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Effect of Cu-chitosan Composite Based Seed Coating on Seed Quality Enhancement and Antifungal Activity

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Abstract

The experiment was conducted during September to November, 2023 at ICAR-IIOR, Rajendranagar, Hyderabad, Telangana (500030), India to develop and evaluate the Cu-chitosan based composite as seed treatment material and its antifungal activity against soil borne pathogen, *Sclerotium rolfsii* Sacc. and compatibility of Cu-chitosan composite with biocontrol agent *Trichoderma*, in order to develop a sustainable approach to enhance seedling growth in groundnut. Chitosan, a natural biopolymer has a significant attention in agriculture due to its versatile properties. Seed coating with chitosan improves germination rate and seedling quality by providing conducive microenvironment and protection against soil borne pathogens. The Cu-chitosan composite solution prepared with 4% chitosan and cross-linker C, showed better film forming ability with stickiness and complete solubility in the water compared to other combinations. Coating of groundnut seeds with Cu-chitosan composite prepared with 4% chitosan resulted in the highest germination percentage and seedling vigour of the seedlings compared to the control. *In vitro* studies revealed that Chitosan based composite (Cu-chitosan composite C) exhibited 100% compatibility with the biocontrol agent, *Trichoderma harzianum* Th4d. Further, it also inhibited the mycelial growth of *S. rolfsii* to the extent of 44.81%. These findings indicate that Cu-chitosan composite can be potentially utilized in combination with the biocontrol agent, *Trichoderma harzianum* Th4d to provide enhanced protection to the seeds against soil borne pathogens and it also improves the seedling growth and development.

Keywords: Antifungal activity, biocompatibility, cu-chitosan composite, seedling vigour

1. Introduction

Modern agriculture primarily focuses on producing high-quality and quantity of the food to meet global demand while restraining environmental impact. Hence, development of sustainable strategies to protect the plants from pests and diseases using biodegradable material is essential. In this regard biopolymers serve the purpose as they are derived from renewable resources like plants, animals and microorganisms (Baranwal et al., 2022). Nowadays, biopolymer-based composites are getting a great deal of attention for various applications in agriculture due to its exceptional benefits like biocompatibility, biodegradability, eco-friendly nature and anti-microbial properties (Tang et al., 2018). These are originated from plants or any other biological sources. Biopolymers are sticky and viscous in nature which is suitable for seed treatment purposes. Among different polymers, chitosan is the most abundantly available natural bio-polymer which is derived fully or partially from the deacetylated chitin. It consists of alternating units of 1–4 linked n-acety glucosamine units, which is an inelastic and nitrogenous polysaccharide (Badawy and Rabea, 2011). It has greater attention in the field of nanocomposites because of its bio-degradability, non-toxicity and antimicrobial properties (Kean and Thanou, 2010; Kumar et al., 2004; Tang et al., 2018). Chitosan is reported to exhibit the inhibition activity on different stages of the pathogen and the production of fungal virulence factors (Xu et al., 2007; Rubina et al., 2017) different stages of pathogen Copper has a high affinity towards chitosan compared to other metal oxides. Composites, nanomaterials and polymer blends synthesized with the combination of Cu-chitosan are being used in various fields (Brunel et al., 2013; Saharan et al., 2013; Saharan et al., 2015). In the agriculture sector, chitosan can be used as growth enhancers and potential biocide agent against the pathogenic fungi and bacteria (Kong et al., 2010; Sahab et al., 2015).

A most recent approach of using chitosan is as seed treatment by maintaining the integrity of seed coat and simultaneously reducing the water solubility (Pirzada et al., 2020). Chitosan alone or integration with any other active ingredient demonstrated promising effect against various soil borne pathogens (Hadrami et al., 2010; Maluin and Hussein, 2020). Cu-chitosan acts as a stabilizing agent and coats uniformly around the seeds thereby regulating the water uptake and protecting the seeds from high humidity and other pathogenic microorganisms. Seed treatment with chitosan was reported to increase the seed germination index, and seedling vigour and also act as an antimicrobial agent (Ahuja et al., 2012; Saharan et al., 2015; Chandrika et al., 2019). In addition, it has nitrogen content of around 9–10% which serves as a macronutrient for the plant (Agarwal et al., 2015). Seed borne diseases *viz*., stem rot, seed rot and collar rot are potential threats to groundnut cultivation and the germination of groundnut seeds greatly depends on the intact seed coat. Slight damage to the seed coat results in less-no germination and can lead to seed rot. Coating the seeds with biopolymers creates a barrier around the seed which can protect the seed from external damage and soil borne pathogens. Chitosan supplemented formulations proved to be effective against several phytopathogens while enhancing the plant growth significantly (Manjula and Podile, 2005).

