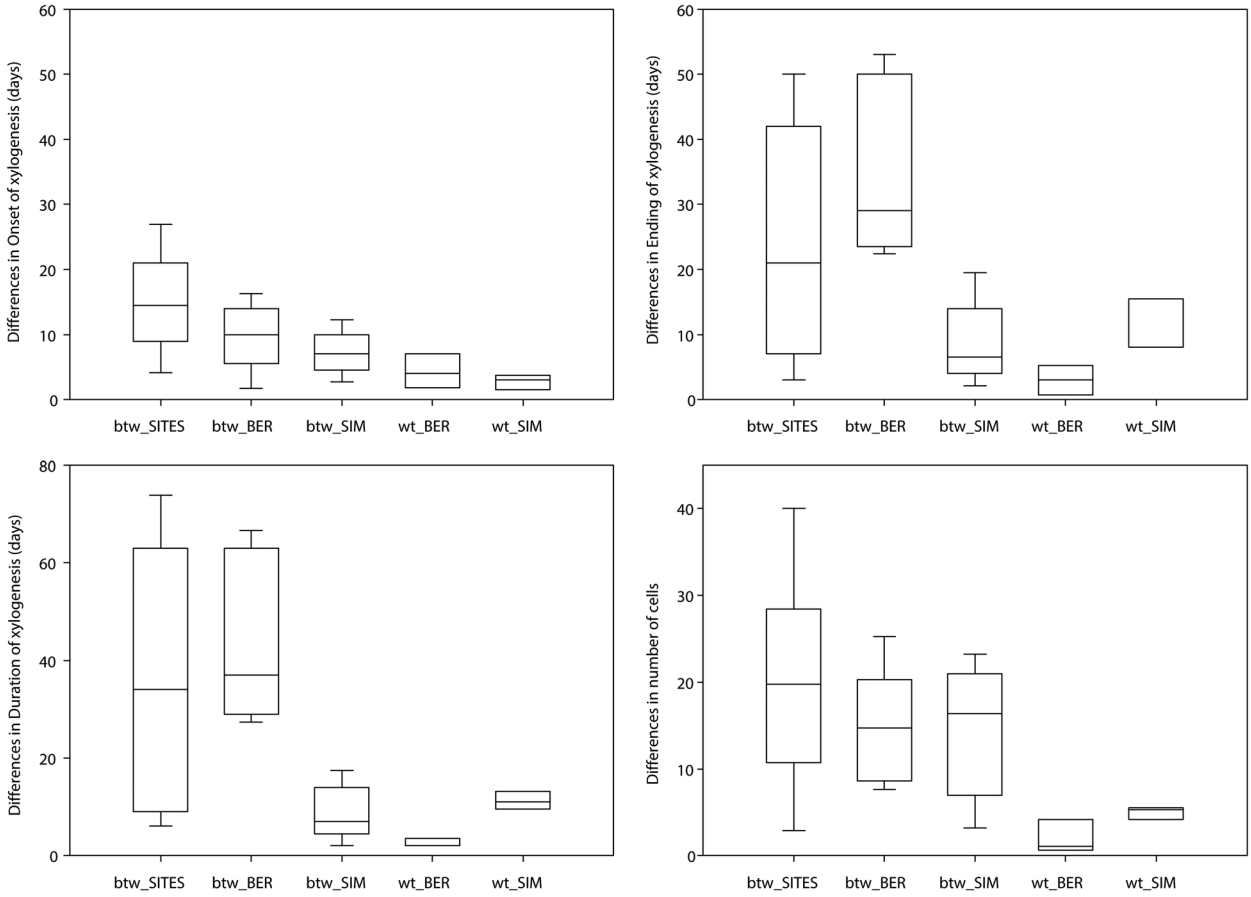


Figure 3. Boxplots of the differences in xylem phenology and cell production between (btw) and within (wt) sites and trees.

both sites and for all the studied variables (Table 2). For the onset of xylogenesis, the highest variation was observed between sites (69.0%), while between- and within-tree variation was 24.9 and 6.1%, respectively. The same pattern was observed for cell production, even if the contribution of the variable tree as a source of variation increased (43.9%), while the variation at the level of site decreased (53.1%). Ending and duration of xylogenesis exhibited a higher variation between trees than between sites, with the smallest values being observed for the within-tree variation.

### *Multidimensional scaling*

The MDS analysis confirmed the previous results on a multivariate level (Figure 4). Data points belonging to the same trees were grouped in clusters, and trees were clearly separated according to the site. Consequently, the differences between sampling paths (within tree) were generally smaller than those between trees, while the two sites were markedly segregated.

