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Synthesis, characterization and efficacy of green synthesized copper nanoparticles from eucalyptus, against *Fusarium* wilt of green gram

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Abstract
Green gram suffers heavy losses in yield due to many diseases especially wilt caused by *Fusarium* sp. Thus, the present study was undertaken with emphasis on development of simple and cost-effective methods for synthesis of nanomaterials and their application in the management of the pathogen. Green synthesis methods, employing plant extracts, have emerged as an eco-friendly and clean alternative to physical and chemical methods. The eucalyptus plant extract mediated copper nanoparticles were synthesized using 0.01M of $CuSO_4$ solutions and characterized by using UV-Visible spectrophotometry, Zeta size analysis, Zeta potential, and Nano Tracking Analysis**.** The synthesized nanoparticle (129.23 nm) were applied at various concentrations to determine their antifungal activities. The result showed the highest inhibition percentage of mycelial growth (80.59%) was recorded on CuNP-E @ 1000 ppm.

Keywords: Wilt, green synthesis, nanoparticles, *Fusarium*, etc.

Introduction
In recent years, Synthesis of metal Nanoparticles using plant leaf extract has attracted attention of many researchers because of easy availability of materials, inexpensive procedure to carry out in any laboratory and use of non-toxic reagent (Kulkarni *et al*. 2015) [4]. Nanomaterial has completely new or improved properties based on specific characteristics such as size distribution and morphology. The two approaches were used for the synthesis of nanoparticles i.e., 'top down" approach and "bottom up" approach (Gaikwad *et al.* 2016) [2]. The confirmation of synthesis and stabilization of nanoparticles were done by various characterization methods. Green gram is one of the important pulse crop and the crop is suffers from many disease among that the wilt caused by *Fusarium* sp. is the most common.
The use of fungicide to manage this disease causes several adverse effect like residuel toxicity, pollution to environment, therefore the present study was undertaken to provide the farmer with the most reliable and advanced measures of nanotechnology for managing the incidence and losses caused by wilt of green gram.

Materials and Methods
1. Collection of sample and isolation of the pathogen
The green gram seed were collected and *Fusarium* sp. isolated from that seeds. Potato dextrose agar media is used for the isolation of pathogen.

2. Preparation of Eucalyptus leaves extract
Eucalyptus (*Eucalyptus globulus* L.) leaves were collected. These plant products were cleaned under running tap water subsequently dried under shade to remove moisture completely, and then it was powdered and stored. 10 g of powdered material were taken in a beaker along with 100 ml of de-ionized water and allowed to boil at 80 °C for 25-30 minutes under reflux condition, cooled down to room temperature. Each prepared solution was initially filtered through Whatman No.42 filter paper and then centrifuged at 3000 rpm for 10 minutes to remove heavy biomaterials and to get clear solution.

Then plant extract was stored at 4 °C for further studies. (Iliger *et al.* 2020) [3].

3. Preparation of Copper sulphate Solution

Accurate amount of 1mm copper sulphate solution can be prepared by dissolving 0.2496 gm of $CuSO_4.5H_20$ in 100 ml of double distilled water and stored in clean, dried reagent bottle.

4. Synthesis of copper nanoparticles

In a typical synthesis of copper nanoparticles from eucalyptus, 10 ml of fresh leaf extracts were added to 100 ml of 0.01 M copper sulphate ($CuSO_4.5H_2O$) aqueous solutions. Tulsi leaves extract 50 ml were mixed with 50 ml aqueous solution of 1mM copper sulphate (1:1 ratio of tulsi extract to copper solution). A prepared mixture was kept at constant stirring on a magnetic stirrer for 2 hrs. The suspended solution was autoclaved and centrifuged at 5000 rpm for 25 min and the supernatant liquids were decanted off and the residues were repeatedly washed with de-ionized water. Centrifugation-decantation-washing processes were repeatedly done several times to remove impurities if any on the surface of the copper nanoparticles. The obtained precipitate was dried in an oven at 60 °C for 24 hr. (Seeram *et al.* 2016 and Mali *et al.* 2020) [9, 6].

