RESEARCH ARTICLE

The structure and ecological function of the interactions between plants and arbuscular mycorrhizal fungi through multilayer networks

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Abstract

1. Arbuscular mycorrhizas are one of the most frequent mutualisms in terrestrial ecosystems. Although studies on plant mutualistic interaction networks suggest that they may leave their imprint on plant community structure and dynamics, this has not been explicitly assessed. Thus, in the context of plant-fungi interactions, studies explicitly linking plant-mycorrhizal fungi interaction networks with key ecological functions of plant communities, such as recruitment, are lacking.

2. In this study, we analyse, in two Mediterranean forest communities of southern Iberian Peninsula, how plant-arbuscular mycorrhizal fungi (AMF) networks modulate plant-plant recruitment interaction networks. We use a new approach integrating plant-AMF and plant recruitment networks into a single multilayer structure. We also develop a new metric (Interlayer Node Neighbourhood Integration, INNI) to explore the impact of a given node on the structure across layers.

3. The similarity of plant species in their AMF communities is positively related to the observed frequency of recruitment interactions in the field. Results reveal that properties of plant-AMF networks, such as plant degree and centrality, can explain about the properties of plant recruitment network, such as in- and out-degree (i.e. sapling bank and canopy service) and its modular structure. However, these relationships differed between the two forest communities. Finally, we identify particular AMF that contribute to integrate the neighbourhood of recruitment interactions between plants.

4. This multilayer network approach is useful to explore the role of plant-AMF interactions on recruitment, a key ecosystem function enhanced by fungi. Results provide evidence that the complex structure of plant-AMF interactions impacts functional and structurally plant-plant interactions, which in turn may potentially

influence plant community dynamics, through their effects on the structure of the recruitment network.

1 | INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) interact with around 75% of vascular plant species (Van der Heijden et al., 2015), constituting one of the most frequent symbiotic mutualisms in terrestrial ecosystems (Brundrett & Tedersoo, 2018; Lanfranco et al., 2016). Plants provide photosynthetic carbon to fungi which, in turn, supply plants with nutrients (e.g. phosphorus and nitrogen) and other services, like defence against pathogens, herbivores or abiotic stresses (Barea et al., 2011; Bennett & Bever, 2007; Delavaux et al., 2017). AMF effects on plants are well known in cultivated plants and artificial settings (Klironomos, 2003; Smith & Read, 2008). However, it has been only recently, mainly due to the advance in genetic sequencing techniques, that a number of studies have started to explore the complex structure of plant-AMF interaction networks in natural communities (Montesinos-Navarro et al., 2012a; Van der Heijden et al., 2015). The description of the structure of ecological networks is important, but we need to transcend this mere description if we seek to understand how their complexity translates its effects to plant community dynamics. It is becoming increasingly evident that plant mycorrhizal interactions play fundamental roles in plant community dynamics (Tedersoo et al., 2020). Despite this, evidence that the structure of plant-AMF interactions modulates plant–plant interactions in ways that influence plant community dynamics is lacking.

The ecological services that AMF provide to plants are particularly critical during early recruitment stages. Current knowledge is fundamentally based on experiments showing that the performance of seedlings and saplings is improved in the presence of AMF (Bennett et al., 2017; Koorem et al., 2012; Van der Heijden, 2004; Zhen et al., 2014). Plant-AMF interactions may affect plant recruitment both in direct and indirect ways. AMF directly provide nutrients to the young recruiting individuals, enhancing their growth and survival (Smith & Read, 2008), although AMF may also draw carbon from the recruit what may be detrimental to recruiting plants depending on environmental circumstances (Selosse et al., 2006). In an indirect way, in natural communities, plant root systems are connected by common mycorrhizal networks (CMN) that act as efficient avenues for interplant carbon, water and nutrients exchanges between conspecific and heterospecific individual plants of different ages (Smith & Read, 2008). This mediates the competitive or facilitative outcome of plant–plant interactions (Brown et al., 2020; Montesinos-Navarro et al., 2012b, 2019; Selosse et al., 2006) and may provide adaptability and resilience to the whole community (Simard et al., 2012; Tedersoo et al., 2020).

The study of plant-AMF interactions in natural communities can be approached from an ecological network perspective (Montesinos-Navarro et al., 2012a; Sepp et al., 2019; Toju et al., 2014). Plant-AMF interactions are typically studied as undirected bipartite networks formed by two types of nodes (plants and AMF), with links indicating a given AMF colonizing the roots of a given plant species. From a functional perspective, these links could be interpreted as carbon, water or nutrient trades between plants and fungi. Similarly, the recruitment function in plant communities can be studied using a network approach. The direct and indirect interactions between established plants and plants recruiting in their neighbourhood (i.e. canopy-recruit interactions) constitute a so-called recruitment network that acts as an important driver of plant community dynamics (Alcántara et al., 2015; Alcántara & Rey, 2012). Recruitment networks are unipartite (i.e. with a single node type) and directed (i.e. links point from canopy to recruit species), indicating that the canopy plant provides a suitable recruitment microhabitat for the recruit species. The detailed analysis of these networks let us describe two functional properties characterizing recruitment in plant communities: the canopy service, which represents the abundance of saplings of any species under each species in the community; and the sapling bank, which represents the abundance of saplings of each species in the community (Alcántara et al., 2018).

