Polygalacturonase gene \textit{pgxB} in \textit{Aspergillus niger} is a virulence factor in apple fruit

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Abstract

\textit{Aspergillus niger}, a saprophytic fungus, is widely distributed in soil, air and cereals, and can cause postharvest diseases in fruit. Polygalacturonase (PG) is one of the main enzymes in fungal pathogens to degrade plant cell wall. To evaluate whether the deletion of an exo-polygalacturonase gene \textit{pgxB} would influence fungal pathogenicity to fruit, \textit{pgxB} gene was deleted in \textit{Aspergillus niger} MA 70.15 (wild type) via homologous recombination. The \textit{ΔpgxB} mutant showed similar growth behavior compared with the wild type. Pectin medium induced significant higher expression of all pectinase genes in both wild type and \textit{ΔpgxB} in comparison to potato dextrose agar medium. However, the \textit{ΔpgxB} mutant was less virulent on apple fruits as the necrosis diameter caused by \textit{ΔpgxB} mutant was significantly smaller than that of wild type. Results of quantitative-PCR showed that, in the process of infection in apple fruit, gene expressions of polygalacturonase genes \textit{pgaI}, \textit{pgaII}, \textit{pgaA}, \textit{pgaC}, \textit{pgaD} and \textit{pgaE} were enhanced in \textit{ΔpgxB} mutant in comparison to wild type. These results prove that, despite the increased gene expression of other polygalacturonase genes in \textit{ΔpgxB} mutant, the lack of \textit{pgxB} gene significantly reduced the virulence of \textit{A. niger} on apple fruit, suggesting that \textit{pgxB} plays an important role in the infection process on the apple fruit.

Introduction

Pectinases are the most important pathogenic factor in plant pathogenic bacteria and fungi [1–4]. They are responsible for pathogens to decompose pectin in plant cell wall. Pectin hydrolysis not only weakens the cell wall to facilitate penetration and colonization of the host, it also provides the fungus carbon sources for its growth [5]. Pectinases are consisted of polygalacturonase (PG), pectin lyase (PNL), pectate lyase (PL), pectinesterase (PE), pectin methyl esterase (PME). Polygalacturonase is one of the major members of pectinases which cleaves \(\alpha\)-1,4-glycosidic of D-galacturonic acid in pectin and it is classified into endo- and exo-polygalacturonase on the basis of the way of eliminating galacturonic acid [6,7].
Polygalacturonase gene pgxB in Aspergillus niger

The production of PG by pathogenic fungi is critical for their success and survival during host infection [8]. It has been confirmed that the loss of a polygalacturonase gene in some fungi would result in decreased pathogenicity. Shieh et al [9] showed that a polygalacturonase gene is related to the infection of Aspergillus flavus in cotton bolls. The disruption of endopolygalacturonase gene Bcpg1 or Bcpg2 in Botrytis cinerea reduced its virulence on different hosts [10]. It is also reported that the loss of pectin methyl esterase gene Bcpme1 reduced virulence on apples, and pectin lyase pelB was an important virulence factor in Colletotrichum gloeosporioides when attacking avocado [11,12]. PG is also required for infection by Phytophthora capsici and Alternaria citri [13,14]. However there are also studies demonstrated that disruption of some polygalacturonase genes in fungi did not directly affect virulence, for example, deletion of PG1, PG5, PGX4 in Fusarium oxysporum led to no virulence difference in tomato [15,16] and endopolygalacturonase PGN1 is not required for pathogenicity of Cochliobolus carbonum on maize [17]. Mutants lacking both polygalacturonase genes cppg1/cppg2 in Claviceps purpurea did not affected vegetative properties, but they are nearly nonpathogenic on rye [18].

Aspergillus niger is a saprophytic fungus which degrades plant cell wall polysaccharides and leads to the decay of fruits and vegetables [19]. Since the 1990s, technical advances in molecular biology speed up the operation mechanism of A. niger and A. niger gene sequencing has been completed [20,21]. Deletion of kusA gene in A. niger dramatically improved homologous integration efficiency and facilitated gene knockout in A. niger [22]. However, whether polygalacturonase contributes to the pathogenicity of A. niger on fruit is still unclear. Here we constructed pgxB deletion mutant in A. niger MA 70.15 via homologous recombination and the pathogenicity were evaluated in ΔpgxB strain.

