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Effects of Zinc Oxide Nanoparticles (ZnO NPs) and some Plant Pathogens on the Growth and Nodulation of Lentil (*Lens culinaris* Medik.)

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Effects of ZnO nanoparticles (NPs) were studied on lentil plants inoculated with *Alternaria alternata*, *Fusarium oxysporum* f. sp. *lentis*, *Xanthomonas axonopodis* pv. *phaseoli*, *Pseudomonas syringae* pv. *syringae* and *Meloidogyne incognita*. Plant growth, chlorophyll, carotenoid contents, nitrate reductase (NR) activity and nodulation of lentil both in the presence and absence of *Rhizobium* sp. were examined in a pot test. Inoculation of plants with *A. alternata* / *F. oxysporum* f. sp. *lentis* / *X. axonopodis* pv. *phaseoli* / *P. syringae* pv. *syringae* or *M. incognita* caused a significant reduction in plant growth, number of pods per plant, chlorophyll, carotenoids and NR activity over uninoculated control. Inoculation of plants with *Rhizobium* sp. with or without pathogen increased plant growth and number of pods per plant, chlorophyll, carotenoids and NR activity. When plants were grown without *Rhizobium*, a foliar spray of plants with 10 ml solution of 0.1 mg ml^{-1} of ZnO NPs per plant caused a significant increase in plant growth and number of pods, chlorophyll, carotenoid contents and NR activity in both inoculated and uninoculated plants. Spray of ZnO NPs to plants inoculated with *Rhizobium* sp. caused non significant increase in plant growth, number of pods per plant, chlorophyll, carotenoid contents and NR activity when plants were either uninoculated or inoculated with pathogens. Numbers of nodules per root system were high in plants treated with *Rhizobium* sp. but foliar spray of ZnO NPs had adverse effect on nodulation. Inoculation of plants with test pathogens also reduced nodulation. Spray of ZnO NPs to plants reduced galling, nematode multiplication, wilt, blight and leaf spot disease severity indices.

Keywords: Root nodulation *Rhizobium* sp., disease management, plant pathogens.

Lentil (*Lens culinaris* Medik.) is a bushy and annual shrub. Lentils are nutritious and provide a good source of minerals, containing about 25% proteins, 56% carbohydrates, 1.0% fat in seeds. Lentil is one of the best and cheapest sources of vegetable proteins (Adsule et al, 1989). Lentils indulge in the nitrogen fixation process that helps the soil to revive its nitrogen content. The lentils are an important source of essential amino acids, fatty acids and trace mineral (Haq et al., 2011).

Unfortunately, there are some constraints of pests and pathogens in the successful cultivation of lentil (Taylor et al., 2007). Fungal diseases on lentil includes Alternaria blight (*Alternaria alternata*), Fusarium wilt (*Fusarium oxysporum* f. sp. *lentis*). Bacterial

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diseases of lentil are bacterial leaf spot caused by *Xanthomonas axonopodis* pv. *phaseoli* and bacterial blight caused by *Pseudomonas syringae* pv. *syringae*. Root knot nematode *Meloidogyne incognita* is also a major pathogen.

Root nodulation is a complex symbiotic process between host plant and *Rhizobium* sp. *Rhizobium* is a genus of Gram-negative soil bacteria that fix nitrogen. It forms an endosymbiotic nitrogen fixing association with roots of legumes. The association of Rhizobia with plant pathogens in the rhizosphere is not always detrimental since it sometimes leads to stimulation of nodulation (Hussey and Barker, 1976). The presence of rhizobia in the rhizosphere may also protect the host root from damage caused by pathogens (Siddiqui and Husain, 1992; Siddiqui and Mahmood, 1995).

Nanoparticles can offer green and eco-friendly alternatives for plant disease management (Alghuthaymi et al., 2015) because of increased antimicrobial activity and decreased ecological toxicity (Neal, 2008). Zinc oxide nanoparticles (ZnO NPs) have remarkable optical, physical, and antimicrobial properties (Sabir et al., 2014) and have potential to enhance agriculture production due to active oxygen species generated by these NPs.

During the course of survey of lentil fields of Aligarh district of U.P., we found frequent occurrence of *Meloidogyne incognita* (Kofoid and White) Chitwood, *Alternaria alternata* (Fr.) Keissler, *Fusarium oxysporum* f. sp. *lentis* Gordon, *Xanthomonas axonopodis* pv. *phaseoli* (E. F. Smith) Dowson and *Pseudomonas syringae* pv. *syringae* van Hall. Plants infected with these pathogens were poor in growth and showed typical disease symptoms. These pathogens were highly destructive and considered as major constraints in the successful cultivation of lentil.

In the present study an attempt was made to use ZnO NPs for the management of *M. incognita*, *A. alternata*, *F. oxysporum* f. sp. *lentis*, *X. axonopodis* pv. *phaseoli* and *P. syringae* pv. *syringae* on lentil both in the presence and absence of *Rhizobium* sp.

Materials and Methods

Root and soil samples were collected from lentil fields of Aligarh district, U.P., India. These samples were collected in polythene bags and stored in a refrigerator at 4 °C until processing began. The samples were examined for the presence of the root-knot nematode *Meloidogyne* spp., fungi and plant pathogenic bacteria.

Isolation and identification of root-knot nematode M. incognita

Lentil roots were examined for root-knot symptoms. Root galls were dissected to take out the females of root-knot nematodes. Identification of *Meloidogyne* sp. was made on the basis of perineal patterns (Taylor and Sasser, 1978). Soil samples were also processed for the nematodes isolation by Cobb's sieving and decanting technique followed by a Baermann funnel (Southey, 1986). Identification of root-knot juveniles were also made from the nematode suspension.

