



Phytohormones mediated antifungal resistance against *Fusarium oxysporum*

Lucky Duhan¹ · Raman Manoharlal² · Deepak Kumar³ · Deepika Kumari¹ · Mamta Saini¹ · G. V. S. Saiprasad² · Anil K. Chhillar⁴ · Ritu Pasrija¹

Received: 3 October 2022 / Revised: 17 March 2023 / Accepted: 30 January 2024 / Published online: 6 March 2024

© The Author(s) under exclusive licence to Franciszek Górski Institute of Plant Physiology, Polish Academy of Sciences, Kraków 2024

Abstract

This study aims to evaluate the in vitro as well as in vivo antifungal activities of different phytohormones (PHs) against the hemibiotroph fungus, *Fusarium oxysporum* using black gram [*Vigna mungo* (L.) Hepper] as a model system. The potential antifungal activities were tested using PHs viz. salicylic acid (SA), methyl jasmonate (MeJA), melatonin (MT), brassinolide (BL), indole-3-acetic acid (IAA), gibberellic acid₃ (GA₃), ethephon (ET), and abscisic acid (ABA), by determining the minimum inhibitory concentration (MIC) and minimum effective concentration (MEC) end point in a microtiter plate-based assay. The results suggested significant antifungal activity for all the tested PHs, wherein SA and MeJA showed potency even at the lowest concentration tested, with corresponding MIC₉₀ values of 0.312 mM and 0.625 mM, respectively. Likewise, a similar MEC profile was also observed for both SA and MeJA, with a corresponding value of 0.078 mM and 0.312 mM, respectively. The microtiter results were corroborated using spore germination and mycelial susceptibility assays. The in vivo antifungal efficacy of PHs was assessed by recording the germination characteristics in SA and MeJA-primed *V. mungo* seeds that were already exposed to *F. oxysporum* spores. The PHs-primed seeds displayed a characteristic longer seedling length and higher seed vigor index (SVI), in concomitant with relative enhanced ROS-scavenging activity. The priming of *V. mungo* seeds with SA and MeJA seems to induce a defense mechanism against *F. oxysporum* infection, which also improved its germination characteristics.

Keywords *Fusarium oxysporum* · Stress · Phytohormones · Microtiter plate assay · *Vigna mungo* (L.) Hepper · Priming

Abbreviations

PHs	Phytohormones	MGT	Mean germination time
SA	Salicylic acid	SL	Seedling's length
MeJA	Methyl jasmonate	SVI	Seedling Vigor Index
<i>F. oxysporum</i>	<i>Fusarium oxysporum</i>	ANOVA	Analysis of variance
GP	Germination percentage	ROS	Reactive oxygen species
		O ₂ ⁻	Superoxide
		H ₂ O ₂	Hydrogen peroxide
		MDA	Malondialdehyde
		NBT	Nitroblue tetrazolium
		DAB	3,3'-Diaminobenzidine
		DPPH	2,2-Diphenyl-1-picrylhydrazyl
		AA	Ascorbic acid
		SOD	Superoxide dismutase
		CAT	Catalase
		APX	Ascorbate peroxidase
		PAL	Phenylalanine ammonia-lyase
		ICS	Isochorismate synthase
		SAR	Systemic acquired resistance
		ISR	Induced systemic resistance

Communicated by A. Piotrowska-Niczyporuk.

✉ Ritu Pasrija
ritupasrija@yahoo.com

- ¹ Department of Biochemistry, Maharshi Dayanand University, Rohtak, India
- ² ITC Limited, ITC Life Science and Technology Centre (LSTC), Peenya Industrial Area, 1st Phase, Bengaluru, Karnataka, India
- ³ Department of Botany, Institute of Science, Banaras Hindu University, Varanasi, Uttar Pradesh, India
- ⁴ Centre for Biotechnology, Maharshi Dayanand University, Rohtak, India

Introduction

In plants, various hormones like auxin, gibberellic acid (GA), cytokinin (CK), ethylene (ET), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA), brassinosteroids (BRs), melatonin (MT), and strigolactone (SL), etc. exist at different physiological concentrations (Jang et al. 2020). Phytohormones (PHs) contribute to diverse activities throughout the plant's life cycle. Notably, the physiological impact of PHs is often multifaceted, as they exert their impact either directly or through cross-talk with other PHs (Alazem and Lin 2014). For instance, CKs, auxin, GAs, SLs, and BRs are vital for modulating photosynthetic levels in normal situations. On the contrary, ABA, ETs, SA, and JAs significantly influenced photosynthesis under various stress conditions. Hence, an integrated hormonal response offers an adequate photosynthetic percentage during regular and stressful conditions (Bari and Jones 2009; Muller and Munne-Bosch 2021). Thus, ascertaining the biological role of a single PH becomes complicated. In the past, attempts were made to assign the specific function(s) of PHs. Besides auxin, CK, GA, ET, and ABA, regarded as standard PHs; BR, SL, and other peptides have also been added to this list of PHs playing essential roles in plant growth, and development (Muller and Munne-Bosch 2021). PHs also provide tolerance against abiotic stress and resistance to biotic stress. For instance, it has been reported that ABA levels get elevated during abiotic stresses, such as salinity, drought, heat, and cold stresses (Sah et al. 2016). On the other hand, SA, JA, and ET become more prominent during biotic stresses (Jang et al. 2020).

The biotic stress caused by phytopathogens (bacteria, fungi, viruses, parasites, and pests) is studied on different plants. The PHs trigger signaling pathways and generate either induced systemic resistance (ISR) or systemic acquired resistance (SAR) against pathogen attacks (Walters et al. 2008; Worrall et al. 2012). Among these, ISR is induced by beneficial soil microbes interacting with plant roots like rhizobacteria; which although non-specific, imparts long-lasting endurance. It employs JA and ET-mediated signaling (except SA). On the contrary, SAR is initiated after a pathogen's attack and involves SA accumulation (Conrath 2006; Worrall et al. 2012). During these signaling cascades, different PHs interact either synergistically or antagonistically with each other. For example, JA and SA act antagonistically with each other and the induction of one attenuates the other (Shigenaga and Argueso 2016). Upon exposure to biotic stress, different plants showed diverse process(es) of tolerance, which varies with both pathogen(s) and their mode of attack. Pathogens can be either necrotrophs or biotrophs. The

necrotrophs damage host tissues and feed on remains, which is possible with programmed cell death (PCD) and assist in ISR, whereas biotrophs feed on living tissues and articulate a hypersensitive response (HR) with resistance and generate SAR. A long-standing notion posits that SA and JA/ET are associated with biotroph and necrotrophs resistance, respectively (Chowdhury et al. 2017). Notably, fungal pathogens also employ a strategy to switch from biotrophic to necrotrophic and vice versa, which is often accompanied by the corresponding adjustment in their host's defense strategy. For instance, it has been reported that SA-mediated signaling in the early phase changes to JA-mediated signaling in the later phases of infection. This alteration is more prompt in the tolerant hosts rather than in susceptible ones, which in turn contributes to their better disease endurance (Shigenaga and Argueso 2016). The plant physiological responses against biotic stress(es) mainly involve PHs biosynthesis, which helps in enduring the pathogen strike. The PHs-mediated protection in plants can be mimicked with their exogenous application as well (Jiao et al. 2017; Kepczynska and Krol 2012; Portu et al. 2015). The MeJA priming triggers protection against *Fusarium oxysporum* f. sp. *lycopersici*, involving accumulation of 12-oxo-phytodeinoic acid, SA, and flavanol, in the tomato plants. This signaling concomitantly involves an increase in the expression of genes, including phenylalanine ammonia-lyase5 (*PAL5*), chalcone synthase (*CHS*), and flavonol synthase/flavanone 3-hydroxylase (*FLS*) (Krol et al. 2015). Likewise, SA foliar application on common bean (*Phaseolus vulgaris* L.) augments SAR against *F. oxysporum* f. sp. *phaseoli* (Fop) infection by increasing the activity of *PAL* and *POX*-encoded proteins (Xue et al. 2014).

With this background, our present study aims to evaluate the antifungal activity of different PHs against the hemibiotrophic fungus, *F. oxysporum*. Notably, the biotrophic stage of the *F. oxysporum* infection cycle is followed by the necrotrophy stage, causing vascular wilt in plants, including pulses. *F. oxysporum* initiates infection through the roots and moves toward the vasculature during the biotrophic phase. Through plant vasculature, fungi travel upwards, resulting in a congregation of fungal mycelia and defense-related compounds in the xylem, causing vascular wilting (Lyons et al. 2015). With the progression of infection, it switches to necrotrophy, causing foliar necrosis, lesions development, and eventually plant death. During its life cycle in the plant, *F. oxysporum* secretes many phototoxic compounds, resulting in root cell collapse, veinal chlorosis in leaves, and reprogramming of the host for inducing senescence (Dong et al. 2014; Lyons et al. 2015). It impacts the seedling stages and causes up to 100% yield losses in the case of legumes.

To combat *Fusarium* infection, the activity of PHs against *Fusarium* was evaluated with different in vitro methods, viz. microtiter assay, fungal spore germination inhibition, and agar-based mycelial growth inhibition. The in vitro competent PHs against *F. oxysporum* were further used for in vivo analysis on *Vigna mungo* (L.) Hepper (black gram or urd bean). The protective effect of hormo-priming on *V. mungo* seeds, pre-treated with the fungus was compared with the control. Based on the results, we propose the practical implication of hormo-priming in imparting endurance and resistance to plants against *F. oxysporum*.

Materials and methods

Chemicals, seeds, and fungal culture

Media and chemicals, including potato dextrose agar (PDA), potato dextrose broth (PDB), Tween 80, methanol, ethanol, dimethyl sulphoxide (DMSO), nitroblue tetrazolium (NBT), 3,3'-diaminobenzidine (DAB), 2,2-diphenyl-1-picrylhydrazyl (DPPH), ascorbic acid (AA), trichloroacetic acid (TCA), potassium phosphate monobasic (KH₂PO₄), potassium phosphate dibasic (K₂HPO₄), potassium iodide (KI), thiobarbituric acid (TBA), methionine, riboflavin, PHs- SA, MeJA, MT, BL, IAA, ET, GA₃, and ABA were procured from HiMedia and Sigma Aldrich. *Vigna mungo* seed variety UH-1 (commercial variety) was procured from Chaudhary Charan Singh Haryana Agricultural University (CCSHAU), Hisar, Haryana, India. *F. oxysporum* MTCC 9913 culture was obtained from the Institute of Microbial Technology (IMTECH), Chandigarh, India.

Preparation of fungal culture

The fungal culture was grown on PDA slants for 7 days at 28 °C. Later, the spore/conidial suspension was collected by adding 2–3 ml of sterile normal saline with 0.05% Tween 80 (NST) and scraping with a sterile loop. The spore suspension was filtered through a sterile gauze, collected in 1.5 ml screw-cap tubes, and stored at 4 °C, until further use.

Methodology

In vitro antifungal activity: The in vitro susceptibility of PHs against *F. oxysporum* was tested through different methods. Each experiment was performed with three technical replicates and repeated twice.

