Eur J Plant Pathol
https://doi.org/10.1007/s10658-021-02257-0

The antimicrobial peptide AsR416 can inhibit the growth, sclerotium formation and virulence of *Rhizoctonia solani* AG1-IA

Zohreh Nassimi · Parissa Taheri · Xi Kong · Wubei Dong · Saeed Tarighi

Accepted: 10 March 2021

Abstract Plant antimicrobial peptides (AMPs) as a part of plant defense responses, are small soluble defense molecules which can inhibit the growth of pathogens. This study evaluates the effect of an antimicrobial peptide obtained from *Allium sativum* (AsR416) on *Rhizoctonia solani* (AG1-IA) the causal agent of rice sheath blight, in vitro and in vivo conditions. Firstly, the obtained results revealed that AsR416 (100 mg ml^{-1}) inhibited the growth and sclerotia production of *R. solani* AG1-IA. Furthermore, these results showed the mode of action and mechanisms of AsR416 effect in inhibiting sclerotia formation of *R. solani* AG1-IA via metabolomics tools. AsR416 decreased the biomass of *R. solani* AG1-IA in liquid culture. In addition, nitro blue tetrazolium and evans blue staining methods revealed that the antimicrobial peptide induced O_2^- formation in the hyphal cells and mycelia cell death of *R. solani* AG1-IA, respectively. AsR416 delayed the pathogen infection process and decreased the severity of rice sheath blight disease in vitro and in vivo conditions. AsR416 reduced activity of cellulase, which is one of the virulence factors of this pathogen. The number of sclerotia decreased on plants treated with AsR416 after 2 months compared with the control. Considering the need to reduce application of hazardous synthetic fungicides against pathogenic fungi, using AMPs could be a successful method to increase rice production and reduce the use of chemical fungicides against sheath blight disease.

Keywords Antimicrobial peptide · Biocontrol · *Oryza sativa* · *Rhizoctonia solani*

Z. Nassimi · P. Taheri (✉) · S. Tarighi
Department of Plant Protection, Faculty of Agriculture, Ferdowsi University of Mashhad, P. O. Box: 91775-1163, Mashhad, Iran
e-mail: p-taheri@um.ac.ir

Z. Nassimi · X. Kong · W. Dong
Department of Plant Pathology, College of Plant Science and Technology, Key Lab of Crop Disease Monitoring and Safety Control, Huazhong Agricultural University, Wuhan, Hubei Province, China

Introduction

The AsR416 is an antimicrobial peptide (AMP) obtained from *Allium sativum* with 34 amino acids, molecular weight of 3799.52 Da, and 1 cysteine disulfide, which has α-helix and β-sheet in its secondary structure (Xi et al., 2018). The AMPs are peptides with short sequence (usually less than 50 amino acids), which can be produced in living organisms such as bacteria (Cooter et al., 2005; Ferre et al., 2006; Jack & Jung, 2000), fungi (Degenkolb et al., 2003), insects (Bulet et al., 2004), marine invertebrates (Tincu & Taylor, 2004), amphibians and mammals (Andreu & Rivas, 1998; Zasloff, 2002) and plants (Lay & Anderson, 2005). Chemical and biological stresses such as drought, cold, heavy metals, pollutants, and biotic stresses caused by various pathogens trigger a broad range of resistance responses in plant species. In response to biotic stresses, plants display upregulation of different defense-related genes involved in resistance response (Stintzi et al., 1993). The AMPs obtained from

different plant tissues have inhibitory activity not only on phytopathogens, but also against human pathogens (Montesinos, 2007). Different types of AMPs are capable of inactivating prokaryotic cells by targeting essential or metabolic processes at extracellular area, plasma membrane, and intracellular space (Tam et al., 2015; Yount & Yeaman, 2013).

As a part of resistance responses, plants produce various types of toxic molecules, including AMPs, which can inhibit the growth of various types of pathogens by interaction with phospholipids and membrane permeabilization (Datta et al., 2015). General resistance is the result of secondary metabolites production like polyphenolics, phytoalexins, tannins, and pathogenesis-related proteins (PRs) (Taheri, 2018; Taheri & Tarighi, 2011). The AMPs are small soluble defense-related molecules, which can inhibit the growth of pathogens by membrane permeabilization of the microbial cells and inactivation of cytoplasmic targets. The AMPs are produced by proteolytic processing of one or more precursor peptides. Active peptides contain 10 to 50 amino acids, generally having lysine and arginine amino acids and being positively charged, but they also consist of a considerable proportion of hydrophobic residues (Swidergall & Ernst, 2014). The AMPs, known as defense constituents, are classified into two groups. The first group of AMPs, such as cecropins and magainins, consist of the short linear peptides with 20 to 40 amino acids, which form amphipathic α- helices in solution. The second type of AMPs is formed by the cysteine - rich peptides (Broekaert et al., 1997). All AMPs are classified based on structural characteristics. Lipopeptides are the linear and cyclic peptides that linked to fatty acid chains and pseudopeptides are the peptides which are linked to other molecules (Montesinos, 2007).

Every year, diseases caused by fungal pathogens affect different crops and decrease the quality, quantity, and safety of consuming agricultural products (Taheri et al., 2007). Recently, the antimicrobial effect of several AMPs has been investigated for protecting plants against various types of pathogens (Lopez-Garcia et al., 2012; Montesinos, 2007). For example, the Mi-AMP1, isolated from *Macadamia interifolia,* has antimicrobial effect against fungal phytopathogens, oomycetes and gram-positive bacteria (Lopez-Garcia et al., 2012; McManus et al., 1999). The 2S albumins AMPs, isolated from monocotyledonous and dicotyledonous seeds, have antimicrobial activity against fungal phytopathogens such as *Fusarium oxysporum*, *F. solani* and *Colletotrichum* spp. (Candido et al., 2011). Another example of AMPs is antimicrobial peptides with four-cysteines, which revealed inhibitory influence on several filamentous fungi, yeasts, and gram-positive bacteria (De Lucca et al., 1998). Shepherin I and shepherin II are two glycine-rich cysteine-free antimicrobial peptides, isolated from the root of shepherd's purse plants, which have antimicrobial effect against *Erwinia herbicola* and *F. culmorum* (Egorov et al., 2005). The Mj-AMPs, isolated from *Mirabilis jalapa*, have antimicrobial effect against phytopathogenic fungi including *Botrytis cinerea*, *Colletotrichum lindemathianum, Venturia inaequalis*, and also against gram-positive bacteria like *Bacillus megaterium* (Cammue et al., 1992; Lopez-Garcia et al., 2012). Also, PAFP-s is a knottin-like peptide obtained from the seeds of *Phytolacca americana*, with antifungal activity against *F. oxysporum*, *F. graminearum*, *Alternaria tennuis* and *Magnaporthe oryzae* (Gao et al., 2001; Lopez-Garcia et al., 2012).

Plants have established various mechanisms to defend themselves against pathogens and pests (Islam et al., 2020; Thaler et al., 1993). Compatible (susceptible) or incompatible (resistant) reactions are outcomes for plant-pathogen interactions. In the compatible interaction, infection occurs. But, in the incompatible interaction, several defense responses are activated in plants. Accumulation of structural proteins and antimicrobial compounds are among the most important outcomes of changes in the host plant tissues in response to various pathogens. As well as local response to infections, these defense-related proteins and compounds can also accumulate in uninfected parts of the plant (Lay & Anderson, 2005).

Different types of AMPs, such as plant defensins (Terras et al., 1992), thionin (Lay & Anderson, 2005), lipid transfer proteins (Cammue et al., 1995), snakins (Segura et al., 1999), hevein- type (Broekaert et al., 1992), knottin-like family (Craik et al., 2007), β-barrelins (Lopez-Garcia et al., 2012), 2S albumins (Lopez-Garcia et al., 2012), four-cysteine antimicrobial peptides (Lopez-Garcia et al., 2012), glycine-rich cysteine- free antimicrobial peptides (Lopez-Garcia et al., 2012) macadamia (McManus et al., 1999), impatiens (Tailor et al., 1997), and cyclotide (Jennings et al., 2001) are known to be active against several fungal and bacterial phytopathogens.

Thionins, as plant derived AMPs, can be induced by several stress factors. Infection of barley with *Erysiphe graminis* f. sp. *hordei* led to incorporation of leaf thionins into papillae in incompatible interactions (Ebrahim-Nesbat et al., 1993). The thionin obtained

from *Pyrularia pubera* has antimicrobial effect against plant pathogenic fungi, including *F. oxysporum* f. sp. *conglutinans*, *Plectosphaerella cucumerina*, *Botrytis cinerea* and gram-negative bacteria (Vila-Perello et al., 2003). Other example of AMPs with antifungal effect is the AMPs obtained from radish seeds, which have small cysteine-rich AMPs with antifungal activity against *Alternaria longipes*, *Fusarium culmorum* and *Pyricularia oryzae* (Terras et al., 1995). Defensins are another type of AMPs with antifungal effect. The DRR230 family of defensins, as an AMP isolated from pea, can reduce the growth of *Trichoderma reesei* and *Ascochyta pisi* (Lai et al., 2002).

The AsR416, as a new AMP obtained from *Allium sativum*, has antimicrobial activity against *Phytophthora capsici*, gram-negative bacteria such as *Xanthomonas campestris* pv. *oryzicola*, *Agrobacterium tumefaciens*, *Escherichia coli* DE3, *Ralstonia solanacearum* and gram-positive bacteria including *Clavibacter fangii*, *Clavibacter michiganensis*, *Bacillus anthraci*, *Bacillus subtilis*, and *Bacillus cereus* (Xi et al., 2018). However, the AsR416 effect is not widely studied on phytopathogenic true fungi species.

