LAESI mass spectrometry imaging as a tool to differentiate the root metabolome of native and range-expanding plant species

Purva Kulkarni¹ · Rutger A. Wilschut¹ · Koen J. F. Verhoeven¹ · Wim H. van der Putten^{1,2} · Paolina Garbeva¹

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Abstract

Main conclusion LAESI-MSI, an innovative high-throughput technique holds a unique potential for untargeted detection, profiling and spatial localization of metabolites from intact plant samples without need for extraction or extensive sample preparation.

Our understanding of chemical diversity in biological samples has greatly improved through recent advances in mass spectrometry (MS). MS-based-imaging (MSI) techniques have further enhanced this by providing spatial information on the distribution of metabolites and their relative abundance. This study aims to employ laser-ablation electrospray ionization (LAESI) MSI as a tool to profile and compare the root metabolome of two pairs of native and range-expanding plant species. It has been proposed that successful range-expanding plant species, like introduced exotic invaders, have a novel, or a more diverse secondary chemistry. Although some tests have been made using aboveground plant materials, tests using root materials are rare. We tested the hypothesis that range-expanding plants possess more diverse root chemistries than native plant species. To examine the root chemistry of the selected plant species, LAESI-MSI was performed in positive ion mode and data were acquired in a mass range of m/z 50–1200 with a spatial resolution of 100 μm . The acquired data were analyzed using in-house scripts, and differences in the spatial profiles were studied for discriminatory mass features. The results revealed clear differences in the metabolite profiles amongst and within both pairs of congeneric plant species, in the form of distinct metabolic fingerprints. The use of ambient conditions and the fact that no sample preparation was required, established LAESI-MSI as an ideal technique for untargeted metabolomics and for direct correlation of the acquired data to the underlying metabolomic complexity present in intact plant samples.

Keywords Mass spectrometry imaging · Ambient imaging · Comparative metabolomics · Plant root · Metabolic profiling · Range expansion

Abbreviations

MS Mass spectrometry
MSI Mass spectrometry imaging
LAESI Laser-ablation electrospray ionization

CJ *Centaurea jacea* L
CS *Centaurea stoebe* L
GM *Geranium molle* L
GP *Geranium pyrenaicum* Burm
 m/z Mass by charge
GC Gas chromatography
TOF Time-of-flight
PCA Principal component analysis
PC Principal component
FC Fold change

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✉ Purva Kulkarni
P.Kulkarni@nioo.knaw.nl

¹ Netherlands Institute of Ecology (NIOO-KNAW),
Droevendaalsesteeg 10, 6708 PB Wageningen,
The Netherlands

² Laboratory of Nematology, Wageningen University
and Research Centre, PO Box 8123, 6700 ES Wageningen,
The Netherlands

Introduction

Detection of plant metabolites is extremely challenging, as there is no single-instrument platform available to effectively measure their overall coverage. During the last decade, mass spectrometry imaging (MSI) has emerged as a valuable tool, with numerous applications in the field of biological sciences. This analytical technique enables label-free, high-resolution spatial mapping of a large variety of biomolecules along with providing qualitative and quantitative chemical information, in a single experiment (Petras et al. 2017). Identical to traditional mass spectrometry, during MSI it is important to ionize the sample to form ions suitable for mass analysis. Different ionization methods exist for MSI; however, many of them require artificially altering the native biochemical status of the system under study, for example, by the application of a matrix, and are mainly operated under vacuum. Recently developed ambient ionization approaches such as laser-ablation electrospray ionization (LAESI) allow direct analysis of biological samples in a matrix-free, native atmospheric condition with minimal to no sample preparation, in a significantly shorter analysis time (Cooks et al. 2006; Wu et al. 2013). This opens up possibilities for in situ chemical analysis in biological systems.

LAESI-MSI is particularly tailored for biological samples that are rich in water content (Nemes and Vertes 2007). In this technique, the sample under investigation is mounted on a sample stage and is ablated using a focused mid-infrared laser pulse, under atmospheric conditions. This ablation ejects a mixture of molecules, clusters, and particulate matter in microscopic volumes from the sample, in the form of a plume. The catapulted biomolecules then coalesce with charged droplets, produced by an electrospray to become ionized (Chen et al. 2006; Chen and Vertes 2008). MSI using the LAESI ionization approach is realized by rastering the sample surface at pre-defined coordinates with a laser beam, where at each coordinate position the generated ions pass through the mass analyzer and a mass spectrum is recorded. LAESI-MSI has shown considerable success in revealing the lateral and cross-sectional distribution of primary and secondary metabolites for a range of plant-related samples, along with providing chemical information from deeper parts of the tissue section (Bjarnholt et al. 2014). LAESI equipped with a sharpened optical fiber tip has also been widely used to perform in situ metabolic profiling of single cells from plant and animal samples (Shrestha and Vertes 2009).

