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## RESEARCH ARTICLE

# Early impacts of marginal land-use transition to *Miscanthus* on soil quality and soil carbon storage across Europe

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## Abstract

*Miscanthus*, a C<sub>4</sub> perennial rhizomatous grass, is a low-input energy crop suitable for marginal land, which cultivation can improve soil quality and promote soil organic carbon (SOC) sequestration. In this study, four promising *Miscanthus* hybrids were chosen to evaluate their short-term potential, in six European marginal sites, to sequester SOC and improve physical, chemical, and biological soil quality in topsoil. Overall, no differences among *Miscanthus* hybrids were detected in terms of impacts on soil quality and SOC sequestration. SOC sequestration rate after 4 years was of +0.4 Mg C ha<sup>-1</sup> year<sup>-1</sup>, but land-use transition from former cropland or grassland showed contrasting SOC sequestration trajectories. In unfertilized marginal lands, cultivation of high-yielding *Miscanthus* genotypes caused a depletion of K (−216 kg ha<sup>-1</sup> year<sup>-1</sup>), followed by Ca (−56 kg ha<sup>-1</sup> year<sup>-1</sup>), Mg (−102 kg ha<sup>-1</sup> year<sup>-1</sup>) and to a lesser extent of N. On the contrary, the biological turnover of organic matter increased the available P content (+164 kg P<sub>2</sub>O<sub>5</sub>

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ha<sup>-1</sup> year<sup>-1</sup>). SOC content was identified as the main driver of changes in biological soil quality. High input of labile plant C stimulated an increment of microbial biomass and enzymatic activity. Here, a novel approach was applied to estimate C input to soil from different *Miscanthus* organs. Despite the high estimated plant C input to soil (0.98 Mg C ha<sup>-1</sup> year<sup>-1</sup>), with significant differences among sites and *Miscanthus* hybrids, it was not identified as a driver of SOC sequestration. On the contrary, initial SOC and nutrients (N, P) content, as well as their elemental stoichiometric ratios with C, were the key factors controlling SOC dynamics. Introducing *Miscanthus* on marginal lands impacts positively soil biological quality over the short term, but targeted fertilization plans are needed to secure crop yield over the long term as well as the C sink capacity of this perennial cropping system.

#### KEYWORDS

C input, marginal lands, *Miscanthus* hybrids, soil organic carbon sequestration, soil quality

## 1 | INTRODUCTION

*Miscanthus*, a perennial herbaceous crop with C<sub>4</sub> photosynthesis, has received considerable attention as a multi-purpose crop that can provide large amounts of biomass for the growing bioeconomy and simultaneously offering environmental benefits. Novel *Miscanthus* hybrids are being developed in Europe as promising biomass crops for the bioenergy and bio-based industry, rural diversification, to face climate changes and global energy security (Clifton-Brown et al., 2017). Its nearly year-round soil cover reduces soil erosion and run-off, while the deep and dense rooting system ensures a more efficient use of nutrients and water, making this crop a low-input species that minimizes nutrient losses (Anderson et al., 2011; Ferrarini et al., 2017). Moreover, its strong tolerance to environmental stresses makes *Miscanthus* a suitable crop for marginal land and degraded soils affected by heavy metal contamination, erosion, flooding, drought, salinity, and heavy clay soils (Brami et al., 2020; Islam et al., 2023; Pidlisnyuk et al., 2014; Xu et al., 2021). On such lands, *Miscanthus* enhances soil quality by improving soil structure (Schrama et al., 2016; Winkler et al., 2020), increases soil organic carbon (SOC) content (Clifton-Brown et al., 2007; Pidlisnyuk et al., 2014; Richter et al., 2015), microbial biomass (MB) (Emmerling et al., 2017), and the overall belowground functioning (Chen et al., 2020; Hargreaves & Hofmockel, 2014; Kane et al., 2023). However, several studies highlighted that the effects of *Miscanthus* on soil quality are site-dependent and related to soil properties (Brami et al., 2020; Emmerling et al., 2017; Rowe et al., 2016; Ruf et al., 2018), intensity of contamination (Brami et al., 2020) and previous land use

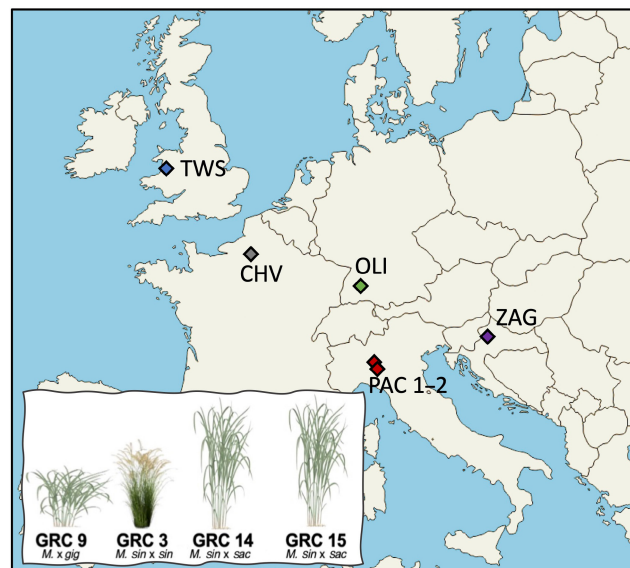
(Brami et al., 2020; Rowe et al., 2016; Ruf et al., 2018). For instance, land-use change from grasslands to commercial rhizome-based *Miscanthus* × *giganteus* negatively impact soil quality, while land-use change from intensively managed arable lands is more likely to have a beneficial impact on soil quality (Emmerling et al., 2017; Ruf et al., 2018). This is mainly related to changes in SOC content, as priming of existing soil organic matter (SOM) in response to the input of easily decomposable carbon from this crop occurred in grasslands soils characterized by high level of initial SOC (Richter et al., 2015; Rowe et al., 2016; Ruf et al., 2018; Zatta et al., 2014). However, on the long term, these authors suggested that establishment of perennial bioenergy crops on degraded and marginal lands could provide long-term improvements to SOC sequestration and soil quality. Most of these studies addressed the potential contribution of *Miscanthus* cultivation to deliver climate and other ecosystem benefits using the standard *Miscanthus* × *giganteus* genotype (Don et al., 2012). However, there is a significant knowledge gap on the soil carbon storage potential of novel *Miscanthus* hybrids. In particular, the contribution of plant-based C inputs of high-yielding hybrids to soil C sequestration and overall, to soil quality improvement is unknown. *Miscanthus* hybrids can act as a sink or even a source of atmospheric carbon according to their plant growth habit, rooting pattern, as well as the original SOC content and nutrient availability of the site before *Miscanthus* plantation (Agostini et al., 2015; Richter et al., 2015). Only few recent studies compared the carbon storage capacity of *Miscanthus sinensis* and *M. sacchariflorus* with that of the commercially grown *Miscanthus* × *giganteus* (Ouattara et al., 2021; Richter et al., 2015).

Advances in *Miscanthus* domestication are needed to breed novel genotypes to suit a wider range of ecological conditions and increase the efficiency of carbon capture and thus soil C sequestration (Clifton-Brown et al., 2007). Within the BBI demonstration GRACE project (grant agreement ID 745012) a number of novel rhizome- and seed-based *Miscanthus* hybrids were cultivated across European marginal lands to assess their potential to secure feedstock for the bio-based economy (Awty-Carroll et al., 2022; Clifton-Brown et al., 2023; Impollonia, Croci, Ferrarini, et al., 2022; Impollonia, Croci, Martani, et al., 2022; Magenau et al., 2022, 2023; Shepherd et al., 2023). In this study, four promising *Miscanthus* hybrids (two novel seed-based hybrids *M. sinensis* × *sinensis* [*M. sin* × *sin*] and *M. sacchariflorus* × *sinensis* [*M. sac* × *sin*]; a novel *M. sacchariflorus* × *sinensis* [*M. sac* × *sin*] rhizome-based clone; and a standard *M. × giganteus* clone [*M* × *g*]) were chosen to evaluate their short-term potential (4 years after land-use change from marginal land) to (1) sequester soil C in topsoil (0–30 cm) and (2) improve chemical, biological and physical soil quality. A set of representative European marginal sites were chosen to identify the effects of land-use change both from grasslands and arable lands to *Miscanthus* cultivation. In southerly sites, some hybrids reached mature yield levels at the end of the second growing seasons, while in more northerly sites mature yield levels required 3–4 years depending on soil and climatic factors (Awty-Carroll et al., 2022; Magenau et al., 2022). Once successfully established, standard *Miscanthus* × *giganteus* performed well in all locations, while *M. sin* × *sin* hybrids were found to be better adapted to Northern Europe, and two novel *M. sac* × *sin* hybrids were more productive in Southern Europe. In this work it was hypothesized that after 4 years from establishment higher-yielding hybrids across diverse pedoclimatic conditions would have the highest contribution to soil C sequestration thanks to their higher belowground plant C input to soil. To evaluate the impact of these novel *Miscanthus* genotypes on soil quality, multiple parameters were assessed including soil physical (bulk density [BD] and porosity), chemical (content and stock of SOC, total N [TN], P, and K, plant available P and K, Ca, and Mg), and biological (MB and enzymatic activity [EA]) indicators.

## 2 | MATERIALS AND METHODS

### 2.1 | Experimental design and sampling strategies

The field experiment was established in plot scale trials in 2018, at six locations across Europe, arranged along a



**FIGURE 1** Map of the sites over a range of European marginal lands. Sites in order of decreasing latitude: TWS (Aberystwyth, Wales, UK) [Aberystwyth University, IBERS], CHV (near Paris, France), OLI (near Stuttgart, Germany) [University of Hohenheim], ZAG (Zagreb, Croatia) [University of Zagreb], PAC 1 and 2 (Po Valley, Italy) [Università Cattolica del Sacro Cuore]. The panel at the bottom left of the figure show the four high-yielding *Miscanthus* hybrids selected for this study: GRC9, a standard rhizome-based clone of *M. × giganteus*; GRC 3 (*M. sin* × *sin*) and GRC 14 (*M. sac* × *sin*), two novel seed-based hybrids; GRC15 (*M. sac* × *sin*), a novel rhizome-based clone.

