X-ray Microdensitometry for Assessing the Resistance of Hybrid Larch to Fungus *Coniophora puteana*

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X-ray microdensitometry was performed on small wood samples after fungal exposure to assess wood density loss and its variability within a hybrid larch population. Microdensitometry was applied to thin wood slices obtained from increment cores collected from standing trees before and after 4 weeks of exposure to *Coniophora puteana* fungus. Density loss rates were measured and compared to mass loss percentages calculated on wood blocks following a 16-week decay test according to CEN TS 15083-1 (2005). The relationship between the standard mass loss and the density loss percentages was weak. Several explanations for the results are presented in this work, and methodological improvements are suggested to achieve a more accurate comparison. The proposed screening test has several advantages over the current standard method, as it is less invasive for the tree, less time consuming, and is therefore better suited for genetic studies and breeding. As predicted, due to extractive content variation and fungus behaviour, density loss after 4 w was more important in earlywood than in latewood and in inner heartwood than in outer heartwood. The new method also better determined the differences in decay among trees within the larch population than the standard method at 16 w.

Keywords: Coniophora puteana; Larix spp; X-ray microdensitometry; Natural durability

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INTRODUCTION

Natural wood durability is highly variable and difficult to determine in timber species. Although it is defined as an intrinsic characteristic of wood (Eaton and Hale 1993) related to chemical and anatomical composition, it depends principally on the service conditions of wood (Van Acker *et al.* 2003; Råberg *et al.* 2005; CEN EN 335 2013).

The genus *Larix* is represented by a few species that are widely distributed in boreal and temperate forests and in mountainous regions, where it is either native or introduced in artificial plantations. The wood of the species *Larix decidua* has high mechanical strength, durability, and an attractive reddish colour. According to CEN EN 350 (2016), *L. decidua* is classified as between moderately durable (durability class 3) and slightly durable (durability class 4). The early and abundant extension of the heartwood justifies the traditional outdoor use of larch wood in continental climates (houses, roof shingles, and bridges) and its indoor applications (floorings and wall paneling).

In differentiated species, such as larch, the resistance against basidiomycetes fungi is principally localized in the heartwood and is due to extractives. Chemically, the extractives are terpenoids, tropolones, flavonoids, stilbenes, and other aromatic compounds

(Scheffer and Cowling 1966; Hills 1972; Pometti *et al.* 2010). The extractives are associated with natural durability or intrinsic resistance and with the colour of wood (Gierlinger *et al.* 2004a; Moya and Berrocal 2010). However, some authors claim that wood colour depends on the interaction between extractives and sunlight (Hills 1987). Windeisen and Wegener (2003) found that the most important extractive compounds in *Larch* spp. were Dihydrokaempferol (DHK) and Dihydroquercetin or Taxifolin (Tax).

Many studies based on European standards have been carried out to test the natural durability of larch wood (Gierlinger *et al.* 2003; Gambetta *et al.* 2004; Venäläinen *et al.* 2006; Jebrane *et al.* 2014; Plaschkies *et al.* 2014). The variability in larch heartwood durability is affected by genetic (species, genetic origin within species, and individuals within origin) and environmental factors (site and forestry practices), the position within the trunk, and the age of the tree. This finding has been documented in heartwood extension (Pâques 2001; Gierlinger and Wimmer 2004c), the content of extractives (Gierlinger *et al.* 2004b; Venäläinen *et al.* 2006; Pâques *et al.* 2013), natural durability (Curnel *et al.* 2008), wood colour (Gierlinger *et al.* 2004a), and modulus of elasticity (Pâques *et al.* 2009).

Natural wood durability is related to heartwood formation (Taylor *et al.* 2002). It is a desirable trait for genetic improvement and selection programmes, especially when the wood is ultimately utilized for commodities and structures. In these programmes, it is essential to have access to fast, reliable, and less-invasive or non-invasive tests to screen large sets of living trees (several hundreds to thousands) for durability.

The existing European standard tests for assessing natural durability (CEN TS 15083-1 2005; CEN EN 113 2006) require the logging of trees and the tedious preparation of wood specimens. They consist of exposing wood samples of specified dimensions to specified fungal strains for a certain time period and finally evaluating the average mass loss of the samples. These tests are time and material consuming. To perform a standard test, approximately three trees per species must be logged to obtain approximately 30 samples collected from different areas of the trunk (*e.g.*, sapwood and inner and outer heartwood). Furthermore, the duration of the tests is 16 w. Due to these conditions, standard tests are inappropriate for determining wood durability on the large scale required by genetic studies and breeding programmes.

The literature describes several accelerated methods, such as the use of mini-blocks (30 mm x 15 mm x 10 mm) based on the mass loss percentage after shorter fungi exposure times (6 w to 8 w) (Bravery 1979; Van Acker *et al.* 2005; Palanti *et al.* 2012). In the last 15 years, several studies have demonstrated the ability of near infrared spectroscopy (NIRS) to detect extractive content within the heartwood and predict natural durability in certain cases (Gierlinger *et al.* 2003; Taylor *et al.* 2008). However, due to the difficulty and time-consuming nature of calibration, the currently available NIRS models are not appropriate for routine use.

