2.1 Plant and seed materials

*Cicer arietinum* seeds of cultivar JG62 and Digvijay (Fig. 2.1) were obtained from Mahatma Phule Krishi Vidyapeeth (MPKV), Rahuri, Maharashtra, India. JG62 (pedigree-selection from germplasm) is highly susceptible to wilt, and shows features like twin podding and early maturity. It has medium sized seeds. Digvijay (pedigree-Phule G-91028 × Bheema) shows high average yields (19.00 q/ha; which is higher by 14.44% than Vijay and 17.81% than Vishal) and is highly resistant to Fusarium wilt as compared to the other varieties such as Vijay and Vishal. It has attractive yellowish brown coloured bold seeds (24.0g/100 seeds) and is suitable for optimum sowing, well irrigated and late sown conditions.

![JG62 seeds and Digvijay seeds](image)

**Fig. 2.1:** Seeds of chickpea cultivars, JG62 and Digvijay

2.2 Fungal strain

*F. oxysporum* f. sp. *ciceri* (*Foc*) standard race 1 (NRRL 32153) was obtained from the International Crops Research Institute for Semi Arid Tropics (ICRISAT), Patancheru, India. The culture was maintained on Potato Dextrose Agar (PDA) slants with regular sub-culturing. Fig. 2.2 shows the growth patterns of *Foc* race 1 on PDA plates.
2.3 Methodologies involved in chickpea-*Fusarium* interaction

Seeds of Fusarium wilt resistant (Digvijay, DV) and susceptible (JG62, JG) chickpea cultivars were surface sterilized by sodium hypochlorite (4%) treatment prior to sprouting on wet filter paper and sowing in Soil-Rite. The DV and JG plants were further grown in greenhouse [25°C/16 h light (06:00-22:00) and 25°C/8 h dark (22:00-06:00) cycle] for seven days and divided into two groups (control or mock inoculated and Foc inoculated). The scheme for Foc inoculation and workflow for data collection/analysis is shown in Fig. 2.3. Foc inoculum (*F. oxysporum* f. sp. *ciceri* race 1) was used at a concentration of 10⁶ spores/mL, while sterile water was used for mock inoculation. As the pathogen is reported to colonize the xylem vessels two days post inoculation (Gupta *et al.*, 2010, Jimenez-Fernandez *et al.*, 2013), the plant tissues (roots) were collected at various time interval such as 2, 4, 8 and 12 DAI. In each group, a pool of 10 plants comprised one biological replicate. Roots of Mock inoculated and Foc inoculated JG62 and DV roots of these time points were washed with sterile milli-Q water and cut at the hypocotyl region. The harvested root tissues were snap-frozen in liquid nitrogen and stored at -80°C till further use. Plant root tissue was ground well in liquid nitrogen using bead beater (Retsch GmbH, Germany). For proteomics analyses, tissues from three biological replicates were analyzed, for NMR based metabolomics analysis, tissues from ten biological replicates while for UHPLC-Orbitrap based untargeted metabolomics three independent biological replicates with three technical replicates of each were analyzed.
2.4 Protein extraction and Mass spectrometry analysis

Total proteome of root tissue was extracted as described by Isaacson et al., (2006). In brief, this includes, removal of phenolic compounds by protein precipitation in 10% TCA/Acetone resuspension of pallet in 10 ml of extraction buffer containing 0.7 M sucrose; 0.1 M KCl; 0.5 M Tris-HCl, pH 7.5 and 50 mM EDTA. The reducing agent beta-mercaptoethanol should be added to a final concentration of 2% (vol/vol). Samples were vortexed and incubated by shaking for 10 min on ice. Afterwards, an equal volume of Tris-buffered phenol (pH = 8.0) was added, and solutions were incubated on a shaker for 10 min at room temperature. The phenol phase was carefully transferred to a new tube.
and four volumes of precipitation buffer, consisting of ammonium acetate in ice-cold methanol and samples were incubated overnight at – 20 °C. After centrifugation, the pellets were washed three times with ice-cold precipitation buffer and finally, pellets were dried under air at room temperature (Fig 2.4). Further, protein pellets were solubilized in 50 mM ammonium bicarbonate buffer containing 0.1% Rapigest (Waters, USA). The dissolved proteins were reduced and alkylated by DTT and iodoacetamide, respectively followed by overnight tryptic hydrolysis at 37°C using Promega sequencing grade trypsin. The digested peptides were analyzed with LC-MS²E workflow using nano-ACQUITY online coupled to a SYNAPT HDMS system (Waters, USA). Nano-LC separation was performed with symmetry C18 trapping column (180 μm x 20 mm, 5 μm) and bridged-ethyl hybrid (BEH) C18 analytical column (75 μm x 250 mm, 1.7 μm). The binary solvent system comprised solvent A (0.1% formic acid in water), and solvent B (0.1% formic acid in acetonitrile). Each sample (500 ng) was initially applied to the trapping column and desalted by flushing with 1% solvent B for 1 minute at a flow rate of 15 μL/min. Elution of the tryptic digested sample was performed at a flow rate of 300 nL/min by increasing the solvent B concentration from 3% to 40% over 90 min. Before data acquisition, the mass analyzer was calibrated using Glu-fibrinopeptide B (Sigma-Aldrich, USA) from m/z 50 to 1990. The Glu-fibrinopeptide B (GFP-B) was delivered at 500 fmole/µL to the mass spectrometer via a NanoLockSpray interface using the auxiliary pump of the nano-ACQUITY system at every 30 second interval for lock mass correction during data acquisition. Data independent acquisition was performed (LC-MS²E) as described by Patel et al., (2009).