Material and methods

Fungal strains and growth conditions

A. niger MA 70.15 (ΔpyrG, pyrG encodes orotidine-5-phosphate decarboxylase, cell lacking this enzyme cannot grow without exogenous uridine, but can resist toxicity of 5-Fluoroorotic acid) was used as wild type strain in this work. A. niger was grown on potato dextrose agar medium (PDA) (per liter: 200 g of potato; 20 g of agar; 20 g of glucose and 10 mM uridine) at 28°C. Therefore all medium used in this study were supplemented with 10 mM uridine except where stated. For in vitro growth evaluation, A. niger spores were suspended in sterile water and adjusted to 10⁶ spores per mL. To test whether the absence of pgxB affected mycelial growth, PDA andpectolytic enzyme-inducing medium (PEIM) (per liter: 20 g of agar; 20 g of pectin; MM medium (5 g of KNO₃; 0.3 g of KCl; 2 g of MgSO₄·7H₂O; 5 g of KH₂PO₄; 0.008 mg of Na₂B₄O₇·10H₂O; 0.16 mg CuSO₄·5H₂O; 0.256 mg of FeCl₃·6H₂O; 0.1213 mg of MnSO₄·4H₂O; 0.16 mg of NaMoO₄·2H₂O; 2.85 mg of ZnSO₄·6H₂O; 0.08 mg of MgCl₂·6H₂O) plus 10 mM uridine) [23] were spot-inoculated with 5 μL spore suspension of wild type and ΔpgxB mutant. All strains were inoculated onto three plates of each medium and colony diameter was measured daily.

For the determination of growth curve, 1 mL spore suspension of wild type and ΔpgxB mutant were inoculated in 100 mL Erlenmeyer flasks containing 30 mL potato dextrose medium or PEIM at 30°C, 150 rpm. Mycelium was harvested on a quantitative filter paper at the time of 12, 24, 36, 48, 60, 72, 84, 96 h and weighed.

Vector construction and transformation

The exo-polygalacturonase gene pgxB in A. niger was deleted following gene knockout method of Delmas et al [24]. Upstream and downstream DNA fragments AB (548 bp) and CD (564 bp) flanking pgxB gene were amplified by polymerase chain reaction (PCR) from A. niger
MA 70.15 genomic DNA. Primers were designed using the genome database *A. niger* CBS 513.88 and the upstream and downstream fragments contained a common *Hind*III restriction site to ligate them together and *Eco*RI and *Xho*I restriction sites were used for cloning the joined fragment into the plasmid pC3 [24] to create pC3-*An*Δ*pgxB* integrative plasmid. Primers are shown in Table 1.

*A. niger* protoplast preparation and transformation were carried out by the method of Baltz et al [23]. 4-day-old mycelia grown from PDA were harvested and were digested with 0.4 g Lyticase (Sigma) to obtain fungal protoplasts. Protoplasts were transformed with 10 μg pC3-*An*Δ*pgxB* (un-linearized) in 50 μL polyethylene glycol 6000. Transformations were inoculated on upper layer of transformation medium without uridine (per liter: upper medium: MM; 6 g of agar; 0.95 M sucrose; lower medium: MM; 12 g of agar; 0.95 M sucrose) to select for the integration of the plasmid carrying *pyrG* on the chromosome. Transformants were purified by breeding them twice successively on the same transformation medium but lacking sorbitol (per liter: MM, 20 g of agar). Transformants were then propagated twice on PDA medium containing 10 mM uridine to release the selective pressure on the integrated plasmid. For selecting clones that had excised the plasmid (Δ*pyrG*), spores were then spread on MM-Uri-5-FOA medium (per liter: MM; 20 g of agar; 10 g of glucose; 1.6 mM uridine; 750 μg/mL 5-fluoro-orotic acid). Clones from last medium were cultured in MM-Uri-5-FOA liquid medium at 250 rpm at 30˚C for 3 d, and mycelia were harvested for genomic DNA extraction.

**DNA extraction and PCR confirmation of Δ*pgxB* strain**

Genomic DNA was extracted using Master Pure Yeast DNA Purification Kit (Epicentre). Primers *pgxB*-A, *pgxB*-D and another primers internal to the *pgxB* gene *pgxB*-E1, *pgxB*-E2 (PCR product size: 953 bp) were designed using Primer Premier 5.0 software. Primers are shown in Table 1.

