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Antifungal effects of *Kurthia gibsonii* Mb 126 chitinase as a seed treatment on seed-borne fungi of rice seed on germination percentage and seedling vigor

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ABSTRACT

Soil and seed-borne phytopathogenic fungi are the main factors limiting crop yield in India's agricultural sector. They attack the root of the seed before germination or seedling after germination resulting in huge deprivation in crop yield. In this scenario, it is crucial to control phytopathogenic fungi to ensure sustainable food production to the ever-increasing world population. The antifungal property of purified chitinase of *Kurthia gibsonii* Mb 126 was investigated by isolating fungi infected with seeds of various rice samples and then studying the effect of purified chitinase of *K. gibsonii* Mb 126 on these isolated fungi. The effect of *K. gibsonii* Mb 126 purified chitinase on the germination of rice seed infested with these isolated fungi was also investigated. Eight fungi (*Aspergillus niger, Aspergillus flavus, Curvularia lunata, Fusarium moniliforme, Rhizopus oryzae, Trichoderma harzianum, Rhizoctonia solani*, and *Fusarium subglutinans*) were isolated and identified from the different rice varieties of Kerala, India, viz Aswathy (PTB 37), Jaya, Sabari (PTB 40), Ahalya, Onam, Makam, Triveni (PTB 38), Swarnaprabha, Kairali, Pavizham, and Ponni, The frequency of isolated fungi ranged from 46% to 100% (present in all the 20 samples). The isolated fungi *C. lunata, A. flavus, R. solani*, and *A. niger* were predominated. Seeds treated with the *K. gibsonii* Mb 126 chitinase enzyme had a strong germination response. *K. gibsonii* Mb 126 chitinase proved beneficial in eliminating seed-borne fungus, boosting seed germination percentage, and seedling vigor. Farmers of our country should be aware of soil and seed-borne fungi, and they should do seed treatment with chitinase before sowing in the agricultural field, and through this strategy, they will be more benefited.

1. INTRODUCTION

Rice (*Oryza sativa L.*) is the primary food source for India, China, Nepal, Pakistan, Bangladesh, and Malaysia [1]. 60% of people in the world use rice as the staple food. Demand for rice is increasing day by day worldwide, but the yield of rice was reducing due to fungal diseases of rice. Seed-borne fungal diseases cause approximately 10% yield loss of rice every year [2]. There is no adequate information on the seed-borne fungi and their adverse effects on rice seed germination. Phytopathogenic fungi are devastating because they are very hard to control as the hyphae of the fungi get established. Seed-borne fungal decreases seedling vigor, and wane out the plant in its initial seedling stages. If a seed that was already infected or contaminated by a pathogen is sown in non-infested soil, the pathogen may also be spread to that soil. Seed-borne pathogens and fungi grown on stored seeds may reduce seed germination, discoloration of seed, reduce seed weight, and produce toxic components that may be harmful to humans and animals [3]. Chemical fungicides and pesticides are extensively used to protect seeds against diseases. However, their utilization is of concern recently since the chemical fungicides and pesticides are highly toxic. They can cause environmental contamination, and/or the presence of fungicide/pesticides residues in food products induce pathogen resistance.

Seeds carry most of the infectious diseases of rice. The seed treatment process reduces the number of phytopathogens associated with seeds. This strategy can be used as a curative and preventive method against soil and seed-borne pathogens. Chitinase enzymes have been reported in the biocontrol of soil-borne pathogens because they degrade fungal cell walls whose major component is chitin. Chitinase (EC 3.2.1.14) are glycosyl hydrolases that can hydrolyze β-1,4-glycoside bond present in chitin, the highly insoluble homopolymer of N-acetyl glucosamine [4]. Chitinase-producing microorganisms can act as biocontrol agents for different kinds of fungal diseases of plants [5-8]. Biological control using chitinolytic microorganisms offers an alternative better strategy for bio-controlling phytopathogens. Hence, the objective of this study was to analyze the potential of chitinase of *Kurthia gibsonii* Mb

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126 against fungi that inhibit rice seed germination. No chitinolytic microorganism has yet been reported for use in biocontrol of fungi which inhibit rice seed germination. However, a few reports of studies on the germination of seeds of other crops.

2. MATERIALS AND METHODS

Different rice varieties of Kerala, India, viz Aswathy (PTB 37), Jaya, Sabari (PTB 40), Ahalya, Onam, Makam, Triveni (PTB 38), Swarnaprabha, Kairali, Pavizham, and Ponni were collected from different locations of Kerala, India. The chitinous waste was collected and powdered. Analytical grade reagents were used.

