Carbohydrate Polymers 111 (2014) 670–682

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Antimicrobial and inhibitory enzyme activity of *N*-(benzyl) and quaternary *N*-(benzyl) chitosan derivatives on plant pathogens

Mohamed E.I. Badawy[a,*], Entsar I. Rabea[b], Nehad E.M. Taktak[c]

[a] Department of Pesticide Chemistry and Technology, Faculty of Agriculture, 21545-El-Shatby, Alexandria University, Alexandria, Egypt
[b] Department of Plant Protection, Faculty of Agriculture, Damanhour University, 22516 Damanhour, Egypt
[c] Department of Tropical Health, High Institute of Public Health, Alexandria University, Alexandria, Egypt

ARTICLE INFO

Article history:
Received 6 February 2014
Received in revised form 26 April 2014
Accepted 28 April 2014
Available online 4 May 2014

Keywords:
Antibacterial activity
Antifungal activity
Biochemical studies
N-(benzyl) and quaternary *N*-(benzyl) chitosan derivatives
Spectroscopy

ABSTRACT

Chemical modification of a biopolymer chitosan by introducing quaternary ammonium moieties into the polymer backbone enhances its antimicrobial activity. In the present study, a series of quaternary *N*-(benzyl) chitosan derivatives were synthesized and characterized by ^{1}H-NMR, FT-IR and UV spectroscopic techniques. The antimicrobial activity against crop-threatening bacteria *Agrobacterium tumefaciens* and *Erwinia carotovora* and fungi *Botrytis cinerea*, *Botryodiplodia theobromae*, *Fusarium oxysporum* and *Phytophthora infestans* were evaluated. The results proved that the grafting of benzyl moiety or quaternization of the derivatives onto chitosan molecule was successful in inhibiting the microbial growth. Moreover, increase water-solubility of the compounds by quaternization significantly increased the activity against bacteria and fungi. Exocellular enzymes including polygalacturonase (PGase), pectin-lyase (PLase), polyphenol oxidase (PPOase) and cellulase were also affected at 1000 mg/L. These compounds especially quaternary-based chitosan derivatives that have good inhibitory effect should be potentially used as antimicrobial agents in crop protection.

1. Introduction

Synthetic microbicides are effective in reducing the damage to plants caused by bacterial and fungal pathogens but repeated applications of these pesticides are harmful to the environment (Houeto, Bindoula, & Hoffman, 1995). Furthermore, chemical control of plant pathogens has only been partially successful due to the continual appearance and establishment of resistant strains. Therefore, the development of safely alternatives to harmful pesticides is a goal of considerable interest within the framework of a sustainable and economically profitable agriculture (Tripathi & Dubey, 2004). Among the new alternatives known to date, chitosan, a polysaccharide including both β-1,4-anhydroglucosamine and *N*-acetyl-β-1,4-anhydroglucosamine units, has the best prospects as a biocontrol agent (El Ghaouth, Arul, Grenier, & Asselin, 1992; El Ghaouth et al., 1994; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003). Chitosan has attracted people's attention for its physiochemical characteristics and its bioactivities (Allan & Hadwiger, 1979; No & Meyers, 1997; Badawy & Rabea, 2011; Rabea et al., 2003). The antimicrobial properties of chitosan and its derivatives have been widely explored (Shahidi, Arachchi, & Jeon, 1999; Badawy & Rabea, 2011; Pedro et al., 2013; Guo et al., 2014). To improve the antimicrobial activity of chitosan, researchers have prepared many derivatives which showed higher antimicrobial activity than unmodified chitosan, but the antimicrobial activities of these materials are still lower than those of the antimicrobial agents currently used (Muzzarelli & Tanfani, 1985; Guo et al., 2006, 2014). Moreover, the antimicrobial activity of chitosan was prominent only in acidic medium because of its poor solubility in neutral and basic media where chitosan start to lose its cationic nature (Liu et al., 2001; Zhang, Gen, Jiang, Li, & Huang, 2012). To overcome this obstacle, several chitosan derivatives with high solubility in water have been prepared (Muzzarelli, Tanfani, Emanuelli, & Mariotti, 1982; Muzzarelli & Tanfani, 1982,1985; Domard, Rinaudo, & Terrassin, 1986; Grant, Blair, & Mckay, 1988; Holme & Perlin, 1997; Jia, Shen, & Xu, 2001; Ramos et al., 2003; Rúnarsson et al., 2007; Badawy, 2010; Li, Zhanyong, & Pingan, 2010; Badawy & Rabea, 2012; Tan, Ma, Lin, Liu, & Tang, 2013).

The present work describes the preparation of a series of quaternary *N*-(benzyl) chitosan derivatives to enhance the antimicrobial activity of chitosan molecule against some important crop-threatening bacteria *Agrobacterium tumefaciens* and *Erwinia carotovora* and fungi *Botrytis cinerea*, *Botryodiplodia theobromae*, *Fusarium oxysporum* and *Phytophthora infestans*. The ultimate

* Corresponding author. Tel.: +20 1004902923/+20 39575269; fax: +20 35972780.
E-mail address: m_eltaher@yahoo.com (M.E.I. Badawy).

http://dx.doi.org/10.1016/j.carbpol.2014.04.098
0144-8617/

Table 1
Chemical structures of chitosan, *N*-(benzyl) chitosans and their quaternized derivatives.

N-(benzyl) chitosans	DDA[a]	DS[b]	Quaternary *N*-(benzyl) chitosans	DQ[c]
Ch	0.91	–	QCh	0.20
C1	0.73	0.15	QC1	0.18
C2	0.67	0.17	QC2	0.22
C3	0.75	0.14	QC3	0.17
C4	0.81	0.14	QC4	0.15
C5	0.76	0.21	QC5	0.17
C6	0.70	0.23	QC6	0.08
C7	0.74	0.16	QC7	0.09
C8	0.66	0.52	QC8	0.09
C9	0.49	0.51	QC9	0.17

[a] DDA is a degree of deacetylation.
[b] DS is a degree of substitution and it was based on the ratio between the integral areas of the protons in the phenyl moiety and the integral area of the protons in pyranose unit.
[c] DQ is a degree of quaternization and it was generally calculated between the integral area of methyl protons in —[$N^+(C_2H_5)_2R$] and the integral area of the protons in pyranose unit.

purpose of our research is to provide an alternative treatment to control plant pathogens that negatively affect on the quality and production of fruits and vegetables.

2. Experimental

2.1. Materials

Chitosan of low molecular weight (3.60×10^5 Da) was purchased from Sigma-Aldrich Co. (USA). Nine chemically modified chitosan compounds name as *N*-(benzyl) chitosan derivatives were previously prepared and characterized by Rabea et al. (2005) and Rabea, Badawy, Steurbaut, and Stevens (2009). The compounds with the indicated functional groups and different substituents are shown in Table 1. Ethyl iodide, deuterium oxide, deuterated acetic acid, pyrocatechol, thiobarbituric acid (TBA), citrus pectin, carboxymethyl cellulose (CMC), dinitrosalicylic acid (DNS), sodium–potassium tartarate, Folin–Ciocalteu phenol reagent and Bovine Serum Albumin (BSA) were purchased from Sigma-Aldrich Chemical Co. (USA) and used without further purification. Nutrient agar (NA) and Potato Dextrose Agar (PDA) were purchased from Oxoid Ltd. (Basingstoke, Hampshire, UK).

2.2. Instrumentation

^{1}H-NMR measurements were performed on a JEOL A-500 NMR Spectrometer under a static magnetic field of 500 MHz at 25 °C. IR spectra were recorded with KBr discs in the range of 4000–400 cm^{-1} with resolution of 4.0 cm^{-1} on Perkin Elmer FT-IR Spectrophotometer. UV–vis absorption spectra of the chitosan compounds were recorded on Alpha-1502 UV–vis Spectrophotometer in 0.5% (v/v) aqueous acetic acid solution at the range of 200–340 nm. A quartz cell path length of 1 cm was employed.

2.3. Synthesis of quaternary N-(benzyl) chitosan derivatives

The quaternization of *N*-(benzyl) chitosan derivatives was achieved as shown in Scheme 1 according to the method of Saxena, Kumar, and Shahi (2006) with some modifications. In brief, 2.5% (w/v) of chitosan or *N*-(benzyl) chitosan derivative was dispersed in 42% methanol/water and the mixture was kept under constant stirring for 1 h at 30 °C. Then 5% (v/v) ethyl iodide was added dropwise under constant stirring and the reaction mixture was refluxed at 70 °C overnight. 5% NaCl (w/v) was added and the reaction was extra stirred for 2 h at 70–80 °C. Quaternary ammonium of chitosan [*N*,*N*,*N*-(triethyl) chitosan] or quaternary ammonium of *N*-(benzyl) chitosan derivatives [*N*,*N*-(diethyl),*N*-(benzyl) chitosans] were then precipitated and washed several times by acetone and then dried overnight under vacuum at 50 °C.

2.4. Solubility tests

The chitosan derivatives were tested for solubility in water and aqueous acetic acid solutions (0.1, 0.5 and 1%, v/v). In each case, the solubility was evaluated at the concentration of 10 mg/mL in each solvent and checked after standing for 24 h at room temperature.

2.5. Pathogens and cultures

Two bacterial strains *A. tumefaciens* and *E. carotovora* and four fungal strains *B. cinerea*, *B. theobromae*, *F. oxysporum* and *P. infestans* were provided by Microbiology Laboratory, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Egypt. The bacterial strains were maintained on NA medium at 37 °C and fungi were maintained on PDA medium at 27 °C.

