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# Development of Integrated Pest Management Techniques Using Biomass for Organic Farming (I)

## Suppresssion of Late Blight and Fusarium Wilt of Tomato by Chitosan Involving Both Antifungal and Plant Activating Activities

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# 유기농업에서 무공해 생물자원을 이용한 병충해 종합방제 기술개발 (I)

## 키토산의 항균 및 병저항성관련 유전자 유도에 의한 토마토 역병 및 시들음병 억제효과

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**ABSTRACTS: Effects of chitosan on growth of tomato plant, and suppression of Fusaruim wilt caused by *Fusarium oxysporum* f. sp. *lycopersici* and late blight caused by *Phytophthora infestans*, were examined. Both late blight and fusarium wilt were suppressed by spray and irrigation of chitosan, respectively. Inhibition of mycelial growth was not greatly affected by molecular size of chitosan but, concentration dependent effect was observed. Ninty percent of *P. infestans* and 80% of *F. oxysporum* f. sp. *lycopersici* of mycelial growth was inhibited by 1,000 ppm of chitosan (MW 30,000~50,000) when amended in plate media. Induction of defense-related gene expression in plant by chitosan treatments were observed when chitosan treated tobacco and tomato RNA samples were hybridized with several defense-related genes as probes. The results revealed that β-1,3-glucanase and chitinase genes were strongly induced, while pathogenesis-related protein-1, 3-hydroxy-3-methylglutaryl coenzyme A reductase, anionic peroxidase, phenylalanine ammonia lyase genes were weakly induced by chitosan treatment. These results suggest that chitosan have dual effects on these host-pathogen interactions. Possible roles of chitosan in suppression of tomato diseases by inhibition of mycelial growth and activation of plant defense responses are discussed.**

***Key words*: chitosan, inhibition of mycelial growth, activation of plant defense responses, late blight, fusarium wilt, tomato.**

Chitosan, a mostly deacetylated β-1,4 linked D-glucosamine polymer, is a component of the cell wall of phytopathogenic fungi (9), the cuticle of insects (11), and the chitin of crustacean shell wastes, including crab and shrimp shell (10).

Chitosan is known to have various biological functions; a) antifungal activity against several phytopathogenic fungi (1, 11, 13, 20), b) an elicitor of phytoalexins, including pisatin inducing activity (22), c) a potential elicitor or inducer of many plant defense responses including the accumulation of β-1,3-glucanase (4, 15) and chitinase (5, 16), d) and synthesis of proteinase inhibitor in tomato leaves (22), and e) an accelerator of lignification (18).

Recently, several works have demonstrated that chitosan treatment affected suppression of crown and root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici* (2, 3, 14), which delays the disease development by fungicidal effects, and suppresses root rot of cucumber caused by *Pythium aphanidermatum* during aqueous and nutritional culture (8), and controls decay of strawberry caused by *Botrytis cinerea* and *Rhizopus stolonifer* during storage (7).

Chitosan is known to exhibit direct antifugal properties against *Fusarium solani*, and other plant pathogens (1, 13). When tested *in vitro*, chitosan inhibited the radial growth of major post-harvest pathogens, indicating a marked effect at high concentrations (7). Allan and

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Hadwiger (1) reported that chitosan was effective in reducing the radial growth of most fungi tested, except those containing chitosan as a major cell wall component (i.e., Zygomycetes). Inhibitory effect of chitosan also was demonstrated with several phytopathogenic fungi including soil-borne pathogens (11, 20). Kendra and Hadwiger (13) suggested that the maximal antifugal and pisatin-inducing activities of chitosan were exhibited by chitosan oligomers of seven or more residues.

Furthermore, chitosan is also known to be a potential elicitor of many plant defense response such as accumulation of pathogenesis-related protein, and phytoalexin biosynthesis genes. Chang et al. (4, 5) reported that defense-related genes, chitinase and β-1,3-glucanase, were induced by chitosan treatment on pea plant. Chitosan treatment also induced expression of phenylalanin ammonia lyase (22), which associated with accumlation of phytoalexin pisatin (13), a major metabolite of the PAL pathway.

