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Seed treatment and foliar application of methyl salicylate (MeSA) as a defense mechanism in rice plants against the pathogenic bacterium, *Xanthomonas oryzae pv. oryzae*

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ABSTRACT

Methyl salicylate (MeSA) is a volatile biological compound synthesized from salicylic acid (SA) and is a plant hormone that helps defend against pests and pathogens. A major bacterial pathogen of rice, *Xanthomonas oryzae* pv. *oryzae (Xoo)* causes severe disease. Seed and plant treatments with MeSA can stimulate the defense enzyme peroxidase (POD) in plants. Response of peroxidase activity in rice (*Oryza sativa* L) cultivars IR 20, IR 50, IR 64, ASD 16, ASD 19 and ADT 46 to MeSA were measured under greenhouse conditions. Treatments of rice seedlings with MeSA at 50 and 100 mg L^{-1} significantly upregulated POD expression in the plants. The activity of POD was also significantly upregulated when plants were inoculated with bacterial blight. Effects were stronger in ASD 16, ASD 19 and ADT 46 and were more pronounced in high dose treatment (100 mg L^{-1}) when inoculated with bacterial blight condition and the effects were dose dependent, although the relationship between dose and rice varieties were not always linear. The pathogenic related (PR) protein bands at 33 kDa and 14 kDa were identified in treatments of 100 mg L^{-1} MeSA in the presence of bacterial blight disease. Band intensity was estimated to be twice that of those from pathogen induce MeSA levels in rice plants. These results suggest that treatment with MeSA can significantly increase the POD defense related enzyme by altering the plant physiology in ways that may be beneficial for crop protection.

1. Introduction

Rice (*Oryza sativa)* is an essential agricultural food crop. Economic sustainability in some countries depends upon improving rice productivity. Rice production is threatened by insect pests and bacterial pathogens like bacterial blight disease, *Xanthomonas oryzae pv. oryzae* (*Xoo*) (Stout et al., 2006a; Zhu et al., 2013; Kalaivani et al., 2016). Losses from insects and diseases are typically controlled by the use of chemical pesticides (Lanka et al., 2017; Senthil-Nathan, 2015). The heavy use of pesticides results in the evolution of chemical resistance in pests (Preston and Malone, 2014). Agricultural researchers today recognized the advantages of plant hormones and their roles to reduce pests and disease symptoms (Mithöfer and Boland, 2012). When plants are attacked by pathogens, immune mechanisms are triggered by chemical elicitors. The three main plant signaling molecules are: salicylic acid (SA), jasmonic acid (JA) and ethylene, which can increases the level of resistance against various pathogens (Kalaivani et al., 2016; Thomma et al., 1998; Kunkel and Brooks, 2002; Senthil-Nathan et al., 2009; Nisha et al., 2012; Yang et al., 2013; Senthil-Nathan, 2019), and insects (Preston and Malone, 2014; Mithöfer and Boland, 2012).

SA is a phenolic compound and plant hormone that enhances plant development and immunity (Lattanzio et al., 2006; Dempsey et al., 2011), while signaling other plant chemical compounds that defend against pathogenic microbes and insect pests (Sticher et al., 1997). It is essential for both local defense response and systemic acquired resistance (SAR). *Salicylic acid* and *methyl salicylate, MeSA,* upregulation often occur simultaneously in response to insect feeding (Frost et al., 2008; Arimura et al., 2011). Volatile chemical signaling in plants provides broader interplant communication among related and unrelated plant species across distances to upregulate their defenses (Song et al., 2010; López et al., 2012). Long distance signaling mechanisms often use systemic acquired resistance (SAR) immune responses (Klessig, 2012; Shah and Zeier, 2013).

A large family of class III plant peroxidases, POD, are responsible for

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many physiological and biochemical functions in plants, such as cell-wall growth (Senthil-Nathan, 2013; Passardi et al., 2004), elongation (Macadam et al., 1992), lignification (Hammerschmidt and Kuc, 1980), auxin catabolism (Gaspar et al., 1982), expression of defense-related proteins, RP (Van Loon et al., 2006; Duan et al., 2014), wound healing and defense mechanisms (Lagrimini, 1991; Hiraga et al., 2001). Several reports have shown that SA and JA play vital roles in triggering the induced pathogenesis-related, PR, resistance proteins in plants (Stout et al., 2006b; Umemura et al., 2009; Sinha et al., 2014; Jiang et al., 2015).