X-ray densitometry is a well-known method that has been frequently applied in dendroclimatology and in wood quality studies (Rozenberg *et al.* 1999; Briffa *et al.* 2013) to evaluate ring sizes and density variation. Though some studies require no pretreatment (Bergsten *et al.* 2001; Helama *et al.* 2010; Nocetti *et al.* 2011), the extraction of wood samples with organic solvents, such as ethyl alcohol, benzene, acetone, or pentane, especially for resinous species, is a standard pretreatment method. Extraction is an important process because wood tissue includes not only lignin, cellulose, and hemicelluloses, but also other natural chemical components (Polge 1970; Schweingruber *et al.* 1978). The use of X-ray densitometry with non-extracted samples before and after sample decay appears attractive; the loss of density may be a proxy for mass loss, as it

reflects the loss of matter (cellulose – lignin) per unit of volume. Such a technique has been tested to evaluate fungal decay in a kinetic study with two non-durable species (beech and Scots pine) (Macchioni *et al.* 2007). Mass loss during increasing periods of exposure to fungi was well correlated with corresponding density losses. This study and that by Bucur *et al.* (1997), also demonstrated the utility of the X-ray approach to monitor the decay within rings at a finer scale than traditional approaches. Recently, Hervé *et al.* (2014) mapped decay in beech using X-ray computed tomography.

The aim of this study was thus to evaluate whether the technique based on X-ray microdensitometry is suitable to determine natural durability and its variability within a hybrid larch population by analyzing thin radial wood slices obtained from increment cores.

This paper also discussed the pros and cons of using X-ray microdensitometry to evaluate decay due to fungal attack and whether it could be proposed as a new screening method useful for standing trees.

The utilization of only one fungus, *C. puteana*, the most suitable for softwood is justified by the large number of samples necessary to make the standard procedure even for a fungus, and principally to methodological investigation of this research.

EXPERIMENTAL

Larch Population

The original larch population consisted of 98 hybrid trees (*Larix x eurolepis*) (trees numbered 1 to 27, 28 to 73, and 76 to 102), two European larches (*L. decidua*) (trees 74 and 75), and two Japanese larches (*L. kaempferi*) (trees 103 and 104). The hybrids belonged to 20 full-sib families developed at INRA by control crosses (EL x JL) and sampled from a factorial mating design with 12 European larches (EL, Sudetan/Alps origins) as mothers and 12 Japanese larches (JL, several origins) as fathers; European and Japanese larches were included as controls. This population was planted on April 2, 1985, with 1+1 bare-root seedlings at 3 m x 3 m spacing with an incomplete randomized block design (bl: 40, one-tree plot). The site is located at Beaumont-du-Lac (West Massif Central Range, France; 1°50'E–45°45'N) at an altitude of 540 m. This experimental plantation was thinned for the first time in 2002, and further thinning was delayed because of several scientific issues that required the largest number of trees/progeny possible. Plantation growth seriously suffers from delays in thinning. The trees sampled in this study were obtained from a thinning in 2013.

Resistance against *Coniophora puteana* According to CEN TS 15083-1 (2005) Standard

From each of the 102 trees, a 30-cm-thick disk was collected at breast height to obtain the wood blocks for the natural durability assessment test. Disks were cut in a radial direction passing across the pith to obtain two short diametral boards per tree. The boards were dried in a conditioned room with a relative humidity (RH) of 60% at 20 °C to a constant mass before preparing the wood blocks. The larch standard wood blocks (50 (L) x 25 (R) x 15 mm^3(T) were obtained from the five outer rings of the heartwood.

The experimental design of the decay test was carried out according to CEN TS 15083-1 (2005), but for each tree, only six replicates (instead of ten) were used, and only one fungus was tested (instead of three). In particular, *Coniophora puteana* Schumacher

ex Fries, strain Karsten (BAM) (Ebw. 15) obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures GmbH (Leibniz, Germany) was tested. A further set of six replicates per tree was dried at 103 °C to calculate the moisture content (MC) to determine the theoretical initial dry mass (M_i). The value of M_i was calculated with Eq. 1,

$$M_i = M_1 \times 100/(100 + MC) \quad (1)$$

where M_1 represents the initial conditioned mass (unit) at 65 ± 5 °C and 20 ± 2 °C.

Before starting the biological tests, all the samples were conditioned at 65 ± 5% RH and 20 ± 2 °C and sent to an Irradiation Centre (Gammatom, Como, Italy) for sterilization *via* γ rays.