Chitosan, therefore, appears to play a dual function by interferring directly with fungal growth and also by activating several biological process in plant tissue, especially induction of defense-related gene expressions.

In this study, our purpose were focused to investigate the mode of actions of chitosan on suppression of two major diseases of tomato, fusarium wilt and late blight. Antifungal activity of chitosan against two phytopathogenic fungi, and role of chitosan in increasing expression of defense-related genes in plant were investigated. Possible mode of action of chitosan on suppression of fusarium wilt and late blight of tomato in relation to its antifungal and plant defence-inducing activities are presented.

## MATERIALS AND METHODS

**Fungal culture and preparation of chitosan.** *Phytophthora infestans* causing late blight and *Fusarium oxysporum* f. sp. *lycopersici* causing fusarium wilt of tomato plant were incubated at 20±1°C and 26±1°C under dark condition for 7 days in V-8 juice agar (V-8 juice 200 ml, $CaCO_3$ 3 g, agar 20 g, distilled water 1000 ml) and PDA (Potato dextrose broth 24 g, agar 20 g, distilled water 1000 ml) medium, respectively. A 5-mm-diameter mycelial plug taken from the margin of a 5-day-old *F. oxysporum* f. sp. *lycopersici* and/or *P. infestans* culture were used as inoculum in this study.

Chitosan solution was prepared according to previously reported method (3). Chitosan was dissolved in 0.25 N HCl with heating at 70°C and then removed pellets after centrifuging at 3000 rpm. Chitosan solution was adjusted pH 5.5 to 6.0 with 0.5 N NaOH. Molecular size of chitosan solution was estimated as previously reported method (19), and found that molecular size of the chitosan was 30,000 to 50,000. Concentration of chitosan was used as 250, 500, 1000, and 2000 ppm for mycelial growth inhibition and plant activation studies, respectively.

**Plants and treatments.** *Nicotiana tobacum* cv. Samsun NN, and *Lycopersicon esculentum* cv. Seukwang grown in the greenhouse under a regime of 14 hr light and 10 hr dark at 25±2°C were used as plant materials in this study. Chitosan solution (0 to 2000 ppm) prepared as described above was sprayed onto tobacco and tomato leaf surface. Control was treated only with water. Plant tissues were sampled and total RNAs were extracted from leaf tissues of those plants, and expression of the genes were monitored by northern blot hybridization.

**Effect of chitosan on protection against fusarium wilts of tomato seedlings.** Tomato seeds (cv. Seukwang) sterilized by immersion in 1% sodium hyperchlorite and rinsed in sterile distilled water were air-dried in a sterile cabinet for use. Seeds were sown in a mixture of peat/vermiculite (1:1/v:v) amended or not with chitosan (250, 500, and 1000 ppm). Thirty plants per each treatment were maintained in a growth chamber at about 25±2°C and a RH of 75%. Chitosan treatment was begun at two leaf stage of tomato seedlings and irrigated 5 times with every 7 days over a period of 50 days. Spore suspension ($1\times10^7$ microconidia/ml) of *Fusarium oxysporum* f. sp. *lycopersici* was inoculated 3 times with every 10 days over same period. Degree of root rot and wilt were investigated when the control plants were started wilting.

**Effect of chitosan on suppression against late blight of tomato plants.** Leaves of tomato seedlings (three to four leaf stage) were sprayed with different concentration of chitosan equivalent to 10~2000 ppm. After being sprayed with chitosan, wetted leaves were air-dried in natural condition. Tomato plants were inoculated at the four or five leaf stage, about 4 weeks after potting. Inoculation was performed on leaves by spraying zoosporangial suspension ($1\times10^3$ zoosporangia/ml) on the total surface of each leaf. The surface of leaves on control plants were sprayed with distilled water under identical conditions. After inoculations, plants were incubated in a growth chamber for 48 hr in the dark at

20±1°C and 95% RH, and then transferred to a 14/10 hr, light and dark photoperiod at 20±1°C and 95% RH, for 24 hr, and returned at 85% RH. Three plants were used for each treatment, and the experiment was repeated twice. Percentage of infected leaf area was measured 7 days after inoculation.

