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Soil biochemical activities after the application of pyroligneous acid to soil

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Pyroligneous acid is a complex aqueous liquid fraction produced during the combustion of woody biomasses that can provide beneficial or toxic effects on soil and plants, depending on the doses used.

The effects of pyroligneous acid on the soil microbial community have been investigated, in order to gather further information on its toxicity or stimulation.

Our results indicate that pyroligneous acid has no negative effects on the soil, but only if it is distributed at doses up to 1% dilution.

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Abstract

Pyroligneous acid (PA) is produced during the combustion of woody biomass and is a complex aqueous fraction resulting from the thermochemical rupture of the components of the vegetable biomass. We evaluated the effect of PA on the soil microbial community and activity in order to assess the applicability of this acid on soil and to gather further information on the mechanisms of its toxicity or stimulation. Five concentrations of PA solution (0%, 0.5%, 1%, 2% and 5%) were selected to monitor the biochemical parameters of the soil. The respirometric test showed that the increase in the evolved CO_2-C was not due to a release of carbon from the organic soil of the native organic C, but only from the organic compounds of PA. The highest values of microbial biomass content were found in the soil treated with the lowest doses, while it decreased with an increase in the dose of PA. At higher application doses (2% and 5%), there was a decrease in most enzymatic activities and a loss of soil quality. When PA is applied in doses of up to 1%, our results indicate that it has no negative effects on soil biology and that there is even an improvement.

Keywords: Wood vinegar – Soil respiration - Soil microbial biomass – Soil enzymes

Introduction

The global production of woody biomass produced by agriculture and forestry has been estimated at 146 billion tons per year (Demirbas 2002). Its combustion degrades the soil and releases into the atmosphere a large number of particles, volatile organic carbon and semi-volatile organic carbon compounds, ash, sulphate aerosols, and trace gases. These pollutants increase greenhouse

gas emissions, which can contribute to many serious environmental problems on an overall scale such as the increase in global climate change, the extinction of biodiversity as well as serious socio-economic and health problems. Therefore, it is important to minimize combustion or waste plant biomass and, instead, to develop means of reducing pollution at a low cost along with sustainable technologies to convert it into useful bioproducts.

Pyrolysis is a thermochemical process that leads to the thermal degradation of materials in the absence or near absence of oxygen (Balat et al. 2009). During slow pyrolysis, the organic bonds are decomposed, and the plant biomass is converted into organic vapors and solid charcoal. The gaseous products of pyrolysis are water vapor, tar and volatiles that are condensed and collected using filters and cold traps (Mansur et al. 2013). The condensed organic vapors form an aqueous liquid fraction rich in oxygenated compounds (Mathew et al. 2014) called pyroligneous acid or wood vinegar.

Pyroligneous acid (PA) is a complex aqueous liquid fraction which results from the thermochemical rupture of the components of the plant biomass such as cellulose, hemicellulose and lignin. PA is produced during the combustion of woody biomasses, when the gases from the oven are channeled in such a way as to allow the condensation of the steam.

PA is a yellowish brown or dark brown liquid that usually includes a complex mixture of different classes of organic compounds, namely aldehydes, ketones, alcohols, organic acids, esters, furan and pyran derivatives, phenols, hydrocarbons and nitrogen compounds, in which the main ones are organic and phenolic acids (Souza et al. 2012). The high concentration of acids (up to 25% by weight) gives PA a low pH (pH<3). These substances may have positive effects if correctly applied in terms of quantity and application time.

The beneficial effects of PA on soil and plants are well known (Benzon et al. 2015; Kang et al. 2012; Prasertsit et al. 2011; Rui et al. 2014; Souza et al. 2012). PA is used in various areas, such as an antioxidant, antimicrobial, anti-inflammatory, plant growth stimulator, a coagulant for natural rubber and as a termiticidal agent and pesticide (Loo et al. 2007). The results obtained by

Mmojieje and Hornung (2015) demonstrate the benefits of PA through its application as a pesticide against mites and red spiders at a 10% dilution. These results suggest a potential role in crop protection. Furthermore, in the same work a phytotoxic effect was reported on its application in dilutions above 20%.

