Impact of Soil Management on the Functional Activity of Microbial Communities associated to Cork Oak Rhizosphere

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***Abstract*—The microbial ecology of cork oak rhizosphere was investigated using the Biolog Community Level Physiological Profile (CLPP) that provides a unique metabolic fingerprint helpful for the characterization of complex microbial communities. Microbial populations from the rhizosphere of cork oak plants growing at three different sites within the same area were characterized using CLPP and compared. The sites were distinguished by a different soil management under the tree cover and, in general terms, by a different anthropogenic impact. The comparison of metabolic fingerprints of the different microbial populations showed the existence of a relationship between general microbial activity and functional biodiversity in the rhizosphere and the level of anthropogenic impact. Particularly the presence of grazing animals, soil tillage and fire could be identified as the main factors affecting both the general microbial activity and the structure of microbial populations from cork oak rhizospheres.**

Keywords: *soil management; microorganisms; environmental factors; forestry.*

INTRODUCTION

Cork oak (*Quercus suber* L.) is a common tree species in Mediterranean countries being present in Italy, France (including Corsica), Tunisia, Algeria, Spain, Portugal and Morocco [1]. The presence of different pasture species under the tree cover (hence the possibility to breed grazing animals), the cork production attitude, as well as the fire resistance allowed a wide distribution of this species in Sardinia [2].

During the last decades a widespread and progressive decline of the phyto-sanitary conditions of *Q. suber* influenced not only the quali-quantitative cork production attributes but also threatened plant survival [3]. Nowadays in several Sardinian areas, cork oak trees show general stress conditions most likely caused by different biotic (e.g. pathogenic fungi) and abiotic factors often leading to plant dessication [4]. This phenomenon is commonly referred to as cork oak decline and human activities, most likely, can significantly contribute to this. In particular soil tillage, fertilization and not-rationale above ground management can be identified as key factors affecting plant health status possibly through a major influence on plant-microbes relationships that finally govern plant nutrient availability.

Identifying the structural and functional features of microbial communities inhabiting cork oak forest soils that show a different management history, can be helpful in order to define the impact of anthropogenic activities on microbial diversity and activity. This seems quite important since these latter govern plant and ecosystem fitness.

The aim of this work was to investigate the structure, general activity and function of rhizosphere microbial communities in different cork oak stands characterized by diverse above ground management histories. The Biolog Community Level Physiological Profile (CLPP) approach has been used for this purpose.

MATERIALS AND METHODS

Three different sampling sites were selected in Sardinia taking into account different above ground management histories. All the sites were characterized by the presence of cork oak stands and were located on a relatively small area with homogeneous geo-morphological features. The main features of the sites were as follows: site 1, presence of a heavily-grazed pastureland under the tree cover, surface tillage, absence of the shrub component. Site 2, cork oak stand with sparse *Q. pubescens* trees, not-grazed pastureland under the tree cover, presence of a shrub component, subjected to fire in the past. Site 3, presence of moderately-grazed pastureland under the cork oak cover. The organic matter content at these sites was determined (in duplicate) following the Walkley and Black method while pH was detected in water (1:2,5 soil/water ratio) using standard procedures [5]. Six soil samples of about 1 Kg each were randomly collected within a surface of 100 m^2 (with a homogeneous appearance) from each site. In the laboratory they were bulked together, sieved to <2 mm and a 10 g sub-sample used for subsequent chemical analysis.

Six rhizosphere samples of 500 g each were collected from these sites between 5 and 20 cm depth and the community structure and functional diversity of the microbial populations were investigated using the Biolog Community Level

