Songklanakarin J. Sci. Technol.
36 (5), 507-512, Sep. - Oct. 2014

SJST SONGKLANAKARIN JOURNAL OF SCIENCE AND TECHNOLOGY
http://www.sjst.psu.ac.th

Original Article

Effects of chitosan treatments on para rubber leaf fall disease caused by *Phytophthora palmivora* Butler - a laboratory study

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Received: 11 October 2013; Accepted: 1 July 2014

Abstract

Leaf fall is a serious disease of para rubber seedlings, known to cause economic losses in several nurseries of southern Thailand. It is caused by a phytopathogenic fungal-like organism *Phytophthora*. Chitosan is a biological polymer used as a plant growth stimulator, and used against several phytopathogenic fungi on many crops. This study aimed to determine the effects of chitosan in controlling leaf fall disease of para rubber seedlings caused by *Phytophthora palmivora,* both *in vitro* and *in vivo*. Various concentrations of chitosan were applied on seedlings of para rubber clone RRIM 600. The growth rate of *P. palmivora* on potato dextrose agar was determined with various chitosan concentrations, and a concentration of 1 mg/ml was sufficient to reduce the radial growth of *P. palmivora* by about 91% on agar plates. This concentration also reduced the size of necrotic lesions at 24 and 48 hours post inoculation (hpi), but not after 72 hpi. The results suggest that chitosan (i) directly inhibits the growth of *P. palmivora*, and (ii) reduces the growth rate of necrotic lesions, but may not completely be able to control the leaf fall disease of para rubber seedling.

Keywords: chitosan, leaf fall, para rubber, *Phytophthora*

1. Introduction

Para rubber (*Hevea brasiliensis* Muell. Arg.) is an economically important crop worldwide because of its multiple uses in numerous industries. Several pathogens cause devastating infections of para rubber trees, especially in the nursery stage, but also in immature and mature trees (Rao, 1965; Begho, 1995; Jayasinghe *et al*., 1995; Jayasinghe, 2000). Para rubber diseases are mainly caused by fungal pathogens (Igeleke, 1988; Begho, 1990) which can infect the para rubber at any growth stage. In Thailand leaf fall epidemics occur during the long period of high rain fall from June to December, and can cause serious losses of yield and market value to para rubber seedlings (Chantarapratin *et al*., 2001). Three species of *Phytophthora* common in Thailand are *Phytophthora palmivora* Butler (Tsao *et al*., 1976), *P. botryosa* Chee (Suzuki *et al*., 1979), and *P. nicotinae* var. *parasitica* (Rubber Research Institute of Thailand, 2010). Signs of leaf fall disease include coagulated latex in a central lesion of petiole, and later dieback of terminal branches. Zoospores of this pathogen are spread by rain splash from infected leaves to the trapping panel (Johnston, 1989). A 75% defoliation of para rubber tree may reduce latex yield by up to 30-50% (Chee, 1969). Jayarathnam *et al*. (1994) suggested a 25%-defoliation as an indicator for a need to intervene and manage the leaf fall disease.

Application of fungicide is the most effective method to control *Phytophthora* diseases. However, the high cost of fungicide, fungicide-resistant strains of the pathogen, as well as effects on human health have raised concerns in many countries. Biological products are widely used in agricultural production and are considered environmentally friendly and sustainable. Chitosan, a natural polymer, and chitosan derivatives, have been widely used as growth

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stimulators to increase various crops, such as orchids (Nge *et al*., 2006), faba beans (El-sawy *et al*., 2010), cucumbers (Shehata *et al*., 2012), and corn (Boonlertnirun *et al*., 2011; Lizarraga-Paulin *et al*., 2011). Aside from its role as plant growth promoter, chitosan has also been effective in the biological control against several fungi (Allan and Hadwiger, 1979; Hirano and Nagao, 1989; Kendra *et al*., 1989; Stossel and Leuba, 1984; Benhamou, 1996; Sunpapao and Pornsuriya, 2013). In addition, chitosan can induce the accumulation of phytoalexins that have antifungal effects and enhance resistance to further infection (Uthairatanakij *et al*., 2007).

Para rubber clone RRIM 600 is susceptible to *Phytophthora* spp. infection (Narayanan and Mydin, 2011). To investigate the effects of chitosan in controlling para rubber leaf fall disease, *P. palmivora* was used as the causal agent in seedlings. Agar plates were prepared with various concentrations of chitosan and radial growth of *P. palmivora* was examined. A treatment concentration of chitosan was selected based on these *in vitro* data, and treatment effects were evaluated in para rubber leaves. We found that chitosan reduced the progress of necrotic lesions, but did not completely control the para rubber leaf fall disease.

