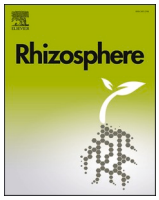
Rhizosphere 34 (2025) 101039

Contents lists available at ScienceDirect

Rhizosphere

journal homepage: www.elsevier.com/locate/rhisph



Development of whole-soil microbial inoculants based on solid-phase fermentation for the regeneration of the functioning of vineyard soils

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ARTICLE INFO

Keywords:
Solid-phase fermentation
Biofertilizer
Inoculum
Soil microorganisms
Enzyme activity
Rhizosphere

ABSTRACT

Achieving the sustainability of modern agriculture will require, among other actions, an improvement of the soil and its associated microbiota. One way to achieve this is through the inoculation with beneficial soil microbial communities. In this study, we used solid-phase fermentations to produce 25 distinct microbial inoculants based on complex communities obtained from the rhizosphere of 23 European vineyards. For this purpose, we mixed 0.1 g of donor rhizosphere soil and 25 g of ground and sterilized growing substrate composed of winemaking byproducts in 15-cm Petri dishes. Aerobic fermentations were carried out for a period of 56 days and the activity of microbial enzymes linked to the biogeochemical cycling of carbon, nitrogen, phosphorus, and sulfur was evaluated every two weeks. We then carried out a common garden experiment where the inoculants were tested in pots containing vineyard soil and planted with Tempranillo vines. During the fermentation, the enzyme activity of the inoculants evolved from no activity to high-activity values. Carbon- and phosphorus-linked enzymes tended to show higher activity after 14 days of incubation and then decreased or remained constant, while nitrogen-linked enzymes tended to show their highest values after 28 days of incubation. Despite these general patterns, inoculants developed from different rhizosphere communities followed different trajectories in terms of activity. In addition, we observed significant relationships between the enzyme activity of donor rhizosphere soils and the enzyme activity of inoculants, especially after 28 days of incubation. We also found a significant relationship between the enzyme activity of the inoculants and the enzyme activity measured in the soil of pots containing vines. Our results suggest the possibility of predicting the metabolic potential of the inoculants from

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https://doi.org/10.1016/j.rhisph.2025.101039
Received 14 October 2024; Received in revised form 19 December 2024; Accepted 11 February 2025
Available online 20 February 2025

the metabolic potential of their donor soil sample, as well as the possibility of transferring these metabolic capabilities to soils, with likely applications for the regeneration of the functioning of vineyards.

1. Introduction

Vineyards are one of the oldest perennial cultivation systems on the planet, as grapevines have been planted since Neolithic times (McGovern, 2013). Despite their societal, economic, and environmental relevance, vineyards have recently suffered a process of degradation due to the intensive use of phytosanitary products and ploughing, affecting their health and long-term sustainability (Newton et al., 2010). Soils play a fundamental role in the health of vineyards (Fournier et al., 2022), as the complex relationships established between the plant and the microbes that inhabit the soil are vital for the proper functioning of both (Ahemad et al., 2009; Ahmad et al., 2008). For example, soil microbial communities play a key role for plant health through their ability to recycle nutrients from the soil (Berendsen et al., 2012). Extracellular enzymes produced by microbes are key for the decomposition and mineralization of organic matter, in turn, allowing for a better plant growth (Ahemad and Khan, 2011; Lugtenberg and Kamilova, 2009). The associations that are established between plants and soil communities also contribute to proper plant development and defence against pathogenic microbes. For example, some filamentous fungi can create a dense network of hyphae around plant roots that can trap herbivorous nematodes that try to feed on them (Mixter, 2013). Since the rhizosphere, the small portion of soil that is in intimate contact with the fine roots, is a hotspot of microbial biodiversity (Das and Varma, 2010), it has been frequently argued that these organisms and the processes that they carry out may be key to the development of nature-based solutions aimed at soil regeneration in agroecosystems.

One strategy involving the use of microbes for the regeneration of agroecosystems is the development and application of inoculants that, when added to degraded soils, can contribute to improve their functioning (Elliott and Lynch, 1995; Gu et al., 2020). Currently, most microbial inoculants are based on the culturing of pure strains, which can be added alone or as part of a more complex formulation. However, it has been argued that inoculants based on whole-soil communities may represent a much more powerful solution (Toju et al., 2018), somewhat equivalent to faecal microbiota transplantation vs. the use of probiotics in medicine (Cammarota et al., 2014). There are two main strategies used for the development and production of these whole-soil inoculants, namely solid-phase fermentation (Bhargav et al., 2008; Thomas et al., 2013) and composting (de Bertoldi et al., 1983). Although there are more studies dealing with composting, growing microbial communities using solid-phase fermentation techniques has several advantages, including more flexible growing conditions that can be adapted to the particularities of the complex communities to be replicated.

Solid-phase fermentation allows the production of microbial inoculants using a solid organic substrate as culture medium (Mitchell et al., 2000). This fermentation technique appeared with the aim of producing pharmaceutical compounds, but as the technology has advanced, it has been used for different industries such as the food or plant protection industries (Hölker and Lenz, 2005). Solid-phase fermentation typically aims to recreate the ideal conditions in humid and solid environments for the proliferation of all kinds of micro-organisms, especially filamentous fungi (Hölker and Lenz, 2005). In contrast to unicellular organisms, filamentous fungi tend to grow in greater proportion in solid environments without the presence of liquid water (Raghavarao et al., 2003). Fermentations, especially those carried out by fungi, produce enzymes and other metabolites that can be used for multiple purposes, including promoting plant growth. For example, *Aspergillus terreus* solubilises inorganic phosphates that are assimilated by plants (Vassileva et al., 2021). Solid-phase fermentation may also allow to create inoculants that harbour selected communities of microorganisms with positive effects on plants through their ability to produce extracellular enzymes linked to organic matter mineralization, thus allowing a more efficient nutrient release from soil pools and organic amendments (Vassilev and de Oliveira Mendes, 2018; Vassilev and Malusà, 2021).

In this study, we used solid-phase fermentations to develop inoculants based on complex communities from rhizosphere soil samples collected from 23 European vineyards, which allowed us to test our capacity to transfer the metabolic potential of soils to complex formulations. Inoculants were developed from agro-food industry residues, specifically from the wine industry, and these residues were chosen to maximize the growth of fungi over bacteria based on the carbon (C): nitrogen (N) ratio of the growing media (30:1) (Berg and Laskowski,