As PA is used to control pests and diseases, we hypothesize that toxic effects might reduce the soil microbial population size, activity and population growth, at least in the most concentrated doses. Soil biochemical activities are commonly used as indicators of soil quality as they are more sensitive to changes in management than the physical or chemical properties of the soil, they measure the key microbial reactions involved in the soil nutrient cycles and can be easily measured (Nannipieri et al. 2002). However, there are very few publications on the effects of adding PA to soil enzyme activities. We thus selected various biochemical properties for analysis based on their importance in the decomposition of organic matter and several enzymes involved in different nutrient cycles were finally chosen.

We also calculated the geometric mean of enzyme activities (GMea) in order to estimate soil quality considering that it is sensitive to changes in soil management (García-Ruiz et al. 2008). We also monitored soil basal respiration and microbial biomass in order to assess the effects of management practices (Steiner et al. 2004) or toxic agents (Beck and Bengel 1992) on the soil microbial community.

We assessed the effects of PA on the soil microbial community in order to evaluate the applicability of PA to soil and to gather further information on the mechanisms of its toxicity or stimulation. In fact, many researchers have shown that PA enhances harvest yield in many plant species through the enhancement of seed germination, plant growth, fruit size, fruit weight and the quality of many fruits and vegetables (Zulkarami et al., 2011).

The starting hypothesis of this research is that the PA has a positive effect on the quality of the soil, if applied in the appropriate doses. The aim of this work is to investigate whether PA can improve soil quality by verifying the effects on microbial activity at various application doses.

Materials and Methods

Soil

The surface (0–15 cm) of a soil, classified as a loamy sand Typic Xerorthent, was collected from a dedicated agricultural area at Pontasserchio, which is located at a distance of approximately 9 km from the sea (43°45′51" N, 10°23′23" E) and 1 m above sea level (Pisa, Italy). The soil samples were collected in March 2019 and consisted of 10 cores measuring 5 cm dia x 15 cm depth. These were air-dried and passed through a 2 mm sieve to remove large residue fragments. The main soil characteristics were determined by standard methods (SISS 1995): 77% sand (2 - 0.05 mm), 14% silt (0.05 - 0.002 mm), 9% clay (< 0.002 mm), 7.9 pH, 10.0 g kg^{-1} total organic C (TOC), 1.03 g kg^{-1} total N, 16.4 mg kg^{-1} available P, 43.8 mg kg^{-1} available K, 13.7 cmol (+) kg^{-1} cation exchange capacity, and 44% maximum water-holding capacity.

Pyroligneous acid

Pyroligneous acid (PA), also called wood vinegar or wood distillate, was produced by RM Impianti srl (Arezzo, Italy) and was obtained from native forest plant essences with the same physiological water through pyrolysis. The manufacturer recommends using the product in the open field by a fertigation system at a 0.5% dilution. We tested higher doses of the product (up to 5%) to investigate the effects on the biochemical activities of the soil. The main PA characteristics were: 2.8 pH, 33.8 g l^{-1} total organic C, 0.43 g l^{-1} total N. The composition of PA is detailed in previous works (Mathew and Zakaria 2015; Grewal et al. 2018).

Treatments of soil with pyroligneous acid

In 250ml microcosms, the experiment was conducted in triplicate with five treatments (15 microcosms total) to differentiate between the influence of PA doses. Five dilutions of PA were selected to monitor the soil parameters: control (C) only water, low (L) 0.5%, medium-low (ML) 1%, medium-high (MH) 2% and high (H) 5% doses. The samples were watered with the different