**Antifungal assays.** Antifungal assays were carried out on PDA or V-8 juice agar plates amended with chitosan at different concentrations (0, 10, 100, 250, 500, 1000, and 2000 ppm). Five PDA or V-8 juice agar plates per chitosan concentration were seeded with a 5-mm-diameter mycelial plug taken from the margin of a 5-day-old *F. oxysporum* f. sp. *lycopersici* and/or *P. infestans* culture. Treatments were arranged in a randomized complete block design and, the experiment was repeated twice. Plates inoculated were incubated at 26± 1°C (*F. oxysporum* f. sp. *lycopersici*) and/or 20±1°C (*P. infestans*) in the dark. Fungal growth was recorded at intervals of 1-day until the mycelia of the control (0 ppm of chitosan) plates reached to the edge of the plate. Growth inhibition is expressed as the percentage of mycelial growth relative to the control. Means and Duncan's multiple range test ($P$=0.05) were estimated on the basis of the observation of 5 plates per chitosan concentration.

**Total RNA isolation and RNA blot analysis.** Total RNA was isolated from treated leaf tissues according to previously described methods (17). Frozen and powdered plant material was thawed in the solution containing 0.8% tri-isopropylnaphthalenesulfonic acid, 4.8% p-aminosalicylic acid, 250 mM Tris-HCl (pH 9.0), 250 mM NaCl, 50 mM EDTA, and 50 mM 2-mercaptoethanol: phenol. The aqueous phase was extracted with phenol/chloroform and precipitated with 0.5 volume of isopropanol. Pellets were resuspended in TE buffer, 2 M LiCl, and reprecipitated by centrifugation, and resuspended in TE buffer completely. RNA concentration was estimated spectrophotometically ($A_{260}$) and 20 μg of the RNA was fractionated by electorphoresis through 1% agarose gels containing formaldehyde and transfered to nytran membranes. Loadding of equal amount of RNA was checked by staining of the membranes using methylene blue solution after exposure. Hybridizations of RNA blots with $^{32}$P-labelled cDNA probes were carried out in 5×SSC containing 50% formamide at 42°C, and washed with same condition except 2×SSC using standard procedures. The probes used in the northern blot analyses were prepared from PR-1, β-1,3-glucanase (23), HMGR (6), chitinase, PAL and anionic peroxidase DNA using random priming methods (12).

## RESULTS

**Effect of chitosan on growth of tomato plant and protection against *Fusarium oxysporum* f. sp. *lycopersici*.** Chitosan treatment was effective in controlling the incidence of root rot or fusarium wilts caused by *F. oxysporum* f. sp. *lycopersici*. Tomato plants grown in the presence of different concentrations (250, 500 and 1000 ppm) of chitosan and inoculum of *F. oxysporum* f. sp. *lycopersici* microconidia ($1\times10^7$ spore/ml) remained-healthy, and did not exhibit symptoms of wilting throughout the experiment (50 days, Fig. 1B and D). Plants grown in the presence of inoculum alone (control), started showing symptoms of wilting within 40 days after inoculation. By fifty day, control plants appeared to be not only yellowing of leaves and wilting but also reached to an advanced stage of root decay (Fig. 1, A and C). However, chitosan treated plants (500 or 1000 ppm) significantly promoted the growth of plants (Table 1) and protected plants against fusarium wilt even in lower concentration of chitosan treatment(250 ppm). The wilting and root rot symptoms of tomato by *F. oxysporum* f. sp. *lycopersici* were slowly developed when compared with non-treatment (data not shown).

