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# Implications of mistletoe parasitism for the host metabolome: A new plant identity in the forest canopy

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## Abstract

Mistletoe–host systems exemplify an intimate and chronic relationship where mistletoes represent protracted stress for hosts, causing long-lasting impact. Although host changes in morphological and reproductive traits due to parasitism are well known, shifts in their physiological system, altering metabolite concentrations, are less known due to the difficulty of quantification. Here, we use ecometabolomic techniques in the plant–plant interaction, comparing the complete metabolome of the leaves from mistletoe (*Viscum album*) and needles from their host (*Pinus nigra*), both parasitized and unparasitized, to elucidate host responses to plant parasitism. Our results show that mistletoe acquires metabolites basically from the primary metabolism of its host and synthesizes its own defence compounds. In response to mistletoe parasitism, pines modify a quarter of their metabolome over the year, making the pine canopy metabolome more homogeneous by reducing the seasonal shifts in top-down stratification. Overall, host pines increase antioxidant metabolites, suggesting oxidative stress, and also increase part of the metabolites required by mistletoe, which act as a permanent sink of host resources. In conclusion, by exerting biotic stress and thereby causing permanent systemic change, mistletoe parasitism generates a new host-plant metabolic identity available in forest canopy, which could have notable ecological consequences in the forest ecosystem.

**KEYWORDS**
ecometabolomic, mistletoe–host system, oxidative stress, permanent and systemic effects, plant–plant interaction, seasonality

## 1 | INTRODUCTION

Plants react to biotic and abiotic stress, causing a wide range of well-known biotic changes, for example by modifying plant ecophysiology, growth, reproduction and phenology (Pérez-Ramos et al., 2020; Strauss & Zangerl, 2002). These responses could be almost instantaneous in response to a pulse disturbance or could cause a permanent reaction, leaving a long-lasting fingerprinting and, eventually, causing a generalized effect throughout the system over time (Bender, Case, & Gilpin, 1984; Sutherland, 1981). Thus, while insect outbreaks and some abiotic disturbances (e.g., episodic drought events, wildfires and strong storms) have short-term implications, the

case of parasitic plants such as mistletoe represents a long-term host–parasite interaction that might cause a permanent host reaction (Lázaro-González, Hódar, & Zamora, 2019a).

Mistletoe are long-lived plants with a perennial endophytic system called haustorium, which is embedded in the host xylem system and serves to parasitize by extracting water and minerals from the host (Ehleringer et al., 1985; Hawksworth & Wiens, 1996; Marshall & Ehleringer, 1990). Vast literature is available on the visible changes that mistletoe cause to their host, such as growth and reproductive changes (Kuijt, 1955; Pennings & Callaway, 2002; Press & Phoenix, 2005), as well as to their neighbouring plants (Hartley et al., 2015; Hódar, Lázaro-González, & Zamora, 2018; Mellado & Zamora, 2017) and insect community (Hartley et al., 2015; Lázaro-González, Hódar, & Zamora, 2019b; Mellado, Hobby, Lázaro-González, & Watson, 2019). However, less evident effects, such as chemical profile alterations, have been less studied, with attention usually focused on a single compound or group of metabolites (e.g., Anselmo-Moreira, Teixeira-Costa, Ceccantini, & Furlan, 2019; Lázaro-González et al., 2019a).

The first response of a plant to biotic or abiotic stress starts with their phenotypical response including physiological and metabolic acclimation. These metabolite changes could be episodic or permanent according to the nature of the stress factor (e.g., Peters et al., 2018 and references therein). The challenge of studying chemical and physiological plant responses to environmental stress is the extraordinary variety of traits that can be altered, as well as the range of analytical methods that researchers need in order to disentangle the situation. A consequence of this complexity is that most of the research studies using traditional techniques focus on a single compound or a group of compounds, such as chemical defence by toxins and deterrents (e.g., Chen, 2008; Sampedro, Moreira, & Zas, 2011). Plant metabolite profiles comprise a complex set of primary metabolites (sugars, amino acids, nucleotides, etc.) and secondary ones (terpenoids, phenolics, etc.), jointly called the metabolome, which is synthesized by the system of plants and which shapes the real functionality of plants at a specific time (Fiehn, 2002; Tomita & Nishioka, 2005; Weckwerth, 2003). For this reason, metabolomic techniques that have great sensitivity have been developed, allowing us to combine ecological and biochemical studies on plants and to capture these ecophysiological and functional changes in a dynamic way at the finest metabolite level (Allwood, Clarke, Goodacre, & Mur, 2010; Bundy, Davey, & Viant, 2008; Gargallo-Garriga et al., 2017; Lima et al., 2010; Peñuelas & Sardans, 2009a; Sardans, Peñuelas, & Rivas-Ubach, 2011).

Biotic stress exerted by mistletoe parasitism could alter pine metabolomic identity in different ways. As modular organisms, pine trees could have a high phenotypic plasticity, adjusting the response of the entire module population against environmental conditions. In addition, tree canopies offer a stratified top-down trait because they are exposed under a vertical gradient of different microclimatic conditions (e.g., light availability, wind speed, air temperature), generating top-down differences in ecophysiological properties (Brooks, Flanagan, Varney, & Ehleringer, 1997; Lewis, McKane, Tingey, & Beedlow, 2000; Parker & Brown, 2000). On a temporal scale, the metabolome of any organism is dynamic and highly susceptible to change under variations in environmental conditions. For example, in spring, new shoots start a burst of growth, and therefore, their metabolic requirements differ from those of more mature needles, which contain compounds from other pathways (Gargallo-Garriga et al., 2015; Meijón et al., 2016). In a typical Mediterranean climate, two crucial and metabolically different periods for current pine needles could be early summer, after the first elongation, and early autumn, after a stress period of hot temperatures and drought. For these reasons, pine stratification and the time period become essential for researchers to analyse correctly the diversity and spatio-temporal consistency of metabolic profile on the whole host–parasite system.

