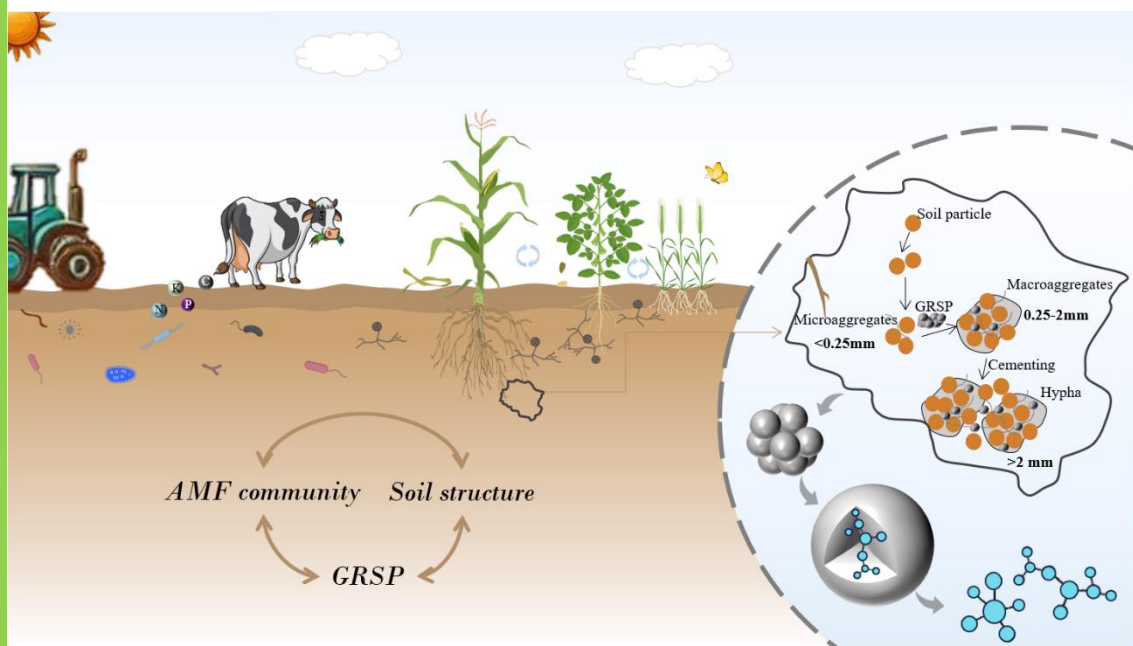


Characteristics of glomalin-related soil proteins accumulation and its response to fertilization and tillage in typical cropland soil

Hongbo Yang



Promoteurs: Prof. Gilles Colinet & Prof. Jeroen Meersmans
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Characteristics of glomalin- related soil proteins accumulation and its response to fertilization and tillage in typical cropland soil

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Abstract

Intensive cultivation and improper human management practices in cropland have led to the deterioration of the soil environment, including low soil organic carbon (SOC) content, nutrient imbalances, and land degradation, all of which hinder sustainable agricultural development. Glomalin-related soil proteins (GRSP), derived from arbuscular mycorrhizal fungi (AMF), are considered a stable component of SOC and plays a crucial role in improving soil structure and increasing SOC sequestration. Optimal management strategies such as organic fertilization, diversified cropping, and conservation tillage have been reported to enhance GRSP levels, but the mechanisms behind its accumulation remain unclear. Moreover, different soil orders result in distinct responses of GRSP to management practices. Therefore, understanding the distribution characteristics of GRSP and its accumulation mechanisms is essential for sustainable soil management in agricultural production. In this study, we collected soil samples from 14 long-term (>25 years) field experiments across different temperature gradients in eastern China, and investigated GRSP levels, soil properties, microbial attributes, and climate variables to study the characteristics and distribution differences of GRSP. Additionally, we also analyzed the climatic and microbial factors causing differences in GRSP accumulation on a large scale. Finally, we selected specific-site to explore the potential mechanisms of long-term fertilization and tillage in enhancing GRSP and its relationship with SOC accumulation. Our results are as follows:

GRSP content ranged from 1.0 to 5.1 mg g⁻¹ soil, with the trend: mid-temperate zone (mid-tem) > warm-temperate zone (warm-tem) > subtropics. In the warm-tem and subtropics, organic fertilization (OF) significantly increased GRSP contents compared to no fertilization (CK) and chemical fertilization (CF), but there was no significant difference among fertilization treatments in the mid-tem. In cropland soils, clay content, mean annual temperature (MAT), pH, and nutrients were identified as the key factors influencing GRSP accumulation. Random forest (RF) model showed that clay content was the primary determinant of GRSP levels in the middle temperate zone, while soil available phosphorus (AP) was the limiting factor in the subtropics and warm-tem. Partial least squares path modelling (PLSPM) further indicated that OF could indirectly enhance AMF biomass by increasing nutrient availability, thereby mitigating the negative impact of temperature on GRSP accumulation. This study highlights the importance of organic fertilizer application for soil stability and SOC sequestration in the context of climate change.

GRSP contents increased over years across fertilization treatments. Compared with CK and simple rotation (wheat-maize), long-term manuring (M, pig manure; NPKM, mineral N, P, K+M), straw returning (NPKS, NPK+straw) and diversified cropping (wheat-sweet potato/soybean) (NPKMR) significantly increased bulk soil GRSP by 100%, 80% and 39%, respectively, and altered the chemical composition of GRSP by increasing the recalcitrant (aromatic) C proportion. The proportion of aromatic C in GRSP was positively correlated with AMF biomass and diversity (Shannon and Chao1), indicating that the chemical composition of GRSP could be regulated by

AMF community and diversity. Moreover, manuring facilitated the formation of macro-aggregates ($>250\ \mu\text{m}$), thus increasing the physical protection of GRSP. The PLSPM further demonstrated that GRSP content was positively regulated by soil macro-aggregates, AMF biomass and diversity and their linkage with GRSP chemical composition.

GRSP contained 37-49% C and 6-9% N, respectively. Compared with CK and NPK, the C and N proportions in GRSP significantly increased under M and NPKM, with a concurrent decrease in the C/N ratio. Using a parallel factor analysis model, four fluorescent components of GRSP were identified: a fulvic acid-like component (C2), a tyrosine-like component (C4), and two humic acid-like components (C1 and C3). Under M and NPKM treatments, the fluorescent intensity and relative percentage of the C4 component decreased, while those of the C1 component increased relative to CK and NPK. Moreover, the GRSP humification index (HIX) under M and NPKM treatments was higher than CK and NPK, indicating that organic fertilization could enhance the stability of GRSP. PLSPM revealed that fertilizer-induced changes in edaphic properties (i.e. pH, SOC, AP) increased AMF biomass and diversity, thereby altering GRSP composition.

No-tillage (NT) significantly increased SOC and GRSP contents by 1.14-1.46 mg g^{-1} and 0.43-0.72 mg g^{-1} in the bulk soil relative to rotary tillage (RT) and deep tillage (DT), respectively. The contribution of GRSP-C to SOC under NT was higher than RT and DT, especially in $>53\ \mu\text{m}$ particle size. Additionally, NT increased AMF diversity and the abundance of *glomerales* and *diversisporales*, all showing a strong positive correlation with GRSP, indicating their regulatory role in GRSP production. The positive correlations between GRSP and the mass percentage of the $>53\ \mu\text{m}$ particle size fraction and mean weight diameter (MWD) suggest that NT may drive macroaggregates and microaggregates ($>53\ \mu\text{m}$) formation and enhance aggregate stability through GRSP levels. Overall, increased AMF diversity and keystone taxa abundance at the order level via NT promoted SOC accumulation through the production of GRSP and the protection of macroaggregates and microaggregates.

Overall, organic fertilization, straw return and diversified cropping can enhance content and stability of GRSP, and/or facilitate the formation and stability of soil macro-aggregates. No-tillage promotes SOC accumulation and GRSP production by increasing AMF diversity and the abundance of keystone taxa. Future research should further explore the micro-mechanisms of profile GRSP accumulation and the relationship between GRSP and SOC dynamics under different management practices. This will provide scientific evidence for developing more effective soil management strategies to promote sustainable agricultural development.

Keywords: Long-term fertilization, tillage, aggregate, glomalin-related soil proteins, arbuscular mycorrhizal fungi

Résumé

La culture intensive et les pratiques de gestion humaine inadéquates dans les terres agricoles ont conduit à la détérioration de l'environnement du sol, y compris une faible teneur en carbone organique du sol (COS), des déséquilibres nutritionnels et la dégradation des terres, ce qui entrave le développement agricole durable. Les protéines du sol liées à la glomaline (GRSP), dérivées des champignons mycorhiziens arbusculaires (AMF), sont considérées comme une composante stable du COS et jouent un rôle crucial dans l'amélioration de la structure du sol et l'augmentation de la séquestration du COS. Des stratégies de gestion optimales telles que la fertilisation organique, la diversification des cultures et le travail de conservation ont été rapportées pour améliorer les niveaux de GRSP, mais les mécanismes de son accumulation restent flous. De plus, différents ordres de sols entraînent des réponses distinctes des GRSP aux pratiques de gestion. Par conséquent, comprendre les caractéristiques de distribution des GRSP et ses mécanismes d'accumulation est essentiel pour une gestion durable des sols en production agricole. Dans cette étude, nous avons collecté des échantillons de sol provenant de 14 essais de terrain à long terme (> 25 ans) à travers différents gradients de température dans l'est de la Chine, et nous avons étudié les niveaux de GRSP, les propriétés du sol, les attributs microbiens et les variables climatiques pour étudier les caractéristiques et les différences de distribution des GRSP. De plus, nous avons également analysé les facteurs climatiques et microbiens causant des différences dans l'accumulation des GRSP à grande échelle. Enfin, nous avons sélectionné des sites spécifiques pour explorer les mécanismes potentiels de la fertilisation et du travail à long terme dans l'amélioration des GRSP et leur relation avec l'accumulation de COS. Nos résultats sont les suivants:

La teneur en GRSP variait de 1.0 à 5.1 mg g⁻¹, avec une tendance de contenu : zone tempérée moyenne > zone tempérée chaude > subtropicales. Dans les régions tempérées chaudes et subtropicales, la fertilisation organique a considérablement augmenté les teneurs en GRSP par rapport à l'absence de fertilisation et à la fertilisation minérale, mais il n'y avait pas de différence significative entre les traitements de fertilisation dans la zone tempérée moyenne. Dans les sols agricoles, la teneur en argile, la température moyenne annuelle (TMA), le pH et les nutriments ont été identifiés comme les facteurs clés influençant l'accumulation de GRSP. Le modèle de forêt aléatoire a montré que la teneur en argile était le principal déterminant des niveaux de GRSP dans la zone tempérée moyenne, tandis que le phosphore disponible du sol (PA) était le principal moteur dans les zones subtropicales et tempérées chaudes. La modélisation par équation structurelle a également indiqué que la fertilisation organique pouvait indirectement augmenter la biomasse des AMF en augmentant la disponibilité des nutriments, atténuant ainsi l'impact négatif de la température sur l'accumulation de GRSP. Cette étude souligne l'importance de l'application d'engrais organiques pour la stabilité du sol et la séquestration du carbone dans le contexte du changement climatique.

Les teneurs en GRSP ont augmenté au fil des années dans tous les traitements de fertilisation. Comparé à l'absence de fertilisation et à la monoculture (blé-maïs), l'application de fumier à long terme, le retour de la paille et la diversification des cultures (blé-patate douce/soja) ont augmenté de manière significative la GRSP dans le sol en vrac de 100 %, 80 % et 39 %, respectivement, et ont modifié la composition chimique du GRSP en augmentant la proportion de C récalcitrant (aromatique). La proportion de C aromatique dans le GRSP était positivement corrélée avec la biomasse et la diversité des AMF (Shannon et Chao1), indiquant que la composition chimique du GRSP pouvait être régulée par la communauté et la diversité des AMF. De plus, le fumier a facilité la formation de macro-agrégats (> 250 µm), augmentant ainsi la protection physique du GRSP. La modélisation par équation structurelle a également démontré que le contenu en GRSP était positivement régulé par les macro-agrégats du sol, la biomasse et la diversité des AMF et leur lien avec la composition chimique du GRSP.

Le GRSP contenait respectivement 37-49 % de C et 6-9 % de N. Comparé à CK et NPK, les proportions de C et de N dans le GRSP ont considérablement augmenté sous M et NPKM, avec une diminution simultanée du rapport C/N. En utilisant un modèle d'analyse factorielle parallèle, quatre composants fluorescents du GRSP ont été identifiés : un composant de type acide fulvique (C2), un composant de type tyrosine (C4) et deux composants de type acide humique (C1 et C3). Sous les traitements M et NPKM, l'intensité fluorescente et le pourcentage relatif du composant C4 ont diminué, tandis que ceux du composant C1 ont augmenté par rapport à CK et NPK. De plus, l'indice de humification du GRSP (HIX) sous les traitements M et NPKM était supérieur à CK et NPK, indiquant que la fertilisation organique pourrait améliorer la stabilité du GRSP. La modélisation par équation structurelle a révélé en outre que les changements induits par les engrais dans les propriétés édaphiques (c'est-à-dire le pH, le COS, le PA) augmentaient la biomasse et la diversité des AMF, modifiant ainsi la composition du GRSP.

Le non-labour (NT) a considérablement augmenté les teneurs en COS et en GRSP de 1.1-1.5 mg g⁻¹ et 0.4-0.7 mg g⁻¹ dans le sol en vrac par rapport au labour rotatif (DR) et au labour profond (DT), respectivement. La contribution du C-GRSP au COS sous NT était supérieure à celle du DR et du DT, en particulier dans les fractions de taille des particules > 53 µm. De plus, NT a augmenté la diversité des AMF et l'abondance des glomérales et des diversisporales, toutes montrant une forte corrélation positive avec le GRSP, indiquant leur rôle régulateur dans la production de GRSP. Les corrélations positives entre le GRSP et le pourcentage massique de la fraction de taille des particules > 53 µm et le DGM suggèrent que le non-labour peut entraîner la formation de grands agrégats (> 53 µm) et améliorer la stabilité des agrégats par les niveaux de GRSP. Globalement, l'augmentation de la diversité des AMF et de l'abondance des taxons clés au niveau de l'ordre via le non-labour a favorisé l'accumulation de COS par la production de GRSP et la protection des grands agrégats.

Dans l'ensemble, la fertilisation organique, le retour de la paille et la diversification des cultures peuvent améliorer le contenu et la composition chimique du GRSP, et

faciliter la formation et la stabilité des macro-agrégats du sol. Le non-labour favorise l'accumulation de COS et la production de GRSP en augmentant la diversité des AMF et l'abondance des taxons clés. Les recherches futures devraient explorer davantage les micro-mécanismes de l'accumulation du GRSP dans le profil et la relation entre le GRSP et la dynamique du COS sous différentes pratiques de gestion. Cela fournira des preuves scientifiques pour développer des stratégies de gestion des sols plus efficaces pour promouvoir le développement agricole durable.

Mots-clés: fertilisation à long terme, travail du sol, agrégat, protéines du sol liées à la glomaline, champignons mycorhiziens arbusculaires

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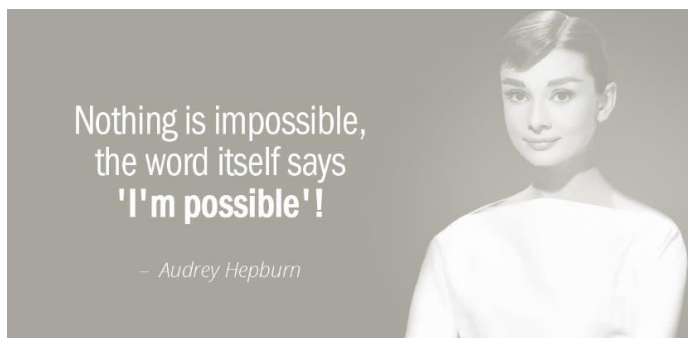
List of acronyms

(T-) GRSP	Total glomalin-related soil proteins
EE-GRSP	Easily extractable glomalin-related soil proteins
DE-GRSP	Difficult extractable glomalin-related soil proteins
GRSP-C	Carbon content in GRSP (mg C g ⁻¹ Soil)
AMF	Arbuscular mycorrhizal fungi
PLFA	Total phosphor lipid fatty acid
SOC	Soil organic carbon
TN	Total nitrogen
AN	Available nitrogen
AP	Available phosphorus
MWD	Mean weight diameter
PCA	Principal component analysis
VPA	Variance partitioning analysis
RF	Random forest model
PLSPM	Partial least squares path modelling
GOF	Good of fit
CK	No fertilization
NPK	Mineral nitrogen, phosphorus, and potassium fertilizers
NPKS	NPK fertilizers plus straw returning
M	Pig manure
NPKM	NPK fertilizers plus pig manure
NPKMR	NPKM plus leguminous rotation
NT	No tillage
DT	Deep tillage
RT	Rotary tillage
FA (F)	Fallow, nature succession
CF	Chemical fertilizers
OF	Organic fertilizers
MAT	Mean annual temperature
MAP	Mean annual precipitation
Mid-tem	Middle temperate zones
Warm-tem	Warm temperate zones

Chapter 1

General Introduction

“Nothing is impossible. The word itself says ‘I’m possible!’”
—Audrey Hepburn



1. Background

The increasing global demand for food and bioenergy has led to the conversion of natural ecosystems into agricultural land, resulting in significant losses of soil organic carbon (SOC) and exacerbating soil degradation due to improper practices (Smith, 2008). Since the 1950s, approximately 60% of soil ecosystem services have experienced varying degrees of degradation (León and Osorio, 2014). As the largest carbon (C) reservoir in terrestrial ecosystems, soil is influenced by both climate and human activities (Schlesinger, 1990). Even small changes in soil C can lead to global climate fluctuations (Lal, 2004). Cropland soils, which are among the most active C reservoirs (Flach et al., 2019), possess substantial C sequestration potential, particularly in soils with large yield gaps or significant SOC losses (Amelung et al., 2020). Tillage and the extensive input of organic and inorganic materials affect the mineralization, turnover, and sequestration of SOC (Sun et al., 2016). As a key indicator of soil quality (Šarapatka et al., 2019), the sequestration and transformation of SOC in cropland soils impact soil fertility and crop yields, making it a critical focus in agricultural production. Therefore, studying the sequestration of SOC in agricultural ecosystems is essential for stabilizing food production and mitigating climate change.

Glomalin-related soil protein (GRSP), a hydrophobic and adhesive glycoprotein primarily produced by arbuscular mycorrhizal fungi (AMF), is closely linked to the stabilization of SOC (Wright and Upadhyaya, 1998). On the one hand, the stability and persistence of GRSP in soil make it a significant component of the recalcitrant (biochemical stability) C pool within SOC. On the other hand, the adhesion of GRSP promotes the formation and stabilization of large soil aggregates (Wright and Upadhyaya, 1998), which helps prevent the decomposition of SOC (Guo et al., 2020). AMF are a crucial microbial group in the formation and stabilization of soil aggregates, as its hyphae bind small aggregates to form stable large ones, providing physical protection for organic matter (Cheng et al., 2012). Both AMF and GRSP are highly susceptible to agricultural management practices (Guo et al., 2019; Thomopoulos et al., 2023). These effects may differ significantly across regions and aggregate sizes. China, particularly its eastern regions, has extensive arable land resources. This area spans three temperature zones—the mid-temperate, warm temperate, and subtropical zones, creating a broad environmental gradient with varying soil properties and microbial communities. Studying the accumulation of GRSP and SOC on a regional scale allows for a comprehensive assessment of interactions between different factors. To ensure agricultural productivity and security, it is necessary to implement region-specific management practices, such as organic fertilization and conservation tillage (Kuhn et al., 2016; Tingyu et al., 2020). In this context, it is essential to comprehend how agricultural management practices promote GRSP and SOC accumulation in cropland soils.

Long-term experimental sites are invaluable in sustainable agriculture research. They provide important data for understanding how agricultural practices can adapt to and mitigate the effects of climate change. These sites also allow for the observation

of the cumulative effects of different agricultural practices over extended periods, leading to deeper insights in soil health, crop productivity, and environmental remediation. Based on this premise, our study focuses on typical cropland soils in eastern China. By examining how various management practices affect the production and accumulation of GRSP, we aim to optimize these practices to enhance soil carbon storage, improve soil health, and develop sustainable agricultural strategies.

2. Literature review

2.1. Source and characteristics of GRSP

GRSP is a metal-rich class of glycoproteins secreted by AMF. It was first discovered on the surface of the hyphae belonging to *Glomus intraradices* by soil scientist Wright and colleagues in 1996. This previously unknown protein, which reacts immunofluorescently with the monoclonal antibody MAb32B11, was initially thought to be a specific gene product secreted by AMF of the genus *Glomus* and was named glomalin (Wright and Upadhyaya, 1996). In 1998, Wright et al. conducted further experiments, extracting glomalin from soil using a sodium citrate solution at pH 8.0 and a concentration of 50 mmol·L⁻¹ under high pressure at 121°C. They compared this glomalin with proteins extracted from AMF hyphae using polyacrylamide gel electrophoresis (SDS-PAGE) and found that their protein bands were essentially identical. This finding confirmed that extracting glomalin from soil with a neutral to alkaline sodium citrate solution under high temperature and pressure was feasible and provided initial evidence that glomalin was produced by AMF. However, in 2004, Rillig and colleagues discovered that this protein also contained some non-glomalin components, leading to its renaming as glomalin-related soil protein (GRSP) (Rillig, 2004).

GRSP is a mixture containing humic substances, lipids, inorganic materials, and other compounds (Gillespie et al., 2011). Previous studies have shown that AMF are not the sole source of GRSP (Wang et al., 2015; Zhong et al., 2017). Although the exact origins of GRSP remain uncertain, many experiments have demonstrated that the growth of AMF and the content of GRSP change in tandem. Therefore, the complex chemical characteristics and origins of GRSP remain a focal point for future research. Numerous scientists have investigated the chemical composition of GRSP (Wang et al., 2014; Zhong et al., 2017). Different soil types and management practices may lead to changes in the elements and components of soil GRSP. Results indicates that GRSP contains a wide range of elements, including C, N, P, O, and Fe. Specifically, GRSP has been found to contain 3-5% N, 36-59% C, 0.03–0.1% P, 33-49% O, and 0.8-8.8% Fe (Lovelock et al., 2004; Rillig et al., 2001). Using ¹³C NMR, GRSP extracted from peat soils contains 42-49% aromatic C, 24-30% carboxyl C, and 4-11% low-fat C (Schindler et al., 2007). Zhong et al. (2017) used Fourier transform infrared spectroscopy to determine that GRSP contains functional groups such as O—H and N—H stretching vibration bands, C—H stretching vibration bands, CO and COO— stretching vibration bands, and C—O and Si—O—Si stretching vibration bands (Figure 1-1). Wang et al. (2014) identified seven compounds using three-

dimensional fluorescence spectroscopy (EEM): tyrosine-like protein, tryptophan-like protein, fulvic acid, humic acid, soluble microbial byproducts, nitrobenzoxadiazole, and calcite compounds. However, Guo et al. (2023) identified four components from coastal wetland soils. These findings indicate that there are differences in the fluorescent components of GRSP across different soil types. Despite these variations, the composition of GRSP can be broadly categorized into distinct compound classes and functional groups, each playing specific roles in soil structure and function: (i) Polysaccharides: long chains of monosaccharides linked by glycosidic bonds, rich in hydroxyl groups (-OH), essential for stabilizing soil aggregates. (ii) Proteins: polymers of amino acids connected by peptide bonds, containing carboxyl (-COOH) and amine (-NH₂) groups, which provide GRSP with adhesive and binding properties. (iii) Humic substances (e.g., humic acids, fulvic acids): complex aromatic compounds with multiple functional groups, such as phenols and carboxyls, which enhance soil stability and nutrient retention. (iv) Functional groups: groups such as hydroxyl (-OH), carboxyl (-COOH), and amide (-CONH₂) govern reactivity of GRSP and its ability to interact with soil minerals and organic matter. These components highlight the complexity of GRSP and its significant role in soil structure and function, particularly in fertilized cropland soils.

The humification process, which transforms organic matter into humic substances, is central to soil structure. Aromaticity, a key feature of humic acids and fulvic acids, enhances the stability and reactivity of these compounds in the soil. Humic substances interact with clay minerals via electrostatic forces and ligand exchange, contributing to stable soil aggregates. Calcium ions further promote this process by facilitating the binding of humic substances to clay minerals, thus strengthening soil structure and supporting organic matter sequestration. Therefore, understanding the composition of GRSP in fertilized soils is essential for elucidating its role in soil aggregation and stability.

2.2. Function of GRSP

2.2.1 SOC sequestration and soil aggregation

GRSP plays a crucial role in regulating the SOC pool, aggregate stability, and maintaining soil fertility (Figure 1-1; Preger et al., 2007). Some studies have reported a significant positive correlation between GRSP content and SOC content (Singh et al., 2017; Yang et al., 2024). GRSP is a substance with an aromatic C structure. Using ¹⁴C tracing techniques, Rillig et al. (2001) found that GRSP has a high C concentration of 36-59%, and can persist in soil for 6–42 years, making it an important source of organic carbon in the soil while contributing to the stability of the SOC pool.

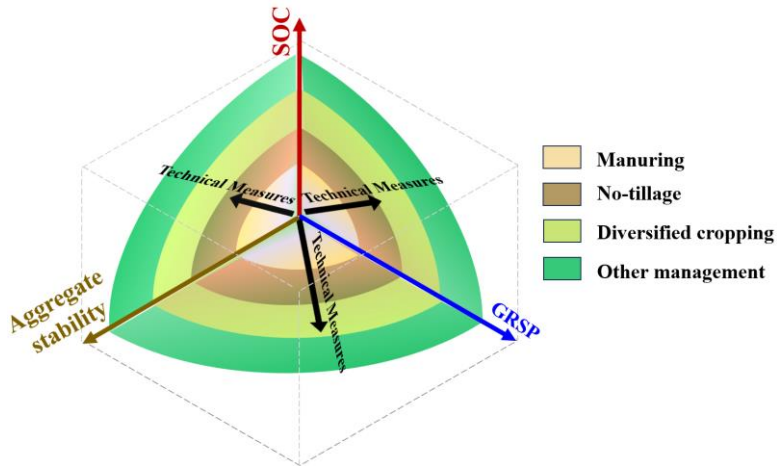


Figure 1-1 The diagram illustrates the relationships between GRSP, SOC, and aggregate stability. The central convergence of arrows labeled as "Technical Measures" highlights the integration of various practices, indicating a positive correlation among these indicators. These technical measures collectively enhance SOC, GRSP, and aggregate stability.

The sources of SOC include plant and microbial sources. The stabilization of SOC in soil involves complex mechanisms that are influenced by various factors, including soil microbial activity, mineral interactions, and the physical protection of organic matter (Figure 1-2). One of the key mechanisms of C stabilization is the formation of organo-mineral complexes. GRSP can bind soil particles to form aggregates, enhancing their stability by increasing the bond energy of the aggregates (Ji et al., 2019), thereby improving soil physical and chemical properties (Gao et al., 2019). Soil aggregates protect organic matter from microbial decomposition (Bronick and Lal, 2005). Many studies indicate a positive correlation between GRSP and aggregate stability (Singh, 2012; Wright and Upadhyaya, 1996).

In addition to forming aggregates, GRSP contributes to microbial-derived stabilization mechanisms. Through its binding properties, GRSP facilitates the interaction between soil microbes and organic matter. The role of microbial residues in C stabilization is well-established, with microbial biomass and exudates forming part of the stable C pool. Microbial-derived compounds, including fungal residues, are integrated into soil aggregates, where they persist longer than free organic matter (Lal, 2004). Moreover, GRSP may directly interact with microbial by-products, promoting their stabilization within aggregates or on mineral surfaces.

The mineral association of organic matter is another critical mechanism for C stabilization. GRSP interacts with minerals, particularly clay and iron/aluminum oxides, forming organomineral complexes that protect organic carbon from decomposition (Six et al., 2002). These complexes are relatively resistant to microbial degradation due to the tight binding of organic molecules to the mineral surfaces, which reduces their accessibility to microbial enzymes. The mineral fraction of soil,

including the iron and aluminum oxides, plays a crucial role in maintaining the long-term stability of SOC (Kleber et al., 2015).

In summary, the stabilization of carbon in soils involves multiple interconnected mechanisms, including the physical protection of organic matter within aggregates, chemical binding to minerals, and microbial processes that collectively maintain the long-term persistence of organic carbon. GRSP contributes to all these processes by binding organic matter, enhancing aggregate stability, and interacting with soil minerals and microbial products, thus playing a central role in the stabilization of SOC.

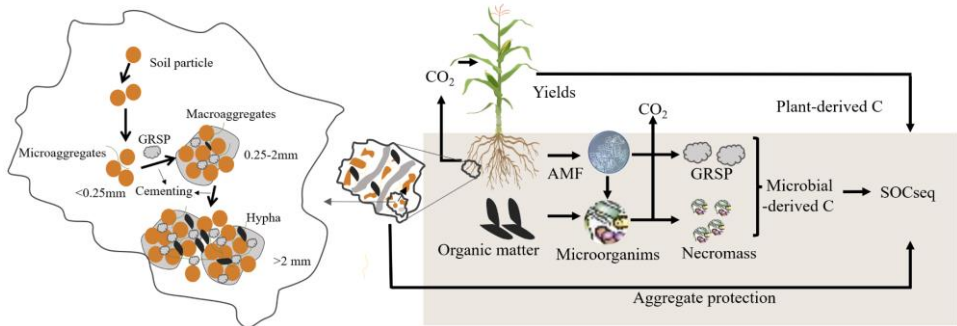


Figure 1-2 Schematic representation of the role of GRSP in SOC sequestration within cropland ecosystems. This illustration shows how GRSP, produced by AMF, acts as a binding agent for soil particles, promoting the formation of stable micro- and macroaggregates. These aggregates protect organic matter from decomposition, allowing for both plant-derived and microbial-derived C to be stored as SOC. The diagram also depicts the interactions between organic matter inputs (e.g., root residues and microbial necromass), microbial activity, and the role of GRSP in stabilizing SOC through aggregate protection. The cycling of C through these processes contributes to SOC sequestration.

2.2.2 Biostabilization

GRSP can also immobilize heavy metals in the soil (e.g., Mn, Cd, Pb, Cr, etc.), reducing the bioavailability of these toxic elements (Aguilera et al., 2011; Gonzalez-Chavez et al., 2004). For example, Wu et al. (2014) demonstrated that GRSP can bind Pb and Cd in the soil. Further studies have found that the amount of Pb adsorbed by GRSP is positively correlated with the total Pb content in the soil, whereas the adsorption of Zn by GRSP is negatively correlated with the total Zn content in the soil. When both Pb and Zn levels are high, GRSP adsorbs more Pb (Vodnik et al., 2008). In particular, some ecosystems use the correlation between GRSP and heavy metal content to indicate soil pollution levels, such as in wetland ecosystems (Wang et al., 2019). Therefore, the chelation of GRSP with heavy metal ions can immobilize potential heavy metals in the soil, providing a basis for plant-microbe remediation techniques.

2.2.3 Regulation of plant stress resistance

Abiotic stresses such as drought, salinity, and extreme temperatures lead to soil degradation and pose significant threats to agricultural production (Wang et al., 2003). Beneficial soil microorganisms, such as AMF, can mitigate adverse effects and

improve plant functions under stressful environmental conditions (Aroca and Ruiz-Lozano, 2009). AMF can enhance plant growth and physiological status by increasing the absorption of mineral salts and improving tolerance to both biotic and abiotic stresses (Clark and Zeto, 2000). During AMF symbiosis, plants prioritize the uptake of minerals such as P and Zn to support their growth. In this process, the demand for N is relatively lower. As a result, plants allocate a larger proportion of their organic C to the AMF, facilitating the symbiotic exchange of nutrients. This transfer of carbon from the plant to the fungi enhances the ability of AMF to provide essential minerals to the plant (Fellbaum et al., 2014; Kafle et al., 2019). This transfer promotes hyphal development and the production of more GRSP. The increased GRSP resulting from environmental stress helps soil ecosystems better resist adverse conditions. Current studies have shown that plants response to stress is accompanied by increased GRSP levels in the soil. When plants experience adverse abiotic stress, AMF can indirectly improve the plant's metabolism of substances by enhancing the absorption of soil moisture and mineral nutrients through the AMF symbiont in the plant roots. This process increases the production of substances (such as soluble sugars, amino acids, and soluble proteins) that help plants withstand unfavorable environments and generate GRSP, thereby improving soil resistance and stability (Clark and Zeto, 2000).

2.3. Analysis of GRSP

Currently, GRSP can be quantitatively analyzed using the Bradford colorimetric method (Coomassie Brilliant Blue) or enzyme-linked immunosorbent assay (ELISA) (Figure 1-3; Wright et al., 1996). The Bradford method is a common protein quantification technique (Bradford, 1976). The extraction steps for GRSP are as follows: Weigh 0.25 g of soil, add 2 ml of 50 mmol·L⁻¹ sodium citrate solution with a pH of 8.0, and extract at 121°C for 90 minutes. Immediately centrifuge the extract at 10,000 rpm for 6 minutes, remove the supernatant, add an equal volume of extractant, and repeat the extraction at the same temperature for 60 minutes. Centrifuge and remove the supernatant under the same conditions, and continue the extraction until the supernatant is colorless or pale yellow. Combine the supernatants for further analysis. 300 µL of the clear supernatant and 700 µL of dH₂O were drawn up into the sampling tank, and then 5 mL Coomassie bright blue (CBB) solution was added. This method is based on the principle that the dye (Coomassie Brilliant Blue G-250) binds with protein, changing the color from red to blue. The absorbance is read using a spectrophotometer at a wavelength of 595 nm, and the GRSP concentration is calculated using a standard protein concentration curve.

The supernatant was precipitated by adding a high concentration of hydrochloric acid, chilling in an ice bath for 1h, and centrifuging at 10,000 ×g for 6 minutes. The sediment was dialyzed in dH₂O for 60 hours after being dissolved in 0.1 M sodium hydroxide. After dialysis, in order to remove any insoluble residue, the dialysate was centrifuged at 10,000 ×g for 6 minutes. The supernatant was then freeze-dried using a freeze-dryer to obtain solid GRSP. Solid GRSP can be used to measure the elemental composition, chemical structure and functional groups of GRSP based on elemental

analyzer, three-dimensional fluorescence spectroscopy and Fourier transform infrared spectroscopy techniques (Figure 1-1).

