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REPORT

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Plant metabolic response to stress in an arid ecosystem is mediated by the presence of neighbors

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Abstract

Plant neighbors in arid environments can ameliorate abiotic stress by reducing insolation, but they also attract herbivores and pathogens, especially when neighbors are close relatives that share similar antagonists. Plants' metabolic profiles provide a chemical fingerprint of the physiological processes behind plant responses to different environmental stresses. For example, abscisic acid and proline, mainly involved in stomatal closure and osmotic adjustment, can induce plant responses to abiotic stress, while jasmonic acid and salicylic acid primarily regulate plant defense to herbivory or pathogens. Neighbor plants can generate contrasting ecological contexts, modulating plant responses to abiotic and biotic stresses. We hypothesize that plant metabolic profile is modulated by its neighbors in a vegetation patch, expecting a higher investment in metabolites related to biotic-stress tolerance (i.e., herbivory or pathogens) when growing associated with other plants, especially to phylogenetically close relatives, compared to plants growing alone. We show that plants from five species growing with neighbors invest more in biotic-stress tolerance while their conspecifics, growing alone, invest more in abiotic-stress tolerance. This tendency in plants' metabolic profiles was not affected by the phylogenetic diversity of their neighborhood. Linking physiological snapshots with community processes can contribute to elucidating metabolic profiles derived from plant–plant interactions.

KEYWORDS
arid environments, drought, herbivory, phylogenetic neighborhood, phytohormones, plant–plant interactions

INTRODUCTION

The presence of neighbors triggers a complex balance of positive and negative effects on sessile organisms such as terrestrial plants (Valiente-Banuet & Verdú, 2013). In arid ecosystems where the abiotic stress is severe, the presence of neighbors can alleviate plant abiotic stress by providing shade, moisture, and nutrients, facilitating the

establishment of other species (Foronda et al., 2019). Although plants can find a milder abiotic microhabitat close to neighbors, neighbors might also impose increasing biotic stress, and the outcome of these interactions can depend on the phylogenetic diversity of the neighborhood (Castillo et al., 2010; Valiente-Banuet & Verdú, 2007). For example, increased biomass in vegetation patches, compared to plants growing on the open ground, can attract generalist herbivores (Novotny & Basset, 2005) or pathogens with low host specificities (Gilbert & Webb, 2007; Spear & Mordecai, 2018). However, the effects of vegetation patches on a focal plant can depend on the diversity and the phylogenetic composition of the neighborhood conditioning the attraction of specialist and generalist antagonists (Salazar et al., 2016). The likelihood that a pathogen infects two plant species decreases with the phylogenetic distance between them (Gilbert & Webb, 2007), while the overlap of herbivores also decreases gradually with increasing phylogenetic distance between host plants (Novotny et al., 2002). Nevertheless, the positive effect of the distantly related species might be diluted by the occurrence of multihost pathogens with low specialization (Spear & Mordecai, 2018) or compensated because closely related species may also share defenses and resources (Agrawal, 2007; Dickie et al., 2002).

Plants deal with these abiotic and biotic stresses through different physiological mechanisms that leave chemical fingerprints. On one hand, abiotic stresses such as salt or drought can be faced by plants through stomata closure, of which abscisic acid (ABA) is a major and evolutionarily conserved regulator (Cai et al., 2017), and/or by proline accumulation (Kishor et al., 2005), which leads to osmotic adjustment by a reduction of osmotic potential (Bohnert & Shen, 1998). On the other hand, plant responses to biotic stress, such as herbivory or pathogens, are frequently regulated by the phytohormones jasmonic acid (JA) and salicylic acid (SA) (Erb et al., 2012). However, plants' responses to simultaneous biotic and abiotic stresses are not independent and may lead to physiological trade-offs that can be phylogenetically conserved (Montesinos-Navarro et al., 2020). In any case, plant responses to abiotic and biotic stresses can be tracked by their metabolic profiles that reflect the combination of physiological processes that each species counts on to face the different stresses.

