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## Research Article

# Saponins in soil, their degradation and effect on soil enzymatic activities

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## ABSTRACT

Saponins can be potential candidates for the development of safe biopesticides, due to their widely acknowledged insecticidal, fungicidal and nematocidal activity, but information on their effects on soil biological properties is still limited. This study was aimed to investigate short-term fate of saponins from *Medicago sativa* in soil and their dose-effect relationship with microbial biomass and selected enzyme activities in soils with different origin, physical and chemical properties. Microbial degradation of total saponins ranged from 46% to 91%, according to soil characteristics, within 28 days from their incorporation into the soil. Both saponin glycosidic chains and triterpenic aglycones were also microbially degraded, though by dynamics changing among the different soils. In all soils, *M. sativa* saponins significantly reduced microbial biomass at rates of 10 and 20 mg saponin mixture per g of soil. Microbial enzymatic activities were less affected as indicating an adaptive response of soil microbial communities to the presence of saponins.

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## Introduction

1 Saponins are a class of plant specialized metabolites with sev-  
2 eral biological properties, though commonly distinguished by  
3 their surfactant and hemolytic activities (Rao and Gurfinkel,  
4 2000; Tava and Avato, 2006). However, these phytochemicals  
5 have been also documented for a broad spectrum of other bi-

ological and pharmacological properties such as fungicidal, 6  
molluscicidal, antibacterial, antiviral, anthelmintic and anti- 7  
tumor activities, as well as also recognised as allelochemical 8  
compounds (Oleszek et al., 1992; Rao and Gurfinkel, 2000; Tava 9  
and Avato, 2006; Avato et al., 2006, 2017; Podolak et al., 2010; 10  
Maestrini et al., 2020). Chemical, physical and physiological 11  
traits of plant saponins and their wide spectrum of proper- 12  
ties have already led to different industrial applications and a 13  
number of commercial products (Güçlü-Üstündağ and Mazza, 14  
2007; Tamura et al., 2012; Bezerra et al., 2018). 15

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Moreover, aqueous solutions of plant saponins have been also applied for biodegradation of polycyclic aromatic hydrocarbons and heavy metals in contaminated soils and water, due to their amphipathic properties and ability to increase the solubility of several chemicals, including lipophilic pollutants (Kobayashi et al., 2012; Kaczorek et al., 2016). These positive experimental data indicated a possible use of plant saponins as washing agents and biodegradation enhancers in environmental friendly strategies for detoxification of contaminated soils (Kobayashi et al., 2012; Kaczorek et al., 2016; Ye et al., 2017; Yu and He, 2018). Soil microbial parameters, such as microbial biomass and enzyme activities, must be taken into account to understand mechanistic responses of below-ground carbon and changes of soil organic matter properties. Evaluation of soil microbial biomass can provide information on soil ecological status, due to the involvement of soil microorganisms in fundamental soil processes, mainly regulation of carbon, nitrogen and nutrient cycle and, therefore, to their key role in soil ecosystem sustainability (Rengel, 2007). Enzyme activity is an expression of microbial function, with extracellular enzymes affecting nutrient transformation, especially the cycling of carbon, nitrogen, phosphorus and sulphur as well as carbon sequestration. Soil enzyme activity provides information on soil ability to perform biogeochemical reactions and can be an indicator of impacts of anthropogenic management or pollution on soils (Nannipieri et al., 2018). Study of soil microbial extracellular enzyme activities (EEAs) could also play an important role in monitoring microbial response to climate change under field conditions, as reflecting metabolic requirements of microbial community (Caldwell, 2005). Enzymes most widely assayed are those involved in degradation of lignocellulose (e.g.,  $\beta$ -glucosidase), proteins, chitin, and peptidoglycan (e.g., leucine aminopeptidase), or in hydrolysis of nucleic acids, phospholipids, and other ester phosphates (e.g., acid or alkaline phosphatase). In particular,  $\beta$ -glucosidases, are considered good soil quality indicator, as widely distributed in nature and associated with carbon cycle (Sinsabaugh et al., 2008).

Information on the effect of plant saponins on soil properties is quite poor, as generally limited to the role of saponins as a source of carbon for microorganisms possessing catabolic enzymes (Sugiyama, 2021) and soil microbiota. Similarly, effects of plant saponins on soil microbial communities are still not well known. Literature data reported plant saponin effects on growth, diversity and function of soil microorganisms (Yu and He, 2018; Nakayasu et al., 2021), as well as a relationship between structural type of saponins and their effects on soil bacterial communities (Nakayasu et al., 2021). In addition, some studies suggest that saponins may control soil rhizosphere bacteria in soil through rhizodeposition mechanisms (Fons et al., 2003; Bao et al., 2023). Zhang et al. (2022) reported a bactericidal toxicity of tea saponins from *Camellia oleifera* and lethality of their accumulation in soil to some soil bacteria involved in litter degradation, as well as a significant association of other soil bacteria, in particular *Acinetobacter*, with saponin degradation.

Stability of different metabolites in the rhizosphere varies depending on their chemical structure and soil microbial communities. Specialized metabolites as saponins are reported as rather stable in soil, in contrast to degradation of general

metabolites within few minutes (Gunina and Kuzyakov, 2015; Sugiyama, 2021). Saponins released into soil are degraded by soil microbes but microorganisms specifically responsible of this degradation process are not yet well known (Sugiyama, 2021). In addition to degradation by soil microbes, distribution of metabolites in the rhizosphere is affected by organic matter and soil biosorbents such as humic substances and clay minerals. In particular, humic components containing hydroxyl and phenolic hydroxyl groups are involved in the formation of complexes with organic metabolites (Gan and Li, 2013).

In recent years, plant saponins have been also gaining an increasing attention as a potential source of safe crop pesticides, due to their insecticidal, nematicidal and fungicidal properties (Tava and Avato, 2006; De Geyter et al., 2007; D'Addabbo et al., 2011; T. 2020; Abbruscato et al., 2014; Morcia et al., 2022, Quasim et al., 2020; Iovinella et al., 2023). In particular, saponins from *Medicago* spp. have been proved to have a toxic effect on soil pests such as phytoparasitic nematodes (D'Addabbo et al., 2011, 2020), but no data are available about quantitative effects of such class of compounds on soil microbiota and enzymatic activity. At the same time, at the best of our knowledge, there is no information on chemical/physical behavior of *Medicago* saponins in soil environment, where adsorption is a key factor regulating the effective biological activity of any substance. Nor we found any data describing rate of hydrolysis of saponin glycosidic bonds, as well as of any possible structural transformation of saponins in soil. According to this lack of information, this study was aimed to investigate short-term extractability and chemical transformations of saponins from *Medicago sativa* aerial parts incorporated to seven soils with different origin, chemical and physical properties, as well as to assess dose-effect relationship of saponin addition with both soil microbial biomass and selected enzyme activity.

## 1. Materials and methods

### 1.1. Soil samples

Seven soils differing largely for their physical and chemical properties were sampled from different locations in Italy (Table 1). Each soil sample was air-dried, sieved to 2 mm and stored dry until experiments. Particle-size analysis was assessed following the pipette procedure according to Indorante et al. (1990); pH was determined in soil:distilled water extracts (1:10, W/V) by using a GLP 21 pH-meter (Crison, Barcellona, Spain). Total carbon (C) and nitrogen (N) contents were determined in dried samples, using a CHN analyser (Perkin Elmer, Milano, Italy). Total phosphorous (P) was calculated according to Iatrou et al. (2014); ashes were determined after soil incineration with a muffle furnace at 550 °C for 5 h. Chemical-physical properties of selected soils are reported in Table 1.

### 1.2. Preparation and characterization of the saponin mixture

Saponins were extracted from the aerial parts of *M. sativa* and purified according to previously reported methods (Tava et al., 1993; Biały et al., 1999). Purity of the saponin mixture was

**Table 1 – Composition and physico-chemical characteristics of the different soils used in this study.**

Origin	Sand (%)	Silt (%)	Clay (%)	pH	Ash (% DM)	Total C (% DM)	Organic C (% DM)	Total N (% DM)	Olsen-P (mg/kg)
Bari01 Monopoli Open field silty-clay	15 ± 3	54 ± 4	31 ± 4	7.9 ± 0.1	92.0	2.18 ± 0.04	2.00 ± 0.02	0.18 ± 0.03	170 ± 10
Bari02 Zapponeta Greenhouse sandy	86 ± 4	7 ± 1	7 ± 3	8.5 ± 0.1	97.5	3.66 ± 0.01	0.53 ± 0.06	0.03 ± 0.00	15 ± 1
Bari03 Copertino Greenhouse sandy-loam	55 ± 3	34 ± 3	11 ± 3	7.4 ± 0.1	94.0	1.41 ± 0.09	1.26 ± 0.06	0.16 ± 0.02	155 ± 5
Mode01 Modena Greenhouse silty-clay	8 ± 2	51 ± 4	41 ± 4	8.1 ± 0.1	91.2	3.20 ± 0.10	1.99 ± 0.07	0.22 ± 0.01	35 ± 2
Lodi01 Lodi Permanent sandy-loam meadow	65 ± 4	29 ± 4	5 ± 2	6.2 ± 0.1	94.3	2.18 ± 0.16	2.11 ± 0.13	0.28 ± 0.01	7 ± 1
Lodi02 Lodi Greenhouse sandy-loam	71 ± 3	25 ± 2	4 ± 1	7.9 ± 0.2	96.2	2.11 ± 0.21	1.96 ± 0.22	0.17 ± 0.00	41 ± 2
Lodi03 Lodi Open field sandy-loam	67 ± 4	28 ± 2	5 ± 1	6.7 ± 0.1	97.2	1.36 ± 0.28	1.24 ± 0.20	0.08 ± 0.00	23 ± 1

DM: dry matter.

129 evaluated by an external standard method using a calibration  
130 curve obtained by HPLC with previously purified saponins  
131 from *Medicago* spp. (Biały et al., 2006; Tava et al., 2005, 2009,  
132 2011; A. 2020).

133 Saponins were analyzed by HPLC using a Perkin Elmer  
134 (Milano, Italy) chromatograph equipped with a LC250 binary  
135 pump and a DAD 235 detector. Separation was performed  
136 on a Discovery C18 column (Supelco, Milano, Italy, 5  $\mu$ m,  
137 4.6  $\times$  250 mm) with the following mobile phase, solvent A:  
138 CH<sub>3</sub>CN 0.05% CF<sub>3</sub>COOH; solvent B: H<sub>2</sub>O 1% MeOH 0.05%  
139 CF<sub>3</sub>COOH. Chromatographic runs were carried out under gra-  
140 dient elution from 25% (5 min isocratic condition) to 90% of  
141 solvent A in 50 min. Ten  $\mu$ L of methanolic solutions (1 mg/mL)  
142 of saponin samples were injected. Saponins were eluted at  
143 1.0 mL/min and detected by UV monitoring at 215 nm (Ap-  
144 pendix A Fig. S1). Composition of saponin mixture was per-  
145 formed using pure saponins, previously purified and identified  
146 from several *Medicago* spp.

147 Saponin extract was carefully powdered using an agate  
148 mortar and pestle before its addition to soil samples. This  
149 saponin mixture (purity 84.3%) was used in all the experi-  
150 ments at different concentrations: D0 (no saponins), D01 (0.1%  
151 W/W, 1 mg saponin mix per g of soil, corresponding to 0.75  
152  $\mu$ mol total sapogenins per g of soil), D10 (1% W/W, 10 mg  
153 saponin mix per g of soil, corresponding to 7.5  $\mu$ mol total sa-  
154 pogenins per g of soil) and D20 (2% W/W, 20 mg saponin mix  
155 per g of soil, corresponding to 15  $\mu$ mol total sapogenins per g  
156 of soil).