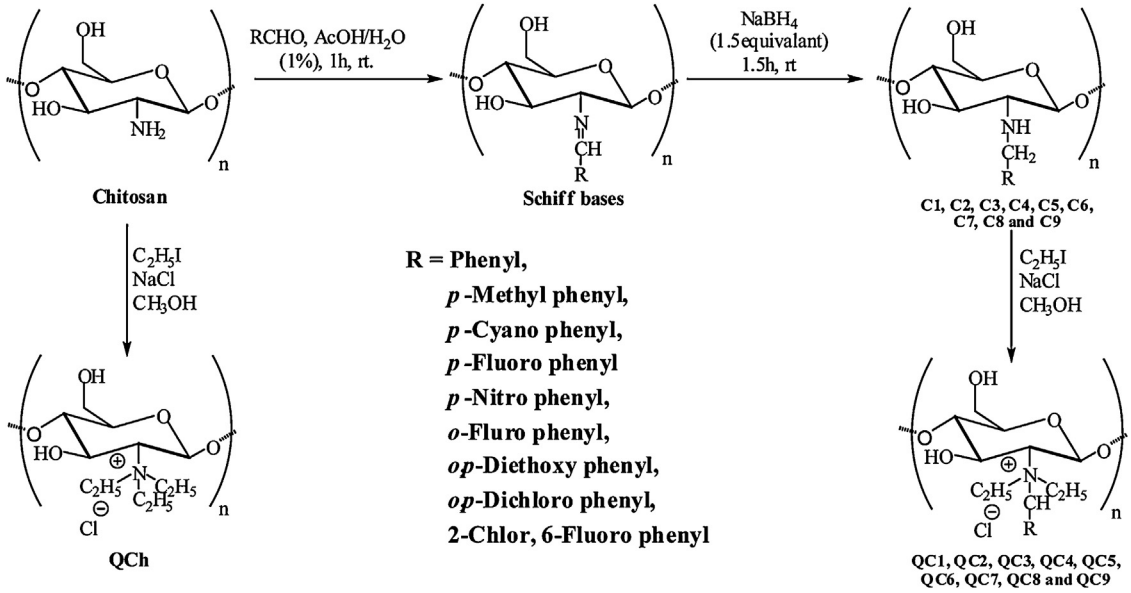
2.6. Antibacterial assay

The minimum inhibitory concentration (MIC) of chitosan compounds was determined using NA dilution method (EUCAST, 2000) against *A. tumefaciens* and *E. carotovora*. Chitosan compounds were dissolved in 0.5% (v/v) aqueous acetic acid and added to NA medium immediately before it was poured into the Petri dishes at a temperature of 40–45 °C. The compounds were tested at concentrations ranged from 100 to 3000 mg/L and each concentration was tested in triplicate. Parallel controls were maintained with distilled water and 0.5% (v/v) aqueous acetic acid mixed with NA medium. One loopful of microorganism suspensions in Nutrient Broth (NB) medium (≈6 μL) was spotted on the surface of NA medium (ten spots per plate) then incubated at 37 °C for 24 h. Each concentration was tested in triplicate. The MIC was recorded in each case as the minimum concentration of compound which inhibited the growth of tested microorganism after incubation at 37 °C for 24 h. From the MIC observed, the intermediate concentrations between MIC values were prepared by suitable dilution of stock solution and the accurate MIC values were determined.

To further characterize the antibacterial activity of chitosan compounds, we carried out a parallel study using autoclaved liquid medium (NB, pH 6.8) supplemented with 1000 mg/L of each compound. 20 mL of the autoclaved medium containing 1000 mg/L of the tested compounds was inoculated with 100 μL of bacterial suspension of 24 h culture ($O.D_{550}$ = 0.90) and incubated at 37 °C for 6 days with shaking at 150 rpm. At the end of incubation period, the biomass was separated by centrifugation at 6000 rpm for 10 min. The supernatant was used in determination of protein and exocellular enzymes including polygalacturonase (PGase) and pectin-lyase (PLase). Protein concentration was determined by Lowry, Rosebrough, Farr, and Randall (1951) method. PGase and PLase activities were determined colorimetrically by thiobarbituric acid (TBA) reagent according to the modified method by Nedjma, Hoffmann, and Belarbi (2001).

2.7. Antifungal assay

The antifungal activity of chitosan compounds against *B. cinerea*, *Bd. theobromae*, *F. oxysporum* and *P. infestans* was tested using mycelia radial growth technique according to El Ghaouth et al. (1992). The polymers solution were prepared at pH 5.5 in acetic acid and added at concentrations of 0.0 (control plate), 100, 250, 500, 1000, 1500, 2000, 2500 and 3000 mg/L to the melted PDA medium



Scheme 1. Synthetic rout to quaternary *N*-(benzyl) chitosan derivatives. **QCh**: *N*,*N*,*N*-(triethyl) chitosan, **QC1**: *N*,*N*-(diethyl),*N*-(benzyl) chitosan, **QC2**: *N*,*N*-(diethyl),*N*-(p-methyl benzyl) chitosan, **QC3**: *N*,*N*-(diethyl),*N*-(*p*-cyano benzyl) chitosan, **QC4**: *N*,*N*-(diethyl),*N*-(*p*-fluoro benzyl) chitosan, **QC5**: *N*,*N*-(diethyl),*N*-(*p*-nitro benzyl) chitosan, **QC6**: *N*,*N*-(diethyl),*N*-(*o*-fluoro benzyl) chitosan, **QC7**: *N*,*N*-(diethyl),*N*-(*o*,*p*-diethoxy benzyl) chitosan, **QC8**: *N*,*N*-(diethyl),*N*-(*o*,*p*-dichloro benzyl) chitosan and **QC9**: *N*,*N*-(diethyl),*N*-(2-chloro,6-fluoro benzyl) chitosan.

and then transferred to Petri dishes. After solidification, the mixtures were inoculated with a 5 mm in diameter mycelium fungi at the center of Petri dishes and these were incubated in the dark at 27 °C. The fungal growth was measured when the control had grown to the edge of the plate. Growth inhibition was calculated as the percentage of inhibition of radial growth relative to control. The effective concentration that inhibited 50% of the mycelial growth (EC_{50}) and its corresponding 95% confidence limits were estimated by SPSS 17.0 software according to the probit analysis (Finney, 1971).

To further characterize the antifungal activity of chitosan compounds, we carried out a parallel study using autoclaved liquid medium (PDB, pH 5.5) supplemented with chitosan compounds at final concentration of 1000 mg/L. Fungal inoculates were prepared by harvesting spores from 7-day-old PDA plates of the tested fungi in sterilized distilled water containing 0.1% (v/v) Tween-80. The concentrations of spore suspensions were determined in a hemacytometer and adjusted to 1.0×10^6 spore/mL. Each tube contains 20 mL PDB was inoculated with 100 μL of fungal spores and the tubes were incubated for 8 days at 27 °C in a shaking incubator (160 rpm). At the end of the incubation period, the content of each tube was filtered through Whatman filter paper No. 1 to separate the mycelium from the culture filtrate. Three replicates of each tube were used for each fungus and the mycelial mat was oven dried at 80 °C till constant weight to obtain the dry weight expressed as mg/mL growth medium. The filtrate was then centrifuged at 5000 rpm for 15 min at 4 °C and the supernatant was used in determination of protein and exocellular enzymes including PGase, PLase, polyphenol oxidase (PPOase) and cellulase. Protein concentration was determined by Lowry et al. (1951) method. PGase and PLase activities were determined according to the Nedjma et al. (2001). The activity of PPOase was determined according to Zhi-qing, Xiu-ling, Jun-tao, Guang-ze, and Xing (2008) by mixing of 1.5 mL of 0.2 mol/L pyrocatechol, 1.4 mL of 0.05 mol/L phosphate buffer (pH 6.8) and 0.1 mL enzyme extract, respectively. The mixture was incubated at 25 °C for 25 min and the absorbance was measured at 420 nm by Spectrophotometer. The specific activity of PPO was calculated as $OD_{420}.30\ min^{-1}\ (mg\ protein)^{-1}$. Cellulase activity was measured using spectrophotometer by dinitrosalicylic acid (DNS) method (Mandels, 2005). This was done by centrifuging the culture broth at 8000 rpm for 15 min and then 0.5 mL of the culture filtrate, one mL of 1% CMC in 50 mM citrate buffer (pH 4.8) incubated for 30 min at 50 °C then reacting with 0.5 mL of DNS reagent (96 mM) and boiled for 5 min and the absorbance was measured at 540 nm by Spectrophotometer. The specific activity of cellulase was calculated and expressed as $OD_{540}.30\ min^{-1}\ (mg\ protein)^{-1}$.

3. Results and discussion

3.1. Structure and characterization of quaternary N-(benzl) chitosan derivatives

The formation of quaternary ammonium salt into chitosan and *N*-(benzl) chitosan derivatives backbone was carried out by nucleophilic substitution of chitosan amine protons with ethyl groups of ethyl iodide leading to water-soluble chitosan compounds (QCh and QC1–QC9) as shown in Scheme 1. The counterion I^- was exchanged in the presence of sodium chloride in a methanol/water medium with Cl^- to obtain *N*,*N*,*N*-(diethylbenzyl) chitosans chloride having higher solubility than the iodide counterpart. The chemical structures were well confirmed by ^{0}H-NMR and FT-IR spectroscopy. All the ^{1}H-NMR spectra of the compounds exhibited the main characteristic pattern of chitosan molecule, *i.e.*, the peak at 2.09–2.12 ppm represent protons of the methyl group in of GlcNAc unit, broad singlet at δ 3.15–3.30 due to the resonance of H-2 proton of the GlcN unit and multiplet peaks at δ 3.57–4.10 ppm due to the resonance of H-3, H-4, H-5 and H-6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit (Sashiwa and Shigemasa, 1999; Badawy & Rabea, 2013). The ^{1}H-NMR spectra of the synthesized chitosan derivatives exhibited singlet or broad peak around at δ 1.23 to 1.96 ppm which confirm the presence of the methyl protons in the quaternized chitosan compounds and also the peaks around at δ 7.24 to 8.90 ppm that represent the presence of the phenyl ring.

