

# Unravelling metabolomics and antioxidant potential of sweet orange cultivar Pusa Sharad grafted on various citrus rootstocks under sodium chloride stress

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## ABSTRACT

Rootstock as grafting material mitigates sodium chloride (NaCl) stress by altering physiological, metabolite, and gene expression patterns across different genotypes, cultivars, or species. In this study, we investigated the antioxidant and metabolic responses of sweet orange cultivar Pusa Sharad (PS) grafted onto various rootstocks: Jatti Khatti (JK), X-639 (X9), CRH-12 (C12), NRCC-1 (N1), NRCC-2 (N2), NRCC-3 (N3), NRCC-4 (N4), NRCC-5 (N5), Troyer citrange (TC), CRH-47 (C47), and Cleopatra mandarin (CM). These responses were assessed under different salinity stress levels. Biochemical parameters, including sugars, proline, phenol, soluble protein, hydrogen peroxide ( $H_2O_2$ ), superoxide radicals ( $O_2^-$ ), lipid peroxidation, catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POD), vitamin C, vitamin E, organic acids, and fatty acids were analysed. Results demonstrated that the accumulation of  $H_2O_2$ ,  $O_2^-$ , and malondialdehyde (MDA) was upregulated in PS grafted onto the TC, JK, N2, and C12 rootstocks. Conversely, the CAT, SOD, APX, vitamin C, and vitamin E contents were notably higher in PS grafted onto CM, X9, and C47 under 60 mM NaCl stress. Metabolomic analysis indicated that trehalose, raffinose, sucrose, D-galactose, myo-inositol, piperazine, acetic acid, malonic acid, palmitic acid, stearic acid, and pentanoic acid played crucial roles in metabolic adjustments under increasing NaCl stress. Furthermore, PS grafted onto CM, C47, X9, N1, or N3 showed greater tolerance to NaCl compared to those grafted onto JK, C12, N2, N4, N5, or TC, making these combinations adaptable upto 60 mM NaCl concentrations. This study highlights the role of potential metabolites and its use in enhancing NaCl tolerance through grafting onto tolerant rootstocks.

## Introduction

Citrus, a genus belonging to the Rutaceae family is grown commercially across the globe and have economic significance (Talon et al., 2020). Different species of citrus grown in India accounts for 12.13 % of global citrus production (Agricultural statistics at a glance, 2023). Its cultivation holds a prominent position in agriculture, contributing significantly to the world's agricultural output. However, citrus cultivation faces multifaceted challenges, including biotic and abiotic stresses, which impede optimal production (Vincent et al., 2020). Among these challenges, salt stress has emerged as a critical factor

affecting citrus growth, yield, and quality (Shankar et al., 2023). *Citrus* species are sensitive to salt levels such as sweet orange have a threshold of 10 mM, rough lemon and sour orange can be grown upto 15 mM, and Rangpur lime upto 25 mM (Storey and Walker, 1998; FAO, 1988). Limited range of thresh hold limit area expansion of citrus cultivation in areas having higher salinity levels. Alterations in antioxidants and metabolites due to salt stress in citrus plants have been documented by many researchers (Ruiz et al., 1997; Tozlu et al., 2000; Alam et al., 2020). High levels of sodium chloride (NaCl) in the soil solution create an osmotic imbalance between the soil and plant cells (Ferguson and Grattan, 2005), which disrupts water uptake by roots, leading to

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dehydration of plant tissues (Kronzucker et al., 2013). Excessive accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  ions disrupts ion homeostasis within cells. Sodium ions can replace potassium ions in enzyme activation, leading to enzyme dysfunction (Page and Di Cera, 2006). NaCl stress induces reactive oxygen species (ROS), such as superoxide radicals ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and hydroxyl radicals ( $\text{OH}^-$ ), within plant cells and damages cellular components, such as lipids, proteins, and DNA, leading to oxidative stress (Bose et al., 2017). Citrus plants respond to oxidative stress by activating antioxidant defense mechanisms, including enzymes such as superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD), as well as nonenzymatic antioxidants such as glutathione, tocopherol and ascorbate (Gueta-Dahan et al., 1997). Understanding the mechanisms underlying salt stress and exploring effective mitigation strategies are of paramount significance.

