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Combining effects of ozone and *Xanthomonas oryzae* pv. oryzae on antioxidants and phytoalexins in rice (*Oryza sativa* L.)

Nithiwadee Buawat[1], Morifumi Hasegawa[2], Chanin Umponstira[1]*

[1]Department of Natural Resources and Environment Faculty of Agriculture Natural Resources and Environment, Naresuan University, Muang, Phitsanulok, 65000 Thailand
[2]College of Agriculture, Ibaraki University,3-21-1Chuo, Ami, Ibaraki 300-0393, Japan

***Corresponding author: chaninum99@gmail.com**

Abstract

Ozone is the most oxidative air pollutant and considered as an abiotic stress which harm to vegetation and crop. Similarly, biological stressor such as plant pathogen could cause severe damage to crops. Combination effects from both stresses will potentially lead to economic loss. Basically, plants will response to both abiotic stress and pathogenic stressor by generate antioxidants and phytoalexins. The concentration of these chemical indicate stress level in plant and also defence system response. This research is aimed to investigate the combination effects of ozone and *Xanthomonas oryzae* pv. *oryzae* on antioxidants and phytoalexins in rice (*Oryza sativa* L. cv. KDML 105). In this study, 45-day-old rice plants were used. Ozone was elevated at concentrations of 100, 200 and 300 ppb in the fumigating chambers for 12 hours. *Xanthomonas oryzae* pv. *oryzae* which caused bacterial blight disease in rice was selected as plant pathogen. Combining effects of ozone and *Xanthomonas oryzae* pv. *oryzae* were examined using ozone at a concentration of 100 ppb and *Xanthomonas oryzae* pv. *oryzae* $1x10^8$ cells/ml inoculation. The experimental design was a completely randomized design with 2×2 factorials treatment and three replications required. Temperature, relative humidity and light intensity in fumigating chambers were controlled. The results of the combining effects showed the amount of antioxidants and phytoalexins were higher than those in single treatment. The experimental data indicated that the combining effects will result in more stress in rice. This suggests that, in rice plantation area with experience of ozone episode, pathogenic resistance rice strain should be considered to reduce combining effects.

Keywords: ozone; bacterial blight disease; antioxidants; phytoalexins; combining effects.
Abbreviations: ROS_reactive oxygen species, SOD_superoxide dismutase, APX_ascorbate peroxidise, H_2O_2_hydrogen peroxide, $O_2^{\bullet-}$_superoxide anion, CF_charcoal-filtered, HR_hypersensitive responses.

Introduction

The crop production was affected by the climate change (Liu et al., 2013; Padakandla, 2016; Sarker et al., 2014) due to increasing of several air pollutant, including ozone (Zhan et al., 2008; Zhang et al., 2008; Ariyaphanphitak et al., 2005; Wang et al., 2012; Danh et al., 2016). Ozone was the most reactive air pollutant. Production of forests and cultivation of crops around the world, such as wheat, soybeans, rice and maize, were severely affected by high ozone concentration (Vaultier and Jolivet, 2015; Dingenen et al., 2009; Tai et al., 2014; Avnery et al., 2011). There have been several studies indicating that ozone could cause severe effects on plant physiology, for instance, the reduction of photosynthesis and visible injury due to changing of lipid metabolism, membrane permeability and nutrient leaching consequently decreasing the crop production (Feng et al., 2016; Sicard et al., 2016; Díaz-De-Quijano et al., 2012). Generally, when ozone enters plant leaves through stomata, it would quickly filtrate and react with apoplastic fluid in cell membrane causing free radicals and other reactive oxygen species (ROS), which damages structure and function of cell membrane (Vaultier and Jolivet, 2015; Heath, 2008). Strong oxidants ROS that also act as signal molecules in plants would initiate hypersensitive responses (HR), including the activation ethylene, salicylic acid jasmonate and the programmed cell death in response to ozone (Kangasjärvi et al., 1994). Complex antioxidatives comprising of non-enzymatic and enzymatic antioxidant in higher plants' defense system helps with protection to avoid oxidative damage (Sharma et al., 2012; Caverzan et al., 2012). Similarly, biotic stresses such as pest and diseases also cause the yield loss of vegetation and crop production (Al-Whaibi, 2011). Bacterial blight disease was one of the common diseases found in the rice paddy fields which originally infected by *Xanthomonas oryzae* pv. *oryzae* (Lorenzo et al., 2016; Thein and Prathuangwong, 2010; Xu et al., 2015; Islam et al., 2016). Bacterial blight disease symptoms were the results of *Xanthomonas oryzae* pv. *oryzae* entering through wounds or hydathodes, multiplying in the epitheme and moving to the xylem vessels (Chithrashree and Srinivas, 2012). Plant have evolved secondary metabolites and specific proteins against pathogen and herbivores (Walling, 2000; Howe and Jander, 2008; Mithofer and Boland, 2012). Salicylic acid, jasmonic acid and ethylene are plant hormones that have essential roles during systemic defense signaling (Glazebrook, 2005; Robert-Seilaniantz et al., 2011). In addition, plants active defense mechanisms are generated by phytoalexins, which

