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PROTOCOL NOTE

Applications in Plant Sciences

A sick plot–based protocol for dry root rot disease assessment in field-grown chickpea plants

Vadivelmurugan Irulappan[1] | Komal Vitthalrao Mali[1] | Basavanagouda S. Patil[2] | Hanumappa Manjunatha[3] | Saifulla Muhammad[3] | Muthappa Senthil-Kumar[1]

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

[2]ICAR-IARI Regional Research Center, P. B. Road, Dharwad 580001, India

[3]Department of Plant Pathology, College of Agriculture, University of Agricultural Sciences, GKVK, Bengaluru 560065, India

Correspondence

Muthappa Senthil-Kumar, National Institute of Plant Genome Research, Aruna Asaf Ali Marg, P.O. Box No. 10531, New Delhi 110067, India.
Email: skmuthappa@nipgr.ac.in

Present address

Hanumappa Manjunatha, Department of Plant Pathology, College of Agriculture, Karekere, Hassan 573 225, India.

Abstract

Premise: A comprehensive field-based screening protocol is lacking for dry root rot (DRR) disease in chickpea, which is caused by *Macrophomina phaseolina* (formerly referred to as *Rhizoctonia bataticola*). Here, we describe a protocol for establishing a sick plot for DRR to enable disease assessment of a large number of chickpea plants during the natural growing season.

Methods and Results: We used a chickpea plot with >30% DRR incidence, and enriched the inoculum by cultivating highly susceptible chickpea plant genotypes and incorporating infected plant material into the soil. The chickpea plants were then subjected to infection in developed sick plots with various levels of soil moisture under natural field conditions.

Conclusions: Our protocol provides a robust way to impose *M. phaseolina* infection on chickpea plants under natural field conditions and to investigate plant responses to the infection at morphological, physiological, and molecular levels. This method can also be used to screen for other soil-borne diseases in a variety of plants.

KEYWORDS

Cicer arietinum, combined stress, dry root rot, *Macrophomina phaseolina*, *Rhizoctonia bataticola*, sick plot technique

Macrophomina phaseolina (Tassi) Goid (formerly referred to as *Rhizoctonia bataticola*) is a soil-borne necrotrophic fungal pathogen (asexual form) that causes dry root rot (DRR) disease in chickpea (*Cicer arietinum* L.). Similarly, *M. phaseolina* strains cause foliage blight in cowpea (*Vigna unguiculata* (L.) Walp.), root rot in mung bean (*Vigna radiata* (L.) R. Wilczek), and charcoal rot in corn (*Zea mays* L.) and soybean (*Glycine max* (L.) Merr.) (Gupta et al., 2012a; Gupta and Sharma, 2015; Sharma et al., 2015). Microsclerotia and hyphae are the forms of inoculum in the soil. The mycelia infect the roots of the plant, followed by penetration through the epidermal cells ultimately causing necrosis (Singh and Mehrotra, 1982; Sharma et al., 2015). The taproot is blackened, with signs of rotting and loss of lateral roots. The roots become brittle, and conspicuous, minute, dark microsclerotia can be observed on the inside of the bark of split roots (Sharma et al., 2015; Sinha et al., 2019; Khaliq et al., 2020). Under field conditions, disease symptoms appear during the crop's reproductive phase as straw-colored leaves and petioles and, finally, dried foliage.

DRR disease occurrence has been reported in all chickpea-growing areas of the United States, Spain, India, Egypt, Ethiopia, Mexico, Lebanon, and other South Asian countries (Beniwal et al., 1992; Khaliq et al., 2020), with yield losses up to 90% globally (Sharma et al., 2015; Sinha et al., 2019). Disease management by crop rotation with non-host crops, removal of infected plants, application of fungicides, seed inoculation with *Rhizobium* sp., and optimum irrigation are required to overcome the high yield losses caused by DRR. Screening methods are indispensable to identify and evaluate DRR-resistant germplasm lines as a part of resistance breeding programs. At this time, planting resistant varieties has been found to be the best sustainable solution for managing DRR (Rao and Haware, 1987). Disease phenotyping data constitute an indispensable resource for the breeding of DRR-resistant

varieties. Screening using the blotting paper and sick pot techniques is routinely used for laboratory studies (Nene et al., 1981; Halila and Strange, 1997; Gangwar et al., 2002; Pande et al., 2006; Pandey et al., 2020; Irulappan and Senthil-Kumar, 2021), and a few resistant ICRISAT (International Crops Research Institute for the Semi-Arid Tropics) crop chickpea (ICC) lines (e.g., 1710, 2242, 2277, 11764, 12328, and 13441) have been identified using these two techniques (Nene et al., 1981; Gangwar et al., 2002; Pande et al., 2006; Gupta et al., 2012b; Sharma et al., 2015). The blotting paper technique involves inoculation of the fungal inoculum on chickpea seedlings and assaying the disease progression on blotting paper, whereas the sick pot technique involves growing the chickpea plants in soil or potting medium that has been inoculated with the fungus (Pande et al., 2012; Irulappan and Senthil-Kumar, 2021). Both techniques are executed under controlled conditions. However, the field-based screening method involving sick plots (i.e., fields with fungal inoculum capable of causing up to 100% disease incidence in susceptible chickpea plant genotypes) is the most widely used and best-suited method for disease phenotyping for breeding programs (Nene et al., 1981; Pande et al., 2006). The sick plot method is also normally used to evaluate varieties before their release for cultivation.

Sick plot–based screening of chickpea genotypes for DRR has been reported in several studies (Gupta et al., 2012b; Pande et al., 2012; Reddy et al., 2016; Manjunatha and Saifulla, 2018; Sinha et al., 2019, 2021). However, the detailed steps involved in sick plot development, disease assessment, assays for trait-based parameter scoring, and the procedure for extensive screening of breeding lines for DRR resistance in chickpea have not been reported in the literature. Hence, there is an immediate need for a comprehensive field-based screening protocol. Similar to sick plot–based screening for DRR, field-based screening methods have been successfully used to test resistance against various other root-infecting pathogens such as *Aphanomyces euteiches*, which causes root rot in pea (*Pisum sativum* L.) (Infantino et al., 2006); *Rhizoctonia solani* Kühn (sexual stage: *Thanatephorus cucumeris* (Frank) Donk), which causes wet root rot in chickpea (Infantino et al., 2006); *Fusarium oxysporum* f. sp. *ciceri*, which causes wilt in chickpea (Halila and Strange, 1997); and *Fusarium* spp., which causes wilt in castor (*Ricinus communis* L.) (Shaw et al., 2016).