North-East to South-West transect (Figure 1). The six trial sites are located:

- at Trawsgoed (TWS) near Aberystwyth, UK (52°24'59.8'' N, 4°04'02.6'' W);
- at Oberer Lindenhof (OLI) near Stuttgart, Germany (48°28'42.1'' N, 9°18'41.0'' E);
- at Chanteloup (CHV) near Paris, France (48°58'34.9'' N, 2°01'57.9'' E);
- near Zagreb (ZAG), Croatia (45°85'05.55'' N, 16°17'77.7'' E);
- near Piacenza at PAC1 (45°00'11.70'' N, 9°42'35.4'' E) and PAC2 (44°50'40.32'' N, 9°35'04.9'' E).

Experimental sites had different land uses before *Miscanthus* transplanting: PAC1, OLI, and ZAG were marginal arable lands, while TWS and PAC2 were semi-improved permanent marginal grasslands. CHV was a polluted site by 100 years of irrigation with raw wastewater coming from Paris and its suburbs on sandy soils occupied by market gardens and orchard. Information on climatic conditions, previous land uses, soil type, and marginality factors are thoroughly described in Awty-Carroll et al. (2022) and Magenau et al. (2022).

Field trials were planted with 14 *Miscanthus* seed- and rhizome-based hybrids in a randomized complete block design with four replicates. This study analyses the impact of four of these 14 hybrids on soil properties: GRC 3 (*M. sin* × *sin*) and GRC 14 (*M. sac* × *sin*), two novel seed-based hybrids; GRC15, a novel rhizome-based clone (*M. sac* × *sin*); and GRC9, a standard rhizome-based clone of *M. × giganteus*. Detailed hybrid information is presented in (Awty-Carroll et al., 2022; Magenau et al., 2022), including planting density, biomass yield, growth rate, and nutrients offtake. An initial soil sampling was conducted in autumn 2017 ( $T_0$  sampling) before the establishment of field trials, collecting six soil cores at 30 cm depth in each field trial which were then divided into 0–10 and 10–30 cm sections. At each location BD samples were collected and measured for the same depth intervals. In spring 2022, 4 years after establishment ( $T_4$  sampling), soil and BD sampling was performed by extracting four replicates of 0–30 cm soil cores for each *Miscanthus* hybrid. Two soil cores were collected in three different plant positions (inter-row, edge of the plant and center of the plant) according to the sampling strategy described by Martani et al. (2020). Soil cores collected were then split into 0–10 and 10–30 cm sections and combined to have one sample per each soil depth layer per plot. Soil samples were taken to the laboratories, where one subsample was air-dried and sieved at 2 mm for physicochemical analysis and another subsample was stored at  $-18^{\circ}\text{C}$  for microbiological analysis.

## 2.2 | Soil quality indicators

### 2.2.1 | Soil quality physicochemical indicators

Air-dried soil samples were weighted and analyzed by Dumas's combustion method with elemental analyzer (SoliTOC; Elementar) to determine SOC and TN concentration ( $\text{g kg}^{-1}$ ) respectively. Total phosphorous (P), potassium (K), calcium (Ca), and magnesium (Mg) were analyzed through microwave digestion method and read with an Inductively coupled plasma optical emission spectrometry, while plant available phosphorous ( $\text{P}_2\text{O}_5$ ) and plant available potassium ( $\text{K}_2\text{O}$ ) were analyzed with the ammonium lactate method (Egnér et al., 1960). Soil BD ( $\text{g cm}^{-3}$ ) was calculated dividing dry soil weight by the volume of the known cylinder used, while soil porosity (Por) was calculated from BD ( $1 - \text{BD}/2.65$ ). Variations in SOC and soil nutrients concentration ( $\text{g kg}^{-1}$ ) were calculated as the difference between the values measured after 4 years from plantation

and the values measured before the beginning of the experiment.

### 2.2.2 | Soil quality biological indicators

Soil biological quality was evaluated by measuring soil enzymes activities and MB. The effect of *Miscanthus* spp. cultivation on soil microbial community activity was determined via measurements of EA. The assay based on the procedure of Ferrarini et al. (2021) tested 16 hydrolytic enzymes involved in the principal nutrient cycles, namely:  $\beta$ -glucosidase (betaG),  $\beta$ -1,4-glucanase (cell),  $\beta$ -1,4-xylanase (xilo), involved in C cycling; *N*-acetyl- $\beta$ -D-glucosaminidase (chit), leucine amino-peptidase (leu), arginin amino-peptidase (argi), and aspecific protease (aprot) involved in N cycling; acid- (acP) and alkaline- (alkP) phosphomonoesterase, phosphodiesterase (bisP), pyrophosphate-phosphodiesterase (piroP), inositol-P phosphatase (inositP) involved in P cycling; arylsulfatase (aryS); butyrate esterase (butir) and fluorescein diacetate hydrolysis (fda) involved in the hydrolysis of ester bonds; and peroxidase (perox) involved in redox reaction of SOM cycle. All measurements were taken in triplicate and the activities were expressed as nanomoles of 4-methylumbelliferone (MUF) (or 7-amino-4methyl coumarine [AMC])  $\text{g}^{-1}$  dry soil  $\text{h}^{-1}$ . MB was determined using the double-stranded DNA (dsDNA) content as a proxy (Bragato et al., 2016). DNA was extracted as described by (Fornasier et al., 2014). The soil dsDNA content was corrected for soil moisture content and expressed as nanograms dsDNA  $\text{g}^{-1}$  dry soil.

## 2.3 | SOC and nutrients stock changes calculation

Soil organic carbon and soil nutrients stocks ( $\text{Mg ha}^{-1}$ ) were calculated across sites using the equivalent soil mass (ESM) approach proposed by Wendt and Hauser (2013). ESM's ( $\text{Mg ha}^{-1}$ ) were calculated using a cubic spline function to provide estimates that correspond to 0–10 cm and 10–30 cm soil depth across all sites to ensure comparable SOC and nutrients stock estimates responding to changes in BD between sites and the two sampling times  $T_0$  (time zero) and  $T_4$  (year 4). Stocks values were calculated by multiplying the ESM of the soil layer by its C or nutrient concentration. Stocks were calculated also as cumulative soil mass of SOC and nutrients in the 0–30 cm soil layer. In this study, SOC sequestration is expressed both as (1) SOC stock variation ( $\Delta\text{SOC}$  stock in  $\text{Mg ha}^{-1}$ ), calculated as the difference between SOC stock measured after 4 years from plantation and the SOC stock measured

before the beginning of the experiment and (2) yearly carbon sequestration rate ( $\text{Mg ha}^{-1}\text{year}^{-1}$ ), calculated dividing the  $\Delta\text{SOC}$  stock  $\text{Mg ha}^{-1}$  by the years of *Miscanthus* cultivation. We used here the term “SOC sequestration” as SOC stock measured change from time zero as discussed in Don et al. (2023), by assuming that SOC stock is under equilibrium for the business-as-usual scenario with no land-use change to *Miscanthus*.

## 2.4 | Plant C input to soil calculation

Plant C input to soil from above- and belowground biomass components were estimated as described in Martani et al. (2023) for perennial biomass crops with the addition of the formula to estimate C input from rhizomes (Table S3). Total C input to soil was calculated on a yearly basis as the sum of the C input from the following plant organs: C from stubble (Cs), C from roots (Cr), C from root exudates (Ce), and C from rhizomes (Crhiz). C input from weeds (Cw) was considered negligible and was excluded from the calculation. Cs, Cr, and Ce were calculated as the sum of the four growing seasons while Crhiz was calculated only for three growing seasons since in the first year of establishment the rhizome biomass (RB) is negligible. In order to calculate belowground C input from Cr and Ce in the 0–10 and 10–30 cm layers, the “beta” root distribution model was applied using a  $\beta$  coefficient value of 0.967 (see Chimento & Amaducci, 2015). In order to calculate Crhiz, RB was estimated using an allometric function derived from literature where RB and cumulative aboveground biomass over the growing seasons (CumAGB) have been measured. A detailed description of RB estimation is reported in the Supporting Information. To apply the formula of Table S3 the data on annual yields were obtained from Awty-Carroll et al. (2022) and Magenau et al. (2022) for the same plot/hybrid/year/site combination, while information about harvest index was obtained from Martani et al. (2023) and root to shoot (R:S) ratio values from dedicated roots sampling at PAC1 and OLI sites.

## 2.5 | Statistical analysis

Soil variables (BD, Por, SOC, TN, TP, TK,  $\text{P}_2\text{O}_5$ ,  $\text{K}_2\text{O}$ , Ca, and Mg concentration and stock) as well as single EA and MB were analyzed using a three-way mixed-model ANOVA for complete randomized block design. Analyses were performed using R: *nmls* (Pinheiro et al., 2012) and *emmeans* (Lenth et al., 2018) packages. *Miscanthus* hybrids (Hybrid), site (Site), and block were considered as random effects to represent the experimental designs of the different trials while Site  $\times$  Hybrid  $\times$  sampling time

interactions were used as fixed factors. Mixed model was run independently for each soil layer (0–10, 10–30, and 0–30 cm). Annual rate of SOC and nutrients stock changes were analyzed with a two-way mixed-model ANOVA for complete randomized block design. *Miscanthus* hybrids, site, and block were considered as random effects while their interaction as fixed effect. Mixed model was run independently for each soil layer (0–10, 10–30, and 0–30 cm). Log transformations or square root transformations were performed to satisfy assumptions of normality and heteroskedasticity when needed. If the transformation did not meet the criteria, “function weights” were used in the linear mixed-model effect formula to consider the non-homogeneous variance structure introduced by the factors studied. The function *emmeans* was used to estimate the marginal means and Tukey HSD post hoc tests were used to assess significant differences between treatments when the *F*-tests indicated statistically significant effects. All test results were considered statistically significant at  $p \leq 0.05$ .

