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Antimicrobial activity of recombinant thanatin against some of the major bacterial plant pathogens under *in vitro* and greenhouse conditions

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Abstract

The control of fungal and bacterial plant diseases mainly relies on the application of mineral and chemical fungicides/bactericides, which are considered to be environmental pollutants and toxic to humans. Moreover, the prolonged use of bactericides and antibiotics has led to the development of resistance among pathogens, as well as an increase in environmental and health threats. Therefore, the development of non-toxic and non-polluting treatments to control plant diseases has been the focus of extensive research in agriculture. Synthetic antimicrobial peptides have recently received extensive attention as the potential alternatives to other conventional methods in terms of their strong broad-spectrum antimicrobial activity. *In vitro* assays using various chemically synthesized peptides showed that the broad-spectrum peptide thanatin derived from the spined soldier bug (*Podisus maculiventris* (Say)) had the greatest potential for eliminating aflatoxigenic fungi. However, the antibacterial effect of thanatin against bacterial plant pathogens was less studied so far. The thanatin encoding sequence was codon optimized for expression in *Pichia pastoris*. This coding sequence cloned into *P. pastoris* expression vector pPICZαA and used for synthetizing the recombinant thanatin. Then, antibacterial activities of the constructed peptide were studied under *in vitro* and *in vivo* condition. The result showed that, all construction, cloning and expression processes were successfully performed in yeast. The results of the MIC and MBC tests showed that the growth rate of the majority of bacterial plant pathogens including gram-negative and gram-positive bacteria was inhibited by recombinant thanatin under *in vitro* conditions.

MIC values for different bacterial isolates ranged from 0.016 to 2.048, while the MBC values for the same bacterial isolates were determined to be between 0.064 and 4.096 µg/mL. These amounts were significantly lower than the MIC and MBC values obtained by applying copper oxychloride. The application of 4.09 µg/mL of recombinant thanatin against rice leaf blight splendidly controlled this bacterial disease under greenhouse conditions. However, this promising antimicrobial peptide requires further investigation for the development of novel molecules for the control of plant pathogens.

Key words: Antimicrobial peptides, Pichia pastoris, rice leaf blight, Xanthomonas oryzae pv. oryzae.

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فعالیت ضد میکروبی تاناتین نوترکیب علیه برخی از بیمارگرهای باکتریایی گیاهان در شرایط آزمایشگاهی و گلخانه‌ای

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چکیده

کنترل بیماری‌های قارچی و باکتریایی گیاهان عمدتاً مبتنی بر کاربرد قارچ‌کش‌ها و باکتری‌کش‌های معدنی و شیمیایی است که از آلاینده‌های محیطی و سمّی برای انسان محسوب می‌شوند. علاوه بر این، استفاده طولانی مدّت از ترکیبات فوق و آنتی‌بیوتیک‌ها منجر به ایجاد مقاومت در بین بیمارگرها گردیده و همچنین موجب افزایش مخاطرات زیست‌محیطی و بهداشتی شده‌است. از این رو، توسعه تیمارهای غیرسمّی و غیر-آلاینده برای کنترل بیماری‌های گیاهی، هدف پژوهش‌های گسترده‌ای در علوم کشاورزی بوده است. پپتیدهای ضدمیکروبی نوترکیب اخیراً به دلیل فعالیت ضد میکروبی با طیف وسیع و قوی خود به عنوان جایگزین‌های بالقوه برای سایر روش‌های کنترلی مرسوم مورد توجه گسترده‌ای قرار گرفته‌اند. سنجش‌های آزمایشگاهی روی پپتیدهای مختلف ساخته شده شیمیایی نشان می‌دهد که پپتید تاناتین منشأ گرفته از سن شکارگر ***Podisus maculiveventris*** با طیف عمل گسترده بیشترین پتانسیل را در از بین بردن قارچ‌های مولد آفلاتوکسین دارا می‌باشد. با این حال، اثرات ضد باکتریایی تاناتین علیه بیمارگرهای باکتریایی گیاهی تاکنون کمتر مورد بررسی قرار گرفته است. در این مطالعه، توالی کد کننده تاناتین برای بیان در ناقل ***Pichia pastoris*** بهینه سازی گردید. سپس این توالی کدکننده در وکتور بیانی **pPICZαA** همسانه‌سازی شد و برای ساخت تاناتین نوترکیب مورد استفاده قرار گرفت. فعالیت ضد باکتریایی پپتید ساخته شده در شرایط ***in vitro*** و ***in vivo*** مورد بررسی قرار گرفت. نتایج نشان داد که تمامی مراحل ساخت، شبیه سازی و بیان در مخمر با موفقیت انجام شده‌است. نتایج آزمون‌های تعیین حداقل غلظت بازدارندگی (**MIC**) و حداقل غلظت باکتری‌کشی(**MBC**) نشان داد که رشد اکثر بیمارگرهای باکتریایی گیاهی اعم از گرم منفی و مثبت توسط تاناتین نوترکیب در شرایط آزمایشگاهی مهار شد. میزان **MIC** ارزیابی شده برای جدایه‌های مختلف باکتریایی بین ۰/۰۱٦ تا ۲/۰٤۸ میکروگرم در میلی لیتر بود در حالی که مقادیر **MBC** برای جدایه‌های باکتریایی مشابه بین ۰/۰٦٤ تا ٤/۰۹٦ میکروگرم در میلی لیتر تعیین شد. این مقادیر بسیار کمتر از مقادیر **MIC** و **MBC** بدست‌آمده با استفاده از اکسی‌کلرید مس بود. کاربرد ٤/۰۹ میکروگرم بر میلی لیتر تاناتین نوترکیب علیه بیماری باکتریایی سوختگی برگ برنج، این بیماری را در شرایط گلخانه ای به خوبی کنترل کرد. با این حال، این پپتید ضد میکروبی امیدوارکننده نیاز به بررسی بیشتر برای توسعه مولکول‌های جدید برای کنترل بیمارگرهای گیاهی دارد.