DRR incidence and disease development are further aggravated by drought stress (Sharma and Pande, 2013; Sinha et al., 2019, 2021). This suggests that DRR screening should be performed under moderate drought stress conditions in order to identify genotypes suitable for cultivation in field conditions, where combined stresses are common. The sick plot–based method described here is ideal for growing plants under relatively low soil moisture conditions. We report a systematic field-based methodology for sick plot development for large-scale germplasm screening and validation of laboratory-based results under field conditions. Sick plot–based comprehensive field screening includes assessing the impact of environmental conditions on plants, including changing climatic factors, diverse soil microorganisms, soil environment, the form and concentration of the inoculum, and the physiological growth stage of the plant (Ghosh et al., 2017). Here, we present a detailed description of sick plot development and assessment, and present an experimental plan for testing chickpea varieties and screening a large number of genotypes. We also describe the assays related to the morphological and agronomic traits associated with plant resistance.

METHODS AND RESULTS

Identification of the location for sick plot development

We first reviewed research stations located in DRR disease hotspot regions to identify a suitable location for developing the sick plot. The methodologies and results from previously developed sick plots or natural hotspots (Sinha et al., 2019) are detailed below. As a first step, hotspots with 20–30% DRR incidence reported due to the naturally occurring pathogen were identified by measuring disease incidence, followed by morphological confirmation of the fungus isolated from an affected plant root and subsequent confirmation of the fungal ITS sequence by PCR. Pathogen identity (National Center for Biotechnology Information [NCBI] nucleotide sequence MH509971.1) was confirmed in the selected field (250 m^2), and the field plot was earmarked (Figures 1, 2A, 2B; Appendices S1, S2). In this study, we developed or assessed three locations: Location 1 (naturally present inoculum): National Institute of Plant Genome Research, New Delhi, 28.5307°N, 77.1665°E; Location 2 (sick plot): University of Agricultural Science, Bengaluru, 13.0784°N, 77.5793°E; Location 3 (naturally infected hotspot): Indian Council of Agricultural Research–Indian Agricultural Research Institute (ICAR-IARI), regional station at Dharwad, 15.4889°N, 74.9813°E.

The survival of the DRR pathogen and its ability to infect plants is influenced by edaphic factors such as soil type, pH, matric potential, and macronutrient content (Jordan et al., 1984; Sinha et al., 2021). In this regard, the soil's physical properties, such as organic carbon (0.66%), pH (7.31), electrical conductivity (0.29 μS/m), soil type (sandy clay), and water-holding capacity (40.7), as well as its chemical properties, namely, nitrogen (125 kg/ha), phosphorus (39.8 kg/ha), and potassium (282 kg/ha), were analyzed in the designated plots. The physical properties of the soil were similar in the sick plot and the control plot (Appendix S3) (Sinha et al., 2019). In the control plot, the chickpea crop had well-established roots with active root nodules (Appendix S4A, C), whereas in the sick plot DRR disease symptoms (Appendix S4B, D) were evident during the flowering or pod-forming stage, as previously described (Nene et al., 1981; Sharma et al., 2015; Sinha et al., 2019).

