



Effect of antimicrobial peptides and monoterpenes on control of fire blight

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Abstract

Aim of study: Antimicrobial peptides and monoterpenes are safe compounds that have been used for control of many plant diseases. Herein, the effects of two recombinant antibacterial peptides (AMPs) were compared with two monoterpenes for control of *Erwinia amylovora* directly or via induction of plant defense enzyme guaiacol peroxidase (GPOD).

Area of study: The experiments were performed at the Ferdowsi University of Mashhad (Iran).

Material and methods: The central composite design (CCD) method was used to study the effect of mixing the compounds and copper compound (Nordox) in controlling the pathogen. The resistance level was studied on shoots of tolerant ('Dargazi') and semi-susceptible ('Spadona') pear cultivars treated with the antibacterial compounds.

Main results: Thanatin and 1,8-cineole showed the highest and lowest antibacterial effects. All treatments reduced *E. amylovora* pathogenicity on blossom. The CCD analysis revealed that the best reduction in colony number obtained by mixing Lfc, thanatin, thymol, 1,8-cineole and Nordox at concentrations of 32, 16, 24, 250 and 250 µg/mL. Thymol and 1,8-cineole at 500 µg/mL decreased disease severity significantly compared to that of AMPs. The level of GPOD enzyme in 'Dargazi' was higher than in 'Spadona'. All treatments increased the GPOD levels in both cultivars. Furthermore, resistance level and GPOD ratio were negatively correlated.

Research highlights: Antimicrobial peptides showed better effect on growth inhibition of *E. amylovora* than monoterpenes. Mixing of these peptides and monoterpenes at special dosage enhanced their antimicrobial efficacy against *E. amylovora*; that could represent a new method in control of fire blight disease.

Additional keywords: antibacterial; biological control; central composite design; *Erwinia amylovora*; guaiacol peroxidase; induced resistance.

Abbreviations used: AMP (antibacterial peptides); CCD (central composite design); DAI (days after inoculation); DS (disease severity); EO (essential oil); GPOD (guaiacol peroxidase); LB (Luria-Bertani); Lfc (lactoferricin); Lfp (lactoferrampin); MBC (minimum bactericidal concentration); MIC (minimum inhibitory concentration); NA (nutrient agar); ROS (reactive oxygen species); RSM (response surface methodology)

Authors' contributions: Technical and material support: ST and PT. Performed the experiment and analyzed the data: MA. All authors conceived and designed the experiment, wrote and approved the final manuscript.

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Introduction

The fire blight disease with the bacterial agent *Erwinia amylovora* (Burrill) Winslow *et al.* (1920) is undoubtedly one of the most vicious diseases of pome fruit trees worldwide imposing heavy and irreparable damage each year (Gusberti *et al.*, 2015). This disease is the most destruc-

tive bacterial disease, particularly on pears and apples, the two most economically important members of Rosaceae (Emeriewen *et al.*, 2019). The main symptoms of fire blight infection are burnt-like appearance of infected tissues, bacterial ooze released from the infected tissues, shepherd's crook, wilting, and water soaked appearance (Awais Khan *et al.*, 2011). In the last two centuries, this

pathogen has been spread globally and, consequently, *E. amylovora* has been classified as a quarantine organism in some countries, where it is subject to phytosanitary legislation. Recently, it has been included in the top 10 plant pathogenic bacteria (Mansfield *et al.*, 2012; Piqué *et al.*, 2015). The regular use of chemicals poses a challenge to the control of this disease, which can be performed by integrated management based on using copper compounds and antibiotics (Cabrefiga & Montesinos, 2017). Application of copper compounds during blooming has been recommended for the disease control, but these chemicals sometimes cause damage on blossoms and fruits (Steiner, 2000; Karami-Osboo *et al.*, 2010; Akhlaghi *et al.*, 2018). The use of antibiotics is prohibited in many countries due to the risk of developing resistance in the target and non-target populations of bacteria (Iacobellis *et al.*, 2005). Nevertheless, developing new active compounds with low phytotoxicity, decreased environmental effect and broad spectrum of activity is still essential (Cabrefiga & Montesinos, 2017). There are small peptides and proteins in the nature that exhibit antimicrobial activity. Antimicrobial peptides (AMPs) are emerging as a novel class of compounds for preventing plant diseases (Cabrefiga & Montesinos, 2017). AMPs are capable of being natural antibiotics created by animals, plants, protozoa, fungi, and bacteria (Biswaro *et al.*, 2018). The antimicrobial role of this group of compounds is well recognized and their application in the agriculture has been discovered. In recent years developments in the synthesis and screening of AMPs are crucial to the antimicrobial use of these compounds (Marcos *et al.*, 2008). The lack of AMPs in natural resources, along with extraction and purification costs, prompted scientists to use the recombinant DNA technology for achieving these valuable bio-products. Due to advances in genetic engineering, the AMPs can be produced in a cost-effective manner compared to natural resources and industrial biochemical production (Li, 2011).

Lactoferricin (Lfc) and lactoferrampin (Lfp) are two AMPs, which are the primary components of lactoferrin in milk. Lactoferrin is known as iron-binding glycoprotein with 80-kDa molecular weight; it has several biological activities, such as defense against pathogens, regulating the iron uptake, controlling the immune system, and promoting cellular growth (Fukuta *et al.*, 2012). It has been shown that the antimicrobial effect of Lfc+Lfp is more potent than that of Lfp or Lfc alone (Wang *et al.*, 2016). The thanatin peptide is also derived from the spined soldier bug (*Podisus maculiventris* S.) insect that possesses antimicrobial properties. Thanatin contains two amino acids, including two cysteine residues, forming a disulfide bridge (Mandard *et al.*, 1998). It is the first inducible insect peptide having diverse activities against bacteria and fungi at various concentrations (Mamarabadi *et al.*, 2018). Former research attempted to develop new antimicrobial agent paving the way for identifying peptides with

activity against some plant pathogenic bacteria, including *Erwinia amylovora*, *Pseudomonas syringae* pv. *syringae* (van Hall) and *Xanthomonas axonopodis* pv. *vesicatoria* (Doidge) (Cabrefiga & Montesinos, 2017).

In addition to the antimicrobial influence of AMPs for bacterial control, other alternative ways like applying active ingredients in essential oils (EOs) have been proposed as well. Currently, there is a new trend to look for antimicrobial compounds from medicinal plants. These sources may include EOs and plant extracts. An important feature of EOs and their active ingredients are hydrophobicity enabling them to partition with the lipids present in the cell membrane of bacteria making them more permeable by unsettling the cell structures (Chouhan *et al.*, 2017). In general, more phenolic compounds in the EO lead to higher antimicrobial properties. Thymol is a well-known component of many EOs. A greater quantity of this monoterpene is found in *Lamiaceae* plants, including *Thymus* spp., *Monarda* spp., and *Origanum* spp. (Salehi *et al.*, 2017). Thymol and carvacrol are similar in terms of construction, but they differ in terms of the position of the hydroxyl group of the phenolic ring. Eucalyptol, also known as 1.8-cineole, is a monoterpene found in the EOs of some herbs such as eucalyptus, daphne, cardamom, rosemary, and sage (Brown *et al.*, 2017). This compound has antioxidant, antimicrobial and anti-inflammatory properties and its antimicrobial effects on gram negative and gram positive bacteria have been shown (Hassanzadeh, 2005; Ji *et al.*, 2005).

Fire blight disease severity ranges from widespread damage on a highly susceptible host to limited symptoms, or the lack of symptoms on a resistant host (Viljevac *et al.*, 2009). The symptom severity is a result of different plant resistance levels that could be associated with the histochemical and/or biochemical variations such as accumulation of reactive oxygen species (ROS) and activity of antioxidative enzymes (Viljevac *et al.*, 2009). It is believed that ROS accumulation is one of the first defense responses to the bacterial infection (Mittler, 2002; Viljevac *et al.*, 2009). Several kinds of ROS such as superoxide anion, hydrogen peroxide, and hydroxyl radical have direct antimicrobial activities, which lead to decreased pathogen viability. However, the improved ROS production could also result in the oxidative damage of pigments, proteins, nucleic acids and lipids (Mandal *et al.*, 2008; Viljevac *et al.*, 2009).

Host plants have developed effective protective mechanisms against oxidative stress using enzymatic and non-enzymatic antioxidants for balancing ROS levels. The main enzymatic antioxidants involved in response to pathogen attack are peroxidases (ascorbate peroxidase and guaiacol peroxidase (GPOD)), superoxide dismutase and catalase. Considering the potential antimicrobial effects of the above-mentioned compounds, the present study seeks to examine the inhibitory effects of these

compounds against *E. amylovora*. In this study, antibacterial effects of two recombinant antimicrobial peptides (Lfc+Lfp and thanatin) and two monoterpenes (thymol and 1,8-cineole) against *E. amylovora* were investigated. Many studies have explored diverse aspects of these compounds on human microorganisms. But scant attention has been paid to the effects of these compounds on plant pathogens. In addition, the level of GPOD enzyme changes in two pear cultivars, tolerant ‘Dargazi’ and semi-susceptible ‘Spadona’, under treatment with four antibacterial compounds was assayed.

