



# Rapid springtime leaf osmotic adjustment, but low late-seasonal and interannual variation in leaf turgor loss points in three temperate tree species

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Received: 24 September 2024 / Accepted: 6 November 2024 / Published online: 18 March 2025  
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**Abstract** Leaf turgor loss point has been recognized as an important plant physiological trait explaining a species' drought tolerance ( $\pi_{tlp}$ ). Less is known about the variation of  $\pi_{tlp}$  in time and how seasonal or interannual differences in water availability are affecting  $\pi_{tlp}$  as a static trait. I monitored the seasonal variation of  $\pi_{tlp}$  during a drought year starting in early spring with juvenile leaves and assessed the interannual variation in  $\pi_{tlp}$  of fully matured leaves among years with diverting water availability for three temperate broad-leaved tree species. The largest seasonal changes in  $\pi_{tlp}$  occurred during leaf unfolding until leaves were fully developed and matured. After leaves matured, no significant changes occurred for the rest of the vegetation period. Interannual variation that could be related to water availability was only present in one of the three tree species. The results suggest that the investigated species have a rapid period of osmotic adjustment early in the growing season followed by a period of relative stability, when  $\pi_{tlp}$  can be considered as a static trait.

**Keywords** Drought · Leaf drought tolerance traits · Tree mortality · Turgor loss point

## Introduction

In the last two decades, numberless studies provided supporting evidence that the leaf turgor loss point ( $\pi_{tlp}$ ) describes a plant's response to drought (e.g. Brodrribb et al. 2003; Baltzer et al. 2008; Bartlett et al. 2014). From a physiological perspective,  $\pi_{tlp}$  refers to the leaf water potential at which a leaf loses its turgidity and wilts irreversibly (Eisley and Wolfe 2024). Hence,  $\pi_{tlp}$  could be linked to a variety of other physiological important variables describing plant responses to water limitation across species such as the leaf water potential when 50% of the hydraulic and/or stomatal conductance is lost (Auge et al. 1998; Brodrribb et al. 2003; Bucci et al. 2004). Not surprisingly,  $\pi_{tlp}$  partly predicts, not only species habitat preference along local topographic and regional rainfall gradients (e.g. Maréchaux et al. 2015; McFadden et al. 2019; Medeiros et al. 2019), but also species distribution across biomes (Bartlett et al. 2012, 2016; Zhu et al. 2018; Vargas et al. 2022).  $\pi_{tlp}$  has further been found to explain growth reduction in response to drought (McGregor et al. 2021; Kunert et al. 2024) and to even foresee plant survival during and after intensive drought stress (Sun et al. 2020; Álvarez-Cansino et al. 2022; Petek-Petrik et al. 2023). Approaches to explain this predictive power of  $\pi_{tlp}$  refer to its physiological function in plants.  $\pi_{tlp}$  describes the degree of water limitation at which the plant can sustain turgor pressure in its cells (Zhu et al. 2018). Along with a progressing water scarcity, turgor pressure declines and stomata are closing. This induces, in turn, a reduced stomatal conductance and thus gas exchange. Accordingly, plant species that are characterized by a 'more' negative  $\pi_{tlp}$ , can take up CO<sub>2</sub> under drier conditions and maintain growth with increasing water scarcity compared with species characterized by a 'less' negative  $\pi_{tlp}$ .  $\pi_{tlp}$  has thus received much attention in assisting species selection for climate-change-resistant urban

The online version is available at <https://link.springer.com/>.

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forest ecosystems, forest production systems and agroforest systems (Sjöman et al. 2018; Kunert 2020; Kunert and Brändle 2022).

Despite the aggravating evidence of  $\pi_{\text{tlp}}$  being a key trait describing drought tolerance, the assumption of  $\pi_{\text{tlp}}$  to be a stable trait throughout time has received some criticism due to possible osmotic adjustment of leaves to altering seasonal growth conditions or in response to drought (e.g. Sjöman et al. 2018; Sueltenfuss et al. 2020; Hesse et al. 2023). In theory, plants can adjust the cytoplasmic solute concentrations of fully developed leaves under drought stress or to flush new leaves with a higher cytoplasmic solute concentration (Meyer and Boyer 1972; Bartlett et al. 2014). The higher cytoplasmic solute concentration enables the plant to maintain turgidity in the leaves, which is necessary to support the pressure gradient for the transpiration stream between the soil, the plant and the atmosphere (Eisley and Wolfe 2024). Therefore, the osmotic adjustment by the active accumulation of solutes has been described to play a crucial role in maintaining turgor pressure and thus stomatal opening and gas exchange (Hsiao et al. 1976). Active osmotic adjustment in response to changing water availability means, in turn, that the  $\pi_{\text{tlp}}$  values measured under certain environmental conditions might vary considerably and might not represent an intersessional and interannual static trait. This would limit the comparability of  $\pi_{\text{tlp}}$  measurements conducted at different times.