The duration of the biological test was 16 w at 70 ± 5 % RH and 25 ± 2 °C. Then, the wood blocks were weighed and placed in the oven at 103 °C for 24 h, and the final dry mass (M_f) was measured. The mass loss percentage (*ML*) was calculated using Eq. 2 and interpreted according to Table 1:

$$ML = \frac{(M_i - M_f)}{M_i} \times 100 \quad (2)$$

The virulence of *C. puteana* was determined on 8 specimens of Scots pine (*Pinus sylvestris* L.) with the same dimensions as the larch samples. The efficacy of fungus was determined for testing the activity of fungus on reference wood, sapwood of Scots pine for softwood, information important for being sure that the fungus utilized is active and the results are reliable.

Table 1. Range of Median Mass Loss (ML) Percentage and Durability Class (DC) Resulting from CEN TS 15083-1 (2005)

Durability Class (DC)	Median ML (%)
Very durable (1)	≤ 5
Durable (2)	5 < x ≤ 10
Moderately durable (3)	10 < x ≤ 15
Slightly durable (4)	15 < x ≤30
Not durable (5)	x > 30

Resistance against *C. puteana* Through X-ray Microdensitometry Analysis

Wood slices (2.3 mm thick and 14 mm wide) were obtained from the 102 trees from a log just beneath the one used for the standard decay test. The slices showed transversal surfaces of the tree along the radial direction, and two opposite radii per tree were obtained, which were called radius a and radius b. The slices were composed of all radii, and both radii were put in contact with fungus on plastic spacing mesh for 4 w at 70 ± 5 % RH and 25 ± 2 °C. Before fungal exposure, the sterilization of the slices consisted of exposure to UV radiation for 30 min. Then, the slices were maintained at 65% RH and 20 °C until reaching a constant mass, and they were then exposed to an X-ray source (Gilardoni Radiolight, Lecco, Italy for 15 min at the following conditions: 25 kV, 3 mA, and a source-surface distance of 1.60 ± 5 ± 2 m. The sets of slices were directly laid on Carestream Healthy M100 X-ray film (Milano, Italy) with the calibration wedge, which had 13 steps and a density of 1.254 g/cm^3. The calibration wedge was built from a 5.0-mm-thick cellulose acetate film, in which 13 steps were carved at different thicknesses. Manual development was performed with Kodak products using the following time conditions: 5

min of development, 5 min of water rinsing, and 5 min of fixation. After initial radiography, the slices were then exposed to fungal decay by *C. puteana* for 4 weeks on Petri dishes. After 4 w, the mycelium was gently removed, and the slices were conditioned again as above described and X-rayed.

The X-ray images of the slices were analysed before and after fungal exposure with WinDENDRO software (Regent Instruments, version 2005, Chemin Sainte-Foy, Canada). For each radius (a and b), the density profiles before and after the fungal attack of the heartwood rings were obtained. The mean density of the non-decayed (mean initial density (mD_i)) and decayed (mean final density (mD_f)) slices were obtained, and the density loss percentage (DL) was determined according to Eq. 3:

$$DL = \left[\frac{(mD_i - mD_f)}{mD_i}\right] \times 100 \quad (3)$$

The year that corresponded to the most external rings of heartwood was recorded by visually observing colour change. The density loss percentages obtained from two slices from both radii were averaged and compared with the mass loss percentages of the standard wood blocks.

STATISTICAL ANALYSES

All the statistical analyses was obtained through the open source software. The basic statistical analyses was calculated for both methods, the standard one following the CEN TS 15083 and the innovative ones based on X-ray microdensitometry. Further, the dispersion of percentage mass loss obtained in standard wood blocks in the trees population was determined, using software R. A two-way analysis of variance of mass loss in standard wood blocks (with family and tree nested within family) was evaluated. Determinations included the correlation curve the percentages of mass and density losses and the Pearson correlation coefficient obtained respectively with two different methods by the mean of excel Microsoft software.

RESULTS AND DISCUSSION

The efficacy (mean value) of *C. puteana* on scots pine, sapwood, was more than 30% (37.6 ± 2.3%). This means that, in accordance with the followed standard for determining the biological resistance (CEN TS 15083) of larch, the fungus used was sufficiently active and consequently the results were reliable. The efficacy test was performed according to the standard.

The average mass loss of the standard wood blocks was 25.6% (Table 2). The box plots of the tree samples with respect to mass losses (Fig. 1) showed great dispersion in the set of samples. The trees are arranged in the graph in order of increasing median mass loss.

A two-way analysis of variance (with family and tree nested within family) found highly significant differences between families ($p < 2.2\ e^{-16}$***) and between trees ($p < 2.337\ e^{-11}$***) for mass loss. The variance components showed high within-tree variation (68.1% of the total variance), which was followed by that at the tree (20.4%) and finally the family (11.4%) levels.