Microtiter assay

Minimum inhibitory concentration (MIC)/Minimum Effective Concentration (MEC) of different PHs against

F. oxysporum, was determined according to the Clinical & Laboratory Standards Institute (CLSI) broth microdilution method (Al-Hatmi et al. 2017). The MIC₉₀ is the minimum concentration that could inhibit 90% growth, whereas MEC is the lowest concentration at which the fungal spores show stunted growth with swollen tips (Borman et al. 2017). Both MICs/MECs were determined in 96-well microtiter plates, having 100 µl of twofold serial dilution of PHs in broth. Subsequently, the fungal spore suspension having 1 × 10⁶ spores ml⁻¹ was 50-fold diluted, and 100 µl of spore suspension was added in pre-filled wells to achieve a final concentration of 1–5 × 10⁴ spores ml⁻¹. Growth control and sterility control were taken for comparison. The plates were incubated for 72 h at 28 °C to obtain the end-point. The readings were taken through both a microplate reader and a plate reader mirror of 3 × magnification.

Spore germination inhibition assay

Spore germination inhibition assay was done by the cavity slide technique method as described earlier (Kepczynska and Krol 2012). The spore suspension was adjusted to 1 × 10⁶ spores ml⁻¹ and droplets (~50 µl) were placed on slides, kept in petriplates, and lined with moistened filter paper. Spores were mixed with 50 µl of water (control) or PHs solution to get the desired final concentrations. The germinated spores were counted under a light microscope, where > 300 spores were taken on a slide. For each treatment, six droplets were used. The germination process was ceased by adding 5% TCA at time intervals of 3 h, 6 h and 9 h.

Mycelial growth inhibition on agar

The effect of PH-mediated reduction in fungal growth observed in microtiter plate-based assay was also validated by the agar-plate inhibition assay (Krol et al. 2015). Briefly, different concentrations of PHs were taken and mixed in agar, which was poured into 90 mm Petri plates. A 5 mm diameter mycelial disk of the fungus was placed at the center of these agar plates and incubated in the dark at 28 °C for 7 days. The results were analyzed by measuring the diameter of fungal growth, and expressed as percentage growth inhibition in comparison with control using the following equation:

$$\frac{DC - DT}{DC} \times 100$$

Here, DC is the diameter of the fungal colony in control and DT is the diameter of the fungal colony with PHs treatment.

Priming of seeds and germination assay

V. mungo seeds were stored in a dry and dark environment. For surface sterilization, the seeds were immersed in 0.1% HgCl₂ for 3 min and then washed numerous times with double distilled water (ddH₂O). For a completely randomized study, the sterilized seeds were divided into 4 sets (50 seeds per set), namely control (C), fungal (F) treated, hormone (H) treated and both fungal and hormonal (F + H)-treated; hereafter referred as set I–IV, respectively. The first set of seeds (I), was treated with ddH₂O (hydro-primed) for 8 h. The second set (II), was incubated with *F. oxysporum* spores (10⁶ spores ml⁻¹) for 8 h, and then dried out. The third set (III), was soaked with PHs at two different concentrations for 8 h. The fourth set (IV), includes 8 h fungal-infected dried seeds (from set II), subsequently primed for 8 h with PHs (as in set III). Following the specific incubation period, the seeds were air-dried in the laminar flow hood before being allowed to germinate.

The seeds were placed on filter paper in sterile glass Petri plates (10 seeds/plate), covered with a layer of absorbent cotton, and blotting paper. These plates were kept in a seed germinator (REMI) and incubated at 28 °C under 16 h light/8 h dark, and watered 2 ml day⁻¹, until the end of test-period. The different germination criteria were recorded, where ‘germination’ referred to the emergence of a radical protrusion (≤ 2 mm) and recorded by daily observing the growth. The germination percentage (GP) and the mean germination time (MGT) were calculated after 3 days of sowing seeds. In contrast, seedling length (SL) and seedlings vigor index (SVI) were calculated after 7 days of sowing with the help of the below-mentioned formulas (Lemmens et al. 2019; Shiade and Boelt 2020).

Germination percentage(GP) = (Germinated seeds/total no of seeds) × 100

Mean germination time (MGT) = $\frac{\sum \text{Number of seeds germinated on the day of measurement} \times \text{number of days from the beginning of the experiment}}{\text{total no of seeds germination at the termination}}$

Seedling length (cm) = Mean seedling length of five normal seedlings in cm

Seedling vigor index(SVI) = Germination(%) × Seedling length(cm)

For biochemical analysis, seedlings obtained after the test period were immediately snap-frozen in an ultra-deep freezer (− 80 °C), until further use.

Histochemical detection of reactive oxygen species (ROS)

Accumulation of superoxide (O₂⁻) and hydrogen peroxide (H₂O₂) under stress conditions was detected by staining the *V. mungo* seedlings roots with nitroblue tetrazolium (NBT) and 3,3'-diaminobenzidine (DAB), respectively (Fryer et al. 2002). The root samples of 5-day-old seedlings were washed thoroughly with ddH₂O. For NBT staining, the roots were soaked overnight in 50 mM phosphate buffer, containing 6 mM NBT (pH 4.8). The NBT reacts with O₂⁻ and generates a formazan complex, appearing as deep-blue visible patches in roots, which was digitally captured. For H₂O₂ detection, roots were soaked overnight in a solution of DAB (1 mg ml⁻¹, pH 3.8). Owing to fungal infection, H₂O₂ was produced, which in turn binds to DAB resulting in the generation of reddish-brown visible color in roots, which was again digitally captured.

Measurement of malondialdehyde (MDA) content

The MDA content in seedlings was determined as described previously (Jakhar and Mukherjee 2014). Briefly, 5-day-old plant material (~0.5 g) was powdered in 0.1% (w/v) TCA solution and centrifuged at 10,000×g for 20 min at 4 °C. An aliquot of 1 ml supernatant was mixed with 4 ml solution having both TBA (0.5%) and TCA (20%). The mixture was vortexed thoroughly and heated for 30 min at 95 °C. The reaction was stopped by keeping the samples in ice for 5 min and centrifuging at 10,000×g for 10 min. The absorbance of the supernatant was measured at 532 nm and 600 nm. The concentration of MDA was determined by deducting the non-specific absorptions at 600 nm from 532 nm and applying an absorbance coefficient of extinction of 155 mM⁻¹ cm⁻¹.

MDA content(μmol g⁻¹FW) = $(A_{532} - A_{600}) \times V \times 1000 / 155 \times W$

here, A₅₃₂ and A₆₀₀ are the absorbances of the reaction mixture at 532 nm and 600 nm respectively, V is the extraction volume and W is the weight of the plant sample.

Measurement of hydrogen peroxide (H₂O₂) content

To determine the H₂O₂ amount, 0.4 g of 5-day-old plant seedlings was homogenized in 1 ml of 0.1% (w/v) TCA, then centrifuged at 12,000×g for 15 min. The supernatant (0.5 ml) was combined with 0.5 ml of 10 mM potassium phosphate buffer (pH 7.0), and 1 ml of 1 M KI. The final reaction mixture absorbance was measured at 390 nm (Velikova et al. 2000).

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay

Total free radical scavenging activities of PHs-primed seedlings were evaluated according to the previously described method, with slight modifications (Rajurkar and Hande 2011). A solution was prepared by adding 2.4 mg DPPH to 100 ml methanol. A methanolic sample solution (5 µl) of 7-day-old seedlings was mixed with the 3.995 ml of freshly prepared methanolic DPPH and incubated for 15 min, in the dark, at 37 °C. The discoloration of the reaction mixture was recorded at 517 nm. Methanol was taken as solvent control, while AA was used as a reference. DPPH radical scavenging activity (%) was determined using the following equation:

$$\left[1 - \left(\frac{A_{\text{test}}}{A_{\text{control}}} \right) \right] \times 100$$

where A_{test} and A_{control} are the absorbances of test and solvent control, respectively (Manoharlal and Saiprasad 2019).

Measurement of antioxidant enzymes activity

7-day-old plant material (0.5 g) was homogenized in 10 ml of 0.1 M ice-cold potassium phosphate buffer (pH 7.5) in a mortar and pestle. The homogenate was filtered after centrifuging it at 10,000×g for 30 min at 4 °C, and the supernatant was then utilized for the various tests for antioxidant enzymes.

Superoxide dismutase (SOD) activity

SOD activity was determined as mentioned in an earlier described method (Kumar et al. 2013). The assay was conducted in a 3 ml mixture volume, comprising 100 µl of enzyme extract, 50 mM potassium phosphate buffer (pH 7.8), 13 µM methionine, 63 µM NBT, and 1.3 µM riboflavin. The tubes were shaken and placed in light for 15 min. The inhibition of photochemical reduction of NBT was measured spectrophotometrically at 560 nm. One

unit of SOD activity is the point at which NBT reduction decreased to 50%.

Catalase (CAT) activity

CAT activity was determined following the earlier described procedure (Aebi 1984). The assay was carried out in a reaction mixture of 3 ml containing 3.2 mM H₂O₂, 50 mM phosphate buffer (pH 7.0), and 100 µl of enzyme extract. The activity was estimated by calculating the decrease in absorbance of H₂O₂ at 240 nm. Catalase activity was expressed as µmol of H₂O₂ decomposed min⁻¹.

Ascorbate peroxidase (APX) activity

APX activity was assayed according to the previously described method (Chen and Asada 1989). A 1 ml reaction mixture containing 50 mM phosphate buffer (pH 7.0), 0.1 mM ascorbate, 0.3 mM H₂O₂, and 50 µl of enzyme extract was used to measure the activity. The absorption was observed at 290 nm. The quantity of enzyme required to reduce 1 mol of H₂O₂ per min is referred to as one unit of APX activity.

Statistical analysis

The results are illustrated as mean ± SDs of three independent replicates for each data set. In order to conduct the statistical analysis, ANOVA (analysis of variance) followed by least significant difference (LSD) and Duncan's multiple range test (DMRT) was done using SPSS (Statistical Package for the Social Sciences). Similar and sharing letters denote statistically non-significant differences, while different letters indicate statistically significant differences ($p \leq 0.05$) in the outcomes.

Results

Phytohormones (PHs) exhibited antifungal activity against *F. oxysporum*

Fusarium genus contains hundreds of species that possesses broad-spectrum trans-kingdom pathogenicity. The CLSI clinical guidelines and standards adopted for assessing *Fusarium* susceptibility against the test chemicals in the in vitro methods were extended to this study.

