J. For. Res. (2018) 29(3):823–831
https://doi.org/10.1007/s11676-017-0481-9

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ORIGINAL PAPER

A study on JA- and BTH-induced resistance of *Rosa rugosa* 'Plena' to powdery mildew (*Sphaerotheca pannosa*)

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Received: 19 December 2016 / Accepted: 5 March 2017 / Published online: 19 September 2017
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Abstract Different concentrations of jasmonic acid (JA) and benzothiadiazole (BTH) were sprayed on 2-year-old *Rosa rugosa*'Plena' seedlings. The induced resistance of JA and BTH to *Sphaerotheca pannosa* (Wallr.) and the changes of their related physiological indices were investigated. Results showed that JA and BTH treatments had inhibitory impacts on *S. pannosa* infection. The optimal concentration of JA and BTH was 0.5 mmol/L for the disease-resistance induction of the leaves, its inductive effect was up to 66.36% for BTH and 54.49% for JA. Our results confirmed that exogenous JA and BTH significantly improved *R. rugose* 'Plena' resistance to *S. pannosa*. When treated with JA and BTH, activities of the three defense enzymes (POD, PPO, and PAL) increased significantly. Contents of total phenolics, flavonoids, and lignin also increased significantly. It is inferred from these results that exogenous JA and BTH could improve the resistance of *R. rugose* 'Plena' to *S. pannosa* through enhancing activities of the defensive enzymes and accumulation of secondary metabolites in the leaves.

Project funding: This work was supported by the Science Foundation of Heilongjiang Province of China (No.QC2014C012) and the Fundamental Research Funds for the Central Universities (NO. 2572016CA11).

The online version is available at http://www.springerlink.com

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Keywords Benzothiadiazole · Defensive enzyme · Jasmonic acid · Powdery mildew · *Sphaerotheca pannosa* (Wallr.) · *Rosa rugosa*'Plena' · Secondary metabolism

Introduction

Roses, one of the most important commercial crops, have a long history in the service of humankind (Rout et al. 1999; Uggla and Carlson-Nilsson 2005; Pati et al. 2006). *Rosa rugosa* 'Plena', belonging to the genus *Rosa*, is a perennial shrub with multiple values such as for ornamentals, and sources of food and medicines (Zhang et al. 2014). In recent years, the exploitation and industrial production of *Rosa*-based products have been growing rapidly. However, with the extensive cultivation and application of *R. rugose* 'Plena', it has been reported to be affected by severe pests and diseases (Yan et al. 2017). Powdery mildew, *Sphaerotheca pannosa* (Wallr.), is one of the most common and important plant diseases and has a significant negative impact on plant development, even leading to death of the plant (Pasini et al. 1997; Mortensen and Gislerød 2005). Traditional control by pesticides on the pathogenic bacteria pollutes environment and damages its pharmacological efficacy and edible value (Zhao et al. 2017). Studies on the induction of plant disease resistance provide new ideas for exploring environmentally friendly methods of disease control (Zhao et al. 2003; Wang 1994; Chen et al. 2007; Terry and Joyce 2004; Walters et al. 2005).

Peroxidase (POD) is an important active oxygen scavenger in plant cells. It catalyzes the oxidation reaction of phenolic substances and synthesis of coniferyl alcohol, a precursor of lignin. It participates in a polymerization reaction at the final step of lignin synthesis, so it has a close correlation with plant disease resistance (He 2001;

Shivakumar et al. 2003; Yanti 2015). Phenylalanine ammonia-lyase (PAL) is another defensive enzyme and involved in the generation and sediment of lignin in cells. Polyphenol oxidase (PPO) can catalyze the oxidation of phenolic substances to quinones which have a higher toxicity to pathogenic bacteria as compared with phenolic substances. The PAL and PPO both promote the lignification of cell walls to defend against various pathogenic bacteria (Wang et al. 2005; Babu et al. 2015). A previous study showed that increasing PAL, PPO and POD activities could reflect the dynamics of plant disease resistance and be considered as a biochemical index (Ran et al. 2004; Duzan et al. 2005).

Phenolic substances can accumulate in plants that have been infected by pathogenic bacteria or were treated by inductors. These phenolic substances could inhibit the growth and spore production of pathogenic bacteria and in addition, they would induce the generation of flavonoids such as pisatin and phaseolin to prevent secondary infection by some pathogenic bacteria (Chen et al. 2010a, b; Aires et al. 2011). When infected by pathogenic bacteria, treatment by a biological agent or induction by physical and chemical factors increases the lignin content significantly, leading to the thickening of cell walls which is beneficial to prevent the spread of infection of pathogenic bacteria (Ren et al. 2007). Plant secondary metabolites play important roles in improvement of disease resistance.

