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ORIGINAL PAPER

Automated quantification of intra-annual density fluctuations using microdensity profiles of mature *Pinus taeda* in a replicated irrigation experiment

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Abstract

Key message **We developed a new automated method to use wood microdensity profiles to detect and quantify intra-annual wood density fluctuations in earlywood of pine trees.**

Abstract We developed a new, automated method to use X-ray wood microdensity profiles to detect and quantify intra-annual density fluctuations within the earlywood region (IADFe) of each annual growth ring. The method quantifies the number and area of IADFe by detecting variations in wood density beyond the limits of "normal" values observed during periods without environmental stress. As a case study, we examined the effect of water availability and water stress associated with the formation of IADFe in irrigated and non-irrigated 11-year-old loblolly pine trees. As expected, non-irrigated trees formed significantly more IADFe than irrigated trees. Strong relationships were observed between IADFe formation (as a proportion of total earlywood area or as the number of IADFe formed each year) and the minimum monthly Palmer Drought Severity Index for the earlywood growing season (February to July). When compared against visual detection, the number of IADFe detected optically was significantly fewer than the number detected with densitometry. This difference likely comes from the higher resolution and more objective criteria that confidently detected and counted more very small IADFe than was possible optically at 10× magnification. The method to detect and quantify IADFe described in this study can allow climate analysis in long-lived species prone to producing intra-annual growth zones and false rings.

Keywords Growth rings · X-ray densitometry · False ring · Specific gravity · Water availability · Palmer drought severity index · Loblolly pine

Introduction

The annual formation of secondary xylem in temperate trees can provide valuable long-term records of climatic variability. However, understanding the genetic mechanisms and environmental factors controlling intra-annual variation in wood anatomy is crucial to establishing valid relationships between annual wood growth and climate (Fritts 1976; Wimmer et al. 2000; Larson et al. 2001). The best studied intra-annual variation in secondary xylem is the differentiation of cambial meristem derivatives into earlywood and latewood. Compared with earlywood, latewood tracheids and vessels have narrower diameters and lumens and thicker secondary walls (Fritts 1976; Larson et al. 2001).

In temperate conifers, the temporal dynamics of differentiation into earlywood and latewood tracheids within a growing season are under environmental and genetic control, and the climate drivers affecting wood formation can differ between early and late growing seasons (Parker 2014). In the southeastern United States, Henderson and Grissino-Mayer (2009) reported that earlywood formation

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was related to water availability during spring and Larson (1994) concluded that shortened photoperiod can induce latewood formation. Harley et al. (2012) similarly reported that seasonal variation in solar radiation is the main control of intra-annual growth dynamics for *Pinus elliottii* var *densa*. Evidence for the importance of water availability on intra-annual variation in tracheid density comes from the analysis of tracheids in false rings, which have morphologies similar to latewood, suggesting it is possible that water stress prematurely induces the same signal involved in annual latewood formation.

Intra-annual density fluctuations in earlywood (IADFe) are discrete regions of latewood-like cells in the earlywood portion of a tree's annual growth ring (Larson 1963; Fritts 1976; Young et al. 1993). The number and timing of IADFe is significantly correlated with reduced water availability (extended drought) during the early part of the growth season before regular latewood formation starts (Larson 1963; Campelo et al. 2007; de Luis et al. 2007; Sánchez-Vargas et al. 2007; Copenheaver et al. 2010; Edmonson 2010; de Luis et al. 2011). Based on these correlations, it has been suggested that IADFe are useful tool to reconstruct annual climatic information, and several authors have used IADFe as environmental indicators of early season drought or water stress in different tree species (Ewel and Parendes 1984; Young et al. 1993; Priya and Bhat 1998; Villalba and Veblen 1996; Masiokas and Villalba 2004; Wimmer et al. 2000; Sánchez-Vargas et al. 2007; Rossi et al. 2009; Gruber et al. 2010; Vieira et al. 2010; Olivar et al. 2012). However, causative evidence for the induction of IADFe by water stress is still non-conclusive and the results are mixed. For example, for *Pinus baknsiana*, Copenheaver et al. (2006) found no relationship between IADFe formation and climatic factors but others studying a variety of species (e.g., Copenheaver et al. 2010; de Luis et al. 2011; Battipaglia et al. 2014) concluded that drought was the main factor behind IADFe formation. Some of this variation in the cause of IADFe formation is likely due to differences in which environmental factors control growth across the tested sites (Battipaglia et al. 2010, 2014; Novak et al. 2013). In addition, formation of IADF in pines has been reported to be also dependent on tree age (Campelo et al. 2013) and size (Novak et al. 2013).

The width of annual growth rings reflects the environmental conditions that the woody plant experienced during the year the ring was formed (Fritts 1976; Bräuning 1999). For a variety of pine species, several authors reported associations between variations in ring width, presence of IADFe and drought, e.g., Bräuning (1999), Chernavskaya et al. (1999), Ogle et al. (2000), Wimmer et al. (2000), Rigling et al. (2001, 2002), Campelo et al. (2007), de Luis et al. (2007, 2011), Sánchez-Vargas et al. (2007), Rozas et al. (2011) or Olivar et al. (2012). In all these studies water availability was not controlled and the associations reported lack of statistical correlation or an explicit model to estimate ring width variations or IADFe production as function of environmental variables.

To establish causation, experimental drought treatments of seedlings or saplings have been used to examine IADFe responses (Larson 1963; Glerum 1970; Lloyd et al. 1996; Priya and Bhat 1998; de Luis et al. 2011). However, mixed results have been reported and as yet no controlled common garden field study with large trees has been reported that causatively links water availability with IADFe production. In addition, few standardized protocols have been established to objectively identify and quantify IADFe occurrence (Koubaa et al. 2002; De Micco et al. 2012); most researchers rely on subjective visual identification of IADFe, combined with statistical cross-dating techniques. In recent years, X-ray densitometry (Steppe et al. 2004; Mora et al. 2007) and tomography (Van den Bulcke et al. 2009) had been used to analyze wood anatomy. Our objective was to develop a new, automated method with X-ray densitometry profiles to detect and quantify IADFe in pine trees. This novel method uses variations in wood density beyond the limits of the normal values under non-environmental stress to identify and quantify IADFe number and area. As a case study, we examined the effect of water availability on intra-annual growth zones, determining the amount of water stress that trigger IADFe formation through the analysis of wood density profiles from loblolly pine (*Pinus taeda* L.) growing in a long-term irrigation experiment. Here we report on the first controlled experiment with large, field grown pine trees. We hypothesized that (1) irrigated trees would produce fewer IADFe than rainfed trees, and (2) the intensity of IADFe would be correlated to changes in water stress.