The Microtiter plate assay: It is employed for obtaining the exact quantity of PH required to stop fungal growth/kill the pathogen (as described in the material and methods). MIC₉₀ is an indicator of ≥ 90% killing of the fungus. In contrast, MEC is the concentration that stops the germination of spores/conidia, a critical stage in the fungus life cycle,

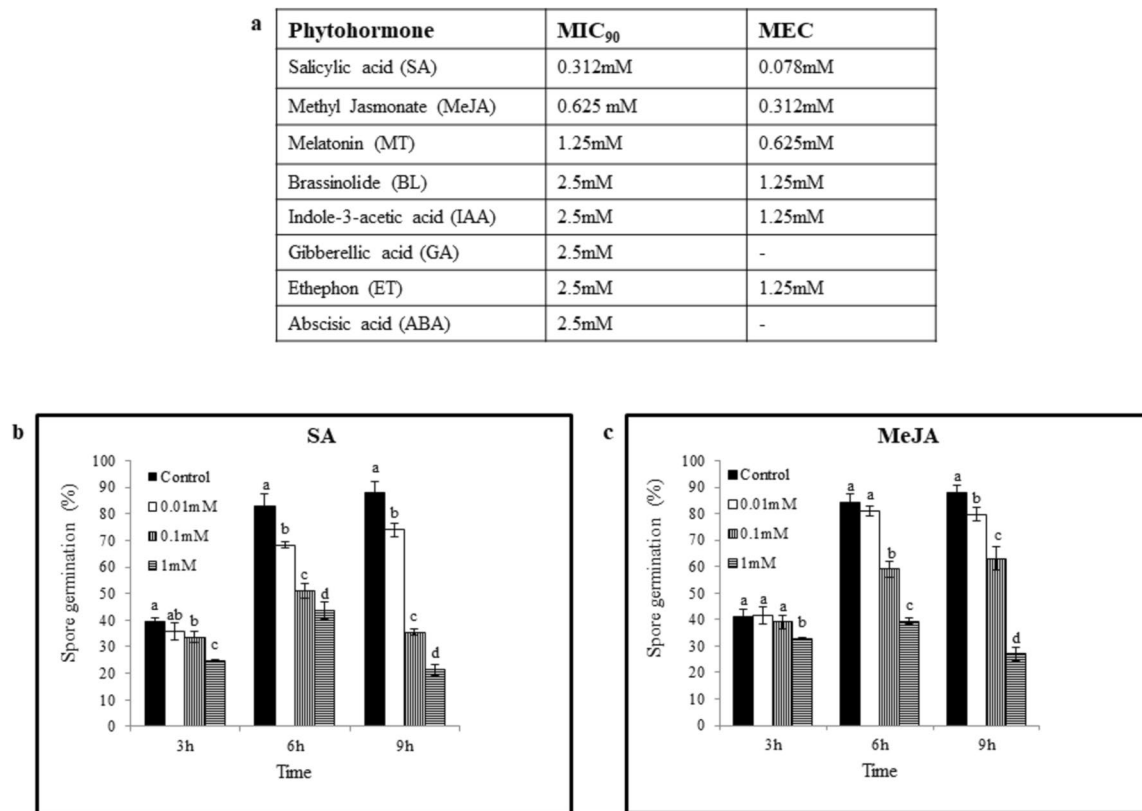


Fig. 1 The in vitro antifungal activity of different PHs against *F. oxysporum* 9913. **a** microtiter plate-based assay done for determining the Minimum Inhibitory Concentration (MIC₉₀) and Minimum Effective Concentration (MEC), end-points were recorded after 72 h. **b**, **c** The fungal spore germination in the presence of SA, and MeJA was evaluated with both PHs at different concentrations, viz 0.01 mM,

0.1 mM, and 1 mM. The results taken at regular 3 h intervals are the mean of three independent experiments, and vertical bars indicate the standard deviation (\pm SD). Data marked with different letters show significance ($p \leq 0.05$), whereas similar and sharing letters show insignificant deviations

but logically lower than the MIC end-point. The various PHs- SA, MeJA, MT, BL, IAA, GA₃, ET, and ABA were tested to calculate the MIC₉₀ and MEC end-point against *F. oxysporum*. Our results showed that among the different PHs tested, SA was most effective against *F. oxysporum*, with a corresponding MIC₉₀ and MEC end-point at 0.312 mM and 0.078 mM, respectively (Fig. 1a). The second-best activity was observed for MeJA, with a corresponding MIC₉₀ and MEC at 0.625 mM and 0.312 mM, respectively (Fig. 1a). MT did fairly well with a corresponding MIC₉₀ and MEC value 1.25 mM and 0.625 mM, respectively. Notably, the rest of the PHs (BL, IAA, GA, ET, and ABA) had comparable activity at 2.5 mM (MIC₉₀) and 1.25 mM (MEC) (Fig. 1a).

Fungal spore germination: The antifungal activity of each PH against the fungal spores was corroborated through spore germination assay. To gage the activity of PHs on spore germination, we limited ourselves to SA and MeJA. As anticipated, the germination of the spores was sensitive to the SA and MeJA (Fig. 1b, c). Both the tested PHs inhibited the germination of spores at all three different concentrations tested (0.01 mM, 0.1 mM and 1 mM). As can be seen in

Fig. 1b, after 3 h, the spore germination started showing an inverse trend with the increasing SA concentration. The germination of fungal spores was 35.7%, 33.7%, and 24.6% at 0.01 mM, 0.1 mM, and 1 mM of SA respectively, compared to the control (39.3%). These results suggested that although the inhibition at 0.01 mM was not significant after 3 h incubation, although 0.1 mM and 1 mM of SA, showed a significant reduction in spore germination. The results even got better after 6 h incubation, wherein comparison to 83% spore germination (control); 0.01 mM, 0.1 mM, and 1 mM of SA caused 68.3%, 51%, and 43.7% spore germination, respectively (Fig. 1b). The trend was essentially similar after 9 h, where percentage spore germination remained at 74%, 35.33%, and 21.33%, at the concentration of 0.01 mM, 0.1 mM, and 1 mM, respectively, as compared to control (88%).

In the case of MeJA, an inhibition in spore germination was also observed (Fig. 1c). After 3 h incubation, the sub-inhibitory concentration, 0.01 mM and 0.1 mM of MeJA did not bring any significant inhibition of spore germination. However, 1 mM caused a significant (~8.3%) reduction

in spore germination. Interestingly, at 6 h, the results got engaging, wherein even 0.1 mM MeJA showed a substantial (~25%) spore germination inhibition. Notably, spore germination inhibition showed a remarkable improvement with an increment in PH concentration, as well as incubation time. Here, although all three concentrations showed marked inhibition, the prominent suppression was observed at 1 mM, wherein only 27% spores' germination was recorded at 9 h than 88% spore germination in control (Fig. 1c). Overall, these results concluded that both SA and MeJA significantly inhibited the *Fusarium* spore's germination.

Mycelium inhibition (%): *F. oxysporum* is a spore-forming fungus. However, invasion and spread to different regions in the plant happen through mycelia formation. Indeed, the disease-causing potential of fungus is directly correlated with its mycelia formation. The inhibitory effect of SA and MeJA on fungal mycelia formation was tested by placing a fungal growth disk on agar, having desired PH concentration and results were recorded after 7 days of incubation at 28 °C (Fig. 2a, b). The diameter of the fungal growth region (mm) was measured and calculated as percentage growth inhibition (Fig. 2B). Our results demonstrated that SA causes 13.0%, 40.6%, and 97% inhibition at 0.01 mM, 0.1 mM, and 1 mM, respectively. Similarly, MeJA also caused substantial inhibition of 8.8%, 23%, and 96.7% at 0.01 mM, 0.1 mM, and 1 mM, respectively (Fig. 2b). These results also corroborated the microtiter and fungal spore germination inhibition assays. The experiments with the rest of the PHs were also

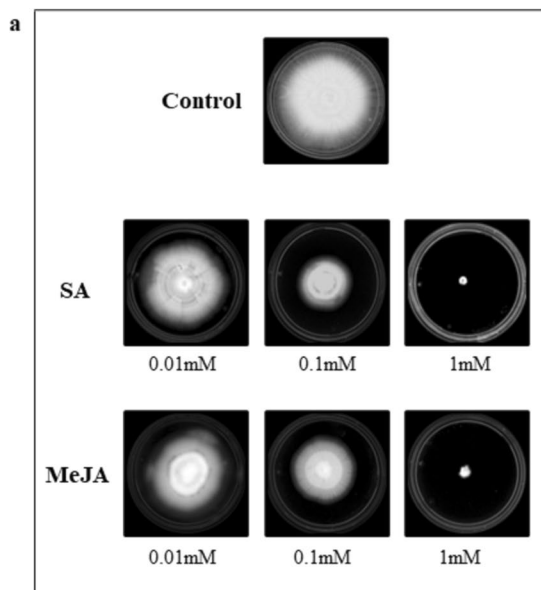
performed (Fig. S1a, b). Overall, the data suggested that SA and MeJA showed the highest potency against *F. oxysporum*.

Further, to assess the role of PHs in imparting defense against *F. oxysporum*-generated stress, it was imperative to investigate the consequence of exogenously supplied PHs on fungal-infected seeds. Consequently, SA and MeJA were chosen for in vivo activity against one of the well-characterized *Fusarium* host plants, *V. mungo*, wherein an impact on distinctive germination parameters and antioxidant levels was monitored.

SA and MeJA priming improved germination parameters in fungal-treated seeds

SA and MeJA competence in tackling *Fusarium* intervened stress was determined through four different seed germination parameters i.e., germination percentage (GP), mean germination time (MGT), seedlings length (SL), and seedlings vigor index (SVI). For this, the seeds were subjected to four different treatments, namely set I–IV (as explained earlier).

The GP in all four sets of seeds, as recorded after 3 days of seeding (Fig. 3a). Our results suggested that set I (control; black bar) witnessed 96% germination, whereas F-treated II set (vertical lined bar), had a substantially lower GP (~78%), confirming that fungal infection mediated biotic stress significantly reduced the seed germination. From these results, it can be extrapolated that *Fusarium* infestation impairs the plant growth from the germination stages to probably the yield and other quality parameters in the later growth stages



Phytohormone	Concentration (mM)	Mycelial growth (mm)	Inhibition (%)
Control	-	77.4 ± 0.6	nd.
SA	0.01	67.4 ± 3.0	13.0 ± 5.9
	0.1	46 ± 5.2	40.6 ± 7.8
	1	2.4 ± 0.6	96.9 ± 1.8
MeJA	0.01	70.6 ± 2.8	8.8 ± 4.7
	0.1	59.6 ± 3.2	23.0 ± 5.1
	1	2.6 ± 1.0	96.7 ± 2.2

Fig. 2 Agar-based plate assay for showing the effect of PHs on inhibiting *F. oxysporum* growth. **a** *F. oxysporum* mycelial plug of equal size were placed in the control plate, as well as plates having different concentrations: 0.01 mM, 0.1 mM, and 1 mM of SA and MeJA, after

7 days of inoculation. **b** The diameter of the growth zone in control and test plates is represented in mm. The growth inhibition percentage of *F. oxysporum* was measured for comparison

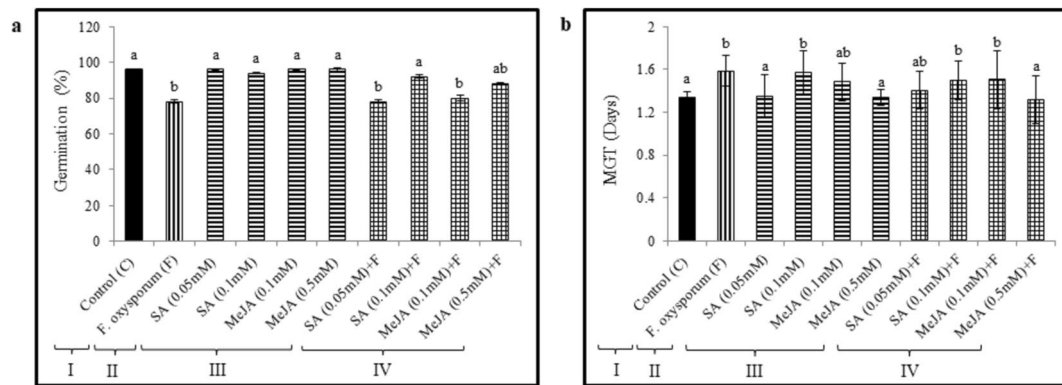


Fig. 3 Comparison of various germination parameters of 3-day-old *V. mungo* seedlings in four different sets. Here growth control (black bar), fungal infection control (vertical lined bar), hormones treatment (horizontal lined bars), and fungal infection followed by hormone treatment (squares filled bars) are calculated and compared **a** Ger-

mination Percentage (GP), and **b** Mean Germination Time (MGT) of seedlings. The results are the mean of three independent experiments, and vertical bars indicate the standard deviation (\pm SD). Data marked with different letters show significance ($p \leq 0.05$), whereas similar and shared letters signify insignificant changes

as well. *Fusarium* infestation in the *V. mungo* seedlings was validated by performing the root's histochemical sectioning (Fig. S2). For set III, hormo-priming at sub-inhibitory concentrations of SA and MeJA was employed and the consequent effect on germination in *V. mungo* seeds was recorded (horizontal lined bars). The SA (0.05 mM and 0.1 mM) and MeJA (0.1 mM and 0.5 mM) showed GP (~95%), comparable with the control. Thus, both defense hormones did not augment germination in seeds more than in the control. However, the IV set of fungal exposed and PHs primed seeds showed promising results (squares filled bars), wherein a ~14% increment with 0.1 mM SA was recorded than F exposed set II. Although, the lower concentration of SA (0.05 mM) did not show any significant effect. MeJA priming at 0.1 mM and 0.5 mM was not conclusive for a positive statement. Thus, it can be summarized that priming with SA augmented germination in fungal-infected seeds, possibly by alleviating the fungal-induced stress by eliciting defense signaling.

