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Quantifying the impacts of genetically engineered crops and deep soil C cycling on the sustainability of bioenergy crop production

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Abstract

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Bioenergy can help mitigate climate change by providing a carbon-neutral fuel source. However, multiple challenges exist to achieving carbon neutrality including converting lignocellulosic materials to fuel and enhancing soil C sequestration during the growth of the feedstocks. To address these challenges, there have been recent efforts to genetically modify feedstocks to produce more energy dense oils that increase fuel conversion efficiency and to cultivate deep-rooted perennial feedstocks that can enhance soil C storage. However, the C consequences and efficacy of these solutions remain largely uncertain.

To examine the C consequences of enhancing oil content of bioenergy feedstocks, I examined the impact of Sugarcane litter decomposition on soil carbon (C) formation and loss and determined if the genetic modifications to produce Oilcane alter these dynamics. To do this, I traced the fate of Sugarcane and Oilcane litter in protected and unprotected soil C pools. I found that both crops led to net soil C gains dominated by an accumulation of the litter as particulate organic carbon (POC) and that the genetic modifications to Oilcane did not substantially alter soil C dynamics. To investigate the efficacy of deep-rooted perennial feedstocks to build soil C, I linked depth gradients in root biomass with microbial activity and soil C stocks down to 1 meter to determine the predictors of soil C and soil C fractions with depth. I also performed a lab experiment where I examined differences between depths in the ability of simple C inputs to prime or build soil C. In the field, I excavated quantitative 1 m deep soil pits under 20-year-old Miscanthus plots and quantified, fine root biomass, total soil C, mineral-associated organic C (MAOC), particulate organic C (POC), microbial respiration, net nitrogen cycling, and enzyme activities. In the lab, I experimentally followed the fate of ^{13}C labeled glucose into soil C fractions at each depth. I found that soil C and MAOC declined with depth and were best predicted by fine root biomass, representing inputs, microbial respiration, representing losses, and NAG activity, representing the recycling of microbial necromass. I also found that deep soils had a greater potential to minerally stabilize new simple C inputs than shallow soils due to the C inputs having a greater stabilizing than priming effect below 50cm. Collectively, my research shows that sustainable bioenergy solutions such as lipid enhanced Oilcane and growing deep-rooted perennial feedstocks may lead to enhanced soil C.

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Chapter 1: Introduction

Bioenergy can help mitigate climate change by providing a carbon-neutral fuel source through reducing our reliance on fossil fuels and enhancing soil carbon (C) sequestration during the growth of the feedstocks (Mathews, 2008). However, multiple challenges exist to achieving carbon neutrality. First, most varieties of perennial bioenergy crops are not able to be efficiently converted to fuel owing to long standing difficulties in transforming lignocellulose materials (Cheng and Timilsina, 2011). Second, the most widely used feedstock in the U.S is corn, which under conventional management practices, has been shown to reduce soil C (Lu et al., 2018). Therefore, there is a critical need to determine solutions that can enhance our ability to turn feedstocks into fuels and increase soil C storage to ensure the C neutrality of bioenergy.

To enhance our ability to turn feedstocks into fuels, there have been recent efforts to genetically engineer bioenergy crops to contain higher amounts of energy dense materials such as oils (Limayem et al., 2012, Parajuli et al., 2020 and Zale et al., 2015). One successful effort is the development of Oilcane, a genetically modified version of Sugarcane, which has altered litter chemistry owing to genetically enhanced oil production in the stems and leaves (Cheng & Timilsina, 2011; Limayem & Ricke, 2012; Parajuli et al., 2020; Zale et al., 2016). The development of oilcane raises an important question: Do the genetic modifications in Oilcane alter soil microbial activity and soil C storage? (Cerri et al., 2011; de Resende et al., 2006).

There are multiple reasons why Oilcane may differ from Sugarcane in litter decomposition and the resulting impacts on micobial activity and soil C storage. Sugarcane litter has a high C:N ratio, decomposes slowly, and can increase soil C stocks by remaining in the soil as undecomposed or partially decomposed particulate organic C (POC; Phukongchai et al., 2022). However, POC is largely unprotected and subject to further decomposition making it

less ideal for building stable soil C stocks (Cotrufo et al., 2013). By contrast, Oilcane litter is likely of higher quality than Sugarcane owing to a lower C:N ratio due to a 44%–59% reduction in soluble lignin resulting in more rapid decomposition (Parajuli et al., 2020). This rapid decomposition has the potential to both prime soil C losses through greater respiratory losses of soil C (Strickland et al., 2015; Talbot et al., 2012) or increase the stabilization of simple, microbially derived C in mineral-associated organic C (MAOC) that is highly stable and physically protected from microbial attack (Cotrufo et al., 2013; Lehmann & Kleber, 2015).

To increase soil C storage to ensure the carbon neutrality of bioenergy, recent research suggests that growing deep-rooted perennial crops may be an effective solution (Thorup-Kristensen et al., 2020). Compared to corn, growing deep-rooted perennial crops may increase soil C sequestration due to less physical soil disturbance through reduced tilling and their deeper root systems can increase C inputs to deeper soil layers where it may have a greater potential to be stabilized (Chimento et al., 2016). However, most of this research has focused on shallow soils (i.e., <30 cm), ignoring the importance of deep soils that have low C concentrations, but due to their volume contain nearly half of all soil C (Jobbagy et al., 2000; Dietzel et al., 2017). This focus on shallow soils has created unknowns in what drives depth gradients and whether simple C inputs would prime or stabilize soil C. On one hand, decomposing roots and the active release of root exudates can stimulate microbial activity and the production of enzymes to have a priming effect leading to the decomposition and respiratory loss of SOC that is accessible to microbes, known as particulate organic matter (POC) (Shahzad et al., 2018; Fontaine et al., 2007; Wang et al., 2014; Tian et al., 2016). On the other hand, dead roots and root exudates can drive soil C gains by having a stabilizing effect through enhancing the production of microbial biomass and products that are preferentially sorbed onto clay mineral surfaces to form

microbially inaccessible C, known as mineral-associated organic C (MAOC) (Cotrufo et al., 2013). Enhancing the root stabilization effect to increase the amount of MAOC while minimizing the priming effect to reduce C losses of POC is essential to build and store soil C to achieve carbon neutrality for bioenergy. However, the balance between root priming vs. stabilization and how it changes with depth is currently unknown.

Due to these unknowns, the overarching goal of my master's research is to examine whether these potential sustainable solutions lead to soil C gains or losses by answering the following research questions:

Solution 1: *Genetically modifying feedstocks to enhance our ability to turn them into fuels.*

1) To what degree does Sugarcane litter differ from Oilcane litter in their impacts on microbial activity and their ability to form new soil C?

Solution 2: *Cultivating deep rooted perennial crops to enhance soil C storage.*

2) What factors influence depth gradients in C stocks in Miscanthus, a deep-rooted perennial grass?
3) Do deep soils differ from shallow soils under Miscanthus in their potential to stabilize new simple C inputs?

To address these research questions, I used a combination of lab incubations and field observations. For solution 1, I traced the fate of Sugarcane and Oilcane litter into respiratory losses and soil C pools in a jar incubation using natural ^{13}C abundance differences between C_4 litter and C_3 forest soils. For solution 2, I observationally quantified depth gradients in roots, microbial activity, and soil C pools down to 1 meter under Miscanthus and experimentally tested the ability of deep root exudates to build MAOC by adding ^{13}C glucose to soils from each depth in a jar incubation.

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Chapter 2: Lipid-enhanced Oilcane does not impact soil carbon dynamics compared with wild-type Sugarcane

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2.1 Abstract:

The carbon neutral potential of bioenergy relies in part on the ability of feedstocks to sequester carbon (C) in the soil. Sugarcane is one of the most widely used bioenergy crops, yet there remain unknowns about how it impacts soil C dynamics. In addition, Oilcane, a genetically modified version of Sugarcane has been produced to accumulate more energy-dense oils and less soluble lignin, which enhances conversion efficiency but may also impact soil C cycling. Thus, our objectives were to examine the impact of Sugarcane litter decomposition on soil C formation and losses and determine if the genetic modifications to produce Oilcane alter these dynamics. To do this, we incubated bagasse (processed stem litter) and leaf litter from Sugarcane and Oilcane in microcosms with forest soil for 11 weeks. We used differences in natural abundance δ13C between C3 forest soil and C4 litter to trace the fate of the litter into respiratory losses as well as stable and unstable soil C pools. Our results show that genetic modifications to Oilcane did not substantially alter soil C dynamics. Sugarcane and Oilcane litter both led to net soil C gains dominated by an accumulation of the added litter as unstable, particulate organic C (POC). Oilcane litter led to small but significantly greater net soil C gains than Sugarcane litter due to greater POC formation, but the formation of stable, mineral associated organic matter (MAOC) did not differ between crop types. Sugarcane and Oilcane had opposing effects on tissue type where Sugarcane bagasse formed more MAOC, while Oilcane leaves preferentially remained as POC which may have important management implications. These results suggest that genetic modifications to Sugarcane will not significantly impact soil C dynamics; however, this may not be universal to other crops particularly if modifications lead to greater differences in litter chemistry.

2.2 Introduction:

Maximizing the carbon-neutral potential of bioenergy as a fuel source relies on the ability of the feedstocks to sequester carbon (C) in the soil (Mathews, 2008). Currently, Saccharum officinarum (herein Sugarcane) is one of the most widely used bioenergy feedstock due to its high biomass yields, conversion efficiency, and adaptability for a wide range of growing conditions (Hoang et al., 2015; Lam et al., 2009). The conventional Sugarcane harvest method of burning the field to remove the leaves for easier harvest of the stems diminishes the crop's carbon-neutral potential by increasing C emissions. Recently, there has been a shift toward more sustainable harvesting practices that leave the leaf litter on the field rather than burning it as well as adding the processed stem litter (bagasse) back as a soil amendment. These sustainable harvesting practices significantly increase the amount of biomass left on the field which has the potential to increase soil C stocks. However, it remains unknown whether these sustainable practices significantly enhance soil C (Cerri et al., 2011; de Resende et al., 2006). In addition, there have been recent efforts to genetically modify Sugarcane to enhance its conversion efficiency and optimize the bioenergy crop (Lam et al., 2009). One successful effort is the development of Oilcane, which has altered litter chemistry owing to genetically enhanced oil production in the stems and leaves (Cheng & Timilsina, 2011; Limayem & Ricke, 2012; Parajuli et al., 2020; Zale et al., 2016). As litter chemistry regulates the microbial activity and shifts soil C dynamics (Cotrufo et al., 2015), it is critical to quantify and compare the abilities of both Sugarcane and Oilcane litter to form soil C.

