

Carbon sequestration potential in perennial bioenergy crops: the importance of organic matter inputs and its physical protection

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Abstract

To date, only few studies have compared the soil organic carbon (SOC) sequestration potential between perennial woody and herbaceous crops. The main objective of this study was to assess the effect of perennial woody (poplar, black locust, willow) and herbaceous (giant reed, miscanthus, switchgrass) crops on SOC stock and its stabilization level after 6 years from plantation on an arable field. Seven SOC fractions related to different soil stabilization mechanisms were isolated by a combination of physical and chemical fractionation methods: unprotected (cPOM and fPOM), physically protected (iPOM), physically and chemically protected (HC- μ s + c), chemically protected (HC-ds + c), and biochemically protected (NHC-ds + c and NHC- μ s + c). The continuous C input to the soil and the minimal soil disturbance increased SOC stocks in the top 10 cm of soil, but not in deeper soil layers (10–30; 30–60; and 60–100 cm). In the top soil layer, greater SOC accumulation rates were observed under woody species ($105 \text{ g m}^{-2} \text{ yr}^{-1}$) than under herbaceous ones ($71 \text{ g m}^{-2} \text{ yr}^{-1}$) presumably due to a higher C input from leaf-litter. The conversion from an arable maize monoculture to perennial bioenergy crops increased the organic C associated to the most labile organic matter (POM) fractions, which accounted for 38% of the total SOC stock across bioenergy crops, while no significant increments were observed in more recalcitrant (silt- and clay-sized) fractions, highlighting that the POM fractions were the most prone to land-use change. The iPOM fraction increased under all perennial bioenergy species compared to the arable field. In addition, the iPOM was higher under woody crops than under herbaceous ones because of the additional C inputs from leaf-litter that occurred in the former. Conversion from arable cropping systems to perennial bioenergy crops can effectively increase the SOC stock and enlarge the SOC fraction that is physically protected within soil microaggregates.

Keywords: biomass crops, C sequestration, intra-aggregates C, litter C-input, particulate organic matter, rhizomatous crops, root C-input, short rotation coppice, soil organic carbon

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Introduction

Biofuel production is a key component of renewable energy strategies in Europe and around the world (McLaughlin & Adams Kszos, 2005; Ragauskas *et al.*, 2006; EU, 2009), but the sustainability of biofuel cropping systems is currently under debate (Fargione *et al.*, 2008; Gelfand *et al.*, 2013; Guzman & Lal, 2014). To produce sustainable bioenergy, the Renewable Energy Directive 28/2009 (hereafter RED) promotes small-scale power or combustion plants that use a local supply of feedstock to release the pressure on global food markets while preventing further land-use change. Decentralized energy production would favor the exploitation of local

biomass resources, increasing local energy security, and reducing energy transmissions losses. In addition, the RED promotes the production of biofuels from biomass obtained on marginal lands. In this regard, perennial bioenergy crops can be successfully cultivated in marginal areas leading to several environmental benefits (Guzman & Lal, 2014). Where soil fertility is low and water availability is limited, perennial rather than annuals bioenergy crops are preferred, because of their higher yield potential and lower input requirements in terms of fertilizers and pesticides (Tilman *et al.*, 2006; Beringer *et al.*, 2011). This could relieve the pressure on fertile lands where food is produced while restoring degraded lands if soil organic carbon (SOC) sequestration is promoted by the implementation of perennial bioenergy crops. Both herbaceous and woody perennial bioenergy crops can provide several environmental

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benefits such as SOC sequestration due to minimal soil disturbance by tillage operations and the continuous plant C input into the soil. However, to date there is no agreement on the role of bioenergy crops from a climate mitigation perspective. While some authors suggest that the change in SOC should be considered when quantifying the environmental impacts of bioenergy crops (Dondini *et al.*, 2009), some others argue that SOC sequestration potential of a perennial crop has a modest impact on greenhouse gas balance compared to the use of its biomass to replace fossil fuels (Bransby *et al.*, 1998). It is also important to consider that most of the SOC sequestered over the bioenergy crop lifetime could be lost when these bioenergy crops are reconverted to arable land. The comparison of perennial biomass crops under the same environmental (e.g., soil, climate) and management conditions can be helpful for clarify their SOC sequestration potential. In fact, limited information is available to compare the effect of different perennial bioenergy crops on SOC dynamics and only two studies have compared the SOC storage capacity of herbaceous and woody crops under the same soil properties and microclimatic conditions (Ceotto & Di Candilo, 2011; Bonin & Lal, 2014). Woody bioenergy crops are supposed to have a high SOC sequestration potential compared to herbaceous bioenergy crops due to the combined role of roots and leaf-litter C inputs. In herbaceous crops, where whole aboveground biomass is removed during harvesting, the main C inputs to the soil derive from the root system, while in woody crops both roots and leaf-litter inputs are important contributors to SOC accumulation on top soil (Tolber *et al.*, 2002; Hangs *et al.*, 2014). In addition, aboveground plant litter, more easily decomposable than root material, has positive effects in stimulating the soil fauna (micro, meso, and macro), which plays a major role in the translocation and/or decomposition of fresh detritus, and therefore it accelerates C sequestration mechanisms in the first soil cm depth (Conant *et al.*, 2001; Fonte *et al.*, 2012).