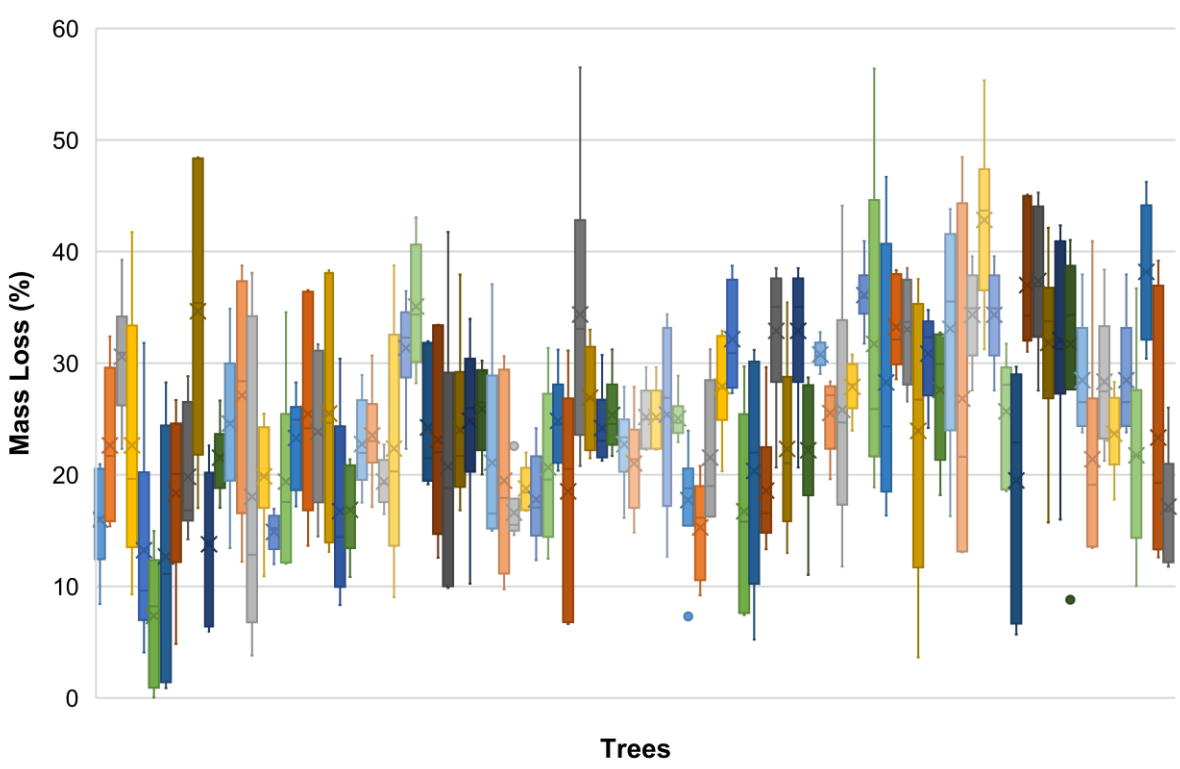

Fig. 1. Box and whiskers plot of the mass loss percentages of the set of samples, that originated from the different trees of the tested population. The rectangle (the "box") is delimited by the first and third quartiles and divided inside by the median. The segments (the "whiskers") are delimited from the minimum and maximum values.

This biological test had highly heterogeneous results at the tree level, which was reflected by an intra-class correlation coefficient (ICC) of less than 0.35. As reported by Venäläinen *et al.* (2003), in Scots pine small samples obtained from different areas and sections inside the trunk that were exposed to *C. puteana* for 6 w, the mass loss variation was high within the radial sections. Further, mass loss variation was even higher within the most durable section, which was the outer heartwood. This variation could not be explained satisfactorily by wood characteristics, such as extractives, wood density, or chemical parameters. Therefore, the activity of the fungus may have been dependent on unknown factors.

When considering the mass loss percentage grouped only on the criterium of the species, it is apparent that European larch represented the most durable group, followed by Hybrid larch and then the less durable was Japanese larch.

For comparison, the mass loss of the same standard samples using a robust NIRS calibration model developed by Sykacek *et al.* (2006) from spectra captured on the tangential face of the blocks. The average predicted mass loss for these samples was similar (24.4%) to the observed mass loss, but the Pearson correlation coefficient between the observed and predicted mass loss values was weak (0.301). Once a robust NIRS model has been obtained, its predictions are highly reliable, and it often allows for the detection of experimental errors or weaknesses. For NIRS prediction (Simenot *et al.* 2015), the intra-class correlation coefficient was much higher and reached 0.705 (Table 2).

Table 2. Basic Statistics (Number of Samples (n_i), Mean, Coefficient of Variation (CV)), and Intra-class Correlation Coefficients (ICC) for the Two Different Methods Utilized to Characterize the Larch Population

	n_i	Mean	CV (%)	Min-max	ICC
Standard Test Mass Loss					
Sample	585	25.6	34.0	-2.9 to 63.3	0.329
Tree	102	25.6	22.7	4.8 to 56.5	
NIRS Predicted Mass Loss					
Sample	592	24.1	19.8	6.9 to 37.4	0.705
Tree	102	24.4	12.8	16.7 to 37.4	
Density Loss					
Sample	197	9.3	141.3	-62.8 to 48.0	0.226
Tree	100	11.6	101.3	29.2 to 46.3	