In this context, citrus scion grafted onto tolerant rootstocks has emerged as a promising approach for alleviating the effects of salinity stress. Rootstocks have been recognized for their ability to mitigate the effects of salinity (Shankar et al., 2023) and exert specific effects on the size and overall development of the scion, as well as abiotic stresses (Warschefsky et al., 2016). The variable response imparted by the different citrus species may be attributed to cellular compartmentation, physiological and biochemical adjustments (Singh and Sharma, 2018), nutrient imbalances, and the expression of specific proteins and enzymes (Awasthi et al., 2015). Researchers have conducted extensive studies on various citrus rootstock genotypes, including Cleopatra mandarin, Rangpur lime, and *Severinia buxifolia*, to assess their respective abilities to limit the transport of salts to scions (Banuls and Primo-Millo, 1992, 1995; Khoshbakht et al., 2015). Numerous citrus investigations have underscored the genotypic or rootstock-dependent nature of salt tolerance (Alam et al., 2020; Vives-Peris et al., 2023). For example, Kinnow mandarin scions grafted onto salt-tolerant rootstocks exhibited lower level of ROS and enhanced activities of antioxidant enzymes (superoxide dismutase, peroxidase, catalase, ascorbate peroxidase, guaiacol peroxidase, and glutathione reductase) and greater accumulation of osmolytes (sugars and glycine betaine) (Shahid et al., 2019). Vives-Peris et al. (2023) grafted salt sensitive Navelina orange onto Carrizo citrange exhibited an increase in MDA content (byproduct of membrane lipid peroxidation), compared to *Citrus microphylla*, which is moderately tolerant.

Metabolites, including sugars, organic acids, sugar alcohols, fatty acids, and volatile compounds, play essential roles in imparting stress tolerance (Chen et al., 2022). Even among citrus cultivars, volatile compounds in citrus leaves play a significant role in increasing stress tolerance (Killiny et al., 2017). The selection of citrus rootstock is vital for influencing the levels of flavonoids and phytohormones in citrus plants subjected to dehydration and rehydration (Santos et al., 2017). Various metabolites are intricately involved in the biosynthesis of crucial pathways that help maintain cellular homeostasis by mitigating the impact of ROS (Argamasilla et al., 2014). In a study by Melgarejo et al., (2022), a thorough analysis revealed 19 different metabolites in Citrus limon cv. leaf samples from Verna. The research involved manipulating growth conditions using three different rootstocks and implementing three unique culture media. Among the identified metabolites, nine were amino acids, five were organic acids, three were sugars, and two were intermediate metabolites. This indicates that the metabolic responses of lemon trees are robust to various growing conditions, highlighting their importance in physiological adaptation. Several metabolites, such as citric acid, nobletin, malic acid, and phenylalanine, have been identified as biomarkers associated with salinity stress (Hung and Wang, 2018). The different volatile organic compounds (VOCs) released from the leaves of the mandarin hybrid Sugar Belle grafted on sour orange, C-35, and US-897 responded differently to pest infestation (Jones and Killiny 2021). Members of the MATE (multidrug and toxic compound extrusion) gene family directly or indirectly participate in secondary metabolite metabolism under salinity stress in dragon fruit (Khan et al., 2024). An increase in the levels of

phenylpropanoid metabolites was reported in Cleopatra mandarin in response to stress (Zhang et al., 2022), while scopolin has been suggested to play a role in defense mechanisms (Siwinska et al., 2014). The accumulation of flavonols, as well as glycosylated and polymethoxylated flavones such as tangeritin, in response to mitigated oxidative damage has been reported in Cleopatra mandarin (Zandalinas et al., 2017). Sugar alcohols such as myo-inositol protect Sugar Belle mandarin from stress (Killiny et al., 2017). Ghanbari et al. (2023) reported the upregulation of secondary metabolites through exogenous application of nano-selenium and selenium that alleviates salt stress in Verbena cultivar of lemon under salinity stress. The present study examined the impact of salinity stress on citrus scions grafted onto various interspecific and intergeneric rootstocks. However, our study opens a new roadmap that how such nanomaterial (Ghanbari et al., 2023) can be used in citrus to upregulate the different metabolites involved in NaCl tolerance. These findings enhance our understanding of NaCl stress tolerance and its implications for other fruit crops, which could be mitigated through grafting.

