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Original Article

Phytoalexin production of lettuce (*Lactuca sativa* L.) grown in hydroponics and its *in vitro* inhibitory effect on plant pathogenic fungi

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Abstract

A series of experiments were conducted to investigate phytoalexin production from five varieties of lettuce in hydroponics using abiotic (2.5, 5% $CuSO_4$; 0.5, 1% $AgNO_3$) and biotic elicitors (non-pathogenic *Pythium* sp.) at different plant ages. It showed that phytoalexin was successfully induced in tested lettuce grown in hydroponics after elicitation with abiotic elicitors throughout the trial. Phytoalexin showed yellow fluorescent spot under 365 nm UV light with R_f 0.45-0.48 and clear inhibition zone where *Aspergillus niger* failed to develop on TLC plate. For biotic elicitors, no yellow fluorescent spot on TLC plate was observed from tested lettuce varieties however inhibition zone at R_f 0.9 was detected at 8-9 weeks from red oak, green oak, and red coral. Moreover, crude extract of lettuce elicited with abiotic elicitors possessed *in vitro* antifungal activity against *C. gloeosporioides*, *C. lunata*, *F. oxysporum*, and *P. aphanidermatum* probably due to phytoalexin (lettucenin A) in the extract.

Keywords: phytoalexin, biotic elicitor, abiotic elicitor, lettuce, hydroponics.

1. Introduction

In Thailand, the growing demand worldwide for safe foods has given rise to the increased awareness over environmentally friendly agricultural production especially on fresh agricultural product. Food safety covers from the production in farm up to the consumer's table. Lettuce (*Lactuca sativa* L.) described as the "queen of the salad plants" (Martin & Ruberte, 1975) is *most often* grown as a leaf *vegetable both in soil and soilless cultivation.* It is certainly the most commonly used salad vegetable since it is important for its nutrient content. In recent years, demand for high quality lettuce with minimal or no pesticide residues, has also risen sharply to serve local consumption and for export. Therefore, better agricultural practices for disease management with consideration on maintaining a more sustainable and healthier crop eco-system should be taken. Developing alternative strategies to improve plant disease resistance and control of pathogens would be promoted.

Phytoalexin production has received much attention especially on its importance in plant defense (Ahuja *et al.*, 2012). Accumulation and production of phytoalexin occur in healthy plant cells surrounding wounded or infected cells and are stimulated by alarm substances produced and released by the damaged cells and diffusing into the adjacent healthy cells (Deverall, 1982). Abiotic elicitors are usually capable to induce phytoalexin in many crops (Angelova *et al.*, 2006, Yean *et al.*, 2009) while biotic elicitors such as microorganisms are also reported to elicite phytoalexins as well (Liu *et al.*, 1995). Lettucenin A was first found and reported to be the principal phytoalexin in soil-grown lettuce after being

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elicited by abiotic elicitors (Takasugi *et al.*, 1985). The isolation of lettucenin A by Takasugi *et al.* (1985) have opened up the path for further researches on this compound in lettuce plant for the purpose of improving alternative plant disease management. Lettucenin A is believed to have a role in the resistance of lettuce to microbial colonization in a sufficient concentration and at the right time (Yean *et al.*, 2009). However, most researches have been done to look into the response of lettuce in production of lettucenin A only in soil cultivation. Hence, there is no evidence so far to show whether this compound can be produced in lettuce in hydroponic cultivation or not. Nowadays, hydroponic cultivation of lettuce in Thailand has become popular and its market has increased significantly over the years (Damsteegt, 2015). On this regard, we have therefore chosen to study lettucenin A phytoalexin in lettuce in hydroponic cultivation using abiotic and biotic elicitors. The idea of using fungal biotic elicitors has been risen since most of indigenious non-pathogenic *Pythium* spp. commonly found in hydroponic s ystem (Koohakan *et al.*, 2004; Talubnak *et al.*, 2014) were reported to be the beneficial isolates promoting the growth of lettuce (Talubnak *et al.*, 2010) and some were proven to be biological control agents against several plant pathogenic fungi (Bala *et al.*, 2009).

