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Use of Neem leaves as soil amendment for the control of collar rot disease of chickpea

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Abstract

Chickpea (*Cicer arietinum* L.) is an important grain legume that is attacked by the fungal pathogen, *Sclerotium rolfsii*, responsible for collar rot disease. In the present study, the pathogen was isolated from diseased chickpea seedlings and identified on molecular basis using internal transcribed spacer (ITS) and β-tubulin markers. To control this pathogen, an in vivo study was carried out, using Neem (*Azadirachta indica* A. Juss.) leaf dry biomass (1, 2, and 3%) as soil amendment. A broad-spectrum fungicide mancozeb was selected as a reference and the data regarding plant growth and mortality rates were calculated. In positive control, the highest seedling mortality (56%) was recorded in comparison to negative control (0%) after 30 days of sowing. In 1, 2, and 3% concentrations of *A. indica* dry leaf biomass, the seedling mortalities were 49, 38, and 38%, respectively. On the other hand, the mancozeb-treated seedlings showed the lowest plant mortality rate (28%). Soil treatments with mancozeb as well as with 1 and 2% leaf biomass concentrations showed marked differences in root and shoot dry biomasses over positive control. In a laboratory bioassay, methanolic leaf extract of Neem of 0.5 to 3.5% concentrations reduced biomass of *S. rolfsii* by 86–90% over control. The present study concluded that 2% *A. indica* leaf amendment was the most useful concentration for management of collar rot disease of chickpea.

Keywords: *Azadirachta indica*, Chickpea, Collar rot, Natural fungicide, Soil amendment

Background

Chickpea (*Cicer arietinum* L.) is an important grain legume providing an enormous source of minerals, fibers, and proteins both for humans and animals (Varol et al. 2020). It is considered as the drought resistant winter season annual crop grown widely in Pakistani and Indian subcontinent regions (Ilyas et al. 2007). In Pakistan, the total area under chickpea cultivation is 978,000 ha with an annual production of 340,000 t (Pakistan Economic Survey 2017-18). Also, the production of chickpea is very low due to many factors like pathogenic attacks and environmental stresses such as heat, cold, and salinity. A number of pathogens have been reported to attack the crop. Among these, a soil-borne fungal pathogen *Sclerotium rolfsii* is responsible for collar rot disease of chickpea. It was reported that the pathogen attacks the crop at seedling stage causing severe yield losses in tropical and sub-tropical areas of the world (Javaid and Khan 2016 and Tarafdar et al. 2018). Seedling mortality due to this disease ranges from 55 to 95% (Kokub et al. 2007). The pathogen was reported from Asia, South East Asia, Indian subcontinent, Australia, and America (Maurya et al. 2007). It can cause diseases in 500 host plant species belonging to 100 families (Remesal et al. 2013). It forms appreciable number of sclerotia, which can remain in soil for several years (Farr et al. 2007).

For the control of collar rot disease, generally chemical control is considered the most practical strategy (Javaid and Khan 2016). However, synthetic agrochemicals besides controlling pathogens also have toxic effects in soil, water, and food products; thus, their substantial use should be discouraged (Bundschuh et al. 2016). These are also detrimental to many non-target organisms. In addition, use of fungicides also leads to the emergence of many new fungicide resistant strains, resulting in disturbance in ecological balance (Weber et al. 2018).

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There is a need to replace synthetic agrochemicals with alternative environment friendly methods. In the recent years, many successful attempts have been made in this regard and scientists have reported that use of plant dry biomasses of *Chenopodium album*, *C. murale*, *Coronopus didymus*, *Eucalyptus citriodora*, *Eruca sativa*, and *Sisymbrium irio* as soil amendment can effectively control soil-borne pathogens namely, *Fusarium oxysporum*, *Sclerotium rolfsii*, *Rhizoctonia solani*, and *Macrophomina phaseolina* (Javaid et al. 2017a,b; Bajwa et al. 2019; Hadi et al. 2019; Naqvi et al. 2019 and Javaid et al. 2020).

