EFFECT OF NITRATE LEVELS AS A FERTILIZER OR AS A FUNGAL NUTRITION ON THE AGGRESSIVENESS OF *RHIZOCTONIA SOLANI* ON FABA BEAN

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ABSTRACT

The effect of sodium nitrate level as a fertilizer on aggressiveness of the fungus *Rhizoctonia solani* on faba bean plants was studied either in pots experiment or *in vitro*. Plants grown in infested soil amended with different levels of sodium nitrate showed that under zero amended nitrate or under high level of nitrate disease severity was very severe which reflected on decreasing the number of emerging plants , weight of foliage system, root weight , plant height and number of plant leaves . The levels of nitrate in between showed decreasing of disease severity. *In vitro* studies was carried out by putting peeled germinated seeds on *R. solani* fungal growth growing on Czapek's Dox agar medium supplemented with different levels of sodium nitrate. The fungus becomes more virulent when grown on media with increasingly higher concentration of nitrate. This was evident when disease was determined either visually or by determination of Polyphenol oxidase (PPO) activity of infected germinated seeds.. Transverse cross sections of faba bean radicals subjected to the fungus showed that increasing nitrate level raised the number and intensity of the developed infection cushions. The obtained results clearly monitor that higher level of nitrate in growth medium clearly enhanced the aggressiveness of the fungus which resulted in severe damage of the outer cortical layers of faba bean radical just after 16 h. from subjecting the host to the pathogen.

Keywords: light microscope , polyphenol oxidase (PPO), , pots experiment, root rot. *Vicia faba L.*

INTRODUCTION

The effect of sodium nitrate level as a fertilizer on aggressiveness of the fungus *Rhizoctonia solani* on faba bean plants was studied either in pots experiment or *in vitro*. Plants grown in infested soil amended with different levels of sodium nitrate showed that under zero amended nitrate or under high level of nitrate disease severity was very severe which reflected on decreasing the number of emerging plants , weight of foliage system, root weight , plant height and number of plant leaves . The levels of nitrate in between showed decreasing of disease severity. *In vitro* studies was carried out by putting peeled germinated seeds on *R. solani* fungal growth growing on Czapek's Dox agar medium supplemented with different levels of sodium nitrate. The fungus becomes more virulent when grown on media with increasingly higher concentration of nitrate. This was evident when disease was determined either visually or by determination of Polyphenol oxidase (PPO) activity of infected germinated seeds.. Transverse cross sections of faba bean radicals subjected to the fungus showed that increasing nitrate level raised the number and intensity of the developed infection cushions. The obtained results clearly monitor that higher level of nitrate in growth medium clearly enhanced the aggressiveness of the fungus which resulted in severe damage

of the outer cortical layers of faba bean radical just after 16 h. from subjecting the host to the pathogen.

MATERIALS AND METHODS

Pathogen isolation and pathogenicity

Rhizoctonia solani was isolated from the naturally diseased faba bean (*Vicia faba* L.) plants collected from Qualiobya Governorate, Egypt. Samples were rinsed with tap water then cut into small pieces (2-5 mm), washed three times in sterile distilled water and blotted to dry on sterile filter paper. Pieces were placed on 2% water agar (WA) and incubated at 25 C° for 2 days. Emerging hyphal tips were transferred on potato sucrose agar (PSA: 200 g of potato, 20 g sucrose and 20 g agar) and pure culture was transferred to PSA slants.

Pathogenicity test of *R. solani* isolates was carried out by putting sterilized germinated faba bean seeds on the fungal growth of tested isolates growing on PSA media. The most aggressive isolate on faba bean cotyledons was chosen for further studies. This isolate was found belonging to AG4.

Effect of nitrate level on *R. solani* virulence

Pots experiment

Preparation of R. solani inocula and soil infestation

Acid washed sand was poured in Petri dishes (9 cm in diameter). Dishes were autoclaved at 121° C for 30 min, after cooling dishes were wetted with autoclaved Czapek's Dox medium and infested with actively growing hyphal growth of *R.solani* grown on PSA medium. Dishes were incubated at 25 ± 1° C for 2 weeks. Sand contained fungal growth was used to infest autoclaved clay soil poured in plastic pots (15 cm in diameter) by a ration Dish/ pot. Clay soil was amended with different sodium nitrate levels i.e. 0 level, 0,015 gm, 0.03 and 0.06 gm/ pot, this amounts equivalent to 15kg,30 kg and 60kg nitrate/fedan (one fedan is 4200 m².), the recommended dose is 30 kg/fedan.

