THESIS

RARE SUGARS IN SOILS: INSIGHTS ON THEIR PRESENCE, PERSISTENCE, AND POTENTIAL FOR CARBON SEQUESTRATION

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ABSTRACT

RARE SUGARS IN SOILS: INSIGHTS ON THEIR PRESENCE, PERSISTENCE, AND POTENTIAL FOR CARBON SEQUESTRATION

Carbon (C) is a fundamental element in the biosphere, cycling through all its natural pools. However, due to human activity, the flux of C into the atmosphere has accelerated, impacting the climate in significant and consequential ways. Awareness of this has prompted world-wide research into different mitigation strategies, including both reducing the flux of C into the atmosphere and active carbon dioxide removal (CDR) from the atmosphere. Soil represents a substantial C reservoir with the capacity to store large amounts of C. Our research focuses on the role of chiral molecules, specifically rare sugars, to enhance the storage of C in soil. To assess the feasibility of this idea, we designed an experiment to test whether soil microorganisms were able to consume and respire 14 rare sugars. We found that some rare sugars showed very little or repressed respiration, but that most showed moderate or high respiration rates. This finding prompted the hypothesis that soil microorganisms have evolved the capacity to grow on rare sugars because those rare sugars are present in the soil. To test this, we designed another experiment to check for the presence of rare sugars, specifically rare hexoses, in soils.

Hexose sugars are among the most important small molecules in nature, in part because they are essential sources of energy for most cells. D-glucose, the most abundant hexose, is well-known due to its roles in both cellular respiration and photosynthesis; however, D-glucose is far from the only hexose in nature. Plants and microorganisms produce not only D-glucose, but also D-fructose, D-galactose, and D-mannose, and they contribute these hexoses to soils in different proportions. These four hexoses are considered common in soil and have been incredibly well-studied, but they represent only a small fraction of the hexoses possible in soil systems. In this study, we used gas chromatography-mass spectrometry (GC-MS) to measure hexoses in diverse soils crowdsourced from across the contiguous United States. In addition

to the four common hexoses, we identified a fifth hexose: a rare ketohexose corresponding to the overlapping retention times of psicose and tagatose ("PsiTag"). This rare ketohexose, or possible mixture of these two rare ketohexoses, was present in every soil we sampled. To the best of our knowledge, this is the first time a rare ketohexose has been identified in a soil extract. The ubiquitous presence of a rare hexose in soils shifts the paradigm and challenges the narrative about which hexoses are truly common.

These two experiments explore the potential of rare sugars for soil C sequestration via bio-transformative CDR (BtCDR) by examining their presence and the persistence in soils. As a result of our investigation, we determined that each rare sugar falls into one of two categories: having high turnover in soil, and therefore low C sequestration potential, or having high recalcitrance in soil and high C sequestration potential. Experimental data suggest that PsiTag and L-fructose have high recalcitrance in soil, but that L-glucose has high turnover in soil. Further research is needed to verify these findings and explore additional rare sugars. Although preliminary data indicate that rare sugar monosaccharides would not serve as effective long-term C sinks in soil, we believe that rare sugars may have a yet unknown role to play in soil C dynamics. This thesis sheds new light into the previously uninvestigated presence of rare sugars in soil, their implications for sustainable C storage, and their potential contributions to a holistic approach to climate change mitigation.

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"If I have seen farther than others, it is because I have stood on the shoulders of giants."

-Isaac Newton

DEDICATION

To Nichole, Joe, Mikey, Alley, and Daniella: may you stand on my shoulders and see even farther.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER I: THE POTENTIAL OF RARE SUGARS FOR SOIL CARBON SEQUESTRATION

1.1 INTRODUCTION

Carbon (C) is one of the most important atoms in the biosphere because it permeates all parts of it, cycling among many living and nonliving pools. Some of the most notable C pools include the atmosphere, lithosphere, oceans, soil, and plant and animal biomass.[1] Nearly all natural systems have inherent mechanisms in place that regulate the fluxes among them.[2–7] However, human activities, especially those since the Industrial Revolution, have increased the flux of C into the atmosphere at much faster rates than nature is able to remove it.[8,9] This increased flux has dramatically influenced atmospheric concentrations of carbon dioxide (CO_2) and other greenhouse gases.[10–12]

The causes and consequences of increasing concentrations of atmospheric CO_2 are indisputable and distressing, so scientists are actively engineering many mitigation strategies that allow us to reverse the disastrous and unexpected effects of our actions.[12,13] These mitigation strategies will help us achieve the goal of staying below 2 °C of warming set by the United Nations Framework Convention on Climate Change (UNFCCC) according to research by the Intergovernmental Panel on Climate Change. Proposed mitigation strategies include those that would both slow the flux of CO_2 into the atmosphere by reducing fossil fuel usage (conventional mitigation) and those that would actively capture more CO_2 from the atmosphere (negative mitigation).[14] Many models show that conventional mitigation alone would be insufficient at meeting the UNFCCC goal and that negative emissions are essential.[14] Scholars around the world have proposed removal criteria of at least 1 Gt C annually.[14] Other models show that while this goal is possible, it will be challenging, requiring a clever strategy, substantial financial investment, and global cooperation.[15]

The feasibility of negative mitigation strategies considers many factors, including efficiency, scalability, cost effectiveness, technical potential, institutional capacity, long-term sustainability, regulatory framework, ethical concern, public acceptance, and ecological risk. Most climate engineering strategies fall

short in at least one, if not several, of these categories. While all these factors are critical, one of the most important criteria is scalability, because the most favorable mitigation strategies are those that have high throughput. Another criterion of incredible consequence is long-term self-sustainability. Fortunately, this combination of high throughput and self-sustainability is found naturally in plants. Plants fix about 200 Gt C annually through photosynthesis.[16,17] Plant-fixed C consists of molecules including sugars, amino acids, and organic acids, and these molecules are either utilized by the plant or exuded from its roots into the soil.[18] Once in the soil, a majority of fixed C is typically consumed rapidly by soil microbial activity, and that C is released back into the atmosphere.[18,19] However, if the fixed C were exuded from plant roots in a more stable form that remained in the soil, the bio-transformation of C by processes such as photosynthesis and the subsequent C storage in soil would be an incredibly effective C sink. For these and other reasons, one of the most promising and novel negative mitigation strategies for C sequestration is soil management in conjunction with bio-transformative carbon dioxide removal (BtCDR), a strategy that utilizes biological processes, including the inherent C sequestration mechanisms present in plants and microorganisms, to remove CO_2 from the atmosphere and transform it to a stable material.[13,20]

Soil, well known for being an extremely heterogeneous and diverse system, contains one of Earth's most important C pools and holds incredible potential for C sequestration. Depending on the mineral content, the top 1 m of soils can hold around 100 Pg C by sorption, and soils globally have the potential to hold over 1000 Pg C, but some argue that these values might be underestimates of the true C capacity in soil.[21–23] Soil C exists in two pools: the labile pool and the recalcitrant pool. Labile C is readily consumed and respired by soil microorganisms.[24] Recalcitrant C compounds comprise a small but stable pool that can persist and accumulate in soils for centuries, or even millennia, because they are inaccessible or otherwise resistant to degradation by soil microorganisms.[25] These persistent compounds are often characterized by high molecular weight and functional complexity and include compounds such as terpenoids, waxes, and biochar.[26] Substrates that have shorter initial decomposition times are also linked with soil persistence.[27] In addition to these characteristics, we hypothesize that chirality may also play a role in a molecule's persistence in soil.

Chiral molecules, including important biomolecules such as DNA, amino acids, and sugars, lack a plane of symmetry and therefore occupy a characteristic and unique three-dimensional shape in space. These important chiral biomolecules exist almost exclusively in nature in one form or the other: amino acids are predominantly the L-form, and sugars, including the sugar groups present in RNA and DNA, are predominantly the D-form. This is the principle of homochirality. The origins of homochirality are still largely unknown, but it is a popular field of study for scientists who wish to discover the origins of life on Earth.[28,29] Chiral amino acids and sugars are small, labile molecules and provide good sources of food for soil microorganisms.[19,30] However, due to the unique three-dimensional shapes of chiral sugars and amino acids, microorganisms that have adapted to utilize L-amino acids and D-sugars are generally not able to metabolize D-amino acids and L-sugars.[31]

There are over 100 naturally occurring sugars, but only a small handful of those is considered common; sugars that are not found commonly in nature are considered rare sugars.[32,33] Because they are rare, natural systems are less equipped to metabolize them.[31] For example, sucrose, a common disaccharide of D-glucose and D-fructose, is 100% digestible in humans, but D-psicose, a rare monosaccharide, is only 0.3% digestible by comparison.[34] By extrapolating this lack of digestibility to soil microorganisms, we hypothesize that soil microorganisms may be just as unable to digest rare sugars, and that rare sugars could thus be an avenue for C storage in soil. Very little work has been done regarding rare sugars as a food source for soil microorganisms, so led by Dr. Bethany Avera and Dr. Peter Baas, I assisted members of my research group in conducting a study to see if we could answer the question of whether soil microorganisms could consume rare sugars.

1.2 EXPERIMENT AND INTERPRETATION

We collected soils from around the country (for a full description of soils collected, see Chapter 2) and performed the MicroResp™ experiment as described by Campbell *et al*.[35] We incubated samples of each soil with one of 19 different testing conditions: 14 L-sugars (L-allose, L-altrose, L-fructose, L-galactose, L-glucose, L-gulose, L-idose, L-mannose, L-psicose, L-ribose, L-ribulose, L-sorbose, L-talose, and L-xylose), two D-sugars (D-glucose and D-fructose) and an achiral sugar alcohol (galactitol) as positive

controls, perfluorooctanoic acid (PFOA) as a negative control, and water-only agricultural soil to measure the background soil C respiration (full details of MicroResp™ experiment will be published by Boot *et al.* at a later date). Figure 1.1 shows the cumulative respiration of each rare sugar and control substance over 10 days. The positive control sugars (D-glucose and D-fructose) had the highest cumulative respiration profiles of all tested sugars. Galactitol, a common sugar alcohol, also showed moderately high respiration, though not as high as D-glucose and D-fructose. Most of the sugars, including L-altrose, L-glucose, L-gulose, L-idose, L-mannose, L-ribose, L-ribulose, L-sorbose, L-talose, and L-xylose, showed moderate to high respiration as compared with our positive controls. However, four rare sugars (L-allose, L-fructose, L-galactose, and L-psicose) showed very low respiration, similar to PFOA, or even suppressed respiration, seen in the case of L-fructose.