The ^{1}H-NMR spectra were used to calculate the degree of deacetylation (DDA), degree of substitution (DS) and degree of qutarnization (DQ) in according to the methods of Hirai, Odani, and Nakajima (1991) and Lavertu et al. (2003). DDA was estimated from

δ δ 3.20 ppm (x) that attributed to H-2 of GlcN unit *vs* 3.40–4.40 ppm ($6-x$) which attributed to H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit. DS value was based on the ratio between the areas of the protons in the phenyl substituent and the protons of the pyranose unit (Rabea et al., 2009). DQ was generally calculated between the integral area of one hydrogen from the methyl protons in—[$N^+(C_2H_5)_2$R] at the range of δ 1.30–1.90 ppm and the integral area of one hydrogen from the protons of H-1,2,3,4,5 and 6 of GlcN unit in the range of δ 3.15–4.20 ppm in ^{1}H-NMR spectra of the quaternary ammonium chitosan compounds. DQ was ranged from 0.08 to 0.22 (Table 1).

Further evidence for confirmation the chemical structure was obtained from FT-IR spectroscopy. The spectral data of the quaternized derivatives were similar to those of their corresponding *N*-substituted benzyl precursors except for the presence of absorption band at the region of 1426–1480 cm^{-1} due to C—H symmetric bending of the methyl groups on the quaternary ammonium moieties. In addition, the absorption peak of the amino group at 1510–1590 cm^{-1} considerably decreased due to the reaction of amino group with aldehydes and ethyl iodide, and a new peak by the methyl groups appeared at about 1426–1480 cm^{-1} (Jia et al., 2001; Badawy, 2010). These results demonstrate that the quaternized chitosan derivatives were obtained.

The UV spectra of the quaternary *N*-(benzyl) chitosan derivatives indicated a broad absorption bands between 200 and 260 nm due to the presence of aromatic ring and the maximum absorption peaks (λ_{max}) were found at 227, 225, 232, 232, 227, 230, 240, 228 and 220 nm for QC1, QC2, QC3, QC4, QC5 QC6, QC7, QC8 and QC9, respectively. However, the maximum absorption peaks for *N*-(benzyl) chitosan derivatives were found at 255, 241, 257, 255, 272, 262, 278, 259 and 251 nm for C1, C2, C3, C4, C5, C6, C7, C8 and C9, respectively. It can be noticed that the quaternization of *N*-(benzyl) chitosan compounds showed shifting in λ_{max} to the lowest values. Unmodified chitosan had no absorption peaks from 200 nm to 340 nm. It is transparent in the UV and visible region, and so its conformation is hard to characterize by spectroscopy methods.

3.1.1. Spectral data of chitosan (Ch)

^{1}H-NMR (25 °C): δ 2.09–2.12 (br s, NHCO$\underline{CH_3}$), 3.15–3.30 (br m, H-2 of GlcN residue), 3.57–4.10 (br m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit), 4.88–5.00 (m, H-1 of GlcN and GlcNAc units). **IR (KBr):** ν 3400–3460 cm^{-1} (br, OH/NH), 2855–2930 cm^{-1} (m, C—H of CH_2 and CH_3 groups), 1656 cm^{-1} (>C=O stretching, amide I) and 1570 cm^{-1} (NH_2 stretching, amide II), 1380–1420 cm^{-1} (C—H stretching of methyl groups in GlcNAc units), 944–1150 cm^{-1} assigned to the saccharide structure.

3.1.2. Spectral data of N,N,N-(triethyl) chitosan (QCh)

^{1}H-NMR: δ 1.36–1.40 (br t, CH_3), 2.0.9–2.12 (s, $NHCOCH_3$), 3.15–3.20 (br m, H-2 of GlcN residue), 3.30–3.40 (br s, tertiary *N*-CH_2), 3.76–3.94 (br m, H-3,4,5,6 of GlcN unit and H-2,3,4,5,6 of GlcNAc unit), 4.75 (s, H-1 of GlcN unit) and 4.85–4.94 (m, H-1 of GlcNAc unit). **IR (KBr):** ν 3400–3450 cm^{-1} (br, OH/NH), 2860–2950 cm^{-1} (m, C—H of CH_2 and CH_3 groups), 1633 cm^{-1} (>C=O stretching, amide I), 1510 cm^{-1} (NH_2 stretching, amide II), 1480 cm^{-1} (tertiary *N*-$(C_2H_3)_3$), 1380 cm^{-1} (C—H stretching of methyl groups in GlcNAc units), 1260 cm^{-1} (C—N stretching) and 1000–1150 cm^{-1} assigned to the saccharide structure. The **UV λ_{max}** was 227 nm.

3.1.3. Spectral data of N,N-(diethyl),N-(benzyl) chitosan (QC1)

^{1}H-NMR: δ 1.29 (CH_3), 2.05 (s, NHAc), 3.10–3.15 (br s, H-2 of GlcN residue), 3.30 ($C\underline{H_2}$–NH), 3.49–3.88 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN units), 4.58 (br s, H-1 of GlcNAc residue), 4.72 (br s, H-1 of GlcN residue), 7.38 (m, 3*H*, phenyl ring) and 7.49 (m, 2*H*, phenyl ring). **IR (KBr):** ν 3427 cm^{-1} (br, OH/NH), 2923 cm^{-1} (m, C—H of CH_2 and CH_3 groups), 1631 cm^{-1} (>C=O stretching, amide I), 1543 cm^{-1} (NH_2 stretching, amide II), 1459 cm^{-1} (ethyl group of quaternary -N^+-$(C_2H_5)_2$), 1350 cm^{-1} (C—H stretching of methyl groups in GlcNAc units), 1250 cm^{-1} (C—N stretching) and 950–1155 cm^{-1} assigned to the saccharide structure. The **UV λ_{max}** was 227 nm.

3.1.4. Spectral data of N,N-(diethyl),N-(p-methyl benzyl) chitosan (QC2)

^{1}H-NMR: δ 1.23–1.32 (CH_3), 1.34–1.36 (CH_3, on the phenyl ring), 2.07–2.10 (s, NHAc), 3.19–3.25 (br s, H-2 of GlcN residue), 3.25–3.30 ($C\underline{H_2}$–NH), 3.55–4.05 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.60 (br s, H-1 of GlcNAc residue), 4.80 (br s, H-1 of GlcN residue) and 7.40–7.45 (br, 4*H* of the phenyl ring). **IR (KBr):** ν 3400 cm^{-1} (br, OH/NH), 2874 cm^{-1} (m, C—H of CH_2 and CH_3 groups), 1654 cm^{-1} (>C=O stretching, amide I), 1570 cm^{-1} (NH_2 stretching, amide II), 1457 cm^{-1} (ethyl group of quaternary -N^+-$(C_2H_5)_2$), 1380 cm^{-1} (C—H stretching of methyl groups in GlcNAc units), 1260 cm^{-1} (C—N stretching) and 950–1130 cm^{-1} assigned to the saccharide structure. The **UV λ_{max}** was 225 nm.

3.1.5. Spectral data of N,N-(diethyl),N-(p-cyano benzyl) chitosan (QC3)

^{1}H-NMR: δ 1.80–1.92 (br s, CH_3), 1.98–2.07 (br s, NHAc), 3.01–3.10 (br s, H-2 of GlcN residue), 3.10–3.30 ($C\underline{H_2}$–NH), 3.40–4.03 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.49 (br s, H-1 of GlcNAc residue), 4.68 (br s, H-1 of GlcN residue) and 7.24–7.38 (m, 4*H* of the phenyl ring). **IR (KBr):** ν 3390–3450 cm^{-1} (br, OH/NH), 2800–2900 cm^{-1} (m, C—H of CH_2 and CH_3 groups), 1635 cm^{-1} (>C=O stretching, amide I), 1570 cm^{-1} (NH_2 stretching, amide II), 1457 cm^{-1} (ethyl group of quaternary -N^+-$(C_2H_5)_2$), 1380 cm^{-1} (C—H stretching of methyl groups in GlcNAc units), 1225 cm^{-1} (C—N stretching) and 1000–1180 cm^{-1} assigned to the saccharide structure. The **UV λ_{max}** was 232 nm.

3.1.6. Spectral data of N,N-(diethyl),N-(p-fluoro benzyl) chitosan (QC4)

^{1}H-NMR: δ 1.86–1.89 (m, CH_3), 1.99–2.01 (br s, NHAc), 2.91–3.10 (br s, H-2 of GlcN residue), 3.10–3.16 (br m, $C\underline{H_2}$–NH), 3.34–4.05 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.40–4.45 (br s, H-1 of GlcNAc residue), 4.52–4.67 (br s, H-1 of GlcN residue) and 7.50–7.61 (br m, 4*H* of the phenyl ring). **IR (KBr):** ν 3415 cm^{-1} (br, OH/NH), 2852–2919 cm^{-1} (m, C—H of CH_2 and CH_3 groups), 1641 cm^{-1} (>C=O stretching, amide I), 1534 cm^{-1} (NH_2 stretching, amide II), 1450 cm^{-1} (ethyl group of quaternary -N^+-$(C_2H_5)_2$), 1384 cm^{-1} (C—H stretching of methyl groups in GlcNAc units), 1225 cm^{-1} (C—N stretching) and 898–1156 cm^{-1} assigned to the saccharide structure. The **UV λ_{max}** was 232 nm.

