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# Chitosan beads combined with *Terminalia nigrovenulosa* bark enhance suppressive activity to *Fusarium solani*

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ABSTRACT

Chitosan beads (CTB) and *Terminalia nigrovenulosa* bark (TNB) were prepared using chitosan powder combined with *T. nigrovenulosa* powder (TNP) and *T. nigrovenulosa* extract (TNE) to obtain CTB + TNP (0, 1, 3, and 5%) and CTB + TNE (0, 0.1, 0.3, and 0.5%), respectively. The chitosan beads combined with TNB were tested for their antifungal activity against *Fusarium solani*. *In vitro* studies indicated that a mixture of 20 chitosan beads and 3–5% TNP inhibited *F. solani* mycelial growth the greatest. Mixtures of 10–20 chitosan beads and 0.3–0.5% TNE showed the greatest inhibition of *F. solani* mycelial growth. Total inhibition of *F. solani* mycelial growth was achieved with CTB and 5% TNP and CTB and 0.5% TNE after a 6 day incubation. Induction of pathogenesis-related proteins such as chitinase, β-1,3-glucanase, and guaiacol peroxidase were tested in cucumber seedlings. Enzyme accumulation was significantly higher in CTB and 0.5% TNE treated cucumber plants compared to that in control plants. We effectively demonstrated the biological activities of the CTB and TNP and CTB and TNE treatments. These results suggest that applying chitosan beads combined with TNB could be useful to control soil-borne diseases of cucumber seedlings.



## 1. Introduction

Diseases caused by *Fusarium solani* are a limiting factor in plant production, and yield quantity. *F. solani* causes the death of young and adult plants, with consequent economic losses (Kim and Jee, 1998). Several effective fungicides have been recommended for use against pathogens, but they are not considered long-term solutions due to expense, exposure risk, fungicide residue, and environmental hazard concerns. Therefore, alternative control methods that can be safely and effectively used are urgently required (Kavino et al., 2008). Applying plant extracts and biocontrol agents is eco-friendly and effective against many plant pathogens (Kagale et al., 2004). A number of plant species possess natural substances that are toxic to many pests and pathogens (Akhtar et al., 2008; Harish et al., 2008). These pesticides are generally considered non-persistent under field conditions, as they are readily transformed by light, oxygen, or microorganisms into less toxic products. Therefore, fewer residues are expected from those products (Akhtar et al., 2008).

Up to date, various plant extracts exert different levels of antifungal activity *in vitro* against phytopathogenic fungi (Soylu et al., 2006; Jasso de Rodríguez et al., 2005; Bajwa and Iftikhar, 2005). Chitosan is obtained by partial deacetylation of chitin (Muzzarelli, 2009; Jayakumar et al., 2010). Chitosan has been used as a swollen bead support for preparing immobilized enzymes and conjugate drug carriers (Juang et al., 2001; Yu et al., 2011). Chitosan as fungicides are effective in inhibiting spore germination, germ tube elongation and mycelial growth of fungal phytopathogens, such as *Phytophthora capsici* and *Alternaria solani* (Xu et al., 2007), *Fusarium* (Eweis et al., 2006), and *Alternaria kikuchiana* and *Physalospora piricola* (Meng et al., 2010). Additionally, combinations of chitosan and *Cornus officinalis* seed extract have been showed high antifungal activities, especially against *Rhizoctonia solani* and *F. solani* (Seo et al., 2013).

Plants in the genus *Terminalia*, family *Combretaceae*, comprising some 250 species, are widely distributed in tropical areas of the world (Fabry et al., 1998). A methanol extract of *Terminalia arjuna* and *T. superba* bark shows nematicidal and antifungal activities against *Haemonchus contortus*, *Trychophyton rubrum*, and *Microsporum audouinii* (Bachaya et al., 2009; Kuete et al., 2010). Additionally, the presence of phenolic acids such as gallic acid, ellagic acid, and corilagin acid is one of the reasons for the antifungal nature of the *Terminalia chebula* Retz (Rangsriwong et al., 2009).

In our previous studies, *Terminalia nigrovenulosa* bark (TNB) showed strong biocontrol properties against *F. solani* (Nguyen et al.,

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2013b) and *Meloidogyne incognita* (Nguyen et al., 2013a) *in vitro*. In this study, chitosan beads were used as a carrier to immobilize the agent showing both antifungal and nematicidal activities of TNB. Applying TNB to swollen chitosan beads has not been studied extensively. This study was designed to investigate the activities of TNB combined with swollen chitosan beads against the phytopathogens *F. solani*.

## 2. Materials and methods

### 2.1. Materials

Chitosan powder [90% deacetylation, 10 cPs (in 0.5% acetic acid + 0.5% chitosan solution, at 20 °C)] was purchased from Keumho Chemical Co., Ltd. (Seoul, Korea). TNB was collected from DakLak province, Vietnam. *F. solani* KACC 40384 was obtained from the Korea Agricultural Culture Collection (Suwon, Korea). All other chemicals were of analytical grade.

### 2.2. Preparation of chitosan beads (CTB) combined with T. nigrovenulosa powder (TNP) and CTB combined with T. nigrovenulosa extract (TNE)

TNB was cut into 0.5–1.0 cm pieces and powdered using a mortar and pestle to obtain TNP. The TNB was also cut into 2–3 cm pieces and extracted with 99% methanol at a ratio of 1:5 (v/v, dry bark/methanol) at 30 °C with shaking at 150 rpm for 7 days. The filtrate was evaporated to dryness under a vacuum at 40 °C (Eyela N-1000) and then lyophilized to obtain the TNE. The mixtures of CTB and TNP were prepared by dissolving chitosan powder (7 g) in 100 mL of 7% acetic acid and then adding it to 0, 1, 3, and 5 g TNP, respectively. Similarly, mixtures of CTB and TNE were prepared by dissolving chitosan powder (7 g) in 100 mL of 7% acetic acid and then 0, 0.1, 0.3, and 0.5 g of TNE was added, respectively. The resulting viscous solutions of CTB and TNP and CTB and TNE were de-gassed under vacuum and dropped into 200 mL of an alkali coagulating solution ($H_2O$–MeOH–NaOH = 4:5:1, w/w/w) to prepare highly swollen 3.5 mm average diameter spherical beads (Mitani et al., 1991). The chitosan beads were collected and thoroughly washed with distilled water.

