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Functional wood traits: xylogenesis, tree rings and intra-annual density fluctuations (IADFs) in Mediterranean species

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GENERAL OVERVIEW

Nowadays the relationships between wood anatomical traits and environmental factors are more and more investigated to predict plants' responses to expected climate changes and assess the vulnerability of forests to the increasing severity of climatic stresses, such as drought (McDowell et al., 2008; Gea-Izquierdo et al., 2014). In Mediterranean ecosistems woody species are able to cope with abrupt changes in climatic conditions, including summer drought, thanks to the high plasticity of their wood functional traits that allows to achieve a trade-off between the need to maintain high conductivity, when water is available, and the need to prevent phenomena of embolism during periods of severe drought (Cherubini et al., 2003; Sperry, 2008; Meinzer et al., 2010; De Micco and Aronne, 2012). Many wood traits within the tree rings (e.g. size and thickness of the cell walls of the conductive elements and pits) are very sensitive to environmental variability and are modulated in order to contribute to the safety–efficiency tradeoff of water transport (Carlquist, 1984; De Micco et al., 2008; Martínez-Vilalta et al., 2012; Lens et al., 2013; Gleason et al., 2016). Environmental variability can thus be recorded in wood traits within the tree rings that represent in this way archives which can provide information on eco-physiological state of the trees during their growth period. Indeed, lumen size is generally considered as an indicator of water availability which guides turgor-driven cell enlargement, while cell-wall thickness is treated as indicator of photosynthate availability (Cuny et al., 2015). In fact, wood traits are determined by the cambial activity and the subsequent phases of cell differentiation. In the Mediterranean species, xylogenesis often shows a bimodal pattern in response to the "double stress" due to the alternation of summer aridity and low temperature in winter (Mitrakos, 1980; Lev-Yadun et al., 2000). This double stress is recorded within the tree ring as abrupt changes in the density defined "Intra-annual Density Fluctuations" (IADFs) (De Micco et al., 2016a). The analysis of IADFs in tree-ring series has recently awakened the interest of scientific community since they represent a proxy to provide information with intra-annual resolution on the relationship between environmental conditions and eco-physiological processes occurred during the period of wood formation (Battipaglia et. al, 2010; De Micco et al., 2014). However the relationships between IADFs and environmental conditions are not always straightforward; moreover, they seem to be species-, age- and site-dependent (Battipaglia et al., 2016; Zalloni et al., 2016).

To achieve a complete understanding of IADF ecological meaning, two approaches can be applied: a) a retrospective approach based on the analysis of tree-ring series to unravel how plants have reacted to past environmental changes; b) an *in-vivo* analysis of xylogenesis to follow the formation of the tree rings *in real-time*. The retrospective approach on tree-ring series has been applied on several Mediterranean trees and shrubs and the combination of dendrochronology, quantitative wood anatomy and climate data has been proven to be valuable to make hypotheses on the environmental factors triggering IADF formation (de Luis et al., 2007; De Micco et al., 2007; Battipaglia et al., 2010; Vieira et al., 2010; De Micco et al., 2014; Zalloni et al., 2016). More recently, the need to support such hypotheses with the investigation of cambial activity and IADF formation has been highlighted (De Micco 2016b; Pacheco et al., 2016; Ziaco & Biondi 2016).

Understanding the different adaptive ability of the different species to cope with environmental constraints through the modulation of xylem functional traits linked to efficiency and safety of water transport can help forecasting plant behaviour and vegetation dynamics in the context of expected environmental changes.

In this framework, the main objectives of the presented thesis are: *a)* to assess the attitude of different Mediterranean species, both softwood and hardwood, to form IADFs in different growth condition, identifying which wood functional traits are the most appropriate to highlights their presence within the tree-ring series; *b)* to evaluate the relationships between IADFs and environmental conditions to elucidate their functional role to contribute to the safety–efficiency tradeoff of water transport. To pursue these aims, the two approaches based on retrospective analysis of tree-ring chronologies and xylogenesis were applied on different Mediterranean species growing at different sites in Italy.

Our results proved the occurrence of IADFs in all the species considered namely *Pinus halepensis* Mill., *Pinus pinaster* Aiton, *Pinus pinea* L., *Erica arborea* L., and *Arbutus unedo* L.

In tree rings of *Erica arborea* L. and *Arbutus unedo* L. growing at a xeric and a mesic site on the Elba Island we identified the occurrence of different types of IADFs (Chapter 2). In particular, *E. arborea* formed *E*-IADFs (characterised by the occurrence of latewood-like conduits in earlywood) in both sites, in response to summer drought to promote safety in water transport. *A. unedo*, instead, formed *E*-IADFs in the xeric site and *L*-IADFs (characterised by the occurrence of earlywood-like conduits in latewood) in the mesic site. *L*-IADFs were formed to promote efficient water transport in response to rain events following the summer drought. Moreover we assessed that vessels and fibres parameters show the same sensitivity to environmental fluctuations, and represent good indicators of summer drought to describe and interpret the ecological meaning of IADFs in tree rings. In *Pinus pinaster* and *Quercus ilex* growing at the same sites on the Elba island we also highlighted the occurrence of *L*-IADFs appearing as an extra-band of wood formed in latewood (Chapter 3). Such IADFs were characterised by earlywood-like tracheids in the latewood of the softwood, while appeared as an additional band of wood with earlywood-like vessels in the oak. In *Pinus halepensis*, *Pinus pinea* and *Arbutus unedo*growing at two sites in Southern Italy, we analysed xylogenesis and detected the periods of IADFs formation during the calendar year, also linking the different phenological phases of xylem formation to their triggering environmental factors (Chapters 4 and 5). Although the species showed different timing of cambial activity, in all of them *L*-IADF occurred, promoted by rainfall events following a period of drought. Correlations with climatic parameters combined with the retrospective approach in *Pinus pinea* and *Arbutus unedo* finally suggested that IADF timing of formation in *Arbutus unedo* and specific anatomical traits confer to this species a high adaptation capability to the frequent intra-seasonal variations of water availability, often resulting in the formation of more than one IADF per year (Chapter 6).

In addition to the main aims of the thesis, two supplementary topics have been investigated. The first regarded the study of conduit occlusion, a natural phenomenon occurring during heartwood formation or in response to stress-induced embolism in sapwood (Chapter 7). The second regarded the application of the study of wood functional traits in horticultural science in order to verify whether a different plasticity of hydraulic anatomical traits can explain the different growth behaviours of an ornamental crop under conditions of water stress in order to manage deficit irrigation treatments (Chapter 8).

INTRODUCTION AND STATE OF THE ART

In recent years, forest ecology researchers have showed increasing interest in studying wood anatomy in trait-based ecology (Kattge et al., 2011). Wood-trait analysis linked to classical ecological wood anatomy and dendrochronology represents a powerful tool to understand plant's ability to cope with rapid environmental changes, including anthropogenic climate changes (McDowell et al., 2008; Martinez-Vilalta et al., 2008; Beeckman, 2016). Ecological wood anatomy has been applied since the end of 20th century (Carlquist, 1975, 1980, 1984; Baas, 1976; Wheeler et al., 2007), with the aim to elucidate the relations between wood anatomical features of plant species and the conditions of the ecosystems in which they grow. Some research lines focused the attention on phenotypical modifications of the wood structure in response to different growing environmental conditions (Baas & Miller, 1985); such discipline is referred as "wood anatomical trait-based ecology" (Beeckman, 2016).

In wood anatomical trait-based ecology, dendrochronology and quantitative wood anatomy (QWA) methodologies are often applied. For instance, the intra-annual variability of traits such as size and abundance of vessels can reflect interesting environmental signals as precipitation seasonality, allowing to treat them as dendrochronological variables (Verheyden et al., 2004; García-González & Fonti, 2006; Campelo et al., 2010).

Dendrochronology is the study of events through time that are recorded in tree ring features. The main aim of dendrochronology is to develop chronologies through the principle and the methodology of cross-dating assuring that every single ring is associated to the calendar year when it was formed. QWA is the methodological approach based on the measurement of anatomical traits of the wood cells (e.g. density and thickness of cell walls, lumen area of tracheas or tracheids, form and frequency of xylem elements, etc.), analyzed in time and used to characterize the relationships between plants growth and various environmental factors. Combining these methodologies it is possible to obtain time series of intra-annual variability of anatomical traits, providing information on the functional role of xylem plasticity under environmental constrains over the life span of trees (De Micco et al., 2012; Abrantes et al., 2013).

Involving wood anatomy into a trait-based ecology presupposes good definitions since is based on a quantitative approach. Violle et al. (2007) defined functional traits as “morpho-physio-phenological traits which impact fitness directly via their effects on growth, reproduction and survival”. Recently, traits have been defined by Lachenbruch and McCulloh (2014) as “any morphological or chemical measure that does not change over the period in which a performance is examined” where “performance” should be understood as a “quantifiable level at which a plant unit accomplishes a function over a particular period of time.” The latter highlights in a better way the fundamental link between trait functions and the period during these functions are expressed, needed to define wood traits. The terminology of wood anatomical features is well defined in the IAWA feature lists for hardwood and softwood identification (IAWA Committee 1989, 2004) and it should be taken into consideration in project including wood traits analysis.

Trait-based ecology assumes morphological traits as determiners of physiological performance, which influences vital rates and determines individual fitness and life history evolution (Alder, 2014). In Mediterranean forests, cross-species relationships between functional traits and the survival and growth rates of individuals has been found (Alder, 2014).

Mediterranean species, growing in environments characterised by drought periods, show structural alterations that are mainly related to the efficiency and safety in the water transport in order to achieve a trade-off between the need to maintain high conductivity, when water is available, and to prevent phenomena of embolism in arid conditions (Cherubini et al., 2003; Sperry, 2008; Meinzer et al., 2010; De Micco and Aronne, 2012). Some wood traits, such as the size of conductive elements and cells wall tickness, are proportionally related to the efficiency and safety of water transport (Carlquist and Hoekman, 1985; Tyree and Ewers, 1991; Carlquist, 2001; Tyree and Zimmermann, 2002; De Micco and Aronne 2007; Wheeler et al., 2007; Sperry et al., 2008; Martínez-Cabrera et al., 2009; Poorter et al., 2010). Narrower conduits are less efficient in water transport compared to wider ones, but such higher efficiency is also linked to lower vulnerability to embolism. On the contrary, larger conduits are more efficient in water flow but are more vulnerable to embolism. However, under stressful conditions, primed by either biotic or abiotic factors, embolism can occur and there happens a compartmentalization process culminating in vessel occlusion by tyloses or gums, depending on the size of conduits. In xeric conditions, short conductive elements with reduced lumen area, thicker walls and simple perforations are favoured. Xeromorfic woods are also characterized by an increase of vessel frequency, known as "redundancy". In dry conditions, the redundancy of conductive elements ensures the transport of water even if a part of them is embolised (Carlquist 1975; Baas et al. 1983). In relation to the size of vessel lumen, the conductivity increases in proportion with the fourth power of the vessel radius (Van den Oever et al., 1981; Zimmermann, 1983), thus vessels with reduced lumen area only allow a slow water flow but on the other hand they assure the transport of water even when the vessels with the wider lumen are embolised (Carlquist, 1975). The type of pit between adjacent vessel is a genetically-controlled character to solve the conflict between the need to minimise vascular resistance and limit the occurrence of embolism phenomena (Lens et al., 2011). Reduced porosity decreases the probability of cavitation even in an indirect way considered that the pore size is proportional to the diameter of the elements of the vessels (Wheeler et al., 2005). Vessel grouping is also positively related with the resistance to cavitation (Lens et al., 2011). If in a group of embolized vessels, one of them remains active, it still provides water transportation. The same role can be played by vasicentric tracheids, distributed around the vessel.

At the tree ring level, earlywood reflects the traits of highly efficient wood, characterized by large lumen vessels with thin cell walls. Latewood, instead, reflects the traits of a safe wood with narrow vessels characterised by thick cell walls. The transition from earlywood to latewood is perceptible by a variation in the wood density, as happen between tree ring boundaries. Variations in the growth conditions can determine thus changes in density within the ring referred as Intra-annual Density Fluctuation (IADFs). IADFs are regions within a tree ring where abrupt changes in density occur (De Micco et al., 2016a). They can occur as either a dark band of dense latewood-like cells within the earlywood or a light band of less dense earlywood-like cells within the latewood. In some species, the occurrence of specific anatomical features, such as the increased incidence of thin-walled parenchyma cells, narrow marginal parenchyma bands, and resin canals, can help to distinguish IADFs from true ring boundaries (Campelo et al., 2007a; Schweingruber, 2007); however the more reliable method to identify IADFs is the cross- dating of tree-ring series.

IADFs can be formed whithin the tree ring in response to environmental changes during the calendar year that affect cambial activity and cell differentiation. Their appearance and their radial position within the ring is thus determined by the time in which the triggering factor occurred (Campelo et al., 2007a) and their analysis may provide accurate information at the intra-seasonal level (Bräuning, 1999; Campelo et al., 2007b; Battipaglia et al., 2010, 2014; De Micco et al., 2012), allowing a more detailed climate analysis within the growing season (Wimmer et al., 2000; Novak et al., 2013a,b, 2016). The occurrence of IADFs has been found in different biomes but they have been study mostly in Mediterranean ecosystems. A complete description and classification of IADFs is reviewed in De Micco et al. (2016a). IADFs occurring in Mediterranean conifers were mainly classified into four types by Campelo et al. (2007b, 2013) according to their relative position within the earlywood and latewood. More specifically: 1) *E*-IADF occurs in the first half of the ring as a band of latewood-like cells in the earlywood; 2) *E*+-IADF occurs at the end of the earlywood, as cells with features intermediate between true earlywood and true latewood cells, called transition cells, but are often more similar to latewood-like cells; 3) *L*-IADF is found in the second half of the ring as earlywood-like cells in the latewood; 4) *L*+-IADF occurs between the latewood and earlywood of the next tree ring as a band of earlywood-like cells with narrower lumen and thicker walls than true earlywood, and are similar to transition cells. IADFs in Mediterranean hardwoods were classified into three types according to their position within the tree ring (Battipaglia et al., 2010; De Micco et al., 2012, 2014): 1) early IADFs, occurring in the first third of the annual ring as latewood-like cells in the earlywood; 2) middle IADFs, occurring in the second third of the annual ring as latewood-like cells in the earlywood; and 3) late IADFs, occurring in the third part of the annual ring as earlywood-like cells in the latewood. A simplification has been recently proposed, by considering the early and middle IADFs as corresponding to *E*-IADFs, while late IADF to *L*-IADF; the other IADFs would be considered only as transition wood (De Micco et al., 2016a).

Many studies aimed to identify the environmental conditions triggering IADFs formation, but it is still challenging to draw more straightforward conclusions on cause-effect relations for species growing in different environments. Indeed, how trees react physiologically to identical environmental stressors is also affected by their age, size and growth rate, as well as genetic and morphological traits. *L*-IADF formation as been related to the return of favourable conditions for tree growth after the start of latewood formation, i.e. more water availability due to autumn rainfall after a summer drought period (Abe et al., 2003; Battipaglia et al., 2010; De Luis et al., 2011a,b; Novak et al., 2013a,b). In *L*-IADFs, the presence of conducting cells with lumens larger than those of true latewood would increase the hydraulic conductivity or the available volume of wood for water storage (Battipaglia et al., 2010; Campelo et al., 2013). Conversely *E*-IADFs in Mediterranean woods have been considered the consequence of reduced cell expansion during a summer drought event, which induces the formation of tracheids or vessels with narrow lumens (Vieira et al., 2010; De Luis et al., 2011a,b; Novak et al., 2013a; Battipaglia et al., 2014). This type of IADF is formed in response to stomatal closure under drought stress, when the tree appears to make a sort of hydraulic adjustment to reduce its vulnerability to cavitation, at the cost of lower hydraulic conductivity (De Micco et al., 2007; Campelo et al., 2013; Battipaglia et al., 2014). The formation of different types of IADFs appear to be species- and site-specific phenomenon (Battipaglia et al., 2010; De Luis et al., 2011b; Novak et al., 2013a,b; Nabais et al., 2014). Indeed, in two nearby study sites on Elba Island in Italy, *Arbutus unedo* formed two types of IADFs depending on water availability at the growth site. In *A. unedo* growing under xeric conditions, Battipaglia et al. (2010) reported that *E*-IADFs (middle-IADFs) were formed, while under mesic conditions, *L*-type IADFs were formed. The physiological and ecological role of IADFs has been evaluated by retrospective approach on tree ring series in several species by combining dendrochronological, quantitative wood anatomy techniques and climate data showing their potential to detect the environmental factor triggering IADF formation (de Luis et al., 2010; Battipaglia et al., 2010; Vieira et al., 2010; De Micco et al., 2014; Zalloni et al., 2016). However, since the formation of different IADFs can be triggered by multiple factors, beside a retrospective approach, there is common agreement on the need to further investigate the climate-growth relationships with xylem cell features during their formation with the *real-time* analysis of xylogenesis (Rossi et al., 2008; De Micco 2016b; Pacheco et al., 2016; Ziaco & Biondi, 2016).

AIMS

The occurrence of long and severe summer drought periods, forecasted for the Mediterranean basin (IPCC, 2013), is expected to affect cambial activity and wood formation, thus altering tree growth and vegetation dynamics thus affecting the productivity of forests (Sarris et al., 2007; Martinez del Castillo et al., 2016).

In such a context, is important to understand the strategies in water utilisation exploited by different Mediterranean tree and shrub species in response to variation in the climate conditions. Such knowledge would allow to predict the response of Mediterranean ecosystems to climate change and help in decision-making for a focused forest management.

The general aim of the thesis is to acquire knowledge on the adaptation strategies operated at the xylem level by Mediterranean species to cope with the intra-annual variation of environmental conditions, and in particular with summer drought. This main aim has been pursued through the study of wood functional traits within tree-ring series, focusing in tree rings presenting Intra-annual density fluctuations (IADFs). The starting hypothesis of this Doctoral Thesis is that the Mediterranean woody species could respond to the seasonal distribution of precipitations and temperature patterns, showing a bimodal model of xylem growth identifiable by main trends in the wood functional traits, attested by the occurrence of IADFs within tree rings.

The specific objectives of the presented thesis are: *i)*to assess the attitude to form IADFs in different Mediterranean species, in different sites characterized by a period of summer drought in Italy; *ii)* to analyse the patterns of variation of different xylem traits along tree rings and determine which wood functional traits are the most appropriate to highlight IADF presence in the different species; *iii)* to evaluate the type of IADFs formed in different species and analyze the relationships with environmental conditions; *iv)* to identify the precise timing of IADF formation within tree ring during the calendar year in reference to climatic data to evaluate which factors play a major role in their formation; *v*) to study compartmentalization phenomenon occurring in response to stress-induced embolism in sapwood resulting in conduit occlusion by tyloses and gums; vi) to evaluate the application of the study of wood functional traits in crop science.

To achieve these purposes, two different approaches have been followed: a) the retrospective approach based on the reconstruction of long-term anatomical features of tree-ring series through the combination of dendrochronological and quantitative wood-anatomy techniques with climate data; b) the *in-vivo* approach based on the analysis of xylogenesis to monitor *real-time* cambium activity.

Research activities were carried out at different sites characterized by Mediterranean climate in Central and Southern Italy and were focused on the following case studies:

The analysis of the intra-annual variability of different parameters of vessels and fibres in the wood of two species of the Mediterranean maquis, namely *Arbutus unedo* L. and *Erica arborea* L. in order to compare the different sensitiveness of various wood anatomical traits to the intra-annual variability of environmental conditions by analysing tree rings with and without IADFs, in plants growing at sites characterised by different water availability in Central Italy (Chapter 1).

The characterization of IADFs and the analysis of the relations between the intra-annual variability of conduit size and climatic drivers in *Pinus pinaster* Aiton. and *Quercus ilex* L. growing at sites characterized by different water availability in Central Italy (Chapter 2).

The investigation of xylogenesis during summer period in one softwood and one hardwood species, *Pinus halepensis* Mill. and *Arbutus unedo* L., co-occurring at a site on Mt. Faito, in Southern Italy in order to : (a) evaluate whether and which type of IADFs were formed, and (b) highlight which weather conditions were concomitant or closely preceding IADF formation (Chapter 3).

The investigation of xylogenesis throughout one year in a softwood and a hardwood species, *Pinus pinea* L. and *Arbutus unedo* L. co-occurring at the same site in Mt. Vesuvius, Southern Italy, in order to: (a) identify the calendar of main xylem phenological phases; (b) analyse how many, when and which type of IADFs were formed in the two species (c) to evaluate which factors play the major role in their formation (Chapter 4).

The analysis of xylem plasicity in *Pinus pinea* L. and *Arbutus unedo* L. through the analysis of anatomical features of IADFs in long-term tree-ring series. Knowing the timing of xylogenesis in the two species (ref. to chapter 4) allowed us to gain clearer indications on the relations of IADFs features with monthly precipitation and temperature parameters (Chapter 5).

In addition to the main aims of the thesis, two supplementary topics have been investigated. The first regarded the study of conduit occlusion by tyloses and gums, a compartmentalization phenomenon occurring in response to stress-induced embolism in sapwood (Chapter 6). The second regarded the evaluation of the application of the study of wood functional traits in crop science with a study case aimed to verify whether a different plasticity of hydraulic anatomical traits can explain the different growth behaviours of an ornamental crop under conditions of managed water stress (Chapter 7).

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ENVIRONMENTAL-DRIVEN INTRA-ANNUAL VARIABILITY OF TRAITS OF WOOD FIBRES AND VESSELS IN TREE RINGS WITH INTRA-ANNUAL-DENSITY FLUCTUATIONS (IADFS) IN MEDITERRANEAN SPECIES

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Abstract

Forecasted environmental changes are likely to increase the frequency of intra-annual density fluctuations (IADFs) in Mediterranean tree rings. The interpretation of intra-annual anatomical variability of tree rings with IADFs can be useful to understand plant-growth response to environmental changes with seasonal resolution. We analysed the intra-annual variability of quantitative traits of both vessels and fibres in the wood of *Arbutus unedo* L. and *Erica arborea* L. to compare the sensitivity of different cell types to environmental variations. We applied digital image analysis on microphotographs of semi-thin sections of tree rings formed at sites with different soil water availability. Wood of both species showed good adaptability that allows anatomical traits of vessels and fibres to be harmonised by changing the water transport capacity and wood strength, promoting the efficiency or safety of water transport according to water availability during wood formation. The size of fibres showed trends of variation similar to vessels. Not all parameters of vessels were accurate indicators of the IADF presence. In conclusion, parameters of fibres, which offer advantages during automatic measurement, showed the same sensitivity to environmental fluctuations as vessels. Thus, they could be good indicators of summer drought to describe and interpret the ecological meaning of IADFs in tree rings.

Introduction

Forecasted environmental changes will likely lead to longer periods of drought and an increasein intensity and frequency of extreme events in Mediterranean ecosystems (IPCC, 2001, 2007; Giorgi & Lionello, 2008). To cope with drought conditions, Mediterranean species show morphological adaptations mainly related to water saving and mechanical reinforcement of tissues (Shields, 1950; Fahn, 1964; De Micco & Aronne, 2007, 2012). Moreover, a balance between efficient conductivity, when water is available, and safety against embolism, during water shortage, is regulated through the harmonisation of many wood anatomical parameters including conduit size, vessel grouping, intervessel pitting and traits linked to mechanical strength (Carlquist, 1989; Martínez-Vilalta *et al.*, 2002; Jansen *et al.*, 2003; *Baas et al.*, 2004; Mc Culloh & Sperry, 2005; Sperry *et al.*, 2006; Jacobsen *et al.*, 2007; De Micco *et al.*, 2006, 2008; Pratt *et al.*, 2007; De Micco & Aronne, 2009).

Vessel size is directly related to theoretical hydraulic conductance per leaf area unit, which decreases in arid conditions (Villar-Salvador *et al.*, 1997). The low water conductivity of narrow vessels can be compensated by the co-occurrence of higher vessel frequency (Fahn *et al.*, 1986). Resistance to drought-induced embolism is positively correlated to parameters of wood strength: the reinforcement of the fiber matrix is needed when conduits are subjected to high negative pressures which propagate into the fibres (Hacke *et al.*, 2001; Baas *et al.*, 2004).

The high plasticity of Mediterranean woody plants, in response to fluctuating environmental conditions, is responsible for the intra-annual variability of anatomical traits in tree-rings which form intra-annual-density-fluctuations (IADFs). IADFs are mainly formed as the consequence of a peculiar cambial activity induced by dry conditions (De Luis et al., 2007; De Micco & Aronne, 2009). Forecasted climate change will likely determine more pronounced periods of summer drought (Herting & Jacobeit, 2008; Girogi & Gao, 2008; Gibelin & Deque, 2003) which may have a severe impact on IADFs occurrence in Mediterranean woods. IADFs, also known as false or double rings, are recently attracting more and more interest because they can provide morpho-functional information with a seasonal resolution and can be used for understanding ecological processes, such as those related to species-specific sensitivity to drought (Rigling *et al*., 2002; Cherubini *et al*., 2003; Camarero *et al*., 2010; De Micco *et al*., 2014).

IADFs are formed due to an unusual change in wood density due to the interruption of the normal pattern of cambial activity during the growing season (Tingley, 1937). IADFs can be found at different positions within the ring and can be characterised by different anatomical and isotopic features depending on the environmental signal that caused their formation (Rigling et al. 2002; De Micco et al. 2007; Hoffer and Tardif 2009; Battipaglia et al. 2010; Griffin et al. 2011, Battipaglia et al. 2014). IADFs can be either observed as latewood-like cells (appearing as a dark band) within earlywood or earlywood-like cells (appearing as a light band) within the latewood, and are caused by different climatic, sometimes extreme, events depending on temperature and precipitation regimes (Campelo et al., 2006; De Luis et al., 2007). Mediterranean woods will likely have different aptitude to form IADFs (Camarero et al. 2010).The relationship between IADF formation and climate has been studied in various tree species, particularly in conifers such as *Pinus* spp. (Wimmer *et al*., 2002; Rigling *et al*., 2002; Masiokas & Villalba, 2004; Campelo *et al*., 2006, 2007; De Luis *et al*., 2009; Rozas *et al*., 2011; Vieira *et al*., 2014). In the Iberian Peninsula, IADFs in the latewood of *Pinus pinea* L., *Pinus halepensis* Mill. and *Pinus pinaster* Ait. were induced by late-summer or autumn rainfall (Campelo *et al*., 2007; De Luis *et al*., 2007; Vieira *et al*., 2009 ; Rozas *et al*., 2011). The frequency of IADFs in *P. halepensis* has been found to enhance according to increasing aridity (De Luis *et al*., 2011).Different ability to modulate xylogenesis patterns (leading to density fluctuations in tree-rings) has been also described in sympatric *Pinus* and *Juniperus* species co-occurring in xeric and mesic sites, suggesting that species having more plasticity during wood formation can better adapt to a wide range of climatic conditions (Camarero *et al*., 2010).

IADFs in some Mediterranean hardwoods have been recently identified and classified according to their position in the tree rings (Battipaglia *et al*., 2010, 2014a; De Micco *et al*., 2012, 2014). Early-and Middle-IADFs were latewood-like cells at the beginning or in the middle of the annual ring respectively, while Late-IADFs were earlywood-like cells in latewood (Battipaglia *et al*., 2010). In *Arbutus unedo*, Battipaglia *et al*. (2010) verified that the type and position of IADFs along the tree rings mostly depend on water availability at the site where plants are growing, whereas in plants of *Erica arborea*co-occurring at the same sites, IADFs were mainly located in the middle of the ring independently from the soil water availability (Battipaglia *et al*., 2014a).

Various wood anatomical features measured in tree-rings have been used to better understand the complexity of wood formation and IADF occurrence also in response to specific environmental events (e.g. insect infestation, fires, soil erosion, landslide, flooding and drought) (e.g. Schweingruber, 2007; De Micco *et al*., 2013; Gea-Izquierdo *et al*., 2013; Battipaglia *et al*., 2014b; Vieira *et al*., 2014). In comparison to softwoods, still only few studies have been carried out on hardwoods, although such studies would be very useful, especially in Mediterranean shrubs and their peculiar anatomical traits are responsible for species ability to endure severe dry conditions where trees usually do not survive (Carlquist, 1975; Baas *et al*., 2004; De Micco *et al*., 2006, 2008). Softwood species are the most investigated not only because softwoods are simpler models than hardwoods, but also because the position of tracheids in exact and ordered raws makes them suitable woods for the application of specific image analysis tools, such as ROXAS, which recognise conduits and build centuries-long chronologies of tracheid lumen area (von Arx & Carrer, 2014; Brunel *et al*., 2014). Such tools automatically measure anatomical parameters in rows of tracheids in short time, also reducing the subjectivity of the operator (Von Arx & Carrer, 2014). In hardwoods, this is not easily obtained because vessels have different patterns of distribution and grouping along tree-rings in various species, and surely dependent on the type of wood porosity. Many wood anatomical features, including 3D characteristics, could be calculated but, since their calculation is consuming in terms of resources (e.g. traits at the ultrastructural level) and time, wood density and size of water conducting cells have been the most explored wood anatomical parameters (Scholz *et al*., 2013) as indicators of environmental and mainly climatic information recorded in tree-rings. Although difficult to analyse, hardwoods could be considered archives of environmental information richer than softwoods because different anatomical parameters can be characterized by various sensitivity to environmental variations and could furnish additional ecological information for the interpretation of IADFs.

In this paper, we compare the intra-annual variability of different parameters of vessels and fibres in the wood of two species of the Mediterranean maquis, namely *Arbutus unedo* L. and *Erica arborea* L., growing at sites characterized by different water availability. We aim to evaluate whether parameters of non-conducting cells show similar sensitivity to the variability of environmental conditions if compared to conducting cells. Indeed, different anatomical features are often highly inter-correlated (Wimmer, 2002) and the choice of which parameter to use in ecological studies depends not only on the easiness of measurement but also on potential use of obtained data. The use of fibres as indicators of environmental fluctuations in tree-rings in hardwoods would help overcoming technical difficulties in the application image analysis tools on such complex woods.

Materials And Methods

Study site and plant material

The study was conducted on plants of *Erica arborea* L. and *Arbutus unedo* L. growing on Isola d'Elba, an island in the Tyrrhenian Sea (Central Italy). The climate is Mediterranean, with a mean annual temperature of 16.4°C and mean annual precipitation of 375 mm (Battipaglia *et al.*, 2014). Two sampling sites characterized by different amount of soil moisture were selected: a xeric and a mesic site. The xeric site, located on Monte Perone at 420 m a.s.l. (42°46'N,10°12'E), was characterized by more open and scattered vegetation with a higher frequency of xeric species and shrubs than the MS, located in the Nivera Valley at 460 m a.s.l. (42°46'N, 10°11'E). Details on site characteristics are given in Battipaglia *et al.* (2010).

Tree-ring sampling, sectioning and microscopy

At both sites, 5 plants of *E. arborea* L. (2-3 m in height, 4-8 cm in diameter) and 5 plants of *A. unedo* L. (3-5 m in height, 5-10 cm in diameter) were sampled. In particular, three cross-sections were taken from the thickest main stem for each plant. Semi-thin sections (15 µm thick) were obtained from each sample with a sliding microtome: each section corresponded to a tree-ring chronology from 1 plant. The sections were double stained with safranin O (1 g in 65 ml of 100% ethanol and 30 ml of distilled water) and counterstained with astra blue (1 g in 100 ml of ethanol at 100 % and 5 ml of distilled water) according to Vasquez-Cooz & Meyer (2002). Then, the sections were dehydrated, immersed in xylene and mounted on glass slides with Canada balsam (Schweingruber, 1978; Gartner *et al*., 2001). The sections were analysed under a light microscope (BX60, Olympus, Germany) in order to identify tree-ring boundaries and IADFs.

Identification and classification of IADFs, and Quantitative Wood Anatomy

We identified IADFs in the sections using reference tree-ring chronologies (Battipaglia *et al*., 2010, 2014). The most represented types of IADFs were selected in each species and site. More specifically, in the tree-ring chronologies of *E. arborea* from both sites and of *A. unedo*from the xeric site, we considered earlywood IADFs (EW-IADFs) as those occurring as latewood-like cells in earlywood in the middle of the tree ring. In *A. unedo* plants growing at mesic site, we considered latewood IADFs (LW-IADFs) as those occurring as earlywood cells in latewood.

In brief, the following tree rings were considered: a) 15 rings (from 1992 to 2006) in *E. arborea* per each plant at both sites; b) 13 rings (from 1994 to 2006) in *A. unedo* per each plant at both sites.

The variability of the anatomical features along the width of each ring was quantified with digital image analysis techniques. For each ring, micro-photographs were acquired with a digital camera (CAMEDIA C4040, Olympus) in specific regions selected in critical areas of each ring, as reported in De Micco *et al*. (2014). For *E.arborea*, in rings withIADFsfrom both sites, four regions were selected proceeding from the beginning towards the end of each ring: 1) Region1, selected in earlywood (EW); 2) Region 2, selected in the area where IADF begins, in correspondence of the dark band (FL1); 3) Region3, selected in the area of the fluctuation in correspondence of the light band (FL2); 4) Region4, selected in latewood (LW).

For control rings, the same four regions were selected: EW and LW regions were selected respectively at the beginning and ending of the ring, while FL1 and FL2 were selected as two consecutive regions in the middle of EW. Since data collected in FL1 e FL2 were not significantly different, they were pooled to consider FL1 and FL2 as a single central region of the ring defined as potential-fluctuation (PFL): the region of the ring where the fluctuation could have been potentially found.

As regards *A. unedo*, in tree-ring chronologies of the xeric site, the same regions as in *E. arborea* were selected in both rings with and without IADFs. For sections from the mesic site, according to De Micco *et al*. (2012), only three regions were identified for the rings with IADFs proceeding from the beginning towards the end of the ring: 1) Region1, in earlywood(EW); 2) Region 2, in latewood (LW); 3) Region3, in the fluctuation zone which appears as alight band of earlywood-like cells in the latewood (FL). For control rings, the same three regions were selected, named EW, LW and PFL at the end of the ring in the area where IADF should potentially occur.

The anatomical parameters were analyzed in each region through the Analysis 3.2 software (Olympus). The following parameters, related to the characteristics of efficiency and safety of water transport, were analyzed: vessel and fibre size measured as maximum, medium and minimum Feret diameters (distance between the parallel lines tangent to the perimeter of the particle); vessel frequency (the number of vessels per mm^2, determined by counting the vessels present in a known area); thickness of fibre cell walls (avoiding cell corners).

These anatomical parameters were measured in at least 10 elements per region. All abnormal vessels and fibers or collapsed cell walls were excluded from the analysis. All data were processed with one-way ANOVA and withLSD and Student-Newman-Keuls coefficients for multiple comparison tests ($p < 0.05$), using SPSS® (SPSS Inc., Chicago, IL, USA).

Results

In rings without IADFs, the wood of *E. arborea* L. was characterized at both sites by diffuse porosity, prevalence of solitary vessels and gradual transition from earlywood, with wide vessel lumen area, towards latewood, often reduced in width, with narrower vessels. At both sites, tree-rings showed a higher occurrence of EW-IADFs (Fig. 1a), appearing as a dark band formed by latewood-like cells, corresponding to the beginning of fluctuation(FL1), which is followed by a clear band, with earlywood-like cells (FL2).

Considering that there were missing rings in some plants and that tree rings with more than one IADF were avoided, in summary we analysed: a) 47 rings with EW-IADFs and 25 control rings (without IADFs) in *E. arborea* at the xeric site, and b) 46 rings with EW-IADFs and 29 control rings in *E. arborea* at the mesic site.

Wood was denser at the beginning of the fluctuation (FL1) and in latewood as compared with other tree-ring regions (FL2 and earlywood). Contrary to tree-rings without IADFs, where density (percentage of cell walls over total xylem area) increased towards latewood, tree-rings with IADFs showed a similar density in FL1 and LW which was significantly higher than EW and FL2.

In *A. unedo* rings without IADFs, xylem was characterized in both sites by typical diffuse porosity, vessels often arranged in radial rows of four or more elements, vessel lumen with angular boundaries and size gradually decreasing from earlywood to latewood. Predominant IADF type varied as a function of the site. Tree rings with IADFs from the xeric site presented the same appearance as EW-IADFs described in *E. arborea*. On the contrary, in wood of the wet site, LW-IADFs were the most represented (Fig. 1b). This type of IADF was characterized by a gradual decrease in vessels lumen from earlywood to latewood, followed by a sudden increase of vessel lumen appearing as earlywood-like cells in latewood. A new reduction of vessel lumen established the real boundary of the ring. As for *E. arborea*, because of the occurrence of missing rings and rings with more than one IADF, in *A. unedo* we analysed: a) 22 rings with EW-IADFs and 34 control rings at the xeric site, and b) 13 rings with LW-IADFs and 39 control rings at the mesic site. At the xeric site, the trends of variation of wood density in tree rings with and without IADFs were the same as found in *E. arborea*. At the mesic site, in tree-rings with LW-IADFs, wood density increased significantly from EW to LW, but wood was significantly lighter in the IADFs zone as compared to control rings.

In *E. arborea*, Feret diameters of vessels and fibres presented the same trends of intra-annual variation which varied according to the presence/absence of IADFs. In the absence of fluctuation, Feret diameters of both conducting and non-conducting cells significantly decreased from EW to LW with no differences between sites. In the presence of IADFs, intra-annual variability of feret diameters of both conducting and non-conducting cells was similar for the two sites with a strong decrease from EW to the region where the fluctuation starts (FL1), followed by a significant increasein the region FL2. In LW, diameters of both conducting and non-conducting cells decreased again reaching values almost comparable to those found in the regionFL1.

In *A. unedo*, Feret diameters of vessels and fibres presented the same trends of intra-annual variation which varied according to the presence/absence of IADFs and to the site. In the absence of IADFs, feret diameters of both conducting and non-conducting cells decreased significantly from the beginning to the end of the ring in both sites. In presence of IADFs, the trend was different in the two sites. At the xeric site, we observed a severe decrease from EW to FL1, followed by a subsequent significant increase in FL2. In LW, diameters of both conducting and non-conducting cells decreased again reaching values not significantly different from those found in the region FL1. At the mesic site, we observed a significant decrease in diameters of both conducting and non-conducting cells from EW to LW, followed by a significant increase at the end of the ring in the FL region.