Studies on plant mutualistic interaction networks suggest that these interactions (e.g. pollination and seed dispersal) can leave their imprint in the structure and dynamics of plant communities (Valdovinos, 2019, and references therein). However, studies explicitly linking plant-mycorrhizal fungi networks with key ecological functions of plant communities are lacking. To gain a more complete view of how mycorrhizal fungi participate in plant community structure and dynamics, we must understand how plant-mycorrhizal fungal networks modulate recruitment network properties. Thus, we propose a multilayer network approach (Aleta & Moreno, 2019) with layers defined by organism type, that is plant or fungus (Figure 1). One layer would be integrated by plant species, with canopy-recruit interactions as intralayer connections, while the other one would be formed by AMF. Plant-AMF interactions would be represented by interlayer connections. This multilayer structure would be the combination of two subnetworks sharing the same set of plant species: a plant-AMF subnetwork (e.g. Montesinos-Navarro et al., 2012a; Toju et al., 2018) and a plant recruitment subnetwork (e.g. Alcántara et al., 2019). The adoption of multilayer approaches to study complex ecological systems is an emerging and active field (Aleta & Moreno, 2019; García-Callejas et al., 2018; Kivelä et al., 2014; Pilosof et al., 2017). Most studies so far have started by combining several
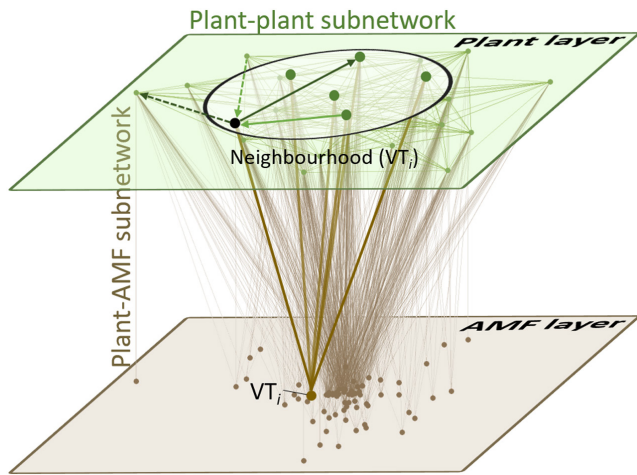
FIGURE 1 Conceptual multilayer network approach of this study and interpretation of the Interlayer Node Neighbourhood Integration (INNI) index. This scheme represents the plant (green nodes) and the AMF (brown nodes) layers, as well as the plant-AMF (brown links) and the plant–plant recruitment (green links) subnetworks. Black circle gathers the plant nodes constituting the neighbourhood of the AMF VT i. In the plant layer, solid arrows are examples of links interconnecting nodes within the neighbourhood of VT i, while dashed arrows are examples of links connecting nodes of the neighbourhood of VT i with the rest of nodes of the recruitment subnetwork. Depending on whether we consider all the links among the nodes of its neighbourhood or only incoming or outgoing links, a particular AMF may exhibit different contributions to structuring the interactions in the recruitment network. These contributions can be defined by the INNI, $INNI_{IN}$ and $INNI_{OUT}$ values, respectively. INNI is the difference between the connectance among the nodes of the VT i neighbourhood (solid arrows) and the connectance among the nodes of the whole recruitment subnetwork (solid and dashed arrows). It represents the contribution of VT i to the overall connectance of the recruitment subnetwork. $INNI_{IN}$ is the difference between the mean in-degree of the nodes within the VT i neighbourhood (solid arrows) and their mean in-degree considering all the nodes. $INNI_{OUT}$ is the difference between the mean out-degree of the nodes within the VT i neighbourhood (solid arrows) and their mean out-degree considering all the nodes.

networks that share some common nodes (Kéfi et al., 2015, 2016). These studies provide new ways to describe ecological systems with increasing levels of complexity. However, we are still far from understanding the ecological mechanisms underlying the integration of multiple networks and its consequences for the constituent species. Here, we use a multilayer approach to explore, not only how plant-AMF interactions are assembled in natural forest communities, but also to understand how this assembly modulates the plant recruitment function in these communities.

We build different predictions with the aim to understand the influence of plant-AMF interactions on plant recruitment. This is performed by assessing the effect of different properties of the plant-AMF interaction subnetwork on functional and structural properties of the plant–plant recruitment subnetwork. The benefit of plant-AMF interactions for plant recruitment arises at the initial stages of this process. Seedling growth and survival benefit from early establishment of associations with mycorrhizal fungi (Zhen et al., 2014), thus a seed germinating in the vicinity of a plant that uses some of the same AMF will be more likely to establish the association. Therefore, (i) the similarity between plants in AMF communities should enhance the recruitment efficiency of these plants. The probability of sharing more similar AMF, with its entailed benefits in terms of plant recruitment, should be higher between plant species with more diverse AMF communities; thus, (ii) plant species hosting a higher number of AMF species should be more abundant in the sapling bank and should also provide a better canopy service. If this is the case, a plant species with a large number of AMF should be located in network positions concentrating a higher number of connections. Thus, (iii) plant species more centrally located in the plant-AMF subnetwork should be more abundant in the sapling bank. Finally, if sharing AMF favours recruitment of one plant in the vicinity of another, (iv) the similarity of plant AMF communities between plants would contribute to explain which species recruit in the vicinity of which other, thus affecting key structural properties of the recruitment network, such as its modularity and plant species neighbourhood.

2 | MATERIALS AND METHODS

2.1 | Study area and sampling design

The study was conducted in two mixed pine–oak forest communities (regions, hereafter; area where no permits were needed) of southern Iberian Peninsula separated 100 km: Sierra de Segura (Segura, hereafter; permit ref.: 174-CV-22, Junta de Andalucía), characterized by mixed forests *of Pinus nigra* subsp. *salzmanii*, *Quercus faginea* and *Q. pyrenaica*, and Sierra Sur de Jáen (Jáen, hereafter), characterized by mixed forests of *P. halepensis*, *Q. ilex* and *Q. faginea*. Despite a wide dominance of upper canopies by pines and oaks, understories of subcanopy trees and tall shrubs (with species of *Acer*, *Crataegus*, *Juniperus*, *Sorbus*, *Prunus*, *Phillyrea* and *Pistacia*) and a layer of small shrubs (with species of *Thymus*, *Cistus*, *Phlomis*, *Genista* and *Rosmarinus*) are behind the high species richness of these forests. Sierra de Jaén has mean annual temperatures of 14.1°C and mean annual rainfall of 715 mm, while Sierra de Segura has mean annual temperatures of 11.6°C and mean annual rainfall of 890.5 mm.

To describe the recruitment subnetwork in each region, we monitored five sites in Jaén (separated 0.78–3.69 km from each other) and four in Segura (1.10–3.58 km). At each site, one 50×50 m plot was established, where we registered the abundance of each woody species and the frequency of canopy-recruit interactions. Species abundance was estimated by the total canopy cover (i.e. the canopy projection in m^2) of each species in the plot. The frequency of canopy-recruit interactions was estimated by performing a systematic search of saplings of all woody species throughout each plot, registering their number and identity, and the identity of the canopy species where they were growing beneath (see Alcántara et al., 2019

for further sampling details). Data were pooled across sites within each region to build their respective interaction networks. We used the available dataset from Alcántara et al. (2018) but only analysing AMF-associated plant species.

Root sampling was performed for characterizing the AMF communities associated with 32 plant species with relevant roles in the plant communities' assemblage of each region (Alcántara et al., 2018, 2019; Pulgar et al., 2017). They represented 27 genera, 16 families and 12 orders, and covered 80% of the woody species and 91% of the canopy-recruit interactions of these communities. In the present study, we focus on plants that associate with AM fungi (Bueno et al., 2017), but some (e.g. *Crataegus* sp., *Juniperus communis* or *Cistus albidus*) may show dual AM-ectomycorrhizal colonization (Comandini et al., 2006; Wang & Qiu, 2006). In Autumn 2016 and Spring 2017, root samples were collected from individual plants at nine sites (Table S1) distributed across both regions, accounting for a total of 407 samples and averaging 12.72 (6–24) samples per plant species (Table S2). Out of these, 268 were taken at Jaén and 139 at Segura, averaging, respectively, 11.65 (6–15) and 11.58 (8–15) samples per species. Since AMF communities change seasonally (Dumbrell et al., 2011; López-García et al., 2014), 206 samples were taken in autumn and 201 in spring, averaging respectively, 6.28 (3–12) and 6.43 (3–12) samples per species.

To check the presence of AMF in each samples, 2 g of roots were stained with trypan blue (Phillips & Hayman, 1970) and examined under a compound light microscope. Samples showed mycorrhizal symbiotic structures.