5. Characterizations of synthesized nanoparticles

UV-Visible spectrophotometry, Zeta size analysis, Zeta potential, and Nano Tracking Analysis, were used to characterize the synthesized copper nanoparticles.

6. *In-vitro* efficacy of synthesized nanoparticles

Evaluation of synthesized nano-particles were carried out under controlled conditions through poison food technique with carbendazim 50 WP (500 ppm), mancozeb (2500 ppm) and thiram(500 ppm) served as standard checks while as water served as control. CuNP-E including three standard fungicides for each were tested against the *F. oxysporum* f. sp. *Vigni* under study. The nanoparticles were evaluated at 25, 50, 100, 150, 250, 500 and 1000 ppm concentrations with three replications each in a Completely Randomized Design (CRD). The efficacy of nanoparticles prepared from eucalyptus against the test fungus was expressed as inhibition of mycelial growth over control percent calculated by using the formula given by Vincent (1947) [12].

$$\text{Percent Inhibition (I)} = \frac{C - T}{C} \times 100$$

Where,

C = Growth (mm) of test fungus in untreated control plate.

T = Growth (mm) of test fungus in treated plate.

Results and Discussion

1. Isolation, Purification, and identification of the pathogen

The seeds which showing the symptoms of disease after examination, were selected for further research work. The agar plate method was used for the isolation of the pathogen.The incubated Petri dishes at 22-25 °C were shows mycelial growth of the fungus after 3 days. The pure culture of fungi was obtained by transfer of hyphal tip of actively growing mycelium at 7 days after incubation.

Sandhyarani *et al*. (2020) [8] and Deshmukh *et al*. (2019) [1] utilized similarly the agar plate method (ISTA, 1999) for the isolation of seed mycoflora associated with green gram seeds.

2. Green synthesis of nanoparticles

The biosynthesis of copper nanoparticles was carried out using copper sulphate pent hydrate ($CuSO_4.5H_2O$) solution mixedwith leaf extracts of eucalyptus and leaf extracts. The leaf extract act as a capping and stabilizing agent and copper sulphate pent hydrate act as a reductant chemical. The results estimated that the 100 gm. of eucalyptus leaves (powder) produce 0.12 gm. of copper nanoparticles, which was in solid form.

The green synthesis of CuNP from the eucalyptus leaves was conducted by Illiger *et al.* (2020) [3] and Kulkarni *et al.* (2015) [4], they utilize copper sulphate pent hydrate ($CuSO_4.5H_2O$) as the reducing agents.

3. Characterizations of synthesized nanoparticles

3.1 Visual observation

The reaction of nanoparticles synthesis started when the leaf extract of eucalyptus leaves was added into 1mM aqueous copper sulphate pent hydrate ($CuSO_4.5H_2O$) solution. The Copper nanoparticle solution was autoclaved to increase the intensity of nanoparticles in the solution. The aqueous copper sulphate solution turned blue to dark brown within 2 hours indicates the formation of copper nanoparticles in the solution.

The similar results were reported by Illiger *et al.* (2020) [3] where Copper sulphate solution (aqueous) which was blue in colour changed to brown colour within 2 hours after addition of leaf extract of Eucalyptus shows the presence of Cu nanoparticles in the solution.

3.2 UV-Visible spectrophotometry

Absorption spectra of copper nanoparticles formed in the reaction has sharp absorbance peak at 345 nm which can be attributed to absorption by Nano size copper particle confirming the presence of copper nanoparticles by UV-Visible spectroscopy.

Similar observations were reported by Shende *et al.* (2016) [10], where the presence of copper nanoparticle was confirmed by UV-visible spectroscopy technique.