The MGT of *V. mungo* seeds was also recorded after 3 days of seedling growth (Fig. 3b). The MGT in set II seeds was longer (1.55 days), than in control (1.34 days), indicating that fungal infection causes a delay in seed germination (vertical lined bar vs black bar). For SA priming (set III, horizontal lined bars), no significant improvement in MGT was observed as compared to the control (set I). The MGT of 0.05 mM SA-treated seeds was 1.35 days (comparable to the control), which became longer at 1.57 days in 0.1 mM treated seeds. Although this trend was not followed in the case of MeJA-primed control seeds, wherein MGT was 1.49 days for 0.1 mM MeJA-primed seeds, however, the germination time was reduced to 1.34 days at its higher concentration (0.5 mM). Similarly, in set IV, infected seedlings primed with 0.05 mM and 0.1 mM of SA did not show any

significant improvement (MGT of 1.41 days and 1.5 days, respectively). On the contrary, MeJA-primed fungal-infected seeds showed a lower MGT value of 1.32 days at its higher concentration (0.5 mM). However, the lower concentration of MeJA (0.1 mM) was not as effective in lowering the MGT of infected seeds (1.5 days) (set IV, squares filled bars). These results further strengthen the earlier reports, wherein MeJA was favored more as a defense hormone, rather than a growth hormone.

The effect of fungal attack on *V. mungo* seedlings and the protective effect of SA and MeJA was also observed by mapping the morphological growth characteristics in 7 days old seedlings (Fig. 4a, b). It appears that F-treated seedlings (set II) had a relatively compromised length than set I of control seeds. The F-infected seedlings length was 12.09 cm, whereas in control, it was 15.37 cm (Fig. 4c, vertical lined bar vs black bar). The PHs priming alone did not improve the seedling length, except for a slight increase with 0.1 mM of SA (upper row in Fig. 4a and horizontal lined bars in Fig. 4c). However, the SA treatment significantly helped in overcoming the negative impact of fungal infection on seedlings' growth at 0.1 mM (14.76 cm) (squares filled bars in Fig. 4c). Notably, the lower concentration of SA (0.05 mM) did not augment the significant length of the infected seedlings. The two different concentrations, 0.1 mM and 0.5 mM of MeJA (set III), also had comparable growth to control seedlings. However like SA, only the higher concentration of MeJA (0.5 mM) could improve (14.6 cm) the infected seedlings' length (Fig. 4c).

For SVI, the infected seedlings (Set II) had a much lower SVI (953) than the control (set I) (1447.17) (Fig. 4d, vertical lined bar vs black bar). Although in the SA and MeJA primed seedlings, SVI was equivalent to the control (set III, horizontal lined bars). In set IV, the lower concentration

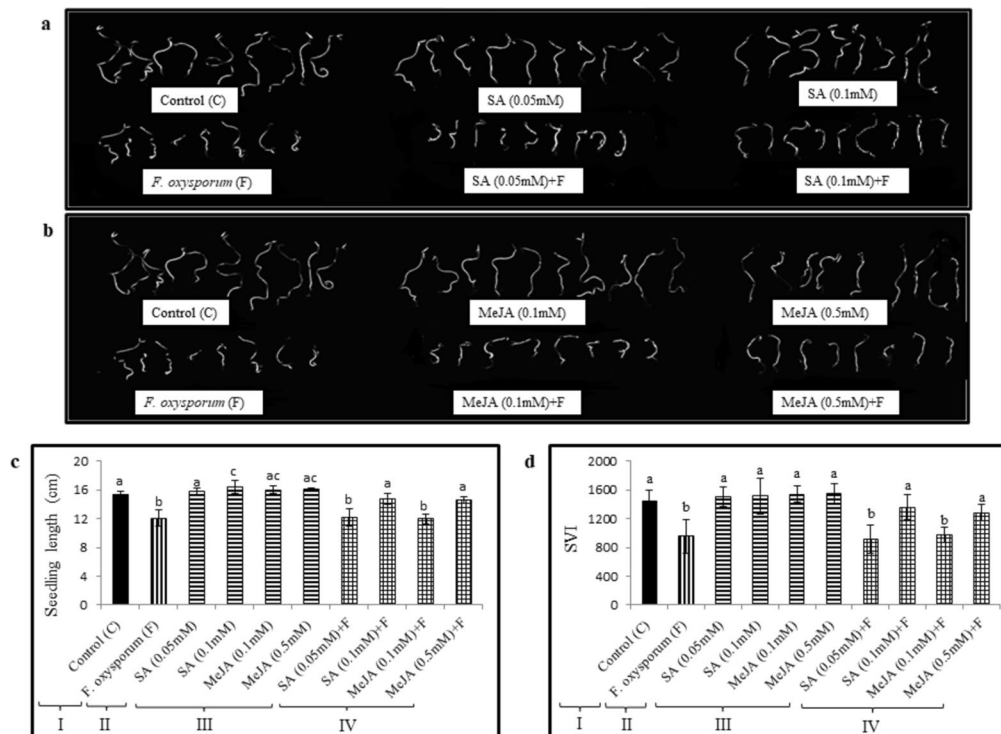


Fig. 4 The in vivo effect of PH was recorded by observing morphological growth characteristics in seedlings. **a** The SA, and **b** MeJA-primed seeds were grown and seedlings were collected after 7 days observing the seedling's morphological growth characteristics, taken in four different sets (explained earlier), including growth control, fungal infection control, hormones treatment, and fungal infection combined with hormone treatment. The figure shows 7 days old seedlings of *V. mungo*, showing the different lengths of radicle and

plumule. **c** The seedling length was compared in different sets and **d** seedling vigor index (SVI) was calculated. The four different sets include growth control, fungal infection control, hormones treatment, and fungal infection combined with hormone treatment. The mean of three independent experiments is the result, and vertical bars indicate the standard deviation (\pm SD). Data marked with different bars letters show significance ($p \leq 0.05$), whereas similar and sharing letters signify insignificant changes

of both SA (0.05 mM) and MeJA (0.1 mM) did not result in much surge in the SVI than fungal-infected alone (set II); however, the higher concentration of SA (0.1 mM) and MeJA (0.5 mM), could reclaim SVI back to control (set IV, squares filled bars). These results suggested that PHs priming effectively counters the fungal infection in a concentration-dependent manner.

SA and MeJA priming reduced ROS accumulation in infected *V. mungo* seedlings

The plant defense against pathogens involves an oxidative burst, characterized by a surge in ROS levels (Kim and Hwang 2014; Yang et al. 2017). Thereby, an increase in ROS levels was also expected in *Fusarium*-mediated infection. This was confirmed with histochemical staining with ROS-sensitive dyes, nitroblue tetrazolium (NBT), and 3,3'-diaminobenzidine (DAB) (Fig. 5a, b) and quantitative ROS levels in seedlings extract (Fig. 6a, b).

During seedlings staining, NBT and DAB dyes infiltrate through plant roots and react with accumulated O_2^- and H_2O_2 respectively, thereby resulting in root-staining (Samatly et al. 2018). The intensity of coloration correlates with ROS accumulation. NBT staining of 5 days old seedling roots was performed, and an overnight incubation showed O_2^- buildup in control seedlings, seen as deep blue patches around roots (Fig. 5a). Notably, fungal-treated set II roots showed a negative effect on seedlings' growth, corresponds well with a more intense coloration than the control roots (Fig. 5a, set I vs II). The SA and MeJA priming was done to ascertain their effect on O_2^- levels. Seedlings of 0.1 mM SA-treated seeds showed lesser color intensity than 0.05 mM. Similarly, seedlings with 0.1 mM and 0.5 mM MeJA primed seeds had lesser color intensity than the control, as can be compared in sets III and I. These results suggested a reduction of O_2^- levels in SA and MeJA primed seedlings than in control. The IV set seedlings were observed to have lower staining intensity than F alone (set II vs IV). This suggests that seed priming with SA and MeJA diminished

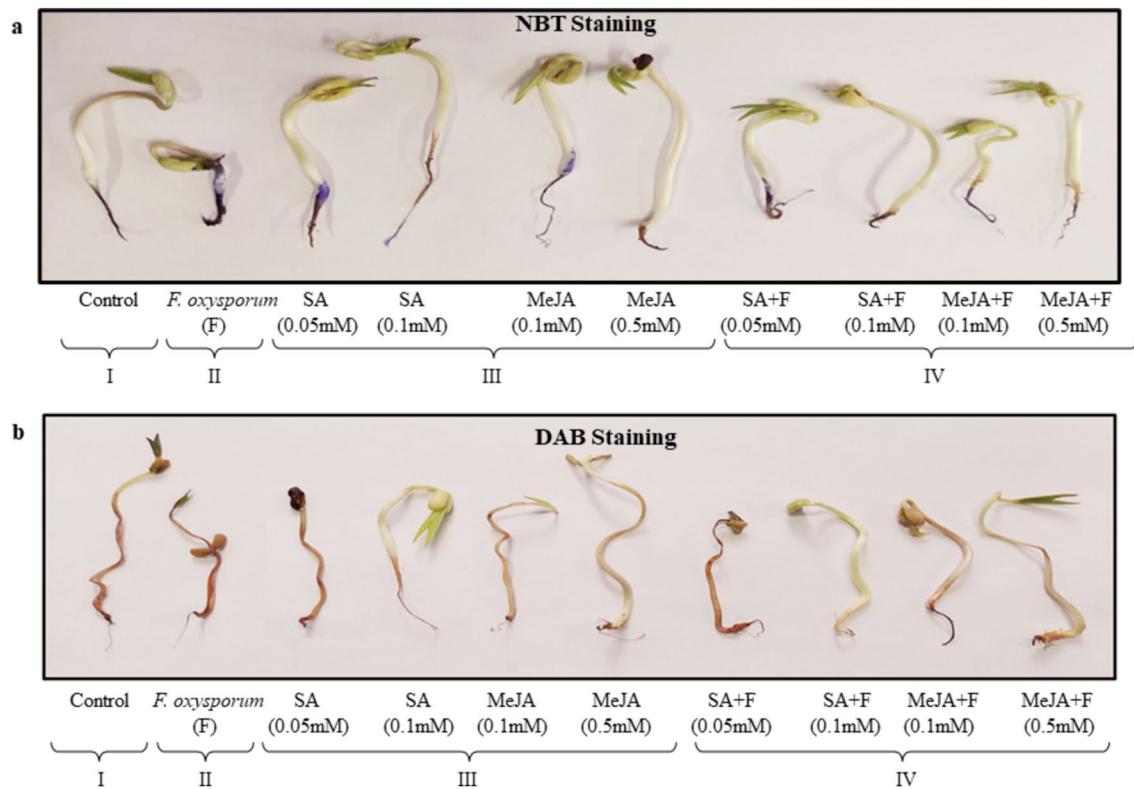


Fig. 5 The reactive oxygen species (ROS) accumulation was tested by staining 5-day-old *V. mungo* seedlings. **a** Superoxide (O_2^-) accumulation was detected with nitroblue tetrazolium (NBT), and **b**

