

Evaluating the *In vivo* Efficacy of Copper-Chitosan Nanocomposition for Treating Vascular Wilt Disease in Date Palm

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Abstract— Date palm, *Phoenix dactylifera*, as one of the most important fruit crops in Egypt and many other countries, can be affected by many fungal diseases, among which the vascular wilt disease, caused by the fungal pathogen *Fusarium oxysporum*, is considered the most deteriorating one. This study aims at evaluating the efficiency of Copper-Chitosan Nanocomposition for treating the vascular wilt disease in date palm. The study relies mainly on beleaguering the disease via the double-role functionality of copper-chitosan nanocomposition, i.e. its potential antifungal effect on the fungal pathogen, besides its capability to enhance the immune responses of the infected plant. In this regard, chitosan nanoparticles were prepared according to the ionic gelation method, whereas copper nanoparticles were prepared according to the chemical reduction method. Physicochemical characterization of both chitosan and copper nanoparticles was performed using dynamic light scattering (DLS), transmission electron microscopy (TEM), fourier transform infrared spectroscopy (FTIR) and x-ray diffraction (XRD). Copper-chitosan nanocomposition could significantly reduce the vascular wilt disease severity; this means that the nanocomposition can be used in the future for developing new nano-fungicides to control such pathogens.

Keywords—Copper nanoparticles, Chitosan nanoparticles, Date palm, *Fusarium oxysporum*, Vascular wilt.

I. INTRODUCTION

Date palm, *Phoenix dactylifera*, is considered one of the most important fruit crops in many arid regions including the Middle East; This is due to its versatility, as it has a variety of uses includes eating fresh fruit and benefiting from its high nutritional value and its richness of carotenoids, citric acid, folic acid, and provitamins; this in addition to the antiviral, antibacterial, antifungal, antiulcer, antitumor and immunomodulatory properties of phenolic compounds detected in dates. Moreover, this crop has a great potential as a source of renewable energy by producing biofuel due to the high carbohydrates

content in the fruits. Also, seeds are used in animal feeding, cosmetics, source of oxalic acid and charcoal, besides using them as a paste to relieve ague (Al-Shahib and Marshall, 2003).

The worldwide production of dates reached **7600315** tons annually. In this context, Egypt is considered the largest producer of dates all over the world with annual production of **1465030** tons (FAO, 2014). This massive production of dates makes date palm a promising potential source of national income in Egypt and other large producers. But, this important crop, like all other crops, is threatened with many fungal and bacterial phytopathogens.

Generally speaking, more than 70% of the crop diseases result from fungal pathogens (Agrios, G.N., 2005), this indicates to the importance of controlling such virulent phytopathogens. In this regard, surveys showed that among these fungal pathogens, the fungal pathogen *Fusarium oxysporum*, which causes vascular wilt disease (also known as fusarium wilt disease), is considered the most common and most virulent one (Flood, 2006).

In this context, there are many traditional chemicals that are used to control such pathogenic fungi. But many phytopathogens have exhibited resistance against many traditional chemicals that are used to control them (Hide *et.al*, 1992; M. J. Hajipour *et al.*, 2012; A. J. Friedman, 2013; Patel *et.al.*, 2014). Thus, there is a dire need to develop newer and more effective controlling agents. In this regard, nanotechnology may provide more efficient alternatives for the current fungicides.

Among different types of nanomaterials, metal nanoparticles have gained a considerable attention and have a wide range of applications due to their unique catalytic, electric, magnetic and structural properties (M. Sahu and P. Biswas, 2011; H. Tian *et al.*, 2014 and H. M. Yadav *et al.*, 2014). In this regard, copper nanoparticles (CuNPs) are considered a very important type of transition metals, which have many potential applications including but not limited to catalysis of some dyes such as rose Bengal dye (S. T. H. Sherazi *et al.*, 2013), printing in electronics (S. Magdassi *et al.*, 2010)

and its antimicrobial effect (A. D. Karthik and K. Geetha, 2013; Prachi. K. *et al.*, 2013; R. Betancourt-Galindo *et al.*, 2014 and A. M. Muthukrishnan *et al.*, 2015).