## Materials and methods

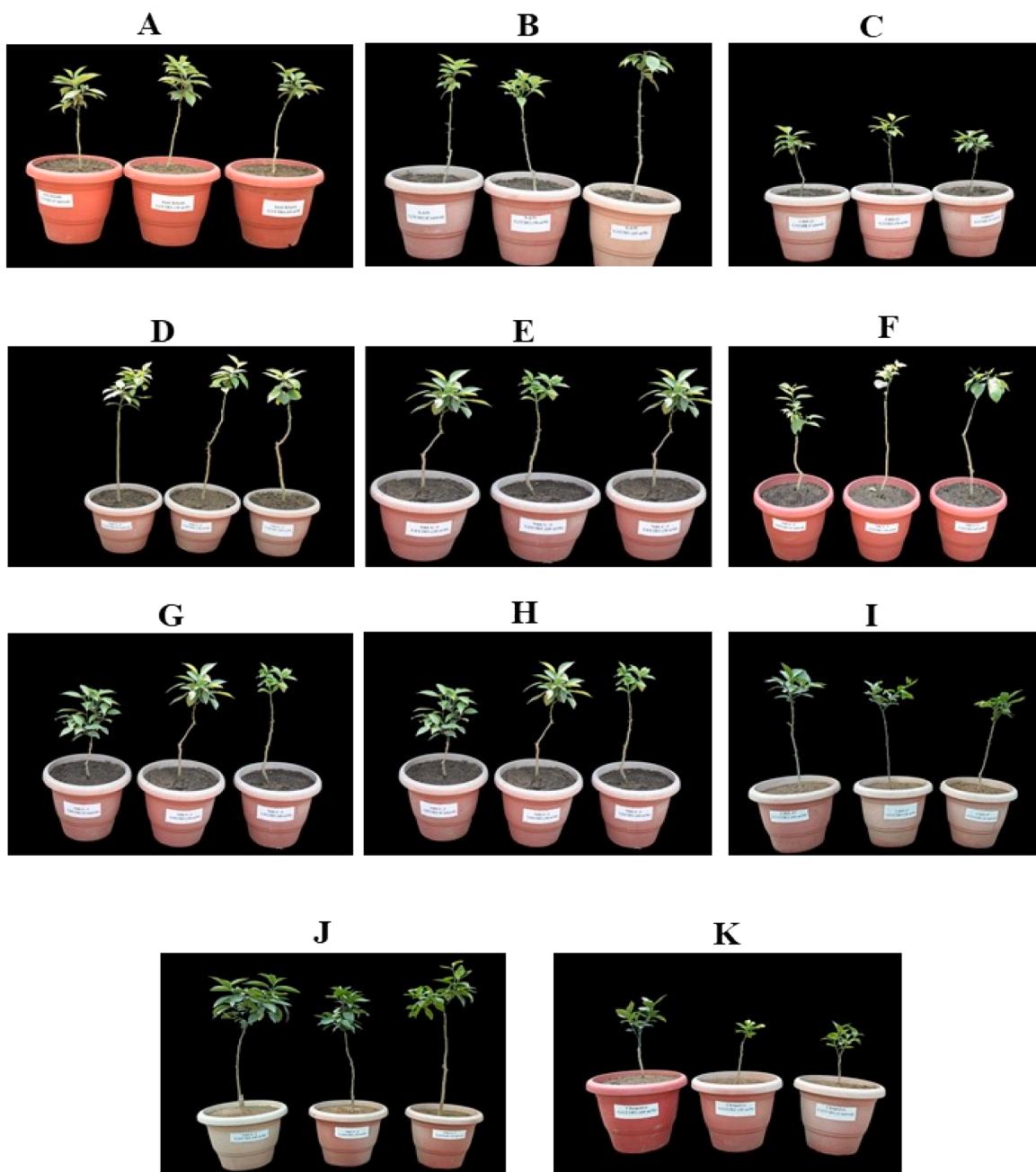
### Plant materials and stress treatment

The fruits from the improved rootstock were obtained from the Central Citrus Research Institute (CCRI), Nagpur, and the germplasm repository of the Division of Fruits & Horticultural Technology (F&HT), ICAR-IARI, New Delhi. The fruits were collected in December 2019 to raise the rootstocks. In July 2021, eleven rootstocks (18 months old) (Table 1) were grafted with the sweet orange scion cultivar PS (Fig. 1). After seven months, the grafted plants were transferred to terracotta pots with the following dimensions: a top diameter of 35 cm, height of 35 cm, and bottom diameter of 23 cm. These pots were filled with a 13 kg mixture of orchard soil and well-composted farmyard manure at a ratio of 4:1 (pH 6.8). Each pot was filled with water up to saturation to determine irrigation water volume. The pots were designed without drainage holes to maintain the standardised saturation. The plants were allowed to acclimate until establishment, which lasted 35 days, after which salinity stress was imposed. The soil and water (Singh et al., 2005) characteristics are detailed in Tables 2 and 3, respectively. The experimental setup consists of 33 combinations of scions-rootstock arranged in factorial completely randomized design (CRD) with three replications. This study was conducted in the shade net house of the nursery unit in the Division of Fruits and Horticultural Technology at ICAR-IARI in New Delhi, India, from February to April 2022. The plants were irrigated with

**Table 1**

List of rootstocks grafted with sweet orange CV. Pusa Sharad (*Citrus sinensis* L. Osbeck).