Material and methods

Bacterial strain and culture conditions

The highly virulent *E. amylovora* isolate used in this study was obtained from the microbial collection of the Dept. of Plant Prot., Ferdowsi Univ. Mashhad, Iran. This strain was isolated from a garden of pear trees in Mashhad, Khorasan-Razavi province, Iran (Akhlaghi *et al.*, 2018). When it was needed, fresh bacterial culture was prepared in Luria-Bertani (LB) broth medium (Liofilchem, Teramo, Italy, 25 g/L) overnight at 28 °C. The concentration of bacterial cells was adjusted to an optical density (OD) of 0.2 (108 CFU/mL) at 600 nm on LB broth (Bellemann *et al.*, 1994).

Antibacterial compounds

The recombinant chimeric peptide quaternary Lfc+Lfp was constructed by camel Lfc and Lfp (Tanhaeian *et al.*, 2018). Lfc+Lfp and thanatin peptides were obtained from Dept. of Biotechnol. Plant Breed., Fac. Agr., Ferdowsi Univ. of Mashhad, Iran. The Lfc+Lfp peptide production platform was the human embryonic kidney 293 cells. Cells were transformed with a vector (pcDNA™3.1(+)) containing a secret signal and a recombinant chimer coding sequence. The thanatin peptide was transformed from a *Pichia pastoris* yeast with a pPICZα vector containing the sequence of thanatin (Tanhaeian, 2018). Thymol crystal (5-methyl-2-(propan-2-yl) phenol) was purchased from Merck (Darmstadt, Germany) company (≥99.5 % purity). Also, 1,8-cineole (1,3,3-trimethyl-2-oxabicyclo [2.2.2] octane) was purchased from Sigma-Aldrich (Brussels, Belgium). Each compound was sterilized using millipore filter (0.22 μm) (Mirzaei-Najafgholi *et al.*, 2017).

Disk diffusion assay

Fresh culture of the bacterium in LB broth medium with a concentration of 10⁸ CFU/mL was distributed on

the surface of nutrient agar (NA) (Quelab, Montreal, Canada, 22 g/L) medium. After 15 min, 10 μL of each compound in final concentration of 250, 500, 1000, 1500 μg/mL were loaded over sterile blank paper discs (6 mm). Subsequently, plates were incubated at 28 °C for 48 h and the diameters of bacterial growth inhibition zones around the discs were measured. The experiments were performed in triplicate (Sokovic & Van Griensven, 2006).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC and MBC for each compounds were determined in 96-well plates containing NA medium as described previously (Mirzaei-Najafgholi *et al.*, 2017). These assays were carried out in triplicate in three independent experiments.

In vitro flower inoculation assay

Pear flowers (*Pyrus communis* cv. ‘Spadona’) at balloon stage (a day before opening) were transferred into a sterile 10% sucrose solution. Fresh culture at the concentration of 10⁷ to 10⁸ bacteria was made overnight in a sterile 0.05 M Na/KPi buffer containing 0.03% Tween 20 (pH=7). Four microliters of bacterial suspension was placed on the cup (hypanthium) of each flower. The bacteria were sprayed after 4 h of treatment with the antibacterial compounds at the MIC concentration. Na/KPi buffer treatment (without bacteria) in hypanthium and water spray (negative control) and Na/KPi buffer treatment (with bacteria) and water spray (positive control) were used. The flower was incubated at 24 to 26 °C with a humidity of 80%. After 4 days, disease symptoms were investigated on the flowers (Fischer *et al.*, 2012). Four types of marks were used for grading: A, flowers were healthy and without color change; B, color change in hypanthium and darkening of the tissue; C, tissue and color degradation in the hypanthium, style and filament; and D, blackened whole flower, ooze production, spreading symptoms to pedicle (Lelliott & Stead, 1987; Gerami *et al.*, 2013).

Experimental design and optimization for mixing the compounds

In this study, response surface methodology (RSM) was used to optimize mixing of the antimicrobial compounds with the copper compound (Nordox cuprous

oxide (Cu₂O)). The RSM is an advantageous method to investigate the impact of several variables on the responses by changing them concurrently and performing a restricted number of tests. No studies employing the experimental design and optimization modeling approach for mixing the AMPs, monoterpene and copper compounds have been reported in the literature, so far. The central composite design (CCD) is an effective design for sequential experimentation letting a sensible amount of information to test the lack of fit while excluding a remarkably large number of design points. The CCD method was used to determine the mixing ratio of different antibacterial compounds. Therefore, 43 mixing states of the four antibacterial compounds were obtained with Nordox. Subsequently, concentrations (1/2 MIC, MIC, 3/2 MIC) of Lfc+Lfp 16, 32 and 47 µg/mL, thanatin and thymol 8, 16, and 24 µg/mL, 1,8-cineole 125, 250 and 375 µg/mL, Nordox 250, 500 and 750 µg/mL were prepared. The mixing method of the tested materials was used in the LB broth medium in the microplate. For this purpose, the concentrations were prepared and mixed in equal volumes (30 µL) in the LB broth and 10 µL of bacteria were added to each well with the concentration of 10⁸ CFU/mL. After 24 h, 100 µL of bacterial cultures (concentration of 10⁻³ CFU/mL) was distributed on the surface of NA medium. The number of colonies grown after incubation (24 h) was counted (Demirel & Kayan, 2012).

Disease severity on tolerant and semi-susceptible pear cultivars treated with compounds

Two-year-old pear (*Pyrus communis*) trees cv. 'Spadona' and 'Dargazi' were used for greenhouse experiments. These cultivars were obtained from Horticultural Science Research Institute (HSRI), Karaj, Iran. All tests were done on pears in the active growth phase of terminal shoots. The trees were kept to grow in separate pots in a greenhouse at 22 to 25 °C under humidity and natural photoperiod. Pear seedlings used in this assay included plants sprayed with distilled water and inoculated with *E. amylovora* by placing 50 µL of the bacterial suspension on the shoots using a syringe (10⁸ CFU/mL) (zero time). Plants were sprayed with antibacterial compounds (24 h before inoculation) in the 125, 250 and 500 µg/mL concentration and inoculated with the pathogen. Disease severity (DS) was monitored 16 days after inoculation and the symptoms were recorded. Total shoot and lesion lengths were measured for each shoot. Shoot susceptibility to fire blight was computed as follows: $DS = (\text{Length of blighted shoot} / \text{Total shoot length}) \times 100$. The experiment was carried out with three replications (Bell *et al.*, 2004; Ozrenk *et al.*, 2011).

Activity of guaiacol peroxidase in two pear cultivars

The experiment was performed as described by Sklodowska *et al.* (2011) with some modifications. Two-year-old pear (cvs. 'Spadona' and 'Dargazi') seedlings were obtained from HSRI, Karaj, Iran. The growth conditions of seedlings and inoculations were similar to those of the previous stage. Pear seedlings used in this assay include: control plants sprayed with distilled water, plants sprayed with distilled water and shoots were punctured by a needle connected to a syringe containing the bacterial suspension (10⁸ CFU/mL), at the axil of the third leaf below the shoot tip (zero time). Plants sprayed with antibacterial compounds (24 h before inoculation) in the 500 µg/mL concentration and inoculated with *E. amylovora*. Guaiacol peroxidase activity was measured at 0, 1, 3, 5 and 7 days after inoculation.

Collection and preparation of leaf samples as well as determination of GPOD activity and protein content were done as described earlier (Kar & Mishra, 1976; Viljevac *et al.*, 2009). To extract the enzyme, three replicates were obtained from the control and infected leaves. Each sample included one leaf. After removing the main veins and adding polyvinyl pyrrolidone, leaf tissue was macerated into fine powder with liquid nitrogen using the pestle and mortar. Leaf powder (500 mg) was extracted with 3 mL of 100 mM potassium phosphate buffer, pH=6.8. After centrifugation for 15 min at 15000 g and 4 °C, the supernatant was taken for the GPOD assays.

Determination of the GPOD (EC 1.11.1.7) activity was done as described by Plewa *et al.* (1991) and Zhang *et al.* (2009). The GPOD activity was calculated by observing the increased absorbance at 470 nm over 2 min. The GPOD activity was measured using the guaiacol precursor and measuring tetraguaiacol absorption rate. In this method, the complex reaction consisted of 1.4 mL of 50 mM phosphate buffer (pH=7), 800 µL of guaiacol 2%, 0.5 mL of 1% hydrogen peroxide and 200 µL of the extracted solution.