Experimental evidence of any seasonal effects of osmotic adjustment on  $\pi_{\text{tlp}}$  and interannual assessments are still rare in the literature limiting our understanding of seasonal patterns of phenological drought tolerance (Grossman 2023). The existing seasonal comparisons suggest a decline in  $\pi_{\text{tlp}}$  to “increase the drought tolerance by increasing the water potential gradient from the soil to the leaf” (Teskey et al. 1984). Such a decline in  $\pi_{\text{tlp}}$  has been found in trees growing in an urban environment. Those trees significantly increased their leaf drought tolerance through osmotic adjustment leading to a more than 50% higher tolerance in the late vegetation period compared to the early vegetation period (Sjöman et al. 2018; Hiron et al. 2020). Grossman et al. (2024) compared the change in  $\pi_{\text{tlp}}$  of 27 woody species from early to late Northern Hemispheric summer and found a decrease of  $\pi_{\text{tlp}}$  between 12 and 72% in 20 species, but in seven species no change could be detected. The same study describes an acclimatory decline in  $\pi_{\text{tlp}}$  when water was withheld artificially in potted *Acer palmatum* saplings. In turn, trees were found to downregulate their drought tolerance after being released from an artificial drought stress treatment (Hesse et al. 2023). Whereas no short-term osmotic adjustment in response to the rewetting was present in the study by Hesse et al. (2023), trees downregulated  $\pi_{\text{tlp}}$  by 0.70 MPa aligned with the values found for the control trees within several weeks. Similarly, Bartlett et al. (2014) revealed in a global

meta-analysis of almost 300 different plant species, that  $\pi_{\text{tlp}}$  can drop on average 0.44 MPa in response to water limitation. Much lower responses to seasonal water limitation were shown recently by Eisley and Wolfe (2024). Besides a less negative  $\pi_{\text{tlp}}$  of trees in a well-watered floodplain habitat than a drier upland habitat, they described a seasonal osmotic adjustment of 0.08 MPa only to be present in the water-limited habitat. Reviewing the existing literature, there might be a high plasticity of  $\pi_{\text{tlp}}$  in time and in response to water limitations that could potentially justify the criticism of  $\pi_{\text{tlp}}$  as a static trait (compare Sueltenfuss et al. 2020; Sorek et al. 2022; Grossman 2023).

However, most of the mentioned studies present only a few point measurements of  $\pi_{\text{tlp}}$  in time e.g. early and late growing season. In this study, I investigated how static  $\pi_{\text{tlp}}$  is over time by assessing (1) the seasonal variation of  $\pi_{\text{tlp}}$  during a drought year starting in early spring with juvenile leaves, and (2) the interannual variation in  $\pi_{\text{tlp}}$  of fully developed leaves among years with divergent water availability. Therefore, I monitored the  $\pi_{\text{tlp}}$  of three temperate tree species throughout an entire vegetation period starting with leaf unfolding until the first discoloration of the leaves in fall. Further, I compared  $\pi_{\text{tlp}}$  measurements on the same trees from an extremely dry year, a moderately wet year, and an extremely wet year.

## Materials and methods

### Study site and botanical material

The botanical material for this study was collected in an approximately 20-ha large forest patch in the rural district of Fürth in Middle Franconia, Germany (49° 24' 9.9" N, 10° 57' 11.09" E). A variety of autochthonous tree species grow in the forest patch with European beech (*Fagus sylvatica* L.), pedunculate oak (*Quercus robur* L.) and European hornbeam (*Carpinus betulus* L.) being the most abundant broadleaved tree species. The first two species are the most important broad-leaved species in Germany, covering 28% of the overall forested areas (BMEL 2024). The forest patch was also enriched with Scots pine (*Pinus sylvestris* L.) for economic reasons and silviculturally promoted over the last century leading to a dominance of Scots pine especially in the western part of the forest patch. For this study, I focused on the above mentioned three broadleaved tree species. I chose four mature tree individuals per species for the seasonal monitoring of  $\pi_{\text{tlp}}$ . Individuals were all located around six permanent tree stands of local hunters. Tree stands were evenly distributed throughout the forest patch. All trees had a diameter of breast height between 18 and 35 cm. From those tree stands, branches were collected with a 10 m long pruner allowing to collect branches from the upper canopy. I sampled only branches that

were directly exposed to the sun. I wrapped moist tissue paper around the cuts of the branches to keep them hydrated and placed the branches in opaque plastic bags. All samples were taken to the laboratory to be processed for the turgor loss point measurements immediately after sampling. Sampling started in spring when the leaves started unfolding (European hornbeam on the 8th of April 2020, DOY 99; European beech and pedunculate oak on the 17th of April 2020, DOY 108) and ended as soon as I noticed a first discoloration of the leaves (26th of September 2020). During the entire sampling period, all leaves were visually assessed for their development stage and maturity. The leaves were defined as mature when they were fully expanded and when they reached their final stage of coloration (dark green). I repeated the sampling and the measurements in intervals between seven and thirteen days. Each time, one branch was sampled per tree. In 2023 and 2024 (25th of June 2023, DOY 177; and 1st of July 2024, DOY 183), I revisited the same trees to sample branches again. However, not all tree individuals were still alive, thus sample size was reduced to three tree individuals. For comparison between years, I took the measurements in 2020 around the same date as the measurements in 2023 and 2024, hence I used the data sampled on the 26th of June 2020 (DOY 178).