Rhizoctonia solani is a soil-borne necrotrophic phytopathogen (teleomorph: *Thanatephorus cucumeris*) with a wide host range in monocots and dicots. This fungal species is divided into 14 anastomosis groups, including AG1 to AG13 and AGBI (Carling et al., 2002). The AG1 of *R. solani* contains three main intraspecific groups. The subgroup AG1-IA is highly virulent on rice (*Oryza* spp.), which causes sheath blight disease (Taheri et al., 2007). It is also the causal agent of banded leaf, aerial blight and brown patch in many plants, including more than 27 families of monocots and dicots (Zheng et al., 2013). Every year, sheath blight disease can reduce the rice yield up to 50% worldwide (Groth, 2008). This fungus is also considered to be a destructive phytopathogen for other economically important crops, including corn, bean, and soybean (Ciampi et al., 2008). Control strategies against diseases caused by *R. solani* are limited and most of the strategies have relied mainly on application of hazardous chemical fungicides (Ciampi et al., 2008; Zheng et al., 2013).

This study provides cellular and metabolic information on the mode of action of the AsR416 in inhibiting growth and sclerotia formation of *R. solani* AG1-IA. In addition, we showed that the AsR416 decreased virulence of this pathogen in vitro and in vivo conditions by decreasing the activity of cell wall degrading enzyme produced by the pathogen and changing membrane permeability in hyphal cells of *R. solani* AG1-IA, as the causal agent of rice sheath blight disease.

Materials and methods

The fungal isolate and pathogenicity tests

The virulent isolate of *R. solani* AG1-IA was obtained from the culture collection of Ferdowsi University of Mashhad, Iran. *Oryza sativa* cv. Ming hui 63 was used as a host plant. Rice seeds were surface sterilized by 1% sodium hypochlorite solution for 5 min and 75% ethanol for 5 min, then washed three times with sterile distilled water. Finally, rice seeds germinated in sterile petri dish at room temperature. Germinated seeds were sown in trays containing potting compost and grown under greenhouse conditions at 25 ± 5 °C with a 16/8 h light/dark photoperiod. Forty-five days old plants were inoculated with inoculum consisting of toothpicks, 2 cm in length that had been sterilized and colonized with *R. solani* AG1-IA over 1 week. One colonized toothpick was placed into the lowest inner sheath of the main tiller. After one week of inoculation with the pathogen, symptoms and severity of the disease were evaluated (Taheri et al., 2007). Disease evaluation was performed based on the method described by Taheri et al. (2007) at 1, 2, 3, and 4 days post inoculation (dpi).

Transformed *Bacillus subtilis* with antimicrobial genes of *Allium sativum* (*AsR1036*, *AsR1064*, *AsRl11*, *AsR413*, *AsR864*, *AsR1205*, *AsR1205*, *AsR498* and *AsR416*) from a previous study (Xi et al., 2018) were obtained from bacterial collection of Huazhong Agricultural University, Wuhan, Hubei, China. The bacterial strains were maintained for long-term storage in LB with glycerol at −80 °C. Bacterial strains were grown on solid LB with kanamycin (10 mg ml^{-1}). A single colony was transferred to 250 mL flask containing LB and kanamycin and grown aerobically in flasks on a rotating shaker (170 rpm) for 3 days at 37 °C. Grown bacteria were diluted in sterile distilled water to final concentration of 10^6 CFU ml^1. The resulting bacterial suspensions were used to treat rice plants.

Extraction of AMPs

Bacillus subtilis, which was cultured on solid medium containing kanamycin (50 mg ml^1), was cultivated in

liquid LB medium containing kanamycin (50 mg ml $^{-1}$) and shaken at 170 rpm for 3 days at 37 °C. The supernatants were obtained by centrifugation at 10000 rpm for 25 min at 4 °C. The supernatant was collected and precipitated with saturated ammonium sulfate at 4 °C overnight. Then, centrifuged again at 10000 rpm for 20 min at 4 °C.

Extraction of intracellular protein

After 3 days growth in LB at 37 °C and 170 rpm of rotation, the bacterial cells were collected by centrifugation at 10000 rpm/ 4 °C/ 20 min. Then, the cells were washed twice, suspended in cold PBS buffer and transferred to sonication tubes. Sonication was performed for 20 min and then centrifuged at 10000 rpm and 4 °C for 20 min. The supernatant was used for other experiments.

Effect of AMPs on fungal growth and sclerotia formation

The agar-based diffusion method was used to investigate the effect of AMPs (1036, 1064, l11, 413, 864, 1205, 498, 416) on growth of *R. solani* AG1-IA. In this method, stainless steel cylinder was used to produce holes on the surface of potato dextrose agar (PDA) medium. Then, 500 µLL of AMPs (100 mg mL^{-1}) was added in each hole and the *R. solani* AG1-IA was cultured at the center of the plate (Hadacek & Greger, 2000). The WB800, without the genes encoding AMPs, was used as a control.

Minimun inhibitory concentration

Effect of AsR416 on *R. solani* AG1-IA growth in PDA was tested. Different concentrations of the AsR416, including 100, 50, 25, 12.5 and 6.25 mg ml^{-1} were prepared in PBS buffer (pH 7) and tested by agar-based diffusion method in PDA medium for determining minimum inhibitory concentration (MIC). The WB800 was used as control.

Effect of AMPs on fungal biomass

Effect of AMPs (AsR416 and WB800) on fungal biomass (fresh and dry weight of mycelia) and sclerotia production was investigated according to the method described by Granade et al. (1985), with some modifications. Briefly, mycelial plugs (0.5 mm diameter) of *R. solani* AG1-IA were grown in potato dextrose broth (PDB) medium containing each AMP at 100 mg ml^{-1} concentration. The WB800 (without antimicrobial genes) was used as the control in this assay. After 3 days and before production of sclerotia, the mycelia were harvested. For determining fresh weight, the samples were put on filter paper for 30 min and then were weighed. For determining dry weight, the samples were dried at 35 °C overnight and weighed.

Effect of AMPs on apoptosis

Effect of AMPs on reactive oxygen species (ROS) production in R. solani AG1-IA

Nitro blue tetrazolium (NBT) was used for detecting superoxide anion (O_2^-) as a main type of ROS in the hyphal cells of *R. solani* AG1-IA, according to the method described by Semighini and Harris (2010), with some modifications. The NBT (2.5 mM) solution was prepared in Hepes buffer (25 mM, pH 7.6). Mycelial plugs (5 mm) of active colony were placed on coverslips. The coverslips were placed in petri dishes containing a moist filter paper and the petri dishes were sealed with parafilm. The hyphae were treated with the AsR416. After 24 h, 500 µl of NBT solution was added to the corner of coverslip without disturbing the hyphae. Then, the fungal hyphae were incubated in the dark at room temperature for 30 min. The coverslips were observed using light microscopy (Nikon Eclipse 80i, Melville, USA) and the hyphal cells containing blue NBT formazan deposits were counted.

Effect of AMPs in induction of cell death in R. solani AG1-IA

Evans blue staining was used for detecting cell death in the hyphae of *R. solani* AG1-IA, according to the method described by Semighini and Harris (2010) with some modifications. In this method, Evans blue solution 1% was prepared in the PBS buffer. A mycelial plug (5 mm) of active fungal colony was placed on each coverslip. The coverslips were placed in petri dishes containing a moist filter paper and the petri dishes were sealed with parafilm. The hyphae were treated with the AsR416. After 24 h, 500 µL of Evans blue was added without disturbing the hyphae. Staining was performed for 5 min at room temperature. The coverslips were washed three times with the PBS buffer. The samples were visualized by fluorescence microscope (Nikon Eclipse 80i,

Melville, U. S.A), using 40× objective and a UV filters and the photos were taken by Nikon DS Ri1. Intensities of fluorescence were quantified by the Image J software version 1.6.2–24 (National Institutes of health, USA).

Effect of AMPs on membrane permeabilization

The pathogen was grown in PDB for 72 h at 28 °C, then treated with 1 ml of the AsR416 and/or WB800 (as a control) at 100 mg ml^1 concentration for 1 min. Then, the fungal protoplast was generated from each treatment as described by Hamlyn et al. (1981) and stained with propidium iodine (PI) using the Annexin V-FITC kit (BestBio, china). Images were obtained by a fluorescent microscope (Nikon Eclipse 80i, Melville, USA) and photos were taken by a Nikon DS Ri1 camera.

Effect of AMPs on activity of cellulase, as a cell wall degrading enzyme

Cellulase activity was investigated using a medium containing carboxymethyl cellulose (4.6 g), yeast extract (5.0 g), peptone (5.0 g), and K_2HPO_4 in 1 L of distilled water with pH 7.2, as described by MacMillan and Voughin (1964). The AsR416 was added to sterilized medium in each flask. Then, the flask was inoculated with a mycelial plug (5 mm diameter) of active colony of *R. solani* AG1-IA. Cellulase activity was determined after 10 days of incubation at 28 °C. The supernatants were obtained by centrifugation (5000 g for 15 min at 4 °C). Three flasks were used as replicates for each treatment as well as the control with the experiment repeated three times. The amount of reducing glucose is the base of determining cellulase activity. Dinitrosalicylic acid colorimetric method described by Miller (1959) and Ghose (1987) with some modifications was used for determining cellulase activity. According to this method, 0.5 ml of cell free supernatant was incubated with 0.5 ml carboxymethyl cellulose (2%) in 0.05 M acetate buffer with pH 4.8 and the reaction mixture was incubated at 50 °C for 30 min in static condition. Then, 3 L of 3, 5-dinitrosalicylic acid (DNS) reagent was added and the mixture was boiled for 5 min in vigorously boiling water bath. Then, the tubes were transferred immediately to the cold water bath. The mixture was diluted by adding 20 ml of deionized water and absorbance was measured at 540 nm. Absorbance of the samples were expressed as glucose production during the reaction using a glucose standard curve (Ghose, 1987).

Effect of AMPs on disease index in detached leaf bioassay

The first leaves of 4-week-old rice plants were detached and used in this assay. Each leaf segment with upper side was placed inside of petri dishes containing moist whatman filter papers. A mycelial disc of active colony of *R. solani* AG1-IA (5 mm diameter) was placed on the leaf surface. The petri dishes were incubated under laboratory conditions (25 °C; 12 h of light and 12 h of darkness). Symptom development was evaluated at 4 dpi as described by Taheri and Tarighi (2010). Intensity of the disease symptoms was graded into five classes based on the leaf area infected: 0 = no infection, 1 = 1–25%, 2 = 26–50%, 3 = 51–75%, and 4 = 76–100% infected leaf area. Disease index (DI) was calculated for each treatment by the formula $DI = [(0n_0 + 1n_1 + 2n_2 + 3n_3 + 4n_4)/4\ N]\ \ 100$, where n_0 is the number of plants with score 0, n_1 the number of plants with score 1, n_2 the number of plants with score 2, n_3 the number of plants with score 3, n_4 the number of plants with score 4, and N the total number of plants used in the experiment.

