An efficient protein extraction method for proteomic analysis of black pepper (*Piper nigrum* L.) and generation of protein map using nano LC-LTQ Orbitrap mass spectrometry

Palaniyandi Umadevi¹ and Muthuswamy Anandaraj²

¹Division of Crop Improvement and Biotechnology, ICAR- Indian Institute of Spices Research, Kozhikode, Kerala, India
²ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India

*Corresponding author: umamanicar@rediffmail.com

Abstract

Information on black pepper proteins can accelerate the basic understanding of crop response to various biotic, abiotic stresses as well as the crop improvement programmes. Here, we present the protocols for total leaf protein extraction from this non model, recalcitrant plant species *P. nigrum* after evaluating 5 different standard methods. The efficiency and suitability of the methods were evaluated using important parameters such as protein quantity and quality, solubility in IEF compatible buffer and streak free results, number of spots in 2DE gel. Modified lysis buffer and phenol method were found to be applicable methods for protein extraction from black pepper. Among two applicable methods, the phenol protocol is time-consuming, demanding the toxic phenol. Hence, we propose the modified lysis buffer method as superior, rapid method with no polyphenol contamination for total leaf protein extraction. This is a very first report which evaluated different methods and optimized the standards for the analysis of black pepper leaf proteome. Successful identification of randomly selected spots by nano LC-LTQ Orbitrap proves its compatibility to high throughput MS analysis which leads to the generation of leaf protein map, the first of its kind.

Keywords: 2DE, extraction methods, recalcitrant plant, proteogenomics.

Abbreviations: CHAPS_3-(3-cholamidopropyl dimethylammonio)-1-propanesulfonate; 2DE_two dimensional gel electrophoresis, DTT_dithiothreitol; IEF_isoelectric focusing; IPG_immobilised pH gradient; KDa_kilo Dalton; MW_molecular weight; PAGE_polyacrylamide gel electrophoresis; SDS_sodium dodecyl sulphate; TCA_trichloroacetic acid.

Introduction

Known as the “King” of spices, black pepper (*Piper nigrum*), a perennial crop of the tropics, is economically the most important and the most widely used spice crop of the world. Though the information on essential oils, oleoresin, piperin and polyphenol profiles are reported, the knowledge on proteins is still in its infancy. Proteomic studies especially quantitative/expression proteomics requires 2D gels with well resolved proteins with reproducible patterns. To achieve this, sample preparation forms crucial step prior to electrophoresis. The major drawback in protein extraction with plant tissues are the presence of proteases and interfering compounds such as phenols, lipids and carbohydrate (Carpentier et al., 2005). Handling tissues from tropical plant species brings even greater challenges as the cell wall and vacuole make up large volume of cell mass, with less representation of cytosol. Therefore, these plant tissues have relatively low protein volume content when compared to bacteria and animal tissues (Islam et al., 2004).

Many recalcitrant plants have secondary metabolites that combine with proteins by covalent condensation which make protein charge heterogeneity and streaks in 2D gels. On the other hand, the carbohydrates block the gel pores leading to extended IEF time and sample loss. Terpenoids, lipids, nucleic acids, etc. affect the reproducibility. Though, major optimal conditions are required for sample preparation from plant tissues for IEF and 2DE purposes (Saravanan and Rose 2004). There is no single method of sample preparation that can be commonly applied to all species of plant samples (Weiss and Gorg 2008). The efficiency of extraction method will vary based on the plant species. Earlier proteomic studies suggest that extraction protocols need to be standardized for different plant sample as different plant species and its tissues vary in the amount and types of non-protein interfering compounds (Carpentier et al., 2005; Ramesh Sundar et al., 2010). After extracting proteins, precipitation and solubilisation in a compatible solution suitable for IEF to yield reasonable protein patterns on 2 D gels is another challenge, when using recalcitrant, non-model plant as plant source. The IEF buffers are not able to solubilise the hydrophobic proteins which lead to precipitations of these proteins at their isoelectric point. The low abundant proteins are not detected by standard proteomic techniques (Ephritikhine et al., 2004). Black pepper (*Piper nigrum*) is a flowering plant in the family piperaceae, cultivated for its fruit, which is usually dried and used as a spice. This crop is native to south India, and is extensively cultivated in India and elsewhere in tropical regions. Black pepper is affected by many biotic and abiotic factors which lead to low productivity (Anandaraj et al., 1989; Sarma and Kalloo 2004). With the lack of whole genome sequence the crop improvement programme becomes more challenging. As the direct executors for physiological and biochemical reactions, proteins becomes key players in biological system. Proteomics technology has unravelled a great number of proteins which play crucial roles in plant growth/
development and adaptation to environmental stresses. Functional analyses of those proteins will contribute to develop biotic/abiotic stress-resistant/tolerant crops and artificially regulated crops (Eldakak et al., 2013). This may help to find the functionally important proteins or their modifications that could not be found through the other studies at any other levels. The present study aimed to find a procedure to reach a well-resolved protein for 2-D gels by optimizing the technical steps for extraction of total protein from leaf and also the downstream analysis and identification of proteins by nanLC-LTQ Orbitrap with the view to initiate the generation of protein map.