The present study aimed to control collar rot disease of chickpea by practicing Neem leaf dry biomass as soil amendment.

Materials and methods

Molecular characterization of *S. rolfsii*

S. rolfsii was isolated from diseased seedlings of chickpea. Molecular characterization was done to confirm its identity with internal transcribed spacer (ITS) and beta tubulin markers through PCR amplification (Table 1). Pure culture of *S. rolfsii* was scratched from 2-week-old malt extract agar (MEA) plates and DNA was isolated by CTAB method (Doyle and Doyle 1990), using the fungal DNA extraction kit of GeneAll Biotechnology Co., Ltd. PCR was performed in a 30-μl reaction system with ITS and beta tubulin primer pairs for rDNA amplification. Amplified PCR products were got sequenced from 1st Base Sequencing Singapore Co., Ltd.

In vitro antifungal activity of methanolic leaf extract

For preparation of extract and antifungal bioassays, protocol given by Ali et al. (2017a) was adopted with little modifications. *A. indica* leaves were collected from Lahore, Pakistan, dried and crushed. Finely crushed material (200 g) was used for extraction in 1.0 l of methanol for 10 days and filtered subsequently through a Whatman No. 1 filter paper. Next, the filtrate was undergone to a rotary evaporator at 45 °C to get 20 g of thick gummy material.

For the preparation of stock solution, 11.2 g of crude methanolic extract was dissolved in 6 ml of dimethyl sulfoxide (DMSO) and raised the volume up to 14 ml by adding distilled water. A control solution was prepared simultaneously with the same amount of DMSO and water, but without the addition of plant extract. Seven concentrations *viz.* 3.5, 3.0, 2.5, 2.0, 1.5, 1.0, 0.5, 0 g 100 ml^{-1} were formulated by the addition of 3.5, 3.0, 2.5, 2.0, 1.5, 1.0, 0.5, and 0 ml of stock solution and 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 ml control solution, respectively, to different flasks having 76.5 ml of malt extract broth then mixed well and poured into 4 equal aliquots in 100 ml flasks, each was run simultaneously as a replicate. Mycelial plugs (5 mm) of 7-day-old *S. rolfsii* culture were added in each conical flask and left to stand for 7 days at 25 ± 2 °C. Thereafter, the fungal biomass was filtered and dried in an electric oven at 60 °C (Javaid et al. 2017c).

Pot trial

S. rolfsii inoculum was prepared on 1.0 kg boiled and autoclaved seeds of pearl millet in three 500 ml of conical flasks and kept for 15 days in an incubator at 25 ± 2 °C until profuse sporulation occurred. Freshly prepared 15 g of fungal inoculum was then mixed in 1 kg of fumigated sandy loam soil in 10-cm deep and 15-cm diameter plastic pots, except in negative treatment which was supplied only by 15 g of boiled pearl millet and left for 10 days. After that, the soils of the respective pots were amended with *A. indica* dry leaf powder at different concentrations *viz.* 1, 2, and 3% (w/w) and irrigated. The pots were left for 1 week for leaching of soil (Javaid and Saddique 2011). Mancozeb fungicide concentration of 150 ppm was prepared and applied (0.5 l pot^{-1}) to pots of respective treatment.

The following 6 treatments, with 5 replicates each were arranged in a completely randomized design:

T_1 Negative control (without *S. rolfsii* or dry leaf biomass)

T_2 Positive control [inoculated with *S. rolfsii* (SR)]

T_3 SR + mancozeb

T_4 SR + 1% dry leaf biomass (DLB) of *A. indica*

T_5 SR + 2% DLB of *A. indica*

T_6 SR + 3% DLB of *A. indica*

Chickpea variety CMS-2118-2508 was obtained from National Agriculture Research Centre (NARC) Islamabad, Pakistan. Seeds were surface sterilized for 3 min in 1% sodium hypochlorite solution, followed by consecutive washings in autoclaved distilled water. Eight seeds were sown in each plastic pot and irrigated on regular basis. The pots were monitored regularly for the development of collar rot symptoms, which were