Isolation of fungi from lentil

Roots and shoots with fungal disease symptoms were transferred to sterilized Petri dishes containing sterilized distilled water and gently freed of soil particles. These parts were transferred to other Petri dishes and the process was repeated until all adhering soil particles were removed. Later, these infected parts were cut into approximately 5 mm pieces and transferred to a Petri dish containing 0.1% sodium hypochlorite (NaOCl) solution. After one minute the pieces were washed at least thrice in distilled water and dried on filter paper. Five of these pieces were then plated in Petri dishes containing PDA (Potato dextrose agar) using sterilized forceps under aseptic conditions. Petri dishes were incubated at 25 ± 2 °C for 10 days. The fungus that developed from infected root and shoot pieces were examined and identified. For confirmation of identity as *Alternaria alternata* and *Fusarium oxysporum* f. sp. *lentis*, pure cultures of these fungi were prepared. Identification of *A. alternata* was made based on morphological characters (Simmons, 2007), while F. *oxysporum* was identified using the key proposed by Nelson et al. (1983). Pathogenicity and characterization of the isolate as *forma specialis lentis* was tested by inoculating the susceptible lentil cultivar P 651. The pure cultures were stored at 5 °C until used. The composition of PDA medium was as follows:

Potato infusion from	200 g
Dextrose	20 g
Agar agar	15 g
Distilled water	1000 ml

Isolation of bacteria from lentil

Collected lentil plants showing symptoms of bacterial blight or leaf spot were used for the isolation of bacteria. Plant parts having rot or blight symptoms were transferred to a sterilized Petri dishes containing sterilized distilled water and gently freed of soil particles. These parts were transferred to another Petri-dish and process was repeated till all adhering soil particles were removed. Later, the plant parts were cut into 5 mm pieces and then surface sterilized with 0.1% sodium hypochlorite (NaOCl) solution, followed by three repeated washings with distilled water and dried on filter paper. Five of these pieces were then plated in each of the Petri dishes containing nutrient agar medium with the help of sterilized forceps under aseptic condition. Petri dishes were then incubated at 30 + 2 °C for 24–48 h. The bacteria that developed from infected pieces were examined and identified. *Pseudomonas syringae* pv. *syringae* was identified using biochemical and physiological tests (Ashorpour et al., 2008) while *X. axonopodis* pv. *phaseoli* was identified according to Nunes et al. (2008). To confirm identity as *Pseudomonas syringae* pv. *syringae* and *Xanthomonas axonopodis* pv. *phaseoli*, pure cultures of these bacteria were prepared and stored at 5 °C. The composition of nutrient agar medium was as follows:

Beef extract	3.0 g
Peptone	5.0 g
Agar agar	15.0 g
Distilled water	1.0 liter

Preparation of zinc dioxide nanoparticles solution

Zinc oxide, dispersion nanoparticles (ZnO NPs), < 100 nm particle size (TEM), ≤ 40 nm average particle size, 20 wt. % in H_2O, pH 7.5 ± 1.5 was obtained from Sigma-Adrich (Product No. 721077-100G). Solution of ZnO NPs was prepared by dissolving 0.50 ml ZnO NPs in one litre distilled water to obtain a final concentration of 0.1 mg ml^{-1} for the foliar spray. Ten ml suspension of ZnO NPs was used as foliar spray per plant.

Preparation and sterilization of soil mixture

Sandy loam soil is collected from a field belonging to the Department of Botany, A.M.U., Aligarh and passed through a 10 mesh sieve. The soil, river sand and organic manure were mixed in the ratio 3:1:1 and 15 cm diameter clay pots were each filled with 1 kg of the mixture. A little water was poured into each pot to just wet the soil surface before sterilization at 137.9 kPa for 20 minutes. Sterilized pots were allowed to cool at room temperature before use.

Raising and maintenance of test plants

Lentil (*Lens culinaris* L.) seeds of cultivar K-75 were surface sterilized with 0.1% sodium hypochlorite (NaOCl) for 2 minutes and rinsed three times with sterile water. Half of the seeds were treated with *Rhizobium* sp. while the other half remained untreated before sowing. To treat the seeds with *Rhizobium* sp., commercial culture of *Rhizobium* sp. was obtained from Quarsi Agriculture farm, Aligarh. For seed treatment, 100 g commercial culture of *Rhizobium* lentil strain was placed on butter paper and mixed with 50 g sucrose. Later, 50 ml distilled water was poured over culture and mixed. Hundred g seeds were mixed with commercial culture and left to dry at room temperature. Sowing of *Rhizobium*-treated and untreated seeds were done separately. Three seeds were sown in the 15 cm diameter clay pots and one week after germination thinning was done to retain single seedling per pot. Seedlings were subjected to the treatments and uninoculated plants served as a control and plants were kept in a glass house at 20 ± 2 °C. Pots were arranged in a randomize block design and each treatment was replicated 5 times. Pots were watered as needed and experiment was terminated 90 days after inoculation.

Inocula of fungi

For obtaining sufficient inocula, *A. alternata* and *F. oxysporum* f. sp. *lentis* were separately cultured on Richard's liquid medium having following composition:

Potassium nitrate	10.0 g
Potassium dihydrogen phosphate	05.0 g
Magnesium sulphate	02.5 g
Ferric chloride	0.02 g
Sucrose	50.0 g
Distilled water	1000 ml

The medium was prepared and filtered through muslin cloth, sterilized in an autoclave at 103.4 kPa for 15 minutes in 250 ml Erlenmeyer flasks each containing 80 ml

liquid medium. *A. alternata* and *F. oxysporum* f. sp. *lentis* were separately inoculated in each flask with the help of inoculation needle. Inoculated flasks were incubated at 25 ± 1 °C for about 15 days to allow sufficient growth of each fungus. The mycelium of each fungus was collected on blotting papers separately to remove excess water and nutrients. The inoculum of each fungus was prepared separately by mixing 50 g mycelium in 500 ml distilled water and blending it for 30 seconds in Waring blender. Ten ml of this suspension containing 1 g fungus was used as inoculum. Pure cultures of *A. alternata* and *F. oxysporum* f. sp. *lentis* were continuously maintained on PDA by re-inoculation after every 15 days.

