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RESEARCH ARTICLE

IN VITRO EFFECT OF COPPER OXYCHLORIDE NANOPARTICLES ON FUSARIUM WILT DISEASE RESISTANCE IN SOLANUM LYCOPERSICUM THROUGH SEEDLING ROOT TREATMENT

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Abstract

Tomatoes *(Solanum lycopersicum)* are one of the most extensively produced vegetables globally. Fusarium wilt is caused by the fungus *Fusarium oxysporum*, which is a major pathogen of tomato vascular wilt and a soil-borne pathogen that causes yield losses. In earlier studies, copper (nanoparticles) NPs were reported by many researchers for the management of diseases in crops. To overcome this wilt problem in tomatoes, attempts were made to identify the significant use of copper oxychloride (COC). Here, in the present report, the further enhancement of nanofungicide is reported using copper oxychloride NPs and silver (Ag)-doped copper oxychloride NPs, which were studied against the wilt pathogen. The antifungal activity and the minimum inhibitory concentration of COC and Ag-doped COC was discussed. The root dip method demonstrates that COC NPs and Ag-doped COC NPs were used to treat tomato seeds. The seedlings coated with 8 mg of COC NPs and Ag-doped COC NPs in the presence of ***Fusarium oxysporum*** showed excellent growth in both root and shoot length, with only a very small amount of wilting observed at this concentration after 2 weeks. Chlorophyll and carotenoid estimation were done to compare the differences between the COC-coated and *Fusarium oxysporum*-infected seedlings. In contrast, the control seedlings without any treatment showed wilting within one week. The result revealed that the NPs at an 8 mg concentration combined with *Fusarium oxysporum* showed a synergistic effect in inducing disease resistance in tomato seedlings at the early stage of wilt resistance. As a result, it is a simple and rapid method for screening induced resistance at an early stage, which will help evaluate bioagents for their effectiveness.

Introduction:-

Tomato (*Solanum lycopersicum*) is considered the most significant crop for vegetables that are widely farmed and grown in both temperate and tropical regions due to its wide adaptability and nutritional value. Tomatoes are the second most widely marketed product after potatoes and one of the most consumed vegetables globally. It is one of

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the most significant cash crops cultivated in India due to its high consumption and production (Subba et al., 2024). However, numerous fungal infections can infect tomato plants from the soil, especially through seeds. These diseases substantially damage crop productivity, resulting in significant economic loss.

Fusarium is a significant fungal genus found in farmland soil, including several phytopathogenic species. It is a common soil-borne disease that infects a variety of foods, including tomatoes, potatoes, peppers, and eggplants (El-Abeid et al., 2024). *Fusarium oxysporum*can persist in the soil for up to two decades and is one of the most devastating soil-borne fungi that affects the majority of crops. The disease *Fusarium oxysporum*affects plants through their roots and travels to the stems and leaves, limiting water supply and causing the leaves to wilt and turn yellow. Fusarium wilt causes modest vein clearing on the outer portion of young leaves, usually on one side of the plant or shoot. Frequently before the plant reaches maturity, successive leaves begin to shrink, turn yellow, and eventually die. Plants become stunted and produce little to no fruit as the disease spreads. The diagnosis of Fusarium wilt is characterized by the browning of the vascular system.

By lowering chemical inputs, encouraging plant development, and enhancing biomass production to help meet global demands, nanotechnology has recently helped to mitigate issues in plant disease management (Elmer and White 2018; Eid et al. 2021). Copper is a vital micronutrient for plants and has a role in photosynthesis, respiration, carbon and nitrogen metabolism, and oxidative stress resistance. Copper NPs can act as both antifungal agents and plant growth enhancers. Copper-based NPs have the potential to improve crop nutrition and disease management. They have been used as antimicrobials since 2000 B.C. and continue to be used today. Copper is a crucial component in several inorganic fungicides used in agriculture (Lopez-Lima et al., 2021). Cu-based fungicides, particularly Bordeaux mixture ($CuSO_4$ + CaO), and other Cu-based salts have accumulated in soils due to the long-term use of Cu treatments for agricultural disease management since 1850 (Poggere et al., 2023).

In the current work, copper oxychloride NPs and Ag-doped copper oxychloride were used to enhance antimicrobial resistance against *Fusarium oxysporum*in tomato seedlings through the root dip method, which has been adopted for screening disease resistance.