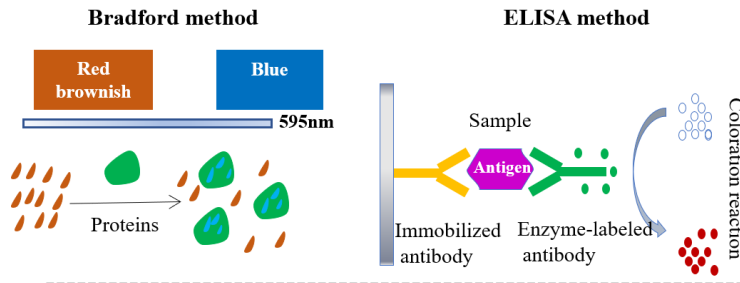


Figure 1-3 Comparison of methods for detecting glomalin-related soil protein (GRSP) using Bradford and ELISA (enzyme-linked immunosorbent assay) assays. The Bradford method is a colorimetric assay that detects total protein content, where the sample color changes from red-brown to blue upon binding with proteins, with maximum absorbance at 595 nm. This simple, rapid method gives a general measure of protein concentration, including GRSP. In contrast, the ELISA method provides a more specific detection of GRSP by using an antigen-antibody interaction. Immobilized antibodies bind to GRSP in the sample, followed by a secondary enzyme-labeled antibody, producing a coloration reaction upon substrate addition. This specific and sensitive approach allows for precise quantification of GRSP in complex soil samples.

Based on the Bradford method, GRSP is classified into two types: easily extractable glomalin-related soil protein (EE-GRSP, newly secreted fresh protein) and total glomalin-related soil protein (T-GRSP, accumulated stable protein). The EE-GRSP is extracted with 20 mM sodium citrate buffer (pH 7.0) followed by centrifugation. The T-GRSP needs a long autoclaving time and 50 mM citrate buffer (pH 8.0). This process is repeated until the color is gone or slightly colorless. However, the Bradford method cannot completely exclude interference from non-GRSP components, which may cause slight deviations in the results. ELISA can also measure these two types of proteins, requiring specific monoclonal antibodies (MAb32B11) (Rillig, 2004). The portions of EE-GRSP and T-GRSP that react immunologically with the monoclonal antibody are referred to as immunoreactive EE-GRSP and immunoreactive T-GRSP, respectively. This method provides a precise measurement of GRSP content, as the antibody shows negligible reaction with non-GRSP components in the soil. However, organic residues in the soil can reduce the sensitivity of ELISA (Tian et al., 2009). Additionally, many scientific laboratories lack the facilities to perform ELISA, which is costly and involves complex procedures. Rosier et al. (2006) compared ELISA and the Bradford method and found that ELISA is suitable for measuring GRSP in soils with low organic matter content. However, at higher organic matter levels, the Bradford method is simpler and easier to operate (Wright et al., 1996; Wright and Upadhyaya, 1996) and can be widely applied in GRSP quantification experiments.

2.4. Influencing factors of GRSP

Multiple factors affect the content of GRSP in soil, such as climate, soil properties, soil management, and AMF communities. The level of GRSP in soil is mainly influenced by the balance between AMF production and microbial decomposition. Additionally, GRSP is protected by soil aggregates, which shield it from microbial decomposition.

2.4.1 Climate

Higher temperatures can increase carbon allocation to AMF plants, subsequently boosting AMF biomass and promoting GRSP production. However, higher temperatures also promote the decomposition of GRSP by microorganisms (including some saprophytic fungi and bacteria), thereby reducing its levels (Adame et al., 2012). Studies by Vasconcellos et al. (2016) have found a negative correlation between GRSP content and soil water content. However, Adame et al. (2012) found that GRSP increases with annual rainfall in coastal (intertidal) seagrass meadow soils. Stress conditions such as drought stimulate GRSP production by increasing the allocation of assimilates to AMF as a plant survival strategy byproduct (Emran et al., 2012).

2.4.2 Edaphic factors

Soil pH affects the composition and content of GRSP because soil acidity has a strong impact on AMF (Wang et al., 2014). Among different soil types, GRSP content is highest in acidic soils and decreases as soil pH increases. Higher salinity reduces both AMF biomass and GRSP content (Krishnamoorthy et al., 2014) and alters the structure of GRSP (Zhang et al., 2017). Lower soil bulk density is associated with higher GRSP content (Zhong et al., 2017). The content of GRSP in soil is related to organic matter and clay content (Nichols and Wright, 2005), and it also shows a positive correlation with soil nutrients such as total nitrogen, total phosphorus, and available phosphorus. Infrared functional groups (IR-II, IR-V) and fluorescent substances (tyrosine-like and humic acid-like) are directly affected by soil moisture, pH, and electrical conductivity, thereby influencing the composition of GRSP (Zhong et al., 2017).

The interaction between clay and organic matter, mediated by calcium ions, plays a key role in soil aggregation. Organic compounds bind to calcium ions on mineral surfaces through electrostatic or ligand exchange, forming stable calcium-organic-mineral complexes (Ren et al., 2021). Calcium ions enhance the binding strength of these complexes, thereby facilitating the aggregation of organic matter and minerals (Chen et al., 2014). These processes underscore the critical role of calcium in organic matter retention and its influence on soil structure.

2.4.3 The ecology of AMF

The ecological characteristics of AMF are strongly influenced by soil factors such as nutrient availability and pH. These fungi are particularly sensitive to environmental conditions, and their ability to produce GRSP is closely linked to the nutrient composition and pH levels of the soil. Acidification tends to reduce AMF biomass and diversity globally, as low pH negatively affects spore germination and hyphal growth (Oehl et al., 2010). However, some AMF taxa, such as *Acaulospora*, exhibit

greater tolerance to acidic soils compared to others like *Funneliformis*, which are more sensitive to changes in pH (He et al., 2016). In the slightly alkaline soils (pH 7.8-8.6) of the upper Ganges Plain in India, the dominant AMF species is found to belong only to *Glomus*, while *Acaulospora* is completely absent (Ghosh et al., 2019).

Changes in global soil nutrient levels significantly impact AMF biomass, diversity, and their symbiotic relationships with plants. In nutrient-deficient soils, AMF population and diversity are quite high (Smith et al., 2010). AMF symbiosis and activity generally reduced in a nutrient-rich soils, particularly under high P (Lin et al., 2012). Fertilizer application can negatively impact AMF species composition in soil (Ferreira et al., 2012), often leading to reduced diversity compared to natural ecosystems (Oehl et al., 2003). While this trend is generally observed, exceptions exist. For instance, in arid and hot ecosystems, AMF diversity was found to be higher in a low-input agricultural site than in an adjacent natural site (Li et al., 2007). This highlights the variability of AMF responses to agricultural practices and the importance of context-specific factors such as climate, soil type, and land-use history in determining AMF community dynamics.

2.4.4 Tillage practices

Tillage affects not only soil aggregates but also the distribution of AMF and GRSP within aggregates. Generally, tillage is unfavorable for GRSP accumulation because it disrupts AMF hyphae and reduces AMF species richness (Dai et al., 2015; Oehl et al., 2003). Additionally, tillage breaks down large soil aggregates, reducing their ability to physically protect GRSP. Tillage practices can also alter the composition and function of soil microbial communities, significantly changing soil processes. Previous studies have shown that no-till and reduced tillage increase AMF biomass, diversity, and spore density, thereby enhancing GRSP production. Undisturbed soil maintains the stability of the hyphal network (Castillo et al., 2006; Wright et al., 2007). This stabilization promotes the formation and stability of aggregates mediated by hyphal networks and GRSP, preventing GRSP and organic carbon from coming into contact with saprophytic fungi and bacteria, thus providing a protective effect. Contradictory studies have also been reported; for instance, Brito et al. (2012) found that conventional tillage reduced AM fungal richness, while Hu et al. (2015) found no effect of conventional tillage on AM fungal richness in a four-year maize-wheat rotation system in northern China. Therefore, the impact of different tillage practices on AMF and GRSP levels requires further investigation.

2.4.5 Fertilization regimes

Studies have shown that applying appropriate nitrogen fertilizer can increase GRSP content in grassland, farmland, and forest ecosystems (Sun et al., 2018; Zhang et al., 2023). However, exogenous nitrogen deposition has been found to reduce GRSP production in Chinese forests (Jia et al., 2022). Additionally, adding phosphorus to soil has a more pronounced negative impact on AMF biomass and activity than adding nitrogen. In ecosystems with sufficient phosphorus, adding more phosphorus has little effect on GRSP content. However, in phosphorus-deficient ecosystems, adding phosphorus can increase GRSP content (Cissé et al., 2021). Nonetheless, research has

also found that GRSP production is not related to soil available phosphorus levels. Compared to conventional agricultural systems, organic farming (with added fertilizers) and composting, as well as straw incorporation, increase AMF abundance, species richness, and GRSP content (Turgay et al., 2015). Organic fertilization (e.g., animal manure) often promotes the growth and spore formation of AM fungi in agricultural systems (Yang et al., 2018). The rate of fertilizer application has a significant impact on AMF communities. For example, increased N fertilizer application under low P conditions enhanced AMF colonization benefits, the benefits sharply declined at higher nitrogen fertilizer rates (Bååth and Spokes, 1989). Application of 150 kg N ha⁻¹ may have a direct negative impact on AMF function. However, Tian et al. (2013) found that long-term nitrogen fertilization at 300 kg N ha⁻¹ could negatively affect AMF metabolism, but no negative effects were observed at 150 kg N ha⁻¹. Zeng et al. (2021) concluded that under the 0-300 kg N ha⁻¹ fertilization range, there was no significant effect on the AMF diversity (Shannon-Wiener index) in a maize-soybean intercropping system. The reason may be that the increase in nitrogen fertilizer use was not enough to make a significant difference. Therefore, in resource-limited agricultural fields, the amount of fertilizers (N, P) may promote or not affect AMF colonization (Higo et al., 2020; Qian et al., 2024). However, at higher fertilizer inputs, it becomes crucial to study their potential impacts on AMF communities and their symbiotic relationships with plants.

2.4.6 Cropping systems

Cropping systems play a crucial role in influencing AMF communities and GRSP content by shaping soil conditions and plant-microbe interactions. Intercropping systems, particularly those involving legumes and cereals, have demonstrated the ability to enhance GRSP concentrations and improve soil aggregate stability, a key factor in soil structure maintenance and carbon sequestration (Sekaran et al., 2020). Different crops, however, exert variable effects on AMF community composition and diversity, depending on their root traits and exudate profiles (Wang et al., 2022). For instance, Zhang et al. (2020) explored maize/soybean intercropping and observed that while the intercropping system did not significantly alter AMF α -diversity in the maize rhizosphere, it notably increased AMF α -diversity in the soybean rhizosphere. This indicates that legumes, such as soybean, can promote AMF diversity due to their nutrient-enriching properties and specific root exudates, which are attractive to a wider range of AMF taxa, including *Acaulospora* and *Scutellospora*. These taxa are known to enhance nutrient cycling and GRSP production, further contributing to soil health (Smith and Read, 2010). In contrast, wheat and maize are commonly associated with *Glomus* species, which are dominant in agricultural systems due to their robust nutrient acquisition capabilities (Luo et al., 2021). Such monoculture systems, while effective for nutrient uptake, may limit AMF diversity compared to multi-species cropping systems. Research by Selvakumar et al. (2018) showed that crop rotation could maintain AMF biomass and enhance GRSP content, underlining the importance of diversifying cropping practices for sustainable soil management. Similarly, Burrows (2014) reported that plots with multiple plant species had higher GRSP concentrations compared to monocultures, likely due to the complementary roles of

varied root systems in supporting AMF communities. Therefore, adopting intercropping and crop rotation practices, especially those involving legumes, appears to be an effective strategy to promote AMF diversity, increase GRSP production, and improve soil aggregate stability, thereby contributing to long-term soil fertility and resilience.

3. Objectives

GRSP is one of the critical indicators affecting aggregate stability and SOC sequestration. Cropland soil, due to human disturbances, has lower GRSP levels compared to grassland and forest soils (Agnihotri et al., 2022). The primary objective of this study is to understand the response of GRSP to fertilization and tillage in cropland, elucidate the accumulation process of GRSP, and identify the main controlling factors. Our study area spans three temperature zones with varying environmental gradients, allowing for a comprehensive investigation of the factors influencing GRSP accumulation. The technical roadmap of our study is illustrated in Figure 1-4. We selected 14 long-term fertilization experiment sites across three temperature zones. Theoretical analysis will be conducted on the distribution and influencing factors of GRSP (Chapter II), followed by selecting specific sites to reveal the effects of fertilization on GRSP accumulation (quantity) (Chapter III). Simultaneously, long-term tillage experiments will be utilized to uncover the carbon sequestration mechanisms of GRSP under different tillage practices (Chapter IV). Finally, the compositional traits of GRSP (quality) will be investigated under fertilization regimes (Chapter V). The specific objectives are as follows:

- (1) Clarify the distribution characteristics of GRSP at spatial and aggregate scales, as well as identify the main controlling factors.
- (2) Reveal the accumulation mechanisms of GRSP in bulk soil and aggregates under long-term fertilization.
- (3) Investigate the effects of long-term fertilization on the compositional traits of GRSP and the key influencing factors.
- (4) Uncover the microbiological mechanisms of SOC accumulation under different tillage practices, focusing on the role of AMF.

Characteristics of glomalin-related soil proteins accumulation and its response to fertilization and tillage practices in typical cropland soil

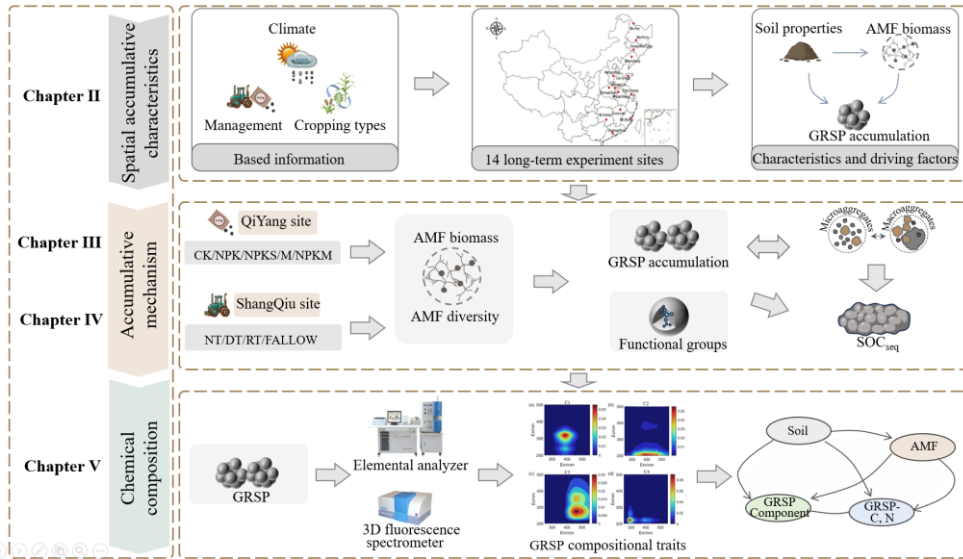


Figure 1-4 Technical route. This foundational information include climate, management practices and cropping types. Climate: Information on temperature, rainfall and other climatic factors affecting the experimental sites. Management: Agricultural practices, including fertilization, tillage techniques used at the experimental sites. Cropping types: Types of crops grown in the study areas, which can impact soil properties, microbial communities, and GRSP accumulation.

4. Overview of the chapters

Chapter I General introduction

We provided a comprehensive overview covering the source, compositional traits, function, determination method and influencing factor of GRSP. In addition, we also reviewed the effects of long-term fertilization (including organic and inorganic fertilizers), tillage, and cropping systems on GRSP accumulation, as well as the key influencing factors. Finally, we presented our research objectives and the framework of the thesis.

Chapter II Response of glomalin-related soil proteins accumulation to fertilization over three decades across eastern China

In order to elucidate the accumulation characteristics and main controlling factors of GRSP in cropland, we collected soil samples from 14 long-term experimental sites across eastern China, and investigated the GRSP contents in bulk soil and aggregates under fertilization regimes and combined with climate variables (MAT, mean annual temperature; MAP, mean annual precipitation), soil properties (SOC; TN, total nitrogen; AP, available phosphorus), and microbial parameters (AMF, arbuscular mycorrhizal fungi; PLFA, total phosphor lipid fatty acid) to reveal the main controlling factors.

Chapter III Long-term manuring facilitates glomalin-related soil proteins accumulation by chemical composition shifts and macro-aggregation formation

To understand the effects of long-term fertilization regimes on GRSP accumulation and related AMF communities, we investigated the dynamics of GRSP contents, AMF biomass and diversity based on a 29-year fertilization experiment (including fallow, control, mineral fertilization, manuring and straw returning treatments) in Qiyang, China.

Chapter IV No-tillage facilitates soil organic carbon accumulation by increasing arbuscular mycorrhizal fungi-related soil proteins and aggregation

In order to reveal mechanisms driving the accumulation of SOC, especially concerning the regulation of AMF communities and diversity in SOC sequestration. We analyzed AMF taxa at order levels, GRSP, and SOC within bulk soil and aggregates across four tillage treatments (i.e. FA, fallow; DR, rotary tillage; DT, deep tillage; NT, no-tillage) based on a 7-year tillage experiment.

Chapter V Long-term manuring enhanced compositional stability of glomalin-related soil proteins through arbuscular mycorrhizal fungi regulation

The changes in compositional characteristics of GRSP were evaluated under different fertilization regimes. We identified GRSP compositional traits using three-dimensional fluorescence spectroscopy (EEM) combined with parallel factor analysis (PARAFAC), and also investigated carbon (C) and nitrogen (N) contents in GRSP based on a 29-year fertilization including no fertilizer (CK), chemical fertilizer (NPK), manure (M) and manure combined with chemical fertilizer (NPKM) treatments.

Chapter VI General discussion, conclusions, and perspective.

We discussed the results of this thesis from three aspects. i.e. 1. GRSP distribution and driving factor; 2. Effects of fertilization regimes on GRSP contents and compositional traits; 3. Effects of tillage practices on SOC and GRSP contents. We also presented the answers to the main questions, perspective and the new knowledge for this study.

Chapter 2

Response of glomalin-related soil proteins accumulation to fertilization over three decades across eastern China

From: Hongbo Yang, Guanmo Li, Xin Li, Qiong Xiao, Caroline De Clerck, Jeroen Meersmans, Gilles Colinet, Wenju Zhang. Response of glomalin-related soil proteins accumulation to fertilization over three decades across eastern China (Submitting)

“Where there is a will, there is a way”

—Fan Ye

有志者，事竟成

—范晔



Abstract

Glomalin-related soil proteins (GRSP), a microbial metabolite related to arbuscular mycorrhizal fungi (AMF), plays an important role in soil health and C cycle dynamics. Although regional-scale storage of GRSP has been studied in natural ecosystems, our understanding of the spatial variation of GRSP in cropland and its response to fertilization regimes remains unclear. Therefore, based on soil samples from 14 long-term experimental sites in eastern China, we investigated the GRSP contents under fertilization regimes and combined with climate (MAT, mean annual temperature; MAP, mean annual precipitation), soil (SOC; TN, total nitrogen; AP, available phosphorus), and microbial (AMF; PLFA, total phospholipid fatty acid,) factors. Results showed that the GRSP content ranged from 1.0 to 5.1 mg g⁻¹ soil, with a trend of content: mid-temperate zone > warm-temperate zone > subtropics, showing a decrease followed by an increase with increasing latitude. In the warm-temperate and subtropical regions, soils treated with organic fertilizer had significantly higher GRSP content compared to unfertilized and chemical fertilizer-treated soils, but there was no significant difference in GRSP content among fertilization treatments in the mid-temperate zone. In cropland soils, clay content, MAT, pH, and nutrients were identified as the main factors influencing GRSP accumulation, while microbial factors (AMF) had a relatively minor impact. Concretely, physicochemical protection provided by clay dominated GRSP storage in the mid-temperate zone, while nutrient availability (especially phosphorus) played a significant role in regulating GRSP levels in the warm-temperate zone and subtropics. Structural equation modeling further indicated that temperature had a negative effect on GRSP accumulation, which could be alleviated by organic fertilizer application. This study highlights the importance of organic fertilizer application for soil stability and carbon sequestration in the context of climate change.

Keywords: Long-term fertilization; Climate zone; Spatial scale; Arbuscular mycorrhizal fungi; Cropland

1. Introduction

Glomalin-related soil protein (GRSP) derived from arbuscular mycorrhizal fungi (AMF) plays a significant role in soil quality and productivity, (Driver et al., 2005; Rillig, 2004; Wright and Upadhyaya, 1998), which could bind with soil particles to form stable structures, promoting soil aggregation, increasing soil porosity and water retention capacity (Wright and Upadhyaya, 1996). GRSP, with aromatic structure (Schindler et al., 2007; Yang et al., 2024), can adsorb organic C molecules, preventing their decomposition and oxidation. Therefore, the accumulation of GRSP contributes to the stability of soil aggregates and soil organic carbon (SOC) sequestration. However, some research indicate that agricultural management activities, such as fertilization (Cissé et al., 2021; Guo et al., 2019), leguminous intercropping (Sekaran et al., 2020) and conservation tillage (Thomopoulos et al., 2023) may impact the production and storage of GRSP in soils, and this impact might be significantly different across regions. Therefore, understanding the spatial distribution of GRSP in cropland soils and its response to agricultural practices is essential for optimizing management regimes to enhance soil structure and carbon sequestration, thereby maintaining the ecological functionality of cropland soils.

The accumulation of GRSP may be influenced by various factors, including climate variables, soil nutrients, clay, pH, and crop types (Chen et al., 2023; Commatteo et al., 2023; Rillig, 2004; Treseder and Turner, 2007). Studies have indicated that the application of nitrogen (N), phosphorus (P) and/or manure can affect AMF activity, promoting the synthesis and stability of GRSP in arable soils with low-fertility (Wu et al., 2012; Yang et al., 2024). However, they are also studies suggest that the accumulation of GRSP is unrelated to soil nutrient levels (Alguacil et al., 2014; Wu et al., 2014). These inconsistent results imply that other factors largely regulate the influence of nutrients on soil GRSP levels. Additionally, GRSP increases with increasing clay content due to the strong adsorption of proteins on mineral surfaces, unaffected by chelating ligands (Cheshire et al., 2000; Demyan et al., 2012; Quiquampoix and Burns, 2007). Soil pH is also a significant regulator on GRSP stabilization, with alkaline conditions typically inhibiting its synthesis and stability, while neutral to slightly acidic conditions favor its accumulation and stability (Chen et al., 2023; Xue-li et al., 2008). It is believed that soil pH can change the structure and function of AMF and other microbial communities in soil (Aliasgharzad et al., 2010). While, there is also contradictory result indicates that higher pH value is often associated with higher GRSP content (Peng et al., 2015).

At the spatial scale, GRSP accumulation is often jointly regulated by multiple factors (Li et al., 2020). Studies have indicated that in the warm temperate coastal regions of China, GRSP accumulation is driven by rainfall, while in subtropical regions, GRSP is regulated by pH and clay content. All these reflect the complexity of factors that affect GRSP accumulation (Chen et al., 2023). In particular, climate change such as rising temperature may lead to the decomposition of GRSP (Rillig and Steinberg, 2002), potentially damaging soil health and agricultural sustainability. Different fractions of GRSP may respond differently to climate (Fokom et al., 2012;

Singh et al., 2016), where in mollisols, easily extractable GRSP (EE-GRSP) is more sensitive to climate (temperature and rainfall) changes compared to difficult extractable GRSP (DE-GRSP) (Li et al., 2020). Therefore, the control factors of GRSP are considered comprehensively to gain a further understanding of the interaction mechanism between fertilization management, climate, and GRSP dynamics.

Although there has been research focusing on the distribution and influencing factors of GRSP in ecosystems such as forests and grasslands (Li et al., 2015; Wang et al., 2018; Wu et al., 2012). For example, in forested soils, GRSP content decreases with increasing soil depth, and its spatial distribution is influenced by mycorrhizal colonization, soil carbon compounds, β -glucosidase, and protease (Wu et al., 2012). In grasslands, plant diversity can affect the AMF community and GRSP levels (Li et al., 2015). However, in intensively managed agriculture ecosystem, the accumulation of GRSP may differ from other natural ecosystems because they are regulated by climate conditions, fertilization regimes, and cropping systems and their interaction. To address this knowledge gap, this study systematically analyzed the difference of GRSP response to fertilization based on 14 long-term experimental trials across eastern China with a gradient of temperature from mid-temperate to subtropical zone. We aimed to explore the key driving factors on the accumulation of GRSP in response to various fertilization. We hypothesize that (i) fertilization may be more conducive to increasing GRSP levels in warm temperate zone and subtropics, as higher temperatures promote higher AMF activity, which is beneficial for GRSP production, and (ii) clay content and temperature jointly regulate the patterns of GRSP to fertilization.

2. Materials and methods

2.1. Study sites and experimental design

This study selected 14 long-term (>29 years) field experimental sites spanning across the northern to southern regions of eastern China. These sites cover different temperature zones: middle temperature zone (HH, Heihe; HEB, Harbin; GZL, Gongzhuling; SY, Shenyang), warm temperature zone (HS, Hengshui; LY, Laiyang; ZZ, Zhengzhou; XZ, Xuzhou; MC, Mengcheng), subtropical zone (YJ, Yanjiang; JX, Jinxian; QY, Qiyang). The mean annual temperature (MAT) and the mean annual precipitation (MAP) in these sites ranged from 1.5 °C to 18.1 °C and 510 to 1537 mm, respectively. There are eight different soil orders in these sites, as per the FAO soil taxonomy system. Each site has different fertilization treatments, including CK (no fertilizer), CF (one or more chemical fertilizers of N, P and K), OF (organic fertilizers with/without CF). The chemical fertilizers for N, P, and K were urea ($\text{CO}(\text{NH}_2)_2$), superphosphate ($\text{Ca}(\text{H}_2\text{PO}_4)_2$) and potassium chloride (KCl), respectively. The organic fertilizers were manure and/or straw residue. Three replications per plot were designed to be completely random. These sites have different cropping system, including three systems: continuous cropping (GZL, SY, JX), non-legume rotation (HS, LY, ZZ, XZ, QY) and legume rotation (HH; HEB, MC, YJ). More detailed information is shown in [Table 2-S1](#).

2.2 Soil sampling

Soil samples (0–20 cm) were collected from 14 experimental sites after crop harvest during July to October of 2019. A total of 258 soil samples under different fertilization were sieved through a 2mm mesh and divided into two parts, one part was stored at -80°C refrigerator for microbial parameters determination, and the other part was air-dried to determine soil properties. The soil pH was assessed using a 1:2.5 ratio of soil to water. Specifically, a pre-determined weight of air-dried soil was combined with the corresponding volume of deionized water and mixed thoroughly. The mixture was allowed to stand for 30 minutes to stabilize the solution. A calibrated pH meter was then used to measure the pH, ensuring accurate readings. The elemental content of soil SOC and TN was determined using elemental analyzer (EA3000, Milan, Italy). Before analysis, soil samples were air-dried, ground, and sieved through a 2 mm mesh to ensure uniformity. The samples were combusted in the elemental analyzer at 950°C to determine SOC and TN concentrations. Calibration was performed using known standard samples (e.g., alanine) to ensure precision and accuracy in the measurements. Soil available phosphorus (AP) was analyzed via the molybdenum-antimony resistance colorimetric method. The procedure involved weighing air-dried soil samples and adding 0.5 mol/L NaHCO₃ solution as the extractant at a 1:10 soil-to-extractant ratio, with the solution pH adjusted to 8.5. Extraction was conducted at 25°C for 30 minutes, followed by filtration with Whatman filter paper to obtain a clear filtrate. The filtrate was then reacted with ammonium molybdate and ascorbic acid to form a blue phosphomolybdate complex, and absorbance was measured at 885 nm to calculate available phosphorus concentration. Clay was analysed by the pipette method. First, the soil samples were pre-treated with an oxidizing agent, such as hydrogen peroxide, to remove organic matter. A dispersing agent, such as sodium hexametaphosphate, was then added to disperse soil particles. The samples were allowed to settle for a specified time under controlled temperature conditions, and a pipette was used to extract a specific depth of suspension. The suspension was then dried and weighed, and the percentage of clay was calculated using sedimentation time equations.

2.3 Analysis of glomalin-related soil proteins (GRSP)

The determination of GRSP followed the method described by Wright et al. (1998). In brief, easily extractable GRSP (EE-GRSP) was extracted from 0.25 g of air-dried soil using a solution composed of 2 mL of 20 mmol L⁻¹ sodium citrate (pH=8.0). The samples were subjected to autoclaving at 121 °C for 30 min and then centrifuged at 10,000 ×g for 6 minutes. Difficult extractable GRSP (DE-GRSP) was extracted from the residues in tube using 2 mL of 50 mmol L⁻¹ sodium citrate (pH=8.0) at 121°C for 60 min. This process was repeated four times for each sample. Subsequently, the supernatants were combined and subjected to an additional centrifugation step before quantification. For quantification, 300 µL of the clear supernatant and 700 µL of dH₂O were transferred into the sampling tank, followed by the addition of 5 mL Coomassie bright blue (CBB) solution. Following a 2–3 min color reaction period (Liu et al., 2021), the optical density (OD) value of GRSP was measured at 595 nm

using a microplate reader (Biotek Synergy H1, USA), with bovine serum albumin (BSA) serving as the standard. The total GRSP content is the sum of EE-GRSP and DE-GRSP.

2.4. Determination of microbial biomass

AMF biomass was characterized using C16:1 ω 5 phospholipid fatty acids (PLFAs) as described by Olsson et al. (1999). PLFAs were extracted with a slightly adapted Bligh-Dyer method, as detailed in Tian et al. (2022). Briefly, 2 grams of freeze-dried fresh soil samples were placed in a Teflon centrifuge tube. The extracting solution, composed of citric acid buffer solution (with a ratio of chloroform to methanol to citric acid of 1:2:0.8), along with the unesterified internal standard (C19: 0.1 ng μL^{-1}), was added.

2.5. Statistical analysis

The data were examined for homogeneity and normal distribution of variance. Differences (Duncan's test) in GRSP content, edaphic, and microbial factors among fertilization treatments were analyzed using one-way ANOVA with IBM SPSS 19.0 software. Multi-ways ANOVA was used to analyze the effects of climate, fertilization, and planting on GRSP. The relationship between GRSP and climate, nutrients, microbial factors were assessed using the Pearson's correlation coefficient test. The redundancy analysis (RDA) was performed to explore the relationship between GRSP and factors in this model. The variance partitioning analysis (VPA) and random forest (RF) were used to quantify the relative importance of clay, edaphic and microbial factors on GRSP. The partial least squares path modelling (PLSPM) was constructed to clarify the pathways regulating GRSP properties. All statistical analyses were conducted using R 4.3.0.

3. Results

3.1. Spatial distribution patterns and differences of GRSP under fertilization regimes across eastern China

Different ecosystem types significantly influence GRSP content, with levels typically observed as forest land > grassland > cropland (Table 2-S2). Thus, enhancing GRSP content in cropland is particularly crucial for agricultural production. However, GRSP accumulation in agricultural fields is further shaped by factors such as climate zones, fertilization regimes, and cropping systems. A multi-way analysis of variance revealed that among these factors, climate exerts the most pronounced effect on GRSP levels (Table 2-S3). Notably, the distribution patterns of GRSP displayed spatial variability (Figure 2-1a). Specifically, GRSP contents ranged from 1.0 to 5.1 mg g^{-1} (Figure 2-1c). EE-GRSP ranged from 0.2-1.7 mg g^{-1} (Figure 2-1b). Moreover, GRSP contents decreased first and then increased with the increase of latitude (Figure 2-1d), suggesting a significant pattern of mid-temperate zone > subtropics > warm temperate zone (Figure 2-1f). In the warm temperate zone and subtropics, EE-GRSP and T-GRSP levels were higher under organic fertilization (OF) treatment compared to the no fertilization (CK) treatment (Figure 2-1e, f). Conversely,

in the mid-temperate zone, organic fertilization (OF) significantly increased EE-GRSP levels, while showing no significant effect on T-GRSP (Figure 2-1e, f). Chemical fertilizer (CF) application significantly enhanced EE-GRSP and T-GRSP content in the subtropics (Figure 2-1e, f). Moreover, the rate of GRSP increase under organic fertilization (OF) treatment was higher than that under chemical fertilization (CF) treatment. Additionally, the response of GRSP to climate zones and cropping systems demonstrated a trend of mid-temperate zone < warm temperate zone < subtropics (Figure 2-6a), with continuous cropping \geq non-leguminous rotation > leguminous rotation (Figure 2-6b).

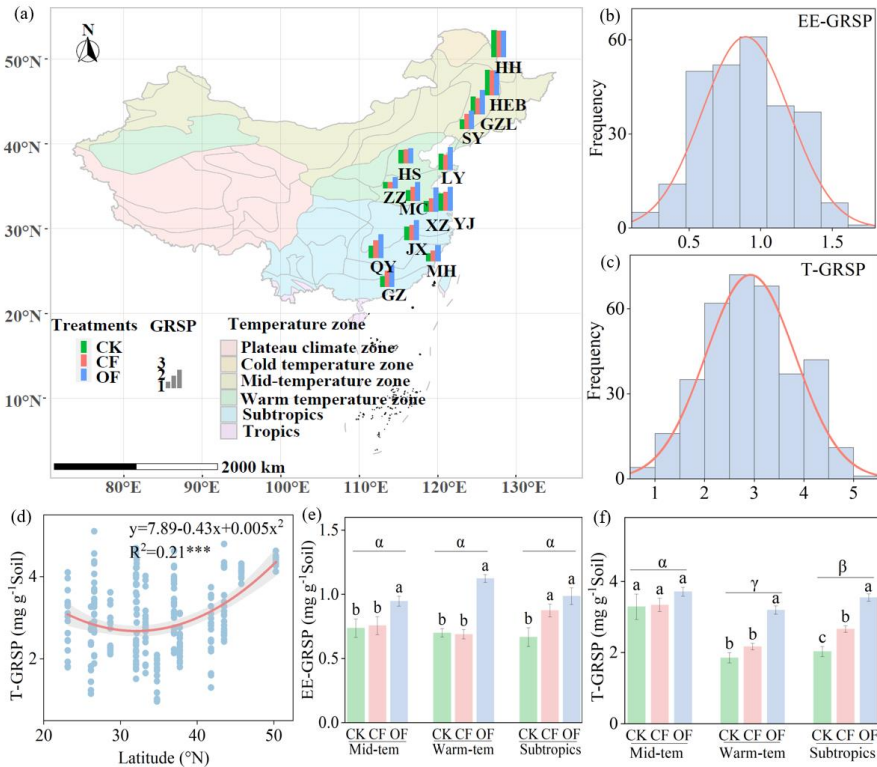


Figure 2-1 Total glomalin-related soil proteins (T-GRSP) contents in 14 long-term field experiment sites across eastern China (a). Frequency distribution of EE-GRSP (b) and T-GRSP (c). Relationship between GRSP and latitude under long-term fertilization regimes (d), and differences of EE-GRSP (e) and T-GRSP (f) contents under temperature zones and fertilization regimes. Different English letters and Greek letters represent significant differences under different fertilization regimes and climate zones (Duncan's test, $p < 0.05$). HH, Heihe. HEB, Harbin. SY, Shenyang. GZL, Gongzhuling. SY, Shenyang. HS, Hengshui. LY, Laiyang. ZZ, Zhengzhou. XZ, Xuzhou. MC, Mengcheng. YJ, Yanjiang. JX, Jinxian. QY, Qiyang. MH, Minhou. GZ, Guangzhou. Mid-tem: middle temperate zone; Warm-tem: warm temperate zone. CK: no fertilizer; CF: chemical fertilizer; OF: organic fertilizer. T-GRSP: Total glomalin-related soil proteins; EE-GRSP: easily extractable glomalin-related soil proteins.

