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石灰土易提取球囊霉素相关土壤蛋白的实验条件优化

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摘要：在水-二氧化碳-碳酸盐岩-生物的相互作用下，全球形成 8.24×10^8t C/a 的岩溶碳汇，其中部分岩溶碳汇以土壤有机质的形式固存，进而在国家双碳目标中发挥重要作用。全球分布广泛的丛枝菌根真菌分泌的球囊霉素相关土壤蛋白 (Glomalin-related soil protein，GRSP) 性质稳定、不易分解，是土壤有机质的重要组成部分。提取合格的 GRSP 是深入研究岩溶土壤有机碳汇的基础，然而以往研究因 GRSP 提取量不高、提取不充分、产物不专一等问题，以至其在有机质中发挥的作用机制无法深入开展研究，因此，提高 GRSP 提取量对于探究岩溶土壤有机质的形成和稳定机制具有重要意义。本文选取岩溶区黑色、棕色、黄色和红色四种石灰土，通过温度和时间正交实验，筛选出适用于岩溶土壤颗粒态有机质 (POM) 和矿物结合态有机质 (MAOM) 易提取球囊霉素相关土壤蛋白 (EE-GRSP) 的最佳提取条件。实验结果表明，POM 和 MAOM 在 123℃ 和 80min 条件下提取时，EE-GRSP 提取量最高。应用于四类石灰土后，EE-GRSP 增量范围为 4.6%～34.2%。相较于全土，MAOM 具有更高的稳定性与更强的有机碳保护能力。因此，随着温度和提取时间的提高，MAOM 中的 EE-GRSP 得到更完全的释放，提取量显著提高。由此可见，EE-GRSP 提取条件优化对深入研究岩溶土壤碳汇潜力及其稳定机制具有重要意义。

关键词：易提取球囊霉素相关土壤蛋白；提取条件优化；颗粒态有机质；矿物结合态有机质

要点：

(1) 提高温度、延长提取时间可提高石灰土易提取球囊霉素相关土壤蛋白提取量。

(2) 通过正交实验进行提取条件探索，并将优化条件应用于石灰土实际样品，实验假设得到验证。

(3) 石灰土颗粒态有机质和矿物结合态有机质的易提取球囊霉素相关土壤蛋白提取条件区别于全土，需要提高提取温度、延长提取时间。

中图分类号：S151.9　　**文献标识码：**A

随着全球变暖, 全球大气中 CO_2 浓度不断上升, “减源增汇”成为国内外学术界研究的热点问题。土壤碳汇具有很大的碳中和潜力, 阐明土壤有机质的稳定机制对于准确预测陆地碳循环与气候变暖之间的反馈关系十分重要[1]。中国岩溶面积达 340 万平方公里以上[2], 占国土面积 1/3。石灰土由岩溶地区的碳酸盐岩风化成土, 具有土层薄、有机质含量高、结构稳定性强等特点[3]。据统计, 中国裸露岩

收稿日期：2024−02−06；**修回日期：**2024−06−13；**接受日期：**2024−07−06
基金项目：国家自然科学基金项目 (42172341)；广西重点研发计划项目 (桂科 AB21196050)；中国地质科学院岩溶地质研究所基本科研业务费项目 (2023020)
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溶区土壤有机碳储量约 1.261×10^7 吨、埋藏岩溶区土壤有机碳储量约 1.602×10^7 吨, 约占中国土壤有机碳储量的 32.11%[3]。岩溶土壤有机碳作为碳汇的重要组成部分, 在陆地碳汇系统中发挥着不可忽视的作用。现阶段国内外关于碳汇的研究主要集中于碳储量、碳封存等方面[4-5]。丛枝菌根真菌 (Arbuscular mycorrhizal fungi, AMF) 分泌的球囊霉素相关土壤蛋白 (Glomalin-related soil protein, GRSP) 由于性质稳定、不易分解, 其含量占土壤有机碳的百分比约为 17%～22%, 因此在土壤碳固存方面具有重要的作用[6]。为此, GRSP 对土壤有机碳汇的贡献是近年来关于碳封存领域中的热点。然而, 目前关于岩溶区 GRSP 与土壤有机碳之间的相互作用机制未有深入的研究。本课题组基于岩溶区石灰土富含钙离子特点[7], 将土壤矿物结合态有机质与 GRSP 联系起来, 研究岩溶区 GRSP 在土壤有机质保存中的作用。因此, 对土壤 GRSP 进行提取后, 不但可以直接分析其在研究区域的分布情况, 而且还由于 GRSP 含量与土壤团聚体稳定性、土壤有机碳等具有相关关系, 可将 GRSP 作为评价这些性质的指标。

