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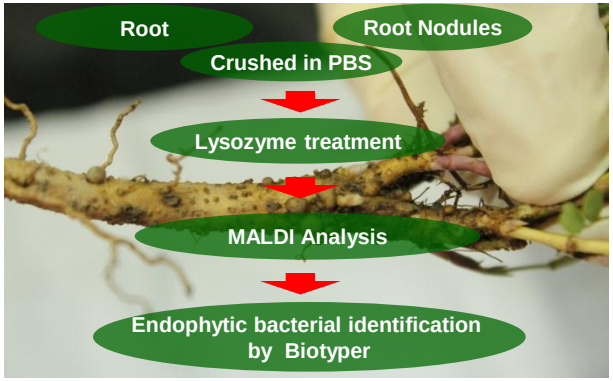

Rapid endophytic bacterial detection by enzyme incorporated MALDI MS

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Abstract

In this paper we have attempted the use of MALDI-MS for the detection of bacteria from complex real world samples such as the root nodules of plants. We have employed lysozyme as the enzyme biosensor to enrich the bacterial molecular signatures from endophytic bacteria during MALDI-MS analysis. We used root nodules of *Arachis hypogaea*, L. as an endophytic model system to prove the enrichment of the endophytic bacterial signals for the rapid and direct identification of the endophytic bacteria. We have been able to demonstrate the functionality of lysozyme as enzyme biosensor during mass

spectrometric analysis from complex plant tissues such as root nodules leading to culture-free detection of the endophytic bacteria. We also report the direct identification of the specific dominant endophytic isolate using MALDI-MS combined with Biotyper software analysis and validation.

Key words: MALDI-MS; plant root nodule; endophytic bacteria; enzyme biosensor; biotyper; identification

Introduction

Organisms from microbial kingdom are always known to be the critical key players for the existence of life on earth. They participate in numerous processes that drive life on biosphere. Furthermore, they are powerful enough to even to influence global climate change [1]. These tiny inhabitants exist widespread and impart both beneficial and harmful effects to plants and animals. Plants harbor a vast variety of bacterial species over the rhizosphere (rhizobacteria), phyllosphere (epiphytes) and inside of the plant tissues (endophytes). Endophytic bacteria are reported to reside within internal tissues of plants without causing damage to their hosts [2]. Endophytic bacterial community are advantageous to plants [3], since endophytic bacteria can contribute to plant development by producing phytohormones [4] and siderophores [5], increasing resistance to pathogens [6] and parasites [2], promoting biological nitrogen fixation [7] and antibiotic production [8]. Thus, each individual plant is most probably a host to one or more endophytic bacteria [9]. However, identification of the endophytic bacteria is a huge challenging task which involves laborious and time consuming processes.

The general practice in bacterial identification relies on basic culturing of bacteria and their responses to various physiological, serological and biochemical tests [10]. However, these methods have a limitation which makes their identification rather complicated or misleading. Analysis of the signature molecules (such as fatty acids) of each bacterial group seems to have influence on the bacterial identification systems [11]. However, once again that method too involves culture based approaches and complicated sample preparation protocols. Phylogenitic methods based on nucleotide sequence similarity and hybridization came into existence to circumvent many problems in culture based approaches. Highly conserved 16S rRNA/DNA sequences of bacterial isolates are given supreme importance in phylogenetic identifications. Moreover the sequences of RNA polymerase (rpoB) [12], or elongation factors [13] were also been used as other targets in bacterial identification. PCR has also been advantageous for the identification of slow growing bacteria and uncultivable bacteria. Although this technique is considered to be one of the most reliable methods for bacterial identification, discriminating to species level is always not possible with one target sequence. Moreover, this technique involves expensive chemicals and technical expertise. Therefore, always alternative identification methods are sought for, for surpassing routine tedious protocols.

MALDI TOF-MS has turned out to be an alternative tool in bacterial identification by utilizing the proteome obtained from the whole cells. The differences in the surface proteome may lead to the identification and differentiation of microorganisms, based on the peaks appearing between the mass range 2000 and 20000Da [14]. MALDI MS has been employed to identify bacteria [15], archaea [16], micro algae and fungal species [17].

While there is continuing interest in using MALDI-MS for characterization and identification of microorganisms, always a pure culture of the bacterial species is essential for its identification to genus/species level. In real world samples, however, such identification may not be practical. To overcome this difficulty of bacterial identification from the real samples, the technique of affinity mass spectrometry was developed, to selectively concentrate trace amounts of bacteria from biological fluids [18-19]. This technique will be more reliable if the culture is in liquid phase. However, the direct endophytic bacterial identification by MALDI MS analysis is extremely difficult, since these bacteria exist inside the plant tissues. Therefore MALDI MS that target selectively on bacterial protein/molecules is required for endophytic bacterial identification amidst plant proteome. Such bacterial protein enrichment could be obtained either through selectively breaking the bacterial cell wall or altering the structural integrity of the plant cell wall in order to release the endophytic bacteria during MALDI MS. This technique would be helpful in the identification of dominant endophytic bacteria directly from plant tissues by MALDI MS analysis, since MALDI MS signal is proportional to the bacterial numbers [17, 20].

Therefore in this paper, we have employed lysozyme as the enzyme biosensor to enrich the bacterial signals of endophytic bacteria during MALDI-MS analysis. We used root nodules of *Arachis hypogaea,* L. as an endophytic model system to prove the enrichment of the endophytic bacterial signals for the rapid and direct identification of endophytic bacteria. We have demonstrated the functionality of lysozyme as an enzyme biosensors during mass spectrometric analysis from complex plant tissues such as root nodules.

Materials and method

Chemicals

Trifluoroacetic acid (TFA) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3, 5-Dimethoxy-4-hydroxycinnamic acid (SA) was purchased from Alpha Aesar (UK). Acetonitrile (MeCN) and acetone were purchased from J.T. Baker, Phillipsburg, NJ, USA. Hen Egg white Lysozyme was purchased purchased from Sigma-Aldrich (St. Louis, MO, USA) from Sigma-Aldrich. Deionized water purified by a Milli-Q reagent system (Millipore, Milford, MA, USA) was used for all experiments.

Collection of endophytic plant material

Root nodules are known for the presence of endophytic bacteria inside the plant tissue. Hence, in our present study, a nodule bearing plant species, *Arachis hypogaea*, L. was used (Fig. S1). Twenty days old *Arachis* plants were carefully uprooted from an agricultural field from Pingtung county and brought to the laboratory in an ice box. The whole plants were washed under running water using a paint brush until the sand particles were removed. Stems and roots were cut into sections of 2 cm long. The tissues were rinsed thoroughly using a series of sterile water followed by sterilization using 70% ethanol (30 seconds). The plant tissues were then surface sterilized using 0.1 % $HgCl_2$ for 3 minutes and rinsed thoroughly with sterile distilled water twice [21] and dried using sterile filter paper.

