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Interactive effects of tree species composition and water availability on growth and direct and indirect defences in *Quercus ilex*

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Plant diversity has often been reported to decrease insect herbivory in plants. Of the numerous mechanisms that have been proposed to explain this phenomenon, how plant diversity influences plant defences via effects on growth has received little attention. In addition, plant diversity effects may be contingent on abiotic conditions (e.g. resource and water availability). Here, we used a long-term experiment to explore the interactive effects of tree species composition and water availability on growth, direct (i.e. phenolics) and indirect (i.e. volatile organic compounds – VOCs) defences and leaf herbivory in *Quercus ilex*. We quantified herbivory by chewing insects, phenolic compounds and VOCs in *Q. ilex* trees growing in stands differing in tree species composition (*Q. ilex*, *Q. ilex* + *Betula pendula*, *Q. ilex* + *Pinus pinaster* and *Q. ilex* + *B. pendula* + *P. pinaster*) and water availability (irrigated versus control). Both direct and indirect defences were affected by tree species composition, but such changes were not mediated by changes in tree stem diameter. *Quercus ilex* trees growing in stands with *P. pinaster* had the lowest concentration of both direct and indirect defences. Importantly, the effects of tree species composition on VOCs were exacerbated on irrigated blocks. Despite variation in defences, tree species composition did not affect herbivory in *Q. ilex*. Accordingly, we did not find any association between defences and insect herbivory. Our results suggest that changes in the micro-environment rather than growth-defence associations may mediate tree diversity effects on defences. In addition, reduced defensive investment in more diverse stands could negatively impact tree resistance masking the beneficial effects of species diversity at reducing insect herbivory.

Keywords: chemical defences, insect herbivory, phytochemical diversity, *Quercus ilex*, tree diversity, VOCs



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Introduction

Plant species diversity determines biotic interactions and ecosystem processes (Scherber et al. 2010, Cardinale et al. 2011), and has particularly strong effects on arthropod community composition and overall species richness at higher trophic levels (Crutsinger et al. 2006, Haddad et al. 2009) thus affecting ecosystem functioning. In the context of plant–insect herbivore interactions, an increase in plant diversity most generally results in a decrease of insect herbivory on focal plant species in both agricultural and forestry systems, a phenomenon known as associational resistance (Barbosa et al. 2009, Jactel et al. 2020). Different mechanisms have been identified as drivers of associational resistance. There are evidences that plant species diversity can disrupt host-finding processes through density-dependent processes (Jactel and Brockerhoff 2007, Hambäck et al. 2014, Field et al. 2020) whereby an increase in the diversity of heterospecific neighbours reduces the probability of a focal plant being located and colonized by its specialist herbivores ('resource concentration hypothesis') (Root 1973). This effect is further strengthened when heterospecific neighbours produce physical and chemical cues that reduce the apparency of the focal plant ('host apparency hypothesis') (Castagneyrol et al. 2013). Furthermore, there is also evidence that due to increased availability of refuges or alternative resources, plant species diversity should favour greater abundance and diversity of predators and parasitoids contributing to strengthen the top–down control on herbivores ('the enemies hypothesis') (Elton 1958, Staab and Schuldt 2020). However, several studies have also reported no changes (Cardinale et al. 2011, Haase et al. 2015) or even increased insect herbivory when mixing plant species (i.e. associational susceptibility – Schuldt et al. 2010, 2015, Castagneyrol et al. 2018). An alternative mechanism explaining associational susceptibility might be that polyphagous insects may benefit from higher dietary resource diversity in tree species mixtures (nutrient balance theory) causing higher herbivore damage (Westoby 1978, Schuldt et al. 2015). Thus, there is a need to re-evaluate the mechanisms determining the direction and strength of associational effects.

The above predictions are based on the idea of associational resistance or susceptibility where plant species diversity influences consumer foraging behaviour through increased habitat heterogeneity. However, host-finding is only one of the dimensions of plant–insect herbivore interactions and so

far, little attention has been paid to the trait-mediated effects of diversity on insect herbivory.

Plant species diversity can modify plant nutritional status and plant secondary metabolites that could potentially act as defensive traits mediating herbivory (Moreira et al. 2016a, Castagneyrol et al. 2017, 2018) in at least two independent ways. First (H1, Table 1), greater plant species diversity is expected to increase plant growth due to more efficient resource use (i.e. via resource partitioning and facilitation between a focal species and their neighbours) (Loreau and Hector 2001). In line with this, several studies have found mixed forest to be more productive and show higher biomass (Vilà et al. 2007, Zhang et al. 2012, Huang et al. 2018, Jactel et al. 2018). Following this thread, the growth–differentiation balance hypothesis predicts that under conditions of resource limitation where plants show reduced growth defensive investment is maximized, based on the expression of growth–defence tradeoffs (Herms and Mattson 1992). If growth and defences tradeoff this may lead to decreased allocation to defences by plants in mixed forest and, consequently, to increased herbivory (i.e. associational susceptibility). Second (H2, Table 1), tree diversity effects on trait expression could be driven by concomitant changes in the micro-environment (Muiruri and Koricheva 2017, Muiruri et al. 2019). Leaves exposed to direct light generally have lower nitrogen content and higher concentrations of anti-herbivore defences (Dudt and Shure 1994). In a mixed forest, light availability for a focal tree will depend on the growth rates of their heterospecific neighbours and this can determine the nutritional quality and anti-herbivore defences on the leaves. For instance, Castagneyrol et al. (2013) found lower production of phenolics by oak trees growing in mixed forest with taller faster-growing neighbours.

The few studies exploring the variability of plant defences with plant species diversity in trees, have reported contrasting effects, ranging from positive (Moreira et al. 2014, Poeydebat et al. 2021), negative (Castagneyrol et al. 2018, Muiruri et al. 2019) or no effect of tree diversity on plant traits (Castagneyrol et al. 2017, Muiruri et al. 2019), indicating that the mechanisms driving the magnitude and direction of trait-mediated associational effects on plant–insect herbivore interactions are not clear.

One of the reasons behind these inconsistencies may be that previous studies have not considered the multivariate nature of plant chemistry. In particular, secondary metabolites

Table 1. Summary of the main hypothesis explaining trait mediated effects of tree species diversity on insect herbivory. The table includes potential mechanisms and expected predictions for each case.

