American Journal of Agriculture and Forestry
2016; 4(2): 15-22
http://www.sciencepublishinggroup.com/j/ajaf
doi: 10.11648/j.ajaf.20160402.12
ISSN: 2330-8583 (Print); ISSN: 2330-8591 (Online)

Inhibitory Effects of Oligochitosan on Pathogenic Fungi Isolated from *Zanthoxylum bungeanum*

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To cite this article:

Peiqin Li, Zhou Wu, Tao Liu, Yanan Wang. Inhibitory Effects of Oligochitosan on Pathogenic Fungi Isolated from *Zanthoxylum bungeanum*. *American Journal of Agriculture and Forestry.* Vol. 4, No. 2, 2016, pp. 15-22. doi: 10.11648/j.ajaf.20160402.12

Received: February 13, 2016; **Accepted**: February 27, 2016; **Published**: April 9, 2016

Abstract: To explore nontoxic degradable natural substances which could be used to control *Zanthoxylum bungeanum* diseases, the effects of oligochitosans, i.e., OCHA and OCHB, on pathogenic fungi *Pseudocercospora zanthoxyli, Fusarium sambucinum* and *Phytophthora boehmeriae* were investigated. Excellent inhibitory effects of OCHA and OCHB on the growth of all tested pathogens were observed, which were calculated by RGI and BGI. The highest inhibitions for *P. zanthoxyli* and *F. sambucinum were induced by* 1.0 mg/mL OCHB with the corresponding RGI values as 51.25% and 95.69%, and BGI values as 44.76% and 92.34%. For *P. boehmeriae*, the maximum values of RGI and BGI were induced by 1.0 mg/mL OCHA with the corresponding values as 82.35% and 53.24%. Desirable results obtained from the present research might establish the foundation for the utilization of oligochitosan for the nuisanceless control of *Z. bungeanum* diseases.

Keywords: Oligochitosan, Pathogenic Fungi, Growth Inhibition, *Zanthoxylum bungeanum*

1. Introduction

These years, the control of plant diseases and pests has been facing a huge challenge. Although utilization of chemical pesticide to control plant disease could bring great advantages, the excessive use has taken its toll on human health and natural environment [1-4]. Furthermore, the registration procedures of broad-spectrum pesticides but low-toxic to human are extremely complicated and time-consuming, while the resistance of plant pathogen to pesticide is also one main problem faced by growers [5, 6]. Hence, there is a worldwide trend to explore new alternatives for synthetic pesticides, which can not only protect plant against the infection of pathogen but also avoid negative and side effects on human health because of abuse of chemical pesticides.

Oligochitosan (OCH), which is the degraded product of chitosan or chitin and abundant in nature, has aroused an increasing attention for its various biological properties, such as anti-tumor and antioxidant activities, antimicrobial activities, immuno-modulating effects, and so on [7]. Of them, the antimicrobial activity of OCH has been especially concerned by many plant pathologists because of its nontoxic and degradable characteristics [8]. The inhibitory effects of OCH on the growth of plant pathogen have been frequently reported in previous researches, such as *Fusarium solani* [9], *Puccinia arachidis* [10], *Alternaria alternate* [11], *Aspergillus niger* [12], *Botrytis cinerea* [13, 14], and so on. However, up till now, there are no related researches on the inhibitory effects of OCH on the pathogenic fungi isolated from *Zanthoxulum bungeanum*.

*Z. bungeanum*is an aromatic plant of Rutaceae *Zanthoxylum* as shrubs or small trees and native to southwestern China, which has been considered as an important economic crop for its special seasoning function [15, 16]. However, *Z. bungeanum*is frequently infected by different kinds of plant pathogenic fungi during its growth process, which has generated serious effects on the yield and quality of *Z. bungeanum* [17]. The most reported pathogenic fungi isolated from *Z. bungeanum* were *Pseudocercospora*

zanthoxyli, *Fusarium sambucinum* and *Phytophthora boehmeriae* in Shaanxi and Gansu districts, which respectively resulted in prickly-ash leaf mold, stem blight and dry rot [18-20]. Up until now, the main method of controlling the above mentioned pathogenic fungi is still utilization of chemical pesticide [21]. Although the application of chemical pesticides has brought great advantages, the side effects of synthetic pesticides on crops should not be neglected [6, 22]. Hence, it is very necessary to explore new nontoxic and efficient alternatives to synthetic pesticides to control plant pathogenic fungi.