Hydrogen peroxide (H_2O_2) was identified by 3,3'-diaminobenzidine (DAB) staining, as described in the material and methods

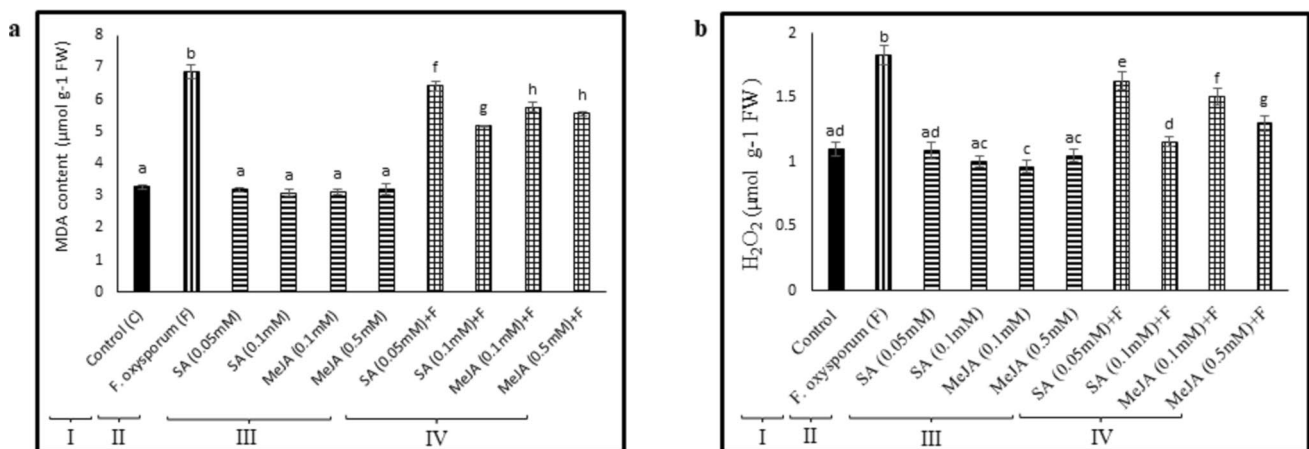


Fig. 6 The quantitative ROS generation in fungal infected and PHs (i.e., SA and MeJA) priming. **a** Malondialdehyde (MDA) levels and **b** hydrogen peroxide (H_2O_2) accumulation, in the extract of 5-day-old

V. mungo seedlings. The data is the mean \pm SD of three replicates per set. Here, different lowercase letters on the bars represent statistically significant differences ($p \leq 0.05$)

O_2^- accumulation and improved overall germination characteristics despite fungal infection (Fig. 5a).

The H_2O_2 levels in all four sets were also estimated by overnight immersing the seedlings in the DAB solution and visualizing their color intensity, which appeared as brown

patches. As can be seen in Fig. 5b, the F seedlings (set II) showed intense DAB staining. Pale staining was observed for 0.1 mM SA, and 0.1 mM and 0.5 mM of MeJA-primed seedlings (set III). For the IV set, the higher concentrations of SA (0.1 mM) and MeJA (0.5 mM) indicated reduced H_2O_2

levels due to faint staining. Although, lower concentrations (i.e. 0.05 mM and 0.1 mM of SA and MeJA, respectively) of both the PHs did not show any clear reduction of ROS in infected seedlings. Thus, it can be concluded that 0.1 mM and 0.5 mM of SA and MeJA, respectively, reduced H_2O_2 levels in fungal-infected seedlings.

Although the histochemical staining results indicated qualitative differential ROS levels; still to statistically validate the results, we further performed a quantitative estimation of ROS indicators in seedlings' extract. For this, MDA accumulation, as well as H_2O_2 content in fungal-infected and PH-treated *V. mungo* seedlings were measured (Fig. 6a, b). The MDA content is an indicator of increased cell membrane damage, which results in excessive ROS accumulation (Fig. 6a). As anticipated, the MDA content increased by more than two-fold in the fungal-infected seedlings (set II, vertical lined bars), than in the control (set I, black bar). Under the absence of any biotic stress, the MDA levels in SA and MeJA (set III, horizontal lined bars) treated seedlings were comparable with non-primed control seedlings (set I). Further, in the fungal-infected seedlings of set IV (squares filled bars), both SA and MeJA significantly reduced the MDA content. However, priming with SA showed the best results and its 0.1 mM concentration reduced the MDA accumulation by ~25%, more than F-infected seedlings in set II (Fig. 6a, set IV vs II).

The H_2O_2 content in seedlings extract was measured with KI in 5 days old *V. mungo* seedlings (Fig. 6b). The comparison of different sets suggested that fungal infection elevated the H_2O_2 levels (set II, vertical lined bars), compared to the healthy non-infected seedlings (set I, black bar). Like the trend with earlier results, PHs priming alone did not contribute to H_2O_2 levels (set III; horizontal lined bars). However, 0.1 mM of MeJA seedlings treatment did result in a decrease in H_2O_2 levels. This suggested that MeJA does have a positive impact on H_2O_2 level reduction. In set IV (squares filled bars), the PHs treatment significantly lowered the H_2O_2 levels in the fungal-infected seedlings, although the finest results were seen with 0.1 mM of SA. At this concentration, SA decreased the H_2O_2 content by ~37% in the infected seedlings and managed to counter the ROS accumulation (Fig. 6b).

SA and MeJA treatment elevated ROS-scavenging

Plants maintain redox homeostasis by generating compounds with antioxidative properties (Dumanovic et al. 2021; Samsatly et al. 2018). To assess the protective role of PHs, we measured the ROS-scavenging activity through DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. We observed that the infected F-treated seedlings displayed a relatively enhanced antioxidant potential (57.7%), compared to the control (53.3%) (Fig. 7, black and vertical lined bars for

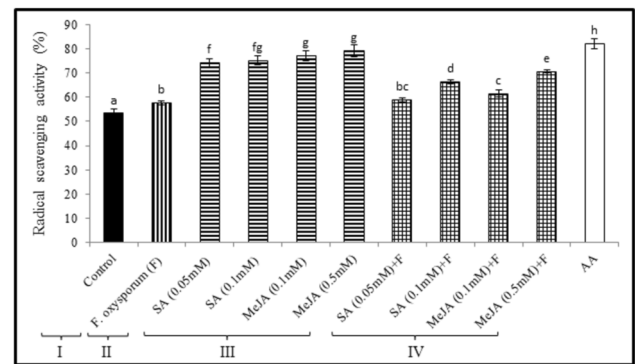


Fig. 7 The DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay for assessing the effect of SA and MeJA priming. The extract of 7 days old seedlings was prepared, and DPPH scavenging activity was measured at 517 nm. Ascorbic acid (AA) was used as a reference. Vertical bars indicate \pm SD, $n=3$. Means with separate letters were found significant ($p \leq 0.05$), whereas similar and sharing letters did not signify considerable change

set I & II, respectively). In set III (horizontal lined bars), the SA-primed control seedlings had an antioxidant activity of 74.23% and 75.2%, at concentration of 0.05 mM and 0.1 mM, respectively. Similarly, the MeJA-primed seedlings had a scavenging activity of 77.1% and 79.3% at 0.1 mM and 0.5 mM concentrations, respectively. Interestingly, these values were comparable with the natural antioxidant ascorbic acid, AA (82.2%) (white bar, Fig. 7). In the IV set (squares filled bars), a significant boost in the antioxidant activity with 0.5 mM MeJA gave 70.7% activity. 0.1 mM of SA enhanced the antioxidant level to 66.3% in seedlings. Notably, the lower concentration of MeJA (0.1 mM) was also effective in significant increase of the antioxidant level of the pre-infected seedlings. Although, SA at its lower concentration of 0.05 mM, did not significantly elevate the scavenging process.

The antioxidant enzymes activity increased in fungal-infected PH-primed seedlings

The cells have different antioxidant enzymes for controlling ROS levels. Major physiological antioxidant enzymes include- SOD, CAT, and APX. As fungal stress elevates the ROS levels, cellular homeostasis tries to detox them by increasing the activity of these enzymes. For this, 7-day-old seedlings extract was prepared and the activity of these three antioxidant enzymes was determined. The results were apparent that indeed plant cells were trying to reduce the ROS levels, evident with increasing antioxidant enzyme activity. The SOD activity increased by 64% in the *Fusarium*-infected seedlings (set II, vertical lined bar) than control (set I, black bar) (Fig. 8a). The PHs protective role was evident in primed non-infected seedlings (set III, horizontal

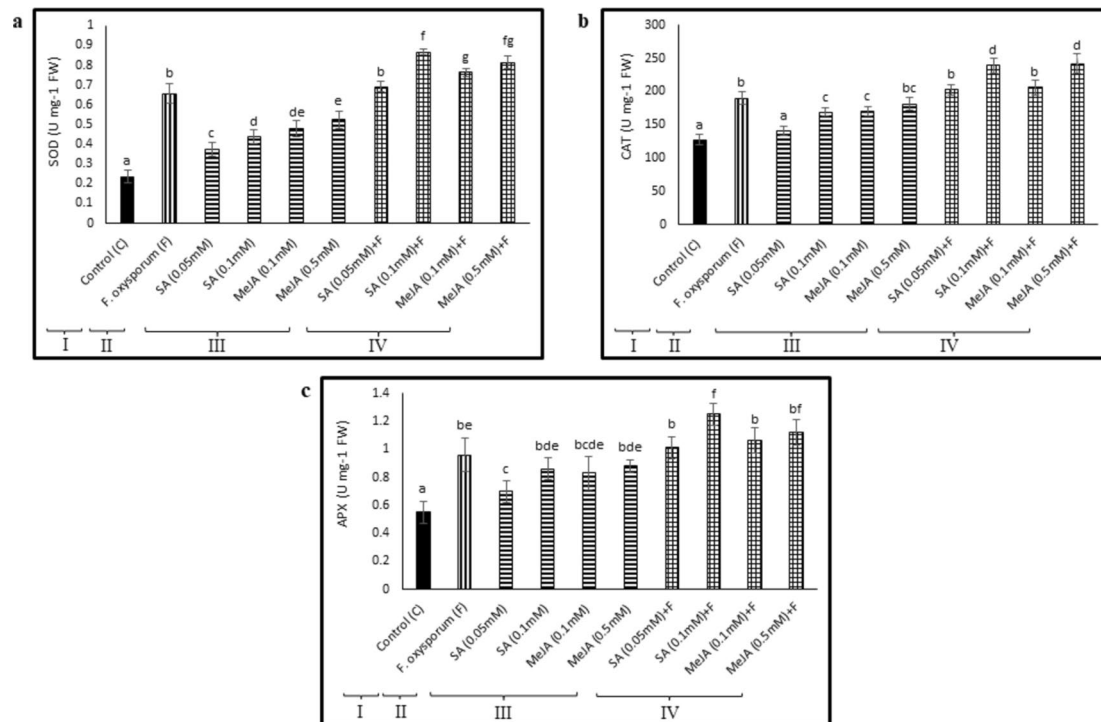


Fig. 8 The effect of SA and MeJA priming on the different antioxidant enzymes. The 7 days old fungal-infected *V. mungo* seedlings were assessed by measuring the activity of **a** superoxide dismutase (SOD), **b** catalase (CAT), and **c** ascorbate peroxidase (APX) in dif-

ferent treatment sets and explained in methods. The column represents \pm SD, $n=3$. Different lowercase letters indicate a statistically significant difference ($p \leq 0.05$), whereas similar or sharing letters signify insignificant differences

ferent treatment sets and explained in methods. The column represents \pm SD, $n=3$. Different lowercase letters indicate a statistically significant difference ($p \leq 0.05$), whereas similar or sharing letters signify insignificant differences

lined bars). Thus, it was likely that PH protected fungal-infected seedlings, which was validated with set IV results. The PH-treated and infected seedlings showed increased SOD action and 24% raise was seen with 0.1 mM of SA (squares filled bars) than set II. It was followed by MeJA-led 19% and 13% increased activity with 0.5 mM and 0.1 mM concentrations, respectively (Fig. 8a).