GRSP 是一类具有热稳定性的糖蛋白[8], 其发现始于单克隆抗体 MAb32B11 对 AMF 破碎孢子上的一个未知表位研究[9]。根据提取方法的不同, GRSP 可分为易提取球囊霉素相关土壤蛋白 (EE-GRSP) 和总球囊霉素相关土壤蛋白 (T-GRSP)。目前对 GRSP 组成和结构进行了深入探究, 例如郭雪佳等[10]通过刀豆球蛋白凝集素亲和层析分离技术, 发现 GRSP 中部分蛋白具有糖蛋白特性。柴立伟等[11]通过三维荧光光谱分析纯化的 GRSP, 将土壤类球囊霉素蛋白分为 5 个荧光组分。Zhang 等[12-13]采用傅里叶变换红外光谱技术和 X 射线光电子能谱技术发现 GRSP 具有羟基、羧基等官能团。GRSP 提取多采用基于 Wright 等[14]提出的柠檬酸盐高温提取法。譬如, 多数学者使用 121℃、高压灭菌 30min 提取 EE-GRSP 或者 121℃、高压灭菌 60min 提取 T-GRSP[15-18]。此外, 对于 GRSP 提取时间的控制多有不同, 已有部分研究者采用 30min、60min、90min 对 EE-GRSP 进行提取[19-26]。尽管对 GRSP 的生态功能认识已逐步深入, 但 GRSP 提取仍存在产物含有非球囊霉素成分的问题。这是因为最初对于 GRSP 提取的假设为除 GRSP 以外的所有蛋白质在提取程序中完全变性, 但近年来有研究者对此提出质疑[27-28]。譬如, 高瑞等[29-30]和王国禧等[31]关于 EE-GRSP 的提取量集中于 0.5～1.5mg/g。如何优化 GRSP 提取条件、提高 GRSP 提取量是亟待解决的问题。

虽然已有诸多学者对提取时间和离心力进行了改进, 但是鲜见对提取温度和时间进行正交优化。岩溶区石灰土富钙偏碱的特点使其富钙和固碳作用相互影响[32], 因此, 钙与有机物质相互作用形成的腐植酸钙使有机质得到积累[33]。此外, 土壤有机质可以划分为颗粒态有机质 (POM) 与矿物结合态有机质 (MAOM)[34], 且 GRSP 能与金属离子结合并形成稳定的结构[35]。由此可见, 岩溶土壤 POM 和 MAOM 采用 121℃ 条件则不能充分提取 EE-GRSP。为此, 本文在已有提取方法基础上, 依次进行温度、时间与双因素正交实验, 对 EE-GRSP 提取温度与提取时间进行优化。完成优化条件筛选后, 以岩溶区 4 种类型石灰土 (黑色石灰土、棕色石灰土、黄色石灰土和红色石灰土) 为研究对象, 进行提取条件的实用性验证。通过对不同提取条件下 POM 和 MAOM 中 EE-GRSP 提取量进行对比分析, 建立适用于岩溶土壤 POM 及 MAOM 的提取方法。