MALDI MS based direct endophytic bacterial analysis

The overall scheme for the identification of endophytic bacteria directly from the plant tissues by biosensor application is given in Fig. S2. 500µg of root and root nodules were crushed in 500µL Tris HCl buffer (100mM; pH-7.2) using a pre-sterilized mortar and pestle (1 inch radius) and the samples were treated with different concentrations of lysozyme such as 5, 50, 100, 200 and 500µg. The samples were vortexed for 5 minutes for obtaining suspension with uniform distribution and incubated at 37ºC for 30 minutes. The samples were then vortexed again for 10 minutes and centrifuged at 10000×g for 5 minutes. 4µL from the supernatant was spotted onto the stainless steel MALDI target plate (Bruker Daltonics Inc., Germany). For validation of the spectral peak of bacterial origin in the lysozyme treated samples, the bacterial species from the crushed samples were grown on manitol agar medium (Fig S3). Then a colony of the predominant bacterial species was picked, washed and resuspended in 100µL sterile distilled water. 4µL from the bacterial suspension was spotted onto the stainless steel MALDI MS target plate (Bruker Daltatonics Inc., Germany). Each spot was overlaid and mixed with 4µL of matrix SA (0.05M Sinapinic Acid (SA) in 3:1 Acetonitrile:Water containing 0.1% TFA for the analysis of bacterial proteins (> 2000 Da). All experiments were performed in triplicates.

MALDI-MS analysis

Positive ion mode was used to obtain MALDI-MS spectra using MALDI mass spectrometer (Microflex, Bruker Daltonics, Bremen, Germany). The MALDI-MS source was equipped with a nitrogen laser (337 nm), a 1.25m flight tube and the sample target having the capacity to load 96 samples simultaneously. All mass spectra were acquired with the following parameters set on the MALDI-MS: IS1, 19.0 kV; IS2, 16.15 kV; lens,

9.35 kV and reflector at 20.0 kV. The laser energy was adjusted to slightly above the threshold to obtain good resolution and single-to-noise ratios at 60 Hz. 200 laser shots were maintained for obtaining each spectrum. Mass spectra from 2000Da to 12,000Da were acquired in the positive/linear mode with a laser energy of 63.2 μJ using SA as matrix.

Spectral analysis

Statistical analyses of the MALDI MS spectra of the lysozyme treated and untreated plant tissues were carried out using ClinPro Tools 2.0 (Bruker Daltonics). Spectra of different classes were loaded into the software for offline analysis. Baseline correction was achieved using a top hat algorithm with a 10% minimal baseline width. Normalization was done for all spectra to their own TIC (total ion count) by the default programming. Thus, for each spectrum the TIC was determined. The TIC was calculated based on the total sum of intensities from all data points in the spectrum Subsequently, all data point intensities of this spectrum were divided by the obtained TIC value bringing all intensities into the range of [0,1]. To identify the differentially displayed peaks, statistical comparison of the peak intensities of each class of spectrum was obtained through ANOVA (analysis of variance) analysis. Those peaks showing 0.05 P values according to Kruskal-Wallis test and Anderson darling test were considered to present statistically significant differences. The reproducibility of the mass spectral peaks was demonstrated by the stack gel view.

MALDI Biotyper 1.0 software (Bruker Daltonics) with mass spectral library consisted about 967 strains of bacteria was used for the identification of unknown bacterial MS spectrum. The identification was done by using the default settings. The identification was carried out in the automatic mode without any user intervention. Total number of 100 peaks

was picked up from each bacterial spectrum by the software. The peak lists generated were used for matches against the reference library, by directly using the integrated pattern matching algorithm of the software.

Result and discussion

Fig S1 shows the morphology of the plant and the root nodules, which are the sources from where the bacterial endophytic association was obtained. The morphology of the *Arachis* (Fig S1b) plant above the ground level is shown in the photograph. After uprooting the plants from the agricultural fields, we can clearly see the distinct structures, known as root nodules (Fig. S1b) occurring throughout the tap root system of the *Arachis* plants. These root nodules are the sites which actually the endophytic bacteria are housed. The present study involved the direct identification of these endophytic bacteria within these root nodules.

In the present study, lysozyme was used as enzyme biosensor for selectively enriching the bacterial peaks during MALDI-Ms analysis. Further, the bacterial peaks enriched by the lyzozyme treatments were compared with the MALDI MS spectrum obtained from the pure dominant colony of bacterium isolated from the root nodule (Fig S3). Fig. 1 shows the MALDI MS spectrum of crushed root nodule (Fig. 1a), the root nodules treated with different concentrations of lysozyme (Fig 1b-f) and the bacterial isolate from the root nodule of the *Arachis* plant (Fig 1g). Crushed root nodules of *Arachis* which had not been treated with lysozyme showed less number of MALDI MS peaks. Further, in those samples, we could obtain MALDI MS peaks upto m/z 7494.070. The lowest concentration

of lysozyme treatment (5µg) exhibited few bacterial peaks (indicated by star) such as 9358.993, 10941.509 and 13279.655 along with the plant peaks (indicated by a spherical symbol) (Fig 1(b)). On increasing the concentration of lysozyme (50, 100, 200 and 500µg) for enriching the bacterial peaks from the crushed samples, increase in bacterial peak numbers and in their intensity was observed (Fig. 1(c-f)). Almost 9 and 13 bacterial mass spectral peaks were observed while treating the crushed nodule with highest concentration of lysozyme such as 200 and 500µg respectively (Fig 1(e-f); Fig S4). Till date, MALDI MS has been successfully employed for bacterial identification of axenic cultures isolated by traditional culture based approach [15]. In few other reports, nanoparticle (NP) based sensors have been used for the enrichment of bacterial signals in MALDI MS applications [22, 17]. The NP based sensors work through their affinity towards intact bacterial membranes and aid better ionization of the bacterial proteins upon laser irradiation during MALDI MS [23, 24]. But these NPs will be ineffective for bacterial detection in a complicated system such as plant roots/nodules where the bacteria lie encased within plant tissues [24]. More over the penetration of NP based sensors into the inter and intracellular spaces of plant cells where the bacteria are housed, is highly limited. On the contrary, lysozyme is an unique functional biosensor, which selectively cleaves the glycoside bonds between the N-acetyl glucosamine and N-acetyl muramic acid of bacterial cell wall [25, 26] and thereby presumably enrich the endophytic bacterial signals. It can easily penetrate into the intercellular spaces and selectively act on endophytic bacteria and release the bacterial surface signature molecules which can be mobilized out from plant tissues by simple pretreatments such as vortexing and centrifugation and detected using MALDI MS.

Fig. 2 shows the statistical reproducibility of the mass spectral peaks of the nodule (with no lysozyme treatment), which we consider as the untreated control (Fig. 2a) versus the lysozyme treatment (at highest concentration) (Fig. 2b). The protein peaks observed in the spectra within m/z differences ±10 were considered reproducible. Thus in all the plant spectra without lysozyme treatment, we observed that the higher intensity peaks appeared below m/z 8000 (Fig 1a and Fig 2a). Dominant bacterial isolate obtained from the *Arachis* nodule were used for comparison and identification of bacterial peaks from the biosensor treated nodule samples (Fig 1g and Fig 2c). MALDI MS analysis of bacteria lead to wide spread appearance of m/z peaks upto 13280, these peaks were very useful in identifying the bacterial peaks directly from the real sample, which in this case is the root nodule. Lysozyme treated root nodules exhibited peaks from plant origin as well as enriched bacterial mass spectral peaks (Fig 2b). Thus, the clear distinction of bacterial peaks enrichment was observed upon treatment with lysozyme biosensor which aided to the identification of the predominant bacterium.

