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ORIGINAL PAPER

Enhancing cucumber transplanting immunity against Rhizoctonia root rot using micronutrients and antioxidants

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ABSTRACT

Cucumber is one of the most popular and favorite vegetable crops cultivated under greenhouse in Egypt. A greenhouse experiment was carried out to reduce cucumber transplants root rot. Soaking cucumber seeds before sowing in different concentrations of micronutrients, antioxidants, and their combinations were evaluated for controlling *Rhizoctonia solani* root rot. The results indicated that the highest seedling stand 73.4% after 12 days was recorded after soaking cucumber seeds in sodium selenium solution at 1 ppm. In artificially infested pots with *R. solani* seed treated with potassium tartrate and boric acid had the highest seedling stands with a clear increase in total phenol contents. Potassium tartrate combined with boric acid showed the highest reduction in radial growth of *R. solani* by 88.9%. The highest reduction of oxalic acid produced by *R. solani* on PDA medium supplemented with boric acid were measured using high performance liquid chromatography. Our findings demonstrated an effective approach for inducing resistance in cucumber transplants to Rhizoctonia root rot utilizing micronutrients and free radical scavengers.

Keywords: Cucumber, *Rhizoctonia solani*, Antioxidants, Micronutrients

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INTRODUCTION

Cucumber plants growing in plastic houses in Egypt are frequently affected by root rot and wilt diseases, which lead to significant fruit yield losses (**Abd-El-Kareem, 2009).** Compared to open field, greenhouse cucumbers have an added benefit of a premium pricing because to their high output, seasonal availability, and fruit quality, especially fruit symmetry and firmness. *Rhizoctonia solani* Kuhn, responsible for damping-off and seedling blight, causes significant losses in vegetable crop transplant production, both in a greenhouse and in the field, once asymptomatically infected seedlings are transplanted. (**Morsy *et al*., 2009**). *R. solani* is known for causing yield reductions across a wide range of commercial crops globally, affecting both agricultural and horticultural sectors (**Dhingra *et al*., 2004**). The fungus is active as fine hyphae that survive in soil. Resting structures of *R. solani* sclerotia consist of hard masses of fungal threads that are resistant to hard conditions and are unable to eradicate with chemical fungicides (**Aydin, 2022**). Additionally, this pathogen and other sclerotium-producing fungi produce oxalic acid, a key factor in their pathogenicity (**Nagarajkumara *et al.*, 2005**). The growing worry over the use of pesticides in terms of human health and the natural environment has brought greater interest in the usage of alternatives that have a positive influence on the environment **(Ali, 2021).** In this context, employing micronutrients as a strategy for inducible plant defence presents a promising alternative to fungicide application. Micronutrients can enhance plant resistance to a broad range of diseases and offer a more environmentally sustainable approach. (**Dordas, 2008 and Zhang *et al.*,2021**). Essential nutrients play a crucial role in plant growth and development, as well as in the defense against diseases by affecting various aspects of the disease pyramid (**Huber and Haneklaus, 2007**). These compounds are inexpensive, readily available, easy to apply, and environmentally safe. Additionally, they have been shown to induce resistance in many plants against pathogens in nursery, potentially increasing profitability in the seedling production. Plant nutritional status has a significant impact on disease susceptibility, which has been used to reduce a range of diseases (**Christos, 2008**). Plants require an antioxidative procedure that protects them from oxidative stress induced by internal variables such oxygen radicals created during the metabolism process or as a result of infections **(Yousef *et al.*, 2016).** Furthermore, several antioxidant substances have been demonstrated to inhibit the effects of free radicals produced during the pathogenicity of different fungi (**Weigend and Lyr, 1996 and EL-Korany and Mohamed, 2008). Kheyri and Taheri (2021),** reported that boric acid, zinc sulfate, and manganese sulfate at a concentration of 1000 mg/L completely suppressed the linear growth of *R. solani*. So, this study was conducted to demonstrate how a combination of effectively antioxidants and micronutrients can increase plant resistance against *R. solani* and increasing cucumber seedling health.

MATERIALS AND METHODS

1- Greenhouse Experiments

1.1 Evaluation of micronutrients for controlling cucumber seedling root rot.

1.1.1. Source of pathogen: -

The pathogenic fungus, *Rhizoctonia. solani* isolate (MZ267232) known for its high virulence, was previously isolated and identified by (**Ali, 2021**).

1.1.2. Preparation of pathogen inoculum and soil infestation: -

R. solani inoculum was produced by growing the fungus on corn sand meal medium, 1:3 w: w and 40% water, and enriching with 2% peptone (**Abd El-Moity, 1985**). Bottles containing the medium were inoculated with equal 5 mm disks of *R. solani* from 5 day old cultures. The inoculated bottles were incubated at 26°C for ten days. Loaded fungal growth was mixed with the plastic bag mixture (peat-vermiculite-perlite mix at ratio 2:1:1by volume) at the rate of 10 g/ kg soil (each g contains 8×10^5 CFU).

