Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(7):837-843

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Rice-induced resistance, elicited by β-glucanase from *Peanopacillus polymxa*, in controlling blast disease

Wafaa M. Haggag

Plant Pathology Department, National Research Centre, Dokki, Giza, Egypt, 12622

ABSTRACT

β-1,3-glucanase from Peanopacillus polymxa was tested against Pyricularia oryzae , the causels pathogen of blast disese of rice. A glucan-producing P. polymxa, was purified and showed antagonistic activity to Pyricularia oryzae . The minimum fungicidal concentration of β-1,3-glucanase against Pyricularia oryzae, was determined to be 250 µg/ml. Under greenhouse condations, β-1,3-glucanase at concentrations of 0, 250 and 500 µg/ml and culture filtrate of P. polymxa remarkably reduced disease incidence by enhancing perioxdase and total phenols defense. The activity of perioxdase and total phenols increased several folds by the elicitors. Field experiments incidcated that rice seedlings treated with β-1,3-glucanase at 500 µg/ml and and culture filtrate of P. polymxa significantly decreased blast development by 89.1% and 92.3%, respectively, compared to the untreated and increase in rice grain yield. The obtained results suggest that the β-1,3-glucanase from P. polymxa can be used as an biofungicide for the purpose of enhancing plant immunity.

Keywords: β-1,3-glucanase, *Paenibacillus polymyxa*, Rice, Blast disease.

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the world's most important strategic crops[1][2]. Rice blast causes between 11% and 30% crop losses annually, this represents a loss of 157 million tonnes of rice [3] . Rice is among the most important field crops in Egypt[4] [5]. It occupies about 0.5 million h /year with 7.9 t/ha as a national average [4] . However, rice blast caused by *Pyricularia oryzea* Cav. is considered as the major constraint to Egypt's rice production [4][5][6]. Currently, the rice diseases are controlled by the use of chemicals and resistant cultivars[5][6]. However, the lack of durable resistance, the existence of pathogenic variability, and concerns regarding chemical resistance have limited the potential of such strategies for the disease management [5][6]. So, environment friendly and more effiency biofungicides are needed for controlling of the rice disease [7][8]. Many antagonistic microorganisms are potential to produce diverse antifungal compounds and enhane plant resistance against diseases [9][10]. Among these *Peanopacillus polymxa* know to produce glucans,which are of particular interest in the context of biocontrol that exhibit successful biocontrol effects not only by inhibiting fungal pathogen growth, but also by re-inforcing the host resistance potential through the induction of plant defense responses [11][12][13][14][15]. Specifically, β-glucan, a glucose homopolymer, has shown high immunomodulating effects [16][17][18], thus promoting its utility in the food and pharmaceutical Industries. b-glucan-degrading enzymes are produced by a wide variety of organisms. Even though several bacteria, including *Bacillus* spp. and others, are reported to produce glucans [16][18] . Therefore, in industry, glucans from microorganisms are widely used.

The aim of this study was to investigate the role of a *β-1,3-glucanase* obtained from against *Pyricularia oryzae* and the ability to elicit rice- resistance to blast disease.

EXPERIMENTAL SECTION

Pyricularia oryzae was originally isolated from diseased rice plants grown in El Kalubia governorate. Fungal cultures were subcultured on Potato dextrose agar (PDA) at 28°C for routine use and stored at 4°C until use. For the preparation of pathogens inoculums, pathogen wa inoculated into an PDA plates and incubated at 28°C for 6 days. At that time, the cells on the surface of the plate were collected by washing with sterile H2O and diluted to OD600 = 0.1 (approximately 10^4 CFU/ml).