Here, we focus on the European mistletoe (*Viscum album* subsp. *austriacum* Wiesb. Vollman, hereafter *V. album*), an evergreen, epiphytic and dioecious parasitic plant native of most regions of Europe, which specializes on conifers (Zuber, 2004). Part of changes in the chemical profile of the main host, the black pine, *Pinus nigra* subsp. *salzmannii* (Dunal) Franco (hereafter *P. nigra*), caused by *V. album*, have recently been studied (Lázaro-González et al., 2019a). This prior study shows how highly parasitized pines react against mistletoe parasitism, provoking changes in the concentrations of nitrogen and defence compounds in pine needles. However, the overall metabolic profile (the complete set of metabolites) of the plant host–mistletoe interaction has not yet been examined. Thus, a higher-level resolution in the analyses of host metabolic profile could help to elucidate the diversity and spatio-temporal consistency of metabolic profile of the host–parasite system. In addition, metabolomics is a powerful tool for improving our understanding of the changes in metabolism and biochemical composition of organisms, that is the ultimate phenotypic response to environmental changes (Fiehn et al., 2000; Peñuelas & Sardans, 2009b). It is increasingly applied to ecological studies in what has been called ecometabolomics (Gargallo-Garriga et al., 2016, 2018, 2020; Sardans et al., 2014, 2020). Ecometabolomics approaches have specially been applied in plant–animal, plant–fungus and plant–microbe interactions, but this is the first time such an approach has been used in a plant–plant interaction, which are involved a host–parasite system with two long-lived plants (Peters et al., 2018).

Our general hypothesis is that parasitized pines react permanently to mistletoe due to the chronic parasitism, changing their metabolome over the year. Thus, we expect the following: (a) parasitized pines compared with unparasitized pines will increase the concentration of metabolites according to mistletoe requirements, and therefore, these metabolites of parasitized pines would show more similar concentrations to mistletoes than those of non-parasitized pines to mistletoe, and (b) parasitized pines will promote their secondary metabolism to bolster the production of defence compounds against mistletoe parasitism. In addition, due to the intimate connection of the haustorium with the vascular vessels of the pine and the long-lasting attack of the mistletoe, we expect (c) these changes in metabolomics to manifest themselves systemically throughout the parasitized pine canopy. This study advances our

understanding of plant–parasitism ecology and the plant–host responses at the finest metabolic level in two long-lived plants, a relationship that in turn can promote far-reaching ecological consequences in forest ecosystems.

## 2 | MATERIALS AND METHODS

### 2.1 | Study zone

This study was conducted in a Mediterranean pine forest in Sierra de Baza (Granada, south-eastern Spain, 2°51′ 48″ W—37°22′ 57″ N), which has an altitudinal gradient of 850–2,269 m a.s.l. and represents the southernmost limit of the *V. album* subsp. *austriacum* geographical distribution. The climate is typically Mediterranean with a mean annual temperature of 15.5°C (CMAOT, 2017) and annual mean (±*SE*) precipitation of 495 ± 33 mm (1991–2006 period; Cortijo Narváez meteorological station, 1,360 m a.s.l.) concentrated in spring and autumn, hot and dry summers (June–September) and cold winters (December–March). This site is dominated by conifers (43%), especially *P. nigra* Arn., which is the main host and frequently parasitized by *V. album* (Mellado & Zamora, 2020). There are other species of pines, such as Aleppo (*Pinus halepensis* Mill.), maritime (*Pinus pinaster* Ait.) and Scots pine (*P. sylvestris* L.), as well as oaks (*Quercus ilex*, *Q. coccifera*, 9%) and an ensemble of shrubs and herbaceous areas (23%; CMAOT, 2008).

### 2.2 | Experimental design

The study was conducted in 2015 in a stand of afforested *P. nigra* (57.3 ± 3.2 trees $ha^{-1}$) located at 1450 m a.s.l. in Sierra de Baza. These trees have the same age (~40 years old) and similar architecture (*M* [±*SE*] DBH: 48.4 ± 2.6 cm, and height: 6.1 ± 0.3 m). In addition, due to the self-reinfection system of mistletoe (Mellado & Zamora, 2014), pine hosts have a wide range of mistletoe parasite loads, from mistletoe-free pines to heavily parasitized ones. We selected 10 unparasitized pines and 10 highly parasitized ones (>50% of canopy foliage occupied by mistletoe), paired by their structural similarities (i.e., canopy configuration and size) and spatial proximity. The pines were selected from within a maximum distance of 30 m and a minimum of 10 m in order to ensure that the trees constituted independent sampling units. We collected three samples of current-year needles from terminal twigs in each pine, located at different cardinal points with equivalent distances between them, per strata and at three different strata within pine canopy (upper, medium and bottom third of canopy). All samples were collected at the morning (9–10 hr) and repeated in two seasons (early summer [July] and early autumn [October]). Each sample was formed by mixing different terminal twigs from the same canopy stratum. Therefore, the experimental design contained a total of 120 pine-needle samples: 10 pine trees per treatment (parasitized and unparasitized), two sampling seasons (summer and autumn) and three strata for pine-needle samples due to their vertical gradient (upper, medium and bottom third of the canopy). In addition, current-year mistletoe leaves (MLs) of three mistletoes randomly selected from the upper part of the parasitized pines canopy were collected in both seasons.

### 2.3 | Collection and preparation of tissue samples

The samples were frozen immediately in liquid nitrogen and then lyophilized and stored in plastic cans at −80°C. At the laboratory, the samples were ground with a ball mill (Mikrodismembrator-U, B. Braun Biotech International, Melsungen, Germany) at 1700 rpm for 4 min, producing a fine powder that was stored at −80°C. Finally, the powdered samples were extracted with a mix of 80% of methanol and 20% of water. The rest of the sample preparation is described in detail by Rivas-Ubach et al. (2013) and Gargallo-Garriga et al. (2014).

### 2.4 | Analysis by liquid chromatography-mass spectrometry (LC–MS)

The LC–MS platform (all from ThermoFisher Scientific, San Jose, CA, USA, unless otherwise noted) consisted of an Accela U-HPLC system with quaternary pumps, an HTC PAL autosampler (CTC Analytics AG, Zwingen, Switzerland), a Keystone hot pocket column heater and an Exactive Orbitrap mass spectrometer controlled by Xcalibur 2.1. Reversed-phase LC separation used a Synergy Hydro-RP column (100 × 2 mm, 2.5 μm particle size, Phenomenex, Torrance, CA, USA) with the ion-pairing agent tributylamine in the aqueous mobile phase to enhance retention and separation. The LC used a column with a small particle size (2.5 μm instead of 4 μm) to reduce peak widths and expedite analysis. The total run time was 25 min, and the flow rate was 200 μl/min. Solvent A was 97:3 water:methanol with 10 mM tributylamine and 15 mM acetic acid; solvent B was methanol. The gradient was 0 min, 0% B; 2.5 min, 0% B; 5 min, 20% B; 7.5 min, 20% B; 13 min, 55% B; 15.5 min, 95% B; 18.5 min, 95% B; 19 min, 0% B; 25 min, 0% B. Afterwards, the column was washed and stabilized for 5 min before the next sample was injected. Other LC parameters were autosampler temperature, 4°C; injection volume, 10 μl; and column temperature, 25°C. HESI (heated electrospray ionization) was used for MS detection. All samples were injected twice: once with the ESI operating in negative ionization mode ($^{-}$H) and once in positive ionization mode ($^{+}$H). The Orbitrap mass spectrometer was operated in FTMS (Fourier transform mass spectrometry) full-scan mode with a mass range of 50–1,000 m/z and high-mass resolution (60,000). The resolution and sensitivity of the spectrometer were monitored by injecting a caffeine standard after every 10 samples, and the resolution was further monitored with lock masses (phthalates). Blank samples were also analysed during the sequence. The assignment of the metabolites was based on standards, with the retention time and mass of the assigned metabolites in both positive and negative ionization modes.

