



Plant Pathology Journal

ISSN 1812-5387

science
alert

ANSI*net*
an open access publisher
<http://ansinet.com>

Chitosan Inhibits the Growth of *Phytophthora botryosa*: The Causal Agent of Para Rubber Leaf Fall Disease

Anurag Sunpapao and Chaninun Pornsuriya
Department of Pest Management, Faculty of Natural Resources,
Prince of Songkla University, Songkhla 90112, Thailand

Abstract: The most destructive disease of Para rubber seedling is a leaf fall caused by *Phytophthora botryosa*. The bioactivity of chitosan against *P. botryosa* infection was studied *in vitro*. The growth rates of *P. botryosa* in potato dextrose agar were determined with different concentrations of chitosan. A chitosan concentration of 0.5 mg mL⁻¹ was sufficient to cause clear growth inhibition (about 87% reduction in diameter). Mycelia and oogonia wilt were observed in scanning electron micrographs of *P. botryosa*. The biodegradable chitosan therefore inhibits the *in vitro* growth of *P. botryosa* and may have potential for practical disease management of leaf fall.

Key words: Biological control, chitosan, leaf fall, Para rubber, *Phytophthora*

INTRODUCTION

Para rubber is one of the most important economic crops in Thailand. The major rubber-production areas are in southwest and northeast of Thailand. Like most other cultivated crops, rubber is facing serious problems from several diseases: after long periods of high rainfall, the leaf fall disease becomes a problem. Two species of *Phytophthora* are known as destructive diseases in rubber: both *Phytophthora palmivora* and *P. botryosa* are common.

The infection process of a soil borne pathogen, *Phytophthora*, starts at an immature pod resulting in pod rot and becomes the source of inoculum (Chantarapratin *et al.*, 2001). Spores of the pathogen are spread by rain splash from the infected leaves to the trapping panel (Johnston, 1989). In Thailand, leaf fall epidemics occur from June to December (Chantarapratin *et al.*, 2001). An obvious sign of *Phytophthora* caused leaf fall is the coagulated latex in a central lesion of the petiole (Fig. 1). However, lesions can occur anywhere along the length of the petioles. Heavy defoliation may lead to dieback of terminal branches (Chee, 1968; Turner, 1969; Johnston, 1989).

Application of fungicides, such as metalaxyl and fosetyl-Al, is the most effective method to control *Phytophthora* disease. However, chemical control may select for fungicide-resistant strains of these pathogens and is under review in many countries also due to human health concerns. Bioactive substances are actively

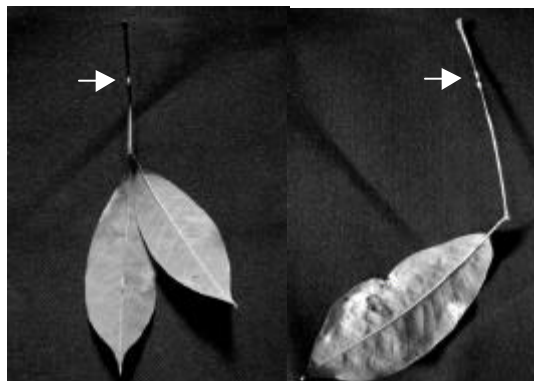


Fig. 1: Para rubber leaf fall symptoms. The white arrows indicate coagulated latex in central lesions on petioles

pursued for an alternative approach. The application of chitosan or derivatives of chitosan has been effective as a biological control against several fungi in several hosts (Allan and Hadwiger, 1979; Hirano and Nagao, 1989; Kendra *et al.*, 1989; Stossel and Leuba, 1984). Chitosan has reduced the disease severity and incidence of *Puccinia pimpinellae* (Saber *et al.*, 2009) and it had inhibitory effects on soil borne phytopathogenic fungi (Stossel and Leuba, 1984; Hernandez-Lauzardo *et al.*, 2011).

For these reasons the native form of low molecular weight chitosan was selected to test its activity against

the causal agents of para rubber leaf fall disease. Agar plates were prepared with various concentrations of chitosan and the mycelia and oogonia grown on the plates were also characterized from Scanning Electron Microscope (SEM) micrographs.

