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Dry root rot disease assays in chickpea: a detailed methodology

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TITLE:

Dry root rot disease assays in chickpea: a detailed methodology

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KEYWORDS:

Cicer arietinum, *Rhizoctonia bataticola*, sick pot, blotting paper, drought stress, mycology, plant biology, fungal infection, screening protocol, molecular biology, pathology, disease resistance

SUMMARY:

This study presents methodologies to study the pathomorphological and molecular mechanisms underlying chickpea–*Rhizoctonia bataticola* interaction. The blotting paper method is useful to rapidly study chickpea genotype responses, while the sick pot-based method can be used to simultaneously impose drought and *R. bataticola* infection and screen for tolerant genotypes.

ABSTRACT:

Dry root rot (DRR) disease is an emerging biotic stress threat to chickpea cultivation around the world. It is caused by a soil-borne fungal pathogen, *Rhizoctonia bataticola*. In the literature, comprehensive and detailed step-by-step protocols on disease assays are sparse. This article provides complete details on the steps involved in setting up a blotting paper technique for quickly screening genotypes for resistance to DRR. The blotting paper technique is easy and less expensive. Another method, based on the sick pot approach, is a mimic of natural infection and can be applied to study the interacting components—plant, pathogen, and environment—involved in the disease triangle.

Moreover, in nature, DRR occurs mostly in rainfed chickpea cultivation areas, where soil moisture recedes as crop growth advances. Drought stress is known to predispose chickpea plants to DRR disease. Pathomorphological and molecular understanding of plant-pathogen interaction under drought stress can pave the way for the identification of elite DRR-resistant varieties from the chickpea germplasm pool. This article provides a stepwise methodology for the preparation of a sick pot and subsequent disease assay. Overall, the information presented herein will help researchers prepare *R. bataticola* fungal inoculum, maintain this pathogen, set up the blotting paper technique, prepare sick culture and sick pot, and assess pathogen infection in chickpea plants.

INTRODUCTION:

Dry root rot (DRR) is one of the economically significant diseases in chickpea[1,2]. It is a root-specific disease caused by *Rhizoctonia bataticola* (teleomorph, *Macrophomina phaseolina*). Infected plants lack lateral roots and possess brittle taproots and yellow foliage[1,3]. DRR under drought stress has been reported to be an emerging threat to chickpea cultivation[1–3]. Moreover, DRR incidence is reported to be aggravated under drought stress under field conditions[1–3]. DRR is more prevalent in rainfed areas than in irrigated fields[4]. The utilization of resistant varieties is the way to overcome the disease and circumvent fungicide use[1,13]. Because chickpea germplasm available across the globe harbors genetic variation for the trait[5], the screening and identification of resistant/susceptible genotypes are critical for molecular breeding for crop improvement.

Robust, easy, and cost-effective disease assays are essential to investigate *R. bataticola* infection patterns in chickpea. The primary disease assay used to observe the response of chickpea genotypes to *R. bataticola* infection is the blotting paper technique[1,4]. It is a simple technique and can be executed using liquid fungal inoculum, seedlings with roots, and sterile blotting paper. However, this technique has not been utilized to its maximum because no step-by step-protocol is available in the literature.

Meanwhile, the sick pot technique involves the preparation of a potential sick culture and the imposition of drought stress. Given that drought stress aggravates DRR disease incidence[3], it is essential to study the plant-pathogen interaction under drought stress[6, 7]. The sick pot technique provides the platform for such a simultaneous study, promoting better possibilities for germplasm screening and understanding the mechanistic basis of the interaction. Pathomorphological changes such as an increase in root length and reduction in lateral root number—inherent to DRR disease—can be addressed using the sick pot technique[1, 3, 7].

Herein, a detailed protocol for blotting paper and sick pot techniques, which can be used to study the interaction between chickpea and *R. bataticola* and screen chickpea germplasm, is presented. The details of the materials used in the study are given in **Table of Materials**.

PROTOCOL:

1. Isolation of *R. bataticola* and storage

1.1 Details of the chickpea genotype and DRR symptoms

1.1.1. Use chickpea plants (genotype, JG 62) that generally show typical DRR symptoms, such as dry, brittle primary root with no lateral roots and microsclerotia beneath the bark and inside the pith[1,3].

1.2 Collection and washing

1.2.1 Uproot plants showing symptoms dry straw-colored foliar and brittle primary root with microsclerotia under the epidermis layer. While uprooting, a major part of the roots will remain

inside the soil as the infected roots are brittle. Remove the coarse soil debris attached to the root. Cut the roots separately and collect them in paper envelopes (27.5 cm x 12 cm) with the proper label and place them in a sample collection box.

1.2.2 After transporting samples to the laboratory, place the roots in a 200 mL beaker and cover with mesh (Nylon mesh with the pore size of 3 mm diameter) and wash the roots thoroughly with running tap water to remove adhering soil particles.

1.2.3 Use reverse osmosis (RO) water to rinse once at the end.

1.3 Surface sterilization

1.3.1 Break the roots into a four pieces with 2 cm length each with a scalpel blade and put them in a clean 200 mL beaker.

1.3.2 Assemble autoclaved RO water, 2% NaOCl, discarding jar, and autoclaved blotting paper (5 cm^2) inside the laminar flow chamber.

1.3.3 Wash the roots with 50 mL of autoclaved RO water thrice and then with 50 mL of 2% NaOCl for 10 min. Wash the roots with 50 mL of RO water three times again to remove the NaOCl.

1.3.4 Blot dry the roots by placing the roots on autoclaved blotting paper and leave until the roots are dried.

1.4 Media and incubation

1.4.1 Remove the edges of the roots with a sterilized scalpel blade. Split the roots using the blade and use the sterilized forceps to place them on a potato dextrose agar (PDA) media Petri plate containing streptomycin sulfate (50 mg L^{-1}) and ampicillin (50 mg L^{-1}).

1.4.2 Close the plate, seal with parafilm, and incubate in an incubator at 28 °C for two days in the dark.

1.5 Hyphal tip method

1.5.1 After two days of incubation, bring the plates into the laminar flow chamber. Cut the tip of the hyphal growth[8] under a stereomicroscope (Leica EZ4 educational stereomicroscope) and transfer it into a fresh PDA plate with streptomycin sulfate and ampicillin.

1.5.2 Incubate the plates in the incubator at 28 °C for ten days in the dark.

1.6 Storage and maintenance of *R. bataticola* fungal inoculum

1.6.1 Make PDA slants in test tubes and transfer a fungal agar plug from a ten-days-old culture plate using the flame sterilized inoculation loop. Seal the test tube cap with parafilm.

1.6.2 Incubate the slants at 28 °C for ten days in the dark.

1.6.3 Seal the cap with parafilm again and store at 4 °C in the refrigerator for the future use.

1.6.4 Subculture every six months to maintain the fungus.

1.7 Maintenance of virulence

1.7.1 Infect the plants with pure fungal inoculum prepared as mentioned in the sick pot technique[3]. Isolate the same fungus from the infected plants (Koch's postulates)[9] and use for further experiments.