A distance-based Redundancy Analysis (dbRDA) based on Bray–Curtis distance was used to assess the effects of depth and time on soil enzyme activities and how they are affected by soil properties (SOC, TN, TP, and MB). dbRDA was performed separately on each site and run on a three-step basis as described in Ferrarini et al. (2021). A similarity percentage (SIMPER), coupled with Wilcoxon signed-rank tests with  $n = 9999$  permutations, was used to determine the enzyme activities that contributed most to the Bray–Curtis dissimilarity between  $T_0$  and  $T_4$  sampling times.

A backward stepwise multiple linear regression analysis was applied according to Ferrarini et al. (2021) to discriminate and rank the most important soil variables (initial SOC, TN, TP and TK concentration, C:N, C:P, N:P, and C input) in explaining the SOC sequestration ( $\Delta\text{SOC}$  stock in  $\text{Mg ha}^{-1}$ ) at two soil depths, 0–10 cm and 10–30 cm, and for two different environments: the Northern environment (NE) that includes TWS, OLI, and CHV sites and the Southern environment (SE) that includes ZAG, PAC1, and PAC2 sites. Moreover, a correlation analysis was conducted to identify significant relationships between changes in SOC concentration ( $\Delta\text{SOC g kg}^{-1}$ ) and enzymatic activities ( $\Delta\text{EA}$ ).

## 3 | RESULTS

### 3.1 | Effects of *Miscanthus* cultivation on soil quality indicators

Four years after planting *Miscanthus* on the six widely distributed European sites, most of the soil quality indicators

were significantly affected ( $p < 0.05$ ) by the land use transition (Table S4a,b). “Site” effect was significant for all soil physical, chemical, and biological indicators, while the effect of “Time” was significant for 7 out of 10 soil physicochemical indicators and for 3 out of 10 biological indicators. Moreover, no significant “Hybrid” effect was detected for any soil quality indicators, therefore they will not be considered hereafter.

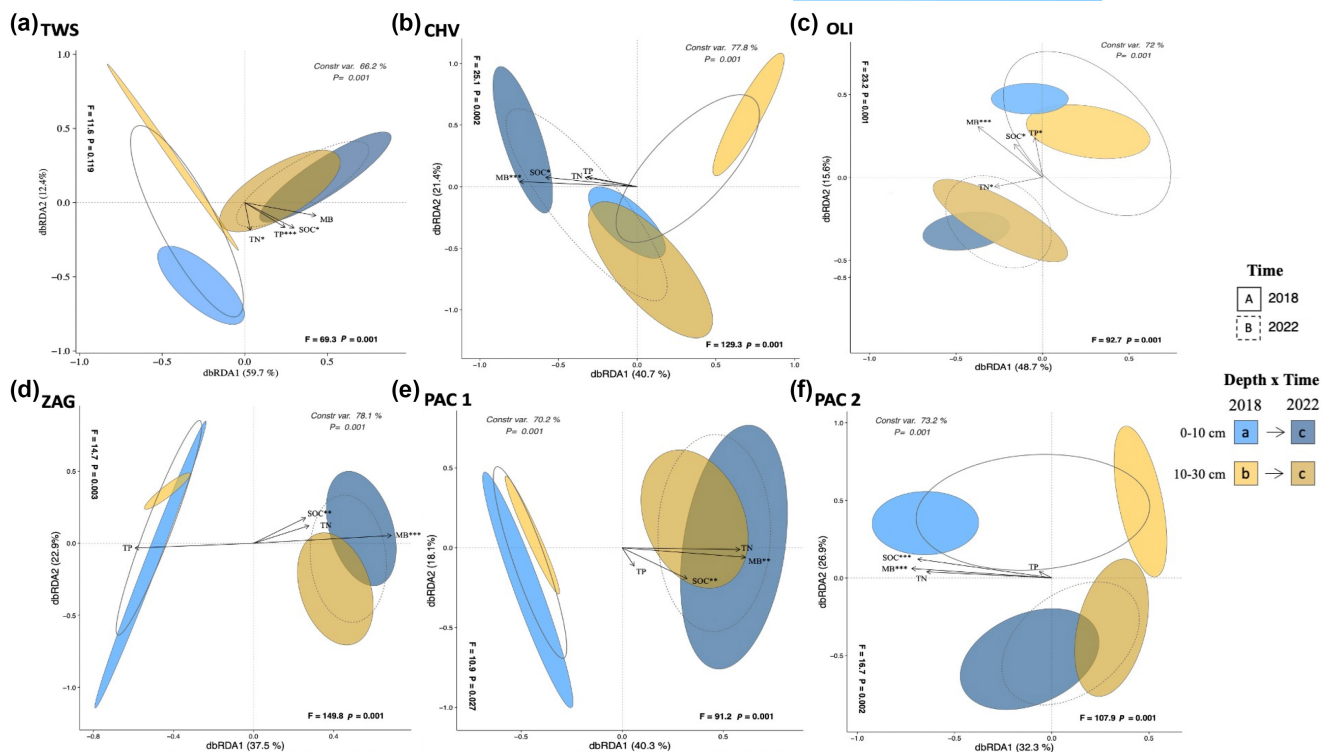
### 3.1.1 | Effects of *Miscanthus* cultivation on soil physical and chemical indicators

The BD increased significantly at ZAG and PAC2 sites both in 0–10 cm (+11.7% and +83.8%, respectively) and 10–30 cm (+14.4% and +10.7%, respectively) layers (Figure S2a,c) and OLI in the top layer (+16.4%), while in all other sites its variation was not significant. Estimates of soil porosity changes have an opposite behavior to BD (Figure S2b,d). Overall, across sites, SOC concentration ( $\text{g kg}^{-1}$ ) decreased by  $-2.9\%$  ( $-1.4 \text{ g kg}^{-1}$ ) in the 0–10 cm layer and increased by  $+6.4\%$  ( $+1.5 \text{ g kg}^{-1}$ ) in the 10–30 cm layer (Figure S3a,e). In the 0–10 cm layer, SOC content significantly decreased in TWS, by  $-5\%$  ( $-2.6 \text{ g kg}^{-1}$ ) and PAC2 by  $-37\%$  ( $-8.6 \text{ g kg}^{-1}$ ), while significantly increase in CHV by  $+13\%$  ( $+3.8 \text{ g kg}^{-1}$ ) (Figure S3a). In addition, SOC content increased by  $+33\%$  at TWS in the 10–30 cm layer ( $+10.1 \text{ g kg}^{-1}$ ) (Figure S3e). Similarly to SOC, concentration of TN ( $\text{g kg}^{-1}$ ) was reduced in the upper layer by  $-4.9\%$  ( $-0.3 \text{ g kg}^{-1}$ ) and enhanced in the lower layer by  $+19\%$  ( $+0.2 \text{ g kg}^{-1}$ ). In TWS and PAC2, TN content was reduced by  $-24.3\%$  ( $-1.2 \text{ g kg}^{-1}$ ) and  $-32.5\%$  ( $-0.7 \text{ g kg}^{-1}$ ) in the first 0–10 cm respectively, while in the 10–30 cm layer TN increased by  $+39.2\%$  ( $+0.9 \text{ g kg}^{-1}$ ) and  $+49\%$  ( $+0.4 \text{ g kg}^{-1}$ ), respectively (Figure S3b,f). In contrast, at CHV TN content ( $\text{g kg}^{-1}$ ) significantly decreased in both layers by  $-16\%$  ( $-0.3 \text{ g kg}^{-1}$ ) on average (Figure S3b,f). PAC1 was the only site where TN content significantly increased in both layers, on average by  $+13.8\%$  ( $+0.2 \text{ g kg}^{-1}$ ). Generally, across sites, total P content increased in the soil profile by  $+11.6\%$  ( $+0.06 \text{ g kg}^{-1}$ ) with significant increases at CHV ( $+30.1\%$ ;  $+0.2 \text{ g kg}^{-1}$ ), OLI ( $+8.7\%$ ;  $+0.09 \text{ g kg}^{-1}$ ), and PAC1 site ( $+34.8\%$ ;  $+0.1 \text{ g kg}^{-1}$ ). However, TP significantly decreased in ZAG  $-15.6\%$  ( $-0.1 \text{ g kg}^{-1}$ ) and in TWS in 10–30 cm layer by  $-5\%$  ( $-0.08 \text{ g kg}^{-1}$ ) (Figure S4a,e). Available P significantly increased in all sites by  $+129\%$  ( $+0.2 \text{ g kg}^{-1}$ ), with the highest increment in PAC1  $+418.9\%$  ( $+0.5 \text{ g kg}^{-1}$ ) (Figure S3c,g). Moreover, a significant depletion of total K concentration was observed with *Miscanthus* cultivation across sites corresponding to  $-21\%$  ( $-0.3 \text{ g kg}^{-1}$ ), which was significant in TWS ( $-49.3\%$ ;  $-0.7 \text{ g kg}^{-1}$ ), ZAG ( $-45.7\%$ ;  $-0.3 \text{ g kg}^{-1}$ ), PAC1 ( $-25.3\%$ ;  $-0.3 \text{ g kg}^{-1}$ ) and PAC2 ( $-39.9\%$ ;  $-0.3 \text{ g kg}^{-1}$ )