3.3 Zeta size Analysis

The characterization of copper nanoparticles in terms of average particle diameter was recorded in nanometer from the intensity distribution analysis by using Zeta sizer as shown in Fig.1 The result obtained shows that sample of eucalyptus copper nanoparticle is with average particles diameter 129.23 nm size.

The similar method was used by Usha *et al.* (2017) [11] for the characterization of nanoparticles. Zeta sizer was used by her to examine average particle diameter (nm) of biosynthesized copper nanoparticles and it revealed that three biosynthesized samples with average particles diameter were 37.61 nm.

Plate 1: Chanes in the colour of mixture

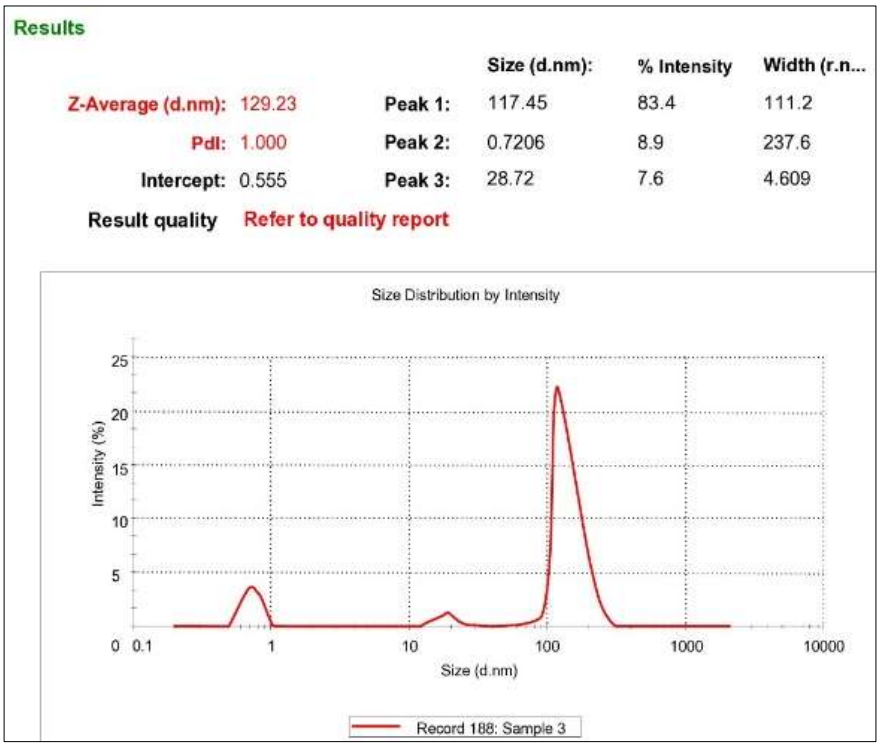

Fig 1: Size distribution report by intensity of eucalyptus sample

3.4 Zeta potential

zeta potential analysis of biosynthesized CuNP-E was founded -2.41 mV. The results proved that during investigation copper nanoparticles are having high stability. The above finding was similar to the work done by Manikandan *et al.* (2015) [7] in which he synthesized stable CuNP and the zeta potential was -29.0 mV.

