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Cold stress and the role of signalling hormones: A preliminary study on cold-tolerant high-altitude Himalayan rice genotypes

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Abstract
Salicylic acid, methyl salicylate, jasmonic acid, and methyl jasmonate are important signaling hormones that play a significant role during plant stress. In the present study, the effect of cold stress on rice (*Oryza sativa* L.) leaves at the seedling stage was studied with regard to the level of these signaling hormones. Rice genotypes of Kashmir Himalayas were screened for cold tolerance under controlled conditions. The two contrasting (cold tolerant/susceptible) genotypes were selected to find whether these phytohormones play some role in cold stress. The seedlings of the two rice genotypes were subjected to cold stress at 5 °C and samples were collected at different time points. The level of Jasmonic acid and methyl jasmonate decreased sharply under cold treatment in both the contrasting genotypes. However, the concentration of these signalling hormones (JA and MeJA) was much higher in the resistant genotype than in the susceptible genotype. In the susceptible genotype, salicylic acid decreased sharply under cold treatment, while in the resistant genotype, it increased under cold. The concentration of methyl salicylate increased in both susceptible and resistant genotypes under cold stress.

Keywords: Rice, cold stress, jasmonic acid, salicylic acid, HPLC

Introduction
Drought and low temperatures are the most common environmental stress factors that cause a detrimental effect on plant growth and development globally. Rice (*Oryza sativa* L.) is a semi-aquatic plant that is cold and drought-sensitive (Shimono *et al.,* 2012, Jagadish *et al.,* 2012, Luquet *et al.,* 2012, Wang *et al.,* 2013) [12, 13, 24, 43, 50, 55]. However, there exist substantial differences in the sensitivity range against these two stress factors within this species. Being a thermophilic crop, cold stress is a major issue in rice farming, with over 15 Mha of land globally being unfit for rice cultivation (Kaw and Khush, 1998; Li *et al*., 2006) [15]. 25°C is the optimum temperature for rice germination, and temperatures below 15°C at the germination stage have harmful consequences. These include a decrease in germination and vigor, delayed seedling emergence, delayed initial growth, seedling establishment, the appearance of yellowing on the leaves, overall retardation in growth, and late and limited tillering, and most importantly high mortality in seedlings (Cruz & Milach, 2000; Fujino 2004; Cruz *et al*., 2013) [6-8]. Losses have been recorded around 0.5 to 2.5 t/ha with grain drop of about 26% (Lee *at al.,* 2001; Singh *et al*., 2005; Ye *et al*., 2009; Cruz *et al*., 2013) [6, 7, 20, 36, 37, 46].

Many changes occur in the plant during cold acclimatization that includes physiological, molecular, and biochemical changes. These changes include alterations in signal perception, transduction, gene expression, and metabolomic variations (Agarwal *et al*., 2006) [1]. A schematic representation of the significant changes is shown in Fig 1 (Lata and Prasad, 2011; Harfouche *et al*., 2013; Awasthi *et al*., 2016; Sami *et al*., 2016; Ruan *et al*., 2019; Sharma *et al*., 2020) [3, 10, 19, 34, 35, 39, 40]. Plant hormones play vital roles in setting up signalling networks for regulating the plant stress response. Jasmonic acid (3-oxo-2-20-cis-pentenyl-cyclopentane-1-acetic acid, abbreviated as JA) is an important signaling molecule linked with the tolerance of plants against abiotic stresses. JA is generally involved in physiological and molecular responses under abiotic stress. JA and its methyl ester (MeJA) and isoleucine conjugate (JA-Ile) are derivatives of fatty acids. These are collectively called jasmonates. Methyl jasmonate (MeJA) regulates plant morphogenesis and responses to abiotic and biotic stresses (Karpets *et al.,* 2014; Wasternack *et al.,* 2013) [14, 27, 51, 52]. Various reports confirm that jasmonates are the fast responding signaling

molecules in plant stress and wounding signalling pathways (Gundlach *et al.,* 1992, McConn *et al.,* 1997, Wasternack and Parthier 1997, Xie *et al.,* 1998, Sanders *et al.,* 2000) [9, 25, 27, 36, 51-53].

Salicylic acid (SA; chemically 2-hydroxy benzoic acid) is a phenolic compound, having an aromatic ring with a hydroxyl group synthesized by the plant. It regulates physiological and biochemical processes by acting as a regulatory signal, mediating the plant response to abiotic stresses. The hydroxyl