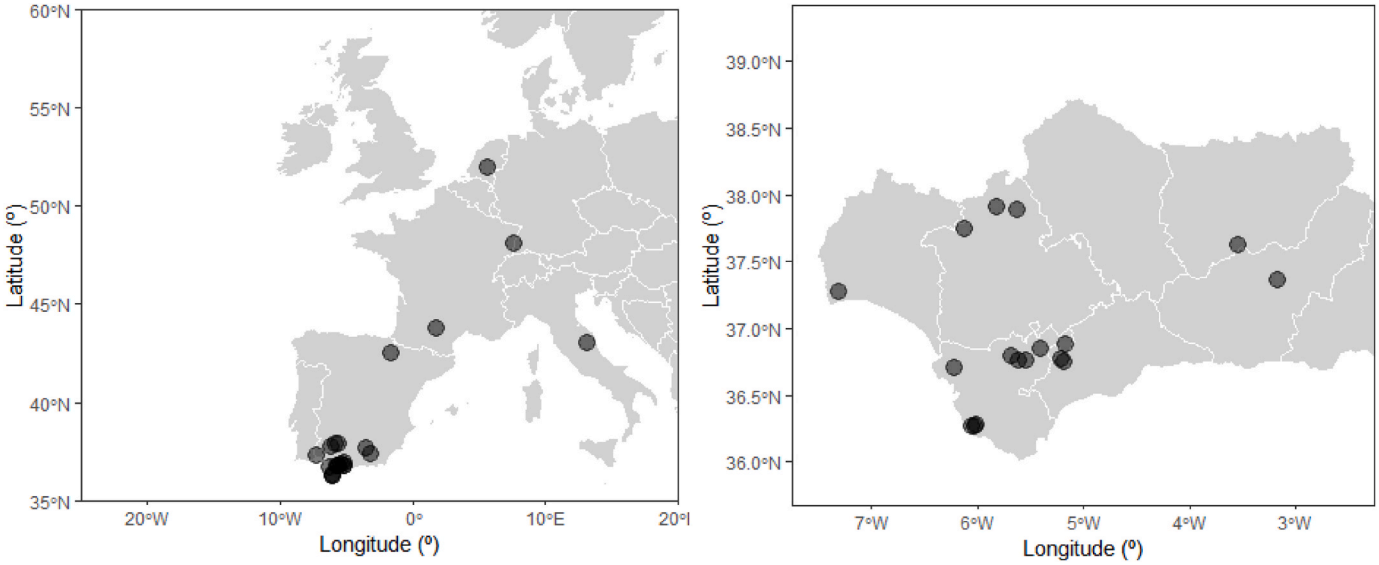

Fig. 1. Distribution of vineyards where donor rhizosphere soils were collected. Left panel: European vineyards. Right panel: Close-up look to vineyards from Andalucia (south of Spain).

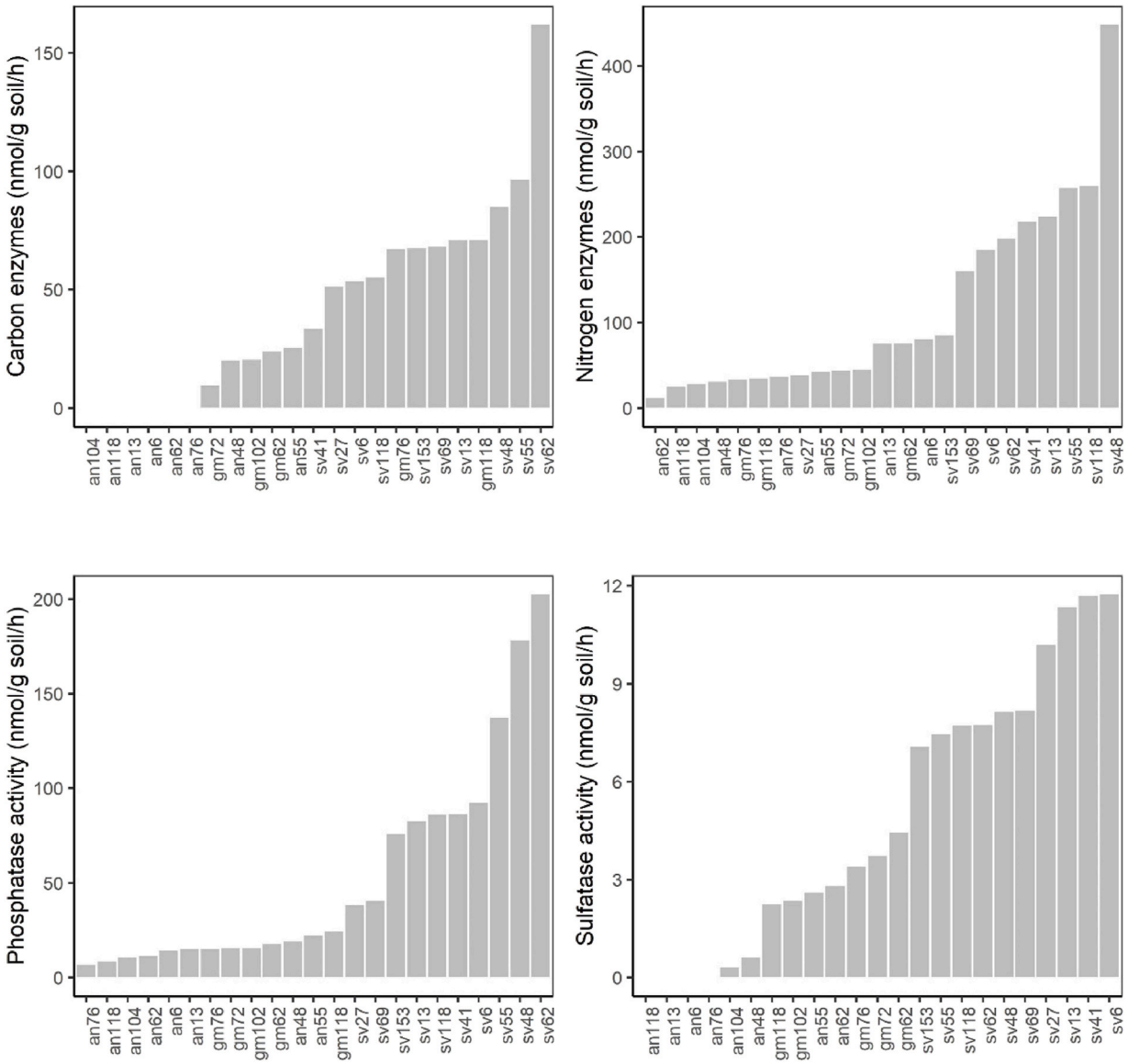

Fig. 2. Enzyme activity in 23 soils from Cádiz (sv), Andalusia (an), and the rest of Europe (gm). Activities are ordered from lowest to highest.

2005; De Boer et al., 2005; Meidute et al., 2008).

First, we evaluated the transferability of the metabolic potential (enzyme activity) from the donor soils to the inoculated Petri dishes. We then tested the transfer potential from the inoculants to the soil of pots containing Tempranillo grapevines grown under common garden conditions. We expected that, by using different soils from different locations, the fermentation trajectory will be influenced by the metabolic potential of the initial microbial community, and that we would also be able to transfer part of this metabolic potential to soils supporting the growth of plants, thus allowing us to transfer the metabolic potential of the donor rhizosphere sample to a recipient soil via an intermediate step of solid-phase fermentation.