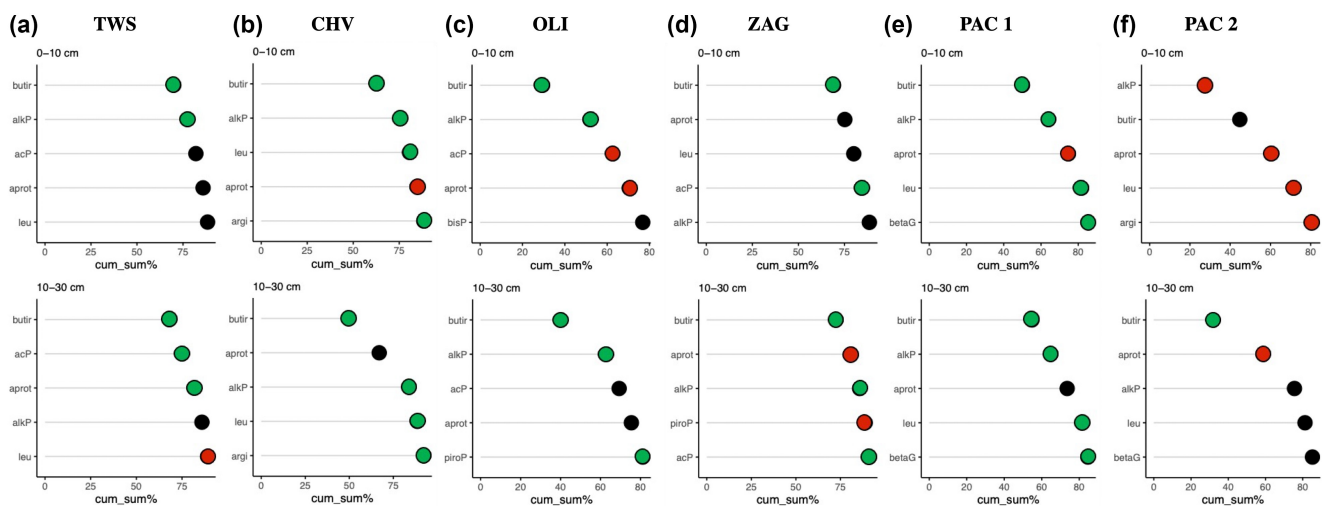
(Figure S4b,f). Available K generally increased in the first layer, on average by  $+31.8\%$  ( $+0.1 \text{ g kg}^{-1}$ ), while it decreased in the 10–30 cm layer by  $-11.5\%$  ( $-0.06 \text{ g kg}^{-1}$ ), except at PAC1 where it had the highest increment in both layers ( $+71.3\%$ ;  $+0.2 \text{ g kg}^{-1}$ ) (Figure S3d,h). Ca content ( $\text{g kg}^{-1}$ ) was not significantly affected by *Miscanthus* cultivation (Figure S4c,g), while Mg concentration significantly decreased in the 10–30 cm layer in TWS, PAC1 and PAC2 sites with an average decrease in  $-5.1\%$  ( $-0.4 \text{ g kg}^{-1}$ ) (Figure S4d,h).

### 3.1.2 | Effects of *Miscanthus* cultivation on soil biological indicators

Cultivation of *Miscanthus* positively affected biological soil quality measured as MB (measured as dsDNA content) and EA (Table S4b). In TWS, CHV, ZAG, and PAC1 sites, the MB significantly increased along the soil profile, showing the highest increment in CHV ( $+10.9\%$ ) followed by TWS ( $+6.3\%$ ), PAC1 ( $+3.8\%$ ) and ZAG ( $+3.5\%$ ) (Figure S5a,b). However, MB decreased significantly in OLI ( $-2.9\%$ ) and PAC2 ( $-15.5\%$ ) mainly in the 0–10 cm, while no significant differences were detected in the 10–30 cm layer in PAC2. Considering the previous land uses of these sites, in managed grassland such as TWS and PAC2, the differentiation of EA was more evident in the top layer with separation along axis 1 accounting for  $59.7\%$  in TWS ( $F: 69.3$ ;  $p=0.001$ ) and  $32.3\%$  in PAC2 ( $F: 107.9$ ;  $p=0.001$ ) (Figure 2a,b). Differently, land use change from former arable lands such as OLI, ZAG and PAC1 showed a clearer differentiation in the entire soil profile considered, with the separation along axes 1 ( $F$  range:  $91.2$ – $149.8$ ;  $p=0.001$ ) and accounting for  $37.5\%$ – $48.7\%$  of the total variance. dbRDA results from multivariate analysis on C-, N-, P-, and S-cycling enzymes indicated that the soil parameters that had the highest influence on EA distribution across sites were mostly MB and SOC (Figure 2). MB and SOC were in turn positively correlated in all sites as also highlighted by the correlation analysis between SOC and EA changes (Table S5). A south–north environmental gradient controlling EA was observed (Figure 2). EA was found to be mainly governed by MB and SOC in SEs, while availability of total nutrients (TN and TP) significantly controlled EA in NE such as OLI and TWS. On average, the activities of C-acquiring, esterase, and P-acquiring enzymes under *Miscanthus* cultivation were significantly increased in most of the sites (Figure 3). However, PAC2 showed a reduction for all EA in the 0–10 cm and for N-acquiring enzymes in the 10–30 cm layer. In addition, in TWS most of the EA decreased in the 10–30 cm layer, while in OLI N-acquiring enzymes were reduced in the top layer (Table S6). SIMPER analysis



**FIGURE 2** dbRDA plots showing shifts in C-, N-, P- and S-cycling enzyme activities ( $\text{MUF}$  or  $\text{AMC}_{\text{g dry soil}}^{-1} \text{h}^{-1}$ ) among marginal sites ([a] TWS, [b] CHV, [c] OLI, [d] ZAG, [e] PAC1, [f] PAC2) in topsoil layers (0–10 cm and 10–30 cm) from  $T_0$  to  $T_4$ . The interaction of the factors “Depth” and “Time” is positioned as multivariate centroids surrounded by 95% confidence interval ellipsoids. Soil variables (SOC, TN, TP and MB), represented as arrows that significantly contributed to EA differentiation along axis are indicated with: \*, \*\*, \*\*\* for  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$  respectively. dbRDA, distance-based Redundancy Analysis; EA, enzyme activity; MB, microbial biomass; SOC, soil organic carbon; TN, total nitrogen; TP, total phosphorus



**FIGURE 3** SIMPER analysis showing enzyme activities that contributed most (>80%) to the Bray–Curtis dissimilarity between  $T_0$  and  $T_4$  sampling times, in 0–10 cm (figures above) and 10–30 cm (figures below) soil layers, among marginal sites ([a] TWS, [b] CHV, [c] OLI, [d] ZAG, [e] PAC1, [f] PAC2). Different colors indicate a significant positive (green) or negative (red) contribution to the Bray–Curtis dissimilarity.

showed that esterase activity (measured as butir) was the EA that increased significantly in both soil layers and contributed the most to overall EA dissimilarities between  $T_0$

and  $T_4$  sampling time (52.5% in 0–10 and 55% in 10–30 cm layer). AlkP was significantly increased and contributed to the overall dissimilarities in TWS, CHV, OLI, and PAC1



sites in the top layer as well as in the lower layer in CHV, OLI, ZAG, and PAC1 sites (Figure 3). At PAC2, EA were significantly decreased after 4 years of cultivation, in particular in the 0–10 cm layer were alkP and three N-cling enzymes (aprot, leu, and argi) contributed to 80% of the overall dissimilarity. Furthermore, a significant decrease of N-acquiring enzymes activity like aprot in the top layer was observed in CHV, OLI, PAC1, PAC2 and in the lower layer of ZAG and PAC2 sites (Figure 3).

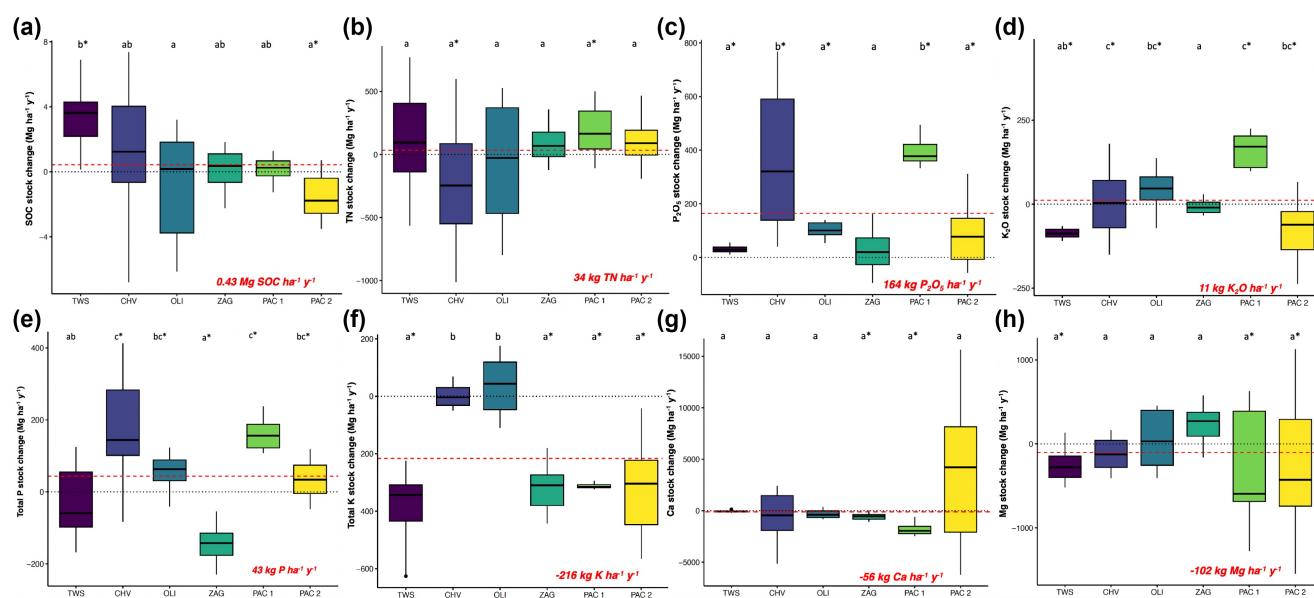
### 3.1.3 | Soil nutrients stock variation after 4 years of *Miscanthus* cultivation

