

Research paper

Induced post-invasive defenses in the nonhost plant *Parthenium hysterophorus* L. prevent root cortical colonization by *Macrophomina phaseolina* and impart resistance to dry root rot

Rishabh Mirchandani, Manu Kandpal, Aashish Ranjan , Senjuti Sinharoy, Muthappa Senthil-Kumar 

National Institute of Plant Genome Research, Aruna Asaf Ali Marg, New Delhi 110067, India

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ABSTRACT

Dry root rot (DRR) of chickpea is caused by the broad-range necrotrophic fungus *Macrophomina phaseolina*. Chickpea germplasm does not provide durable resistance to DRR, which is particularly devastating under drought. Even moderately resistant chickpea varieties become susceptible under combined stress. We hypothesized that nonhost resistance (NHR) is durable even under combined stress. Using the blotter paper assay and stereomicroscopic observations, we identified the asterid weed *Parthenium hysterophorus* as a potential nonhost of *M. phaseolina* among 82 potential nonhosts. Epidermal necrotic lesions were prevented in *P. hysterophorus*. *In planta* fungal load was 0.195 and 0.007 ng/ng total DNA in chickpea and *P. hysterophorus*, respectively. *M. phaseolina* could not colonize the *P. hysterophorus* root while up to 6 cortical cell layers were colonized in chickpea. Further, NHR was durable under combined stress. Dual RNA sequencing revealed that *M. phaseolina* actively attempted to infect the nonhost and activated specific genes in the xenobiotics degradation pathway. *P. hysterophorus* also showed an active defense response with 1958 and 2294 differentially expressed genes at 2 and 4 DAI, respectively, with 363 upregulated at both time points. Differential expression of cell wall synthesis, phytohormone signaling, and other defense response pathways likely contributes to NHR. Few genes in the phenylpropanoid biosynthesis pathways in *P. hysterophorus* were also upregulated, possibly because these metabolites are linked to the distinct changes in the fungus during nonhost infection. We therefore conclude that *P. hysterophorus* exhibits post-invasive NHR to *M. phaseolina* and that general defense, phytohormone signaling and secondary metabolic pathways contribute to NHR.

1. Introduction

Cicer arietinum L. (commonly known as garbanzo or chickpea) is the second most widely cultivated grain legume species in the world (FAO, 2021). However, several abiotic and biotic factors limit chickpea yield, particularly dry root rot (DRR) caused by *Macrophomina phaseolina* (Tassi) Goid. can lead up to 80–100% yield losses in chickpea (Mirchandani et al., 2023; Rai et al., 2022). The resting structure and the primary propagule of the pathogen are the microsclerotia present in the soil. Under favorable conditions, mycelia with a germ tube like appearance emerge from the microsclerotia and attach to the host root surface, subsequently leading to epidermal necrosis followed by cortical colonization (Irulappan et al., 2022; Rai et al., 2022). Later stages of the infection are marked by endodermal breaching by the pathogen and

vascular plugging leading to nutrient and water deficiency in the shoot of the plant. This stage marks the onset of aerial symptoms where shoots become entirely straw-colored and dry. Concurrently, there is a total loss of lateral roots and extensive necrotic lesions on the primary root. Furthermore, DRR is particularly aggressive under concurrent drought and heat stress (Chilakala et al., 2022; Irulappan et al., 2022; Sharath Chandran et al., 2021; Sinha et al., 2021, 2019). Disease incidence and severity increase significantly under concurrent drought stress (Chilakala et al., 2022; Irulappan et al., 2022). Owing to its cell wall-degrading enzymes and non-specific cytotoxins, *M. phaseolina* can infect over 500 plant species (Marquez et al., 2021), although only 97 of which are confirmed reports (Pennerman et al., 2024). Nonetheless, such a broad host range, with most hosts being crops, renders crop rotation as a management strategy ineffective. Further, chemical control

* Corresponding author.

E-mail address: skmuthappa@nipgr.ac.in (M. Senthil-Kumar).

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does not adequately alleviate the losses caused by DRR (Marquez et al., 2021), and despite extensive screening of the chickpea germplasm, no robust resistance to DRR has been found (Gangwar et al., 2002; Pande et al., 2004; Sharma et al., 2015). However, biocontrol of *M. phaseolina* has been used (Iqbal et al., 2014; Iqbal and Mukhtar, 2020a, 2020b) against various isolates of *M. phaseolina* (Iqbal and Mukhtar, 2014). In addition, extensive screening studies have not been conducted under combined stress conditions. Further, field studies have shown that even

moderately resistant or tolerant chickpea varieties become susceptible under combined stress (Chilakala et al., 2022; Rai et al., 2022; Sinha et al., 2021). These studies indicate a need for an alternate and robust source of resistance capable to defending against broad-spectrum fungal pathogens, particularly under drought.

Nonhost resistance (NHR), a broad-spectrum, species-level resistance could be an alternative source of resistance to DRR. NHR is a multifaceted and durable phenomenon, encompassing a wide range of resistance

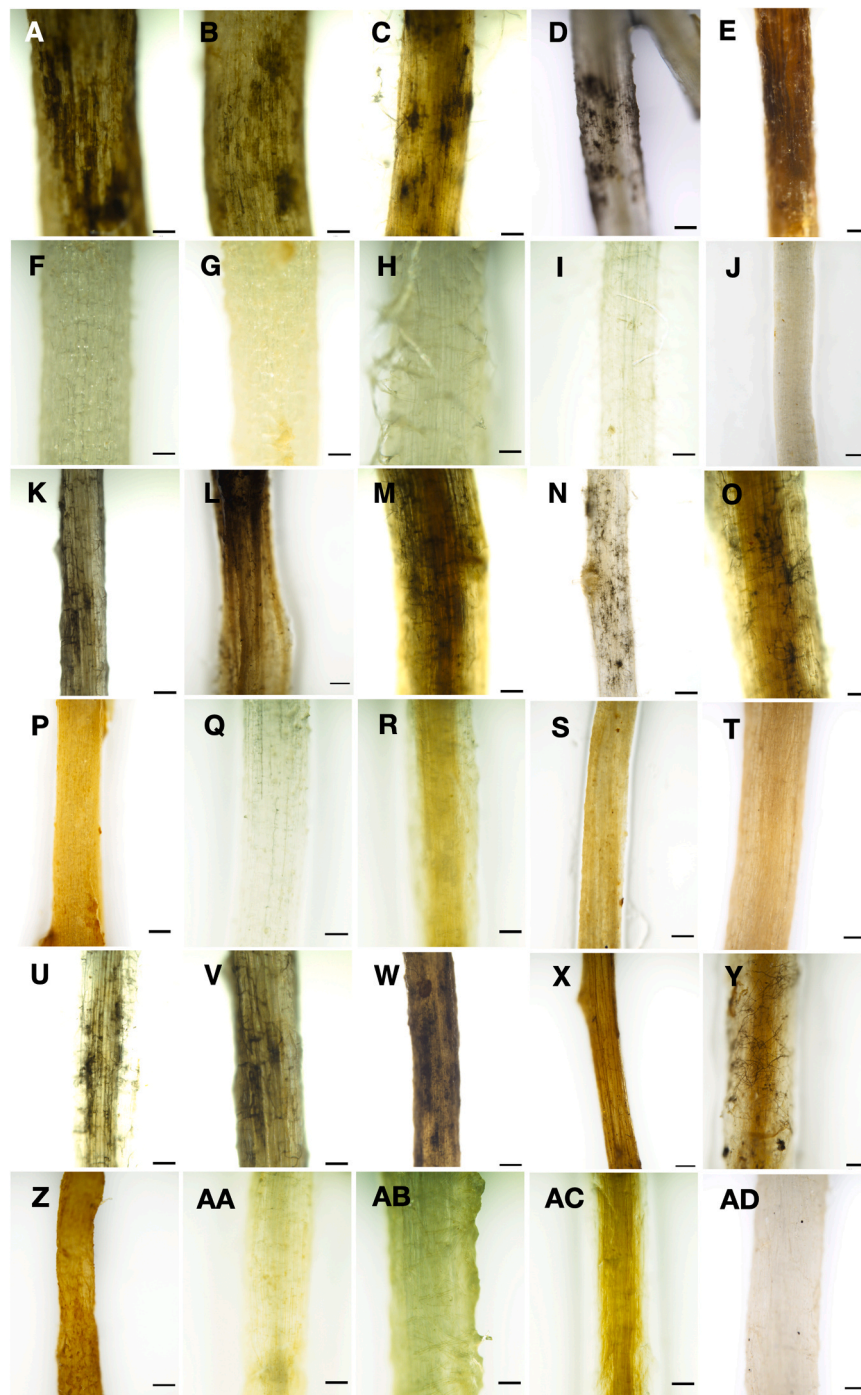


Fig. 1. – Disease response of various potential nonhosts inoculated with *M. phaseolina*. The blotter paper assay was conducted to screen the 12 potential nonhost species. The samples were collected at 7 DAI and observed under a stereomicroscope. Stereomicroscopic observations of the root surface to visualize necrosis. Images depict an inoculated section of the roots of the screened plants. Pathogen inoculated roots of A) *C. arietinum* BG 212, B) *C. arietinum* ICC 4958, C) *Helianthus annuus*, D) *Hordeum vulgare*, E) *Triticum monococcum*, and F-J) their respective controls. Pathogen inoculated roots of K) *Cynodon dactylon*, L) *Chenopodium album*, M) *Amaranthus retroflexus*, N) *Cyperus rotundus*, O) *Tridax procumbens* and P-T) their respective controls. Pathogen inoculated roots of U) *Pennisetum glaucum*, V) *Euphorbia hirta*, W) *Ageratum conyzoides*, X) *Triticum turgidum*, Y) *Parthenium hysterophorus* and Z-AD) their respective controls. Scale bars – 250 μ m.