In this regard, the present study aims to develop a seed coating agent by using natural biopolymer and different copper salt based cross-linker which has uniform film forming ability. Standardization was carried out by considering different physical parameters like stickiness, film forming ability, yield efficiency and solubility. The effect of Cu-chitosan composite on seed germination and seedling vigour of groundnut was evaluated. Further, its compatibility with *Trichoderma* and antifungal activity against soil-borne pathogens was evaluated under *in vitro* conditions.

2. Materials and Methods

The present study was conducted during September to November, 2023 at ICAR-Indian Institute of Oilseeds Research, Rajendranagar, Hyderabad, Telangana (500 030), India.

2.1. Materials used

Chitosan, 38–200 kDa with >=75% degree of deacetylation, (from shrimp cells, Hi-Media, PCT0817); Cross-linkers [two organic acid based cross-linkers; Span 80 (RM10823, Hi-Media); Copper salt]; Acetic acid (AS119, EMPARTA®); Potato dextrose media (PDA) from Hi-Media. The organisms that are used for antifungal activity and biocompatibility tests *viz., Sclerotium rolfsii* Sacc. and *Trichoderma harzianum* Th4d, respectively were collected from the Plant Pathology section of ICAR-IIOR, Rajendranagar, Hyderabad–500 030.

2.2. Preparation of Cu-chitosan polymer composite

Chitosan solution of different concentrations was prepared using a 1% acetic acid solution. To synthesize Cu-chitosan composite copper salt was added after complete dissolution of chitosan. Cross-linkers (A, B, C and D) were added in separate batches to the above solution and homogenized by maintaining the under-reaction conditions as per the ICAR-IIOR standardized protocol. The resulting solution was centrifuged to collect the precipitate of the Cu-chitosan composite. Precipitate was used as such for further studies.

2.3. Solubility test and film forming ability

Solubility of the synthesized polymer composite was tested at the concentration of 0.1 mg 10 ml^{-1} and the solubility rating was given as described in earlier studies (Dixit and Puthli, 2009; Anonymous, 2010). Fim forming ability was evaluated by using solvent casting method and the rating has been given as described in the earlier studies.

2.4. Effect of Cu-chitosan composite on seed germination and seedling vigour

Groundnut seed were surface sterilized with 2% sodium hypochlorite solution and subsequently washed in distilled water three times and dried at room temperature under shade. Surface sterilized seeds were coated with a solution of Cu-chitosan composite by tumbling method. Coated seeds were placed on autoclaved germination paper towels (10 seeds per paper towel) and rolled tightly without disturbing the seed placing and moistened with water. Germination was checked after 7 days of incubation in a growth chamber at 25±2°C. Data on seedling vigour was recorded after 15 days. The germination percentage, vigour index and disease incidence were calculated by using the formulae mentioned below;

Germination Percentage=(Number of germinated seeds)/(Total number of seeds)×100

Vigour Index-I=Per cent germination×Seedling length

[Seedling length (cm)=Shoot length+Root length]

Vigour Index-II=Per cent germination×Seedling dry weight (g)

2.5. Compatibility test with Trichoderma harzianum Th4d

The compatibility of Cu-chitosan composite was tested with *T. harzianum* by following the poison food technique. The cu-chitosan composite solution was mixed with potato dextrose media (1 ml 15 ml^{-1} of media) at different concentrations. The mycelial disc of *T. harzianum* was placed on the center of the Petri plate containing solidified potato dextrose media mixed with Cu-chitosan composite. Each treatment is replicated thrice. Mycelial growth was measured after 4 and 7 days of incubation and percent compatibility was calculated by using the formula given by Vincent, 1947.