Metabolomics analysis

Mycelial plugs (5 mm) from active colony of *R. solani* AG1-IA were inoculated onto fresh PDB in 50 mm vials and incubated at 28 °C. Cultures were divided into 2 groups. One group treated with AsR416 protein and other group untreated with the protein (as the control) the vials were kept at 28 °C until sclerotia formation in the control. Each group consisted of 5 replicates and each replicate consisted of mycelia from 2 vials. After sclerotia formation in the control, while mycelium treated with AsR416 did not produce sclerotia, all samples were placed into a vacuum lyophilizer for 8 h and stored at −80 °C. Methanol stock solution (HPLC purified, 0.1% *v*/v formic acid) (1.5 ml) was added to 50 mg of each sample. After 2 min vortexing, the samples were shaken at 120 rpm and 24 °C for 4 h. After 20 min of sonication and the first centrifugation (4 °C at 14000 g for 20 min), 1 ml of supernatant was transferred into a new microtube and centrifuged for the second time. From each sample, 0.9 ml of supernatant from the second centrifugation was filtered using a 0.22 μm filter and transferred into the vials.

The Waters Ultra Performance Liquid Chromatography (UPLC) system used for metabolomics was equipped with an ACQUITY C18 column (10 cm 2.1 mm, particle

size 1.7 μm, Waters, USA). The column was eluted according to the following sequence with acetonitrile solution (A: B; A = water with 0.1% formic acid, B = acetonitrile): 1 min, 99: 1; 2 min, 80: 20; 5 min, 60: 40; 6 min, 45: 55; 13 min, 20: 80; 15 min, 5: 95; 17 min, 5: 95; 20 min, 99: 1. The gradient duration was 20 min at a flow rate of 0.4 ml min^{-1} with the column temperature set at 45 °C. One μl of each sample was injected into the column. Mass spectrometry was performed on the Waters QTOF MS and operated in positive ion mode with a scan range from 50 to 1200 m z^{-1}. The de-solvation gas flow was set to 500 l h^{-1} at a de-solvation temperature of 400 °C. The cone gas was set to 50 l h^{-1} and the source temperature was 100 °C. The capillary voltage and cone voltage were set to 1000 and 30 V, respectively. The QTOF MS acquisition rate was set at 0.3 s with a 0 s inter-scan delay. Tune page was used to regulate the sample cone voltage. In the MS/MS experiments, argon was employed as the collision gas, and the collision energy was set to 6 eV. The MS collision low and high energy were set at 15.0 and 45.0 eV, respectively. Data were collected in centroid mode. All analyses were performed using the lock spray, which was set at 20 s to ensure accuracy and reproducibility, leucine - enkephalin was used as the lock mass at a concentration of 0.8 ng μl^{-1} and a flow rate of 10 μl min^{-1}.

Statistical analysis

Effects of the AsR416 on biomass parameters, sclerotia formation and virulence of *R. solani* AG1-IA were analyzed by MSTATC version 1.42 (Michigan State University, UAA). All assays were repeated three times with three replications in each repetition. Experimental data were processed by analysis of variance (ANOVA) procedures. Means of the data obtained for different time points were separated by Duncan's Multiple Range test at $P < 0.05$. Significant differences were presented by various letters on the figures. The experiments were performed using completely randomized design (CRD).

Results

Effect of AMPs on fungal growth, sclerotia formation and fungal biomass

We examined the inhibitory effect of 8 intra and extracellular proteins, including AsR1036, AsR1064, AsRL11, AsR413, AsR864, AsR1205, AsR498 and AsR416 produced by 8 genes of *Allium sativum* and the WB800 (without antimicrobial genes) was used as a control. Among these proteins, only the AsR416 had inhibitory effect on the mycelial growth of *R. solani* AG1-IA compared to the WB800 (Fig. 1a). Therefore, the AsR416 was used for further cellular and chemical investigations. The AsR416 inhibited sclerotia production of the pathogen in vitro. After 1 week, sclerotia were not formed in the part of plate containing 100 mg ml^{-1} concentration of the AsR416, whereas numerous sclerotia were produced in the part of the same plate containing the WB800 (Fig. 1b). Determining fresh and dry weight of *R. solani* AG1-IA mycelia in liquid medium showed that the AsR416 was effective in reducing fungal biomass, as biomass of *R. solani* AG1-IA significantly decreased in the presence of AsR416 protein compared to the control and WB800 (Fig. 1c).

Minimum inhibitory concentration (MIC)

Among different concentrations of the AsR416 (100 to 6.25 mg ml^{-1}) used in this assay, minimum concentration with inhibitory effect (MIC) on growth of *R. solani* AG1-IA was 100 mg ml^{-1}. In lower concentrations of the AsR416, inhibitory effect against *R. solani* AG1-IA was not observed.

Effect of AMPs on apoptosis

Effect of AMPs on ROS production in R. solani AG1-IA

Hyphae obtained from active colony of *R. solani* AG1-IA were treated with the AsR416 and/or WB800, then they were stained with Nitro Blue Tetrazolium (NBT) to investigate the effect of the AsR416 on O_2^- production. Presence of blue cells revealed O_2^- production in the hyphal cells of *R. solani* AG1-IA. Production of O_2^- in fungal hyphae in presence of the AsR416 increased compared to the WB800 as control. The percentage of blue cells in hyphae treated with the AsR416 was more than in the hyphae treated with the WB800 and control (Fig. 2a and b).

Effect of AMPs on viability of hyphal cells in R. solani AG1-IA

The hyphae of *R. solani* AG1-IA treated with the AsR416 and/or WB800 were used for detecting dead

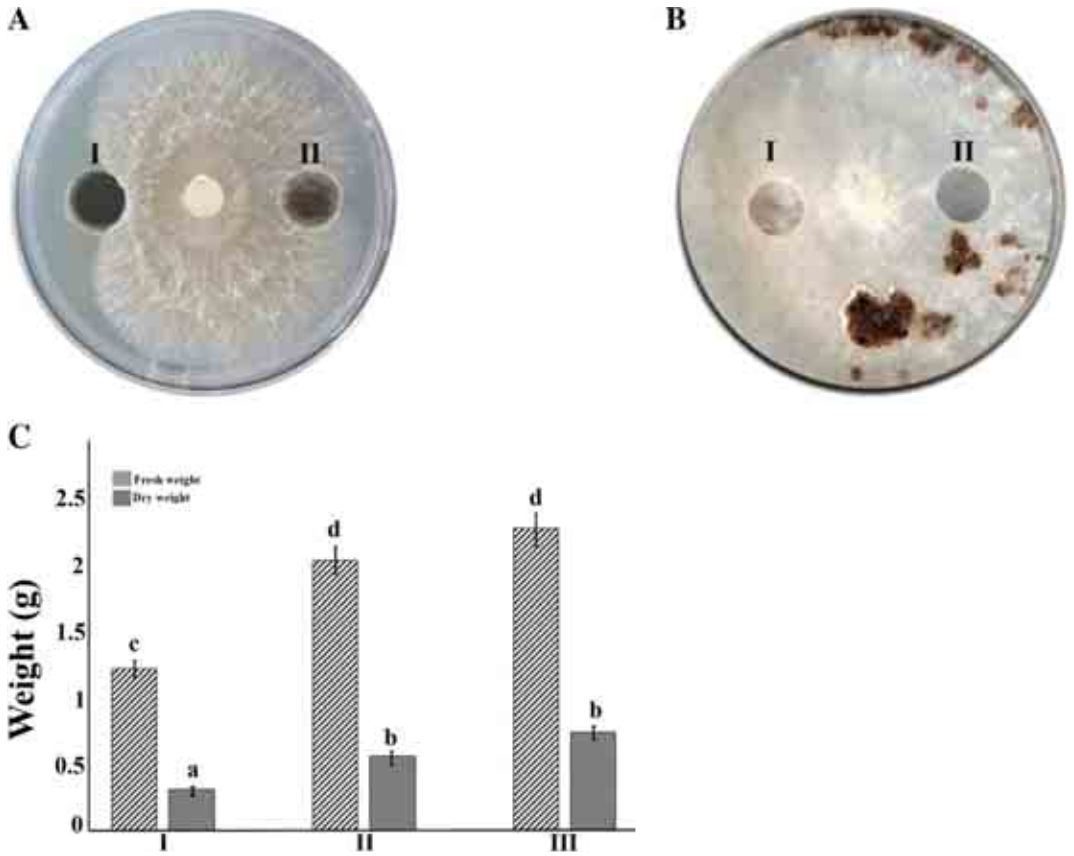

Fig. 1 Effect of AsR416 and WB800 (as a control without antimicrobial genes) on growth and sclerotia formation in *Rhizoctonia solani* AG1-IA in potato dextrose agar and fungal biomass in potato dextrose broth. A, Growth of mycelia around the hole containing WB800 compared to AsR416 after 2 days; B, formation of sclerotia around the hole containing WB800 after 10 days is visible; C, fungal biomass of *R. solani* AG1-IA in the liquid medium. The means obtained for various treatments with significant differences were separated by different letters based on Duncan's multiple range test procedure at $p<0.05$. I: AsR416, II: WB800, III: Control

cells using Evans blue staining. Evans blue penetrated dead cells and stained them, which can be visualized using a fluorescence microscope (Nikon Eclipse 80i, Melville, USA). Intensity of fluorescence color in the hyphal cells treated with the AsR416 had significant difference with the hyphae treated with WB800 and control (Fig. 2c and d).