2. Materials and Methods

2.1 Culture and growth conditions of *Phytophthora palmivora*

The *P. palmivora* was provided by S. Chuenchit, Department of Pest Management, Faculty of Natural Resources, Prince of Songkla University. The fungi were stored in potato dextrose agar (PDA) slants at 4°C. The fungal mycelia were routinely maintained on PDA (200 g potato, 20 g dextrose, and 15 g agar in 1,000 ml distilled water) at room temperature for five days, prior to testing with chitosan.

2.2 Chitosan preparation

The stock solution (1%, w/v) of chitosan was prepared by dissolving chitosan in 0.5% (v/v) glacial acetic acid as described by Benhamou *et al.* (1998). The pH of stock solution was adjusted to 5.6 by 1N NaOH and it was sterilized with autoclave (120°C, 20 min) prior to use in the assays.

2.3 Effect of chitosan on radial growth of *Phytophthora*

To find an effective concentration of chitosan for inhibiting *Phytopthora*, PDA plates were amended with chitosan at different concentrations (0.125, 0.25, 0.5, 1 and 2 mg/ml) as in Laflamme *et al.* (2000). Unamended PDA plates with 0.05% final concentration of acetic acid (pH 5.6) served as negative controls. Comparison to these controls shows the activity of chitosan and excludes the effects of acidic conditions. Four replicate plates at each chitosan concentration were inoculated in the center with a plug (5 mm diameter) from the edge of a 3-5 day-old colony of *P. palmivora*. The colony radii were measured seven days after the inoculations (Sunpapao and Chaninun, 2013). The experiments were repeated twice. Percent inhibition of diameter growth (PIDG) was calculated from treatment-average diameters in an experiment using the following formula:

$$\text{Percent inhibition of diameter growth (PIDG)} = \frac{A-B}{A} \times 100$$

where A is the diameter of control colonies, and B is the diameter of treated colonies.

2.4 Effect of chitosan on expansion of necrotic lesions

A zoospore suspension was obtained by rinsing with 15 ml sterilized DW into culture plates with *Phytophthora*. Culture plates were incubated at 4°C for 15 minutes and then placed at room temperature for 30 min to release zoospores. Zoospores were then counted in a Petroffhausser counting chamber prior to inoculating the para rubber leaves. Individual para rubber leaves were split into two parts, to ensure the control and treated samples were as similar as possible. Treatment was by immersion into 0.5, 1 or 2 mg/ml chitosan solution for 1 hr, while immersion into 0.05% acetic acid served as control. The treated para rubber leaves were cleaned with sterilized distilled water (DW). The Para rubber leaves were wounded with detached leaf method. The $1x10^5$ zoospores/ml concentration was inoculated into chitosan treated para rubber leaves, then incubated in moist chamber for 24, 48, and 72 hrs. The lesions from *Phytophthora* penetration in leaves were measured. Percent inhibition was calculated using the formula shown above.

2.5 Statistical analyses

The completely randomize design (CRD) was repeated twice for each experiment. Data were analyzed by analysis of variance (ANOVA), and statistical significance of mean differences between chitosan and control treatments was assessed with Duncan's Multiple Range Test (DMRT) for multiple comparisons.

3. Results

3.1 Effect of chitosan on radial growth of *Phytophthora palmivora*

In order to determine the inhibitory effect of chitosan on radial growth of *P. palmivora*, agar plates with five concentrations of chitosan (0.125, 0.25, 0.5, 1, and 2 mg/ml) were tested against this pathogen. The PDA amended with 0.05% acetic acid served as control, and this in fact showed no growth differences to a plain medium without acetic acid (data not shown). All treatments reduced the growth of *P. palmivora* over seven days post inoculation, and the growth inhibition consistently increased with concentration (Table 1). The photos in Figure 1 illustrate the effects on radial

Table 1. Effect of chitosan concentration on growth rate of *P. palmivora*.

Chitosan concentration (mg/ml)	Inhibition rate (%)
0.125	45.65±4.56^{d}
0.25	57.64±3.63^{c}
0.5	87.65±1.18^{b}
1.0	90.35±0.53^{ab}
2.0	93.24±0.59^{a}

% inhibition rate is relative to control. Different superscripts indicate statistically significant differences (P<0.01).