### 157 1.3. Analysis of sapogenin composition

158 Saponins were also characterized based on their aglycone  
159 moieties, sapogenins, by GC/FID and GC/MS analyses. Sa-  
160 pogenins identification and quantitation were achieved using  
161 uvaol as internal standard and was based on all the peaks,  
162 including artefact compounds derived after acid hydrolysis  
163 of the corresponding saponins (Tava et al., 2017). In all the  
164 experiments, sapogenins were identified by GC methods as  
165 their methyl-silyl derivatives. Sapogenins from saponins acid

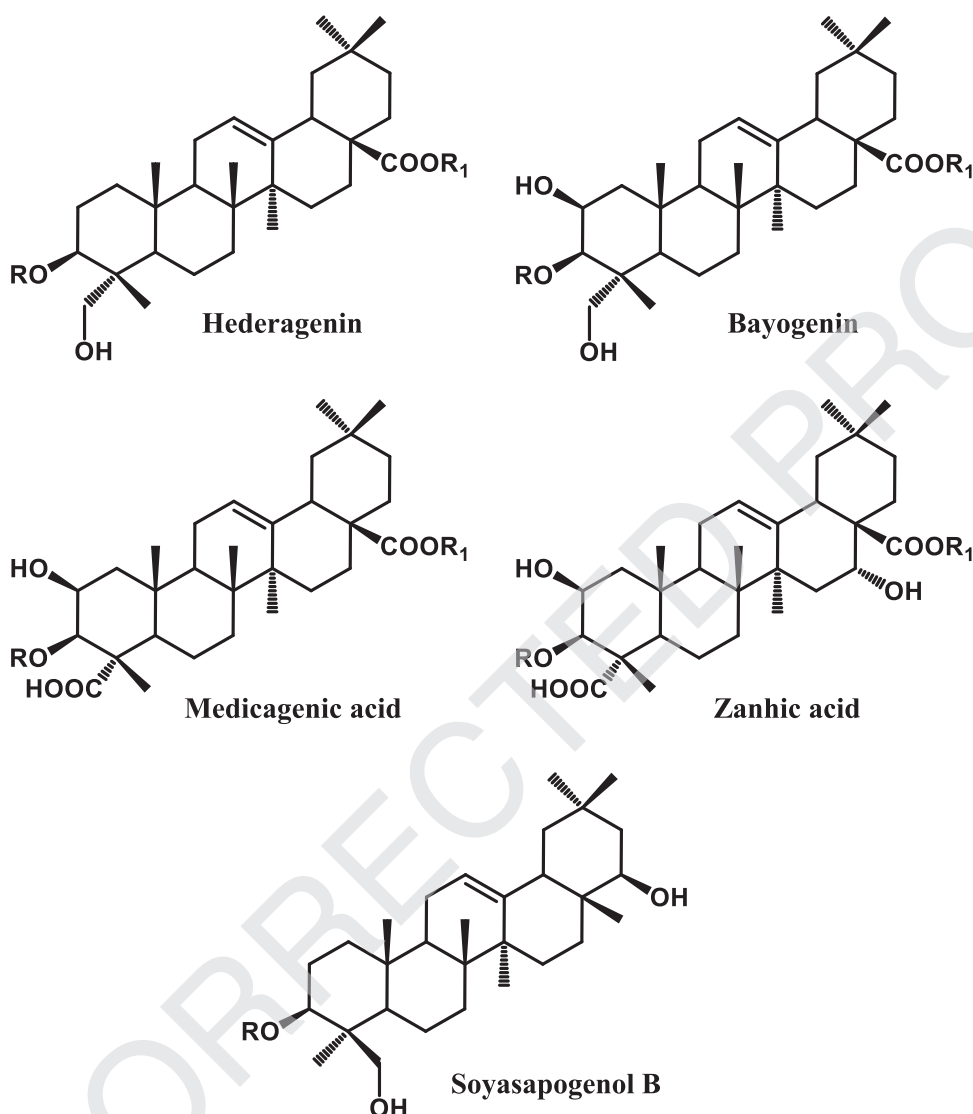
166 hydrolysis were dissolved in 0.5 mL of MeOH, treated with  
167 CH<sub>2</sub>N<sub>2</sub> at room temperature for 15 min and then the solvent  
168 was eliminated under a stream of nitrogen. Silylation was per-  
169 formed on the methylated sapogenins using 0.2 mL of a mix-  
170 ture of pyridine/hexamethyldisilazane/chlorotrimethylsilane  
171 (2:1:2, V/V/V) at 70 °C for 10 min. Reacted samples were diluted  
172 with isooctane and analysed by GC/FID and GC/MS.

173 GC/FID analyses were carried out using a Perkin-Elmer (Mi-  
174 lano, Italy) Clarus 500 GC equipped with a 30 m  $\times$  0.32 mm i.d.,  
175 0.25  $\mu$ m, DB-5 capillary column. Injector and detector temper-  
176 atures were set at 350 °C, and the oven temperature program  
177 was 90 °C for 5 min, increased at 20 °C/min to 250 °C for 1 min  
178 and then increased at 4 °C/min to 350 °C for 15 min. Samples  
179 (1  $\mu$ L) were injected in the 'splitless' mode. Helium was the  
180 carrier gas with a head pressure of 12.3 psi.

181 GC/MS analyses were carried out using a Perkin-Elmer (Mi-  
182 lano, Italy) Clarus 500 GC equipped with a MS detector and  
183 a 30 m  $\times$  0.32 mm i.d., 0.25  $\mu$ m, Elite-5MS capillary column us-  
184 ing the same chromatographic conditions as for GC/FID. Mass  
185 spectra were acquired over a 50–850 amu range at 1 scan/s  
186 with ionizing electron energy of 70 eV; transfer line 300 °C;  
187 and carrier gas He 1.2 mL/min. Retention times and MS spec-  
188 tra were compared with those of previously identified sa-  
189 pogenins. Sapogenins identified in the hydrolysed saponin  
190 mixture were soyasapogenol B (non-hemolytic) and hedera-  
191 genin, bayogenin, medicagenic acid, zanhic acid (hemolytic)  
192 (Tava et al., 2017) (Fig. 1).

### 193 1.4. Evaluation of the effects of saponins on soil enzyme 194 activity

195 Two g of dried and sieved soil were added with 0 (untreated), 2,  
196 20 and 40 mg of saponin mixture in a corked test tube and vig-  
197 orously mixed, as to obtain 0% (D0), 0.1% (D01), 1% (D10) and  
198 2% (D20) W/W test rates, respectively. Saponin mixture rates  
199 applied in both experimental assays were in a range consis-  
200 tent with use of natural bioactive compounds in controlled  
201 (greenhouses, etc.) or field conditions for agrochemical pur-  
202 poses (D'Addabbo et al., 2011; T. 2020; Abbruscato et al., 2014;



**Fig. 1 – Chemical structures of the most abundant identified saponins ( $R = R_1 = H$ ). Saponins:  $R = \text{sugar/sugar chain}$ ,  $R_1 = H$  or  $R = R_1 = \text{sugar/sugar chain}$ .**

203 Iovinella et al., 2023). Water (0.5 mL per test tube) was added  
 204 to bring soil to 50% water-holding capacity, and then incu-  
 205 bated at 21 °C. For each soil sample 27 replicates were pre-  
 206 pared (3 doses  $\times$  3 sampling time  $\times$  3 replicates). Aliquots of  
 207 300 mg of moist soil were taken from each test tube at time  
 208 0 (T0), 3 (T03), and 14 (T14) days for determination of soil mi-  
 209 crobial biomass and enzyme activities. All experiments were  
 210 performed in triplicate.

211 Four enzyme activities were determined in soil:  
 212  $\beta$ -glucosidase (EC.3.2.1.21), leucine amino-peptidase  
 213 (EC.3.4.11.1), alkaline phosphomonoesterase (alkP, EC.3.1.3.1)  
 214 and arylsulphatase (EC.3.1.6.1). Enzymes were extracted from  
 215 the soil samples by heteromolecular exchange according to  
 216 Cowie et al. (2013). Briefly, 0.3 g of moist soil were placed in a  
 217 2 mL corked test tube, together with 1.4 mL of a solution  
 218 containing 3% lysozyme and microbeads. Samples were  
 219 then subjected to bead-beating at 30 strokes/s for 3 min,  
 220 using a Retsch (Dusseldorf, Germany) 400 MM beating mill,  
 221 then centrifuged at 20,000  $\times g$  for 2 min. Enzyme assay was

performed according to Bardelli et al. (2017) containing 4- 222  
 methyl-umbelliferone (MUF) and 7-amino-4-methyl coumarin 223  
 (AMC) as fluorogenic substrates: supernatant (containing des- 224  
 orbed enzymes) was dispensed into 384-well transparent 225  
 microplates with the appropriate buffers and fluorogenic 226  
 substrates. Enzyme activity was expressed as  $\mu\text{mol}$  of MUF (or 227  
 AMC)  $\text{h}^{-1} \text{g}^{-1}$  dry soil. Fluorescence detection was performed 228  
 using a Synergy HT (BIO-TEK, Milano, Italy) microplate reader. 229

#### 1.5. Determination of soil microbial biomass 230

Microbial size was determined as double-strand DNA (dsDNA) 231  
 content (Fornasier et al., 2014). The extraction procedure was 232  
 similar to that of the study of enzyme activities, except for the 233  
 extraction buffer, 0.12 mol/L sodium phosphate, pH 7.8. dsDNA 234  
 in the supernatant was quantified using PicoGreen (Life Tech- 235  
 nologies, Milano, Italy) reagent. dsDNA was corrected for soil 236  
 moisture content and expressed as micrograms dsDNA  $\text{g}^{-1}$  237

238 dry soil. Fluorescence measurements were performed using  
239 a Synergy HT (BIO-TEK, Milano, Italy) microplate reader.

### 240 1.6. Determination of environmental fate of saponins

241 Five hundred mg of dried and sieved soil were added to 5 mg  
242 of saponin mixture (1% W/W, D10 rate) in a corked test tube  
243 and vigorously mixed. Each sample was added with 0.125 mL  
244 of water (to bring soil to 50% water-holding capacity), and then  
245 incubated at room temperature. For each soil sample 15 repli-  
246 cates were prepared (5 sampling time  $\times$  3 replicates) and the  
247 saponin/sapogenin content was then evaluated at different  
248 incubation time: immediately after (T0), and 3 (T03), 7 (T07), 14  
249 (T14) or 28 (T28) days after saponin addition. All experiments  
250 were performed in triplicate.

251 Soil samples were sequentially extracted to determine, in  
252 order: i) free sapogenins, ii) free saponins, and iii) adsorbed  
253 saponins/sapogenins. i) Free sapogenins were extracted from  
254 soil samples with AcOEt/CHCl<sub>3</sub> (V/V, 9:1, 3  $\times$  3 mL). Samples  
255 were vigorously shaken for 10 min, centrifugated at 3000  $\times$  g  
256 for 5 min and combined organic solutions added with 50  $\mu$ g  
257 of uvaol as internal standard. ii) Free saponins were evalu-  
258 ated on the same soil sample previously used for the free sa-  
259 pogenin determination. Soil samples in 3 mL of H<sub>2</sub>O/MeOH 1:1  
260 (V/V) solution, were vigorously shaken for 10 min and then  
261 centrifuged at 3000  $\times$  g for 5 min. Supernatant was removed,  
262 and the samples were treated again two times with the same  
263 solution as above. The combined solutions (3  $\times$  3 mL) were fil-  
264 tered to remove soil impurities, added with 9 mL of H<sub>2</sub>O/MeOH  
265 1:1 (V/V), 4 mol/L HCl and then treated under reflux for 8 h.  
266 After cooling, 100  $\mu$ g of uvaol were added as internal stan-  
267 dard, MeOH was removed under vacuum and aglycones were  
268 extracted with AcOEt/CHCl<sub>3</sub> 9:1 (V/V) (3  $\times$  5 mL). iii) Adsorbed  
269 saponins/sapogenins were evaluated on each soil sample pre-  
270 viously extracted for free saponins. The residual soil sample  
271 was transferred in a 50 mL glass and treated with 20 mL of  
272 H<sub>2</sub>O/MeOH 1:1 (V/V) and 2 mol/L HCl under reflux for 8 h. Af-  
273 ter cooling, 50  $\mu$ g of uvaol were added as internal standard,  
274 MeOH was removed under vacuum and aglycones were ex-  
275 tracted with AcOEt/CHCl<sub>3</sub> 9:1 (V/V) (3  $\times$  5 mL). In all the three  
276 procedures, processed samples were concentrated to dryness  
277 by a rotary evaporator, derivatized (see below) and used for GC  
278 analyses.

