Soil Biology and Biochemistry 153 (2021) 108116

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: http://www.elsevier.com/locate/soilbio

Perspectives Paper

Glomalin – Truths, myths, and the future of this elusive soil glycoprotein

Jiří Holátko [a,b], Martin Brtnický [a,b,c], Jiří Kučerík [c], Michala Kotianová [d], Jakub Elbl [e,f], Antonín Kintl [b,f], Jindřich Kynický [g], Oldřich Benada [d], Rahul Datta [b], Jan Jansa [d,*]

[a] Institute of Geology and Pedology, Faculty of Forestry and Wood Science, Mendel University in Brno, Zemědělská 3, 61300, Brno, Czech Republic
[b] Department of Agrochemistry, Soil Science, Microbiology and Plant Nutrition, Faculty of AgriSciences, Mendel University in Brno, Zemědělská 1, 61300, Brno, Czech Republic
[c] Institute of Chemistry and Technology of Environmental Protection, Faculty of Chemistry, Brno University of Technology, Purkyňova 118, 61200, Brno, Czech Republic
[d] Institute of Microbiology, Czech Academy of Sciences, Vídeňská 1083, 14220, Praha, Czech Republic
[e] Department of Agrosystems and Bioclimatology, Mendel University in Brno, Zemědělská 1, 61300, Brno, Czech Republic
[f] Agricultural Research Ltd, Zahradní 1, 66441, Troubsko, Czech Republic
[g] BIC Brno, spol.s.r.o, Purkyňova 648/125, 61200, Brno, Czech Republic

ARTICLE INFO

Keywords:
Arbuscular mycorrhizal fungi (AMF)
Extracellular polymeric substances (EPS)
Glomalin-related soil proteins (GRSP)
Glycoprotein
Microorganism
Soil organic matter (SOM)

ABSTRACT

The term "Glomalin" was originally used to describe a hypothetical gene product of arbuscular mycorrhizal fungi (AMF) that was assumed to be a nearly ubiquitous, thermostable and highly recalcitrant glycoprotein, deposited in soils in large amounts, and deemed to indicate soil health and quality. It was defined operationally as the fraction of soil organic matter (SOM) extractable by a hot citrate buffer and assessed either by Bradford assay or by cross-reactivity with monoclonal antibody MAb32B11. Later, it was recognized that the extracts contained a variety of compounds, including some of non-AMF origin, cross-reactive with both Bradford assay and the monoclonal antibody. This led to re-describing the pertinent (and still only operationally defined) SOM as "glomalin-related soil proteins (GRSP)", albeit without any substantial change in the underlying concepts. Consequently, a great deal of confusion in this area arose among researchers in soil, plant, and environmental sciences. Glomalin or GRSP (often used interchangeably) has previously been linked to various soil features, including stability of soil aggregates, size of soil C and N pools, sequestration of heavy metals, and alleviation of various plant stresses. GRSP concentrations in soil often, but not always, have been correlated with AMF biomass measured by alternative (mainly microscopic) approaches. GRSP formation, deposition, and/or decomposition in soils seem to be largely dependent on a multitude of interactions among plants, AMF, and other soil microorganisms, including prokaryotes. The chemical structure of GRSP extracted from soil remains unclear and generally complex. That is due to the unspecific mode of its extraction and purification, as well as the great variety of analytical approaches that have been used heretofore to assess it. Future research needs to elucidate the exact composition of this operationally defined SOM fraction, the controls over its production and accumulation in soils, and its exact role in soil ecology generally and soil food webs in particular. Furthermore, novel and independent tools should be established to more specifically (as compared to current glomalin assays) assess AMF biomass and functioning in roots and soil and its involvement in soil processes.

1. History and current understanding

"Glomalin" was first described by Sara Wright and co-workers in the 1990s (Wright et al., 1996; Wright and Upadhyaya, 1996) as an abundant, nearly ubiquitous, and highly thermostable (glyco)proteinaceous material that was presumed to be produced by arbuscular mycorrhizal fungi (AMF, see Box 1). Glomalin was assumed to be responsible for stabilization of soil aggregates (see Box 2), to have very long turnover times of years or more, therefore to accumulate in soils in large concentrations, and, as a consequence, to make up a significant portion of the soil organic matter (SOM, see Box 3) (Rillig et al., 2001a, 2001b; Halvorson and Gonzalez, 2006). It has traditionally been measured as a concentration of proteins extracted by singly or repeatedly autoclaving a soil sample in citrate buffer (or, alternatively, in other extraction solutions) to obtain easily extractable or total glomalin fractions, respectively (Wright and Upadhyaya, 1998; Halvorson and Gonzalez, 2006;

* Corresponding author.
E-mail address: jansa@biomed.cas.cz (J. Jansa).

https://doi.org/10.1016/j.soilbio.2020.108116
Received 13 August 2020; Received in revised form 16 December 2020; Accepted 18 December 2020
Available online 22 December 2020

Janos et al., 2008). Technically, it has been assessed in the extracts either by unspecific Bradford protein assay using Coomassie Brilliant Blue G-250 dye or as the fraction of the extracts immunoreactive with the monoclonal antibody MAb32B11, previously raised against crushed (non-sterile) spores of *Glomus intraradices* FL208 (Wright et al., 1996; Rillig, 2004), currently known as *Rhizophagus intraradices* (Stockinger et al., 2009; Schüßler and Walker, 2010). This antibody is reportedly cross-reactive with spores of a number of AMF species and has been shown to correlate with intensity of root colonization by various AMF (Wright et al., 1996; Rosier et al., 2008). It has been demonstrated, however, that the fraction of SOM yielded under repeated autoclaving of soil in citrate buffer contains a mixture of various proteins and also other compounds, such as humic acids (Driver et al., 2005; Gillespie et al., 2011). Although the MAb32B11 cross-reactive protein fraction was identified as a structure closely related to the heat shock protein Hsp60 with predicted chaperone function (Gadkar and Rillig, 2006), there is some experimental evidence that the antibody MAb32B11 may not be sufficiently specific as to react only with a single molecular type but may potentially cross-react with more than one protein within the soil extracts (Bolliger et al., 2008). In fact, the antibody does cross-react, among others, with proteins or other compounds derived from non-mycorrhizal plants (Rosier et al., 2008).