The ability of sustainable harvesting practices to increase soil C stocks relies on the degree to which the litter and bagasse inputs lead to a net priming or a net stabilization effect. Overall, the resulting balance between priming and stabilization is likely driven by differences in

litter quality between Sugarcane and Oilcane (Figure 2.1). Lower-quality Sugarcane litter with a high C:N ratio decomposes slowly and can increase soil C stocks by remaining in the soil as undecomposed or partially decomposed particulate organic C (POC; Phukongchai et al., 2022). However, POC is largely unprotected and subject to further decomposition making it less ideal for building stable soil C stocks (Cotrufo et al., 2013). By contrast, Oilcane litter is likely of higher quality than Sugarcane owing to a lower C:N ratio due to a 44%–59% reduction in soluble lignin resulting in more rapid decomposition (Parajuli et al., 2020). This rapid decomposition has the potential to both prime soil C losses and stabilize soil C. On the one hand, Oilcane litter may prime the decomposition of soil organic C (SOC) and lead to greater respired C losses (Strickland et al., 2015; Talbot et al., 2012). On the other hand, enhanced decomposition in Oilcane may increase the stabilization of simple, microbially derived C in mineral-associated organic C (MAOC) that is highly stable and physically protected from microbial attack (Cotrufo et al., 2013; Lehmann & Kleber, 2015). Recent advances in soil stabilization theory suggest the more microbially accessible Oilcane litter may promote a higher microbial C use efficiency (CUE) and enhance the production of microbial products that preferentially form MAOC in grassland and crop ecosystems (Angst et al., 2021; Bradford & Crowther, 2013; Liang et al., 2017; Manzoni et al., 2012). Therefore, the sustainable harvesting practices with Sugarcane versus Oilcane litter may have disparate priming and stabilizing effects due to differences in litter quality and the resulting impacts on microbial decomposition.

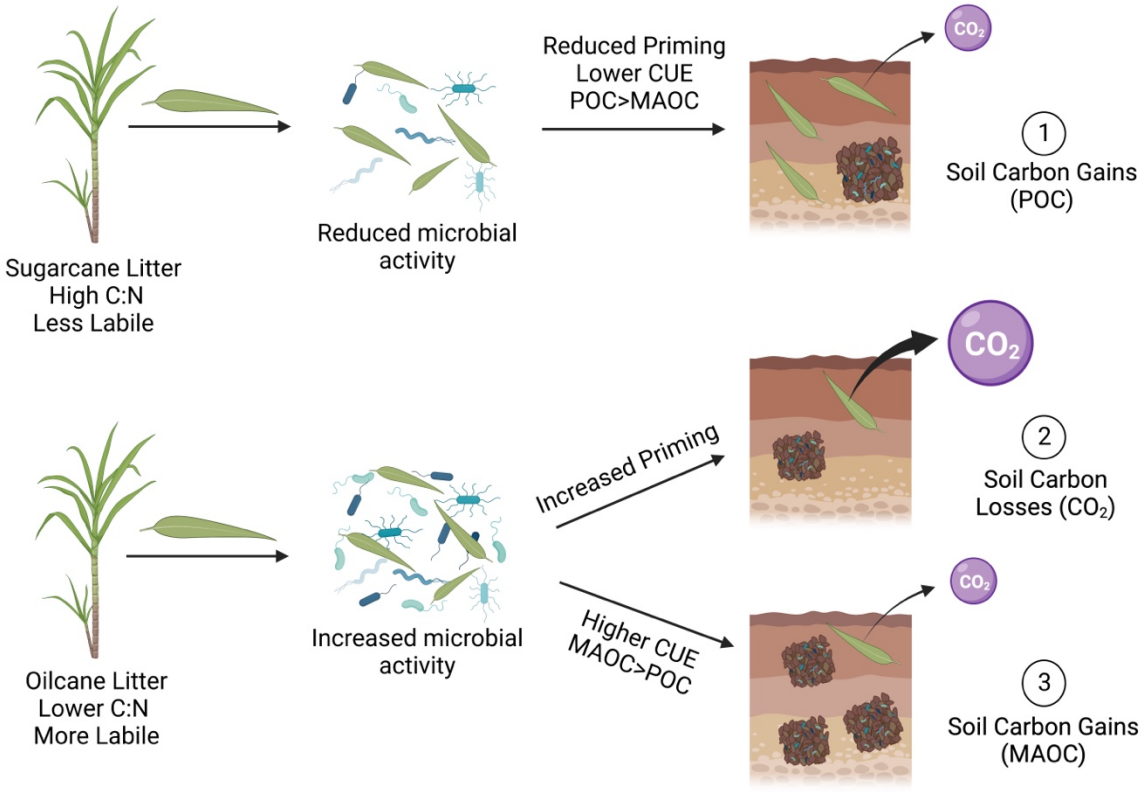

Fig. 2.1: Hypothesized impacts of Sugarcane and Oilcane litter on soil carbon stocks. (1) Lower-quality Sugarcane litter will lead to soil carbon gains that are mostly particulate organic C (POC). (2) Higher-quality Oilcane litter will lead to increased soil carbon losses of primed soil organic matter. (3) Higher-quality Oilcane litter will be decomposed with a higher carbon use efficiency by the microbial communities resulting in soil carbon gains that are mostly mineral associated organic matter (MAOC). Created with BioRender.com.

Given the potential for sustainable harvesting practices to enhance soil C, it is important to investigate how Sugarcane litter builds stable soil C and impacts existing SOC. As such, our first objective was to quantify the balance between Sugarcane litter priming the loss of existing soil C stocks versus forming new stable SOC. In addition, genetic alterations to increase the energy density may increase the lability of the litter and impact microbial activity. Therefore, our second objective was to quantify differences between Sugarcane and Oilcane litter in the balance of soil C priming losses versus stable soil C gains. To meet our two objectives, we conducted a

lab incubation experiment to trace the fate of Sugarcane and Oilcane bagasse and litter and tested the following hypotheses: (1) Due to its high C:N ratio, Sugarcane litter will reduce microbial decomposition resulting in more of the litter forming POC than MAOC compared with Oilcane litter. Due to the two competing mechanisms in which Oilcane litter could either enhance or reduce soil C stocks, we have two competing hypotheses: (2) The lower soluble lignin content of Oilcane will result in faster decomposition than the Sugarcane litter and result in greater priming-induced losses of soil C as a result of increased microbial activity, or (3) The greater decomposition of the more labile Oilcane litter will result in a higher microbial CUE and greater accumulation of MAOC.

2.3 Methods

Experimental design

To test our hypotheses, we compared the decomposition of Sugarcane and Oilcane litter in a laboratory microcosm experiment. We did this by incubating C_4 Sugarcane and Oilcane bagasse and leaf litter in C_3 forest soils that differed in their natural abundance ^{13}C isotopic signatures. Our study included control of soil with no litter (S) and 4 litter treatments with 10 replicates each (total of 50 incubations): soil and Sugarcane bagasse (S-B), soil and Sugarcane leaves (S-L), soil and Oilcane bagasse (O-B), and soil and Oilcane leaves (O-L). We measured the concentration and $\delta^{13}C$ signature of CO_2 to quantify how much of the added litter versus existing SOC was respired. After 11 weeks, we density fractionated the soil to quantify the added litter C in POC and MAOC by assessing the $\delta^{13}C$ signature of these soil C fractions.

Soil collection

We collected 10 kg of fine-loamy, mixed, active, mesic Ultic Hapludalfs soil in September 2021 from the top 15 cm of multiple locations in a 20 × 20 m forest plot dominated by

the arbuscular mycorrhizal (AM) tree species, sugar maple (Acer saccharum), and tulip poplar (Liriodendron tulipifera), at Tom's Run Nature Preserve in Morgantown, West Virginia. We brought the soil back to the lab and sieved it through a 2-mm sieve to remove large roots and rocks and to homogenize the soil. We sieved the soil within 1 week of collection and stored it at 5°C prior to incubation.

Litter processing

The Oilcane was genetically modified as detailed by Parajuli et al. (2020). The Sugarcane and Oilcane were grown through direct organogenesis in media, transferred to a temperature-controlled greenhouse (27 ± 2°C) for 2 months, and then transplanted into the field at the Plant Science Research and Education Unit in Citra, FL where they grew from April 2020 until October 2020. The crops were harvested in the Fall of 2020. During harvest, the leaves were separated from the stems for collection. In the lab, the bagasse was produced by extracting the juice from the stems using a benchtop juice extractor (JuiceMattic SC-3, Juicernet, FL, USA). Both the bagasse and leaves were oven dried at 50°C and then ground using a hammer mill with a 2-mm sieve (W-8-H, Schutte-Buffalo Hammermill). Lipid content, C:N ratio, and $\delta^{13}C$ are provided in Table 2.1. Complete details on the growth and processing of the litter can be found in Maitra et al. (2022).

Table 2.1- Litter and soil properties

Litter type	Total lipid content (%/gdw)[1]	C:N	$\delta^{13}C$
Sugarcane bagasse	3.19 ± 0.11	267[a]	-12.77
Sugarcane leaves	2.96±0.06	123[b]	-12.86
Transgenic Oilcane 1566 bagasse	3.59±0.21	163[c]	-12.75
Transgenic Oilcane 1566 leaves	3.27±0.02	183[c]	-12.58
C_3 forest soil	N/A	11[2]	-26

Note: Values are mean ± SE and letters represent significant differences.

[1] Data from Maitra et al. (2022).

[2] Data from Raczka et al. (2021).

Microcosm incubation

For the incubation, we used wide-mouthed glass mason jars (930 mL) with rubber septa installed into each lid. To set up the incubation, we mixed the sieved soil and added 70 ± 5.0 g of field moist soil (45% gravimetric water content) to all 50 jars. For the 10 control jars, we did not add any litter. For the remaining 40 jars, we added 0.5 g of dry litter according to the treatment (10 jars received Sugarcane bagasse, 10 jars received Sugarcane leaves, 10 jars received Oilcane bagasse, and 10 jars received Oilcane leaves). We sealed the mesocosms with the lids and incubated for 11 weeks in the dark (under blackout curtains) at room temperature (~22°C).

Respiration and $\delta^{13}C$ of CO_2 measurements

To determine the amount of added litter C and SOC that was respired, we sampled the microcosm headspace weekly for 11 weeks including measurements on days 1, 3, and 10, for a total of 13 time points. We measured the total microbial production of CO_2 using an infrared gas analyzer (LI-6400, LI-Cor Biosciences Inc.) and the $\delta^{13}C$ signature of the respiration with a Picarro G2201 (Picarro Inc.). Due to the C isotopic differences between the C_3 forest soil and

C_4 litter, we were able to use the $\delta^{13}C$ signature to calculate the proportion of the total respiration that came from the added litter versus SOC using the two end-member mixing model using the following equation:

(1) $pCO_{2\ litter} = (\delta^{13}C_{\ sample} - \delta^{13}C_{\ soil})/(\delta^{13}C_{\ litter} - \delta^{13}C_{\ soil})$

where pCO2 litter is the proportion of the total CO2 attributed to microbial respiration of the litter, the δ13C signatures of each sample and the soil controls were measured weekly on the Picarro, and the δ13C signature of the litter was obtained using a Thermo Fisher Delta V+ isotope ratio mass spectrometer interfaced with a Carlo Erba NC2500 Elemental Analyzer at the University of Maryland Center for Environmental Science Appalachian Laboratory. To calculate the amount of CO2 attributed to the respiration of the added litter, we multiplied the pCO2 litter by the total CO2 respired. We then calculated the CO2 attributed to the respiration of soil organic matter by subtracting the CO2 derived from the added litter from the total CO2 respired (Morrissey et al., 2017; Ridgeway et al., 2022).