The objective of this study was to test the antimicrobial effects of chitosan on *P. botryosa*, the causal agent Para rubber leaf fall disease, *in vitro*.

MATERIALS AND METHODS

Cultures and growth conditions: The *P. botryosa* was kindly provided by Asst. Prof. S. Chuenchit (Department of Pest Management, Faculty of Natural Resources, Prince of Songkla University). The fungi were stored on Potato Dextrose Agar (PDA) (HiMedia, Mumbai, India) slants at 4°C. The fungal culture was routinely maintained on PDA made of 200 g potato, 20 g dextrose and 15 g agar, at room temperature (25-30°C) for 5 days, prior to testing with various concentrations of chitosan solution.

Preparation of chitosan: Low molecular weight (10253 kDa) chitosan was purchased from Aldrich, China. Purified chitosan was prepared as described by Benhamou *et al.* (1998). The stock solution (1%, w/v) of chitosan was prepared by dissolving purified chitosan in 0.5% (v/v) glacial acetic acid under continuous stirring and the pH was adjusted to 5.6 using 1 N NaOH (Du *et al.*, 1998). The chitosan solution was autoclaved (120°C, 20 min) prior to use in assays.

Effect of chitosan on radial growth: To evaluate the effect of chitosan on *Phytophthora* growth, PDA plates were amended with chitosan at different concentrations (0.125, 0.25, 0.5, 1 and 2 mg mL⁻¹) as by 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. The most prevalent para rubber pathogenic microorganism, *P. botryosa*, was assessed for its growth inhibition. Five 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. botryosa*. The colony radii were measured 7 days after the inoculations. The experiments were repeated twice. Percent Inhibition of Diameter Growth (PIDG) was calculated using the following formula:

$$\text{PIDG (\%)} = \frac{A - B}{A} \times 100$$

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

Statistical analyses: A Complete Randomized Design (CRD) was repeated twice for each experiment. Statistical analyses were run with SPSS software. Prior to such analyses, the growth effects of chitosan treatments were normalized to percentages relative to control. The differences between means from chitosan treatments and controls were tested for statistical significance by Duncan's Multiple Range Test (DMRT) for multiple comparisons.

Microscopic analysis: Mycelia and oogonia of *P. botryosa*, either untreated or treated with chitosan, were fixed by immersion in 2.5% glutaraldehyde in 1 mol L⁻¹ Phosphate Buffer (PB) at 4°C. They were then fixed with 0.1% osmium tetroxide in PB for 30 min at room temperature. The samples were dehydrated gradually with alcohol solutions (50, 70, 80, 95 and 100%) and then air-dried for one week before coating with gold/palladium. Finally they were transferred to Scanning Electron Microscope (SEM) stubs. Three random fields of view per sample were photographed.

RESULTS AND DISCUSSION

Chitosan solutions in five concentrations (0.125, 0.25, 0.5, 1 and 2 mg mL⁻¹) were tested for their inhibitory effect of *P. botryosa* linear growth. The antimicrobial activity of chitosan against *P. botryosa* is shown in Fig. 2 and Table 1. All concentrations reduced the growth of *P. botryosa* at 7 days post inoculation and the growth inhibition consistently increased with concentration. Effective inhibition was obtained with 0.5, 1 and 2 mg mL⁻¹ (87.5, 90.6 and 91.3% linear growth reduction, respectively). When the concentration was 0.5 mg mL⁻¹, the antimicrobial activity of chitosan was already at an acceptable level. The PDA amended with 0.05% acetic acid served as control. No growth differences were observed between this control and plain medium without acetic acid (data not shown).