Sowing

Apparently healthy germinated seeds of faba bean (cv. Geiza 1) were surfsly sterilized in 5% sodium hypochlorite for 5 min., then washed in sterilized distilled water. Germinated seeds were sown in infested pots by 3 seeds/ pot. Pots were irrigated when it needed and left for 20 days under plastic house. Ten replicates were used for each particular treatment.
Determination of disease severity and morphological features of plants.

After 20 days from sawing, number of emergence plants was calculated. Plants were taken, and then roots were washed to remove adhered soil. Plant height (cm.), foliage weight (gm.), root weight (gm.) and leaves number/ plant were determined. Moreover disease severity on polants was determined according to the method adopted by Abawi *et al*. (2006). This experiment was repeated three times.

In vitro study

Rhizoctonia solani was grown in Petri dishes (9 cm). The dishes contained Czapex-Dox agar media with different levels of sodium nitrate. The basal synthetic medium consisted of 3g

$NaNO_3$, 1g K_2HPO_4, 0.5g $MgSO_4.7H_2O$, 0.5g KCl, 0.01g $FeSO_4$, 1ml of 1% $ZnSO_4$, $CuSO_4$, 30g sucrose and 20g agar per liter of distilled water. The variation of the basal media prepared by different levels of sodium nitrate was: half level: 1.5g/L, normal level: 3g/L, double level: 6g/Liter. Dishes were left at 25°±1 for one week to insure that the fungus completely filled the dishes

Surface sterilized of germinated seeds of faba bean were prepared as mentioned before. Apparently, healthy germinated seeds were peeled and put on the fungal growth. Dishes contained germinated seeds were incubated in dark at 25 ± 1 C° for three days, then disease incidence was estimated using adopted scale ranged from 1 to 5 as follow:

1: apparently healthy seeds; 2: very weak infection; 3: moderate infection; 4: very severe infection; 5: seeds were completely covered by fungal mats and died (Fig 7). Three dishes (each contains 5 seeds) were used as replicates for each treatment. This experiment was repeated at least three times. Samples of the aforementioned germinated seeds were taken for studying both PPO activity and histopathic alteration as well.

Determination of polyphenol oxidase (PPO) activity

Polyphenol oxidase activity was determined 2 days after subjecting the cotyledons to the pathogen. Five seeds of each treatment were taken and grinded in phosphate buffer solution (1:2, w:v – pH 5.5). Suspensions were centrifuged at 3000 g for 10 min at 4°C. Supernatant was taken for determination of PPO activity using catechol as substrate. (Arnnok *et al*., 2010). The absorbance at 410 nm was recorded at 15 second intervals using ultraviolet-visible spectrophotometer, (Unico UV-2100) USA.

Light microscopy

Seed samples of each nitrate levels (1.5, 3, 6 g/L) were collected 8 and 16 hr. after they were subjected to the fungal growth. Collected samples were cut into suitable pieces where killing and fixation were achieved in formalin-acetic acid-ethanol 70 % (FAA) for 24 hr. then dehydrated in ascending ethanol. Paraffin wax was infiltrated and samples were embedded (Johanson 1940). Cross sections were microtomed (10 µm) mounted to glass slides and stained with safranin-fast green schedule (Sander 1993). Sections were examined and photographed with a light microscope (Leica DM 2500) equipped with image analysis system in Histopathology Unit, Department of Plant pathology, Faculty of Agriculture, Ain Shams University.

Statistical analyses

For all obtained means, Standard deviation between experiments was calculated according to Ghahramani 2002.

RESULTS
Effect of nitrate level on R. solani virulence
Pots experiment

Under plastic house condition- in plastic pots- germinated faba bean seeds were sown in clay soil amended with different levels of sodium nitrate ranging from zero level to double recommended dose of fertilization with nitrate.

Data obtained indicated that zero level gave the highest degree of infection (Fig. 1).This degree of infection reflected on number of emerged plants (Fig. 2), plant height (cm) (Fig, 3), foliage system weight (Fig. 4), root system weight (Fig. 5) . number of leaves/plant (Fig.6),

By increasing the level of sodium nitrate till half or recommended dose, disease severity was declined which led to significant increase of all determined parameters. In case of double level of sodium nitrate, disease severity progressively increases once again, leading to reduction of all determined parameters.