### 279 1.7. Statistical analyses

280 Microbial biomass and enzymatic activities were analyzed by  
281 means of a two-way (Tables 2a and 3a) and a three-way (Tables  
282 2b and 3b) ANOVA using the general linear model (GLM) pro-  
283 cedure in SAS software version 8 (SAS Institute Inc., Cary, NC,  
284 USA). Linear contrasts implemented in the GLM procedure  
285 were used for comparisons of specific means. Dynamics of  
286 saponin degradation in soils was analyzed by means of a one-  
287 way (Table 4a) and a two-way (Table 4b) ANOVA with the same  
288 procedure. The correlations among microbial biomass and en-  
289 zymatic activities were analyzed by the proc CORR in SAS  
290 software. A principal component analysis (PCA) on 24 vari-  
291 ables, from both the assays on saponin effects on soil micro-  
292 bial biomass and enzymatic activity (5 variables) and on dy-  
293 namics of saponin degradation in soils (19 variables), was un-

dertaken at D10 saponin rate and T03 and T14, as time points  
common to both the assays, using the PRINCOMP procedure  
in SAS software.

## 2. Results

### 2.1. Characterization of the saponin mixture and recovery from soil samples

By using a standard procedure usually adopted in our labo-  
ratory, a purified mixture of saponins was extracted from the  
aerial parts of *M. sativa* with a yield of 1.7% dry matter. Its  
purity was evaluated as 84.3% based on HPLC analysis. De-  
termination of sapogenin content and composition allowed to  
detect: medicagenic acid (47% of the total sapogenins), zanhic  
acid (39%), soyasapogenol B (7%), bayogenin (6%) and hedera-  
genin (1%) (Fig. 1). Recovery of saponins/sapogenins from the  
different type of soils was quoted as 85%–90% of the total  
saponins added to the soils.

### 2.2. Effect of saponin mixture on soil microbial biomass and enzymatic activity

Soils used in this study significantly differed in their initial  
microbial biomass (Fig. 2, D0, Table 2 and Appendix A Ta-  
ble S1): silty-clay soils Bari01 from Apulia and Mode01 from  
Emilia showed the highest microbial biomass content, 192.5  
and 179.5  $\mu$ g dsDNA/g dry soil, respectively, while the low-  
est microbial biomass was detected in sandy soil Bari02 and  
sandy-loam soil Lodi03, 23.0 and 50.2  $\mu$ g dsDNA/g dry soil over  
time average, respectively (Appendix A Table S1, D0). The D01  
rate of saponin mixture had a significant positive effect on mi-  
crobial biomass of soils Bari03, Lodi01 and Bari02 (Fig. 2 and  
Appendix A Table S1). Adversely, the D10 and D20 rates of  
saponin mixture resulted in a significant decrease of micro-  
bial biomass compared to D0 in all the soils but Bari03 at D10  
rate (Table 2, linear contrasts D0 vs D10 within soil and Fig. 2).  
In contrast to all the other soils, microbial biomass of Bari03  
significantly increased after 14 days from the addition of the  
D10 saponin rate, as suggesting the development of a specific  
microflora able to efficiently use saponins for metabolic pro-  
cesses and growth during that specific time interval. As a mat-  
ter of fact, Bari03 was the only soil with a clear increasing  
trend of  $\beta$ -glucosidase activity, i.e. the hydrolyzation of  $\beta$ -D-  
glucosyl residues, at D10 and D20 rates (Fig. 3a).

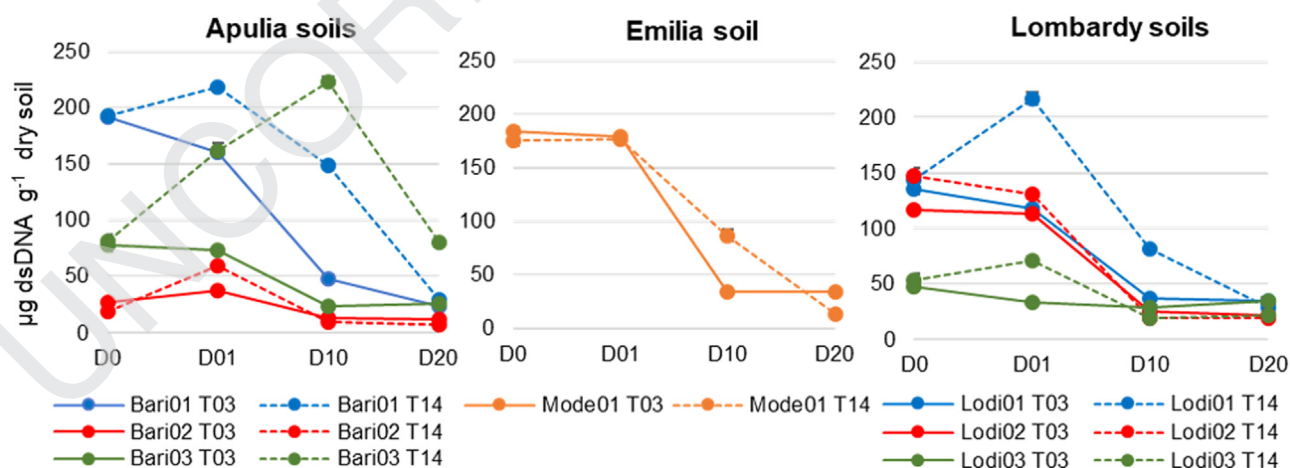
The effect of time on soil microbial biomass (comparison  
of T03 vs T14 within soil  $\times$  time interaction, Table 2) was, in  
general, significant and positive (T14 > T03) for all the soils but  
Bari02, which showed the most reduced of initial microbial  
biomass (Table 2 and Fig. 2). This positive trend indicates an  
adaptation of soil microflora to saponin presence in the time  
interval T03-T14.

Due to the differences among microbial biomass content  
of different soils, enzyme activities were evaluated both as  
absolute (Fig. 3 and Table 2) and relative values, referred to  
dsDNA unit (Fig. 4 and Table 3). The  $\beta$ -glucosidase enzyme,  
involved in the hydrolysis of  $\beta$ -D-glucosyl residues, showed  
generally low absolute activity values in all the untreated soils  
(Fig 3a, D0), though with a significant ranking from Lodi01 (58.2

**Table 2 – Absolute values of microbial biomass (estimated by  $\mu\text{g dsDNA g}^{-1}$  dry soil) and enzymatic activities (estimated by  $\mu\text{mol of MUF (or AMC) h}^{-1} \text{g}^{-1}$  dry soil, see experimental) in the soils studied: analysis of variance (ANOVA) results (F values and significance). A) Soils without saponin mixture (D0) at the T03 and T14 timepoints. B) Soils treated with increasing rates of saponin mixture (D0, D01, D10, D20 rates) at the T03 and T14 timepoints.**

Sources of variation	df	dsDNA	$\beta$ -Glucosidase	Leucine amino-peptidase	Alkaline phosphomonoesterase	Arylsulphatase
A) Soil	6	354.8****	391.4****	357.4****	366.3****	1016.7****
Time	1	3.5 ns	1.1 ns	36.6****	125.0****	193.5****
Soil $\times$ time	6	3.7*	8.6****	10.5****	29.1****	100.8****
B) Soil	6	957.8****	90.7****	383.3****	95.8****	209.5****
Treatment	3	2506.8****	32.3****	119.5****	96.6****	5.5***
Time	1	830.8****	113.9****	6.8*	5.3*	573.0****
Soil $\times$ treat	18	203.6****	25.2****	39.4****	10.9****	10.1****
D0 vs D10 in Bari01 <sup>c</sup>	1	733.8****	9.8***	3.6 ns	25.3****	0.1 ns
D0 vs D10 in Bari02 <sup>c</sup>	1	10.6**	0.2 ns	15.9****	0.0 ns	2.5 ns
D0 vs D10 in Bari03 <sup>c</sup>	1	158.0****	20.4****	0.1 ns	0.1 ns	0.0 ns
D0 vs D10 in Mode01 <sup>c</sup>	1	1187.3****	0.1 ns	8.1**	72.7****	1.4 ns
D0 vs D10 in Lodi01 <sup>c</sup>	1	544.4****	155.5****	80.7****	4.1*	10.9***
D0 vs D10 in Lodi02 <sup>c</sup>	1	1001.0****	0.4 ns	12.6****	13.3****	0.3 ns
D0 vs D10 in Lodi03 <sup>c</sup>	1	56.4****	1.4 ns	296.8****	3.1 ns	75.3****
Soil $\times$ time	6	158.5****	34.5****	8.6****	9.8****	97.7****
T03 vs T14 in Bari01 <sup>d</sup>	1	284.1****	0.8 ns	1.1 ns	11.8***	7.1**
T03 vs T14 in Bari02 <sup>d</sup>	1	0.3 ns	0.1 ns	2.5 ns	0.3 ns	18.1****
T03 vs T14 in Bari03 <sup>d</sup>	1	1242.1****	121.6****	0.6 ns	0.0 ns	6.6*
T03 vs T14 in Mode01 <sup>d</sup>	1	5.0*	1.1 ns	6.1*	4.3*	905.6****
T03 vs T14 in Lodi01 <sup>d</sup>	1	228.6****	195.9****	43.6****	42.5****	151.7****
T03 vs T14 in Lodi02 <sup>d</sup>	1	17.1****	0.1 ns	3.7 ns	4.8*	53.0****
T03 vs T14 in Lodi03 <sup>d</sup>	1	4.6*	1.6 ns	1.0 ns	1.0 ns	17.2****
Time $\times$ treat	3	216.0****	18.1****	63.4****	11.0****	39.5****
Soil $\times$ time $\times$ treat	18	57.8****	11.5****	23.5****	1.5 ns	12.4****

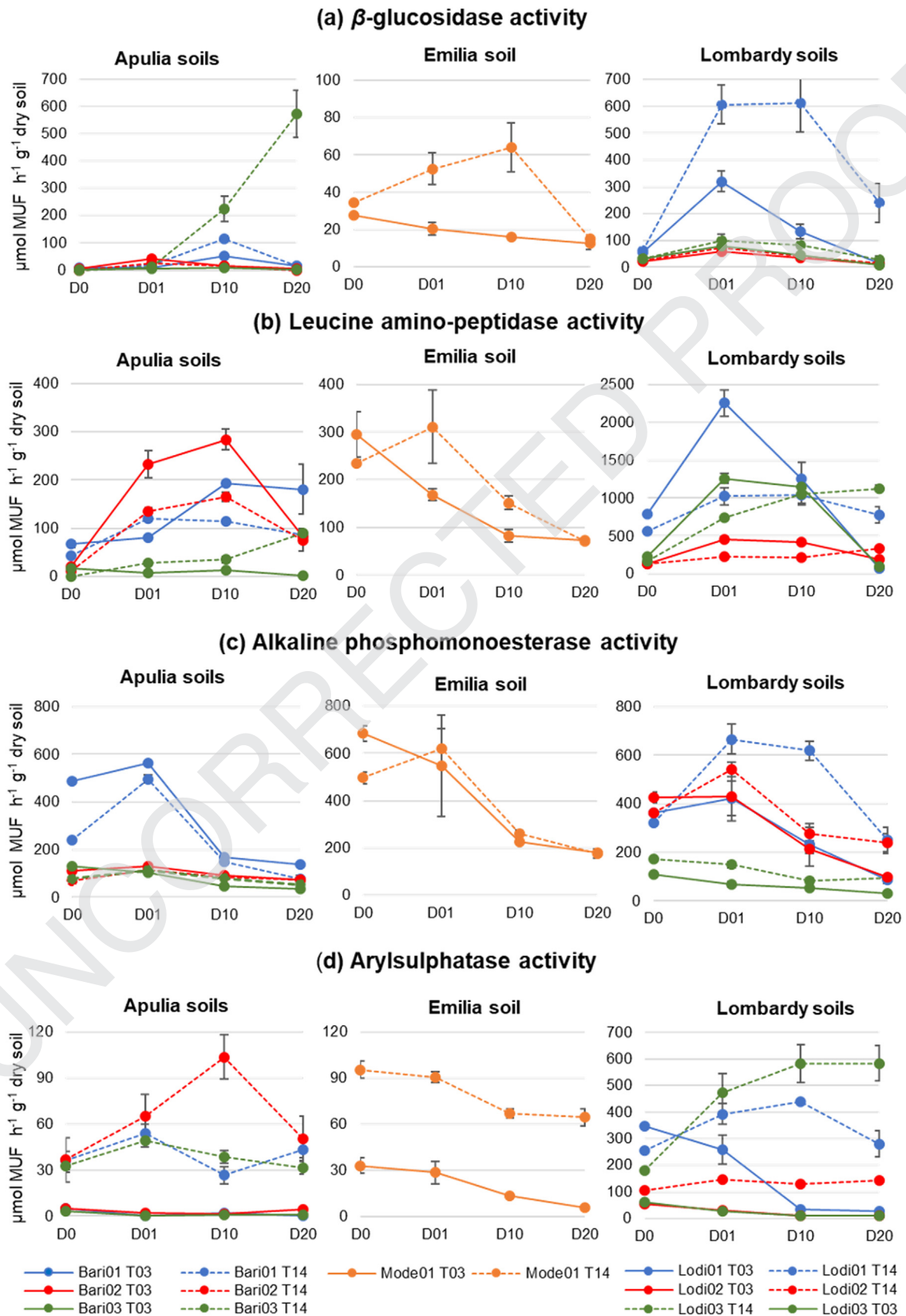
\* Significant at 0.05 level; \*\* significant at 0.01 level; \*\*\* significant at 0.005 level; \*\*\*\* significant at 0.001 level; ns, not significant.  
<sup>c</sup> Comparisons between D0 and D10 rate means within soil  $\times$  treat interaction by linear contrasts.  
<sup>d</sup> Comparisons between T03 vs T14 time points within soil  $\times$  time interaction by linear contrasts. df, degrees of freedom.