## 2.5 | Statistical analyses

First, the normality of each metabolite signal-intensity data was tested by Kolmogorov–Smirnov tests. The data for all metabolites followed a normal distribution, except 5 unidentified compounds (0.25%), which were removed from the data set. Then, a permutational multivariate analysis of variance (PERMANOVA) was performed to test differences between pine needles, from parasitized (PPN) and unparasitized pines (UPN), and MLs in both seasons. Thus, treatment (PPN, UPN and ML) and season (summer and autumn) were included as fixed factors and pine tree individual as a random factor. In the same way, a partial least squares discriminant analysis (PLS-DA) was also performed to determine general trends on a sample ordination, and a linear mixed model and Tukey post hoc test with score coordinates of the two first PLS-DA components were used to test differences among metabolomes of ML, PPN and UPN for summer and autumn. Finally, one-way ANOVAs were performed for each individual metabolic compound to identify any statistical differences between ML, PPN and UPN metabolomes.

Second, the whole metabolomic profile of *P. nigra* needles (1991 metabolites), including 55 identified from our metabolite library, was analysed in order to test global effects of mistletoe parasitism (parasitized and unparasitized pines), canopy modularity (upper, middle and bottom third of the pine canopy) and season (summer and autumn). These three factors were run on a PERMANOVA using the Euclidean distance, with 10,000 permutations, as fixed independent factors and each pine tree as random factors. One-way ANOVAs between treatment and season were also performed for each individual metabolic compound. Multivariate ordination PLS-DAs were also performed to detect general patterns of sample ordination in the metabolomes. The PLS-DAs allowed us to reduce the dimensionality of the entire data set of identified and unidentified metabolites and to project our samples and variables on a biplot. Therefore, we were able to identify metabolomic trends of parasitized and unparasitized *P. nigra*, seasons and canopy modularity. To test differences among the metabolome of different groups across the scores coordinates of two first components of the PLS-DAs, we used an linear mixed model (LMM) for each component and a Tukey post hoc test, with three factors as fixed and pine tree as the random factor.

All statistical analyses were conducted with R software (R Core Team, 2020) and were performed to detect shifts in both the metabolomes and individual metabolites as well as the variables controlling them. The PERMANOVA was conducted with the *adonis* functions in "vegan" package (Oksanen et al., 2019). One-way ANOVAs and the Kolmogorov–Smirnov test were performed by *aov* and *ks. test* functions in "stats" package (R Core Team, 2020). PLS-DA was conducted with the *plsda* function in the "mixOmics" package (Rohart, Gautier, Singh, & Lê Cao, 2017). All data were scaled for the PLS-DA by setting the parameter "scale = TRUE" in the function. Finally, LMM and Tukey post hoc tests were performed with the *lme* and *lsmeans* functions of the "nlme" (Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2020) and "lsmeans" (Lenth, 2016) packages, respectively.

# 3 | RESULTS

## 3.1 | Metabolomic profile differences between pine host and its hemiparasite

All metabolites detected in pine needles (1991 compounds in total) were found in both pine treatments, PPN and UPN. However, the metabolic profile of ML lacked 17 and 15 of metabolites when compared to pine needles in summer and autumn season, respectively, 5 of these being absent in all cases. The PERMANOVA of the entire data set indicated differences in the overall metabolomes among treatments ($F_1 = 32.21$; $p < .001$), seasons ($F_1 = 43.39$; $p < .001$) and their interaction ($F_1 = 4.73$; $p < .001$).

Overall, the ML metabolome differed markedly from that of pine needles, and although PPN metabolome was displayed close to UPN, their metabolic profile was statistically different, being PPN more similar to ML than UPN to ML. When all the data were analysed at once, these differences were displayed on component 2 of PLS-DA (Table 1, Figure 1). The one-way ANOVAs of all metabolic compounds showed that the ML metabolome differed from UPN in 80% of the compounds (1,542 out of 1991), whereas the metabolic profile of PPN showed fewer differences with regard to ML (72.5%, 1,444 compounds). The concentration of 933 and 842 metabolites was higher in UPN and PPN, respectively, than that in ML, and the rest (609 and 602 compounds) proved higher in ML (see Figure 2 and Table S1 for identified compounds). Thus, the metabolic profile of the ML showed a higher proportion of most amino acids, most sugars, organic acids

**TABLE 1** Post hoc results from LMMs of two first components from PLS-DA between treatment and season

| | Component 1 | | | | | Component 2 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ML-S | ML-A | PPN-S | PPN-A | UPN-S | ML-S | ML-A | PPN-S | PPN-A | UPN-S |
| ML-A | **<0.001** | – | – | – | – | **<0.001** | – | – | – | – |
| PPN-S | 0.898 | **<0.001** | – | – | – | **<0.001** | **<0.001** | – | – | – |
| PPN-A | **<0.001** | **<0.001** | **<0.001** | – | – | **<0.001** | **<0.001** | **<0.001** | – | – |
| UPN-S | 0.444 | **<0.001** | **0.013** | **<0.001** | – | **<0.001** | **<0.001** | **<0.001** | **<0.001** | – |
| UPN-A | **<0.001** | **<0.001** | **<0.001** | **0.038** | **<0.001** | **<0.001** | **<0.001** | 0.677 | **<0.001** | **<0.001** |

*Note*: Bold type indicates significant effects ($p < .05$).
Abbreviations: A, autumn; ML, mistletoe leaves; PPN, parasitized pine needles; S, summer; UPN, unparasitized pine needles.

