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1 **SOIL ENZYME ACTIVITIES RECOVERY AFTER ORGANIC**
2 **TREATMENTS OF DEGRADED AREAS WITHIN VINEYARDS**

3
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6
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14
15
16 **Abstract**

17 Soil enzymes were used to assess the impact of different treatments applied in four
18 farms, each one with three vineyards as replicates, on soil functionality. 8 enzymes
19 related to C, N, S and P cycling were measured and functional diversity index was
20 estimated. Three treatments were compared: compost, green manure and dry
21 mulching with respect to degraded and non-degraded soil. The four vineyards
22 showed different enzymatic patterns and response to treatments. Vineyards with
23 the largest difference between degraded and non-degraded soil have benefited more
24 largely from the treatments. Among treatments, dry mulching and compost seemed
25 to be effective to recover soil functionality in degraded vineyards. However, the
26 effect might be limited in the short term.

27
28 **Keywords:** *soil enzymes, functional diversity, substrates decomposition, vineyards*
29 *degradation*

30
31 **Introduction**

32
33 Soil enzyme activities are proximal driver of soil functioning, contributing to
34 biogeochemical cycling, organic matter transformations and nutrient availability
35 and are widely accepted as indicators of soil health, responding in a sensitive,
36 quantitative and predictable manner to different land use and management (Aon et
37 al., 2001; Badiane et al., 2001; Vepsäläinen et al., 2001). Soil enzymatic activities
38 are closely related to microbial activity or biomass as they catalyse biochemical
39 reactions and nutrient cycling in the soils. Furthermore, being synthesized by
40 microorganisms, roots and soil micro- and meso-fauna such as earthworms or
41 nematodes, enzymes can be a valid tool to present and manage complex
42 information in a simple and informative manner.

43 The most studied group of soil enzymes that have ecological importance in soil are
44 hydrolases, which are involved in the main biogeochemical cycling of elements
45 and release C compounds as well as N, P and S. These enzymes exist in soil either
46 intracellularly or extracellularly, free in soil solution or immobilized on the surface
47 of organic and inorganic soil components.

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48 Several soil enzyme assays have been developed to detect the total potential
49 activity against a specific substrate. Fluorometry has been proved to be more
50 sensitive than are the colorimetric methods (Marx et al., 2001; Moscatelli et al.,
51 2011) and has become more common since the adoption of microplates that
52 facilitate the rapid measurement of a large number of enzymes and samples. In this
53 context, measuring the activity of several soil enzymes could be useful to
54 understand the organic matter turnover and the availability of inorganic nutrients
55 and could give indications on the function and quality of an ecosystem and on the
56 interaction among subsystems (Dick and Tabatabai, 1993).
57 Within this work, fluorimetric approach was used for the determination of
58 hydrolase activities related to the main biogeochemical cycling. In particular,
59 enzymes degrading cellulose (β -glucosidase, cellulose), hemicellulose (β -
60 xylosidase), chitin (N-acetyl- β -D-glucosaminidase) phosphate (acid phosphatase)
61 and sulphate (arylsulphatase) esters have been assessed, together with two
62 unspecific endo-cellular enzymes (butyrate and acetate esterase).

63

64 Materials and methods

65

66 **Soil sampling**

68 Soil samples were collected in four farms, each one with three vineyards as
69 replicates, before (2015) and after (2016 and 2017) organic treatments application.
70 Two farms are located in France (Maison Blanche, Saint Émillion – MB and Pech
71 Redon, La Clape - PR) and two in Italy (Fontodi, Panzano in Chianti – FON and
72 San Disdagio, Civitella Marittima - SD). In each vineyard, an area characterized by
73 soil degradation was selected. Each degraded area was subdivided into 4 plots,
74 where different strategies of organic soil management were implemented: (COMP)
75 composted organic amendment; (GM) green manure with winter legumes and
76 cereal; (DM) reseeded legumes, mown and leaved on the ground as dry mulching;
77 (CONTR) only tillage once per year. A reference plot, characterized by optimal
78 soil functionality (ND, non-degraded) was selected in each vineyard. For further
79 details on climate and pedological characteristics and for treatments type and
80 application see D'Avino et al. (this issue).

