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Efficacy of green synthesized copper nanoparticles towards leaf spot disease and its effects on vase life of chrysanthemum cv. Snowball

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Abstract
An investigation was carried out during 2015-2018 to study the efficacy of green synthesized copper nanoparticles in managing leaf spot disease of chrysanthemum (*Dendranthema grandiflora*) cv. Snowball. The fungal infection was studied in chrysanthemum in pot experiment and the synthesized CuNPs were targeted to the fungus in lab condition. Further, the CuNPs were also tested in vase solution of cut chrysanthemum.
A novel fungus was isolated from infected leaf of chrysanthemum (*Dendranthema grandiflora*) and was cultured in PDA media. The isolate was identified as *Corynespora dendranthema* that is reported for the first time as foliage pathogen of chrysanthemum. The efficacy of green synthesized CuNPs against the fungus was evaluated using Agar well technique. The result showed that the green synthesized CuNPs exhibited mycelial growth inhibition at 60.52%, 57.13% and 52.44% for Night jasmine, Allamanda and Yellow oleander CuNPs respectively against the mycelial growth inhibition by traditional of fungicide (48.57%). The CuNPs treatments significantly enhanced the vase life of cut chrysanthemum. Night jasmine mediated CuNPs and Allamanda mediated CuNPs at 75 ppm found to be best among all other treatments that increase vase life of cut chrysanthemum than traditional practice.
From the study inference can be drawn that green synthesis of nanoparticles from ornamental plants *viz*. Night jasmine, Yellow oleander and Allamanda may be superior method for management of leaf spot disease in chrysanthemum and enhancing vase life of cut chrysanthemum. However, Night jasmine mediated CuNPs was found to be more effective.

Keywords: Chrysanthemum, copper nanoparticles, leaf spot, green synthesized

Introduction
The recent development and implementation of nanotechnology have led to a new era called nano-revolution. It unfolds the role of plants in green synthesis of nanoparticles for synthesizing stable nanoparticles. Nanoparticles can be synthesized using various approaches including chemical, physical and biological. Nanotechnology has two major aspects; the first aspect is synthesis of nanosize materials and second is the application of nano materials for the desired objectives. Among the various nanoparticles, metal nanoparticles assume special importance because of their easier and cheaper mode of synthesis and promising in applications. Copper nanoparticles (CuNPs) have recently attracted special attention as it proved its superior antimicrobial activity against various bacterial and fungal strains reported by many researchers. Many studies confirmed that metal nanoparticles are effective against plant pathogens, insects and pests (Choudhury *et al*., 2010) [2].
Chrysanthemum (*Dendranthema grandiflora*) the golden flower is one of the most beautiful and perhaps the oldest flowering plants commercially grown in different parts of the world. Chrysanthemum belongs to the family Asteraceae. It is important both as cut flower and as potted plant in the international market. Chrysanthemum is preferred practically due to its vast range of shapes and sizes of flowers, brilliance of colour tones, long lasting floret life, diversity of height and growth habit of the plant, exceptionally hardy nature, relatively easy to grow all the year round and versatility of use. It is one of the most important traditional flowers of India, mainly used as a potted plant, loose flower, cut flower and as border plant in the garden. There has been constant demand for chrysanthemum flowers particularly from European markets during winter months and throughout the year in one country. India is bestowed with varied types of agro-climatic conditions besides, the availability of land and cheap skilled labour.

Hence there is a great potential for production of chrysanthemum on a commercial scale in India. However, it is difficult to get good quality exportable blooms, higher yields and long-lasting post-harvest life of the cultivars under open conditions. The most important factors responsible are the diseases like leaf spot, leaf blight, rust, wilt, bacterial blight etc. Among these diseases leaf spot is one of the most destructive diseases, commonly prevailing in almost all chrysanthemum growing pockets of India, which cause heavy losses under field as well as market conditions. Though various information were available about the antimicrobial activity of various CuNPs synthesized from plants, report on synthesis of nanoparticles from ornamental plants is very meagre. So, the research work was carried out to know the efficacy of synthesized nanoparticles towards leaf spot disease of chrysanthemum.

Materials and Method

Method of antifungal evaluation

Diseased sample having typical symptom of the targeted fungal infection were collected from Experimental farm, Department of Horticulture, Assam Agricultural University, Jorhat, Assam. Freshly infected leaves from the chrysanthemum plants with typical diseased symptom were isolated and pathogenicity test was done on leaves.

The antifungal study of CuNPs was carried out by Agar well method using 100% concentration of the copper nanoparticles synthesized from Night jasmine (CuNP-1), Yellow oleander (CuNP-2) and Allamanda (CuNP-3) which was compared with commercial fungicide (Blitox @ 2g/litre of water. After incubation of fungi on culture medium containing CuNPs and fungicide, radial growth of fungal mycelium was recorded. Mycelial growth inhibition was calculated when growth of mycelia in the control plate reached the edge of the petridish. The following formula was used for calculation of inhibition rate (%).

$$\text{Mycelial growth inhibition (\%)} = \left(\frac{R - r}{R}\right) \times 100$$

Where, R is the radial growth of the fungal mycelia on the control plate and r is the radial growth of fungal mycelia on the treated plate.