Cell-wall thickness of fibres showed the same trends of intra-annual variation as feret diameters. For *E. arborea* and *A. unedo*, in the rings without IADFs at both sites, wall thickness of fibres decreased gradually from EW to LW with the most evident decrease in LW of *E. arborea*. In the rings with fluctuations of *E. arborea*, high values of fibre wall thickness were found in EW and FL2 regions; then, there was a significant decrease in FL1 and LW regions. The same trend occurred in *A. unedo* tree-rings with EW-IADFs, while in presence of LW-IADFs, fibre wall thickness decreased from EW to LW, and increased significantly in FL region.

Vessel frequency showed a general increase, not always significant, in the middle of the ring in both species, at the two sites, independently from the presence of IADFs. More specifically, in *E. arborea*, vessel frequency showed no significant changes between the various regions of the rings with and without IADFs for plants at the xeric site, except in LW where it underwent a significant decrease in the presence of IADFs. For samples from the mesic site, in the rings without IADFs, there was a significant increase in the vessel frequency in PFL region and a subsequent marked decrease in the LW region. Instead, in rings with IADFs this increase occurred in FL1 with subsequent significant decrease before in FL2 region and then in LW region.

For *A. unedo* at the xeric site, the vessel frequency increased in the regions PFL and FL1 of tree rings with and without IADFs respectively. In the same species growing at the mesic site vessel frequency significantly increased in LW of both wings with and without IADFs.

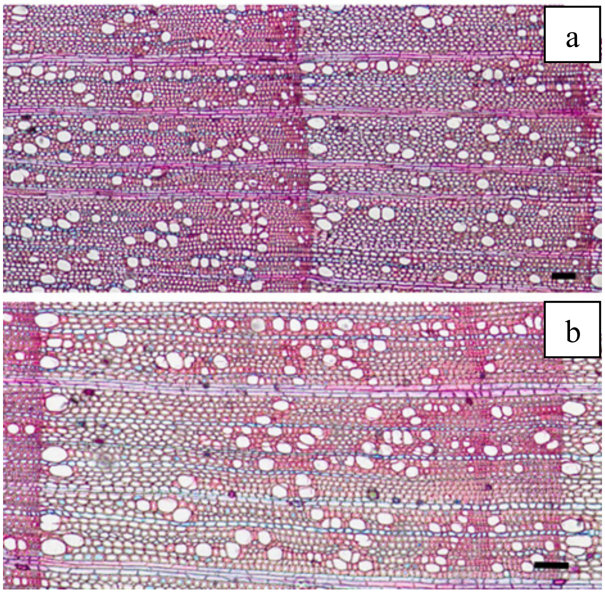

Fig. 1. Light-microscopy views of cross sections showing a: rings with EW-IADFs; b: ring with LW-IADFs. Rings are oriented with earlywood on the left. Bar = 100 μm.

Discussion

In this study, we highlighted that parameters of non-conducting cells can be used to understand the effect of water availability on tree-ring growth, thus to describe and interpret the ecological significance of IADFs. Indeed, fibres can furnish the same ecological information as gained from the analysis of vessel size.

IADFs can be used as tools to better understand the effect of environmental fluctuations on wood, provided that the physiological and ecological meaning of anatomical “signals” are correctly elucidated. Untl now, the analysis of intra-annual variability of tree rings has been mainly based on the measurement of wood density and tracheid size in softwoods that are ideal models for the application of image analysis tools, such as ROXAS, which automatically detect and measure tracheids in ordered rows along tree-rings (von Arx & Carrer, 2014). Based on the assumption that water availability triggering IADFs would mostly affect water-conducting cells, studies on intra-annual variability of hardwood tree-rings have been mainly focused on the analysis of vessel features (Battipaglia *et al.*, 2010, 2014). Our results indicate that the size and cell wall thickness of fibres could be used instead of vessel size to analyse the intra-annual variability of tree rings in relation with environmental fluctuations, especially drought occurrence. The use of fiber parameters in automatic measurements allows overcoming technical constraints due to different vessel arrangement in hardwoods. Indeed, non-conducting cells in hardwoods form the ground tissue in which vessels are spread with various aggregations. Although fibres in hardwoods are not always precisely arranged in ordered rows as tracheids in softwoods, it is still possible to follow the variability of their traits by tracing continuous transects along the ring width. This helps cell detection and automatic measurement.

The wood of *E. arborea* and *A. unedo* is very plastic and often form IADFs: the mechanism triggering their appearance has been recently hypothesised by applying a multidisciplinary approach combining dendro-ecological, wood anatomical and isotopic analyses (Battipaglia *et al.*, 2010, 2014a). Quantitative wood anatomy allowed a fine characterisation of wood traits of the IADFs occurring in tree-rings formed in two different conditions of water availability in the two species. The intra-annual variability of wood density and of the size of vessels and fibres follows the same species- and site-specific trends of variation. Indeed, the values of wood density and of Feret diameters of both vessels and fibres in the tree-rings with EW-IADFs encounter a significant increase or decrease respectively in the middle of the ring that marks the beginning of IADFs. The beginning of EW-IADFs is characterised by high safety against embolism due not only to the reduced vessel lumen size but also to the reduced fibre size and increased density, that improve wood strength. In the same region, the decrease in fibre cell wall thickness indicates that there is more volume available for cell elements, leading to the formation of a stiffer wood made of more numerous and smaller elements: such characteristics are sign of a wood less prone to drought-induced embolism (Hacke *et al.*, 2001). The decrease in vessel and fibre feret diameters at the beginning of the IADFs zone is around 50% in all cases and seems to be independent on cell type. This could be a mechanical consequence of the reduced turgor-driven cell expansion due to drought (Hsiao *et al.*, 1976; Sperry *et al.*, 2006). Moreover, the decrease in fibre cell wall thickness suggests a reduction in cell wall synthesis due to reduced carbon uptake following stomata closure (Chaves *et al.*, 2002). This is in agreement with increased δ^{13}C or water use efficiency found in the same species at the beginning of the IADF zone (Battipaglia *et al.*, 2010, 2014a). The onset of a drought period in the two species leads to the formation of a timber in which safety features are privileged over hydraulic efficiency. In both species, safe water transport is also favoured by the occurrence of tracheids which still are capable to transport water even when part of vessels are embolized (Baas *et al.*, 2004). The same phenomenon in *A. unedo* is permitted also by vessel grouping since it is rare that embolism would spread towards all vessels of a group, especially because reduced vessel size also means decreased size of the membrane pores in intervessel pitting (Baas *et al.*, 2004; Wheeler *et al.*, 2005). The valuable increase in size of both vessels and fibres in the second part of the EW-IADFs indicates that the wood of the two species reacts by following the principle that the survival of Mediterranean species after a period of summer water deficit may be strongly influenced by their ability to recover by quickly absorbing water after a rain pulse (Gratani & Varone, 2004 Gallé *et al.*, 2007).

As regards the values of vessel frequency, in the two species at both sites, the trends of variation in tree-rings with IADFs appear to follow the normal pattern found in the rings without IADFs. The lack of control of vessel frequency by water availability suggests that these species do not implement an adaptation strategy in favour of hydraulic safety through the "redundancy" of the conductive elements. This is in contrast with the typical increment of vessel frequency that occurs moving from mesic to xeric conditions (Fahn *et al.*, 1986; Carlquist, 1989). This wood trait might be among those features mainly controlled by genetic factors (Wimmer, 2002). In fact, the IADFs are generally produced in response to physiological, genetic and climatic factors (Larson, 1960; Wimmer, 2002). Many reasons, such as genetic factors or differences at the root level, could explain the lack of EW-IADFs in *A. unedo* tree-rings formed at the mesic site. Moreover, in these plants, the occurrence of LW-IADFs could be possibly ascribed to a longer duration of cambial activity. Whatever the reason, the onset of such type of IADFs in *A. unedo* at the mesic site seems to be triggered by conditions other than those acting at the xeric site (Battipaglia *et al.*, 2010). Also in this type of IADF, the size of both vessels and fibres as well as fibre cell wall thickness, follow similar trends of variation opposite to wood density, which suggest a recovery in wood growth soon after latewood formation. This restoration of wood growth, occurring at the same extent in vessels as in fibres, can be ascribed to possible rain events favouring turgor-driven cell enlargement at the end of the growing season after the period of water stress that primed the formation of latewood. Indeed, when in late summer, rainfall is more abundant than normal seasonal trends, the dormancy induced by water stress in cambial activity could be interrupted and cambium reactivated to produce new larger cells (Masiokas & Villala, 2004). This hypothesis is supported by the findings that $\delta^{13}C$ was reduced in the zone of the fluctuation indicating a growth of the rings in the last part of the growing as response to unexpected rain events (Battipaglia *et al.*, 2010). However, many other factors (including the duration of the day, availability of carbohydrates and hormonal regulators) would ultimately affect the properties of vessels and fibres by also influencing cell enlargement and maturation.

The overall analysis of two species at the two sites indicates that the tree-rings of *E. arborea* and *A. unedo* have good plasticity that allows harmonising anatomical traits of both conducting and non-conducting cells by changing the capacity of water transport and wood strength to promote the efficiency of transport when water is available, while favouring the traits of hydraulic safety in conditions of stress. The finding that vessel and fibre size show the same sensitivity to water availability (e.g. similar trends and extent of variation) suggests that no cell type is privileged when water availability is scarce. Consequently, anatomical characteristics of both conducting and non-conducting cells can be used to reconstruct climatic information. From a methodological viewpoint, this finding is interesting because the use of fibre parameters instead of vessels would help overcoming technical constraints in the automatic measurements of the variation of wood traits in hardwoods. Indeed, the distribution of fibres along tree-rings from earlywood to latewood follows more ordered and regular rows than vessels, in a structure more similar to tracheids in softwoods. This would help achieving more continuous reconstructions of the trends of variation of cell size within harwood tree-rings.

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XYLEM PLASTICITY IN *PINUS PINASTER* AND *QUERCUS ILEX* GROWING IN MESIC AND XERIC SITES IN MEDITERRANEAN REGION: RELATIONS BETWEEN IADFS AND ENVIRONMENTAL CONDITIONS

Chapter based upon a manuscript in preparation with the collaboration of De Micco V., Battipaglia G., Aronne G., Cherubini P.

Abstract

Fluctuations in climatic conditions during the growing season are recorded in Mediterranean tree rings and often result in Intra-annual density fluctuations (IADFs). Dendroecology and quantitative wood anatomy analysis were used to characterize IADFs and the relations between the intra-annual variability of xylem traits and climatic drivers in *Pinuspinaster* Aiton and *Quercus ilex* L. growing at sites characterized by different water availability on the Elba island in Central Italy. Our results showed that both species present high xylem variability resulting in the formation of *L*-type IADFs, consisting in earlywood-like cells in latewood, whose occurrence have been linked to the restoration of favourable conditions of water availability after rain events following conditions of summer drougth stress. The formation of *L*-IADFs in both species seems increase the hydraulic conductivity, due to their larger lumen area than “true latewood”, late in the growing season.Similarities between species were more marked under higher climatic constraint determined by the arid conditions. Vessel area and frequency, wood density and IADF’s frequency proved to be efficient proxies to encode climate signals recorded in IADFs. The response of these anatomical traits to climatic variations was found to be species- and site- specific.

Introduction

Mediterranean ecosystems are characterized by seasonal fluctuations in water availability amount, often leading to drought stress in summer. Those seasonal climatic fluctuations are expected to become more and more marked as a consequence of the ongoing climate change (Sanchez et al., 2004). Therefore, increasing frequency of severe summer drought and heat waves episodes will likely affect woody plant growth and productivity of forests (Sarris et al., 2007; Gea-Izquierdo et al., 2014).

Mediterranean woody species are adapted to deal with the typical double stress of the Mediterranean climate due to low winter temperature and summer aridity (Mitrakos, 1980; De Micco et al., 2008). Their attitude to cope with seasonal Mediterranean conditions resides in the ability to adjust their functional anatomical traits, at the whole plant level, to the prevailing environmental condition (De Micco and Aronne, 2012). Adaptations at the level of plant hydraulics are fundamental in Mediterranean environment to allow efficient water transport when water is available, while maintaining safety against embolism when water availability in the soil is reduced (Fahnet al., 1986; Carlquist, 1989; De Micco et al., 2008). Functional traits in wood of Mediterranean species is the result of peculiar patterns of xylogenesis allowing cambial cells to differentiate in more efficient but less safe earlywood than latewood alternating them more than twice a year (Tyree et al., 1994; De Micco&Aronne, 2007; Sperry et al., 2008; Camarero et al., 2010; Meinzer et al., 2010). The number of conductive elements produced by the cambium, their lumen area, frequency and the thickness of cell walls are controlled by both physiological processes and environmental conditions (Vaganov et al., 1996; Nicault et al., 2001; De Micco et al., 2007; Rossi et al., 2008) and can affect the functionality of the xylem. Fluctuations in climatic conditions during the growing season are thus recorded in xylem and often result in “anomalies” changing the anatomical appearance and functionality of the typical tree rings formed in temperate environments where every tree ring is annual and is made of a band of earlywood alternating with a band of latewood (De Micco et al., 2016a). Such anomalies, called Intra-annual density fluctuations (IADFs) have been recently classified and considered within functional wood traits (Beeckman, 2016; De Micco et al. 2016a). IADFs are regions within a tree ring where abrupt changes in density occur (Rigling et al., 2001; De Micco et al., 2016). Different types of IADFs have been detected in Mediterranean species and their position within the ring is determined by the time in which the triggering factor occurred (Campelo et al., 2007b). The most frequent type of IADFs occurring in tree rings of Mediterranean species are *L*-IADFs and *E*-IADFs (De Micco et al., 2016a). *L*-type IADFs, appear as earlywood-like cells in the latewood, and their occurrence have been linked to the restoration of favourable conditions of water availability after rain events following conditions of drought stress (Campelo et al., 2007b; Battipaglia et al., 2010; de Luis et al., 2011a,b). *E*-type IADFs, conversely, appear as latewood-like cells in earlywood, and their formation has been hypothesized to be due to stomata closure under summer drought stress in order to reduce the embolism risk (De Micco et al., 2007; Battipaglia et al., 2014). However the physiological mechanism triggering IADFs formation and how tree ring anatomy responds to climate pressures has been non completely unraveled yet. One of the challenges in the studying IADFs to define general main trends in their formation is the high variability in their occurrence and their dependency on many factors, both intrinsic and external (Battipaglia et al., 2016; De Micco et al., 2016a). Several studies have demonstrated the dependency of IADF's frequency on species, cambial age, climatic and microclimatic conditions (Rigling et al., 2001; Vieira et al., 2008; Battipaglia et al., 2010;Camarero et al., 2010; Novak et al., 2013 a,b; Nabais et al., 2014; Zalloni et al., 2016). Since wood anatomy and plant hydraulics play a key role in understanding specie responses and ability to cope with rapid environmental change (McDowell et al., 2008; Fonti et al., 2010), understanding the ecological meaning of IADFs is crucial to comprehend the adaptive capability of plants based on xylem plasticity. The analysis of quantitative anatomical traits of IADFs can help obtaining information about the relationships between environmental factors and tree growth at the seasonal level (Battipaglia et al., 2010; Campelo et al., 2010; De Micco et al., 2015). However, the quantification of xylem cell characteristics (e.g. lumen area, density, frequency) can result time consuming and represent a constrain, although to reduce the time of measurements, different semi-automatic approaches have been developed (Corcuera et al., 2004; Fonti et al., 2010). The tracheidogram method has been developed to identify the relation between environmental conditions and growth in conifer on the basis of the diameter and wall thickness of tracheids (Vaganov et al., 1991). In hardwoods, the study of specific region within tree rings was used to study the influence of environmental conditions on vessel lumen area throughout the growing season (Sass and Eckstein 1995; Battipaglia et al., 2010; Abrantes et al., 2013). Recently the concept of tracheidogram was also applied to analyze vessels in hardwoods which allow linking vessel features to climate (Schume et al., 2004; Grabner, 2005;Micco et al., 2012).

In this study, we analyzed xylem plasticity of two of the most widespread evergreen species in Mediterranean area, *Pinus pinaster* Aiton and *Q .ilex* L., which are species prone to form IADFs when growing under Mediterranean conditions (Cherubini et al., 2003;De Micco et al., 2007; Campelo 2007a, 2010; Viera et al., 2009; Rozas et al., 2011; Campelo et al., 2013). Tree-ring series of the two species were analyzed from trees growing at two sites with contrasting soil water availability, namely a mesic (Wet) and a xeric site (Dry). Previous studies at these sites showed that the aptitude to form different types of IADFs was species-specific; in particular, *Arbutus unedo* formed two different types of IADFs according to different water availability at the two sites, while *Erica arborea* always formed the same type of IADFs (Battipaglia et al., 2010, 2014). Hence, to completely understand IADFs ecological role in response to climate, comparative studies on intra-annual xylem plasticity of different species growing in different conditions are useful also to evaluate plant growth responses which affect possible future ecosystem dynamics.

Our specific aims were: 1) to verify whether IADFs occur in both species and compare their aptitude to form them; 2) to classify IADFs in both species in order to verify whether water availability at the two sited affected IADF frequency and type; 3) to link intra-annual variation of anatomical traits with climate parameters at seasonal scale. To achieve our goals we used a multidisciplinary approach combining dendrochronology, quantitative wood anatomy (QWA) and climatic correlations. In particular we used a novel approach for IADFs analysis, based on the measurement of vessel size data *in continuum*, within the tree ring, on digital images (De Micco et al., 2012). We also quantified other wood traits, namely wood density, in both species, and vessels frequency, in *Q. ilex*, along tree-ring width to assess their potentiality to be used as indicators of intra-annual variations of climate parameters.

Materials and methods

Species and study site

The study was conducted on *Pinuspinaster* Aiton and *Quercus ilex* L. trees growing on Elba Island in the Tyrrhenian Sea (Central Italy). The site was characterized by Mediterranean climate, with a mean annual temperature of 15.41 °C and precipitation mainly concentrated in autumn and winter, with an average of 417 mm during the period 1960–2007. We selected two sampling sites with different amounts of soil moisture: the first xeric (Dry), located on Monte Perone (42°46’ N, 10°12’ E, 420 m a.s.l.), presented xeric species and shrubs; the second mesic (Wet), located in the Nivera Valley (42°46’0N, 10°11 E, 460 m a.s.l.) presenting more mesic species. Precipitation and temperature data were obtained from the Portoferraio meteorological station located at 10km from the sites (42°49’N, 10°20’ E, 25 m a.s.l.). Details on site characteristics are given in Battipaglia et al. (2010).

Tree-ring data and IADFs frequency

We selected 10 trees per species at both sites, sampling for each plants twin cores at breast height, with a Pressler increment borer. Since our sites were placed in the National Park of the Tuscan Archipelago we were allowed to sample the minimum number of specimens. However, we could obtain reliable chronologies, assessing strength of the chronologies by the 'expressed population signal' (EPS; Wigley et al., 1984). The cores were dried and polished in order to obtain visually cross-dating (Stokes & Smiley, 1968) and allow IADFs identification and classification according to De Micco et al. (2016). Tree-ring width was then measured using a LINTAB system with a resolution of 0.01 mm and individual series were crossdated and detrended (20-year spline) to remove non-climatic signals using the Dendrochronology Program Library in R (dplR; Bunn 2010).

We detected *L*-IADFs in tree-ring series of both *P. pinaster* (Fig 1a) and *Q. ilex* (Fig. 1b). Their relative frequency (IADFs yr–1) was calculated as the number of trees that present IADFs in a given year, divided by the total number of sampled trees in that year. Stabilized IADF's frequency was calculated according to Osborn et al. (1997) as f=Fn 0,5. The frequency was calculated on ten cores for each species.

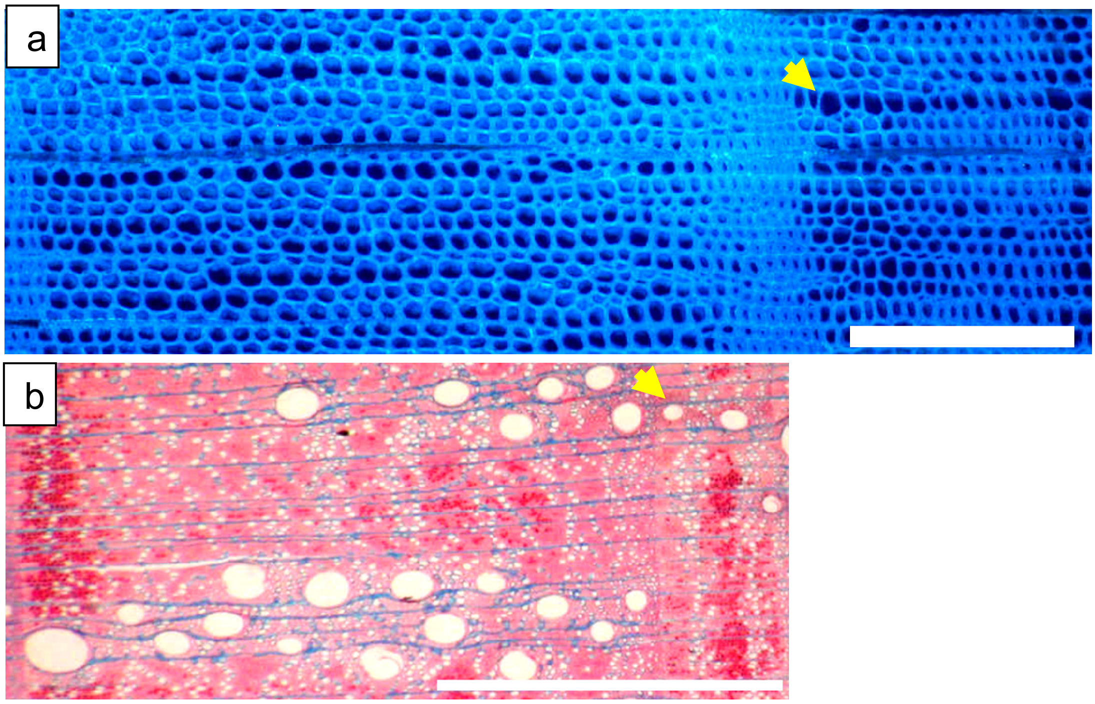


Fig 1. Tree ring with *L*-IADF in *P. pinaster* (a) and tree rings with *L*-IADFs in *Q. ilex* (b). Yellow arrows indicate the beginning of earlywood-like cells in the IADFs. Bar=500 μm

Microscopy and QWA

Microsections of cores (15μm thick) were obtained with a sliding microtome. Sections were dehydrated through an ethanol series (70 , 90, 95 and 100%),stained with a safranin (0.04 %) and astra blue (0.15 %) water solution (van der Werf et al., 2007) and mounted in Euparal (Bioquip Rancho Dominguez,California). Each tree-ring was analyzed under a light microscope (Olympus BH-2, Hamburg, Germany), equipped with a photo-micro adapter (Olympus OM-Mount) and a camera (Olympus OM101). Microphotographs of each whole tree-ring were taken and digitized to perform quantification of anatomical traits. For the analysis, we considered 5 plants per species at both sites; in particular, we analyzed tree-rings corresponding to the following periods: *(a)* from 1969 to 2007 in *P. pinaster; (b)* from 1990 to 2007 in *Q. ilex*. The variability of the anatomical features along the width of each ring was quantified with digital image analysis techniques using

Analysis 3.2 software (Olympus). Lumen area of vessels of *Q. ilex* and tracheids of *P. pinaster* were semi-automatically quantified in all elements encountered moving from the beginning to the end of each tree ring in a chosen horizontal transects, including one radial line of tracheids in *P. pinaster* and vessels in a radial bundle in *Q.ilex,* following standardized progressive number method described by De Micco et al. (2012). During the measurement, the progressive number of each element was recorded in order to build dispersion graphs with Y and X as coordinates of each conductive element in order to visually compared rings with and without IADFs in the Wet and in the Dry site. The progressive number of vessel was standardized dividing by the total number of vessels encountered along the transect and multiplying by 100, obtaining a series of vessels/tracheids size by the same principle of tree-ring width chronologies. For each of the considered parameters, a dispersion graph was drawn, where each conduit was characterised by two coordinates: Y, corresponding to the measured lumen parameter and, X, corresponding to the distance from the beginning of the ring, expressed as percentage of the total ring width. The patterns of vessels/tracheids size variability along ring width were visually compared in rings with and without IADFs. In the standardized data series we calculated interpolation equations (fourth-order polynomial curve) with confidence intervals using the option Non-linear curve fitting in R system (R Development Core Team, 2008).

Then, obtained series of tracheids/vessels lumen area, the tree-ring without IADFs were partitioned in 4 regions (each region corresponding to the 25% of total ring width) while tree-ring with IADFs in 5 regions (each region corresponding to the 20% of total ring width) along the radial direction from the beginning (Region 1) to the end (Region 4 for tree rings without IADFs; Region 5 for tree rings with IADFs). We considered the mean value of vessels/tracheids lumen area for each region of the ring to perform climate correlations with intra-annual resolution.

Further, we analyzed wood density (measured as the percentage of cell walls over total xylem area) for both species. In *Q. ilex*, we calculated vessel frequency [the number of vessels per mm^2, determined by counting the vessels present in a known area, according to Wheeler et al. (1989)] , in the same tree rings considered for measurements of tracheids/vessels lumen area. To compare mean values of anatomical traits between different sectors of tree rings, each tree ring without IADFs was partitioned in the above-reported 4 regions, while for tree-rings with IADFs, a sort of standardization of the conduit position was performed by making the 80% ring width of tree rings with IADFs coinciding with 100 % ring width of tree rings without IADFs. In detail, tree-ring width in the presence of IADFs were divided into the above-reported 5 regions: the first fourth regions were considered coinciding with those of rings without IADFs and relative data were pooled, while the fifth region was considered as an additional growth layer containing the IADF.

Intra-annual trend of variations in the two species at the two sites was processed with an one way ANOVA, using Student–Newman–Keuls coefficient for multiple comparison tests (p\0.05). The SPSS statistical package was used (SPSS Inc., Chicago, IL, USA).

Climate signal

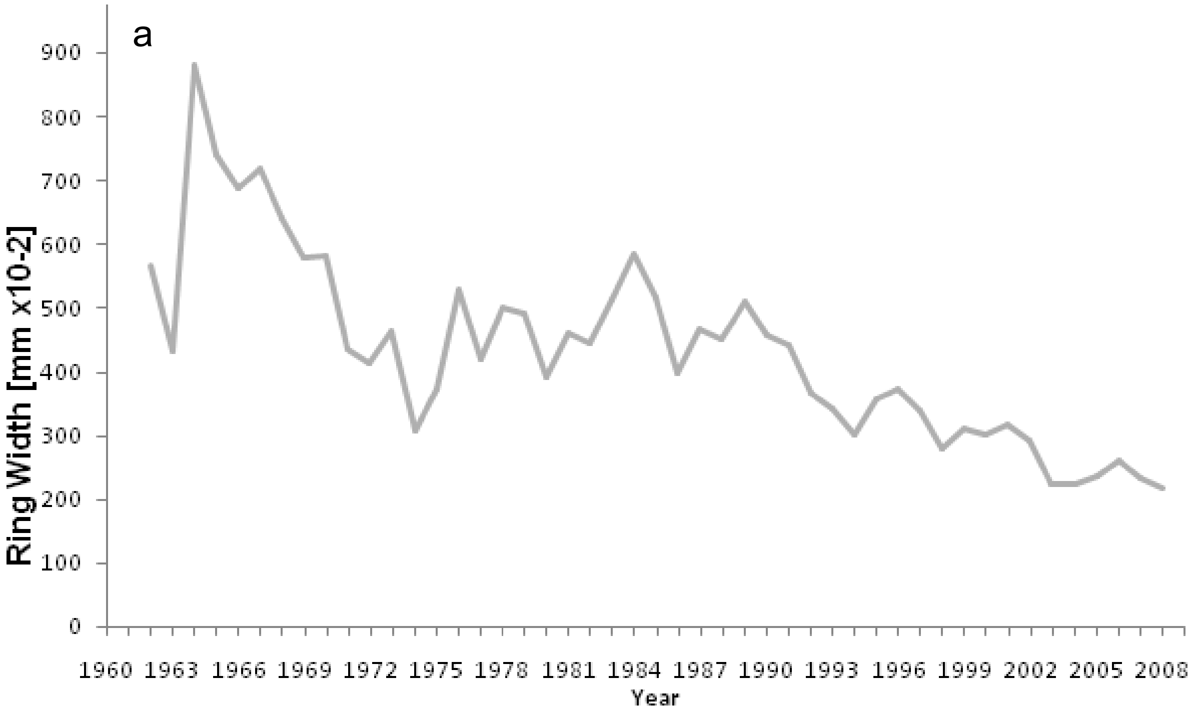
We correlated measured anatomical traits and stabilized IADF's frequency to meteorological data by computing Pearson coefficients ($p < 0.05$) considering the following periods: a) 1969-2007 for *P.pinaster*in both sites; b) 1994-2007 in Dry site and 1975–2007 in the Wet site for *Q.ilex*. We considered the mean value of vessels/tracheids lumen area, wood density and vessel frequency in different regions within the tree ring in order to obtain climate correlation with intra-annual resolution. Precipitation and maximum temperature (Portoferraio meteorological station,42°49′N, 10°20′E, 25 m.a.s.l.) at three months scale from September of previous year to December of the current year, were used in the analysis. All correlations were calculated using the treeclim R package (Zang&Biondi 2015).

Results

Tree-Ring Dating and IADFs occurrence

Mean chronologies of *P. pinaster* (Fig. 2a) covered the period from 1964 to 2007 (mean age 38 years, GLK 0,65, rbar 0,47, EPS= 0.73) for the trees at Wet site and from1960 to 2007 (mean age 32 years, GLK 0,55, rbar 0,29, EPS= 0.87) for the trees at the Dry site. For *Q. ilex* (Fig. 2 b)the total time span of the chronologies extended from 1994 to 2007 (mean age 11, GLK0,65, rbar 0,39, EPS=0.81) for the trees at the Dry site and from 1975 to 2007 (mean age 24, GLK 0,59, rbar 0,17, EPS= 0.76) for those at the Wet site.

P. pinaster showed higher frequency of IADFs than *Q. ilex* in both site. In both species IADF's frequency varied according to the site. Indeed, in the case of *Q. ilex* in the Dry site the occurrence of IADFs was higher (*f* = 1.0) than in the Wet site (*f* = 0.5). Conversely *P. pinaster* trees in the Wet site appeared to be more prone of IADFs (*f* = 2.1) than those at the Dry site (*f* = 1.2).



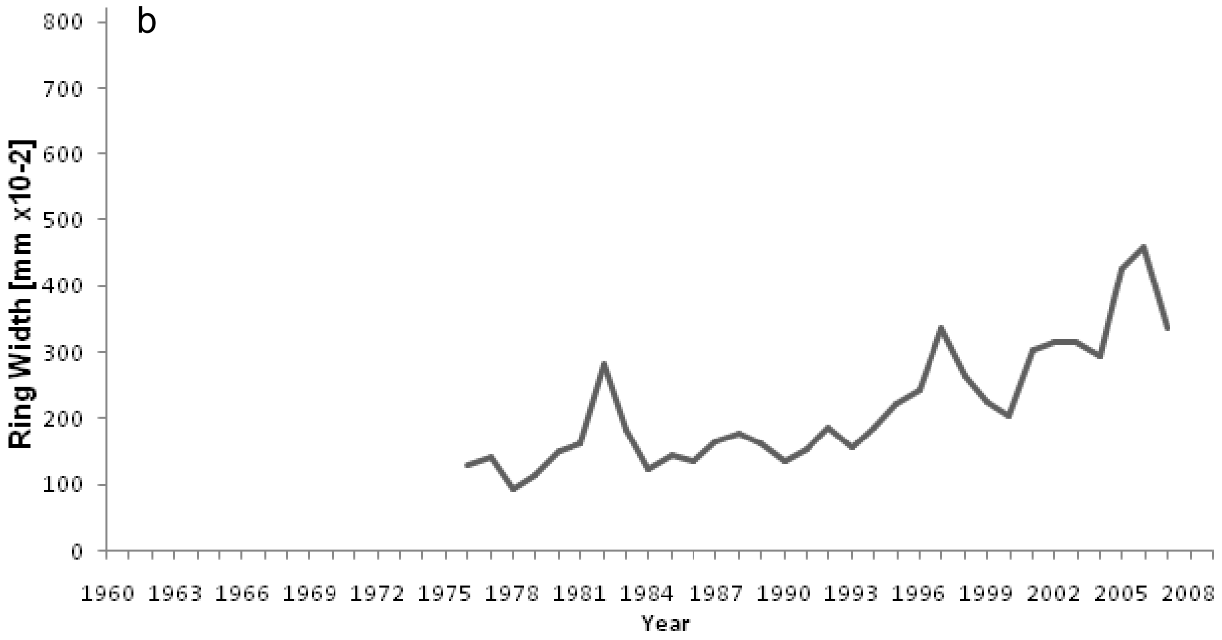


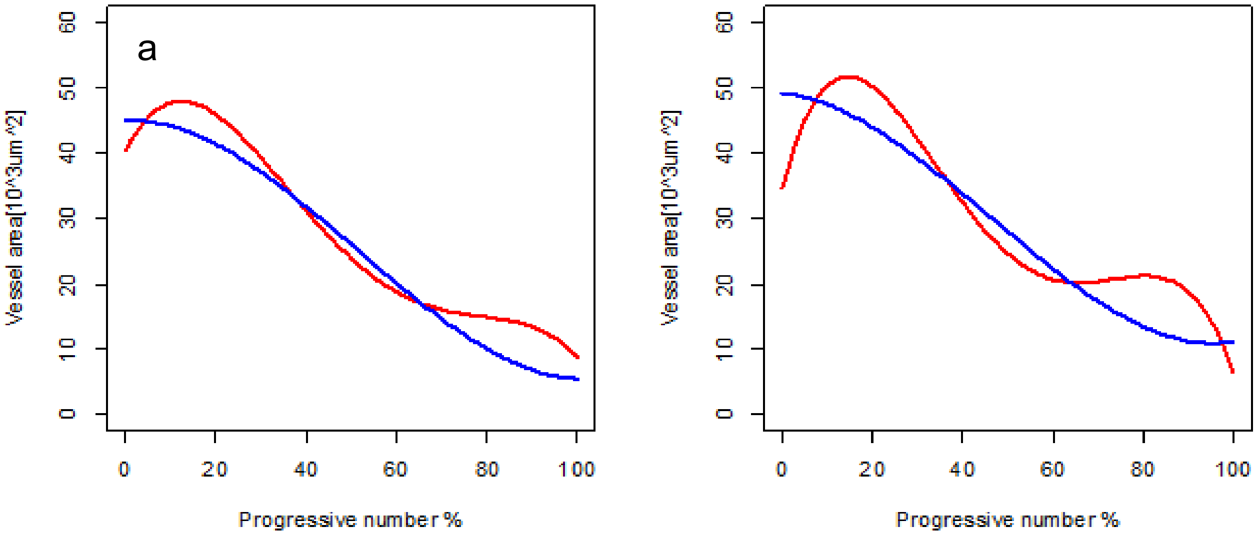
Fig 2. Average ring width chronologies of *P. pinaster* (a) and in *Q.ilex* (b).

Xylem traits

Series of lumen area of tracheids obtained using the standardized progressive number method in *P. pinaster* showed basically a common pattern in both sites, clearly bimodal in the rings with IADFs. Lumen area of tracheids in ring without IADFs decreased from earlywood to latewood (Fig. 3a, b blue line) while in the rings with IADFs increased around the 70% (Fig. 4a, b red line) of the tree ring width, where erlywood-like cells are present. Tracheids lumen area values in rings with IADFs in the Wet site (Fig. 3b, red line) increased more markedly at the end of the ring than in those in the Dry site (Fig. 4a, red line)(r^2 = 0.362 for rings with IADFs, r^2 = 0.410 for rings without IADFs in the Wet site; r^2 = 0.497 for rings with IADFs, r^2 = 0.596 for rings without IADFs in the Dry site).

Wood density measurements showed an opposite patterns than tracheids lumen area. In the rings without IADFs (Fig. 4a) in both sites wood density gradually increase until reaching the maximum values at the end of the ring. In rings with IADFs, instead, density decreased at the end of the ring in particular more markedly in the Wet site (Fig. 4d) than in the Dry site (Fig. 4c).

Trends of lumen area vessel of *Q. ilex* also showed differences between rings with and without IADFs in the two sites. In the Wet site vessels lumen area at the beginning of ring without IADFs (Fig.3d, blue line) showed higher values than those in ring with IADFs (Fig.3d, red line). In the Dry site (Fig.3c), both curves presented lumen area values relatively low, which raised around 15% of the ring. In this case, values of vessel lumen area in rings with IADFs (Fig.3c, red line) showed an increase at the end of the ring (90%). In rings without IADFs (Fig.3c, blue line), vessel lumen area decreased gradually from the beginning to the end of the ring. Rings with IADFs in Dry site (Fig.3c, red line) showed a peak in the values of lumen area at the end of the ring that was not present in rings without IADFs in the Wet site (Fig.3d, blue line). As in *P. pinaster*, also in *Q. ilex* in both sites density increased gradually from the beginning to the end of the ring when the IADFs was not present (Fig. 4c). However in contrast to *P .pinaster*, in the ring with IADFs of *Q. ilex* wood density did not decrease at the end of the ring (Fig. 5d). Vessel frequency (data not shown) showed higher values in the Wet site than in the Dry site and in general the highest values were detected in the middle of the ring. Frequency decreased always at the end of the ring but more markedly in rings with IADFs.