Hypothesis	Mechanism	Predictions
(H1) Tree species composition affects tree defences via effects on growth	Growth–defence tradeoffs	In mixed forest tree growth should be favoured over investment in anti-herbivore defences, ultimately leading to higher insect herbivory (i.e. associational susceptibility).
(H2) Tree species composition affects tree defences via changes in micro-environment conditions	Changes in abiotic conditions (e.g. light and temperature) may affect the production of anti-herbivore defences	In mixed forest, trees growing with faster-growing heterospecific neighbours should have less access to light and thus produce lower levels of defences

that mediate plant–insect herbivore interactions (i.e. direct and indirect defences) must be considered. Phenolic compounds are secondary metabolites widely produced by plants and involved in many ecological processes. These compounds have been described to act as direct plant defences (i.e. plant traits that deter or reduce herbivore feeding) against insects and pathogens (Feeny 1970, Matsuki 1996) and to be involved in associational susceptibility processes in mixed forest (Castagneyrol et al. 2017). In addition, volatile organic compounds (VOCs) are also phytochemicals from the secondary metabolism that play an important role in mediating plant–insect herbivore interactions in different ways. VOCs can act as chemical cues mediating host-finding processes (Massad et al. 2017) and as indirect defences (i.e. plant traits that recruit natural enemies of herbivores such as predators and parasitoids) (Kessler and Baldwin 2001, Pearse et al. 2020). In addition, VOCs may also play an important role in plant-to-plant communication (Karban et al. 2014a, b). Plants can recognize VOCs emitted by their neighbours in response to herbivory and adjust their defensive phenotype (i.e. priming effect) (Karban et al. 2000, Karban and Shiojiri 2009). Despite these compounds are recognized to play relevant roles in ecological processes, there is an important research gap at exploring the role of VOCs mediating associational effects (Moreira et al. 2016a, Jactel et al. 2020).

Recent studies have suggested that the effects of plant species diversity on plant–insect herbivore interactions might be modulated by environmental factors (Ratcliffe et al. 2017, Jactel et al. 2018). Specifically, trait-mediated effects of plant diversity on insect herbivores could be modulated by climatic factors (Muiruri et al. 2019, Poeydebat et al. 2021). Among climatic factors, water availability is particularly influential on plant–insect herbivore interactions as it modifies the chemical content of leaves (Walter et al. 2012) and was found to modulate the effect of plant diversity on insect herbivory (Castagneyrol et al. 2018, Field et al. 2020). Yet, the mechanisms underlying the interactive effects of plant diversity and water availability on plant defences and insect herbivory remains to be deciphered (Castagneyrol et al. 2018, Poeydebat et al. 2021).

In this study, we tested for the independent and interactive effects of tree species composition and water availability on growth, defences and insect herbivory in the holm oak *Quercus ilex*. In addition, we further tested whether plant growth and defences were associated (and potentially mediated) with tree species composition and water availability effects on leaf herbivory. For this, we quantified height and diameter, direct (phenolic compounds) and indirect (VOCs) chemical defences, and insect leaf herbivory (i.e. the percentage of leaf area removed) in *Q. ilex* trees growing in stands with different tree species composition under contrasting irrigation treatments (irrigated versus non-irrigated). We expect that tree species diversity would have a positive effect on tree growth, compromising the investment in defences and leading to increased levels of herbivory, particularly under low water availability conditions due to higher resource constraints. Overall, this study builds towards a more

comprehensive understanding of the mechanisms driving associational effects on plant–insect herbivore interactions.

Material and methods

Natural history

The holm oak *Quercus ilex* is an evergreen, slow-growing and shade-tolerant species native to central-western Mediterranean basin (Rodà et al. 1999, De Rigo and Caudullo 2016). Its distribution is mostly limited to the Mediterranean basin and extends from Portugal to Turkey, with some populations in the Atlantic coast of France. This species grows in different habitats from mixed forest, scrublands or pure stands and under different climates, from semi-arid to very humid. *Quercus ilex* is a sclerophyllous species with dark coriaceous leaves covered by white hairs on the abaxial surface that reduces transpiration improving tree resistance to drought. However, it has been reported that the species is a bad competitor with other deciduous oaks and with conifers under extreme water stress (Barbero et al. 1992), circumstances under which *Q. ilex* trees can even suspend the vegetative activities during drought periods (De Rigo and Caudullo 2016).

Across its distribution range, a wide array of specialist and generalist insect herbivores commonly feed on this species, including leaf chewers, miners and skeletonizers (Southwood et al. 2005, Ruiz-Carbayo et al. 2017, Hernández-Agüero et al. 2022). In response to these attacks, *Q. ilex* produces a wide variety of secondary metabolites in leaves that potentially act as direct or indirect defences against biotic and abiotic stressors, including phenolic compounds (Moreira et al. 2019b, 2020) and VOCs (Holzinger et al. 2000). Although the function of VOCs as indirect defences has not been tested yet in *Q. ilex*, these compounds have been found to attract natural enemies of herbivores in other *Quercus* species (Ghirardo et al. 2012, Pearse et al. 2013).

Experimental design

This study was conducted in the ORPHEE experimental trial established in 2008 in south-west France (44°44'N, 00°46'W) (Castagneyrol et al. 2017). The experimental design consisted of eight blocks and 32 plots within each block. Each plot represented a tree species composition treatment, corresponding to 31 possible combinations of one to five tree species (*Betula pendula*, *Quercus robur*, *Q. pyrenaica*, *Q. ilex* and *Pinus pinaster*) and an additional plot replicate of the five species mixture. Each plot contained 10 rows of 10 trees planted 2 m apart (100 trees on 400 m²). Tree species mixtures were established according to a substitutive design, keeping tree density of tree neighbours equal across plots. Within plots, individual trees from different species were planted in a regular alternate pattern, such that a tree from a given species had at least one neighbour from each of the other species within a 2-m radius.

From 2015 four out of the eight experimental blocks were allocated to an irrigation treatment consisting of sprinkling

the equivalent of 3 mm precipitation from a 2 m height pole in the centre of each irrigated plot. Blocks were irrigated on a daily basis, at night, from May to October. The four remaining blocks were kept as controls (Castagneyrol et al. 2017). Pre-dawn water potential (Ψ) measured in two *Q. ilex* trees in three irrigated and three control monocultures in July 2019 was almost three times lower (i.e. greater drought stress) on average (mean $\pm$ SE, $n = 12$) in control ($\Psi = -0.493 \pm 0.08$) than in irrigated plots ($\Psi = -0.175 \pm 0.02$).

