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Chitosan nanoparticle induced defense responses in fingermillet plants against blast disease caused by *Pyricularia grisea* (Cke.) Sacc.

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ABSTRACT

The in vitro antifungal properties of chitosan nanoparticle and its role in protection of fingermillet plants from blast disease were evaluated. Chitosan nanoparticle inhibited the radial growth of *Pyricularia grisea* indicating the antifungal property. Application of chitosan nanoparticle delayed blast symptom expression on fingermillet leaves for 25 days while it was on 15 day in control plants. Chitosan naoparticle was able to induce the reactive oxygen species and the level of peroxidase actvitiy in leaves of fingermillet, which might be the reason for delayed symptom. The treated plants showed reduced disease incidence when compared to untreated control plants. These results suggested the role of chitosan nanoparticle in protecting fingermillet plants from *P. grisea* infection.

1. Introduction

Finger millet (*Eleusine coracana* Gaertn.) is one of the most important cereal crops, supporting the lives of millions of people across the globe and particularly in the developing countries (Dida et al., 2007; Gashaw, Alemu, & Tesfaye, 2014). Although it is adapted to a wide range of environments, including high temperature, moisture deficit and water stagnation, biotic stress cause heavy losses and can damage entire crop. Of the biotic stress that affect finger millet crop, blast disease caused by *Pyricularia grisea* (Cke.) Sacc. is the most important diseases of finger millet growing areas which results in reduction of physiological maturity, biomass and yield of the crop (Lenne et al., 2007). The disease affects the crop at all growth stages from seedling stage, causing lesions and premature drying of young leaves, affecting panicle causing neck/finger blast. The average loss due to finger millet blast has been reported to be around 28% and in endemic areas it is as high as 80–90% (Kumar, 2011). *P. grisea,* is also known to infect other cereal crops world wide (Agrios, 2000). Farmers use chemical fungicides for effective control of this disease, however, it is not economical and also cause severe damage to the environment (Giannousi, Avramidis, & Dendrinou-Samara, 2013). Hence, a better way of managing the disease could be through inducing host plant resistance.

Reactive oxygen species (ROS) are key molecules in plant defense (Lamb & Dixon, 1997; Nanda, Andrio, Marino, Pauly, & Dunand, 2010) and in particular H_2O_2 is known to be a local signal, mediating the induction of plant defense responses (Knight & Knight, 2001; Orozco-Cardenas & Ryan, 1999), expression of PR (pathogen-related) genes (Jones & Dangl, 2006; Huckelhoven, 2007). Peroxidases was classified as PR 9 (Van Loon, Pierpoint, Voller, & Conejero, 1994) which are known to be activated in response to biotic and abiotic stress (Huckelhoven, Dechert, Trujillo, & Kogel, 2001) and play a significant role in the plant defense mechanism (Anusuya & Sathiyabama, 2015a; Hiraga, Sasaki, Ito, Ohashi, & Matsui, 2001).

Chitosan was recognized as elicitors (Li et al., 2009) of plant defense, which can induce various defense reactions, including changes in membrane permeability, production of reactive oxygen species, defense related enzymes, biosynthesis of jasmonic acid, lignifications, ion flux etc. (Bueter, Specht, & Levitz, 2013; Hadwiger, 2013; Lin, Hu, Zhang, Rogers, & Cai, 2005; Prapagdee, Kotchadat, Kumsopa, & Visarathanonth, 2007; Sathiyabama & Balasubramanian, 1998; Sathiyabama, Bernstein, & Anusuya, 2016). However, the insolubility of bulk chitosan in aqueous media limits its wide spectrum of application (Saharan et al., 2013; Sathiyabama & Charles, 2015). Therefore, various strategies have been employed to enhance its solubility. The chelating property of chitosan towards organic and inorganic compounds renders suitable for improvement in solubility, biocidal activity (Shukla,

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Mishra, Arotiba, & Mamba, 2013) and leads to the preparation of chitosan nanoparticles.

Chitosan based nanoparticles are used worldwide for various applications including agriculture due to their biodegradability, solubility, high permeability, non-toxicity to human and cost-effectiveness (Ali, Rajendran, & Joshi, 2011; Brunel, Gueddari, & Moreeshbacher, 2013; Manikandan & Sathiyabama, 2015) and are found more effective than bulk material (Du, Niu, Xu, Xu, & Fan, 2009; Sathiyabama & Parthasarathy, 2016; Smitha, Nisha, Maya, Biswas, & Jayakumar, 2015). The improved activity is due to high surface-to-volume ratio, size-dependent qualities and unique optical properties (Anusuya & Sathiyabama, 2015b, 2015c; Saharan et al., 2015). Chandra et al. (2015) reported that application of chitosan nanoparticle to leaves of *Camellia sinensis* showed enhanced defense response and also high accumulation of defense enzymes. They also demonstrated that chitosan nanoparticle act as an effective plant defense elicitor and showed better immuno modulatory effect when compared to natural chitosan. Recently, we have reported that application of chitosan nanoparticle to detached rice leaves protected it from *P. grisea* under in-vitro condition (Manikandan & Sathiyabama, 2016). Therefore, the present study aims to evaluate chitosan nanoparticle as agrochemical to protect finger millet plants from blast fungus by the way of products that reinforce the natural defense mechanism.