The main aim of this research is to investigate the effects of OCH on the growth of pathogenic fungi of *Z. bungeanum*, i.e., *P. zanthoxyli*, *F. sambucinum*and *P. boehmeriae*, which would establish the basement for the nuisanceless prevention and management of *Z. bungeanum* diseases.

2. Materials and Methods

2.1. Chemicals

OligochitosanA (OCHA) and oligochitosan B (OCHB) were purchased from Qindao BZ-Oligo Biotech Co. Ltd (Qiandao, China). OCHA was the hydrolyzate mixture of chitosan by acid, but OCHB was the hydrolyzate mixture of chitosan by enzyme. All the other chemicals were purchased from JieCheng Chemical and Glass Company (Yangling, Shaanxi, China). The preliminary structural analyses of OCHA and OCHB were demonstrated in Section of 3.1.

2.2. Plant Pathogenic Fungi

The tested pathogenic fungi of *Pseudocercospora zanthoxyli*, *Fusarium sambucinum* and *Phytophthora boehmeriaewere* isolated and preserved in our lab by previous study [18-20], all of which were cultured and preserved on Potato Dextrose Agar (PDA) medium.

2.3. Determination of the Effects of OCHA and OCHB on the Radial Growth of Tested Pathogenic Fungi

The inhibitory effects of OCHA and OCHB on the tested pathogenic fungi were firstly conducted by checking the growth of radial colony on PDA. Both of OCHA and OCHB were separately dissolved in sterile distilled water, and then filtered through a sterile filter membrane (pore size, 0.45 um). For the radial colony growth determination, the sterile oligochitosan solution was added into PDA at 60ºC with the final concentrations of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL, respectively, which were then mixed rapidly and poured into Petri dishes (diameter, 9 cm). The same volume of sterile distilled water was added into PDA as the control. After the PDA plate cooled, a 5-mm-diameter mycelial plug of *tested pathogen* was inoculated on the center of the PDA plate and incubated at 25ºC in dark. When the tested pathogenic fungi were cultured for seven days, the diameter (R) of each colony was measured respectively [23]. Each treatment was carried out for three duplicates. Radial growth inhibition (RGI) was calculated according to the following formula:

$$RGI\ (\%) = (R_0\text{-}R) \times 100\%/R_0 \quad (1)$$

where R_0 is the diameter of colony of control, R is the diameter of colony of treatment.

2.4. Evaluation of the Effects of OCHA and OCHB on Mycelial Biomass Growth of Tested Pathogenic Fungi

To determinate the effects of oligochitosan on mycelial biomass of *tested pathogenic fungi*, the submerged culture was carried out in PDB liquid medium. Each 1000-mL flask was filled with 300 mL PDB medium, and then sterile oliogochitosan solution was added into PDB. The final concentrations of both OCHA and OCHB in PDB were separately 0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL. And then, 0.5 mL seven-day-old suspension culture of *tested pathogen* in PDB was taken as the inoculum and injected into each flask containing PDB liquid medium with oligochitosan supplementation. All the flasks were maintained on a rotary shaker at 150 rpm at 25ºC. Seven days after inoculation, the mycelial biomass of each tested pathogen in each flask was respectively by filtrating under vacuum to obtain the mycelia, which were further lyophilized to a constant dry weight (DW) and expressed as gram per liter [24]. The effect of oligochitosan on mycelial biomass of each tested pathogenic fungus was calculated by the biomass growth inhibition (BGI) according to the following formula:

$$BGI\ (\%) = (DW_0\text{-}DW) \times 100\%/\ DW_0 \quad (2)$$

where DW_0 is the mycelial dry weight of control, DW is the mycelial dry weight of treatment.

2.5. Monitoring of Time Dynamics of RGI and BGI Respectively for the Three Tested Pathogenic Fungi

To investigate the effects of OCHA and OCHB on the radial colony and biomass growth of *P. zanthoxyli, F. sambucinum*and *P. boehmeriae* as culture time varied under certain concentration, time dynamics of RGI and BGI were calculated. The selected concentrations were respectively 0.5 and 1.0 mg/mL. And the checking time were designated on the culture day of 2, 4, 6, 8, 10, 12 and 14, separately. The evaluations of RGI and BGI were the same as described above.