Sr. No	Genotype	Code	Parentage
1.	Jatti Khatti	JK	<i>Citrus jambhiri</i> Lush
2.	X-639	X9	<i>C. reshni</i> Hort. Ex Tanaka x <i>Poncirus trifoliata</i> (L.) Raf.
3.	NRCC-1	N1	<i>C. jambhiri</i> Lush x [ <i>C. sinensis</i> (L.) Osbeck x <i>P. trifoliata</i> (L.) Raf.]
4.	NRCC-2	N2	<i>C. jambhiri</i> Lush x [ <i>C. sinensis</i> (L.) Osbeck x <i>P. trifoliata</i> (L.) Raf.]
5.	NRCC-3	N3	<i>C. jambhiri</i> Lush. x <i>P. trifoliata</i> (L.) Raf
6.	NRCC-4	N4	<i>C. jambhiri</i> Lush x <i>P. trifoliata</i> (L.) Raf.
7.	NRCC-5	N5	<i>C. jambhiri</i> Lush x [ <i>C. sinensis</i> (L.) Osbeck x <i>P. trifoliata</i> (L.) Raf].
8.	CRH-12	C12	<i>Citrus limonia</i> (L.) Osbeck x <i>Poncirus trifoliata</i> (L.) Raf.
9.	Troyer Citrange	TC	<i>C. sinensis</i> (L.) Osbeck x <i>P. trifoliata</i> (L.) Raf
10.	CRH-47	C47	<i>Citrus reshni</i> Hort. ex Tan. x <i>Poncirus trifoliata</i> (L.) Raf.
11.	Cleopatra Mandarin	CM	<i>Citrus reshni</i> Hort. ex Tan.



**Fig. 1.** Eleven different citrus rootstocks of the sweet orange cultivar Pusa Sharad (PS) were grafted for seven months. A. Jatti Khatti B. X-639; C. CRH-12; D. NRCC-1; E. NRCC-2; F. NRCC-3; G. NRCC-4; H. NRCC-5; I. Troyer citrange; J. CRH-47; K. Cleopatra mandarin

**Table 2**

Properties of soil used.

Content	N	P	K	Ca	Mg	Na	Fe	Mn	Cu	Zn	EC	pH	OC	OM
Quantity	212.56	47.04	338.40	746	436	1.09	11.45	27.45	2.67	6.24	0.46	7.18	0.69	1.18

N: mineralizable nitrogen ( $\text{kg ha}^{-1}$ ), P: available phosphorus ( $\text{kg ha}^{-1}$ ), K: ammonium acetate potassium ( $\text{kg ha}^{-1}$ ), Ca: ammonium acetate calcium ( $\text{mg kg}^{-1}$ ), Mg: ammonium acetate magnesium ( $\text{mg kg}^{-1}$ ), Na: ammonium acetate sodium ( $\text{me 100 g}^{-1}$ ), Fe: iron ( $\text{mg kg}^{-1}$ ), Mn: manganese ( $\text{mg kg}^{-1}$ ), Cu: copper ( $\text{mg kg}^{-1}$ ), Zn: ( $\text{mg kg}^{-1}$ ), EC: electrical conductivity ( $\text{dS m}^{-1}$ ), OC: organic carbon (%), OM: organic matter (%).

**Table 3**

Quality of water used in the study.

Irrigation quality	$\text{HCO}_3^-$ (me/L)	$\text{CO}_3^{2-}$ (me/L)	$\text{Ca} + \text{Mg}$ (me/L)	Na (me/L)	RSC	SAR	$\text{Cl}$ (me/L)	EC (dS/m)	pH
Content	9	Nil	10	9.05	Nil	4.77	12	0.29	6.8

30 mM or 60 mM NaCl to induce salt stress. Salt solutions were applied with each irrigation, up to saturation. The NaCl solution was prepared based on molecular weight, resulting in concentrations of 1753.2 mg/L for 30 mM NaCl and 3506.4 mg/L for 60 mM NaCl in distilled water. The control group received distilled water with a pH of 6.8 and an EC of 0.29 dS m<sup>-1</sup>. The watering schedule was set at four-day intervals, ensuring that the plants were consistently maintained at field capacity. After the treatments, the data were recorded 42 days after salt stress initiation, and fresh samples were collected, snap frozen in liquid nitrogen and kept at -80 °C for further metabolite analysis.