After the gas samples were taken, we aerated the jars for 20 min before resealing them and then placing them back under a blackout curtain at room temperature (~22°C). At the end of the 11 weeks, we terminated the incubation and air-dried the soils for 2 months at room temperature (~22°C).

Recovery of Sugarcane and Oilcane litter in soil organic carbon fractions

We density fractionated the dry soils to determine the amount of litter C that ended up in the POC versus the MAOC fractions as outlined in Lavallee et al. (2020). We performed a water extraction followed by an extraction with 1.85 g/mL sodium polytungstate (SPT) solution to separate out the light fraction of the POC. We then wet-sieved the remaining heavy fraction using a 53-μm sieve to separate the heavy fraction of the POC and MAOC (POC > 53 μm, MAOC < 53 μm). We dried each fraction at 60°C and weighed them to calculate the recovery. We calculated mass recovery by comparing the total mass recovered in all three fractions to the

initial sample mass prior to fractionating. All the samples had recoveries of 100% ± 5% except for 3 that were 100% ± 10%. Lastly, we ground and analyzed each fraction for %C, %N, and $\delta^{13}C$ using a Thermo Fisher Delta V+ isotope ratio mass spectrometer interfaced with a Carlo Erba NC2500 Elemental Analyzer at the University of Maryland Center for Environmental Science Appalachian Laboratory. We scaled the results to the mass of dry soil in each jar to determine the proportion of total added litter that ended up in each fraction.

Estimation of the balance between soil C losses versus new soil C gains

To determine if the treatments had net soil C losses or gains, we calculated the difference between soil C lost through priming and the new soil C gained from the added litter. We calculated the soil C losses through priming by subtracting the average cumulative respiration in the control jars without litter from the cumulative soil respiration in each jar with litter. Finally, we calculated the new soil C gains from the added litter by summing the litter C recovered in the POC and MAOC fractions.

Statistical analysis

To determine the extent to which the genetic modifications to oilcane altered microbial respiration of the litter and SOC as well as the fate of the litter into the different soil C fractions, we performed a two-way analysis of variance (ANOVA) in R Studio Posit Cloud (Copyright © 2022 Posit Software, PBC). In this analysis, we used crop (Oilcane vs. Sugarcane), tissue type (bagasse vs. leaves), and their interaction as factors. When there was a significant interaction or a trend between crop and tissue type, we made post hoc multiple comparisons using the Tukey–Kramer HSD test. We categorized significance as having a p-value <0.05 and p-values <0.1 were

considered to be a trend. Data points above or below two standard deviations of the mean were removed as outliers.

2.4 RESULTS

Respiratory losses and soil priming effect of added litter

After 11 weeks of respiration measurements, we found no differences in the cumulative total (soil + litter), soil, or litter respiration between Oilcane and Sugarcane. However, there was more total respiration in jars with bagasse than leaves (Figure 2.2a, Tissue Type $p < 0.01$), which was due to significantly more soil-derived respiration (Figure 2.2c, Tissue Type $p < 0.0001$). We also found there was a trend between crop and tissue type on the amount of leaf litter and bagasse litter respired in which we identified a trend for Oilcane but not for Sugarcane (Figure 2.2b, Crop * Tissue Type $p < 0.1$). We found that litter additions led to the priming of existing SOC. All the litter addition treatments had greater respiration of SOC than the control treatment (Figure S1; $p < 0.0001$). In addition, this priming varied by tissue type with greater SOC priming in jars with bagasse than leaves (Figure 2.2d, Tissue Type $p < 0.0001$).

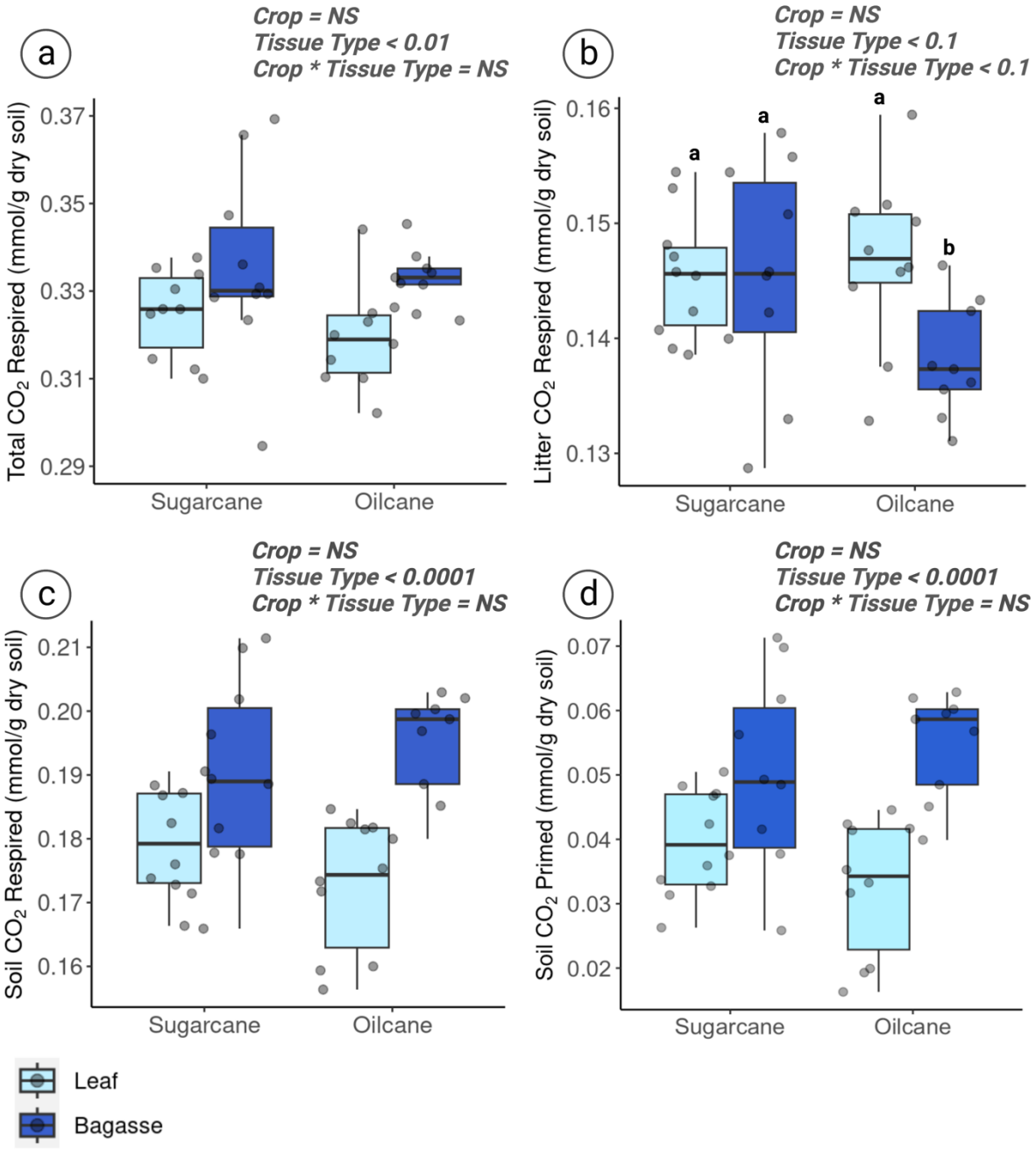

Fig. 2.2: (a) Total respiration (soil + litter). (b) Microbial respiration of the added litter. Microbial respiration of soil organic C (SOC; c). SOC priming (difference between soil CO2 respired in each treatment jar versus the average of the control jars; d). Data shown are 10 replicates excluding outliers for each treatment. Created with BioRender.com.

Recovery of the added litter in soil C fractions

On average, we recovered 98% of the added litter C after fractionating the soil organic matter. Across all treatments, the greatest amount of litter C was recovered in the POC (~55%), followed by CO_2 (~37%), and then MAOC (~7%).

Of the proportion of added litter C that was respired, there was an interactive effect between crop and tissue type. There was significantly more litter C respired with Oilcane leaves than Oilcane bagasse (Figure 2.3a, Crop * Tissue Type $p < 0.001$). There were main effects of crop and tissue type on the proportion of litter that was recovered in the POC fraction (Figure 2.3b, Crop $p < 0.05$, Tissue Type $p < 0.05$). However, there was an interactive effect of crop and tissue type where the proportion of litter C recovered in POC was significantly higher for Oilcane leaves than the other treatments (Figure 2.3b, Crop * Tissue Type $p < 0.05$). Lastly, tissue type had a significant main effect on the proportion of litter C recovered in the MAOC fraction (Figure 2.3c, Tissue Type $p < 0.0001$). In addition, there was a significant interactive effect of crop and tissue type (Figure 2.3c, Crop * Tissue Type $p < 0.05$) where there was a significant difference in the proportion of the litter C recovered between Sugarcane but not Oilcane tissue types. Sugarcane bagasse led to significantly more MAOC formation than Sugarcane leaves.