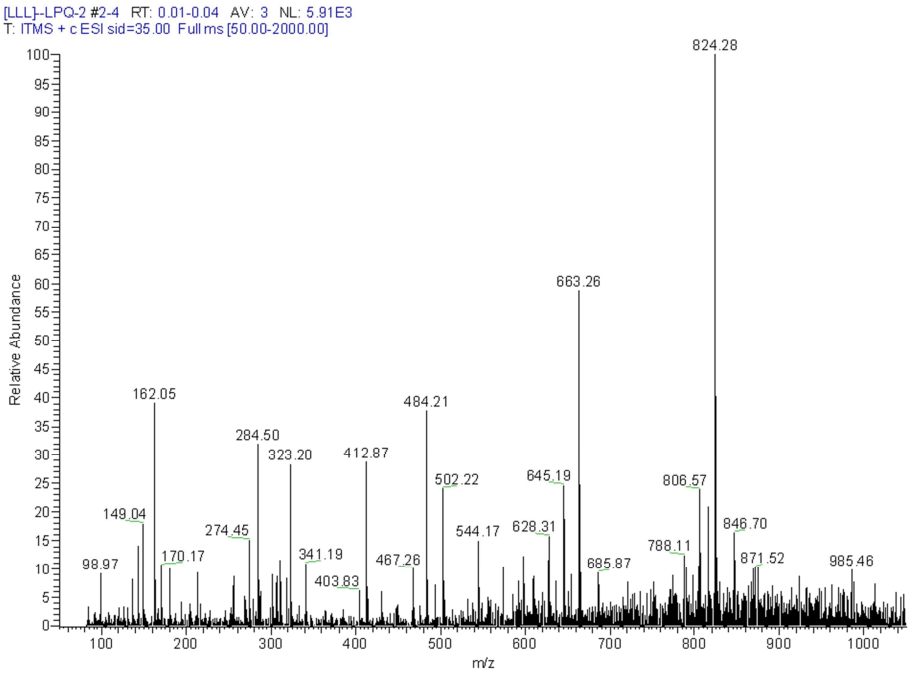
3. Results and Discussion

3.1. ESI-MS and IR Analyses of OCHA and OCHB

The ESI-MS spectra of OCHA and OCHB were presented in Figure1. As shown in Figure 1, the molecular weight of OCHA or OCHB was below 1000 Da, and some fragement ion peaks of oligochitosan were observed. Figure 1A showed the oligosaccharide nature of OCHA with one oligochitosan residue molecular weight ($C_6H_{11}O_4N$, 161 Da) differences among the ion peaks with the m/z respectively at 985.46,

824.28, 663.26, 502.22 and 341.19, or 806.57, 645.19, 484.21, 323.20 and 162.05. For OCHB, one oligochitosan residue molecular weight ($C_6H_{11}O_4N$, 161 Da) difference was also observed among the ion peaks with the m/z respectively at 824.39, 663.26 and 502.23, or 789.46, 645.27, 484.19, 323.18 and 162.06 (Figure 1B).

The FT-IR of OCHA and OCHB were shown in Figure 2, which demonstrated the characteristic absorption peaks of oligochitosan, including those peaks respectively at 3448 cm^{-1} (the absorption of stretching vibration of associated –OH groups), 2932 cm^{-1} (the absorption of stretching vibration of C–H bond), 1632 cm^{-1} (the absorption of flexural vibration of N–H bond), 1410 cm^{-1} (the absorption of flexural vibration of O–H bond), 1340 cm^{-1} (the absorption of stretching vibration of C–N bond), 1070 cm^{-1} (the absorption of stretching vibration of C–O–C bond) and 1020 cm^{-1} (the absorption of stretching vibration of C–OH bond). Besides, the FT-IR spectrum of OCHA was extremely similar to that of OCHB, which might indicate the consistency of chemical components of OCHA and OCHB.