3.2. Associations of GRSP properties with climatic, edaphic and microbial factors

The GRSP properties exhibited close relationships with climatic, edaphic, and microbial factors (Figure 2-2, 8, 9), and demonstrated variations across different climatic zones. In the mid-temperature zone and subtropics, GRSP showed negative correlations with MAT and MAP, decreasing with rising temperature and increasing precipitation (Figure 2-2a, b). Similarly, GRSP significantly increased with microbial factors (AMF and total PLFA) and nutrients (SOC, TN, AP) across three climatic zones (Figure 2-2c-g). In addition, clay content showed a significant positive correlation with GRSP in the mid-temperature zone (Figure 2-2h). pH exhibited a positive/negative correlation with GRSP in the subtropics/warm temperate zone, indicating an initial increase followed by a decrease in GRSP with increasing pH (Figure 2-2i).

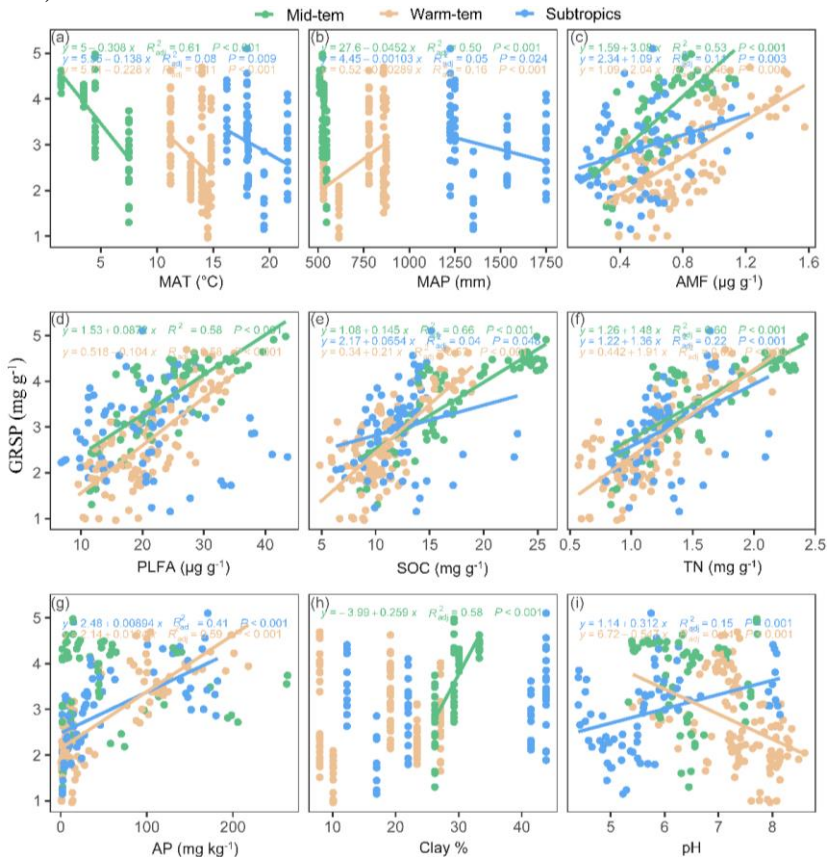


Figure 2-2 Pearson correlations between GRSP and factors, including MAT (a), MAP (b), AMF (c), PLFA (d), SOC (e), TN (f), AP (g), clay (H) and pH value (i). MAT: mean annual temperature; MAP: mean annual precipitation; TN: total nitrogen; SOC: soil organic carbon; AP: available phosphorus; AMF: arbuscular mycorrhizal fungi; PLFA: phosphorus lipid fatty acid.

3.3. Main controlling factors driving GRSP accumulation in different climatic zones

Redundancy analysis (RDA) indicated that the differences and distribution patterns of GRSP are primarily affected by clay content, MAT, nutrients (SOC, TN, AP), and pH. Among these factors, clay content, pH and nutrients emerged as the most crucial controlling factors in the soil, while MAT was a significant climatic factor. The first axis and the second axis accounted for 95.48% of the total variation (Figure 2-3a, b). Variance partitioning analysis (VPA), together with random forest analysis, demonstrated that the primary driving factors of GRSP were different across climatic zones (Figure 2-3c, d, e). In the mid-temperature zone, clay content exhibited the most substantial impact on GRSP components, explaining 12% of the variation. However, in the warm temperate and subtropical zones, nutrients (SOC, TN, AP) were most important for GRSP, explaining 20% and 27% of the variation, respectively. Among these nutrients, Available P was the most influential factor regulating GRSP variation (Figure 2-10).

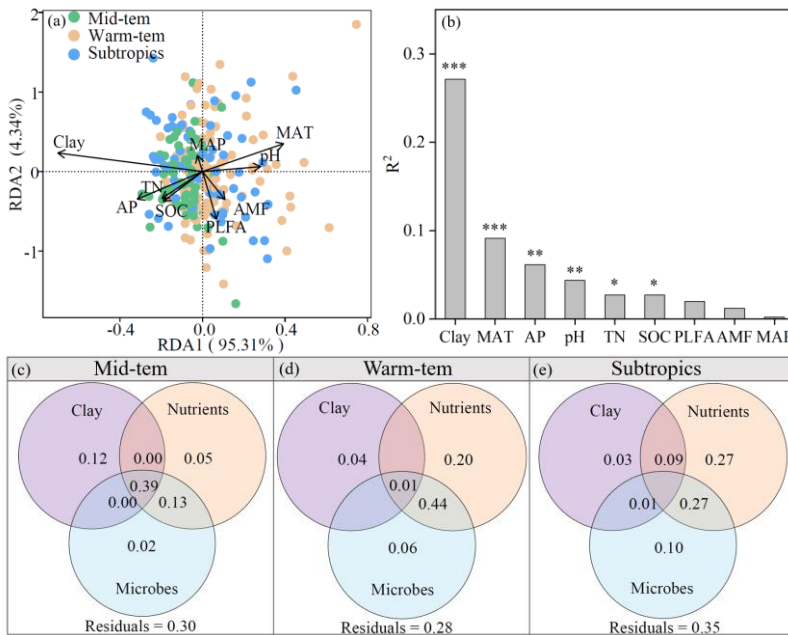


Figure 2-3 Redundancy analysis (RDA) illustrating environmental factors impact on GRSP properties under climate zones (a). The bar chart showing the degree of significance of each environmental factor (b). Variance partitioning analysis (VPA) to distinguish the relative importance each environmental factor under middle temperature zone (c), warm temperature zone (d), subtropics (e). MAT: mean annual temperature; MAP: mean annual precipitation; SOC: soil organic carbon; TN: total nitrogen; AP: available phosphorus; AMF: arbuscular mycorrhizal fungi; PLFA: total phospholipid fatty acid; Mid-tem: middle temperate zone; Warm-tem: warm temperate zone.

The partial least squares path modelling (PLSPM) further elucidated the pathways of climate (MAT) and fertilization on GRSP accumulation combined with edaphic and microbial factors (pH, nutrients, clay, microbes) (Figure 2-4). Climate and agricultural management practices had both direct and indirect effects on GRSP (Figure 2-4b). Specifically, climate predominantly negatively influenced GRSP through its impact on clay content and nutrients, agricultural management practices positively contributed to GRSP accumulation via their effects on nutrients and clay (Figure 2-4a). Overall, agricultural management practices countered the negative effects of climate on nutrients, clay and pH, subsequently exerting a positive influence on AMF and other microbes, thereby favoring GRSP accumulation.

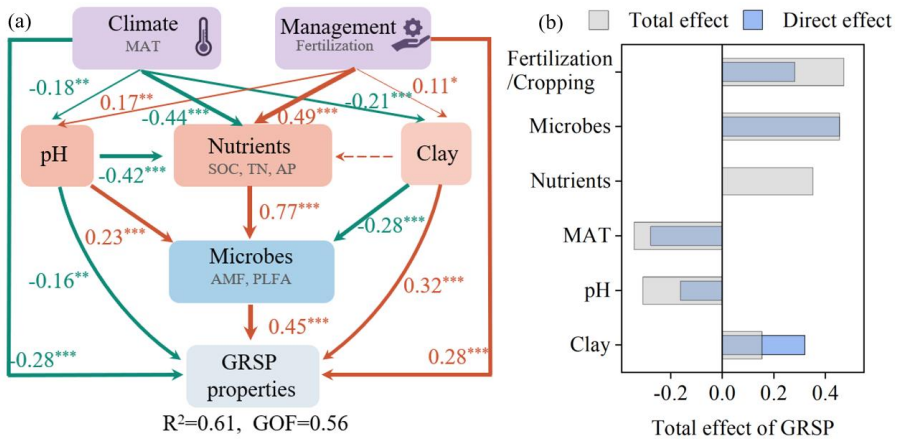


Figure 2-4 Partial least squares path modelling (PLS-PM) displaying the impacts of climate and agricultural management practices on GRSP properties (a), and the total and direct standardized effects on GRSP properties (b). The boxes denote the first component from the PCA performed for Nutrients, microbes and GRSP properties. Red arrows represent positive effects, while green arrows represent negative effects. These effects are standardized and quantified.

4. Discussion

Based on soil samples under different fertilization at long-term experiment sites, we evaluated the levels and main drivers of GRSP in cropland across eastern China with multiple statistical methods. First, we found a lower levels of GRSP in cropland relative to forests and grassland ecosystems (Table 2-S3). This phenomenon might be that agricultural management practices (fertilization, tillage, cropping) disturb on GRSP production (Fokom et al., 2012). Although we found that fertilization increased the content of GRSP (Figure 2-1), these practices are not sufficient to compensate for the decomposition of GRSP. Furthermore, climate also had a significant impact on the GRSP content (Figure 2-1), and the differences among temperate zones may be attributed to specific factors (Figure 2-7). Therefore, this study elucidated the spatial distribution characteristics of GRSP in cropland soils and the primary driving factors.

4.1. Distribution characteristics of GRSP and its response to fertilization

GRSP is believed to be produced in the form of EE-GRSP by AMF and accumulated in the soil (Rosier et al., 2006; Wu et al., 2014), hence the level of EE-GRSP can reflect the production potential of GRSP. In this study, the level of EE-GRSP ranged from 0.2 to 1.7 mg g⁻¹ soil (Figure 2-1b). The application of organic fertilizer significantly increased the content of EE-GRSP in mid-temperate, warm-temperate, and subtropical zones, while the effect of chemical fertilizer was only evident in the subtropics (Figure 2-1e). The reason may be that soil environmental disturbances created new habitats for AMF dispersal and growth (Camargo-Ricalde, 2002; Qin et al., 2015). In agricultural fields, nutrients are often in a state of depletion or imbalance due to production activities. Balancing fertilization (NPK) and applying organic fertilizers can increase AMF colonization (Luo et al., 2021), which is beneficial for the production of EE-GRSP. Nitrogen fertilization also actively promoted the accumulation of GRSP (Guo et al., 2019; Zhang et al., 2023), while the cultivation of leguminous crops enhanced the accumulation of GRSP even more (Figure 2-11), possibly as a result of the long-term nitrogen fixation by legumes (Sekaran et al., 2020). Singh et al. (2018) also found that incorporating legumes such as chickpeas in crop rotation increased root deposition, thereby stimulating the growth of AMF and GRSP production.

In this study, the level of GRSP ranged from 1.0 to 5.1 mg g⁻¹ soil (Figure 2-1d). The application of organic fertilizer increased the GRSP content in different degrees, with great differences observed among sampling sites (Figure 2-1a). From the perspective of GRSP content alone, there was a trend of first decreasing and then increasing with increasing latitude (Figure 2-1b), showing: temperate zone > subtropical zone > warm-temperate zone (Figure 2-1c). The reasons for this trend may be as follows: (i) Increasing temperature can accelerate the decomposition rate of GRSP, but in subtropics, acidic soils and Fe-Al oxides reduce their degradation capacity (Wang et al., 2014). (ii) In warm-temperate zones, the low clay content in the soil weakened the mineral protection compared to other regions (Helassa et al., 2011); (iii) Although AMF biomass was higher in this zone than in other zones, EE-GRSP did not increase significantly (Figure 2-1e); (iv) GRSP was an alkaline-soluble protein material (Vodnik et al., 2008), and the soils in warm-temperate zones were mainly alkaline (Figure 2-2i). From the point of view of the improvement of GRSP by fertilization, organic fertilizer significantly increased the magnitude of GRSP enhancement in cropping systems and climatic zones (Figure 2-5). Fertilization had a more pronounced effect on GRSP enhancement in warm-temperate and subtropical zones (Figure 2-1f), confirming the first hypothesis. This phenomenon may be due to the fact that under low-temperature conditions, microbial decomposition of GRSP was limited, resulting in slower turnover rates and higher accumulation levels (Woignier et al., 2014). Additionally, the higher organic matter content in this region leded microbes (r-strategy) to preferentially utilize readily decomposable organic matter (Blagodatskaya et al., 2004; Salome et al., 2010). However, the metabolic activity of

AMF is higher in regions with higher temperature (Xiao et al., 2021), and nutrients addition may be more conducive to the growth of AMF, thereby promoting GRSP accumulation. The magnitude of GRSP increase through organic fertilization under leguminous crop rotation is lower than that under continuous cropping and non-leguminous crop rotation (Figure 2-6b), possibly because non-legumes and legumes contain distinct AMF communities (Scheublin et al., 2004), and these respond differently to fertilization. Therefore, planting legumes or applying organic fertilizer in warmer regions may promote GRSP accumulation, counteracting the negative effects of temperature and contributing to maintaining soil health.

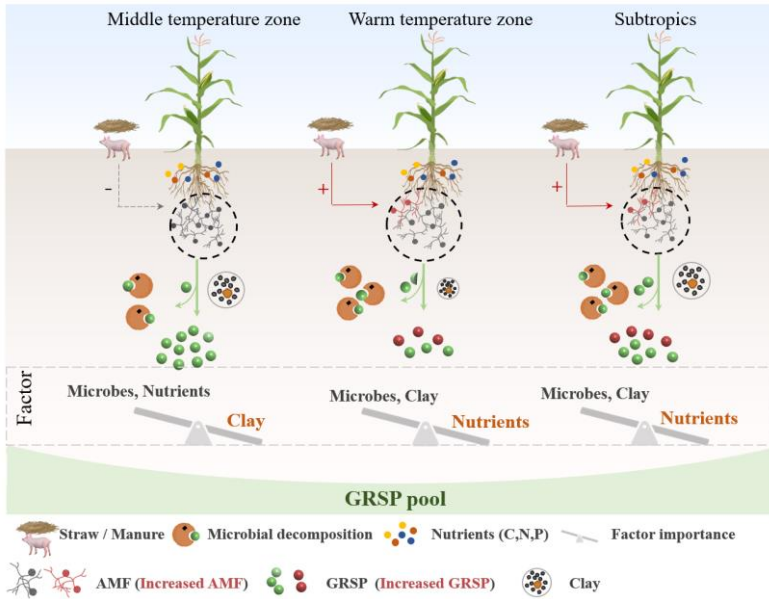


Figure 2-5 Schematic illustration of GRSP accumulation and driving factors in different climatic zones.

4.2. Factors influencing the accumulation of GRSP in cropland

Since the GRSP content in agricultural ecosystems is lower than that in forest or grassland ecosystems (Agnihotri et al., 2022), the factors influencing GRSP accumulation between agricultural ecosystems and other ecosystems may differ. Some studies have indicated that factors such as AMF species and abundance, vegetation types, climate, and soil physicochemical properties may influence GRSP content to varying degrees (Rillig, 2004; Wang et al., 2018). In this study, through the analysis of climate, soil, and microbial factors, we found differences in the relationships between GRSP and various factors across different climatic zones (Figure 2-2,8,9). It is noteworthy that MAT and GRSP content exhibited a negative correlation under three climatic zones (Figure 2-2a), indicating that increasing temperatures induce GRSP decomposition. Mean annual precipitation (MAP) showed positive and negative relationships with GRSP in warm-temperate and subtropical regions, respectively (Figure 2-2b), suggesting that moderate rainfall can alleviate soil

water-air contradictions and provide a favorable habitat for AMF. Our results also show that AMF (PLFA16:1 ω 5) exhibits a positive correlation with GRSP in all regions, consistent with previous studies (Agnihotri et al., 2022; Li et al., 2020; Wang et al., 2022). Total GRSP and EE-GRSP are significantly positively correlated with total PLFA, indicating that the microbial community also plays an important role in GRSP. As GRSP serves as an energy source for microbial growth and dispersal, the changes in GRSP content in soil can be reasonably explained by the contemporaneous AMF and total PLFA. This also explains why indicators such as SOC, TN, and AP were significantly positively correlated with GRSP (Figure 2-2e,f,g), as nutrient addition in low-nutrient environments such as agricultural fields promoted AMF growth and dispersal, thereby facilitating GRSP accumulation (Bonser et al., 1996; Liu et al., 2008). Additionally, the relationship between pH and GRSP varies in different temperature zones. In warm-temperate zones, pH was negatively correlated with GRSP, while in subtropical regions, it was positively correlated (Figure 2-2i). The reason may be that pH regulates AMF growth. AMF generally grow and disperse best under slightly acidic to neutral conditions. In warm-temperate regions of China, soil pH is >7 , while in subtropical regions, it is <7 . Therefore, higher pH levels are unfavorable for AMF growth, thereby reducing GRSP production (Chen et al., 2023; Peng et al., 2015; Zhang et al., 2017). Another significant factor is soil clay content. Our results indicated a close relationship between clay and GRSP in mid-temperate zones. This positive correlation had also been found in other ecosystems (Nichols and Wright, 2005; Rillig and Steinberg, 2002; Treseder and Turner, 2007). Similar to general SOM, GRSP can be considered protected by clay mineral association, thereby accumulating in soil (Woignier et al., 2014). However, no significant relationship was found between clay and GRSP in temperate forest soils in France, possibly because the effect of tree types may overshadow the protective effect of clay (Cissé et al., 2023). Therefore, the accumulation of GRSP in agricultural ecosystems is the result of the combined effects of multiple factors.

The redundancy analysis (RDA) revealed that MAT, clay, nutrients, and pH significantly influence GRSP properties (Figure 2-3a,b). It is noteworthy that variance partitioning analysis (VPA) and random forest analysis (RF) indicated different dominant factors affecting GRSP accumulation in distinct climatic zones (Figure 2-3c, d, e). In mid-temperate zones, clay served as the primary determinant regulating GRSP levels (Figure 2-3c, 10a), while in warm-temperate and subtropical zones, nutrients played dominant roles in regulating GRSP levels (Figure 2-3d,e, 10b,c). The reasons may be that higher temperature exacerbated the risk of GRSP loss in warm-temperate and subtropical zones (Rillig and Steinberg, 2002). In addition, AMF may shift life-history traits (Horsch et al., 2023), which could affect GRSP production as AMF were highly sensitive to soil nutrients. Generally, soil P is a major limiting nutrient for microbes and plants (Hou et al., 2020). Studies have shown that P fertilization significantly increases GRSP content. However, in P-rich soils, P application may not have a significant effect on GRSP content or even have a negative impact (Cissé et al., 2021; Yang et al., 2023). Therefore, nutrients to some extent drive GRSP accumulation by AMF regulation. Structural equation modelling also

confirmed this process. However, in mid-temperate zones, there were higher clay content, with predominance of 2:1 clay minerals, mainly montmorillonite (Abrar et al., 2020), providing more binding sites due to the small size and surface properties of clay particles, thus having greater chelating capacity for soil organic matter (e.g., GRSP) (Balabane and Plante, 2004; Wagai and Mayer, 2007). The adsorption capacity of clay minerals for GRSP varies, which in turn affects GRSP accumulation; for example, montmorillonite may preferentially adsorb GRSP and have a higher adsorption capacity than kaolinite (Chen et al., 2019; Quiquampoix and Burns, 2007). Therefore, soil nutrition is not the primary factor limiting GRSP levels in mid-temperate zones.

The partial least squares path modelling (PLSPM) can clearly illustrate the role of fertilization in mediating the impact of climate on the dynamic processes of GRSP in cropland. Fertilization primarily influenced the level of GRSP by indirectly regulating soil nutrients to promote the increase of AMF biomass, thereby facilitating GRSP production. However, the effect of climate (MAT) on GRSP was directly negative, while climate also indirectly affected pH, nutrient and clay, thus influencing on AMF and GRSP. In summary, fertilization regimes play positive roles in the accumulation of GRSP, mitigating the losses caused by temperature. These findings can guide the rational application of organic fertilizers in regions with higher temperature to alleviate nutrient limitations, while also counteracting the negative effects of warming in the context of global climate change.

4.3. Implications and perspective

GRSP is a crucial “bridge” between soil microorganisms and organic matter. In-depth study of GRSP synthesis and accumulation mechanism can better understand the function and stability of soil ecosystems, and promote the maintenance and improvement of soil health. In the future, we can further research in the following aspects: (i) quantitative assessment of GRSP influencing factors in a large scale. Statistical analysis methods such as regression analysis or principal component analysis (PCA) will be used to quantify the influence extent of factors on GRSP spatial variations. The dominant factors and their mechanisms will be further explained by constructing models to predict the relationship between GRSP content and factors like climatic conditions (e.g., temperature, precipitation), soil properties (e.g., texture, organic matter content), and land use practices (e.g., crop types, fertilization). (ii) Long-term monitoring and dynamic changes. Integrating long-term experimental platforms to collect and analyze soil samples from different locations annually, and documenting GRSP content changes over time. This approach can help reveal the seasonal, interannual, and long-term trends of GRSP, thus better understanding its dynamic characteristics. (iii) Evaluation of ecosystems functionality. To assess the impact of GRSP on soil moisture retention, nutrient cycling, crop growth and yield, as well as regulation of SOC storage and greenhouse gas emissions. (iv) The effects of type and quantity of manure on GRSP synthesis and accumulation. Manure application rates should be normalized (e.g., based on equal nitrogen input) to ensure comparability across treatments. However, this was not feasible in our study due to

the constraints of using data from a pre-existing long-term experiment established by others. While this limitation does not undermine the validity of our findings, it should be acknowledged in discussions to contextualize the results. Future research could address this gap by designing new experiments with standardized manure application rates to isolate and evaluate the specific effects of manure type and dosage on GRSP dynamics. This evaluation can provide scientific basis for ecosystem management and policy formulation.

5. Conclusion

This study revealed the spatial variation of GRSP in cropland soils across eastern China and its response to fertilization regimes. The content of GRSP exhibited a quadratic trend with increasing latitude, that is, mid-temperate zone > subtropical zone > warm temperate zone. In the warm-temperate and subtropical regions, organic fertilizer application significantly increased GRSP content, whereas there was no significant difference among fertilization regimes in the mid-temperate zone. Additionally, clay content, MAT, pH, and soil nutrients were identified as the main factors influencing GRSP accumulation. Specifically, clay content predominantly determined GRSP levels in the mid-temperate zones, while soil nutrients primarily drove GRSP in the subtropical and warm-temperate zones. Structural equation modeling further indicated a negative effect of temperature on GRSP accumulation, which could be mitigated by organic fertilizer application through indirect improving nutrient availability AMF biomass, thereby promoting GRSP accumulation. In summary, this study emphasized the importance of organic fertilizer application for maintaining soil stability and carbon sequestration in the context of climate change. Future research could explore the effects of different soil types and crop species on GRSP.

6. Acknowledgements

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7. Supplementary figures and tables

Table 2-S1 Geographical information, climate, soil order and temperature zones of the sampling sites.

Site name	Latitude (N°)	Longitude (E°)	MAP (mm)	MAT (°C)	Crop system	Soil order(USDA)	Ecosystem types	Clay %	Fertilization application (C, N, P ₂ O ₅ kg ha ⁻¹)
HH	50.2	127.5	510	1.5	Wheat/Soybean	Alfisols		33.26	CF: 0, 75-150, 75-150 OF:1350, 95-170, 83-158
HEB	45.8	126.6	533	3.5	Wheat/Soybean/Maize	Mollisols	Middle temperate	30.09	CF: 0, 150, 75 OF: 558, 28, 11
GZL	43.5	124.8	525	4.5	Maize	Mollisols		29.26	CF: 0, 0-165, 0-82 OF: 2300-3375,165, 175
SY	41.8	123.4	547	7.5	Maize	Alfisols		26.21	CF: 0, 120, 60 OF:858-1717,75-271,112-284
HS	37.7	115.7	530	13.0	Wheat-Maize	Alfisols		23.41	CF: 0, 360, 240 OF:2025, 0-392, 0-252
LY	37.0	120.7	779	11.2	Wheat-Maize	Inceptisols	Warm temperate	19.20	CF: 0, 138-276, 0-90 OF:600-1200,150-426, 60-90
ZZ	34.8	113.7	615	14.5	Wheat-Maize	Inceptisols		10.10	CF: 0, 353, 176 OF:1485-3300, 165-447, 93-242
XZ	34.3	117.2	860	14.0	Wheat-sweet potato	Inceptisols		8.00	CF: 0, 300, 0-150 OF:2362, 118-418, 96-245
MC	33.3	116.6	872	14.8	Wheat-Soybean/Maize	Vertisols		27.14	CF: 0, 180, 90 OF:1350-3375,206-315, 100-240
YJ	32.1	120.6	1250	16.2	Barley/cotton-Wheat/Rice-Broad beans/Maize	Inceptisols		35.20	CF: 0, 225, 112 OF:5400, 270-495, 153-266
JX	28.4	116.2	1537	18.1	Maize	Inceptisols	Subtropics	41.45	CF: 0, 120-240, 60-120 OF:1080, 53-174, 45-105
QY	26.6	111.8	1225	18.0	Wheat-Maize	Inceptisols		43.86	CF: 0, 0-300, 0-120 OF:1485-4208,210-330,84-204
MH	26.2	119.1	1351	19.5	Rice	Entisols		17.00	CF: 0, 103, 27 OF:855-1690, 153-163, 60-120
GZ	23.9	112.0	1750	21.6	Rice	Ultisols		21.99	CF: 0, 550, 60 OF:855, 550, 60

Abbreviations: HH, Heihe. HEB, Harbin. SY, Shenyang. GZL, Gongzhuling. SY, Shenyang. HS, Hengshui. LY, Laiyang. ZZ, Zhengzhou. XZ, Xuzhou. MC, Mengcheng. YJ, Yanjiang. JX, Jinxian. QY, Qiyang. MH, Minhou. GZ, Guangzhou. MAT, mean annual temperature. MAP, mean annual precipitation. “-” indicates crops grown in same year. “/” means crops grown in different years.

Table 2-S2 Investigation of GRSP content in different ecosystems across China.

Ecosystems	GRSP (mg g ⁻¹)	Reference
Forest	4.5-7.0	Sun et al., 2024
Forest	6.7	Gu et al., 2024
Forest	3.6-6.9	Zhang et al., 2022
Forest	4.4 - 7.3	Qin et al., 2017
Forest	5.5-6.7	Zhang et al., 2023
Grassland	4.1-6.5	Li et al., 2015
Grassland	10.1	Bedini et al., 2007
Grassland	4.5	Xu et al., 2017
Grassland	4.5-6.2	Hou et al., 2022
Grassland	5.5	Liu et al., 2020
Grassland	0.6-1.6	Yang et al., 2023
Cropland	2.5-5.1	Liu et al., 2020
Cropland	1.9-4.2	Yang et al., 2024
Cropland	5.2	Gu et al., 2024
Cropland	0.3-3.4	Guo et al., 2019
Cropland	1.0-5.1	Our study

Table 2-S3 Multi-way analysis of variance (MANOVA) of GRSP properties under different climate zones, fertilization and cropping system (type III tests).

	T-GRSP		EE-GRSP		DE-GRSP	
	F value	<i>p</i>	F value	<i>p</i>	F value	<i>p</i>
Temperature zone	15.53	< 0.001	1.39	0.25	12.78	< 0.001
Fertilization	0.06	0.94	0.14	0.87	0.15	0.86
Cropping system	12.27	< 0.001	0.42	0.52	10.49	< 0.01

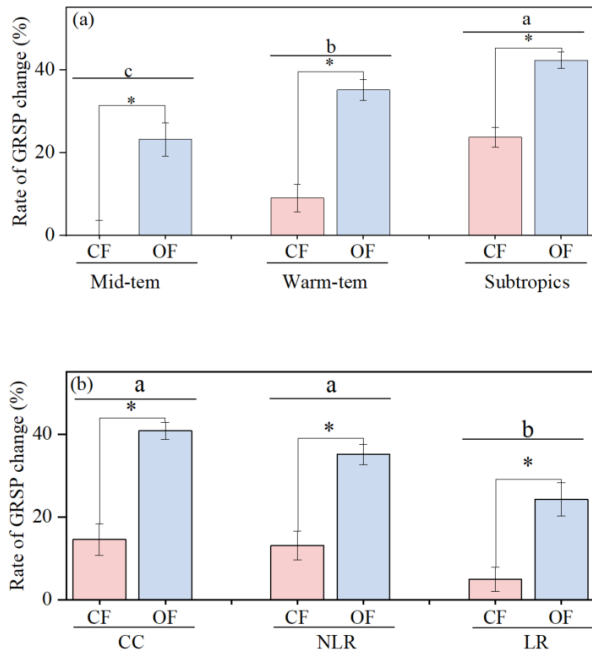


Figure 2-6 Differences in GRSP change rates under climate zones (a) and cropping systems (b). Mid-tem: middle temperature zone; Warm-tem: warm temperature zone. CC: continuous cropping; NLR: non-legume rotation; LR: legume rotation; CF: chemical fertilizer; OF: organic fertilizer. The total GRSP content is the sum of EE-GRSP and DE-GRSP. The response ratio of GRSP was calculated as follows: Response ratio of GRSP = GRSP_{fertilizer} / (GRSP_{no fertilizer}), where GRSP_{fertilizer} is the content of GRSP under fertilization treatments. GRSP_{no fertilizer} is the content of GRSP under no fertilization treatment.

Characteristics of glomalin-related soil proteins accumulation and its response to fertilization and tillage practices in typical cropland soil

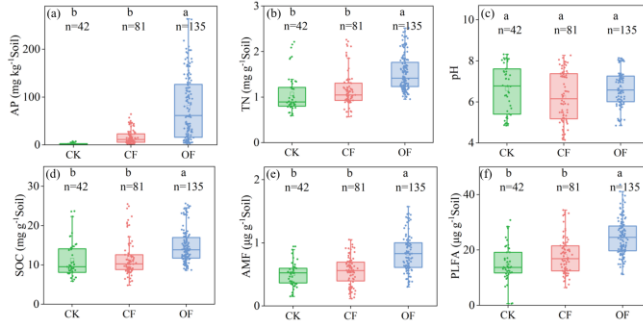


Figure 2-7 The difference comparison of soil properties (AP, TN, SOC, pH) and microbial biomass (AMF, PLFA) under fertilization regimes. CK: no fertilizer; CF: chemical fertilizer; OF: organic fertilizer; SOC: soil organic carbon; TN: total nitrogen; AP: available phosphorus; AMF: arbuscular mycorrhizal fungi; PLFA: total phospholipid fatty acid.

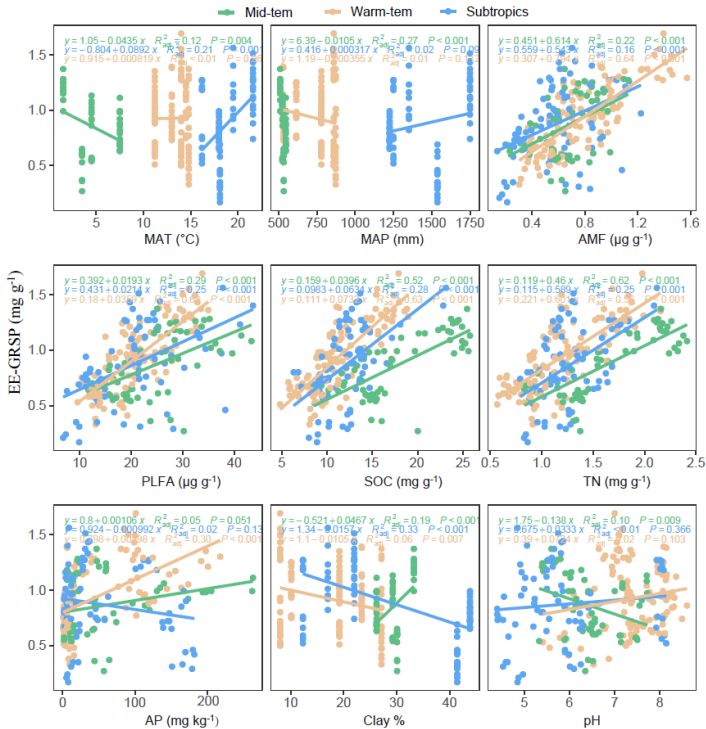


Figure 2-8 Relationships between EE-GRSP content and factors of climate (MAT, MAP), soil properties (pH, SOC, TN, AP, clay) and microbial biomass (AMF, PLFA). EE-GRSP: easily extractable glomalin-related soil proteins; Mid-tem: middle temperature zone; Warm-tem: warm temperature zone; SOC: soil organic carbon; TN: total nitrogen; AP: available phosphorus; AMF: arbuscular mycorrhizal fungi; PLFA: total phospholipid fatty acid; MAT: mean annual temperature; MAP: mean annual precipitation.

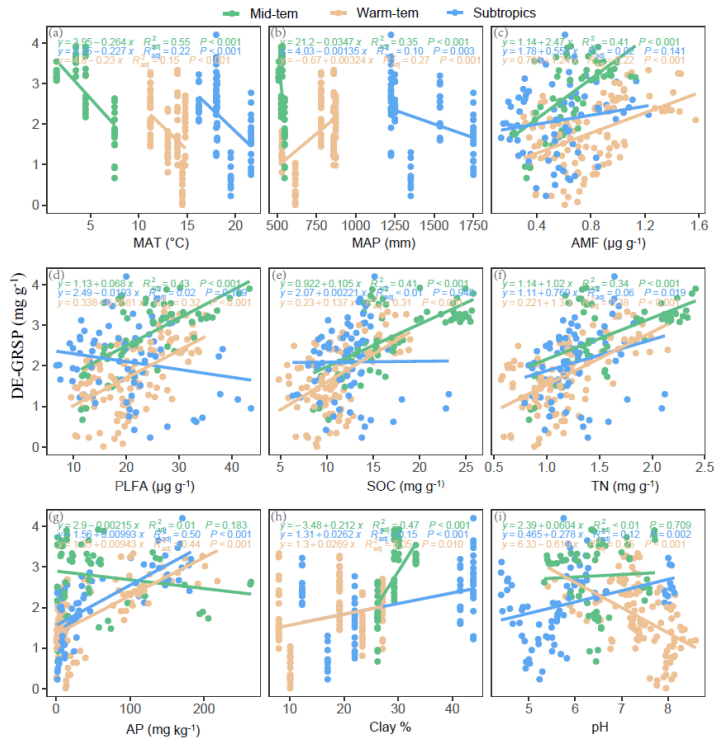


Figure 2-9 Relationships between DE-GRSP content and factors of climate (MAT, MAP), soil properties (pH, SOC, TN, AP, clay) and microbial biomass (AMF, PLFA). DE-GRSP: difficult extractable glomalin-related soil proteins; Mid-tem: middle temperature zone; Warm-tem: warm temperature zone; SOC: soil organic carbon; TN: total nitrogen; AP: available phosphorus; AMF: arbuscular mycorrhizal fungi; PLFA: total phospholipid fatty acid.