were antimicrobial substances of low molecular weight, promptly produced after plants were exposed to pathogen or stress (Cho and Lee, 2015; Jeandet et al., 2013; Ahuja et al., 2012). The ozone and *Xanthomonas oryzae* pv. *oryzae* similarly activated ROS, causing oxidative damage. Plant cells would react to ROS by producing some antioxidant enzymes (Sharma et al., 2012) such as superoxide dismutase (SOD) and ascorbate peroxidase (APX). Generally, SOD plays an important antioxidant role to scavenge superoxide anion ($O_2^{\bullet-}$) by transforming into hydrogen peroxide (H_2O_2) (Alscher et al., 2002). Continuously, APX reacted with H_2O_2 to provide water (H_2O) (Caverzan et al., 2012; Chao et al., 2015). Plant also produces secondary metabolites, such as phytoalexins to protect themselves from disease or stress (Ahuja et al., 2012). Growth and development of pathogens at infection site are inhibited by antimicrobials compound of phytoalexins (Hain et al., 1993; Kuc', 1995). Accumulation of rapid phytoalexins is associated with resistance in plants disease caused by bacteria and fungi (Kuc', 1995). Nineteen phytoalexins were found in rice, including momilactones A-B, phytocassanes A-F, oryzalexins A-F, oryzalexin S, ent-10-oxodepressin, N-benzoyltryptamine, N-cinnamoyltryptamine (Horie et al., 2016), and sakuranetin (Shimizu et al., 2012).

Xanthomonas oryzae pv. *oryzae* is a common plant pathogen in paddy fields and severe damage to the production of rice in Thailand. Presently, agriculture areas have been affected by ozone, which precursor nitrogen oxides (NO_x) and volatile organic compounds (VOC_s), generated from industry and traffic. This research was aimed to study the combinatory effects of ozone and *Xanthomonas oryzae* pv. *oryzae* on antioxidants, as well as the phytoalexins by using ozone and *Xanthomonas oryzae* pv. *oryzae*. Rice (*Oryza sativa* L.) the major crop of Southeast Asia was selected as the plant sample. Two experiments were conducted in this study: (1) rice exposed to four levels of ozone concentrations: charcoal-filtered (CF<10 ppb), 100, 200, and 300 ppb (2) inoculated and non-inoculated with *Xanthomonas oryzae* pv. *oryzae* applied on rice leaves and combination of ozone exposure at a concentration of 100 ppb and *Xanthomonas oryzae* pv. *oryzae* inoculation. The results presented the analysis of antioxidants and their product e.g. SOD, APX and H_2O_2 and phytoalexins e.g. momilactone A and sakuranetin. The findings from this research may provide a better understanding of the rice immune system reacting to oxidative stress and pathogenic stress.

Results

Dose-dependent effects of ozone on antioxidants and phytoalexins

The value of SOD, APX and H_2O_2 in plant samples were gradually increased from day 1 to day 4 and started to decline on day 5 after fumigation by ozone at three concentration levels, 100, 200 and 300 ppb for 12 hours (Table 1). There was no significant change of those values from day 1 to day 5 in the control group. The highest dose of ozone concentration level at 300 ppb caused plant samples response by producing more SOD, APX and H_2O_2 than the ozone concentration level at 200 and 100 ppb. The average of five days value of SOD, APX and H_2O_2 found in ozone-fumigated plant samples at 300 ppb were 11337.13±831.70 units/g FW, 890.13±102.32 units/g FW and 13.08±1.11 nmol/g FW, respectively, which were significantly different from the other groups. However, no momilactone A and sakuranetin was detected when plant samples were fumigated by ozone concentration at 100 and 200 ppb levels along with control group. Surprisingly, ozone concentration level at 300 ppb could significantly induce momilactone A and sakuranetin synthesis in plant samples. The values were gradually increased from day 1 to day 4 and declined on day 5 (Table 2) and the highest value of momilactone A and sakuranetin were observed on day 4 with values of 42223.11±939.46 ng/g FW and 23360±5714.97 ng/g FW, respectively. There was a strong correlation between SOD, APX and H_2O_2 with momilactone A and sakuranetin when plant samples were fumigated by ozone concentration at 300 ppb level (Fig. 2a-f).