Our research was therefore conducted to investigate the production of phytoalexin in lettuce especially grown in hydroponics with three main objectives: i) To determine the possible occurrence of phytoalexin in hydroponically-grown butterhead lettuce after elicitation with abiotic elicitors at different plant ages; ii) To determine the effect of biotic elicitors (e.g. indigenous non-pathogenic *Pythium* sp. from hydroponics) and abiotic elicitors (0.5, 1% $AgNO_3$ and 2.5, 5% $CuSO_4$) on phytoalexin production of five varieties (red oak, green oak, red coral, cos, and butterhead) of lettuce grown in hydroponics at different plant ages; and iii) To assess the *in vitro* antifungal activity of crude extract of elicited lettuce against conidial germination of four plant pathogenic fungi (namely, *Colletotrichum gloeosporioides*, *Curvularia lunata*, *Fusarium oxysporum*, and *Pythium aphanidermatum*).

2. Materials and Methods

Our researches were comprised of three experiments and conducted by using the completely randomized design.

Plants preparation

Lettuce seeds were germinated on moist sponge in a tray at room temperature. Butterhead seeds were used in the 1st experiment while seeds from five varieties (red oak, red coral, green oak, cos, and butterhead) were tested in the 2nd experiment. After seven days, seedlings were transplanted into nutrient film technique (NFT) system with nutrient solution (Modified from Benoit, 1992). Plants were grown in greenhouse (28-30°C) and be prepared for elicitation according to the experiments.

2.1 Possible occurrence of phytoalexin in butterhead in hydroponics after abiotic elicitation

Elicitation of phytoalexin: Four-week-old seedlings were used for elicitation. Leaves of lettuce were sprayed every seven days up to 11-week-old seedlings with 1% $AgNO_3$ and 5% $CuSO_4$. Sterilized water was used as control. After spraying, lettuces were placed in greenhouse for three days before extraction.

Extraction of phytoalexin: Treated leaves of lettuce were washed in running tap water and homogenized using mortar and pestle before addition of 60% ethanol with ratio of 10 ml of solvent per gram of tissue and left overnight in the dark at room temperature. The homogenate was filtered through Whatman No.1 filter paper and the residue was again re-extracted as described before. The extracts were pooled and evaporated at 46°C using a rotary evaporator until about 30% remain from the total volume and extracted three times with chloroform. The extract was detected for phytoalexin by TLC bioassays (Ong & Chong, 2009).

Detection of phytoalexin: Twenty microliter of crude extract of lettuce was dropped on thin layer chromatography (TLC) plate (Merck kiesel 60 F254 silica gel). Plates were developed in hexane:ethyl acetate (1:1 v/v). When the solvent reached 7 cm from the starting point, the plates were taken out and examined under UV light at 365 nm wavelength. Lettucenin A gives off a greenish yellow florescent at R_f 0.45 (Ong & Chong, 2009). For direct detection of antifungal activity zones, the developed TLC plates were sprayed with spore suspension of *Aspergillus niger* $4x10^6$ spore/ml suspended in potato dextrose broth (PDB). The inoculated TLC plates were kept in moist chamber plastic boxes and incubated at 25°C in the dark for three days. Inhibition zones and R_f value were measured.

2.2 Determination of phytoalexin production in five lettuce varieties after biotic and abiotic elicitation

Four-week-old seedlings were used for elicitation. Leaves of lettuce were sprayed every 7 days up to 11-week-old seedlings with 0.5, 1% $AgNO_3$ and 2.5, 5% $CuSO_4$ and 3 isolates of non-pathogenic *Pythium aphanidermatum* (NPA1, 2, and 3). The *P. aphanidermatum* was obtained from hydroponics system (Talubnak *et al.*, 2014). Sterilized water was used as control. After spraying, lettuces were placed in greenhouse for three days before extraction. Extraction and detection of phytoalexin were mentioned above.

2.3 Assessment of antifungal activity of crude extract derived from elicited lettuce

To determine the effect of crude extract of lettuce on conidia of all tested plant pathogenic fungi such as *Curvularia lunata*, *Fusarium oxysporum*, *Colletotrichum gloeosporioides*, and *Pythium aphanidermatum*. Fifty microliter of conidia suspension (10^5 spore/ml) was dropped on

well slide containing 50 ul of crude extract. Slides were incubated in moist chamber plastic boxes at room temperature (25°C) and examined for conidia germination and abnormal conidia at 30 min, 12, 24, and 72 hrs. Germination percentage was determined by counting total conidia and germinated conidia under light microscope. Percentage data were calculated and analyzed by ANOVA and means were separated by Duncan's multiple range test.