A similar trend was seen with CAT, and it was apparent that to counter the infection; CAT activity increased by more than 30% in set II of infected seedlings extract (Fig. 8b; vertical lined bar). The PH treatment also increased CAT activity in healthy seedlings; although only 0.1 mM of SA (~25%) resulted in a significant increase. On the other hand, both 0.1 mM and 0.5 mM of MeJA were effective and a 25% and 30% increase was recorded respectively (set III, horizontal lined bars) than control seedlings (set I, black bar). Contrarily, in set IV (squares filled bars), only higher concentrations of both SA and MeJA (0.1 mM and 0.5 mM of SA and MeJA, respectively), boost the CAT activity significantly, which was close to 22% in both extracts (Fig. 8b).

The APX activity also filled the gaps in validating the increased activity observation with SOD and CAT. The APX activity increased by ~43% in the fungal-infected seedlings (Fig. 8c, set II, vertical lined bar) than in set I (black bar). Further, in set III (horizontal lined bars), all PHs treatments

significantly enhanced the APX activity more than the control (set I). Still, PH treatment in set IV (squares filled bars) seedlings extract did not augment APX activity worth consideration, except, for a 23% surge in APX activity in the 0.1 mM SA-primed seedlings than set II (Fig. 8c).

Thus, results suggested that SA and MeJA increased the scavenging activity and antioxidant levels in primed *Fusarium*-infected seedlings.

Discussion

The ever-increasing population compels for escalating crop productivity; thereby, newer farming methods and technologies are embraced to develop high-produce varieties. However, crop growth and yield are threatened by various abiotic and biotic factors, which adversely affect plant growth and development.

In plants, (a)biotic stresses are modulated by well-characterized PHs, i.e., IAA, GA, ABA, CK, ET, JA, SA, MT, SL, and BL. However, the majority of exogenous PHs therapies on various crops besieged abiotic threats, including- salinity, drought, heat, heavy metal, oxidative stress, etc. (Fahad et al. 2015; Li et al. 2021; Saini et al. 2021; Ullah et al. 2018). For instance, ABA application alleviates wheat tolerance in

saline conditions (Li et al. 2020). Su et al. (2020) observed that BL priming augments salinity tolerance via regulation of Na^+ (K^+)/ H^+ antiporters transcription in apples (Su et al. 2020). SA spraying on seedlings improves salt tolerance in pistachio (Bastam et al. 2013). ET reduces glucose sensitivity and inverts photosynthetic suppression in salt-stressed wheat by optimizing glutathione production (Sehar et al. 2021). MeJA, methyl ester of JA, exogenously sprayed on *Brassica napus*, *B. campestris*, and *B. juncea* modulates glyoxalase systems and bestows salt resistance (Alam et al. 2014). Exogenous MT treatment could protect peanut seedlings against salinity stress (ElSayed et al. 2020). Analogously, a combination of PHs was initiated to map their interactions with each other. In chickpeas, JA in combination with GA_3 enhances tolerance to cadmium metal and oxidative stress (Ahmad et al. 2021). MeJA and SA combined exogenous application through foliar spray on maize mitigate salt stress (Tayyab et al. 2020). GA and JA combination does the same in summer squash (Al-harhi et al. 2021). Similarly, SL (through synthetic SL analog GR_{24}) and SA foliar spray help in drought tolerance accomplishment in wheat by lowering electrolyte leakage and controlling water content (Sedaghat et al. 2017). The external plant analysis in these studies advocates that exogenous PHs assist in stabilizing metabolic activities to normal levels and are evident in improved growth parameters. However, transcriptomic studies suggest complex changes involving alteration in gene expression of many genes, including both over-expression and repression (Hartmann et al. 2022; Li et al. 2022). Here, it is pertinent to mention that PHs interact antagonistically or synergistically with each other, creating a multiplex system that could be difficult to interpret and require a more profound understanding.

Over the last two decades, SA, JA, and ET have particularly gained attention for their role in defense against phytopathogens. These defense PHs interact with each other, as well as with other general growth hormones, and modulate different signaling cascades. However, the capacity of these defense hormones to mitigate biotic stress remains largely unexplored. It could be the reason that the general antifungal activity of the PHs and their analogs was never studied in detail. Consequently, our study measured *in vitro* antimicrobial activities of different PHs against *Fusarium*. Among the various PHs tested, SA and MeJA showed promising results at sub-micromolar concentrations (Figs. 1, 2). SA and MeJA functions in imparting plant defense were anticipated during biotic stress. For example, tomato seeds primed with MeJA trigger the protection against the necrotroph fungus, *Alternaria porri* f. sp. *solani* in concomitant with better growth parameters (Kepczynska and Krol 2012). Similar results have been demonstrated in tomatoes against *F. oxysporum* f. sp. *lycopersici* infection when primed with MeJA via accumulation of 12-oxo-phytodeinoic acid, SA

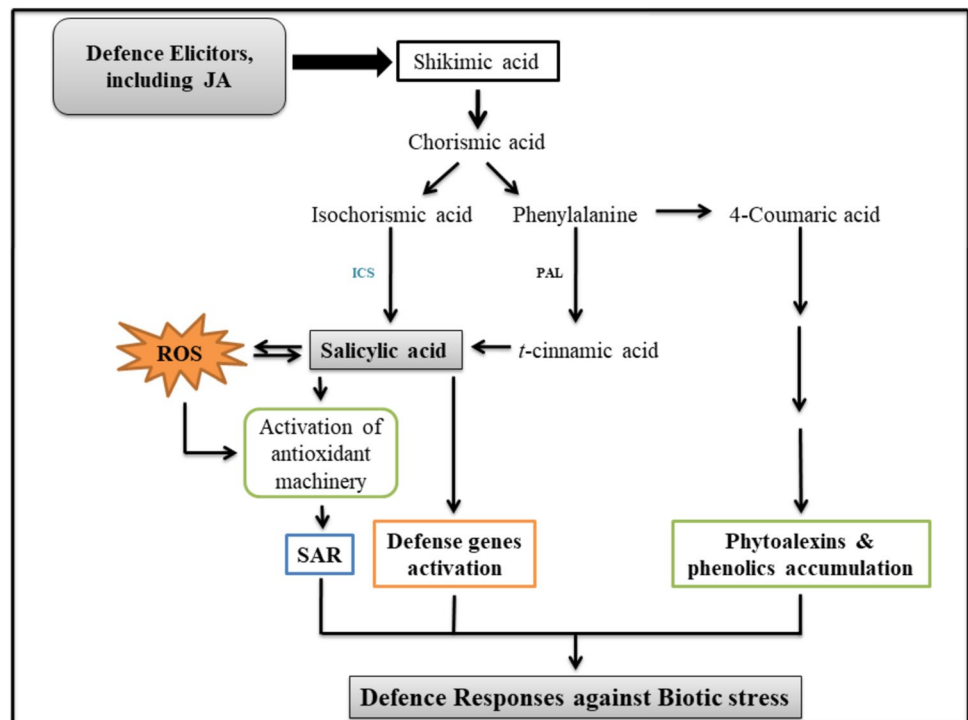
and flavanol, etc. (Krol et al. 2015). Besides growth, these studies also reported the elevation of many phenolic compounds, such as SA, kaempferol, and quercetin (Krol et al. 2015). Hence, hormo-priming with SA and JA offers protection against infections and simultaneously provides resistance and tolerance.

To explore this possibility in the present study, we used *V. mungo* as the host plant. For *in vivo* effectiveness, the sub-inhibitory concentration of SA and MeJA was used separately as priming material. Our results suggested that although both these PHs did not significantly assist in seed germination in control seedlings, but substantially inhibited the *Fusarium* mycelia formation and spore germination in infected seedlings (F). Thus, SA and MeJA augmented the germination characteristics of *Fusarium*-exposed *V. mungo* seedlings.

The role of SA in defense seems to be related to its biosynthesis via the shikimic acid pathway, induced by different defense PHs, particularly SA, MeJA, and ET, which assist in the production of secondary compounds, like- SA, phytoalexins, phenolic compounds, flavonoids, alkaloids, terpenoids, etc., that are involved in plants' defense (Fig. 9) (Krol et al. 2015). Thus, we presume that SA enrichment through exogenous treatment also assists in improving the metabolic activities and stimulating explicit reactions in plant's defense, like retarding ethylene synthesis, fortification of cell membranes, antioxidants, osmoprotectant, and photosynthetic pigments, *i.e.*, chlorophyll (Krol et al. 2015; Nazar et al. 2015; Torun et al. 2020).

The SA biosynthesis also occurs through the cinnamate route, controlled by the product of phenylalanine ammonia-lyase (*PAL*), which seems to play a role in PH priming as well (Fig. 9). This branch is also known to trigger oxidative burst and ROS accumulation, generating resistance and imparting defense against pathogens (Lefeverre et al. 2020). Our results suggested an increase in ROS levels during fungal infection, with apoplastic O_2^- and H_2O_2 accumulation (Figs. 5, 6). However, this increased ROS triggers hypersensitive programmed cell death (PCD), as reported by Xing et al., and accompanies chloroplast dysfunction, *PAL* up-regulation, and SA accumulation (Xing et al. 2013). Excessive ROS can ionize membrane lipids, generating lipid peroxides, and leading to the formation of damaging intermediates, such as MDA (Singh and Upadhyay 2014). This might result in the seedling's downfall, and thus, controlling ROS levels would be critical for plants' survival. Thus, it seems probable that an appropriate equilibrium between ROS accumulation and abrogation is essential for both pathogen removal and host survival. These observations were again validated in our results, and low levels of MDA, O_2^- and H_2O_2 were recorded in SA-primed infected seedlings, compared to the non-primed infected seedlings in set II (Figs. 5, 6). In DPPH

Fig. 9 Schematic model for defense responses in the plants against biotic stress. The hormo-priming of PHs elevates different defense elicitors which ultimately induce defense gene activation, antioxidants, phenolics, and phytoalexin accumulation against the biotic stress in the plant



and antioxidant enzymes assays, the SA-treated infected seedlings (set IV) exhibited better ROS-scavenging activity than the F-infected (Figs. 7, 8). This supports that SA contributes to managing ROS levels by reducing the oxidative damage induced by fungal stress. Thus, it is suggested that exogenous SA application would play a protective role by initiating a negative feedback loop, thereby limiting ROS levels and, avoiding PCD, which could be the reason behind an increase in recorded GP and other germination parameters in set IV (Figs. 3, 4).