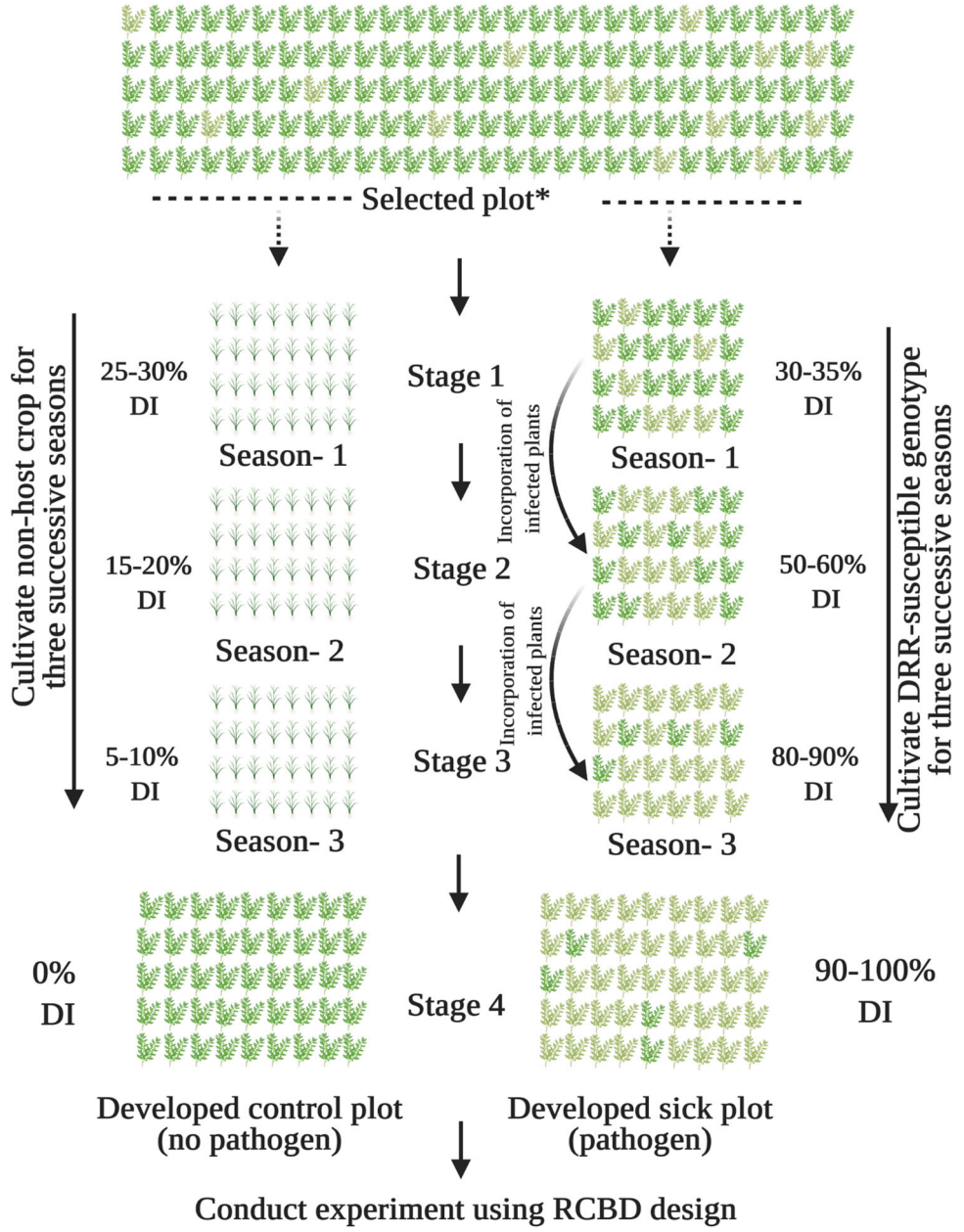

FIGURE 1 Pictorial representation of the steps to develop the sick plot. The steps are as follows: select the chickpea-growing region and test for soil characteristics. Identify a field area with >30% dry root rot (DRR) disease incidence and one with <30% disease incidence (DI). Mark the fields as sick and control, respectively, and quantify the inoculum by plant mortality rate. Sick and control plots (no target pathogen) can be developed in four stages. For control plots, sow non-host crops for three consecutive crop seasons. To develop the sick plot, enrich the inoculum by adding infected plant roots from other fields followed by sowing of highly DRR-susceptible genotypes for three successive crop seasons. After 80–90% plant mortality is ensured in the sick plot, it can be used for screening and for experiments such as imposing drought. *Test the physical and chemical properties of the soil in the designated control and sick plots. Note: Green-colored plants in the illustration represent healthy chickpea plants, and pale-yellow-colored plants indicate chickpea plants with DRR disease symptoms. RCBD = randomized complete block design

Assessment and confirmation of DRR disease and its causal pathogen

Plants exhibiting DRR disease symptoms were examined for root symptoms such as necrotic spots and brittle primary roots devoid of lateral roots and for foliar symptoms such as straw-colored leaves. DRR-infected plants first showed necrotic symptoms in the roots, before exhibiting foliar symptoms (Appendix S4B, D). Thus, any DRR-symptomatic plants, including those with only infected roots, were counted as infected plants. Plants were uprooted and examined at the reproductive stage, and the percentage of disease incidence was calculated using the following formula:

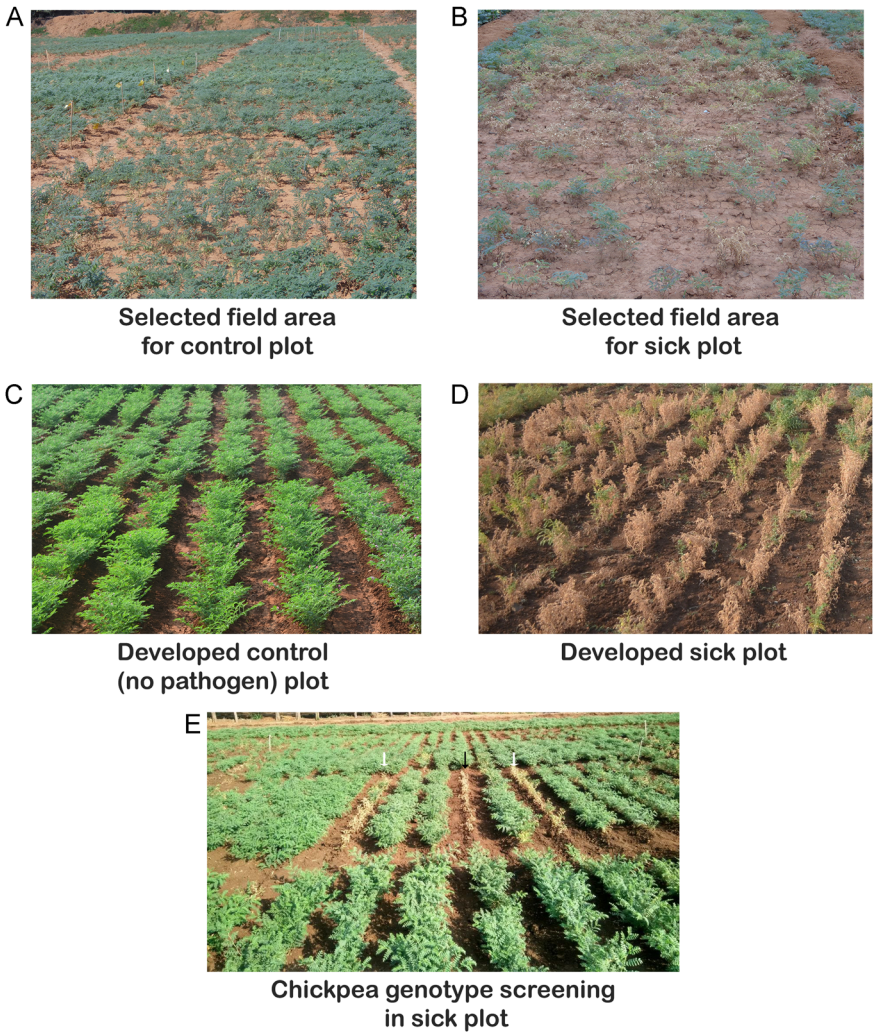

FIGURE 2 Photos of representative control and sick plots for testing dry root rot in chickpea. (A, B) A chickpea-growing region with optimum soil physical and chemical characteristics was identified. A chickpea field with <30% disease incidence was earmarked as the control (A), whereas fields with >30% disease incidence were earmarked as sick plots (B). (C, D) The control (C) and sick plots (D) (>80–90% disease incidence, stage 4) were developed by sowing appropriate genotypes, with a susceptible variety sown every 10 rows. (E) Genotype screening with BG 212 seeds sown every 10th row. White arrows indicate the diseased plants and black arrows indicate the susceptible check variety

$$\text{Disease incidence}(\%) = \frac{\text{Number of infected plants}}{\text{Total number of plants}} \times 100$$

In addition to disease incidence, the disease index was also measured at around 80 days after sowing, following a scoring system (Appendix 1) with scores from 0 to 5 (Appendix S5A), where 0 indicates resistance and 5 indicates the highest susceptibility. At this time, the number of growing degree days (GDD, calculated as the total number of days and the average minimum and maximum temperatures on these days) was approximately 1104.3. The DRR-susceptible genotypes (e.g., BG 212) had a high disease incidence, with a disease score of 4–5 (Appendix 1, Appendix S5B). In contrast, the resistant genotype ICC 4958 had a lower disease incidence, with diseased plants only exhibiting root symptoms (disease score of 0–1).