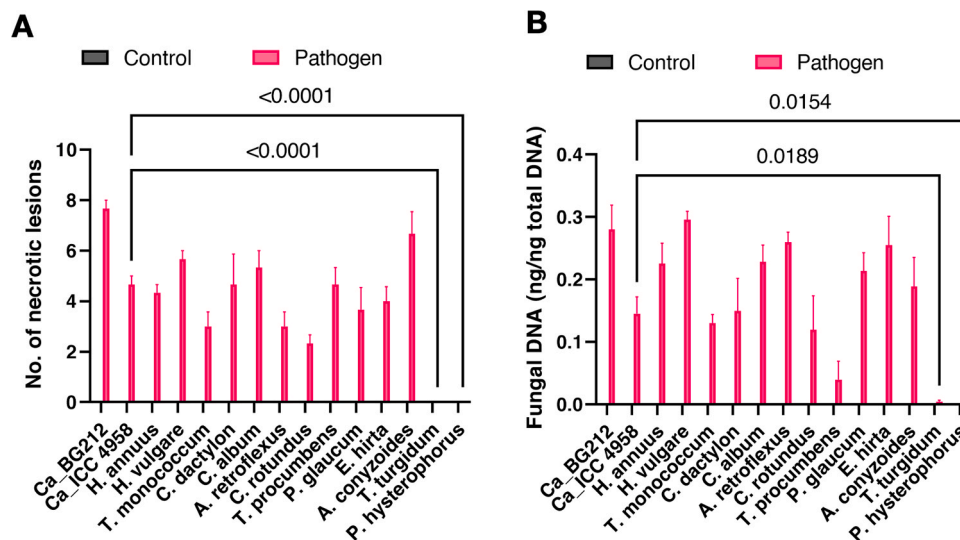


Fig. 2. – Necrotic lesions and *in planta* fungal load in potential nonhost plants inoculated with *M. phaseolina*. The blotter paper assay was conducted to screen the 12 potential nonhost species. The samples were collected at 7 DAI and observed under a stereomicroscope. Number of necrotic lesion were quantified and the *in planta* fungal DNA load was estimated. **A)** Quantification of the number of necrotic lesions on the plant roots screened at 7 DAI. **B)** *In planta* fungal load estimation at 7 DAI. Experiments were repeated at least twice with similar results observed. N = 3, Error bars on the graphs represent standard error of mean. Significance of differences was calculated using two-way ANOVA. P-values were computed using Tukey post hoc for multiple comparisons test after two-way ANOVA. P-values are indicated on the graph between variables which were compared. DAI – days after inoculation.

mechanisms that ward off a plethora of potential non-adapted pathogens (Oh and Choi, 2022; Panstruga and Moscou, 2020). NHR can involve physical barriers, chemical defenses, effector triggered immunity (ETI) and pattern triggered immunity (PTI) (Lee et al., 2017; Panstruga and Moscou, 2020). These can also be classified as pre- and post-invasive NHR. PTI seems to be more prominent in nonhosts which are distantly related to the host while ETI is the common mechanism of NHR in closely related nonhost-host pairs (Schulze-Lefert and Panstruga, 2011). Physical and chemical defenses can consist of constitutive or induced defenses while PTI and ETI are generally induced. Variations in wax, cuticle and membrane lipid composition can lead to the failure of non-adapted pathogens in colonizing a nonhost while cell wall apposition and cytoskeletal rearrangements can constitute induced physical defenses (Lee et al., 2017). Similarly, phytoanticipins are constitutive defensive compounds while phytoalexins are rapidly induced in responses to pathogens in several nonhost species (Lee et al., 2017). Further, while NHR to fungal leaf pathogens such as *Alternaria brassicae* have been studied (Fatima et al., 2019), to our knowledge, no reports of NHR are available for necrotrophic root pathogens. Nonetheless, components of both pre- and post-invasive NHR are active in plant roots as well. Such NHR mechanisms once elucidated in nonhost species can be effectively deployed to fend off pathogens causing severe economic losses in cultivated species (Ayliffe and Sørensen, 2019).

We hypothesized that NHR can be a viable and robust alternative to host resistance in the case of chickpea-DRR, primarily due to the pathogen's broad host range, lack of resistance sources in chickpea and the drought-mediated augmentation of the disease. Thus, our study aimed to identify a nonhost species resistant to DRR; elucidate the morphopathological, transcriptomic, and metabolomic characteristics of its NHR to DRR. Elucidation of such NHR mechanisms and its subsequent translational research in future will aid in alleviating DRR-induced economic losses in chickpea as well as other host crops. R-gene transfer between host-nonhost pairs have been used to benefit from NHR in plant breeding (Panstruga and Moscou, 2020; Stam et al., 2014). This strategy can be effectively implemented as the pathways downstream of the R-genes are largely conserved, even across dicotyledons and monocotyledons (Wulff et al., 2011). Further, nonhost specific metabolite production in transgenic hosts has shown potential to alleviate diseases caused by adapted pathogens (Hain et al., 1993; Lee et al.,

2017; Tattersall et al., 2001). Mechanisms identified from our study have the potential for exploitation of NHR in plant breeding for crop protection from DRR.

2. Materials and methods

2.1. Plant material and growth conditions

The details of the 12 potential nonhost species screened experimentally are available in [Supplementary Table 1](#). For the initial screening, *C. arietinum* varieties BG-212 and ICC-4958, susceptible and moderately resistant to DRR, respectively, were used. ICC-4958 was used for subsequent experiments. *Helianthus annuus* variety KBSH-44 was used as an Asteraceae host for positive control. *P. hysterophorus* seeds were obtained from the All India Coordinated Research Project on Weeds (Pondicherry University, Karaikal, India). The ecotype Ph-1 was used for our study. For detailed information, refer to the [Supplementary Materials](#) and Methods.

2.2. Fungal infection assays

M. phaseolina (SKMPt70; NCBI ID OM674336) was isolated from Guntur, Andhra Pradesh (16° 23' 19.2048" N, 80° 25' 41.9772" E) (Chilakala et al., 2022). A microsclerotial suspension was used as the fungal inoculum for the blotter paper assays. Fungal inoculum was prepared by scraping potato dextrose agar plate cultures of *M. phaseolina*.

For the blotter paper assays, plants at the 8–12 leaf stage were uprooted from the nurseries and washed. The plants were then placed on one half of wet blotting sheets (12 × 8 in.) in plastic trays (16 × 12 in.) ([Supplementary Figure 1](#)). The 2 cm region below the seed attachment point was inoculated with 250 µL microsclerotial suspension. The trays were kept in a growth chamber and watered once every day. Samples were collected at 7 days after inoculation (DAI) for the initial screening, at 1, 4, and 7 DAI for the pathogen colonization studies and at 4 DAI for the combined stress studies. Combined stress blotter paper assay was conducted as previously shown (Irulappan et al., 2022). The weight of the wet blotting sheet (2 × 2 cm) was maintained at 102 ± 4 mg (100 % water) for the control and pathogen treatments while it was maintained

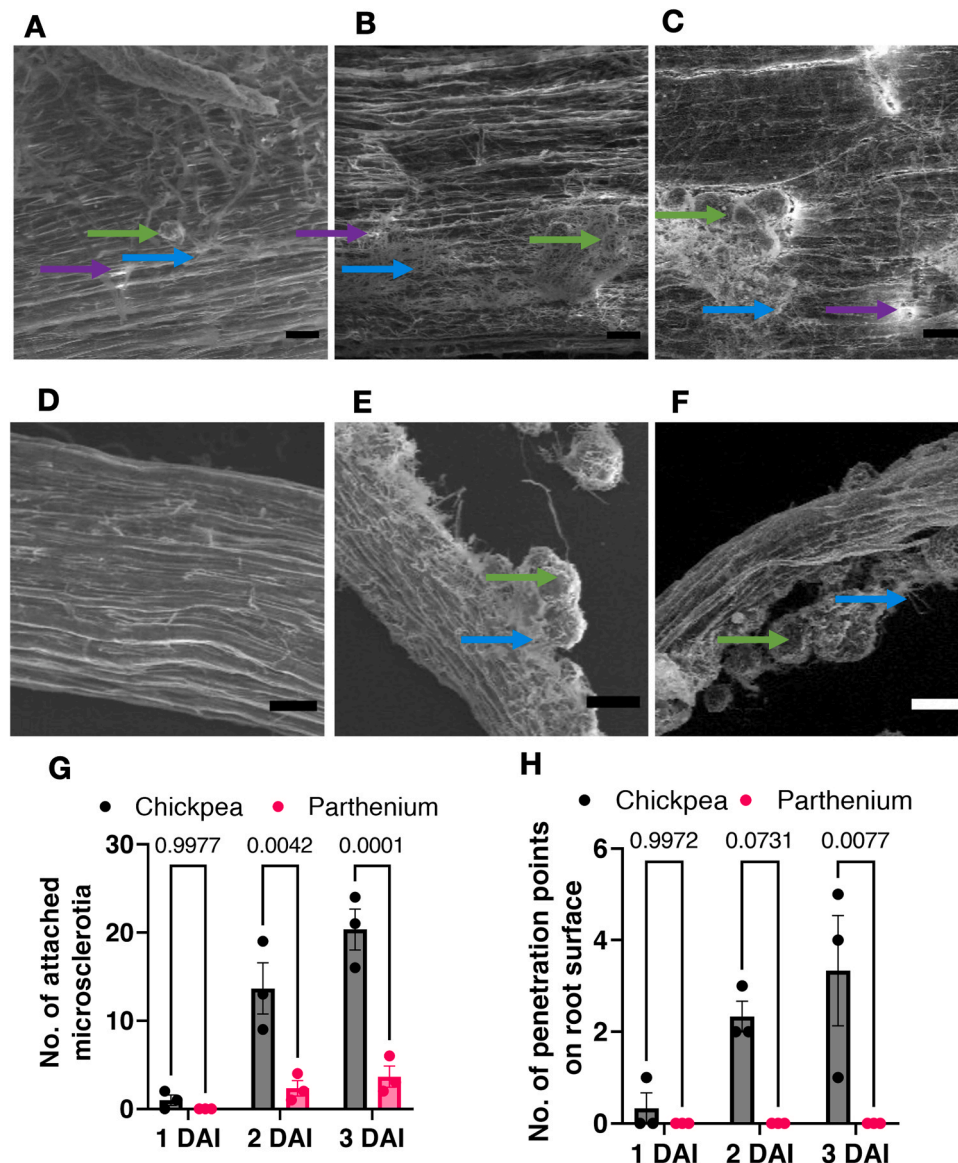


Fig. 3. – *M. phaseolina* has an altered attachment and penetration frequency on the surface of nonhost roots. Samples were collected from the blotter paper assay at three time points and fixed in glutaraldehyde and dried in acetone before observing under a scanning electron microscope. Scanning electron micrographs showing the microsclerotia and mycelia on the root surface of chickpea at A) 1 DAI, B) 2 DAI, C) 3 DAI, and Parthenium at D) 1 DAI, E) 2 DAI, F) 3 DAI, Green arrow – microsclerotia, Blue arrow – mycelia, Purple arrow – penetration point, Scale bars – 100 μm. G) Quantification of the microsclerotial attachment on host and nonhost roots, H) Quantification of penetration events by *M. phaseolina* on the root surface of chickpea and Parthenium. Experiments were repeated at least twice with similar results observed. $n = 3$, Error bars on the graphs represent standard error of mean. Significance of differences was calculated using two-way ANOVA. P-values were computed using Tukey post hoc test for multiple comparisons test after two-way ANOVA. P-values are indicated on the graphs between variables which were compared. DAI – days after inoculation.

at 65 ± 7 mg (36.27 % decrease) for the drought and combined stress treatments.

For soil assays, 14-day-old chickpea and Parthenium plants from nurseries were used. The plants were then transferred to plastic tubs ($16 \times 16 \times 12$ in.) with soil containing *M. phaseolina* inoculum. The tubs were kept in the greenhouse, and samples were collected at 14 and 28 days after transplantation (DAT).