**Effect of chitosan on the suppression of late blight caused by *Phytophthora infestans* of tomato plants.** Leaf pre-treatment of tomato plant with different concentration of chitosan solution (10 to 2000 ppm) sup-

**Table 1.** Effects of chitosan treatment on growth of tomato seedling and protection of fusarium wilt[a]

| Conc. of chitosan (ppm) | Plant biomass | | Protection of fusarium wilt |
|---|---|---|---|
| | Fresh wt. (g) | Dry wt. of root (g) | Degree of root infection |
| 250 | 118.2[b] | 1.7 | ++[c] |
| 500 | 135.8 | 1.8 | + |
| 1000 | 157.6 | 2.0 | ± |
| Control | 91.2 | 1.4 | +++ |

[a] Tomato seedlings were grown in growth chamber at 26±1°C, for 50 days after inoculation with *F. oxysporum* f. sp. *lycopersici* for 3 times on every 10 days, and irrigation with 250, 500, 1000 ppm of chitosan solution for 5 times on every 7 days. Control was irrigated with tap water.

[b] Total weight of 30 plants

[c] -; healthy, +; weak infection, ++; moderate infection, +++; severe infection

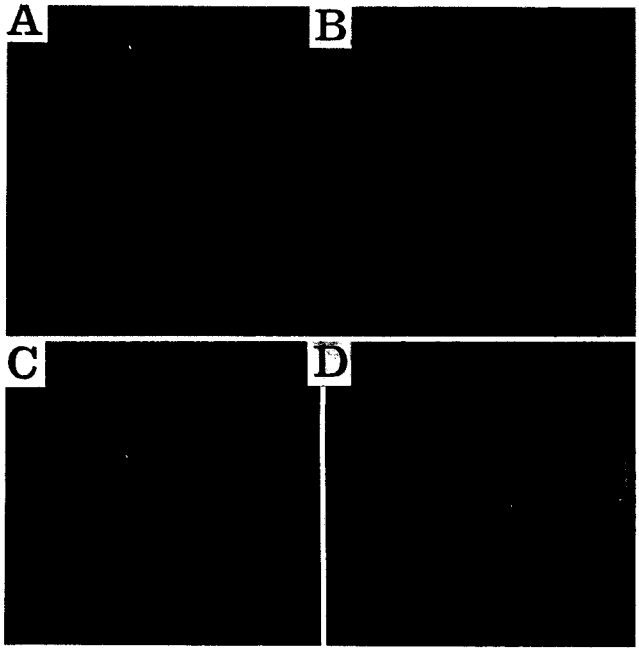


**Fig. 1.** Effects of chitosan on growth of tomato plant and protection against *Fusarium oxysporum* f. sp. *lycopersici*. Tomato plant were grown in growth chamber at 26±1°C for 50 days after inoculation with *F. oxysporum* f. sp. *lycopersici* for 5 times on every 10 days, and irrigation with 1000 ppm of chitosan solution for 3 times on every 7 days (A: Control, B: 1000 ppm of chitosan treatment). Comparison of tomato roots treated with water (C) or 1000 ppm of chitosan (D).