NOTE: In this study, a field isolated strain of the fungus was used (GenBank: MH509971.1 https://www.ncbi.nlm.nih.gov/nuccore/MH509971)

2. Blotting paper technique

NOTE: The blotting paper technique entails the preparation of liquid fungal inoculum, seedling preparation, and disease assessment.

2.1 Preparation of liquid *R. bataticola* inoculum

NOTE: Liquid *R. bataticola* fungal inoculum contains both mycelia and microsclerotia. Both these structures act as primary inoculum.

2.1.1 Prepare 500 mL of PDB media in a 1,000 mL flask and autoclave it at 121 lbs for 15 min.

2.1.2 After the media is cooled down, inoculate the broth with a loopful of fungal agar plug from a fungal slant culture and incubate at 28 °C for five days in a shaker at 180 rpm in the dark.

2.1.3 Assemble an autoclaved glass or plastic funnel (10 cm), one-liter conical flask, and two layers of mesh (10 cm^2 size) (Nylon mesh with the pore size of 3 mm diameter) on the table. Filter out the fungal mycelia and microsclerotia using the mesh. Blot dry the fungus in autoclaved blotting paper.

2.1.4 Weigh 100 g mycelia for 50% inoculum and keep at room temperature.

2.2 Preparation of chickpea plant

2.2.1 Select 50 healthy seeds (in this study, the DRR-susceptible genotype JG 62 was used), place them in a 100 mL beaker and cover with a mesh.

2.2.2 Wash the seeds with tap water first to remove soil debris.

2.2.3 Take the beaker with seeds into the laminar flow chamber and wash them with sterilized 50 mL of RO water thrice for 1 min per wash.

2.2.4 Sterilize the seeds with 50 mL of 2% aqueous NaOCl for 2 min with continuous shaking and wash the seeds with 50 mL of sterilized water five times for 1 min each.

2.2.5 Fill a polythene bag (47.5 cm x 25 cm) with Soilrite (a mixture of horticulture grade expanded perlite, Irish Peat moss, and exfoliated vermiculite in equal ratio i.e., 1/3:1/3:1/3), sow the surface-sterilized 50 seeds two cm deep, and keep in a growth chamber/room with 28 °C ± 2 °C temperature, 16 h photoperiod with a light intensity of 150 µmol m^{-2} s^{-1}, and relative humidity of 70%. Water them with RO water and uproot the plants eight days after sowing.

2.2.6 Wash the roots in tap water to remove the Soilrite particles. Rinse the roots with sterilized RO water, and keep them in the RO water in a 1000 mL beaker.

2.3 Preparation of blotting paper in trays

2.3.1 Take a blotting paper and cut pieces (30 cm × 23 cm) in sufficient numbers to meet the replications.

2.3.2 Pack the sheets in autoclavable polythene bad and autoclave them at 121 lbs for 15 minutes and keep it in a hot air oven for drying.

2.3.3 Fold each blotting paper sheet once from left to right.

2.3.4 Place the paper on a clean, spirit-wiped plastic tray, as shown in the **Figure 2i-iv**.

2.4 Plant inoculation by dipping

2.4.1 Dissolve 100 g of fungal inoculum in 200 mL autoclaved RO water in a 200 mL beaker to obtain 50% inoculum.

2.4.2 Dip the plant roots in the prepared inoculum for 1 min with intermittent up-and-down movement to ensure uniform attachment of fungal inoculum.

2.5 Placing the plants in the blotting paper

2.5.1 Wet the bottom side of the blotting paper placed on the tray with sterile RO water. Place the plants on the paper in a way where only the roots are covered by the paper, and shoots are left out.

2.5.2 Close it by folding the top side of the blotting paper and wet the entire paper to provide enough water to sustain plant growth.

2.5.3 Water the tray once a day and keep the trays at 28 °C. Observe the symptoms such as necrosis, root rot, and leaf yellowing daily.

3. Sick pot technique

NOTE: The sick pot technique entails the preparation of virulent inoculum and a sick pot, maintenance of moisture level, and assessment of disease symptoms.

3.1 Preparation of substrate

3.1.1 Take one kg of any commercially available chickpea seeds. Remove infected seeds (seeds with fungal growth, infection spots, and insect eaten). Place them in 5 L plastic beaker and wash with tap water thoroughly 3–4 times to remove major debris.

3.1.2 Wash the seeds with 2 L of RO water thrice and soak the 1 kg seeds in a 5 L beaker with threefold more water for five h.

3.1.3 Once the seeds imbibed the water, rewash them with RO water thrice to remove the seed exudates.

3.1.4 Remove the water completely and pack the seeds in jam bottles (300 mL, 12 cm height, 6 cm diameter, and 155 g weight) to about $1/4^{th}$ capacity (100 g per jam bottle) and close the bottles with caps. Pack ten jam bottles in an autoclavable plastic bag (48 cm x 30 cm) autoclave twice at 121 lbs for 15 min continuously.

3.1.5 Dry the seeds at 40 °C in an oven overnight to remove the water droplets inside the bottle.

3.2 Preparation of sick culture

3.2.1 Turn on the BSL-2 level laminar flow chamber. Wipe the floor thoroughly with 70% ethanol, and turn on UV for 15 min. Then, keep the seeds inside the laminar flow chamber.

3.2.2 Take 10-day-old freshly isolated *R. bataticola* culture (part 1). Then, take three fungal agar plugs (4 mm diameter) using a sterile pipette tip or cork borer, and put them into jam bottles aseptically. Cover the bottles with caps and seal with parafilm.

3.2.3 Shake the bottles to mix the fungal disc with the chickpea seeds uniformly.

3.2.4 Incubate the bottles at 30 °C for 15 days in the dark.

3.2.5 Take jam bottles with black fungal growth (**Figure 4iii**) and transfer the sick culture (seeds with microsclerotia) from jam bottles to a glass Petri plate using sterile forceps. Dry them at room temperature for two days in a glass Petri plate.

3.2.6 Powder the fungal mass using a mortar and pestle, and store the powder at 4 °C.

3.2.7 Autoclave Soilrite twice at 121 lbs for 15 min.

3.2.8 Dry the autoclaved Soilrite mix under a sunshade.

3.2.9 Mix the fungal powder with Soilrite at 50% w/w, fill the pots with the mixture, and keep them at room temperature for a day.

3.2.10 Sow a surface-sterilized DRR-susceptible chickpea seed per pot (10 cm round pots) (**Table of Materials**) and maintain a moisture level of 80% field capacity (FC).

3.2.11 Observe the symptoms such as necrosis and root rot by uprooting the plants after germination.

3.3 Assessment of sick pot efficiency

NOTE: Plants show yellow foliar symptoms when the roots are completely rotten.