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Physiological Profile (CLPP) approach [6]. Prior to analysis rhizosphere samples from each site were bulked, sieved to <2 mm and 20 g sub-sample used for CLLP. CLLP was performed on three different soil sub-samples for each site. Microbial populations from the different rhizosphere samples were extracted, serially (ten-fold) diluted and then 120 µl of a standardized cell suspension (approx. 2-5 x 10^3 cells) were added to every wells of a Biolog microtiter Ecoplate [7]. This latter uses 31 different carbon substrates each one present in a well of a microtiter plate. A tetrazolium dye was also presence in each well to reveal oxidative catabolism. The inoculated Ecoplates were incubated in the dark at 25°C for up of 168 h. The assay was based on a daily measurement of the oxidative catabolism of the substrates (optical density readings at 590 nm, OD_{590}) by the microbial community in the wells. Optical density data were processed as previously described [8] to determine: the overall rate of colour development (AWCD) and the Shannon-Weaver index (*H'*) for each microbial community. The Average Well Color Development (AWCD), that is a measure of general microbial activity, has been calculated for each time-point using the following formula:

$$\mathbf{AWCD} = \sum_{(i=1,\,31)} \mathbf{(Ri\text{-}C)/31}$$

where Ri is the optical density value at 590 nm (OD_{590}) for a specific carbon source and C is the optical density of the control well. The Shannon's Index of Diversity (*H'*) that is a measure of the potential functional diversity of the microbial populations has been calculated using the following equation:

$$H' = -\sum_{i=1}^{N} p_i \cdot \ln p_i$$

where *pi* is the ratio of the activity on each substrate to the sum of activities on all substrates.

Richness, defined as the number of carbon sources utilized by each microbial community has been also determined. Patterns of carbon source utilization from the 96 h data were used to compare soil communities by Principal Component Analysis (PCA). Richness, *H'* and AWCD were analysed using One Way ANOVA (P<0.05) for mean comparisons.

Results and Discussion

In this study a Community Level Physiological Profile (CLPP) has been used to investigate both structure and function of rhizosphere microbial communities associated to cork oak trees present in stands with a different management history. For this aim metabolic fingerprints of each microbial community were obtained using Biolog Ecoplates and then compared. The use of metabolic fingerprints from Biolog Ecoplates, that contain 31 different carbon substrates (see Table 1), has been previously revealed very helpful for the characterization of microbial communities from different environmental samples [9], [10]. Furthermore several studies have reported distinctive multivariate profiles of carbon source utilization among a variety of microbial community [11]. In more general terms the pattern of substrates used by each community, at a given incubation time, can provide a unique metabolic fingerprint allowing the characterization of the microbial community analyzed.

This can be very helpful in order to track any functional and/or structural change of a microbial community after a disturbance event or, importantly, to evaluate the impact of agricultural practices on soil microbial activity and/or catabolic versatility.

TABLE I
CARBON SOURCES PRESENT IN THE WELLS OF A BIOLOG ECOPLATE

SUGAR DERIVATES.		
Well	G 1	d-cellobiose
	H 1	alfa-d-lactose
	A 2	b-methyl-d-lucoside
	B 2	d-xilose
	C 2	i-erythritol
	D 2	d-mannitol
	E 2	n-acetil-d-glucosamine
SUGAR PHOSPHATES		
Well	H 2	d,l-alfa-glicerol-phosphate
	G 2	glucose-1-phosphate
CARBOXILIC ACIDS.		
Well	B 1	pyruvic acid methyl ester
	F 2	d-glucosaminic-acid
	A 3	d-galactoni acid g-lactone
	B 3	d-galacturunic acid
	C 3	2-hydroxy benzoic acid
	D 3	4-hydroxy benzoic acid
	E 3	g-hydroxy butiric acid
	F 3	itaconic acid
	G 3	a-ketobutiric acid
	H 3	d-malic acid
AMINOACIDS		
Well	A 4	l-arginine
	B 4	l-asparagine
	C 4	l-phenylalanine
	D 4	l-serine
	E 4	l-threonine
	F 4	glycyl-l-glutamic cid
	G 4	phenylethyl-amine
	H 4	putrescine
POLYMERS		
Well	C 1	tween 40
	D 1	tween 80
	E 1	alfa-cyclodextrin
	F 1	glycogen