Table 1 List of oligonucleotide primers used for the characterization of *Sclerotium rolfsii* at molecular level

No.	Primer name	5′ to 3′ sequence	Annealing temperature
1	ITS 1 forward	TCCGTAGGTGAACCTGCGG	60 °C
2	ITS 4 reverse	TCCTCCGCTTATTGATATGC	
3	β-tubulin forward	GGTAACCAAATCGGTGCTGCTTTC	62 °C
4	β-tubulin reverse	ACCCTCAGTGTAGTGACCCTTGGC	

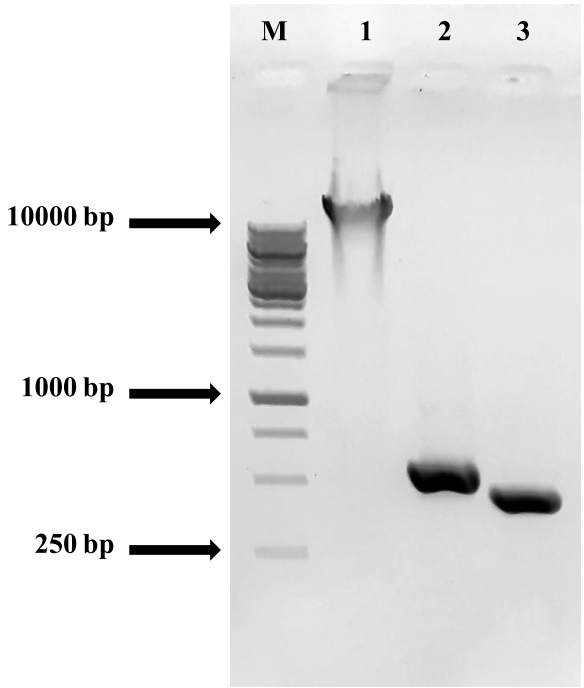

Fig. 1 *Sclerotium rolfsii* (M), 1 kb DNA standard marker; (1), Genomic DNA; (2), ITS1/ITS4 amplified PCR product; (3), Bt_{2a}/Bt_{2b} amplified PCR product

confirmed later on 30th day after the seed sowing. Then the plants were uprooted to measure shoot and root lengths and fresh and dry weights. Data regarding plant mortality were recorded carefully after 15 and 30 days of seed sowing by applying the following formula:

$$\text{Mortality } (\%) = \frac{\text{No.of plants died due to disease}}{\text{Total No.of plants}} \times 100$$

Statistical analysis

All the data were analyzed by ANOVA, followed by Tukey HSD test ($P \leq 0.05$), using computer software Statistix 8.1.

Results and discussion

Identification based on rDNA sequence analysis

Nucleotide sequence analyses of ITS and beta tubulin regions of rDNA were carried out to characterize the *S. rolfsii* genetically. PCR amplified products of ITS and beta tubulin rDNA yielded approximately 527 and 448 bp amplicon sizes, respectively, when visualized at 1% agarose gel (Fig. 1). Nucleotide sequences of ITS and beta tubulin amplified rDNA were deposited in GenBank under the accession No. MT573510 and MN736404, respectively.

In vitro antifungal bioassays

In vitro effect of methanolic leaf extract against the growth of *S. rolfsii* was illustrated in Fig. 2. It clearly