Effect of AMPs on membrane permeability

Propidium iodide (PI) is a kind of nucleic acid dye, which does not penetrate intact cells but can bind to the nucleus through cell membranes in the necrotic cells. The PI staining showed that plasma membrane in the hyphae treated with the WB800 and control remained intact, as the PI dye was not detected in the mycelia with these treatments. In contrast, the plasma membrane of protoplast in the mycelia treated with the AsR416 lost permeability compared to the control and the hyphae treated with the WB800 (Fig. 2e).

Effect of AMPs on progress of the disease caused by R. solani AG1-IA

Detached rice leaves were used for investigating effect of the AsR416 on development of the disease caused by *R. solani AG1-IA* in vitro conditions. Symptom development was evaluated at 1, 2, 3, and 4 days post inoculation (dpi) with *R. solani* AG1-IA. Among the three different treatments used in this assay, growth of the fungal mycelia began at 2 dpi on the leaves treated with the AsR416, whereas on the leaves treated with the

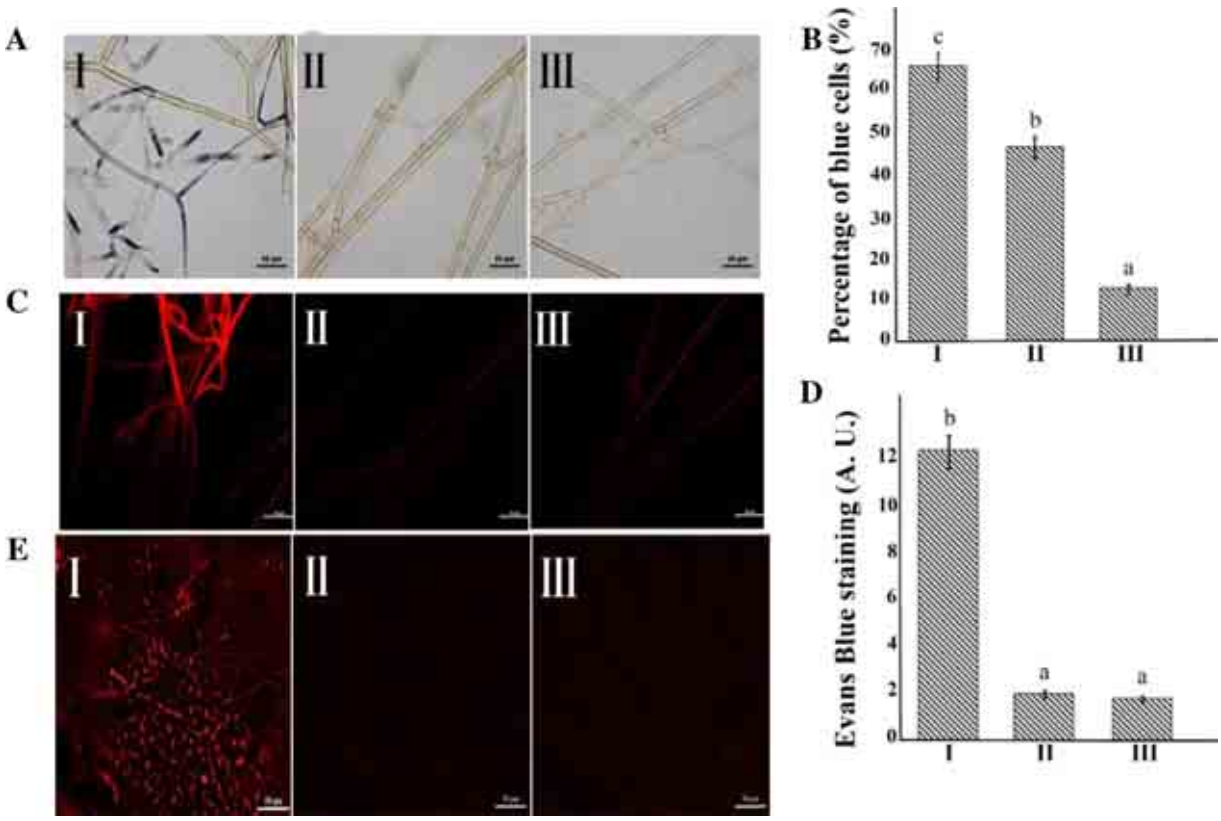

Fig. 2 Effect of AsR416 on apoptosis feature in *Rhizoctonia solani* AG1-IA. A, Detection of O^{-}_{2} in the hyphal cells of *R. solani* AG1-IA. Accumulation of formazan after Nitro Blue Tetrazolium staining was visualized for detecting O^{-}_{2}. B, Percentage of blue cells in the hyphae after treatment with AMPs. C, Detection of dead cells in *R. solani* AG1-IA using Evans blue staining. Fluorescence dead cells were detected in *R. solani* AG1-IA. D, Intensities of fluorescence in presence of AsR416 and WB800 and in the control were quantified by Image J software. The means obtained for various treatments with significant differences were separated by different letters based on Duncan's multiple range test procedure at $P < 0.05$. E, Effect of AsR416 on membrane permeability of *R. solani* AG1-IA. I: AsR416, II: WB800, III: Control

WB800 and controls, mycelial growth was observed earlier, at 1 dpi. Appearance of necrotic symptoms on control and the leaves treated with the WB800 was observed at 1 dpi. But, on the leaves treated with the AsR416, necrosis was not observed until 3 dpi and mild necrosis just around the mycelial plug was observed at 4 dpi. However, in other treatments, including WB800 and control, necrosis completely developed on the leaves. At 3 dpi, sclerotia were formed on the leaves treated with the WB800 and control leaves, while sclerotia production was not observed on the leaves treated with the AsR416 at any time point investigated in this assay (Fig. 3a). At the third and fourth days after inoculation disease index was not significantly different between the leaves treated with the WB800 and control (Fig. 3b).

For in vivo assay, effect of the AMPs on progress of the disease caused by *R. solani* AG1-IA on four-week-old rice plants was investigated in greenhouse conditions. The plants inoculated with *R. solani* AG1-IA were treated with the AsR416 and WB800 proteins. Plants untreated with the proteins and plants treated with the WB800 were used as controls in this assay. After 4 days, severe symptoms appeared in untreated plants and plants treated with WB800 protein, while in plants treated with the AsR416 mild symptoms were observed. The disease symptoms included greenish gray and oval-shaped lesions with yellow to brown margins mostly formed on the leaf sheaths. Analysis of diseased area by Image J software showed that lesion area in the plants treated with the AsR416 were less than that of untreated controls and plants treated with the WB800 (Fig. 4a and b).

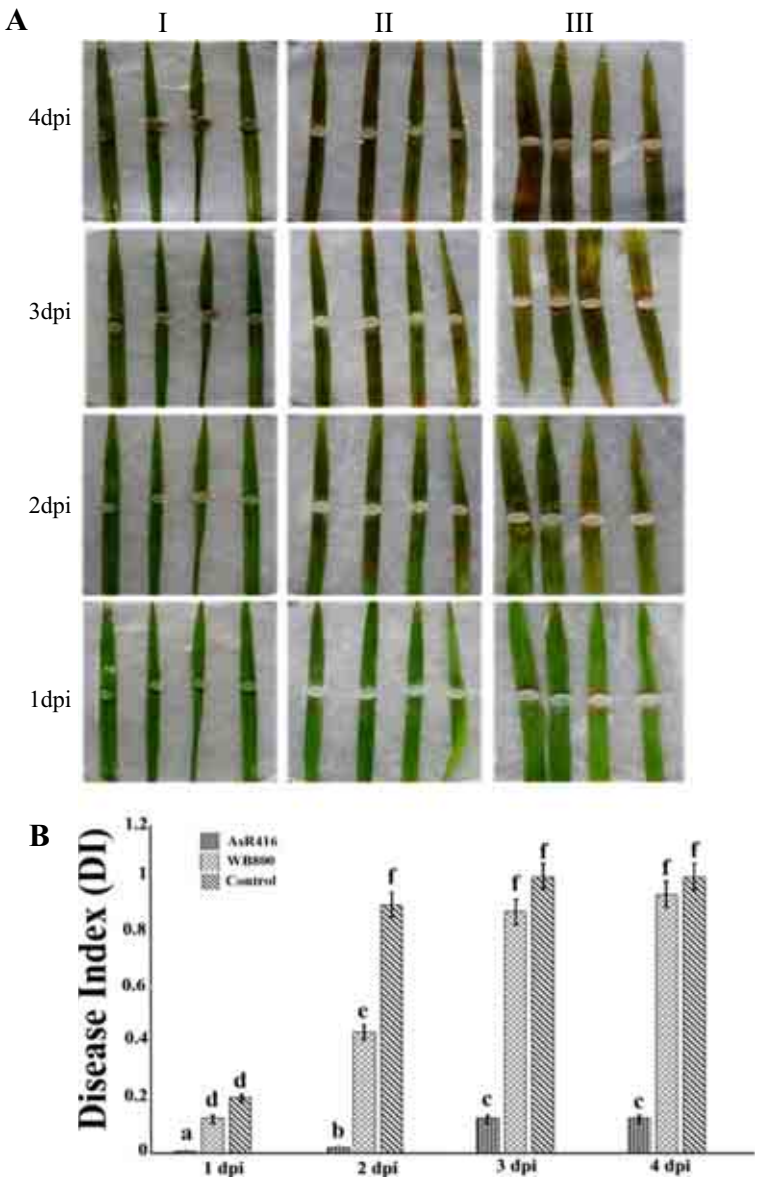

Fig. 3 Disease progress in detached leaves of greenhouse-grown rice plants. **a**, Symptom development in presence of AsR416, WB800 after 1, 2, 3 and 4 days post inoculation (dpi). Control is the leaf inoculated with *Rhizoctonia solani* AG1-IA without using AMPs. **b**, Symptom development was assessed at 1, 2, 3 and 4 dpi. The means obtained for various treatments with significant differences were separated by different letters based on Duncan's multiple range test procedure at $P < 0.05$. Each experiment was repeated three times using at least four replications of each treatment with similar results. I: AsR416, II: WB800, III: Control

Effect of AMPs on activity of cell wall degrading enzyme

Cellulase activity analysis showed that *R. solani* AG1-IA without AMPs had the maximum cellulase activity in vitro among all treatments in liquid culture. The lowest cellulase activity was observed for *R. solani* AG1-IA treated with AsR416. The results revealed that cellulase activity of *R. solani* AG1-IA decreased in presence of AsR416 (Fig. 4c).