81 Soils were sampled at 0-30 cm depth in French sites in 2015. In French sites in
82 2016 and 2017 and in Italian sites in the three years, they were sampled at 0-10 and
83 10-30 cm depths. Averaged activities at 0-30 cm depths are shown.

84

85 **Enzyme activities measurement**

86
87 Enzyme activities were measured according to the methods of Marx et al. (2001)
88 and Vepsäläinen et al. (2001). N-acetyl- β -glucosaminidase (NAG), β -glucosidase
89 (β G), butyrate esterase (BUT), acid phosphatase (AP), arylsulphatase (ARYL), β -
90 xylosidase (XYL), cellulose (CELL) and acetate esterase (AC) activity were
91 measured using fluorogenic methylumbelliferyl (MUF) conjugated surrogate
92 substrates (Sigma, St Louis, MO, USA). Briefly, 2 g soil sample was weighed into
93 a sterile jar and incubated for 24 hours at 20% soil moisture. A homogenous

94 suspension was obtained by homogenizing samples with 50 mL deionized water
95 with UltraTurrax at 9600 rev / min for 3 min. Aliquots of 50 µL were withdrawn
96 and dispensed into a 96 well microplate (3 analytical replicates/sample/substrate).
97 50 µL of Na-acetate buffer pH 5.5 was added to each well. Finally, 100 µL of 1
98 mM substrate solution were added giving a final substrate concentration of 500
99 µM. Fluorescence (excitation 360 nm; emission 450 nm) was measured after 0, 30,
100 60, 120, 180 min of incubation at 30 °C with an automated fluorimetric plate-
101 reader (Fluoroskan Ascent).

102 **Statistical analysis**

103
104 Analysis of variance was performed to assess the effect of treatments, years and
105 their interactions on soil enzyme activities using Statistica package (StatSoft inc).
106 The order of magnitude of the values obtained for the different enzymatic
107 responses varies considerably depending on the specific activity being determined,
108 thus leading to some enzyme having more weight than others. To resolve this
109 problem, the sum of the percentage of the maximum value found for a specific
110 enzymatic response across all enzymes was used for the calculation of the sum of
111 enzymes (SUM). From this percentage of maximum enzyme activities, the
112 Simpson-Yule index was calculated following the equation $E = 1/\sum p_i^2$, as indicated
113 by Bending et al. (2004), where p_i is calculated as the enzymatic response to a
114 substrate as a proportion of enzymatic responses summed across all substrates for a
115 soil. Discriminant function analysis (DFA) was performed using the percentage of
116 maximum value for each enzyme to show separation among the four sites. Squared
117 Mahalanobis distances between group centroids were determined. Two significant
118 discriminatory roots were derived and the results of DFA were graphically
119 presented in two dimensions.
120

121 **Results and discussion**

122
123 Overall, the four sites were significantly different in terms of soil enzymatic pattern
124 (Fig. 1), with the greatest enzyme activities observed on average in Pech Redon
125 and Fontodi, followed by San Disdagio and Maison Blanche. Differences among
126 sites can be ascribed to several abiotic (climate, pH, carbonates, etc.), and biotic
127 factors (organic matter, microbial biomass and activity, fauna and roots, etc.).
128 Greater enzyme activities were observed in ND soils with respect to CONTR in all
129 sites along the three years of observations (Fig. 2 and Table 1).
130 Indeed, this difference was larger in the first year, as also reported in a previous
131 work on the same sites before treatments application (Costantini et al., in press). In
132 the second and third years the increase was reduced and remained significant in
133 Maison Blanche and San Disdagio until the end of measurements.
134
135
136

137 **Table 1:** Mean activities of enzyme activities in the four sites in plots without treatments
 138 (CONTR), treated with compost (COMP), green manure (GM), mulching (DM) and non-
 139 degraded (ND) before (2015) and after (2016 and 2017) treatments.
 140