#### Determination of biochemical parameters

The biochemical studies involved measuring the levels of reactive oxygen species (ROS), enzymatic and nonenzymatic antioxidants and osmolytes.

#### Reactive oxygen species, osmolytes, enzymatic and nonenzymatic antioxidants

To determine H<sub>2</sub>O<sub>2</sub>, 0.1 g of leaf tissue was homogenized with 2 mL of acetone and centrifuged. Titanium reagent was added to the supernatant, followed by the addition of 17 M ammonia solution. The precipitates were separated, washed with acetone, and dissolved in 3 mL of 2 N H<sub>2</sub>SO<sub>4</sub> (Patterson et al., 1984). The absorbance of the solution was measured at 410 nm using Lambda 365, UV-visible spectrophotometer (Perkin Elmer, Waltham, Massachusetts, United States). The O<sub>2</sub><sup>-</sup> generation rate was determined following the method described by Elstner and Heupel (1976). In this method, a 0.1 g leaf sample was mixed with 0.5 mL of phosphate buffer, 1 mL of xanthine oxidase, and 0.1 mL of hydroxyl ammonium chloride and then incubated at 25 °C for 20 min. Subsequently, 0.5 mL of this mixture was blended with 0.5 mL of sulfanilic acid and 0.5 mL of α-neptylamine and thoroughly shaken. The mixture was left to stand at room temperature for 20 min, after which the optical density was measured at 530 nm using Lambda 365, UV-visible spectrophotometer (Perkin Elmer, Waltham, Massachusetts, United States). Malondialdehyde (MDA) is a widely accepted biomarker for assessing the extent of lipid peroxidation, and its concentration is an indicator of oxidative stress-induced damage (Dayal et al., 2014). Lipid peroxidation (LPO), a critical cellular process, involves the oxidative degradation of lipids, resulting in the generation of reactive oxygen species (ROS) and ultimately causing damage to cellular membranes. LPO was assessed by measuring the levels of malondialdehyde (MDA) and thiobarbituric acid (TBA) according to the methods described by Heath and Packer (1968). Equal volumes of leaf extract and 0.5% (w/v) TBA solution containing 20% (w/v) trichloroacetic acid (TCA) were mixed. The mixture was then heated to 95 °C for 30 min and rapidly cooled in an ice-filled cooling bath. Afterwards, the mixture was centrifuged at 3000 × g for 10 min. The absorbance of the supernatant was measured at 532 and 600 nm. To determine enzymatic antioxidant activity, the leaf samples were ground using liquid, suspended in 50 mM phosphate buffer (PB) at a pH of 7.0 and centrifuged at 15,000 × g for 20 min at 4 °C. The supernatant was then separated through a two-layered filter paper, and this supernatant was promptly used to measure enzyme activity. The activities of enzymatic antioxidant enzymes, including SOD (Fridovich 1975), APX (Nakano and Asada 1981), CAT (Aebi 1984), GTR (Smith et al., 1988) and POD (Thomas et al., 1982), were determined following established methods using Lambda 365, UV-visible spectrophotometer (Perkin Elmer, Waltham, Massachusetts, United States). SOD rapidly scavenges oxygen radicals through oxidation-reduction cycles involving transition metal ions at its active site and catalyzes the decomposition of O<sub>2</sub><sup>-</sup> into H<sub>2</sub>O<sub>2</sub> and molecular oxygen (O<sub>2</sub>) (Zheng et al., 2023). CAT enzymes breakdown hydrogen peroxide into water and oxygen, hence reducing oxidative stress, upregulating catalase activity and helping plants detoxify ROS and prevent oxidative damage at the cellular level (Ibrahim et al., 2018). GTRs maintain the pool of reduced

