EFFECT OF FOLIAR APPLICATION OF SALICYLIC ACID, CHITOSAN AND BENZOIC ACID IN ELEVATING TOTAL PHENOL AND H_2O_2 CONTENT IN RICE LEAVES TO MODULATE RESISTANCE AGAINST BLAST DISEASE

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ABSTRACT

Rishad, M. B., Sultana, A., Chakraborty, S., Humaira, R. K. and Khokon, M. A. R. 2021. Effect of foliar application of salicylic acid, chitosan and benzoic acid in elevating total phenol and H_2O_2 content in rice leaves to modulate resistance against blast disease. Bangladesh J. Plant Pathol. 37(1&2):7-14

Efficacy of foliar application of three elicitors *viz.* salicylic acid, benzoic acid and chitosan was investigated to manage blast disease of rice. Salicylic acid, benzoic acid and chitosan were sprayed @ 0.1% at 15, 30 and 45Days After Transplanting (DAT) of rice seedlings cv. BRRI dhan28.Artificial inoculation was done to ensure disease development in the pots under net house. At 21 DAT the lowest disease incidence and severity was recorded in case of benzoic acid (11.97 %, 6.00 %) and chitosan (11.80 %, 6.00 %) application followed by salicylic acid (17.41 %, 12 %) compared to control. Application of elicitors elevated total phenol content at 7 Days After Treatment (DAT) and gradually elevated up to 21 DAT compared to its constitutive level while the highest phenol content (2885.147µg/g) in rice leaves was found by chitosan application. Likewise, a gradual increment of H_2O_2 content was also observed in all elicitors treated rice leaves up to 21 DAT. Foliar spraying of chitosan showed the highest H_2O_2(98.525 mol/g) content at 21 DAT followed by benzoic acid and salicylic acid. The findings of the present investigation provide evidence of accumulation of total phenol and H_2O_2 in rice leaves which might play important roles in suppressing incidence and severity of blast disease of rice.

Key words: Salicylic acid, Chitosan, Benzoic acid, H_2O_2, Phenol, Blast disease, Rice

INTRODUCTION

Rice (*Oryza sativa* L.) is an important crop for providing food for more than half of the world's population (Talbot and Foster 2001). However, rice blast, is one of the most destructive diseases caused by a filamentous ascomycete fungus *Magnaporthe oryzae*, reduces rice yield significantly every year (Skamnioti and Gurr 2009). The excessive and indiscriminate use of chemicals for controlling diseases may result in environmental pollution, ecological imbalance in soil and ailing effects on human health (Kaur 2014) and also develops resistant pathotypes and races. Therefore, strategies for the reduction of yield losses in an effective, economical and environmentally sustainable way need to be implemented urgently. Scientists around the world are now investigating the plant innate ability to be used intensively for controlling various constraints including pest and pathogens (Gaikwad and Balgude 2016). Compared with traditional strategies, inducing resistance offer the most effective and environmentally safe option for managing this pathogen. Plants can enhance their level of resistance either localized or systemic by inducers of biotic and abiotic origins, and thus become resistant/tolerant to subsequent infections. These compounds can be used depending on their efficacy in fields either alone or combined with fungicides.

Elicitors in plant biology include both substances of pathogen origin and compounds released from plants by the action of the pathogen (Ebel and Cosio 1994, Boller1995). Elicitors are now commonly used for compounds stimulating any type of plant defence (Ebel and Cosio 1994, Nürnberger 1999). Recent research has found remarkable similarities between the defensive mechanisms caused by general elicitors and

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animal innate immunity, leading to the enticing hypothesis that recognition of general elicitors contributes to plant innate immunity (Nürnberger and Brunner 2002). At low concentrations, elicitors serve as signal compounds, providing information to the plant that allows it to initiate defense and distinguishing it from toxins, which may affect the plant detrimentally at higher concentrations without active plant metabolism.