For the current study, we focused on *Q. ilex* as target species and selected six blocks (three irrigated and three control) and four plots (tree species composition treatments) in each block corresponding to the monoculture of *Q. ilex* and its combinations with *B. pendula* and *P. pinaster* (*Q. ilex* + *B. pendula*, *Q. ilex* + *P. pinaster* and *Q. ilex* + *B. pendula* + *P. pinaster*). Therefore, a total of 24 experimental plots (4 tree species composition treatments $\times$ 2 irrigation treatments $\times$ 3 blocks) were included in the study. *Quercus ilex* trees were on average 2.9 ± 0.108 m tall (mean $\pm$ SE; $n = 431$), being smaller than *B. pendula* (7.2 ± 0.9 m; $n = 140$) and *P. pinaster* trees (11.6 ± 0.15 m; $n = 143$) (based on additional data gathered in 2020). It follows that *Q. ilex* was under the cover of birch and pine and was therefore less apparent in mixed stands than in monocultures.

Sampling and measurements

At the end of the growing season (September 2019), we haphazardly selected four *Q. ilex* trees in each of the 24 plots ($n = 96$ trees). Trees in the plot margins were not selected to avoid border effects. First, we estimated total height and basal diameter (± 30 cm aboveground) in all experimental trees with a tape-measure and a digital caliper respectively. After tree growth measurements, we collected VOCs for each tree following (Rasmann et al. 2011). Briefly, we bagged one branch of each tree with a 1 l nalophan bag and we trapped the compounds on a charcoal filter by pulling air through the filter using an air-sampling pump for 2 h at a rate of 250 ml min^{-1} . Importantly, we sampled air VOCs in empty bags (one bag placed in the middle of each plot within each block) as controls, in order to identify compounds that may contaminate the blend of VOCs taken from the focal trees (e.g. VOCs emitted by neighbour species). After collecting the VOCs, we stored the filters at -80°C until chemical analyses.

Right after VOCs collection, we haphazardly collected 20 fully expanded and developed leaves throughout the tree's canopy. Importantly, because *Q. ilex* is an evergreen species, sampled leaves may have consisted of one to three cohorts of leaves (i.e. produced between 2017 and 2019; up to two-years old). For each leaf, we visually estimated the percentage of leaf area removed by insect herbivores (mostly leaf chewers) using the following scale: 0 = no damage; 1 = 1–5% damaged; 2 = 6–10% damaged; 3 = 11–25% damaged; 4 = 26–50% damaged; 5 = 51–75% damaged; 6 $\geq$ 75% damaged ('leaf herbivory' hereafter) (Galmán et al. 2021). We averaged class values across all leaves to obtain a mean value per tree for statistical analyses. We selected a subset of 4–5 leaves with

little or no evidence of herbivory for further chemical analyses of phenolic compounds (Moreira et al. 2019a). Leaves were oven-dried for 48 h at 40°C .

Chemical analyses

Quantification of volatile organic compounds (VOCs)

To analyse VOCs, we performed gas chromatography and mass spectrometry analyses. To extract the compounds from the charcoal traps, we first added 5 μl of naphthalene (20 ng ml^{-1}) as an internal standard to the traps (Pellissier et al. 2016), and then eluted their contents with 400 μl of dichloromethane. We then injected 2 μl of the extract for each sample into a gas chromatograph (GC) coupled with a mass selective detector (MSD) fitted with a $30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \text{ mm}$ film thickness HP-5MS fused silica column. We operated the GC in splitless mode with helium as the carrier gas (constant flow rate 0.9 ml min^{-1}). The GC oven temperature program was: 1 min hold at 40°C , and then $10^\circ\text{C min}^{-1}$ ramp to 240°C . We identified individual volatile compounds (i.e. terpenes) using Kovats retention index from published work (Supporting information), the NIST Standard Reference Database 1A v17, and by comparison with commercial standards when available. Volatile emissions are reported as nanograms naphthalene equivalents. For subsequent analyses, we selected VOCs identified as either monoterpenes or sesquiterpenes (Supporting information). We quantified individual monoterpenes and sesquiterpenes relative to the internal standard and used for statistical analyses those exhibiting a relative abundance higher than 1%. Importantly, for those compounds present in both the samples and the corresponding control, we only consider those which intensity in the sample was at least double than in the control. Finally, we quantified the total concentration of VOCs as the sum of concentrations of all individual compounds.

Quantification of phenolic compounds

We extracted phenolic compounds from 20 mg of dry leaf tissue with 1 ml of 70% methanol in an ultrasonic bath for 15 min, followed by centrifugation (Moreira et al. 2020) and transferred the extracts to chromatographic vials. To analyse the phenolic compounds, we performed chromatographic analyses using ultra-high performance liquid chromatography equipped with a Nexera SIL-30AC injector and one SPD-M20A UV/VIS photodiode array detector. The compound separation was carried out on a Kinetex $2.6 \mu\text{m}$ C18 $82\text{--}102 \text{ \AA}$, LC Column $100 \times 4.6 \text{ mm}$, protected with a C18 guard cartridge. The flow rate was 0.4 ml min^{-1} and the oven temperature was set at 25°C . The mobile phase consisted of two solvents: water–formic acid (0.05%) (A) and acetonitrile–formic acid (0.05%) (B), starting with 5% B and using a gradient to obtain 30% B at 4 min, 60% B at 10 min, 80% B at 13 min and 100% B at 15 min. The injection volume was 15 μl . For phenolic compound identification, we used an ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry. We identified four groups of phenolic compounds:

flavonoids, ellagitannins and gallic acid derivatives ('hydrolysable tannins' hereafter), proanthocyanidins ('condensed tannins' hereafter) and hydroxycinnamic acid precursors to lignins ('lignins' hereafter). We quantified flavonoids as rutin equivalents, condensed tannins as catechin equivalents, hydrolysable tannins as gallic acid equivalents and lignins as ferulic acid equivalents (Moreira et al. 2020, Galmán et al. 2021). The quantification of these was conducted by external calibration using the corresponding calibration curve at 0.25, 0.5, 1, 2 and 5 $\mu\text{g ml}^{-1}$ for each of the four standards used (rutin, catechin, gallic acid and ferulic acid). We expressed phenolic compound concentrations in mg g^{-1} tissue on a dry weight basis.