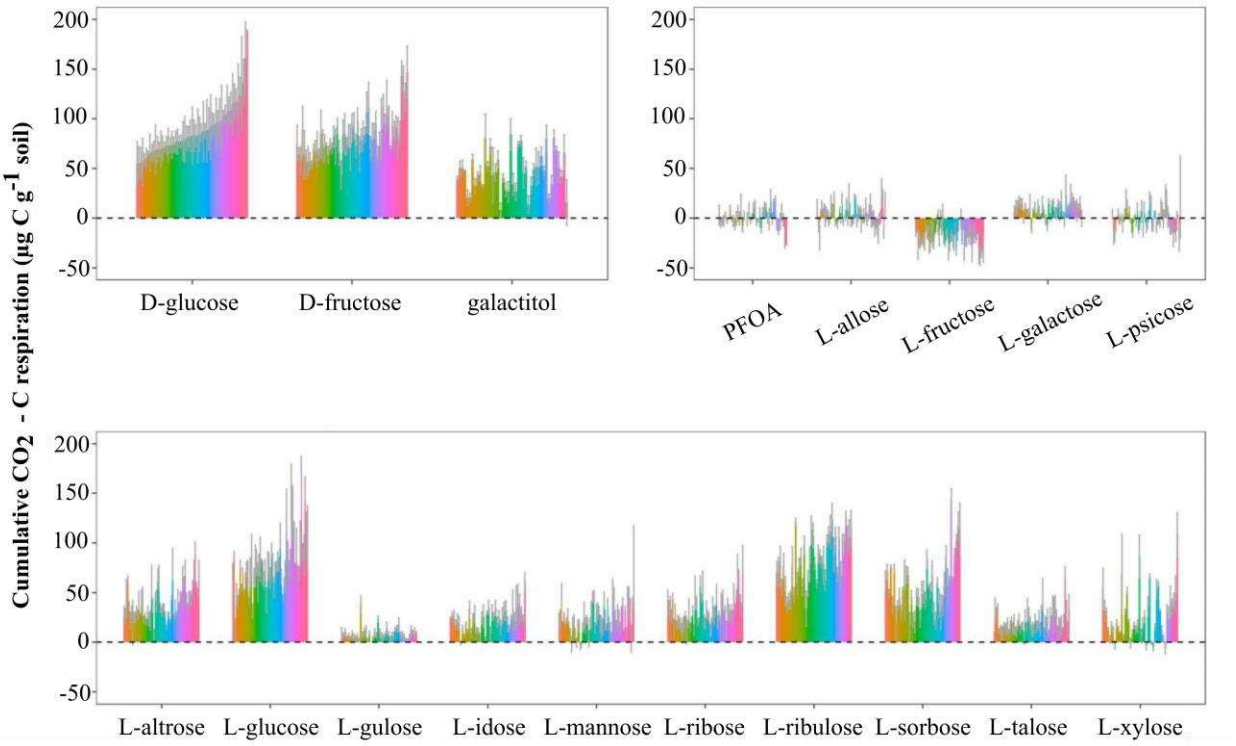

Figure 1.1. Cumulative respiration of 14 L-sugars, one common sugar alcohol, two common D-sugars, and PFOA. Each colored bar represents a unique soil sample. D-glucose and D-fructose show significant respiration, and galactitol shows moderate respiration (top left). L-altrose, L-glucose, L-gulose, L-idose, L-mannose, L-ribose, L-ribulose, L-sorbose, L-talose, and L-xylose show moderate to significant respiration (bottom). L-allose, L-fructose, L-galactose, and L-psicose all show minimal or suppressed respiration, consistent with PFOA (top right). Figure created by Dr. Bethany Avera.

The results from the 10-day MicroResp™ incubation study highlight the potential of rare sugars for C sequestration. The sugars that had low respiration might be good candidates for BtCDR, but the sugars that had moderate or high respiration raised even more questions. If these sugars are rare in nature, why were soil microorganisms able to respire them? There are many possible answers to this question. However, Occam's Razor, a heuristic principle from philosophy, emphasizes the need to avoid unnecessary complexity and suggests that when there are multiple possible explanations of a phenomenon, the simplest explanation is likely the correct one. Therefore, it follows that soil microorganisms were able to consume and respire rare sugars because they had previously encountered those rare sugars. Of the many possible reasons for this, the hypothesis that best describes the dataset from the MicroResp™ 10-day incubation experiment is that soil microorganisms can consume and respire rare sugars because those rare sugars are already present in soil systems. This hypothesis became the focus of my master's work and is the subject of Chapter 2 of this thesis.

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CHAPTER II: REDEFINING COMMON: A RARE KETOHEXOSE IDENTIFIED IN 100% OF SOILS SAMPLED

2.1 INTRODUCTION

Of the four classes of biological macromolecules, carbohydrates are widely considered to be the most structurally and functionally diverse.[1–3] This diversity is due to the more than 100 naturally-occurring monosaccharides, spanning a range of sizes and functional types, and the countless different ways they polymerize.[3,4] Carbohydrates play many roles in nature, but one of their main functions is energy storage.[5,6] Sugars, particularly hexose sugars (with the formula $C_6H_{12}O_6$), serve as the primary energy source for most cells, making them critical for cellular function and therefore extremely abundant in biological systems.[7–9]

There are 24 possible hexose sugar stereoisomers consisting of two chiral sets of 12 sugar enantiomers: eight aldohexoses (glucose, galactose, mannose, allose, altrose, gulose, idose, and talose) and four ketohexoses (fructose, psicose, sorbose, and tagatose) (Figure 2.1). In aqueous solutions, most hexoses exist predominantly in the six-membered ring (pyranose) form; however, in non-aqueous solutions, some hexoses may be more commonly found in the five-membered ring (furanose) form.[10–13] In fact, in any system, most hexoses exist in a unique equilibrium between pyranose, furanose, and linear forms.[12] Whether a hexose will adopt one conformation over another depends on the energetics of the system that occur resulting from the phase of the sugar or, if in the liquid phase, the identity of the solvent.[13,14] Because natural conditions involve primarily aqueous systems, for the purposes of this study, we discuss each hexose only in the context of their enantiomeric forms and do not consider their ring-chain tautomerization potential.

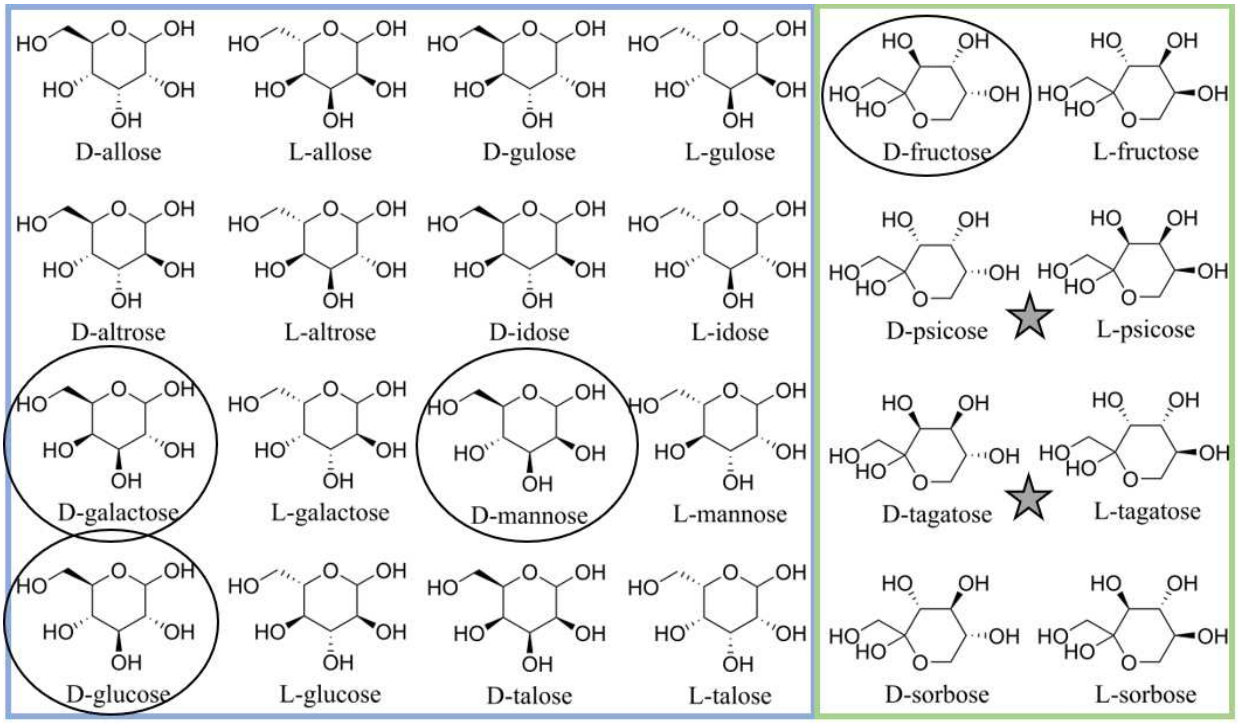

Figure 2.1. The structures and functionalities of 24 hexoses, shown as pyranoses for simplicity. The aldohexoses, left, are shown in the blue box, and the ketohexoses, right, are shown in the green box. The four common hexoses are circled, and the two rare hexoses of interest in this study, psicose and tagatose, are starred.

Of the 24 hexose sugars, only D-glucose, D-galactose, D-mannose, and D-fructose are common; the other 20 hexoses are considered rare sugars, defined by the International Society of Rare Sugars as "monosaccharides and their derivatives being rare in nature."[15–17] Rare hexoses, especially L-hexoses, are challenging and/or expensive to isolate as natural products or synthesize chemically, so their applications are not as well studied.[18,19] Despite this, global interest in rare sugars is piquing; many fields including food and health, medicine, pharmaceuticals, and agriculture are looking toward rare sugars to serve purposes such as replacements for dietary sucrose and as pesticidal amendments to crop systems in lieu of traditional chemical applications.[18–20] Contrary to rare sugars, common sugars are ubiquitous in natural systems, and their presence is well-catalogued. All photosynthesizing plants produce D-glucose and D-fructose; D-galactose and D-mannose derivatives are found in cell wall polysaccharides including pectin and mannan, respectively.[21–24] Because polysaccharides comprise more than half the dry mass of plants, this makes plant polysaccharides a critical primary input of sugar carbon (C) to soil.[25]

In soil, sugars are a significant component of the C pool. Soil polysaccharides can become sticky, coalescing with minerals and organic particles to form microaggregates, increasing the turnover time of polysaccharides in soil to be on the order of decades to centuries.[25–28] Eventually, however, all polysaccharides do decompose, passing through the labile soil organic C (SOC) pool as free monosaccharides, creating biogeochemical hotspots and supporting fast fluxes in the C cycle.[25,26,29] Free monosaccharides can also enter the soil C pool through plant root exudation. Free sugars are a valuable component of plant root exudates and have been shown to be beneficial to soil systems by stimulating the growth of rhizosphere bacterial colonies and microbial communities.[30–33] The dominant free hexoses present in most root exudates are glucose, fructose, and galactose.[34–38] Surprisingly, despite these two seemingly significant sugar contributions, only 20% of the total free hexose concentrations in soils are plant-derived; a majority of the sugars that plants contribute are pentoses (with the formula $C_5H_{10}O_5$), which are known to exist in soils in concentrations roughly proportional to D-galactose and D-mannose.[26,29,39–41] The remaining 80% of free soil hexoses come from secondary sources including microorganisms, which are known to produce all four common hexoses.[24,25,27,42–45] Perhaps it is no surprise that microorganisms contribute hexoses to the soil, primarily through their metabolism, because hexoses are their most critical energy source.[25,42]

Many soil studies have successfully measured common sugars, including both hexoses and pentoses, using a variety of analytical methods. Initial studies used spectrophotometry for qualitative total sugar detection, often paired with quantitative methods such as gas-liquid chromatography.[26,46–48] Other types of spectroscopy, including infrared and nuclear magnetic resonance spectroscopies, have also been historically popular techniques.[26,49] Chromatography is the most common method of studying sugars, with a majority of studies using methods including high-performance anion-exchange chromatography[50], gas chromatography[26,28,29,39,42,45,51–58], high- and ultra-high performance liquid chromatography[40,59–62], and ion chromatography.[63] The findings from all these studies report identification and quantitation of the common sugars and their polymers in soils. However, to the best of our knowledge, free, unbound hexoses have not

been studied independently of polysaccharide-bound hexoses, and rare hexoses have not been identified or quantified in soils.

In the study presented here, we investigated a collection of soils with the goal of identifying and measuring free, unbound soil hexoses. We used gas-chromatography mass-spectrometry (GC-MS) to, first, quantitatively measure concentrations of hexoses in soils and, second, to separate the D- and L-enantiomers of glucose and fructose to determine whether either L-hexose contributed to the total concentrations. This study provides novel insight into the presence and concentrations of hexoses in soils and addresses the need for more research in this area to lay a foundation that will bolster our understanding of free hexose metabolism in soils.