1　实验部分

1.1　实验仪器

立式压力蒸汽灭菌器 (YXQ-SⅡ, 上海博讯医疗生物仪器股份有限公司); 台式高速离心机 (TGL-16, 湖南湘仪实验室仪器开发有限公司)。

紫外分光光度计 (T6 新世纪, 北京普析通用仪器有限责任公司); 15mL 无菌离心管; 1000μL 移液枪; 2mL 无菌离心管 [生工生物工程 (上海) 股份有限公司]。

1.2　标准溶液和主要试剂

牛血清蛋白 (BSA) 标准溶液 (1000μg/L): 称取 100mg 牛血清蛋白溶于蒸馏水中, 定容至 100mL。

柠檬酸钠溶液 (20mmol/L, pH=7): 称取 5.882g 柠檬酸钠溶于 500mL 离子水中, 定容至 1L。

考马斯亮蓝 G-250 染色剂: 称取 0.1g 考马斯亮蓝 G-250 溶于 50mL 的 95% 乙醇中, 加入 100mL 850g/L 浓磷酸, 定容至 1L, 放入棕色瓶中保存。

1.3　土壤样品采集与处理

实验土壤采自广西壮族自治区弄岗国家级自然保护区。该保护区位于桂西南龙州县城北面, 地跨龙州、宁明两县, 占地 100.775km^2, 保存有完整的热带季雨林, 是世界上 14 个重要的陆生生物多样性区域之一。区域内母岩以泥盆系、石炭系和二叠系碳

酸盐岩为主。

本研究采集黑色石灰土(pH=7.10, SOM 含量 316.84g/kg, 交换性钙离子含量 69.11cmol/kg)、棕色石灰土(pH=7.44, SOM 含量 83.44g/kg, 交换性钙离子含量 23.24cmol/kg)、黄色石灰土(pH=6.34, SOM 含量 32.31g/kg, 交换性钙离子含量 11.25cmol/kg)和红色石灰土(pH=8.00, SOM 含量 21.39g/kg, 交换性钙离子含量 17.91cmol/kg)四类土壤。原始样品剔除石砾、残根等杂物,混合均匀。全土风干过 10 目筛;POM 和 MAOM 运用湿筛法分离全土获得:将全土土样置于 3、2、1、0.5、0.25 和 0.053mm 套筛的最上层,放置于土壤团粒结构分析仪中,筛分过程中用镊子或夹子将土块轻轻掰开。设定频率为 2min 上下 50 次,振幅大约 3cm。筛分结束后收集 0.053~2mm 筛上的样品,即为 POM。筛分仪桶中的悬浊液于 4000r/min 转速下离心 10min,弃去上清液,得到 MAOM(<0.053 mm)。POM、MAOM 通过湿筛法获得后烘干,过 10 目筛。

提取温度与时间优化实验:选取棕色石灰土和红色石灰土的全土与筛分后的颗粒态有机质,各设置三个平行处理;实际样品提取实验:选取筛分后的四类石灰土颗粒态有机质与矿物结合态有机质,各设置四个平行处理。

1.4 EE-GRSP 提取

1.4.1 影响因素探索实验

选取石灰土演替过程中具有代表性的棕色石灰土和红色石灰土进行探索实验。称取筛分后的棕色石灰土-颗粒态有机质、红色石灰土-颗粒态有机质样品各 0.5g 于 15mL 离心管中。按照样品与提取液 1∶8 比例,管内加入 20mmol/L 柠檬酸钠溶液(pH=7),混合均匀,样品一半进行开盖处理,一半进行关盖处理。放入高温灭菌锅,分别在 121℃ 条件下提取 40min、60min、80min。灭菌结束后 9500r/min 离心 10min。4℃ 保留上清液测定 EE-GRSP 含量。

1.4.2 提取温度、时间优化实验

称取棕色石灰土、红色石灰土:全土、POM 各 0.5g。按照 121℃、123℃、125℃ 温度梯度和 40min、60min、80min 时间梯度进行正交试验。所有样品开盖放入高温灭菌锅。其余步骤同 1.4.1 节。