On the other hand, chitosan has gained much attention in many different applications, including but not limited to pharmaceutical, medical, agricultural, nutritional and industrial applications, this is due to its superior characteristics such as non-toxicity, biodegradability and biocompatibility (Harish P. and Tharanathan, 2007). Also, chitosan nanoparticles (CsNPs) have a potential ability to enhance the immune responses of plants (Chandra, S. *et al.*, 2015), this makes CsNPs a potential competitor in formulating the protective compositions directed toward enhancing the plant immunity.

In this work, the *In vivo* efficacy of copper-chitosan nanocomposition (CuCs) will be evaluated to treat the vascular wilt disease in date palm. The idea relies upon exploiting the dual functionality of this nanocomposition, i.e. its ability to induce and augment the innate immune responses in the plant and its potential antifungal activity against the fungal pathogen itself. This can hinder the fungal growth inside the vascular system of the infected plant and hence beleaguering the vascular wilt disease.

II. MATERIALS AND METHODS

All chemicals used were analytical grade of purity, and were used as received without any further purification.

2.1. Preparation of CsNPs and CuNPs

2.1.1. Preparation of CsNPs

CsNPs were synthesized in accordance with the ionic gelation method (Qi, L. *et al.*, 2004) with a simple modification, in which:

- 2 g of chitosan powder (Degree of Deacetylation \geq 90%; water content \leq 8.0%; Carl Roth, Germany) were dissolved into 1 liter of 1% acetic acid solution.
- 0.5 g of sodium tripolyphosphate (TPP) (Sigma Aldrich) was dissolved into 1 liter of distilled water.
- TPP solution was added dropwise under stirring to the chitosan solution in 1:10 volumetric ratio.
- Temperature was kept at 25 °C and pH was adjusted at 4.

2.1.2. Preparation of CuNPs

CuNPs were prepared according to the chemical reduction method (Mustafa B. and Ilkay S., 2010), in which L-ascorbic acid (0.11M) (Future Modern Co., Egypt) was added into aqueous solution of CTAB (0.09M) (Sigma-Aldrich, Egypt). NaOH solution was used to adjust pH at 6.8; temperature was kept at 85 °C. During stirring of the previous mixture of CTAB and L-ascorbic acid, Copper

sulfate pentahydrate (0.03M) (Elnasr Pharmaceuticals Co., Egypt) solution was introduced. The reaction was continued until reddish brown color was developed. Then, CuNPs were collected by centrifugation for further characterization and application.

2.2. Characterization of CsNPs and CuNPs

2.2.1. Fourier Transform Infrared Spectroscopy (FTIR) of CsNPs

Fourier Transform Infrared Spectroscopy (FTIR) was performed to confirm the successful ionic gelation between chitosan and TPP. In this step, solutions of chitosan and CsNPs were firstly lyophilized, using the lyophilizer (EDWARDS Freeze Dryer), then FTIR analysis was performed on the lyophilized chitosan and CsNPs using FTIR spectrophotometer (Model Jasco 4100, Japan; 400 – 4000 cm^{-1}).

2.2.2. UV-Vis spectroscopy of CuNPs

The characteristic surface plasmon resonance of CuNPs was detected using Helios Gamma Spectrophotometer.

2.2.3. X-Ray Diffraction (XRD) of CuNPs

The characteristic X-Ray Diffractogram of CuNPs was recorded using Philips PW1840 X-Ray Diffractometer, USA ($\lambda = 1.54056 \text{ \AA}$; 40 kV; 25 mA). The range of recording was from 5° to 70°, with a rate of 2°/minute.

2.2.4. Determining Particle Size Distribution of CsNPs and CuNPs

Dynamic light scattering (DLS) (Zetasizer nano series (Nano ZS), Malvern, UK) was used to measure the particles size of the synthesized CsNPs and CuNPs.

2.2.5. Transmission Electron Microscopy (TEM) of CsNPs and CuNPs

Transmission Electron Microscopy (Tecnai G20, Super twin, double tilt, FEI, Netherland) was used to figure out the shape of the synthesized CsNPs and CuNPs.

2.3. The Fungus, *Fusarium oxysporum*

Fusarium oxysporum (FO1 isolate), which was isolated from infected date palm, was obtained from Department of Date Palm Pathology, Central Laboratory for Date Palm Research & Development, Agricultural Research Center, Giza, Egypt.