3.1.7. Spectral data of N,N-(diethyl),N-(p-nitro benzyl) chitosan (QC5)

^{1}H-NMR: δ 1.84–1.91 (br s, CH_3), 2.04–2.11 (br s, NHAc), 2.95–3.14 (br s, H-2 of GlcN residue), 3.14–3.18 (br m, $C\underline{H_2}$–NH), 3.44–3.96 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.07 (s, H-1 of GlcNAc residue), 4.54 (s, H-1 of GlcN residue) and 7.29–7.43 (br m, 4*H* of the phenyl ring). **IR (KBr):** ν 3420 cm^{-1} (br, OH/NH), 2970 cm^{-1} (m, C—H of CH_2 and CH_3 groups), 1654 cm^{-1} (>C=O stretching, amide I), 1560 cm^{-1} (NH_2 stretching, amide II), 1430–1460 cm^{-1} (ethyl group of quaternary —N^+-$(C_2H_5)_2$), 1379 cm^{-1} (C—H stretching of methyl groups in GlcNAc units), 1255 cm^{-1} (C—N stretching) and 1000–1140 cm^{-1} assigned to the saccharide structure. The **UV λ_{max}** was 227 nm.

Table 2
Solubility of chitosan, chitosan, *N*-(benzyl) chitosans and their quaternized derivatives.

Compound	Solubility			
	Distilled water	0.1% Aqueous acetic acid	0.5% Aqueous acetic acid	1% Aqueous acetic acid
Ch	Insoluble	Gel	Soluble	Soluble
C1	Insoluble	Swelling	Gel	Soluble
C2	Insoluble	Swelling	Gel	Soluble
C3	Insoluble	Swelling	Gel	Soluble
C4	Insoluble	Swelling	Gel	Soluble
C5	Insoluble	Swelling	Gel	Soluble
C6	Insoluble	Swelling	Gel	Soluble
C7	Insoluble	Swelling	Gel	Soluble
C8	Insoluble	Swelling	Gel	Soluble
C9	Insoluble	Swelling	Gel	Soluble
QCh	Soluble	Soluble	Soluble	Soluble
QC1	Gel	Soluble	Soluble	Soluble
QC2	Gel	Soluble	Soluble	Soluble
QC3	Gel	Soluble	Soluble	Soluble
QC4	Gel	Soluble	Soluble	Soluble
QC5	Gel	Soluble	Soluble	Soluble
QC6	Gel	Soluble	Soluble	Soluble
QC7	Gel	Soluble	Soluble	Soluble
QC8	Gel	Soluble	Soluble	Soluble
QC9	Gel	Soluble	Soluble	Soluble

3.1.8. Spectral data of N,N-(diethyl),N-(o-fluoro benzyl) chitosan (QC6)

^{1}H-NMR: δ 1.90–1.96 (br, CH_3), 2.03–2.14 (br s, NHAc), 2.98–3.20 (br s, H-2 of GlcN residue), 3.30 ($C\underline{H_2}$–NH), 3.42–4.16 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.04–4.55 (br s, H-1 of GlcNAc residue), 4.67–4.90 (br s, H-1 of GlcN residue), 7.89–8.00 (br s, 1*H*, phenyl ring), 8.50–8.61 (br s, 2*H*, phenyl ring) and 8.78–8.90 (br s, 1*H* of the phenyl ring). **IR (KBr):** ν 3394 cm^{-1} (br, OH/NH), 2924 cm^{-1} (m, C—H of CH_2 and CH_3 groups), 1652 cm^{-1} (>C=O stretching, amide I), 1565 cm^{-1} (NH_2 stretching, amide II), 1426–1461 cm^{-1} (ethyl group of quaternary $-N^+$-$(C_2H_5)_2$), 1347 cm^{-1} (C—H stretching of methyl groups in GlcNAc units), 1260 cm^{-1} (C—N stretching) and 945–1131 cm^{-1} assigned to the saccharide structure. The **UV λ_{max}** was 230 nm.

3.1.9. Spectral data of N,N-(diethyl),N-(o,p-diethoxy benzyl) chitosan (QC7)

^{1}H-NMR: δ 1.88 (CH_3), 2.09 (s, NHAc), 3.05–3.20 (br s, H-2 of GlcN residue), 3.37 ($C\underline{H_2}$–NH), 3.49–4.25 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), The peak of ethoxy group on the phenyl ring was covered and included in the peaks at about δ 3.91–4.12, 4.55 (br s, H-1 of GlcNAc residue), 4.95 (br s, H-1 of GlcN residue), 7.32–7.39 (br s, 1*H*, phenyl ring), 7.50–7.62 (br s, 1*H*, phenyl ring) and 7.83–7.95 (br s, 1*H* of the phenyl ring). **IR (KBr):** ν 3472 cm^{-1} (br, OH/NH), 2890 cm^{-1} (m, C—H of CH_2 and CH_3 groups), 1654 cm^{-1} (>C=O stretching, amide I), 1575 cm^{-1} (NH_2 stretching, amide II), 1457 cm^{-1} (ethyl group of quaternary $-N^+$-$(C_2H_5)_2$), 1380 cm^{-1} (C—H stretching of methyl groups in GlcNAc units), 1255 cm^{-1} (C—N stretching) and 980–1179 cm^{-1} assigned to the saccharide structure. The **UV λ_{max}** was 240 nm.

3.1.10. Spectral data of N,N-(diethyl),N-(o,p-dichloro benzyl) chitosan (QC8)

^{1}H-NMR: δ 1.79–1.85 (br s, CH_3), 1.98–2.06 (br s, NHAc), 2.91–3.12 (br s, H-2 of GlcN residue), 3.37 ($C\underline{H_2}$–NH), 3.25–3.90 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.54 (br s, H-1 of GlcNAc residue), 4.60–75 (br s, H-1 of GlcN residue), 6.76–6.89 (br s, 1*H*, phenyl ring), 7.04–7.26 (br s, 1*H*, phenyl ring) and 7.26–7.39 (br s, 1*H* of the phenyl ring). **IR (KBr):** ν 3362 cm^{-1} (br, OH/NH), 2921 cm^{-1} (m, C—H of CH_2 and CH_3 groups), 1649 cm^{-1} (>C=O stretching, amide I), 1573 cm^{-1} (NH_2 stretching, amide II), 1459 cm^{-1} (ethyl group of quaternary $-N^+$-$(C_2H_5)_2$), 1347 cm^{-1} (C—H stretching of methyl groups in GlcNAc units), 1254 cm^{-1} (C—N stretching) and 944–1131 cm^{-1} assigned to the saccharide structure. The **UV λ_{max}** was 228 nm.

3.1.11. Spectral data of N,N-(diethyl),N-(2-chloro,6-fluoro benzyl) chitosan (QC9)

^{1}H-NMR: δ 1.35 (CH_3), 2.08 (s, NHAc), 3.05–3.20 (br s, H-2 of GlcN residue), 3.37 ($C\underline{H_2}$–NH), 3.51–4.10 (br m, H-2 of GlcNAc and H-3,4,5,6 of GlcN unit), 4.58 (br s, H-1 of GlcNAc residue), 4.69 (br s, H-1 of GlcN residue), 7.20 (br s, 1*H*, phenyl ring), 7.45 (br s, 1*H*, phenyl ring) and 7.60 (br s, 1*H* of the phenyl ring). **IR (KBr):** ν 3350–3900 cm^{-1} (br, OH/NH), 2900 cm^{-1} (m, C—H of CH_2 and CH_3 groups), 1653 cm^{-1} (>C=O stretching, amide I), 1590 cm^{-1} (NH_2 stretching, amide II), 1457 cm^{-1} (ethyl group of quaternary -N^+-$(C_2H_5)_2$), 1378 cm^{-1} (C—H stretching of methyl groups in GlcNAc units), 1246 cm^{-1} (C—N stretching) and 1000–1246 cm^{-1} assigned to the saccharide structure. The **UV λ_{max}** was 220 nm.

3.2. Solubility of the chitosan derivatives

The solubility of chitosan derivatives was evaluated in water and aqueous acetic acid solutions (0.1, 0.5 and 1%, v/v) and the data are shown in Table 2. Because of the presence of hydrophobic moiety in chitosan backbone in C1–C9 derivatives, the water solubility is negligible and these compounds start to swell in diluted acetic acid (0.1%), whereas they formed gel in 0.5% and were completely soluble in 1% aqueous acetic acid. However, after conversion of these compounds to quaternary ammonium forms, QCh and QC1–QC9 have favorable water solubility. Moreover, the quaternary chitosan compounds have better solubility in diluted acetic acid (0.1, 0.5 and 1%) compared to unmodified chitosan and *N*-(benzl) chitosan derivatives. With a quaternization process, the derivatives turn water soluble, owing to the presence of positively charged groups imparting polycation character to the polymer chain.

3.3. The in vitro antimicrobial activity of chitosan derivatives

3.3.1. The MICs of chitosan derivatives for bacteria

The MICs of chitosan compounds on *A. tumefaciens* and *E. carotovora* are listed in Table 3. The results showed that chitosan had the lowest antibacterial activity under the experimental conditions (MIC > 3000 mg/L). The antibacterial activity of quaternary ammonium chitosan compounds were significantly increased approximately two folds compared to *N*-(benzyl) chitosan

Table 3
The *in vitro* antibacterial activity of chitosan and chitosan derivatives against *A. tumefaciens* and *E. carotovora*.