The SOC sequestration effectiveness also depends on C stabilization mechanisms, which ultimately affect the SOC turnover rates and the residence time in soil. The inherent biochemical recalcitrance of organic matter compounds (plant or soil fauna derived) has been considered one of the main properties leading to SOC stabilization in the soil. Recently, it has been found that the soil residence time for recalcitrant molecules is comparable to that of molecules that are more easily degraded by the soil microbial community (Marschner *et al.*, 2008). The importance of biochemical recalcitrance has been therefore reconsidered, and it was concluded that other factors rather than recalcitrance are involved in SOC stabilization. Schmidt *et al.* (2011) proposed that

the persistence of OC in the soil is mainly governed by the ecosystem properties (i.e., chemical composition of the parental material, microenvironmental conditions, management practices, etc.) rather than by the molecular composition of organic matter. For example, OC interacts with the parental material (Cotrufo *et al.*, 2013): The surface charge of the organic compounds binds to the silt and clay particles and to the soil metal oxides leading to chemical protection of the SOC (Lutzow *et al.*, 2006) and to the formation of a soil aggregate structure which ensures physical protection (Six *et al.*, 2004). The chemical and physical protection of SOC, provided by the mineral soil matrix, have been highlighted as the most important SOC stabilization mechanisms (Allison & Jastrow, 2006; Marschner *et al.*, 2008). Oades (1984) identified soil microaggregates (30–250 μm) as the main soil structures involved in the physical protection of SOC due to the spatial inaccessibility of OC to microbes and exo-enzymes (Allison & Jastrow, 2006; Dungait *et al.*, 2012). In addition, the microbial degradation of OC contained within microaggregates is inhibited by limiting oxygen concentration as the microorganisms can only survive on the external surface of the microaggregates (Sexstone *et al.*, 1982). Gunina & Kuzyakov (2014) using C isotopic analysis revealed that the main OC flow in soil occurred from macroaggregates (250–2000 μm) toward microaggregates, confirming the theory of SOC incorporation within the microaggregates (Oades, 1984; Six *et al.*, 2000, 2004). As a consequence, the physical protection of SOC within the microaggregates is considered an important SOC sequestration mechanism.

Six *et al.* (2002) proposed a methodology where a special device (microaggregate isolator) is used to isolate the soil microaggregates. This method, adopted in this study, combines physical and chemical soil fractionation to isolate seven SOC fractions related to specific stabilization mechanisms and turnover rates. Three particulate organic matter (POM) fractions are associated with the soil macro- and micro-aggregate dynamics (2000–250 μm and 250–53 μm respectively). Coarse POM (cPOM) and fine POM (fPOM) are unprotected SOC fractions, that are not occluded within microaggregates, and they represent an active C pool. The turnover of unprotected C is fast and it depends on environmental factors affecting microbial activity (humidity, temperature, pH, etc.). The third POM fraction, indicated as intra-microaggregate POM (iPOM), is physically protected within the soil microaggregates and is part of the slow C pool. The proportion of OC which is associated with silt and clay particles of the microaggregates is protected biochemically by inherent recalcitrance (passive C pool), while the rest is stabilized through the adsorption and chemical binding onto mineral soil

surfaces. This latter is subjected to both chemical and physical protection due to spatial inaccessibility from microorganisms and adsorption on the soil mineral surface (slow C pool). Likewise, part of the C released by the disruption of macroaggregates is biochemically protected (passive C pool), while the fraction lost after acid hydrolysis, which is bound to mineral soil particles (silt and clay and metal oxides), is chemically protected and represent part of the slow C pool. Both the unprotected and protected POM in microaggregates are the most sensitive C fractions to management practices and land-use change (Six *et al.*, 2002). In particular, variation in the SOC fraction which is physically protected within microaggregates has been proposed as an early indicator for SOC stock changes (De Gryze *et al.*, 2004).

In this study, the SOC stock and the abovementioned seven SOC fractions under six bioenergy crops were compared with those of an adjacent arable field to identify the bioenergy crop with the greatest SOC sequestration potential. Main objectives were: (i) to assess the SOC stock variation under six perennial bioenergy crops at the sixth year from plantation (three woody crops *Populus spp.* (poplar), *Robinia pseudoacacia* (black locust), and *Salix spp.* (willow) – and three herbaceous crops – *Arundo donax* L. (giant reed), *Miscanthus x giganteus* (miscanthus), and *Panicum virgatum* (switchgrass) in comparison with an arable field cropped with continuous maize under conventional tillage for more than 30 years; and (ii) to determine the relative contribution of the seven SOC fractions to SOC stabilization under the perennial bioenergy crops.

Materials and methods

Study site and experimental design

The experimental area is located in Gariga di Podenzano (PC), in the Po Valley, Italy (44°58'N 9°41'E; 118 m a.s.l.). The climate is continental Mediterranean, with a mean annual precipitation of 890 mm and a mean temperature of 12.2 °C. The soil in the study site has a silt loamy texture (sand 12%, silt 64%, and clay 24%) and is classified as *Chromic Luvisol*, according to soil taxonomy (FAO, 2006). Overall, the soil has a low percentage of carbonates, a subacid or neutral pH (6.9) and the CEC is 14.9 meq/100 g.

To compare the SOC sequestration potential of three herbaceous (*Arundo donax* L., *Panicum virgatum*, and *Miscanthus x giganteus*) and three woody bioenergy crops (*Populus spp.*, *Salix spp.*, and *Robinia pseudoacacia*) a field trial was established in winter 2006 on a field that had hosted a maize monoculture for 30 years. The experimental layout was a randomized complete block design with three blocks and a single plot size of 450 m² (15 m × 30 m). To compare the SOC storage capacity of the perennial bioenergy crops with that of the continuous maize cropping system (hereafter arable field), three additional plots,

one per block, were established in the same field, where the continuous maize monoculture had continued. No irrigation or fertilization was applied under any of the bioenergy crops, and biomass harvesting was carried out at the end of each winter period for the herbaceous species, while the woody species were harvested on the second and fourth year after the onset of the experiment.