Statistical analyses

We used linear mixed models to test for the individual and interactive effects of tree species composition and irrigation on growth traits (height and diameter), the quantitative profile (concentration) of direct (i.e. phenolics) and indirect (i.e. VOCs) defences and leaf herbivory. Models included tree species composition, irrigation and their interaction as fixed effect factors. The block by irrigation interaction and the plot (nested within block) were included in the model as random factors to control for potential spatial variation. Concentration of VOCs was log-transformed to meet normality assumptions. When a significant interaction between tree species composition and irrigation was observed, we computed contrasts of least squared means among treatment groups (for both tree species composition and irrigation factors). Linear mixed models were run using the *lmer* function from the *lmerTest* package in R (Kuznetsova et al. 2017). We considered significant effects those with an associated p-value < 0.05 .

We also explored whether tree species composition and irrigation affected the qualitative profile of indirect defences (i.e. diversity and composition of VOCs). First, to explore variation patterns in the phytochemical diversity of VOCs we computed a Shannon diversity index (SDI) for each tree as a proxy of VOC's diversity (Wetzel and Whitehead 2020). The SDI accounts for both the number of unique compounds (i.e. richness) and their relative abundance (i.e. evenness) in each sample. The individual and interactive effects of tree species composition and irrigation on SDI were tested by fitting the same linear mixed model described above. Second, we used a permutational multivariate analysis of variance (PERMANOVA) to explore the individual and interactive effects of tree species composition and irrigation on the composition of VOCs using data of individual compounds. The PERMANOVA included tree species composition, irrigation and their interaction as fixed factors. When a significant interaction between tree species composition and irrigation was observed, we ran separate models for each irrigation treatment in order to evaluate whether the effects of tree species composition differed between irrigation treatments. PERMANOVA is analogous to an ANOVA, but partitions similarity matrices between treatments and uses

permutation tests with pseudo F-ratios. The PERMANOVA was based on 10 000 permutations using the *adonis* function from the *vegan* package in R (Oksanen et al. 2010). We considered significant effects those with an associated p-value < 0.05 . To visualize the results of this analysis, we used pairwise Bray–Curtis dissimilarities as input to a principal coordinate analysis (PCoA; unconstrained ordination). The result of this analysis was then visualized in two dimensions. We selected influential mono- or sesquiterpenes for which $R^2 > 0.40$ ($p < 0.05$) for associations with the first two ordination axes and displayed them using biplot arrows with length scaled to R^2 values. We used the *capscale* function to run the PCoA and the *envfit* function to obtain vector (arrow) values, both functions from the *vegan* package in R.

Finally, we fitted a piece-wise structural equation model (pSEM) at the tree level to test whether plant growth and defences were associated and potentially mediated tree species composition and water availability effects on *Q. ilex* leaf herbivory. For this, we tested for the direct effects of height and diameter on defensive traits showing variation among tree species composition or irrigation treatments, and the direct effects of such defensive traits on leaf herbivory while accounting for the effects of the random factors (block by irrigation interaction and the plot (within block)). We also tested for the indirect effects of growth on herbivory (i.e. the effects of height or diameter on leaf herbivory mediated by defensive traits). Direct effects were estimated as standardized partial regression coefficients, while indirect associations were calculated by fitting a multiple regression model between the two variables of interest (growth measures and defences) with any conditioning variables included as covariates (i.e. defensive traits). Significance of direct and indirect coefficients were assessed with t-tests and considered significant when $p < 0.05$. The goodness of fit of the general model was evaluated with a 'test of direct separation' based on Fisher's C-test (Lefcheck 2016). The pSEM analysis was performed using the *psem* function from the *piecewiseSEM* package in R (Lefcheck 2016).

Results

Tree species composition and irrigation effects on tree growth

Quercus ilex height was not affected by tree species composition (Table 2), but there was a trend towards larger tree diameter in the monoculture (3.57 ± 1.63 cm; mean $\pm$ SE), with the lowest value in the two species mixture including *P. pinaster* (2.06 ± 0.77 cm). However, the effect of tree species composition on tree diameter was only marginally significant (Table 2, Supporting information). The irrigation treatment significantly affected tree growth (Table 2). In particular, *Q. ilex* were on average 1.3 times taller in irrigated (3.14 ± 0.16 m) than in non-irrigated plots (2.27 ± 0.15 m) (Fig. 1A). There was no significant effect of the interaction

Table 2. Results of linear mixed models showing the effect of tree species composition (four levels; Q: *Quercus ilex* monoculture; Q + B: *Q. ilex* + *Betula pendula*; Q + P: *Q. ilex* + *Pinus pinaster*; Q + B + P: *Q. ilex* + *B. pendula* + *P. pinaster*), irrigation (two levels, control versus irrigated) and their interaction on 1) growth (height and diameter), 2) the concentration of direct (phenolic compounds: condensed tannins, hydrolysed tannins, lignin and flavonoids) and indirect (VOCs – volatile organic compounds identified as either monoterpenes or sesquiterpenes) defences, 3) the diversity of indirect defences (Shannon diversity index computed for VOCs; SDI) and 4) leaf herbivory. F-values (F), degrees of freedom (numerator, denominator) and associated p-values (p) are shown. Significant p-values (< 0.05) are highlighted in bold.

	Irrigation			Tree species composition			Irrigation × tree species composition		
	df _(Num, Den)	F	p	df _(Num, Den)	F	p	df _(Num, Den)	F	p
Height	1,16	8.10	0.012	3,16	0.26	0.857	3,16	1.21	0.337
Diameter	1,16	2.59	0.127	3,16	3.08	0.057	3,16	1.00	0.416
Condensed tannins	1,87	3.41	0.068	3,87	0.80	0.494	3,87	0.52	0.664
Hydrolysed tannins	1,87	3.70	0.057	3,87	0.48	0.700	3,87	2.00	0.120
Lignin	1,87	0.63	0.430	3,87	2.54	0.062	3,87	0.76	0.522
Flavonoids	1,87	0.00	0.972	3,87	10.52	< 0.001	3,87	1.06	0.372
VOCs	1,88	10.34	0.002	3,88	20.77	< 0.001	3,88	3.05	0.033
SDI	1,4	0.97	0.381	1,84	17.55	< 0.001	1,84	1.88	0.139
Leaf herbivory	1,88	0.01	0.908	3,88	0.80	0.495	3,88	1.46	0.232

between tree species composition and irrigation treatments on tree growth (Table 2).