Jasmonic acid (JA) is a key hormone in plant resistance to pathogenic bacteria and pests. It is a signal molecule inducing the expression of resistance genes (Niu et al. 2011; Liu et al. 2006; Gaige et al. 2010). When plants are infected by fungi, jasmonic acid can induce physiological changes and form a defensive structure (Lorenzo et al. 2004). Benzothiadiazole (BTH) is an artificially synthesized inductor, the most commonly used chemical systemic-acquired resistance (SAR) inducer (Perazzolli et al. 2008). It does not display any noticeable direct activity against pathogens, but does increase crop resistance to diseases caused by viruses, bacteria and fungi by activating SAR-signaling pathways (Bovie et al. 2004) and plant priming (Kohler et al. 2002). Recent studies have shown that BTH has significant induced-resistance to some fungal diseases of crops such as: downy mildew in cucumber, powdery mildew in melon, *Sclerotinia sclerotiorum* in cauliflower, *Alternaria solan* in potato, *Peronospora hyoscyami* in tobacco (Bokshi et al. 2003; Perez et al. 2003; Cheng et al. 2006; Chen et al. 2011; Sun et al. 2012; Sillero et al. 2012). However, it is still not well known if induced-resistance to powdery mildew of *R. rugosa*'Plena' could be induced by applying JA or BTH, and how their resistance-physiological index might be changed.

In this study, we investigated the effects of JA and BTH on *R. rugosa*'Plena' resistance. The results obtained will provide a reference to the study of disease resistance inducing of *R. rugosa*'Plena' and might provide new ideas for exploring environmentally friendly methods of plant disease control.

Materials and methods

Materials

2-year-old healthy *R. rugosa*'Plena' seedlings with similar growth vigor were used.

Induction and inoculation methods

Based on preliminary experimental results, three concentrations of JA and three of BTH were used in this experiment. JA and BTH at concentrations of 0.1, 0.5 and 1.0 mmol/L were sprayed on the leaves of *R. rugosa*'-Plena'. The inductive agents were evenly sprayed on the leaves at a dosage of 20 ml per seedling. Each concentration treated 40 seedlings. *S. pannosa* inoculation was carried out by leaf spraying 2 days (48 h) after spraying with JA or BTH. Spores of *S. pannosa* were obtained from powdery mildew-infected leaves on outdoor cultivated *R. rugosa*'Plena' and were diluted with distilled water to a suspension liquid with a concentration of 3×10^4 spores/ml for inoculation. Distilled water spraying alone was used as the control 1 (CK1), and distilled water + spores inoculation was control 2 (CK2). Three replications were conducted for each treatment.

Leaf samples were collected on the 1st, 3rd, 5th, 7th, 9th, 11th and 14th day the second day after inductive agents were sprayed for 24 h, as well as on the 1st, 3rd, 5th, 7th, 9th and 12th day after *S. pannosa* was inoculated. Samples were immediately sealed into plastic bags, stored in a freezer at −80 °C for measurement.

Index measured and methods

Statistics on disease injury

The third day after *S. pannosa* inoculation, morbidity was investigated at intervals of 2 days, including the number of leaves at different infection classifications, as well as morbidity ratios, disease index, and inductive effects. Diseased leaf classification index was based on "Plant Disease Research Methods" (Yan et al. 2013a, b). In this study, disease degree was classified into five classes (Table 1).

Table 1 Rank of *S. pannosa* infected leaves of *Rosa rugosa*'Plena'

Rank	Disease resistance	Morbidity ratio	Representative value
1	Immune	Asymptomatic	0
2	Highly resistant	Lower than 1/4 of the ratio disease spots area to total leaf area	1
3	Moderately resistant	1/4–1/2 of the ratio disease spots area to total leaf area	2
4	Moderately sensitive	1/2–3/4 of the ratio disease spots area to total leaf area	3
5	Highly sensitive	More than 3/4 of the ratio disease spots area to total leaf area	4

Percentage of infected leaves = number of infected leaves/total number of leaves × 100%; Disease index = $\sum$ (number of the leaves at each infection rank × representative value at the corresponding rank)/(total number of leaves × the highest-rank representative value) × 100%; Inductive effect = (disease index for the control − disease index for the treatment)/disease index for the control × 100%

Physiological parameters

PAL was measured according to the method described by Hu et al. (2009) and Yan et al. (2013a, b). A 0.2 g leaf sample was weighed and ground in a mortar with liquid nitrogen, and 5-ml of an ice-cold borate buffer (5 mmol/L, pH 8.8) was added. The homogenates were centrifuged at 6000 r min^{-1} for 20 min at 4 °C. The supernatant was used as a crude extract for PAL activity assay. A 0.05 ml supernatant was mixed with 1.0 ml of 0.02 mol/L phenylalanine and 2.95 ml distilled water to produce a 4-ml reaction system. The mixtures were shaken well and transferred to a quartz cuvette and measured at 290 nm. The mixtures were placed in a thermostatic water bath at 30 °C for 30 min, and measured again at 290 nm. The reaction system without substrate had 1.0 ml distilled water added and was taken as the control. The results were expressed as U min^{-1} g^{-1} Fw.