***In vitro* studies**

The fungus *R. solani* was growing on Czapex-dox agar media with different levels of sodium nitrate. Germinated faba bean seeds were put on fungal growth. Obtained data indicated that the fungus on media with high nitrate level resulted in significant increase of disease severity compared with that growing on media with normal or lower level of nitrate (Fig. 8).

In order to make sure that increasing of nitrate level increases the aggressiveness of the pathogen, PPO activity was determined in severely infected five seeds subjected to pathogen. The activity of PPO was significantly increased in seeds subjected to pathogen growing on higher level of nitrate (Fig. 9).
Light Microscopy

Penetration processes

Epidermal and outer cortical cell layers were invaded by the hyphae of *R. solani* (Fig.10). Numerous fine infection pegs were produced from the infection cushions formed by *R. solani* hyphae. Higher levels of nitrate enhance the aggressiveness of the fungus. Increasing of nitrate caused higher number and intensity of developing infection cushions and tissue damage as well (Fig. 10 e & f).

Post-penetration processes

The fungal hyphae were severely dispersed inter-and intracellularly in the radicle tissues. The higher level of nitrate caused a severe aggressiveness of *the fungus*. The outer layers of cortical cells appeared severely collapsed 16 hr after inoculation (Fig.11). Invaded cortical tissues appeared darkened and collapsed, and ultimately were sloughed and cell masses of *Rhizoctonia* become encased in the sloughed cortical cells (Fig. 11 e, f). However, the half level of nitrate showed slight collapsing of cortical cells, as compared to those caused by *R. solani* in normal nitrate level.

DISCUSSION

As mentioned earlier plants require – for nutrition- many macro- and micro-elements. Nitrogen fertilization considers one of the most critical applications. Nitrogen deficiency causes much deviation from normal growth and high levels cause dramatic increase of many diseases (Agrios, 2005).

In the present study, the effect of sodium nitrate levels on the infection of faba bean plants with the fungal pathogen *R. solani* was carried out under pots experiment; faba bean seeds

were cultivated in infested or non infested soil with *R. solani*. Soil was amended with different levels of sodium nitrate ranged from zero level till double recommended dose of nitrate fertilization.

Data obtained clearly indicated that the behavior of the *R. solani* on faba bean plant under different levels of sodium nitrate take curve shape of two peaks . From one hand, non nitrate amended soil showed very severe infection. From the other hand, double level of nitrate returned the high level of disease severity. Levels in between (i.e half and normal recommended levels) led to decrease disease severity. How could explain the reason of this behavior? Concerning nitrate limitation, it was speculated that nutritional limitation of various types, in particular of nitrogen, appears to affect pathogensis. Talbot *et al.* ,1977, have found that bacterial and fungal genes are both induced during pathogensis and under nitrogen limiting conditions in artificial media suggest that during growth in *planta* there is limited nitrogen available for pathogens. In a previous study (Maha H. Mohamed *et al*., 2014), it was found that starvation of *R. solani* caused dramatic increase of disease severity on faba bean plants and by increasing starvation the aggressiveness of the pathogen significantly increased.

Findings of Talbot *et al*., 1997 explained the cause of increasing disease severity in case of zero level of nitrate, but how could explain the reason of increasing disease severity in case of high level of sodium nitrate? The phenomenon of increasing disease severity due to high level of nitrate was studied *in vitro.*

In the present study, growing of *Rhizoctonia solani* in increasing concentrations of sodium nitrate dramatically increase radicles canker when germinate seeds were put directly on the surface of fungal growth. The increase of disease severity due to *R. solani* infection was evident either visually or by determination of polyphenol oxidase activity in infected cotyledons. Hence, increasing of PPO activity is a function of increasing disease severity (Shetty *et al.*, 2001; Parihar *et al*. 2012).

The question arised from these results does such increase of disease severity refer to factor(s) concern with fungus or concern with the host. Results obtained indicated that increasing nitrate in the media is the factor provokes pathogenicity of *R.solani* on faba bean cotyledon.

In order to prove this suggestion, a thin sections in radicles subjected to the pathogen were carried out. Data obtained indicated that after 8 hours of subjecting germinated seeds to fungal growth on highly concentration of nitrate a heavily infection cushions were found surrounded the radicles and after 16h radicle cortex was completely collapsed.