کلیدواژه‌ها: پپتیدهای ضد میکروبی، سوختگی برگ برنج، Pichia pastoris، Xanthomonas oryzae pv. oryzae

دبیر تخصصی: دکتر رسول رضائی

Introduction

Plant pathogens continuously attack plants and cause numerous problems in agriculture. The management of fungal and bacterial diseases in plants relies primarily on mineral compounds and synthetic chemicals. However, these substances are known to be environmental pollutants and may pose risks of toxicity or carcinogenicity to humans (Makovitzki et al., 2007). Moreover, the prolonged use of pesticides and antibiotics has led to the development of resistance among plant pathogens, and other pests, as well as an increase in environmental and health risks (Keymanesh et al., 2009). Therefore, the development of non-toxic and non-polluting treatments to control plant diseases has been the focus of extensive research in agriculture (Datta et al., 2015). Synthetic antimicrobial peptides have recently received extensive attention as potential alternatives to other conventional methods in terms of their strong broad-spectrum antimicrobial activity. These peptides have also been used in plant disease control as an alternative to conventional treatment methods, which are polluting and dangerous to the environment and human health (Keymanesh et al., 2009, Datta et al., 2015).

Small biological molecules with molecular weight of less than 10 kDa and antimicrobial activity, provide effective microbial protection for all kind of organisms including bacterial pathogens (Sang and Blecha, 2009, Guaní-Guerra et al., 2010). Natural gene encoded antimicrobial peptides are a group of innate immune molecules found in all organisms. These mature antimicrobial peptides typically contain 12–100 amino acid residues, possess a net positive charge and an amphipathic structure that facilitates association with adversely charged microbial layers or other cell targets (Linde et al., 2008, Sang and Blecha, 2009, Mahlapuu et al., 2016).

Among diverse animals, insects are a major source of antimicrobial peptides. Several antimicrobial peptides have been found in insects up to now (Yi et al, 2014, Jozefiak and Engberg, 2017). Most insect antimicrobial peptide are small and cationic, and their antimicrobial activities have been reported against different organisms including bacteria, fungi and other parasites as well as viruses (Jansen and Kogel, 2011, Andres, 2012, Zhang and Gallo, 2016).

Thanatin is a 2.4 kDa, 21–residue antimicrobial peptide isolated from the spined soldier bug (*Podisus maculiventris*) that possesses both antibacterial and antifungal properties (Schubert et al., 2015). *In vitro* assays with different chemically-synthesized peptides confirmed that, thanatin has the greatest potential to inhibit aflatoxigenic fungi (Schubert et al., 2015). The mode of action of thanatin has not been clearly understood. However, by observing the morphological changes in clinical isolates of extended-spectrum β-lactamase producing *Escherichia coli*, it has been demonstrated that thanatin causes membrane permeabilization and depolarization in both outer and inner membranes (Hou et al., 2011, Ma et al., 2016).

In addition to directly killing the bacteria, thanatin causes significant bacterial aggregation, although its underlying mechanism remains unclear. It is possible that the cationic peptide binding leads to a decrease in the surface charge density of the lipopolysaccharide, thereby reducing the electrostatic repulsion between bacteria (Hou et al., 2011). It was shown that the disulfide bond in thanatin is not necessary for its antimicrobial activity in both *in vivo* and *in vitro* conditions (Ma et al., 2016).

Like other peptides, thanatin can be produced through artificial protein synthesis, but this method is laborious and expensive. Recombinant production offers an efficient and fast alternative, allowing peptides to be produced on a large scale. In recent years, there was a significant focus on using yeast, such as *Pichia pastoris* and *Saccharomyces cerevisiae* as systems for recombinant protein production (Li, et al., 2007, Kim et al., 2015). This yeast-based system was successfully utilized for the production of various recombinant heterologous proteins, highly toxic products, and the expression of several antibacterial peptides (Mao et al., 2015, Tanhaeian et al., 2018). The control of plant diseases caused by bacterial pathogens is difficult due to limited efficacy of biological and chemical agents and also restricted use of conventional antibiotics (Sledz et al., 2015). However, copper was used in agriculture to control oomycetes, fungi and bacteria for over a century and it plays important roles in integrated diseases management, but using this heavy metal may have long-term consequences due to its accumulation in the soil, which appears incompatible with organic farming's purposes (La Torre et al., 2018).