### Turgor loss point measurements

I followed the protocol for the rapid estimation of the leaf turgor loss point ( $\pi_{\text{tlp}}$ ) established by Bartlett et al. (2012). The method allows to convert the solute concentration value  $c_0$  (in  $\text{mmol kg}^{-1}$ ) of a fully rehydrated leaf sample to  $\pi_{\text{tlp}}$ .  $c_0$  was measured with a vapor pressure osmometer (VAPRO 5520, Wescor, Logan, UT, USA) and  $\pi_{\text{tlp}}$  calculated as:

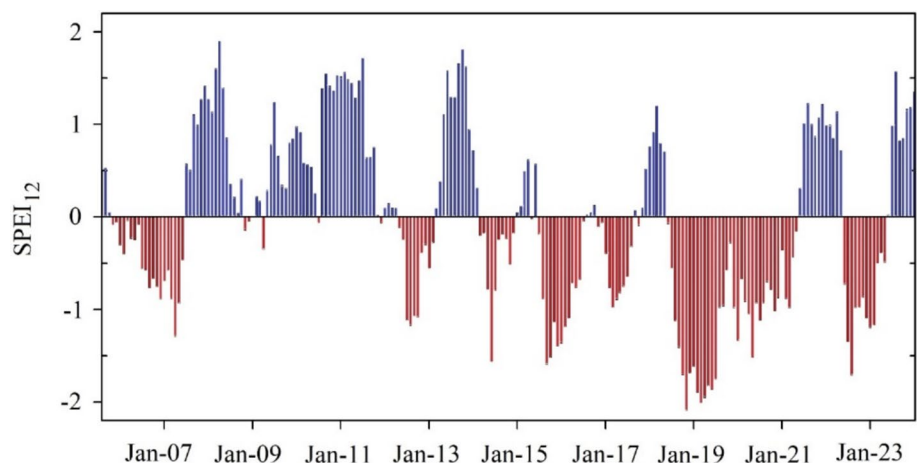
$$\pi_{\text{tlp}} = \frac{0.832(R \times T)}{1000} \times c_0^{-0.631} \quad (1)$$

where  $R$  represents the ideal gas constant, and  $T$ , the temperature in Kelvin. The rehydrated leaf samples for the measurements were prepared as follows, first all branches were recut under water to remove any embolism. They were then placed into buckets with water and covered with opaque plastic bags for rehydration. I collected two leaves per branch and individual. I used a 4-mm cork borer to punch out a small leaf disc from each leaf. The leaf disc was packed in aluminum foil and shock-frozen in liquid nitrogen ( $\text{LN}_2$ ). Afterwards the leaf disc was mechanically perforated with a dissection needle. The disc was then placed in the standard chamber well of the osmometer. The osmometer was configured to run in auto-repeat mode. I assumed equilibrium when  $\Delta c_0$  reached  $5 \text{ mmol kg}^{-1}$  or smaller.

### Data and statistical analysis

I defined the three different sampling years as wet, intermediate and drought year after the prevailing rainfall conditions and using the Standardized Precipitation Evapotranspiration Index (SPEI) as reference (Fig. 1). In the months used for the interannual comparison, SPEI was 1.53, 0.03 and  $-0.94$  in the year 2024, 2023 and 2020, respectively. SPEI was compiled with the 'spei' package (Vicente-Serrano et al. 2010). Besides using the compiled SPEI for the area as a definition, 2020 is the last year of a multiple year drought beginning in the spring 2018 with no significant rain until fall in 2020 (Rakovec et al. 2022; van der Wiel et al. 2023). The data analysis was conducted using the R program, version 4.3.1 (R Core Team 2023). Microclimatic parameters such as air temperature and rainfall were available from a nearby weather station (Kunert 2024). Differences among different measuring dates were assessed with a paired Student's *t*-test. Differences between years were compared using a non-parametric test (Kruskal–Wallis test followed by a Dunn's post hoc comparison). Please note, for the interannual comparison all measurements on leaf level were included, resulting

**Fig. 1** Drought conditions in the study area for the years 2004–2024. Bars represent the monthly Standardized Precipitation Evapotranspiration Index (SPEI) accounting for the weather conditions of the previous 12 months ( $\text{SPEI}_{12}$ ). Monthly mean temperature and monthly precipitation for the period 2004–2024 was provided by the German Meteorological Service (DWD, Station number 03668, retrieved from <https://opendata.dwd.de>)



in sample sizes of  $n=8$ ,  $n=6$ , and  $n=6$  in the years 2020, 2023, and 2024, respectively. The student's  $t$ -test and the Kruskal–Wallis test were performed in JASP, version 0.19.0 (JASP Team 2024).

