Spatial localization of monoterpenoid indole alkaloids in Rauvolfia tetraphylla by high resolution mass spectrometry imaging

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ABSTRACT

Monoterpenoid indole alkaloids (MIAs) are a large group of biosynthetic compounds, which have pharmacological properties. One of these MIAs, reserpine, was discovered in the 1950s and has shown properties as an anti-hypertension and anti-microbial agent. Reserpine was found to be produced in various plant species within the genus of Rauvolfia. However, even though its presence is well known, it is still unknown in which tissues Rauvolfia produce reserpine and where the individual steps in the biosynthetic pathway take place.

In this study, we explore how matrix assisted laser desorption ionization (MALDI) and desorption electrospray ionization (DESI) mass spectrometry imaging (MSI) can be used in the investigation of a proposed biosynthetic pathway by localizing reserpine and the theoretical intermediates of it. The results show that ions corresponding to intermediates of reserpine were localized in several of the major parts of Rauvolfia tetraphylla when analyzed by MALDI- and DESI-MSI. In stem tissue, reserpine and many of the intermediates were found compartmentalized in the xylem. For most samples, reserpine itself was mainly found in the outer layers of the sample, suggesting it may function as a defense compound.

To further confirm the place of the different metabolites in the reserpine biosynthetic pathway, roots and leaves of R. tetraphylla were fed a stable-isotope labelled version of the precursor tryptamine. Subsequently, several of the proposed intermediates were detected in the normal version as well as in the isotope labelled versions, confirming that they were synthesized in planta from tryptamine. In this experiment, a potential novel dimeric MIA was discovered in leaf tissue of R. tetraphylla.

The study constitutes to date the most comprehensive spatial mapping of metabolites in the R. tetraphylla plant. In addition, the article also contains new illustrations of the anatomy of R. tetraphylla.

1. Introduction

Rauvolfia tetraphylla L. (Apocynaceae), also known as ‘Devil Pepper’ and ‘Be Still Tree’, is a plant which has received attention due to its ability to produce compounds with medical properties. R. tetraphylla appears as a combination between a tree and a shrub that can grow up to the height of 1.8 m (Gupta et al., 2012; Iqbal et al., 2013; Verma et al., 2012). R. tetraphylla has been used in traditional medicine in several areas of India, often as a substitute for Rauvolfia serpentina (Malalakshmi et al., 2019). Traditional medicine made from R. tetraphylla has been used to treat headache (Ganesh and Sudarsanan, 2013), skin diseases, snake bite, insect bite (Kumar et al., 2018), stomachache (Malaia, 2016), blood pressure (Babu et al., 2010), and diarrhea (Morshed and Nandini, 2012). Indeed, Rauvolfia sp. Synthesize secondary metabolites known as monoterpenoid indole alkaloids (MIAs) displaying significant biological activities. MIAs are a large group of biosynthetically related metabolites, with Rauvolfia sp. Known for production of MIAs like reserpine (Muller et al., 1952), ajmaline (Stöckigt et al., 2007), alstonine (Elisabetsky and Costa-Campos, 2006) and yohimbine (Kumar et al., 2016) used as drugs. MIAs in general have a wide variety of complex

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structures, which make them challenging to produce by conventional chemical synthesis at industrial scales. Exploitation of these compounds mainly relies on extraction from plant tissue which is complicated since many MIA are produced in very low levels in planta (Deshmukh et al., 2012; Miettinen et al., 2014).

MIAs result from long and complex biosynthetic pathways that also exhibit a complex organization at both the cellular and subcellular levels (de Bernonville et al., 2015; Pan et al., 2016; Yamamoto et al., 2019). They have previously been localized in the tissues of the well-known MIA producing plant Catharanthus roseus (also of the Apocynaceae family) using immunostaining (St-Pierre et al., 1999) and MALDI-MSI (Yamamoto et al., 2016).

All MIAs derive from the same common precursor, strictosidine, which is synthesized by strictosidine synthase from secologinin and tryptamine (St-Pierre et al., 2013; Treimer and Zem, 1979). Secologinin derives from the seco-iridoid pathway, which has been thoroughly studied in C. roseus. The seco-iridoid pathway has been shown to take place initially in internal phloem-associated parenchyma cells and finalized in epidermis cells (Burlat et al., 2004; Miettinen et al., 2014), where strictosidine synthase is located (St-Pierre et al., 1999). This implies that strictosidine precursors are being transported to the epidermal cells. However, more recent studies have found that strictosidine accumulates in idioblasts and laticifer cells in C. roseus (Yamamoto et al., 2016, 2019), which suggest that it is transported there from the epidermis. Many post-strictosidine steps have been discovered in C. roseus; in Rauvolfia sp., however, only the ajmaline pathway has been investigated (Dang et al., 2017, 2018).