The antagonist bacterium *Peanopacillus polymxa* was isolated from health rice plant. The storage and cell maintenance conditions was subcultured onto aniline blue agar (per L; 20 g sucrose, 5 g yeast extract, 20 g agar, 0.05 g aniline blue, 3 g CaCO3) [19] for the isolation of glucan producers. All bacteria were stored in 15% glycerol stocks held at -4^{o}C for longterm preservation. Bacteria was adjusted to 10^6 CFU/ml. Extraction and Purification of B-glucane from *Peanopacillus polymxa*. The seed cells were cultivated on nutrient broth (Difco,USA) at 30°C for 12 h. Ten milliliters of seed culture was inoculated into 1 L of mineral salt medium (MSM; per L; 1.74 g KH2PO4, 0.015 g CaCl2·2H2O, 0.49 g K2HPO4, 0.01 g MnCl2·4H2O, 3.7 g Na2SO4·10H2O, 0.21 g citrate, 0.25 g MgCl2·6H2O, 1.5 g NH4Cl , 0.024 g FeCl3·6H2O) that was supplemented with 10% sucrose as a carbon source to induce glucan production, and cultured at 30°C for 3 day with shaking at 180 rpm.

-1,3-Glucanase assay

Enzyme activity was measured by mixing 50 µl of sample with 100 µl of 50 mmol l^{-1} acetate buffer (pH 5.0), containing 0.25% laminarin (Sigma). The mixture was incubated at 40°C for 30 min and the reducing sugar produced was determined by the method described by Miller [20]. One unit (U) of β-1,3-glucanase activity was defined as the amount of enzyme that produced 1 µmol of reducing sugar min^{-1}under the above conditions. Protein concentration was determined by the method of Lowry [21], using bovine serum albumin as standard.

Enzyme purification

To separate the glucan produced by *P. polymyxa*, the culture broth was centrifuged at 5,000 rpm for 15 min to collect the culture supernatant. From the supernatant, the glucan was precipitated by incubating at 4oC for 12 h after adding 3 volumes of ice-cold ethanol. The pellet recovered from the mixture by centrifugation was washed 3 times with sterilized distilled water, and then lyophilized to obtain the crude glucan. The harvested crude glucan was dissolved in 0.1 M NaCl solution, loaded onto a Sephadex G-100 column, and fractionated with 0.1 M NaCl at a flow rate of 0.1 mL/min. The glucan content of each fraction was determined by measuring the total carbohydrate content, using the phenolsulfuric acid method [21]. The glucan fractions having a high content of carbohydrate were pooled and lyophilized to give the purified glucan.

Evaluation of the Antifungal Activity of β-1,3-Glucanase and culture filtrate of *Peanopacillus polymxa* against pathogen was evaluated by the filter paper disc method as previously described [22]. The assay was performed at least in triplicates.

Estimation of Total Phenolics from seedlings of rice

The total phenolic content was determined as described by Mandal et al. [23] using Folin-Ciocalteau reagent. The reaction mixture contained 100 L of methanolic extract of rice seedlings leaves tissues and 200 L sterile distilled water with 500 L of Folin-Ciocalteau reagent. After 5 min, 800 L of 20% sodium carbonate was added, and after 1 h of incubation, the absorbance was measured at 254 nm in a BioMate 3 spectrophotometer (Thermo Spectronic, USA). Standard curve was prepared with p-hydroxybenzoic acid in 50% (v/v) methanol. The total phenolic content was expressed as micrograms of p-hydroxybenzoic acid equivalent/g FW of rice seedlings leaves tissues.

OD Activity Assay in seedlings of rice

Enzyme extraction steps were carried out at 4°C. 1 g fresh weight of rice seedlings leaves tissues was crushed in liquid nitrogen in presence of 1 g polyvinyl pyrophosphate and then extracted with 5 mL of 200 mM Tris-buffer (pH 8.0). The suspension was homogenized for 1 min and then centrifuged at 20 000 g for 20 min. Peroxidase activity was determined from the crude enzyme extract (supernatant) after concentration through Amicon Ultra-4 CFU membrane (Millipore, Bedford, USA) using an assay system consisting of 20 mM guaiacol (0.5 mL), 0.1 mM acetate buffer (pH 5.0) (2.1 mL), 40 mM hydrogen peroxide (H_2O_2) (0.2 mL), and the enzyme extract (0.2 mL) with a final volume of 3 mL (modified from Chance and Maehly [24]). Oxidation of guaiacol was measured by the increase in absorbance at 470 nm. One unit of enzyme activity represented the amount of enzyme catalyzing the oxidation of 1 mol of guaiacol in 1 min.