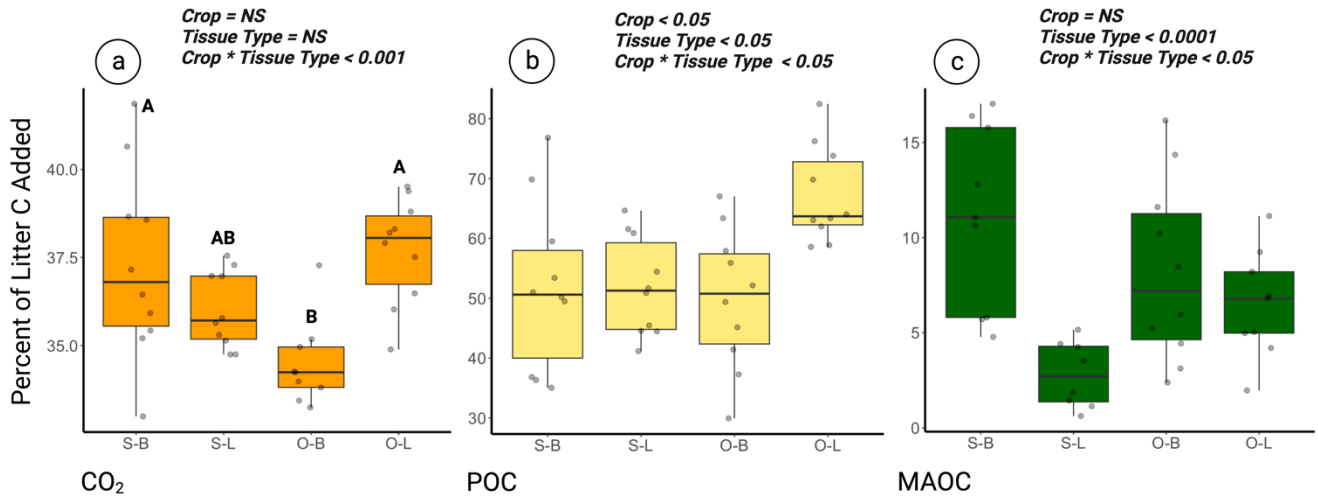

Fig. 2.3: Added litter C recovered in CO2 respired (a), particulate organic C (POC; b), and mineral associated organic matter (MAOC; c). Sugarcane bagasse (S-B), Sugarcane leaves (S-L), Oilcane bagasse (O-B), and Oilcane leaves (O-L). Data shown are 10 replicates excluding outliers for each treatment. Created with BioRender.com.

Net soil C gains

All litter treatments had net soil C gains, where the litter C incorporated into SOC exceeded the soil C respired. Overall, Oilcane had greater net soil C gains than Sugarcane (Figure 2.4, Crop $p < 0.05$). In addition, there was an interactive effect between crop and tissue type where leaves had greater net soil C gains than bagasse for Oilcane (Figure 2.4, Crop * Tissue Type $p < 0.05$).

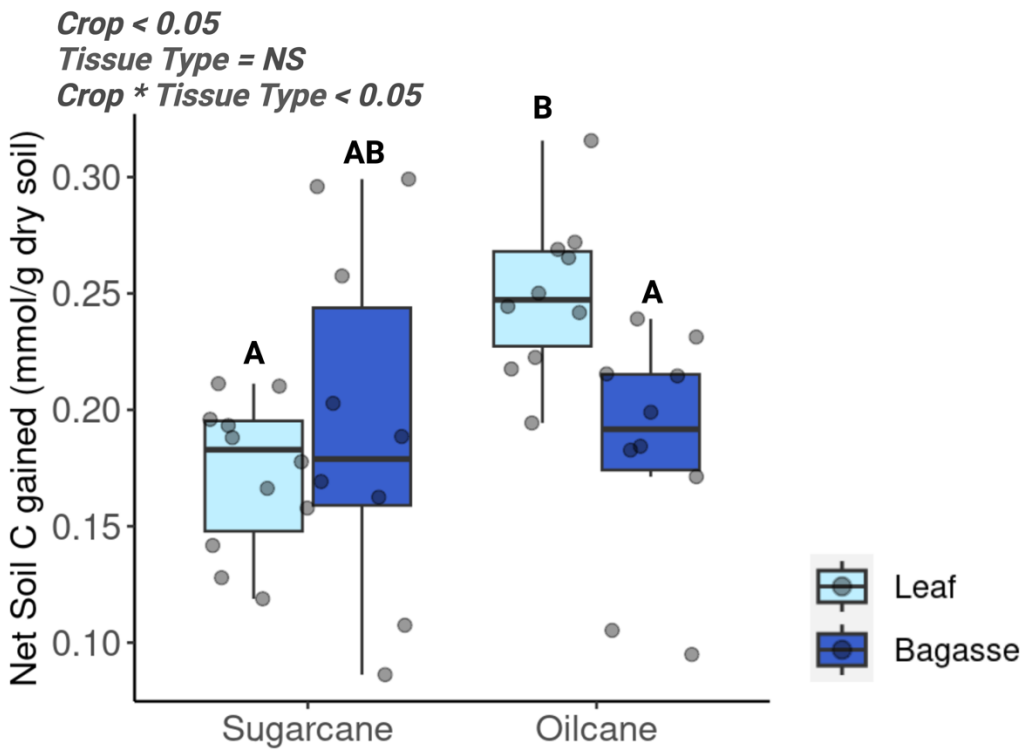

Fig. 2.4: Difference between soil C lost through priming and new soil C from the added litter. Data shown are 10 replicates excluding outliers for each treatment. Created with BioRender.com.

2.5 DISCUSSION:

In support of H1 that Sugarcane would enhance the POC pool, we found that the addition of Sugarcane litter led to net soil C gains that were primarily driven by an accumulation of undecomposed litter in the POC fraction (Figure 2.3b). This finding is most likely explained by the high C:N ratio of the Sugarcane litter slowing microbial decomposition of the substrate. In support, previous research shows that the beginning stage of Sugarcane bagasse decomposition is dominated by microbes degrading the more labile parts of the litter, leaving behind the more recalcitrant components (Phukongchai et al., 2022). In addition, the priming losses of soil C we observed (Figure 2.2d) may be driven by the decomposition of the labile litter components which may have reduced the energetic constraints of soil microbes (Nottingham et al., 2009). Although

the added Sugarcane litter led to priming losses of soil C (Figure 2.2d), these losses were outweighed by the gains of new litter C in the POC fraction (Figure 2.3b), resulting in net soil C gains (Figure 2.4). These findings are consistent with longer-term (>1 year), in-situ Sugarcane decomposition experiments (Cerri et al., 2011; Galdos, Cerri, & Cerri, 2009; Robertson & Thorburn, 2007) as well as Sugarcane decomposition models (Brandani et al., 2015; Galdos, Cerri, Cerri, Paustian, et al., 2009; Silva-Olaya et al., 2017), which also report increases in soil C following the shift from harvest via burning to leaving the Sugarcane litter on the field. However, it is uncertain how long the recalcitrant components of the litter in the POC fraction will remain undecomposed and stable in the soil. Recalcitrant POC is highly susceptible to loss under conditions that may enhance microbial decomposition, like increasing soil temperature or changes in N availability (Benbi et al., 2014; Li et al., 2018). While our findings suggest that the initial addition of Sugarcane bagasse and litter enhances soil C stocks, the stability of this soil C over longer time scales is uncertain.

Our findings did not support H2 that Oilcane would increase priming losses of soil C or H3 that Oilcane would build soil C in stable MAOC. Instead, we found that Oilcane did not differ from Sugarcane in priming (Figure 2.2d) or the incorporation of litter into MAOC (Figure 2.3c). The lack of support for these hypotheses likely reflects marginal differences in the C:N ratios between the two plants. Although the genetic modifications to Oilcane decreased the plant's C:N ratio by 11.5% compared with Sugarcane (Table 2.1), these ratios remain relatively high, and a marginal C:N reduction may not be enough for microbes to overcome their nitrogen limitations and increase decomposition of the substrate or SOC (Moorhead & Sinsabaugh, 2006). In addition, the greater lipid content in the Oilcane litter could have increased the hydrophobicity of the litter and led to reductions in the ability of microbes to decompose Oilcane litter (Lützow

et al., 2006). In support, we found that Oilcane litter additions led to greater soil C gains than Sugarcane with more of the Oilcane litter remaining undecomposed in the light POC fraction (Figure 2.4). Regardless of the exact mechanisms, our results show that in the short term, the genetic modifications to Oilcane did not lead to soil C losses compared with Sugarcane and may even enhance soil C gains.

Sugarcane and Oilcane had opposing effects of tissue type (i.e., bagasse vs. leaf litter) on the fate, priming, and net soil C gains that may have important management implications for building stable soil C. We found that Sugarcane bagasse formed more MAOC than Sugarcane leaves while Oilcane leaves preferentially remained as POC (Figure 2.3b,c). These differences have the potential to impact the amount of new litter C that can be stored and its residence time in the soil. MAOC is more stable than POC, but there is an upper limit to building MAOC because the available mineral surfaces can become saturated (Cotrufo et al., 2019). By contrast, there is not a clear saturating limit to POC formation, but POC may have a shorter residence time depending on litter chemistry and microbial activity (Burns et al., 2013; Cotrufo et al., 2019; Stewart et al., 2009). Therefore, soils that are saturated or close to MAOC saturation may have the potential to build more soil C by adding Oilcane leaf litter while soils that have low C and high mineral content may have the potential to enhance soil C stocks by adding Sugarcane bagasse litter (Castellano et al., 2015). In addition to the interactive differences in litter fate, bagasse litter additions led to greater priming of native SOC than leaf litter additions (Figure 2.2d). As a result, Oilcane leaves led to greater net soil C gains than Oilcane bagasse, which indicates that there may be a tradeoff between building new stable MAOC and losing SOC through priming with bagasse litter additions in certain crops. The differences observed in bagasse and leaf litter are likely attributed to structural differences between the two tissue types

(e.g., C:N ratio, lipid content, and differences in the proportion of and structural characteristics of cellulose, hemicellulose, and lignin; Nottingham et al., 2009; Schmatz et al., 2020). However, regardless of the exact litter chemistry control, these results suggest that the tissue type of litter you amend the field with may impact the stability, retention time, and net effect of new soil C gains.

Sugarcane and Oilcane may build less MAOC and more POC in comparison with bioenergy crops with lower C:N ratio litter. In a similar experiment with corn and miscanthus, 22%–29.3% of the added litter C formed MAOC compared with only 7.22%–7.39% with Sugarcane and Oilcane (Ridgeway et al., 2022). In addition, Sorghum aboveground litter in the field formed more MOAC than POC (Fulton-Smith & Cotrufo, 2019). While differences in experimental conditions limit direct comparisons, our results suggest that the recalcitrant nature of Sugarcane and Oilcane litter may be limiting MAOC formation and enhancing POC to a greater degree than other, lower C:N bioenergy crops. Overall, this comparison indicates that there are emergent differences between bioenergy crops in how they build soil C.

While our results point to important differences in how Sugarcane and Oilcane build soil C, we acknowledge that there are limitations to our study. First, our study was conducted in a microcosm that excludes seasonal and diurnal fluctuations in soil temperature and moisture as well as living roots that may have an impact on litter decomposition and soil C dynamics. Although these field conditions were absent, microcosm experiments have proven important in identifying and isolating mechanisms that can help explain observations and experimental results from the field (Benton et al., 2007; Cortez et al., 1996; Craig et al., 2022; Nicolardot et al., 2007; Sokol & Bradford, 2019; Strickland et al., 2009). Second, we ran our incubation experiment for

11 weeks which likely only captured the initial stages of litter decomposition. However, the initial stage of decomposition is an important indicator of long-term stabilization patterns (Craig et al., 2018). Future research should examine the long-term dynamics of new MAOC or POC formation in Sugarcane systems by following the fate of litter enriched in ^{13}C and ^{15}N in the field. Finally, although both Sugarcane and the forest stands we sampled are associated with AM symbionts, adding Sugarcane and Oilcane to forest soils may have influenced microbial responses by introducing these microbes to novel substrates (Palozzi & Lindo, 2018). While studies with isotopically enriched litters and agricultural soils can better represent real-world soil microbial community differences (e.g. Fulton-Smith & Cotrufo, 2019; Ridgeway et al., 2022), our experimental design allowed us to use leaves and bagasse substrates that directly reflect real-world amendments. Moreover, we also point to the successful use of soil transplants (i.e., C_4 soils in C_3 ecosystems) to estimate root-derived SOC in forest systems (Huang et al., 2021; McCloskey et al., 2021). Despite these limitations, our results identify important mechanisms that lay the foundation for future large-scale field experiments.

