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# Control of the chickpea blight, *Ascochyta rabiei*, with the weed plant, *Withania somnifera*

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## Abstract

**Background:** *Ascochyta* blight caused by a fungal pathogen, *Ascochyta rabiei*, is a serious disease of chickpea in most chickpea growing areas of the world. The disease can be controlled by fungicides to reduce the environmental pollution.

**Main body:** This study reports the control of *Ascochyta* blight by using extracts and dry biomass of a weed plant, *Withania somnifera* (Family Solanaceae). In a laboratory bioassay, a 0.2% concentration of methanolic leaf extract of *W. somnifera* significantly inhibited the pathogenic fungal growth. Methanolic leaf extract was fractionated with 4 organic solvents of different polarities namely *n*-hexane, chloroform, ethyl acetate, and *n*-butanol. The effect of different concentrations (3.125, 6.25, 12.5, 25, 50, 100, 200 mg ml<sup>-1</sup>) of the 4 organic solvent fractions was assessed on in vitro growth of the pathogen. Among these, ethyl acetate and *n*-butanol fractions completely inhibited the fungal growth. Ethyl acetate fraction was further subjected to thin layer chromatography to separate the potent antifungal compounds A and B. Compound A was found highly effective against the targeted fungal pathogen with MIC 31.25 µg ml<sup>-1</sup>, followed by B with MIC value of 250 µg ml<sup>-1</sup> as compared to 7.81 µg ml<sup>-1</sup> MIC of a commercial fungicide mancozeb. In pot trial, the soil was amended by 1, 2, and 3% dry leaf material of the test plant species. Disease incidence was significantly reduced in 3% dose.

**Conclusions:** This study concludes that *W. somnifera* contains potent antifungal compounds that can be effectively exploited for the control of *A. rabiei*.

**Keywords:** *Ascochyta rabiei*, Bioassays guided fractionation, *Cicer arietinum*, *Withania somnifera*

## Background

Chickpea is an excellent drought-tolerant grain legume (Merga and Haji 2019). It plays a vital role in fulfilling the nitrogen requirement through symbiotic N<sub>2</sub> fixation and increasing soil fertility (Girma et al. 2017). *Ascochyta rabiei* is considered a very devastating fungal pathogen responsible for *Ascochyta* blight to chickpea (Deokar et al. 2019). The disease starts from distal plant portion which may result in wilting with eventual death of the infected plant (Mahmood et al. 2019). The diseased plant shows

dark brown to black lesions of variable sizes on pods, seeds, branches, stem, leaflets, and stalk.

The pathogen remains viable for more than 2 years on infected seeds and crop residue (Kaiser 1981). It can be effectively managed through chemical practices such as seed and foliar applications of synthetic fungicides (Namriboi et al. 2018). However, due to ill effects of fungicides on environment, use of natural plant-based products against this pathogen is being explored (Shuping and Eloff 2017). Many of the plants are rich in secondary metabolites that have inhibitory effects against the fungal pathogens (Bottger et al. 2018; Akhtar et al. 2020; Javaid et al. 2020). Moreover, the exploitation of phytochemicals is safe,

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eco-friendly, and economical in use when compared to fungicides (Palanichamy et al. 2018).

*Withania somnifera* (Family Solanaceae) is an important wild plant being used in folk medicines a thousand years ago (Dhanani et al. 2017). It is a small woody shrub native to Indian subcontinent, Southern Europe, and North Africa (Aslam et al. 2017). It contains interesting chemical constituents' profile that are biologically active such as alkaloids, withanone, withasomniferols A-C, steroidal compounds, steroidal lactones, withanolides A-Y, withaferin A, withasomniferin-A, and withasomnidienone (Tiruveedi et al. 2018). This plant has been studied widely to explore antifungal, antibacterial, antimicrobial, pharmaceutical, antioxidant, and anti-inflammatory properties (Shuaib et al. 2019). Previous studies have shown that many members of family Solanaceae exhibited antifungal activities against various fungal plant pathogens (Nino et al. 2006). However, studies regarding the use of *W. somnifera* against *A. rabiei* are lacking. Therefore, the present study was carried out to evaluate the activity of leaf extract and dry biomass of *W. somnifera* for in vitro and in vivo control of *A. rabiei*.