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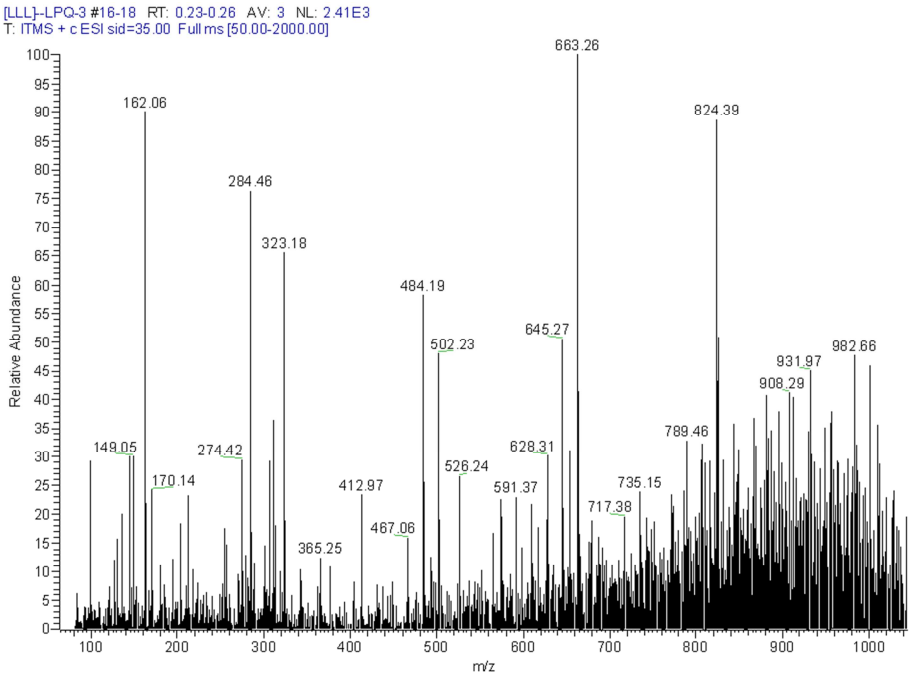

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Figure 1. *The positive ion ESI-MS spectra of OCHA (A) and OCHB (B).*

Both of OCHA and OCHB were crude samples not pure compounds. Hence, we just conducted their ESI-MS and IR experiments. However, all the above analyses proved the oligochitosan nature of OCHA or OCHB.