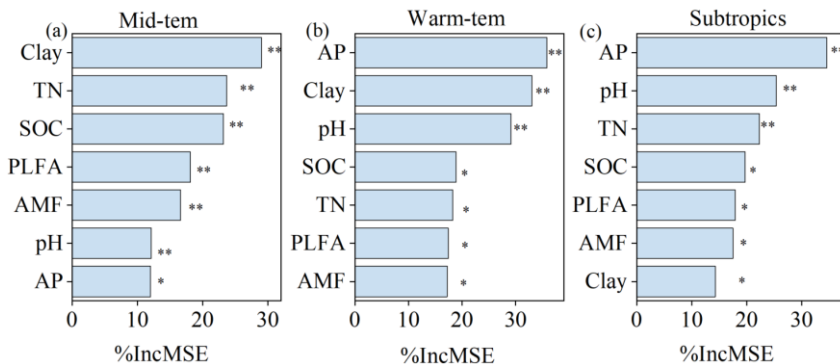


Figure 2-10 Analysis of the relative importance of each factor to GRSP in different temperature zones.

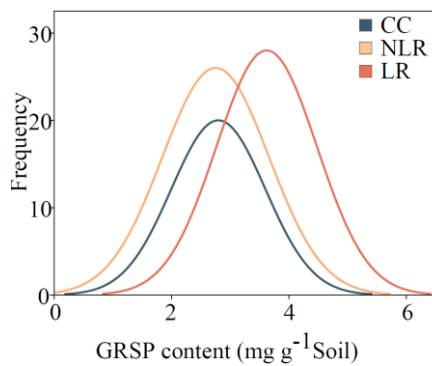


Figure 2-11 Frequency distributions of GRSP under different cropping systems. CC: continuous cropping; NLR: non-legume rotation; LR: legume rotation

Chapter 3

Long-term manuring facilitates glomalin-related soil proteins accumulation by chemical composition shifts and macro-aggregation formation

From: Yang, H., Xiao, Q., Huang, Y., Cai, Z., Li, D., Wu, L., Meersmans J., Colinet, G, Zhang, W. (2024). Long-term manuring facilitates glomalin-related soil proteins accumulation by chemical composition shifts and macro-aggregation formation. *Soil and Tillage Research*, 235, 105904.

“Hold on to belief, face challenges, always move forward without fatigue;
Firmly grasp faith, pierce through darkness, sincerity will shine even brighter”

— Beyond band, 'Hold on to Belief'

坚持信念，迎接挑战，只向前永不倦
紧握信念，划破黑暗，真挚诚会更光

—黄家驹“坚持信念”



Abstract

Glomalin-related soil proteins (GRSP), derived from arbuscular mycorrhizal fungi (AMF), contributes significantly to soil stability and carbon sequestration. However, the responses of GRSP accumulation and associated AMF community and diversity to long-term fertilization regimes remain unclear. Here, we investigated the dynamics of GRSP contents, AMF biomass and diversity based on a 29-year fertilization experiment (including control, mineral fertilization, manuring and straw returning treatments). Results showed that GRSP contents increased over years across fertilization treatments. Compared with no fertilization, long-term manuring and straw returning significantly increased bulk soil GRSP by 100% and 80%, respectively, and altered the chemical composition of GRSP by increasing the recalcitrant (aromatic) C proportion. The proportion of aromatic C in GRSP was positively correlated with AMF biomass and diversity (Shannon and Chao1), indicating that the chemical composition of GRSP could be regulated by AMF community and diversity. Moreover, manuring facilitated the formation of macro-aggregates ($>250\ \mu\text{m}$), thus increasing the physical protection of GRSP. The structural equation modeling further demonstrated that GRSP content was positively regulated by soil macro-aggregates, AMF biomass and diversity and their linkage with GRSP chemical composition. Collectively, long-term manuring could facilitate GRSP accumulation by shifts in AMF-mediated GRSP chemical composition (aromatic C) along with enhanced protection of macro-aggregates. This study highlights a feasible way forward for soil quality improvement and carbon sequestration for sustainable agriculture.

Keywords: Long-term fertilization; Glomalin-related soil proteins; Chemical composition; Aggregate stability; Arbuscular mycorrhizal fungi

1. Introduction

Glomalin-related soil proteins (GRSP), as metabolites from arbuscular mycorrhizal fungi (AMF) assimilation (Wright et al., 1998; Driver et al., 2005), contributes to soil C sequestration and aggregate stability. GRSP is a mixture of compounds that contains humic, lipid, inorganic materials, etc. (Gillespie et al., 2011). It is estimated that the organic C in GRSP accounts for 4–15% of SOC (Rillig et al., 2001; Singh et al., 2016; Wang et al., 2020), and its residence time in soil is around 35 years (Harner et al., 2004). Moreover, GRSP, by virtue of its cohesiveness and hydrophobicity, can enhance soil water retention and permeability, improve soil stability by promoting the bonding of soil mineral particles, thus reducing the risk of soil erosion and debris flows (Wright and Upadhyaya, 1996). To maintain the human demand for food production, amounts of fertilizers are used in agricultural practices. Fertilization significantly affects GRSP in the soil (Cissé et al., 2021a; Cissé et al., 2021b). However, the underlying mechanism of GRSP accumulation in response to fertilization remain limited. Therefore, studying the dynamics and controlling factors of GRSP accumulation under various fertilization managements is of significant importance for C sequestration and food stability.

Fertilization greatly alters the community composition and diversity of AMF (Xiao et al., 2019; Wang et al., 2022), which might had predominant effects on GRSP accumulation (Bedini et al., 2007; Holátko et al., 2021). Large numbers of fertilizer application studies have been performed to analyze the impacts of nutrients on AMF. However, there is no consensus on the response of AMF to fertilization, with positive (Porrás-Alfaro et al., 2007; Zheng et al., 2014) and negative (Zhu et al., 2016; Williams et al., 2017; Luo et al., 2021) effects being observed. For example, Mineral fertilization (e.g., N, P) causes soil acidification, thus decreasing AMF colonization and diversity (Liu et al., 2021b). Manure application decreases AMF richness and diversity (Garó et al., 2022), possibly due to reduced allocation of plant C to AMF (Johnson et al., 2013). However, some studies have found the opposite. N, P and organic fertilizer addition have beneficial effects on AMF biomass and diversity by increasing nutrient availability (Lee et al., 2008; Dia et al., 2013; Camenzind et al., 2016). These contradictions indicate that our understanding of the effects of fertilization on AMF and GRSP content is still unclear and needs further research. In addition, differences in chemical composition of GRSP can also affect its accumulation in soil due to the different decomposition rates of composition. The main chemical composition of similar humic substances can be obtained by Fourier transform infrared reflection (FTIR) and/or nuclear magnetic resonance (NMR), such as aromatic C, aliphatic C, polysaccharides and hydrocarbons in GRSP (Tivet et al., 2013; Wang et al., 2020; Guo et al., 2022; Zhang et al., 2022). The aromatic C contained in GRSP is responsible for its stable character. (Schindler et al., 2007). However, studies on how fertilization affects GRSP composition through altering AMF diversity are still lacking. Additionally, the chemical composition of GRSP could potentially be modified through the manipulation of soil physicochemical properties (e.g., pH) (Zhong et al., 2017). Therefore, it is crucial to reveal the

mechanisms of changes in GRSP composition and its linkage with AMF biomass and diversity under different fertilization.

In addition, fertilization regimes could influence GRSP through the promotion of stable soil aggregates, which can protect GRSP from decomposition (Sekaran et al., 2020). Based on the hierarchical model of aggregate formation concept, primary mineral particles are combined with bacteria, fungi, GRSP and plant residues to form microaggregates (<250 μm), which then become macroaggregates (>250 μm) through binder, such as roots and AMF hyphae (Tisdall and Oades, 1982; Rillig and Mummey, 2006). Consequently, GRSP is crucial for the formation of aggregates, and aggregation can likewise protect GRSP from degradation. However, the results presented by multiple studies considering the impact of fertilization on aggregate stability are inconsistent. For instance, Bottinelli et al. (2017) found that manure application increases the formation of large aggregates and aggregate stability (e.g., mean weight diameter (MWD)), whereas Guo et al. (2019) showed different results. In addition, the regulation mechanisms of GRSP accumulation by changes in aggregate stability after long-term fertilization are still lacking. Hence, further research on GRSP distribution among different aggregate size fractions is required to shed light on how its accumulation in aggregates responds to long-term fertilization regimes.

In this study, the aim was to clarify the response of GRSP content and its chemical composition to long-term fertilization regimes (29-year). We hypothesized that long-term manuring could facilitate GRSP accumulation by enhancing its recalcitrance of GRSP due to increased AMF biomass and diversity, and promoting the formation of macro-aggregates.

2. Materials and methods

2.1. Site description and soil sampling

A long-term field experiment was established on the basis of a cropland soil located in Qiyang County, Hunan Province, China (26°45'N, 111°52'E) in 1990. The site has a subtropical climate with mean annual temperature and mean annual precipitation of 18.1°C and 1431 mm, respectively. The parent material of the soil is quaternary red clay, and classified as Ferralic Cambisol according to the FAO classification. The soil texture was loamy clay (i.e. clay 43.9%; silt 31.9%; sand 24.2%), with soil pH of 5.70, and SOC 8.58 g kg⁻¹, total nitrogen (TN) 1.07 g kg⁻¹, and total phosphorus (TP) 0.45 g kg⁻¹. The main cropping system is winter wheat-summer maize rotations after the experiment was launched. The experiment was a completely randomized design with two repetitions, each with two sub-replicates. To ensure sample adequacy, we selected three replicates for measurement. The treatments included: no fertilizer (CK); mineral fertilizer (NPK, mineral N, P, K fertilizer); manure (M, pig manure; NPKM, NPK plus pig manure; NPKMR, NPKM plus wheat-soybean-sweet potato rotation of three crops a year); Straw returning (NPKS, NPK plus straw); Fallow (F, natural succession). The fertilizer application schedule is shown in Figure 3-7. Soil samples (0-20 cm in depth) were selected after crop harvest in September of 1990, 2006, 2012,

and air-dried (Zhang et al., 2009; Xiao et al., 2021). Part of the fresh soil in 2019 was stored in a -80 °C refrigerator.

2.2. Determination of soil physicochemical properties

The pH of the soil was tested at a soil-to-water ratio of 1:2.5. An elemental analyzer (EA3000, Milan, Italy) was used to measure the contents of SOC and TN. Nitrate nitrogen (NO_3^- -N) and ammonium nitrogen (NH_4^+ -N) content were measured by continuous flow analyzer (San⁺⁺ system, Netherlands). Soil available phosphorus (AP) was tested by molybdenum-antimony resistance colorimetric method. The soil properties are shown in Table 3-1.

2.3. Determination of soil water-stable aggregates

According to Cambardella and Elliott (1993), soil aggregates were tested using the wet sieve method, considering four particle size classes (i.e. <53 μm , 53-250 μm , 250-2000 μm , and >2000 μm), and using a grain-size analyzer (Damon, Shanghai, China) (Table S3). The MWD was calculated to assess the aggregates stability for each treatment (Zhang and Horn, 2001):

$$MWD = \sum_1^{n+1} \frac{r_{i-1} + r_i}{2} \times m_i$$

where m_i is the weight percentage of aggregate still on the i^{th} sieve. r_i is the aperture size (mm) of the i^{th} mesh. n value refers to the fractions of soil aggregate size that represent 2.00 mm.

2.4. Determination of AMF biomass and diversity

AMF biomass was characterized by C16:1 ω 5 phospholipid fatty acids (PLFAs) (Olsson, 1999). PLFAs were extracted using a slightly modified version of the Bligh-Dyer method (Tian et al., 2022). Briefly, 2 g of the freeze-dried fresh soil samples were placed in a Teflon centrifuge tube, after which the extracting solution (citric acid buffer solution, V(chloroform): V(methanol): V(citric acid) = 1:2:0.8) and the unesterified internal standard (C19: 0.1 ng μL^{-1}) were added.

AMF diversity was analyzed by high-throughput sequencing. From around 0.5 g of soil stored at -80°C. The soil DNA genome was extracted by using the FastDNATM SPIN Kit for Soil (MP Biomedicals). After extraction, nested PCR was used to amplify the target fragment. AML1F (5'-ATCAACTTTCGATGGTAGGAT AGA-3')-AML2R (5'GAACCCAAACACTTTGGTTTCC-3') primers were used for the first round of PCR, and subsequently, AMV4.5NF (5'-AAGCTCGTAGTTGAATTT CG-3')-AMDGR (5'-CCCAACTATCCCTATTAATC AT-3') primers were used for the second round of PCR (Van Geel et al., 2014). The amplicon sequencing was performed by the Magigene platform (<http://cloud.magigene.com>). AMF α -diversity was calculated by the Shannon-Wiener index and Chao1 index.

2.5. GRSP determination and purification

GRSP was determined by a method provided by Wright et al. (1998). Briefly, total GRSP was extracted by using 2 mL of 50 mmol L^{-1} sodium citrate (pH=8.0) for 0.25 g of air-dried soil. The extractions were autoclaved for 1 h at 121°C and

centrifuged for 6 min at 10,000×g. This process was repeated four times on each sample, after which the supernatants were combined and centrifuged once more before quantification. 300 μL of the clear supernatant and 700 μL of dH₂O were drawn up into the sampling tank, and then 5 mL Coomassie bright blue (CBB) solution was added. After 2–3 minutes of color reaction (Liu et al., 2021a; Liu et al., 2021b), the optical density (OD) value of the GRSP was measured at 595 nm with a microplate reader (Biotek Synergy H1, USA) using bovine serum albumin (BSA) as the standard. All the collected supernatant was precipitated by titrating with hydrochloric acid (1 mol L⁻¹), cooling in an ice bath for 1 h, and then centrifuging at 10,000×g for 6 min. The resulting sediment was solubilized in 0.1 M sodium hydroxide (NaOH) and dialyzed in dH₂O for 60 h (dialysis bag, molecular weight cut off=10,000 Da, USA). Following dialysis, the dialysate was centrifuged for 6 min at 10000×g to eliminate insoluble residue, the supernatant was freeze-dried with a freeze-dryer (FD-1A-50, Beijing BoYiKang Laboratory Instrument Co., Ltd., China).

2.6. GRSP functional groups determination

The functional groups of GRSP were determined by the KBr pellet method (Schindler et al., 2007; Guo et al., 2022). Analysis method and instrument parameters were as follows: The KBr was dried in a drying oven at 60°C for 6 hours to ensure no residual water vapor, then ground and passed through a 100-mesh sieve. Subsequently, 1 mg of freeze-dried GRSP sample and 200 mg of KBr (≥99%) were weighed, respectively, both components were carefully placed into an agate mortar and ground in a 1:200 ratio to ensure proper mixing. After achieving a homogeneous mixture, the resulting blend was pressed into pellets. These pellets were scanned by infrared spectrometer (BrukerVertex70, Germany). The resolution of Vertex70 was 4 cm⁻¹, the sample scan time was 32 s, the background scan time was 32 s, and the wavenumber range was 400 cm⁻¹ to 4000 cm⁻¹. Baseline and ambient corrections (i.e. H₂O, CO₂) were applied to every obtained spectra. We found four functional groups by wavenumber matching, which belong to four chemical compositions: polysaccharides, aliphatic C, aromatic C, hydrocarbons. To estimate the proportion of functional groups that correspond to each peak in the spectrum, the area of each peak was estimated by integration to obtain semi-quantitative data for functional groups (Wang et al., 2014). It is demonstrated how functional features and peak wavenumbers correspond. The matching of functional characteristics and peak wavenumbers are shown in Table 3-S2.

2.7. Statistical analysis

Statistical analyses were conducted after all data had been examined for homogeneity and normal distribution (K-S test). A one-way ANOVA was used with IBM SPSS 19.0 statistical software to evaluate the differences (Duncan's test) in soil nutrients, soil aggregates, AMF, GRSP, and its chemical compositions among fertilization treatments. The correlation between GRSP content and nutrients, AMF and MWD were assessed using the Pearson's correlation coefficient test. The partial least squares path modelling (PLSPM) was applied to identify the regulatory pathways of factors on GRSP, we substituted independent variables with the first component

PC1 by principal component analysis (PCA) (Table 3-S4). Variance partitioning analysis (VPA), random forest (RF) and PLSPM were all performed in R 4.0.5.

3. Results

3.1. Soil properties, GRSP contents and chemical composition

The soil physicochemical properties showed great difference across fertilization treatments (Table 3-1). More specifically, pH value was significantly higher in the M, NPKM and F treatments relative to CK, whereas this was significantly lower in the NPK, NPKS treatments. SOC, TN, AN and AP contents under M, NPKM and NPKMR were higher than that of CK and NPK treatments. However, SOC, TN contents were significantly higher under NPK and NPKS treatments than that under CK treatment. N accumulation showed a trend of increasing over time (Figure 3-8).

Table 3-1 Soil properties subjected to 29-year various fertilization (CK, no fertilizer; NPK, mineral nitrogen, phosphorus, potassium fertilizer; M, pig manure; NPKM, NPK plus pig manure; NPKS, NPK plus Straw; NPKMR, NPKM plus wheat-soybean/sweet potato rotation; F, Fallow, natural succession).

Treatments	pH	SOC (g C kg ⁻¹)	TN (g N kg ⁻¹)	AN (mg N kg ⁻¹)	AP (mg P kg ⁻¹)
CK	5.33 ± 0.06c	6.77 ± 0.25c	1.21 ± 0.02d	10.21 ± 0.46d	1.93 ± 0.26e
NPK	4.41 ± 0.01d	9.99 ± 0.12b	1.50 ± 0.02c	6.63 ± 0.56e	43.34 ± 2.47d
NPKS	4.50±0.04d	9.55 ± 0.31b	1.46 ± 0.04c	7.54 ± 0.60de	56.43 ± 4.25c
M	6.26 ± 0.01b	14.07 ± 0.33a	2.01 ± 0.07a	38.40 ± 0.18a	141.63 ± 2.99b
NPKM	6.12 ± 0.07b	13.55 ± 0.20a	1.77 ± 0.02b	30.02 ± 0.66b	147.94 ± 1.53b
NPKMR	5.83±0.04c	14.26 ± 0.50a	1.99 ± 0.02ab	28.95 ± 2.04b	167.57 ± 1.82a
F	6.52±0.08a	13.57 ± 1.51a	1.46 ± 0.18c	24.40 ± 1.01c	7.03 ± 0.88e

Note: SOC, soil organic carbon; TN, total nitrogen; AN, available nitrogen; AP, available phosphorus; Values represents the means with standard errors (n=3). Different letters indicate significant differences among various fertilization for each parameter using Duncan's test ($p < 0.05$).

The GRSP accumulated in all fertilizer and fallow treatments over time from 1990 to 2019, with values increasing from 1.9 mg g⁻¹ in 1990 to 3.2-4.7 mg g⁻¹ in 2019, whereas under the CK treatment this remained constant (Figure 3-1a). Long-term fertilization significantly altered GRSP contents ($p < 0.05$, Fig. 1b). Compared with CK treatments, NPK, straw returning (NPKS) and manuring (M, NPKM, NPKMR) increased GRSP content by 55%, 80% and 100%, respectively. When considering manure application, diversified cropping (NPKMR) facilitated significantly higher GRSP accumulation in bulk soil than in wheat-maize rotation (NPKM). Fertilization also strongly affected the content of GRSP across aggregate size fractions ($p < 0.05$, Figure 3-1b). GRSP mainly accumulated in the 250-2000 μm particle size fractions. Compared with CK treatment, NPK and NPKS treatments increased GRSP only significantly in aggregate size fractions larger than 53 μm (1.0-1.6 mg g⁻¹ soil),

whereas NPKM and NPKMR treatments increased GRSP across all particle sizes (1.3-2.6 mg g⁻¹ soil).

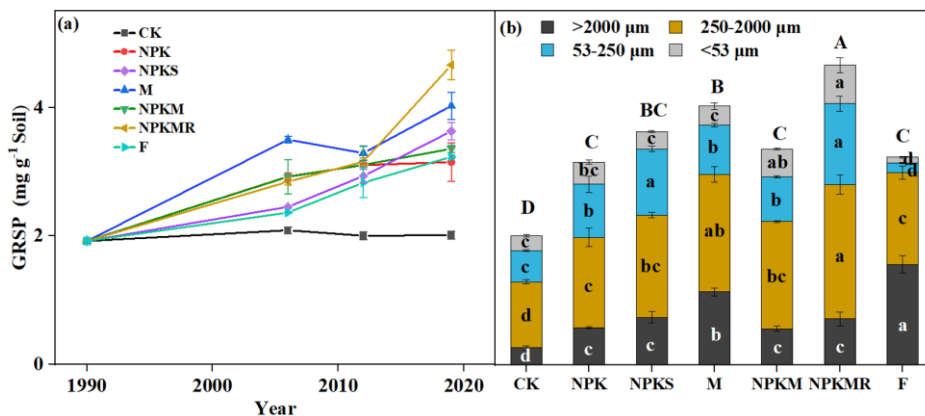


Figure 3-1 Temporal glomalin-related soil protein (GRSP) trends between 1990 and 2019 (a). GRSP accumulation in bulk soil and aggregates (b) (i.e., >2000 μm, 2000-250 μm, 250-53 μm, <53 μm) under various fertilization treatments (CK, no fertilizer; NPK, mineral nitrogen, phosphorus, potassium fertilizer; M, pig manure; NPKM, NPK plus pig manure; NPKS, NPK plus Straw; NPKMR, NPKM plus wheat-soybean/sweet potato rotation; F, Fallow). Data represents the means with standard errors (n=3). Different lowercase and uppercase letters respectively indicate significant differences in aggregates and the bulk soil under different fertilization treatments (Duncan's test, $p < 0.05$).

Manuring (M, NPKM, NPKMR) increased the absorbance of each chemical composition compared to CK and NPK (Figure 3-2a). According to peak area, the relative proportion of aromatic C under manuring and straw returning was 21%-73% higher than that of no/mineral fertilization ($p < 0.05$, Figure 3-2b). NPKMR treatment resulted in a relative chemical composition of GRSP in which the aromatic C was 43% higher, but the polysaccharides were 12% lower than in the NPKM treatment. Finally, when considering the fallow treatment it is notable that the aromatic C was higher and polysaccharide was lower than all other treatments.

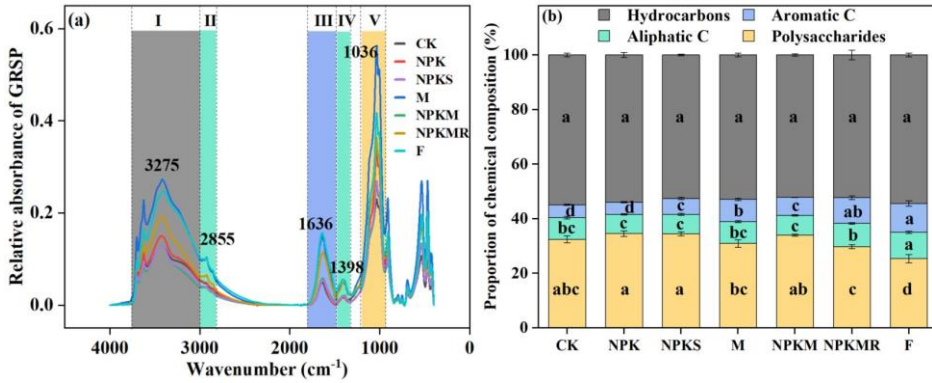


Figure 3-2 Fourier transform infrared (FTIR) spectra (a) and relative percentage of compositional traits of glomalin-related soil protein (GRSP) (b) in soils subject to 29-year various fertilization treatments (CK, no fertilizer; NPK, mineral nitrogen, phosphorus, potassium fertilizer; M, pig manure; NPKM, NPK plus pig manure; NPKS, NPK plus Straw; NPKMR, NPKM plus wheat-soybean/sweet potato rotation; F, Fallow). Data represents the means with standard errors (n=3). Different letters indicate significant differences in each compositional trait under the various fertilization treatments.

3.2. AMF community composition and diversity and their relationship with GRSP

According to the results of the non-metric multidimensional scaling (NMDS) analysis, mineral fertilization (NPK), manuring (M, NPKM, NPKMR) and straw returning (NPKS) greatly altered the AMF community and diversity compared to no fertilization (CK) as well as fallow (F) (Figure 3-3a). The AMF biomass (PLFA 16:1ω5) and total PLFA was significantly higher under manuring (M, NPKMR) treatments compared to the CK treatment. However, there was no significant difference in AMF and PLFA between NPK, NPKS versus CK (Figure 3-3b, 10). In addition, manuring (M, NPKM, NPKMR) and straw returning (NPKS) resulted in a significantly higher AMF alpha diversity index (Shannon and Chao1) compared with no and mineral fertilization (CK, NPK) (Figure 3-3c-d). The heatmap results showed that AMF biomass was positively correlated with GRSP in bulk soil as well as GRSP in large aggregates (>250 μm), and positively correlated with aromatic C composition and negatively correlated with polysaccharides. The AMF alpha diversity index (both Shannon and Chao1) showed a significant positive correlation with aromatic C ($p < 0.05$, Figure 3-3e).

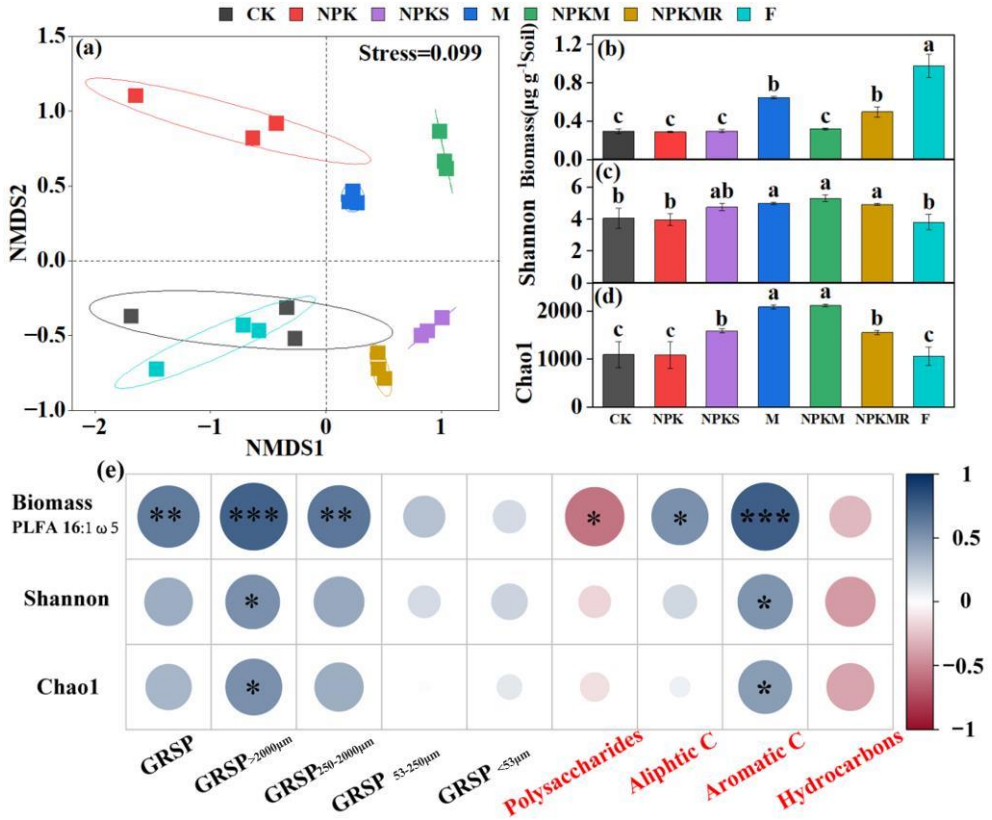


Figure 3-3 Non-metric multidimensional scaling (NMDS) ordination plots based on operational taxonomic units (OTUs) (a), biomass (b), Shannon index (c) and Chao 1 index (d) of arbuscular mycorrhizal fungi (AMF) under various fertilization treatments (CK, no fertilizer; NPK, mineral nitrogen, phosphorus, potassium fertilizer; M, pig manure; NPKM, NPK plus pig manure; NPKS, NPK plus Straw; NPKMR, NPKM plus wheat-soybean/sweet potato rotation; F, Fallow), and their relationships with glomalin-related soil protein (GRSP) contents (in bulk soil and different aggregates) and compositional traits (e). Data represents the means with standard errors ($n=3$). Different letters indicate significant differences between fertilization treatments (Duncan's test, $p<0.05$).

3.3. The relationship between MWD, SOC and GRSP

Fertilization had a great effect on aggregates stability. Compared with no and mineral fertilizer treatments (with a MWD around 1.3 mm), manuring (M) and fallow (F) treatments had a significantly higher MWD resulting in a value of 1.9 mm and 1.7 mm, respectively ($p<0.05$; Figure 3-11a), whereas NPKS, NPKM and NPKMR treatments had no significant impact on MWD. In addition, the relationship between GRSP in bulk soil and MWD was weak ($p>0.05$; Figure 3-11b), while MWD was positively correlated with the GRSP in 250-2000 µm and >2000 µm particle size fractions ($p<0.01$; Figure 3-4a). SOC content was positively correlated with GRSP ($p<0.01$; Figure 3-4b).

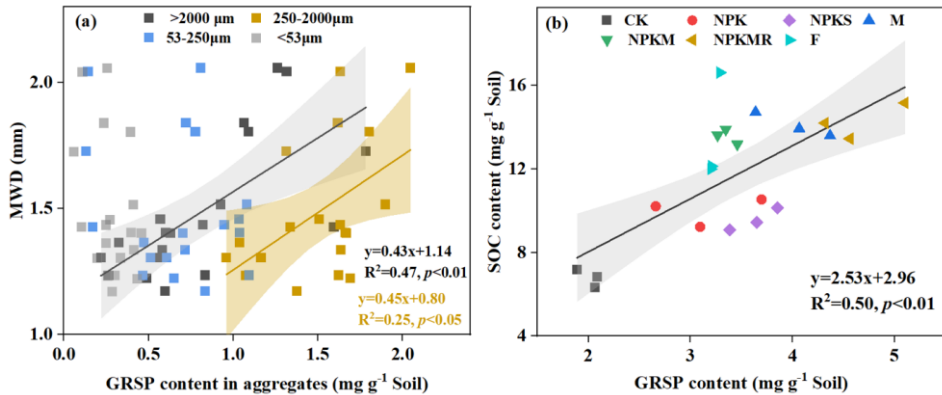


Figure 3-4 The relationship between glomalin-related soil protein (GRSP) in aggregates and mean wight diameter (MWD) (a), and the relationships between glomalin-related soil protein (GRSP) in bulk soil and soil organic carbon (SOC) (b). (CK, no fertilizer; NPK, mineral nitrogen, phosphorus, potassium fertilizer; M, pig manure; NPKM, NPK plus pig manure; NPKS, NPK plus Straw; NPKMR, NPKM plus wheat-soybean/sweet potato rotation; F, Fallow).

3.4. Effect of fertilization on GRSP via regulating AMF and aggregate stability

The partial least squares path modelling (PLSPM) showed that pH had a significant positive effect on nutrients (pc (path coefficients)=0.82, $p<0.001$), but is not directly related to AMF and aggregate stability. However, nutrition significantly impacted AMF in a positive direction ($pc=0.76$, $p<0.01$). In turn, AMF was positively correlated with soil aggregate stability and GRSP composition ($pc=0.70$, $p<0.01$; $pc=0.68$, $p<0.05$). GRSP accumulation was positively linked with soil aggregate stability and GRSP composition ($pc=0.87$, $p<0.001$; $pc=0.44$, $p<0.05$) (Figure 3-5a). In addition, our analysis suggests that the GRSP composition had the greatest positive effect on GRSP accumulation (total standardized effects = 0.87), followed by AMF (total standardized effects = 0.60) (Figure 3-5b). Therefore, soil aggregate stability and GRSP composition were the main controlling factors of the GRSP accumulation process in the studied cropland ecosystems. These results were consistent with variance partitioning analysis (VPA). Random forest analysis (RF) showed that the proportion of aromatic C in GRSP dominated the accumulation of GRSP, followed by the protective effect of aggregates (MWD and 0.25-2 mm) (Fig. 5c).

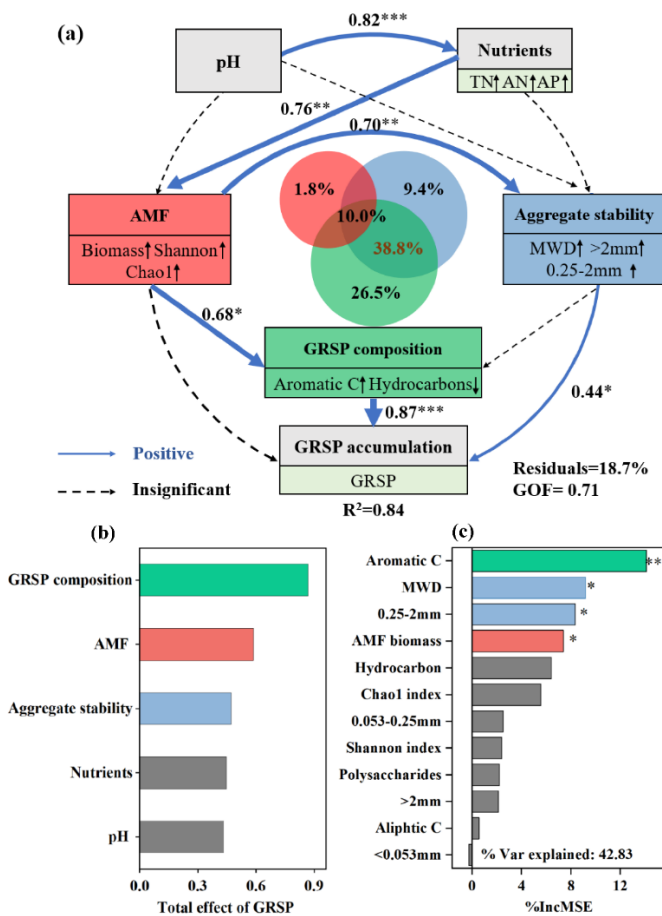


Figure 3-5 Partial least squares path modelling (PLS-PM) displaying the effects of abiotic and biotic factors on glomalin-related soil protein (GRSP) accumulation (a), and the standardized effects on GRSP accumulation derived from PLS-PM (b). Variance partitioning analysis (VPA) and random forest to distinguish the relative importance of aggregate stability, GRSP chemical composition and AMF (c). Blue and black arrows represent positive and negative effects, significant and insignificant correlations are denoted by continuous and dashed arrows, respectively. The thickness of the arrow is positively correlated to the strength of the relationship. Two-layer rectangle boxes denote the first component from the PCA performed for AMF, soil aggregate stability and GRSP composition. The up arrow “↑” denotes a positive correlation between the variables and the first component of PCA, the opposite “↓” is negative.