Site	Treatment	Year	nmol MUF g ⁻¹ h ⁻¹								
			CELL	AP	bG	NAG	XYL	BUT	AC	ARYL	
Maison Blanche	CONTR	2015	33	247	223	53	33	232	748	24	
		2016	8	149	86	23	13	249	382	15	
		2017	20	198	187	43	27	411	588	27	
	COMP	2015	31	256	239	55	34	272	869	27	
		2016	11	134	103	21	14	287	453	17	
		2017	37	211	249	69	36	519	721	37	
	GM	2015	19	224	179	56	16	228	749	24	
		2016	11	159	99	24	14	267	482	18	
		2017	29	181	205	43	28	398	545	32	
	DM	2015	30	225	173	47	26	244	849	25	
		2016	16	195	119	33	18	281	516	20	
		2017	32	225	211	52	38	454	664	38	
	ND	2015	36	249	378	76	39	331	1035	29	
		2016	19	175	163	38	21	360	550	25	
		2017	45	171	337	55	39	511	602	42	
	ANOVA										
	Year			***	*	***	***	***	***	***	***
	Treatment			**	n.s.	*	*	**	n.s.	n.s.	*
Y * T			n.s.	n.s.	n.s.	*	n.s.	n.s.	n.s.	n.s.	
Pech Redon	CONTR	2015	9	123	141	57	17	472	1101	20	
		2016	7	96	107	21	14	365	667	11	
		2017	33	84	173	49	35	563	1045	45	
	COMP	2015	11	115	133	35	16	596	1048	14	
		2016	5	80	58	17	8	302	513	8	
		2017	32	80	171	46	32	505	1028	42	
	GM	2015	20	133	215	46	23	685	1322	21	
		2016	8	98	88	23	11	364	612	9	
		2017	35	71	203	48	32	518	971	45	
	DM	2015	12	111	110	41	13	536	991	12	
		2016	7	93	93	18	10	352	635	10	
		2017	33	68	214	53	36	580	1029	42	
	ND	2015	17	123	198	39	31	690	1096	18	
		2016	9	110	127	24	17	441	763	13	
		2017	31	72	186	44	34	521	895	44	
	ANOVA										
	Year			***	***	***	***	***	**	***	***
	Treatment			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Y * T			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

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 142
 143

Table 1 (to be continued)

Site	Treatment	Year	nmol MUF g ⁻¹ h ⁻¹								
			CELL	AP	βG	NAG	XYL	BUT	AC	ARYL	
Fontodi	CONTR	2015	15	118	164	56	18	465	801	34	
		2016	24	131	226	47	28	709	1041	32	
		2017	11	51	122	33	18	390	562	34	
	COMP	2015	21	126	185	76	21	605	984	33	
		2016	38	156	236	71	31	823	1123	35	
		2017	17	66	176	44	18	480	535	43	
	GM	2015	24	133	165	77	23	556	893	38	
		2016	37	160	270	53	33	770	1136	41	
		2017	17	86	133	32	16	331	458	36	
	DM	2015	22	142	204	76	26	678	1056	33	
		2016	20	143	178	38	29	651	953	33	
		2017	14	71	151	33	19	351	462	34	
	ND	2015	21	134	184	85	30	559	934	37	
		2016	43	165	285	51	31	788	1097	41	
		2017	15	66	125	39	14	347	474	32	
	ANOVA										
	Year			***	***	***	***	***	***	***	n.s.
	Treatment			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	Y * T			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
	San Disdagio	CONTR	2015	10	138	92	35	16	500	949	20
			2016	12	113	88	21	14	432	870	15
2017			6	96	71	22	14	439	996	25	
COMP		2015	8	133	72	26	14	385	917	16	
		2016	16	130	105	27	19	536	916	15	
		2017	9	79	67	18	15	353	887	19	
GM		2015	11	119	87	30	15	416	816	17	
		2016	19	148	189	37	28	608	1016	19	
		2017	11	85	68	25	14	322	813	17	
DM		2015	10	106	63	27	12	348	713	12	
		2016	17	160	167	40	25	593	1057	18	
		2017	11	92	132	31	19	499	959	22	
ND		2015	22	171	177	55	23	595	1099	33	
		2016	36	182	269	51	37	692	1166	40	
		2017	21	84	117	33	16	360	568	29	
ANOVA			10	138	92	35	16	500	949	20	
Year			*	***	**	*	**	**	n.s.	n.s.	
Treatment			***	n.s.	***	*	n.s.	n.s.	n.s.	***	
Y * T			n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	

CELL=cellulose; AP=acid phosphatase; βG=glucosidase; NAG=N-acetyl-β-glucosaminidase; XYL=β-xylosidase; BUT=butyrate esterase; AC=acetate esterase; ARYL=arylsulphatase