Rice PR proteins are produced in response to wounding by insect herbivories, the environment, or pathogens (Sinha et al., 2014; Jiang et al., 2015). Since previous research has reported that upregulation of PR proteins occurs with applications of MeSA, this research was designed to examine the biochemical responses in MeSA treated rice seedlings using increasing concentrations across a time interval. Furthermore, we analyzed the POD activity in rice plants to high stressors caused by the bacterial pathogen, *Xanthomonas oryzae pv oryzae*, (*Xoo*). Analyses of MeSA applications during stress treatments were conducted to determine if plant health could be improved.

2. Material and methods

2.1. Plant material used for MeSA treatments

Six rice varieties were used in this study (*i.e.* IR 20, IR 50, IR 64, ASD16, ASD19 and ADT46). IR 20, IR 50 and IR 64 are more susceptible to blight disease, conventional, semi-dwarf, long-grain aromatic variety with relatively high seedling vigor. However, ASD16, ASD19 and ADT46 are resistant to blight disease conventional, semi-dwarf long grain variety with low seedling vigor.

2.2. Plant culture

Rice plant culture was carried out according to methods in Schmelz et al. (Senthil-Nathan, 2019). Rice seeds were sown in a pot with diameter of 9 cm and height of 12 cm, comprising the ratio of sand and peat moss (2:1), five seeds per pot with five replicates to each treatment. Seedlings were grown in a greenhouse with day/night cycle of 14 h/10 h, at 30 °C /25 °C, respectively; sunlight was ambient with natural ventilation and watered as required.

2.3. Seed treatment with MeSA

MeSA (Sigma-Aldrich) was dissolved in a small amount of ethanol (0.25% in the final solution) and brought to the desired concentration with distilled water: (0, 50, and 100 mg L^{-1} equivalent to 0.65, 3.2 and 6.5 Mm). Rice seeds were disinfected with 2% sodium hypochlorite for 2 min and rinsed with purified water, and dried with tissue paper. The solution of MeSA (0, 50 and 100 mg L^{-1}) was prepared with a stock solution of MeSA 100 mg L^{-1}, which was used for successive dilutions in distilled water respectively. The seed treatment was carried out according to the method of Tavares et al. (Tavares et al., 2014). Rice seed, 0.2 kg, were agitated in MeSA solutions for 3 min and then dried for 24 h at room temperature.

2.4. Inoculation of Xanthomonas oryzae pv. Oryzae, Xoo

Xanthomonas oryzae inoculum were cultured for 48 h on nutrient broth medium, grown culture were centrifuged at 8000 $\times g$ for 15 min and the resulting sample suspended was diluted with sterile distilled water at 2×10^7 CFU ml^{-1}. *Xoo* was inoculated to rice plants 28 days after sowing (fourth-leaf stage). The leaves of rice cultivar in each experimental pot were inoculated through scissors-dip method. Scissor tips were tipped into the *Xoo* suspension and leaf tip was cut. Plants were grown at 28–32 °C (light, 12 h), 28–32 °C (dark, 12 h), 90% relative humidity (Cai et al., 2008).

2.5. MeSA exogenous application

Exogenous applications of MeSA were sprayed on the rice plants at Zadoks' growth stage 4.5 (Zadoks et al., 1974; Rahman et al., 2009) with increasing concentrations from 0, 50 and 100 mg L^{-1}. The treatments were sprayed uniformly at the rate of 12–15 ml to each plant by a regulator-controlled sprinkler. Control plants were treated with water.