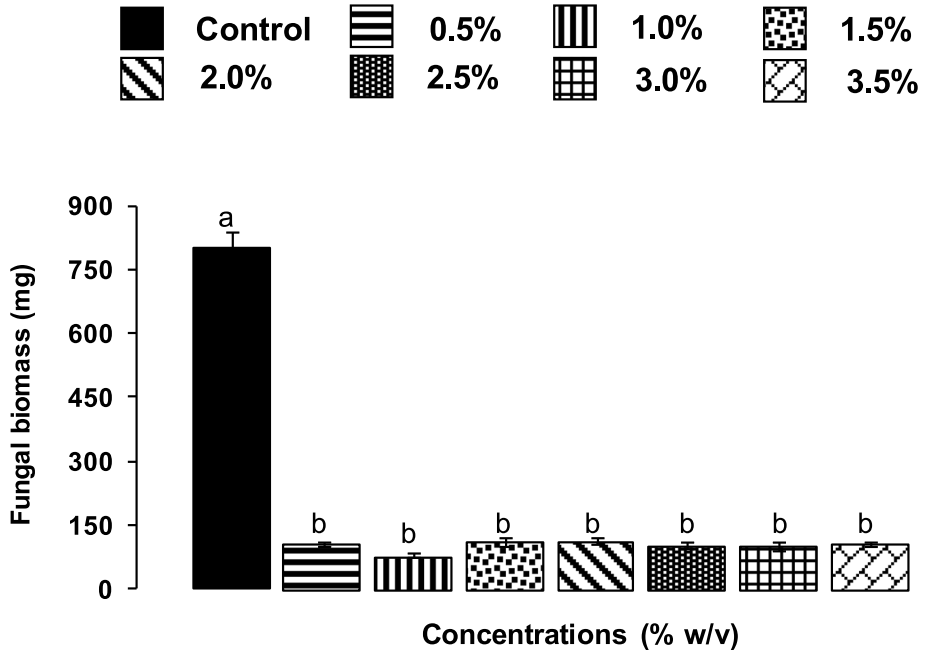

Fig. 2 Effect of different concentrations of methanol leaf extract of *Azadirachta indica* on biomass of *Sclerotium rolfsii*. Vertical bars show standard errors of means of five replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by Tukey HSD test

indicated that all the concentrations significantly ($P \leq 0.05$) reduced the fungal biomass than the control. Different concentrations were found to reduce the fungal biomass by 86–90% over control treatment. These findings are in the favor of work of Obongoya et al. (2010) who evaluated the antifungal efficacy of Neem extract against *Fusarium oxysporum* with promising results. Generally, plant extract efficacy was influenced by the level of concentrations used in experimental work; however, in the present study, the increase in concentration did not result in a parallel decline in mycelial growth of the pathogen. Likewise, Akaeze and Aduramigba-Modupe (2017) checked in vitro efficacy of Neem plant extracts against *F. oxysporum*, where all the concentrations of methanolic extract were found almost equally effective against *F. oxysporum.*

Pot trial

After 15 days of sowing, the highest seedling mortality (42%) was recorded in positive control, which increased to 56% after 30 days of sowing. Mancozeb application was highly effective in reducing plant mortality. In this fungicidal treatment, the plant mortality was just 3% after 15 days of sowing that was increased to 28% after 30 days of sowing. The lowest concentration (1%) of dry leaf biomass (DLB) did not show any remarkable effect as there were 33 and 49% disease incidences after 15 and 30 days of sowing, respectively. However, 2 and 3% DLB significantly reduced disease incidences over positive control both after 15 and 30 days of sowing (Fig. 3).

In pot trials, the collar rot of chickpea was managed through the use of a fungicide namely, mancozeb and Neem dry leaf biomass. Disease highly occurred in positive control, whereas negative control was disease free. The disease incidence was reduced significantly in Neem dry leaf biomass and mancozeb-treated soils. The fungicide gave better results than the dry leaf biomass. There are reports that fungicides are highly effective for the control of target fungus *S. rolfsii* (Shirsole et al. 2019). Mancozeb proved to be a broad-spectrum fungicide effective against downy mildew, collar rot, foot rot, damping-off, stem rot, root rot, southern blight, bulb rot, and wilt diseases caused by *Lagenaria siceraria*, *Alternaria solani*, *Rhizoctonia solani*, *Sclerotium rolfsii*,