Inocula of bacteria

For obtaining sufficient inocula, *P. syringae* and *X. axonopodis* were separately cultured on Nutrient agar medium. Medium was poured in sterilized Petri dishes and these dishes were later streaked separately with a pure colony of *P. syringae* / *X. axonopodis* and incubated at 30 ± 1 °C for 24 h. Single colonies from a 24-h-old pure culture of *P. syringae* / *X. axonopodis* were inoculated separately into nutrient broth flasks and incubated at 30 ± 1 °C for 72 h. Cell density was determined following Sharma (2001) to achieve 1.2×10^5 colony-forming units (CFU) / ml. Ten ml of this suspension was added to each pot around a lentil seedling.

Preparation of nematode inoculum

Meloidogyne incognita was collected from lentil roots and multiplied on the roots of egg plants (*Solanum melongena* L.) using single egg mass. Large numbers of egg masses from heavily infected eggplant roots were hand-picked with the help of sterilized forceps from the previously maintained pure culture of *M. incognita.* The egg masses were washed with distilled water and placed in a small sieve (9 cm diameter with 1-mm pore size) containing crossed layers of tissue paper. The sieve was placed in a Petri-dish containing distilled water deep enough to contact the egg masses. A number of these assemblies were kept in an incubator running at 25 ± 1 °C in order to obtain the required number of second-stage juveniles for inoculation. The hatched second-stage juveniles were collected from the Petri-dishes every 24 h, fresh water was added, and the process was repeated. For counting nematode juveniles, an average of 5 counts was made to determine the density of nematodes in the suspension. The inoculum was adjusted to 200 ± 5 nematodes per ml. Ten ml of this suspension (i.e. 2000 freshly hatched juveniles) was added to each pot around a lentil seedling.

Inoculation techniques

Ten days after germination, inoculations were performed on well-established healthy seedlings. For inoculation of *M. incognita,* fungi and bacteria, the soil around the roots was carefully moved aside without damaging the roots. The inoculum suspension was poured around the roots before the soil was replaced. As a control treatment, water was used. There were 2 sets of experiments 1. Plants with *Rhizobium* sp. 2. Plants without *Rhizobium* sp. Each set comprised of six treatments, i.e. (A) Control (without

pathogen); (B) *M. incognita*; (C) *P. syringae* pv. *syringae*; (D) *X. axonopodis* pv. *phaseoli*; (E) *A. alternata*; (F) *F. oxysporum* f. sp. *lentis* (6 × 2 = 12 treatments). These 12 treatments were combined with (I) water (control, without ZnO NPs); (II) ZnO NPs 0.50 ml/L (12 × 2 = 24). Each treatment was replicated 5 times (24 × 5 = 120 pots). Experiment was conducted in 2017–2018.

Chlorophyll and carotenoid estimation

The chlorophyll and carotenoid contents in the fresh leaf was estimated 90 days after inoculation following Mackinney (1941). One g of freshly cut leaves was ground to fine pulp using a mortar and pestle after adding 20 cm^3 of 80% acetone. The mixture was centrifuged at 5,000 rpm for 5 minutes. The supernatant was collected in 100 cm^3 volumetric flask. The absorbance was read at 645 and 663 nm for chlorophyll and 480 and 510 nm for carotenoid against the blank (80% acetone) on spectrophotometer (Shimadzu UV-1700, Tokyo, Japan).

Estimation of Nitrate Reductase (NR) activity

Nitrate reductase (NR) activity (EC 1.7.1.1) was estimated 90 days after inoculation following Jaworski (1971). The amount of nitrite formed was determined spectrophotometrically. Fresh leaves (0.2 g) were chopped and transferred to a plastic vial; each vial contained 2.5 mL of phosphate buffer (pH 7.5), 0.5 mL of 0.2 M potassium nitrate solution, and 2.5 mL of 5% isopropanol. These vials were incubated for 2 h at 30 °C in dark. Then 0.4 mL of the incubated mixture was transferred to a test tube and 0.3 mL each of 1% sulfanilamide and 0.02% N-(1-naphthyl) ethylenediamine dihydrochloride solution was added. Finally, the vials were kept at room temperature for 20 min for colour development and the vial content was diluted to a volume of 5 mL with double distilled water. The optical density (OD) of the content was recorded at 540 nm using the spectrophotometer (UV-1700, Shimadzu, Japan). A blank was run simultaneously with each sample. Standard curve was plotted by using known graded concentrations of $NaNO_2$ (sodium nitrite) solution. NR activity was expressed as nano-moles of nitrite produced per gram fresh weight of leaf tissue per hour (nM NO_2^- g^{-1} FW h^{-1}).

Observations

The plants were harvested 90 days after inoculation. Data on plant length, plant fresh weight, plant dry weight, no. of pods per plant and number of nodules per root system were recorded. The length of plant was recorded in centimeter from the top of the first leaf to the end of the root. Excess water was removed by blotting before weighing the plant. The plants were cut with knife above the base of the root emergence zone to separate shoot and root. Shoot and root were kept in envelope at 60 °C for 4 days before weighing for dry weight determination. Number of galls per root system were counted. For nematode population, a 250 g subsample of well-mixed soil from each treatment was processed by Cobb's sieving and decanting technique followed by Baermann funnel extraction (Southey, 1986). Nematode suspension was collected after 24 h and the numbers of nematodes were counted in five aliquots of 1 ml of suspension from each sample. The

means of five counts were used to calculate the population of nematodes per kg soil. To estimate the number of juveniles, eggs and females inside the roots, a 1 g subsample of roots was macerated in a Waring blender and counts were made from the suspension thus obtained. Numbers of nematodes present in roots were calculated by multiplying the number of nematodes present in 1 g of root by the total weight of root. Disease severity caused by fungal and bacterial pathogens were also recorded. Wilt, leaf blight, bacterial blight and leaf spot were determined by scoring the severity of disease. Disease rating was on a scale from 0 to 5 where 0 = no disease (no wilt / leaf blight / leaf spot / bacterial blight symptoms observed); 1 = wilt / leaf blight / leaf spot / bacterial blight symptoms up to 12.5% on leaf; 2 = wilt / leaf blight / leaf spot / bacterial blight symptoms 12.6 to 25% on leaf; 3 = wilt / leaf blight / leaf spot / bacterial blight symptoms 25.1 to 37.5% on leaf; 4 = wilt / leaf blight / leaf spot / bacterial blight symptoms 37.6 to 50% on leaf, and 5 > 50% wilt / leaf blight / leaf spot / bacterial blight symptoms on leaf.