Tree species composition and irrigation effects on direct and indirect defences

Tree species composition treatment significantly affected the concentration of flavonoids, but had no significant effects on any other group of phenolic compounds (Table 2). In particular, the concentration of flavonoids was the highest in the monoculture ($7.79 \pm 0.62 \text{ mg g}^{-1}$), and the lowest in the two ($4.85 \pm 0.30 \text{ mg g}^{-1}$) and three ($4.58 \pm 0.34 \text{ mg g}^{-1}$) species mixtures including *P. pinaster* (Fig. 1B).

Both tree species composition and irrigation affected the quantitative profile (i.e. concentration) of indirect defences, but the effect of both factors was interactive (significant tree species composition by irrigation interaction) (Table 2). Specifically, there was a larger effect of tree species composition on the concentration of VOCs on irrigated blocks, where the concentration of VOCs was the lowest in the two (1571.82 ± 464.20) and three (3410.59 ± 947.13) species mixtures including pines (Fig. 1C). Similarly, irrigation reduced the concentration of VOCs in *Q. ilex* trees but only when growing in mixed stands with pines (Fig. 1C).

Tree species composition treatments significantly affected VOC diversity (Table 2). In particular, the diversity of VOCs was the highest in the two (0.11 ± 0.005) and three (0.09 ± 0.007) species mixture with *P. pinaster* and the lowest in the *Q. ilex* monoculture (0.07 ± 0.003) (Fig. 1D). Irrigation and its interaction with tree species composition did not significantly affect VOC diversity (Table 2).

The PERMANOVA analysis revealed qualitative effects of both tree species composition and irrigation on indirect defences (Table 3). In particular, tree species composition explained 18% of variation in VOCs composition, while the irrigation treatment only explained as little as 3% of the variation (Table 3). The first two axes of the ordination together accounted for ~54% of the variation in VOCs among tree

species composition treatments (37% and 16.99% respectively, Fig. 2A), which was primarily associated with variation in the relative amount of α -pinene ($R^2=0.56$, $p < 0.05$), sabinene ($R^2=0.44$, $p < 0.05$) and α -thujene ($R^2=0.40$, $p < 0.05$) (Fig. 2A). In particular, the presence/absence of *P. pinaster* trees in a plot discriminated individuals with different VOCs composition profiles. In the case of the irrigation treatment, the first two axes of the ordination together accounted for ~50% of the variation in VOCs (30.3% and 19.76% respectively, Fig. 2B), which was primarily associated with variation in the relative amount of α -pinene and sabinene ($R^2=0.40$, $p < 0.05$ for both compounds) (Fig. 2B).

Again, we observed a significant tree species composition × irrigation interaction on the composition of VOCs (Table 3). PERMANOVAS performed separately for each irrigation treatment showed that tree species composition effect was stronger in irrigated plots (pseudo-F=7.33, $R^2=0.33$, $p < 0.001$) than in non-irrigated control plots (pseudo-F=3.95, $R^2=0.21$, $p=0.001$).

Tree species composition and irrigation effects on herbivory

We did not observe any significant effect of tree species composition, irrigation and their interaction on insect leaf herbivory (Table 2).

Direct and indirect effects of growth on defences and herbivory

The pSEM revealed a significant direct negative association between height and the concentration of flavonoids (standardized partial regression coefficient = -0.29 ; $p=0.038$) (Supporting information). We did not find any significant direct association between defences and herbivory, neither a significant indirect association between growth and herbivory mediated by defensive traits (Supporting information).