The present study aimed to the cloning and recombinant expression of thanatin in *P. pastoris* and evaluation of its antimicrobial activity against some of the major plant bacterial pathogens under *in vitro* and greenhouse conditions.

Materials and methods

Gene cloning

The strain DH5α (Invitrogen, USA) was used as a bacterial host for vector construction and amplification according to the protocol suggested by manufacturer. Recombinant thanatin was expressed in *P. pastoris* strain KM71H (Invitrogen, USA). pPICZαA (Thermo Fisher Scientific, USA) was used as a vector for cloning and extracellular protein expression in yeast. PCR primers were synthesized by Macrogen Company (South Korea).

Culture media condition

Luria-Bertani (LB) medium (Merck, Germany) was used for *E. coli* (DH5α) propagation. LB medium containing Ampicillin (50 µg/mL) was used for selection of transformant DH5α. The *P. pastoris* KM71H strain was cultured in yeast extract peptone dextrose (YPD) medium (Jones et al., 2017). The growth and induction media were buffered minimal glycerol-complex medium (BMGY) contained 1% (w/v) yeast extract, 2% (w/v) peptone, 0.1 M phosphate buffer pH 6.0, 1.34% (w/v) yeast nitrogen base, 4×10^{-5} % (w/v) biotin and 1% (w/v) glycerol and buffered minimal methanol-complex medium (BMMY) same as BMGY excluding glycerol which was replaced by 0.5% (v/v) methanol, respectively.

Vector construction up to transformant confirmation

There are 21 amino acids in the sequences of thanatin. This sequence was retrieved from the antimicrobial peptide database (http://aps.unmc.edu/AP/main.php) with the following identification number AP00102. The thanatin encoding sequence was codon optimized for the proper expression and cloned into *P. pastoris* expression vector pPICZαA by Genscript® (USA). The pPICZαA vector was digested with *Eco*RI and *Xba*I and the obtained product was purified by electrophoresis on 1% agarose gel and ligated into the *Eco*RI-*Xba*I digested thanatin at 16 °C for 24 h using T4 DNA ligase. The ligation mixture was transformed

into DH5α by heat shock method. Positive clones were taken for LB plus 25 µg/mL Zeocin and screened by colony PCR using *AOX1* gene primers (forward AOX1: 5′-GACTGGTTCCAATTGACAAGC-3′, and reverse AOX1: 5′-GCAAATGGCATTCTGACATCC-3′). The PCR condition was: initial denaturation at 94 °C for 5 min, followed by 30 cycles (94 °C, 1 min; 58 °C, 1 min, and 72 °C, 2 min) and a final elongation at 72 °C for 10 min. The plasmid DNA from positive clones were purified and subjected for DNA sequencing.

A *P. pastoris* electro-competent cells strain KM71H was prepared according to the method suggested by Invitrogen (USA). The pPICZαA-thanatin vector was linearized with *Pme*I and purified afterward. 10 µg of linearized vector were mixed with 80 µL of electro-competent cells, transferred into an 0.2 cm electroporation cuvette and incubated on ice for 5 min. Cells were then pulsed by electricity and 1.0 mL of 1 M ice cold sorbitol was immediately added to the cuvette and the mixture incubated at 30 °C for 1 h. Then 1mL YPD media was added to the Micro-tubes and incubated at 30 °C for 1 h. 200 µL of each Micro-tube aliquots were spread on YPDS agar plates containing 100 µg/mL Zeocin and incubated at 30 °C until colonies appeared (about 2-4 days). The integration of thanatin in the genome of recombinant *P. pastoris* was confirmed by genomic PCR using thanatin specific primers (forward: 5′-GCTGAATTCC ATGGGCAGCAAGAA-3′, and reverse: 5′-TTCTAGATTACATCCGCTGG CACTT-3′). The PCR condition was: initial denaturation at 94 °C for 5 min, followed by 30 cycles (94 °C, 1 min; 60 °C, 1 min, and 72 °C, 2 min) and a final elongation at 72 °C for 10 min.

Expression of recombinant thanatin

The positive colony of transformant *P. pastoris* was cultivated in 5 mL of BMGY medium at 30 °C for 24 h. The obtained cells were then further cultured in 100 mL of BMGY, incubated at 30 °C and 250 rpm for 20 h until the optical density at 600 nm (OD600) come to 2–6. Cells were collected by centrifugation at 3000×*g* at room temperature for 5 min. The cell pellet was then re-suspended in 25 mL of BMMY medium and transferred into a 500 mL flask. The culture was shaken at 30 °C for 96 h. Absolute methanol was added every 24 h to a final concentration of 5 µL/mL. Cultures were centrifuged at 5000×*g* at 4 °C for 5 min and the final supernatant was harvested.