2. Materials and methods

2.1. Study area

The study area includes vineyards located in: (1) the province of Cádiz, south Spain (nine vineyards), (2) the rest of Andalusian provinces, also in the south of Spain (nine vineyards), and (3) the rest of mainland Spain and several European countries (five vineyards in total distributed between Spain, Italy, France, the Netherlands, and Germany) (Fig. 1). Therefore, the studied vineyards originate from areas with contrasting climatic and soil conditions and include different cultivation systems. These vineyards were chosen among a greater number of vineyards (90) sampled from these regions because they showed higher enzymatic activity values (data not shown).

2.2. Sampling

Rhizosphere soil samples to be used as donor soils were collected during the veraison season between 2020 and 2021. To collect the samples, we dug a hole 40 cm deep at the base of five randomly selected vines within a 30 m × 30 m plot in each vineyard. We looked for fine roots and collected the thin fraction of soil attached to them, which is what we considered as rhizosphere soil. Soils were kept refrigerated during fieldwork and transportation and then were frozen at −20 °C upon arrival to the laboratory.

2.3. Preparation of growing substrate for solid-phase fermentation

The material used as culture medium for the development of

Table 1
ANOVA table showing the effects of inoculation and time on the enzymatic activity of the inoculants (top), as well as the effects of inoculation on the enzyme activity of soils in the pot assay (bottom). SPF = solid-phase fermentation study. Pots = inoculation study. C = sum of all enzymes linked to C cycling. N = sum of all enzymes linked to N cycling. BG: β-1,4-glucosidase. XYL: β-xylosidase. CB: β-D-cellobiohydrolase. AG: α-1,4-glucosidase. NAG: β-1,4-N-acetylglucosaminidase. LAP: leucine-aminopeptidase. PHOS: phosphatase. AS: arylsulfatase.

	df	BG		CB		NAG		PHOS		XYL		AG		LAP		AS		C		N	
		F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Fermentations																					
Location	25, 338	3.29	<0.01	4.36	<0.01	3.11	<0.01	4.30	<0.01	3.68	<0.01	1.02	0.44	4.87	<0.01	2.17	<0.01	3.85	<0.01	3.03	<0.01
Time	1, 338	97.63	<0.01	25.51	<0.01	23.50	<0.01	57.60	<0.01	0.65	0.41	58.04	<0.01	2.45	0.11	38.43	<0.01	67.96	<0.01	22.98	<0.01
Location:Time	25, 338	2.19	<0.01	2.28	<0.01	1.12	0.31	3.14	<0.01	1.77	0.01	1.03	0.43	0.79	0.74	1.1	0.34	2.28	<0.01	1.09	0.34
Pots																					
Inoculation	26, 130	5.03	0.58	2.62	<0.01	8.23	<0.01	2.71	<0.01	18.48	<0.01	40.68	<0.01	2.1	<0.01	34.61	<0.01	2.10	<0.01	34.6	<0.01

inoculants originated from an organic vineyard in Jerez de la Frontera, south of Spain (IFAPA Rancho de la Merced), where pruning remains, grape stems, pomace, and lees were collected. All these compounds constitute the standard wastes from wine production.

Prior to solid-phase fermentation, residues were oven-dried at 60 °C and ground using a blade mill (Restch GM200), and a toothed rotor and sieve mill (Restch ZM200) in order to have a homogeneous substrate and avoid coarse fractions. Once the different materials were dry and ground, we analysed their C and N content in order to adjust the C:N ratio of the mixture to 30:1. The final mix was composed of 88.5% pruning wood, 4% grape stems, 6.5% pomace, and 1% lees, and had a pH of 4.5. This mixture was then rehydrated to 100% water holding capacity and sterilized in an autoclave at 120 °C for 1 h. Finally, we introduced 25 g of mixture into 15-cm diameter Petri dishes in order to carry out the fermentations.

2.4. Solid phase fermentation

We diluted 0.1 g of each of the 23 donor soils into 1 mL of distilled water and used these slurries to inoculate Petri dishes containing 25 g of hydrated and autoclaved substrate. In addition to the 23 inoculants corresponding to the 23 sampled sites, we also prepared two additional inoculants based on the mixture of 9 locations in Cádiz and 9 locations in Andalucía, for a total of 25 inoculants. Petri dishes were sealed using Parafilm in order to allow the flow of air but not water. A total of 312 Petri dishes were prepared, which were distributed among the 25 donor soils in addition to control Petri dishes (that is, without added inoculum) and four sampling times (after 14, 28, 42, and 56 days of incubation). Each treatment and sampling time was replicated 3 times. The incubations were carried out at room temperature (21–24 °C) and in the dark. After the destructive sampling of the Petri dishes (3 per sampling time and per donor soil), a fraction of inoculant was frozen at −20 °C for subsequent enzyme analyses.

2.5. Pot inoculation experiment

In order to test the potential of the inoculants developed to steer the functioning of vineyard soils, we carried out a common garden experiment. For this, we grew Tempranillo grapevines on 110-R rootstocks (Vitis Navarra nursery, Larraga, northern Spain) in 10 L pots. Pots were filled with a mixture of 60% vineyard soil and 40% commercial peat. This blend of vineyard soil and peat was necessary to facilitate the handling of the soil volume used for the implementation of the experiment. Vineyard soils were collected from an organic vineyard at IFAPA Rancho de la Merced (Jerez de la Frontera, Cádiz, Spain). Soils in this vineyard are known as *albariza* and are characterised by a high pH (> 8.2) and calcium content, and a whitish colour; these soils are also characterised by their high content of siliceous marine fossil remains (mainly diatoms) (Paneque et al., 2000). We grew a total of 156 plants that were distributed among the 26 inoculation treatments (25 inoculants + 1 control without inoculation, with n = 6 replicates per treatment). These inoculants were based on the 56-day fermentations, when microbial communities were stable.

Before the initiation of the common garden experiment, we excluded the possible existence of pathogens in our inoculants by applying them to roses and looking for signs of infection. We initiated the experiment in June 2022, after verifying the lack of symptoms. Once vines were planted, we placed 0.1 g of ground inoculant at a depth of 10 cm inside each pot. In November 2022, that is, after 145 days of experimental duration, we collected 3 soil samples per pot using a soil corer and homogenised them. A fraction of these samples was frozen at −20 °C for subsequent enzyme activity analyses.