**Determination of PG activity**

For determination of polygalacturonase activity, 100 μL of conidia (1×10^6 spores per mL) were inoculated into a 100 mL Erlenmeyer flask containing 30 mL of liquid PEIM and cultured at 30˚C for 5 days. Culture medium after suction filtration was used for PG activity assay following the method described by Miller [25]. Reaction mixture was consisted of 1 mL of 50 mM acetic acid-sodium acetate at pH5.5, 0.5 mL of 10 g/L pectin solution and 0.5 mL of crude enzyme or enzyme boiled for 5 min followed by incubation at 40˚C for 30 min. Reactions were terminated by adding 1.5 mL of DNS (3,5-dinitrosalicylic acid) followed by a 5 min incubation in a boiling water bath. The reaction mixture was cooled to room temperature, and distilled water was added to a final volume of 25 mL. Absorbance at 540 nm was measured. One unit of PG was defined as 1 μg of galacturonic acid produced by pectin per hour and expressed as U/mL enzyme extract.

PG activity was also determined by plate assay. The wild type and Δ*pgxB* mutant were inoculated on PEIM and cultured for 3 days at 30˚C. Thereafter the colonies were rinsed off the plates with distilled water before staining the plates with 0.05% ruthenium red. Pectinase production was evaluated by ratio of diameter of clear zone formed around colonies relative to diameter of mycelia [26,27].

**Virulence assay of *A. niger* on apple fruit**

Apple fruits were washed with tap water and then surface-sterilized with 75% ethanol. Five different sites on the surface of apples were wounded (2 mm diameter and 5 mm deep) and injected with 5 μL wild type or Δ*pgxB* mutant spore suspension (10^6 spores per mL).
| Primer | sequence |
|--------|----------|
| pgbx-B (forward) | 5'-TTGC GGCCGCTT TTGCGTCTT GATTGTGA G-3' |
| pgbx-B (reverse) | 5'-CGAC AGACCCAA GCTTTGATG TGGGTAGA TGCGTAG- 3' |
| pgbx-C (forward) | 5'-TTAA GCTTGGGT CTGTCGTTG ATGATTT- 3' |
| pgbx-D (reverse) | 5'-TTAC TAGTTGTT CGAGAAGGG TGGTTTT- 3' |
| pgbx-E1 (forward) | 5'-GCTTTGCGGCTCTCAATCT-3' |
| pgbx-E2 (reverse) | 5'-TTCCGCCAGAAGCAGCTC-3' |

For quantitative PCR:
- First line: forward
- Second line: reverse

| Primer | sequence |
|--------|----------|
| pgbx | 5'-TTCCGCCAGAAGCAGCTC-3' |
| pgaI | 5'-CATGAACCTTGCGTGTTGCT-3' |
| pgaII | 5'-TGATCCGCTTTGGATGCTGA-3' |
| pgaA | 5'-CGTATGACCTTGCGTGTTGCTA-3' |
| pgaB | 5'-GGACCCTTACCAATTCCTTGTA-3' |
| pgaC | 5'-TTCCACCCCTCCTCTCTTG-3' |
| pgaD | 5'-TGCTCAAGAAGGCTATGAGCC-3' |
| pgaE | 5'-ACTTGCCCTTCGCTCTC-3' |
| pgaX | 5'-TGGAGCCGCTTCTCTTGTA-3' |
| pgaA | 5'-GCTCTTCACTCTATTCCTTGTA-3' |
| pgaX | 5'-TGCTACCTCTTGGACCC-3' |
| pgaA | 5'-GGACGAGCTTTCTCTGCT-3' |
| pgaB | 5'-GGACGAGCTTTCTCTGCT-3' |
| pelA | 5'-AGCCGAGCCTTCTTGGACCC-3' |
| pelB | 5'-AGCCGAGCCTTCTTGGACCC-3' |
| pelC | 5'-ACATTTTCTCTTGGACCC-3' |
| pelD | 5'-ACATTTTCTCTTGGACCC-3' |
| pelF | 5'-ACATTTTCTCTTGGACCC-3' |
| plyA | 5'-GCTGCTTCGTTGCTCGCTTGC-3' |
| pmeA | 5'-GCTGCTTCGTTGCTCGCTTGC-3' |
| An04g09690 | 5'-TGGTTGCCTCTTGGACCC-3' |
| An02g12505 | 5'-CTCCCGCTCGCTCGCTC-3' |
respectively. After air-drying, three replicates were put in sealed container with H2O at the bottom at 25°C. The necrosis diameter were measured daily after inoculation. Spores grown from wounds were used for RNA extraction and quantitative PCR.

### RNA extraction and quantitative PCR

RNA was extracted from frozen mycelium grown on PDA, PEIM or apples ground in liquid nitrogen with TRNzol RNA Reagent kit (Tiangen). After DNase treatment, cDNA were obtained according to the manufacturer’s instruction of PrimeScript RT Master Mix (TaKaRa, Japan). Primers used for quantitative PCR are shown in Table 1. Relative quantification was processed using the method of Delta-Ct.