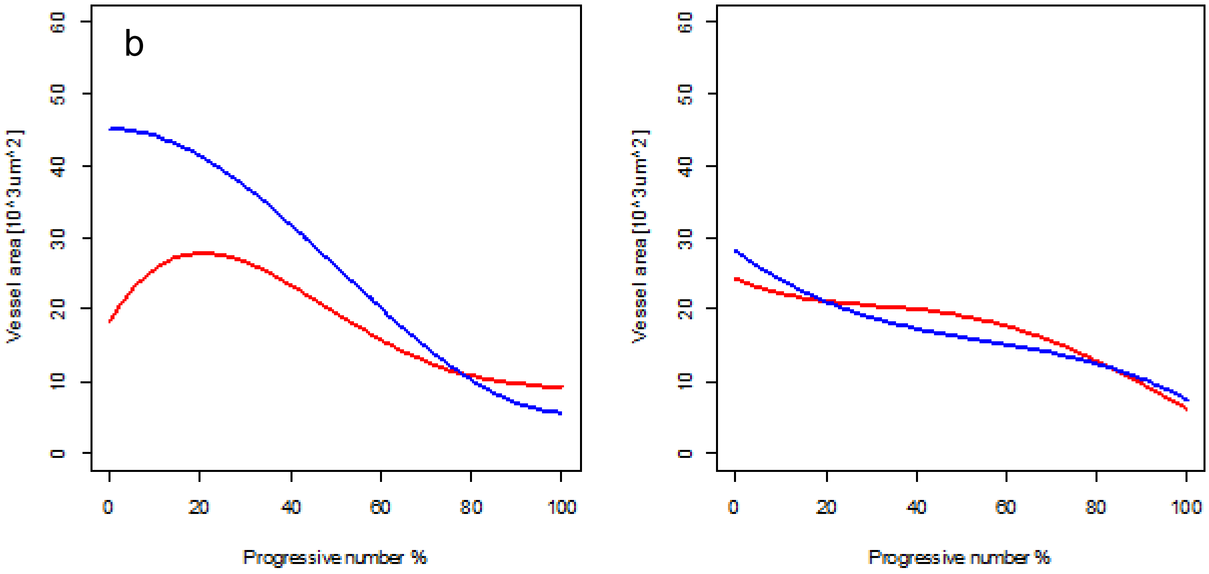
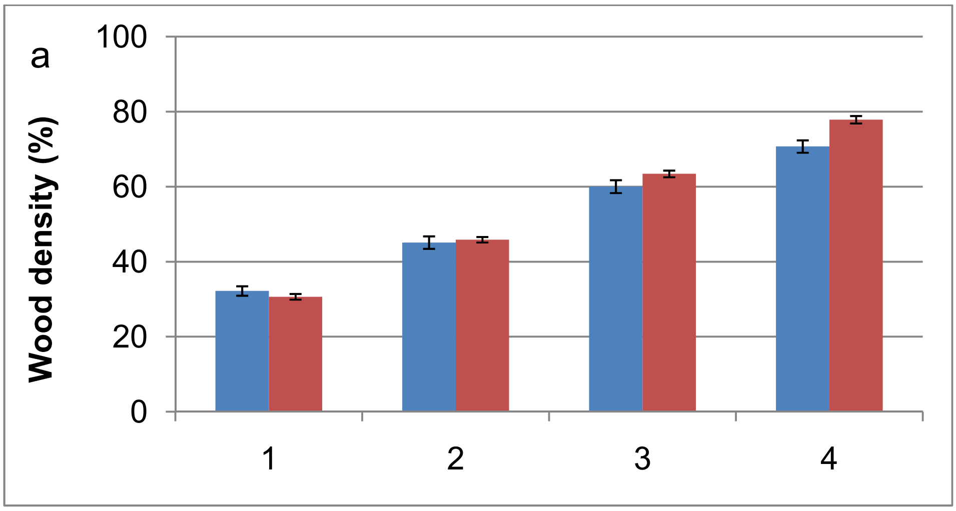


Fig 3. Variation in tracheid/vessel size along ring width shown by plotting theset of the tracheid/vessel-lumen-area standardized data of a replicate of rings with IADFs (red line) and without IADFs (blue line) for *P. pinaster* at Dry (a) and Wet site (b) and *Q. ilex* at Dry (c) and Wet site (d).



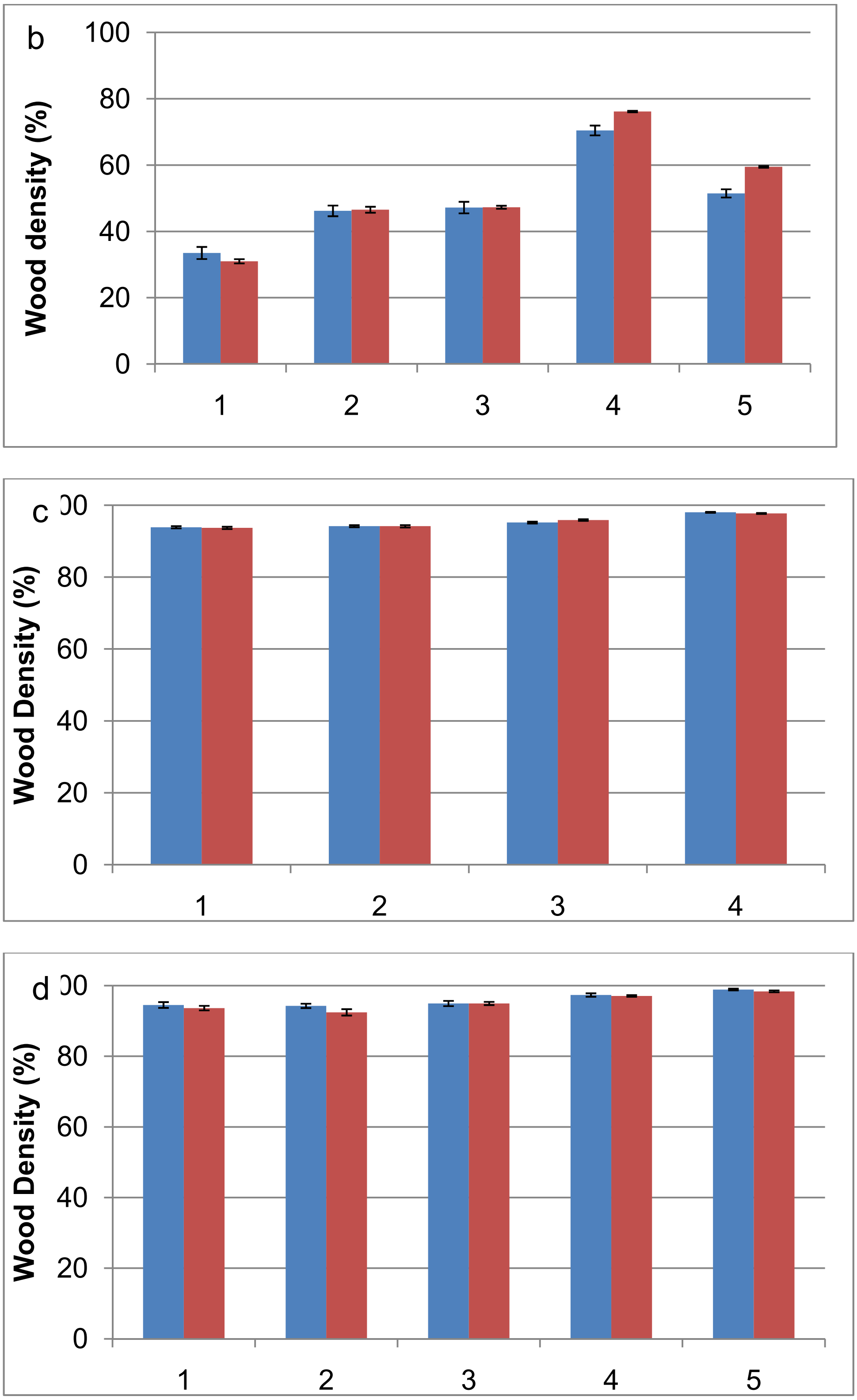


Fig. 4Wood density (measured as percentage of cell walls over total xylem area) in tree rings of *P pinaster*without (a) and with (b) IADFs at the Dry (red) and Wet sites (blue), and of *Q. ilex* without

Climate correlations

Correlations between anatomical traits and climate data (data not shown) showed that in *P. pinaster* growing at the Wet site there was a negative relation between lumen area of latewood tracheids and precipitation from October to December of the previous year, while a positive relation occurred between precipitation from current October to December and tracheid lumen area in the region of IADF. A negative correlation was found between earlywood lumen area and maximum temperature from October to December of the previous year. In the Dry site, there were not significant correlations between lumen area tracheids and precipitation, while maximum temperature from April to June was positively correlated with earlywood lumen area tracheids. In the Wet site, there were not significant correlations between wood density and precipitation while a positive correlation between wood density in earlywood and maximum temperature from January to March was found. In the Dry site, wood density in earlywood was positively correlated with precipitation from January to March while negatively correlated with maximum temperature from December to October of the previous year. Latewood density was negatively related to maximum temperature from July to September. In *Q. ilex* growing in Wet site, we found a positive correlation between lumen area of latewood vessels and precipitation in the period from October to December of the previous year and a positive correlation between lumen area of IADFs vessels and precipitation from October to December of the current year. No significant correlations were found between vessels lumen area and maximum temperature in this site. At the Dry site, vessel lumen area of latewood was negatively correlated with precipitation and positively with maximum temperature in the period from April to June. In the Wet site, latewood density was negatively correlated with precipitation in the period from October to November, while precipitation during these months was positively correlated with wood density in the IADFs region. Maximum temperature in the same months showed a positive correlation with wood density at the end of earlywood and beginning of latewood. In the Dry site, latewood density was negatively correlated with precipitation in the period from April to June, while wood density in the region of IADF was negatively correlated with maximum temperature in the period from January to March. In the Wet site, we found positive correlation between vessels frequency in earlywood and precipitation in the period from June to September. Vessel frequency at the beginning of latewood was negatively correlated with maximum temperature in the period from January to March, while vessel frequency in second part of the latewood was negatively correlated with maximum temperature in the period from October to December. In the Dry site, vessels frequency in the earlywood and at the beginning of latewood was positively correlated with precipitation in the period from April to July. Vessels frequency in IADF region was positively correlated with the maximum temperature in the above- mentioned months.

Concerning the correlation between *L*-IADFs stabilized frequency and climate data, we found that in *P. pinaster*in the Wet site their frequency was negatively correlated to precipitation in November of the previous year and positively correlated with maximum temperature in September. Instead, at the Dry site, the occurrence of *L*-IADFs was positively correlated with the maximum temperature in December. In *Q. ilex* growing at the Wet site, *L*-IADF frequency was negatively correlated with maximum temperature of the previous September and current November. At the Dry site, there were not significant correlations between *L*-IADF frequency and data of precipitation and maximum temperature.

Discussion

Wood anatomical features, such as vessels (or tracheids) lumen area and vessels density, measured in long tree-ring series can be used as climatic proxies with high temporal resolution (Schume et al., 2004; Tardif and Conciatori 2006; Kames et al., 2011), thus improving information traditionally gained from tree-ring width (Fonti and Garcia-Gonzalez 2008; Fonti et al., 2010; Campelo et al., 2010).

In our study, the collection of vessel size data *in continuum* within the tree-ring chronologies (De Micco et al. 2012) and the quantification of wood density and vessels frequency in specific regions within the tree rings allowed to show the patterns of xylem variability of *P. pinaster* and *Q. ilex* in mesic (Wet) and xeric (Dry) conditions. We identified IADF's frequency in both species, classifying them as *L*-type according to De Micco al., (2016).

P. pinaster series of lumen area tracheids showed a clear bimodal pattern due to the occurrence of *L*-IADFs already identified in this species growing at other Mediterranean sites (Bogino& Bravo 2009; Vieira et al., 2009; Rozas et al., 2011). Tree rings of *P. pinaster* growing in the Wet site appeared to be more prone to form *L*-IADFs than those at the Dry site. In both sites, tree rings with IADFs showed an increase of tracheids lumen area combined to a decrease in wood density at the end of tree ring width. However, at the Wet site the increase of vessel lumen area and the decrease of wood densitywas more pronounced than at the Dry site. The hypothesis is that at the dry site, environmental conditions determined by rainfall events in autumn were not sufficiently favorable to promote so marked increase in wood growt has in the Wet site. Indeed, for the Wet site climatic correlations showed a positive relation between the tracheids lumen area measured in the IADFs region and precipitation in autumn (October-December) that is in agreement with findings by Rozas et. al, (2011). Autumn precipitation would have acted on cell differentiation by increasing the turgor of xylem cells during the enlargement phase, consequently producing earlywood-like cells within latewood (Wimmer et al., 2000). In trees growing at the Dry site, such a correlation was not found. On the contrary, we found a positive correlation between earlywood tracheid lumen area and maximum temperature in late spring. At the Wet site, wood density seemed to be not affected by precipitation, but was more sensitive to maximum temperature from January to March. At the Dry site, instead, precipitation from January to March positively influenced earlywood density combined to the increase in temperature in the previous autumn (October-December). The difference of water availability at the two sites, thus seemed to influence the plasticity of xylem that responded in different ways to the variations in climatic condition as also found in other species growing at the same sites (Battipaglia et al., 2010, 2014). The higher water availability at the Wet site likely promoted the formation of tracheids with larger lumen in he second peak of growth. The formation of such tracheids with wide lumen seemed to be enhanced by the occurrence of warm/dry conditions during most of the growing season, in particular from March to May, followed by a wet October. The stabilized frequency of IADFs in the Wet site was negatively correlated with the precipitation of the previous November, in agreement with similar relations found also by Campelo et al. (2013)in *P. pinaster* growing in the central coast in Portugal linking IADF's frequency to low precipitation in November of the previous year. In this respect, scarce winter precipitation seems likely reduce moisture availability during the next growing season, increasing water stress, especially noticeable during the summer, slowing down cambial activity and causing an earlier start of latewood formation (Campelo et al., 2013). Current summer and high precipitation in autumn enhance IADFs occurrence (Vieira et al., 2009, 2010; Rozas et al., 2011; Campelo et. al, 2013, 2015). Since at Wet site water availability seems not to be a limiting factor, IADF formation could be triggered by warm condition in September combined with the peak in rain events in autumn. Such finding are in agreement with those of a previous study on *Arbutus unedo* growing in a mesic and a xeric site on Elba Island in which was already reported the importance of mild temperature in September associated with higher water availability for the plant to form *L*-IADFs in the wet site (Battipaglia et al., 2010).

At the Dry site, the second peak in the cell lumen area was not so pronounced as at the Wet site probably due to the lower water availability. IADFs frequency at Dry site was positively correlated with maximum temperature in December. Such relation suggests the hypothesis of a prolonged cambial activity, lasting in December, with the formation of *L*-IADFs after autumn favourable conditions.

As regards *Q. ilex* series of vessel lumen area, they did not show a clear bimodal pattern although *L*-IADFs were found within the tree ring. Only in rings with IADFs at the Dry site there was a clear increase of vessel lumen area at the end of the ring following the gradually decrease of values from earlywood to latewood. This increase in vessel lumen was accompanied by a decrease in vessel frequency, while wood density seemed to maintain constant values. The increase of vessel lumen area at the end of the ring seemed to be promoted by wet and warm condition from April to June, period in which the most of the tree ring is formed in this specie (Nabais et al.,1998-1999; Abrantes et al., 2013), that could promoted resumption of cambial activity in autumn leading to the IADF formation. Wet and warm condition from April to June also seemed to have negative influence on vessels frequency, while density at the end of the ring was negatively correlated with winter temperature. Wood density at beginning of latewood seemed to be negatively related to precipitation from April to June, while the increase in temperature in these months positively influenced vessel frequency. These results are in agreement with Abrantes et al. (2013) who also found a positive correlation between vessel size and spring precipitation, and negative correlation between density and precipitation in early summer. Precipitation during the summer could be related to the formation of new leaves in *Q. ilex* with the deposition of an extra growth band of xylem (Corcuera et al., 2004; Campelo et al., 2007a, 2010). Correlation between climatic data and stabilized IADFs frequency at Dry site were not significant, which suggests that the formation of IADFs in the dry condition could be triggered by different interconnected factors from those acting during wet conditions.

At the Wet site in *Q. ilex*, as in *P. pinaster*, vessel lumen area and wood density at the end of the ring was positively affected by autumn precipitations that instead influenced negatively vessel frequency. The higher amount of water availability in this case seemed to induce the formation of more efficient wood, with larger lumen and lower wood density and vessels frequency in comparison with the Dry site. The stabilized IADFs frequency showed to be negatively correlated with maximum temperature of the previous September and current November that probably can affect cambial activity at beginning and at the end of the vegetative period. The formation of *L*-IADFs in both species could increase the hydraulic conductivity late in the growing season or increase the water storage capacity of trees due to their larger lumen area (Campelo et al., 2013).Indeed, tracheids of latewood IADFs are earlywood-like cells, thus with larger diameters and higher hydraulic efficiency than ‘true’ latewood cells (Domec and Gartner 2002; Deslauriers et al., 2008).Similarities between species were more marked at the Dry site than Wet site, indicating the higher climatic constraint determined by the arid conditions (Camarero 2010).

In conclusion, not only tracheid and vessel area, but also frequency and wood density showed to be efficient proxies to encode climate signals recorded in IADFs. Moreover the degree of plasticity of these traits to the seasonal climatic condition, as well as IADFs frequency, resulted to be linked to water availability of the growing site.

Both species formed a similar type of IADF, but factors triggering their formation were site- and species- specific, thus confirming findings in other species at other Mediterranean sites. The incorporation of IADFs in dendroecolgy can improve the resolution of the climate signal within the growing season (Wimmer 2002) obtaining information about the physiological and ecological significance of IADFs as adaptive traits (Rozas et al., 2011). To obtain a detailed insight, we planned moreover further investigation through a xylogenesis approach to detect the timing of cambial activity and thus to reach a precise synchronization between wood formation and concurrent climatic factors needed to fully disclose IADFs ecological rule in the two species.

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Timing of False Ring Formation in *Pinus halepensis* and *Arbutus unedo* in Southern Italy: Outlook from an Analysis of Xylogenesis and Tree-Ring Chronologies

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Mediterranean tree rings are characterized by intra-annual density fluctuations (IADFs) due to partly climate-driven cambial activity. IADFs are used as structural signals to gain information on relations between environmental conditions and eco-physiological processes during xylogenesis, with intra-annual resolution. To reach an unbiased synchronization of the IADF position within tree rings and seasonal fluctuations in environmental conditions, it is necessary to know the timing of cambial activity and wood formation, which are species- and site-specific processes. We applied the microcoring technique to analyze xylogenesis in *Pinus halepensis* and *Arbutus unedo*. To the best of our knowledge, this is the first attempt to study xylogenesis in a hardwood species forming frequent IADFs. Both species co-occur at a site in southern Italy characterized by a Mediterranean climate. To facilitate tree-ring dating and identification of IADFs, we performed traditional dendroecological analysis. We analyzed xylogenesis during summer, which is considered a constraint for xylogenesis and a trigger for IADF formation. We followed the different phases of cell development in the current wood increment with the aim of evaluating *whether* and *which* type of IADFs were formed. We additionally analyzed the same phases again in September and in winter to verify the possible formation of IADFs in fall and whether cell production and differentiation was completed by the end of the calendar year. Both species formed the same type of IADFs (earlywood-like cells within latewood), due to temporary growth restoration triggered by rain events during the period of summer drought. At the end of the calendar year, no cells in the phases of enlargement and secondary cell wall deposition occurred. *A. unedo* was more sensitive than *P. halepensis* because IADFs were formed earlier in the season and were more frequent in the tree-ring series. The dendro-anatomical approach, combining analysis of tree-ring series and of xylogenesis, helped to detect the period of IADF formation in the two species. Results are discussed in functional terms, highlighting the environmental conditions triggering IADFs, and also in methodological terms, evaluating the applicability of xylogenesis analysis in Mediterranean woods, especially when the formation of IADFs is not uniform around the stem.

Keywords: cambial activity, cambial phenology, mediterranean climate, intra-annual density fluctuations, tree rings

INTRODUCTION

Tree rings are well-established climate proxies: environmental information can be extracted from dated tree-ring series by analyzing the variability in tree-ring width, earlywood and latewood widths, wood density and functional anatomical traits (e.g., Eckstein and Schmidt, 1974; Schweingruber, 1978; Eckstein et al., 1979; Tardif, 1996; Cherubini et al., 2003; Grudd, 2008; Fonti et al., 2010; Esper et al., 2012; Beeckman, 2016). The analysis of tree-ring series is relatively easily applied in plants from temperate regions characterized by a clear seasonality inducing a dormancy in cambial activity once a year (e.g., Prislan et al., 2013a). Under such conditions, each ring corresponds to one calendar year, with earlywood and latewood, respectively, linked to spring and summer climatic conditions (Fritts, 1976).

The advancement of tools of digital image analysis has raised new interest in the application of quantitative wood anatomy to tree-ring series to study a plant's response to environmental changes (Fonti et al., 2010; von Arx and Carrer, 2014). The analysis of tree-ring series is more and more applied in various climatic regions worldwide and has a great potential to reconstruct environmental information with seasonal or intra-seasonal resolution, especially under conditions promoting an alternation of growth flushes and dormancy during the year (De Micco et al., 2016a). Within this context, wood of Mediterranean species is particularly interesting because frequent fluctuations in climatic factors exert a control on cambial activity, thus triggering the formation of intra-annual density fluctuations (IADFs) in tree rings (Cherubini et al., 2003; De Micco and Aronne, 2009; De Micco et al., 2016a). The increasing drought and changes in the frequency of precipitation and extreme events forecasted for the Mediterranean basin (IPCC Working Group I et al., 2013) will likely influence trends in cambial phenology and xylogenesis, thus the frequency and structural features of IADFs (Vieira et al., 2010). Since different species can show different sensitivities to fluctuating environmental conditions and can be differently prone to form IADFs in various environments, understanding the patterns and processes of xylem formation in response to variable environmental conditions is valuable for forecasting species growth fitness and adaptation capability (Camarero et al., 2010), which are ultimately linked to forest dynamics, biomass production and biogeochemical cycles (Cuny et al., 2015; Xia et al., 2015; Pacheco et al., 2016).

IADFs have been considered a constraint in dendrochronology until recently but they have been finally accepted as "positive anomalies" in tree rings because their analysis furnishes information on the relations between environmental conditions and eco-physiological processes during wood formation, with intra-annual resolution (Campelo et al., 2007a,b, 2013; de Luis et al., 2007, 2011a; De Micco et al., 2007, 2012, 2014; Battipaglia et al., 2010, 2014; Vieira et al., 2010; Rozas et al., 2011). In the last decade, numerous studies have analyzed IADFs in Mediterranean softwoods and hardwoods, also raising hypotheses on the factors responsible for their formation (De Micco et al., 2016a). Several classifications of IADFs have been proposed based on their position within the ring and on anatomical traits (e.g., lumen diameter and cell-wall thickness) of the xylem conduits in the IADF zone (Campelo et al., 2007a,b, 2013, 2015; Battipaglia et al., 2010, 2014; De Micco et al., 2012, 2014). In Mediterranean conifers growing at coastal sites in south-eastern Spain, the most common IADFs are classified as type-*L* (large-lumen and thin-walled earlywood-like cells within narrow-lumen and thick-walled latewood conduits), whose formation has been linked to the reactivation of cambial activity, due to favorable conditions in fall after a period of summer drought (de Luis et al., 2011a,b; Campelo et al., 2013; Novak et al., 2013a,b, 2016; Carvalho et al., 2015; Vieira et al., 2015). Type-*E* IADFs (narrow-lumen and thick-walled latewood-like cells within large-lumen and thin-walled earlywood conduits) have been described in *Pinus pinaster* growing in Italy and are considered a response to summer drought conditions inducing stomata closure (De Micco et al., 2007). Both types of IADFs have also been found in a few Mediterranean hardwoods (Campelo et al., 2007a, 2010; Battipaglia et al., 2010, 2014; De Micco et al., 2012, 2014).

Hypotheses on the reason for IADF formation derive from indirect evidence, namely correlations with climate variables (i.e., temperature and precipitation) but knowledge gaps still remain to be filled (Battipaglia et al., 2016; De Micco et al., 2016a; Zalloni et al., 2016). The formation of IADFs in Mediterranean species has been mainly linked to water availability, which affects the turgor-driven expansion of xylem cells (Sperry et al., 2006; De Micco et al., 2016a). However, to confirm such a hypothesis, the study of IADFs during their formation is needed through analysis of xylogenesis aimed at unraveling *how* and *when* wood with specific anatomical traits is formed (Vaganov et al., 2006; Camarero et al., 2010; de Luis et al., 2011b; Vieira et al., 2014; Novak et al., 2016). The analysis of xylogenesis in woods forming IADFs with high frequency is useful for achieving precise synchronization of the IADF position within the tree ring and specific environmental fluctuations triggering them. Analysis of cambial activity has been widely applied through micro-coring techniques, mostly in conifers and hardwoods growing in temperate climates (Rossi et al., 2003, 2006, 2008, 2012; Čufar et al., 2011; Prislan et al., 2013b; Gričar et al., 2014; Pérez-de-Lis et al., 2016). Analysis of xylogenesis has also recently been applied to tree rings forming IADFs in *Pinus* species (de Luis et al., 2011a; Vieira et al., 2014, 2015; Novak et al., 2016). However, to the best of our knowledge, there are no reports dealing with the study of IADF-genesis in hardwood species.

In this study, we analyzed cambial activity in a softwood and a hardwood species, *Pinus halepensis* Mill. and *Arbutus unedo* L., co-occurring at a site in southern Italy, characterized by a Mediterranean climate. The work aimed at: (1) evaluating *whether* and *which* type of IADFs were formed during summer, and (2) highlighting which weather conditions were concomitant or closely preceding IADF formation. Together with the two ecological aims, we also pursued a third methodological issue. In view of the fact that the formation of IADFs is a variable phenomenon along the stem circumference in Mediterranean woods (Cherubini et al., 2003), we aimed to verify the degree of applicability of the micro-coring technique to the two species, especially considering that *A. unedo* is a hardwood species. Thus, we applied common dendroecological techniques to analyze tree-ring chronologies and evaluate their variability within and between plants.

We performed microcore sampling and microscopy analysis of thin cross sections during the period of summer aridity (which is considered a factor limiting plant growth and predisposing the occurrence of IADFs) to detect the time of IADF formation. In order to do this, we first evaluated the onset of latewood formation during the development of tree rings with and without IADFs, then continued the analysis of xylogenesis, searching for earlywood-like cells, until we detected the formation of an IADF in the 2014 tree-ring. This was based on the hypothesis that IADFs in both species are formed during the summer months, triggered by water stress followed by the temporary restoration of growth as a consequence of favorable conditions. We therefore hypothesized that both species experience a bimodal pattern of cambial activity, as reported in Camarero et al. (2010), thus completing their ring growth by the end of the calendar year. To verify this hypothesis, we also analyzed xylogenesis at additional dates until the end of the calendar year. This helped us to verify that ring growth had been completed by the end of the calendar year, as commonly assumed in traditional dendrochronology, and to verify the possible formation of other IADFs primed by rain events in fall.

MATERIALS AND METHODS

Species and Study Area

The study was conducted in 2014 on plants of *Pinus halepensis* Mill. and *Arbutus unedo* L. co-occurring at a site at Quisisana, Castellammare di Stabia (Naples) in southern Italy. The sampling site (40′683 N, 14′481 E, 346 m a. s. l) is characterized by typical Macchia vegetation, with shrub and tree species including *Quercus ilex* L., *P. pinaster* Aiton, *P. halepensis* Mill., *Castanea sativa* Mill., *Fraxinus ornus* L., *Acer opalus* Mill. subsp. *neapolitanum*, *Erica arborea* L., *Laburnum anagyroides* Medik, *A. unedo* L., and *Ruscus aculeatus* L. The climate at the site is Mediterranean, with hot, dry summers followed by mild, wet winters. According to data recorded at the closest meteorological station, 10 km from the sampling site (Pimonte, 40′672N, 14′50E, 370 m a. s. l), during the year of sampling 2014, annual mean temperature was 15.5°C with the hottest month being August (monthly average mean temperature of 23.3°C) and the coldest month being January (monthly average mean temperature of 9.8°C). The cumulative annual precipitation was 1020 mm; the wettest month was January, with a cumulative monthly precipitation of 447 mm, while the lowest value was reached in August (cumulative precipitation of 1.4 mm). The worst aridity period lasted from June to the beginning of September (**Figure 1**). Longer meteorological series are not available for the Pimonte site. Meteorological series from other nearby stations in the Campania region were analyzed and showed that meteorological conditions in 2014 did not deviate considerably from those registered in the period 2005–2014.

Tree-Ring Data

Tree-ring chronologies were built through common dendroecological techniques to facilitate the synchronization of tree rings and IADFs in the collected microcores.

Core sampling was carried out in March 2015 on 15 dominant trees of *P. halepensis* and 15 plants of *A. unedo*. Diameter at breast height (DBH) was measured and two cores were taken at breast height as well from each tree (west and east directions) with an increment borer (diameter 5 mm). The cores were transported to the laboratory and air dried. The surface of the cores was polished using sand paper of different grain-sizes, and tree-ring width (TRW) measurements were made at a resolution of 0.01 mm, using LINTAB measurement equipment fitted with a stereoscope and equipped with TSAP Win software (Frank Rinn, Heidelberg, Germany). Tree-ring series were visually cross-dated and compared using standard dendrochronological techniques (Stokes and Smiley, 1968). The

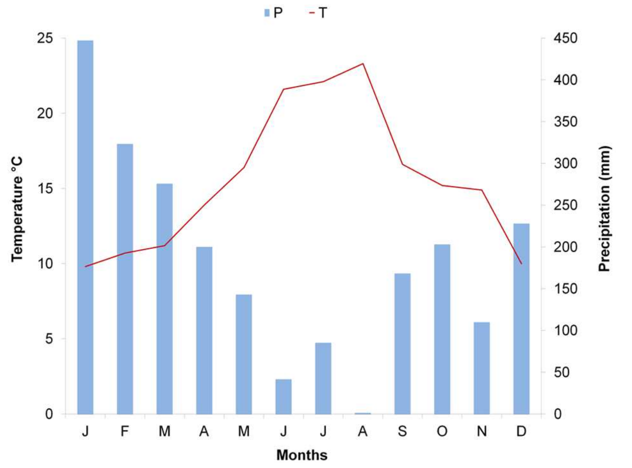

FIGURE 1 | Climatic diagram of Pimonte meteorological station: monthly average mean temperature and total monthly precipitation for 2014.

cross-dating accuracy was then checked using the program COFECHA (Holmes, 1983). The program ARSTAN (Cook, 1985) was used to remove growth trends related to tree age and competition, producing standardized tree-growth indices. Series were detrended with a 10-year spline to remove long-term growth trends embedded in the raw tree-ring series, which were thought to be induced by non-climatic influences, such as aging and competition between trees (Fritts, 1976). Once all series had been validated, tree-ring chronologies were constructed. Descriptive statistics were computed, including standard deviation (SD), which estimates the variability of measurements and the expressed population signal (EPS), thus indicating the level of coherence of the constructed chronology and how it portrays a hypothetical perfect population chronology.

The occurrence of IADFs was quantified in each core by considering each ring as a growth increment and distinguishing the true annual rings from IADFs through visual analysis of the features of boundaries (e.g., abruptness of changes between earlywood and latewood cells) and considering data from cross dating in cases of doubt (Cherubini et al., 2003; De Micco et al., 2016a). We finally calculated the frequency of IADFs in each plant as the ratio between the number of IADFs and the total number of increment growth. The Chi-square test was used to compare the occurrence of IADFs between the two species (two-way contingency table).

Microcore Sampling and Microscopy

Microcores (1.8 mm in diameter) were collected with a Trephor tool (Rossi et al., 2006) from six trees of *P. halepensis* and eight plants of *A. unedo* at breast height, following a spiral with a distance of 2 cm between consecutive samples. Since we were interested in following xylogenesis throughout the aridity period, microcores were collected at weekly intervals starting from June. Sampling was interrupted in August because the observation of microcores (as reported below) showed that IADFs had been already formed. Further microcore collections were done after rain events at the beginning of September 2014 and at the end of the calendar year to check whether other IADFs had been formed in August or during fall.

Microcores were immediately fixed in 70% ethanol and stored at 4°C. Microcores were then embedded in paraffin using a Leica TP 1020-1 (Nussloch, Germany) tissue processor for dehydration in alcohol series (70, 90, 95, and 100%) and bio-clear (d-limonene) for paraffin infiltration. Paraffin blocks were cut with a semi-automatic rotary microtome RM 2245, Leica, (Nussloch), thus obtaining cross sections (9 μm thick), which were flattened on slides pre-treated with albumin. The slides were dried at 70°C for 30 min and cleaned of residual paraffin by washing with bio-clear and ethanol. The sections were then stained with a water solution of safranin and astra blue (Werf van der et al., 2007) and permanently mounted on glass slides in Euparal (Bioquip Rancho Dominguez, California). The sections were observed under a BX61 transmission light microscope (Olympus, Hamburg, Germany), while the quantification of anatomical parameters was performed through a Nikon Eclipse 800 microscope equipped with Nis Elements BR3 (Melville, NY, USA) image analysis software on microphotographs captured with a Nikon DS-Fi1 digital camera.

In *P. halepensis*, we focused on the development of tracheids, using visual criteria based on lumen size and wall thickness (de Luis et al., 2007, 2011a; Novak et al., 2016) measured by means of the eyepiece micrometer while looking through the microscope. The distinction between earlywood and latewood was based on the application of Mork's definition (Mork, 1928). In *A. unedo* we focused on the development of vessels and imperforate tracheary elements, using visual criteria based not only on lumen size and wall thickness but also on vessel frequency; the transition between earlywood and latewood was often diffuse but latewood could be distinguished by the presence of narrower (halved lumen diameter) and less frequent vessels than earlywood (De Micco et al., 2016b). The following phases of cell development were considered according to de Luis et al. (2007) and Čufar et al. (2008, 2011): cambial cells (CC), post cambial cells (PC), cells with developing secondary wall (SW) and mature cells with lignified secondary wall (MC) (**Figure 2**). CC were radially flattened, with thin cell walls that stained blue. PC were enlarging cells in the phase of postcambial growth, which also stained blue. SW were immature xylem derivatives with developing (thickening and lignifying) secondary walls. SW cells showed birefringence under polarized light and stained blue and light red, depending on the progress of lignification. MC were cells without any trace of protoplast in the lumen and had fully deposited and lignified cell walls that colored intense red by safranin. The cambium was considered productive when PC were detected.

In *P. halepensis*, the number of cells in different developmental phases was counted. In *A. unedo*, since xylem consisted of conduits and fibers that were not arranged in ordered radial rows, the width of the cambium zone and developing xylem corresponding to the various developmental stages was measured as in Čufar et al. (2008, 2011). Measurements were taken in the cambium and in the developing xylem ring along three radial rows. Measurements along three radial rows were averaged.

Finally, in order to detect the time of IADF formation, we analyzed the microsections from subsequent microcores by focusing on cells in SW and MC phases to highlight changes in cell lumen size and wall thickness marking the transition from earlywood to latewood and vice versa. For each series of microcores, we classified SW and MC cells into four categories: earlywood (EW), latewood (LW), earlywood-like (EW-like), and secondary production of latewood (SLW) to distinguish them from customary LW. When the transition from earlywood to latewood was detected only once, and latewood formation was maintained until the end of the calendar year, the ring was classified as "not having an IADF". In contrast, when the transition from earlywood to latewood was followed by additional transition from latewood to earlywood (EW-like) and from EW-like to latewood (SLW), then the ring was classified as "having an IADF". We calculated the percent of plants showing SW and MC cells in each category per each date.

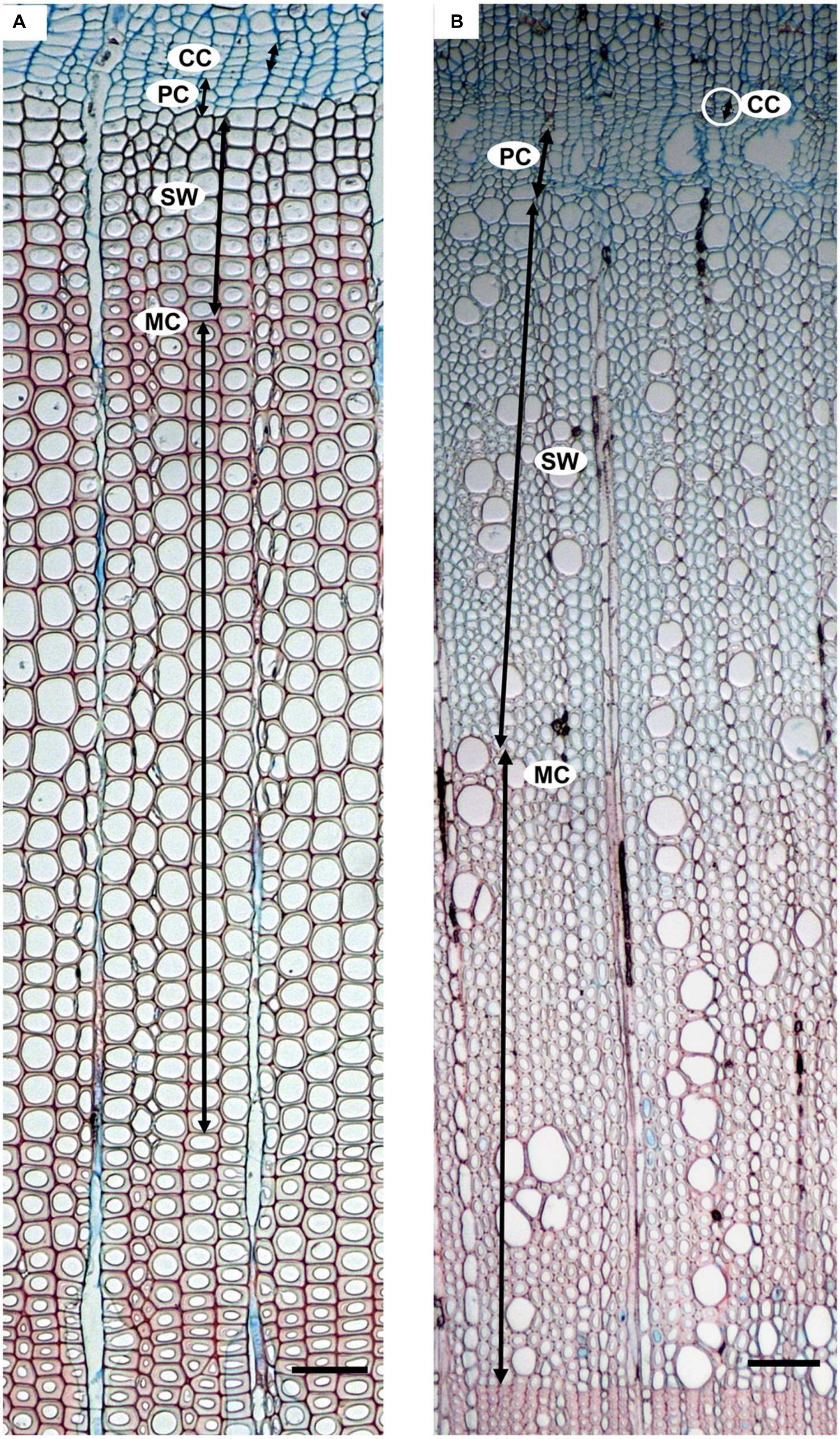

FIGURE 2 | Developing xylem in *Pinus halepensis* (A) and *Arbutus unedo* (B). Moving from the cambial zone toward the center of the stem, the following cells are encountered: cambial cells (CC), enlarging post cambial cells (PC), cells developing secondary walls (SW), and mature cells with lignified secondary wall (MC). Scale bars = 100μm.