Postharvest evaluation

Chrysanthemum cv. Snowball was evaluated for shelf life studies by placing in 250 ml Erlenmeyer flask containing different treatment combinations consisting of three biologically synthesized CuNPs and distilled water as control. The mouths of the conical flask were covered with plastic film to minimize evaporation. CuNPs solutions were prepared at the beginning of the experiment. Nine treatments with three replicates were applied. The different treatments used in the experiments were as follows:

T_1= CuNP-1@ 25ppm, T_2= CuNP-2@ 25ppm, T_3= CuNP-3@ 25ppm, T_4= CuNP-1@ 50ppm, T_5= CuNP-2@ 50ppm, T_6= CuNP-3@ 50ppm, T_7= CuNP-1@ 75ppm, T_8= CuNP-2@ 75ppm, T_9= CuNP-3@ 75ppm, T_{10}= Control

Where,
CuNP-1 = Night jasmine mediated CuNPs, CuNP-2 = Yellow oleander mediated CuNPs, CuNP-3 = Allamanda mediated CuNPs, Control = Distilled water

Ten treatments were compared and replicated thrice. The whole experiment was arranged in Completely Randomized Design (CRD) and observations were recorded.

Result and Discussion

The results of antifungal screening of CuNPs synthesized from Allamanda, Night jasmine and Yellow oleander was screened by agar well technique (Shanmuga *et al*., 2002) [10] to select out best CuNPs on the criterion of more than 50% inhibition of radial growth of the targeted fungus. It was observed that all the synthesized CuNPs treatment significantly inhibited 50% growth of fungus by plant mediated CuNPs compared to commercial fungicide. This may be due to the fact that particle size of fungicide (~5µ) is more than the size of nanoparticles (1-100nm) which fundamentally affecting all of its properties.

The treatment CuNP-1 showed maximum percent mycelia growth inhibition compared to other treatments. The antimicrobial activity of CuNPs is associated with ions which are released from nanoparticles. The activity is further intensifying by its small size and high surface to volume ratio that it enables them to interact closely with microbial membranes (European commission, 2007) [4]. In oxidation state, CuNPs has a tendency to alternate between its cuprous-Cu (I) and cupric-Cu (II) which enhances its antimicrobial activity.

Copper produces hydroxyl radicals which subsequently bind with DNA molecules and lead to disorder of the helical structure by cross-linking within and between the nucleic acid strands and damage essential proteins and there by denaturing the protein it makes the enzyme ineffective (Yoon *et al*., 2007) [14]. Ananthi and Kala (2017) [1] reported antifungal activity of green synthesized CuNPs against different fungal strains *viz. Candida albicans*, *Candida tropicalis*, *Aspergillus fumigatus* and *Aspergillus niger*. CuNPs are known to exhibit wide range of antibacterial activity against different strains of gram positive and gram negative bacteria. The CuNPs synthesized using plant extract of *Magnolia*, *Syzygium aromaticum*, *Tridax procumbens* were tested against *Escherichia coli*, they showed higher antibacterial activity on cells after 24hr growth (Lee *et al*., 2011; Subhankari and Nayak, 2013) [8, 11, 12]. CuNPs synthesized by *Zingiber officinale* extract showed the zone of inhibition against *E. Coli* of 15 mm (Subhankari and Nayak, 2013) [11, 12]. Yoon *et al*. (2007) [14] demonstrated antibacterial effects of silver and copper nanoparticles using single representative strain of *E. coli* where the CuNPs showed superior antibacterial activity compared to the silver.

Regarding vase life evaluation, Days taken for discolouration of flower head and first fall of petal were found to be maximum in 75ppm CuNP-1 and 75ppm CuNP-3. Discoloration of flower head is due to water balance and production of ethylene during process of senescence which leads to discolouration, wilting and ultimately reduction in the ornamental value of flowers. Vase life termination for cut flowers is characterized by wilting which is due to loss of water from cells (He *et al*., 2006) [6]. Water balance is a major factor which determines the quality and longevity of flowers. It is influenced by water uptake and transpiration and balance between two mentioned processes (Da Silva, 2003) [3]. When the amount of transpiration exceeds the volume of water uptake is mainly due to occlusion located in the basal stem end (He *et al.*, 2006) [6] and microbes that cause stem end blockage (Van Doorn, 1997) [13]. Many agents have been used in vase solutions of cut flowers which extends vase life by improving water uptake (Fujino *et al.*, 1983) [5]. Untreated flowers produces higher ethylene that is responsible for deterioration related to oxidative stress and ethylene mediates the production of reactive oxygen species (ROS). ROS are generated when plant tissues are exposed to environmental stresses and can cause damage to cellular macromolecules and hence flower senescence (Zhou *et al.*, 2014) [15]. In this experiment CuNPs treated cut flower shows delay in discolouration of flower head and first fall of petal this might be due to that CuNPs act as anti-ethylene agents which slowed down the process of senescence of flowers.