2.1. Microorganism and Chitinase Production

Chitinase enzyme was extracted and purified from *K. gibsonii* Mb126, one of the best chitinase producing bacteria isolated previously from coastal regions of "Cochin, Kerala, India". *K. gibsonii* Mb126 was cultured under solid substrate fermentation method where dried, milled chitinous waste was the solid substrate. Fermentation was carried out with 75% moisture content, 40°C temperature, 4–5 × 10^9 CFU/mL inoculum, and pH was 8. After 72 h, the chitinase enzyme was extracted and purified from the culture supernatant by four-step reaction process at 4°C such as ammonium sulfate precipitation, affinity adsorption, ion-exchange chromatography, and gel filtration chromatography.

2.2. Assay of Chitinase

N-acetyl Glucosamine, the product of chitinase activity, was estimated by dinitrosalicylic acid method [9]. One unit of chitinase activity can be calculated as the amount of chitinase enzyme that liberates one micromole of N-acetyl Glucosamine per mL per minute under standard assay conditions.

2.3. Effect of Chitinase on Phytopathogenic Fungi

2.3.1. Isolation of fungi from rice

Rice seed samples were collected from different locations. One gram of rice from each sample was taken inoculated into potato dextrose agar medium and incubated at 28°C for 8 days. The plates were then examined for fungal growth.

2.3.2. Identification of fungi

The fungi were identified by studying the colony characteristics and microscopic morphology. The microscopic morphology was studied by lactophenol cotton blue staining of needle mount preparations. For identification, the guidelines of Hoog *et al.* were followed [10]. The frequency of different species of fungi in rice seeds was calculated as follows;

$$\text{Frequency }(\%) = \frac{\text{Number of seeds infected with fungal species}}{\text{Total number of seeds}} \times 100$$

2.3.3. Effect of chitinase on phytopathogenic fungi

A loopful of test fungi was inoculated into 5 ml of sabouraud's dextrose broth and incubated at 28°C for 3 days. Then, the mycelium from each was inoculated in the center of freshly prepared PDA plates with different concentrations of chitinase (0.2–1 U/ml). In the control plates, distilled water was used in place of the enzyme solution. The plates were incubated at 28°C for 12 days, and the radial growth was recorded. The inhibition percentage was calculated by following the equation.

Inhibition (%) = [(C-T)/C] × 100.

C – Mycelial diameter in the control plate (mm).
T – Mycelial diameter in the treatment plate (mm).

2.4. Effect of Chitinase on the Rice Seed Germination

2.4.1. Preparation of spore suspension

The fungi were cultured on PDA plates for 7 days at 25°C, fungal mycelia from pure culture were aseptically transferred to sabouraud's dextrose broth, and cultured for 10 days at 25°C. Spores were taken and suspended in sterile water have 0.05% Tween 80. The concentration of spore suspension was kept at 10^4–10^5/ml.

2.4.2. Seed germination test

The germ inability of seed was analyzed according to Nghiep and Gaur [11]. This study used a completely randomized design. Rice Seeds were subjected to surface sterilization with 0.1% Mercuric chloride for 4 min and then washed in sterile water 4 times. After surface sterilization, seeds were put in spore suspension of each fungus for 12 h then transferred to each treatment solution of chitinase containing 0.2, 0.4, 0.6, 0.8, and 1 U/ml, incubated for 12 h. The seeds were then placed on a moistened Whattman filter paper (No. 1) carefully layered in Petri plates. Ten seeds were placed per plate. Seeds were then covered with another layer of moistened Whattman filter paper (No. 1). Two types of controls were included. In control (a), seeds were soaked with distilled water for 24 h and were neither spore nor chitinase treated. In control (b), the seeds were treated with spore suspension only. The plates were then wrapped with wax paper and kept in a germinator set at 25°C. Germ inability and seedling vigor index were determined according to the recommended method by ISTA [12]. Each treatment was examined for seedlings. The number of seeds germinated was taken starting from the 3rd day till the 7th day, and the formula can calculate the germination percentage;

$$\text{\% germination} = \frac{\text{Total number of germinated seeds as on } 7^{\text{th}}\text{day}}{\text{Total number of seeds inoculated}} \times 100$$

The formula can also calculate seedling vigor index;

Percent Germination × Seedling length = Seedling vigor index.