Microscopic examination of eggs and second stage juveniles of M. incognita

Effect of ZnO NPs was observed on eggs and second stage juveniles of *M. incognita*. Forty ml of ZnO NPs solution (0.1 mg ml^{-1})was placed in five Petri dishes and ten egg masses of *M. incognita* were added for hatching. Twenty four hours after incubation, juveniles and eggs were observed under microscope.

Statistical analysis

Plant length, plant fresh weight, plant dry weight, chlorophyll, carotenoid and nitrate reductase activity were analysed statistically using analysis of variance in the statistical software R (R development core team 2011; package library agricolae). Least significant differences (L.S.D.) were calculated at p = 0.05. Duncan's multiple range test (DMRT) was applied to denote the significant differences between the treatments.

Results

Analysis of variance revealed that *Rhizobium,* ZnO NPs and pathogen exposure significantly ($P = 0.001$) affected plant length, plant fresh weight, plant dry weight, no. of pods, no. of nodules, chlorophyll and carotenoid contents and nitrate reductase activity (Table 1). Interaction of *Rhizobium* × NPs was significant at $P = 0.05$ on plant length, plant fresh weight, no. of pods, no. of nodules, chlorophyll, carotenoid and nitrate reductase activity. Interactions of NPs × pathogens and *Rhizobium* × NPs × pathogens were found non-significant on all parameters studied (Table 1).

Effects of pathogens and ZnO NPs on plants growth in the absence of Rhizobium *sp.*

In plants sown without *Rhizobium* sp., inoculation with *P. syringae* pv. *syringae* / *X. axonopodis* pv. *phaseoli* / *M. incognita* / *A. alternata* or *F. oxysporium* f. sp. *lentis* caused a significant reduction in plant growth and number of pods / plant (Table 2). Among these pathogens, *M. incognita* and *F. oxysporium* f. sp. *lentis* resulted in relatively

Table 1

Analysis of variance (ANOVA) showing the effect of *Rhizobium*, ZnO nanoparticles, pathogens and their interaction on the growth and physiological parameters of lentil

Parameters	Treatments	Df	Sum of square	Mean of square	F value	Pr (>F)
Plant length	*Rhizobium*	1	135.00	134.90	43.280	2.49e-09 ***
	Nanoparticles (NPs)	1	93.00	92.80	29.777	3.78e-07 ***
	Pathogens	5	6302.00	1260.40	404.272	< 2e-16 ***
	Rhizobium × NPs	1	18.00	17.80	5.717	0.0187 *
	Rhizobium × pathogens	5	9.00	1.80	0.564	0.7274ns
	NPs × pathogens	5	1.00	0.20	0.060	0.9975ns
	Rhizobium × NPs × pathogens	5	2.00	0.40	0.120	0.9876ns
	Residuals	96	299.00	3.10		
Plant fresh weight	*Rhizobium*	1	79.80	79.80	64.683	2.31e-12 ***
	Nanoparticles (NPs)	1	47.20	47.20	38.255	1.51e-08 ***
	Pathogens	5	3136.30	627.30	508.510	< 2e-16 ***
	Rhizobium × NPs	1	3.60	3.60	2.881	0.0929*
	Rhizobium × pathogens	5	3.30	0.70	0.532	0.7513ns
	NPs × pathogens	5	0.60	0.10	0.104	0.9911ns
	Rhizobium × NPs × pathogens	5	0.50	0.10	0.076	0.9957ns
	Residuals	96	118.40	1.20		
Plant dry weight	*Rhizobium*	1	20.10	20.13	31.115	2.24e-07 ***
	Nanoparticles (NPs)	1	10.80	10.83	16.739	8.93e-05 ***
	Pathogens	5	862.10	172.42	266.498	< 2e-16 ***
	Rhizobium × NPs	1	1.70	1.67	2.579	0.112ns
	Rhizobium × pathogens	5	0.40	0.08	0.116	0.989ns
	NPs × pathogens	5	0.20	0.04	0.068	0.997ns
	Rhizobium × NPs × pathogens	5	0.20	0.04	0.064	0.997ns
	Residuals	96	62.10	0.65		
No. of pods	*Rhizobium*	1	649.00	648.70	61.586	5.96e-12 ***
	Nanoparticles (NPs)	1	400.00	399.70	37.946	1.70e-08 ***
	Pathogens	5	9037.00	1807.40	171.598	< 2e-16 ***
	Rhizobium × NPs	1	52.00	52.00	4.938	0.0286 *
	Rhizobium × pathogens	5	34.00	6.90	0.653	0.6601ns
	NPs × pathogens	5	4.00	0.90	0.083	0.9947ns
	Rhizobium × NPs × pathogens	5	4.00	0.80	0.077	0.9956ns
	Residuals	96	1011.00	10.50		
No. of nodules	*Rhizobium*	1	51875.00	51875.00	62720.06	< 2e-16 ***
	Nanoparticles (NPs)	1	500.00	500.00	60.479	8.40e-12 ***
	Pathogens	5	3672.00	734.00	88.791	< 2e-16 ***
	Rhizobium × NPs	1	317.00	317.00	38.312	1.48e-08 ***
	Rhizobium × pathogens	5	2779.00	556.00	67.189	< 2e-16 ***
	NPs × pathogens	5	24.00	5.00	0.569	0.723ns
	Rhizobium × NPs × pathogens	5	22.00	4.00	0.529	0.754ns
	Residuals	96	794.00	8.00		

Table 1 cont.