Statistical analysis

The ANOVA test was conducted on a factorial experiment based on a completely randomized design with three replications. After ensuring the normal distribution of the data, ANOVA was performed using SPSS (V22.0). The means were compared using the Duncan's multiple rang test at 5% probability level. The charts were drawn by Excel 2016. To correlate the disease severity and GPOD activity levels and avoid any possible errors, the ratios were calculated between values obtained for control and treated plants. The Pearson correlation coefficient between the data obtained from the GPOD activity assay and disease

severity was calculated using SPSS (V22.0) software during three days.

Results

Screening antibacterial activity of various compounds against *E. amylovora*

The results of analyzing the antibacterial effects of AMPs and monoterpene compounds suggest their potential antibacterial effect against *E. amylovora* (Table 1). Regarding the inhibition zone diameter, thanatin peptide had the highest antibacterial activity at 1500 µg/mL against *E. amylovora* with 16.17 mm inhibition zone. The Lfc peptide and thymol with 12.67 and 12.33 mm diameter inhibition zones were placed in subsequent positions. The lowest inhibition was observed for the 1,8-cineole compound, where the mean diameter of inhibition hole was 8.67 mm (similar to the Nordox). AMPs had less antibacterial effect than thymol at concentrations of 250 and 500 µg/mL. Improved antibacterial properties of compounds were directly related to the increased

concentrations. According to the results of antibacterial activity screening, the AMPs had stronger antimicrobial effect than that of monoterpene compounds.

Determination of MIC and MBC

Microplate dilution is an accurate and sensitive method for determining the antimicrobial effects of various compounds. The MIC results revealed that thanatin and thymol at concentration of 15.62 µg/mL restricted the growth of *E. amylovora* and the MBC was 31.5 µg/mL. The highest levels of MIC were obtained for Nordox and 1,8-cineole at 250 µg/mL and 500 µg/mL, respectively. The highest amount of MBC was obtained at 1000 µg/mL for the Nordox as a copper compound (Table 2).

In vitro flower inoculation assay

Considering the effect of antimicrobial compounds on pear flowers, all compounds were capable of slowing down the disease progress compared to the control (Type

Table 1. Antimicrobial activity of antimicrobial peptides and monoterpene against *Erwinia amylovora* by paper disk diffusion method

No.	Compounds	Inhibition zone (mm) ⁽¹⁾			
		250 µg/mL	500 µg/mL	1000 µg/mL	1500 µg/mL
1	Lfc+Lfp	7.67±0.57b	8.33±0.57c	10.17±0.76b	12.67±0.57b
2	Thanatin	7.17±0.29c	9.50±0.50a	11.17±0.76a	16.17±0.76a
3	Thymol	9.00±0.00a	9.33±0.57ab	11.00±1.00ab	12.33±0.57b
4	1,8-Cineole	0.00±0.00d	0.00±0.00d	7.33±0.57c	8.67±0.57c
5	Nordox	0.00±0.00d	0.00±0.00d	7.00±0.00d	8.67±0.57c

Lfc: lactoferricin. Lfp: lactoferrampin. ⁽¹⁾Diameter of the zone of inhibition includes disk diameter (6 mm). The presented values are the means ± standard deviation. The same letter indicates no statistical difference at $p < 0.05$

Table 2. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of antimicrobial peptides and monoterpene compounds against *Erwinia amylovora*

No.	Compounds	<i>E. amylovora</i>	
		MIC (µg/mL)	MBC (µg/mL)
1	Lfc	31.50	62.50
2	Thanatin	15.62	31.50
3	Thymol	15.62	31.50
4	1,8-Cineole	250.00	500.00
5	Nordox	500.00	1000.00

D) as shown in Fig. 1. The lowest disease symptoms were observed on the flowers treated with both of thanatin (type B) and thymol (type B). The highest level of disease symptom was observed on flowers treated with the 1,8-cineole (Type C). The results of this test suggested that, unlike AMPs, the two monoterpene compounds used in this study had phytotoxicity on flower petals (Table 3).

Optimization analysis for mixing the antibacterial compounds

The results of mixing the antibacterial compounds were compared using a completely randomized design with the CCD method (Table 4). The results indicate that all of the mixed ratios could reduce bacterial growth compared to the control (medium contain bacteria: 237.3 colony). The comparison results showed that the mixing ratios of 32, 16, 24, 250 and 250 $\mu\text{g/mL}$ for Lfc+Lfp, thanatin, thymol, 1,8-cineole and Nordox (third row; 41 colonies) and mixing ratios of 32, 16, 16, 375 and 250 $\mu\text{g/mL}$ for Lfc+Lfp, thanatin, thymol, 1,8-cineole and Nordox (fifteenth row; 43 colonies) led to the maximum

reduction of bacterial colony number in NA (Table 4). Other results also showed that the mixing ratio of 32, 8, 16, 250 and 750 $\mu\text{g/mL}$, (seventeenth row, 104 colonies) had the least effect on Lfc+Lfp, thanatin, thymol, 1,8-cineole and Nordox respectively. The antibacterial effect of AMPs and copper compound during mixing of all treatments was more than monoterpenes (Table 4).

Effect of antimicrobial compounds on protecting the shoots of pear cultivars against *E. amylovora*

The results of disease severity assay revealed that the percentage of damage caused by *E. amylovora* on cv. 'Dargazi' (tolerant) (21.87%) was lower than in 'Spadona' (semi-susceptible) (58.85%). Application of all antibacterial compounds tested on 'Spadona' and 'Dargazi' reduced the DS compared to the Nordox treatment and control (Fig. 2). The results indicate that the Nordox was not significantly different in reducing the DS in comparison with the control (water spray) in the three concentrations tested. The results of DS and concentration showed that in three concentrations (125, 250 and 500 $\mu\text{g/mL}$)

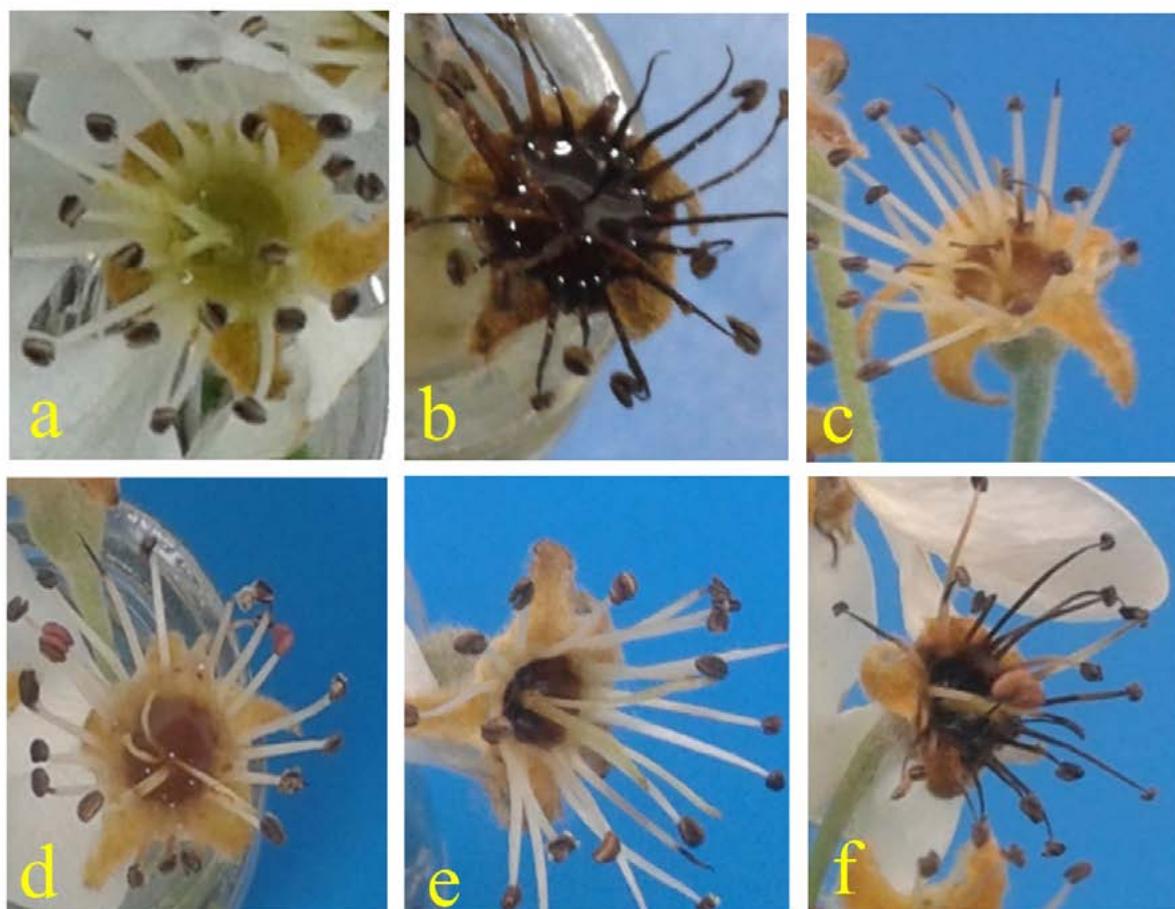


Figure 1. Effect of treating pear flowers with the antimicrobial peptides and monoterpene at MIC concentration against *Erwinia amylovora*. Flowers treated with: a) distilled water (negative control); b) bacterial suspension (positive control); c) thanatin; d) thymol; e) Lfc+Lfp; and f) 1,8-cineole.