129 solutions at the 60% maximum water holding capacity (26.4ml.100g^{-1}), which we considered to
130 be optimal for soil biological activities. They were then closed with parafilm to permit a gaseous
131 exchange, and incubated at 25±1°C for 10 days. After 10 days, the samples were refrigerated at 4
132 °C for the analyses.
133
134 *Analyses*
135 A short-term (21 days) aerobic incubation was used to determine the potential of the samples to
136 mineralize organic C. The CO_2 evolution was monitored daily between days 1 and 21 : 50 g of soil
137 was placed in 250-ml glass containers closed with rubber stoppers, moistened with the various
138 solutions of PA at 60% of the maximum water holding capacity (13.2ml.50g^{-1}) and incubated at
139 25±1°C; the CO_2 evolved was trapped in NaOH solution and the alkali excess was titrated with
140 HCl (Levi-Minzi et al. 1990). The results, normalized with respect to time, were expressed as mg
141 of C mineralized.100g^{-1} of dry soil.
142 Soil microbial biomass C (MB-C) was determined according to Vance et al. (1987) with the
143 extraction of organic C from fumigated and unfumigated soils by 1 N K_2SO_4. The organic C was
144 then measured using a QBD1200 Laboratory TOC Analyser (Hach Company, USA). An
145 extraction efficiency coefficient (Kc) of 0.45 was used to convert the difference in soluble C
146 between the fumigated and unfumigated soils into microbial biomass C.
147 Specific respiration of biomass (qCO_2) was calculated as follows: the CO_2 evolved during the 15th
148 day of incubation (Fig. 1) was used as basal respiration value because, after that period, the soil
149 reached a constant rate of CO_2 production. The specific respiration of biomass (µg CO_2-C basal
150 h^{-1} µg biomass C^{-1}) represents the microbial respiration per biomass unit (Schnurer et al. 1985).
151 Dehydrogenase activity (DH) was assayed following Tabatabai (1994), based on a colorimetric
152 assay of 2,3,5 triphenylformazan (TPF) produced by the microorganism reduction of 2,3,5
153 triphenyltetrazolium chloride (TTC).

β-glucosidase activity (GL) was assayed by a colorimetric method, using 4-nitrophenyl-ß-D-glucopiranosyde as a substrate: soil samples were incubated at 37°C for 60 minutes; the reaction product p-nitrophenol was determined at 410 nm (Eivazi and Tabatabai 1988).

Following Eivazi and Tabatabai (1977), Alkaline phosphatase activity (AP) was based on the hydrolysis of p-nitrophenyl phosphate added to soil samples. This phosphate releases p-nitrophenol, which can be detected colorimetrically.

Arylsulphatase activity (AS) was determined by a colorimetric method, using p-nitrophenyl sulfate as a substrate: soil samples were incubated at 37°C for 1 hour and the reaction product (p-nitrophenol) was extracted by dilute alkali ($CaCl_2$ 0.5M and NaOH 0.5M) and determined at 400 nm (Tabatabai and Bremner 1970).

The geometric mean (a general index to integrate information from variables that possess different units and range of variation) of the assayed enzyme activities was calculated for each sample as: $GMea = (DH \times GL \times AP \times AS)^{1/4}$ (Paz-Ferreiro et al. 2012).

The hydrolysis rate of fluorescein diacetate (FDA) was estimated as reported by Dick et al. (1996), by determining the concentration of fluorescein released by FDA ($\mu g.g^{-1}.2h^{-1}$) at 490 nm.

The determination of catalase activity (CA) was based on the rates of recovery of H_2O_2 by titration with $KMnO_4$ in the presence of sulphuric acid (Jin et al. 2009).

Urease activity (UR) was determined according to Kandeler and Gerber (1988), based on the spectrophotometric measurement of released ammonia after a 2-hour incubation of soil samples with urea substrate at 37°C.

Statistical analysis

Statistica 7.0 (StatSoft Inc., Tulsa, Oklahoma, USA) was used for the statistical analysis. Data were expressed on the basis of the oven-dry weight of the soil. Results were the means of determinations carried out on three replicates. The differences between treatments were analyzed

using a one-way analysis of variance (ANOVA). Significantly different means were separated at the 0.05 probability level by Tukey's test (Steel et al. 1997).

Results and discussion

The pH of the soil with the addition of the H dose of PA dropped by only 0.9 points compared to the control (from 7.9 down to 7.0, data not shown). We believe that this limited variation does not explain any changes in the properties that we discuss below.