$I=(C-T)/C \times 100$

Where, I= Per cent inhibition, C=Mycelial growth in diameter (mm) in control, T=Mycelial growth in diameter (mm) in treatment

2.6. In vitro antifungal activity test

The antifungal activity of Cu-chitosan composite was tested against *S. rolfsii* under *in vitro* conditions using the poison food technique. The antifungal activity of reactants used for composite preparation were also tested. Mycelial disc (5 mm) of *S. rolfsii* was taken from 7 days old culture and inoculated separately at the center of the petri dish containing solidified PDA media mixed with Cu-chitosan composite and reactants. Inoculated plates were incubated at 25°C. For each treatment five replications were maintained. Per cent mycelial growth inhibition was calculated using the formula given by Vincent, 1947 (Section.2.5)

2.7. Statistical analysis

In vitro antifungal activity experimental results were represented as mean±standard error. The statistical data was analyzed by one-way ANOVA using SPSS software (version 22, IBM Corporation, USA).

3. Results and Discussion

3.1. Preparation of Cu-chitosan composite

Cu-chitosan composite was prepared by mixing copper salt solution in to chitosan solution and after homogenization precipitate was collected through centrifugation at high speed. Stickiness and film forming ability increased with the increase in the chitosan concentration (Table 1). Highest yield efficiency was observed with 4 % chitosan with the addition of cross-linker B, but it showed no solubility in the water. Cu-chitosan composite with 4% concentration added with cross-linker C showed 97% yield efficiency with 90% solubility. Cu-chitosan composite synthesized with 3% and 4% chitosan concentration with A, C and D cross-linkers showed better yield efficiency along with solubility (50–90%) (Table 1, Figure 1). Stickiness and film forming ability are the important factors to create uniform barrier around the seeds when coated. Cu-chitosan composite synthesized with 3% and 4% chitosan concentration (with A, C and D cross-linker) has formed a uniform coating on groundnut seeds. Uniform film coating on seeds is very important to protect the seeds from various biotic and abiotic stresses which helps the absorption of water at an optimized rate and protects the seeds from high relative humidity (Chandrika et al., 2019; Chandrika et al., 2017).

Table 1: Standardization of polymer concentration based on different physical parameters

Concentration of polymer	Cross-linker	Stickiness of the solution	Film forming ability	Yield efficiency (%)	Water solubility
1%	A	-	-	59.4	-
	B	-	-	86.62	-
	C	-	-	53.85	-
	D	-	-	60.69	-
2%	A	++	+	80.00	-
	B	+	-	93.33	-
	C	+	+	73.33	-
	D	+	-	80.00	-
3%	A	+++	+	88.23	++
	B	++	+	88.23	-
	C	++	+	76.47	+++
	D	++	+	82.35	++
4%	A	+++	+	85.00	++
	B	+++	+	100.00	-
	C	+++	+	97.00	+++
	D	+++	+	95.00	++

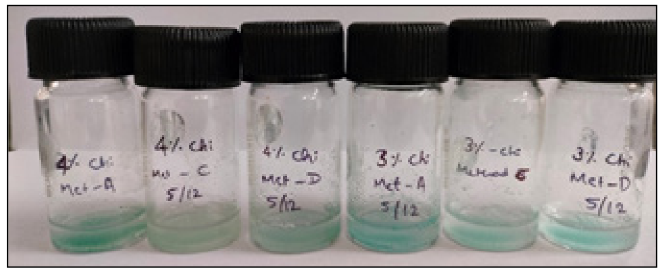

Figure 1: Solubility of Cu-chitosan composite in water

Scoring for film forming ability: No film formation (-); Film formation (+). Scoring for solubility: All coacervates were given scoring for solubility in the form ++++ scale i.e., no solubility (-); 25–40% solubility (+); 40–60% solubility (++); 65–90% solubility (+++); 90–100% solubility (++++) as per earlier studies (Dixit and Puthli, 2009; Anonymous, 2010). Synthesis parameters: Ratio of polymer A to polymer B is 1:1 for B, C, D and 1:1/2 for A; Crosslinker; V_{sol} 50 to 76 ml g^{-1} of feed used depending on the concentration of reactants; Homogenization was done at 700 rpm and 70°C for 10 min.