DRR-affected plants were confirmed by studying the disease phenotype and pathogen morphological characteristics using an SMZ25 Research Stereomicroscope (Nikon Instruments Inc., Melville, New York, USA), as well as at the molecular level using pathogen-specific ITS primers (Appendix S4G). In the DRR-infected fields, symptomatic plants (i.e., with straw-colored leaves) and prematurely dead plants (Appendix S4B) were scattered throughout the field, and some green plants also present. The diseased plants were further examined for necrosis in the roots and microsclerotia. The DRR-affected plants showed brittle primary taproots with dark microsclerotia appearing as dark granules (Appendix S4D). These plants lacked lateral roots, and most of the roots remained in the soil upon uprooting. The presence of the causal agent of DRR was confirmed by observing the pigmentation pattern of microsclerotia in a potato dextrose agar (PDA) plate from one to five days after incubation (Appendix S4E). Microscopic observation confirmed microsclerotia with dark pigmentation and a diameter of ~60 μm, as well as cross wall formation at the beginning of the branching (Sinha et al., 2019). *Macrophomina phaseolina* (asexual form in chickpea; see Appendix 2 for details on taxonomic classification) possesses the typical characteristics of right-angle branching, myceliogenic germination (Coley-Smith and Cooke, 1971; Marquez et al., 2021), and multinucleate hyphal cells. It should be noted that some of these characteristics are specific to the fungus in chickpea.

The genomic DNA of the fungus was isolated using the DNAzol method (Thermo Fisher Scientific, Carlsbad,

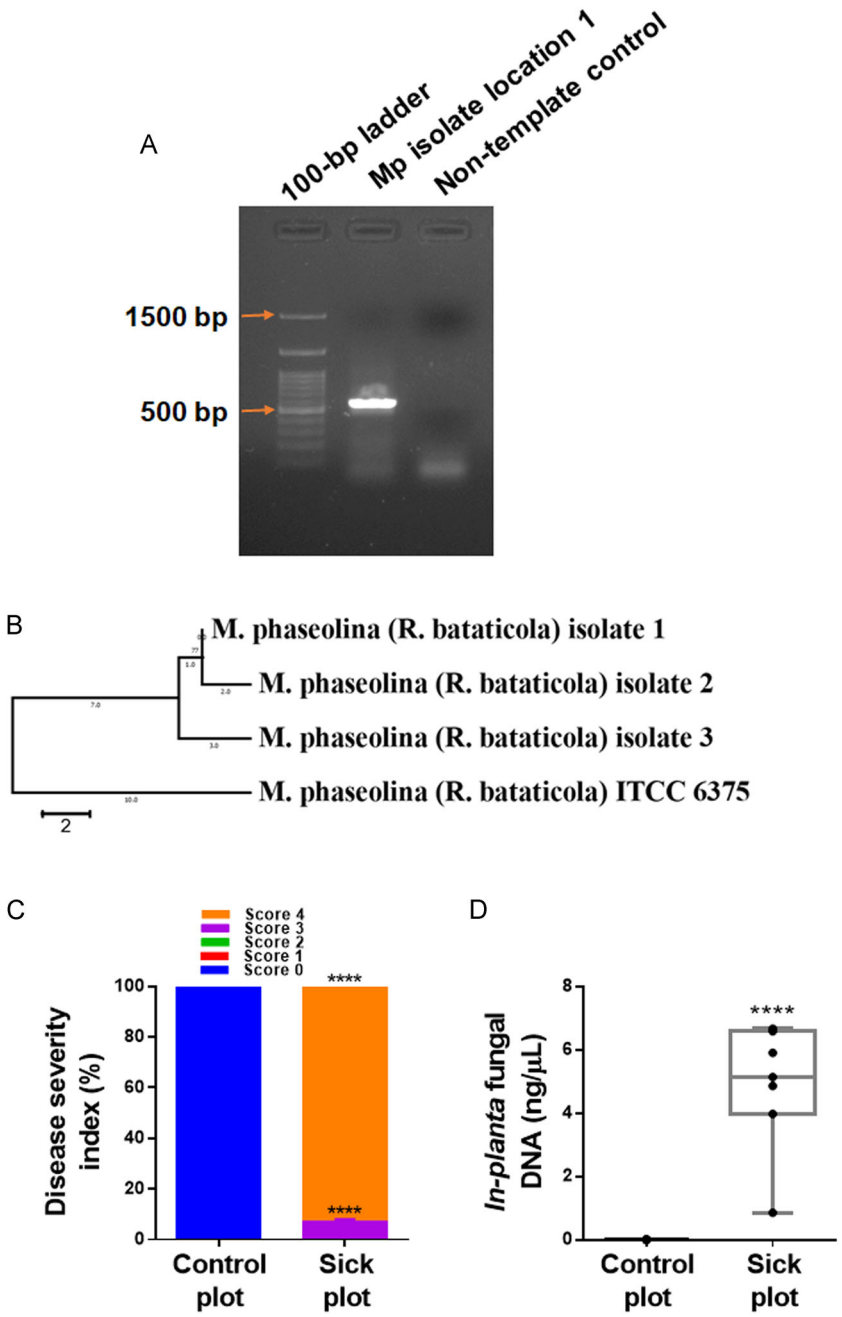

FIGURE 3 Pathogen confirmation and measurement of disease incidence and yield. (A) A representative PCR gel of one *Macrophomina phaseolina* isolate. Genomic DNA of the DRR disease-causing pathogen was isolated from the sick plot, and PCR was used for pathogen confirmation. Universal ITS primers were used to amplify the rDNA-ITS sequence. (B) Phylogenetic tree showing the evolutionary distance among *M. phaseolina* (chickpea strain/asexual stage) strains collected across selected chickpea-growing fields in India based on the ITS sequence. (C) Comparison of the disease severity index between the control and sick plots. (D) Comparison of the in planta pathogen load quantified from DNA (10 ng/µL) of an infected plant from the sick plot. The comparison was performed using qPCR, and a two-way analysis of variance (ANOVA)/unpaired *t*-test was used to analyze the data. The asterisk indicates the significance level at $P < 0.0001$. Error bars indicate the standard error of the mean

California, USA; Sinha et al., 2019) and sequenced. Basic Local Alignment Search Tool (BLAST) analysis of PCR products amplified using universal ITS primers targeting rDNA (White et al., 1990; Gardes and Bruns, 1993) confirmed its molecular identity (Figure 3A, B). The results showed 99% similarity to the existing *M. phaseolina* ITS sequences. The field isolate's nucleotide sequence was submitted to NCBI (MH509971.1).