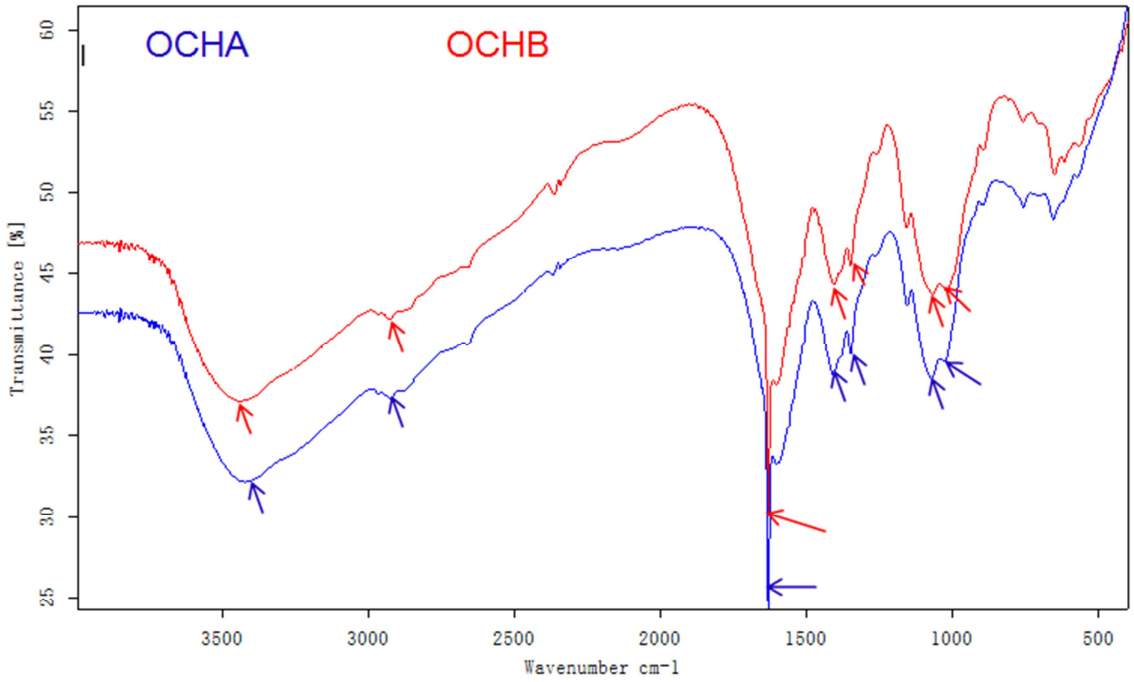


Figure 2. *The Fourier transform infrared spectra of OCHA and OCHB.*

3.2. Effects of OCHA and OCHB on Radial Colony Growth of Tested Pathogenic Fungi

The effects of OCHA and OCHB on the radial colony growth were summarized in Table 1, which displayed that both of OCHA and OCHB could inhibit the radial colony growth of all three tested pathogenic fungi. For P. zanthoxyliand F. sambucinum, OCHA showed more effective inhibition than that of OCHB when both of them were used at low concentrations. However, when both of OCHA and OCHB were applied at the concentration of 0.6 mg/mL or higher than o.6 mg/mL, OCHB exhibited stronger inhibitory effects on P. zanthoxyliand F. sambucinum. For P. boehmeriae, OCHA always displayed higher inhibitory effects than that of OCHB among all tested concentrations. The inhibitory effects of both of OCHA and OCHB on all tested pathogenic fungi showed concentration-dependent relationship, which became more obvious as the concentration of OCHA or OCHB was higher. The strongest inhibitory effects were induced by 1.0 mg/mL oligochitosan, when the corresponding highest RGI values induced by OCHA were respectively 40.18%, 78.92% and 77.85% for P. zanthoxyli, F. sambucinum and P. boehmeriae. For OCHB, the maximum RGI values for P. zanthoxyli, F. sambucinum and P. boehmeriae were separately 47.58%, 81.27% and 68.25%. By comprehensive analyzing Table 1, OCHA or OCHB showed the strongest inhibitory effects on the radial colony growth of F. sambucinum, P. boehmeriae subsequently, and the weakest inhibition on P. zanthoxyli.

Table 1. *Effects of COHA and OCHB on radial colony growth of P. zanthoxyli, F. sambucinum and P. boehmeriae on the 7th day.*

Oligo-chitosan	Concentration (mg/mL)	RGI (%)		
		P. zanthoxyli	*F. sambucinum*	*P. boehmeriae*
OCHA	0.2	26.52 ± 3.06f	41.23 ± 1.27g	36.00 ± 1.27h
	0.4	27.11 ± 2.01f	52.14 ± 1.79e	45.01 ± 0.56f
	0.6	29.26 ± 2.41e	63.58 ± 1.97d	61.04 ± 0.83d
	0.8	35.57 ± 2.31d	72.32 ± 2.71c	70.24 ± 1.01b
	1.0	40.18 ± 1.27c	78.92 ± 1.58b	77.85 ± 1.39a
OCHB	0.2	20.56 ± 0.37h	33.57 ± 1.57h	28.33 ± 0.56i
	0.4	25.00 ± 4.07g	49.25 ± 2.06f	37.96 ± 2.74g
	0.6	35.74 ± 1.98d	73.58 ± 2.27c	57.59 ± 2.32e
	0.8	42.35 ± 1.24b	78.27 ± 2.27b	61.34 ± 1.58d
	1.0	47.58 ± 1.45a	81.27 ± 3.80a	68.25 ± 1.21c

Note: Each value was expressed as mean ± standard deviation (n = 3). The significant difference analyses of RGI for each pathogen were respectively carried out at p = 0.05 level. Different letters indicated significant differences under different treatments for each pathogen.

3.3. Effects of OCHA and OCHB on Mycelial Biomass of Tested Pathogenic Fungi

To determine the effects of OCHA and OCHB on the mycelia biomass growth, the submerged liquid culture of all three tested pathogenic fungi were conducted in PDB medium supplemented with OCHA or OCHB, which was calculated by the indicator of BGI (%). Table 2 summarized all the BGI values caused by OCHA and OCHB respectively for P. zanthoxyli, F. sambucinum and P. boehmeriae.