2.3. In planta fungal load estimation

The fungal load was estimated using an aprotocol described earlier (Irulappan et al., 2022; Supplementary Materials and Methods). Total DNA was isolated using the cetyltrimethylammonium bromide method of Allen et al., (2006). In planta fungal load was quantified based on the ITS4 region using a standard curve of fungal DNA from broth culture.

2.4. Microscopic observations

Samples were collected at 7 DAI for the initial screening and combined stress assays at 1, 4, and 7 DAI for the pathogen colonization studies; at 14 and 28 DAT for the sick-soil assay; and at 1, 2, and 3 DAI for scanning electron microscopy. Microscopy sample preparation and observations were performed according to Irulappan et al. (2022) with certain modifications. Wheat germ agglutinin conjugated with fluorescein (Cat. W834, Invitrogen™, Thermo Fisher Scientific™, Waltham, MA, U.S.A.) was used to stain the fungus (details in Supplementary Materials and Methods).

2.5. RNA isolation, library preparation, and RNA sequencing

The blotter paper assay was used to collect samples for RNA-seq.

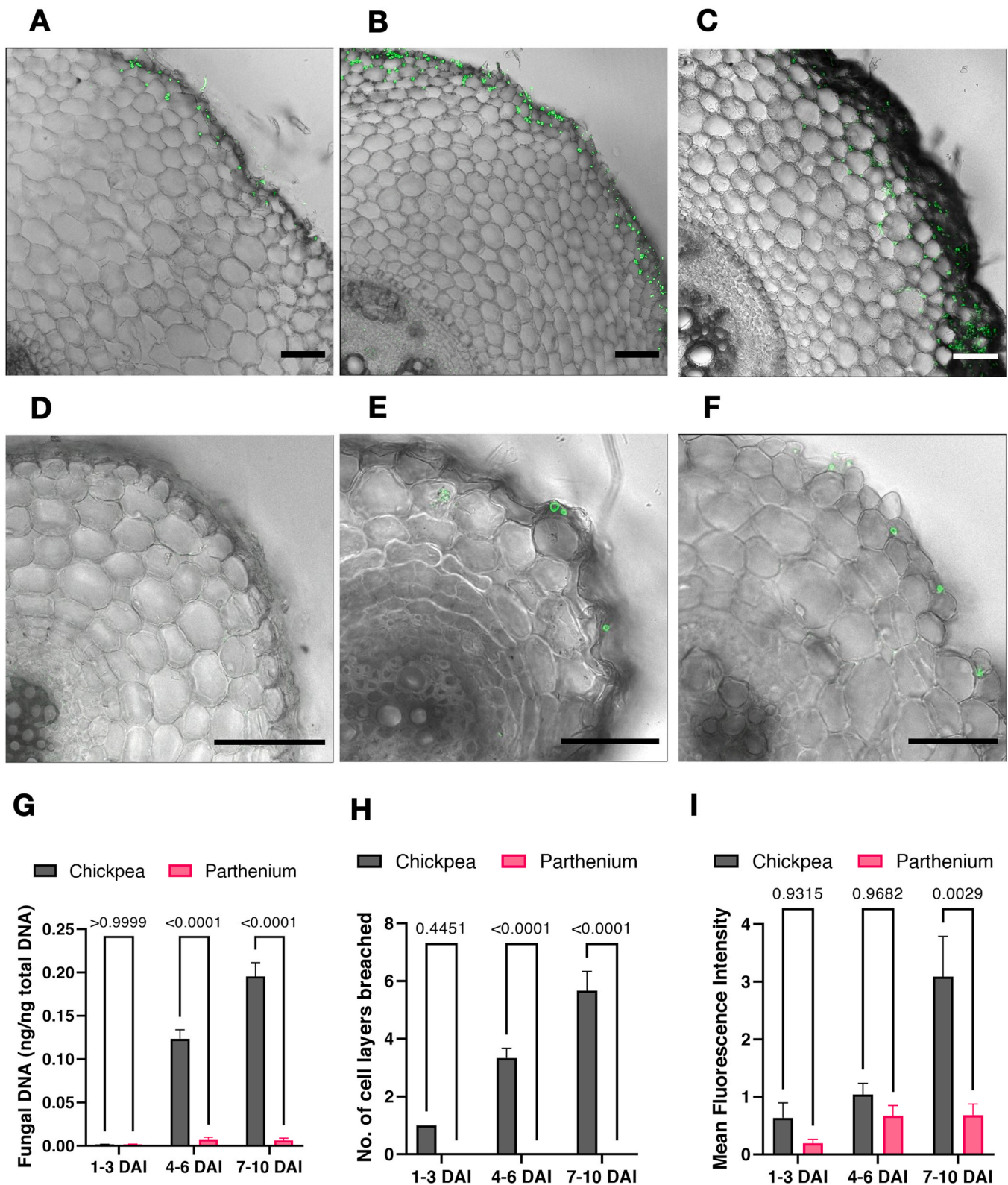
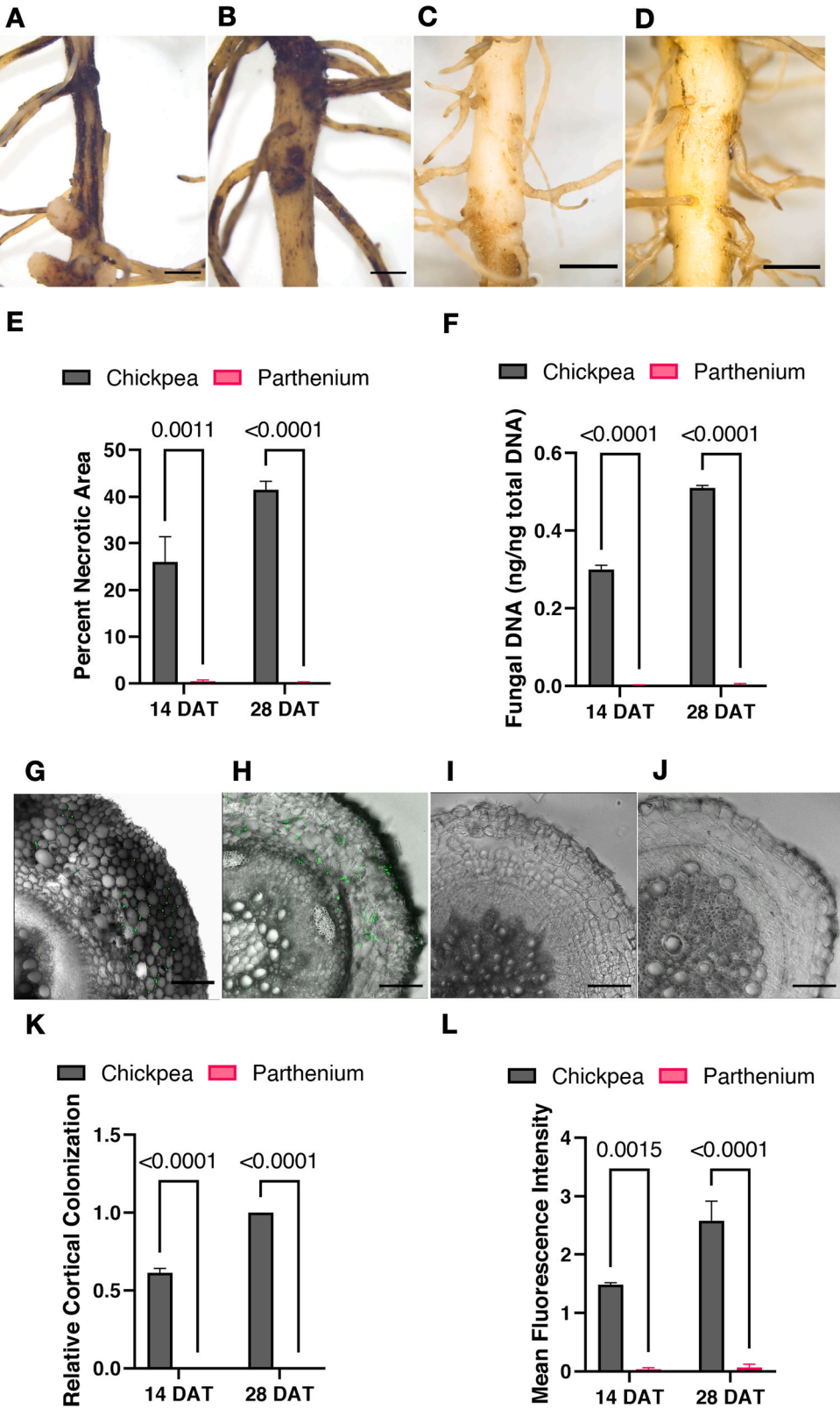


Fig. 4. – Nonhost plant restricts *M. phaseolina* propagation in root cortical cells. Blotter paper assay was conducted to study the cortical colonization by *M. phaseolina* in the host and nonhost roots. Root samples were collected at three time points. Transverse sections were cut using a vibratome and observed under a confocal laser scanning microscope after staining with the chitin specific stain wheat germ agglutinin conjugated with fluorescein. Confocal microscopic observations in chickpea at A) 1 DAI (Time window 1), B) 4 DAI (Time window 2), C) 7 DAI (Time window 3) and in Parthenium at D) 1 DAI (Time window 1), E) 4 DAI (Time window 2), F) 7 DAI (Time window 3). Scale bars – 100 μ m. G) *In planta* fungal load estimation in chickpea and Parthenium at the three time points studied. H) Number of cortical cell layers colonized in chickpea and Parthenium at the three time points studied. I) Fluorescence quantification of confocal images. Graphs indicate the mean fluorescence intensity quantified using ImageJ. Experiments were repeated at least thrice with similar results observed. $n = 3$, Error bars on the graphs represent standard error of mean. Significance of differences was calculated using two-way ANOVA. P-values were computed using Tukey post hoc for multiple comparisons test after two-way ANOVA. P-values are indicated on the graph between variables which were compared. DAI – days after inoculation.



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Fig. 5. – NHR is durable at advanced vegetative stages. To study the pathogen colonization in the host and nonhost roots at relatively mature stages, the disease assay was conducted in soil with *M. phaseolina* inoculum. Samples were collected at 14 and 28 DAT. The samples were then subjected to stereomicroscopic and confocal microscopic observations and *in planta* fungal load estimation. Stereomicroscopic observations of the roots of chickpea at A) 14 DAT, B) 28 DAT and Parthenium at C) 14 DAT, D) 28 DAT. Scale bars = 2000 μ m. E) Quantification of percent necrotic area using ImageJ, F) *In planta* fungal load estimation in chickpea and Parthenium, Fungal colonization in the root cortex of chickpea at G) 14 DAT, H) 28 DAT and Parthenium at I) 14 DAT, J) 28 DAT, Scale bars = 200 μ m. K) Relative cortical colonization in the roots of chickpea and Parthenium. L) Fluorescence quantification of confocal images. Graphs indicate the mean fluorescence intensity quantified using ImageJ. Experiments were repeated at least twice with similar results observed. n = 3, Error bars on the graphs represent standard error of mean. Significance of differences was calculated using two-way ANOVA. P-values were computed using Tukey post hoc for multiple comparisons test after two-way ANOVA. P-values are indicated on the graph between variables which were compared. DAT – days after transplantation.