Materials and methods

Site and stand description

The study took place in an irrigation experiment established in January 1995 by International Paper, Inc. in the Upper Coastal Plain 22 km west of Bainbridge, GA, USA (30°48′N latitude and 84°39′W longitude). Soils at this location were classified as well-drained Grossarenic Paleudults, with 0.5 m sandy loam over sandy clay loam (Samuelson 1998).

The study included two water availability treatments: a water irrigation treatment with drip irrigation from March to November and a non-irrigated control treatment (Samuelson 1998). Yearly water additions ranged between 210 mm in 1996 and 1127 mm after year 1999 (at year 2003 the irrigation system was not functioning, at year

Table 1 Age 11 mean diameter at breast height (DBH), mean tree height, basal area (BA) and leaf area index (LAI) for Irrigation treatments. Values in parenthesis are standard error ($n = 3$)

	Control	Water availability	$P > F$*
DBH (mm)	205.3 (3.1)	220.7 (4.2)	**0.002**
Height (m)	15.7 (0.3)	17.5 (0.4)	**0.009**
BA (m^2/ha)	33.5 (0.7)	39.8 (1.9)	**0.003**
LAI (m^2/m^2)	4.3 (0.1)	4.5 (0.2)	0.348

Mean at age 11 (Dec-2005), LAI was measured on Oct-2005. *P* values <0.05 are shown in bold. * *P* values using mixed procedure

2004 the irrigation system worked only intermittently, and at year 2005 the irrigation system started in June; Gonzalez-Benecke et al. 2010). The water availability treatments selected for this study represent a subset of the whole experiment, which also included additional genetic sources, fertilization and pest control treatments. Within each water availability treatment we collected data from two plots containing different genetic sources. The data from the two genetic source plots was pooled obtaining a single value for each water availability treatment within each block. As was reported previously by Gonzalez-Benecke et al. (2010), there was no irrigation by genetics interaction effect in growth (stem diameter, height, basal area and leaf area index) or wood properties. The experimental design contained three replicates in a complete randomized block design. The measurement plot areas were 0.026 ha, containing 28 sample trees planted at 2.4 × 3.7 m spacing and surrounded by two buffer rows. Additional site and experimental details have been reported by Samuelson et al. (2004, 2008). Table 1 summarizes the stand characteristics at age 11 and shows that irrigation significantly increased growth and basal area.

Meteorological measurements

Long-term meteorological measurements from stand establishment (January 1995) until the wood properties sampling date (April 2006) were obtained from a weather station installed in an open area adjacent to the study site (30°49′N latitude and 84°′W longitude). Monthly values of Palmer drought severity index (PDSI; Palmer 1965) were obtained from the national climatic data center of NOAA (http://www.ncdc.noaa.gov/temp-and-precip/drought/historical-palmers.php). Monthly average values of Palmer drought severity index (PDSI) indicate that the site experienced long periods of water deficit (Fig. 1a). Between April 1998 and May 2002, PDSI averaged −2.47 (classified as “moderate drought” in Palmer 1965), and reached a minimum of −4.02 during August 2000 (classified as “extreme drought” in Palmer 1965), with no single month average above zero. Between January 1995 and December

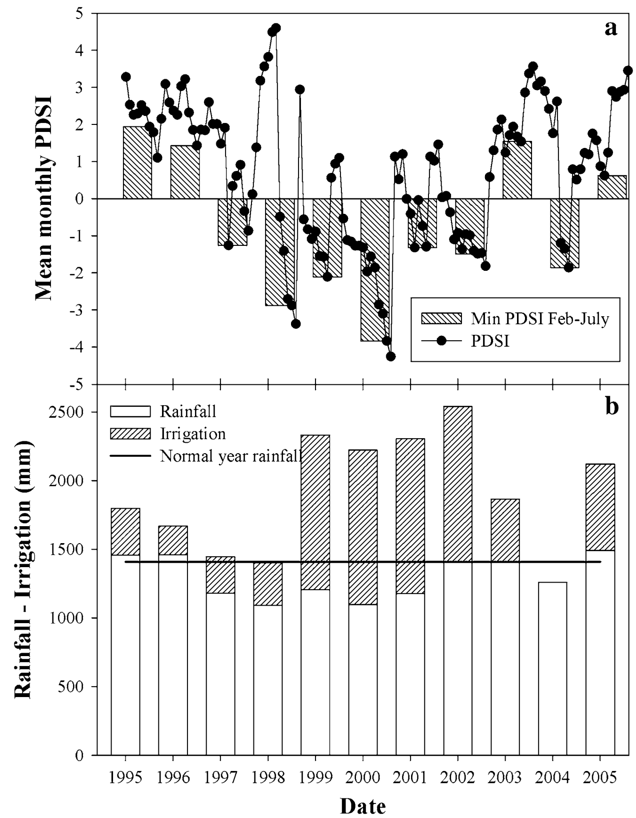

Fig. 1 **a** Monthly average Palmer drought severity index (PDSI) (*line with black circles*) and minimum monthly mean PDSI between February and July and **b** total rainfall (*open bar*) and water added by drip irrigation (*dashed bar*) during each year from 1995 through 2005 on the study site. Thirty-year rainfall mean for the site (1,408 mm) is indicated with a *straight line* on **b**

2005, 53 % of the time PDSI was negative. Yearly rainfall records from 1995 to 2006 indicated that precipitation was in most cases lower than normal, being 15–22 % lower from 1997 to 2001, and 3.5–5.8 % lower in 1995, 1996 and 2005 (Fig. 1b).