![Grind chickpea root tissue to extremely fine powder](#) [TCA/Acetone wash][Phenol/SDS extraction][Protein pallet]

**Fig. 2.4:** Schematic presentation of plant proteome extraction
As accuracy and reproducibility in mass measurement are critical in data acquisition during large-scale proteomic experiments, Principle Component Analysis (PCA) was used to assess the quality of the measurement in terms of replicate similarity of unit variance (UV) scaled data using Metaboanalyst software. ProteinLynxGlobal server (PLGS) quality control outputs suggest that the replicates of each sample were clustered together reflecting inherent similarities between like samples (Fig. 2.5A). In addition, linear response and reproducibility of measurement of the quantitative proteomic data acquisition were tested by plotting two replicates (Fig. 2.5B), whereas data were acquired below 3 ppm mass accuracy (Fig. 2.5C). Further, the percent coefficient of variance of retention time was calculated to assess the separation stability and coefficient of variance of 0.3 minutes, which also suggested stability in chromatographic separation (Fig. 2.5D).

Fig. 2.5: Protein quality control measurements; PCA plot indicating clear separation between control and inoculated samples (A), reproducibility of intensity in replicates of samples (B), majority of ion counts with less than 3 ppm error (C) and percent coefficient of variance of retention time (% CV RT) (D).
2.5 Analysis of quantitative proteomics data

The acquired LC-MS\textsuperscript{E} data were processed using the ProgenesisQI for Proteomics software (Waters, USA). Protein identifications were obtained by searching the genomic databases of chickpea (http://www.icrisat.org/) and *Fusarium oxysporum* (http://www.broadinstitute.org/). LC-MS\textsuperscript{E} data were searched with a fixed carbamidomethyl modification for cysteine residues, along with a variable modification for oxidation of methionine, N-terminal acetylation, deamination of asparagine and glutamine and phosphorylation of serine, threonine and tyrosine. The ion accounting search algorithm within ProgenesisQI for Proteomics software was used which has been developed specifically for searching data-independent MS\textsuperscript{E} data sets and described in detail by Li *et al.*, (2009). The ion accounting search parameters were- precursor and product ion tolerance: automatic setting, minimum number of product ion matches per peptide: 3, minimum number of product ion matches per protein: 7, minimum number of peptide matches per protein: 1, and missed tryptic cleavage sites: 1. False positive rate was set at 1%. Search results of the proteins and the individual MS/MS spectra with a confidence level at or >95\% were accepted. Label free quantitation of identified proteins was done on the basis of spiked bovine serum albumin (BSA) protein.