Materials and Methods:-

Seeds of the tomato variety S-22 were obtained from the local market of Tumakuru, Karnataka, India, and were used in this experiment to determine the efficiency of COC and Ag-doped COC NPs, and *Fusarium oxysporum*effects. Tomato seeds were surface sterilized for 10 minutes with a 1% sodium hypochlorite solution, then gently washed three times with sterile distilled water. (SamreenNaz GS et al.,2024).

For the MIC assay, *Fusarium oxysporum*was procured from the Microbial Type Culture Collection and Gene Bank (MTCC), CSIR-Institute of Microbial Technology, Sector 39-A, Chandigarh-160036, India, and was utilized in this study. Sabouraud's dextrose agar powder from HiMedia (M286), Sabouraud's dextrose broth from HiMedia (MH033), and *Fusarium oxysporum*(strain ATCC 62506) were used.

For the Zone of inhibition assay, *Fusarium oxysporum*was used. The fungal strain (ATCC 62506) was cultured on Potato dextrose agar (HiMedia, M286) and incubated at 25°C for 5–7 days to ensure active growth. To prepare the fungal inoculum, a loopful of actively growing *Fusarium oxysporum*culture was transferred into potato dextrose broth (HiMedia, MH033) and incubated under shaking conditions at 120 rpm for 48 hours at 25°C, as shown in Fig. 1. The fungal suspension was then adjusted to the required concentration for further experimental analysis.

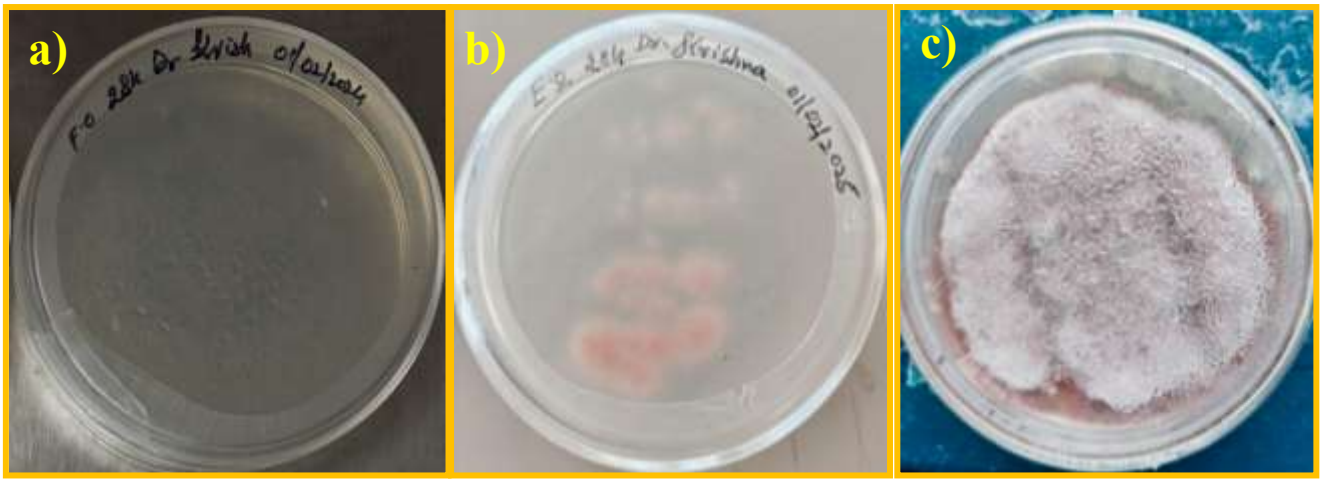

Fig. 1:- Preparation of the pathogen fusarium oxysporum.

MIC of COCNPs and Ag-doped COCNPs

Inoculum preparation:

Before the initiation of the experiment, an aliquot of glycerol stockof *Fusarium oxysporum*(ATCC 62506 strain) was thawed and inoculated into sterile Sabouraud's dextrose agar (SDA) plates, then incubated for 8 days at 25ºC to reach the exponential phase. Post-incubation, the culture was identified, and the optical density was adjusted to approximately 1.0×10^8 spores/mL. The suspension was then diluted to ~5×10^5 spores/mL and used for the assay.