3. RESULTS

3.1. Isolation of Fungi from Rice

The frequency of isolated fungi is plotted in Figure 1**.** Eight different fungi (*Aspergillus niger, Aspergillus flavus, Curvularia lunata, Fusarium moniliforme, Rhizopus oryzae, Trichoderma harzianum, Rhizoctonia solani,* and *Fusarium subglutinans*) were isolated from the 20 samples. The frequency of isolated fungi ranged from 46% to 100% (present in all the 20 samples). The isolated fungi *C. lunata, A. flavus, R. solani*, and *A. niger* were predominated.

3.2. Effect of Chitinase on Phytopathogenic Fungi

The result of mycelium growth inhibition recorded at 12 days after inoculation at 27°C showed that chitinase treatment could inhibit fungal mycelium extension of *A. niger, A. flavus, Fusarium subgultinans*, and *T. harzianum*. The percentage of mycelial inhibitions is shown in Figures 2-5. The photos of mycelial inhibitions are shown in (Figures 6-9).

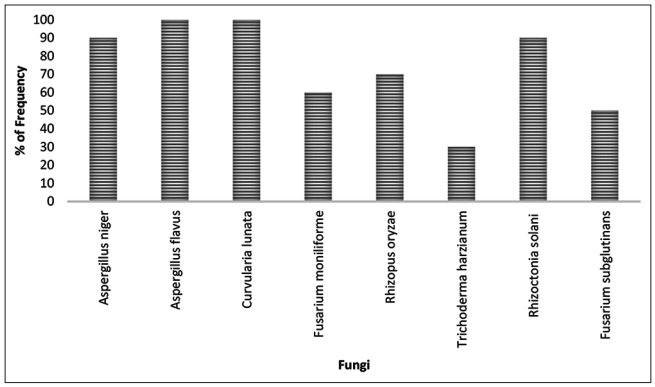

Figure 1: The frequency of isolated fungi.

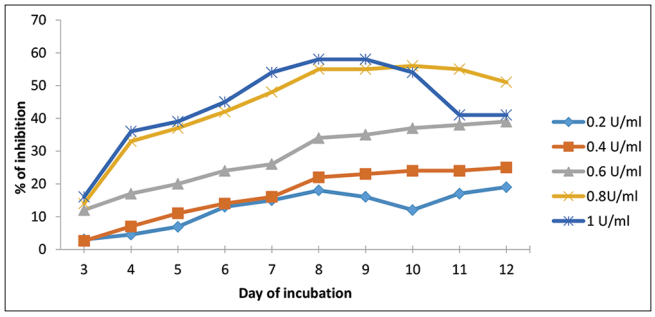

Figure 2: Effect of chitinase on *Aspergillus flavus.*

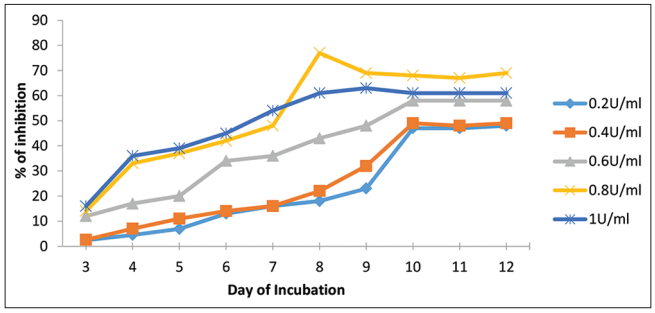

Figure 3: Effect of chitinase on *Trichoderma harzianum.*

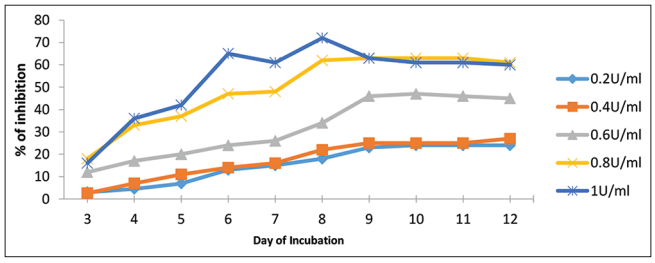

Figure 4: Effect of chitinase on *Aspergillus niger.*

In the case of *A. flavus*, the inhibition percentage was above 50 by applying 0.8, and 1 U/ml chitinase on the 8th day of incubation. After 10th day of incubation, the percentage of inhibition decreases with 1 U/ml chitinase. However, with 0.8 U/ml chitinase, the percentage of inhibition was steady.