2.2 | Characterization of AMF communities

Root samples were stored, processed for DNA extraction, DNA amplified and sequenced for AMF identification following standard methods detailed in S4. Raw sequences were quality-filtered and amplicon sequence variants (ASV, hereafter) were inferred using the pipeline known as DADA2 v.1.8. (Callahan et al., 2016), followed by a LULU algorithm analysis (see Frøslev et al., 2017), yielding a total of 897 corrected ASV. Since DADA2 and LULU infer unique original sequences in the DNA template, the result could correspond to infra-specific levels. Thus, the 897 ASV were compared against the MaarjAM database (Öpik et al., 2010; accessed 01/2019), and eventually named as their corresponding virtual taxa (VT, hereafter) when ≥97% identity. Those ASV not reaching a 97% identity in MaarjAM database were aligned and clustered (97%) together with VT-named ASV: those ASV clustering with VT-named ASV were added to the existing VT; the clusters that did not include any VT-named ASV were considered as new VT. Sequencing depth per sample was evaluated with rarefaction curves, representing the number of detected VT per number of reads, and were visualized with the *Rarecurve* function of vegan R package (Oksanen et al., 2019). We calculated the accumulation curves of fungal VT per host plant species, using iNEXT R package (Hsieh et al., 2016). This analysis indicates that our sampling reached a high coverage (Figure S3b). This allows us to assume that we obtained a representative sample of the fungal partners to correctly estimate interaction networks. This is relevant for an optimal balance in the sampling design. The highly balanced spatiotemporal structure of our sampling design seeks to properly study plant-AMF networks, trying to maximize link detection at each study site. Moreover, an appropriate balance in sample number per site, season and plant species is critical to correctly estimate the interaction strengths magnitude and other network metrics (Blüthgen, 2010).

2.3 | Subnetwork analyses

Our multilayer network can be defined as a quadruplet $\{V_M, E_M, V, L\}$ (Kivelä et al., 2014). L describes the set of elementary layers, two in this study: one including all plants and another including all AMF (see Figure 1 for our conceptual multilayer approach, and Figure 2a,b, for its specific representation for Jaén and Segura, respectively). *V* is the full set of nodes, which in this case is the sum of plants and AMF (101 in Jaén and 75 in Segura). V_M is a set of elements identifying the occurrence of each node in different layers. Here, species only occur

FIGURE 2 Graphic representation of the different interaction networks analysed in this study. Multilayer networks for Jaén (a) and Segura (b) show the relationships between the recruitment (i.e. plant–plant) subnetwork (green), representing the intralayer links, and the plant-arbuscular mycorrhizal fungi (AMF) subnetwork (brown), representing the interlayer edges. Plant-AMF undirected bipartite subnetworks for Jaén (c) and Segura (d) show how plants (numbered list beneath) interact with AMF virtual taxa. Nodes coordinates are defined by metric multidimensional scaling using the shortest paths between nodes (i.e. closeness centrality) as distance matrix. Node colour indicates their adscription to the modules defined by the unipartite modularity estimated for the plant-AMF subnetwork. Node size indicates the degree. Recruitment (i.e. plant–plant) directed unipartite subnetworks for Jaén (e) and Segura (f) show how canopy plant species recruit different plant species beneath (in arrow direction), with arrows pointing from the canopy to the recruit species. Node coordinates (i.e. groups) are defined by the unipartite directed modularity estimated for the recruitment subnetwork. Node colour indicates their adscription to the modules defined by the unipartite modularity estimated for the plant-AMF subnetwork. Node size indicates the plant degree in the plant-AMF subnetwork. Edge colour indicates source module. Edge width indicates recruitment frequency. Numbered list of plant species: *Acer monspessulanum* (1), *Amenlachier ovalis* (2), *Berberis hispanica* (3), *Crataegus laciniata* (4), *Crataegus monogyna* (5), *Cytisus scoparius* (6), *Daphne gnidium* (7), *Daphne laureola* (8), *Genista cinerea* (9), *Juniperus communis* (10), *Juniperus oxycedrus* (11), *Juniperus phoenicea* (12), *Lavandula latifolia* (13), *Olea europea* (14), *Phillyrea angustifolia* (15), *Phillyrea latifolia* (16), *Phlomis purpurea* (17), *Pistacia lentiscus* (18), *Pistacia terebinthus* (19), *Prunus spinosa* (20), *Rhamnus lycioides* (21), *Rosa sp* (Jaén) (22), *Rosmarinus officinalis* (23), *Rosa sp* (Segura) (24), *Ruscus aculeatus* (25), *Sorbus torminalis* (26), *Thymus mastichina* (27), *Thymus zygis* (28), *Ulex parviflorus* (29), *Viburnum tinus* (30), *Teucrium fruticans* (31).
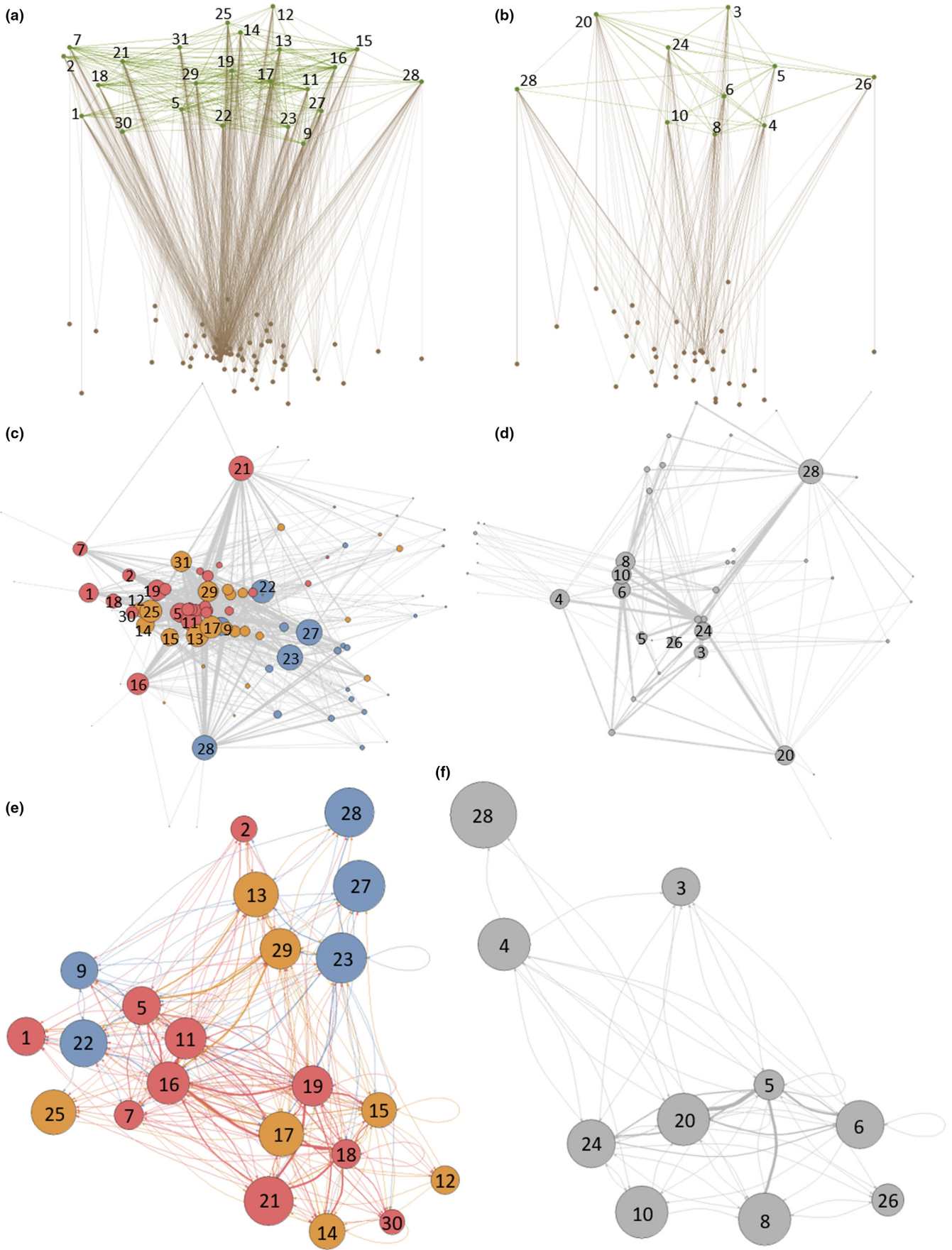
in one layer, so $V_M = V$. E_M is the full set of edges (links) in the entire network, including intra and interlayer levels. Here, these types of edges correspond to two different subnetworks: the intralayer edges correspond to the plant–plant recruitment subnetwork, and the interlayer edges, the plant-AMF subnetwork.