Similar to SA, MeJA priming alone did not show much change in the germination parameters of control seedlings (set III vs set I). On the contrary, *Fusarium*-infected seedlings showed significant improvement in the GP, MGT, SL, and SVI at 0.5 mM of MeJA. It could be due to the reason that JA biosynthesis (octadecanoid pathway) itself starts in response to biotic stress. It activates the genes that code for enzymes which produce volatile compounds, or secondary substances like nicotine, a variety of phenolics, and other molecules implicated in defense (Ruan et al. 2019). Interestingly, jasmonates are tangled with the circadian clock and their levels change to optimize plant growth and fitness (Thines et al. 2019). This explains an increase in SAR post-fungal attacks. It is also reported that JA signaling results in the activation of PAL and chalcone synthase (CHS), involved in flavonoid synthesis and has a protective role (Krol et al. 2015).

Further, in Figs. 5 and 6, low ROS levels (and more scavenging) were observed in MeJA-treated set IV than in set II

of infected seedlings, especially at 0.5 mM concentration. Thus, like SA, MeJA priming also improved the antioxidant activities in the biotically stressed seedlings (Figs. 7, 8). However, besides the anti-pathogen activity of ROS, it is reported to act as a secondary messenger in signaling pathways, increasing plants' tolerance (Torres et al. 2006). Thus, it appears that fungal infection and MeJA bring ROS at the interface between deleterious effects and signaling, maintaining a controlled 'oxidative window' of ROS generation and improving germination and growth.

It is worth mentioning here that the understanding of SA and JA production inside plants is not straightforward, as genes are not continuous in the genome, and thus end up being differentially regulated (Chowdhury et al. 2017; Krol et al. 2015). This could also be the reason that external PHs applications showed promising results in different studies. However, a confirmed statement can be made only after the quantification of endogenous PHs levels in little mature seedlings. However, this preliminary study cannot rule out the possibility of the involvement of other mechanisms and leaves a knowledge gap, which can be completed with further work. A comparative transcriptomic analysis of separate PHs and infection control, combined with both infection and PH treatment, would suggest the differences in the abiotic and biotic-challenges. Isolated studies show PHs treatment, including SA and MeJA, imparts resistance in grapevine, against powdery mildew (*Erysiphe necator*), by stimulating MAPK signaling, leading to secondary metabolites synthesis, including- phenolic acid, stilbenes, and flavonoids

production (Jiao et al. 2017; Portu et al. 2015). A similar mechanism in hullless barley infected with powdery mildew (*Blumeria graminis*) is reported, and enhanced levels of JA, SA, ABA, and CKs drive MAPK and toll-like receptor signaling (Sang et al. 2021).

It should be kept in consideration that PHs role in fungal pathogen attack is crucial, as many pathogens' origin protein effectors are known to alter plant hormone regulation, which supports infection (Chanclud and Morel 2016). It is reported that JA and SA antagonize each other and can also be exploited by a pathogen to promote disease (Chowdhury et al. 2017). However, our in vitro results showed no antagonism (data not shown). Interestingly, pathogens, including fungi, are also identified to produce PHs like- auxin, CK, or similar molecules to favor infection (Robert-Seilaniantz et al. 2007). The IAA synthesis in fungi is testified but depends on external tryptophan uptake (Tsavkelova et al. 2012).

Overall, the effect of exogenously supplied PH on plant growth and metabolic activities seems promising. Although, little is known about the possible negative effects of the PHs on the plant's growth and yield in fields. Still, it is observed that the varying PHs dose on separate plant species might display different impacts on the photosynthesis parameters. High SA concentrations (1–5 mM) decrease the activity of RuBisCO and the photosynthetic rate in barley plants (Inada and Shimmen 2000). Similarly, higher concentrations of exogenous IAA and GA₃ hamper the elongation of root segments in *Lemna minor* (Pancheva and Popova 1998). In another study, JA moderately and ABA severely suppressed the growth of *L. minor* in a concentration-dependent manner (Utami et al. 2018). Still, to combat the challenge of feeding an ever-increasing population, PHs seem better alternatives than pesticides. PHs use in seeds' priming is meticulous in increasing both ISR and SAR, with better handling capacity, controlled timing, and lower toxicity than spraying on leaves or adding to soil, which might be costly, along with adverse environmental impact.

Conclusions and future perspectives

The present study concludes that tested PHs showed antifungal activity against the pathogenic fungi, *F. oxysporum*, wherein the defense PHs-SA, and MeJA showed the highest antifungal potential. Based on these results, the protective role of hormo-priming of *V. mungo* seeds, with a sub-inhibitory concentration of these two PHs were evaluated. The in vivo results suggested that SA and MeJA seed priming imparts protection, improves GP, SL, and SVI, and reduces MGT than fungal-treated seeds. Biochemical evaluation revealed that SA and MeJA priming were also observed to activate the antioxidant machinery to counter

the ROS. Overall, the study advocates an eco-friendly and safer method in alleviating *Fusarium*-mediated biotic stress in plants, wherein seed hormo-priming could serve as an effective tool. Although, these PHs work in a dose-dependent manner, so should be used within safe biological levels.

Author contribution statement All authors contributed to the study conception and design. R. Pasrija and R. Manoharlal conceived the idea. L. Duhan, D. Kumari, and M. Saini carried out experimental and initial data analysis and prepared the initial draft of the manuscript. R. Pasrija, D. Kumar and R. Manoharlal performed major data analysis and subsequent discussion of results and revised the draft of the manuscript. R. Pasrija, D. Kumar, A. K. Chhillar and G.V.S. Saiprasad assisted in data analysis and figure/graphs preparation. R. Pasrija was involved in correspondence with the journal. All authors read and consented on the manuscript.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11738-024-03659-3>.

Funding R. Pasrija acknowledges Science & Engineering Research Board (SERB) (CRG/2020/004986) under Department of Science and Technology and Maharshi Dayanand University, Radhakrishnan (R. K. Fund) minor research project (Sanctioned amount: Rs. 50,000) for funding [Grant no. DSW/2020/411]. L. Duhan acknowledges the CSIR, New Delhi, for the financial assistance, the Junior Research Fellowship (JRF) and Senior Research Fellowship (SRF) (09/382(0249)/2019-EMR-I).

Data availability No separate data available with the authors.

Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval The study requires no ethical approvals. The manuscript complies with ethical standards. An ethics statement was not required for this study type; no human or animal subjects or materials were used.

Consent to participate Not applicable.

Consent to publish Consent to publish has been received from all authors.

References

- Aebi H (1984) Catalase in vitro. *Methods Enzymol* 105:121–126. [https://doi.org/10.1016/s0076-6879\(84\)05016-3](https://doi.org/10.1016/s0076-6879(84)05016-3)
- Ahmad P, Raja V, Ashraf M et al (2021) Jasmonic acid (JA) and gibberellic acid (GA₃) mitigated Cd-toxicity in chickpea plants through restricted cd uptake and oxidative stress management. *Sci Rep* 11:19768. <https://doi.org/10.1038/s41598-021-98753-8>

- Alam MdM, Nahar K, Hasanuzzaman M, Fujita M (2014) Exogenous jasmonic acid modulates the physiology, antioxidant defense and glyoxalase systems in imparting drought stress tolerance in different *Brassica* species. *Plant Biotechnol Rep* 8:279–293. <https://doi.org/10.1007/s11816-014-0321-8>
- Alazem M, Lin N (2014) Roles of plant hormones in the regulation of host–virus interactions. *Mol Plant Pathol* 16:529–540. <https://doi.org/10.1111/mpp.12204>
- Al-harhi MM, Bafeel SO, El-Zohri M (2021) Gibberellic acid and jasmonic acid improve salt tolerance in summer squash by modulating some physiological parameters symptomatic for oxidative stress and mineral nutrition. *Plants* 10:2768. <https://doi.org/10.3390/plants10122768>
- Al-Hatmi AMS, Curfs-Breuker I, de Hoog GS et al (2017) Antifungal susceptibility testing of *Fusarium*: a practical approach. *J Fungi* 3:19. <https://doi.org/10.3390/jof3020019>
- Bari R, Jones JDG (2009) Role of plant hormones in plant defence responses. *Plant Mol Biol* 69:473–488. <https://doi.org/10.1007/s11103-008-9435-0>
- Bastam N, Baninasab B, Ghobadi C (2013) Improving salt tolerance by exogenous application of salicylic acid in seedlings of pistachio. *Plant Growth Regul*. <https://doi.org/10.1007/s10725-012-9770-7>
- Borman AM, Fraser M, Palmer MD et al (2017) MIC distributions and evaluation of fungicidal activity for amphotericin B, itraconazole, voriconazole, posaconazole and caspofungin and 20 species of pathogenic filamentous fungi determined using the CLSI broth microdilution Method. *J Fungi* 3:27. <https://doi.org/10.3390/jof3020027>
- Chanclud E, Morel J-B (2016) Plant hormones: a fungal point of view. *Mol Plant Pathol* 17:1289–1297. <https://doi.org/10.1111/mpp.12393>
- Chen GX, Asada K (1989) Ascorbate peroxidase in tea leaves: Occurrence of two isozymes and the differences in their enzymatic and molecular properties. *Plant Cell Physiol* 30:987–998. <https://doi.org/10.1093/oxfordjournals.pcp.a077844>
- Chowdhury S, Basu A, Kundu S (2017) Biotrophy-necrotrophy switch in pathogen evoke differential response in resistant and susceptible sesame involving multiple signaling pathways at different phases. *Sci Rep* 7:17251. <https://doi.org/10.1038/s41598-017-17248-7>
- Conrath U (2006) Systemic acquired resistance. *Plant Signal Behav* 1:179–184. <https://doi.org/10.4161/psb.1.4.3221>
- Dong X, Xiong Y, Ling N et al (2014) Fusaric acid accelerates the senescence of leaf in banana when infected by *Fusarium*. *World J Microbiol Biotechnol* 30:1399–1408. <https://doi.org/10.1007/s11274-013-1564-1>
- Dumanovic J, Nepovimova E, Natic M et al (2021) The significance of reactive oxygen species and antioxidant defense system in plants: a concise overview. *Front Plant Sci*. <https://doi.org/10.3389/fpls.2020.552969>
- ElSayed AI, Boulila M, Rafudeen MS et al (2020) Melatonin regulatory mechanisms and phylogenetic analyses of melatonin biosynthesis related genes extracted from peanut under salinity stress. *Plants* 9:854. <https://doi.org/10.3390/plants9070854>
- Fahad S, Hussain S, Matloob A et al (2015) Phytohormones and plant responses to salinity stress: a review. *Plant Growth Regul* 75:391–404. <https://doi.org/10.1007/s10725-014-0013-y>
- Fryer MJ, Oxborough K, Mullineaux PM, Baker NR (2002) Imaging of photo-oxidative stress responses in leaves. *J Exp Bot* 53:1249–1254. <https://doi.org/10.1093/jexbot/53.372.1249>
- Hartmann A, Berkowitz O, Whelan J, Narsai R (2022) Cross-species transcriptomic analyses reveals common and opposite responses in *Arabidopsis*, rice and barley following oxidative stress and hormone treatment. *BMC Plant Biol* 22:62. <https://doi.org/10.1186/s12870-021-03406-7>
- Inada S, Shimmen T (2000) Regulation of elongation growth by gibberellin in root segments of *Lemna minor*. *Plant Cell Physiol* 41:932–939. <https://doi.org/10.1093/pcp/pcd018>
- Jakhar S, Mukherjee D (2014) Chloroplast pigments, proteins, lipid peroxidation and activities of antioxidative enzymes during maturation and senescence of leaves and reproductive organs of *Cajanus cajan* L. *Physiol Mol Biol Plants* 20:171–180. <https://doi.org/10.1007/s12298-013-0219-x>
- Jang G, Yoon Y, Choi YD (2020) Crosstalk with jasmonic acid integrates multiple responses in plant development. *Int J Mol Sci*. <https://doi.org/10.3390/ijms21010305>
- Jiao Y, Wang D, Wang L et al (2017) VqMAPKKK38 is essential for stilbene accumulation in grapevine. *Hortic Res* 4:17058. <https://doi.org/10.1038/hortres.2017.58>
- Kepczynska E, Krol P (2012) The phytohormone methyl jasmonate as an activator of induced resistance against the necrotroph *Alternaria porri* f. sp. *solani* in tomato plants. *J Plant Interact* 7:307–315. <https://doi.org/10.1080/17429145.2011.645169>
- Kim DS, Hwang BK (2014) An important role of the pepper *phenylalanine ammonia-lyase* gene (*PAL1*) in salicylic acid-dependent signalling of the defence response to microbial pathogens. *J Exp Bot* 65:2295–2306. <https://doi.org/10.1093/jxb/eru109>
- Krol P, Igielski R, Pollmann S, Kepczynska E (2015) Priming of seeds with methyl jasmonate induced resistance to hemibiotroph *Fusarium oxysporum* f.sp. *lycopersici* in tomato via 12-oxo-phytodienoic acid, salicylic acid, and flavonol accumulation. *J Plant Physiol* 179:122–132. <https://doi.org/10.1016/j.jplph.2015.01.018>
- Kumar D, Yusuf MA, Singh P et al (2013) Modulation of antioxidant machinery in α -tocopherol-enriched transgenic *Brassica juncea* plants tolerant to abiotic stress conditions. *Protoplasma* 250:1079–1089. <https://doi.org/10.1007/s00709-013-0484-0>
- Lefevere H, Bauters L, Gheysen G (2020) Salicylic acid biosynthesis in plants. *Front Plant Sci*. <https://doi.org/10.3389/fpls.2020.00338>
- Lemmens E, Deleu LJ, De Brier N et al (2019) The impact of hydro-priming and osmo-priming on seedling characteristics, plant hormone concentrations, activity of selected hydrolytic enzymes, and cell wall and phytate hydrolysis in sprouted wheat (*Triticum aestivum* L.). *ACS Omega* 4:22089–22100. <https://doi.org/10.1021/acsomega.9b03210>
- Li X, Li S, Wang J, Lin J (2020) Exogenous abscisic acid alleviates harmful effect of salt and alkali stresses on wheat seedlings. *Int J Environ Res Public Health* 17:3770. <https://doi.org/10.3390/ijerph17113770>
- Li N, Euring D, Cha JY et al (2021) Plant hormone-mediated regulation of heat tolerance in response to global climate change. *Front Plant Sci* 11:627969. <https://doi.org/10.3389/fpls.2020.627969>
- Li L, Zhu T, Song Y et al (2022) Salicylic acid fights against *Fusarium* wilt by inhibiting target of rapamycin signaling pathway in *Fusarium oxysporum*. *J Adv Res* 39:1–13. <https://doi.org/10.1016/j.jare.2021.10.014>
- Lyons R, Stiller J, Powell J et al (2015) *Fusarium oxysporum* triggers tissue-specific transcriptional reprogramming in *Arabidopsis thaliana*. *PLoS ONE* 10:e0121902. <https://doi.org/10.1371/journal.pone.0121902>
- Manoharlal R, Saiprasad GVS (2019) Assessment of germination, phytochemicals, and transcriptional responses to ethephon priming in soybean [*Glycine max* (L.) Merrill]. *Genome* 62:769–784. <https://doi.org/10.1139/gen-2019-0013>
- Muller M, Munne-Bosch S (2021) Hormonal impact on photosynthesis and photoprotection in plants. *Plant Physiol* 185:1500–1522. <https://doi.org/10.1093/plphys/kiab119>
- Nazar R, Umar S, Khan NA, Sareer O (2015) Salicylic acid supplementation improves photosynthesis and growth in mustard through changes in proline accumulation and ethylene formation under