1.1.3. Evaluation of micronutrients on managing *Rhizoctonia solani*:

After one week from infestation, the cucumber seeds were sown. Twenty seeds were used per replicate, with five plastic bags (25 cm diameter) per replicate. three replicates were used for each treatment. All used micronutrients in these experiments were purchased from Piochem for laboratery Chemical Co., Egypt. The concentrations of micronutrients were based on previous research with some modifications (**Devchand *et al.*, 2010; Yousef *et al.*, 2013 and Toorray, 2023**). Cucumber seeds, cultivar Beit Alpha, susceptible to *R. solani* were soaked for 12 hours in the following treatments:

1. Tap water (control).
2. Zinc sulphate ($ZnSO_4.7H_2O$): 96% purity, solution at the rate (250, 500 and 1000 ppm), (Zn).
3. Manganese sulphate ($MnSO_4.H_2O$): 95% purity, solution at the rate of (250, 500 and 1000 ppm), (Mn).
4. Boric acid (BH_3O_3): 98% purity, solutions at the rate of (75, 150 and 300 ppm), (B).
5. Copper sulphate ($CuSO_4$.5H2O): 99% purity, solution at the rate of (100, 200 and 400 ppm), (Cu).
6. Sodium selenite (Na_2SeO_3): 98% purity, solution at the rate of (0.5 and 1 ppm), (Se).

All treatments were conducted under greenhouse conditions at central lab of organic agriculture, Agricultural Research Center, Giza, Egypt. In each treatment, the percentage of pre-emergence damping off was recorded after 6 days and seedlings stand after12 days from sowing.

1.2. Effect of using Antioxidants (free-radical scavengers) on cucumber seedling Rhizoctonia root rot.

This experiment was carried out to determine the most effective antioxidant and the best concentration on improving seedling stand percentage, in soil infested with *R. solani*. Preparation and infestation of *R. solani* were done as described in the previous experiment. The experiment included 22 treatments, 3 concentrations (5, 10 and 15 ppm) of 7 antioxidants plus check treatment. Cucumber seeds were soaked for 12 hours in aqueous antioxidants solutions of benzoic acid, potassium tartrate, di potassium hydrogen citrate, hydroquinone, salicylic acid, ascorbic acid and thiamin. All used antioxidants in these experiments were purchased from El-Gomhoria Chemical Co. (Mansoura, Egypt). The percentage of pre-emergence damping off was recorded after 6 days and seedlings stand after12 days after sowing.

1.3. Impact of mixing potassium tartrate with various micronutrients on cucumber seedling Rhizoctonia root rot.

This experiment evaluated the effectiveness of potassium tartrate (K tartrate) at 300 ppm as the best antioxidant combined with various micronutrients on cucumber seedling stand and total phenol content. The treatments were:

a) Tap water (control)
b) Zn sulphate 500 ppm + K tartrate
c) Mn sulphate 500 ppm + K tartrate
d) Cu sulphate 100 ppm + K tartrate
e) Boric acid 100 ppm + K tartrate
f) Sodium selenite 1.0 ppm + K tartrate

cucumber seeds were sown in infested soil with the virulent isolate of *R. solani*. Data were analyzed separately for pre-emergence damping off and seedling stands after 6 and 12 days, respectively. Additionally, total phenol content in fresh infested roots of cucumber seedlings was determined after 6 days from planting.

1- Laboratory Experiments

2.1. Effect of using various micronutrients and potassium tartrate at different concentrations on reducing *R. solani* mycelial growth

The micronutrients (Manganese sulphate, Zinc sulphate, Copper sulphate, Boric acid and Sodium selenite) and potassium tartrate were incorporated separately into PDA agar medium supplemented with three concentrations, 250, 500, and 1000 ppm of Zn and Mn; 100, 200, and 400 ppm of Cu; Boric acid at 75, 150, and 300 ppm and 0.5, 1 and 2 ppm of Se. Potassium tartrate at concentration of (5, 10, and 15 mM) was tested. Three chemical-free petri dishes were used as a check. After solidification of the medium, each dish was inoculated in the middle with a mycelial disc (5-mm diameter) obtained from the periphery of actively growing colonies of *R. solani* on PDA agar medium (three replicate plates per concentration of chemicals). All of the Petri dishs were incubated in the dark at 26 ± 2˚C. The diameter of each colony was measured after 5 days, when the control plate had covered the whole petri dishs. Mycelial growth inhibition was determined as follows:

%Hyphal growth inhibition = 100 – (r2 / R2) × 100

where **r** is the colony radius of the pathogen on PDA medium incorporated with the different micronutrients and K tartrate. R is the colony radius of the fungal pathogen on PDA medium without treatments.