3. Results and Discussion

3.1 Occurrence of phytoalexin in butterhead in hydroponics after abiotic elicitation

The phytoalexin production examined at the different age of butterhead lettuce grown in hydroponics with abiotic elicitors is presented in Table 1. The results revealed that phytoalexin could be produced in butterhead lettuce grown in hydroponics after elicitation with abiotic elicitors (5% $CuSO_4$ and 1%$AgNO_3$) at the plant age of 4 till 11 weeks. On TLC bioassays, phytoalexin showed yellow fluorescent spot under 365 nm UV light with R_f value of 0.45-0.48 (Figure 1). Both $CuSO_4$ and $AgNO_3$, abiotic chemical elicitors, showed the same pattern of phytoalexin production. Clear inhibition zone (about 0.5-1.1 cm) where the fungi *Aspergillus niger* failed to develop on TLC plates dipped in hexane: ethyl acetate (1:1, v/v) was observed at the same R_f. Furthermore, the inhibition zones (0.9 and 1.1 cm) were greatest particularly at the plant age of 10 weeks using 5%$CuSO_4$ and 1%$AgNO_3$, respectively (Figure 1, Table 1). Control (unelicited) plant did not show either the yellow fluorescent spot under 365 nm UV light or inhibition zone of *A. niger* on TLC plates throughout the experiment. From our findings, phytoalexin detected so far from lettuce could probably be lettucenin A since having the same pattern of yellow fluorescent spot at the same R_f with clear inhibition zone against *A. niger* (Ong & Chong, 2009). This result was supported by the finding of the highest phytoalexin lettucenin A accumulation at different age from week nine to week twelve after elicitations with chemical elicitors, silver nitrate ($AgNO_3$) and copper sulfate ($CuSO_4$) (Ong & Chong, 2009). Moreover, phytoalexin in lettuce was

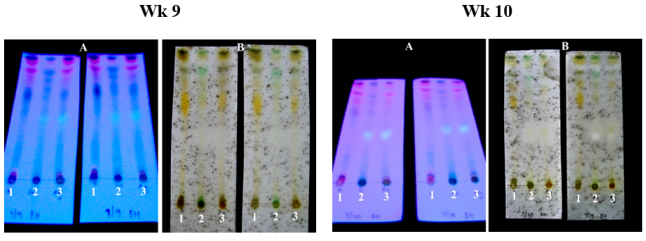

Figure 1. TLC plate bioassay with 365 nm UV light (A) and *Aspergillus niger* (B) detects phytoalexins (at R_f 0.45) in leaves tissue of lettuce elicited with SW (Lane 1); 5% $CuSO_4$ (Lane 2) and 1% $AgNO_3$ (Lane 3) at the age of 9 and 10 weeks.

Table 1. Effect of abiotic elicitors on phytoalexin production of leaves of butterhead lettuce grown in hydroponics and detection of antifungal activity zones by TLC bioassays.

		Plant age at which being treated with elicitors																					
		Wk4		Wk5			Wk6			Wk7			Wk8			Wk9			Wk10			Wk11	
	UV[1/]	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone	
	R_f[2/]	Size[3/]	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f
SW	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
5% $CuSO_4$	0.45	0.5	0.45	0.45	0.6	0.45	0.42	0.8	0.42	0.43	0.5	0.43	0.43	0.7	0.43	0.45	0.8	0.45	0.45	0.9	0.45	0.45	0.5	0.45
1% $AgNO_3$	0.48	0.7	0.48	0.46	1.0	0.46	0.43	0.7	0.43	0.45	0.6	0.45	0.45	0.8	0.45	0.45	0.7	0.45	0.45	1.1	0.45	0.45	0.5	0.45

[1/]UV light 365 nm; [2/]R_f value as the ratio of the distance travelled by compound and the distance travelled by solvent front; [3/]Size in centimeter.

reported to be induced from five day-old plants until harvest stage (Mai & Glomb, 2014).