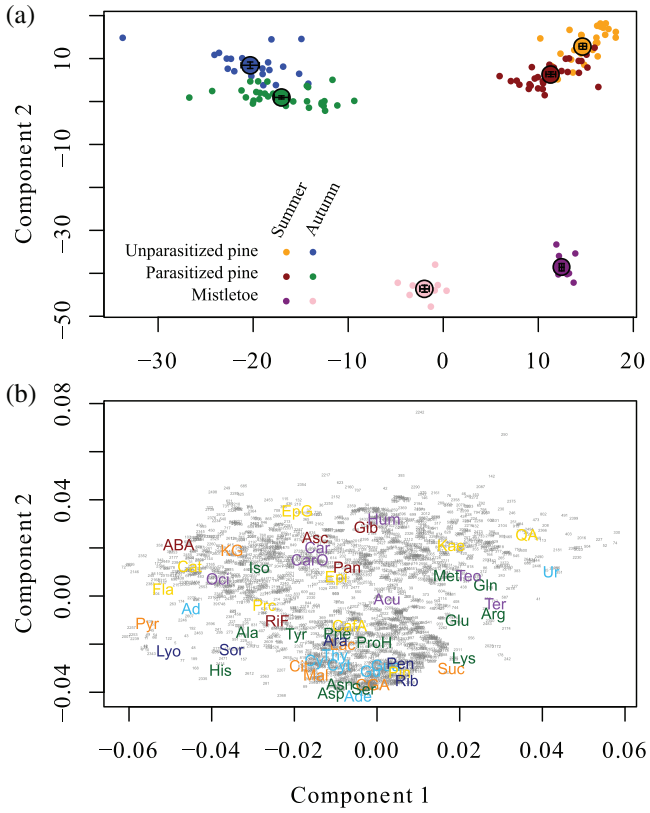


**FIGURE 1** Component 1 versus Component 2 of the partial least squares discriminant analysis (PLS-DA) conducted with all metabolome of parasitized and unparasitized pine needles, and mistletoe leaves. Biplots of the two first components of (a) the PLS-DA of metabolomic data presenting the scores (*M* ± *SE*) of the *Pinus nigra* needles (dark red and green, parasitized pines; orange and blue, unparasitized pines) and mistletoe leaves (purple and pink), and summer and autumn season. (b) The various metabolomic families are represented by colours: green, amino acids; cyan, nucleotides; orange, organic acids related to the tricarboxylic acid cycle; red, other secondary metabolites; dark blue, sugars; yellow, phenolics; purple, terpenes; and grey, unknown metabolites. Aspartic acid (Asp), serine (Ser), lysine (Lys), asparagine (Asn), arginine (Arg), tyrosine (Tyr), methionine (Met), histidine (His), glutamine (Gln), glutamic acid (Glu), isoleucine (Iso), phenylalanine (Phe), hydroxyproline (ProH), alanine (Ala), adenine (Ad), uracil (Ur), guanosine (Gua), guanine (Gu), cytidine (Cy), cytosine (Cyt), adenosine (Ade), thymine (Thy), α-ketoglutaric acid (KG), chlorogenic acid (CGA), citric acid (anhydrous) (Cit), L-(−)-malic acid (Mal), lactic acid (Lac), pyruvic acid (Pyr), succinic acid (Suc), D-(+)-arabitol (Ara), pentose (Pen), 2-deoxy-D-ribose (Rib), D-(−)-lyxose (Lyo), D-(+)-sorbose (Sor), gibberellic acid (GA3) (Gib), ascorbic acid (Asc), abscisic acid (ABA), riboflavin (RiF), pantothenic acid hemicalcium salt (Pan), quinic acid (QA), (+)-catechin hydrate (anhydrous) (Cat), epigallocatechin (EpG), 5,7-dihydroxy-3,4,5-trimethoxyflavone (Fla), epicatechin (Epi), protocatechuic acid (Prc), caffeic acid (CafA), kaempferol (Kae), D-pinitol (Pin), α-humulene (Hum), aucubin (Acu), α-terpinene (Ter), caryophyllene oxide (CarO), ocimene (Oci), α-terpineol (Teo) and carvone (Car)

associated with the Krebs cycle and a higher proportion of most of the nitrogenous bases (Figure 2 and Table S1). The metabolic profile of pine needles showed higher concentrations of most of the defence compounds such as here determined phenolic compounds and terpenes, some amino acids and other secondary metabolites (Figure 2 and Table S1).

## 3.2 | Metabolomic responses of pine host to mistletoe

All of the metabolites detected in pine needles were found in both treatments (parasitized and unparasitized *P. nigra*) and seasons (summer and autumn), but with different concentrations and ratios. The PERMANOVA of the entire data set revealed significant differences in the overall metabolomic profile between parasitized and non-parasitized pines. Moreover, seasonality and the interaction between treatments (parasitized vs. non-parasitized pines) x season were also significant (Table 2).

Differences between UPN and PPN were displayed in Component 2 of PLS-DA (Figure 3). One-way ANOVAs show that mistletoe presence was related to a shift in the concentrations of 26% of metabolites detected in *P. nigra* needles (518 out of 1991). Approximately half of these metabolites (239) presented higher concentrations in parasitized pines, whereas concentrations of the rest (279) was lower (see Figure 4a for identified compounds). Therefore, the metabolic profiles of the PPN had higher concentrations of most amino acids, a higher proportion of the guanine nitrogenous bases, some sugars such as arabitol and some secondary metabolites such as phenols (Figure 4a). The UPN had higher concentrations of Vit. B5 (pantothenic acid) and some phenols among the determined metabolites (Figure 4a).

## 3.3 | Seasonality of the host–mistletoe system and the vertical within-canopy gradient

The concentrations of 38.3% (761 out of 1986) of the total detected metabolites in the ML metabolome changed between seasons, whereas the overall metabolic profile of the pine needles showed a difference of 65.4% (1,303 out of 1991). The differences in seasonality between parasite–host metabolome were displayed on Component 1 of PLS-DA (Figure 1 and Table 1). For MLs, the one-way ANOVA identified a trend in which the concentration of 276 metabolites (13.9% of the total detected metabolites) was higher during summer, including few amino acids, sugars and defence compounds. Conversely, another 485 compounds (24.4%), including most amino acids, some nucleotides, compounds associated with the Krebs cycle, and growth factors such as Vit. B5 and gibberellic acid, increased their concentrations in autumn.