Samples were collected at 2 and 4 DAI for Parthenium and at 2 DAI for chickpea. Root tissue (2 cm) from each plant was collected (Supplementary Figure 3), and five individual plant roots were pooled to make one replicate. Three replicates of each treatment were used for sequencing. Total RNA was isolated from the plant tissue sample using the conventional TRIzol method. RNA-seq paired-end sequencing libraries were prepared from the QC-passed RNA samples using NEB-Next® Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs™, (NEB), Ipswich, MA, U.S.A.). After the Qubit concentration for the libraries and the mean peak sizes from Agilent Tape Station profile were obtained, the PE Illumina libraries were loaded onto NovaSeq6000 for cluster generation and sequencing. The quality of raw sequences was evaluated using FastQC (version 0.11.5). For the detailed protocol, refer to the Supplementary Materials and Methods.

2.6. De novo assembly and differential expression analysis

The remaining reads of Parthenium were concatenated into a single set input to generate a single reference, Trinity (Supplementary Figure 2). CD-HIT-EST (Li and Godzik, 2006) was used to cluster transcripts with at least 90 % identity, and the representative sequences were chosen for further analysis. Finally, TransDecoder (<https://github.com/TransDecoder/TransDecoder/wiki>) was used to predict coding sequences (CDS). The assembly quality was accessed by mapping the RNA-seq reads back to the assembly and computing the contig N50 values. BLASTX (NCBI Blast +2.8.1) was used to annotate CDS-predicted transcripts against the NCBI Ref sequence protein databases. The BLAST results were processed using BioBamOmicbox (v1.3), and GO ID and description were assigned to each transcript, using default parameters. Differential expression analysis was performed using DEseq2 (Love et al., 2014) in the R package with FDR $\leq$ 0.05.

2.7. Metabolome analysis

Samples for flavonoid extraction were collected from the blotter paper assay at 2 DAI for chickpea and Parthenium. The flavonoids were extracted with 80 % methanol overnight at room temperature under brief agitation. The resulting extracts were subjected to centrifugation and supernatants were transferred to a fresh reaction tube and then vacuum-dried at 65 °C using a SpeedVac. Finally, the dried form of extracts was resuspended in 80 % methanol. The analysis of target flavonoids in the plants was performed as described in previous studies (Naik et al., 2021).

2.8. Antifungal assays

Varying concentrations of naringenin (1–100 μ g/mL) were made in ethanol. 100 μ L of each concentration in five replicates was loaded into the well of a 96-well cell culture plate. 100 μ L of ethanol was used as control. 100 μ L of potato dextrose broth was added to each well. A micropipette tip was pricked into a 7-day old *M. phaseolina* potato dextrose agar plate and the tips were used to inoculate each well. The turbidimetric method was used to quantify growth after 24 h using a spectrophotometer. Percentage inhibition was calculated relative to the control. The treated mycelia were then used as inoculum for the subsequent blotter paper assays.

2.9. Statistical analysis

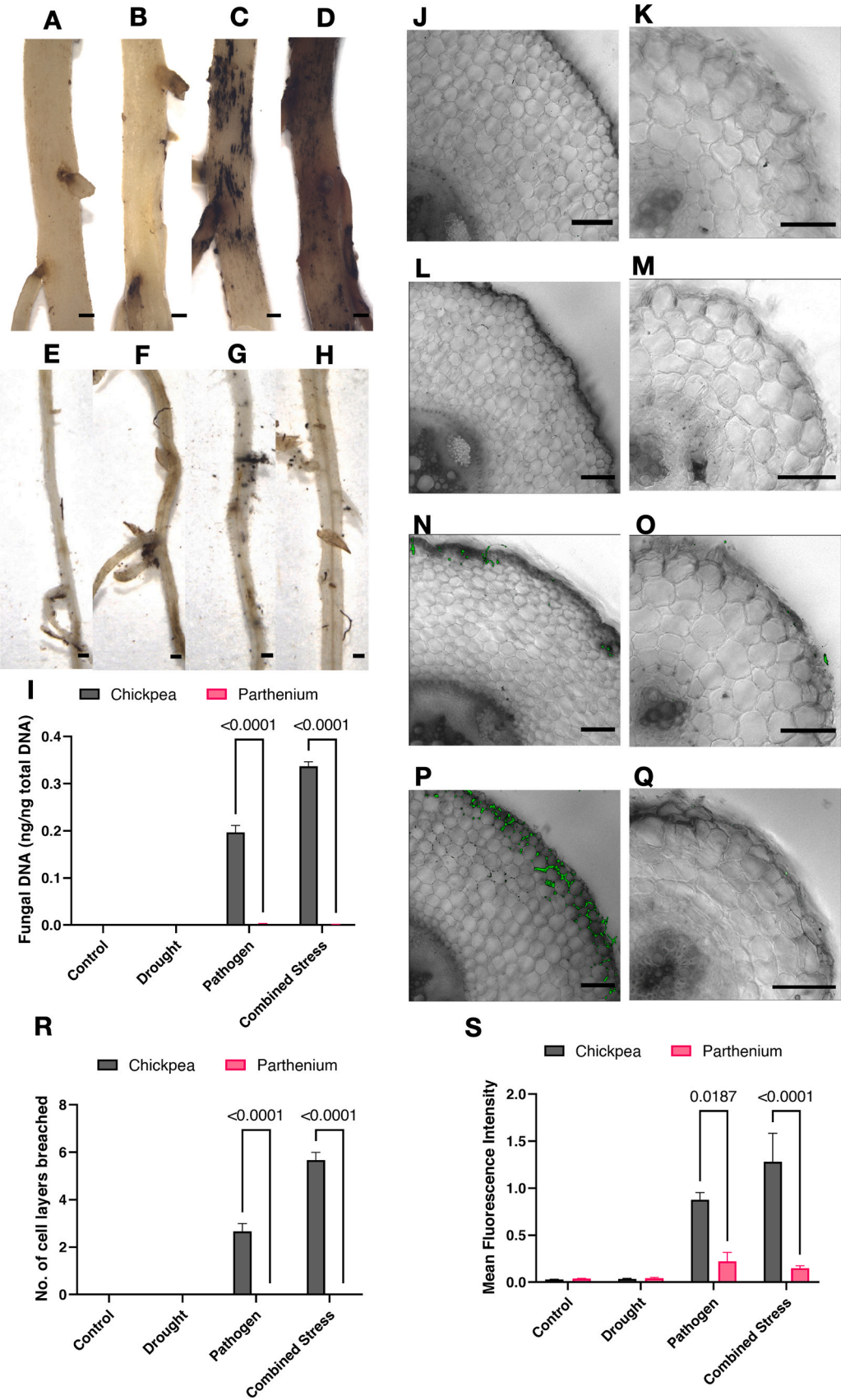
Two-way analysis of variance (ANOVA) was conducted to compute the significance differences among the treatments. Tukey's HSD test to compute multiple comparisons between the means of all treatments. P-values are indicated on the graphs between the treatments that were compared. For the naringenin antifungal assays, one-way ANOVA followed by Tukey's HSD test was used. All analyses and plots were computed using the GraphPad Prism software for Microsoft Windows (Version 9.3.1, GraphPad Software, San Diego, CA, USA). The heatmaps and the bar charts for the RNA-seq data were generated using the ggplot2 package in R. The letter 'n' is used in the figure legends to denote the number of biological replicates.

3. Results

3.1. Parthenium hysterophorus is a nonhost plant species resistant to DRR

A pipeline for shortlisting potential nonhost species was generated (Supplementary Figure 4, Supplementary Materials and Methods). Two databases were used: 1) the USDA Fungal Database (Farr and Rossman, 2024) (Supplementary Table 2), for listing the reported host species of *M. phaseolina*, and 2) the FAOSTAT Crop Production Database (FAO, 2021; Supplementary Table 3), for listing commercially cultivated species around the world. These databases consisted of 530 and 134 species, respectively. From the fungal database, 396 non-cultivated species were removed based on FAOSTAT 2021 Crop Production Data consisting of 134 species. Only cultivated host species were selected, as these species can be used as sources to identify other species cultivated or found near these hosts. From the cultivated species (FAO, 2021), 87 species were removed based on previous reports of *M. phaseolina* infection, either from the USDA fungal database or from literature, and the remaining 47 species made up the initial crop nonhost pool (Supplementary Table 3). Parallely, from the remaining 87 host species (Farr and Rossman, 2024), 69 were removed based on the global area under cultivation, using the chickpea area under cultivation as a threshold (Supplementary Table 4). A literature survey was conducted using the remaining 18 host species to identify the hosts' common intercrops and weeds. We identified 36 weed species, and 18 intercrops/rotated crops found in the host species' fields (Supplementary Tables 5 and 6). From these, 3 weed species and 14 crop species had reports of *M. phaseolina* infection and were subsequently removed (Supplementary Table 7). The remaining 33 weed species, 4 intercropped species, along with the 47 species from the initial crop nonhost pool together comprised the final nonhost pool of 84 species. From these, 4 crop species with greater area under cultivation than chickpea and the 8 most abundant weed species observed in the field study conducted by Chilakala et al., (2022) were selected for experimental screening in this study (Supplementary Table 7, Supplementary Figure 5).

To further shortlist a plant species for morpho-pathological studies of NHR, fungal assays were conducted using the blotter paper assay. Stereomicroscopic observations showed that all but two species had necrotic lesions on the root surface at 7 DAI, implying that the fungus could initiate colonization by killing root epidermal cells (Fig. 1A–AD). There were significant differences in lesion numbers among different species (Fig. 2A). The average number of lesions in the microscope field



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Fig. 6. Robustness of nonhost resistance under combined stress. To study the durability of NHR in combined stress conditions, a modified version of the blotter paper assay with lowered watering frequency was used. Samples were collected at 7 DAI. Samples were then observed using a stereomicroscope and confocal laser scanning microscope followed by *in planta* fungal load estimation. Stereo-microscopic observations of *M. phaseolina* infection under combined stress in roots of chickpea under A) Control, B) Drought, C) Pathogen, D) Combined stress and in Parthenium under E) Control, F) Drought, G) Pathogen, H) Combined stress. Scale bars = 500 μm . I) *In planta* fungal DNA quantification in chickpea and Parthenium, Confocal microscopic observations of root cortical colonization in chickpea under J) Control, K) Drought, L) Pathogen, M) Combined stress and in Parthenium under N) Control, O) Drought, P) Pathogen, Q) Combined stress. Scale bars = 100 μm . R) Number of cortical cell layers colonized by the fungal mycelia under various treatments in chickpea and Parthenium. S) Fluorescence quantification of confocal images. Graphs indicate the mean fluorescence intensity quantified using ImageJ. Experiments were repeated at least thrice with similar results observed. $n = 3$, Error bars on the graphs represent standard error of mean. Significance of differences was calculated using two-way ANOVA. P-values were computed using Tukey post hoc for multiple comparisons test after two-way ANOVA. P-values are indicated on the graph between variables which were compared. DAI – days after inoculation.

of view (FOV) varied from 0 (no observable necrotic lesions) in *Triticum turgidum* (Fig. 1X) and *P. hysterophorus* (Figs. 1Y) to 7.66 in the *C. arietinum*-susceptible genotype BG-212 (Fig. 2A). A similar trend was observed with *in planta* fungal load quantification. *P. hysterophorus* had the lowest *in planta* fungal load at 0.0018 ng/ng total plant DNA, while *Hordeum vulgare* had the highest at 0.29 ng/ng total DNA. Furthermore, *T. turgidum* and *P. hysterophorus* had no significant change relative to their respective controls. In addition, *Triticum monococcum*, *Cyperus rotundus*, and *Tridax procumbens* had significantly lower *in planta* fungal load relative to the moderately resistant *C. arietinum* genotype ICC-4958 (Fig. 2B). Hence, both *T. turgidum* and *P. hysterophorus* are nonhost species for *M. phaseolina*. However, we selected *P. hysterophorus* for this study because being a dicot herb, it is relatively closer to chickpea than the monocot *T. turgidum* (Li et al., 2019). *P. hysterophorus* is referred to as Parthenium henceforth in this manuscript, while the moderately resistant genotype of *C. arietinum* ICC-4958 is referred to as chickpea.