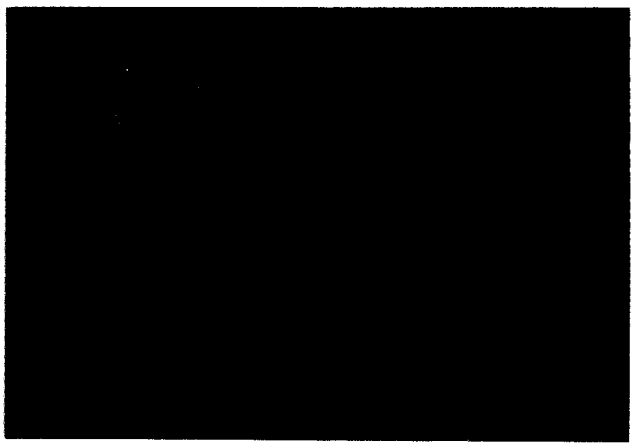

**Fig. 2.** Effect of chitosan treatment on the late blight of tomato plant (left: untreated control, right: 1000 ppm chitosan treated). Inoculation of *Phytophthora infestans* ($1\times 10^3$ sporangia/ml) was performed after 24 hr of chitosan treatment. After all inoculation, plants were incubated in a growth chamber for 48 hr in the dark at 20±1°C and 95% RH, and then transferred to a 14/10 hr, light and dark photoperiod at 20±1°C and 95% RH, for 24 hr, and returned at 85% RH. Disease severity was measured 7 days after inoculation.

pressed the late blight disease caused by *P. infestans* (Fig. 2 and Table 2).

After spraying with chitosan, tomato plants were inocu-

**Table 2.** Effect of leaf treatment of chitosan on late blight of tomato seedlings in pots[a]

| Conc. of chitosan (ppm) | Disease severity (%)[b] | Control value (%) |
|---|---|---|
| 10 | 21.4[c] b[z] | 14.4 |
| 100 | 21.0 b | 16.0 |
| 500 | 11.2 ab | 55.2 |
| 1000 | 7.2 a | 71.2 |
| 2000 | 6.3 a | 72.6 |
| Control | 25.0 b | – |

[a] Inoculation of *P. infestans* ($\times 10^3$ sporangia/ml) was performed after 24 hr of chitosan treatment.

[b] Disease severity was calculated based on the following equation

$$\text{Disease severity } (\%) = \frac{(0\times a)+(1\times b)+(2\times c)+(3\times d)+(4\times e)}{4(a+b+c+d+e)}\times 100$$

where 0, 1, 2, 3, and 4 indicate the infection category, 0% (healthy leaf), 0.1~5%, 5~20%, 20~40%, 40~100% leaf lesion area, respectively, and a, b, c, d, and e are the number of leaf which fall into the categories of 0, 1, 2, 3, and 4, respectively.

[c] Values are average of 6 replications.

[z] Means carrying same letters in a column are not significantly different ($P$=0.05) according to Duncan's multiple range test.

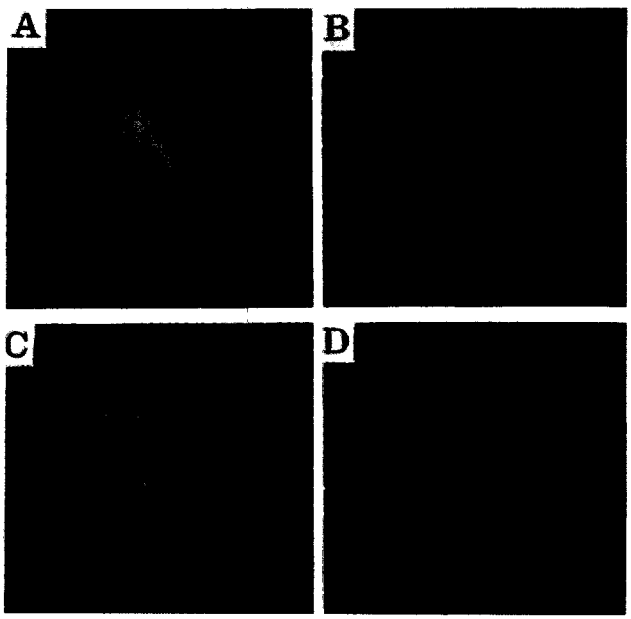


**Fig. 3.** Effects of chitosan on mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* on PDA, and *Phytophthora infestans*. *F. oxysporum* and *P. infestansn* were grown on PDA of V-8 juice agar, respectively. A and C are media alone (control), B and D plate contain 1000 ppm chitosan. *P. infestans* was grown at 20±1°C for 8 days, and *F. oxysporum* at 26±1°C for 7 days.

