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A metabolomic protocol for plant systematics by matrix-assisted laser-desorption/ionization time-of flight mass spectrometry

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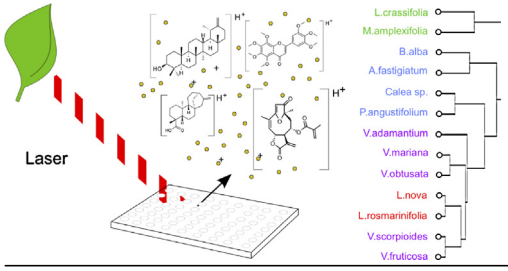
HIGHLIGHTS

- Metabolic fingerprinting of plant by MALDI-TOF MS.
- Multivariate data analysis by in-house algorithms.
- Taxonomic classification of plants from different genera, families and orders.

GRAPHICAL ABSTRACT



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ABSTRACT

Matrix-assisted laser desorption/ionization time-of flight mass spectrometry (MALDI-TOF MS) has been widely used for the identification and classification of microorganisms based on their proteomic fingerprints. However, the use of MALDI-TOF MS in plant research has been very limited. In the present study, a first protocol is proposed for metabolic fingerprinting by MALDI-TOF MS using three different MALDI matrices with subsequent multivariate data analysis by in-house algorithms implemented in the R environment for the taxonomic classification of plants from different genera, families and orders. By merging the data acquired with different matrices, different ionization modes and using careful algorithms and parameter selection, we demonstrate that a close taxonomic classification can be achieved based on plant metabolic fingerprints, with 92% similarity to the taxonomic classifications found in literature. The present work therefore highlights the great potential of applying MALDI-TOF MS for the taxonomic classification of plants and, furthermore, provides a preliminary foundation for future research.

1. Introduction

Plants exhibit a very wide variety of secondary metabolites and their classifications are traditionally also based on their chemical constituents. Secondary metabolites have been used as taxonomic makers for nearly 200 years [1,2], predating the emergence of chemotaxonomy – the taxonomic classification of plants based on their chemical features – in the 1960s [2,3]. With the rapid expansion of metabolomics research, which is defined as the measurement of all the metabolites (the metabolome) in a given system (cell, tissue, or organism) under a given set of conditions [4], several studies have aimed to taxonomically classify plants based on their metabolic fingerprints or profiles,

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representing a more holistic approach compared with traditional chemotaxonomic methods, which typically focus on one class of secondary metabolites. Such studies, however, are restricted to plants of the same genus and primarily utilize nuclear magnetic resonance (NMR) or hyphenated mass spectrometry methods, such as mass spectrometry coupled to gas or liquid chromatography (GC/LC-MS) [5–9], for data acquisition.

The use of MALDI-MS in plant metabolomics research remains relatively unexplored. Fraser et al. [10] described a targeted approach for the measurement of carotenoids, and several studies have proposed new matrix substances for MALDI-MS with potential applications in metabolomics studies [11,12]. Furthermore, the potential of MALDI-MS has also been seen in MALDI imaging for the investigation of the spatial distributions of metabolites in a plant organ [13–15].

MALDI-TOF MS has been widely applied to the taxonomic classification and identification of microorganisms, due to its rapid turnaround time, high throughput and reliable results between others [16–18]. However, the classification of microorganisms by MALDI-TOF MS is based on proteomic rather than metabolic fingerprints. To the best of our knowledge, there neither exist metabolomics studies on the chemotaxonomic classification of plants from different genera, nor has MALDI-TOF MS ever been applied for the taxonomic classification of plants based on their metabolic fingerprints.

Compared to the more traditional techniques applied in metabolomics research, MALDI-TOF MS offers advantages including simple and rapid sample preparation, low sample consumption and high throughput. Furthermore, less ion suppression is observed in compound mixtures for MALDI-MS compared with electrospray ionization mass spectrometry (ESI-MS) [19], the ionization technique that is most commonly applied in mass spectrometric plant metabolomics studies.

The disadvantages of MALDI-MS for plant metabolomics studies largely overlap with the disadvantages of direct injection mass spectrometry. The differentiation of isomers is not possible, less chemical information is obtained than in, for example, hyphenated mass spectrometry methods. To increase the chemical information obtained from analysis by MALDI-TOF MS, the spectra of the plant extracts in the present study were acquired in both negative and positive ionization modes and with three different matrix substances, including 4-nitroaniline (4-NA), α-cyano-4-hydroxycinnamic acid (CCA) and lithium 2,4-dihydroxybenzoate (LiDHB). By merging the data acquired with different matrices and using careful algorithms and parameter selection, we demonstrate that a close taxonomic classification can be achieved based on plant metabolic fingerprints. The present study therefore represents a first step toward the fully automated taxonomic classification of plants based on their metabolic fingerprints by MALDI-TOF MS.