PPO measurement is based on Yan et al. (2013a, b). A 0.5 g leaf sample was weighed and ground under liquid nitrogen, and 5-ml of 0.05 mol/L phosphate buffer (pH 7.0) added. The homogenate was centrifuged at 4000 r min^{-1} for 15 min at 4 °C. A 0.1 ml supernatant was mixed with 1.5 ml of 0.02 mol/L pyrocatechol and 1.5 ml phosphate buffer. The reaction system was left standing at 30 °C for 2 min and then measured at 398 nm. One unit of enzyme activity was defined as an increase in absorbance of 0.01/min.

Peroxidase (POD) was measured as described by Yan et al. (2013a, b) and Li et al. (2016). A 0.3 g leaf sample was weighed and placed into a pre-cooled mortar for grinding and 5 ml of 0.2 mol/L sodium phosphate buffer (pH 7.4) was added. The homogenate was centrifuged at 3000 r min^{-1} at 4 °C for 10 min. The supernatant was used as a crude enzyme solution and 0.1 ml of the supernatant and 2 µl guaiacol were transferred to a quartz cuvette. Optical density was record at 470 nm immediately within 2 min after adding 0.6% H_2O_2. The control mixture contained no hydrogen peroxide. Enzyme activity was measured at an absorbance of OD470, one unit of enzyme activity was defined as an increase in absorbance of 0.01/min. Enzyme activity was expressed as U min^{-1} g^{-1} Fw.

Total phenolics and flavonoids were measured according to the measurement of Lin et al. (1995) with a minor modification. A 0.2 g leaf sample was ground in a pre-cooled mortar with 5 ml of a 1% HCl-methanol solution. The liquid was centrifuged at 6000 r min^{-1} at 4 °C for 20 min, and then measured at 280 nm after standing at 4 °C for 24 h. Total phenolic content was calculated on the basis of a gallic acid standard curve ($y = 0.0066x + 0.0077$, $R^2 = 0.9963$), expressed by mg g^{-1}. Flavonoid content was measured at 325 nm, expressed by mg g^{-1}.

Lignin content was measured according to Yu et al. (2013) and Wang et al. (2015) with a minor modification. A 0.2 g leaf sample was ground in 95% ethyl alcohol. The liquid was filtered and washed in 95% ethyl alcohol three times and subsequently three times in a mixture of ethanol and n-hexane (1:2, V/V). The sediment obtained was dissolved in 25% acetyl bromide glacial acetic acid, and then placed in a water bath of 70 °C for 30 min. A 0.9 mL of 2 mol/L NaOH, 3 mL glacial acetic acid and 0.1 mL 7.5 mol/L hydroxylamine hydrochloride were added in sequence to the reaction system and centrifuged at 4500 r min^{-1} for 10 min. A 20 µL supernatant was diluted by adding three mL of distilled water and measured at 280 nm.

Data analysis

The data were analyzed using SPSS 19. One-Way ANOVA and LSD testing was used for significance analysis of difference.

Results and analysis

Effects of JA and BTH on leaf morbidity ratio

As shown in Table 2, the disease indices of leaves treated by different concentrations of JA and BTH were lower than

Table 2 Disease index of *S. pannosa* on *R. rugosa*'Plena' leaves treated with JA and BTH after inoculation