2.6 Conclusion

Our results showed that the genetic modifications to Sugarcane had modest impacts on soil C dynamics in the initial stages of litter decomposition but did not negatively alter soil C stocks. These results indicate that transitioning to genetically engineered Oilcane may enhance bioenergy fuel conversion efficiency without unintended consequences on soil C cycling. However, testing how other genetically modified crops alter soil C cycling remains critical, particularly when modifications lead to greater litter chemistry differences. In addition, our observations that Sugarcane bagasse amendments formed more MAOC while Oilcane leaves preferentially remained as light POC may be important to consider when deciding how to

manage bioenergy agricultural systems to meet sustainability goals. Overall, we highlight the potential for Oilcane to sustainably displace Sugarcane as a bioenergy feedstock and emphasize the remaining need to examine whether genetic modification will alter soil C dynamics over longer durations in the field, across different management strategies, and for other genetically modified bioenergy crops.

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Chapter 3: Unlocking plant-microbial interactions in deep soils: Linking depth gradients in roots, microbial activity, and soil carbon

3.1 Abstract:

Deep-rooted plants may offer the potential to build soil carbon (C). However, most research has focused on shallow soils, creating unknowns about how shifts in the balance between decomposition and inputs drive soil C formation with depth. Thus, our objectives were to: (1) link depth gradients in root biomass with microbial activity and soil C stocks down to 1 meter and (2) examine differences between depths in the ability of simple C inputs to prime or build soil C. To meet our objectives, we dug five, quantitative soil pits under 20-year-old Miscanthus plots in Champaign-Urbana. Observationally, we measured fine root biomass, total soil C, mineral-associated organic C (MAOC), particulate organic C (POC), microbial respiration, net nitrogen cycling, and enzyme activities. Experimentally, we added ^{13}C labeled glucose to soils from each depth in a lab incubation and followed its fate into different soil C fractions. We found significant declines with depth in fine root biomass, total soil C, MAOC, POC, and microbial activity. POC declined more rapidly with depth than MAOC leading to an increase in the ratio of MAOC-to-POC. We found that fine root biomass, n-acetyl-glucosaminidase (NAG) activity, and microbial respiration explained 98% of the variability in soil C and MAOC, while POC was only dependent on fine root biomass. Fine root biomass, representing inputs, and NAG, representing microbial recycling of dead cell walls, had positive effects while microbial respiration had a negative effect on soil C and MAOC. All depths had a similar ability to transfer simple C inputs into MAOC. However, this transfer led to net MAOC losses in shallow soils vs gains in deep soils. Collectively, these results suggest that soil C represents a balance between inputs, decomposition, and the recycling of microbial necromass. Moreover, in deeper soil horizons, increases in root C inputs may have the potential to build stable MAOC.

3.2 Introduction:

The cultivation of deep-rooted perennial crops has the potential to build soil C in agricultural soils (Thorup-Kristensen et al., 2020). Although, most of this research has focused on shallow soils (i.e., < 30 cm). This focus on shallow soils ignores the importance of deep soils, which have low C concentrations, but due to their volume contain nearly half of all soil C (Jobbagy et al., 2000; Dietzel et al., 2017). Moreover, limited research on the deep soil environment and the impact of deep roots leads to a critical unknown about what drives soil C formation with depth. At the center of this unknown is how depth gradients in abiotic (i.e. temperature, moisture, oxygen availability, soil texture) and biotic factors (i.e. plant inputs, microbial activities) alter the balance between soil C decomposition and persistence. Thus, we lack a mechanistic understanding of how shallow and deep soils differ in the form of soil C and how depth gradients in inputs, decomposition, and available mineral surfaces may drive these differences.

While shallow soils have greater soil C concentrations than deep soils, they likely have a lower ratio of mineral associated organic C (MAOC) to particulate organic carbon (POC). Mechanistically, the potential prevalence of POC in shallow soils, minimally decomposed plant litter fragments, reflects a balance between inputs and decomposer capacity. Shallow soils receive the majority of plant litter because they are the primary location of leaf litter entry and they contain nearly 70% of fine root biomass (Dietzel et al., 2017). Decomposer capacity is also high in shallow soils, but POC likely still accumulates due to energetic constraints on decomposing complex plant inputs and accessibility constraints when POC becomes locked into aggregates (Stewart et al., 2009). By contrast, MAOC, mineral bound organic carbon, is limited by the availability of mineral surfaces in shallow soils (Stewart et al., 2009; Mikutta et al., 2019).

MAOC primarily relies on the sorption of microbial decomposition products and to a lesser degree on the direct sorption of dissolved organic carbon, which are both more abundant in shallow soils (Chari and Taylor, 2022) As such, new MAOC formation is limited not by the size of the pool of organic C that can be sorbed onto mineral surfaces, but instead is limited by the supply of mineral surfaces that are not already occupied by organic C to stabilize this pool.

On the other hand, deep soils likely have a greater ratio of MAOC-to-POC due to low inputs, slow decomposition, and more available mineral surfaces. Due to a lack of plant litter inputs to form POC, deep soil C is dominated by dissolved organic carbon (DOC) and microbial decomposition products transported from shallow to deep soil depths (Leinemann et al., 2018). DOC and microbial decomposition products have a greater potential to form MAOC because they are highly decomposed molecules that can be directly sorbed onto mineral surfaces (Sokol et al., 2019). In addition, the deep soil environment also has unique biophysical properties (e.g., low O_2, buffered temperature, and moisture) that are thought to limit microbial decomposition (Balesdent et al. 2018; Mikutta et al., 2019). Overall, these potential differences in the ratio of MAOC-to-POC between shallow and deep soils are consequential because MAOC is thought to be more persistent, have a longer residence time, and be less susceptible to loss with global change than POC.

In addition to the unknowns on the prevalence of different C forms with depth, there remains an open question on the extent to which increasing root inputs to deep soils are an effective tool to build soil C. Lab incubation studies have shown that simple C inputs can prime microbial activity and drive soil C losses in deep soils (Shahzad et al., 2018; Fontaine et al., 2007; Wang et al., 2014; Tian et al., 2016). However, many of these studies looked at relative differences in priming or simply the potential to mobilize old C and did not examine the ability

of simple C inputs to build new soil C. A more parsimonious hypothesis may be that the same factors that drive differences in the ratio of MAOC-to-POC may lead to deep soils having a greater potential to stabilize simple C inputs than shallow soils. In shallow soils, priming likely dominates due to the greater abundance of POC that is energetically protected and the lack of available mineral surfaces to stabilize the resulting microbial products. In deep soils, stabilization could occur to a greater degree owing to a lack of POC that can be primed and the availability of mineral surfaces that can stabilize microbial products.

Given the management potential for growing deep rooted crops to build soil C, it is critical to determine what drives soil C formation with depth and test the ability of root inputs to build deep soil C. Therefore, our objectives were to link depth gradients in root biomass with microbial activity and soil C stocks down to 1 meter and examine differences between depths in the ability of simple C inputs to prime or build soil C. We used *Miscanthus x giganteus* (herein Miscanthus) as a model system because it is a deep-rooted perennial bioenergy crop that has been shown to build soil C in the top 30 cm (Chimento et al., 2016). To meet these objectives, we tested the following hypotheses: (1) Total soil C and the ratio of MAOC-to-POC will decline with depth. (2) Depth gradients in soil C and fractions will be driven by a balance between inputs and microbial decomposition. (3) Deep soils will have a greater capacity to form new stable soil C from root inputs than shallow soils.

3.3 Methods

Field Sampling

We dug five, quantitative soil pits in June 2022 under Miscanthus plots established in 2002 at the SoyFACE Farm in Champaign-Urbana, Illinois. We extracted all the soil and root biomass in a 1 m x 0.3 m x 1 m volume separated into five depths (0-15, 15-30, 30-50, 50-70, and 70-100 cm) for a total of 25 root and soil samples (5 pits x 5 depths = 25 samples).

In the field, we sieved all the extracted soil through a 5-mm sieve at each depth to homogenize and separate the roots. After weighing all the soil and roots from each depth, the sieved soil from each depth was thoroughly mixed and a composite sample was taken for lab analyses.

We washed and oven-dried all the roots to calculate the total dry root biomass. We then separated the roots and rhizomes from the 0-15 cm depths to get dry weights of fine roots versus rhizomes. We also oven-dried soil samples from each depth to determine the dry weight of the soil. We then calculated bulk density by dividing the total dry weight of soil from each depth by the total volume for each depth.

Soil Carbon and Carbon Pools

To quantify total C with depth, we sent out 3 replicates of each air-dried soil and ground root sample to be analyzed for %C using a Thermo Fisher Delta V+ isotope ratio mass spectrometer interfaced with a Carlo Erba NC2500 Elemental Analyzer at the University of Maryland Center for Environmental Science Appalachian Laboratory.

To determine the proportion of soil C as POC vs MAOC with depth, we density fractionated 5 ± 1.0g of each air-dried soil sample as outlined in Lavallee et al. (2020). To separate the light POC fraction, we performed two extractions, first with water and then with

1.85g/mL sodium polytungstate (SPT). To separate the heavy fraction into heavy POC and MAOC, we wet-sieved the remaining sample using a 53 μm sieve (POC>53 μm, MAOC<53 μm). We then oven-dried each fraction at 60 °C and recorded the dry weights to calculate the recovery. We determined mass recovery by calculating the difference between the initial sample mass prior to fractionating and the total mass recovered in all three fractions. All the samples had recoveries of 100 ± 10%. Lastly, we ground and sent out each fraction to be analyzed for %C, %N, and δ13C using a Thermo Fisher Delta V+ isotope ratio mass spectrometer interfaced with a Carlo Erba NC2500 Elemental Analyzer at the University of Maryland Center for Environmental Science Appalachian Laboratory. The results were scaled up to the mass of dry soil at each depth.

Microbial Respiration

To calculate microbial respiration, we incubated 25 ± 5.0 g of field moist soil from each depth in wide-mouthed glass mason jars (930 ml) with rubber septa installed into each lid (25 incubations total). We extracted 15 ml of the headspace from each jar after 1, 3, and 7 days and stored the gas in 10 mL Wheaton serum vials. After each gas sample was taken, we aerated the jars for 20 minutes before resealing and then placing them in the dark at room temperature (~22 °C). At the end of the week, we terminated the incubation. We measured the total microbial production of CO_2 using a Picarro G2201 (Picarro Inc.).