Wood properties

In April 2006, 12 mm increment wood cores that crossed from bark to bark (passing through the pith) were extracted at breast height from eight trees per plot (same 96 trees used for diameter and sapflow measurements reported by Gonzalez-Benecke and Martin (2010) and Gonzalez-Benecke et al. (2010)]. The cores were split at the center of the pith and dried at 40 °C, then were glued to core holders and sawn into ~2 mm thick strips (Clark et al. 2006). One radius was stored as a backup for further analysis and the other was sent to the USDA-Forest Service Forestry Sciences Laboratory in Athens, GA, USA for X-ray densitometry analysis (Hoag and Krahmer 1991; Clark et al. 2006; QTRS-01X, Quintek Measurement Systems, Knoxville, TN, USA). A radial

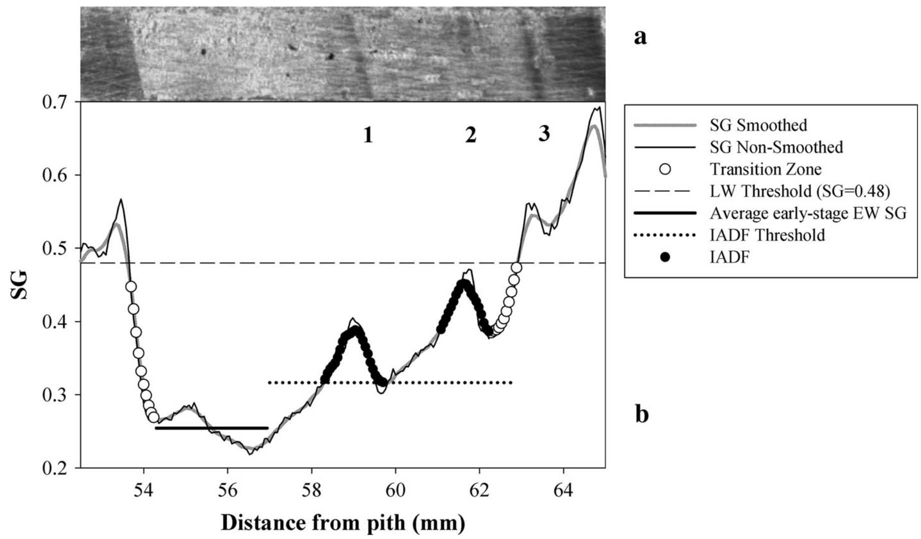

Fig. 2 Optical image of a selected earlywood ring of a tree growing under non-irrigated conditions (**a**), and example of basic specific gravity (SG) profile demonstrating the delineation of intra-annual density fluctuations in earlywood (IADFe) for the same earlywood ring (**b**)

profile in basic specific gravity (SG) from each radial strip was determined for each ring on all 96 sample trees at 60 μm intervals, and a SG threshold value of 0.48 was used to distinguish earlywood from latewood. This threshold method to differentiate earlywood from latewood is the standard used for wood properties analysis using X-ray densitometry for loblolly pine in US (Clark and Saucier 1989; Clark et al. 2004, 2006; Jordan et al. 2007, 2008; Antony et al. 2009). Latewood SG (SG_L), earlywood SG (SG_E), latewood width (W_L, mm), earlywood width (W_E, mm), ring width (W_R, mm), latewood percentage (LW %, %) and whole-ring SG (SG_R) were determined for each ring on all 96 sample trees and reported in Gonzalez-Benecke et al. (2010).

Determination of number and area of IADFe

We defined IADFe as regions of increased density with latewood-like tracheids that form prematurely within the season when genetic and environmental factors are conducive to earlywood and before normal latewood formation. Our definition of IADFe differs slightly from the traditional definition of Kaennel and Schweingruber (1995): "a layer of cells within a tree ring identified by different shape, size and wall thickness". Prominent IADFe are delineated regions of higher density in the earlywood portion of the annual growth ring, and have been predominantly detected optically by differences in color. Although a difference in color facilitates counting, it is not adequate for quantifying their area. To objectively quantify IADFe number and area requires microscopy of sections or microdensitometry with standardized thresholds for wall thickness to lumen ratio (Denne 1988) or minimum density definitions that can be used to objectively delineate latewood-like regions within earlywood. We developed a

Table 2 P values from time series analysis ANOVA for wood properties of rings formed between year 1999 and 2005, for Irrigation and Control treatments

Trait	$P > F$*		
	Water availability	Year	Water availability × year
SG_E	0.68	**<0.001**	**<0.001**
$SG_{E\text{-}E}$	0.56	**<0.001**	**<0.001**
SG_{IADFe}	0.56	**<0.001**	**<0.001**
SG_{Th}	0.63	**<0.001**	**<0.001**
IADFe $_{\%}$	**<0.001**	**<0.001**	**<0.001**
$IADFe_{c\text{-}od}$	**0.001**	**<0.001**	**<0.001**
$IADFe_{c\text{-}\rho}$	**0.010**	**<0.001**	**0.001**

Analysis for IADFe was carried out with transformed values

P values <0.05 are shown in bold

SG_E specific gravity of earlywood, $SG_{E\text{-}E}$ specific gravity of early-season earlywood, SG_{IADFe} specific gravity of intra-annual density fluctuations in earlywood, SG_{Th} threshold specific gravity to determine intra-annual density fluctuations in earlywood, *IADFe %* area percentage of intra-annual density fluctuations, $IADFe_{c\text{-}od}$ total count of intra-annual density fluctuations in earlywood using optical detection, $IADeF_{c\text{-}\rho}$ total count of intra-annual density fluctuations in earlywood. * *P* values using mixed procedure

method to automate the counting and area measurements of IADFe using microdensity data. To estimate the area of each ring, we assumed that the stem of the pine tree is circular. For each sample analyzed, the number and area of IADFe of each ring was determined as follows:

1. The radial SG profile was smoothed using moving averages, estimating, for each point of the profile, a smoothed SG as the average of four previous and four posterior values along the radial profile (Fig. 2);
2. The mean and standard deviation of early-season earlywood SG ($SG_{E\text{-}E}$) was calculated for each EW

ring. This early-season zone was defined as the points within the first third of distance between the transition zone from LW of the previous year to EW of the current year, and the transition zone from EW to LW of the current year (Fig. 2). The means of $SG_{E\text{-}E}$ and standard deviation of SG_E across treatments and years were 0.2822 and 0.0086, respectively (Table 2). With this criterion we estimated for each ring an average "baseline" SG of EW cells produced, assuming that environmental signals that tend to increase SG_E are rare in the early growing season;