SDS-PAGE analysis

Fifteen µL from the cultural medium located on the top of each transfected cells were run on SDS-PAGE in Tris/Tricin/SDS buffer using a 16% polyacrylamide gel stained by Coomassie Brilliant Blue according to the protocol suggested by manufacturer (Schägger et al., 1988). The quantification of peptide band was carried out by NIH ImageJ Wiki software (https://imagej.net/nih-image/).

Plant pathogenic bacterial isolates

The same plant bacterial isolates (listed in the Table 1) mentioned in our pervious study (Tanhaeian et al., 2018) were also used for thanatin antibacterial activity assay. They have already been provided by the department of Plant Protection, Ferdowsi University of Mashhad.

MIC and MBC determination test

The Minimum Inhibitory Concentrations (MIC) for the bacterial isolates listed in Table 1 were determined using microbroth dilution method (Vipra et al. 2013). The test strains were grown overnight on Nutrient agar and discrete colonies were hand-picked from the plates and suspended in Nutrient broth to prepare the 0.5 McFarland scale (~1 × 10^8 cfu/mL), then distributed in 100 µL

volumes into a 96-well microtiter plate. The stock solution of thanatin and a well-known commercial bactericide named copper oxychloride (Arya Chemical Company Tehran, Iran) was serially diluted to prepare different concentrations. One hundred µL from each dilution were added to the wells holding bacterial cells. Associated bacterial and media controls were included. The microtiter plates were incubated overnight at 28 °C. The lowest concentration of thanatin or copper oxychloride representing of no bacterial turbidity was designated as MIC for different bacterial strains. The wells which resulted in the inhibition of the bacterial growth were then sub cultured onto nutrient agar plates and examined for bacterial growth to give minimum bactericidal concentration. The Minimum Bactericidal Concentration (MBC) was defined as the lowest concentration of thanatin or copper oxychloride that eliminated 99.9% of bacterial cells within 24 h (Sledz et al., 2015). Experiments were performed in triplicate.

Plant growth condition in the greenhouse

The rice seeds were first surface sterilized with NaClO 1% for 1 min and then washed three times with sterilized distilled water consecutively, each time for 30 seconds. The seeds were then transferred to sterile petri dishes covered with wet paper and placed at 25 °C for one week. Germinated seeds were planted in the pot containing autoclaved soil (including equal volume of field soil and sand). These pots were kept in a greenhouse at 25-30 °C under the photoperiod of 14/10 h (light/darkness) and 60% relative humidity. Thirty days old rice seedlings possessing an average of 3–5 leaves were used for bacterial inoculation.

Thanatin antibacterial evaluation under *in vivo* condition

Xanthomonas oryzae pv. *oryzae*, the causal agent of rice bacterial leaf blight was selected as a test bacterial pathogen. Rice seedlings were used as host plants in order to investigate the potential antibacterial activity of thanatin under greenhouse condition. The antibacterial activity of thanatin was determined by leaf clipping method in the greenhouse as has previously been described by Barker, 2002. The fresh bacterial culture was prepared overnight in nutrient broth medium (NB). A bacterial suspension containing 10^9 cfu /mL (equivalent to an absorbance equal to one at a wavelength of 600 nm) was provided in sterilized distilled water and used for inoculation (Sukhwinder et al., 2003).

Table 1: Plant bacterial isolates used for thanatin antibacterial activity test and their other related information

Name	Strain number	Isolation source	Disease
Pseudomonas syringae pv. *syringae*	B728a	*Prunus persica*	Canker
Pseudomonas syringae pv. *phaseolicola*	ATCC 21781	*Phaseolus vulgaris*	Halo blight
Pseudomonas syringae pv. *porri*	Local isolate	*Allium cepa*	Leaf spot
Pseudomonas viridiflava	LMG 5399	*Phaseolus vulgaris*	Soft rot
Pseudomonas tolaasii	ATCC 33618	*Agaricus bisporus*	Blotch
Xanthomonas translucens pv. *cerealis*	CFBP 4165	*Bromus* sp.	Leaf streak
Xanthomonas oryzae pv. *oryzae*	Local isolate	*Oryza sativa*	Leaf blight
Xanthomonas perforans	DSM 18975	*Solanum lycopersicum*	Leaf spot
Erwinia amylovora	ATCC 49946	*Malus domestica*	Fire blight
Pectobacterium carotovorum	ATCC 15713	*Solanum tuberosum*	Soft rot
Agrobacterium tumefaciens	Local isolate	*Vitis vinifera*	Crown gall
Agrobacterium rhizogenes	Lab strain 1586	*Prunus* sp.	Hairy root
Brenneria nigrifluens	Local isolate	*Juglans regia*	Bark canker
Rhodococcus fascians (gram positive)	Local isolate	*Pelargonium* sp.	Leafy gall