## Discussion

In this paper, the variability in xylem phenology and cell production was determined by different components belonging to site and tree. The results provide important information on the range of variability that should be expected in intra-annual analyses of xylem formation. The variability within tree was always lower than that between trees and sites, and very

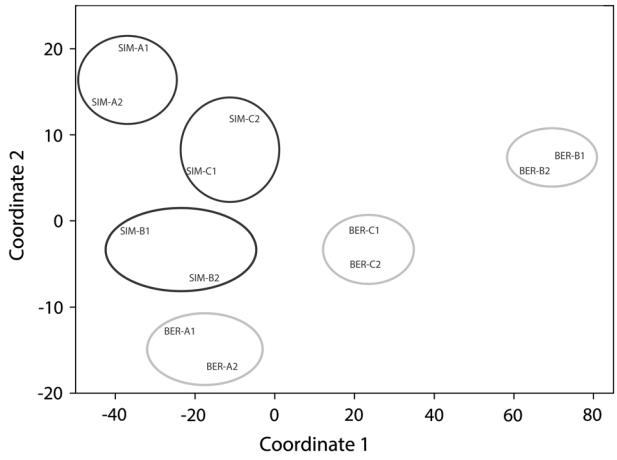


Figure 4. Metric multidimensional scaling based on xylem phenology and cell production. Site, tree id and spiral id are used for labelling. Hand drawn circles highlight the main clusters. At each site, the same letter indicates that the two spirals (1 and 2) come from the same tree.

similar growth patterns were observed between the two sampling paths. These findings demonstrated that one sample collected from the stem is sufficiently representative of the cell production of a tree. It could be concluded that the current methods for estimating the dynamics of xylem formation are accurate and suitable for evaluating xylogenesis at tree level.

For the onset of xylogenesis, the variation within tree (2.7–4.3 days) was less than half of that observed between trees (7.3–9.5 days), and <1 week, which is usually the time interval at which sampling is carried out (Lupi et al. 2010). This pattern was confirmed for the ending of xylogenesis in BER, the colder and less productive site, while SIM showed similar variability within and between trees, ranging between 9.0 and 11.3 days. Based on these findings, samplings with resolutions <1 week and >4 days (corresponding to the within-tree variability) would be able to improve the precision of the estimations for the onset of xylogenesis. On the contrary, a higher frequency of sampling (i.e., increasing time resolution) for the ending of xylogenesis may be ineffective when within-tree variation is >7 days, as observed in SIM, where to improve accuracy higher sample sizes would be required (e.g., more sample collected for each tree and for each sampling day, thereby increasing spatial resolution).

Substantial differences were observed between the phases of xylem formation. In particular, for the onset of xylogenesis, a higher proportion of variation was attributed to site. This probably indicates that growth resumption was mainly triggered by environmental factors varying between sites (e.g., climate, more likely temperature in our sites) than by differences between trees (e.g., social status, cambial age, genetic or microclimatic conditions) (Rossi et al. 2008, 2011, Gauchat 2011, Rathgeber et al. 2011). The trees were selected from even-aged stands and were dominant or co-dominant, and thus the effects of social status or age were expected to be very limited. However, the difference in age between sites may in part explain differences in productivity, together with the warmer temperatures at SIM, the site where more tracheids were produced (Rossi et al. 2008). Concerning the ending of xylogenesis, greater differences were observed between trees than between sites, contrary to the onset of xylogenesis, perhaps pointing to a stronger genetic control on ending than on onset of xylogenesis. Interestingly, studying genetically selected trees in plantations, Gauchat (2011) observed than the families diverged more for the ending of xylogenesis than for other phenological variables.

The variability in cell production between sites and trees may be related to the causal relationships proposed by Lupi et al. (2010) and Rossi et al. (2012) between phenology and cell production. According to these authors, the onset of xylogenesis would affect the number of tracheids produced, which in its turn would influence the timing of ending of xylogenesis. Thus, cell production would be in part controlled by site-specific factors and in part by between-trees differences that would be expressed throughout the growing season and affect the ending of xylem differentiation.

For the first time, an investigation has been carried out that quantified the variability at different levels (from within tree to between sites) in xylem phenology and cell production, the variables most commonly considered in recent papers on xylogenesis. Our findings for the ending of xylogenesis showed more between-tree variability for this variable than for the onset of xylogenesis and cell production, and possibly explain why the mechanisms controlling the ending are less well defined than those controlling the onset of xylogenesis. We clearly showed how the variability in phenological variables tends to decrease from trees in different sites to trees in the same site and, finally, within the same tree, and that current sampling methods are sufficiently reliable, even if the estimates of ending of xylogenesis may be improved by increasing the sampling (at two or more points) in autumn.

## Conflict of interest

None declared.

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