3.2. Parthenium root surface showed reduced microsclerotial attachment and penetration frequency

To study the interaction between the fungal propagules and the nonhost root surface, plant samples were observed under a scanning electron microscope. Samples were collected at 1, 2, and 3 DAI from the blotter paper assay. Results indicate that *M. phaseolina* microsclerotia can attach to the root surface of Parthenium. However, the frequency of attachment was significantly lower in the nonhost. A significant rise in microsclerotial attachment was observed in chickpea across the three time points (Fig. 3A–C). This trend was, however, not observed in Parthenium (Fig. 3D–F). Attachment on the root surfaces per microscopic FOV in chickpea and Parthenium was maximum at 3 DAI, at an average of 20.33 and 3.66 microsclerotia, respectively (Fig. 3G). The number of penetration events on the root surface, despite an apparent similar trend of steady rise in chickpea, was not statistically significant across the subsequent time points (Fig. 3H). No penetration events were observed at the time points studied on the surface of Parthenium roots. Various reasons for lower microsclerotial attachment and mycelial penetration were explored in subsequent experiments.

3.3. *M. phaseolina* is unable to colonize nonhost root cortical cells

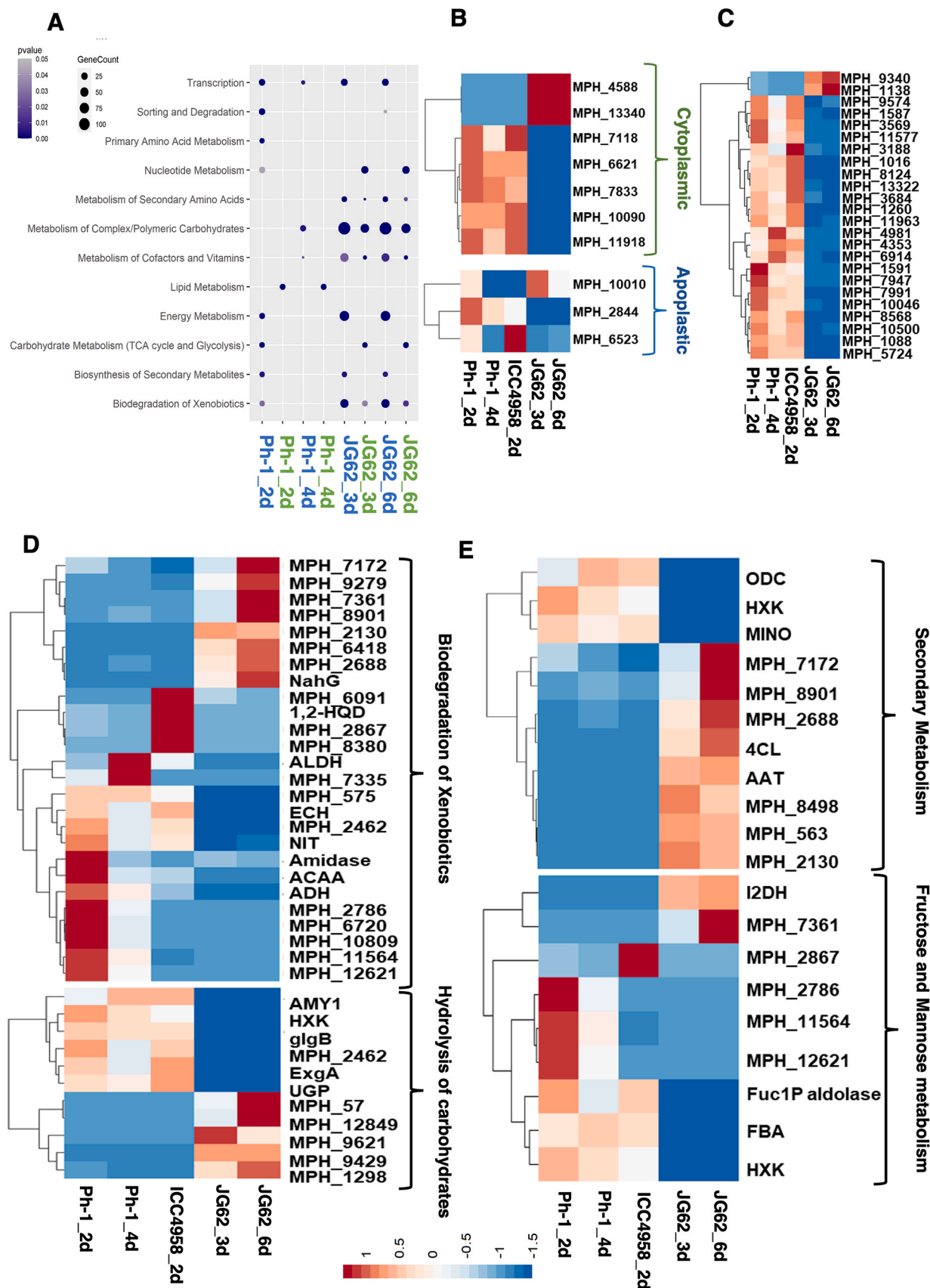
To study the differences between pathogen colonization in the roots of the host and nonhost, three time windows were studied via confocal microscopy and *in planta* fungal load estimation. The *in planta* fungal DNA was 0.001, 0.123 and 0.195 ng/ng total plant DNA during 1–2 DAI, 3–6 DAI, and 7–10 DAI, respectively, in chickpea. Meanwhile, Parthenium had total fungal DNA loads of 0.001, 0.007, and 0.006 ng/ng total plant DNA at the respective time points, indicating little to no mycelial load in its root tissues. A significant increase in the number of root cell layers (epidermis and cortex) colonized was observed under the confocal microscope at 1–2 DAI, 3–6 DAI, and 7–10 DAI in chickpea (Fig. 4A–C, H, Supplementary Figure 6). The average numbers of cell layers breached were 1, 3.33, and 5.66 at 1–2, 3–6, and 7–10 DAI, respectively. The extent of colonization was even higher in a susceptible host genotype, BG-212 (Supplementary Figure 7). In Parthenium, only the

epidermal layer was breached at a much higher concentration of inoculum used. No cortical tissue breaching by *M. phaseolina* was observed in Parthenium at any of the time points studied (Fig. 4D–F, H). A similar trend was observed while quantifying the fluorescence in confocal images using ImageJ. The fluorescence observed is due to the mycelia in the epidermal layer and on the root surface. The mean fluorescence intensity rose from 0.634 to 1.037 and 3.083 at 1–2, 3–6, and 7–10 DAI, respectively, in chickpea (Fig. 4I). However, much lower average fluorescence intensities, 0.194, 0.175, and 0.191, were observed at 1–2, 3–6, and 7–10 DAI, respectively, in Parthenium (Fig. 4I). Therefore, after the initial penetration of the epidermis, *M. phaseolina* is unable to propagate within the roots of the nonhost plant even at later time points.

As aerial symptoms and peak DRR symptoms in the roots are predominantly observed towards the advanced vegetative stage of the plant under field conditions, we conducted sick-soil assays. Sick-soil with *M. phaseolina* inoculum was used as the medium to grow the host and nonhost species. Roots collected at 14 and 28 DAT were observed. Stereomicroscopic results showed that chickpea had large, black necrotic spots on the root surface at both time points (Fig. 5A, B, Supplementary Figure 8). However, no such necrotic spots were observed on the roots of Parthenium (Fig. 5C, D). Using ImageJ, the root necrotic area in chickpea was found to increase from 25.98 % to 41.46 % at the two time points observed (Fig. 5E). However, Parthenium had very low necrotic areas at both time points, with no increase (0.499 % and 0.252 %, respectively) (Fig. 5E). Chickpea leaves started becoming straw-colored, marking the onset of aerial disease symptoms, while no typical aerial symptoms were observed in the nonhost plant. A similar trend was observed with *in planta* fungal load estimation (Fig. 5F). The fungal loads were 0.30 and 0.51 ng/ng total DNA at 14 and 28 DAT, respectively. However, no significant *in planta* fungal load was observed in Parthenium. Confocal microscopic observations were consistent with the above results (Fig. 5G–J). Relative cortical colonization in chickpea rose from 0.613 to 1 at the two time points evaluated (Fig. 5K). In other words, within 28 days, the mycelia could completely colonize the host root cortex and reach the endodermal tissue. This event marks the beginning of the final stage of infection (vascular colonization) in the host roots. However, no mycelia were observed in the root sections of Parthenium at any time point, demonstrating no relative cortical colonization at either of the time points (Fig. 5K). As expected, fluorescence quantification showed consistent results. Mean fluorescence intensity in chickpea root sections rose from 1.48 to 2.58 at 14 and 28 DAT, respectively (Fig. 5L), while it was negligible for Parthenium root sections, at 0.039 and 0.071, respectively (Fig. 5L). These results indicate that no fungal mycelia or propagule was present in Parthenium root sections. This is due to the significantly lower fungal inoculum present in the soil compared to that used for the blotter paper assay. Thus, Parthenium might be resistant to *M. phaseolina* even at relatively mature plant growth stages.