Here, we aim to demonstrate the potential of LAESI-MSI as an analytical technique for the direct metabolite profiling of plant samples. We applied LAESI-MSI in a comparative metabolic profiling study on two pairs of non-native, range-expanding plant species and congeneric native plant species.

In response to recent climate warming, many plant species have expanded their range to higher latitudes and altitudes (Walther et al. 2002; Le Roux and McGeoch 2008). It is thought that plant secondary chemistry is an important factor determining the invasive success of exotic plant species. The novel chemistry of invasive exotic plant species may effectively control defenses against insect herbivores and other natural enemies (Cappuccino and Arnason 2006). Such ‘novel chemistry’ has been shown to potentially suppress native plant species directly through allelopathy (Callaway and Aschehoug 2000) or indirectly through the suppression of the fungal mutualists of native plant species (Marler et al. 1999; Stinson et al. 2006). Moreover, due to this difference in chemistry, native generalist herbivores may perform less well on exotic plant species than on related native plant species (Schaffner et al. 2011), potentially leading to a reduced herbivore pressure on exotics compared to natives. The poor performance of generalist herbivores has also been linked to the high diversity of metabolites produced by exotic plant species compared to native plant species (Macel et al. 2014). This suggests that chemically diverse plant species may be prone to become abundant when they are introduced in a new area where the local herbivores are poorly adapted to neutralize, or circumvent the novel defenses.

In this study, we use LAESI-MSI as a high-throughput tool for untargeted comparative metabolomics of intact plant roots of native and range-expanding plant species. For this, we use two range-expanding plant species that are currently expanding in northwestern Europe, *Centaurea stoebe* L. and *Geranium pyrenaicum* Burm. f., and their respective congeneric native species *Centaurea jacea* L. and *Geranium molle* L. With this study, we demonstrate the suitability of LAESI-MSI for untargeted metabolomics profiling and we give insights in the potential chemical novelty of range-expanding plant species in comparison to congeneric-related native plant species.

Materials and methods

Plant species and root collection

The seeds used for all four plant species originated from natural populations in natural areas in The Netherlands, where the range expanders are immigrating. Seeds of *G. molle* and *C. stoebe* were collected directly from the field. For *C. jacea*, seeds were collected from plants growing in an experimental garden, whereas the mother plants were germinated from field-collected seeds. Seed production company Cruydt-hoeck (Groningen, The Netherlands), that grows plants originating from field-collected seeds, delivered the seeds for *G. pyrenaicum*. For all plant species, the seeds were surface-sterilized by washing for 3 min in a 10% bleach

solution, followed by rinsing with demineralized water, after which they were germinated on glass beads. After 20 days, the seedlings were collected for LAESI analysis.

LAESI mass spectrometry imaging

The LAESI-MSI of intact roots collected from the seedlings was carried out on a Protea Biosciences DP-1000 LAESI system (Protea Bioscience Inc., Morgantown) coupled to a Waters model Synapt G2S (Waters Corporation) mass spectrometer. The LAESI system was equipped with a 2940-nm mid-infrared laser yielding a spot size of 100 μm . The laser was set to fire ten times per x - y location (spot) at a frequency of 10 Hz and 100% output energy. The system was set to shoot at 105 locations per plant root (grid of 21×5 positions). A syringe pump was delivering the solvent mixture of methanol/water/formic acid (50:50:0.1% v/v) at 2 $\mu\text{L}/\text{min}$ to a PicoTip (5 cm $\times$ 100 μm diameter) stainless steel nanospray emitter operating in positive ion mode at 3800 V. The LAESI was operated using LAESI Desktop Software V2.0.1.3 (Protea Biosciences Inc.). The Time of Flight (TOF) mass analyzer of the Synapt G2S was operated in V-reflectron mode at a mass resolution of 18,000–20,000. The source temperature was 150 $^{\circ}\text{C}$, and the sampling cone voltage was 30 V. The data were acquired in a mass range of m/z 50–1200. The acquired MS data were lock mass corrected post-data acquisition using leucine enkephalin ($\text{C}_{28}\text{H}_{37}\text{N}_5\text{O}_7$, $m/z=556.2771$), which was added in the spray as an internal standard.