Parameters	Treatments	Df	Sum of square	Mean of square	F value	Pr (>F)
Chlorophyll content	*Rhizobium*	1	0.0328	0.03284	36.024	3.45e-08 ***
	Nanoparticles (NPs)	1	0.0091	0.00910	9.984	0.00211 **
	Pathogens	5	0.9556	0.19113	209.691	< 2e-16 ***
	Rhizobium × NPs	1	0.0043	0.00426	4.674	0.03311 *
	Rhizobium × pathogens	5	0.0042	0.00085	0.931	0.46443ns
	NPs × pathogens	5	0.0013	0.00026	0.284	0.92093ns
	Rhizobium × NPs × pathogens	5	0.0016	0.00031	0.343	0.88553ns
	Residuals	96	0.0875	0.00091		
Carotenoids content	*Rhizobium*	1	0.000213	0.0002133	33.247	9.85e-08 ***
	Nanoparticles (NPs)	1	0.000101	0.0001008	15.714	0.000142 ***
	Pathogens	5	0.006924	0.0013848	215.818	< 2e-16 ***
	Rhizobium × NPs	1	0.000041	0.0000408	6.369	0.013292 *
	Rhizobium × pathogens	5	0.000014	0.0000028	0.442	0.818431ns
	NPs × pathogens	5	0.000017	0.0000033	0.519	0.760970ns
	Rhizobium × NPs × pathogens	5	0.000007	0.0000013	0.208	0.958482ns
	Residuals	96	0.000616	0.0000064		
Nitrate reductase activity	*Rhizobium*	1	2839.00	2839.00	48.415	4.23e-10 ***
	Nanoparticles (NPs)	1	1560.00	1560.00	26.593	1.35e-06 ***
	Pathogens	5	31362.00	6272.00	106.9581	< 2e-16 ***
	Rhizobium × NPs	1	647.00	647.00	11.038	0.00126 **
	Rhizobium × pathogens	5	826.00	165.00	2.817	0.02037 *
	NPs × pathogens	5	138.00	28.00	0.470	0.79806ns
	Rhizobium × NPs × pathogens	5	128.00	26.00	0.437	0.82144ns
	Residuals	96	5630.00	59.00		

* = F values are significantly different at $P = 0.05$, ** = F values are significantly different at $P = 0.01$, *** = F values are significantly different at $P = 0.001$ and ns = F values are not significant.

higher reduction in plant growth and number of pods / plant, than *A. alternata*. Inoculation of plants with *P. syringae* pv. *syringae, X. axonopodis* pv. *phaseoli*, *M. incognita, A. alternata* and *F. oxysporium* f. sp. *lentis* resulted in a 43.18, 45.23, 49.42, 38.69 and 48.91 % reductions in plant dry weight, respectively (Table 2).

In the absence of *Rhizobium* sp., a spray of ZnO NPs to plants inoculated with either test pathogens resulted in a significant increase in plant growth and number of pods / plant (Table 2). Spray of ZnO NPs to uninfected plants grown without *Rhizobium* sp. resulted in a 3.93% increase in plant dry weight. Similarly, ZnO NPs treatment caused 9.52, 9.62, 10.17, 8.60 and 13.43% increases in plant dry weight upon inoculation with *P. syringae* pv. *syringae, X. axonopodis* pv. *phaseoli*, *M. incognita, A. alternata* and *F. oxysporium* f. sp. *lentis*, respectively (Table 2).

Nodulation was very poor (1 to 5 per root system) in plants without *Rhizobium*, irrespective of pathogen presence or ZnO NPs treatment (Table 2). Plants inoculated with *P. syringae* pv. *syringae, X. axonopodis* pv. *phaseoli*, *A. alternata* and *F. oxysporium* f. sp. *lentis* showed severe symptoms of bacterial blight, bacterial leaf spot, fungal blight and wilt (with severity indices of 4, 4, 3 and 4, respectively) (Table 2). Spray of ZnO NPs to plants inoculated with *P. syringae* pv. *syringae, X. axonopodis* pv. *phaseoli*, *A. alternata*

Table 2

Effect of ZnO NPs on *M. incognita* (M), *A. alternata* (A), *F. oxysporum* f. sp. *lentis* (F), *P. syringae* pv. *syringae* (P) and *X. axonopodis* pv. *phaseoli* (X) on the growth and nodulation of lentil both in the presence and absence of *Rhizobium* sp.