2.6. Enzyme activity analyses

To assess the metabolic potential of the donor rhizosphere soil,

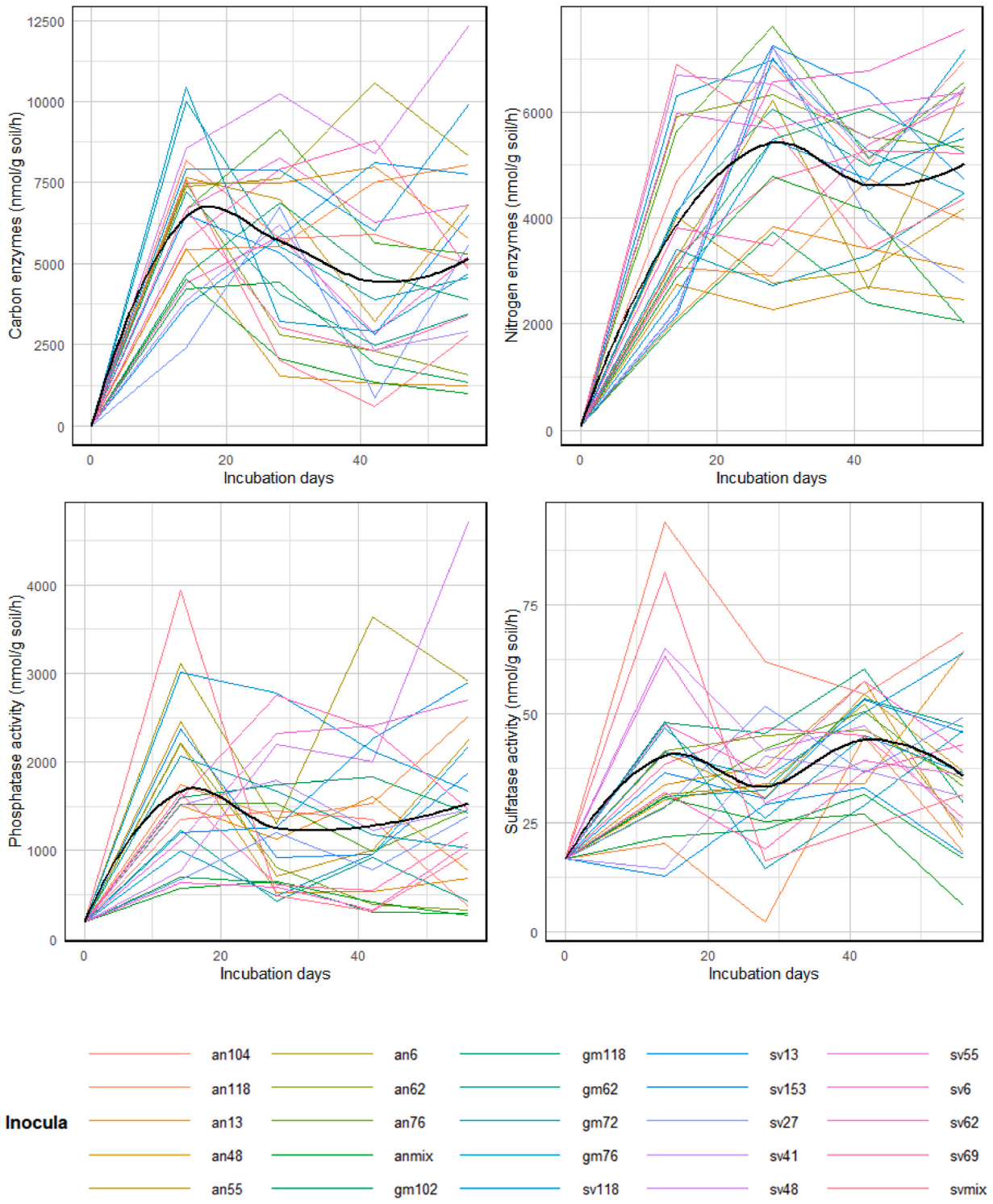

Fig. 3. Evolution of solid-phase fermentations over the 56-day incubation period. The black solid line indicates the evolution of all plates when these are considered together, while the colored lines refer to the different locations that served as soil donors. Carbon enzymes = BG + CB + AG + XYL. Nitrogen enzymes = NAG + LAP.

inoculants, and potted soils, we evaluated the activity of eight hydrolytic enzymes. We measured: (i) four enzymes linked to the C cycle: β-1,4-glucosidase (BG; substrate: 4-methylumbelliferyl α-D-glucopyranoside), β-xylosidase (XYL; substrate: 4-methylumbelliferyl-β-D-xylopyranoside), β-D-cellobiohydrolase (CB; substrate: 4-methylumbelliferyl β-D-cellobioside) and α-1,4-glucosidase (AG; substrate: 4-methylumbelliferyl β-D-glucopyranoside); (ii) two enzymes linked to the N cycle: β-1,4-N-acetylglucosaminidase (NAG; substrate: 4-Methylumbelliferyl N-acetyl-β-D-glucosaminide), and leucine-aminopeptidase (LAP; substrate: L-leucine hydrochloride-7-amido-4-methylcoumarin); (iii) one enzyme linked to the phosphorus (P) cycle: phosphatase (PHOS; substrate: 4-methylumbelliferyl phosphate); and (iv) one enzyme linked to the sulfur (S) cycle: arylsulfatase (AS; substrate: 4-methylumbelliferyl sulfate). Enzyme activity analyses were carried out on frozen soil/inoculum samples.