Prior to analysing each subnetwork, we tested whether different plant species interact with different AMF assemblages. If plants and AMF interact randomly, we would not find differences among plant species and would not make sense exploring the properties of the interlayer plant-AMF connections. To explore variation in AMF communities' composition among plant species ($n=32$), sites ($n=9$), seasons ($n=2$) or regions ($n=2$), we performed a PERMANOVA by means of the *adonis* function of VEGAN R package (Oksanen et al., 2019) with 9999 permutations. We used VT presence/absence in a given sample and Morisita-Horn as dissimilarity measure (Barwell et al., 2015).

In the plant-AMF bipartite subnetwork, the interaction weight was expressed as incidence: the proportion of root samples of a given plant species with the presence of each VT. Previous studies on plant-fungus interaction networks used the number of reads as their estimate of interaction weight. However, in the context of the AMF effect on plant recruitment, it is more informative to use the incidence of plant-AMF interactions, since it estimates how likely it is that a plant recruiting under a randomly chosen individual of a given canopy species will find a particular AMF. In turn, the recruitment subnetwork is unipartite and directed (i.e. edges have a specific directionality). The interaction weight (F_{ij}) represents the observed frequency of individuals of recruit species i, under canopy species j, (i.e. F_{ij} is the frequency of canopy-recruit interactions). The recruitment matrix corresponding to a given recruitment network has canopy species as columns and recruit species as rows (see Figure S5). This matrix allows to obtain two functional properties of the nodes (i.e. plants) of the recruitment network: the canopy service and the sapling bank. The canopy service represents the abundance of saplings of any species under each species in the community and is the vector of column sums of the recruitment matrix. The sapling bank represents the abundance of saplings of each species in the community and is the vector of row sums of the recruitment matrix. Considering links' directionality, the canopy service of a node would be represented by its outgoing links (i.e. the so-called out-degree), while sapling bank would be represented by its incoming links (i.e. in-degree).

To characterize the plant-AMF subnetwork, we obtained species degree, closeness centrality and interaction push-pull index as node properties, all calculated using BIPARTITE R package (Dormann et al., 2009). We also calculated the modularity of this network through simulated annealing (Guimera & Amaral, 2005) using the RNETCARTO R package (Doulcier & Stouffer, 2015). Additionally, we obtained overall descriptive parameters. To characterize the recruitment subnetwork, we used two functional node properties: canopy service and sapling bank. We also calculated modularity of this subnetwork which, given its directed nature, was estimated by using a modification of the Louvain algorithm (Blondel et al., 2008) to handle

directed networks (Dugué & Pérez, 2015). Detailed explanations on these metrics can be found in S6.

To test whether modularity departs from values expected by chance, we designed a multinomial probabilistic null model specifically tailored for each type of subnetwork, defined by our study system and sampling design, that delimits appropriately our random expectations (see S8 for further details). Plant-AMF and recruitment subnetworks were represented by means of IGRAPH R package (Csardi & Nepusz, 2006), while multilayer representations were performed using also GRAPHLAYOUTS (Schoch, 2020) and GGRAPH (Pedersen, 2020) R packages.

2.4 | AMF effects on plant recruitment through the multilayer structure

2.4.1 | Effects of AMF communities' similarity between plants on the efficiency of canopy-recruit interactions

We analysed whether the frequency of canopy-recruit interactions (F_{ij}) was affected by the similarity in the AMF communities associated to each plant species (prediction i). Alcántara et al. (2018) showed, for the same communities of the present study, that intraspecific interactions tend to inhibit recruitment, while interspecific interactions tend to enhance it. These differences cannot be related to the effect of similarity of AMF between plants, since conspecifics have the maximum similarity. This is more likely related to the occurrence of antagonists acting in a density-dependent way (e.g. specialist pathogens). Therefore, for this analysis, we focused on inter-specific canopy-recruit interactions.

Fij was fitted by a generalized linear mixed model (glmm, hereafter) with negative binomial distribution and log link function, by means of the R package GLMMTMB v.1.0.2.1 (Brooks et al., 2017). As predictors, we included the cover (canopy projection in m^2) of both canopy and recruit species, the plant dissimilarity in AMF communities (dissimilarity between canopy-recruit species pairs; as Morisita-Horn distances), region (Jaén and Segura) and region×plant dissimilarity in AMF communities. As random effects we included site, and canopy and recruit species' identity. By including the abundance of both interacting species, the effects of the rest of predictors can be interpreted in terms of recruitment interaction "efficiency", that is number of recruits of species i under species j, per unit cover of both species in the community.

2.4.2 | Relationships of node (i.e. plant) properties across layers

The relationships between the plant properties in the plant-AMF subnetwork (i.e. degree and centrality; predictions ii and iii, respectively) and the functional properties of the recruitment network (canopy service and sapling bank) were calculated using Spearman

correlation (ρ) analyses. Their significance was assessed by the Spearman critical values, at $\alpha = 0.05$, for $n = 22$ in Jaén and $n = 11$ in Segura.

To explore whether the effect of the plant-AMF interaction goes beyond that on recruitment efficiency, eventually crystallizing in the structure of the recruitment subnetwork (prediction iv), we first assessed to what extent AMF communities' similarity between plant species (i.e. canopy-recruit species pairs) increased the probability of both plants belonging to the same recruitment subnetwork module. Similarly to previous analysis, we fitted a *glmm* with binomial distribution and logit link function. Our dependent variable indicated whether both plants belonged to the same recruitment subnetwork module or not. As fixed effects we included plant dissimilarity in AMF communities, region and region by AMF dissimilarity interaction. As random effects we included canopy and recruit species' identity.