Among the pathways beyond strictosidine that have still not been explored are the biosynthetic pathways of reserpine and rescinnamine in R. tetraphylla. Reserpine and rescinnamine were isolated for the first time in 1952 and 1954, respectively (Klohs et al., 1954; Muller et al., 1952). They share similar structure and have both been shown to have sedative and antihypertension effects (Bakris and Frohlich, 1989; Fife et al., 1960). Reserpine has also been shown to possess antimicrobial properties (Mullin et al., 2004; Stavri et al., 2007; Vaou et al., 2021). A proposed biosynthetic pathway for reserpine and rescinnamine, based on previously elucidated MIA pathways (G Connor and Marash, 2006) is shown in Fig. 2. Intermediates shown in the proposed biosynthetic pathway are named RPI 2–7.

To gain insights into reserpine and rescinnamine metabolism, more information is needed about the spatial distribution of the different metabolites. A mass spectrometry based technique known as Mass Spectrometry Imaging (MSI) constitute a powerful tool to analyze the spatial location of reserpine, rescinnamine and their intermediates in situ (Bjarnholt et al., 2014; Boughton et al., 2016). MSI enables mass-specific imaging of numerous compounds in a sample in one experiment. It is performed by rastering a sample below an ionization spot such that a mass spectrum is acquired in every point on the samples. Subsequently,
Fig. 2. Theoretical biosynthetic pathway of reserpine and rescinnamine. The brackets indicate which intermediates should be considered as tentative.
heatmaps of ion intensities can be extracted for each compound of interest, with every recorded mass spectrum corresponding to a pixel in the final image. In this study, both Matrix Assisted Laser Desorption Ionization (MALDI)-MSI and Desorption Electrospray Ionization (DESI)-MSI was used. In brief, MALDI-MSI enables spatial resolution as high as 1–5 µm while DESI-MSI typically enables spatial resolution of 50 µm or lower. MALDI-MSI uses a UV laser for ionization and requires application of a layer of UV-absorbing matrix molecules onto the sample, while DESI uses solvent spray for ionization and can analyze the samples directly after sample preparation such as cryo-sectioning or imprinting (Feenstra et al., 2017; Li et al., 2016). MSI has been utilized to propose a biosynthetic pathway in *Linum usitatissimum* seeds (Dalissy et al., 2015) based on co-localization of metabolites and transcripts. In *C. roseus*, MALDI-MSI was used on leaves (Yamamoto et al., 2019) and stem tissue (Yamamoto et al., 2016) to decipher MIA pathway organization with a spatial resolution of 10–20 µm. DESI-MSI was used to study MIAs in leaf, stem, root and fruit tissue of *R. tetraphylla* at a spatial resolution of 250 µm (Kumara et al., 2019).

In the present study, the MIA distribution in *R. tetraphylla* is further investigated by high-resolution MALDI-MSI in combination with DESI-MSI, focusing on the biosynthetic pathways of reserpin and rescinnamine. The proposed pathway was investigated in leaf, stem, root and fruit tissue of *R. tetraphylla*. Compared to the previous MSI study of *R. tetraphylla*, imaging was performed at a significantly higher spatial resolution (15 or 20 µm), which enabled much more detailed visualization, including imaging of cross sections of leaves.

### 2. Results and discussion

The present study represents a comprehensive analysis of the distribution of reserpine and theoretical intermediates of reserpine biosynthesis in the main structures of the *R. tetraphylla* plant, including root, stem, leaf and fruit tissue. Most of the imaging experiments have been carried out by MALDI-MSI due to its superior spatial resolution, but the lateral distribution of the metabolites in leaf was investigated by DESI-MSI due to its capabilities for direct analysis of imprints on porous
Fig. 4. MALDI-MS images of R. tetraphylla primary stem tissue. (A) Microscope image. (B) PC (34:2). (C) Di-hexose. (D) Strictosidine. (E) Strictosidine aglycone. (F) Isorauhimbine. (G) RPI2. (H) RPI3/RPI6. (I.A) RPI6-OH. (I) RPI5. (J) RPI4. (K) RPI7. (L) Reserpine. The pixel size is 20 μm. Scale bars: 2 mm.
Fig. 5. MALDI-MS images of R. tetraphylla secondary stem tissue. (A) Microscope image. (B) PC (34:2). (C) Di-hexose. (D) Strictosidine. (E) Strictosidine aglycone. (F) Isorauhimbine. (G) RPI2. (H) RPI3/RPI6. (H.A) RPI6-OH. (I) RPI5. (J) RPI4. (K) RPI7. (L) Reserpine. The pixel size is 20 μm. Scale bars: 1 mm.
PTFE surfaces. MALDI- as well as DESI-MSI was carried out on an Orbitrap mass spectrometer providing high resolution MS analysis with a mass accuracy of 2 ppm, excluding most compounds of similar, but not identical masses.

