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Trade-offs among leaf toughness, constitutive chemical defense, and growth rates in oaks are influenced by the level of leaf mass per area

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Abstract

Key message Among the variety of leaf defensive strategies to counteract herbivory attacks, the oak species analyzed in this study maximize investment in no more than one, with high-LMA oaks developing very tough leaves and low-LMA oaks favoring between high concentrations of condensed tannins or high growth rates.

Context Plants develop a variety of defense strategies to counteract herbivory attacks, from physical and chemical defenses to tolerance strategies. Tradeoffs between strategies have been widely assessed from a resource allocation perspective, but there is a need to consider eventual interactions among them.

Aim We evaluate the among-species tradeoff between three main constitutive plant defense traits, while considering the leaf mass per area ratio (LMA) as a proxy of leaf construction investment on area basis.

Methods Leaf toughness measured as work of fracture, condensed tannins, and relative growth rate (RGR) were analyzed in a set of 19 *Quercus* L. species with contrasting LMA.

Results Most species had low values either in two or in the three traits analyzed. Moreover, the highest values of work of fracture appeared in the species with high LMA; the highest values of condensed tannins were found in the species with the lowest LMA; and high values of RGR were measured in species with intermediate or lower values of LMA.

Conclusion Oaks showed a trade-off among leaf defensive strategies influenced by LMA. Oaks with high LMA developed very tough leaves while oaks with low LMA presented lower values of toughness but favored between one of the two other strategies.

Keywords Condensed tannins, Defensive traits, Relative growth rate, *Quercus*, Work of fracture

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1 Introduction

Plant herbivory, referred to the consumption of all or a part of a plant, is a key ecological process affecting primary production, vegetation shape, and composition, which is likely to intensify in temperate regions due to global warming (Schowalter 2006; Coley and Barone 1996; Schemske et al. 2009; Currano et al. 2010; Birkemoe et al. 2016; Nakamura et al. 2021). To counteract herbivory attacks, plants have developed a variety of defense mechanisms (Rasman et al. 2014; Dostálek et al. 2016). Direct defenses are the first kind and comprise physical and chemical defenses that increase plant resistance and reduce plant palatability (Schoonhoven et al. 2005). The second category, referred to as indirect defenses, is the attraction with volatile organic compounds of the herbivores' natural enemies (Dicke and Baldwin 2010; Lehrman et al. 2013). The third type of defense mechanism is the tolerance of plants to herbivory, which measures how plants are able to overcome herbivory damage (Strauss and Agrawal 1999; Schoonhoven et al. 2005).

Focusing on the leaf, the most common physical defenses analyzed in herbivory studies are those associated with its mechanical resistance, specifically, the leaf toughness (Pearse and Hipp 2009; 2012; Sanson et al. 2001; Onoda et al. 2011). Leaf toughness is defined as the resistance of the leaf to crack propagation and is a measure of the energy required to fracture the leaf. The calculation of the toughness is associated with the measurement of the work of fracture (WF) which needs a simultaneous measure of the punch strength—maximum force at which the leaf breaks—and the punch displacement (Vincent 1992; Wright and Vincent 1996). Leaves with higher toughness are those with higher values of work of fracture, and therefore, leaves with an improved mechanical resistance to predation (Vincent 1991). Work of fracture has also been correlated with leaf mass per area ratio (LMA) (e.g., Alonso-Forn et al. 2023). Due to this association, LMA has sometimes been used as an estimator of leaf toughness. However, leaves can show higher WF values than expected at a given LMA (Alonso-Forn et al. 2023). Thus, there is an inability of LMA to account adequately for the wide variation in WF indicating that there are variations in the mechanical properties that do not contribute to an increase in the accumulated mass per surface area (Alonso-Forn et al. 2023).