Secondly, we developed a multilayer index that we named as Interlayer Node Neighbourhood Integration (INNI), describing the influence of each AMF on the connectance of plants in the recruitment subnetwork. This index can be applied to any pair of connected layers (A, B) in a multilayer network, as long as, at least, one of them has intralayer edges. A given node i in layer A (AMF layer in our networks; Figure 1) has a set of partners in layer B (plant layer in our networks), which forms the neighbourhood of i in the layer B (Figure 1). If the interactions between nodes within this neighbourhood are favoured by node i, then the connectance among these nodes (i.e. neighbourhood connectance) should be higher than the overall connectance of the layer B (i.e. layer connectance). Then, a positive difference between these two connectances would indicate that node i contributes to structuring the interactions in layer B. The connections between nodes within the plant layer are represented by the recruitment subnetwork, while the connections between nodes of the plant layer and nodes of the AMF layer are represented by the plant-AMF subnetwork. In the case of the recruitment subnetwork, due to its directed nature, layer connectance can be decomposed into the connectance of the nodes with incoming links (incoming connectance) and the connectance of the nodes with outgoing links (outgoing connectance). In other words, layer connectance can be decomposed into the connectance relative to the sapling bank and the connectance relative to the canopy service. This, generates three different versions of the INNI index: INNI, $INNI_{IN}$ and $INNI_{OUT}$. An AMF with a positive INNI value would indicate that the plants of its neighbourhood are more interconnected among them than the average plant in the layer. An AMF with a positive $INNI_{IN}$ value would indicate that the plants of its neighbourhood are more likely to recruit under other plants of the neighbourhood than under the rest of plants of the layer (i.e. the AMF would contribute to structure the sapling bank of its hosts). An AMF with a positive $INNI_{OUT}$ value would indicate that plants of its neighbourhood are more likely to allow the recruitment of other plants of the neighbourhood than of the rest of plants in the layer (i.e. the AMF would contribute to the structure of the canopy service of its hosts). To assess whether the INNI, $INNI_{IN}$ and $INNI_{OUT}$ contributions of each

AMF to the structure of the recruitment subnetwork could emerge by chance, we performed 2000 simulations of a null model where a simulated AMF interacts with a random subgroup of k plants (with k ranging between 2 and n-2 plant species) chosen from a multinomial distribution where each plant has a probability proportional to its observed number of associated AMF. Thus, we consider that an AMF has a significant contribution to the structure of the recruitment network if its observed INNI value falls beyond the 95% percentile of the simulated distribution.

All statistical analyses were performed in the R-environment version 4.0.2 (R Development Core Team, 2020) used by means of RStudio IDE (RStudio Team, 2020), and graphically represented by means of R package GGPLOT2 v.3.3.3 (Wickham et al., 2016).

3 | RESULTS

3.1 | AMF sequencing

The number of samples analysed from each plant species captured a large proportion of the local AMF richness associated with each one, with a mean sampling coverage of 87.20%, ranging between 65.28% and 95.80% (Figure S3).

Samples averaged 7296.67 sequences, ranging from 28 to 60,010 reads. VT averaged 33,212.42, ranging from 5 to 341,214 reads. We finally obtained 86 VT from 2,898,073 reads, with five of them (191a, 191b, 191c, 135a, and 153b) considered as new VT (see S4 for further bioinformatics details and Figure S9 for VT phylogenetic relationships). They represented four orders [Glomerales (67), Diversisporales (13), Archaeosporales (3) and Paraglomerales (3)], nine families [Glomeraceae (60), Claroideoglomeraceae (8), Diversisporaceae (6), Acaulosporaceae (3), Gigasporaceae (3), Paraglomeraceae (3), Archaeosporaceae (2), Ambisporaceae (1), and Pacisporaceae (1)] and 11 genera [Glomus (59), Claroideoglomus (6), Diversispora (6), Acaulospora (3), Paraglomus (3), Archaeospora (2), Scutellospora (2), Ambispora (1), Gigaspora (1), Pacispora (1) and Septoglomus (1)].

3.2 | Subnetwork analyses

Variation in AMF community composition was mainly due to differences among plant species within each site (34.3% variance; Table S10a). Spatial variation in AMF communities was small, with region and site within region accounting for 4.7 and 7.6% of the variance, respectively. Season showed no effect. When analysing each region separately, results were highly consistent. The analysis yielded the same qualitative results when using number of reads instead of presence/absence data and applying variance stabilizing transformation (Table S10b).

The plant-AMF subnetwork consisted of 24 plant species and 76 AMF VT in Jaén (Figure 2c), and on 12 and 63 in Segura (Figure 2d). Connectance values indicated that 48.4% of all possible plant-AMF

TABLE 1 Modularity estimates of the plant-AMF and plant–plant networks studied at Sierra de Jaén and Sierra de Segura. Modularity was estimated by simulated annealing considering networks as unipartite, using a directed unipartite modularity measure. Weighted versions were also calculated. Bold type indicates that the observed values are higher than the confidence interval estimated by the null model. A multinomial probabilistic null model was used for plant-AMF networks and a canopy abundance-based version of it was used for plant–plant networks. See Section 2 for further details on null models and metric calculations.

	Observed values		Probabilistic null model	
Metric	Jaén	Segura	Jaén	Segura
Plant-AMF networks				
Unweighted modularity	0.1197	0.1933	0.1341–0.1493	0.2085–0.2323
Weighted modularity	**0.1533**	0.1824	0.1340–0.1489	0.2090–0.2340
Plant–plant networks				
Unweighted modularity	**0.0942**	0.0758	0.0456–0.0688	0.0494–0.1150
Weighted modularity	**0.1583**	0.0243	0.0304–0.0523	0.0235–0.0746

interactions were realized in Jaén and 40% in Segura. Weighted modularity was significant in the plant-AMF subnetwork at Jaén (Table 1), with three modules detected. Mean interaction push-pull was threefold in Segura (0.15) than in Jaén (0.05). See Table S7 for additional descriptive plant-AMF subnetwork parameters.

The recruitment subnetworks (Figure 2e,f) consisted of 23 species in Jaén and 12 species in Segura. They were highly connected both at Jaén (0.60) and Segura (0.55). Both considering weighted and unweighted data, the network was modular at Jaén but not at Segura (Table 1). Three modules were detected in Jaén for weighted data and four for unweighted. Weighted data were used for subsequent analyses.

3.3 | Effect of AMF on plant recruitment through the multilayer structure

3.3.1 | Effects of AMF communities' similarity on the efficiency of canopy-recruit interactions

The *glmm* revealed that plants with more similar AMF communities had a higher recruitment efficiency, after controlling for canopy and recruit abundances, for spatial variation between sites, and for recruit and canopy plant species identity (Table 2a, Figure 3). This effect was consistent across regions, given the absence of region and region by AMF dissimilarity interaction effects.