Here we assess the effect of the neighborhood on the metabolic profiles of five plant species in a plant community structured in vegetation patches. We hypothesize that conspecifics modulate their metabolic responses predictably based on whether they live associated with neighbors in a vegetation patch or are growing alone (Figure 1). We expect conspecifics will invest more in metabolites related to biotic-stress tolerance than abiotic-stress tolerance when they grow in a vegetation clump, especially when they co-occur with close relatives.

METHODS

Study site and field sampling design

The study is performed in a gypsum outcrop located in the south of Alicante (Spain) (38°29′ N, 0°44′O; elevation: 568 m) within a flat area of 0.5 km^2. The climate is semi-arid, with an annual mean rainfall of 414 mm and a variation of 55 mm between the driest and the wettest months. Mean daily maximum and minimum temperatures range from 3.3 to 13.3°C in January and from 18.4 to 30.6°C in August (data for the year of the study in Appendix S1: Figure S1). The plant community is mainly scrubland and chamephytes such as *Helianthemum squamatum* (L.) Dum. Cours., *Teucrium libanitis* Schreb., and *Helianthemum syriacum* (Jacq.) Dum. Cours. (Delalandre & Montesinos-Navarro, 2018).

For this study we selected five representative chamephyte species in this gypsum outcrop *Fumana ericoides*, *H. squamatum*, *H. syriacum* (all Cistaceae), *Stipa parviflora* (Poaceae), and *T. libanitis* (Lamiaceae) (Appendix S1: Figure S2 and Table S1). As it is not possible to measure and control for all the sources of spatial heterogeneity in field studies (such as soil texture, water, nutrients, and microbial communities), we used a paired design in which each replicate consists of two focal plants, one associated with others in a vegetation patch and the other growing alone, without contact with any other plant in the adjacent open ground, on average within 1 m. This design ensures that soil texture, nutrients, water, and many other variables difficult to assess are likely similar within each pair, and differences between the two plants are more likely explained by their ecological condition (i.e., associated with others vs. growing alone) than other factors. In this paired design we holistically control for all possible microenvironmental variables by relativizing the responses of the associated plant to its paired plant growing alone (see *Statistical analyses* section for a detailed explanation of how the neighbor-induced changes are characterized).

In October 2019, we selected 20–50 pairs of individuals for each species (183 pairs; 366 plants total) (Appendix S1: Figure S2). We maximized the range of phylogenetic distances between the focal plant and the other species in the neighborhood, selecting vegetation patches in which the closest relative to the focal species belonged to the same species, genus, family, class (monocots/dicots), or