Data processing, peak detection and chemometrics

All the acquired Waters .raw data files were first pre-processed to remove noise and to make the data comparable. Since the root samples used in this study were tiny, many LAESI ablation spots constituted the background on which the root samples were placed. To avoid including the mass spectra purely consisting of spectral signals from the background, 50 ablation spots per sample replicate, present on the root section were selected manually. The selected ablation spots for every sample replicate are displayed in Online Resource 1 as Supplementary Fig. 1. The mass spectra arising from the spots colored in green are included in the study whereas those in red have been excluded.

The spectra from all the 50 selected spots for each replicate were averaged. Processing of these mass spectra involved multiple steps. An overview of the data processing steps applied is provided in Fig. 1. First, square root transformation was applied to the data to stabilize the variance. Then, baseline correction was performed to enhance the contrast of peaks to the baseline. For better comparison of intensity values and to remove small batch effects, Total-Ion-Current (TIC)-based normalization was applied. This was followed by spectral alignment and peak detection to extract a list of significant mass features for each sample replicate. In the end, a mass feature matrix was generated with sample replicates in columns and mass features in rows. This feature matrix was used to perform chemometric analysis. The pre-processing and peak-detection steps were applied using R scripts developed in-house and the functions available within the MALDIquant R package (Gibb and Strimmer 2012).

To perform multivariate analysis, the feature matrix was imported into Metaboanalyst 3.0 (Xia et al. 2015). Principal

Fig. 1 Data preparation and processing steps applied post-acquisition. **a** Optical image for the intact roots of a single replicate of *C. jacea* with labeled ablation spots. **b** Ablation spots present on the root

selected (in green) for further analysis. **c** Averaged spectra acquired from all the 50 selected spots per replicate. **d** Data pre-processing and peak-detection steps applied to all spectra for a sample

component analysis (PCA) was initially applied to visualize the intrinsic spectral differences in the non-native, range-expanding plant species and congeneric native plant species. To get an overview of the differences amongst the samples, a dendrogram showing clustering of the sample replicates was generated using the Euclidean distance measure and the Ward's clustering algorithm. To visualize the number of differential metabolites in non-native, range-expanding plant species and congeneric native plant species, a pairwise comparative analysis was performed. To graphically illustrate these differences volcano plots were generated. Metabolites with a fold change (FC) threshold of 2 on the x axis and a t tests threshold (p value) of 0.1 on the y axis were considered significant. Box plots for selected significant metabolites were created to display changes in the concentration of native and range-expanding species. Corresponding accurate ion intensity maps (± 1 ppm) displaying spatial distribution for these selected mass features were created using the ProteaPlot software V2.0.1.3 (Protea Biosciences Inc., Morgantown, WV). The intensity values for the selected ion maps were normalized to the maximum intensity within the image, measured for each mass value individually. Venn diagrams

were drawn using the jvenn tool (Bardou et al. 2014) to plot the number of shared and unique metabolites for each pair of samples.

Results and discussion

Untargeted metabolite profiling and multivariate analysis

Untargeted metabolomic studies are exploratory in nature and usually result in extremely large and multi-dimensional datasets. Analyses of such datasets using chemometric tools can hugely aid data interpretation.

The representative averaged pre-processed spectra for each replicate belonging to the different plant species exhibit some visual distinction in mass spectra between the two plant genera (Fig. 2). This distinction between *Centaurea* and *Geranium* samples was further confirmed by unsupervised hierarchical clustering of the mass feature matrix (Fig. 3a). Within the two genera, the different plant species were mostly clearly separated based on their chemical

Fig. 2 Metabolic profiling and comparison of LAESI-MS spectra from native and range-expanding plant species. Each representative mass spectra is generated by averaging and pre-processing the signals acquired in positive ion mode, arising from the 50 ablation spots pre-

sent on the imaged root sample for each replicate. The averaged pre-processed mass spectra are displayed for the three replicates of native species (*C. jacea* and *G. molle*) and the three replicates for range-expanding plant species (*C. stoebe* L. and *G. pyrenaicum*)

Fig. 3 Dendrogram and Principal component analysis (PCA) score plot for the selected native and range-expanding species. **a** Species clustering represented as a dendrogram (distance measure used is Euclidean and clustering algorithm is ward). Each node in the dendrogram corresponds to a single replicate belonging either to the

range-expanding or to the congeneric native plant species. **b** The PCA score plot displays the total explained variance of >70% for component 1 and component 2. Ovals represent 95% confidence intervals. Each oval represents a sample group and each point represents a single sample

Table 1 Overview of the number of metabolites detected in each sample replicate after pre-processing and peak detection of the acquired LAESI-MSI datasets

Replicate	<i>C. jacea</i> (CJ)	<i>C. stoebe</i> (CS)	<i>G. molle</i> (GM)	<i>G. pyrenaicum</i> (GP)
1	283	332	143	129
2	204	301	151	127
3	286	286	131	122

features, with the exception of one of the *C. stoebe* replicates (Fig. 3a).