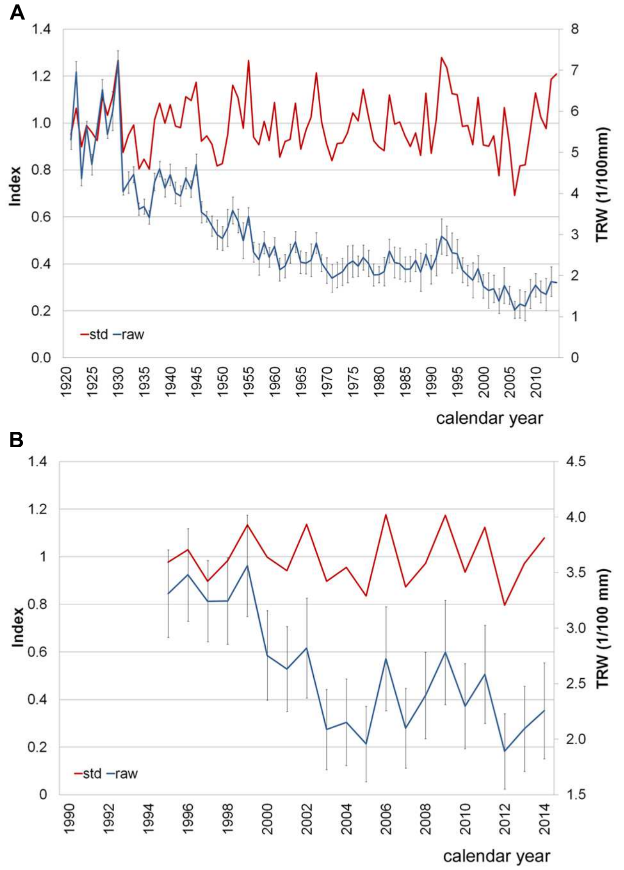

FIGURE 3 | Average ring-width chronologies using either raw (raw, blue line) or detrended data (std, standardized data, red line), in *P. halepensis* (A) and *A. unedo* (B). Standard deviation (SD) is shown for raw data.

RESULTS

Tree-Ring Chronologies and IADF Occurrence

The *P. halepensis* trees had a DBH of 52.24 ± 5.41 cm (mean value ± SD) and belonged to the same age class, with a mean of 90 ± 12 years (**Figure 3A**). High EPS values (>0.85) for the period of 1921–2014 indicated that the mean chronology was representative of radial growth variations of the whole population of trees (Wigley et al., 1984). The MS value (0.25) and the *r* bar value (0.82) showed a strong common growth signal among individuals. Thus, the variability among individuals and between twin cores from the same tree was not high. Despite the occurrence of IADFs, it was still possible to recognize, measure and cross-date the rings and build a robust mean chronology (**Figure 3A**). The percentage of IADFs in *P. halepensis* was 20.19 ± 2.63% (mean value ± SE), with a minimum of 6.19% and a maximum of 34.56%. In 2014, 58.3% of the trees formed IADFs appearing as earlywood-like cells within latewood (**Figures 4A,B**).

A different situation was found in the *A. unedo* plants. They were younger than the *P. halepensis* trees, with DBH of 7.97 ± 1.78 cm and related tree-ring chronologies spanning from 1995 to 2014 (**Figure 3B**). Very high variability was found among individuals and between twin cores from the same plant, as shown by high values of standard deviation (**Figure 3B**). Furthermore, the *A. unedo* cores contained a very high frequency of IADFs, hampering cross-dating. In this shrub hardwood, the percentage of IADFs was 36.65 ± 3.02% (mean value ± SE), with a minimum of 21.43 and maximum of 48.39%. In some cases, the tree-ring from the same calendar year contained more than one IADF. In 2014, 72.3% of the plants formed IADFs appearing as earlywood-like cells within latewood (**Figures 4C,D**).

The analysis of the two-way contingency table showed that the percentage of IADFs was significantly higher ($p < 0.0001$) in *A. unedo* than in *P. halepensis* tree-ring series.

Cambial Productivity

As expected, xylem formation in *P. halepensis* and *A. unedo* had started prior to the first sampling on 5 June. At the beginning of June, in *P. halepensis* the cambial zone (CC) consisted of 5.81 ± 0.14 cells (mean value ± SE) and the current xylem growth ring consisted of 11.72 ± 1.65 cells in different phases of differentiation (PC and SW) and included 1.99 ± 0.81 fully differentiated cells (MC). The number of CC slightly increased in July (**Figure 5A**). The number of PC was highest on 12 June, remained more or less stable until the end of July and was very low at the beginning of September. The number of SW and MC cells varied due to the usual variability around the stem. In December, CC consisted of 5.00 ± 0.23 cells and no differentiating PC or SW cells were observed, so the current tree-ring consisted of MC cells only.

At the time of the first sampling, the CC of *A. unedo* was 18.35 ± 0.82 μm wide (mean value ± SE) and on average consisted of three cells (**Figure 5B**). Its width slightly increased thereafter and reached a maximum on 26 June (34.10 ± 1.10 μm). The thickness of the PC zone reached its maximum on 12 June (63.57 ± 18.67 μm), whereas almost no PC cells were observed on 31 July or at the beginning of September. The zone of SW cells remained wide throughout the summer. In September, the current tree-ring mainly consisted of SW with a small proportion of MC cells. At the end of December, we could observe no cell production and almost all cells of the current ring were fully differentiated. The width of the currently formed tree ring also varied around the stem in *A. unedo*.

Wood Formation and IADFs

The overall analysis of the microcore data showed that at the time of first sampling, only 33.4 ± 4.9% (mean value standard error) and 46.5 ± 6.2% of the xylem increment (tree ring) of the current year had been already formed in *P. halepensis* and *A. unedo*, respectively. In June, it consisted of mainly SW cells with earlywood characteristics.

The analysis of anatomical characteristics of wood formed during summer 2014 was much easier in *P. halepensis* than in

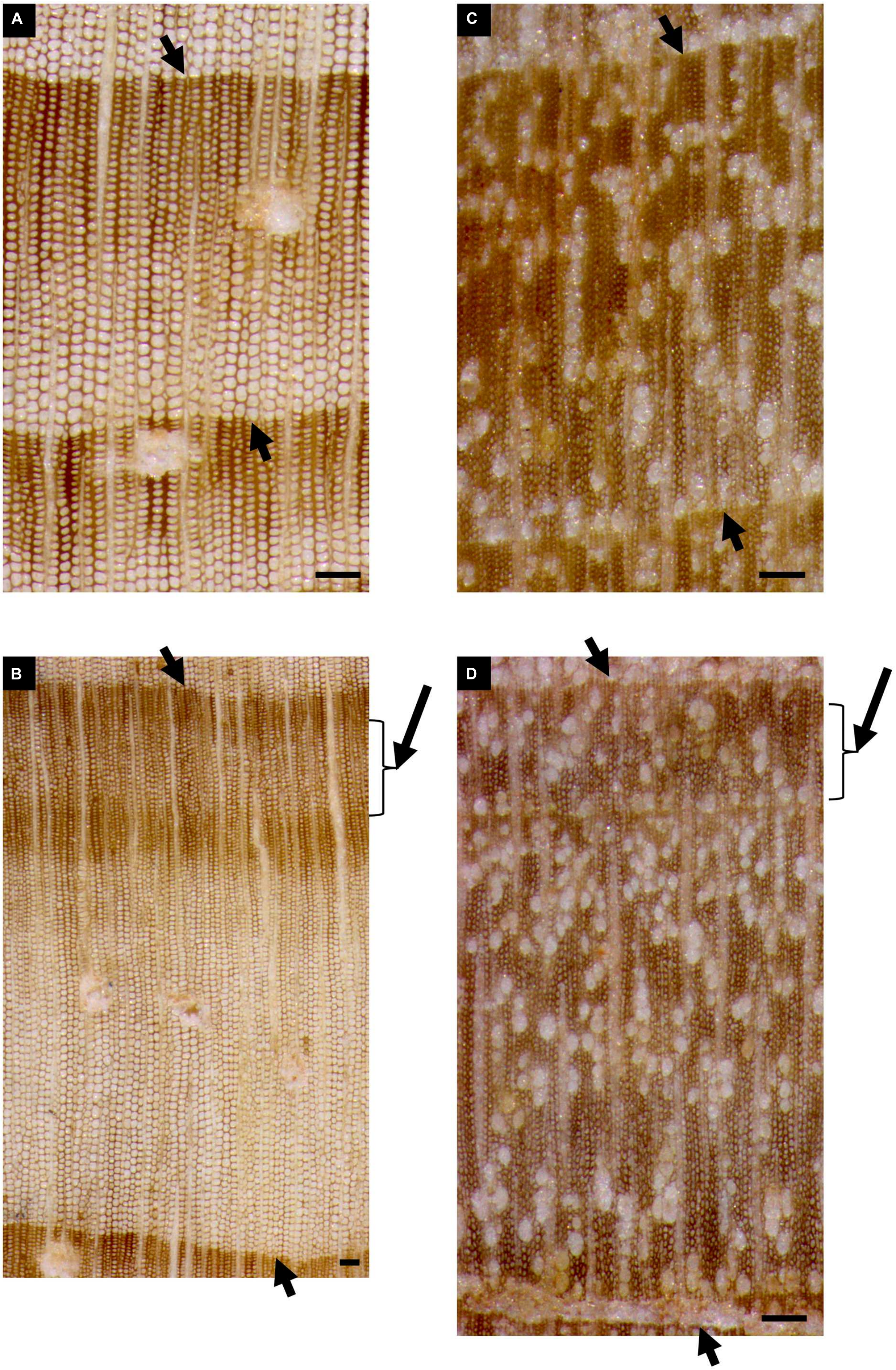

FIGURE 4 | Reflected light microscopy view of the tree ring corresponding to the 2014 calendar year in cores of *P. halepensis* and *A. unedo* with and without IADFs. Tree rings of: *P. halepensis* without IADF **(A)**, *P. halepensis* with IADF **(B)**, *A. unedo* without IADF **(C)**, *A. unedo* with IADF **(D)**. Short arrows point to tree-ring boundaries; long arrows indicate the IADF. Bars = 100 μm.

A. unedo. In *P. halepensis*, two (out of six) analyzed trees, had a "normal" 2014 tree ring, consisting of earlywood followed by latewood with no IADFs (**Figures 4A** and **6A**). In the other four trees, IADFs were formed during the summer (**Figures 4B** and **6B**). As summarized in **Table 1**, in the two trees without 2014-IADF, MC in the increment growth consisted of earlywood tracheids until mid-July (**Figures 4A** and **6A,E**). The first latewood tracheids appeared on 24 July and the production of latewood tracheids continued until the completion of the annual ring (**Table 1**). In the other four trees, the first latewood tracheids appeared completed between 24 and 31 July, and latewood production was followed again by earlywood-like cells, with large lumina and thin cell walls on the successive dates. In samples collected in September, the first mature earlywood-like tracheids were observed (**Figures 6F,G**; **Table 1**) while current PC and SW cells evolved into latewood cells, as found in the last date analyzed (**Figures 4B** and **6B,F**; **Table 1**). From mid-September until the end of the year, only latewood cells were produced. It is worth highlighting that if IADFs formed in 2014 were classified by visual analysis of the complete tree ring without considering the timing of cambial activity, they could have been classified as either *E*-type IADF (latewood-like cells within earlywood) or *L*-type IADF (earlywood-like cells within latewood). By adding the information on xylogenesis, as well as temperature and precipitation of the corresponding calendar year, the rings with IADFs would be classified as *L*-IADFs.

In the case of *A. unedo*, the formation of the different types of wood showed high variability among plants. It was possible to follow the genesis of different wood structures in six (out of the eight) analyzed plants. More specifically, in the other two plants, consecutive microcores (taken in spiral along the stem) showed very different growth increments. Due to the great variability of ring width around the stem it was not always possible to differentiate unequivocally between false and very narrow "normal" tree-rings. These plants were also characterized by very diverse twin cores and corresponding tree-ring series, which could not be cross-dated. We therefore excluded them from the analysis. Of the remaining six plants, two did not form IADFs. As summarized in **Table 1**, in these plants, the first mature latewood vessels were evident on 12 and 19 June and the production of this cell type continued until the completion of the annual ring (**Figures 4C** and **6C**; **Table 1**). In the other four plants, MC observed until the middle of June were of the earlywood type (**Figure 6C**; **Table 1**). The first mature latewood vessels appeared progressively from 19 June to 17 July in the different plants (**Figures 6H,I**, black arrowhead, **Table 1**). In these four plants, after the formation of the first latewood vessels, new earlywood-like cells were formed starting from the middle of July (**Figures 6D,I** blue arrowhead, **Table 1**). A successive production of very narrow latewood was recorded progressively starting from 24 July. Similar to *P. halepensis*, also in *A. unedo* we classified the 2014-IADFs as *L*-IADFs. From September until the end of the year, only latewood cells were produced and no other IADFs were formed. Only in one plant did we finally record two *L*-IADFs in the 2014 ring.

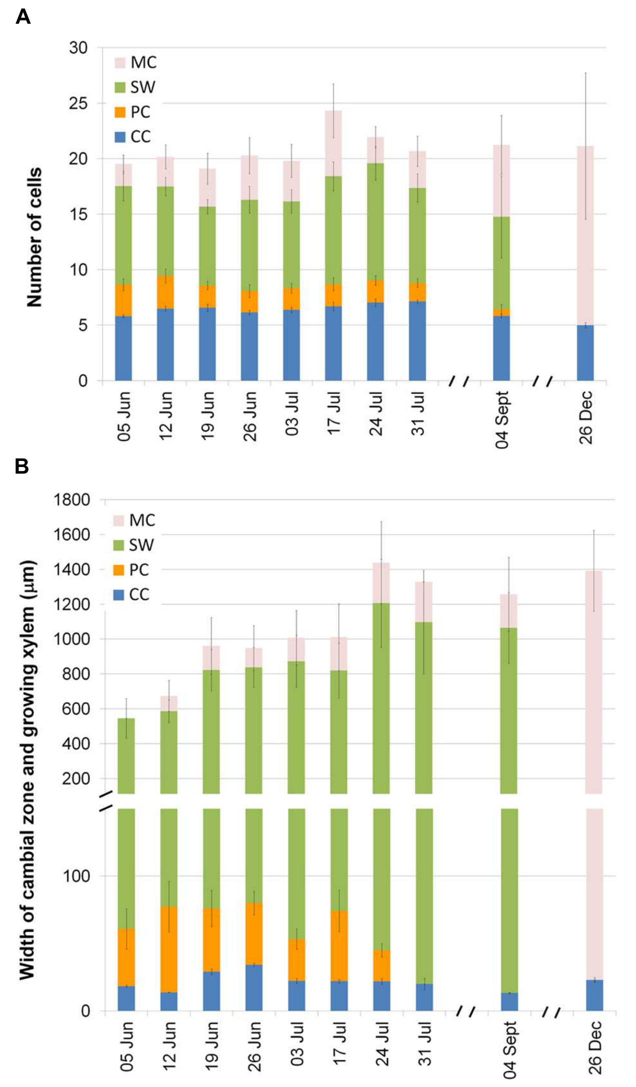

FIGURE 5 | Number of cells in various phases of xylem formation in 6 trees of *P. halepensis* (A) and width of different developmental xylem zones in five plants of *A. unedo* (B): cambial cells (CC), enlarging post cambial cells (PC), cells developing secondary walls (SW), and mature cells with lignified secondary wall (MT). Data from rings both forming and not forming IADFs in 2014 were considered together. Mean values and standard errors are shown.

DISCUSSION

Analysis of cambial activity supported by dendro-anatomical investigations in the softwood *P. halepensis* and the hardwood *A. unedo* allowed us to reconstruct the timing of IADF formation and to hypothesize possible reasons for their formation.

Intra-annual density fluctuation identification and classification is still mainly done by visual analysis of cores and/or microsections under the microscope. When analyzing a tree ring for the presence of an IADF, the operator considers the sequence of wood types (earlywood, latewood) along the tree-ring width. A calendar tree-ring contains an IADF when

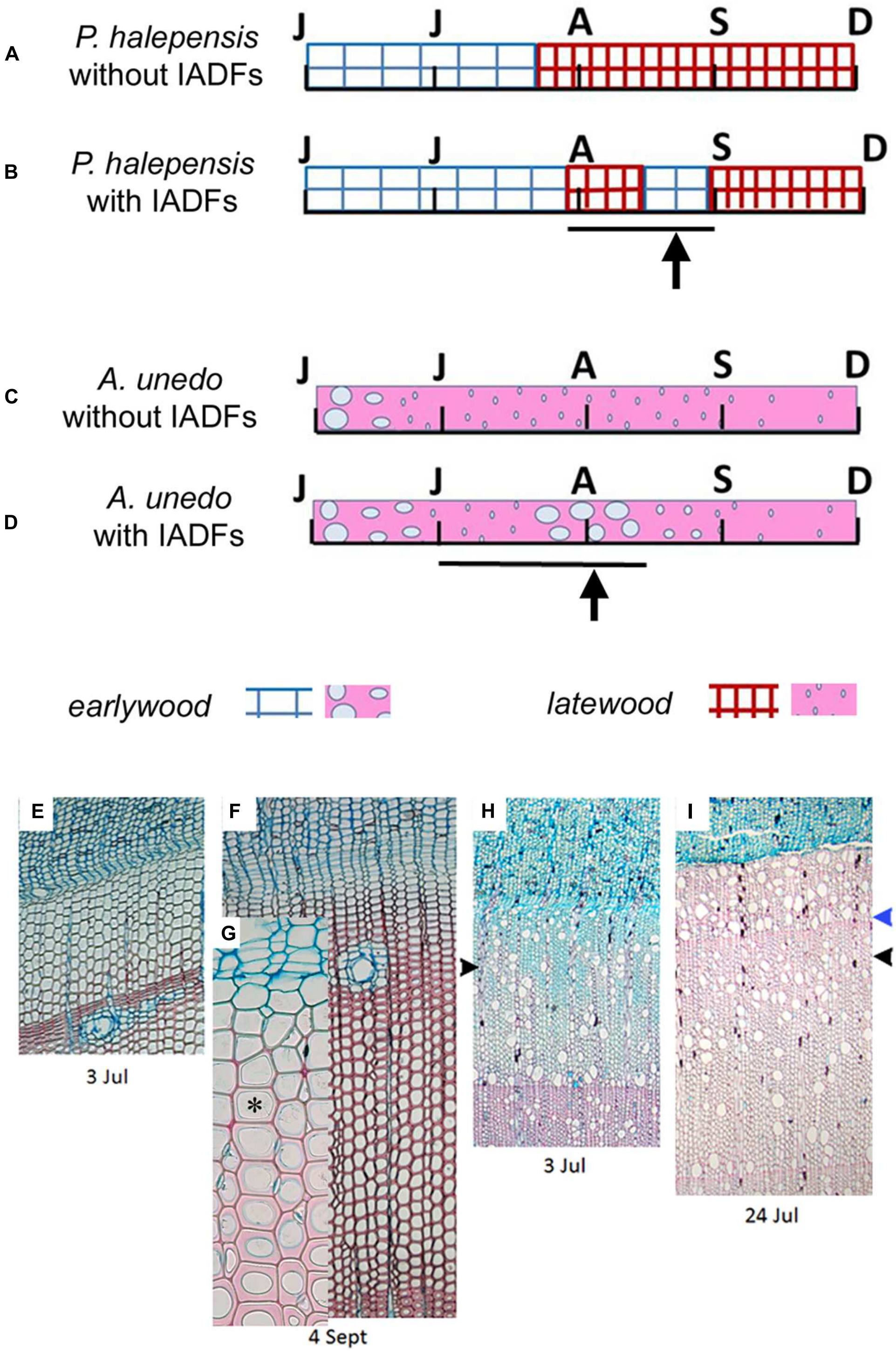

FIGURE 6 | (A–D) Schematic qualitative presentation of different types of developing xylem in tree rings of *P. halepensis* **(A,B)** and *A. unedo* **(C,D)** with and without IADFs. Letters correspond to the months from June to December. Bar and arrow indicate the IADF position. **(E–I)** Light microscopy view of microsections of microcores of *P. halepensis* and *A. unedo* plants forming IADFs in the 2014 tree ring. E. *P. halepensis* increment growth consisting of cells in different phases of differentiation and MC of the sole earlywood-type. **(F–G)** *P. halepensis* increment growth with a band of latewood and first rows of maturing earlywood-like cells; asterisk indicates an earlywood-like tracheid with lignifying secondary cell wall (light red staining due to safranin) and lumen larger than the previously formed mature latewood-like tracheids. **(H)** *A. unedo* increment growth consisting of cells in different phases of differentiation and MC of the earlywood-type cells and first rows of latewood vessels (black arrowhead). **(I)** *A. unedo* increment growth with a band of latewood cells detected starting from 3 July (black arrowhead) and a band of earlywood-like cells detected starting from 17 July (blue arrowhead).

TABLE 1 | Percent of *Pinus halepensis* (PIHA) and *Arbutus unedo* (ARUN) plants whose cells in SW and MC phases were classified as earlywood (EW), latewood (LW), earlywood-like (EW-like), or secondary latewood production (SLW) in the 2014-tree rings without (No) or with (Yes) IADFs, per each analyzed week (day of the year is reported in parenthesis below the date).

Species	IADF	SW-MC	Percent of plants producing specific wood type (%)										
			05 June (156)	12 June (163)	19 June (170)	26 June (177)	03 July (184)	10 July (191)	17 July (198)	24 July (205)	31 July (212)	04 September (247)	26 December (360)
PIHA	No	EW	100	100	100	100	100	100	100	0	0	0	0
		LW	0	0	0	0	0	0	0	100	100	100	100
		EW-like	0	0	0	0	0	0	0	0	0	0	0
		SLW	0	0	0	0	0	0	0	0	0	0	0
PIHA	Yes	EW	100	100	100	100	100	100	100	75	0	0	0
		LW	0	0	0	0	0	0	0	25	100	0	0
		EW-like	0	0	0	0	0	0	0	0	0	100	0
		SLW	0	0	0	0	0	0	0	0	0	0	100
ARUN	No	EW	100	50	0	0	0	0	0	0	0	0	0
		LW	0	50	100	100	100	100	100	100	100	100	100
		EW-like	0	0	0	0	0	0	0	0	0	0	0
		SLW	0	0	0	0	0	0	0	0	0	0	0
ARUN	Yes	EW	100	100	75	50	25	25	0	0	0	0	0
		LW	0	0	25	50	75	75	75	50	25	0	0
		EW-like	0	0	0	0	0	0	25	25	25	25	0
		SLW	0	0	0	0	0	0	0	25	50	75	100

the sequence of wood types encountered from the beginning of the tree ring toward the cambium is the following: earlywood, latewood (or latewood-like cells), earlywood (or earlywood-like cells) and latewood. The IADF can then be classified into *E*- or *L*-type IADF (De Micco et al., 2016a). It is clear that the classification into one type or the other depends on identification of the region of the ring in which the true latewood begins. Analysis of relations between IADFs and climatic data also helps their classification because the two types of IADFs, can occur at different positions within the growth ring and have completely different functional significance. For example, *A. unedo* growing at a site in Southern Italy has been recently shown to be able to form two types of IADFs, according to water availability at the growing sites (Battipaglia et al., 2010, 2014). More specifically, under xeric conditions, this species forms *E*-IADFs, meaning that the "anomaly" is the formation of latewood-like cells in a period when earlywood is expected, thus triggered by a particularly severe or unexpected period of drought (Battipaglia et al., 2010). Under more mesic conditions, *A. unedo* developed *L*-IADFs, meaning that the "anomaly" was the formation of earlywood-like cells in a period when latewood should have been formed; such *L*-IADFs were ascribed to rain events occurring during fall after a period of summer drought. This mechanism of *L*-IADF formation has also been highlighted as typical of *Pinus* species (Zalloni et al., 2016). Such a seasonal dynamics of wood development follows the bimodal pattern described for *P. halepensis* from arid and semi-arid ecosystems in Spain (de Luis et al., 2011a), and also reported recently by Camarero et al. (2010) in *Juniperus thurifera* from sub-humid and semi-arid Mediterranean continental sites in Spain, and in the co-occurring *P. halepensis* only in the more xeric site. Such a pattern has been also reported for *P. pinaster* growing on sand dunes in Portugal by Vieira et al. (2015). The bimodal pattern of wood growth is due to cambial reactivation triggered by spring and fall precipitations, which control cell enlargement and cell wall deposition after winter low-temperatures and summer drought stresses (Camarero et al., 2010; Pacheco et al., 2016). In our study, to classify IADFs objectively in the two analyzed species and to evaluate the period of their formation, xylogenesis proved to be a valuable tool because it allowed identification of the starting moment for latewood formation, as well as observation of the progressive formation of IADFs while they were "under construction" (de Luis et al., 2011b; Novak et al., 2016). Considering the timing of IADF formation and keeping in mind the precipitation pattern observed in 2014, we confidently classified the 2014 IADFs as *L*-type, following a bimodal pattern of xylogenesis. However, in our case, the second growth flash, leading to the second earlywood formation during the calendar year, already occurred in summer and not in fall as hypothesized for Mediterranean species (De Micco et al., 2016a). We could speculate that, in this specific case, fall precipitations can lead to more than one IADF per ring. This is sometimes observed in *A. unedo* where such additional IADFs would be better classified L^{+}-IADFs according to Campelo et al. (2007b, 2013). The high variability of behavior of different plants in various environments strengthen the need for a common and unambiguous classification of IADFs, also considering their position within tree rings, to achieve a correct functional interpretation of them (De Micco et al., 2016a).

In the two analyzed species, the formation of IADFs appeared irregularly in time and different individuals showed different predispositions to IADF development in different years, in agreement with other studies (Cherubini et al., 2003). This phenomenon indicates the capacity of these two species to switch from a unimodal pattern of xylogenesis toward a bimodal wood growth, and vice versa, from one year to another. The two species responded with the same strategy (i.e., the formation of *L*-IADFs) to fluctuating environmental conditions, but their sensitivity was different. The onset of latewood formation and the appearance of IADFs was shifted a couple of weeks earlier in *A. unedo* than in *P. halepensis*. However, the occurrence of mature latewood cells in *A. unedo* was still delayed compared with the few studied broadleaved deciduous species such as chestnut and beech (Čufar et al., 2008, 2011). *A. unedo* appeared to be more sensitive than *P. halepensis* also because of a higher frequency of IADFs and because several growth rings were often formed per year. This would indicate a better and quicker ability to induce dormancy and activation of the cambium after fluctuating environmental conditions. This different sensitivity is not surprising because of the different size, age, and growth and reproductive strategies of the two species which dictate different hydraulic constraints and resource use/allocation. For instance the younger age of *A. unedo* plants compared with *P. halepensis* can partly explain their higher tendency to form IADFs. Indeed, IADF frequency is generally higher in younger than in older trees, probably not only because young plants have shallow root systems and may thus be less able to access deep soil layers but also because young plants show increased tree-ring width, which is positively correlated to IADF frequency (Rigling et al., 2001, 2002; Cherubini et al., 2003; Bogino and Bravo, 2009; Hoffer and Tardif, 2009; Rozas et al., 2011; Novak et al., 2013b; Campelo et al., 2015; Pacheco et al., 2016).

The appearance of the first mature latewood conduits in the two analyzed species occurred well after the onset of the summer drought period (May). Indeed, wood formation is the result of the integration of complex cellular processes, in which cell-wall thickening and lignification lags behind cell enlargement by as much as a month or more, according to the recent model by Cuny et al. (2015). The time-lag observed between the increase in xylem size (linked to cell enlargement) and accumulation of woody biomass (due to cell wall thickening and lignification) shows differences between earlywood and latewood, and it has been quantified in a range of 27–49 days in different climates, with maximum values in the Mediterranean region (Cuny et al., 2014, 2015). The application of this time-lag principle to our samples would indicate that latewood conduits are produced by cambium activity from the beginning of the drought period but, given that cell-wall deposition and maturation requires several weeks (up to ≈ 7) (Prislan et al., 2009, 2013a; Cuny et al., 2014), latewood thick-walled cells become evident only later. Similarly, in tree rings showing IADFs, a new production of earlywood may have been primed by rain pulse events during July (which was characterized by almost twice as much precipitation as June). Increased cell enlargement in July thus possibly triggered the formation of earlywood-like cells, which appeared mature only at the end of July and August, based on the time required for cell wall thickening (up to$\approx$ 4 weeks) as estimated by Prislan et al. (2009, 2013b) and Cuny et al. (2014).

The two analyzed species showed wood production during the summer drought period, which is considered limiting for growth, also perhaps inducing a halt in cambial productivity in some species (Liang et al., 2006; Camarero et al., 2010; Pacheco et al., 2016). However, this could be explained by temporary favorable conditions, specific resource use efficiency and strategies, or might have another basis, phylogenetic or biogeographical, given that many Mediterranean species perform costly metabolic processes precisely under unfavorable summer drought periods (Aronne and Wilcock, 1994).

From a methodological viewpoint, the micro-coring technique and analysis of xylogenesis were easily applied to tree rings showing IADFs of *P. halepensis*, whereas it was more difficult to apply them in *A. unedo* because of the very high variability of IADFs among and within plants in different years. However, cross-dating was a helpful tool for identifying the beginning of the last growth increment (2014) and for verifying wood growth variability in the same stem. We thus suggest that, when studying xylogenesis in Mediterranean plants forming a high frequency of IADFs and more than one IADF per calendar year, it is useful to perform a preliminary tree-ring analysis in order to predict the applicability of micro-coring in single plants, thus excluding from the micro-coring experiment those trees having high variability of wood growth around the stem and those having clear anomalies.

To conclude, with the support of dendroecological analysis, micro-coring allowed the identification of the period of IADF formation in the two Mediterranean species confirming the hypothesis of the occurrence of a bimodal pattern of cambial activity. Both species were prone to form IADFs that were classified as *L*-type, indicating a period of growth flash due to favorable environmental conditions for growth occurring during summer, and not in fall as reported for other Mediterranean species (e.g., Camarero et al., 2010; de Luis et al., 2011b; Campelo et al., 2013; Carvalho et al., 2015; Zalloni et al., 2016). A possible explanation, still to be verified is that different kinds of *L*-IADFs exist (*L*- and L^+-type); they would be triggered by temporary favorable conditions occurring during summer and fall, respectively.

The formation of *L*-IADFs can be considered a way of improving the hydraulic conductivity of wood (Sperry et al., 2006) when water is unexpectedly available after a period of severe drought. As a consequence, species showing high plasticity in cambial productivity, thus prone to form *L*-IADFs, promptly after a positive climatic event (e.g., unexpected summer rain pulse) following a period of severe drought (e.g., dry periods at the end of spring), should have an advantage under fluctuating environmental conditions over those not able to form IADFs.

AUTHOR CONTRIBUTIONS

VDM, AB and GB made a substantial contribution to the conception and design of the study. AB performed sample collection. AB and MM performed sample preparation. VDM, AB, JG, KC and GB made a substantial contribution to the analysis of tree-ring series and anatomical signals in microsections and in data analysis. VDM, AB, KC and GA contributed to the interpretation of the overall data. VDM, KC, MM, GA and GB contributed to the analysis tools. VDM wrote the main part of the manuscript. AB and GB contributed to writing the text. All authors contributed to final revisions of the manuscript and read and approved the submitted version of the manuscript.

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THE ANALYSIS OF XYLOGENESIS REVEALS THE GENESIS AND ECOLOGICAL MEANING OF IADFS IN *PINUS PINEA* L. AND *ARBUTUS UNEDO* L.

Chapter based upon a manuscript in preparation with the collaboration of De Micco V., Battipaglia G., Aronne G., Cufar K.

Abstract

Wood formation in Mediterranean species shows various temporal patterns and durations. Cambial activity in Mediterranean woods can have one or more stops per year, depending both on environmental conditions and species sensitivity. Such patterns of cambial production lead to Intra-annual density fluctuations (IADFs) in tree rings.

The aim of this paper was to analyse xylogenesis in stone pine (*Pinus pinea* L.) and strawberry tree (*Arbutus unedo* L.) co-occurring at a site on Mt. Vesuvius, in order to identify the periods of cambial productivity and IADF formation. Microcores were collected weekly for one year, (starting from January 2015), from the stem of the two species. Microscopy analysis of the cross sections of microcores showed that cambial production in *P. pinea* was active throughout the calendar year. In the period January–March 2015, the cambium produced post cambial cells differentiating into earlywood-like cells, thus producing the so-called transition tracheids, while cells produced in April differentiated into true earlywood. IADFs were formed in autumn, with earlywood-like cells in latewood (*L*-IADFs). Cambial activity in *A. unedo* showed a double stop, in summer and winter, leading to *L*-IADFs in autumn as well. The analysis of xylogenesis demonstrated that the two species, although having different timing of cambial production, form L-IADFs in the same period of the year. Furthermore, in *P. pinea* the beginning of the tree ring was not marked by a sharp boundary between latewood of the previous year and the new xylem produced, because of the lack of a true cambial dormancy. Finally, in *A. unedo* the formation of more than one IADF was observed in some plants in the analysed period. Such peculiar patterns of cambial production and the species-specific sensitivity need to be taken into account in dendroecological studies to better interpret climatic signals in Mediterranean woods.

Introduction

Environmental signals are summarized in anatomical features of tree rings whose formation is driven by genetic and climatic factors acting on the different phases of cambial cell division, cell differentiation and programmed cell death (Scarpella and Meijer, 2004; Fonti et al., 2009; Ružička et al., 2015). The general increase in drought, forecasted for the Mediterranean basin (IPCC, 2013), is expected to alter cambial phenology and xylogenesis, thus affecting growth and productivity of forests (Sarri et al., 2007), vegetation dynamics (Martinez del Castillo et al., 2016)and ultimately influencing biogeochemical cycles (Xia et al., 2015). The plasticity of secondary growth in trees, which is responsible for their adaptation to specific environmental changes, is evident in the different possible patterns of cambial activity in Mediterranean tree and shrub species. Such patterns are species- and site-specific (Camarero et al., 2010): they result in the formation of Intra-annual density fluctuations (IADFs) in tree rings, mainly driven by seasonal variations in temperature and water availability (Battipaglia et al., 2016; De Micco et al., 2016a). IADFs appear as regions where abrupt, unexpected, changes in density occur and have been recently re-classified according to their position and anatomical traits in the latewood or earlywood of tree rings (De Micco et al., 2016a). *L*- IADFs are frequent in Mediterranean woods and consist in the occurrence of a band of earlywood-like cells within latewood (Campelo et al., 2007, 2013; Battipaglia et al., 2010, 2016; De Micco et al., 2014, 2016a; de Luis et al., 2011a; Novak et al., 2013a,b). At the *L*-IADF level, the presence of conducting cells with larger lumen than those of true latewood has been related to an extension of the period of wood formation due to the return of favourable conditions for tree growth after the start of latewood formation, primed by a bimodal wood growth with two peaks in cambial activity, respectively in spring and autumn (De Micco et al., 2016a; Zalloni et al., 2016).

The analysis of IADF genesis can help encoding the factors triggering their formation: indeed, division of cambial cells and cell differentiation are guided not only by genetic factors, but also by the physiological status of plants. They depend on availability of water and sugar which, in turn, are affected by climatic conditions (Deslauriers and Morin 2005; Steppe et al., 2015; Vieira et al., 2015; Cuny et al., 2015; Prislan et al., 2016). All the phases of cambial cell production and differentiation can be affected by climatic factors and there is evidence that the onset, duration and ending of the different phenological phases of xylogenesis are variable among study sites and years (Martinez del Castillo et al., 2016). This phenomenon has clearly emerged by several studies conducted on *Pinus sylvestris* and *Fagus sylvatica* throughout their geographical distribution and in different biomes: it has been linked to variations in temperature, water availability, photoperiod and leaf phenology (Martinez del Castillo et al., 2016; Ziaco and Biondi 2016; Prislan et al., 2016; Rossi et al., 2013; Cuny et al., 2012,2014; Gruber et al., 2010; Swidrak et al., 2014; Cufar et al., 2008).

Recently, the interest at analysing xylogenesis in tree rings prone to form IADFs has increased in the framework of understanding the species capability to adapt to expected climate changes in Mediterranean forest and scrub biomes (Camarero et al., 2010). IADF formation in *Pinushalepensis* has been followed through the analysis of xylogenesis in Mediterranean sites in Spain and Italy (De Luis et al., 2011a;De Micco et al., 2016b). In both cases, *L*-IADFs were formed in latewood after a period of reduced cambial activity due to summer drought which was followed by the restoring of active cambial production due to precipitations occurring either in autumn or summer at Spanish and Italian sites respectively (De Luis et al., 2011a; De Micco et al., 2016b). Indeed, the restoring of cambial activity allows the extension of the wood formation period that may continue till the end of

December (Novak et al., 2016) if the conditions (i.e. temperature above a certain threshold) are favourable.

IADFs genesis in Mediterranean hardwoods is still poorly explored. The reason for IADF formation in Mediterranean hardwoods, indeed, has been hypothesized from retrospective analyses of anatomical and isotopic traits in tree-ring series (Battipaglia et al., 2010, 2014; De Micco et al., 2012, 2014, 2016c). Recently De Micco et al. (2016b) described for the first time the formation of IADFs in tree rings of the Mediterranean small tree *Arbutus unedo* at a site in Southern Italy, through the analysis of xylogenesis focused in summer months. As shown for *P. halepensis* growing at the same site, the genesis of *L*-IADFs in *A. unedo* did not occur in autumn, but happened earlier, due to rain events happening in summer (July-August) after a period of drought. To the best of our knowledge, the cambial production throughout the year in Mediterranean hardwoods, which are also prone to form frequent IADFs, still remains unexplored.