After 4 years of *Miscanthus* cultivation, total K, Ca and Mg stocks were significantly reduced compared to time zero sampling in the 0–30 cm layer (Figure 4F–H; Table S7b). Total K stock showed the highest cumulative reduction ( $-0.2 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ) across sites, which was significant in TWS ( $-0.4 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ), ZAG, PAC1 and PAC2 ( $-0.3 \text{ Mg ha}^{-1} \text{ year}^{-1}$  in all three sites). Ca stock overall decreased on average by  $-0.06 \text{ Mg ha}^{-1} \text{ year}^{-1}$  among sites, but significantly only at ZAG ( $-0.6 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ) and PAC1 ( $-1.8 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ). Moreover, Mg stock was lowered by  $-0.1 \text{ Mg ha}^{-1} \text{ year}^{-1}$ , but significantly only in the 10–30 cm layers (Figure S7; Table S7a). The variation of Mg stock was significant in the lower layer at TWS ( $-0.2 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ), PAC1 ( $-0.2 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ) and PAC2 ( $-0.4 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ). On the contrary, available P, available K, TN, and TP stocks cumulative

increased over time in the 0–30 cm layer, respectively, by  $+0.16 \text{ Mg P}_2\text{O}_5 \text{ ha}^{-1} \text{ year}^{-1}$ ,  $+0.01 \text{ Mg K}_2\text{O ha}^{-1} \text{ year}^{-1}$ ,  $+0.03 \text{ Mg TN ha}^{-1} \text{ year}^{-1}$ , and  $+0.04 \text{ Mg TP ha}^{-1} \text{ year}^{-1}$  (Figure 4B–E; Table S7b). The highest increment was found for available P, which resulted significantly enhanced in all sites and substantially at CHV and PAC1 (both  $+0.4 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ). Increased stocks of available K were found mainly in the upper layer ( $+0.03 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ) and decreased in the lower layer ( $-0.02 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ) (Figure S6; Table S7a). Moreover, significant variations in TN and TP stocks were different across sites. TN was enhanced only in the 0–30 cm at PAC1 ( $+0.2 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ), while decreased at CHV ( $-0.2 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ). In addition, at TWS and PAC2, TN stock was reduced in the upper layer ( $-0.2 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ), while it was increased in the lower layer ( $+0.3 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ) (Figure S6; Table S7a). Similarly, for TP stock a significant increase in 0–30 cm was found at CHV ( $+0.2 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ), OLI ( $+0.06 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ) and PAC1 ( $+0.2 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ), while it was reduced at ZAG ( $-0.1 \text{ Mg ha}^{-1} \text{ year}^{-1}$ ) (Figure S7; Table S7a).

## 3.2 | Effects of *Miscanthus* cultivation on SOC stock and drivers of soil C sequestration

After 4 years of *Miscanthus* cultivation SOC stocks ( $\text{Mg SOC ha}^{-1}$ ) showed significant variations on former grasslands (TWS, CHV, and PAC2) with opposite behaviors



**FIGURE 4** Rate of ([a] SOC stock [ $\Delta$  stock  $\text{Mg ha}^{-1} \text{ year}^{-1}$ ] and soil nutrients, [b] total N, [c] available P, [d] available K, [e] total P, [f] total K, [g] Ca, [h] Mg) stock variation ( $\Delta$  stock  $\text{Mg ha}^{-1} \text{ year}^{-1}$ ) in the topsoil layer [0–30 cm], in 4 years of *Miscanthus* cultivation among marginal sites. Different letters denote statistically different means among marginal sites (Tukey's test,  $p < 0.05$ ), while (\*) show statistically different variations from  $T_0$  to  $T_4$  (Tukey's test,  $p < 0.05$ ) within the same site. SOC, soil organic carbon.

in the upper and lower layers (Figure 4A; Figure S6; Table S7a,b). In the upper layer of TWS and PAC2, SOC stock was significantly reduced by  $-0.5$  and  $-2.1 \text{ Mg ha}^{-1} \text{ year}^{-1}$ , respectively, while in CHV SOC increased by  $+1.0 \text{ Mg ha}^{-1} \text{ year}^{-1}$  (Figure S6). On the contrary, SOC stock significantly increased in the 10–30 cm layer by  $+3.7$  and  $+0.6 \text{ Mg ha}^{-1} \text{ year}^{-1}$ , respectively, at TWS and PAC2, while no significant differences were detected at CHV (Figure S6). In other marginal sites, that were former arable lands, SOC stock after *Miscanthus* establishment did not show significant changes (Figure 4A; Figure S6). Nevertheless, it was estimated that *Miscanthus* cultivation had an average plant C input to soil of  $3.9 \text{ Mg C ha}^{-1}$  in 4 years across sites (Table 1). Estimated plant C input values (as a sum of above and belowground inputs) varied greatly across sites and within genotypes, showing the greatest values at PAC1 ( $5.3 \text{ Mg C ha}^{-1}$ ). Generally, the standard clone  $M \times g$  (GRC9) showed the lowest average plant C input over four growing seasons ( $2.6 \text{ Mg C ha}^{-1}$ ), with the lowest value at CHV ( $0.3 \text{ Mg C ha}^{-1}$ ) and the highest at OLI ( $4.0 \text{ Mg C ha}^{-1}$ ). *M. sac \times sin* hybrids, such as GRC14 and GRC15 showed the highest plant C input in SE like ZAG ( $5.1 \text{ Mg C ha}^{-1}$  GRC 15), PAC1 ( $7.5 \text{ Mg C ha}^{-1}$  GRC 14), and PAC2 ( $6.1 \text{ Mg C ha}^{-1}$  GRC 14). In contrast, the *M. sin \times sin* hybrid (GRC3) showed higher C input values in NE such as CHV ( $4.5 \text{ Mg C ha}^{-1} \text{ year}^{-1}$ ), OLI ( $6.5 \text{ Mg C ha}^{-1} \text{ year}^{-1}$ ) and TWS ( $5.3 \text{ Mg C ha}^{-1} \text{ year}^{-1}$ ). Overall, the contribution to the estimated plant C input to soil from *Miscanthus* derives mainly from roots (47%), followed by roots exudates (21%) and rhizomes (20%) (Table S8). Despite considerable estimated values of plant C added to the soil by *Miscanthus* hybrids across sites in 4 years, other factors were identified as drivers of soil C sequestration (measured as  $\Delta \text{SOC}$  stock changes in  $\text{Mg SOC ha}^{-1}$  after 4 years of *Miscanthus* cultivation compared with initial SOC storage). The results of the multiple

**TABLE 1** Estimated total C input to soil ( $\text{Mg ha}^{-1}$ ) as the sum of C inputs from the following *Miscanthus* organs: C from stubble (Cs), C in roots (Cr), C in root exudates (Ce), and C in rhizomes (Crhiz).

	GRC 14	GRC 15	GRC 3	GRC 9	
TWS	2.67 <sup>a</sup>	3.58 <sup>a</sup>	5.33 <sup>b</sup>	2.92 <sup>a</sup>	3.62 <sup>AB</sup>
CHV	3.25 <sup>bc</sup>	2.49 <sup>b</sup>	4.46 <sup>c</sup>	0.30 <sup>a</sup>	2.63 <sup>A</sup>
OLI	3.10 <sup>a</sup>	4.59 <sup>b</sup>	6.55 <sup>c</sup>	4.07 <sup>ab</sup>	4.58 <sup>BC</sup>
ZAG	3.89 <sup>ab</sup>	5.07 <sup>b</sup>	3.41 <sup>a</sup>	3.10 <sup>a</sup>	3.86 <sup>B</sup>
PAC 1	7.48 <sup>c</sup>	5.31 <sup>b</sup>	4.90 <sup>ab</sup>	3.47 <sup>a</sup>	5.29 <sup>C</sup>
PAC 2	6.05 <sup>c</sup>	3.78 <sup>b</sup>	2.59 <sup>ab</sup>	1.93 <sup>a</sup>	3.59 <sup>AB</sup>
	4.41 <sup>A</sup>	4.14 <sup>A</sup>	4.54 <sup>A</sup>	2.63 <sup>B</sup>	3.93

Note: Lowercase letters denote significant differences ( $p > 0.05$ , Tukey test) among hybrids in a particular site, while uppercase letters denote significant differences ( $p > 0.05$ , Tukey test) among sites and hybrids.

linear stepwise regression showed overall good  $R^2$  values of the models for the northern and southern European environments, except in 10–30 cm in south Europe (Table 2). In NE,  $R^2$  were 35.4% in 0–10 cm and 64.1% in 10–30 cm layer, while in SE  $R^2$  were 92.8% in 0–10 cm and 13.9% in 10–30 cm depth (Table 2). In both environments, in the top layer the initial concentration of soil carbon was identified as the main driving factor of soil C sequestration, showing a relative importance (% to  $R^2$ ) of 28% in northern Europe and 31% in southern Europe and a significant ( $p < 0.001$ ) negative correlation. Moreover, initial values of TN and C:P ratio (SOC:TP stoichiometric ratio of concentration values) had a positive correlation with  $\Delta \text{SOC}$  stock in both environments. In addition, initial TP values had a positive correlation in NE (27% to  $R^2$ ) and N:P a negative correlation in SE (19% to  $R^2$ ). In the lower layer of northern Europe, TK had the highest relative contribution to the model (38%) followed by C:N (19%), TP (18%), C (15%), and N:P (10%). Moreover, considering the correlation between variations of SOC concentration ( $\Delta \text{SOC g kg}^{-1}$ ) and changes in EA ( $\Delta \text{EA}$ ), it is worth noting that a significant positive correlation ( $p < 0.0001$ ) was found with a redox enzyme ( $\Delta \text{perox}$ ) (Table S5).