Effect of AMPs on survival of *R. solani* AG1-IA in plant debris

To evaluate the AMPs (AsR416 and WB800) effect on survival of *R. solani* AG1-IA in plant debris, the number of sclerotia in plant debris was counted one month after the pathogen inoculation. The obtained data revealed that in plants treated with the WB800, *B. subtilis* WB800, and more than 100 sclerotia were formed on each control plants (Fig. 5a). In contrast, significantly

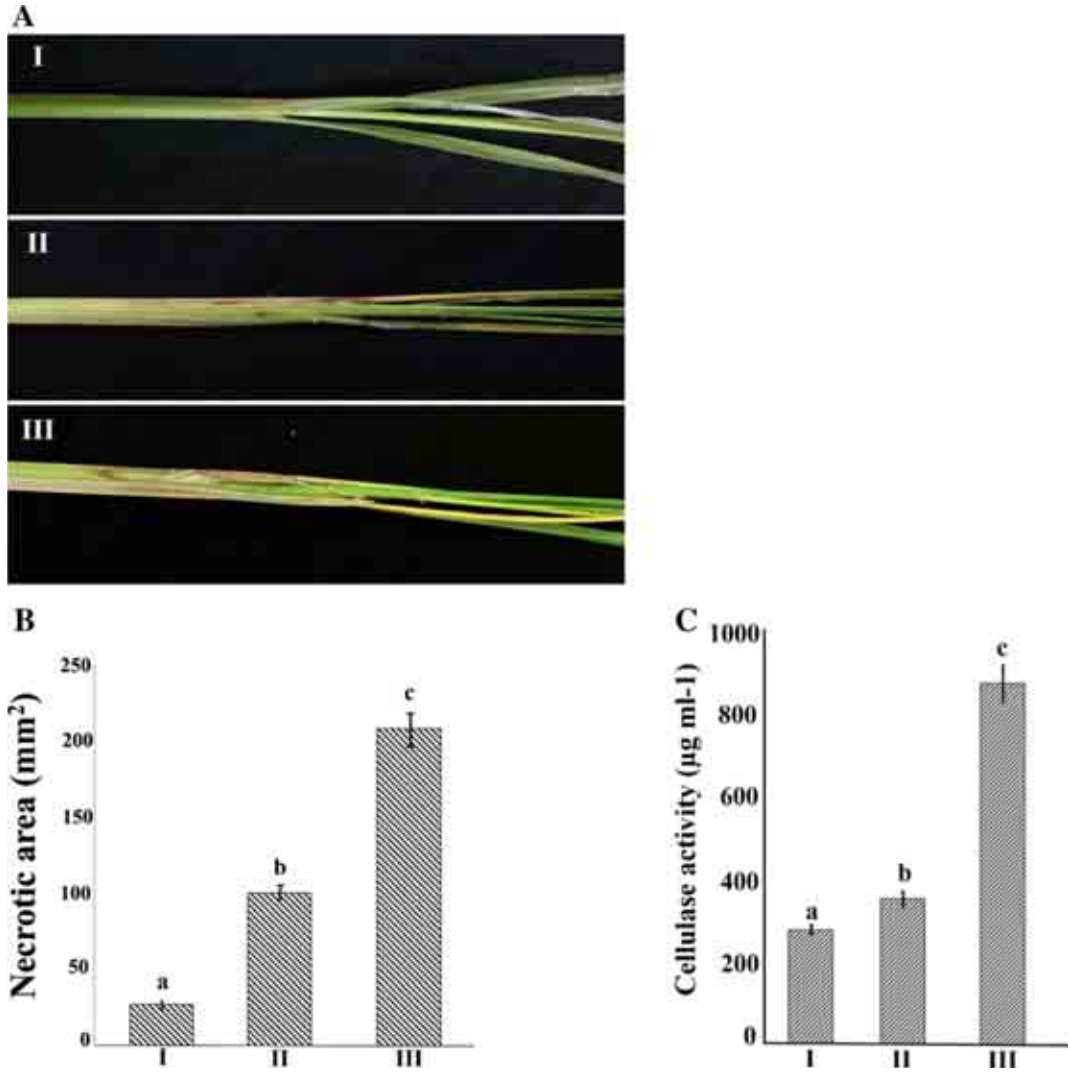

Fig. 4 Effect of AsR416 and WB800 on the progress of sheath blight on rice in greenhouse conditions (25 °C, 12 h of light and 12 h of darkness) and cellulase activity as a virulence factor of *Rhizoctonia solani* AG1-IA. Rice plants were inoculated with *Rhizoctonia solani* AG1-IA (into the lowest inner sheath of the main tiller.). **a** Symptom development of sheath blight in greenhouse condition. **b** Necrotic area was evaluated at 4 days post inoculation (dpi) by Image J software. **c** Analysis activity of cellulase produced by *Rhizoctonia solani* AG1-IA in presence of AMPs (AsR416, WB800) and effect of AsR416 in decrease of cellulase activity in *Rhizoctonia solani* AG1-IA. I: AsR416, II: WB800, III: Control

lower number of sclerotia were formed on the plants treated with the AsR416 compared to those treated with *B. subtilis* WB800, the WB800, and control plants without bacterial or AMP treatments (Fig. 5b).

Metabolomics analysis

As various metabolic pathways are involved in fungal growth, survival and virulence, metabolomics based on mass spectrometry technique by UPLC-QTOF-MS was used to investigate the intracellular metabolic changes in *R. solani* AG1-IA in presence and absence of AsR416. Raw data were analyzed by MZmine software (USA) and chromatogram was obtained after mass detection. For identification of different compounds, online database search was performed. The results revealed that the number of intracellular metabolites in mycelia without AsR416 treatment (control) was more than metabolites in mycelia treated with AsR416. Some metabolites were detected in all treatments, whereas other metabolites were found only in the control. Therefore, production of some metabolites was suppressed by the AsR416

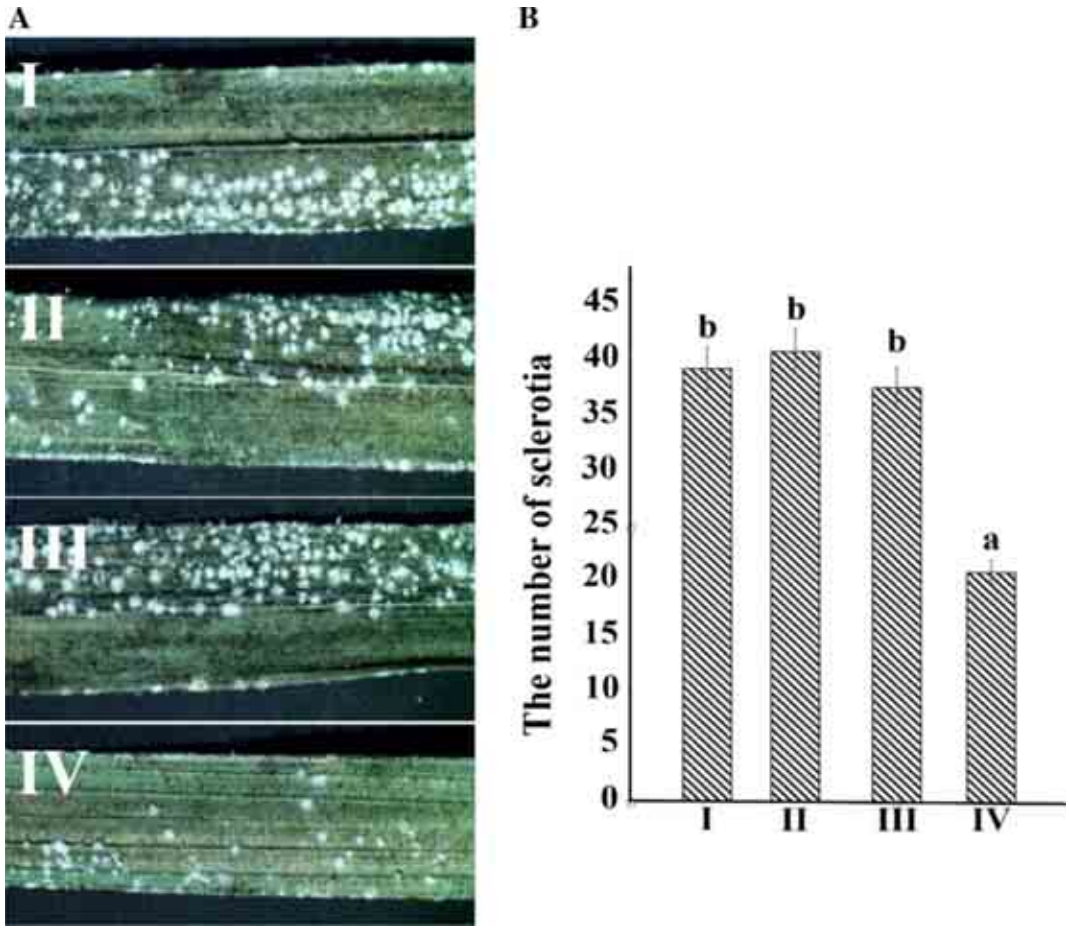

Fig. 5 Effect of antimicrobial peptides (AMPs) and *Bacillus subtilis* on the sclerotia formation of *Rhizoctonia solani* AG1-IA in plant debris at one month after inoculation. A, Sclerotia formation on rice plant debris; B, the number of sclerotia produced on the plants with different treatments at one month after the pathogen inoculation. I: rice plant inoculated with *R. solani* AG1-IA, no AMPs, no *B. subtilis* II: plant inoculated with *R. solani* AG1-IA and treated with *B. subtilis* WB800, III: plant inoculated with *R. solani* AG1-IA and treated with the WB800, IV: plant inoculated with *R. solani* AG1-IA and treated with the AsR416

treatment (Table 1). Production of some metabolites, which are known to be involved in sclerotia formation, was suppressed by the AsR416 in *R. solani* AG1-IA (Table 1).