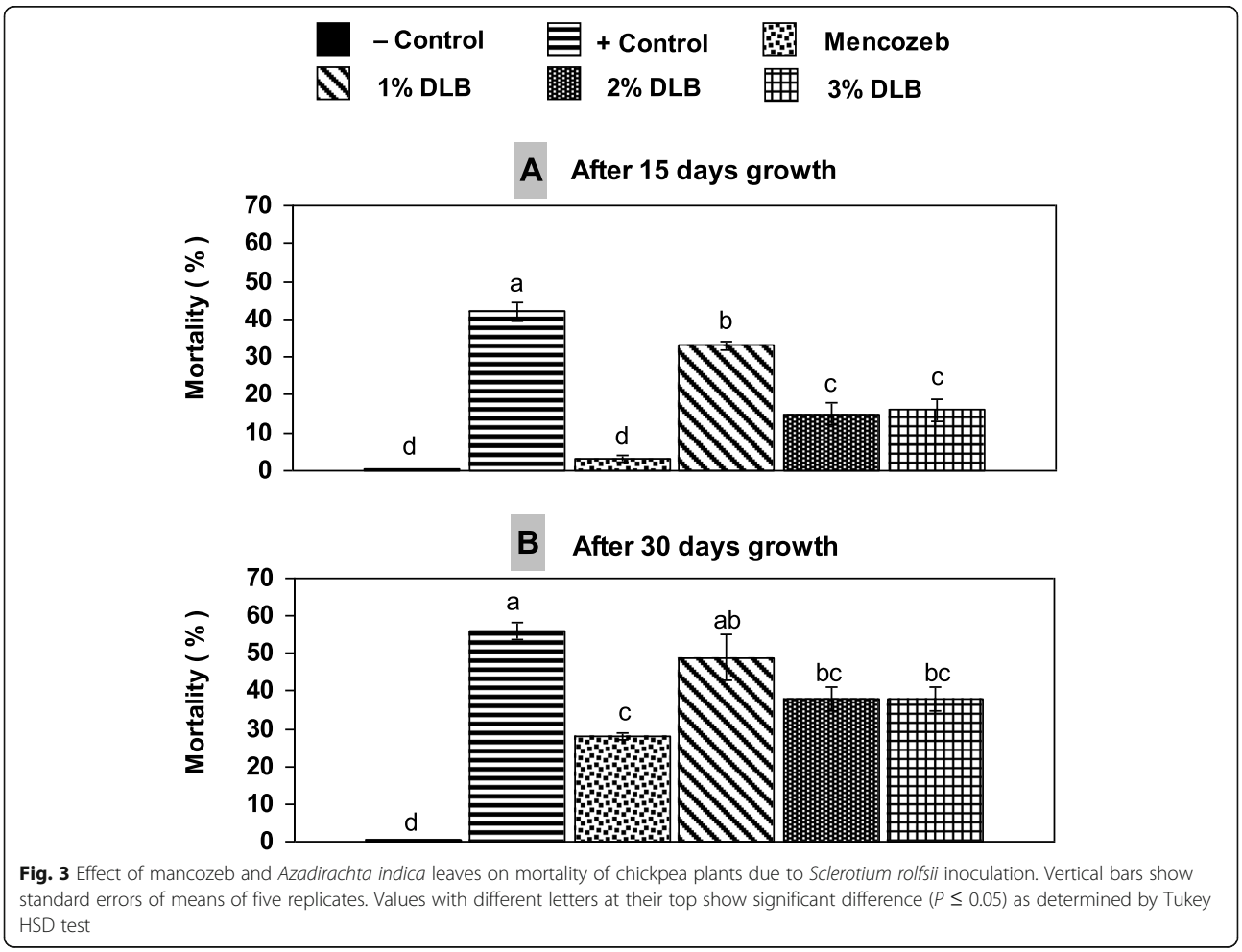

Fig. 3 Effect of mancozeb and *Azadirachta indica* leaves on mortality of chickpea plants due to *Sclerotium rolfsii* inoculation. Vertical bars show standard errors of means of five replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by Tukey HSD test

