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RESPONSES OF FUSARIUM OXYSPORUM F. SP. RADICIS-LYCOPERSICI (FORL) TO ZNNO3 UNDER IN VITRO CONDITIONS

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ABSTRACT

Fusarium oxysporum f. sp. radicis-lycopersici (FORL) is a problematic soil-borne pathogen that is widely reported to cause severe losses in tomatoes. Studies on the use of trace elements to suppress different F. oxysporum biotypes and various other soil-borne diseases has yielded contrasting results. This study was carried out to analyze differential response of FORL to different $ZnNO_3$ concentrations. Four concentration levels of $ZnNO_3$: 0, 86, 172 and 258 mg/ml were investigated. Malt Extract Agar media plated petri dishes were infused with 1 ml of the respective four $ZnNO_3$ concentrations These treatments were replicated three (3) times making a total of twelve experimental units. Ten (10) mm diameter mycelial discs of FORL were placed at the center of each of respective experimental units. Mycelial growth in the experimental units incubated at 27^0C was assessed by measuring mycelial discs diameters. The mycelial radial growth assessment indicated a general trend of increasing growth inhibition with increasing $ZnNO_3$ concentration. Regression analysis was carried out to determine statistically significant differential response of FORL to different $ZnNO_3$ concentrations. There were statistically significant responses ($P<0.05$) to $ZnNO_3$ concentrations, with LC_{50} established at 170mg/ml. As this LC_{50} was established based on FORL it would not assumed to be the same for other FO biotypes. There is therefore the need to establish LC_{50} for the various FO biotypes i.e., FOL (races 1,2,3) and the endophytic FO. With Zn reported to have phyto-toxicity effect on some plants at certain levels, it is further recommended that LC_{50} of $ZnNO_3$ for tomato, and various other vegetable seeds should be determined.

Keywords: Fusarium oxysporum, Biological control, Endophytic, Myco-ecology

INTRODUCTION

Fusarium oxysporum (FO) is a problematic soil-borne pathogen that is widely reported to cause severe losses in tomato seedlings. This disease is often caused by two forms of biotypes i.e. F. oxysporum f. sp. radicis-lycopersici (FORL) Jarvis et Shomaker (ATCC 60095) that causes crown and root rot, and the other biotype F. oxysporum f. lycopersici (FOL) that causes vascular wilt disease in tomatoes (Edel-Hermann *et al*., 2011). Although both Fusarium biotypes infect the same host plant, FOL and FORL have strict host varietal specificity probably influenced by the variation in location of resistance genes in respective hosts. For instance, it has been established that FOL occurs in form of three physiological races (1, 2, and 3). These three races were differentiated on the variation in their infectivity for different tomato cultivars with different resistance loci (Mes *et al*., 1999). In addition to these pathogenic FO biotypes, there is a non-pathogenic strain that occurs in the same myco-ecology. This non-pathogenic FO has endophytic properties and host plant defensive response against the pathogenic strain of FO and various other soilborne fungal pathogens (de Lamo and Takken 2020). One of the interesting differences that has been noted between the pathogenic (FOL) and non-pathogenic strain is the pattern of colonization, even though both pathogenic and non-pathogenic strains colonize the root cortex, their post-cortex infection progression differ. The

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endophytic biotype's weak aggression is as a result of low generation of effector properties i.e., it is restricted not to go beyond root cortex, and the FOL pathogenic strains with higher effector properties goes further to invade the vascular system. It is the infection of the xylem that results in wilting symptoms (Gordon, 2017).

Fusarium wilts and various other soil borne diseases have generally been managed by use fungicides and trace elements. Suppression of soil borne diseases by use of macro and micro-elements has been investigated by various workers and their results have been variable (Robson 1993 and Chandrashekara *et al*., 2012). Copper and Zn have particularly been reported to have suppressive effect on certain soil-borne diseases (Marschner, 1995). Effect of these soil borne disease management techniques on beneficial FO endophytic strains, and other beneficial fungi such as mycorrhizal fungi have not however been extensively investigated. Use of these trace elements in the management of soil borne disease could be adopted if their safety on both beneficial soil borne fungi and vegetable seeds is established.