2.2. Reduction of *R. solani* mycelial growth using micronutrients mixed with Potassium tartrate

The most effective concentrations of tested micronutrients (Manganese sulphate, Zinc sulphate, Copper sulphate, Boric acid and Sodium selenite) were mixed with potassium tartrate (as antioxidant). PDA medium was supplemented with each of alone 500 ppm of Zn and Mn. Cu as 100 ppm, Boric acid as 100 ppm and Se, as 1 ppm. Also, Potassium tartrate was used at concentration 300 ppm. Three replicates of each concentration had been used, while three Petri dishes containing just PDA agar media were used as control. After the medium had solidified, each dish was inoculated in the center with a mycelial disc (5-mm diameter) obtained from the outer edge of actively growing *R. solani* colonies. Plates were incubated at 26 ± 2˚C in the dark. The diameter of each colony was measured 5 days later. Mycelial growth inhibition was assessed as previously described.

2.3. Using micronutrients to reduce oxalic acid producing by *R. solani*

This paper describes a method for measuring oxalic acid production in *R. solani* broths with and without micronutrient supplementation.

Preparation and Inoculation: A 5-mm mycelial disc was obtained from a 5-day-old PDA culture of *R. solani* and transferred to a conical flask containing 100 ml of potato dextrose broth as the control. Flasks containing 100 mL of potato dextrose broth were enriched with Selenium (1 ppm), Boric Acid (100 ppm), and Copper Sulphate (200 ppm). ten days later of inoculation at 26 ˚C, mycelial mats were filtered through two thicknesses of cheesecloth.

Microscopic Examination:

The mycelial mats were examined for structural deformation in *R. solani* hyphal cells using a light microscope (CHS, Olympus Optical Co. Ltd.) at 40x magnification.

Oxalic Acid Extraction and Analysis:

The liquid media were centrifuged at 2000 rpm for 20 minutes. The supernatant was filtered using 0.45 µm non-sterile 4mm micro filter syringes. The sample preparation technique and chromatographic conditions were improved so that the fermentation process could be monitored. Oxalic acid concentration was determined using HPLC methods of **Van Hees *et al.*, (1999)**. The oxalic acid was determined using high performance liquid chromatography (HPLC), (Agilent 1200). The column temperature of the HPLC (Agilent 1100 DAD) was 30 degrees Celsius. The mobile phase was composed of basic ammonium and methanol in a ratio of 99:1. The pH was adjusted to 2.6 using phosphoric acid at a flow rate of 0.5 ml/min. A UV detector tuned to 214 nm was used for detection. Quantification was achieved by comparing the absorbance in the chromatograms to external standards.

Statistical analysis

Data were statistically evaluated using the Statistic Analysis System package (SAS institute, Cary, NC, USA). Differences between treatments were investigated using Fisher's least significant difference (L.S.D.) test and Duncan's multiple range test (**Duncan, 1965**). All analyses were conducted at the P 0.05% level.

RESULTS AND DISCUSSION

Plant nutrition is an important factor in sustainable agriculture, because it is usually environmentally friendly to control plant diseases without pesticide residues in plants.

1-Evaluation of micronutrients on seedling establishment and managing *Rhizoctonia solani*

Data presented in Fig. (1) clear that Selenium at 1 ppm was the most effective nutrient compared to other micronutrients individually and provided significant protection to cucumber seedlings against Rhizoctonia root rot. Also, 73.4% seedling stand after 12 days was recorded in pots infested with *R. solani*. Boric acid (150 ppm) came in the second rank and reduced the disease incidence and allowed of 71.0% seedling survival (seedling stand) compared to 33.0% in the untreated infested control.

Micronutrients can, also effectively reduce disease levels at a lower cost, as demonstrated by previous studies (**Christos, 2008)**. These nutrients appear to induce a systemic effect, enhancing plant resistance to other diseases. This concept is founded on the belief that certain diseases can be controlled with mineral fertilization regimes. In the majority of cases, the minerals act effectively to reduce the inoculum potential, improving host tolerance, or both (**Broembsen and Deacon 1997**). Boric acid has been proven to decrease the severity of various diseases due to its impact on cell wall structure, plant metabolism, enzyme activity (peroxidase and polyphenoloxidase), and total phenol content. Increases in enzymatic activity and total phenol concentrations may be responsible for the control of certain diseases. (**Tohamey and Ebrahim, , 2015**). **Reuveni *et al.* (1997)** suggested that nutrients such as manganese (Mn), copper (Cu), and boron (B) facilitate the release of calcium cations to be released from cell walls, which react with salicylic acid and activate systemic acquired resistance pathways. Zinc is a cofactor of several enzymes and influences a variety of biological processes, including photosynthetic reactions, nucleic acid metabolism, protein and carbohydrate production (**Marschner, 1996, Wadhwa *et al*., 2014**). Furthermore, selenium (Se) has