Data obtained in this study indicated that nitrate play its role through the pathogen more than the host. The question arises from the obtained results is how excess of nitrate increased *R. solani* pathogenicity and provoke it to growth quickly around the host root. This phenomenon may be related to the major positively-acting regulatory genes as *are* A in *Aspergillus nidulars* and *nit*-2 (*Neurospora crassei*) which mediate global nitrogen repression and de repression (Marzluf, 1997). Both AREA and NIT2 activate the expression of many genes whose products are required for the utilization of nitrogen from various secondary sources.
It is well established that *R. solani* utilizes nitrate by its reduction to nitrite then to ammonia (Geisseler *et al*., 2010), and by increasing nitrate level, the level of reduced agents will increased. Does the increase of reducing nitrogen led to up stream of pathogenecity genes? In a study (the author, unpublished data) it was found that preparation of *R. solani* inocula on

ammonium sulfate instead of sodium nitrate led to increase the aggressiveness of *R. solani* on faba bean.

It could be conclude from the obtained results in the present study that nitrogen metabolism and its regulation and its relation with pathogenicity in *R. solani* requires further studies.

REFERENCES

Abawi, G.S.; Ludwig, J.W. & Gugino, B.K (2006). Bean root rot evaluation protocols currently used in New York. Ann. Rep. Bean Improv. Coop. 49: 83-84.

Agrios, G.N(2005). *Plant Pathology* 5th.Elsevier Academic Press, San Diego, California, 384p.

Anderson, N. (1982). The genetics and pathology of *Rhizoctonia solani*. Annu. Rev. Phytopathol. 20: 329-347.

Arnnok,P;Ruangviriyachai,C; Mahachai, R; Techawongstien, S. & Chanthai, S.(2010). Optimization and determination of polyphenol oxidase and peroxidase activities in hot pepper (*Capsicum annuum* L.) pericarb. International Food Research Journal 17: 385-392.

Baker, K.F (1970). Types of *Rhizoctonia* diseases and their occurrence. Pages 125-148 in: *Rhizoctonia solani*: Biology and Pathology. J. R. Parmeter, Jr., ed. University of California Press, Berkeley. 255 pp.

Balestra, G.M & Varvaro, L (1997). Influence of nitrogen fertilization on the colonization of olive phylloplane by *Pseudomonas syringae* subsp. *savastanoi*. In: Rudolph K, Burr TJ, Mansfield JW, Stead D, Vivian A and von Kietzell J (eds) Pseudomonas syringae Pathovars and Related Pathogens (pp 88–92) Kluwer Academic Publishers, Dordrecht, The Netherlands.

Ballini, E., Nguyen, T.T & Morel, J (2013). Diversity and genetics of nitrogen-induced susceptibility to the blast fungus in rice and wheat. Rice, 6:32.

Christou, T. (1962). Penetration and host-parasite relationships of *Rhizoctonia solani* in been plants. Phytopathology, 52: 381-389.

Dodman, R.L., Barker, K.R. & Walker, J.C.(1968a). A detailed study of the different modes of penetration by *Rhizoctonia solani.* Phytopathology, 58: 1271:1276.

Dodman, R.L; Barker, K.R. & Walker, J,C. (1968b). Modes of penetration by different isolates of *Rhizoctonia solani*. Phytopathology 58: 31-33.

Geisseler, D.; Horwath, W.R; Jeorgensen, R.G. & Ludwig, B (2010). Pathways of nitrogen utilization by soil microorganisms- A review. Soil Biology and Biochemistry 42(12): 2058-2067.

Ghahramani,Saeed (2002). Fundamentals of Probability (2ed Edition). Prentice Hail: New Jersey. P. 438.

Hanounik, S. B(1978). Diseases of major food legumes in Syria. Pages 98-102 in G. C. Hawtin and G. J. Chancellor, eds., Food Legumes Improvement and Development. ICARDA/IDRC, Ottawa, Canada.

Johanson, D.A (1940). Plant Microtechnique. McGraw-Hill, Book Company, Inc New York and London.523pp.

Lecompte, F., Abro, M.A & Nicot, P.C (2013). Can plant sugars mediate the effect of nitrogen fertilization on lettuce susceptibility to two necrotrophic pathogens: *Botrytis cinerea* and *Sclerotium sclerotiorum*? Plant and Soil, 369 (1-2): 387-401.

Long, D.H (2000). Effect of nitrogen fertilization on disease progress of rice blast on susceptible and resistant cultivars. Plant disease, 84(4): 403-409.