2.6. Plant enzyme preparation

To measure POD activity, leaves were collected and kept in freezer under -20 °C, until processed. The methods followed Macadam et al. (Macadam et al., 1992) with slight modifications. Samples were taken from the first to sixth day (0, 24, 48, 72, 96, 120 and 144 h) after treatment. For the seed treatment, rice leaves were taken after 14 days of emergence. Weighed leaf tissue of 1 g was cut into small pieces and ground with 100 mM sodium phosphate buffer (pH 6.8) using a pre-cooled mortar and pestle. The ground mixture was centrifuged at 12,000 $\times g$ for 20 min at 4 °C. The supernatant was collected and used for POD assays.

2.7. POD assay

The reaction mixture contained 0.1 ml enzyme extract, 12 mM H_2O_2, and 7.2 mM guaiacol in 50 mM phosphate buffer (pH 5.8). The POD activity was calculated by the increase in the absorbance of tetra-guaiacol at 470 nm. The oxidation product was determined in a spectrophotometer (Lambda 25, UV/Vis spectrometer, Perkin Elmer) at 470 nm and the POD activity calculated as $\Delta 470\ min^{-1}\ g^{-1}$ fresh weight.

2.8. Isolation and separation of PR proteins through gel electrophoresis

The isolation and separation of protein mixture in rice plants exposed to MeSA applications were carried out through a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Laemmli, 1970)

2.9. Statistical analysis

Effects of MeSA treatments on POD activity and the *Xoo* inoculated on POD activity in rice plants were subject to three-way factorial ANOVA wherein time courses and treatment (control, low and high) were considered as variables while the effect of MeSA seed treatment on POD activity of rice seed varieties data were subject to one-way ANOVA (Minitab® version 17; State College, Pennsylvania, USA). Where necessary, log or square root transformations were used. The Tukey's HSD test ($\alpha = 0.05$) was used for multiple comparison (WINKS SDA version 7, Texasoft Cedar Hill, Texas, USA).

3. Results

The MeSA spray on all rice varieties (either susceptible or resistance) increased POD activity significantly when compared with the control plant. The data shows a significant increase in POD activity for all varieties at high MeSA (ASD 16- $F_{2,\ 12} = 38.01$; $P < 0.0001$, ADT 46- $F_{2,\ 12} = 19.22$; $P < 0.0001$, ASD 19-$F_{2,\ 12} = 11.32$, $P < 0.002$) (Fig. 1).

MeSA foliar treatments increased POD activity in both susceptible and resistant of rice varieties (Fig. 2). The POD activity in ASD 16 rice variety was significantly greater at 48 h post treatment at the highest concentration of MeSA ($F_{2,\ 12} = 31.36$; $P < 0.0001$) than at 0 h ($F_{2,\ 12} = 0.05$; $P < 0.952$). The POD activity reached the maximum level at 96 h ($F_{2,\ 12} = 144.62$; $P < 0.0001$) being about four-fold greater in