For soil samples, 0.5 g of soil was deposited in a Falcon tube and 15

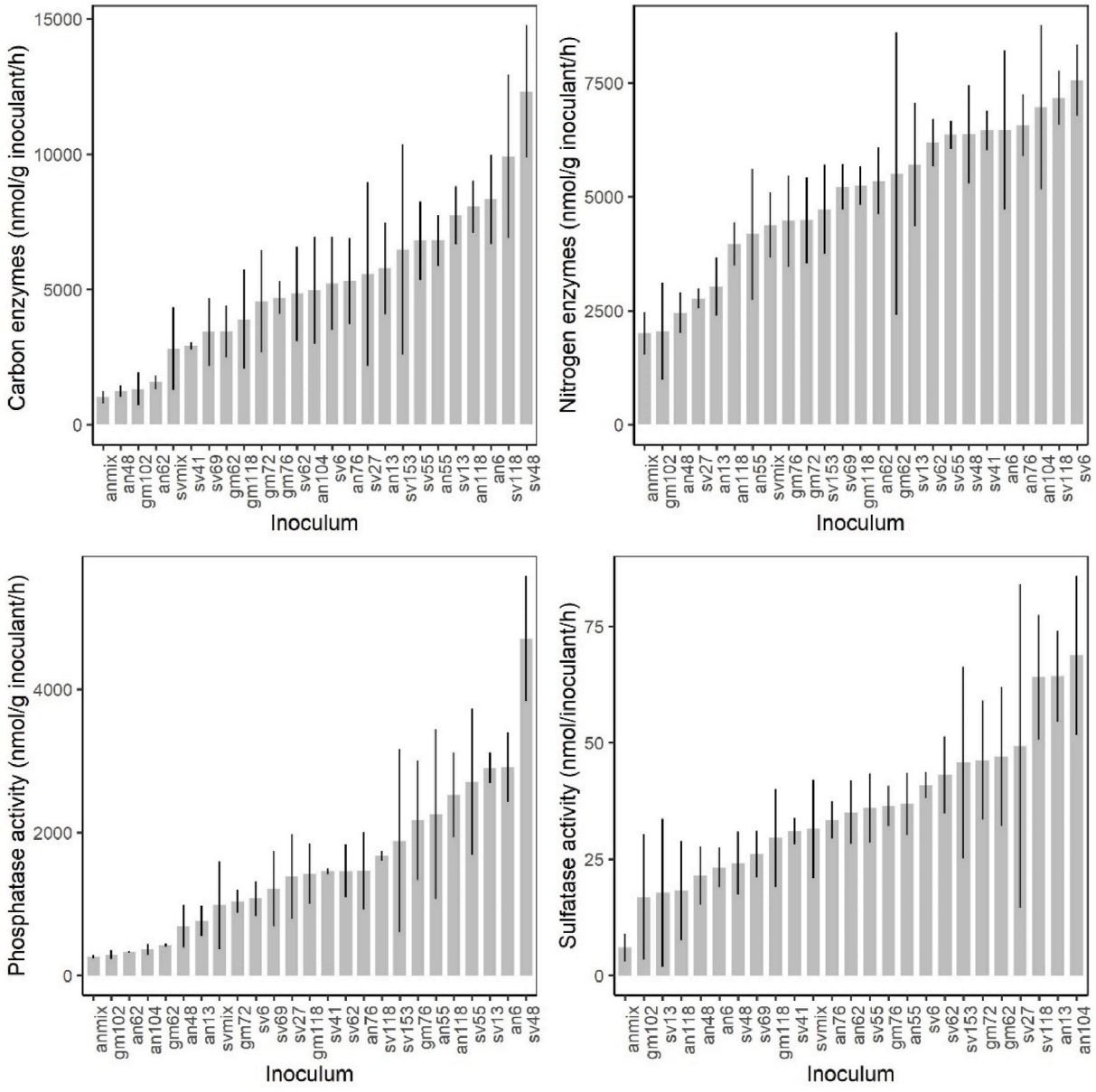

Fig. 4. Enzyme activity measured in the ferments after 56 days of incubation. Activities are ordered from lowest to highest. Means ± SEs are presented.

mL of distilled water was added. For inoculants, 0.5 g of frozen inoculant was suspended in 30 mL of distilled water. After 30 min of shaking on an orbital shaker, samples were transferred to black 96-well plates, to which the fluorescent substrates of the enzymes to be measured were added according to the protocol described by Bell et al. (2013). The corresponding calibration lines were made using 4-methylumbelliferone (MUB) and L-leucine-7-amino-4-methylcoumarin (MUC). Finally, the plates with the samples were incubated for 1.5 h at 35 °C in the dark, and they were measured in a fluorescence microplate reader (BMG FLUOStar Omega), using an excitation wavelength of 355 nm and a wavelength emission of 460 nm.

2.7. Numerical calculations and statistical analyses

Numerical calculations and statistical analyses were done in R version 4.2.2 (R Core Team, 2022). We used general linear models to evaluate the effect of the identity of the donor sample and the incubation time on the enzyme activity of the inoculants. Likewise, we used linear models to evaluate the effect of the inoculants on the enzymatic activity of the soil of pots. For this, we used the lm function from the *stats* package (R Core Team., 2022). In addition, we used Pearson correlations to evaluate the relationship between the enzyme activity of the donor soil and the inoculants throughout the incubation period. For this, we used the rcorr function of the *Hmisc* package (Frank et al., 2022). Finally, we used structural equation modelling to assess the hypothetical causality of the transferability potential of the enzyme activity from the donor soil to the inoculant, and from the inoculants to soils in pots. For this, we used the psem function of the *piecewiseSEM* package (Lefcheck et al., 2016). Good model fit was assumed when the P-value of the Fisher's C was greater than 0.05.

3. Results

3.1. Development of inoculants through solid-phase fermentation

Rhizosphere soils from Cádiz showed, in general, the highest values of enzyme activity for the four groups of enzymes (Fig. 2 and S1). Enzymes corresponding to the N cycle showed the highest activity values,

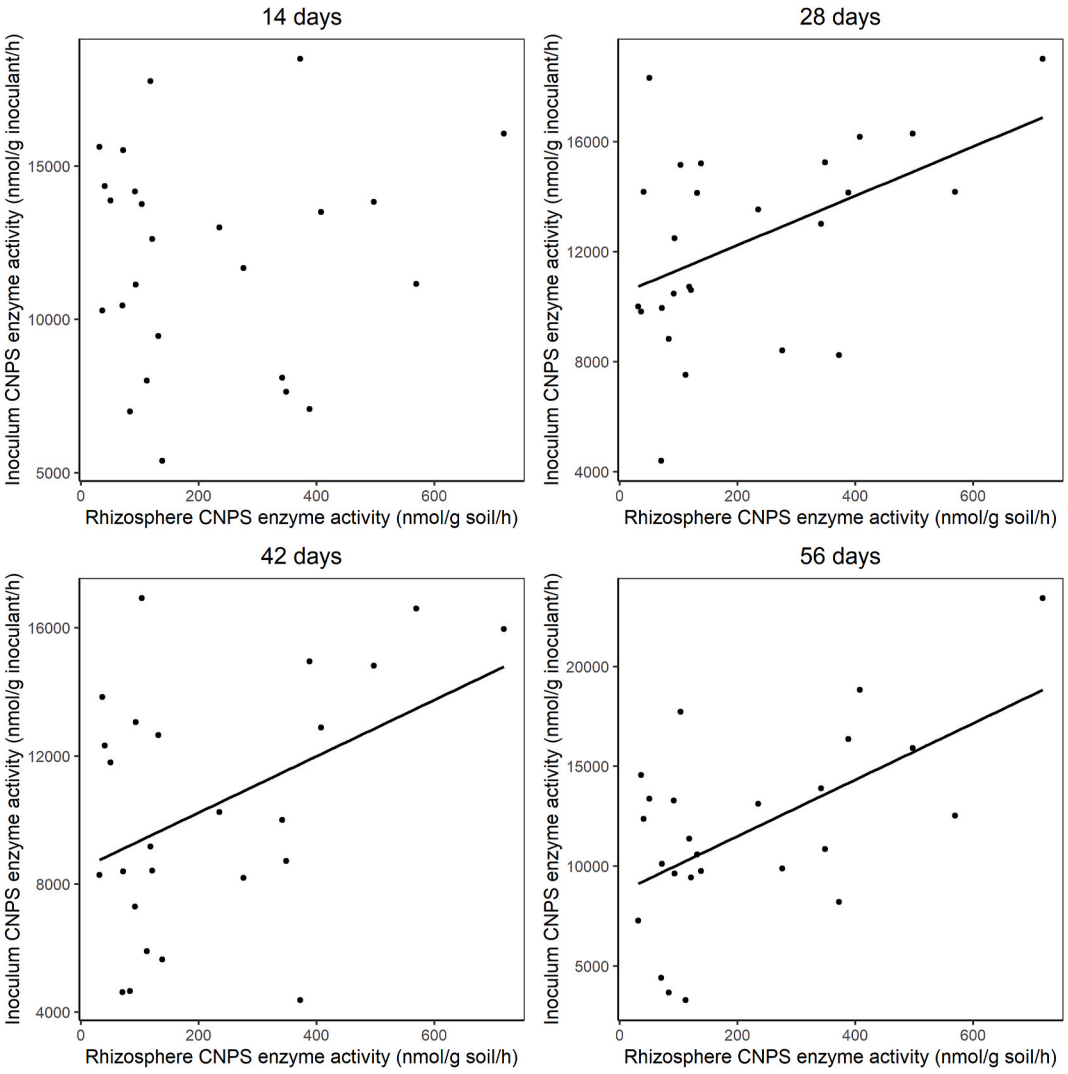

Fig. 5. Relationships between the enzymatic activity of the donor rhizosphere soils and that of the inoculants (i.e., ferments) prepared based on them during the 56 days of incubation. CNPS = sum of all enzymes related to the C, N, P and S cycles.

with an average of 114.34 nmol/g soil/h, while enzyme linked to the S cycle showed the lowest values, with an average of 4.58 nmol/g soil/h.