3.4. Parthenium NHR is durable under combined stress

DRR symptoms are significantly more severe under concurrent drought stress. To identify the effect of drought on NHR to *M. phaseolina*, we conducted the blotter paper assay under pathogen-only and



(caption on next page)

Fig. 7. – Global gene expression analysis of *M. phaseolina* during the infection of host and nonhost species. Root samples for RNA-seq were collected at 2 and 4 DAI for Parthenium (Ph-1) and at only 2 DAI for chickpea (ICC 4958) from the blotter paper assay. RNA-seq data from Irulappan et al., (2022) was used for comparison with the susceptible genotype of chickpea (JG62). **A)** Bubble chart represents the enriched pathways for all treatments. The gradation of the shade represents the *p*-value. The size of the bubble represents the number of genes in each pathway. Labels in green colour represent downregulated pathways while those in blue represent upregulated pathways. All treatments in the chart are compared to the moderately resistant chickpea variety (ICC 4958) at 2 DAI. **B)** Heat map represents the differential expression of the putative effector genes targeted to plant cytoplasm and apoplast predicted using the EffectorP 3.0 tool. Colour grade represents adjusted *z*-score signifying upregulation (red) or downregulation (blue). **C)** Heat map represents the predicted fungal genes with putative interacting partners in plants. Plant-fungus interacting partners were predicted using the Pathogen-Host Interaction database. Heat maps represent the genes in the **D)** biodegradation of xenobiotics and the hydrolysis of carbohydrates, **E)** secondary metabolism and the fructose and mannose metabolism pathway classes. Colour grade in all the heat maps represents adjusted *z*-score signifying upregulation (red) or downregulation (blue). *Ph-1_2d* – Parthenium at 2 DAI, *Ph-1_4d* – Parthenium at 4 DAI, *ICC4958_2d* – Chickpea ICC4958 at 2 DAI, *JG62_3d* – Chickpea JG62 at 3 DAI, *JG62_6d* – Chickpea JG62 at 6 DAI.

combined stress conditions. Root necrotic lesions increased significantly in chickpea under combined stress, as evident from stereomicroscopic observations (Fig. 6A–D). However, no necrotic lesions were observed in Parthenium (Fig. 6E–H). *In planta* fungal DNA in chickpea was an average of 0.196 and 0.336 ng/ng total plant DNA under pathogen-only and combined stress treatments, respectively (Fig. 6I). However, no significant rise was observed in Parthenium (Fig. 6I). Confocal microscopic observations paint a similar picture. Fungal colonization in the root of chickpea was significantly greater under combined stress, although no such trend was observed in Parthenium (Fig. 6J–Q). Cortical colonization by the fungus significantly increased in chickpea from an average of 2.66 and 5.66 cortical layers under pathogen-only and combined stress, respectively (Fig. 6R). No cortical colonization was observed in Parthenium at the microsclerotial suspension concentration used for the experiment (Fig. 6R). Mean fluorescence intensity quantified from the confocal images increased from 0.875 to 1.28 in chickpea under combined stress, while no significant change was observed in Parthenium (Fig. 6S).

3.5. Co-transcriptome analysis

Dual RNA-seq was conducted on root samples from the blotter paper assay. Samples were collected at 2 and 4 DAI for Parthenium and at only 2 DAI for chickpea (tolerant genotype ICC-4958). The analyzed data were also compared to the RNA-seq data for the susceptible chickpea genotype JG-62 from Irulappan et al., (2022). An average of 3.18 % and 2.11 % reads from the chickpea and Parthenium samples, respectively, mapped to the *M. phaseolina* MS6 genome (Islam et al., 2012). All comparisons of the fungal transcriptome were between the early time points of both species. DEGs were estimated relative to the early time point of chickpea ICC 4958. When infecting Parthenium, *M. phaseolina* had 477 DEGs (164 upregulated and 313 downregulated), (Supplementary File 1, RNA-seq Dataset 1). This was significantly lower than the number of DEGs observed in JG-62, which had 5542 (1552 upregulated and 3900 downregulated) (Supplementary File 1, RNA-seq Dataset 1). Enrichment analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Significant enrichment was observed in several pathways in *M. phaseolina* when infecting chickpea JG-62. Among the most significantly enriched pathways were energy metabolism, metabolism of complex/polymeric carbohydrates, nucleotide metabolism, and transcription and biodegradation of xenobiotics. Enrichment was largely similar across upregulated and downregulated genes in JG-62 (Fig. 7A). However, relative to ICC-4958, fewer pathways were enriched in Parthenium (Fig. 7A). Furthermore, most enriched pathways were upregulated. Only the lipid metabolism pathway was found to be significantly downregulated. The metabolism of complex/polymeric carbohydrates and transcription and metabolism of cofactors and vitamins pathways were significantly upregulated (Fig. 7A). EffectorP 3.0 was used to predict the putative effector genes in *M. phaseolina* using the MS6 genome (Islam et al., 2012; Sperschneider and Dodds, 2022)). Using the default parameters, 1482 effectors were predicted. These included effectors targeted either to the plant cytoplasm or the plant apoplast. Only highly significant effectors were further analyzed after a stringent threshold for EffectorP 3.0 parameters

and a log fold change of 2 (Supplementary File 1-RNA-seq dataset 1). Ten putative effectors were observed, of which seven were cytoplasmic, while three were apoplastic (Fig. 7B). All the cytoplasmic effectors had a similar upregulated expression trend in Parthenium compared to ICC-4958, while these genes were significantly downregulated in the susceptible genotype. A broadly similar trend was observed in the case of apoplastic effectors. Only one putative effector gene had an opposite trend, being significantly upregulated in the susceptible genotype (Fig. 7B). Further, Pattern Hit Initiated (PHI) Basic Local Alignment Search Tool (BLAST) was used to predict the fungal proteins that putatively interact with certain plant proteins (Fig. 7C). A similar trend of expression was observed with both upregulated and downregulated genes in the fungus associated with Parthenium. Expression patterns were similar between Parthenium and ICC-4958 but generally had an opposite trend in JG-62 (Fig. 7C). Analysis of certain pathways possibly involved in the offensive and nutrient acquisition strategies employed by the fungus also had similar trends. Four major pathways—biodegradation of xenobiotics, biosynthesis of secondary metabolites, hydrolysis of carbohydrates and mannose, and fructose metabolism—showed similar expression patterns as observed previously (Fig. 7D, E). However, gene expression patterns of one or more gene clusters in the biodegradation of xenobiotics and the mannose and fructose metabolism pathways differed between Parthenium and ICC-4958, showing an opposite trend to what was previously observed (Fig. 7D, E). Further, in all but one such cluster, genes were significantly upregulated compared to ICC-4958. Global expression pattern and enrichment analysis was compared between ICC-4958 and JG-62 and is represented in Supplementary Figure 9 and orthology analysis is presented in Supplementary Figure 10.

To attain a broader view of the mechanistic aspects of NHR to *M. phaseolina*, we analyzed the global transcriptomic profile of Parthenium (Fig. 8). Comparisons for the global analysis were first done with each treatment's respective control. DEG analysis revealed that Parthenium had 1958 and 2294 significant DEGs at 2 and 4 DAI, respectively, under pathogen stress. At 2 DAI, the numbers of upregulated and downregulated DEGs were 929 and 1029, respectively, while at 4 DAI, the numbers were 1854 and 4,41, respectively (Fig. 8A). While comparing the two time points, we found that 363 genes were commonly upregulated genes at both time points, while only 23 common downregulated genes were found (Fig. 8B). Enrichment analysis done via gene ontology (GO) revealed that downregulation was more prominent at 2 DAI (Fig. 8C, D). Few pathways such as transcription and its regulation, response to other organisms, and multicellular processes were upregulated at 2 DAI, while cellular processes and secondary metabolite processes were significantly downregulated. At 4 DAI, however, the upregulation of genes was equally prominent (Fig. 8E, F). Pathways involved in signal transduction, defense response, and protein modification processes were significantly upregulated (Fig. 8E). Few gene clusters in these pathways were significantly upregulated at 4 DAI. A slight upregulation in the major secondary metabolite pathway genes was observed; however, very few genes were observed (Fig. 9A, Supplementary File 1, RNA-seq dataset 3). It appears that Parthenium indeed activates an active immune response against *M. phaseolina* attack, highlighting a potential role of induced defenses in NHR in