Maha H. Mohamed; Gado, E.A.M., El Deeb, S.H. & Mostafa, M.H (2014). Behaviour of fungus Rhizoctonia solani under faba bean cotyledons when away from host and the effect of its starvation on aggressiveness. Journal of Yeast and Fungal Research 5 (1): 26-32.

Marzluf, G.A (1997). Genetic regulation of nitrogen metabolism in the fungi. Microbiol. Mol. Biol. Rev., 61: 17-32.

Mubarak, H.M (2003). Control of root rot and damping-off disease of bean. PhD Thesis Tanta Univ. Botany Depart. Egypt.

Ogoshi, A. (1987). Ecology and pathogenicity of anastomosis and intraspecific groups of *Rhizoctonia solani* Kühn. Annu. Rev. Phytopathol. 25: 125-143.

Parihar, P.S., Prakash, O., & Punetha, H. (2012). Investigation on defensive enzymes activity of *Brassica juncea* genotypes during pathogenesis of *Alternaria* blight. Nature and Science, 10(2): 63-68.

Salt, G.A. (1982). Factors affecting resistance to root rot and wilt diseaes. Pages 259-267 in G. C. Hawtin and C. Webb, eds., Faba Bean Improvement. Martinus Nijhoff Publishers, The Netherlands.

Shetty, H.S., Vasanthi, N.S., Sarosh, B.R. & Kini, K.R (2001). Inheritance of downy mildew resistance, ß-1, 3-glucanases and peroxidases in pearl millet [*Pennisetum glaucum* (L.) R. Br.] crosses. Theor. Appl. Genet. 102: 1221-1226.

Talbot, N.J., McCafferty, H.R.K., Ma, M., Moore, K. & Hamer, J.E. (1997). Nitrogen starvation of the rice blast fungus *Magnaporthe grisea* may act as an environmental cue for disease symptom expression. Physiol Mol Plant Pathol 50:179–195.

Wallwork, H. (1996). Cereal root and crown diseases. Kondinin Group, Perth, Australia.

Walters, D.R. & Bingham, I.J.(2007). Influence of nutrition on disease development caused by fungal pathogens: implications for plant disease control. Annals Appl. Biol. 151:307-324.

Yoshida, M.,Nakajima,T. & Tonooka, T. (2008). Effect of nitrogen application at anthesis on *Fusarium* head blight and mycotoxin accumulation in breadmaking wheat in the western part of japan. Journal of General Plant Pathology 74(5): 355-363.

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Fig.5: Effect of nitrate levels on root weight (gm) of faba bean plants grown in infested soil with *Rhizoctonia solani* amended with different levels of sodium nitrate.

Fig.6: Effect of nitrate levels on number of leaves/plant of faba bean plants grown in infested soil with *Rhizoctonia solani* amended with different levels of sodium nitrate.

Fig. 7: The scale of disease incidence expressed on faba bean germinated seeds.
1: apparently healthy; 2: very weak infection; 3: moderate infection; 4: very severe infection; 5: completely covered by fungal mats and died.

Fig. 8: Effect of nitrate levels on the aggressiveness of *Rhizoctonia solani* on germinated faba bean seeds. Average of three distinct experiments.

Fig. 9: The activity of polyphenol oxidase of faba bean seeds infected with *R. solani* grown on different nitrate level.Enzyme activity was determined as ▲changes of optical density at 410 nm.
Average of three distinct experiments.

Fig. 10: Light micrographs of faba bean radicle cross-sections illustrating the *R. solani* hyphal penetration 8 hours after inoculation. a&b: half level; c&d: normal level; e&f: double nitrate level.

Fig. 11: Light micrographs of faba bean radicle cross-sections illustrating the effect of nitrate levels on post-penetration process of *R. solani*, 16 hours after inoculation. a&b: half nitrate level; c&d: normal nitrate level; e&f: double nitrate level.