Preparation of test substance and Ketoconazole

A 12,800 µg/mL stock solution of the test compounds (COC NPs and Ag-doped COC NPs) was prepared by adding 12.8 mg of the test compounds to 1 mL of DMSO. After adding DMSO, the compounds were sonicated for 10 minutes to ensure complete solubility, obtaining the Master Stock (MS) solution. This MS (12.8 mg/mL) was then serially diluted two-fold, as shown in Table 1, to obtain a series of working stock solutions. The final concentration of the solvent in the assay was 5%, and the assay volume was 200 µL (Gaber et al., 2020).

Positive control

A 3,200 µg/mL solution of ketoconazole was prepared by adding 3.2 mg of the test compound to 1 mL of DMSO, obtaining the Master Stock (MS) solution. These stock solutions were serially diluted two-fold, as shown in Table 2, to obtain a series of Working Stock (WS) solutions. The final concentration of the solvent in the assay was 2%, and the assay volume was 200 µL. The details of the final concentrations assayed are shown in Tables 1 and 2

Antifungal activity

An 8 mg/mL solution of COC NPs and Ag-doped COC NPs was prepared by adding 8 mg of the test compounds to 1 mL of dimethyl sulfoxide (DMSO) and sonicated for 30 minutes to ensure complete solubility, obtaining the master stock (MS) solution. This solution was then added to a Petri plate containing *Fusarium oxysporum*, and the results were recorded (AlHarethi et al., 2024).

Table1:-Preparation of working standard dilutions of test compounds.

Dilution	Conc. (µg/mL)	Vol.(ml)	Diluent (mL)	WS Conc. (µg/mL)
1	Master Stock solution	-	-	12800
2	Dilution 1 (12800)	0.05	0.05	6400
3	Dilution 2 (6400)	0.05	0.05	3200
4	Dilution 3 (3200)	0.05	0.05	1600
5	Dilution 4 (1600)	0.05	0.05	800
6	Dilution 5 (800)	0.05	0.05	400
7	Dilution 6 (400)	0.05	0.05	200

8	Dilution 7 (200)	0.05	0.05	100
9	Dilution 8 (100)	0.05	0.05	50
10	Dilution 9 (50)	0.05	0.05	25
11	Dilution 10 (25)	0.05	0.05	12.5
12	Dilution 11 (12.5)	0.05	0.05	6.25
13	Dilution 12 (6.25)	0.05	0.05	3.125

Table 2:-Preparation of working standard dilutions of Ketoconazole.

Dilution	Conc. (µg/mL)	Vol.(mL)	Diluent (mL)	WS Conc. (µg/mL)
1	MasterStock solution	-	-	3200
2	Dilution 1 (3200)	0.05	0.05	1600
3	Dilution 2 (1600)	0.05	0.05	800
4	Dilution 3 (800)	0.05	0.05	400
5	Dilution 4 (400)	0.05	0.05	200
6	Dilution 5 (200)	0.05	0.05	100
7	Dilution 6 (100)	0.05	0.05	50
8	Dilution 7 (50)	0.05	0.05	25
9	Dilution 8 (25)	0.05	0.05	12.5
10	Dilution 9 (12.5)	0.05	0.05	6.25
11	Dilution 10 (6.25)	0.05	0.05	3.125
12	Dilution 11 (3.125)	0.05	0.05	1.5625
13	Dilution 12 (1.5625)	0.05	0.05	0.78125

MIC assay

The MIC assay was performed in a 96-well microtiter plate with a total assay volume of 200 µL(Table 3 and Table 4). Each well containing different concentrations of test compound and inoculated with 50 µL of fungal culture (10^5 spores/mL) along with culture control (CC, culture in broth), broth control (BC, broth only), and vehicle control (VC, solvent in broth plus culture). The plate was incubated at 26±1ºC for 9 days. Post incubation, the plate was visually examined for turbidity, and the optical densities (OD) at 520 nm were measured. The experiment was done in replica. (Abdelaziz et al.,2022)

Table 3:-Preparation of MIC plates and final assay concentrations of test compounds.

Sl. No	WS Conc. (µg/mL)	Volume WS in assay well (µL)	Vol of RPMI (µL)	Vol of Culture (µL)	Final Assay Vol. (µL)	Final conc. (µg/mL)
1	12800	4	146	50	200	256
2	6400	4	146	50	200	128
3	3200	4	146	50	200	64
4	1600	4	146	50	200	32
5	800	4	146	50	200	16
6	400	4	146	50	200	8
7	200	4	146	50	200	4
8	100	4	146	50	200	2
9	50	4	146	50	200	1
10	25	4	146	50	200	0.5
11	12.5	4	146	50	200	0.25

Table 4:-Preparation of MIC plates and final assay concentrations of Ketoconazole.