### Statistical analysis

Statistical significance was tested by one-way analysis of variance (ANOVA), and the results are expressed as the mean values ± standard deviation (SD) of three independent experiments. Fisher’s least significant differences (LSD) were calculated following a significant \( (P < 0.01 \) or \( P < 0.05 \) \) t test.

### Results

#### Gene disruption of pgxB in A. niger

548-bp upstream and 564-bp downstream DNA fragments AB and CD were amplified by PCR from A. niger MA 70.15 genomic DNA. Fragment ABCD (1094 bp) by the ligation of AB and CD were cloned into plasmid pC3 to generate the recombinant plasmid pC3-AnΔpgxB (6959 bp).

Then the plasmid pC3-AnΔpgxB was transformed and integrated into A. niger MA 70.15 protoplast and the ΔpgxB mutant was confirmed by genomic PCR. As shown in Fig 1, genomic DNA was used as template for PCR confirmation with primers pgxB-A and pgxB-D which flank pgxB gene and primers on the ORF of pgxB gene pgxB-E1 and pgxB-E2. 1100 bp band for ΔpgxB and 3500 bp for the wild type were obtained by the primers pgxB-A and pgxB-D, and a 953-bp band for the wild type and no amplification for ΔpgxB with the primers pgxB-E1 and pgxB-E2. All of these results confirmed that the pgxB gene was disrupted in ΔpgxB.

#### Growth analysis of ΔpgxB mutant strain

Pectinases in some fungi can be induced by pectin [28]. To evaluate whether the lack of pgxB would affect its growth on pectin medium, we compared growth of ΔpgxB and A. niger MA 70.15 on PDA (no pectin) and pectin medium (PEIM). The radial growth was measured on solid media. It was found that growth rate, estimated as colony diameter, showed no difference between the ΔpgxB mutant and wild type on PDA (Fig 2A and 2B) or pectin medium (Fig 2C.

| Primer | sequence |
|--------|----------|
| Actin  | 5’-ACGTTGGACTGGCTCTC-3’ |
| Actin  | 5’-CAATGTTTCGGGATAGTGC-3’ |

Underlined letters refer to restriction enzymes site.

doi:10.1371/journal.pone.0173277.t001

Table 1. (Continued)
and 2D). Besides, no significant difference in growth curve was found between ΔpgxB mutant and the wild type in liquid medium with or without pectin (Fig 2E and 2F).

**Inducing effect of pectin on the expression of pectinase genes in A. niger**

To study whether the expression of pectinase genes were induced by pectin, relative expression of different pectinase genes in ΔpgxB and A. niger MA 70.15 on PDA and pectin medium (PEIM) were determined by quantitative PCR. As shown in Fig 3A, expression of pgxB gene in wild type was significantly enhanced by pectin medium, while the expression was not detected in ΔpgxB, further confirming that pgxB gene was deleted in the mutant. For wild type, most of pectinase genes like PG genes pgaII, pgA, pgB, pgD, pgE, pgX, pgx, pgXC, PL genes pelA, pelC, pelD, pelF, pLyA, PME gene An04g09690, PE gene An02g12505 showed enhanced expression on PEIM than those on PDA, suggesting that the expression of pectinases genes were induced by pectin (Fig 3B and 3C). Similarly, enhanced gene expression of PG genes pgaII, pgA, pgB, pgD, pgE, pgX, pgx, pgXC, PL genes pelA, pelC, pelD, pelF, pLyA, PME gene An04g09690, PE gene An02g12505 was also observed in ΔpgxB mutant (Fig 3D and 3E).

**Polygalacturonase activity analysis**

*pgxB* gene is a member of polygalacturonase family found in *A. niger*. To study whether *pgxB* gene actually contributes to the whole activity of polygalacturonase in *A. niger*, polygalacturonase activity was determined in ΔpgxB mutant. The ΔpgxB mutant and *A. niger* MA 70.15 were grown in liquid PEIM for 3 days and the activity of secreted polygalacturonase in the medium was analyzed. As shown in Fig 4A, ΔpgxB exhibited lower PG activity (by 5.8%) compared with the wild type. Secreted PG activity by *A. niger* was also assayed on plate. As shown in Fig 4B and 4C, the ratio of diameter of clear zone (Dc) which showing the degradation of pectin by secreted PG and mycelia (Dm) produced by ΔpgxB was smaller (by 6.5%) than that of wild type, indicating that ΔpgxB mutant produced less pectinase than the wild type *A. niger* MA 70.15.