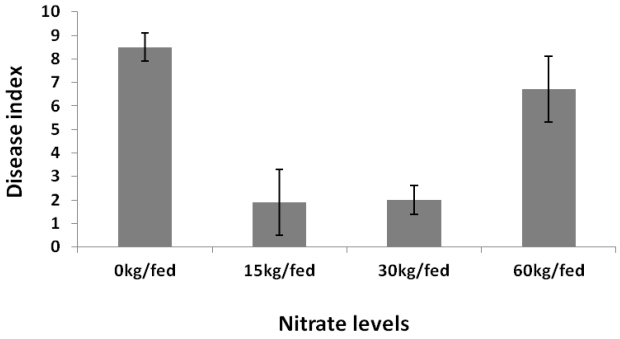
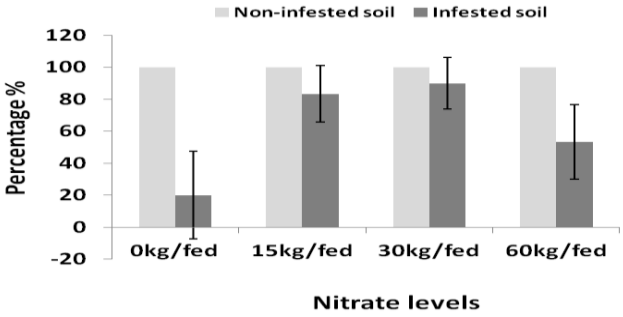


Fig. 1



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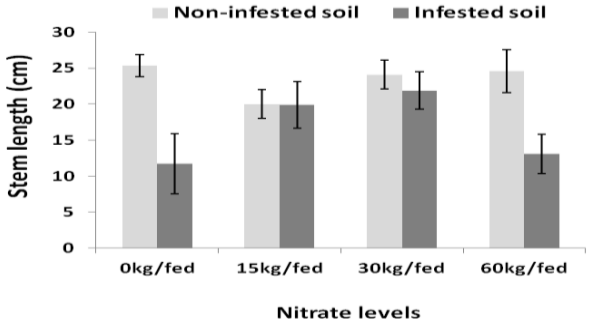

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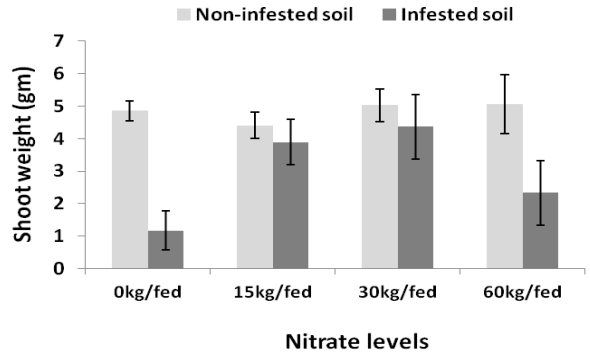

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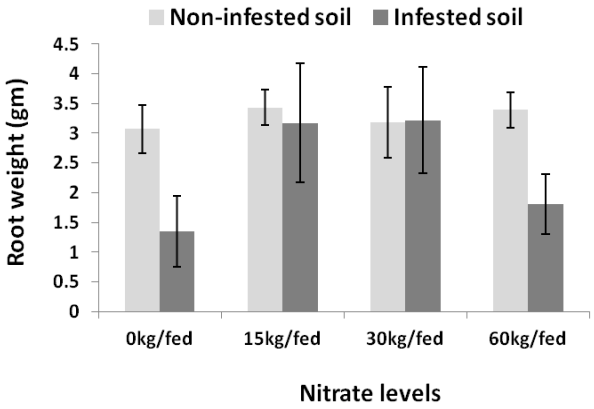
Non-infested soil
Infested soil
Root weight (gm)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0kg/fed
15kg/fed
30kg/fed
60kg/fed
Nitrate levels

Fig. 5

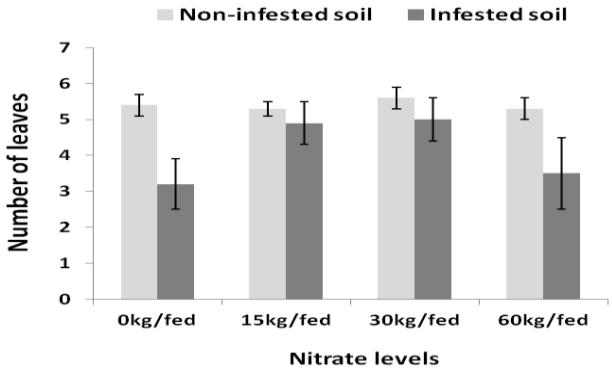
Non-infested soil
Infested soil
Number of leaves
7
6
5
4
3
2
1
0
0kg/fed
15kg/fed
30kg/fed
60kg/fed
Nitrate levels