lated with zoosporangial suspension ($1\times10^3$ zoosporangia/ml) on the surface of leaf. When diseased leaf area was compared with control plants after 7 days of inoculation, 55 to 75% of control values were obtained, depending on concentrations of chitosan treatments (Table 2). Increase of chitosan concentration from 1000 to 2000 ppm did not increase the protection against late blight (Table 2). This result may imply the effective maximum concentration of chitosan against late blight is around 1000 ppm. Furthermore, tomato plants treated with different concentrations of chitosan did not show any apparent phytotoxicity symptoms during the course of the experiment even at the hightest chitosan concentration (data not shown). These results lead us to investigate how does chitosan treatment suppress two major disease of tomato caused by different taxa of fungal pathogens.

**Effect of chitosan on mycelial growth of *Fusarium oxysporum* f. sp *lycopersici* and *Phytophthora infestans*.** *In vitro* antifungal activity of chitosan was determined by investigating its direct inhibitory effect on mycelial growth of fungal pathogen. Antifungal assays were carried out on PDA or V-8 juice agar plates amended with chitosan with different concentrations (0 to 2000 ppm). As a results, We observed that chitosan significantly inhibited the mycelial growth of *P. infestans* and *F. oxysporum* f. sp. *lycopersici* with a marked effect at concentrations ranging from 500 to 2000 ppm (Fig. 3). At the 1000 ppm concentration, chitosan inhibited the mycelial growth of *P. infestans* and *F. oxysporum* f. sp. *lycopersici* by more than 90% and 80%, respectively (Fig. 4). By 7 days after inoculation, mycelial growth on chitosan-amended plates with 2000 ppm was almost halted, whereas the mycelial growth on control plates developed actively. Chitosan appeared to be more effective in inhibiting mycelial growth of *P. infestans* than *F. oxysporum* f. sp. *lycopersici*. The 50% growth inhibition concentrations of chitosan against *Fusarium* and *Phytophthora* were 450 and 250 ppm, respectively (Fig. 4).

**Induction of defense-related genes by chitosan treatment.** To test the possiblity of chitosan treatment on activating plant defense mechanism, several defense-related gene expressions was monitored following challenge of tobacco plants with different concentration of chitosan. Total RNA was extracted from leaves harvested 6 to 48 hr after chitosan treatment and healthy leaves of plant. Total RNA (20 μg) was fractionated on 1% agrose gel transferred to nytran membrane and hybridized with several cDNA clones labelled with $^{32}P$ as a probe. As a result, we observed that chitosan treatments can induce expression of pathogenesis-related protein genes such as PR-1, PR-2 (β-1,3-glucanase), PR-3 (chitinase), and plant secondary metabolism genes such as phenylalanine ammonia lyase (PAL), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), and anionic peroxidase gene (APOD). Among PR-protein genes, PR-1 was specifically induced upon chitosan treatment. Highest level of PR-1 expression was detected 6hr after chitosan treatment and induced levels of mRNA were detected until 24 hr after treatment (Fig. 5). Expression of glucanase and chitinase mRNA were detected both treated and untreated tobacco tissues but the treated leaves have more induced level of both mRNAs (Fig. 5). Chitosan treatment also affect the expression of secondary metabolism related gene expressions including PAL and HMGR (Fig. 5). This result may imply that enhanced level of fungitoxic secondary metabolites may be accumulated in plants by chitosan treatment. Peroxidase gene expression, which considered as a biochemical marker of induced resistance in some pathosystem, is also induced by chitosan treatment (Fig. 5). All together, these results may suggest the possible roles of chitosan on activating plant defense mechanism and contributing suppression of tomato diseases caused by *F. oxysporum* f. sp. *lycopersici* and *P. infestans*.