Days after inoculation (d)	Concentration (mmol/L)	Percentage of infected leaves (%)		Disease index (%)		Inductive effects (%)	
		JA	BTH	JA	BTH	JA	BTH
3	CK2	8.73	8.24	3.56	3.21	–	–
	0.1	7.45	6.87	2.07a	1.78a	41.85	44.55
	0.5	5.74	5.31	1.62a	1.08a	54.49	66.36
	1.0	6.86	6.54	1.94a	1.31a	55.81	59.19
5	CK2	16.37	15.34	8.24	7.17	–	–
	0.1	10.71	10.84	6.03a	4.78a	26.82	33.33
	0.5	8.86	8.12	3.94a	2.95a	52.18	58.86
	1.0	10.85	9.51	5.59a	3.94a	32.16	45.05
7	CK2	40.64	38.33	18.21	12.21	–	–
	0.1	30.56	28.42	13.52a	8.44a	25.76	30.88
	0.5	21.58	17.67	10.94a	5.47a	39.92	55.20
	1.0	25.67	20.26	12.98a	6.87a	28.68	43.73
9	CK2	57.86	55.48	27.74	23.95	–	–
	0.1	48.36	45.33	22.98a	17.67a	17.16	26.22
	0.5	39.25	34.26	19.37a	13.12a	30.17	45.22
	1.0	46.54	42.78	22.84a	15.46a	17.66	35.45
12	CK2	72.33	68.33	40.18	32.27	–	–
	0.1	60.36	54.29	35.79b	27.49b	10.79	14.39
	0.5	48.89	41.28	29.18a	20.46a	27.37	36.36
	1.0	52.51	49.43	34.39a	24.78a	14.35	22.88

"a" indicates significant difference ($p < 0.01$) between CK2 and treatments at the same time point; "b" indicates significant difference ($p < 0.05$)

those in CK2, indicating inhibitory effects of JA and BTH treatment on the *S. pannosa*. The inductive effect of 0.5 mmol/L BTH was optimal on the third day after inoculation, reaching 66.36%. After this point, it decreased with increasing time; on the 12th day it dropped to 36.36%. The 0.5 mmol/L JA had the optimal inductive rate of 54.49% on the third day, with an inductive effect of 27.37% on the 12th day. The disease indices of JA- and BTH-treated leaves on the 3rd to 9th day were significant lower than that of CK2 ($p < 0.01$); although the inductive effects of 0.1 mmol/L JA and/or BTH both decreased significantly on the 12th day, the disease index of the treated groups was still lower than that of CK2 ($p < 0.05$). In summary, JA and BTH treatments had inhibitory impacts on powdery mildew on *R. rugosa*'Plena', among them, the inductive effect of 0.5 mmol/L BTH was better than the other treatments.

Effects on defensive enzymes of *Rosa rugosa* 'Plena' induced by JA and BTH

PAL activity

Figure 1 shows that the activity of PAL in leaves treated with different concentrations of JA and BTH increased at first and then decreased subsequently. For the JA-treated leaves, PAL activity of 1.0 mmol/L JA was significantly higher than that of CK1 and CK2 ($p < 0.05$) at the same point in time. The maximum value of PAL activity occurred on the seventh day (the fifth day after inoculation with *S. pannosa*) after spraying with 1.0 mmol/L JA which was 1.58 times higher than that of CK1 and 1.29 times higher than that of CK2. Leaves treated with 0.5 mmol/L BTH showed higher PAL activity. The maximum value occurred on the seventh (fifth) day, 1.72 times as high as that of CK1 and 1.41 times as high as CK2. PAL values of 0.5 mmol/L BTH treated in the other time periods were also significantly higher than that of CK1, and CK2 ($p < 0.05$).

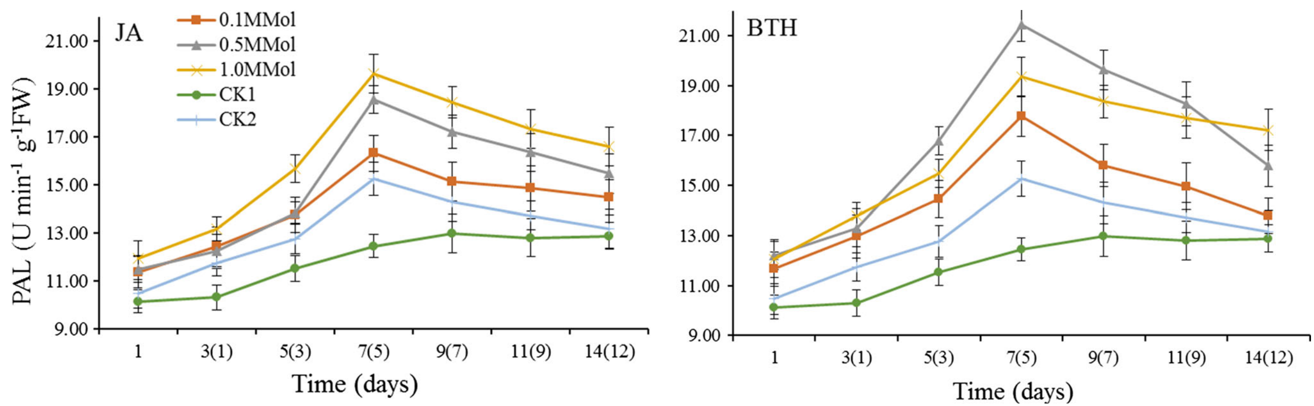

Fig. 1 PAL activity of *R. rugosa*'Plena' induced by JA and BTH (Parentheses indicated the numbers of days after *S. pannosa* was inoculated)