3.3.1 Develop a disease score based on the lesion (necrotic spots) (**Supplementary Figure 2C**) numbers and severity of root rot. Sick pots showing 90% plant death can be used further.

3.4 Genotype screening

3.4.1 Prepare sick culture in large quantities and mix it either with sterilized Soilrite or field soil (5% w/w) in 30 cm-tall pots. Water the pots to wet the surface and leave them undisturbed for seven days.

3.4.2 Sow one surface-sterilized seed per 10 cm round pot and three seeds per 30 cm round pots and water them adequately.

3.4.3 Observe the yellow foliar and root rot symptoms.

3.5 Combined drought and *R. bataticola* infection

3.5.1 Impose drought stress by following the protocols mentioned in Sinha et al. (2019).

REPRESENTATIVE RESULTS:

This study aimed to demonstrate techniques such as blotting paper and sick pot techniques to facilitate pathomorphological and molecular understanding of plant-pathogen interaction under

drought stress. To accomplish this, plants exhibiting DRR symptoms[1,3,4] were collected from a chickpea field, and the fungus was isolated using the hyphal tip method[8]. *R. bataticola* fungal culture appears dark gray on the PDA plate four days after incubation and on the slant (**Figure 1 A & C**) and darker in grey color in PDB medium five days after incubation (**Figure 1B**). *R. bataticola* has septate mycelia (**Figure 1D**), and it produces microsclerotia (**Figure 1E**), which act as primary inoculum in the soil[10].

The steps given in **Figure 2** were followed to execute the blotting paper technique. Eight-day-old plants were infected with liquid inoculum (50%), and eight days after infection, plants were observed for symptoms. Plants showed root rot because of extensive necrosis, as well as leaf yellowing, which is the typical root and foliar symptoms of DRR disease (**Figure 3B** and **Supplementary Figure 1A**).

The sick pot technique was carried out using the steps shown in **Figure 4**. The concentrations of fungal inoculum in Soilrite and field soil was 10% and 5%, respectively. DRR-susceptible seeds show the typical DRR symptoms such as root rot, lack of lateral roots, leaf yellowing, and premature death, as compared to control plants (**Figure 5A and B**). Plants subjected to infection in the sick pot made with Soilrite died and showed root rot seven days after sowing (**Figure** 5C and **Supplementary Figure 2C**). Meanwhile, plants growing in the sick pot made with field soil showed typical foliar symptoms, i.e., straw-colored foliage 48 days after sowing (**Figure 5E**).

The influence of drought on DRR disease was also studied in the sick pot under laboratory conditions. Drought stress was imposed by withholding water[3]. The plants under drought stress (30% FC) showed aggravated disease incidence as compared to the pathogen-only-treated (90% FC) plants (**Figure 6A and B**). Control and drought-treated plants did not show any symptoms (**Figure 6A and B**). Roots under combined stress had more necrotic spots and rot as compared to pathogen only-plants (**Figure 6B**).

FIGURE LEGENDS:

Figure 1. Characteristic morphological features of *Rhizoctonia bataticola*. The causal agent of dry root rot (*Rhizoctonia bataticola*, ITCC 8635) was isolated from the field (National Institute of Plant Genome Research, New Delhi, 28.6139°N, 77.2090°E). From the cultures, the fungus was isolated using the hyphal tip method[8]. Images show the 4-day-old *R. bataticola* culture in potato dextrose agar in a Petri plate (**A**), 5-day-old liquid culture (**B**), and 10-day-old slant culture (**C**). Fungal mycelia (**D**) and microsclerotia (black arrows) (**E**) was teased on a microscope slide and stained with WGA-FITC and aniline blue, respectively. Images (**E, F**, scale bar, 20, and 50 µm) were captured under the 20x and 40x objective lens of an epifluorescent microscope. White arrows show the cross walls in the mycelia.

Figure. 2 Steps involved in *R. bataticola* inoculation and DRR assays in the blotting paper technique. Surface sterilize the seeds by passing them through running tap water and washing with 2% sodium hypochlorite, followed by washing with sterile RO water 3–4 times (step 1). Then, sow 30 seeds in 15-cm-high pots containing Soilrite (step 2) and allow the seeds to grow for eight days in a growth room with 28 °C ± 2 °C temperature, 16 h photoperiod with a light intensity of

150 µmol m^{-2} s^{-1}, and relative humidity of 70% (step 3). Uproot the plants and wash them with sterilized water (step 4). Prepare the fungal inoculum by inoculating 500 mL PDB media with fungus (step 5). For the infection, use 5-day-old fungal broth culture. Then, inoculate the plants by dipping the roots into the fungal inoculum in a beaker for 30 s and remove excess inoculum by touching the inner sidewall of the beaker (step 6). After infection, place fungal-inoculated and mock-inoculated plants in different blotting papers in separate clean trays (step 7). Moisten the blotting paper with adequate sterile water daily and observe the symptoms, viz., shedding off of lateral roots, yellowing and wilting of plant leaves, rotten seeds, and root rot at eight days post-infection (step 8) (**A**). Images represent essential steps (steps 3–7) (**B**).

Figure 3. DRR disease symptoms in chickpea-based on the blotting paper technique. Following the protocol depicted in Figure 2, DRR-susceptible plants (genotype, JG 62) were subjected to infection using the blotting paper technique, and symptoms were captured eight days after infection. Images show the representative control plants (mock-inoculated) with healthy shoots (red arrow) and roots with more lateral roots (yellow arrow) (**A**). Images show the representative infected plants with typical symptoms, such as wilting, yellowing, and drying of leaves (blue arrows) and dried/necrotic roots with fewer lateral roots (white arrows) (**B**). The scale bar is 1 cm. Experiments were repeated at least five times.

Figure. 4 Overview of sick pot preparation for *R. bataticola* inoculation and DRR disease assays. Prepare the fungal inoculum by inoculating a PDA plate with a 5 mm fungal disc of actively growing fungal culture and incubate at 28 °C for ten days. Then, for preparing the substrate, wash the chickpea seeds with tap water, soak the seeds in water overnight, and autoclave at 121 lbs for 15 min. Then, inoculate 100 g of the substrate with three agar plugs from 10-day-old culture and mix well. Then, incubate the inoculated substrate at 30 °C for 15 days in an incubator. Crush the sick culture (fungal grown substrate), dry and powder it, and store at 4 °C. Then, mix 50 g of sick culture with 100 g of dry Soilrite thoroughly (sick pot) (**A**). Then, sow the surface-sterilized susceptible chickpea seeds and observe the symptoms, viz., tap root rot, lateral root necrosis, and leaf yellowing. Assimilate the plants showing symptoms in the same pot. For further experiments, use the pots showing 90% infection as the susceptible genotype. Images represent the inoculum (i), the substrate (ii), and control and inoculated sick culture (iii) (**B**).