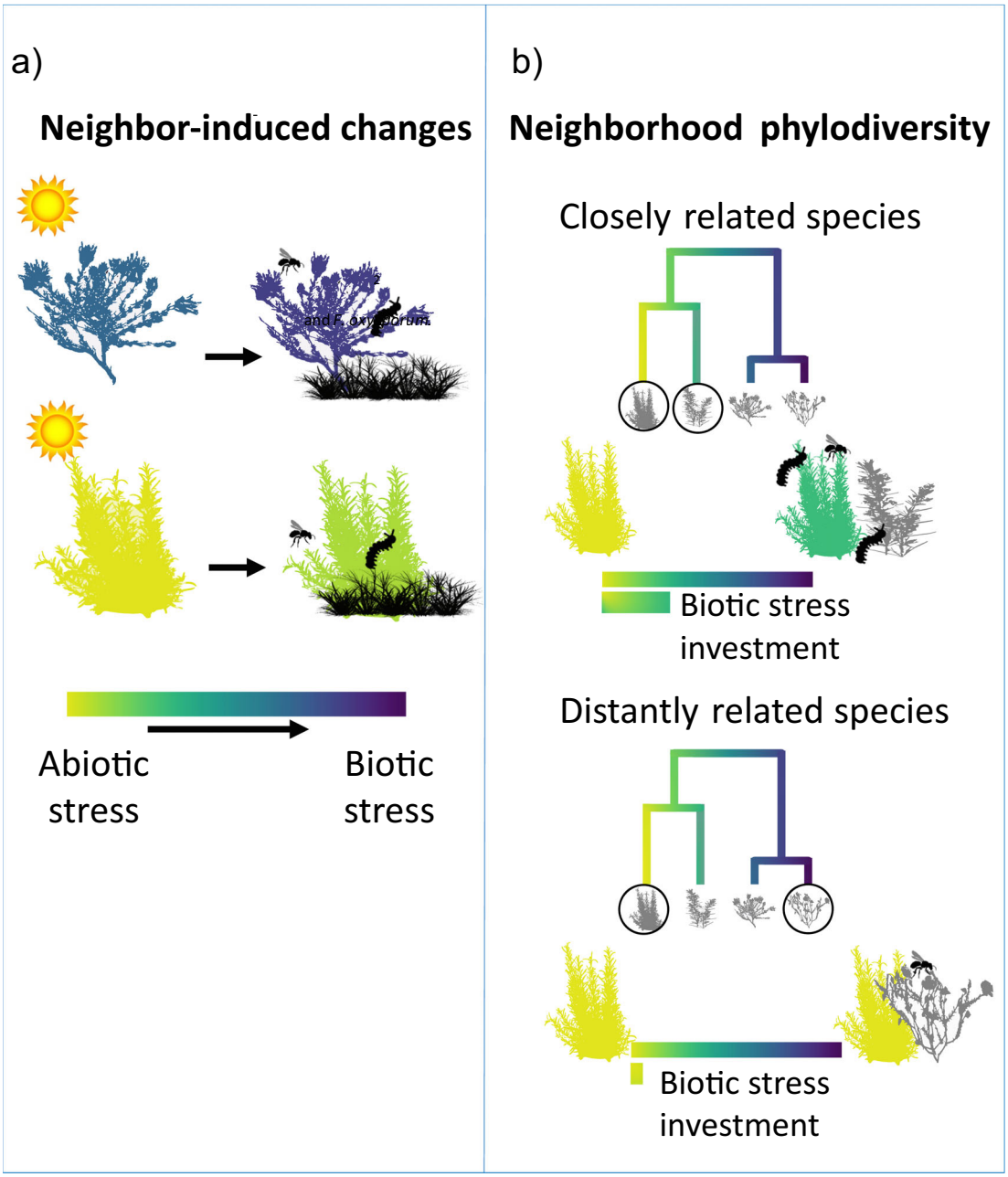

FIGURE 1 Schematic representation of tested hypotheses. Plant colors represent a continuum between investment in response to abiotic (greenish colors) versus biotic (bluish colors) stresses. We test two hypotheses: (a) Neighbors induce changes in metabolic strategies with individuals living in vegetation patches investing less in abiotic-stress tolerance (i.e., solar radiation) and more in biotic-stress tolerance (i.e., insect herbivory) than congenerics in open ground. The modulation of the metabolic profile is represented as a right shift in the color of the individuals. (b) The phylogenetic diversity of the neighborhood (gray plants) conditions the metabolic profile changes of the focal species (nongray). Living with close relatives that tend to share the same herbivores, induces larger metabolic changes (more contrasting color shifts) toward biotic-stress response investment than living with distantly related species. Illustrative material created by Ricardo Sánchez-Martín, Miguel Verdú, and Alicia Montesinos-Navarro.

phylum (gymnosperms/angiosperms). In some cases, the specific composition of the site prevented finding a particular species combination (Appendix S1: Table S1a). Once selected, we recorded the height, the maximum, and the minimum diameter of each of the two focal plants and the spatial distance between them. Also, we recorded the species composition of the vegetation patch and the height and maximum and minimum diameter of the plant with the largest biomass of the vegetation patch, when it was a different one from the focal individual.