3.3.2 | Relationships of node (i.e. plant) properties across layers

Plant species degree in the plant-AMF subnetwork was significantly correlated with the canopy service of the recruitment subnetwork differently at each region (Figure 4a,b). While at Jaén, weighted and unweighted plant species degrees were positively correlated with canopy service ($\rho = 0.37$, and 0.32, respectively; not significantly in the latter case), at Segura, both were negatively correlated ($\rho = -0.64$ and −0.53, respectively). This effect was mainly due to subnetwork

topology, since the weighted estimates modified the values of the correlation very slightly. Weighted and unweighted plant species degrees were not correlated with sapling bank, neither at Jaén ($\rho = 0.18$ and 0.17, respectively) nor at Segura ($\rho = -0.27$ and −0.31).

Plant species centrality in the plant-AMF subnetwork was positively correlated with the sapling bank of the plant–plant subnetwork in Jaén ($\rho = 0.47$), but not in Segura ($\rho = 0.12$; Figure 4c). This effect was affected by AMF incidence since unweighted centrality was not correlated with sapling bank neither in Jaén ($\rho = 0.01$) nor in Segura ($\rho = 0.12$). Canopy service was not correlated with weighted or unweighted centrality estimates at any region ($\rho < 0.25$ in all cases). Plants belonging to the same recruitment subnetwork module were more similar in their associated AMF communities. This effect was consistent across regions, given the absence of region and region by AMF dissimilarity interaction effects (Table 2b). However, results of the individual AMF contributions to the structure of the interactions in the recruitment network through the INNI index analyses, did clearly vary between regions. In Jaén, we detected four AMF with significant INNI, and INNI_{OUT} contributions, two of them also showing a significant INNI_{IN} contribution (Figure 5). In Segura, we detected one AMF with significant INNI, INNI_{IN} and INNI_{OUT} contributions. All AMF detected with significant contributions (VT72, VT105, VT191, VT191b, VT418) belong to the genus *Glomus* (Figure S9). Excepting AMF 191b, which was detected only in Jaén, all occurred in both sites and were relatively abundant (ranging between 3228 and 68,518 reads) and occurred in 54% to 83% of the plant species.

4 | DISCUSSION

We have explored the interaction between 86 AMF and 32 woody plant species that characterize two Mediterranean forest communities, by means of a multilayer network approach. We show that properties of the plant-AMF interaction subnetwork, like similarity of AMF communities between plants and presence of keystone AMF (i.e. those potentially impacting disproportionately the structure of plant–plant interactions, regardless of their abundance in the

TABLE 2 Generalized linear mixed model testing the effect of the dissimilarity in AMF communities between canopy and recruit plant species on (a) the recruitment efficiency (i.e. the number of saplings of the species i recruiting under canopy species j (F_{ij}), relative to plant species abundance) and on (b) the plants belonging to the same or different module of the recruitment subnetwork. Significant fixed effects are represented in bold type.

| Fixed effects	(a)				(b)			
	Estimate	**Std. error**	**Z value**	**p**	**Estimate**	**Std. error**	**Z value**	**p**
AMF dissimilarity	**−0.7146**	**0.3327**	**−1.148**	**0.032**	**−1.5211**	**0.5606**	**−2.713**	**0.0066**
Recruit sp abundance	**0.2812**	**0.0375**	**7.493**	**<0.0001**				
Canopy sp abundance	**1.0848**	**0.0609**	**17.797**	**<0.0001**				
Region	−0.3720	0.5839	−0.637	0.5241	0.8965	0.5063	1.771	0.0766
AMF dissimilarity × Region	−0.3775	0.8945	−0.422	0.6730	−0.7724	1.0002	−0.772	0.4399
Random effects	**Variance**	**Std. dev**			**Variance**	**Std. dev**		
Site	0.4023	0.6343						
Recruit sp	0.7722	0.8788			9.81×10^{-10}	3.13×10^{-5}		
Canopy sp	0.0599	0.2449			9.81×10^{-10}	3.13×10^{-5}		
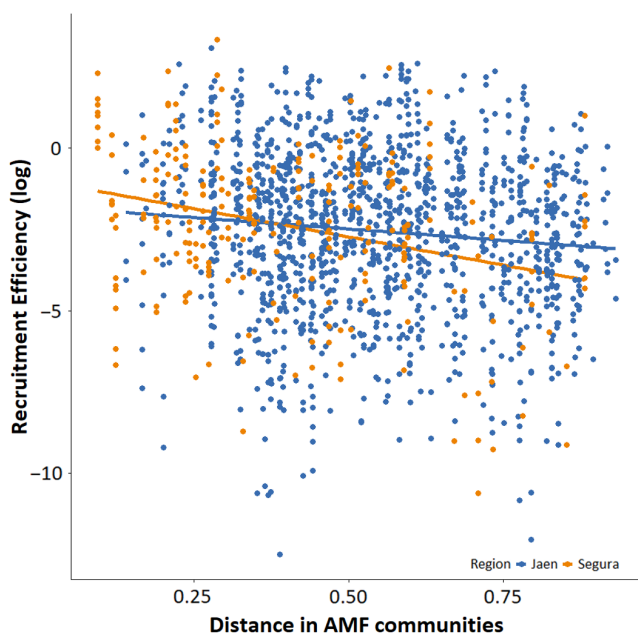
FIGURE 3 Relationship between the similarity in arbuscular mycorrhizal fungi (AMF) communities between canopy and recruit species, and the recruitment efficiency of a given canopy-recruit pair of species. Distance in AMF communities is measured by Morisita-Horn distances. Recruitment efficiency is measured as the recruitment frequency (F_{ij}) of a species i recruiting under a species j, relative to the abundance of both i and j species; and it is shown as the partial residuals of the recruitment frequency from a generalized linear mixed model (see Section 2 for details).

community), leave their imprint on the structure of the plant–plant recruitment subnetwork.

Sample coverage indicated that only less than 13% of links in plant-AMF subnetworks remained undetected. Completeness studies of ecological interaction networks show that it is much more difficult to achieve a high coverage in link number than in nodes number (Chacoff et al., 2012; Henriksen et al., 2019; Pulgar et al., 2017),

since link number grows to the square with node number. On the other hand, links remaining undetected even after reaching a high coverage should correspond to very low frequency interactions, likely playing a minor role in the network. Thus, our coverage level suggests that plant-AMF networks were properly sampled and described at both study regions.

4.1 | Effects of plant-AMF interactions on plant recruitment

We have found clear signals that plant-AMF interactions play a relevant role on the assembly of plant–plant recruitment interactions at the community level in two natural forest stands. As expected by the eminently mutualistic nature of plant-AMF interactions, our results show that the similarity between canopy and recruit species in their associated AMF enhances the recruitment efficiency in both forests, supporting Prediction (i). Despite the moderate predictive power of the relationship (i.e. Figure 3), the fact that we could detect this effect in spite of the stochastic noise imposed by the multifarious processes involved in canopy-recruit interactions (Alcántara et al., 2018) suggests that plant-AMF interactions influence the recruitment process in the wild.