R. bataticola, and *F. oxysporum* (Majumder et al. 2016; Gowari et al. 2017; Bagri et al. 2019; Michel-Aceves et al. 2019 and Vani et al. 2019). Rather et al. (2012) concluded that many fungicides like tebuconazole, penconazole, vitavax, hexaconazole, thiophanate methyl, and mancozeb were effective against *S. rolfsii*. Similarly, Madhavi and Bhattiprolu (2011) reported that in field experiments, mancozeb was highly effective for the control of *S. rolfsii* responsible of dry root rot in chilies. In a previous study, it was also reported that *F. oxysporum* isolated from the cucumber-infected seeds was completely inhibited even at lower concentrations of mancozeb (Sultana and Ghaffar 2013).

All the concentrations of Neem dry leaf biomass significantly reduced the pathogen growth and disease incidence over control. In a previous study, Obongoya et al.

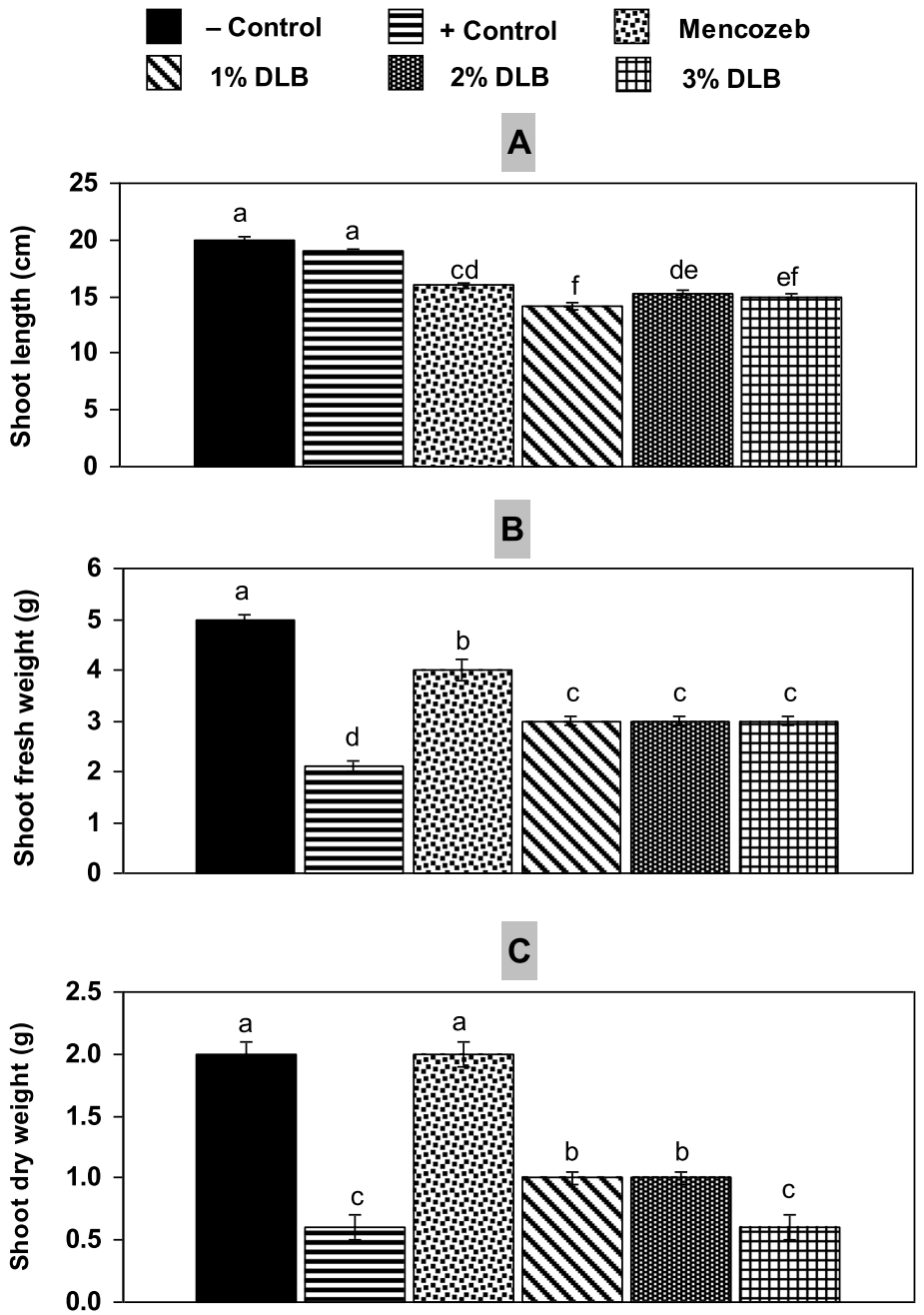

Fig. 4 Effect of mancozeb and *Azadirachta indica* leaves on shoot growth of chickpea under biotic stress of *Sclerotium rolfsii*. Vertical bars show standard errors of means of five replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by Tukey HSD test

(2010) revealed that *F. oxysporum*, the pathogen of yellow disease, can effectively be managed through the botanicals isolated from Neem plant. In their study, soil amendment with Neem was also observed to be effective in enhancing the plant size and seed germination with minimum seedling mortality by *F. oxysporum*. Ezeonu et al. (2018) also gave similar report regarding the effectiveness of Neem seed, bark, and leaf ethanolic extracts against fungal pathogens namely, *Aspergillus oryzae*, *A. niger*, *Rhizopus