N-(benzyl) chitosans	MIC[a] (mg/L)		Quaternary *N*-(benzyl) chitosans	MIC[a] (mg/L)	
	A. tumefaciens	*E. carotovora*		*A. tumefaciens*	*E. carotovora*
Ch	>3000	>3000	QCh	750	800
C1	1550	1200	QC1	650	700
C2	1780	1550	QC2	875	940
C3	1375	1200	QC3	700	850
C4	1450	1225	QC4	725	900
C5	800	700	QC5	500	600
C6	1350	1100	QC6	600	650
C7	1475	1250	QC7	750	975
C8	1500	1300	QC8	650	700
C9	1650	1450	QC9	800	875

[a] MIC is a minimum inhibitory concentration. MIC is the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation.

derivatives (C1–C9). MICs of C1–C9 ranged from 800 to 1780 mg/L against *A. tumefaciens* and from 700 to 1550 mg/L against *E. carotovora*. However, the MICs of quaternary *N*-(benzyl) chitosan derivatives (QC1–QC9) ranged from 500 to 875 mg/L for *A. tumefaciens* and from 600 to 975 mg/L for *E. carotovora*. *N*-(*p*-nitro benzyl) chitosan (C5) showed the highest antibacterial activity among *N*-(benzyl) chitosan compounds with MICs 800 and 700 mg/L on *A. tumefaciens* and *E. carotovora*, respectively. It was also noticed that a substitution of hydrogen atom by methyl group on the phenyl moiety (see C1 *vs*. C2) led to decrease of the activity. However, substitution of hydrogen atom by cyano, fluoro, nitro, ethoxy or chloro on the phenyl moiety led to enhance the antibacterial activity (see C1 *vs*. C3–C9). When we consider the susceptibility of the microorganisms, another point deserves awareness; *E. carotovora* was more sensitive to C1–C9 compounds than *A. tumefaciens*. In contrast, *A. tumefaciens* was more sensitive to QC1–QC9 compounds than *E. carotovora*.

3.3.2. The EC_{50}s of chitosan derivatives for fungi

As indicated in Table 4, all *N*-(benzyl) chitosan derivatives (C1–C9) showed better antifungal activity than unmodified chitosan. *N*-(*p*-nitro benzyl) chitosan (C5), *N*-(*o*-fluoro benzyl) chitosan (C6), *N*-(*o*,*p*-dichloro benzyl) chitosan (C8) and *N*-(2-chloro,6-fluoro benzyl) chitosan (C9) have the highest antifungal activity against *B. cinerea* (EC_{50} = 820, 989, 520 and 951 mg/L, respectively). However, *N*-(*p*-cyano benzyl) chitosan (C3) was the lowest active one among these derivatives against *B. cinerea* (EC_{50} = 1215 mg/L). C1, C6, C7 and C8 were the most potent compounds against *Bd. theobromae* (EC_{50} = 790, 939, 993 and 724 mg/L, respectively). However, C3 was the lowest active one among these derivatives against *Bd. theobromae* (EC_{50} = 1337 mg/L). For *F. oxysporum*, all *N*-(benzyl) chitosan derivatives indicated high antifungal activity with EC_{50} lower than 1000 mg/L except of C3, C6 and C9 which showed moderate activity (EC_{50} = 1412, 1194 and 1480 mg/L, respectively). The biological activity of these compounds against *P. infestans* proved that all products indicated high antifungal activity where the EC_{50} values were lower than 1000 mg/L except of C1 (EC_{50} = 1166 mg/L).

As indicated in Table 5, quaternary ammonium chitosan (QCh) and quaternary *N*-(benzyl) chitosan derivatives (QC1–QC9) showed better antifungal activity than the unmodified chitosan (Ch) and *N*-(benzyl) chitosan derivatives (C1–C9) which shown in Table 4. The biological activity of these compounds against *B. cinerea* proved that all the derivatives have promising activity with EC_{50} ranged from 512 to 1060 mg/L. *N*,*N*-(diethyl),*N*-(*o*,*p*-dichloro benzyl) chitosan (QC8) was the most active (EC_{50} = 512 mg/L). *N*,*N*,*N*-(triethyl) chitosan (QCh) exhibited much better antifungal activity (EC_{50} = 1060 mg/L) than chitosan (EC_{50} > 3000 mg/L). All compounds exhibited high activity against *Bd. theobromae* and QC4 and QC7 were the most active compounds with EC_{50} of 564 and 547 mg/L, respectively. For *F. oxysporum*, all the derivatives proved high antifungal activity with EC_{50} lower than 1000 mg/L except of QC3 and QC6 (EC_{50} = 1083 and 1070 mg/L, respectively). QC2 and QC4 were the most active compounds against *F. oxysporum* (EC_{50} = 641 and 647 mg/L, respectively). The data against *P. infestans* proved that all the derivatives had high antifungal activity where the EC_{50} values were lower than 1000 mg/L except of QC1 which showed EC_{50} of 1023 mg/L.

3.4. The in vivo antimicrobial activity of chitosan derivatives

3.4.1. The inhibitory effects on bacterial enzymes

The *in vivo* inhibitory effects of chitosan compounds on PGase and PLase activities in *A. tumefaciens* and *E. carotovora* after 6 days of the treatment at 1000 mg/L in NB medium are shown in Fig. 1. Most of the compounds inhibited PGase and PLase in *A. tumefaciens* more than *E. carotovora*. It can be noted that the quaternary chitosans (QCh and QC1–QC9) exhibited higher effect than of the *N*-(benzyl) chitosan derivatives (C1–C9). C3 and C8 exhibited the highest inhibitory effect on PGase (6.76 and 7.92 U/mg protein, respectively) and PLase (16.45 and 17.99 U/mg protein, respectively) in *A. tumefaciens* among the series of *N*-(benzyl) chitosan derivatives compared to the untreated control (27.77 and 40.33 U/mg. protein for PGase and PLase, respectively). However, C1 was the most active one against the enzymes produced from *E. carotovora* with specific activities of 7.66 and 18.77 for PGase and PLase, respectively, compared to 50.64 and 60.02 for PGase and PLase, respectively, in the untreated sample. Regarding to the series of quaternary *N*-(benzyl) chitosan, QC9 was the most active compound in inhibition of enzyme activities in *A. tumefaciens* with specific activities of 4.24 and 5.09 for PGase and PLase, respectively. However, QC1 and QC9 showed the highest inhibition on both enzymes in *E. carotovora*.

3.4.2. The inhibitory effects on fungal biomass

The dry weight of the fungal biomass declined with application of chitosan compounds as shown in Fig. 2 compared to the controls. However, the level of fungal growth inhibition never reached 100%, which led us to assume that the fungal growth was affected by chitosan compounds but not fully inhibited. Clearly, grafting of benzyl moiety or quaternization of the derivatives onto chitosan molecule was successful in inhibiting the fungal growth. It can be seen that the descending order in fungal biomass of four fungi without treatment was *B. cinerea*, *Bd. theobromae*, *F. oxysporum* and then *P. infestans* (18.0, 12.4, 8.8 and 4.0 mg/mL growth medium, respectively). QCh and QC1–QC9 exhibited a higher effect on the biomass compared to the C1–C9 compounds.

Table 4
In vitro antifungal activity of chitosan and *N*-(benzyl) chitosan derivatives against *B. cinerea*, *Bd. theobromae*, *F. oxysporum* and *P. infestans* using mycelial growth inhibition method.

Compound	EC_{50}[a] (mg/L)	95% confidence limits (mg/L)		Slope[b] ± SE	Intercept[c] ± SE	(χ^2)[d]
		Lower	Upper			
			B. cinerea			
Ch	>3000	–	–	–	–	–
C1	1168	1068	1277	3.59 ± 0.35	−11.02 ± 1.07	3.14
C2	1118	1009	1234	3.15 ± 0.33	−9.59 ± 1.01	0.58
C3	1215	1096	1352	2.99 ± 0.33	−9.24 ± 1.01	0.64
C4	1173	1041	1325	2.57 ± 0.31	−7.88 ± 0.95	3.75
C5	820	356	1248	5.30 ± 0.43	−15.45 ± 1.28	10.15
C6	989	869	1111	2.58 ± 0.31	−7.72 ± 0.94	2.90
C7	1019	923	1117	3.41 ± 0.33	−10.26 ± 1.01	1.22
C8	520	446	585	3.99 ± 0.43	−10.84 ± 1.25	2.88
C9	951	573	1324	3.84 ± 0.35	−11.42 ± 1.06	5.34
			Bd. Theobromae			
Ch	>3000	–	–	–	–	–
C1	790	584	964	1.50 ± 0.28	−4.35 ± 0.87	0.05
C2	1154	1004	1330	2.18 ± 0.30	−6.70 ± 0.92	1.74
C3	1337	1142	1620	1.85 ± 0.29	−5.79 ± 0.92	0.43
C4	1128	999	1274	2.54 ± 0.31	−7.75 ± 0.95	2.82
C5	1129	994	1282	2.42 ± 0.30	−7.39 ± 0.94	0.98
C6	939	811	1066	2.35 ± 0.30	−7.01 ± 0.91	3.71
C7	993	867	1123	2.44 ± 0.30	−7.31 ± 0.93	1.61
C8	724	620	817	2.84 ± 0.32	−8.12 ± 0.96	2.85
C9	1194	952	1540	1.33 ± 0.28	−4.09 ± 0.87	2.01
			F. oxysporum			
Ch	>3000	–	–	–	–	–
C1	980	858	1103	2.54 ± 0.31	−7.62 ± 0.93	1.50
C2	722	580	844	2.11 ± 0.29	−6.03 ± 0.90	0.78
C3	1412	1267	1600	2.85 ± 0.33	−8.97 ± 1.03	1.27
C4	856	750	956	2.87 ± 0.31	−8.43 ± 0.95	2.96
C5	744	626	850	2.48 ± 0.30	−7.14 ± 0.92	3.16
C6	1194	1059	1352	2.53 ± 0.31	−7.79 ± 0.95	3.72
C7	838	729	940	2.76 ± 0.31	−8.09 ± 0.94	3.58
C8	894	528	1232	3.78 ± 0.34	−11.17 ± 1.04	5.13
C9	1480	1310	1721	2.50 ± 0.32	−7.95 ± 1.01	0.03
			P. infestans			
Ch	>3000	–	–	–	–	–
C1	1166	1015	1345	2.18 ± 0.30	−6.70 ± 0.92	0.57
C2	911	609	1196	3.91 ± 0.35	−11.58 ± 1.06	4.07
C3	924	822	1023	3.04 ± 0.32	−9.02 ± 0.96	3.45
C4	727	326	1048	4.71 ± 0.41	−13.49 ± 1.2	7.75
C5	648	353	874	3.93 ± 0.37	−11.05 ± 1.11	4.37
C6	806	700	912	2.81 ± 0.39	−8.17 ± 1.17	1.68
C7	746	342	1066	4.40 ± 0.38	−12.64 ± 1.14	7.19
C8	685	622	745	4.72 ± 0.42	−13.40 ± 1.23	3.04
C9	776	662	885	2.60 ± 0.39	−7.53 ± 1.16	1.01

[a] The concentration causing 50% mycelial growth inhibition.
[b] Slope of the concentration-inhibition regression line ± standard error.
[c] Intercept of the regression line ± standard error.
[d] Chi square value.