Table 3. *In vitro* assays on the phytotoxicity of different antimicrobial peptides and monoterpenes on the external parts of the bloom

No.	Treatment	Blossom phytotoxicity	
		on petals	on sepal and pedicle
1	Lfc+Lfp	-	-
2	Thanatin	-	-
3	Thymol	+	-
4	1,8-Cineole	+	-
5	Nordox	W	-

+: phytotoxic, -: no reaction, W: weak reaction

Table 4. Using central composite design to optimize the mixing ratio and the results of mixing antimicrobial peptides (lactoferricin, thanatin) and monoterpene treatments (thymol, 1,8-cineole) at different concentrations (1/2 MIC, MIC, 3/2 MIC) with Nordox

No.	Lfc	Tha.	Thy.	Cin.	Nor.	No. of colonies	No.	Lfc	Tha.	Thy.	Cin.	Nor.	No. of colonies
1	32	8	16	125	500	70.70 ^{i-r}	24	32	24	16	250	750	68.00 ^{m-s}
2	32	24	24	250	500	66.30 ^{o-s}	25	16	16	16	250	250	68.00 ^{m-s}
3	32	16	24	250	250	41.00 ^v	26	47	16	16	375	500	80.00 ^{h-j}
4	32	8	8	250	500	71.00 ^{i-p}	27	47	16	16	250	250	71.30 ^{l-p}
5	47	16	24	250	500	74.00 ^{j-n}	28	32	8	16	250	250	74.30 ^{j-m}
6	16	8	16	250	500	94.70 ^c	29	32	24	16	375	500	64.00 ^{q-s}
7	47	16	16	125	500	72.70 ^{k-o}	30	32	16	16	125	250	78.30 ^{i-k}
8	16	16	8	250	500	88.70 ^{de}	31	32	8	24	250	500	81.70 ^{f-i}
9	16	16	16	250	750	94.00 ^{cd}	32	32	16	8	375	500	63.70 ^s
10	47	8	16	250	500	93.70 ^{cd}	33	16	24	16	250	500	87.30 ^{ef}
11	32	16	8	250	750	64.30 ^{rs}	34	32	16	24	250	750	66.00 ^{p-s}
12	32	24	16	125	500	70.30 ^{l-r}	35	32	16	24	375	500	68.70 ^{l-s}
13	32	16	16	250	500	70.70 ^{l-r}	36	16	16	24	250	500	83.30 ^{e-i}
14	32	16	8	125	500	73.30 ^{k-n}	37	32	16	16	250	500	69.30 ^{l-s}
15	32	16	16	375	250	43.00 ^v	38	32	8	16	375	500	72.00 ^{k-p}
16	32	16	16	250	500	67.70 ^{n-s}	39	32	16	24	125	500	86.00 ^{e-h}
17	32	8	16	250	750	104.00 ^b	40	47	16	8	250	500	81.00 ^{g-i}
18	32	24	16	250	250	73.30 ^{k-n}	41	32	16	16	125	750	74.70 ^{j-l}
19	16	16	16	125	500	87.00 ^{e-g}	42	32	16	8	250	250	49.00 ^u
20	32	24	8	250	500	67.70 ^{n-s}	43	47	24	16	250	500	75.00 ^{i-k}
21	32	16	16	375	750	57.30 ^t	44	Culture medium (bacteria)					237.30 ^a
22	47	16	16	250	750	73.70 ^{k-n}	45	Culture medium (alone)					0.00 ^w
23	16	16	16	375	500	81.70 ^{f-i}							

The same letter indicates no statistical difference at $p < 0.01$

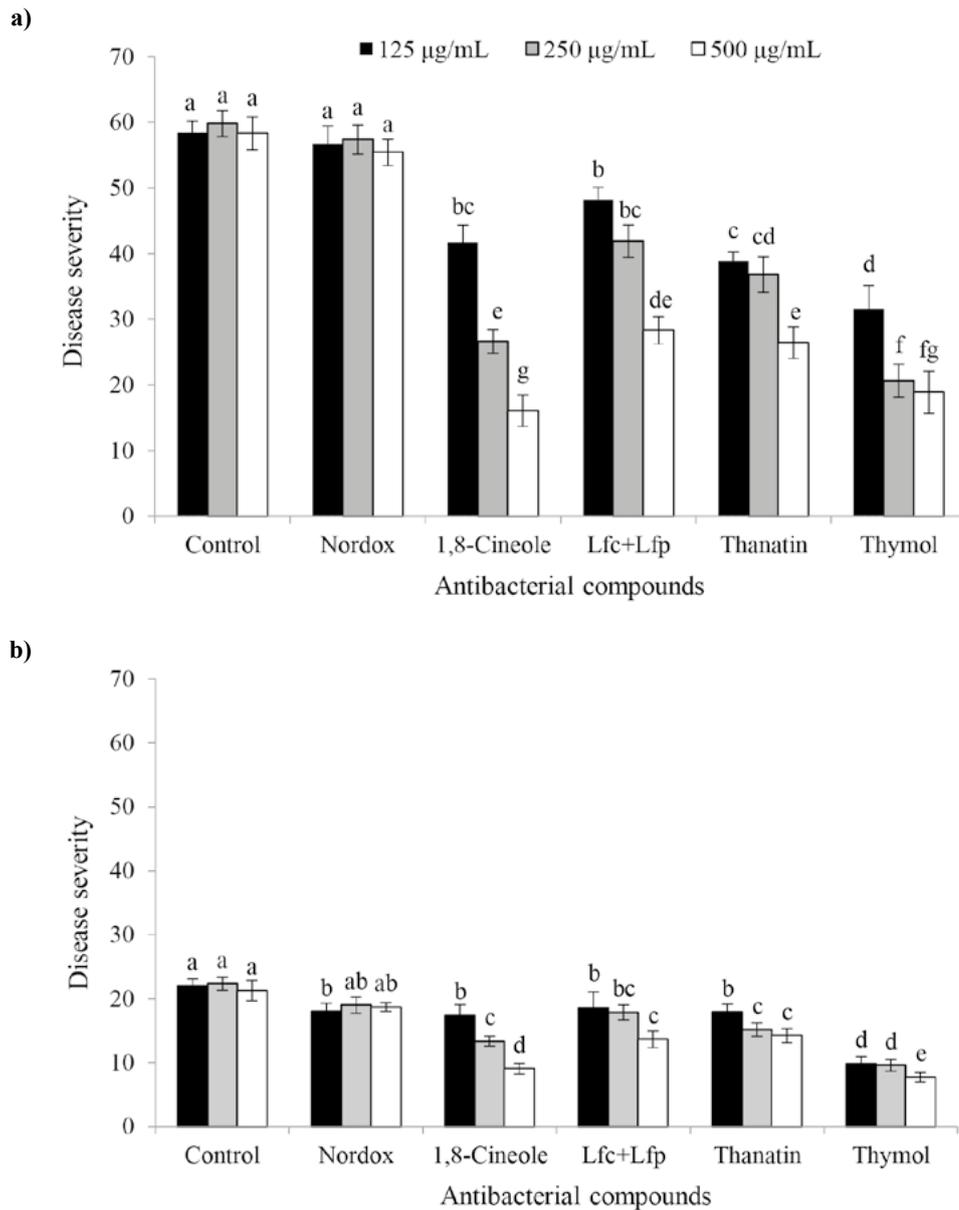


Figure 2. Effect of antimicrobial peptides and monoterpene treatments on disease severity (DS) at three concentrations (125, 250 and 500 µg/mL) on: a) 'Spadona' and b) 'Dargazi' cultivars

used in this assay, the maximum DS reduction was observed at concentrations of 500 µg/mL. The maximum reduction of DS on 'Spadona' was obtained using the 1,8-cineole (16.05%) and thymol (18.88%) at concentrations of 500 µg/mL (Fig. 2). 1,8-cineole and thymol had a greater role in reducing the percentage of necrosis on the shoot and there was no statistically significant difference in the in-vivo effect of two tested AMPs on this cultivar at 500 µg/mL. The maximum reduction of DS on cv. 'Dargazi' was obtained using the thymol (7.71%) and 1,8-cineole (9.05%) at concentrations of 500 µg/mL (Fig. 2). There was no statistically significant difference with the application of 250 µg/mL concentrations of each treatment on 'Dargazi' except thymol. However, the differences between the effect of Lfc+Lfp, thanatin and 1,8-cineole on

'Dargazi' were not significant compared to Nordox treatment. The least effect on reducing the DS was associated with the application of the lowest concentrations (125 µg/mL) of each compound, which was more pronounced for AMPs, although they did not significantly differ. Other results revealed the fact that there is a direct correlation between concentration of antibacterial compounds and reduction in the percentage of shoot necrosis.