## DISCUSSION

In the present study, we have suggested that chitosan have dual effects on host-pathogen interaction both in inhibition of mycelial growth against two phytopathogenic fungi, and activation of plant defense response such as induction of several defense-related gene expressions. In recent years, there has been an increasing interest in the use of chitosan as a protective agent in agriculture (21) and several biological functions have been reported (7, 8). Previous works showed that chitosan treatment suppressed fusarium crown and root rot of tomato caused by *F. oxysporum* f. sp. *radicis-lycopersici* (2, 3), and protected root rot by *Pythium aphanidermatum* of cucumber (8), and also reduced the incidence of decay of strawberry fruits by *Botrytis cinerea* and/or *Rhizopus stolonifer* (7).

We observed that chitosan treatments were significantly suppress disease incidense in plant against fusarium wilt caused by *F. oxysporum* f. sp. *lycopersici*,

and late blight caused by *P. infestans* of tomato plant (Fig. 1 and 2).

Chitosan, a positively-charged compound has applied as a seed treatment, a soil-drenching additive, and a foliar spray. The soil-irrigation of chitosan (500 and 1000 ppm) significantly suppressed wilting symptoms and root rots than untreated tomato plants against same pathogen (Table 1). Foliar spray of chitosan (1000 ppm and 2000 ppm) enhanced a level of protection to the disease incidence about 70% by inoculation of late blight pathogen *P. infestans* without causing phytotoxicity (Table 2). To investigate the mode of action of chitosan on suppression of two major tomato disease, we studied on the two aspects.

First, we examined *in vitro* antifungal activities to determine whether chitosan showed direct inhibition activity on both pathogen. In vitro antifungal assay demonstrated concentration dependent inhibition of the mycelial growth of *P. infestans* and *F. oxysporum* f. sp. *lycopersici* when amended in plate media (Fig. 4). Also, increase of the concentration of chitosan had signigficant inhibitory effect on spore germination of *F. oxysporum* f. sp. *lycopersici* (data not shown). The inhibitory property of chitosan is well-known and has been demonstrated by Hirano and Nagao (11). When chitosan was tested *in vitro*, chitosan inhibited the mycelial growth of several phytopathogenic fungi including soil-borne pathogens, with a marked effect according to molecular size. Chitosan is also known to interfere with the growth of various fungi including *B. cinerea* (7) and to induce severe morphological changes in *F. oxysporum* (3). However, the basis of the antifungal activity of chitosan is not yet clear, it seems to consist of more than one mode of action. Therefore, the property of the antifungal activity by chitosan may particulary contribute to suppress two diseases of tomato.

Also, several defense-related gene expression such as pathogenesis-related protein and phytoalexin biosynthesis genes were monitored. We showed that chitosan treatment (1000 ppm) non-specifically induced PR-proteins such as PR-1, β-1,3-glucanase, and chitinase mRNA expression, and also induced phytoalexin biosynthesis genes and APOD gene by time and concentration dependent manner (Fig. 5).