The daily emission of carbon dioxide (Fig. 1) shows that on the first day, the treatment with the highest dose of PA induced a low production of CO_2, indicating a temporary stress of the microflora immediately after the PA addition. From the second day, the pattern showed a clear influence of PA on the CO_2 emission, with values that were constantly higher than the other treatments throughout the whole incubation period (21 days), as a result of a greater amount of mineralizable organic C. This means that the H dose had an initial inhibiting effect on the activity of microbial biomass, which was probably due to the high concentration of phenolic compounds and organic acids of PA (Grewal et al. 2018); in fact, at certain doses PA is an effective antimicrobial solution (Lee et al., 2011).

Figure 2 shows the cumulative emission of CO_2 from PA-soil systems. The curves seem to suggest that the positive effects on C mineralization were due to the incremental addition of PA and the difference between the five treatments was the quantity of organic matter added to soil. The addition of readily available substrate following the incorporation of the different doses of PA had either stimulated microbial activity or put it under stress. In fact, the highest C mineralization was detected in the H treatment (77.66 mg CO_2-C.100g^{-1}, Table 1). After the first phase of toxicity and inhibition, revealed by the patterns shown in Fig. 1, in H treatment a resiliency was evidently followed which stimulated the activity of the remaining biomass.

The regression line describing the cumulative C loss from PA–soil systems as a function of different loading rates of pyroligneous acid-C was calculated (Fig. 3). This enabled us to estimate,

without having to use isotopically labelled biochar, the influence of the material on the native organic C decomposition in the soil. This procedure consists of comparing the value of the intercept of the regression line (Fig. 3) with the Y-axis, corresponding to CO_2-C produced at the application of 0 g/100 g TOC, with the CO_2-C actually measured in the control (Levi-Minzi et al. 1990). The value of the priming ratio, (0.95) obtained by dividing the CO_2-C value of 33.09 mg.100g^{-1} estimated by the intercept of the line with the Y-axis for the value of CO_2-C of 34.80 mg.100g^{-1} actually measured in the control, indicates a negative priming effect following the PA addition, i.e. a reduction in the microbial decomposition of native organic carbon. This result indicates that the higher values of enhanced CO_2-C in the treated samples were not due to a carbon release of native soil organic matter (Cardelli et al. 2016).

Values of microbial biomass carbon ranged from 495 to 773 µg.g^{-1} of soil (Table 2). The highest values of MB-C were found in the L and ML soil, treated with the lowest doses of PA. This is in line with results described by Rui et al. (2014), who reported promoting effects on total microbial quantities exhibited by soils treated with low doses (0.3%) of PA. In our work, MH and H presented lower quantities of microbial biomass, probably due to a repressive dose-effect of the toxic components of PA.

If the basal respiration rates are related to the biomass size, the specific respiration of biomass (qCO_2) thus obtained represents the CO_2–C produced per unit biomass and time. This parameter indicates how efficiently the microbial biomass uses available C for biosynthesis rather than for maintenance of respiration. Anderson and Domsch (1993) reported that qCO_2 increases when the ecosystem is stressed, polluted or in adverse climatic conditions.

In our study, the specific respiration activity of biomass was influenced by the different doses of PA and showed an inverse relation with the biomass content (Table 2). The highest value of qCO_2 was in H soil with 9.67 µg CO_2-C basal h^{-1} µg biomass C^{-1}, while L soil had the minimum value of 4.09 µg CO_2-C basal h^{-1} µg biomass C^{-1}. The high qCO_2 value of H suggests an intense competition for the available C accessible after the treatment. Moreover, Islam and Weil (2000)

reported that perturbed systems, such as H, favored bacteria with a low efficiency in C assimilation, while more efficient fungi tended to dominate in natural non-stressed systems. It is interesting to note that the low microbial pool size of H (Table 2) corresponded to the highest level of specific respiration activity of biomass (qCO_2), and a high amount of microbial biomass of L soil corresponded to the lowest qCO_2. These results suggest, in line with Nsamibana et al. (2004), the predominance of more active microorganisms in H. Anderson and Domsch (1993) also found an inverse relation between qCO_2 and soil reaction. Accordingly, in our work the highest qCO_2 was in the most acidic soil H (data not shown).

Dehydrogenase activity is an important component of the enzymatic system of every microorganism. A significantly lower DH activity was found in the MH and H doses compared with the control, L and ML doses (Table 2). This suggests that the microorganisms are unable to use PA as a substrate because they are inhibited by the toxic effect of phenolic compounds and volatile acids, which are abundant in PA.