4. Discussion

Our study elucidates the response of GRSP accumulation to long-term fertilization regimes (Figure 3-6). GRSP content increased over time across fertilization, suggesting long-term fertilization is advantageous for the accumulation of GRSP (Guo et al., 2019). However, there was obviously divergences in the increasing trends in response to 29-year fertilization regimes (Figure 3-1). Long-term manure and straw

returning significantly promoted GRSP accumulation, confirming our hypothesis (Figure 3-1a), which agreed with most previous studies (Zhang et al., 2014; Bertagnoli et al., 2020; Choudhary et al., 2021; Wang et al., 2022). The application of manure and straw into agricultural production strategies might hold significant value in achieving soil stability and sustainable development. In this study, structural equation modeling showed that the accumulation of GRSP could be attributed to the synchronized regulation by (1) the production by AMF biomass and diversity, (2) the stabilization via soil aggregates protection and recalcitrance of GRSP.

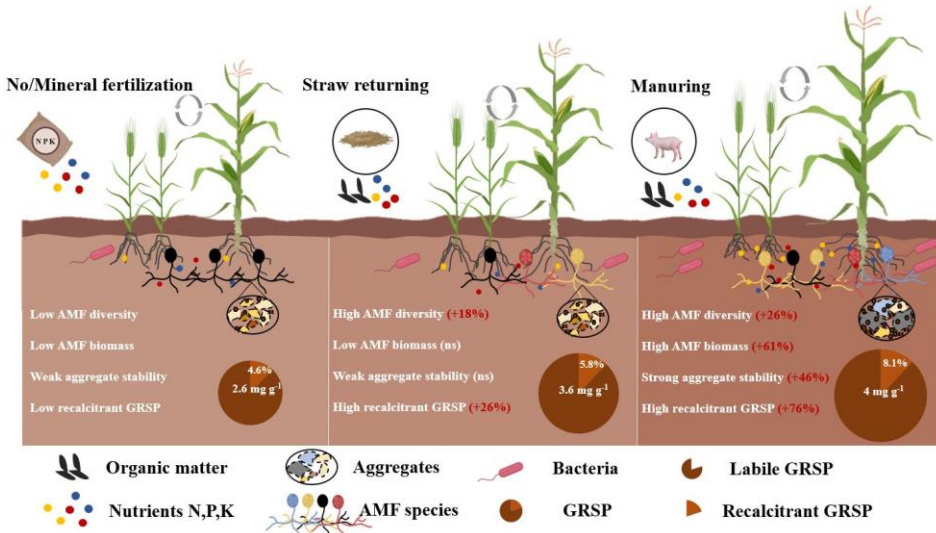


Figure 3-6 Conceptual figure showing differences in glomalin-related soil protein (GRSP) accumulation under 29-year various fertilization treatments. ns indicates no significant difference. The size of the pie chart represents the GRSP content.

4.1. Production mechanism of GRSP

The positive relationship between GRSP content and AMF diversity and biomass indicated that long-term fertilization regimes can directly or indirectly affect GRSP accumulation via regulated AMF diversity and/or biomass (Figure 3-3c-e, Agnihotri et al., 2022). Manuring and fallow increased GRSP through AMF biomass by following pathways (Figure 3-3b). First, Manuring led to an improvement in soil pH and nutrient levels (e.g., TN, AN, AP) (Table 3-1), alleviating competition for nutrients between host and AMF (Johnson, 2010). Second, manure application can decrease soil bulk density and increase soil porosity (Ozlu et al., 2019), providing AMF with a sufficient supply of oxygen and water (Wang et al., 1993). This reduces the obstruction of AMF hyphal elongation (Ma et al., 2006), promoting mycorrhizal development (Delavaux et al., 2017; Šarapatka et al., 2019). Third, AMF biomass was positively correlated with the GRSP content in bulk soil and in macroaggregates (>250 μm) ($p < 0.01$; Figure 3-3e). This indicates that manuring-induced increases in AMF biomass directly enhanced the GRSP content in macroaggregates. Because soil aggregates provide a suitable habitat for microorganisms (Gupta and Germida, 2015;

Trivedi et al., 2017). Malusà et al. (2016) showed that manure can promote the growth and metabolism of multiple AMF species, which has also been supported by more recent studies (Baltruschat et al., 2019; Song et al., 2015; Huo et al., 2022; Liu et al., 2022). In this study, the experimental location is situated in a subtropical region with significant soil acidification (Figure 3-7; Zhang et al., 2009) and a serious imbalance of soil nutrients. Improving the living environment (N availability, pH) through organic fertilization can mitigate microbial nitrogen limitation, stimulating AMF species interactions (Yu et al., 2013), which results in the activation of previously inactive AMF species and contributes to their growth (Aggarwal et al., 2011). Low nutrients (i.e. N, P) under fallow treatment caused nutrient competition among AMF species and other microbes, resulting in low AMF diversity. However, fallow treatment had higher AMF biomass and total PLFA compared to fertilization, probably because as undisturbed natural succession progresses, the soil develops and becomes richer in organic matter (Table 3-1). The latter provides a favorable environment for growth of AMF, leading to higher biomass, meanwhile, hypha has a good environment to grow without disturbance.

Notably, straw returning and mineral fertilizer with manure had no significant effect on AMF biomass and total PLFA (Figure 3-3,10). The explanation for these results is that straw returning significantly decreased soil pH (Table 3-1), while AMF prefer to grow in neutral or alkaline soils (Toljander et al., 2008). Therefore, low pH counteracts the positive regulatory effect of nutrients on AMF. Mineral fertilizer with manure increased P availability (Table 3-1), which could possibly suppress AMF growth (Lin et al., 2012). In addition, the content of added N in this experiment is equal between manure with/without mineral fertilizer, which may result in non-significant differences. Therefore, soil pH, N, P availability and their interactions could impact the AMF growth (Qin et al., 2020; Williams et al., 2017; Luo et al., 2021).

4.2. Stabilization mechanism of GRSP

The peak intensity of each functional group and fingerprints of GRSP altered under different fertilization treatments (Figure 3-2a), suggesting that fertilization could change the chemical composition of GRSP. Specifically, Manure and fallow increased the proportion of aromatic C, but had no significant effect on hydrocarbons, enhancing recalcitrance of GRSP by altering its chemical composition (Figure 3-2b). Organic matter from manure and straw decomposed to release mineral forms of nitrogen and phosphorus, which become available for plant and AMF uptake/utilization, hereby increasing AMF biomass. Meanwhile, an increase in soil pH following manuring and fallow can strongly alter the AMF community and their interactions (Wang et al., 2014). Furthermore, organic fertilizer application may activate dormant AMF species in the rhizosphere (Zhu et al., 2016). These might influence GRSP chemical composition. Therefore, soil nutrient levels and pH could potentially be significant factors influencing the chemical composition of GRSP (Zhong et al., 2017).

Another key factor is the soil aggregate protection mechanism (Chen et al., 2019). Fertilization regimes affect the redistribution of GRSP in aggregates. In our study, manuring increased MWD values (Figure 3-11a) and the mass percentage of the large aggregate size fractions (Table 3-S2). These results are supported by other findings (Van-Camp et al., 2004; Duan et al., 2021). Manure application reduces macroaggregate breakdown by improving hydrophobicity and the interparticle cohesiveness of aggregates. Generally, aggregate stability increases or decreases synergistically with GRSP content (Liu et al., 2021a). However, our study found that the relationship between MWD and GRSP in bulk soil was not significant (Figure 3-11b). The reason might be that the method of sweet potato harvesting and tillage systems interfered with the aggregate–GRSP relationship. By investigating GRSP contents in aggregates, we observed a strong correlation between MWD and GRSP in both the >2000 μm and 250–2000 μm particle size fractions (Figure 3-4a), indicating that the formation of large aggregates facilitated the accumulation of GRSP in soil. The winding effect of hyphae and the bonding capacity of GRSP synergistically promote the transformation of small aggregates into large aggregates, thereby increasing the stability of aggregates (Bedini et al., 2009; Kohler et al., 2017; Ji et al., 2019). In turn, large aggregates protect GRSP from decomposition. Moreover, large aggregates provide a stable environment for the formation of microaggregates, increasing the ability to absorb or surround more unprotected organic matter (e.g., GRSP) (Liu et al., 2020).

In summary, shifts in the AMF community and diversity directly and indirectly mediated the effects of fertilizers on GRSP accumulation, via two paths (Figure 3-5a): (1) Changes in pH and/or nutrients regulated the recalcitrance of GRSP by increasing the AMF biomass and diversity, thus affecting the accumulation of GRSP (Figure 3-9). (2) AMF regulated the stability of aggregates, thereby increasing the physical protection of GRSP. Therefore, GRSP composition (e.g., aromatic C) had a huge overall effect on GRSP accumulation. VPA and RF modelling (Figure 3-5c) together supported the reliability of structural equation modelling, and concluded that the stabilization mechanisms of GRSP dominate its accumulation.

4.3 Implications

Previous studies have indicated that AMF diversity associated with GRSP accumulation will be influenced by plant diversity through natural succession (Wang et al., 2022; Li et al., 2023). In this study, we demonstrated that legume cropping considering a wheat, soybean and sweet potato rotation (NPKMR) was more beneficial for the accumulation of GRSP than a classical wheat-maize rotation (NPKM). The possible explanations were that (1) different AMF species and populations have been detected in legume and non-legume roots (Scheublin et al., 2004), the nitrogen fixation of rhizobium provides more favorable conditions for AMF growth and multiplication under legumes compared to non-legumes (Xavier and

Germida, 2003), and as a consequence can increase GRSP accumulation. (2) Low C:N ratios and labile available substances, such as sugars and amino acids, found in leguminous crops and their root exudates can encourage AMF activity (Dinesh et al., 2004). (3) Legume cropping made the soil AMF community richer (Guzman et al., 2021). Therefore, legume cropping may be a sustainable way to increase sequestration and stability of soil C in cropland soils and could be considered as an interesting strategy to combat climate change. However, there are still some limitations in this paper, and further research is needed to investigate the differences in various crop rotations under mineral amendments.

Long-term manuring and straw returning significantly increased SOC (Table 3-1; Figure 3-4b), although their extent of increase differed, indicating that the contribution of straw and/or root exudates and microbial necromass to SOC may be less than that of manure. An explanation is that pH under manuring is higher than that of straw returning (Table 3-1), increased pH in acid soil increased bacterial. Faster turnover of bacteria can promote accumulation of microbial necromass, which is beneficial to SOC accumulation (Prommer et al., 2020). In addition, the relationships of GRSP and SOC with soil physicochemical traits are not fully consistent. The factors in controlling GRSP and SOC formation might be different and need further research. Therefore, in future research, we can pay more attention to the impact of different carbon source inputs on the recalcitrance of GRSP and SOC.

5. Conclusion

Manuring and straw returning strongly promoted GRSP accumulation, mainly attributed to changes in AMF biomass, diversity, proportion of recalcitrant (aromatic) C in GRSP and macro-aggregates. Manuring and straw returning increased the AMF diversity through improving nutrients and/or alleviating acidification, therefore making its metabolites (GRSP) more difficult to decompose. In addition, manuring also increased the physical protection of GRSP through the formation of macro-aggregates. In summary, the application of organic fertilizer could promote the growth of AMF as well as aggregate stability, and finally facilitate GRSP accumulation. Our findings contribute to clarifying the process of GRSP accumulation under long-term fertilization regimes.

6. Acknowledgements

This study was financed by the National Key R&D program of China (No. 2021YFD1901201), and National Science & Technology Fundamental Resources Investigation Project of China (2021FY100501). We thank the CAAS-Gembloux Agro-Bio Tech joint training program, Innovation Program of Chinese Academy of Agricultural Sciences and the China Scholarship Council for their support.

7. Supplementary Figures and Tables






Winter-Spring (Oct-May) $\xleftrightarrow{29 \text{ years}}$ Summer-Autumn (Jun-Sep)		Treatments	Fertilization (kg hm ⁻²)			
			N	P ₂ O ₅	K ₂ O	Manure/Straw
Wheat 	Maize 	CK	-	-	-	-
		NPK	300	120	120	-
Wheat 	Soybean/ Sweet potato 	M	-	-	-	M: 60000
		NPKM	90	120	120	M: 42000
		NPKS	300	120	120	S: All
Fallow land (Natural succession) 		NPKMR	90	120	120	M: 42000
		F	-	-	-	-

Figure 3-7 Fertilizer application amounts under different treatments and cropping systems. CK, no fertilizer; NPK, mineral nitrogen, phosphorus, potassium fertilizer; M, pig manure; NPKM, NPK plus pig manure; NPKS, NPK plus Straw; NPKMR, NPKM plus wheat-soybean/sweet potato rotation; F, Fallow.

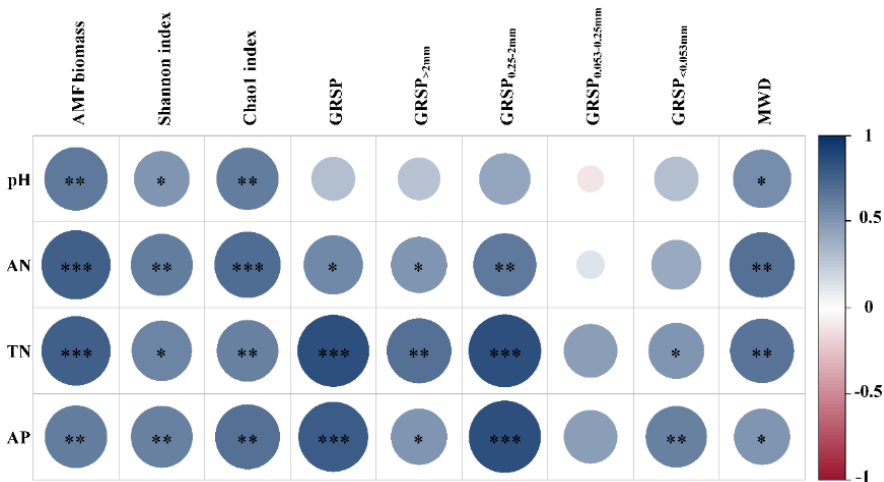


Figure 3-8 Pearson correlation heatmap between soil properties (pH, TN, AN, AP) with AMF biomass and diversity, GRSP contents (in bulk soil and different aggregates level). GRSP_{>2mm}, GRSP_{0.25-2mm}, GRSP_{0.053-0.25mm}, GRSP_{<0.053mm} represent GRSP in different aggregates, respectively.

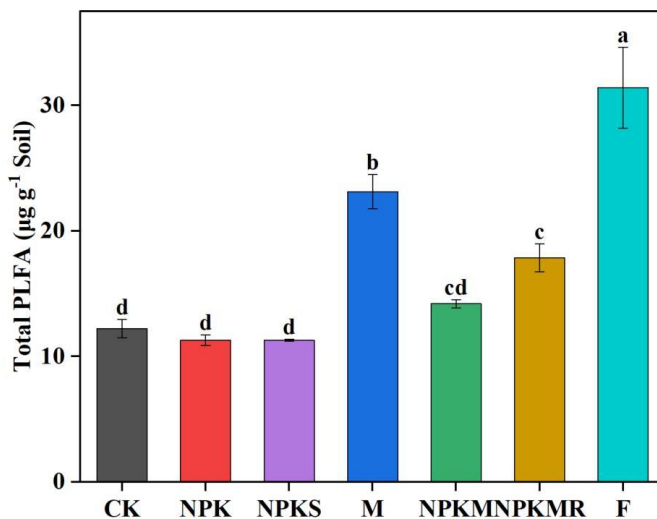


Figure 3-9 The total phospholipid fatty acid (PLFA) contents under 29-year various fertilization (CK, no fertilizer; NPK, mineral nitrogen, phosphorus, potassium fertilizer; M, pig manure; NPKM, NPK plus pig manure; NPKS, NPK plus Straw; NPKMR, NPKM plus wheat-soybean/sweet potato rotation; F, Fallow, natural succession).

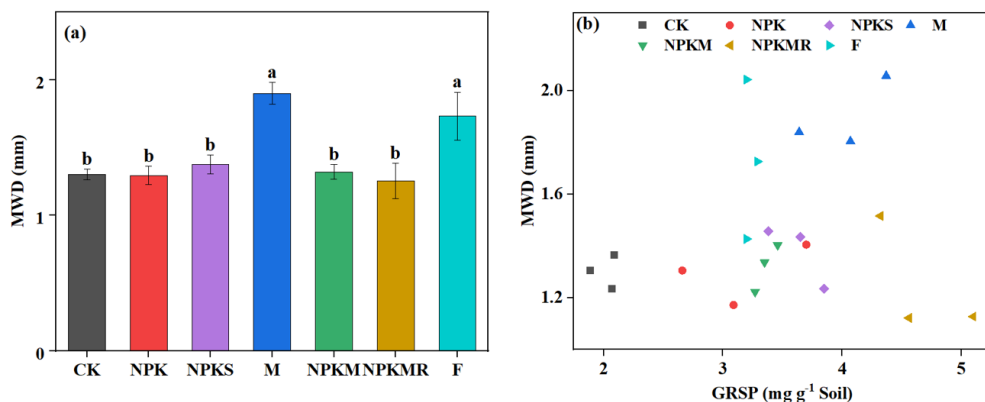


Figure 3-10 Mean weight diameter (MWD) values in soils subject to 29-year various fertilization treatments (a) and its relationship with glomalin-related soil protein (GRSP) under bulk soil (b). Data represents the means with standard errors (n=3). Different letters indicate significant differences between fertilization treatments (Duncan's test, p<0.05).

Characteristics of glomalin-related soil proteins accumulation and its response to fertilization and tillage practices in typical cropland soil

Table 3-S1 The matching of functional characteristics and peak wave numbers (Tivet et al., 2013; Wang et al., 2020).

Absorption band (cm ⁻¹)	Trait	Chemical composition	Functional group
3275-3624-3699	I	Hydrocarbons	O-H stretching of hydroxyl groups.
2928	II	Aliphatic C	Aliphatic asymmetric C-H stretch,
2855			Aliphatic symmetric C-H stretch.
1636	III	Aromatic C	Aromatic C=C vibrations and C=O vibrations of carboxylic acid anions.
1398	IV	Aliphatic C	Aliphatic C-H deformation of CH ₂ and CH ₃ bending, C-OH deformation of COOH, COO ⁻ symmetric stretch.
1036	V	Polysaccharides	Si-O-Si in-plane stretching mode of kaolinite, OH vibrations of gibbsite, and C-O stretching of polysaccharides.
563-694-752-800-912			O-H bending modes
			Al ³⁺ , Si-O stretch
			Si-O-Al
			O-H stretching of gibbsite and Si-O stretch of quartz
			Al ³⁺ ions, Si-O-Si deformations.

Table 3-S2 Soil aggregate fraction (%) under 29-year various fertilization (CK, NPK, M, NPKM, NPKS, NPKMR, F)

Treatments	Soil aggregate fraction			
	>2 mm (%)	0.25-2 mm (%)	0.053-0.25 mm (%)	<0.053 mm (%)
CK	14.09 ± 0.84b	49.43 ± 0.55a	24.77 ± 1.05bc	11.71 ± 0.70abc
NPK	14.63 ± 1.88b	45.76 ± 2.46ab	29.90 ± 0.50ab	9.73 ± 0.98bc
M	27.88 ± 1.62a	41.81 ± 0.57bc	22.30 ± 0.72c	8.02 ± 1.43c
NPKM	16.28 ± 0.52b	45.96 ± 1.18ab	24.79 ± 1.63bc	12.98 ± 0.69a
NPKS	17.23 ± 1.26b	41.76 ± 1.06bc	32.21 ± 1.63a	8.77 ± 0.50bc
NPKMR	20.14 ± 2.71b	41.22 ± 1.03bc	27.73 ± 1.32ab	10.94 ± 2.72ab
F	24.61 ± 3.33a	41.26 ± 1.44c	22.62 ± 3.10c	11.52 ± 1.77abc

Note: Values represents the means with standard errors (n=3). Different lowercase letters indicate significant differences among various fertilization for the same aggregate size using Duncan's test ($p < 0.05$).

Table 3-S3 Principal components analysis (PCA) results of Nutrients, AMF parameters, soil aggregates and GRSP composition.

	PC1 (Correlation)
AMF Parameters	
AMF biomass	0.66**
Shannon index	0.94***
Chao1 index	0.93***
Explanation (%)	72.4%
Nutrients	
TN	0.95***
AN	0.97***
AP	0.98***
Explanation (%)	93.1%
Soil aggregates	
MWD	0.93***
>2mm	0.90***
0.25-2mm	-0.76**
Explanation (%)	75.6%
GRSP composition	
Aromatic	0.86**
Hydrocarbons	-0.86**
Explanation (%)	74.8%

Chapter 4

No-tillage facilitates soil organic carbon accumulation by increasing arbuscular mycorrhizal fungi-related soil proteins and aggregation

From: Yang, H., Wang, G., Wang, J., Xiao, Q., Li, Z., De Clerck, C., Meersmans, J., Colinet, G., Zhang, W., 2024. No-tillage facilitates soil organic carbon sequestration by enhancing arbuscular mycorrhizal fungi-related soil proteins accumulation and aggregation. CATENA, 245, 108323.

Living without an aim is like sailing without a compass.

— Alexandre Dumas

生活没有目标就像航海没有指南针

—大仲马



Abstract

No-tillage is known to optimize soil structure and enhance soil organic carbon (SOC) stocks in cropland. However, the exact mechanisms driving the accumulation of SOC are still unclear, especially concerning the regulation of arbuscular mycorrhizal fungi (AMF) communities and diversity in SOC sequestration. Here, this study aims to elucidate the intricate relationship between AMF community, glomalin-related soil proteins (GRSP), and SOC within bulk soil and aggregates across four tillage regimes (i.e. FA, fallow; DR, deep rotary tillage; DT, deep tillage; NT, no-tillage) based on a 7-year tillage experiment. Results showed that the contents of SOC and GRSP were significantly higher by 1.14-1.46 mg g⁻¹ and 0.43-0.72 mg g⁻¹ in the bulk soil under NT relative to DR and DT, respectively. The contribution of GRSP-C to SOC under NT was also higher than DR and DT, especially in >53 μm particle size. Additionally, NT increased AMF diversity and the abundance of *glomerales* and *diversisporales*, all showing a strong positive correlation with GRSP, indicating their regulatory role in GRSP production. The positive correlations between GRSP and the mass percentage of the >53 μm particle size fraction and MWD suggest that no-tillage may drive large aggregates (>53 μm) formation and enhance aggregate stability through GRSP levels. Overall, increased AMF diversity and keystone taxa abundance at the order level via no-tillage promoted SOC accumulation through the production of GRSP and the protection of large aggregates. This study highlights that no-tillage is an effective and sustainable soil management strategy for enhancing soil quality in agricultural ecosystems.

Keywords: Long-term no-tillage; Soil organic carbon; Glomalin-related soil proteins; Aggregate stability; Arbuscular mycorrhizal fungi.

1. Introduction

Soil organic carbon (SOC) is the largest carbon (C) reservoir in the terrestrial ecosystems (Schlesinger, 1990). Even relatively small changes in SOC directly impact atmospheric CO₂ concentrations and the global C cycle (Sakschewski et al., 2016). Hence, in theory, improving SOC storage can be an effective measure to combat climate change. Notably, human activities like fertilization and tillage significantly impact SOC storage in cropland soil (Mazzoncini et al., 2011; Smith et al., 2005). Conservation tillage, especially no-tillage, is recognized for reducing soil disturbance and erosion, maintaining soil structure, and enhancing the accumulation of SOC in the upper 20 cm depth (Choudhary et al., 2013). Compared to conventional tillage, no-tillage promotes SOC accumulation in topsoil (0-20 cm) through physical protection of large aggregates (Six et al., 2000) and association with soil minerals (Kan et al., 2022). No-tillage also maintains stable soil environment and microbial activity (Jiang et al., 2011), thereby impacting the distribution and stability of SOC in aggregates (Kan et al., 2022). Consequently, it is crucial to elucidate how tillage regimes influence SOC dynamics at the aggregate scale.

A key factor affecting SOC is the role of glomalin-related soil proteins (GRSP), mainly derived from arbuscular mycorrhizal fungi (AMF) (Rillig, 2004; Wright et al., 1998). GRSP can enhance SOC stability due to aromatic C structure (Rillig et al., 2001; Yang et al., 2024). Meanwhile, the adhesive properties of GRSP also promote the formation and stability of large aggregates (Wright and Upadhyaya, 1998), thereby providing physical protection to SOC (Guo et al., 2020). Previous studies have explored the impacts of tillage regimes on GRSP accumulation, but the findings have been inconsistent (Agnihotri et al., 2022; Helgason et al., 2010). For example, Wright et al. (2007) reported significantly higher GRSP content in untilled bulk soils and large aggregates compared to tilled soils in Ultisols, while Curaqueo et al. (2011) found no increase in GRSP content after ten years of no-tillage compared to conventional tillage in a thermic Entic Haploxeroll (sandy clay alluvial soil). These contradictions may arise from differences in soil types and climate effects, causing changes in the complexity and diversity of AMF network (Yang et al., 2022), as well as their interaction with the surrounding soil environment (pH and nutrients) (Avio et al., 2013; Zhang et al., 2017). Further research on the effect of tillage on GRSP is needed. Furthermore, different aggregate sizes and pores provide distinct habitats for microorganisms. This can lead to varied distributions of AMF within the soil microstructure (Lehmann et al., 2017), thereby influencing GRSP levels. Therefore, deeper understanding of the distribution of GRSP within aggregates is crucial for the accumulation of SOC under tillage regimes.

In addition, compositions of AMF communities significantly influence the decomposition, resynthesis, transformation, and stabilization of SOC (Wu et al., 2023). The role of AMF in promoting SOC sequestration within aggregates remains a controversial research topic, with positive (Frey, 2019) and negative effects (Cheng et al., 2012) reported. Differences in life-history strategies between *glomerales* and

diversisporales within AMF are notable, with *glomerales* orders demonstrating a higher R-strategy, and *diversisporales* orders leaning towards a more K-strategy (Hart and Reader, 2002; Sýkorová et al., 2007). Promoting specific AMF orders may enhance C sequestration strategies. It is noteworthy that the impact of conventional tillage on AMF communities and diversity is inconsistent (Brito et al., 2012), possibly due to varying responses of AMF taxonomic groups to tillage practices concerning their activities and functions (Kabir, 2005). Further study is needed to understand these dynamics. Given the interrelation between the community and diversity of AMF and GRSP accumulation (Yang et al., 2024), elucidating how different tillage practices affect AMF diversity and keystone taxa richness is crucial for deciphering their roles in regulating SOC storage.

The fluvo-aquic soil (Fluvents, USDA), formed by the alluvial sediments from the Yellow River in China, faces issues such as poor soil structure, low SOC content, and high pH (He et al., 2021). Traditional tillage regimes exacerbate soil erosion and C loss in this region, making it a focal area for conservation tillage research. The objectives of this study were to: (i) explore the differences of GRSP, SOC and AMF communities in bulk soil and aggregates under tillage regimes; and (ii) identify AMF communities associated with GRSP accumulation. Our hypotheses were that (i) no-tillage would increase the content of SOC and GRSP in topsoil (0-20 cm), along with the contribution of GRSP to SOC within large aggregates; (ii) no-tillage may promote an increase in AMF diversity than biomass and the abundance of taxa (*glomerales* and/or *diversisporales*) contributing to GRSP production.

2. Materials and methods

2.1. Site description

A long-term field experiment was launched on the basis of a cropland soil in Shangqiu city, Henan Province, China (34°31'13" N, 115°35'30" E) in October 2012. The site is located in a sub-humid, warm temperate monsoon climate. The annual agrometeorological data show averages of 708 mm for precipitation, 13.9 °C for temperature, and 1735 mm for evapotranspiration. The parent material of the soil is alluvial sediments from the Yellow River and the soil texture is fluvo-aquic soils (i.e. clay 14.3%; silt 40.8%; sand 44.9%, classified as Fluvents in the USDA taxonomy). The soil in top 30 cm layer contains 5.35 g kg⁻¹ SOC, 0.86 g kg⁻¹ total nitrogen (TN), 157 mg kg⁻¹ available nitrogen (AN) and 11 mg kg⁻¹ available phosphorus (AP) and 8.7 soil pH. The main cropping system is winter wheat-summer maize rotations. Winter wheat is sown in mid-to-late October with a seeding rate of approximately 225 kg ha⁻¹; summer maize was planted in early June with a seeding rate of 37.5 kg ha⁻¹, resulting in a density of 75,000 plants ha⁻¹ when five leaves have been formed.

2.2. Experimental design

The experiment included four treatments: rotary tillage to a depth of 10-15 cm (DR), deep tillage to a depth of 30-35 cm (DT), no-tillage (NT), and fallow (FA, undisturbed soil). The plot area for the DR, DT and NT treatments is 12m × 75m. The FA treatment

is located in a nearby plot that had been cropland before 2007, and has remained in natural state of weeds and/or small shrubs since the experiment was established, with no fertilization, planting and tillage (Figure 4-6). Maize was continuously planted after the previous crop in the DR, DT, and NT plots. The straw residue from both wheat and maize has been returned to the field for full coverage. Compound fertilizer has been used, with 375 kg ha^{-1} (N:P₂O₅:K₂O = 25:18:7) applied before planting, and 600 kg ha^{-1} (30:5:5) applied 35-45 days after planting. For winter wheat, 600 kg ha^{-1} (25:18:7) is applied before planting, followed by 375 kg ha^{-1} (30:5:5) during the greening stage (135-150 days after planting).

2.3. Soil sampling and soil physicochemical properties analysis

To ensure comparability and consistency with other studies and to account for the majority of impacts from both different tillage depths, soil samples (the top 20 cm) were collected after crop harvest in October 2019. Each treatment included three replicates, with each replicate consisting of a composite sample from 4 points (Figure 4-6). One portion of the soil was air-dried, while the other fresh soil was stored in a -80 °C refrigerator. The physico-chemical properties of air-dried soil were determined. SOC in bulk soil and aggregates were determined using the sulfuric acid-potassium dichromate oxidation method, SOC content was corrected by factor of 1.3 (Walkley and Black, 1934). Determination of total nitrogen (TN) in bulk soil was made using the Kjeldahl method for nitrogen digestion and distillation. Total phosphorus (TP) and available phosphorus (AP) in bulk soil were measured using molybdenum-antimony resistance colorimetric method. The soil pH was measured at a soil-to-water ratio of 1:2.5 (FE30, Mettler Toledo).

2.4. GRSP determination

The method from Wright et al. (1998) was used to determine GRSP. In brief, for every 0.25 g of air-dried bulk soil/aggregates, 2 mL of 50 mmol L⁻¹ sodium citrate (pH=8.0) was used to extract the total GRSP. The extracts underwent a 1-hour autoclaving process at 121°C and a 6-min 10,000 ×g centrifugation. Each sample was performed this procedure four times, following which the supernatants were mixed and centrifuged once more in order to prepare them for measurement. Using bovine serum albumin (BSA) as the standard, the optical density (OD) value of the GRSP was measured at 595 nm using a microplate reader (Biotek Synergy H1, USA) after two to three minutes of color reaction (Liu et al., 2021). For the detailed steps of GRSP determination and purification, please see Yang et al. (2024).

2.5. AMF biomass and diversity measurements

AMF biomass was quantified using C16:1ω5 phospholipid fatty acids (PLFAs) (Olsson, 1999). 2 g of the freeze-dried fresh bulk soil samples were used to measure PLFA. Detailed determination procedures are described in Yang et al. (2024). The AMF community was measured in 0.5 g of soil stored at -80°C. Soil DNA was extracted using the FastDNA™ SPIN Kit for Soil (MP Biomedicals). Nested PCR

was employed to amplify the target fragment. For the first round of PCR, AML1F (5'-ATCAACTTTCGATGGTAGGATAGA-3')-AML2R(5'GAACCCAAACACTTTG GTTTCC-3') primers were utilized, while AMV4.5NF(5'-AAGCTCGTAGTTGAAT TTCG-3')-AMDGR (5'CCCAACTATCCCTATTAATCAT-3') primers were used for the second round of PCR (Van Geel et al., 2014). AMF α -diversity (including the Shannon-Wiener index and Chao1 index.) was calculated using the Magigene platform (<http://cloud.magigene.com>).

2.6. Determination of soil water-stable aggregates

The fractionation technique suggested by Cambardella and Elliott (1993) was used to separate the soil aggregates into four particle size classes (i.e. <53 μm , 53-250 μm , 250-2000 μm , and >2000 μm). Mean weight diameter (MWD) can be used to characterize the stability of aggregates. The MWD was calculated as:

$$MWD = \sum_{i=1}^n x_i \times m_i$$

Where m_i represents the mass percentage of aggregate remaining on the i^{th} sieve, x_i is the mean diameter of particle size fraction and n is the number of fractions.

2.7. Statistical analysis

The data were examined for homogeneity and normality using Kolmogorov-Smirnov test. Duncan's test was used to evaluate the effect of tillage regimes on the dependent variables SOC, GRSP, AMF, MWD. The relationship between GRSP content and SOC, keystone species of AMF, and MWD were evaluated using Pearson's correlation coefficient. Analysis of Similarities (ANOSIM) was performed to evaluate the significance of differences in the composition of AMF communities. Random forest (RF) was used to analyze the importance of different species on GRSP accumulation. RF, non-metric multidimensional scaling (NMDS), and chord diagram were all performed using the statistical software R v.4.3.0.

3. Results

3.1. Soil properties

The different tillage regimes exhibited significant differences in physicochemical properties ($p < 0.05$, Table 4-1). The pH value was significantly lower in NT treatment compared with DT, DR, and FA treatments. Total phosphorus (TP) and available phosphorus (AP) showed no significant differences among the DR, DT, and NT treatments. Total nitrogen (TN) is higher under the NT treatment than under the DT and FA treatments. However, tillage regimes (DR, NT) resulted in higher TN, TP, AP contents compared to fallow treatment, with increases of 13-23%, 26-38%, and 270-331%, respectively.

Table 4-1 Soil physicochemical properties under tillage regimes (FA: fallow; DR: deep rotary tillage; DT: deep tillage; NT: no-tillage).

Treatments	pH	TN (g kg ⁻¹)	TP (g kg ⁻¹)	AP (mg kg ⁻¹)
FA	8.18 ± 0.06 a	1.07 ± 0.13 b	0.77 ± 0.04 b	2.50 ± 0.60 b
DR	7.88 ± 0.11 b	1.21 ± 0.03 ab	1.06 ± 0.04 a	9.24 ± 1.50 a
DT	7.80 ± 0.02 b	1.10 ± 0.06 b	0.97 ± 0.05 a	9.61 ± 0.16 a
NT	7.44 ± 0.07 c	1.32 ± 0.00 a	1.02 ± 0.05 a	10.77 ± 1.06 a

Notes: TN, total nitrogen; TP: total phosphorus; AP, available phosphorus; Values represents the means with standard errors (n = 3). Different letters indicate significant differences among tillage regimes for each parameter using Duncan’s test (p < 0.05).