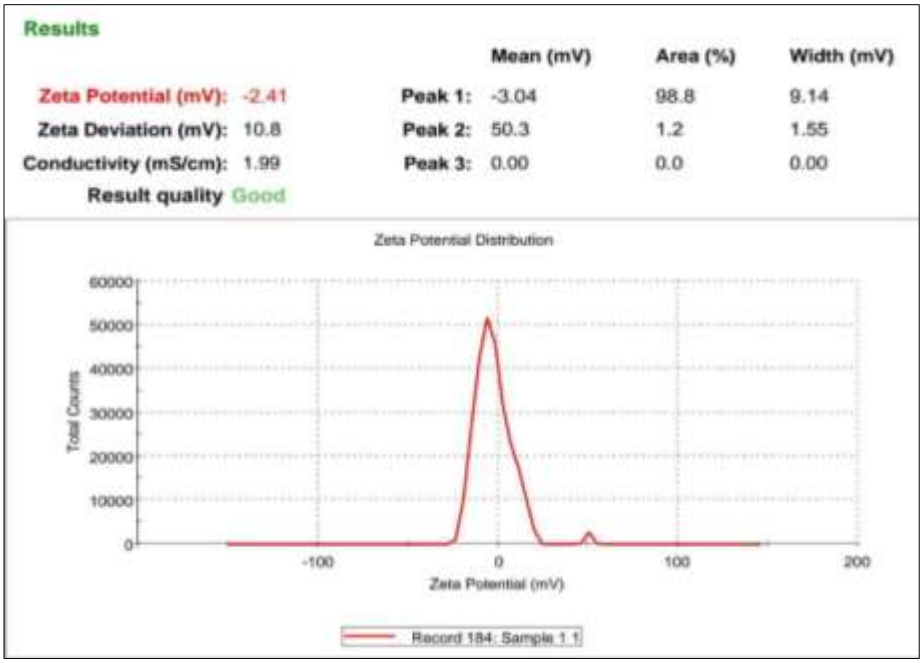

Fig 2: Zeta potential report of eucalyptus sample

3.5 Nano Tracking Analysis (NTA)

During the analysis of sample, the sample was tested at two different camera level and the variation is observed among them. When the sample of CuNP-E was captured at camera level 12 (NTA 3.0 Levels) the 149.5 nm size of nanoparticles is observed (Fig 3). When the sample of CuNP-E is captured at camera level 11 (NTA 3.0 Levels) the 196.5 nm size of nanoparticles is observed (Fig 4).

The characterization of synthesized nanoparticle was done similarly using Nano tracking analysis technique by Shende *et al.* (2016) [10], where nanoparticles tracking analyzer measurements showed average particle size of copper nanoparticles was 25 nm.