The combining effects of ozone and Xanthomonas oryzae pv. *oryzae on antioxidants and phytoalexins*

Plant samples were inoculated by *Xanthomonas oryzae* pv. *oryzae* expressed SOD, APX and H_2O_2 increasingly from day 1 to day 4. They declined from day 5 (Table 1), when compared with the non-inoculation group but the value was remarkably less than ozone fumigated plants. Interestingly, the average of five days value of SOD, APX and H_2O_2 were 7143.87±519.44 units/g FW, 512.40±34.29 units/g FW and 5.90±0.48 nmol/g FW, respectively. These values were close to the average of five days value of SOD, APX and H_2O_2 in ozone fumigated group at 100 ppb. However, there was an inconsistent result of momilactone A and sakuranetin induction when plant samples were subject to only *Xanthomonas oryzae* pv. *oryzae* inoculation due to infection development. For instance the little amount of momilactone A found on day 2 and day 5 and sakuranetin on day 3 and day 5 (Table 2).

The combining effect of ozone concentration level at 100 ppb and *Xanthomonas oryzae* pv. *oryzae* inoculation found the value of SOD, APX and H_2O_2 gradually increasing from day 1 to day 5 (Table 1). The average five days value of those was similarly or slightly higher than both single treatments. However, the amount of momilactone A was detectable from day 3, 4 and 5 (Table 2) as value as 97.51±16.78 ng/g FW, 85.64±11.59 ng/g FW and 64.71±5.08 ng/g FW, respectively, and sakuranetin on day 3 and 5 with values 4640±85.72 ng/g FW and 3866.67±100.96 ng/g FW, respectively. Notably, the value of sakuranetin which was found from the combing effect treatment was significantly higher than those from the single treatment of *Xanthomonas oryzae* pv. *oryzae*. Nevertheless, this value was still less than the value which has been found during fumigation by ozone concentration level at 300 ppb.

Discussion

The results indicate that ozone concentration at 300 ppb was the most effective to stimulate both SOD and APX antioxidants in rice cultivar 105 which is considered sensitive to ozone (Phothi et al., 2016). The high level of ozone affects protein activity, gene expression and metabolism in plant cell quickly (Vainonen and Kangasjärvi, 2015; Ueda et al., 2013). This is a typical reaction of plant cell during exposure to ozone, which induces an oxidative products in plant cells, resulting in the generation and accumulation of ROS such as H_2O_2, $O_2^{\bullet-}$ and hydroxyl radicals ($^{\bullet}OH$) (Sharma et al., 2012; Heath, 2008; Langebartels et al., 2002). The abundance of SOD and APX was also measured including their metabolic product as H_2O_2 during ozone exposure. The consistent results occurred in other ozone concentrations at 200 and 100 ppb but only differences in less amount of SOD, APX and H_2O_2 when compared with treatment of ozone concentration

at 300 ppb. Notably, SOD, APX and H_2O_2 were dramatically increased after exposure to ozone from day 1 to day 4 and started declining at day 5. Under significant stress, most plants quickly react at early stages by generating antioxidants to encounter ROS (Gundel et al., 2015). Ishii et al. (2004) and Agrawal and Rai (2008) found that SOD and APX were increased in rice to eliminate ROS after detoxification proceeded and its product e.g. H_2O_2 decreased along with reduction of SOD and APX. In an ozone sensitive rice cultivar the APX, monodehydroascorbatereductase (MDHAR) and dehydroascorbatereductase (DHR) were increased in the first stage during the fumigation, which decreased later (Wang et al., 2014).