Table 1 accounts the differentially displayed peaks in all the three groups of samples with their statistical significance. A total number of 39 peaks were picked up, based on the default programming, from 3 groups of samples. Out of peaks picked, 21 peaks were detected with differential expression in the 3 groups of samples by their significant level. Eight peaks of plants were also found in MALDI MS spectra of axenic bacterial culture isolated from root nodules (m/z 5733.54; 6361.; 3995.56; 3265.07; 4071.03; 5632.22; 5946.51; 3980.33) with extremely low intensity (negligible level at bacteria), thus signify their plant origin. Twelve bacterial peaks (m/z 9800.83; 8266.79; 8932.59; 7371.33; 9359.11; 12668.05; 3822.48; 6800.28; 13279.68; 7854.45; 10284.81 and 7859.45) were

found to express in very low levels in root nodule without lysozyme treatment. The lysozyme treatment significantly (PTTA<0.000001) increased the MALDI MS peaks in the root nodules, which again confirms the sensing effect of lysozyme. We also extend our strategy to the roots which might harboured very less bacterial entities compared with that of nodule. Figure 3 shows the MALDI MS spectra for the control (Fig. 3a), biosensor (lysozyme) treated root tissues (Fig. 3b-f) and axenic culture of root nodule bacteria (Fig. 3g). The control and biosensor treatment had not resulted in the enrichment of bacterial peaks which could be attributed to the extremely low quantities of endophytic bacteria.

From the extended library of Biotyper1 software from Bruker Daltonics, the spectra obtained from the predominant colony from the root nodule was compared with spectra obtained from axenic cultures of different strains of nitrogen fixing bacteria. The bacteria showed highest mass homology with *Bradyrhizobium* sp.USDA 3187 (Fig. 4), followed by a local *Rhizobium* sp and another *Rhizobium* sp USDA 3138 **(Table S1)**. Further to identify the sensor assisted peak enrichment for sensing the predominant endophytic bacteria, the MALDI MS spectra obtained from the root nodule treated with lysozyme (500µg) were subjected to the Biotyper 1 identification tool. The spectra exhibited highest similarity again with Bradyrhizobium sp. USDA 3187 (Fig. 5), however the score value are comparatively lower than the MALDI MS spectra of axenic culture (Table S2) and thus the identity of the lysozyme treated nodules affirmatively enriched more MALDI MS peaks of bacterial origin (Fig 1 and Table1), thereby easing the identification of the predominant bacterium in the real sample like root nodule. Biotyper software is already proven to be very effective for diversity and ecological studies applicable for the analysis of large

populations of isolates allowing the differentiation of strains, species and genera with an effectiveness [27, 28]. The use of this software combined with the lysozyme incorporated approach for selective release of bacterial proteins has led to the successful detection and identification of the endophytic bacteria in the root nodules of the peanut plant.

Conclusions

We have reported the application of lysozyme biosensor for the MALDI MS peak enrichment of endophytic bacteria directly from the root nodules of *Arachis hypogeaea* plant. This enzyme biosensor selectively acts on the bacterial cell wall and thereby alters the bacterial cell wall structure without agitating plant cells. Thus the MALDI MS signal of the endophytic bacterial proteins had been enriched. The enriched bacterial signals were validated using the dominant bacterial isolates obtained from the root nodule. The root nodule bacteria had been identified as the close relative of *Bradyrhizobium* sp USDA 3187. Enrichment of the bacterial MALDI MS signals from the real samples (root nodules) treated with lysozyme biosensors pays a lead for the rapid analysis of endophytic bacteria from plant tissues without complex cultivation and additional confirmation steps.

Acknowledgements

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Figure captions

Fig. 1. MALDI MS analysis of endophytic bacteria from the root nodule of the plant *Arachis hypogeaea*. Spectra obtained from (a). Crushed root nodule; (b) crushed root nodule with 5µg lysozyme ; (c) crushed root nodule with 50µg lysozyme; (d) crushed root nodule with 100µg lysozyme; (e) crushed root nodule with 200µg lysozyme; (f) crushed root nodule with 500µg lysozyme; (g) Bacterial isolate from the root nodule (symbol ● denotes peaks of plant origin; ★ - peaks of bacterial origin)

Fig. 2. Mass spectral reproducibility of (a) crushed root nodule; (b) crushed root nodule treated with 500µg of lyzozyme; (c) bacterial isolate isolated from root nodule of *Arachis hypogeaea* plant represented by gel view of clin pro software

Fig. 3. MALDI MS analysis of endophytic bacteria from the root of *Arachis hypogeaea* plant. Spectra obtained from (a). Crushed root; (b) crushed root with 50µg lysozyme; (c)

crushed root with 100µg lysozyme; (d) crushed root with 200µg lysozyme; (e) crushed root with 500µg; (f) Bacterial isolate from the root nodule

Fig. 4. Identification of root nodule bacteria isolated from *Arachis hypogeaea* plant from the MALDI MS library constructed using Biotyper 1 software. Spectrum above the X-axis signify the unknown endophytic bacteria. Spectum below the X axis denote most similar spectrum of the library (Bradyrhizobium sp). Mass spectral peak of the test spectra having similar mass spectral peak from the spectral library is highlighted in Green (mismatched peaks shown in red).

Fig. 5. Identification of nodule bacteria directly from *Arachis hypogaea* nodules after lysozyme treatment using the MALDI MS library constructed using Biotyper 1 software. Spectrum above the X-axis signify the unknown endophytic bacteria. Spectum below the X axis denote most similar spectrum of the library (*Bradyrhizobium* sp). Mass spectral peak of the test spectra with similar mass spectral peak from the spectral library is highlighted in Green (mismatched peaks shown in red).

Supplementary figure

Fig. S1. Photograph of the source plants of the root nodules used in the study (a). *Arachis*; (b) root nodule of *Arachis hypogaea*

Fig. S2. Schematic of bacterial peak enrichment by enzyme incorporated MALDI MS analysis of endophytic bacteria from root nodules of *Arachis hypogaea*

Fig. S3. Photograph of colony morphologies of bacterial isolates obtained from root nodules belonging to *Arachis hypogaea*

Fig. S4. Histogram showing the statistical variation (Error bars) of MALDI MS peaks from *Arachis* plant. Peaks in red represent mass profile of bacterial isolates; Green peaks represent mass profile of root nodule treated with lysozyme; Blue colored peaks represent mass profile of root nodule

Table-1 Statistical analysis of MALDI MS peaks from plant tissue treated with biosensors and authentic bacterial strain obtained from *Arachis* root nodule

Index	Mass	[a] DAve	[b] PTTA	[c] PWKW	[d] PAD	Average Nod Bacteria	Average Biosensor treatment	Average Nodule	StdDev 1	StdDev 2	StdDev 3
1	5733.54	74.07	< 0.000001	0.000499	0.182	6.17	50.71	80.24	4.03	18.63	5.76
2	9800.83	174.53	< 0.000001	0.000702	0.151	213.86	137.99	39.33	19.43	57.01	8.36
3	8266.79	334.19	< 0.000001	0.00106	0.185	422.42	291.98	88.24	43.06	116.67	21.41
4	8932.59	149.12	< 0.000001	0.000655	0.206	188.03	119.29	38.91	19.48	42.45	9.92
5	7371.33	163.11	< 0.000001	0.000935	0.182	259.89	190.79	96.78	10.31	46.41	13.93
6	9359.11	822.72	< 0.000001	0.000499	0.327	900.98	422.58	78.26	97.54	145.62	16.28
7	6361	504.19	< 0.000001	0.000499	0.143	13.74	279.04	517.93	1.18	134.39	37.74
8	12668.05	242.8	< 0.000001	0.00106	0.0908	297.21	204.16	54.41	30.41	68.35	7.04
9	3996.81	44.07	< 0.000001	0.000499	0.132	1.19	20.85	45.27	0.62	10.77	3.8
10	5632.22	47.04	< 0.000001	0.00252	0.0908	10.87	45.21	57.91	6.74	18.18	4.25
11	5946.51	584.7	< 0.000001	0.000499	0.183	20.51	331.53	605.21	5.79	168.23	55.39
12	3980.33	163.49	< 0.000001	0.000499	0.177	5.59	80.85	169.08	2.56	41.38	16.5
13	3995.56	132.28	< 0.000001	0.000499	0.177	4.08	65.61	136.36	1.11	32.32	14.57
14	3822.48	48.59	< 0.000001	0.000499	0.327	9.85	33.43	58.44	4.54	12.06	7.1
15	6800.28	98.98	< 0.000001	0.000499	0.177	26.6	80.91	125.58	3.08	29.58	13.02
16	13279.68	748.25	< 0.000001	0.000499	0.191	787.42	373.97	39.17	130.82	188.4	8.13
17	7854.45	366.53	< 0.000001	0.00127	0.0286	395.25	152.34	28.72	61.67	131.21	6.75
18	10284.81	347.32	< 0.000001	0.000935	0.191	437.9	276.71	90.57	65.15	118.38	15.3
19	7859.45	208.22	< 0.000001	0.000655	0.143	237.74	110.67	29.52	38.3	59.22	8.96
20	3265.07	65.08	< 0.000001	0.000655	0.183	4.53	38.4	69.61	2.2	21.3	8.75
21	4071.03	76.13	< 0.000001	0.000499	0.268	21.06	58.58	97.19	3.94	19.47	11.75
22	5943.72	360.96	2.16E-06	0.000499	0.154	22.58	207.31	383.54	8.31	98.55	57.13

