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# Induction of defense response of *Oryza sativa* L. against *Pyricularia grisea* (Cooke) Sacc. by treating seeds with chitosan and hydrolyzed chitosan

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**Abstract**

Seeds of rice (*Oryza sativa* L.) were treated with chitosan and hydrolyzed chitosan at 100, 500 and 1000 mg $L^{-1}$. After 18 days of germination, spore suspension of *Pyricularia grisea* was applied. The enzyme activity of phenylalanine ammonia-lyase, β-1-3-glucanase, chitinase and chitosanase in leaves of rice seedlings was evaluated after 24, 72, 120 and 168 h of inoculation. Blast affected area (%) was evaluated 7 and 14 days after spraying spore suspension. Chitosan performance to elicit defense response induction was associated with the concentration and type of chitosan. The activity of most of the enzymes tested was induced in leaves of treated seeds with chitosan and hydrolyzed chitosan at 1000 and 500 mg $L^{-1}$, respectively. The highest enzyme activities were observed with hydrolyzed chitosan after 72 h however, compared to chitosan, the activity was not maintained during the entire post-inoculation period. The highest control (0 = no lesions) of *P. grisea* in rice seedlings was observed at 1000 mg $L^{-1}$ in both chitosan and hydrolyzed chitosan treated leaves. Symptoms of infection by *P. grisea* were evident after 14 days evaluation date, but according to the standard scale proposed by the International Rice Research Institute, these symptoms fell into the resistance category of blast diseases.


*Keywords:* Rice blast; Leaf blast; Poly-(1-4)-β-D-glucosamine; PAL; Chitosanase; β-1-3-Glucanase; Chitinase

## 1. Introduction

The rice blast disease, originated by the fungus *Pyricularia grisea* (teleomorph = *Magnaporthe grisea*) occurs in most rice growing areas around the world. It is considered as one of the most important diseases of rice because of its worldwide distribution, destructiveness and high degree of pathogenicity [24,25]. Most infections occur in leaves but seeds may also be infected. In leaves, symptoms include enlarged elliptical or spindle lesions, showing grey or white spots surrounded by brown to reddish-brown margins, eventually killing the leaves [17]. In Cuba, incidence of disease in leaves is closely related to environmental conditions. In rice-growing areas, relative humidities greater than 85% and temperatures of 26–28 °C may provoke yield loses up to 80% [32].

Numerous strategies have been followed to control rice blast disease of leaves such as breeding and phytosanitary programs and application of systemic fungicides [37,5]. Nevertheless, a significant reduction of rice blast disease has not been successfully achieved. One example is the application of systemic fungicides such as Hinosan (edifenfos), Fuji One (isoprothiolane), Silvacur Combi (tebuconazole + triadimenol) and Kitazin CE (iprobenfos) [35]. Seed treatments are also carried out by using expensive chemicals such as benomyl, carbendazim (banned in some

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countries) tebuconazole and their combinations. It is pointed out that their effectiveness is acceptable, even though they are toxic to humans and environment [30,31].

An alternative to control *P. grisea* might be the application of natural compounds that control blast disease by fungicidal effects or induction of resistance. Inhibition of mycelial growth of *P. grisea* up to 77% [6] and spore formation [21,7] by chitosan application was reported. In plants, defense mechanisms against pathogens have drawn special attention. Elicitors are a wide range of compounds which are able to induce host responses normally associated with resistance response [15]. Chitosan and its derivatives have been found to induce defense responses against pathogenic microorganisms of several agricultural commodities [29,36].