Treatments			Plant length (Cm)	Plant fresh weight (g)	Plant dry weight (g)	No. of pods	No. of nodules	No. of galls	Nematode population	Disease index
Without *Rhizobium*	Without ZnO NPs	Control	63.24b	32.82c	15.74c	54c	5i	–	–	–
		P	46.90fgh	21.88ijkl	8.92kl	36hijk	3i	–	–	4
		X	45.15hi	20.74klmn	8.62lm	35jkl	4i	–	–	4
		M	41.90j	17.38p	7.96m	29m	2i	29a	8740a	–
		A	48.65def	23.18ghi	9.65ghij	40fghi	3i	–	–	3
		F	44.52hi	18.52op	8.04m	31lm	2i	–	–	4
	With ZnO NPs	Control	66.19a	34.26b	16.36bc	60b	4i	–	–	–
		P	49.26cdef	23.42fghi	9.77fghi	41efgh	1i	–	–	3
		X	48.36def	22.16hijk	9.45hijk	39ghij	2i	–	–	3
		M	44.16i	19.25no	8.77kl	34kl	3i	18c	5410c	–
		A	50.85cd	24.90def	10.48ef	45de	1i	–	–	2
		F	46.72fgh	20.12mn	9.12ijkl	36ijk	2i	–	–	3
With *Rhizobium*	Without ZnO NPs	Control	67.36a	35.28ab	16.72ab	62ab	68a	–	–	–
		P	49.65cde	23.58fgh	9.96fgh	41efgh	47cd	–	–	3
		X	48.42def	22.55ghij	9.60hij	41efgh	45d	–	–	3
		M	44.72hi	19.48no	8.90kl	35jkl	36g	21b	6350b	–
		A	50.62cd	25.12de	10.86de	45de	50c	–	–	2
		F	46.94fgh	20.36lmn	9.22ijkl	37hijk	41ef	–	–	3
	With ZnO NPs	Control	68.41a	36.35a	17.16a	65a	62b	–	–	–
		P	50.76cd	24.06efg	10.32efg	43defg	39fg	–	–	2
		X	49.15def	23.42fghi	10.06fgh	44def	36g	–	–	2
		M	45.84ghi	20.52lmn	9.05jkl	37hijk	30h	10d	3170d	–
		A	51.76c	25.96d	11.22d	47d	44de	–	–	1
		F	47.72efg	21.52jklm	9.64ghij	39ghij	32h	–	–	2
L.S.D. $P = 0.05$			2.22	1.39	0.71	4.0	3.6			

*Value in the same column followed by different letters are significantly different at P=0.05 based on DMRT

and *F. oxysporium* f. sp. *lentis* decreased disease severity indices to 3, 3, 2 and 3, respectively. Without *Rhizobium* sp., inoculation of plants with *M. incognita* caused 29 galls per root and high nematode population. Galling and nematode population was reduced by 37.93 and 38.10%, respectively, followed by a spray application of ZnO NPs (Table 2).

Effects of pathogens and ZnO NPs on plants growth in the presence of Rhizobium *sp.*

Inoculation of *Rhizobium* resulted in a significant increase in plant growth and number of pods / plant compared to plants without *Rhizobium* (Table 2). Inoculation of *Rhizobium*-treated plants with *P. syringae* pv. *syringae, X. axonopodis* pv. *phaseoli*, *M. incognita, A. alternata* and *F. oxysporium* f. sp. *lentis* resulted in a significant reduction in plant growth and number of pods / plant. Inoculation of plants with *P. syringae* pv. *syringae, X. axonopodis* pv. *phaseoli*, *M. incognita, A. alternata* and *F. oxysporium* f. sp.

lentis resulted in a 40.43, 42.58, 46.77, 35.04 and 44.85% reductions in plant dry weight in plants treated with *Rhizobium.*

Spray of ZnO NPs to *Rhizobium*-inoculated plants resulted in a non-significant increase in plant growth and number of pods / plant in both pathogen inoculated and uninoculated plants. ZnO NPs treatment of plants inoculated with *Rhizobium* and either *P. syringae* pv. *syringae, X. axonopodis* pv. *phaseoli*, *M. incognita, A. alternata* or *F. oxysporium* f. sp. *lentis* and control (without pathogens) resulted in 3.61, 4.79, 1.68, 3.31, 4.55 and 2.63% increases in plant dry weight which is statistically non-significant (Table 2).

Higher numbers of nodules were observed in plants with *Rhizobium*, however, inoculation of these plants with test pathogens resulted in a significant reduction in nodulation (Table 2). ZnO NPs treatment of *Rhizobium* inoculated plants reduced the nodulation in plants either or not inoculated with pathogens (Table 2).

Disease severity was found to be reduced in plants inoculated with *Rhizobium* and challenged with *P. syringae* pv. *syringae*, *X. axonopodis* pv. *phaseoli, A. alternata* or *F. oxysporium* f. sp. *lentis* (disease indices were 3, 3, 2 and 3, respectively, Table 2). Disease indices were further reduced in plants treated with both ZnO NPs and *Rhizobium*. Root galling and nematode multiplication was reduced by 27.58 and 27.34%, respectively, over plants without *Rhizobium.* Galling and nematode multiplication was further reduced to 52.38 and 50.07%, respectively, by the spray application of ZnO NPs (Table 2).

Effects of pathogens and ZnO NPs on chlorophyll, carotenoids and NR activity in the absence of Rhizobium *sp.*

Inoculation of *Rhizobium*-untreated plants with *P. syringae* pv. *syringae* / *X. axonopodis* pv. *phaseoli* / *M. incognita* / *A. alternata* or *F. oxysporium* f. sp. *lentis* caused a significant reduction in chlorophyll and carotenoid contents and NR activity (Table 3). *P. syringae* pv. *syringae, X. axonopodis* pv. *phaseoli*, *M. incognita, A. alternata* and *F. oxysporium* f. sp. *lentis* infections resulted in 33.33, 37.01, 46.22, 27.99 and 41.98% reductions in chlorophyll contents, respectively, and 26.56, 28.12, 35.93, 23.43 and 31.25% reductions in carotenoid contents, respectively. Similarly, *P. syringae* pv. *syringae, X. axonopodis* pv. *phaseoli*, *M. incognita, A. alternata* and *F. oxysporium* f. sp. *lentis* infections resulted in 9.83, 9.20, 15.17, 7.25 and 11.51% reductions in NR activity, respectively (Table 3).

In the absence of *Rhizobium* sp., a spray of ZnO NPs to plants inoculated with one of the test pathogens resulted in a significant increase in chlorophyll and carotenoid contents and NR activity (Table 3). Spray of ZnO NPs to uninoculated plants without *Rhizobium* sp. resulted in 9.02, 6.25 and 4.92% increases in chlorophyll and carotenoid contents and NR activity, respectively. Similarly, 3.59, 5.84, 11.98, 9.46 and 6.98% increases in chlorophyll were observed by the spray of ZnO NPs to plants inoculated with *P. syringae* pv. *syringae, X. axonopodis* pv. *phaseoli*, *M. incognita, A. alternata* and *F. oxysporium* f. sp. *lentis*, respectively. Similarly, 4.08 to 9.75% increases in carotenoids and 3.61 to 3.90% increases in NR activity was observed when ZnO NPs was sprayed on plants inoculated with test pathogens (Table 3).