2.2 METHODS

2.2.1 Soil Collection and Storage

Soil collection kits included a Cooliner envelope, a ShockWatch indicator, five 50-mL plastic centrifuge tubes, a mini soil corer, instructions for collecting the soil (Appendix B, Figure B1), and a data collection worksheet to record soil information (Appendix B, Figure B2). In summer 2020, we sent these collection kits to our friends and families living in many different locations across the United States, avoiding USDA/APHIA soil quarantine areas. We requested that the soil be collected from one turf grass location, then four samples from any other location of their choice. We received many samples from grasses, trees, and flowers; one soil from a vegetable garden; and two compost soils. Because of this, we organized the soils into four plant cover type categories: trees, grass, flowers, and multiple cover types (consisting of vegetable soils, compost, or any combination of trees, grass, and flowers). After collecting the soils, participants packed them in the Cooliner envelope with the ShockWatch indicator to monitor for extreme shipping conditions. Upon receiving the soils, we sieved them to 6 mm and stored them at 4 °C.

2.2.2 TOC and Water Content Determination

We dried subsamples of each soil at 60 °C for 24 h to determine the water content, and we collected total carbon data on the dried soils using a Delta V IRMA coupled to a Costech ECS 4010 elemental analyzer and a Precon Device.

2.2.3 Soil Extraction

We performed a hot water soil extraction using up to 2 g dry soil as suggested by the University of Georgia Complex Carbohydrate Research Center.[64] We weighed our soils using an analytical balance (Denver Instrument APX-100, d=0.1mg), and we prepared soil solutions of approximately 0.5 g/mL in 50-mL conical tubes (Fisherbrand) using HPLC-grade water (Avantor, Macron Fine Chemicals). We heated the conical tubes in a 50 °C water bath for 20 min total, briefly vortexing the tubes at 10 min and returning them to the water bath. We centrifuged the samples at 1600×g for 10 min (Thermo Scientific Sorvall Legend X1R) and then transferred the supernatant into new vials by filtering it through a pipette containing a glass wool plug. To the supernatant, we added enough ethanol (Fisher Scientific, 90%, reagent-grade) to bring the final volume to ~80% (v/v) ethanol in water. We vortexed these samples briefly and then centrifuged again at 1600×g for 10 min. This centrifugation produced an ethanol-soluble supernatant containing the free hexose fraction and a pellet containing the ethanol-insoluble, polysaccharide-bound fraction. We stored the ethanol-insoluble pellet containing the polysaccharide-bound sugars for later analysis. We transferred the ethanol-soluble supernatant into new 20-mL glass scintillation vials (VWR) and dried the samples overnight at 25 °C and 15 torr using a Savant® SPD121P SpeedVac® concentrator equipped with a universal vacuum system with Vapornet® (hereafter referred to as "SpeedVac"). We redissolved these samples in 1.5 mL HPLC-grade water and transferred the contents into 1.8-mL autosampler vials (VWR), dried them overnight using the SpeedVac, and stored them in a refrigerator at 5 °C.

2.2.4 Sugar Standards and Retention Time Determination

We obtained reagent grade D-allose, D-altrose, D-galactose, D-gulose, D-idose, D-psicose, D-sorbose, L-glucose, L-fructose, L-galactose, D-xylose, L-xylose, L-ribose, and L-ribulose from Omicron; D-glucose and D-fructose from Fisher; D-tagatose, D-talose, and L-psicose from TCI; and D- and L-mannose from Sigma. Isotopically labeled internal standards (IS) D-glucose-$^{13}C_6$ and D-fructose-$^{13}C_6$ came from Cambridge Isotope Labs.

We performed a non-chiral, quantitative derivatization to determine the retention time for each hexose and pentose standard, excluding the labeled IS. We prepared 1 mg samples of each sugar in 1.8-mL

autosampler vials and dried them overnight using the SpeedVac. Our analysis confirmed that D- and L-enantiomers do not separate by the quantitative method and that pentoses elute much sooner than hexoses. We achieved maximum resolution with a standard mixture that included glucose, fructose, galactose, mannose, and psicose, so we selected these five hexoses as the ideal standard mixture for our method.

2.2.5 GC Derivatization

To assess the soil extracts, we performed two distinct GC derivatizations. We first performed a non-chiral, quantitative derivatization for absolute hexose quantitation, and after assessing the total hexose quantities of each soil, we selected a sub-group with high concentrations of total hexoses for the subsequent two-step chiral derivatization: a time-stable reductive amination using a chiral derivatizing agent, then a time-sensitive silylation.

For the quantitative derivatization, we first added 500 ng D-glucose-$^{13}C_6$ and 500 ng D-fructose-$^{13}C_6$ as IS into each vial and dried them overnight using the SpeedVac. To the dry vials, we added 50 µL of a 25 mg/mL methoxyamine hydrochloride (LiChropur™, Sigma Aldrich, 97.5-102.5% (AT)) in pyridine (Sigma-Aldrich, ≥99.5%) solution to the dry samples. We then incubated the samples at 60 °C for 45 min, sonicated the samples for 10 min, and incubated again at 60 °C for 45 min. After centrifuging the samples briefly using the SpeedVac, we added 50 µL of silylating mixture I according to Sweely for GC Derivatization[65] (hereafter referred to as "Tri-Sil reagent", Sigma-Aldrich, quality level 100), and we incubated again at 60 °C for 40 min. We centrifuged the samples to remove the precipitate and transferred the reaction mixture to a glass GC insert (Agilent) for GC-MS analysis. We also prepared a true blank containing no sugar, a method blank (containing only 500 ng D-glucose-$^{13}C_6$ and 500 ng D-fructose-$^{13}C_6$ IS), a pooled quality control (QC) sample containing a mixture of aliquots from a subset of soil extracts, a nine-point calibration curve containing equal parts D-glucose, D-galactose, D-mannose, D-fructose, and D-psicose ranging from 15.6 ng each hexose to 4000 ng each hexose, and three QC standards containing equal parts of our five-hexose mix at known concentrations: 25 ng each hexose, 100 ng each hexose, and 750 ng each hexose. All calibration curve and QC standards contained 500 ng D-glucose-$^{13}C_6$ and 500 ng D-fructose-$^{13}C_6$ IS.

After the quantitative analysis, we selected soils that we identified as having high quantities of total hexose and prepared them for chiral derivatization. To the dry samples, we added 50 µL of 0.1% acetic acid (Mallinckrodt AR Select®, >80%) in ethanol (Greenfield Global, 200 proof). We then prepared a solution of 30% (v/v) (R)-(+)-α-methylbenzylamine (RMBA, Aldrich, 98%) in ethanol, handling the RMBA using a glass pipette. Ten minutes after adding the acetic acid solution, we added 50 µL of the RMBA solution. We heated the samples at 60 °C for 4 h, let them sit at room temperature overnight, and then dried the samples under nitrogen. Once dry, we added 150 µL of a 10 mg/mL sodium borohydride (Sigma-Aldrich, ReagentPlus®, 99%) in 50% ethanol solution to each vial. We left them to sit for 2.5 h with loose caps due to gas evolution, shaking them occasionally. Then, we added acetic acid dropwise until the effervescence ceased and dried the vials under nitrogen. Next, we added 200 µL methanol (Fisher Scientific, 99.8%) and dried again under nitrogen. We then performed three 200-µL additions of methanol plus 2 drops acetic acid, drying completely under nitrogen each time. Finally, immediately prior to performing the GC analysis, we added 100 µL Tri-Sil reagent, heated at 60 °C for 40 min, centrifuged, and transferred the supernatant into a GC insert to measure within the next 10 h. We prepared 5 mg solutions of D- and L-glucose, fructose, galactose, mannose, and psicose in the same manner. The chiral derivatization was not quantitative, so we did not include IS in the analyte soils or the standards.

2.2.6 GC-MS

We analyzed the samples using a Thermo Scientific TSQ 8000 Evo Triple Quadrupole GC-MS/MS (Thermo Fisher Scientific, Waltham, MA, USA) equipped with Thermo Trace 1310 GC, Triplus RSH autosampler, and split/splitless injector. We performed the separation using a ZB-5HT Inferno™ column (Zebron™, 30 m × 0.25 mm, 0.25 µm in film thickness). We operated the MS in EI mode (70 eV) and used helium (Airgas, UHP) as carrier gas with flow rate of 1.000 mL min^{-1}.

We optimized two GC-MS methods to use in this analysis: one for the quantitative derivatization and another for the chiral derivatization. For the quantitative derivatization, we set the GC-MS interface, ion source, and injector temperatures to 330 °C, 260 °C, and 285 °C, respectively. We started the column temperature program at 80 °C for 1 min, increased by 25 °C min^{-1} to 170 °C and maintained for 1 min,

increased by 5 °C min^{-1} to 215 °C, and then increased by 25 °C min^{-1} to 330 °C and maintained for 3.3 min. We injected all samples in splitless mode at a volume of 0.5 µL. We used water, acetonitrile, and hexane as needle pre-wash solvents (2 cycles at 1 µL) and water and methanol as the needle post-wash solvents (4 cycles at 1 µL). We selected *m/z* (217.12, 319.16) and *m/*z (217.12, 307.16) as quantitation fragment ions for the determination of aldoses and ketoses, respectively. For the chiral derivatization, we set the GC-MS interface, ion source, and injector temperatures to 300 °C, 300 °C, and 240 °C, respectively. We started the column temperature program at 60 °C for 1 min, increased by 20 °C min^{-1} to 180 °C and maintained for 5 min, increased by 20 °C min^{-1} to 210 °C and maintained for 15 min, increased by 20 °C min^{-1} to 215 °C and maintained for 10 min, increased by 20 °C min^{-1} to 300 °C and maintained for 5 min, and decreased by 50 °C min^{-1} to 50 °C. We injected all samples in splitless mode at a volume of 1 µL. We used acetonitrile and hexane as needle pre-wash solvents (2 cycles at 1 µL) and water and chloroform as needle post-wash solvents (2 cycles at 1 µL). We selected *m/z* of (134.12, 217.11, 332.17, 421.2, 540.28) and *m/z* (132.06, 217.11, 236.15, 542.3) as qualification fragment ions for the determination of aldoses and ketoses, respectively.

2.2.7 Data Processing and Analysis

We processed our GC-MS data using Chromeleon 7.2.10 and Skyline 23.1.0.455. We used JMP ® Pro 17.0.0 for statistical analysis, and we used Igor Pro 9.05 64-bit and Inkscape 1.3.2 to visualize our data.

As this was a collaborative project, Dr. Allison Haase performed the following parts of the procedures. She optimized the quantitative GC-MS separation method, selected characteristic quantitative and qualitative fragment ions for each standard, confirmed the retention times for the hexose standards, input IS concentrations, analyzed and optimized the calibration curve, interpreted and manually selected hexose analyte peaks, normalized the hexose analyte peaks using the IS peaks, determined the limit of detection (LOD) and limit of quantitation (LOQ) for each hexose, checked the quality of the measurements using the QC known concentration standards and pooled QC samples, and reported all absolute hexose concentrations in tabulated format. Allison also developed the GC-MS separation method that we used for chiral derivatization. Full details regarding these portions of the research methods appear in Dr. Haase's

dissertation (Allison Haase, 2024, Chemistry, dissertation chapter "Quantitative and Qualitative Analysis of Hexose from Environmental Samples using GC-MS").

The quantitative derivatization produced two peaks for each hexose because of a tautomerization that occurred in the derivatization process. To most accurately report the concentrations of hexoses present, we selected only the peak that had the lower limit of detection for each sugar. In the chiral reductive amination and silylation, the derivatization of ketohexoses also created two peaks, and the derivatization of aldohexoses created one peak. When standards did not yield peaks in the expected range but only one peak was present in the analyte samples, we interpreted that peak as the common D-hexose. QC results showed passing results (IS > 10× blank) in 95.9% of soils; individual hexoses had passing QC results in 94.2% of cases. All blank samples containing IS and 97.0% of standards had passing QC results as well. We found the LOD for each hexose by adding three times the standard deviation to the signals present at each retention time in our method blank, and we determined the LOQ for each hexose by setting bias and coefficient of variance parameters to 20% max. The LODs for glucose, fructose, galactose, mannose, and psicose standards were 0.009, -0.016, 0.014, 0.033, and 0.013 ng, respectively, and the LOQs were 15.6, 15.6, 62.5, 31.25, and 31.25 ng, respectively. Because the negative value obtained for the fructose LOD is nonsensical, we substituted 0.002 ng for the fructose LOD. Each hexose had some samples that were below the LOQ, so we performed some manual data manipulation: for samples that measured below the LOQ but above the LOD, we replaced those measured values with the calculated average concentration found for each hexose; for samples that were below the LOD, we replaced their measured values with half the LOD for each hexose. We tested $\log_{10}$-transformed data for normality using Welch's test and for differences in variance using Levene's and Bartlett's tests, but the log transformation did not meet the requirements necessary to perform ANOVA. To determine pairwise comparisons on our nonparametric data, we used the Wilcoxon each pair test. We report all statistical significance using $\alpha = 0.05$.