2.4. Preparing copper-chitosan nanocomposition (CuCs)

CuCs was prepared in four gradient concentrations (0.5, 1.0, 1.5 and 2.0 g/l) through the admixture of CuNPs and CsNPs with the respective concentration from both components.

2.5. Evaluating the Antifungal Activity of CuCs

Spores` inoculum of the fungus was prepared; then the microscopic enumeration technique was used to adjust the fungal inoculum size at 1.0×10^6 spores/ml. After that, 100 μ l of the inoculum suspension were used to inoculate each of potato dextrose broth (control) and potato dextrose broth containing different concentrations of the nanocomposition. Both the control and treatments were kept at 30 °C for 2 days. Then, the optical densities (OD) of the cultures were detected using Uv-Vis Spectrophotometer (ORION AQUAMATE 8000) at 530 nm (Eva Petrikkou *et al.*, 2001). Percentage of inhibition at each concentration was calculated according to the following formula (Ling Yien Ing *et al.*, 2012).

$$\text{Inhibition rate} = (1 - \text{OD}_{\text{treatment}} / \text{OD}_{\text{control}}) \times 100$$

(1)

2.6. Evaluating the Immune responses of Date Palm Seedlings caused by CuCs

Four groups of date palm seedlings, Sewi cultivar, (10 months) were used. Each group consists of 20 healthy seedlings. Each group was irrigated with 50 ml of CuCs with the respective concentration (0.5, 1.0, 1.5 or 2.0 g/l) per seedling. The fifth group of seedlings was used as a control, in which seedlings were irrigated with 50 ml water per seedling. Leaves from each seedling were dried, then the total phenolics and enzymes were extracted (kâhkônén *et al.*, 1999).

- **The level of total phenolics** in the extracts was quantified by the modified Folin – Ciocateau method (Singelton and Rossi, 1965). Gallic acid standard (5 g%) was used, and the total phenolic content was expressed as milligram Gallic Acid per gram dry weight of the original sample (mg GA/g dw).
- **Phenoloxidase activity** was determined according to (Ishaaya, 1971). The phenol oxidase activity was determined as optical density (OD₄₀₅) units $\times 10^{-3}$ at an absorbance of 405 nm.
- **Peroxidase activity** was determined according to (Vetter *et al.*, 1958). The enzyme activity was expressed as the change in absorbance at 430 nm (Δ OD₄₃₀)/minute/g fresh weight.

2.7. Evaluation of the *In vivo* Efficiency of CuCs in Treating Fusarium Wilt Date Palm Seedlings In Comparison With Rizolex™

2.7.1. Initiation of Fusarium Wilt Disease

Firstly, the fungal culture was raised on Richard's liquid medium and incubated at 26°C for 2 weeks. Thus, the fungal mat is formed (Riker and Riker, 1936). After that, the fungal inoculums were prepared through mixing

of 10 g of the mycelial mat with 100 mL of distilled water in a blender (S. Ansari *et al.*, 2012). Each seedling was inoculated through adding 50 mL of the fungal inoculum in its root zone.

2.7.2. *In vivo* treatment

In this experiment, three groups of the inoculated date palm seedlings, Sewi cultivar, (10 months) were used. Each group consists of 20 inoculated seedlings. 10 days after the fungal inoculation, the treatment began as follow:

The first group of inoculated seedlings was used as a treatment, in which 50 ml of CuCs with different concentrations was uniformly applied to root zone of each inoculated seedling.

The second group of inoculated seedlings was used as a positive control. In which each inoculated seedling was treated with 50 ml of 3 g/l Rizolex™ fungicide.

The third group of the inoculated seedlings was used as a negative control, in which 50 ml of water was added to the root zone of each seedling.

2.7.3. Assessment Disease Severity

Disease progression was observed through two successive weeks. Symptoms of Fusarium wilt disease on leaves (wilting and yellowing) of the date palm seedlings were used to measure the disease severity. In this regard, a standard rating (Campbell and Madden, 1990) was used to assess the disease severity for each group. Disease severity (DS) was calculated (Chester *et al.*, 1959; Wheeler, 1969) as follow:

$$\text{Disease Severity (DS)} = ((\text{sum of all individual disease rating}) / (\text{total number of leaf assessed} \times \text{maximum rating})) \times 100.$$

(2)

2.8. Statistical analysis

SPSS 22 software was used at $P \leq 0.05$ to distinguish between DS, antifungal efficiencies and levels of total phenolics and activity of phenoloxidase and peroxidase in different groups. The whole experiments were repeated twice (McDonald, 2008).