glutathione (GSH) by converting oxidized glutathione (GSSG) back to its reduced form (GSH) (Csizsar et al., 2016). This regeneration of GSH is crucial because GSH acts as a powerful antioxidant that can directly scavenge ROS or serve as a cofactor for other antioxidant enzymes (Gill et al., 2013). Peroxidase helps maintain cell membrane integrity by reducing lipid peroxidation and preventing cell membrane damage by triggering signaling mechanisms that adjust peroxidase activity in the leaves as a part of a systemic stress response (Farooq et al., 2009). The nonenzymatic vitamin C content was quantified following the method suggested by Hedges et al. (1996), while the vitamin E content was determined according to Schmieden and Wild (1994). In this method, a 0.5 g leaf sample was homogenized in 8 mL of ethanol containing 0.1 g of soluble polyvinyl pyrrolidone (PVP) and 0.2 g of sodium hydroxide. The resulting homogenate was centrifuged at 5000 × g for 5 min at 4 °C, and the supernatant was filtered through a cellulose acetate filter and maintained at 70 °C. Measurements were taken at 292 nm. The quantities of α-tocopherol were determined using α-tocopherol acetate. The total soluble sugars were quantified using anthrone colorimetry (Riazi et al. 1985), in which 1 gram of fresh leaves was ground in 10 mL of 80% ethanol and then centrifuged, and the extract was filtered. Then, 1 mL of the supernatant was mixed with 6 mL of anthrone reagent (150 mg of anthrone + 72% H<sub>2</sub>SO<sub>4</sub>) and heated in a 100 °C water bath for 10 min. The cooled samples were then read at 625 nm. The proline content was determined as outlined by Bates et al. (1973). Initially, 0.5 g of leaf sample was ground in 5 mL of 3% sulpho-salicylic acid. The resulting mixture was centrifuged at 7826 × g for 10 min at 4 °C, after which the resulting supernatant was diluted to 10 mL with double-distilled water. Next, 0.1 mL of the supernatant was mixed with 5 mL of acid ninhydrin reagent and 5 mL of glacial acetic acid, and the mixture was heated for 1 hour at 100 °C in a hot water bath. The reaction was then terminated in an ice bath, followed by extraction with 4 mL of toluene and vortexing for 20–30 s. The toluene layer, containing the chromophore (appearing light pink), was aspirated from the aqueous phase and brought to room temperature, and the absorbance was measured at 520 nm, with pure toluene as a blank. The proline concentration in the sample was determined using a standard curve prepared with analytical grade proline. The Bradford method was also used to assess the soluble protein content (Bradford, 1976) using coomassie brilliant blue (CBB). In this method, a 0.1 gram leaf sample was homogenized in 50 mM phosphate buffer (pH 7.8), and the supernatant (25 μL) was added to distilled water (475 μL) and 3 mL of CBB (0.01%). The mixture was then thoroughly shaken and incubated for 5 min at room temperature for color development. The absorbance was measured at a wavelength of 595 nm. The total phenolic content was determined using the Folin-Ciocalteu method as described by Singleton and Rossi (1965). For the assay, 20 μL of leaf extract or gallic acid standard was mixed with 0.9 mL of 10% Folin-Ciocalteu reagent. Subsequently, 0.6 mL of 7.5% (w/v) sodium carbonate solution was added. The mixture was left to stand at room temperature for 60 min. After this incubation period, the absorbance was read at 760 nm using Lambda 365, UV-visible spectrophotometer (Perkin Elmer, Waltham, Massachusetts, United States). Aqueous solutions of gallic acid, ranging from 100 to 600 ppm, were used to create a calibration curve. The results were expressed as milligrams of gallic acid equivalents of fresh weight (FW).

#### Metabolite analysis

The leaves were snap-frozen in liquid nitrogen and stored at -80 °C until further examination. Methanol, hexanol, water and chloroform (HPLC grade) were purchased from Himedia. The internal standards ribitol (adonitol), methoxamine hydrochloride, and N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) were obtained from Sigma, and pyridine was also obtained from Sigma.