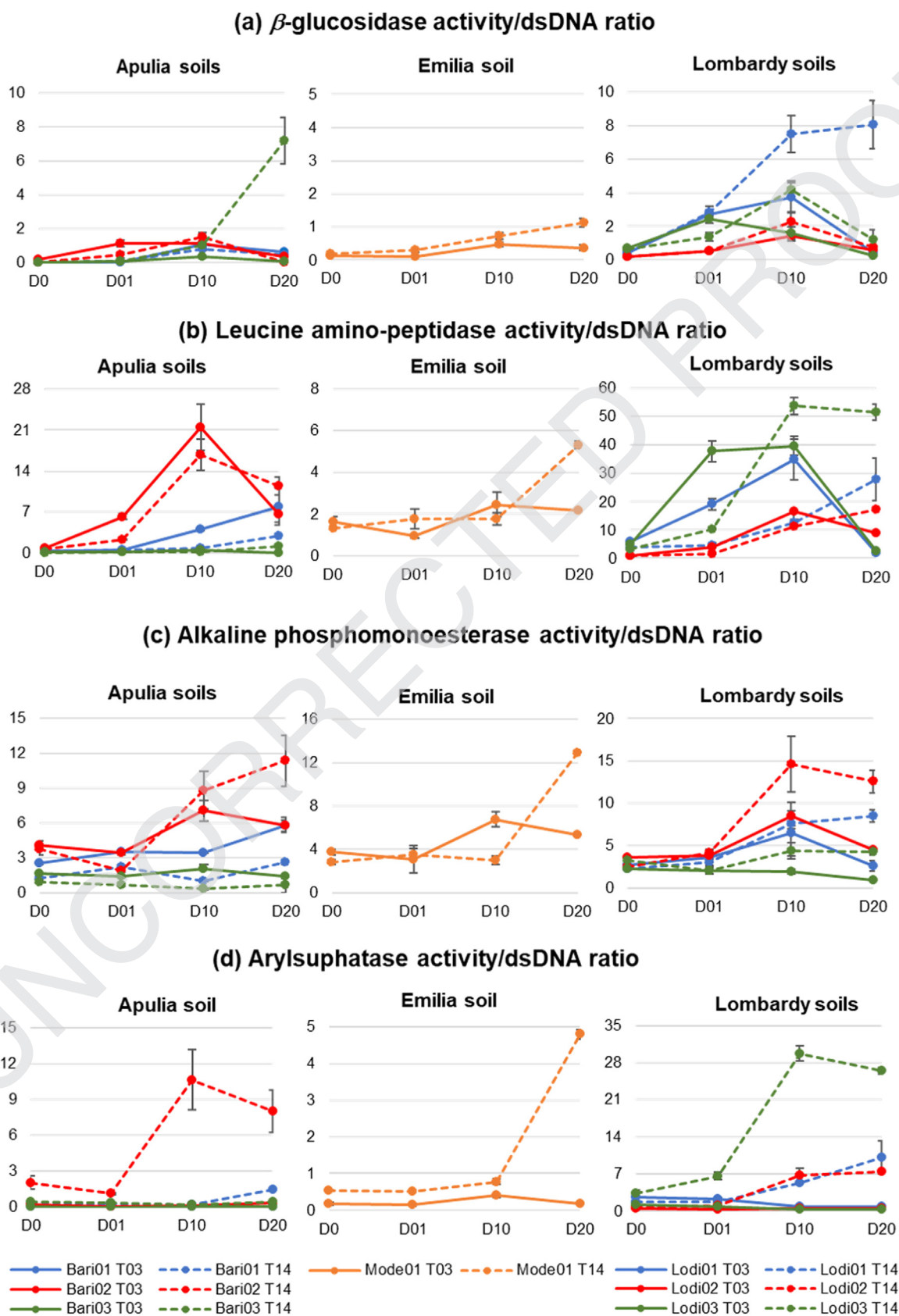
**Fig. 2 – dsDNA content (as  $\mu\text{g dsDNA g}^{-1}$  dry soil) estimated in seven different soils, from three different Italian regions. Saponin rates (W/W): D0, 0% (untreated); D01, 0.1%; D10, 1%; D20, 2%. Time points of treatments: T03, 3 days; T14, 14 days.**

347  $\mu\text{mol of MUF h}^{-1} \text{g}^{-1}$  dry soil, averaged over time) to Lodi03  
 348 and Mode01 (33.0 and 31.0, respectively) to Lodi02 (22.7), down  
 349 to all the Apulia soils, ranging from 4.2 to 1.5 (Table 2 and  
 350 Appendix A Table S1). No significant time course effect was  
 351 found at D0 for  $\beta$ -glucosidase activity (Table 2). Compared to  
 352 the untreated controls, the addition of *M. sativa* saponins did  
 353 not significantly modify the absolute  $\beta$ -glucosidase activity in

any soils except for a significant increase in Lodi01 and Bari03  
 (Table 2, D0 vs D10 linear contrast, Fig. 3a and Appendix A Table  
 S2). In soil Lodi01, originating from a permanent meadow  
 and with the highest organic C content (Table 1),  $\beta$ -glucosidase  
 activity already increased at D01 saponin rate and remained  
 stable up to D10, then decreasing only at D20 (Fig. 3a). Trend  
 of  $\beta$ -glucosidase activity in Lodi01 suggests the presence of



**Fig. 3 – Absolute values of enzyme activities (as  $\mu\text{mol}$  of MUF (or AMC)  $\text{h}^{-1} \text{g}^{-1}$  dry soil) estimated in seven different soils, from three different Italian regions. Saponin rates (W/W): D0, 0% (untreated); D01, 0.1%; D10, 1%; D20, 2%. Time points of treatments: T03, 3 days; T14, 14 days.**



**Fig. 4** – Relative values of enzyme activities (as  $\mu\text{mol}$  of MUF (or AMC)  $\text{h}^{-1} \text{g}^{-1}$  dry soil) per unit of dsDNA in seven different soils, from three different Italian regions. Saponin rates (W/W): D0, 0% (untreated); D01, 0.1%; D10, 1%; D20, 2%. Time points of treatments: T03, 3 days; T14, 14 days.

**Table 3 – Relative values of enzymatic activities (estimated by  $\mu\text{mol}$  of MUF (or AMC)  $\text{h}^{-1} \text{g}^{-1}$  dry soil, see experimental) per unit of dsDNA in the soils studied: analysis of variance (ANOVA) results ( $F$  values and significance). A) Soils without saponin mixture (D0) at the T03 and T14 timepoints. B) Soils treated with increasing rates of saponin mixture (D0, D01, D10, D20 rates) at the T03 and T14 timepoints.**

Sources of variation	df	Ratio $\beta$ -glucosidase/ dsDNA	Ratio leucine amino-peptidase/ dsDNA	Ratio alkaline phosphomono esterase/ dsDNA	Ratio arylsulphatase/ dsDNA
Soil	6	69.2****	229.1****	27.3****	41.8****
Time	1	2.3 ns	49.0****	19.7****	31.8****
Soil $\times$ time	6	1.6 ns	10.4****	5.2**	12.5****
Soil	6	58.5****	172.1****	47.1****	153.8****
Treatment	3	59.1****	132.5****	55.8****	104.7****
Time	1	66.9****	0.8 ns	14.1****	587.5****
Soil $\times$ treat	18	14.5****	22.5****	9.3****	24.0****
D0 vs D10 in Bari01 <sup>c</sup>	1	5.9*	1.2 ns	0.2 ns	0.0 ns
D0 vs D10 in Bari02 <sup>c</sup>	1	11.1***	94.0****	23.7****	41.1****
D0 vs D10 in Bari03 <sup>c</sup>	1	3.1 ns	0.0 ns	0.0 ns	0.0 ns
D0 vs D10 in Mode01 <sup>c</sup>	1	2.4 ns	0.1 ns	3.8 ns	1.4 ns
D0 vs D10 in Lodi01 <sup>c</sup>	1	104.9****	98.5****	30.9****	2.1 ns
D0 vs D10 in Lodi02 <sup>c</sup>	1	1.8 ns	46.7****	108.0****	20.3****
D0 vs D10 in Lodi03 <sup>c</sup>	1	0.0 ns	504.3****	0.2 ns	364.2****
Soil $\times$ time	6	34.5****	8.0****	9.6****	97.7****
T03 vs T14 in Bari01 <sup>d</sup>	1	0.2 ns	2.4 ns	12.6***	1.1 ns
T03 vs T14 in Bari02 <sup>d</sup>	1	0.6 ns	0.5 ns	5.5*	124.7****
T03 vs T14 in Bari03 <sup>d</sup>	1	53.9****	0.0 ns	2.7 ns	0.4 ns
T03 vs T14 in Mode01 <sup>d</sup>	1	0.5 ns	6.1*	2.1 ns	9.2**
T03 vs T14 in Lodi01 <sup>d</sup>	1	120.5****	5.6*	6.9*	45.3****
T03 vs T14 in Lodi02 <sup>d</sup>	1	1.0 ns	0.0 ns	32.9****	58.7****
T03 vs T14 in Lodi03 <sup>d</sup>	1	5.5*	39.8****	8.8**	1145.8****
Time $\times$ treat	3	18.1****	67.8****	21.6****	124.2****
Soil $\times$ time $\times$ treat	18	11.5****	23.9****	4.5****	27.5****

\* Significant at 0.05 level; \*\* significant at 0.01 level; \*\*\* significant at 0.005 level; \*\*\*\* significant at 0.001 level; ns, not significant.

<sup>c</sup> Comparisons between D0 and D10 rate means within soil  $\times$  treat interaction by linear contrasts.

<sup>d</sup> Comparisons between T03 vs T14 time points within soil  $\times$  time interaction by linear contrasts. df, degrees of freedom.

361 a cellulolytic microflora in the untreated soil, activated by  
362 the addition of saponins whose glycosidic chains could act  
363 as substrate for  $\beta$ -glucosidase. Adversely, soil Bari03 showed  
364 an increasing trend of  $\beta$ -glucosidase activity from D10 to D20  
365 saponin rate (Fig. 3a), suggesting a specific adaptation of soil  
366 microflora to the highest saponin mixture rates both as activi-  
367 ty (Fig. 3a) and as biomass (Fig. 2).

368 Absolute activity at D0 of leucine amino-peptidase, in-  
369 volved in the cleavage of N-terminal residues from proteins  
370 and peptides, ranked from Lodi01 (679.2  $\mu\text{mol}$  of MUF (or AMC)  
371  $\text{h}^{-1} \text{g}^{-1}$  dry soil, averaged over time) to Mode01 (265.0), Lodi03  
372 (199.2) and Lodi02 (134.0), with the lowest values in Apulia  
373 soils, not significantly different among them (Fig. 3b, D0 and  
374 Appendix A Table S1). In untreated soils Lodi01, Lodi03 and  
375 Mode01, a significant decrease of leucine amino-peptidase ac-  
376 tivity occurred at T14 compared to T03 (Appendix A Table S2).  
377 Overtime averaged effect of the addition of increasing rates of  
378 saponin mixture on leucine amino-peptidase activity varied  
379 from positive in Lodi03, Lodi01, Bari02, Lodi02 to negative in  
380 Mode01, while was not significant for Bari03 and Bari01 (Table  
381 2, D0 vs D10 linear contrasts and Fig. 3b).