3. The transition zone from LW of the previous year to EW of the current year was determined as the area between EW start (first point with SG lower than 0.48) and the point at which SG no longer declined with no break in that trend (left tail of the EW ring in Fig. 1) for a minimum of seven points (420 µm). This minimum number of seven consecutive points was determined empirically to properly account for the transition zone;
4. The transition zone from current year EW to next year LW was determined as the area between LW start (last point with SG lower than 0.48) and the point when SG starts to increase with no break in that trend (right tail of the EW ring in Fig. 2) for a minimum of seven points (420 µm);
5. A zone of potential IADFe was determined, for each EW ring, as the points with SG larger than a threshold value of baseline $SG_{E\text{-}E}$ plus three standard deviations, excluding the transition zones. The three standard deviations criterion establishes a buffer zone that ensures that any point delimited as IADFe will have less than 1 % chance to have SG within the normal values for early-season EW;
6. After identifying the zone of potential IADFe and the starting point of the transition zone from current year EW to next year LW, any point with SG larger than that point will be considered as a IADFe until SG reaches a value lower than the break point (transition zone starting point). All the points with SG lower than the break point are not considered as IADFe, but as part of the normal trend increasing SG from EW to LW. If a new break in that trend is found, any point with SG larger than that new break point will be considered as a new IADFe until SG reaches a value lower than the second break point. A minimum of four consecutive points (240 µm) are required to be considered an IADFe. This minimum number of four consecutive points was determined empirically. Even though the moving averages smoothing procedure reduced errors in X-ray densitometry data, some "false" IADFe were detected and iteratively eliminated until getting the adequate threshold number of four points. The same procedure continues until analyzing all points in the potential IADFe zone;
7. The area of each annulus that corresponds with each measurement point at 60 µm intervals was determined, using the center of the pith as the starting point;
8. The area of each IADFe determined in step v, was calculated as the sum of all annulus areas that were tagged as IADFe points;
9. The IADFe proportion was estimated for each earlywood ring as the fraction between earlywood ring area and IADFe area.

Figure 2 shows an example of the image, using reflection microscopy image (Fig. 2a), and the delineation of IADFe, using X-ray densitometry (Fig. 2b), of a selected growth ring. All values below the threshold value of 0.48 (dashed line) were considered earlywood. Within the earlywood zone, IADFe (black-filled circles) were considered all points above the IADFe threshold (dotted line) that do not comply with the normal trend of increasing SG from EW to LW (step vi of IADFe determination procedure) and are not included in transition zones (white-filled circles). Intra-annual density fluctuations present in the selected growth ring showed in Fig. 2 were identified with numbers 1, 2 and 3. It is important to remark that sometimes sample preparation is not optimum, making difficult the visual detection (2). Also intra-annual density fluctuations in latewood can be confounded with IADFe, inducing to incorrect IADFe identification (3). Under these circumstances, the use of X-ray densitometry improves the identification, reducing the detection errors.

Given the quite consistent $SG_{E\text{-}E}$, we defined IADFe as any area wider than 240 µm (≥8 cells) in the earlywood portion of the ring having greater than three times the standard deviation above the baseline SG_E of the early season earlywood and that does not comply with the normal trend of increasing SG from EW to LW. Implementing this approach is complicated by the need to distinguish between IADFe and the transition to normal latewood. The transition zones (from LW of the previous year to EW of current year, and from current year EW to LW) were determined as the points between EW start (or ending, in the case of EW-to-LW transition) and the point at which SG no longer declined with no break in that trend for a minimum of seven points (420 µm). Yearly trends in SG for control and irrigated plots are shown in Fig. 3.

The occurrence of IADFe was also measured macroscopically for rings formed between years 1998 and 2005, using a 10x scaled magnifier by counting the darker regions within each earlywood ring.

The first three growth years (1995 and 1997) were eliminated from the analysis because: (1) wood cores were sampled at 1.4 m stem height and only 29 % of the trees reached

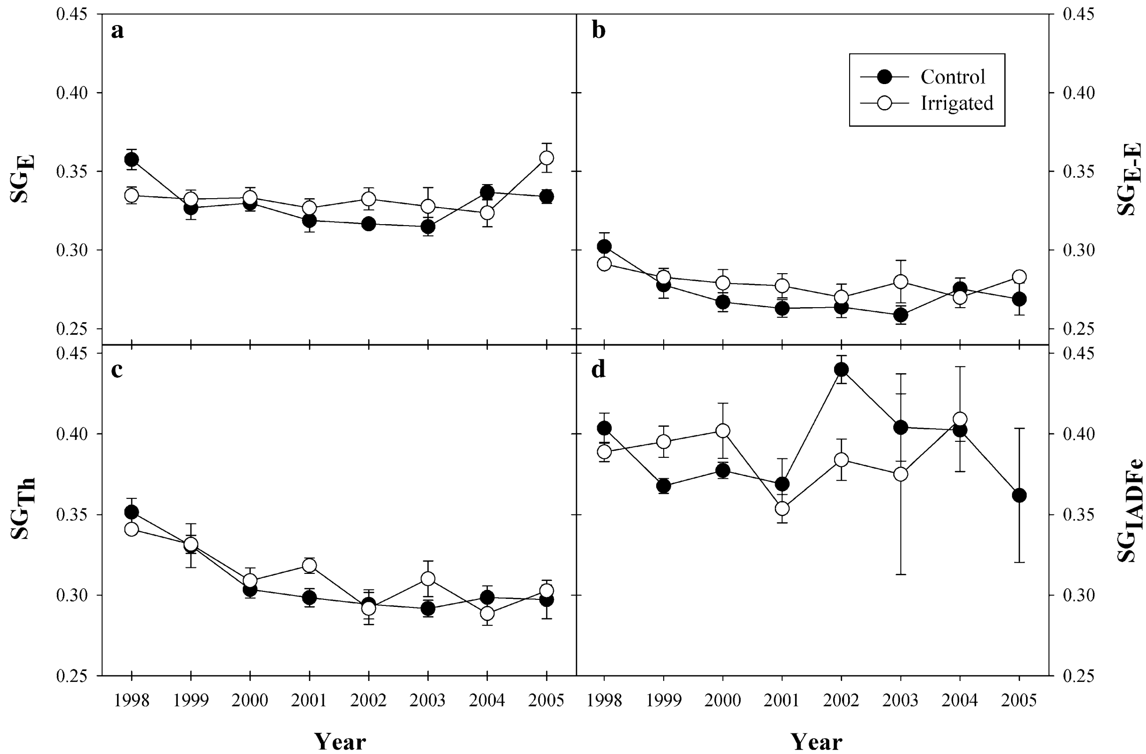

Fig. 3 Annual earlywood SG (SG_E) (**a**), early-season earlywood SG ($SG_{E\text{-}E}$) (**b**), IADFe threshold SG (SG_{Th}) (**c**), IADFe SG (SG_{IADFe}) (**d**) during each year from 1998 through 2005 for water availability treatments

that height in 1995, year 1; and (2) even though around 94 % of the trees showed a growth ring in the sampled wood core in 1997 (for 65 % of the trees that was the first "ring"), those rings were mostly pith-associated wood (Larson et al. 2001). Gonzalez-Benecke et al. (2010), reported for the same samples analyzed in this study, that irrigation significantly affected ring SG and LW %, but had no effect on wood velocity stiffness. Weighted whole-core SG of earlywood (SG_E), early-season earlywood ($SG_{E\text{-}E}$), IADFe (SG_{IADFe}) and IADFe threshold (SG_{Th}) were calculated as the product of the sum of the specific gravity for all earlywood rings by the corresponding earlywood ring basal area and divided by total tree earlywood basal area (Jordan et al. 2007).