Guaiacol peroxidase activity in two pear cultivars

The results of GPOD activity assay showed that the primary level of enzyme in 'Dargazi' (tolerant) was higher than the enzyme level in 'Spadona' (semi-susceptible).

Moreover, the GPOD activity pattern was not similar in the two cultivars (Fig. 3). Generally, in 'Dargazi' the enzyme level showed an increasing trend up to 3 days after inoculation (DAI), and then decreased. In 'Spadona' an increasing trend was observed for the GPOD activity at all five time points (Fig. 3). In 'Dargazi', the control treatment (water spray) showed a constant trend, while the treatment with bacteria tested showed a rising trend (up to 3 DAI, the enzyme level showed an increasing trend, followed by a decreasing one). The GPOD activity increased until 3 DAI, then showed a decreasing trend until the 5 DAI, and the enzyme level increased again until the 7th day (Fig. 3). In 'Dargazi' all antibacterial compounds increased the level of GPOD over a period of three days. The highest level of the enzyme was obtained by treatment with 1,8-cineole (Fig. 3d) and thymol (Fig. 3c). The rate of GPOD activity under Lfc+Lfp (Fig. 3a) and thanatin (Fig. 3b) treatments was lower than that of the plants treated with 1,8-cineole and thymol. However, the enzyme level in the plants treated with thanatin was close to the bacterial treatment at 5 DAI, but in case of Lfc+Lfp, a lower decrease in the level of enzyme was observed at 5 DAI (Fig. 3). In 'Spadona' the control treatment (water spray) had a constant trend, while treatment with bacteria showed an increasing trend until 7 DAI. In this cultivar, all antibacterial compounds increased the level of GPOD for three to seven days. The highest level of enzyme activity was observed in the plants treated with 1,8-cineole (Fig. 3d) and thymol (Fig. 3c) over a period of 5 to 7 days. Like in 'Dargazi', the activity of GPOD enzyme in plants treated with Lfc and thanatin was at the lowest level during the time period investigated compared to 1,8-cineole and thymol and did not show considerable differences with Nordox and control (Fig. 3).

Discussion

Fire blight causes serious damage to pome fruit trees in Iran and other countries, so it is necessary to combat *E. amylovora* bacteria to increase yield. In addition to antibiotics, copper compounds and other biological controlling products have also been applied to control fire blight; nevertheless, applying these materials is restricted due to their unpredictable control effects and application of copper compounds can lead to phytotoxicity (Patel *et al.*, 2017). On the other hand, it is difficult to assess the effect of copper compounds on fire blight and the ineffectiveness of other chemical compounds in controlling the disease due to the resistance or tolerance of microorganisms (Al-Daoude *et al.*, 2009; Ivanović *et al.*, 2016).

Every year, approximately 2.5 million tons of pesticides are used on crops worldwide to fight plant disease (Koul *et al.*, 2008; Ben Kaab *et al.*, 2019). Natural

products derived from plants and AMPs have no harmful effects of chemical compounds on human health and the environment. In addition to medical applications, the AMPs can also be used in plant protection. The reduced yield, quality, and safety of agricultural products caused by plant diseases can be overcome using AMPs (Holaskova *et al.*, 2015). Large amounts of high purity peptides are required for basic research and clinical trials. In comparison with the process of separation from natural sources and chemical synthesis, the recombinant expression method suggests an effective and economic means to produce peptide in high yield. In this regard, using recombinant AMPs (naturally occurring in camel milk and insect), we showed that they have unique antibacterial effect on *E. amylovora*.

To date, several studies have been performed on thanatin. For instance, the transgenic rice (*Oryza sativa* L.) encoded thanatin peptide was resistant to blast disease (*Magnaporthe grisea* M.E. Barr) in field evaluations (Imamura *et al.*, 2010). Other study reported that the transgenic *Arabidopsis thaliana* encoded thanatin was resistant to the phytopathogens (Wu *et al.*, 2013). Great antifungal activity of the AMP was observed at μg level under both *in vitro* and *in vivo* conditions against five fungal phytopathogens (Mamarabadi *et al.*, 2018). Although no comprehensive research has been conducted on the anti *E. amylovora* role of thanatin, this study showed that the peptide possesses high antimicrobial activity similar to that of previous studies. The antimicrobial effect of Lfc peptide on plant pathogenic bacteria has been evaluated in Lfc of cow milk against *Xanthomonas campestris* pv. *phaseoli* (Smith) Constantin *et al.*, *Ralstonia solanacearum* (Smith) Yabuuchi *et al.*, and *Pseudomonas syringae* pv. *phaseolicola* (van Hall) (Fukuta *et al.*, 2012). Tahaian *et al.* (2018) studied the effect of lactoferrin+lactopharmin on 14 plant pathogenic bacteria, including *E. amylovora*, and found that the MIC for the bacterium is 1.31 $\mu\text{g}/\text{mL}$. Compared to previous studies, the results of this study showed that the MIC for the *E. amylovora* was higher (62.5 $\mu\text{g}/\text{mL}$), which is probably due to the type of *E. amylovora* isolate, which was highly virulent. Other researchers reported that the antibacterial activity of a recombinant chimeric peptide is significantly stronger than its non-recombinant peptide (Bolscher *et al.*, 2009; Tang *et al.*, 2012). The results of this study revealed that, contrary to previous studies, thanatin (a non-recombinant chimeric peptide) has more antibacterial properties than the Lfc peptide (a recombinant chimeric peptide). It seems that the role of the N-terminal tail of recombinant peptides in antibacterial activities is more important than the fusion of two peptides. Considering that peptide is edible and has high durability (temperature resistance of 90 °C), the use of this peptide to control bacterial pathogens can be considered as a new strategy in the biological control.

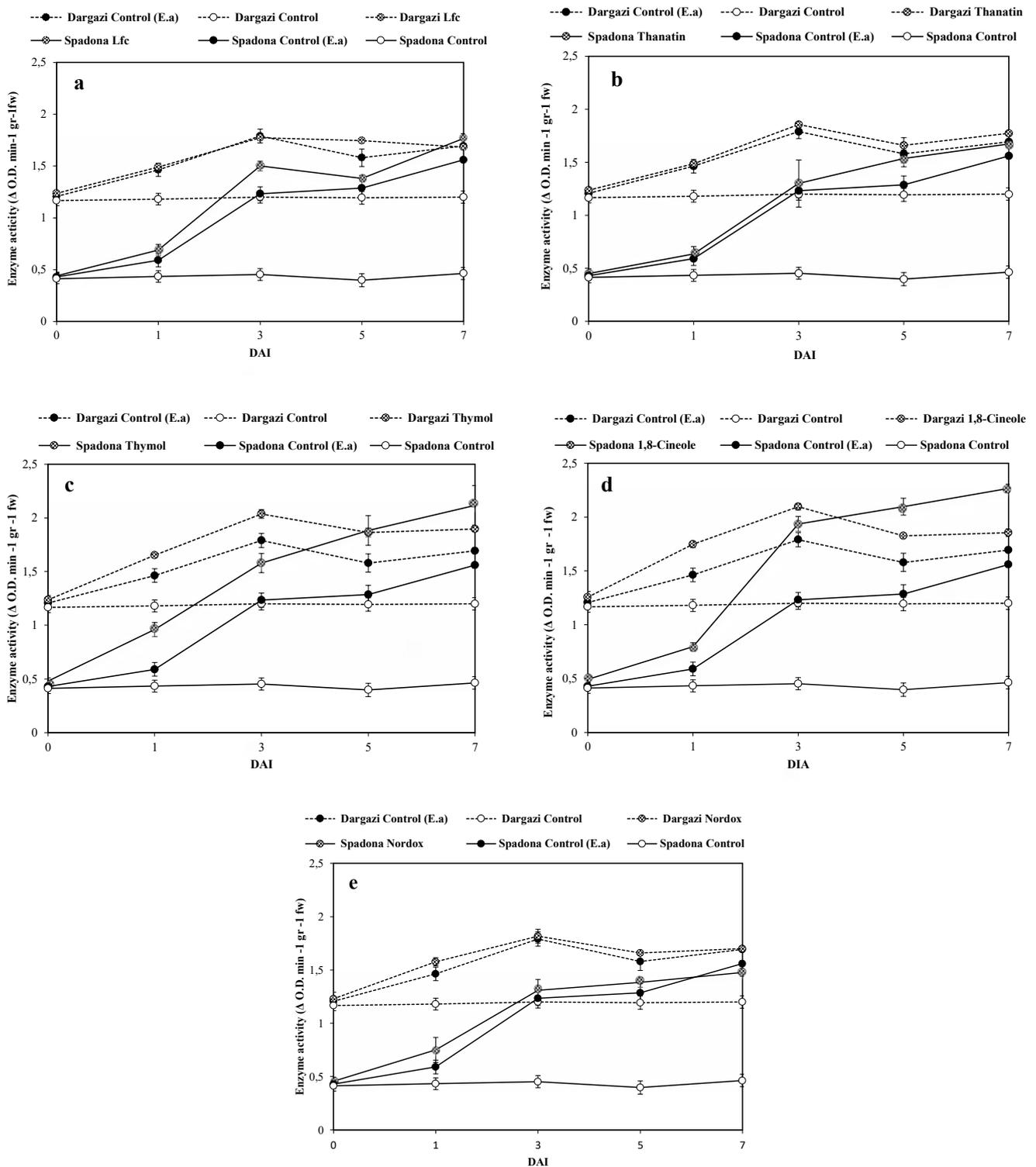


Figure 3. The results of the GPOD enzyme modification in 'Dargazi' and 'Spadona' cultivars treated with: Lfc+Lfp (a), thanatin (b), thymol (c), 1,8-cineole (d), and Nordox (e)

As an alternative approach for preventing the spread of plant diseases, it is possible to use natural compounds of plants as a source of new pesticides (Yumlembam & Borkar, 2014). Thymol is generally used as a general disinfectant or additive in cosmetics, food industry, or medicine.