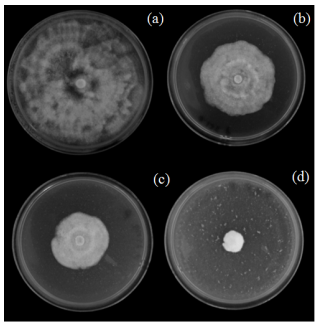

Figure 1. Effects of chitosan on radial growth of *P. palmivora*. PDA amended with 0.05% acetic acid served as control (a), for comparison with chitosan solutions at 0.5 (b), 1 (c) and 2 mg/ml (d).

growth of *Phytophthora*, and the effects were statistically significant as detailed in Table 1. After establishing growth inhibition *in vitro*, the chitosan concentrations 0.5, 1, and 2 mg/ml were subjected to *in vivo* tests on para rubber leaves.

3.2 Chitosan delayed necrotic lesions caused by *Phytophthora palmivora*

After treatments with chitosan concentrations of 0.5, 1 and 2 mg/ml, *P. palmivora* specimens were inoculated on para rubber leaves. Necrotic lesions would indicate that *P. palmivora* had invaded the plant tissue. The necrotic lesion diameters were measured at 24, 48 and 72 hours post inoculation (hpi), and the growth curves are shown in Figure 2. The lesions consistently grew over time, regardless of treatment group (Figure 2A and 2B), but the growth curves for actual treatments are below that for control (Figure 2B). Effect of chitosan on inhibition of *Phytophthora* invasion to para rubber leaves is shown in Table 2. A chitosan solution at a concentration of 2 mg/ml showed the highest level to inhibit *Phytophthora* (up to 67%). There were four replicates of each treatment in an experiment, and the experiments were repeated twice. The growth inhibition effects were statistically significant, as detailed in Table 2.

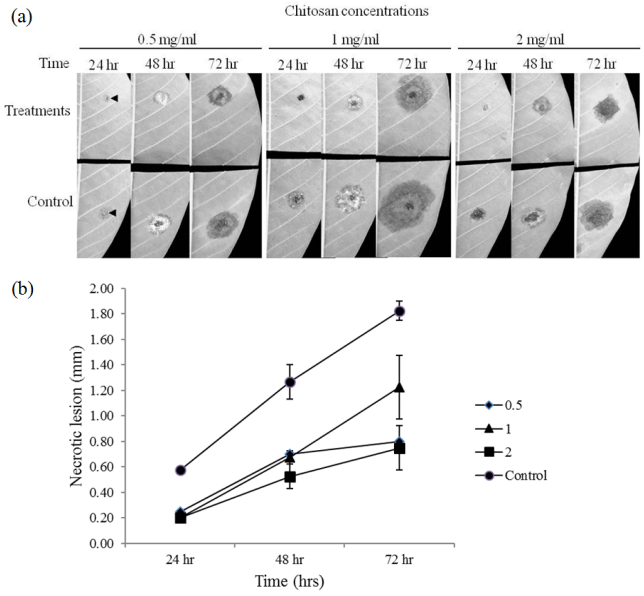

Figure 2. Growth of necrotic lesions caused by *P. palmivora* on para rubber leaves, with various chitosan treatments compared to control (a). Growth curves of necrotic lesion diameters (b) on para rubber leaves, for the various treatment groups. Actual treatments applied 0.5, 1 and 2 mg/ml chitosan, while 0.05% acetic acid was used as control.

Table 2. Effect of chitosan concentration on development of necrotic lesions and percent inhibition of *P. palmivora* on Para rubber leaves.

Chitosan (mg/ml)	Necrotic lesion (cm)			% inhibition		
	24 hrs	48 hrs	72 hrs	24 hrs	48 hrs	72 hrs
control	0.58^{a}	1.27^{a}	1.83^{a}	-	-	-
0.5	0.25^{b}	0.70^{b}	0.80^{c}	52.50^{a}	47.56^{ab}	48.50
1	0.20^{c}	0.68^{b}	1.23^{b}	65.00^{b}	41.96^{a}	37.48
2	0.20^{c}	0.53^{b}	0.75^{c}	67.86^{b}	58.84^{b}	60.58

Within the column, different superscripts indicates statistically significant difference for necrotic lesion ($p<0.01$), and for percent inhibition ($p<0.05$).