Previous researches revealed that some synthetic elicitors have been used as inducers of resistance in plants against pathogens, including chitosan (Bohland *et al.*1997, Reddy *et al.* 1999), salicylic acid analogues (Benhamou and Bélanger1998, Brisset *et al*. 2000), benzoic acid, 2, 6-dichloroisonicotinic acid (INA) and potassium salts. These products can modulate various cellular functions including reactive oxygen production, ion channel activity through phosphorylation and de phosphorylation of target protein, stomatal movement, up regulation of pathogenesis related genes (Khokon *et al*. 2010).Based on the previous findings, it is highly likely that salicylic acid, benzoic acid and chitosan can be used *in vitro* and foliar application of these components can induce resistance to overcome diseases as well as increasing final crop production (Mondal *et al.* 2012).

Chitosan has been used to some extent as an inducer of systemic acquired resistance in some crops. Antifungal activity of chitosan against some pathogen has also been investigated. Properties of chitosan for inhibition of pathogenic bacteria and fungi in antimicrobial films and edible coatings are used (Yarahmadi *et al.* 2014). Salicylic acid (2-hydroxybenzoic acid) is an important signal molecule involved in the improving of plant tolerance against abiotic and biotic stresses and plays a crucial role for the regulation of physiological and biochemical processes (Saruhan and Kadioglu 2012). In fact, salicylic acid does not only play an important role in SAR (Grant and Lamb 2006) but also has been used successfully to control several plant diseases (Jabnoun-Khiareddinie *et al.* 2015) especially against biotrophic pathogens (Campos *et al.* 2014).Under field conditions, spraying of benzoic acid led to a significant reduction in disease severity (DS) and disease incidence (DI) on the plant leaves, in addition to a significant increase in the grain yield and its components (Shabana *et al.* 2008).

Following elicitor perception, the activation of signal transduction pathways generally leads to the production of active oxygen species (AOS), phytoalexin biosynthesis, reinforcement of plant cell wall associated with phenyl propanoid compounds, deposition of callose, synthesis of defense enzymes, and the accumulation of pathogenesis-related (PR) proteins, some of which possess antimicrobial properties (Van Loon and Van Strien1999). Generation of Reactive Oxygen Species (ROS) are the prerequisite for initiation of the signalling network that will trigger the overall defense response (Hammond-Kosack and Jones 1996). Phenolic compounds are considered as secondary plant metabolites that are present in all tissues of higher plants, play an important role as a signal molecule in certain symbiotic relationships, and act as defense molecules against soil pests and pathogens (Makoi and Ndakidemi 2007).

Since use of elicitors as inducer of resistance in rice against blast pathogen has never been tried. Therefore, it is important to investigate the effectiveness of utilizing elicitors to manage blast disease of rice and their underlying mechanism. The research was undertaken to study the effect of organic and inorganic elicitors in reducing incidence and severity of blast disease of rice and examine whether these elicitors can accumulate total phenol and H_2O_2 which are related to developing resistance against disease.

MATERIALS AND METHODS

Isolation of blast pathogen

M. oryzae was isolated by picking up a single conidium following the method described by Jia (2009) with some modifications. At first, the infected portions of rice plant containing typical blast symptoms were cut into small pieces and surface sterilization was done by using 10% chlorox. Infected plant parts were placed on moist filter paper with the support of pipette tips to allow the sample not to come in direct contact with moist surface of filter paper placed inside the petri dish followed by covering with a lid, and incubated for 48 h. A part of infected plant samples was examined under a light microscope to check for sporulation of *M. oryzae*. A few conidia of *M. oryzae* were then allowed to the sterilized needle having agar layer in tip by touching sample surface placed on the stage of a stereo microscope. Conidia on

the needle tip were subsequently transferred onto PDA plates and spread with a glass rod. Plates were incubated for 2 days at 25C. Under a stereo microscope, a single germinated conidium was marked with a needle by making a stretch around it and then agar block containing the germinated conidium was cut and transferred to another PDA plate for mycelial growth. Then necessary multiplication of the pathogen was done.