Considering that plants are prone to trace elements phyto-toxicity that exceed acceptable safe levels, the objective of this study was therefore to carry out in-vitro sensitivity test of FORL to various $ZnNO_3$ concentrations levels. The lowest effective $ZnNO_3$ concentration against FORL would thereafter be evaluated for its phyto-safety on various vegetable seeds, and biosafety on various ecological beneficial endophytes and other beneficial fungi.

MATERIALS AND METHODS

Experimental description

Preparation of spore suspension*:* F. oxysporum f. sp. radicis-lycopersici (FORL) isolate sourced from Agricultural Research Council Plant Protection Institute, (South Africa) was cultured and incubated at 28 ºC for a period of eight (8) days. Mycelia grown in petri dishes were gently loosened by spatula and 5 ml of distilled water was added into each respective petri dish . Mycelial suspension of FORL isolate from the respective petri dishes were transferred into 250 ml beaker filled with distilled water creating a mycelia/spore suspension. The suspension was homogenized by an electric magnet stirrer for 30 minutes.

Fungal spore viability test*:* A syringe was used to draw 5 ml of mycelial/spore suspension from the beaker. Five (5) drops of 0.05 ml each of suspension were placed into each of the four MEA plated petri dishes. Dishes were wrapped with a para-film and incubated in the dark at 28°C for 7 days. Plates were inspected for single spore germination at an interval of two days starting two days after inoculation.

FORL pathogenicity test*:* Five (5) millilitre of FORL suspension (10^6 spores/ ml) was simultaneously subjected to pathogenicity test by inoculating tomato seedlings at three weeks after emergence.

Assessment of mycelial disc growth response to different $ZnNO_3$ *concentrations:* Parallel to the FORL inoculum preparation, different $ZnNO_3$ concentration solutions were prepared i.e., 0; 86; 172 and 258 mg/ml were dissolved in distilled water. This made the following Zn concentration levels: 0; 29,67; 59,34; 89,01 mg/ml. The twelve MEA media plated petri dishes were infused with respective four $ZnNO_3$ concentrations at a rate of ml/plate. These were kept in a lamina flow until complete absorption. The 10 mm diameter mycelial discs were cut from the FORL cultures and placed at the center of each of the respective $ZnNO_3$ infused MEA growth media. Each of the respective treatments i.e., four treatments ($ZnNO_3$ concentrations) i.e., 0; 86; 172 and 258 mg/ml were replicated three times making a total of 12 experimental units.

The twelve (12) experimental units with mycelial discs inserted in the center of respective petri dishes were arranged in a Completely Randomized Design (CRD) in an incubator set at 28°C. Mycelial growth of FORL in the twelve experimental units were assessed by measuring mycelial discs (cm) at their perpendicular diameters by use of a caliper. The mycelial radial growth assessment was carried out at three (3) day interval, for a period of 12 days starting three (3) days after incubation.

Determination of Inhibition ratio*:* Growth inhibitory effect of the different $ZnNO_3$ concentrations were analysed by calculation of inhibition ratio for the respective treatments. Inhibition effect may be explained as the measure of inhibitory influence by subtracting inhibited growth from normal growth of the same object and dividing by the normal growth of the same object. Inhibition ratios for the respective treatments were determined by use of the following formula:

$$Mycelia\ Disc\ Diameter\ Area = \pi r^2 = \pi\left(\frac{Average\ D}{2}\right)^2$$

$$Inhibition\ Ratio = \frac{(Average\ Control\ Area - Average\ Treatment\ Area)}{Average\ Control\ Area}$$

STATISTICAL ANALYSIS

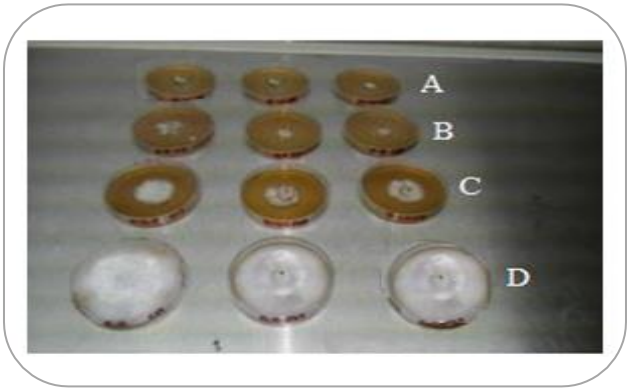
Statistical Analysis Software (SAS, 2010) was used to carry out regression analysis of the effect of different $ZnNO_3$ concentrations on the mycelial growth of FORL.