Fig. 6

1 2 3 4 5

Fig. 7

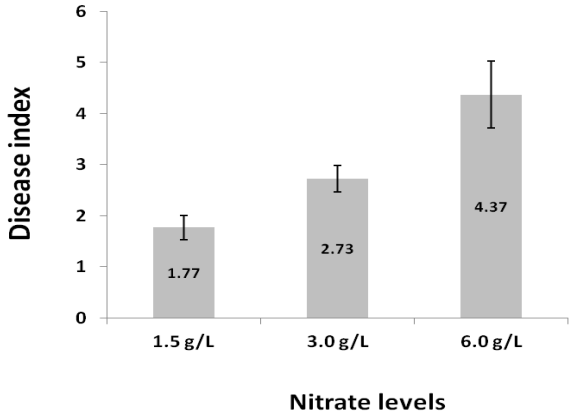

Fig. 8

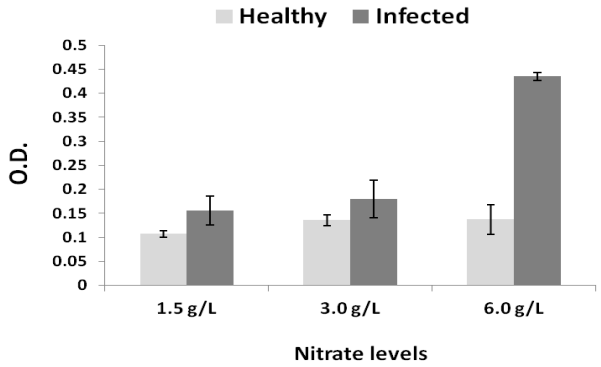