groups are involved in signaling defense activation, lignin biosynthesis, and disease resistance by inducing antimicrobial defense compounds called phytoalexins. SA is converted to methyl salicylate (MeSA) by salicylic acid carboxyl methyltransferase. MeSA is volatile and plays a significant role in plant systemic acquired resistance by transmitting the signal over long-distance (Vlot *et al.*, 2008; Yusuf *et al.,* 2013) [35, 48, 58].
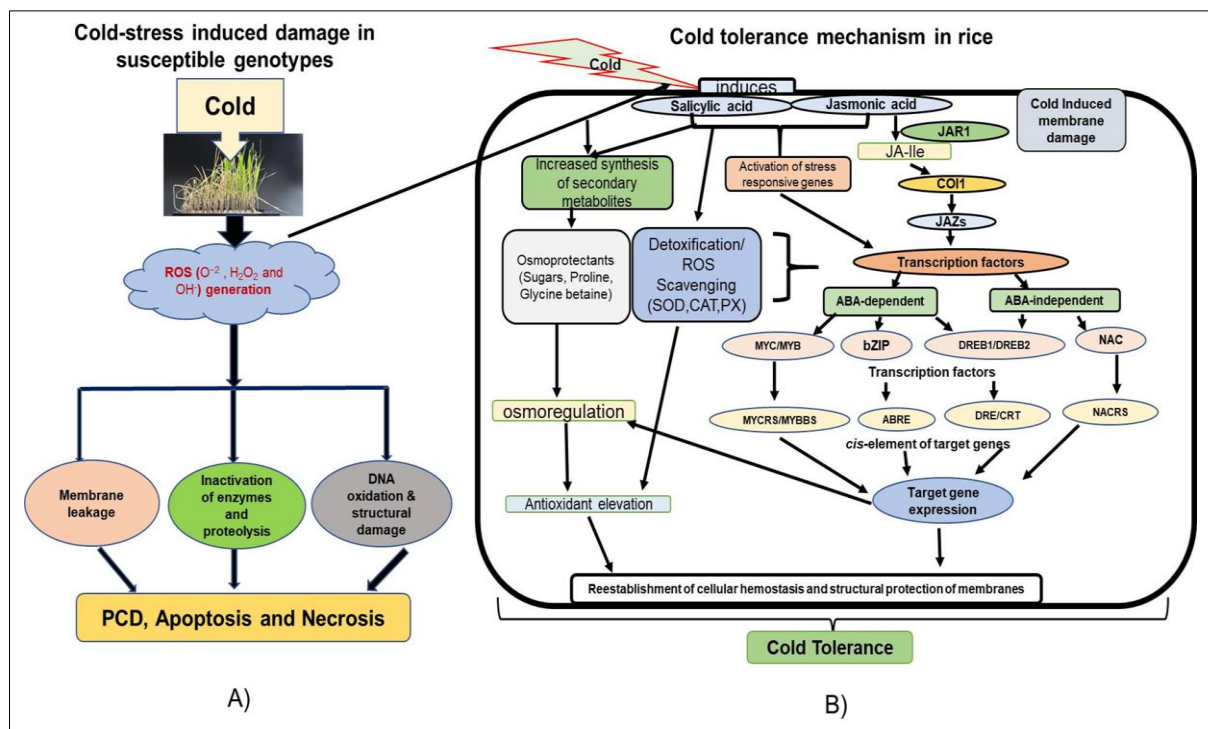

Fig 1: Schematic representation of the regulatory cascade of cold signal perception, transduction, downstream gene regulation, and the consequent biochemical response leading to cold tolerance.

A) Cold stress causes the accumulation of reactive oxygen species (ROS) such as O^{-2}, H_2O_2 and OH^-, bringing out oxidative stress response in cells. Exposure of cells to severe oxidative stress causes damage to DNA, lipids and protein structure/integrity and elicits lethal response pathways ultimately leading to programmed cell-death; **B)** Spatial and temporal changes in ROS levels play a multitude of signaling roles in a complex regulatory system encompassing plant hormones and signaling molecules (e.g., salicylic acid and jasmonic acid) ultimately causing overproduction of ROS-scavenging enzymes {like catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and glutathione reductase (GR)} and some non-enzymatic antioxidants (like osmoprotectants, osmolytes and secondary metabolites). Compatible solutes Pro, GB, and sugars mitigate stress by osmoregulation and the release of downstream transcription factors. Salicylic acid is involved in the modulation of antioxidant metabolism and regulation of various plant metabolic processes. Jasmonic acid is involved in physiological and molecular responses which include activation of the antioxidant system and interactions with TFs. JA gets converted to the biologically active JA-Ile by JAR1. JA-Ile perception by its receptor COI1 triggers the degradation of JAZ repressors, leading to the release of downstream transcription factors such as myelocytomatosis oncogene (MYC), myeloblastosis oncogene (MYB), basic leucine zipper (bZIP), NAM, ATAF, and CUC (NAC), dehydration responsive element binding (DREB). These TFs interact with cis-elements in the promoter regions of various stress-related genes to up-regulate the expression of many downstream genes causing activation of stress-responsive mechanisms for re-establishing cellular homeostasis and to protect and repair damaged proteins and membranes, thus imparting stress tolerance.

The SA-and ET/JA-mediated defense signalling pathways act both synergistically and antagonistically. Treatment with low concentrations of SA and JA results in the synergistic expression of both the SA target gene PR1 and the JA marker gene PDF1.2, whereas higher concentrations of SA and JA produce the antagonistic expression of these genes (Mur *et al.,* 2006) [27].

