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The Pharma Innovation

ISSN (E): 2277-7695
ISSN (P): 2349-8242
NAAS Rating: 5.23
TPI 2023; 12(8): 53-56

www.thepharmajournal.com
Received: 01-06-2023
Accepted: 07-07-2023

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Biogenesis of Zinc oxide nanoparticles from mustard seed extract and its antifungal activity against *Colletotrichum falcatum* causing red rot in sugarcane

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Abstract
The green method of nanoparticle synthesis, which is an eco-friendly way to synthesize nanoparticles was employed to synthesis zinc oxide nanoparticles using mustard seed extracts as reducing agents, and it was tested against the red rot pathogen *Colletotrichum falcatum* and biocontrol agent *Trichoderma harzianum.* Mustard seed-based ZnNPs (MS-ZnNPs) at five different concentrations (1, 2, 5, 10 and 15 mM) and precursor zinc nitrate at 15 mM were tested against *C. falcatum* and *T. harzianum* by dual culture assay. MS-ZnNPs showed maximum mycelial growth inhibition of *C. falcatum*at at 1 mM (76.1%), followed by 2 mM (67.1%) concentration. Whereas *T. harzianum* was not inhibited by synthesized Zinc nanoparticles at all tested concentrations.

Keywords: Mustard, Zn nanoparticles, *Colletotrichum falcatum*, inhibition

Introduction
Sugarcane is one of the most important and popular cash crops grown in India between the latitude 36.7° north and 31.0° south of the equator extending from tropical to subtropical zones. It is mostly vulnerable to the fungal disease "red rot," which is caused by *Colletotrichum falcatum*, also known as the cancer of sugarcane crops, and can wipe out entire standing crops (Nayyar *et al* 2017; Sharma and Tamta (2015) [1, 2]. The management of red rot has been a challenging task for pathologists and sugarcane breeders. Inspite of effective screening for disease resistance and deploying resistant varieties, the cultivars succumb to disease in the field due to the development of newer races, which results in breakdown of red rot resistance (Viswanathan, 2021) [3]. Hence, management practices are recommended to sustain the released elite cultivars by using fungicides (Malathi *et al*., 2004 and 2017) [4, 5] biocontrol agents (Viswanathan and Samiyappan, 2008; Elamathi *et al*., 2016) [6, 7] and their combination (Malathi *et al*., 2004) [4]. Recently the biologically based techniques for management of plant diseases are emerging as a competitive alternative to physical and chemical methods due to their simplicity, low cost, and environmental safety (Khatami *et al* 2015) [8]. The green synthesis of nanoparticles is the novel technology for the management of plant diseases (Tabrez *et al*. 2016) [9]. Many researchers suggested using biologically reducing agents like bacteria, fungi and plants, which are natural, inexpensive, and environmentally friendly, for manufacturing nanoparticles (NPs) for diverse nanotechnological applications to avoid the use of hazardous and toxic solvents (Soltani-Nejad *et al* 2016; Medda *et al*. 2015) [10, 11]. Among all biological sources, plants are the most preferred one to synthesize NPs as they are easily available, and this leads to large-scale synthesis and stable production with varied shape and size of NPs (Qu *et al*. 2011a) [12]. Extensive methodologies for the green synthesis of ZnNPs from natural sources such as plant parts, algae, and microorganisms have been developed. Plants are a rich source of numerous bioactive compounds such as polysaccharides, polyphenolic compounds, vitamins, amino acids, flavonoids, tannins, terpenoids, and alkaloids that can be used to synthesize ZnNPs in a cost-effective and environmentally friendly manner. These secondary metabolites, in particular, act as both reducing agents (i.e., reducing metal ions or metal oxides to zero-valent metal NPs) and capping or stabilization agents (Qu *et al*. 2011b) [13]. Of all inorganic metal oxides like Cuo, TiO_2 and ZnO, nanoparticles synthesized from ZnO attracted much attention as they are cost effective in production, easy to prepare and safe (Jayaseelan *et al*. 2013) [14].