RESULTS

Pathogenicity test for FORL*:* Pathogenicity of FORL was determined by occurrence of crown and root rot as shown in Figure 1. This differentiated it from F. oxysporum f. lycopersici (FOL; 1.2.3) infections which affects fine roots and vascular systems result*ing in wilting of the seedlings.*

Fig*ure* 1. Tomato seedlings showing crown and root rot symptoms (FORL biotype symptoms).

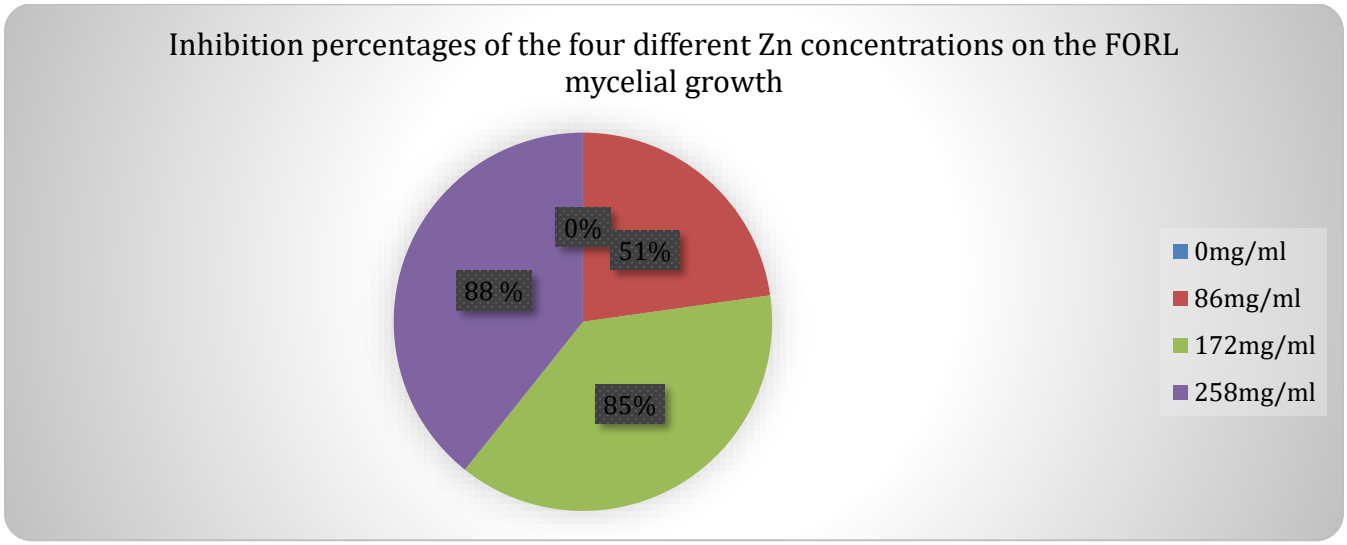
Analysis of FORL sensitivity to different $ZnNO_3$ concentrations*:* Figure 2 shows effect of four $ZnNO_3$ concentrations on FORL mycelial growth.



Fig*ure* 2. FORL mycelial growth in $ZnNO_3$ infused MEA and Control: 12 days of incubation.

KEY: Row A-258mg/ml; Row B -172mg/ml; Row C - 86mg/ml and Row D - 0 mg/ml (Control)

Although Fig. 2 exhibits evidence that there is variation in the sensitivity of FORL to the different $ZnNO_3$ concentrations, it was however necessary that this variation be quantitatively determined and statistically analyzed. This was undertaken by determination of Inhibition ratios and carrying out regression analysis of effect of $ZnNO_3$ concentration on the FORL mycelial growth. Figure 3 shows inhibition effect of different $ZnNO_3$ concentration on FORL mycelial growth. Of the four $ZnNO_3$ concentration levels, 258mg/ml had the highest inhibition ratio which converted *to inhibition percentage of 88%*.