2.3 RESULTS

2.3.1 Method Development

Due to their high degree of structural similarity, there was considerable overlap of retention times among the 12 D-hexose standards; however, we successfully separated the common hexoses using standards for D-glucose, D-fructose, D-galactose, and D-mannose (Figure 2.2). As a result of a tautomerization that occurred during the quantitative derivatization, each hexose standard had two forms and, therefore, eluted at two distinct retention times. Absolute retention times for all 12 D-hexose standards vary from system to system, but, on our system, the retention times ranged from 13.25 min to 14.45 min. This window was distinct from the retention time window for pentoses (8.32 min to 10.58 min). The retention time window from 13.25 min to 13.70 min was unique to D-psicose and D-tagatose. Except D-psicose and D-tagatose, all other hexoses had at least one peak that overlapped with a common hexose peak. Because overlapping hexoses are impossible to distinguish between in a mixture, we included D-glucose, D-fructose, D-galactose, D-mannose, and D-psicose in our standard analyte mixture. For quantitation, we selected the peak with the lower limit of detection, shown in pink (Figure 2.2). We achieved good resolution between the psicose and fructose peaks (separation factor of 1.01), despite the apparent overlap between them (Allison Haase, 2024, Chemistry, dissertation chapter "Quantitative and Qualitative Analysis of Hexose from Environmental Samples using GC-MS").

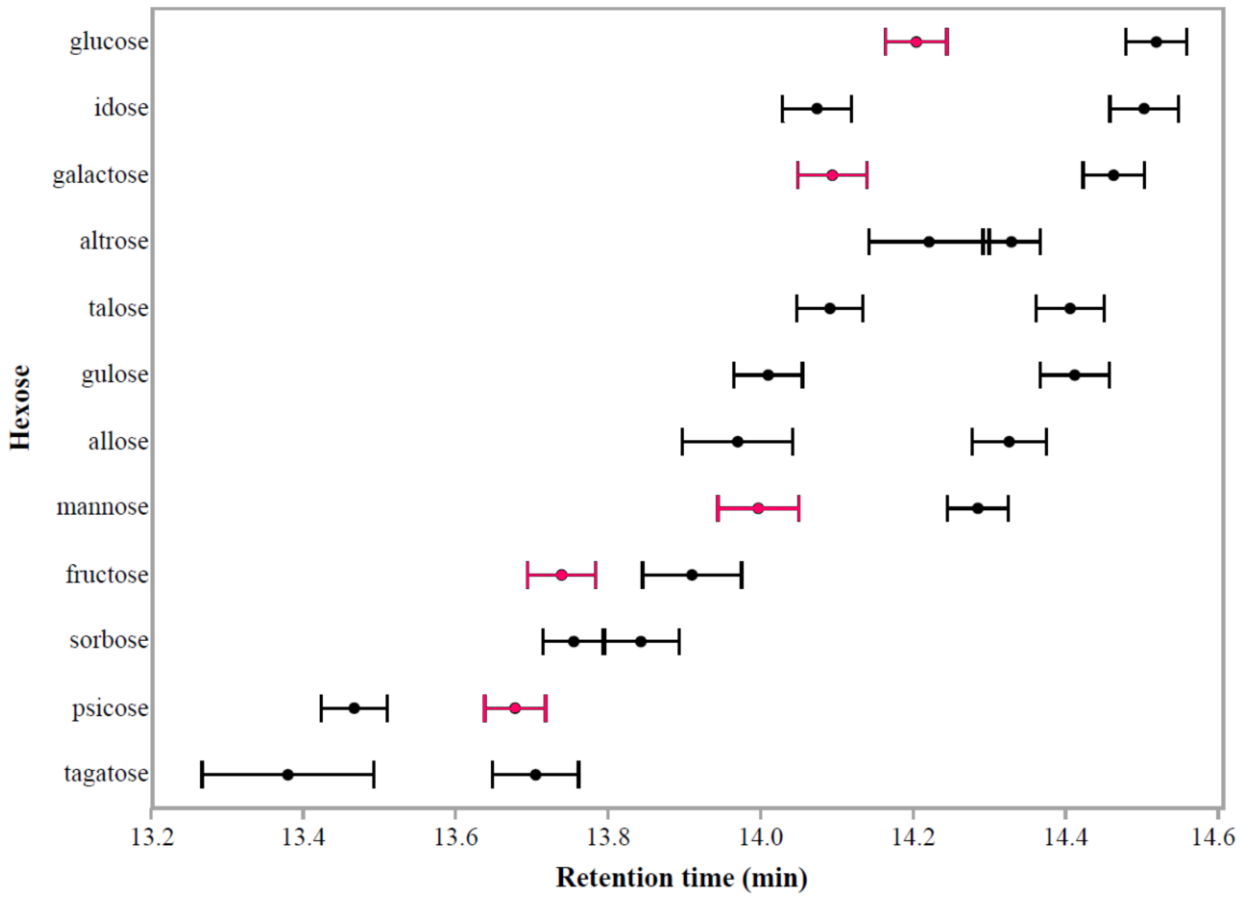

Figure 2.2. Retention times for quantitative derivatizations of D-hexose standards. Error bars represent the chromatogram peak width and show potential overlap between peaks. The peaks we used for quantitation are highlighted in pink.

2.3.2 Experimental Results

We detected the four common hexoses in many soils, but the only hexose we observed in every soil sample eluted at 13.45 and 13.65 min. Because this falls in the overlapping region between psicose and tagatose, we identify this peak as a rare ketohexose and hereafter refer to this rare ketohexose as "PsiTag". While 100% of soils had detectable quantities of PsiTag, only 87.1% of soils had detectable amounts of fructose, the only common ketohexose. We found glucose, galactose, and mannose in 97.1% of soils. Figure 2.3 shows the measured concentrations of PsiTag, glucose, fructose, galactose, and mannose. PsiTag had by far the smallest concentrations in soils (mean 5.64 µg hexose C g^{-1} soil) and comprised the smallest fraction of the total hexoses present (mean 5.55%). Glucose had the highest mean concentration, followed by fructose, galactose, and mannose (766.86, 45.67, 21.76, and 12.13 µg hexose C g^{-1} soil, respectively).

The average relative contributions of glucose, fructose, galactose, and mannose to the total hexose concentrations were 67.44%, 11.85%, 8.83%, and 6.32%, respectively. Mannose and galactose did not have significantly different mean concentrations in soils ($p = 0.6126$), but all other sugar pairs did ($p \leq 0.0159$). Soils from multiple cover types had statistically different total hexose concentrations than soils from trees, flowers, and grass ($p = 0.0001$, $p = 0.0055$, and $p = 0.0002$, respectively), but soils from trees, flowers, and grass did not have statistically different hexose concentrations from each other ($p \geq 0.4043$), so any variations in total hexose concentration were not correlated with plant cover type (Appendix A, Figure A1).

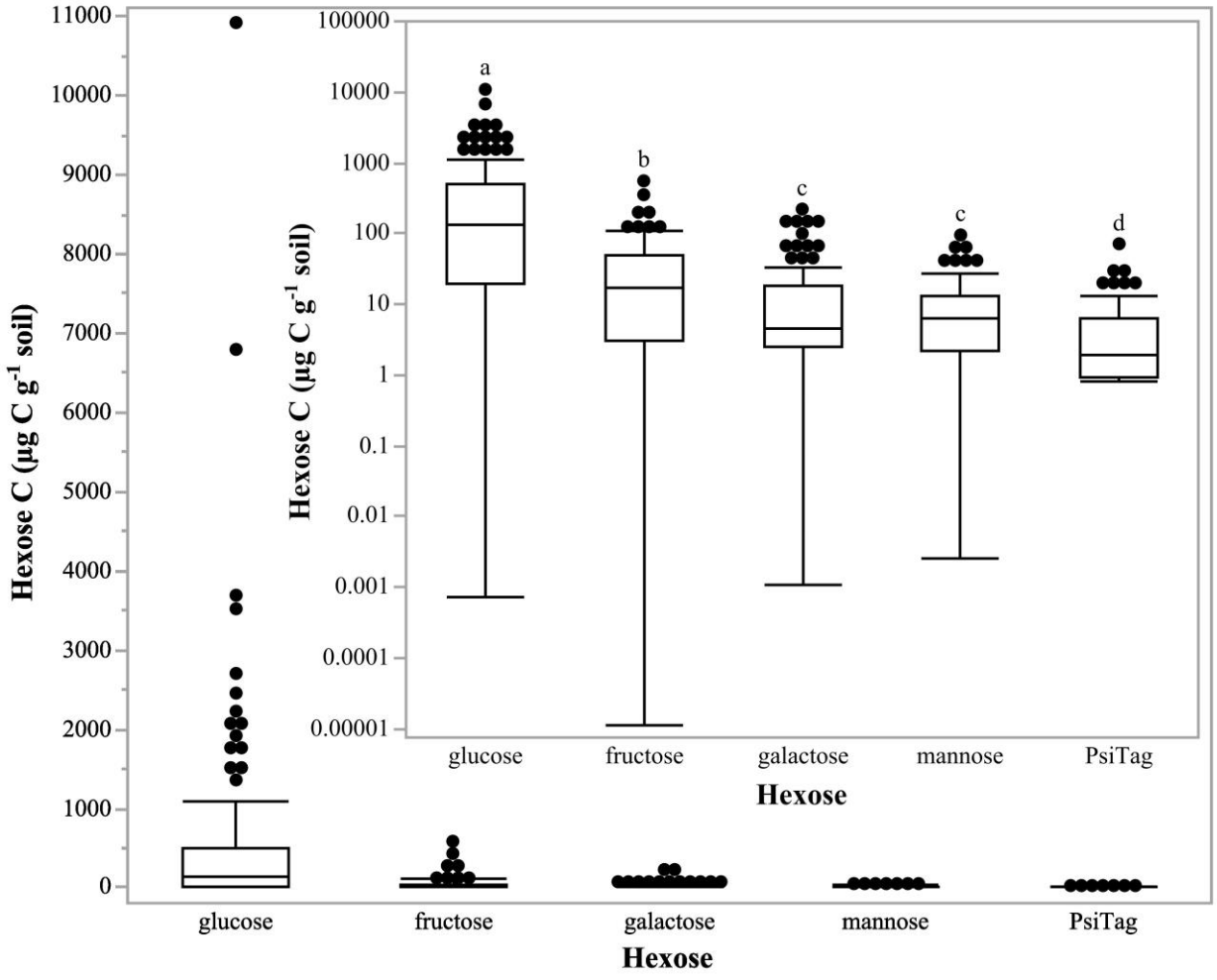

Figure 2.3. Quantitative concentrations of free hexoses in our soil extracts. The concentration means for each hexose (µg hexose C g^{-1} soil) are as follows: glucose = 766.86, fructose = 45.67, galactose = 21.76, mannose = 12.13, and PsiTag = 5.64. Inset: data presented on a log-axis. All pairs of means are significantly different ($p \leq 0.0159$) except for galactose and mannose ($p = 0.6126$).