382 Alkaline phosphomonoesterase, which mediates phos-  
383 phorous mineralization in soil through the hydroly-  
384 sis/transphosphorylation of organic phosphorus compounds,

385 showed the highest absolute values in untreated silty-clay soil  
386 Mode01 (591.0  $\mu\text{mol}$  of MUF (or AMC)  $\text{h}^{-1} \text{g}^{-1}$  dry soil, averaged  
387 over time). The other soils were grouped in two clusters, the  
388 first including Lodi02 (394.0), Bari01 (364.0) and Lodi01 (342.5)  
389 and the second Lodi03 (141.5), Bari03 (104.7) and Bari02 (90.5)  
390 (Fig. 3c and Appendix A Table S1). A significant decrease of the  
391 activity of this enzyme was found in untreated soils at T14  
392 with respect to T03, except for the overtime increased activity  
393 in soil Lodi03 (Fig. 3c, D0 and Appendix A Table S2). Effect of  
394 soil addition with increasing amounts of *M. sativa* saponins on  
395 alkaline phosphomonoesterase activity varied from slightly  
396 positive for Lodi01 to negative for Mode01, Bari01 and Lodi02,  
397 while Bari02, Bari03 and Lodi03 resulted not significantly  
398 affected (Table 2, D0 vs D10 with linear contrasts and Fig. 3c).

399 Absolute values of the activity of arylsulphatase (Fig. 3d), in-  
400 volved in soil sulfur cycling, significantly differed among the  
401 untreated soils (Table 2 and Fig. 3d, D0). Soils from Lombardy  
402 showed the highest enzymatic activities with a significant in-  
403 ternal ranking (Lodi01 > Lodi03 > Lodi02), while soil from Emilia  
404 showed an intermediate activity and Apulia soils resulted in  
405 the lowest activity values (Fig. 3d, D0 and Appendix A Table  
406 S1). Dynamics of arylsulphatase activity was characterized by  
407 the highest increase from T03 to T14 in all the untreated soils  
408 (Appendix A Table S2). Addition of saponin mixture produced

**Table 4 – Dynamics of saponin degradation in the soils studied at T0 and after 3 (T03), 14 (T14) and 28 (T28) days from saponin mixture addition (D10 rate equivalent to 1% W/W) for the variables free saponins, adsorbed saponins/sapogenins, free sapogenins and total saponins/sapogenins expressed in  $\mu\text{mol sapogenin g}^{-1}$  dry soil: analysis of variance (ANOVA) results (*F* values and significance). A) Soils at the T0 timepoint (immediately after saponin mixture addition). B) Dynamics of saponin degradation at the T0, T03, T07, T14 and T28 timepoints.**

Sources of variation	df	Free sapogenins	Free saponins	Adsorbed saponins/sapogenins	Total saponins
A) Soil	6	absent	0.93 ns	8.75**	0.34 s
B) Soil	6	34.51****	18.01****	32.50****	22.44****
Time	4	38.30****	131.21****	39.02****	156.62****
Soil $\times$ time	24	9.35****	1.71 ns	15.40****	3.49****
T0 vs T03 in Bari01 <sup>c</sup>	1	39.28****	32.87****	0.42 ns	20.43****
T0 vs T03 in Bari02 <sup>c</sup>	1	13.95****	2.24 ns	0.07 ns	0.22 ns
T0 vs T03 in Bari03 <sup>c</sup>	1	21.69****	9.77**	33.81****	0.35 ns
T0 vs T03 in Mode01 <sup>c</sup>	1	121.98****	35.08****	48.27****	2.22 ns
T0 vs T03 in Lodi01 <sup>c</sup>	1	0.12 ns	10.86**	0.19 ns	12.56**
T0 vs T03 in Lodi02 <sup>c</sup>	1	0.71 ns	6.36*	0.05 ns	5.80*
T0 vs T03 in Lodi03 <sup>c</sup>	1	0.19 ns	1.36 ns	2.45 ns	2.29 ns
T0 vs T07 in Bari01 <sup>c</sup>	1	10.84**	48.98****	17.45****	57.68****
T0 vs T07 in Bari02 <sup>c</sup>	1	62.27****	10.28****	0.07 ns	1.57 ns
T0 vs T07 in Bari03 <sup>c</sup>	1	0.30 ns	23.55****	46.49****	45.24****
T0 vs T07 in Mode01 <sup>c</sup>	1	60.50****	51.51****	8.20**	22.77****
T0 vs T07 in Lodi01 <sup>c</sup>	1	3.44 ns	17.73****	0.30 ns	16.99****
T0 vs T07 in Lodi02 <sup>c</sup>	1	6.42*	19.26****	2.48 ns	12.93****
T0 vs T07 in Lodi03 <sup>c</sup>	1	0.68 ns	13.14****	1.93 ns	15.97****
T0 vs T14 in Bari01 <sup>c</sup>	1	12.05****	61.16****	53.94****	84.73****
T0 vs T14 in Bari02 <sup>c</sup>	1	49.19****	17.73****	0.71 ns	7.41**
T0 vs T14 in Bari03 <sup>c</sup>	1	1.78 ns	45.76****	4.19*	80.60****
T0 vs T14 in Mode01 <sup>c</sup>	1	54.52****	64.87****	73.00****	25.08****
T0 vs T14 in Lodi01 <sup>c</sup>	1	4.19*	66.07****	2.14 ns	72.15****
T0 vs T14 in Lodi02 <sup>c</sup>	1	17.62****	26.25****	8.10**	12.93****
T0 vs T14 in Lodi03 <sup>c</sup>	1	4.25*	30.63****	1.90 ns	36.32****
T0 vs T28 in Bari01 <sup>c</sup>	1	0.88 ns	59.64****	46.93****	94.11****
T0 vs T28 in Bari02 <sup>c</sup>	1	38.13****	47.04****	0.16 ns	30.19****
T0 vs T28 in Bari03 <sup>c</sup>	1	0.00 ns	58.36****	60.52****	101.88****
T0 vs T28 in Mode01 <sup>c</sup>	1	4.37*	91.19****	0.98 ns	102.74****
T0 vs T28 in Lodi01 <sup>c</sup>	1	0.10 ns	67.21****	3.71 ns	84.18****
T0 vs T28 in Lodi02 <sup>c</sup>	1	18.79****	53.54****	0.50 ns	46.02****
T0 vs T28 in Lodi03 <sup>c</sup>	1	14.09****	64.94****	3.98 ns	61.08****

\* Significant at 0.05 level; \*\* significant at 0.01 level; \*\*\* significant at 0.005 level; \*\*\*\* significant at 0.001 level; ns, not significant.

<sup>c</sup> Comparisons T0 vs T03, T07, T14 and T28 timepoints within soil  $\times$  time interaction by linear contrasts. df, degrees of freedom.

a significant increase of arylsulfatase activity in soils Lodi03 and Lodi01, which showed the highest values for this enzymatic activity even when untreated, while increasing saponin rates did not significantly affect D0 activity values in all the other soils (Table 2, linear contrasts D0 vs D10 within soil and Fig. 3d, D10 rate).

When considering enzyme activities per unit of microbial biomass (Fig. 4 and Table 3), Bari02 and Mode01 soils showed the most relevant differences in the response to saponin addition compared to absolute values. In Bari02 an increasing trend of the activity of all the enzymes was evident at D10 rate (Table 3, D0 vs D10 linear contrasts and Fig. 4) and persisted at D20 except for  $\beta$ -glucosidase activity (Appendix A Tables S3 and S4). In the same soil, a positive effect of time was also found for alkaline phosphomonoesterase and arylsulfatase activities (Table 3, T03 vs T14 linear contrast and Fig. 4), indicating that saponin presence can stimulate an adaptive response even in soil with the least abundant microflora

(Fig. 2) and lacking in sorption capacity. In Mode01 soil, where an abundant microflora was severely affected by the presence of high rates of saponin mixture (Fig. 2), relative activities of all the enzymes remained stable at D10 (Table 3, D0 vs D10 linear contrasts and Fig. 4), while showed a significant increase of alkaline phosphomonoesterase and arylsulfatase at D20 (Fig. 4 and Appendix A Tables S3 and S4). This trend could be due to a high buffering capacity of clay soil from Emilia towards saponin mixture, as resulting in stable enzymatic activities also in the presence of an adaptive response of its microflora.

Soil microbial biomass was highly significantly ( $P < 0.01$ ) and positively correlated with alkaline phosphomonoesterase activity in untreated soils (D0,  $r_{T03}=0.92$  and  $r_{T14}=0.77$ ) and at D01 rate ( $r_{T03}=0.86$  and  $r_{T14}=0.71$ ) at both time points T03 and T14 (Appendix A Table S5). At the same saponin concentrations and time points, highly significant and positive correlations were found among the activities of  $\beta$ -glucosidase, leucine amino-peptidase and arylsulfatase enzymes (T03)

445 and between  $\beta$ -glucosidase vs leucine amino-peptidase and  
 446 leucine amino-peptidase vs arylsulfatase (T14) (Appendix A  
 447 Table S5). At the D10 rate, no correlation was found be-  
 448 tween microbial biomass and enzymatic activities at both  
 449 time points, as confirming the detrimental effect of 1% w/w  
 450 saponin mixture on soil microbial population (Appendix A Ta-  
 451 ble S5). Positive and highly significant relationships between  
 452  $\beta$ -glucosidase vs leucine amino-peptidase and arylsulfatase  
 453 were present at T03 and between  $\beta$ -glucosidase vs alkaline  
 454 phosphomonoesterase and leucine amino-peptidase vs aryl-  
 455 sulfatase at T14 time point (Appendix A Table S5). At the D20  
 456 rate no significant correlations were found among the enzyme  
 457 activities except for leucine amino-peptidase vs arylsulfatase  
 458 at T14 ( $r = 0.97^{****}$ ) (Appendix A Table S5). Interestingly, a posi-  
 459 tive and highly significant correlation between soil microbial  
 460 biomass and  $\beta$ -glucosidase activity was observed at T14 (Ap-  
 461 pendix A Table S5), as driven by soil Bari03 that showed the  
 462 highest values for both the variables (Appendix A Table S2 and  
 463 Fig. S2).

### 464 2.3. Dynamics of saponins degradation in the soils

465 No free sapogenins were detected at T0, since soil extrac-  
 466 tion was performed immediately after the addition of the  
 467 saponin mixture. Free saponin content, averaging 86.7% of  
 468 total saponins at T0, did not significantly differ among the  
 469 soils (Table 4a and Appendix A Table S6). Adversely, the ad-  
 470 sorbed saponin/sapogenin fraction showed a significant dif-  
 471 ference among soils, as indicated by the statistically higher  
 472 content in Bari03 and Bari01 soils compared to Bari02, Lodi03  
 473 and Lodi01 (Table 4a and Appendix A Table S6). Free saponins  
 474 were promptly degraded, with a concomitant significant in-  
 475 crease in free sapogenin content, in soils presenting the high-  
 476 est amount of microbial biomass, i.e. Mode01 and Bari01 (Fig. 2,  
 477 Table 4b, T0 vs T03 linear contrasts and Fig. 5). A similar trend  
 478 was found in Bari03 soil in which a significant increase of mi-  
 479 crobial biomass (Fig. 2) and  $\beta$ -glucosidase activity (Fig. 3a) oc-  
 480 curred at D10 and D20 saponin rates. A significant and prompt  
 481 decrease of free saponin content occurred also in Lodi01 with-  
 482 out any significant variation of content of free sapogenins and  
 483 adsorbed saponins/sapogenins at all the time points (Table 4b  
 484 and Fig. 5), as suggesting a similar rate of depletion of glycosy-  
 485 lated chains of *M. sativa* saponins and their aglycones by the  
 486 microflora of the soil. In the above four soils (Bari01, Mode01,  
 487 Bari03, Lodi01), depletion of total saponins during the 28 days  
 488 of the assay ranged from 81.4% (Lodi01) to 91.2% (Bari03) of  
 489 initial mixture (Fig. 4). It is noteworthy that the highest deple-  
 490 tion rate occurred in Bari03, i.e. the soil with the highest mi-  
 491 crobial biomass at D10 rate of saponin addition (Fig. 2, Table 2),  
 492 as well as with the highest initial saponin sorption capacity,  
 493 1.38 and 2.19  $\mu\text{mol}$  sapogenin  $\text{g}^{-1}$  dry soil at T0 and T03 time  
 494 point, respectively (Appendix A Table S6).