Statistical analysis of the antifungal effect of chitosan concentration against *P. botryosa* is shown in Table 1. The activity increases with chitosan concentration, with the highest concentrations having a significantly different (p<0.01) effect from the lowest ones (and control). The plant pathogenic fungus-like organism *P. botryosa* was

Table 1: Effects of different concentrations of chitosan on radial growth of *P. botryosa*

Chitosan conc. (mg mL ⁻¹)	Inhibition rate
0.125	47.77±6.09 ^c
0.25	60.94±5.48 ^b
0.5	87.53±1.06 ^a
1.0	90.59±0.00 ^a
2.0	91.29±0.64 ^a

% inhibition rate is relative to corresponding control. Different superscripts indicate values that are significantly different (p<0.01)

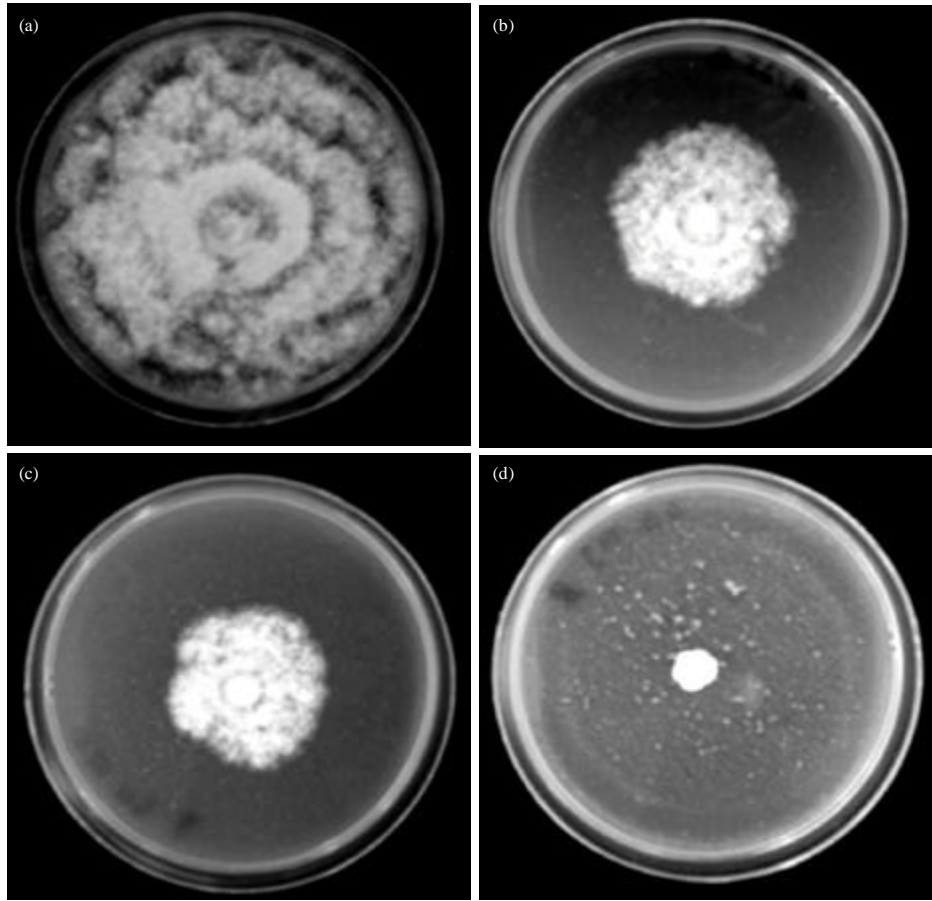


Fig. 2(a-d): Effects of chitosan on radial growth of *P. botryosa*. PDA amended with 0.05% acetic acid served as (a) A control, for comparison with chitosan solutions at (b) 0.125, (c) 0.25 and (d) 0.5 mg mL^{-1}

clearly sensitive to chitosan, so these *in vitro* results suggest that chitosan may be an effective growth inhibitor of *P. botryosa*.

Chitosan has been reported as an effective biocompound against several bacterial and fungal strains (Liman *et al.*, 2011). The results of the present study demonstrate that the plant pathogenic fungus-like organism *P. botryosa* is also sensitive to chitosan. Chitosan has great potential as a biodegradable substance. Recent studies have shown that chitosan is not only effective in inhibiting the growth of the pathogen but also in eliciting activities (Benhamou, 1996; El Ghaouth *et al.*, 1999; Barka *et al.*, 2004). It has been shown that mycelia growth of fungi is inhibited by chitosan. The level of inhibition was highly correlated with chitosan concentration (in the range 0.75-6.0 mg mL^{-1}), decreasing the radial growth of *Alternaria alternata*, *Botrytis cinerea*,