Microbial Extracellular Enzyme Production

We assayed the potential activities of microbial extracellular enzymes at each depth. We measured the potential activities of enzymes that degrade labile C (β-glucosidase [BG]), N (n-acetyl-glucosaminadase [NAG]), and P (acid phosphatase [AP]) (Saiya-Cork et al., 2002). All assays were run using a 1 g soil sample from each depth homogenized in a pH 5.0 sodium acetate

buffer. NAG, AP, and BG are hydrolytic enzymes, and these activities were determined using a fluorometric microplate assay with methylumbelliferone-linked substrates (Brzostek et al., 2015; Saiya-Cork et al., 2002).

Net Nitrogen Mineralization and Net Nitrification

We also measured the net nitrogen mineralization and net nitrification at each depth. This was done by extracting NO_3^- and NH_4^+ from 5 g of soil with 10 ml of 2 M KCl. We performed one extraction within 24 hours of sample collection (day 0) and then incubated another sample for 2 weeks in the dark at room temperature (day 14). We measured NO_3^- and NH_4^+ concentrations in the KCl extracts from day 0 and day 14 using a SEAL AQ300 Discrete Analyzer (SEAL Analytical, Inc.). N mineralization was calculated as the difference between NO_3^- and NH_4+ and net nitrification was calculated as the difference between NO_3^- from day 0 to day 14 (Finzi et al., 1998)

Potential to Form New Soil C from δ13C Glucose Additions

To determine the rate at which new glucose additions form soil C, we incubated 25 ± 5.0 g of field moist soil from each depth with 400 ug C/ 1 g soil of 99.9 atomic % ^{13}C glucose in wide-mouthed glass mason jars (930 ml) for 7 days. At the end of the 7 days, we opened the jars to let the soil air dry. We then density fractionated the soils as outlined in Lavallee et al. (2020) and detailed above to trace the fate of the glucose additions into the MAOC pool.

We calculated the proportion of ^{13}C glucose C recovered in the MAOC fraction using the following two end-member mixing model:

$$(1)\quad pC_{glucose} = (^{13}C\ Atm\%_{\ MAOC} - {}^{13}C\ Atm\%_{\ soil})/(^{13}C\ Atm\%_{\ glucose} - {}^{13}C\ Atm\%_{\ soil})$$

Where $pC_{\ glucose}$ is the proportion of ^{13}C glucose C recovered in the MAOC fraction and the ^{13}C Atm% of each MAOC fraction and the control soil were obtained using a Thermo Fisher

Delta V+ isotope ratio mass spectrometer interfaced with a Carlo Erba NC2500 Elemental Analyzer at the University of Maryland Center for Environmental Science Appalachian Laboratory. We then multiplied the proportion of C attributed to the ^{13}C glucose by the total C in the MAOC fraction to determine the total amount of ^{13}C glucose C recovered in MAOC. We calculated the percentage of the total MOAC C pool derived from new ^{13}C glucose additions by dividing the glucose C recovered in MAOC by the total MAOC. We also calculated the priming caused by ^{13}C glucose additions by subtracting the total MAOC in jars with glucose from control jars without glucose.

Statistical Analysis

To determine statistically significant differences in soil C, MAOC, POC, the ratio of MAOC-to-POC, fine root biomass, N cycling (nitrogen mineralization and nitrification), enzyme activities (β-glucosidase [BG], n-acetyl-glucosaminadase [NAG], and acid phosphatase [AP]), and microbial respiration between depths, as well as differences in the MAOC pool between control incubations and incubations with added ^{13}C glucose, we performed a two-way analysis of variance (ANOVA) and used the Tukey HSD test in R to do post hoc multiple comparisons in R Studio Posit Cloud (Copyright © 2022 Posit Software, PBC). We categorized significance as having a p-value <0.05 and p-values <0.1 were considered marginally significant.

We used a linear mixed effects model framework including fixed and random effects to determine the drivers of total soil C, MAOC, and POC, where fine root biomass, N cycling (nitrogen mineralization and nitrification), enzyme activities (β-glucosidase [BG], n-acetyl-glucosaminadase [NAG], and acid phosphatase [AP]), and microbial respiration were considered fixed effects and soil pit and depth, were considered random effects. We used the multi-model inference MuMIn R package to examine all possible combinations of models (N = 192) to

determine the most parsimonious model of soil C, MAOC, and POC using AIC criteria. We also determined the standardized regression coefficients to determine the relative contribution of each factor.

3.4 Results

From our observational quantitative soil pit measurements, we found that total soil C declines with depth where the top two depths (0-30 cm) had the greatest amount of total C, the bottom two depths (50-100 cm) had the lowest amount of total C, and the total C in the middle depth 30-50 cm was more similar to the 15-30 cm horizon above than the 50-70 cm horizon below (Figure 1A). Both the MAOC and POC pools declined with depth, but the POC pool declined to a greater extent than the MAOC pool (Figure 3.1b and 3.1c). The greater decline in POC with depth than MAOC resulted in the ratio of MAOC-to-POC increasing with depth where the top depth (0-15 cm) had the lowest ratio of MAOC-to-POC and the bottom two depths (50-100 cm) had the highest ratio of MAOC-to-POC.

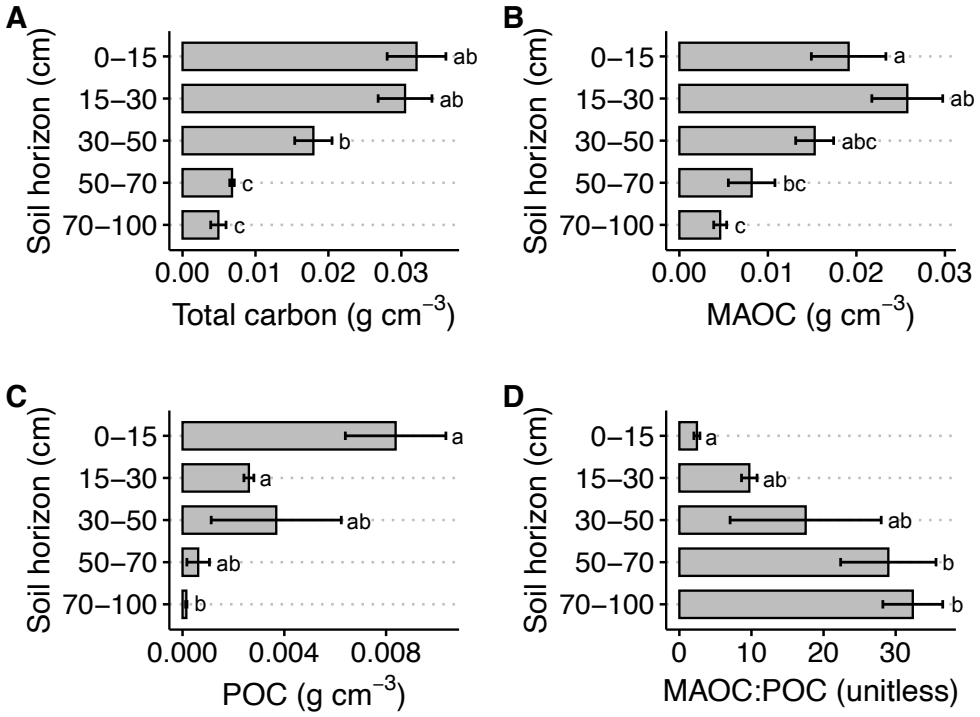

Figure. 3.1: Mean soil carbon (A), MAOC (B), POC (C), and MAOC:POC (D) across soil horizons. Error bars indicate the standard error of the mean for each group. Within each panel, differences in group means are denoted by dissimilar connecting letters, while similar letters indicate no differences among group means.

Fine root biomass, microbial respiration, and nitrogen cycling (net nitrogen mineralization and net nitrification) were significantly greater in the top 15 cm than in the bottom 85 cm (Table 3.1). Rhizomes were only present in the top 15 cm and weighed 540.65 ±86.89g. Enzyme activities (β-glucosidase [BG], n-acetyl-glucosaminadase [NAG], and acid phosphatase [AP]) declined more gradually with depth where enzyme activity was greatest in the top 30 cm, with a decline between 50-70 cm and the lowest below 70 cm (Table 3.1).

Table 3.1: Depth gradients in roots and microbial activity

Measurement	Depth					p-value
	0-15 cm	15-30 cm	30-50 cm	50-70 cm	70-100 cm	
Fine root biomass (g/depth volume)	114.60(11.74)[a]	29.23(5.53)[b]	14.66(3.85)[b]	13.06(2.66)[b]	8.74(0.68)[b]	p<0.0001
Microbial respiration (mmol CO_2/g soil)	0.011(0.003)[a]	0.005(0.001)[b]	0.004(0.001)[b]	0.004(0.000)[b]	0.004(0.001)[b]	p<0.01
Net nitrogen mineralization (ug N/g soil/day)	0.70(0.20)[a]	0.20(0.11)[b]	0.04(0.08)[b]	-0.02(0.06)[b]	0.00(0.07)[b]	p<0.01
Net nitrification (ug N/g soil/day)	0.75(.10)[a]	0.24(0.08)[b]	0.07(0.05)[b]	0.05(0.047)[b]	0.04(0.03)[b]	p<0.0001
NAG activity (umol/g/hr)	0.018(0.003)[a]	0.013(0.004)[ab]	0.013(0.004)[ab]	0.004(0.001)[b]	0.001(0.000)[b]	p<0.01
BG activity (umol/g/hr)	0.30(0.07)[a]	0.19(0.06)[ab]	0.14(0.02)[b]	0.09(0.02)[b]	0.10(0.01)[b]	p<0.05
AP activity (umol/g/hr)	0.290(0.101)[a]	0.187(0.038)[ab]	0.139(0.034)[ab]	0.087(0.023)[ab]	0.036(0.009)[b]	p<0.05

Our linear mixed effects model showed that 72% of the variation in total soil C and 42% in MAOC was explained by fine root biomass, NAG activity, and microbial respiration. When random effects were included, fine root biomass, NAG activity, and microbial respiration explained 98% of the variation in total soil C and 99% in the MAOC, showing that 26% of the model variability for total soil C and 57% for MAOC was explained by differences between soil pit and horizons. Fine root biomass and NAG activity had a positive effect on soil C and MAOC while microbial respiration had a negative effect on soil C and MAOC (Fig 3.2). The standardized regression coefficients suggest that fine root biomass had the largest impact on soil C and MAOC, followed by soil respiration, then NAG activity (Fig 3.2). Variations in the POC pool were only explained by fine root biomass, where 42% of the variability in POC was explained by fine root biomass and 99% of the variability was explained when random effects were included.