Results and Discussion

Quantitative and qualitative differences of proteins

The evaluation of the efficiency and suitability of the selected methods on two dimensional profiling was analyzed by some important factors viz., protein quantity and quality, solubility in IEF compatible buffer and streak free results, number of spots in 2DE gel. The efficient precipitation of protein was found only in 0.1M ammonium acetate in 80% methanol (both at 2 hrs and overnight incubated samples). The pellets obtained from all the methods by TCA/acetone precipitation were difficult to solubilise when compared to pellets obtained from ammonium acetate/methanol precipitation. The difficulty to re-dissolve TCA precipitated proteins was also reported in earlier studies (Nandakumar et al., 2003; Carpentier et al., 2005).

Solubility of proteins with good all-purpose buffer (buffer I) and with strong buffer (buffer II) produced clear cut result in selection of solubilisation buffer for black pepper proteins. The pellets dissolved in buffer I found to be superior, compared to solubilisation buffer II, in which the latter produced 2D gel pattern with high horizontal streaking (data not shown).

The quantification using 2D quant kit as well as manual method (Ramagui and Rodriguez 1985) yielded same pattern of protein concentration. The protein yield using TCA-acetone method was extremely low. The dense SDS, PVP/ TCA-acetone method yielded next least protein concentration but also had quantifiable poly phenol contamination (Fig 2). The modified lysis buffer method and the classical phenol method resulted in high amount of protein with very less poly phenol contamination. The major reason for low protein yield in TCA/Acetone method of extraction may be due to the insolubility of the pellet in IEF buffer (Chen and Harmon 2006), compared to other methods. Though PVP is known to be effective in absorbing poly phenols, it has been found ineffective in extraction of proteins in black pepper. This could be due to high pH of extraction buffer, at which phenols get oxidized and it cannot be absorbed by the PVP. Similar finding was reported by Carpentier et al., (2005) on protein extraction from banana meristems. On contrary to Wang et al (2003), in our study the addition of SDS to the extraction buffer did not improve extraction rather it contained poly phenols as contaminant. Similar result was reported by Carpentier et al., (2005) in banana meristem, reporting inefficiency of SDS for improved solvent action.

The protein separation by 1D SDS-PAGE (Fig 3) shows the well separated pattern of proteins with molecular weight ranging from below 14 KDa to above 95KDa in lysis buffer method and phenol methods, respectively. The other methods proved to be less efficient for black pepper leaves. The good separation and distribution of low molecular weight proteins between 20 KDa to below 14 KDa denotes the suitability of these two extraction methods for this plant species. Based on the quantitative and qualitative analysis by SDS-PAGE, only the modified lysis method and the phenol methods were used for further downstream analysis, while the other three methods were omitted. Though the one step protocol of denaturation extraction in lysis buffer is restricted to “clean” samples (Carpentier et al., 2005), it is not suitable for recalcitrant plants, we introduced precipitation step with addition of solvents to remove interfering substances which yielded quality protein suitable for downstream processing in our study. The precipitation step with methanolic ammonium acetate was very efficient in removing all interfering compounds from the sample. The 2D gel pattern of proteins from modified lysis buffer method yielded excellent streak-free results with Coomassie, colloidal coomassie and also with silver staining when compared to phenol method derived samples which had prominent streak in the gel even in colloidal coomassie stained gel (Fig 5). In general, the phenol has the tendency to dissolve some polysaccharides and nucleic acids. This could be the reason for prolonged focusing time and streaks in the high pH range in 2D gels (Carpentier et al., 2005).