Leaf chemical protection includes secondary compounds known by their antiherbivore actions (Seigler 1998; Lokvam et al. 2004; 2006; 2007). The most common and widespread secondary metabolites analyzed in herbivory studies are the tannins, including the condensed tannins also known as proanthocyanidins (Kursar et al. 2009; Pearse and Hipp 2009; Barbehenn and Constabel 2011; Eichenberg et al. 2015; Solla et al. 2016; Galmán

et al. 2019; Fyllas et al. 2022). Condensed tannins cause oxidative damage within the epithelial cells of an insect's midgut by acting as toxins and feeding inhibitors (Barbehenn and Constabel 2011), although the level of such effects may depend on how the digestive system of the herbivore species interact with the plant-specific tannins (Barbehenn and Constabel 2011; Salminen and Karonen 2011; Büchel et al. 2016). Condensed tannins have been shown to most affect chewing insects damage in a set of oak species (Pearse and Hipp 2009) or have driven a latitudinal gradient in leaf herbivory when studying *Quercus robur* (Moreira et al. 2018). Condensed tannins have also been reported as a tolerance, rather than a chemistry resistance factor in aspens, indicating that condensed tannins may benefit plants under extreme herbivory through the improvement of the nutrient cycling (Madritch and Lindroth 2015).

The relative growth rate (RGR), or the rate of accumulation of new dry mass per unit of existing dry mass, has been shown to be the best predictor of tolerance to herbivory (Gianoli and Salgado-Luarte 2017). Having higher levels of relative growth rate, not only reduce leaf predation on the more vulnerable, young, expanding leaves (Aide and Londoño 1989; Moles and Westoby 2000) but also has reported to increase the tolerance to leaf herbivory through a greater ability to recover lost material (Danckwerts 1993; Strauss and Agrawal 1999; Gianoli and Salgado-Luarte 2017). Relative growth rate is an indirect measurement of the rate of resource acquisition. Thus, the faster a species accumulates biomass, the more carbon is available to increase growth of roots, shoots, and photosynthetic material for greater access to resources, which in turn promotes further biomass accumulation. In this sense, Gianoli and Salgado-Luarte (2017) found a strong positive correlation between RGR and tolerance to herbivory in a set of tree species from a temperate rainforest showing, effectively, that a higher RGR entails a higher tolerance.

Tradeoffs between the different defensive strategies have been widely assessed in herbivory studies to evaluate the investments from the perspective of resource allocation (Rohner and Ward 1997; Adams et al. 2009; Cárdenas et al. 2014). It has been proposed that resistance and tolerance are alternative plant strategies to deal with herbivore damage because if plant resistance mechanisms are relatively more costly or ineffective, herbivory might result in selection for tolerance because resistance is not a viable strategy (van der Meijden et al. 1988). In addition, it is also in agreement that maximizing multiple defensive strategies might not occur in the same individual as it is costly for a plant and allocating in defensive traits is assumed to reduce resource availability for other processes (Heil and Bostock 2002; Koricheva et al. 2004;

Ballhorn et al. 2008). In this regard, some previous studies showed that tree growth was negatively correlated with chemical defense, concluding that production of defensive compounds had a growth cost (Kosola et al. 2004; Stevens et al. 2007; Paul-Victor et al. 2010; Züst and Agrawal 2017; Cope et al. 2021; Perkovich and Ward 2021). Other studies found tradeoffs within resistance strategies, i.e., between physical and chemical defenses (e.g., Eichenberg et al. 2015; Wang et al. 2016). However, some other studies did not find any tradeoff or found weak correlations between defensive traits (Gianoli and Salgado-Luarte 2017; Moreira and Pearse 2017). So far, most studies have only analyzed two of the three most important strategies (e.g., growth vs. chemistry, or physical vs. chemical defense), thus there is a need to consider eventual interactions among the three defense strategies.

The aim of our study was to evaluate the among-species tradeoff between three of the most important factors involved in plant defense described above: leaf toughness (physical defense), condensed tannins (chemical defense), and relative growth rate (tolerance strategy), in a set of *Quercus* L. species growing in a common garden. The genus *Quercus* is an excellent system to perform this research, since it not only minimizes phylogenetic variation (in comparison to studies conducted across diverse species) but also exhibits significant trait variation among species (Pearse and Hipp 2009; Sancho-Knapik et al. 2021). We analyzed the interspecific tradeoff by studying the constitutive levels (i.e., basal levels expressed at leaf maturation) of defensive traits (Karban 2011) while considering the leaf mass per area ratio as a proxy of leaf construction investment on an area basis (Escudero et al. 2017). We hypothesized that, due to tradeoffs between the alternative strategies, each oak species would tend to maximize one single of the three strategies. We also hypothesized that the strategy maximized by the oak species would depend on the species' leaf mass area and therefore on the species' leaf construction cost on area basis.