Statistical analysis

As IAFDe area percentage (IADFe %) and count ($IADFe_c$) are not normally distributed, natural-logarithm and logit transformation were carried out for both traits as follows:

$$\ln(\mathrm{IADFe_c}) = \ln(\mathrm{IADFe_c} + 0.05)$$

$$\mathrm{logit}(\mathrm{IADFe\%}) = \frac{\mathrm{IADFe\%} + 0.05}{1 - \mathrm{IADFe\%} + 0.05}$$

Analysis of variance (ANOVA) was used to analyze the effects of irrigation on IADFe production and wood property traits, including Bonferroni adjustments for differences in least square means (PROC MIXED, SAS Inc., Cary, NC, USA). The linear model for the analysis was:

$$Y_{ij} = \mu + b_i + I_j + (bI)_{ij} + p_{ij} + \varepsilon_{ij}$$

where Y_{ij} is the parameter value of the plot of the *j*th irrigation treatment in the *i*th replicate; $i = 1,2$ and 3 for replications; $j =$ control and irrigated; and

- μ population mean,
- b_i random variable of replication $\sim$NID $(0,\sigma_b^2)$, (NID, normally and independently distributed),
- I_j fixed effect of irrigation (control or irrigated),
- $(bI)_{ij}$ fixed effect for replication × irrigation interaction $\sim$NID $(0, \sigma_{bI}^2)$,
- p_{ij} random effect of plot $\sim$NID $(0, \sigma_p^2)$,
- ε_{ij} error term $\sim$NID $(0,\sigma_\varepsilon^2)$

Repeated measures analysis was used to analyze time series data. Several co-variance structure models were used for the time series analysis (power, heterogeneous first-order autoregressive, compound symmetry, heterogeneous Toeplitz, unstructured and uncorrelated models) and the model with the lowest Schwartz's Bayesian information criterion (BIC) was selected for each variable analyzed (Littell et al. 1996). Empirical R^2 (Myers 2000) was determined using data from the adjusted model determined from the mixed procedure.

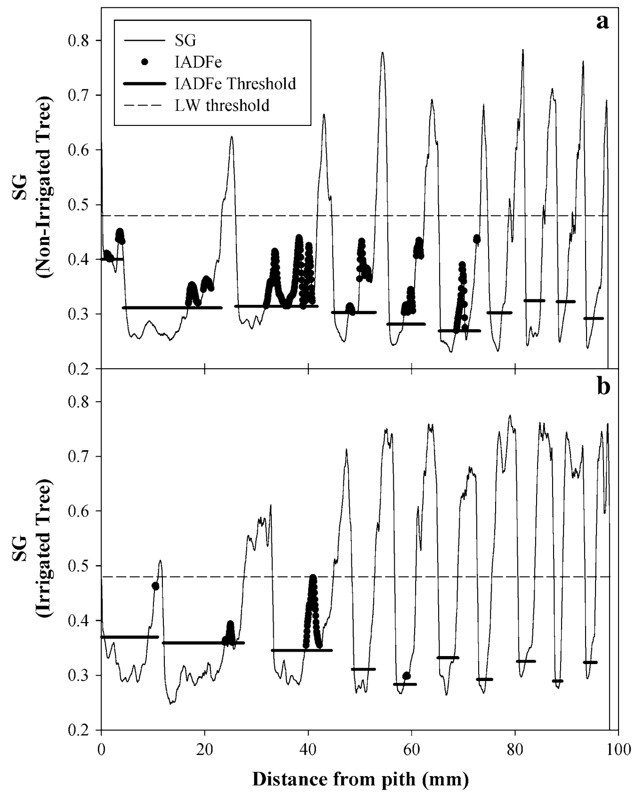

Fig. 4 Example microdensity profiles showing IADFe determination for non-irrigated (**a**) and irrigated (**b**) trees of similar size. In other studies, all values below the 0.48 threshold value (*dashed line*) have been considered earlywood. Within earlywood, IADFe (*black-filled circles*) were considered all points above the IADFe threshold (*solid line*) that do not comply with the normal trend of increasing SG from EW to LW and are not included in transition zones

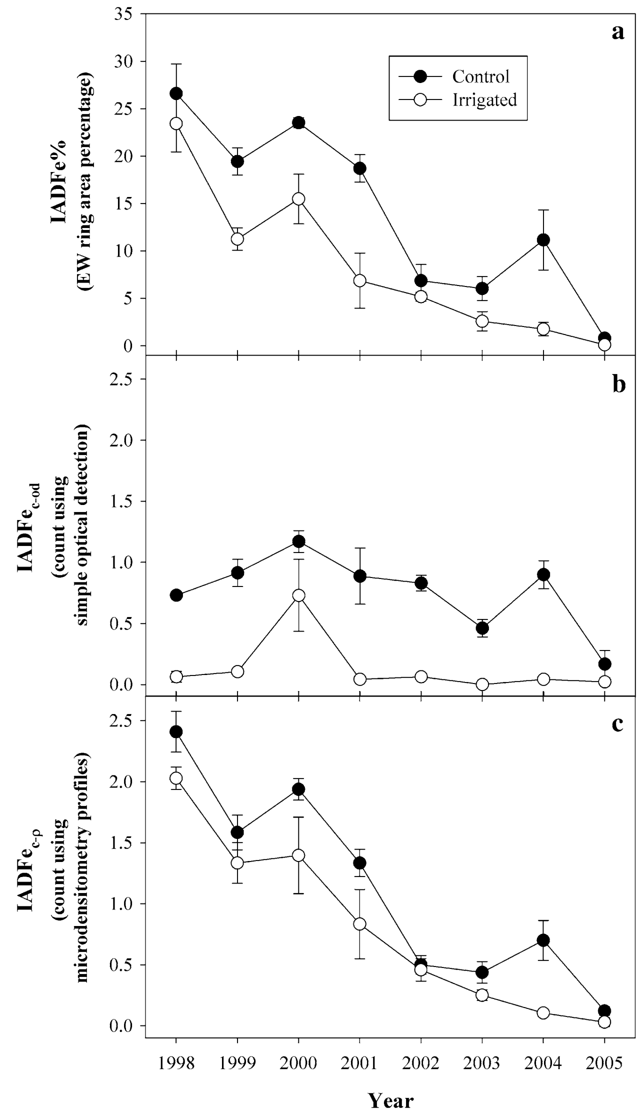

Fig. 5 Average IADFe area percentage (**a**), IADFe count using simple visual detection (**b**) and IADFe count using densitometry profiles detection (**c**) during each year from 1998 through 2005 for water availability treatments

Results

Determination of IADFe

The procedure developed and used in this study effectively detected IADFe in microdensitometry profiles of juvenile wood from loblolly pine trees (Fig. 4). Analysis of our density profiles showed many IADFe above the standard earlywood density but below the minimum specific gravity threshold of 0.48 SG used to define LW in loblolly pine (Clark et al. 2006; Jordan et al. 2007).