Evaluation of the Biocontrol Effect of *Peanopacillus polymxa* –Induced Rice Disease Resistance

The rice variety, Sakha 101 was used in this study. The rice seeds were germinated for 3 days at room temperature. The seedlings were then planted in 15-cm-diameter pots containing sterile moist soil (clay: sand, 3:1) dipped in 1/2-diluted Hoagland micronutrients. The planted seedlings were kept in a greenhouse at 26 ± 2oC with a relative humidity of 80 ± 5% for another 15 days. After the 15 days, the seedlings were challenged by inoculation with freshly prepared cell suspensions of pathogen . One day after inoculation, the seedlings were uniformly sprayed with a 1 ml volume of purified β-1,3-Glucanase (0.0, 250, and 500 µg/ml) or sterile H2O (as a pathogen inoculation control) on the leaf surface of every seedling. The seedlings were then returned to greenhouse conditions for disease development. At day 15 after pathogen challenge, the number of leaves exhibiting disease symptoms and the lesion size (lesion length/leaf length) were recorded. For each treatment, 30 seedlings were used, and the assay was repeated at least three times in independent experiments.

Natural Infection in the Field

Field experiments were condacted using Sakha 101 cultivar, to control of blast disease in Kalubia govarnorate. Rice cultivar was sown in 5x5 m plots. A randomized complete block design with five replicates for each treatment was used. Irrigation was carried out as recommended. Seeds and seedlings were uniformly sprayed with a 1 ml volume of purified β-1,3-Glucanase (500 µg/ml) or sterile H2O after 30 days of transplanting as previously description. Disease incidence of leaves was assessed monthly using the (0-9) scale of IRRI [25] as follows:
0) No lesions observed Highly resistant; 1) Small brown specks of pinpoint size or larger brown specks without sporulating center, Resistant; 2) Small roundish to slightly elongated, necrotic gray spots, about 1-2 mm in diameter, with a distinct brown margin, Moderately resistant ; 3) Lesion type is the same as in scale 2, but a significant number of lesions are on the upper leaves; Moderately resistant; 4) Typical susceptible blast lesions 3 mm or longer, infecting less than 4% of the leaf area, Moderately susceptible; 5) Typical blast lesions infecting 4-10% of the leaf area , Moderately susceptible; 6) Typical blast lesions infection 11-25% of the leaf area , Susceptible; 7) Typical blast lesions infection 26-50% of the leaf area ; Susceptible; 8) Typical blast lesions infection 51-75% of the leaf area and many leaves are dead; Highly susceptible; 9) More than 75% leaf area affected ; Highly susceptible.

Mean number of spores /cm^{-2} were counted during growth periods using hemocytometer. Resistant reaction was based on no visible infection and no conidia produced from affected tissue. Susceptible reaction was based on a lesion size greater than 3 cm in length, visible infection, and conidia evident in affected tissue . The intensity of blast infection was measured for each plot by calculation of the Area Under the Disease Progress Curve (AUDPC) using numerical integration**.** The largest length (L) and width (W) of each lesion were measured and used to calculate the lesion area (A); (A=0.25πLW). AUDPC is expressed as units of 'percentage months'. Data were recorded per 10 plants, 20 days intervals on randomly selected lines per replicate**.** At harvest stage, plant height and grain dry weight were measured.

Statistical Analysis

The percentages of disease severity were transformed before analysis of variance to improve homogeneity of variance. Analysis of variance was performed to analyze the transformed data. The completely randomized method Snedecor and Cochran [26], was used for analysis of variance and LSD (0.05).

RESULTS AND DISCUSSION

Exo-β-1,3-glucanase asassy

We have purified a β-1,3-glucanase produced by *Peanopacillus polymxa* , using two steps procedures: gel filtration on Sephacryl S-100 and ion exchange chromatography on Q-Sepharose. Gel filtration resulted in the separation of two peaks of proteins with β-glucanase activity, and the first peak was used for further purification of the enzyme on a Q-Sepharose. The enzyme was purified 34.6-fold with a recovery of 9.4% (Table 1).