Figure 5. DRR disease symptoms in chickpea-based on the sick pot technique. For making a sick pot, the protocol depicted in Figure 4 was used. Then, surface-sterilized DRR-susceptible chickpea genotype (JG 62) seeds were sown in the control (**A**) and sick pot (**B**). Each plant in the pot represents one replicate, and dead or stunted plant growth was observed in the sick pot. Plants on the right side of the panel (**C**) indicate the presence and absence of lateral roots (yellow arrow) in the control and treatment, respectively. The graph shows the number of dead plants in the sick pot treatment as compared to the control (**D**). The sick pot was prepared on sterilized field soil collected from the NIPGR field, and sick culture was mixed. Surface-sterilized seeds were sown, and disease symptoms were captured 48 days after sowing. The image shows the control plants (E), and the typical DRR foliar symptoms, namely, the drying of plants, are indicated (white arrows). Statistical significance was determined using Student's *t*-test. The bar represents the SEM of nine biological replicates, and the asterisk denotes a statistically significant value at P <

0.0001. The yellow arrow indicates necrotic/rotten, dried primary root without any lateral roots. Experiments were repeated at least ten times, with similar results.

Figure 6. The sick pot method is useful to study the influence of drought stress on plant-pathogen interaction. A pot experiment was conducted to study the effect of drought on *R. bataticola* infection. The experiment comprised control, drought-only, pathogen-only (*R. bataticola*, pathogen), and combined drought and *R. bataticola* stress (combined stress). Plants were grown in a growth room with 28 °C ± 2 °C temperature, 16 h photoperiod with a light intensity of 150 µmol m^{-2} s^{-1}, and 70% relative humidity. The sick pot was prepared following the protocol depicted in Figure 4. Then, surface-sterilized DDR-susceptible chickpea genotype (JG 62) seeds were sown in control and sick pots. Control and pathogen treatments were irrigated throughout the experiment. Drought stress was imposed on plants under drought and combined stress treatment. Water was withheld 18 days after sowing, and the desired drought level was reached 24 days after sowing. Plants were observed for symptoms 29 days after sowing. The image shows the foliar and root changes under treatments (**A**). Images show unstained plant roots observed under the 0.5X objective lens of an SMZ25/SMZ18 research stereomicroscope (**B**). The red arrow indicates the uninfected lateral roots, and the black arrows indicate the infected lateral roots. Experiments were repeated at least ten times, with similar results.

Supplementary Figure S1. Inoculum size and reduction in lateral root number in chickpea

Supplementary Figure S2. Foliar symptoms of DRR in chickpea in soilrite

DISCUSSION:

The blotting paper technique provides a straightforward approach to screen chickpea genotypes under laboratory conditions. Dip inoculation enables the investigation of interaction on a temporal basis with easy control over inoculum load (**Supplementary Figure 1**) and facilitates in vitro screening. Furthermore, even young seedlings can be used. Five-day-old fungal culture (**Figure 1B**) can yield enough inoculum to infect the plants. Liquid inoculum contains both mycelia and microsclerotia (**Figure 1D & E**). Root rot symptoms (**Figure 3B**) can be used to score the disease and identify resistant genotypes. DRR occurrence is majorly influenced by drought stress[1,3,5]. However, with this technique alone, drought stress imposition is impossible, and screening with this technique will not reflect natural responses.

The sick pot technique allows the study of the interactions among plants, pathogens, and drought stress. It provides a way to screen the genotypes under combined drought and pathogen stress to identify resistant genotypes. In a sick pot, drought stress can be imposed at any age of the plant and screen the plants. Plants show typical DRR symptoms (**Figure 5C & F** and **Supplementary Figure 2B & C**) in the sick pot method. Plants subjected to combined drought and pathogen infection showed severe root rot as compared to pathogen-only treatment. This implies that available chickpea germplasm needs to be screened to identify a resistant genotype to not only pathogen but also combined pathogen and drought stress. Several studies were attempted earlier to screen the genotypes but by using blotting paper technique[11,12]. Besides, field screening has also been conducted but without imposing drought stress[11]. It is crucial to

impose drought stress during different stages of chickpea and assess the genotype response.

For the blotting paper technique, the volume of the inoculum in the beaker should be at the level where the entire plant root is dipped. Additionally, excess watering will lead to wet rot of the plant roots. Do not use tap water for watering the plants because it can cause contamination.

For the sick pot technique, Desi chickpea varieties are preferable. The number of seeds can vary depending upon the researchers' needs. The volume of water used to soak seeds should be threefold more because the seeds imbibe water. Bacterial growth will occur if the washing is not done correctly. Non-autoclaved water can be used to soak the seeds. The color of the seeds after autoclaving should be black instead of brown, which indicates improper autoclaving. Over drying in a hot air oven leads to the drying of seeds; such seeds cannot be used for fungal inoculation. Inoculating the bottles outside the laminar flow may cause contamination. White fungal growth in inoculated chickpea meal is a sign of improper autoclaving. Biosafety precautions should be followed in discarding the used inoculum, blotting paper, and infected plants.

DISCLOSURES:

We have nothing to disclose

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500

Figure 1

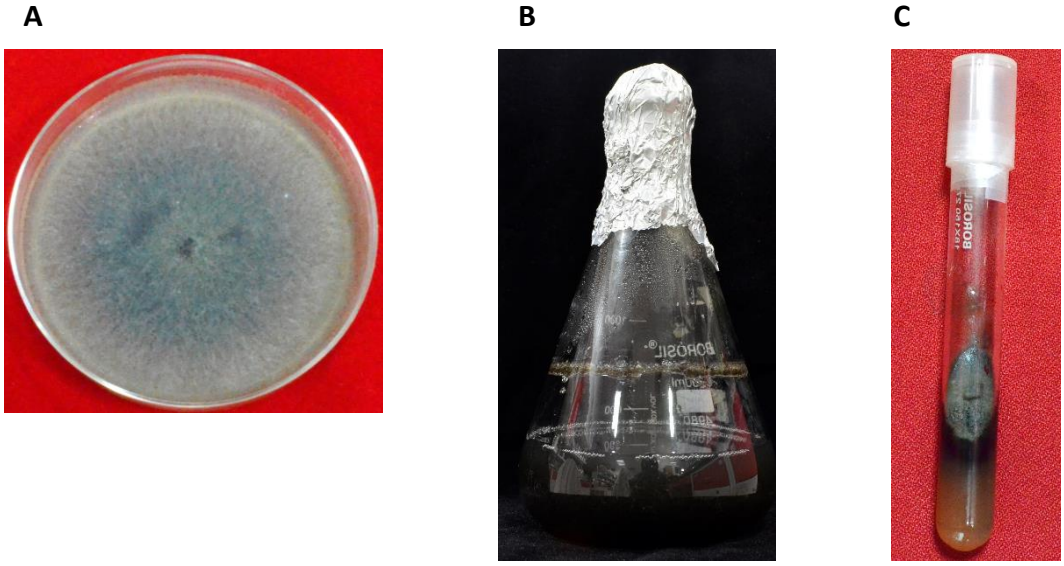


Figure 2

A

Step 1: Surface sterilize the chickpea seeds with 2% sodium hypochlorite

⇩

Step 2: Sow the seeds in 15-cm height pot containing Soilrite

⇩

Step 3: Uproot the 8 days old seedlings, wash under running tap water and rinse with sterile water twice