Our analysis showed that the mean SG of earlywood formed in the early part of the season ($SG_{E\text{-}E}$) across all treatments and all years was 0.2695 with a narrow standard deviation of 0.0086 (Table 2; Fig. 4). Within each year, the basic specific gravity of earlywood, early season earlywood, the threshold for IADFe and the IADFe were similar for irrigated and non-irrigated treatments (Table 2).

Water availability effects on IADFe

Formation of IADFe was highly affected by water availability, using both simple optical detection and counting of IADFe (Fig. 5b) and our new automated IADFe area proportion method with SG microprofiles (Fig. 5a, c). When IADFe were detected with densitometry profiles and expressed as the percentage of earlywood ring area (IADFe %), in which SG departed from early-season earlywood SG (assumed as non-water-stressed), a strong effect of irrigation on IADFe % ($P < 0.001$) was observed (Fig. 5a; Table 2). The significant year by irrigation interaction ($P < 0.001$) in response to water availability was due to irrigation and control plots having similar IADFe area in year 2000, when PDSI showed extreme drought, and in years 2003 and 2005 (Fig. 5), when the irrigation treatments were intermittent or stopped. The number of IADFe and IADFe % estimated with densitometry profiles were strongly, positively correlated ($P < 0.0001$), IADFe count explained 95 % of variability in IADFe % (data not shown).

A significant effect of irrigation on IADFe count based on optical detection ($IADFe_{c\text{-}od}$) was found; however, the response to water availability depended on the year of growth ($P < 0.001$ for year by irrigation interaction; Fig. 5a; Table 2). In 2000, when PDSI was below −2 for the whole year, irrigated and control plots had the same number of IADFe (mean $IADFe_{c\text{-}od} = 1.32$), and in 2005 (mean $IADFe_{c\text{-}od} = 0.24$), when irrigation started on June 15, affecting mostly latewood formation, and PDSI was always positive, irrigated and control plots had also similar number of IADFe. $IADFe_{c\text{-}od}$ for control and irrigated plots averaged 1.13 and 0.32, respectively. When IADFe count was carried out with SG microprofiles ($IADFe_{c\text{-}\rho}$), the effect of water availability on the number of IADFe also depended on the year of growth ($P < 0.001$; Table 2). Irrigated trees formed fewer IADFe than non-irrigated trees (expressed as a percentage of total earlywood area IADFe %, or as total number of IADFe). When the number of IADFe detected optically and using densitometry was compared, the number of IADFe detected with densitometry was significantly larger than number of IADFe detected optically, on both, irrigated and non-irrigated trees ($P < 0.001$). There was a significant year by irrigation interaction in the difference between both detection methods ($P < 0.001$). In year 2000, when PDSI showed extreme drought, and in years 2003 and 2005, when the irrigation treatments were intermittent or stopped, there was no difference in the number of IADFe detected using both methods.

The difference likely comes from the greater sensitivity of the densitometry with higher resolution and more objective criteria implemented that confidently detected and counted more and very small IADFe than was possible optically at 10× magnification.

On a weighted whole-core basis, irrigation showed no effect on earlywood SG (Table 3), averaging across irrigation treatments, values of 0.33, 0.27 and 0.35, for SG_E, $SG_{E\text{-}E}$ and SG_{IADFe}, respectively. On the other hand, irrigated trees produced fewer IADFe than non-irrigated trees (expressed as a percentage total earlywood area or as total number). For non-irrigated trees, the number of IADFe detected optically and using densitometry did not differ ($P = 0.279$). However, for irrigated trees, the number of IADFe detected optically was significantly fewer than the number detected with densitometry ($P = 0.005$).

Table 3 Means for whole-core wood properties on earlywood ring formed between year 1999 and 2005, for irrigation and control treatments

Trait	Control	Water availability	$P > F$*
SG_E	0.324	0.330	0.73
$SG_{E\text{-}E}$	0.267	0.277	0.785
SG_{IADFe}	0.381	0.396	0.608
SG_{Th}	0.302	0.307	0.885
IADFe $_{\%}$	13.8	7.9	**0.002**
$IADFe_{c\text{-}od}$	5.2	1.0	**<0.001**
$IADFe_{c\text{-}\rho}$	6.5	4.7	**<0.001**

Analysis for IADFe was carried out with transformed values

P values <0.05 are shown in bold

SG_E SG of earlywoo, $SG_{E\text{-}E}$ SG of early-season earlywood, SG_{IADFe} SG of intra-annual density fluctuations in earlywood, SG_{Th} threshold SG to determine intra-annual density fluctuations in earlywood, *IADFe %* area percentage of intra-annual density fluctuations in earlywood, $IADFe_{c\text{-}od}$ total count of intra-annual density fluctuations in earlywood using optical detection, $IADFe_{c\text{-}\rho}$ total count of intra-annual density fluctuations in earlywood using SG variations. * P values using mixed procedure