## 4 | DISCUSSION

### 4.1 | Effects of *Miscanthus* on soil quality indicators

The effect of land-use transition to *Miscanthus* on soil physical parameters was site-specific. Overall, BD increased in the upper layer mainly in those sites characterized by heavy textured soils such as OLI, PAC2 (clay loam texture) and ZAG (silty loam texture) (Awty-Carroll et al., 2022). Likewise, in these sites soil porosity was reduced, and this might negatively impact soil structure, gas exchange, and water movement (Winkler et al., 2020). The increase in BD during *Miscanthus* cultivation is a consequence of the no tillage regime of this crop (Guzman et al., 2019) and also of the use of heavy machinery during harvesting operations under wet soil conditions (Hargreaves et al., 2019; Zatta et al., 2014). However, TWS, CHV (sandy-loam soils) and PAC1 (clay loam soil) did not show any significant variation of soil physical properties. These results confirm that the impact of the no-tillage perennial crop regime on soil physical quality is site-specific. Indeed, other studies revealed either a reduction (Das et al., 2016; Kahle et al., 1999; Lemus & Lal, 2005; Martani et al., 2020), or an increase (Brami et al., 2020; Guzman et al., 2019) of BD after planting *Miscanthus*, making difficult to establish which is the key factor determining the impact on physical soil quality.

**TABLE 2** Summary statistics of the multiple regression models obtained for SOC sequestration (measured as  $\Delta$ SOC stock changes in  $\text{Mg C ha}^{-1}$  after 4 years of *Miscanthus* cultivation compared to initial SOC storage) for Northern (TWS, OLI, CHV) and Southern (ZAG, PAC1, PAC2) environments.

Type of environment	Depth (cm)	Variable	Prop. of variance %	Residual SE	Adjusted $R^2$	F-statistic	p-Value	Significant predictors (% relative importance)*
Northern	0–10	$\Delta$ SOC stock ( $\text{Mg C ha}^{-1}$ )	35.42	6.075	0.2773	4.607	0.0019	C content (28%) <sup>a</sup>
								Total P content (27%) <sup>b</sup>
Southern	0–10		64.10	3.627	0.5937	13.57	1.294e-07	Total N content (18%) <sup>b</sup>
								C:P ratio (14%) <sup>b</sup>
	10–30							C input (13%)
								K content (38%) <sup>a</sup>
	0–10			92.84	1.517	0.9199	109	C:N ratio (19%) <sup>b</sup>
								Total P content (18%) <sup>b</sup>
	10–30			13.94	1.58	0.0369	1.361	C content (15%) <sup>a</sup>
								N:P ratio (10%) <sup>a</sup>
	0–10							C content (32%) <sup>a</sup>
								C:P ratio (24%) <sup>a</sup>
10–30							N:P ratio (19%) <sup>a</sup>	
							Total N content (19%) <sup>a</sup>	
0–10							K content (6%) <sup>b</sup>	
							C:N ratio (30%) <sup>b</sup>	
10–30							C content (26%) <sup>b</sup>	
							Total N content (22%) <sup>b</sup>	
0–10							Total P content (11%)	
							N:P ratio (11%)	

Note: Initial values (as  $\text{g kg}^{-1}$ ) of carbon and nutrients at  $T_0$  sampling were considered as predictors.

Abbreviations: SE, standard error; SOC, soil organic carbon.

\*Metrics are normalized to sum to 100% of adjusted  $R^2$ . Superscript lowercase letter denotes significant differences among predictors (<sup>a</sup> $p < 0.001$ , <sup>b</sup> $p < 0.01$  Bonferroni test) as assessed by bootstrap ( $n = 1000$ ) measures of relative importance.

In this study, after 4 years of cultivation, variations of SOC content in marginal lands were significantly influenced by previous land uses as confirmed by several studies (Brami et al., 2020; Dondini et al., 2009; Emmerling et al., 2017; Rowe et al., 2016; Ruf et al., 2018; Zatta et al., 2014; Zimmermann et al., 2013). SOC content significantly decreased in former grasslands, which were characterized by higher contents of SOC, while in former arable lands SOC concentration did not vary significantly. Several studies have reported that variations in SOC content can be site-dependent and related to mean annual temperature (Emmerling et al., 2017; Poeplau & Don, 2014), or soil texture (Brami et al., 2020; Emmerling et al., 2017; Kahle et al., 2001; Ruf et al., 2018). In this study, under marginal land conditions, the trend of SOC sequestration during *Miscanthus* cultivation was affected by initial SOC content as identified in other non-marginal lands converted to *Miscanthus* (Emmerling et al., 2017; Rowe et al., 2016; Ruf et al., 2018; Zatta et al., 2014; Zimmermann et al., 2013). Generally, ploughing operations conducted before planting *Miscanthus* on grassland, along with the subsequent disruption of soil aggregates, is considered a contributing factor to the significant SOC loss in C-rich soils (Poeplau & Don, 2014; Zimmermann et al., 2013) which can be accelerated by the input of easily decomposable organic substrate, a phenomenon ascribed to the priming effect (Dondini et al., 2009; Kuzyakov, 2002; Richter et al., 2015; Zatta et al., 2014). Variation of SOC content after land-use changes to *Miscanthus* was also identified as the main driver of changes of physical and biological soil quality indicators. For instance, aggregate stability (Brami et al., 2020; Emmerling et al., 2017; Ruf et al., 2018; Winkler et al., 2020), MB, and microbial activity (Brami et al., 2020; Emmerling et al., 2017; Ruf et al., 2018), were higher after conversion from arable lands to perennial crops, but lower when converted from grasslands. This is confirmed also in this study, where SOC concentration was significantly correlated to MB ( $p < 0.001$ ) in most of the sites as well as to EA variations in all sites (Figure 2). The high quantity of estimated plant C input to soil, especially the more easily decomposable roots and root exudates (on average  $0.67 \text{ Mg C ha}^{-1} \text{ year}^{-1}$ ), may have stimulated an increase of the microbial turnover, which resulted in a significant increment of the MB in most of the sites in 4 years of *Miscanthus* cultivation. However, at PAC2 site, land-use change from a 15-year-old meadow to *Miscanthus* significantly depleted SOC and MB agreeing with other reports (Brami et al., 2020; Ruf et al., 2018). This study also highlighted the importance of plant C input in activating the overall belowground functioning. The physiological shift of the microbial community was mainly governed by the stimulation of the activity of esterase (butir), involved in the hydrolysis of ester bonds, and

alkaline phosphomonoesterase (alkP), involved in organic P mineralization to inorganic P available for plant uptake. The increase in esterase (butir) activity could reflect an increment of the organic matter decomposition rate, which was probably related to the large amounts of unprotected fresh root litter from fine roots turnover and production of root exudates (Wittmann et al., 2004). The availability of easily utilizable C led also to a reduction of extracellular protease (“aprot”) production due to catabolic repression (Vranova et al., 2013). Indeed, fresh root turnover enriches soil with free amino acids, inhibiting microbial proteolytic activity (Vranova et al., 2013). Production of P-acquiring enzymes was instead indicative of a microbial community with high P demands. Indeed, to gain access to P, microbes synthesize extracellular enzymes that decompose organic matter and release plant available P. Moreover, the high correlation between MB and the EA ( $p < 0.001$  in dbRDA) was reflected in the reduction of the microbial community physiological capacity in PAC2, where the activity of all enzymes analyzed decreased in the top layers as well as the enzymes associated with N cycling in the 10–30 cm layer.

In this short-to-medium field experiment, where no fertilization was applied at transplanting nor during the growing seasons, *Miscanthus* cultivation led to a significant depletion of some soil macronutrients, while others were significantly enhanced. Among all sites, total K was the plant macronutrient that showed the greatest depletion ( $-860 \text{ kg K ha}^{-1}$ ), followed by Mg ( $-410 \text{ kg ha}^{-1}$ ) and Ca ( $-240 \text{ kg Ca ha}^{-1}$ ) (Figure 4F–H). On the contrary, top-soil under *Miscanthus* were on average enriched of available P, TP, TN, and available K respectively by  $+640 \text{ kg P}_2\text{O}_5 \text{ ha}^{-1}$ ,  $+160 \text{ kg P ha}^{-1}$ ,  $+120 \text{ kg N ha}^{-1}$ , and  $+40 \text{ kg K}_2\text{O ha}^{-1}$  (Figure 4B–E). However, soil nutrient stocks varied greatly among sites depending on initial soil nutrient content and crop yield. Indeed, the extent of the nutrient recycling under *Miscanthus* cultivation depended on soil nutrient content available before conversion to *Miscanthus* plantation and by nutrient offtake during the growing seasons (Magenau et al., 2022). If *Miscanthus* is harvested in the spring time when the plants are most senescence only a small proportion of nutrients is removed by harvesting because 60%–80% is remobilized in rhizomes and recycled in the system through fallen leaves and the turnover of rhizomes and roots (Amougou et al., 2011; Beuch et al., 2000; Christian et al., 2006; Magenau et al., 2022; Ruf et al., 2017; Strullu et al., 2011). For nutrient budget calculations to determine fertilizer requirements, the seasonal dynamics of nutrient contents in the aboveground biomass and the harvest time must be taken into account (Cadoux et al., 2012). In this study, total K stock is highly depleted after four growing seasons at all sites, as K is the primary nutrient absorbed by