In this study the matrix of optical density data recorded at each incubation time, and quantifying the usage of each of the

carbon source in the microtiter Ecoplate (see Table 1), has been processed in order to obtain few simple indexes defying microbial activity and diversity at the investigated sites. This can be considered the typical approach used to separate the community-level response in the Biolog plate. The indexes obtained provided a measure of the overall rate of activity, the diversity and pattern of soil microbial communities. The overall rate has been estimated by the rate of average well color development (AWCD) calculated as stated above. Importantly, this index is a function of inoculum density and community composition [12]. As highlighted by Garland [8] differences in the overall rate of color development among samples (i.e. inoculum density) will produce variations in the diversity of color development independent of any change in the types of organisms present. Furthermore differences in community composition are expected to be resolved with different AWCD. Failure to account for differences in the rate of AWCD can result in classification based on the density rather than C source profile of the community. To eliminate confounding effects we standardized the inoculum density as previously suggested [8].

The evolution of the AWCD for the different rhizosphere microbial communities is presented in Fig. 1.

In this case differences in the AWCD kinetics are apparent (Fig. 1). In particular, the microbial community from site 3 reached the highest AWCD value showing a greater microbial activity with respect to the other sites. This was consistently observed for all the time-points considered even if mean differences were found significant until the 120 h reading (One-way ANOVA, $P<0.05$). Microbial communities from site 2 and 3 did not show any significant difference in their AWCD for all the time points, most likely presenting comparable metabolic activity (see Table II for details).

The number of substrates oxidized (Richness) by the different microbial communities was also considered (Fig. 2) using an OD_{590} of 0.25 as threshold for positive response. In this case we minimize the occurrence of weak false positive responses.

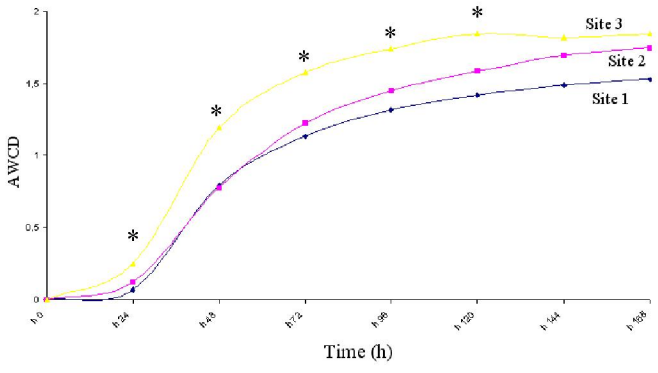

Fig. 1. Evolution of the Average Well Color Development (AWCD) for the different microbial communities extracted from cork oak rhizospheres. Asterisks denote significant difference between Site 3 and Sites 1 / 2 (One-way ANOVA, $P< 0.05$) at specific time points. Sites 1 and 2 did not show any statistical difference for all the time points considered (see Table II for details).

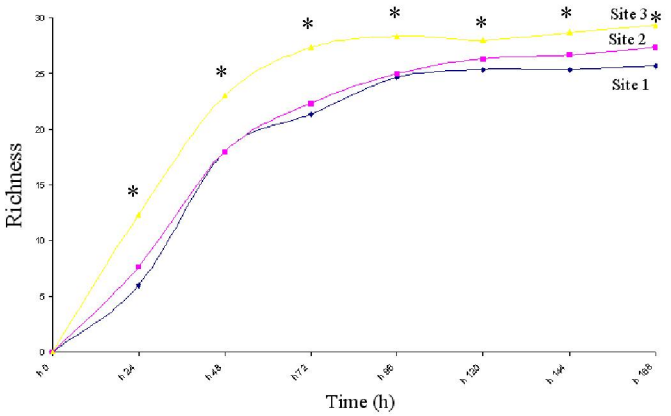

Fig. 2. Evolution of the number of carbon sources utilized (Richness) by the different microbial communities extracted from cork oak rhizospheres. Asterisks denote significant difference between Site 3 and Sites 1 / 2 (One-way ANOVA, $P< 0.05$) at specific time points. Sites 1 and 2 did not show any statistical difference for all the time points considered (see Table II for details).

The number of positive wells also followed, for all the communities, a sigmoidal curve with positive responses observed in > 64% of the wells within the first 72 h incubation. Richness data presented in Figure 2 confirmed an apparent superior metabolic potential of the microbial population from site 3.