495 In Lodi02 and Lodi03 soils, free saponins degradation was  
 496 nearly linear and parallel to increase of free sapogenins,  
 497 as well as statistically significant from T07 (Lodi02) or T14  
 498 (Lodi03) time point (Table 4 and Appendix A Table S6). This  
 499 degradation pattern indicates a higher prompter depletion  
 500 rate of saponin glycosylated chains compared to aglycones,  
 501 resulting in 72.5% and 62.4% total saponins degradation  
 502 in Lodi03 and Lodi02, respectively, after 28 days. Bari02, i.e.

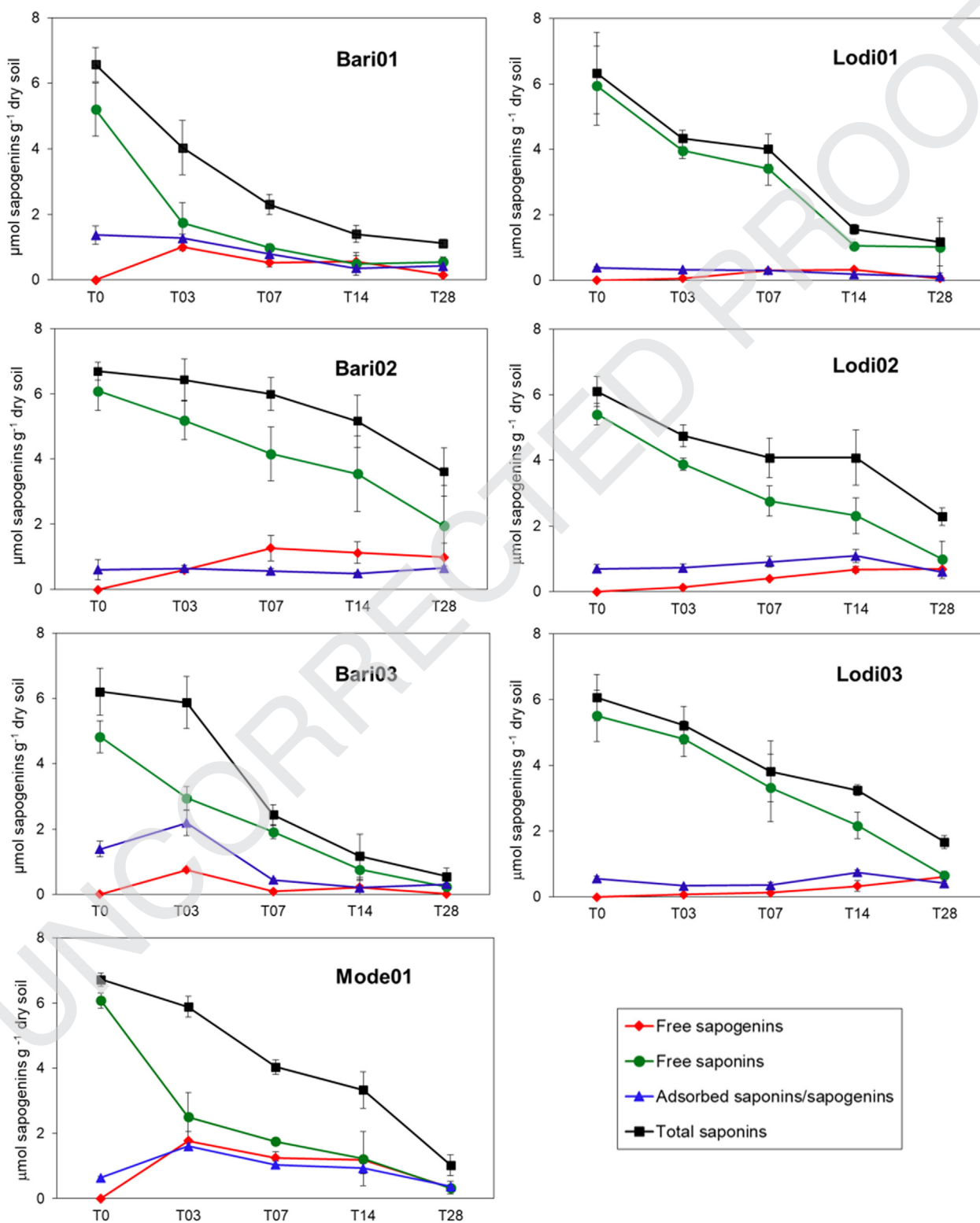
503 the soil with the lowest microbial biomass at T0 (Fig. 2,  
 504 Appendix A Table S1), showed also the lowest depletion  
 505 rate of total saponins and consequently the lowest degra-  
 506 dation percentage, 46.1%, at T28. A parallel significant in-  
 507 crease of free sapogenins, accounting for 14.8% of initial to-  
 508 tal saponins at T28 time point, was evident from the T03  
 509 time point (Table 4, Fig. 5, and Appendix A Table S6). Sandy-  
 510 loamy soils from Lombardy showed low values of the adsorbed  
 511 saponin/sapogenin fraction at T0, with not significant vari-  
 512 ations along all the time points but Lodi02 at T14. A simi-  
 513 lar trend of saponins/sapogenins adsorption was displayed by  
 514 sandy soil Bari02 (Table 4 and Fig. 5). Adversely, Mode01, i.e. the  
 515 soil with the highest clay content (41%), presented a low ini-  
 516 tial level of adsorbed saponins/sapogenins which significantly  
 517 increased at T03, T07 and T14 (Table 4 and Fig. 5).

518 Comparison of soil sapogenin composition at T0, i.e. im-  
 519 mediately after saponins addition, and T28 (Fig. 6) was based  
 520 on all the different aglycones expressed as percentage of to-  
 521 tal sapogenins, i.e. the sum of free sapogenins, free saponins  
 522 and adsorbed saponins/sapogenins (Fig. 5, black lines). In this  
 523 comparison, main significant differences were found for soils  
 524 Bari01, Bari03 and Mode01, in which percentage of soyasa-  
 525 pogenol B (non-hemolytic sapogenin) increased from 14.1%  
 526 (T0) to 36.6% (T28) (on average). Adversely, total hemolytic sa-  
 527 pogenins decreased from 85.9% to 63.4% (Fig. 6) due to a de-  
 528 crease of medicagenic acid in Bari01 and Mode01 (from 46.6%  
 529 to 26.1% on average) and of zanhic acid in Bari03 (from 34.7%  
 530 to 23.7%). Interestingly, these three soils were characterized by  
 531 the highest amount of clay and silt fractions (Table 1), as sug-  
 532 gesting a role of soil sorption in dynamics of saponin degra-  
 533 dation depending on different aglycones.

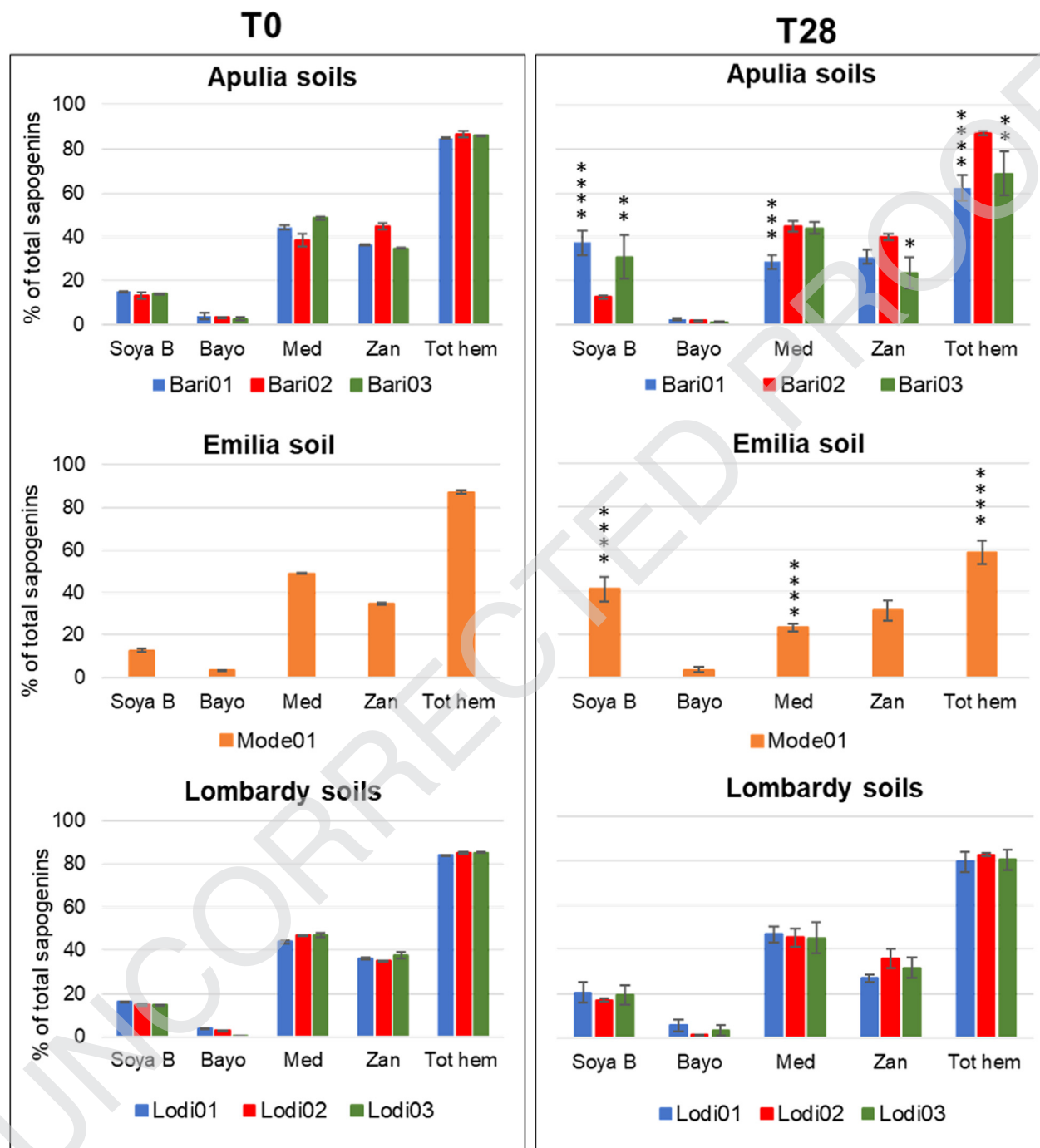
### 534 2.4. Results from the PCA analysis of all data from 535 saponin effects in soil

536 Percentage of the total variation represented by Prin1 and  
 537 Prin2 variables was 73.8% and 72.0% at T03 and T14, respec-  
 538 tively (Fig. 7). At T03, Prin1 (49.4% of the total variation) clearly  
 539 separated sandy and sandy-loam soils Lodi02, Bari02, Lodi01  
 540 and Lodi03 from soils with the highest values of clay and silt  
 541 fractions, i.e. Mode01, Bari03 and Bari01. Prin1 was related posi-  
 542 tively ( $r > 0.5$ ) to the adsorbed saponin/sapogenin fractions  
 543 and negatively to the free saponin fractions (Appendix A Fig.  
 544 S3a). Prin2 (24.4% of the total variation) was related negatively  
 545 to microbial biomass and enzymatic activities and positively  
 546 to total soil saponin content (Appendix A Fig. S3a) and separ-  
 547 ated soils with high microbial biomass and enzymatic activ-  
 548 ity and a fast total saponin degradation (Bari01 and Lodi01)  
 549 from soils characterized by opposite features, Bari02, Bari03  
 550 and Lodi03 (Fig. 7).