The relative water content was found to increase in 75ppm CuNP-3; this might be due to the fact that at 75ppm concentration of CuNP-3, the microbial contamination might be less than that of control. Due to this the blockage at the stem end may be prevented for which uptake of water is more compare to control. The short vase life of cut flowers was caused by poor water relations which is in associated with a lower uptake of water. This is probably due to growth of microbes causing vascular blockage, high rate of transpiration and water loss. The data indicated that 75ppm CuNP-1 increased vase life of cut chrysanthemum than in control. The increase in vase life may be due to the phytochemicals and concentration of CuNPs. Kumar *et al.* (2017) [7] reported various phytochemicals such as oleanic acid, tannic acid, benzoic acid, lupeol, flavanol glycoside etc. are present in Night jasmine that have significant hepatoprotective, antiviral, antifungal, antibacterial, antioxidant activity. A positive effectiveness of CuNPs can be ascribed to antimicrobial effect of copper containing solution by reducing population of microbes which help to increase vase life. Positive effect of CuNPs on bacterial contamination is due to the antimicrobial properties of copper ion on microorganism (Liu *et al*. 2009) [9].

Table 1: Mycelial growth inhibition of fungus by biosynthesized CuNPs

Treatments	Mycelial growth inhibition (%)
CuNP-1	60.52^{a}
CuNP-2	52.44^{c}
CuNP-3	57.13^{b}
Blitox	48.57^{d}
S.Ed(±)	0.88
$CD_{0.05}$	1.56

Same superscript letters are not statistically different at $p \leq 0.05$

Table 2: Effect of biosynthesized CuNPs on relative water content (%) of cut chrysanthemum cv. Snowball

Treatments	Relative water content (%)
T_1	83.00^{def}
T_2	83.00^{def}
T_3	82.33^{def}
T_4	81.33^{ef}
T_5	83.67^{de}
T_6	84.33^{bcd}
T_7	86.33^{ab}
T_8	86.00^{abc}
T_9	88.00^{abc}
T_{10}	78.00^{g}
S.Ed(±)	1.14
$CD_{0.05}$	2.39

Same superscript letters are not statistically different at $p \leq 0.05$

Table 3: Effect of biosynthesized CuNPs on discolouration of flower head and first fall of petal of cut chrysanthemum cv. Snowball

Treatments	Discolouration of Flower head (days)	First fall of petal (days)
T_1	11.00^{defg}	12.33^{c}
T_2	12.00^{cdef}	12.67^{bc}
T_3	9.33^{g}	12.00^{c}
T_4	11.33^{defg}	13.67^{abc}
T_5	12.67^{bcde}	14.33^{abc}
T_6	13.00^{bcd}	12.67^{bc}
T_7	15.67^{a}	16.00^{a}
T_8	14.33^{abc}	15.00^{ab}
T_9	15.00^{ab}	16.00^{a}
T_{10}	6.33^{h}	8.00^{d}
S.Ed(±)	1.21	1.20
$CD_{0.05}$	2.53	2.51

Same superscript letters are not statistically different at $p \leq 0.05$

Table 4: Effect of biosynthesized CuNPs on determination of vase life of cut chrysanthemum cv. Snowball

Treatments	Determination of vase life of floret (days)
T_1	14.00^{d}
T_2	14.33^{d}
T_3	14.00^{d}
T_4	15.67^{bcd}
T_5	16.00^{abcd}
T_6	15.00^{cd}
T_7	18.33^{a}
T_8	17.33^{abc}
T_9	18.00^{ab}
T_{10}	10.00^{e}
S.Ed(±)	1.19
$CD_{0.05}$	2.49

Same superscript letters are not statistically different at $p \leq 0.05$

Table 5: Effect of biosynthesized CuNPs on microbial count in vase solution of cut chrysanthemum cv. Snowball

Treatments	Microbial count (log_{10} cfu/ml)
T_1	5.39^{f}
T_2	5.41^{f}
T_3	5.24^{de}
T_4	5.22^{cde}
T_5	5.24^{de}
T_6	5.18^{bcd}
T_7	4.94^{a}
T_8	5.12^{bc}
T_9	5.09^{b}
T_{10}	5.52^{g}
S.Ed(±)	0.06
$CD_{0.05}$	0.10

Same superscript letters are not statistically different at $p \leq 0.05$

Conclusion

All CuNPs treatment significantly enhanced the vase life of cut flowers. In evaluating the vase life of flowers, parameters like relative water content, days taken for discolouration of flower head, days taken for first fall of petal, determination of vase life of floret and microbial count in the vase solution were studied in the present investigation. The treatment 75 ppm CuNP-1 and 75 ppm CuNP-3 was found to be best among all other treatments.

Out of the studied CuNPs, Night jasmine mediated CuNPs was found to be more effective against the *Corynespora dendranthema* and enhancing vase life of cut chrysanthemum cv. Snowball.

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