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Reactive Oxygen Species Play a Role in the Infection of Rice by Bacterial Leaf Blight Pathogen *Xanthomonas oryzae* pv. *oryzae*

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ABSTRACT: *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causing bacterial leaf blight of rice is a global problem in rice production. Reactive oxygen species (ROS) are produced as a normal product of plant cellular metabolism. Various environmental stresses including pathogen attack trigger excessive production of ROS causing progressive oxidative damage leading to programmed cell death and ultimately activating the host defense responses. Inoculation of *Xoo* by leaf clipping method resulted in more lesion length in the susceptible cultivar TN-1 compared to resistant cultivar BPT-5204. Further, quantification of ROS *viz.,* Hydrogen peroxide (H_2O_2) and Superoxide (O_2^-) by 3, 3- Diaminobenzidine (DAB) and Nitro blue tetrazolium (NBT) assays respectively showed increased levels of both H_2O_2 and O_2^- in the resistant cultivar BPT-5204 at 6 days post inoculation (dpi) and it was highest in the leaf tissues collected at 10dpi. The levels of H_2O_2 and O_2^- decreased significantly after 2 dpi in the control plants of both cultivars BPT-5204 and TN-1. In the susceptible cultivar TN-1, the levels of H_2O_2 and O_2^- decreased significantly from 4dpi. There was 78% fold increase in the production of H_2O_2 and 70% fold increase in the production of O_2^- in the resistant cultivar compared to the susceptible cultivar. Oxidative burst or the rapid production of ROS in response to pathogenic invasion aids the host plant in establishing various defensive barriers against infections. As a result, pathogen infections cause rapid accumulation of ROS, which are important in restricting pathogenic entry, inducing signal transduction of various defence responses or programmed cell death. We speculate that the increased production of the ROS *viz.,* H_2O_2 and O_2^- in the resistant variety trigger the defense pathways in the host and thereby limit the disease.

Keywords: *Xanthomonas oryzae* pv. *oryzae*, Reactive oxygen species, hydrogen peroxide, superoxide.

INTRODUCTION

Rice is one of the most fundamental staple crops world-wide, and provides sustenance and nutrition for over half the global population. It is one of the most important food crops throughout the world growing in various agro-climatic conditions (Debnath *et al*., 2013). *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) causing bacterial leaf blight (BLB) disease is a potential threat to rice cultivation throughout the world including India (Mondal *et al*., 2016).

Bacterial leaf blight (BLB) of rice caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is the most destructive disease of rice, the staple food of almost half of the world's population, causing yield loss of 20-30 per cent (Amin *et al*., 2022). BLB is one of the oldest recorded rice diseases, which was first found by a farmer in the Fukuoka area of southern Japan in 1884 (Nino-Liu *et al*., 2006). *Xoo* enters leaf through the hydathodes or wounds and multiplies in the intercellular spaces of the underlying epitheme, thereby propagating to reach the xylem vessels. The bacteria move through the veins of leaves and spread throughout the plant. Water-soaked spots at the leaf tips and margins are the initial symptoms and later the leaves become chlorotic and necrotic along the leaf veins (Lee *et al*., 2011).

Reactive oxygen species (ROS) are the by-products of different metabolic pathways, which are produced by the incomplete reduction of oxygen. They are highly reactive and reduced forms of oxygen molecules, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and singlet oxygen (O_2) (Yang *et al*., 2013; Gilroy *et al*., 2014; Camejo *et al*., 2016). They are essential for cellular functioning and their level in cellular system is tightly regulated by the antioxidant defense system.

ROS play important roles in maintaining normal plant growth, and improving their tolerance to stress (Huang *et al*., 2019). They are regarded as by-products of plant aerobic metabolism and are generated in several cellular compartments such as chloroplasts (Dietz *et al*., 2016), mitochondria (Huang *et al*., 2016), and peroxisomes (Sandalio and Romero-Puertas 2015). ROS not only cause irreversible DNA damage and cell death, but also function as important signalling molecules that regulate normal plant growth, and responses to stress. This suggests that ROS have a dual role *in vivo* depending on

different levels of reactivity, sites of production and potential to cross biological membranes (Miller *et al*., 2010).