2 Materials and methods

2.1 Study system, plant material, and field site

To test our hypotheses, we used the dataset of Alonso-Forn et al. (2023), which included oak species with contrasting leaf toughness and leaf mass area. In addition, plant material for condensed tannins and relative growth rate was sampled on 19 oak species during the same year and in the same specimens as those described in Alonso-Forn et al. (2023). Briefly, plant material was collected from a living collection of oak species, maintained in an experimental field under uniform nutrient supply and light conditions, and with no water restrictions, located at CITA de Aragón (41.728° N, 0.821° W, 218 m a.s.l.,

Zaragoza, Spain). The common garden features Mediterranean climatic conditions with a long-term mean annual temperature of 15.4 °C and total annual precipitation of 298 mm. The study site was in a river terrace, with clayey silt soil of alluvial origin. Oak trees were ca. 20 years old; they were drop irrigated twice per week from spring to autumn. Plant material was collected from south-exposed branches of one individual per species during the early morning, stored in sealed plastic bags and carried to the laboratory in portable coolers. For condensed tannins we collected a set of 9 current year, fully developed, mature leaves per species. For growth analysis, we sampled two sets of 10 developing shoots (including leaves and the stem) per species spaced in time (see below for details).

2.2 Leaf toughness

Leaf toughness was directly obtained from the values of work of fracture (WF, J m⁻¹) that appear in the dataset of Alonso-Forn et al. (2023) which are openly available in TRY Plant Trait Database at <http://doi.org/10.17871/TRY.86>. This previous study performed punch and die tests, which consisted on punching a hole through the leaf lamina and generating force–displacement curves. These mechanical tests were taken at room temperature on 10 full-hydrated leaves per species, with measurements avoiding major veins. The analysis of the force–displacement curves allowed to obtain the work of fracture as the area below the curve between the initial contact of the punch with the leaf and the highest load value registered.

2.3 Condensed tannins

Condensed tannin concentrations (CT, mg g⁻¹ dry weight) of oak leaves were determined colorimetrically following the butanol–HCl assay (Porter et al. 1986; Pearse and Hipp 2009; Grabber et al. 2013). Briefly, 500 mL of leaf extract were incubated with 3 mL of acidified butanol (BuOH-HCl) and 100 mL of ferric reagent at 100 °C for 60 min. The mixture was cooled to room temperature and the absorbance of the mixture was read at 550 nm.

The dataset generated and the code used for the analyses during the current study are available in the citaREA repository (Sancho-Knapik et al. 2023).

2.4 Relative growth rate

Relative growth rate (RGR, d⁻¹) was measured for each species by making two harvests separated by a time-interval (Paul-Victor et al. 2010). For each species, the first harvest took place 7 days after bud bursting (t_1); the second harvest took place 28 days after bud bursting (t_2). Figure 1 shows the time-interval period for each species as well as the temperature throughout the growing

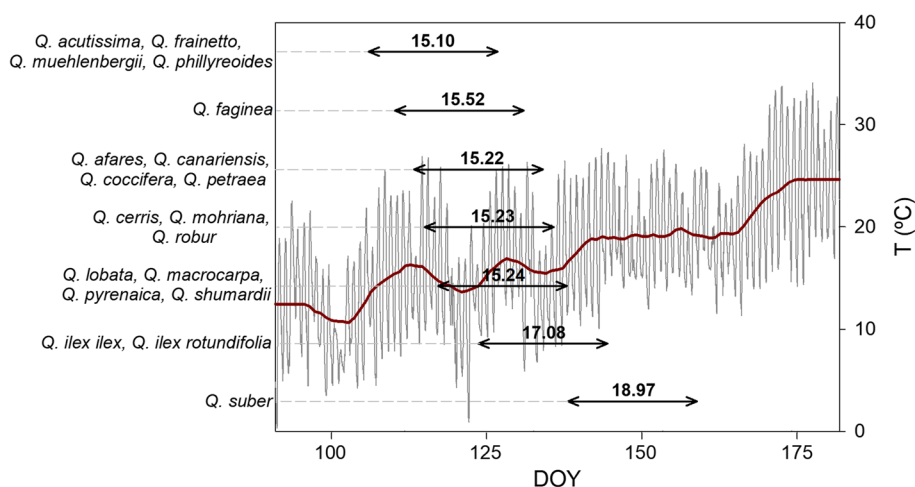


Fig. 1 Temperature (T) throughout the growing season (grey line) and smothering of the data (red line). Arrows indicate the 21-day-period for the RGR calculation of each species. Values above arrows are the mean temperatures of each period

season and the growth mean temperature for each species (climatic data are from the nearest weather station, 1 km away).