⇩

Step 4: Inoculate Potato dextrose broth with *R. bataticola* and incubate at 28°C for 5-days with 180 rpm in a shaker

⇩

Step 5: Dip only the roots in inoculum and remove the excess inoculum

⇩

Step 6: Place pathogen inoculated and mock (water)-inoculated plants in separate blotter paper

⇩

Step 7: Keep the trays with plants at 28±2°C for eight days with 16 h artificial light and relative humidity at approximately 70% and moisten the plants every day with adequate water

⇩

Step 8: Inspect for root damage on 8 days after inoculation

B

Step 3- Nursery

Step 4- Uprooted plants

Step 5- Fungal inoculum

Step 6- Dip inoculation

i. ii.

iii. iv.

Step 7- Blotting paper set up

Figure 3

Figure 4

A

Substrate
Soak chickpea seeds overnight and autoclave

Inoculum
10 days old actively growing *R. bataticola*

Sick culture
Inoculate the substrate (100 g) with five 5 mm fungal discs and incubate at 30°C for 15 days

↓

Sick pot
Mix 50 g of sick culture with 100 g of soilrite uniformly

↓

Sow the susceptible genotype seeds in sick pot and allow them to grow

↓

Assess the symptoms in roots and foliar

↓

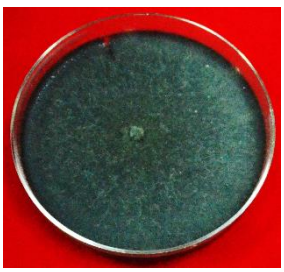
Chop down infected plants and assimilate into the same sick pot

↓

Use the sick pot which is showing 90% of infection for further experiment

B

i.

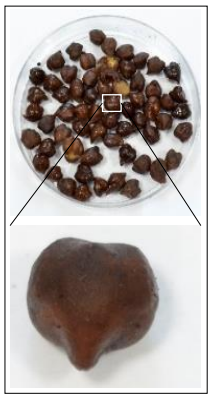


Inoculum

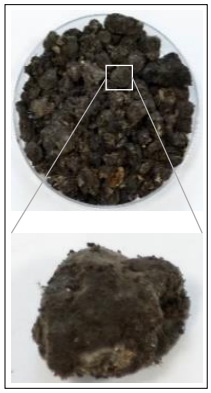
ii.

Substrate

iii.



Control
No fungal growth



Inoculated
Fungal growth

Figure 5

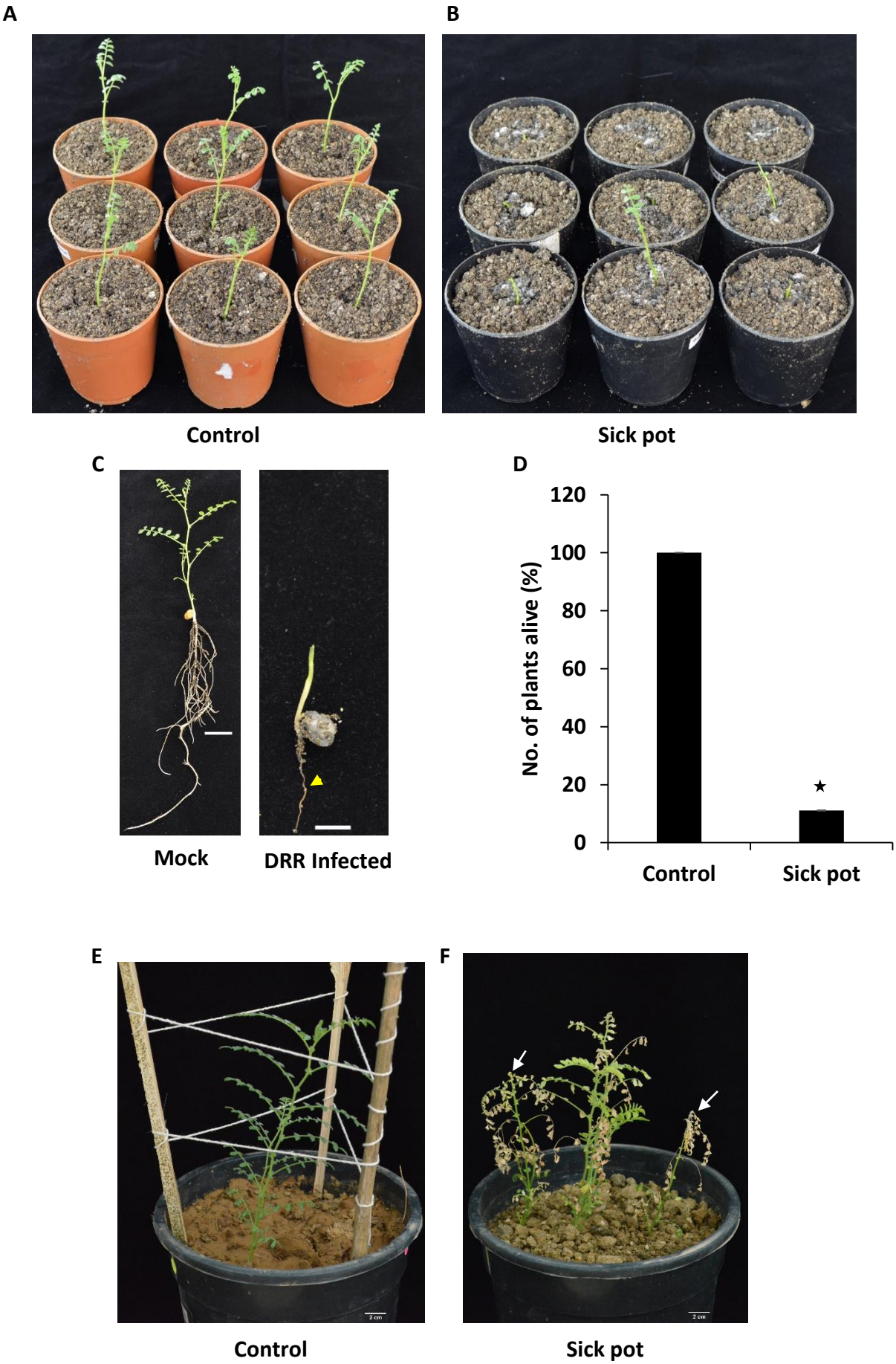
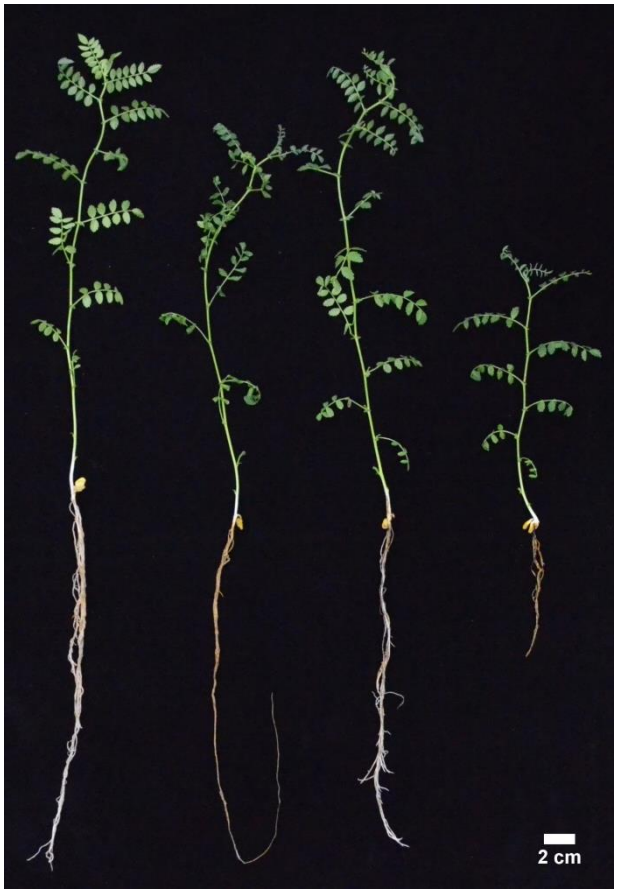