Plants are exposed to a variety of biotic stresses as they grow and develop, which has a greater impact on crop productivity. Plants that encounter biotic stresses change in a variety of physiological, biochemical, molecular, and metabolic ways. ROS production is one of the significant alterations that is seen in response to pest and pathogen attack. Although chloroplasts, mitochondria and peroxisomes are the main locations where ROS are generated, endoplasmic reticulum, cell walls, cell membranes and apoplast are secondary locations where ROS are produced under stress. Free radicals (O_2^-, OH^-) and nonradicals (H_2O_2, $^1O_2$) are both included in ROS (Suman *et al*., 2021).

Doke (1983) first reported the oxidative burst, demonstrating that potato tuber tissue generated super-oxide that is rapidly transformed into hydrogen peroxide following inoculation with an avirulent race of *Phytopthora infestans*. Similar H_2O_2 production is also observed during avirulent interaction between the bacteria *Pseudomonas syringae* strain DC3000 and *Arabidopsis* (Alvarez *et al*., 1998). Radwan *et al.* (2010) observed higher H_2O_2 concentrations in *Vicia faba* leaves infected with Bean Yellow Mosaic Virus than those of the corresponding controls. A concentration of 0.1mM H_2O_2 completely inhibited the growth of cultured bacteria *Pectobacterium carotovorum* subsp. *Carotovorum* and resulted in >95% inhibition of *Phytophthora infestans* growth (Wu *et al*., 1995). PAMPs from gram-negative bacteria like flg22 (a peptide derived from flagellin), harpin or lipopolysaccharides can all induce a transient but robust production of ROS (Felix *et al*., 1999; Krause and Durner 2004).

Rboh (*Respiratory Burst Oxidase Homologs*) genes were first identified to generate ROS in response to biotic stress. Study on mutant and antisense lines of *Rboh*genes *Atrboh D* and *Atrboh F*, gave the proof of production of oxidative burst by RBOH in pathogen infection in *Arabidopsis* plants against Pseudomonas syringae pv. tomato DC3000 (Torres *et al*., 2002). O_2^- or its subsequently resulting products are also capable of potentiating a signaling process, activating defence responses. Oxidative burst suppresses the bacterial growth in the plant through induction of HR mechanism (Dey *et al*., 2020).

Plant NADPH oxidases are homologs of mammalian RESPIRATORY BURST OXIDASE HOMOLOGs (RBOHs) with the apo- plastic oxidase domain generating O_2^- to the apoplast and the N- terminal regulatory domain directed to the cytoplasm (Marino *et al*., 2012). Superoxide anion is the first free radical generated by partial reduction of O_2 within the thylakoid membrane-bound to photosystem I (Gill and Tuteja 2010). The mechanistic phenomenon involves functional regulation of two main ROS-producing enzymes, i.e., cytochromec oxidase and alternative oxidases that catalyze the transfer of electrons to O_2, resulting in the formation of O_2^- and H_2O_2 (Akter *et al*., 2015). The RBOH-mediated ROS burst involved in plant defense against pathogens by activation of the hypersensitive response and regulation of innate immunity (Torres *et al*., 2002).

In this study, we have investigated the production of ROS molecules *viz*., H_2O_2 (Hydrogen peroxide) and O^{2-} (superoxide) in two contrasting rice cultivars against *Xoo*- BPT-5204 (resistant) and TN-1 (susceptible). We found out that the resistant cultivar produced significantly higher amounts of both H_2O_2 and O^{2-} thereby limiting the pathogen growth in host cells when compared to susceptible cultivar TN-1.

MATERIALS AND METHODS

Bacteria and plant growth conditions. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*)isolate KA from Gangavathi region of Karnataka was used in this study. Two rice cultivars (*Oryza sativa* L. cultivar BPT-5204 and TN-1), which are resistant and susceptible to *Xoo* respectively, were used. Rice seeds were imbibed in sterilized water at 28°C for overnight, germinated on moist tissue paper for 3 days, and transferred to soil in a growth chamber at 28°C (16/8 h day/light cycle, 70% relative humidity). Rice at the tillering stage (35-40 days after sowing) was used for *Xoo* inoculation.