Soil and roots sampling and processing

Soil samples were collected in May 2012, 6 years after the bioenergy crops were planted. Two intact soil cores were collected in each plot per crop treatment at a depth of 0–100 cm pressing with the hydraulic arm of a digger a self-constructed 'Shelby' tube sampler of known volume (7 cm in diameter) in the inter-row of each crop (Amaducci *et al.*, 2008). The two 1 m depth soil cores were divided into four sections (0–10; 10–30; 30–60; and 60–100 cm). In addition, to represent the variability within the plot, eight additional soil cores per plot were collected with a smaller soil auger from two top soil layers (0–10 and 10–30 cm). The two 1 m depth soil cores from each plot were combined in one composite sample according to the respectively depth. A soil subsample was oven dried at 105 °C until constant weight for residual moisture measurement. Bulk density (corrected for stone content) was calculated dividing dry soil weight by the volume of the soil sample (Table 1). The eight soil cores (the one divided in 0–10 and 10–30 cm soil depth) and the two soil cores (divided in 0–10; 10–30; 30–60; and 60–100 cm soil depth) were pooled to have one representative soil sample for each soil layer and plot. Each soil sample was air-dried, and sieved at 2 mm for further analysis.

At the end of September 2012, the self-constructed 'Shelby' tube sampler was used to collect soil samples for root biomass determination at 0–10 cm depth. Five samples per plot for the woody species and three samples for the herbaceous species were extracted and combined in one composite sample per plot. Before root separation and analysis, soil samples were stored at –20 °C. To separate roots from soil, samples were immersed in oxalic acid (2%) for 2 h, and then washed in a hydraulic sieving-centrifuge device (Monti & Zatta, 2009). Once cleaned, roots were hand recovered from the water using a 2 mm mesh sieve, oven dried at 105 °C until constant weight, and weighted. The dry root weight was divided by the whole soil sampled volume and reported as g of roots per m² of soil.

Soil organic carbon (SOC) stock

After sieving the soil at 2 mm, one subsample (20 g) for each plot per treatment, and depth was ground until all material was passed through a 0.5 mm sieve. About 1 g of soil per each subsample was then weighed and analyzed by Dumas combustion method with an elemental analyzer varioMax CN for carbon (C) and nitrogen (N) determination (VarioMax CNS, Elementar, Germany). Soil carbonates removal was not necessary due to the low carbonate content in the soil. SOC stock (g m⁻²) in each soil layer (0–10; 10–30; 30–60; and 60–100 cm) was computed as the product of total soil organic carbon (TSOC) concentration, bulk density and the depth of each soil

Table 1 Soil bulk density (g cm^{-3}) at different soil depths and for each bioenergy crop. Values indicate mean \pm SD error ($n = 3$). Different letters within the same column indicate significant differences ($P < 0.05$) among crop types, according to Tukey's test

Depth (cm)	Woody			Herbaceous			Arable field	F	P
	Black locust	Poplar	Willow	Miscanthus	Switchgrass	Giant reed			
0–10	1.55 \pm 0.01abc	1.49 \pm 0.04bc	1.49 \pm 0.06bc	1.62 \pm 0.04ab	1.68 \pm 0.01a	1.70 \pm 0.06a	1.42 \pm 0.03c	8.16	0.001
10–30	1.67 \pm 0.01a	1.69 \pm 0.03a	1.62 \pm 0.02a	1.65 \pm 0.03a	1.74 \pm 0.02a	1.66 \pm 0.03a	1.42 \pm 0.03 b	17.57	< 0.001
30–60	1.67 \pm 0.01abc	1.64 \pm 0.04bc	1.71 \pm 0.00ab	1.70 \pm 0.03ab	1.74 \pm 0.01a	1.69 \pm 0.03ab	1.58 \pm 0.01c	7.43	0.001
60–100	1.55 \pm 0.02bc	1.65 \pm 0.04ab	1.68 \pm 0.01a	1.67 \pm 0.03a	1.67 \pm 0.01a	1.67 \pm 0.01a	1.53 \pm 0.03c	8.65	< 0.001

layer. Annual changes in SOC stock ($\text{g C m}^{-2} \text{yr}^{-1}$) were estimated subtracting the SOC stock value for each perennial bioenergy crop from those determined in the arable field (as the reference site) and dividing the obtained difference by 6 (the number of years from crop establishment).

SOC fractions isolation

Soil organic matter fractionation was only performed on samples from 0–10 and 10–30 cm soil layers. In a first step, coarse nonprotected particulate organic matter (cPOM, 2000–250 μm), microaggregates (250–53 μm), and easily dispersed silt + clay C (<53 μm) were isolated from 2 mm air-dried sieved soil using an isolator device developed by Six *et al.* (2000) (Fig. 1). Approximately, 50 g of 2 mm air-dried sieved soil were slaked in deionized water for 30 min. Subsequently, each soil sample was transferred, with 50 glass beads, on the top of a 250- μm sieve into the microaggregate isolator. Shaking at 120 rpm and a constant flux of deionized water was applied to disrupt all the macroaggregates (250–2000 μm) without breaking up the microaggregates. All the material having a diameter lower than 250 μm was flushed through the sieve, the material retained by the mesh (2000–250 μm) consists of coarse sand and POM (cPOM), while the flushed material consists of microaggregates and fine POM (fPOM) (250–53 μm), which are retained on the top of a 53- μm sieve, and the easily dispersed silt and clay fraction (< 53 μm), that passes through the sieve (Fig. 1). The isolated cPOM was separated from the sand by floatation in deionized water. The microaggregates collected on the top of a 53- μm sieve were manually wet sieved applying 50 strokes in 2 min to separate the easily dispersed silt and clay fraction from the water stable aggregates.