3.2 Phytoalexin production in five lettuce varieties after biotic and abiotic elicitation

The result showed that all five tested varieties (red oak, green oak, red coral, cos, and butterhead) of hydroponically grown lettuce could produce phytoalexin after elicitation with abiotic elicitors whereas only green oak lettuce was shown to produce this phytoalexin quite constantly throughout the plant ages until maturity stage (4 to 12 weeks) (Table 2). Phytoalexin showed yellow fluorescent spot under 365 nm UV light with R_f value of 0.38-0.6 as well as antifungal activity against *A. niger* on TLC plate which is in line with the previous experiment of butterhead lettuce. Among the chemical abiotic elicitors, all lettuces elicited with half concentrations of both chemical (2.5% $CuSO_4$ and 0.5% $AgNO_3$) could produce phytoalexin throughout the trial but less constant compared to those elicited with reference dose (5% $CuSO_4$ and 1% $AgNO_3$). Moreover, the present study revealed among the different weeks tested, 8 and 9 weeks-old lettuces seemed to produce phytoalexin more constantly chemical abiotic elicitors. This was in line with the previous researches of Bestwick *et al.* (1995) suggesting different ages of plant produce different amount of phytoalexins. Ong and Chong (2009) also reported that lettucenin A significantly increased at week 9 to 12. Elicitation using three isolates of non-pathogenic *Pythium* sp. as biotic elicitors, surprisingly, no yellow fluorescent spot on TLC plate under 365 nm UV light was observed from all five tested varieties of lettuce however inhibition zone on TLC plate against *A. niger* at the R_f 0.9 was detected at the plant age of 8-9 weeks from three varieties of lettuce namely red oak, green oak, and red coral. In addition, sizes of inhibition zones were in the range of 0.5-1.5 cm. In this regard, our result on using non-pathogenic *Pythium* as biotic elicitors was not satisfactory. Unlike, there was a report (Liu *et al.*, 1995) on the success of using *Pythium ultimum* and *P. sylvaticum* as biotic elicitors to produce three kinds of phytoalexins (kievitone, phaseollinisoflavan, and phaseollin) in root tissue of bean seedlings (*Phaseolus vulgaris* L.). Moreover, there were few reports on phytoalexin accumulation in lettuce induced by biotic elicitors such as *Pseudomonas cichorii* (Takasugi *et al.*, 1985), *Botrytis inerea*, *Bremia lactucae*, and *Pseudomonas syringae* (Bennett *et al.*, 1994).

3.3 Assessment of the *in vitro* antifungal activity of crude extract of elicited lettuce against conidial germination of four plant pathogenic fungi

The conidia germination of 4 plant pathogenic fungi were examined at 30 min, 12, 24, and 72 hrs after incubation with crude extract from lettuce elicitation with $CuSO_4$ and $AgNO_3$. The results presented that in control treatment, conidia germination of all tested fungi except *P. aphanidermatum* started after 30 min and increased continuously with the increasing the incubation times and reached the highest percentage of germination at 72 hrs (55.63% for *C. gloeosporioides*, 58.59% for *C. lunata* and 65.2% for *F. oxysporum*). Whereas, the tested conidia except *P. aphanidermatum* in both crude extract treatments did not germinate or slightly germinated throughout incubation times which were not significantly different among the crude extract treatments (Figure 2). In addition, abnormal conidia observed under light microscope were only noted in the crude extract treatments. Abnormalities of conidia included damaged cell, swelling, lysis, deformation, and a granular cytoplasm with an intense vacuolization at 72 hrs (Figure 3). Regard to *P. aphanidermatum*, observation on germination of sporangium, zoospore and oospore was made but no data were obtained either in control or in crude extract treatments due to the difficulties in detecting. However, changes in sporangium, oogonium, antheridium, and oospore were detected such as lysis of sporangium and antheridium, granular cytoplasm with an intense vacuolization in oogonium and oospore. Lysis was