## Results

### Seasonal course of $\pi_{\text{lp}}$

The least negative  $\pi_{\text{lp}}$  was found with leaf unfolding in early spring during the first measurements on DOY 99 for European hornbeam and on DOY 108 for European beech and pedunculate oak (Fig. 2). The least negative  $\pi_{\text{lp}}$  during the first measurements were present in European hornbeam with  $-1.38 \pm 0.13$  MPa, followed by pedunculate oak with  $-1.54 \pm 0.07$  MPa. European beech had the most negative  $\pi_{\text{lp}}$  in the early leaves with  $-1.87 \pm 0.11$  MPa.  $\pi_{\text{lp}}$  decreased almost gradually in all species until DOY 152 when the most negative values were found. On DOY 152,  $\pi_{\text{lp}}$  of pedunculate oak was at  $-2.71 \pm 0.15$  MPa, of European beech at  $-2.82 \pm 0.27$  MPa and of European hornbeam at  $-3.24 \pm 0.26$  MPa. In the following two weeks the last significant rain fell for the rest of the summer (97 mm between DOY 155 and DOY 168 compared to less than 5 mm per week until the week between DOY 239 and DOY 245). Within those two weeks, the leaves of most tree individuals fully matured to their final state and toughness. During those two weeks  $\pi_{\text{lp}}$  of all species decreased resulting in  $-2.60 \pm 0.10$  MPa in pedunculate oak,  $-2.43 \pm 0.07$  MPa in European beech and  $-2.52 \pm 0.08$  MPa in European hornbeam on DOY 169. However, it took several days longer for European beech to reach the static summer plateau with  $-2.65 \pm 0.10$  MPa on DOY 178. From there on, despite minor fluctuations,  $\pi_{\text{lp}}$  remained relatively constant until the end of the measurements on DOY 270 with  $-2.52 \pm 0.08$  in pedunculate oak,  $-2.70 \pm 0.13$  in European beech, and  $-2.63 \pm 0.17$  MPa in European hornbeam. There were significant differences between the different measuring dates in European hornbeam, for example between the early dates DOY 99 and DOY 152 ( $t(7)=21.9$ ;  $p<0.001$ ) and DOY 152 and DOY 169 ( $t(7)=-5.03$ ;  $p=0.002$ ), however, no significant differences were indicated between DOY 169 and DOY 270 ( $t(7)=0.99$ ;  $p=0.353$ ) after leaves were fully matured. In European beech all consecutive dates were significant different (DOY 99 and DOY 152:  $t(7)=8.20$ ;  $p<0.001$ ; DOY 152 and DOY 169:  $t(7)=-3.58$ ;  $p=0.009$ ; DOY 169 and DOY 270:  $t(7)=4.17$ ;  $p=0.004$ ). In contrast, a significant difference was only present in pedunculate oak between the first two sampling dates (DOY 99 and DOY 152:  $t(7)=34.51$ ;  $p<0.001$ ), whereas there was no significant difference between the other sampling dates including DOY 178 (DOY 152 and DOY 169:  $t(7)=-1.69$ ;  $p=0.135$ ;

DOY 169 and DOY 270:  $t(7)=-1.58$ ;  $p=0.159$ ; DOY 178 and DOY 270:  $t(7)=0.934$ ;  $p=0.377$ ).

### Interannual differences in $\pi_{\text{lp}}$

European hornbeams had mean values of  $\pi_{\text{lp}}$  of  $-2.57 \pm 0.12$  MPa,  $-2.57 \pm 0.35$  MPa, and  $-2.51 \pm 0.15$  MPa in 2020, 2023, and 2024, respectively (Fig. 3a). There was no significant difference in  $\pi_{\text{lp}}$  between the three different years in European hornbeam ( $H(2)=0.605$ ,  $p=0.739$ ; Fig. 3a) and no effect of the prevailing moisture condition four weeks prior to the measurements. In European beech,  $\pi_{\text{lp}}$  was  $-2.65 \pm 0.10$  MPa in 2020,  $-2.79 \pm 0.18$  MPa in 2023 and  $-2.54 \pm 0.08$  MPa in 2024. A significant differences between years was detected ( $H(2)=7.890$ ,  $p=0.019$ ), with the years 2023 and 2024 ( $p=0.005$ ) being significant different and no significant difference between 2020 and 2023, as well as 2020 and 2024 ( $p=0.246$  and  $p=0.07$ , respectively). Pedunculate oak was characterized by a  $\pi_{\text{lp}}$  of  $-2.54 \pm 0.18$  MPa in 2020,  $-2.38 \pm 0.13$  MPa in 2023, and  $-2.11 \pm 0.13$  MPa in 2024 and a significant difference between groups ( $H(2)=7.534$ ,  $p=0.023$ ) however, only the years 2020 and 2024 were significantly different ( $p=0.007$ ). Nevertheless,  $\pi_{\text{lp}}$  of oak was correlated positively with SPEI<sub>12</sub> of the preceding month before the measurement (Fig. 3c).