A scissor was first disinfected using 70% ethanol and used for inoculation. The scissors were then immersed in bacterial suspension and incisions were made up to one cm in the upper part of the leaves. The initial concentration of thanatin in the stock solution was 5 µg/mL. To prepare the test solutions, the stock solution was diluted in 5 mL dimethylsulfoxide (DMSO) followed by dilution with the water containing Tween-20 (250 µg/mL). Three hours after bacterial inoculation, 10 mL from different concentrations which were equal to 0.51, 1.02, 2.04 and 4.09 µg/mL (according to the evaluated MIC and MBC) were individually sprayed on the rice seedlings as foliar application. Three replications were maintained for each treatment; each replication consisted of five pots and in each pot three plants were maintained in a completely randomized design under glasshouse conditions. The inoculated plants were kept under plastic bags for 24 hours and then moved to a greenhouse with a photoperiod of 14/10 h (light/darkness) at 25- 30 °C and 70% relative humidity. Negative control plants were treated with double sterilized distilled water and positive control plants were treated with bacterial suspension without thanatin. Disease development was recorded 15 days after inoculation. The severity of leaf blight was recorded for each treatment and scored from 0 to 6 according to the following rating system (0 = healthy; 1 = 1–5%; 2 = 6–10%; 3 = 11–25%; 4= 26–50%; 5 = 51–75%; and 6 ≥ 76% of the leaf area infected) suggested by Latha et al. (2009) with minor modification. Five leaves from each plant were randomly checked for disease severity evaluation. The whole experiment was repeated twice. The disease severity was estimated as a value on the interval scale which was subsequently used to determine the disease severity index (DSI) as described by Mckinney's (1923) where DSI (%) = (Σ of ratings of infected leaves observed / Number of leaves observed × Maximum disease grade) ×100.

Data analysis

All the analyses were performed in triplicate and datasets were subjected to analysis of variance (ANOVA) and the Tukey's honest significant difference (HSD) test using SPSS 24 software. In all cases, a P value of ≤ 0.05 was considered significant. The diagrams were drawn using Microsoft Office Excel 2013.

Results

Recombinant vector construction and synthesize of thanatin

The sequence of thanatin was codon optimized to improve gene expression. Stop codon and two restriction sites for the enzymes *Eco*RI and *Xba*I were found on the sequence in order to transfer the construct in pPICZαA vector (Figure 1). Thanatin encoding sequence was virtually cloned and the obtained schematic figure was presented in Figure 2.

Transfection, gene expression and SDS-PAGE analysis

The pPICZαA vector and the coding sequence of thanatin were successfully digested with restriction enzymes and transformed into DH5α competent cells after ligation. The positive colonies form LB medium plus Zeocin were confirmed by PCR with *AOX1* gene primers (Figure 3a colony PCR 1). The sequencing result showed that, thanatin fragment was successfully cloned in the correct frame without any mutation. The pPICZαA plasmid carrying the coding sequence of thanatin was linearized with *Pme*I restriction enzyme and purified later (Figure 3a linear pPICZαA). The linearized vector was transfected into *P. pastoris* electro-competent cells strain KM71H. Thanatin was integrated in the genome

of recombinant *P. pastoris* as confirmed by genomic PCR using thanatin specific primers (Figure 3a colony PCR 2). Expression of recombinant thanatin in *P. pastoris* was evaluated by SDS-PAGE analysis. As expected, a 2.345 KD band corresponding to the size of thanatin peptide was observed on the gel (Figure 3b).

MIC and MBC evaluation tests

The results of MIC and MBC evaluation revealed the outstanding antibacterial activity of thanatin against tested plant bacterial pathogens (Table 2).

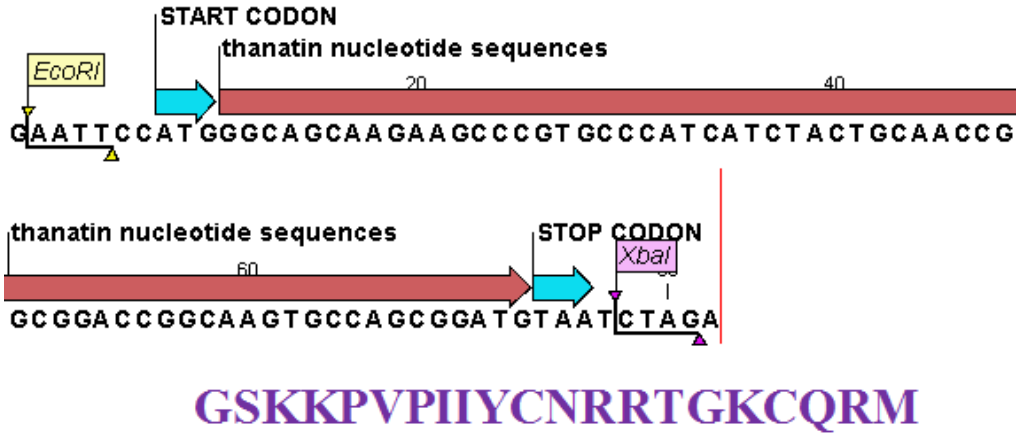


Figure 1. Schematic representation of thanatin nucleotide and amino acid sequences.