Therefore, although the analytical tools to detect and quantify glomalin were originally developed and validated using AMF biomass, the evidence provided so far for glomalin detected in soil being a direct metabolic product of AMF is still merely correlative (Bedini et al., 2007; Wilson et al., 2009). Numerous positive correlations have been reported between AMF hyphal length or spore densities and glomalin concentrations in soil, thus implying causal links (Lutgen et al., 2003; Steinberg and Rillig, 2003). Nevertheless, it is still not completely clear how and whether such a molecular proxy truly relates to the recent and/or past AMF abundance in soil (Feeney et al., 2004; Treseder and Turner, 2007) or whether the correlation is indirect or merely coincidental. This is also because the correlation between glomalin concentration in soil and AMF abundance is not always positive (Lutgen et al., 2003; Rillig et al., 2003; da Silva et al., 2012). Indeed, sometimes no significant correlation has been observed and occasionally even a negative correlation has been recorded (see supplementary tables for a thorough survey of the relevant literature). This may be because glomalin production rates are not linearly correlated with AMF hyphal biomass under all conditions and/or that the turnover rates for glomalin and AMF differ (Rillig, 2004; Hammer and Rillig, 2011). It also is possible that not all AMF species produce the same amount of glomalin per unit of biomass or length of their hyphae, although to the best of our knowledge this has not been specifically tested. Moreover, the complexity of soil food webs could further affect the results, for example through proteins co-detected in the soil extracts and originating from prokaryotes living on AMF exudates and/or on the dead AMF cell walls (Redmile-Gordon et al., 2014; Bukovská et al., 2018; Jansa et al., 2020). This could also involve organisms or metabolic products thereof living in biofilms on surface of AMF hyphae (Fig. 1).

Total glomalin concentrations in soil certainly have tight linkages to total SOM concentrations (Rillig et al., 2001b; Wuest et al., 2005; Wright et al., 2007; Zbíral et al., 2017; Li et al., 2020), as also documented by a literature survey presented in the supplementary tables accompanying this article. AMF have sometimes been reported to stabilize SOM by promoting cleavage of mineral nutrients such as N from the organic moieties, thereby leaving condensed organic matter without much mineral nutrients left over and resembling what has previously been referred to as a Gadgil effect (Leifheit et al., 2015; Verbruggen et al., 2016). Operationally-defined glomalin reportedly contains 28–45% C, 0.9–7.3% N, and 0.03–0.1% P (Sousa et al., 2012; Wang et al., 2014), and in various soils it may also contain metal ions within a wide range of concentrations (Gadkar and Rillig, 2006; Wu et al., 2014). This indicates

Box 1
Arbuscular mycorrhizal symbiosis

The term “arbuscular mycorrhizal (AM) symbiosis” is used for one of the globally most widespread and often mutually beneficial associations between roots or rhizoids of many (>70%) terrestrial plant species and specialized soil fungi from the Glomeromycotina and Mucoromycotina clades (Brundrett and Tedersoo, 2018; Field and Pressel, 2018). This symbiosis is considered evolutionarily primordial (Parniske, 2008), dating back to the Ordovician (Redecker et al., 2000), and showing little specificity in interaction between a particular plant and fungal species (Sanders, 2003). This symbiosis provides direct interconnection between roots and surrounding soil, as well as between plant individuals belonging to the same or different species through the hyphae of the same fungus interlinking different root systems and forming so-called common mycorrhizal networks (Simard and Durall, 2004; Smith and Read, 2008; Walder et al., 2012). This association has repeatedly been shown to be significantly implicated in plant acquisition from soil of nutrients having low mobility, such as P and Zn (Mosse, 1957; Jansa et al., 2003; Smith et al., 2004). In return, the AM fungi (AMF) obtain a significant share of plant C budget (usually between 4% and 15% of plant net photosynthetic production) in the form of either simple sugars or fatty acids (Bravo et al., 2017; Konvalinková et al., 2017). This AM symbiosis is therefore an important ecosystem player as it redistributes (often asymmetrically) symbiotic benefits and costs amongst individual members of a plant community (Walder et al., 2012; Merrild et al., 2013; Weremijewicz et al., 2016). By affecting both plant nutrition and C balance, AM symbiosis influences the quantity and quality of root exudation and creates an independent pathway for C fluxing from plant to soil (de Boer et al., 2005; Lambers et al., 2009; Lendenmann et al., 2011; Mechri et al., 2014). The AMF hyphae exude various organic compounds into their surrounding soil (i.e., the hyphosphere) ranging from small organic molecules such as fructose to more complex compounds (e.g., lipochitooligosaccharides, peptides, glycoproteins) with signalling or other (often putative) functions. Through uptake of soluble nutrients from soil solution and exudation of specific chemical compounds (acting as attractants or deterring compounds), the AMF fungi may possibly shape their hyphal microbiome, with consequences for a multitude of soil processes including organic N mineralization and nitrification (Bukovská et al., 2018; Zhang et al., 2018; Jansa et al., 2020). The fungi efficiently explore the soil for mineral nutrients at distances as much as 10 cm from roots (i.e., much further than root hairs could reach), whereby they overcome the depletion zones forming around roots for diffusion-limited nutrients (Smith et al., 2001; Jansa et al., 2005). Because they possess only a very limited gene repertoire for potent exoenzymes, the AMF appear to team with other soil microbes to gain efficient access to nutrients present in such organic forms as P in phytate or N in chitin (Hodge and Fitter, 2010; Tisserant et al., 2013; Bukovská et al., 2018; Wang et al., 2019). Details of communication and/or reciprocal rewards in such inter-microbial interactions between AMF and other soil microbes remain mostly unknown in spite of growing interest in AMF hyphal microbiome composition and function (Jansa et al., 2013; Kaiser et al., 2015; Zhang et al., 2018). By building up extensive hyphal networks in soil, and possibly also through their exudation (either directly or indirectly via their microbial companions), the AMF contribute to enmeshment and bonding of soil particles into aggregates and thus stabilize soil structure (Rillig and Mummey, 2006). Vigorous soil disturbance and use of mineral fertilizers (particularly mineral phosphates) often suppresses AMF abundance and diversity in soils used for agricultural purposes (Jansa et al., 2002; Oehl et al., 2010; Verbruggen et al., 2010).