Quantification of pathogen inoculum load in the soil and enrichment of pathogen inoculum in the sick plot

Inoculum load can be assessed by quantifying the pathogen levels or by testing the extent of the disease in a susceptible variety (Nene et al., 1981). In this study, quantitative PCR (qPCR) was used for pathogen quantification. A species-specific primer was designed to distinguish *M. phaseolina* from other fungus present in the root, and the rDNA region of the fungus was PCR amplified (Appendix 1; Sinha et al., 2019) and the PCR product was sequenced. The ITS sequence of the pathogen used for species-specific primer design (using GenScript^{R}; GenScript, Piscataway, New Jersey, USA) had an amplicon size of approximately 150 bp. Fungal genomic DNA from the field-isolated strain was used to quantify the inoculum using qPCR (Figure 3D) with the standard curve method (Appendix S4F). To assess the disease in susceptible genotypes, the DRR-susceptible genotype was sown and the disease incidence was measured. The designated subplots

of the sick plot showed 70–90% DRR disease incidence, whereas the control plots did not show any disease (Figure 3C). In the field, DRR is often observed in patches, presumably because of the uneven distribution of fungal inoculum. Hence, it is necessary to ensure uniform pathogen distribution in sick plots by plowing, distributing topsoil uniformly across treatment subplots, and cultivating highly susceptible varieties.

The pathogen load in the sick plot was enriched over three successive crop seasons (Figure 1; Appendices S1, S2). At the beginning of the first crop season, 30% disease incidence was observed. Over the next three crop seasons, the inoculum was enriched by cultivating DRR-susceptible genotypes and incorporating infected plant material into the soil. At the end of the third crop season, the disease incidence and uniformity in distribution across the plot were assessed, and the results showed 70–90% disease incidence with near-uniform distribution across the plots (Figure 2). Parallel to this, in the control plot, the pathogen load can be reduced by cultivating nonhost plants over consecutive crop seasons. After three crop seasons, the disease incidence in the control plot was found to be near 0% (Figure 1; Appendices S1, S2).

Confirmation of sick plot establishment

The efficiency of the sick plot was assessed by sowing the DRR-susceptible chickpea genotypes BG 212 and JG 62 (Appendix S6). Plants were examined for foliar and root symptoms at the vegetative, flowering, and mature stages. The overall percentage of disease severity was 80–90% (Figure 3C). Furthermore, the pathogen load (quantity of fungal genomic DNA) was 4.8 ± 0.7 ng/10 ng of total DNA in plant roots collected from the sick plot, and 0 ng/10 ng of total DNA in plants collected from the control plot (Appendix 1, Figure 3D).

DRR occurrence under drought stress

DRR disease incidence has been known to increase due to drought stress (Sharma and Pande, 2013; Sinha et al., 2019). In this experiment, drought-only, pathogen-only, and combined drought and pathogen treatments were maintained, along with a no-pathogen control. In addition, seeds for the control and drought stress treatment were treated with fungicides (Bavistin and SAAF [see Appendix 1 for details]). For the control and pathogen stress treatments, full irrigation was provided. For the drought stress treatment and combined stress treatment, irrigation was withheld (Sinha et al., 2019). Plants under the severe drought stress treatment were irrigated every 30 days, whereas the control and pathogen plots were irrigated every 10 days. The soil moisture content was measured using a Lutron soil moisture meter (Lutron Electronic Enterprise Co. Ltd., Taipei, Taiwan); drought-stressed plots had <10% soil moisture content, whereas well-irrigated plots had ~40% soil moisture content. Our study showed that the combined drought and pathogen stress treatment led to ~50% disease incidence, whereas the pathogen-only treatment resulted in ~20% disease incidence (Figure 4A). The pod yield under the combined stress treatment decreased 1.8-fold compared with the pathogen-only treatment (Figure 4B).

Comparing disease resistance among the tested chickpea genotypes

A randomized complete block design (RCBD) was employed to compare disease resistance among the different chickpea genotypes used in this study (Appendix S7) (Clewer and Scarisbrick, 2013; Casler, 2015). The genotypes showing DRR susceptibility and moderate resistance were sown in four rows with 30 cm inter-row distance (per standard cultivation practice), and a fifth row was sown with DRR-susceptible genotypes to establish the uniform inoculum prevalence. Four blocks with subplots were designed as required for the statistical design according to the total area available. The contrast genotypes were sown in each block, but in one subplot only one genotype was sown with four block replicates to decrease the error. Disease incidence, disease index, and yield were measured. In our experiments, ICC 4958, a moderately DRR-resistant variety, showed scores from 0 to 2, whereas the DRR-susceptible genotypes JG 62 and BG 212 showed scores from 3 to 5 (Figures 3C, 4A). These results indicate that the sick plot can be used successfully to screen germplasm lines. For genotype screening, it is recommended that a susceptible genotype be sown between each group of genotypes to confirm the efficiency of the sick plot and the uniformity of the fungal load distribution (Appendices S7, S8).

Statistical analysis

One-way and two-way ANOVA followed by post hoc analysis (i.e., Sidak's multiple comparisons test or uncorrected Fisher's LSD) was performed using GraphPad Prism 6

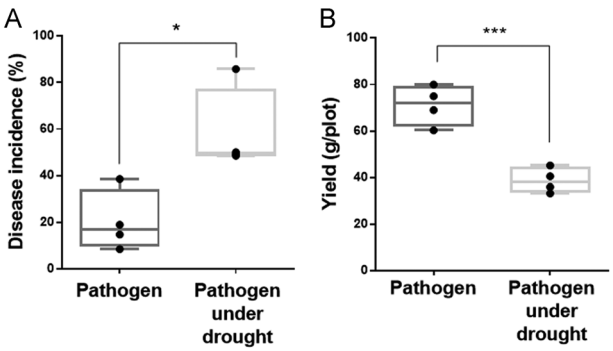

FIGURE 4 Disease incidence and yield of plants (JG 62 genotype) subjected to well-watered and drought stress conditions in the sick plot. (A) Disease incidence under pathogen-only and combined stress conditions. (B) Yield data of plants under pathogen-only and combined stress conditions. An unpaired *t*-test was used to analyze the data. Asterisks indicate statistical significance at $^{*}P < 0.05$ and $^{***}P < 0.001$. Error bars indicate the standard error of the mean

(GraphPad Software, San Diego, California, USA). RCBD analysis was performed using Statistix 10 software (Analytical Software, Tallahassee, Florida, USA; Sinha et al., 2019) with the least significant difference.