3.2. SOC and GRSP content in aggregates and their relationship

Tillage strongly affected the content of SOC in bulk soil and aggregates ($p < 0.05$, Figure 4-1a,b,c). In the bulk soil, the SOC content was 1.14, 1.46, and 2.24 mg g⁻¹ higher in the NT treatment than in the DT, DR, and FA treatments, respectively (Figure 4-1a). In aggregates, SOC contents in >53µm particle size was 0.64, 1.00 mg g⁻¹ higher in the NT treatment than in the DR and DT treatments, respectively ($p < 0.05$, Figure 4-1b). However, SOC contents in <53µm particle size was lower in the NT treatment than in the DR and DT treatments ($p < 0.05$, Figure 4-1c). NT treatment significantly increased the SOC content in >53µm particle size, but had no significant effect in <53µm particle size as compared to the FA treatment. (Figure 4-1b,c).

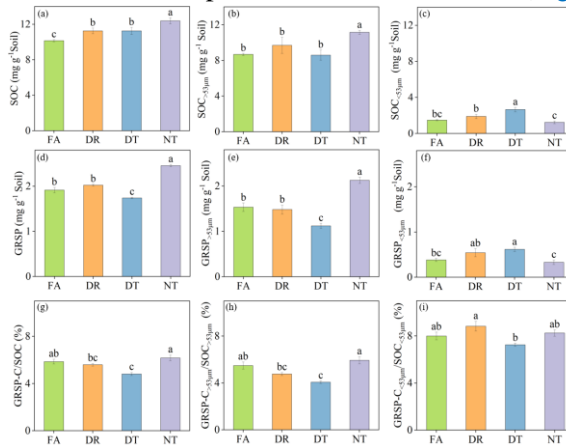


Figure 4-1 The concentrations of SOC (a), SOC >53µm (b), SOC <53µm (c), GRSP in bulk soil (d), GRSP >53µm (e), GRSP <53µm (f), contribution of GRSP-C in bulk soil to SOC (g), contribution of GRSP-C >53µm to SOC >53µm (h), and contribution of GRSP-C <53µm to SOC <53µm (i) under different tillage regimes (FA: fallow; DR: deep rotary tillage; DT: deep tillage; NT: no-tillage). SOC: soil organic C; GRSP-C: C contents in glomalin-related soil proteins. Data represents the means with standard errors (n=3). Different lowercase letters indicate significant differences under different tillage treatments (Duncan's test, $p < 0.05$).

Tillage also changed GRSP content and chemical composition. Polysaccharides was significantly higher in NT treatment compared to DT treatment ($p < 0.05$, Figure 4-9b). GRSP content in bulk soil was 0.43, 0.72, and 0.54 mg g⁻¹ significantly higher in the NT treatment as compared to the DR, DT, and FA treatments, respectively ($p < 0.05$, Figure 4-1d). In aggregates, the GRSP content in >53µm particle size was higher under NT treatment than that under DR and DT treatments ($p < 0.05$, Figure 4-1e). However, GRSP in <53µm particle size was higher under DR and DT treatments than that under NT treatment ($p < 0.05$, Figure 4-1f). Compared with the FA treatment, GRSP was significantly lower in the DT treatment and higher in the NT treatment ($p < 0.05$, Figure 4-1e). In addition, there was no significant influence of tillage regimes on the concentration of C in GRSP, so calculating the average resulted in a GRSP-C content of 31% (Figure 4-8). The ratio of GRSP-C in SOC under NT treatment was significantly higher than that under DR and DT treatments in bulk soil and >53µm particle size ($p < 0.05$, Fig. g, h), there was no significant difference in <53µm particle between NT and other treatments (DR, DT, FA) (Fig. 1i).

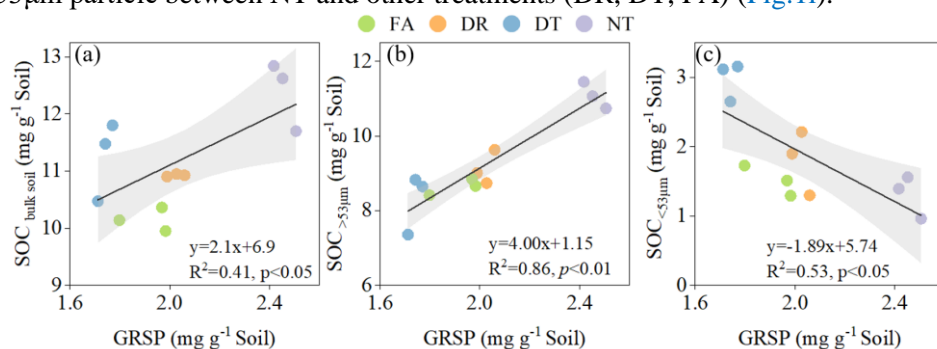


Figure 4-2 The relationship between soil organic carbon (SOC) in bulk soil (a), >53µm (b), <53µm (c) and glomalin related soil proteins (GRSP) under tillage regimes (FA: fallow; DR: deep rotary tillage; DT: deep tillage; NT: no-tillage).

Regression analysis showed that SOC content in bulk soil increased linearly with an increase in GRSP content ($p < 0.05$, Figure 4-2a). Specifically, GRSP had a positive relationship with SOC in >53µm particle size fraction ($p < 0.01$, Figure 4-2b), but a negative correlation with SOC in <53µm particle size fraction ($p < 0.05$, Figure 4-2c).

3.3. AMF community composition, diversity, keystone taxa abundances and its relationship with GRSP

NMDS analysis showed that DT, DR, and NT treatments significantly altered the AMF community structure compared to the FA treatment ($p < 0.05$, Figure 4-3a). However, the changes in AMF communities among DR, DT, and NT treatments were not significant (Figure 4-3a). There was no significant difference in the biomass of AMF (PLFA 16:1ω5) among different tillage treatments (Figure 4-3b), but AMF diversity, especially chao1 index, was significantly higher in NT than in DR and DT

treatments ($p<0.05$, Figure 4-3d). The Venn diagram showed the number of AMF species at the order level under different tillage regimes.

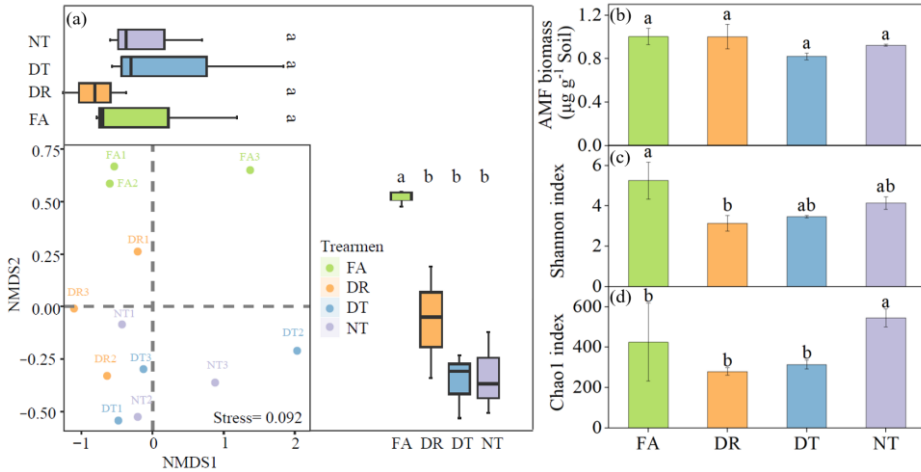


Figure 4-3 Ordination plots for non-metric multidimensional scaling (NMDS) based on operational taxonomic units (OTUs), box plots represent the differences between different tillage regimes (a). The biomass (b), Shannon index (c) and Chao 1 index (d) of arbuscular mycorrhizal fungi (AMF) under tillage regimes. Different lowercase letters indicate significant differences under tillage regimes (FA: fallow; DR: deep rotary tillage; DT: deep tillage; NT: no-tillage). Data represents the means with standard errors (n=3). Different lowercase letters indicate significant differences under different tillage treatments (Duncan's test, $p<0.05$).

This study selected the top 10 species under order level based on their relative abundance. The chord diagram showed that *Glomerales* and *Others* dominate in the AMF community, followed by *Diversisporales* and *Spizellomyces* species (Figure 4-4a). We also observed differences in relative abundance under different tillage regimes. Specifically, compared to DR and DT treatments, the relative abundance of *Glomerales* and *Diversisporales* increased under NT treatment by 81-134%, 226-380%, respectively., and found that *Glomerales* exhibited the highest and statistically significant importance on GRSP, followed by *Collembola*, *Diversisporales*, *Mortierellales* (Figure 4-4b). Among them, the abundances of *Glomerales* and *Diversisporales* showed significant positive correlations with GRSP ($p<0.05$, Figure 4-4c-d, 10).

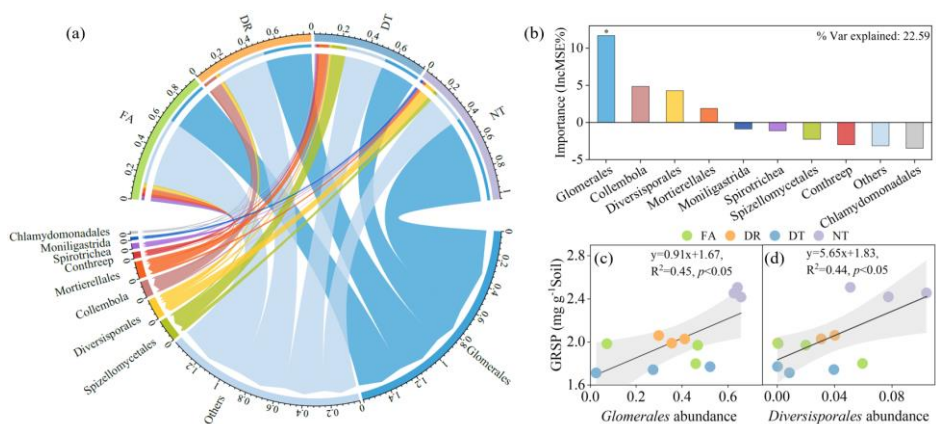


Figure 4-4 Chord diagram illustrated the distribution of arbuscular mycorrhizal fungi (AMF) order level of each tillage regime (DR, DT, NT, FA) (a). The bar chart displayed the relative importance of different taxa at the order level to glomalin related soil proteins (GRSP) (b). The correlation between GRSP and the abundances of keystone taxa (*Glomerales* (c), *Diversisporales* (d)), respectively. FA: fallow; DR: deep rotary tillage; DT: deep tillage; NT: no-tillage.

3.4. Aggregate fractions, stability and their relationship with GRSP

When comparing the different treatments, NT and FA treatments resulted in a significant increase in the mass percentage of $>2000\mu\text{m}$ particle size fraction relative to DR and DT treatments, and a noticeable decrease in the mass proportion of $<53\mu\text{m}$ particle size fraction under the NT treatment ($p < 0.05$, Figure 4-5a). Compared to DR treatment, NT and FA treatments exhibited higher MWD values, whereas MWD value was significantly lower under DT treatment ($p < 0.05$, Figure 4-5c). GRSP was significantly positively correlated with MWD value and mass percentage of $>53\mu\text{m}$ aggregates ($p < 0.01$, Figure 4-5b, d).

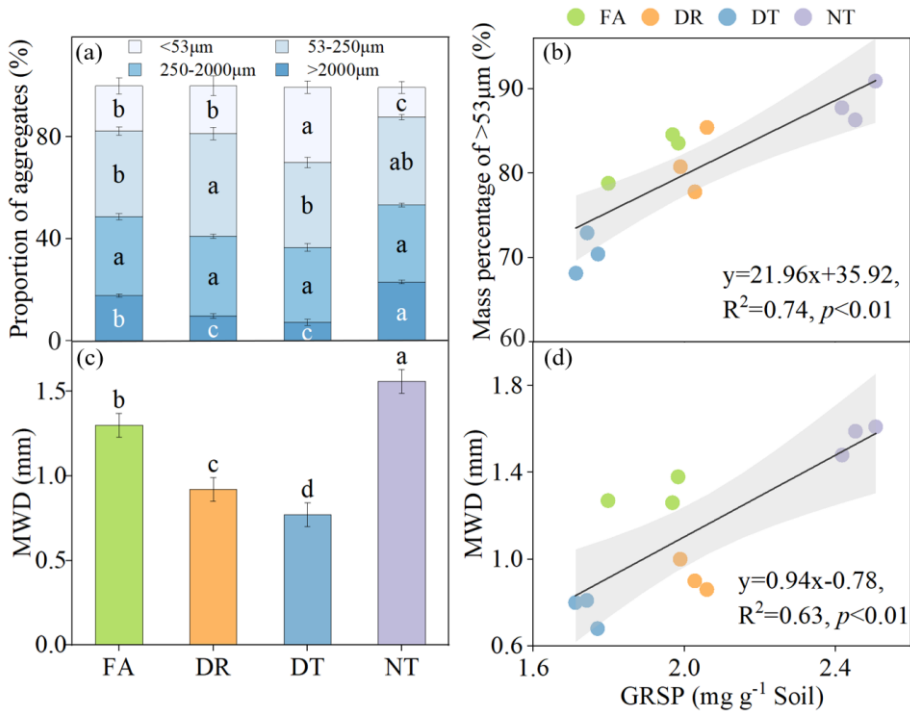


Figure 4-5 The bar chart showed the mass percentage of aggregates (a) and the mean weight diameter (MWD) values (b) under tillage regimes (FA: fallow; DR: deep rotary tillage; DT: deep tillage; NT: no-tillage). The correlation between glomalin related soil proteins (GRSP) and the mean weight diameter (MWD) (a), mass percentage of >53µm particle size fraction. Data represents the means with standard errors (n=3). Different lowercase letters indicate significant differences under different tillage treatments (Duncan's test, $p < 0.05$).

4. Discussion

4.1. Effects of tillage regimes on GRSP and SOC in bulk soil and aggregates.

We found that long-term (7-year) no-tillage significantly increased SOC and GRSP contents compared to FA treatment (7-year) and conventional tillage (DR, DT) regimes (Figure 4-1a, d), which is in line with our first hypothesis (Figure 4-6). Similarly, no-tillage also enhanced SOC and GRSP within in >53µm aggregates compared to FA treatment and conventional tillage (Figure 4-1b, e), indicating that changes in SOC and GRSP contents were primarily driven by alterations in large aggregates. These results are consistent with previous research findings (Singh et al., 2016; Wright et al., 2007; Zhang et al., 2012). Soil aggregates are considered one of the primary mechanisms governing the sequestration of SOC (Six et al., 2004). Reducing soil disturbance helps large aggregates formation and physically enclose free carbon, such as polysaccharides (Figure 4-9), inducing an increase in labile carbon by minimizing contact with decomposing microbes, and as such facilitates

SOC accumulation (Chenu et al., 2019; Six et al., 2000). However, conventional tillage practices, like DT or DR treatments, resulted in higher SOC concentrations in <math><53\mu\text{m}</math> particle size fractions (Figure 4-1c, f). It might be that tillage disrupted large aggregate, leading to an increase in the proportion of small aggregates, and a small part of the exposed free C was adsorbed onto the surface of mineral particles by cation bridging (Franzluebbers et al., 1996), resulting in a longer residence time for SOC (Rabbi et al., 2014). GRSP and SOC contents under the FA treatment were lower than under the NT treatment but higher than under the DT treatment in large aggregates (>math>53\mu\text{m}</math>) (Figure 4-1b, e), while no significant difference was observed in small aggregates (<math><53\mu\text{m}</math>) (Figure 4-1c, f). This discrepancy might be attributed to external nutrient (fertilization) and organic matter (straw returning) input under NT treatment in field soils, increasing the activity of roots and AMF, thereby facilitating GRSP and SOC accumulation in large aggregates (Yang et al., 2024). However, DT greatly damaged the fungal hyphae. This damage offsets any positive effects of the nutrients on AMF, thus inhibiting GRSP production. Notably, the positive correlation between GRSP and SOC in bulk soil and >math>53\mu\text{m}</math> particle size suggested that they have a similar accumulation pattern (Figure 4-2a, b). Because GRSP with aromatic structure can be used as a recalcitrant component in the SOC pool. On the other hand, according to the hierarchical model, both GRSP and SOC were binding agents that firmly hold primary particles together to form microaggregates, which amalgamate to form large aggregates (Oades, 1984; Wright et al., 1998). In turn, large aggregates can provide physical protection to slow down the decomposition of organic matter.

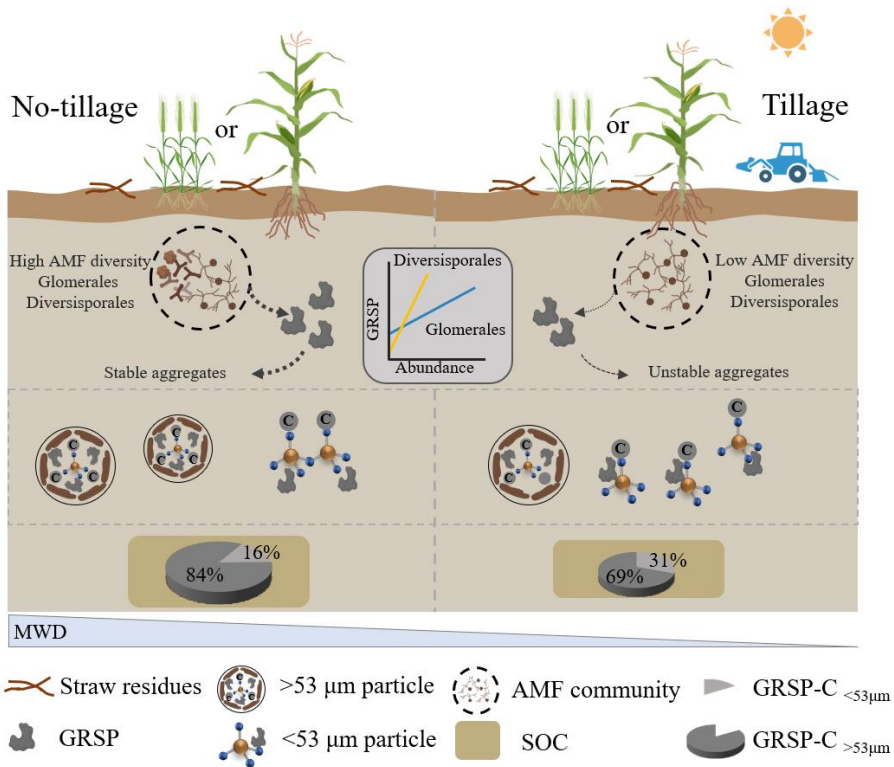


Figure 4-6 Schematic illustration of the impact of tillage on glomalin related soil proteins (GRSP) and soil organic carbon (SOC) in aggregates through the regulation of arbuscular mycorrhizal fungi (AMF) community and diversity.

Our study found that no-tillage increased GRSP-C/SOC in bulk soil which is mainly reflected in >53µm particle size, implying a faster accumulation rate of GRSP relative to SOC in >53µm particle size (Figure 4-1g, h). The possible reason is that no-tillage preserves the structure and integrity of the soil, which supports the hyphal networks and activity of AMF (Kabir, 2005). This preservation likely enhances the production and accumulation of GRSP. However, SOC might be more influenced by diverse microbial communities (composed by microorganisms as saprophytic fungi and bacteria), whose growth rates may not be affected by tillage practices (Van Groenigen et al., 2010). This was also supported by our results of microbial total phospholipid fatty acid (PLFA) biomass under different tillage regimes (Figure 4-12). In <53µm particle sizes, the contribution of GRSP-C to SOC remained unaffected by different tillage treatments (Figure 4-1i), indicating stability in the organo-mineral complexes formed by organic matter and clay, which are less susceptible to microbial attack (Nyamadzawo et al., 2009).

Previous studies have highlighted the positive linear correlation between GRSP and SOC, which in turn suggested similar relationships with various soil physicochemical properties (Singh et al., 2016). Our study found that no-tillage reduced soil pH by 0.36 to 0.74 units (Table 4-1), which was negatively correlated

with SOC and GRSP content (Figure 4-11). These results may be due to low precipitation (708 mm) and high evaporation (1735 mm) at the study site, salts migrate upwards with the water, causing carbonates to accumulate on the soil surface, causing an alkaline soil environment. Typically, the AMF sporulation is inhibited in alkaline soil (Isobe et al., 2007). No-tillage benefits the secretion of organic acids by roots and other microorganisms (Dakora and Phillips, 2002), improving the adaptation (pH ranging from 4.5 to 7.5) of AMF to the soil environment by lowering pH (Bücking and Kafle, 2015), thereby favoring the production of GRSP. Tillage practices had no significant effect on nutrient levels (TP, AP) (Table 4-1), likely due to consistent fertilization amounts. Thus, pH, TN, AP, and related factors may directly or indirectly affect residue and root inputs, as well as the activity of AMF, ultimately influencing GRSP and SOC accumulation.

4.2. Effects of tillage regimes on community and diversity of AMF, Soil Aggregates and their relationships with GRSP and SOC.

Our results showed no significant differences in AMF biomass among various tillage systems (Figure 4-3b), which contrasted with the findings of Agnihotri et al. (2022), who reported that no-till strongly increased AMF biomass. This observation might be attributed to the crop rotation or the application of organic matter that help maintain the biomass of AMF, possibly making it less susceptible to significant impacts from tillage practices (Thomopoulos et al., 2023). Therefore, further research and investigation will be needed to more fully explain the relationship between AMF biomass and GRSP. However, we discovered that tillage altered AMF community composition (Figure 4-3a). Compared to conventional tillage (DT, DR), no-tillage increased AMF diversity, particularly the Chao1 index (Figure 4-3d), which was positively correlated with GRSP (Figure 4-11). This result is consistent with other studies (Brito et al., 2012; de Pontes et al., 2017), which found that reduced or minimal soil disturbance promoted AMF growth and reproduction, fostering a richer community (Kabir, 2005). The reason may be that soil pH strongly affects AMF spore germination and hyphal growth, impacting the diversity and structure of AMF communities (Sun et al., 2016). In fact, the soils are alkaline in this study, and some species could have disappeared due to the alkaline environment (Xu et al., 2016). Therefore, reducing pH through no-tillage can effectively maintain or increase AMF diversity and GRSP content. We further screened the taxa at order levels, revealing that no-tillage increased the abundance of *Glomerales* and *Diversisporales* species in AMF (Figure 4-4a). This phenomenon could be explained by the fact that *Glomerales* might thrive in a relatively undisturbed soil environment, appearing more apt to form a closer symbiotic relationship with plant roots (Banerjee et al., 2019). Additionally, *Glomerales* and *Diversisporales* were more important orders involved in GRSP accumulation (Figure 4-4b), showing a positive correlation with GRSP (Figure 4-4c, d). This indicated that the greater presence of *Glomerales* and *Diversisporales* under no-tillage regimes might explain the higher soil content in GRSP, which confirms our second hypothesis (Figure 4-6). Some studies have also supported this view. For

example, Magurno et al. (2019) identified specific gene sequences in AMF species, such as those in the genus *Glomus*, that are responsible for GRSP production. Furthermore, Wang et al. (2023) demonstrated that GRSP is positively correlated with *Glomeraceae* family, highlighting their crucial role in GRSP accumulation. Overall, the positive impact of no-tillage on GRSP accumulation primarily stems from the diversity and keystone species abundance rather than biomass of AMF (Figure 4-6).

The stability of soil aggregates influences its capacity to withstand external environmental changes and maintain a stable state (Oades, 1984). In this study, tillage regimes significantly affect the stability of soil aggregates (MWD) (Figure 4-5c, 6). No-tillage showed an increase in the percentage of large aggregates (>250 μ m) and a decrease in the percentage of small aggregates (<53 μ m) (Figure 4-5a), suggesting no-tillage induced the transfer of small aggregates to large aggregates. This shift can be attributed to reduced mechanical disturbance, increased available organic substrates, and improved microenvironment under no-tillage, favoring the formation of fungal hyphal networks (Strickland and Rousk, 2010). These networks intertwined soil particles, facilitating the formation and stability of macroaggregates (Peng et al., 2013). Furthermore, SOC and GRSP act as binding agents in stabilizing soil aggregates (Bronick and Lal, 2005; Six et al., 2004). Therefore, the increase in AMF and GRSP in undisturbed soil may be the result of soil aggregate stability mediated by traditional agricultural practices. However, contrary to prior research, our study did not observe a significant positive relationship between aggregate stability (MWD) and SOC (Figure 4-11). This being said, some other studies support our findings (Spohn and Giani, 2010; Zhang et al., 2012). Correspondingly, a strong positive correlation was found between GRSP and aggregate stability (MWD) and the mass percentage of large aggregates (Figure 4-5b, d), indicating that GRSP could be a robust predictor of aggregate stability, while the influence of exogenous organic matter input often weakens the relationship between SOC and aggregates.

4.3. Future directions and implications of agricultural management

Our work emphasizes the role of no-tillage on AMF communities and diversity, exploring the changes in GRS and SOC at the aggregate scale, providing new insights into SOC sequestration mechanisms. The study offers a more nuanced perspective on soil C management practices. In the future, we need to highlight the synergistic impact of tillage regimes with other agricultural management practices to improve our understanding of SOC sequestration and agricultural sustainability. Some studies have suggested that tillage regimes may not significantly alter microbial abundance, diversity, and SOC levels (Yin et al., 2017), with other factors such as cover crop rotations potentially weakening tillage effects (Somenahally et al., 2018). In addition, Angers et al. (1997) indicated that in the surface soil (0-10 cm depth), C and N contents were higher under no-tillage compared to moldboard plowing, whereas the reverse was true at deeper levels (>20 cm depth). Because the AMF biomass and community composition in the subsoil (20-40 cm) differ significantly from those in

the topsoil (0-20 cm) (Luo et al., 2021; Oehl et al., 2005) due to limitations in oxygen and/or nutrients (Zhang et al., 2018). Therefore, we need to explore the differences in SOC sequestration at different depths.

In addition, given the mechanisms by which AMF regulates SOC accumulation may be different in various study sites. Future research should build on these findings by exploring large-scale and long-term effects of different tillage practices on AMF diversity and SOC sequestration across various soil types and climatic conditions. It is crucial to investigate the specific roles of different AMF taxa in GRSP production and SOC stabilization, as well as the potential interactions between AMF and other soil microorganisms under varying tillage regimes. By addressing these areas, we can develop more effective strategies for optimizing SOC storage and improving soil health in agricultural landscapes.

5. Conclusion

Our study revealed that AMF-mediated soil C sequestration mechanisms under different tillage regimes. No-tillage significantly enhances the accumulation of SOC and GRSP, as well as the contribution of GRSP to SOC, especially in $>53 \mu\text{m}$ particle size. This effect is primarily attributed to the influence of *glomerales* and *diversisporales* of AMF and aggregate stability. Specifically, (i) no-tillage increased AMF diversity and keystone species abundance of *glomerales* and *diversisporales*, which can regulate the production of GRSP(C). (ii) No-tillage also increases the physical protection of SOC through GRSP-regulated large aggregates formation and soil stability. Overall, no-tillage promotes GRSP accumulation along with large aggregates formation by enhancing AMF diversity, thus showing potential for enhancing SOC sequestration and soil stability, warranting further investigation into its long-term implications for soil health and agricultural ecosystem sustainability.

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7. Supplementary Figures and Tables

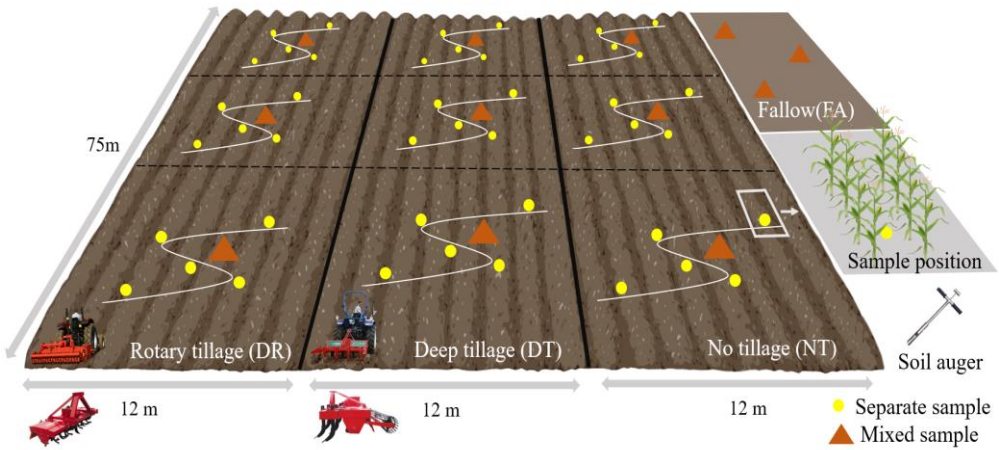


Figure 4-7 The visual diagram presents plot area, various tillage treatments, and sampling procedures and the type of farm machineries used in each tillage practice (FA: fallow; DR: rotary tillage; DT: deep tillage; NT: no-tillage).

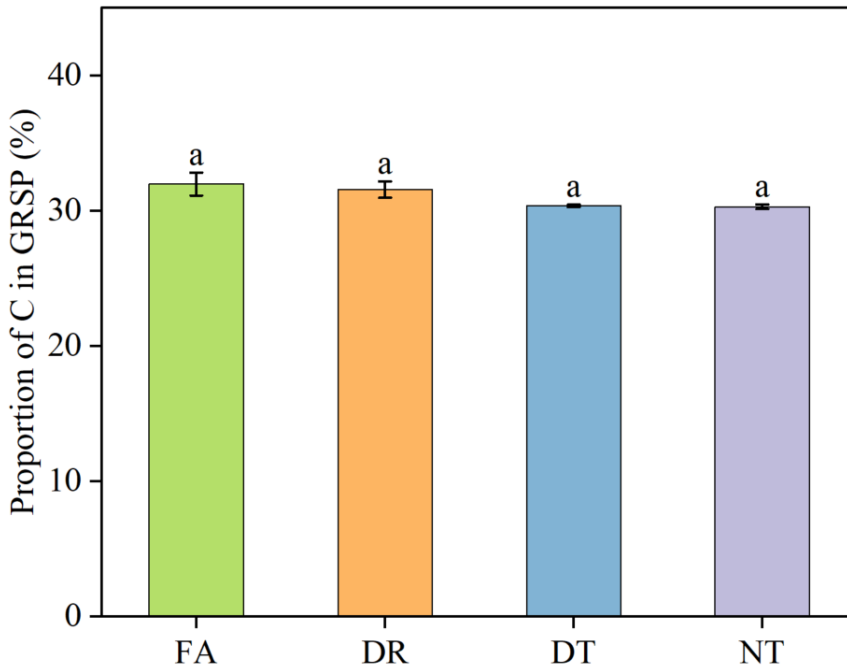


Figure 4-8 The proportion of carbon in GRSP under tillage practices (FA: fallow; DR: rotary tillage; DT: deep tillage; NT: no-tillage). Different lowercase letters indicate significant differences under different tillage treatments (Duncan's test, $p < 0.05$).

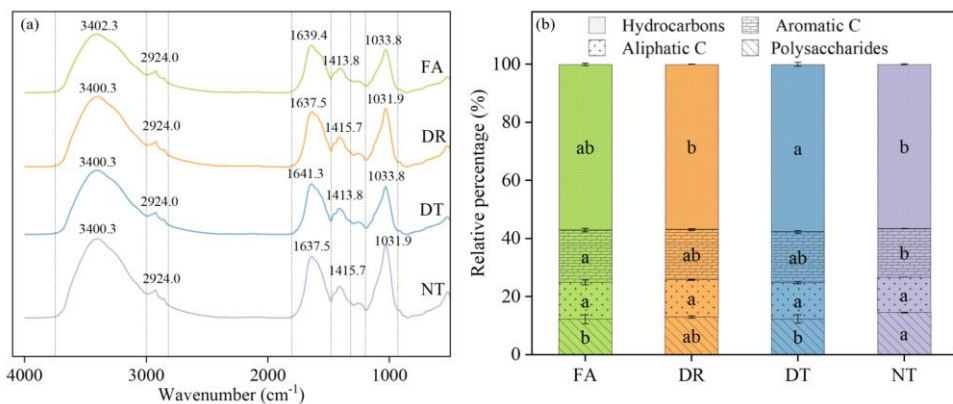


Figure 4-9 Fourier transform infrared (FTIR) spectra (a) and relative percentage of compositional traits of glomalin-related soil protein (GRSP) (b) under different tillage regimes (FA: fallow; DR: deep rotary tillage; DT: deep tillage; NT: no-tillage). Roman numerals from I to V represent hydrocarbons, aliphatic C1, aromatic C, aliphatic C2 and polysaccharides of GRSP components, respectively. Different lowercase letters indicate significant differences under different tillage treatments (Duncan's test, $p < 0.05$).

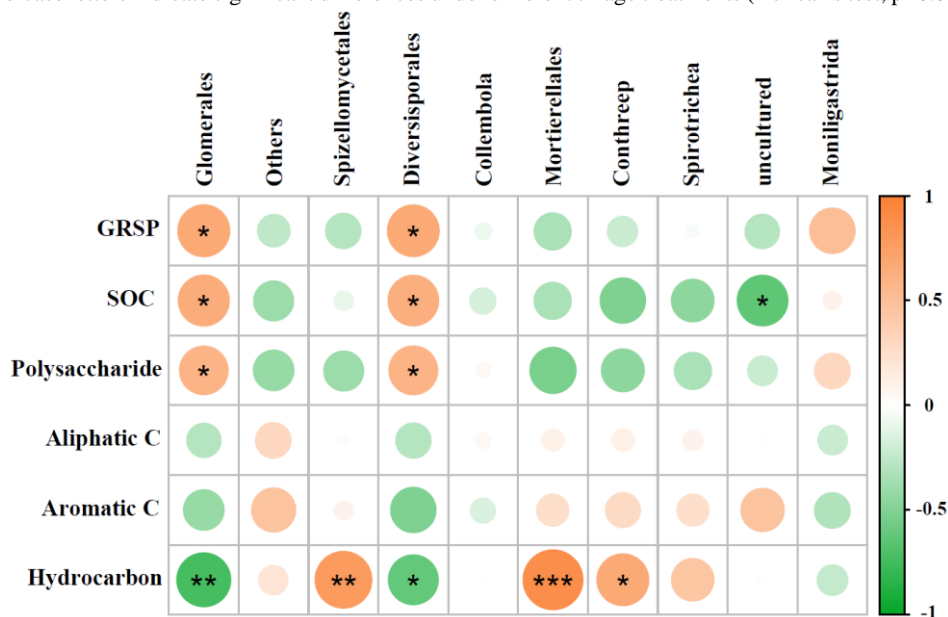


Figure 4-10 The relationships between top 10 dominant orders of AMF and glomalin-related soil protein (GRSP) and compositional traits.