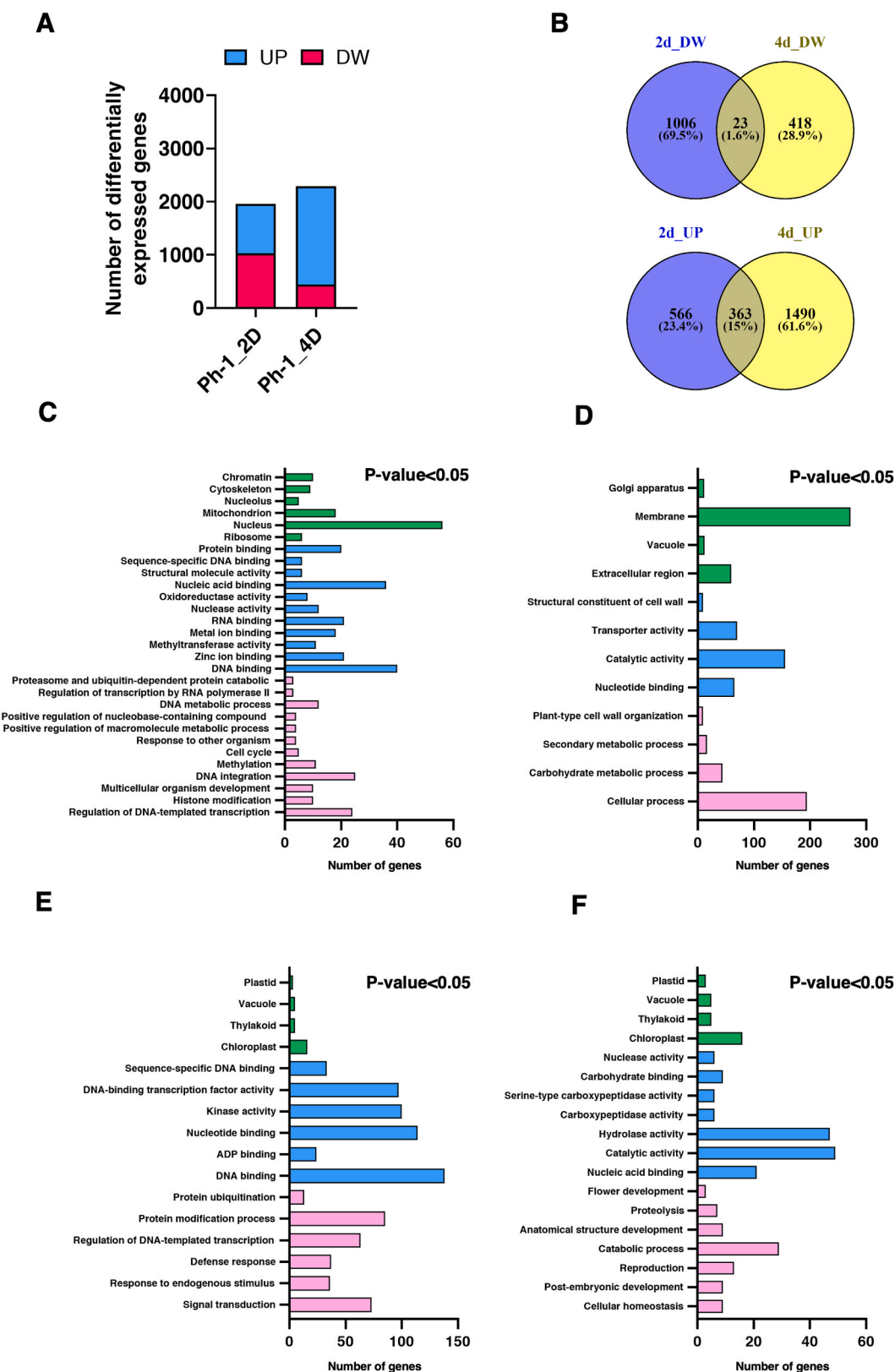
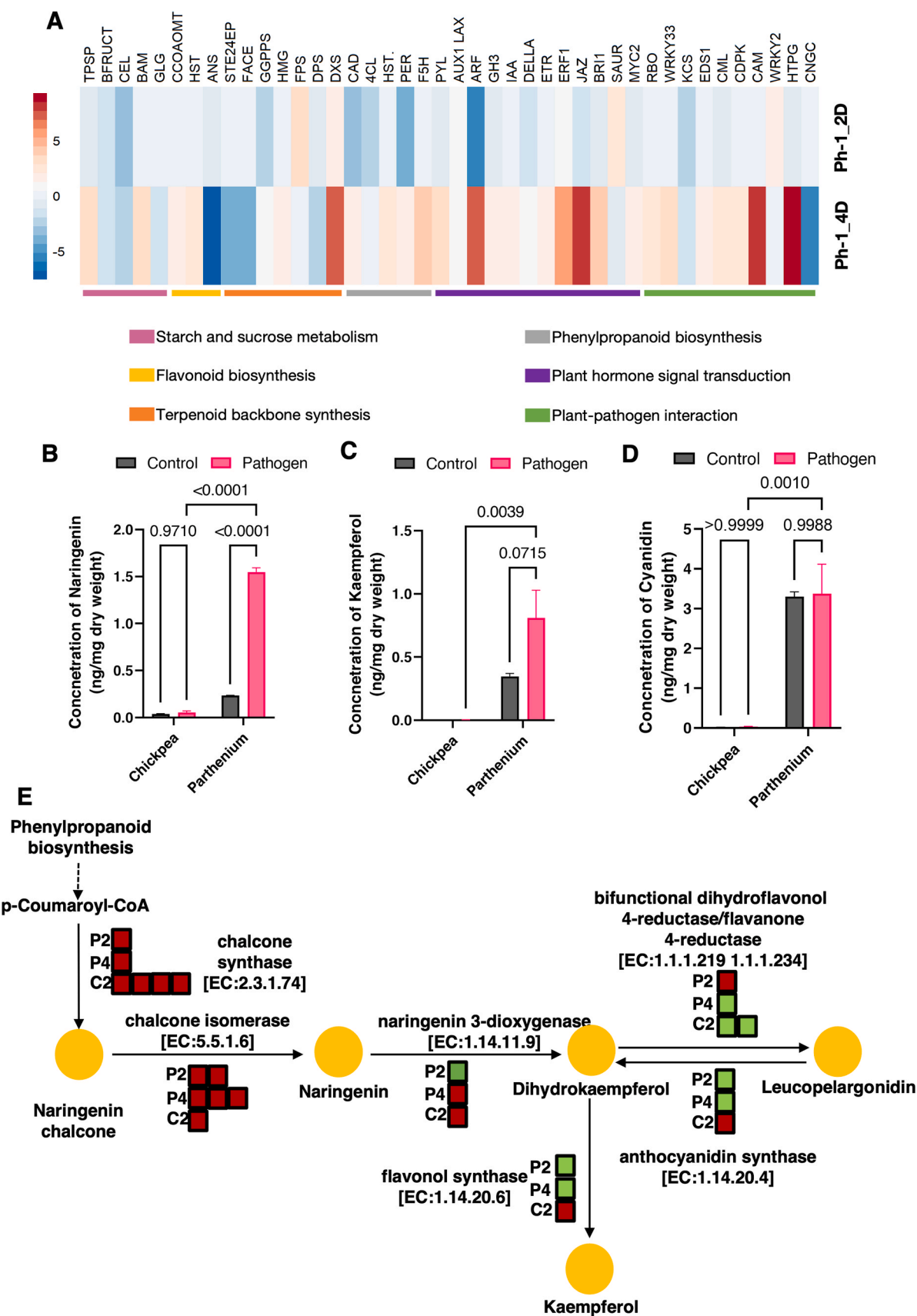


Fig. 8. – Global gene expression analysis of Parthenium root tissue after inoculation with *M. phaseolina*. Blotter paper assay was used to conduct the experiment. Samples were collected at 2 and 4 DAI. **A)** Bar chart represents the number of significantly differentially expressed genes (DEGs) at the two time points. Red bar represents the upregulated genes while the blue bar represents the downregulated genes. **B)** Venn diagram represents the number of unique or common DEGs across the two time points. Labels in red represent upregulated genes while labels in blue represent downregulated genes. Bar charts illustrate the GO enriched pathways at 2 DAI that are **C)** upregulated, and **D)** downregulated, and at 4 DAI that are **E)** upregulated, and **F)** downregulated. Pink bars represent cellular processes, blue represent molecular functions and the green bars represent the cellular component. *2d_UP* – Genes upregulated at 2 DAI, *4d_UP* – Genes upregulated at 4 DAI, *2d_DW* – Genes downregulated at 2 DAI, *4d_DW* – Genes downregulated at 4 DAI. *Ph-1_2d* – Parthenium at 2 DAI, *Ph-1_4d* – Parthenium at 4 DAI. DAI – days after inoculation.



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Fig. 9. – Differential expression of enriched pathways and putative regulation and biosynthesis of naringenin and kaempferol in response to *M. phaseolina*. Blotter paper assay was used to conduct the experiment. Samples were collected at 2 and 4 DAI. Certain pathways with putative role in defence are represented here. Only pathways having significant GO enrichment were selected. Differential expression was calculated using DESeq2. For flavonoid estimation, plant samples were collected from the blotter paper assay at 2 DAI. 80 % methanol extract was used for flavonoid estimation. Flavonoid estimation was done via LC/MS using the UHPLC-MS/MS (QTRAP 6500) system. Standards of 13 known flavonoids were used for estimation. Only 9 flavonoid species had a detectable signal in at least one treatment (Supplementary Table 10), among which the absolute quantification of three species is depicted in the figure. A) Heat map depicting the log fold change values of the significantly enriched pathways in response to *M. phaseolina*. Heat map was generated based on the log fold change values respective to each time point's uninoculated controls. Bars next to the heat map are colour coded according to the GO pathways. TRINITY IDs and respective results from BLASTx are available in Supplementary File 1 (RNA-seq dataset 3). *Ph-1_2d* – *Parthenium* at 2 DAI, *Ph-1_4d* – *Parthenium* at 4 DAI. DAI – days after inoculation. B-D) Absolute quantification of flavonoids (Naringenin, Kaempferol and Petunidin) in root samples of chickpea and *Parthenium* inoculated with *M. phaseolina*. B) Naringenin, C) Kaempferol, D) Cyanidin, E) The chart depicts a brief part of the flavonoid biosynthesis pathway in plants focusing on naringenin and Kaempferol production. The name of the enzyme along with its code is mentioned at each step. The relative fold change of each treatment is depicted as heatmaps next to each step. Fold change represented is relative to each treatment's respective uninoculated control. Red box represents upregulation while green box represents downregulation. *M. Experiments were repeated at least twice with similar results observed. n = 3, Error bars on the graphs represent standard error of mean. Significance of differences was calculated using two-way ANOVA. P-values were computed using Holm-Sidak post hoc test for multiple comparisons test after two-way ANOVA. P-values are indicated on the graphs between variables which were compared.*

Parthenium to the DRR pathogen.

3.6. Flavonoids play a putative role in NHR

To identify the potential roles of flavonoids in NHR in *Parthenium*, targeted flavonoid estimation was conducted using LC/MS/MS. Only naringenin was significantly upregulated at 2 DAI in *Parthenium* (Fig. 9B, Supplementary Figure 11), while kaempferol and cyanidin, although not significantly upregulated, were significantly higher in concentrations relative to the host roots in both uninoculated and inoculated conditions (Fig. 9C, D). The genes involved in the flavonoid pathway, particularly those involved regulating the levels of naringenin and kaempferol were identified from the transcriptome. (Fig. 9E). The putative role of naringenin was tested by conducting antifungal assays on *M. phaseolina* using varying naringenin concentrations. Naringenin displayed significant fungal inhibition at concentrations of 50 and 100 µg/mL. Furthermore, naringenin treated fungus had significantly lower virulence than the mock when inoculated on chickpea roots (Fig. 10).

Further, the untargeted analysis revealed that terpenoids and fatty acids were the most prominent group of compounds identified in the ethyl acetate extracts from GC/MS. The terpenoid group was more extensive in pathogen treated *Parthenium* roots. (Supplementary Figure 12).

4. Discussion

M. phaseolina has a broad host range as it uses cell wall-degrading enzymes and toxins to kill host cells (Marquez et al., 2021). Based on our pipeline, we identified 73 potential nonhosts. However, a majority of these probably have not encountered the fungus in natural conditions, likely due to their geographical distribution (Rai et al., 2022). Thus, we shortlisted 12 species based on the greater likelihood of these species having encountered *M. phaseolina* in chickpea fields (Supplementary Figure 1; Chilakala et al., 2022). The 12 species shortlisted were either cultivated with chickpea or were found as weeds in chickpea fields, i.e., species that were more likely to have encountered chickpea-infecting *M. phaseolina* (Rai et al., 2022). We subsequently conducted detailed morpho-pathological and molecular studies on *Parthenium*.

Major mycelial penetration of the host roots was found to begin beyond 3 DAI. However, in the nonhost roots, it was delayed even further. Scanning electron microscopy results indicated a lowered frequency of microsclerotial attachment and mycelial penetration on the root surface of *Parthenium*. This indicated possible pre-invasive NHR. However, when we increased the fungal load, a few epidermal cells were breached by the fungus (Fig. 4). However, *M. phaseolina* was still unable to colonize the cortical cells even at later time points. Defenses at the cortical layer comprise post-invasive defenses and are most likely induced. Further, assays under combined stress conditions indicated

that NHR is durable under drought as well, highlighting that the possible mechanism of NHR is independent of the pathways leading to drought-induced susceptibility or that drought and pathogen combined stress do not affect *Parthenium* (Irulappan et al., 2022; Pandey et al., 2015; Ramegowda and Senthil-Kumar, 2015).