By treating 0.8 U/ml chitinase on *T. harzianum*, more than 75% of inhibition was noticed on the 8th day of incubation. Concentration above 0.8U/ml has no considerable effect on the percentage of inhibition**.**

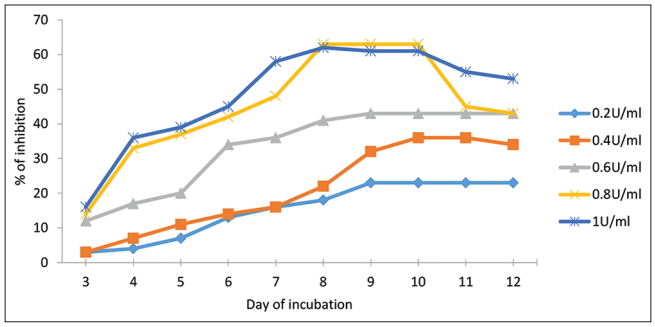

Figure 5: Effect of chitinase on *Fusarium subgultinans.*

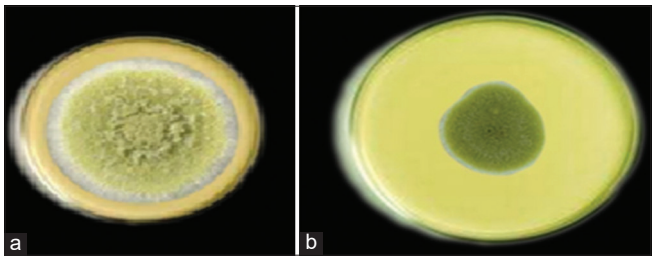

Figure 6: Effect of chitinase on *Aspergillus flavus* (after 8 day inhibition) (a) *Aspergillus flavus* (Control) (b) *Aspergillus flavus*+*Chitinase.*

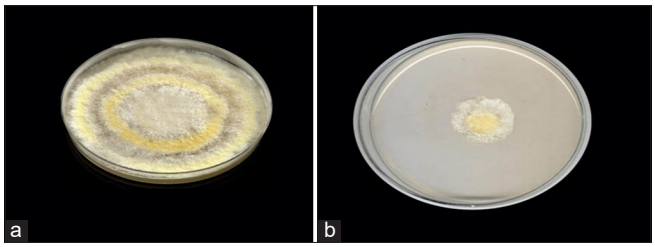

Figure 7: Effect of chitinase on *Trichoderma harzianum* (after 8 day inhibition) (a) *Trichoderma harzianum* (control) (b) *Trichoderma harzianum*+*Chitinase.*

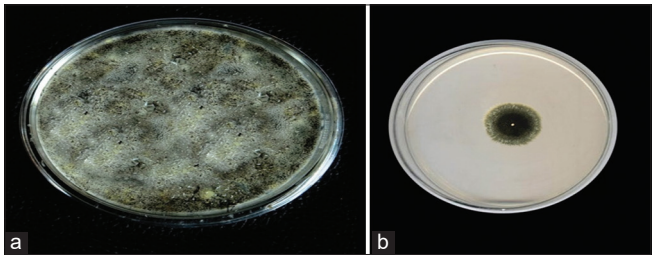

Figure 8: Effect of chitinase on *Aspergillus niger* (after 8 day inhibition) (a) *Aspergillus niger* (control) (b) *Aspergillus niger* +chitinase.

By treating 1 U/ml chitinase on *A. niger*, more than 70% of inhibition was noticed on the 8th day of incubation. In the case of *F. subgultinans*, the percentage of inhibition was above 60 by the treatment of 0.8, and 1 U/ml chitinase on 8th day of incubation. After 10th day of incubation, the percentage of inhibition decreases to 30% with 0.8 U/ml chitinase. But with 1, inhibition slightly decreased.