Visual comparison of the representative mass spectrum for each sample group can be used to broadly study the differing metabolic profiles. To further examine these differences and similarities between the root metabolic profiles of the four plant species, we employed PCA. The first two selected principal component axes explain over 75% of cumulative variance amongst the samples (Fig. 3b). Samples from different plant genera were strongly separated along the first PC-axis (~57%), whereas the separation along the second PC-axis (~18%) corresponded with within-genus variation (Fig. 3b). Together with hierarchical clustering (Fig. 3a), these results indicate a strong phylogenetic signal

in root chemistry, as between-genus variation is considerably stronger than within-genus variation (Senior et al. 2016).

The number of mass features detected for each LAESI-MSI acquisition after performing data pre-processing and peak detection clearly shows that there are more mass features detected for the replicates of *Centaurea* as compared to those of *Geranium* (Table 1). The two *Centaurea* species shared 314 metabolites, whereas 53 metabolites were unique to either one of the species (Fig. 4a). Interestingly, 49 of these metabolites were unique for range-expanding *C. stoebe*, whereas only four were unique for native *C. jacea*. In contrast, for native *G. molle* more unique metabolites were detected than in range-expanding *G. pyrenaicum* (Fig. 4b). These results are in line with a previous study in which only root volatiles were examined (Wilschut et al. 2017) and indicate that range-expanding plants do not necessarily possess a more unique root chemistry than related natives.

To visualize the statistically significant metabolites for the two *Centaurea* species, a volcano plot was constructed (Fig. 5a). As seen in Fig. 5a, in total 367 metabolites were detected in genus *Centaurea*. Within this, ten mass features (shown in green) that are located in upper right quadrant of the plot, indicate that their concentration is significantly higher in native species *C. jacea* than in range-expanding species *C. stoebe*. The five mass features (shown in red)

Fig. 4 Venn diagram showing overlapping and unique metabolites associated with native and range-expanding plant species. **a** Venn diagram for *C. jacea* (CJ) and *C. stoebe* (CS). **b** Venn diagram for *G.*

molle (GM) and *G. pyrenaicum* (GP). To construct the Venn diagram, a single mass feature was considered even if it was present in only one replicate for a specific sample species

that are observed in the upper left quadrant indicate that their concentration is significantly lower in native species *C. jacea* than in range-expanding species *C. stoebe*. To examine the differences in metabolite concentrations for the *C. jacea* and *C. stoebe* pair, box-and-whisker plots were realized for four statistically significant metabolites chosen based on the volcano plot (Fig. 5b). The box-and-whisker plots and the ion intensity maps reveal that m/z 84.9607, m/z 159.0520 and m/z 557.290 are highly abundant in native species *C. jacea*, whereas m/z 272.9550 are highly abundant in range-expanding species *C. stoebe*. Additionally, the corresponding ion intensity maps for these metabolites were also generated to visualize the changes on the spatial level in the imaged roots. The ion intensity maps can be seen alongside the box-and-whisker plots in Fig. 5b. Each ion map is plotted on the same color scale (depicted below the ion maps) ranging from 0 (blue meaning least intense) to 1 (red meaning most intense), to allow comparison of relative ion intensity between images.