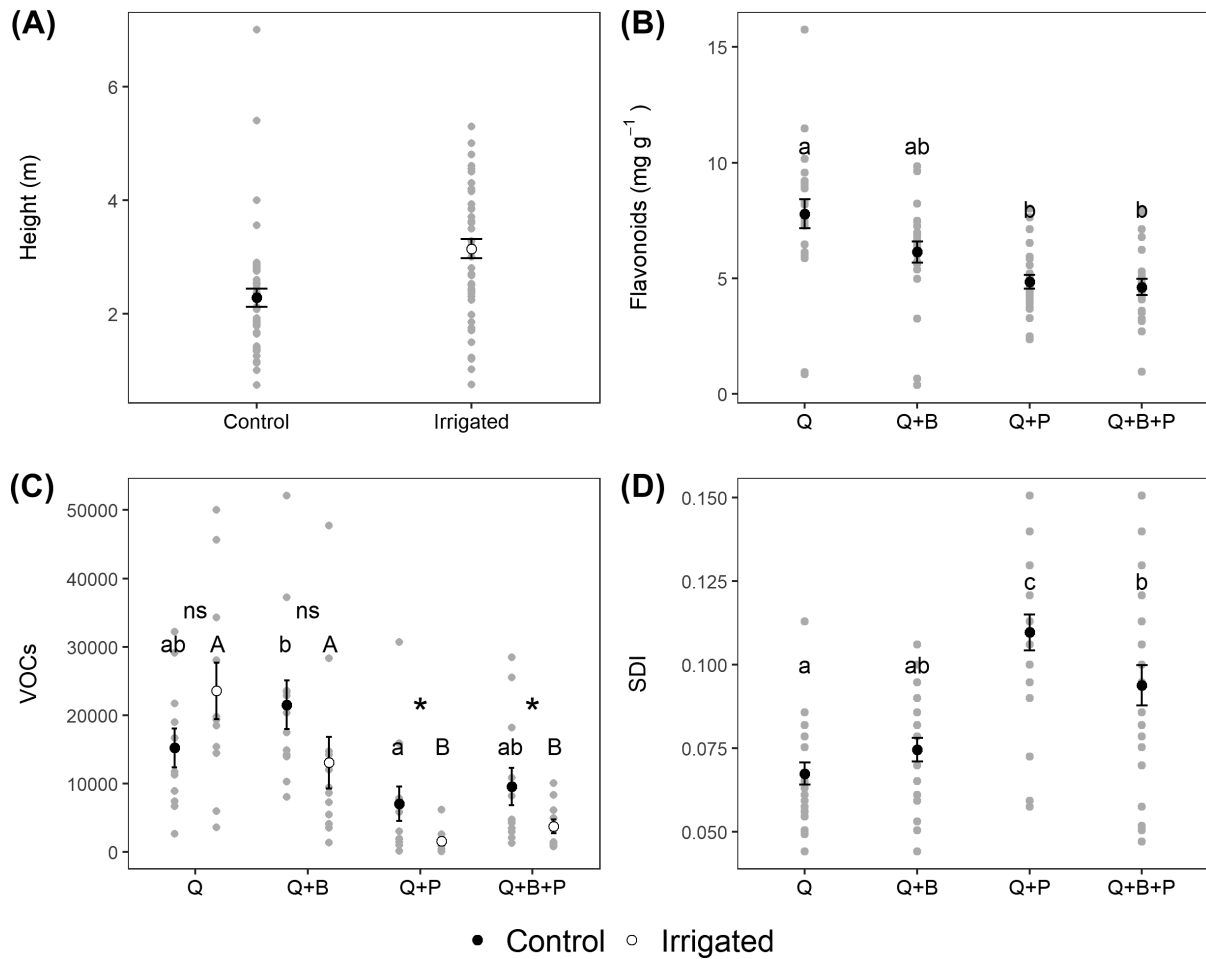


Figure 1. (A) Height (m) of *Quercus ilex* trees growing in control and irrigated stands. (B) Concentration of flavonoids (mg g⁻¹). (C) Concentration (naphthalene equivalents) of VOCs (volatile organic compounds identified as either monoterpenes and sesquiterpenes). (D) Shannon diversity index (SDI) calculated for VOCs. Dots represent means ± standard errors for each categorical group of irrigation (irrigation versus control) and/or tree species composition (Q: *Quercus ilex* monoculture; Q+B: *Q. ilex* + *Betula pendula*; Q+P: *Q. ilex* + *Pinus pinaster*; Q+B+P: *Q. ilex* + *B. pendula* + *P. pinaster*). Grey dots in the background represent the concentration of flavonoids of each *Q. ilex* tree (n=96). Same letters indicate non-significant differences among groups. In (C), black and open (white) dots represent means ± standard errors for control and irrigated trees respectively in each tree species composition group. Lowercase letters refer to comparison among tree species composition treatments in the irrigated trees. Capital letters refer to comparison among tree species composition treatments in the control trees. Significant irrigation effects in each tree species composition treatment are indicated with stars (p < 0.05 = *; p > 0.05 = ns).

Table 3. Results of the PERMANOVA analysis showing the effect of tree species composition (four levels: *Quercus ilex* monoculture; Q+B: *Q. ilex* + *Betula pendula*; Q+P: *Q. ilex* + *Pinus pinaster*; Q+B+P: *Q. ilex* + *B. pendula* + *P. pinaster*), irrigation (two levels, control versus irrigated) and their interaction on the qualitative profile (composition) of VOCs (volatile organic compounds identified as either monoterpenes and sesquiterpenes). Pseudo F-values (pseudo-F), R² values and p-values (p) are shown for each factor. Significant p-values (p<0.05) are highlighted in bold.

	Pseudo-F	R ²	p
Irrigation	3.64	0.03	0.008
Tree species composition	7.01	0.18	< 0.001
Irrigation × tree species composition	1.78	0.05	0.045

Discussion

We found that tree species composition and water availability interactively shaped direct and indirect chemical defences in *Q. ilex* leaves. However, and contrarily to our expectations, we found no evidence that these changes were mediated by growth-defence associations, or that had consequences on insect herbivory.

Tree species composition and irrigation effects on growth

Despite significant effects of tree species composition on tree growth, *Q. ilex* trees in the monoculture tended to have the largest diameter. This result runs counter our expectations,