In a second step, fine nonprotected (fPOM) and protected (iPOM), which were collected together with the microaggregates on the sieve, were separated by density flotation and dispersion (Fig. 1). A subsample (5 g) of oven-dried material was placed in a 50-ml centrifuge tube with approximately 45 ml of sodium polytungstate ($\rho = 1.85 \text{ g cm}^{-3}$) and hand shook with 10 strokes. After 10 min of vacuum (138 kPa) and 20 min of equilibration the sample was centrifuged at 20 $^{\circ}\text{C}$ for 1 h, according to Six *et al.* (1998). Floating material (fPOM) was filtered on a preweighed 20- μm nylon filter. The remaining material, the heavy fraction (HF: iPOM + sand + microaggregates), was then dispersed in 100 ml of 5 g l^{-1} sodium hexametaphosphate. After shaking for 18 h on a reciprocal shaker, the dispersed heavy fraction was passed through a 53- μm sieve. The

intra-aggregate (iPOM) material and sand retained on the sieve were collected, while the silt and clay fraction released by microaggregate dispersion, passed through the 53- μm sieve and transferred to a preweighed aluminum tray. The three fractions obtained in this step were placed in preweighed aluminum trays, oven dried at 60 $^{\circ}\text{C}$ until constant weight, weighed, and ground for further C and N analysis. The sand content was determined after complete organic matter oxidation with H_2O_2 (30 m/m in water), according to Gee & Or (1986). For more details on this step, see Stewart *et al.* (2009) and Six *et al.* (1998).

Although the acid hydrolysis method has been recently claimed to overestimate the biochemically protected SOC fraction (Greenfield *et al.*, 2013), it has been extensively used to distinguish the chemically and physico-chemically protected C vs. the biochemically protected C (nonhydrolyzable). According to Plante *et al.* (2006) silt- and clay-sized fractions, obtained from both the density flotation (microaggregate-derived silt- and clay-sized fractions, $\mu\text{s} + \text{c} < 53 \mu\text{m}$) and the initial physical fractionation (easily dispersed silt- and clay-sized fractions, $\text{ds} + \text{c}$, <53 μm), were subjected to acid hydrolysis (Fig. 1). About 0.5 g of mineral soil fraction was immersed in 25 ml of 6 M HCl and refluxed at 95 $^{\circ}\text{C}$ for 16 h. After refluxing, the sample was filtered on preweighed glass fiber filters, rinsed at least three times with deionized water, oven dried at 60 $^{\circ}\text{C}$ and weighed. These fractions (< 53 μm) represent the nonhydrolyzable C fractions: nonhydrolyzable easily dispersed (NH-ds + c) and microaggregate derived (NH- $\mu\text{s} + \text{c}$) silt and clay-sized fraction. The hydrolyzable fractions (<53 μm) were then estimated by difference between the total organic C content of the fractions and the C contents of the nonhydrolyzable fractions: hydrolyzable easily dispersed (H-ds + c) and microaggregate derived (H- $\mu\text{s} + \text{c}$) silt and clay-sized fraction.

In summary, the combination of different soil fractionation methods (physical, density, and hydrolysis analysis) was used to distinguish seven SOC fractions: cPOM, fPOM, iPOM, H-ds + c, H- $\mu\text{s} + \text{c}$, NH-ds + c, and NH- $\mu\text{s} + \text{c}$ (Fig. 1). The organic carbon and nitrogen content were analyzed in the bulk soil and separately for each soil fraction using an elemental analyzer (Vario Max CNS, Elementar, Germany).

Statistical analysis

To evaluate the effects of bioenergy crops at each soil depth layer, data were analyzed using a one-way ANOVA analysis for a randomized complete block design for each soil depth layer

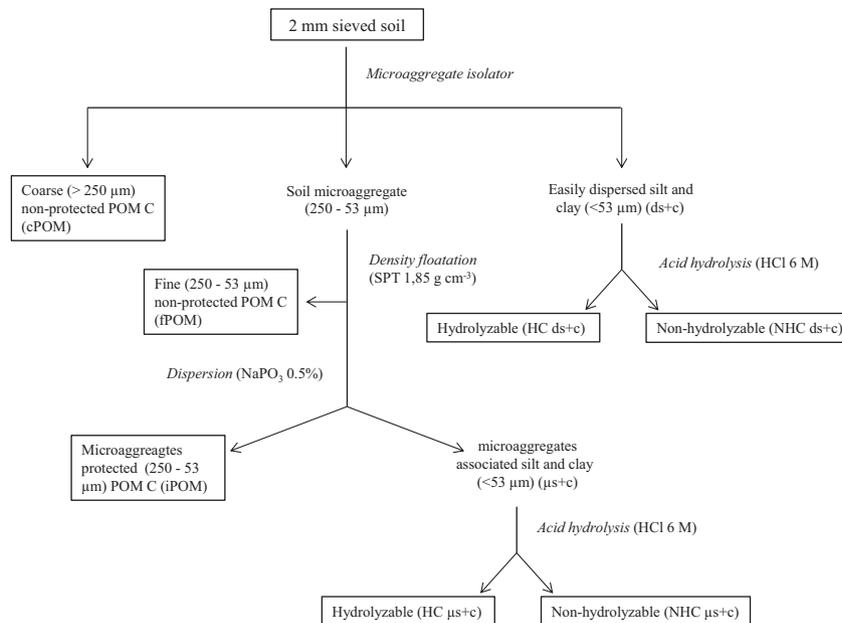


Fig. 1 Representation of the soil fractionation scheme adopted to identify seven soil C fractions related to four stabilization mechanisms: unprotected (cPOM and fPOM), physically protected (iPOM), physically and chemically protected (HC $\mu\text{s} + \text{c}$), chemically protected (HC $\text{ds} + \text{c}$), physically and biochemically protected (NHC $\mu\text{s} + \text{c}$), and biochemically protected (NHC $\text{ds} + \text{c}$), according to Six *et al.* (2002) and Stewart *et al.* (2009).

separately, in which crop type and block were considered as fixed factors. Significant differences between crop types were identified at the 0.05 probability level of significance using Tukey's test carried out using the agricolae package of R software. Prior to the analyses, the data were examined for normality by the Kolmogorov–Smirnov test and for homogeneity of variances by the Levene's test.