1.4.3 实际样品提取实验

称取黑色石灰土、棕色石灰土、黄色石灰土和红色石灰土:全土、POM 及 MAOM 各 0.5g,置于 15mL 无菌离心管中。提取条件:优化前 121℃、40min;优化后:全土 121℃、40min;POM 和 MAOM 123℃、80min。样品开盖放入高温灭菌锅。其余步骤同 1.4.1 节。

1.5 EE-GRSP 测定

EE-GRSP 含量测定参照 Bradford 蛋白测定法[36]。样品稀释 10 倍,加入 2mL 考马斯亮蓝 G-250 染色剂,显色 5min 后于 595nm 波长下比色[37-39]。用牛血清白蛋白(BSA)作标准液,考马斯亮蓝法显色,绘制标准曲线,以 1.00g 风干土壤中蛋白质的毫克数表示 EE-GRSP 的含量。

2 结果与讨论

2.1 影响 EE-GRSP 提取因素的探究

温度和时间都是影响 EE-GRSP 提取的因素。严苛的温度条件使 EE-GRSP 提取过程中多数蛋白质组分变性,一定程度上减少了其他物质的干扰。

由于未有对 EE-GRSP 提取过程中样品管开盖/关盖的明确规定。棕色石灰土和红色石灰土作为研究对象,一半样品进行开盖处理,一半样品进行关盖处理,进而对比棕色与红色石灰土 EE-GRSP 含量(图 1)。关盖后,棕色石灰土的 EE-GRSP 含量从 0.591mg/g 降至 0.105mg/g;红色石灰土的 EE-GRSP 含量从 0.071mg/g 降至 0.011mg/g。EE-GRSP 含量显著降低,约为开盖提取量的 10%。从提取量的差异可以看出,与环境接触的充分程度影响了样品的提取量。开盖时样品与环境之间既有物质的传递,也有以热和功的形式传递能量[40],能更快地达到目标温度,以至于管内温度和压强都与灭菌锅内保持一致。关盖阻碍了管内外的能量传递,影响 GRSP 提取过程,进一步导致 GRSP 提取量降低。

固定 121℃ 为提取温度,进行 40min、60min 和

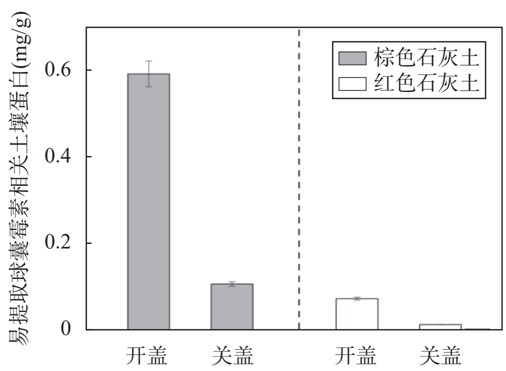


图1 开盖/关盖条件下棕色石灰土、红色石灰土中易提取球囊霉素相关蛋白含量

Fig. 1 EE-GRSP content of brown calcareous soil and red calcareous soil under open/closed lid condition.

80min 时间梯度提取实验。棕色石灰土和红色石灰土样品，随着提取时间的延长，EE-GRSP 含量相应增加 (图 2)。初步认为，提取温度为 121℃ 时，棕色石灰土-颗粒态有机质在 60min 处达到提取量的峰值；红色石灰土-颗粒态有机质呈现出 EE-GRSP 含量与提取时间同步升高趋势。从该结果可以看出，提取时间的延长在一定程度上可以提高 EE-GRSP 提取量。谢小林等[41]指出，在 GRSP 提取定量前进行高压灭菌，测量值显著提高，该结果与本文延长提取时间效果一致。