**Pathogenicity assay on apple fruit**

Whether pectinase was a pathogenic factor in *A. niger* infection on fruit is still unknown, thus we studied the virulence of ΔpgxB mutant on fruit. Apple fruits were inoculated with conidial
suspension of ΔpgxB and the wild type A. niger MA 70.15. Lesion development was monitored daily and the diameter was measured. As shown in Fig 5A and 5B, ΔpgxB disruption resulted in a reduction of decay development as the lesion diameter caused by ΔpgxB was about 20% smaller than that of wild type on 4, 5, 6, 7 days and 15% smaller on 10, 11 days, suggesting that the virulence of ΔpgxB on apple fruit was significantly lower than that of wild type.

To study why the virulence decreased in ΔpgxB, expression of various pectinase genes in ΔpgxB and wild type in the process of infecting apples was determined by quantitative PCR. Expression of PG genes pgaI, pgaII, pgaA, pgaC, pgaD, pgaE, pgaX in ΔpgxB were higher in ΔpgxB than those in wild type, while there was no significant difference in expression of PG genes pgaB, pgaX, pgaC, PL genes pelA, pelB, pelC, pelD, pelF, pfaA and PME genes pmeA,
between ΔpgxB mutant and wild type (Fig 5C and 5D). The increased expression of PG genes in ΔpgxB suggested a possible compensation effect in pgxB deletion mutant.

**Discussion**

Four exo-polygalacturonase genes were found in *A. niger*, including *pgxA*, *pgxB*, *pgxC* and *pgaX* [29]. Besides, endo-polygalacturonase gene *pgal*, *pgalI*, *pgalA*, *pgalB*, *pgalC*, *pgalD*, *pgalE*, pectin lyase gene *pelA*, *pelB*, *pelC*, *pelF*, pectin methylesterase gene *pmeA*, *An04g09690*, *plyA* and *An02g12505* in *A. niger* MA 70.15 on PDA and PEIM medium respectively for 4 days; C and D, relative expression of *pgal*, *pgalI*, *pgalA*, *pgalB*, *pgalC*, *pgalD*, *pgalE*, *pgaX*, *pgaA*, *pgaX*, *pgxA*, *pgxC*, *pelA*, *pelB*, *pelC*, *pelD*, *pelF*, *pmeA*, *An04g09690*, *plyA* and *An02g12505* in ΔpgxB mutant on PDA and PEIM medium respectively for 4 days. * and ** in this figure and following ones stand for a significant difference between two data at $P < 0.05$ and $P < 0.01$, respectively.

doi:10.1371/journal.pone.0173277.g003

*An04g09690* between ΔpgxB mutant and wild type (Fig 5C and 5D). The increased expression of PG genes in ΔpgxB suggested a possible compensation effect in pgxB deletion mutant.

In this paper, we described the construction of a mutant disrupted in the *pgxB* gene in *A. niger*. ΔpgxB exhibited no growth rate reduction on PDA and pectin medium compared with wild type, which means the disruption of *pgxB* did not weaken its ability of decomposing pectin as carbon source (Fig 2). In contrast, *Bcpme1* mutant in *B. cinerea* and *pelB* mutant in *Colletotrichum gloeosporioides* showed reduced growth on pectin medium [11,12]. Pectinase was induced by pectin, polygalacturonic acid or galacturonic acid and was repressed by glucose and polygalacturonase-inhibiting protein (PGIP) [37,38]. Quantitative PCR results showed that expression of most of pectinase genes such as PG genes *pgalI*, *pgalA*, *pgalB*, *pgalD*, *pgalE*, *pgaX*, *pgxA*, *pgxC*, PL genes *pelA*, *pelC*, *pelD*, *pelF*, *plyA* were up-regulated on pectin medium.
compared that on PDA (Fig 3), confirming that the expression of pectinase genes were
induced by pectin. We also found that \( \Delta \text{pgxB} \) mutant grown in liquid and solid PEIM showed
significant lower PG activity than the wild-type strain, suggesting that the loss of \( \text{pgxB} \) reduced
the production of PG (Fig 4).