The aim of this study was to analyse cambial productivity, xylem formation and differentiation in two Mediterranean species, the softwood *Pinus pinea* L. (stone pine) and the hardwood *Arbutus unedo* L. (strawberry tree), co-occurring at the same site in Southern Italy, characterised by a period of summer drought. We analysed xylogenesis in the two species throughout one year in order to highlight the genesis of IADFs in their tree rings. We aimed to: a) identify the onset of cambial activity, earlywood and latewood formation; b) evaluate whether the key phenological periods of cambial activity coincided in the two species; c) analyse how many, when and which type of IADFs were formed in the two species. Cambium phenology and IADFs genesis was compared between the two species also in reference to climatic data to evaluate which factors play a role in IADF formation. Findings are discussed also in reference with hypotheses raised from retrospective analyses of tree-ring series performed throughout the Mediterranean region.

Materials and methods

Species, study site and climatic conditions

The study was conducted on trees of stone pine (*Pinus pinea* L.) and strawberry tree (*Arbutus unedo*L.), co-occurring at a site in Southern Italy. The study area, located on the Vesuvius Montain (40’782 N, 14,425 E, 200 m.a.s.l.) consists in a pine stone forest with strawberry tree bushes. The climate at the site is Mediterranean,withhot and dry summers followed by mild and wet winters. Microcoring sampling to analyse the cambial productivity and xylem formation in the two species was carried out in the year 2015 which was characterised by annual mean temperature of 17.8 °C. The hottest month was July (monthly average mean temperature of 28.5°C), while the coldest month was February (monthly average mean temperature of 9.4°C). The cumulative annual precipitation was 1054 mm; the wettest month was October with a cumulative monthly precipitation of 286 mm, while the lowest value was recorded in July (cumulative precipitation of 9.4 mm). The aridity period lasted from May to July. A review of long-term meteorological series from the same site, obtained from KNMI Climate Explorer (Trouet and Van Oldenborgh, 2013), indicated that meteorological conditions in 2015 did not deviate considerably from those registered in the previous 20 years (1995–2014).

Tree-ring identification

Dendrochronological techniques were applied to build tree-ring chronologiesto allow the synchronization of tree-rings in order to identify the xylem formed in the year 2015. Core sampling was performed on 20 trees of *P. pinea* and 20 plants (not multi stem) of *A. unedo*, by taking two cores at breast height from each tree (west and east directions) with an increment borer (diameter 5 mm). Cores were air-dried, mounted in grooved wooden blocks, and their surface was polished with a graded series of sandpaper. Tree-ring series were visually crossdated by comparison of signature rings and calendar dates assigned to the year of formation of each ring to facilitate the synchronization of tree rings and IADFs, through dendrochronological techniques (Stokes and Smiley, 1968). Tree-ring width (TRW) measurement was done to the nearest 0,01 mm, using LINTAB measurement equipment and TSAP Win software (Frank Rinn, Heidelberg, Germany). The quality of crossdating was checked using R package CRAN (Bunn, 2008). Descriptive statistics were calculated as well as the expressed population signal (EPS) which indicates the level of coherence of the constructed chronology (Wigley et al., 1984).

Microcore sampling and microscopy

For collection of microcores, 6 healthy trees/plants per species were selected with similar size and age. For *P. pinea*, stem diameter at breast height was in the range of 47–59 cm, height was about 16 m and age was about 95 years. For *A. unedo*, stem diameter at breast height ranged between 10 and 25 cm, high was 4-8 m, and age was about 20 years. Microcores (1.8 mm diameter) were collected using a Trephor tool (Rossi et al., 2006) at weekly intervals from January 2015 to January 2016.In order to avoid wound effects, the points of microcore extractions were chosen following a spiral with a distance of 3-4 cm between consecutive samples. Each microcore contained inner phloem, cambium and xylem (at least three of the last-formed xylem rings). The extracted microcores were immediately fixed in 70% ethanol and stored at 4°C. The samples were dehydrated in a graded ethanol series (70 , 90, 95 and 100%), infiltrated with bio-clear (D-limonene) and embedded in paraffin blocks using a Leica TP1020-1 (Nussloch, Germany) tissue processor. Cross-sections (9 µm thick) were cut with a semi-automatic rotary microtome RM 2245, Leica (Nussloch), using low profile microtome blades. The sections were flattened on glass slides pre-treated with albumin and dried at 70°C for 30 minutes. After removing the residual paraffin through bio-clear and ethanol, the sections were stained with a safranin (0.04 %) and astra blue (0.15 %) water solution (van der Werf et al., 2007) and mounted in Euparal (Bioquip Rancho Dominguez, California). The sections were observed under a Nikon Eclipse 800 light microscope and microphotographs were taken by means of a DS-Fi1 digital camera with a NIS-Elements BR 3 image analysis system (Melville, NY, USA). The sections were analyzed to identify and measure cambial cells and xylem cells using visual criteria of proportional dimension and wall thickness (De Luis et al. 2007;Čufar et al. 2008, 2011; De Luiset al. 2011a; De Micco et al. 2016b). Sections from samples collected every two weeks were measured, but in periods of high cambial production sections collected every week were considered. Tracheids in *P. pinea* were classified as earlywood or latewood by applying the Mork's definition (Mork, 1928). In *A. unedo*, the transition between earlywood and latewood was often diffuse, but latewood was characterized by the presence of narrower(halved lumen diameter) and less frequent vessels than earlywood (De Miccoet al., 2016c). The following cell types were considered to identify the different phases of cell development: cambial cells (CC), expanding post-cambial xylem derivatives (PC), immature xylem derivatives with developing secondary walls (SW) and mature cells with lignified secondary walls (MT) (Čufar et al. 2011; De Luiset al. 2011a; De Micco et al. 2016b). CC were tangentially elongated, with thin cell walls that stained blue. PC were cells radially enlarging, whose cell walls also stained blue.

SW were immature xylem derivatives with thickening and lignifying secondary walls which stained blue and lightred, depending on the progress of lignification .The polarized light helped to discriminate between PC and SW cells, as described in Rossi et al. (2006b), in so far as SW cells showed birefringence. MT were cells with completely thickened and lignified cell wall, staining completely red and without any trace of protoplast in the lumen. Active production of xylem cells by the cambium was indicated by an increase in the number and width of CC and the presence of PC phases.

In *P. pinea*, the number of cells was counted along three radial rows for all phenological phases (CC, PC, SW and MT). Considering that the boundary limiting the end of the tree ring corresponding to the previous year (2014) was not always clear, in order to count the number of MT cells formed in the analysed period, we established a sort of point zero by counting the number of all mature cells starting from the boundary between the 2013 and 2014 in the sections of the first microcore sampling. This point-zero number was subtracted by all MT counted in the successive microcores with the same principle (all mature cells starting from the boundary between the 2013 and 2014). In *A. unedo*, since vessels and fibres were not arranged in ordered radial rows, thewidthof all the zones occupied by the various cell types in the different phenological phases was measured according to De Micco et al., (2016b). Measurements along the three radial rows were averaged.

To avoid the influence of variability around the circumference, the number, for *P. Pinea*, and the width for *A.unedo*, of cells in the previous xylem ring were quantified to normalize the measurements (Rossi et al., 2003, Prislan et al., 2013). We focused on cells in SW and MC phases to detect the beginning of IADFs formation marking the transition among earlywood, latewood, earlywood-like and latewood-like cells according to De Micco et al. (2016b).

Results

Tree-ring series

The statistics of the tree-ring chronologies are reported in Table 1 and refer to the six trees for each species selected for xylogenesis activity. *A. unedo* trees were much younger than *P. pinea* plants. The TRW chronologies of the two species showed quite different trends, with no significant correlations between them.

The mean ring widths (Table 1) of *A. unedo* were larger than rings of *P. pinea* for the whole chronologies ($P<0.05$) and for the 2015 year ($P<0.001$). Both chronologies have EPS value>0.85 indicated that the chronologies were representative of radial growth variations of the whole population of trees. All the statistics listed in Table 1 showed the strong common growth signal between individuals of the same species and high year-to-year radial growth variability associated with inter-annual changes in climatic conditions, especially for *A. unedo* that presents highest mean sensitivity.

Table 1. Summary of the statistics of the tree-ring chronologies for the analysed trees of *P. pinea* and *A. Unedo*. EPS= expressed population signal; GLK= Gleichläufigkeit; MS=mean sensitivity.

	Pinus pinea	***Arbutusunedo***
TRW mean	222 ± 106	372 ± 141
TRW mean2015	175± 57	317 ± 115
EPS	0.842	0.957
GLK	0.957	0.842
r-bar	0.717	0.312
MS	0.13	0.28

Cambial productivityand phenology of xylem formation

The status of cambial productivity on the first sampling date(29 January) was different in the two analysed species, being active in *P. pinea* (Fig. 1a) and dormant in *A.unedo* (Fig. 2a). The curves of cambial cells (CC) (black), enlarging cells (PC) (blue)(Fig. 3a, c) and secondary-wall thickening cells (SW) (Fig. 3b, d black) followed a characteristic bimodal shape, with the second peak more evident in *P. pinea* than in *A. unedo*. As regards the curve of mature cells (MT), it showed a typical sigmoid shape in *A.unedo* (Fig. 3d blue), while the typical sigmoid curve was preceded by a peak in *P. pinea* (Fig. 3b blue).

More in detail, in the developing xylem of *P. pinea*, CC consisted of 7.30 ± 0.66 cells (mean value ± SD), while PC consisted of 1.94 ± 0.82 cells in the period from the end of January until the end of March (Fig. 3a).On the end of March (DOY 86), the number CC and PC cells started to increase (8.46 ±1.06 and 3.94 ±1.65 respectively). The peak of CC was detected on 6 May (DOY 127) (9.16 ±0.69), while for PC it occurred on 21 April (DOY 112) and corresponded to 6.0 ±1.07 cells. Afterwards, the number of CC and PC cells declined, reaching minimum values during the summer months (Fig. 1 b, c). Indeed, on 15 July (DOY 189), CC and PC consisted of 6.0 ± 1.11 and1.11 ± 0.40 cells respectively. A second peak in the number of CC (9.44± 0.93) and PC (3.2 ± 1.57) cells occurred on 22 October(DOY 296)after which they decreased again reaching a minimum value on 4 January 2016 (6.22± 0.45 and 0.88 ± 0.40 respectively). The curve of SW cells showed a similar pattern compared to CC and PC cells, although shifted of some weeks (Fig. 3b black). The number of SW cells was on average 3.2 ±0.88 and started to increase in the middle of April, reaching the maximum value (12.22 ±3.27) on 21 May (DOY 142).A second peak of SW cells was detected in November (DOY 317). As regards MT cells, they were detected since the first sampling: their number was low (6.49±4.78) and started to increase in mid May (DOY 142) (10.64 ±1.87), reaching a maximum values (37.47 ±13.71) in August (DOY 225) which were mantained until the end of the year (Fig. 3b blue).

Regarding *A. unedo*, on the first sampling date, the cambium was still dormant (CC width was 14.76± 2.78µm) (Fig. 2a, 3c blue). The first signs of activity appeared on 10 April (DOY100), with production of PC cells (22.93 ± 6.33 µm) (Fig. 3c blue). Cambial production reached the maximum peak in May (DOY 142) (37.73± 8.55). High presence of PC cells was also found in April-June with a peak on 7 May (DOY 127) (103.60± 61.95) (Fig. 3c blue).

At the beginning of July (DOY 189), we observed a decrease of CC (22.24± 2.23); such low values were maintained until the beginning of October (DOY 282) (Fig. 3c black). In the second half of July and until the beginning of October, PC cells were scarce with a width comparable to values found at the beginning of the year, suggesting a new period of dormancy (Fig. 3c blue). All the samples showed a second peak of growth around 28 October (DOY 296) (CC 24.14± 9.53), with a new significant increase of PC (23.6±18.3) (Fig. 2b, 3c). Cambial productivity stopped again in December (DOY 345), when the ring consisted of MC cells only.

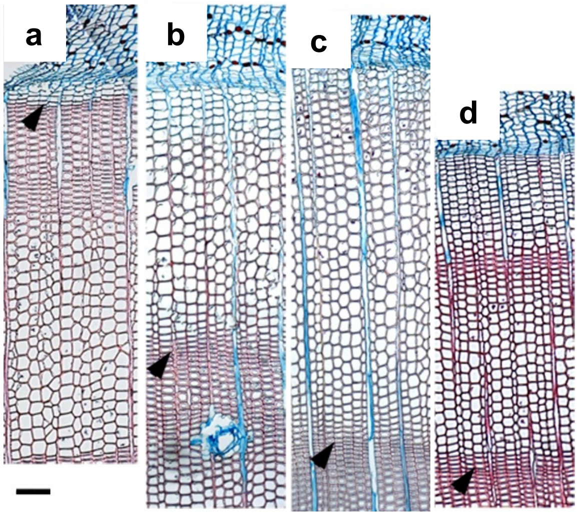


Fig. 1. Light microscopy views of microsections of microcoresof *P. pinea* trees forming IADFs inthe 2015 tree-ring. a, 29 January; b, 18 June; c, 8 July; n, 11 December. Arrowheads point to the boundary between the 2014 and 2015 tree rings. Images are all at the same magnification. Bar = 100µm.

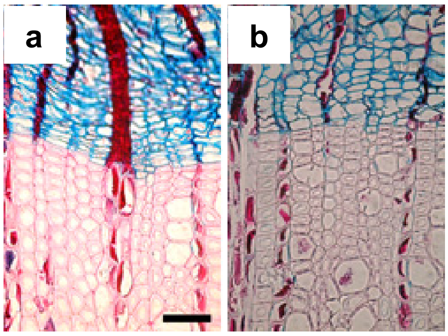
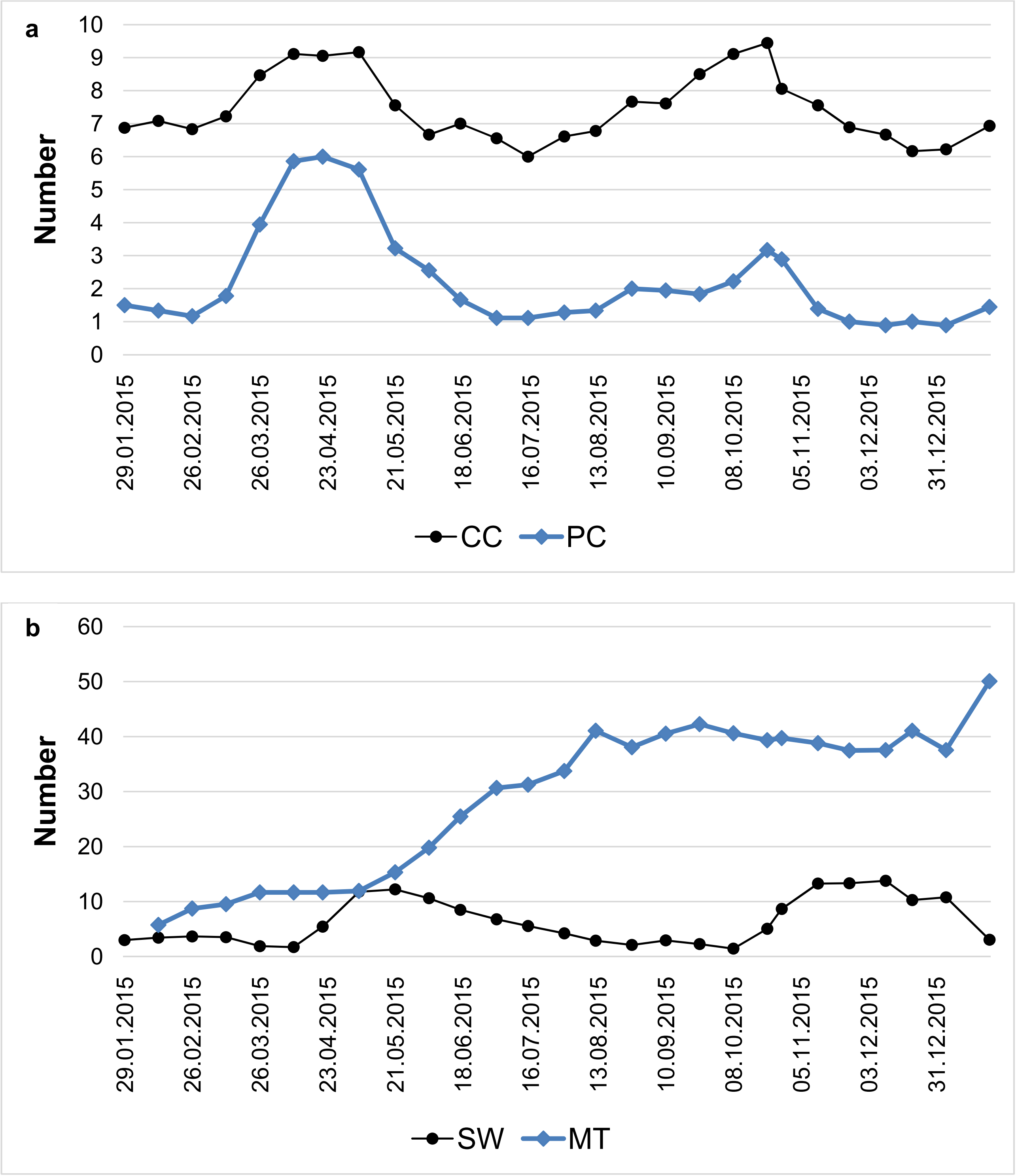


Fig. 2. Light microscopy views of microsections of microcores of *A. unedo* plants forming IADFs in the 2015 tree-ring. a, 29 January; 28 October. Images are all at the same magnification. Bar = 50 mm.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26.02.2015
26.03.2015
23.04.2015
21.05.2015
18.06.2015
16.07.2015
13.08.2015
10.09.2015
08.10.2015
05.11.2015
03.12.2015
31.12.2015
SW
MT

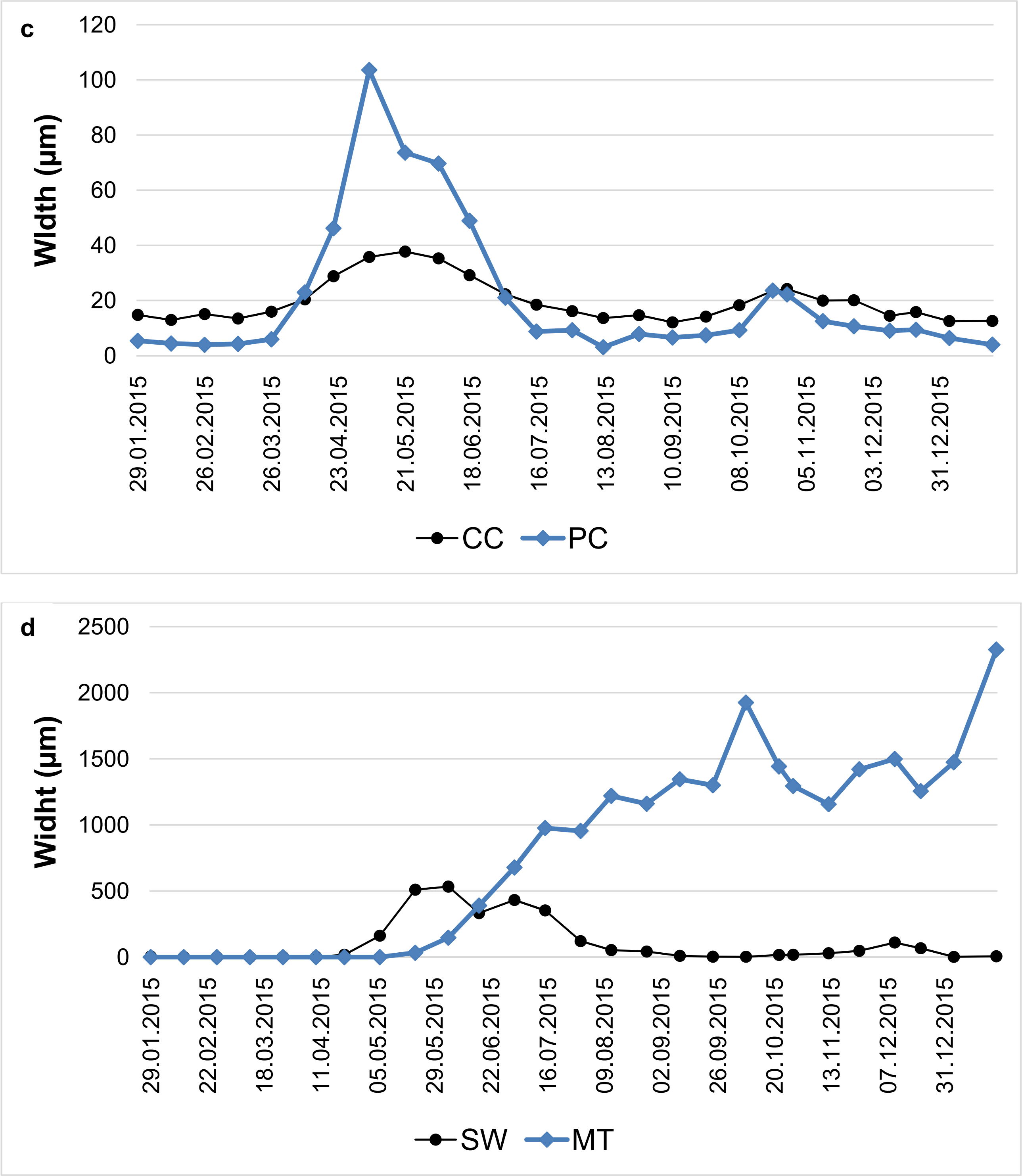

Fig. 3. Number of cells in various phases of xylem formation in *P. pinea* trees (a,b) and width of different developmental xylem zones in *A. unedo* plants (c-d): cambial cells (CC), enlarging postcambial cells (PC) (a,c), cells developing secondary walls (SW), and mature cells with lignified secondary wall(MT) (b,d). Mean values and standard errors are shown.

IADFs genesis

Microscopy observation of the cross sections of the microcores sampled at the beginning of 2016 showed that in the year of the investigation (January 2015 - January 2016) and in the two previous years, tree rings showed IADFs in both *P. pinea* and *A. unedo*. Such IADFs were of the type-*L*, being characterised by the presence of earlywood-like cells in latewood in the end of the tree ring

In *P. pinea*, since the first sampling, cambium was productive and the previous tree ring (corresponding to 2014) did not end with true latewood cells: indeed, in the months January-March 2015 (DOY 29 - 71),“earlywood-like cells” were formed, with narrow lumen and thick cell walls, similar to the so-called transition tracheids. True earlywood mature cells were evident in the end of March until the beginning of July; then the production of latewood cells started and lead to mature latewood cells in the second half of July (DOY 211). Latewood tracheids were formed and differentiated until the beginning of October (DOY 281).The genesis of *L*-IADFs occurred in middle October: the first mature earlywood-like cells were evident on 22 October (DOY 295) and their production continued until middle December. Then the production of latewood restarted and mature true latewood cells were evident on 22 December (DOY 356). Thus, in *P. pinea*cambial activity did not stop throughout the year.

In *A. unedo*, no cambial production was found from January to April (DOY 100), when the production of earlywood started and lasted until the beginning of July when mature latewood cells became evident (DOY 183). The production of latewood cells continued until middle October. All the samples showed the formation of *L*-type fluctuation with occurrence of mature earlywood-like cells on 22 October (DOY 295); such cells were produced throughout November. Latewood maturation was evident again on 10 December (DOY 344), while cambium production stopped at the beginning of January again. Moreover, in 3out of 6 samples, the formation of more than one IADF was found in the year of investigation with additional earlywood-like cells mature in August

(DOY 239) and a new production of latewood cells in September (DOY 257) or even earlier just at the beginning of the ring production.

Discussion

Mechanistic models of xylogenesis have been proposed to link the kinetics of tracheid formation in the "typical" tree rings formed by conifer species in temperate climates in the absence of major factors of environmental stresses (Cuny et al., 2014). Such models, based on an unimodal pattern of xylogenesis, are not directly transferable to softwoods and hardwoods developing in Mediterranean climates, where different patterns of cambial activity occur in several species and in various microclimate conditions (Camarero et al., 2009; Martinez del Castillo et al., 2016). Recently, Martinez del Castillo et al., (2016) showed that different tree species, namely *Pinus sylvestris* and *Fagus sylvatica*, although growing at the same site, have different phenologies of xylem formation, likely depending on their different life strategies and adaptive capability under limiting environmental conditions. In our study, the analysis of xylogenesis in two completely different models of plant species, specifically a conifer tree and a hardwood shrub/tree, partially supported this idea. Indeed, the two species showed different timings of cambial production and different patterns of tree-ring chronologies, although a bimodal xylogenesis leading to the formation of the same type of IADF in latewood (*L*-IADF), was found in both of them. Duration of cambial production was longer in *P. pinea* than in *A. unedo* under the same climatic conditions, confirming that duration of xylogenesis is species-specific. Indeed, *P. pinea* did not show a complete stop in cambial activity in winter, as assumed for *Pinus* spp. and other species where microcoring studies are focused from spring to autumn (de Luis et al., 2007; Cufar et al., 2011;Martinez del Castillo et al., 2016). Recently, it has been shown that cambial production of PC cells in *P. halepensis* may continue until the end of December and that its reactivation may be observed as early as in February or March (Novak et al., 2016). In *P. pinea* growing on the Vesuvio mountain, we found that although slowly, xylogenesis was active from January to March, while in *A. unedo* cambial production was restored in April after the winter stop. However, in the two species, the first peak of cambial production (highest incidence of CC and PC cells) occurred in the same period, namely between the end of April and beginning of May, when air temperature increases. This is in agreement with several studies, also based on experiments of artificial heating of stem, which indicate the increase in spring temperature as the main factor triggering the onset of xylogenesis (Begum et al., 2010; Prislan et al., 2011; Vieira et al., 2014). On the other hand, the onset of latewood in the two analyzed species seems to be triggered by a strong decrease in precipitation, coinciding with the highest value of average mean temperature (July). In *A. unedo*, the appearance of mature latewood conduits occurred 1-2 weeks earlier than in *P. pinea*. A similar temporal shift in the onset of latewood was also found between *A. unedo* and *P. halepensis* co-occurring at the same site in a previous study in Southern Italy, suggesting that *A. unedo* has a faster, more plastic control of cambial processes than *Pinus* spp. (De Micco et al., 2016b). The second peak of cambial production seems to be strongly controlled by the precipitations of October that were very abundant in the year of investigation (mm of rains were more than double in October if compared with September). The production of earlywood-like cells in latewood continued until middle December, when temperature decreased again reaching minimum values. The occurrence of these *L*-IADFs in *P. pinea* and *A. unedo*is in line with findings in other Mediterranean pines, in *Juniperus thurifera* and *A. unedo* itself, developing in Mediterranean ecosystems in Spain, Portugal and Italy, where *L*-IADFs were ascribed to an extended period of growth due to autumn precipitations creating favourable conditions for wood growth (Camarero et al., 2010; de Luis et al., 2011b; Vieira et al., 2015; Zalloni et al., 2016). The mechanism triggering *L*-IADFs is linked with the increased cell enlargement due to high turgor pressure in enlarging cells due to high water availability after abundant autumn rain (Sperry et al., 2006; De Micco et al., 2016a; Pacheco et al., 2016). The same mechanism has been reported for the formation of *L*-IADFs in *P. halepensis* and *A. unedo*, although in a different period of the year: a second flesh of growth in summer after rain episodes (De Micco et al., 2016b).

In *A. unedo*, the high sensitivity of the rhythm of cambium production in response to changing environmental conditions would be responsible for extended xylogenesis and frequent occurrence of more than a single IADF per calendar year as found in this investigation and in agreement with previous observations (Cherubini et al., 2003; De Micco et al., 2016b). The high predisposition of *A. unedo* to form IADFs could be also due to the young plant age which is correlated to larger tree rings which, in turn, are positively correlated with IADF frequency (Rigling et al., 2001, 2002; Cherubini et al., 2003; Campelo et al., 2015; Pachecoetal.,2016; Zalloni et al., 2016). An extension of xylogenesis has also been ascribed to mild autumn temperatures in *P. sylvestris* (Martinez del Castillo et al., 2016). Latewood is then produced again in winter when low temperature is limiting the processes of cell production and differentiation, and can lead to dormancy as found in *A. unedo*. The low sensitivity of *P. pinea* to winter temperature would explain the onset of latewood delayed of one week, if compared to *A. unedo*, and its lack of a true cambium dormancy leading to the formation of a few rows of transition wood made of earlywood-like tracheids before the production of true earlywood tracheids. Such a phenomenon explains why the boundary between successive tree rings is not always abrupt in successive years and is probably due to a winter temperature (reaching minimum values of about 9°C) not enough low as to induce cambium dormancy. In *P. pinea*, the lack of cambium dormancy due to mild winter conditions is in agreement with findings reported in *P. radiata* growing in New Zealand and in *P. halepensis* at a Mediterranean site in Spain (Barnett, 1971; Prislan et al., 2016). In the recent study by Prislan et al. (2016), a temperature of 10°C in January did not induce cambial dormancy in *P. halepensis*. The production of earlywood-like tracheids has been also found in tree-ring chronologies of *P. pinea* and *P. pinaster* growing in Portugal: classified as an L^+-IADF, such a wood type has been associated with above-average precipitation in early autumn (Campelo et al., 2007), leading to a decrease in the deposition of cell wall (Antonova and Stasova, 1997). In our case, we prefer to refer to these earlywood-like cells as a transition wood according to a more recent re-classification of IADFs (De Micco et al. 2016a), but from a genesis viewpoint they could be assimilated to L^+-IADF reported by Campelo et al. (2007) because they were formed at the beginning of the calendar year when rain was abundant after a period of low precipitation in December. Such a transition wood makes the boundary between tree rings less abrupt and attention should be paid in referring to it as the wood formed at the beginning of the calendar year (January-March in our case) and not to the last months of the previous year. Indeed, this becomes a critical point in tree-ring dating.

Whatever the triggering factors, the occurrence of IADFs or of transition wood in the tree rings are responsible for the adjustment of the balance between efficiency and safety of water transport. While the formation of narrow conduits in latewood formed in summer allows slow, still continuous, water transport under conditions of soil aridity, the formation of an extra-band of earlywood-like cells in latewood guarantees increased efficiency of water transport when water is largely available again after autumn rain according to conductivity physical laws (Sperry et al., 2006). Such a strategy is in line with anatomical peculiarities allowing adaptive regulation of water flow in Mediterranean species (De Micco et al. 2008). The control of the balance efficiency/safety of water transport is critical for plant adaptation in Mediterranean ecosystems, which are prone to frequent fluctuations in soil water content. Optimization of efficiency/safety of water transport can be achieved through a plastic control of conduit size during wood formation and through the occurrence of peculiar anatomical traits especially in hardwoods (e.g. presence of tracheids, vessel redundancy, occurrence of vessels with different class diameters, etc.) (De Micco et al., 2008; 2009).

In conclusion, the occurrence of true earlywood in *P. pinea* was detected earlier than the onset of cambial activity in *A. unedo*, but in both species the appearance of mature latewood, of earlywood-like cells and of latewood again after the IADF were quite well synchronized. This indicates that, despite they have completely different wood models and different life strategies, the production of earlywood, latewood and earlywood-like cells is strongly controlled by climatic factors. Moreover, the lack of a true winter dormancy, the slightly delayed onset of latewood in *P. pinea* compared to *A. unedo*, the occurrence of more than one IADF per year in *A. unedo*, and the delayed onset of latewood after the IADF, are all signs indicating a lower sensitivity of the softwood species and the higher plasticity of the hardwood species in response to variations in temperature and water availability. Indeed, the presence of IADFs in tree rings could be considered a consequence of plant plasticity in response to environmental fluctuations typical of Mediterranean climates and would confer a competitive advantage in species forming frequent IADFs if compared with species less prone to form IADFs.

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CLIMATE INFLUENCE ON XYLEM ANATOMY AND IADF OCCURRENCE IN TREE RINGS OF *PINUS PINEA* AND *ARBUTUS UNEDO* CO-OCCURRING AT A MEDITERRANEAN SITE.

Chapter based upon a manuscript in preparation with the collaboration of De Micco V., Battipaglia G., Aronne G.

Abstract

Mediterranean trees and shrubs form Intra-annual density fluctuations (in tree rings) as the sign of plasticity in wood formation in response to intra-annual variations in environmental conditions.
Dendroecology and quantitative wood anatomy analysis were used to characterize IADFs in *Pinus pinea* and *Arbutus unedo* co-occurring at a site in Southern Italy which is characterized by Mediterranean climate. The relations between climate parameters and intra-annual tree-ring traits (i.e. IADF frequency and conduit lumen size) were analyzed to highlight the main triggers of xylem formation.

Data showed that both species are characterised by high plastic response to climate and formed a high frequency of *L*-IADFs (earlywood-like conducting cells in latewood) in tree rings. The two species, although forming the same types of IADFs, showed different sensitivity to environmental factors. *P. pinea* showed higher dependence of tracheid size on temperature than *A. unedo*, while the latter was more sensitive to precipitation. *A. unedo* was more sensitive to precipitation in spring and autumn and was able to respond promptly to subsequent rainfall events during these periods, also with the formation of more than one IADF per year.

The combination of dendroecological and anatomical analyses was useful to compare the aptitude of the two species in forming IADFs in tree rings and to highlight the factors priming their

formation. Gained information is useful to understand wood growth reactions to environmental drivers and to evaluate tree adaptive capability, thus forest reactions in the context of climate changes.

Introduction

Wood research is more and more involved in trait-based ecology: there is increasing interest in investigating the plasticity of wood anatomical traits in response to environmental constraints to evaluate the adaptive capability of plants under climate change scenarios (Beeckman 2016). This need arises from the concern about the increasing frequency of extreme events (e. g. severe drought, flooding, heat weaves) that can negatively impact wood growth, ultimately leading to tree mortality and forest decline (Sarris et al. 2007; Gea-Izquierdo et al., 2014).

The cambial rhythm and dynamics of xylogenesis are characteristic of the species and thus genetically determined: in trees growing in favourable environmental conditions, such genetic factors can be considered the main trigger for wood formation (Fisher et al., 2007; Cocozza et al., 2011). In case of harsh conditions, climatic factors can have a prominent role in wood formation and quantitative traits of conductive and non-conductive cells can be modulated, thus recording climatic trends during the growing season (Fromm, 2013; De Micco et al., 2016). In temperate environments, the tree rings, formed in response to genetic and climatic factors, show the typical alternation of earlywood and latewood bands due to the occurrence of one main flush of growth in spring (Fritts 1976). However, deviations in the xylogenesis course, due to the action of abiotic and biotic factors during plant life, can determine structural “anomalies” in tree rings, called Intra-annual density fluctuations (IADFs)which have been reported in many environments, including boreal and tropical, but still are more frequent in species growing in Mediterranean ecosystems (De Micco et al. 2016b). Actually, in Mediterranean woody species, IADFs are more the rule than the exception: their functional role and genesis have been described in different species, both softwood and hardwood (Campelo et al., 2007b, 2013; Battipaglia et al., 2010, 2014; De Micco et al., 2014, 2016a, c; Martinez del Castillo et al., 2016; Prislan et al., 2016). The formation of IADFs is interpreted as an adaptive strategy of the plants to cope with the intra-seasonal alternation of favourable and stressful periods (De Micco et al., 2016b). It is likely that the ability to promptly adjust the rhythm of the cambial activity according to environmental factors confers a competitive advantage under stressful climatic conditions to those species prone to form IADFs. Indeed, tree rings with IADFs are made of successive intra-annual bands of earlywood and latewood that allow more efficient water transport (large earlywood cells) when environmental conditions are favourable, while maintaining safety against embolism (narrower cells with thicker walls) under limiting conditions (Sperry et al., 2006; Battipaglia et al., 2016; De Micco et al., 2016b).

It has been shown that IADFs formation in some Mediterranean species is consequent to the bimodal xylogenesis guided by the typical double-stress due to winter low temperature and summer drought (Camarero et al., 2010; de Luis et al., 2011a,b; Battipaglia et al.,2014; Vieira et al.,2015; Battipaglia et al., 2016; De Micco et al., 2016b).Recent studies on xylogenesis showed that these two stressors can induce either cambium dormancy twice a year or just a slowing down of xylogenesis in two periods during the calendar year (De Micco et al., 2016c; Prislan et al., 20016; Please refer also to Chapter 5). In the last decade, many studies have demonstrated that the relations between IADFs formation and environmental fluctuations are species- and site-specific (de Luis et al., 2011b; Vieira et al., 2014; Zalloni et al., 2016). Different types of IADFs and different IADF's frequency can be found in different species co-occurring at the same site or even in the same species subjected to different environmental conditions (Battipaglia et al., 2010; Camarero et al., 2010, de Luis et al., 2011b; Campelo et al., 2013). In Mediterranean pines *L*-IADFs, which consist of earlywood-like cells in latewood, are frequent, and generally occurring in the second half of the ring (Campelo et al., 2007b, 2013).Such IADFs have been ascribed to the occurrence of favourable conditions of water availability in early autumn following a period of summer drought which temporarily reduce cambial activity (Abe et al., 2003; Battipaglia et al., 2010; de Luis et al., 2011a,b; Novak et al., 2013a,b; Carvalho et al., 2015; Pacheco et al., 2015). In *Pinus pinea* L. growing under a Mediterranean climate at a site in Portugal, the occurrence of earlywood-like cells within latewood was positively correlated to above-average precipitation in September and October (Campelo et al., 2007). A recent survey of IADFs in *Pinus* species at regional scale suggested the occurrence of a common climatic driver, namely abundant water availability in late summer/early autumn, for the formation of IADFs in Mediterranean pines (Zalloni et al., 2016). The same conditions are considered starters for *L*-IADF formation in *Juniperus thurifera* and *Pinus halepensis*(Pacheco et al., 2015). Also in the hardwood *A. unedo* growing in Italy, *L*-IADFs were found, being positively correlated with precipitations of the period June-September which induced a regrowth of the last part of the ring with the formation of wider xylem cells than true latewood (Battipaglia et al., 2010; De Micco et al., 2016c). The same species, under different microclimatic conditions formed *E*-IADFs, which were localized in the middle of the ring and were characterised by the occurrence of latewood-like cells in earlywood (Battipaglia et al., 2010). *E*-IADFs were detected also in other species, such as *Erica arborea*, *Pinus pinaster* and *Pinus halepensis*, and were ascribed to reduced cell expansion caused by stomata closure under summer drought conditions (De Miccoet al.,2007; Campeloetal.,2013; Battipagliaetal.,2014; Vieira et al.,2010; de Luis et al.,2011a,b; Novak et al.,2013a).An increase in wood safety against embolism is consequent to the formation of *E*-IADFs (Sperry et al., 2006; De Micco et al., 2016b). In all these recent studies, the combination of dendrochronological and wood anatomical data with climate information has furnished valuable information on how climate variations affect wood grow at the seasonal scale.