3.4. Effect of Chitinase on the Germination of Rice Infested by Different Fungi

The effect of chitinase on the percentage of rice seed germination by different fungi is summarized in Table 1, and the seedling vigor

is shown in Table 2. Treatments with spores of *A. flavus, A. niger*, and *F. subgultinans* affected germination percentage, but spores of *Trichoderma* had no inhibitory effect on seed germination. The percentage of seed germination was increased significantly with the treatment of chitinase on *A. flavus* infested seeds**.** In most treatments, there is an increase in the percentage of seed germination with the increase in concentration of chitinase used. On statistical analysis *P*-value in the case of *A. flavus* is found to be <0.0001, indicating the effect is extremely significant. There is no significant effect of chitinase treatments on germination of rice seeds inoculated with *Trichoderma* spores, and the $P = 0.2157$. In the case of *A. niger* none of the seeds in control didn't germinate. Seed germination rate significantly increased up to 0.8 U/ml, and the $P < 0.0001$, considered highly significant. A significant increase in seed germination was noted with 0.8 and 1 U/ml chitinase in the case of *F. subgultinans* infested rice seeds, and the $P < 0.0001$, considered extremely significant. Similar to a percentage of germination treatments with spores of *A. flavus, A. niger*, and *F. subgultinans* significantly affected seedling vigor index but spores of *Trichoderma* had no inhibitory role on seed germination. Seedling vigor index in the case of *A. niger* (control b) was 0. By treating chitinase, seedling vigor index increased significantly in *A. flavus, A. niger,* and *F. subgultinans*, and the $P < 0.0001$, considered extremely significant. The P-value in the case of *T. harzianum* is 0.1001, considered not significant.

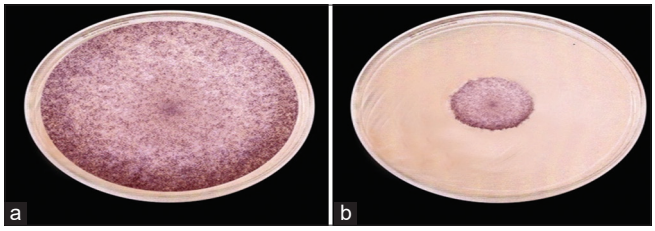

Figure 9: Effect of chitinase on *Fusarium subgultinans* (after 8 day inhibition) (a) *Fusarium subgultinans* (control) (b) *Fusarium subgultinans*+chitinase.1

4. DISCUSSION

In this study, Eight fungi (*A. niger, A. flavus, C. lunata, F. moniliforme, R. oryzae, T. harzianum, R. solani, and F. subglutinans*) were isolated. Mia *et al.* [13] isolated *Bipolaris oryzae, Trichoconis paddwickii, F. moniliforme, Fusarium oxysporum, Fusarium semitectum, Pyricularia grisea, C. lunata,* and *Alternata* from rice seeds. Fungi such as *F. miniliforme, F. semitectum, F. oxysporum, F. solani Alternaria alternata, A. padwickii, A. longissima, A. niger, Curvularia oryzae, C. lunata, Drchslera oryzae, Pyricularia oryzae*, and species of *Phoma, Cercospora, Chaetomium, Sclerotium, Pecicillium, Myrothecium*, and *Colletotrichum* have been isolated from different varieties of rice seeds [14-17].

A. flavus, C. lunata were isolated from all samples collected. This may lead to the deterioration of rice grains. Bad odors and discoloration of grains may also happen. It also leads to a reduction in milling yield. *Aspergillus* sp. can produce large quantities of a carcinogenic toxic product, aflatoxin, in stored grains. *Fusarium* sp., the major soil-borne fungi, create severe economic damage in the agricultural production sector. *Fusarium* sp. cause *Bakanae* disease in rice. *Fusarium* produces significant quantities of gibberellic acid, resulting in hypertrophy and "foolish seedling disease," the plants become etiolated, infertile, chlorotic, and die. *Curuvularia* sp. are known as casual agents of kernel rot, seedling blights, leaf spots, leaf blights, root rot, grain deformation of rice, grain discoloration, and grain lesions. *R. solani* causes sheath blight disease characterized by water-soaked lesions and sclerotia in leaf sheaths [18]. To manage these dreadful fungi, the formulation of fungicide is essential. These fungi are soil and seed-borne; the removal through chemical fungicide is ineffective and expensive. Chitinolytic microorganisms have been suggested for the biocontrol of soil-borne phytopathogens. Formulating bio fungicides may be possible using chitinases having different modes of action from different microorganisms. By keeping these factors, this experiment was carried out to analyze the inhibitory effect of chitinase on seed-borne pathogens. The purified chitinase from *K. gibsonii* Mb 126 could

Table 1: Effect of chitinase on the percentage of rice seed germination infested with various fungi.