The results indicated that this larch population was characterized by a low natural durability against *C. puteana*. This fact was supported by both methods considered. Slightly over 30% of the samples were evaluated as being moderately to highly durable. At the tree level, only 4 trees belonged to durability class (DC) 3, 72 belonged to DC 4, and 24 belonged to DC 5 (Fig. 2). These results differed from CEN EN 350 (2016), in which *Larch* spp. is considered to belong to DC 3 to DC 4. The classification from CEN EN 350 (2016) was based on mature trees (100 y to 200 y) grown in the Alps, a region where larch wood is traditionally used for external cladding, terraces, and shingles in traditional alpine cottages. In contrast, the trees in the larch population utilized in this study were young (under 30 y old) and cultivated at 540 m above sea level in much milder environmental conditions. The trees in this study were characterized by large rings due to their faster growth than the alpine trees. The results obtained with X-ray microdensitometry and fungal decay, which were elaborated using WinDENDRO software, are shown in Fig. 2.

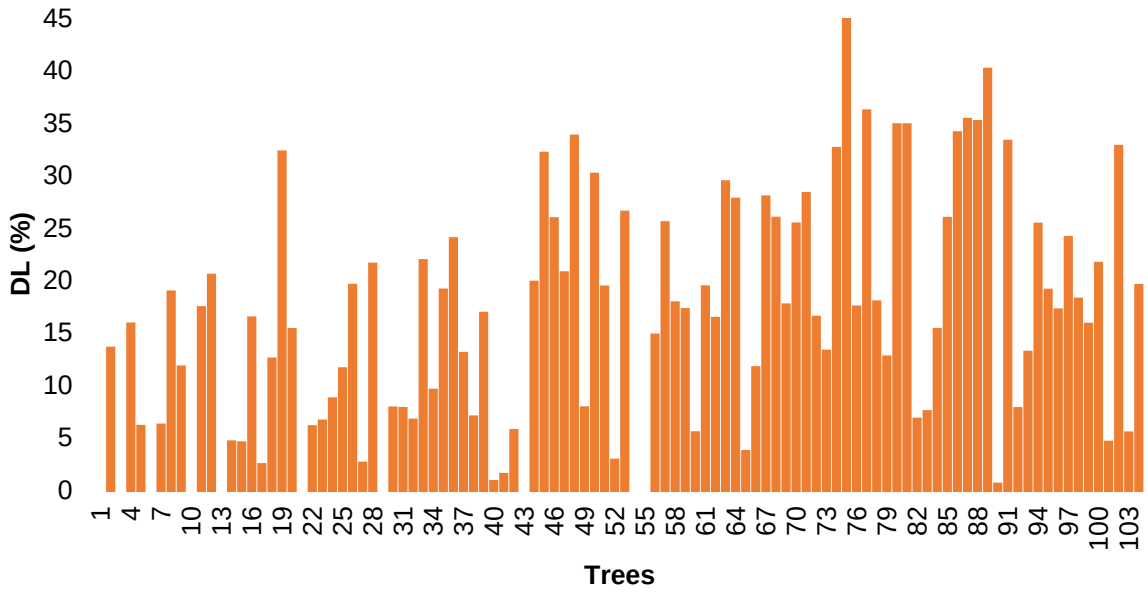

Fig. 2. Average density loss percentages of the set of samples (two radii) for the trees tested

The percentage of average density loss reached 9.3%. Its variability among samples and among trees was larger than that for mass loss, and its ICC was low. Significant differences were found among families ($p < 0.007$) but not among trees ($p = 0.308$). The highest source of variation was within trees (89.3% of total variance) and across families (10.7%).

Figure 3a shows that the loss of density along the thin wood slices progressively decreased on average from the pith to the heartwood-sapwood border.

