Original article

Visualizing the spatial distribution and alteration of metabolites in continuously cropped Salvia miltiorrhiza Bge using MALDI-MSI

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Salvia miltiorrhiza Bge (SMB) has long been used in traditional Chinese medicine to treat cardiovascular and cerebrovascular diseases. Growing clinical usage has led to a huge demand for artificial planting of SMB. Thus, continuous cropping of SMB is an important challenge that needs to be addressed. Continuous cropping can alter the metabolic profile of plants, resulting in poor growth and low yield. In this study, we tried to image the spatial location and variation of endogenous metabolites in continuously cropped SMB using matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI). Spatially resolved expressions of tanshinones, salvianolic acids, polyamines, phenolic acids, amino acids, and oligosaccharides in normal and continuously cropped SMB roots were compared. The expressions of dihydrotanshinone I, tanshinone II A, dehydromiltirone, miltirone, dehydrotanshinone IIA, spermine, salvianolic acid B/E, tetrasaccharide, and pentasaccharide in continuously cropped SMB roots were much lower than those in normal roots. There was little difference in the expressions of caffeic acid and salvianolic acid A in normal and continuously cropped SMB roots. Ferulic acid was much more widely distributed in xylem of normal SMB but strongly expressed in xylem, phloem, and cambium of continuously cropped SMB. The spatially resolved metabolite information enhances our understanding of the metabolic signature of continuously cropped SMB and also provides insights into the metabolic effects of continuous cropping in other plants.

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1. Introduction

Salvia miltiorrhiza Bge (SMB) is a high-value medicinal plant that is widely used in China and many other countries worldwide [1,2]. The dried root and rhizome of SMB have long been used to treat cardiovascular and cerebrovascular diseases, diabetes, and liver fibrosis [3–5]. However, continuous cropping of SMB in the same land can easily cause continuous cropping obstacles, which in turn lead to poor growth, serious insect diseases, and low yield [6]. Moreover, continuous cropping obstacles also lead to significant changes in the metabolite profile of SMB. There is growing evidence that the roots of plants can secrete metabolites into the surrounding environment during their growth and that the secreted metabolites can change the properties of the soil as well as its microbial communities [7–9]. Altered soil and microbial environment have been recognized as important contributors to continuous cropping obstacles [10,11]. Therefore, exploring changes in metabolites because of continuous cropping of SMB will greatly enhance our understanding of the metabolic mechanisms underlying SMB continuous cropping obstacles.

Liquid chromatography has been previously used to characterize metabolic changes because of continuous cropping of SMB. For example, Liu et al. [6] investigated the impacts of continuous planting on the appearance and active metabolite contents of SMB roots. The tanshinone and salvianolic acid contents, weight of fresh roots, and main root diameter exhibited a tendency to decline with the increasing continuous planting years. We also compared the expression of dihydrotanshinone, cryptotanshinone, tanshinone IIA, miltirone, rosmarinic acid, and salvianolic acid B in normal and...
continuous-cropping SMB [12]. The levels of most of the above compounds were significantly downregulated in the continuously cropped SMB. However, plant metabolic networks are complex. In addition to tanshinones and salvianolic acids, SMB contains amino acids, low-molecular-weight organic acids, polyamines, carbohydrates, and other metabolites. Whether continuous cropping of SMB leads to any changes in these metabolites is still unknown. More importantly, the SMB root is highly heterogeneous, and consists of epidermis, cortex, pith, xylem, and cambium. The synthesis and accumulation of metabolites in different parts of SMB are also different. Therefore, a technique that enables a direct comparison and mapping of metabolites in normal and continuously cropped SMB will enhance our understanding of the metabolic alterations of SMB due to continuous cropping.

Matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI) can simultaneously map the spatial distribution and characterize the relative contents of metabolites [13–21], thus offering a non-targeted approach to the screening of differentially expressed metabolites in plant tissue sections. Previously, we developed a highly sensitive MALDI-MSI method and successfully imaged the distribution of amino acids, polyamines, phenolic acids, fatty acids, oligosaccharides, and tanshinones in normal SMB roots and stems [22]. Here, we used the MALDI-MSI technique to map and compare the expressions of metabolites in normal and continuously cropped SMB. Unsupervised principal component analysis showed that the underlying metabolite profiles of normal and continuously cropped SMB varied greatly. Furthermore, we performed targeted MALDI-MSI experiments and screened out many key metabolites that differentially expressed between normal and continuously cropped SMB. The expressions of dihydrotanshinone I, tanshinone II A, dehydromiltirone, miltirone, dehydrotanshinone IIA, spermine, spermidine, salvianolic acid B/E, tetrasaccharide, and pentasaccharide in continuously cropped SMB significantly downregulated compared with those in normal SMB. The expressions of some salvianolic acids (such as salvianolic acid A) and some low-molecular-weight phenolic acids (such as caffeic acid) in normal and continuously cropped SMB showed little difference. Ferulic acid mainly expressed in the xylem of normal SMB but strongly expressed in the xylem, phloem, and cambium of continuously cropped SMB. The expression of phenylalanine in continuously cropped SMB was much higher than that in normal SMB. The expression of phenylalanine in continuously cropped SMB is higher than that in normal SMB. However, plant metabolic networks are complex. In addition to tanshinones and salvianolic acids, SMB contains amino acids, low-molecular-weight organic acids, polyamines, carbohydrates, and other metabolites. Whether continuous cropping of SMB leads to any changes in these metabolites is still unknown.

### 2. Materials and methods

#### 2.1. Solvents and reagents

1,5-naphthalenediamine (1,5-DAN) and 1,1'-binaphthyl-2,2'-diamine (BNDM) were purchased from Aladdin Reagent (Shanghai, China). Formaldehyde-acetic acid-ethanol fixative (70% ethanol), safranin O stain dye, and fast green (SOFG) dye were obtained from Servicebio Biological Technology Co., Ltd. (Wuhan, China). Trifluoroacetic acid (TFA) was purchased from Sigma (St. Louis, MO, USA). Acetonitrile (ACN) was obtained from Tedia (Fairfield, OH, USA). Purified water was purchased from Wahaha (Hangzhou, China). Cryo-Gel compound media used for embedding frozen tissues were provided by Leica Biosystems Richmond, Inc. (Richmond, IL, USA).

#### 2.2. Sample preparation

Normal and continuously cropped SMB roots were collected from Laigu Ecology Park Co., Ltd. (Jinan, China). The photos of normal and continuously cropped SMB are shown in Fig. S1. Fresh SMB roots were stored at ~80 °C. Prior to MALDI-MSI analysis, the roots of normal and continuously cropped SMB were cut into 12 μm tissue sections at ~20 °C using a cryostat microtome (NX50; Thermo Fisher Scientific Inc., Bremen, Germany). One set of root sections was fixed in formaldehyde-acetic acid-ethanol fixative and then dyed with SOFG for histological observation. Two sets of root sections were vacuum-dried in a desiccator for approximately 15 min before matrix coating and MALDI-MSI analysis.

#### 2.3. Matrix coating

1,5-DAN and BNDM were optimized as MALDI matrices in positive ion mode and negative ion mode, respectively. 1,5-DAN (2.5 mg/mL) and BNDM (1.0 mg/mL) in ACN/H2O/TFA (70:30:0.1, V/V/V) was sprayed onto the surface of the SMB root section using HTX TM-Sprayer™ (HTX Technologies, Carrboro, NC, USA). The flow rate of the matrix solution was 50 μL/min. The nozzle temperature and nitrogen gas pressure were set to 60 °C and 10 psi, respectively. The track spacing and track speed of the spray solvent were set to 3 mm and 800 mm/min, respectively. The nozzle-to-target distance was 4 cm. Six passes of 1,5-DAN and 12 passes of BNDM were made over each SMB root section.

#### 2.4. MALDI-MSI

After coating the matrix, MALDI-MSI analysis was carried out using a rapifleX MALDI Tissuetyper™ TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). The repetition rate of the beam laser was 5000 Hz. The number of laser shots was 200, and the beam parameter was selected as a single. The spatial resolution...
was 75 \( \mu \text{m} \). SciLS Lab 2018b software (GmbH, Bremen, Germany) and FlexImaging 5.0 software (Bruker Daltonics, Bremen, Germany) were used to construct MALDI-MS images of metabolites in SMB root sections. To compare the spatial characteristics and relative content of different metabolites in normal and continuously cropped SMB roots more intuitively, we imported the MSI data of normal and continuously cropped SMB roots into one.sl file using the SciLS Lab software.