Table 2. *Effects of COHA and OCHB on mycelial biomass of P. zanthoxyli, F.sambucinum and P. boehmeriaeon the 7th day.*

Oligo-chitosan	Concentration (mg/mL)	BGI (%)		
		P. zanthoxyli	*F. sambucinum*	*P. boehmeriae*
OCHA	0.2	18.35 ± 0.97h	33.33 ± 1.03i	21.12 ± 1.32i
	0.4	20.34 ± 1.34g	57.35 ± 2.38g	27.24 ± 1.04g
	0.6	24.32 ± 1.67f	67.78 ± 1.65e	39.35 ± 2.14e
	0.8	33.25 ± 1.27d	76.25 ± 2.56c	45.27 ± 1.87c
	1.0	40.57 ± 2.01b	81.25 ± 3.57b	50.24 ± 1.37a
OCHB	0.2	16.34 ± 0.97i	39.78 ± 1.97h	20.24 ± 0.78i
	0.4	20.47 ± 1.54g	64.32 ± 2.76f	25.37 ± 1.47h
	0.6	27.25 ± 1.25e	72.21 ± 2.13d	34.37 ± 0.94f
	0.8	35.67 ± 1.29c	82.35 ± 2.47b	42.27 ± 1.39d
	1.0	43.25 ± 1.57a	87.25 ± 3.04a	47.25 ± 2.34b

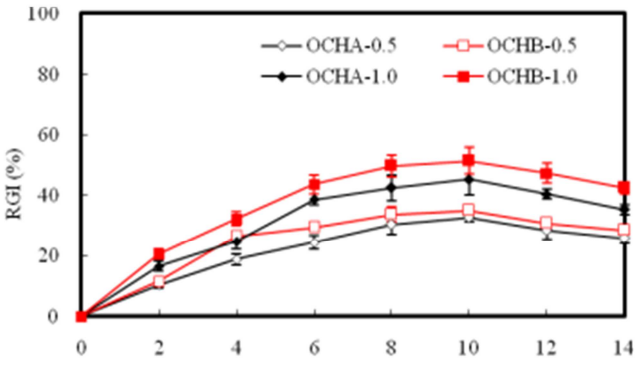
Note: Each value was expressed as mean ± standard deviation (n = 3). The significant difference analyses of RGI for each pathogen were respectively carried out at p = 0.05 level. Different letters indicated significant differences for every pathogen under different treatments.

Either OCHA or OCHB could inhibit the mycelia biomass growth of all tested fungi significantly. Furthermore, BGI showed a concentration-dependent relationship with OCHA or OCHB. The larger the concentration of OCHA or OCHB was, the higher the BGI was. The maximum BGI values induced by OCHA respectively for P. zanthoxyli, F. sambucinum and P. boehmeriae were 40.57%, 81.25% and 50.24% when OCHA was employed at 1.0 mg/mL. For OCHB, the highest BGI were separately 43.25%, 87.25% and 47.25% when 1.0 mg/mL OCHB was respectively treated P. zanthoxyli, F. sambucinum and P. boehmeriae. As demonstrated in Table 2, OCHA or OCHB showed the strongest inhibitory effects on the mycelia biomass growth of F. sambucinum, P. boehmeriae subsequently, and the weakest inhibition on P. zanthoxyli, which was coincident with their effects on radial colony growth.

3.4. Time Dynamics of RGI and BGI for Tested Pathogenic Fungi

To investigate the effects of OCHA and OCHB on the radial colony and mycelia biomass growth of tested pathogenic fungi on different cultured time, their RGI and BGI were respectively calculated on the cultured days of 2, 4, 6, 8, 10, 12 and 14. The time dynamics of RGI and BGI for the tested pathogenic fungi were graphed in Figure 3. As presented in Figure 3A, the radial colony growth of P. zanthoxyli was inhibited by OCHA or OCHB during examining period, and the RGI value was improved as the cultured time from the beginning to the 10 day and then decreased slightly. The variation tendency of BGI of P. zanthoxyli was similar to that of RGI, and the maximums of BGI were observed on the 8 day as shown in Figure 3B. The time dynamics of RGI and BGI of F. sambucinum were exhibited in Figure 3C and 3D. From Figure 3C, it was seen that when F. sambucinum was treated by OCHA or OCHB at the concentration of 0.5 mg/mL, the maximum of RGI was observed on the 4 day, and then kept relatively constant till to the 8 day, after which the RGI was decreased. However, when the concentration of OCHA or OCHB was increased to 1.0 mg/mL, RGI was significantly increased from the beginning and reached to the maximum on the day of 10. The variation trend of BGI of F. sambucinum was similar to its RGI as presented in Figure3D. The dynamics of RGI and BGI of P. boehmeriae were shown in 3E and 3F, which indicated RGI or BGI reached the maximum either on the 8 day or 10 day and OCHA exhibited stronger inhibitory effects than that of OCHB.