In this context, the aim of this study was to assess how IADF's frequency and wood anatomical traits are affected by variations in climatic conditions in tree rings of two Mediterranean species, namely *Pinus pinea* and *Arbutus unedo,* co-occurring at the same site in Southern Italy. We analyzed intra-annual variations of anatomical traits in tree-ring series, comparing rings with and without IADFs. Anatomical data were correlated to temperature and precipitation data. We aimed to compare the two species to assess: 1) whether they had the same aptitude to form IADFs; 2) which type of IADFs were formed in each of them; 3) whether there are common climatic drivers in the formation of IADFs in the two species not withstanding their different life strategies. Xylogenesis data reported in the Chapter 5 helped the interpretation of the correlations between anatomical and climatic data. All data were discussed with reference to general trends in IADF formation in Mediterranean environments, in terms of their influence on plant functionality, theirrole in plants adaptation strategies and consequences on vegetation dynamics.

Materials and methods

Study area

The study was conductedon the Vesuvius volcano, near Naples (40'782 N, 14'425 E, 200 m a.s.l.) in Southern Italy. The site consists in a *P. pinea* forest mixed with *A. unedo* shrubs and small trees. The climate is typically Mediterranean, with mean average summer (June-August) and winter (December-February) temperatures of 24.6 and 9.3°Crespectively, while mean total precipitation in autumn (September- December) and in summer is 99.2 mm and 27.6 mm for the period 1979-2015 (Fig 1). The drought period lasted from June to August. Temperature data were derived from the E-OBS gridded datasets, with1°spatial resolution (14.250-14.500 E, 40.750-41.00 N), while monthly precipitation data were from the GPCC gridded dataset, with 1° spatial resolution (14.00-15.00 E, 40.00-41.00 N).

Plant material and Tree-ring data

In January 2016, twenty dominant and healthy trees of *P. pinea* (47–59 cm diameter at breast height, 16 m height) and twenty healthy shrubs (not multistem) of *A. unedo* (10–25 cm diameter at breast height, 4-8 height) were selected for the study. Two cores per each plant were sampled at breast height with a Pressler increment borer. The cores were dried and polished; tree rings were visually identified and distinguished from IADFs (Stokes and Smiley, 1968). Then tree-ring width was measured using a LINTAB system with a resolution of 0.01 mm. The occurrence and position of IADFs within the annual rings were recorded and classified according to De Micco et al., (2016b). The program COFECHA (Holmes, 1983) was run to validate the cross-dating and measurements and to find potential errors. Once all measurement sseries had been validated, tree-ring chronologies were developed. Individual series were crossdated and detrended (20-year spline) to remove non-climatic signals (Fritts, 1976) and then individual chronologies were averaged (Fig.2) using the Dendrochronology Program Library in R (dplR; Bunn, 2010).

Microscopy and QWA

Five cross-dated cores per species were cut in order to obtain thin sections (15 μm thick) using a sliding microtome. Sections were stained with a safranin and astra blue solution (van der Werf et al., 2007), and mounted with mineral oil for microscopy.

Sections were analyzed under a transmitted light microscope (BX60, Olympus) and microphotographs of each tree-ring were taken with a digital camera (CAMEDIA C4040, Olympus). Each microphotograph corresponded to a single whole tree ring from the beginning of earlywood to the end of latewood. In *P. pinea*, tree rings from the year 1979 to 2015 were analysed for a total of 130 tree rings without IADFs and 50 with IADFs. In *A. unedo*, tree rings from the year 2000 to 2015 were analysed for a total of 26 tree rings without IADFs and 49 with IADFs.

In transects extended for the whole length of the tree ring, including three radial line of tracheids in *P. pinea* and vessels in radial multiples at least of four in *A. unedo*, tracheids and vessels were automatically detected starting from the inside border of the tree ring (beginning of earlywood)towards the outside ring boundary (ending of latewood). Lumen area of all encountered conduits was measured. During the measurement, the progressive number of each element was recorded moving within the transect from earlywood to latewood and used to standardise the position of each conduit in terms of distance from the beginning of the ring, the whole ring width being considered equal to 100% (De Micco et al., 2012). For each of the considered parameters, a dispersion graph was drawn, where each conduit was characterised by two coordinates: Y, corresponding to themeasured lumen parameter and, X, corresponding to thedistance from the beginning of the ring, expressed as percentage of the total ring width. The patterns of vessel/tracheid size variability along ring width were visually compared in rings with and without IADFs. In the standardized data series we calculated interpolation equations (fourth-order polynomial curve) with confidence intervals using the option Non-linear curve fitting in R system (R Development Core Team, 2008). For the calculation of correlations between conduit size and climate variables, each tree ring without IADFs was partitioned in 4 regions (each region corresponding to the 25% of total ring width) while in tree-rings with IADFs, a sort of standardization of the conduit position was performed by making the 80% ring width of tree rings with IADFs coinciding with 100 % ring width of tree rings without IADFs. In detail, tree-ring width in the presence of IADFs were divided into 5 regions (each region corresponding to the 20% of total ring width): the first fourth regions were considered coinciding with those of rings without IADFs and relative data were pooled, while the fifth region was considered as an additional growth layer containing the IADF. Mean values of vessel/tracheids lumen area were computed per each region of the ring and correlation with maximum temperature and total precipitation of months from September of the previous year to December of the current year were calculated using Pearson coefficient($p < 0.05$).

Analysis of IADFs

The relative frequency of IADFs per year was calculated as the number of trees that present IADFs in a given year, divided by the total number of sampled trees in that year. Stabilized IADF's frequency was calculated according to Osborn et al. (1997) as $f=Fn^{0,5}$. The frequency was calculated on 20 cores (10 twin cores) for each species.

The relationship between climate data and stabilized IADF's frequency, was calculated with Pearson coefficient ($p < 0.05$). We included in the analysis vessel chronologies of 5 trees per species and the chronology of stabilized IADF's frequency considering the period 1982–2015 for *P. pinea* and 2000-2015 for *A. unedo*. Gridded data of precipitation and maximum temperature at monthly scales, from September of previous year to December of the current year, were used. We selected the aforementioned months for analysis according to the cambial activity calendar of both species outlined in our previous work (unpublished data, please refer to chapter 5). To avoid losing information we considered the conditions also of the previous three months to the current year. All correlations were calculated using the treeclim R package (Zang&Biondi, 2015).

Results

Tree-Ring Dating and IADF occurrence.

P. pinea chronologies have a mean of 96 years (Fig.1a) with EPS of 0.94 and GLK >60, showing high correlation between the mean chronology and the radial growth variation of trees population. The r bar value of 0.35 indicated a strong relationship among individual series. *A. unedo* plants were younger than the *P. pinea* with average chronologies of 20 years, an ESP of 0.96, GLK >

60,and r bar of 0.53 which indicated a good synchronization between individual trees (Fig.1b). In both species, a high frequency of IADFs was found.

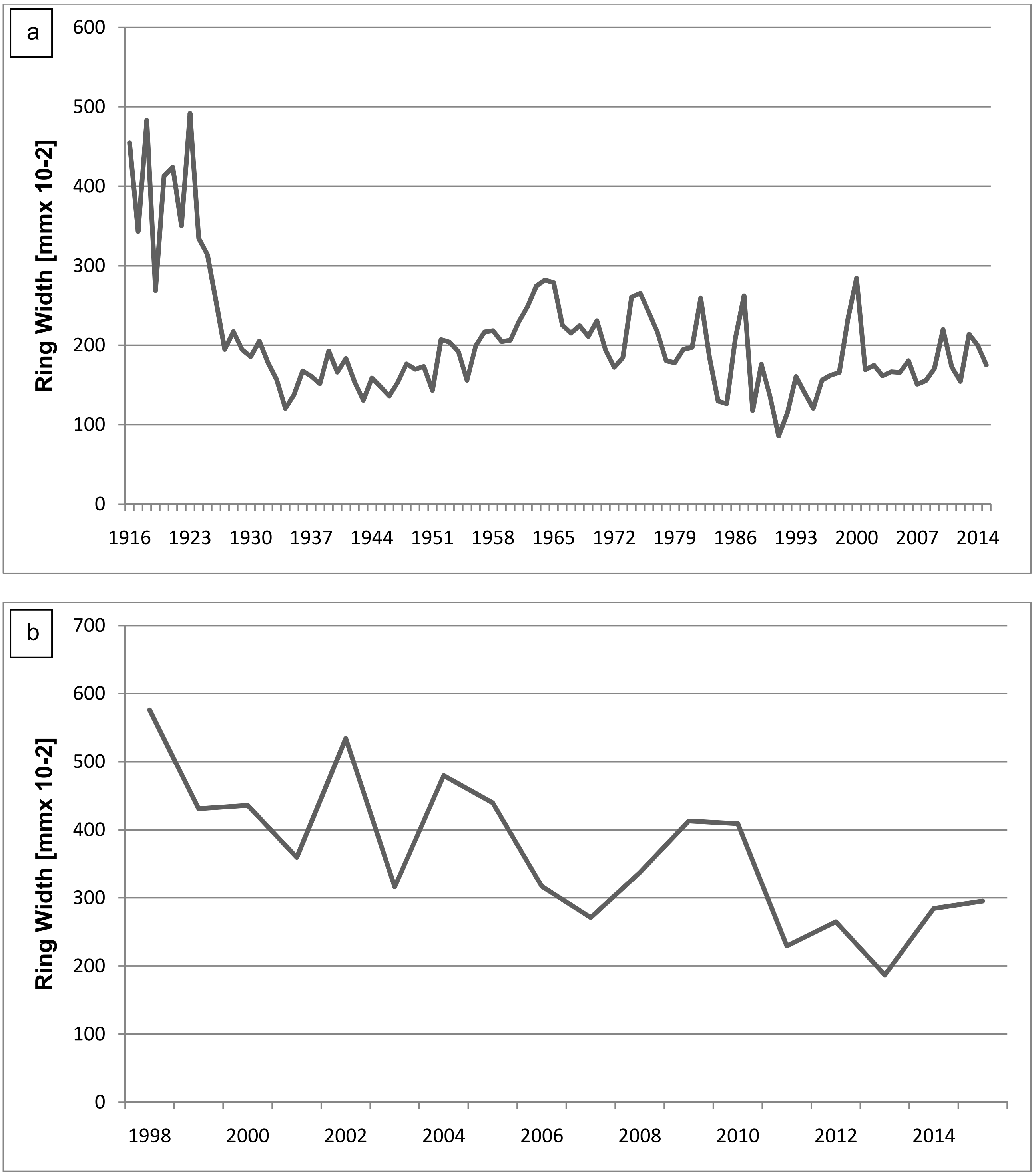

Fig 1. Average ring width chronologies of *P. pinea* (a) and *A. unedo* (b).

In figure 2, microphotographs of tree rings with *L*-IADF of *P. pinea* (Fig. 2a) and *A. unedo* (Fig. 2b) are shown. In the rings without IADFs, tracheid size varied along the ring following the usual trend, narrowing lumen and thickening cell walls moving from EW to LW.IADFs were classified as: 1)*L*-IADFs, consisting in earlywood-like cells in the latewood; 2) L^+-IADF or transition wood, consisting in earlywood-like cells between the latewood and earlywood of the successive tree ring. In *A. unedo*, wood was diffuse-porous, but latewood was distinguishable in the last quarter of the ring width. In this hardwood species, IADFs were classified only as *L*-IADFs; sometimes tree rings formed more than one *L*-IADF which appeared as two or more successive bands of earlywood like cells occurring in latewood.

L-type IADFs occurred in 28% of rings of *P. pinea* while L^+-IADFs/transition tracheids were found only in 0.28% of the rings. *A. unedo* showed a higher percentage of *L*-type IADFs (66% of total rings) while double IADFs were found in 21% of the total rings.

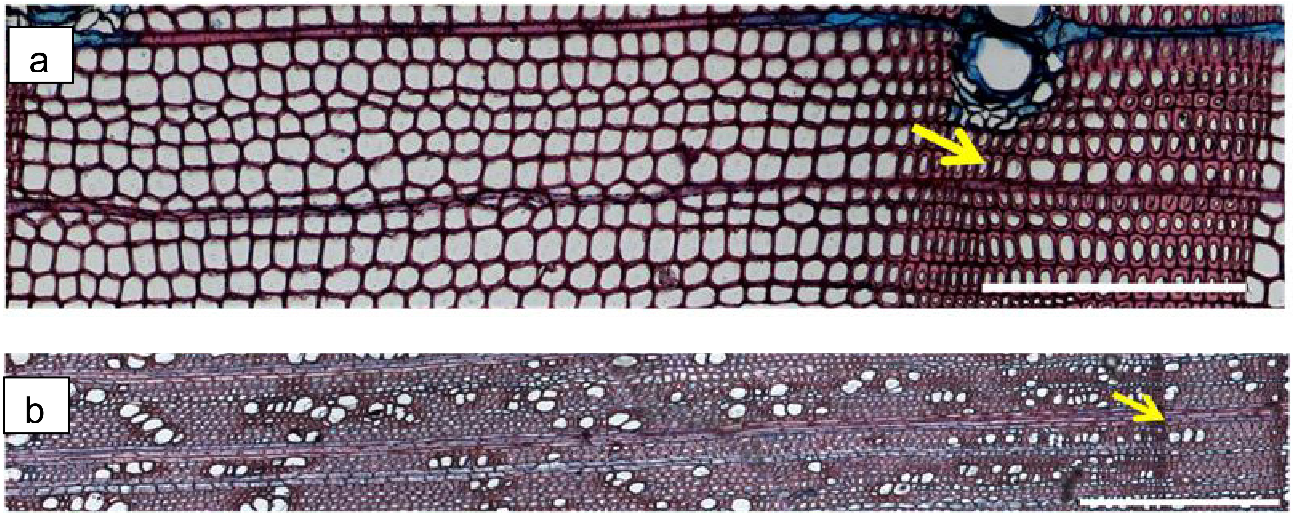

Fig 2. Microphotographs of cross sections of tree rings with *L*-IADF of *P. pinea* (a) and *A. unedo* (b). Yellow arrows indicate the beginning of earlywood-like cells. Bar=500 µm.

Lumen area chronologies of conductive elements

The graphs in Fig. 3 report the non-linear regression lines based on a fourth-order polynomial function for tracheid lumen area variation along tree-ring width, for rings without(Fig. 3a, c)and with IADFs (Fig. 3b, d)of *A. unedo*(Fig. 3a,b) and *P. pinea* (Fig. 3 c,d) In *P.pinea* in the first half of the ring, earlywood cells were characterized by larger lumen in rings with IADFs if compared with those without IADFs. The two curves coincided at 50-70% of the ring width. Then, tracheid size continued to decrease in rings without IADFs, while it increased again in rings with IADFs which showed larger earlywood-like tracheids in the LW region (at 80-100% of ring width).The variability between rings was lower in rings without IADFs but the correlation coefficient was similar in the two types of rings ($r^2 = 0.267$ for rings with IADFs; $r^2 = 0.324$ for rings without IADFs).

In *A. unedo* at the beginning of the ring (until about 10% width), earlywood vessels were characterised by larger lumen in rings with IADFs if compared with those without IADFs. Vessel size progressively decreased in rings without IADFs from earlywood to latewood. On the opposite, in tree rings showing IADFs, vessels size showed a first not significant decrease at a distance of about 20% from the beginning of the ring and a second, more severe, significant decrease in the region around 50-70% of the ring width, compared to rings without IADFs. Vessel lumen size increased again in rings with IADFs reaching maximum values in the LW region (at about 90%of ring width).

The variability between rings was similar in rings with and without IADFs while correlation coefficient was higher in rings with IADFs ($r^2 = 0.198$) than in those without IADFs ($r^2 = 0.151$).

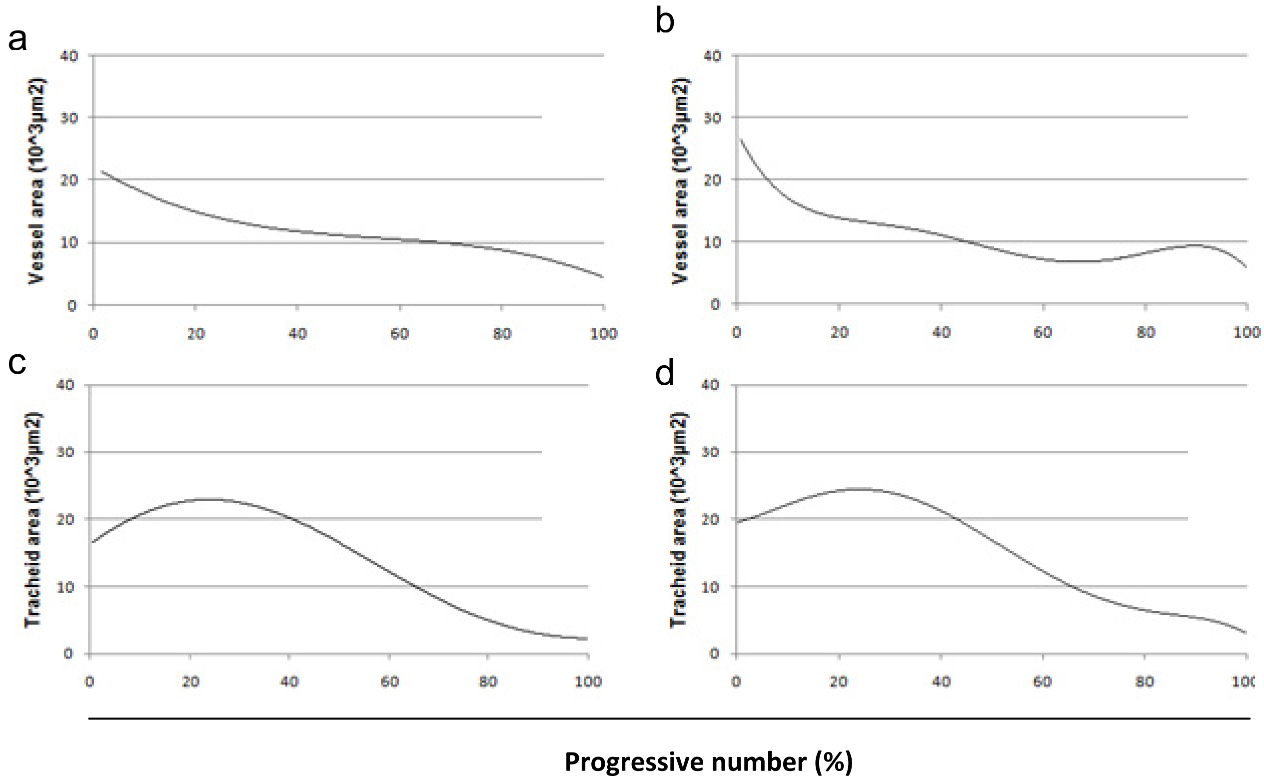

Fig 4. Variation in [illegible], b) and tracheid (c, d) size along ring width shown by plotting the set of the standardized data of tree rings with(b) and without (d)IADFs for *A. unedo* (a, b)and *P. pinea* (c, d)

Climate influences on xylem and IADF formation.

Climatic analysis (data not shown) at monthly scale over the periods 1982–2015 for *P. pinea* and 2000-2015 for *A. unedo* revealed a different influence of temperature and precipitations on the lumen area of conductive elements and on IADF occurrence in the two species. In *P. pinea*, tracheid lumen area resulted more sensitive to the temperature than precipitation. Lumen area of tracheids at the beginning of earlywood was positively correlated with April maximum temperature and the whole earlywood was positively affected by the maximum temperature of the previous autumn months. Tracheids of latewood were negatively influenced by the maximum temperature of May and negatively correlated to the precipitation of August and December. Lumen area of the IADF region was positively influenced by maximum temperature of April and December and by June precipitation.

In*A.unedo*, a positive correlation was found between lumen area of earlywood vessels (Region 1) and temperature in April. Vessels of the transition xylem between earlywood and latewood were positively influenced by April temperature and precipitation in February and October. Vessel lumen in the latewood and IADF region was positively correlated with January and February precipitation, and April and July precipitation and negatively to March precipitation.

Correlations between climatic data and stabilized IADF's frequency in *P. pinea* showed a negative relation between the occurence of *L*- IADF and precipitation of December of previous year. A positive relation was found with the maximum temperature of August and October. The occurrence of L^+-IADF was instead correlated negatively with precipitation of the previous October and positively with the temperature of December.

Regarding *A. unedo, L*-IADF occurrence was positively correlated with precipitation in September and negatively correlated with maximum temperature in previous September and current August. Double *L*-IADFs occurrence was positively correlated to precipitation of March, June and

November and maximum temperature of April, while negatively correlated with precipitation of August and maximum temperature in June.

Discussion

The analysis of tree rings of *P. pinea* and *A. unedo* growing at a same site in Southern Italy showed that the two species, although being completely different wood models, namely softwood and hardwood, and having different life strategies, react to the constraints of the Mediterranean climate through the formation of the same type of IADFs. Indeed, both species form earlywood-like conducting cells in latewood in agreement with previous studies, thus confirming the steadiness of the formation of *L*-IADFs as a qualitative response to abundant precipitation after periods of drought (De Micco et al., 2016b; Zalloni et al., 2016). However, deeper analysis of IADF's frequency, anatomical traits and relations with climate also demonstrated that the two species have different degrees of xylem plasticity. *A. unedo* seems to be more sensitive to variations of environmental fluctuations in so far as it shows a higher frequency of IADFs together with the occurrence of more than one IADF per year. Moreover, in *A. unedo*, the increase in vessel size found in the last part of latewood preceded by a significant decrease in the previous sector of the ring which is not evident in *P. pinea*. The higher sensitivity of *A. unedo* compared to *P. pinea* can be due not only to a genetic predisposition, but also to the younger age of the plants. Indeed, younger plants generally show wider tree-ring width which in turn is correlated with higher IADF's frequency (Bogino and Bravo, 2009; Vieira et al., 2009; Novak et al., 2013; Campelo et al., 2015). The young age of *A. unedo* plants can also be related to a shallow root system, likely being more sensitive to fluctuating water availability in upper soil layers (Cherubini et al. 2003).

The *L*-IADFs found in the two species suggest the occurrence of a bimodal xylogenesis which is considered an adaptive strategy of Mediterranean plants to cope with low winter temperature and summer drought (Camarero et al. 2010). The bimodal cambial production in *P. pinea* and *A. unedo* was confirmed by the analysis of xylogenesis in the year 2015 which showed the occurrence of two main peaks of cambial activity in spring and autumn, alternating with two moments of cambial dormancy or slowed xylogenesis in winter and summer (Please refer to chapter 5).

Considering the stabilized frequency of IADFs in *P. pinea*, the main drivers for the formation of *L*-IADFs resulted to be the high temperatures in August and October, together with high precipitation in the previous winter. Indeed, the presence of high water availability in the soil would promote wood growth and the formation of wider rings which are more prone to form IADFs in agreement with the mechanism proposed for *L*-IADF formation in many *Pinus* species growing in Mediterranean areas (Zalloni et al., 2016). Moreover, the positive relation with temperature of August and October suggests that their occurrence is related to summer drought (induced by the combination of high temperature and low precipitation) and to warm temperature in October which, in the absence of water limitations thanks to autumn rains, would promote again the development of earlywood-like cells. This hypothesis is in agreement with xylogenesis data which demonstrate the formation of IADFs in the middle of October (Please refer to chapter 5). In a recent study, Zalloni et al. (2016) reported that the increase in temperature throughout the year plays an important positive role for *L*-IADF formation in *P. pinea* from a network across the Mediterranean Basin. Moreover, a previous study on *P.pinea* in Portugal showed that *L*-IADF stabilized frequency is related to high temperature in March and May and precipitation in October and November (Campelo et al., 2007b). This variability of results confirms that, although triggered by the same mechanism, IADFs formation in the Mediterranean region presents a high year-to-year variability, strongly dependent on climate (Vieira et al., 2014) and growing site conditions. The positive relation found between IADF's frequency and the temperature of December suggests that warm conditions in late autumn/winter play a fundamental role for the formation also in the case of L^+-IADFs. Such conditions would allow the prolongation of wood formation, avoiding the usual period of cambium dormancy (Campelo et al., 2013; de Luis et al., 2011). Vieira et al. (2009) also reported positive correlations between December temperature and L^+-IADF's frequency in *P. pinaste r*suggesting that cells can continue differentiation until the end of the year or even all year round. Moreover, also in *P. halepensis* latewood tracheids were still differentiating in December, demonstrating that warm winters could extend the period of xylogenesis (Novak et al., 2016; de Luis et al., 2011).Our xylogenesis data (Please refer to chapter 5)confirmed that the formation of L^+-IADFs, consisting in the so-called transition tracheids, occurred in winter months, when temperature was not below the threshold inducing cambial dormancy. The positive correlation between December temperature and tracheid lumen size supports the idea of the winter temperature as a trigger for the formation of earlywood-like cells after the normal latewood production. Temperature more than precipitation guided tracheid size in the earlywood in *P. pinea*. Warm conditions in April, at beginning of true earlywood formation (Please refer to chapter 5), determine the differentiation of tracheids with wide lumen. This suggests that, at the beginning of the active vegetative season, air temperature has the main influence on cambium activity and cell enlargement, while precipitation is not a crucial factor, probably because the soil moisture supply is not limiting after the winter/spring period (Antonova and Stasova, 1993). Lumen area of latewood tracheids in *P. pinea* is negatively affected by the increase in the temperature of May. As we reported in chapter5, the production of latewood cells in *P. pinea* started at the beginning of June. This suggests that latewood is formed as a response to the increasing temperature of May: high temperatures would be a signal to induce the formation of narrower tracheids to improve safety of water transport (Sperry et al. 2006). The significant correlation between wet conditions in June and the occurrence of larger lumen tracheids formed in autumn in the regions of the *L*-IADFs suggests that summer rain events can promote the formation of earlywood cells in a second peak of cambial activity in autumn, probably due to the positive role played in soil refilling. Finally the negative relation between the amount of precipitation in some months (i.e. August and December) and tracheid size in latewood is in line with the strategy to produce larger conduits when water availability is limited. Such a strategy, considered as a way to improve conductivity, while allocating less carbon to cell walls under conditions of water deficit, has been described in Mediterranean pines and other species (Liang and Eckstein, 2006; Maherali and De Lucia, 2000; De Micco et al. 2017).

As regards *A. unedo*, *L*-IADF's stabilized frequency seems to be positively related to precipitation in September. This is in agreement with findings in the same species growing at a site in Central Italy where positive correlations found between the stabilized frequency of IADFs and the precipitation occurring in the period June-September were considered as a driver needed to allow the species to resume xylem production in autumn after summer dormancy (Battipaglia et al. 2010). Moreover, the finding that the occurrence of double *L*-IADFs was positively correlated with precipitation in March, June, and November, while negatively correlated with precipitation in August, suggests the role played by fluctuating water availability during the year to activate/accelerate and deactivate/slow down cambial production more than once a year. The occurrence of more than one flash of xylem growth during the calendar year is in agreement with previous findings in *A. unedo* and with our xylogenesis data (Cherubini et al., 2003; please also refer to chapter 5).

As for *P. pinea*, also for *A.unedo* the temperature in April was positively correlated with conduit size of both earlywood and latewood, suggesting the role of this factor not only on the onset of xylogenesis (please refer to chapter 5), but also on the phase of cell enlargement. Vessel size in latewood was also positively controlled by precipitation in February and October which would assure enough water reserves in the soil especially before the summer drought period. Vessel size in the region of the IADF showed the more numerous significant correlations with precipitation, either positive or negative, suggesting their role in modulating cell production, enlargement and differentiation promptly at the intra-annual scale. A previous study in southern France already highlighted the sensitiveness of *A. unedo* to precipitation in the months from April to July showing a positive tree-growth response (Nijland et al., 2011) and indicating a high dependence of the trees on rainfall during these months. The high sensitivity of *A. unedo* to precipitation amount, suggested by the correlation with climatic parameters, indicates that the species is very adapted to the frequent intra-seasonal variations of water availability and shows high plasticity in xylem formation. This is in agreement with previous studies where this species was demonstrated to form different types of IADFs in study sites characterised by different soil water availability (Battipaglia et al., 2010).

In conclusion, the overall analysis showed that both species are characterised by high plastic response to climate, although with different degree and timing. *P. pinea*trees were demonstrated to be able, as other Mediterranean pines, to sustain low growth rates during the summer dry period and winter (De Micco et al., 2016b; Camarero et al., 2010). The low dependence of tracheid size on precipitation suggests that the trees of this species can reach deeper soil-water stocks and are less influenced by short rain events if compared to *A. unedo*. On the other hand *A. unedo* was demonstrated to be more sensitive to precipitation in spring and autumn and to be able to respond promptly to subsequent rainfall events during these periods, also with the formation of more than one IADF per year. These results demonstrate that although forming the same types of IADFs, species co-occurring at the same site can show different sensitivity to environmental factors. Therefore, main triggers for IADFs formation can vary according to the species.

The different ability of different species to cope with environmental constraints by modulating xylem anatomy determines their efficiency and safety against embolism (Sperry et al., 2006) and therefore affects their growth. Consequently, vegetation dynamics could be primed by climate changes because of the different capability of different species to perform plastic adjustment of xylogenesis that seems to be crucial for their adaptation and survival in a climate change scenario.

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TYLOSES AND GUMS: A REVIEW OF STRUCTURE, FUNCTION AND OCCURRENCE OF VESSEL OCCLUSIONS

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ABSTRACT

Vessel occlusion through tyloses or gums is a natural phenomenon occurring with aging and heartwood formation, and in sapwood in response to vessel embolism. These types of vessel occlusion play a crucial role to limit the spread of pathogens and wood decay organisms, also as part of compartmentalization after wounding. In the sapwood, they can be considered to be an effective stress response.Here we review the literature on tyloses and gums in hardwoods, starting with the detailed 19th century account on tyloses by Hermine von Reichenbach. The structural diversity of tyloses (from thin-walled to sclerotic) and gum deposits is highlighted and illustrated. Our understanding of the development of vessel occlusions through vessel contact cells of the ray and axial parenchyma has greatly increased over the last decades, also thanks to ultrastructural and immunocytological studies. We critically discuss the postulated relationships between vessel-to-ray parenchyma pit size and vessel size and the incidence of either tyloses or gums and review the occurrence of these types of vessel occlusions in extant and fossil dicots. All factors identified in the literature as stimuli for vessel occlusion probably act through vessel embolism as a single direct trigger. Attempts in the literature to relate vessel occlusion with mechanisms of vessel refilling and embolism repair are controversial and invite more experimental research.

Keywords: Compartmentalization, embolism, heartwood, woundwood, protective layer.

INTRODUCTION

In recent decades, there has been increasing interest in the study of the relationships between functional wood anatomy and environmental factors, especially for forecasting plant responses to climate change and extreme events. Plant survival depends on the ability to harmonise anatomical traits of leaves, stem and root systems, and regulate physiological mechanisms at the whole plant level to allow water flow through the xylem (Holbrook & Zwieniecki 2005; Kim *et al*. 2014).

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Each anatomical trait, or combination of traits, plays a role in favouring one or more xylem functions, namely water transport, mechanical stability, biological defence, and storage and mobilisation of metabolites (Beeckman 2016). As a woody plant ages, sapwood (peripheral wood active in water transport) loses its main functions of water flow as well as transport and storage of carbohydrates, and is converted to heartwood (central wood, often dark coloured, no longer active in water flow and carbohydrate transport) whose main functions are decay resistance and mechanical support. The transition from sapwood to heartwood can be gradual or abrupt and is marked by various phenomena including senescence and death of parenchyma as well as vessel occlusion. Conduits that in sapwood serve water flow are unable to do so in the heartwood.

Vessel occlusion can be due to the formation of tyloses or to the deposition of gums. Although a common feature of heartwood, these organic occlusions can also occur in sapwood both normally and after wounding (Gerry 1914; Klein 1923; Murmanis 1975). There is common agreement that the prerequisite for occlusion is cavitation, which would also explain the more frequent tylosis formation in large vessels that are more prone to embolism than narrow ones (Esau 1965; Evert 2006; Kitin & Funada 2016).

According to the "IAWA list for microscopic features for hardwood identification", deposits in vessels include "tyloses common", "tyloses sclerotic" and "gums and other deposits in heartwood" (IAWA Committee 1989). In the IAWA list, "other deposits in heartwood vessels" include a variety of chemical compounds, which have various colours. According to the subjective criterion "common", these features have only been scored positive in the literature for a minority of all woody species (35%, Wheeler *et al.* 2007) and in the InsideWood database (2004-onwards); however, gums or tyloses can occur in the heartwood of virtually *all* species specifically reviewed for the occurrence of vessel occlusions (Chattaway 1949; Bonsen & Kučera 1990; Bonsen 1991; Saitoh *et al.* 1993). Tyloses themselves can contain a wide variety of organic and mineral compounds, including gums, resins, starch, crystals and phenolic compounds.

This review of the literature is focused on tyloses and gums as complementary "functional traits" in tree biology and briefly summarises what we know of their occurrence in extant and fossil woods.

FROM THE FIRST DESCRIPTION OF "SMALL BAGS IN VESSELS" TO THE ULTRASTRUCTURE OF TYLOSES

Zimmermann (1979) has narrated the discovery of tyloses and their development. Malpighi (1686) in his "*Opera Omnia*" was the first to report "balloon-shaped sacs" in heartwood vessels. Later on, such structures were named "Thyllen" (tyloses), deriving from the Greek word indicating "bag" or "container", in an anonymous paper in 1845, which has been ascribed to Hermine, Baroness von Reichenbach of Vienna (Winckler 1854). This Viennese lady observed peculiar structures in vessels that she confidently described as outgrowths of neighbouring wood parenchyma cells passing through the pits, since they always originated close to a region of the vessel wall adjacent to axial or radial parenchyma cells and never in regions where two vessels were in contact (Fig. 1). In the following decades, this view was supported or questioned by several

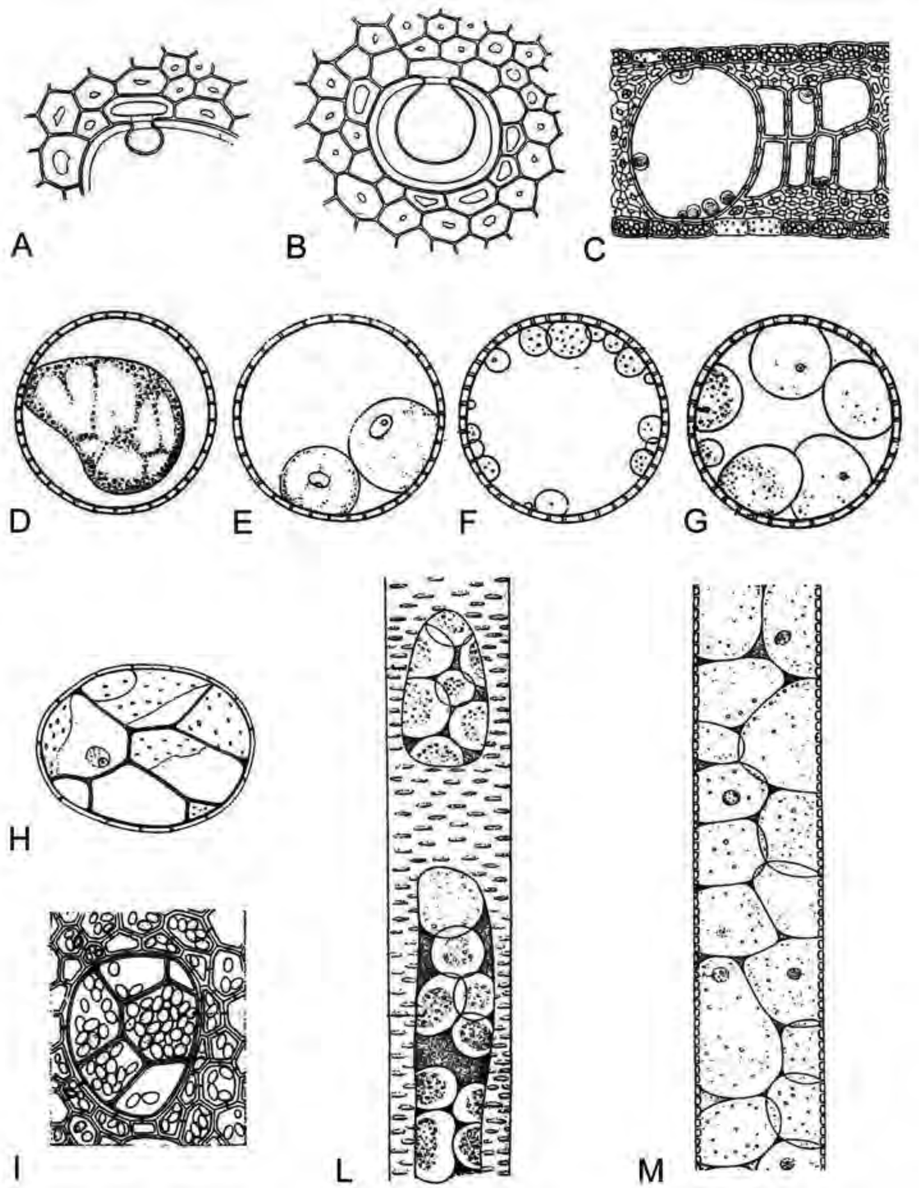

Figure 1. Selection of drawings from the original plates of Reichenbach's paper reported in Zimmermann (1979). – A, B: Bulging tyloses and respective mother cells in a cross section of *Vitis vinifera*. – C: Young tyloses in vessels of one-year-old shoot of *Robinia pseudoacacia*. – D–G: Vessels of *Cucurbita pepo* with tyloses with cytoplasmic streaming (D), evident nuclei (E), translucent (young tyloses) or granular (old tyloses) content (F, G). – H: Vessel of *Robinia pseudoacacia* filled with many tyloses with evident walls with pits and nuclei. – I: Vessel of *Vitis vinifera* with tyloses containing starch. – L, M: Vessels of *Cucurbita pepo* in longitudinal section.

authors, but Hermine von Reichenbach's views have been generally accepted (Gerry 1914; Koran & Côté 1965; Esau 1965; Zimmermann 1979; Bonsen & Kučera 1990; Saitoh *et al*. 1993; Tyree & Zimmermann 2002).