III. RESULTS & DISCUSSION

3.1. Confirming successful preparation of CsNPs by the Ionic Gelation Method

FTIR analysis showed that chitosan has two main characteristic peaks at 3433 cm^{-1} and 1644 cm^{-1} which correspond to stretching vibrations of the primary amine group ($-\text{NH}_2$) and the amide group ($-\text{CONH}_2$), respectively; as shown in figure (1). On the other hand, FTIR analysis of chitosan nanoparticles showed that both peaks were shifted to 3428 cm^{-1} and 1580 cm^{-1} , respectively; as shown in figure (2).

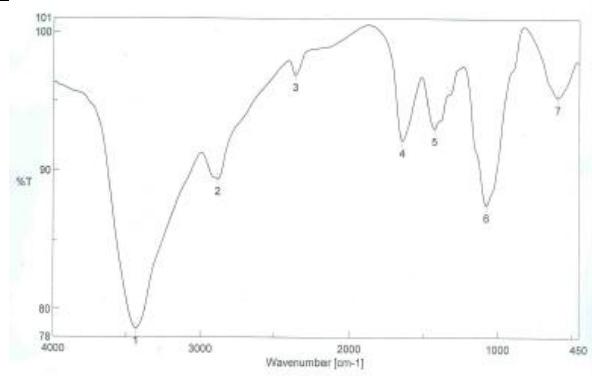


Fig.1: FTIR spectrum of chitosan

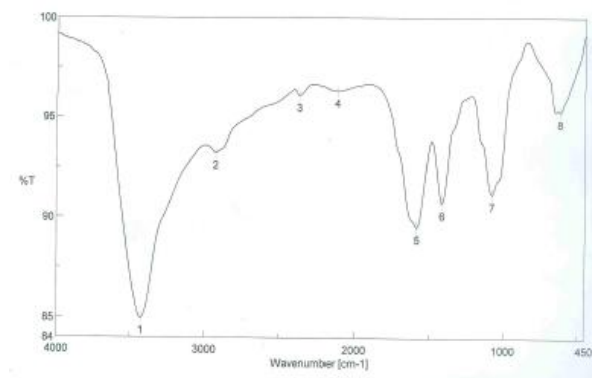


Fig.2: FTIR spectrum of chitosan nanoparticles

This reduction in stretching frequency indicates successful interaction of the polyphosphoric groups of sodium tripolyphosphate with the ammonium groups of chitosan and the more hydrogen bonding in chitosan-TPP complex.

Also, it is clear that the amine peak in chitosan nanoparticles (at 3428 cm^{-1}) is sharper than the amine peak in chitosan (at 3433 cm^{-1}), which indicates that the hydrogen bonding in chitosan nanoparticles was enhanced. (A. Anitha *et al.*, 2009; Xu, Yongmei and Du, Yumin, 2003). Hence, chitosan nanoparticles were successfully synthesized by the ionic gelation method.

3.2. Characterization of CsNPs

Size distribution by number using dynamic light scattering revealed that the synthesized CsNPs have an average particle size about 50 nm as shown in figure (3). In addition, transmission electron microscopy showed spherical shape of the synthesized CsNPs, as shown in figure (4).

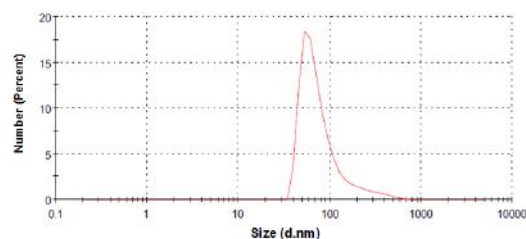


Fig.3: Particle size distribution of the synthesized CsNPs

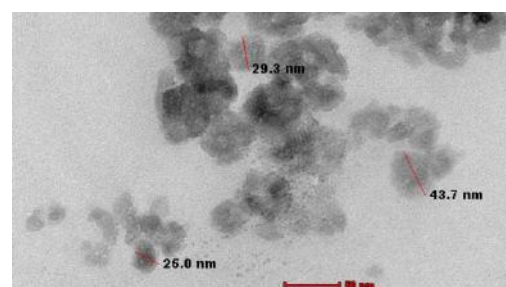


Fig.4: Transmission electron micrograph of the synthesized CsNPs, showing its spherical shape.