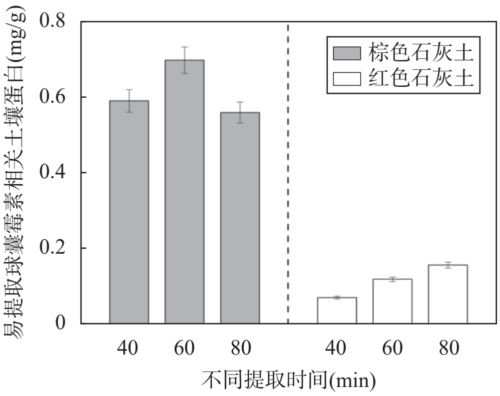


图2　不同提取时间下棕色石灰土、红色石灰土的 EE-GRSP 含量

Fig. 2　EE-GRSP content of brown calcareous soil and red calcareous under different extraction time.

2.2　提取时间和温度正交优化结果

探明提取温度、时间是 EE-GRSP 提取过程的关键因素后，以棕色石灰土、红色石灰土全土与 POM 为研究对象，设置 121℃、123℃、125℃ 温度梯度和 40min、60min、80min 时间梯度进行正交实验，每个提取条件设置三个平行处理，结果见表 1。对比 EE-GRSP 提取量，POM 在 123℃、80min 条件下，提取量达到峰值，显著高于其他提取条件 ($p<0.05$)。棕色石灰土和红色石灰土全土的 EE-GRSP 没有呈现一致的规律，为此本课题组还是建议采用 121℃、40min 的提取方法。

2.3　实际样品提取结果

为探究筛选出的提取条件对实际样品的提取效果，对石灰土演替过程中的四类石灰土 POM 与 MAOM 进行 EE-GRSP 提取。实验分为两部分：未优化条件提取与优化条件提取。对 POM(图 3a) 和 MAOM(图 3b) 使用未优化条件(121℃，40min)和优化条件(123℃，80min)分别进行提取。

与未优化前相比，提高提取温度和增加提取时间后，黑色石灰土、棕色石灰土、黄色石灰土样品的 EE-GRSP 含量增加。黑色石灰土-颗粒态有机质的 EE-GRSP 含量由 0.833mg/g 增加至 1.118mg/g；红色石灰土 POM、MAOM 和全土的 EE-GRSP 含量皆低于优化前。这是由于岩溶环境具有土层浅薄、土被不连续、岩石裸露等异质性强的特点[42]。此外，长期雨水淋溶使初育的黑色石灰土向棕色石灰土、黄色石灰土、红色石灰土等土壤类型演替。红色石灰土形成于古风化壳的基础上，土壤富铝化作用明显，蛭石减少，并有大量三水铝矿形成。土壤受到强烈的淋溶作用，盐类大量淋失，有机质的含量较低，因此异质性较强[43-45]。红色石灰土处于石灰土演替的最终阶段，在该阶段土壤钙离子大量流失，土壤中游离状态 EE-GRSP 没有被完好地保存。因此，

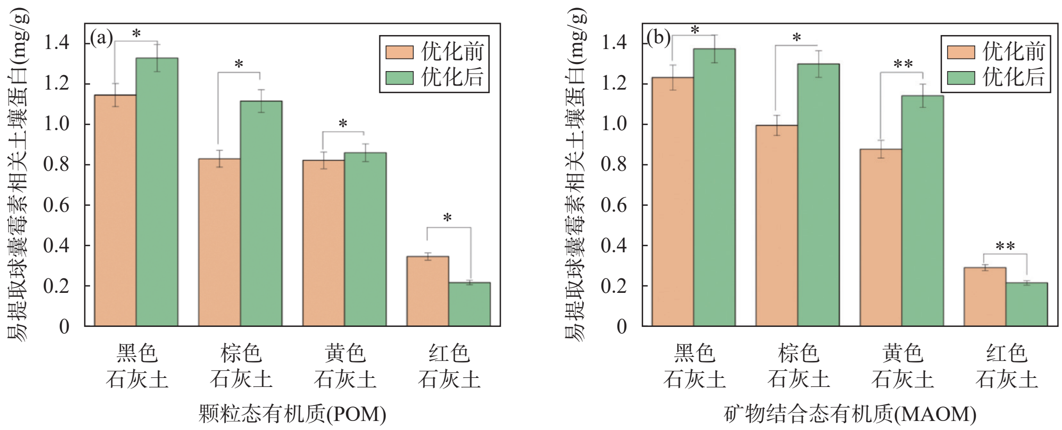
表 1　不同提取时间和提取温度下棕色石灰土、红色石灰土的 EE-GRSP 提取量