Free hexose and SOC relationships varied depending on the identity of the hexose and the relative contribution of total hexose C to SOC (given as the ratio of total hexose C to SOC, Hex:SOC). The relative contribution of glucose to total hexose (Glc:Hex) increased as Hex:SOC increased, and glucose was nearly always the major component of total hexose C regardless of Hex:SOC (Figure 2.4). Mannose (Man:Hex), followed the same trend. A power law fit,

$$y = Ax^B,$$

for the Man:Hex relationship was significant ($p < 0.0001$) and explained 73% of the variation in the data. Even though Gal:Hex and PsiTag:Hex had significant correlations, only 34% and 51% of the variability, respectively, was described by the power law relationship between their relative contributions to total hexose concentrations and Hex:SOC. The Glc:Hex trend approached a significant power law relationship ($p = 0.063$), but only explained just under half the variability in the data, indicating there are other factors contributing to the relationship that we did not measure. Fru:Hex did not follow a power law relationship with Hex:SOC at all. Interestingly, there was no overall relationship between total hexose C and SOC (Appendix A, Figure A2). There was also no relationship between the individual non-glucose hexose C and SOC, however, there was a notable decrease in glucose C as SOC increased (Appendix A, Figure A3).

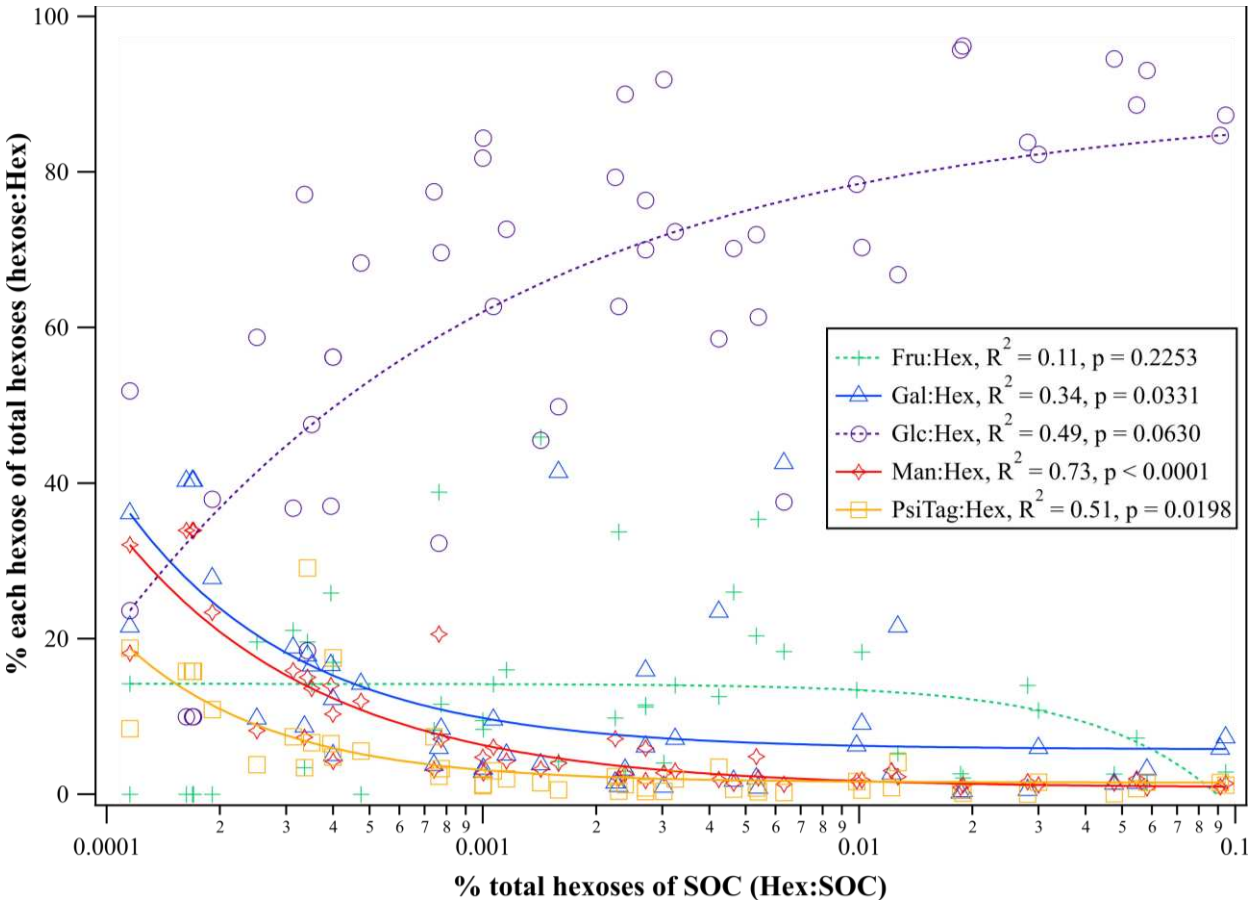

Figure 2.4. The relative contribution of each free hexose (fructose, galactose, glucose, mannose, and PsiTag) as a function of the total hexose concentration (hexose:Hex) compared to the total hexose contribution to SOC (Hex:SOC) shown as points fit to a power law (lines). Solid lines indicate significant relationships ($p < 0.034$) while dashed lines indicate insignificant relationships ($p > 0.05$).

PsiTag, the smallest component of the total hexoses, was also typically the smallest component of the non-glucose hexoses, though in some cases PsiTag had a more substantial contribution to the non-glucose hexose total. In many of the soils sampled (38.6%), PsiTag comprised 5% or less of the non-glucose hexoses, and in most of the soils (84.3%), PsiTag was only as much as 18% of the non-glucose hexoses (Figure 2.5). Occasionally, however, in 4% of soils, more than half of the non-glucose hexoses were PsiTag (Figure 2.5); in these soils, the PsiTag contribution ranged from 51.2% to 58.8% of the non-glucose hexoses (Appendix A, Figure A4). PsiTag abundance patterns did not correlate with plant cover type. Fructose, galactose, and mannose contributed to non-glucose hexoses in the same way that they did to total hexose concentrations: fructose had the highest contribution and greatest range in contributions, and galactose and mannose had statistically similar contributions ($p = 0.1546$) (Appendix A, Figure A4). Other than galactose

and mannose, all pairs of non-glucose hexoses had statistically significant differences in their total contributions to non-glucose hexoses in soils ($p \leq 0.0002$) (Appendix A, Figure A4).

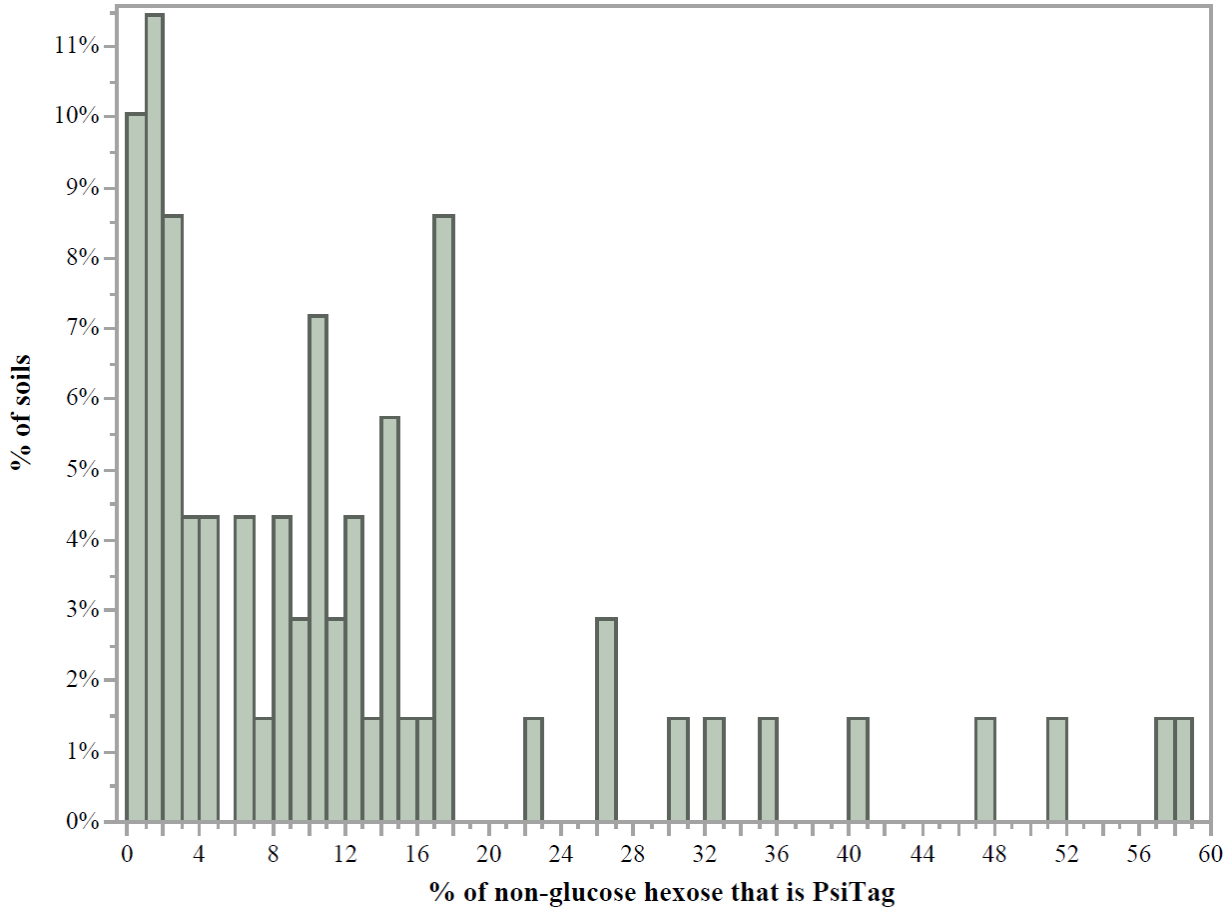

Figure 2.5. Relative percentages of the PsiTag contribution to total non-glucose hexoses in each soil.

Despite finding high concentrations of glucose and fructose in our quantitative derivatization, our chiral derivatization showed that there was no L-glucose or L-fructose present in quantities detectable by our method. On our system, D-fructose has retention times of 30.00 and 30.80 min, and L-fructose has retention times of 29.33 and 30.50 min (Figure 2.6). Chiral glucose standards did not derivatize as expected, so we do not have standard peaks for comparison in the chromatogram, but the aldohexose region of the chiral-derivatized soil extracts had only one peak, which we interpreted to be the extremely common D-glucose. Soils with concentrations above 100 µg fructose C g^{-1} soil showed peaks only in the D-fructose regions, and soils with concentrations below 100 µg fructose C g^{-1} soil did not show any peaks corresponding to either D-fructose or L-fructose (Figure 2.6). Due to complications, we collected the soil

extract and standards on different days so there is slight peak shifting, but the soil extract shows peaks at 30.05 and 30.90 min, consistent with D-fructose. Small signals in the soil extract at 29.45 and 30.65 min were below the signal to noise required to reach a conclusive determination.