stolonifer*, *A. ochraceus*, and *Lasiodiplodia theobromae* responsible for rot diseases in cocoyam and yam plants. Obtained results agree with the findings of Ali et al. (2017b) who discovered that nano emulsions of Neem represent excellent antifungal activity against many phytopathogenic fungi such as follows: *S. rolfsii* and *Rhizoctonia solani* that cause collar rot, damping-off, wilting, and dry root rot under field conditions.

The effect of *A. indica* DLB and fungicide on the shoot growth of chickpea is shown in Fig. 4. There was insignificant difference in shoot length between positive and negative control treatments. Applications of 1, 2, and 3% DLB of *A. indica* and mancozeb significantly reduced shoot length over negative and positive controls. In contrast, shoot fresh weight at 1 and 2% DLB amended treatments showed significant increases over positive control, whereas in mancozeb treatment, it was significantly lower than negative control. Shoot dry biomass in 1% amendment was at par with negative control. However, as the concentration of DLB was increased, it adversely affected the production of shoot biomass.

The effect of DLB and fungicide on the root growth is shown in Fig. 5. Significant reductions in positive and negative control root lengths with enhanced dry root weights were recorded in comparison to mancozeb application. The root lengths and dry biomasses increased significantly in 1, 2, and 3% concentrations of DLB over positive control. The effect of 1% DLB was more pronounced than the effect of higher concentrations of 2 and 3%. In general, increase in DLB dose resulted in reduction of root growth parameters.