Fig*ure* 3. *Inhibition percentage of four Zn concentrations of FORL mycelial growth *Inhibition percentages were calculated from Inhibition ratios.

The Analysis of Variance (Table 1) shows that FORL was significantly sensitive to the tested $ZnNO_3$ levels ($P \leq 0.05$). The R-squared value of 0,92 [Table 1 (b)] shows that influence of ZnNO3 concentration on FORL mycelial growth was significantly strong.

Table 1 (a). Analysis of variance for regression analysis for the effect of ZnNO3 on the FORL mycelial growth

	DF	SS	MS	F	Significance F
Regression	1	1,92	1,92	22,43	0,041
Residual	2	0,17	0,08		
Total	3	2,09			

Table 1 (b). Summary of Regression analysis for the effect of $ZnNO_3$ on the FORL mycelial growth

Regression Statistics	
Multiple R	0,927471
R Square	0,860203
Adjusted R Square	0,790305
Standard Error	0,601714
Observations	4

Figures 4 (a)) gives a scatter graphical presentation of the linear relationship between $ZnNO_3$ concentration levels and FORL mycelial growth. Figure 4 (b) shows the linear relation (Predicted Y-values) between the $ZnNO_3$ concentrations and mycelial growth.

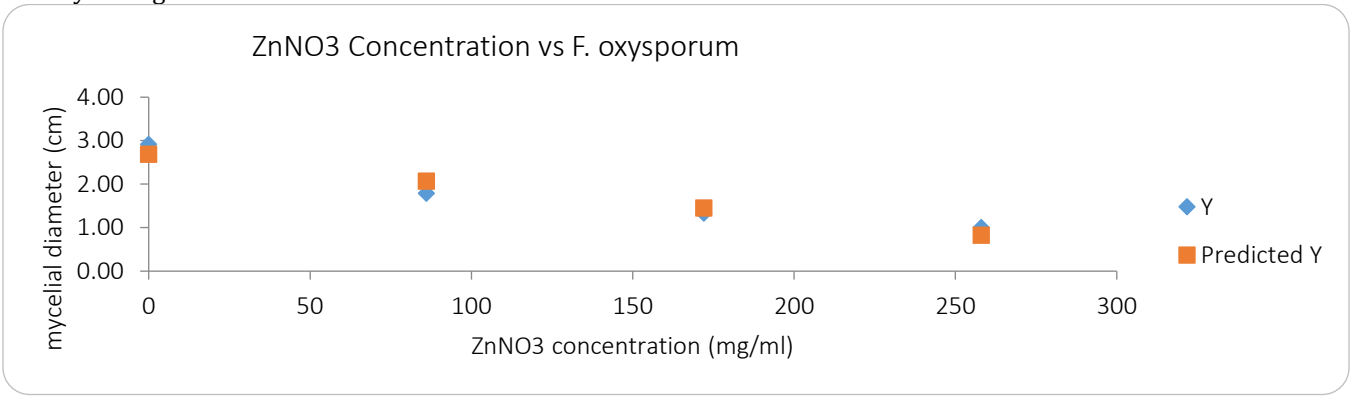


Figure 4 (a). Regression analysis of effect of Zn concentration on FO mycelial growth