As mentioned above, 10 shoots (including leaves and the stem) per species were collected from the southern exposure of the tree for each harvest. Then, shoots were oven dried for 3 d at 70 °C and weighed using an analytical balance (Sartorius AG Model BP221S; Sartorius AG, Goettingen, Germany) obtaining the dry mass (M) of each shoot. RGR was then calculated as (Paul-Victor et al. 2010):

$$\text{RGR} = \frac{\log(M_2/M_1)}{t_2 - t_1}$$

where M_1 and M_2 are the mean dry mass values of the 10 shoots recollected for each species during the first and the second harvest, respectively. Individual shoot dry mass values for each species are available in the citaREA repository (Sancho-Knapik et al. 2023).

2.5 Statistical analysis

For the evaluation of the results, species were classified according to the value of leaf mass per area (LMA, g m^{-2}) reported in Alonso-Forn et al. (2023). Oak species with values of LMA higher than 151 g m^{-2} were considered species with high LMA and, therefore, species with a high construction cost on an area basis. Species with values of LMA between 150 and 101 g m^{-2} were considered species with intermediate values of LMA, and oak species with values of LMA lower than 100 g m^{-2} were considered species with low values of LMA and, therefore, species with a low construction cost on an area basis.

Relationships between work of fracture, condensed tannins and RGR were first related two by two. Then,

values of the three defensive traits were normalized between 0 and 1 and were plotted in a three-dimension graph. To assess the probability to find a combination of two or more defensive traits, we divided the species in three categories, according to their position within the range of values (0=none of the variables in the upper half of the range; 1=one variable in the upper half of the range; 2=two or more variables in the upper half of the range). Significance differences in the abundance of species among defense strategies were tested with a general linear model assuming a Poisson distribution using the basal function “glm” in R version 4.2.1 (R Core Team 2022). Finally, a principal components analysis (PCA) with two components was carried out to summarize the multivariate relationships among defensive traits and *Quercus* species.

3 Results

The three plant defense strategies showed a wide range of variation within our set of *Quercus* species studied. Leaf toughness, measured as work of fracture, ranged from the 0.07 J m^{-1} found in *Q. lobata* to the 1.08 J m^{-1} showed by *Q. ilex* subsp. *rotundifolia* (Fig. 2). Condensed tannins ranged from 9.6 mg g^{-1} of dry weight measured in *Q. petraea* to 109 mg g^{-1} d.w. found in *Q. lobata* (Fig. 2). RGR ranged from 0.037 day^{-1} found for *Q. lobata* to 0.160 day^{-1} measured in *Q. muehlenbergii* (Fig. 2). When arranging the oak species from highest to lowest leaf mass area (LMA), the highest values of work of fracture were measured in the species with the highest LMA, whereas the highest values of condensed tannins were found in the species with the lowest LMA (Fig. 2). By contrast, high values of RGR were measured in species with intermediate or lower values of LMA (Fig. 2). It