3.3. Confirming successful preparation of CuNPs

The Uv-vis spectroscopy of copper nanoparticles showed their characteristic resonance band at 572 nm, as shown in figure (5).

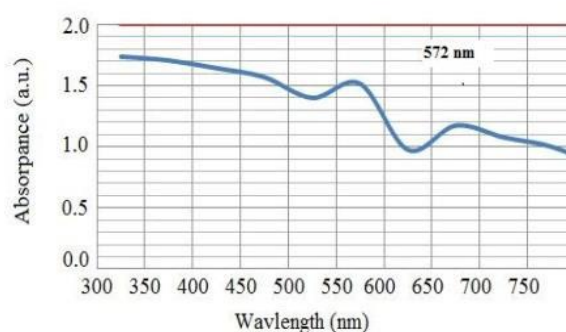


Fig.5: Uv-Vis spectrum of CuNPs showing its characteristic resonance peak at 572 nm.

Also, X-ray diffraction pattern as shown in figure (6) revealed the main characteristic diffraction peaks at $2\theta = 43.36, 50.47$ and 74.13 degrees, which correspond to the (1 1 1), (2 0 0), and (2 2 0) crystal faces of copper (X. Zhu *et al.*, 2012). It is also noteworthy that there are other peaks at $2\theta = 36.45$ and 61.39 degrees, that are characteristic for Cu_2O (M. S. M. Suan *et al.*, 2011) which indicates the formation of a Cu_2O shell covering the Cu core due to surface oxidation of CuNPs.

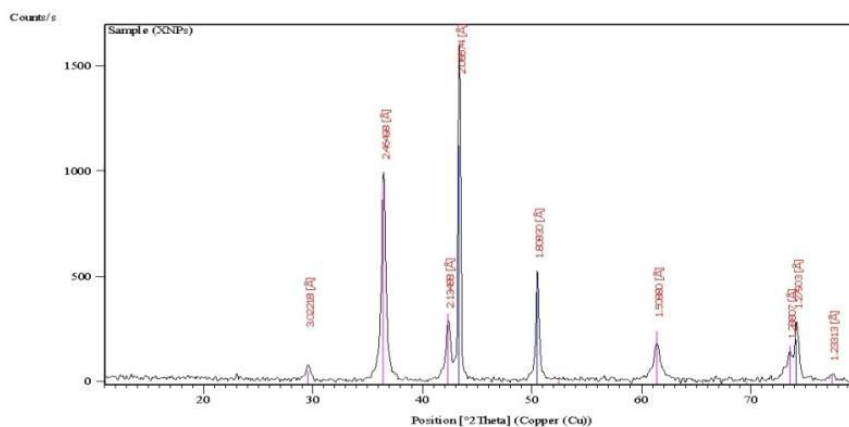


Fig.6: X-Ray Diffractogram of the synthesized CuNPs

3.4. Characterization of CuNPs

Transmission electron microscopy showed that the synthesized CuNPs have spherical shape, as shown in figure (7). Moreover, dynamic light scattering revealed that the average size of CuNPs was about **100 nm**, as shown in figure (8).

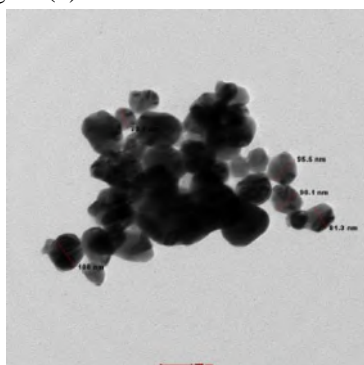


Fig.7: Transmission electron micrograph of copper nanoparticles, showing its spherical shape.