Plant leaf sampling for metabolic analysis was conducted in November 2019. For each focal individual in the 183 pairs, we selected fully developed leaves without any sign of damage. We collected 10 g of fresh leaves, which were kept refrigerated in a cooler with ice in the field, for less than 1 h, and frozen at −20°C once in the lab until the samples were analyzed. We collected each pair of samples of any given species simultaneously and all the samples between 2 and 6 p.m. on a single day. This procedure was used after testing with a pilot experiment, and the results do not significantly differ from freezing the samples immediately with liquid nitrogen (Appendix S1: Table S2).

Metabolite quantification

We carried out hormone extraction and analysis on a subsample of the frozen leaves collected from each individual following the procedure described in Durgbanshi et al. (2005), with slight modifications. Phytohormones were eluted with a gradient of methanol and 0.01% acetic acid (CH_3COOH) in water that started from 10:90 (v/v) and linearly reached 60:40 (v/v) in 10 min. In the following 4 min, the gradient increased to 80:20 (v/v). Isocratic conditions of 80:20 were then retained during the last 2 min of the run. The initial conditions were restored and allowed to equilibrate for 5 min, giving a total time of 21 min per sample. The solvent flow rate was 0.3 mL/min, with working pressures around 70–100 bar. Briefly, 100 mg of ground frozen leaf tissue was spiked with 50 ng [$^2H_6$]-ABA, [$^{13}C_6$]-SA, and dihydro jasmonic acid (DHJA) and homogenized with 2 mL ultrapure water using a mill ball (MillMix20, Domel, Železniki, Slovenija). Samples were centrifuged at 4000 × *g* for 10 min at 4°C after the extraction, and supernatants were recovered and pH adjusted to 2.8–3.2 with acetic acid. We partitioned the extracts twice against 2 mL diethyl ether, and the organic layer evaporated under vacuum in a centrifuge concentrator (Speed Vac, Jouan, Saint Herblain Cedex, France). The residue was resuspended in 0.5 mL methanol:water 10:90 and filtered through 0.22-µm polytetrafluoroethylene membrane syringe filters (Kinesis, Germany). The filtrate was diluted 1:4 (v:v) with 90:10 (v:v) water:methanol and injected into the UPLC-MS system (Xevo TQ-S, Waters Corp., Milford, MA, USA). Chromatographic separations were carried out on a reversed-phase C_{18} column (50 × 2.1 mm, 1.6 µm particle size, Luna Omega, Phenomenex, Torrance, CA, USA), using a gradient of ultrapure water and acetonitrile, both supplemented with 0.1% formic acid, with a constant flow rate of 300 µL min^{-1}. Quantification was achieved through a standard curve prepared with commercial standards using the internal standards mentioned previously and processed with the software MassLynx version 4.2 (Waters Corp., Milford, MA, USA). Quality assurance procedures are provided as supplementary material (Appendix S1: Section S3).

We analyzed proline using the method described by Bates et al. (1973), with some modifications. We extracted 50 mg ground frozen leaf tissue in 5 mL 3% sulfosalicylic acid (Panreac, Barcelona, Spain) by sonication for 30 min. Samples were centrifuged at 4000 × *g* for 20 min at 4°C after the extraction, and 1 mL supernatant was mixed with 1 mL glacial acetic acid and ninhydrin reagent (Panreac) in a 1:1 (v:v) ratio. The reaction mixture was incubated in a water bath at 100°C for 1 h and subsequently centrifuged at 2000 × *g* for 5 min at 4°C. Finally, absorbance was read at 520 nm on a Spectronic Genesys 10 (Thermo Scientific, Waltham, MA, USA). A standard curve was assayed with pure proline (Sigma-Aldrich, St. Louis, MO, United States).