The enzyme activity of the substrate in the Petri dishes evolved from the absence or practically absence of activity to high activity values (Table 1 and Figs. 3 and 4, S2, and S3). The enzymes that showed the greatest overall activity were those linked to the C cycle (with an average of 4,413.9 nmol/g soil/h), followed, in order of activity, by the enzymes linked to the N (3,775.8 nmol/g soil/h), P (1,215 nmol/g soil/h), and S cycle (34 nmol/g soil/h). Enzymes linked to C and P showed a general trend of greater activity after 14 days of incubation, to subsequently decrease or remain constant, while N-linked enzymes tended to show their highest values after 28 days of incubation. Despite these general patterns observed, the samples corresponding to each of the different inoculated communities followed a different trajectory with different enzyme levels (Table 1 and Figs. 3 and 4, S2, and S3). These results showed a period of high initial activity during which the microbial communities are established, and a second phase from 14 to 28 days in which the activity is maintained in a steady state. We also observed that a longer incubation time seemed to be related to a greater variability in enzyme activity values, especially in the case of C- and P-linked enzymes.

We found significant relationships between the initial activity of the donor rhizosphere soils and the enzymatic activity of the inoculant (Fig. 5, Table 2). These relationships started to emerge from day 28 of incubation and mainly involved those enzymes linked to the N and P cycles, and to a lesser extent the C cycle. This relationship became more evident after 56 days of incubation and was especially apparent when all the enzymes were considered together (i.e., as the sum of the enzymes linked to the C, N, P, and S cycles).

3.2. *Common garden experiment to test the effects of inoculants on soil functioning*

The enzyme activity of the soil in the pots differed depending on the identity of inoculant (Table 1, Fig. 6 and S4). The inoculants with the highest activity were SV27 and AN6, and those with the lowest activity AN55 and SV41. The highest soil activity in the pots was mainly related to N- and P-degrading enzymes and the lowest to C- and S- degrading enzymes.

3.3. *Testing the potential transfer of metabolic properties of donor soils to inoculants and back to agricultural soils*

Supporting previous correlational analyses, our structural equation models showed a significant and positive relationship between the total enzymatic activity of the donor rhizosphere soil and that of the inoculum, which, in turn, translated into an increase in the total enzymatic activity of the potted soils (Table 3). Enzymes like BG, PHOS, LAP

Table 2
Pearson correlations between the enzyme activity of microbial communities present in the donor rhizosphere soils and the activity of the inoculants prepared based on them over the 56-day incubation period. Correlation coefficients in bold indicate significant relationships ($P < 0.05$). C.enz = BG + AG + CB + XYL; N.enz = NAG + LAP; CNPS = sum of all enzymes; S = rhizosphere soil enzymes; I = inoculant enzymes after 14, 28, 42, and 56 days of fermentation.

	14 days					28 days					42 days					56 days				
	C.enz.I	N.enz.I	PHOS.I	AS.I	CNPS.I	C.enz.I	N.enz.I	PHOS.I	AS.I	CNPS.I	C.enz.I	N.enz.I	PHOS.I	AS.I	CNPS.I	C.enz.I	N.enz.I	PHOS.I	AS.I	CNPS.I
C.enz.S	−0.103	0.201	−0.098	0.137	0.007	0.224	0.105	**0.434**	−0.097	0.279	0.155	**0.457**	0.268	−0.098	0.315	0.216	0.276	0.364	−0.006	0.306
N.enz.S	−0.003	0.252	−0.054	0.202	0.096	**0.423**	0.222	**0.548**	−0.054	**0.486**	0.319	0.374	0.356	−0.171	**0.429**	**0.507**	**0.481**	**0.583**	−0.026	**0.600**
PHOS.S	−0.089	0.234	−0.074	0.183	0.035	**0.409**	0.129	**0.589**	−0.116	**0.442**	0.300	**0.413**	0.337	−0.176	**0.422**	0.390	**0.418**	**0.471**	0.005	**0.483**
AS.S	−0.315	−0.021	−0.089	0.000	−0.207	0.109	0.396	0.197	0.061	0.294	−0.158	**0.399**	−0.023	−0.216	0.004	0.187	0.362	0.253	0.060	0.294
CNPS.S	−0.039	0.237	−0.056	0.196	0.069	**0.412**	0.203	**0.557**	−0.057	**0.472**	0.303	**0.401**	0.356	−0.162	**0.425**	**0.473**	**0.468**	**0.558**	−0.024	**0.569**

showed a greater transfer potential between the donor soil towards the inoculant. On the other hand, when individually considered, enzymes did not show signs of transfer potential from the inoculant to the pots (Table 3). However, when all enzymes were jointly considered (i.e., as the sum of the enzymes linked to the C, N, P, and S cycles) within the context of our SEM, we found evidence of transfer potential from the inoculants to potted soils (Table 3).

4. Discussion

In this study, we carried out solid-phase fermentations using by-products of winemaking and rhizosphere soils from donor vineyards for the production of inoculants based on whole-soil microbial communities. We demonstrated the capacity of these inoculants to promote, in turn, the functioning of vineyard soils, at least under controlled conditions using a common garden experiment. This suggests the possibility of regenerating the functioning of degraded vineyards through the development and application of inoculants based on complex microbial communities from selected fields that show greater rates of soil functioning, but this potential should be validated in field trials under a variety of experimental conditions and cultivars. Furthermore, we focused only on the enzymatic potential of inoculants and soils, but we acknowledge that understanding the composition of the microbial communities that develop during the fermentation could generate additional insights that may be particularly useful to drive the fast regeneration of agricultural soils aided by microbes.

The production of enzymes, in general, showed an exponential growth around the first two weeks of incubation, and then was either maintained or even decreased slightly. These results suggest a period of high initial activity during which the microbial communities are established, and a second phase from 14 to 28 days in which the activity is maintained in a steady state, possibly due to a greater stability of the microbial communities developed from the donor soil. In fact, visual inspection of the Petri dishes suggests that once they were fully colonized, the communities remained stable (Fig. S5). Therefore, this period of 14–28 days seems critical for the establishment of communities of fungi (especially) and bacteria, which will later thrive in the culture medium and, therefore, this would be a phase in which the competition between species dominates (Rousk and Bååth, 2007). This also suggests that understanding this phase may be a critical step to produce inoculants with stable characteristics.