The 2D patterns of the proteins extracted by modified LB method showed good resolution of proteins in the entire range of pH 3-11 (Fig 8). In phenol method, the alkaline range of proteins was found to be very less and only acidic range had good number of separated proteins (Fig 7). Approximately 233 detectable spots as estimated by Image Master Platinum Software were obtained by phenol method, while 620 spots observed in modified lysis buffer method (Fig 4). Quantitative proteomics requires minimal variation between replicates. Our results show it applicability as all the replicate gels had the same pattern of protein spot profile. Hence, the protocol would be useful for any kind of expression studies in black pepper.

To test the effectiveness and applicability of the modified lysis buffer method for other genotypes, we extracted proteins from black pepper variety Subhakara (Fig 10) and from wild black pepper P. colubrinum (Data not shown). We found the greatest applicability of this method. Our results suggested that modified lysis buffer method may provide enhanced proteomic information for leaves of black pepper as it is very less time consuming compared to other methods and does not demand toxic phenol for the extraction.

Nano LC analysis and protein identification

Since the phenol extraction showed presence of poly phenols, we expected the charge heterogeneity of proteins. In this method protein identification was done using gels from lysis method. The replicated gels were aligned/merged to get a master gel to select the spots. The gel similarities were checked with the scatter plots for matched spots Corr=1. It indicates that the spot values for all matched spots are the same in the three replicate gels. Histogram representing spot values were also deduced (Fig 6). The 15 spots selected (Fig 9) for downstream analysis consisting of both high intensity and low intensity was successfully identified and listed (Table 1).

The spot1 was identified as ATP synthase subunit, ATPase alpha/beta chains family membrane protein which is involved in production of ATP from ADP in the presence of a proton gradient across the membrane (Kenji and Katsuyuki 1992). The spot2 is a heat shock protein 60-2 functions in copper ion
binding. ATP binding involved in, response to salt stress. Spot 3 is a chaperonin belongs to Hsp 60 family with the role in protein folding and assisting re-folding (Boston 1996). The spot 9 is NBD Sugar Kinase Hsp 70 family. These are abundant chaperones that are required for the proper folding and/or transport of proteins and also stress responsive proteins (Rod et al., 1994). Spot 10 is Actin, apart from acting as building blocks of cytoskeleton also assumed to play a role in plant innate immunity against fungi and Oomycetes (Jessica et al., 2013).

Spot 11 is an ATP synthase beta subunit fragment. The ATPase alpha/beta chains family membrane protein is involved in production of ATP from ADP in the presence of a proton gradient across the membrane (Kenji and Katsuyuki 2013). The spot 12 and 15 are subunits of Rubisco activase which is denoted as Rubisco's catalytic chaperone. Rubisco activase has been detected in all plant species examined so far and is a member of the AAA+ superfamily, whose members perform chaperone like functions (Portis 2013). Rubisco activase occurs in two isoforms which result from the alternative splicing of a single gene transcript. From our study it is evident that black pepper has 2 subunits with 34 and 39KD. Spot 13 is fructokinase and is involved in fructose function as sugar sensors and seems to play a central metabolic role in vascular tissues, controlling the amounts of sugars allocated for vascular development (Granot et al., 2013). Spot 14 is a membrane kinase involved in phosphorylation and signal transduction. Spot 16 is Epoxy hydrolase. In plants, it is involved in the metabolism of epoxy fatty acids and mediating defense responses (Stefan et al., 2002). Spot 17 is a chloroplast lipocalin protein, which transport small hydrophobic molecules such as steroids, bilins, retinoids, and lipids. Lipocalins are a group of proteins that have been characterized in bacteria, invertebrate, and vertebrate animals. However, very little is known about plant lipocalins. Spot 18 is Glyoxalase also known as lactoylglutathione lyase that catalyzes the first step of the glyoxal pathway. It is involved in the glyoxalate system in stress tolerance and in oxidative defense system. It detoxifies methylglyoxal (MG), a cytotoxic byproduct of glycolysis, to S-lactoylglutathione. The level of MG, which is produced ubiquitously in all living organisms, is enhanced upon exposure to different abiotic stresses in plants which is controlled by the glyoxalase (Yadav et al., 2005). Spot 19 is proteosome subunit alpha type and Spot 36 is Rubisco large chain (rbcL) involved in major step of carbon fixation.