Statistical analyses

We used the "prcomp" function in base R version 4.3.1 (R Core Team, 2021) to obtain a principal component analysis (PCA) that explains the maximum variation in the metabolic profile of plants growing associated and alone based on linear combinations of the four focal metabolites. This approach allows for detecting trade-offs among metabolites considering their interdependencies. Only pairs of plants in which we were able to quantify all four metabolites in both individuals were considered in the multivariate analyses (153 pairs out of the originally selected 183 pairs of species, Appendix S1: Table S1b). The tendency to invest more in some metabolites than others, hereafter "metabolic strategy," of these 306 individuals from the five focal species was defined by their scores in the first axis of the PCAs (Appendix S1: Table S3, Question 0).

We characterized the neighbor-induced changes in the metabolic strategies by calculating, within each pair of plants, the difference in the principal component 1 (PC1) score between the plant growing in association with others and the plant growing alone (i.e., "difPC1" = PC1 associated—PC1 alone). To test for the presence of neighbor-induced changes in metabolic strategies, we ran an intercept-only general linear mixed model with "difPC1" as the dependent variable and the species as a random, grouping factor (Appendix S1: Table S3, Question 1). We consider species as random factor because we are interested in whether there is a pattern

generalizable to all species, such that plants associated with others invest more in metabolites related to biotic stress. If the presence of neighbors modulates the metabolic strategy, then we would expect to see a significant difference from 0 in the fitted intercept. In follow-up analyses, we used separate linear models to test whether "difPC1" was explained by potential confounding factors such as the spatial distance between the members of a pair, the area of the vegetation patch in which the "together" plant resided, or the size of the largest plant in the patch; each model included species identity as a random factor and log10-transformed predictors (Appendix S1: Table S3, Question 2).

Finally, we checked whether the neighbor-induced modulation of the metabolic strategy of a species depended on the phylogenetic diversity of the neighborhood of the focal plant. First, we constructed the phylogenetic tree of the species in the community using the V.Phylomaker R package (Jin & Qian, 2019), using the phylogeny GenBank taxa with a backbone provided by Open Tree of Life (GBOTB extended tree) as a backbone phylogeny and grafting the missing species following Scenario 3. Then, several phylogenetic distances (PDs) between species present in each patch were calculated with the "cophenetic.phylo" function in the ape R package (Paradis & Schliep, 2019). We characterized three relevant PDs for plant performance according to Castillo et al. (2010): (1) the PD of the focal plant to the largest species in the patch, (2) the PD of the focal plant to the most closely related species, and (3) the mean PD between the focal plant and the different species in the vegetation patch. We then fit separate general linear mixed models with "difPC1" as the dependent variable and each of the three PDs as a continuous predictor variable, again with species as a random effect (Appendix S1: Table S3, Question 3).

All statistical models were fit using the lmerTest R package (Kuznetsova et al., 2017), with degrees of freedom based on the Satterthwaite approximation (Satterthwaite, 1946). The marginal R-squared (R^2_m) for mixed models, which represents the variance explained by the fixed effects, was estimated with the function "*r*.squaredGLMM" in the MuMIn R package (Bartoń, 2023), based on Nakagawa and Schielzeth (2013). Assumptions for all models were evaluated using the R package DHARMa (Hartig, 2022). All the analyses were performed in R version 4.3.1.

RESULTS

The first principal component axis (PC1) containing the four metabolites measured in 306 individuals of five species explained 30% of the variance, while the second (PC2) explained 26%. We focus here on PC1, since it represents an interpretable combination of species' investment in response to abiotic versus biotic stresses: Individuals with negative PC1 scores showed high levels of metabolites related to abiotic stress (proline and ABA loading factors of −0.65 and −0.44, respectively; Appendix S1: Table S3, Question 0), while individuals with positive PC1 scores had higher levels of biotic-stress-related metabolites (loading factors for SA and JA of 0.18 and 0.59, respectively) (Figure 2a). The actual levels of each metabolite are presented in Appendix S1: Table S4, and the data are available in Montesinos-Navarro et al. (2023). In contrast, positive scores of PC2 represent high concentrations of SA and proline (loading factors 0.84 and 0.38, respectively) and negative scores high levels of ABA and JA (loading factors −0.37 and −0.11, respectively) (Figure 2a), which are more difficult to interpret in terms of a trade-off in the metabolic response to biotic versus abiotic stress.