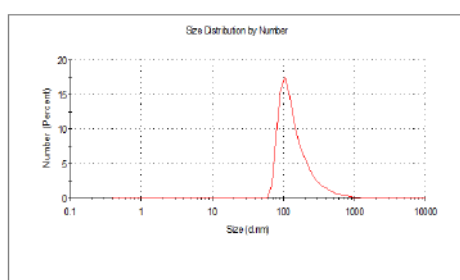


Fig.8: Size distribution by number of the synthesized CuNPs.

3.5. *In vitro* antifungal efficacy of copper-chitosan nanocomposition

All concentrations of CuCs had significant inhibition percentages, as shown in table (1); this demonstrates the potential antifungal efficacy of CuCs against the fungal pathogen, *Fusarium oxysporum*. Data express the average over triplicates.

Table.1: inhibition percentages caused by different concentrations of CuCs

Concentration (g/L)	Inhibition %
0.00 (Control)	00.00
0.50	61.94%
1.00	77.11%
1.50	89.47%
2.00	100.00%

3.6. Evaluating the Immune responses of Date Palm Seedlings caused by CuCs

Table (2) shows the positive immunomodulatory effect of CuCs with different concentrations on date palm seedlings. From the data shown, it was clear that CuCs can significantly enhance the immune responses of date palm seedlings to a great extent. Data express the average over triplicates selected randomly from each group of seedlings.

Table.2: Positive immunomodulatory effect of CuCs on date palm seedlings

Parameter	Concentration of CuCs (g/l)				
	0.00 Control	0.50	1.00	1.50	2.00
Total phenols (mg/gdw)	2.70	2.92	3.29	3.62	4.05
Phenoloxidase (OD units x10 ³ /min/gdw)	31.4	33.83	40.22	47.10	62.73
Peroxidase (ΔOD x10 ³ /min/gdw)	40.33	66.03	96.52	109.85	121.53

3.7. Evaluation of *In vivo* Efficiency of Copper-Chitosan Nanocomposition in Treating Fusarium Wilt-Date Palm Seedlings In Comparison With Rizolextm

Table (3) shows the summary of disease severity (DS) of the inoculated seedlings, which were treated with different concentrations of CuCs in comparison with

those treated with water (-ve control) and with RizolexTM (+ve control).

Statistical analysis showed that the disease severity of the inoculated date palm seedlings treated with different concentrations of the nanocomposition was significantly lower than that of the inoculated date palm seedlings which were treated with **RizolexTM** (+ve Control) and water (-ve Control), except the concentration of 0.50 g/l.

Table.3: DS of the inoculated date palm seedlings treated with nanocomposition, RizolexTM and water.

	Composition concentrations (g/l)				Rizolex TM (3.00 g/l) +ve Control	Water -ve Control
	0.50	1.00	1.50	2.00		
DS	70.67	58.33	46.67	34.33	69.33	84.33

IV. CONCLUSION

To this point, it was clear that copper-chitosan nanocomposition may provide a competitive candidate for treating vascular wilt disease in date palm, which clearly manifested significantly higher efficiency than the commercially available fungicide at lower concentrations. This can be interpreted by virtue of two main axes:

Firstly, the positive immunomodulatory effect of copper-chitosan nanocomposition on date palm seedlings as shown from its ability to increase peroxidase, phenoloxidase and total phenols levels, which were considered main constituents of the plant innate immune response against the invading fungal pathogen;

Secondly, the antifungal effect of the nanocomposition on the fungal pathogen itself.

Hence, this copper – chitosan nanocomposition with its dual complementary functionality may provide a potential approach to beleaguer the fusarium wilt disease in date palm via enhancing the plant immune responses at the same time of inhibiting the fungal growth. Thus, this nanocomposition can be used in developing new nanofungicides to control such pathogens. But, further research should be undertaken in order to investigate its potential toxicity on plant, human and environment; Thus, determining the optimal concentration that can be used in field without considerable toxicity consequences.