## Discussion

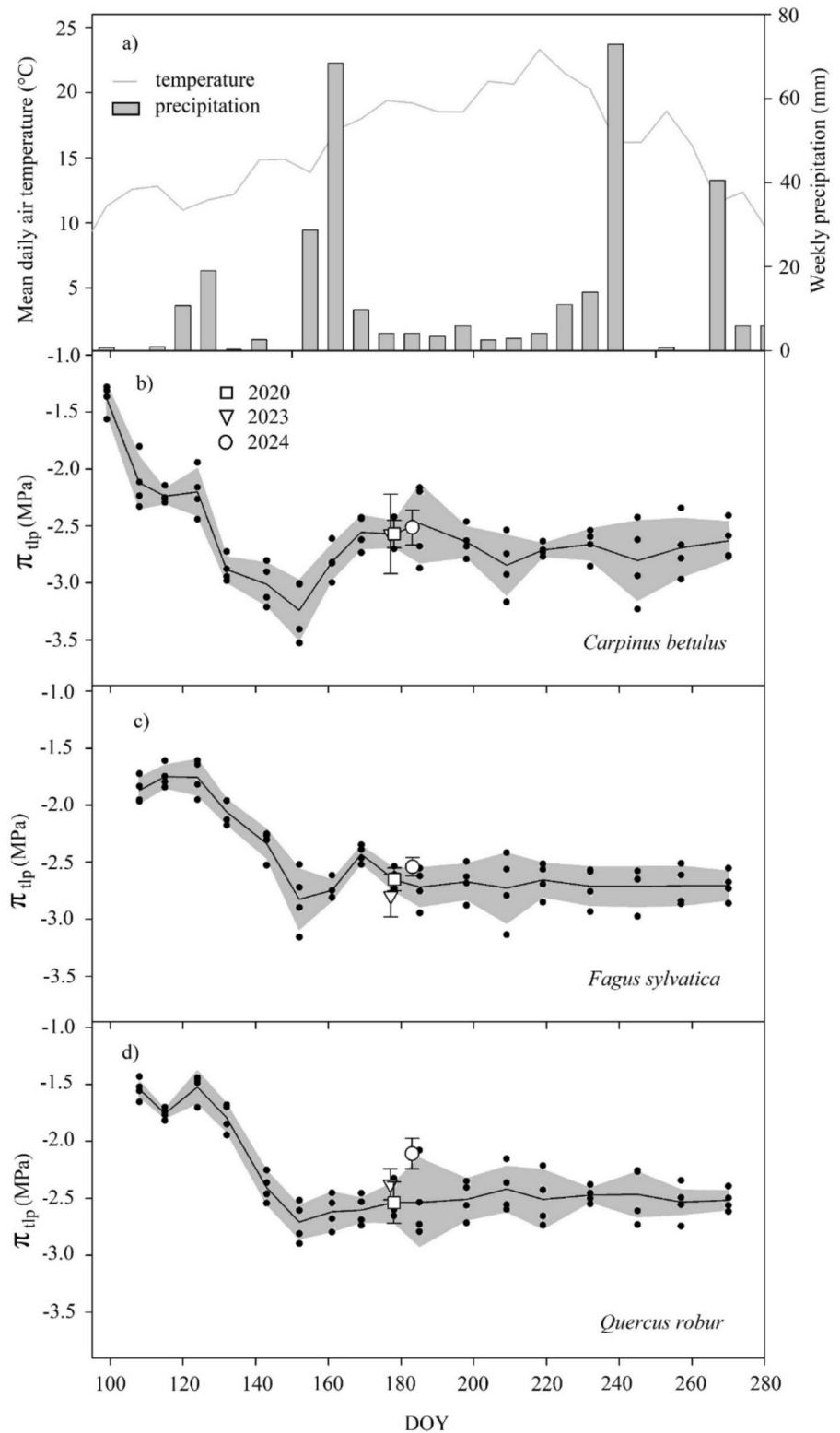
### Overview

The largest seasonal changes in  $\pi_{\text{lp}}$  occurred in the three tree species during leaf-unfolding until leaves were fully developed and matured. After the leaves matured, there was a 'static summer plateau' with no significant changes and further osmotic adjustment of  $\pi_{\text{lp}}$  to persisting water limitation during the summer. Interannual variation of  $\pi_{\text{lp}}$  that could be related potentially to varying water availability among years was only present in pedunculate oak. The other two species, namely European beech and European hornbeam showed no significant difference in  $\pi_{\text{lp}}$  over the three years with varying water availability.