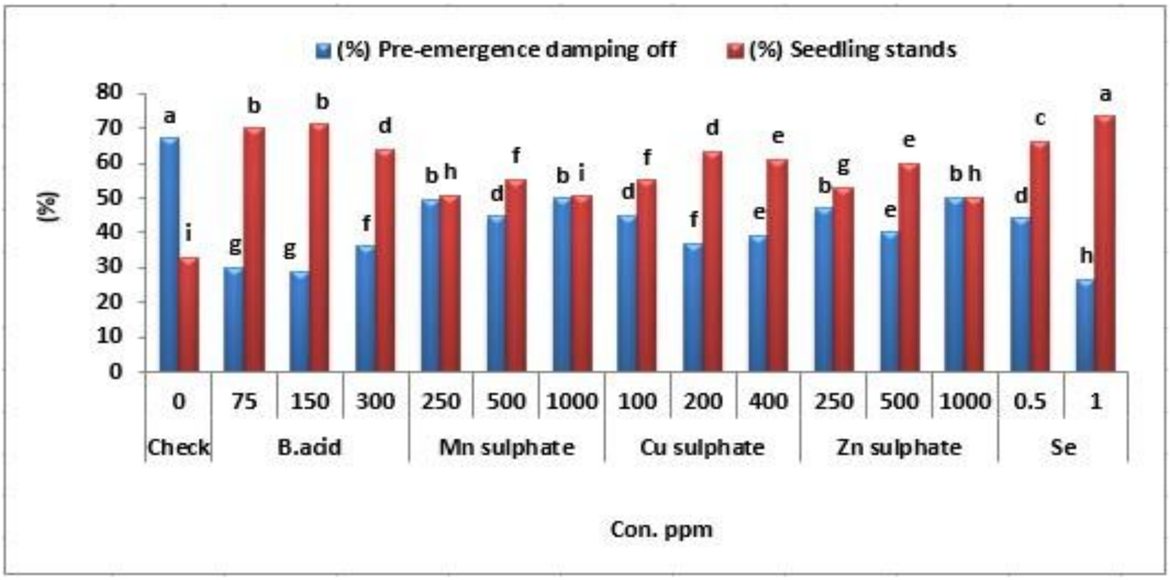

Fig. (1): Effect of seed treatment with different concentrations of micronutrients under greenhouse conditions on pre-emergence damping off and cucumber seedlings stand in soil infested with *Rhizoctonia solani*

been reported to enhance amino acid metabolism in *Sclerotinia sclerotiorum* infected leaves **Jiayang et al. (2020).** These findings suggested that Selenium might serve as an effective eco-fungicide for plant protection.

2-Improving seedling stand and management of Rhizoctonia root rot using Free- radical scavengers

Treatment of cucumber seeds with seven different antioxidant compounds significantly improved seedling protection against *R. solani*. As shown in Fig. (2) benzoic acid at 15 mM and Potassium tartrate at 300 ppm reduced seedling pre-emergence damping off and allowed to maximum seedling stands (79%). Salicylic acid (SA) and ascorbic acid at 5 mM reduced seedling mortality in cucumber and produced seedling stands 75-76% as compared to 33%in the control treatment. The mechanisms by which antioxidants provide protection have been extensively studied in numerous host-pathogen interactions. **Chen *et al*., (1993)** reported that salicylic acid interacts with tobacco and inhibits catalase, leading to increased concentrations of hydrogen peroxide (H_2O_2), which has a direct antimicrobial activity against many pathogens. A further mode of action of antioxidants, can form free radicals through the inhibition of certain enzymes, (**Durner *et al*., 1997**). Potassium (K) salts stimulated the synthesis of jasmonic acid (JA), which improved the plant's resistance to fungal infections. **Davis *et al*., (2018)** found that the transcript levels of JA-responsive genes in K-sufficient plants reflected a natural tip-to-base K concentration gradient within their leaves. The application of inducers under greenhouse conditions significantly decreased disease progression on treated plants relative to untreated ones. Thiamine and zinc sulfate considerably decreased the disease index and enhanced growth measures when compared to the controls **(Kheyri and Taheri, 2021).**

3- Impact of mixing potassium tartrate with various micronutrients on cucumber Rhizoctonia root rot.

The application of resistance inducers and micronutrients has a critical role in reducing disease incidence. In artificially infested pots with *R. solani* **,** seed treated with potassium tartrate and Born had the highest seedling stands, followed by potassium tartrate and selenium or copper sulphate (Fig.3). Boric acid (B) has been previously studied for its effects on bacterial wilt in tomatoes, with findings suggesting that its mechanism involves increasing the rate of hydrogen peroxide (H_2O_2)