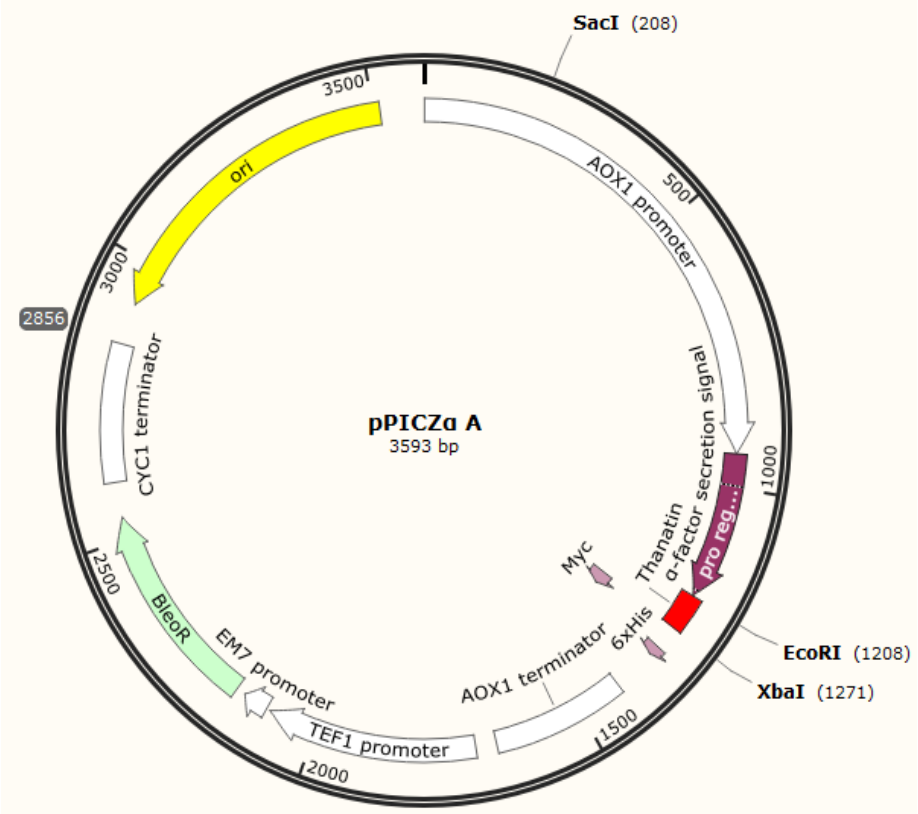
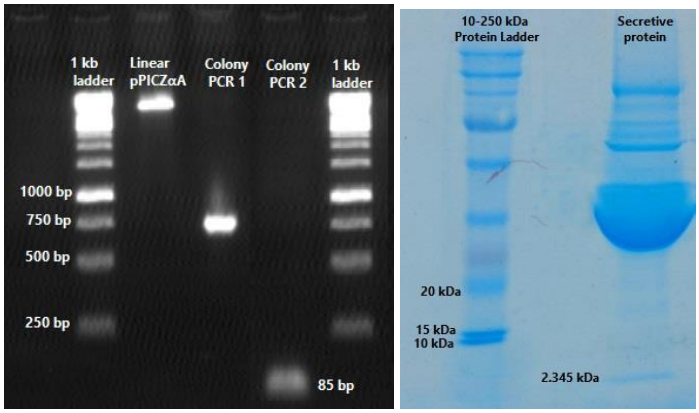


Figure. 2. Schematic figure from the virtual clone of thanatin encoding sequences in pPICZαA.



(a) **(b)**

Figure 3. The positive colonies were confirmed by PCR with *AOX1* gene primers (Figure 3a colony PCR 1). The linearized pPICZ*α*A carrying the coding sequence of thanatin (Figure 3a linear pPICZ*α*A). Integration of thanatin in the genome of recombinant *P. pastoris* confirmed by PCR using thanatin specific primers (Figure 3a colony PCR 2). SDS-PAGE analysis of *P. pastoris* secreted protein in cultural medium as seen a peptide band with the size of 2.345 kDa represented the recombinant thanatin (b).

Table 2. The evaluated MIC and MBC of thanatin compered to copper oxychloride against 14 plant bacterial isolates

Bactericides →	Thanatin		Copper Oxychloride	
Plant pathogenic bacterial isolates ↓	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
Pseudomonas syringae pv. *syringae*	0.032	0.064	625	1250
Pseudomonas syringae pv. *phaseolicola*	0.512	1.024	625	1250
Pseudomonas syringae pv. *porri*	0.032	0.064	312.5	625
Pseudomonas viridiflava	0.032	0.064	312.5	625
Pseudomonas tolaasii	0.064	0.128	312.5	625
Xanthomonas translucens	0.256	0.512	312.5	625
Xanthomonas oryzae pv. *oryzae*	2.048	4.096	78	156
Xanthomonas perforans	0.512	1.024	1250	2500
Erwinia amylovora	0.512	1.024	1250	2500
Pectobacterium carotovorum	0.064	0.128	312.5	625
Agrobacterium tumefaciens	0.128	0.256	1250	2500
Agrobacterium rhizogenes1586	0.512	1.024	2500	5000
Brenneria nigrifluens	0.064	0.128	156	312
Rhodococcus fascians	0.032	0.064	625	1250

Greenhouse antibacterial evaluation

The symptoms of bacterial disease were first observed in the form of water-soaked lesions on the upper parts of inoculated leaves. After 5-7 days in the positive control samples, the entire leaves changed to a yellow color and began to dry out. No disease symptom was observed in the negative control samples (Figure 4). Disease development was recorded 15 days after inoculation. The antibacterial activity of thanatin against *Xanthomonas oryzae* pv. *oryzae* was determined on the rice seedlings under greenhouse condition.