that it scarcely can be a uniform proteinaceous material, but rather that it is a mix of various compounds, potentially of different origins, with other components (e.g., metal ions) acquired only when deposited into the soil. Environmentally very important seems to be its Fe-complexing capacity (Lovelock et al., 2004). Glomalin also sequesters several other potentially toxic metals, in particular Cu, Pb, Zn, Cd, Al, and Mn, thus alleviating their potential negative effects on various life forms in soils, including plants (Gonzalez-Chavez et al., 2004; Chern et al., 2007; Vodnik et al., 2008).

2. Physical and chemical properties of glomalin – what is it actually?

An abundant glycoprotein (with its lectin-reactivity being the indication of glycosylation), previously referred to as glomalin, was originally detected within the cell walls of AMF spores or living AMF hyphae during active colonization of plant roots (Wright et al., 1996; Rosier et al., 2008). Later, a glycoprotein isolated from surfaces of soil aggregates showed strong similarity to the previously reported glomalin (Wright and Upadhyaya, 1996, 1998; Lee and Eom, 2009). This protein was then partly characterized as thermostable and recalcitrant to microbial degradation, with part of its amino acid sequence showing similarity to the Hsp60 from AMF fungal isolate *Glomus intraradices* DAOM197198, currently known as *Rhizophagus irregularis* (Stockinger et al., 2009; Schüßler and Walker, 2010). The corresponding gene was deposited in GenBank under accession number ABE02805.1 (Gadkar and Rillig, 2006). This class of proteins (i.e., heat shock proteins) is produced by various organisms, including fungi, and their expression is often upregulated in response to various biotic and abiotic stresses (Ferreira et al., 2007; Hammer and Rillig, 2011; Raggam et al., 2011). In addition to their thermostability, these proteins often show ability to self-polymerize and adhere to other proteins, thereby protecting the other proteins from heat denaturation (Wright and Anderson, 2000; Lau et al., 2013).

Although long turnover times of (total) glomalin have been reported in multiple studies (Rillig et al., 2001b; Steinberg and Rillig, 2003), concentration in soil of easily extractable glomalin appears quite vulnerable to environmental changes and agricultural management practices (Lutgen et al., 2003; Nie et al., 2007). Further, the amount of the immunoreactive fraction of the easily extractable glomalin (assumed to be the young, recently produced, and faster degrading glomalin) clearly fluctuates in time more than total glomalin in some tropical forest soils does (Steinberg and Rillig, 2003; Lovelock et al., 2004). This immunoreactive glomalin has been postulated to be coupled with increased turnover of AMF biomass in fertile soils having high concentrations of Ca, P, and K; high N:P ratios; and low C:N ratios (Lutgen et al., 2003; Lovelock et al., 2004). These types of soils may display greater microbial degradation activity, which could be one of the possible mechanisms preventing efficient sequestration of glomalin and/or SOM (Blanco-Canqui and Lal, 2004; Wilson et al., 2009; Fokom et al., 2012; Ghosh et al., 2018; Restovich et al., 2019). Total glomalin concentrations found in most tropical soils are indeed much higher than are those detected in temperate forest and arable soils, as the latter frequently feature higher levels of P and Ca availabilities and often relatively low C:N ratio (Lovelock et al., 2004). Higher C:N ratio is often coupled with higher bacterial exudation rates (Redmile-Gordon et al., 2015). Bacteria, then, contribute to SOM build-up by producing diverse extracellular polymeric substances (EPS). These substances seem to be easily co-extracted by customary glomalin isolation methods (Redmile-Gordon et al., 2014), potentially increasing the ratio between immunoreactive (presumably not affected by the EPS) and total (potentially biased by the EPS) glomalin stocks measured in such soils. It long has been known that autoclaving of soil samples in citrate buffer does indeed yield a complex mixture of proteins and other organic compounds, some of which have

Box 2
Soil aggregates and their stability.