2.6 Clustering of identified proteins

Data were normalized by spiked BSA (50 fmoles) and relative accumulation differences were determined for proteins having differential expression. Sum of three replicates of inoculated samples was divided by that of the respective controls. This established a ratio of fold change of a protein in plants upon Foc infection in relation to that in mock-inoculated control plants. The log\textsubscript{2} transformed ratio (susceptible/control and resistant/control) pairs were clustered by the application of SplineCluster (Heard *et al.*, 2006), a Bayesian model-based hierarchical clustering algorithm for time series data.
2.7 Gene ontology enrichment analysis

Protein functional annotation was determined using Blast2GO (Conesa et al., 2005) and for each cluster, GO enrichment analysis was carried out using BiNGO 2.3 plugin tool in Cytoscape version 2.8 (Maere et al., 2005). Overrepresented GO Biological Process categories were identified using a hypergeometric test with a significance threshold of 0.05 after Benjamini and Hochberg false discovery rate correction (Benjamini and Hochberg, 1995) using the annotated chickpea genome as the reference set. Whole chickpea genome GO term for biological process was extracted from Blast2go software and was used to make customized annotation file for chickpea as explained in Maere et al., (2005).

2.8 Metabolite extraction and NMR measurement

Plant root tissue was ground well in liquid nitrogen by using bead beater (Retsch GmbH, Germany) and lyophilized. The powdered root tissue (~ 50 mg) was extracted with 0.75 mL of CD3OD and 0.75 mL of 10 mM KH2PO4 buffer (pH 6.0) containing sodium3-trimethylsilyl [2,2,3,3-D4] propionate (TSP) as described previously (Kim et al., 2010). After ultrasonication for 20 min and centrifugation at 12,000g for 10 min at room temperature (~25°C), 0.5 mL of supernatant was collected for NMR detection (Fig. 2.6). 1H NMR spectra of root extract were acquired at 25°C on a Bruker AV II 500 spectrometer (Bruker Biospin, Germany) operating at 500.13 MHz for 1H. A standard water-suppressed one-dimensional NMR spectrum was recorded using noesyprld pulse sequence (RD-90°-t1-90°-tm-90°-acquisition) with the recycle delay of 6 s and the mixing time (tm), of 50 ms. Typically, 90° pulse was set to about 15μs and 256 transients were collected into 48K data points for each spectrum with a spectral width of 16 ppm. All spectra were referenced to chemical shift of TSP (δ=0.00). For the metabolite assignment purpose, a range of 2DNMR spectra were recorded for selected samples including 1H-1H correlation spectroscopy (COSY), 1H-1H total correlation spectroscopy (TOCSY), 1H-13C heteronuclear single quantum coherence spectroscopy (HSQC), and 1H-13C heteronuclear multiple-bond correlation (HMBC). In COSY and TOCSY experiments, respective 64
and 32 transients were collected into 2 K data points for each of 256 increments with the spectral width of 2426 Hz for both dimensions. Magnitude mode was used with gradient selection for the COSY experiments whereas the \textit{mlevgpphw5} pulse program was employed as the spin-lock scheme in the phase sensitive mode, with the mixing time of 60 ms, for TOCSY. Both HSQC and HMBC spectra were acquired using the gradient-selected sequences. In HSQC experiment, 80 transients were collected into 1k data points for each of 140 increments. In HMBC experiment, 160 transients were collected into 2k data points for each of 256 increments. The spectral widths were 2426 Hz for $^1$H and 9809 Hz for $^{13}$C in HSQC and HMBC experiments.

\textbf{Fig. 2.6:} Metabolite extraction protocol and data acquisition methodology; (Source: modified from Kim et al., 2010)
2.9 NMR spectra processing and multivariate data analysis

All the $^1$H NMR spectra were manually corrected for phase and baseline distortions using TOPSPIN (v2.1, Bruker Biospin), and calibrated for chemical shift drifting by in-house developed script for MATLAB (The Mathworks, USA). The spectral region $\delta$ 0.5-9.5 was divided into bins with width of 0.002 ppm (1.0 Hz) using AMIX software (v3.8.3, Bruker Biospin GmbH, Germany). The region $\delta$ 4.727-5.089 ppm was discarded to remove the effects of imperfect water pre-saturation. The areas of the remaining bins were normalized to total sum of intensity for each spectrum to compensate for the overall concentration differences prior to statistical data analysis. Multivariate data analyses were carried out with SIMCA-P+ v 12.0 software package (Umetrics, Sweden). PCA was performed on the mean-centered NMR data to inspect overall data distributions and possible outliers. Using the NMR data as the X-matrix and group information as Y-matrix, orthogonal projection to latent structures discriminant analysis (OPLS-DA) was carried out with unit variance scaling (Tyrgg 2002; Xiao et al., 2008). The OPLS-DA models were 7-fold cross-validated and the quality of the model was described by the parameters $R^2_X$, representing the total explained metabolic variables, and $Q^2$, indicating the model predictability. The models were further evaluated with a CV-ANOVA approach ($p<0.05$) and permutation tests. To facilitate interpretation of the results, back-transformation (Cloarec et al., 2005) of the loadings generated from the OPLS-DA was performed prior to generating the loadings plots, which were color-coded with the Pearson linear correlation coefficients of variables (or metabolites) using an in-house developed script for MATLAB (The Mathworks, USA) (Wang et al., 2007). The color-coded correlation coefficient indicates the significance of the metabolite contribution to the class separation, with hot colors (e.g., red) being more significant of the metabolite contributions to the group classification than the cold ones (e.g., blue). In this study, a correlation coefficient cutoff value of 0.602 (i.e., $N=10$, $|r| > 0.602$) was used for the statistical significance based on the discrimination significance at the level of $P<0.05$, which was determined according to the discriminating significance of the Pearson’s product-moment correlation coefficient (Cloarec et al., 2005).
2.10 Methodology and experimental design for non-targeted metabolomics with UHPLC