Materials and Methods

Plant sample preparation

Black pepper leaf from variety IISR Shakhi grown under greenhouse condition was used for this study. The second and fourth leaf samples were ground in liquid nitrogen to extract the total proteins.

Protein extraction protocols

The schematic outline of methods for protein extraction from leaf is shown in Fig 1. Each method was repeated four times to check the reproducibility. Method I is the modified TCA/Acetone method (Damerval et al., 1986) with some modification. Method II is the Dense SDS/Phenol method (Wang et al., 2003), Method III is the PVP/TCA acetone method (Shen et al., 2002) with some modification. Method

<table>
<thead>
<tr>
<th>Spot ID</th>
<th>NCBI Accessions</th>
<th>Protein identity</th>
<th>Score</th>
<th>Coverage</th>
<th>AA</th>
<th>MW/PI</th>
<th>Plant Species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>A8ASH6</td>
<td>ATP Synthase Subunit (Membrane Protein)</td>
<td>384.6</td>
<td>41</td>
<td>487</td>
<td>98/5.1</td>
<td>Piper Kadsura</td>
</tr>
<tr>
<td>2</td>
<td>AEC 08800</td>
<td>Heat Shock protein 60-2 family</td>
<td>472.26</td>
<td>11.27</td>
<td>577</td>
<td>61/5.45</td>
<td>Arabidopsis thaliana</td>
</tr>
<tr>
<td>3</td>
<td>A9RKJ5</td>
<td>Heat shock protein 60 family</td>
<td>166.69</td>
<td>7.66</td>
<td>548</td>
<td>57.95/5.325</td>
<td>Physcomitrella patens</td>
</tr>
<tr>
<td>9</td>
<td>AT5G09810</td>
<td>NBD Sugar Kinase Hsp 70 family</td>
<td>94</td>
<td>15.75</td>
<td>292</td>
<td>39/5.1</td>
<td>Nicotiana tabaccum</td>
</tr>
<tr>
<td>10</td>
<td>K7VB13</td>
<td>Putative actin family protein</td>
<td>113.07</td>
<td>15.22</td>
<td>368</td>
<td>40.56/5.37</td>
<td>Zea mays</td>
</tr>
<tr>
<td>11</td>
<td>R46L12</td>
<td>ATP synthase beta subunit (Fragment)</td>
<td>123.8</td>
<td>10.55</td>
<td>379</td>
<td>40/5.4</td>
<td>Pythcomonium cygisetum</td>
</tr>
<tr>
<td>12</td>
<td>D4N5G1</td>
<td>Rubisco activase (AAA Superfamily)</td>
<td>915.4</td>
<td>13.5</td>
<td>474</td>
<td>39/4.8</td>
<td>Glycine max</td>
</tr>
<tr>
<td>13</td>
<td>I0J1B8</td>
<td>Fructokinase (Ribo kinase)</td>
<td>100.7</td>
<td>7.43</td>
<td>323</td>
<td>34.7/4.99</td>
<td>Oryza australiensis</td>
</tr>
<tr>
<td>14</td>
<td>B9MY45</td>
<td>Kinase (Membrane protein)</td>
<td>107.6</td>
<td>7.32</td>
<td>328</td>
<td>35.25/5.1</td>
<td>Populus trichocarpa</td>
</tr>
<tr>
<td>15</td>
<td>C5JO3</td>
<td>Chloroplast rubisco activase (AAA Superfamily)</td>
<td>103.8</td>
<td>4.91</td>
<td>611</td>
<td>34/4.6</td>
<td>Cucumis sativus</td>
</tr>
<tr>
<td>16</td>
<td>I3T7A2</td>
<td>Epoxy Hydrolase</td>
<td>102.3</td>
<td>6.71</td>
<td>313</td>
<td>34.84/5.59</td>
<td>Lotus japonicas</td>
</tr>
<tr>
<td>17</td>
<td>Q38JB4</td>
<td>Chloroplast lipocalin</td>
<td>114.86</td>
<td>8.71</td>
<td>333</td>
<td>37.5/5.99</td>
<td>Solanum tuberosum</td>
</tr>
<tr>
<td>18</td>
<td>M4C3J0</td>
<td>Glyoxalase</td>
<td>111.66</td>
<td>12.02</td>
<td>341</td>
<td>37.87/6.5</td>
<td>Brassica rapa</td>
</tr>
<tr>
<td>19</td>
<td>A9REG6</td>
<td>Proteosome Subunit alpha type</td>
<td>89.47</td>
<td>9.57</td>
<td>282</td>
<td>30.7/5.64</td>
<td>Physcomitrella patens</td>
</tr>
<tr>
<td>36</td>
<td>B1NF06</td>
<td>Rubisco large chain (rbcL)</td>
<td>138.3</td>
<td>20.91</td>
<td>220</td>
<td>24.38/6.39</td>
<td>Piper Kadsura</td>
</tr>
</tbody>
</table>