To aid with interpretation of the presented MSI data, illustrations are provided in Fig. 1, which show the anatomy of root, stem and leaf material of R. tetraphylla. These illustrations were inspired by the several studies of the anatomy of Rauvolfia sp. and other members of Apocynaceae (Baratto et al., 2010; Court et al., 1957; Metcalfe and Chalk, 1950). Compounds included in the reserpine and rescinnamine pathway in Fig. 2 have been imaged by MALDI-MSI and DESI-MSI in root, stem, and leaf tissues as shown in Figs. 3-9. The identification of the compounds is based on the accurate mass of the compounds, but some of these compounds have one or more known isomers, which in principle could contribute to the imaged signals. These potentially interfering compounds have been listed in Table 1. For each MSI experiment, an image of phosphatidylcholine 34:2 (PC (34:2)) or di-hexose is included to show the contour of the analyzed tissue. Rescinnamine was not detected in any tissue of R. tetraphylla and is therefore not included in any of the figures. The findings from the MSI experiments have been summarized in Table 2, which provides an overview of the parts of the plant in which reserpine and the intermediates of reserpine and rescinnamine were detected. Additional experiments were made on immature and mature fruits, and the results from these experiments are found in the supporting information.

It should be noted that results from MSI experiments are generally to be considered of qualitative nature. From the reported images it can thus only be inferred where the compounds were observed, but not in which quantity. Nor can the relative abundances of different compounds be assessed, as different compounds may have different ionization efficiencies.

2.1. Root tissue

MALDI-MS images of a cross section of a R. tetraphylla root is found in Fig. 3. MALDI-MS images of PC (34:2) and di-hexose (Fig. 3B-C) show that PC 34:2 and di-hexose are not observed in the entire root tissue, but they give an indication of the xylem tissue. Most reserpine/rescinnamine intermediates are observed in the secondary phloem. However, as is more clearly seen in the overlaid image in Fig. 3M, RPI2 is observed in the phelloderm compared to the other intermediates. From the merged MS image of RPI2, RPI3 and PC 34:2, it is observed that RPI2 (Green) and RPI3 (Red) are localized in two different layers (the phloem and the phelloderm), which could indicate some transport of either RPI2 or RPI3 and the rest of the intermediates between the two layers. The intermediates RPI5 and RPI7 (Fig. 3 I & K) were not detected. This could indicate that instead of accumulating, the compounds are rapidly further metabolized, thus not reaching high enough amounts to be detected. Root tissue was the only tissue where RPI7 was not detected. The reason could be that its further conversion into reserpine and/or rescinnamine is particularly efficient in the roots, preventing its accumulation. Reserpine is mostly present in the phloem, phelloderm, cork and around the xylem. In the study by Kumara et al. (2019), MIAs are also observed in what is referred to as cortex, which is similar to the area of secondary

Fig. 6. MALDI-MS images a cross section of R. tetraphylla young leaf tissue. (A) Microscope image. (B) PC(34:2). (C) Di-hexose. (D) Strictosidine. (E) Strictosidine aglycone. (F) Isorauhinbime. (G) RPI2. (H) RPI3/RPI6. (H–OH) RPI6-OH. (I) RPI5. (J) RPI4. (K) RPI7. (L) Reserpine. The pixel size is 15 μm. Scale bars: 1 mm.
Fig. 7. MALDI-MS images of R. tetraphylla old leaf tissue. (A) Microscope image. (B) PC (34:2). (C) Di-hexose. (D) Strictosidine. (E) Strictosidine aglycone. (F) Isorauhinmine. (G) RPI2. (H) RPI3/RPI6. (H.A) RPI6-OH. (I) RPI5. (J) RPI4. (K) RPI7. (L) Reserpine. The pixel size is 20 μm. Scale bars: 1 mm.
phloem and phelloderm. These findings suggest that the biosynthetic pathway of reserpine and rescinnamine occurs in the cortex of the root.

2.2. Stem tissue

Fig. 4 shows MALDI-MS images of primary stem tissue of *R. tetraphylla*, and Fig. 5 shows the corresponding images of secondary stem tissue. In the primary stem tissue, the reserpine and rescinnamine intermediates are observed in small compartments in the pith and xylem region, indicating existence of specialized cells responsible for MIA production in primary stem tissue. In addition to this, RPI2 was also observed in the epidermal layer of primary stem tissue (Fig. 4G), which
is in contrast to secondary stem tissue where it was only observed in small compartments close to the internal phloem (Fig. 5G). In general, secondary stem tissue appears quite distinct from the primary stem, as most intermediates are localized around the pith and internal phloem in secondary stem (Fig. 5). However, as seen for strictosidine aglycone, RPI2, 3 and 6 (Fig. 4E, G and H), there seem to be smaller compartments close to or within the internal phloem in primary stem, which could suggest that it has similar specialized cells.