Preparations of elicitors for *in-vivo* application

To prepare 0.1% chitosan, 0.1g powder was dissolved in concentrated acetic acid solution adding drop by drop then diluted with distilled water up to volume of 100 mL. Again, 0.1g of salicylic acid or benzoic acid was dissolved in ethanol (drop by drop) and volume up to 100 mL by adding distilled water to get 0.1% concentration.

Pot experiment

Seedlings of BRRI dhan28 were collected from Pathology Field Laboratory and transplanted in pots under controlled environment at Professor Golam Ali Fakir Seed Pathology Centre following Completely Randomized Design maintaining three replications. The doses of NPK fertilizers were applied as recommended by Adhunik Dhaner Chas Handbook (BRRI 2017) and other cultural operations were done.Salicylic acid, benzoic acid and chitosan were sprayed @ 0.1% at three stages *viz.* 15 DAT, 30 DAT, 45 DAT(Days after transplanting) of rice seedlings cv. BRRI dhan28.

Artificial inoculation of *Magnaporthe oryzae*

Isolates of *Magnaporthe oryzae* were grown in OMA petriplates for 15 days at room temperature. Conidial suspension was prepared by collecting the mycelia from the agar surface of each plate into 15 mL distilled water. Then the suspension was filtered through cheese cloth to remove the mycelial fragments and lumps of agar (Zhang *et al.* 2014). The inoculum was sprayed through hand sprayer at every 3 days after treatment application and covered with transparent polythene sheet.

Assessment of blast disease incidence and severity

All the leaves and spikes were examined for recording incidence following the formula given by James (1974):

Percent Disease Incidence =

$$\frac{\text{Number of Diseased leaves} \times 100}{\text{Total Number of Leaves}} \times 100$$

and Percent Disease Severity =

$$\frac{\text{Sum of all disease ratings}}{\text{Total No of Number of grade} \times \text{Maximum disease grade}} \times 100.$$

For the seedling inoculation tests, disease severity index was calculated using an ordinal scale from 0 to 5 as described by Urashima *et al.* (2005).

Determination of total phenolic contents

The grinded shoot samples were analyzed by using the following procedures as per the method developed by Sadsivam and Manickam (1996). Preparation of plant extracts was done by using 80% Ethanol, 10% Folin-Ciocalteu's reagent and 20% sodium-bicarbonate ($NaHCO_3$) reagents. The concentration of phenolics in shoot extracts were determined using spectrophotometric (Model: UH5300 Hitachi, Japan) method (Singleton *et al.* 1999). Methanolic solutions of the extract in the concentration of 1 mg/mL were used in the analysis. The samples were prepared in triplicate for each analysis and the mean values of the absorbance were obtained. The standard curve was prepared by plotting the catechol concentration on the X-axis and the absorbance values on Y- axis. The working standards were prepared by dissolving 100 mg catechol in 100 mL of distilled water and diluted to 10 times from the working standards, different concentrations ranging from 0.1 to 1.0 mL were prepared. From the standard curve, concentration of total phenols in terms of mg phenols/ 100 g plant material were estimated and converted to percent.

Reactive Oxygen Species (ROS) Determination

Determination of Hydrogen peroxide (H_2O_2) activity was done by using 0.1% Tri-chloro-acetic acid (TCA), Phosphate buffer (10mM) and Potassium iodide (1 M) reagents. Fresh leaf sample of 0.1 g was homogenized in a mortar with pestle using 1 mL of 0.1% tri-chloro-acetic acid (TCA) and homogenizes it at 4 C. After centrifugation at 10000 rpm for 15 min, keep the supernatant in dark for 1 h after mixing with phosphate buffer (10 mM, pH 7.0) and potassium iodide (1 M)

(adding in the ratio 0.5 mL: 0.5 mL: 1 mL). Absorbance of the resulting solution was recorded at 390 nm. All the steps were performed at 4C except absorbance measurement. The peroxide concentration H_2O_2 (µmole g^{-1} FW) was determined using Extinction Coefficient of H_2O_2 = 0.28 $\mu M^{-1}cm^{-1.}$

Data collection and analysis

Disease incidence and severity, phenolic contents and ROS were determined at 7, 14 and 21 days after every treatment application. All collected data were statistically analyzed by using WASP package program. The significance of the difference among the mean was calculated by LSD (Least Significance Difference) test.