In agriculture, the effect of thymol against microorganisms and insects has been depicted (Calvet *et al.*, 2001; Ji *et al.*, 2005). However, there are few studies about the use of thymol in greenhouses and farms. Applying thymol as a fumigant in laboratory and greenhouse conditions leads

to a significant reduction in the population of *Ralstonia solanacearum* bacteria in the soil (Ji *et al.*, 2005). The analysis of thymol effect in this study showed its antibacterial effect on *E. amylovora*, which is consistent with the results of previous studies in the control of gram-negative and gram-positive bacteria (Hassanzadeh, 2005; Ji *et al.*, 2005). The study showed that thymol has potential to control *E. amylovora*. However, further research is required to investigate the possibility of direct application of thymol on flowers due to the sensitivity of tissue, the possibility of damage to pollination process and formation of fruits.

The 1,8-cineole is used as a component of some aromatic plants such as coriander, oregano, rosemary, thyme and ginger (Van Vuuren & Viljoen, 2007). The results of this study, consistent with other studies, revealed the antibacterial effects of 1,8-cineole against *E. amylovora*. The antibacterial effect of 1,8-cineole on flowers and fruits was lower than thymol, which is related to the structural differences between the two compounds. Identical to thymol, 1,8-cineole also had improved effects on reducing the disease on the fruits compared to the blossom, and showed a phytotoxicity rate lower than that of thymol. Although reduction in disease symptoms on pear blossoms treated with 1,8-cineole was not clear (because of the phytotoxicity of this compound on the blossoms), but 1,8-cineole inhibited the disease progress of *E. amylovora* on pear branches better than other tested compounds when used at 500 µg/mL. In natural conditions, initial infection of pear trees occurred by epiphytic populations of *E. amylovora* that become established on the stigmas of flowers. The pathogen spreads from stigmas to the hypanthium in surface moisture and invades through the nectarthodes (Thomson, 1986). Therefore, application of copper compounds or antibiotics either before or immediately after the pathogen reaches to the pear flowers or shoots would be very useful because of direct contact of applied materials with the pathogen. Once *E. amylovora* enters the host xylem or cortical parenchyma and spreads in the endophytic phase of pathogenesis (Koczan *et al.*, 2009), external control methods become ineffective. Plant resistance inducers and biological control agents are potential alternatives to antibiotics and copper (Johnson & Temple, 2013). The two tested AMPs, Lfc+Lfp and thalatin, reduced disease symptoms caused by *E. amylovora* on pear flowers and did not show phytotoxicity, while the two monoterpenes used in this study, specially 1,8-cineole, showed high phytotoxicity. However, 1,8-cineole and thymol reduced disease symptoms on branches. Therefore, we suppose these compounds could be new alternatives for management of fire blight disease in epiphytic or endophytic growth phase of *E. amylovora*.

Another part of this study focused on applying the RSM and finding an applicable approximating function for the ratio of mixing antibacterial compounds (antimi-

crobial peptides and monoterpene) with a copper compound (Nordox) for finding the most effective antibacterial MIC (1/2 MIC, MIC, 3/2 MIC) concentration. RSM is a type of mathematical and statistical method to design experiments, create models, assess the relative significance of several independent variables, and determine the optimum conditions for necessary responses (Zhang & Zheng, 2009; Demirel & Kayan, 2012). The two most common designs widely applied in RSM are the CCD and the Box-Behnken design. The CCD is perfect for sequential experimentation paving the way for a sensible amount of information to test lack of fit while excluding remarkably considerable number of design points (Somayajula *et al.*, 2011; Demirel & Kayan, 2012). In this study, the mixing conditions of tested compounds were determined using the CCD method. Using the mentioned statistical method was better than other methods due to reduced number of experiments and the simultaneous analysis of the parameters. More studies have been done on the mixing of antimicrobial compounds and toxins, antibiotics, essential oils and plant extracts against various pathogens (Langeveld *et al.*, 2014). In another study, the synergistic effects of piperacillin antibiotic with cinnamon essential oil and chloramphenicol antibiotic with oil of savory were assayed against *E. coli* (Bassolé & Juliani, 2012). However, the effects of mixing between new antimicrobials agents and copper compounds were not assayed and discussion on comparing the antibiotic, essential oils and plant extracts with poison was important. Various biotic and abiotic stresses activate a series of reactions leading to resistance in plants. Applying protective agents and chemical activators of plant defense system for decreasing the disease damage are novel methods favored by researchers in recent years. Plants are capable of increasing their resistance against plant pathogens agent. This phenomenon is known as induced resistance activated by some microorganisms, natural or artificial chemicals, or by lesions. The GPOD activity assay in this study showed that in tolerant and semi-susceptible cultivars, the GPOD activity was significantly different. Ebadi *et al.* (2014) found that in resistant cultivars such as 'Dargazi' and 'Harrow Sweet', the GPOD level reached to its maximum level at 3 DAI. But in the susceptible cultivars 'Bartlett' and 'Mohamdali', the GPOD activity peaked later at 6 DAI, followed by a decreasing trend until 12 DAI, which is in agreement with the results of the present study. It seems that antioxidant activity (such as peroxidase and catalase) in early days after inoculation of bacteria in resistant pear cultivars causes a hypersensitive response. In resistant cultivars, the hypersensitive response (which is a mechanism, used by plants, to prevent the spread of infection) is a local, defensive, and quick reaction leading to the cell death in the infected area inhibiting the spread of bacteria in the host tissue. As the results of this study showed, the tested antibacterial compounds play a role in

the induction of resistance, which is more evident in monoterpene compounds. In susceptible or semi-susceptible cultivars, the free radicals are very high after the bacterial attack, but the defense system was unable to decompose them due to the lower level of antioxidant enzymes and the bacteria developed more rapidly inside the plant (Ebadi *et al.*, 2014). The use of antibacterial compounds, especially monoterpenes, in this study showed that like some natural and artificial chemical compounds, monoterpenes could play an important role in increasing the antioxidant enzymes, such as GPOD. So far, the mechanism of action of monoterpene compounds and AMPs in induction of resistance was not fully understood, but it seems that they activate some of the defense related mechanisms in plants.

The correlation coefficient is one of the most often used statistical tools for analyzing the associations among the traits. Pearson's correlation coefficient is a statistical method of quantifying the association, or "coherence", between two variables. Therefore, it is a very popular tool to analyze data many scientific disciplines like biology (Soares *et al.*, 2011). The results of this study revealed that the GPOD enzyme has a negative correlation with disease severity (-0.701, $p=0.0001$). This study is the first report on the use of antibacterial compounds, such as 1,8-cineole, thymol and two AMPs (thanatin and Lfc) on blooms, fruits and seedlings of pear. In this study, for the first time, statistical methods for mixing new antibacterial compounds with common copper compounds were used to control fire blight. It seems that the antibacterial compounds used in this study, in addition to their antibacterial role, can also play a role in activating defense responses in the pear tree. If these materials provide a higher level of protection against the fire blight pathogen before the induction of resistance using their antimicrobial properties, the effective control of the disease will occur. The results of this research could be helpful for applying novel alternative compounds in fire blight disease management.