3.2. Effect of Cu-chitosan composite on seed germination

Cu-chitosan composite D with 4% chitosan showed the highest germination percentage (100%) followed by Cu-chitosan composite D with 3% chitosan (97%) compared to other treatments (Table 2, Figure 2). Cu-chitosan coated seeds

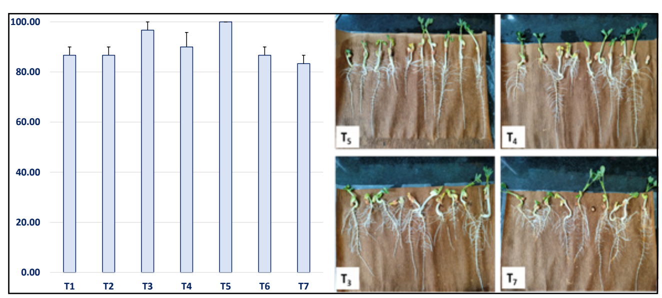

Figure 2: Effect of Cu-chitosan composite as seed treatment on germination of groundnut seeds. Treatment details: T_1: Cu-chitosan composite A (3% chitosan), T_2: Cu-chitosan composite C (3% chitosan), T_3: Cu-chitosan composite D (3% chitosan), T_4: Cu-chitosan composite A (4% chitosan), T_5: Cu-chitosan composite C (4% chitosan), T_6: Cu-chitosan composite D (4% chitosan), T_7: Control

Treatments	Percent germination	Shoot length (cm)	Root length (cm)	Fresh weight (g)	Dry weight (g)	Vigour index I	Vigour index II
T_1	87^{bc}	7.5^{c}	24.2^{a}	3.2^{c}	0.42^{ab}	2750.3^{c}	36.08^{bc}
T_2	87^{bc}	8.4^{b}	23.5^{ab}	3.2^{c}	0.39^{b}	2766.7^{c}	33.73^{c}
T_3	97^{ab}	9.0^{b}	24.3^{a}	3.8^{ab}	0.43^{a}	3223.3^{b}	41.86^{ab}
T_4	90^{abc}	8.5^{b}	21.6^{b}	3.4^{bc}	0.41^{ab}	2694.0^{c}	36.87^{bc}
T_5	100^{a}	10.4^{a}	24.9^{a}	4.0^{a}	0.45^{a}	3536.7^{a}	44.73^{a}
T_6	87^{bc}	8.6^{b}	22.0^{b}	3.7^{ab}	0.43^{ab}	2653.7^{c}	37.13^{bc}
T_7	83^{c}	7.0^{c}	18.8^{c}	2.1^{d}	0.23^{c}	2146.7^{d}	18.83^{d}
SEm±	3.34	0.27	0.72	0.15	0.016	92.51	2.29
C.D (p≤0.05)	10.81	0.798	2.22	0.362	0.044	296.02	7.09
CV	6.85	5.35	5.57	6.22	6.41	5.98	11.37

Table 2: Effect of Cu-chitosan composite seed treatment on seedling growth parameter and vigour index

Mean values in a column followed by same letters indicates no significant difference between the treatment according to DMRT, p≤0.05

showed healthy seedling growth compared to control (Figure 2). Seed treatment with Cu-chitosan composite D prepared with 4% chitosan showed highest shoot length, root length, vigour index-I and vigour index-II compared to all other treatments and was significantly improved the seedling vigour over control (Table 2). Similar results are reported by earlier researchers that the application of chitosan positively impacts the seed germination index and seedling vigour (Chandrika et al., 2019; Malarkodi et al., 2024; Behboudi et al., 2018; Vijaykumar et al., 2024). Cu-chitosan treatment enhances the mobilization of stored food thereby positive influencing seed germination and growth (Saharan et al., 2015).

3.3. Compatibility of Cu-chitosan composite with Trichoderma harzianum Th4d

Initially, up to 4 days of inoculation slow growth of *Trichoderma* was observed in the treatments compared to the control and there was a significant difference between the treatments, whereas at 7 days after inoculation, there was no significant difference between the treatments and all tested Cu-chitosan composite combinations showed 100% compatibility with *T. harzianum* Th4d irrespective of chitosan concentration and type of cross-linker at 7 days (Table 3, Figure 3). Chitosan is having good biocompatibility with biocontrol agents which provides a congenial environment for the growth of *Trichoderma* (Chandrika et al., 2019). Other researchers reported that the tolerance of *Trichoderma* for chitosan is associated with low membrane fluidity of chitosan (Chittenden and Singh, 2009; González et al., 2016). *Trichoderma* is also reported to have tolerance with Cu (Ladi et al., 2020 and Yap et al., 2011). *Trichoderma* is a well-known biocontrol agent that has great potential to control a wide range of fungal pathogens and it is found to be fully compatible with Cu-chitosan composite, it can be blended together to improve the seedling growth and protect the seeds from soil borne and seed borne pathogens.