PPO activity

As shown in Fig. 2, PPO activity in the leaves treated by different concentrations of JA and BTH took on a trend of increasing first and decreasing subsequently. The maximum activity of PPO was in leaves treated with 0.5 mmol/L JA and BTH. PPO activity was significantly higher than that of CK1 and CK2 ($p < 0.05$) from the first day after spraying, reaching the maximum value on the seventh (fifth) day. The maximum value treated with 0.5 mmol/L JA was 1.50 times as high as that of CK1 and 1.29 times as high as that of CK2. In the 0.5 mmol/L BTH treatment, it was 1.58 times as high as that of CK1 and 1.35 times as high as that of CK2.

POD activity

POD activity of the treated leaves also took on a trend of increasing first and decreasing subsequently (Fig. 3). POD activity was highest in the 1.0 mmol/L JA and BTH treated groups, increasing from the first day after spraying, reaching the maximum value on the seventh (fifth) day, and then starting to decrease. During the whole sampling period, the POD values were higher than that of CK1 and CK2 ($p < 0.05$). The maximum POD value of 1.0 mmol/L JA was 1.89 times as high as that of CK1, and 1.43 times as high as CK2. The maximum POD value of 1.0 mmol/L BTH was 2.18 times as high as that of CK1, and 1.65 times as high as CK2. POD activity of the 0.5 mmol/L BTH treatment was highest on the seventh (fifth) day, being significantly higher than that of CK1 and CK2 ($p < 0.05$), and 1.75 and 1.33 times higher than CK1 and CK2, respectively. At the concentration of 0.5 mmol/L, the POD value of the BTH treatment was higher than that of the JA treatment during the sampling period but with no significant difference.

Effects on total phenolic content of *Rosa rugosa* 'Plena' induced by JA and BTH

As shown in Fig. 4, the total phenolic content of *R. rugosa*'Plena' treated with JA and BTH showed a trend of increasing first and decreasing subsequently; the optimal concentration for inducing was 0.5 mmol/L for both inductive agents.