Comparing the basic level of signalling molecules, which regulate other stress-induced changes like the production of protective compounds, can throw a lot of light on the causes behind the differences in tolerance level. Under physiological conditions, JA, MeJA, SA, and MeSA are usually at low concentrations and are highly volatile. These characteristics increase the difficulty in their extraction and estimation. The

present study was conducted with the twin objective of standardizing and validating a protocol for the estimation of JA, MeJA, SA, and MeSA from rice leaves, and then comparing their level for developing a better understanding of their role in cold stress in high altitude irrigated rice.

Materials and Methods
Plant material
Two rice genotypes GS-74 (cold-resistant) and SR-4 (cold-sensitive) grown at 2200 m amsl or above were selected from a set of 90 genotypes of high-altitude regions of western Himalayas (30.25° to 35.20° N latitude and 74° to 75.25° E longitude). The seeds of these genotypes were germinated in petri-plates inside a seed germinator at 28 ± 2 °C and relative

humidity of 60-80 percent. Germinated seeds were then sown in plastic pots (in triplicate) filled with clay soil (0.5 kg each) and kept under controlled conditions (25±2 ºC, relative humidity of 60-80 percent, photoperiod of 16/8 h day/night, and light intensity of 3000 lm / m^2) in a walk-in plant growth chamber (Blue Star), Genome Engineering lab, Division of Plant Biotechnology, SKUAST-K Shalimar. Pots were watered to field capacity (saturation) thrice a week. After two weeks of growth, each seedling (single) was transplanted in a pot containing clay: sand: FYM in 2:1:1 (5 pots for each accession), and were grown inside the growth chamber at 25±2 °C and 60 percent relative humidity. Cold stress (5ºC) was given to 14 days old rice seedlings for 24 hours, and leaf samples were collected as per details (Table 1). The leaf samples were washed with double-distilled autoclaved water and stored at -80 ºC in a deep-freezer.

Table 1: Samples were collected at the following time points under 5 °C cold treatment for 24 hours

Treatments	Leaf samples collected at	Sample label
T0	0 hrs before cold stress at 25 ºC	(S0, R0)
T1	2 hrs during cold stress at 5 ºC	(S2, R2)
T2	6 hrs during cold stress at 5 ºC	(S6, R6)
T3	24 hrs during cold stress at 5 ºC	(S24, R24)
T4	24 hrs after stress recovery at 25 ºC	(PS24, PR24)

Reagents
HPLC grade (95%) Jasmonic acid (JA), Methyl Jasmonate (MeJA), Salicylic acid (SA), and Methyl salicylate (MeSA) standards were purchased (Sigma, Germany). HPLC grade methanol, acetonitrile, and orthophosphoric acid 85% were obtained from Merck, Germany. Solid-phase extraction (SPE) cartridges (Sep-pak C18) were procured from Waters, USA. High-purity water (18 MΩ) was employed throughout.