After 2 days of inoculation by *Xanthomonas oryzae* pv. *oryzae*, the significant amount of SOD, APX and H_2O_2 were detected higher than the non-inoculated group. Interestingly, the amount of SOD, APX and H_2O_2 were less than the ozone treatment group at 200 and 300 ppb. Nevertheless, these amounts were higher than the ozone treatment group at 100 ppb. Therefore, *Xanthomonas oryzae* pv. *oryzae* as a pathogenic stress had lower capacity to stimulate antioxidants and their product than ozone. This could be attributed to the the fact that longer exposure/incubation time is required for *Xanthomonas oryzae* pv. *Oryzae* infection. Before that minimum time plant may not react to infestation. Generally, rice could be infested by *Xanthomonas oryzae* pv. *oryzae* within 24-72 hours, in which rice react through its self-defense mechanism (Niño-Liu et al., 2006). In this experiment, the results showed that plant samples under stresses either treated by ozone or inoculated by *Xanthomonas oryzae* pv. *oryzae* fully reacted and produced more both SOD and APX antioxidants to eliminate ROS. Nevertheless, stress caused by ozone was fast and strong rather than *Xanthomonas oryzae* pv. *oryzae* because the infection required longer incubating time and slow process.

The results of combination of stresses between ozone 100 ppb and *Xanthomonas oryzae* pv. *oryzae* showed SOD, APX and H_2O_2 values higher than single treatment of both *Xanthomonas oryzae* pv. *oryzae* and ozone concentration at 100 ppb. This could be explained by the co-reaction of stresses when plants were firstly affected by ozone causing plant samples injury, wound and plant pathogen. The ROS rapidly increased in plant cells due to stresses. However, the values were still greater than combining results when higher levels of ozone concentration treatments such as 200 and 300 ppb were applied. The effects of combining abiotic and biotic stresses were commonly found in several crops. Darmanti et al. (2016) found that in soybean the enzymatic antioxidants such as SOD activity was increased to eliminate ROS in plant cell under combining stresses of *Cyperus rotundus* L. and mild drought. Therefore, antioxidants played an important role in plant tolerance to stress combination both abiotic and biotic stresses (Suzuki et al., 2014).

The momilactone A and sakuranetin were significantly induced in rice leaves when plant samples were fumigated by high concentration of ozone at 300 ppb. Nevertheless, no momilactone A and sakuranetin were found in plant samples under lower ozone concentration fumigated at 200 and 100 ppb. These results were similar to the experiment of Eckey-Kaltenbach et al. (1994) reported higher ozone concentration at 200 ppb could induce Furanocoumarin phytoalexin rather than the ozone concentration at 100 ppb. The ozone affected the secondary metabolites in plant cell such as alkaloids, isopenoid and phenylpropanoid, which were directly stimulated to phytoalexin biosynthetic (Iriti and Faoro, 2009). The phytoalexin biosynthetic requires longer time of incubation than the other antioxidants. This was due to phytoalexin has more complex synthetic process of mevalonate or methylerythritol phosphate pathways (Liao et al., 2016; Dubey et al., 2003; Hunter, 2007). Both processes needed isopentenyldiphosphate and dimethylallyldiphosphate as the important components for phytoalexin synthesis (Okada et al., 2007).

When compare between phytoalexins and antioxidants biosynthesis, the results showed that phytoalexin need longer time and complex process to response to the stresses. The higher doses of ozone influenced and significantly induced phytoalexin production faster, compared with lower dose of ozone. Similar to ozone, *Xanthomonas oryzae* pv. *oryzae* the causing agent of bacterial blight disease in rice could induce momilactone A and sakuranetin production. The results showed momilactone A and sakuranetin were detectable only in the treatment group with pathogenic inoculation and not found in the non-inoculation or the control group. According to phytoalexin role as an antimicrobial substance, plant cell could generate phytoalexin to defense any intruder pathogen (Ahuja et al., 2012; Ejike et al., 2013; Jeandet et al., 2014; Pedras and Ahiahonu, 2005). Normally, the high amount of phytoalexin was found close to damaged area from the disease attack (Großkinsky et al., 2012). Phytoalexins would directly damage cell wall of intruding microorganisms by inhibiting growth, interfering metabolism and retarding colonizing process of pathogenic disease (Kanno et al., 2012). This experiment showed that after rice leaves were clipped and inoculated by *Xanthomonas oryzae* pv. *oryzae*, the substantial amount of momilactone A and sakuranetin were detectable in rice leaves after 24 hours for counter act with the microbes. Similarly, Li et al. (2012) found that in Japanese rice (*Oryzae sativa* subsp. *japonica*) the accumulation of phytoalexin occurres in 24 hours after inoculated by *Xanthomonas oryzae* pv. *oryzae*. Also the amount of phytoalexin both momilactone A and sakuranetin were dramatically increased from day 1 to day 3 after inoculation. This was due to incubation period of disease infection which the phytoalexin could be continuously generated to prevent further damage of infestation.