Yamamoto et al. (2016) analyzed stem tissue of *C. roseus* and found MIAs to be present around the vascular bundle, idioblast cells and laticifer cell by MSI. However, analysis of stem tissue from *Rauvolfia sellowii* found idioblast cells present in cortex and pith and laticifer cells only present in the pith (Baratto et al., 2010). Therefore, it is unlikely that the MIA compartments in xylem seen in Figs. 4 and 5 are due to idioblast or laticifer cells (assuming that the anatomy of *R. tetraphylla* is similar to the one of *R. sellowii*).

Yamamoto et al. (2016) found the majority of strictosidine present in the idioblast and laticifer cells in the stem tissue of *C. roseus*. However, while in the present study strictosidine was detected in both primary and secondary stem tissue, due to highly scattered and low intensity it is not possible to conclude specifically where the molecule is localized (Figs. 4D and 5D). RPI5 was not detected in primary stem tissue, however in the secondary stem tissue it was observed around the internal phloem, like the other intermediates. RPI7 is the only intermediate not observed in the xylem, phloem or pith and is only observed in the epidermis/suber area of both primary and secondary stem tissue. This could mean that the last step of the reserpine/rescinnamine pathway takes place in the epidermis/suber; however, more evidence for the pathway is required before this can be concluded. In primary stem tissue, reserpine was observed in compartments in pith and xylem as the intermediates. In secondary tissue reserpine was observed in the xylem, however not in compartments like in the primary tissue. In both primary and secondary stem tissue, reserpine was also observed in the epidermal layer and in external phloem/cortex. The reason why it is present in the epidermal cells could be that it serves as a defense compound, something which is well in line with its antimicrobial effects previously described (Bakris and Frohlich, 1989; Mullin et al., 2004; Stavri et al., 2007; Vaou et al., 2021). Given its presence in external phloem, it could be that it is being transported via these cells, however no evidence for transporters of reserpine in this tissue have been documented before.

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**Fig. 9.** MALDI-MS images showing isotope labelled reserpine and its intermediates in leaf cross-sections of *R. tetraphylla*. MS images of two leaves are shown. Leaf A was administered 10 μl 1 mM tryptamine and Leaf B was administered 10 μl 1 mM tryptamine-d4. The MS images are divided into two groups, the unlabeled and labelled. m/z values of the labelled and unlabeled masses can be viewed in Table 1. The pixel size is 20 μm. Scale bars: 1 mm.
Table 1
Table 1
Rauvoldine

2.3. Leaf tissue
Leaf tissues were analyzed by MALDI-MS and DESI-MS. MALDI was used to analyze cross sections of young and old leaf tissue and in the
DESI-MS images of young leaf tissue. In both MALDI-MS and DESI-MS images of young leaf tissues, most of the rhespine and rescinnamine intermediates were detected. DESI-MS images of strictosidine (Fig. 6C) shows its presence in most of the leaf tissue except the veins. This is seen more clearly in the cross section images by MALDI (Fig. 6D), where strictosidine was present in the mesophyll area but completely devoid from the midrib and veins. Most of the MALDI-MS images of old leaf tissue (Fig. 7) showed that strictosidine was observed in the mesophyll area (like in young leaf tissue) but also in the mesophyll close to the epidermis in midrib area. The common theory is that strictosidine is biosynthesized in the epidermis cells in leaf tissue. However, this is also based on early findings in C. roseus (St-Pierre et al., 1999), where Yamamoto et al. (2019) found strictosidine not only in epidermis, but also in idioblast cells, laterider cells as well as spongy and palisade mesophyll. These papers together with the findings in this paper could suggest that strictosidine is being transported across cells. It remains, however, to be proven that strictosidine is biosynthesized in the epidermis of Rauvoldia sp. As it is in C. roseus.

The next metabolite, strictosidine aglycone, was detected with both DESI and MALDI. Strictosidine aglycone was detected in the midrib and veins of both old and young leaf tissue (Figs. 6E and 7E). In old leaf tissue, strictosidine aglycone was also detected around the xylem, suppesdedly in the phloem area (Fig. 7F). This further supports the theory that reserpine biosynthetic intermediates are transported between tissues of R. tetraphylla.

RPI2 and RPI5 were not detected by DESI and MALDI in young leaf tissue, but they were detected in old leaf tissue. DESI-MS images of the old leaf tissue show both RPI2 and RPI5 (Fig. 5I and H) in the midrib and veins. RPI6 was not detected by MALDI, but RPI6 is found within the phloem of the midrib (Fig. 7G). The difference between the DESI and MALDI detection of RPI6 could be ascribed to DESI being more sensitive than MALDI in this instance, perhaps also owing to the sample preparation in the DESI experiments where the soluble leaf contents are extracted into the pores of the PTFE surface.