REFERENCES

- [1] **D. Karthik and K. Geetha, (2013).** Synthesis of copper precursor, copper and its oxide nanoparticles by green chemical reduction method and its antimicrobial activity, *Journal of Applied Pharmaceutical Science*, vol. 3, no. 5, pp. 16–21.
- [2] **M. Muthukrishnan, (2015).** Green synthesis of copper-chitosan nanoparticles and study of its antibacterial activity, *Journal of Nanomedicine & Nanotechnology*, vol. 6, no. 1.
- [3] **Agrios, G.N. (2005).** Plant Pathology, Elsevier Academic Press, San Diego, California, USA.
- [4] **Al-Shahib W. and R. J. Marshall. (2003).** the fruit of the date palm: its possible use as the best food for the future. *International Journal of Food Sciences and Nutrition*, 54 (4): 247–259.
- [5] **Anitha, A., Rani, V. D., Krishna, R., Sreeja, V., Selvamurugan, N., Nair, S. V., ... & Jayakumar, R. (2009).** Synthesis, characterization, cytotoxicity and antibacterial studies of chitosan, O-carboxymethyl and N, O-carboxymethyl chitosan nanoparticles. *Carbohydrate Polymers*, 78(4), 672–677.
- [6] **Campbell, C.L. and Madden, L.V. (1990).** Introduction to Plant Disease Epidemiology, John-Wiley & Sons, New York, NY, USA.
- [7] **Chandra S.; Chakraborty, N.; Dasgupta, A.; Sarkar, J. Panda, K. and Acharya, K. (2015).** Chitosan nanoparticles: A positive modulator of innate immune responses in plants. *Sci. Rep.* 5, 15195; doi: 10.1038/srep15195 .
- [8] **Chester, K. S.; Horsfall, J. G. and Diamond, A. E. (1959).** Plant Pathology: An Advances Traits, 1, Academic Press, New York, NY, pp. 199–242.
- [9] **Eva Petrikkou, Juan L. Rodríguez-Tudela, Manuel Cuenca-Estrella, Alicia Gómez, Ana Molleja, and Emilia Mellado. (2001).** Inoculum Standardization for Antifungal Susceptibility Testing of Filamentous Fungi Pathogenic for Humans. *J. Clin. Microbiol.* vol. 39 no. 4 1345-1347.
- [10] **F.A.O (2014).** Dates Statistics 2014. Food and Agriculture Organization of the United Nations.
- [11] **Flood J. (2006).** A review of Fusarium wilt of oil palm caused by Fusarium oxysporum f. sp. Elaeidis. *Phytopathology*, 96:660-662.