The solid phase of soil is composed of differently sized primary particles (either mineral particles classified, according to their size, as clay, silt, or sand, or particulate soil organic matter, SOM) arranged into aggregates of different sizes and with different mechanical and temporal stabilities. How the particles are assembled into aggregates defines soil structure (Bronick and Lal, 2005). Aggregation is further conditioned by the rearrangement, flocculation, and cementation of the primary particles. Since the early 1900s, knowledge has gradually grown as to the main determinants of soil aggregation, which consist in the following factors: (1) soil fauna, (2) soil microorganisms, (3) plant roots, (4) inorganic binding agents, and (5) the environment (Six et al., 2004). These factors also interact, either synergistically or disruptively. The most intensively studied groups of soil fauna with relevance to soil aggregate formation are earthworms and termites, both of which produce so-called biogenic aggregates such as earthworm casts and termite sheetings (Young et al., 1998; Bossuyt et al., 2005; Jouquet et al., 2012). The most important soil microorganisms relevant to formation and stabilization of soil aggregates are probably the mycorrhizal and saprotrophic fungi (Tisdall et al., 1997, 2012; Rillig and Mummey, 2006), but prokaryotes can also have a strong effect on soil aggregation, especially at the microscale (Gupta and Germida, 2015; Lehmann et al., 2017). There are two ways how prokaryotes could be involved in the formation of soil aggregates. First, because a large share of soil prokaryotes live in biofilms (Flemming and Wuertz, 2019), where they often produce large amounts of extracellular polymeric substances (exopolysaccharides and/or other organic compounds), they could effectively glue the particles together (Alami et al., 2000; Redmile-Gordon et al., 2020). Second, depending on soil pH and composition of the walls or outer membrane, prokaryotic cells have been shown to develop a net negative electrostatic charge on their surfaces (Huysman and Verstraete, 1993). The charge enables microorganisms to adhere to clay and silt particles, mediating their agglomeration and holding them together through adhesion via interaction with positively charged surfaces such as metal oxides (Mills, 2003; Lehmann et al., 2017). Roots and fungal hyphae can also assemble soil particles, both by mechanical forcing while realigning them and by releasing various organic compounds that glue the particles together (Czarnes et al., 2000; Hallett et al., 2009; Milleret et al., 2009; Galloway et al., 2018). Clay-sized particles mainly mediate aggregation through rearrangement and flocculation, but swelling clays can disrupt aggregates. Organo-metallic compounds and cations bridge the soil particles. The SOM enhances aggregation by bonding soil particles together (Bronick and Lal, 2005). The extent of the SOM effect depends upon its concentration, composition, and chemical nature, which in turn are dependent on its physical and chemical protection from microbial degradation (Ellerbrock et al., 2005). Soil inorganic C increases aggregation mainly in semiarid environments (Fernandez-Ugalde et al., 2011). The formation of secondary carbonates is influenced by the presence of soil organic C and Ca^{2+} and Mg^{2+} ions (Bronick and Lal, 2005; Rowley et al., 2018). In addition to carbonates, the precipitation of (hydr)oxides and phosphates also supports soil aggregate build-up. Cations such as Si^{4+}, Fe^{3+}, Al^{3+}, and Ca^{2+} promote precipitation of compounds that act as bonding agents for primary particles (Six et al., 2004; Bronick and Lal, 2005). Soil aggregate stabilization can thus exert important feedback effects upon soil C sequestration, which, in turn, could be counteracted by mechanical disturbance such as tillage or by reliance upon plant mineral nutrient replenishment approaches, as used in modern production agriculture, depending mainly upon mineral fertilizer inputs (Six et al., 2000; Blanco-Canqui and Lal, 2004; Jastrow et al., 2007).

significant potential to interfere with the total glomalin quantification and therefore to bias the Bradford quantification of total glomalin (Halvorson and Gonzalez, 2006; Schindler et al., 2007; Whiffen et al., 2007). Furthermore, the realization that the soil citrate extracts contained a great diversity of compounds of various origins and potential functions led to the materials previously termed "glomalin" being described as "glomalin-related soil proteins (GRSP)" and a proposal to reserve the name "glomalin" or "Glomalin" for a putative gene product (or group of gene products) of AMF only (Rillig, 2004; Janos et al., 2008). The two designations (i.e., glomalin and GRSP) have been used in the subsequent literature almost interchangeably, however, and without any substantial shift in the underlying concepts and results interpretation. The option has almost entirely been neglected to use Glomalin written with an initial capital to designate the true product of AMF and to distinguish it from the operationally defined SOM fraction (the glomalin). This has created a great deal of confusion in the scientific community. For reasons of consistency, we have used the term "glomalin" in the text up to this point and in the title because it has been referred to as such in most of the pertinent literature (particularly before 2004), although it should, in most cases, be designated as GRSP (which itself is confusing inasmuch as it contains the word "glomalin"). Hereinafter, we will distinguish carefully between "glomalin" when describing a gene product of AMF and "GRSP" when referring to the operationally defined pool of SOM extractable by hot citrate buffer from soil.

3. Origin of GRSP: who is actually responsible?

Detailed structural characterization of GRSP extracts have revealed (Gillespie et al., 2011; Walley et al., 2014), as previously expected (Rillig, 2004), a consortium of various proteins (along with assorted lipids and phenolic compounds) that could not be distinguished from one another when using the unspecific Bradford assay for measuring protein concentration. The results from proteomics analysis of such soil extracts have suggested that GRSP also contain, among others, proteins possessing a thioredoxin-like domain, a feature encountered in some chaperones (Kern et al., 2003; Gillespie et al., 2011). It also has been shown that a significant fraction of GRSP contained proteins of non-mycorrhizal origin (Gillespie et al., 2011) and that bacteria (particularly some thermophilic taxa) contributed significantly to the GRSP fraction of SOM (Bolliger et al., 2008; Gillespie et al., 2011; Walley et al., 2014). Some soil microorganisms (and particularly those associated with AMF hyphae, Fig. 1) may contribute to forming such proteins on their own or via transformation of AMF exudates and/or necromass by extracellular enzymes that they produce (Martens and Frankenberger, 1992; Gonzalez-Chavez et al., 2008). Such compounds could then become a part of soil EPS rich in proteinaceous compounds and eventually land in the GRSP extracts. Due to their association with AMF hyphae, and even if most of the GRSP were not strictly of AMF origin but actually produced by such hyphae-associated bacteria, the GRSP concentration in the soil would nevertheless (coincidentally) correlate with the AMF hyphal length and/or spore densities as observed in some, albeit not all, studies (see supplementary tables).