## Materials and methods

### Isolation of the fungal pathogen

Chickpea pods infected with *Ascochyta* blight were dried and surface-treated with 1% sodium hypochlorite solution for 1 min and sterile water for 1 min. The diseased samples were cut into small pieces and placed on potato dextrose agar (PDA)-containing Petri dishes supplemented with chloromycetin ( $0.5 \text{ g l}^{-1}$ ) and chickpea flour ( $10 \text{ g l}^{-1}$ ) and incubated at  $24^\circ\text{C}$  for 2 weeks (Bahr et al. 2016). The growing fungus was subcultured on PDA and was identified based on its phenotypic characters (Aveskamp et al. 2010).

### Bioassay-guided fractionation

Leaves of *W. somnifera* were collected from Lahore, Pakistan, washed and air dried. Dried leaf material (2 kg) was dipped in methanol (7.0 l) for 10 days, followed by a filtration procedure. The filtrate was evaporated on a rotary evaporator in order to get a concentrated gummy extract. After that, 2.4 g of the concentrated extract was mixed with autoclaved distilled water to prepare 3 ml of a stock solution. Five concentrations, viz., 0.2, 0.4, 0.6, 0.8, and 1.0 g,  $100 \text{ ml}^{-1}$  were prepared by addition of 0.2, 0.4, 0.6, 0.8, and 1.0 ml stock solution and 0.8, 0.6, 0.4, 0.2, and 0 ml autoclaved  $\text{d.H}_2\text{O}$ , respectively, in each 100-ml conical flask and raised the volume up to 80 ml by adding malt extract (ME) broth and divided into 4 equal aliquots. Similarly, a control set was also prepared without addition of plant extract. Actively growing *A. rabiei* culture mycelial plugs (diameter 5 mm) were added aseptically in all the conical flasks and kept for 10

days at  $18 \pm 2^\circ\text{C}$ . After that, fungal mats from each flask were harvested, dried, and weighed (Iqbal and Javaid 2012).

Three hundred milliliters of autoclaved  $\text{d.H}_2\text{O}$  was mixed with 247 g of methanolic leaf extract, followed by a partitioning process with *n*-hexane ( $2 \times 500 \text{ ml}$ ), chloroform (600 ml), ethyl acetate (400 ml), and *n*-butanol (300 ml) in a separating funnel; then, the solvents on a rotary evaporator were evaporated. Each of the extract fractions (1.2 g) was dissolved in dimethyl sulfoxide (1 ml) and raised volume up to 6 ml with the addition of ME broth to make a stock solution. Different concentrations 200, 100, 50, 25, 12.5, 6.25, and  $3.125 \text{ mg ml}^{-1}$  were formed by a serial double dilution with 3 replicates each. A control solution was also prepared in a similar way by adding 1 ml of dimethyl sulfoxide (DMSO) and ME broth (5 ml). *A. rabiei* conidial suspension ( $20 \mu\text{l}$ ) was added to each test tube and left for 10 days at  $18 \pm 2^\circ\text{C}$ . Then, the fungal mats from each test tube were harvested, dried, and weighed (Khan and Javaid 2020).

Thin layer chromatography (TLC) was performed by the ethyl acetate fraction, followed by a preparative TLC for the separation of the volatile organic antifungal compounds. The solvent system used for TLC was comprised of *n*-hexane and ethyl acetate in 3:2 ratio. Compounds A and B were separated from the tested fraction with 0.763 and 0.842  $R_f$  values, respectively. Both the isolated compounds and mancozeb were used against the pathogen to evaluate the MIC values. For this, mancozeb and the compounds (8 mg) were dissolved in DMSO (0.25 ml) with subsequent addition of ME broth (1.75 ml) serving as a stock solution. Further concentrations, viz., 2000, 1000, 500, 250, 125, 62.5, 31.25, 15.62, and  $7.81 \mu\text{g ml}^{-1}$ , were formed from the stock solution of  $4000 \mu\text{g ml}^{-1}$  by serial double dilutions. A control was also prepared simultaneously by adding DMSO (0.25 ml) and ME broth (1.75 ml) with 3 replicates of each. One drop of *A. rabiei* conidial suspension was added in each glass tube. After 72 h, data regarding MIC was recorded by visual determination of the fungal mycelial growth.