Sl. No	WS Conc. (µg/mL)	Volume WS in assay well (µL)	Vol of RPMI (µL)	Vol of Culture (µL)	Final Assay Vol. (µL)	Final conc. (µg/mL)
1	3200	4	146	50	200	64

2	1600	4	146	50	200	32
3	800	4	146	50	200	16
4	400	4	146	50	200	8
5	200	4	146	50	200	4
6	100	4	146	50	200	2
7	50	4	146	50	200	1
8	25	4	146	50	200	0.5
9	12.5	4	146	50	200	0.25
10	6.25	4	146	50	200	0.125
11	3.125	4	146	50	200	0.0625

Data Analysis

The MIC was defined as the lowest concentration of test compound that prevented fungal growth (lack of turbidity by OD at 520 nm and visual inspection relative to no growth control).

The MFC (minimum fungicidal concentration) is the lowest concentration of the test compound resulting in a greater than 90% reduction in the number of viable fungi compared to the initial inoculum, as shown in Figs 2 and 3.

Table 5 shows the MIC of Test compounds against *Fusarium oxysporum*(ATCC 62506)

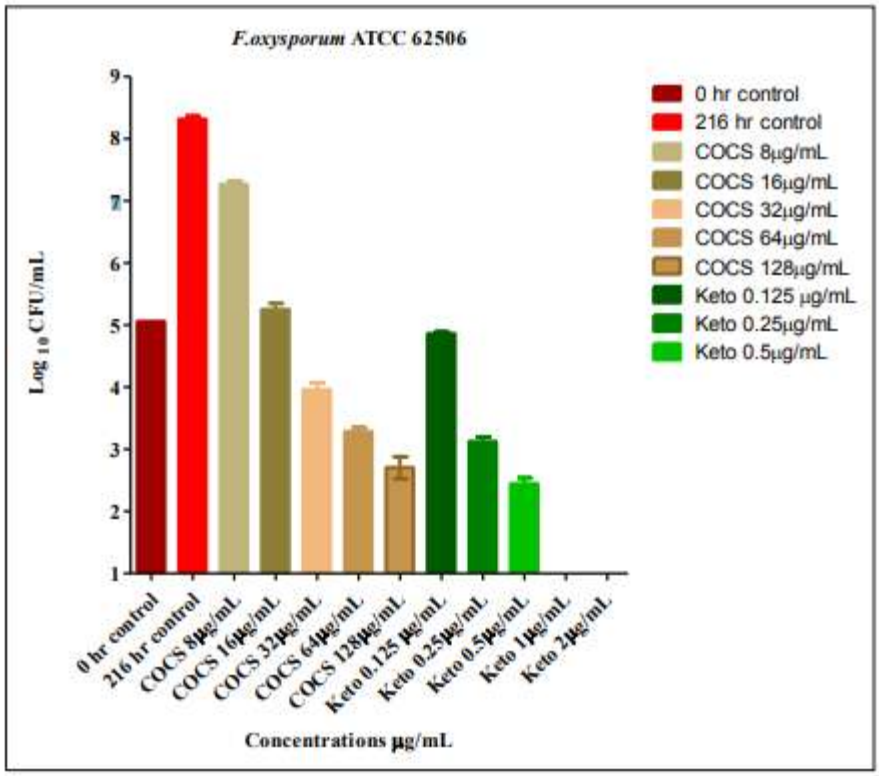

Fig. 2:-MIC activity of COC NPs.