Extraction and purification using direct SPE column method
HPLC protocol as described by Liu *et al.,* 2010 [23, 48, 55] was followed with some modifications. 500 mg of each deep-freezed plant sample dipped in liquid N$_2$ and ground to a fine powder with a mortar and pestle. Following the addition of 600 μL of methanol (Merck, Germany), homogenates were mixed and kept at 4 °C overnight, then centrifuged at 4800 × *g* for 10 min. The supernatant was transferred to a new 5 mL glass tube, and the residue was re-extracted with 200 μL of methanol. 3000 μL of ddH$_2$O was added to the combined extracts and the solution was applied onto the Waters Sep-pak C18 cartridge. The cartridge was washed with 200 μL of 20 percent methanol and 250 μL of 30 percent methanol. Finally, the cartridge was eluted with 300 μL of 100 percent methanol, and the eluted solution was collected as the samples extract ready for analysis.
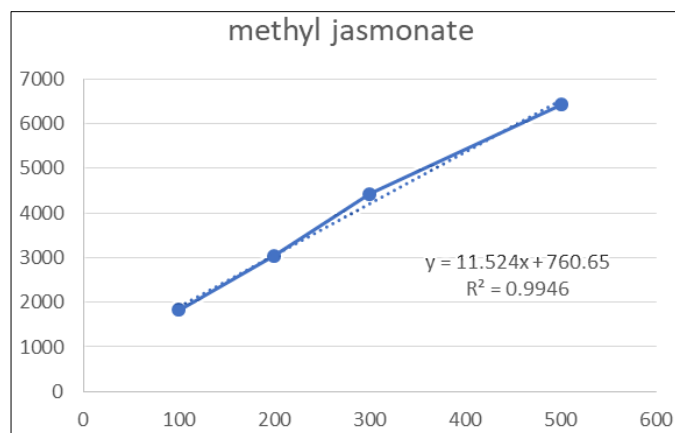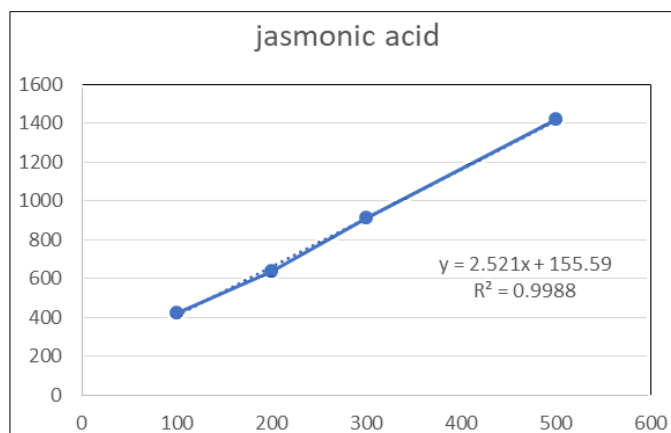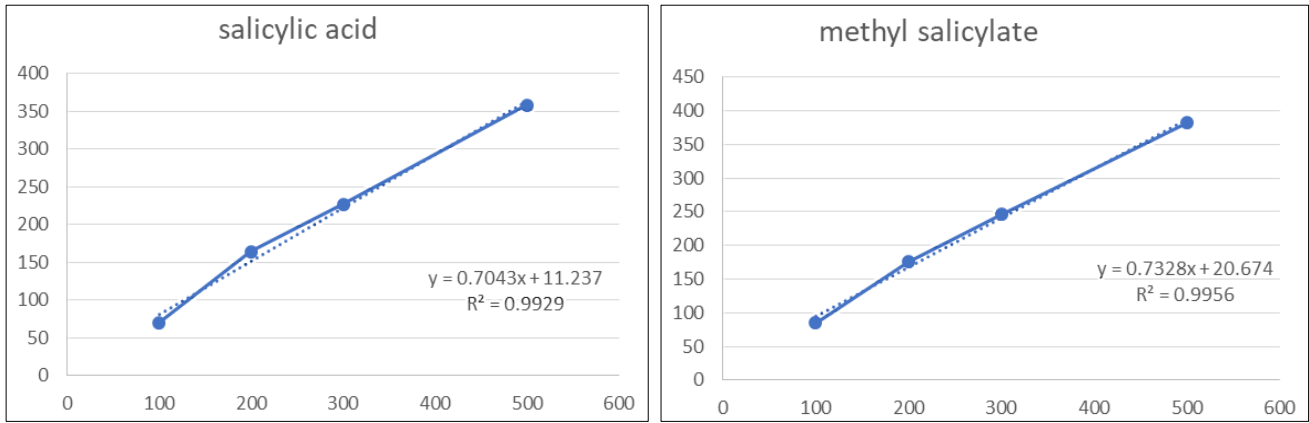
Instrumentation
HPLC measurements were performed on an Agilent 1100 HPLC with a G1314A UV detector. Chromatographic separation was performed on a Waters C18 250 mm×4.6 mm, 5 μm column at 35°C. The mobile phase was a binary gradient: distilled water with 0.1% (v/v) orthophosphoric acid 85 percent and acetonitrile. The linear gradient was 15 percent acetonitrile for 5 minutes. The flow rate was 1 mL/min, and the injection volume was 10 μL. The detection wavelength was 210 nm for JA and MeJA while as for SA and MeSA detection wavelength was 300 nm.
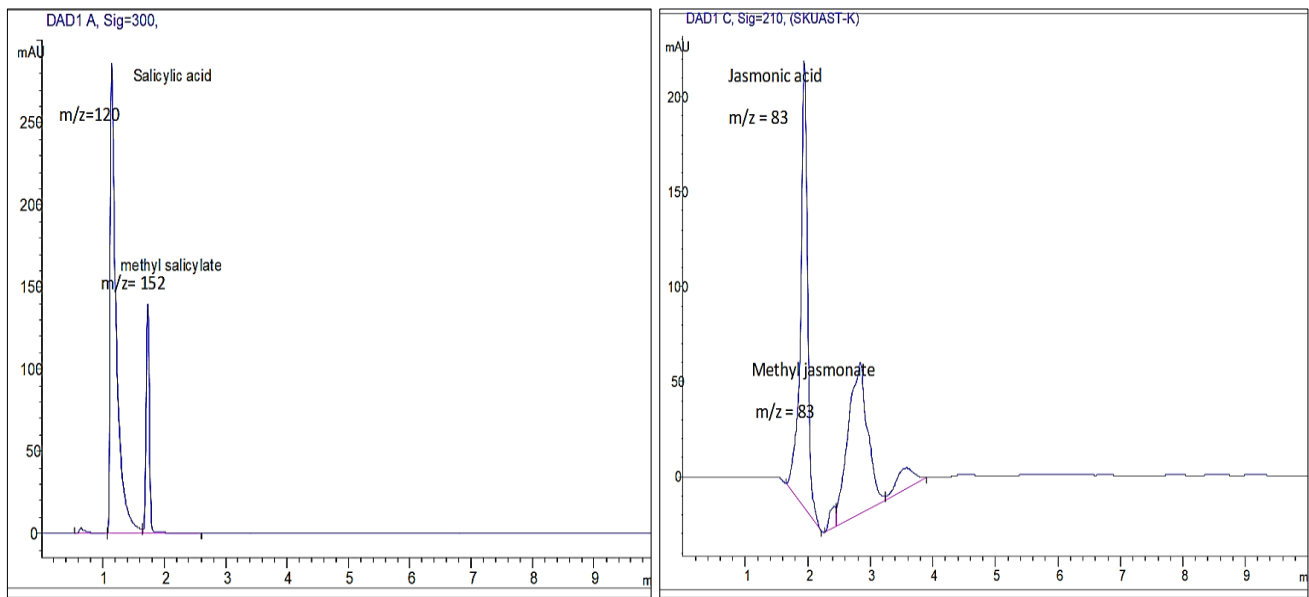
Quantification
To generate standard curves, an individual standard stock solution containing 1 μL mL^{-1} of JA, MeJA, SA, and MeSA was prepared by diluting the pure compound (1 μL) with methanol in a 1.5 ml centrifuge tube. Solutions were stored at −20 °C. Working solutions were obtained from standard stock solutions by appropriate dilution in methanol before use (500, 300, 200, and 100 μl ml^{-1}) (Supplementary fig. 1, 2). Using C18 columns JA, MeJA, SA, and MeSA were separated. The retention time of each compound was recorded and compared with the standard.