Arising mostly from ray cells and to a much lesser extent from axial paratracheal parenchyma cells, tyloses expand into the adjacent vessel through pits (Pearce 1996; Sun *et al*. 2008). Braun (1967) has coined the term "contact cells", synonymous with the "vessel-associated cells" of Czaninski (1977) to indicate parenchyma cells that have the potential to form tyloses or secrete gums into vessels (Catesson & Moreau 1985; see also Morris & Jansen 2016). Tyloses are most commonly found in pitted vessels of metaxylem and secondary xylem, and more rarely in protoxylem elements with annular or spiral secondary walls (Zimmermann 1979, citing Hermine von Reichenbach).

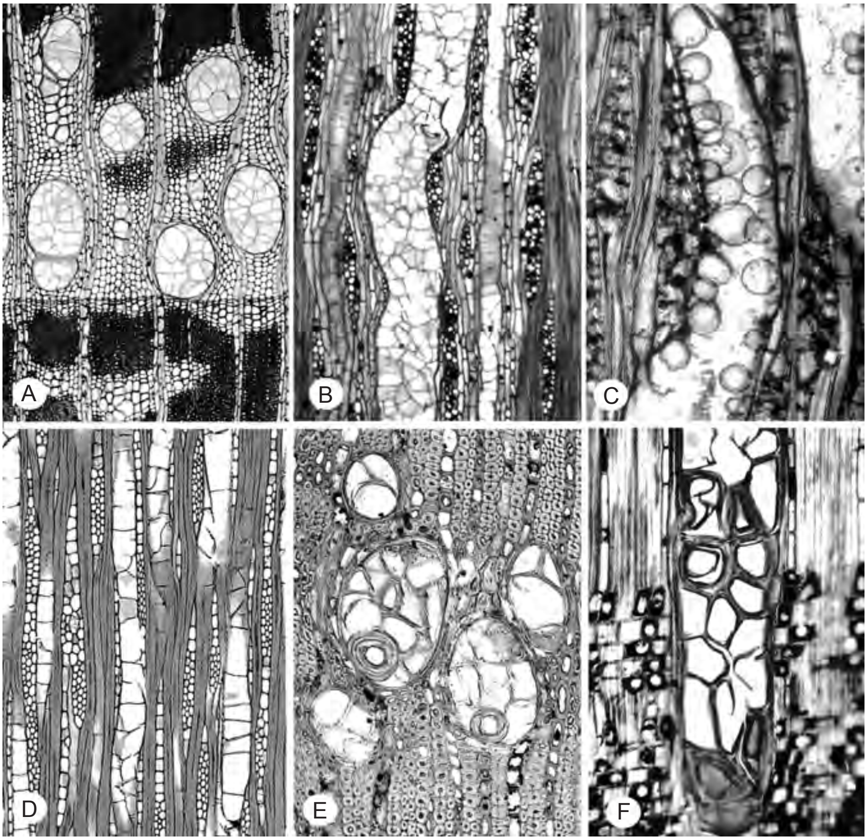

Figure 2. Tyloses, light microscope images. – A, B: Ring-porous wood with tyloses abundant in wide earlywood vessels and less common and less crowded in narrower vessels, *Maclura pomifera* (Moraceae), photos Els Bakker. – C: Tyloses formation from ray parenchyma in a fossil wood, *Cinnamomoxylon oleiferum* (Lauraceae), middle Eocene (c. 44 million years). – D: Widely spaced tyloses, appearing to segment vessel elements, *Altingia excelsa* (Altingiaceae). – E: Some tyloses sclerotic, with thick, layered walls. Note: very thick-walled fibres also present, *Henriquezia verticillata* (Rubiaceae), photo A.M.W. Mennega. – F: Sclerotic tyloses, *Pouteria guianensis* (Sapotaceae), photo R.B. Miller.

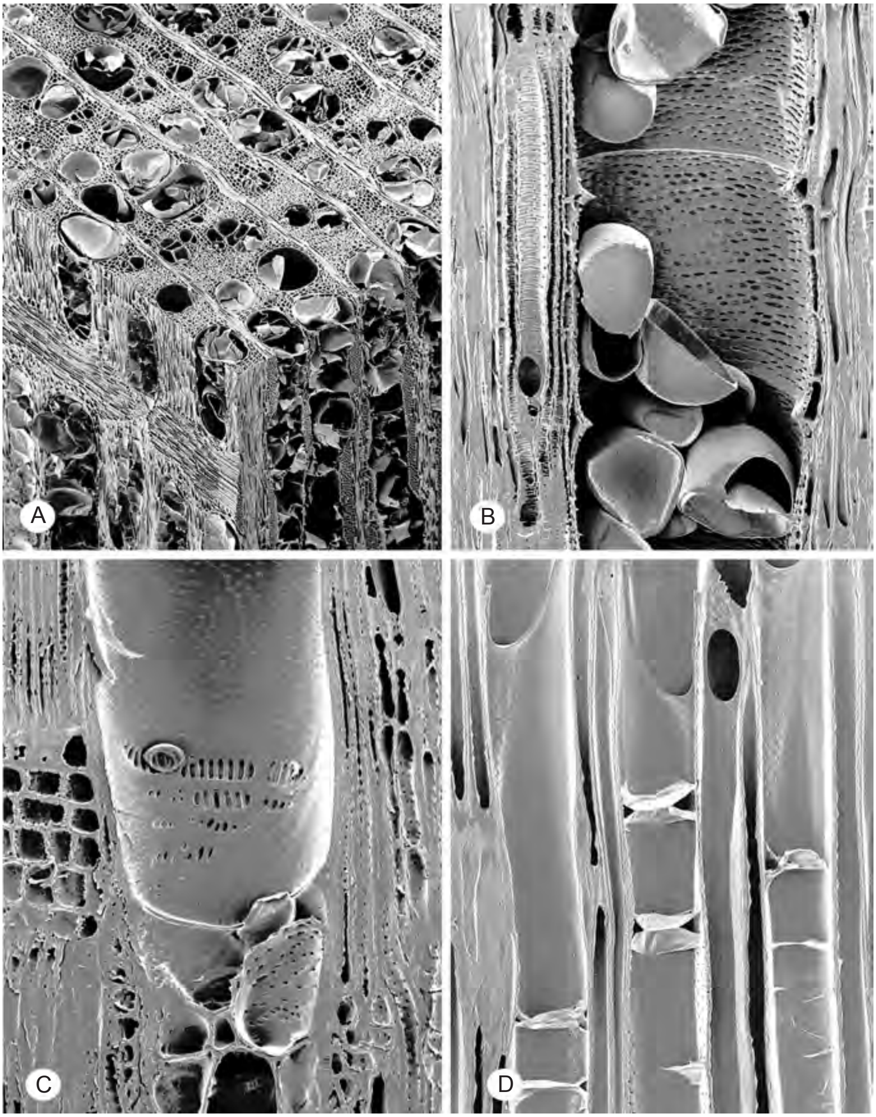

Figure 3. Tyloses, SEM images. – A: Tyloses abundant in wide vessels, *Morus australis* (Moraceae). – B: Wide vessel with tyloses, narrow vessel without tyloses, *Celtis sinensis* (Cannabaceae). – C: Tyloses bud emerging from vessel-ray parenchyma pit, *Quercus salicina* (Fagaceae). – D: Septa-like tyloses, *Acanthopanax sciadophylloides* (Araliaceae). Photos by courtesy of the Forestry and Forest Products Research Institute, Tsukuba, Japan.

Moreover, they can appear as a single tylosis or as groups of tyloses crowded together, as thin- or thick-walled structures, sclerified or unsclerified, pitted or unpitted, and with or without inclusions in the form of starch, crystals, resins, gums, gels or other storage products (Fig. 2 & 3) (IAWA Committee 1989; Bonsen & Kučera 1990). The vessel lumen can be partially or completely filled by tyloses.

Although tyloses typically develop in vessels, they also occur occasionally in tracheids of both softwoods and hardwoods and in the fibres of hardwoods (Chrysler 1908; Gerry 1914; Esau 1965; Gottwald 1972; Peters 1974).

The fine structure of tyloses has been well studied, although in a few species only (Kato & Kishima 1965; Koran & Côté 1965; Meyer 1967; Sachs *et al*. 1970; Shibata *et al*. 1982; Parameswaran *et al*.1985; Bonsen 1991). During the formation of a tylosis, the primary wall component of the pit membrane is not merely stretched and pushed into the vessel, but new wall materials, including cellulose, hemicellulose, pectins, suberin and lignin, are actively deposited (Foster 1967; Pearce & Holloway 1984; Barnett *et al*. 1993).

In the contact cells, a special layer of pecto-cellulosic composition, called "protective layer" overlies the pit membrane and cell wall portions that are in contact with the vessel (Fig. 4) (Foster 1964; Czaninski 1977; Gregory 1978; Mueller & Beckman

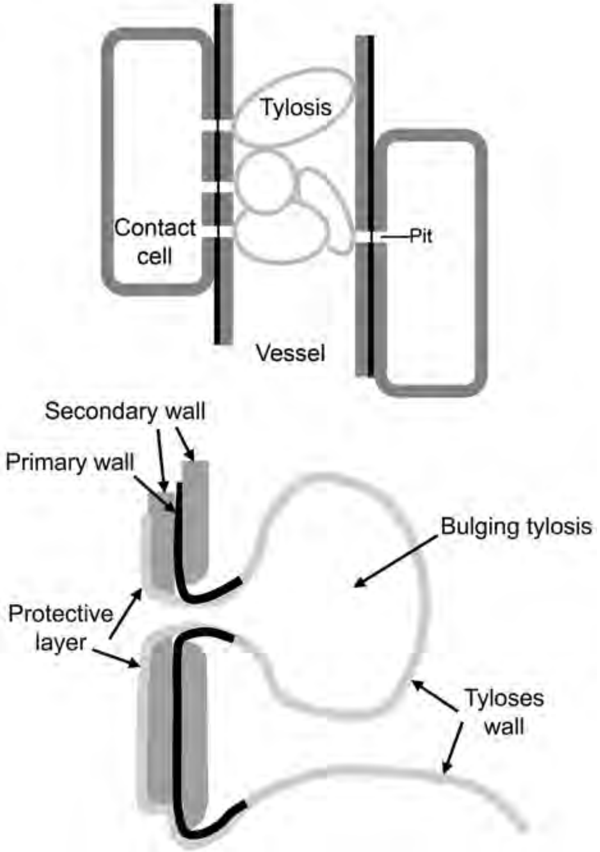

Figure 4. Scheme of bulging tyloses from parenchyma cells to vessel lumen, through pits. The tylosis wall belongs to the protective layer which coats the secondary wall of the contact cell and the primary wall at the pit level before protruding towards the vessel lumen. Overview (above) and detail (below).

1984; van Bel & van der Schoot 1988; Evert 2006). The protective layer, also referred to as "tylose-forming layer", "amorphous layer" or "isotropic layer", is firstly deposited between the protoplast and the pit membrane of the vessel-ray parenchyma pits. Then it "balloons" into the vessel lumen giving rise to the tylosis wall (Schmid 1965; Meyer & Côté 1968; Esau 1977; Fujii *et al*. 1980, 1981; Schaffer & Wisniewski 1989).

A budding tylosis is first transparent, while starch grains, a nucleus and nucleolus may appear later in the cytoplasm. Then, ultrastructural changes may occur, including the accumulation of phenolic compounds and crystals in the cytoplasm (Ranjani & Krishnamurthy 1988; Schmitt & Liese 1993; Rioux *et al*. 1998). Tyloses maintain symplastic continuity with the contact cells which are the only cells supplying resources to them since vessels are embolised.

Tyloses can also undergo cell divisions (Schmitt & Liese 1994). Without obstacles, tyloses can either enlarge up to vessel lumen limits as in *Quercus alba*, or remain small without completely blocking the vessel as in *Kalopanax pictus* (Sachs *et al*. 1970; Sano & Fukazawa 1991). Different tyloses bulging into the same vessel constrain each other; in narrow vessels, tyloses may misleadingly give the impression of septate vessel elements (*e.g. Liquidambar styraciflua,* latewood vessels in *Robinia pseudoacacia*, and *Acanthopanax sciadophylloides*) (Fig. 3D).

When tylosis expansion is complete, secondary wall formation begins and pit pairs can develop in the zone of contact between tyloses. Tylosis walls have been described as two-layered: the outer layer is amorphous and may be smooth or granular, while the inner surface shows microfibrillar structure which, more than the secondary wall of the contact cell, resembles a thickened primary wall (Foster 1964; Bonsen 1991; Schmitt *et al*. 1997). The fibrillar layer surrounding the tylosis has also been reported as similar to the compound middle lamella in several species including *Pinus balsamifera*, *Ulmus americana* and *Quercus rubra* (Koran & Côté 1965; Sachs *et al*. 1970; MacDonald & McNabb 1974; Robb *et al*. 1979; Ouellette 1980; Parameswaran *et al*. 1985; Bonsen & Kučera 1990; Rioux *et al*. 1995). In *Eucalyptus, Kalopanax, Quercus*, and *Robinia*, cellulose microfibrils are oriented randomly in the outer layer of the tylosis wall, while they are parallel in the inner layer (Koran & Côté 1965; Foster 1967; Sachs *et al*. 1970; Shibata *et al*. 1982; Parameswaran *et al*. 1985; Sano & Fukazawa 1991). At the completion of the tylosis expansion, a multi-layered wall is distinguishable which can be encrusted with suberin or lignin in a sort of secondary wall (Sachs *et al*. 1970; Ouellette 1980; Obst *et al*. 1988; Evert 2006). Histochemical tests showed suberised tyloses in *Pinus balsamifera* and *Ulmus americana* (Rioux & Ouellette 1991). Vessels in *Fagus*, *Quercus*, and *Populus* contain suberised tyloses, while those in *Betula* contain amorphous and fibrillar materials deposited after wounding (Schmitt & Liese 1990). Multilayered cell wall deposition and lignification of the secondary wall can convert a normal tylosis into a sclereid (sclerified tylosis) (IAWA Committee 1989; Evert 2006). Lignin and suberin make the tylosis wall impermeable, thus constraining water transfer (Dimond 1955; Parameswaran *et al*. 1985; Schmitt & Liese 1993; Parke *et al*. 2007; Collins *et al*. 2009). Sano & Fukazawa (1991) found a unique type of tylosis wall in *Fraxinus mandshurica* var. *japonica*, poor in microfibrillar cellulose and rich in lignin in the inner sapwood, and collapsing and crumbling in the heartwood.

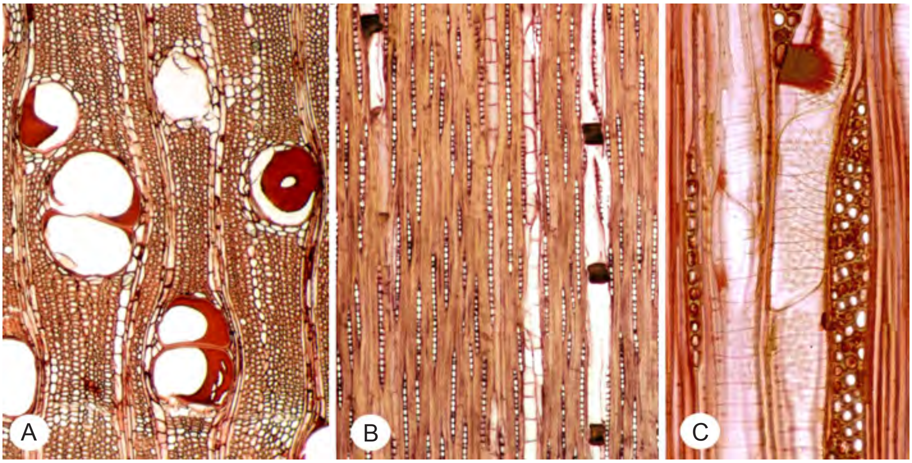

Figure 5. – A: Gum deposits concentrated near vessel–ray contact areas, *Khaya senegalensis* (Meliaceae), photo Els Bakker. – B: Gum deposits accumulating at perforation plates, *Trichilia cipo* (Meliaceae), photo Els Bakker. – C: Gums being secreted from ray parenchyma, *Prunus serotina* (Rosaceae).

GUMS IN VESSELS: WITH AND WITHOUT TYLOSES

In the literature both "gum" and "gel" have been used to indicate materials occluding vessels, mainly composed of polysaccharides and pectins, thus creating some confusion (Koran & Yang 1972; Rioux *et al*. 1998) (Fig. 5). Gums have been reported to form where gels were firstly formed after pathogen-released pectinolytic and cellulolytic enzymes have attacked walls of cells surrounding vessels (Agrios 1969). Here we enter a dichotomy between the anatomical and physiological literature: since gels easily dissolve, they are not normally recorded in anatomical descriptions. Solidified gum deposits (responsible for heartwood colours, and discoloration of woundwood) may well have had unreported gels as their precursors.

Gums have been described as amorphous material occluding vascular tissues or intercellular spaces (Catesson & Moreau 1985). Hillis (1987) reported that many secondary metabolites stored in vessels have been referred to as gums, although they are not water-soluble compounds as gums are by definition. Currently, the technical term gum is used to indicate a very wide range of secondary metabolites that vary tremendously in their chemistry and can be of chemotaxonomic interest (Hillis 1987).

Like tyloses, gums occluding vessels derive from contact parenchyma cells (Bonsen & Kučera 1990; Bonsen 1991). Gums in vessels can be either produced by the secretory activity of surrounding living cells (mainly ray cells) and deposited directly into the adjacent vessel lumen or, more rarely, can be secreted by a previously formed tylosis as shown in *Eucalyptus blakleyi* by Chattaway (1949). Individual tyloses may also be filled with gums (Chattaway 1949). The occlusion of vessels by gums is different from the secretion of gums in ducts. The gums causing vessel occlusion have a different chemical composition from those produced in ducts, which may also contain components originating from the lysis of cells located near the gum canal lumen or from cell-wall decomposition (Groom 1926; Rioux *et al*. 1998; Evert 2006). Based on these considerations, distinguishing between the true gums, mainly made of pectic material occluding vessels, and gums secreted in gum ducts, Rioux *et al*. (1998) proposed using only the term 'gel' to indicate the pectin occlusions secreted by parenchyma cells into conduits.

Like tyloses, gums have been reported by many authors as occurring both in heartwood and sapwood, either due to natural aging or to factors triggering embolism. The appearance of gums in heartwood has been categorised into four groups: 1) partitions across the vessel lumina, 2) irregular lumps on the inner vessel wall, 3) small droplets on the vessel-parenchyma pits, and 4) thin layers lining the inner vessel wall (Saitoh *et al*. 1993).

Immunocytochemical studies of *Populus basalmifera*, *Ulmus americana,* and *Quercus rubra* led Rioux *et al*. (1998) to hypothesise that gum production can be related to tylosis formation. They found that antibodies directed against pectins labelled a material both present at the external layer of the tylosis middle lamella and accumulating outside it, particularly in pit chambers. The same authors suggested a mechanism of secretion of pectic substances across the tylosis primary wall since both the latter and some other compounds within the tylosis were intensely labelled for pectins. Such a mechanism could be similar to that associated with the formation of gums in vessel elements (Rioux *et al*. 1998).

In gums produced in response to fungal attack, phenolic fungitoxic substances have been reported both filling the tylosis and in the suberised layer (Rioux *et al*. 1998). Phenolics, anti-feeding deterrents, can be variously located in plant tissues and have been observed also in vessels of young twigs of *Rhamnus californica* (De Micco & Aronne 2012).

DO STRUCTURAL LIMITS AFFECT THE TYPE OF VESSEL OCCLUSION?

Chattaway (1949) reported that the relation between vessel-parenchyma pit size and the type of vessel occlusion was known since the beginning of the 1900s. She noticed that the development of tyloses occurs almost universally in woods with large vessel-ray pitting (in which the apertures are large and the borders reduced or insignificant), but it rarely or never occurs in woods with small bordered vessel-parenchyma pits (Chattaway 1949). In woods with small bordered vessel-ray pits, the occlusion of the vessels in the heartwood is mainly ascribed to the secretion of gums. After analysing 1100 genera of higher plants, she concluded that whether tyloses or gums are formed is related to the maximum width of the vessel-ray pit apertures: if more than 10 μm, then tyloses are formed; if less than 10 μm, then gums are formed. Within the same family, genera with simple pits, whose width exceeds 10 μm, were characterised by tylosis formation, while those with bordered pits, whose width is lower than 10 μm, did not form tyloses (Chattaway 1949). Bonsen and Kučera (1990) also analysed the relations between the type of vessel occlusion and the vessel-ray pit aperture diameter in trees and shrubs common in the Swiss flora (both native and introduced) belonging to 65 species from 45 genera and 24 families. Instead of maximum aperture size, they focused on minimum size and established 3 μm as the threshold size for occlusion by tyloses or by gums. Other authors found similar results (Saitoh *et al.* 1993; Fujii *et al.* 2001). Bonsen and Kučera (1990) also found a strong correlation between maximum vessel diameter, which is somewhat related to pit size, and tylosis formation. In the woody flora of Central Europe they studied, species with a maximum vessel diameter greater than 80 µm had tyloses, while those with narrower vessels had gum plugs, *Magnolia* being the only exception of a narrow-vesseled species with tyloses. However, vessel-ray pit apertures over 10 µm accompany narrow vessels in *Magnolia* (InsideWood 2004-onwards). Similarly, the rule associating large-diameter vessels and tylosis occurrence does not apply for many tropical species, *e.g.*, members of the Meliaceae and Fabaceae *s.l.* which combine wide vessels with narrow pit apertures and gum deposition in the vessels (InsideWood 2004-onwards). Analysing correlations between vessel diameter and presence of tyloses or gums in > 6000 woody taxa worldwide only very partially supports these generalised trends. In the InsideWood database (2004-onwards; Wheeler 2011), the incidence of the feature "tyloses common" is present in about 17 % of all world woods (Wheeler *et al.* 2007), and increases from 4% in very narrow vessel taxa (< 50 /µm), via 10 % (50–100 /µm) and 20 % (100–200 /µm) to 25 % in very wide vesseled taxa (> 200 /µm). However, along this gradient of vessel diameters the incidence of gum deposits also increases from 11, to 13, 17 to 24 %. These percentages for both types of vessel occlusion are possibly too low, because numerous coded descriptions are probably based on sapwood samples.

VESSEL OCCLUSION AS AGING PHENOMENON OR STRESS RESPONSE

Vessel occlusion can happen either naturally with xylem aging or in response to various stresses in the sapwood (Chattaway 1949; Ranjani & Krishnamurthy 1988; Rioux *et al.* 1995; Dute *et al.* 1999). Several hypotheses have been suggested to explain the reasons triggering vessel occlusion by tyloses and gums. Whether vessel occlusion is the reason for or the consequence of embolism has been extensively investigated. Hermine von Reichenbach already hypothesised that tylosis formation is not the cause, but the result of the cessation of water conduction (Zimmermann 1979). Her hypothesis was experimentally proven when Klein (1923) observed that tyloses develop when the vessels are air-filled (Zimmermann 1979). Although obvious, it has often been reported that the presence of tyloses affects water movement in living trees and is responsible for a decline in hydraulic conductivity over time (Panshin & De Zeeuw 1980; Sano & Fukazawa 1991; Davison 2014). More recently, some authors challenged the long-standing notion that tyloses are induced by embolisms, claiming that vessel embolism is not required for wound-induced tylosis development in grapevine (Sun *et al.* 2007). Many studies about the relations between embolism and tylosis formation date back to years when cavitation and embolism processes were still poorly understood and the methods used to verify the occurrence of embolism often suffered from artefacts, leading to controversial results (Cochard *et al.* 2000, 2010). At present, it is commonly accepted that embolism precedes vessel occlusion (Brodersen *et al.* 2010). Therefore, all the reasons traditionally claimed for the formation of tyloses and gums can be considered indirect factors primarily responsible for embolism. According to this view, all factors causing the interruption of the water column can prime compartmentalization processes culminating in vessel occlusion by tyloses or gums. Biotic and abiotic stresses reported to cause vessel occlusion include: freezing, drought, mechanical wounding (including pruning), flooding, insect attack or pathogen infection (for examples refer to: Chattaway 1949; Dimond 1955; Ouellette 1980; Beckman & Talboys 1981; Davison & Tay 1985; Shah & Babu 1986; van der Molen *et al.* 1987; Cochard & Tyree 1990; Schmitt & Liese 1990, 1994; Pearce 1996; Stevenson *et al.* 2004; Sun *et al.* 2008; Davison 2014).

The mechanisms for vessel occlusion are reported to be mediated by hormonal signals. For example, the biosynthesis of ethylene is reported in response to many of the same biotic and abiotic factors triggering tylosis formation (Abeles *et al.* 1992; Taylor *et al.* 2002; Saniewski *et al.* 2006), suggesting that ethylene could be a coordinating factor in the development of such occlusions (Sun *et al.* 2007; McElrone *et al.* 2010). Auxin is also reported to induce tylosis formation, probably through the stimulation of ethylene production (Abeles & Rubinstein 1964; Lieberman 1979; Yu & Yang 1979).

Embolism-related tylosis formation is considered as a quick response to stressful conditions and explains why tyloses are frequent in vessels close to wounds or around sites of pathogen inoculation, and in large vessels of the earlywood that are most vulnerable to embolism (Ellmore & Ewers 1985; Cochard & Tyree 1990; Pearce 1991; Hargrave *et al.* 1994; Davis *et al.* 1999). In the case of biotic infections, vessel occlusion by tyloses or gums plays an important role in slowing down and preventing the spread of pathogens, embolism and in reducing water loss in non-functional xylem; thus vessel blockage can be considered an example of active disease resistance. The success of vessel-blocking as a barrier against pathogens depends on the speed of tylosis initiation and growth, on the presence of suberised walls and, also in the case of gum-occlusions, on possible accumulation of anti-microbial compounds such as tannins, catechol, flavonoids and coumarins that prevent spreading of pathogens in the infected part of the plant (Del Rio *et al.* 2001).When tylosis formation is the main mechanism for vessel occlusion to impede fungal colonisation, the suberisation of tylosis walls is common and facilitates sealing of vessels, while gummosis is generally not accompanied by suberisation processes even in species showing a strong compartmentalization wall 4 barrier zone according to the CODIT model (Shigo & Marx 1977; Pearce & Woodward 1986; Bonsen & Kučera 1990; Pearce 1990).

During tylosis formation, in defence mechanisms against vascular pathogens, the protective layer seems to have an active role in so far as it represents a protection layer formed by the living cell as a defence against the adjacent dead cell element (Meyer 1967; Meyer & Côté 1968; Mueller & Beckman 1984). Moreover, it could also be involved in solute exchange and act as sugar buffer against oscillations in the hydrostatic pressure in the vessels (van Bel & van der Schoot 1988). It has been suggested that the fibrillar pecto-cellulosic protective layer allows much easier solute access to the symplast as compared with lignified walls (Gregory 1978; van Bel & van der Schoot 1988). Moreover, the contact cells themselves seem to be involved in the metabolically controlled release of sucrose into unoccluded vessels (Sauter *et al.* 1973).

According to some authors, tylosis formation or gel/gum depositions might have a role in embolism repair by contributing to osmotic-related mechanisms for refilling of embolised vessels (Canny 1997; Crews *et al.* 2003; Brodersen *et al.* 2010; Cochard *et al.* 2010). However, in this review, we refrain from entering the controversial debate on the role of tyloses and gums in vessel refilling.

OCCURRENCE IN MODERN AND FOSSIL WOODS

It is well known that tyloses form in both ring-porous (*e.g. Castanea*, *Fraxinus*, *Juglans*, *Ulmus*, *Robinia*, and *Quercus*) and diffuse-porous woods (*e.g. Fagus*, *Populus*, and *Salix*). Tyloses in heartwood vessels were reported by Malpighi in 1686. It was only much later that Gerry (1914) described tyloses in sapwood. Klein (1923) showed that tyloses could be produced artificially in sapwood as the consequence of embolism after wounding. In some genera (*e.g. Quercus*, *Robinia,* and *Castanea*), tyloses form naturally in heartwood and sapwood. Genera, such as *Acer* and *Swietenia*, which are not able to form tyloses, form instead other types of vessel plugs, referred to as gum deposits (Chattaway 1949; Bauch *et al.* 1980; Saitoh *et al.* 1993).

A survey on the occurrence of tyloses and secretion of gums in the wood of over 1,100 genera was made by Chattaway (1949). Since this first extensive study, several authors have reported tyloses in many species, grouping information at the genus and family levels. Saitoh *et al.* (1993) studied tyloses in sapwood and heartwood in 50 Japanese hardwoods and found their occurrence in the middle sapwood, near the transitional region from sapwood to heartwood, or in the inner regions of the wood in species lacking heartwood.

Like tyloses, gums may occur in both ring-porous (*e.g. Zelkova, Phellodendron,* and *Maackia*) and diffuse-porous woods (*e.g. Betula*, *Prunus*, and *Tilia*) (Koran & Yang 1972; Schmitt & Liese 1990).

A search of the InsideWood modern wood database (2004-onwards; Wheeler 2011) indicates that "common tyloses" occur in 111 families and sub-families, "sclerotic tyloses" in 27 and "gums or other occlusions" in 106. Table 1 and Table 2 respectively list the families having tyloses and gums commonly occurring; 19 out of 111 families having "common tyloses" also have the other two types of occlusions. "Common tyloses" are present in 17% of world woods, with much higher percentages in India and Australia, and very low values in the Mediterranean basin, temperate Europe and New Zealand. Gums and other deposits in vessels are present in 18% of world woods, with high percentages in the Mediterranean basin, India, Australia and Tropical Africa.

The fossil record shows that the production of these protrusions has been a common process in woody plants since at least the late Paleozoic. The earliest reports of tyloses in fossil plants are from the Carboniferous, and include a progymnosperm *Protopitys buchiana* (Scheckler & Galtier 2003) and several ferns (Williamson 1876; Weiss 1906; Phillips & Galtier 2005). Tyloses or tylosis-like structures have also been described in the Triassic gymnosperm wood, *Protocedroxylon mineense*, in the Permian fossil wood of *Shenoxylon mirabile*, in the Jurassic woods *Metacedroxylon scoticum* Holden and *Xenoxylon morrisonense* Medlyn & Tidwell, and in some Cretaceous and Cenozoic permineralized angiosperm woods (for examples, refer to: Jeffrey 1904; Holden 1915; Bailey 1924; Medlyn & Tidwell 1975; Manchester 1983; Takahashi & Suzuki 2003;

Table 1. List of Families in which common and sclerotic tyloses occur as reported in the Modern Woods in the InsideWood database (August 2015) (InsideWood 2004-onwards; Wheeler 2011).

Tyloses in Modern Woods			
"Normal" tyloses common			Tyloses sclerotic (in very few species only)
Achariaceae	Euphorbiaceae	Myrtaceae	Achariaceae
Adoxaceae	Eupteleaceae	Nothofagaceae	Anacardiaceae
Anacardiaceae	Fagaceae	Nyctaginaceae	Bonnetiaceae
Apocynaceae	Gentianaceae	Ochnaceae	Calophyllaceae
Araliaceae	Hamamelidaceae	Olacaceae	Cannabaceae
Asteropeiaceae	Hernandiaceae	Oleaceae	Celastraceae
Barbeyaceae	Humiriaceae	Onagraceae	Chrysobalanaceae
Begoniaceae	Hydrangeaceae	Oxalidaceae	Connaraceae
Bignoniaceae	Hypericaceae	Pandaceae	Convolvulaceae
Bixaceae	Icacinaceae	Passifloraceae	Dipterocarpaceae
Bonnetiaceae	Irvingiaceae	Paulowniaceae	Euphorbiaceae
Boraginaceae	Ixonanthaceae	Pentaphylacaceae	Hydrangeaceae
Brunelliaceae	Juglandaceae	Peraceae	Icacinaceae
Burseraceae	Kirkiaceae	Phyllanthaceae	Irvingiaceae
Calophyllaceae	Lamiaceae	Picrodendraceae	Ixonanthaceae
Calycanthaceae	Lauraceae	Piperaceae	Lauraceae
Cannabaceae	Lecythidaceae	Platanaceae	Lecythidaceae
Capparaceae	Leguminosae Caesalpinioideae	Rhamnaceae	Leguminosae Papilionoideae
Caryocaraceae	Leguminosae Papilionoideae	Rhizophoraceae	Melastomataceae
Casuarinaceae	Linaceae	Rhoipteleaceae	Monimiaceae
Celastraceae	Loganiaceae	Rosaceae	Moraceae
Centroplacaceae	Lythraceae	Rubiaceae	Myristicaceae
Cercidiphyllaceae	Magnoliaceae	Rutaceae	Olacaceae
Chrysobalanaceae	Malpighiaceae	Salicaceae	Peraceae
Clusiaceae	Malvaceae Bombacoideae	Santalaceae	Rubiaceae
Combretaceae	Malvaceae Brownlowioideae	Sapotaceae	Sapotaceae
Connaraceae	Malvaceae Byttnerioideae	Sarcolaenaceae	Stemonuraceae
Convolvulaceae	Malvaceae Dombeyoideae	Solanaceae	
Cornaceae	Malvaceae Grewioideae	Stemonuraceae	
Cunoniaceae	Malvaceae Helicteroideae	Theaceae	
Daphniphyllaceae	Malvaceae Sterculioideae	Torricelliaceae	
Didiereaceae	Melastomataceae	Ulmaceae	
Dipterocarpaceae	Monimiaceae	Urticaceae	
Elaeagnaceae	Moraceae	Verbenaceae	
Elaeocarpaceae	Moringaceae	Violaceae	
Ericaceae	Myoporaceae	Vitaceae	
Erythroxylaceae	Myristicaceae	Vochysiaceae	

Table 2. List of Families in which gums and other organic occlusions commonly occur as reported in the Modern Woods in the InsideWood database (August 2015) (InsideWood 2004-onwards; Wheeler 2011).

Gums and other deposits common in Modern Woods
Acanthaceae
Achariaceae
Anacardiaceae
Anisophylleaceae
Annonaceae
Aphloiaceae
Apocynaceae
Asteraceae
Betulaceae
Bignoniaceae
Boraginaceae
Burseraceae
Buxaceae
Calophyllaceae
Canellaceae
Cannabaceae
Capparaceae
Cardiopteridaceae
Caryocaraceae
Casuarinaceae
Celastraceae
Chenopodiaceae
Cistaceae
Clusiaceae
Combretaceae
Convolvulaceae
Ctenolophonaceae
Cunoniaceae
Dilleniaceae
Dipterocarpaceae
Ebenaceae
Elaeagnaceae
Elaeocarpaceae
Ericaceae
Escalloniaceae
Eucommiaceae
Euphorbiaceae
Goodeniaceae
Goupiaceae
Himantandraceae
Huaceae
Humiriaceae
Hypericaceae
Icacinaceae
Juglandaceae
Koeberliniaceae
Lamiaceae
Lauraceae
Lecythidaceae
Leguminosae Caesalpinioideae
Leguminosae Mimosoideae
Leguminosae Papilionoideae
Loganiaceae
Lythraceae
Magnoliaceae
Malpighiaceae
Malvaceae Bombacoideae
Malvaceae Brownlowioideae
Malvaceae Byttnerioideae
Malvaceae Dombeyoideae
Malvaceae Grewioideae
Malvaceae Helicteroideae
Malvaceae Malvoideae
Malvaceae Sterculioideae
Melastomataceae
Meliaceae
Moraceae
Myricaceae
Myristicaceae
Myrtaceae
Nitrariaceae
Nothofagaceae
Nyctaginaceae
Ochnaceae
Olacaceae
Peraceae
Phyllanthaceae
Picramniaceae
Picrodendraceae
Polygalaceae
Proteaceae
Putranjavaceae
Quiinaceae
Resedaceae
Rhamnaceae
Rhizophoraceae
Rosaceae
Rubiaceae
Rutaceae
Salicaceae
Salvadoraceae
Santalaceae
Sapindaceae
Sapotaceae
Sarcobataceae
Simaroubaceae
Styracaceae
Surianaceae
Tamaricaceae
Tetrameristaceae
Thymelaeaceae
Ulmaceae
Urticaceae
Verbenaceae
Vochysiaceae
Zygophyllacea

InsideWood 2004-onwards; Feng *et al*. 2010; Boonchai *et al*. 2015). The earliest large angiosperm tree, *Paraphyllanthoxylon*, has abundant tyloses (Bailey 1924; Wheeler & Lehman 2009). Of the 1,800 records of fossil angiosperm woods in InsideWood (2004-onwards; Wheeler 2011), "common tyloses" occur in over 500 records from 64 families and sub-families plus other woods (mostly Cretaceous in age) whose familial affinities are not known. The apparently more common occurrence of tyloses in fossil woods (30 %) is because, unless fossil wood descriptions specifically stated that tyloses were rare, the phrase "tyloses present" was recorded as IAWA feature 56 "tyloses common".

In this review, we have not included tyloses and gums in woody monocots such as palms, but they certainly occur there, especially in metaxylem vessels in leaf traces in the stem prior to leaf shedding (Tomlinson *et al*. 2011; Tomlinson, personal communication 2016).

CONCLUSIONS

Since the first reports on the occurrence of tyloses or gums in vessels, numerous studies have been conducted in which many different causes triggering vessel occlusion have been considered. In many studies, the understanding of either tylosis formation or gum deposition was not the main goal, and different approaches and methods have been used sometimes leading to contrasting opinions. The development of more and more sophisticated methods and instruments have unambiguously demonstrated that vessel occlusion is a consequence of cavitation, thus all causes claimed for vessel occlusion primarily trigger embolism. The questions whether tyloses and gums in vessels always lead to permanent vessel occlusion or whether partially occluded vessels can refill remain to be answered. The formation of tyloses is considered an irreversible occlusion of conduits because, as cellular structures, they would require a very complex process to be degraded. Whether gums can be dissolved and serve as osmoticum probably depends on "their stage of development". Precursory gels (Crews *et al*. 2003) might be dissolved in refilling vessels; for more or less solid gums in discoloured woundwood this is almost impossible to envision, but further experimental studies are needed.