On the other hand, the PERMANOVA of the entire data set of PPN and UPN also reflected a significant interaction between seasonality and parasitism status (Figure 3 and Table 2). The PLS-DAs of the entire data clearly separated their component according to the PERMANOVA results, where Component 1 separated the cases by seasons (Figure 3). One-third part of seasonally altered metabolites of

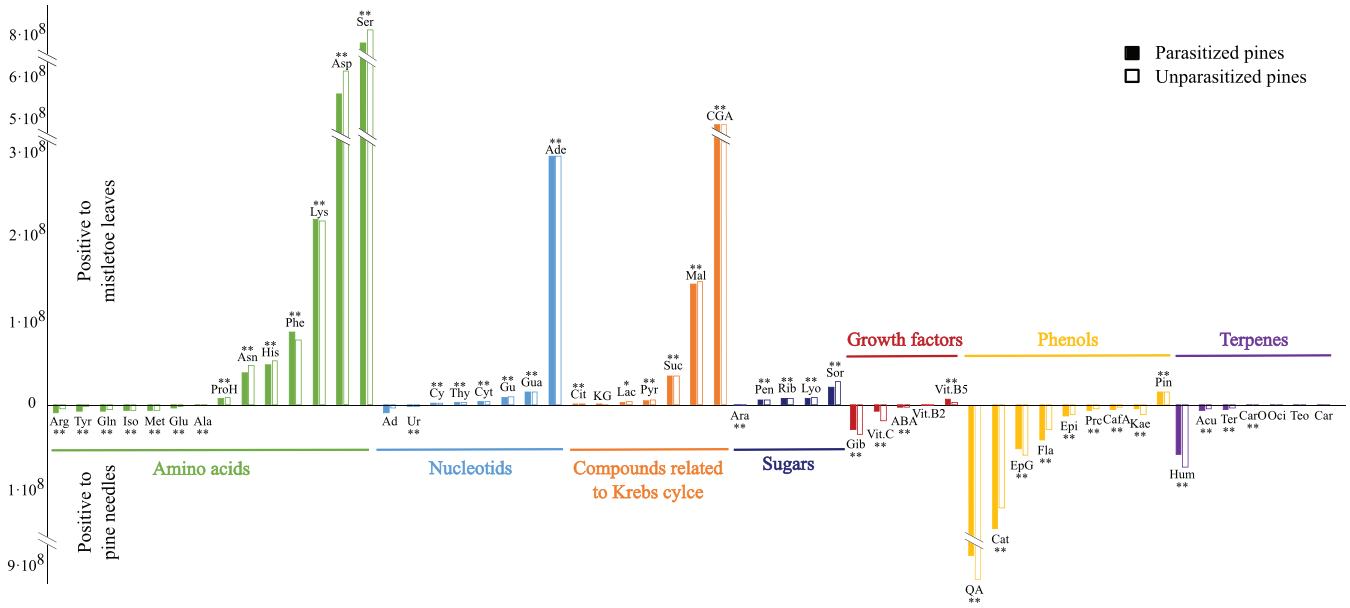


**FIGURE 2** Differences in total intensities of parasitized (filled bars) and unparasitized pine needles (open bars) of all identified compounds with respect to mistletoe–leaf intensities. Bars show mean quantities (intensities) of $N = 10$ samples, where the bars above zero corresponds to greater metabolite intensities in MLs, whereas the bars below zero correspond to greater metabolite intensities in pine needles. The asterisks indicate significant results from the one-way ANOVA ($p < .05$ **; $p < .1$ *). Different metabolomic families are coloured and described in the caption of Figure 1, and *SE* values are given in Table S1 [Colour figure can be viewed at wileyonlinelibrary.com]

**TABLE 2** Effects of treatment (parasitized and unparasitized pines), and stratification level of canopy (upper, medium and bottom third part), and season (summer and autumn) in a complete set of the metabolome of pine needles

| Factors | *Df* | *F*.Model | $R^2$ | Pr(>*F*) |
|---|---|---|---|---|
| Treatment | 1 | 4.62 | .031 | **<0.001** |
| Stratification | 2 | 0.45 | .006 | 0.596 |
| Season | 1 | 39.32 | .263 | **<0.001** |
| Treatment * stratification | 2 | 0.18 | .003 | 0.979 |
| Treatment * season | 1 | 4.03 | .027 | **0.048** |
| Season * stratification | 2 | 0.25 | .003 | 0.892 |

*Note*: The results come from the PERMANOVA model, including all metabolomic variables (1991 compounds). Bold type indicates significant effects ($p < .05$).

pine needles (420 out of 1,303) had higher concentrations in summer, and two-thirds (883 of 1,303) had higher concentration values in autumn (see Figure 4b and Table S1 for identified metabolites). Overall, pine needles in autumn had lower relative concentrations in some amino acids, nucleotides and terpenes, but higher relative concentrations of other nucleotides, organic acids typically related to the Krebs cycle, sugars, phenolics compounds here determined, terpenes and growth factors such as abscisic acid (Figure 4b).

In addition, the post hoc test from LMM, realized with score coordinates of the two first PLS-DA components, showed an interaction between treatment, season and canopy strata (Table 3). This interaction showed that the metabolome from both parasitized and unparasitized pines was homogeneous within the pine canopy during summer, whereas the chemical profile of pine needles differed from the bottom to the upper part of pine canopy, especially in unparasitized pines in autumn (Figure 3 and Table 3).

## 4 | DISCUSSION

This study provides for the first time an integral view of overall shifts in the metabolic profile caused by European mistletoe, *V. album* subsp. *austriacum*, on its main host black pine, *P. nigra* subsp. *salzmannii*, in a Mediterranean forest. Mistletoe parasitism has a systemic effect, making the pine host a more unitary rather than modular organism in space and time. Overall, by causing shifts in host metabolism, mistletoe is able to convert its host into a new plant metabolomic identity available in the forest canopy. In addition, our results strongly suggest that mistletoe acquires resources, derived from primary metabolism, directly from their host, and changes in the metabolic profile of parasitized pines closely fits the hemiparasite metabolome. This indicates that the pine host works for mistletoe, constituting a sink of host resources.