Virulence assay on apple fruit showed that the deletion of \( \text{pgxB} \) gene has a profound effect
on lesion development in the infection of apple as lesion diameter caused by \( \Delta \text{pgxB} \) was smaller
than that of wild type. A similar reduction in maceration ability has been observed with pecti-
nase-deficient mutants of phytopathogenic bacteria such as \( \text{Erwinia} \), \( \text{Pseudomonas} \) and \( \text{Ralsto-
nia} \) species [39,40]. Besides, Öser et al. [18] found that mutant in both \( cpg1 \) and \( cpg2 \) are

Fig 4. Activity evaluation of secreted polygalacturonase (PG) from \( A. \ niger \) MA 70.15 and \( \Delta \text{pgxB} \)
mutant. A, the wild type \( A. \ niger \) MA 70.15 (WT) and \( \Delta \text{pgxB} \) strains were grown in liquid pectolytic
enzyme-inducing media (PEIM) for 5 days and 0.5 mL supernatant of PEIM cultures was sampled for
polygalacturonase activity assay. B, The wild type and \( \Delta \text{pgxB} \) mutant were inoculated on pectolytic enzyme-
inducing media (PEIM) and cultured for 3 days at 30˚C and the plates were stained with 0.05% ruthenium red
and photographed. C, ratio of diameter of activity zone (clear zone, \( D_c \)) relative to the diameter of mycelia
(\( D_m \)).

doi:10.1371/journal.pone.0173277.g004
nearly non-pathogenic on rye using a gene-replacement approach. However, pectinases are usually encoded by multiple genes; thus, mutation in one pectinase gene might not affect the virulence on fruits [15–17]. The disruption of either the pelA or pelD gene in Nectria hemato-cocca alone causes no detectable decrease in virulence, whereas disruption of both pelA and pelD drastically reduces virulence [41]. In order to understand the decreasing virulence of ΔpgxB mutant, expression of some pectinase genes were assayed, and we found that PG genes pgaI, pgaII, pgaA, pgaC, pgaD, pgaE and pgaX were expressed more highly in ΔpgxB mutant than in the wild type. Deletion of one gene in a gene family might result in higher expression of other genes with the same function as polygalacturonases of *A. niger* are encoded by a family of diverged genes [42]. A similar phenomenon that disruption of serine proteinase caused an increase in metalloproteinase has also been found in *Aspergillus flavus* [43]. Even so, the lack of pgxB still dramatically reduced lesion diameter on apples. Our results demonstrate that pgxB is a virulence factor which partially contributes to the virulence of *A. niger* on apple fruit, thus highlighting the need for further research to elucidate the roles of other pectinase genes in *A. niger*.

**Acknowledgments**

We are grateful to Arthur F. J. Ram at University of Leiden for providing the strain *Aspergillus niger* MA 70.15 and David B. Archer at University of Nottingham for providing the plasmid pC3.
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References

1. Herron SR, Benen JA, Scavetta RD, Visser J, Jurnak F. Structure and function of pectic enzymes: virulence factors of plant pathogens. Proceedings of the National Academy of Sciences. 2000; 97(16): 8762–8769.
2. Have AT, Tenberge KB, Benen JAE, Tudzynski P, Visser J, van Kan JAL. The contribution of cell wall degrading enzymes to pathogenesis of fungal plant pathogens. Springer Berlin Heidelberg. 2002; 341–358 p.
3. Schmalhorst PS, Krappmann S, Vervecken W, Rohde M, Müller M, Braus GH, et al. Contribution of galactofuranose to the virulence of the opportunistic pathogen Aspergillus fumigatus. Eukaryotic Cell. 2008; 7(8): 1268–1277. doi: 10.1128/EC.00109-08 PMID: 18552284
4. Reignault P, Valette-Collet O, Boccara M. The importance of fungal pectinolytic enzymes in plant invasion, host adaptability and symptom type. European Journal of Plant Pathology. 2007; 120(1): 1–11.
5. Kars I, Krooshof GH, Wagemakers L, Joosten R, Benen JAE, van Kan JAL. Necrotizing activity of five Botrytis cinerea endopolygalacturonases produced in Pichia pastoris. Plant Journal. 2005; 43(2): 213–225. doi: 10.1111/j.1365-313X.2005.02436.x PMID: 15998308
6. Kars I, van Kan JAL. Extracellular enzymes and metabolites involved in pathogenesis of Botrytis. Botrytis: Biology, Pathology and Control. 2007; 99–118 p.
7. Jayani RS, Saxena S, Gupta R. Microbial pectinolytic enzymes: a review. Process Biochemistry. 2005; 40 (9): 2931–2944.
8. Lorenzo GD, Ferrari S. Polygalacturonase-inhibiting proteins in defense against phytopathogenic fungi. Current Opinion in Plant Biology. 2002; 5(4): 295–299. PMID: 12179962
9. Shieh MT, Brown RL, Whitehead MP, Cary JW, Cotty PJ, Cleveland TE, et al. Molecular genetic evidence for the involvement of a specific polygalacturonase, P2c, in the invasion and spread of Aspergillus flavus in cotton bolls. Applied & Environmental Microbiology. 1997; 63(9): 3548–3552.
10. Have AT, Mulder W, Visser J, van Kan JAL. The endopolygalacturonase gene Bcg1 is required for full virulence of Botrytis cinerea. Molecular Plant-Microbe Interactions. 1998; 11(10): 1009–1016. doi: 10.1094/MPMI.1998.11.10.1009 PMID: 9768518
11. Valette-Collet O, Cimerman A, Reignault P, Levis C, Boccara M. Disruption of Botrytis cinerea pectin methylesterase gene Bcpme1 reduces virulence on several host plants. Molecular Plant-Microbe Interactions. 2003; 16(4): 360–367. doi: 10.1094/MPMI.2003.16.4.360 PMID: 12744465
12. Yakoby N, Beno-Mouallem D, Keen NT, Dinoor A, Pines O, Prusky D. *Colletotrichum gloeosporioides* peiB is an important virulence factor in avocado fruit-fungus interaction. Molecular Plant-Microbe Interactions. 2001; 14(8): 988–995. doi: 10.1094/MPMI.2001.14.8.988 PMID: 11497471