Results

Changes in bulk SOC content and stock

After 6 years from perennial crops plantation, total soil organic carbon concentration (TSOC, in g kg^{-1} of soil) under bioenergy crops, compared to that in the arable field, increased significantly only in the top soil layer (0–10 cm) (Table 2). The larger increments in TSOC content were observed under the woody crops and under miscanthus. For these crops TSOC values differed significantly from that measured in the arable field (Table 2). On the contrary, TSOC under switchgrass and giant reed did not differ significantly from that in the arable field (Table 2).

Soil organic carbon stocks were significantly higher for all bioenergy crops, with the exception of giant reed, compared to that in the arable field in the top layer (0–10 cm), while a marginal significant difference ($P = 0.093$) was observed between bioenergy crops and arable field in the subsurface soil layer (10–30 cm). No

differences were observed in SOC stocks between the bioenergy crops and the arable field below 30 cm of depth, in both the single layers (30–60 cm and 60–100 cm), or considering the whole soil profile (0–100 cm) (Table 2).

In the top soil layer (0–10 cm), the highest annual increment of SOC stock was observed under black locust, with an annual increase of $120 \text{ g C m}^{-2} \text{ yr}^{-1}$ compared to the arable field. On the contrary, the lowest SOC stock accumulation rates occurred under miscanthus and switchgrass ($80 \text{ g C m}^{-2} \text{ yr}^{-1}$ for both species). Interestingly, the increase in SOC stock was larger for woody than for herbaceous species. Mean annual increase in SOC stock under woody crops (black locust, willow, and poplar) was higher ($105 \text{ g C m}^{-2} \text{ yr}^{-1}$) than that observed under herbaceous crops (miscanthus, switchgrass, and giant reed; $71 \text{ g C m}^{-2} \text{ yr}^{-1}$) ($F = 9.39$; $P = 0.008$).

Changes in SOC fractions

In the top soil layer, the observed C in the POM fractions was higher in the woody compared to the herbaceous crops (729 vs. 633 g C m^{-2} , respectively; $F = 4.35$, $P = 0.05$; Fig. 2) while in the subsurface soil layer no significant differences were detected ($F = 0.44$; $P = 0.51$). Regarding the top layer, the unprotected POM fraction (cPOM + fPOM) was considerable higher under bioenergy crops than under the arable field, with

Table 2 Soil organic carbon stock (SOC, g m⁻²) under the adjacent arable field and each of the bioenergy crops at different soil depths and for the entire soil profile after 6 years of establishment. Results of total soil organic carbon (TSOC, g kg⁻¹ of soil) at different soil depths are also reported. Values represent mean ± standard error (*n* = 3). Different letters within the same row indicate significant differences (*P* < 0.05) among crops, according to Tukey's test

Depth (cm)	Woody			Herbaceous			Arable field	<i>F</i>	<i>P</i>
	Black locust	Willow	Poplar	Miscanthuss	Switchgrass	Giant reed			
SOC stock									
0–10	1900 ± 46a	1769 ± 120ab	1749 ± 117ab	1654 ± 8ab	1659 ± 19ab	1504 ± 33bc	1177 ± 67c	10.06	< 0.001
10–30	2618 ± 90	2714 ± 184	2740 ± 145	2648 ± 71	2806 ± 91	2449 ± 25	2354 ± 135	2.39	0.093
30–60	2739 ± 118	2607 ± 157	2560 ± 246	2438 ± 68s	2994 ± 272	2507 ± 192	2271 ± 84	1.32	0.322
60–100	1770 ± 127	1918 ± 97	2180 ± 240	2094 ± 146s	2421 ± 178	2484 ± 835	1943 ± 47	0.63	0.706
0–100	9026 ± 110	9008 ± 330	9230 ± 276	8833 ± 177	9880 ± 343	8944 ± 977	8114 ± 496	1.07	0.430
TSOC									
0–10	12.2 ± 0.3a	11.8 ± 0.7ab	11.7 ± 0.5abc	10.2 ± 0.2bcd	9.9 ± 0.2cde	8.9 ± 0.3de	8.3 ± 0.3e	18.07	< 0.001
10–30	7.9 ± 0.3	8.4 ± 0.5	8.1 ± 0.5s	8.0 ± 0.3	8.1 ± 0.2	7.4 ± 0.2	8.3 ± 0.3	0.89	0.53
30–60	5.5 ± 0.3	5.1 ± 0.3	5.2 ± 0.5	4.8 ± 0.1	5.7 ± 0.5	5.0 ± 0.3	4.8 ± 0.2	1.10	0.41
60–100	2.9 ± 0.2	2.9 ± 0.1	3.3 ± 0.3	3.1 ± 0.2	3.6 ± 0.3	3.7 ± 1.2	3.2 ± 0.0	0.44	0.84

the exception of giant reed (Table 3). The cPOM under bioenergy crops was significantly higher compared to that found in the arable field, with the exception of poplar (Table 3). Among bioenergy crops, switchgrass had a higher C content in the cPOM fraction compared to poplar, whereas the other crops had intermediate values (*F* = 3.86; *P* = 0.032) (Table 3). Regarding fPOM, no significant differences were observed in the top soil layer among bioenergy crops and the arable field, but when pooled by crop type, herbaceous crops showed a lower C content in the fPOM compared to the woody crops (*F* = 5.87; *P* = 0.029) (Fig. 2).

In the subsurface soil layer (10–30 cm), only switchgrass showed a higher C content in the unprotected POM fraction compared to the arable field (Table 3). Willow showed a higher C content in the fPOM fraction compared to the arable field, while no significant differences between bioenergy crop types and arable field for the cPOM were observed (Table 3).