jasmonic acid

$y = 2.521x + 155.59$
$R^2 = 0.9988$

methyl jasmonate

$y = 11.524x + 760.65$
$R^2 = 0.9946$

salicylic acid

$$y = 0.7043x + 11.237$$
$$R^2 = 0.9929$$

methyl salicylate

$$y = 0.7328x + 20.674$$
$$R^2 = 0.9956$$

Supplementary Fig 1: Calibration curves of JA, MeJA, SA and MeSA standards

DAD1 A, Sig=300,

Salicylic acid
m/z=120

methyl salicylate
m/z= 152

DAD1 C, Sig=210, (SKUAST-K)

Jasmonic acid
m/z = 83

Methyl jasmonate
m/z = 83

Supplementary Fig 2: Chromatograms of JA, MeJA, SA and MeSA showing retention time

Results
In the present study, we successfully quantified the levels of JA, MeJA, SA, and MeSA from rice seedlings under cold stress. The retention time of JA (3.58 min), MeJA (2.84 min), SA (1.17 min), and MeSA (1.73 min) was recorded (Supplementary table 1). The levels for JA ranged from 0.095-11.880 μg/g f.w., for MeJA (0.142-95.790 μg/g f.w.), for SA (0.588-7.390 μg/g f.w.) and for MeSA (1.410-1.600 μg/g f.w.), respectively (Supplementary table 2). Salicylic acid concentration decreased sharply under cold treatment in susceptible genotype. In contrast, in the resistant genotype, it increased during cold treatment (Fig 2a). However, methyl salicylate concentration in both susceptible genotype and resistant genotype increased under cold stress (Fig 2b). Jasmonic acid and methyl jasmonate decreased sharply under cold treatment in both the genotypes, but the concentrations of JA and MeJA in the resistant genotype are much higher than the susceptible genotype (Fig 3a, b).

Salicylic acid
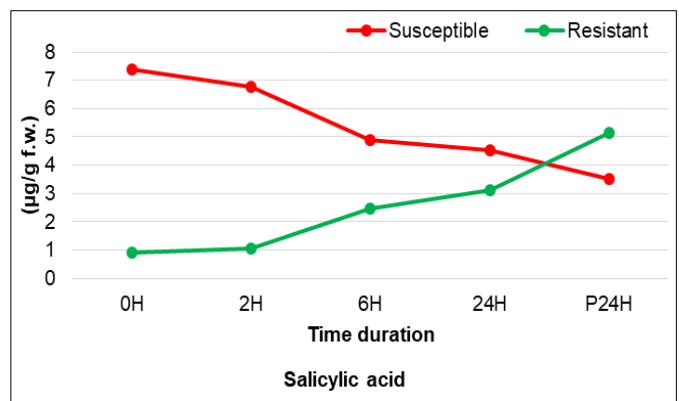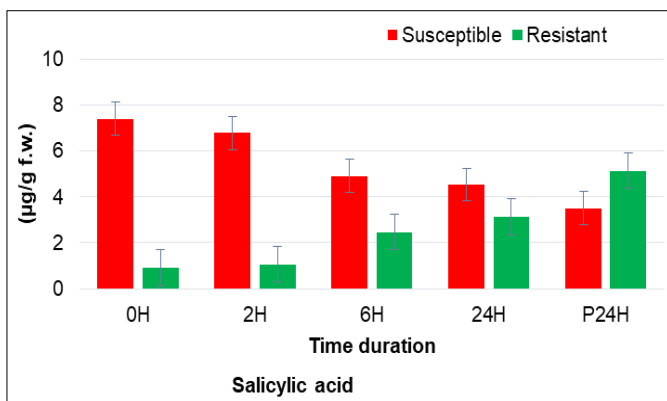
Salicylic acid