In *B. cinerea*, the biomass declined from 18.0 mg/mL growth medium in untreated control to 2.2 mg/mL in QC8 (87.88% growth inhibition). C6, C7, C8 and C9 showed promising activity compared to other *N*-(benzyl) chitosan compounds where 4.0, 4.6, 4.2 and 3.2 mg/mL dry biomass were obtained. However, QC5, QC6, QC7, QC8 and QC9 were the most inhibited compounds among quaternary compounds (3.4, 2.6, 3.8, 2.2 and 3.0 mg/mL, respectively). The results for *Bd. theobromae* showed that the biomass declined from 12.4 mg/mL growth medium in the untreated control to 0.8 mg/mL (93.55% growth inhibition). C1 was the most active (4.2 mg/mL) among the *N*-(benzyl) chitosan compounds, whereas all quaternary *N*-(benzyl) chitosan compounds exhibited the most activity in inhibition of the fungal mass (dray weight ranged from 3.2 to 0.8 mg/mL). The results for *F. oxysporum* showed that the biomass declined from 8.8 mg/mL growth medium in the untreated control to 1.0 mg/mL in QC6 (88.64% growth inhibition). The biomasses of 1.4 to 7.2 mg/mL were found with the treatments of C1–C9. However, 1.0 to 5.0 mg/mL was found with the treatments of QC1–QC9. The biomass of *P. infestans* declined from 4.0 mg/mL in the untreated control to 0.4 mg/mL in QC6 (90.0% growth inhibition). C4 and C6 were the most inhibited compounds among *N*-(benzyl) chitosan compounds (2.2 and 2.0 mg/mL, respectively). All quaternary *N*-(benzyl) chitosan compounds exhibited the most activity in inhibition of the fungal mass (dray weight was ranged from 0.4 to 1.8 mg/mL).

3.4.3. The inhibitory effects on fungal enzymes

The effect of chitosan compounds at 1000 mg/L on exocellular enzymes PGase, PLase, PPOase and cellulase activities in *B. cinerea*, *Bd. theobromae*, *F. oxysporum* and *P. infestans* after 8 days of the treatment is shown in Fig. 3. In *B. cinerea*, C1–C9 proved higher inhibition than QC1–QC9 compounds. High levels of the enzymes were observed in the untreated controls with specific activities of 42.10, 47.67, 18.64 and 4.44 for PGase, PLase, PPOase and cellulase, respectively. C7 and C9 were the most inhibited compounds among *N*-(benzyl) chitosan derivatives for PGase (15.93 and 8.21 U/mg protein, respectively) and PLase (19.96 and 9.36 U/mg protein, respectively). However, QCh was the most active among

Table 5
In vitro antifungal activity of quaternary *N*-(benzyl) chitosan derivatives against *B. cinerea*, *Bd. theobromae*, *F. oxysporum* and *P. infestans* using mycelial growth inhibition method.

Compound	EC_{50}[a] (mg/L)	95% confidence limits (mg/L)		Slope[b] ± SE	Intercept[c] ± SE	(χ^2)[d]
		Lower	Upper			
			B. cinerea			
QCh	1060	876	1265	1.68 ± 0.29	−5.10 ± 0.89	0.71
QC1	837	715	950	2.45 ± 0.30	−7.18 ± 0.92	2.33
QC2	994	892	1098	3.14 ± 0.32	−9.40 ± 0.58	1.24
QC3	998	873	1126	2.48 ± 0.30	−7.45 ± 0.93	3.49
QC4	967	848	1086	2.60 ± 0.31	−7.77 ± 0.94	0.61
QC5	814	335	1212	4.30 ± 0.37	−15.51 ± 1.11	8.33
QC6	976	861	1093	2.68 ± 0.31	−8.04 ± 0.94	1.78
QC7	869	303	1352	3.24 ± 0.33	−9.53 ± 0.98	6.71
QC8	512	354	628	2.08 ± 0.39	−5.64 ± 1.16	0.41
QC9	870	329	1336	3.47 ± 0.33	−10.20 ± 1.01	7.07
			Bd. theobromae			
QCh	955	798	1113	1.92 ± 0.29	−5.73 ± 0.89	0.13
QC1	689	505	838	1.67 ± 0.29	−4.74 ± 0.88	0.89
QC2	991	843	1142	2.07 ± 0.29	−6.20 ± 0.90	2.35
QC3	845	726	956	2.50 ± 0.30	−7.34 ± 0.92	1.68
QC4	564	409	689	1.94 ± 0.30	−5.34 ± 0.91	3.25
QC5	630	534	715	3.03 ± 0.33	−8.48 ± 0.99	3.12
QC6	607	441	741	1.82 ± 0.29	−5.07 ± 0.89	2.31
QC7	547	422	650	2.36 ± 0.32	−6.48 ± 0.95	1.50
QC8	601	163	870	2.84 ± 0.33	−7.89 ± 0.98	4.04
QC9	897	653	1123	1.28 ± 0.28	−3.79 ± 0.86	3.11
			F. oxysporum			
QCh	767	652	871	2.57 ± 0.31	−7.42 ± 0.93	1.18
QC1	919	821	1016	3.15 ± 0.32	−9.33 ± 0.97	3.74
QC2	641	520	743	2.46 ± 0.31	−6.91 ± 0.93	0.06
QC3	1083	991	1178	3.81 ± 0.35	−11.59 ± 1.08	1.68
QC4	647	506	762	2.19 ± 0.39	−6.18 ± 1.14	0.00
QC5	670	532	786	2.22 ± 0.39	−6.29 ± 1.14	0.09
QC6	1070	956	1217	3.10 ± 0.41	−9.40 ± 1.25	0.01
QC7	817	678	956	2.16 ± 0.38	−6.29 ± 1.14	0.52
QC8	787	354	1143	4.35 ± 0.37	−12.59 ± 1.12	7.65
QC9	720	599	827	2.43 ± 0.31	−6.96 ± 0.92	1.61
P. infestans						
QCh	954	806	1100	2.06 ± 0.29	−6.13 ± 0.9	0.60
QC1	1023	886	1167	2.26 ± 0.30	−6.81 ± 0.92	0.71
QC2	879	321	1376	3.74 ± 0.34	−11.02 ± 1.03	8.12
QC3	893	706	1117	1.63 ± 0.37	−4.81 ± 1.12	0.64
QC4	681	197	1010	4.10 ± 0.38	−11.64 ± 1.12	7.77
QC5	636	532	726	2.81 ± 0.32	−7.89 ± 0.97	1.95
QC6	729	576	863	2.00 ± 0.38	−5.73 ± 1.13	0.01
QC7	720	623	811	3.04 ± 0.40	−8.68 ± 1.19	0.00
QC8	608	543	668	4.47 ± 0.43	−12.46 ± 1.25	2.19
QC9	698	291	991	3.99 ± 0.37	−11.36 ± 1.09	6.27

[a] The concentration causing 50% mycelial growth inhibition.
[b] Slope of the concentration–inhibition regression line ± standard error.
[c] Intercept of the regression line ± standard error.
[d] Chi square value.

the quaternary chitosan compounds with 16.93 and 18.41 U/mg protein for PGase and PLase, respectively. C2, C3 and C9 were exhibited high inhibition in PPOase with 3.70, 2.73 and 4.18, respectively, compared to 18.64 in the control. However, QCh and QC5 were the most active in the quaternary compounds (8.41 and 7.56 specific activity, respectively). C1–C9 exhibited good inhibition in cellulase activity in *B. cinerea* and the specific activity was ranged from 0.12 to 2.96 compared to 4.44 in the untreated control. However, QCh, QC3, QC4, QC5 and QC9 were the most active compounds among the series of quaternary *N*-(benzyl) chitosan derivatives.

In *Bd. theobromae*, C3 and C5 were the most active among the series of *N*-(benzyl) chitosan derivatives to PGase (9.63 and 10.18, respectively) and PLase (11.19 and 11.69, respectively) compared to 26.79 and 31.95 specific activities of PGase and PLase, respectively in the untreated control. However, QC4 and QC9 exhibited the highest inhibition in a series of quaternary *N*-(benzyl) chitosan derivatives to PGase (5.82 and 7.70, respectively) and PLase (7.82 and 8.85, respectively). PPOase activity was significantly declined from 11.01 in the control to 1.49 in QC5. In addition, most of *N*-(benzyl) chitosan derivatives significantly reduced the cellulase activity and C5 was the most active one (specific activity = 0.13) compared to 6.96 in the control.

In *F. oxysporum*, C5, QC3 and QC7 proved the highest inhibition to PGase and PLase compared to the untreated control and the specific activity was reduced from 25.05 in the control to 7.22 for PGase and from 26.36 in the control to 8.41 for PLase in QC7. QC7 also the most active compound in inhibition of PPOase with activity of 4.92 compared to 10.28 in the control. However, most of *N*-(benzyl) chitosan derivatives were most active in inhibition of cellulase activity compared to the quaternary *N*-(benzyl) chitosan derivatives and the cellulase activity declined from 6.25 in the control to 0.12 with C5. *P. infestans* was the lowest active fungus in production of PGase, PLase, PPOase and cellulase activities compared to *B. cinerea*, *Bd. theobromae* and *F. oxysporum*. As the same manner in the previous fungi, QC1–QC9 derivatives proved the high efficiency in inhibition of the enzymes except cellulase which more inhibited by *N*-(benzyl) chitosan derivatives than the quaternary forms.