RESULTS AND DISCUSSION

Effect of foliar application of elicitors on the incidence and severity of blast disease

Salicylic acid, benzoic acid and chitosan @ 0.1% were applied on the foliage at three growth stages *viz*. 15, 30 and 45 Days after transplanting to examine the effect on the incidence of blast disease in rice cv. BRRI dhan28 under artificial inoculated condition (Table 1). Chitosan and benzoic acid treatments showed the lowest blast incidence compared to untreated control at 7, 14 and 21 Days After Treatment. Likewise, incidence, severity of blast disease also varied significantly at different days after foliar application of elicitors (Table 1). Chitosan and benzoic acid showed the lowest severity of blast disease compared to untreated control at 7, 14 and 21 DAT. Salicylic acid also reduced incidence and severity of blast significantly compared to control treatment. These findings are in agreement with the findings of Yarahmadi *et al.* (2014) where they reported that chitosan has antifungal activity against *R. stolonifer*. Shabana *et al*. (2008) where they reported that benzoic acid, salicylic acid and hydroquinone had significantly reduced disease severity and disease incidence of brown spot of rice. Yoon*et al.* (2012) also showed the antifungal activities of benzoic acid against *M.oryzae* on rice plants, *P. infestans* on tomato plants and *P. recondita* on wheat plants.

Table 1. Effect of foliar application of elicitors on the incidence and severity of blast of rice at different days after Transplanting (DAT) of rice under artificial inoculated condition in net house

Treatments	Incidence of blast disease (%)			Severity of blast disease (%)		
	7 DAT	14 DAT	21 DAT	7 DAT	14 DAT	21 DAT
T_0 (control)	23.14 a	22.14 a	22.24 a	19.62 a	19.62 a	19.61 a
T_1 (0.1% SA)	18.36 b	17.36 b	17.41 b	11.40 b	11.40 b	12 b
T_2 (0.1% BA)	12.65 c	11.68 c	11.97 c	6.10 c	6.00 c	6.00 c
T_3 (0.1% CH)	12.89 c	12.39 c	11.80 c	6.00 c	6.10 c	6.00 c
CV (%)	4.28	3.92	4.06	0.57	6.22	6.95
$LSD_{0.05}$	1.99	1.73	1.78	1.94	1.98	0.17

DAT= Days After Treatment, SA= Salicylic Acid, BA= Benzoic Acid, CH= Chitosan

Effect of foliar application of elicitors on total phenol content of rice leaves at different DAT

Phenol is one of the important signal molecules that indicate the induction of resistance of host plants. Here, also the total phenol was quantified in the treated leaves at different time intervals and compared with untreated control (Table 2). Total phenol content significantly increased by foliar application of elicitors at all growth stages compared to control. An increasing trend of phenol content found up to 14 Days After Treatment application and after that the amount started to decline. The highest phenol content (76.61 %) was recorded in chitosan (0.1 %) over control followed by benzoic acid (72.74 %) and salicylic acid (67.21 %) at 14 DAT. Kulbat *et al.* (2016) reported that phenolic compounds are plant secondary metabolites playing important roles in plant resistance. Yao*et al.* (2007) reported that phenolic compounds are important plant secondary metabolites for plant defense. Phenolic compounds and lignin surrounding epidermal cells are deposited by the increased activity

of PAL activity in inoculated leaves of resistant cultivars of melon (Ge*et al*. 2013). Nikraftar *et al*. (2013) reported that phenolics production in tomato plants act as effective mechanism in tomato - *R. Solani* pathosystem. Taoutaou *and Emma* (2013) identified some phenolic compounds that determine resistance in potato against late blight agent *P. infestans*. The decrease in phenolic content can be attributed to oxidative polymerization of phenolics into melanin in necrotic tissues or incorporation of phenols into lignin (Thompson 1964).