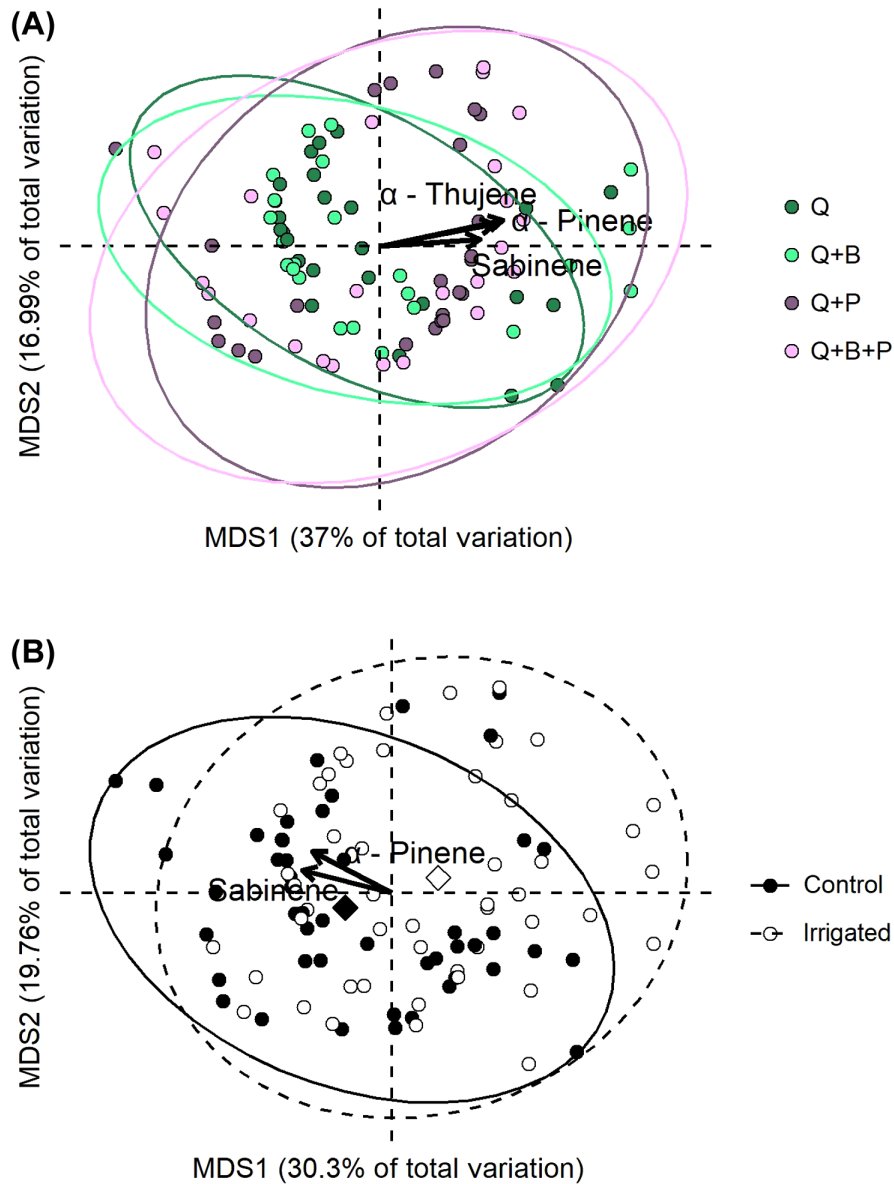


Figure 2. (A) Unconstrained ordinations (principal coordinates analysis – PCoA) of tree composition effects on VOCs (volatile organic compounds identified as either monoterpenes or sesquiterpenes). Biplot arrows show associated linear trends with terpenes, scaled to reflect relative magnitude of effects based on R^2 values ($R^2 > 0.40$, $p < 0.05$). The ordination displays 95% ellipses associated to tree composition treatment centroids (Q: *Quercus ilex* monoculture; Q+B: *Q. ilex*+*Betula pendula*; Q+P: *Q. ilex*+*Pinus pinaster*; Q+B+P: *Q. ilex*+*B. pendula*+*P. pinaster*). PERMANOVA indicates that 19% of variation in the composition of VOCs is explained by the tree composition treatment. Overall, the first two axes of ordination accounted for ~54% variation. (B) Unconstrained ordinations (principal coordinates analysis – PCoA) of irrigation effects on VOCs (volatile organic compounds identified as either monoterpenes or sesquiterpenes). Biplot arrows show associated linear trends with terpenes, scaled to reflect relative magnitude of effects based on R^2 values ($R^2 > 0.40$, $p < 0.05$). The ordination displays irrigated and control centroids (black and white diamonds respectively) and corresponding 95% ellipses (solid and dashed lines respectively). PERMANOVA indicates that 3% of variation in the composition of VOCs is explained by the irrigation treatment. Overall, the first two axes of ordination accounted for ~50% of variation. Irrigated ($n = 48$) and control ($n = 48$) trees are depicted as closed and open circles, respectively.

since plant diversity (including trees) has usually been associated with increased plant growth and productivity (Vilà et al. 2007, Moreira et al. 2012, Huang et al. 2018, Jactel et al. 2018). However, although theory predicts that tree species diversity increases tree growth throughout niche complementarity, it may also strengthen competition by means

of physical (i.e. light or resource competition) or chemical (i.e. allelopathy) interferences with other tree species (Fernandez et al. 2016), in particular for slow growing species (e.g. *Q. ilex*) surrounded by faster growing neighbours (e.g. pines, and birches). Finally, we found that *Q. ilex* trees grew taller in irrigated blocks than in control blocks. This result

was expected, since irrigation reduced drought stress in comparison to control blocks (lower water potential in control than irrigated blocks).

Tree species composition affected direct defences

We found that anti-herbivore direct defences (i.e. phenolics) in *Q. ilex* were lower in mixed stands than in monocultures. A handful of studies have explored the effects of tree diversity on direct defences in a focal plant species (Moreira et al. 2014, Castagneyrol et al. 2017, 2018, Rosado-Sánchez et al. 2018, Muiruri et al. 2019, Poeydebat et al. 2021). In agreement with our results, Castagneyrol et al. (2018) found that *B. pendula* trees had lower concentration of phenolic compounds when growing in mixed stands. Alternatively, Poeydebat et al. (2021) found that the production of phenolic compounds in *B. pendula* increased with tree species richness, and Moreira et al. (2014) found a positive effect of plant diversity on polyphenolics and condensed tannins in *Swietenia macrophylla*. Finally, other studies have reported neutral effects of tree diversity on direct chemical defences (Castagneyrol et al. 2017, Muiruri et al. 2019). Contrasting results from different studies thus prevent any generalization of the effects of tree diversity on tree direct defences.

We predicted that variation in chemical defences among tree species composition treatments would be mediated by carry-over effects on tree growth as predicted under a growth-defence tradeoff scenario (H1). Counter to this prediction, we found no evidence that the effect of tree species composition on chemical defences was related to changes in tree growth patterns. However, it is noteworthy that we lack data on total belowground or aboveground tree biomass, traits that may be better proxies of tree growth and could thus show stronger associations with defences than tree height or diameter. An alternative is that the effects of tree species composition on direct defences are mediated by changes in the abiotic environment (H2). Considering that companion trees in mixed stands (namely *P. pinaster* and *B. pendula*) were taller than *Q. ilex*, lower light availability in mixed stands likely led to lower photosynthetic rates and ultimately lower production of defences (Koricheva et al. 1998, Roberts and Paul 2006). This may also help to explain the fact that only flavonoids were affected by tree species composition. Flavonoids are also involved in photo-protection against ultraviolet light (Harborne and Williams 2000). Thus, these compounds may be more sensitive to changes in tree diversity (and especially to the effect of micro-environment) than other groups like tannins, which act mainly as herbivore deterrents (Barbehenn and Constabel 2011).

Tree species composition and irrigation interactively affected the quantitative and qualitative profile of indirect defences

We found that tree species composition quantitatively affected indirect defences (i.e. VOCs), but that effect was contingent on the level of abiotic stress (i.e. water availability). More

specifically, when trees were irrigated, the concentration of VOCs was the lowest in mixed stands with pines. Plant diversity has been previously reported to lead to quantitative changes on the emission of VOCs in annual plants. For instance, Kigathi et al. (2013) found that the concentration of VOCs in *Trifolium pratense* was higher when growing with heterospecific than conspecific neighbours. Again, we speculate that quantitative effects of tree species composition on VOCs could be related to changes in the abiotic environment as discussed above for direct defences, since the emission of VOCs is strongly affected by abiotic factors such as light and temperature (Penuelas and Llusà 2001). This may also help to explain the fact that tree species composition effects on indirect defences were stronger on irrigated blocks, since positive effects of irrigation on companion species could have enhanced shading on *Q. ilex* in mixed stands, thus exacerbating tree species composition effects on tree defences in irrigated blocks.