The mustard plant (*Brassica indica*) has long been used as a biofumigant (Abdallah *et al*. 2020) [15] in the management of soil-borne plant pathogens as the seeds of mustard contain crucial amounts of polyphenolic and phenolic compounds (Chan *et al*. 2014) [16]. In our earlier study on effect of mustard seed bio-fumigation against *C.falcatum* gave cent percent mycelial inhibition at 0.5g/plate concentration under *in vitro* study(unpublished) (Fig. 1). If we synthesize nanoparticles using this mustard seed, we can reduce the amount of seed utilization (raw material), and on a large scale, it will be cost effective. With this in mind, we designed a study to synthesise zinc oxide nanoparticles using mustard seed extracts as reducing agents and test their efficacy against the red rot pathogen *C. falcatum* and its effective biocontrol agent *Trichoderma harzianum.*

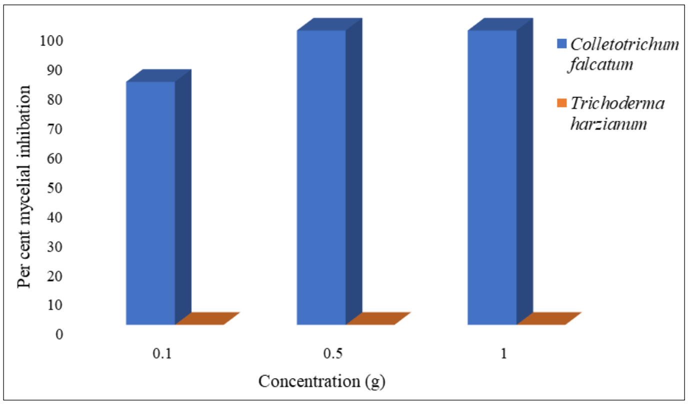

Fig 1: Effect of mustard volatiles on *C. falcatum* and *T. harzianum*

Materials and Methods

Preparation of mustard seed extract

Brown mustard seeds were purchased from the market and washed with tap water and then in distilled water. They were air-dried to remove the water and then the seeds were ground in a food processor for about 2 minutes. The ground sample was mixed with sterile distilled water (1:2 w/v), and subjected for centrifugation for 10 minutes at 10,000 rpm. The supernatant was taken and filtered through Whatman No. 40 filter paper. The final extract was used for the synthesis of zinc-based nanoparticles.

Preparation of precursor solution

Zinc nitrate hexahydrate [Zn (NO3)2.6H2O] (Nice Chemicals Pvt. Ltd.) was used as a precursor to synthesize ZnO nanoparticles from *Brassica indica* in the present study. A 100 mM zinc nitrate solution was prepared using sterile, double-distilled water and stored at 4 °C for further use. From a 100 mM stock solution, 1mM, 2mM, 5 mM, 10 mM, and 15 mM zinc nitrate solutions were prepared.

Synthesis and characterization of mustard seed based Zinc nanoparticles (MS-ZnNPs)

The ZnO nanoparticles from mustard seed extracts were prepared by the method described by Shekhawat *et al*. (2014) [22] with slight modifications. To 20 ml of each millimolar concentration of zinc nitrate, 20 ml of extract was added (1:1) in a sterile bottle and kept under magnetic stirring. After 10 minutes, 200 µl of 0.2 M NaOH solution were added to promote the synthesis of nanoparticles. Then the reaction medium was subjected to microwave oven heating at 800 watts for 60 seconds. The yellow-to-cream-white precipitate formation of the reaction mixtures indicated the formation of zinc nanoparticles. For homogenization, the medium was kept under magnetic stirring at 500 rpm for 4 hours. The synthesized mustard seed-based zinc nanoparticles (MS-ZnNPs) were confirmed and characterized by UV-Visible spectrophotometer (wave length scan between 200nm and 700 nm).

Evaluation of MS-ZnNPs against *C. falcatum* and *T. harzianum*

The poison food technique was followed to test the efficacy of the above-mentioned nanoparticles against red rot pathogen *C. falcatum* and a biocontrol agent *T. harzianum*. Twenty ml of OMA medium initially mixed with MS-ZnNPs at different concentrations *viz*., 1, 2, 5, 10 and 15 mM were poured into Petri dishes. OMA plates with 15 mM precursor zinc oxide and plates without any treatment were maintained as controls. After solidification, 5 mm discs of the above-mentioned fungi were placed separately at the centre of the plate. Each experiment was repeated three times, and the plates were incubated at 25± 2 °C. Percent inhibition of mycelial growth was calculated using the formula given by Vincent (1947) [17].