*Miscanthus* (Cadoux et al., 2012; Magenau et al., 2022; Pidlisnyuk et al., 2014). Using plant nutrient concentration measured by Magenau et al. (2022) and plant yield measured by Awty-Carroll et al. (2022) in the same sites and for the same genotypes, on average K offtake was 96, 89, and 226 kg ha<sup>-1</sup> respectively for *M* × *g*, *M. sin* × *sin* and *M. sac* × *sin* hybrids after 4 years of *Miscanthus* cultivation. Overall, the highest nutrient offtakes were found for the higher-yielding *Miscanthus* hybrids, GRC14 (304.8 kg ha<sup>-1</sup>) followed by GRC15 (146.3 kg ha<sup>-1</sup>) (Magenau et al., 2022). During four growing seasons, the highest nutrients requirement of the *M. sac* × *sin* hybrids resulted also in an offtake of N (120 kg N ha<sup>-1</sup>) and P (29 kg P ha<sup>-1</sup>) compared to *M* × *g* (56 kg N ha<sup>-1</sup> and 13 kg P ha<sup>-1</sup>, respectively) and *M. sin* × *sin* (80 kg N ha<sup>-1</sup> and 17 kg P ha<sup>-1</sup>, respectively). We also observed that soils with high initial total K content, such as those previously occupied with grasslands (TWS and PAC2), led to luxury uptake compared to poorer soils (CHV and OLI). On these sites, it was indeed observed the most significant depletion of TK stock compared with other sites with an average depletion of -1.4 Mg K ha<sup>-1</sup>, while in CHV and OLI no significant variations were detected. According to Cadoux et al. (2012), nutrients removed at winter harvest are greatest, by proportion, for K (38%–57%) and approximately equal for N and P (36%–40%). These values confirm the findings on soil nutrient depletions and on nutrients offtake and clearly show that most of the nutrients are translocated during senescence in belowground biomass. In addition, nutrient remobilization from rhizome to aboveground plant biomass were lowest for K (7%–14%), N (9%–21%) and P (18%–36%), while the proportion of nutrients that were recycled either by translocation or leaf fall were lower for K (50%) and higher for N and P (60%). This indicates that, in the long term, *Miscanthus* hybrids have a higher nutrient requirement for K followed by N and P, and that K might become a limiting factor for growth on marginal site where no fertilization at planting is applied. In this study, plant available K was yearly accumulated in the topsoil (+30 kg ha<sup>-1</sup> year<sup>-1</sup>) as a result of the nutrient mineralization from plant litter deposited on soil surface after harvesting (Figure S6) (Cadoux et al., 2012; Kahle et al., 2001). However, the simultaneous reduction in the 10–30 cm layer (-20 kg ha<sup>-1</sup> year<sup>-1</sup>) indicates that this mechanism of nutrient recycling did not occur in this layer, where the amount of nutrients absorbed by the plant is greater than that derived from the decay of litter and roots (Figure S6). The increment of available P (+160 kg P<sub>2</sub>O<sub>5</sub> ha<sup>-1</sup> year<sup>-1</sup>) among all sites indicate that *Miscanthus* has the potential to sustain P recycling by increasing the amount of plant available phosphorous in upper soils layers. This was suggested also by Ferrarini et al. (2021) who found an increase of available P

(+240 kg P ha<sup>-1</sup> year<sup>-1</sup>) under *Miscanthus* cultivation of which averaged 30% from organic sources. Ferrarini et al. (2021) and Stutter et al. (2015) proposed that under unfertilized perennial crops, with high C-input, the increase of available P in soil is dominated by the increase of organic P forms deriving from the biological turnover of plant litter, roots and rhizome. This highest organic P input coupled with low P uptake by *Miscanthus* (Cadoux et al., 2012; Magenau et al., 2022), results in an accumulation of organic P forms that can be made bioavailable by microbial activity. This is confirmed also by the increased *alkP* activity, involved in the mineralization of organic P, which was stimulated by plant C input from decomposing litter (Figure 3a–e; Tables S5 and S6) (Renella et al., 2006). Despite the increment of total P (+43 kg P ha<sup>-1</sup> year<sup>-1</sup>) in four out of six sites remain unclear, it might be explained by external factors such as events of Saharan dust atmospheric deposition which occurred more frequently as winter storm (Gammoudi et al., 2024). The study's results showed that SOC variations were closely associated with changes in TN stock, highlighting that soil C:N ratios remain stable during *Miscanthus* cultivation (Kahle et al., 2001; Richter et al., 2015; Ruf et al., 2018). In previously managed grasslands, with land-use change to *Miscanthus*, both SOC and TN were significantly reduced in the topsoil layer (Figure S6). Previous studies suggested that the conversion from grasslands with C and nutrient-rich soils to perennials, can accelerate the degradation of existing SOC through the priming effects (Richter et al., 2015; Zatta et al., 2014). With this accelerated SOM degradation, also TN is reduced thorough SOM mineralization, by the soil microbial community, to bring nutrient and C supply close to microbial element demand (Bastida et al., 2019; Kirkby et al., 2013, 2014). However, the increase of TN stock in PAC1 and PAC2 sites (on average +150 kg ha<sup>-1</sup> in 0–30 cm), where SOC stock was reduced, can be explained by N<sub>2</sub> fixation under *Miscanthus* cultivation. The hypothesis of N<sub>2</sub> fixation has only recently been confirmed to occur in *Miscanthus* (Cadoux et al., 2012; Martani et al., 2020) where the presence of potentially N<sub>2</sub>-fixing bacteria was detected (Chen et al., 2020; Davis et al., 2010; Pidlisnyuk et al., 2014). Regarding stock variation of other plant secondary macronutrients such as Ca and Mg, in 4 years of *Miscanthus* cultivation, Ca offtake was higher than Mg as found also by (Dzeletovic & Glamoclija, 2015). Ca offtake estimated values for *M. sac* × *sin* hybrids (1.30 Mg Ca ha<sup>-1</sup>) followed by *M. sin* × *sin* (0.87 Mg Ca ha<sup>-1</sup>) and *M* × *g* (respectively 0.44 Mg Ca ha<sup>-1</sup>) confirmed that across locations, that soil nutrient depletion is affected by yield × initial level interaction. The greatest Ca depletion that was found at PAC1 site was the consequence of the highest biomass production (2.4 Mg Ca ha<sup>-1</sup>), and the highest availability of this

nutrients in soil among sites. In contrast, Mg offtake was similar across *Miscanthus* hybrids with an average value of  $13 \text{ kg ha}^{-1}$ . These Ca and Mg offtake values, their ratio and their correlation with soil stock variations confirmed the values found for *Miscanthus* by Dzeletovic and Glamoclija (2015), Pisani et al. (2024) and Hillel and Hatfield (2005).

## 4.2 | SOC sequestration and its drivers

Our findings show that after 4 years of marginal land conversion to *Miscanthus* cultivation, SOC was sequestered with an annual rate of  $+0.4 \text{ Mg C ha}^{-1} \text{ year}^{-1}$ , but this rate was highly influenced by previous land uses. In former arable lands, SOC did not vary significantly ( $-0.2 \text{ Mg C ha}^{-1} \text{ year}^{-1}$ ), while a significant SOC sequestration was found in former grasslands ( $+1.0 \text{ Mg C ha}^{-1} \text{ year}^{-1}$ ), but with different behaviors in the upper and the lower layer. *Miscanthus* establishment in two managed old grasslands, led to a significant reduction of SOC stock in the 0–10 cm layer ( $-1.3 \text{ Mg C ha}^{-1} \text{ year}^{-1}$ ) and enhanced in the 10–30 cm by  $+2.2 \text{ Mg C ha}^{-1} \text{ year}^{-1}$ . These changes are presumably due to redistribution of the soil in the profile by the inversion tillage occurred before *Miscanthus* plantation, where the decomposition of plant (grass) biomass has boosted SOM formation in 10–30 cm layer. Several studies throughout Europe have reported that the potential of soil C sequestration after land conversion to *Miscanthus* cultivation in marginal sites is still uncertain, showing rates of SOC loss/sequestration between  $-2.9$  and  $+4.5 \text{ Mg C ha}^{-1} \text{ year}^{-1}$  (Agostini et al., 2015; Dondini et al., 2009; Ouattara et al., 2021; Poeplau & Don, 2014; Richter et al., 2015; Rowe et al., 2016; Zimmermann et al., 2013). These contrasting results have been ascribed to site-specific factors like mean annual temperature (Emmerling et al., 2017; Poeplau & Don, 2014), soil texture (Brami et al., 2020; Emmerling et al., 2017; Kahle et al., 2001; Ruf et al., 2018), and on previous land uses (Emmerling et al., 2017; Poeplau & Don, 2014; Rowe et al., 2016; Ruf et al., 2018; Zatta et al., 2014; Zimmermann et al., 2013). Don et al. (2012) performed a meta-analysis of published literature and calculated an average SOC accumulation of  $+0.66 \text{ Mg C ha}^{-1} \text{ year}^{-1}$  for *Miscanthus* cultivated on former croplands, while there was no or even negative SOC stock change ( $-0.09 \text{ Mg C ha}^{-1} \text{ year}^{-1}$ ) if grasslands were converted to *Miscanthus*. Moreover, Wu et al. (2024) identified crop age as the most important driving factors of the SOC sequestration rate, which was estimated reaching a peak in 9 years.