References

- Akhlaghi M, Tarighi S, Taheri P, 2018. Evaluating antibacterial effect of plant extracts against *Erwinia amylovora* and their role in resistance induction in pear. *BCPPD* 7 (2): 31-47.
- Al-Daoude, A, Arabi M, Ammoun H, 2009. Studying *Erwinia amylovora* isolates from Syria for copper resistance and streptomycin sensitivity. *J Plant Pathol* 91: 203-205.
- Awais Khan M, Zhao Y, Korban SS, 2011. Molecular mechanisms of pathogenesis and resistance to the bacterial pathogen *Erwinia amylovora*, causal agent of fire blight disease in Rosaceae. *Plant Mol Biol Rep* 30: 247-260. <https://doi.org/10.1007/s11105-011-0334-1>
- Bassolé IHN, Juliani HR, 2012. Essential oils in combination and their antimicrobial properties. *Molecules* 17: 3989-4006. <https://doi.org/10.3390/molecules17043989>
- Bell AC, Ranney TG, Eaker TA, 2004. Resistance to fire blight among flowering pear and quince. *Hortscience* 40 (2): 413-415. <https://doi.org/10.21273/HORTSCI.40.2.413>
- Bellemann P, Bereswill S, Berger S, Geider K, 1994. Visualization of capsule formation by *Erwinia amylovora* and assays to determine amylovoran synthesis. *Int J Biol Macromol* 16: 290-296. [https://doi.org/10.1016/0141-8130\(94\)90058-2](https://doi.org/10.1016/0141-8130(94)90058-2)
- Ben Kaab S, Rebey IB, Hanafi M, Berhal C, Fauconier ML, de Clerck C, Ksouri R, Jijakli H, 2019. Rosmarinus officinalis essential oil as an effective antifungal and herbicidal agent. *Span J Agric Res* 17 (2): 1-9. <https://doi.org/10.5424/sjar/2019172-14043>
- Biswaro LS, Da Costa Sousa MG, Rezende TMB, Dias SC, Franco OL, 2018. Antimicrobial peptides and nanotechnology, recent advances and challenges. *Front Microbiol* 9: 855. <https://doi.org/10.3389/fmicb.2018.00855>
- Bolscher JG, Adão R, Nazmi K, van den Keybus PA, van't Hof W, Nieuw Amerongen AV, Bastos M, Veerman EC, 2009. Bactericidal activity of LF chimera is stronger and less sensitive to ionic strength than its constituent lactoferricin and lactoferrampin peptides. *Biochimie* 91 (1): 123-132. <https://doi.org/10.1016/j.biochi.2008.05.019>
- Brown SK, Garver, WS, Orlando RA, 2017. 1,8-cineole: An underappreciated anti-inflammatory therapeutic. *J Biomol Res Ther* 6 (154): 1-6. <https://doi.org/10.4172/2167-7956.1000154>
- Cabrefiga J, Montesinos E, 2017. Lysozyme enhances the bactericidal effect of BP100 peptide against *Erwinia amylovora*, the causal agent of fire blight of rosaceous plants. *BMC Microbiol* 17 (39): 1-10. <https://doi.org/10.1186/s12866-017-0957-y>
- Calvet C, Pinochet J, Camprubi A, Estaun V, Rodriguez-Kabana R, 2001. Evaluation of natural chemical compounds against root lesion and root-knot nematodes and side-effects on the infectivity of arbuscular mycorrhizal fungi. *Eur J Plant Pathol* 107: 601-605. <https://doi.org/10.1023/A:1017954315942>
- Chouhan S, Sharma K, Guleria S, 2017. Antimicrobial activity of some essential oils present status and future perspectives. *Medicines* 4 (58): 1-21. <https://doi.org/10.3390/medicines4030058>
- Demirel M, Kayan B, 2012. Application of response surface methodology and central composite design for the optimization of textile dye degradation by wet air oxidation. *Int J Ind Chem* 3 (24): 1-10. <https://doi.org/10.1186/2228-5547-3-24>
- Ebadi A, Erfani J, Abdollahi H, Fattahi Moghadam J, 2014. Investigation of changes in antioxidant

enzyme and total phenol level in some pear cultivars inoculated with fire blight disease. *Ir J Hort Sci* 45 (2): 127-136.

Emeriewen OF, Wöhner T, Flachowsky H, Peil A, 2019. *Malus* hosts-*Erwinia amylovora* interactions: strain pathogenicity and resistance mechanisms. *Front Plant Sci* 10 (551): 1-7. <https://doi.org/10.3389/fpls.2019.00551>

Fischer TC, Gosch C, Mirbeth B, Gselmann M, Thallmair V, Stich K, 2012. Potent and specific bactericidal effect of juglone (5-hydroxy-1, 4-naphthoquinone) on the fire blight pathogen *Erwinia amylovora*. *J Agr Food Chem* 60: 12074-12081. <https://doi.org/10.1021/jf303584r>

Fukuta S, Kawamoto KI, Mizukami Y, Yoshimura Y, Ueda JI, Kanbe M, 2012. Transgenic tobacco plants expressing antimicrobial peptide bovine lactoferricin show enhanced resistance to phytopathogens. *Plant Biotechnol* 29: 383-389. <https://doi.org/10.5511/plantbiotechnology.12.0619a>

Gerami E, Hassanzadeh N, Abdollahi H, Ghasemi A, Heydari A, 2013. Evaluation of some bacterial antagonists for biological control of fire blight disease. *J Plant Pathol* 95 (1): 127-134.

Gusberti M, Klemm U, Meier MS, Maurhofer M, Hunger-Glaser I, 2015. Fire blight control: The struggle goes on. A comparison of different fire blight control methods in Switzerland with respect to biosafety, efficacy and durability. *Int J Environ Res Public Health* 12: 11422-11447. <https://doi.org/10.3390/ijerph120911422>

Hassanzadeh N, 2005. The essential oil of thyme as a natural plant extract for fire blight control. *Proc 1 Int Conf on biological control of bacterial plant diseases, Seeheim/Darmstadt (Germany)*, Oct 23-26. pp: 290-292.

Holaskova E, Galuszka P, Frebort I, Oz MT, 2015. Antimicrobial peptide production and plant-based expression systems for medical and agricultural biotechnology. *Biotechnol Adv* 33: 1005-1023. <https://doi.org/10.1016/j.biotechadv.2015.03.007>

Iacobellis NS, Lo Cantore P, Capasso F, Senatore F, 2005. Antibacterial activity of *Cuminum cyminum* L. and *Carum carvi* L. essential oils. *J Agric Food Chem* 53: 57-61. <https://doi.org/10.1021/jf0487351>

Imamura T, Yasuda M, Kusano H, Nakashita H, Ohno Y, Kamakura T, Taguchi S, Shimada H, 2010. Acquired resistance to the rice blast in transgenic rice accumulating the antimicrobial peptide thanatin. *Transgenic Res* 19: 415-424. <https://doi.org/10.1007/s11248-009-9320-x>

Ivanović M, Gasić K, Prokić A, Kuzmanović N, Zlatković N, Obradović A, 2016. Screening for copper and antibiotic resistance in *Erwinia amylovora* population from Serbia. *Acta Hort* 1139: 715-720. <https://doi.org/10.17660/ActaHortic.2016.1139.122>

Ji P, Momol MT, Olson SM, Pradhanang PM, Jones JB, 2005. Evaluation of thymol as bio fumigant for control of bacterial wilt of tomato under field conditions. *Plant Dis* 89: 497-500. <https://doi.org/10.1094/PD-89-0497>

Johnson KB, Temple TN, 2013. Evaluation of strategies for fire blight control in organic pome fruit without antibiotics. *Plant Dis* 97: 402-409. <https://doi.org/10.1094/PDIS-07-12-0638-RE>

Kar M, Mishra D, 1976. Catalase, peroxidase and polyphenol oxidase activities during rice leaf senescence. *Plant Physiol* 57: 315-319. <https://doi.org/10.1104/pp.57.2.315>

Karami-Osboo R, Khodaverdi M, Ali-Akbari F, 2010. Antibacterial effect of effective compounds of *Satureja hortensis* and *Thymus vulgaris* essential oils against *Erwinia amylovora*. *J Agr Sci Tech* 12: 35-45.

Koczan JM, McGrath MJ, Zhao Y, Sundin GW, 2009. Contribution of *Erwinia amylovora* exopolysaccharides amylovoran and levan to biofilm formation: implications in pathogenicity. *Phytopathol* 99: 1237-1244. <https://doi.org/10.1094/PHTO-99-11-1237>

Koul O, Walia S, Dhaliwal GS, 2008. Essential oil as green pesticides: Potential and constraints. *Biopestic Int* 4: 63-84.

Langeveld WT, Veldhuizen EJA, Burt SA, 2014. Synergy between essential oil components and antibiotics: a review. *Crit Rev Microbiol* 40 (1): 76-94. <https://doi.org/10.3109/1040841X.2013.763219>

Lelliot RA, Stead DE, 1987. Methods for the diagnosis of bacterial diseases of plants. In: *Methods in plant pathology*; Preece TF (eds.). pp. 1-216. Blackwell Sci Publ, Oxford, UK.