Colletotrichum gloeosporioides and *Rhizopus stolonifer* (El Ghaouth *et al.*, 1992b). The same effect was found on *Rhizoctonia solani* (Elmer and LaMondia, 1994) and *Sclerotium sclerotiorum* (Cheah *et al.*, 1997). Furthermore, seed coating with modified chitosan has inhibited *Sphacelotheca reiliana*, a causal agent of head smut of corn (Zeng *et al.*, 2010). The results on the radial growth of *P. botryosa* (a fungus-like organism) with full growth inhibition in 0.5% chitosan-amended PDA, are in agreement with to these prior observations.

Examination of mycelia and oogonia from *P. botryosa* exposed to chitosan (0.5 mg mL^{-1}) showed that they had suffered. SEM images of 0.5% chitosan treated and untreated mycelia and oogonia are shown in Fig. 3. Antimicrobial effects of chitosan are seen on *P. botryosa* as reduction of growth and wilt (Fig. 3b, d, f), when compared to control (Fig. 3a, c, e).

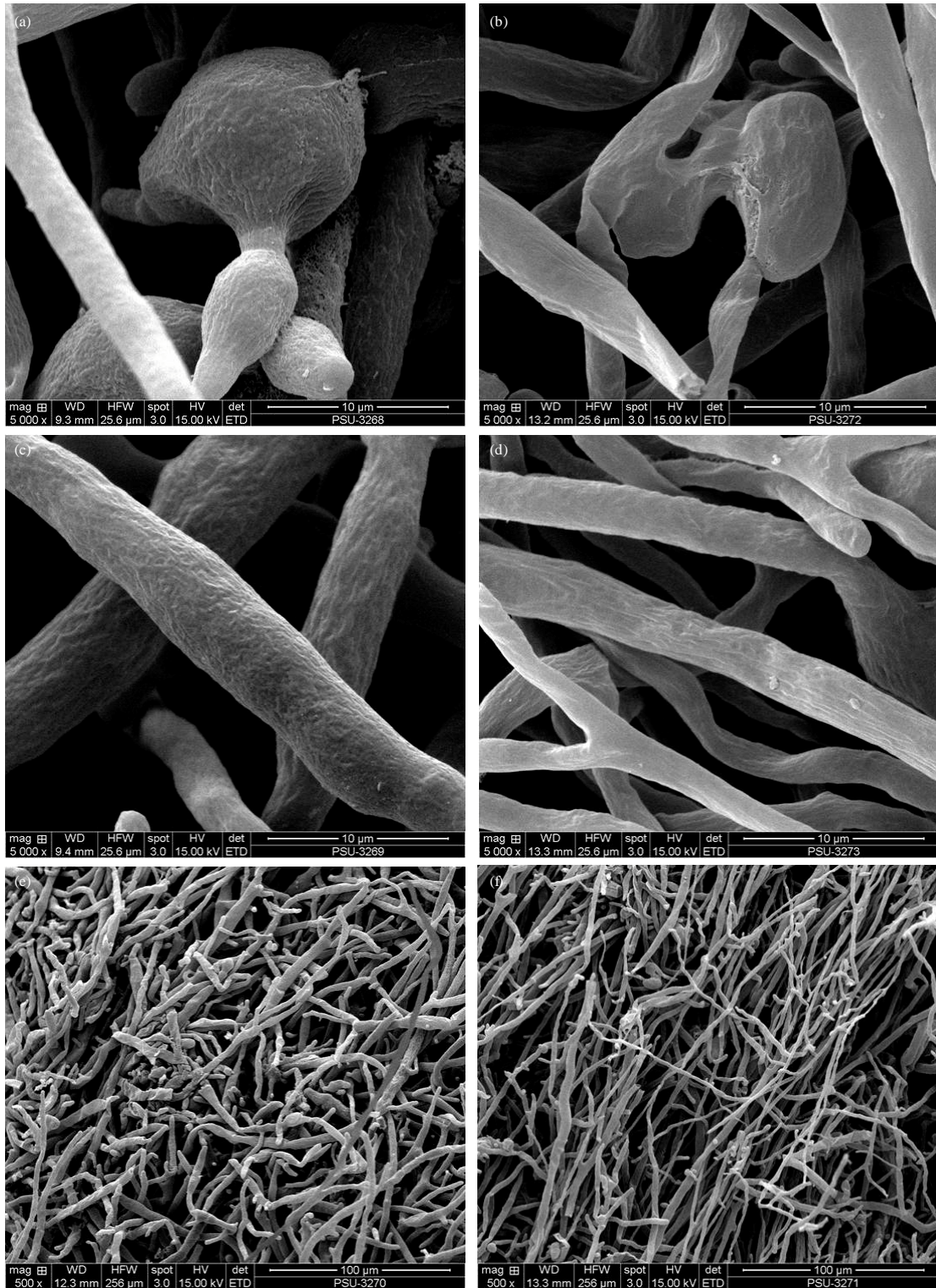


Fig. 3(a-f): Scanning electron micrographs of *Phytophthora botryosa* in PDA (a, c and f) and in PDA amended with 0.5 mg mL⁻¹ of chitosan (b, d and f)