13. Wen XS, Yong JJ, Bao ZF, O’Neill NR, Xiao PZ, Xie BY, et al. Functional analysis of Pcg2 from the straminipilous plant pathogen *Phytophthora capsici*. Genesis. 2009; 47(8): 535–544. doi: 10.1002/pcg.20530 PMID: 19422018

14. Isshiki A, Akimitsu K, Yamamoto M, Yamamoto H. Endopolygalacturonase is essential for citrus black rot caused by *Alternaria citri* but not brown spot caused by *Alternaria alternata*. Molecular Plant-Microbe Interactions. 2001; 14(6): 749–757. doi: 10.1094/MPMI.2001.14.6.749 PMID: 11386370

15. Pietro AD, Madrid MP, Caracuel Z, Delgado-Jarana J, Roncero MIG. *Aspergillus niger*: exploring the molecular arsenal of a vascular wilt fungus. Molecular Plant Pathology. 2003; 4(5): 315–325. doi: 10.1046/j.1364-3703.2003.00180.x PMID: 20569392

16. Meyer V, Arentshorst M, Elghezal A, Drews AC, Kooistra R, CavDH, et al. Highly efficient gene targeting in the molecular arsenal of a vascular wilt fungus. Molecular Plant Pathology. 2003; 4(5): 315–325. doi: 10.1046/j.1364-3703.2003.00180.x PMID: 20569392

17. Scott Craig JS, Panaccione DG, Cervone F, Walton JD. Endopolygalacturonase is not required for pathogenicity of *Cochliobolus carbonum* on maize. Plant Cell. 1990; 2(12): 1191–1200. doi: 10.1105/tpc.2.12.1191 PMID: 2152162

18. Oeser B, Heidrich PM, Müller U, Tudzynski P, Tenberge KB. Polygalacturonase is a pathogenicity factor in *Claviceps purpurea* Irye race. Fungal Genetics & Biology. 2002; 36(3): 176–186.

19. de Vries RP, Visser J. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. Microbiology & Molecular Biology Reviews. 2001; 65(4): 497–522.

20. Schuster E, Dunncoleman N, Frisvad J, Dijkstra PV. On the safety of *Aspergillus niger* – a review. Applied Microbiology & Biotechnology. 2002; 59(4–5): 426–435.

21. Pel HJ, de Winde JH, Archer DB, Dyer PS, Hofmann G, Schwaab P, et al. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nature Biotechnology. 2007; 25(2): 221–231. doi: 10.1038/nbt1282 PMID: 17259976

22. Meyer V, Arentshorst M, Elghezal A, Drees AC, Kohostra R, Ca VDH, et al. Highly efficient gene targeting in the molecular arsenal of a vascular wilt fungus. *Aspergillus niger* mutants. Journal of Biotechnology. 2007; 128(4): 770–775. doi: 10.1016/j.jbiotec.2006.12.021 PMID: 17275117

23. Baltz RH, Davies JE, Remi AL, Baltz RH, Davies JE, Remi AL. Manual of industrial microbiology and biotechnology. ASM Press. 2010.

24. Delmas S, Llanos A, Parrou JL, Kokoski M, Pullan ST, Shunburne L, et al. Development of an unmarked gene deletion system for the filamentous fungi *Aspergillus niger* and *Talaromyces versatilis*. Applied & Environmental Microbiology. 2014; 80(11): 3484–3487.

25. Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry. 1959; 31(3): 426–428.