In the top soil layer, the C content of the iPOM was higher for all the bioenergy crops, with the exception of giant reed, compared to that under the arable field (Table 3). Interestingly, the C content in the iPOM fraction under woody bioenergy species was significantly

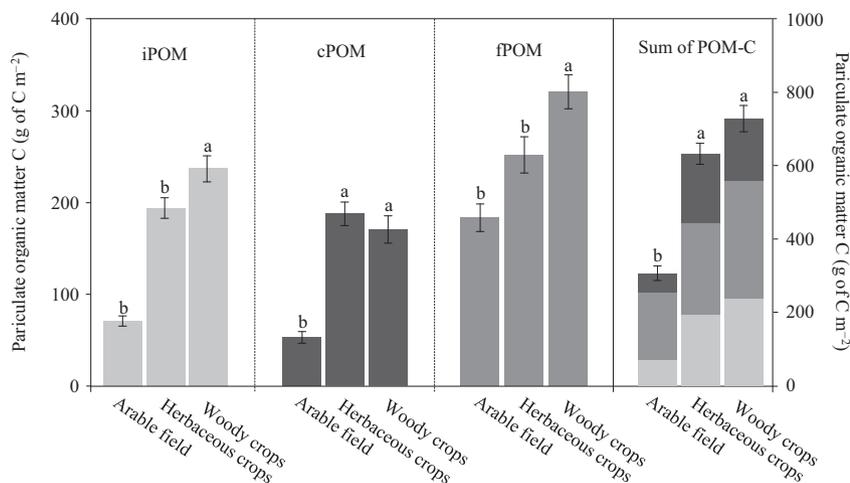


Fig. 2 Carbon content in the different particulate organic matter fractions (iPOM: intramicroaggregate POM; cPOM: coarse POM; and fPOM: fine POM) and the sum of all of them (POM-C) observed under each bioenergy crop in comparison to the arable field (reference site) after 6 years from plantation. Different letters indicate significant differences (*P* < 0.05) between crop types, according to Tukey's test.

Table 3 Carbon content (g C m^{-2}) in each fraction in the 0–10 and 10–30 cm soil depths (mean \pm standard error, $n = 3$). The means separation with Tukey test at α value of 0.05 was performed when ANOVA for the comparison among species was significant. Mean sharing common letters are not statistically different

Pools	Soil protection status	SOC fractions	Woody species				Herbaceous species				F	P
			Black locust	Willow	Poplar	Miscanthus	Switchgrass	Giant reed	Arable field			
0 to 10 cm soil depth												
Active	Unprotected	cPOM + fPOM	510 \pm 43a	523 \pm 48a	440 \pm 13a	440 \pm 22a	483 \pm 41a	396 \pm 55ab	236 \pm 16b	6.8	0.002	
		cPOM	202 \pm 9ab	179 \pm 18ab	131 \pm 31bc	192 \pm 9ab	225 \pm 16a	145 \pm 3ab	53 \pm 7c	12.1	< 0.001	
Slow	Physically	fPOM	309 \pm 49	344 \pm 30	309 \pm 18	247 \pm 28	258 \pm 28	251 \pm 55	183 \pm 15	2.0	0.139	
		iPOM	247 \pm 41a	242 \pm 19a	224 \pm 14a	215 \pm 31a	195 \pm 6a	170 \pm 5ab	71 \pm 6b	7.1	0.002	
	Physically and chemically	HC $\mu\text{s}^+\text{c}$	113 \pm 24a	132 \pm 1a	160 \pm 24a	134 \pm 19a	76 \pm 29ab	118 \pm 22ab	40 \pm 8b	4.5	0.013	
		Chemically	HC ds+c	277 \pm 10	248 \pm 54	313 \pm 47	474 \pm 67	430 \pm 20	321 \pm 55	279 \pm 61	2.5	0.080
Passive	Physically and biochemically	NHC $\mu\text{s}^+\text{c}$	140 \pm 19	102 \pm 5	108 \pm 12	130 \pm 17	157 \pm 43	109 \pm 17	109 \pm 13	0.8	0.563	
	Biochemically	NHC ds+c	528 \pm 97	569 \pm 52	467 \pm 79	385 \pm 37	407 \pm 53	476 \pm 56	418 \pm 34	1.0	0.491	
10 to 30 cm soil depth												
Active	Unprotected	cPOM + fPOM	503 \pm 75b	842 \pm 89a	678 \pm 82ab	577 \pm 130ab	778 \pm 82ab	486 \pm 87b	473 \pm 31b	4.73	0.01	
		cPOM	143 \pm 18	153 \pm 43	90 \pm 10	117 \pm 11	183 \pm 21	123 \pm 22	106 \pm 12	1.87	0.168	
Slow	Physically	fPOM	360 \pm 62b	689 \pm 121a	589 \pm 79ab	460 \pm 122ab	595 \pm 74ab	364 \pm 94b	367 \pm 30b	4.37	0.014	
		iPOM	193 \pm 14ab	223 \pm 16a	191 \pm 14ab	204 \pm 27ab	213 \pm 5a	197 \pm 16ab	142 \pm 11b	3.96	0.020	
	Physically and chemically	HC $\mu\text{s}^+\text{c}$	656 \pm 476	171 \pm 43	619 \pm 219	159 \pm 9	642 \pm 391	382 \pm 186	80 \pm 15	1.08	0.426	
		Chemically	HC ds+c	709 \pm 137	430 \pm 77	657 \pm 7	743 \pm 104	542 \pm 131	799 \pm 58	557 \pm 122	1.80	0.182
Passive	Physically and biochemically	NHC $\mu\text{s}^+\text{c}$	230 \pm 21	265 \pm 43	366 \pm 79	302 \pm 85	239 \pm 45	194 \pm 47	219 \pm 27	1.55	0.241	
	Biochemically	NHC ds+c	811 \pm 163	1132 \pm 124	720 \pm 193	922 \pm 98	1163 \pm 122	771 \pm 82	835 \pm 68	2.79	0.062	

higher than that under herbaceous bioenergy crops ($F = 5.52$; $P = 0.034$) (Fig. 2). In the subsurface soil layer, the effect of bioenergy crops on the C content of the iPOM fraction was not as evident as in the top soil layer (Table 3). Willow and switchgrass were the only bioenergy species showing a higher C content in the iPOM fraction compared to the arable field.