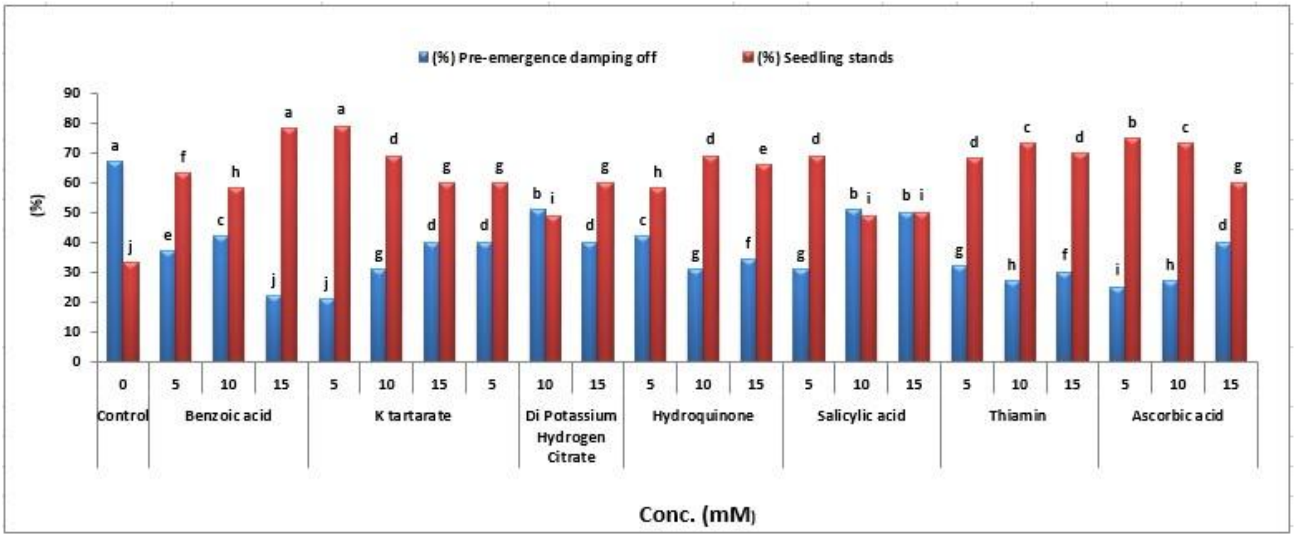

Fig (2): Effect of seed treatment with different concentrations of free- radical scavengers on (%) cucumber pre- emergence damping off and seedling stand in soil infested with *Rhizoctonia solani*.

accumulation and enhancing the activities of peroxidase (POD) and polyphenol oxidase (PPO) (**Jiang *et al.*, 2016).** Selenium (Se) plays an important role in the defense response of many plants toward the pathogen attacks. It has been shown to control postharvest losses in fruits and vegetables by causing significant damage to the conidia plasma membrane and leading to the loss of cytoplasmic components from hyphae. Applying Se (0.5 mg/kg) to soil reduced *S. sclerotiorum* lesion dimension in rape leaves (**Borsani *et al*., 2001, Wu *et al*., 2015 and Jiayang *et al*., 2020)**. Elicitor-induced resistance is thought to be an environmentally beneficial way to enhance plant defense against pathogen attacks. In this study, we investigated the effect of potassium tartrate on induced resistance in cucumber plants to *R. solani* and assessed the potential defensive mechanisms involved. Data in Fig. (**3**) show that total phenol contents in fresh roots of cucumber was higher in seeds treated with the mixture of micronutrient's and potassium tartrate. On the other hand, potassium tartrate combined with copper sulphate recorded the lowest level in total phenols content compared with other treatments. The treatments with the highest phenol levels included the potassium tartrate-boric acid combination and potassium tartrate-selenium combination, with boric acid showing the highest total phenol content.

Previous research supports the role of total phenols in plant defence. For instance, selenium and zinc sulphate, both individually and in combination, have been shown to induce resistance against *R. solani*, associated with increased antioxidant activity and higher levels of lignin accumulation and total phenolic content **(Yousef *et al*.,2016)**. **Kheyri and Taheri (2021)** also demonstrated the efficacy of chemical resistance inducers in protecting bean plants from Rhizoctonia root rot. Although selenium is not essential for higher plants. **Saleh (2013)** found that selenium plays an important role in many plants' defense mechanisms against infection by pathogens. According to **Hansen *et al*. (2003),** selenium protects plants from fungal diseases. Additionally, **Bhalerao *et al.* (2019)** found that boric acid, particularly at concentrations of 1% and 2%, was highly effective in reducing the mycelial growth of *Alternaria solani*.

Lab experiments:

4- Effect of using various five micronutrients and potassium tartrate on the radial growth of *Rhizoctonia solani*

Data presented in Fig (4) demonstrate that *R. solani* mycelial growth was completely inhibited by 1000 ppm of zinc sulfate (Zn) and 300 ppm of boric acid (B). Additionally, 75 ppm and 150 ppm of boric acid achieved an 88.8% inhibition of mycelial growth. In contrast, potassium tartrate, selenium at all tested concentrations, and manganese sulfate (Mn) at 250 ppm did not exhibit significant effects on fungal growth inhibition. Zn appears to be responsible for controlling the growth of the invading fungus by hiding the active groups that are required for interaction with the fungus' enzymes (**Kalim, 2003).** Antioxidative enzymes, such as polyphenol oxidase, peroxidase, and tyrosine ammonialyase, are involved in phenylpropanoid metabolism and can contribute to plant defence mechanisms. Zn acts as a coenzyme for these enzymes, hence it can be concluded that Zn may be utilized as a soil nutritive agent for improving resistance in plants against fungal infections. **(Wadhwa *et al*., 2014). Kheyri and Taheri (2021),** stated that applying boric 1000 and 2000 mg L^{-1} concentrations completely inhibited the growth of *R. solani*.