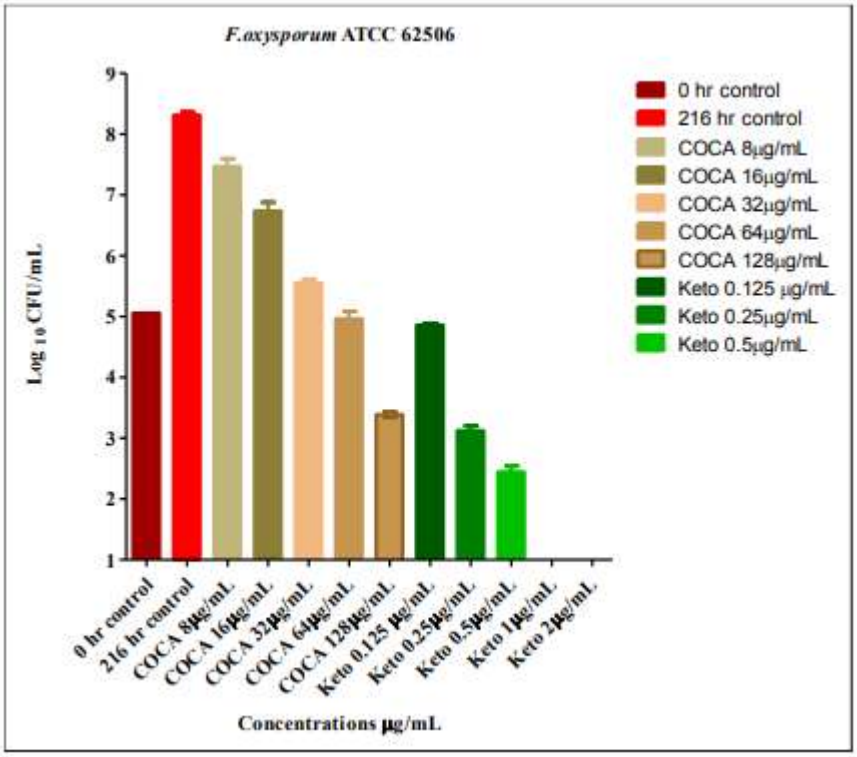

Fig. 3:-MIC activity of Ag-doped COC NPs.

Table 5:-MIC of Test compounds against *Fusarium oxysporum*(ATCC 62506).

Compound name	Organism name	MIC (µg/mL)
Ag COC NPs	**Fusarium oxysporum(ATCC 62506)**	64
COC NPS		32
Ketoconazole		0.25

Effects of COC and Ag-doped COC NPs on the growth of Fusarium oxysporum (a fungal strain)
COC and Ag-doped COC NPs were applied to S22 tomato seeds for 24 hours, while an untreated set of seeds served as a control. After being placed in pot trays with sterilized coco peat, the NP-coated seeds were let to grow for a week. The seedlings in pot trays were carefully uprooted after a week, given a gentle wash with distilled water, and then placed in 2 mL vials with a fungal culture suspension for a full day. After being carefully cleaned with sterile distilled water, tomato seedlings at the two-leaf stage were placed in vials with varying concentrations of COC and Ag-doped COC NPs.

In the current study, the following in vitro treatments were performed.

T1 - Fusarium oxysporum	P1 – Fusarium oxysporum
T2 -2mg COCNPs	P2 -2mg Ag doped COCNPs
T3 - 4mg COC NPs	P3-4mg Ag doped COC NPs
T4 - 8mg COCNPs	P4 -8mg Ag doped COCNPs
T5 - 16mg COC NPs	P5 -16mg Ag doped COC NPs
T6 -32mg COC NPs	P6 -32mg Ag doped COC NPs

Photosynthetic pigments
Chlorophyll and carotenoid content were assessed by homogenizing 0.3 g of different NP-coated fresh leaf samples with 80% acetone. After that, the mixture was centrifuged for 15 minutes at 40 °C at 5000 rpm. The supernatant was collected and analyzed using a UV-visible spectrophotometer, with measurements of absorbance taken at 645 nm,

663 nm, and 470 nm. The amount of chlorophyll and carotenoid was calculated using the formula. (Raliyaet al.,.2017,Shankramma et al., 2016 and Pavithra et al.,2020)

$$\text{Chl a (mg/g of fresh weight)} = \frac{[19.3 \text{ X } A663 - 0.86 \text{ X } A645] \text{ X } V}{1000 \text{ X } W}$$

$$\text{Chl b (mg/g of fresh weight)} = \frac{[19.3 \text{ X } A645 - 3.6 \text{ X } A663] \text{ X } V}{1000 \text{ X } W}$$

$$\text{Total Chlorophyll content (mg/g of fresh weight)} = \text{Chl a} + \text{Chl b}$$

$$\text{Total carotenoids} = \frac{1000 \text{ X } A470 - 22.7(\text{Chl a}) - 81.4 (\text{Chl b})}{227}$$

Where Chl a = Chlorophyll a, Chl b = Chlorophyll b, V= Volume of extract in mL, W= fresh weight of leaves in gm A663 = solution absorbance at 663 nm A645 = solution absorbance at 645 nm.