RPI4 was not detected by MALDI in young leaf tissue, but in the DESI-MS images of young leaf tissue it was detected in both mesophyll, vein and midrib regions. The same distribution of RPI4 in DESI-MS and MALDI-MS images was observed in old leaf tissue. MALDI-MS images of old leaf tissue showed that RPI4 shared the same distribution of stric-
tosidine aglycone, isoaurahimine, RPI3 and RPI6.

DESI-MS and MALDI-MS images of RPI7 show it to be present in the mesophyll tissue in young as well as in old leaves. MALDI-MS images also show RPI7 to be present in the phloem in young leaves (Fig. 5K) but not in the old leaves (Fig. 7K). This could indicate that RPI7 is being converted or transported to other parts of the plant in the young stages of leaf development.

DESI analysis of old and young leaf tissue did not give indications of the spatial location of reserpine. MALDI analysis of young leaf tissue showed presence of reserpine internally in young leaf tissue. However, in some MALDI analyses, reserpine was detected around the midrib, in the epidermal layer (Fig. 4L). In the MALDI-MS images of young leaves, reserpine was also observed outside the tissue area, which might suggest presence of reserpine in the cuticle. The MALDI analysis of old leaf tissue showed the presence of reserpine in the veins and phloem in midrib. This could suggest that reserpine is accumulated in or transported via the phloem in the leaves.

2.4. Fruit tissue
MALDI-MS images of mature and immature fruit cross-sections (Figs. S3 and S4) show that most intermediates were detected in the fruits, primarily in the epidermis or the pedicel. This is in agreement with Kumara et al. (2019) who also found most MAs in the epidermis of fruits. Strictosidine differs from this pattern as it is observed primarily around the seed and in the pericarp in the immature fruit (Fig. S3D). In the mature fruit tissue, strictosidine was observed in the epidermis tissue similar to the other intermediates (Fig. S4D). RPI2 was faintly observed in some MS images of both the mature and immature fruit tissue around the pedicel; however, due to the inconsistency of its detection, it is
Table 2
Overview of the observed localisations of reserpine and the different reserpine and rescinnamine intermediates.

<table>
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<th>Berry</th>
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<td></td>
<td></td>
<td></td>
<td>(immature)</td>
</tr>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>Pedicle</td>
</tr>
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</tr>
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Table 2

uncertain whether it is actually present in fruit tissue. This could indicate that it does not accumulate in fruit tissue due to rapid conversion and that perhaps the conversion is slower in the pedicel. RPI5 was not observed in any of the fruit tissues, and RPI7 was only observed in the pedicel of mature and immature fruit tissue, which could have the same explanation as for RPI2.

Reserpine, on the other hand, was detected in the pericarp of both mature and immature fruit tissue. In some images of young fruit tissues, reserpine was found to be present in the seed, which could indicate reserpine is either synthesized in or transported to the seed during the maturation of the fruit.

Overall, the present study presents several novel findings compared to the previously published MSI work on R. tetraphylla by Kumara et al. (2019), owing among other factors to the difference in instrumentation with regards to spatial resolution, selectivity and sensitivity. In particular, the main MIA precursor, stricosidine, was detected in most MSI analyses in this study unlike in the study by Kumara et al. where stricosidine was not detected at all. Reserpine was observed by Kumara et al. in the in root and stem tissue, where it was mostly observed in pit tissue of roots. In this work, the root tissue did not have any pit region, which could be due to the young age of the plants. Besides the lack of pith in the MS images in this work (Fig. 3), the distribution in root tissue is similar to the findings in the previous study.

2.5. Feeding experiments with tryptamine-d4

In order to further investigate the proposed biosynthetic intermediates of reserpine and rescinnamine, tryptamine-d4 was administered to roots, with the aim of determining whether the proposed metabolites were found in their d4-labelled versions, as this would indicate that the compounds are formed from tryptamine and potentially be parts of the biosynthetic pathway. The m/z values of the relevant d4-labelled intermediates are found in Table 1.

Among the various metabolites and intermediates, only strictosidine and reserpine were observed in their deuterium labelled versions; importantly, these two d4-labelled compounds were not detected in control root tissue but administered tryptamine-d4 (Fig. 5B). Since it is already well established that tryptamine is efficiently incorporated into strictosidine and all MIA's, colocalisation of strictosidine and strictosidine-d4 signals further confirm molecular identity of these m/z values (St-Pierre et al., 2013). The reason for the lack of detectable d4-labelled intermediates may be that the accumulated amounts were too low. The amounts may be increased in future experiments by increased the amount of administered tryptamine-d4.

A similar labelling experiment was carried out with young leaves; the results from this experiment are shown in Fig. 9. As seen in Fig. 9, almost no ions corresponding to d4-labelled molecules were detected in the unlabeled control leaf tissue. Only strictosidine aglycone was observed both as unlabeled and d4-labelled in the control leaves. This signal from strictosidine-aglycone-d4 must therefore be ascribed to an interfering ion which is not related to the MIA's. In both labelled and unlabeled leaves, strictosidine was observed in the mesophyll area (Fig. 9), similar to what was found in Fig. 6D, and in addition, strictosidine-d4 was observed to have the same distribution in leaves administered tryptamine-d4.