On the other hand, the addition of PA up to 1% dilution did not affect the DH activity compared to the control. Given that PA causes significant increases in the quantities of bacteria in soils, but it does not change the characteristics of the soil bacterial composition (Rui et al. 2014), the higher respiration values coupled with lower DH activity in H may indicate that the soil microorganisms were less efficient under high doses of PA. This behavior was also true for GL and AS activities, while AP activity did not seem to be affected at all. In fact, AP activity exhibited no difference between any of the treatments analyzed in our work (Table 2). This result would seem to indicate that P cycling in the used soil is unaltered by the addition of PA. To the best of our knowledge there are no works on the effect of PA addition to soil in these enzyme activities to compare our data with.

The geometric mean of enzyme activities (GMea) has proved to be a good index for estimating soil quality as its values are related to other physical, chemical and biological properties of the soil (García-Ruiz et al. 2008).

In our study, GMea was statistically different in the MH and H treatments compared to the others. The lower GMea values in MH and H suggest that the application of high doses of PA is harmful for the soil microorganisms and results in a decrease in soil quality. The L and ML doses showed higher values of GMea than the MH and H soil thus indicating that the quality of the soil was preserved.

The rate of hydrolysis of fluorescein diacetate (FDA) by soil samples has been considered as an index of the overall microbial activity (Schnurer & Rosswall 1982), because this hydrolysis is carried out by active cells and is due to a variety of enzymes. Table 3 shows no noticeable influence of the doses of PA on FDA in the L, ML and MH treatments, while there was a clear decrease in H. This confirms the results from GMea regarding a general decrease in soil quality, but only when the H dose was used.

The addition of PA to soil did not affect the catalase activity (CA), in any of the treatments (Table 3), probably as a result of the increase in the biologically active remaining microflora.

Urease activity (UR), appeared to be very active only at the highest dose (H), while it was lower at the doses L, ML and MH. The light soluble organic material contributed by the PA and exploitable by UR, could explain the increase in enzyme activity in the thesis H. As mentioned regarding AP activity, there are no reported works on the effect of PA addition to soil also for UR activity, and several hypotheses could possibly explain the behavior of this enzyme which also occurs in an extracellular form.

Conclusions

The respirometric test showed a slight negative priming effect and that the increase in CO_2-C in the treated samples was not due to a carbon release from the native soil organic matter but to the mineralization of PA organic C. This implies that the application of PA to the soil does not accelerate the decomposition of the native organic matter.

We found that the microbial biomass decreased quantitatively with increasing doses of PA, but the efficiency of the remaining part improves.

At higher application doses (2% and 5% dilution) there was a decrease in most of the enzymatic activities. Even GMea indicates a loss of soil quality at those doses.

Phosphatase and catalase activities were not affected by PA, while the urease appears to be stimulated by the addition of PA to soil.

Our results indicate that PA has no negative effects on the soil, but only if it is distributed at doses up to 1% dilution. In our opinion this quantity could be recommended by the producers of the PA, although it will require further investigation to allow the development of this product on a commercial scale.

Further studies are needed to investigate the application of PA to soil and the influence of PA in agriculture, as this type of material could prove to be of great interest for the sustainability of cropping systems.

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Tab. 1. CO_2-C evolved after 21 days, soil microbial biomass and specific respiration (qCO_2) in the different treatments of soil.

Treatment	C mineralized	Biomass-C	qCO_2
	mg CO_2-C. $100g^{-1}$ soil	µg C g^{-1} soil	µg CO_2-C h^{-1} . µg biomass C^{-1} .10^2
Control	34.80	660.23 *b*	5.74 *bc*
L	36.54	773.88 *a*	4.09 *c*
ML	41.04	721.95 *a*	4.96 *bc*
MH	50.58	582.23 *bc*	5.80 *b*
H	77.66	495.57 *c*	9.67 *a*

Means followed by the same letter in a column were not significantly different ($P < 0.05$) according to Tukey's test.