- [12] **H. M. Yadav, S. V. Otari, V. B. Koli (2014).** Preparation and characterization of copper-doped anatase TiO₂ nanoparticles with visible light photocatalytic antibacterial activity, *Journal of Photochemistry and Photobiology A: Chemistry*, vol. 280, pp. 32–38.
- [13] **H. Tian, X. L. Zhang, J. Scott, C. Ng, and R. Amal, (2014).** TiO₂- supported copper nanoparticles prepared via ion exchange for photocatalytic hydrogen production. *Journal of Materials Chemistry A*, vol. 2, no. 18, pp. 6432–6438.
- [14] **Hajipour, M. J.; Fromm, K. M. and Akbar Ashkarran A. (2012).** Antibacterial properties of nanoparticles. *Trends in Biotechnology*. 30: 499–511.
- [15] **Harish Prashanth KV and tharanathan RN (2007).** Chitin/chitosan: modification and their unlimited application potential – an overview. *Trends Food Sci. Techno.* 18: 117-131.
- [16] **Hide G. A., P. J. Read and S. M. Hall (1992).** Resistance to thiabendazole in *Fusarium* species isolated from potato tubers affected by dry rot. *Plant Pathology*, 41, 745–748.
- [17] **Ishaaya, I. (1971).** Observations on the phenoloxidase system in the armored scales *Aonidiella aurantii* and *Chrysomphalus aonidum*. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 39(4), 935-943.
- [18] **Kâhkönen, M.P.; Hopia, A.I.; Vuorela, H. J.; Rauha, J. P.; Pihlaja, K.; Kujala, T. S. and Heinonen, M. (1999).** Antioxidant activity of plant extracts containing phenolic compounds. *J.Agric.food chem.*, 47 :3954-3962.
- [19] **Ling Yien Ing, Noraziah Mohamad Zin, Atif Sarwar, and Haliza Katas (2012).** Antifungal Activity of Chitosan Nanoparticles and Correlation with Their Physical Properties. *International Journal of Biomaterials*, doi:10.1155/2012/632698
- [20] **M. S. M. Suan, M. R. Johan, N. L. Hawari, and H. A. Ching, (2011).** Annealing effects on the properties of copper oxide thin films prepared by chemical deposition. *International Journal of Electrochemical Science*, vol. 6, no. 12, pp. 6094–6104.
- [21] **M. Sahu and P. Biswas. (2011).** Single-step processing of copper-doped titania nanomaterials in a flame aerosol reactor. *Nanoscale Research Letters*, vol. 6, no. 1, article 441, pp. 1–14.
- [22] **McDonald J.H. (2008).** Handbook of Biological Statistics Sparky House Publishing, Baltimore.
- [23] **Mustafa B. and Ilkay S. (2010).** Controlled synthesis of copper nano/microstructures using ascorbic acid in aqueous CTAB solution. *Powder Technology*, 198, 279–284.
- [24] **Patel N., P. Desai, N. Patel, A. Jha and H. K. Gautam (2014).** Agronanotechnology for plant fungal disease management: a review. *Int J Curr Microbiol App Sci*, 3:71–84.
- [25] **Pelgrift, R. Y. and Friedman, A. J. (2013).** Nanotechnology as a therapeutic tool to combat microbial resistance. *Advanced Drug Delivery Reviews*. 65: 1803–1815.
- [26] **Prachi. K; Sonal, B.; Swapnil, G.; Aniket, G.; Amedea, B.; Seabrab, O.; Rubilarc, D.; Nelson; Durane, F. and Mahendra R. (2013).** In vitro antifungal efficacy of copper nanoparticles against selected crop pathogenic fungi. *Materials Letters*, 115, 13-17.
- [27] **Qi, L., Xu, Z., Jiang, X., Hu, C. and Zou, X. (2004).** Preparation and antibacterial activity of chitosan nanoparticles. *Carbohydr. Res.* 339, 2693–2700.
- [28] **R. Betancourt-Galindo, P. Y. Reyes-Rodriguez, B. A. Puente Urbina et al., (2014).** Synthesis of copper nanoparticles by thermal decomposition and their antimicrobial properties. *Journal of Nanomaterials*, vol. 2014, Article ID 980545, 5 pages.
- [29] **Riker, A.J. and Riker, R.S. (1936).** Introduction to research on plant diseases. John's Swift Co., St. Louis, Chicago, New York and Indianapolis, 117 p.
- [30] **S. Magdassi, M. Grouchko, and A. Kamysny, (2010).** Copper nanoparticles for printed electronics: routes towards achieving oxidation stability. *Materials*, vol. 3, no. 9, pp. 4626–4638.
- [31] **S. T. H. Sherazi, R. A. Soomro, S. Uddin, and N. Memon, (2013).** Synthesis and characterizations of highly efficient copper nanoparticles and their use in ultra fast catalytic degradation of organic dyes. *Advanced Materials Research*, vol. 829, pp. 93–99.
- [32] **Safiuddin Ansari¹; Sheila Shahab; Mohd. Mazid and Dania Ahmed. (2012).** Comparative study of *Fusarium oxysporum* f. sp. *lycopersici* and *Meloidogyne incognita* race-2 on plant growth parameters of tomato. *Agricultural Sciences* 3: 844–847.
- [33] **Singleton, V. L. and Rossi, J. A. (1965).** Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *Am.J.Enol.Vitic.*, 16:144-158.
- [34] **Vetter, J. L., Steinberg, M. P., & Nelson, A. I. (1958).** Enzyme assay, quantitative determination of peroxidase in sweet corn. *Journal of Agricultural and Food Chemistry*, 6(1), 39-41.
- [35] **Wheeler, B.E.J. (1969).** An Introduction to Plant Disease, John Wiley and Sons Limited, London. pp 301.

- [36] X. Zhu, B. Wang, F. Shi, and J. Nie, (2012). Direct, rapid, facile photochemical method for preparing copper nanoparticles and copper patterns. *Langmuir*, vol. 28, no. 40, pp. 14461–14469.
- [37] Xu, Yongmei and Du, Yumin (2003). Effect of molecular structure of chitosan on protein delivery properties of chitosan nanoparticles. *International journal of pharmaceutics*, Volume 250, Issue 1, 2 January 2003, Pages 215-226.