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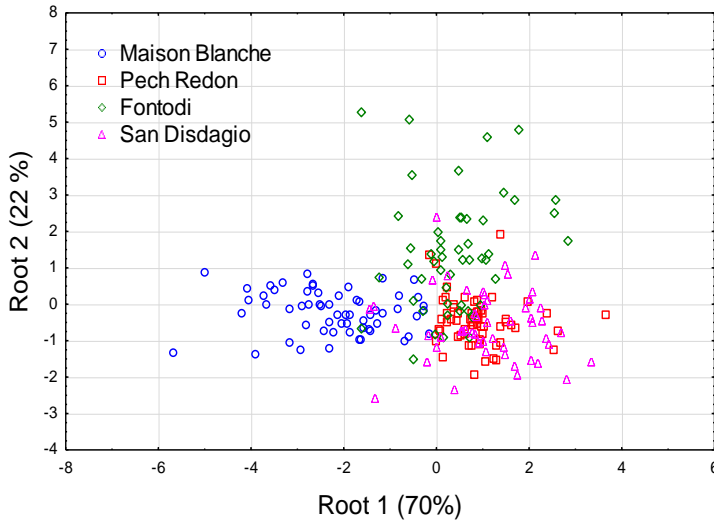


Figure 1
Discriminant Function Analysis showing separation among the four sites on the basis of enzyme activities (percentage of maximum value for each enzyme).

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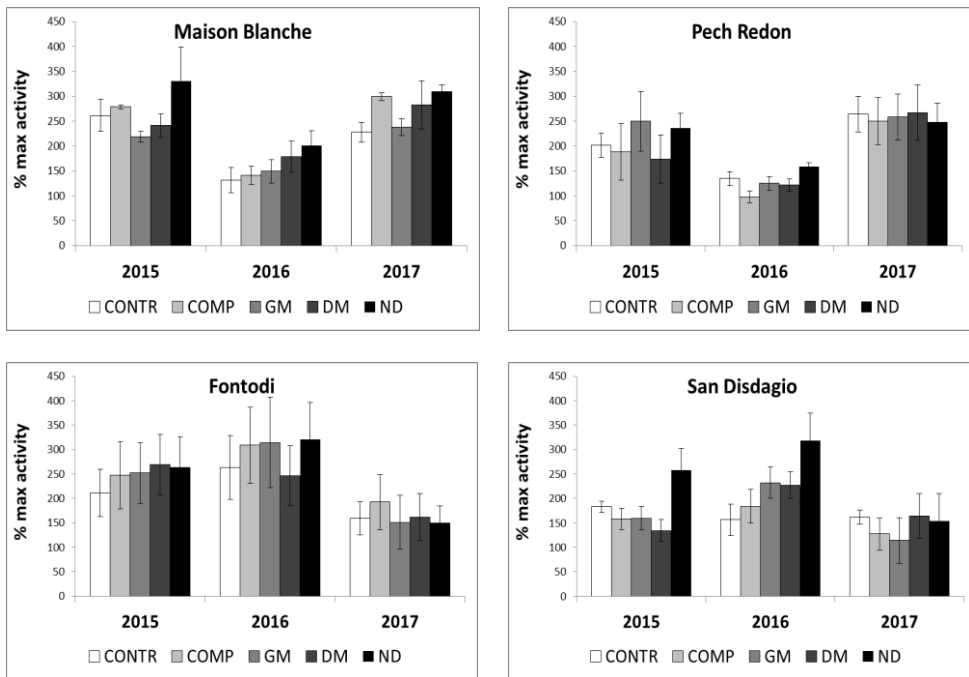


Figure 2. SUM of enzyme activities in the four sites in the three sampling years before (2015) and after (2016 and 2017) treatments. Error bars are reported.