### 4.1 | Metabolomic profile differences between the pine host and its hemiparasite

Previous studies have shown that the concentration of functional chemical groups (basically defence compounds) in the European MLs

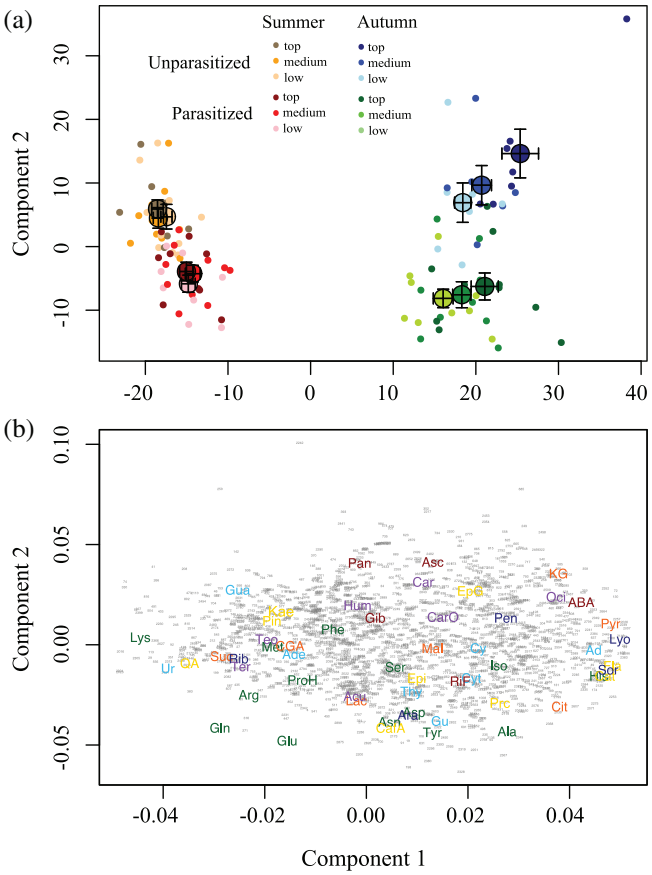


**FIGURE 3** Component 1 versus Component 2 of the partial least squares discriminant analysis (PLS-DA) of the changes of the metabolomes of pine-needle samples. Biplots of the two first components of the PLS-DA of (a) metabolomic data presenting the scores (*M* ± *SE*) of the different *Pinus nigra* treatments (red and green, parasitized *P. nigra*; brown and blue, *P. nigra* uninfected) and different season (red and brown, summer; green and blue; autumn). The different intensities of the colour indicate the height (high intensity indicated the top, the medium indicated the medium, and the lowest indicated the bottom). (b) Different metabolomic families are coloured and described in the caption of Figure 1 [Colour figure can be viewed at wileyonlinelibrary.com]

and black pine needles sharply differs (Lázaro-González et al., 2019a). According to this, our study shows that these differences are evident not only at the level of functional chemical groups (i.e., defence compounds), but also at the finest metabolic level (i.e., amino acids, nucleotides). Overall, the main metabolome differences between MLs and pine needles concern a high concentration of amino acids, nucleotides, compounds related to the Krebs cycle and sugars, and therefore involve a higher up-regulation of primary metabolism (Figure 2). All these changes suggest, on the one hand, that the hemiparasite requires high amounts of metabolic resources to invest in their development in comparison with their plant host. Interestingly, mistletoe acquires at least a part of these resources (Pate, True, & Kuo, 1991; Stewart & Press, 1990), derived from primary metabolism, directly from their host. At the same time, the host accumulates extra amounts of these compounds, benefitting mistletoe, by acquiring part of them (Pate et al., 1991; Stewart & Press, 1990), rather than the host itself, for instance, showing a reduction in host primary and secondary growth (Mellado & Zamora, 2020). In addition, mistletoes have high transpiration rates and low hydric potential (Ehleringer et al., 1985; Schulze & Ehleringer, 1984; Schulze, Turner, & Glatzel, 1984), guaranteeing the unidirectional flow from host to hemiparasite plant, especially for carbohydrates and amino acids (Glatzel & Geils, 2009; Lamont, 1983; López-Sáez, Catalán, & Sáez, 2002). Our results reinforce the idea that the mistletoe–host relationship is a one-way flow system—an asymmetrical relationship where the pine host is forced to work for mistletoe becoming an irreversible sink of resources and water, this being consistent with results of previous non-ecometabolomic studies (Glatzel & Geils, 2009; López-Sáez et al., 2002; Schulze et al., 2019).

On the other hand, our results show that mistletoe has a weak secondary metabolism relative to the pine host, where the concentration of mostly secondary metabolites, especially defence compounds (e.g., flavonoids, tannins and terpenes), is practically absent in mistletoe (Figure 2, Table S1, Lázaro-González et al., 2019a). According to Lázaro-González et al. (2019a), these results reinforce the idea that *V. album* does not benefit from the pine host by acquiring anti-herbivory properties. However, the higher concentrations of free amino acids in mistletoe coming from the plant host are consistent with the higher concentrations of N-rich herbivore deterrent substances in this parasite. These amino acids correspond to a series of compounds necessary to synthesize the sequence of their own proteins, which are toxic for animal cells, such as viscotoxin (Olson & Samuelsson, 1972; Samuelsson, 1973; Samuelsson & Pettersson, 1971) and lectins (Soler et al., 1996; Soler, Stoeva, & Voelter, 1998). Lysine, for instance, required for the synthesis of viscotoxins, shows higher concentrations in ML than in pines (Figure 2). Thus, by generating a net flux of primary metabolites from the host, mainly related to a source of matter and energy (C and nutrients), but not to secondary plant compounds, mistletoe leads its efforts to synthetize their own anti-herbivore defences rather than anti-abiotic stress compounds.

## 4.2 | Metabolomic responses of pine host to mistletoe

Mistletoe can modify the metabolic profile of their pine host by altering the concentration of a quarter of their metabolome (26% of the metabolites analysed). Overall, parasitized pines increase the concentration of most of the primary metabolites intercepted by the mistletoe such as amino acids, nucleobases, compounds related to the Krebs cycle and carbohydrates, while decreasing the concentration of secondary metabolites such as vitamins and certain determined phenolic compounds (Figure 4a and Table S1). Therefore, as a consequence of mistletoe requirements and their inability to take up essential resources from soil, parasitized pines respond by enhancing the concentrations of metabolites especially rich in nitrogen.