26. Cotty PJ, Cleveland TE, Brown RL, Mellon JE. Variation in polygalacturonase production among *Aspergillus flavus* isolates. Applied & Environmental Microbiology. 1990; 56(12): 3885–3887.

27. Strauss MLA, Jolly NP, Lambrecht GS, Rensburg PV. Screening for the production of extracellular hydrolytic enzymes by non- *Saccharomyces* wine yeasts. Journal of Applied Microbiology. 2001; 91(1): 182–190. PMID: 1144279

28. Maldonado MC, Strasser dSAM, Callieri DA. Regulatory aspects of the synthesis of polygalacturonase and pectinesterase by *Aspergillus niger* sp. [pectinase, pectinolytic enzymes, inducible synthesis of enzyme]. Sciences Des Aliments. 1989.

29. Martens-Uzunova E, Zandleven J, Benen J, Awad H, Kools H, Baldman GA, et al. A new group of exoacting family 28 glycoside hydrolases of *Aspergillus niger* that are involved in pectin degradation. Biochemical Journal. 2006; 400(1): 43–52. doi: 10.1042/BJ20060703 PMID: 16822322

30. Benen JAE, Kester HCM, Visser J. Kinetic characterization of *Aspergillus niger* N400 endopolygalacturonases I, II and C. European Journal of Biochemistry. 1999; 259(3): 577–585. PMID: 10092840

31. Paříčková L, Benen JAE, Kester HCM, Visser J. pgA and pgB encode two constitutively expressed endopolygalacturonases of *Aspergillus niger*. Biochemical Journal. 2000; 345(3): 637–644.

32. Paříčková L, Kester HCM, Benen JAE, Visser J. Characterization of a novel endopolygalacturonase from *Aspergillus niger* with unique kinetic properties. Fems Letters. 2000; 467(2–3): 333–336. PMID: 10675564

33. Paříčková L, Benen JAE, Kester HCM, Visser J. pgE encodes a fourth member of the endopolygalacturonase gene family from *Aspergillus niger*. European Journal of Biochemistry. 1998; 251(1–2): 72–80. PMID: 9492270
34. Gysler C, Harmsen JAM, Kester HCM, Visser J, Heim J. Isolation and structure of the pectin lyase D-encoding gene from Aspergillus niger. Gene. 1990; 89(1): 101–108. PMID: 2373363

35. Someren KV, Flippini M, Graaff LD, Broeck HVD, Kester H, Hinnen A, et al. Characterization of the Aspergillus niger pelB gene: structure and regulation of expression. Molecular and General Genetics. 1992; 234(1): 113–120. PMID: 1495474

36. Harmsen JAM, van Someren MAK, Visser J. Cloning and expression of a second Aspergillus niger pectin lyase gene (pelA): indications of a pectin lyase gene family in A. niger. Current Genetics. 1990; 18(2): 161–166. PMID: 2225145

37. Solís-Pereira S, Favela-Torres E, Viniegra-González G, Gutiérrez-Rojas M. Effect of different carbon sources on the synthesis of pectinase by Aspergillus niger in submerged and solid state fermentations. Applied Microbiology & Biotechnology. 1990; 39(1): 36–41.

38. Liu N, Ma X, Zhou S, Wang P, Sun Y, Li X, et al. Molecular and functional characterization of a polygalacturonase-inhibiting protein from Cynanchum komarovi that confers fungal resistance in Arabidopsis. Plos One. 2015; 11(1): e0146959.

39. Barras F, And VG F, Chatterjee AK. Extracellular enzymes and pathogenesis of soft-rot Erwinia. Annual Review of Phytopathology. 2003; 32: 201–234.

40. Collmer A, Keen NT. The role of pectic enzymes in plant pathogenesis. Annual Review of Phytopathology. 2003; 24: 383–409.

41. Rogers LM, Kim YK, Guo W, González-Candelas L, Li D, Kolattukudy PE. Requirement for either a host- or pectin-induced pectate lyase for infection of Pisum sativum by Nectria hematococca. Proceedings of the National Academy of Sciences. 2000; 97(17): 9813–9818.

42. Bussink HJD, Buxton FP, Fraaye BA, Graaff LHD, Visser J. The polygalacturonases of Aspergillus niger are encoded by a family of diverged genes. European Journal of Biochemistry. 1992; 208(1): 83–90. PMID: 1511691

43. Ramesh MV, Kolattukudy PE. Disruption of the serine proteinase gene (sep) in Aspergillus flavus leads to a compensatory increase in the expression of a metalloproteinase gene (mep20). Journal of Bacteriology. 1996; 178(13): 3899–3907. PMID: 8682796