Tab. 2. Dehydrogenase, β-glucosidase, alkaline phosphatase, arylsulphatase and geometric mean index (GMea) in the different treatments of soil.

Treatment	Dehydrogenase	β-glucosidase	Phosphatase	Arylsulphatase	GMea
	µmol TPF.g^{-1}.h^{-1}	µmol p-nitrophenol.g^{-1}.h^{-1}			
Control	0.126 *a*	0.425 *a*	1.486 *a*	0.224 *a*	0.365 *a*
L	0.122 *a*	0.406 *ab*	1.535 *a*	0.209 *ab*	0.355 *a*
ML	0.123 *a*	0.418 *a*	1.720 *a*	0.216 *ab*	0.372 *a*
MH	0.081 *b*	0.392 *b*	1.592 *a*	0.201 *bc*	0.317 *b*
H	0.084 *b*	0.387 *b*	1.691 *a*	0.180 *c*	0.315 *b*

Means followed by the same letter in a column were not significantly different ($P < 0.05$) according to Tukey's test.

Tab. 3. FDA-ase, catalase and urease activities in the different treatments of soil.

Treatment	FDA-ase	Catalase	Urease
	Fluorescein µg.g^{-1}.$2h^{-1}$	$KMnO_4$ µmol.g^{-1}.h^{-1}	NH_4-N µg.g^{-1}.$2h^{-1}$
Control	96.20 *a*	288.9 *a*	14.07 *b*
L	95.65 *a*	283.4 *a*	11.68 *c*
ML	99.41 *a*	282.5 *a*	11.28 *c*
MH	102.50 *a*	281.4 *a*	10.21 *c*
H	55.08 *b*	280.7 *a*	18.71 *a*

Means followed by the same letter in a column were not significantly different ($P < 0.05$) according to Tukey's test.

455 Figure captions.

456

457 Fig. 1. Daily C loss over 21-day incubation in PA–soil systems at different application rates.

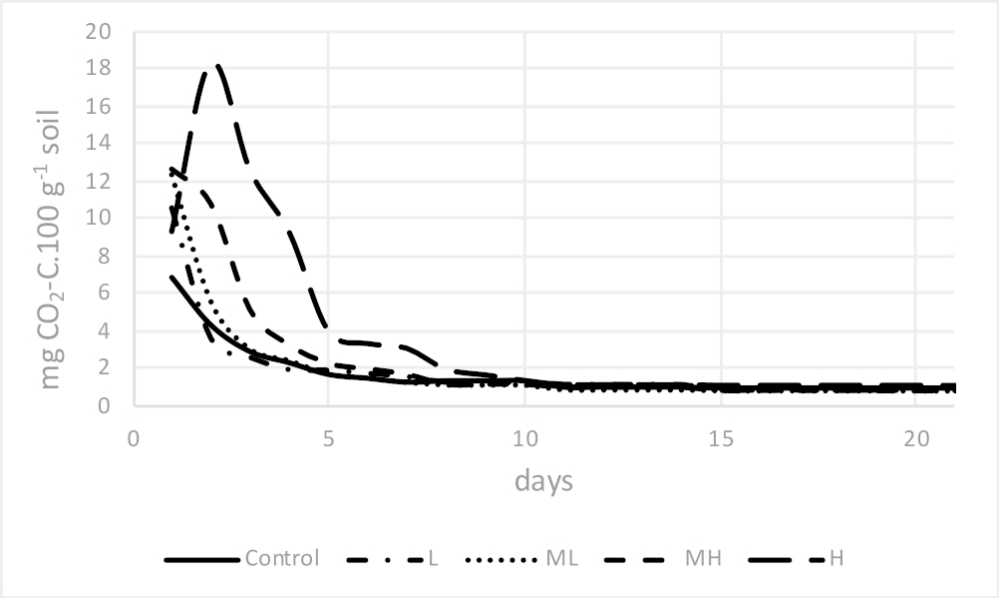
458

459 Fig. 2. Cumulative C loss over 21-day incubation in PA–soil systems at different application rates.

460

461 Fig. 3. Cumulative evolution of CO_2-C from soil systems as a function of different application

462 rates of PA.