4. Ecosystem relevance – what does it do?

Stabilization of soil aggregates (see Box 2 for more details) is undoubtedly one of the most crucial features of GRSP. Their role in improving soil quality has been demonstrated in several field studies and in a couple of manipulative pot experiments (Wright et al., 1996, 2007; Bedini et al., 2007, 2009; Wang et al., 2015; Gao et al., 2019). The GRSP also seem to influence soil wettability via decrease in water permeability of soil surfaces by increase in their hydrophobicity, thereby leading to slower water loss and better soil moisture maintenance (Knorr et al., 2003; Lutgen et al., 2003; Steinberg and Rillig, 2003; Feeney et al., 2004). GRSP are apparently important also for plant nutrition, as they play a role as a reservoir of biogenic elements, chiefly C and N (Rillig et al., 2001a; Nie et al., 2007; Schindler et al., 2007), thus affecting soil microbial activity and stabilizing soil nutrient pools. The GRSP constitute one of the largest pools of soil N and contain almost one-third of soil C and 1–9% of bound iron within the extractable SOM (Nichols and Wright, 2005). The importance of GRSP for ecosystem C cycling has

Box 3
Soil organic matter

Soil organic matter (SOM) is defined, for the purpose of this perspective paper, as any kind of organic material of natural origin, not being an integral part of any living organism, deposited in any layer of the soil profile (Tisdall and Oades, 1982). It can be directly originating from plants (which usually are the dominant primary producers in terrestrial ecosystems) or from any other member of the soil food webs, such as animals, fungi, protists, or prokaryotic microbes. The SOM includes residues of dead biomass of diverse organisms at various stages of decomposition, various exudates and/or excretes of the living organisms, as well as other particulate or dissolved organic inputs. The latter encompass biochar and organic inputs carried in with atmospheric depositions or sediments. We purposefully exclude from this definition any materials being exclusively produced by human activities (plastics, recalcitrant organic pollutants such as polychlorinated or fluorinated compounds, or endogenic disruptors) for which we reserve the term "xenobiotics". The SOM comes in a variety of forms, states, and origins, ranging from fresh and non-degraded materials (e.g., plant litter, animal corpses) to heavily degraded or stabilized SOM. The soil organic C pool contained in SOM for the entire land area of the Earth, excluding C in the litter layer and charcoal, totals 1462–1548 Pg (10^{15} g) of C in the upper 100 cm (Batjes, 1996) and about 2344 Pg C in the upper 300 cm (Jobbagy and Jackson, 2000). Global amounts of soil N in the same layer (of which a great majority is bound either in plant or microbial biomass or SOM) are estimated to be 133–140 Pg of N for the upper 100 cm (Batjes, 1996). Mean C:N ratios for SOM range from 9.9 for arid Yermosols to 25.8 for Histosols, whereas the individual compounds can show even a broader range of values (e.g., from 5.5 for pure proteins such as RUBISCO to compounds lacking any N, such as cellulose, for which the C:N ratio would be indefinitely high). The SOM consists of diverse compounds ranging from chemically reactive and mobile forms with turnover times measurable in seconds or minutes within a soil environment (e.g., glucose, citric acid) to stable recalcitrant forms with turnover times measurable in years, such as lignins, waxes, or humic acids (Marschner et al., 2008). The different compounds can be protected against decomposition by various mechanisms (Oades, 1989; Six et al., 2002), namely by their chemical natures or physical stabilization by bonding tightly with silt and clay particles or location inside soil aggregates with specific micro-environmental conditions (Sexstone et al., 1985; Prove et al., 1990). Certain compounds can be assigned unequivocally to specific organismal groups, such as certain polysaccharides, lignins, tannins, and waxes to plants, whereas other compounds are exclusively produced by microorganisms, such as ether lipids, certain fatty acids, amino sugars or specific glycoproteins (Messner, 2004; Ruess and Chamberlain, 2010; Knappy et al., 2015). SOM is critically important for ecosystem functioning because of its feedback effects on soil quality, microbial activity, and maintenance of ecosystems services related to soil aggregate stabilization, water-holding capacity, soil–plant relations, and slow release of plant nutrients, among others (Vanveen and Kuikman, 1990; Paterson, 2003; Ding et al., 2015).