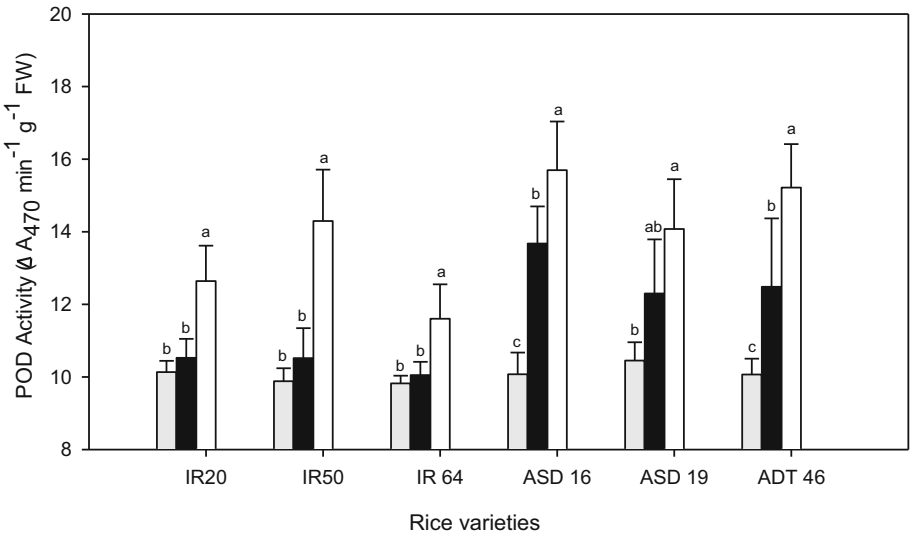

Fig. 1. Effect of MeSA seed treatment on POD activity of rice seed varieties IR 20, IR 50, IR 64, ASD 16, ASD 19 and ADT 46. Plants were grown in greenhouse condition. Rice seed treated with control, low (50 mg/L^{-1}) and high (100 mg/L^{-1}). Mean (± SEM) followed by the same letter in an individual experiment indicate no significant difference ($P < 0.05$) in a Tukeys test [(□-Control; ■-low (50 mg/L^{-1}); ▭ high (mg/L^{-1})].

plants treated at the highest dose of MeSA compared with the control plants. The experiment showed that at 24 h after MeSA treatment the rice plant POD activity was significantly different between the control and high dose.

In the majority of the rice varieties, MeSA was more effective at the higher dose of 100 mg L^{-1}, which induced greater POD activity than the lower doses of 50 mg L^{-1} at 96 h. These treatment doses though produced significantly different level of POD activity ($F_{2,\ 12} = 53.55$, $P < 0.001$ in IR 50, $F_{2,\ 12} = 40.49$, $P < 0.0001$ in IR 64 and $F_{2,\ 12} = 55.35$, $P < 0.005$ in ASD 16). The level of POD activity for the lowest doses were not significant ($F_{2,\ 12} = 17.11$, $P < 0.090$ in IR 20, $F_{2,\ 12} = 65.23$, $P < 0.096$ in ASD19 and $F_{2,\ 12} = 29.81$, $P < 0.105$ in ADT 46).

Plants inoculated with *Xoo* and activated by MeSA treatments showed significantly increased POD activity in both susceptible and resistant rice varieties at 96 h and 120 h (Fig. 3). Concentrations reached a peak with all rice varieties remaining constant up to 144 h post exposure. Significant differences of POD activity in rice varieties were observed after 48 h of treatment in all rice varieties between every 24 h time period ($F_{2,12} = 19.92$, $P < 0.0001$ in IR 20, $F_{2,12} = 13.82$, $P < 0.001$ in IR 50, $F_{2,12} = 16.78$, $P < 0.0001$ in IR 64 and $F_{2,12} = 49.35$, $P < 0.0001$ in ASD 16, $F_{2,12} = 37.82$, $P < 0.0001$ in ASD 19, and $F_{2,12} = 38.18$, $P < 0.0001$ in ADT 46).

The difference in POD activity measured at 24 h after MeSA treatment on *Xoo* inoculated rice plant was insignificantly different between control and low dose ($F_{2,12} = 17.14$, $P < 0.725$ in IR 20, $F_{2,12} = 13.82$, $P < 0.064$ in IR 50, $F_{2,12} = 16.30$, $P < 0.660$ in IR 64 and $F_{2,12} = 7.01$, $P < 0.562$ in ASD 16). With increasing hours post treatment *Xoo* with MeSA, produced similar results to those observed at 72 h. There were significant differences between controls and low dose (50 mg L^{-1}) treatments across all rice varieties, except ASD16 ($F_{2,12} = 45.93$, $P < 0.0001$). When compared the POD activity in control and higher doses of MeSA treated rice plant was more pronounced in *Xoo* inoculated plant at 72 h ($P < 0.001$). The POD activity increased quickly and maintained a tendency to increase compared to all the rice varieties (Fig. 3). In the controls, no such increase in enzyme activity was observed (Fig. 3). At 120 h post treatment, the maximum level of POD activity was recorded in the ASD 16 rice variety with significant POD activity ($P < 0.001$).