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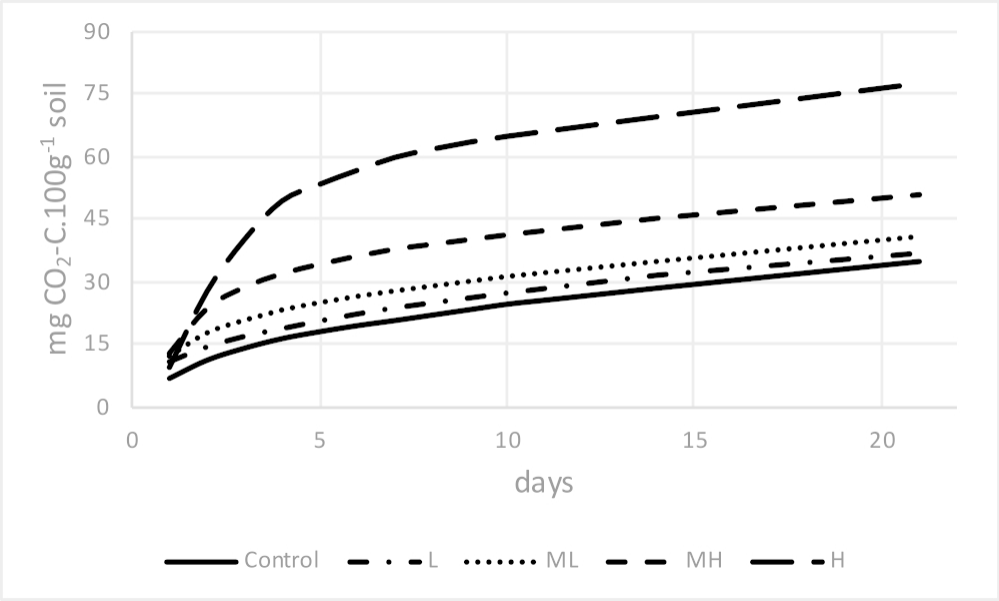

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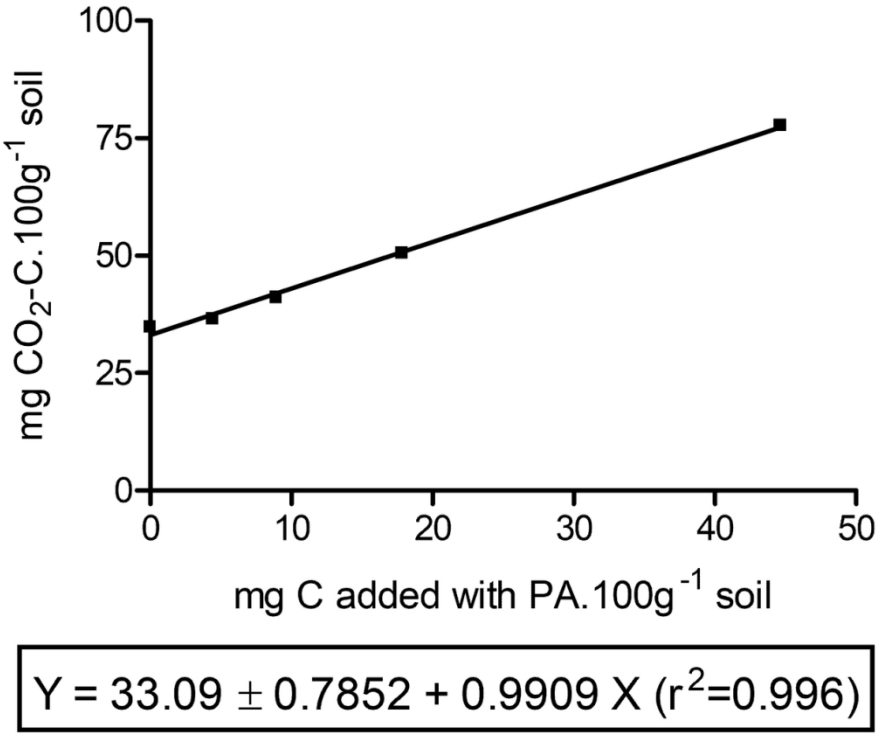

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