### Limited seasonal osmotic adjustment

During springtime a rapid osmotic adjustment was observed. Within 53 days all three tree species osmotically adjusted their still developing leaves to a low  $\pi_{\text{lp}}$ . After this leaf developing phase,  $\pi_{\text{lp}}$  in all three tree species reached a static plateau once the leaves were fully matured. There was no further significant change in  $\pi_{\text{lp}}$  over several weeks, despite no significant rainfall during this time period. I see

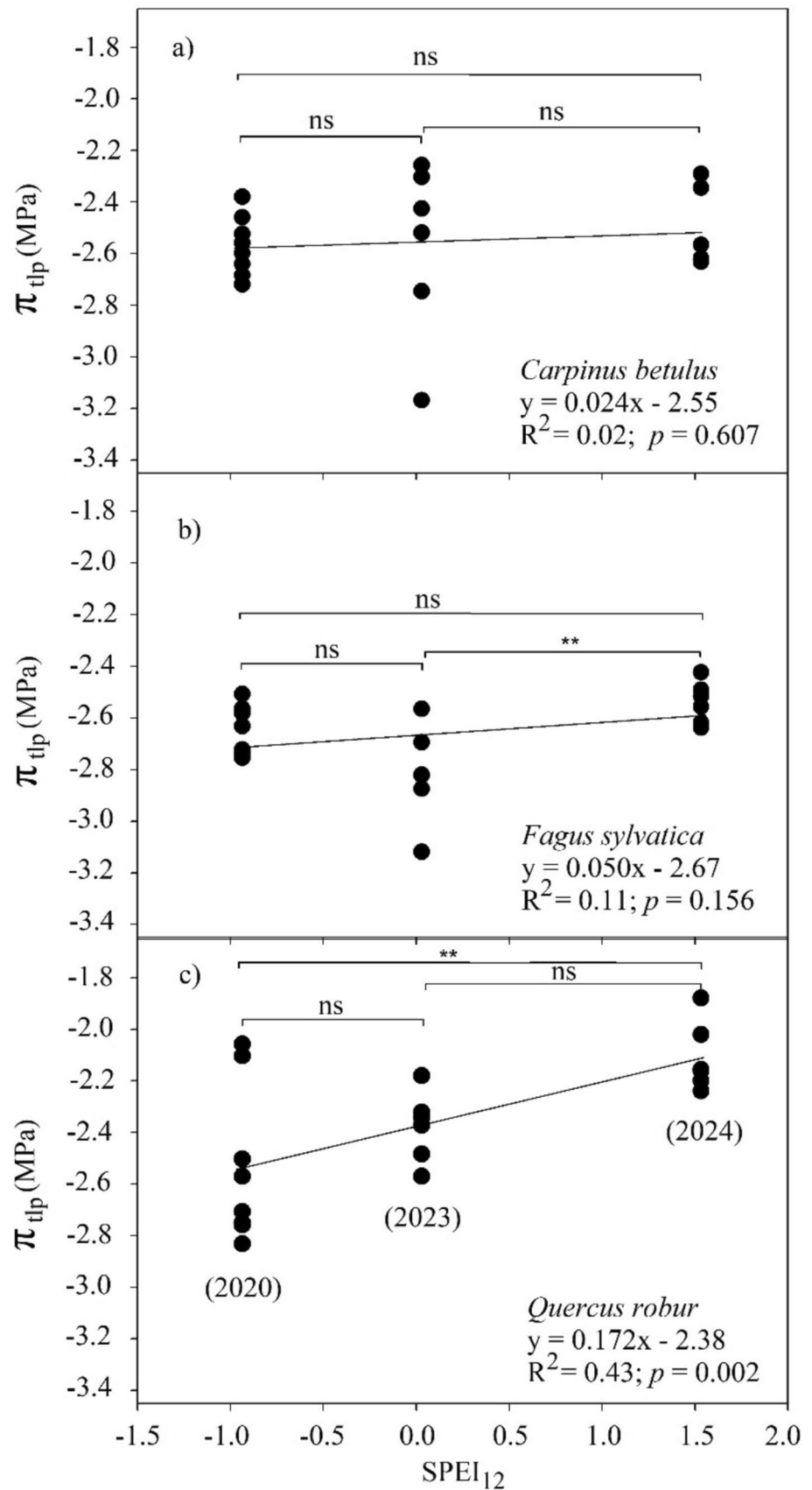
**Fig. 2** Seasonal course of the a) environmental parameters such as mean air temperature and weekly precipitation prevailing during the study period. Panel b), c), and d) show the seasonal course of  $\pi_{\text{tlp}}$  of the three tree species (black line). Dots represent the value of each single leaf disc and the gray band the 95% confidence interval. The large open square, the open triangle and the open dot show the mean  $\pi_{\text{tlp}}$  measured in the years 2020, 2023 and 2024. Error bars on the open symbols represent the standard deviations



two possible reasons as explanation for the limited osmotic change during this period. The first one is that the three investigated tree species have only a limited ability to further adjust their leaf drought tolerance when limited water

availability persisted for the rest of the growing season. The second, that the limited change of  $\pi_{\text{tlp}}$  during this period was due to legacy effects as trees, were pre-exposed to drought stress for the two previous years (Fig. 1). The two