Effects of pathogens and ZnO NPs on chlorophyll, carotenoids and NR activity in the presence of Rhizobium *sp.*

Inoculation of plants with *Rhizobium* resulted in a significant increase in chlorophyll, carotenoid contents and NR activity over plants without *Rhizobium* (Table 3). Inoculation of *Rhizobium*-treated plants with *P. syringae* pv. *syringae, X. axonopodis* pv. *phaseoli*, *M. incognita, A. alternata* and *F. oxysporium* f. sp. *lentis* resulted in a significant reduction in chlorophyll, carotenoid contents and NR activity. Inoculation of *Rhizobium* to plants without test pathogen resulted in 13.44, 7.81 and 7.94% increases in chlorophyll, carotenoids and NR activity, respectively. Inoculation of *Rhizobium* to plants infected with *P. syringae* pv. *syringae, X. axonopodis* pv. *phaseoli*, *M. incognita, A. alternata* and *F. oxysporium* f. sp. *lentis* resulted in 6.90, 8.47, 17.12,12.02 and 14.92% increases in chlorophyll contents, respectively. Similarly, 6.12 to 12.19% increases in carotenoids and 3.30 to 7.94% increases in NR activity were observed when *Rhizobium*-treated plants

Table 3

Effect of ZnO NPs on *M. incognita* (M), *A. alternata* (A), *F. oxysporum* f. sp. *lentis* (F), *P. syringae* pv. *syringae* (P) and *X. axonopodis* pv. *phaseoli* (X) on chlorophyll, carotenoids and nitrate reductase activity of lentil both in the presence and absence of *Rhizobium* sp.

Treatments			Chlorophyll content (mg $^{-1}$ FW)	Carotenoids content (mg $^{-1}$ FW)	NR activity (nM NO_2 g^{-1} FW h^{-1})
Without *Rhizobium*	Without ZnO NPs	Control	0.543b	0.064b	303.37c
		P	0.362efgh	0.047efgh	273.52jkl
		X	0.342fghi	0.046fgh	275.43ijkl
		M	0.292j	0.041i	257.32m
		A	0.391de	0.049cdef	281.37fghij
		F	0.315ij	0.044hi	268.43l
	With ZnO NPs	Control	0.592a	0.068a	318.32b
		P	0.375efg	0.050cde	283.42efghij
		X	0.362efgh	0.048defg	285.40defghi
		M	0.327hij	0.045gh	267.38l
		A	0.428cd	0.051cd	291.65cdef
		F	0.337ghi	0.047efgh	278.26hijkl
With *Rhizobium*	Without ZnO NPs	Control	0.616a	0.069a	327.47ab
		P	0.387ef	0.050cde	286.43cdefghi
		X	0.374efg	0.049cdef	284.52defghij
		M	0.342fghi	0.046fgh	268.32l
		A	0.435c	0.052c	293.37cde
		F	0.362hij	0.048defg	279.43ghijk
	With ZnO NPs	Control	0.623a	0.070a	330.23a
		P	0.391de	0.052c	290.52cdefg
		X	0.372efg	0.050cde	287.43cdefgh
		M	0.344fghi	0.047efgh	270.26kl
		A	0.437c	0.051cd	295.23cd
		F	0.380efg	0.048defg	281.26fghij
L.S.D. $P = 0.05$			0.037	0.003	9.61

*Value in the same column followed by different letters are significantly different at P=0.05 based on DMRT

were inoculated with test pathogens (Table 3). Spray of ZnO NPs to plants with *Rhizobium* and without pathogens resulted in a non-significant increase in chlorophyll, carotenoid contents and NR activity (Table 3). Spray of ZnO NPs to plants with *Rhizobium* also resulted in statistically non-significant effect on chlorophyll, carotenoid contents and NR activity upon inoculation with *P. syringae* pv. *syringae, X. axonopodis* pv. *phaseoli*, *M. incognita, A. alternata* or *F. oxysporium* f. sp. *lentis* (Table 3).

Microscopic examination of 2nd stage juveniles and eggs of M. incognita

Adverse effect of ZnO NPs was also observed on *M. incognita* eggs and second stage juveniles by microscopic examination. Shapes of eggs and second stage juveniles were deformed in 0.1 mg / ml ZnO NPs solution within 24 h of incubation and hatching of second stage juveniles from egg masses was inhibited by more than 60 percent.

Discussion

Use of ZnO NPS as a foliar spray was found beneficial in improving plant growth, chlorophyll, carotenoid contents and NR activity in both pathogen-inoculated and un-inoculated plants. The increase in vegetative growth, chlorophyll, carotenoids and NR activity of lentil might be due to fundamental role of Zn in protection and maintenance of structural stability of cell membranes (Welch et al., 1982) and also its use in protein synthesis, membrane function, cell elongation and tolerance (Cakmak, 2000). ZnO NPs are also known to increase the shoot dry matter and leaf area (Taheri et al., 2015) and higher values for seeded fruit per umbel, seed weight per umbel and 1000-seed weight were observed as compared to control (Laware and Raskar, 2014). ZnO NPs treatments (25 nm mean particle size) at 1000 ppm concentration promoted seed germination, seedling vigor, and plant growth (Prasad et al., 2012). In their study, plants sprayed with NPs showed early flowering as compared to control. Faizan et al. (2017) concluded that presence of ZnO NPs stimulated the antioxidant systems and increased proline accumulation that could provide stability to plants, and improved photosynthetic efficiency.