In particular the microbial community from this latter was able to use a number of carbon substrates always significantly greater than the other two populations (One-way ANOVA, $P<0.05$). The microbial communities from Site 2 and 3 did not show significant differences (One-way ANOVA, $P<0.05$; see also Tab. II). The other index calculated from the matrix of optical density data is the Shannon's Index of Diversity (H') that is a measure of the potential functional diversity of the microbial populations. Among the range of diversity indexes that have been used with bacterial communities the Shannon index is perhaps the more frequently applied. This index takes into account both the number of substrates used and their utilization degree. It is positively correlated with substrate richness and gives more weight per individual community to rare than common carbon sources. The interpretation of this index is very simple and typically limited to point out that samples with highest H' appear to be the most diverse. In our case in terms of catabolic versatility.

The differences previously highlighted among different microbial communities seem to disappear when H' was considered (Fig. 3 and Tab. II). The different microbial populations showed similar biodiversity index even if they were showing a significantly different metabolic activity (AWCD) and Richness for most of the time points considered. However a higher diversity of the microbial community from site 3 was still observed but only after few hours of incubation (Fig. 3 and Tab. II). Principal component analysis (PCA, Fig. 4) applied to OD_{590} data showed substantial difference between the different communities for substrates utilization. Furthermore soil acidity was found as

positively correlated with microbial C. Considering that the three sites were all present in the same relatively small homogeneous area, the differences observed in the CLPP could be possibly linked to the soil management. In particular, because of the high tree density, Site 3 has never been subjected to soil tillage (incidentally it was showing a pH value of 5). To the contrary Site 1 and Site 2, whose microbial populations were showing lower catabolic versatility, were respectively subjected to soil tillage and fire (they were showing pH values of 5,8 and 5,2 respectively). Also the different level of soil organic mater registered at the different sites may be the result of a different soil management. Site 3 with the higher organic matter content (5.5%) was showing higher microbial activity and catabolic versatility while microbial populations from site 1 (4.2%) and 2 (4.8%) revealed a lower activity and functional diversity. Despite statistic analysis was not carried out, the organic matter content seems to correlate in some way with the microbial activity and in some extent with the diversity of the rhizosphere microbial populations. Of course this needs substantial confirmation even if this relationship was previously highlighted [13] [14]. It was shown by these authors that land use and management increasing soil organic matter tend to improve the catabolic diversity of soil microbial community [13] [14].

The results from this study showed that rhizosphere microbial communities from soils subjected to different management or anthropogenic impact (e.g. fire) were showing different activity, and functional biodiversity.

In particular the unsustainable presence of grazing animals (their high number per hectare) and soil tillage (Site 1) and a recent fire event (Site 2) possibly exerted similar influence on both the general microbial activity and the functional diversity. The AWCD, Richness and *H'* of microbial communities from Sites 1 an2 were indeed statistically indistinguishable at all the time points considered (Figs. 1-3).

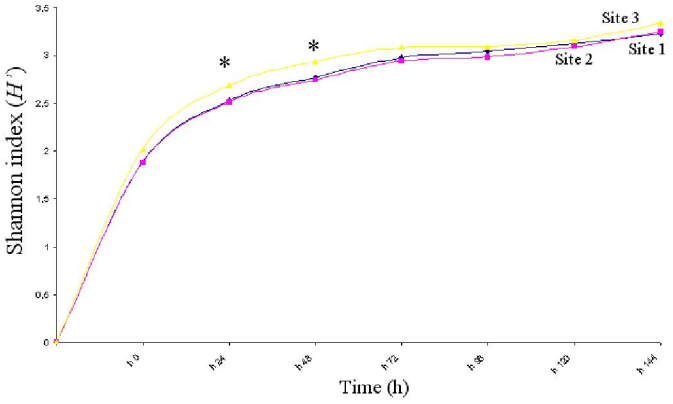

Fig. 3. Evolution of the Shannon Index (*H'*) for the different microbial communities extracted from cork oak rhizospheres. Asterisks denote significant difference between Site 3 and Sites 1 / 2 (One-way ANOVA, $P< 0.05$) at specific time points. Excluding these latter the microbial communities from the different sites did not show any statistical difference (see also Table II for details).