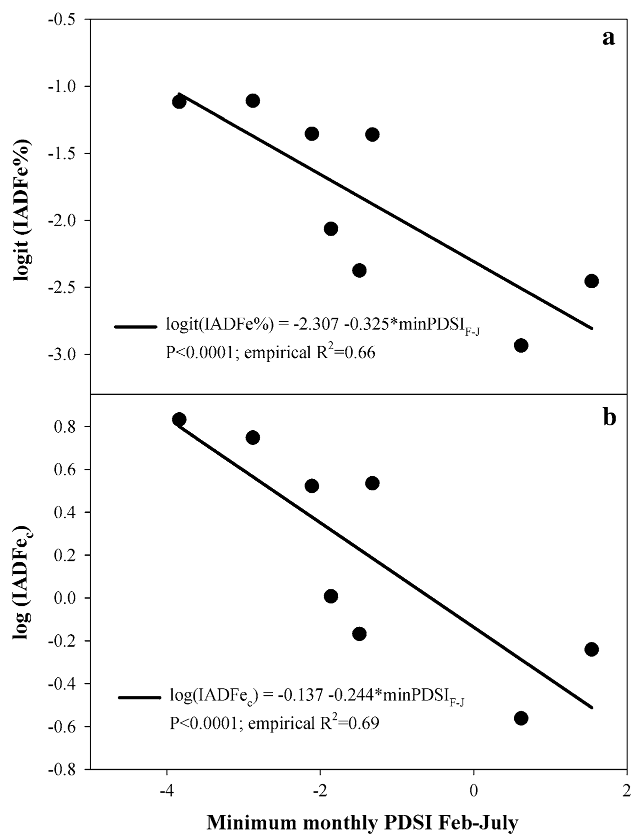

Fig. 6 Relationship between **a** logit-transformed average proportion of IADFe area in earlywood [logit-transformed; logit (IADFe %) and **b**] log-transformed average IADFe count [log($IADF_c$)] for non-irrigated trees and the minimum monthly Palmer drought severity index during February–July ($minPDSI_{F\text{–}J}$) for years 1998–2005

Correlation between PDSI and IADFe

For non-irrigated trees, there was a strong effect of the mean monthly minimum PDSI between February and July ($PDSI_{F-J}$) and the area percentage of IADFe (IADFe %) produced in that season ($P < 0.001$; empirical R^2 was 0.66; Fig. 6a). That period from February to July has been defined as the earlywood growing season for loblolly pine in Southeast US (Blanche et al. 1992; Jayawickrama et al. 1997). Significant but weaker effects were also observed between IADFe % and mean monthly PDSI during the earlywood growing season (February to July) (empirical $R^2 = 0.39$) and mean monthly PDSI during the whole year (empirical $R^2 = 0.40$) (data not shown). The frequency of IADFe ($IADFe_c$; log-transformed) was also affected by PDSI ($P = <0.001$; empirical $R^2 = 0.69$; Fig. 6b). The models predict that if minimum PDSI during the earlywood growing season is −4, then loblolly pine trees will average around 2.3 IADFe, corresponding to 37 % of the early-wood ring area. For years when minimum PDSI between February and July is 0, the average number of IADFe should be around 0.8, corresponding to 5 % of the early-wood ring area.

Discussion

Determination of IADFe

To quantify the area of IADFe, we developed a new automated method that uses X-ray densitometry profiles. In this method, the specific gravity threshold for IADFe classification was set at three standard deviations above the average earlywood specific gravity of the first third of the new season's growth for each ring, where genetic and environmental conditions are most favorable for earlywood formation. The selection of the first third of the ring as the early-earlywood was based on the consistency of SG in our samples and from the results of Park et al. (2006), who in their method for multiple intra-ring demarcation describe an early-earlywood zone that corresponds to the cells with a wall-lumen ratio of 0.125, located approximately within the first 40 % of intra-ring position. In our method, this early season baseline average was calculated independently for each annual growth season for all wood samples. Also supporting our assumption about the stability of SG_{E-E}, Bouriaud et al. (2005) reported that wood density of *Picea abies* was not affected by growth rate and climatic conditions during the first part of the growing season, but highly affected during the second half.

Our rationale for using the three standard deviation threshold rather than the typical LW threshold of 0.48 SG (Clark et al. 2006; Jordan et al. 2007) is that in loblolly pine, the average early-season EW density was consistent within a growth season and varied little across treatments and cambial ages (see Table 3). Koubaa et al. (2002) and Park et al. (2006) suggested adjusting the threshold for EW and LW demarcation when analyzing intra-ring variability using inflexion point and error zone methods, respectively. In the error zone method, intra-ring variations in cell wall to lumen ratio classifies cells as EW or LW and establishes a buffer zone defined as 1/20 of the standard threshold of cell wall to lumen ratio of 0.25 (Denne 1988). Another complication in quantifying IADFe relates to the normal timing of LW differentiation. To avoid misclassifying IADFe close to the boundary of LW, a transition zone from EW to LW was determined between LW start (first point with SG larger than 0.48) and the point when SG starts to increase with no break in that trend. Even though the smoothing of data helped reduce micro-variations in SG due to measurement errors, we defined a minimum number of four points to be considered as IADFe. This approach highly reduced erroneous assignments of IADFe. This new method automatically detects and quantifies IADFe area with microdensity profiles, allowing the analysis of narrow rings with small number of cells, similar to high resolution optical methods (Schweingruber 1996; Speer 2010).

Water availability and IADFe

While numerous observational studies suggest that decreased water availability during the early part of the growing season affects IADFe formation (Larson 1963; Jayawickrama et al. 1998; Rozenberg et al. 2002; Sánchez-Vargas et al. 2007; De Luis et al. 2007; De Micco et al. 2012; Parker 2014), many of these studies suffer from analysis of few trees, single year of analysis, and the difficulty in establishing the timing of the drought relative to the IADFe formation (Camarero et al. 1998; Rigling et al. 2001; Bouriaud et al. 2005). Moreover, in data collected from natural stands at different locations, genetic differences confound the environmental conditions. In our study, we experimentally manipulated soil water availability in large trees of known genetic origin growing in a common garden field site and evaluated its effect on IADFe formation.

For the same trees included in this study, Gonzalez-Benecke and Martin (2010) reported large differences in water status between irrigated and non-irrigated trees. For example, on rainfed plots, where available water of the whole soil profile (0–2 m depth) reached values lower than 10 % for long periods (see Figs. 3, 5 in Gonzalez-Benecke and Martin 2010), canopy stomatal conductance and whole-tree sapwood-specific hydraulic conductance were reduced to less than 1/3 of irrigated plots. The ratio between stand transpiration and potential evapotranspiration was decreased from around 80 % on irrigated plots to around 30 % on non-irrigated plots. In our study a consistent effect of soil water availability on intra-annual SG fluctuations was observed. Irrigated loblolly pine trees always produced less IADFe than non-irrigated trees. The presence of IADFe in earlywood from year 2000 in irrigated trees can be explained by irregular irrigation application at that time (Samuelson et al. 2004), and 12 months (July 1999–July 2000) with PDSI lower than −2.5. Fewer IADFe were detected optically than with densitometry profiles. This difference is explained by the fact that small alterations in cell size are reflected in subtle variations in wood density and are detected by densitometry, but are too subtle to confidently detect optically.