149

150 **Table 2.** Percentage difference of enzyme activities with respect to Control in the four sites
 151 after treatments application in 2016 and 2017. Significant differences are reported in bold.
 153

Site	Year	Treatment	% difference with respect to control									
			CELL	AP	bG	NAG	XYL	BUT	AC	ARYL	SUM	S-Y
Maison Blanche	2016	COMP	37	-10	20	-7	8	15	18	17	8	14
		GM	39	7	15	8	6	7	26	21	14	3
		DM	99	31	38	46	36	12	35	34	36	7
		ND	140	17	89	67	61	44	44	72	53	17
	2017	COMP	86	7	33	61	33	26	23	35	31	8
		GM	46	-8	10	1	3	-3	-7	18	5	1
		DM	61	14	13	20	39	10	13	39	24	3
		ND	128	-14	80	29	44	24	2	55	36	3
Pech Redon	2016	COMP	-33	-17	-46	-19	-43	-17	-23	-4	-27	-4
		GM	6	2	-18	8	-24	0	-8	-1	-7	-1
		DM	2	-3	-14	-16	-33	-4	-5	-1	-10	-1
		ND	30	15	18	15	20	21	14	3	18	3
	2017	COMP	-3	-5	-1	-6	-10	-10	-2	2	-5	2
		GM	6	-16	18	-2	-8	-8	-7	2	-2	2
		DM	1	-20	24	8	1	3	-2	1	1	1
		ND	-8	-15	8	-10	-4	-7	-14	0	-6	0
Fontodi	2016	COMP	55	19	4	50	8	16	8	3	17	3
		GM	52	22	20	12	17	9	9	-1	19	-1
		DM	-16	9	-21	-19	4	-8	-8	-6	-6	-6
		ND	78	26	26	8	9	11	5	1	22	1
	2017	COMP	45	28	45	31	5	23	-5	-6	21	-6
		GM	49	69	9	-6	-10	-15	-19	-19	-5	-19
		DM	24	38	24	-1	9	-10	-18	-8	1	-8
		ND	36	29	3	16	-22	-11	-16	-2	-6	-2
San Disdagio	2016	COMP	31	15	20	32	33	24	5	6	17	6
		GM	61	30	116	78	97	41	17	13	48	13
		DM	39	41	90	95	75	37	21	8	45	8
		ND	197	61	207	146	154	60	34	19	103	19
	2017	COMP	55	-19	-6	-20	2	-19	-11	-8	-21	-8
		GM	78	-12	-5	13	-5	-27	-18	-16	-29	-16
		DM	73	-4	85	41	32	14	-4	0	1	0
		ND	249	-13	64	47	9	-18	-43	-6	-5	-6

154 These two sites showed also the largest impact of treatments (Table 2), however a
155 different response was observed in the four vineyards (Table 1 and 2):

156 **Maison Blanche**

157 In the first year DM showed to be the most effective treatment, able to increase
158 most of the enzyme activities considered. This effect decreased in the second year,
159 and was maintained for enzymes related to cellulose and hemicellulose degradation
160 and arylsulphatase only, suggesting a short-term effect of this treatment
161 application, more evident and permanent for C-cycling enzymes. In the second
162 year COMP showed the maximum increase with respect to CONTR, for all
163 enzymes. GM increased cellulase activity only, in both years.

164 **Pech Redon**

165 The treatments did not affect significantly enzyme activities, with the exception of
166 β -glucosidase in the second year after dry mulching. This vineyard showed also the
167 lowest difference between CONTR and ND soils, suggesting that soil functionality
168 was i) less responsive to degradation or ii) degradation was not so strong.

169 **Fontodi**

170 In the first year GM increased cellulolytic enzymes and acid phosphatase and this
171 effect persisted in the second year. However, other enzymes were not affected by
172 this treatment. In the second year COMP application positively affected enzyme
173 activities related to C and P cycling, and also N cycling with DM. This vineyard
174 seemed to be slower in the response to treatments, even if after the second year of
175 treatments the activities were comparable to those of ND soil.

176 **San Disdagio**

177 This vineyard showed the highest percentage effects of treatments, in particular in
178 the first year, when GM and DM almost doubled enzyme activities with respect to
179 CONTR, though without reaching the values of ND soils. This effect was evident
180 for most enzymes of C, N, S, and P cycling. In the second year the effect persisted
181 for cellulase with all treatments and also for chitin and hemicellulose degrading
182 enzymes with DM.

183

184 **Conclusions**

185
186 Overall, treatments application showed to improve soil enzyme activities, although
187 to different extent depending on vineyard type and treatment. Maison Blanche and
188 San Disdagio were the two vineyards most responsive to treatments, possibly as a
189 consequence of the largest difference between degraded and non-degraded soil
190 found in these two sites. Among treatments, DM and Compost seemed to be
191 effective to recover soil functionality in degraded vineyards. However, the effect
192 might be limited in the short term.

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