Table 1. Summary of the purification steps of the exo-β-1,3-glucanase produced by *Peanopacillus polymxa*

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude enzyme	1.486	0.314	0.217	1	100
Sephacryl S-100	0.023	0.047	2.040	9.3	15.0
Q-Sepharose	0.003	0,030	7.648	34.6	9.4

Biocontrol Potential of β-1,3-glucanase from *Peanopacillus polymxa* against *Pyricularia oryzae*

β-1,3-glucanase was purified from *P. polymxa* and tested against *Pyricularia oryzae* at different concentratios by using the paper disc method (Fig.1). β-1,3-glucanase showed highly antagonistic activity towards *P. oryzae* at low

concentration 250µg/ml. For in vitro antagonism of culture filtrate of *P.polymxa* toward pathogen exhibited antifungal ability in bioassaysin compared with water as control.

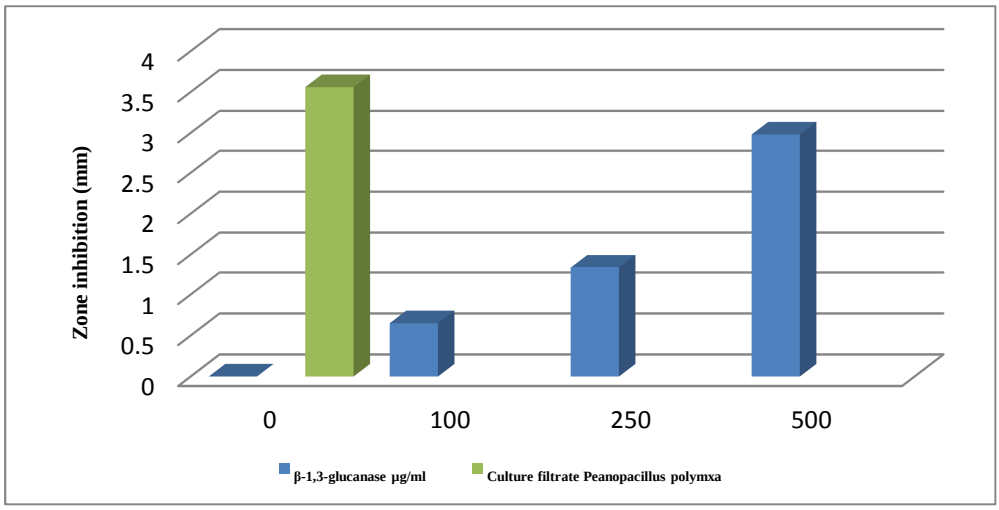

Fig. 1. Biocontrol Potential of β-1,3-glucanase from *Peanopacillus polymxa* against *Pyricularia oryzae*

Evaluation of the Biocontrol Effect of *Peanopacillus polymxa* –Induced Rice Disease Resistance

Bioassays for the elicitation of ISR in rice using different concentrations of β-1,3-glucanase produced by *P. polymxa* was tested 15 days after leaves rice seedlings treated with the pathogen *P. oryzae* (Fig. 2). The seedlings treated with 100, 250, or 500 ug/ml of β-1,3-glucanase were 18.03%, 12.56%, and 8.0%, respectively. Foliar symptoms of rice blast disease and the average lesion size was greater reduced by 89%. In control seedlings, the symptoms on leaves of these rice seedlings were detected, indicating that *P. oryzae* is the virulent causal agent of rice blast disease. The lesion sizes in untreated control were significantly lower than those of the control rice seedlings challenged with only the pathogen (Fig. 2). These results indicated that β-1,3-glucanase effectively protects against rice disease caused by *P. oryzae* under the tested concentrations compared with culture filtrate of *P. polymxa* .