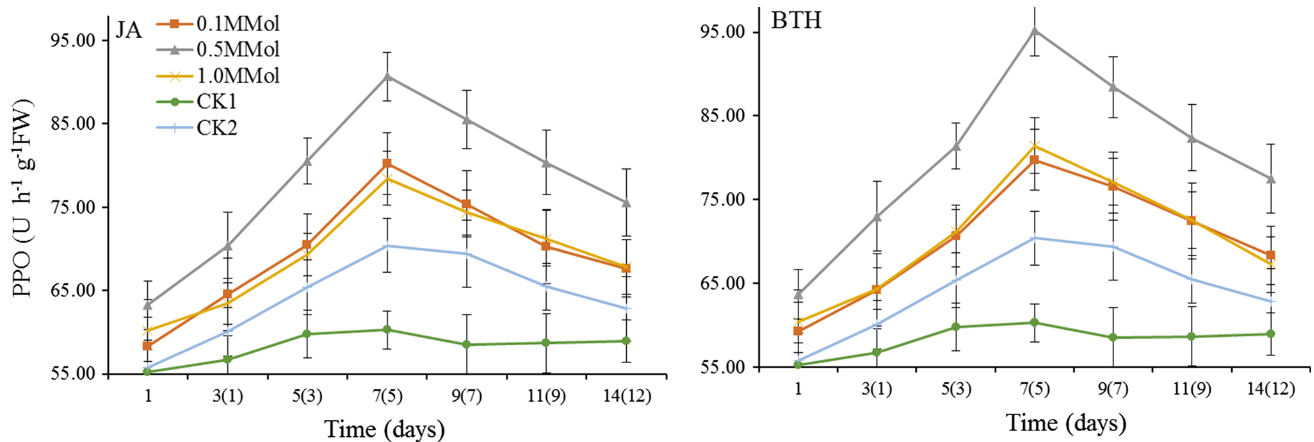

Fig. 2 PPO activity of *R. rugosa*'Plena' induced by JA and BTH

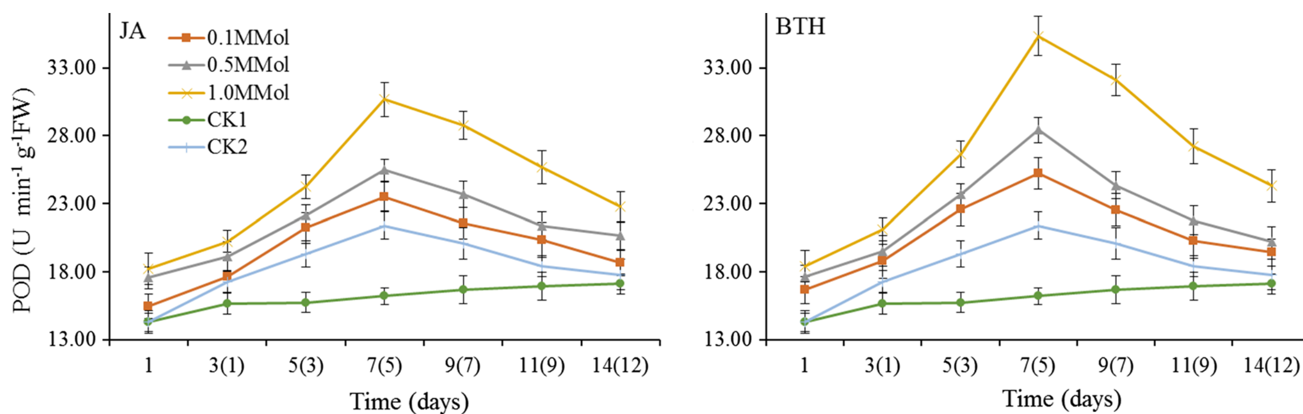

Fig. 3 POD activity of *R. rugosa*'Plena' induced by JA and BTH

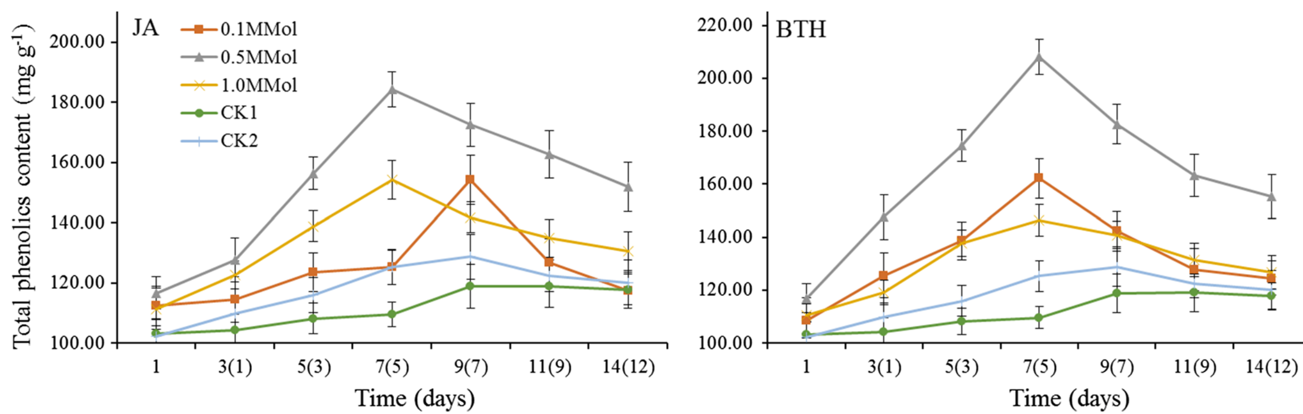

Fig. 4 Total phenolic content of *R. rugosa*'Plena' induced by JA and BTH

The total phenolic content of the 0.5 mmol/L JA treatment was higher than that of CK1 and CK2 at all time periods ($p < 0.05$). The maximum value was on the seventh (fifth) day which was 1.68-times higher than CK1 and 1.47-fold higher than CK2. Total phenolic content of the 0.5 mmol/L BTH treatment showed a similar trend to that of the 0.5 mmol/L JA treatment, total phenolic content was 1.90- and 1.66-fold higher than CK1 and CK2 ($p < 0.05$), respectively.

Effects on flavonoid content of *Rosa rugosa* 'Plena' induced by JA and BTH

Flavonoid content of *R. rugosa*'Plena' treated with JA and BTH increased first and decreased subsequently (Fig. 5). During the sampling period, the flavonoid content in the 0.5 mmol/L JA treatment was significantly higher than in the CK1 and CK2 treatments ($p < 0.05$), and reached a maximum value on the seventh (fifth) day. This was 2.5- and 1.42-fold higher than CK1 and CK2, respectively. After treatment with 1.0 mmol/L BTH, there was an induced rapid increase in flavonoid content which was significantly higher than CK1 and CK2 ($p < 0.05$). The maximum value occurred on the seventh (fifth) day which was 2.37 and 1.34 times higher than CK1 and CK2, respectively. In summary, the 0.5 mmol/L JA treatment produced the highest flavonoid content of all the treatments.