A
B
Control
Sick pot
C
Mock
DRR Infected
D
No. of plants alive (%)
120
100
80
60
40
20
0
Control
Sick pot
E
F
Control
Sick pot

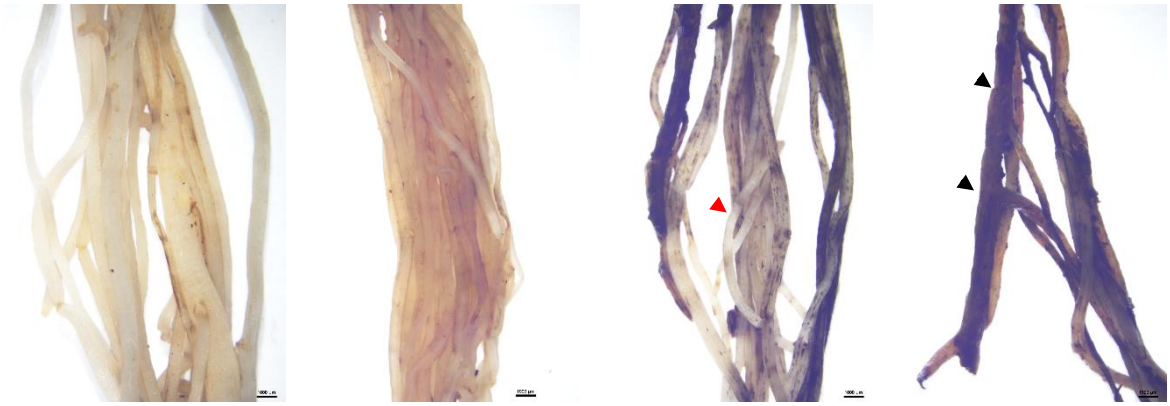
Figure 6

A



Control Drought Pathogen Combined stress

B



Control Drought Pathogen Combined stress

Table 1. List of materials and resources used in this study and their details.

S. No	Name of chemicals/instruments/ materials	Key usage
1	Fungus- *Rhizoctonia bataticola*	Pathogen inoculum
2	Soilrite mix	Soil medium in the lab
3	Filter paper	Blotting paper to support the plant growth
4	Pot	Growing plants
5	Potato dextrose agar/broth	Culture and maintain the fungus
6	Incubator	Culture the fungus
7	Growth chamber	Growing plants in controlled condition
8	Laminar airflow	Carrying out aseptic exercises
9	Mesh	Filtering the fungal mycelia
10	Autoclave	Autoclaving media and chickpea seeds
11	Microscopes	Visualizing the infection ang fungal mycelia
12	Weighing balance	Weighing fungus and chemicals

Model/company name

Indian Type Culture Collection No. 8365
Keltech Energies Limited, Bangalore, India
Himedia
10 and 30 cm size pots
Cat# 213400, DifcoTM, MD, USA
LOM-150-2, S/N AI13082601-38, MRC, incubator, and shaker
Model No. A1000, Conviron, Canada
Telstar, Bio II advance, Class II cabinet, EN-12469-2000
Nylon mosquito net
Autoclave
SMZ25 / SMZ18, Research Stereomicroscopes, Leica EZ4 educational stereomicroscope
Sartorius Electronic Weighing Balance, BSA 4202S-CW

Details/link

GenBank: MH509971.1, ITCC 8635 (https://www.iari.res.in/index.php?option=com_content&view=article&id=125
http://www.keltechenergies.com/
http://himedialabs.com/catalogue/chemical2017/index.html#374
Routinely used nursery pots, for example, https://dir.indiamart.com/impcat/nursery-pots.html
https://www.fishersci.com/shop/products/bd-difco-dehydrated-culture-media-potato-dextrose-agar-3/p-490194
http://www.mrclab.com/productDetails.aspx?pid=91131
https://www.conviron.com/products/gen1000-reach-in-plant-growth-chamber
https://www.telstar.com/lab-hospitals-equipment/biological-safety-cabinets/bio-ii-advance-plus/, http://www.at
Mesh with 0.6-1 mm diameter pore size
http://www.scientificsystems.in/autoclave
https://www.microscope.healthcare.nikon.com/products/stereomicroscopes-macroscopes/smz25-smz18
https://www.sartorius.com/en/products/weighing/laboratory-balances

51&Itemid=1370)

6

lantisindia.co.in/laminar-air-flow.html

Response to editor and reviewers (JoVE61702R1)

Editorial and production comments:
Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Proof reading has been done.

2. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

Response: Yes. As suggested, we have provided the additional details wherever needed.

3. 1.2.1: Please specify the symptoms. What are the paper envelope dimensions?

Response: The disease symptoms are specified. The envelope details are provided.

4. 1.3.1: Break into pieces of what size? What is the size of the beaker?

Response: the length of the root pieces is mentioned in the text. The size of the beaker is also mentioned.

5. How much volume is used for the washes?

Response: The volume is mentioned.

6. 1.7.1: Please provide a citation for the infection. Is reference 9 for the infection or the isolation?

Response: Citation for the infection is given now as asked. Reference 9 is for testing the Koch's postulate (re-isolation).