Infested Fungi	Percentage of seed germination (mean±S.D)					
	Without chitinase treated (control b)	Treated with chitinase at different concentrations				
		0.2 U/mL	0.4 U/mL	0.6 U/mL	0.8 U/mL	1 U/mL
Aspergillus flavus	13.30+3.333	40±0.000	40±5.774	46.666±3.333	73.333±6.667	76.666±3.333
Trichoderma harzianum	86.666±5.77	73.333±11.547	90±0.000	70±26.458	90±0.000	93.333±11.547
Aspergillus niger	0±0.000	40±0.000	40±0.000	53.333±15.275	60±0.000	53.333±5.774
Fusarium subgultnans	20±0.000	20±10.000	23.333±5.774	33.333±5.774	46.666±5.774	66.666±5.774

Percentage of seed germination in control a (neither spore nor chitinase treated)- 83.33±3.33

Table 2: Effect of chitinase on the seedling vigor index of rice seed infested with various fungi.

Infested Fungi	Seedling vigor index (mean±S.D)					
	Without chitinase treated (control b)	Treated with chitinase at different concentrations				
		0.2 U/ml	0.4 U/ml	0.6 U/ml	0.8 U/ml	1 U/ml
Aspergillus. flavus	14.666±6.351	44±0.000	48±12.000	53.666±6.640	88±13.856	92±6.928
Trichoderma harzianum	104±6.928	88±13.856	108±0.000	87.5±33.072	114.31±0.000	123.2±15.242
Aspergillus niger	0±0.000	44±0.000	48±0.000	64±18.330	66±0.000	64±6.928
Fusarium subgultinans	24±0.000	24±12.000	28±6.928	40±6.928	56±6.928	80±6.928

Seedling vigor index in control a (neither spore nor chitinase treated)-99.96±0.0

inhibit fungal mycelium growth of *A. niger, A. flavus, F. subgultinans*, and *T. harzianum*. Hence, these fungal strains were selected for rice seed germination studies. In the present study, *T. harzianum* itself was found to be promoting seed germination. Seedling emergence was higher in *T. harzianum* treated seeds than the control. The promotion of plant growth induced by *Trichoderma* sp. has been reported to be due to the control of minor pathogens and the production of growth-regulating factors. *Trichoderma* sp. could produce enzymes that detoxify waste cyanide produced by pathogens, which leads to an increase in plant growth [19].

In this present study *A. niger*, and *A. flavus* decreased the seed germination to 0% and 13.3, respectively. Seed vigor was also decreased significantly (zero for *A. niger*). Treatment with *Fusarium* sp. seed germination % was 20, and the seed vigor was 24. Seeds treated with enzyme chitinase exhibited a positive response for germination. Percentage of germination, and seed vigor increased considerably. This ranged from 60% for seeds infested with *A. niger* to 76% for seeds infested with *A. flavus*. Seed vigor index was 64 for seeds infested with *A. niger*, and 92 for seeds infested with *A. flavus*. Seedling vigor is the criteria of the healthy condition of seeds, which upon planting allows rapid germination in different environmental parameters [20]. Several factors like the nutritional influence seed vigor, and environmental condition of the mother plant, genetic constitution, seed weight, mechanical integrity, seed size, maturity at harvest, ageing, and deterioration of seeds, and pathogens also. The seed which shows higher seed vigor index is considered to be more vigorous.

The results of the present investigation using the purified chitinase of *K. gibsonii* Mb 126 in controlling fungal growth show that this may be a promising method of biocontrol of phytopathogenic fungi of rice. Limited study reports are available on the similar application of chitinase in other crops to improve seed germination by controlling the seed-infested fungi. There is a successful report of increased seed germination by applying chitinase on soya bean seeds infested by *S. rolfsii* [21]. The massive application of chitinase for such seed treatment requires large-scale chitinase production and the use of a waste product of the prawn peeling industry would be helpful for the production of cost-effective chitinase enzyme.

5. CONCLUSION

Chitinase of *K. gibsonii* Mb 126 effectively reduced the seed-borne fungi and increased the percentage of seed germination and seedling vigor. This study revealed that pre-treatment of seeds with chitinase before germination reduced fungal infection and enhanced the seed viability and vigor. The application of seed treatment can be used as a preventive and curative method against plant pathogens.

6. AUTHORS' CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

7. ACKNOWLEDGMENTS

None.

8. FUNDING

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9. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

10. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

11. DATA AVAILABILITY

Not Applicable.

12. PUBLISHER'S NOTE

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