Several fungi treated with chitosan had changes of morphology of the hyphae, when observed by microscopy. In some experiments *Fusarium oxysporum* f. sp. *radicis-lycopersici*, *R. stolonifer* and *S. sclerotiorum* treated with chitosan showed abnormal hyphae shape and size reduction (Benhamou and Theriault, 1992; El Ghaouth *et al.*, 1992a, b; Cheah *et al.*, 1997). Large vesicles or empty cells devoid of cytoplasm in the mycelium of *B. cinerea* and *F. oxysporum* f. sp. *albedinis* were also observed (Barka *et al.*, 2004; El-Hassni *et al.*, 2004). In this study, SEM micrographs showed reduction of size of *P. botryosa* as well as wither of mycelia and oogonia. Further studies are planned to follow up on this *in vitro* study, by assessing the management of leaf fall disease by chitosan application, in a greenhouse or in the field.

As a summary of current results chitosan, as a bioactive compound, directly inhibits the *in vitro* growth of *P. botryosa* and withers its mycelia and oospores.

ACKNOWLEDGMENT

This study was supported by the grants from the Prince of Songkla University, grant number NAT550131S. The copy-editing service of Research and Development Office (RDO) of Prince of Songkla University (PSU), especially the comments and suggestions of Assoc. Prof. Seppo Karrila, are gratefully acknowledged.