Chitinase from soybean seeds was produced when treated with chitosan glutamate at 0.1%, 0.5% and 1.0% concentrations after exposure to conidia of *Rhizopus stolonifer*, *Aspergillus orizae* and *Mucor ruoxii* [27]. In leaves of wheat seedlings (*Triticum aestivum* L.), the levels of enzyme activity induced by the hydrolyzed chitosan at 1.0 mg $mL^{-1}$, following inoculation with *Botrytis cinerea* significantly increased the enzymatic activities of phenylalanine ammonia-lyase (PAL) and peroxidase at the wounded site, reaching their maximum peak after 16 or 42 h, respectively [13]. An induced resistance response against *Puccinia arachidis* was also reported in groundnut (*Arachys hypogea* L.) chitosan-treated leaves, after 10 days of treatment, the maximum peak activities of intercellular chitinase and β-1-3-glucanase were evidenced at 1000 ppm [19]. Similar enzymatic activities were reported in tissues of potato tubers (*Solanum tuberosum* L.), after 12 and 48 h of treatment with low molecular weight water-soluble chitosan at 500 μg $L^{-1}$ and inoculated with *Phytophthora infestans* [26]. In tobacco plantlets, protection towards *P. parasitica* var. nicotiane was obtained by embedding roots in chitosan hydrolyzed at concentrations between 50 and 500 mg $L^{-1}$. In this study, induction of PAL and β-1-3-glucanase was significantly higher than in the untreated roots, these enzymatic activities were also dependent on plant exposition to *P. parasitica* [1]. Treatment of leaves of wheat with fungal elicitors such as chitosan and post-inoculated by *Puccinia graminis* sp. *tritici* induced maximum levels of glucanase after 24 and 48 h [33].

The aim of this study was to evaluate the ability of rice seed treatments with chitosan and hydrolyzed chitosan to induce enzymatic activities associated with resistance to *P. grisea* and its effects on leaf blast severity on seedlings.

## 2. Materials and methods

### 2.1. Seed material

Seeds of rice var. Jucarito (J-104) were obtained from the germplasm bank at the Experimental Rice Research Station, Los Palacios, Pinar del Rio, Cuba.

### 2.2. Inoculum production

*Pyricularia grisea* was isolated from infected leaves of rice var. J104 showing the typical symptoms of this disease at the Experimental Rice Research Station, Los Palacios. Plant recollection and *P. grisea* isolation were carried out following the methodology of [9]. Single-spored *P. grisea* was grown on Petri plates containing media prepared with stems and leaves of rice, pH 5.5–5.6, and incubated at 26–28 °C, alternating light and darkness for 16 and 8 h, respectively [18]. Spore suspension was prepared from 10-day old pure cultures of *P. grisea*. Spores were obtained by scrapping them off the agar with the aid of a glass rod. The resulting suspension was added to a mixture of jelly and distilled water. The solution was shaken and then filtered through a cotton wool. The number of spore $mL^{-1}$ was adjusted to $2 \times 10^5$ using a Neubauer haemocytometer.

### 2.3. Chitosan, hydrolyzed chitosan and identification of oligomers

Chitosan from lobster shells (Pharmaceutical Laboratory 'Mario Muñoz', La Habana, Cuba, degree of acetylation = 63%) was obtained following the methodology of Cabrera et al. [10]. Chitosan was dissolved in 2% (v/v) by stirring overnight in water–acetic acid at 1% solution, and adjusted to pH 5.7 with dilute 2 M potassium hydroxide. Five grams of chitosan dissolved in sodium acetate, pH 5.0, was hydrolyzed with celluclast (Novozymes) at a rate of 1/500 v/v for 24 h followed by a second hydrolysis at a rate of 1/300 v/v for an additional 24 h. The identification of oligomers present in the hydrolyzed chitosan was carried out by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) (Bruker Daltonics AutoFlex™). Five microliters of the hydrolyzed chitosan was diluted in 20 μL of matrix solution (2,5-dihydroxybenzoic acid) and was allowed to air-dry. Peak-fractions were analyzed by MALDI-TOF-MS in the positive ion mode, equipped with nitrogen ($N_2$)-laser emitting at 337 nm. Each mass spectrum was the result of 30–70 laser targets. All spectra were measured in the reflector mode using external calibration.

### 2.4. Induced resistance by chitosan and hydrolyzed chitosan

Rice seeds var J-104 were washed with sodium hypochlorite (3%), for 3 min, rinsed with deionized water and ambient air-dried. Seeds were dipped for 15 min in chitosan or hydrolyzed chitosan at the following concentrations: 100, 500 and 1000 mg $L^{-1}$. Seeds were air-dried and seeded in plastic trays containing sterilized Gley Nodular Ferralitic Concrecionario soil [22]. Trays were incubated in growth rooms at 22–24 °C, 88–90% RH and 450 μmol/s $m^2$ lightness) alternating light–darkness cycles for 16 and 8 h, respectively. Seedlings at growth stage 2 [14] were inoculated by spraying the spore suspension 18 day after plant emergence. Seedlings sprayed only with distilled water or