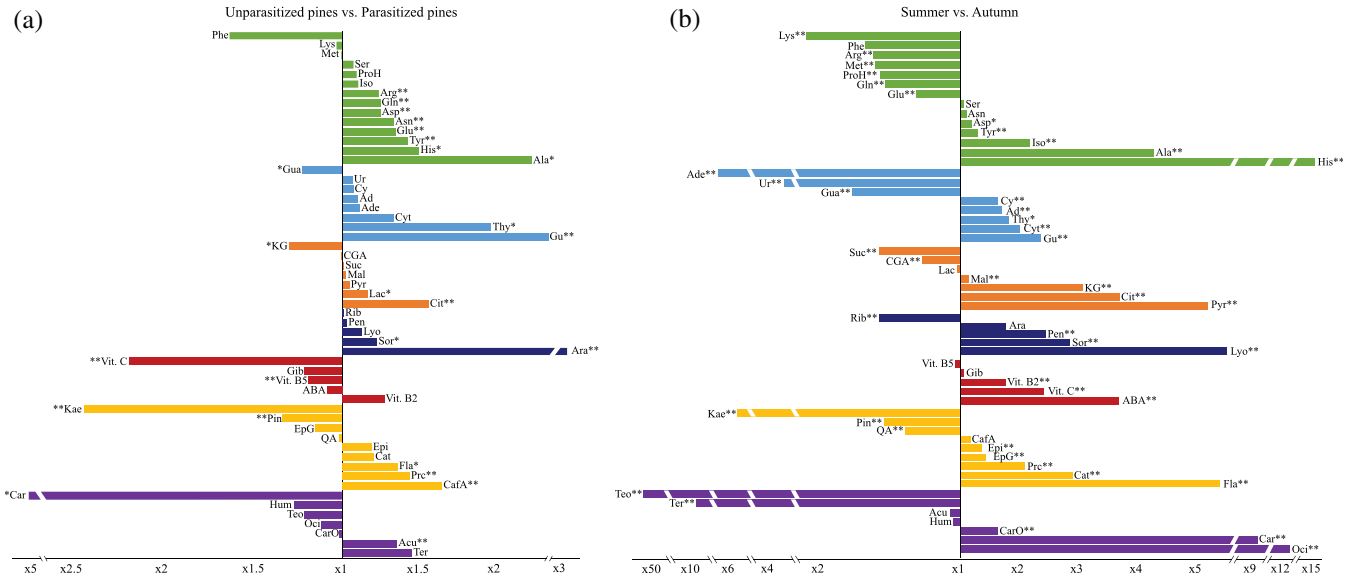


**FIGURE 4** Differences between (a) treatments (parasitized and unparasitized pines) and (b) season (summer and autumn) of compounds identified. Asterisks indicate significant results from one-way ANOVA ($p < .05$ **; $p < .01$ *). Different metabolomic families are coloured and described in the caption of Figure 1 [Colour figure can be viewed at wileyonlinelibrary.com]

In addition, the pine reaction against mistletoe shows common responses to other biotic stressors such as the specialist and more abundant pine-feeding herbivore the pine processionary moth (PPM), which also induces greater concentrations of amino acids, compounds related to the Krebs cycle and carbohydrates (Rivas-Ubach et al., 2016). Besides, PPM generate oxidative stress on pine (Rivas-Ubach, Hódar, et al., 2016), a response commonly induced by folivory in attacked plants (Bi & Felton, 1995). Overall, the metabolic profile of mistletoe-infested pine trees also shows wide similarity with those of pines suffering from water stress. This is because *V. album* keep the stomata open in an almost unregulated way, thus maintaining high transpiration rates under various environmental conditions, leading to drought stress in the host (Escher et al., 2008; Hu et al., 2017; Schulze et al., 1984). As a consequence, primary metabolism is altered by increasing concentrations of soluble sugars and carbohydrate derivatives, and frequently also by elevated concentrations of free amino acids, whereas secondary metabolites, especially flavonoids and terpenes, also commonly exhibited increased concentrations (see Sardans et al., 2020 for a deep analysis on the metabolomic responses to drought in trees). Our results suggest that mistletoe parasitism also provokes oxidative stress, since parasitized pines raise the concentration of some phenols such as flavonoids with antioxidant properties (Figure 4 and Table S1). Despite the similarities of pine responses to PPM attack and mistletoe parasitism, the folivory of PPM causes a slighter effect, with only 12.9% of host metabolome altered (Rivas-Ubach et al., 2016). Meanwhile, mistletoe parasitism has a greater impact on the host pine, modifying 26% of the pine metabolome due presumably to the chronic parasitism and intimate host–parasite relationship.

Parasitized pines increase the relative concentration of aucubin (Figure 4a), an iridoid glycoside known as a secondary defence compound against generalist insect herbivory (Bowers & Puttick, 1988; Nieminen, Suomi, Van Nouhuys, Sauri, & Riekkola, 2003), but also attract specialist lepidopteran species for oviposition and feeding (Harvey, Van Nouhuys, & Biere, 2005; Nieminen et al., 2003; Peñuelas, Sardans, Stefanescu, Parella, & Filella, 2006). Parasitized pines could attract the oviposition of the main pine-feeding specialist, the PPM and, at the same time, provide a low-quality food for caterpillar (Lázaro-González et al., 2019a). As a consequence, pine woodland with mistletoe presence would make pine processionary outbreaks less prevalent. Further studies are needed to assess whether PPM, or other specialist lepidopterans, preferably oviposit upon parasitized pines and whether hatched larvae are able to sequester any defensive compound of pine host for their own defence (Bowers & Collinge, 1992).

### 4.3 | Seasonality of the host–mistletoe system and the vertical within-canopy gradient

MLs and pine needles undergo metabolomic changes from summer to autumn, although MLs are more stable, showing less seasonal variance in their metabolome (38.3% of metabolites change their concentration) than pines (65.4%). This indicates that *V. album* functionality (metabolome) depends less on the environment than on the functionality (metabolome) of the host, as expected from the hemiparasite habit. Thus, both ML and pine-needle metabolomes increase their concentration of primary metabolites such as amino acids associated with chlorophyll synthesis and nutrient assimilation (e.g., lysine and arginine) in summer, whereas other amino acids, nucleotides, compounds associated with the Krebs cycle and vitamins increase in autumn (Table S1). This suggests that the host–parasite system, as

**TABLE 3** Post hoc results from LMMs of two first components from PLSDA between parasitism status, seasons, and stratification level