REFERENCES

- Allan, C.R. and L.A. Hadwiger, 1979. The fungicidal effect of chitosan on fungi of varying cell wall composition. *Exp. Mycol.*, 3: 285-287.
- Barka, E., P. Eullaffroy, C. Clement and G. Vernet, 2004. Chitosan improves development and protects *Vitis vinifera* L. against *Botrytis cinerea*. *Plant Cell Rep.*, 22: 608-614.
- Benhamou, N. and G. Theriault, 1992. Treatment with chitosan enhances resistance of tomato plants to the crown and root rot pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici*. *J. Physiol. Mol. Plant Pathol.*, 41: 33-52.
- Benhamou, N., 1996. Elicitor-induced plant defense pathways. *Trends Plant Sci.*, 1: 233-240.
- Benhamou, N., J.W. Klopper and S. Tuzun, 1998. Induction of resistance against *Fusarium* wilt of tomato by combination of chitosan with an endophytic bacterial strain: Ultra structure and cytochemistry of the host response. *Planta*, 204: 153-168.
- Chantarapratin, U., P. Pattanakul, N. Changreung, A. Rojanasujit, P. Romreunsukarom and A. Ramlee, 2001. Rubber diseases survey on large scale clone trail. Research Report, Rubber Research Institute Thailand, pp: 1-52.
- Cheah, L.H., B.B.C. Page and R. Shepherd, 1997. Chitosan coating for inhibition of sclerotinia rot of carrots. *N. Z. J. Crop Hortic. Sci.*, 25: 89-92.
- Chee, K.H., 1968. *Phytophthora* leaf disease in Malaysia. *J. Rubber Res. Inst. Malaya*, 21: 79-86.
- Du, J., H. Gemma and S. Iwahori, 1998. Effects of chitosan coating on the storability and on the ultrastructural changes of Jonagold apple fruit in storage. *Food Preserv. Sci.*, 24: 23-29.
- El Ghaouth, A., J. Arul, A. Asselin and N. Benhamou, 1992a. Antifungal activity of chitosan on post-harvest pathogens: Induction of morphological and cytological alterations in *Rhizopus stolonifer*. *Mycol. Res.*, 96: 769-779.
- El Ghaouth, A., J. Arul, J. Grenier and A. Asselin, 1992b. Antifungal activity of chitosan on two postharvest pathogens of strawberry fruits. *Phytopathology*, 82: 398-402.
- El Ghaouth, A., J.L. Smilanick, G.E. Brown, A. Ippolito, M. Wisniewski and C.L. Wilson, 1999. Application of *Candida saitoana* and glycolchitosan for the control of postharvest diseases of apple and citrus fruit under semi-commercial conditions. *Plant Dis.*, 84: 243-248.
- El-Hassni, M., A. El-Hadrami, F. Daayf, E. Ait Barka and I. El-Hadrami, 2004. Chitosan, antifungal product against *Fusarium oxysporum* f. sp. *albedinis* and elicitor of defence reactions in date palm roots. *Phytopatho. Mediterranea*, 43: 195-204.
- Elmer, W.H. and J.A. LaMondia, 1994. Chitosan inhibits *Rhizoctonia fragariae* but not strawberry black root rot. *Adv. Strawberry Res.*, 13: 26-31.
- Hernandez-Lauzardo, A.N., M.G. Velazquez-del Valle and M.G. Guerra-Sanchez, 2011. Current status of action mode and effect of chitosan against phytopathogens fungi. *Afr. J. Microbiol. Res.*, 5: 4243-4247.
- Hirano, A. and N. Nagao, 1989. Effects of chitosan, pectic acid, lysozyme and chitinase on the growth of several phytopathogens. *Agric. Biol. Chem.*, 53: 3065-3066.
- Johnston, A., 1989. Diseases and Pests. In: Rubber, Webster, C.C. and W.J.I. Baulkwil (Eds.). Longman Scientific and Technical, New York, USA., pp: 415-458.
- Kendra, D.F., D. Christian and L.A. Hadwiger, 1989. Chitosan oligomers from *Fusarium solani*/pea interactions, chitinase/ β -glucanase digestion of sporelings and from fungal wall chitin actively inhibit fungal growth and enhance disease resistance. *Physiol. Mol. Plant Pathol.*, 35: 215-230.

- Laflamme, P., N. Benhamou, N. Bussieres and M. Dessureault, 2000. Differential effect of chitosan on root rot fungal pathogens in forest nurseries. *Can. J. Bot.*, 77: 1460-1468.
- Liman, Z., S. Selmi, S. Sadok ad A. El Abed, 2011. Extraction and characterization of chitin and chitosan from crustacean by-products: Biological and physicochemical properties. *Afr. J. Biotechnol.*, 10: 640-647.
- Saber, W.I.A., K.M. Ghoneem, M.M. El-Metwally and M.A. Elwakil, 2009. Identification of *Puccinia pimpinellae* on Anise plant in Egypt and its control. *Plant Pathol. J.*, 8: 32-41.
- Stossel, P. and J.L. Leuba, 1984. Effect of chitosan, chitin and some aminosugars on growth of various soilborne phytopathogenic fungi. *J. Phytopathol.*, 111: 82-90.
- Turner, P.D., 1969. Diseases of hevea rubber in Thailand: With particular reference to those associated with *Phytophthora* species. Report No. FAO-AGP--THA/64/515, Rubber Research Center Thailand, pp: 1-86.
- Zeng, D., X. Mei and J. Wu, 2010. Effects of an environmentally friendly seed coating agent on combating head smut of corn caused by *Sphacelotheca reiliana* and corn growth. *J. Agric. Biotechnol. Sustainable Dev.*, 2: 108-112.