Discussion

In this study, antifungal effect of the AsR416 as an AMP on *R. solani* AG1-IA causing rice sheath blight disease was demonstrated. This AMP strongly reduced mycelial growth and inhibited sclerotia formation in this pathogenic fungus. Considering the important role of sclerotia in survival of fungi in the environment (Money, 2016), a natural compound with significant effect on sclerotia formation in *R. solani* AG1-IA has a major role in management of diseases caused by this destructive phytopathogen, causing rice sheath blight.

In the detached leaf assay, symptoms caused by *R. solani* AG1-IA decreased in presence of AsR416 compared to the leaves treated with WB800 and control. Use of AsR416 in rice leaves inoculated with *R. solani* AG1-IA in detached leaf assay and also in greenhouse condition showed that after 4 days the phytotoxicity was negligible. While some of the AMPs obtained from microbial origins have significant phytotoxicity that limits their direct use as plant protection products, some AMPs play a role in protecting plant species against various diseases (Montesinos, 2007; Topman et al., 2018).

The AMPs have different modes of action in cells, including disruption of cell membrane integrity, DNA and RNA biosynthesis inhibition, inhibiting the activity

Table 1 The list of intracellular metabolites detected in the mycelium of *Rhizoctonia solani* AG1-IA treated with AsR416 and without AsR416 using the UPLC-QTOF-MS method. The effective intracellular metabolites in the formation of sclerotia that suppressed in *R. solani* AG1-IA by AsR416 are indicated by *; +: Presence of metabolite; −: lack of metabolite

Intracellular Metabolite	Control	AsR416	Intracellular Metabolite	Control	AsR416
(R)-Mevalonate	+	–	Atropine	+	+
S-Dihydroorotate	+	+	Barbiturate	+	+
14-Demethyllanosterol	+	+	Berbamunine	+	+
1-Methyladenosine	+	+	Bergapten	+	+
2,3,4,5-Tetrahydrodipicolinate	+	+	Biotin	+	+
2,6-Dichloroindophenol	+	+	Bryostatin	+	+
20-Hydroxyecdysone	+	+	Butanoyl-CoA	+	+
2-AminoAMP	+	+	Ceftriaxone	+	+
2-Aminomuconate	+	+	Cephalosporin C	+	+
2-Deoxy-D-ribose 1-phosphate	+	+	Chartreusin	+	+
2- Fluorobenzoyl-CoA	+	+	Choline	+	+
2-Hexaprenyl-3-methyl-6-methoxy-1,4-benzoquinone	+	+	Coenzyme B	+	+
2-Hexaprenylphenol	+	+	Cyanate	+	+
2-Hydroxymuconate semialdehyde	+	+	Cytidine	+	+
2-Hydroxypyridine	+	+	Cytosine	+	+
2-Oxoadipate	+	–	Deoxycytosine	+	+
2-Oxoglutarate	+	+	Destomysin	+	+
3,4-Dihydroxy-L-phenylalanine	+	+	D-Glucarate	+	+
3,7,4-Tri-O-methylquercetin	+	–	D-Glucosamine	+	–
3-Phosphoadenylyselenate	+	+	D-Glucuronate	+	+
3-(3,4-Dihydroxyphenyl)lactate	+	+	D-Mannitol-1-phosphate *	+	–
3,2-Phosphoglycerate	+	+	1-D-myo-Inositol 1, 3, 4-trisphosphate	+	–
3-glycerate	+	+	Dopaquinone	+	+
3-sulfino-L-alanine	+	–	D-Ribose	+	+
4-Aminobutanoate	+	+	D-Xylonate	+	–
4-Deoxy-beta-D-gluc-4-enuronosyl-N-acetyl-D-galactosamine	+	–	Ergosterol	+	+
4-Hydroxybenzoate	+	+	Estrone	+	+
5-Aminoimidazole	+	+	Ferrichrome	+	+
5-Methyltetrahydrofolate	+	+	Ferricyanide	+	–
5-Phosphoribosylamine	+	+	Folate	+	+
Agmatine	+	+	Formyl-CoA	+	+
Alpha-Erythroidine	+	+	Fumarate	+	+
Androsterone	+	+	Glutathione	+	+
Aromatic aldehyde	+	–	Glycocholate	+	+
Atrovastatin	+	+	Guanosine	+	+
Haloperidol	+	+	L-Tyrosine	+	+
Heteropyrithiamine	+	–	Mannitol	+	+
Hypotaurine	+	–	N-D-Glucosylarylamine	+	+
Hypoxanthine	+	+	N-Acetyl-D-glucosamine 6-phosphate *	+	–
Indolepyruvate	+	+	N-Acetylspermidine *	+	–
L-Alanine	+	+	Naringenin	+	–
Aniline *	+	–	Nicotinamide	+	+

Table 1 (continued)

Intracellular Metabolite	Control	AsR416	Intracellular Metabolite	Control	AsR416
L-Arogenate *	+	–	Orthophosphate *	+	–
L-Aspartate *	+	–	Pyridoxine *	+	–
L-Citrulline	+	+	Sulfoacetaldehyde	+	–
L-Cystine	+	+	Tetrahydrobiopterin	+	–
L-Histidine *	+	–	Tyr-Oet	+	–
L-Methionine	+	+	Zeatin	+	–
L-Octanoylcarnitine	+	+	Xylitol *	+	–
L-Phenylalanine	+	+	L-Serine	+	–

of enzymes involved in linking of cell wall structural proteins, inhibition of ribosomal function and protein biosynthesis, and blocking of chaperones necessary for accurate protein folding (Lazzaro et al., 2020; Peters et al., 2010). Mode of AMPs action against various bacteria, fungi and viruses is different. The AMPs have specific characteristics by harboring cationic and amphipathic structures and binding to cell membranes, resulting in the leakage of essential cell ingredients by forming pores or disturbing lipid organization in the membranes (Zhang et al., 2001).

Apoptosis induced by the AMPs has caused releasing cytochrome c, caspase activation, phosphatidylserine externalization, plasma or mithochondrial membrane depolarization, DNA, RNA, and protein damage (Cho et al., 2012; Madeo et al., 1997). Also, the AMPs are capable of inducing cell death in the eukaryotic cells (Aarbiou et al., 2006). The defensin RsAFP2 is an antifungal AMP obtained from radish, which induced apoptosis in *Candida albicans* (Aerts et al., 2009). In this study, we described that the AsR416 is an AMP capable of inducing apoptosis in the hyphal cells of *R. solani* AG1-IA, using three types of staining methods. In the first method, we used Nitro Blue Tetrazolium (NBT), a pale-yellow compound that is reduced by ROS to blue-purple formazan precipitate. When NBT reacted with O_2-, a dark blue insoluble formazan compound is produced, which can be detected microscopically (Baehner et al., 1976). Superoxide was reported to be the major oxidant species responsible for reducing NBT to formazan (Maly et al., 1989). The second method used in this research was detection of cell death by Evans blue staining. Normal hyphae with an intact plasma membrane exclude Evans blue and remain their natural color. Dead hyphae are unable to exclude the dye and stain deep blue. The third method is the PI staining for investigating membrane permeability of *R. solani* AG1-IA in presence of the AsR416. Similar to our findings, it was previously described that the defensin NaD1 from *Nicotiana alata* interacts with the fungal cell surface and increased membrane permeability, which leads to entry of the AMPs into the cytoplasm and increased ROS accumulation (Hayes et al., 2013). These biochemical and histological changes in the fungal cells treated with the AMPs, such as the AsR416, can lead to increased cell death as demonstrated in the present research.

The plant cell wall contains various types of polysaccharides, such as cellulose, hemicelluloses and pectin. Cellulose, as the major cell wall component in most plant species, is composed of β-1, 4 glucan chains (Harholt et al., 2010). For cell wall degradation, exocellular cellulolytic and pectolytic enzymes should be secreted by phytopathogens, which are able to degrade each of these structural polymers in the host plant (Abd-El-Khair & El-Gamal Nadia, 2011). Some plant-pathogenic fungi produce cellulases, which catalyze degradation of the β-1, 4-glycosidic bonds in cellulose (Carpita & Gibeaut, 1993). Cellulase is known as an invasive enzyme produced by phytopathogens, which allow the pathogen to penetrate various plant tissues (Olutiola & Cole, 1976). The rice sheath blight pathogen produces cellulase, which may assist penetration of the fungus into the host cells. Our results revealed that the AsR416 significantly reduced activity of cellulase enzyme produced by *R. solani* AG1-IA, which can be the reason of decreasing the disease symptoms on rice not only in vitro, but also in vivo conditions in presence of this AMP.

Various fungal metabolites are involved in growth, survival and virulence of phytopathogenic fungi such as *R. solani.* Therefore, metabolomics analysis was used to

evaluate the intracellular metabolic changes of *R. solani* AG1-IA caused by the AsR416 treatment. Among the metabolites identified in the control (without the AMP treatment) which were not detected in the mycelia treated with the AsR416, different types of amino acids including N-Acetyl-D-glucosamine 6 phosphate, N1-Acetylspermidine, Aniline, L-Histidine, L-Aspartate, L-Arogenate were detected. Amino acids have effect on sclerotia formation in *Sclerotium rolfsii* (Chet et al., 1966; Henis et al., 1973). Also, Liu and Wu (1971) and Kritzman et al. (1976) reported that the amino acid L-threonine and its derivative induced sclerotia formation in *S. rolfsii*. Xylitol is a sugar alcohol which production was suppressed by the AsR416. α, α-trehalose, 9-(Z)-octadecenoic, 9,12-octadecadienoic, D-glucose, xylitol and glucitol are known to be related with sclerotia formation (Aliferis & Jabaji, 2010). In other words, the AsR416 suppressed sclerotia formation in *R. solani* AG1-IA via its inhibitory effect on production of xylitol as a major metabolite involved in sclerotia production. Also, the data obtained in this research revealed that production of D-mannitol-1-phosphate as the mannitol derivative and a sugar alcohol was suppressed in the presence of AsR416. Mannitol was previously detected in the sclerotia of *Sclerotinia sclerotiorum*, *Claviceps purpurea*, *Claviceps nigricans* and *Sclerotinia curreyana* (Cooke, 1969; Meena et al., 2015). Moromizato et al. (1991) showed that different kinds of phosphorus has effect on sclerotia formation in *R. solani*. Also, we found that the AsR416 suppressed production of orthophosphate in *R. solani*. Pyridoxine is another compound that was suppressed by the AsR416 and detected in mycelia which produced sclerotia. Pyridoxine is known to be involved in sclerotia formation in *Sclerotium hydrophilum* (Hausner & Reid, 1999).