Despite the high variability in the concentration of the different metabolites in plants growing associated versus alone (Appendix S1: Table S4), the paired design allowed us to detect differences between plants in the two contrasting conditions. The difference between the PC1 scores of plants associated with neighbors and those of their conspecifics leaving alone was significantly greater than 0 (Figure 2b) ("difPC1" estimate ± SE = 0.31 ± 0.09; $t_{4.6} = 3.53$; $p = 0.02$; $N = 153$; Appendix S1: Table S3, Question 1), showing that plants associated with neighbors invested more in response to biotic stress. This difference cannot be explained by the spatial distance between the two conspecifics ($t_{109.57} = 0.39$; $p = 0.70$; $R^2_m = 0.001$; $N = 153$; Appendix S1: Table S3, Question 2 and Figure S3), the size difference between the two focal plants ($t_{146.4} = -0.20$; $p = 0.85$, $R^2_m = 0.0002$; $N = 153$), the vegetation patch area, ($t_{118.5} = 0.09$; $p = 0.93$; $R^2_m = 0.00005$; $N = 153$), or the size of the largest plant in the patch (Appendix S1: Table S3, Question 2 and Figure S3) ($t_{151} = -0.60$; $p = 0.55$; $R^2_m = 0.002$; $N = 153$). Similarly, the difference in the PC1 scores of the plants growing in association with a vegetation patch, and their paired conspecifics growing solitary was also not explained by any of the phylogenetic distances evaluated (1) the phylogenetic distance of the focal plant to the largest species in the patch if the focal is not the largest one ($t_{87} = -0.35$; $p = 0.72$; $R^2_m = 0.001$; $N = 89$), (2) the closest related species ($t_{147.64} = 0.02$; $p = 0.98$; $R^2_m = 0.000003$; $N = 153$), or (3) the mean phylogenetic distance between the focal plant and the different species in the vegetation patch ($t_{148.47} = -0.13$; $p = 0.89$; $R^2_m = 0.0001$; $N = 153$) (Appendix S1: Table S3, Question 3 and Figure S4).