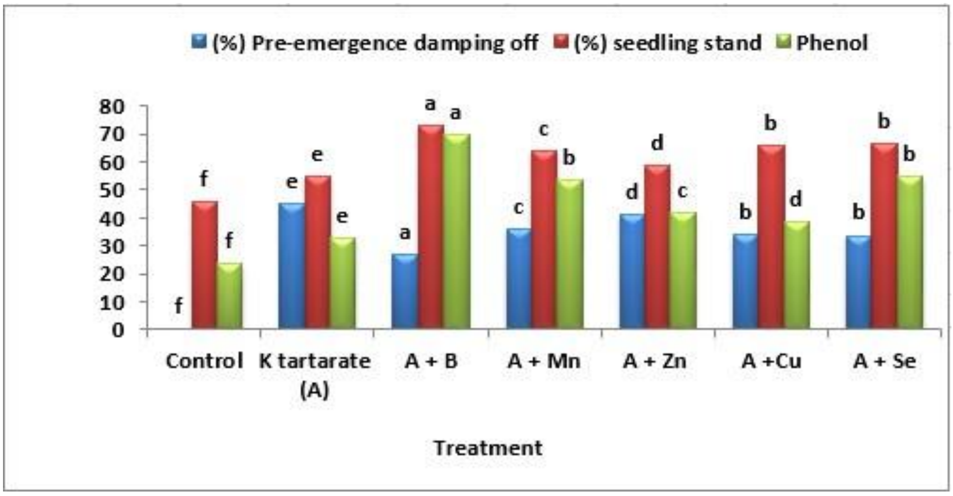

Fig. (3): Effect of seed treatment with Potassium tartrate alone or mixed with microelements on (%) cucumber pre- emergence damping off and seedling stand in soil infested with *Rhizoctonia solani*

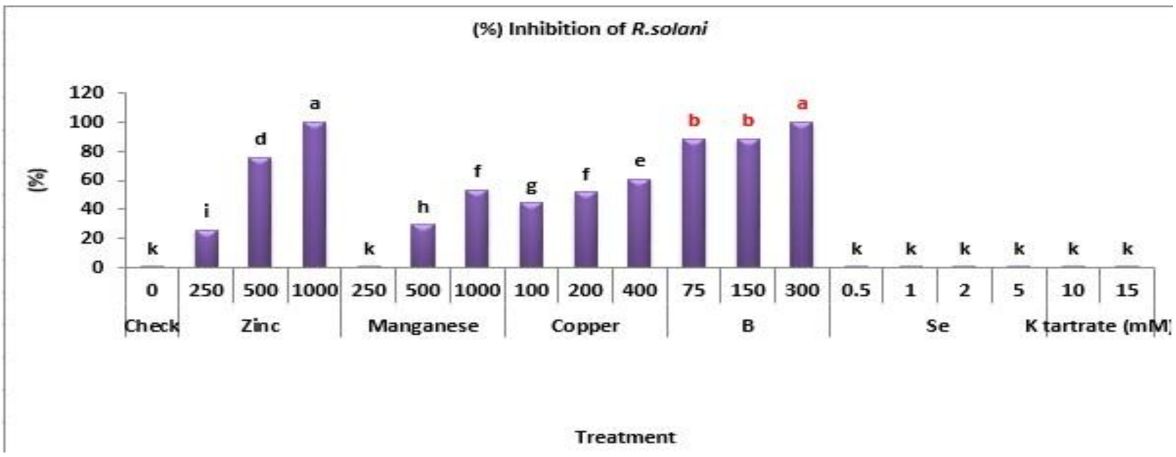

Fig. (4): Effect of using micronutrients and potassium tartrate at different concentrations on the linear growth of *Rhizoctonia solani*

5- Effect of using micronutrients mixed with potassium tartrate on the reduction of *Rhizoctonia solani* mycelial growth

Data presented in Fig. (5) indicate that the combination of potassium tartrate and boric acid achieved the highest inhibition of *R. solani* mycelial growth, with an 88.9% reduction compared to the control. In contrast, potassium tartrate alone or in combination with selenium did not significantly impact fungal growth. Potassium tartrate combined with manganese or zinc resulted in limited inhibition of fungal growth, showing reductions of 28.9% and 22.3%, respectively. Interaction between plants and pathogens results in the production of numerous chemical compounds that are necessary for activating the plant's defense mechanisms. One of the compounds, boric acid, induces systemic acquired resistance (SAR) in plants. The activation of SAR gives a broad-spectrum resistance against a variety of diseases **(Bhalerao *et al.*, 2019)**. Treating *R. solani* with potassium tartrate as antioxidant and a mixture of micronutrients for 48 h showed a great reduction in colony diameter reached to 72% (**Yousef *et al.*, 2013**). **Martinko *et al.*, (2022),** observed that *Alternaria alternata* mycelium cultivated on media supplemented with boric acid exhibited less pigmentation and branching compared to the control, suggesting that boric acid may inhibit sporulation without affecting mycelial development.