In order to further understand the mechanisms underlying the Total phenolic content (expressed as mg 4-hydroxybenzoic acid equivalent/g FW) and peroxidase (POD) activity (nkat/mg protein) triggered by β-1,3-glucanase in rice, experiments on the degree of ISR response produced against infections of the blast pathogen. Data demonstrating the ability to trigger ISR via the enhancening and fold increase of total phenolic content (expressed as mg 4-hydroxybenzoic acid equivalent/g FW) and peroxidase (POD) activity (nkat/mg protein) (Fig. 3).

Effect of β-1,3-glucanase on blast disease of rice under field conditions

β-1,3-glucanase and culture filtrate of *P. polymxa* were tested to study their effect on blast disease incidence of rice plants under field conditions (Table 2). Natural Infection in the field showed that the β-1,3-glucanase at 500 ug/ml effectively protects rice from blast disease caused by *P. oryzae* which significantly reduced the severity of blast spot disease, compared with culture filtrate of *P.polymxa* untreated controls during two growing seasons as compared with untreated plants. Moderate effect was obtained with fungicide treatments which reduced the blast incidence more than 81 % during two growing seasons.

At the same time, application of β-1,3-glucanase at 500 ug/ml effectively increased rice growth and yield, compared with culture filtrate of *Peanopacillus polymxa* and untreated controls during two growing seasons as compared with untreated plants (Table 3). Moderate effect was obtained with fungicide treatments.

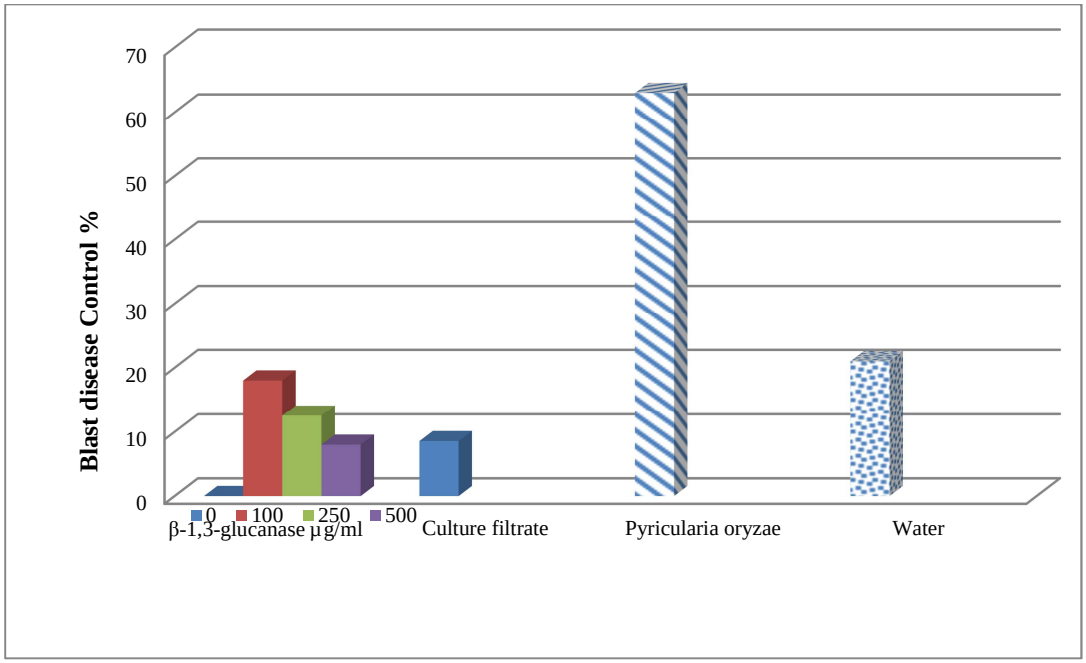

Fig. 2. Evaluation of the biocontrol effect of β-1,3-glucanase from *Peanopacillus polymxa* induced rice blast disease resistance