Briefly, we showed that the AsR416 protein encoded by the *AsR416* gene had inhibitory effect on vegetative growth, survival, and virulence of *R. solani* AG1-IA in both in vitro and in vivo conditions. The most important challenge of using AMPs is their high production costs. Using microbial systems or transgenic plants for production of AMPs might help to reduce the production costs (Lopez-Garcia et al., 2012). Over the last years, the biological and chemical methods have been improved to control plant diseases. Developing environment-friendly management strategies using AMPs seems to be necessary for controlling destructive plant diseases, such as rice sheath blight.

Acknowledgments We thank Ferdowsi University of Mashhad, Iran, for financial support of this research with project number 3/43459 approved on 3 April 2017. Also, this research was partly supported by Huazhong University, China.

Declarations

Ethical statement This manuscript complies to the ethical rules applicable for this journal.

References

Aarbiou, J., Tjabringa, G. S., Verhoosel, R. M., Ninaber, D. K., White, S. R., Peltenburg, L. T. C., & Hiemstra, P. S. (2006). Mechanisms of cell death induced by the neutrophil antimicrobial peptides a-defensins and LL-37. *Inflammation Research, 55*(3), 119–127.

Abd-El-Khair, H., & El-Gamal Nadia, G. (2011). Effect of aqueous extracts of some plant species against *Fusarium solani* and *Rhizoctonia solani* in *Phaseolus vulgaris* plants. *Archives Phytopathology Plant Protection, 44*, 1–16.

Aerts, A. M., Carmona-Gutierrez, D., Lefevre, S., Govaert, G., Francois, I. E., Madeo, F., Santos, R., Cammue, B. P., & Thevissen, K. (2009). The antifungal plant defensing RsAFP2 from radish induces apoptosis in a metacaspase independent way in *Candida albicans*. *FEBS Letters, 583*(15), 2513–2516.

Aliferis, K. A., & Jabaji, S. (2010). 1H NMR and GC-MS metabolic fingerprinting of developmental stage of *Rhizoctonia solani* sclerotia. *Metabolomics, 6*, 96–108.

Andreu, D., & Rivas, L. (1998). Animal antimicrobial peptides: An overview. *Biopolymers, 47*, 415–433.

Baehner, R. L., Boxer, L. A., & Davis, J. (1976). The biochemical basis of nitroblue Tetrazolium reduction in normal human and chronic granulomatous disease polymorphonuclear leukocytes. *Blood, 48*(2), 303–313.

Broekaert, W. F., Cammue, B. P. A., De Bolle, M. F. C., Thevissen, K., De Samblanx, G. W., & Osborn, R. W. (1997). Antimicrobial peptides from plants. *Critical Reviews in Plant Science, 16*(3), 297–323.

Broekaert, W. F., Marien, W., Terras, F. R., De Bolle, M. F., Proost, P., Van Damme, J., Dillen, L., Claeys, M., Rees, S. B., Vanderleyden, J., & Cammue, B. P. A. (1992). Antimicrobial peptides from *Amaranthus caudatus* seeds with sequence homology to the cysteine/glycine-rich domain of chitin-binding proteins. *Biochemistry, 31*, 4308–4314.

Bulet, P., Stocklin, R., & Menin, L. (2004). Antimicrobial peptides: From invertebrates to vertebrates. *Immunological Reviews, 198*, 169–184.

Cammue, B. P. A., Debolle, M. F. C., Terras, F. R. G., Proost, P., Vandamme, J., Rees, S. B., Vanderleyden, J., & Broekaert, W. F. (1992). Isolation and characterization of a novel class of plant antimicrobial peptides form Mirabilis Jalapa L. seeds. *The Journal of Biological Chemistry, 267*, 2228–2233.

Cammue, B. P. A., Thevissen, K., Hendriks, M., Eggermont, K., Goderis, I. J., Proost, P., Damme, J. V., Osborn, R. W.,

Guerbette, F., Kader, J. C., & Broekaerrt, W. F. (1995). A potent antimicrobial protein from onion seeds showing sequence homology to plant liquid transfer proteins. *Plant Physiology, 109*, 445–455.

Candido, E. S., Pinto, M. F., Pelegrini, P. B., Lima, T. B., Silva, O. N., Pogue, R., Grossi-Des-Sa, M. F., & Franco, O. L. (2011). Plant storage proteins with antimicrobial activity: Novel insights into plant defense mechanisms. *FASEB Journal, 25*(10), 3290–3305.

Carling, D. E., Kuninaga, S., & Brainard, K. A. (2002). Hyphal anastomosis reactions, rDNA-internal transcribed spacer sequences, and virulence levels among subsets of *Rhizoctonia solani* anastomosis group-2 (AG-2) and AG-BI. *Phytopathology, 92*, 43–50.

Carpita, N. C., & Gibeaut, D. M. (1993). Structural models of primary cell walls in flowering plants, consistency of molecular structure with the physical properties of the walls during growth. *The Plant Journal, 3*, 1–30.

Chet, I., Henis, Y., & Mitchell, R. (1966). The morphogenetic effect of Sulphur- containing amino acids, glutathione and iodoacetic acid on *Sclerotium rolfsii*. *Journal of General Microbiology, 45*, 541–546.

Cho, J., Hwang, I. S., Choi, H., Hwang, J. H., Hwang, J. S., & Lee, D. G. (2012). The novel biological action of antimicrobial peptides via apoptosis induction. *Journal of Microbiology and Biotechnology, 22*(11), 1457–1466.

Ciampi, M. B., Meyer, M. C., Costa, M. J. N., Zala, M., Macdonald, B. A., & Ceresini, P. C. (2008). Genetic structure of populations of *Rhizoctonia solani* anastomosis group-1 IA from soybean in Brazil. *Phytopathology, 98*(8), 932–941.

Cooke, R. C. (1969). Changes in soluble carbohydrates during sclerotium formation by *Sclerotinia sclerotiorum* and *Sclerotinia trifoliorum*. *Transactions of the British Mycological Society, 58*, 77–86.

Cooter, P. D., Hill, C., & Ross, P. (2005). Bacterial lantibiotics: Strategies to improve therapeutic potential. *Current Protein and Peptide Science, 6*, 61–75.

Craik, D. J., Clark, R. J., & Daly, N. L. (2007). Potential therapeutic applications of the cyclotides and related cysteine knot mini-proteins. *Expert OpinInvestig Drugs, 16*(5), 595–604.

Datta, A., Ghosh, A., Airoldi, C., Sperandeo, P., Mroue, K. H., Jimenez-Barbero, J., Kundu, P., Ramamoorthy, A., & Bhunia, A. (2015). Antimicrobial peptides: Insights into membrane Permeabilization, lipopolysaccharide fragmentation and application in plant disease control. *Scientific Reports, 5*, 11951.

De Lucca, A. J., Jacks, T. J., & Broekaert, W. J. (1998). Fungicidal and binding properties of three plant peptides. *Mycopathologia, 144*(2), 87–91.

Degenkolb, T., Berg, A., Gams, W., Schlegel, B., & Grafe, U. (2003). The occurrence of peptaibols and structurally related peptaibiotics in fungi and their mass spectrophotometric identification via diagnostic fragment ions. *Journal of Peptide Science, 9*, 666–678.

Ebrahim-Nesbat, F., Boh, S., Heitefuss, R., & Apel, K. (1993). Thionin in cell walls and papillae of barley in compatible and incompatible interactions with *Erysiphe graminis f. sp. Hordei*. *Physiological and Molecular Plant Pathology, 43*(5), 343–352.

Egorov, T. A., Odintsova, T. I., Pukhalsky, V. A., & Grishin, E. V. (2005). Diversity of wheat anti-microbial peptides. *Peptides, 26*(11), 2064–2073.

Ferre, R., Badosa, E., Feliu, L., Planas, M., Montesinos, E., & Bardai, E. (2006). Inhibition of plant-pathogenic bacteria by short synthetic cercropin A-melittin hybrid peptides. *Applied and Environmental Microbiology, 72*(5), 3302–3308.

Gao, G. H., Liu, W., Dai, J. X., Wang, J. F., Hu, Z., Zhang, Y., & Wang, D. C. (2001). Solution structure of PAFP-s: A new knottin-type antifungal peptide from the seeds of *Phytolacca americana*. *Biocchemistry, 40*(37), 10973–10978.

Ghose, T. K. (1987). Measurement of cellulose activities. *Pure and Applied Chemistry, 59*, 257–268.

Granade, T. C., Heeann, M. F., & Artis, W. M. (1985). Monitoring of filamentous fungal growth by in situ microspectrophotometry, fragmented mycelium absorbance density and 14C incorporation: Alternative to mycelial dry weight. *Applied and Environmental Microbiology, 49*(1), 101–108.

Groth, D. E. (2008). Effects of cultivar resistance and single fungicide application on rice sheath blight, yield and quality. *Crop Protection, 27*, 1125–1130.

Hadacek, F., & Greger, H. (2000). Testing of antifungal natural products: Methodologies, comparability of results and assay choice. *Phytochemical Analysis, 11*, 137–174.

Hamlyn, P. F., Bradshaw, R. E., Mellon, F. M., Santiago, C. M., Wilson, J. M., & Peberdy, J. F. (1981). Efficient protoplast isolation from fungi using commercial enzymes. *Enzyme and Microbial Technology, 3*, 321–325.

Harholt, J., Suttangkakul, A., & Vibe Scheller, H. (2010). Biosynthesis of pectin. *Plant Physiology, 153*, 384–395.