Effects on lignin content of *Rosa rugosa* 'Plena' induced by JA and BTH

Lignin content in JA- and BTH-treated leaves increased with increasing time after treatment (Fig. 6). The optimal inductive effects for the two agents was 0.5 mmol/L. Lignin content in JA treated leaves was 1.17-, 1.29-, 1.55-, 1.76-, 1.81-, 1.85-, and 1.85-fold higher than CK1 on the 1st, 3rd, 5th, 7th, 9th, 11th and 14th day, respectively, and was significantly higher than CK2 ($p < 0.05$). Similarly, lignin content in BTH treated leaves was significantly higher than in CK1 and CK2 ($p < 0.05$) leaves during the 1st–14th day after treatment, with the maximum value of 6.12 OD g^{-1} FW. The optimal concentration for lignin induction by both JA and BTH was 0.5 mmol/L.

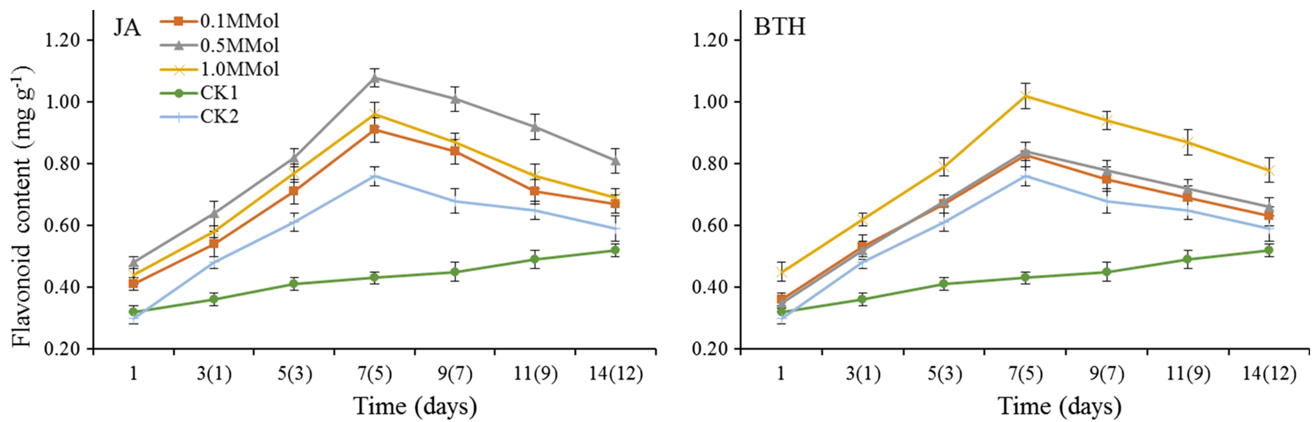

Fig. 5 Flavonoid content in *R. rugosa*'Plena' induced by JA and BTH

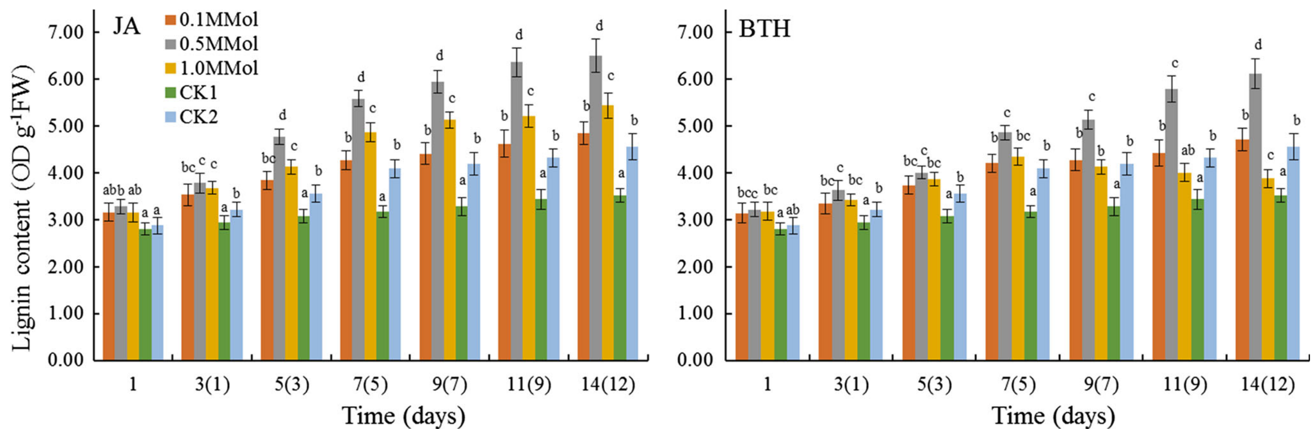

Fig. 6 Lignin content of *R. rugosa*'Plena' induced by JA and BTH Note: different letters indicates significant differences ($p < 0.05$) among JA or BTH treatments