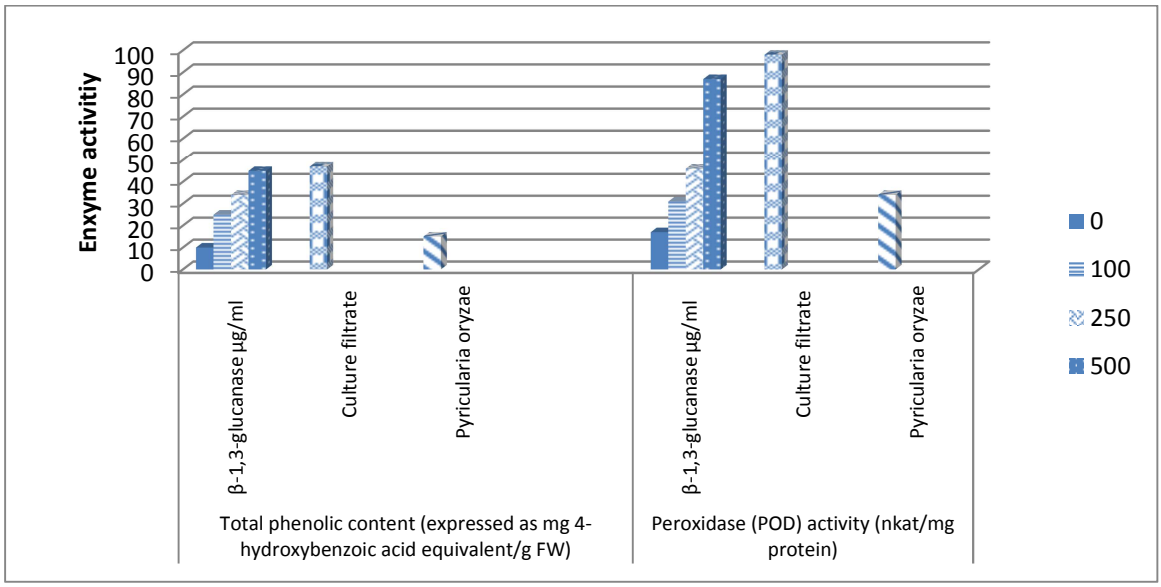

Fig. 3. Total phenolic content (expressed as mg 4-hydroxybenzoic acid equivalent/g FW) and peroxidase (POD) activity (nkat/mg protein) on a time course after elicitation of the rice seedings β-1,3-glucanase from *Peanopacillus polymxa* against *Pyricularia oryzae*

Table 2. Area under disease progress curves (AUDPC) , lesion number and blast disease score of rice cultivar treated with β-1,3-glucanase under natural field conditions after 90 days of growth

Treatment	AUDPC		Lesion number/leaf		Diseased score (0-9)*		Sporulation leaf/cm	
Season	**I**	**II**	**I**	**II**	**I**	**II**	**I**	**II**
β-1,3-glucanase	1.1	1.0	4.43	4.33	2.05	1.9	13.9	11.2
Culture filtrate of *Peanopacillus polymxa*	1.8	1.9	3.61	3.43	1.23	1.26	10.2	10.8
Fungicide	1.9	1.1	5.73	5.43	3.12	3.54	15.2	14.6
Untreated control	5.8	5.8	28.1	22.4	6.17	6.09	59.0	57.6
LSD	0.26	0.25	0.94	0.76	0.66	0.56	2.46	2.37

Analysis of variance was performed to analyze the transformed data. The completely randomized method Snedecor and Cochran (1972), was used for analysis of variance and LSD (0.05).

Table (3): Growth rate and yield of tested rice cultivar treated with β-1,3-glucanase under natural field conditions after 90 days of growth

Treatment	Plant length (cm)		Plant weight (g)		Yield/plant (g/1000 grain)	
Season	**I**	**II**	**I**	**II**	**I**	**II**
β-1,3-glucanase	62.6	63.2	16.1	16.7	32.1	33.4
Culture filtrate of *Peanopacillus polymxa*	65.1	66.8	17.7	17.8	33.2	34.6
Fungicide	61.1	63.5	15.2	15.9	29.1	30.8
Untreated control	50.1	51.4	10.0	11.3	22.1	23.8
LSD	1.18	1.09	1.01	0.98	1.04	0.95

Analysis of variance was performed to analyze the transformed data. The completely randomized method Snedecor and Cochran (1972), was used for analysis of variance and LSD (0.05).