Table 1. Mascot identified proteins with molecular weight and the accessions.
**Fig 1.** Summary of five protein extraction methods tested. (Washing: Washing of pellets was done with ammonium acetate (0.1M), 80% acetone followed precipitation with 70% ethanol).

**Fig 2.** A comparative graph showing protein yield/fresh weight of leaf tissue along with phenol contamination.
**Fig 3.** SDS-PAGE profiles of leaf proteins by different methods (Lanes 1-5: Method I-V; Lane 7: Marker).

**Fig 4.** Average number of protein spots in 2DE gels using modified lysis buffer and phenol extraction protocols.

**Fig 5.** Phenol extracted proteins (60ug) separated on pH 3-11 IPG strip and stained with Colloidal coomassie blue.

**Fig 6.** Histogram on spot intensity in 3 replicate gels.
Fig 7. Phenol extracted proteins (100ug) separated on pH 3-11 IPG strip and stained with coomassie blue.

Fig 8. Modified lysis buffer method extracted proteins (100ug) separated on pH 3-11 IPG strip and stained with coomassie blue.

Fig 9. Leaf protein Map of black pepper (60ug proteins separated on pH 4-7 IPG strips).

Fig 10. Protein profile of black pepper variety Subhakara (60ug proteins separated on pH 4-7 IPG).
IV is the phenol method (Hurkman and Tanaka 1986) with slight modification. Method V is the Lysis buffer extraction (O’Farrell 1975) with some modification.

**Protein precipitation**

Two methods were applied. The TCA and the other ammonium acetate assisted protein precipitation. The protein precipitation was done from the supernatant obtained from all the methods except method I.

**Protein solubilisation**

Protein solubilisation was tested in two buffer (Buffer I and II) combinations separately to obtain the soluble proteins from the pellets. The protein pellets were solubilised in re solubilisation buffer I (8M urea, 2% (w/v) CHAPS, 40mM DTT, traces of bromophenol blue and 0.5% (v/v) IPG buffer) and in re-solubilisation buffer II (7M urea, 2M thiourea, 4% (w/v) CHAPS, 40mM DTT, traces of bromophenol blue and 0.5% IPG buffer) separately, vortexed briefly. The insoluble material was removed by centrifugation at 12,000 g for 5 min at 4°C.

**Protein quantification**

The protein concentration was estimated using 2 D Quant kit (GE health care) and also by modified Bradford method with bovine serum albumin as a standard.

**Phenol estimation**

To test the phenol contamination in the protein extract, the total phenol was estimated using modified Folin-Ciocalteau method. A 0.2 ml of sample was taken and made up to 1 ml with distilled water. A 1 ml of Folin-Ciocalteau reagent was added to it and incubated for 3 min at room temperature. Then 1 ml of 1 M sodium carbonate was added and incubated in a rotary shaker at room temperature. After 45 min, absorbance was measured at 725 nm in a spectrophotometer and quantified against a standard of gallic acid. The total phenolic content was reported as gallic acid equivalents based on a calibration curve.

**Protein separation by SDS-PAGE**

SDS-PAGE was performed in 12% w/v acrylamide gel at 100V for 2 hr and 30 min (Laemmli 1970).

**Iso Electric Focusing (IEF)**

The protein pellets were dissolved in rehydration buffer (8M urea, 2% W/V CHAPS, 18mM DTT, 0.5% W/V IPG buffer pH 3-11 and 0.002% bromophenol blue). The IPG strips non linear (pH 3-11) were rehydrated passively for 16 hr with 100 µg protein. Focusing of proteins was done in Ettan IPGphor system (GE healthcare) with the voltage settings of 300 (200Vh), 1000V (300Vh), 5000V (400Vh) and 5000V (1250Vh) until achievement of a total 5.8kVh and also using IPG strips 4-7 with the voltage settings of 300V (200Vh), 1000V (300Vh), 5000V (4500Vh), 5000V (3600Vh) until the achievement of 8 Kv h.