The rice leaves treated only with MeSA at concentrations of 0, 50 mg L^{-1} to 100 mg L^{-1}, and the plants treated with only *Xoo*, and the water control plants were subjected to SDS-PAGE for the identification of PR proteins (Fig. 4). The protein bands in the control lane and rice leaves of treated plants with the *Xoo* were compared with the lane of the MeSA treatment and water control leaves. Analyses of samples from the treatment MeSA and *Xoo* (Lane A) and control Lane (Lane-E), bands were highly visible in the combined treatment, and not in (or less so in) individual treatments along with *Xoo* lane (C, D-50 and 100 mg L^{-1} MeSA-lane; B- *Xoo* alone). The molecular weights of 14 to 33 kDa corresponded to PR proteins indicating that the defense related enzymes were induced greater in the MeSA plus *Xoo* treatment.

4. Discussion

Salicylic acid and its derived compound MeSA continue to be studied and developed as agricultural products that can induce plant growth regulators enhance defense responses, or improve growth and development (Kalaivani et al., 2016; Rani and Jyothsna, 2010; Le Thanh et al., 2017). The enhanced resistance of MeSA treated rice plants may be due to the elicitation of a set of biochemical defense responses. Hence the biochemical responses *via* POD of the rice plants to MeSA were studied. POD activity was greater when treated with MeSA and infected with *Xoo*, than with either alone. POD induction is regarded as a good indicator of plant immune defense response (Peng et al., 2005; Mandal et al., 2009). The magnitude of effect differed among rice genotypes and effects were rate dependent, although the relationship between seed treatment, foliar application effect was not always linear. Also, effects appeared to be more pronounced under treated with MeSA and infected with *Xoo*.

Increasing the levels of induction of these enzymes by applying SA sprays can increase the overall plant defense. Treatment of seeds with MeSA initiated increased foliar POD activities. Thus, pretreatment of seeds in areas of known disease and pest pressure may provide some degree of advantage. Application of MeSA sprays as rice plants grow also produced increased levels of peroxides activity irrespective of rice variety.

Exogenously applied SA sprays triggered a significant increase of POD activity in treated sunflowers (Noreen and Ashraf, 2009). In antioxidant enzyme activities with heat tolerance, POD activities were greater for leaves than for roots in creeping bent grass (Liu and Huang, 2000). These plant enzymes have long been related with an important role in the plant defense, including mustard seedling (Dat et al., 1998) tomato (Orozco-Cardenas et al., 2001a) soybean (Shirasu et al., 1997), cucumber and rice (Duan et al., 2014). POD activities were shown to be significantly increased in chickpea (*Cicer arietinum* L.) plants sprayed with SA (1.5 mM) (Orozco-Cardenas et al., 2001b). Sauerborn et al. (Sauerborn et al., 2002) reported that an exogenous application of SA reduced the attachment of Sunflower broomrape, a root parasitic plant