The combinatory treatment of two stresses, ozone concentration at 100 ppb and *Xanthomonas oryzae* pv. *oryzae* inoculation, was greater effect to momilactone A and sakuranetin synthesis than the single stress treatments. No momilactone A and sakuranetin were found when plant samples were fumigated by only ozone concentration at 100 ppb. Generally, the plants under ozone stress, the increase in ROS e.g. $O_2^{\bullet-}$ and H_2O_2 was likely expected (Ueda et al., 2013; Vaultier and Jolivet, 2015; Chaudhary and Agrawal, 2015; Kangasjärvi et al., 2005). Nevertheless, ozone concentration at 100 ppb was considered not strong enough to induce plant cell to synthesis phytoalexins. Similarly, in plants during invasion of pathogenic disease, ROS in cell was significantly increased due to stress (Yergaliyev et al., 2016; Baxter et al., 2013; Torres et al., 2006). The results clearly shows the combining effects from two stresses which caused more phytoalexins production particularly sakuranetin. Basically, sakuranetin was one of antioxidant in the group of phenolic phytoalexin (Cho and Lee, 2015). The substances in this group could perform as an antioxidant against ROS (Sakihama et al., 2002). Notably, some researches reported that both sakuranetin and momilctone A could be induced

Table 1. SOD, APX and H_2O_2 values of rice after ozone exposure, inoculation with *Xanthomonas oryzae* pv. *oryzae* and the combining of ozone and *Xanthomonas oryzae* pv. *oryzae*. The data represent the mean ± SE (n = 3). Different letters and * indicate significant differences among treatments at $p \leq 0.05$.

Experiment	Exposure/Inoculation (days)	Antioxidants and product		
		SOD (units/g FW)	APX (units/g FW)	H_2O_2 (nmol/g FW)
Ozone				
CF	1	3670.00±397.44	327.67±39.31	2.18±0.20
	2	3707.00±297.27	332.33±34.08	2.39±0.23
	3	3790.00±679.04	338.00±33.29	2.67±0.42
	4	4342.00±822.70	404.00±59.94	3.13±0.67
	5	3275.00±238.56	400.33±56.53	2.77±0.32
	$\bar{x}$	3756.80±222.58[c]	360.47±19.66[c]	2.63±0.18[d]
100 ppb	1	4425.00±733.73	337.00±49.24	3.77±0.43
	2	6512.33±650.37	372.00±42.58	4.82±0.43
	3	7074.33±127.69	414.00±48.23	6.75±0.39
	4	9499.00±599.50	691.33±41.90	8.41±0.66
	5	7787.00±233.55	594.33±97.35	5.75±0.63
	$\bar{x}$	7059.53±485.56[b]	481.73±43.06[b]	5.90±0.47[c]
200 ppb	1	5287.00±483.50	420.00±91.03	5.50±0.40
	2	7214.00±351.52	431.00±71.25	7.11±0.40
	3	9798.33±602.56	630.00±39.93	9.09±0.37
	4	12529.00±476.38	754.67±34.52	11.43±0.95
	5	8803.00±747.84	693.67±60.91	9.45±0.92
	$\bar{x}$	8726.27±683.95[b]	585.87±43.63[b]	8.52±0.60[b]
300 ppb	1	6674.00±535.05	454.33±57.34	7.10±0.24
	2	9584.67±716.66	471.00±50.33	11.33±0.63
	3	12228.00±714.52	1063.33±81.89	15.25±0.38
	4	15600.00±351.19	1410.33±79.69	19.32±0.99
	5	12599.00±298.92	1051.67±36.99	12.40±0.57
	$\bar{x}$	11337.13±831.70[a]	890.13±102.32[a]	13.08±1.11[a]
Non-inoculation Vs Inoculation				
Non-inoculation	1	3591.00±370.95	304.00±52.81	2.07±0.44
	2	3672.33±346.58	360.33±40.17	2.34±0.48
	3	4033.00±362.08	377.00±31.82	3.25±0.40
	4	4329.00±347.62	392.00±34.70	3.34±0.41
	5	4057.00±396.49	314.67±52.87	2.07±0.51
	$\bar{x}$	3936.47±155.87	349.60±18.82	2.61±0.23
Inoculation	1	4256.33±319.89	311.67±46.93	3.67±0.67
	2	6004.00±452.09	488.00±43.10	4.82±0.66
	3	8571.67±386.61	558.67±60.24	6.17±0.46
	4	9485.67±393.03	625.67±37.75	8.38±0.54
	5	7401.67±469.06	578.00±44.19	6.44±0.47
	$\bar{x}$	7143.87±519.44*	512.40±34.29*	5.90±0.48*
CF+Non-inoculationVs Combining ozone 100 ppb + Inoculation				
CF+Non-inoculation	1	3634.00±345.92	324.67±47.56	2.11±0.39
	2	3781.33±400.54	337.00±29.87	2.34±0.48
	3	4074.00±332.97	346.67±33.33	3.17±0.37
	4	5077.33±417.58	417.00±43.92	3.36±0.41
	5	4289.33±348.06	387.00±46.11	2.85±0.53
	$\bar{x}$	4171.20±194.86	362.47±17.95	2.77±0.21
Ozone 100 ppb+Inoculation	1	4525.00±404.10	333.33±56.59	4.12±0.38
	2	6958.33±332.39	562.67±49.13	5.26±0.43
	3	8768.33±311.61	664.67±57.52	7.25±0.43
	4	9147.00±474.79	744.00±47.29	9.22±0.33
	5	7491.00±509.46	539.00±41.40	6.31±0.41
	$\bar{x}$	7377.93±464.49*	568.73±41.75*	6.43±0.49*