| LMM of component 1 | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | **PPN-SL** | **PPN-SM** | **PPN-ST** | **PPN-AL** | **PPN-AM** | **PPN-AT** | **UPN-SL** | **UPN-SM** | **UPN-ST** | **UPN-AL** | **UPN-AM** |
| PPN-SM | 1 | — | — | — | — | — | — | — | — | — | — |
| PPN-ST | 1 | 1 | — | — | — | — | — | — | — | — | — |
| PPN-AL | **<0.001** | **<0.001** | **<0.001** | — | — | — | — | — | — | — | — |
| PPN-AM | **<0.001** | **<0.001** | **<0.001** | 0.888 | — | — | — | — | — | — | — |
| PPN-AT | **<0.001** | **<0.001** | **<0.001** | **0.021** | 0.671 | — | — | — | — | — | — |
| UPN-SL | 0.808 | 0.604 | 0.878 | **<0.001** | **<0.001** | **<0.001** | — | — | — | — | — |
| UPN-SM | 0.433 | 0.260 | 0.523 | **<0.001** | **<0.001** | **<0.001** | 0.999 | — | — | — | — |
| UPN-ST | 0.392 | 0.231 | 0.479 | **<0.001** | **<0.001** | **<0.001** | 0.999 | 1 | — | — | — |
| UPN-AL | **<0.001** | **<0.001** | **<0.001** | 0.944 | 1 | 0.864 | **<0.001** | **<0.001** | **<0.001** | — | — |
| UPN-AM | **<0.001** | **<0.001** | **<0.001** | 0.289 | 0.948 | 1 | **<0.001** | **<0.001** | **<0.001** | 0.968 | — |
| UPN-AT | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **0.014** | 0.310 | **<0.001** | **<0.001** | **<0.001** | **0.003** | 0.210 |
| **LMM component 2** | | | | | | | | | | | |
| | **PPN-SL** | **PPN-SM** | **PPN-ST** | **PPN-AL** | **PPN-AM** | **PPN-AT** | **UPN-SL** | **UPN-SM** | **UPN-ST** | **UPN-AL** | **UPN-AM** |
| PPN-SM | 0.992 | — | — | — | — | — | — | — | — | — | — |
| PPN-ST | 0.965 | 1 | — | — | — | — | — | — | — | — | — |
| PPN-AL | 0.885 | 0.207 | 0.121 | — | — | — | — | — | — | — | — |
| PPN-AM | 0.985 | 0.440 | 0.294 | 1 | — | — | — | — | — | — | — |
| PPN-AT | 1 | 0.951 | 0.872 | 0.971 | 0.999 | — | — | — | — | — | — |
| UPN-SL | **0.031** | 0.098 | 0.123 | **0.005** | **0.008** | **0.023** | — | — | — | — | — |
| UPN-SM | **0.036** | 0.111 | 0.139 | **0.006** | **0.010** | **0.026** | 1 | — | — | — | — |
| UPN-ST | **0.012** | **0.041** | **0.052** | **0.002** | **0.003** | **0.009** | 0.999 | 0.997 | — | — | — |
| UPN-AL | **0.010** | **0.031** | **0.039** | **0.002** | **0.003** | **0.00** | 0.981 | 0.963 | 1 | — | — |
| UPN-AM | **0.003** | **0.009** | **0.011** | **<.001** | **<.001** | **0.002** | 0.446 | 0.374 | 0.894 | 0.994 | — |
| UPN-AT | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **<0.001** | **0.001** | **0.003** | 0.114 |

*Note*: Bold type indicates significant effects ($p < .05$).
Abbreviations: A, autumn; L, the bottom third of the tree; M, the middle third; PPN, parasitized pine needles; S, summer; T, the treetop; UPN, unparasitized pine needles.

well as mistletoe-free pines, begins to accumulate most primary and some secondary metabolites for the growth period several months before the resources are needed, showing similar responses to seasonality.

At the canopy scale, the pine needles respond permanently to mistletoe parasitism over the year (Figure 3). New needles sprout with a common metabolic profile and shift to a vertical within-canopy gradient in autumn, with changes being more intense from the crown to the bottom part of canopy (Figure 3). However, the vertical gradient in parasitized pines is less accentuated than in unparasitized ones, and therefore, mistletoe parasitism makes the metabolome of parasitized pine needles more homogeneous by softening the stratification during autumn. This suggests that the metabolic responses of pines are systemic at the canopy scale, turning a modular pine tree into an organism with unitary responses. Thus, mistletoe is an agent of systemic changes (see also Cocoletzi, Angeles, Ceccantini, Patrón, & Ornelas, 2016), able to generate a new plant metabolic identity in the host pine with respect to mistletoe-free pines, prompting ecological consequences. Notably, this systemic reaction appears to be mistletoe-specific in *P. nigra*, given that pines attacked by other biotic stressors such as PPM react to folivory more at local level rather than at the systemic level (Rivas-Ubach, Hódar, et al., 2016).

## 4.4 | The ecological consequences of mistletoe parasitism

Mistletoe has a permanent and systemic effect on the metabolic profile of pine hosts needles, as shown by the data presented in Figure 1. On the one hand, mistletoe parasitism causes damage by permanent oxidative stress (Mutlu, Ilhan, & Turkoglu, 2016) and resorbing N-rich compounds from its host over the year in pine needles (Escher, Eiblmeier, Hetzger, & Rennenberg, 2004). On the other hand, the host has a systemic reaction (e.g., Anselmo-Moreira et al., 2019), which prevents minimizing the effects of parasitism by discarding a part of their canopy and acting as a more unitary rather than modular

organism. As a result, the pine-feeding herbivores cannot find safe sites at different times of the year and in different parts of pine canopy free of the mistletoe impact, triggering spatio-temporal tritrophic mediated indirect interactions. For instance, changes in the chemical profile in response to mistletoe have direct detrimental effects, including death, on many pine-feedings herbivores such as the summer folivore beetle *Brachyderes* sp. and the winter folivore PPM (Lázaro-González et al., 2019b), one of the most severe and widespread pests in the Mediterranean forests (Hódar, Castro, & Zamora, 2003; Hódar, Zamora, & Castro, 2002). Thus, mistletoe generates non-trophic links with pine-feeding herbivores, where the permanent and systemic reaction of pine host has indirect effects on arthropod herbivores via changes in the host quality as food (Lázaro-González et al., 2019a, 2019b).

In conclusion, by exerting a press disturbance, mistletoes cause a permanent and long-lasting systemic effect, making the pine host a more unitary rather than modular organism in space and time. By causing shifts throughout the host metabolism, mistletoe is able to generate a new metabolomic identity in host, which increases the complexity and heterogeneity of the forest canopy. This in turn triggers an ecological cascade of consequences, which exert detrimental effects on pine herbivores (Lázaro-González et al., 2019b). Nevertheless, the new identity could mean a novel niche and new opportunities for tolerant and adapted herbivores, promoting the local and regional forest biodiversity at ecosystem level, which can have valuable implications for the conservation and management of pine forests.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHORS' CONTRIBUTIONS

José Antonio Hódar and Regino Zamora conceived the idea; Alba Lázaro-González, José Antonio Hódar and Regino Zamora designed the study; José Antonio Hódar and Alba Lázaro-González performed the field work; Albert Gargallo-Garriga and Alba Lázaro-González performed the laboratory work and statistical analyses; Michal Oravec and Otmar Urban performed the chemical analyses. All authors discussed the results, contributed critically to the drafts and gave final approval for publication.

## DATA AVAILABILITY STATEMENT

The data set generated during the current study is available in the Zenodo repository at https://doi.org/10.5281/zenodo.5387360.

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