***Xoo* culture and infection of rice.** The isolate KA was grown on Modified Nutrient Agar (MNA) plate (Nutrient Agar@ 28g/litre; 0.25% Glucose) at 28°C. For *in vitro* culture, *Xoo* cells were incubated in Modified Nutrient Broth (MNB) medium and shaken at 200 rpm in the dark at 28°C for 48h. Cultured cells were washed twice with distilled water and diluted to 10^8 cells mL^{-1} (OD_{600}= 0.5) with water containing 0.01% tween-20 and 10mM MES buffer. Leaves from 50-day-old (at the tillering stage) rice cultivars were artificially infected with *Xoo* strains (10^8 cells mL^{-1}) by clipping the leaf tips with sterile scissors dipped in *Xoo* suspension (Ke *et al*., 2017). For control plants, leaf tips were cut by dipping the scissors in 10mM MES buffer. Infected and control rice plants were grown under normal light conditions (16 h light/ 8 h dark) with 90% relative humidity in glass house.

ROS detection

A. Quantification of H_2O_2: 0.1 percent of DAB (3, 3^1-diaminobenzidine) solution was prepared by adding 0.1g of DAB in 95ml of autoclaved distilled water; 5ml of 200mM Sodium phosphate dibasic and 50µl of Tween 20. pH was reduced to 3.0 with 0.2M HCl and kept for overnight stirring on magnetic stirrer. DAB solution was prepared freshly the day prior to its use. Rice leaf tissues along with the inoculated end were collected and immediately immersed in DAB solution. Vacuum infiltration was carried out for 5minutes to ensure that DAB solution was uptaken by the rice leaf tissues. After 1h incubation in DAB solution, the chlorophyll was bleached by using bleaching solution (Acetone: Dimethyl Sulfoxide-1:1) and directly visualized for DAB staining and photographs were taken. Later the samples were grinded in liquid nitrogen and homogenised with 0.2M perchloric acid followed by centrifugation for 10minutes @ 12,000 rpm. The supernatant was collected and absorbance was observed @ 450nm.

B: Quantification of O_2^-: 0.2% of Nitro Blue Tetrazolium (NBT) was prepared by dissolving 0.2 g of

NBT in 100ml of 50mM of Sodium phosphate buffer. NBT was freshly prepared prior to use. Rice leaf tissues along with the inoculated end were collected and immediately immersed in NBT solution and incubated for 1h. After 1h incubation in NBT solution, the chlorophyll was bleached by using bleaching solution (Acetone: Dimethyl Sulfoxide-1:1) and directly visualized for NBT staining and photographs were taken. Later the samples were grinded in liquid nitrogen and homogenised with 50% acetic acid followed by centrifugation for 10 minutes @ 12,000rpm. The supernatant was collected and absorbance was observed @ 560nm.

RESULTS AND DISCUSSION

***Xanthomonas oryzae* pv. *Oryzae* infection on BPT and TN-1 rice cultivars:**

Rice cultivars vary in their reaction to pathogenic races and inoculum doses of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). The infection of *Xoo* on the contrasting varieties of rice were observed upto 10 days post inoculation. The resistant cultivar BPT-5204 showed less disease progression with average lesion length of 1cm at 10 days post inoculation (Fig. 1A). The disease severity was more in the susceptible variety TN-1 with average lesion length of 4cm at 10 days post inoculation (Fig. 1B). In the susceptible variety, the leaves showed water soaked lesion on 4th day post inoculation which progressed into yellowing followed by necrosis at 10 days post infection. The symptoms of bacterial leaf blight start with tiny water soaked lesion on the edges of the leaves which late coalesce and turn yellow. The chlorotic lesions enlarge along the veins and turn necrotic (Mizukami and Wakimoto 1969). Bakade *et al*. (2021) studied the reaction pattern of DRR- *Xoo* isolate on the rice cultivars BPT-5204 and TN-1 for upto 12 days and showed that TN-1 exhibited increased necrotic lesion compared to BPT-5204 wherein the symptom was restricted to the cut end of the artificially inoculated leaf.

Quantification of H_2O_2 by using DAB staining:

Hydrogen peroxide (H_2O_2) a moderately reactive ROS is formed when O^{-2} undergoes both univalent reduction as well as protonation. H_2O_2 is produced in plant cells not only under normal conditions, but also by oxidative stress, caused by factors like drought, chilling, intense light, UV radiation, wounding and pathogen infection (Sharma *et al*., 2012).