Foc inoculated and mock inoculated chickpea tissues were collected in similar way as described in section 2.3. For all stages, ten plants per pot were considered as an experimental unit, and three independent biological replicates of all the samples consisting of resistant and susceptible cultivars inoculated with Foc1 and their respective controls as described in Fig. 2.7 were used.

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**Fig. 2.7:** Experimental design for untargeted metabolomics (UHPLC-Orbitrap)
2.11 Extraction of metabolites and UHPLC profiling

Metabolites from 100 mg of each root tissue sample were extracted with 1 mL of 60% ice cold methanol and 0.1% formic acid followed by sonication for 20 min and centrifugation at 4°C at 15,000g for 30 min. Supernatant was filtered with 0.2 micron amicon filter (Millipore, Hessen, Germany) and stored at -80°C until further use. An Accela™ ultra high performance liquid chromatography (UHPLC) system (ThermoFisher, Waltham, USA), coupled online via heated electrospray ionization source (HESI) with a Q-Exactive-Orbitrap mass spectrometer (ThermoFisher), was employed for non-targeted metabolomics profiling with 3 µL sample injection volume. The metabolites were profiled using a C18 Hypersil Gold column (1.9 µm, 2.1 mmX150, ThermoFisher). The temperature of column oven was set at 40°C and the sample manager was maintained at 4°C. The eluents A (water containing 0.1% formic acid) and B (acetonitrile containing 0.1% formic acid) were employed in the electrospray ionization-positive (ESI+) mode and electrospray ionization-negative (ESI−) mode. The flow rate was adjusted at 0.6 mL/min with a linear gradient elution over 15 min. From the start to 0.3 min, eluent B was held at 2%, linearly increased to 30% till 2 min, to 45% during next 5 min, and then to 98% in 12 min. Subsequently, eluent B was returned to 2% in 13.4 min and held for an additional 1.2 min before returning to the initial conditions. The sample sequence was random. In the ESI+ mode, the MS spray voltage was 3.7 KV while it was 2.8 KV in the ESI− mode. The capillary temperature was set at 300°C with the sheath gas at 45 arbitrary units and the aux gas at 5 arbitrary units. The tube lens was set to 45V and the mass scan range was set from 100 to 1000 m/z. The resolution of the Orbitrap was set at 70,000. The tandem mass spectrometry (MS/MS) data were collected with the collision energy between 10 and 35 eV.

2.12 LC-MS stability and reproducibility

Pooled quality control (QC) samples were prepared by mixing all the samples to ensure the quality of metabolic profiling data. Five QC samples were run before analyzing the sample sequence. In addition, one QC sample was run after every 10 sample injections to
monitor the stability of the system during the analysis of sample sequence. To verify the 
ability of the mass spectrometer system to accurately detect changes in metabolite 
abundance, a set of parameters was calculated to assess the reproducibility of the system 
using pooled QC of all the tissue samples. Extracted ion chromatograms (EICs) of 
leucine (Leu) and isoleucine (Ile) were selected to verify the resolution of the mass 
spectrometer, intensity deviation, ppm error and retention time shift. Moreover, the 
retention times, mass accuracies and peak areas of these two selected EICs in the QC 
samples were also determined to validate the system stability.