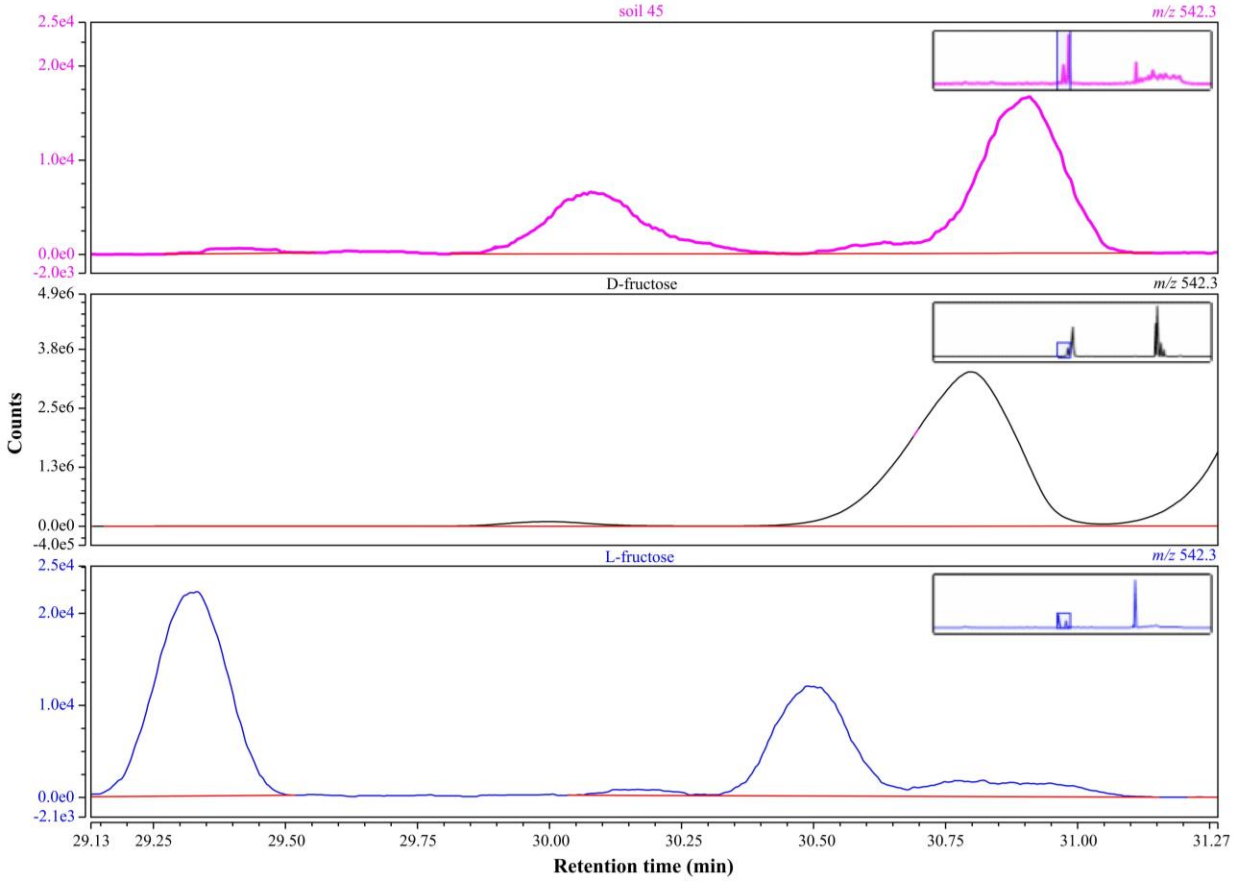

Figure 2.6. Stacked chromatogram of *m/z* 542.3 extracted from total ion chromatograms for D-fructose standard (middle), L-fructose standard (bottom), and a soil extract with high total fructose concentrations (top) showing D-fructose but not L-fructose.

2.4 DISCUSSION

Using GC-MS, we detected and quantified five hexose sugars in soil: glucose, fructose, galactose, mannose, and a rare ketohexose that we label PsiTag. The presence of free PsiTag in soils is a novel finding and, to the best of our knowledge, neither psicose nor tagatose has been previously reported in soil or any other system. Regardless of the identity of the rare ketohexose, its presence in soils suggests interesting and previously unknown biochemical synthesis pathways or possible persistence. Additionally, the ubiquity of PsiTag in our soil samples suggests that other rare sugars could also be present in soils and that further

work is required to identify which free hexoses are present in soils. The study of free hexoses in soils is critical in better understanding potentially unknown pathways, the impacts of free hexoses on soil C cycling, and the possible bioproduction of rare sugars.

Though we cannot distinguish tagatose from psicose using our method, we expect that both psicose and tagatose contribute to the PsiTag analyte peak. D-psicose and D-tagatose are C3 and C4 epimers of D-fructose, respectively, so high fructose concentrations could hypothetically contribute either rare ketohexose to soils.[66] D-psicose is not involved in any known metabolic pathways, soil-related or otherwise.[66] However, D-psicose 3-epimerase, an enzyme found in soil bacterium *Agrobacterium tumefaciens*, has been shown to produce D-psicose from D-fructose with a 32.9% conversion yield.[67] The reason for this transformation by *A. tumefaciens* in soils is unclear, however, soil bacteria are known to consume rare sugars,[68,69] so incubation studies should be performed to determine whether D-psicose could be a food source for this soil bacterium. Conversely, D-tagatose is a metabolite of galactose: D-galactose interconverts with galactitol by the aldose reductase enzyme, and D-tagatose is produced from galactitol by galactitol 2-dehydrogenase.[66] Interestingly, this metabolism is known to occur in soil bacteria including *A. tumefaciens* (conversion yield not reported) and *Enterobacter agglomerans* (86.5% to 93% conversion, depending on substrate concentration).[70,71] Clearly, we can only speculate on the identity of our rare ketohexose, and more targeted studies are needed to determine whether the PsiTag we measured is psicose, tagatose, or some combination of the two. Though PsiTag abundance was low and comprises only a small percentage of the total hexoses, its contribution to the total non-glucose hexoses was substantial in 4% of soils (Figure 2.5; Appendix A, Figure A4). The production, consumption, and biological agents related to PsiTag presence and metabolism in soils are not addressed by this study but remain potentially important for soil energy transformations and should be investigated further.

The presence of one rare hexose coupled with limitations in our analytical method suggests the possibility that other rare hexoses may also be present. If they were present, the overlapping retention times or high detection limits in the derivatization techniques we used would not allow us to detect them. The common hexose peaks present in the quantitative derivatization all overlapped with at least one other

hexose: glucose with idose and altrose; galactose with idose and talose; mannose with allose, gulose, and altrose; and fructose with sorbose and allose (Figure 2.2). As a result, it is possible that the common hexose peaks we measured may represent hexose mixtures impossible to distinguish by this method. To address this uncertainty, we would need to make future improvements in separation, including perhaps a more gradual temperature schedule or repeating the experiment using multiple derivatization techniques.

In our investigation of L-hexoses by chiral derivatization, the limited sensitivity of the method showed that L-hexoses are not present in high concentrations (Figure 2.6), but it is still possible that L-hexoses are present at concentrations below the detection limit for this method (100 μg hexose C g^{-1} soil or greater). For example, despite being a rare aldohexose, L-glucose has been shown to be a food source for multiple bacteria isolated from soil,[68,72,73] implying its presence in soils and suggesting that the lack of measurable L-glucose may be due to its preferential consumption by soil microorganisms. However, to the best of our knowledge, L-fructose has not been identified as a food source for soil microorganisms, implying its absence in soils. Additionally, the low concentrations of fructose in soils (15.41 μg fructose C g^{-1} soil) that we measured in our quantitative derivatization suggested that identifying L-fructose in the soil would be highly unlikely because we expect it to be present in very small quantities if at all. To truly claim the presence or absence of L-glucose and L-fructose using this chiral derivatization method, this study should be repeated using a chiral GC column, greater quantities of soil, and perhaps a longer hot-water soil extraction.

Our study focused only on free hexoses, while other studies simultaneously investigated both free and polysaccharide-bound sugars. Using our quantitative derivatization, we measured free hexoses in the μg- to mg C g^{-1} soil range (Figure 2.3), but other studies used soil extractions that involved an acid hydrolysis step to break down polysaccharides into their sugar monomers and, therefore, reported soil hexoses in much higher concentrations.[25,26,28,29,36,39,40,42–47,50–60,62,63,74] Those studies also showed a positive correlation between hexose C concentrations and SOC, but our results showed no significant relationships for overall total hexose C to SOC (Appendix A, Figure A2) or for individual hexose C to SOC (Appendix A, Figure A3).[25] This indicates that although SOC content does correlate with total soil polysaccharide

concentrations, SOC content does not inform or predict the presence of free hexoses. Figure 2.4 supports this conclusion, showing that the total free hexose contribution to SOC is less than 0.1%. This suggests that a shift in focus from total soil carbohydrates to free soil sugars is necessary to understand this small but rapidly cycling soil C pool. For example, glucose is well known to be the most abundant neutral sugar in soils.[25,28,42,44,45,75] Despite this, the exact metabolism of glucose by soil microorganisms varies greatly and is not fully represented by well-known pathways from industrial strains like *E. coli* and *B. subtilis*.[76–78] Low molecular weight (often <500 Da) organic compounds, including free hexoses, are critical for microbial growth and metabolism in soils, so to accurately describe metabolism in soils, we must first understand the presence and concentration of free hexoses and other low molecular weight organic compounds.[79,80] Measuring this metabolic complexity requires techniques with both high resolution and high sensitivity, so analytical chemistry is an ideal tool to help answer these questions.

The analytical identification of sugar in soils remains a challenge. Except for PsiTag, results from the study presented here do not confirm or deny the presence or absence of most rare hexoses in soil. The complexity of a soil matrix and the inhomogeneity of soil carbon pools makes study-reproducibility a great challenge. Our soil collection is a good representation of the average American yard and garden, which are more likely than natural systems to be amended with fertilizers or other supplements. Although we do see clear evidence of the metabolic capacity for sugar transformations in the residential outdoor spaces we measured, this study does not include natural soil environments, making extrapolation into the field impossible. Additionally, the nuances of experimental design and method development, complicated or time-consuming derivatizations, overlapping retention times, and detection limits higher than the natural abundance of a sugar in soil add layers of difficulty. At the heart of the challenge, however, lies the inherent complexity of carbohydrate research. Between the two functional types (ketoses and aldoses), two enantiomers (D- and L- forms), two anomers (α- and β-forms), two tautomeric rings (pyranoses and furanoses), and a straight-chain, linear form, the 12 hexose sugars total 144 unique isomers. It is not clear at present how this problem will be solved. Improvements in separation and detection, using resources such as chiral chromatography, might be effective. To facilitate high-throughput analysis, the derivatization

process needs to be optimized as well. With the evidence from this study of rare hexose presence in soils, we hope to motivate further analytical investigation of rare sugars in soils and, therefore, shift the paradigm by redefining how we think about “common” and “rare” sugars.

2.5 CONCLUSION

In this study, we identified a rare ketohexose with ubiquitous presence in soils that, to the best of our knowledge, has never before been reported. Though we cannot determine the precise identity of the rare ketohexose using our method, we acknowledge that it may be just psicose or tagatose but is potentially a mixture of psicose and tagatose. The ambiguity about the identity of the rare ketohexose peaks we observed also prompts uncertainty about the common hexose peaks we measured. Because of limitations in the analytical methods used, we conclude that more rare hexoses may be present in soils than we were previously aware of. Further investigation and advances in detection are required before we can conclusively determine whether additional rare hexoses are present. That a rare hexose is present in soils, and that there may be more, is an exciting and novel discovery that challenges our knowledge of small-molecule persistence and pushes our understanding of biogeochemical cycling and microbial C use in soil systems. In future work on this topic, we will analyze the ethanol-insoluble, polysaccharide-bound fraction of our soil extracts and compare the proportions of hexoses contained in the polysaccharide-bound fraction to the proportions of hexoses contained in the free monomer fraction. We will use those data to shed light on both the free hexose metabolism in soils and the ability of rare, small molecules to persist in the soil. This knowledge will better inform us of potentially understudied soil biochemical pathways, and it will enable us to refine the widely accepted definition of rare sugars and their currently unknown presence and persistence in soil.

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CHAPTER III: CLOSING THOUGHTS ON RARE SUGARS FOR SOIL CARBON SEQUESTRATION

Rare sugars in soils represent an understudied and untapped niche for carbon dioxide removal (CDR). In this thesis, I present two of the many studies that the Climate Solutions Lab has designed and performed to test the carbon storage potential of rare sugars.[1] These studies have answered some critical questions about sugars in soils, but many questions remain unanswered, and there is still much to do to determine the true potential of sequestering rare sugars in soil for CDR.