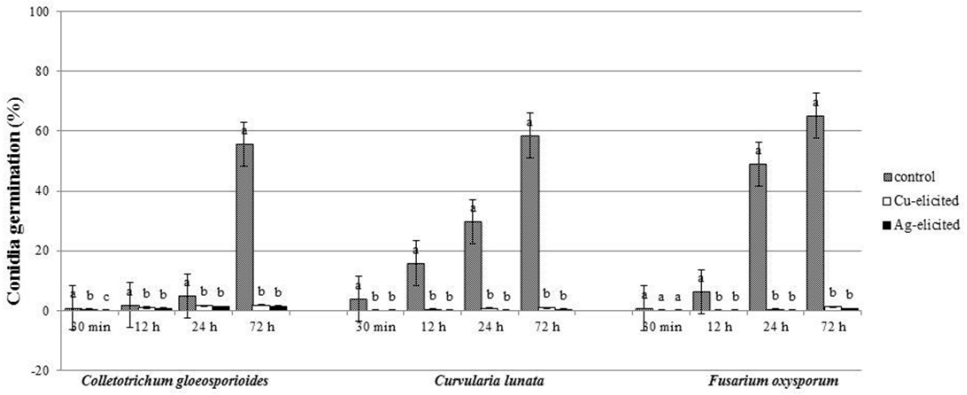

Figure 2. Effect of crude extract of elicited lettuce on conidia germination of plant pathogenic fungi. Each bar represents the percentage of spore germination of *C. gloeosporioides*, *C. lunata* and *F. oxysporum* ($P < 0.05$). Statistically significant differences between type of crude extract and time incubation when the letters are different.

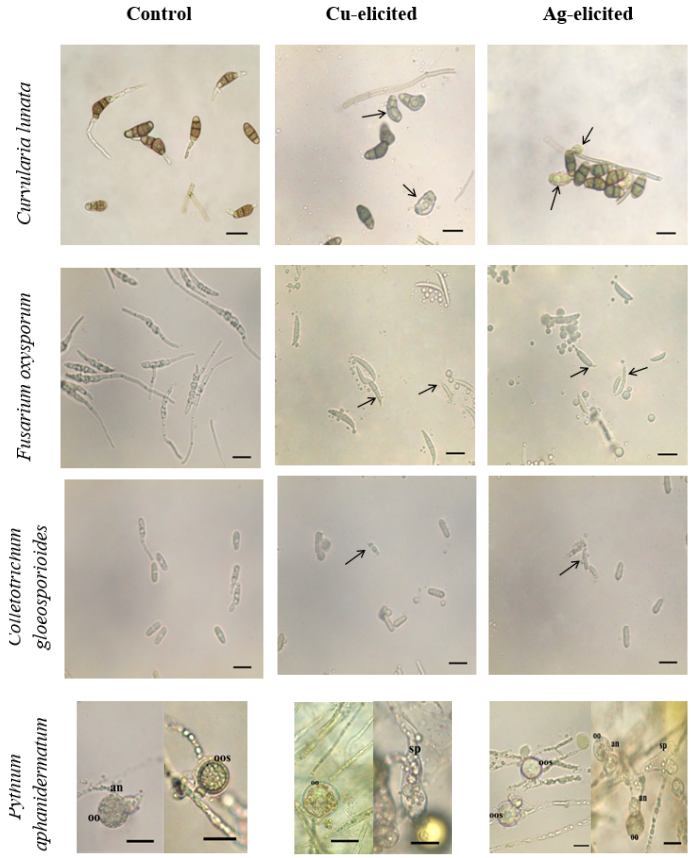

Figure 3. Effect of crude extract from lettuce leaves elicited by abiotic elicitors (5% $CuSO_4$ and 1% $AgNO_3$) on abnormality of conidia and germination of conidia of plant pathogenic fungi for 72 h. Vacuolisation, distortion and degradation were shown. Scale bar = 30 µm. *oo = oogonium; an = antheridium; oos = oospore; sp = sporangium