### Pot trial

The in vivo study was carried out in plastic pots of 10-cm deep inside, filled with sandy loam soil at  $350 \text{ g pot}^{-1}$ . Each pot soil was inoculated by *A. rabiei* inoculum (5 g) prepared on boiled chickpea seeds and left for 7 days for the establishment of the pathogen. The soil was amended with 3 different concentrations of 1, 2, and 3% (w/w) dry leaf biomass of *W. somnifera*. Negative control was prepared by adding sterile boiled chickpea seeds ( $5 \text{ g pot}^{-1}$ ), whereas the positive control was only inoculated by the fungal inoculum ( $5 \text{ g pot}^{-1}$ ) with 10 replicates/treatment. Three surface-sterilized chickpea seeds in sodium hypochlorite solution

(1%) were sown in each pot and watered on regular basis. Data regarding disease incidence and shoot and root growth were recorded after 10 weeks of seed sowing. Disease incidence was calculated by the following formula (Javaid and Iqbal 2014):

$$\text{Disease incidence (\%)} = \frac{\text{No. of diseased plants}}{\text{Total number of plants}} \times 100$$

**Statistical analysis**

Experimental data were analyzed by ANOVA, and the treatment means were separated by Duncan’s multiple range test using software COSTAT at  $P \leq 0.05$ .

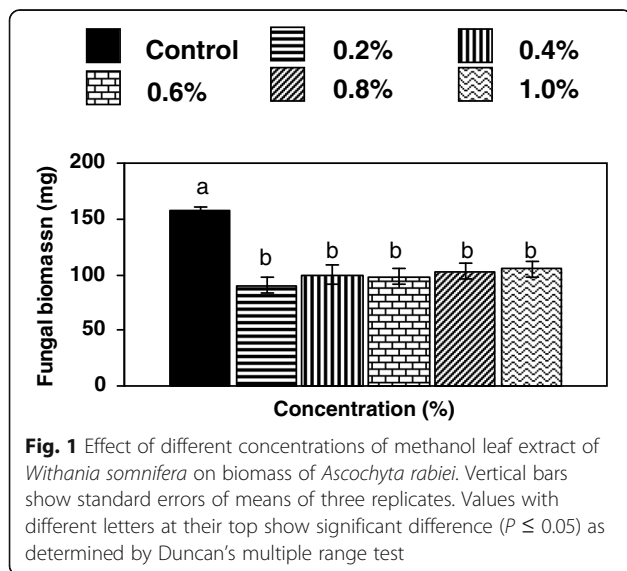
**Results and discussion**

**In vitro bioassay**

*W. somnifera* leaf extract proved to be extremely effective in inhibiting the growth of chickpea blight pathogen. Different concentrations markedly reduced the fungal biomass by 33–43% as shown in Fig. 1. Earlier, Nefzi et al. (2016) studied the antifungal effects of aqueous and organic solvent extract of various parts of *W. somnifera* and reported that a 3% aqueous leaf extract reduced growth of *Fusarium oxysporum* f. sp. *radicis-lycopersici* by 45%. A 200 mg ml<sup>-1</sup> concentration of *n*-butanol fraction of methanolic leaf extract of this plant also significantly reduced growth of *F. oxysporum* f. sp. *cepa*e (Akhtar and Javaid 2018). Aqueous and methanolic extracts of leaves and other parts of this plant have been also reported to have pronounced antifungal activity against a number of fungal species namely: *Candida albicans*, *Curvularia lunata* and *Alternaria alternata*, *A. brassicae*, *A. solani*, and *Phytophthora infestans*. Extracts

of *W. somnifera* had the ability to suppress germination of spores/conidia as well as to arrest the mycelial growth (Baka 2010 and Sailaja 2014). Antifungal activity of *W. somnifera* is related to various alkaloids and steroidal lactones (Singh et al. 2010). There are also many previous reports, where *A. rabiei* showed susceptibility to many other plant extracts. Yilar and Bayar (2019) reported an antifungal potential of *Salvia tomentosa* and *S. officinalis* extracts against *A. rabiei* isolates. Likewise, Amin et al. (2012) demonstrated that the bark and stem methanolic extracts of *Eucalyptus citriodora* also had antifungal efficacy against this pathogen. Similarly, Ghanfar et al. (2011) worked on different plant leaf extracts, viz., *Allium sativum*, *Azadirachta indica*, and *Datura metel*, against *A. rabiei* and found that *A. indica* extract was highly antifungal against the pathogen. Moreover, Onaran (2016) tested different plant extracts namely: *Heracleum platytaenium*, *Vitex agnus-castus*, *Ricinus communis*, *Polygonum cognatum*, and *Isatis glauca* prepared in different organic solvents and found all the extracts highly inhibitory against the mycelial growth of the pathogen. *R. communis* extract caused 100% control of the pathogen.