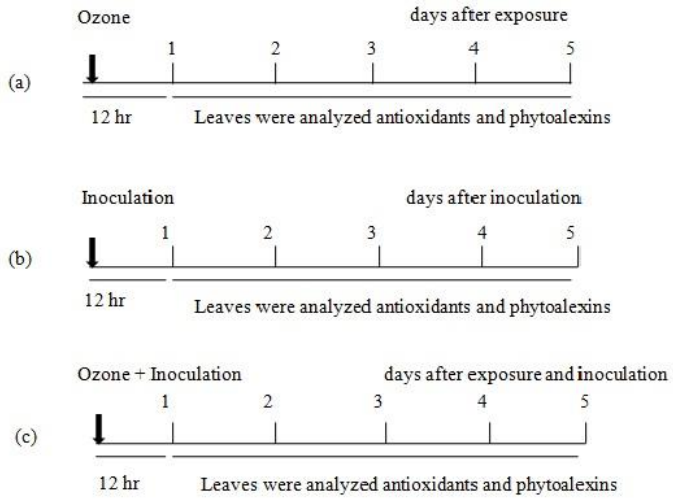

Fig 1. Timeline of the assay with detached leaves in dose-dependent effects of ozone (a), effects of *Xanthomonas oryzae* pv. *oryzae* inoculation (b) and the combining effects of ozone and *Xanthomonas oryzae* pv. *oryzae* (c).

Table 2. Momilactone A and sakuranetin values of rice after ozone exposure, inoculation with *Xanthomonas oryzae* pv. *oryzae* and the combining of ozone and *Xanthomonas oryzae* pv. *oryzae*. The data represent the mean ± SE (n = 3). NA shows "not detected".

Experiment	Exposure/Inoculation (days)	Phytoalexins	
		Momilactone A (ng/g FW)	Sakuranetin (ng/g FW)
Ozone			
CF	1-5	NA	NA
	$\bar{x}$	NA	NA
100 ppb	1-5	NA	NA
	$\bar{x}$	NA	NA
200 ppb	1-5	NA	NA
	$\bar{x}$	NA	NA
300 ppb	1	912.89±255.67	8684.44±3076.52
	2	1401.78±406.58	10344.89±4414.15
	3	3349.33±536.59	18346.67±3744.08
	4	4223.11±939.46	23360.00±5714.97
	5	2735.11±709.48	13937.78±4700.59
	$\bar{x}$	2524.44±401.37	14934.76±2201.30
Non-inoculation Vs Inoculation			
Non-inoculation	1-5	NA	NA
	$\bar{x}$	NA	NA
Inoculation	1	NA	NA
	2	74.49±4.66	NA
	3	NA	58.49±5.19
	4	NA	NA
	5	48.27±8.53	34.67±5.05
	$\bar{x}$	24.56±8.50	18.63±6.54
CF+Non-inoculationVs Combining ozone 100 ppb + Inoculation			
CF+Non- inoculation	1-5	NA	NA
	$\bar{x}$	NA	NA
Ozone 100 ppb+Inoculate	1-2	NA	NA
	3	97.51±16.78	4640.00±85.72
	4	85.64±11.59	NA
	5	64.71±5.08	3866.67±100.96
	$\bar{x}$	49.57±11.73	1701.33±561.16