23	7825.35	240.68	4.07E-06	0.00106	0.177	265.86	157	25.18	54.41	84.43	4.18
24	10941.99	670.67	2.29E-05	0.00106	0.387	846.66	567.05	176	218.43	220.38	25.14
25	6604.22	113.63	0.000035	0.000499	0.177	145.61	79.62	31.98	37.82	29.23	2.01
26	6398.54	147.86	4.96E-05	0.000499	0.183	7.09	76.95	154.95	3.43	37	42.92
27	5421.91	74.7	4.96E-05	0.0023	0.327	100.36	78.45	25.66	34.87	25.59	5.83
28	7317.14	46.5	5.55E-05	0.00123	0.678	55.62	89.74	102.12	8.71	12.76	11.99
29	8344.63	113.5	5.88E-05	0.000499	0.143	169.18	92.87	55.68	43.53	22.29	5.73
30	5260.89	50.59	6.99E-05	0.00127	0.886	30.41	60.97	81	13.2	16.64	9.09
31	3702.16	49.24	0.000171	0.00449	0.237	64.56	44.37	15.33	20.33	18.41	6.69
32	5148.63	34.37	0.000358	0.00559	0.597	61.69	39.93	27.33	12.11	14.55	6.16
33	3082.46	20.32	0.00241	0.0179	0.0908	38.13	37.8	17.81	12.83	19.73	2.78
34	7491.32	140.83	0.00267	0.00988	0.327	333.73	320.78	192.9	68.46	73.88	47.86
35	4875.59	42.34	0.00314	0.115	0.0313	88.24	79	45.9	33.72	29.14	4.72
36	3064.82	9.76	0.0144	0.0122	0.00308	9.41	19.17	13.32	2.72	10.9	2.22
37	4981.97	16.04	0.335	0.18	0.0908	65.04	79.62	81.08	21.36	17.75	10.42
38	5001.64	13.07	0.541	0.525	0.478	106.87	93.8	97.01	20.55	27.18	11.76
39	2750.3	0.49	0.993	0.874	0.886	25.06	25.54	25.14	11.12	6.09	9.07

a

Difference between the maximal and the minimal average peak area/intensity of all classes

b

PTTA is the P-value of t-test ANOVA (<0.01 significant)

c

PWKW is the P-value of Wilcoxon test or Kruskal-Wallis test (<0.01 significant)

d

PAD is the P-value of Anderson-Darling test (1- normal distribution)

Std Dev - Standard deviation of the peak area/intensity average.