For P. zanthoxyli and F. sambucinum, the maximum RGI values were induced by 1.0 mg/mL OCHB on the 10 day, with their corresponding values as 51.25% and 95.69%, while the maximum BGI values were respectively got on the 8 day and 10 day still induced by 1.0 mg/mL OCHB with corresponding values as 44.76% and 92.34%. For P. boehmeriae, the maximum values of RGI and BGI were respectively obtained on the 10 day and 8 day induced by 1.0 mg/mL OCHA, which were separately 82.35% and 53.24%.



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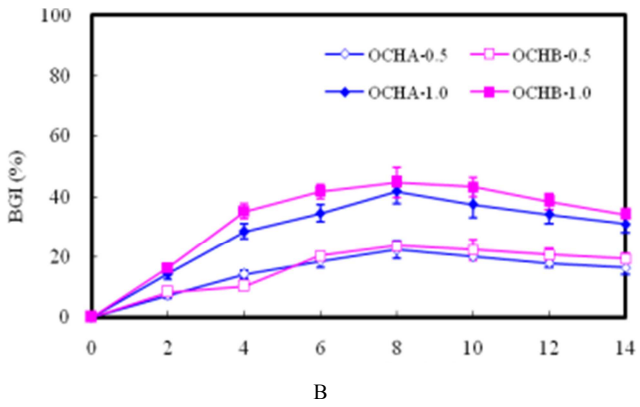

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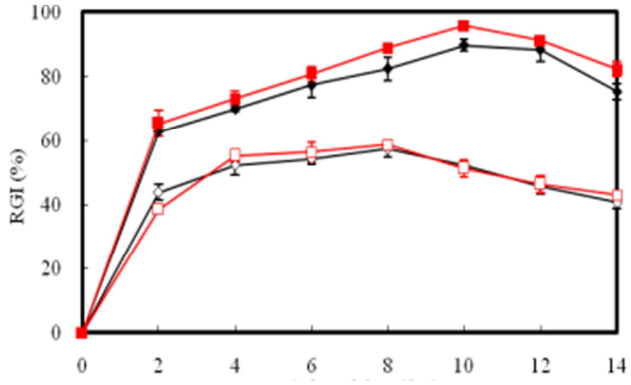

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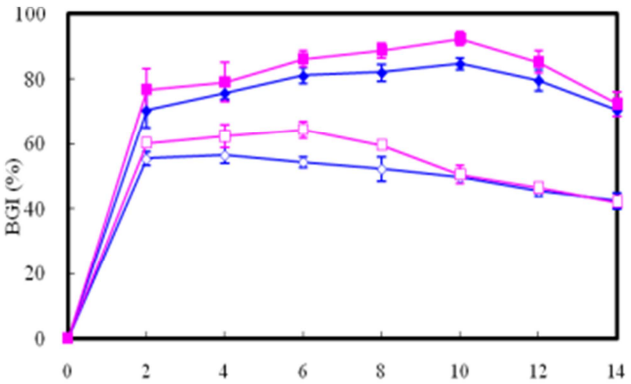

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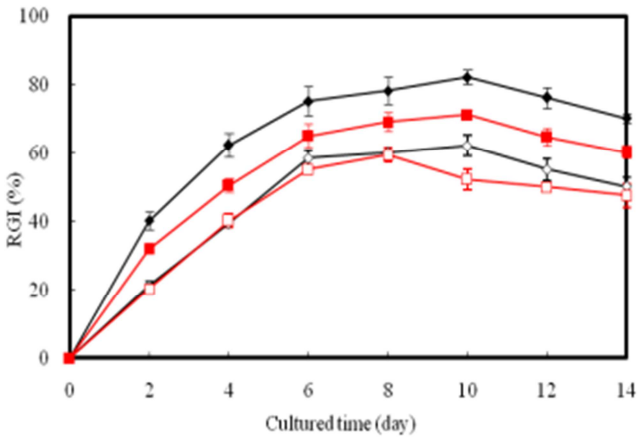