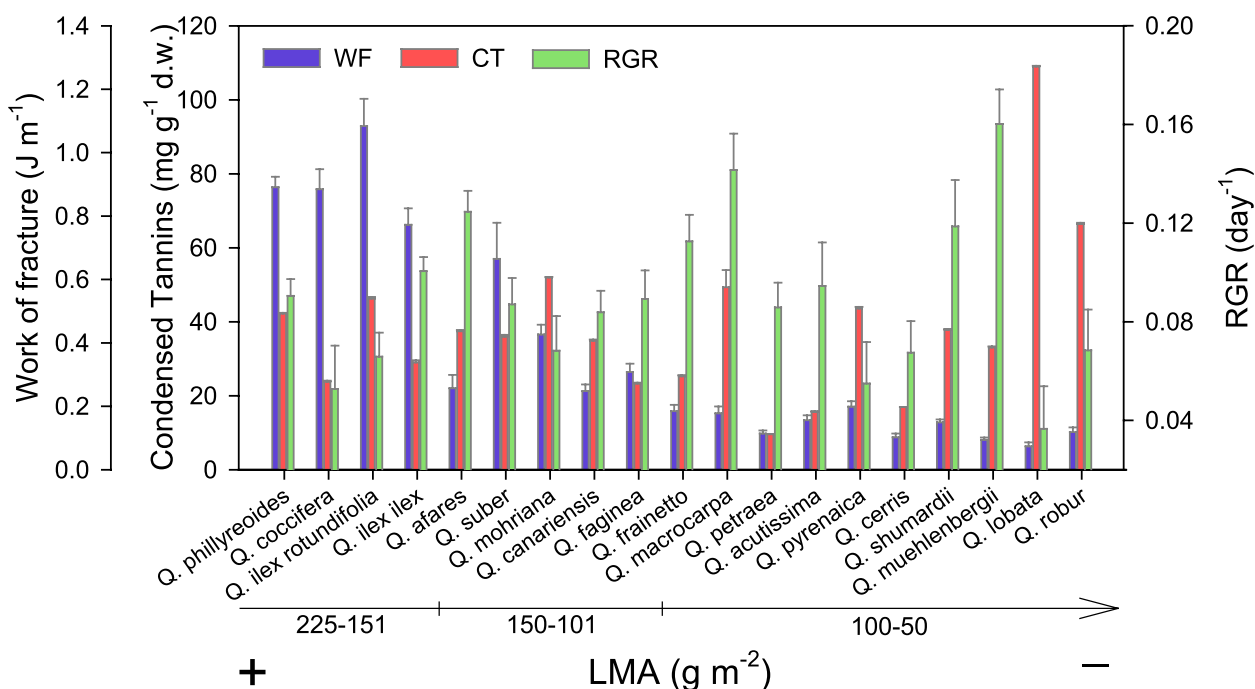


Fig. 2 Mean values and standard errors of work of fracture (WF), condensed tannins (CT) and relative growth rate (RGR) for the 19 oaks studied. Species are arranged from highest to lowest leaf mass area (LMA) and classified in three groups: 225 to 151, 150 to 101, and 100 to 50 g m⁻²

should be noted that, among the species with the lower values of LMA, there was not any oak species that maximized both condensed tannins and RGR (Fig. 2).

When the defensive traits were related two by two (Fig. 3a, b, c) or in the three-dimensional graph (Fig. 3d), we found that most species had relatively low values (i.e., normalized values below 0.5) either in two or in the three traits analyzed. In the categorization of species according to their defense strategies, we found 7 species with a limited defense specialization, i.e., none of the mechanisms in the upper half of the range, 11 species with one of the mechanisms in the upper half of the range (*Q. ilex subsp. ilex*) (Fig. 3d). The basal function “glm” to assess differences between categories showed no significant differences in the number of species with none or one defense mechanism within the upper range ($p=0.3499$) but a marginally significant lower ($p=0.0687$) number of species with a combination of two or more defense mechanisms within the upper range.

The principal component analysis (PCA) among the three defensive traits showed that each trait axis was separated equidistant from the other two by an angle of ca. 120° (Fig. 4). Oak species were distributed along the three-trait vectors, with no species clearly located between any two-trait vectors. Species with high LMA were distributed along the upper range of the work of fracture vector, whereas species with low LMA were

aligned either with the condensed tannins vector or the RGR vector (Fig. 4). Species with intermediate values of LMA were mainly located in the center on the principal component axes (Fig. 4).

4 Discussion

The evaluation of the three constitutive defensive strategies (leaf toughness, condensed tannins and RGR) among the oak species of this study revealed that none of the species have maximum values in more than one defensive trait. That is, species tend to maximize only one of the defensive strategies considered, confirming our first hypothesis. For example, while *Q. ilex subsp. rotundifolia* maximizes leaf toughness, *Q. lobata* and *Q. muehlenbergii* maximize condensed tannins and RGR, respectively (Fig. 2). The existence of an interspecific tradeoff among defensive traits agrees with previous studies in oaks (Mediavilla et al. 2018; Abdala-Roberts et al. 2018; Perkovich and Ward 2021) and other species (Dostálek et al. 2016; Eichenberg et al. 2015), where it is indicated that the co-expression of multiple defense strategies may be too expensive for a particular species, since investing in defensive traits is thought to reduce the resource availability for other processes, such as reproduction (Heil and Bostock 2002; Koricheva et al. 2004). By contrast, some species such as *Q. pyrenaica* or *Q. cerris* presented low values for the three defensive traits analyzed, when compared with the highest