Likewise, unlabeled isorauhimbine was observed both in labelled and control leaves, but isorauhimbine-d4 was only observed in leaves administered tryptamine-d4. Isorauhimbine-d4 was mainly observed in the mesophyll but to a limited extent also around the phloem and xylem in the midrib, whereas unlabeled isorauhimbine was observed in the midrib in the same abundance as in the mesophyll. This could be due to isorauhimbine being initially biosynthesized in mesophyll and subsequently accumulated in the midrib, or the signal in the midrib could potentially come from another interfering compound of the same m/z value.

RPI2-d4, RPI4-d4 and RPI5-d4 were not detected in the leaf tissue that had absorbed tryptamine-d4, and it is therefore not possible to prove their link to tryptamine. Non-labelled RPI2 and RPI5 were detected in leaves treated with tryptamine and tryptamine-d4; this could be stress induced, as they were not observed in the untreated young leaves (Fig. 6). Stress could have arisen from the leaves being excised from the plant.

Ions corresponding to RPI3-d4/RPI6-d4 and RPI7-d4 were detected in the mesophyll outside the midrib. This is in contrast to the non-labelled ions of RPI3/RPI6 and RPI7 that were detected in the phloem and xylem in the midrib as well. Ions corresponding to reserpine-d4 were not detected in leaves treated with tryptamine-d4. It is thus inconclusive whether leaf tissue has the ability to biosynthesize reserpine, even though unlabeled reserpine is vaguely detected in leaf tissue. In conclusion, the precursor experiment has shown that some of the m/z values are related to tryptamine and that the tryptophyll plays a vital role in MIA biosynthesis in young leaves of R. tetraphylla.

Analysis of the MIA biosynthesis in leaves of C. roseus has shown that the seco-iridoid pathway takes place in the internal phloem associated parenchyma and epidermis cells, and metabolism into MIA's takes place in epidermis, idioblast and laticifer cells (de Bernonville et al., 2015; 2016).
Miettinen et al., 2014). In the present study of R. tetraphylla neither reserpine, rescinnamine nor their intermediate intermediates were proven to be present in these afore mentioned cells types, but instead found in the mesophyll and the phloem. Some compounds were more restricted than others, i.e. strictosidine in young leaf tissue, which was not observed in veins or midrib, and RPI7 in old leaf tissue, which was only observed in the mesophyll.

2.5.1. Detection of a potentially novel MIA in R. tetraphylla

In analysis of the MALDI-MSI data from the experiment on the young and old leaves, distinct images could be created of ions with masses m/z 825.40692 and m/z 863.36280 (±5 ppm), corresponding to protonated and potassium adducts of an unknown compound with the molecular formula C_{6}H_{6}N_{4}O_{6} (Fig. S7). This is the same molecular formula as the MIA vincristine, which is a dimeric alkaloid deriving from catharanthine and vindoline and found in C. roseus (de Bernaveille et al., 2015). Vincristine is not known to be produced in Rauvolfia sp., but also in various online libraries (Metlin, ChEMBL, HMDB & BSAChemDB) no other compound matches the exact mass as vincristine within 5 ppm mass accuracy. The signals were observed in compartments in leaf tissue of R. tetraphylla, which could suggest its presence in idioblast or latexifer cells. Idioblast and latexifer cells are also where intermediates of vincristine are observed in leaf tissue of C. roseus (Burati et al., 2004; Roepke et al., 2019; Yamamoto et al., 2019). To investigate this, MALDI-MS analysis was performed of a vincristine standard and a methanol extract of a R. tetraphylla leaf. The MS/MS analysis resulted in two different fragmentation patterns, thus confirming that the detected compound was not vincristine (Fig. S6). In the experiment where d4-labelled tryptamine was administered to a R. tetraphylla leaf, ions corresponding to [C_{6}H_{6}N_{4}O_{6} +H]^+ were observed in young leaves treated with tryptamine-d4 (Fig. S7). This indicates that the compound derives from tryptamine and increases the likelihood that it is indeed a MIA. Although the ions observed in these MS analyses are not of vincristine, it could be a novel, dimeric alkaloid that is isomeric to vincristine. Vincristine is a chemotherapeutic drug, which has seen a shortage in recent times (Rabin, 2019); it would thus be highly beneficial if this new, unidentified MIA of the same molecular formula turned out to have similar pharmacological properties and could be used as a substitute or supplement for vincristine.

3. Conclusions

The use of high-resolution MALDI-MSI on a high-resolution mass spectrometer like the QExactive Orbitrap has enabled mapping of intermediates in the reserpine biosynthesis in root, stem, leaf and berry of R. tetraphylla at unsurpassed detail level and compound selectivity, identifying the location of several theoretical intermediates.