H_2O_2 production was initially more at 2 days post inoculation in all the treatments *viz*., BPT-5204: Control; BPT-5204: *Xoo*; TN-1: Control and TN-1: *Xoo*. This was due to wounding caused by clipping of the leaf tips to inoculate the plants. As the disease progressed, the production of H_2O_2 was reduced in the control plants as well as the susceptible cultivar TN-1 infected with *Xoo*. But production of H_2O_2 was gradually increased in the resistant cultivar BPT-5204 infected with *Xoo*. H_2O_2 oxidises DAB forming dark brown precipitate. The bleached leaves of BPT-5204 showed increased levels of this precipitate compared to TN-1 (Fig. 2). The leaves of BPT-5204 showed higher H_2O_2 levels at all time points from 2 to 10 dpi compared to TN-1 genotype (Fig. 3). Bakade *et al*. (2021) quantified H_2O_2 levels in BPT-5204 and TN-1 varieties of rice and recorded higher absorbance levels in the latter which showed higher H_2O_2 levels in all time points from 2 to 10 dpi compared to TN-1 genotype, suggesting stronger defense responses in BPT-5204 to dampen *Xoo* colonization. In a similar study, Azarabadi *et al*. (2014) reported more H_2O_2 generation in resistant cultivars of pears infected with *Erwinia amylovora* accompanied with slower necrosis progress.

During pathogenesis, the oxidant H_2O_2 is thought to play two distinct roles. The first one includes limiting pathogen growth, while the second one involves stimulating the production of phytoalexins and proteins related to pathogenesis (PR). Many studies back up the concept that H_2O_2 is a possible signal for the induction of a subset of defence genes during pathogenesis. H_2O_2 being relatively most stable form of ROS may act as secondary messenger in several signaling circuits, since it could diffuse from the site of production and subsequently cause microburst of ROS formation (Dey *et al*., 2020).

Quantification of O_2^- by using NBT staining:

Superoxide anion is an atypical ROS which is generated not by electron transfer to O_2, but rather by the reaction of chlorophyll. Environmental stresses like salinity, drought, pathogen attack and heavy metals cause stomatal closure, leading to insufficient intracellular CO_2 concentration. This favors the formation of O_2^- (Das and Roychoudhary 2014).

O_2^-production was initially more at 2 days post inoculation in all the treatments *viz*., BPT-5204: Control; BPT-5204: *Xoo*; TN-1: Control and TN-1: *Xoo*. This was due to wounding caused by clipping of the leaf tips to inoculate the plants. The oxidative burst in response to the pathogen attack occurs in two phases: 15-30 minutes and 2-3 hours after infection. The first phase is apparently an unspecific plant response to diverse stress factors, wounding in particular. Only the second phase is correlated with the establishing of the resistance to the pathogen (Grant and Loake 2000).

With the progression of disease, the production of superoxide was increased in BPT-5204, whereas it was gradually decreased in the control plants of both tolerant and susceptible varieties. In the susceptible cultivar TN-1, the production of superoxide anion was significantly reduced and it was on par with the control treatment at 10dpi (Fig. 4). Superoxide was also quantified in both the varieties by homogenizing the stained leaves with acetic acid. BPT-5204 produced higher superoxide at all the time points from 2dpi to 10 dpi, whereas in the cultivar TN-1, NBT levels were significantly low (Fig. 5). Resistant cultivar BPT-5204 harbors *R* genes which can initiate production of O_2^- and thereby limit the spread of the bacteria, whereas in the susceptible cultivar, due to compatible interaction between *Xoo* and rice, the effector proteins of *Xoo* may nullify or degrade the ROS by production of enzymes like catalase or peroxidase or lipoxygenase. Superoxide levels upon *Xoo* infection was measured using nitro blue tetrazolium (NBT) staining in BPT-5204 and TN-1 varieties. Significantly higher levels of O_2^-were recorded in BPT-5204 compared to the TN-1 genotype (Bakade *et al*., 2021). Kunstler *et al*. (2018) reported O_2^-accumulation to be a pivotal factor governing the development of nonhost resistance in

barley infected with wheat powdery mildew (*Blumeria graminis* f. sp. tritici). O_2^- was localized in the mesophyll chloroplasts of the inoculated leaves and enhanced NADPH oxidase activity was observed leading to transient increase in expression of genes regulating O_2^- levels and cell death.