Li Y, 2011. Recombinant production of antimicrobial peptides in *Escherichia coli*: A review. *Protein Expr Purif* 80 (2): 260-267. <https://doi.org/10.1016/j.pep.2011.08.001>

Mamarabadi M, Tanhaeian A, Ramezany Y, 2018. Antifungal activity of recombinant thanatin in comparison with two plant extracts and a chemical mixture to control fungal plant pathogens. *AMB Expr* 8 (180): 1-12. <https://doi.org/10.1186/s13568-018-0710-4>

Mandal S, Mitra A, Mallick, N, 2008. Biochemical characterization of oxidative burst during interaction between *Solanum lycopersicum* and *Fusarium oxysporum* f. sp. *Lycopersici*. *Physiol Mol Plant Pathol* 72: 56-61. <https://doi.org/10.1016/j.pmpp.2008.04.002>

Mandard N, Sodano P, Labbe H, Bonmatin JM, Bulet P, Hetru C, Ptak M, Vovelle F, 1998. Solution structure of thanatin, a potent bactericidal and fungicidal insect peptide, determined from proton two-dimensional nuclear magnetic resonance data. *Eur J Biochem* 256: 404-410. <https://doi.org/10.1046/j.1432-1327.1998.2560404.x>

Mansfield J, Genin S, Magori S, Citovsky V, Sriariyanum M, Ronald P, Dow M, Verdier V, Beer SV, Machado MA, *et al.*, 2012. Top 10 plant pathogenic bacteria in molecular plant pathology. *Mol Plant Pathol* 13: 614-629. <https://doi.org/10.1111/j.1364-3703.2012.00804.x>

- Marcos JF, Munoz A, Perez-Paya E, Misra S, Lopez-Garcia B, 2008. Identification and rational design of novel antimicrobial peptides for plant protection. *Annu Rev Phytopathol* 46: 273-301. <https://doi.org/10.1146/annurev.phyto.121307.094843>
- Mirzaei-Najafgholi H, Tarighi S, Golmohammadi M, Taheri P, 2017. The effect of citrus essential oils and their constituents on growth of *Xanthomonas citri* subsp. *citri*. *Molecules* 22 (591): 2-14. <https://doi.org/10.3390/molecules22040591>
- Mittler R, 2002. Oxidative stress, antioxidants and stress tolerance. *Trends Plant Sci* 7: 405-410. [https://doi.org/10.1016/S1360-1385\(02\)02312-9](https://doi.org/10.1016/S1360-1385(02)02312-9)
- Ozrenk K, Balta F, Celik F, 2011. Levels of fire blight (*Erwinia amylovora*) susceptibility of native apple, pear and quince germplasm from Lake Van Basin, Turkey. *Eur J Plant Pathol* 132 (2): 229-236. <https://doi.org/10.1007/s10658-011-9866-3>
- Patel RR, Sundin GW, Yang CH, Wang J, Huntley RB, Yuan X, Zeng Q, 2017. Exploration of using antisense peptide nucleic acid (PNA)-cell penetrating peptide (CPP) as a novel bactericide against fire blight pathogen *Erwinia amylovora*. *Front Microbiol* 8 (687): 1-12. <https://doi.org/10.3389/fmicb.2017.00687>
- Piqué N, Miñana-Galbis D, Merino S, Tomás JM, 2015. Virulence factors of *Erwinia amylovora*: A review. *Int J Mol Sci* 16: 12836-12854. <https://doi.org/10.3390/ijms160612836>
- Plewa MJ, Smith SR, Wanger ED, 1991. Diethylthiocarbamate suppresses the plant activation of aromatic amines into mutagens by inhabitation tobacco cell peroxidase. *Mutat Res* 247 (1): 57-64. [https://doi.org/10.1016/0027-5107\(91\)90033-K](https://doi.org/10.1016/0027-5107(91)90033-K)
- Salehi B, Mishra AP, Shukla I, Sharifi-Rad M, Contreiras MDM, Segura-Carretero A, Fathi H, Nasri Nasrabadi N, Kobarfard F, Sharifi-Rad J, 2017. Thymol, thyme, and other plant sources: health and potential Uses. *Phytother Res* 32 (9): 1688-1706. <https://doi.org/10.1002/ptr.6109>
- Skłodowska M, Gajewska E, Kuźniak E, Wielanek M, Mikicinski A, Sobiczewski P, 2011. Antioxidant profile and polyphenol oxidase activities in apple leaves after *Erwinia amylovora* infection and pretreatment with a benzothiadiazole-type resistance inducer (BTH). *J Phytopathol* 159: 495-504. <https://doi.org/10.1111/j.1439-0434.2011.01793.x>
- Soares V, Rodrigues FB, Vieira MF, Silva MS, 2011. Validation of a protocol to evaluate maximal expiratory pressure using a pressure transducer and a signal conditioner. *An Acad Bras Cienc* 83: 967-971. <https://doi.org/10.1590/S0001-37652011005000021>
- Sokovic M, Van Griensven L, 2006. Antimicrobial activity of essential oils and their components against the three major pathogens of the cultivated button mushroom, *Agaricus bisporus*. *Eur J Plant Pathol* 116: 211-224. <https://doi.org/10.1007/s10658-006-9053-0>
- Somayajula A, Asaithambi P, Susree M, Matheswaran M, 2011. Sono-electrochemical oxidation for decolorization of reactive red 195. *Ultrason Sonochem* 19: 803-811. <https://doi.org/10.1016/j.ultsonch.2011.12.019>
- Steiner P, 2000. Integrated orchard and nursery management for the control of fire blight. In: Fire blight: The disease and its causative agent, *Erwinia amylovora*; Vanneste JL (eds.). pp. 339-358. CABI Publ, NY, USA. <https://doi.org/10.1079/9780851992945.0339>
- Sticher L, MauchMani B, Mettraux, JP, 1997. Systemic acquired resistance. *Ann Rev Phytopathol* 35: 235-270. <https://doi.org/10.1146/annurev.phyto.35.1.235>
- Tang XS, Tang ZR, Wang SP, Feng ZM, Zhou D, Li TJ, Yin YL, 2012. Expression, purification, and antibacterial activity of bovine Lactoferrampin-Lactoferricin in *Pichia pastoris*. *Appl Biochem Biotechnol* 166 (3): 640-651. <https://doi.org/10.1007/s12010-011-9455-0>
- Tanhaeian A, Shahriari Ahmadi F, Sekhavati MH, Marabadi M, 2018. Expression and purification of the main component contained in camel milk and its antimicrobial activities against bacterial plant pathogens. *Probiotics Antimicrob Proteins* 10 (4): 787-793. <https://doi.org/10.1007/s12602-018-9416-9>
- Tanhaeian A, 2018. Production of the recombinant chimeric peptide, Lactoferrampin-Lactoferricin of camel milk in different expression systems and its bioactivity evaluation. Doctoral thesis. Univ. Ferdowsi, Mashhad, Iran.
- Thomson SV, 1986. The role of the stigma in fire blight infections. *Phytopathol* 76: 476-482. <https://doi.org/10.1094/Phyto-76-476>
- Van Vuuren SF, Viljoen AM, 2007. Antimicrobial activity of limonene enantiomers and 1,8-Cineole alone and in combination. *Flavour Fragr J* 22: 540-544. <https://doi.org/10.1002/ffj.1843>
- Viljevac M, Dugali K, Stolfa I, Dermi E, Cvjetkovi B, Sudar R, Kovacevi J, Cesar V, Lepedus H, Jurkovic Z, 2009. Biochemical basis of apple leaf resistance to *Erwinia amylovora* infection. *Food Technol Biotechnol* 47 (3): 281-287.
- Wang WY, Wong JH, Ip DTM, Wan DCC, Cheung RC, Ng TB, 2016. Bovine lactoferrampin, human lactoferricin, and lactoferrin 1-11 inhibit nuclear translocation of HIV integrase. *Appl Biochem Biotechnol* 179 (7): 1202-1212. <https://doi.org/10.1007/s12010-016-2059-y>
- Winslow CEA, Broadhurst J, Buchanan RE, Krumwiede C, Rogers LA, Smith GH, 1920. The families and genera of the bacteria. Final report of the committee of the society of American bacteriologists on characterization and classification of bacterial types. *J Bacteriol* 5: 191-229. <https://doi.org/10.1128/JB.5.3.191-229.1920>
- Wu T, Tang D, Chen W, Huang H, Wang R, Chen Y, 2013. Expression of antimicrobial peptides thanatin (S) in transgenic Arabidopsis enhanced resistance to

phytopathogenic fungi and bacteria. *Gene* 527: 235-242. <https://doi.org/10.1016/j.gene.2013.06.037>

Yumlembam RA, Borkar SG. 2014. Assessment of antibacterial properties of medicinal plants having bacterial leaf endophytes against plant pathogenic *Xanthomonads*. *Ind Phytopath* 67 (4): 353-357.

Zhang Z, Zheng H, 2009. Optimization for decolorization of azo dye acid green 20 by ultrasound and

H₂O₂ using response surface methodology. *J Hazard Mater* 172: 1388-1393. <https://doi.org/10.1016/j.jhazmat.2009.07.146>

Zhang Z, Nakano K, Maezawa S, 2009. Comparison of the antioxidant enzymes of broccoli after cold or heat shock treatment at different storage temperatures. *Postharvest Biol Technol* 54: 101-105. <https://doi.org/10.1016/j.postharvbio.2009.05.006>