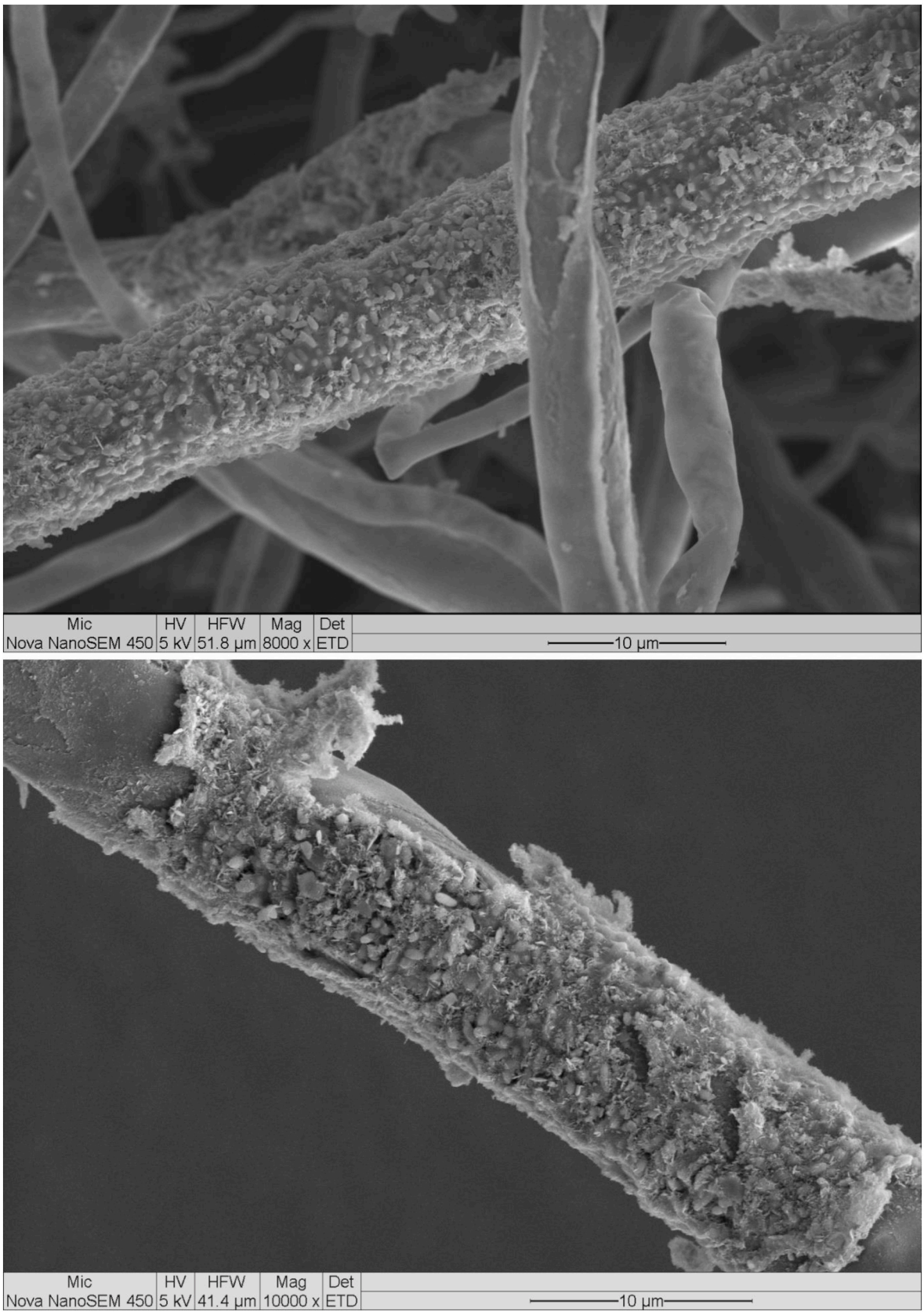

Fig. 1. Scanning electron microscopy of bacterial cells and collapsed extracellular polymeric matrix on surface of arbuscular mycorrhizal fungal (*Rhizophagus irregularis*) hyphae extracted from a root-free compartment of a pot experiment described by Bukovská et al. (2018) after 12 weeks of plant growth. Hyphae were fixed in glutaraldehyde-cacodylate buffer, post-fixed with osmium tetroxide, dehydrated, then sputter-coated with 3 nm of platinum.

been demonstrated by reports that the GRSP are positively affected by higher CO_2 concentrations in the atmosphere, thus following a trend similar to that seen in the development of AMF hyphae (Rillig and Allen, 1999; Rillig et al., 2000; Emran et al., 2012; Zhang et al., 2015). Faster AMF hyphal development and enhanced GRSP production due to increased atmospheric CO_2 levels are possibly coupled also to greater activity of mycorrhiza-helper bacteria in the AMF hyphosphere (Barea et al., 2002; Frey-Klett et al., 2007). It has been suggested also that mineral N fertilization would decrease soil GRSP stocks due to lower reliance of plants on their fungal symbionts for their mineral nutrition, but the experimental results in this area obtained thus far have been contradictory and largely inconclusive (Treseder et al., 2007; Zhang et al., 2015). This is possibly due to rather complex interactions existing among N, P, and C fluxes in plant–AMF–soil systems (Johnson, 2010; Püschel et al., 2016).

The GRSP concentrations in soil respond to various agronomic practices (Wright and Anderson, 2000; Curaqueo et al., 2011; Singh et al., 2017), and particularly to soil tillage and fertilization (Dai et al., 2013; Galazka et al., 2017; see also the literature survey provided as a supplement to this paper). Enhanced concentrations of GRSP have been observed after application of such organic fertilizers as manure, litter, or compost (Valarini et al., 2009; Zhang et al., 2014). Nevertheless, we should emphasize that organic input itself could have increased the amount of proteins, humic acids and tannins, extracted from organic fertilizer-amended soil and subsequently measured by the unspecific Bradford assay, which responds to all of the above-mentioned compounds. Nie et al. (2007) observed increased GRSP concentration in soil after application of a mixture of straw and mineral fertilizer. Generally, larger demand of plants for mineral nutrients such as P, Zn, and to a lesser extent also N, results in larger flux of organic C from plant to AMF (Fellbaum et al., 2014; Bücking and Kafle, 2015; Kafle et al., 2019). Such enhanced plant C allocation to the AMF would promote their hyphal development and subsequent GRSP production. Consequently, this contribution to GRSP production controlled by plants might contribute to ecosystem resistance against various environmental stresses. Namely, it has been demonstrated that plant stress response (e.g., to salinity) has sometimes been accompanied by increased levels of GRSP in soil (Hammer and Rillig, 2011), which could be due to increase in the abundance of AMF or as a result of synergy between the AMF and plant growth-promoting rhizobacteria. For example, enhanced tolerance of *Lactuca sativa* to salinity stress was conveyed by interaction of *Glomus mosseae* (currently known as *Funneliformis mosseae*) and the bacterium *Pseudomonas mendocina* Palleroni in its rhizosphere (Kohler et al., 2010). Salt stress in maize plant was alleviated by co-inoculation of AMF and *Methylobacterium oryzae* CBMB20 (Lee et al., 2015). It would be tempting to hypothesize that the examples cited above provide evidence and support for the interaction between hyphae-associated prokaryotes and AMF hyphae in producing and/or stabilizing GRSP in soil, but the current evidence would be no better than pure correlation inasmuch as there is no mechanistic proof.