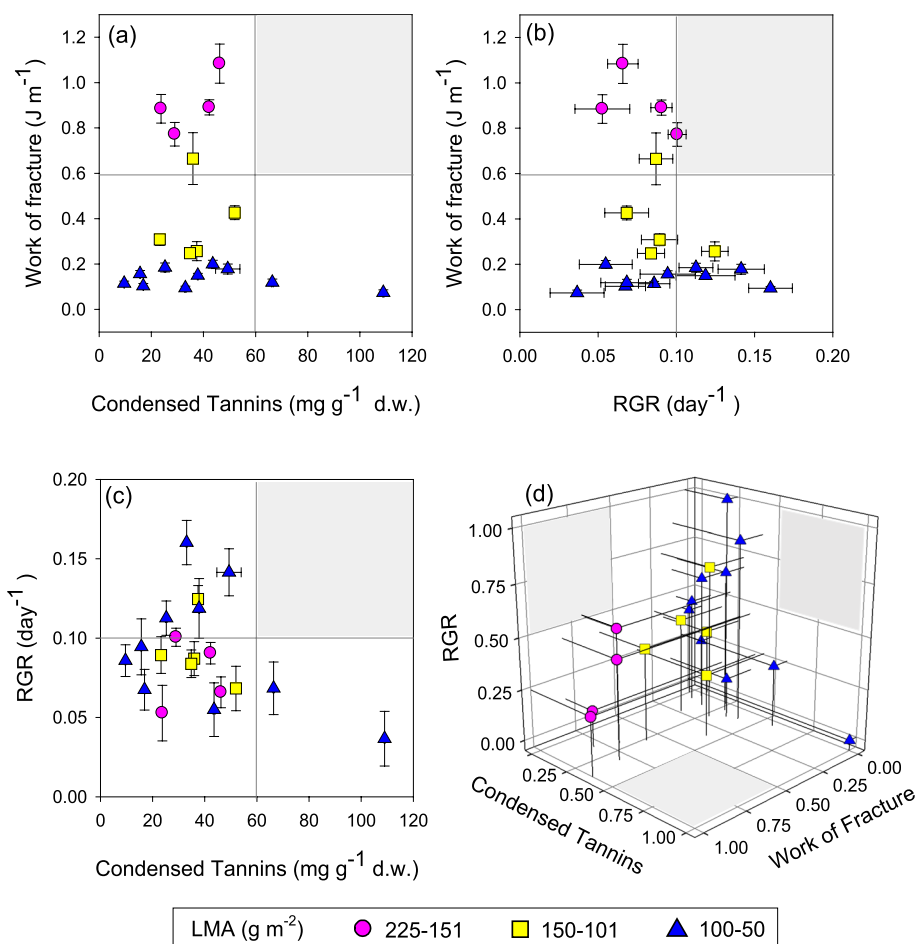


Fig. 3 Two-dimensional relationships between work of fracture, condensed tannins, and relative growth rate (RGR) for the 19 oaks studied ((a), (b), (c)). Three-dimensional relationship between defensive traits using normalized values ((d)). Shadow squares represent the areas with normalized values above 0.5. Species are classified in three levels of leaf mass area (LMA). Data are mean values \pm SE

absolute values of their congeners (Fig. 2). In the case of *Q. pyrenaica*, its leaves have more trichomes than the leaves of all other species of the study (personal observations), being considered leaves densely pubescent, attribute that can act as a defensive barrier against herbivory (Kitamura et al. 2007) and thus compensating the lower constitutive values found in the traits analyzed in this study. In particular, *Q. cerris* seemed to be the most vulnerable oak to herbivory from a constitutive point of view. Perhaps this species allocates resources to other constitutive strategies such as indirect defenses (e.g., attraction of the herbivores' natural enemies with volatile compounds), or relies mostly on induced defense mechanisms, showing low constitutive values (Agrawal and Karban 1999; Lehrman et al. 2013; Moctezuma et al. 2014; Perkovich and Ward 2021). Nevertheless, our results should be interpreted with caution as, due to the restrictions imposed by the common garden, we did not contemplate the intraspecific

variation in the plant defensive traits that might not be negligible (Hahn and Maron 2016).