The highest and the second highest concentrations of DMSO in control solution adversely effected the pathogen growth with 100% inhibition. Therefore, these 2 concentrations were not considered in cases of the 2 highest concentrations (100 and 200 mg ml<sup>-1</sup>) of different fractions of leaf extract. The lowest concentrations of *n*-hexane fraction reduced the fungal biomass by 75–89%, and the highest concentration (50 mg ml<sup>-1</sup>) arrested the pathogen growth by 99%. High concentrations of chloroform fraction, viz., 12.5, 25, and 50 mg ml<sup>-1</sup>, completely retarded the pathogenic growth, while its low concentration of 3.12 and 6.25 mg ml<sup>-1</sup> reduced pathogen biomass by 74 and 77%, respectively. The other 2 fractions, namely, *n*-butanol and ethyl acetate, were the most antifungal, where all the concentrations completely controlled the pathogen growth. Different concentrations of aqueous fraction, viz., 50, 25, 12.25, 6.25, and 3.12 mg ml<sup>-1</sup>, stimulated the growth of the pathogen by 81, 52, 48, 63, and 22%, respectively (Table 1). In the present study, leaf methanolic extract was fractionated by using a variety of organic solvents each having different polarity. This process separated compounds of methanolic extract into different groups depending upon polarity nature of organic solvents. Different organic fractions showed variable antifungal activities when bioassays were carried out against *A. rabiei*. Among these, the most polar organic fractions, viz., *n*-butanol and ethyl acetate, were very effective in reducing the pathogen growth. Both the fractions were effective at very low concentration, i.e., 3.125 mg ml<sup>-1</sup>. Chloroform fraction with MIC of 12.5 mg ml<sup>-1</sup> was moderately effective. None of the applied concentrations of the



**Table 1** Effect of different concentrations of *n*-hexane, chloroform, ethyl acetate, *n*-butanol, and aqueous fraction of methanolic leaf extract of *Withania somnifera* against *Ascochyta rabiei*

Methanolic fraction	Conc. of DMSO (ml ml <sup>-1</sup> )	Extract conc. (mg ml <sup>-1</sup> )	Fungal biomass (mg)
Control	0.1666	0	0 k
	0.0833	0	0 k
	0.0416	0	1.1 hi
	0.0208	0	1.93 g
	0.0104	0	2.56 f
	0.0052	0	3.06 e
	0.0025	0	4.53 c
<i>n</i> -hexane	0.1666	200	0 k
	0.0833	100	0 k
	0.0416	50	0.1 k
	0.0208	25	0.2 k
	0.0104	12.5	0.5 j
	0.0052	6.25	0.63 j
	0.0025	3.125	1.1 i
Chloroform	0.1666	200	0 k
	0.0833	100	0 k
	0.0416	50	0 k
	0.0208	25	0 k
	0.0104	12.5	0 k
	0.0052	6.25	0.7 j
	0.0025	3.125	1.16 hi
Ethyl acetate	0.1666	200	0 k
	0.0833	100	0 k
	0.0416	50	0 k
	0.0208	25	0 k
	0.0104	12.5	0 k
	0.0052	6.25	0 k
	0.0025	3.125	0 k
<i>n</i> -butanol	0.1666	200	0 k
	0.0833	100	0 k
	0.0416	50	0 k
	0.0208	25	0 k
	0.0104	12.5	0 k
	0.0052	6.25	0 k
	0.0025	3.125	0 k
Aqueous	0.1666	200	0 k
	0.0833	100	1.3 h
	0.0416	50	2 g
	0.0208	25	2.93 e
	0.0104	12.5	3.8 d
	0.0052	6.25	5 b
	0.0025	3.125	5.56 a

non-polar *n*-hexane fraction completely controlled the fungal growth. Similar variability was also observed in antifungal activities of different fractions of *Coronopus didymus* leaf extract against *Sclerotium rolfsii* (Iqbal and Javaid 2012), leaf extract of *Datura metel* against *Macrophomina phaseolina* (Javaid and Saddique 2012), radish extract against *Fusarium oxysporum* (Javaid and Bashir 2015), and *Nigella sativa* extract against *M. phaseolina* and *F. oxysporum* (Aftab et al. 2019).