Furthermore, GRSP appear particularly important for detoxification of soil pollutants. The GRSP have been reported to sequester various heavy metals and decrease availability and toxicity risk of these elements for various soil-dwelling organisms, including plants. For example, GRSP have been demonstrated to bind as much as 27.5% of the total Cu in some soils (Cornejo et al., 2008). Higher proportions of GRSP-bound heavy metals such as Cd and Pb through increased content of GRSP was achieved by increasing the CO_2 concentration in the atmosphere (Jia et al., 2016). Along similar lines, it is interesting that sorption of heavy metals can be enhanced by EPS produced by soil bacteria (Mikutta et al., 2012), and thus a role of biopolymers, including those of prokaryotic origin, in the binding capacity of GRSP also should be considered.

5. Open gaps and experimental challenges

Significant research efforts have been invested to date into measuring GRSP in various kinds of samples collected from a broad range of ecosystems and manipulative experiments. Sometimes, the results have been reported as measuring glomalin (i.e., the product of AMF genes sensu Rillig, 2004) while assuming causal links between GRSP and AMF. The big questions remaining to be answered are:

A. Whether tight correlation between the two is common enough to rely on the GRSP as generally valid proxy of AMF abundance, activity and/or legacy, for example if GRSP of non-AMF origin contribute only negligibly to the measured values or whether GRSP of non-AMF origin still correlate with AMF because the relevant bacterial compounds are/were AMF-associated,
B. Whether and to what extent the above assumption has helped us in understanding soils, and
C. What steps should we take to make future research programmes in this direction more useful as compared to the past.

Evidence currently available shows that not only the Bradford-reactive fraction but also the MAb32B11-antibody cross-reactive fraction of GRSP (the latter of which was earlier considered to represent the glomalin sensu Rillig, 2004) contain substances of non-AMF origin (Rosier et al., 2008). Therefore, we still do not have specific analytical tools to measure glomalin (sensu Rillig, 2004) without significant (albeit yet unknown as to its extent and variation) interference from other substances. Although fragments of genes coding for homologs of heat shock protein Hsp60 have successfully been amplified and sequenced from a range of AMF species (Magurno et al., 2019), a detailed proteomics study of all extraradical proteins of *Rhizophagus irregularis* growing in symbiosis with chicory Ri T-DNA transformed roots *in vitro* failed completely to detect overproduction of such gene products (Murphy et al., 2020). Rather, that study reported experimental evidence for presence of Hsp70 and Hsp90 chaperones in the protein extracts but not their overproduction. These recent findings thus seriously call into question the correctness of the previous focus on Hsp60 as presumably the main component of the elusive glomalin. This nevertheless requires independent confirmation (and perhaps the AMF did not overproduce the Hsp60 under the given experimental conditions) before suggesting a refocus of glomalin research onto other gene targets.

Without a doubt, GRSP as currently defined and experimentally assessed appear to comprise a quantitatively significant component of the SOM in a range of soils, tightly correlating with their SOM content in nearly all case studies (see supplementary tables for an overview). GRSP have been shown to relate to a number of ecosystem functions such as water retention and C sequestration, to correlate with soil nutrient contents and bioavailability, and to contribute to alleviating detrimental effects on plants of toxic heavy metals, salinity, and drought (Gonzalez-Chavez et al., 2004; Chern et al., 2007; Sousa et al., 2012). GRSP thus appear to be directly related to sustainability and environmental resilience of agricultural practices (Wright et al., 2007; Lee and Eom, 2009) and GRSP quantification potentially could be used as a proxy for success of remediation technologies (Gonzalez-Chavez et al., 2004; de Souza et al., 2013). However, a recent comparison of GRSP quantification as an indicator of soil quality ranked only mediocre as compared to other well-established indicators of organic C cycling in soils (Thiele-Bruhn et al., 2020). Nevertheless, several fundamental questions related to GRSP still should be tackled by future research as we aim to deepen our understanding of this SOM fraction:

1. What in fact are GRSP? Are they correctly designated and named or are their definition and naming misleading?
2. How do the composition and spatiotemporal dynamics of GRSP vary with environmental context? How are GRSP produced and why?

3. What is the role of GRSP in soil nutrient fluxes? Do GRSP play a specific role in complex food chains at the plant–AMF–bacterial interface or is GRSP sequestration simply an outcome of a rather coincidental metabolic relationship between AMF hyphae and the extremely complex rhizosphere and/or hyphosphere?
4. Could it be that the turnover rates of GRSP have been underestimated and their recalcitrance thus overestimated due to considering them being a homogeneous entity rather than complex mixture of compounds?