### Sample preparation, extraction and derivatization

The processing of samples adhered to the methodology described by Roessner-Tunali et al. (2003). Initially, 250 mg of leaf sample was thoroughly ground with liquid nitrogen, and the metabolites were subsequently extracted in 1.4 mL of prechilled methanol. To serve as an internal standard, 100  $\mu$ L of ribitol (1 mg mL<sup>-1</sup> solution) was added. Following thorough mixing, the samples were incubated for 15 min at 70 °C, after which one volume of HPLC-grade water and 750  $\mu$ L of HPLC-grade chloroform were added. The mixture was vigorously stirred and centrifuged at 22,000  $\times g$  for 15 min. For the derivatization process, 150  $\mu$ L of the aqueous layer was transferred to a fresh vial and vacuum-dried. The resulting residues were then subjected to derivatization using 40  $\mu$ L of methoxyamine hydrochloride in pyridine, with an incubation period of 2 h at 37 °C. Next, 60  $\mu$ L of N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) was added, and the mixture was further incubated for 30 min at 37 °C. A microliter (1  $\mu$ L) of the sample was injected into a 7890A gas chromatography (GC) system (Agilent, Santa Clara, California, USA) equipped with an HP-5MS column (30 m  $\times$  0.25 mm; 0.25  $\mu$ m) directly linked to a triple-axis 1538 HED-EM 5975C mass spectrometer (Agilent, Santa Clara, California, USA). The carrier gas employed was high-purity helium sourced from New Delhi, India, with a head pressure set at 10 psi and a flow rate of 1 mL min<sup>-1</sup>. The initial temperature was maintained at 90 °C for 1 min, and then a gradual increase of 5 °C per minute was applied until the temperature reached 290 °C. Other specified parameters included an interface temperature of 250 °C, an ion source temperature of 200 °C, and an electron impact ionization (EI) of 70 eV. Mass spectra were collected in full scan and selected ion monitoring (SIM) modes. The raw MS data were processed using the MSD productivity chemStation program to generate a refined spectrum, eliminate residual background contaminants, partially elute peaks, and address column bleeds. Compound structures were confirmed by cross-referencing with the instrument's library. The MS acquisition parameters included an interface temperature of 280 °C, an ion source temperature of 200 °C, electron ionization at 70 eV, full scan mode spanning 50–550 mass units, a solvent delay of 3 min, and an electron multiplier voltage of 889.

### Processing of GC-MS data

The identification of compounds relied on their retention times, and different metabolites were discerned through derivatization involving varying numbers of TMS (trimethylsilyl) groups. To facilitate normalization, the peak area of individual metabolites was divided by the peak area of the internal standard (ribitol), resulting in dimensionless values. These unitless values were then employed for subsequent statistical analysis. The assessment of compound fold changes was executed utilizing the normalized peak areas. A standardized dataset was utilized to implement partial least squares-discriminant analysis (PLS-DA). Variables possessing a VIP (variable importance in projection) score surpassing one and achieving a significance level of  $p < 0.05$  were considered statistically significant. To elucidate the pathways associated with the metabolites, an analysis was conducted using the KEGG library of *Arabidopsis thaliana* as a reference.

### Statistical analysis

Data analysis was conducted using analysis of variance (ANOVA) with SAS software. In addition, the Pearson correlation matrix and principal component analysis (PCA) were performed using Python packages, including scikit-learn, bioinfokit, seaborn, scipy, numpy, pandas, and matplotlib. The metabolic profiles were normalized to both the internal standard and the fresh weight of individual samples. These metabolic profiles were analyzed using Metaboanalyst 5.0 software, as described by Xia and Wishart (2011). All metabolic features were subjected to preprocessing steps to ensure uniformity and reliability in

subsequent analyses. This included normalization to the median measurement across all samples, log<sub>10</sub> transformation, and autoscaling to the mean. Following preprocessing, statistical and pathway enrichment analyses were conducted to identify significant metabolite differences between the control and treatment groups via Student's t-test. Furthermore, exploratory data analyses, such as principal component analysis (PCA), heatmap construction, correlation matrix assessment, partial least squares discriminant analysis (PLS-DA) plot generation, and PCA loading plot creation, were performed using MetaboAnalyst 5.0 software (Xia and Wishart, 2011).

### Results

This study investigated the effects of grafting on different citrus rootstocks by investigating the levels of reactive oxygen species (ROS), osmolytes, and enzymatic and nonenzymatic antioxidants (Fig. 2, 2a and 2b) at various NaCl concentrations on the same scion.