The development of complex communities in culture media propagates in most cases at an exponential level that is usually paralleled by a high release of enzymes to the extracellular medium (Gooday et al., 1992; TRINCI, 1971). This implies that as the microbial communities develop in each Petri dish, their metabolic profile also evolves. This is similar to Diaz et al. (2016), who cultivated different fungi using solid-phase fermentation and who also observed an exponential growth in the first days of incubation. This also coincides with Blandino et al. (2002), who cultivated *Aspergillus awamori* using solid phase fermentation in wheat, and who found high enzyme levels at the beginning of incubation followed by a stabilization phase. However, Filipe et al. (2020) found a poorer enzymatic evolution in an incubation with *A. ibericus* in which enzyme production evolved more irregularly during the first phase, observing the disappearance of some enzyme activities during the process. This may be due to the different processes and compounds present in the culture media where the fungal colonies develop. Therefore, we need to understand the relationship between fungal communities and the different compounds present in the culture media that result from the degradation of the original substrate. These results imply that the development of the communities can vary according to the evolution that takes place in the environment in which they are growing, and which establishes what type of taxa, including fungi and bacteria, will develop.

We observed significant relationships between the initial activity of the donor rhizosphere soil sample and the enzymatic activity of the

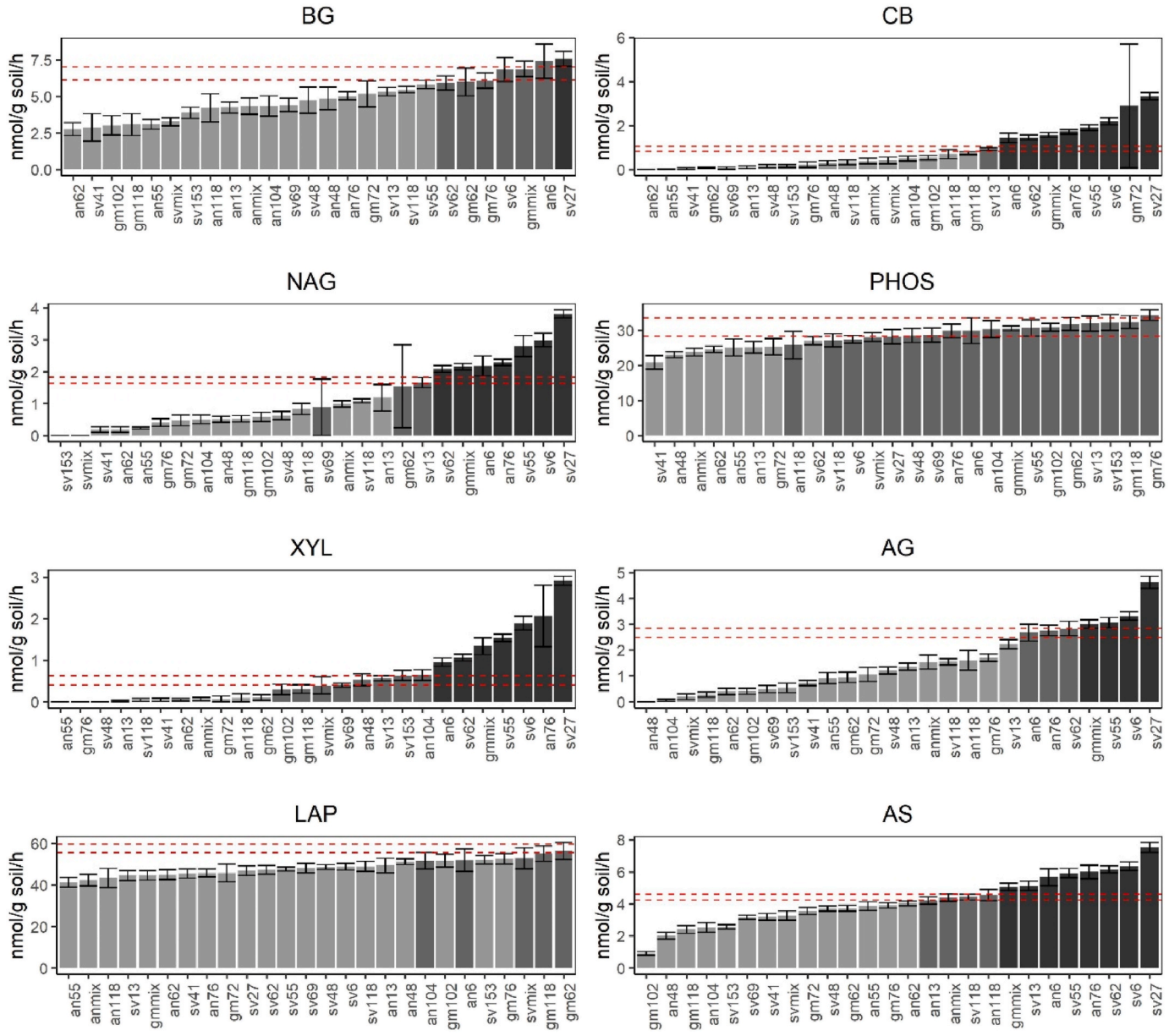

Fig. 6. Effects of inoculation on soil enzyme activity. Means ± SEs are presented. The dotted interval represents the SE of control pots (i.e., no inoculum added). Dark grey = higher activity than control plots; medium grey = no difference with control plots; light grey = lower activity than control plots.

Table 3
Structural equation models testing the causality of the transferability potential from donor rhizosphere soils to the inoculants, and from the inoculants to the pots. C= C enzymes, N= N enzymes, CNPS = sum of all enzymes. The *P*-value is that associated with the standardize effect (i.e., standardized path coefficient). BG: β-1,4-glucosidase. XYL: β-xylosidase. CB: β-D-cellobiohydrolase. AG: α-1,4-glucosidase. NAG: β-1,4-N-acetylglucosaminidase. LAP: leucine-aminopeptidase. PHOS: phosphatase. AS: arylsulfatase.

Directional link	Model	BG	CB	NAG	PHOS	XYL	AG	LAP	AS	C	N	CNPS
Activity donor soil→Activity inoculum	*P*-value	0.49	0.51	0.58	**0.02**	0.22	0.86	**0.05**	0.77	0.30	**0.01**	**0.01**
	Standardised effect	0.14	0.12	0.12	0.44	0.22	0.02	−0.41	**0.05**	0.20	0.49	0.47
Activity inoculum→Activity pots	*P*-value	**0.02**	0.80	0.28	0.16	0.67	0.29	0.35	0.54	0.11	0.41	**0.05**
	Standardised effect	0.46	0.05	0.22	0.28	−0.09	0.22	0.19	0.13	0.32	0.17	0.39
Model statistics												
Fisher's C		0.69	2.27	0.80	1.31	3.49	2.73	0.70	3.38	1.19	0.70	0.27
P-value		0.71	0.32	0.67	0.52	0.18	0.26	0.71	0.18	0.43	0.70	0.88
df		2	2	2	2	2	2	2	2	2	2	2

inoculants. This relationship began to be observed from day 28 of incubation and mainly involved enzymes linked to the N and P cycles. This implies that it is, at least partly, possible to predict the metabolic potential of a digestate from the metabolic potential of the microbial communities contained in the donor sample and that are responsible for carrying out the fermentation. This also suggests the suitability of

carrying out enzymatic activity assays as a fast and cheap method prior to evaluating the fermentative potential of complex microbial communities obtained from rhizosphere samples. In addition, our data suggest that the metabolic potential of these inoculants begins to become evident after 28 days of incubation, and never before 14 days. Our data also support the potential of solid phase fermentation for the elaboration of inoculants based on whole-soil-based complex communities with desirable functional characteristics (for example, high capacity to degrade organic matter as a result of enzyme release). In fact, over the last few years, solid-phase fermentation studies have been increasing and proving the potential of this strategy (Thomas et al., 2013). However, until now, most of these studies focused on the growth of microorganisms for the production of compounds such as enzymes, which can then be isolated from the medium. Therefore, our work represents an advance by developing a system that allows transferring, and multiplying, the metabolic potential of complex whole-soil communities obtained from aliquots of rhizosphere soil collected from highly functional vineyards.