CONCLUSIONS

This study describes a straightforward method to develop an effective DRR sick plot, to be used in tandem with naturally infected plots, to investigate the fungal infection and perform high-throughput screening of chickpea germplasm. Isolation of *M. phaseolina* (chickpea-specific strain/asexual stage) and confirmation at the molecular and morphological levels showed that both the sick plots and naturally infected plots had enriched fungal inoculum and typical disease symptoms. Measurement of the pathogen soil inoculum load confirmed a gradual increase over subsequent cropping seasons. Unlike other techniques like sick pot and paper blot, in which the pathogen culture is introduced (i.e., the fungal biomass is propagated on selective substrates under controlled conditions), the method described here uses naturally occurring pathogen. Our results showed that a sick plot with 70–90% disease incidence in a susceptible variety and 2–10% disease incidence in a resistant variety is adequate for carrying out chickpea genotype screening. The strategy used for developing a control plot (i.e., no target pathogen present) by cultivating non-host crop plants is both quick and sustainable, and facilitates the utilization of land during the off-season. Furthermore, our screening protocol encompasses the imposition of moderate drought stress, and the sick plot can also be used to conduct experiments with combined pathogen and mild drought stress treatments (Sharma and Pande, 2013; Sinha et al., 2019). Using this method, we were able to score the disease at three different plant development stages, and found that the reproductive stage had the highest disease severity. In contrast, such stagewise analysis is not feasible on a large scale using the paper blot or sick pot techniques. Similarly, the sick plot technique allows screening in the presence of the natural soil microorganisms and environmental conditions, which is not possible using the paper blot or sick pot techniques (Erskine and Bayaa, 1996). Alteration in the microbial population (i.e., by introducing *Rhizobium* spp.), along with beneficial microbes already present in the soil, might affect the pathogen population, its interaction with host plants, and DRR incidence, and therefore the sick plot screening method can provide practically relevant results. Furthermore, this method enables large-scale screening with robust statistical design and controls, and also allows yield assessment.

The sick plot technique does, however, have a few limitations. First, sick plot results can be compromised by uneven pathogen distribution, soil texture, variation in time of exposure of the roots to infected soil, and the simultaneous occurrence of multiple soil diseases. To overcome this, validation using controlled environment techniques like sick pot and paper blot should be performed. Second, cultivating susceptible cultivars is necessary to maintain the DRR pathogen virulence and distribution across the sick plot. This is not only cumbersome but might also use field space meant for genotype testing. Third, other diseases may also be present in the developed sick plot; therefore, plants should be closely observed for symptoms of diseases caused by other soilborne pathogens and molecular identification should be performed to confirm (Sinha et al., 2019). Fourth, the physical, chemical, and biological properties of both the control and sick plots may be altered during the process of soil fumigation and solarization. However, these factors can be stabilized over time through subsequent soil amendments (Appendix S3).

This methodology can also be used to develop sick plots for other root diseases that affect chickpea cultivation (e.g., *Fusarium* wilt, black root rot, and collar rot), as well as to develop sick plots to study root diseases of other crop species, particularly those belonging to Leguminosae.

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AUTHOR CONTRIBUTIONS

M.S.-K. conceived the study. M.S.-K. and V.I. designed the experiments. V.I. performed the overall experiments and wrote the first draft of the manuscript. K.V.M., B.S.P., H.M., and S.M. contributed to the field observations and maintenance/data collection of field experiments. M.S.-K. critically reviewed, revised, and finalized the manuscript. All authors gave their approval for the publication of the final revised version.

DATA AVAILABILITY STATEMENT

The nucleotide sequence of the fungus used in this study is available at the National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/nuccore/MH509971.1/). The fungal (ITCC 8635) culture is available at the Indian Type Culture Collection (ITCC; https://www.iari.res.in/index.php?option=com_content&view=article&id=177&Itemid=523). The seeds of the chickpea genotypes are available at the National Bureau of Plant Genetic Resources (NBPGR; http://www.nbpgr.ernet.in/Divisions_and_Units/Exchange.aspx), the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT; https://www.icrisat.org/), the Indian Institute of Pulses Research (IIPR; https://iipr.icar.gov.in/cimprovment.html), and CGIAR (https://www.genebanks.org/resources/crops/chickpea/).

ORCID

Muthappa Senthil-Kumar http://orcid.org/0000-0003-1502-1659

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information tab for this article.

Appendix S1. Flowchart showing the steps in the development of a sick plot.

Appendix S2. Pictorial representation of stages in the development of control (A–C) and dry root rot sick plots (D–F) in three seasons and the extent of disease incidence across different seasons.

Appendix S3. Soil characteristics of the control and sick plots.

Appendix S4. Dry root rot (DRR) disease symptoms (A, B) and morphological (C–E) and molecular confirmation (F–I) of fungal identity using microscopy and PCR-based techniques in chickpea.

Appendix S5. Dry root rot disease severity score (A) and severity index (B) under well-watered and drought-stressed conditions in chickpea.

Appendix S6. Genotypes used in the study and their features.

Appendix S7. Visualization showing the experimental design for screening chickpea genotypes in phase 1 (A) and phase 2 (B) for dry root rot response.

Appendix S8. Disease incidence for genotypes screened in the sick plot.

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APPENDIX 1: RECOMMENDED PROTOCOL FOR SICK PLOT DEVELOPMENT, PATHOGEN CONFIRMATION, AND DISEASE ASSESSMENT

Materials required

- A field area with natural inoculum showing ≥30% dry root rot (DRR) disease incidence (hotspot)
- Chickpea genotype seeds JG 62 (i.e., ICC 4951), BG 212 (i.e., PUSA 212), and ICC 229 (or any similar genotype that is susceptible to DRR but resistant to other diseases) (NBPGR, http://www.nbpgr.ernet.in/Divisions_and_Units/Exchange.aspx; ICRISAT, https://www.icrisat.org/; IIPR, https://iipr.icar.gov.in/; CGIAR, https://www.genebanks.org/resources/crops/chickpea/)
- Nonhost plant seeds (barley and foxtail millet)
- Bavistin (active ingredient: carbendazim 50% WP; Hindustan Antibiotics Ltd., Pune, India)
- SAAF fungicide (active ingredients: 12% carbendazim + 63% WP mancozeb; United Phosphorus Ltd., Mumbai, India)
- Epifluorescence microscope (Eclipse 80i Upright Microscope; Nikon Corporation, Tokyo, Japan)
- Stereomicroscope (SMZ25 Research Stereomicroscopes; Nikon Corporation)
- Laminar flow (Bio II Advance, Class II cabinet, EN-12469-2000; Telstar, Barcelona, Spain)
- Soil moisture meter (Lutron Electronic Enterprise Co., Ltd., Taipei, Taiwan)
- Beakers (200 mL)
- Potato dextrose agar/broth (catalog no. 213400; BD, Franklin Lakes, New Jersey, USA)
- Pipettes (10 µL, 100 µL, 1000 µL)
- Petri plates
- Fresh 50-mL falcon tubes
- Autoclaved reverse osmosis water
- Forceps (small, medium, and long)
- SYBR Green PCR Master Mix (catalog no. 4309155; Thermo Fisher Scientific, Waltham, Massachusetts, USA)
- NanoDrop 2000/2000c Spectrophotometer (Thermo Fisher Scientific)
- Statistix 10 software (Analytical Software, Tallahassee, Florida, USA)
- DNAzol reagent (Thermo Fisher Scientific, Carlsbad, California, USA)
- GraphPad Software (GraphPad Software, San Diego, California, USA)