Results and Discussion:-

In vitro assay of the antifungal activity of NPs

The plates were incubated at 25 °C for 12 days. Post-incubation plates were examined forInhibition zones and diameter were measured. The zone of inhibitions of COC NPs and Ag-doped COC NPs is shown in Fig. 4. The test compound COC NPs and Ag-doped COC NPs showed concentration-dependent mean ZOI against *Fusarium oxysporum*(ATCC62506), the mean ZOI of COC and Ag-doped COC NPs is shown in Table6.

Table 6:-ZOI of the NPs

Compound Name	Fusarium oxysporum (ATCC 62506) ZOI (cm) 8mg/mL
COC NPs	5.2 cm
Ag doped COC NPs	4.8cm

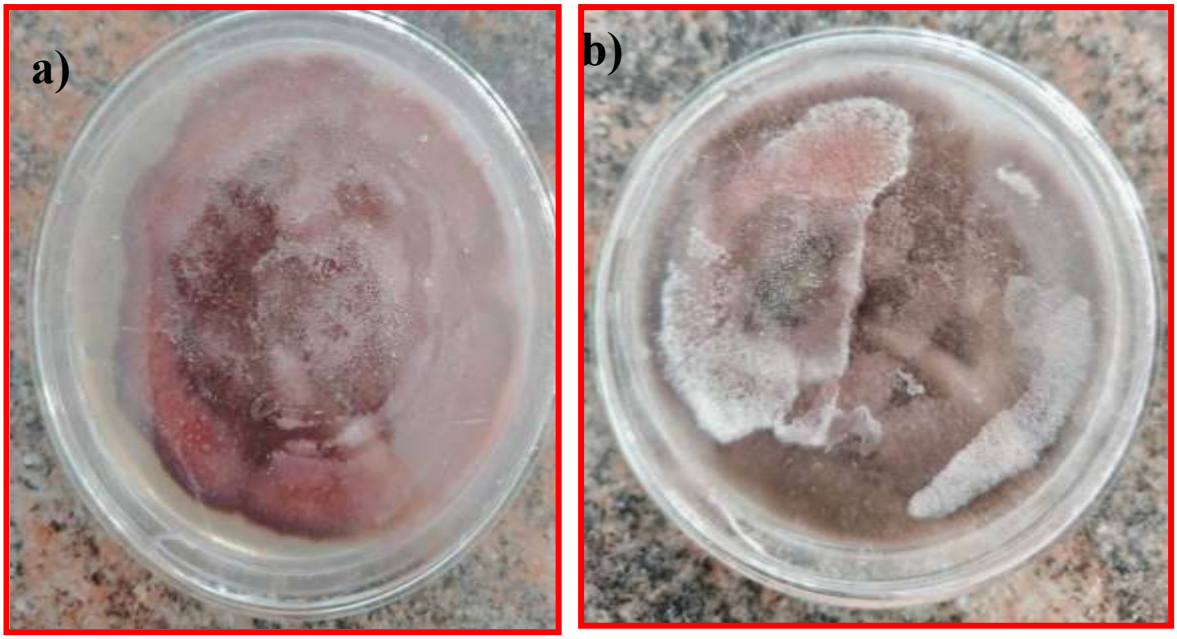

Fig. 4:-Zone of Inhibition of a) COC NPs and b) Ag-doped COC NPs.

Synergistic Effects of Nanoparticle Coating on Seedling Growth and Disease Tolerance (Fusarium Resistance)

This experiment demonstrated that non-treated plants, but root-inoculated with Fusarium spore suspension, showed poor development in both root and shoot compared to the control, COC, and Ag-doped COC NPs. *Fusarium oxysporum*is prominent in displaying necrosis of leaves and roots and the death of seedlings within 15 days. The

images of the wilted seedlings at control and the lower concentration of COC and Ag-doped COC NPs is shown in Fig.5

In the treatment of Ag-doped COC NPs, wilting, and necrosis occurred earlier compared to the COC NPs treatment, whereas wilting appeared after 14 days of sowing in COC NPs compared to Ag-doped NPs at higher concentrations. On the other hand, treatments with COC at an 8 mg/mL concentration survived for 21 days without displaying any symptoms of wilting. Therefore, COC and Ag-doped COC NPs showed good results for wilt disease resistance.

Fig. 5:-Wilted seedlings at control and the lower concentration of COC and Ag-doped COC NPs.