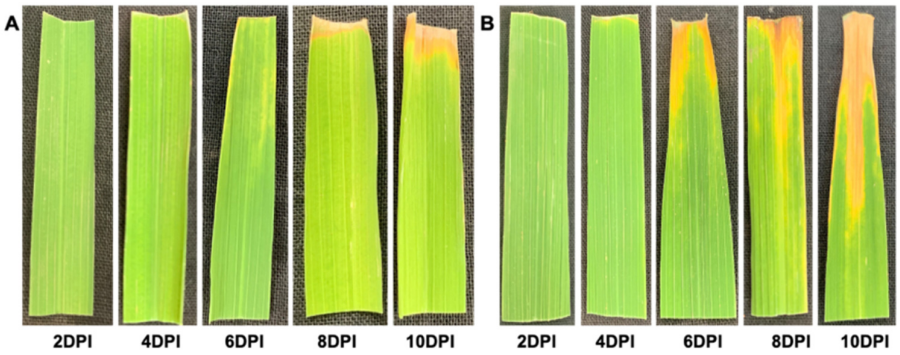

Fig. 1A. *Xoo* infection on BPT-5204; **1B.** *Xoo* infection on TN-1.

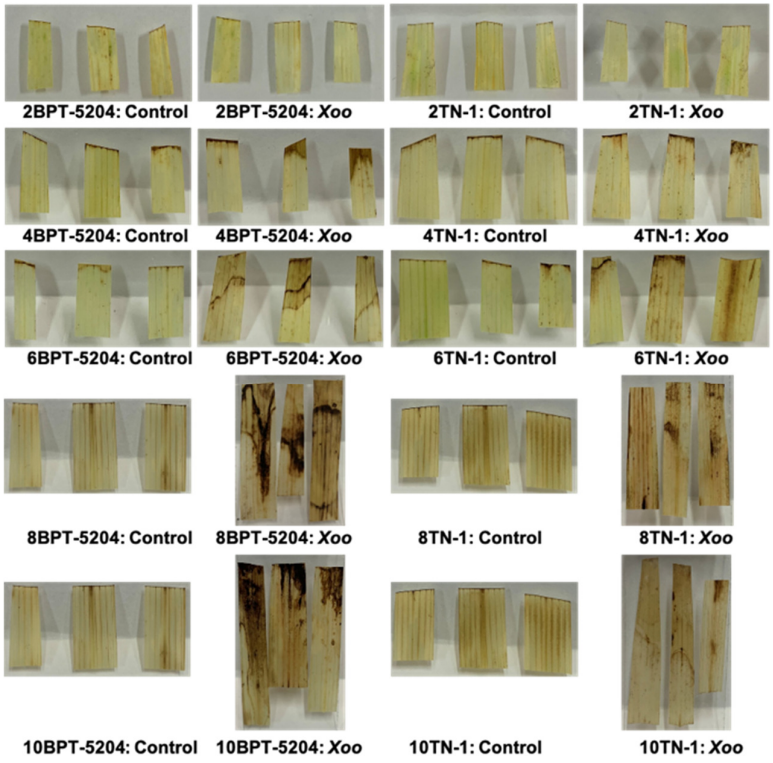

Fig. 2. Rice leaves stained with DAB at 2, 4, 6, 8 and 10 days post inoculation. The production of H_2O_2 was indicated by reddish-brown colour resulting from the polymerisation of DAB.

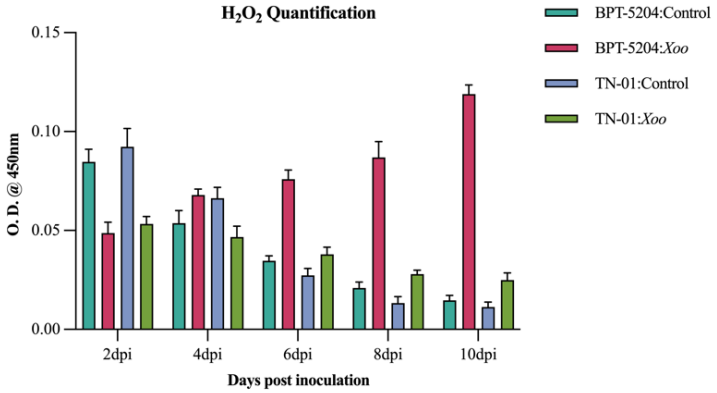

Fig. 3. Quantification of H_2O_2 by DAB assay; infiltrated leaf tissues (at 2, 4, 6, 8 and10 days post infiltration) were homogenised with 0.2M perchloric acid and absorbance at 450 nm was measured for each sample. The absorbance is mean of three replications and absorbance value is directly proportional to H_2O_2 accumulation.