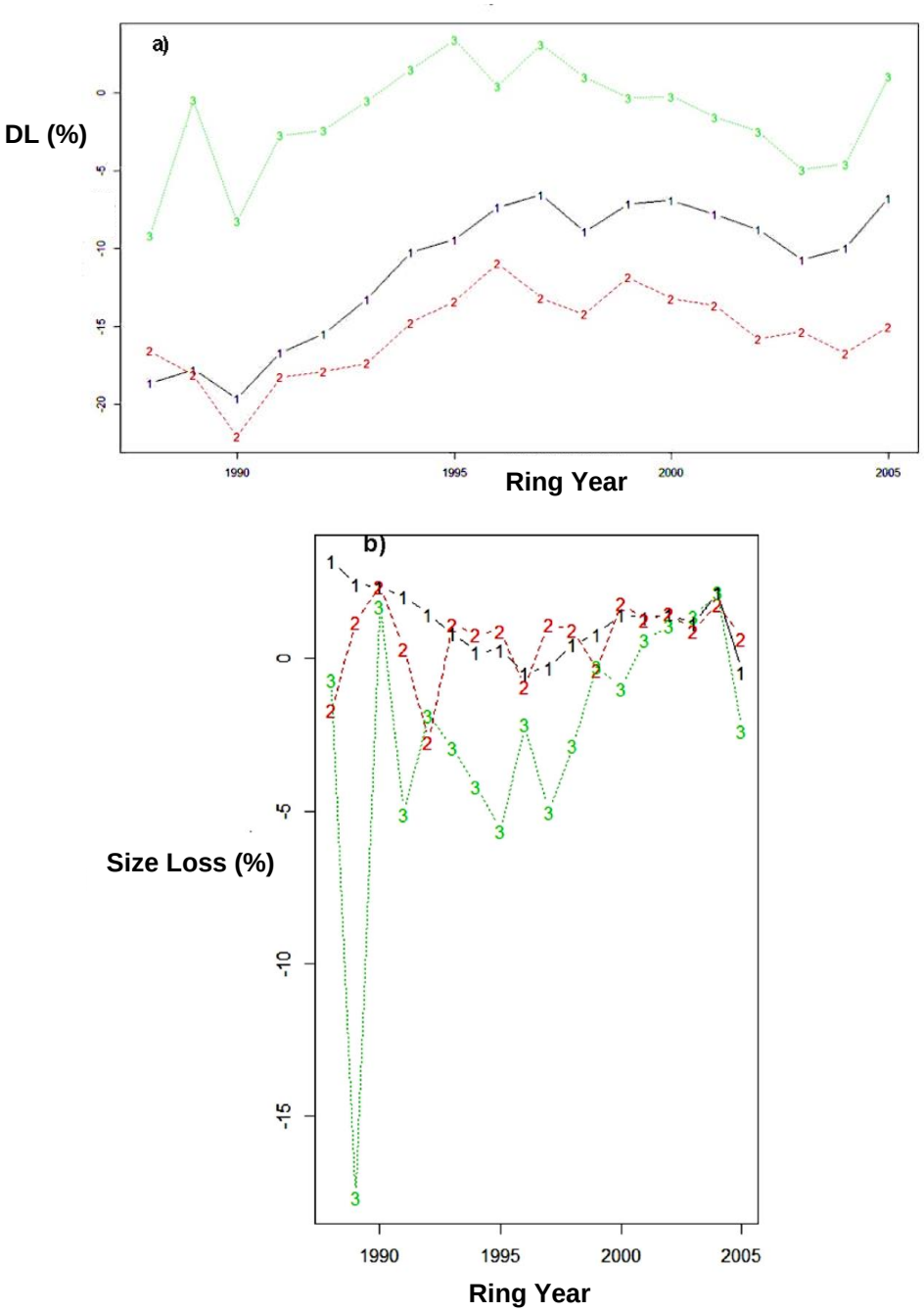

Fig. 3. (a) Average density losses from 1988 to 2005 rings (1, black line) in earlywood (2, red line) and in latewood (3, green line); (b) Mean size loss of slices at ring levels 1 (black overall ring, 2 (red earlywood), and 3 (green latewood)

This was consistent with the fact that extractive content, which is strongly linked to decay resistance, was higher in the outer heartwood than in the inner heartwood (Pâques *et al.* 2013) and continuously increased from the pith to the bark (Gierlinger and Wimmer 2004). Earlywood and latewood also responded differently to decay, and a much greater density loss occurred in the earlywood. In addition to density differences, differences in extractive content and composition (Antonova *et al.* 2012) between earlywood and latewood certainly contribute to such large differences in decay. Early stages of decay by brown-rot fungi were shown by Wilcox (1993) and Fuhr *et al.* (2011) to start in other conifers with the invasion of earlywood by the fungal hyphae and depolymerization of cellulose before the latewood was attacked. The greater availability of nutrients and greater number of pits are likely the main reasons for this phenomenon. However, this result conflicted with the observations by Macchioni *et al.* (2007) regarding Scots pine, as the latewood in this study decayed more than earlywood, and differences in cellulose content were identified.

Further, variation in ring size was also observed after fungal exposure, as shown in Fig. 3b. The latewood rings tended to shrink, whereas the earlywood rings enlarged slightly. The enlargement of earlywood can be explained by the increase in water permeability due to the opening of pits by fungal hyphae (Eaton and Hale 1993).

As can be seen in Table 2, density loss and mass loss had a low intra-class correlation coefficient, which indicated high heterogeneity among samples within trees; in contrast, NIRS predictions are much more homogeneous. The weaker ICC for DL and ML could have been due to the low sample size, as there were only two and six samples per tree, respectively.