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MORPHO-ANATOMICAL AND PHYSIOLOGICAL TRAITS OF BOUGAINVILLEA GENOTYPES GROWN IN TWO SHAPES UNDER FULL AND DEFICIT IRRIGATION CONDITIONS

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Abstract

Bougainvillea genus includes several species cultivated for gardening and landscaping in the Mediterranean region characterized by limited water supply. The usefulness of deficit irrigation to improve water productivity and plant quality has been recently recognized. as strongly influenced by the severity of water stress and by the genotypes. Bougainvillea plants are characterised by a peculiar stem and branch anatomy where secondary xylem and phloem are produced by successive cambia. It is assumed that the large amount of phloem produced by successive cambia, and the presence of other valuable living tissues in stem and branches, can play an important role in water storage and prime adaptation in conditions of changing water availability or frequent drought. Although the general stem anatomical organization is species-specific, wood quantitative traits, such as the number and size of conducting elements, can be severely affected by both environmental conditions and the shape of growth. A greenhouse experiment was conducted to determine the effects of deficit irrigation on two potted Bougainvillea genotypes [B. x buttiana

‘Rosenka’, B. ‘Lindleyana’ (=B. ‘Aurantiaca’)] grown in two shapes, globe and pyramid, on morphological and anatomical parameters. Irrigation treatments were based on the daily water use (C, 100% or DI, 25%). At the end of the experiment total dry biomass, leaf number, leaf area, decreased in response to an increase in water stress with the lowest values recorded in the DI treatment. Our findings indicated that the two genotypes of *Bougainvillea* have several morphological adaptations to cope with imposed water deficit, including the reduction in leaf number, leaf lamina size and dry weights,. In both the analysed genotypes of *Bouganvillea*, the increase in stomatal resistance induced by water deficit is accompanied by the decrease in stomata size, but not by modifications of stomata density. the analysed *Bouganvillea* cultivars would respond to water deficit by increasing the mean vessel size to achieve quick water transport when water is available, while maintaining safety under deficit irrigation, especially at the xylem ring level thanks to increased vessel frequency and grouping

Introduction

In the last decades there has been an increasing interest in the adoption of water saving strategies in horticulture, due to the competition for water resources with other sectors and water scarcity (FAO, 2002). Nevertheless, the general perception is that agriculture water use is often wasteful and highly inefficient (Hsiao et al*.*, 2007).

Water requirements of food crops are well described in literature, whereas less data are available on the irrigation requirements of ornamental species (Grant et al., 2012). Among the factors affecting the quality of ornamental plants in pre- and post- production (i.e. water, light and nutrient quantity and quality), water availability is a key factor. In many areas of potted ornamentals production, water quantity and quality is often a limiting factor, especially because nursery plants use more water per unit of cultivated land than other agricultural crops (Beeson, 2004). To maximize growth, commercial nurseries generally maintain plant available water close to 100% of container capacity

(Beeson, 2006). On the other hand, to minimize water losses, micro-irrigation systems are generally used to irrigate plants in large containers, whereas, frequently, smaller containers are watered with overhead sprinkler systems. Water application efficiency is affected by plant canopy and spatial configuration in addition to application rate, irrigation volume, sprinkler characteristics, growing media and container size (Beeson and Yeager, 2003). The frequency and quantity of irrigation applied on most commercial nurseries is often based on grower's personal experience and irrigation timers (Beeson, 2004). Whilst plant water use can vary greatly from day to day, through the course of growth season, growers rarely respond to such changes by modifying irrigation. Hence, under- or over-irrigation frequently occurs. The large number of species/cultivars, growth habits, water delivery systems, containers and substrates contribute to the difficulty in estimating the water requirements of ornamentals and may negatively affect the efficient use of irrigation water in the nurseries (Beeson and Brooks 2008).

Furthermore, the shape of ornamental plants plays a primary role in the customer satisfaction, since it contributes to match the nursery supply with an increasing demand of diversified products (Kobayashi et al., 2007). Since plant architecture affects the whole hydraulic behaviour of plants, it is likely that training techniques exert control over growth responses to water availability. In some Mediterranean ornamental shrubs, production deficit irrigation (DI) strategies, by applying water below the full evapo-transpiration (ET) requirements, have been developed to enhance water use efficiency (WUE) and to limit negative effects on yield (Costa et al., 2007). DI may be used in potted ornamental plants to improve plant quality, by reducing excessive vigour and promoting a more compact habit (Cameron et al., 2006, 2008). At present, the degree and duration of the water stress to modulate growth habit and plant shape is a critical point (Álvarez et al., 2009). Numerous experimental trials on ornamental plants demonstrated that plant quality decreases as the severity of DI increases (Hansen and Petersen, 2004; Silber et al., 2007; Bernal et al., 2011).

Among ornamental plants, *Bougainvillea* genus is reported to be tolerant to water stress (Pizarro and Bisigato, 2010), but the responses to the application of different levels of DI appeared to be influenced by both the genotype and the shape the plants were trained to (Cirillo et al., 2014). In the last decade, potted *Bougainvillea* plants have been widely used in urban gardening (ISMEA, 2013). *Bougainvillea* genus includes 18 species of flowering plants belonging to the family Nyctaginaceae, native in South America, originated from west Brazil to Southern Argentina (Kent et al., 2007). Several species of *Bougainvillea* are commonly cultivated in the arid lands for their adaptability to different agroclimatic regions around the world (Saifuddin et al., 2010; Suxia et al., 2009).

Bouganvillea species follow a model of secondary growth, typical of mangrove species, which favours adaptation to dry or saline conditions. They are characterised by a peculiar stem and branch anatomy where secondary xylem and phloem are produced by successive cambia (Robert el al. 2011). It is assumed that the large amount of phloem produced by successive cambia, and the presence of other valuable living tissues in stem and branches, can play an important role in water storage and prime adaptation in conditions of changing water availability or frequent drought (Robert et al. 2014). Although the general stem anatomical organization is species-specific, wood quantitative traits, such as the number and size of conducting elements, can be severely affected by both environmental conditions and the shape of growth. Several models (e.g. from the da Vinci's rule to WBE model) (McCulloh and Sperry, 2005) have been proposed to explain patterns of hydraulic architecture and their implications for the efficiency and safety against embolism of water transport, in relation to various plant types and habitats. The relations between vessel diameter and possible environmental drivers have been explored over the past 50 years leading to the consideration that, plant size being equal, under stressful conditions (such as drought) the selection would favour narrow, slowly-transporting but embolism-resistant vessels (De Micco et al., 2008; Carlquist, 2012). However, Olson et al. (2014), taking into account the principles of the Hydraulic Optimality (HO) models, and using data from 256 species with different habits and from various habitats, have recently demonstrated that the main driver of global variation in mean vessel diameter is plant size which explain the 63% of the variation. In addition to the total stem or branch length, the general architecture of the above-ground organs needs to be considered. Indeed, the structure of the crown, which under cultivation is designed through specific training techniques, is known to affect water transport, carbon assimilation and partitioning because it affects light interception and the distribution of nodes and links, thus hydraulic resistances (Zimmerman 1978; Willaume et al., 2004; Souza et al., 2011). The plasticity in modifying quantitative wood traits is responsible for the adaptive capability of a species under stressful conditions. Although general trends in mean vessel diameter and wood density are by far the most analysed in response to water deficit, it is well recognised that different combination of various anatomical parameters (e.g. vessel size and frequency, vessel grouping), along the trade-off between efficiency and safety of water transport, have to be considered of adaptive value more than single traits (Carlquist, 2012; De Micco et al., 2008).

In this paper, we analysed growth, leaf water potentials, stomata traits, twig anatomy and quantitative wood traits of two *Bouganvillea* genotypes, widely diffused on the market, trained at two growth shapes and cultivated under different water availability. We ultimately aimed to verify whether the different growth behaviour in the various conditions can be ascribed to different plasticity of hydraulic anatomical traits.

Materials and Methods

Plant material and experimental conditions

The experiment was conducted from March to October 2013 in a 265 m^2 glass-zinc-coated steel greenhouse situated at the Experimental Station of the University of Naples Federico II, Southern Italy (43°31'N, 14°58'E; 60 m a.s.l.). Plants were grown under a 50% black shading net. The greenhouse was maintained at daily temperature between 16 and 26 °C, and day/night relative humidity of 50/88%.

Two year old plants of two flowering potted *Bougainvillea*genotypes (G), (*B. x buttiana* 'Rosenka' *andB. 'Lindleyana' (=B. 'Aurantiaca')*; names according to the Royal Horticultural Society, London, UK)were obtained from a commercial grower (Vivai Torsanlorenzo, Ardea, Rome, Italy), and transplanted at the beginning of March, into pots (d 20 cm, h 18 cm) containing 4.5 L of peat moss. The pots were placed on three 180 cm wide and 7 m-long troughs, with a plant density of 6 plants$\cdot$m^{-2}. *Bougainvillea* plants were grown in two canopy shapes (S): globe (GS) and pyramid (PS). The *Bougainvillea* globe shape was obtained by regular pruning based on the new shoot thinning and cut back, whereas the pyramid *Bougainvillea* plants were grown as a vine on a tutor and pruned by trimming exceeding shoots.

The treatments, arranged in a randomized complete block design with three replicates, were defined by a factorial combination of two irrigation levels based on the daily water use (100%, or 25%), two *Bougainvillea* genotypes (*B. x buttiana* 'Rosenka', or *B. 'Lindleyana' (=B. 'Aurantiaca'))*, and two canopy shapes (globe, GS or pyramid, PS). Each experimental unit consisted of 15 plants.

Irrigation treatments. The irrigation treatments (I) consisted of: a) control (CI), in which the substrate moisture was maintained close to container capacity and irrigation was applied when 20% of the applied water was leached; and b) deficit irrigation (DI) in which plants were watered with 25% of the amount of water supplied in the control treatment. The electrical conductivity of the water applied was 0.6 dS$\cdot$m^{-1}. The levels of water recovery (100% in CI, and 25% in DI) were obtained using 4, and 1 emitter /s per plant, respectively (flow rate of 2 L$\cdot$h^{-1}).

Plants were fertigated with a nutrient solution containing the following macro- and micronutrients: 1.45 mM N-NO_3, 2.66 mM N-NH_4, 4.36 mM N-ureic, 1.41 mM P, 4.24 mM K, 5.34 μM Fe, 3.45 μM Mn, 0.84 μM Cu, 0.83 μM Zn, 37 μM B and 2.08 μM Mo.

Water potential measurements and growth analysis. During the whole growing cycle, at 171 days after transplanting (DAT), the stomatal resistance to water vapor (s$\cdot$cm^{-1}) was measured between

11:00–13:00 h on the youngest fully expanded leaf (nine plants per treatment, three per each replication) with a diffusion porometer (AP-4, Delta-T Devices, Cambridge, UK). The water potential components of leaves were measured psychrometrically on the same date of the stomatal resistance measurements, using a dew-point psychrometer (WP4, Decagon Devices, Pullman, Washington, USA). Leaf water potential (Ψw) was measured at midday. The osmotic potential ($\Psi\pi$) was measured on frozen/thawed leaf samples and the pressure potential (Ψp) was estimated as the difference between Ψw and $\Psi\pi$, assuming a matric potential equal to 0.

At the end of the growing cycle (225 DAT), four plants per plot were sampled. Above-ground vegetative parts (leaves and stems) and flowers were dried to constant weight in a forced-air oven at 80 °C for 72 h for biomass determination. Leaf area (LA) was measured with an electronic area meter (Li-Cor 3000, Lincoln, NE, USA). The number of leaves and the number of flowers per plant were also recorded.

Microscopy

Three fully expanded leaves and three one-year-old twigs were harvested on three plants per each irrigation treatment and canopy shape in both cultivars. Samples were immediately fixed in FAA (50 ml 40% formaldehyde, 50 ml glacial acetic acid, 90 ml 50% ethanol) for several days.

Stomata characterisation. To analyse stomata frequency and size, the abaxial epidermis of the leaf lamina was carefully peeled off with a pointed tweezer, in a region of the lamina not including the main vein. The epidermis strip was flattened on a glass slide and mounted with mineral oil for miscoscopy.

Three film strips from each leaf were observed under a transmitted light microscope (BX60, Olympus, Hamburg, Germany). Digital images of the epidermis were collected by means of a camera (CAMEDIA C4040, Olympus), avoiding veins.

Twig anatomy. Cross-sections were cut at the base of collected twigs with a sliding microtome and semi-thin sections (15 μm thick) were divided into two groups. The first group of sections were devoted to the quantification of tissues: more specifically, sections were stained with safranin O and astra blue (Vasquez-Cooz and Meyer, 2002; De Micco and Aronne, 2007), or with a saturated solution of Phloroglucinol in 20 % HCl (Jensen, 1962), to distinguish between lignified and un-lignified cell walls. After being mounted with mineral oil, sections were observed under a dissection microscope (SZX9, Olympus). The second group of sections, not stained, were mounted with mineral oil for fluorescence and observed under an epi-fluorescence microscope (BX60, Olympus, Hamburg, Germany) equipped with a Mercury lamp, band-pass filter 330–385 nm, dichromatic mirror 400 nm and above, and barrier filter 420 nm and above. Such settings allow detecting the autofluorescence of lignin (Fukuzawa 1992, Ruzin 1999), also maximising the contrast for subsequent digital image analysis and easing the quantification of xylem properties. Digital images of stained and unstained sections were taken as reported above. Longitudinal section were also cut and analysed through fluorescence microscopy to verify the presence of tracheids.

Digital Image Analysis

All digital images were analysed with the AnalySIS 3.2 (Olympus) software.

Stomata were counted and their frequency (number of objects per surface unit (mm^2) was calculated. The length (guard-cell length, pole to pole) and width (guard-cell width in the middle position) of 10 stomata per image were measured.

The cross-sectional area of twigs (twig cross section - TCS) as well as the area occupied by each tissue was quantified. More specifically the following regions were considered: suberized epidermal and subepidermal layers (SL), cortical parenchyma (CP), xylem ring (XR), pith (Pi), phloem of the vascular bundles in pith (P), xylem of the vascular bundles in pith (X) (Fig. 1). The number of vascular bundles in the pith was also counted.

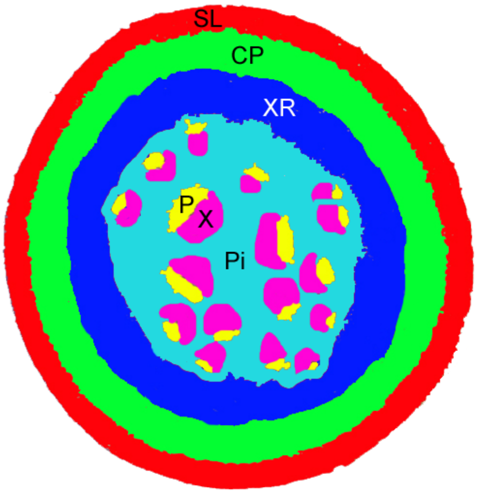

Fig. 1. Scheme of a cross section of a twig showing the different regions analysed: suberised epidermal and subepidermal layers (SL), cortical parenchyma (CP), xylem ring (XR), pith (Pi), phloem of the vascular bundles in pith (P), xylem of the vascular bundles in pith (X).

The following anatomical features were quantified in the region of the xylem ring:

- Vessel frequency: measured as the number of vessels occurring in a given area. This measurement was determined by counting all vessels individually and was repeated in two selected regions per image.
- Percentage of solitary vessels: calculated as the proportion of solitary vessels in relation to the total number of vessels in two selected regions per image.
- Vessel lumen area: measured at least in 20 vessels per twig.

The following anatomical features were quantified in the vascular bundles in the pith:

- Number of vessels per bundle: counted in at least five bundles per twig.
- Vessel lumen area: measured at least in 20 vessels per twig.

Frequency distribution of the vessels in classes of lumen area was calculated in both xylem ring and vascular bundles in pith, to hypothesize how different distributions of lumen areas are related to hydraulic properties.

Statistical analysis. All data were statistically analyzed by three-way analysis of variance (ANOVA) using the SPSS software package (SPSS 13.0 for Windows). Whenever two-way interaction was significant, a one-way ANOVA was performed. Duncan's multiple-range test was performed at p<0.05 on each of the significant variables measured. Shapiro-Wilk and Kolmogorov-Smirnov Tests were performed to check for normality. Percent data were transformed through arcsine function before statistical analysis.

Results

Plant growth, stomatal resistance and water relations.

At the end of the growing cycle the leaf, stem, flower and total dry biomass were significantly affected by genotype (G) x irrigation treatment (I) interaction (Table 1). In detail, irrespective of the shape, the total and stem dry biomass of *B. aurantiaca* (Ba) under water stress (DI) resulted significantly lower than other treatments; the leaf dry biomass was highest in the Ba control (CI) and lowest in the Ba DI plants. The flower biomass was reduced significantly in *B. x buttiana* 'Rosenka' (BxbR), whereas it was not affected in the Ba by the water stress application. The stem dry biomass was significantly reduced in the globe (-46.2%) compared to the pyramid (Table 1).

The leaf, flower and total dry biomass were significantly affected by the G x Shape (S) interaction (Table 1). Indeed, irrespective of the water stress treatment, a reduction of the total dry biomass was recorded in the BxbR grown at globe (G) and Ba both at globe and pyramid (P) compared to the BxbR grown at pyramid shape. On the contrary, the highest leaf biomass was recorded in the Ba G and the lowest in both the BxbR G and Ba P, whereas the BxbR grown at pyramid showed an intermediate value. In both the genotypes the stem biomass was significantly lower in the globe shape than in the pyramid. A different pattern was observed for the flower biomass, where the highest values were recorded for both the shapes of BxbR, whereas in Ba the flower biomass increased in the globe shape compared to the pyramid.

The total number of leaves and the total leaf area per plant were also affected by the G x I interaction (Table 1). Indeed, when averaged overall the shape, the lowest values of number of leaves and leaf area were recorded in the Ba under water stress. The number of leaves was also significantly affected by the shape, with a reduction by 13.3% in the globe shape compared to the pyramid (Table 1). Irrespective of the irrigation level (G x S interaction), the total leaf area per plant showed the highest level in the BxbR at pyramid shape and the lowest in pyramid of Ba.

Table 1 Effects of genotypes (G), irrigation (I), and shapes (S) on dry biomass production and partitioning, and number of leaves and total leaf area of potted *Bougainvillea* plants at the end of the growing cycle.

	Leaf DW (g/plant)	Stem DW (g/plant)	Flower DW (g/plant)	Aerial DW (g/plant)	Leaves (n/plant)	Leaf area cm^2/plant)
G	NS	***	***	***	***	***
I	***	***	NS	***	***	***
S	NS	***	NS	***	*	NS
G x I	***	**	***	*	**	*
G x S	***	NS	***	***	NS	**
I x S	NS	NS	NS	NS	NS	NS
G x I x S	NS	NS	NS	NS	NS	NS

Significance: NS, non-significant; * P ≤ 0.05; **P ≤ 0.01; *** P ≤ 0.001.

The stomatal resistance was significantly increased by 153% under water stress (Table 2). On the contrary, ψw and ψπ decreased significantly under water stress (69.7% and 54%, respectively), but a significant effect of G x S interaction was also observed (Table 2). Indeed, irrespective of the irrigation level (G x S interaction) the lowest level of ψw and ψπ were found for Ba grown at globe shape.

Table 2 Effects of genotypes (G), irrigation (I), and shapes (S) on stomatal resistance (r_S), leaf water potential (ψ_w), leaf osmotic potential (ψ_π), and leaf turgor potential (ψ_P), of potted *Bougainvillea* plants at the end of the growing cycle.

	r_S (s cm^{-1})	ψ_w (MPa)	ψ_π(MPa)	ψ_P (MPa)
G	NS	NS	NS	NS
I	***	***	***	NS
S	NS	NS	NS	NS
G x I	NS	NS	NS	NS
G x S	NS	*	*	NS
I x S	NS	NS	NS	NS
G x I x S	NS	NS	NS	NS

Significance: NS, non-significant; * $P \leq 0.05$; **$P \leq 0.01$; *** $P \leq 0.001$.

Table 3 shows the main effect of the single factors (–genotype – G, irrigation treatment – I, and canopy shape - S) and of their interactions on stomata properties. As far as stomata frequency is concerned, the interaction between the three factors have not a significant effect. The G x S interaction influenced stomata frequency with the highest significance level if compared with other interactions involving the deficit irrigation treatment. Indeed, this parameter was significantly higher in BxbR than in Ba, as well as it was increased in plants grown in a globe shape in comparison with the pyramid shape independently on the irrigation treatment.

The length of guard cells of stomata was significantly affected by genotype (G) x irrigation treatment (I) x canopy shape (S) interaction, while their width was affected by the genotype (G) x canopy shape (S) interaction (Table 3). Ba showed stomata whose cells have longer and narrower guard cells than BxbR; smaller stomata (reduced length and width of guard cells) were found in water-stressed plants than in controls in both species and growth forms (Table 3).

Table 3. Effects of genotypes (G), irrigation (I), and shapes (S) of *Bougainvillea* potted plants (interaction values are mean of three replicates) on stomata features.

	Stomata frequency ($n\ mm^{-2}$)	length (μm)	width (μm)
G	***	***	***
I	NS	***	***
S	***	NS	***
G x I	**	NS	NS
G x S	***	*	***
I x S	*	**	NS
G x I x S	NS	**	NS

Significance: NS, not significant; * $P \leq 0.05$; **$P \leq 0.01$; *** $P \leq 0.001$.

Light and fluorescence microscopy observations of the cross sections of one-year-old twigs of both species (Fig. 2), showed that they are characterised by numerous vascular bundles, part of which are embedded in the pith, the others being enclosed in a complete ring of thick-walled lignified cells.

Phloroglucinol staining and UV-microscopy showed that pith cells are characterised by thin-walled lignified cells. The xylem ring (XR) is surrounded by a cortical parenchyma made of many layers of cells, a multilayered sub-epidermal phellem of suberised cells and an epidermis covered by a cuticle.

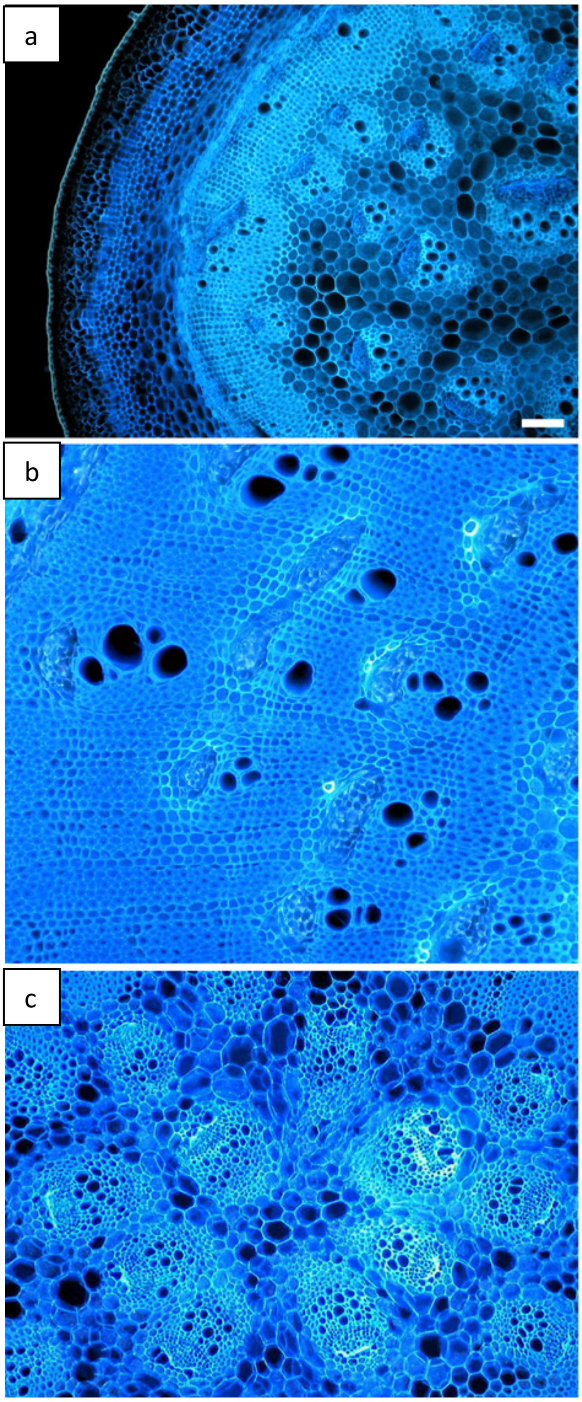

Fig. 2. Fluorescence microscopy views of stem cross sections of *B. x buttiana* 'Rosenka' (BxbR) and *B. 'Lindleyana' (=B. 'Aurantiaca')* (Ba), showing the overall distribution of tissues (a) and details of xylem ring (b) and of vascular bundles in pith (c). Bars = 50 µm.

The basal area of twigs was significantly affected by the genotype (G) x canopy shape (S) interaction (Table 4): the cross section area of twigs of BxbR trained in pyramid shape was 19.42 mm^2, a value significantly higher than twigs of the same species trained in globe (7.50 mm^2) and of Ba grown in both pyramid (7.15 mm^2) and globe (5.72 mm^2). The main effects of species and shape were significant: in general, twig basal area was higher in BxbR than Ba, and it was increased by pyramid training.

The same trends of variations were found in all analysed tissues: each tissue was present in similar percentage over the total twig basal area independently of species, irrigation regime and growth shape (Table 4).

Table 4. Effects of genotypes (G), irrigation (I), and shapes (S) of *Bougainvillea* potted plants (interaction values are mean of three replicates) on the area of twig cross section as well as the area occupied by each tissue. The incidence of each tissue (as percentage) over the total twig cross sectional area are reported in parenthesis.

	TCS (10^6 µm^2)	SL (10^6 µm^2)	CP (10^6 µm^2)	XR (10^6 µm^2)	Pi (10^6 µm^2)	X (10^6 µm^2)	P (10^6 µm^2)
G	***	***	***	***	***	***	*
I	NS	NS	NS	NS	NS	NS	NS

S	***	***	***	***	***	***	*
G x I	NS	NS	NS	NS	NS	NS	NS
G x S	***	***	***	*	***	***	*
I x S	NS	NS	NS	NS	NS	NS	NS
G x I x S	NS	NS	NS	NS	NS	NS	NS

Significance: NS, not significant; * $P \leq 0.05$; **$P \leq 0.01$; *** $P \leq 0.001$.

TC, twig cross-section; SL, suberized epidermal and subepidermal layers; CP, cortical parenchyma ; XR, xylem ring; Pi, pith; P, phloem of the vascular bundles in pith; X, xylem of the vascular bundles in pith.

As regards the water transport system, the number of vascular bundles in the pith was affected by the sole canopy shape: pyramid training determined the formation of a significantly higher number of bundles per twig (Table 5). The number of vessels per vascular bundle in the pith was significantly affected by the genotype (G) x irrigation level (I) x canopy shape (S) interaction, with maximum values found in BxbR plants grown in the pyramid shape with control irrigation treatment. However, the pyramid training induced the formation of narrower vessels in both genotypes (with significant differences in BxbR), in comparison with the globe shape, independently from the irrigation treatment. As far as the xylem ring is considered, vessel frequency was affected by the irrigation regime (I) x canopy shape (S) interaction (Table 5): vessel frequency of twigs of plants trained in pyramid shape without water deficit was 80.69 n/mm^2, a value significantly higher than not-water-stressed twigs of plants trained in globe (60.53 n/mm^2), but not significantly different by twigs of water-stressed plants in both pyramid (75.60 n/mm^2) and globe (74.43 n/mm^2) shapes. As regards the main effects, higher vessel frequency was found in Ba than BxbR, and was triggered by the pyramid shape. Vessel grouping and vessel lumen area were significantly affected by the genotype (G) x canopy shape (S) interaction and were increased by water stress as main effect.

Table 5. Effects of genotypes (G), irrigation (I), and shapes (S) of *Bougainvillea* potted plants (interaction values are mean of three replicates) on xylem parameters of twigs.

	Bundles per twig (n/twig)	Vessels per bundle (n/bundle)	Lumen area of VB (μm^2)	Vessel frequency in XR (n/mm^2)	Vessel Grouping (%)	Lumen area of VXR (μm^2)
G	NS	*	*	**	NS	NS
I	NS	NS	***	NS	*	***
S	*	NS	***	*	NS	NS
G x I	NS	*	*	NS	NS	NS
G x S	NS	*	**	NS	**	***
I x S	NS	NS	***	*	NS	NS
G x I x S	NS	**	NS	NS	NS	NS

Different letters within a column indicate significant differences according to Duncan's multiple range test ($P \leq 0.05$). Significance: NS, not significant; * $P \leq 0.05$; **$P \leq 0.01$; *** $P \leq 0.001$.

VB, vessels in bundles; XR, xylem ring; VXR, vessels in the xylem ring

The distribution of vessels in classes of lumen size in both vascular bundles of pith and xylem ring was similar in the two genotypes with most of the water flow relying on vessels with lumen up to 600 and 800 μm^2 in the two regions of the twig respectively. Interestingly, a safer water transport system was evidenced in pith than in xylem ring. Indeed, in pith, in classes up to 300 μm^2, the frequency of vessels was higher in control than in water-stressed plants, while in classes of larger vessel lumen area, water-stressed plants showed higher values than controls. The opposite trend was found in the xylem ring: in classes up to 300 μm^2, the frequency of vessels was higher in water-stressed than in control plants, while in classes of larger vessel lumen area, control plants showed higher values than water-stressed. In both twig regions, the pyramid shape increased the frequency of vessels in the classes of narrow lumen if compared with plants trained in globe shape.

Discussion

Results from the combined analysis of the growth behaviour and hydraulic anatomical traits of the two *Bouganvillea* cultivars, trained to different plant shape and subjected to different irrigation regimes, fully supports the idea that structural properties play a major role in determining the adaptive capability of plants, thus their productivity and survival.

As far as water transport is concerned, apart from the molecular-biochemical-physiological regulation as every metabolic process, the anatomical properties of the vascular pathway across the plant need to be considered because they exert control over water flow rate, gas exchanges, hence over photosynthetic efficiency and biomass accumulation in both optimal and stressful watering conditions (Brodribb, 2009). Our findings indicate that the two cultivars of *Bougainvillea* have several morphological adaptations to cope with imposed water deficit, including the reduction in plant size, leaf number, leaf lamina size and dry weights, which are in agreement with previous studies on several genotypes of *Bougainvillea* spp. (Cirillo et al., 2014), ornamental herbs (Zollinger et al., 2006), *Callistemon* spp. (Álvarez et al., 2011; Álvarez and Sánchez-Blanco, 2013), and other Mediterranean shrubs (Toscano et al., 2014). Indeed, the minimization of the evaporative surface area is a stress-avoidance mechanism which can be achieved through various strategies as reported in ornamental plants cultivated under limiting conditions of water availability as well as in wild species growing in drought-prone environments (Álvarez et al., 2011; De Herralde et al., 1998; De Micco and Aronne, 2012a; Toscano et al., 2014). In our study, the imposed deficit irrigation level severely decreased shoot dry matter (leaves and stem biomass), likely significantly decreasing water losses (Álvarez et al., 2011; Sánchez-Blanco et al., 2009; Toscano et al., 2014), independently from canopy shape. Morphological responses to water stress varied between canopy shapes and species. In *B. x buttiana* 'Rosenkagrown under water deficit conditions, only the flower biomass was reduced, whereas in *B. aurantiaca* there were reductions in leaf number and area, and shoot dry weight. The two different shapes adopted in the experiment affected differentially the stem biomass, the leaf number and the total leaf area per plant, with lower values of these parameters in the plants grown at globe shape compared to pyramid. Indeed, the compactness of globe shaped plants is mostly due to the frequent shoot cut back applied during the cultivation cycle.

Water stress influences the physiological performances of plant, primarily through stomatal closure, that acts as avoidance mechanism reducing water losses but also inducing osmotic adjustments (Davies et al., 2002). In the analysed *Bouganvillea* cultivars, irrigation deficit of 25% induced substantial increases in stomatal resistance. This is an adaptive mechanism to control the transpiration in several plants, included *Bougainvillea* spp. (Álvarez and Sánchez-Blanco, 2013; Cirillo et al., 2014; Niu et al., 2008). This increase indicates changes in the plant water status: therefore water deficit affected the transpiration rate following the increase in stomatal resistance which, at the same time, may affect plant growth by reducing the photosynthetic rates. Similar responses were described in shrubs under drought conditions (Franco et al., 2006; Scheiber et al., 2007; Álvarez et al., 2011; Álvarez and Sánchez-Blanco, 2013). In both the analysed cultivars of

Bouganvillea, the increase in stomatal resistance induced by water deficit is accompanied by the decrease in stomata size, but not by modifications of stomata density. Smaller stomata are known to have faster dynamic characteristics than larger ones, thus allowing a better control of water losses and regulation of water use efficiency under conditions of reduced water availability (Drake et al. 2013). Quick stomata control in *Bouganvillea* plants growing under water deficit would also reduce the risk of xylem embolism since prompt stomata closure, when the evaporative demand cannot be satisfied by soil water content, is a strategy to avoid xylem hydraulic failure (Meinzer et al. 2009).

In most plants undergoing stress conditions, stomata closure is consequent to water potential adjustment (Ludlow, 1980). Both analysed *Bouganvillea* cultivars showed lowering of leaf water potential and leaf osmotic potential under water deficit, confirming the correlation between leaf water potential and stomatal resistance. Leaf water potential has been used in several recent studies to measure plant responses to drought conditions and water deficiency in ornamental shrubs (Álvarez et al., 2011; Niu et al., 2008; Sánchez-Blanco et al., 2009 Toscano et al., 2014). The reduction in ψ_l values following drought conditions found in this study is consistent with previous studies on *Pittosporum* and *Viburnum* (Toscano et al., 2014), roses (Niu et al., 2008), *Callistemon* (Álvarez et al., 2011) and *Bougainvillea* (Cirillo et al., 2014). It was suggested that drought conditions may cause a decrease in leaf water potential due to reduction in stomatal conductance (Chaves et al., 2002) and that the stomatal closure may occur when there is a decline in the water potential from −0.7 to −1.2 MPa (Sánchez-Blanco et al., 2009). In this study, the increased stomatal resistance was associated with the reduction in leaf number and area as typical of plants undergoing drought stress (Medrano et al., 2002).

Among permanent changes due to water stress other than biomass allocation, also differences in stem diameter have been observed in several shrubs and trees (Ugolini et al., 2014; Corcuera et al., 2004). In our experiment, basal stem diameter was not affected by irrigation regime but was dependent on the cultivar and canopy shape. Although absolute values of stem size may change according to different factors, it seems that allocation among tissues (tissue organisation and percent incidence over the total stem surface) is a quite well conserved feature and proportion among different tissues is maintained independently from the genotype, irrigation regime and canopy shape. This suggests that, while the peculiar stem anatomy of *Bouganvillea* spp. guarantees large volume devoted to living water-storage tissues (i.e. cortical parenchyma, phloem and pith) (Robert et al. 2014) and suberized water-saving tissues (epidermal and sub-epidermal cell layers) (De Micco and Aronne, 2012b), any morpho-anatomical adjustments to cope with changing factors rely not on the increase of such compartments, but on the modification of quantitative wood traits. Actually, xylem features are regulated not only by gene expression and signalling biological molecules, as all processes from cell division in meristems towards cell differentiation (Schuetz et al. 2012), but also by physical drivers (e.g. turgor pressure and cell wall constraints) during cell differentiation (Braidwood et al. 2013). It is generally reported that water stress induces the formation of narrower vessels as consequence of reduced turgor-driven enlargement and as a mechanism to reduce the risk of cavitation (Hacke et al. 2006). However, many species adapted to cope with frequent fluctuations of water availability, such as the Mediterranean shrubs, have also evolved a strategy of hydraulic transport based on the harmonisation of xylem traits to favour water transport efficiency under favourable conditions while maintaining safety against embolism during drought episodes (De Micco et al. 2008). Efficient water transport mainly rely on vessel size; however, in the studies aiming to analyse the behaviour of plants to cope with water scarcity, mean vessel size is not the most appropriate parameter, but it needs to be accompanied by information about the overall distribution of all vessels in classes of size. Indeed the occurrence of even a few large vessels can guarantee quick water transport when water is available, but the presence of a ground tissue of narrow vessels can be fundamental to allow water transport when large vessels are embolized due to water stress (De Micco and Aronne, 2009). Within this context, the analysed *Bouganvillea* cultivars would respond to water deficit by increasing the mean vessel size to achieve quick water transport when water is available, while maintaining safety under deficit irrigation, especially at the xylem ring level thanks to increased vessel frequency and grouping. High vessel frequency and vessel grouping are features promoting resistance to water stress: the first is a sort of redundancy to increase the probability that a sufficient part of vessels would remain active in water transport under embolism-inducing conditions; vessel grouping is a strategy to have all sectors of wood active in water transport because it is unlikely that all vessels in a group would embolize at the same time (Carlquist, 1989). Moreover, vessel distribution in classes of lumen size also suggests that vessels belonging to narrower classes of lumen size are higher in water-stressed *Bouganvillea* plants than in controls in the case of xylem ring, while the opposite is true in pith as if vessels in the xylem ring would be mainly pushed to maintain safety while those in pith vascular bundles would be primed to increase size to favour efficiency. Vessels in pith are able to afford this increase in vessel size without increase the risk of embolism because they reach maximum values of lumen size almost halved than xylem ring vessels. Independently from the irrigation regime, *Bouganvillea* plants trained at pyramid shape are characterised by safer hydraulic system because of the occurrence of narrower vessels (especially at the pith level) and higher redundancy than globe plants. The occurrence of narrower vessels in pyramid- than globe-trained plants is apparently contrasting with the longer main stem typical of pyramid, which should support wider vessels compared to globe canopies, according to the hydraulic optimality models (Olson et al. 2014). However, it fits well with the small need for vessel widening towards the base of the plant to overcome drops in conductivity thanks to the occurrence of few branch junctions which are common sites of decreased diameter of conduits (Tyree and Alexander, 1993; Olson et al, 2014).

The overall analysis suggested that plants of both *Bouganvillea* cultivars harmonise growth behaviour and anatomical hydraulic properties depending on the canopy shape training and irrigation regime. Also under low water regime, their vascular system is designed to promptly benefit from the irrigation episodes by quickly absorbing water through wide vessels, still maintaining wood traits promoting safety. The occurrence of a safer hydraulic system in the pyramid-trained plants suggests a better control of efficiency and safety of water transport, thus supporting better growth performance also under conditions of deficit irrigation if compared with globe-trained plants. This study highlights the importance of knowing structural properties of the hydraulic pathway to comprehend growth behaviour and adaptive capability of ornamental shrubs in order to regulate cultivation factors.

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