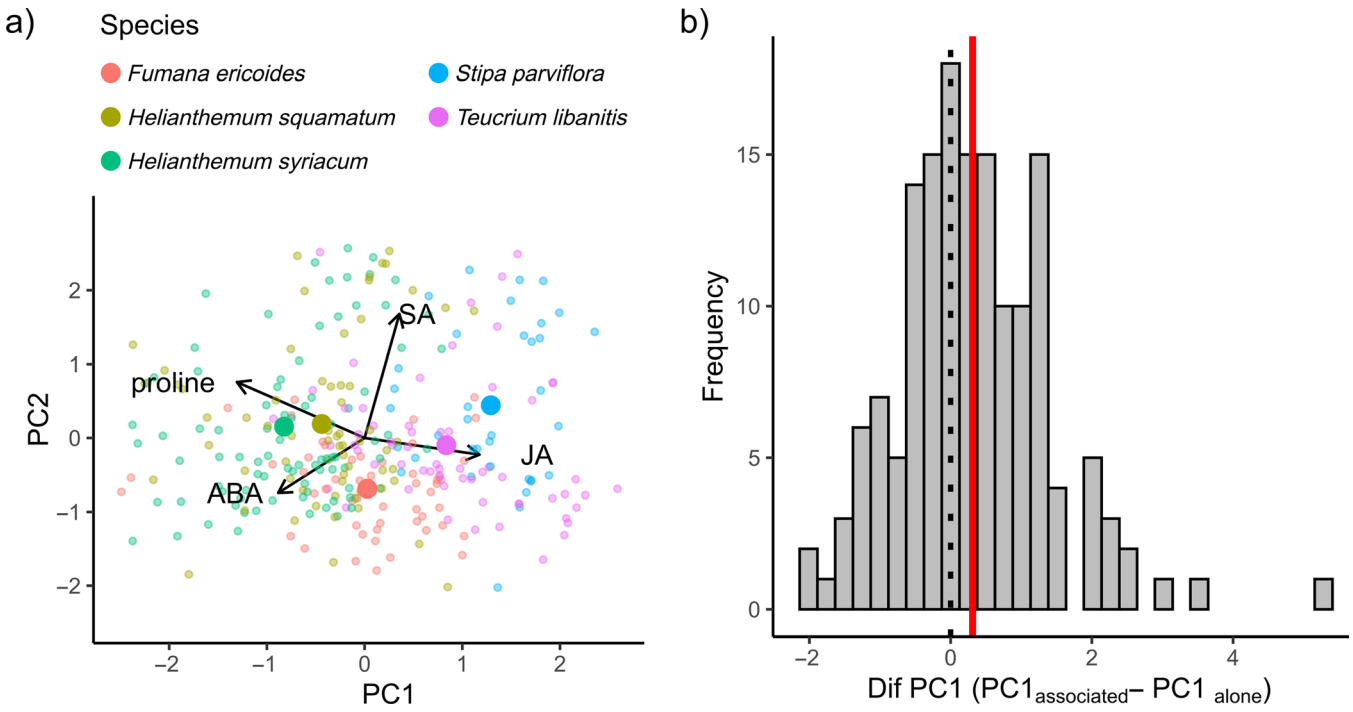

FIGURE 2 (a) Principal component analysis (PCA) of levels of proline, abscisic acid (ABA), jasmonic acid (JA), and salicylic acid (SA). (a) PCA with 306 individuals from five plant species. Each point corresponds to an individual plant, and colors indicate species. (b) Histogram of difference in PC1 score of plants growing associated with neighbors in vegetation patches—growing alone. The mean of the differences (red line) is greater than zero (black dashed line), indicating that the plants growing in association with other plants tend to invest more in metabolites related to biotic-stress tolerance (i.e., SA and JA) and less in abiotic-stress-related metabolites (i.e., proline and ABA) than their paired conspecific growing alone.

DISCUSSION

Our results are consistent with the hypothesis that plants modulate their metabolic profile as a response to the presence of neighbors. Individuals growing with neighbors appeared to invest more in JA and SA, the metabolites commonly associated with plant responses to some biotic stresses, such as tolerance to herbivores or pathogens, while conspecifics growing alone invested more in ABA and proline, metabolites frequently related to abiotic tolerance. However, plants did not alter their metabolic strategy as a response to potential confounding factors or to the phylogenetic diversity of their neighborhood.

Plant–plant interactions have been shown to induce changes in morphophysiological traits involving the leaf economics spectrum (i.e., specific leaf area and water use efficiency) (García-Cervigón et al., 2015) and the production and chemical composition of exudates (Leoni et al., 2021). Our data provide a new perspective regarding changes in the metabolic profiles of plant tissue. Despite the evolutionary conservatism of metabolic strategies among plant species (Montesinos-Navarro et al., 2020), our results suggest that, under field conditions, species can modulate their metabolic profile when they grow alone or associated with neighbors in vegetation patches. Water-stressed plants, such as those growing alone in arid environments, adjust their metabolism to maintain physiological functions by increasing foliar nutrients, proline, antioxidants, or sugars, among other compounds (Rivas-Ubach et al., 2016 and references therein). Similarly, plants may also modulate their levels of specialized metabolites that are toxic or unpalatable for herbivores (Mithöfer & Boland, 2012).