In our study, it is clearly suggested that chitosan

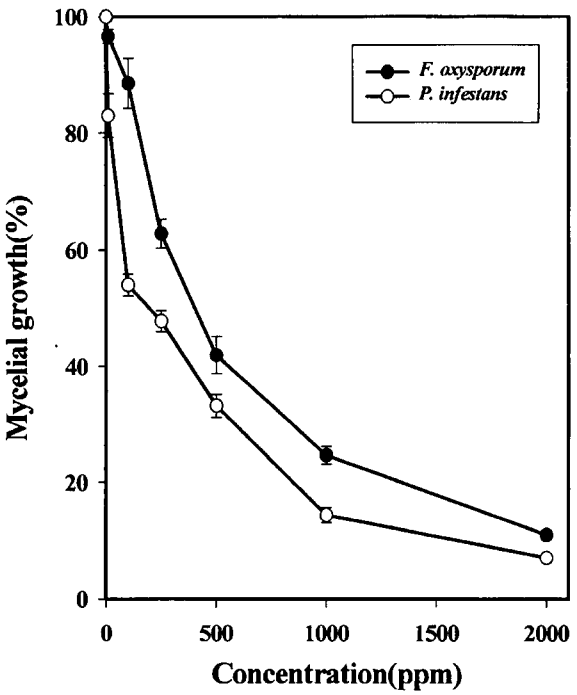


**Fig. 4.** Effect of chitosan on mycelial growth of *Fusarium oxysporum* on PDA (●), and *Phytophthora infestans* on V-8 juice agar (○) containg different concentration of chitosan. Data are presented as means with standard deviations of five individual plates.

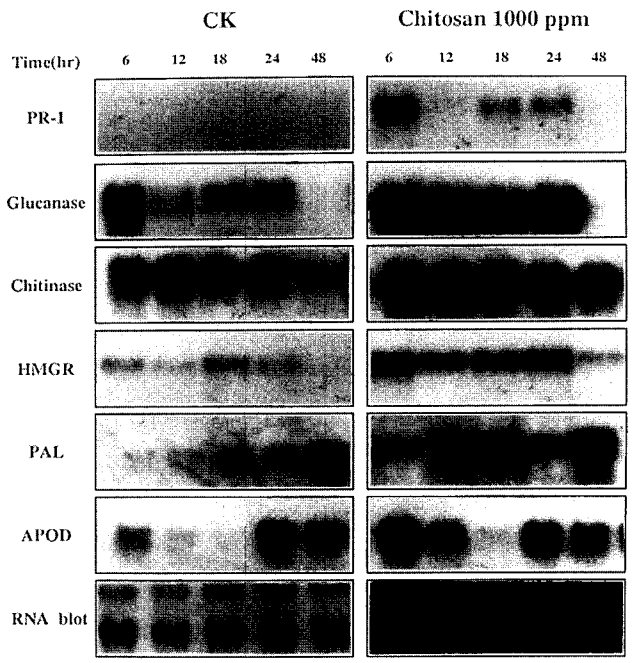


**Fig. 5.** Effect of chitosan on expression of defense-related genes in tobacco (RNA blots of PR-1, β-1,3-glucanase and APOD) or tomato (RNA blots of chitinase, HMGR and PAL). Total RNAs were extracted from tobacco and tomato leaves harvested 6, 12, 18, 24, and 48 hrs after 1000 ppm chitosan treatment. Twenty μg of total RNA samples were bloted on Nytran membrane and hybridized with six probes, respectively.

have dual functions on inhibition of mycelial growth of two pathogens, and induction of expressions of several defense-related genes including PR-protein genes, PAL, HMGR and APOD. All together, these results suggest that both antifungal and plant defense-inducing activities are involved in suppression of two major tomato diseases by chitosan treatment.

## 요 약

키토산 처리가 토마토의 생육 및 시들음병과 역병억제 효과를 조사하였던 바, 분무 또는 관주처리에 의하여 토마토 시들음병 및 역병이 현저하게 억제되었다. 키토산의 시들음병균과 역병균에 대한 균사생육억제는 분자량에 따라 다소 차이는 있었으나 농도가 증가함에 따라 균사생육억제효과가 높았다. 시들음병 및 역병균의 균사생육억제정도는 1000 ppm의 키토산(MW 30,000~50,000) 농도에서 각각 80%와 90% 이상의 억제효과를 보였다. 또한 키토산 처리에 따른 병저항성관련유전자발현 유도효과는 담배 및 토마토의 전체 RNA를 분리하여 Northern blot hybridization 분석을 한 결과, β-1,3-glucanase 및 chitinase 유전자는 강하게 유도되었고, PR-1, PAL, HMGR 및 APOD 유전자는 약하게 유도되었다. 따라서, 토마토 시들음병 및 역병억제효과는 키토산의 균사생육 억제 및 병저항성관련 유전자의 발현유도에 의한 복합작용이 주는 효과로 추정되었다.

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