Observations of COC NPs treated Tomato seedlings and Ag-doped COC NPs treated tomato seedlings at the two-leaf stage after 2 weeksare shown in Fig. 6, and Fig.7, respectively. No growth and wilting are observed in Control and excellent growth in both root and shoot length with no wilting is observed in 8 mg of COC NPs and Ag-doped COC NPs treated seedlings

Fig. 6:-Observation of COC NPs treated Tomato seedlings at the two-leaf stage after 2 weeks: No growth and wilting is observed in Control and excellent growth in both root and shoot length with no wilting is observed in 8 mg of COC NPs treated seedlings.

Fig. 7:-Observation of Ag doped COC NPs treated Tomato seedlings at the two-leaf stage after 2 weeks: very small growth and wilting is observed in Control and excellent growth in both root and shoot length with no wilting is observed in 8 mg of Ag doped COC NPs treated seedlings.

Chlorophyll and Carotenoid content

The chlorophyll content of tomato leaves changed significantly with the application of treatments. The content of Chl a, Chl b, and total chlorophyll increased in the 8 mg of COC NPs treatment. COC NPs + Fusarium (F)-treated seedlings showed slight decreases in chlorophyll content, and Ag-doped COC NPs also exhibited an increase in chlorophyll content compared to Ag-doped COC + Fusarium. The results indicate that COC NPs-treated leaf samples have increased photosynthetic efficiency and potential for better plant growth and health. The analysis of carotenoid content in cultivars treated with COC NPs, when compared with COC + F, Ag-doped COC NPs, and Ag-doped COC + F, showed the highest carotenoid content compared to other concentrations of NPs. In contrast, COC NPs + F and Ag-doped COC + F slightly reduced carotenoid content compared to pure NPs, as shown in Fig. 8 and Fig. 9.

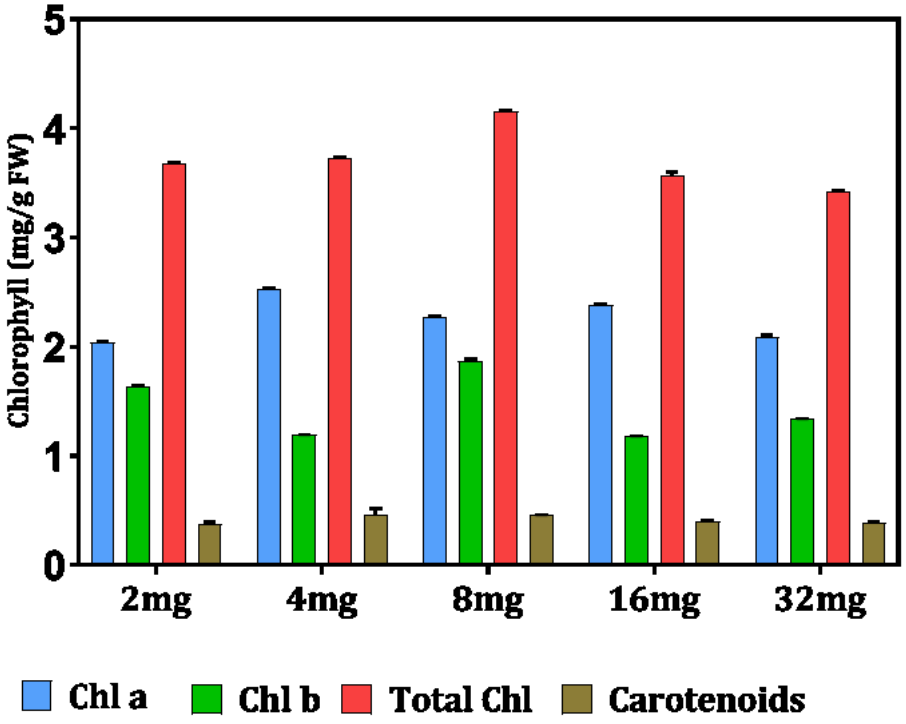

Fig. 8:-Chl a, Chl b, and total chlorophyll contents in the green leaves treated with COC NPs.