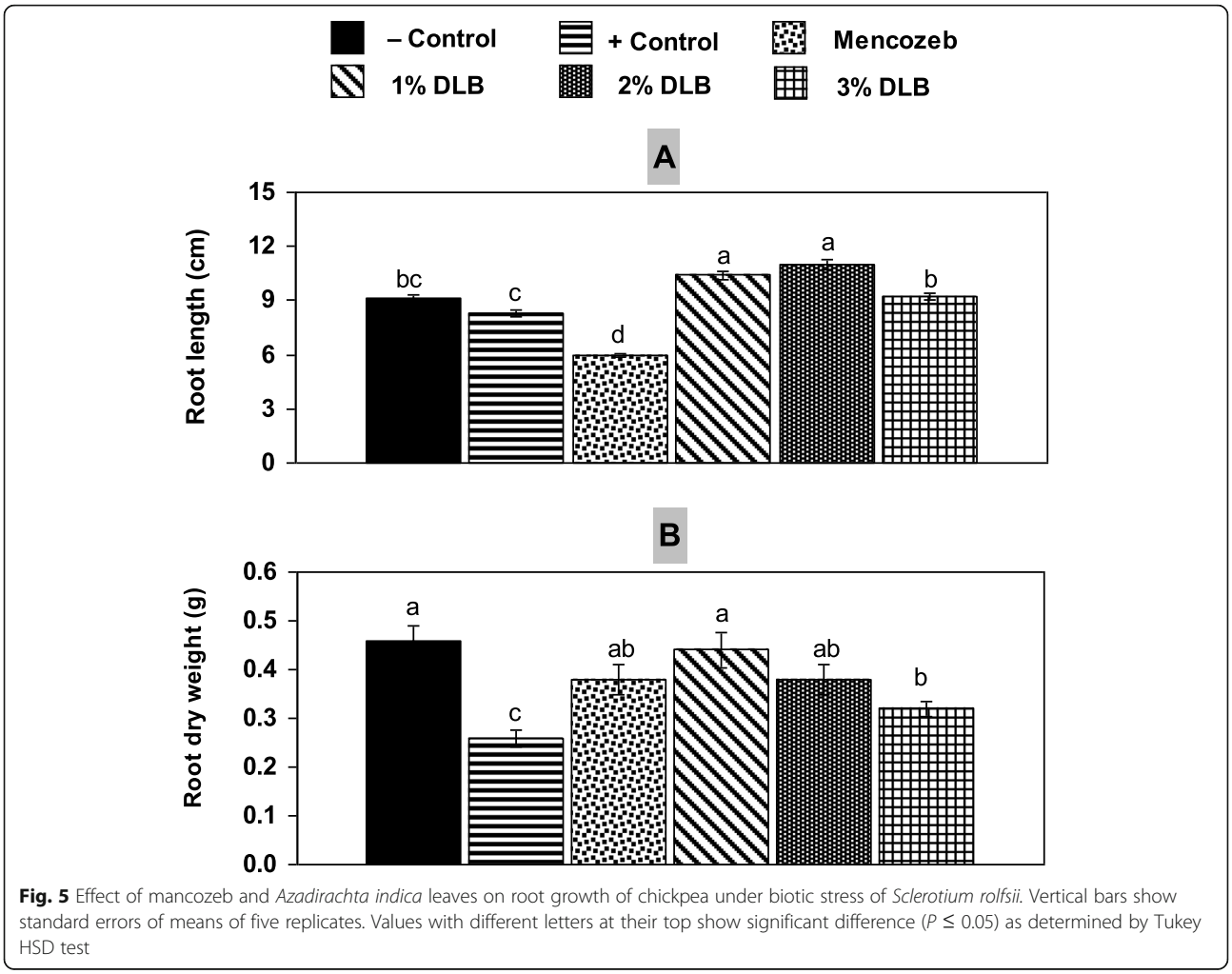

Fig. 5 Effect of mancozeb and *Azadirachta indica* leaves on root growth of chickpea under biotic stress of *Sclerotium rolfsii*. Vertical bars show standard errors of means of five replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by Tukey HSD test

Conclusion

The present findings clearly indicated that Neem leaf extract and soil amendment with leaf dry biomass were highly effective in controlling in vitro and in vivo growth of *S. rolfsii*.

Abbreviations

ITS: Internal transcribed spacer; MEA: Malt extract agar; CTAB: Cetyl trimethyl ammonium bromide; PCR: Polymerase chain reaction; ANOVA: Analysis of variance; DLB: Dry leaf biomass

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Not applicable

Authors' contributions

Miss Iqra Haider Khan conducted the study and wrote a part of the manuscript. Arshad Javaid supervised the work, analyzed the data, and approved the final manuscript. Azher Hameed Al-Taie contributed in paper writing. Dildar Ahmed did GC_MS analysis. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors are agreed to publish this paper.

Competing interests

The authors declare that they have no competing interests.

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