2.13 Data analysis

The raw data alignment and peak picking were performed using the Progenesis QI 
software (Waters and Nonlinear Dynamics) for positive (ESI+) and negative (ESI−) 
ionization modes separately. All the detected ions in each sample with ANNOVA 
$p<0.005$, FDR <1% and minimum fold change of 2 between control and their respective 
inoculated samples, were normalized using total intensity before importing into SIMCA-
P v. 13.0 software (Umetrics) for multivariate data analysis. PCA was performed with 
mean centered data to check the overall pattern and trend in data. Further, OPLS-DA 
as was performed on pareto scaling data to identify the discriminating metabolites between 
control and inoculated samples. The default 7-round cross-validation was applied with 
1/7th of the samples being excluded from the mathematical model in each round. The 
parameters of the models, such as the $R^2_X$, $R^2_Y$, $Q^2_Y$ and the $R^2_{Y^-}$, $Q^2_{Y^-}$-intercepts, were 
analyzed to ensure the quality of the multivariate models and to avoid the risk of over-
fitting using 200 iterations. Further, model validity was assessed with CV-ANOVA (with 
the $p$ value indicating the probability that the model is the result of chance alone). The 
VIP values of all the peaks from the 7-fold cross-validated OPLS-DA model were 
considered as a coefficient for peak selection. Discriminating variables were selected 
according to their highest influence on loading, VIP values (VIP>1.0), S-plot and jack-
knifed-based confidence intervals of OPLS-DA model. Additionally, univariate method 
and the Student’s $t$-test were applied to determine the significance of each metabolite in 
separating the pathogen inoculated and mock inoculated samples. Identification of 
metabolites was carried out by searching the available databases such as KEGG
(http://www.kegg.com), Massbank (http://massbank.imm.ac.cn/MassBank), KNApSAcK (http://kanaya.naist.jp/KNApSAcK) and METLIN (http://metlin.scripps.edu) using exact mass and MS/MS fragmentation patterns. *In silico* prediction of the mass fragmentation of the candidate structures was also performed using Mass Frontier™ software (ThermoFisher) and compared with MS/MS fragmentation pattern of identified metabolites. Commercially available standards were adopted to confirm the structures of some metabolites. Four-way comparison among the identified metabolites was performed using VENNY (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

2.14 RNA extraction, cDNA synthesis and quantitative Real-Time PCR analysis

Total RNA was extracted from 100 mg root tissue by using TRI Reagent (Sigma-Aldrich, USA). First strand cDNA synthesis was performed using the High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA) with 3 µg of DNase1 treated total RNA using oligo (dT) primer following manufacturer’s protocol. Gene specific primers were designed using Primer Express (v2.0) software and listed in Table 2.1. Primer concentration and annealing temperature were optimized before the final analysis. Real time PCR was carried out using 7900HT Fast real-time PCR system (Applied Biosystems, USA) using FastStart universal SYBR green master mix (Roche, USA) with the following conditions: 95°C denaturation for 10 min, followed by 40 cycles of 95°C for 3 s, with primer annealing and extension at 60°C for 30 s. Following amplification, a melting dissociation curve was generated using a 62-95°C ramp with 0.4°C increment per cycle in order to monitor the specificity of each primer pair. The Initiation factor 4α (*IF4α*) gene was used as internal standard or reference gene (Garg *et al.*, 2010). The reactions were performed in triplicate and the results were averaged. PCR conditions were optimized such that the PCR efficiencies of the reference gene and the gene of interest were close to 2.0. PCR efficiencies were calculated using LinRegPCR (Ramakers *et al.*, 2003) software. Relative transcript abundance calculations were performed using the comparative CT (*ΔCT*) method described by Schmittgen *et al.*, (2008).
2.15 Lignin staining

Lignin accumulation within root tissue after Foc inoculation was detected using the phloroglucinol/ hydrochloric acid stain as described by Mauch-Mani and Slusarenko (1996). The control and Foc inoculated chickpea roots were subjected to transverse sections with a scalpel and immersed in 1mL of 1% phloroglucinol in 6N HCl for 5 min and the lignin staining was visualized under light microscope.

Table 2.1: List of primers used in quantitative real-time PCR

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<th>Accession</th>
<th>Gene name</th>
<th>Primer(5’ to 3’)</th>
<th>Product length (bp)</th>
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<td></td>
<td></td>
<td>R: GGGAGCAAAAAACCTGGTGAGAG</td>
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<tr>
<td>XM_004512714</td>
<td>Glutamate dehydrogenase</td>
<td>F: CAAATCACCACGTGACCCA</td>
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<td></td>
<td></td>
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