Fig. 9

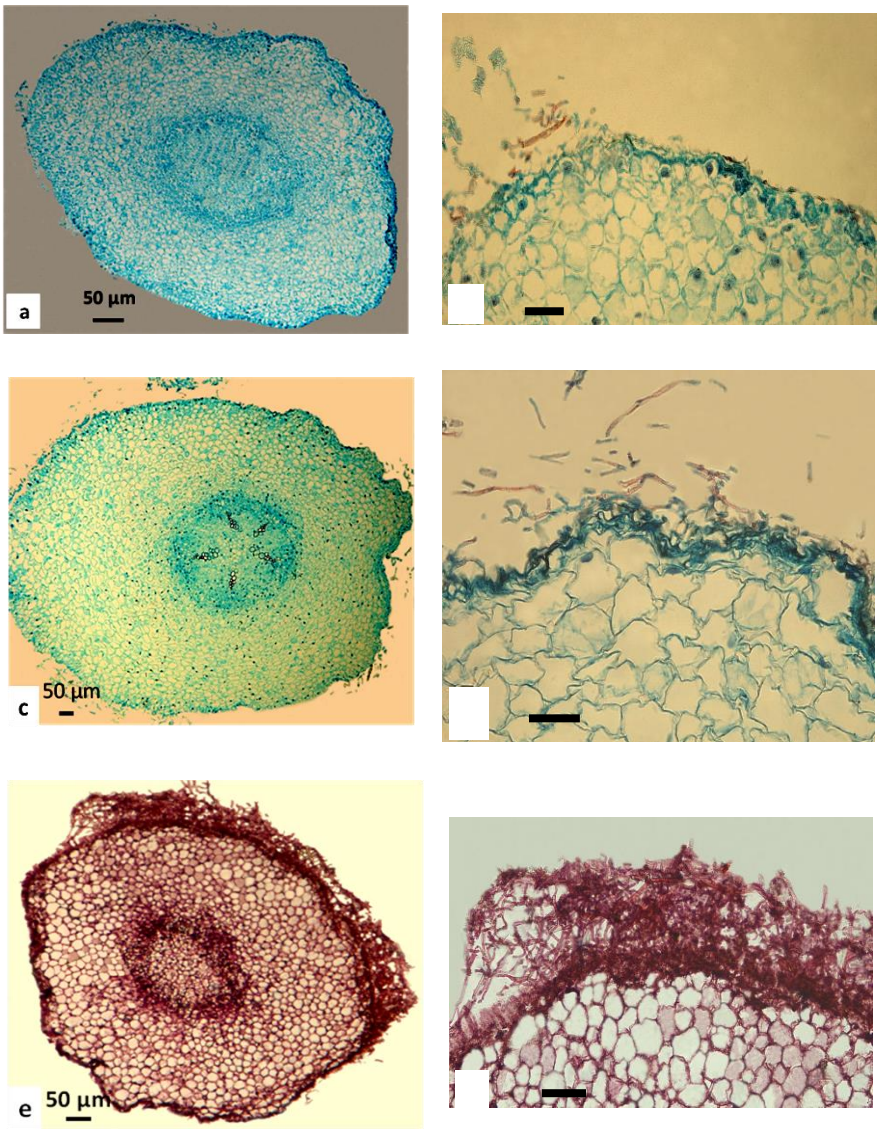

Fig. 10

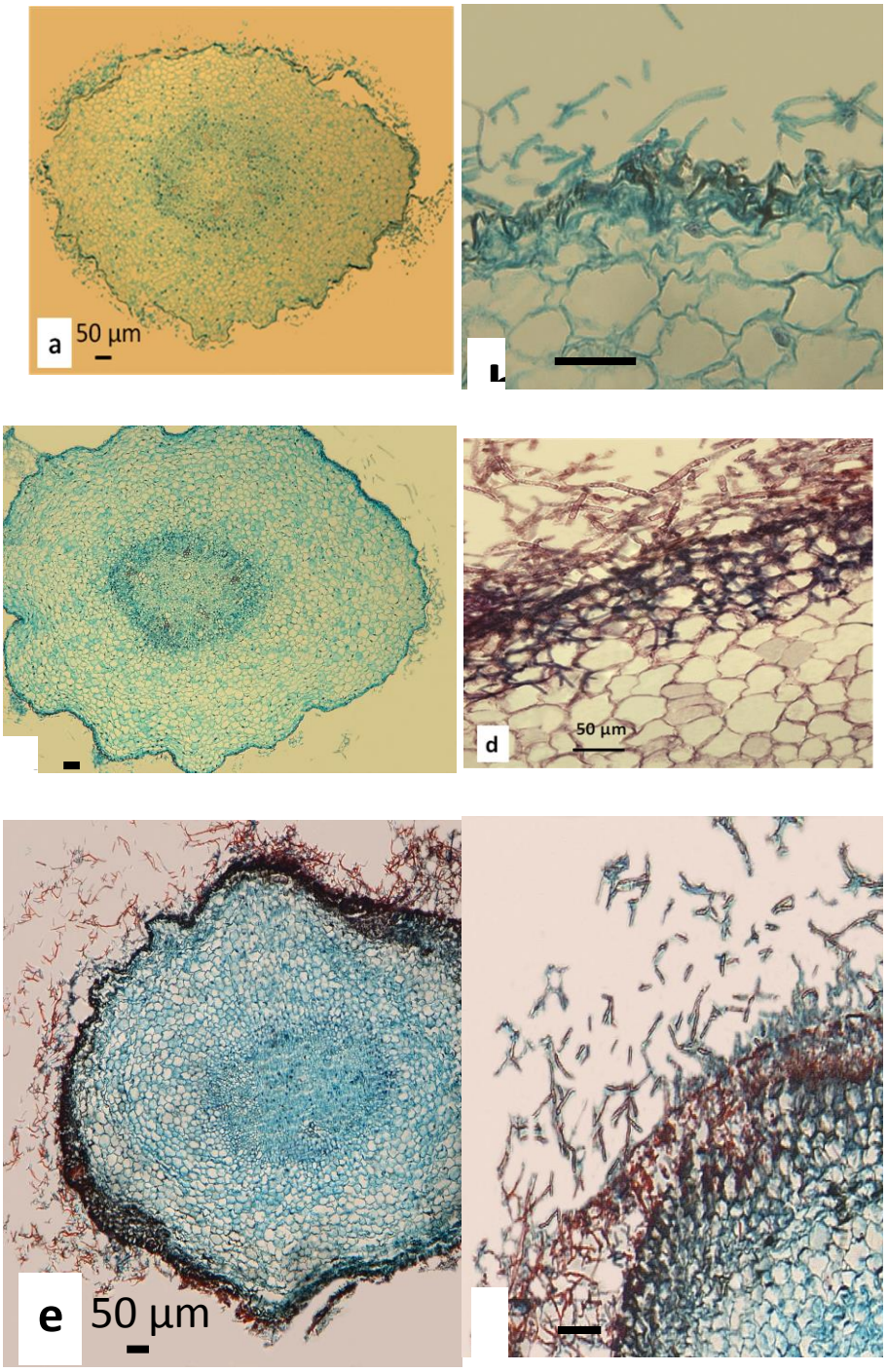

Fig. 11