Taxonomy plays an important role in the biosciences, which deal with the organization and diversity of species [20,21]. Plant taxonomy in specific provides a solid basis in order to estimate the exact distribution of species and the identification of areas of high species richness, which are crucial steps in making informed and efficient decisions for the conservation of biodiversity [20,21]. Due to a decline in expertise and inadequate infrastructure, taxonomy is recognized as a "science in crisis" [20–22]. The development of new tools for the correct taxonomic classification of species suffering from problematic botanical classification is therefore an urgent need.

By possessing almost 41.000 species of plants, fungi and algae [23], Brazil belongs to the group of megadiverse countries [24,25]. However its diversity is threatened due to the loss of habitats with a large amount of endemic species, increasing thus the extinction rates [26]. Therefore, Brazil possesses two biodiversity "hotspots for conservation priority", one in the Atlantic Forest and the other in the Brazilian Savanna (Cerrado) [27]. In this context, Asteraceae is one of the most diverse families of Angiosperms with around 22.000 species [28–30]. The tribe Vernonieae belongs to one of the most diverse tribes in this family and due to many overlapping characters, which complicate the taxonomic delimitation at all levels, also represents one of the most challenging taxa. Out of this reason, Vernonieae is also called the "evil tribe" [31]. The genus *Lychnophora*, which belongs to Vernonieae, is not an exception and exhibits serious challenges to correct botanical identification by traditional taxonomic tools [32,33]. In Brazil, Asteraceae play a key role in plant biodiversity, they are the third most diverse family of Angiosperms in the country [34]. In the Cerrado, Asteraceae is the most diverse family with around 1200 species [35].

In the present study, the metabolic fingerprints of 24 closely related as well as distantly related plant species were acquired by MALDI-TOF MS and were subsequently classified by multivariate data analysis by in-house algorithms implemented in the R environment. All plants were collected in areas of the Brazilian Savanna (Cerrado) and included species from the tribes Vernonieae, Eupatorieae and Heliantheae, in the Asteraceae family and the order Asterales, as well as species from the tribe Microlicieae, in the order Myrtales and the family Melastomataceae. In the tribe Vernonieae, 12 species of the genus *Lychnophora* were included.

2. Materials and methods

2.1. Materials

2.1.1. Plant material

The plants analyzed included species from the Asteraceae family from the tribes Vernonieae, Eupatorieae and Heliantheae, based on the classification of Funk et al. [30] as well as species from the Melastomataceae family and the tribe Microlicieae according to Renner [36] (Table S1, Supplementary data). The species of these two families were chosen because they are phylogenetically distant, with the Asteraceae family belonging to the order Asterales, which is positioned in the Asterid clade, and the Melastomataceae family belonging to the order Myrtales, which is positioned in the Rosid clade [37]. In addition, the selection of the genera from the Asteraceae family was based on their phylogenetic relationships. *Ageratum* L. belongs to the tribe Eupatorieae, and *Bidens* L., *Aspilia* Thouars, and *Porophyllum* Adans. belong to the tribe Heliantheae. Together they belong to the subfamily Asteroideae, clade "Heliantheae alliance" [30]. *Lychnophora* Mart. and *Vernonia* Schreb., however, belong to the tribe Vernonieae in the subfamily Cichorioideae. The genera *Vernonia* and *Lychnophora* were selected because they are positioned in two different subtribes of the tribe Vernonieae; *Lychnophora* in Lychnophorinae and *Vernonia* in Vernonieae. However, they were classified as more closely related in the past, to the extent that some species of *Vernonia* were transferred to *Lychnophora* [33]. The classifications used in the present study were made according to APG III [37] for the orders and families, according to Renner [36] for the subfamilial and tribal classification of Melastamotaceae, according to Panero and Funk [38] for the subfamilial classification of Asteraceae, according to Keeley and Robinson [39] for the subtribal classification of Vernonieae, according to Robinson [40] for the subtribal classification of Heliantheae, and according to Robinson et al. [41] for the tribal classification of *Ageratum*. The botanical identification of the species was realized by Prof. João Semir of the department of Botany, Institute of Biology of the Campinas State University (UEC), and vouchers of each specimen were deposited at the UEC Herbarium. All plants were collected in areas of the Brazilian Savanna (Cerrado), a "hotspot for conservation priority" [27] near Diamantina, Minas Gerais. A detailed list of the plant