A
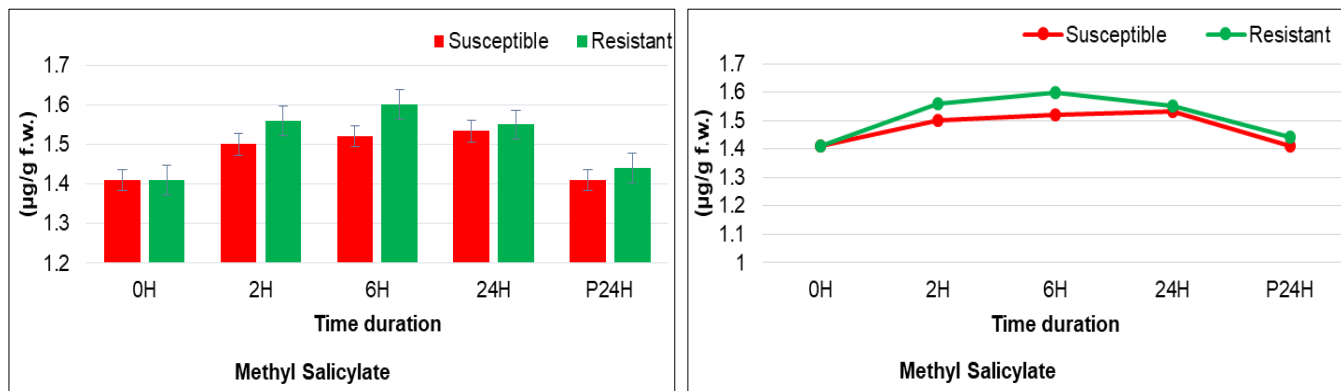
B

Fig 2: Effect of cold stress on salicylic acid and methyl salicylate in rice.

Resistant and susceptible rice genotypes were subjected for 24 hours to cold stress (5 ºC) at seedling stage and leaf samples collected at 0h, 2h, 6h, 24h, and P24 (24h recovery post-chilling at 25 °C). **A)** Salicylic acid consistently increases in resistant genotype with cold stress duration and continuing same trend post-stress, while surprisingly in susceptible genotype it shows an opposite trend; **B)** Methyl Salicylate shows similar trends in the contrasting genotypes, showing a mild increase during cold stress and a quickly regaining the previous level (0h) during recovery period (P24h).
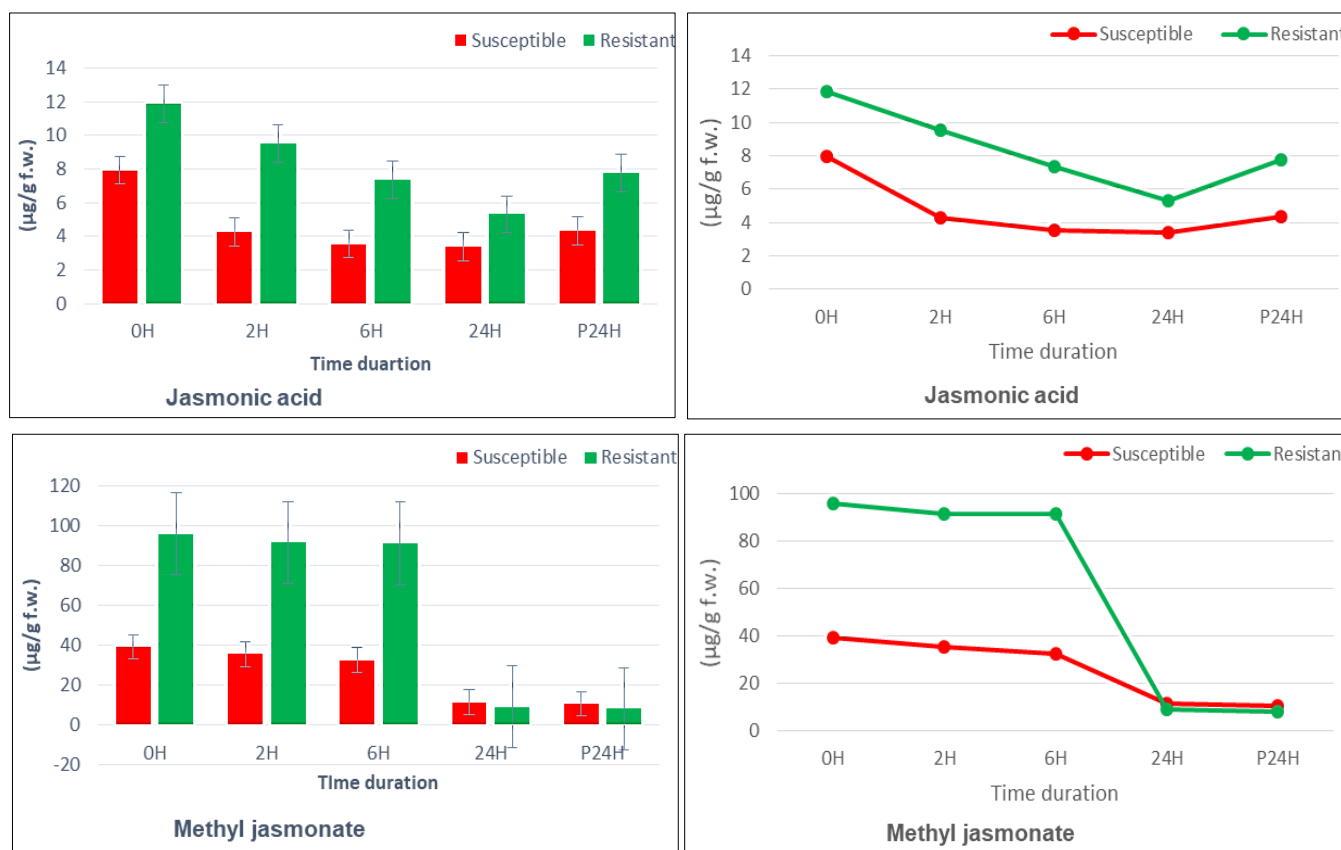