2. Materials and methods

2.1. Biological material

Pyricularia grisea was obtained from the Tamil Nadu Agricultural University, Coimbatore, and maintained in Potato Dextrose Agar (PDA) slants at 4 °C. Seeds of finger millet (susceptible to blast) were obtained from ICRISAT, Patancheru, India.

2.2. Preparation of chitosan nanoparticle

Chitosan nanoparticle was prepared and characterized as described earlier (Manikandan & Sathiyabama, 2016) and used for application to finger millet plants.

2.3. In-vitro antifungal assay

Antifungal assay of chitosan nanoparticle was conducted by radial growth determination of *P. grisea*. The chitosan nanoparticle solution was prepared in distilled water (0.1%) and added to Czapek Dox Agar (CDA) medium at a concentration of 0.5, 1, 2 mg/ml and sterilized. Each CDA plate was seeded with 6 mm diameter mycelial plugs of *P. grisea* and incubated at 28 °C. The fungal growth was measured daily for two weeks. Growth inhibition was expressed as the% inhibition of radial growth relative to the control (Bell, Hubbard, Liu, Davis, & Subbarao, 1998).

2.4. Evaluation of chitosan nanoparticle in protection of finger millet plant from blast disease development

Seeds of finger millet were rinsed with distilled water and surface sterilized with 0.01% (w/v) sodium hypochloride for 2 min and washed thoroughly in distilled water. The seeds (20 seeds/ml) were placed in a sterile petri plate containing chitosan nanoparticle solution (0.1%, optimized) and kept in a rocker overnight. Seeds placed in sterile water served as control. Seeds were sown (5 seeds/pot) in clay pot (27-cm diameter; 26-cm height) containing alluvial soil and grown under glass house condition. After 20 days, the plants were sprayed with chitosan nanoparticle solution (5 ml/plant). This was repeated twice with 10 days interval. Water sprayed plants served as control. For each treatment, approximately 50 plants were used and all the experiments were repeated thrice.

The leaves of 30 d old seedlings were thoroughly cleaned with sterile distilled water and predisposed to nearly 95% humidity for 12 h. The spore suspension from 15 d old *P. grisea* culture (1×10^5 spores/ml) was inoculated by spraying on leaves of untreated (control) and chitosan nanoparticle treated plants. The visible symptom appearance of all finger millet plants was observed daily for the next 50 days. Disease incidence was determined on the basis of disease score, an estimate of the area affected using a scale (0–5) as follows: 0 = No symptoms on the leaves; 1 = small brown specks of pinhead size to slightly elongate (less than 20% affected tissue); 2 = a typical blast lesion elliptical 5–10 mm long (20–40% affected tissue); 3 = a typical blast lesion elliptical 1–2 cm long (40–60% affected tissue); 4 = 60–80% leaf area affected; 5 = complete blast. The percentage of disease incidence was calculated using the formula: Blast incidence = ([Scale × Number of plants infected]/[Highest scale × Total number of plants]) × 100.

The plants were harvested for growth determination of leaf length, leaf number and plant dry weight, at different age level. The yield in terms of grain dry weight/plant was determined at the time of harvest.

2.5. Peroxidase assay

After inoculation, chitosan nanoparticle treated and untreated leaves were collected for peroxidase activity assay at 30,40 and 50 day. They were extracted with 0.01 M potassium phosphate buffer pH 7.0 (1 g × 2 ml) at 4 °C using a pre-cool mortar and pestle. The extract was centrifuged at 10,000 G for 15 min (Eppendrof, Germany). The supernatant was collected and used for estimation of total protein and peroxidase activity assay. Total protein content was determined by the dye binding method of Bradford (1976) using coomassie brilliant blue. Peroxidase activity was quantitatively detected by measuring the change in absorbance at 430 nm at 30-s interval for 3 min at room temperature (Reddy, Subhani, Khan, & Kumar, 1995). The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract, and 0.25 ml of 1% (v/v) H_2O_2. The enzyme activity was expressed as change in absorbance per minute per milligram protein. All experiments were independently repeated at least three times and representative data were shown.

2.6. In gel activity assay of peroxidase

40 μg of proteins was separated on 10% native PAGE gel (Davis, 1964). After electrophoresis, peroxidase isoforms were visualised by soaking the gels in a solution containing 0.03% (w/v) guaiacol and 0.05% (v/v) H_2O_2 in citrate phosphate buffer (0.05 M, pH 5.0) (Birecka & Garrawey, 1975).

2.7. Western blot analysis

Protein (20 μg) samples were separated on 10% SDS-PAGE separation gel and blotted to nitrocellulose membrane. The membrane was blocked with 4% (w/v) Bovine Serum Albumin overnight and then probed with peroxidase antibody (1:1000) in 1% phosphate buffered saline (PBS) for 1 h at room temperature. The membrane was washed thrice with 1% PBS containing Tween-20 about 10 min each. The membrane was incubated with alkaline phosphatase conjugated antirabbit antibody (1 × 10,000) in PBS for 1 h, washed as above and NBT/BCIP was added to localize the peroxidase polypeptides.