551 After 14 days from saponin addition, Prin1 (53.8% of the  
 552 total variation) was positively correlated ( $r > 0.5$ ) with almost  
 553 all saponin fractions in soil solution and negatively with mi-  
 554 crobial biomass and  $\beta$ -glucosidase activity (Appendix A Fig.  
 555 S3b). At this observation time, the Prin1 component clearly  
 556 separated soils Bari03, Lodi01 and Bari01, showing the high-  
 557 est degradation of total saponins at T14 and high micro-  
 558 bial biomass and/or microbial activities, from Bari02, Lodi02  
 559 and Mode01, characterized by a reduced degradation of to-  
 560 tal saponins at T14 (Bari02, Lodi02 in Fig. 7) and a low mi-



**Fig. 5 – Variation of the content of free saponin, free saponin, adsorbed saponin/saponin and total saponin in the seven soil samples at different time points after saponin addition. T0, immediately after saponin addition; T03, 3 days; T07, 7 days; T14, 14 days; T28, 28 days.**

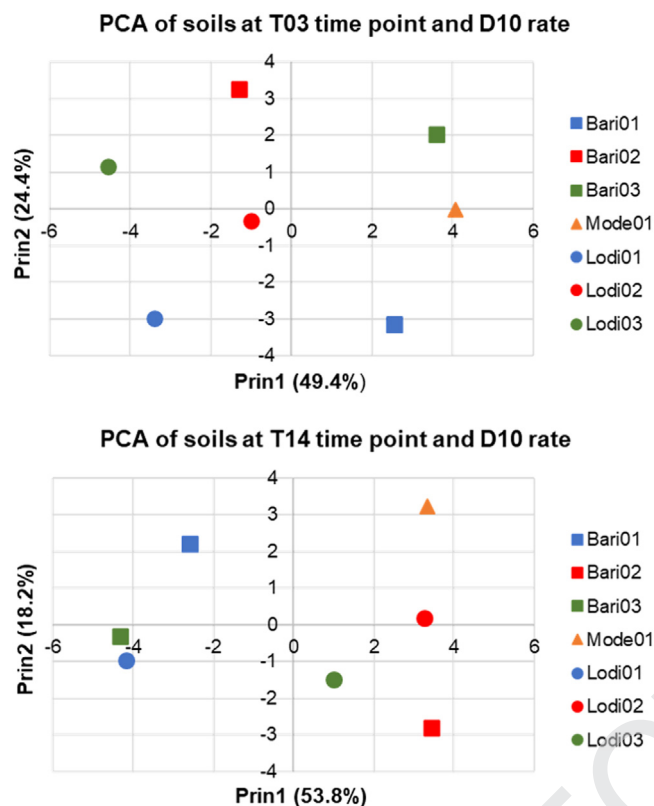


**Fig. 6 – Sapogenin composition of soil extracts at two different time points: T0, immediately after saponin addition, and T28, 28 days, after saponin addition. Values are expressed as percentage of the total sapogenins. Soya B, soyasapogenol B; Bayo, bayogenin; Med, medicagenic acid; Zan, zanhic acid; Tot hem, total hemolytic sapogenins. \* significant at 0.05 level; \*\* significant at 0.01 level; \*\*\* significant at 0.005 level; \*\*\*\* significant at 0.001 level.**

561 microbial biomass or a poor microflora response to saponin ad-  
 562 dition (Mode01 in Fig. 4). Prin2 (18.2% of the total variation)  
 563 was mainly negatively correlated to the free saponin frac-  
 564 tion and positively with adsorbed soyasapogenol B and bayo-  
 565 genin saponins (Appendix A Fig. S3b). Consequently, clay soils  
 566 Mode01 and Bari01 were clearly distinguished from the sandy  
 567 soils Bari02 and Lodi03 (Fig. 7).

### 3. Discussion

Saponins are both constitutively synthesized in *Medicago* spp. 568  
 and triggered by the signal component methyl jasmonate as 569  
 a part of defense-responsive plant metabolites in response 570  
 to biotic/abiotic stresses. Then, detrimental effect of *Medicago* 571



**Fig. 7 – Principal Component Analysis (PCA) performed for 2 time points of treatments: T03 (3 days) and T14 (14 days) at the D10 rate (10 mg of saponin mix per g of soil, 1% W/W).**

saponins on soil microbial biomass at D10 and D20 doses, mainly evident in soils with a high initial microbial biomass as Bari01, Mode01 and Lodi01, is expected as due to their antimicrobial properties (Tava and Avato, 2006). Nevertheless, response of enzymatic activities to increasing rates of saponin mixtures indicated that metabolic changes were activated by soil microbial communities in response to saponin presence. In fact, soil addition with saponin mixture induced few clear negative effects on enzyme absolute activities (leucine aminopeptidase in Mode01 and alkaline phosphomonoesterase in Mode01, Bari01 and Lodi02) and, adversely, constantly positive significant variations of relative enzymatic activities, as indicating that adaptive responses of soil microflora maintained or increased level of relative enzymatic activity of untreated soils. Increase of relative enzymatic activities by increasing saponin rates was particularly evident in sandy and sandy-silty soils (Bari02 and the Lombardy soils), suggesting a protection role of the soil clay fraction towards saponins activity on soil microflora. Consistency of positive relationships, mainly evident at the T03 observation time, among enzymatic activities in untreated soil (D0) and, at D01 and D10 saponin rates, and significant positive (T14 > T03 values) effect of time for microbial biomass, arylsulfatase activity and  $\beta$ -glucosidase activity are further indications of an adaptive response of soil microflora to the presence of saponins.

An adaptive response of soil microflora involving  $\beta$ -glucosidase activity was evidenced in Bari03 soil by the sharp

increase of enzymatic activity from D10 to D20 rate at T14 time point. A two-component system composed of a  $\beta$ -glucosidase and triterpene saponins has been recently described in *M. truncatula* (Lacchini et al., 2023) as a plant mechanism for managing bioactive defence metabolites. Moreover, a  $\beta$ -glucosidase (AMBGL17) obtained from a metagenomic analysis of an Amazonian soil has been proved to catalytically interact with a soybean saponin (Hernandez et al., 2022). It is then possible that in Bari03 a part of soil microflora synthesizing  $\beta$ -glucosidases and effectively interacting with *Medicago* saponins emerged under the selective pressure of high saponin rates.

Microbial degradation of saponins, as an important part of adaptive response to their presence in soil, was evident in all the experimental soils since the T03 time point, though with different dynamics studied. Sugiyama (2021) reported a range of 4.4 to 14.7 days as half-life of most flavonoids and saponins in soil, a degradation pattern consistent with our results in bulk soil. In the T0-T07 time interval, free saponins were the fraction more rapidly degraded and at T03 and T07 their content was always higher than that of free saponin, indicating that both the saponin glycosidic chains and aglycone moieties were used as a C source by soil microflora. Plant root pathogens (bacteria and fungi) are part of soil microflora and interact inside plant root tissues with the plant immunity systems PAMP-triggered (PTI) and effector-triggered (ETI), of which saponins are part as effector molecules (Dodds and Rathjen, 2010). It could be hypothesized that saponins are 'recognized' by potential plant pathogens also in bulk soil and consequently degraded by means of pathways and genes present in this specific microflora. In support of this hypothesis, the saponin fraction most degraded after 28 days in soils Bari01, Bari03 and Mode01 was that of hemolytic saponins, the most abundant ones but also the most biologically active, while the non-hemolytic saponin soyasapogenol B was relatively less degraded. Ability of saponin-treated soils to reshape their microflora by enrichment of bacterial families belonging to plant-growth promoting rhizobacteria, such as Sphingomonadaceae, or potentially pathogenic fungal taxa as *Alternaria* and *Fusarium*, has been recently reported (Fujimatsu et al., 2020; Li et al., 2020; Sugiyama, 2021) and could also play a role in the adaptive response of soil microflora to the presence of saponins.

Sorption of saponins by soil components could reduce their impact on soil microflora and prevent their leaching and water contamination. Sorption ability in our experimental soils resulted as an immediate phenomenon, as present immediately after the addition of saponin mixture (T0), when sandy-silty soil Bari03 and the clay soil Bari01 showed higher values of adsorption compared to the sandy soil Bari02 and the sandy-silty soils Lodi03 and Lodi01. A non-linear relationship between saponin adsorption onto soil surface with time has been reported by a study of Wahab and Yusup (2021), in which a rapid very initial phase was followed by a gradually achieved near-steady state. The same authors concluded that physical sorption, mediated by van der Waals force, is the main phenomenon driving saponin sorption by soils, though chemical sorption can also occur at lower rate mainly in clay soils or in soils with high organic matter content. In our saponin degradation assay, the dynamics of the adsorbed

659 saponin/sapogenin fractions showed a steady or declining  
660 trend across observation times in all the soils but Mode01,  
661 with significantly higher adsorption compared to T0 at T03,  
662 T07 and T14, and Bari03 at T03. It is likely that physical traits  
663 specific of these soils, as the high clay content in Mode01, con-  
664 tribute to enhance the role of chemical sorption.

665 PCA analysis of combined data, referred to common obser-  
666 vation times T03 and T14 and to saponin rate D10 of the two  
667 independent assays, was able to discriminate the analyzed  
668 soils for their ability to react to saponin presence. In particular,  
669 initial response (T03) could be driven by the higher saponin  
670 sorption ability of clay and silty-clay soils (Mode01, Bari03,  
671 Bari01), as enabling a more rapid degradation of free saponin  
672 glycosidic chains compared to sandy and sandy loam soils  
673 (Lodi03, Lodi01, Bari02, Lodi02). After 14 days (T14), higher val-  
674 ues of microbial biomass and enzymatic activities in Bari03,  
675 Lodi01 and Bari01 soils could be responsible for the deeper  
676 degradation of all saponin fractions with respect to Bari02,  
677 Mode01, Lodi02 and Lodi03.

#### 4. Conclusions

678 In the view of using alfalfa saponins as crop biopesticides,  
679 knowledge of their effects on soil microflora is needed to  
680 preserve soil biological fertility parameters. In all the seven  
681 soils under observation, alfalfa saponins reduced soil micro-  
682 bial biomass at doses of 10 and 20 mg saponin mixture per g of  
683 soil corresponding to 7.5 and 15  $\mu\text{mol}$  total sapogenins per g of  
684 soil, respectively. Presence of saponins less affected microbial  
685 enzymatic activities, as indicating an adaptive response of the  
686 soil microbial communities to these compounds. As a matter  
687 of fact, the degradation of total saponins by the soil microflora  
688 accounted for 46% to 91% within 28 days from saponin addi-  
689 tion depending on soil characteristics (clay content, size of soil  
690 microbial biomass, organic matter content). Microbial degrada-  
691 tion was extended to both saponin glycosidic chains and  
692 triterpenic aglycones, according to variable dynamics among  
693 the investigated soils.

#### Uncited reference

694 Qasim et al., 2020.

#### Declaration of competing interest

695 The authors declare that they have no known competing fi-  
696 nancial interests or personal relationships that could have ap-  
697 peared to influence the work reported in this paper.