Experimental studies show that the effect of AMF on seedlings may vary depending on the plant and AMF species identity, on plant species combinations, and on study systems (Montesinos-Navarro et al., 2018; Van der Heijden & Horton, 2009). AMF may enhance seedling establishment by providing fundamental nutrients (Selosse et al., 2006; Van der Heijden, 2004). However, fungi that transfer the most nutrients to plants inevitably require the most energy, and Jakobsen and Hammer (2015) and Kiers et al. (2011) suggest that AMF distribute phosphorus to the plants in accordance with their size or strength as carbon sources. If seedlings and adult plants are connected by AMF mycelia, this could amplify the competition of adult plants towards recruiting seedlings (Weremijewicz &
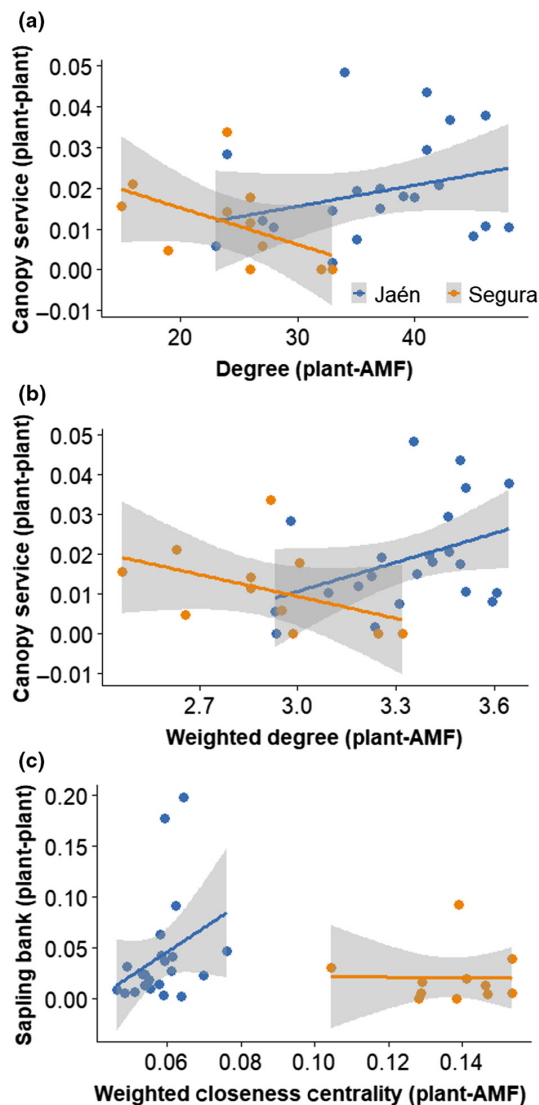
FIGURE 4 Relationships between plant-arbuscular mycorrhizal fungi (AMF) and recruitment subnetworks. Spearman correlations between degree (a), weighted degree (b) and weighted closeness centrality (c) of plant species in the plant-AMF subnetwork, and two aspects of the plant–plant subnetworks, canopy service and sapling bank. All correlations were significant at the $\alpha=0.05$ level, except for degree versus canopy service in Jaén, and weighted closeness centrality versus sapling bank at Segura.

Janos, 2013). Nevertheless, these authors also suggest that this negative effect on recruits would be only temporary and likely to shift to a typical positive mycorrhizal growth response when adult plants loose vigour (senescence, biotic and abiotic interactions), or when recruited plants gain size and become strong enough as carbon sources (Kiers et al., 2011). These changes may explain the contrasting results found here for some aspects of the plant-AMF interaction between the two study regions. The asymmetry of the plant-AMF interaction, measured by the interaction push-pull index, was closer to 0 in Jaén and three times larger in Segura. This indicates that AMF in Segura are more dependent on plants than plants on fungi, while in Jaén, the level of dependence was more similar in both directions.

This suggests that AMF in Segura could be drawing carbon from the plants, which could have negative consequences for small recruiting individuals that could enter in carbon shortage. However, the exchange of nutrients between plants and fungi in Jaén might be more balanced, allowing saplings to benefit from their interaction. This would explain why in Jaén, canopy plant species with a wider set of AMF partners allowed the recruitment of a more abundant sapling bank beneath their canopies. This relationship was reversed in Segura, possibly because saplings interacting with more partners would see their carbon resources depleted by more demanding AMF assemblages. Thus, Prediction (ii) was supported in Jaén but not in Segura.

It may seem counterintuitive that in Segura, the similarity in AMF communities increases recruitment efficiency, while at the same time, canopy plants that interact with more AMF (i.e. showing higher degrees) provide a worse canopy service. However, we found that the similarity in AMF between two species is independent from the degree of the canopy species (see Table S11).

We also found that the abundance of a given species in the sapling bank was higher for plants more centrally located in the plant-AMF network in Jaén but not in Segura. Thus, Prediction (iii) was supported in Jaén but not in Segura. Closeness centrality considers both direct and indirect interactions between plants and AMF. A more centrally located recruiting plant will potentially have access to many AMF partners (i.e. high degree), but it may also benefit indirectly from other AMF benefitting the canopy plants they are recruiting beneath. This system of direct and indirect interactions would behave this way insofar as the mutual dependences between plants and AMF were more symmetric, as it occurs in Jaén. As the system of dependences becomes more asymmetric, only the direct interactions become relevant and centrality loses its added value, as it occurs in Segura.

4.2 | Effects of plant-AMF interactions on the structure of the recruitment network