In order to test whether the proposed biosynthetic pathway for reserpine proceeds via the proposed intermediates, roots and leaves from the plant were administered deuterium labelled tryptamine. Subsequently, several of the intermediates were observed in their unlabeled as well as their labelled version. By analysing the produced data, it was also possible to discover a novel molecule in R. tetraphylla, which shares the exact same mass as the chemotherapeutic drug, vincristine. The molecule was confirmed not to be vincristine, but likely a different and previously undescribed dimeric alkaloid.

MSI results on their own are typically not enough to assess whether the different metabolites have been produced at the sites where they were detected or if they were transported there, but together with immunohistochemical localization of enzymes, it provides a much deeper understanding of where and how the biosynthesis takes place in the plant. This is important information, for example for determining which part of the plant to examine by transcriptomics when the aim is refactoring of plant biosynthesis in bacteria or yeast. Indeed, this is of particular importance for a plant like R. tetraphylla, which produces high-value compounds but only at limited availability.

4. Experimental

4.1. Plant samples for mass spectrometry imaging

Rauvolfia tetraphylla plants were grown from seeds (Konstanz botanical garden, Germany) in greenhouses, at 22–34 °C and 31–86% relative humidity. Plants varied in ages depending on which part was collected. Root samples were ca. Four months old, primary stem tissue were ca. Six months old, and secondary stem samples were ca. Ten months old at the time of harvest. Leaf samples were acquired as “old” and “young” leaves of plants at the age of ca. 12 months. “Old leaves” refers to the leaves first produced by the plant, thus they are some of the largest and most mature leaves. “Young leaves” refers to those on the very tip of the branch, thus they are some of the smallest and least mature leaves. Fruit samples were acquired as immature and mature from a plant age of 12–15 months. Immature fruits were green and had a smooth surface. Mature fruits had changed from green to a dark purple color and had a wrinkled surface.

4.2. Tryptamine-d4 absorption in roots

Seven months old R. tetraphylla plants were carefully up-rooted and rinsed for excess soil. Then, pieces of side-root of approx. 15 mm length were cut with a razor and placed in 1.5 ml Eppendorf tubes containing 150 μL of m tryptamine-d4 (labelled at the two CH_{3} groups, T894602, Toronto Research Chemicals). The roots absorbed the solvent for 30–40 min until the entire volume was absorbed. Then, the lower 1–1.5 cm of the root (from the tip) were cut and left in the closed Eppendorf tube overnight at room temperature. The next day, the roots were snap frozen in liquid nitrogen and prepared for MALDI-MS imaging.

4.3. Tryptamine-d4 absorption in leaves

Young leaves of R. tetraphylla were collected and placed individually in 10 μL of m tryptamine-d4 solution in 5 ml Eppendorf tubes. The tubes were closed and the leaves were set to absorb overnight at room temperature. The next day, the leaves were snap frozen in liquid nitrogen and prepared for MALDI-MS imaging.

4.4. MALDI-MSI - sample preparation

Samples were harvested and placed in 50 mL falcon tubes and immediately suspended into liquid nitrogen for snap freezing. For root samples, the plants were uprooted and washed with tap water to remove the excess dirt before snap freezing. Samples were stored at –80 °C until further preparation.

Cryo-sectioning of samples was performed based on the methods developed by Kawamoto (Kawamoto, 2003; Kawamoto and Kawamoto, 2021) and adapted to plant samples by Montini et al. (2020), but with some modifications. A slurry of n-heptane and dry ice was prepared in a Dewar jar. A 2.5 × 2.0 cm mold (SECTION-LAB Co. Ltd.) was filled with 2% (wt/v) aqueous gel of carboxymethyl cellulose and immersed in the n-heptane dry ice slurry, but not submerged. When the edges of the embedding media were starting to freeze, the sample was placed in the embedding media, and the mold was carefully submerged in the n-heptane until frozen. The frozen block was removed from the mold and stored at –80 °C until cryo-sectioning. Embedded plant samples were cryo-sectioned at –23 °C using a LEICA CM3050S cryo-microtome (Leica Microsystems, Wetzlar Germany). Using the cryo-microtome, the embedded sample was trimmed to the desired section of the sample. Cross sections of the leaves were taken approximately from the longitudinal center of the leaf. To achieve 10 μm sections of the plant tissue, cryo-film (SECTION-LAB Co. Ltd. Hiroshima, Japan) was adhered to the sample prior to sectioning. Once sectioned, the opposite side of the cryo-films were fixated to glass-slides using double-sided carbon tape.
(Electron Microscopy Sciences, Hatfield, PA, USA) and stored at ~80 °C until the MALDI imaging experiment.