#### CRedit authorship contribution statement

698 Aldo Tava: Conceptualization, Data curation, Funding ac-  
699 quisition, Supervision, Writing – original draft, Writing – re-  
700 view & editing. Elisa Biazzzi: Data curation, Formal analysis,  
701 Methodology, Writing – original draft, Writing – review & edit-  
702 ing. Flavio Fornasier: Formal analysis, Methodology, Writing –  
703 original draft. Trifone D'Addabbo: Data curation, Supervision,

Writing – review & editing. Pinarosa Avato: Data curation, Su-  
pervision, Writing – review & editing. Carla Scotti: Data cura-  
tion, Formal analysis, Supervision, Writing – original draft,  
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#### Supplementary materials

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#### REFERENCES

- Abbruscato, P., Tosi, S., Crispino, L., Biazzzi, E., Menin, B.,  
Picco, A.M., et al., 2014. Triterpenoid glycosides from *Medicago*  
*sativa* as antifungal agents against *Pyricularia oryzae*. *J. Agric.*  
*Food Chem.* 62, 11030–11036.
- Avato, P., Bucci, R., Tava, A., Vitali, C., Rosato, A., Bialy, Z., et al.,  
2006. Antimicrobial activity of saponins from *Medicago* sp.:  
structure-activity relationship. *Phytother. Res.* 20, 454–457.
- Avato, P., Migoni, D., Argentieri, M., Fanizzi, F.P., Tava, A., 2017.  
Activity of saponins from *Medicago* species against HeLa and  
MCF-7 cell lines and their capacity to potentiate cisplatin  
effect. *Anti-Canc. Ag. Med. Chem.* 17, 1508–1518.
- Bao, L., Liu, Y., Chen, J., Ding, Y., Shang, J., Li, J., et al., 2023.  
Different effects of six saponins on the rhizosphere soil  
microorganisms of *Panax notoginseng*. *Plant Soil* 487, 1–18.
- Bardelli, T., Gómez-Brandón, M., Ascher-Jenull, J., Fornasier, F.,  
Arfaioi, P., Francioli, D., et al., 2017. Effects of slope exposure  
on soil physico-chemical and microbiological properties along  
an altitudinal climosequence in the Italian Alps. *Sci. Total*  
*Environ.* 575, 1041–1055.
- Bezerra, K.G.O., Rufino, R.D., Luna, J.M., Sarubbo, L.A., 2018.  
Saponins and microbial biosurfactants: potential raw  
materials for the formulation of cosmetics. *Biotechnol. Progr.*  
34, 1482–1493.
- Bialy, Z., Jurzysta, M., Oleszek, W., Piacente, S., Pizza, C., 1999.  
Saponins in alfalfa (*Medicago sativa* L.) root and their  
structural elucidation. *J. Agric. Food Chem.* 47, 3185–3192.
- Bialy, Z., Jurzysta, M., Mella, M., Tava, A., 2006. Triterpene saponins  
from the roots of *Medicago hybrida*. *J. Agric. Food Chem.* 54,  
2520–2526.
- Caldwell, B., 2005. Enzyme activities as a component of soil  
biodiversity: a review. *Pedobiologia (Jena)* 49, 637–644.
- Cowie, A.L., Lonergan, V.E., Rabbi, S.M.F., Fornasier, F.,  
Macdonald, C., Harden, S., et al., 2013. Impact of carbon  
farming practices on soil carbon in northern New South  
Wales. *Soil Res.* 51, 707–718.
- D'Addabbo, T., Carbonara, T., Leonetti, P., Radicci, V., Tava, A.,  
Avato, P., 2011. Control of plant parasitic nematodes with  
active saponins and biomass from *Medicago sativa*.  
*Phytochem. Rev.* 10, 503–519.
- D'Addabbo, T., Argentieri, M.P., Zuchowski, J., Biazzzi, E., Tava, A.,  
Oleszek, W., et al., 2020. Activity of saponins from *Medicago*  
species against phytoparasitic nematodes. *Plants* 9, 443.

- De Geyter, E., Lambert, E., Geelen, D., Smagghe, G., 2007. Novel advances with plant saponins as natural insecticides to control pest insects. *Pest Technol* 1, 96–105. 822
- Dodds, P.N., Rathjen, J.P., 2010. Plant immunity: towards an integrated view of plant-pathogen interactions. *Nat. Rev. Gen.* 11, 539–548. 824
- Fons, F., Amellal, N., Leyval, C., Saint-Martin, N., Henry, M., 2003. Effect of gypsophila saponins on bacterial growth kinetics and on selection of subterranean clover rhizosphere bacteria. *Can. J. Microbiol.* 49 (6), 367–373. 825
- Fornasier, F., Ascher, J., Ceccherini, M.T., Tomat, E., Pietramellara, G.A., 2014. simplified rapid, low-cost and versatile DNA-based assessment of soil microbial biomass. *Ecol. Indicat.* 45, 75–82. 826
- Fujimatsu, T., Endo, K., Yazaki, K., Sugiyama, A., 2020. Secretion dynamics of soyasaponins in soybean roots and effects to modify the bacterial composition. *Plant Direct* 4, 1–12. 827
- Gan, P.P., Li, S.F.Y., 2013. Biosorption of elements. In: Hunt, A.J. (Ed.), *Element Recovery and Sustainability*. The Royal Society of Chemistry, Cambridge UK, pp. 80–114. 828
- Güçlü-Üstündağ, Ö., Mazza, G., 2007. Saponins: properties, applications and processing. *Crit. Rev. Food Sci. Nutr.* 47, 231–258. 829
- Gunina, A., Kuzyakov, Y., 2015. Sugars in soil and sweets for microorganisms: review of origin, content, composition and fate. *Soil Biol. Biochem.* 90, 87–100. 830
- Hernandez, A.I., dos Santos Azevedo, R., Werhli, A.V., dos Santos Machado, K., Nornberg, B.F., Marins, L.F., 2022. Phylogenetic analysis, computer modeling and catalytic prediction of an Amazonian soil  $\beta$ -glucosidase against a soybean saponin. *Integr. Biol.* 14, 204–211. 831
- Iatrou, M., Papadopoulos, A., Papadopoulos, F., Dichala, O., Psoma, P., Bountla, A., 2014. Determination of soil available phosphorus using the Olsen and Mehlich 3 methods for Greek soils having variable amounts of calcium carbonate. *Commun. Soil Sci. Plant Anal.* 45, 2207–2214. 832
- Indorante, S.J., Hammer, R.D., Koenig, P.G., Follmer, L.R., 1990. Particle-size analysis by a modified pipette procedure. *Soil Sci. Soc. Am. J.* 54, 560–563. 833
- Iovinella, I., Barbieri, F., Biazzì, E., Sciandra, C., Tava, A., Mazza, G., et al., 2023. Antifeedant and insecticidal effects of alfalfa saponins in the management of the Japanese beetle *Popillia japonica*. *J. App. Entomol.* 147, 651–660. 834
- Kaczorek, E., Smulek, W., Zdarta, A., Sawczuk, A., Zgoła-Greškowiak, A., 2016. Influence of saponins on the biodegradation of halogenated phenols. *Ecotoxicol. Environ. Saf.* 131, 127–134. 835
- Kobayashi, T., Kaminaga, H., Navarro, R.R., Iimura, Y., 2012. Application of aqueous saponin on the remediation of polycyclic aromatic hydrocarbons-contaminated soil. *J. Environ. Sci. Health Part A* 47, 1138–1145. 836
- Lacchini, E., Erffelinck, M.L., Mertens, J., Marcou, S., Molina-Hidalgo, F.J., Tzfadia, O., et al., 2023. The saponin bomb: a nucleolar-localized  $\beta$ -glucosidase hydrolyzes triterpene saponins in *Medicago truncatula*. *New Phytol* 36, 705–719. 837
- Li, Y., Dai, S., Wang, B., Jlang, Y., Ma, Y., Pan, L., et al., 2020. Autotoxic ginsenoside disrupts soil fungal microbiomes by stimulating potentially pathogenic microbes. *Appl. Environ. Microbiol.* 86, 1–14. 838
- Maestrini, M., Tava, A., Mancini, S., Tedesco, D., Perrucci, S., 2020. *In vitro* anthelmintic activity of saponins from *Medicago* spp. against sheep gastrointestinal nematodes. *Molecules* 25, 242. 839
- Morcia, C., Piazza, I., Ghizzoni, R., Delbono, S., Felici, B., Baima, S., et al., 2022. search of antifungals from the plant world: the potential of saponins and brassica species against *Verticillium dahliae* Kleb. *Horticulturae* 8, 729. 840
- Nakayasu, M., Yamazaki, S., Aoki, Y., Yazaki, K., Sugiyama, A., 2021. Triterpenoid and steroidal saponins differentially influence soil bacterial genera. *Plants* 10, 2189. 841
- Nannipieri, P., Trasar-Cepeda, C., Dick, R.P., 2018. Soil enzyme activity: a brief history and biochemistry as a basis for appropriate interpretations and meta-analysis. *Biol. Fertil. Soils* 54, 11–19. 842
- Oleszek, W., Jurzysta, M., Gorski, P.M., 1992. Alfalfa saponins the allelopathic agents. In: Rizvi, S.J.H., Rizvi, V. (Eds.), *Allelopathy: Basic and Applied Aspects*. Chapman & Hall, London, pp. 151–167. 843
- Podolak, I., Galanty, A., Sobolewska, D., 2010. Saponins as cytotoxic agents: a review. *Phytochem. Rev.* 9, 425–474. 844
- Qasim, M., Islam, W., Ashraf, H.J., Ali, I., Wang, L., 2020. Saponins in insect pest control. In: Mérillon, J.M., Ramawat, K. (Eds.), *Co-evolution of Secondary Metabolites*. Reference Series in Phytochemistry. Springer, Cham, Edinburgh, pp. 1–28. 845
- Rao, A.V., Gurfinkel, D.M., 2000. The bioactivity of saponins: triterpenoids and steroidal glycosides. *Drug Metab. Drug Interac.* 17, 211–236. 846
- Rengel, Z., 2007. Cycling of micronutrients in terrestrial ecosystems. In: Marschner, P., Rengel, Z. (Eds.), *Nutrient Cycling in Terrestrial Ecosystems*. Springer-Verlag, Berlin, Heidelberg, pp. 93–121. 847
- Sinsabaugh, R.L., Lauber, C.L., Weintraub, M.N., Ahmed, B., Allison, S.D., Crenshaw, C., et al., 2008. Stoichiometry of soil enzyme activity at global scale. *Ecol. Lett.* 11, 1252–1264. 848
- Sugiyama, A., 2021. Flavonoids and saponins in plant rhizospheres: roles, dynamics, and the potential for agriculture. *Biosci. Biotechnol. Biochem.* 85, 1919–1931. 849
- Tamura, Y., Miyakoshi, M., Yamamoto, M., 2012. Application of saponin-containing plants in foods and cosmetics. In: Sakagami, H. *Alternative Medicine*. InTech, pp. 85–101. 850
- Tava, A., Oleszek, W., Jurzysta, M., Berardo, N., Odoardi, M., 1993. Alfalfa saponins and sapogenins: isolation and quantification in two different cultivars. *Phytochem. Anal.* 4, 269–274. 851
- Tava, A., Mella, M., Avato, P., Argentieri, M.P., Bialy, Z., Jurzysta, M., 2005. Triterpenoid glycosides from the leaves of *Medicago arborea* L. *J. Agric. Food Chem.* 53, 9954–9965. 852
- Tava, A., Avato, P., 2006. Chemical and biological activity of triterpene saponins from *Medicago* species. *Nat. Prod. Comm.* 1, 1159–1180. 853
- Tava, A., Mella, M., Avato, P., Biazzì, E., Pecetti, L., Bialy, Z., Jurzysta, M., 2009. New triterpene saponins from the aerial parts of *Medicago arabica* (L.) Huds. *J. Agric. Food Chem.* 57, 2826–2835. 854
- Tava, A., Pecetti, L., Romani, M., Mella, M., Avato, P., 2011. Triterpenoid glycosides from the leaves of two cultivars of *Medicago polymorpha* L. *J. Agric. Food Chem.* 59, 6142–6149. 855
- Tava, A., Biazzì, E., Mella, M., Quadrelli, P., Avato, P., 2017. Artefact formation during acid hydrolysis of saponins from *Medicago* spp. *Phytochemistry* 238, 116–127. 856
- Tava, A., Biazzì, E., Ronga, D., Mella, M., Doria, F., Accogli, R., et al., 2020. Triterpene saponins from *Medicago marina* L. *Phytochemistry* 174, 112333. 857
- Wahab, W.R.A., Yusup, S., 2021. Kinetics, isotherm and thermodynamic modeling of liquid phase saponin sorption in soils. *IOP Conf. Series: Earth Environ. Sci.* 765, 012111. 858
- Ye, M., Sun, M., Xie, S., Liu, K., Feng, Y., Zhao, Y., et al., 2017. Feasibility of tea saponin-enhanced soil washing in a soybean oil-water solvent system to extract PAHs/Cd/Ni efficiently from a coking plant site. *Pedosphere* 27, 452–464. 859
- Yu, X.L., He, Y., 2018. Tea saponins: effective natural surfactants beneficial for soil remediation, from preparation to application. *RSC Adv* 8, 24312. 860
- Zhang, S., Kong, J., Chen, L., Guo, K., Zhou, X., 2022. Increased tea saponin content influences the diversity and function of plantation soil microbiomes. *Microbiol. Spectr.* 10 e02324-21. 861