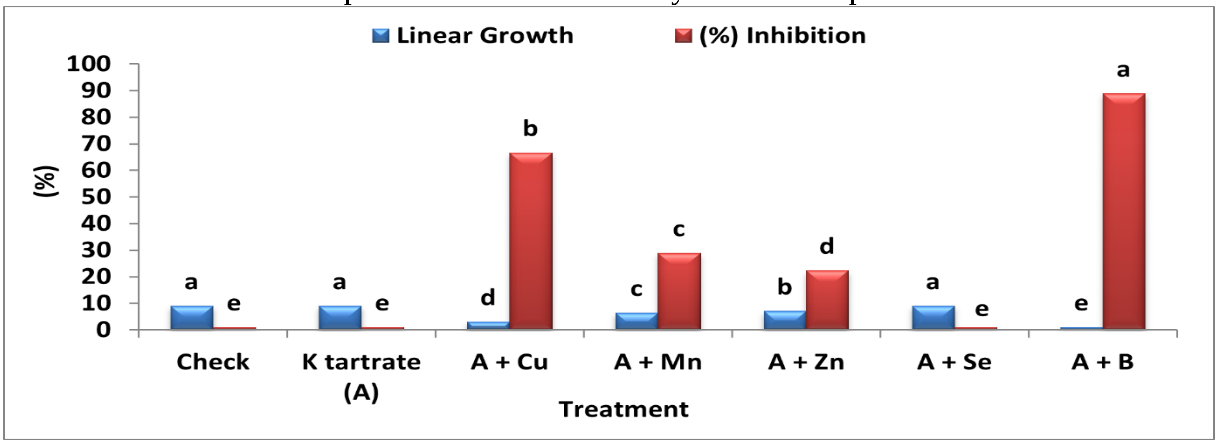

Fig. (5): Effect of using micronutrients with potassium tartrate on the linear growth of *Rhizoctonia solani*

The virulence of *Rhizoctonia solani* isolates varies based on their ability to produce oxalic acid (**Nagarajkumara *et al.*, 2005**). *In vitro* trial effect of growing *R. solani* on PDA medium supplemented with selenium (Se), boric acid (B), and copper sulfate (Cu) on oxalic acid production. Chromatographic profile of oxalic acid production by *R. solani* and mycelium malformation according to growing *R. solani* on PDA medium supplemented with Se, B and Cu appear in Figs. (6 and 7). Data presented in Table (1) show that boric acid supplementation resulted in a 67.2% reduction in oxalic acid production by *R. solani*. Copper sulfate supplementation achieved a 48.8% reduction, while selenium supplementation resulted in a 26.6% reduction. According to **Dutton and Evans (1996),** fungi secrete oxalate to aid in their growth and substrate colonization. Oxalic acid plays a role in pathogenesis by acidifying host tissues and sequestering calcium from host cell walls, which contributes to disease progression.

Table (1): Effect of Selenium, Boric acid and Copper sulfate on oxalic acid production by *Rhizoctonia solani*

No.	Treatment	Oxalic acid (mg/l)	% Reduction
1	Control (PDA medium)	1857.5	00.0
2	PDA medium + Se 1 ppm	1363.7	26.6
3	PDA medium + B 100 ppm	608.7	67.2
4	PDA medium + Cu 200 ppm	951.3	48.8