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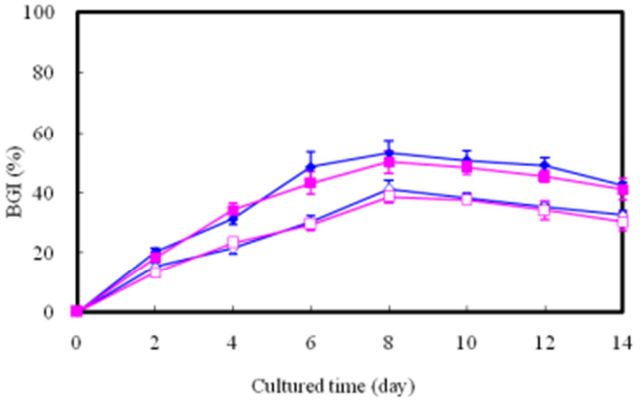

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Figure 3. *Ime dynamics of RGI and BGI respectively for P. zanthoxyli (A and B), F. sambucinum (C and D) and P. boehmeriae (E and F).*

It has been extensively reported that oligochitosan exhibited excellent antimicrobial activities [25]. The present research first studied the inhibitory effects of oligochitosan on pathogenic fungi isolated from Z. bungeanum and desirable results were obtained. Both of OCHA and OCHB processed excellent inhibitory effects on the tested pathogenic fungi. However, the differences of inhibition capabilities between OCHA and OCHB were also observed, which might be attributed to their different preparation methods leading to their different components. Both of OCHA and OCHB had a molecular weight lower than 1000. The comprehensive analyses of their ESI-MS and IR spectra demonstrated the oligosaccharide property. Although both of OCHA and OCHB were not pure oligosaccharide, just crude samples, their obvious inhibitory effects on pathogenic fungi demonstrated their potential as substitute for pesticides to control plant diseases.

Furthermore, it was also observed that the inhibitory effects of OCHA or OCHB on tested pathogenic fungi were related to its concentrations and treated time. The optimal concentration was the highest concentration 1.0 mg/mL in designed ranges, which might indicate a higher concentration of oligochitosan will benefit plant disease control. The maximum inhibitory effects were observed either on the 8 day or 10 day, which demonstrated OCHA or OCHB has long-lasting effective duration. In the present research, OCHA exhibited stronger inhibitory effects on P. zanthoxyli and F. sambucinum than that of OCHB. But for P. boehmeriae, the inhibitory ability of OCHA was slightly weaker than that of OCHB. The present research might provide the illumination for the nuisanceless control of Z. bungeanum diseases. However, all the experiments of the present investigation were carried out in laboratory. The protective effects of OCHA and OCHB on Z. bungeanum in the field against plant diseases are worth further investigating.

4. Conclusion

In conclusion, the excellent inhibitory effects of OCHA and OCHB were observed in laboratory by examining the

variations of RGI and BGI of the pathogenic fungi *P. zanthoxyli, F. sambucinum* and *P. boehmeriae* isolated from *Z. bungeanum*. The highest inhibitions on the growth of radial colony and mycelia biomass of *P. zanthoxyli* and *F. sambucinum were induced by* 1.0 mg/mL OCHB with the corresponding RGI values as 51.25% and 95.69%, and BGI values as 44.76% and 92.34%. For *P. boehmeriae*, the maximum values of RGI and BGI were induced by 1.0 mg/mL OCHA with the corresponding values as 82.35% and 53.24%. Desirable results obtained from the present research might establish the basement for the utilization of oligochitosan for the nuisanceless control of *Z. bungeanum* diseases.

Acknowledgements

This work was financially supported by the National Natural Science Fundation of China (NO.31300542), the Natural Science Foundation of Shaanxi Province (NO.2015JQ3084), the Fundamental Research Funds for the Central Universities of Northwest A&F University (NO.Z109021422; NO.2452015336) and the Scientific Research Start-up Funding supported by Northwest A&F University (NO.Z111021303).

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