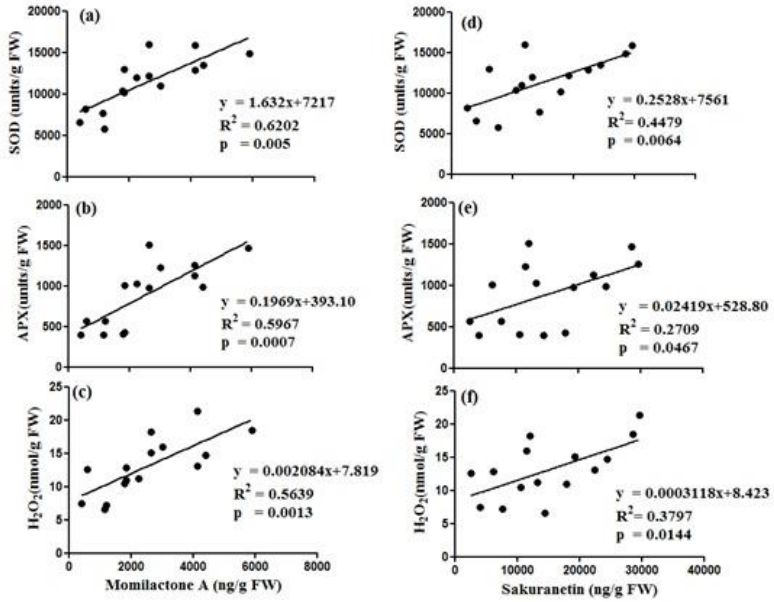

Fig 2. The correlation between SOD and Momilactone A (a); APX and Momilactone A (b); H_2O_2 and Momilactone A (c); SOD and Sakuranetin (d); APX and Sakuranetin (e); and H_2O_2 and Sakuranetin (f) in ozone concentration of 300 ppb. The data represent the mean ± SE (n = 3).

under abiotic and biotic (Dillon et al., 1997; Liu et al., 2012; Kanno et al., 2012), depend on type of stress. In this experiment the results showed that ozone had more capacity to induce sakuranetin rather than momilactone A. In contrast, *Xanthomonas oryzae* pv. *oryzae* showed stronger induction of momilactone A than sakuranetin. Interestingly, the combining effects from low-dose ozone and *Xanthomonas oryzae* pv. *oryzae* could induce sakuranetin more than momilactone A

Materials and Methods

Plant materials and fumigating chambers

Photosensitive rice (*Oryza sativa* L.) cultivar KDML105 was selected as the plant material because the cultivar was considered to show high sensitivity to several stresses (Jankangram et al., 2011; Dongsansuk et al., 2013). Seeds were germinated in soil in a 21×26 cm diameter of plastic tray filled with silt clay soil. Three seedlings of 15-day-old were transferred to 26 cm pot filled with silt clay soil collected from a paddy field. Three seedlings per pot were required. The total samples were setup in 48 pots. Plant samples were placed into the fumigating chambers for 30 days to allow adapting to the new environment before conduction of experiments. The chambers were ventilated with fresh air filtered by charcoal to lower background ozone concentration less than 10 ppb. Temperature was recorded by Testo 608-H1-Thermohygrometer (Testo Limited, UK). The average temperature was 25 °C at night and 30 °C during day time. Light was provided by two 400 W metal halide bulbs whose intensity was 400 PPFD from 30 cm height for 12 hours/day. Additional ozone was generated by an ozone generator model OZ-3020 (Belle, Ltd., Thailand) and monitored by photometric ozone analyzer – Model 400 (A Teledyne Technologies Company, USA).

Plant inoculation and infection

Xanthomonas oryzae pv. *oryzae* was isolated from a paddy field. Identification was made from infected leaves by the previously described method (Ghasemie et al., 2008). The bacterial suspension for inoculation was prepared at a concentration of $1x10^8$ cells/ml. Inoculation on rice leaves was carried out by leaf-clipping method (Shen et al., 2011).