The Chapter 2 finding of a free rare ketohexose, that we label PsiTag, in soil has at least two potential explanations: high recalcitrance or high turnover. By the high recalcitrance explanation, PsiTag was present because it is added to soil by some unknown source, but there is little to no microbial activity to consume it, resulting in its accumulation in soil. This explanation suggests that PsiTag, and potentially other rare sugars, could present potential avenues for C sequestration. Alternatively, by the high turnover explanation, PsiTag was present because it is added to soil by some unknown source, but concentrations were low because of high turnover due to interesting and unknown metabolic pathways or biological usage, resulting in its rapid consumption. This explanation suggests that PsiTag would not be a potential avenue for C sequestration but leaves the door open for other rare sugars. These turnover or recalcitrance explanations can and should be applied to all rare sugars to determine their potential for soil CDR.

PsiTag, specifically, shows evidence for the recalcitrance explanation and may be a good candidate for soil CDR. Preliminary data from the fraction of extracted soil containing the ethanol-insoluble, polysaccharide-bound sugars show much lower relative concentrations of PsiTag than the ethanol-soluble, free monomer fraction we analyzed in Chapter 2. These data suggest that PsiTag may be present in small quantities in polysaccharides and, after hydrolysis, accumulate in soil and persist as a free monomer. The findings from the Chapter 1 MicroResp™ study showed that L-psicose is not actively consumed by soil microorganisms, but D-psicose, D-tagatose, and L-tagatose were not tested. To test PsiTag in the context

of the recalcitrance explanation, the MicroResp™ experiment should be repeated with these three rare hexoses.

Based on evidence we collected in the two experiments described in this thesis, L-hexoses have variable potentials for soil CDR; L-fructose may be a strong candidate, but L-glucose is too highly respired and would likely not be a good C storage molecule in soil. We found no literature reports describing the consumption of L-fructose by soil microorganisms, and we did not find L-fructose in soils in the Chapter 2 GC-MS soil survey experiment, but due to the high limit of detection, we cannot rule out its presence. Its absence, however, is consistent with the results of the Chapter 1 MicroResp™ experiment, which showed extremely suppressed respiration in the soils with added L-fructose. By Occam's Razor, if a sugar cannot be respired by soil microorganisms, then that sugar is likely not present in the soil. These pieces of evidence both suggest that there is likely no L-fructose present in soils, even at concentrations lower than the limit of detection for our method. On the contrary, our MicroResp™ study showed considerable L-glucose respiration, which implies its familiarity to soil microorganisms, but the soil GC-MS survey showed no detectable L-glucose. This suggests that L-glucose is turned over rapidly in soil, though, as mentioned in Chapter 2, our inability to measure these L-hexoses could simply be a result of the high detection limit of the method we used.

D-glucose is a common sugar that is well known to be incredibly consumable by soil microorganisms and is therefore not a candidate for soil CDR, but we noticed curious patterns in D-glucose concentrations that could grant insights into the characteristics of soil turnover and merit discussing D-glucose here. Interestingly, in the GC-MS soil survey, we observed that the absolute concentration of glucose decreased with increasing SOC (Appendix A, Figure A3). Combined with our results from the MicroResp™ experiment (Chapter 1, Figure 1.1), we speculate that decreasing glucose concentrations at high SOC occurs because of increased accessibility to glucose in the soil. In the GC-MS soil survey, we also observed that the relative contributions of each hexose to the total hexose concentration across Hex:SOC (Chapter 2, Figure 2.4) is consistent with known literature for glucose, galactose, and mannose,[2]

suggesting that the turnover of common sugars occurred in the soils proportionally to the concentration of the sugar. This implies that the number of possible fates of a sugar in soil is proportional to the concentration of that sugar.

Unfortunately, other studies conducted by the Climate Solutions Lab have found that most rare sugars can be respired by various microorganisms, so we have determined that using rare sugars to sequester C is likely not a feasible negative mitigation strategy. If it is true that the number of possible fates of a substance is proportional to its concentration, adding rare sugars to soils could seem to sequester C in the short-term but spark unprecedented long-term effects such as the potential speciation of a soil microorganism that can readily consume this sudden untapped source of C. Although we likely cannot use the bioproduction and soil storage of free rare sugars as a negative mitigation strategy to help combat climate change, the pursuit of this incredible idea, and the hope that it brought, has taught us that bio-transformative CDR (BtCDR) is a powerful technique for C sequestration.

Even though rare sugars are not likely to make an efficient C sink in soils, we now know that rare sugars contribute to soil C. I believe that rare sugars have undiscovered but important roles to play in soil C sequestration. We saw that some rare sugars are present in soils and that some are consumed by soil microorganisms. Perhaps if rare sugars capped soil polysaccharides, the polysaccharides might be more resistant to degradation. Perhaps rare sugars are involved in the stability of branched polysaccharides. We know that rare sugars themselves may not be stable, but perhaps the larger structures they belong to have some characteristics of long-term stability. Because polysaccharides are so frequently contributed to the soil by plants, it is clear that plants have the potential to play a pivotal role in sequestering C and that BtCDR is a very promising strategy.

In the end, it will not be BtCDR alone that is responsible for achieving the negative emissions required to meet the United Nations Framework Convention on Climate Change goal of staying below 2 °C of warming; it will be all the additive effects of all the promising mitigation strategies. And just as those implemented climate strategies will work together to help us achieve our climate goals and prevent further warming, the scientists who engineered and applied them will work together to refine them. By

harnessing the collective expertise of interdisciplinary teams, our collaboration can earn us a future without the fear of a changing climate. There is a lot of work still to be done, but every tested hypothesis moves us one step closer to a healthier planet for generations to come.

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APPENDIX A: SUPPLEMENTAL FIGURES

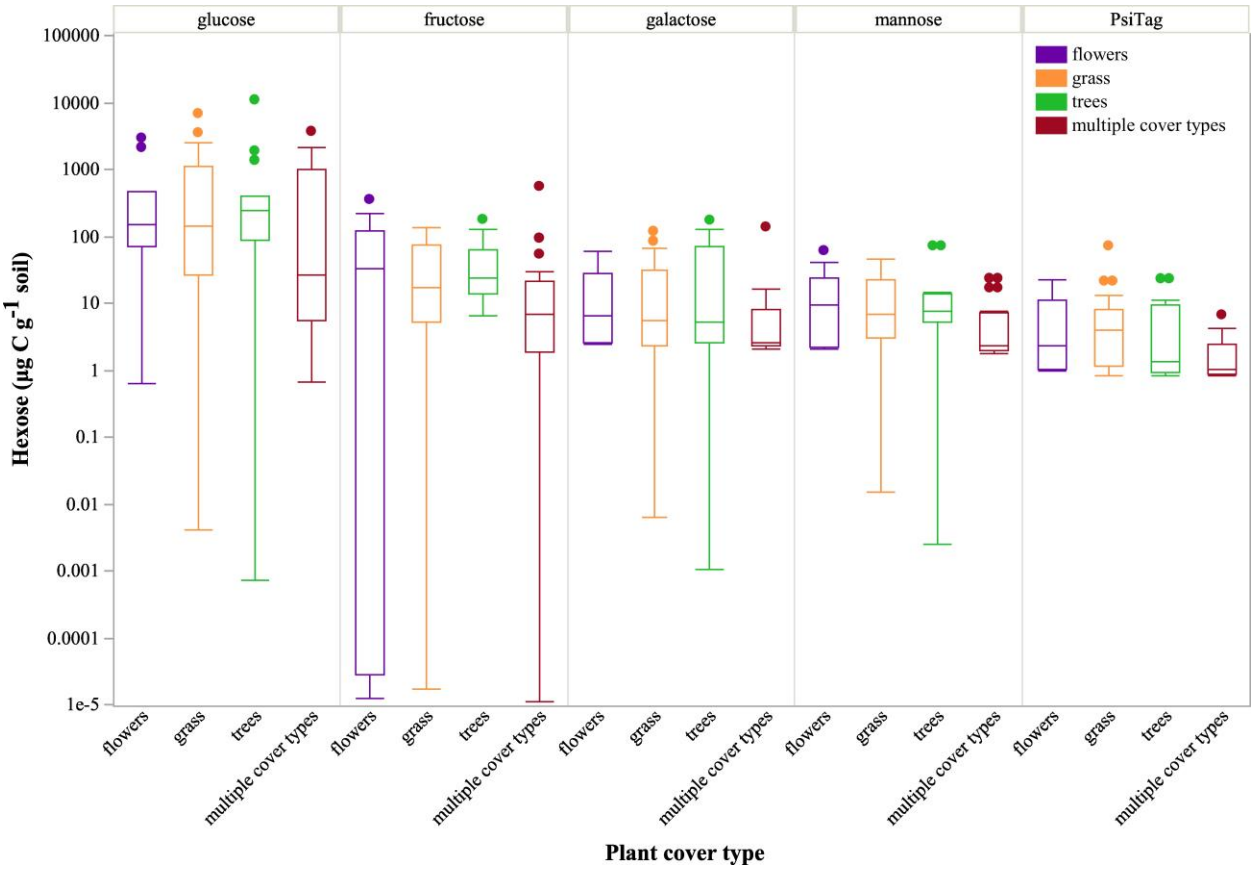

Figure A1. GC-MS-derived concentrations of each hexose in µg hexose C g^{-1} soil sorted by plant cover type and plotted on a log axis. Soils from trees, flowers, and grass did not have statistically different hexose concentration means ($p \geq 0.4043$), but multiple cover types had statistically different hexose concentration means than trees, flowers, and grass ($p = 0.0001$, $p = 0.0055$, and $p = 0.0002$, respectively).