Table 1　EE-GRSP content of brown calcareous soil and red calcareous soil at different extracting time and temperatures.

提取条件	EE-GRSP 提取量 (mg/g)			
	棕色石灰土-颗粒态有机质	红色石灰土-颗粒态有机质	棕色石灰土-全土	红色石灰土-全土
121℃，40min	$0.456±0.070^{d}$	$0.288±0.02^{de}$	$0.7±0.028^{d}$	$0.276±0.012^{cd}$
121℃，60min	$0.688±0.045^{c}$	$0.45±0.027^{b}$	$0.659±0.039^{d}$	$0.467±0.083^{a}$
121℃，80min	$0.733±0.128^{bc}$	$0.38±0.034^{c}$	$0.89±0.082^{c}$	$0.374±0.065^{abc}$
123℃，40min	$0.641±0.083^{cd}$	$0.347±0.023^{cd}$	$0.91±0.074^{bc}$	$0.397±0.062^{ab}$
123℃，60min	$0.610±0.025^{cd}$	$0.294±0.025^{de}$	$1.05±0.041^{ab}$	$0.321±0.066^{bcd}$
123℃，80min	$0.963±0.054^{a}$	$0.594±0.085^{a}$	$1.126±0.114^{a}$	$0.388±0.106^{ab}$
125℃，40min	$0.581±0.052^{cd}$	$0.269±0.019^{e}$	$0.723±0.027^{d}$	$0.256±0.014^{d}$
125℃，60min	$0.892±0.021^{ab}$	$0.387±0.027^{c}$	$1.158±0.164^{a}$	$0.439±0.01^{a}$
125℃，80min	$0.729±0.237^{bc}$	$0.306±0.016^{de}$	$0.653±0.078^{d}$	$0.302±0.005^{bcd}$

注：同列不同小写字母表示差异显著 ($p<0.05$)，含相同字母表示差异不显著。

Note: Different lowercase letters in the same column indicate significant difference ($p<0.05$), and the same letters indicate no significant difference.



优化前：121℃ 和 40min; 优化后：123℃ 和 80min。

图3　四类石灰土颗粒态有机质 (a)、矿物结合态有机质 (b) 的 EE-GRSP 含量对比

Fig. 3　Comparison of EE-GRSP content from POM (a) and MAOM (b) in four types of calcareous soils. Before optimization: 121℃ and 40min. After optimization: 123℃ and 80min.

在应用优化条件提取时, EE-GRSP 含量没有显著增加。

对提取条件进行单一调整后, 不同类型土壤的 EE-GRSP 多集中于 0.5 ~ 1.0mg/g; 何开平等[46]通过延长提取时间, 发现非岩溶土壤 EE-GRSP 提取量为 0.5 ~ 0.6mg/g。沈育伊等[47]提取的棕色石灰土 EE-GRSP 含量在 0.4 ~ 1.17mg/g 之间。通过提取方法的优化, 石灰土演替过程中四种类型土壤的 EE-GRSP 含量最高可达 1.375mg/g。MAOM 中 EE-GRSP 含量的增加, 可能是因为土壤有机质主要通过矿物结合态与团聚体形态保持稳定[48]; GRSP 与土壤矿物之间发生离子交换和表面络合作用[49], 筛分后的 MAOM 相较于全土具有更高的稳定性与更强的有机碳保护能力。随着温度和提取时间的提高, GRSP 与矿物、微生物等形成的稳定结构被打破, MAOM 中的 EE-GRSP 得以更完全地释放, 表现为 EE-GRSP 的含量大量增加。