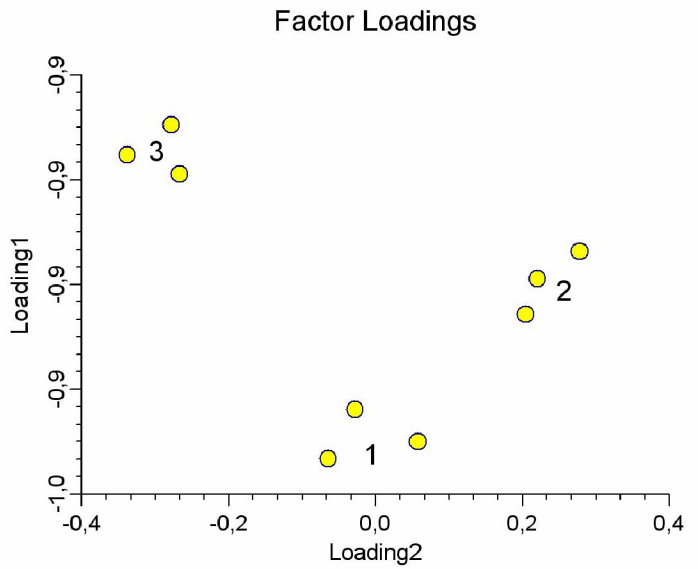

Fig. 4. Ordination biplot of principal component analysis of substrate utilization patterns using Biolog Ecoplates. Numbers refer to the different sites.

However PCA analysis revealed useful to further identify structural differences among these microbial communities and to highlight the functional differences in the microbial communities analyzed.

TABLE II
MEAN VALUES OF BIOLOG INDEXES RECORDED AT DIFFERENT TIME POINTS FOR THE THREE MICROBIAL COMMUNITIES

AWCD	**Reading time**						
Microbial community	**24h**	**48h**	**72h**	**96h**	**120h**	**144h**	**168h**
Site 1	0,066 ^{a}	0,794 ^{a}	1,133 ^{a}	1,316 ^{a}	1,419 ^{a}	1,489 ^{a}	1,528 ^{a}
Site 2	0,121 ^{a}	0,777 ^{a}	1,221 ^{a}	1,448 ^{a}	1,584 ^{a}	1,695 ^{ab}	1,746 ^{a}
Site 3	0,249 ^{b}	1,193 ^{b}	1,574 ^{b}	1,739 ^{b}	1,846 ^{b}	1,816 ^{b}	1,846 ^{a}
Richness	**Reading time**						
Microbial community	**24h**	**48h**	**72h**	**96h**	**120h**	**144h**	**168h**
Site 1	6 ^{a}	17 ^{a}	21 ^{a}	24 ^{a}	25 ^{a}	26 ^{a}	26 ^{a}
Site 2	7 ^{a}	17 ^{a}	22 ^{a}	24 ^{a}	25,5 ^{a}	26 ^{a}	27 ^{a}
Site 3	12 ^{b}	23 ^{b}	27 ^{b}	28 ^{b}	27,5 ^{b}	27,5 ^{b}	29 ^{b}
H'	**Reading time**						
Microbial community	**24h**	**48h**	**72h**	**96h**	**120h**	**144h**	**168h**
Site 1	1,888 ^{a}	2,530 ^{a}	2,772 ^{a}	2,978 ^{a}	3,046 ^{a}	3,125 ^{a}	3,225 ^{a}
Site 2	1,881 ^{a}	2,514 ^{a}	2,747 ^{a}	2,947 ^{a}	2,987 ^{a}	3,088 ^{a}	3,250 ^{a}
Site 3	2,021 ^{b}	2,686 ^{b}	2,939 ^{a}	3,085 ^{a}	3,096 ^{a}	3,162 ^{a}	3,341 ^{a}

Different uppercase letters for each time point denote significant difference (One way ANOVA, $P< 0,05$)

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