Despite the lack of several individuals per species to analyze intraspecific variation, the LMA values used in this study to classify the species in three categories were good representative of the species values when compared with data from other studies (e.g., Niinemets 2001; Cornelissen et al. 2003; Wright et al. 2004; Kleyer et al. 2008), minimizing the phenotypic response to local environmental conditions. Taking into account these LMA categories, our results showed that developing very tough leaves was the main defensive strategy in oaks with high LMA, whereas making leaves with high constitutive concentrations of condensed tannins (chemical defense) or having high growth rates as a tolerance strategy were associated with low LMA values. Therefore, we confirm our second hypothesis: the strategy maximized by oak species would depend on the species' leaf mass area. More specifically, our results suggest that the elevated

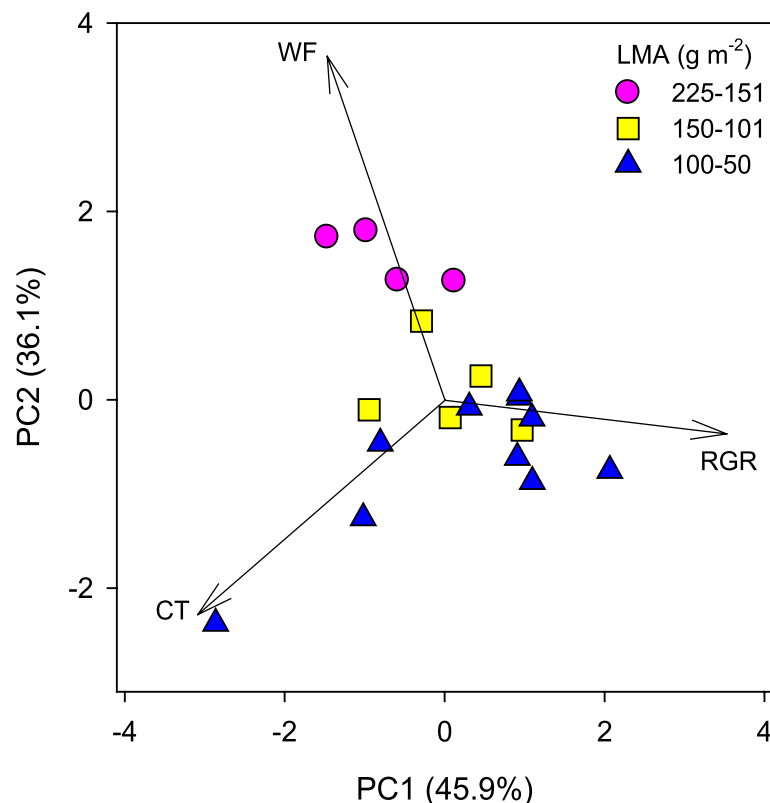


Fig. 4 Principal component analysis (PCA) of defensive traits in the 19 oaks studied. Species are classified in three levels of leaf mass area (LMA). Each dot corresponds to a single species. WF, work of fracture; CT, condensed tannins; RGR, relative growth rate

construction costs associated with high LMA limits the investment in alternative strategies, such as chemical defense or growth.

The higher toughness found in oaks with high LMA ($>151 \text{ g m}^{-2}$) reflects an improvement of a larger structural resistance that was related in previous studies to increased leaf density associated with a higher vein density, thicker cell walls, thicker bundle sheath extensions, and thicker epidermis outer walls (Peguero-Pina et al. 2017; Sancho-Knapik et al. 2021; Alonso-Forn et al. 2023). In addition, high values of LMA and structural resistance have been associated with a longer leaf lifespan (Reich et al. 1991; Wright and Westoby 2002; Onoda et al. 2011; Alonso-Forn et al. 2020). Effectively, the oaks from this group have the longest leaf lifespans of the studied species, from 12 up to 26 months in the case of *Q. ilex* (Alonso-Forn et al. 2020). It has been argued that leaves with higher structural resistance had longer leaf life spans to compensate their costly investment in construction, although the underlying mechanisms are controversial (Turner 1994).