3. Results and discussion

3.1. Imaging the spatial distribution of metabolites in the roots of continuously cropped SMB

As a highly heterogeneous plant tissue, SMB roots can be divided into different microregions according to cell components, namely, epidermis, cortex, pith, xylem, and cambium. Fig. 1A shows the SOFG stain image of the continuously cropped SMB root section. In a previous study, we developed a highly sensitive MALDI-MSI method to map the locations of functional metabolites in normal SMB roots [22]. Here, we carried out MALDI-MSI experiments on continuously cropped SMB roots, and different kinds of metabolites, such as tanshinones (Fig. 1B), salvianolic acids (Fig. 1C), oligosaccharides (Fig. 1D), fatty acids (Fig. 1E), cholines (Fig. 1F), organic acids (Fig. 1G), and polyamines (Fig. 1H), were successfully detected. Miltirone (Fig. 1B, \([\text{M}+\text{H}]\), \(m/z\) 283.2) exhibited higher ion intensity in the epidermis and outer cortex regions. Dihydrotanshinone I (Fig. 1B, \([\text{M}+\text{H}]\), \(m/z\) 279.1) showed higher ion intensity in epidermis, cortex, and xylem regions. The levels of salvianolic acid A (Fig. 1C, \([\text{M}+\text{H}]\), \(m/z\) 493.1) and salvianolic acid B/E (Fig. 1B, \([\text{M}+\text{H}]\), \(m/z\) 717.1) in cambium were much lower than those in epidermis, cortex, pith, and xylem regions. Oligosaccharides such as tetrasaccharide (Fig. 1D, \([\text{M}+\text{H}]\), \(m/z\) 665.2) and pentasaccharide (Fig. 1D, \([\text{M}+\text{H}]\), \(m/z\) 827.3) exhibited higher intensity in xylem and cortex regions. Fatty acids (FAs) are essential for energy metabolism. MALDI-MSI results indicated that FA-16:0 (Fig. 1E, \([\text{M}+\text{H}]\), \(m/z\) 255.2) and FA-18:1 (Fig. 1E, \([\text{M}+\text{H}]\), \(m/z\) 281.2) were more abundant in cortex. Choline (Fig. 1F, \([\text{M}+\text{H}]\), \(m/z\) 104.1) showed strong ion signals in different regions of the continuously cropped SMB root. Phosphocholine (Fig. 1F, \([\text{M}+\text{H}]\), \(m/z\) 184.1) was more concentrated in the outer cortex and xylem. Succinic acid (Fig. 1G, \([\text{M}+\text{H}]\), \(m/z\) 117.0) and malic acid (Fig. 1G, \([\text{M}+\text{H}]\), \(m/z\) 133.0) were mainly in the cortex region.

Spermine and spermidine are two important polyamines in plants, and growing evidence indicates that polyamines play indispensable roles in plant growth and external stress responses [23,24]. Here, the MALDI-MSI data suggest that the expressions of spermidine (Fig. 1H, \([\text{M}+\text{H}]\), \(m/z\) 146.2) and spermine (Fig. 1H, \([\text{M}+\text{H}]\), \(m/z\) 203.2) in the xylem and outer cortex of continuously cropped SMB roots were much higher than those in other root tissue regions. This is inconsistent with our previous study results, which showed that polyamines mainly expressed in the cortex of normal SMB roots [22]. We then collected normal SMB from the same ecological garden and explored the spatial and content differences in polyamines between normal and continuously cropped SMB roots. Fig. 2 shows the MS images and intensity box plots of

![Fig. 2](image-url)
spermine in continuously cropped and normal SMB roots. This suggests that the expression of spermine in continuous-cropping samples was significantly lower than in normal samples. Considering the crucial biological effects of polyamines in plants, significantly downregulated levels of polyamines in continuously cropped SMB may be one of the reasons for the decrease in the growth of root branches, low vitality, and slow growth rate.

3.2. Imaging the metabolic differences between normal and continuously cropped SMB

Accumulating evidence suggests that the metabolic profile of plants changes significantly when continuous-cropping obstacles occur [25,26]. However, the metabolic networks of organisms are extremely complex. In addition to the above-mentioned abnormal levels of polyamines, there may be other metabolic changes after the occurrence of SMB continuous-cropping obstacles. Here, to explore the metabolic changes in continuously cropped SMB, we selected normal and continuously cropped SMB roots with similar diameters to perform MALDI-MSI analysis (Fig. 3A). Then, in situ MALDI-MS data of normal and continuously cropped SMB were extracted to perform unsupervised principal component analysis (PCA)(Fig. 3B). The PCA score plots showed an obvious separation between normal and continuously cropped SMB, suggesting significant metabolite alterations in continuously cropped SMB.