Methodology

Designation of the sick plot and improvement of a natural infection hotspot

1. Locate potential plots where only chickpea or other host plants have been cultivated for the last few successive seasons.
2. Assess the presence of DRR disease–affected plants by examining the disease symptoms, such as a brittle

primary root devoid of most of the lateral roots and the presence of microsclerotia (a hallmark of DRR infection).
3. Calculate the percentage of DRR disease incidence by using the formula provided in the "Assessment and confirmation of DRR disease and its causal pathogen" section. Select a plot (ca. 250 m^2) with approximately 30% disease incidence.
4. Fields with >50% disease incidence (i.e., a natural inoculum hotspot) can be directly used for screening experiments.
5. As a natural inoculum hotspot might have populations of other pathogens, such as *Fusarium oxysporum* and *Sclerotium rolfsii*, the soil should be evaluated and other pathogen populations reduced by cultivating DRR-specific susceptible and wilt-resistant genotypes (e.g., BG 212). The absence of a host for a particular pathogen can result in the reduction of the particular pathogen population whereas the presence of a host plant enriches a particular pathogen inoculum over time.
6. Calculate the disease incidence for other diseases, such as *Fusarium* wilt and collar rot, and take the necessary steps to decrease them to zero levels. A natural hotspot can then be used as a sick plot for further genotype screening.

Note: The chickpea-growing area designated as a sick plot should preferably be isolated from other chickpea-growing areas. *Macrophomina phaseolina* has a wide host range in pulses, causing diseases in many legume plants (Pandey et al., 2020). Foliar symptoms of DRR and *Fusarium* wilt need to be distinguished. Wilt-affected plants possess intact roots with brown discoloration of vascular tissue (a hallmark of wilt infection) and wilted foliage. For the early diagnosis of chickpea-specific *M. phaseolina* (formerly *Rhizoctonia bataticola*) infection, the loop-mediated isothermal amplification assay (Ghosh et al., 2017) may also be used.

Soil testing

1. Collect the composite soil sample in three replicates by following the random sample collection method.
2. Pulverize coarse particles to ensure sample uniformity.
3. Dry the soil samples and perform soil testing. In our study, soil samples collected from field locations were tested for nitrogen, phosphorus, potassium, organic carbon (OC), pH, electrical conductivity, soil type, and water-holding capacity.

Note: The soil's chemical and physical properties should be analyzed, and the properties should be uniform throughout the field. Amendments can be made by following recommended agronomic practices for crop cultivation.

Pathogen isolation

1. Collect root samples from plants with DRR disease symptoms in sample collection pouches and bring them to the laboratory.
2. On the same day that the samples were collected, examine them under a stereomicroscope or compound microscope for microsclerotia in the root.
3. Isolate the fungus according to the steps described in Sinha et al. (2019) and Irulappan and Senthil-Kumar (2021).

Confirmation of *Macrophomina phaseolina* morphology

1. Look for dark septate mycelial growth and microsclerotia in the potato dextrose agar (PDA) plate.
2. Look for the typical branching angle and other morphological features described in the section "Assessment and confirmation of DRR disease and its causal pathogen."

Note: The mycelia of the DRR fungus are septate and multinucleate, with cross walls present at the beginning of newly branching hyphae.

Confirmation of pathogen by sequencing

1. In addition to morphological confirmation, molecular confirmation is also needed to confirm the identity of the pathogen. From the pathogen culture, isolate genomic DNA using the cetyltrimethylammonium bromide (CTAB) method or DNAzol reagent and quantify the concentration using the NanoDrop 2000/2000c spectrophotometer.
2. Amplify the ITS segment using the universal ITS primers (ITS1: 5′-TCCGTAGGTGAACCTGCGG-3′, ITS4: 5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990; Sinha et al., 2019) from the genomic DNA (30–50 ng/μL) by PCR.
3. Separate the amplicons in 1.2% agarose gel to identify the amplicon size (Sinha et al., 2019). Then, excise the amplicon and extract it using the GeneJET Gel Extraction Kit (catalog no. NA1111-1KT; Sigma-Aldrich, St. Louis, Missouri, USA).
4. Sequence the ITS using the same ITS primers and compare the sequence with the existing *M. phaseolina* ITS sequence using the BLAST tool (Altschul et al., 1990).

Crop growth and management

1. Apply the recommended quantities of nitrogen, phosphorus, and potassium, at 20–30 kg/ha, 40–60 kg/ha, and 17–25 kg/ha, respectively (Gaur et al., 2010). Maintain 30 cm distance between rows and 10 cm between seeds. Sow the seeds at 10 cm depth.
2. For no-pathogen (control) plots, treat the seeds with 2 g thiram + 1 g carbendazim per kg seed before sowing.

Additional tips during sick plot preparation

1. Isolate virulent pathogen from an infected plant from the local field using the hyphal tip method. Develop a sick culture using an appropriate fungal substrate such as chickpea, sorghum, or maize meal media and mix 500 g of sick culture with the plot (m^2) soil evenly.
2. Avoid water stagnation in the selected plot, as high moisture conditions deteriorate the microsclerotia structure and reduce the inoculum level.

Note: Preferably, no other crops besides chickpea should be cultivated in the designated sick plot to avoid strain variations specific to phylogenetically diverse plant species.

Preparation of a no-pathogen subplot

1. As with the sick plot area, select a separate control plot with no or <30% DRR disease incidence, because a plot with no pathogen inoculum is essential for comparison.
2. Sow only nonhost crops such as barley and foxtail millet in the control plots before starting chickpea cultivation (Hornby, 1983; Lodha et al., 1990).
3. Before starting the experiment, treat the area by solarization, soil fumigation, and fungicide application.

Note: The cultivation of non-host crops reduces the pathogen inoculum. Soil solarization and application of fungicide/soil fumigation in soil reduces the pathogen population in the field. Soil fumigation has been reported to control soil-borne pathogens (Hornby, 1983; Chauhan et al., 1988; Sinha et al., 2019; Khaliq et al., 2020).

Quantification of pathogen inoculum in the soil

1. Collect fresh field soil samples in replicates from the topsoil and at 10 cm depth in paper pouches (7 cm^2) and bring them to the laboratory.
2. Weigh soil samples of ~1 g.
3. Isolate DNA from the soil samples (using, e.g., the GenElute Soil DNA Isolation Kit [DNB100-50RXN; Sigma-Aldrich, St. Louis, Missouri, USA]).
4. Use specific primers. In our study, we used primers designed from the *M. phaseolina* ITS sequence (Appendix S4G).