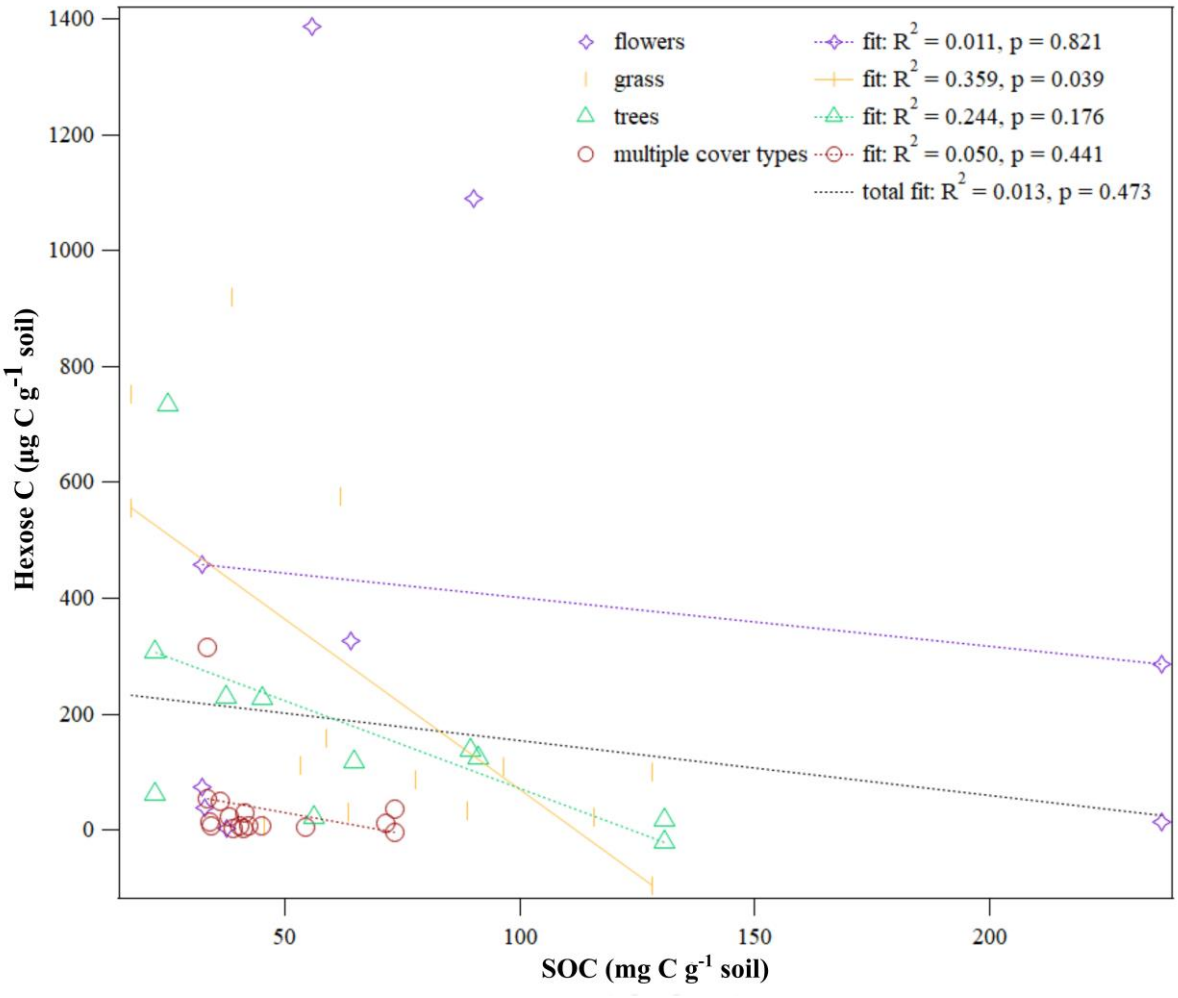

Figure A2. Total concentrations of soil hexose as measured by GC-MS versus SOC. We denoted significant regressions with solid lines and insignificant regressions with dashed lines. There is no relationship between total hexoses and SOC. There is a significant negative relationship between monomer hexose concentration and soil carbon concentration in grass, but this relationship represents only 35.9% of the data.

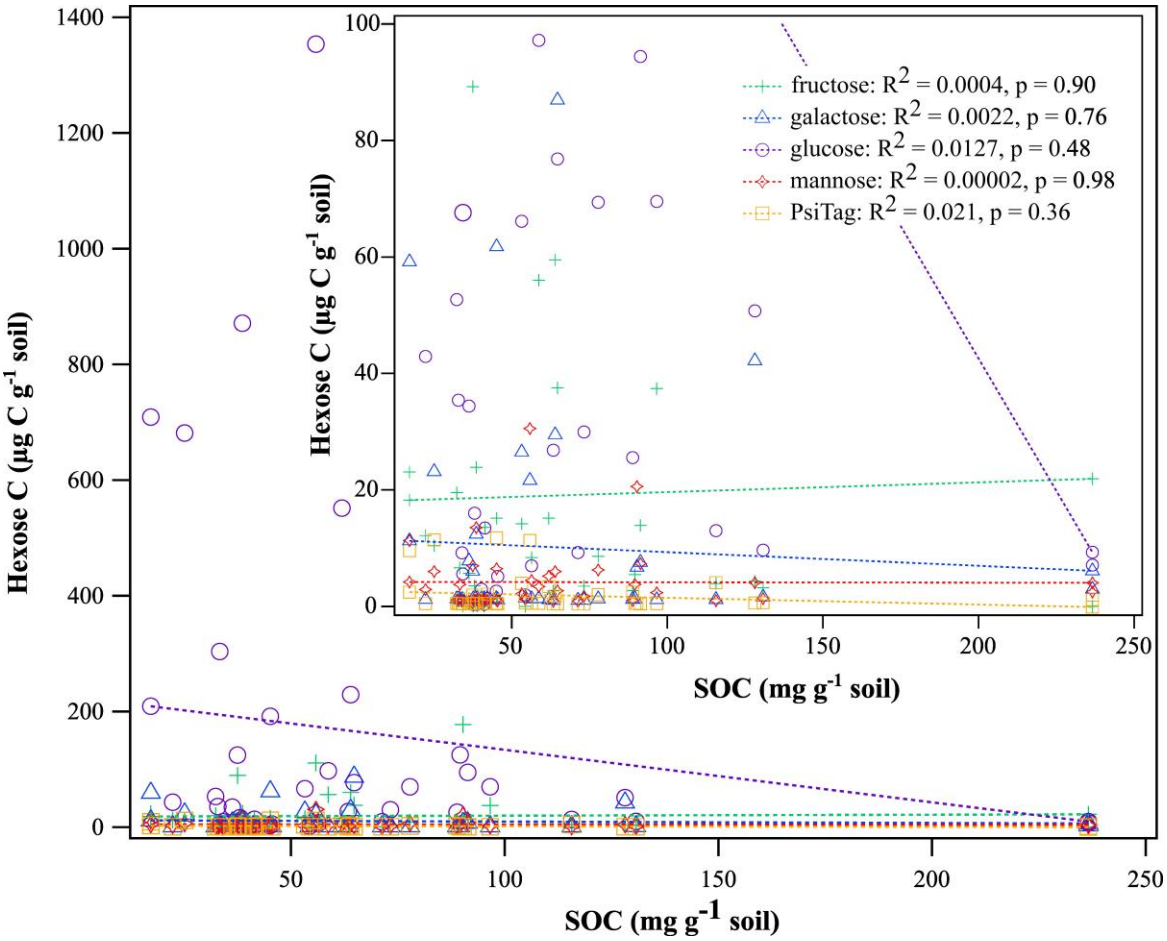

Figure A3. GC-MS derived concentrations of individual hexoses versus SOC. Glucose concentrations decrease with increasing SOC, but concentrations of the other four hexoses stay consistent. However, none of these relationships have a significant p-value or a convincing R^2.

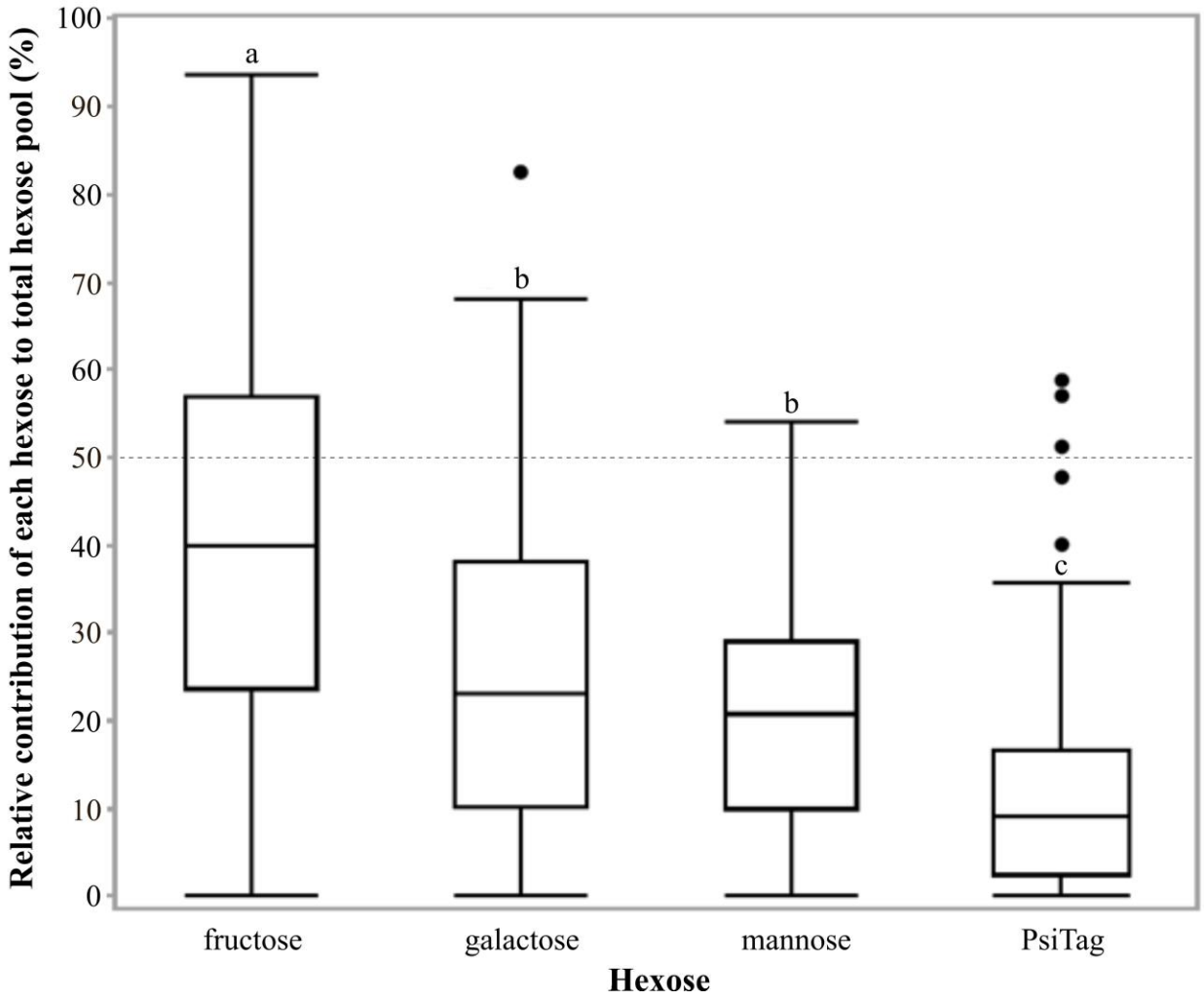

Figure A4. Distributions of the relative contributions of each non-glucose hexose to the total non-glucose hexose concentrations. PsiTag typically comprises the smallest amounts of the total non-glucose hexoses but occasionally contributes more than 50% of the total. Fructose has the greatest range in contributions, and galactose and mannose have statistically similar ranges in contributions (p = 0.1546). The dashed line at 50% highlights the 4% of soils that have >50% PsiTag contribution to non-glucose hexoses.

APPENDIX B: SOIL COLLECTION PROTOCOL

Dear Soil Carbon Moonshot contributor,

We are so excited that you are willing to be a part of our nation-wide effort to gather a variety of soil samples and soil microorganisms! As you know, soils are incredibly diverse and the microbes that inhabit them perform a variety of essential ecosystem processes and have virtually unlimited life styles. The samples you'll be providing us with will help us investigate the diversity of microbial consumption and production of different forms of carbon. Our current scientific understanding on this topic is very limited, yet it has never been more important for our understanding and our ability to mitigate climate change.

Here are some brief instructions for collecting your samples.

1) Collect about three samples in one location using the complementary soil corer and place into the container provided until the container is about ¾ full. On the datasheet, please record the requested information and be sure to add the container number (the containers are labeled 1-5)
2) Do the same for samples 2-5. Try to include at least one turf sample, but otherwise collect soils wherever you think is interesting (under a tree, in the garden, etc). Please note that it is important that the samples are collected on your property.
3) Place all samples in the pre-labeled Cooliner envelope
4) Pull the tab of the ShockWatch indicator and place into the envelope (this will help us understand whether sample was exposed to extreme conditions during shipping).
5) Drop the envelope off at the nearest UPS/Fedex office and please try and drop the envelope of the same day so the sample will be in shipping as briefly as possible.

Thanks for your help!

The Soil Carbon Moonshot team

Richard Conant Peter Baas Stephanie Cardinalli

We hope that the data and soil organisms we gather from these samples will result in scintillating publications, innovative intellectual property, and (we hope) world-changing progress toward a brand new way to sequester carbon in soil. We appreciate your contribution to this effort and your consent to allow us to use these data and materials pursue those ambitions!

Figure B1. The first page of the collection protocol we sent to those who collected crowdsourced soils.

Colorado State University

Sampling Form (Please include in the return envelope)

Sample	Vegetation nearby (check when applicable)				Anything else you'd like to share?
	Turf/Lawn	Trees	Vegetables	Flowers	
1					
2					
3					
4					
5					

Figure B2. The second page of the collection protocol we sent to those who collected crowdsourced soils.