Fig 3: Effect of cold stress on jasmonic acid and methyl jasmonate in rice

Resistant and susceptible rice genotypes were subjected for 24 hours to cold stress (5℃) at seedling stage and leaf samples collected at 0h, 2h, 6h, 24h, and P24 (24h recovery post-chilling at 25℃). **A)** Jasmonic acid level decreased with increasing duration of cold stress in both contrasting genotypes, and increased during post-chilling phase; while maintaining a consistently higher level in resistant genotype compared to the susceptible; **B)** Methyl jasmonate levels are higher in resistant genotype and drop significantly post-6h in both contrasting genotypes, although it is more sharp in resistant genotype

Supplementary table 1: Optimization of chromatography of each compound

Compounds	Retention time (min)	Quantification (m/z)
Jasmonic acid	3.580	83
Methyl Jasmonate	2.840	83
Salicylic acid	1.173	120
Methyl salicylate	1.731	152

Supplementary table 2: JA, MeJA, SA and MeSA contents evaluated in 2 distinct rice genotypes under cold stress

Genotype	Treatment	Jasmonic acid (µg/g f.w.)	Methyl Jasmonate (µg/g f.w.)	Salicylic acid (µg/g f.w.)	Methyl salicylate (µg/g f.w.)
Susceptible (GS-74)	T0	7.930	39.220	7.390	1.410
	T1	4.270	35.460	6.770	1.500
	T2	3.550	32.570	4.900	1.520
	T3	3.390	11.330	4.530	1.533
	T4	4.340	10.560	3.510	1.410
Resistant (SR-4)	T0	11.880	95.790	0.910	1.410
	T1	9.520	91.390	1.070	1.560
	T2	7.340	91.130	2.470	1.600
	T3	5.310	9.100	3.127	1.550
	T4	7.770	8.110	5.140	1.440
C.D.		0.095	0.142	0.588	N/S

Discussion

Plants exposed to cold induce synthesis of cryo-protectants and assemblage of osmoprotectants like proline, sugars (sucrose, maltose, glucose, and fructose) (Lissarre *et al.*, 2010; Sanghera *et al.,* 2011) [22, 37]. These enhance stress tolerance in plants through different activities like aiding in osmotic adjustments, ROS scavenging, and stabilizing the sub-cellular structures (Shen *et al.,* 1999; Ashraf and Foolad, 2007) [41] (Fig. 1). The role of signalling hormones is, however, largely unknown in the cold tolerance of rice.

Salicylic acid (SA) is a well-known signal molecule, which has a role in the protection against biotic (Raskin 1992) [31-33, 45], or abiotic stress (Horvath *et al.,* 2007) [11]. It has been observed that exogenous application of SA (2.0 mM) independently or in combination with methyl jasmonate (10 µM) enhances chilling tolerance of lemon fruit by increasing total phenolics and PAL activity, while inhibiting POD activity (Siboza *et al.,* 2014) [44]. The treatment significantly reduced the membrane lipid peroxidation and chilling induced membrane permeability. Similarly, in *Hordeum vulgare* the exogenous application of SA before cold treatment caused increase in the production of antioxidant enzymes (superoxide dismutase, catalase, and peroxidase), ice nucleation activity, and accumulation of apoplastic proteins; thereby improving cold tolerance in the treated seedlings (Mutlu *et al.,* 2013) [28]. SA pretreatment @ 0.5 mmol/L to banana (*Musa acuminata*) seedlings minimized chilling injury to them by protecting cell ultra-structure (Kang *et al.,* 2007) [13]. The application of SA has been shown to be useful in heat stress as well. Its application @1.0 mM overcame the electrolyte leakage and oxidative stress in cucumber (*Cucumis sativus*) under heat stress (Shi *et al.,* 2006) [42]. Based on a study in SA-deficient transgenic rice lines, SA has been suggested to play a vital role in regulating redox balance and protection from oxidative damage (Yang *et al*. 2004) [23, 55, 56]. SA application (1.5 mmol/L) to rice seedlings at one leave stage increased the content of proline, chlorophyll, and SOD activity, while the malondialdehyde content decreased, thereby enhancing the resistance of rice to cold stress (Xu *et al.,* 2010) [54]. Besides, SA plays a significant role in the induction of PR protiens and SAR, and rice has been shown to possess higher endogenous levels of SA (37.19 *µg/g* fresh weight) in healthy leaves compared to *Nicotiana tabacum* (Tobacco) (Raskin *et al.,* 1990) [31-33, 45]. In the present study, the levels of SA were found to be between 0.588-7.390 µg/g fresh weight. In an earlier study, the free SA concentration in the leaves of rice seedlings M-201 (grown for 35 days) varied from 7.4-15.3 µg/g fresh weight (Silverman *et al.,* 1995) [45]. In another study, the total SA level in rice leaves of different varieties ranged between 10-40 µg/g fresh weight. The reason for a lower value of SA in the present study could be due to methanol-based extraction. Standard extraction procedures with methanol lead to the extraction of many compounds, and since it interferes with subsequent extractions, it is removed by evaporation under vacuum. During this process, free SA and its methyl ester form, methyl salicylate, which is highly volatile, may evaporate (Raskin *et al.,* 1989, Klimes *et al.,* 1992, Meuwly *et al.,* 1993, Chong *et al.,* 2001, Shapiro and Gutsche 2003) [4, 16, 26, 31-33, 38, 45].