The dual RNA-seq approach to study the molecular aspects of NHR indicated an active response from *Parthenium*. The expression patterns indicated that even though *M. phaseolina* is not specifically adapted to *Parthenium*, the fungal pathogen uses its active offensive arsenal in an attempt to infect the nonhost species. These results also imply that the fungus utilizes a specific set of genes for virulence when encountering resistant and susceptible plants. Further, based on certain gene clusters, the nonhost plant *Parthenium* can lead to an amplified response from *M. phaseolina*, even relative to moderately resistant host genotypes. The fungal transcriptome also indicated that *M. phaseolina* sought to infect *Parthenium*. Fungal effector and plant-interacting partner prediction revealed a similar expression pattern between *Parthenium* and ICC-4958. Thus, it appears that *M. phaseolina* likely uses a similar effector repertoire in the host as well as the nonhost. This also points to a putative role of ETI in NHR to *M. phaseolina*. However, ETI is generally considered to have a less significant role in NHR, especially in divergent species (Lee et al., 2017; Schulze-Lefert and Panstruga, 2011). As with the expression patterns of effectors, several other pathways in the fungus have similar expression patterns between ICC-4958 and *Parthenium*. However, interestingly, the xenobiotics biodegradation pathway had a cluster of genes that were specifically expressed while infecting *Parthenium* at the early but were then downregulated at the late time point. This confirms that the pathogen encounters a specific group of metabolites in *Parthenium*, e.g., cytotoxic, phytotoxic, and antimicrobial compounds (Bajwa et al., 2020), which might make up a part of the constitutive pre-invasive NHR. A DEG cluster was also observed in sugar metabolism pathways, suggesting probable acclimatization towards a likely different sugar composition in *Parthenium* roots. Further, while analyzing the transcriptome of *Parthenium*, we observed significant GO enrichment in defense signaling, terpenoid and phenylpropanoid synthesis, and phytohormone signaling pathways. Most of the genes in these pathways were upregulated at the late time point, indicating a putative role in the post-invasive stage of NHR (Supplementary Figure 13). Terpenoid and phenylpropanoid synthesis pathway upregulation indicated that an induced metabolite response also contributes to NHR. Considering these findings together, we hypothesize that secondary metabolite-based defense in *Parthenium* is a major contributor to NHR and is likely involved in both pre-invasive and post-invasive defense. Further, general defense and phytohormone signaling pathways contribute to NHR responses as well. General defense and the phytohormone signaling pathways have also been associated with host responses to *M. phaseolina*. In chickpea, ethylene and jasmonate responsive transcription factors were upregulated in response to the pathogen under combined stress. However, contrary to *Parthenium*,

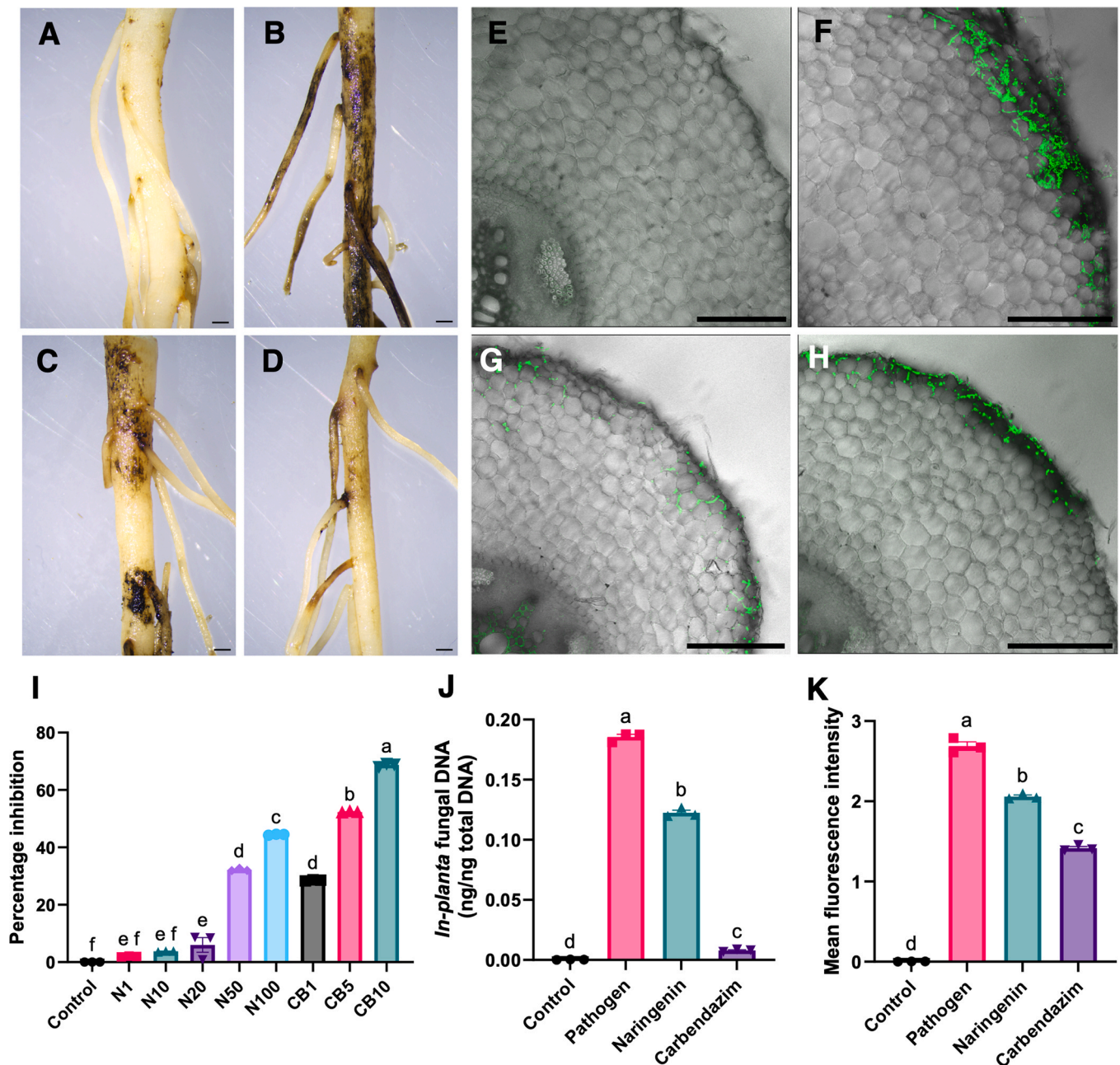


Fig. 10. – Efficacy of naringenin as an antifungal compound against *M. phaseolina*. *M. phaseolina* was treated with varying concentrations of naringenin (1–100 $\mu\text{g/mL}$). The optical density at 450 nm was observed at 24 h post treatment and the percentage inhibition was calculated. Carbendazim was used as a positive control. The treated fungus was used as the inoculum for the blotter paper assay. The stereomicroscopic, confocal microscopic observations and in planta fungal DNA quantification was done at 4 DAI. **A–D)** Stereomicroscopic observations of the necrotic lesions on the roots of chickpea under **A)** Uninoculated, **B)** Untreated pathogen, **C)** Naringenin treated fungus and **D)** Carbendazim treated fungus treatments. **E–H)** Confocal microscopic observations of the cortical colonization in the roots of chickpea under **E)** Uninoculated, **F)** Untreated pathogen, **G)** Naringenin treated fungus and **H)** Carbendazim treated fungus treatments. **I)** Percentage inhibition of *M. phaseolina* at different concentrations of naringenin and carbendazim. **K)** In plant fungal DNA quantification of naringenin and carbendazim treated fungal inoculated chickpea roots at 4 DAI. **L)** Mean fluorescence quantification of the rutin and carbendazim treated fungus in chickpea roots at 4 DAI. $n = 3$, similar results were obtained from two independent experiments. *P*-values were computed using Holm-Sidak post hoc test for multiple comparisons test after one-way ANOVA. Error bars represent standard error of mean. N1 to N100 – Naringenin concentrations from 1–100 $\mu\text{g/mL}$ and CB1 to CB10 – Carbendazim concentrations from 1 to 10 $\mu\text{g/mL}$.

specialized metabolomic and phytohormone signaling processes were downregulated in response to *M. phaseolina* (Irulappan et al., 2022). Additionally, ROS-related genes were also upregulated in pathogen alone and but were downregulated under drought (Irulappan et al., 2022). Further, in soybean, receptor-like kinases are possibly involved in defense responses against *M. phaseolina* (Noor and Little, 2024). A study on transgenic soybean with altered isoflavone synthesis showed impaired glyceollin accumulation and subsequently, increased disease

symptoms in soybean (Lygin et al., 2013). NHR responses in plants to necrotrophic fungi have not been extensively studied. Further, to our knowledge, no report of NHR to necrotrophic fungi in roots is available. Tryptophan-derived compounds produced by a P450 monooxygenase and PEN2 myrosinase are likely involved in NHR to necrotrophic fungi in *Arabidopsis* leaves (Sanchez-Vallet et al., 2010). Further, Azevedo et al., (2008) showed that programmed cell death and NHR are induced following an oxidative burst in pine cell suspensions in response to

Botrytis cinerea.

The role of sesquiterpene lactones (SQLs; a modified group of sesquiterpenoids) in stress tolerance is well studied in *Parthenium* (Bajwa et al., 2020; Das and Das, 1995; de la Fuente et al., 2000). Our RNA-seq data highlighted a few flavonoid pathway genes in response to *M. phaseolina*. However, the role of flavonoids is relatively less explored, particularly in NHR (Iqbal et al., 2022; Shen et al., 1976). Thus, we conducted LC/MS analysis for flavonoid estimation in the root samples of *Parthenium* and chickpea (Fig. 9, Supplementary File 1-Metabolite dataset 1, Supplementary Figure 11). Naringenin was significantly upregulated in response to the pathogen, possibly due to the down-regulation of its downstream pathway. Other flavonoids, albeit not significantly upregulated, had much higher concentrations in *Parthenium* relative to chickpea (Supplementary Figure 11). Naringenin treated *M. phaseolina* had altered virulence even on the host roots with recoded necrosis and in planta colonization. This indicates a putative role of naringenin in induced NHR defenses in *Parthenium*.

In conclusion, pre-invasive NHR in *Parthenium* is likely due to cell-wall composition and metabolites, while phytohormone, oxidation, and secondary metabolite pathways likely contribute to post-invasive NHR (Supplementary Figure 13). Previously, abiotic stresses in *Parthenium* have also been studied (Gul et al., 2024). We present a transcriptomic resource for *P. hysterophorus* L. under *M. phaseolina* stress. We also demonstrate *P. hysterophorus* as an alternate source of resistance to *M. phaseolina* that is robust even under combined stress. This may be exploited to confer resistance to host species and possibly alleviate losses caused by DRR. Global molecular aspects presented in this study can be utilized to attain a deeper understanding of the intricate molecular mechanisms contributing to NHR.

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CRediT authorship contribution statement

Senjuti Sinharoy: Supervision, Resources. **Muthappa Senthil-Kumar:** Writing – review & editing, Supervision, Resources, Funding acquisition, Conceptualization. **Manu Kandpal:** Visualization, Data curation. **Aashish Ranjan:** Supervision. **Rishabh Mirchandani:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation.

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.

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Author-recommended internet resources

<https://nt.ars-grin.gov/fungaldatabases/>
<https://www.fao.org/faostat/en/>
<https://effectorp.csiro.au/>

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envexpbot.2025.106197.

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