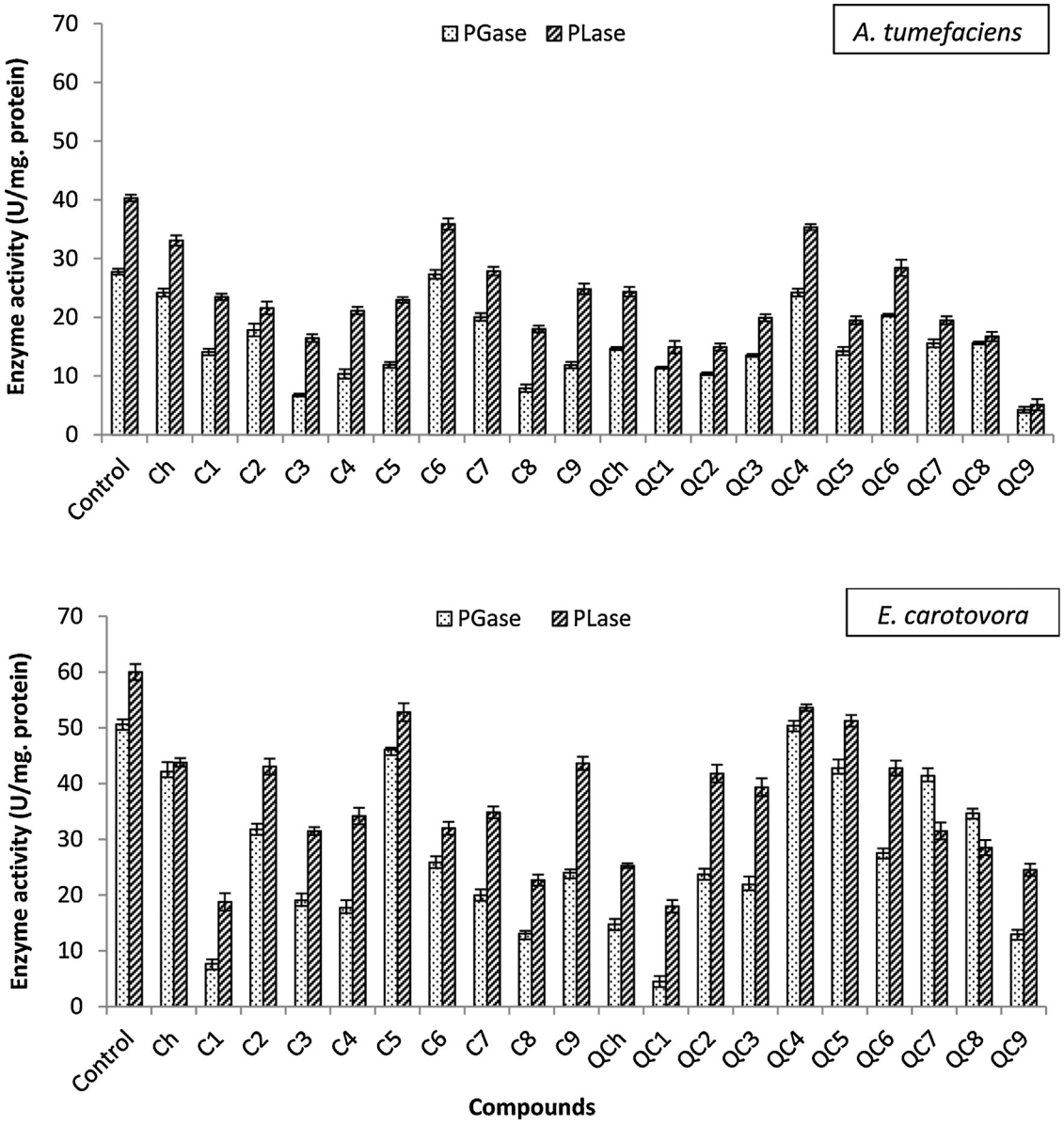


Fig. 1. Effect of chitosan, *N*-(benzyl) chitosan and quaternary *N*-(benzyl) chitosan derivatives on polygalacturonase (PGase) and pectin-lyase (PLase) in *A. tumefaciens* and *E. carotovora* after 6 days of the treatment at 1000 mg/L. Data are means ± SE of three replicates.

The antimicrobial activity of chitosan depends on several factors such as kind of chitosan (*i.e.*, degree of deacetylation and molecular weight), the medium pH, the kind of microorganism, the chemical modification and the solubility. Several hypotheses elucidating the antimicrobial activity of chitosan have been postulated. The most feasible hypothesis is a change in cell permeability due to the interaction between the polycationic chitosan and the electronegative charges on the cell surfaces. This interaction leads to the leakage of intracellular electrolytes and proteinaceous constituents (Young, Kohle, & Kaus, 1982; Papineau, Hoover, Knorr, & Farkas, 1991; Sudarshan, Hoover, & Knorr, 1992; Rabea et al., 2003). Other mechanisms are the interaction of diffused hydrolysis products with microbial DNA, which leads to inhibition of mRNA and protein synthesis (Hadwiger, Kendra, Fristensky, & Wagoner, 1986) and the chelation of metals, spore elements and essential nutrients (Cuero, Osuji, & Washington, 1991).

Chitosan itself has moderate antimicrobial activity against plant pathogens. According to this fact, several research groups have started to modify it to produce high antimicrobial active compounds. Quaternary ammonium chitosan, which was prepared by introducing a quaternary ammonium group on a dissociative hydroxyl group or amino group of the chitosan, exhibited improved water solubility and stronger antimicrobial activity relative to chitosan over an entire range of pH values; thus, this quaternary modification increases the potential applications of chitosan in the field of antimicrobial activity. In the present work, we hypothesized that, the chemical modification of chitosan molecule by introducing benzyl and permanently charged quaternary groups improve the antimicrobial activity of chitosan. The addition of these compounds to the bacterial and fungal media inhibited growth and also the excocellualr enzymes. Comparing the structure and the antimicrobial activity of *N*-(benzyl) chitosan and quaternary *N*-(benzyl) chitosan derivatives, it was obvious that all derivatives showed much better antimicrobial activity than chitosan due to the introduction of the function group of benzyl substituents. It was in accordance with the conclusions of Guo et al. (2006), Rabea et al. (2006, 2009) and Badawy (2008). The MICs and EC_{50}s for chitosan against bacteria and fungi, respectively were higher than 3000 mg/L and this value is within of the range in the literature, which varies from 10 to 5000 mg/L depending on the molecular weight,

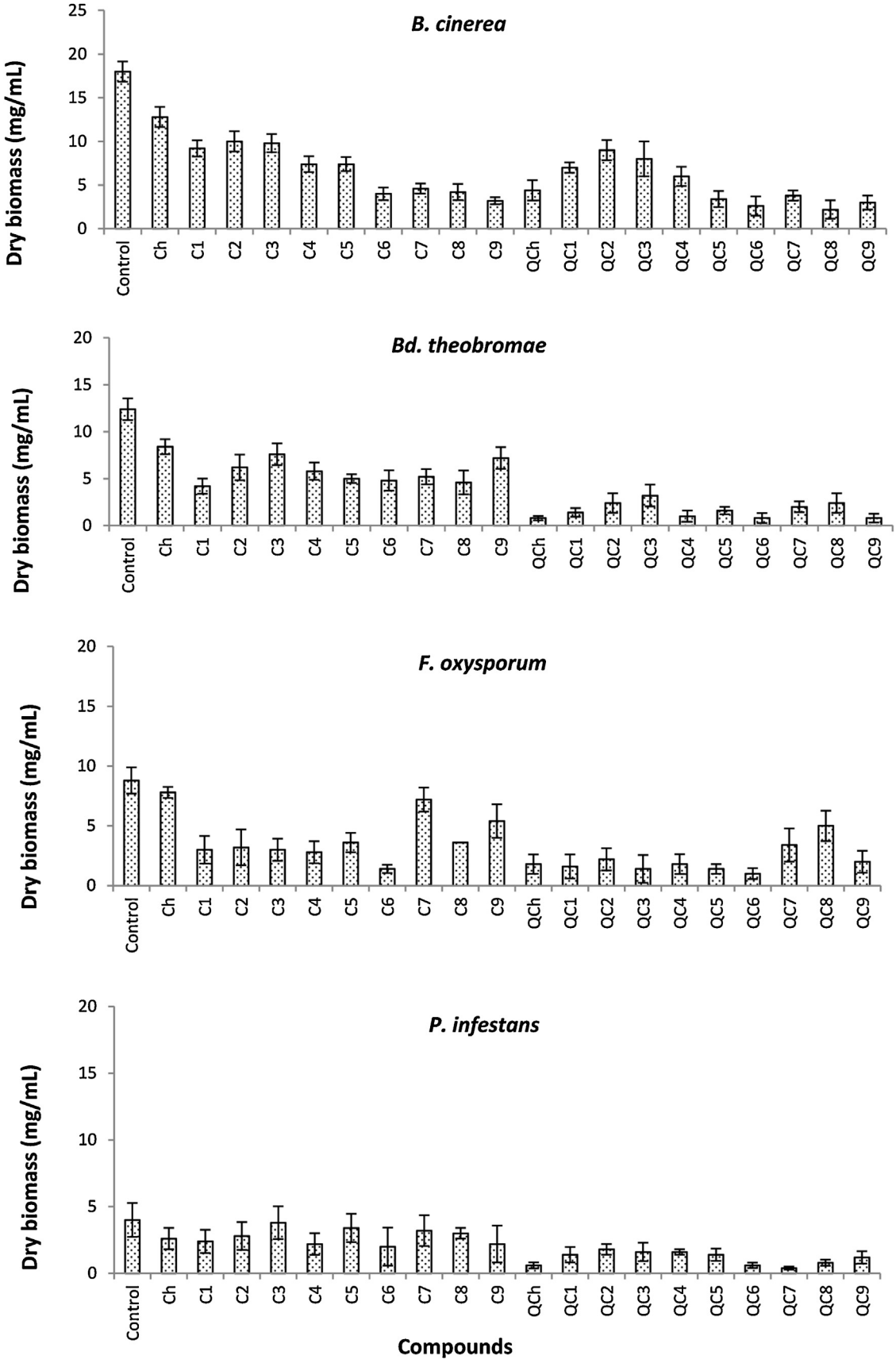


Fig. 2. Effect of chitosan, *N*-(benzyl) chitosan and quaternary *N*-(benzyl) chitosan derivatives on fungal biomass in *B. cinerea*, *Bd. theobromae*, *F. oxysporum* and *P. infestans* after 8 days of the treatment at 1000 mg/L. Data are means ± SE of three replicates.