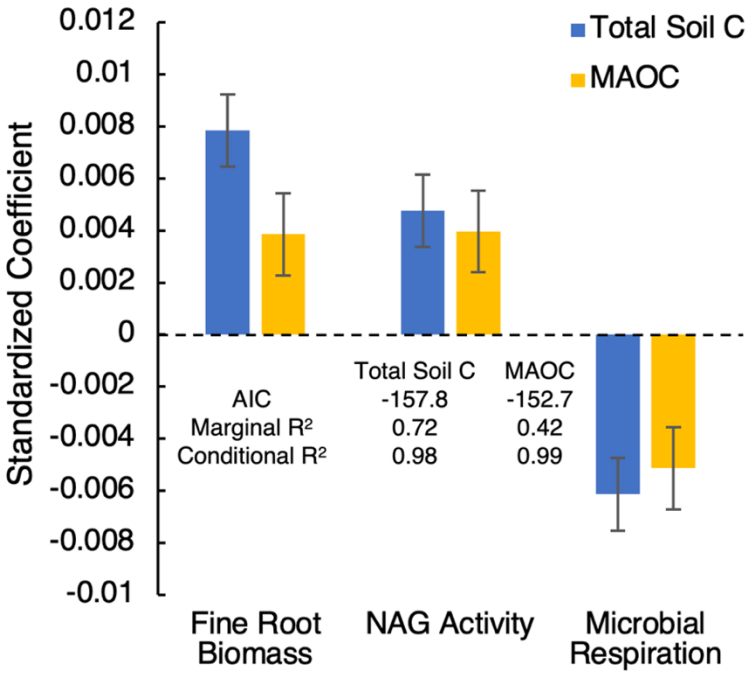

Figure 3.2: The relative contribution of fine roots, NAG, and soil respiration to soil total soil carbon. Shown are standardized regression coefficients for the best model (lowest AIC_c) among all candidate models. Fixed effects account for 72 % of the variability in soil carbon and 42% in MAOC while fixed and random effects (soil horizon, soil pit) combined explain 98 % of total soil carbon and 99% of MAOC variability. Error bars indicate the standard error of the mean for each group.

Table 3.2: Linear mixed effects model outputs

	Fixed Effect	Coefficient	SE	DF	p-value	Variance Inflation Factor
Soil C	Fine root biomass	12.21	2.16	17	<0.0001	2.21
	NAG activity	0.70	0.20	17	0.0033	1.60
	Microbial respiration	-2.30	0.53	17	0.0004	2.24
MAOC	Fine root biomass	5.94	2.42	17	0.0251	2.17
	NAG activity	0.58	0.23	17	0.0219	1.59
	Microbial respiration	-1.91	0.59	17	0.0046	2.21
POC	Fine root biomass	2.86	0.68	19	0.0005	N/A

Through our experimental addition of ^{13}C glucose to microcosm soil incubations, we found that there was no statistically significant difference in the amount of glucose C that was incorporated into the MAOC pool between different depths (Fig 3.3a). However, the percentage of the total MAOC pool that was newly incorporated ^{13}C glucose increased with depth where the top 3 depths (0-50 cm) had the lowest percent of ^{13}C glucose recovered in MAOC, followed by the 50-70cm depth and then the 70-100 cm which had the greatest percentage of ^{13}C glucose in MAOC (Fig 3.3b). The difference between the total MAOC in the microcosms with ^{13}C glucose additions and the control microcosms also significantly differed with depth. The top three horizons (0-50 cm) experienced net losses of MAOC from the ^{13}C glucose additions, while the bottom two depths, 50-100 cm did not experience net MAOC losses, and the 50-70 cm depth had net MAOC gains (Fig 3.3c).

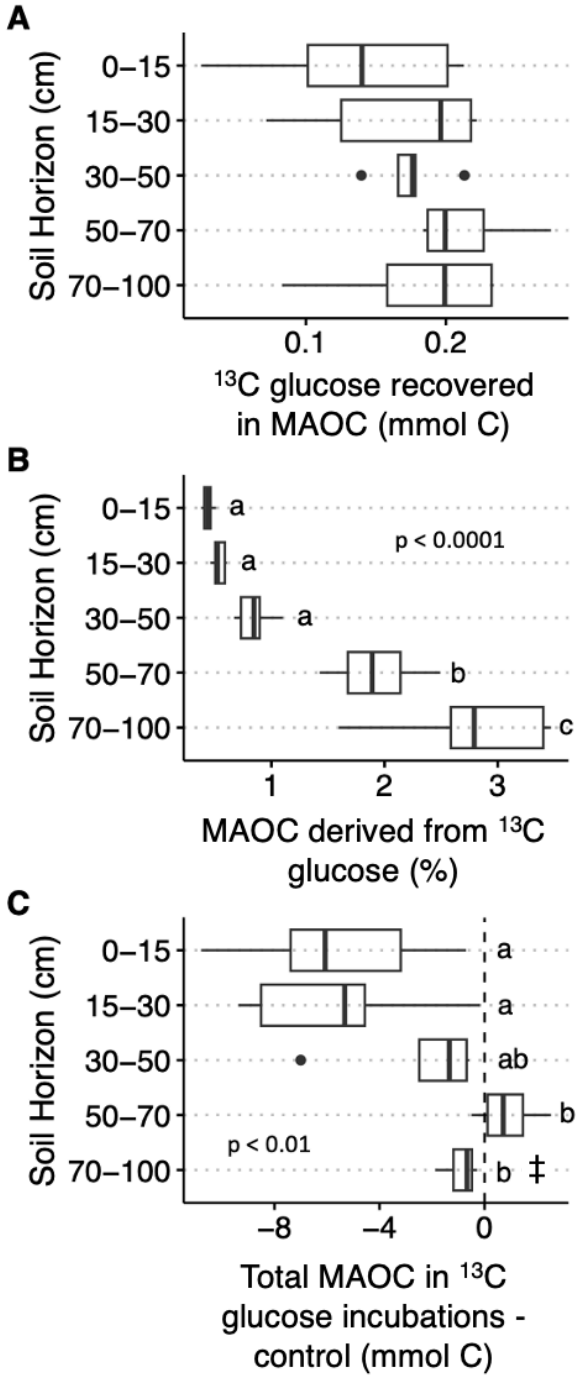

Figure 3.3: 13C glucose recovered in MAOC (A), MAOC derived from ^{13}C glucose (B), total MAOC in ^{13}C glucose incubations - controls (C), across soil horizons. Error bars indicate the standard error of the mean for each group. Within each panel, differences in group means are denoted by dissimilar connecting letters, while similar letters indicate no differences among group means. ‡ Indicates significance of $p < 0.1$.

3.5 Discussion

We quantified depth gradients in roots, microbial activity, and soil C down to 1 meter and examined differences between depths in the ability of simple C inputs to prime or build soil C. We found strong declines in total soil C, MAOC, and POC and an increase in the MAOC-to-POC ratio due to greater declines in POC than MAOC with depth (Fig 3.1). Using a mixed linear effects model, we found that total soil C and MAOC increased with greater fine root biomass and NAG activity and declined with greater microbial respiration (Fig 3.2). These results suggest that soil C gradients with depth represent a balance between inputs, microbial necromass recycling, and microbial decomposition. Experimental additions of ^{13}C glucose showed that although all depths had similar absolute incorporations of the ^{13}C glucose into MAOC (Fig 3.3a), these additions appeared to prime MAOC losses to a greater extent in soils above 50cm than in soils below 50cm (Fig 3.3c). Collectively, our results have important implications for our conceptual understanding of what factors drive depth gradients in soil C stocks and the potential for simple C inputs to build soil C.

In support of H1, we found that total soil C and the ratio of MAOC-to-POC increased with depth (Fig 3.1). Although MAOC was the dominant soil C pool at all depths, the shift in the ratio of MAOC-to-POC was due to a greater depth decline in POC fraction than the MAOC fraction (Fig 3.1) Results from the mixed linear effects model suggest that the strong depth decline in POC is directly linked to the declines in root biomass. Thus, lower root inputs are likely the driver of shifts in the ratio of MAOC-to-POC. Several studies also show that soil C declines with depth (Fang and Moncrieff et al., 2005; Fierer et al., 2003), but few studies have measured how the ratio of MAOC-to-POC changes with depth. Recent studies from forest ecosystems show that the ratio of MAOC-to-POC both increases and remains stable across depth

gradients (Hicks Pries et al., 2018; Ren et al., 2023). These conflicting patterns in forest soils suggest that the pattern we observed in agricultural soils may not be universal and can be altered by shifts in vegetation, climate, and soil type. Moreover, one of these studies also followed the fate of isotopically labeled dead roots and found that in deep soils the roots were largely undecomposed after 30 months. Coupled with these previous *in situ* incubation results, our results which show direct links between POC and root biomass suggest that increasing inputs of fine roots to deep soils may have the potential to increase soil C by enhancing POC (Hicks-Pries et al., 2020). However, future research should examine whether POC at depth is more susceptible to warming losses given that the deep soil environment is more sensitive to global change than previously thought (Rocci et al. 2021).

Our results partially support H2 that depth gradients in total soil C, MAOC, and POC will be driven by a balance between inputs and microbial decomposition. In the mixed linear effects model, we found that total soil C and MAOC increased with greater fine root biomass and NAG activity and declined with greater microbial respiration (Fig 3.2). The similarity in the linear mixed effects model for total soil C and MAOC appears to reflect the dominance of the MAOC pool at all depths. As highlighted above, the only significant predictor of POC was fine root biomass. For the total soil C and MAOC pools, the predictive power of fine root biomass and microbial respiration was anticipated given that fine root biomass is a proxy for inputs and microbial respiration is a proxy for decomposition. As such, these predictors captured the major fluxes in and out of these pools. The predictive power of NAG activity was unanticipated. Commonly, NAG activity is interpreted to be a proxy for microbial investment in enzymes that mobilize N. However, this interpretation is overly simplified because NAG enzymes are primarily responsible for breaking down chitin and peptoglycan polymers that comprise

microbial cell walls into amino sugar monomers that contain both C and N (Mori et al., 2023; Margenot and Wade, 2022). Therefore, the positive effect of NAG activity on both total soil C and MAOC may reflect microbial recycling of dead necromass which has been shown to be preferentially sorbed onto mineral surfaces (Cotrufo et al., 2013). Collectively, our results suggest that the declines in total soil C and MAOC we observed with depth are driven by a balance between plant inputs, microbial decomposition, and necromass recycling. Thus, to build soil C in agroecosystems, there is a clear research need to identify management strategies that can enhance plant inputs and microbial recycling to a greater extent than microbial decomposition.