Fig -1

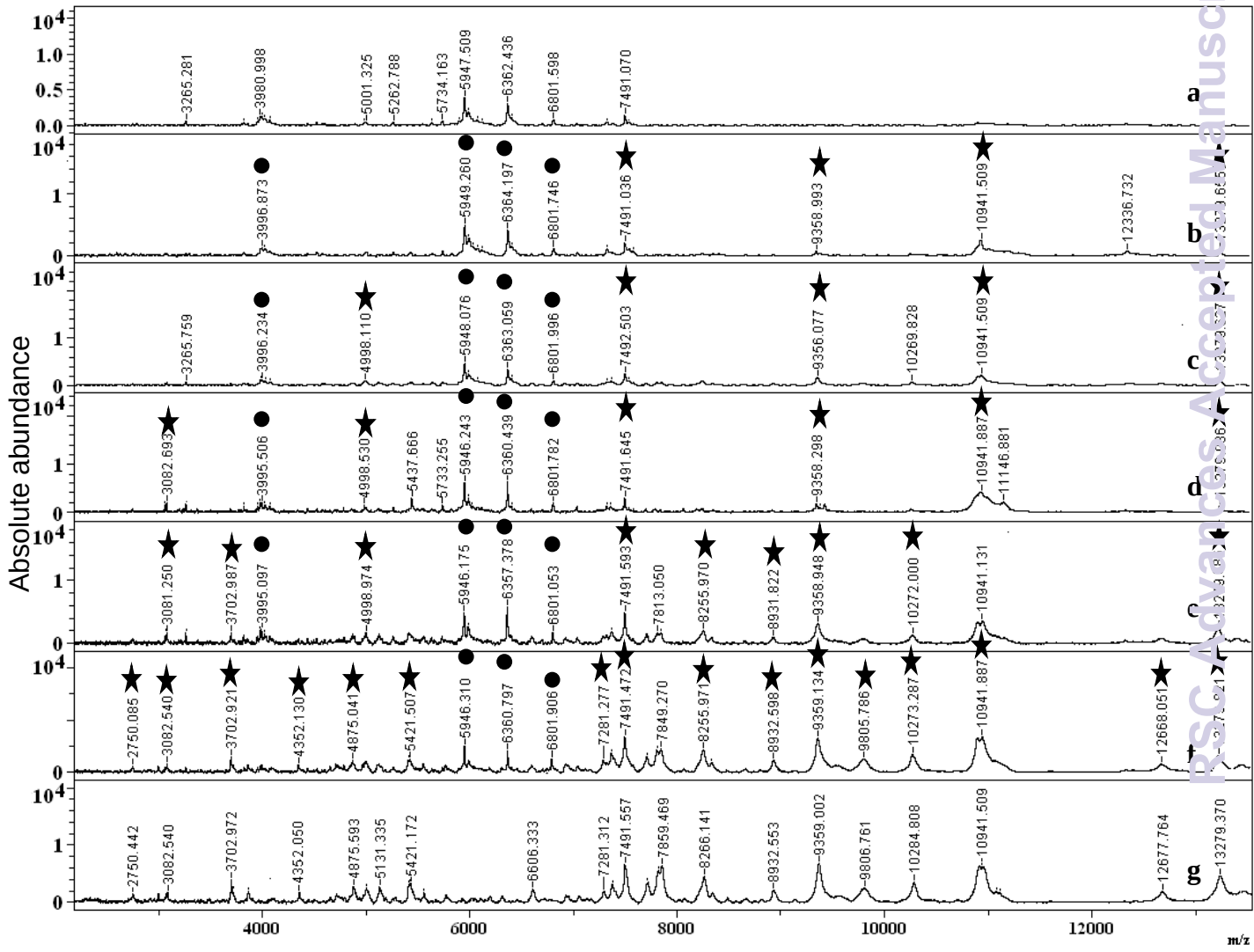


Fig -2

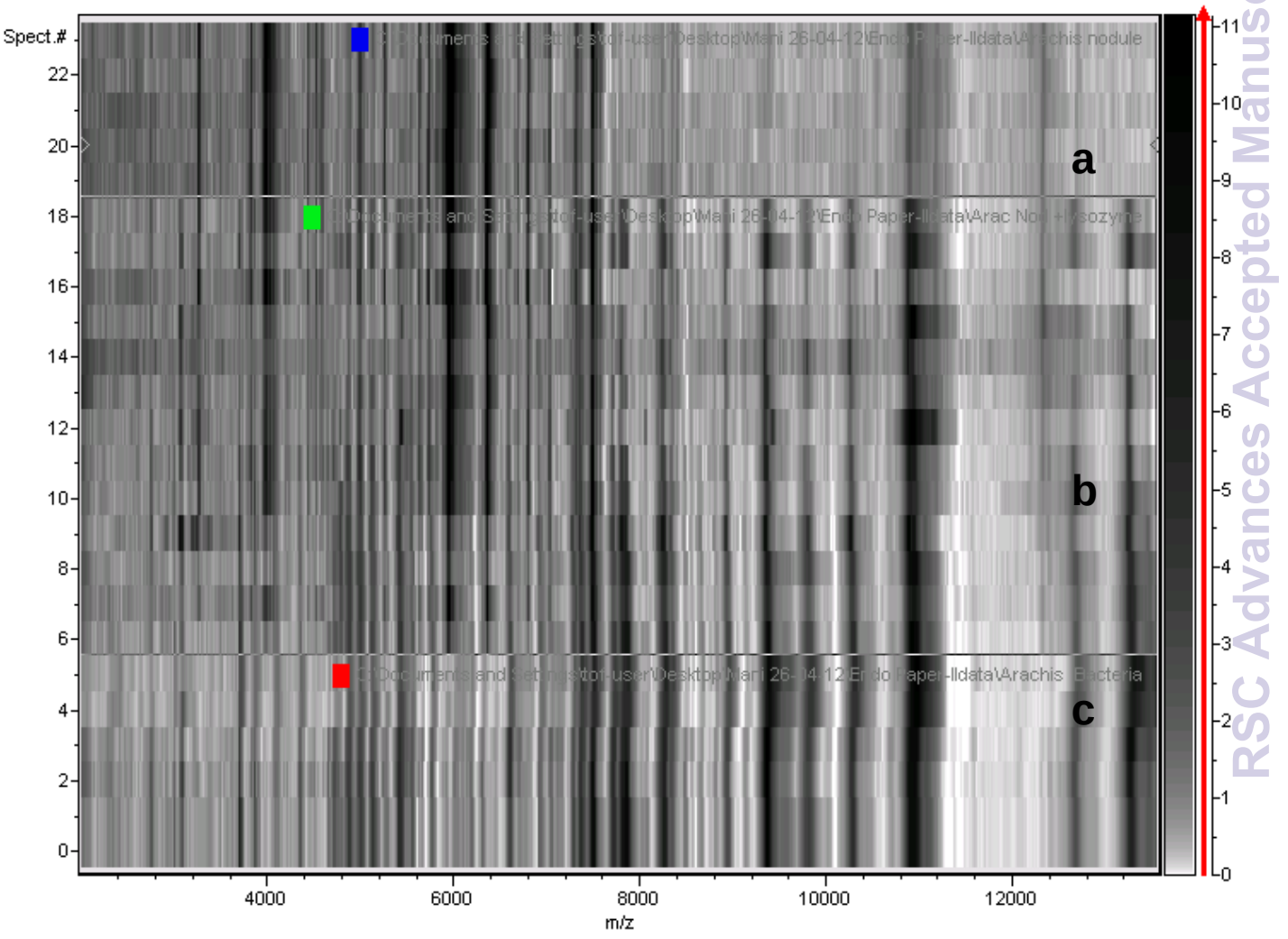


Fig -3