Overall, there were no significant differences between bioenergy crops in the physico-biochemically or biochemically protected C fractions (NHC- μ s + c and NHC ds + c, respectively), or chemically protected C fractions (HC ds + c), while the physico-chemically protected C fraction (HC- μ s + c) was the only C fraction in which the effect of cropping type was significant in the top soil layer, with poplar, willow, and miscanthus showing higher values compared to the arable field (Table 3).

Root biomass content

Among the bioenergy crops studied, root biomass in the top soil layer (0–10 cm) ranged from 82 to 235 g C m⁻², with the highest value reached by switchgrass, followed by willow, miscanthus, poplar, giant reed, and black locust (Fig. 3). Overall, although not significantly different, mean root biomass under herbaceous crops (switchgrass, miscanthus, and giant reed) was higher (185 g m⁻²) compared to that under woody crops (black locust, poplar, and willow; 150 g m⁻²).

Discussion

Since no statistically significant ($P < 0.05$) differences in SOC stocks or fractions were observed between the bioenergy crops and the arable field below 10 cm of depth, the discussion is focused on the top soil layer.

Changes in SOC stocks

The continuous plant C input to the soil and the minimal soil disturbance under perennial bioenergy crops, after 6 years from establishment, increased SOC stocks mainly in the top soil layer (0–10 cm). An increment in SOC stock with respect to the arable soil was observed for black locust, willow, poplar, miscanthus and switchgrass, but not for giant reed. Interestingly, higher annual SOC sequestration rates were observed under woody species (120, 99, and 95 g C m⁻² yr⁻¹ for black locust, willow and poplar, respectively) than under herbaceous species (80 C m⁻² yr⁻¹ for switchgrass and miscanthus). Similar annual SOC stock increments (from 58 g m⁻² yr⁻¹ up to 97.3 g C m⁻² yr⁻¹) were reported in a previous study carried out in the north of Italy, in which the same bioenergy species were confronted to an adjacent ploughed soil after 7 years from plantation

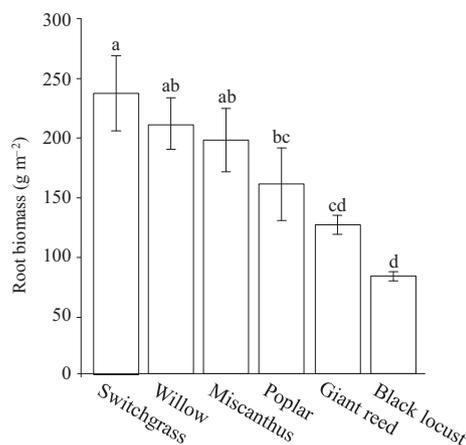


Fig. 3 Root biomass (RB) in the first 10 cm of soil under each bioenergy crop after 6 years from plantation. Different letters indicate significant differences ($P < 0.05$) between crop types, according to Tukey's test.

(Ceotto & Di Candilo, 2011). Although the annual increments of SOC stock under willow and poplar observed in this study were in agreement with those reported by Ceotto & Di Candilo (2011), the annual SOC increment under black locust was double in this study site (120 g C m⁻² yr⁻¹) compared to that previously observed (58 g C m⁻² yr⁻¹). The results herewith reported are in agreement with those obtained by Matos *et al.* (2012), in which an annual SOC stock increment of 150 g C m⁻² yr⁻¹ in the 0–10 cm layer was reported for a black locust plantation after 12 years of establishment on a former marginal land in Germany. The high soil C sequestration rate under black locust can be attributed to the high nitrogen content of its litter, as previously reported for other leguminous species (Johnson & Curtis, 2001; Resh *et al.*, 2002).

The increase in SOC under giant reed progressed at a relatively slow rate (54 g C m⁻² yr⁻¹) compared to what reported in other studies in Italy (Riffaldi *et al.*, 2010; Ceotto & Di Candilo, 2011). These contrasting results could be a consequence of the lack of nitrogen (N) fertilization in this study and the higher below-ground biomass production reported by other authors (Monti & Zatta, 2009). It is widely recognized that the increase in N fertilization rate has a positive effect on soil C sequestration (Guzman & Lal, 2014; Palm *et al.*, 2014). Stewart *et al.* (2014) found that the SOC stock increased with increasing N fertilization application in a nine-year experiment with switchgrass, reporting annual SOC stock rate of 23 g C m⁻² yr⁻¹, 31 g C m⁻² yr⁻¹, and 35 g C m⁻² yr⁻¹ for N application rates of 0, 60, and 120 kg N ha⁻¹, respectively. The annual SOC increment under switchgrass obtained in this study (80 g C m⁻² yr⁻¹) was considerably greater

than those reported by Stewart *et al.* (2014) considering that no N fertilizer was applied. This highlights the important role of specific-site conditions in annual SOC stock accumulation. In fact, this difference could be probably explained by the lower TSOC concentration of the arable field, considered as the reference value in this experiment (8 g kg^{-1} of soil) in comparison to the initial TSOC value (14 g kg^{-1} of soil) reported by Stewart *et al.* (2014). Although the potential of switchgrass for SOC sequestration was highlighted in several studies (Ma, 2000; Liebig *et al.*, 2008; Schmer *et al.*, 2011), the different cropping managements (in terms of fertilization and irrigation) and analytical approaches to estimate the SOC stock variations limit the possibility to compare data across studies. Regarding miscanthus, results found in this study are partially in agreement with those reported by Ceotto & Di Candilo (2011), in which an annual SOC stock increment of $59 \text{ g C m}^{-2} \text{ yr}^{-1}$ was estimated, and are within the range reported by other authors from north Europe (78 and $112 \text{ g C m}^{-2} \text{ yr}^{-1}$; Hansen *et al.*, 2004).