The anatomy of xylem cells are thought to be affected by plant water status (Kramer and Boyer 1995; Tyree and Zimmermann 2002). The larger IADFe of non-irrigated tress seem to be the product of both, tracheids with thicker cell walls and smaller lumen diameters (Kramer and Boyer 1995; Szeifel et al. 2006). For *Pinus sylvestris*, Gruber et al. (2010) and Eilmann et al. (2009, 2011) and for *Abies balsamea* (D'Orangeville et al. (2013) reported that drought produced tracheids with smaller lumen and thicker cell walls. Sheriff and Whitehead (1984) reported, for *Pinus radiata* subjected to dehydration, that water stress reduced lumen diameter and increased cell wall thickness in newly matured stem tracheids. The same authors reported that there was a sharp decline in photosynthetic rate when xylem water potential reached values lower than approximately −1.8 MPa. These results are consistent with our findings, where trees with lower water availability produced EW rings with more IADFe. Gonzalez-Benecke and Martin (2010) reported midday xylem water potential of approximately −1.9 MPa for the same non-irrigated trees.

The formation of IADFe has been related to ecophysiological processes. For example, for *Pinus pinaster*, De Micco et al. (2007) and for *E. arborea*, Battipaglia et al. (2014), concluded that the formation of IADF was correlated with increased water use efficiency. For the same trees used in this study, Gonzalez-Benecke and Martin (2010) reported reduced foliar carbon isotope composition on irrigated trees, indicating higher water use efficiency for trees with reduced water availability that produced more IADF. As earlywood cells have larger lumen diameter than latewood-like IADF cells, it is expected that IADFe will have lower hydraulic conductivity. Domec and Garner (2002) reported that latewood of *Pseudotsuga menziesii* has 11 times less saturated hydraulic conductivity than earlywood, similar resistance to cavitation under well-watered conditions, but higher resistance to cavitation under dry conditions. The same authors also reported that the capacitance of latewood was four times larger than earlywood under well-watered conditions. The authors indicated that the evolutionary significance of forming latewood-like cells after a period of drought during normal earlywood growing season can be explained as a conservative adaptation that reduces water use and decreases embolism under water deficit. Martinez Meier (2008) suggested a similar explanation: as IADFe cells have higher resistance to cavitation due to thicker cell walls and smaller diameter lumen, which may help trees to maintain the integrity of the hydraulic system. Hacke et al. (2001) concluded that thicker cell walls produced under water stress is needed to reduce the risk to cell implosion. It is important to mention that under drought, during the expansion phase of tracheid differentiation, smaller cells are produced because water shortage reduces cell turgor needed for cell enlargement (Plomion et al. 2001). More research is necessary to validate these results, expanding the measurements to bordered pit structure, which has been demonstrated to control drought-induced cavitation in gymnosperms (Domec et al. 2006; Pittermann et al. 2005, 2006).

Relationship between PDSI and IADFe

Palmer Drought Severity Index (PDSI) is an integrated measure of water deficit where temperature and precipitation are included in a water balance model that accounts for soil moisture supply and transpiration rate demand (Palmer 1965). Previous studies reported that PDSI correlates better with ring width than rainfall (Jenkins and Pallardy 1995; Ogle et al. 2000; Eilmann 2009). Ogle et al. (2000) also concluded that ring width was better correlated with growing season PDSI than with annual PDSI. On a recent study on mature *P. elliottii* trees, Parker (2014) concluded that PDSI was the most important factors determining tree growth. Eilmann et al. (2009) found no correlation between radial increment of *P. sylvestris* and temperature, but strong correlations with PDSI. Our rationale to use PDSI is also based on the results of Dougherty et al. (1994) which indicated that stem diameter growth cessation date in *P. taeda* depends on soil moisture content and transpiration rate, and Sheriff and Whitehead (1984) who demonstrated that water deficit reduced lumen diameter and increased cell wall thickness in *P. radiata*. The strong relationship between EW growing season PDSI and IADFe production (Fig. 6) indicates water stress is a main factor affecting intra-annual density fluctuation during EW formation in loblolly pine trees.

The relationships between minimum PDSI during the earlywood growing season and number of IADFe and IADFe area percentage can be used as a tool to estimate drought intensity from intra annual wood density fluctuations. The methodology developed in this study can be used, for example, to quantitatively estimate relationships between IADFe formation and environmental variations in long-living species such as *Taxodium distichum*, which is characterized as readily forming false rings (Young et al. 1993) but has been reported as unsuited for climatic studies (Ewel and Parendes 1984). Further research is underway to evaluate the genetic tendency of different loblolly pine sources to produce IADFe, using water balance from tree transpiration, soil water content and meteorological data, and also assessing the applicability of the method to analyze intra-annual density profiles on trees of different age and species.

Limitations of the method

Even though for *P. taeda* sapwood area production remains relatively constant after canopy closure (Gonzalez-Benecke et al. 2010), ring width tends to decrease with tree age (see Fig. 4). The identifying method reported here requires earlywood rings larger than 840 µm to properly detect IADFe. In our dataset, the thinnest earlywood ring had a width of 300 µm, and more than 97 % of the earlywood rings were wider than 1,000 µm. From a total of 845 earlywood rings analyzed, only 12 rings (1.4 %) were not able to be analyzed (data not shown). We estimate that the developmental characteristic of decreasing earlywood ring width with age is unlikely to affect the ability of our method to detect IADFe for loblolly pine. However, for other slower-growing species, we encourage to use this method for trees with stem radial growth larger than 2 mm per year. In spite of that, the model can be adapted to use higher resolution wood density data, as that from X-ray tomography (Van den Bulcke et al. 2009), allowing for analysis of slow growing species.

Conclusion

We developed a new automated method to detect and quantify IADFe associated to density fluctuations in earlywood of pine trees. Using this methodology we conclude that water stress is a main factor affecting intra-annual density fluctuation in earlywood of *P. taeda* trees. The method described in this study can allow climate analysis in long-lived species prone to produce IADFe. The program for IADFe identification is available in SAS and R+ languages, and will be free distributed upon request to the corresponding author.

Author contribution statement CA. Gonzalez-Benecke (CAG-B) and G.F. Peter (GFP) contributed the idea and design for the research. CAG-B and A.J. Riveros-Walker conducted research. CAG-B, GFP and T.A. Martin wrote manuscript.

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Conflict of interest The authors declare no conflict of interest.

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