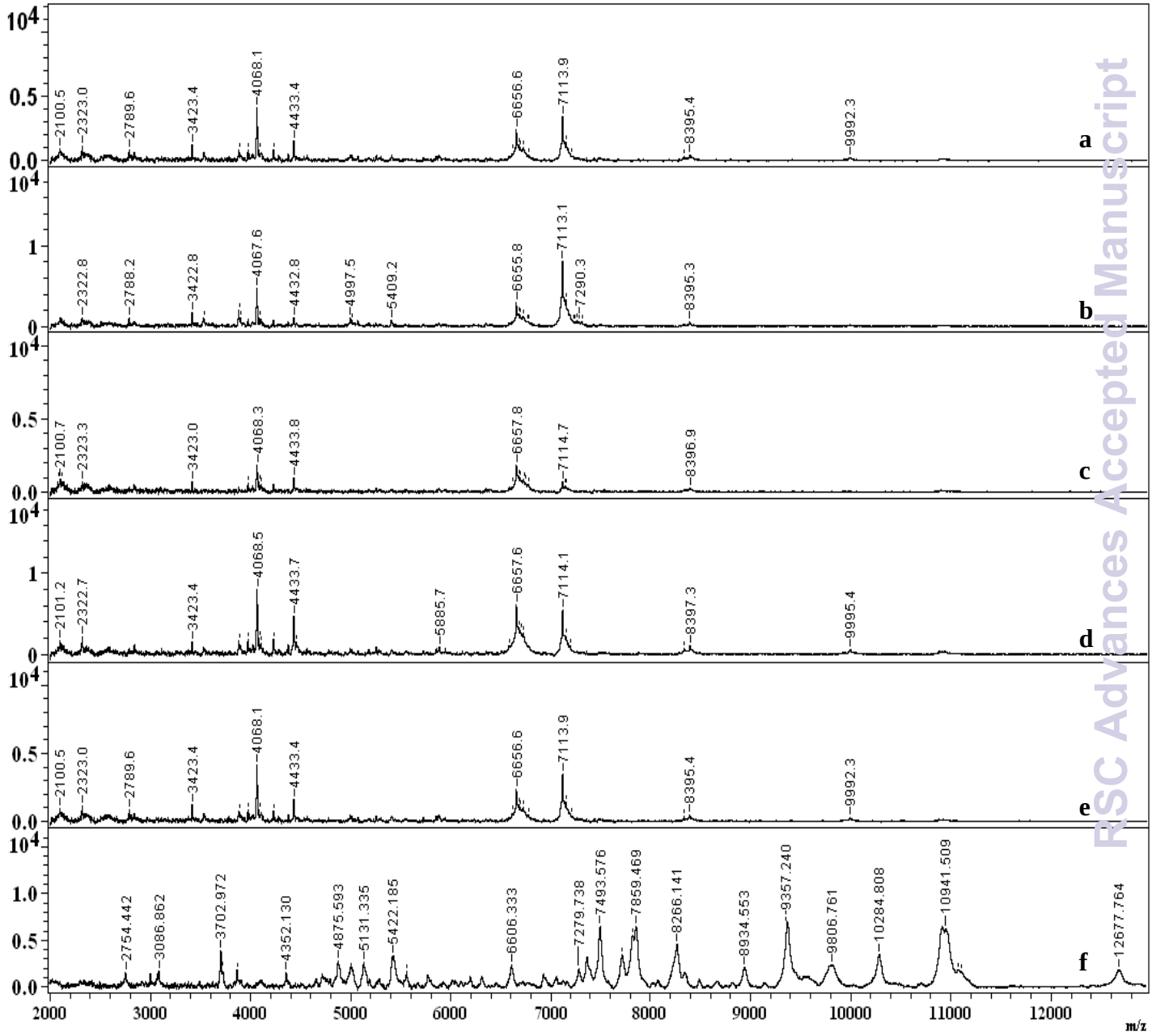


Fig - 4

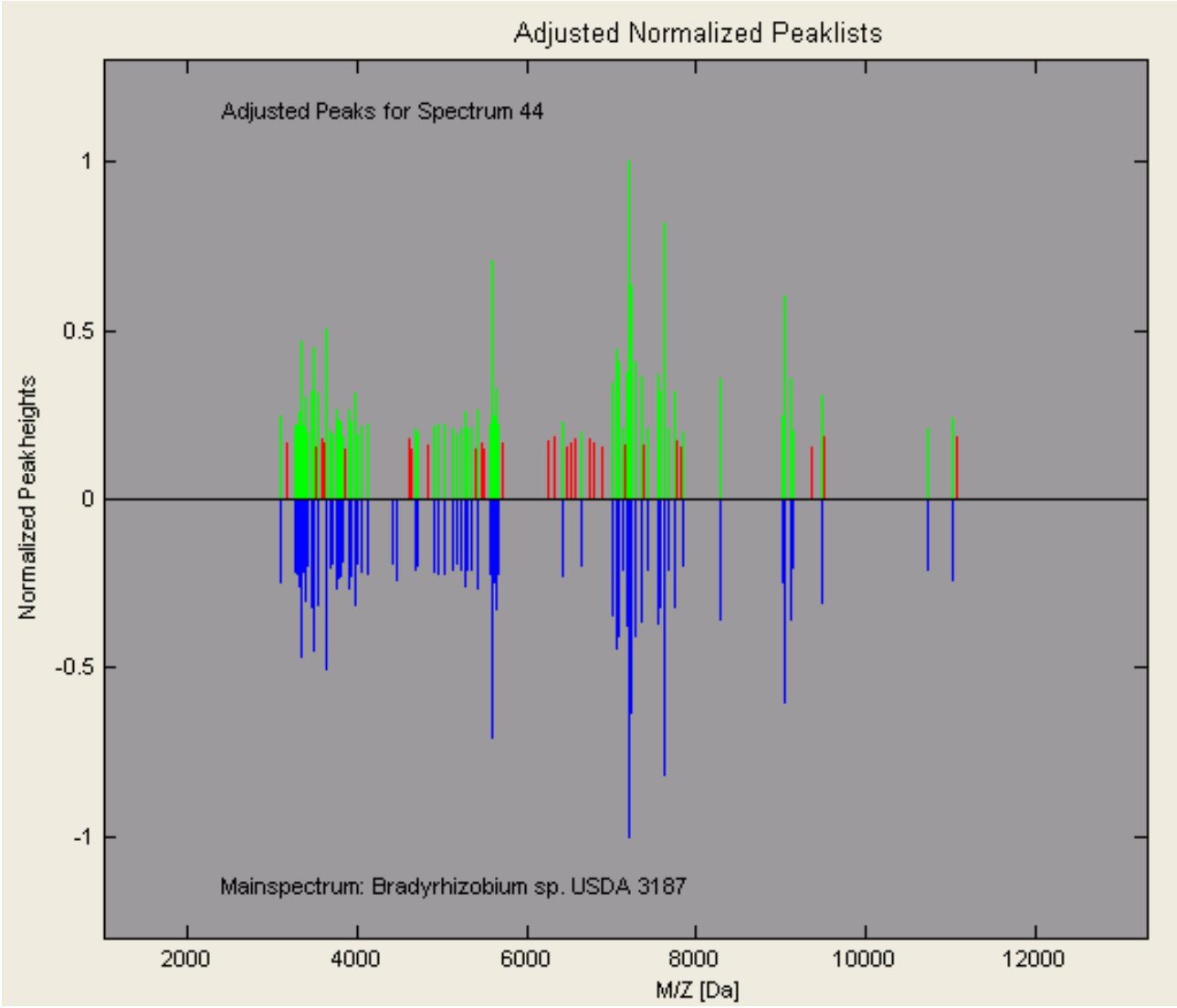


Fig - 5