Mancozeb was highly effective, suppressed the fungal growth with 7.81  $\mu\text{g ml}^{-1}$  MIC. The two compounds of ethyl acetate fraction were less effective against *A. rabiei* than mencozeb. Compound A exhibited better results in inhibiting the growth of fungal pathogen with the minimum inhibitory concentration of 31.25  $\mu\text{g ml}^{-1}$ . Compound B was less toxic with MIC 250  $\mu\text{g ml}^{-1}$  (Table 2). A variety of low molecular weight secondary metabolites was reported in *W. somnifera* including withanolides, tannins, alkaloids, flavonoids, terpenoids, and resins (Dahr et al. 2015). Free and bound flavonoids of this plant suppressed the growth of *C. albicans* (Singh and Kumar 2011). Ghosh (2009) isolated a 30 kDa monomeric acidic lectin-like protein from leaves of *W. somnifera* and found it as effective against fungi as standard lectins.

In vertical column, values with different letters show significant difference ( $p \leq 0.05$ ) as determined by Duncan's multiple range test

#### Pot trial

Data regarding the effect of dry leaf biomass of *W. somnifera* (LBW) on germination of chickpea seeds in *A. rabiei*-inoculated soil is presented in Fig. 2a. The negative control showed the highest germination of 68%, where the pot soil was non-amended and seeds were sown in non-inoculated soil. In positive control, seeds were sown in *A. rabiei*-inoculated pots, and the lowest germination (18%) was recorded in this treatment. Different doses of LBW enhanced the germination of chickpea seeds over positive control. Different doses of LBW evidently effected the germination of test plant as 33, 34, and 50% germination was noted in 1, 2, and 3% doses,

respectively. Positive control showed the highest disease incidence by 66% whereas disease incidence was recorded as 38, 40, and 28% in 1, 2, and 3% LBW, respectively (Fig. 2b). The highest values of dry shoot and root biomasses of chickpea were noted in the negative control treatment and the lowest in the positive control treatment with significant difference between the two. Both shoot and root dry biomasses were gradually increased as the dose of LBW was increased from 1 to 3%. The increase in shoot and root biomasses due to different doses of LBW was 116–283 and 66–333% over positive control, respectively (Fig. 3).

In pot trial, application of different doses of dry leaf biomass of *W. somnifera* variably controlled disease and improved germination and crop growth. The lowest disease incidence and the highest positive effects on germination and seedling growth were recorded in 3% dose of leaf biomass. These findings are in conformity with previous investigation that documented stimulatory action of soil amendments using leaf biomass of *W. somnifera* on growth of onion plant along with suppression in Fusarium wilt disease (Akhtar and Javaid 2018). Observed enhancement in growth of chickpea along with management of the disease may be ascribed to occurrence of many biological active compounds, mainly alkaloids (isopelletierine, anaferrine) and steroids (withanolides, withaferins) in leaves of *W. somnifera* (Pandey et al. 2018). These might have influenced host plant physiological responses triggered by signal transduction process that may lead to extensive cross-talk between pathways to accumulate defense-related compounds upon encountering disease stresses (Isah 2019).

#### Conclusion

This study concludes that the leaf extract of *W. somnifera* was very effective in controlling the growth of *A. rabiei*. Antifungal constituents were mostly present in the 2 polar solvent fractions, viz., ethyl acetate and *n*-butanol. Soil application of dry biomass of *W. somnifera* at 3% w/w concentration had the ability to significantly reduce disease incidence and to improve germination and plant growth in *A. rabiei*-contaminated soil.