Most of the experiments on land-use change to *Miscanthus* compared SOC stock value after a certain number of years of cultivation with those of time zero

before the land use transition. However, it is noteworthy that our study is the first one comparing SOC stock changes after *Miscanthus* establishment among different European sites using the ESM of Wendt and Hauser (2013) approach to calculate SOC stock variation. This approach provides a more accurate estimate of SOC stock variations by reducing errors deriving from a fixed depth sampling on sites showing changes on BD between sampling time points. Indeed, the reduction or the increase in BD over time can respectively results in an underestimation or overestimation of SOC stock changes (Fowler et al., 2023). The adoption of this ESM approach could hence explain the non-significant changes of SOC stock after croplands conversion to *Miscanthus* cultivation differently to other studies where SOC stock is calculated from fixed depth sampling (Lee et al., 2009).

To better understand the mechanisms of SOC sequestration under perennial crops, plant C input to soil from different plant organs need to be calculated (Agostini et al., 2015). In *Miscanthus*, a great quantity of photosynthesized C is allocated belowground in rhizomes, roots and roots exudates (Briones et al., 2023; Martani et al., 2020). Martani et al. (2020) reported that during 11 years of cultivation, *Miscanthus* (*M. × giganteus*) allocated in the belowground biomass  $5.7 \text{ Mg C ha}^{-1}$  of which 38% was allocated in rhizomes and 28% in fine roots. A recent study (Briones et al., 2023), found that *Miscanthus* hybrids with contrasting phenotypic and physiological traits divide different quantities of photo-assimilated C in plant organs that ranged between 16% and 22% in rhizomes and from 6% to 8% in roots. However, no simple allometric equations are available in literature to estimate C input from *Miscanthus* belowground organs to replace the time-consuming work of in-field belowground sampling. In this study, a novel approach was proposed to estimate C input to soil from different *Miscanthus* organs (stubble, roots, root exudates, and rhizomes). An allometric function to estimate RB from cumulative plant yields over the growing seasons was derived using published data. The average estimate of *Miscanthus* carbon input to the topsoil across all sites over 4 years was  $3.93 \text{ Mg C ha}^{-1}$  ( $0.98 \text{ Mg C ha}^{-1} \text{ year}^{-1}$ ). We identified significant differences in plant C input among hybrids as affected by the yield data (Table 1). In Southern Europe, novel *M. sac × sin* hybrids (GRC14 and GRC15) were the most productive, resulting in a plant C input to soil of  $1.32 \text{ Mg C ha}^{-1} \text{ year}^{-1}$ , while in Northern Europe, the *M. sin × sin* hybrid (GRC3) had the best yield performances and the highest C input to soil ( $1.36 \text{ Mg C ha}^{-1} \text{ year}^{-1}$ ). Generally, the standard clone *M × g* (GRC9) had the lowest plant C input to soil ( $0.66 \text{ Mg C ha}^{-1} \text{ year}^{-1}$ ). However, despite significant differences in plant C input among hybrids, no significant differences on SOC sequestration were observed among

hybrids (Tables S3 and S7a,b). In other studies that investigated the SOC sequestration potential of *Miscanthus* hybrids over time [e.g., after 5 (Ouattara et al., 2021), 6 (Zatta et al., 2014) and 14 years (Richter et al., 2015) after planting], no significant contributions of *Miscanthus* genotypes to SOC sequestration were found, despite differences in above- and belowground biomass allocation were observed. Results of the multiple linear stepwise regression showed that plant C input to soil was not a significant factor explaining SOC stock changes ( $\Delta$ SOC stock as  $\text{Mg C ha}^{-1}$ ) (Table 2), but that initial SOC content was the main driving factor of SOC stock change in all environments, especially in the 0–10 cm soil layer. The negative correlation between changes of SOC stock and the initial concentration of SOC suggested the occurrence of negative priming effect of the existing SOM in response to the input of easily decomposable organic substrates from roots and root exudates, which in our study accounted for 47% and 21% of total C input, respectively. This explains the significant depletion of SOC in the topsoil layer in grassland converted to *Miscanthus* cultivation where SOC levels were higher compared to the other sites within the same climatic region. In sites where no significant changes in SOC stock were observed, newly added C to soil from plant litter, roots, and rhizomes turnover, could have compensated the losses of SOC caused by the priming effect, hence compensating the loss of initial SOC as proposed by Richter et al. (2015) and Zatta et al. (2014). However, in sites previously managed as grassland, this mechanism of C compensation requires more time to occur. According to the meta-analysis of Siddique et al. (2023), the change of SOC stock under perennial crops follows a sigmoidal curve over time. The increase in SOC with perennial crops like *Miscanthus* started after approximately 5 years, compensating for losses of original SOC caused by soil disturbance, soil priming and lower productivity of newly established crops. Interestingly, this study highlighted a significant positive correlation between SOC concentration and the increased activity of lignin peroxidase (*perox*). This indicates that the release of oxidative enzymes, which contribute to lignin degradation of plant organs and to the humification process, can lead to a progressive accumulation of stable organic C (Sinsabaugh, 2010) and, in the long term, newly derived plant C could replace the initial SOC loss and enhance SOC stock (Table S5). It is noteworthy to highlight that two significant predictors of SOC change were the initial nutrient content of soils undergoing land use conversion and their elemental stoichiometry ratios with C. Results from the multiple linear stepwise regression (Table 2) identified that initial availability of soil nutrients (N and P), as well as their soil stoichiometry (C:N, C:P and N:P) strongly contributed to determine SOC change trajectories. It is known that the chemical

composition of plant residues and organic matter in soils affects microbial activity, thus controlling SOM cycling (Zechmeister-Boltenstern et al., 2015). This implies that microbial communities, to cope with C, N, and P imbalance, mineralize SOM to bring nutrients and C supply close to microbial element demand (Bertrand et al., 2019; Coonan et al., 2020; Kirkby et al., 2014). In this study, in the topsoil layer, initial TN content was positively correlated to  $\Delta$ SOC stock in both northern and southern climatic conditions. Several authors have reported that low nutrient availability, especially N, inhibits soil C sequestration and, on the contrary, C storage increases with N fertilization (Cadoux et al., 2014; Kirkby et al., 2014; Lemus & Lal, 2005; Tiemann & Grandy, 2015; Zhao et al., 2017). Moreover, both soil C:P and C:N stoichiometric ratios were positively correlated with  $\Delta$ SOC stock. For instance, when phosphate availability is scarce, an increase of soil C:P ratio has the potential to further increase C storage (Satrio et al., 2009). However, if P inhibits the formation of persistent C, but N stimulates it, the combined effect on C sequestration of N and P can be variable (Zhao et al., 2017). Our results revealed an inverse relationship between the N:P ratio and soil SOC stock variation mainly in SE. Nevertheless, the mechanisms by which P and both N and P affect soil C remains unclear. Compared with N and P, the contribution of K to soil C sequestration seems minor but is noticeable. High natural contents of soil K, or potash fertilization, generally promote plant biomass production and increase the root to shoot ratio in terrestrial ecosystems (Zhao et al., 2017). Given the high K demand of *Miscanthus* hybrids, it is important to assess K content before transplanting if both biomass production and soil C storage must be optimized. These results support the general hypothesis that a limitation of one or more of the plant macronutrients (NPK) may place a ceiling on the quantity of SOC that can be stored as stable C with these high-yielding novel *Miscanthus* hybrids.

## 5 | CONCLUSION

Within the GRACE project, a number of novel rhizome- and seed-based *Miscanthus* hybrids were cultivated across six European sites characterized by different marginality factors, pedoclimatic condition, and previous land use histories. This study sought to provide an early evaluation of the potential of four of these *Miscanthus* hybrids to sequester soil C and to improve chemical, biological, and physical soil quality. *Miscanthus* cultivation had a slightly negative impact on soil physical quality, but it remains site-specific. In unfertilized fields, cultivation of high-yielding *Miscanthus* genotypes cause a depletion of K, followed by N and P. The mechanism

of nutrient recycling in the plant–soil system was ineffective for K and, to a lesser extent, for N. Hence, to secure stable *Miscanthus* feedstock in the long-term, K, and N fertilization might be necessary, especially in former arable lands where initial nutrient contents might be low, while the biological turnover of the organic matter maintains or increases the P content, sustaining plant's P demand. After 4 years from *Miscanthus* establishment, although not significantly different from initial SOC stock, average SOC sequestration rate was 0.43 Mg SOC ha<sup>-1</sup> year<sup>-1</sup>. SOC sequestration was highly affected by previous land uses. Soil stoichiometry was a key controlling factor of SOC dynamics. Novel high-yielding *Miscanthus* hybrids provide in the short- to medium-term a secure biomass supply for the bio-based industry, but a longer period of cultivation is needed to substantially ameliorate overall soil quality and quantify the net C sink.

### AUTHOR CONTRIBUTIONS

**Marta Bertola:** Conceptualization; data curation; formal analysis; writing – original draft; writing – review and editing. **Elena Magenau:** Data curation; investigation. **Enrico Martani:** Data curation; investigation. **Mislav Kontek:** Data curation; investigation. **Chris Ashman:** Data curation; investigation. **Vanja Jurišić:** Conceptualization; funding acquisition; methodology. **Isabelle Lamy:** Data curation; investigation. **Jason Kam:** Resources. **Flavio Fornasier:** Data curation; formal analysis; investigation. **Jon McCalmont:** Data curation; investigation. **Luisa M. Trindade:** Resources. **Stefano Amaducci:** Conceptualization; funding acquisition; methodology; writing – review and editing. **John Clifton-Brown:** Conceptualization; funding acquisition; methodology; resources; writing – review and editing. **Andreas Kiesel:** Conceptualization; funding acquisition; methodology; project administration. **Andrea Ferrarini:** Conceptualization; data curation; formal analysis; investigation; methodology; supervision; validation; writing – original draft; writing – review and editing.

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### CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

### DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in Zenodo repository at <https://doi.org/10.5281/zenodo.10987591>.

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## SUPPORTING INFORMATION

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