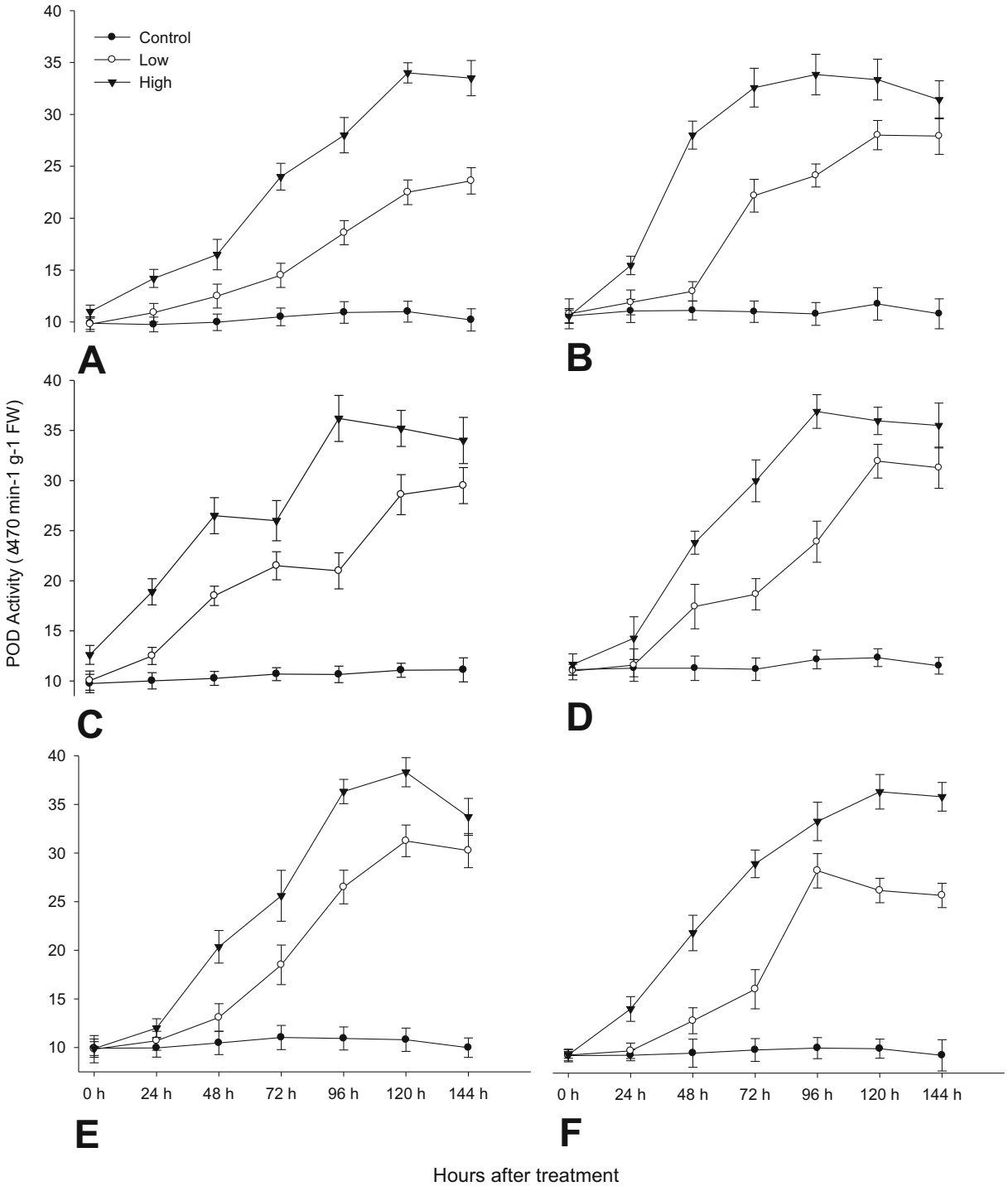

Fig. 2. Effects of MeSA treatments on POD activity in rice plants IR 20, IR 50, IR 64, ASD 16, ASD 19 and ADT 46 were grown in a greenhouse condition. Rice plants were sprayed with control, low (50 mg/L^{-1}) and high (100 mg/L^{-1}). Data represent the means of three replicates of each treatment (–●– control -○ - low -▼ – high) (A-IR 20, B-IR 50, C-IR 64, D-ASD 16, *E*-ASD 19 and F-ADT 46).

on sunflower, which may be the result of the increased phytoalexin. These studies along with the results of the present research demonstrate an increase of POD contents in rice plant after the SA treatments (Figs. 1-3) (Kusumoto et al., 2007).

The POD activity increases in a faster rate in rice varieties compared with control, which shows the deliberate increase in POD activity in response to pathogen infection, as reported in wheat with *Neovossia indica* (Mandal and Gupta, 2016; Gogoi et al., 2001). POD activity in the infected rice plant may lead to the enhanced plant defense mechanism forming a biochemical barrier for development of the pathogen. The results presented here are similar to other cases that have reported similar POD activity following treatment with JA and *Xoo* infections (Hui et al., 2019). POD showed an increased level during our experimental period simultaneously all the rice plants. But it is not linear with rice varieties.