MAOM 中 EE-GRSP 含量可以很好地验证本课题组提出的假设: 对于不同土壤组分, 需要区分 EE-GRSP 提取条件。对于细分后的 POM 和 MAOM 样品, 更高的温度和更长的提取时间可以增加 EE-GRSP 提取量。黑色石灰土-矿物结合态有机质、棕色石灰土-矿物结合态有机质、黄色石灰土-矿物结合态有机质于 123℃、80min 条件下提取, 其 EE-GRSP 含量相较于 121℃、40min 都显著升高。黑色石灰土-矿物结合态有机质的 EE-GRSP 含量由 1.233mg/g 增加至 1.375mg/g, 棕色石灰土-矿物结合态有机质的 EE-GRSP 含量由 0.997mg/g 上升至 1.300mg/g, 黄色石灰土-矿物结合态有机质的 EE-GRSP 含量从 0.879mg/g 增加至 1.143mg/g, 三类样品 EE-GRSP 增量约 11.5% ~ 30.4%。相较于 POM 样品, MAOM 样品的 EE-GRSP 增幅更大。

3　结论

依据粒径将岩溶区石灰土分为 POM 和 MAOM, 通过正交实验对其不同提取时间和温度下的 EE-GRSP 提取量进行比较, 筛选出适用于 POM 和 MAOM 的提取条件: 123℃、80min, 区别于前人使用的 121℃ 条件。通过黑色、棕色、黄色和红色等四种具有代表性的石灰土实际应用, 优化后黑色、棕色和黄色石灰土中 POM 和 MAOM 的 EE-GRSP 含量显著增加, MAOM 的 EE-GRSP 增量皆高于 POM, 表明矿物结合态有机质具有更高的有机质含量与更强的有机质保护能力。

EE-GRSP 作为土壤有机碳库的重要组成部分, 其条件优化为探究 GRSP 生态功能、揭示岩溶区土壤有机碳稳定机制的深入研究提供支持。由此可见, 对岩溶土壤不同粒径的有机质组分应当使用不同的提取条件, 因此本文将岩溶石灰土划分为 POM 和 MAOM 进行条件优化。此外, 未来研究还可以对不同类型、不同粒径土壤的提取条件进行深入优化。

Optimization of Extraction Method for Easily Extractable Glomalin-Related Soil Protein from Calcareous Soil

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HIGHLIGHTS

(1) The amount of easily extractable glomalin-related soil protein from calcareous soil will be increased by increasing extraction temperature and prolonging extraction time.

(2) An optimization condition was selected and applied to four types of calcareous soil based on orthogonal experiments, which verifies the experimental hypothesis.

(3) The extraction condition about easily extractable glomalin-related soil protein from particulate organic matter and mineral-associated organic matter is different from bulk soil, so it is necessary to increase extracting temperature and prolong extracting time.

ABSTRACT: Glomalin-related soil protein (GRSP) secreted by arbuscular mycorrhizal fungi is widely distributed worldwide, has stable properties and is not easily decomposed, which is an important component of soil organic matter. Extracting high quality GRSP is important for in-depth research on organic carbon sinks in calcareous soil from karst areas. However, in previous studies, the mechanism of GRSP in organic matter could not be further studied due to low extraction yield, insufficient extraction and non-specific products. Therefore, the high extraction amount of GRSP is of great significance to explore the formation and stabilization mechanism of organic matter in calcareous soil. This experiment selected four types of calcareous soil from karst areas: black, brown, yellow and red calcareous soil. By using orthogonal experiments of temperature and time, the optimal extraction conditions for easily extractable glomalin-related soil protein (EE-GRSP) related to particulate organic matter (POM) and mineral-associated organic matter (MAOM) in calcareous soil were selected. The experimental results showed that the highest extraction amount of EE-GRSP was achieved when POM and MAOM were extracted at 123℃ and 80min. After application into the four types of calcareous soil, the EE-GRSP contents increased from 4.6% to 34.2%. The BRIEF REPORT is available for this paper at http://www.ykcs.ac.cn/en/article/doi/10.15898/j.ykcs.yk202402060015.