also detected on hyphae. From the above findings, it was suggesting that crude extract of hydroponically grown lettuce elicited with $CuSO_4$ and $AgNO_3$ possessed *in vitro* antifungal activity against *C. gloeosporioides*, *C. lunata*, *F. oxysporum*, and *P. aphanidermatum* probably due to phytoalexin (lettucenin A) in the crude extract. Our results are in accordance with researches reporting on great inhibitory effect of phytoalexin against various plant pathogenic fungi (Huang, 2001). In the group of sesquiterpene lactones phytoalexins, lettucenin A possessed considerable activity against *Botrytis cinerea*, *B. lactucae*, and *Pseudomonas syringae* pv. *phaseolicola* (Bennett *et al.*, 1994), *Ceratocystis fimbriata* (Takasugi *et al.*, 1985), whereas cichoralexin from chicory was reported to completely inhibit the conidial germination of *Bipolaris leerside* (Monde *et al.*, 1990), *B. cinerea*, *Fusarium moniliform*, *P. ultimum*, *Phoma betae*, and *Alternaria* sp. (Mares *et al.*, 2005). Other phytoalexins from various plants were also notified having the antifungal activities, for examples crucifer phytoalexin against *Alternaria brassicicola* and *A. brassicae* (Sellam *et al.*, 2007); momilatones A and B (derived from rice plant) against *B. cinerea*, *F. solani*, *F. oxysporum*, and *C. gloeosporioides* (Fukuta *et al.*, 2007); daidzein (from cowpea) against *F. oxysporum* (Sundaresan *et al.*, 1993).

4. Conclusions

The results of the present study indicated the occurrence of phytoalexin in butterhead lettuce grown in hydroponics after elicitation with 5%$CuSO_4$ and 1%$AgNO_3$ as abiotic elicitors at different plant ages (4-11 weeks). This was also suggesting that lettuce grown in hydroponics can produce phytoalexin in the same pattern as that in soil cultivation. Thereafter, phytoalexin production was further studied on other varieties of lettuce with the use of non-pathogenic *Pythium* sp. as biotic elicitors; it revealed the ability for all five varieties of lettuce elicited with abiotic elicitors to produce phytoalexin in hydroponic cultivation throughout the trial while using biotic elicitors was not satisfactory. From our result, the phytoalexin detected so far from lettuce growing in hydroponics could probably be lettucenin A since showing the same pattern of yellow fluorescent spot with clear inhibition zone against *A. niger* on TLC plate. For *in vitro* antifungal study with 4 plant pathogenic fungi, namely *C. gloeosporioides*, *C. lunata*, *F. oxysporum*, and *P. aphanidermatum,* results showed the crude extract from lettuce elicited with abiotic elicitors possessed antifungal activity against all tested fungi probably due to the impact of phytoalexin (lettucenin A) in

Table 2. Effect of abiotic elicitors on phytoalexin production of leaves of butterhead lettuce grown in hydroponics and detection of antifungal activity zones by TLC bioassays.

	Plant age at which being treated with elicitors																							
	Wk4			Wk5			Wk6			Wk7			Wk8			Wk9			Wk10			Wk11		
	UV[1/]	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone	
	R_f[2/]	Size[3/]	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f
Red oak																								
SW	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.5% $CuSO_4$	-	-	-	-	-	-	-	-	-	-	-	-	0.38	0.5	0.38	-	-	-	-	-	-	-	-	-
5% $CuSO_4$	-	-	-	-	-	-	0.46	0.7	0.5	-	-	-	0.4	0.8	0.4	0.4	0.9	0.4	-	-	-	-	-	-
0.5% $AgNO_3$	-	-	-	0.42	0.9	0.42	-	-	-	-	-	-	0.4	0.8	0.4	-	-	-	0.47	0.8	0.47	-	-	-
1% $AgNO_3$	-	-	-	0.39	1	0.4	-	-	-	0.45	0.8	0.45	0.4	0.8	0.4	-	-	-	0.45	1	0.5	-	-	-
NPA 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8	0.4	-	-	-	-	-	-
NPA 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8	0.4	-	-	-	-	-	-
NPA 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.8	0.4	-	-	-	-	-	-
Green oak																								
SW	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.5% $CuSO_4$	-	-	-	0.46	0.9	0.46	0.44	0.6	0.44	0.68	0.7	0.68	0.35	0.5	0.35	0.52	0.7	0.52	-	-	-	0.38	0.7	0.38
5% $CuSO_4$	-	-	-	0.43	0.8	0.43	0.58	0.9	0.58	0.67	0.7	0.67	0.33	0.8	0.33	0.5	0.65	0.5	-	-	-	0.45	0.7	0.45
0.5% $AgNO_3$	0.5	0.8	0.5	-	-	-	0.42	0.6	0.42	0.67	0.9	0.67	0.33	0.8	0.33	0.54	0.65	0.54	-	-	-	-	-	-
													-	0.8	0.7									
1% $AgNO_3$	0.42	0.9	0.4	-	-	-	-	-	-	0.67	0.6	0.67	0.35	0.9	0.35	0.55	0.7	0.55	0.46	0.8	0.46	0.46	0.7	0.46
													-	0.9	0.7									
NPA 1	-	-	-	-	-	-	-	-	-	-	-	-	-	0.9	0.9	-	-	-	-	-	-	-	-	-
NPA 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NPA 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1/]UV light 365 nm; [2/]R_f value as the ratio of the distance travelled by compound and the distance travelled by solvent front; [3/]Size in centimeter.