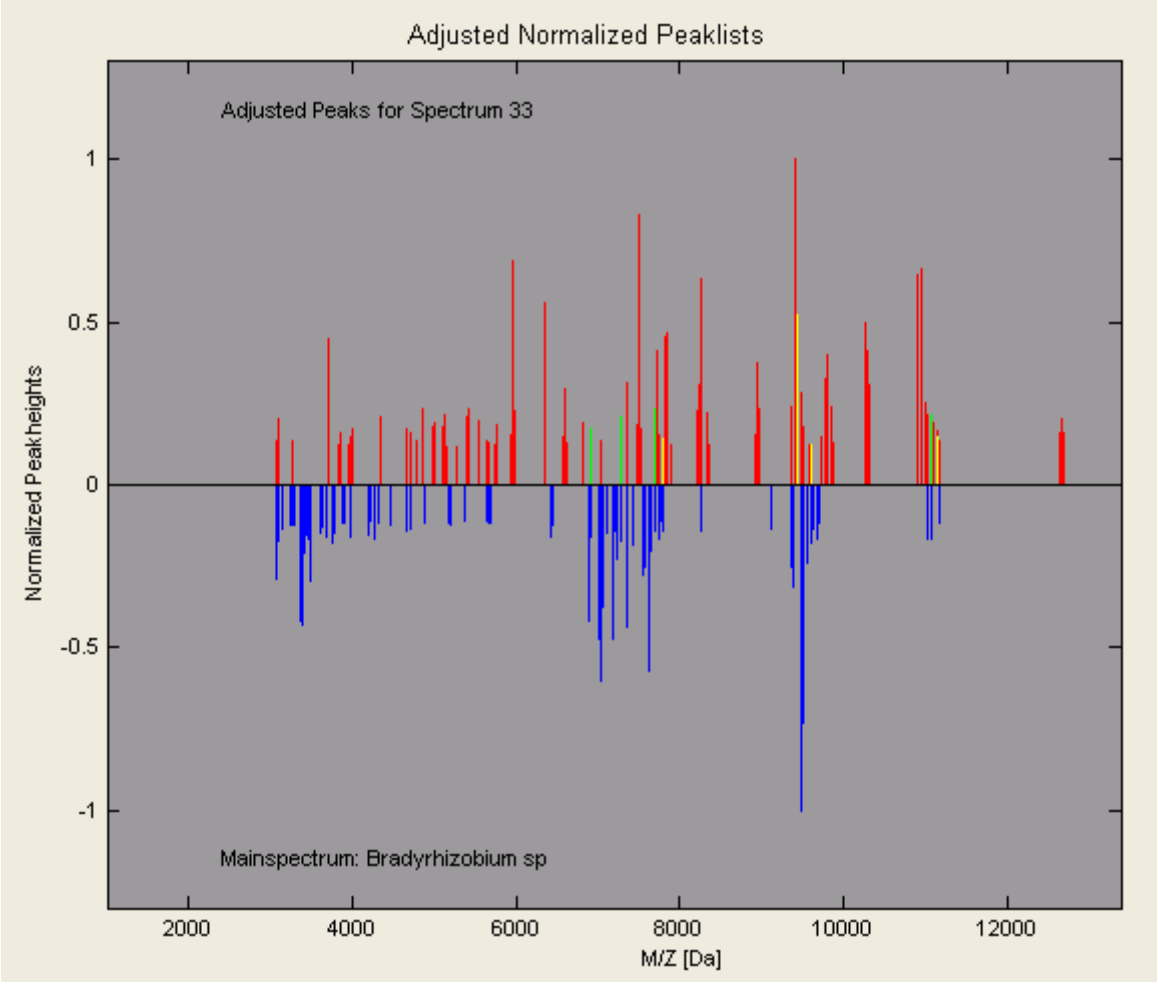


Fig S1

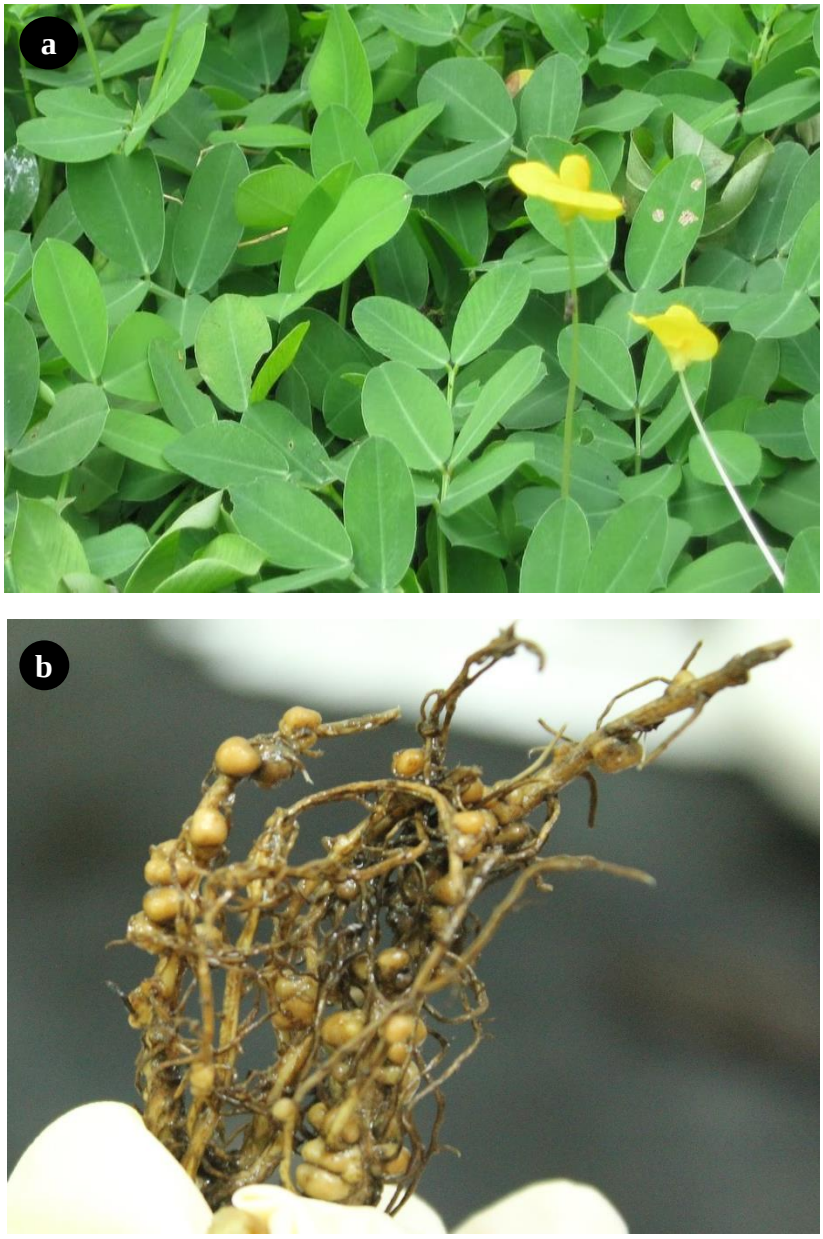
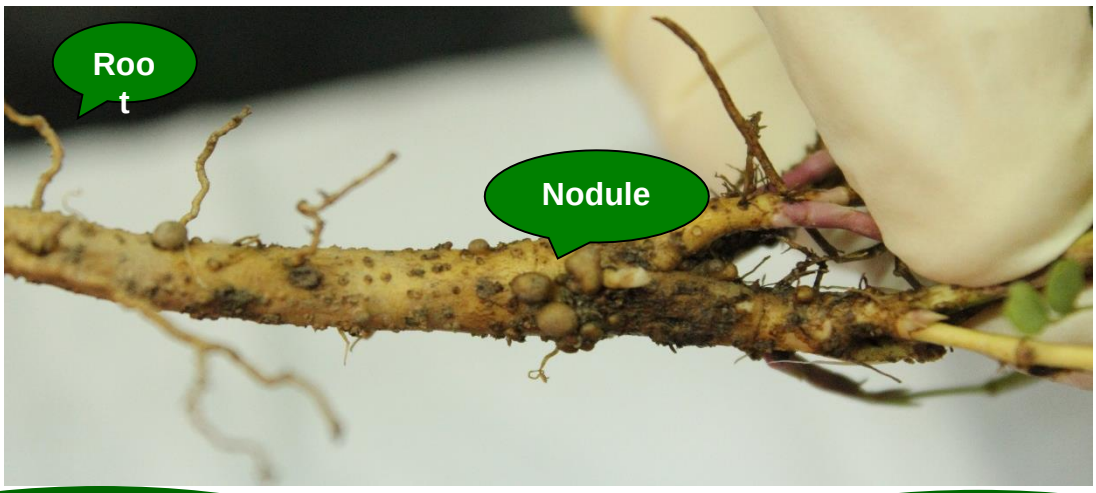