In the present study, β-1,3-glucanase was obtained from the culture supernatant of *P. polymxa* and displayed concentration-dependent antifungicide, effects and involved in antagonism of fungal pathogen [16-18]. The inhibitory effect of *P. polymxa* on the virulence factors of pathogen is indictor that its producer as a control agent to control rice disease caused by *P.oryzae* .

Plant-induced resistance has been recognized as the most attractive type of biocontrol agent for plant disease management in modern agriculture because it could provide plants with long-lasting protection from pathogens [10] [11] [12]. Furthermore, the ability of *P. polymxa* to trigger rice-induced resistance to pathogen attack was investigated by assessing the expression of several well-studied metabolites related to rice defense against pathogen in rice seedlings treated with glucan during the progression of pathogen infection[10-14]. Exo-b-glucanase is a key positive regulator of perioxdase and total phenols -mediated rice immunity, and its ectopic expression in rice conferred high levels of resistance to pathogen [15]. Several types of these enzymes exist, classified according to the type of b-glucosidic linkage that they cleave and the mechanism of substrate attack. They can hydrolyze the substrate by two possible mechanisms, identified by the products of hydrolysis: (*i*) exo-b-glucanase hydrolyze the substrate by sequentially cleaving glucose residues from the nonreducing end, or (*ii*) endo-b-glucanases cleave b-linkage at random sites along the polysaccharide chain, releasing smaller oligosaccharides. Degradation of b-glucan by fungi is often accomplished by the synergistic action of both endo- and exo-b-glucanases [23].

In the present study, applying glucan to rice seedlings significantly reduced the incidence and severity of disease caused by *P. oryzae* when compared with that of rice seedlings only challenged by the pathogen . This result was consistent with the fact that *P.polymxa* possesses multilayered biocontrol-related traits, as descried above. However, the present disease suppression test was performed only on a small scale and under indoor controlled conditions. *Paenibacillus polymyxa* is a plant growth-promoting rhizobacterium (PGPR) commonly found in the rhizospheres of various plant species [11-13] and have demonstrated the beneficial effects plant growth [11] [12]. Such improvements in c crop yield were adopted to involve many characteristics of *P. polymyxa*, including nitrogen fixation, plant-disease suppression, soil phosphorous solubilization, increase in soil porosity, and the production of antibiotics, hydrolytic enzymes, and phytohormones.

In conclusion, β-1,3-glucanase from *P. polymxa* not only strongly inhibited the growth of pathogen but also was capable of protecting rice against *P.oryzae* -induced disease by re-inforcing perioxdase and total phenols defense to the pathogen.These results highlight the potential of *P. polymxa* and its producer as biofungicide to control rice diseases caused by *P. oryzae* .

REFERENCES

[1] Bonman, J.M. (**1992**). Rice Blast. *In:* Compendium of Rice Diseases. Eds. R.K. Webster and P.S. Gunnel. American Phytopathological Society Press. St. Paul, Minnesota. USA. Pages 14-18.
[2] Lee, F.N., R.D. Cartwright, Y. Jia, J.C. Correll, K.A.K. Moldenhauer, J.W. Gibbons, V. Boyett, E. Zhou, E. Boza, and E. Seyran. (**2005**). A preliminary characterization of the rice blast fungus on 'Banks' rice. *In*: R.J. Norman, J.-F. Meullenet, and K.A.K. Moldenhauer (eds.). B.R. Wells Rice Research Studies **2004**. University of Arkansas Agricultural Experiment Station Research Series 529:103-110. Fayetteville, Ark.
[3] Sesma, A; Osbourn, AE**.** *Nature.* 431, **2004,** 582-586
[4] Badawi A.T. (**1995**). Annual report of the National Campaign of Rice in Egypt. Agricultural Res. Center, Ministry of Agriculture Giza, Egypt, p. 24 (in Arabic).
[5] Balal M.S. (**1984**). Rice breeding for resistance to blast in Egypt. In : Proceedings of the 4th National Rice Conference, Feb. 27-March 1, Cairo, Egypt, pp. 1 6 7-1 7 8.
[6] Haggag, Wafaa M. and Mekhail M. *British Biotech.J.,* **2014,** 4(8): 894-903.
[7] Vaiyapuri R. Prabavathy, Narayanasamy Mathivanan', Kandasamy Murugesan. *PM5 Biological Control* . **2006,** 39, 3 , 313–319.