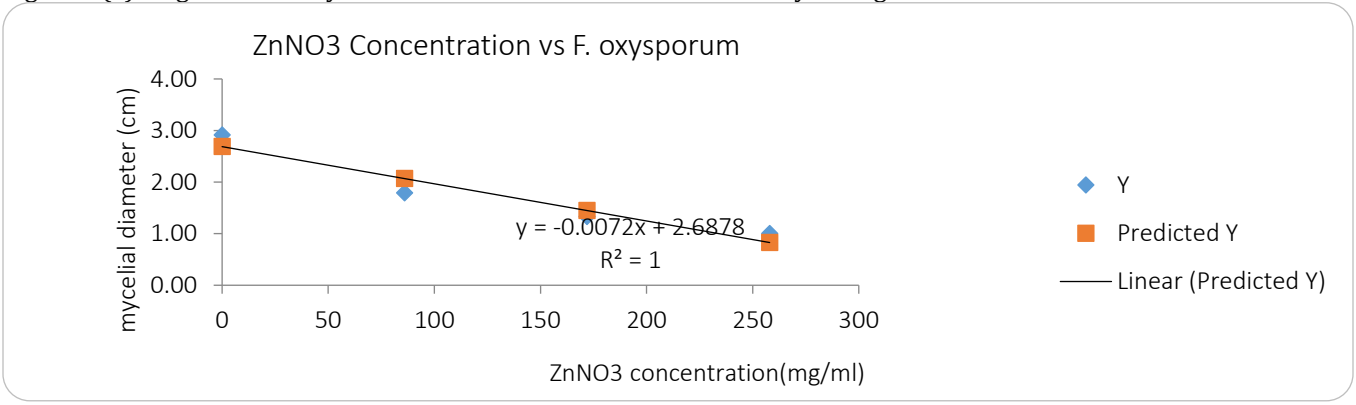


Figure 4 (b). LC_{50} Prediction model for Zn concentration on FO mycelial growth

DISCUSSION

Lethal concentration is concentration of a $ZnNO_3$ mixed in distilled water at which half the FORL mycelial growth is inhibited after a specified duration of exposure. LC_{50} is inversely proportional to toxicity. Estimation of median lethal concentration (LC_{50}) is very valuable tool in analyzing sensitivity tests of anti-microbial solutions. By use of regression equation [Fig 4(b)], it was established that LC_{50} of Zn NO_3 for FORL was 170mg/ml. As FO occurs in various forms of biotypes, it is important that LC_{50} for the different biotypes should be established. If ZnNO3 could be used as seed treatment for the control of all the various biotypes of FO i.e., FOL (race 1,2, 3) and FORL, the rate of seed treatment should have the same LC_{50} of 170mg/ml or higher. With Zn reported to cause phyto-toxicity on seeds (Wang *et al*, 2011), it is important that LC_{50} of $ZnNO_3$ on seeds should also be higher than 170mg/ml so that the FORL or FOL pathogenic strains can be controlled without affecting seed viability.

The growth of the mycelia including the rate of reproduction in the form of sporulation is dependent on metabolic and biosynthesis processes (Papagianni, 2004). Any disruptions in one of the processes could possibly induce suppressive effects on the growth of the mycelia. It is deduced that the Zn infused into the MEA was absorbed by the fungal mycelia in a similar mechanism as how the fungi mycelia acquire carbon and other nutrients from the growth media. Fungi require carbon as food source and this can be available in the form of sugars and starch provided by the growth media such as MEA (Oda *et al*., 2002). The carbon from glucose and starch are utilized by the F. oxysporum for growth, and reproduction (Hedlund and Öhrn, 2000). This absorption process of Zn into the F. oxysporum system could have probably caused disruptions of the biochemical processes of the fungus when it was absorbed at higher concentrations as noted in Fig. 2.

When Zn accumulates in the mycelia cells, it is known to change the enzymatic activities which regulate the biochemical and metabolic processes within the cell (Savi, *et al*., 2013). These biochemical activities could have been disrupted by Zn being bound onto the thiol groups in the cellular membrane of the FORL mycelia (Ahmad *et al*. 2002). This occurrence could probably have resulted in the binding of Zn^{2+} to thiol groups disrupting the formation of the structure of multi-unit proteins (Harper 1977). It is deduced that when these Zn bound thiol groups form strong covalent bonds between different peptide chains in the cytoplasm, they inactivate enzymes which subsequently cause various metabolic damages and reduce the cell vitality (Harper, 1977; Ahmad *et al*. 2002).