7. 2.1.2: How is the inoculation done?

Response: The inoculation steps are now detailed.

8. Please specify all volumes and concentrations used throughout.

Response: Volumes and concentrations are mentioned throughout the manuscript.

9. 2.1.3: Please describe the mesh in more detail.

Response: Additional details are provided in the manuscript as well as in the table.

10. 2.2.5: Is there a generic name for SoilRite?

Response: Details of soilrite composition is provided in the manuscript as suggested.

11. 3.2.2: What are the jam bottles' dimensions?

Response: The dimensions of jam bottles are provided as suggested.

12. 3.3.2: Was a disease score developed and used here? Citation?

Response: Yes, we developed the disease score. This was used in the data analysis. Score details are added in the supplementary figure 2c (revised figure).

13. Please avoid numbered lists in the discussion.

Response: No lists are provided in the discussion.

Changes to be made by the Author(s) regarding the video:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Proof reading has been done.

2. Chapter Title Cards
• 02:20 "II. - Blotting paper technique" remove the hyphen and the spaces around it, having only one space between II. and the title

Response: As suggested, the hyphen and spaces are removed.

• 03:53 "III. - Sick pot technique" remove the hyphen and the spaces around it, having only one space between III. and the title

Response: As suggested, the hyphen and spaces are removed.

• 05:43 "IV. - Conclusion" remove the hyphen and the spaces around it, having only one space between IV. and the title

Response: As suggested, the hyphen and spaces are removed.

3. Video Editing

4. • 01:24 Consider holding on this graphic for a few more moments before moving on to the next part.

Response: As suggested, the flowchart graphic display is extended for 10 seconds more to ease the reading.

5. • 02:50 There is a black flash frame here that should be removed / mended.

Response: the black flash frame is removed.

6. • 02:02 As at 01:24, consider holding on this graphic for another moment as well, so we have the chance to quickly read it.

Response: As suggested, the flowchart graphic is extended for 10 seconds more to ease the reading.

7. • 03:18 The words "and rinse them with autoclaved arro-" are mashed up against "take a blotting paper and fold it once"; sounds like an editing error

Response: The sentence runs as “and rinse with autoclaved RO (water)” with period. Then, starts with “take a blotting paper and fold it once”. The time between these two sentences is increased to have clear pause.

8. • 04:26 Please eliminate "jump cuts" such as this. Use dissolves (as at 04:25) to splice visually similar clips together instead of hard cuts, which creates a distracting "jump" effect on the content in the video. Please replace this style of edit in this and other sections with the dissolve as described. Another example is at 03:27, and there may be a few more.

Response: As suggested, the jump cuts were dissolved wherever applicable.

9. • 05:39 The slow zoom of the root graphic restarts here

Response: As suggested, the restarting graphic is removed.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript "Dry root rot disease assays in chickpea: a detailed methodology" presents the methodology for inoculation of Macrophomina phaseolina in order to test its pathogenicity or to select chickpea genotypes resistant to dry root rot (DRR).

The video may be useful for phytopathologists who intend to work with the fungus M. phaseolina. This fungus is difficult to produce inoculum and, therefore, difficult to be used in research studies in phytopathology.

Major Concerns:
There are two problems with the manuscript:

1. *Rhizoctonia bataticola* is an old and not valid name for this fungus and it must be named as Macrophomina phaseolina, which is the currently accepted name for this fungus.

Response: Fungi listed under ascomycetes and basidiomycetes divisions carry two synonyms representing the anamorph and teleomorph. Teleomorph and anamorph synonyms of dry root rot pathogen are *Macrophomina phaseolina* and *R. bataticola* respectively. Teleomorph specifies the sexual stage where sexual spores are seen, whereas anamorph specifies the asexual stage where vegetative stages like microsclerotia are seen (Agrios, 2004). In chickpea, only the microsclerotia stage is observed and pycnidial stages are not present. Hence, we would like to keep the *R. bataticola* name in our manuscript. Works of literature published on dry root rot in chickpea have been following the name *R. bataticola* (Nene et al., 1981; Sharma et al., 2015; Sinha et al., 2019; Khaliq et al., 2020)

On a related note: A similar name pattern is followed in the case of *Rhizoctonia solani*. Here, the teleomorph synonym is *Thanatephorus cucumeris*. However, *Rhizoctonia solani* has been used in the literature extensively.

2. Some small details of methodology are missing (see the text of the manuscript);
Minor Concerns:
Other minor comments were done in the manuscript text.

Reviewer #2:
Minor Concerns:
1. English language needs to be corrected thoroughly.

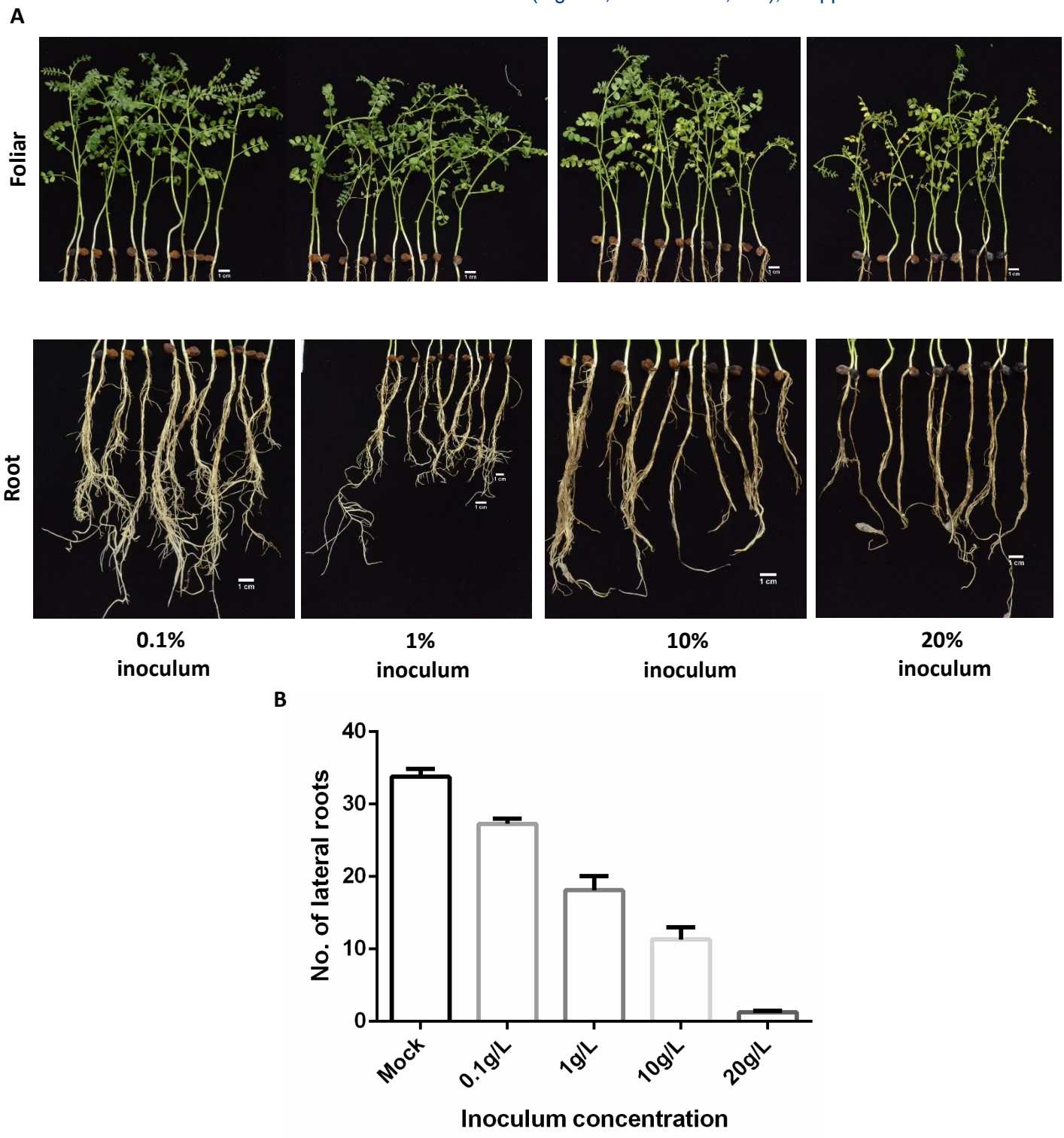
Response: As suggested, the English language is corrected throughout the manuscript.