**2DE**

Prior to second dimension electrophoresis, the strips were equilibrated. The first equilibration was done, for reduction of proteins, in the strip with 2.5ml of equilibration buffer (50 mM Tris Cl pH 8.8, 6.6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, traces of bromophenol blue) containing 0.5% w/v DTT for 20 min and the second equilibration was done to alkylate the proteins, with 2.5 ml equilibration buffer containing 2.5% w/v Iodo acetamide solutions (Gorg et al., 2004) with mild shaking using gel rocker. After equilibration, the strips were dipped for 2min in fresh 1X gel running buffer and then placed on a 12% (40% T, 3% C Acrylamide: Bisacrylamide) continuous SDS PAGE (1mm thick) avoiding any air bubbles in between the strip and the gel. The strip was sealed with agar overlay solution (0.25 mM Tris, 192 mM glycin, 0.1% (w/v) SDS, traces of bromophenol blue, 0.5% agarose). The lower tank buffer was 1x and the upper tank buffer was 2x running buffer and the electrophoresis was done at 80V for 15 min and then 100V till the bromo phenol blue reached the bottom of the gel. Three replicate gels were run under each strip category.

**Protein visualization and image analysis**

Protein patterns were analyzed by coomassie 250 (Neuhoff et al., 1998) and silver staining (Blum et al., 1987). The colloidal CBB staining was done with the kit (Sigma) according to the manufacturer description. Stained gels were scanned and calibrated with Labscan 6.0 software (GE Health care) including IQTL calibration convertor. The Image analysis was carried out with image master platinum 6.0 (GE health care). The total number of spots was obtained automatically by the spot detection parameters wizard using standard parameters with normalization and comparison between spot quantities across gels. Only spots present in each of the three replicate gels with the use of standard deviation graph and histogram pattern on spot value were chosen for subsequent analysis.

**In-gel digestion**

The Protein bands were excised with sterile surgical blade and samples were subjected to in-gel digestion and with additional alkylation and reduction as per Shevchenko et al., 2007).

**Nano LC analysis and database search**

Digested peptides were reconstituted in 15µl of the 0.1% formic acid. 1µl of samples were injected on the column and digested peptides were subjected to 70 minute RPLC gradient, followed by acquisition of the data on LTQ-Orbitrap-MS (Thermo) with collision induced dissociation as the fragmentation method. Peptide mass data was analysed using MASCOT 2.4 as search engine on Proteome discoverer 1.4. The data was searched against Uniprot TrEMBL and Swiss Prot database from NCBI as well with the following parameters. The precursor mass tolerance was at 10ppm and fragment mass tolerance at 0.8 Da with 3 maximum cleavage sites. The dynamic modifications were set as carbamidomethyl(C), Oxidation(M), Carboxymethyl(C), GLN->Pyro-Glu(N-termQ) and Glu->Pyro-Glu(N-term E) with the protein relevance Threshold 20 and protein relevance factor 1. Minimum of two high confident peptides was used as a prerequisite to identify the proteins.

**Statistical analysis**

For plant sample preparation three independent biological replicates were taken and pooled. The pooled sample was
used in 5 different methods of extraction with 4 replications. 2D separation was done with 3 replicate gels. The spot intensity was compared between the replicate gels by the scatter plot method and also by histogram pattern using Image Master Platinum 6.

Conclusion
In this study, we standardized and demonstrated the suitable protocol for efficient extraction of proteins from black pepper along with the initiation on generation of protein map. This proteogenomics of black pepper has yielded a spectrum of proteins with various important biological or molecular significances. With the absence of whole genome sequencing this study forms a research paradigm to explore the candidate protein information for the translational research in black pepper improvement programmes.

Acknowledgement
The authors are thankful to ICAR (Indian Council for Agricultural Research) for funding this research under PhytoFuRa (Outreach project on Phytophthora, Fusarium and Ralstonia Diseases of Horticultural and Field Crops). Their special thanks to Mr. B. Krishnamoorthy, Head, Division of Crop Improvement & Biotechnology, IISR. The authors extend thanks to C-CAMP, mass spectrometry facility, National Center for Biological Sciences, Bengaluru, India for MS services. Her special thanks to Dr. John kennedy Bob (System analyst Wipro GE healthcare, Bengaluru).

References