Application with SA showed an increased level of antioxidative enzymes and it improved impact of H_2O_2, which enhance the POD activity (War et al., 2011). Our investigation provides the biochemical

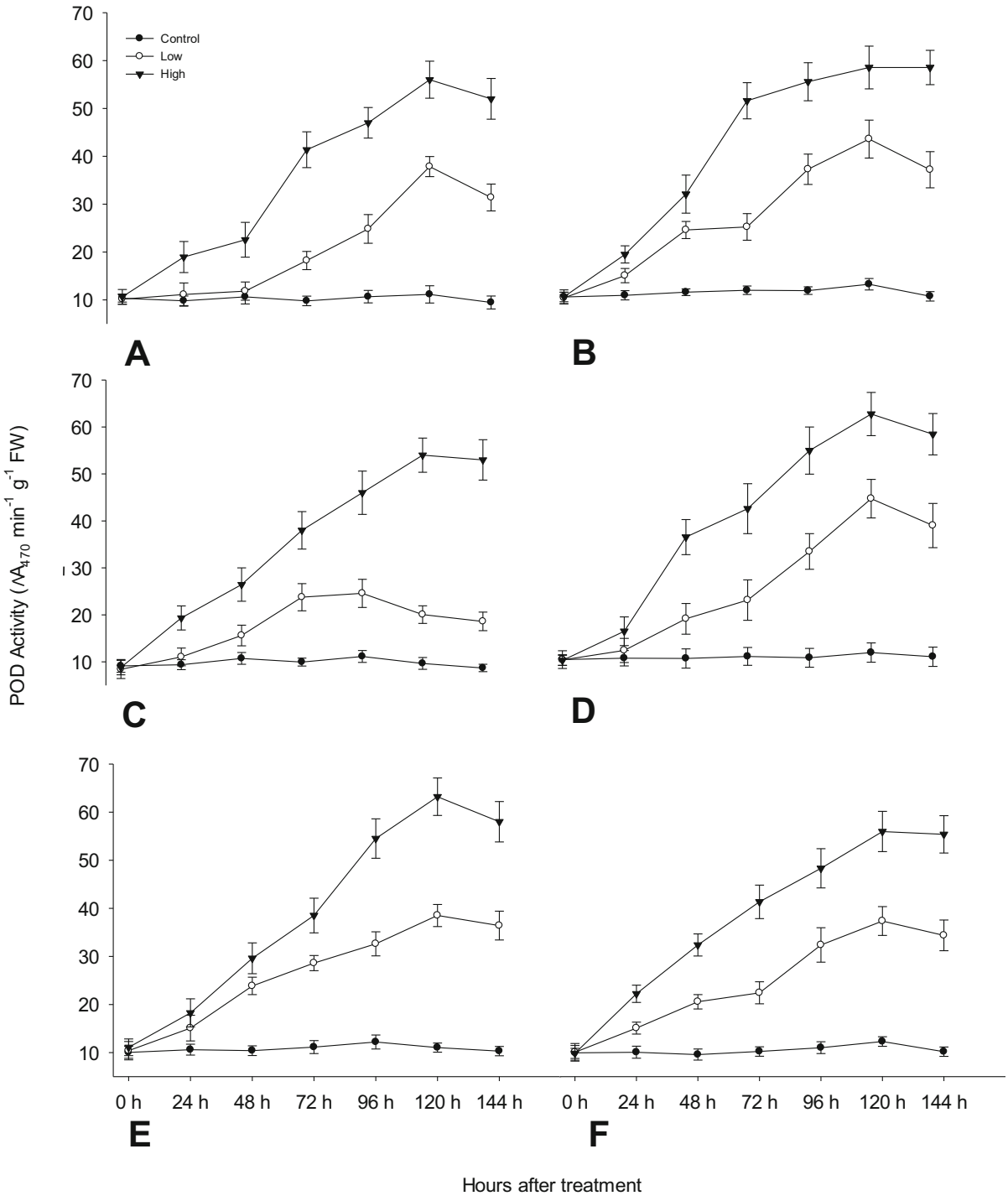

Fig. 3. Effects of MeSA treatments and *Xanthomonas oryzae pv oryzae* inoculated on POD activity in leaves of rice varieties IR 20, IR 50, IR 64, ASD 16, ASD 19 and ADT 46, grown in a greenhouse condition. Plants were inoculated with oryzae (Xoo) when they were 28 days after sowing. Inoculated rice plants were sprayed with control, low (50 mg/L^{-1}) and high (100 mg/L^{-1}) of MeSA. Data represent the means of three replicates of each treatment (– ●– control -○ - low -▼ – high) (A-IR 20, B-IR 50, C-IR 64, D-ASD 16, E-ASD 19 and F-ADT 46).