2. used update reference e.g. Abdul Khaliqa et al 2020; Integrated control of dry root rot of chickpea caused by Rhizoctonia bataticola under the natural field condition.

-Cite this paper to substantiate

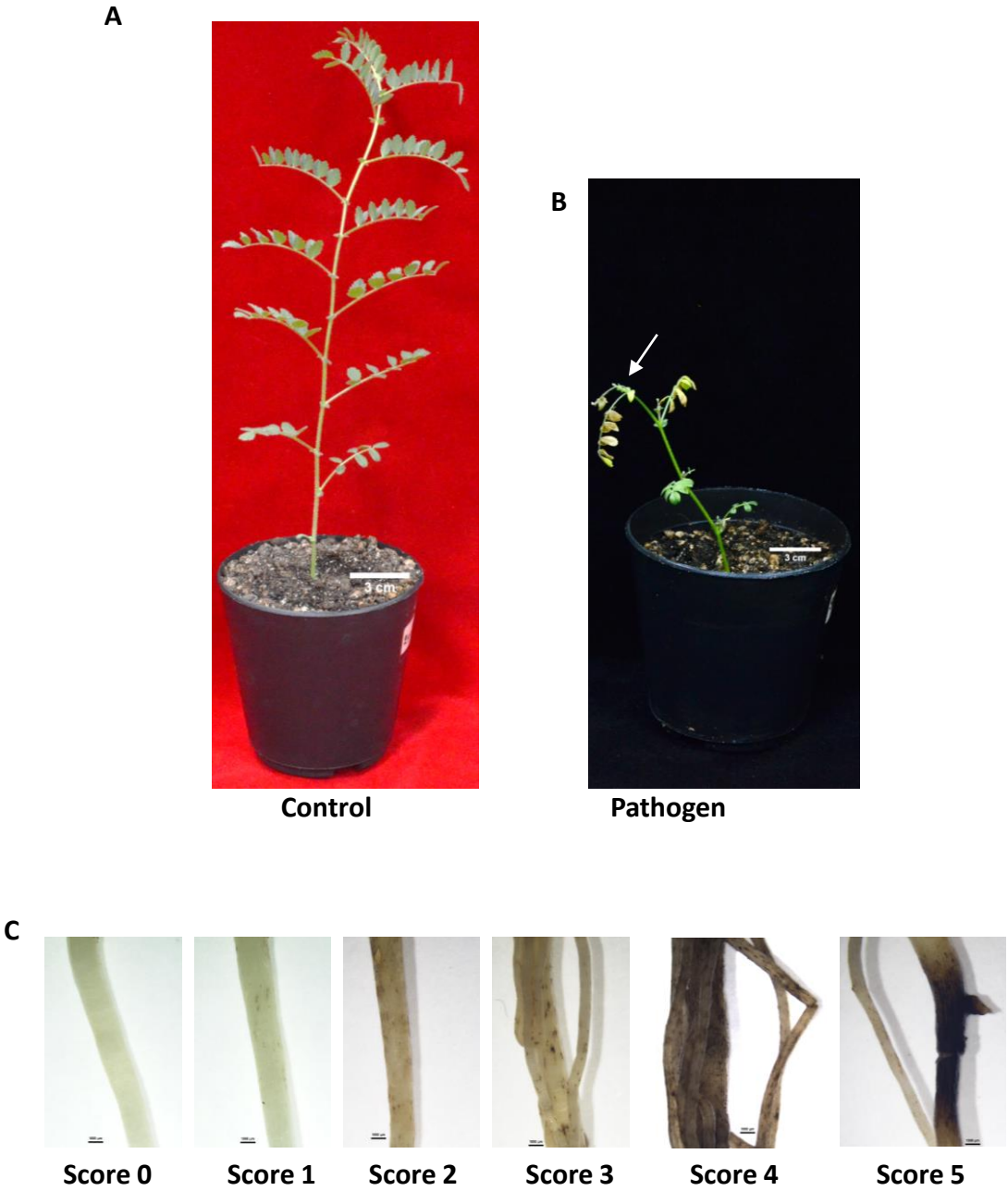
Response: This reference is cited in the manuscript as suggested.

Supplementary Figure 1



Supplementary Figure 1. Inoculum size and reduction in lateral root number in chickpea. Following the protocol depicted in figure 2**,** DRR susceptible plants (genotype, JG 62) were subjected to infection with varied size of inoculum using blotting paper technique, and symptoms were captured eight days after infection. Plants were inoculated with varied inoculum size (0.1%, 1%, 10% and 20% w/v in water)Images show the representative infected plants with typical symptoms such as wilting, yellowing, and drying of leaves and dried/necrotic roots with less lateral roots (B). Graph shows the number of lateral roots in plants under infection with inoculum variation (B). The scale bar is 1 cm. n=10.

Supplementary Figure 2



Supplementary Figure 2. Foliar symptoms of DRR in chickpea in soilrite. Following the protocol depicted in figure 4**, s**urface sterilized seeds were sown, and disease symptoms were captured on 14 days after sowing. The representative image shows the control plants (A), and the picture shows the typical DRR foliar symptoms, namely drying of plants (white arrows) (B). Disease score was developed based on necrotic spots on the roots. The scoring was developed across the days (C). The photographs (A&B) are from the same experiment. Scale bar= 3 cm. n= 10