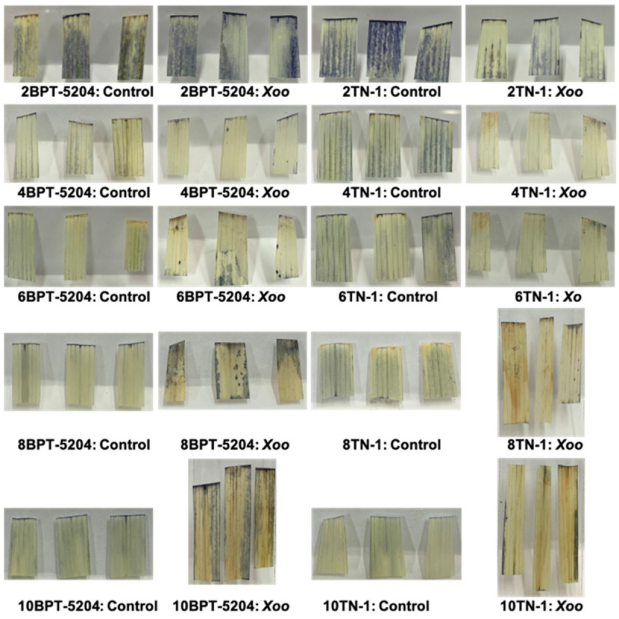

Fig. 4. Rice leaves stained with NBT at 2, 4, 6, 8 and 10 days post inoculation. The production of O_2^- was indicated by blue colour which resulted from the polymerisation of NBT.

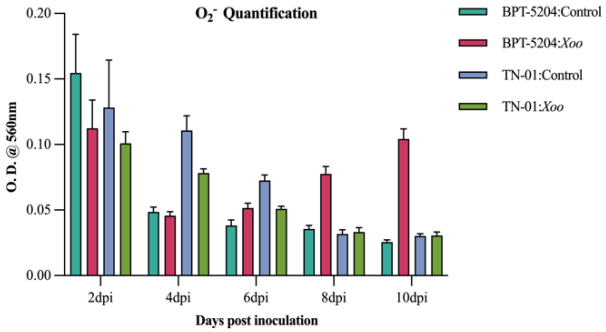

Fig. 5. Quantification of O_2^- by NBT assay; infiltrated leaf tissues (at 2, 4, 6, 8 and 10 days post infiltration) were homogenised with 50% acetic acid and absorbance at 560 nm was measured for each sample. The absorbance is mean of three replications and absorbance value is directly proportional to O_2^- accumulation.

CONCLUSIONS

In nature, plants develop in complex and adaptive environments. Plants must therefore respond efficiently to environmental stressors to maintain homeostasis and enhance their fitness. ROS are considered a double-edged sword for plant life since they regulate various processes such as cell wall synthesis, defense against pathogens, plant aging and programmed cell death, and the behavior of stomata (Chitranashi *et al*., 2022). Reactive oxygen species (ROS) function as critical, fast-acting orchestrators that link biotic responses to plant homeostasis and development (Berrios and Rentsch 2022). Plants respond to pathogen invasion through activation of oxidative burst, which leads to the production of reactive oxygen species (ROS), including superoxide (O_2^-) and hydrogen peroxide (H_2O_2). The production of reactive oxygen species (ROS) is one of the earliest cellular responses following successful pathogen recognition (Mittler, 2017). Thus, ROS production during oxidative burst helps the plants to restrict further spread of the invading pathogens by inducing HR or plant immune responses (Durrant and Dong 2004). We quantified the ROS production in resistant as well as susceptible rice cultivars, particularly in the production of H_2O_2 and O_2^- during rice: *Xoo* interaction. The resistant cultivar BPT-5204 produced H_2O_2 and O_2^- significantly higher compared with the susceptible cultivar TN-1. This may be due to *Xa* genes present in BPT-5204 that contribute to the oxidative burst during pathogenesis by *Xoo* and thereby reducing the infection rate of *Xoo*. ROS are regarded as beneficial messengers that trigger oxidative signalling, systemic acquired acclimation, and systemic acquired resistance. Despite the scientific progress made during the last two decades, the mode of action of ROS is still far from being fully understood. Further elucidation of the genes involved in ROS production and signaling provide doorway to develop varieties resistant to bacterial infection.

Author contribution. Conceived and designed the experiments: MKP. Performed the experiments: SSP. Contributed reagents/materials/analysis tools: MKP.

Wrote the manuscript: SSP. Edited the manuscript: MKP and RSV. All authors read and approved the manuscript for publication.

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