- drought stress. *South Afr J Bot* 98:84–94. <https://doi.org/10.1016/j.sajb.2015.02.005>
- Pancheva TV, Popova LP (1998) Effect of salicylic acid on the synthesis of ribulose-1,5-bisphosphate carboxylase/oxygenase in barley leaves. *J Plant Physiol* 152:381–386. [https://doi.org/10.1016/S0176-1617\(98\)80251-4](https://doi.org/10.1016/S0176-1617(98)80251-4)
- Portu J, Santamaría P, López-Alfaro I et al (2015) Methyl jasmonate foliar application to *Tempranillo* vineyard improved grape and wine phenolic content. *J Agric Food Chem* 63:2328–2337. <https://doi.org/10.1021/jf5060672>
- Rajurkar NS, Hande SM (2011) Estimation of phytochemical content and antioxidant activity of some selected traditional Indian medicinal plants. *Indian J Pharm Sci* 73:146–151
- Robert-Seilaniantz A, Navarro L, Bari R, Jones JD (2007) Pathological hormone imbalances. *Curr Opin Plant Biol* 10:372–379. <https://doi.org/10.1016/j.pbi.2007.06.003>
- Ruan J, Zhou Y, Zhou M et al (2019) Jasmonic acid signaling pathway in plants. *Int J Mol Sci* 20:2479. <https://doi.org/10.3390/ijms20102479>
- Sah SK, Reddy KR, Li J (2016) Abscisic acid and abiotic stress tolerance in crop plants. *Front Plant Sci*. <https://doi.org/10.3389/fpls.2016.00571>
- Saini S, Kaur N, Pati PK (2021) Phytohormones: key players in the modulation of heavy metal stress tolerance in plants. *Ecotoxicol Environ Saf* 223:112578. <https://doi.org/10.1016/j.ecoenv.2021.112578>
- Samsatly J, Copley TR, Jabaji SH (2018) Antioxidant genes of plants and fungal pathogens are distinctly regulated during disease development in different *Rhizoctonia solani* pathosystems. *PLoS ONE* 13:e0192682. <https://doi.org/10.1371/journal.pone.0192682>
- Sang Z, Zhang M, Mu W et al (2021) Phytohormonal and transcriptional response of hulless barley leaf in response to powdery mildew infection. *Agronomy* 11:1248. <https://doi.org/10.3390/agronomy11061248>
- Sedaghat M, Tahmasebi-Sarvestani Z, Emam Y, Mokhtassi-Bidgoli A (2017) Physiological and antioxidant responses of winter wheat cultivars to strigolactone and salicylic acid in drought. *Plant Physiol Biochem* 119:59–69. <https://doi.org/10.1016/j.plaphy.2017.08.015>
- Sehar Z, Iqbal N, Khan MIR et al (2021) Ethylene reduces glucose sensitivity and reverses photosynthetic repression through optimization of glutathione production in salt-stressed wheat (*Triticum aestivum* L.). *Sci Rep* 11:12650. <https://doi.org/10.1038/s41598-021-92086-2>
- Shiade SRG, Boelt B (2020) Seed germination and seedling growth parameters in nine tall fescue varieties under salinity stress. *Acta Agric Scand Sect B Soil Plant Sci* 70:485–494. <https://doi.org/10.1080/09064710.2020.1779338>
- Shigenaga AM, Argueso CT (2016) No hormone to rule them all: interactions of plant hormones during the responses of plants to pathogens. *Semin Cell Dev Biol* 56:174–189. <https://doi.org/10.1016/j.semcdb.2016.06.005>
- Singh VK, Upadhyay RS (2014) Fusaric acid induced cell death and changes in oxidative metabolism of *Solanum lycopersicum* L. *Bot Stud* 55:66. <https://doi.org/10.1186/s40529-014-0066-2>
- Su Q, Zheng X, Tian Y, Wang C (2020) Exogenous brassinolide alleviates salt stress in *Malus hupehensis* Rehd by regulating the transcription of NHX-Type Na⁺(K⁺)/H⁺ antiporters. *Front Plant Sci* 11:38. <https://doi.org/10.3389/fpls.2020.00038>
- Tayyab N, Naz R, Yasmin H et al (2020) Combined seed and foliar pre-treatments with exogenous methyl jasmonate and salicylic acid mitigate drought-induced stress in maize. *PLoS ONE* 15:e0232269. <https://doi.org/10.1371/journal.pone.0232269>
- Thines B, Parlan EV, Fulton EC (2019) Circadian network interactions with jasmonate signaling and defense. *Plants* 8:252. <https://doi.org/10.3390/plants8080252>
- Torres MA, Jones JDG, Dangl JL (2006) Reactive oxygen species signaling in response to pathogens. *Plant Physiol* 141:373–378. <https://doi.org/10.1104/pp.106.079467>
- Torun H, Novak O, Mikulik J et al (2020) Timing-dependent effects of salicylic acid treatment on phytohormonal changes, ROS regulation, and antioxidant defense in salinized barley (*Hordeum vulgare* L.). *Sci Rep* 10:13886. <https://doi.org/10.1038/s41598-020-70807-3>
- Tsavkelova E, Oeser B, Oren-Young L et al (2012) Identification and functional characterization of indole-3-acetamide-mediated IAA biosynthesis in plant-associated *Fusarium* species. *Fungal Genet Biol* 49:48–57. <https://doi.org/10.1016/j.fgb.2011.10.005>
- Ullah A, Manghwar H, Shaban M et al (2018) Phytohormones enhanced drought tolerance in plants: a coping strategy. *Environ Sci Pollut Res Int* 25:33103–33118. <https://doi.org/10.1007/s11356-018-3364-5>
- Utami D, Kawahata A, Sugawara M et al (2018) Effect of exogenous general plant growth regulators on the growth of the duckweed *Lemna minor*. *Front Chem* 6:251. <https://doi.org/10.3389/fchem.2018.00251>
- Velikova V, Yordanov I, Edreva A (2000) Oxidative stress and some antioxidant systems in acid rain-treated bean plants: protective role of exogenous polyamines. *Plant Sci* 151:59–66. [https://doi.org/10.1016/S0168-9452\(99\)00197-1](https://doi.org/10.1016/S0168-9452(99)00197-1)
- Walters DR, Paterson L, Walsh DJ, Havis ND (2008) Priming for plant defense in barley provides benefits only under high disease pressure. *Physiol Mol Plant Pathol* 73:95–100. <https://doi.org/10.1016/j.pmp.2009.03.002>
- Worrall D, Holroyd GH, Moore JP et al (2012) Treating seeds with activators of plant defence generates long-lasting priming of resistance to pests and pathogens. *New Phytol* 193:770–778. <https://doi.org/10.1111/j.1469-8137.2011.03987.x>
- Xing F, Li Z, Sun A, Xing D (2013) Reactive oxygen species promote chloroplast dysfunction and salicylic acid accumulation in fumonisin B1-induced cell death. *FEBS Lett* 587:2164–2172. <https://doi.org/10.1016/j.febslet.2013.05.034>
- Xue RF, Wu J, Wang LF et al (2014) Salicylic acid enhances resistance to *Fusarium oxysporum* f. sp. *phaseoli* in common beans (*Phaseolus vulgaris* L.). *J Plant Growth Regul* 33:470–476. <https://doi.org/10.1007/s00344-013-9376-y>
- Yang C, Li W, Cao J et al (2017) Activation of ethylene signaling pathways enhances disease resistance by regulating ROS and phytoalexin production in rice. *Plant J* 89:338–353. <https://doi.org/10.1111/tpj.13388>

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Springer Nature or its licensor (e.g. a society or other partner) holds exclusive rights to this article under a publishing agreement with the author(s) or other rightsholder(s); author self-archiving of the accepted manuscript version of this article is solely governed by the terms of such publishing agreement and applicable law.