Table 3: Compatibility of *Trichoderma harzianum* Th4d with Cu-Chitosan composite

Treatments	Mycelial growth (%)	
	4 DAI	7 DAI
T_1: Cu-chitosan A (3% chitosan)	61.76	100
T_2: Cu-chitosan C (3% chitosan)	64.71	100
T_3: Cu-chitosan D (3% chitosan)	67.65	100
T_4: Cu-chitosan A (4% chitosan)	61.76	100
T_5: Cu-chitosan C (4% chitosan)	67.65	100
T_6: Cu-chitosan D (4% chitosan)	79.41	100
T_7: Control	91.18	100
SEm±	0.89	-
CD (p≤0.05)	2.49	NS
C.V	2.02	-

*DAI-Days after incoulation, values represented here are mean of three replications

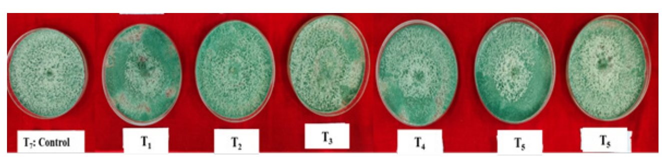

Figure 3: Growth of *Trichoderma harzianum* on the PDA media supplemented with Cu-Chitosan composite

3.4. In vitro antifungal activity of Cu-chitosan composites

Cu-chitosan combination which showed the highest germination percentage and 100% compatibility with *Trichoderma* was further evaluated for its antifungal activity. Cu-chitosan composite C with 4% chitosan showed the highest mycelial growth inhibition of 44.81% followed by Cu-chitosan A with 4% and Cu-chitosan D with 3% chitosan (Table 4, Figure

4). Difference in antifungal activity is may be due to difference in the chitosan concentration. Previous studies also reported the inhibitory effect of chitosan against fungal pathogens. It inhibits the growth of mycelium by interfering with the cell wall of fungi and loss of cytoplasm (Tsai et al., 2002; Ashra et al., 2019; Tang et al., 2018). Similar observations were reported by Rubina et al. (2017) in which they have reported that Cu-chitosan nanocomposite resulted in the loss of cytoplasm, coagulation of cytoplasm and destruction of hyphae.

Table 3: Antifungal activity of Cu-chitosan polymer against *S. rolfsii*

Treatment	Percent mycelial inhibition
Cu-chitosan D (3%)	34.44±0.64
Cu-chitosan A (4%)	38.89±0.64
Cu-chitosan C (4%)	44.81±0.98

*Mean of five replications ±SEm value

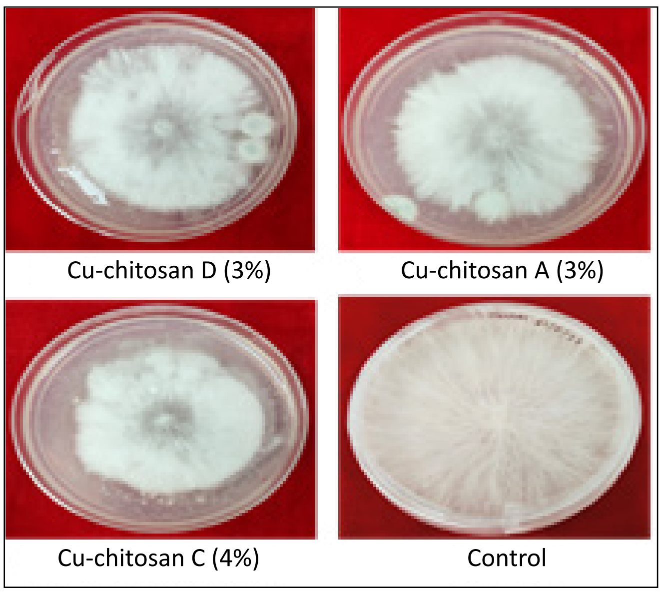

Figure 4: Antifungal activity of Cu-chitosan polymer against *S. rolfsii*

4. Conclusion

Cu-chitosan composite synthesized with 4% chitosan and cross-linker C exhibited ≥90% solubility, high yield efficiency (97%), stickiness, uniform film forming ability, higher seed germination index and seedling vigour. It also showed 100% compatibility with *T. harzianum*, and inhibited the mycelial growth of *S. rolfsii* to the extent of 44%. Results indicate that Cu-chitosan composite can be effectively used for seed treatment along with biocontrol agents to enhance seedling growth and protection of the seeds against soil borne pathogens.

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