Fig S2



Roots

Root Nodules

Crushed in PBS

Lysozyme treatment

MALDI Analysis

Endophytic bacterial identification by Biotyper

Fig S3

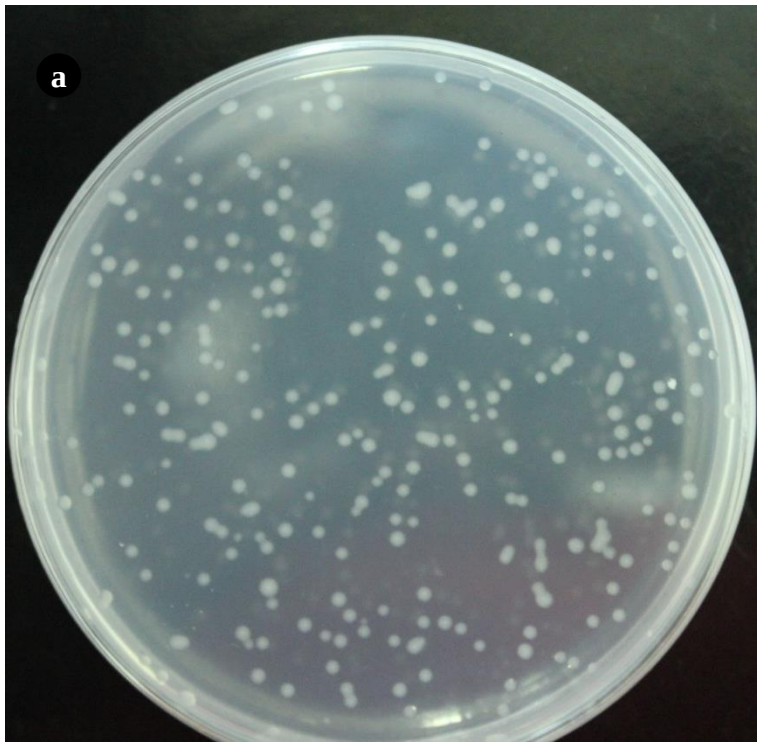


Fig S4

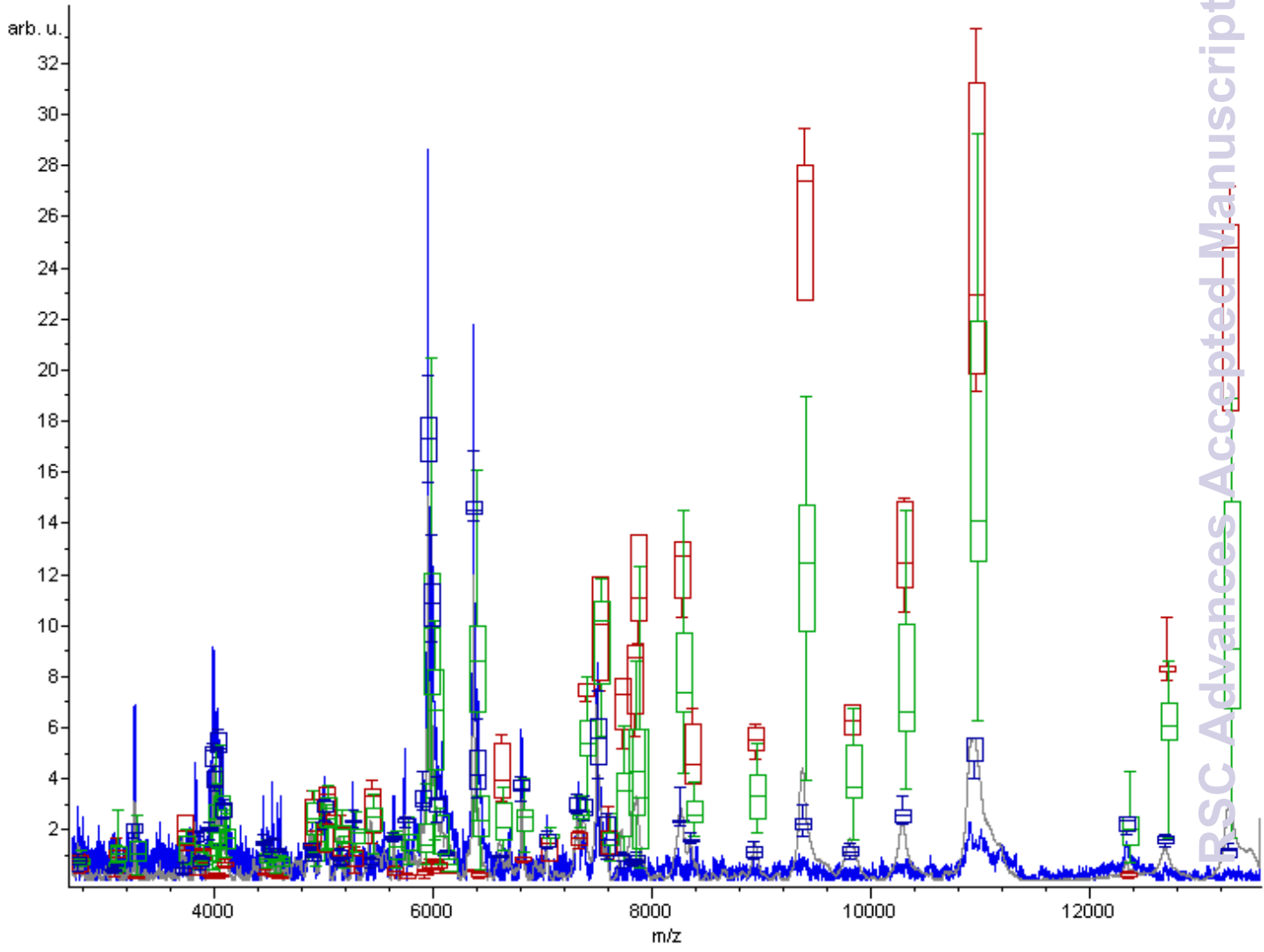
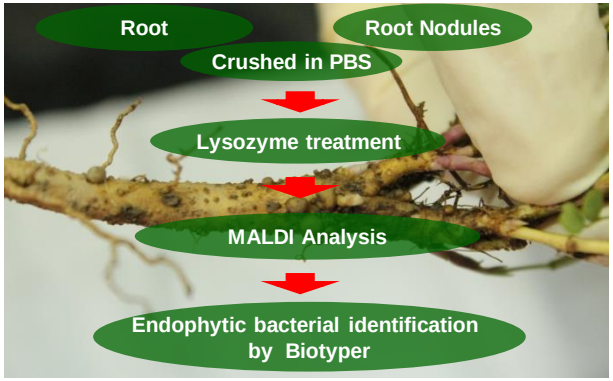


Table S1

Organism resemblance	Act Sc	Max Sc	Rel Score	PN k	PN b	PN m	Rel P-Num	I-Cor	Score
Bradyrhizobium sp. USDA 3187	3046	6640	0.51	30	8	100	0.34	0.88	153
Bradyrhizobium sp. 13562	2602	7000	0.37	15	17	100	0.23	0.79	42
Rhizobium sp. USDA 3138	2298	7000	0.33	15	14	100	0.22	0.75	29
Rhizobium leguminosarum 13541	1936	7000	0.28	14	9	100	0.19	0.65	29
Rhizobium leguminosarum 13544	1728	7000	0.25	5	21	100	0.16	0.60	13
Rhizobium leguminosarum MM105	1601	7000	0.23	8	13	100	0.14	0.65	8
Pseuomonas fluorescence DSM 50090T HAM	1535	7000	0.22	9	12	100	0.15	0.61	7
Pseuomonas fluorescence *DSMZ 50091HAM*	1484	7000	0.21	10	8	100	0.14	0.62	6
Shewanella putrefaciens DSMZ 6067 HAM	912	6717	0.14	7	6	100	0.10	0.39	3

Table S2

Organism resemblance	Act Sc	Max Sc	Rel Score	PN k	PN b	PN m	Rel P-Num	I-Cor	Score
Bradyrhizobium sp. USDA 3187	926	7000	0.13	7	4	100	0.09	0.47	6
Bradyrhizobium sp. 13562	904	6713	0.15	8	4	100	0.1	0.29	4
Rhizobium sp. USDA 3138	834	6852	0.12	6	5	100	0.09	0.35	4
Rhizobium leguminosarum 13541	813	7000	0.12	4	6	100	0.07	0.43	3
Rhizobium leguminosarum 13544	601	6007	0.1	4	5	100	0.07	0.34	2
Arachis bacteria MM105	786	6947	0.11	5	5	100	0.07	0.25	2
Pseuomonas fluorescence DSM 50090THAM	746	6865	0.11	3	7	100	0.07	0.27	2
Shewanella putrefaciens DSMZ 6067 HAM	606	7000	0.09	4	5	100	0.07	0.27	2
Staphylococcus simiae DSM 17637	679	6917	0.1	3	6	100	0.06	0.24	1
Staphylococcus captitis ssp captitis DSM 6180	836	6650	0.13	6	5	100	0.09	0.13	1



Justification

A lysozyme mediated novel and rapid analytical method for direct identification of endophytic bacteria from plant tissues by MALDI MS.