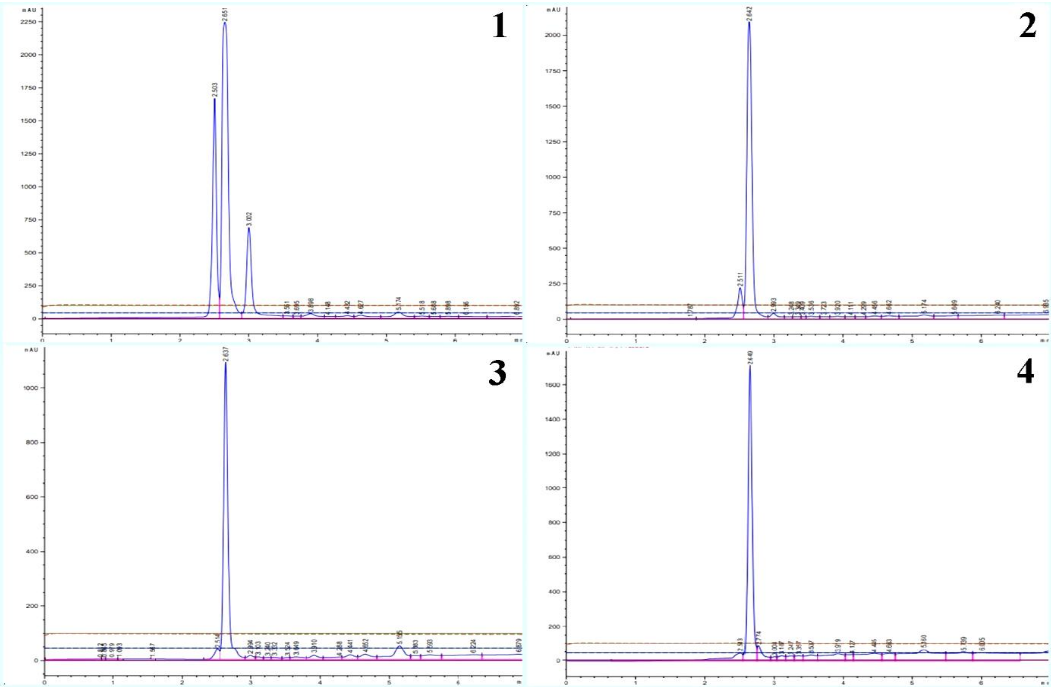

Fig. (6): UV-Vis HPLC chromatographic profile of oxalic acid production by *Rhizoctonia solani* grown on PDA media (Control: 1) supplemented with Selenium (2), Boric acid (3) and Copper sulfate (4).

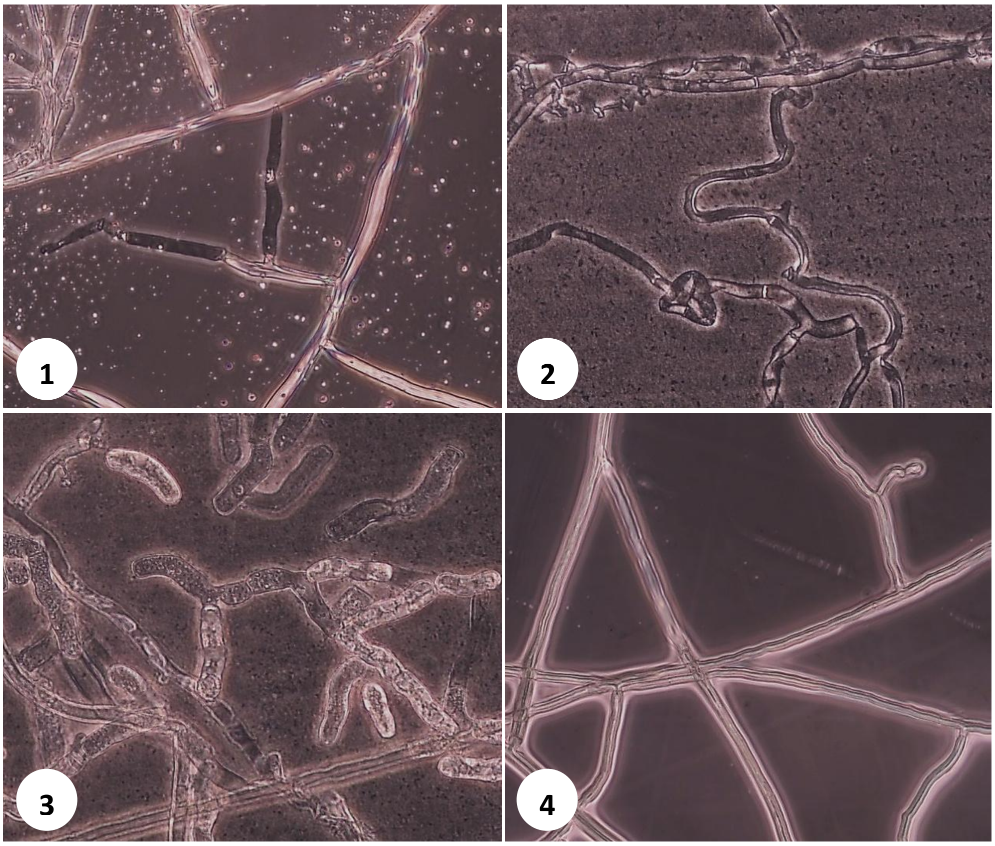

Fig.(7): Microscopical photographs(40X) of malformed *R. solani* mycelium grown on PDA medium supplemented with Se (1), B (2) , Cu (3) and control treatment, without any additives(4).

CONCLUSIONS

This study underscores the dual benefits of utilizing antioxidants and micronutrients in seed treatment not only as a cost-effective approach but also as a crucial factor in reducing disease incidence. The findings highlight the potential of integrating plant nutrition with disease management strategies, revealing new insights into how environmental parameters affect plant-pathogen interactions. By linking plant nutrients, we can develop more sustainable and effective management strategies for controlling *Rhizoctonia solani*. These results suggest that incorporating plant nutrients into an integrated disease management plan could provide a robust approach for achieving optimal disease control and enhancing plant health.

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