[8] Chithrashree AC, Udayashankar AC, Chandra NS, Reddy MS, Srinivas C. *Biocontrol*, **2011,** 59: 114-22.
[9] Perez-Garcia A, Romero D, de Vicente A. *Curr*. *Opin*. *Biotechnol*., **2011,** 22: 187-193.
[10] Haggag Wafaa M., Abouziena H. F. , Abd-El-Kreem F. and El Habbasha S. *Journal of Chemical and Pharmaceutical Research*, **2015**, 7(10):882-889.
[11] Haggag, Wafaa,M., Hussein, M.M., Mehanna, H.M. and Abd El-Moneim D. *Int. J. Pharm. Sci. Rev. Res*., **2014,** 29(2), 292-298.
[12] Haggag, Wafaa, M. and Salme Timmusk. *J. Appl. Microbiol.* (UK)., **2008,** 104 (4): 961-969
[13] Haggag, Wafaa, M. *Archives J. of Phytopathol. and Plant Protection* (German)., **2008,** 41(7): 477 – 491.
[14] Raaijmakers JM, De Bruijn I, Nybroe O, Ongena M. *FEMS Microbiol. Rev.*, **2010,** 34: 1037-1062.
[15] De Vleesschauwer D, Xu J, H◌fte M. *Plant Sci*., **2014,** 5: 611.
[16] Gummadi, S. N. and K. Kumar. *Biotechnol*. *Bioprocess Eng*., **2005,** 10: 546-551.
[17] Park, J. H., M. S. Kang, H. I. Kim, B. H. Chung, K. H. Lee, and W. K. Moon. *Kor. J. Food Sci. Technol.*, **2003,** 35: 488-492.
[18] Seo, H. P., J. M. Kim, H. D. Shin, T. K. Kim, H. J. Chang, B. R. Park, and J. W. Lee. *Kor. J. Biotechnol. Bioeng.*, **2002,** 17: 376-380.
[19] Nakanishi, I., K. Kimura, T. Suzuki, M. Ishikawa, I. Banno, T. Sakane, and T. Harada. *J. Gen. Appl. Microbiol.*, **1976,** 22: 1-11.
[20] Miller G.L. *Anal. Chem.*, **1959,** 31,426–428
[21] Lato M., Brunelli B., Ciuffini G. *J*. *Chromatogr*., **1969,** 39, 407–417.
[22]delaCruz J., Rey M., Lora J.M., HidalgoGallego A., Domínguez F.,PintorToro J.A., Llobell A., Benítez T. *Arc h. Microbiol*., **1993,** 159:316–322.
[23] Mandal, S. , A. Mitra, and N. Mallick. *World Journal of Microbiology and Biotechnology*, **2009,** vol. 25, no. 5, pp. 795–802.
[24] Chance , B. and M. Maehly, (**1995).“**Assays of catalases and peroxidases,” in Methods in Enzymology, S. P. Colowick and N. P. Kaplan, Eds., pp. 764–775, Academic Press, New York, NY, USA,
[25] *IRRI* (International Rice Research Institute). (**1980)**. Standard evaluation system for rice, 2d ed. Los Baños, Philippines.
[26] Snedecor, G. W and W. G. Cockran, (**1972**) . Statistical Methods. 6 ^{6th} Ed, the Iowa State Univ., Press. Ames, Iowa, U.S.A. pp.593.
[27]Pitson, S.M.; Seviour, R.J. and Mcdougall, B.M. *Enzyme Microb*. *Technol*., 1**993, 15**, 178-192.