4. Discussion

In this study, we investigated the potential of chitosan in controlling leaf fall disease of para rubber seedlings, both *in vitro* and *in vivo*. The growth rate of *P. palmivora* was reduced in PDA plates amended with chitosan at 0.5, 1, and 2 mg/ml concentrations (Figure 1). The expansion of necrotic lesions on chitosan treated para rubber leaves was less than in the control group, up to 24 hrs post inoculation, while after that the growth rates appear similar as shown in Figure 2B. Nevertheless, necrotic lesions still spread to cover most of para rubber leaves, demonstrating that chitosan has the potential to inhibit the growth of *P. palmivora* and slow the initial infection spread, but does not prevent or limit the extent of leaf fall disease in para rubber seedlings.

Recently, Sunpapao and Pornsuriya (2013) reported that chitosan directly inhibits the growth of *P. botryosa,* one causal agent of para rubber leaf fall disease that withers its mycelia and oospores. The authors hypothesized that chitosan may have potential for practical disease management of leaf fall in nursery stage. The effects of chitosan against bacterial and fungal strains have been reported (Liman *et al*., 2011), and the inhibition rate correlates with the chitosan concentration (El-Ghaouth *et al*., 1992b). Chitosan has been widely used to reduce the growth of *Rhizoctonia fragariae* (Elmer and LaMondia, 1994), *Sclerotium sclerotiorum* (Cheah *et al*., 1997) and *P. botryosa* (Sunpapao and Pornsuriya, 2013). Furthermore, chitosan changes the pathogen morphologies of *Fusarium oxysporum* f.sp. *radis-lycopersici*, *R. stolonifer* and *S. sclerotiorum,* to abnormal shapes and sizes (Benhamou and Theriault, 1992; El-Ghaouth *et al*., 1992a, b; Cheah *et al*., 1997). However, most of these studies have focused on the efficacy of chitosan in directly inhibiting pathogen growth, not on its potential in controlling phytopathogenic fungi in plants. Therefore, we also examined *in vivo* the potential of chitosan in controlling the leaf fall disease of para rubber seedlings, caused by *P. palmivora*.

The chitosan concentration for the *in vivo* study was selected by tests *in vitro*, so it was effective in controlling the radial growth of *P. palmivora* in agar plates. Chitosan was applied on para rubber leaves prior to inoculation with *P. palmivora*. Necrotic lesions caused by *P. palmivora* were not restricted by the treatments (Figure 2), but the treatment effects match a delay in early infection by about one day. Given time, *P. palmivora* could invade most parts of a leaf, so the treatments did not limit or restrict the extent of infection symptoms. Therefore, chitosan only reduced *P. palmivora* activity in during the early infection state, but could not control leaf fall disease of para rubber seedlings. In contrast to our study, Boonreung and Boonlertnirun (2013) applied chitosan to control dirty panicle disease of rice, caused by *Helminthosporium oryzae*, *Curvularia lunata*, and *Fusarium moniliforme* in plantation fields. They suggested it is possible to control dirty panicle disease by spraying chitosan onto rice plants (Boonreung and Boonlertnirun, 2013).

The application of chitosan might trigger the plant's immune system to limit the expansion of *P. palmivora* during the early infection state, but molecular activities such as the expression of pathogenesis related protein (PR), involved in antifungal activity, were not determined post infection in this study. PR proteins are known to increase resistance of the whole plant against several pathogens' attacks (Adrienne and Barbara, 2006). There are more than 17 different PR protein families categorized by their properties and functions. Among these PR-1, PR-2, and PR-3 play an important role in antifungal defense, and β-1, 3- glucanase and chitinase contribute to defense response against several phytopathogenic fungi. However, no such treatment effects were clarified in this study.

As a summary, chitosan inhibits the growth of *P. palmivora*, and slightly delays the initial onset of infection in leaves, but does not prevent or limit the extent of leaf fall disease in para rubber seedlings. Currently, chitosan is known to control plant diseases caused by several fungi, mostly based on *in vitro* studies. The efficacy in practical application of chitosan *in vivo*, in green houses, and nurseries or in the fields, would further depend on environmental factors that impact disease management in field conditions. The current study attempted to bridge between purely *in vitro* studies and practical conditions, by including a laboratory scale *in vivo* study. For the specific pathogen in this study, the *in vivo* results were clearly discouraging, and relying solely on *in vitro* results would have been misleading. This suggests that an *in vivo* component in laboratory studies of similar nature is a useful low cost screen on seeking alternative pest control treatments.

Acknowledgements

The authors would like to thank Prince of Songkla University for funding and facilities. This work was supported by a grant from Prince of Songkla University (grant number NAT550131S). The authors gratefully acknowledge the editing service of the Research and Development Office, PSU, and helpful comments by S. Karrila were highly appreciated.

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