Tanshinone, an important phenanthraquinone compound in SMB, possesses functional activity in treating cerebrovascular and cardiovascular diseases, and tanshinone content is an indispensable index for evaluating the quality of SMB [27]. After performing MALDI-MSI analysis, we extracted the MS images of tanshinones in

![A] [M–H] MS image of tetrasaccharide, (B) [M–Na] MS image of tetrasaccharide, (C) [M+K]+ MS image of tetrasaccharide, (D) [M–H] MS image of pentasaccharide, (E) [M–Na] MS image of pentasaccharide, and (F) [M+K]+ MS image of pentasaccharide. ***P < 0.001.

Fig. 4. Mass spectrometry (MS) images of [M–H] [M+Na]+ [M+K]+ of tetrasaccharide and pentasaccharide in normal and continuously cropped (CTCP) Salvia miltiorrhiza Bge (SMB) roots. ***P < 0.001. (A) [M–H] MS image of tetrasaccharide, (B) [M–Na] MS image of tetrasaccharide, (C) [M+K]+ MS image of tetrasaccharide, (D) [M–H] MS image of pentasaccharide, (E) [M–Na] MS image of pentasaccharide, and (F) [M+K]+ MS image of pentasaccharide. ***P < 0.001.

Fig. 5. Mass spectrometry (MS) images of (A) salvianolic acid A, (B) salvianolic acid H/I, (C) salvianolic acid B/E, (D) ferulic acid, (E) salvianic acid A, (F) caffeic acid, (G) glutamate, and (H) phenylalanine in normal and continuously cropped (CTCP) Salvia miltiorrhiza Bge (SMB) roots. ***P < 0.001; NS: not significant.

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normal and continuously cropped SMB. As shown in Figs. 3C–G, the contents of all tanshinones, including dihydrotanshinone I (Fig. 3C), tanshinone IIA (Fig. 3D), dehydrodimiltirone (Fig. 3E), miltirone (Fig. 3F), and dehydrotanshinone IIA (Fig. 3G), in continuously cropped SMB were much lower than those in normal SMB. Moreover, the spatial characteristics of miltirone and dehydrotanshinone IIA in continuously cropped SMB were obviously different from those of normal SMB. Miltirone and dehydrotanshinone IIA were mainly present in the epidermis and outer cortex regions of normal SMB, and their distribution in continuously cropped SMB showed little difference (Figs. 3F and G).

As low-molecular-weight saccharide polymers, oligosaccharides are mainly derived from plant cell walls and fungal cell walls [28]. Previous studies have demonstrated that oligosaccharides play important roles in regulating plant development, mediating cell signal transduction, and activating the plant immune system to increase disease resistance [28–31]. In this study, the relative contents and spatial distributions of some oligosaccharides in normal and continuously cropped SMB were successfully characterized using MALDI-MSI. As shown in Figs. 4A and D, the contents of tetrasaccharide (Fig. 4A, [M−H]−, m/z 665.2) and pentasaccharide (Fig. 4D, [M−H]−, m/z 827.3) in continuously cropped SMB significantly decreased by 96.9 and 66.4 times in continuously cropped SMB, respectively (Figs. 4A and C), while the ion signal of [M+Na]+ in tetrasaccharide only decreased by 5.4 times (Fig. 4B). Similarly, the ion signals of [M−H]−, [M+Na]+, and [M+K]+ in pentasaccharide also showed this trend (Figs. 4D–F). This result suggested that the balance between sodium and potassium ions in continuously cropped SMB might be disrupted and that the content of sodium ions in continuously cropped SMB increased. Coincidently, there is growing evidence that the accumulation of sodium ions in plant cells results in deleterious effects on cell metabolism [32,33]. Therefore, abnormally elevated sodium ions may be another reason that continuously cropped SMB is prone to severe diseases, thus resulting in reduced yield.