**Fig. 3** Linear relationship between leaf turgor loss point ( $\pi_{\text{tlp}}$ ) and the Standardized Precipitation Evapotranspiration Index ( $\text{SPEI}_{12}$ ) based on the four weeks before each  $\pi_{\text{tlp}}$  measurement. All leaf disc measurements were included in this analysis (sample size 2020:  $n=8$ ; 2023:  $n=6$ ; 2024:  $n=6$ ). Significant differences between measurements are indicated with brackets (ns: not significant; \*\*:  $p < 0.001$ ). Significant differences between years were evaluated with a Kruskal–Wallis test followed by a Dunn’s post hoc comparison. Note: Regression lines are also indicated for non-significant relationships to highlight the trend in the slopes



previous years have been recognized to be a new benchmark of drought intensity (Rakovec et al. 2022; van der Wiel et al. 2023) inducing significant growth reduction in trees in the study area (Thom et al. 2023; Kunert et al. 2024).

No further adjustment of  $\pi_{\text{tlp}}$  is rather surprising as the investigated species are known for their high potential to adjust leaf properties under water limitation. For example, in irrigation manipulation experiments, closely related oak species have been found to have a high capacity to adjust to drought by osmoregulation (e.g. Aranda et al. 2004). However, these observations are mainly based on predawn leaf water potential measurements and do not investigate changes in  $\pi_{\text{tlp}}$  in response to drought. European beech is known for its' high phenotypic plasticity (Petrík et al. 2020) and  $\pi_{\text{tlp}}$  has been found to vary between  $-1.90$  and  $-2.62$  MPa across different sites (Weithmann et al. 2022). The variation depended strongly on the short-term water deficits in the month prior to the measurement (Weithmann et al. 2022). In contrast, Kunert et al. (2024) report a much lower variation of  $\pi_{\text{tlp}}$  in European beech.  $\pi_{\text{tlp}}$  ranged only between  $-2.58$  and  $-2.79$  MPa on very wet study sites with an annual mean precipitation between 740 and 1060 mm, whereas, Weithmann et al. (2022) included sites with an annual mean precipitation between 497 and 866 mm. The difference between these two studies is that measurements in Kunert et al. (2024) were conducted at the different sites assuming similar climatic conditions during the months prior to measurements, whereas Weithmann et al. (2022) specifically did not filter measuring dates regarding short term water shortage prior to the measurements. In the current study, I found a very low variation of only 0.07 MPa in European beech and 0.12 MPa in pedunculate oak. During the static summer plateau, the variation between measurements reflects more or less the standard deviation of the measurements. Only European hornbeam had a large variation of 0.37 MPa during this period, however, European hornbeam was also characterized by higher standard deviation among the single sampling dates. Nevertheless, a variation of 0.37 MPa between dates is much closer to the osmotic adjustment described by other authors (0.44 MPa on global average in response to drought, e.g. Bartlett et al. 2014). However, I observed no directional adjustment of  $\pi_{\text{tlp}}$  with changing climate conditions in European hornbeam. For example, Hiron et al. (2020) and Sjöman et al. (2018) describe this adjustment to be directional from wet spring conditions to dry summer condition and to have the magnitude of 0.70 MPa in urban trees. Similarly, Grossman et al. 2024 describe an early to late seasonal adjustment of  $\pi_{\text{tlp}}$  in 20 out of 27 species. In the later study, seasonal adjustment depended mostly on the day of year when measurements were taken, rather than on varying water availability between years as measurements across three years could

be pooled. Unfortunately, those studies fail to contextualize how leaf development had deviated in spring versus summer. I speculate that this large difference in those two studies might be due to differences in the development stage, as for example Eisley and Wolfe (2024) found similar mean osmotic adjustment of 0.08 MPa of a variety of temperate tree species between early and late summer.