The results of the HSD test indicated that thanatin was significantly different among the various concentrations that were applied. Obviously, by increasing of thanatin concentration, the length of infected area on the leaves was reduced in all treated samples compared to positive control sample. Notably, the antibacterial effect of thanatin was observed by its application above 0.51 µg/mL concentration (Figure 5).

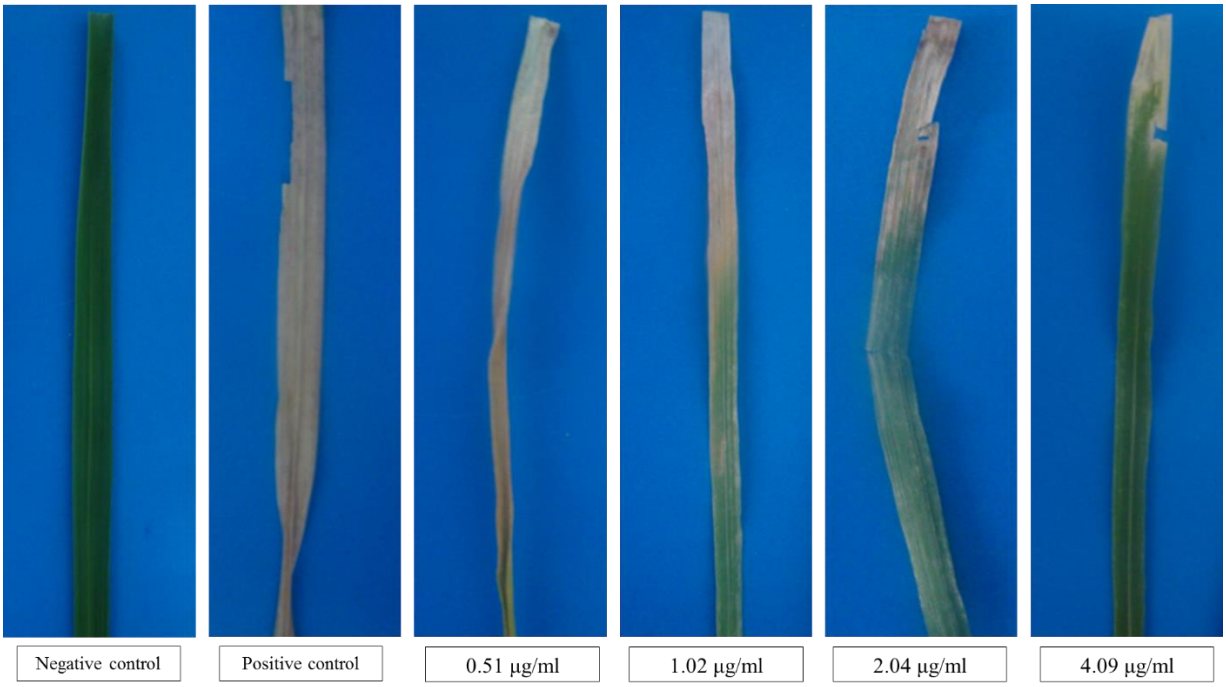


Figure 4. Disease development on rice seedling 15 days after inoculation by *Xanthomonas oryzae* pv. *oryzae*. Three hours after inoculation, the rice plants were sprayed by different concentration of thanatin.

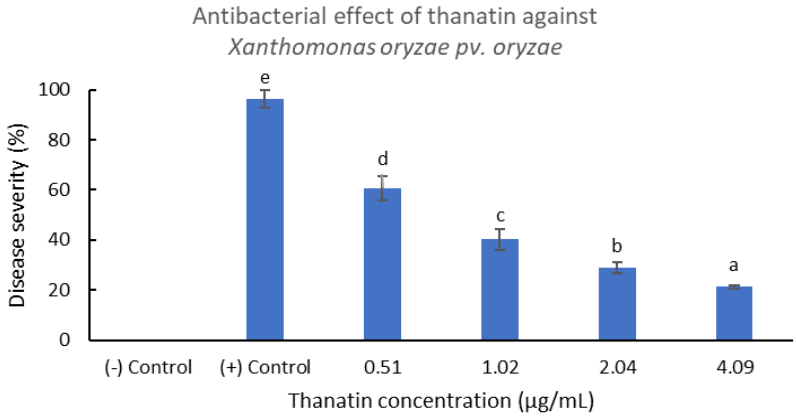


Figure 5. Severity of bacterial leaf blight evaluated on rice seedling 15 days after inoculation by *Xanthomonas oryzae* pv. *oryzae*. Three hours after inoculation the rice plants were treated by different concentration of thanatin as foliar application.