In addition to quantitative effects, tree species composition also led to qualitative changes in the emission of VOCs. More specifically, *Q. ilex* trees emitted different blends of VOCs in the presence of pines, being this pattern particularly striking in irrigated blocks. Interestingly, the diversity of VOCs (i.e. SDI) was higher in mixed stands with pines as compared to monocultures. As far as we know, this is one of the few studies to explore qualitative changes in the emission of VOCs due to plant diversity (but see Dahlin et al. 2018), thus mechanisms underlying such effects remain unknown. Qualitative changes in the emission of VOCs among tree species composition treatments may be explained by multiple factors. First, plant-plant communication may rely on different chemotypes (i.e. composition of VOCs), where conspecific individuals usually share and recognise specific chemotypes (Karban et al. 2014a, 2016, Moreira et al. 2016b). Thus, it may be expected that the blend of VOCs emitted by an individual differs with heterospecific neighbours. Second, mixed stands may harbour larger diversity of insect herbivores (Murdoch et al. 1972, Moreira et al. 2016a). Since the emission of particular and unique VOCs involved in plant-plant communication is specific and contingent on the herbivore species (Moreira et al. 2018, Moreira and Abdala-Roberts 2019), higher diversity of VOCs in mixed stands compared to monocultures may be due to a greater diversity of herbivores. Third, specific blends of VOCs may be involved in the attraction of different herbivore natural enemies (i.e. predators and parasitoids) that provide indirect defence (Hare 2011). Mixed stands usually have higher diversity of herbivore natural enemies (Moreira et al. 2016a, Nell et al. 2018) and thus a larger diversity of VOCs may be involved in indirect defence.

Variation in direct and indirect defences did not mediate herbivory patterns

Contrary to our expectations, we did not find evidence of tree species composition affecting insect leaf herbivory. Our results run counter a recent meta-analysis showing that trees growing in mixed stands suffer, on average, less damage by insect

herbivores than monocultures (i.e. associational resistance) (Jactel et al. 2020). The absence of a clear tree species composition effect in insect herbivory is however not uncommon (Moreira et al. 2014, Haase et al. 2015, Castagneyrol et al. 2017), particularly in young trees (Grossman et al. 2018), when herbivory is assessed as total damage rather than the abundance of damage caused by a well identified – often specialist – herbivore species (Castagneyrol et al. 2014, Jactel et al. 2020).

More surprising is the lack of any association between herbivory and chemical defences. This may be explained by the confounding effects of tree apparency and chemical defences on tree herbivory. In our study, *Q. ilex* trees showed, on average, lower growth than *B. pendula* and *P. pinaster*, thus being less apparent than the other species in the mixed stands. Accordingly, we would expect to observe lower leaf herbivory damage in mixed stands than in monocultures. Alternatively, lower defence production (both direct and indirect defences) in mixed stands may lead to reduced resistance to insects, masking the beneficial effects of associational resistance, and thus leading to a neutral net effect of tree species composition on herbivory. Although this idea could seem over speculative, it suggests that the outcomes of associational effects on insect herbivory will depend on the relative importance of tree species composition effects on the apparency of a focal species and its defensive investment (Poeydebat et al. 2021). In addition, it is noteworthy to mention that anatomical defences may be more relevant than chemical defences at determining herbivory patterns (Carmona et al. 2011). Specifically, previous research has shown that variation in leaf toughness in oak species was negatively associated with herbivore performance (Pearse 2011, Pearse and Karban 2013). Further research exploring defensive traits associated with herbivory patterns should thus consider a wide array of defensive mechanisms, including both anatomical and chemical defences.

Study limitations and future research

Our results do not support the view that variation in chemical defences among tree species composition treatments is mediated by carry-over effects on tree growth (H1), but rather likely associated with changes in the micro-environment (H2). Further research should thus test this hypothesis and explore potential abiotic factors that may mediate tree species composition effects. We also show that variation patterns in the production of direct and indirect defences in *Q. ilex* trees across tree species composition treatments were not associated with insect herbivory. Our results thus recall the importance of taking into account diverse factors that may underlie the mechanistic basis of associational effects on plant–insect herbivore interactions. First, we did not consider the presence of herbivore natural enemies (top–down control of herbivory) and how this may be mediated by the emission of VOCs across tree species composition treatments (Hare 2011, Pearse et al. 2020). Second, the diversity gradient considered in this study was rather small

(monocultures to three species mixtures), and species composition represents one of the many dimensions of species diversity. In addition, we did not consider the understory growing naturally in the experimental trial, and thus species diversity in each plot may have been overestimated. For instance, monocultures of *Q. ilex* showed a denser understory than polycultures with *B. pendula* and *P. pinaster*. Third, associational effects may be more relevant at the community level than those observed in a focal plant species, for both defences and concomitant herbivory patterns. Previous research has shown that the emission and diversity of VOCs at the community level may be higher in more diverse communities (Salazar et al. 2016, Kigathi et al. 2019), and may ultimately affect insect herbivory patterns (Salazar et al. 2016). Further research should thus address the study of plant defences (both direct and indirect) and insect herbivory at the community level. Incorporating a community perspective will lead to a more comprehensive and deep understanding of the mechanistic basis of tree diversity effects on plant–insect herbivore interactions.

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Author contributions

Andrea Galmán and **Carla Vázquez-González** contributed equally to this paper and share first authorship. **Andrea Galmán**: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing – original draft (equal); Writing – review and editing (equal). **Carla Vázquez-González**: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Writing – original draft (equal); Writing – review and editing (equal). **Gregory Röder**: Data curation (supporting); Methodology (supporting); Writing – review and editing (equal). **Bastien Castagneyrol**: Conceptualization (equal); Supervision (supporting); Writing – review and editing (equal).

Data availability statement

Data are available from the Dryad Digital Repository: <<https://doi.org/10.5061/dryad.rjdfn2zwcw>> (Galmán et al. 2022).

Supporting information

The supporting information associated with this article is available from the online version.

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