### 2.3. Testing the antifungal spectrum of TNB using Petri dish

Antifungal assays were performed as previously described by Soylu et al. (2006) with slight modifications. The antifungal spectrum was determined by placing a 4 mm-diameter agar disk cut from the edge of an actively growing colony of the test pathogen onto the center of a new Petri dish containing potato dextrose agar medium. Subsequently, the 5, 10, and 20 CTBs of each concentration were placed carefully around a *F. solani* colony on a PDA plate, respectively. Each combination was tested using three replicates. Zones of inhibition were measured at 2, 4, and 6 days after incubation in the dark at 25 °C. The test was conducted twice.

### 2.4. Antifungal activity of TNB for disease control

Cucumber (*C. sativus* L. Asia Unchun F1) seeds were sown in steam sterilized soil in 5 cm clay pots, and single seedlings were transplanted into each of the pots containing 200 g of sterile soil 1 week after germination. All seedlings were kept in a growth chamber under a 16/8 h light/dark photoperiod, a 26/20 °C day/night and 60–70% relative humidity. The plants were inoculated with spore suspensions ($2 \times 10^5$ spores/mL) of *F. solani*. Subsequently, the cucumber plants were treated with 20 beads of chitosan and TNE (0, 0.1, 0.3, and 0.5%). Three replications were maintained for each treatment, and each replication consisted of three pots in a randomized design under chamber conditions. All procedures were performed under sterile conditions.

### 2.5. Activity measurement of pathogenesis-related (PR) proteins

At 7 days after pathogen inoculation, leaves sample was homogenized with liquid nitrogen in a pre-chilled mortar and pestle. 200 mg of sample was homogenized with 1 mL of 50 mM Tris buffer (pH 6.7), and the homogenate was centrifuged at 10,000 × *g* for 20 min at 4 °C. The supernatant was used as a crude enzyme extract for assaying chitinase, β-1,3-glucanase and guaiacol-peroxidase (GPOD). Protein concentration was determined according to the method described by Bradford (1976) using bovine serum albumin as a standard. Chitinase activity was assayed by measuring the reducing end group, GlcNAc (N-acetyl-β-D-glucosamine), produced by colloidal chitin (Lingappa and Lockwood, 1962). β-1,3-glucanase activity was assayed by measuring the amount of the reducing end group, glucose, produced from laminarin (Yedidia et al., 2000). GPOD activity was assayed by measuring the amount of the reducing end group produced using the method described by Jinmin and Bingru (2001).

### 2.6. Activity staining of PR proteins

Thirty micrograms of enzyme extracts was loaded onto sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and native-PAGE gels to detect β-1,3-glucanase and GPOD activity, respectively. For active staining of β-1,3-glucanase after 12% (w/v) SDS-PAGE, a gel was prepared by dipping it in 0.15% triphenyltetrazolium chloride solution containing 1 N NaOH followed by heating in an oven until red bands appeared (Pan et al., 1989). The gels were soaked in 50 mM Tris buffer (pH 6.8) for 10 min to actively stain GPOD after 10% (w/v) native PAGE, and then they were incubated with 0.46% (v/v) guaiacol and 13 mM $H_2O_2$ in the same buffer until red bands appeared. The gels were fixed in water/methanol/acetic acid (6.5:2.5:1, v/v) (Caruso et al., 1999).

### 2.7. Statistical analysis

Data on the effect of the treatments on the growth of pathogens and activity of enzymes in cucumber plants were compared using Tukey's Studentized range (HSD) test, with a $p \leq 0.05$ indicating statistical significance. All data were analyzed using the Statistical Analysis System 9.1 (SAS Institute, Cary, NC, USA) and are presented as mean ± standard deviation.

## 3. Results and discussion

In this study, mixtures of chitosan beads and TNB inhibited growth of *F. solani*. *F. solani* mycelia grew slowly on PDA plates containing CTB and TNP (Fig. 1a–c). In particular, the mycelial disk of *F. solani* treated with 20 CTB and 3% or 5% TNP did not grow for 2, 4, or 6 days (Fig. 1c, T2 and T3). Inhibition of 5 CTB with all tested concentrations against *F. solani* was only moderate (Fig. 1a). Briefly, mycelial growth was 46.3, 28.7, 18.7, and 18.3 mm at 0, 1, 3, and 5% of TNP, respectively for a 6 day treatment with the 5 CTB (Fig. 1d). Mycelial growth was 46.3, 15.0, 7.7, and 7.7 mm at CTB and 0, 1, 3, and 5% TNP with 10 CTB, respectively after 6 days of treatment (Fig. 1e). The 20 CTB managed to inhibit the mycelial growth of *F. solani* completely (Fig. 1f). Similary, the results of tests with CTB and TNE revealed that the inhibition of the 5 CTB and TNE (0, 0.1, 0.3, and 0.5%) against *F. solani* was only moderate (Fig. 2a and d). The 10 CTB and 0.3 or 0.5% TNE significantly inhibited mycelial growth after 4 days of treatment (Fig. 2b and e). The 20 CTB and 0.3 or 0.5% TNE inhibited mycelial growth completely after 6 days of treatment (Fig. 2c and f).