Discussion

Plants are often threatened by recurring bacterial, fungal and viral infections. Bactericides and fungicides were commonly used to control plant pathogens but their extensive use has contributed to chemical contamination of the environment (Damalas and Eleftherohorinos, 2011). Considering the side effects of chemicals in agriculture, many alternative strategies including antimicrobial peptides were particularly considered (Jansen and Kogel, 2011). The

recent strategy plays the role of innate immunity in living organisms (Pasupuleti et al., 2012). Different peptides and their synthetic derivatives showed their applications as antibacterial, antifungal and therapeutic agents (Laverty et al., 2011, Tanhaeian et al., 2018, Mamarabadi et al., 2018). Transgenic rice with recombinant synthesis of thanatin (Imamura et al., 2016) and transgenic *Arabidopsis thaliana* resistant to different pathogens (Wu et al., 2013) are the good examples of using this peptide against plant pathogens. Using transgenic plants as production platform to produce recombinant protein/peptides have several environmental concerns (Rani and usha, 2013). To use recombinant thanatin extensively against wide range of plant pathogens, it is necessary to use a different production platform such as yeast. The appropriate antibacterial effects of recombinant thanatin against both gram positive and negative bacteria presented in this study are consistent with the other antibacterial evaluations in different studies (Pagès et al., 2003, Ma et al., 2016). Remarkably and consistent with our results, thanatin was found to be highly effective in inhibiting the growth of bacteria and fungi at considerably low concentrations. Moreover, multiple modes of action have been proposed for thanatin in killing bacteria under *in vitro* and *in vivo* conditions (Dash and Bhattacharjya, 2021).

For the first time, the antibacterial mode of action of recombinant thanatin was described in the study made by Wu et al., 2010. As they have suggested, thanatin binds to bacterial lipopolysaccharide and causes pores in the membrane which finally leads to bacterial death. Several studies have demonstrated that thanatin's mode of action may be bacterial cell agglutination. The outer membrane lipopolysaccharide (LPS) of gram-negative bacteria and the cell wall components of gram-positive bacteria are the main sites of interactions of thanatin. However, the lethality mechanisms of thanatin for gram-positive bacteria and fungi are largely indefinable, since these microorganisms do not contain LPS or LPS translocation protein complexes (Dash and Bhattacharjya, 2021). Based on recent studies, the LPS outer membrane and LPS translocation protein complexes are important targets of thanatin in gram-negative bacterial cell inhibition. The dimeric state of thanatin with augmented cationicity and amphipathicity would be essential for outer membrane permeabilization and efficient surface charge neutralization. Permeabilized and charge-neutralized LPS outer membrane, bound with dimeric thanatin, can efficiently induce the cell agglutination process, eventually leading to bacterial cell death (Dash and Bhattacharjya, 2021; Sinha et al., 2017). Apart from its mode of action, the antimicrobial properties of recombinant thanatin could be used for controlling of bacterial pathogens in the plants. As the measured MIC and MBC for plant bacterial pathogens tested in this study were very low, it can be concluded that very small amounts of recombinant thanatin would be sufficient for plant bacterial inhibition in widespread as was already demonstrated for fungal plant pathogens (Mamarabadi et al., 2018). Moreover, our synthetized recombinant thanatin showed a proper antibacterial activity against *Xanthomonas oryzae* pv. *oryzae* on the rice seedlings by its application at µg level under *in vivo* condition. In our previous study we have also shown a good antifungal activity of thanatin in comparison with two plant extracts and a chemical mixture to control fungal plant pathogens under *in vitro* and *in vivo* condition (Mamarabadi et al., 2018).

The side effects of chemical pesticides in agriculture on human health are undeniable, therefore finding proper solutions, such as

antimicrobial peptides derived from nature, is an appropriate alternative in this regard. However, the lack of toxicity of these peptides to humans is an important issue which needs to be confirmed. In the case of thanatin, numerous studies were conducted. The result of a recent study showed that, this nature derived peptide has not shown any toxicity or undesirable effects in human evaluation (Ma et al., 2016). Moreover, unlike the majority of chemical pesticides, no allergy was observed in thanatin human tests (Wu et al., 2011).

Furthermore, the application of this peptide in the field of medicine and treatment has been proven by numerous studies (Pagès et al., 2003; Wu et al., 2011; Ma et al., 2016). Therefore, it is very logical to use such a peptide to inhibit bacterial pathogens.

Thanatin not only has no side effects compared to chemical pesticides, but also has some beneficial effects on human health. However, in this field, the cost of production would be another concern. Considering the synthesis of thanatin in secretion form using a yeast cultural medium a promising future is conceivable for its application as a non-chemical bactericide. Based on the result obtained from this study, the recombinant antimicrobial thanatin could be considered as a safe alternative bactericide against plant bacterial pathogens during integrated disease management programs. However, this promising antimicrobial peptide requires further research for the development of novel molecules for the control of plant pathogens caused by chemical resistant agents.

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