Prior to matrix application, cryo-sectioned samples were freeze-dried overnight. Subsequently, optical images of the plant samples were taken using an Olympus BH-2 microscope with reflected light. 2.5-dihydroxybenzoic acid (DHB, Merck Danmark, Seborg) was used as MALDI matrix and applied to samples using an in-house built pneumatic matrix sprayer. The spraying parameters were as follows: 300 µL of 30 mg/mL DHB dissolved in MeOH/H2O (90:10) was sprayed at a flow rate of 30 µL/min from a distance of 11 cm to the sample, using a nebulizing gas pressure of 2 bar while the sample was rotating at a speed of 600 rpm.

4.5. MALDI MSI - analysis

MALDI-MSI was performed on a Thermo QExactive Orbitrap mass spectrometer equipped with an AP-SMALDI ion source (Thermo Fisher Scientific GmbH, Giessen, Germany). Spectra were acquired in positive ion mode with a scan range of m/z 150–1050, at mass resolving power 140,000 at m/z 200. Two matrix peaks of DHB were used as lock masses (m/z 431.0374 and m/z 721.0800). The pixel size varied between samples, as specified with the presented images.

4.6. DESI-MSI - sample preparation

Leaf tissue of *R. tetraptera* was imprinted on porous PTFE, as described elsewhere (Thunig et al., 2011). Briefly, using a vise the abaxial side of the leaf was pressed towards a piece of porous PTFE together with a piece of tissue paper to absorb excess liquid. After 10–15 s of pressure, the pressure was lifted, and the PTFE imprint was ready for analysis by DESI-MSI. Old – and thus very large – leaves were separated into several sections, imaged individually and merged into a single MS-image.

4.7. DESI MSI - analysis

DESI-MSI was performed on a Thermo QExactive Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a custom built DESI-MSI ion source (Thunig et al., 2011). Spectra were acquired in positive ion mode (scan range m/z 150–1050) at mass resolving power 140,000 at m/z 200. The peak from dioscyrtol phthalate ([C26H34O4 + Na]+, m/z 413.2662), a common contaminant in the background, was used as lock mass.

4.8. Data analysis

Raw data files from DESI- and MALDI-MSI experiments were converted to imzML files (Schramm et al., 2012) using the “RAW – UDP to IMZML” software (v1.6.170; TransMIT, Bremen, Germany), and MS images were generated using MSIRender r.v1.01 (Bokhart et al., 2018) using a mass tolerance of 5 ppm. All MS images were normalized to the Total Ion Current (TIC) and the color scale was adjusted to provide the best possible representation of the compounds of interest. Every MS image of reserpine, rescinamine and their intermediates are summed images of m/z values corresponding to the protonated analytes as well as their sodium and potassium adducts (Table 1). Two primary metabolites, phosphatidylcholine 34:2 (PC (34:2)) ([C24H43NO5P + K]+, m/z 796.52532) and dihe-xose ([C12H25O11 + K]+, m/z 381.07937) were used as tissue markers to show the contours of the tissue. All the presented MS-images are representatives of both experiments.

4.9. MS/MS analysis

Leaves of *R. tetraptera* were freeze-dried overnight and manually mortared to a fine powder. 120 mg leaf powder was extracted in 1 mL of methanol by sonication for 45 min and centrifuged for 10 min at 1120 g. The supernatant was filtered through a 0.22 µm filter (CA syringe filter, Frisicore) prior to analysis. A standard of vincristine (purchased at Merck) was dissolved in methanol to a concentration of 10 ppm.

MS/MS analysis was performed on a Thermo LTQ linear ion trap mass spectrometer. The leaf extract and the vincristine standard were analyzed by electrospray ionization at a flow rate of 5 µL/min using a collision energy of 22 (arb. units).

Short summary

Comprehensive mapping of intermediates in the reserpine pathway was performed by mass spectrometry imaging in root, stem, leaf and fruit tissue of *R. tetraptera*.

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.

Data availability

Raw data in form of imzML files of the presented data has been uploaded to the Metaspace platform (https://nam11.safelinks. protection.outlook.com?url=https%3A%2F%2Fmetaspace2020.eu%2F&data=0%5C7CD1%7C7Ca.hubert%40elsevier.com%7C620321d30d4f7181208db10a1b6%7C7C2724e39425410a9279b15010675dc7%0C7CD1%7C6381344222777961%7C1%7Ckown%7C7CTFwpGZsbd3d9eyJjWioMC4wLjAWMDAIIjCJOiV2uluM2lLCJBTH6k1haWwiLjJKXC16m%037C300%7C7C6%7C&data=XXoPrWucapCWBvVkIr4DMxJr93m%2B7pCeU%2FjVIsa4Qg%3Breserved=0) (Palmer et al., 2017) from where it is freely available.

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Appendix A. Supplementary data

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M.D.B.B. Lorenzen et al.

Phytochemistry 209 (2023) 113620