Salvianolic acid is another effective compound in SMB, which possesses properties like antioxidation, free radical scavenging, and inhibition of cell apoptosis [34]. The spatial distribution and relative contents of representative salvianolic acids in normal and continuously cropped SMB were imaged and are shown in Figs. 5A–C. The results indicated that there was no difference in the expression of salvianolic acid A (Fig. 5A, [M−H]−, m/z 493.1) between normal and continuously cropped SMB. The expressions of salvianolic acid H/I (Fig. 5B, [M−H]−, m/z 537.1) and salvianolic acid B/E (Fig. 5C, [M−H]−, m/z 717.1) in continuously cropped SMB were much lower than those in normal SMB. Low-molecular-weight phenolic acids have been reported to be a class of plant autotoxic compounds that can cause diseases in continuously cropped plants [35,36]. Three low-molecular-weight phenolic acids, namely, ferulic acid (Fig. 5D, [M−H]−, m/z 193.1), salvianolic acid A (Fig. 5E, [M−H]−, m/z 197.1), and caffeic acid (Fig. 5F, [M−H]−, m/z 179.0), were detected and imaged. Ferulic acid and salvianolic acid A showed stronger ion intensities in continuously cropped SMB than in normal SMB. Moreover, it should be noted that ferulic acid was mainly located in the xylem regions of normal SMB, but there was almost no difference in the distribution of ferulic acid in various regions of continuously cropped SMB roots (Fig. 5D). The content of caffeic acid in continuously cropped SMB showed little difference compared to that in normal SMB (Fig. 5F). Additionally, the spatial distribution and relative content of some amino acids were examined in this study. As shown in Fig. 5G, glutamate ([M−H]−, m/z 146.0) was more prevalent in cambium and xylem regions of normal SMB, but was more distributed in xylem and outer phloem regions of continuously cropped SMB. The level of phenylalanine (Fig. 5H, [M−H]−, m/z 164.0) in continuously cropped SMB was significantly higher than that in normal SMB.

We also screened some unknown endogenous metabolites specifically distributed in normal and continuously cropped SMB. Although we failed to identify the structures of these endogenous metabolites, their expressions in normal and continuously cropped SMB varied greatly. For example, the ions of m/z −827.2 and m/z −763.2 were almost exclusively expressed in normal SMB (Figs. 6A and B). Furthermore, receiver operating characteristic (ROC) curve analysis was used to evaluate the levels of m/z −827.2 and m/z −763.2 and discriminate normal and continuously cropped SMB. As shown in Figs. 6A and B, the ROC curve generated from m/z −827.2 and m/z −763.2 showed good differential power with area under curve (AUC) values of 0.992 and 0.984, respectively. The ions of m/z +438.2 were specifically expressed in continuously cropped SMB. The AUC value of the ROC curve built based on the expression of m/z +438.2 was 0.971. In conclusion, this study demonstrated that continuously cropped SMB was prone to severe diseases, thus resulting in reduced yield.
+438.2 in normal and continuously cropped SMB was 0.971, suggesting good discrimination ability of m/z +438.2 (Fig. 6C). The level of m/z +171.1 in continuously cropped SMB was much higher than that in normal SMB, and the AUC value of the ROC curve built based on m/z +171.1 reached 0.927 (Fig. 6D).

4. Conclusion

We successfully mapped the spatial location and variation of metabolites in continuously cropped SMB roots using MALDI-MSI. Compared with normal SMB, continuously cropped SMB had serious metabolic alterations. The expressions of some tanshinones, such as dihydrotanshinone I, tanshinone II A, dehydrortanshinone IIA, in continuously cropped SMB were much lower than those in normal SMB. Levels of polyamines such as spermine and spermidine, which are closely related to plant growth, were significantly downregulated in continuously cropped SMB. The levels of oligosaccharides involved in mediating plant cell signal transduction and activation of the plant immune system in continuously cropped SMB were also significantly lower than those in normal SMB. Salvianolic acid B/E level was significantly downregulated in continuously cropped SMB. This spatially resolved metabolite information enhances our understanding of the metabolic profile of continuously cropped SMB and also provides insight into the metabolic mechanisms of continuous-cropping obstacles in other plants.

CRediT author statement

Chenglong Sun: Methodology, Visualization, Writing - Original draft preparation, Reviewing and Editing; Li Cui: Data Curation, Writing - Reviewing and Editing; Bingqian Zhou: Formal analysis, Resources; Xiao Wang: Investigation, Supervision; Lanping Guo: Project administration, Supervision; Wei Liu: Resources, Project administration.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2021.09.011.

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