In arid environments, growing associated with other plants can affect both water stress and herbivory (Callaway, 2007). Indeed, experimental evidence in *Austrocedrus chilensis* indicates that the presence of shrub cover decreases desiccation mortality of its seedlings growing underneath but increases mortality due to insect predation (Chaneton et al., 2010). Consistent with this expectation, we found that individuals living associated with neighbors tend to invest less in abiotic- and more in biotic-stress tolerance than their congenerics living alone. However, the PC1 axis only explains 30% of the total variance in the production of these metabolites across

individual plants, and many other biotic and abiotic factors due to microenvironmental heterogeneity or legacy effects from previous ecological context can contribute to the rest of the unexplained variation. Unfortunately, some herbivore attacks and especially pathogen infections might not be easily detected from an observational sampling, and therefore, using the metabolites produced by plants in response to these stresses can provide a more adequate resolution to assess the incidence of this stress. Nevertheless, further controlled experiments should be conducted to elucidate the specific underlying mechanisms that are triggering the plant responses observed. The purpose of our study goes beyond this specific question and, in contrast, tries to accommodate other pressures occurring in the field that are difficult to recreate experimentally (e.g., different phylogenetic diversity of plant neighbors, different levels of antagonists and mutualists, legacy effects). Thus, we consider that our approach, while unable to elucidate the underlying mechanisms that are triggering the plant responses, has the added value of describing more accurately the real metabolic changes of plants under different ecological contexts (i.e., growing isolated or not).

Finally, we found that plants did not modulate their metabolic strategy as a result of the phylogenetic diversity of the neighborhood in which they live. Previous works showed that the evolutionary relatedness of the species coexisting in a vegetation patch determines the outcome of plant–plant interactions (Valiente-Banuet & Verdú, 2007). For example, living with distantly related species promotes establishment, but later competition with closely related species in the patch can reduce survival and growth (Castillo et al., 2010). Although we expected metabolic strategies to mirror these phylogenetic patterns, the increase in biotic tolerance investment when living in a patch was not correlated with the evolutionary relatedness of the focal plant with the other components in the vegetation patch. This result, together with the previous ones, suggests that metabolism can respond to contrasting environmental contexts, like those occurring between the open ground and the vegetation patch, but perhaps not to more subtle differences among vegetation patches with different phylogenetic diversity. Alternatively, the lack of a metabolic response to the phylogenetic diversity of the neighborhood might be due to trade-offs among the costs and benefits of living with close relatives. For example, closely related species tend to compete more but also share mycorrhizal fungi that enhance nutrient uptake (Dickie et al., 2002). Similarly, closely related plant species tend to share pests and pathogens (Gougherty & Davies, 2021) but may also have similar defensive chemistry (Agrawal, 2007). Finally, the absence of a relationship between the metabolic strategy and phylogenetic diversity of the neighborhood may come from the limited ability of paired phylogenetic distances to capture the complexity of indirect interactions occurring within the study vegetation patches (Hirn et al., 2022).

CONCLUSIONS

We found that plants modulated their metabolic strategy to cope with the different levels of abiotic and biotic stress imposed by the presence of neighbors, although the phylogenetic diversity of the neighborhood did not strongly affect this modulation. These results can contribute to linking instantaneous snapshots of the physiology of plants with the ecological processes taking place at the community level and understanding the environmental and phylogenetic determinants of the metabolome, which is critical in the current context of global environmental change.

AUTHOR CONTRIBUTIONS

CAS, AGC, MV, and AMN planned and designed the research. AMN and CAS conducted fieldwork. CAS, RMPC, and AGC performed the chemical analyses, and AMN analyzed data. AMN and MV wrote the first draft of the manuscript, and all the authors contributed to the final version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data and code (Montesinos-Navarro et al., 2023) are available in Zenodo at https://doi.org/10.5281/zenodo.8366675.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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