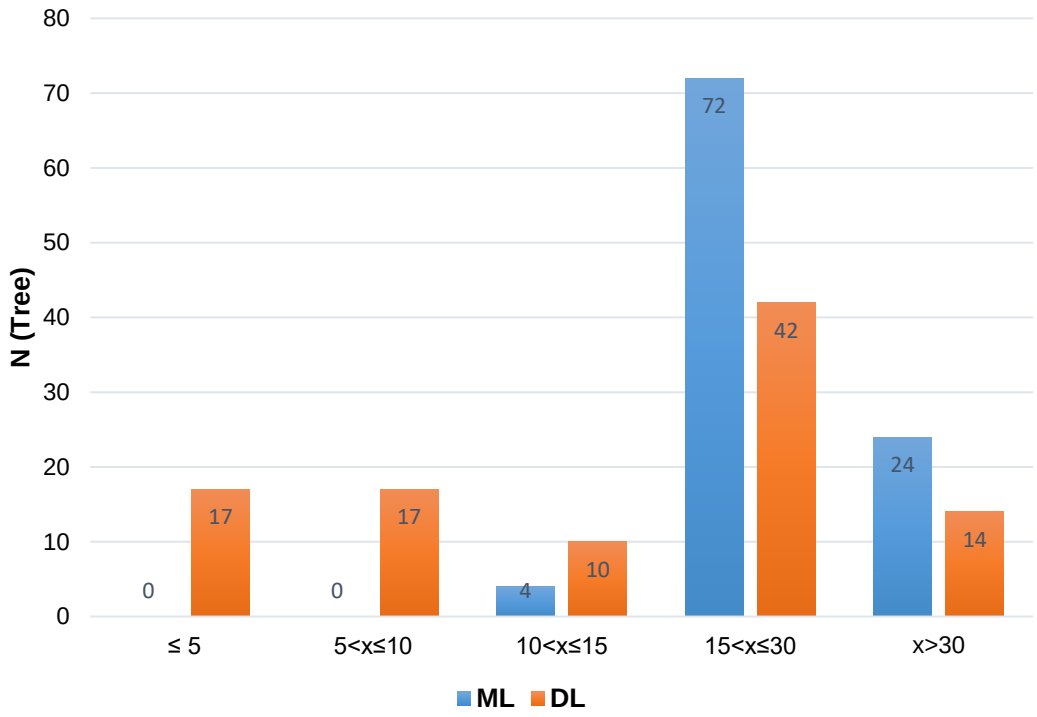

Fig. 4. Distribution of samples among durability classes for two different methods; x represents respectively, mass or density loss percentages in accordance with CEN TS 15083-1 (2015)

Differentiation among trees according to the method based on density loss appeared to be higher than that when based on mass loss. For the latter, most of the trees fell within the classes $15 < x \leq 30$ and $x > 30$, which corresponded to durability classes (DC) 4 and 5. The density loss method seemed to better discriminate among trees of all durability classes, which was similar to what has been reported in the literature for larch species (Gambetta *et al.* 2004; Curnel *et al.* 2008). In Fig. 4, the averages of the set samples, tree masses, and density loss percentages are distributed according to the durability index (x) described in Table 1.

The Pearson correlation coefficient between the means of mass loss percentages and the density loss percentages was low (0.41). The XY dispersion graph is shown in Fig 5. The cloud that represents the values of mass and density loss percentages put in evidence the low correlation found between the two methods.