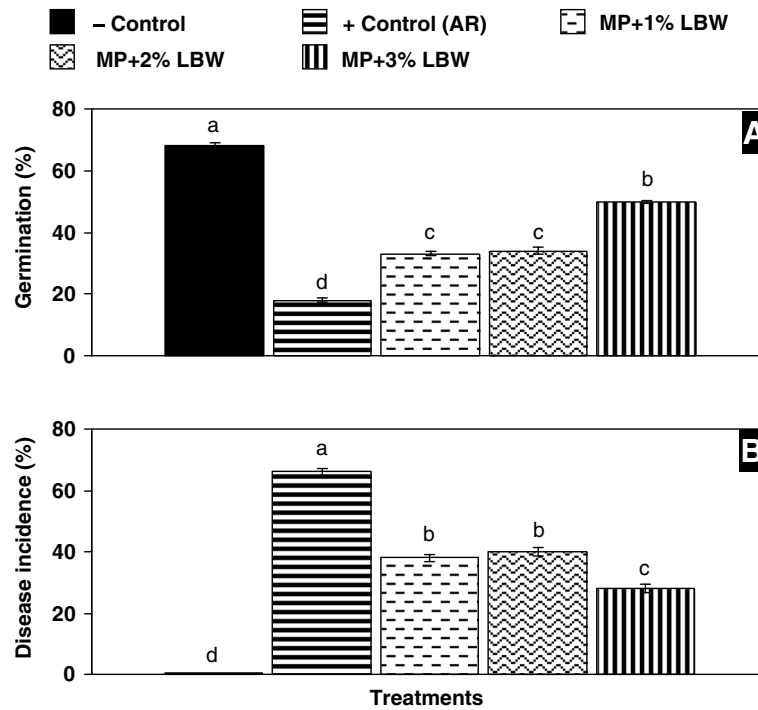
**Table 2** Antifungal activity of isolated compounds against *Ascochyta rabiei*

Treatments	Concentrations $\mu\text{g ml}^{-1}$									
	4000	2000	1000	500	250	125	62.5	31.25	15.62	7.81
<sup>a</sup> Control	+	+	+	+	+	+	+	+	+	+
Mancozeb	–	–	–	–	–	–	–	–	–	–
Compound A	–	–	–	–	–	–	–	–	+	+
Compound B	–	–	–	–	–	+	+	+	+	+

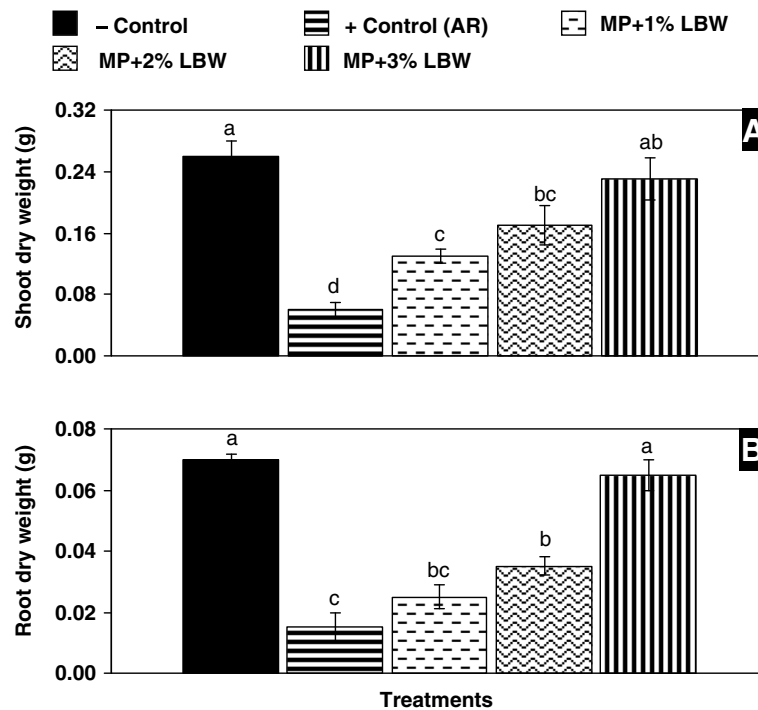
+ Fungal growth appears

– No fungal growth

<sup>a</sup>Concentrations are only for compounds A–D and mancozeb



**Fig. 2** Effect of *Ascochyta rabiei* inoculation and different concentrations of dry leaf manure of *Withania somnifera* (LBW) on germination of chickpea seeds and disease incidence. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ( $P \leq 0.05$ ) as determined by Duncan's multiple range test



**Fig. 3** Effect of *Ascochyta rabiei* inoculation and different concentrations of dry leaf manure of *Withania somnifera* on shoot and root growth of chickpea. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ( $P \leq 0.05$ ) as determined by Duncan's multiple range test

**Abbreviations**

ME: Malt extract; ANOVA: Analysis of variance; LBW: Leaf biomass of *W. somnifera*; TLC: Thin layer chromatography; MIC: Minimum inhibitory concentration

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**Authors' contributions**

RM conducted the study. AJ supervised the work, analyzed the data, and approved the final manuscript. IHK and AS contributed in paper writing. All authors have read and approved the 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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