Table 2. Continued

	Plant age at which being treated with elicitors																							
	Wk4			Wk5			Wk6			Wk7			Wk8			Wk9			Wk10			Wk11		
	UV[1/]	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone		UV	Antifungal zone	
	R_f[2/]	Size[3/]	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f	R_f	Size	R_f
Red coral																								
SW	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.5% $CuSO_4$	0.34	0.5	0.34	0.36	0.6	0.36	0.35	1.1	0.35	0.4	0.5	0.4	-	-	-	-	-	-	0.85	0.9	0.85	-	-	-
							-	0.6	0.6															
5% $CuSO_4$	0.4	0.7	0.4	0.42	0.7	0.42				0.39	0.5	0.39							0.45	1.0	0.45	0.42	0.6	0.42
0.5% $AgNO_3$	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.43	0.8	0.43	-	-	-
1% $AgNO_3$	-	-	-	-	-	-	0.4	0.8	0.4	-	-	-	0.38	0.5	0.38	0.41	0.9	0.41	-	-	-	0.38	0.7	0.38
NPA 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NPA 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NPA 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cos																								
SW	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.5% $CuSO_4$	-	-	-	-	-	-	0.4	1	0.4	-	-	-	0.45	0.5	0.45	0.36	0.7	0.36	-	-	-	0.38	0.8	0.38
5% $CuSO_4$	-	-	-	0.38	0.8	0.38	0.39	0.7	0.39	0.45	1.1	0.45	-	-	-	0.48	0.4	0.48	-	-	-	-	-	-
0.5% $AgNO_3$	-	-	-	-	-	-	-	-	-	0.42	0.9	0.42	-	-	-	-	-	-	0.43	0.9	0.43	0.45	0.5	0.45
																			-	1.8	1.8			
1% $AgNO_3$	0.42	0.9	0.42	-	-	-	-	-	-	0.47	0.4	0.47	0.4	0.8	0.4	0.41	0.8	0.41	0.43	0.5	0.43	0.45	0.6	0.45
NPA 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NPA 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NPA 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Butterhead																								
SW	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2.5% $CuSO_4$	-	-	-	0.38	1.2	0.38	0.34	1.3	0.34	-	-	-	0.38	1.1	0.38	-	-	-	-	-	-	-	-	-
							-	1.2	0.6															
5% $CuSO_4$	-	-	-	0.45	1.1	0.45	-	-	-	0.38	0.6	0.38	0.4	0.7	0.4	0.37	0.6	0.37	-	-	-	0.45	0.9	0.45
0.5% $AgNO_3$	0.45	0.8	0.45	0.37	1.3	0.37	0.46	0.7	0.46	0.41	0.5	0.41	-	-	-	-	-	-	0.36	0.8	0.36	-	-	-
				-	1.3	0.7																		
1% $AgNO_3$	0.47	0.9	0.5	0.4	1.2	0.4	0.36	1.1	0.36	0.4	0.9	0.4	0.45	1	0.45	-	-	-	0.39	0.8	0.39	0.43	1	0.43
				-	1.5	0.7	-	1.6	0.6															
NPA 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NPA 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NPA 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[1/]UV light 365 nm; [2/]R_f value as the ratio of the distance travelled by compound and the distance travelled by solvent front; [3/]Size in centimeter.

the extract. In addition, we are classifying this obtained phytoalexin as the compound.

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