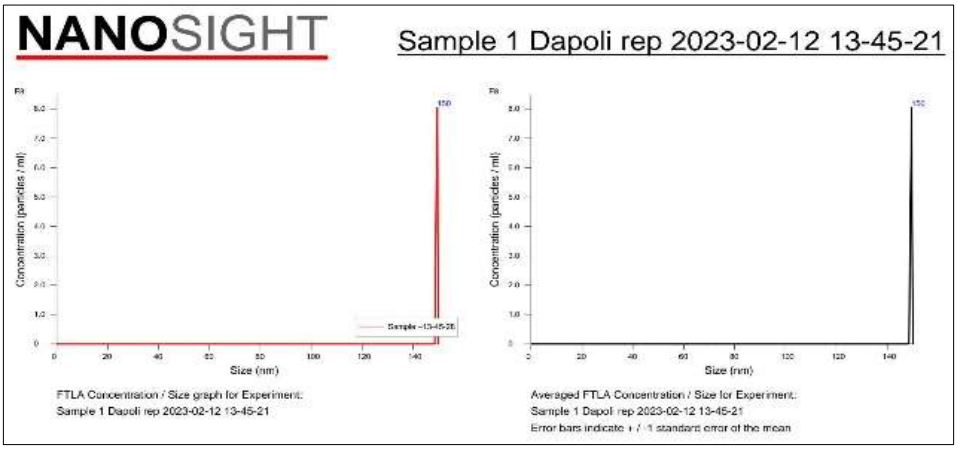

Fig 3: Nano Tracking Analysis report of eucalyptus sample at 12 camera level

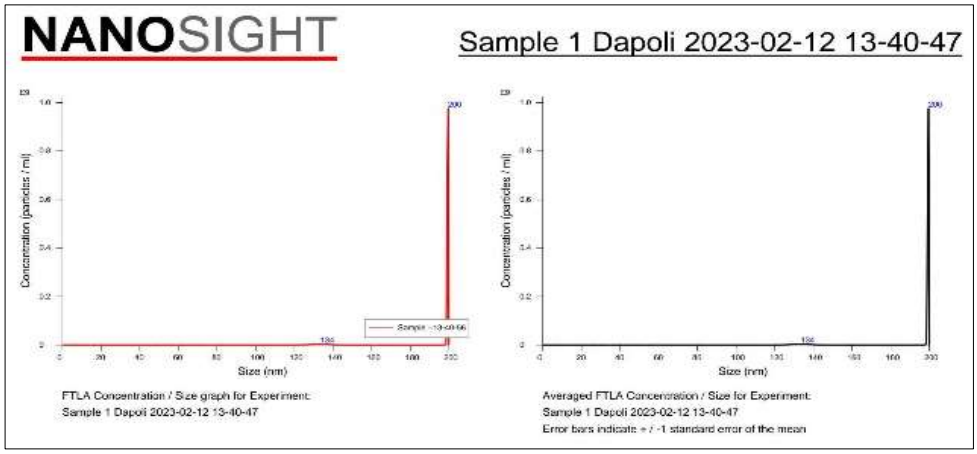

Fig 4: Nano Tracking Analysis report of eucalyptus sample at 11 camera level

4. *In-vitro* efficacy of synthesized nanoparticles

The poison food technique was used to evaluate eucalyptus nanoparticles and the data of their effect on mycelial growth calculating percent inhibition of *Fusarium* sp. was presented in Table 1. Observations on mycelial diameter of *Fusarium* sp. was recorded after ten days of incubation. Mycelial growth of *Fusarium* sp. decreased significantly with increase in the concentration of nanoparticles. Perusal of the data (Table 1) reveals that mycelial growth inhibition (%) of *Fusarium* sp. increased significantly with increase in the concentration of nanoparticles. Highest inhibition percentage of mycelial growth (80.59%) was recorded on CuNP-E @1000 ppm, followed by (41.48%) was recorded on CuNP-E @ 500 ppm.

Highest inhibition percentage of mycelial growth (80.59%) was recorded on CuNP-E @ 1000 ppm, which was significantly higher than the inhibition percent of Thirum (80.41%), Carbendazim (76.44%) and Mancozeb (72.15%) respectively. The data further reveals that the recommended fungicides used at recommended concentrations were not as effective as nanoparticles CuNP-E @1000 ppm. But the inhibition percentage of CuNP-E @ 25 ppm, 50 ppm, 100 ppm, 150 ppm, 250 ppm and 500 ppm was showing lower inhibition percentage than the reccomonded fungicide *viz.* Thirum (80.41%), Carbendazim (76.44%) and Mancozeb (72.15%). This data further reveals that all the CuNP-E when used at lower concentration were not as effective as the recommended fungicides at their recommended concentrations.

The similar research work was conducted by Illiger *et al.* (2020) [3] on impact of copper nanoparticles prepared from eucalyptus on mycelial growth of *C. capsici*. They noted that significantly decreased inmycelial growth of *C. capsici* with increase in the nanoparticle concentration. Lowest growth of pathogen CuNP-E @ 1000 ppm (4.75 mm) was recorded by them.

Table 1: *In vitro* efficacy of CuNP-E on the mycelial growth inhibition of *Fusarium* sp.

Tr. No.	Treatment	Concentration (ppm)	Mycelial diameter (mm)*	Percent inhibition
T_1	CuNP-E	25	80.00	11.11
T_2	CuNP-E	50	77.20	14.22
T_3	CuNP-E	100	74.93	16.74
T_4	CuNP-E	150	71.67	20.37
T_5	CuNP-E	250	60.17	33.15
T_6	CuNP-E	500	52.67	41.48
T_7	CuNP-E	1000	17.47	80.59
T_8	Carbendazim	500	21.20	76.44
T_9	Mancozeb	2500	25.07	72.15
T_{10}	Thiram	400	17.63	80.41
T_{11}	control	-	90	-
S.E(m)±			0.99	
C.D.at 1%			3.93	

Plate 2: *In vitro* efficacy of CuNP-E on the mycelia growth inhibition of *Fusarium* sp.