KEY WORDS: easily extractable glomalin-related soil protein; optimization of extraction method; particulate organic matter; mineral-associated organic matter

BRIEF REPORT

Significance: Under the interaction of water-carbon dioxide-carbonate rocks-organisms, a global karst carbon sink of 8.24×10^8t C/a is formed. Some of the carbon sinks were sequestered in the form as soil organic matter, which plays an important role in the dual carbon goals. Glomalin-related soil protein, as a part of soil organic carbon, is stable and difficult to decompose, and plays an important role in soil carbon sequestration[6]. The extraction process of GRSP remains inadequate. Many scholars have improved the extraction time and centrifugal force, but few of them have optimized the extraction temperature and time by orthogonal optimization. After a single adjustment of the extraction condition, EE-GRSP from soils was mostly concentrated in 0.5−1.0mg/g[46-47]. In this study, temperature, time experiment and two-factor orthogonal experiments were carried out sequentially. After an optimized condition was selected, four types of calcareous soil from the karst area were used to verify the experimental hypothesis. By using this optimization extracting method, EE-GRSP content from calcareous soil can reach up to 1.375mg/g.

Methods: Black, brown, yellow, and red calcareous soil were collected in Nonggang Nature Reserve in Guangxi, China. Bulk samples were mixed well by removing debris such as gravel and stubs. Bulk soil was air-dried and sieved through a 10-mesh sieve. POM and MAOM were obtained by applying the wet sieving method. The 0.5g samples were placed in a 15mL centrifuge tube and parallel samples were set up. According to the ratio of sample and extraction solution (1∶8), 20mmol/L sodium citrate solution (pH=7) was added into the centrifuge tube. Samples were put into an autoclave with the lid open and extracted for 80min at 123℃, and then centrifuged at 9500r/min for 10min after sterilization. The supernatant was retained at 4℃ for the determination of EE-GRSP content.

Data and Results: (1) The main factors affecting EE-GRSP extraction. Sterilization samples were used with open or closed lids. After the lid was closed, EE-GRSP content decreased from 0.591mg/g to 0.105mg/g in brown calcareous soil and in red calcareous soil from 0.071mg/g to 0.011mg/g, as shown in Fig.1. With the extension of extraction time, EE-GRSP content increased in the brown calcareous soil and red calcareous soil sample, as listed in Fig.2.

(2) Results of time and temperature orthogonal optimization experiment. By comparing the extracted amount of EE-GRSP, POM peaked at 123℃ and 80min, which was significantly higher than the other extraction conditions (p<0.05), as seen in Table 1.

(3) Extraction results of actual samples. After increasing the extraction temperature and time, EE-GRSP content of the black calcareous soil-particulate organic matter increased from 0.833 to 1.118mg/g. The previous studies indicated that the extraction amount of EE-GRSP from non-karst area soil was 0.5−0.6mg/g[46], and the EE-GRSP content of brown calcareous soil was 0.4−1.17mg/g[47]. In this study, the EE-GRSP contents of POM and MAOM in black, brown and yellow calcareous soil were significantly increased about 11.5%−30.4% by using this optimization extraction method, as shown in Fig.3. Compared to bulk soil, MAOM had higher stability and stronger organic carbon protection ability. Therefore, by using increased temperature and extraction time, the EE-GRSP content in MAOM is completely released, and the extraction amount can be significantly increased.

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