Discussion and conclusions

Co-actions of multi disease-tolerance factors are usually involved in induced tolerance of plants for protection from infection by pathogens but are not depend on a single disease-resistant factor. Plant disease resistance is commonly induced by enzymatic actions. The activity of protective enzymes is usually increased when plants are infected by pathogens or are treated by inductive agents, which is considered an important mechanism for plant disease resistance induction (Mao et al. 2005). POD, PPO, and PAL are three vital protective enzymes in plant disease resistance reactions. Increasing their activities is considered a biochemical index of plant disease resistance (Chen et al. 2010a, b). POD plays a role in plant disease resistance by catalyzing phenolics to form quinones (Li et al. 2016). PPO and POD participate in lignin synthesis, leading to thickness of cell walls to protect plants from infection. (Shivakumar et al. 2003; Chen et al. 2010a, b; Yanti 2015). PAL is a key and rate-limiting enzyme in catalyzing L-phenylalanine to trans-cinnamic acid, it can improve the disease resistance of plants by promoting the synthesis of phenolic substances and lignin (Xue et al. 1983; Zhang et al. 1987). Total phenolics and flavonoids were higher in disease resistant cultivars than in disease-sensitive ones (Chen et al. 2010a, b); for example, BTH treatment improved cucumber resistance to downy mildew by inducing synthesis of phenolics and lignin (Wang et al. 2005).

In this study, different concentrations of JA and BTH increased activities of PAL, PPO, and POD in *R. rugosa*'Plena' leaves. This is in agreement with that reported by Yu et al. (2013) and Chen et al. (2011). The optimal concentration for both exogenous BTH and JA was 0.5 mmol/L for inducing *R. rugosa*'Plena' resistance to *S. pannosa*. After treatment with BTH and JA, the total content of phenols, flavonoids and lignin were higher than in the controls. The results in this study are consistent with melon resistance to powdery mildew (Chen et al. 2010a, b) and cucumber resistance to downy mildew (Wang et al. 2005), but with a difference in induction time possibly due to differences in the stress response ratio of different plants

to inductive agents. The results suggest that BTH- and JA-induced increase in activities of the protective enzymes and contents of secondary metabolites is a possible mechanism of enhanced resistance of *R. rugosa*'Plena' to *S. pannosa*.

Plant disease resistance levels depend on a response time and the ratio and quantity of accumulated resistant substances after the expression of resistance genes. Inductive agents have functions to induce the expression of disease resistance genes and subsequent synthesis and accumulation of disease resistant chemicals (Chen et al. 2010a, b). An improvement in disease resistance is the optimal selection for plants in defending against pathogen infections (Zeng et al. 2008). Our results demonstrated that different concentrations of JA and BTH induced increased resistance of *R. rugosa*'Plena' leaves to *S. pannosa*. Leaves treated with 0.5 mmol/L BTH had the maximum resistance to *S. pannosa*; its inductive effect reached 66.36%. Leaves treated with 0.5 mmol/L JA showed the optimal induction to plant disease resistance with its inductive effect reaching 54.49%. Both JA and BTH can improve the resistance to powdery mildew of *R. rugosa*'Plena'. This result is similar to the research of Chen et al. (2010a, b) and Niu et al. (2011) on melon and wheat. The optimum concentration of induction was different for different species. To conclude, BTH and JA treatments could increase the resistance of *R. rugosa*'Plena' to *S. pannosa* by improving the activities of the protective enzymes and the accumulation of secondary metabolites.

Plants use different defense signaling pathways to carry out the most effective response to pathogens (Thomma et al. 1998). At present, it is generally accepted that there are two main signaling pathways to induce disease resistance in plants. They are salicylate-dependent defense-response pathways and jasmonate-dependent defence-response pathways (Balbi and Devoto 2008). BTH, as a salicylic acid analogue, can induce the production of the salicylic acid pathway (Sun et al. 2012). Therefore, we concluded that JA- and BTH- induced resistance of *R. rugosa*'Plena' to *S. pannosa* might be related to the activation of salicylic acid and jasmonic acid pathways.

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