In addition to transferring the activity of the rhizosphere soil to the inoculants, in this study we demonstrated the possibility of transferring the functionality of the inoculants to other vineyard soils. In fact, there are already studies that suggest the possibility of inoculating soils with complex microbial communities to redirect ecosystem restoration and the transition to a more sustainable agriculture (Farrell et al., 2020; Wubs et al., 2016). For example, Wubs et al. (2016) used soil from grassland and heathland to successfully redirect and accelerate the ecological succession towards the desired ecosystem type. The potential of inoculating with complex communities from whole-soil inoculants is, however, limited by the amount of soil that can be applied to the recipient agroecosystem. The use of the solid-phase fermentation as a multiplier of complex whole-soil microbial communities may, therefore, represent a key step in this process. In our case, the tightest relationship between the activity of the inoculum and that of the soil was detected when we studied all the activities together, which suggests the possibility of transferring the metabolic capacity of the inoculant in a general way, but less guarantee of success if the aim is to transfer the metabolic potential linked to specific enzymes. In addition, we observed that certain inoculants showed a particularly high potential for enzymatic activity linked to the C cycle, especially XYL and AG activities. For example, XYL, linked to the degradation of xylan from plant cell walls, has been related to a greater abundance of fungi (Knob et al., 2010). In other cases, we observed how the metabolic activity of inoculated soils is lower than in the case of non-inoculated soils, especially in the case of BG and AG enzymes, which suggests that certain communities present in the inoculants have the potential to inhibit the microbial metabolism of the recipient soil. This also suggests that these communities may alternatively be used to slow down nutrient cycles of altered soils, which we believe is a completely underexplored area of research. Similar to our results, Triebwasser et al. (2012) studied the inhibitory potential of leaf litter tannins on certain enzymes. Because our initial substrate used to grow the inoculum may contain such substances, it makes it possible for such inhibitory phenomena to develop. In any case, our data point toward the possibility of regenerating the functioning of vineyard soils through the multiplication of donor communities and their subsequent transfer to crops using solid-phase fermentation as a key intermediate step in this process.

5. Conclusion and future work

In this study, we have demonstrated the potential of solid-phase fermentation of viticulture and winemaking by-products to produce inoculants based on complex rhizosphere-associated microbial communities with similar functional capabilities to those of the donor soils. However, important questions such as the composition of the microbial communities of the inoculants (bacteria, fungi, protozoa and small invertebrates) remain to be explored through DNA extraction and amplicon sequencing (16S for bacteria, ITS for fungi and 18S for eukaryotes and nematodes), as well as the similarity between these communities and those developing naturally within the grapevine rhizosphere. We also recognize the relevance of studying the changes that occur in the chemical composition of the culture media throughout the fermentation process, which may provide further insights as to which compounds, or groups of compounds, may promote the increase of the different nutrient cycles investigated. This research also opens the door to studies under field conditions that assess the ability of these inoculants to promote variables linked to soil functioning (for example, N mineralization and/or increased solubilization of limiting nutrients such as P), improve yield and the nutritional status of plants, or increase the resistance to pests and diseases. These studies could shed light on the usefulness of these microbial inoculants developed from complex and highly functional communities to contribute towards a viticulture that is more based on the natural functioning of ecosystems and, therefore, more sustainable.

CRediT authorship contribution statement

Juan F. Aguiar: Writing – original draft, Software, Resources, Methodology, Data curation, Writing – review & editing. **Isabel M. Liberal:** Supervision, Methodology, Formal analysis, Writing – review & editing. **María L. Muñoz:** Methodology, Writing – review & editing. **Emma Cantos-Villar:** Methodology, Writing – review & editing. **Belén Puertas:** Methodology, Writing – review & editing. **Antonio Archidona-Yuste:** Methodology, Writing – review & editing. **Ramón Casimiro-Soriguer:** Methodology, Writing – review & editing. **Martina Coletta:** Methodology, Writing – review & editing. **Aldo D'Alessandro:** Methodology, Writing – review & editing. **Ana González-Robles:** Methodology, Writing – review & editing. **David Gramaje:** Methodology, Writing – review & editing. **Delphine Renard:** Methodology, Writing – review & editing. **Mélanie Roy:** Methodology, Writing – review & editing. **Antonietta La Terza:** Methodology, Writing – review & editing. **Rubén Tarifa:** Methodology, Writing – review & editing. **Corinne Vacher:** Methodology, Writing – review & editing. **G.F. (Ciska) Veen:** Methodology, Writing – review & editing. **Raúl Ochoa-Hueso:** Writing – review & editing, Validation, Methodology, Funding acquisition, Formal analysis, Data curation.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

R.O.H. was funded by the Ramón y Cajal program of the MICINN (RYC-2017 22032). R.O.H. also acknowledges funding from PID2019-106004RA-I00 and PID2023-150371OB-I00 funded by AEI/10.13039/501100011033; from the program José Castillejo" of the "Ministry of Universities" (CAS21/00125); from the European Regional Development Fund (FEDER) and the Ministry of Economic Transformation, Industry, Knowledge and Universities of the Junta de Andalucía (ERDF Andalucía 2014–2020 Thematic objective "01 - Reinforcement of research, technological development and innovation"): P20_00323 (FUTUREVINES); from the Fondo Europeo Agrícola de Desarrollo Rural (FEADER) through the 'Ayudas a Grupos operativos de la Asociación Europea de Innovación (AEI) en materia de productividad y sostenibilidad agrícolas', References: GOPC-CA-20-0001 (O.G. Suelos Vivos) and GO2022-01 (O.G. Viñas Vivas); from the "Planes complementarios de I + D + I. Marco de Recuperación y Resiliencia. Biodiversidad" (REGENBIOVIN); and from Fundación Biodiversidad (SOILBIO). A.A.-Y. is funded by the Ramón y Cajal program (RYC2021-031108-I), funded by MCIN/AEI/10.13039/501100011033 and UE "Next Generation EU/PRTR".

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.rhisph.2025.101039.

Data availability

The data that has been used is confidential.

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