Miscanthus was the only herbaceous crop to reach TSOC concentration in the top soil layer similar to those found under woody crops (Table 2). This is probably a consequence of the partial shedding of miscanthus leaves during the winter months, that was observed during the field trial and that was reported in other studies (Clifton-Brown *et al.*, 2007; Meehan *et al.*, 2013). On the contrary, in switchgrass and giant reed most of the leaves are still attached to the stems at harvest (Bransby *et al.*, 1998), and therefore leaf-litter inputs from these crops can be assumed to be negligible. It has been widely reported that harvesting time and crop residue removal from the soil surface affects the SOC stocks (Jones & Donnelly, 2004; Stewart *et al.*, 2014). Clifton-Brown *et al.* (2007) demonstrated that the SOC stock under miscanthus was negatively affected by early harvesting (i.e., in autumn) as a consequence of the lower input from leaf fall, while delaying harvesting to the end of winter allowed for a higher accumulation of the aboveground-litter on the soil surface and an improved soil C sequestration potential. Herbaceous crops, that are always subjected to total aboveground biomass removal, have a limited potential to sequester C from leaf-litter. This could explain why in this experiment the average annual increment of SOC stock in the top soil layer under the herbaceous crops ($71 \text{ g C m}^{-2} \text{ yr}^{-1}$) was lower compared to that under the woody crops ($105 \text{ g C m}^{-2} \text{ yr}^{-1}$) ($F = 9.38$; $P = 0.008$), despite no differences were observed in mean root biomass values between the two type of bioenergy crops (Fig. 3). This finding highlights the important role of leaf-litter accumulation in increasing SOC stock under woody crops. The potential of soil C sequestration of

woody bioenergy crops due to leaf-litter accumulation on the surface of the soil has been highlighted by several authors (Rytter 2012; Vesterdal *et al.*, 2013; Hangs *et al.*, 2014), but few works have provided a direct comparison between herbaceous and woody crops in terms of SOC stock variations. Coleman *et al.* (2004) compared SOC stock variations under poplar and switchgrass in relation to those in a nearby arable field and did not find differences between these species. Also Ceotto & Di Candilo (2011) did not report significant differences between herbaceous and woody crops. The results presented in this study are in agreement with those reported by Bonin & Lal (2014), in which lower SOC stock increments were found in switchgrass compared to willow.

Soil C fractions and C sequestration potential of the bioenergy crops

The continuous plant input and the minimal soil disturbance under bioenergy perennial crops led to an increase in the C content of the particulate organic matter (POM) fraction after 6 years of the establishment (Fig. 2), confirming previous evidences that POM-C fraction is the most prone to land-use change (Six *et al.*, 2002; De Gryze *et al.*, 2004). Among bioenergy crops, the POM-C fraction was 38% of the SOC stock, while it only accounted for 26% of the SOC stock under the arable field. This result is in agreement with previous findings (Cambardella & Elliot, 1992; De Gryze *et al.*, 2004). The POM fraction is predominantly plant-derived C and its increase is driven by the increment in quantity and quality of plant C inputs to the soil (Gulde *et al.*, 2008; Cotrufo *et al.*, 2013) and management practices (i.e., tillage cessation) (Six *et al.*, 2000). Therefore, this C fraction can be considered as a useful proxy for plant-derived C input and land management. In particular, in our study the physically protected C (iPOM) was increased passing from arable field to herbaceous crops and woody crops, with the latter having higher iPOM values than the former (Fig. 2). This pattern is explained by the different management and C inputs which occurred within each crop type. Under the arable field, potential C inputs derived from crop residues and roots are affected by annual tillage operations, which decrease the turnover of the macroaggregates limiting the occlusion of POM within microaggregates (Six *et al.*, 2000). On the contrary, the cessation of tillage under bioenergy crops favored the preservation of the POM fractions (Fig. 2). The higher iPOM content observed under woody compared to the herbaceous crops seems to be a consequence of the largest C inputs observed under the former ones. Leaf-litter, being more easily decomposable than root material (Puget & Drinkwater,

2001; Kemp *et al.*, 2003; Austin *et al.*, 2009; D'Acunto *et al.*, 2014), might have accelerated the soil C cycling under woody crops, further enhancing the C content in the fPOM fraction and stimulating the formation of microaggregates, therefore improving the physical protection of C (Helfrich *et al.*, 2008; Cotrufo *et al.*, 2013; Gunina & Kuzyakov, 2014). On the contrary, under herbaceous crops, soil carbon storage was mainly affected by root production and turnover. The lower decomposability of roots compared to leaf-litter resulted in a slower soil carbon cycling and storage under herbaceous crops.

In conclusion, the continuous organic C input to the soil and the minimal soil disturbance under perennial bioenergy crops increased SOC stocks at the top soil layer (0–10 cm) after 6 years from establishment. The greater SOC accumulation rates observed under woody crops ($105 \text{ g m}^{-2} \text{ yr}^{-1}$) than under herbaceous crops ($71 \text{ g m}^{-2} \text{ yr}^{-1}$) were mainly driven by leaf-litter accumulation on the surface of the soil. As a consequence of the minimal soil disturbance, the intramicroaggregate particulate organic matter (iPOM) fraction was increased under the bioenergy crops, with exception of giant reed, compared to conventional arable management. The woody crops reached the higher C increment in the iPOM fraction due to the combined leaf-litter and root C inputs, showing the fastest C sequestration potential.

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