Results and Discussion

The key indicator of nanoparticle synthesis is a change in reaction mixer color. The colour of MS-ZnNPs was changed from yellow to creamish colour (Fig.2). The synthesized NPs were further characterized using a UV-visible spectrophotometer at a wave length between 200 nm and 700 nm. Intensive ultraviolet absorption was observed at 320–350 nm (Fig.3). The falling of the absorption peak at 350 nm reveals the confirmation of zinc oxide nanoparticles from the mustard seed extract reaction mixture.

Fig 2: The colour change in mustard seed extract upon ZnONPs synthesis

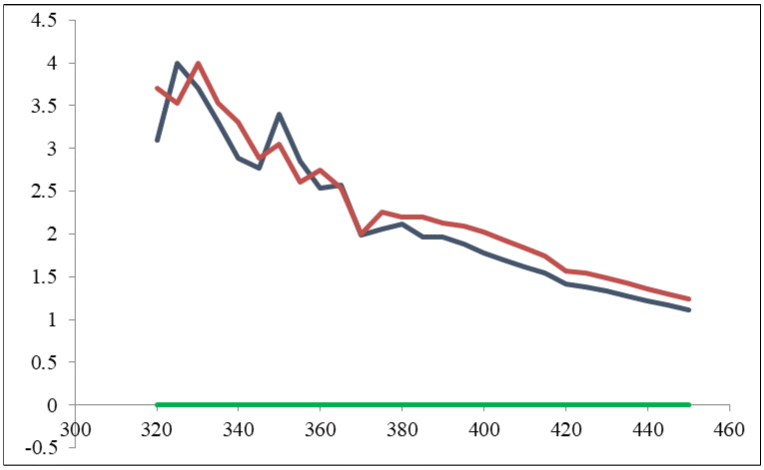

Fig 3: UV-Spectrophotometric absorbance peak of ZnONPs (Blue line indicates chemically synthesized ZnONP, Red line indicates MS-ZnONPs and green line indicates Blank)

The observations regarding the percent inhibition of mycelial growth by mustard seed-based ZnNPs (MS-ZnNPs) at five different concentrations and precursor zinc nitrate at 15 mM are presented in Table 1. A review of the data reveals that MS-ZnNPs significantly inhibited mycelial growth (76.1%) of *C. falcatum*at a 1 mM concentration. Whereas *T. harzianum* was not inhibited by synthesized Zinc nanoparticles. The precursor zinc nitrate (15 mM) showed 9.4% mycelial inhibition of *C. falcatum*at. The results clearly showed that, the percent inhibition of mycelial growth decreases as the concentration of zinc nitrate in nanoparticles increases. The results are in agreement with Al-Dhabaan *et al.* (2017) [18] they also noticed the antifungal activity of zinc nanoparticles against *Rhizoctonia solani*, *Alternaria alternata* and *Botrytis cinerea.* Similar results have been reported against *F. oxysporum* by Wani and Shah (2012) [19], Yehia and Ahmed (2013) [20] and Narendhran and Sivaraj (2016) [21]. The silver nanoparticles were synthesized from the mustard seeds by Khatami *et al.* (2015) [8], but to our knowledge, there are no anti-fungal or anti-microbial studies of mustard seed extract-based nanoparticles in agriculture. And for the management of the red rot pathogen with green synthesized NPs, it is a preliminary study and able to get encouraging results. Hence further studies have been initiated to study the effect of mustard cultivation in combination with soil application of biocontrol agents and application of MS-ZnNPs by different delivery methods on red rot incidence.

Table 1: Effect of MS-ZnNPs against *Colletotrichum falcatum* and *Trichoderma harzianum*

MS-ZnNPs** (mM)	Percent inhibition of mycelial growth	
	C. falcatum	*T. harzianum*
1	76.1 (60.7)*	0.0
2	67.1 (55.0)	0.0
5	35.66 (36.7)	0.0
10	9.30 (17.7)	0.0
15	8.14 (16.7)	0.0
Zinc nitrate@ 15	9.44 (17.9)	0.0
S.Em. ±	0.5	-
C.D. @5%	1.54	-

*Arcsine transformed value, **concentration of MS-ZnNPs

Acknowledgements

Author would like to thank ICAR-Sugarcane Breeding Institute, for the support provided to carry out present work.

Declarations

Conflict of Interest

All contributing authors declare no conflicts of interest.

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