POD activity using the MeSA treated rice plant regulate their defense systems against the *Xoo*. The effect of SA could provide a cost-effective treatment to increase plant growth and biomass (Rivas-San Vicente and Plasencia, 2011; Chandra and Bhatt, 1998; Gunes et al., 2007). Plant breeders could also select for plants that have increased levels of SA or methyl salicylic acid (Guo et al., 2007) by applying certain natural elicitors (Chanthini et al., 2019a; Chanthini et al., 2019b). These pathways also increase the production of H_2O_2 peroxides which activates several physiological and molecular processes in plants that signal the production of various defensive compounds and enzymes that increase plant resistance against pest and disease attack (Kalaivani et al., 2016).

Generally, PR protein present in mostly all type of plant, it protects the plant through activating the defense mechanisms which controls the pathogen infection. While in our study the pathogen infection increases the PR protein activator level in presence of chemical elicitor MeSA in rice plant. PR protein bands were visibly increased in leaf extraction of *X. oryzae* + 50 mg L^{-1} of MeSA when compared with other treatment

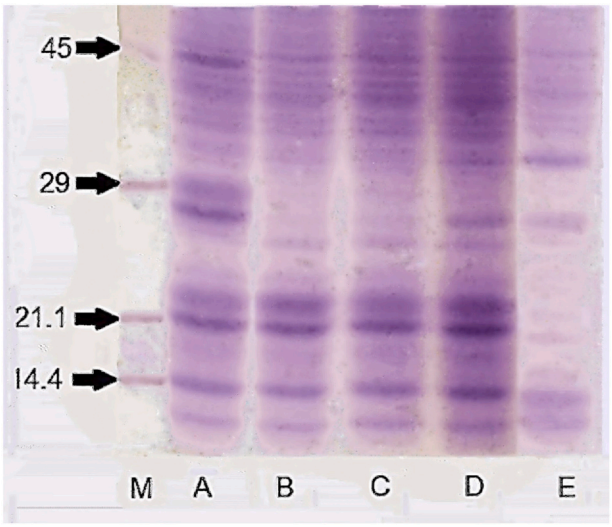

Fig. 4. Rice plant protein SDS-PAGE after treatment with MeSA and after pathogen inoculation (M-Marker; A- *X. oryzae* + 50 mg/L^{-1}MeSA; B- *X.* oryzae; C-50 mg/L^{-1} MeSA; D,- 100 mg/L^{-1} MeSA; E-Control).

and control. GmPRP protein activates the defense activity and considerable inhibition of infected with *Phytophthora sojae* in soybean leaves (Jiang et al., 2015). SA, MeJA and ACC which enhance the PR genes in marker level in leaves, stems and roots of *Malus hupehensis* attacked by pathogen (Maffei et al., 2007). SA derived compound, the chemical elicitor MeSA, showed significantly increased POD activity in *Xoo* inoculation in rice leaves. The accumulation of PR protein in rice plant was also increased by showing protein visible bands in SDS PAGE gel. Thus, the chemical elicitor MeSA play a vital role in induce defense against the plant disease.

5. Conclusion

In conclusion, our study described MeSA increased the POD activity more effectively than the normal condition under *in vivo* and *in vitro*, probably through induced defense enzyme.

While it may be concluded, that POD alone cannot produce a complete plant defense mechanism, POD does produce a physiological effect that improves the plant defense system. The results recommend that 100 mg L^{-1} MeSA could be used for the induction of rice plant that will improve protection against *Xoo*. Thus, MeSA could be used as an effective agent to suppress the *Xoo* infection and increased plant resistance under field condition.

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