Hausner, G., & Reid, J. (1999). Factors influencing the production of sclerotia in the wild rice (*Zizania aquatica*) pathogen *Sclerotium hydrophilum*. *Mycoscience, 40*, 393–400.

Hayes, B. M., Bleackley, M. R., Wiltshire, J. L., Anderson, M. A., Traven, A., & Van DerWeerden, N. L. (2013). Identification and mechanism of action of the plant defensing NaD1 as a new member of antifungal drug arsenal against *Candida albicans*. *Antimicrobial Agents and Chemotherapy, 57*, 3667–3675.

Henis, Y., Okon, Y., & Chet, I. (1973). The relationship between early hyphal branching and formation of sclerotia in Sclerotium rolfsii. *Journal of General Microbiology, 79*, 147–150.

Islam, W., Tayyab, M., Khalil, F., Hua, Z., Huang, Z., & Chen, H. Y. H. (2020). Silicon-mediated plant defense against pathogens and insect pests. *Pesticide Biochemistry and Physiology, 168*, 104641.

Jack, R. W., & Jung, G. (2000). Lantibiotics and microcins: Polypeptides with unusual chemical diversity. *Current Opinion in Chemical Biology, 4*, 310–317.

Jennings, C., West, J., Waine, C., Craik, D., & Anderson, M. (2001). Biosynthesis and insecticidal properties of plant cyclotides: The cyclic knotted proteins from *Oldenlandia affinis*. *PNAS, 98*(19), 10614–10619.

Kritzman, G., Okon, Y., Chet, I., & Henis, Y. (1976). Metabolism of L-threonine and its relationship to sclerotium formation in *Sclerotium rolfsii*. *Journal of General Microbiology, 95*, 78–86.

Lai, F. M., DeLong, C., Mei, K., Wignes, T., & Fobert, P. R. (2002). Analysis of the DRR230 family of pea defensins: Gene expression pattern and evidence of broad host-range antifungal activity. *Plant Science, 163*, 855–864.

Lay, F. T., & Anderson, M. A. (2005). Defensins- components of the innate immune system in plants. *Current Protein and Peptide Science, 6*, 85–101.

Lazzaro, B. P., Zasloff, M., & Rolff, J. (2020). Antimicrobial peptides: Application informed by evolution. *Science*, (6490), 368, eaau5480.

Liu, M. F., & Wu, L. C. (1971). The effect of amino acids on the growth and morphogenesis of *Sclerotium rolfsii*. *Plant Protection Bulletin, Taiwan, 13*, 87–96.

Lopez-Garcia, B., Segundo, B. S., Coca, M. (2012). *Antimicrobial peptides as a promising alternative for plant disease protection*. In: Peptides for disease control. American ChemicalSociety, Washington DC.

MacMillan, J. D., & Voughin, R. H. (1964). Purification and properties of polyglacturonic acid- transeliminase produced by *Clastridium multiformentans*. *Biochemistry, 3*, 564–572.

Madeo, F., Frohlich, E., & Frohlich, K. U. (1997). A yeast mutant showing diagnostic markers of early and late apoptosis. *The Journal of Cell Biology, 139*(3), 729–734.

Maly, F. F., Nakamura, M., Gauchat, J. F., Urwyler, A., Walker, C., Dahinden, C. A., Cross, A. R., Jones, O. T., & de Weck, A. L. (1989). Superoxide- dependent nitroblue tetrazolium reduction and expression of cytochrome b- 245 component by human tonsillar B lymphocytes and B cell line. *The Journal of Immunology, 142*(4), 1260–1267.

McManus, A. M., Nielsen, K. J., Marcus, J. P., Harrison, S. J., Green, J. L., Manners, J. M., & Craik, D. J. (1999). MiAMP1, a novel protein from Macadamia integrifolia adopts a greek key beta-barrel fold unique amongst plant antimicrobial protein. *Journal of Molecular Biology, 293*, 629–638.

Meena, M., Prasad, V., Zehra, A., Gupta, V. K., & Upadhyay, R. S. (2015). Mannitol metabolism during pathogenic fungal-host interactions under stressed conditions. *Frontiers in Microbiology, 6*, 1–12.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry, 31*, 426–428.

Money, N. P. (2016). *Fungal cell biology and development*. In: The fungi. Elsevier 37–66.

Montesinos, E. (2007). Antimicrobial peptides and plant disease control. *FEMS Microbial Letter, 270*, 1–11.

Moromizato, Z., Ishizaki, F., Takara, K., & Tamori, M. (1991). The effect of phosphorus and magnesium on sclerotium formationin *Rhizoctonia solani* Kuhn. *Annual phytopathology Society of Japan, 57*, 649–656.

Olutiola, P. O., & Cole, O. O. (1976). Production of a cellulose complex in culture filtrates of *Aspergillus tamari* associated with mouldy cocoa beans in Nigeria. *Physiologia Plantarum, 37*, 313–316.

Peters, B. M., Shirtliff, M. E., & Jabra-Rizk, M. A. (2010). Antimicrobial peptides: Primeval molecules or future drug? *PLoS Pathogens, 6*(10), e1001067.

Segura, A., Moreno, M., Madueno, F., Molina, A., & Garcia-Olmedo, F. (1999). Snakin-1, a peptide from potato that is active against plant pathogen. *Molecular Plant Microbe Interaction, 12*(1), 16–23.

Semighini, C. P., Harris, S. D. (2010). Methods to detect apoptotic-like cell death in filamentous fungi. IN: Molecular and cell biology methods for fungi, methods in molecular biology. Springer science + Business Media. 317 pp.

Stintzi, A., Heitz, T., Prasad, V., Wiedemann-Merdinoglu, S., Kauffmann, S., Geoffroy, P., Legrand, M., & Fritig, B. (1993). Plant "pathogenesis-related" proteins and their role in defense against pathogens. *Biochimie, 75*, 687–706.

Swidergall, M., & Ernst, J. F. (2014). Interplay between *Candida albicans* and antimicrobial peptide armory. *Eukaryotic Cell, 13*(8), 950–957.

Taheri, P. (2018). Cereal diseases caused by Fusarium graminearum: From biology of the pathogen to oxidative burst-related host defense responses. *European Journal of Plant Pathology, 152*(1), 1–20.

Taheri, P., Gnanamanickam, S., & Hofte, M. (2007). Characterization, genetic structure, and pathogenicity of *Rhizoctonia* spp. associated with rice sheath disease in India. *Phytopathology, 97*(3), 373–383.

Taheri, P., & Tarighi, S. (2010). Riboflavin induces resistance in rice against *Rhizoctonia solani* via jasmonate-mediated priming of phenylpropanoid pathway. *Journal of Plant Physiology, 167*, 201–208.

Taheri, P., & Tarighi, S. (2011). Cytomolecular aspects of rice sheath blight caused by *Rhizoctonia solani*. *European Journal of Plant Pathology, 129*, 511–528.

Tailor, R. H., Acland, D. P., Attenborough, S., Cammue, B. P., Evans, I. J., Osborn, R. W., Ray, J. A., Rees, S. B., & Broekaert, W. F. (1997). A novel family of small cysteine-rich antimicrobial peptides from seed of *Impatiens blasamina* is derived from a single precursor protein. *The Journal of Biological Chemistry, 272*(39), 24480–24487.

Tam, J. P., Wang, S., Wong, K. H., & Tan, W. L. (2015). Antimicrobial peptides from plants. *Pharmaceutical, 8*, 711–757.

Terras, F. R., Eggermont, K., Kovaleva, V., Raikhel, N. V., Osborn, R. W., Kester, A., Rees, S. B., Torrekens, S., Leuven, F. V., Vanderleyden, J., Cammue, B. P. A., & Broekaert, W. F. (1995). Small cysteine-rich antifungal proteins from radish: Their role in host defense. *The Plant Cell, 7*, 573–588.

Terras, F. R., Schoofs, H. M., DeBolle, M. F., Van Leuven, F., Rees, S. B., Vanderleyden, J., Cammue, B. P., & Broekaert, W. F. (1992). Analysis of two novel classes of plant antifungal proteins from radish seeds. *The Journal of Biological Chemistry, 5*(267), 15301–15309.

Thaler, J. S., Fidantsef, A. L., Duffey, S. S., & Bostock, R. M. (1993). Trade-offs in plant defense against pathogens and herbivores: A field demonstration of chemical elicitors of induced resistance. *Journal of Chemical Ecology, 25*, 1597–1609.

Tincu, J. A., & Taylor, S. W. (2004). Antimicrobial peptides from marine invertebrates. *Antimicrobial Agents and Chemotherapy, 48*, 3645–3654.

Topman, S., Tamir-Ariel, D., Bochnic-Tamir, H., Bauer, T. S., Shafir, S., Burdman, S., & Hayouka, Z. (2018). Random peptide mixtures as new crop protection agents. *Microbial Biotechnology, 11*(6), 1027–1036.

Vila-Perello, M., Sanchez-Vallet, A., Garcia-Olmedo, F., Molina, A., & Andreu, D. (2003). Synthetic and structural studies on Pyrularia pubera thionin: A single-residue mutation enhance activity against gram- negative bacteria. *FEBS Letters, 536*, 215–219.

Xi, K., Yang, M., Abbas, H. M. K., Wu, J., Li, M., & Dong, W. (2018). Antimicrobial genes from *Allium sativum* and *Pinellia ternate* revealed by Bacillus subtilis expression system. *Scientific Reports, 8*, 14514.

Yount, N. Y., & Yeaman, M. R. (2013). Peptide antimicrobials: Cell wall as a bacterial target. *Annals of the New York Academy of science, 1277*, 127–138.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature, 415*, 389–395.

Zhang, L., Rozek, A., Robert, E., & Hancock, R. E. (2001). Interaction of cationic antimicrobial peptides with model membranes. *The Journal of Biological Chemistry, 276*, 35714–35722.

Zheng, A., Lin, R., Zhang, D., Qin, P., Xu, L., Ai, P., Ding, L., Wang, Y., & Chen, Y. (2013). The evolution and pathogenic mechanisms of the rice sheath blight pathogen. *Nature Communications, 4*(1424), 1–10.