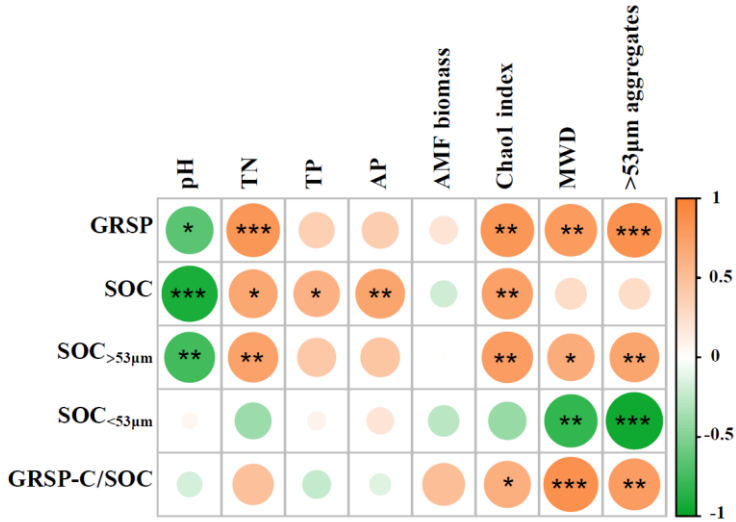


Figure 4-11 The correlation between soil properties, microbes and GRSP, SOC, and the contribution of GRSP-C to SOC.

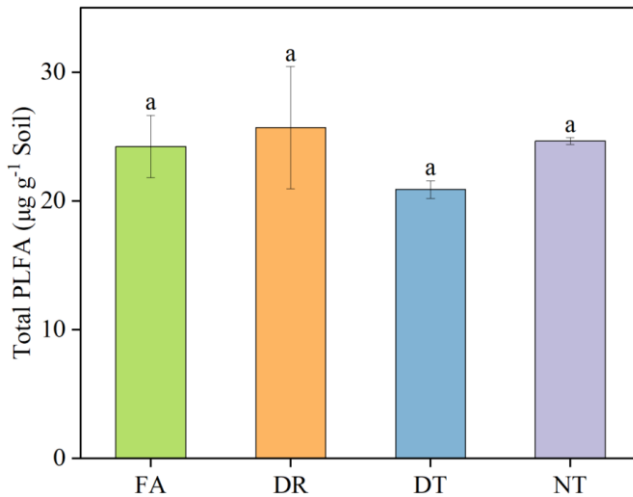


Figure 4-12 The total phospholipid fatty acid (PLFA) contents under tillage practices (FA: fallow; DR: rotary tillage; DT: deep tillage; NT: no-tillage)

Chapter 5

Long-term manuring enhanced compositional stability of glomalin-related soil proteins through arbuscular mycorrhizal fungi regulation

From: Yang, H., Cai, Z., De Clerck, C., Meersmans, J., Colinet, G., Zhang, W., 2024. Long-term manuring enhanced compositional stability of glomalin-related soil proteins through arbuscular mycorrhizal fungi regulation. *Agriculture*, 14(9), 1510.

Success is the sum of small efforts - repeated day in and day out.

—Robert Collier

成功是由日复一日的小努力汇聚而成的

—罗伯特·科利尔



Abstract

Glomalin-related proteins (GRSP) derived from arbuscular mycorrhizal fungi (AMF) is pivotal in enhancing soil structure and quality. Although fertilization generally impacts GRSP levels, its specific compositional characteristic responses to long-term various fertilization practices remain unclear. This study investigated carbon (C) and nitrogen (N) contents and three-dimensional fluorescence characteristics in GRSP based on a 29-year fertilization including no fertilizer (CK), chemical fertilizer (NPK), manure (M) and manure combined with chemical fertilizer (NPKM) treatments. Our findings showed that GRSP contained 37-49% C and 6-9% N, respectively. Compared with CK and NPK, the C and N proportions in GRSP significantly increased under M and NPKM, with a concurrent decrease in the C/N ratio. Using the parallel factor (PARAFAC) model, four fluorescent components of GRSP were identified: one fulvic acid-like component (C2), one tyrosine-like component (C4), and two humic acid-like components (C1, C3). Under M and NPKM treatments, the fluorescent intensity and relative percentage of C4 component decreased, while those of the C1 component increased relative to CK and NPK. Moreover, the GRSP humification index (HIX) under M and NPKM treatments was higher than CK and NPK, indicating that organic fertilization could enhance the stability of GRSP. The content and proportion of GRSP elements (C, N) positively associated with soil organic C (SOC), total N (TN), available phosphorus (AP), AMF biomass and diversity, while the C2 and C4 components showed negative associations. Structural equation modeling further revealed that fertilizer-induced changes in edaphic properties (pH, SOC, TN, AP) increased AMF biomass and diversity, thereby altering GRSP composition. This study has promising implications for understanding the C sequestration mechanisms of GRSP in agroecosystems.

Keywords: Long-term fertilization; Glomalin-related soil proteins; Arbuscular mycorrhizal fungi; Three-dimensional fluorescence

1. Introduction

Glomalin-related proteins (GRSP) is a heat-stable proteins with an aromatic structure, primarily derived from the metabolic activities of microorganisms, notably arbuscular mycorrhizal fungi (AMF) (Gillespie et al., 2011; Wright and Upadhyaya, 1996). GRSP serves as a crucial nutrient source for plants and microbes, containing 20-43% carbon (C) (Agnihotri et al., 2022; J. Zhang et al., 2017), 3-5% nitrogen (N) (Lovelock et al., 2004). In addition, GRSP has important ecological functions in improving soil stability, promoting soil C sequestration, enhancing microbial activity and passivating heavy metals due to its recalcitrance, hydrophobicity, cohesiveness and iron-binding capacity (Singh et al., 2022). These functions largely depend on compositional traits of GRSP, such as functional group, fluorescence components and/or C, N element ratio (Guo et al., 2022, 2023). Studies have shown that molecular weight and element contents in GRSP vary with vegetation types and soil properties (Holátko et al., 2021; Lin et al., 2018). For instance, the C concentration of GRSP from saline soils is reported to be about 2.5 times higher than that from marine sediments of the old Yellow River Delta (Wang et al., 2018a; J. Zhang et al., 2017). In coastal wetland soils, the C/N ratio of GRSP increases with soil depth (Guo et al., 2022). Compared to forest soils, cropland soils exhibit higher concentrations of low molecular weight and aromatic GRSP (Sui et al., 2017). In cropland, fertilization can alter soil properties, thereby affecting GRSP contents (Yang et al., 2024). However, the impacts of different fertilization regimes on the elemental characteristics of GRSP remain unclear.

Research on the chemical composition of GRSP remains limited, with significant differences observed in various soil types. Wang et al. (2014) used three-dimensional fluorescence spectroscopy (EEM) combined with parallel factor analysis (PARAFAC) to identify various organic substances in GRSP samples from forest soil. These substances included compounds similar to tyrosine protein, tryptophan protein, fulvic acid, humic acid, soluble microbial byproduct, nitrobenzoxadiazole, and calcofluor white. However, Guo et al. (2023) identified four components from coastal wetland soils, including one tyrosine-like protein and three humic acid-like components. These findings indicate that GRSP is not a typical glycosylated protein and that there are differences in the fluorescent components of GRSP. Although some progress has been made in forest and/or grassland ecosystems (Agnihotri et al., 2022; Zhong et al., 2017), the fluorescence characteristics of GRSP in cropland, particularly under different fertilization types, remain unclear. Since the 1980s, mineral fertilizer has become mainstream due to its low price and convenience (Wang and Lu, 1998), but long-term mineral fertilization has led to issues such as soil acidification (Tao et al., 2019) and decreased fertility (Zhang et al., 2009), which may largely affect the compositional traits of GRSP (Zhong et al., 2017). Therefore, elucidating the differences in compositional traits of GRSP under various long-term fertilization regimes are crucial for comprehensively understanding its ecological functions and providing scientific guidance for agricultural management.

AMF represent a significant pathway for C transfer from plants to soil (Hawkins et al., 2023). AMF utilize specific membrane transporters in root cortex cells to obtain N, P nutrients, in exchange for plant-derived C (Zhang et al., 2022). However, it remains unclear how fertilization affects AMF communities and diversity, and subsequently, the fluorescence components and C, N element changes in GRSP. Some studies have shown that organic fertilization increases AMF biomass and diversity by regulating pH and nutrients, thereby altering the functional group structures of GRSP and increasing the proportion of aromatic C (Yang et al., 2024). Zhong et al. (2017) have found that soil moisture and electrical conductivity (EC) have a negative influence on tyrosine-like component of GRSP. Therefore, exploring the relationship between GRSP chemical characteristics and edaphic and microbial properties can clarify the key regulatory factors influencing GRSP accumulation in cropland soils under different fertilization regimes.

The acidic nature of the soil can have a profound impact on the growth and activity of arbuscular mycorrhizal fungi, the main producers of GRSP. These fungi have different growth preferences and tolerances to soil pH. Studying GRSP in acidic red soil helps to understand how the fungi adapt to this particular pH environment and how the production of GRSP is affected. In this study, we aimed to unravel the response of GRSP fluorescence components and C, N element changes to fertilization regimes based on a 29-year fertilization experiment. Our hypotheses were that (i) long-term manuring could increase the content and percentage of C and N in GRSP, as well as shift the fluorescence components, and (ii) fertilization-induced changes in soil nutrients and pH would regulate AMF diversity, thereby altering the fluorescence components and C, N concentrations in GRSP.

2. Materials and methods

2.1. Study Site

A long-term field experiment was initiated in 1990 on cropland soil located in Qiyang County, Hunan Province, China (26°45'N, 111°52'E). The site experiences a subtropical climate characterized by a mean annual temperature of 18.1 °C and mean annual precipitation of 1431 mm. The soil originates from quaternary red clay and is classified as Ferralic Cambisol according to the FAO classification. The initial soil pH was 5.70, with soil organic carbon (SOC) at 8.58 g kg⁻¹, total nitrogen (TN) at 1.07 g kg⁻¹, and total phosphorus (TP) at 0.45 g kg⁻¹. The predominant cropping system consists of winter wheat-summer maize rotations (Zhang et al., 2009). The experimental design followed a completely randomized design with two repetitions, each having two sub-replicates. Because one sample is not enough, we had to select three replicates for measurement. The treatments comprised: no fertilizer (CK); mineral fertilizer (NPK, mineral N, P, K fertilizer); manure (M, pig manure, C: N ≈ 21); NPKM (NPK plus pig manure). The fertilizer application can be seen in reference of Yang et al. (2024). We collected post-harvest soil samples in September of 2019 at a depth of 0–20 cm. One portion of the soil was air-dried according to previous protocols, the other portion of fresh soil was stored at -80 °C.

2.2. Soil sampling analysis

The samples were combusted in the elemental analyzer at 950°C to determine SOC and TN concentrations. Calibration was performed using known standard samples (e.g., alanine) to ensure precision and accuracy in the measurements. Soil available phosphorus (AP) was analyzed via the molybdenum-antimony resistance colorimetric method. The procedure involved weighing air-dried soil samples and adding 0.5 mol/L NaHCO₃ solution as the extractant at a 1:10 soil-to-extractant ratio, with the solution pH adjusted to 8.5. Extraction was conducted at 25°C for 30 minutes, followed by filtration with Whatman filter paper to obtain a clear filtrate. The filtrate was then reacted with ammonium molybdate and ascorbic acid to form a blue phosphomolybdate complex, and absorbance was measured at 885 nm to calculate available phosphorus concentration. Soil pH was measured at a soil-to-water ratio of 1:2.5 (FE30, Mettler Toledo). AMF biomass was quantified using C16:1ω5 phospholipid fatty acids (PLFAs). AMF diversity was assessed using high-throughput sequencing. The Magigene platform (<http://cloud.magigene.com>) was used to calculate AMF α -diversity (Shannon-Wiener index).

2.3. GRSP determination

GRSP were measured using the methods described by Wright et al. (1998). Briefly, to extract the total GRSP, 8 mL of 50 mmol L⁻¹ sodium citrate (pH=8.0) was used for every 1 g of air-dried soil. The extracts were centrifuged at 10,000 ×g for 6 minutes after an autoclaving process lasting 1 hour at 121°C. After completing this process four times for each sample, the supernatants were combined and centrifuged one more time to get them ready for measurement. The optical density (OD) values of GRSP were determined at 595 nm with an enzyme microplate reader, using bovine serum albumin (BSA) as the standard.

The supernatant was precipitated by adding a higher concentration of hydrochloric acid, chilling in an ice bath for one hour, and centrifuging at 10,000 ×g for six minutes. The sediment was dialyzed in dH₂O for 60 hours (dialysis bag, molecular weight cut off = 10,000 Da, USA) after being dissolved in 0.1 M sodium hydroxide (NaOH). After dialysis, in order to remove any insoluble residue, the dialysate was centrifuged at 10,000 ×g for six minutes. The supernatant was then freeze-dried using a freeze-dryer to obtain solid GRSP.

2.4. Structural characterizations of GRSP

Elemental analyzer (EA3000, Milan, Italy) was used to analyze atomic ratios of GRSP including C and N elements. In brief, solid GRSP samples of 1-3 mg were weighed using millionth of a gram precision balance, and wrapped in tin foil before determining their C, N content. GRSP-C (-N) was calculated as follows:

$$GRSP - C(N) = C(N) \times GRSP \times 100\% \quad (1)$$

Where $GRSP - C(N)$ stands for the C content of GRSP (mg g⁻¹Soil). $C(N)$ represents the percentage of C in GRSP. $GRSP$ is glomalin-related soil proteins content (mg g⁻¹Soil).

Fluorescence spectra were recorded employing Hitachi F-7000 fluorescence spectrometer with a 700 V xenon lamp (Wang et al., 2014). Specifically, the 1.00 mg solid GRSP samples were placed in centrifuge tubes, then 1ml sodium hydroxide (NaOH) solution (0.1M) and 4 ml dH₂O were added, and the precipitate was completely dissolved by shaking. Excitation-emission matrices (EEMs) were obtained across an emission wavelength range from 250 to 550 nm and an excitation wavelength range from 200 to 500 nm. The excitation-emission slit was maintained at a fixed 2-mm, 5-mm step, respectively, with a scan speed of 2400 nm min⁻¹.

The EEMs data of the samples were analyzed using parallel factor (PARAFAC) model with the dreem6.4 and N-Way toolbox. The “smootheem” function was used to subtract the effects of Raman and Rayleigh scattering to obtain the actual fluorescence intensity. The fluorescence data were compared with the data published in the online spectral library (the OpenFluor website, <http://www.openfluor.org>). The excitation and emission wavelengths of similar fluorescence peaks identified by the OpenFluor database had a significance level greater than 0.95. PARAFAC is a mathematical modeling method based on trilinear decomposition theory, implemented using alternating least squares (Gao et al., 2017). It decomposes the EEMs dataset into a set of trilinear terms and a residual array. The calculation formula of the PARAFAC model is shown in equation (2):

$$X_{ijk} = \sum_{n=1}^N a_{in} b_{jn} c_{kn} + \varepsilon_{ijk} \quad (2)$$

Where X_{ijk} is the fluorescence intensity of the i -th sample at the excitation wavelength k and emission wavelength j ; a_{in} is the proportional concentration of the n -th component in the i -th sample; b_{jn} and c_{kn} are model parameters linearly correlated with the fluorescence quantum efficiency of the n -th component at the emission wavelength j and excitation wavelength k , respectively; N is the number of columns in the loading matrix, representing the number of components needed to correctly fit the model; and ε_{ijk} is the residual matrix.

Based on the EEMs data, we also obtained some fluorescence indices: biological index (BIX), and humification index (HIX) (Equations (3) and (4)). The humification index (HIX) is the ratio of the integrated fluorescence intensity values at an excitation wavelength (λ_{Ex}) of 254 nm for emission wavelengths (λ_{Em}) in the range of 435-480 nm to those in the range of 300-345 nm (Equation (3)) (Zsolnay et al., 1999). The biological index (BIX) is the ratio of the fluorescence intensity at an excitation wavelength (λ_{Ex}) of 310 nm for emission wavelengths (λ_{Em}) at 380 nm and 430 nm (Equation (4)) (Huguet et al., 2009). Here,

I represents the fluorescence intensity value at specific excitation/emission wavelengths.

$$HIX = \frac{\sum_{435nm}^{480nm} I_{\lambda Em}}{\sum_{300nm}^{345nm} I_{\lambda Em}}, \lambda Em=254nm \quad (3)$$

$$BIX = \frac{I_{\lambda Em=380nm}}{I_{\lambda Em=430nm}}, \lambda Em=310nm \quad (4)$$

2.5. Statistical analysis

The data were examined for homogeneity and normality using Kolmogorov-Smirnov test. One-way ANOVA was used to test for differences in C, N content and structural compositions of GRSP among fertilization practices (Duncan's test). The relationship between characterizations of GRSP and edaphic and microbial factors (pH, SOC, TN, AP, AMF) were evaluated using Pearson's correlation coefficient. Spectra graphs were completed using Matlab2020b software, heatmap and structural equation modeling were performed in R 4.3.0.

3. Results

3.1. Differences of C, N content and proportion in GRSP under fertilization regimes

There were significant differences in the C, N proportion in GRSP under various fertilization regimes ($p < 0.05$, Figure 5-1). The percentage of C in GRSP was 31% higher under the M and NPKM treatments than that under the NPK treatment (Figure 5-1a). Similarly, the percentage of N in GRSP was 60% higher under M and NPKM treatments relative to CK and NPK treatments (Figure 5-1b). However, the C/N ratio in GRSP was lower under the M and NPKM treatments than in the CK and NPK treatments (Figure 5-1c).

The contents of C and N in GRSP also varied significantly under fertilization treatments ($p < 0.05$, Figure 5-1d,e). The GRSP-N content was substantially higher under the M and NPKM treatments (0.29-0.35 mg g⁻¹) compared to the CK and NPK treatments (0.12-0.16 mg g⁻¹), with GRSP-N being higher under M than under NPKM. In addition, GRSP-C significantly increased with fertilization (NPK, M, NPKM) treatments compared to CK treatment. GRSP-C content under M and NPKM was also higher than under NPK.

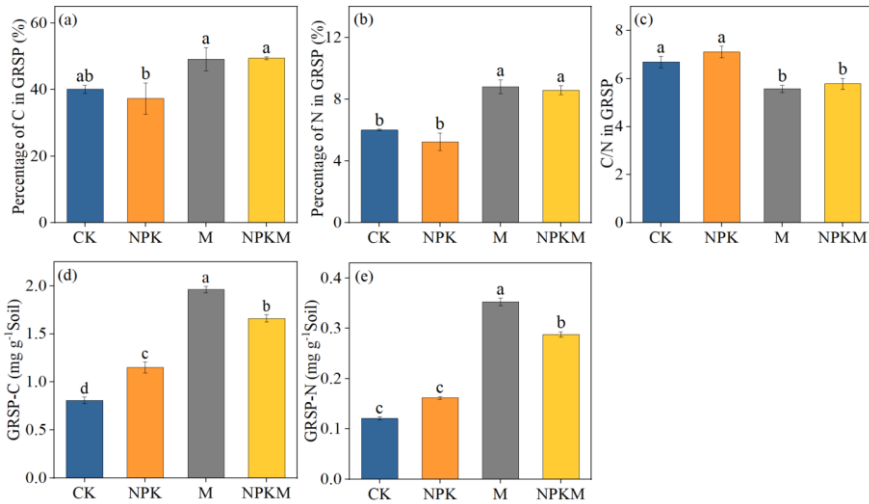


Figure 5-1 The percentage of C (a), N (b), the ratio of C to N (c), and the content C (d), C (e) in GRSP under long-term fertilization regimes. CK, no fertilizer; NPK, mineral N, P, K fertilizer; M, pig manure; NPKM, NPK + pig manure. The data (n = 3) shows the means with standard errors. Significant differences across fertilization treatments are shown by different letters (Duncan's test, $p < 0.05$).

3.2. Fluorescence characteristics analysis of GRSP

The fluorescence characteristics of GRSP were preliminarily identified using excitation-emission matrix fluorescence spectroscopy (EEMs). Three distinct peaks were observed across the fertilization regimes (Figure 5-3). Based on the parallel factor (PARAFAC) model, four fluorescent components of GRSP were identified, including one fulvic acid-like component, one tyrosine-like component, and two humic acid-like components (Table 5-1, Figure 5-2). These components showed significant differences across different fertilization regimes (Figure 5-3, 4). The fluorescence intensity of the fulvic acid-like (C2) and tyrosine-like (C4) components significantly reduced by 50% and 55%, respectively, under M and NPKM treatments compared to CK and NPK, while the intensity of humic acid-like component (C1) increased by 17%. There was no significant difference in the humic acid-like component (C3) among the fertilization regimes (Figure 5-4).

Characteristics of glomalin-related soil proteins accumulation and its response to fertilization and tillage practices in typical cropland soil

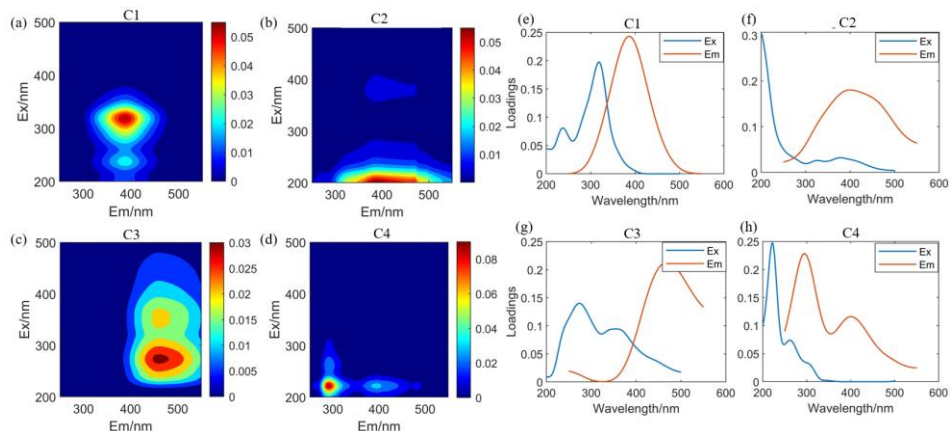


Figure 5-2 Four fluorescent components identified using PARAFAC analysis under fertilization regimes. The spectral shapes of emission and excitation are shown in contour plots (a-d). The split-half validation findings for the appropriate component are presented in line plots on the right side of each contour plot (e-h).

Table 5-1 Characteristics of the four components of glomalin-related soil proteins identified by PARAFAC of excitation-emission matrices.

Component	Excitation max (nm)	Emission max (nm)	Likely structure
C1	320	390	UVA humic acid-like
C2	200	385	Fulvic acid-like
C3	274	460	UVA humic acid-like
C4	222	290	Protein-like substance (tyrosine)

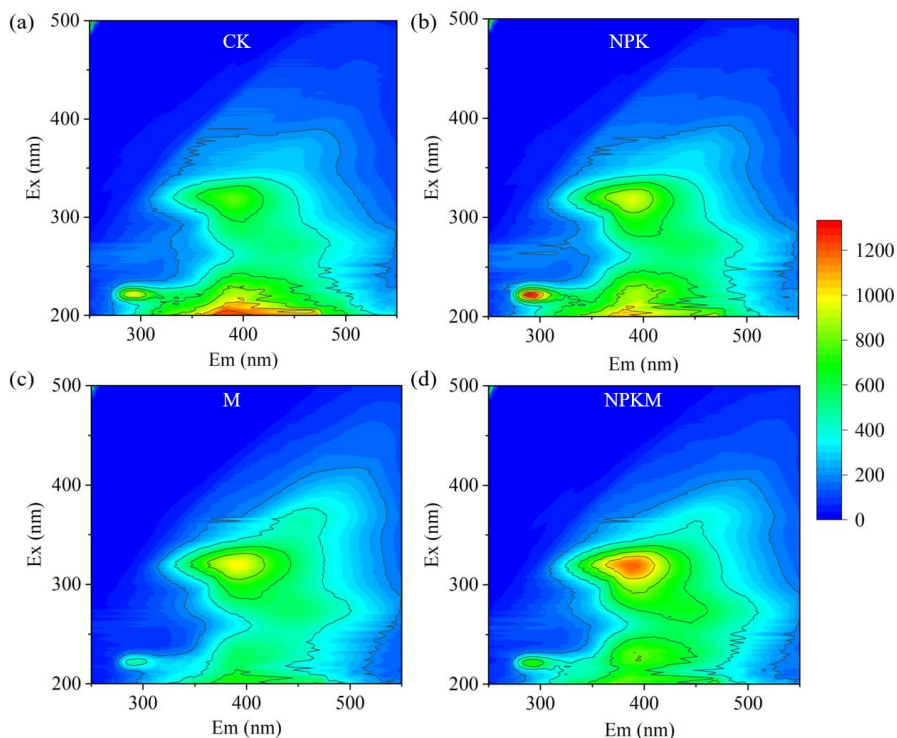


Figure 5-3 Fluorescent excitation-emission matrix fluorescence spectroscopy (EEMs) spectra under different fertilization regimes. CK, no fertilizer; NPK, mineral N, P, K fertilizer; M, pig manure; NPKM, NPK + pig manure.

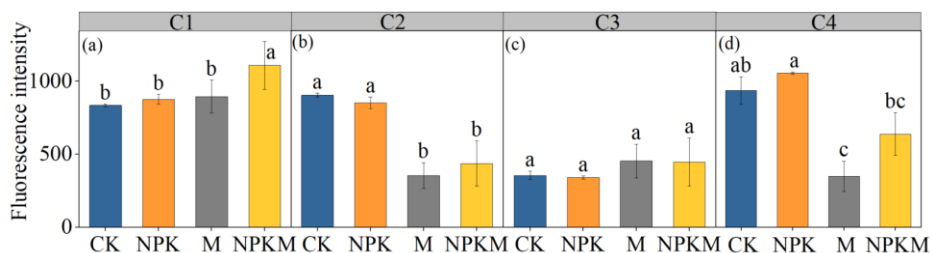


Figure 5-4 Comparison of fluorescence intensity of four components in glomalin-related soil proteins (GRSP) under different fertilization regimes. CK, no fertilizer; NPK, mineral N, P, K fertilizer; M, pig manure; NPKM, NPK + pig manure. The data ($n = 3$) shows the means with standard errors. Significant differences across fertilization treatments are shown by different letters (Duncan's test, $p < 0.05$).

The relative percentage of fluorescence components had strongly difference among the fertilization regimes ($p < 0.05$, Figure 5-5a). The relative percentage of the humic acid-like components (C1, C3) were higher under M and NPKM than under CK and NPK treatments, while tyrosine-like component (C4) was significantly reduced. The relative percentage of the fulvic acid-like component (C2) did not change significantly

under different fertilization conditions. Moreover, the humification index (HIX) ranged from 0.72 to 0.81 (Figure 5-5c). Compared with CK and NPK, M and NPKM significantly increased GRSP humification index (HIX) by 10 %, but there was no effect on the biological index (BIX) between fertilization treatments (Figure 5-5b).

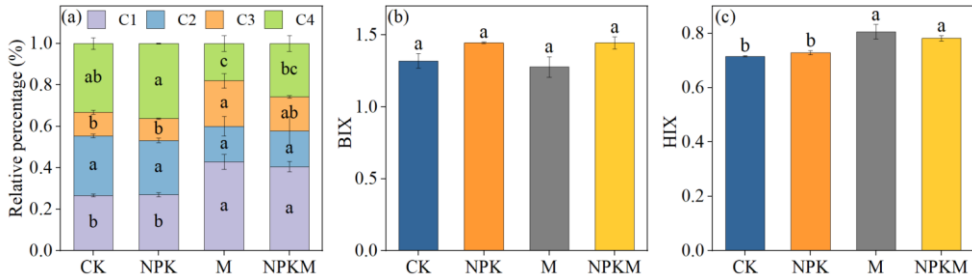


Figure 5-5 Comparison of relative percentage of four components in glomalin-related soil proteins (GRSP) across different fertilization regimes (a). Differences of the humification index (HIX) and the biological index (BIX) across fertilization regimes (b). CK, no fertilizer; NPK, mineral N, P, K fertilizer; M, pig manure; NPKM, NPK + pig manure. The data (n = 3) shows the means with standard errors. Significant differences across fertilization treatments are shown by different letters (Duncan's test, $p < 0.05$).

3.3. The relationship between structural characterizations of GRSP and the properties of edaphic and AMF

The heatmap results indicated that soil pH, soil organic carbon (SOC), total nitrogen (TN), available phosphorus (AP), AMF biomass, and diversity (Shannon and Chao1 indices) were positively correlated with the content of GRSP-N and GRSP-C, as well as their N proportion. Additionally, the proportion of GRSP-C also showed a positive correlation with these properties, except for AMF biomass. Furthermore, the C/N ratio in GRSP was significantly negatively correlated with AMF biomass, pH, SOC, TN, AP. The C2 and C4 components of GRSP showed significant negative correlations with pH, SOC, TN, AP, AMF biomass, and diversity (Shannon and Chao1 indices). The C3 component showed a significant positive correlation only with pH, TN, and AP. The humification index (HIX) index had significant positive correlations with pH, SOC, TN, AP, AMF biomass, and diversity (Shannon and Chao1 indices), while biological index (BIX) had no correlation (Figure 5-6).

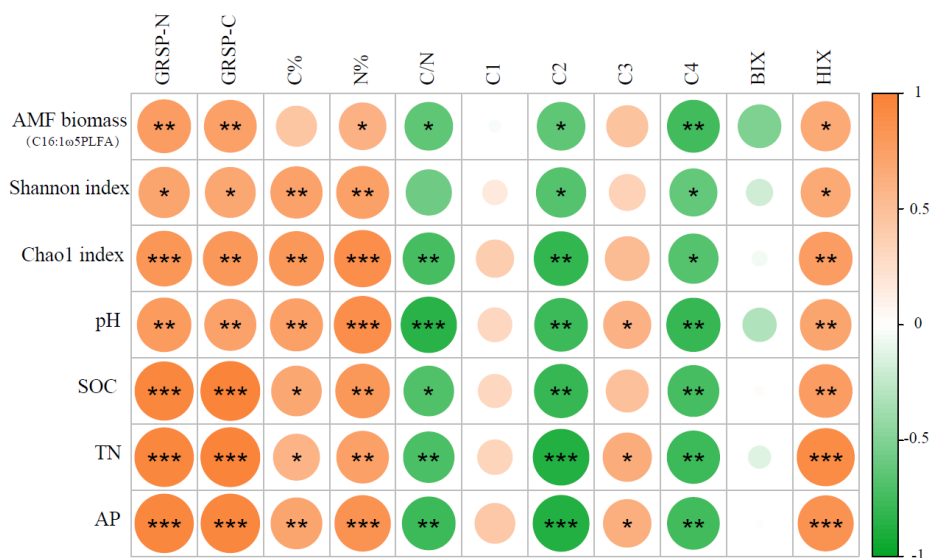


Figure 5-6 Relationships between compositional traits of glomalin-related soil proteins (GRSP) and soil properties. GRSP-C(N), the content of C (N) in GRSP; C% and N%, the percentage of C and N in GRSP; HIX, humification index; BIX, biological index. C1-C4, four fluorescence components in GRSP; SOC, soil organic carbon; TN, total nitrogen; AP, available phosphorus.

Partial least squares path modelling exhibited that fertilization significantly influenced edaphic properties (i.e. pH, SOC, TN, AP) in a positive direction (pc (path coefficients) = 0.89, $p < 0.001$). Subsequently, these edaphic properties had a significant positive effect on AMF biomass (pc = 0.68, $p < 0.05$) and diversity (pc=0.80, $p < 0.01$). In turn, AMF diversity showed a positive correlation with GRSP elements (C, N) (pc=0.66, $p < 0.01$), whereas AMF biomass did not directly affect GRSP elements contents (Figure 5-7a). However, both AMF biomass and diversity were negatively correlated with the intensity of fulvic acid-like (C2) and tyrosine-like (C4) components of GRSP (Figure 5-7b).

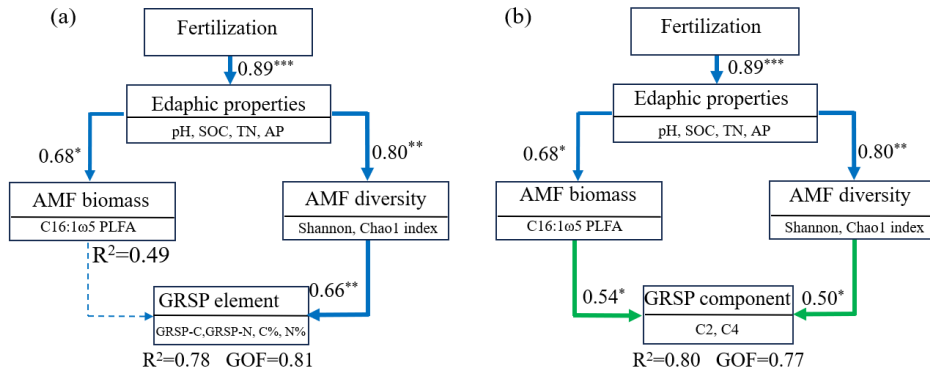


Figure 5-7 Partial least squares path modelling showing impacts of edaphic and biotic factors on element composition (a) and fluorescence components (b) in glomalin-related soil protein (GRSP). Positive and negative effects are shown by blue and green arrows, respectively, and significant and insignificant correlations are displayed by dashed and continuous arrows. The strength of relationship is positively associated with the thickness of arrow. GRSP-C (N), the content of C (N) in GRSP; C% and N%, the percentage of C and N in GRSP; SOC, soil organic carbon; TN, total nitrogen; AP, available phosphorus. GOF, Goodness of fit.

4. Discussion

Previous studies have reported the impacts of fertilization on the content of GRSP in soil (Cissé et al., 2021; Guo et al., 2019; Wu et al., 2011), but there has been less research on the changes in GRSP components, particularly in fertilized agricultural soils. This study clarified the differences in the elemental contents and fluorescent components of GRSP under long-term different fertilization regimes (Figure 5-8). The results revealed that the application of organic fertilizers shifted the elemental contents and fluorescent components of GRSP by increasing the biomass and diversity of AMF.

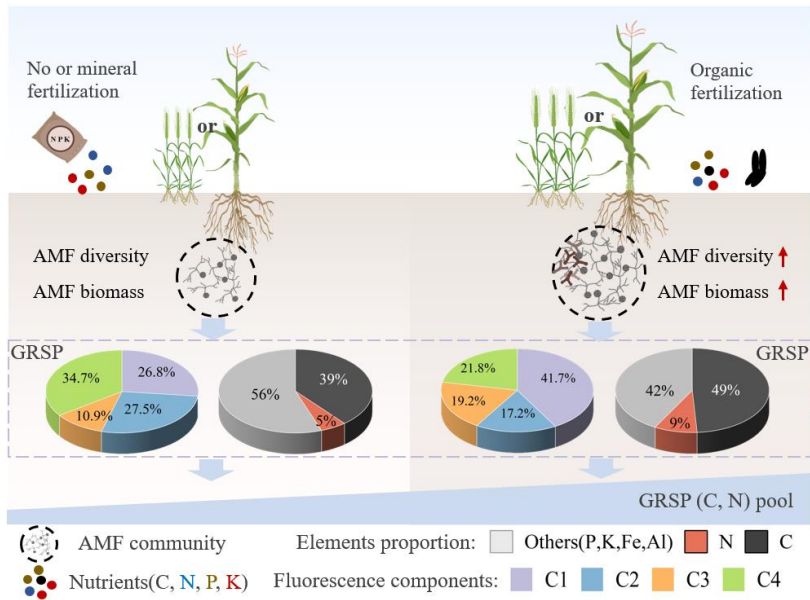


Figure 5-8 Conceptual diagram illustrating variations of fluorescence components and elements in glomalin-related soil protein (GRSP) under 29-year fertilization regimes. The up arrow “↑” indicates a positive response.