Below we share some thoughts related to the above questions:

Ad question 1: Improvement in isolation and purification methods for extraction of soil proteins, EPS, and other non-EPS materials of microbial origin appears to be crucial for furthering our understanding of GRSP composition. Until recently, a nonselective procedure for extracting GRSP from soil was coupled with either a non-specific (Bradford assay) or more selective (Rosier et al., 2006) method for estimating proteinaceous matter. This has been done mostly without further detailed chemical characterization. Attribution of detected GRSP levels to AMF abundance (measured mainly as the standing AMF biomass, AMF hyphal length density or AMF spore density) in soil has not always been successful (Treseder and Turner, 2007). In nearly half of the case studies (see supplementary table for details) GRSP levels did not in fact correlate at all with density of AMF hyphae or spores in soil. Detailed analyses of various GRSP fractions (e.g., easily extractable, immunoreactive, and total GRSP) have revealed a consortium of compounds including various lipids, tannins, and humic acids, all of which could interfere with the Bradford assay (Schindler et al., 2007; Whiffen et al., 2007), as well as thermostable proteins of other than AMF origin, and particularly of bacterial origins (Bolliger et al., 2008; Gillespie et al., 2011). The total protein assessment in the different GRSP fractions and its attribution to AMF is thus currently loaded with a number of potential biases. Because the term glomalin-related soil proteins contains "glomalin" in its name, it could be (and actually often is) confused with glomalin sensu Rillig (2004) or Glomalin sensu Janos et al. (2008). This is utterly incorrect, and therefore we believe citrate extractable soil proteins (CESP) would serve this purpose probably better than GRSP.

Ad question 2: It should be clarified which (if any) of the materials in GRSP actually derive directly from AMF, whether they are actively exuded or only released to the soil environment after hyphal death, as has previously been proposed (Driver et al., 2005; Purin and Rillig, 2008), and whether they are further transformed extracellularly via exoenzymes or ingested by other microbes and then metabolized internally (e.g., by AMF hyphae-associated microbes; de Boer et al., 2005; Hartmann et al., 2009; Baraniya et al., 2016). Soil bacteria are themselves producers of a great variety of thermostable proteins (Diamantidis et al., 2000; Sterner and Liebl, 2001). For example, bacterial chaperonin GroEL is homologous to eukaryotic Hsp60 and thus also to Gi-Hsp60 (Fayet et al., 1989; Gupta, 1995; Sigler et al., 1998). Interaction of these proteins with other organic substances in soil (e.g., humic acids) and mineral sorbents (i.e., clays) may also play a certain role in the stabilization and ecosystem function of these compounds (Marshman and Marshall, 1981). Further, instead of regarding GRSP as a steady and uniformly reacting entity, a more dynamic view of GRSP should be established. That view should incorporate spatiotemporal dynamics of such a complex mix of various compounds and be responsive to the soil and environmental contexts (e.g., drought, salinity, CO_2 levels, aeration of soil, agricultural management, plant and/or microbial invasions) and with regard for both its total content and its composition.

Ad question 3: Positive correlation among AMF abundance, GRSP, and soil organic C levels (Singh et al., 2016; Zhang et al., 2017) generally agrees with the evidence that high fungal-to-bacterial biomass ratio is coupled with increased N availability and litter decomposition and is accompanied by higher C storage potential of the soils (Koranda et al., 2014; Malik et al., 2016). Enhanced N availability increases fungal degradation of cellulose from plant cell walls (Koranda et al., 2014) and subsequent increased glucose availability enhances activity of exoenzymes (including proteases) secreted by saprotrophic fungi (Rineau et al., 2016). Although bacteria in such soils are considered to play a subordinate role as decomposers of smaller molecules that are primarily produced by soil fungi, the hyphal network of AMF offers a specific niche for heterotrophic soil bacteria (Nazir et al., 2010). These facts, together with mounting direct experimental evidence (Hodge et al., 2001; Hodge and Fitter, 2010; Nuccio et al., 2013; Bukovská et al., 2018; Bunn et al., 2019) suggest that the so-called AMF hyphosphere, a zone of soil directly affected by the presence of the AMF hyphae, is very efficient at breaking down various polymers, including proteins. Nevertheless, GRSP seem to remain rather inert to this degradation machinery, and this certainly merits more detailed investigations in future.

Ad question 4: If we consider the possibility that a significant portion of the GRSP in soils is not of AMF origin and that the GRSP-producing microbiota are more diverse and have a broader range of activity than previously assumed, then we can expect dynamic changes in the GRSP pool with environmental conditions and over time. Therefore, we also should examine the fluxes of compounds within the plant–AMF–bacteria system. To further our understanding of GRSP formation, transformation, and degradation will require carefully designed experiments with AMF cultures produced *in vitro* while examining the exact chemistry of AMF's metabolic products and separating them from the products of other soil microbes detected in the GRSP extracts. Although such research actually was initiated long ago (Rillig and Steinberg, 2002), only very recently has it reached a stage where detailed insights into AMF protein chemistry are technically feasible (Murphy et al., 2020). Possibly, future work will reveal that GRSP (or CESP) are too versatile, multifunctional, and chemically complex to be directly connected only to AMF but that a more holistic, whole-soil perspective is needed to explain their function and temporal dynamics in living soils. This would need to include quantitative data on the contributions of other microbial guilds beyond just AMF to this complex's formation and turnover.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Petra Bukovská for providing samples of AMF hyphae with microbial biofilms for microscopy analyses and Olga Kofroňová for her professional support with scanning electron microscopy analyses. This work was supported by the project of the Technology Agency of the Czech Republic TH02030169, by the Ministry of Agriculture of the Czech Republic (institutional project MZE-RO1720), and by the Czech Science Foundation (project 18-04892S). The authors gratefully acknowledge access to the electron microscopy facility, supported by the project LO1509 of the Ministry of Education, Youth and Sports of the Czech Republic. Further support was provided by long-term research development program RVO 61388971. Inspirational suggestions by two anonymous reviewers helped improve the manuscript significantly, and this is gratefully acknowledged.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.soilbio.2020.108116.

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