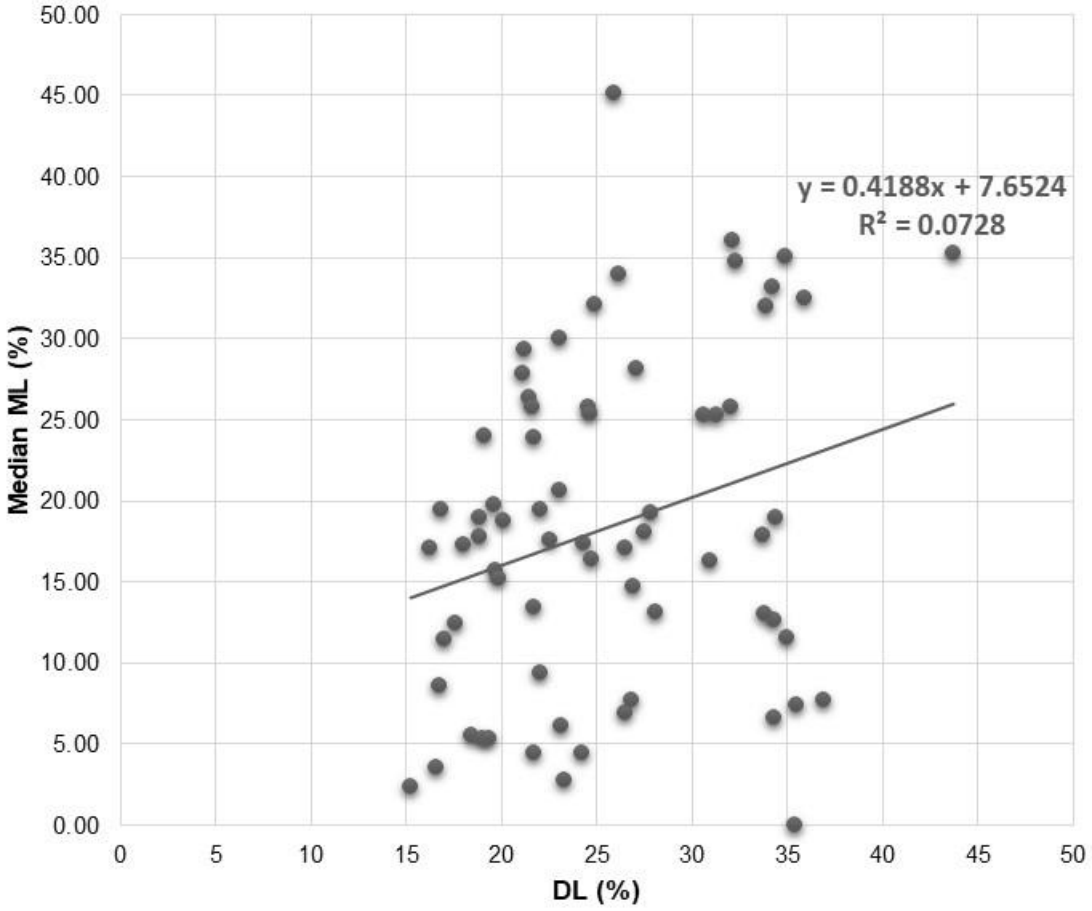

Fig. 5. Scatter graph of the median mass loss and density loss percentages; the equation of the trendline is shown.

Several factors could explain this weak correlation in addition to the limited sample numbers and the high intra-tree heterogeneity. First, in addition to the size of the wood samples, the shapes of the samples and the ways that they were in contact with the fungus were different; most of the slice samples in contact with the fungus were axial, which contrasted with the standard blocks, whose major faces in contact with the fungus were tangential and radial. In fact, a great difference was found in the shape and provenance of

the two sets of samples, which were wood standard blocks obtained from the last five rings area of trees logs and the X-ray microdensitometry samples that contained the entire sequence of heartwood from the transition zone to sapwood boundary. The entire heartwood was exposed to fungal decay to evaluate what happened at the ring level during the fungal attack. This difference may have played an important role in the weak correlation between the two methods.

Second, the durations of contact with the fungus also differed, as fungal exposure lasted for 4 w and 16 w, respectively. However, a shortened standard decay test was not possible due to the shape and dimension of the samples used in the standard test, and the first 4 w to 6 w are necessary to reach the right moisture content of the wood and subsequently permit the mycelium to colonize it from the culture media.

For the thin-slices test, a test duration of only 4 w allowed better observation of the differences among trees, even if they had low overall durability. In a previous trial with slices taken from the same areas within the trees but with a longer duration (8 w) (Palanti *et al.* 2018), the level of decay was much higher, as it sometimes reached wood cell collapse, and the density loss differences among trees were much lower.

However, the meaning of the results regarding this early degradation stage and its relationship with natural durability is questionable. The rather weak correlation (at the tree level) with a parallel mass loss assessment using the standard test CEN TS 15083-1 (2005) is disturbing in a sense but unsurprising. Similarly, the evaluation of MOE from bending tests on standing trees and from the static bending standard test on wood specimens gave rather different results (Pâques *et al.* 2009). Though they were rather close within trees, the samples used in the two decay assessments still differed, and sampling for the mass loss experiment addressed a larger portion of within-tree variability. Further, due to their different sizes, profiling, and positioning towards the fungus, the processes of invasion and later degradation by the fungus are likely quite different. In a future experiment, to obtain a better correlation between the values from the two different experiments (slices and density loss; wood blocks and mass loss), the protocol of sample preparation taking care to saw two samples (a slice and a block) from exactly the same rings at the same position within the trunk and with the same number of samples per tree should be improved.

For further progress, a new experiment should be conducted using i) more precise sampling for the comparison of the two methodologies and ii) a more variable set of genotypes well known for their contrasting decay resistance, as described by Curnel *et al.* (2008).

CONCLUSIONS

1. X-ray microdensitometry in conjunction with WinDENDRO® software was a high-throughput tool that is well adapted for obtaining density profiles from small wood specimens. Increment cores submitted to decay by a fungus could thus be easily assessed for density loss from density profiles before and after fungal attack.
2. Using this approach, this study showed the possibility of differentiating trees within a hybrid larch population for their resistance to *C. puteana* attack after 4 w and demonstrated the within-tree sample variation. The use of a 4-week period was optimal to avoid an overly strong decay incompatible with the microdensitometry approach (due to loss of matter or tracheid collapse).

3. The density loss microdensitometry approach provided information regarding the different behaviours of brown rot *C. puteana* at the ring level. It showed greater density loss and thus a faster expansion of decay in earlywood than in latewood during the first stage of fungal attack.
4. Compared to the standard method, this density loss approach proved efficient for shortening the experimental time and was determined to be the only method available that is suitable for non-destructively estimating the early stages of decay resistance in standing trees. These are two major requirements for genetic studies and breeding that rely on the screening of several hundreds to thousands of samples.

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Statement on Conflict of Interest

The authors declare that there was no conflict of interest.

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