Within the group with low LMA ($<100 \text{ g m}^{-2}$) and in opposition to the previous group, species constructed leaves with the lowest values of toughness and therefore

the lowest mechanical resistance, which agreed with their shorter leaf life span (5 to 8 months, Alonso-Forn et al. 2020). These species, with a low investment in mechanical resistance and reduced construction costs per area, would favor one of the two other strategies, chemical defense or tolerance (Fig. 4). Regarding the chemical defense, the contribution of condensed tannins to LMA seems to be very small, as the two species with the highest concentration of tannins are those with the lowest LMA (Fig. 2). Concerning the tolerance strategy, the species with low LMA tend to have higher leaf area ratio (LAR, plant area/plant dry weight, $\text{m}^2 \text{ g}^{-1}$) (Cornelissen et al. 1996, 1998), which results in higher values of RGR (Antúnez et al. 2001). Another way to increase RGR is to increase the net assimilation rate (NAR) as RGR can also be calculated as $\text{LAR} \times \text{NAR}$ (Antúnez et al. 2001). Growing climatic conditions, especially temperature, can also affect RGR in *Quercus*, although the explanatory power of this factor can be low when compared to other environmental factors such as tree age or tree density (Villar et al. 2017). In our experiment, RGR of most species was obtained for an interval period with a mean temperature ca. $15.2 \text{ }^\circ\text{C}$ (Fig. 1), i.e., growth temperature of most species was similar, minimizing the possible effect

of temperature on RGR. Only three of the species (the Mediterranean evergreens) grew with a higher interval growth temperature (17–19 °C; Fig. 1) due to their late phenology. In any case, despite this higher temperature, RGR of these three species was not remarkable (Fig. 2). By contrast, *Q. muehlenbergii*, a cold-temperate oak, showed the highest RGR with one of the lowest interval temperatures (15.1 °C).

Additionally, we found that those oaks with high condensed tannins tended to have low RGR (e.g., *Q. lobata*, Fig. 2), and conversely, those oaks with high RGR had low to intermediate values of condensed tannins (e.g., *Q. muehlenbergii*, Fig. 2). This is in agreement with previous works reporting a tradeoff between constitutive chemical defense and tolerance (i.e., growth rate) within this group (Kosola et al. 2004; Stevens et al. 2007; Perkovich and Ward 2021). Nevertheless, as pointed out by Perkovich and Ward (2021), species with low constitutive values of chemical defense may develop a strong induced response, which would be compatible with high potential growth rates. Indeed, Perkovich and Ward (2021) found a negative association between growth rates and constitutive chemical defense, partially explained by the phylogeny, but did not find any tradeoff with induced chemical defense. In this regard, we encourage further assessment of the tradeoffs between defense traits, considering both constitutive and induced responses, and the eventual role of phylogenetic constraints, such as those imposed by elevated LMA (e.g., in the species from section *Ilex*, which tend to show high values of LMA; Sancho-Knapik et al. 2021).

5 Conclusion

Oaks within this study showed a trade-off among three leaf defensive strategies: leaf toughness, condensed tannins, and RGR that were associated to different levels of LMA. Oak species with high LMA developed very tough leaves that was associated with a larger structural resistance and longer leaf lifespans. By contrast, oaks with low LMA presented the lowest values of leaf toughness but favored between one of the two other strategies, higher constitutive concentrations of condensed tannins or having high growth rates.

Code availability

Not applicable.

Authors' contributions

Conceptualization: Domingo Sancho Knapik, Eustaquio Gil-Pelegrín, Juan Pedro Ferrio, José Javier Peguero-Pina; methodology: Rubén Martín-Sánchez, David Alonso-Forn, Eustaquio Gil-Pelegrín; formal analysis and investigation: Domingo Sancho Knapik; writing—original draft preparation: Domingo Sancho Knapik; writing—review and editing: Rubén Martín-Sánchez, José Javier Peguero-Pina, Juan Pedro Ferrio, Eustaquio Gil-Pelegrín; funding acquisition:

Juan Pedro Ferrio, Domingo Sancho Knapik; supervision: Eustaquio Gil-Pelegrín. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets generated during and/or analyzed during the current study are available in the citaREA repository, <https://doi.org/10532/6533>.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors gave their informed consent to this publication and its content.

Competing interests

The authors declare that they have no competing interests.

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