Similar analysis was performed for the two *Geranium* species (Fig. 5c). For this pair, in total 175 metabolites were detected. Within these, 15 mass features (shown in green) that are located in the upper right quadrant of the plot, which indicates that their concentrations are significantly higher in native species *G. molle* than in range-expanding species *G. pyrenaicum*. The four mass features (shown in red) that are observed in the upper left quadrant indicate that their concentration is significantly lower in native species *G. molle* than in range-expanding species *G. pyrenaicum*. The box-and-whisker plots for the four statistically significant metabolites selected from the volcano plot for the pair *G. molle* and *G. pyrenaicum* are shown in Fig. 5d. The ion intensity maps for these statistically significant metabolites are shown alongside box-and-whisker plots. As it can be seen, m/z 158.2647 and m/z 250.8271 show high abundance

Fig. 5 Volcano plots and box plots to demonstrate metabolite concentration differences observed in native and range-expanding plant species. **a** Volcano plot for *C. jacea* (CJ) vs. *C. stoebe* (CS). **b** Volcano plot for *G. molle* (GM) vs. *G. pyrenaicum* (GP). Each point in the volcano plot represents one metabolite. Significant metabolites were calculated with a fold change (FC) threshold of 2 on the x axis and a t tests threshold of 0.1 on the y axis. The red and the green dots indicate statistically significant metabolites, and the gray dots below the FC threshold line represent statistically non-significant metabolites. The vertical FC threshold lines indicate an increase or decrease in concentration of metabolites. Negative $\log_2$ (FC) values indicated in red represent lower concentrations in native than in range-expanding species; positive values indicated in green represent higher concentrations of metabolites in native than in range-expanding species. The box plots for the detected metabolites and their corresponding ion intensity maps below each volcano plot display the localization of the selected metabolites that are significantly different in the respective native and range-expanding species. The signal intensity in the ion intensity maps is represented in rainbow color scale, in a mass window of ± 1 mDa

in native species *G. molle*, whereas m/z 172.3829 and m/z 196.5855 display high abundance in range-expanding species *G. pyrenaicum*. All significant metabolites detected for *Centaurea* and *Geranium* samples are listed in Online Resource 1 as Supplementary Table 1.

Taken together, we demonstrated the utility of the unique ambient ionization ability of LAESI coupled with MSI as a high-throughput method to explore the chemical differences in the root metabolome between two pairs of native and range-expanding plant species. This technology provided an in situ analysis method capable of revealing differentially produced metabolites linked to each group. We detected clear differences in root chemical profiles within both pairs of range-expanding plant species and congeneric natives using untargeted LAESI-MSI approach. Interestingly, the range-expanding plant species *Centaurea stoebe* showed a

(a) CJ and CS

(c) GM and GP

(b)

(d)

strongly unique root chemistry, which also may have enabled this species to become invasive in its introduced range in North America (Callaway and Ridenour 2004; Schaffner et al. 2011).

Furthermore, we demonstrated that LAESI-MSI can help to spatially elucidate the metabolite composition of the intact roots with minimal to no sample preparation. Our demonstration did not involve an exhaustive region-specific spatial analysis of the roots, but rather a ‘proof-of-concept’ by lateral profiling of the root samples. This allowed us to establish that LAESI-MSI of whole-root sections could reveal information on location-specific metabolite distribution without the need for any sample preparation. These results can help to reveal the role of single metabolites based on their location within the roots. The statistically significant biomolecules in the processed LAESI mass spectra can be putatively annotated by matching the observed masses with those of known metabolites using database searches. However, for confident compound identification it is best to couple LAESI mass spectrometry imaging with ion mobility separation (Stopka et al. 2017), which allows separation of isobaric species. Apart from this, LAESI-MSI with its feature of spatial mapping can hugely complement conventional extraction-based untargeted hyphenated-MS techniques like LC–MS or GC–MS. This can be further assisted by the application of tandem mass spectrometry for increased selectivity and structure assignments.

Overall, our results illustrate the feasibility of LAESI-MSI as a high-throughput technique for the detection and localization of metabolites from intact plant samples and gaining spatial information without the need for extensive sample preparation. The potential applications of this work could lead to rapid phenotyping of plant tissues as well as comparative untargeted metabolomics of different plant parts, a topic of considerable recent interest for plant research.

Author contribution statement PG, KJFV and RAW devised the project. PG and RAW oversaw the sample collection and the data acquisition. PK planned and performed the bioinformatics analysis, interpretation of results and prepared the figures. PK, RAW and PG wrote the manuscript. WHvdP, PG, KJFV and RAW provided their comments and contributed to substantial revision of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Availability of source code and requirements Project name: LAESI-MSI-Root-Metabolomics. Project home page: <https://github.com/purvakulkarni7/LAESI-MSI-Root-Metabolomics> (DOI: <https://doi.org/10.5281/zenodo.1240843>). Operating system(s): platform independent. Programming language: R. Other requirements: R ($\geq 3.2.0$), MALDIquant package, MALDIquantForeign package. License: GNU General Public License version 2.0 (GPLv2). Any restrictions to use by non-academics: none.

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