The structure of plant recruitment networks has important effects on plant community dynamics (Alcántara et al., 2015; Alcántara & Rey, 2012). Some studies have shown that increasing connectance may lead to decreased stability in communities of competing species (Allesina & Tang, 2012), and that modularity can contribute to their stability (Dormann et al., 2017; Grilli et al., 2016). Despite these important effects, the nature of such mechanisms remains poorly understood. Our results clearly indicate that plant-AMF interactions take part in the mechanisms structuring recruitment networks. However, we also show how the functioning of this mechanism may shift depending on the environmental context (i.e. region), what modifies its influence on the recruitment network structure. Nonetheless, it is to be noticed that AMF may be just partially responsible of the patterns here observed, since plant associates with ECM ectomycorrhizas also play an important role in these temperate forest environments.
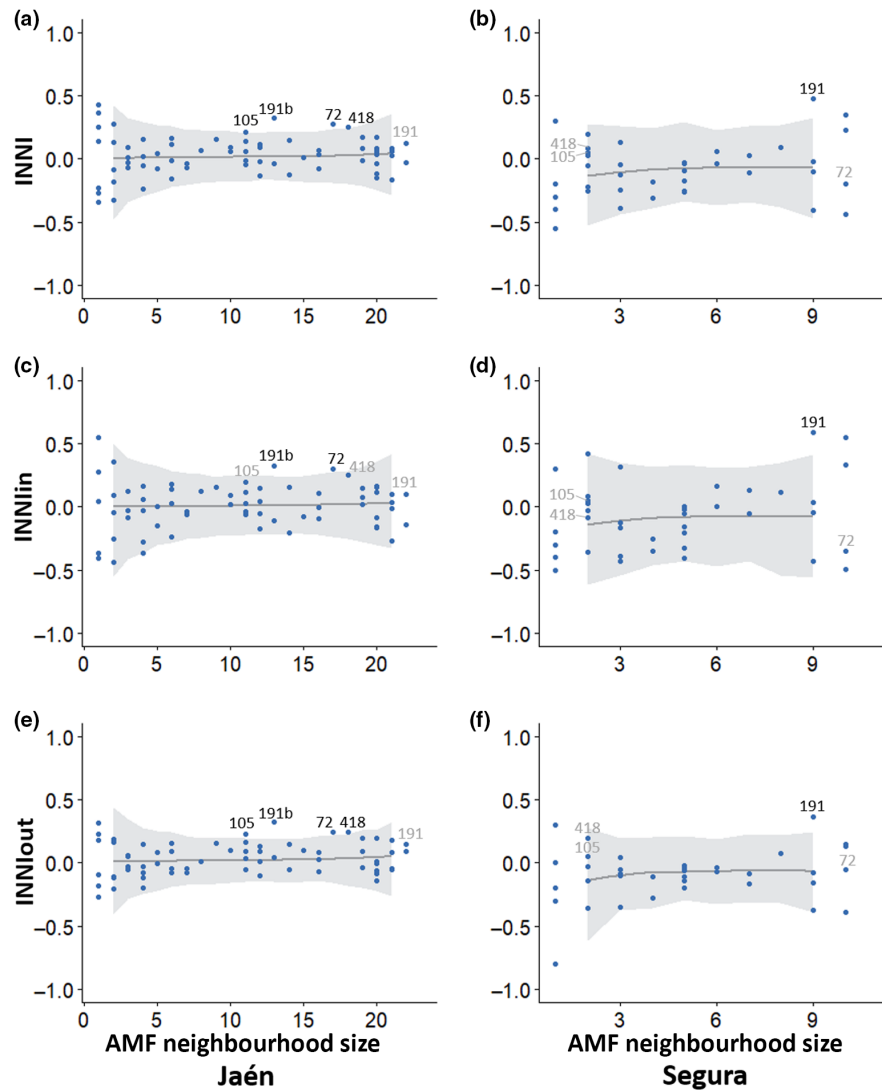
FIGURE 5 Contribution of each arbuscular mycorrhizal fungi (AMF) virtual taxa (VT) in the plant-AMF subnetwork to the structure of interactions among plants in the recruitment subnetwork both at Sierra de Jaén (left panels) and Sierra de Segura (right panels). This contribution is estimated by the Interlayer Node Neighbourhood Integration (INNI) multilayer index. Specifically, it measures whether an AMF VT contributes significantly to the connectance between the plants of its neighbourhood relatively to the rest of plants of the recruitment subnetwork (see Section 2). INNI values indicate the importance of the neighbourhood of VT i respect to the total of interactions in the plant–plant subnetwork. $INNI_{IN}$ values indicate the importance of the neighbourhood of VT i respect to the interactions received by the plants in the plant–plant subnetwork, that is respect to the sapling bank of each plant species in the community. $INNI_{OUT}$ values indicate the importance of the neighbourhood of VT i respect to the interactions sent by the plants in the plant–plant subnetwork, that is respect to the canopy service of each plant species in the community. Overall, we detected 5 AMF VT (72, 105, 191, 191b, and 418) contributing significantly to the structure of the interactions in the recruitment subnetwork. Significance of each AMF VT contribution is assessed by means of 2000 simulations of a null model where a given AMF interacts with k plants from the recruitment networks, chosen depending on their interaction probability with VT. k ranges between 2 and n-2 plant species. Out of the 5 AMF detected, the ones contributing significantly in each case are indicated with black numbers, while the rest are represented in grey. Mean and 95% confidence interval of simulations are grey shaded.

In general, a highly connected network is less likely to show modularity. Despite this, the recruitment network in Jaén (but not in Segura) showed non-random levels of modularity, what points to the action of some ecological processes structuring the network. Accordingly, in agreement with Prediction (iv), the likelihood of two plants belonging to the same module increased with the similarity in their AMF communities. Moreover, we found four AMF VT in Jaén and one in Segura that have a non-random contribution to the structure of the recruitment network. These AMF seem to enhance the connectance, the sapling bank and the canopy service of their hosts. Their effects do not seem to be related to each AMF degree, since in Jaén the largest INNI, $INNI_{IN}$ and $INNI_{OUT}$ corresponded to an AMF with intermediate degree (see VT 191b in Figure 5). Moreover, this AMF, as well as VT 105, occurred with a relatively low incidence in our samples (5.5% and 7.3%, respectively) and both were also the least abundant, out of the 5 significant VT, in terms of number of reads (3228 and 14,742,

respectively). This suggests that they enhance connectance, sapling bank and/or canopy service disproportionately in the few host species where they are present. Interestingly, these potentially keystone AMF occurred mostly in Jaén, even though some of their host plants, for example, *Crataegus monogyna*, *Cistus albidus* and *Thymus mastichina* are also very common in Segura. It is tempting to speculate to which extent the scarcity of potentially keystone AMF may be behind the loose structuring of the recruitment network in Segura. It would be interesting to experimentally assess the effect of potentially keystone AMF, as they could be useful in natural vegetation conservation (Barea et al., 2011).

Recent studies are highlighting the rich and complex nature of plant-fungi interactions present in natural communities (Montesinos-Navarro et al., 2012a; Sepp et al., 2019; Toju et al., 2014, 2015, 2018; Xing et al., 2019). However, if we seek to understand how this complexity translates its effects to plant community dynamics, we must transcend the mere description of the structure of such networks, looking for structural and functional effects of plant-AMF interactions. The multilayer approach here used provides a suitable tool for this purpose, which can be further developed as far as we can obtain information to meaningfully integrate more network features, more network types and/or improve both the extent and multidirectionality of the effects of one network on another.

AUTHOR CONTRIBUTIONS

José L. Garrido, Julio M. Alcántara and Concepción Azcón-Aguilar designed the study. José L. Garrido lead the writing. José L. Garrido and Julio M. Alcántara conducted the statistical analyses and wrote the first draft of the manuscript with contributions of Álvaro López-García. Álvaro López-García and Carmen V. Ozuna processed root samples and performed the bioinformatics analyses. José L. Garrido, Julio M. Alcántara, Concepción Azcón-Aguilar, Álvaro López-García, Antonio J. Perea, Jorge Prieto and Ana Rincón collected field samples. All authors contributed critically to the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest.

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SUPPORTING INFORMATION

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

Table S1. Location, altitude and dominant vegetation at each study site.

Table S2. Samples distribution by species, season, region and site.

Figure S3. Sequencing rarefaction curves and sampling completeness.

Methods S4. Root processing, DNA extraction, sequencing and bioinformatic analyses.

Table S5. A recruitment network represented by its recruitment matrix.

Table S6. Description of main network metrics and indices used throughout the work.

Methods S7. General metrics describing the plant-AMF networks studied.

Methods S8. Null models for modularity.

Table S9. Maximum likelihood tree of the sequences defining the AMF virtual taxa sampled in this study.

Table S10. PERMANOVA exploring the effect of region, season, site and plant species on AMF community composition.

Table S11. Bayesian glmm testing the influence of the degree of canopy plant species on the dissimilarity in AMF communities between canopy and recruit species.