Under high Zn concentrations, mycelial cells lose their ability to replicate and therefore fungal growth is reduced. Yin-dong CHANG *et al*., (2018) also highlighted that Zn suppresses F. oxysporum by interacting with mycelial cell membrane which cause structural change and permeability. There are various other studies which reported that Zn^{2+} may cause structural changes of microbial cell membrane, causing cytoplasm leakage and eventually the death of fungal cells (Sawai and Yoshikawa 2004). The study by Sawai and Yoshikawa, (2004), further reported that increased cell membrane permeability results in leakage of those microbial nucleic acids thereby disturbing the metabolic activities with resultant reduction of cellular growth. Cabot *et al*., (2019) also reported that Zn interacts with microbial nucleic acids, preventing cell replication. These could possibly be the explanation for the reduced mycelial growth noted in growth media treated at 170 LC_{50} [Fig. 4 (b)]

Furthermore, studies by Baldrian, (2009) highlighted that Zn^{2+} ions can bind to aromatic amino acid residues in enzyme molecules and cause oxidative damage of proteins of the fungi. It was also reported that Zn^{2+} can be transported by the absorption mechanism for essential metal ions to the cell where it can accumulate and exert toxic effects at high concentrations possibly as noted in Fig.2. Baldrian, (2003) found that Cu^{2+} and Zn^{2+} were also reported to induce morphological changes in all groups of fungi and influenced the reproduction by reducing cellular multiplication. This could possibly explain the higher efficacy at 170mg/ml LC_{50} [Fig. 4 (b)]. Zn absorbed into the cell wall might have interacted with the cellular metabolites and other enzymatic chemicals within the cell membrane causing possible cell wall deterioration and possible disintegration. It was also reported that Zn^{2+} can easily be absorbed through the cell wall and cytoplasmic membrane inside the fungal cell. These changes in enzymes and metabolites might have caused deformities and malfunctions of the cells by thickening the structures of the hyphae resulting in reduced rate of mycelial growth and development. It is also reported by Brayner *et al*. (2006) that metabolic activities can also result in distorted and damaged conidia and fungal spore germination could be completely inhibited when the mycelia are exposed to certain Zn concentration levels.

Other possible Zn myco-pharmacological processes that have been reported include biochemical reactions of Zn within the cell membrane. This could have triggered the F. oxysporum mycelial cells to produce more carbohydrates and proteins to thicken the hyphae as a defense mechanism against Zn. Various other researchers have reported that fungal mycelial cells produce more carbohydrates and protein in response to high Zn levels (Kim *et al*. 2008a, b). It can therefore be deduced that this increase of carbohydrates may be due to the self-protecting mechanism against Zn^{2+} when fungi are treated with Zn^{2+} at high concentrations of 170 LC_{50}. Other similar studies indicated that an increase of nucleic acid may be due to stress response of fungal hyphae when there is high Zn concentration (Alvarez-Peral *et al*. 2002). It can be assumed that a cellular membrane can only hold and withstand a certain

amount of cytoplasmic fluids over a specific period. Therefore, when there is an oversupply of carbohydrates and nucleic acid the cytoplasm may grow uncontrollably and cause the cell membrane to lose its turgidity. The cell membrane could burst and become permeable and these activities could have eventually led to leakage of essential biochemical compounds for F. oxysporum mycelial growth and development. Leakage of those components could have resulted in F. oxysporum mycelial cell death.

CONCLUSIONS AND RECOMMENDATIONS

With the LC_{50} of ZnNO3 for FORL being established to be 170 mg/ml, it is important that LC_{50} of Zn NO3 for FOL (races 1,2,3) should also be determined. It is particularly of myco-ecological value to establish whether it varies from the level that is effective against FORL. Having established in-vitro LC_{50} for ZnNO3 for FORL to be 170mg/ml, it is recommended that this investigation should be carried out under various in-vivo conditions where various soil factors such as pH, clay particles, other trace elements could have influence on the biochemical properties of ZnNO3 and with possible resultant influence on its efficacy. It is also recommended that LC_{50} of ZnNO3 for beneficial fungi should be investigated. These fungi include the endophytic non-pathogenic FO and mycorrhizal fungi which are often found in the same myco-environment with the pathogenic strains.

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Manny Mathuthu	:	Wrote paper
Jan R. Zeevaart	:	Analyzed the data