4.1. Effects of fertilization regimes on C, N contents in GRSP.

Our previous research found that long-term organic fertilization can increase GRSP levels (Yang et al., 2024). In this study, fertilization also strongly altered GRSP-C and GRSP-N contents, with significant differences between fertilization regimes (Figure 5-1). Compared with no fertilization, chemical and organic fertilization increased GRSP-C by 42% and 123%, and GRSP-N by 33% and 166%, respectively (Figure 5-1d, e). These findings indicated that fertilization, especially organic fertilization, can enhance the stability of soil C and N pools. The substantial increase in GRSP-C and GRSP-N could be attributed to enhanced soil nutrients under fertilization (Yang et al., 2024), which allowed AMF to help plants absorb more nutrients in exchange for plant-derived C (Williams et al., 2017). This mutualistic relationship promoted AMF growth and metabolism, thereby increasing GRSP production. However, chemical fertilization tended to lower soil pH, reducing AMF colonization and diversity (Liu et al., 2021), thereby affecting the chemical composition of GRSP (Zhong et al., 2017). Notably, the percentage of C and N in GRSP significantly increased with the application of organic fertilizer, while chemical fertilization had no significant effect (Figure 5-1a, b), supporting the first hypothesis. Organic fertilization can improve soil organic matter and microbial activity, creating a more conducive environment for AMF colonization and activity (Yang et al., 2024). The results in AMF allocating more resources to produce GRSP with higher C, N proportion under nutrient-rich conditions. Interestingly, the C/N ratio (5.6-5.8) decreased under organic fertilization

relative to no or chemical fertilization (6.7-7.1), indicating a more balanced integration of these elements in the GRSP structure. This shift could reflect enhanced N cycling and availability in soils receiving organic inputs, fostering more efficient assimilation into microbial biomass. Additionally, increased microbial activity, often stimulated by organic fertilization, may preferentially decompose carbon-rich components (Six et al., 2006), leading to a decrease in the C/N ratio.

Environmental factors like soil characteristics, plant types and climatic conditions affect GRSP content and composition (Schindler et al., 2007; Wang et al., 2018b). We found that GRSP-C, GRSP-N and the proportions of C, N in GRSP were significantly positively correlated with soil factors (SOC, TN, AP, pH) and AMF diversity (Fig.6). Organic fertilization increased TN, AP through the decomposition of organic matter, facilitating better AMF symbiosis with host plants (Koorem et al., 2014). This improved nutrient exchange, leading to increased GRSP production. Moreover, organic fertilization typically maintained or increased soil pH at this experimental site. Soil pH is a critical factor affecting T-GRSP composition (Wang et al., 2014). Thus, improving nutrients (C, N, P) availability and maintaining a favorable pH range can support AMF growth and their ability to produce C, N-rich GRSP. Structural equation modeling in our study further revealed that improved soil conditions after fertilization directly influenced AMF characteristics, which in turn enhanced the contents and proportions of C and N in GRSP (Figure 5-7a).

4.2. Effects of fertilization regimes on fluorescence components in GRSP.

This study characterized the fluorescent components of GRSP using three-dimensional fluorescence spectroscopy, complementing our previous research on the chemical composition of GRSP. We identified four fluorescent components of GRSP (Figure 5-2), differing from previous studies that reported up to seven components (Wang et al., 2014). This discrepancy may be due to the lower biomass and diversity of AMF in cropland soils compared to forest and grassland soils (Masebo et al., 2023; Wang et al., 2022). In cropland soils, we found that the application of organic fertilizers significantly reduced the fluorescence intensity of the fulvic acid-like component (C2) and the tyrosine-like component (C4), while increasing the fluorescence intensity of the humic acid-like components (C1, C3) (Figure 5-4). Additionally, there was a decrease in the relative percentage of the tyrosine-like component (C4) and an increase in the proportion of the humic acid-like component (C1) (Figure 5-5a). These may be attributed to the strong binding of GRSP with soil organic matter fractions, such as humic acids, which are co-extracted during acid precipitation and alkaline dissolution, thus interfering with GRSP fluorescence intensity (Agnihotri et al., 2022). This binding is likely regulated by functional groups such as hydroxyl, carboxyl, amide and carbonyl in GRSP, which provide binding sites (Yuan et al., 2024; Z. Zhang et al., 2017). The correlations between GRSP components and pH and SOC in our study supported this explanation (Figure 5-6). Humic and fulvic acids are complex organic molecules derived from the decomposition of plant and microbial materials, with significant structural differences.

Humic acids are larger, with higher molecular weights and aromatic structures enriched with phenolic and carboxylic groups, contributing to their strong chelating capacity for nutrient retention and soil stabilization. In contrast, fulvic acids are smaller, more aliphatic, and have a higher proportion of oxygen-containing functional groups, making them more soluble and mobile in the soil environment. This structural distinction underpins their differing roles in soil processes, with fulvic acids facilitating nutrient transport and availability, while humic acids are more involved in long-term carbon stabilization. Furthermore, the application of organic fertilization increased the humification index (HIX) of GRSP (Figure 5-5c), which was positively correlated with AMF and soil properties (Figure 5-6). This indicated that organic fertilization enhanced GRSP stability by increasing AMF biomass and diversity. This finding complemented our previous research on the mechanisms by which organic fertilization promotes GRSP accumulation.

Both biotic and abiotic factors appear to influence the fluorescent components of GRSP (Wang et al., 2015). We found that the fluorescence components of fulvic acid-like (C2) and tyrosine-like (C4) were significantly negatively correlated with pH, SOC, TN, AP, and AMF biomass and diversity (Figure 5-6). These findings are not entirely consistent with Wang et al. (2015), who reported that GRSP components were little influenced by soil P content. However, AMF are highly sensitive to soil P levels (Van Geel et al., 2017). Within a certain range of P, AMF biomass and diversity increase with the increase of P levels, but internal competition may reduce the AMF groups associated with the C2 and C4 components, such as *Paraglomus* and *Claroideoglomus* species. Sequencing data from our study supported this view, showing that the abundance of *Paraglomus* and *Claroideoglomus* species decreased significantly with organic fertilization and positively correlated with C2 and C4 (unpublished data). Structural equation modeling also indicated that fertilization-induced increases in AMF biomass and diversity reduced the fluorescence intensity of the C2 and C4 components (Figure 5-7b).

4.3. Implications and perspective

The observed changes in GRSP composition under different fertilization regimes have significant implications for soil quality and soil C sequestration in agricultural systems. The increased C and N content in GRSP under organic fertilization suggests a potential for greater SOC storage and improved nutrient cycling. The alterations in GRSP fluorescence components and increased humification indicate a shift towards more recalcitrant organic forms, which are likely to persist longer in the soil. This transformation could enhance the stability of SOC, contributing to climate change mitigation efforts. However, the negative associations of certain fluorescent components (C2 and C4) with SOC and nutrients warrant further investigation to understand the underlying mechanisms and their implications for soil quality and the development of sustainable agroecosystems.

Future research should explore the long-term impacts of various fertilization strategies on GRSP compositional traits across different soil types and climatic zones.

Understanding the interactions between climate, fertilization practices, microbial communities, and GRSP formation will be crucial for developing site-specific fertilization practices that enhance soil fertility and C sequestration. Additionally, advancing techniques can be applied to precisely characterize GRSP composition, providing deeper insights into its role in soil ecosystems. Moreover, GRSP contains 0.8-9.9% Fe and 0.3-0.7% Ca, respectively (Agnihotri et al., 2022; Rillig et al., 2001; Wright et al., 1998). GRSP-bound Fe and Ca, forming a GRSP-Fe (Ca)-OC ternary complex, may play an important role in SOC accumulation and stabilization. Therefore, studying above contents will help us to comprehensively understand the carbon sequestration mechanisms of GRSP in agroecosystems.

5. Conclusion

We elucidated the compositional traits of GRSP under long-term fertilization regimes. Long-term manuring significantly increased the contents and proportions of C and N, as well as the percentage and fluorescence intensity of humic acid-like components (C1) in GRSP, while reducing tyrosine-like components (C4). The C, N contents and proportion of GRSP positively correlated with SOC, TN, AP and the diversity and/or biomass of AMF, but fulvic acid-like (C2) and tyrosine-like components (C4) was negatively correlated with these factors. On the whole, manuring enhanced soil nutrients (SOC, TN, AP) and pH, promoting the diversity and/or biomass of AMF, thereby altering the compositional characteristics of GRSP.

6. Acknowledgements

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Chapter 6

**General discussion, conclusions, and
perspective**

“Keep your face to the sunshine and you cannot see a shadow”.

– Helen Keller

心向阳光，无畏阴影

— 海伦·凯勒



1. General discussion

1.1. Spatial distribution characteristics and influencing factors of GRSP under different fertilization regimes.

Our study found that the GRSP content ($0.3\text{--}5.1\text{ mg g}^{-1}$) in cropland is lower than in forest ($3.6\text{--}7.0\text{ mg g}^{-1}$) and grassland ($0.6\text{--}10.1\text{ mg g}^{-1}$) ecosystems (Table 2-S3). This disparity could be attributed to human interference through fertilization and tillage in cropland, which inhibits AMF hyphal growth, consequently reducing AMF biomass and diversity, thereby hindering GRSP production (Fokom et al., 2012). Furthermore, the increased soil nutrients and porosity induced by fertilization and tillage promote microbial activity, accelerating GRSP decomposition. Spatially, GRSP levels varied considerably under different fertilization, fluctuating between 1.0 and 5.1 mg g^{-1} (Figure 2-1). GRSP tended to decrease first and then increase with increasing latitude, following a pattern of mid-temperate > subtropical > warm-temperate zones. Possible reasons for this trend include: (i) the gradual increase in temperature accelerates GRSP decomposition. Meanwhile, in subtropical zone, acidic or slightly acidic soils with abundant Fe-Al oxides reduce GRSP decomposition (Wang et al., 2014). (ii) In warm-temperate zone, there is low clay content, weakening the mineral protection of organic matter (Helassa et al., 2011). (iii) GRSP is an alkali-soluble protein (Vodnik et al., 2008), and the predominantly alkaline soils in warm-temperate zone lead to protein loss.

GRSP content is also affected by fertilization regimes. In our study, applying organic fertilizers significantly increased GRSP levels in subtropical and warm-temperate zones compared to unfertilized and chemical fertilized soils, but had no significant effect in the mid-temperate zone. This phenomenon could be due to the rapid decomposition of GRSP in warmer zones (warm-temperate and subtropical zones), where organic fertilizer not only mitigates microbial decomposition of GRSP but also promotes the production of GRSP through AMF. However, in the mid-temperate region, high organic matter content leads microorganisms (r-strategists) to prioritize to use easily decomposable organic matter, preserving GRSP. Additionally, the lower temperature limits the decomposition of GRSP by microorganisms, resulting in a slower turnover rate and higher accumulation level of GRSP (Woignier et al., 2014). This makes it possible for the effects of organic fertilizers to be masked in this region. Therefore, applying organic fertilizers in warmer regions can mitigate GRSP loss, while in cooler regions, rational fertilization can be monitored to maintain soil stability.

GRSP levels are regulated by multiple factors, varying across different temperature zones. In the mid-temperate zone, GRSP positively correlated with AMF, PLFA, clay, SOC, and TN, and negatively correlated with MAT and MAP. The reason may be that GRSP is a byproduct of AMF activity, SOC and TN provide the substrate and nutrients necessary for AMF, indirectly supporting GRSP production. Clay minerals provide a hospitable environment for microbial colonization, influencing the diversity and biomass of AMF and their production of GRSP. Clay minerals can also adsorb

and protect GRSP by forming organo-mineral complexes, reducing decomposition rates. In the warm-temperate zone, GRSP positively correlated with MAP, AMF, PLFA, SOC, TN, and AP, and negatively correlated with MAT and pH. In the subtropical zone, GRSP positively correlated with AMF, SOC, TN, AP, and pH, and negatively correlated with MAT and MAP. In addition, VPA analysis revealed that clay is the most critical factor regulating GRSP levels in the mid-temperate zone, while nutrients, particularly AP, are most important in the warm-temperate and subtropical zones. This is because in warmer zones, AMF may alter life history traits (Horsch et al., 2023), affecting GRSP production due to the sensitivity of GRSP to soil nutrients. Generally, soil phosphorus is the main limiting nutrient for microorganisms and plants (Hou et al., 2020). Studies have shown that P fertilization significantly increases GRSP content, but in P-rich soils, it may have no significant impact or even a negative effect (Ciss et al., 2021; Yang et al., 2023). Therefore, nutrients drive GRSP accumulation to some extent through AMF regulation. However, in the mid-temperate zone, high clay content, primarily 2:1 clay minerals such as montmorillonite (Abrar et al., 2020), provides more binding sites due to the small size and surface properties of clay particles (Balabane and Plante, 2004; Wagai and Mayer, 2007), thereby adsorbing GRSP.

1.2. Response of GRSP accumulation and associated AMF community to long-term fertilization regimes.

The subtropical zone, characterized by abundant light, heat, and water, faces significant challenges in agriculture due to severe soil acidification and nutrient imbalance caused by intense weathering and unreasonable fertilization. These factors hinder agricultural productivity and development. Long-term fertilization experiments have been established to observe the impact of different fertilization regimes on soil properties and productivity, providing valuable information for developing sustainable management strategies. Recent studies suggest that applying organic fertilizers and/or diversified cropping can be effective sustainable management practices for improving soil quality. A large amount of exogenous organic matter and nitrogen fixation by legumes improve soil structure and nutrient balance. This process also enhances soil nutrient levels through plant-derived organic matter, benefiting microbial (e.g. AMF) growth. Therefore, organic fertilization and diversified cropping may promote GRSP accumulation and SOC sequestration. Our field trials indicated that nearly 30-year fertilization have increased GRSP levels from 1.9 mg g⁻¹ in 1990 to 3.2–4.7 mg g⁻¹ in 2019. The increase in GRSP was more pronounced with organic fertilizers compared to chemical fertilizers, and the accumulation mainly occurred in >53 μm particle size fractions. Additionally, diversified cropping significantly increased GRSP content (Figure 3-1b), primarily within the 53-250 μm aggregate size fraction. This study mainly explained GRSP accumulation from two perspectives.

Production effects: our study found that organic fertilization and diversified legume cropping significantly increased AMF biomass and/or diversity, which showed a

significant positive correlation with GRSP. This indicated that organic fertilization can directly promote GRSP accumulation by regulating AMF biomass and diversity. First, organic fertilizers improve soil pH and nutrient levels (such as TN, AN, AP) (Table 3-1), alleviating nutrient competition between the host and AMF (Johnson, 2010). Second, manure application can reduce soil bulk density and increase soil porosity (Ozlu et al., 2019), providing adequate oxygen and water for AMF (Wang et al., 1993). This reduces the obstacles to AMF hyphal extension (Ma et al., 2006), promoting mycorrhizal development (Delavaux et al., 2017; Sarapatka et al., 2019).

Stabilization effects: fertilization can alter the functional groups of GRSP, affecting its stability. Aromatic compounds, which are more resistant to decomposition, enhance organic matter stability. Our study found that organic fertilization significantly increased the proportion of aromatic C in GRSP. This is primarily due to the release of mineral forms of nitrogen and phosphorus from the decomposition of manure and straw, which can be absorbed by plants and AMF, increasing AMF biomass. Additionally, organic fertilization may activate dormant AMF species in the soil (Zhu et al., 2016), enhancing AMF diversity. These changes may affect the chemical composition of GRSP. In addition, soil aggregates, as fundamental units of soil, determine soil aeration and water retention capacity, and they can retain organic carbon within the aggregates (Abiven et al., 2009). Different aggregate sizes have different C content and stability, with C in large aggregates (>250 μ m) exhibiting lower decomposition rates (saha et al 2010). Our study found that organic fertilization significantly increased the MWD value and the mass percentage of large aggregate size fractions, which positively correlated with GRSP content in large aggregates. This indicated that the formation of large aggregates protected GRSP from decomposition, thereby facilitating GRSP accumulation. Moreover, large aggregates can provide a stable and suitable environment for microaggregate formation, enhancing the capacity to absorb or encapsulate more unprotected organic matter (e.g., GRSP) (Liu et al., 2020). As a result, GRSP accumulation mainly occurs in large aggregates.

1.3. The mechanism of AMF-mediated SOC sequestration under tillage systems

The primary grain-producing regions of China are the central and eastern regions, which are distinguished by their level topography and fluvo-aquic soils that originate from river alluvium. Nonetheless, the soil in these areas is typically high in pH, light-textured, poorly structured, and low in organic carbon. These adverse factors severely impact soil fertility and agricultural productivity. This study aims to evaluate the effects of different tillage practices on soil structure and organic carbon content, with the goal of identifying feasible measures to optimize soil management and enhance soil fertility. Previous studies have suggested that no-tillage is one way to increase soil SOC. However, the response of AMF to tillage and their subsequent impact on SOC sequestration remains unclear. Our study found that no-tillage significantly increases the content of SOC and GRSP in the topsoil (0-20 cm), and also enhances the contribution of GRSP to SOC. No-tillage helps in forming large aggregates that

encapsulate free carbon and GRSP, thereby providing physical protection. Additionally, no-tillage preserves the structure and integrity of the soil, which supports the hyphal networks and activity of AMF (Kabir, 2005). This preservation likely enhances the production and accumulation of GRSP. However, SOC might be more influenced by diverse microbial communities (composed of microorganisms such as saprophytic fungi and bacteria), whose growth rates are less influenced by tillage practices (Van Groenigen et al., 2010).

Although our study found that tillage did not change the biomass of AMF, it did alter the AMF community composition and diversity. This, in turn, drove changes in the content and functional groups of GRSP to some extent. Compared to conventional tillage (DT, DR), no-tillage increased AMF diversity (i.e. Chao1 index) (Figure 4-3d), which was positively correlated with GRSP (Figure 4-S4). This result is consistent with other studies (Brito et al., 2012; de Pontes et al., 2017), which found that reduced or minimal soil disturbance promoted AMF growth and reproduction, fostering a richer community (Kabir, 2005). The reason may be that soil pH positively affects AMF spore germination and hyphal growth, impacting the diversity and structure of AMF communities (Sun et al., 2016). In fact, the soils are alkaline in this study, and some species could have disappeared due to the alkaline environment (Xu et al., 2016). Therefore, reducing pH through no-tillage can effectively maintain or increase AMF diversity and GRSP content. We further screened the taxa at order levels, revealing that no-tillage increased the abundance of *Glomerales* and *Diversisporales* orders in AMF (Figure 4-3e), which was positively affected the proportion of polysaccharide in GRSP (Figure 4-10). This phenomenon could be explained by the fact that *Glomerales* might thrive in a relatively undisturbed soil environment, appearing more apt to form a closer symbiotic relationship with plant roots (Banerjee et al., 2019).

1.4. Response of GRSP compositional traits and associated AMF community to long-term fertilization regimes

GRSP is a relatively stable organic substance, and understanding its chemical structure can help elucidate the potential mechanisms of GRSP in C sequestration. Previous studies have reported the effects of fertilization on the content of GRSP in soils (Cissé et al., 2021; Guo et al., 2019; Wu et al., 2011), but research on the compositional changes of GRSP, particularly in fertilized agricultural soils, was limited. This study elucidated the differences in elemental content and fluorescent components of GRSP under long-term different fertilization regimes (Figure 5-8). The results show that using a parallel factor analysis model, four fluorescent components of GRSP were identified: a fulvic acid-like component (C2), a tyrosine-like component (C4), and two humic acid-like components (C1 and C3). This result differs from previous reports identifying up to seven components (Wang et al., 2014). This discrepancy may be due to the lower biomass and diversity of arbuscular mycorrhizal fungi (AMF) in agricultural soils compared to forest and grassland soils (Masebo et al., 2023; Wang et al., 2022). In agricultural soils, we found that the application of

organic fertilizer significantly reduced the fluorescence intensity of fulvic acid-like (C2) and tyrosine-like (C4) components, while increasing the fluorescence intensity of humic acid-like components (C1 and C3) (Figure 5-4). Additionally, the relative percentage of the tyrosine-like component (C4) decreased, and the proportion of the humic acid-like component (C1) increased (Figure 5-5a). This may be due to the strong binding of GRSP with soil organic matter components (such as humic acids), which, during acid precipitation and alkaline dissolution processes, leads to the co-extraction of humic acids, thereby interfering with the fluorescence intensity of GRSP (Agnihotri et al., 2022). Moreover, the application of organic fertilizer increased the humification index (HIX) of GRSP (Figure 5-5c), which was positively correlated with AMF and soil properties (Figure 5-6). This suggests that organic fertilizer enhances the stability of GRSP by increasing the biomass and diversity of AMF. This finding complements our previous research on the mechanisms by which organic fertilization promotes the accumulation of GRSP (Yang et al., 2024).

The application of organic fertilizer increased the content of GRSP-C and GRSP-N (Figure 5-1). These results indicate that fertilization, especially organic fertilization, can enhance the stability of soil carbon (C) and nitrogen (N) pools. The significant increase in GRSP-C and GRSP-N can be attributed to fertilization increasing soil nutrients (Yang et al., 2024), which allows AMF to help plants absorb more nutrients in exchange for plant-derived C (Williams et al., 2017). This mutual relationship promotes the growth and metabolism of AMF, thereby increasing the production of GRSP. However, chemical fertilization tends to lower soil pH, reducing AMF colonization and diversity (Liu et al., 2021), thereby affecting the chemical composition of GRSP (Zhong et al., 2017). Notably, with the application of organic fertilizer, the percentage of C and N in GRSP increased significantly, while chemical fertilization had no significant effect (Figure 5-1a, b), supporting the first hypothesis. Organic fertilizer can improve soil organic matter and microbial activity, creating a more favorable environment for AMF colonization and activity (Yang et al., 2024). The results indicate that under nutrient-rich conditions, AMF allocates more resources to producing GRSP with higher C and N ratios. The carbon-to-nitrogen ratio (C/N) of organic fertilization (5.6 to 5.8) is lower than that of no fertilization or chemical fertilization (6.7 to 7.1), indicating a more balanced integration of these elements in the structure of GRSP. This shift may reflect enhanced nitrogen cycling and availability in soils receiving organic inputs, promoting more efficient assimilation of microbial biomass. Furthermore, increased microbial activity, often stimulated by organic fertilization, may preferentially decompose carbon-rich components (Six et al., 2006), leading to a decrease in the C/N ratio.

2. General conclusions

In this study, GRSP content ranged from 1.0 to 5.1 mg g⁻¹, showing a quadratic trend with increasing latitude: middle temperate > subtropical > warm temperate zones. In the warm temperate and subtropical regions, the application of organic

fertilizers significantly increased GRSP content in the soil, whereas this effect was not evident in the middle temperate zone. Additionally, clay content, mean annual temperature (MAT), pH, and soil nutrients were the main factors influencing GRSP accumulation. In the middle temperate zone, clay content was the primary determinant of GRSP levels, while in the subtropical and warm temperate zones, soil available phosphorus (AP) was the main driver. Structural equation modeling further indicated that organic fertilization could indirectly enhance AMF biomass by increasing nutrient availability, thereby mitigating the negative impact of temperature on GRSP accumulation. Future research should further explore the effects of different soil types and crop species on GRSP.

In fertilized agricultural soils, GRSP mainly consists of four functional groups (polysaccharides, aromatic C, aliphatic C, and hydrocarbons) and four fluorescent components (fulvic acid-like, tyrosine-like, and two humic acid-like). The application of organic fertilizers and straw returning increased AMF biomass and diversity by improving nutrient availability and/or alleviating acidification, thereby increasing the proportion of aromatic C and the humification index, making GRSP more resistant to decomposition. Moreover, fertilization also enhanced the physical protection of GRSP through the formation of macroaggregates. In summary, the application of organic fertilizers can promote AMF growth and aggregate stability, improve the quality and quantity of GRSP, and facilitate its accumulation.

No-tillage significantly increased the accumulation of SOC and GRSP, as well as the contribution of GRSP to SOC in the bulk soil and $>53 \mu\text{m}$ particle sizes. This effect was mainly attributed to the influence of AMF *glomerales* and *diversisporales*, as well as aggregate stability. (1) No-tillage increased AMF diversity and the keystone species abundance of *glomerales* and *diversisporales*, thereby regulating GRSP(C) production. (2) No-tillage also enhanced the physical protection of soil organic carbon through the formation of macroaggregates regulated by GRSP and increased soil stability. Overall, no-tillage promoted GRSP accumulation and the formation of macroaggregates by enhancing AMF diversity, demonstrating the potential to enhance organic carbon sequestration and soil stability.

3. Perspectives

3.1. Management Recommendations

Our study highlights practical strategies for enhancing soil stability and sustainability through GRSP management. (1) Encourage organic amendments. Promote the application of organic fertilizers (e.g., manure, compost, straw) to increase GRSP levels by promoting AMF biomass and diversity. These practices can promote carbon stabilization by increasing the carbon input to the soil and fostering the formation of stable organic-mineral complexes. Organic amendments also support microbial activity, which contributes to aggregate formation and improves both the physical and chemical stabilization of carbon. (2) Adopt conservation tillage practices. Practices like no-tillage or minimal tillage can enhance both the chemical and physical stabilization of carbon. By reducing soil disturbance, conservation tillage helps

maintain soil aggregates and prevents the exposure of organic carbon to microbial decomposition. This not only preserves existing carbon stocks but also encourages the accumulation of additional organic matter in the form of stable aggregates and mineral-associated carbon. These practices are cost-effective and reduce labor and energy input while maintaining soil fertility. (3) Adjust soil management according to soil properties. Adapt soil management strategies to account for clay content, phosphorus availability, and soil organic carbon levels, as these factors significantly influence GRSP accumulation. This approach ensures site-specific optimization of soil health measures. (4) Promote diversified cropping systems. Implementing diversified cropping systems (such as crop rotations or intercropping) can increase the production of GRSP, which in turn supports aggregate formation and the stabilization of carbon in the soil. Diverse root systems and organic inputs from a variety of plants also contribute to the overall health of soil ecosystems and enhance carbon storage.

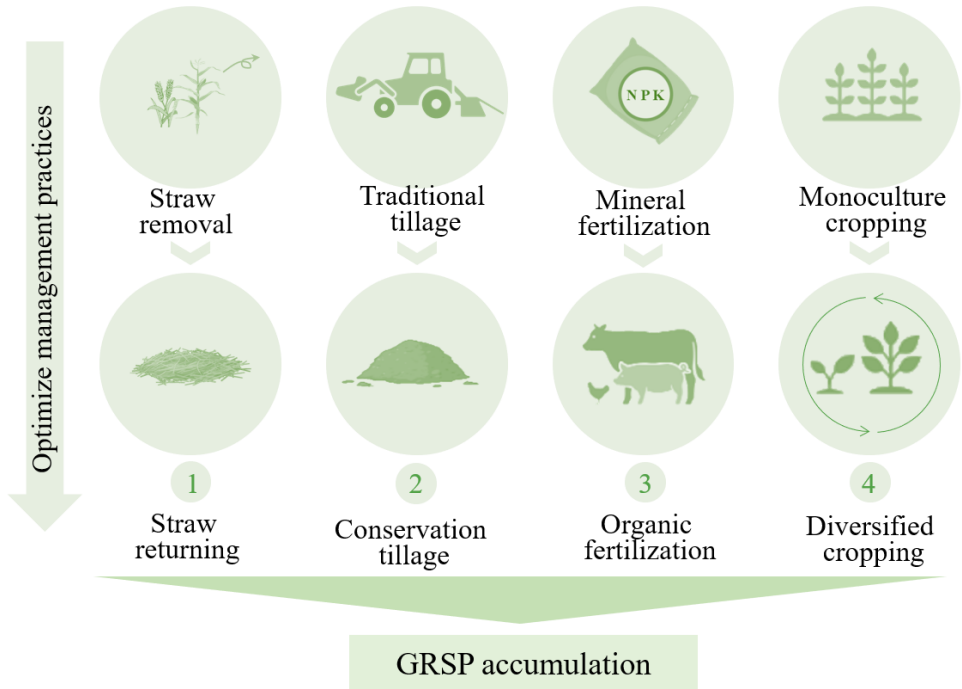


Figure 6-1 GRSP response to shifts in management practices

3.2. Guidance for Policymakers

Policymakers should design subsidies and training programs for organic amendments, no-tillage, and diversified cropping to facilitate adoption by farmers. Policies promoting soil health are key to sustainable agriculture and climate change mitigation. Our study offers several key insights into the mechanisms of GRSP accumulation and its role in soil health. Firstly, the study elucidates the critical role of organic fertilization in enhancing GRSP levels and its chemical stability. By

increasing nutrient availability, organic amendments indirectly promote AMF biomass and diversity, which in turn enhances GRSP accumulation. This finding provides a mechanistic understanding of how organic matter inputs can be strategically used to improve soil health and carbon sequestration. Secondly, our research highlights the importance of considering soil properties such as clay content and available phosphorus in soil management practices. These factors significantly influence GRSP distribution and accumulation, suggesting that soil management strategies should be adapted to the specific properties of each soil type to maximize GRSP levels. Furthermore, the study reveals the potential of no-tillage practices to enhance soil carbon sequestration through increased GRSP production and improved soil aggregate stability. The positive correlations between GRSP and large soil aggregates, as well as the enhanced AMF diversity under no-tillage, provide valuable insights into the benefits of minimal soil disturbance for sustainable agriculture. These scientific insights can be applied to future research aimed at understanding the micro-mechanisms of GRSP accumulation and its interaction with SOC dynamics. By investigating the specific pathways through which GRSP influences soil structure and stability, researchers can develop more effective soil management strategies to promote long-term soil health and productivity.

3.3. Future research

In the future, we should consider the relationships between GRSP, soil health, and crop yields. GRSP, as a nutrient reservoir, may play a crucial role in improving nutrient supply. Future research should focus on how GRSP interacts with microbial communities in various soil types (e.g., clay, sandy, saline-alkali) and its impact on nutrient availability and microbial dynamics to optimize soil management practices for sustainable crop productivity. In addition, understanding how AMF communities in the rhizosphere and bulk soil affect GRSP accumulation is also important, as AMF plays a key role in nutrient uptake and plant stress resilience. Future studies should explore the effects of different AMF populations on GRSP production and their impact on plant growth and yield. Investigating how different crop varieties respond to GRSP will further help identify crops that enhance GRSP production and improve soil health, thereby increasing crop yields and resilience. Moreover, the impact of microbial fertilizers on GRSP dynamics should be explored in greater depth, as these fertilizers have the potential to significantly influence microbial communities and nutrient cycling. Microbial fertilizers, which contain beneficial microbes such as bacteria, fungi, and other microorganisms, can stimulate the growth and activity of soil microorganisms, including AMF and other key microbial communities. These microbes can directly contribute to the production of GRSP, a protein complex produced by AMF that plays a vital role in stabilizing soil aggregates and enhancing soil structure.

Given environmental changes, including extreme weather events such as heatwaves and wildfires, we should investigate whether the accumulation mechanisms of GRSP are affected. Simulation experiments can be established to study the effects of varying temperature and humidity conditions on GRSP levels in the soil during such events.

Long-term monitoring of soil samples following extreme weather can evaluate the recovery capacity and trends of GRSP. Furthermore, integrating climate models to predict GRSP changes under future climate scenarios can provide scientific basis for agricultural management and soil conservation.

3.4. Limitations

In our study, several limitations need to be addressed for a more comprehensive understanding of GRSP and its role in soil health. One of the key challenges in assessing GRSP is its biochemical complexity. GRSP is not a single compound but a heterogeneous mixture of proteins, polysaccharides, and other organic molecules, which makes its quantification and characterization difficult. Different research studies may focus on different fractions of this complex mixture, leading to variations in GRSP measurement methods and the results obtained. This variability in analytical methods complicates the interpretation and comparison of GRSP data across studies. Standardization of GRSP extraction and quantification protocols is therefore needed to improve the consistency and reliability of future research. Another limitation of our study is that we did not consider the impact of tillage practices on GRSP and SOC profiles. Tillage is known to affect microbial communities, soil structure, and organic matter decomposition, which in turn can influence GRSP production and accumulation. Different tillage practices, such as conventional tillage, no-tillage, or minimal tillage, can lead to variations in microbial biomass and activity, thus affecting the dynamics of GRSP and SOC. Future studies should investigate how different tillage practices and soil depths (e.g., topsoil versus subsoil) influence the accumulation of GRSP. A better understanding of the interactions between tillage, microbial communities, and GRSP will provide valuable insights into how soil management practices can be optimized for improved soil health and carbon sequestration.

Furthermore, while this study suggested that diversified cropping could influence microbial communities and GRSP accumulation, the exact regulatory mechanisms remain poorly understood. Diversified cropping systems, such as crop rotations or intercropping, may enhance the diversity and functionality of soil microbial communities, potentially promoting the accumulation of GRSP. However, the specific pathways through which diversified cropping affects microbial communities and GRSP production need to be further explored. Experimental evidence is needed to clarify how different crop combinations and management practices modulate microbial interactions and GRSP dynamics in the soil. This knowledge would help optimize cropping systems to improve soil health and enhance carbon storage.

Finally, the limitations of our study highlight the need for a more systematic, multidisciplinary approach in future research. To fully understand the functions and mechanisms of GRSP, future studies should integrate knowledge from molecular biology, soil ecology, plant physiology, and microbiology. This integrated approach will allow researchers to explore the complex interactions between GRSP, soil microorganisms, and plant roots, and to identify strategies that can enhance GRSP

accumulation and its benefits for soil health and crop yield. By developing a more comprehensive understanding of GRSP, we can create better soil management strategies that promote both environmental sustainability and agricultural productivity.

Chapter 7

Reference and Appendix

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Appendix

1.1 Publications

1. **Yang, H.**, Xiao, Q., Huang, Y., Cai, Z., Li, D., Wu, L., Meersmans, J., Colinet, G., Zhang, W., 2024. Long-term manuring facilitates glomalin-related soil proteins accumulation by chemical composition shifts and macro-aggregation formation. *Soil Tillage Res.* 235, 105904.
2. **Yang, H.**, Wang, G., Wang, J., Xiao, Q., Li, Z., De Clerck, C., Meersmans, J., Colinet, G., Zhang, W., 2024. No-tillage facilitates soil organic carbon sequestration by enhancing arbuscular mycorrhizal fungi-related soil proteins accumulation and aggregation. *CATENA*, 245, 108323.
3. **Yang, H.**, Cai, Z., De Clerck, C., Meersmans, J., Colinet, G., Zhang, W., 2024. Long-term manuring enhanced compositional stability of glomalin-related soil proteins through arbuscular mycorrhizal fungi regulation. *Agriculture*, 14(9), 1510.

1.2 Presentations

1. Long-term manuring enhances glomalin-soil-carbon sequestration by increasing its recalcitrance and macro-aggregation (EGU23, the 25th EGU General Assembly, held 23-28 April, 2023 in Vienna, Austria and Online. Poster presentation).
2. Long-term manuring facilitates glomalin accumulation by chemical composition shifts and macro-aggregation formation (19/12/2022, Soil Science Society of Belgium. Poster presentation).