Deep soils appeared to have a greater potential to form new stable soil C from simple C inputs than shallow soils. In support of H3, we found that soils from the 50-70cm and 70-100cm depths had the highest proportion of the total MAOC pool derived from the ^{13}C glucose additions (Fig 3b). While all the depths had similar absolute incorporations of the ^{13}C glucose into MAOC (Fig 3a), the additions appeared to prime MAOC losses to a greater extent in soils above 50cm than in soils below 50cm (Fig 3c). These differences in MAOC formation and loss with depth likely reflect differences in the size and activity of the microbial community, the availability of other limiting nutrients to support microbial decomposition, and the potential for direct sorption of simple C inputs in deeper soils. The addition of simple C inputs in the shallow soil may lead to a greater priming effect due to the larger and more active microbial community (Li et al., 2021; Keiluweit et al., 2015). We found that N cycling rates declines with depth (Table 3.1) which may limit microbial decomposition despite the influx of labile C in the deeper soils leading to greater MAOC stabilization (Meier et al., 2017). Finally, the greater availability of mineral surfaces (Kaiser and Guggengerger, 2008) and less active microbial community (Table

3.1) in the deeper soils may increase the potential for simple C inputs to be directly sorbed onto mineral surfaces. Regardless of the mechanism, the limited priming of MAOC pools below 50cm suggests that the foraging of roots in deeper soils has the potential to build deep soil C. However, there may be a threshold in deep root inputs where microbial activity and nutrient availability would increase enough for soil C losses to outweigh gains (Tang et al., 2023).

Our results show the quick incorporation of new ^{13}C glucose into the MAOC pool and the loss of MAOC over a short-term incubation, which suggests that MAOC is more dynamic than previously thought. There is a growing consensus in the literature that the MAOC pool may be comprised of a stable and dynamic fraction, an idea that builds upon the "onion model" of Sollins et al. (2006). The stable fraction of MAOC is thought to be the C that is closest to the mineral site, making it tightly bound. On the other hand, the dynamic fraction is thought to be the C that is accumulated and bound to other organic molecules (Biegill et al., 2023), leading to a more dynamic sorption and desorption of the outer layers of the "onion". (Sollins et al., 2006; Kleber et al., 2021, Cotrufo et al., 2023). Following the "onion model", the limited losses of MAOC with the addition of glucose to the deep soils may indicate that deep soils are dominated by more stable, directly mineral-bound MAOC (Fig 3.3c). Shallow soils show the opposite response to glucose additions and in these depths, there is likely a significant portion of the MAOC that is comprised of organic matter that is in the outer layer of the "onion". As such, simple C inputs to deep soils may have a greater potential to build new stable MAOC, while these same inputs to shallow soils may just accelerate the sorption and desorption of the dynamic MAOC fraction. In addition, our results show that the simple C inputs to soils may provide a way to assay dynamic vs. stable MAOC fractions, which may alleviate methodological

limitations to empirical support of conceptual models that classify dynamic and stable fractions of MAOC.

While our study points to important drivers of soil C and building soil C at depth, our results raise important future research directions. First, future research should investigate the extent to which keystone microbial traits vary with depth, including carbon use efficiency, turnover, and growth rate. These data may provide important support for the positive effect of NAG, a proxy for microbial recycling, on total soil C and MAOC stocks. Second, although we were able to show differences in the ability of simple C inputs to build soil C with depth, our results should be classified as a potential rate due to the lab incubations being performed on soils at the same temperature, moisture, and aerobic conditions. Lab incubations that mimic depth gradients in these abiotic conditions may show that they constrain microbial activity in deep soils which could lead to simple C inputs driving larger net gains in MAOC. Finally, our results suggest that there is a clear need for in situ experiments that build off our efforts with simple C inputs in the lab as well as a recent effort that used isotopically labeled, dead roots in the field (Hicks Pries, et al., 2018). Experiments that couple these two approaches in the field may be able to tease apart the net soil C effect of dead roots building POC (Hicks Pries, et al., 2018) and simple C inputs building MAOC.

3.6 Conclusion

Our results show important depth gradients in roots, microbial activity, and soil C that may influence the ability of deep roots to build soil C. We found that increases in fine root inputs and to a lesser extent the recycling of microbial necromass lead to soil C and MAOC gains while microbial respiration leads to soil C and MAOC losses. Therefore, cultivating perennial bioenergy crops that can increase fine root inputs and microbial recycling of necromass without

enhancing microbial respiration is critical to meeting sustainability goals of producing a carbon neutral fuel and building soil C at the same time. We also found that deep soils appeared to have a greater potential to form new stable soil C from simple C inputs than shallow soils. This result raises an important hypothesis that the net effect of deep-root inputs at depth is positive with stable MAOC formation outweighing priming losses. To test this hypothesis, in-situ experiments that couple following the fate of dead roots at depth with the potential priming effects of root exudates are needed.

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Chapter 4: Discussion and Conclusions

This master's thesis examined unknowns surrounding the impacts two potential sustainable bioenergy solutions will have on soil C. To do this, I used a combination of lab incubations and field observations to answer the following questions: (1) To what degree does Sugarcane litter differ from Oilcane litter in their impacts on microbial activity and their ability to form new soil C? (2) What factors influence depth gradients in C stocks in Miscanthus, a deep-rooted perennial grass? (3) Do deep soils differ from shallow soils under Miscanthus in their potential to stabilize new simple C inputs?

Chapter 2: Lipid-enhanced Oilcane does not impact soil carbon dynamics compared with wild-type Sugarcane

To examine the impacts on soil C cycling of enhancing oil content of bioenergy feedstocks, I examined the impact of Sugarcane litter decomposition on soil carbon (C) formation and loss and determined if the genetic modifications to produce Oilcane alter these dynamics. To do this, I used a jar incubation method to trace the fate of C_4 Sugarcane and Oilcane litter in protected and unprotected soil C pools in C_3 forest soil. I found that both crops led to net soil C gains primarily due to an accumulation of the litter as POC and that the genetic modifications to Oilcane did not substantially alter soil C dynamics (Fig 2.3b & 2.4). I also found that Sugarcane bagasse formed more MAOC than Sugarcane leaves while Oilcane leaves preferentially remained as POC (Fig 2.3b & 2.3c). In addition, for both crop types, bagasse additions resulted in greater priming of native soil C than leaves (Fig 2.2d).

These results have important implications for bioenergy feedstock production. First, our results point to being able to enhance plant lipid production through genetic engineering without modifying microbial activity in a way that negatively impacts soil C stocks. Second, the

opposing effects of tissue type for Sugarcane and Oilcane on the fate, priming, and net soil C gains suggests that the tissue type of litter you amend the field with may impact the stability, retention time, and net effect of new soil C gains and that there may be a tradeoff between litter additions forming new stable MAOC and priming soil C losses. These results are meaningful for advancing sustainable bioenergy production as it is imperative to increase crop fuel conversion efficiency for bioenergy to be carbon neutral while producing enough energy to compete with non-renewable fuel sources. In addition, there has been a shift toward more sustainable harvesting practices that leave the leaf litter on the field rather than burning it as well as adding the processed stem litter (bagasse) back as a soil amendment (Cerri et al., 2011; de Resende et al., 2006). Although these sustainable harvesting practices significantly increase the amount of biomass left on the field which has the potential to increase soil C stocks, my research quantified the impact of crop and tissue type on building soil C and their net effect on soil on C stocks. This work will help inform sustainable bioenergy management practices for genetically modifying crops and amending the soil with leftover lignocellulosic material.

Chapter 3: Unlocking plant-microbial interactions in deep soils: Linking depth gradients in roots, microbial activity, and soil carbon

To investigate the potential of deep-rooted perennial feedstocks to build soil C, I linked depth gradients in root biomass with microbial activity and soil C stocks down to 1 meter to determine the predictors of soil C and soil C fractions with depth and experimentally tested the potential of simple C inputs to build soil C at depth. I found that soil C and MAOC declined with depth and were best predicted by fine root biomass, representing inputs, microbial respiration, representing losses, and NAG activity, representing the recycling of microbial necromass while POC also declined with depth but was only predicted by fine root biomass (Fig 3.1 & 3.2 &

Table 3.2). In addition, I found that deep soils had a greater potential to minerally stabilize new simple C inputs than shallow soils due to the C inputs having a greater stabilizing than priming effect below 50cm (Fig 3.3c).

These results can help guide sustainable bioenergy management decisions for growing deep-rooted perennial feedstocks. First, our results identified the most significant predictors of total soil C, MAOC, and POC with depth, which can help inform crop selection for building deep soil C. Our results suggest that deep-rooted crops that enhance fine root biomass inputs and the recycling of microbial necromass in deep soils to a greater extent than microbial respiration have the greatest potential for building deep soil C. Second, our research indicates that simple C root exudates in deep soil horizons may have a greater stabilizing than priming effect which is positive for growing deep rooted perennial crops for building deep soil C. In addition, these results provide important future research directions. While our results showed that simple C inputs may lead to a greater stabilizing than priming effect in deeper soils, future research is needed to assess the collective impact of roots and their exudates on soil C stabilization with depth.

Future directions for sustainable bioenergy research

Collectively, my research shows that sustainable bioenergy solutions such as lipid enhanced Oilcane and growing deep-rooted perennial feedstocks may have the potential to enhance soil C. Although these are promising results for advancing bioenergy closer to carbon neutrality, there is still much work to be done. For solution 1, Genetically modifying feedstocks to enhance our ability to turn them into fuels, I believe future research should focus on the impact of growing Oilcane roots on soil C cycling. For solution 2, Cultivating deep rooted

perennial crops to enhance soil C storage, future research should focus on in-situ experiments that investigate the combined impact of roots and natural exudates on deep soil C cycling.

4.1 Literature Cited

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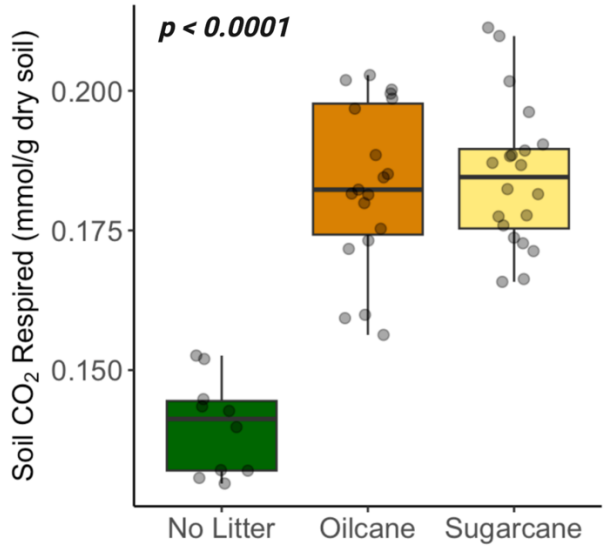
Appendix: Supplementary Tables and Figures



SI fig. 1: Soil CO_2 respired in jars with no litter, Oilcane litter, and Sugarcane litter.