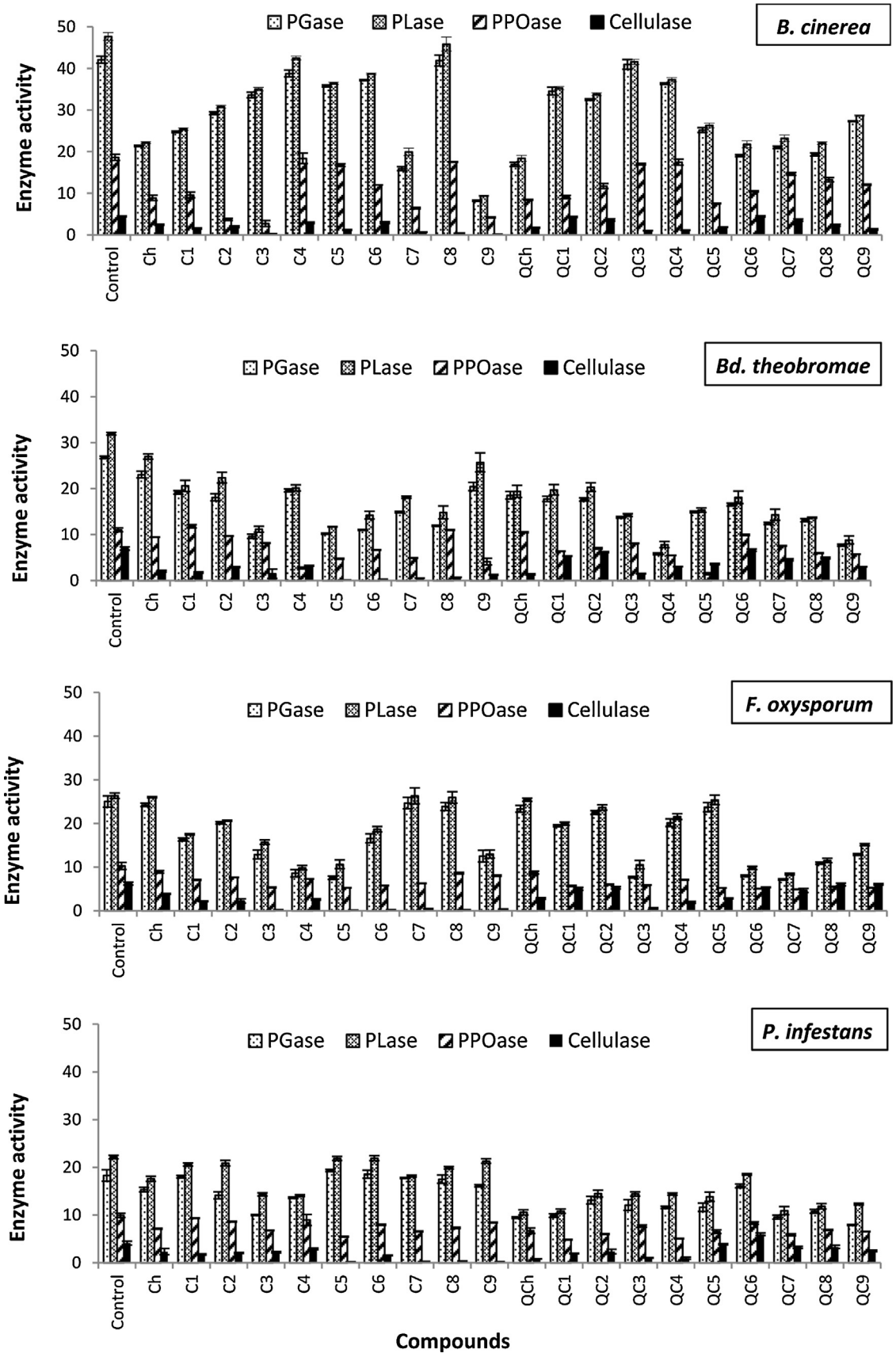


Fig. 3. Effect of chitosan, *N*-(benzyl) chitosan and quaternary *N*-(benzyl) chitosan derivatives on polygalacturonase (PGase), pectin-lyase (PLase), polyphenol oxidase (PPOase) and cellulase activities in *B. cinerea*, *Bd. theobromae*, *F. oxysporum* and *P. infestans* after 8 days of the treatment at 1000 mg/L. Data are means ± SE of three replicates.

chemical modification, degree of substitution, degree of acetylation and the type of microorganism (Rabea et al., 2003; Badawy & Rabea, 2011; Tan et al., 2013; Guo et al., 2006, 2014). Similar responses as obtained in the current study were observed by other authors and the MICs of quaternized chitosan compounds were decreased with the degree of substitution (DS), which can be explained based on the interaction of these groups with negatively charged cell surface membrane of the microorganism, preventing nutrients from entering the cell (Young et al., 1982; Guo et al., 2006).

The present results indicate good antibacterial activity of quaternary *N*-(benzyl) chitosan derivatives against the tested bacteria comparing to the other derivatives that previously reported in our laboratory. For example, Badawy (2010) reported that the *N*,*N*,*N*-(dimethyl alkyl) chitosans showed inhibitory activity ranged from 750 to 2415 mg/L against *A. tumefaciens* and 1225 to 2680 mg/L for *E. carotovora*. Badawy and Rabea (2012) added that *N*-(4-carboxybutyroyl) chitosans at different DS values ranged from 0.10 to 0.53 proved MIC values of 800 to 1200 against *A. tumefaciens* and 725 to 1100 mg/L for *E. carotovora*. The presence of benzyl in the quaternary group could improve the binding capability of the derivative with bacteria and restrained the movement of microbiological substances; therefore, all the derivatives in the present study showed higher antimicrobial activity than the alkyl compounds tested in the previous studies (Badawy, 2010; Badawy & Rabea, 2012). As the same manner, Jia et al. (2001) reported that the quaternary ammonium chitosan derivatives had better antibacterial activities against *Escherichia coli* than unmodified chitosan, which was attributed to the high charge density of quaternized chitosan. These results are in agreement with the present study indicates that all quaternary *N*-(benzyl) chitosan compounds have better antimicrobial activity than of *N*-(benzyl) chitosan compounds. As shown in Scheme 1, the cationic charge of quaternized chitosan derivatives is higher than that of chitosan and *N*-(benzyl) chitosan and thus has the best antimicrobial activity. Another study presented *N*-methylation of *N*-arylated chitosan derivatives containing *N*,*N*-dimethylaminophenyl and pyridyl substituents as water-soluble compounds over all pH ranges. These compounds displayed good antibacterial activity against *Staphylococcus aureus* and *E. coli* and their MIC values were in the range of 32–128 μg/mL against both bacteria (Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2008).

The data of the enzyme activities revealed that most of the compounds tested at 1000 mg/L had good inhibitory effects against PGase, PLase, PPOase and cellulase. This finding indicates that these enzymes may be the main targets of the chitosan compounds. It is known that bioactivities of chitosan are mainly due to their reactions with proteins and some enzymes. It is also important to note that the antimicrobial activity of the tested compounds is based on their change in cell permeability of the cell wall of the tested fungi and bacteria (Young et al., 1982; Hadwiger et al., 1986).

Although quaternized chitosan derivatives have been regarded as effective antimicrobial agents, their modes of action need to be further studied in depth. Investigations have recently focused on the morphological changes of the microorganism with respect to its antimicrobial property and mechanism. The methods used to evaluate the antimicrobial phenomena have been limited to the biological conception only. Therefore, future work should aim at demonstrating the molecular details of the underlying mechanisms and their relevance to the antimicrobial activity of quaternized chitosans.

4. Conclusion

Quaternized *N*-(benzyl) chitosan derivatives were prepared by the reaction between *N*-(benzyl) chitosans and ethyl iodide. Compared with the *N*-(benzyl) chitosan derivatives (C1–C9), quaternized *N*-(benzyl) chitosan derivatives (QC1–QC9) showed much better inhibitive capability against both plant pathogenic bacteria and fungi, it indicated that there was a synergistic effect between benzyl moiety and quaternary ammonium group on the antimicrobial activity. The grafted of substituted benzyl group into chitosan with quaternization improved the antimicrobial activity of chitosan greatly. Substituted benzyl group especially with chloro-, fluoro-, cyano-, and nitro benzyl are widely used in most synthetic pesticides. However, such pesticides have pronounced toxicities and their residues in the environment have already aroused serious environment problems. When these groups are grafted onto chitosan, they could be released slowly which will largely avail the environmental issue. So, this kind of chitosan derivatives and similar compounds are worthy of further studying. The synthesis and characterization of chitosan derivatives having biologically active groups are ongoing in our laboratory aiming to obtain products with promising antimicrobial activity. This study suggests that the use of such compounds could be a promising handling as antimicrobial agents to partially substitute for the harmful synthetic bactericides and fungicides.

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