Most existing studies on variation of  $\pi_{\text{tlp}}$  were conducted when leaves were fully expanded as stated in the methods established by Bartlett et al. (2012). Therefore, how  $\pi_{\text{tlp}}$  of young and still expanding leaves develops remains largely unknown and is a new aspect of this study. I could show that  $\pi_{\text{tlp}}$  upon unfolding is much lower between (European hornbeam  $-1.38$  MPa, pedunculate oak  $-1.54$  MPa and European beech  $-1.87$  MPa) than  $\pi_{\text{tlp}}$  of fully expanded and mature leaves (average over all values after DOY 169: European hornbeam  $-2.67 \pm 0.11$  MPa, pedunculate oak  $-2.51 \pm 0.05$  MPa, European beech  $-2.66 \pm 0.09$  MPa). Lakso et al. (1984) found that mature leaves had lower osmotic potential (note: that I used the osmotic potential of rehydrated leaves to estimate  $\pi_{\text{tlp}}$ ) than the expanding leaves and they blame the less negative osmotic potential on the incapacity of immature leaves to synthesize certain enzymes supporting osmotic adjustment. Hence, I conclude that the less negative  $\pi_{\text{tlp}}$  and the inability to rapidly adjust to drier conditions make leaves much more susceptible to drought during their early life in springtime than in the late season.

Finally, and even if I considered legacy effects to be responsible for low seasonal osmotic adjustment during the static summer plateau, I would like to reject this hypothesis since there were only significant interannual differences for one species. Briefly, legacy effects have been described as post-drought morphological alterations, including changes in specific leaf area or physiological responses such as lower leaf water potential and net photosynthesis (Bushal et al. 2021). Hence, stress legacy is assumed to modify phenotypes of plant individuals in response to stress (Quan et al. 2022). Please note, 2020 was the last year of three consecutive years without significant rainfall and without full soil water re-storage during the winter months in Central Europe causing a significant growth reduction in most native tree species (Thom et al. 2023; Kunert et al. 2024). Taking this extreme dryness into consideration, I did not observe a significantly less negative  $\pi_{\text{tlp}}$  in the following years with high moisture availability and the time between the dry year in 2020 and the wet year in 2023 should have been enough time for the trees to downregulate  $\pi_{\text{tlp}}$ . For example, Hesse et al. (2023) describe an alignment of  $\pi_{\text{tlp}}$  between drought-treated trees after rewetting and control trees within 22 days. The interannual measurements were three, respectively four, summers apart, any adjustment to better water availability should have been expressed by a change in  $\pi_{\text{tlp}}$ . Accordingly, legacy effects might not have caused the investigated tree

species to aim for a high level of drought tolerance, reflected by a more negative  $\pi_{\text{tlp}}$ , due to pre-exposing to drought stress in the years prior to the measurements.

### Interannual variation in $\pi_{\text{tlp}}$

Interannual drought related osmotic adjustment was only detected in pedunculate oak (Fig. 3), whereas  $\pi_{\text{tlp}}$  in the other two species did not scale with SPEI. There was a significant difference between the drought year and the wettest year in pedunculate oak. The difference in the mean annual values had a magnitude of 0.43 MPa. Despite the clear linear relationship that described the variation of  $\pi_{\text{tlp}}$  among years with different SPEI (Fig. 3c), I want to note that the less negative  $\pi_{\text{tlp}}$  in 2024 compared to 2020 might have come from a heavy infection of all oak individuals with oak powdery mildew (*Erysiphe alphitoides* (Griffon & Maubl.) U. Braun & S. Takam.). Powdery mildew has been reported as a fungus that might change the cytoplasmic solute concentration in different ways. First, powdery mildew is known to reduce carbon acquisition of the host leaves (Marçais and Desprez-Loustau 2014) and to enhance the accumulation of secondary non-osmotically active metabolites such as phenols and lignin in the host cell (Grzebyta et al. 2005). Further, powdery mildew feeds on nutrients from living host cells via haustoria (Divon and Fluhr 2007) what can reduce potentially the solute concentration in the vacuoles. All these characteristics of powdery mildew might alter the osmolarity of the cytoplasmic solution and affect the osmotic potential of the leaves and thus  $\pi_{\text{tlp}}$ . Therefore, I cannot entirely exclude any effects of the infection on  $\pi_{\text{tlp}}$  values of European oak from 2024. I found no plausible explanation for the significant difference in the mean values of European beech of 2023 and 2024. Overall and despite the limited sample size, I conclude that there is little variation of  $\pi_{\text{tlp}}$  between years in the three investigated species and ecosystem if  $\pi_{\text{tlp}}$  is measured at a certain time after leaves fully matured.

### Conclusion

This study provides evidence that  $\pi_{\text{tlp}}$  can be considered a static trait once leaves are fully matured and expanded in the investigated species and ecosystem. The results suggest that the three species have a rapid period of osmotic adjustment early in the growing season followed by a period of relative stability. This opens the way for direct interannual comparison of  $\pi_{\text{tlp}}$  values as they seem to be static across years with different rainfall conditions if measure on entirely healthy leaves.

**Acknowledgements** NK was supported by an EU-Mobility grant to collect data in Fürth. NK is thankful to his family, particularly his dad,

for helping with the data collection during 2020. The author would like to thank Elena Düsterhöft for performing the measurements in the laboratory in July 2024.

**Funding** Open Access funding enabled and organized by Projekt DEAL. The study was supported by the European Union as a mobility grant.

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