Primer sequence	Primer length	Forward/ reverse	Starting positions	T_m (°C)	Amplicon size
CCCGCCAG AGGACT ATCAAA	20	Forward	93	56.20	129
TTATCGCA TTTCGC TGCGTT	20	Reverse	202	56.41	129

Note: In the literature, for pathogen quantification, the serial dilution technique (on a selective medium of PDA containing chloroneb 100 µg active ingredient/mL and streptomycin sulfate 250 µg active ingredient/mL) (Mihail and Alcorn, 1982) and quantitative PCR (qPCR; Fierer et al., 2005; Höppener-Ogawa et al., 2007) are commonly used. A standard curve should be developed from a diluted known DNA quantity. The quantity range should be wide enough to be used for both in planta fungal DNA and soil DNA quantification.

Quantification of pathogen inoculum inside the plant root

1. Collect root samples from the control plot and sick plot.
2. Isolate genomic DNA using the CTAB method (Clarke, 2009).
3. Quantify the concentration by qPCR using the standard curve with the known concentration.
4. Follow the steps described in the "Quantification of pathogen inoculum load in the soil and enrichment of pathogen inoculum in the sick plot" section.

Plant phenological stage and assessment of percent disease incidence

1. Uproot 20 plants at 15, 30, 40, 60, and 120 days after sowing.
2. Examine the roots for the presence of necrotic spots and microsclerotia at the epidermis, cortex, and pith regions.
3. Examine the foliage for straw-colored leaves.
4. Calculate the percentage of disease incidence.

Note: Disease incidence can be observed at any plant stage. However, the flowering or fruiting stage is preferable as the typical DRR disease symptoms appear at this stage. Moreover, other diseases such as collar rot and wilt can occur simultaneously at other plant stages, and can interfere with DRR assessment. At the susceptible plant stage (i.e., flowering), count the number of dead plants exhibiting DRR symptoms.

Score for calculating the disease index

1. Develop a disease score by examining the root and foliar symptoms.
2. The following suggestions for the disease score can be used (also see Appendix S5A)
   Score 0 = zero infection
   Score 1 = necrotic spots on the primary and lateral roots
   Score 2 = rotting of half of the lateral roots
   Score 3 = rotting of all lateral roots and partial straw-colored foliage
   Score 4 = root rot and completely straw-colored foliage
   Score 5 = dry root rot and dead plant

Note: The randomized complete block design (RCBD) design can be followed for genotype screening, and the disease index can be calculated by following the disease score. Then, use ANOVA to calculate the difference between the scores followed by Sidak's multiple comparisons test (Figure 3C) and uncorrected Fisher's LSD (Appendix S5B). Overall, the efficiency of the sick plot can be assessed by estimating disease incidence, disease index, and pathogen load in the soil and/or the plant roots.

Experimental design for imposing combined drought and pathogen stress

1. Assign the treatments (i.e., control, drought, pathogen, and combined drought and pathogen) in the statistical design.
2. Drought can be imposed by withholding irrigation. The drought level can be measured using a Lutron soil moisture meter (Lutron Electric Enterprise Co. Ltd., Taipei, Taiwan) at a soil depth of 15 cm from the surface for all the treatment plots. Observations should be made

at a minimum of three different spots for every treatment plot.

3. Canopy temperature can also be used to assess the plant response to drought stress. Canopy temperature can be measured using the Fluke Infrared Thermometer (Fluke Ti32; Fluke Corporation, Everett, Washington, USA) between 11:00 a.m. and 2:00 p.m., when ambient temperature is at its maximum. The relative water content (RWC) for the leaves, leaflets, or roots can be measured by determining the following: (A) the fresh weight, (B) the turgid weight (by soaking in water for 4 h), and (C) the dry weight (by drying in the oven at 60°C for two days) (Gupta et al., 2016; Sinha et al., 2019). The percentage of leaf RWC can be calculated using the following formula:

$$\text{Relative water content}(\%) = \frac{(\text{Fresh weight} - \text{dry weight})}{(\text{Turgid weight} - \text{dry weight})} \times 100$$

Reclamation of the sick plot for regular cultivation, pathogen containment, and biosafety

Macrophomina phaseolina belongs to the risk group-1 category (per the "List of microorganisms corresponding to different risk groups" released periodically by the Department of Biotechnology, Government of India; https://dbtindia.gov.in/). Proper safety measures should be followed while handling the fungus and fungus-infected plant samples in the laboratory and the field. The soil and pathogen samples should be discarded after incineration or autoclaving, and safety measures should be taken while transporting samples from field to laboratory as per the standard operating procedure of the local biosafety authorities. The inoculum in the designated sick plot can be eliminated, and sick plots reclaimed to regular cultivation, by following the steps mentioned in the "Preparation of a no-pathogen subplot" section.

APPENDIX 2: TAXONOMIC CLASSIFICATION INFORMATION FOR DRY ROOT ROT CAUSATIVE FUNGUS

The fungus causing dry root rot belongs to the following classification:

Name of the fungus referred to in this study: *Macrophomina phaseolina* (asexual/chickpea-specific strain)

Kingdom: Fungi
Division: Ascomycota
Class: Dothideomycetes
Order: Botryosphaeriales
Family: Botryosphaeriaceae
Genus: *Macrophomina*
Species: *phaseolina*

In the current literature, *Rhizoctonia bataticola* is incorrectly used as a synonym for *M. phaseolina* (chickpea strain). After the taxonomic re-classification (http://www.indexfungorum.org/; Kirk et al., 2008), *M. phaseolina* is the recently accepted taxonomic name. However, the following points should be noted for clarity. (1) *Rhizoctonia bataticola* continues to be used even in recent literature, stating that it has been observed in the sclerotial phase only in chickpea. The literature that used this name reported only asexual stages (i.e., hyphae and microsclerotia) in chickpea, without any spores. This usage is incorrect. (2) *Macrophomina phaseolina* has only the pycnidial stage in other host plants (stage not reported in chickpea) and produces single-celled spores (not reported in chickpea). (3) Because *Rhizoctonia* belongs to Basidiomycota and *Macrophomina* belongs to Ascomycota, these two genus names cannot be used together to refer to the DRR-causing fungus. Hence, to rectify the confusion and to follow the "one fungus one name" rule (Turland et al., 2018), in this article, we refer to the causal agent of chickpea DRR disease as *M. phaseolina* (chickpea strain; asexual stage). For more details, please refer to Farr and Rossman (2021).