In the present study, SA consistently increased in resistant genotype with cold stress duration and continued the same trend in the post-stress recovery phase of 24 hours (Fig 2a). An earlier study has reported that the concentration of SA increased in cold-tolerant wheat genotype during cold acclimation, acquiring a maximum value of 750 pmol/g fresh weight on the 7th day (Kosova *et al.,* 2012) [18]. Surprisingly, in the present study, susceptible genotype SA showed the opposite trend and depleted with increasing time cold stress. The SA concentration in grape berries under cold stress has been shown to increase rapidly (4.6 folds) in the initial 40 minutes, while it regresses to base level at 60 minutes. SA influences intra- and inter-plant communication through its methyl ester form, methyl salicylate, which is highly volatile (Sharma *et al.,* 2020) [39, 40]. MeSA has been shown to induce antioxidant production. It is an important regulatory signal intervening in plant responses to abiotic stress (Tang *et al.,* 2015) [31, 47, 57]. In the present study, the MeSA production trend in the contrasting genotypes was found to be similar, showing a mild increase during cold stress and quickly regaining the base level (0 hrs) during the recovery period (P24 hrs) (Fig 2b).

Jasmonates (JA, MeJA and their derivatives) are extensively distributed in higher plants (Creelman and Mullet, 1997) [5, 25]. These compounds are produced when plants experience environmental stresses like wounding or pathogen attack. As a result of these JA signals, a substantial change in gene expression occurs. Subsequently, critical changes in important regulatory pathways occur, which induce biosynthesis of defense responsive genes (Pauwels *et al.,* 2009) [30]. In the present study, JA levels were recorded between 0.095-11.880 µg/g f.w. JA level decreased with increasing duration of cold stress in both contrasting genotypes, and increased during the post chilling phase. JA maintained a consistently higher level in resistant genotype than the susceptible (Fig 3a). In a study on *Arabis alpine,* the concentration of JA was high at room temperature in both sensitive and cold-tolerant plants. Under cold stress (4ºC), the concentration of JA didn't change in the tolerant plants, while as in the susceptible plants its level reduced 10-folds. However, under frost conditions (below 0ºC), the JA level decreased in the tolerant plants also

(Kolaksazov *et al.,* 2013) [17]. The free acid JA may further be converted into methyl jasmonate by jasmonate methyltransferase or by jasmonate-amido synthetase activity into JA-isoleucine conjugate (JA-Ile). In response to environmental signals, the expression of the genes involved in JA metabolism is dynamically regulated, leading to changes in endogenous JA levels and stress responses (Yang *et al.*, 2019) [23, 55, 56]. In the present study, the levels of MeJA were recorded between 0.142-95.790 µg/g f.w. MeJA levels were higher in the resistant genotype and dropped significantly post 6 hours in both contrasting genotypes, although it was sharper in the resistant genotype (Fig 3b).

Conclusion
The present study shows that jasmonic acid and salicylic acid play a significant role in cold response signaling. The level of Jasmonic acid and methyl jasmonate decreased sharply under cold treatment in both the contrasting genotypes. However, the concentration of these signaling hormones (JA and MeJA) was much higher in the resistant genotype than in the susceptible genotype. In the susceptible genotype, salicylic acid decreased sharply under cold treatment, while in the resistant genotype, it increased under cold. The concentration of methyl salicylate increased in both susceptible and resistant genotypes under cold stress.
Further, previously reported HPLC methods had mainly focused on the estimation of JA and SA after their exogenous application on crops. However, in the present study, an HPLC method was validated to estimate the 'endogenous' base levels of JA, MeJA, SA, and MeSA in rice. Jasmonic acid, methyl jasmonate, salicylic acid, and methyl salicylate were well separated within 20 min. The HPLC method, described herein, can be utilized for routine analysis of these signalling molecules in rice genotypes.

Acknowledgment

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