Foliar spray of NPs resulted in increased permeability of lipophilic organic molecules through the cuticle (Laware and Raskar, 2014). Hence, ZnO NPs have more chances to penetrate the leaf surface and release ions across the cuticle compared to water soluble ions (Da Silva et al., 2006). Prasad et al. (2012) observed that nano size and lower water solubility of ZnO NPs resulted in higher bioavailability of these NPs, which may be responsible for higher yields. In *Cucurbita pepo*, NPs were observed both in the extracellular spaces and inside the cells (Gonzalez-Melendi et al., 2008). Therefore, foliar spray of ZnO NPs may be responsible for increased plant growth in our experiments.

Sawai and Yoshikawa (2004) used 100 mg / ml ZnO suspension to obtain inhibitory effect on fungal growth but in our study 0.1 mg per ml showed antifungal activity. In our study, foliar spray on lentils was performed, while Sawai and Yoshikawa (2004) used higher concentration in an indirect conductimetric assay. The differences in results may be due to differences in assay conditions and fungal pathogens used. ZnO NPs generally interfere in cell functioning, cause deformation in fungal hyphae and inhibit the growth of fungi (He et al., 2011). In this study, application of ZnO NPs increased plant growth,

chlorophyll, carotenoid levels and NR activity in addition to reducing disease severity indices, which may be due to the antifungal effect of ZnO NPs on *A. alternata* and *F. oxysporum* f. sp. *lentis*.

The antibacterial activity of ZnO is mainly associated with the generation of highly reactive oxygen species such as OH $^{\bullet}OH_{,}$ H_2O_2 and $^{\bullet}O_2^-$ (Anita et al., 2010). Reactive oxygen species that are released from the surface of ZnO may cause damage to microorganisms (Sunada et al., 1998). The generation of H_2O_2 depends strongly on the surface area of ZnO, and smaller nanoparticles produce more reactive oxygen species with the increased surface area and enhanced antibacterial activity (Yamamoto et al., 2008). Continuous release of membrane lipids and proteins is induced by the integration of ZnO NPs with bacteria which changes membrane permeability of bacterial cells and later causes cell lysis (Brayner et al., 2006; Zhang and Chen, 2009). The bacterial cells were completely deformed by ZnO NPs, probably due to the lysis of cell wall, which was observed under microscope. Disease severity of bacterial blight was reduced in plants treated with ZnO NPs. This result confirms the antibacterial property of ZnO NPs.

Adverse effect of ZnO NPs was also observed on *M. incognita* eggs and second stage juveniles in this study by microscopic examination. Intestine is the major target tissues of NP toxicity which is revealed by distributional pattern of ZnO-NPs (Gupta et al., 2015). Similarly, galling and nematode multiplication was reduced in plants sprayed with ZnO NPs. Sudanophilic lipids are rich in nematodes cuticle (Sood and Kalra, 1977) and nematode cuticle also contain weakly acidic mucopolysaccharides. Lipids and glycogen are present in hypodermis and muscles of nematodes. In addition, hypodermis also consists of acidic mucopolysaccharides (Sood and Kalra, 1977). Toxicity of ZnO NPs is due to dissolved-zinc that caused toxicity plus nanoparticle-specific effects also contribute to toxicity (Savoly et al., 2016). It is possible the ZnO NPs had adverse effect on both cuticle and hypodermis of nematodes by affecting lipid, glycogen and mucopolysccharides.

Inoculation of *Rhizobium* sp. increased plant growth, chlorophyll and carotenoid coontents and NR activity compared to uninoculated control by increasing the nitrogen status of the soil. Antifungal activity of *Rhizobium* sp. was demonstrated by *in vitro* tests (Drapeau et al., 1973), and plants inoculated with *Rhizobium* sp. suffered less damage by pathogens than uninoculated plants (Bopaiah et al., 1976; Tu 1978, 1980). The pathogens tested had adverse effect on nodulation caused by *Rhizobium* sp., which is in line with previous findings (de Souza et al., 2016). Plant pathogens are also able to attack their opponents including symbionts using molecular weapons (Duffy et al., 2003), which include hydrogen cyanide (Benizri et al., 2005), alkaloids (Antunes et al., 2008), and bacteriocins (Holtsmark et al., 2008). Most of the secondary metabolites produced by plant pathogens can modify the structure and abundance of soil microbial populations, including plant symbionts (Mrabet et al., 2006; Liu et al., 2010; Chihaoui et al., 2012), causing deleterious impacts for both plant and microorganism.

Spray application of ZnO NPs to *Rhizobium*-inoculated plants resulted in reduced root nodulation. ZnO NPs at 0.5 g kg^{-1} significantly altered soil bacterial communities (Ge et al., 2014). The presence of ZnO NPs in the rhizosphere affected root nodulation, delayed the onset of nitrogen fixation, and caused early senescence of nodules (Huang et al., 2014). Consistent with previous findings, spray of ZnO NPs to plants treated with *Rhizobium* was not able to increase plant growth and number of pods per plants. ZnO NPs have toxic effect to many different bacteria tested in the laboratory (Dorobantu et

al., 2015). Similarly, Moghaddam et al. (2017) reported that in the presence of NPs, the total length of treated plants and the number of nodules were decreased by increasing the concentration of NPs (1.25 to 10 μg/ml of Ag NPs and 12.5 to 100 mg/ml of ZnO NPs) compared to the control plants ($P \leq 0.05$). Gene expression of *nif* (nitrate fixing) gene was decreased in the presence of sub minimum inhibitory concentration of NPs. Non-significant increase in the growth and number of pods in plants treated with both ZnO NPs and *Rhizobium* sp. can be attributed to a decrease in root nodulation and adverse effect on nitrogen fixation.

In conclusion, ZnO NPs exhibited antifungal and antibacterial activities in lentil plants and inhibitory effect on nematodes. The application of ZnO NPs on lentil resulted in improved plant growth, reduced galling and nematode multiplication upon inoculation by *M. incognita* and reduced disease severity when challenge inoculated with bacterial or fungal pathogens. This clearly demonstrates that ZnO NPs may be used for the management of these pathogens on lentil.

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