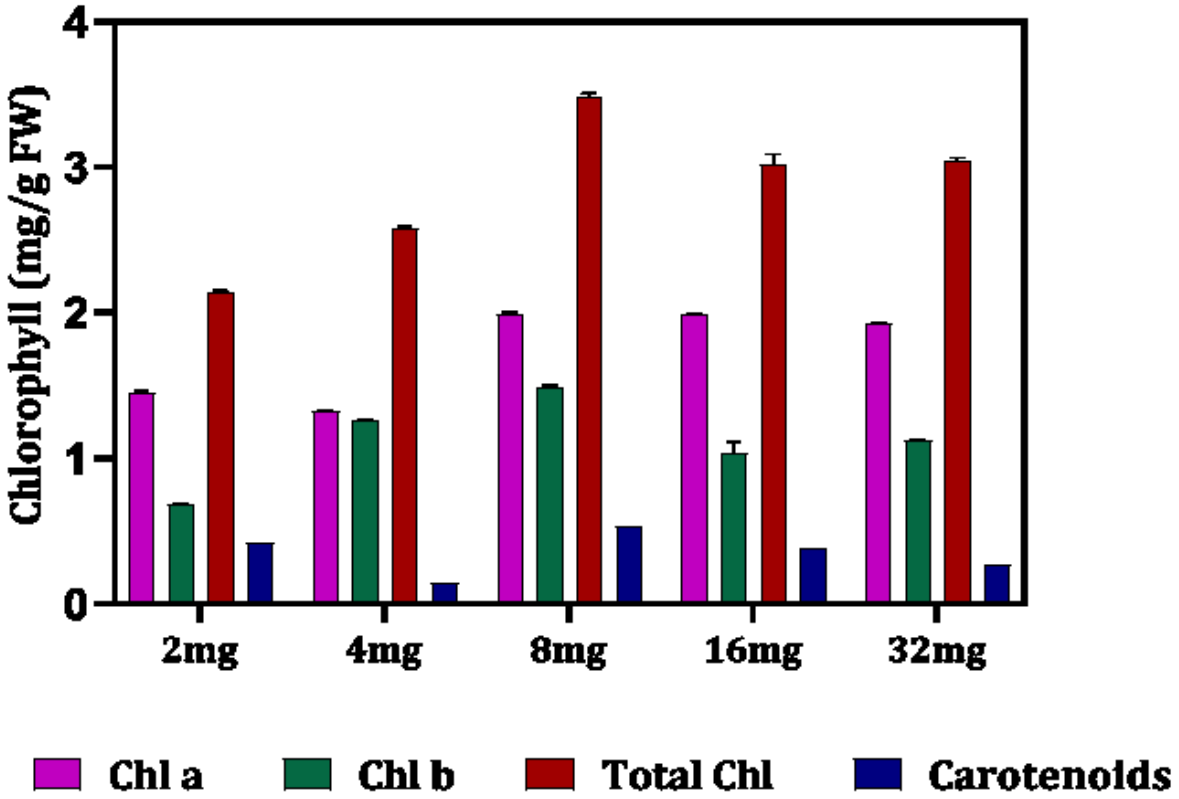

Fig. 9:-Chl a, Chl b, and total chlorophyll contents in the green leaves treated with Ag-doped COC NPs.

Conclusion:-

This study explores the potential of copper oxychloride (COC) and Ag-doped COC NPs in managing the disease. The antifungal activity and minimum inhibitory concentration of these NPs were evaluated, demonstrating their effectiveness in enhancing tomato seedling resistance.COC NPs against *Fusarium oxysporum*showed a minimum inhibitory concentration at 32 µg/mL, while Ag-doped COC NPs showed microbial resistance at 64 µg/mL. The plating was done from the lowest concentration to the highest concentration (from 1 µg/mL to 128 µg/mL) to confirm the minimum fungicidal activity in the test compounds. A simple method was used to evaluate the efficacy of COC and Ag-doped COC NPs against *Fusarium oxysporum*. This method depends on the response of NP-coated plants to *Fusarium oxysporum*at various concentrations. As a result, the seedlings without treatment were more sensitive, while the NP-treated seedlings showed resistance even at low concentrations to pathogen spore inoculation. In addition, COC-treated NPs enhanced the shoot growth of the treated seedlings. These results showed the potential of NP-treated seedlings for inducing disease resistance against *Fusarium oxysporum*.

CRediT authorship contribution statement

SamreenNazG. S: Data curation, Writing – original draft, Writing – review & editing, Visualization, Software, Validation, Methodology, and Formal analysis. **Soundarya T. L.**: conceptualization, Writing – review &editing, Methodology. **Dr Krishna**: Supervision, Validation, Methodology and conceptualization.

Declaration of competing interest

The authors declare that they have no identified competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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