Assays for antioxidants and hydrogen peroxide (H_2O_2)

The youngest expanded leaf (0.1 g fresh weight) was collected in 2-ml cap-locked tube with 67 mM phosphate buffer (pH 7.0) which was prepared form 1 mM EDTA and100% w/w PVPP. The sample was centrifuged at 10,000 g, 4 °C for 10 minutes. The supernatant was used for the following assays:

Superoxide dismutase (SOD; EC 1.15.1.1) was determined by the method previously described (Winterbourn et al., 1975).

Ascorbate peroxidise (APX; EC 1.11.1.11) was determined by the method previously described (Nakano and Asada, 1981).

Hydrogen peroxide (H_2O_2) was determined by the method previously described (Umponstira et al., 2006).

Quantification of phytoalexins

Leaves were cut into ca. 1-cm sections, and the leaves and 80% methanol (4 ml/0.1 g leaves) were put into a screw-capped test tube. The tube was shaken overnight by using a shaker. The supernatant was filtered through a 0.22-µm membrane filter. A 500 µl of the solution was analyzed using LC/MS/MS (Shimadzu, Japan) by the method previously described (Inoue et al., 2013).

Experimental design

Experiment 1: Dose-dependent effects of ozone.
Rice samples were subjected to ozone at concentrations of 100, 200 and 300 ppb. Rice was fumigated in the ozone fumigating chambers for 12 hours (light period 6.00 am-6.00 pm). The control group was grown in the charcoal-filtered (CF) air which ozone concentration was less than 10 ppb (Fig. 1a).

Experiment 2: The combining effects of ozone and Xanthomonas oryzae pv. oryzae.
Rice leaves were inoculated with *Xanthomonas oryzae* pv. *oryzae* ($1x10^8$ cells/ml) by clipping method. The control group (Non-inoculated) was prevented from the infection by using sterile technique (Fig. 1b).

The experimental design was arranged as 2×2 factorials treatment in completely randomized design and three replications. 45-day-old (tillering stages) rice samples were grown in the fumigating chamber under 100 ppb ozone combining with *Xanthomonas oryzae* pv. *oryzae* concentration at $1x10^8$ cells/ml. The control group (CF + Non-inoculated) plants were grown in the charcoal filter chamber with no elevated ozone and *Xanthomonas oryzae* pv. *oryzae*. The rice samples were taken at the end of the ozone fumigation period of 12 hours for antioxidants and phytoalexins analyses, when carried out once a day during the following 5 days (Fig. 1c).

Statistical analysis

The data was analyzed by one-way ANOVA with the Duncan's Multiple Range Test (DMRT) and independent Sample t-test. The SPSS software (SPSS Inc., version 19) was utilized for statistical tests.

Conclusion

This experiment clearly showed that both antioxidants and phytoalexins in rice could be induced by ozone and *Xanthomonas oryzae* pv. *oryzae* as the key triggers of abiotic and biotic stresses. The high concentration of ozone at 300 ppb was the most effective dose for phytoalexin induction, which took shorter incubating time than pathogenic infection. Ozone concentration at this level commonly initiated program cell death in rice which was similar to the pathogenic invasion, consequently, rice response by producing phytoalexin to cope with the stress. Even though ozone concentration at 100 and 200 ppb could only induce antioxidant production in rice but it was not enough for stimulating phytoalexin production. The combination of fumigating with low concentration of ozone at 100 ppb and inoculating *Xanthomonas oryzae* pv. *oryzae*, activated rice against stress by producing both antioxidants and phytoalexins better than single stress. The results from combining stress showed that both momilactone A and sakarunetin were occurred faster than sole stress of *Xanthomonas oryzae* pv. *oryzae*. Comparison between sakuranetin and momilactoneA induction in rice found that sakuranetin was generated better than momilactone A. Therefore, Sakuranentin, in the group of phenolic phytoalexin, plays an important role for plant defense mechanism as an antioxidant and anti-bacteria in rice. Finally, both ozone and *Xanthomonas oryzae* pv. *oryzae* as abiotic and biotic stresses could stimulate antioxidant and phytoalexin in rice responding to the stresses. The combining effect showed promising results which the further experiment required to understand the relations of sakuranetin with the other antioxidant pathways.

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