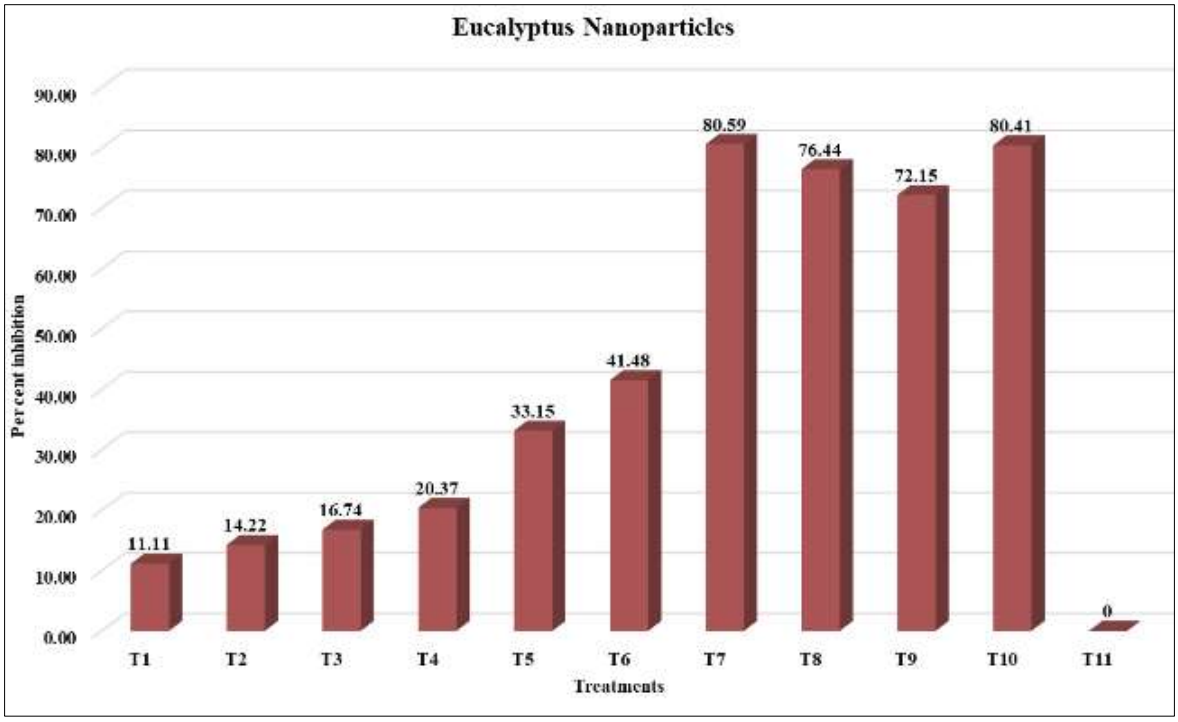

Fig 3: *In vitro* efficacy of CUNP-E on the mycelial growth inhibition of *Fusarium* sp.

Conclusions

Green synthesis of nanoparticles was done by using aqueous plant extracts of eucalyptus with inorganic salts of copper ($CuSO_4.5H_20$). This method of green synthesis has merits over other methods of nanoparticles synthesis. The characterization of nanoparticles were done by various analytical techniques *viz.*, UV visible electroscopy, Zeta sizer, Zeta potential, Nano tracking analyser. The synthesize nanoparticles were effective in inhibiting mycelial growth of *Fusarium* sp. *in vitro.* The highest inhibition 80.59% is observed @1000 ppm concentration of CuNP-E.

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