Table 2. Effect of foliar application of elicitor on total phenol content in rice leaves at different DAT

	Total Phenol (µg/g)		
Treatments	7 DAT	14 DAT	21 DAT
T_0 (Control)	1708.193 d	1554.352 d	1791.856 d
T_1 (0.1% SA)	1805.124 c	2598.964 c	2282.628 c
T_2 (0.1% BA)	2405.142 b	2685.114 b	2396.370 b
T_3 (0.1% CH)	2885.147 a	2745.164 a	2484.063 a
CV(%)	0.034	0.030	0.031
$LSD_{0.05}$	1.98	2.02	1.87

DAT= Days After Treatment, SA= Salicylic Acid, BA= Benzoic Acid, CH= Chitosan

Effect of foliar application of elicitors on ROS content of rice leaves at different DAT

Reactive oxygen species is another important signalling intermediate for induction of resistance to defend pathogen invasion. Hydrogen peroxide (H_2O_2) was quantified in treated rice leaves at 7, 14 and 21 Days After Treatment (Table 3). H_2O_2 increased gradually with times in all treatments, but the highest elevation was recorded in case of chitosan at 21 DAT (63.65 %) followed by benzoic acid (22.32 %) and salicylic acid (1.89 %) over the control. Mandal *et al*. (2013) reported similar results in tomato plants against *R. solanacearum.* They revealed that the reduced disease incidence in tomato by SA and CHT might be a result of cell wall strengthening through deposition of lignin and induction of defense enzymes. Fungicidal activity of chitosan has already been reported by several researchers. Chitosan inhibits *in vitro* mycelial growth, sporulation, spore viability, germination and the production of fungal virulence factors of many pathogenic fungi like *B. cinerea*, *A. alternata*, *C. gloeosporioides*, *R. stolonifer* (Badawy and Rabea 2011). Xing *et al*. (2015) reported that chitosan acted as a powerful biotic elicitor at low molecular weight, ready to prompt plant protection responses and to initiate various pathways that induce the crop resistance to diseases. Around the infection site, the induction of the hypersensitive response is mainly caused by chitosan which results into the programmed cell death (Vasil'ev *et al*. 2009). These finally synthesize and accumulate different secondary metabolites with dynamic roles in defence: phenolic compounds such as lignin, callose, phytoalexins, PR proteins. Chitosan persuades the expression of PR proteins (NPR1) not only in roots (Lopez-Moya *et al*. 2017) but also in leaves (PR1 and PR5) (Beatrice *et al*. 2017). From the present experiment, it is clear that elicitors could be an effective basis of increasing Phenol and H_2O_2 content in rice leaves which is in the long term responsible for inducing resistance against *M. oryzae.*

Table 3. Effect of foliar application of elicitors on ROS content in rice leaves at different DAT

Treatments	ROS content		
	H_2O_2 (nmol/g FW)		
	7 DAT	14 DAT	21 DAT
T_0 (Control)	7.650 c	27.527 c	60.203 d
T_1 (0.1% SA)	8.079 c	40.540 b	61.345 c
T_2 (0.1% BA)	10.653 b	49.978 a	73.645 b
T_3 (0.1% CH)	13.513 a	50.121 a	98.525 a
CV(%)	7.086	1.689	0.007
$LSD_{0.05}$	1.95	1.75	1.87

DAT= Days After Treatment, SA= Salicylic Acid, BA= Benzoic Acid, CH= Chitosan

ACKNOWLEDGEMENT

The study was funded by the Ministry of Education (MoE), The peoples' Republic of Bangladesh, we gratefully acknowledge for awarding research fund (Project no. 2018/504/MoE - LS 2017357).

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