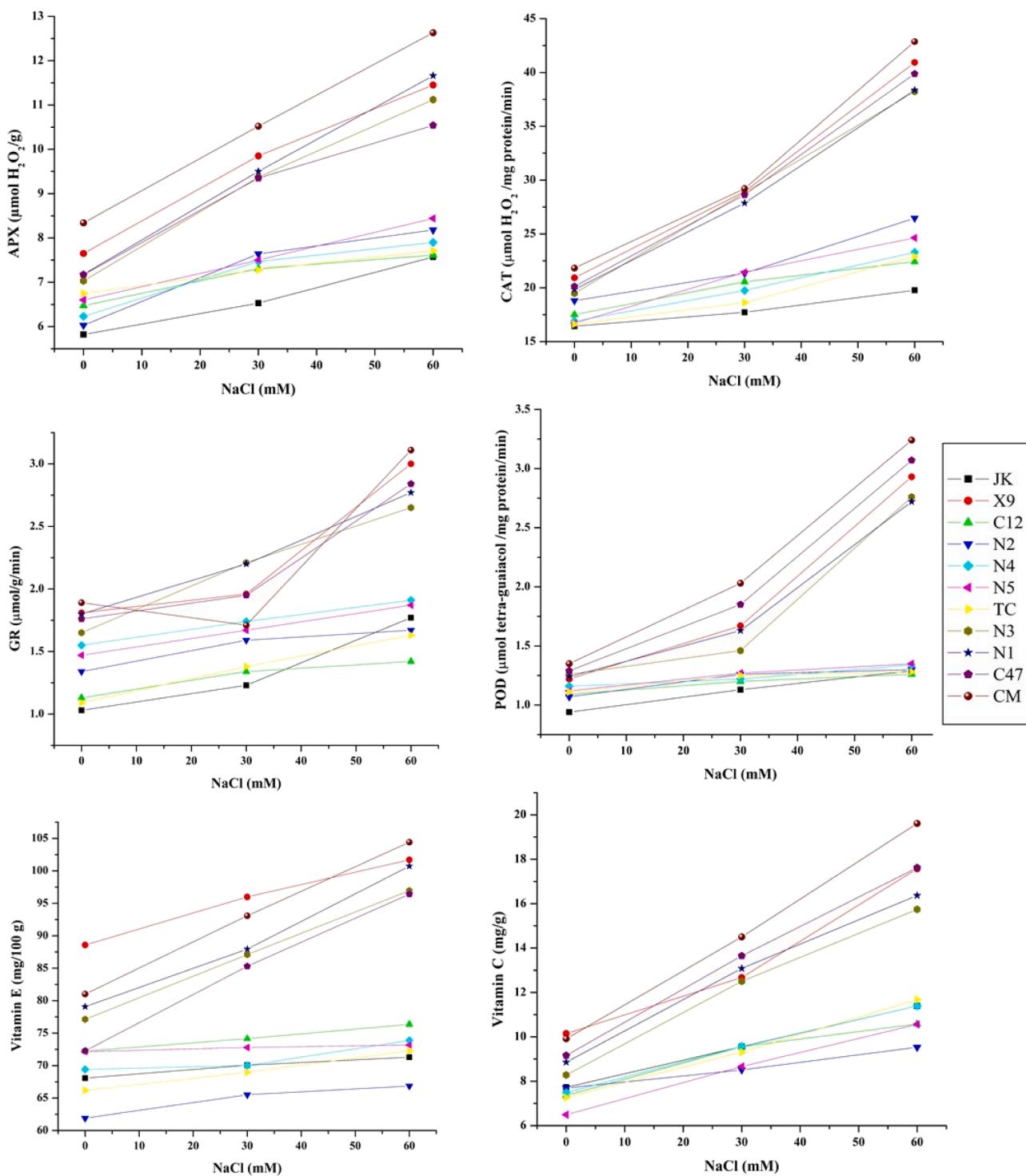
#### Reactive oxygen species (ROS)

The maximum H<sub>2</sub>O<sub>2</sub> levels were recorded in TC (103.00  $\mu$ mol/g), N5 (98.33  $\mu$ mol/g) and N4 (95.67  $\mu$ mol/g) under 60 mM NaCl stress, while the lowest levels were observed in CM (29.33  $\mu$ mol/g) under control conditions. Compared with those in the control group, the number of PS plants grafted on C12 and TC plants significantly differed under 30 mM and 60 mM NaCl stress. The highest levels of O<sub>2</sub><sup>-</sup> were observed when the stress level increased to 60 mM in PS grafted on JK (6.57 nmol/min/g), followed by N2 (6.43 nmol/min/g) and C12 (6.33 nmol/min/g), while the stress level was least affected in PS grafted on X9 (4.77 nmol/min/g), CM (4.83 nmol/min/g) and C47 (5 nmol/min/g). Lipid peroxidation in the leaf tissue of PS plants varied significantly among the treatments. The lipid peroxidation activity at 30 mM NaCl was greatest for N5 (38 nm MDA/g), TC (36 nm MDA/g), and C12 (35 nm MDA/g), which were also affected by 60 mM NaCl. However, it was least disturbed at the same level on the PS plants grafted onto the CM and X9 rootstocks.

#### Osmolytes, enzymatic and nonenzymatic antioxidants

SOD activity was determined to be high, reaching 27.25 and 24.70 Units/mg protein in PS grafted on CM and C47, respectively, under 60 mM NaCl stress. Conversely, it was lowest in PS grafted on JK (12.55 Units/mg protein) under 60 mM NaCl stress. PS grafted on N2 or N4 were subjected to 30 mM or 60 mM NaCl stress, and the activity levels were 11.63, 12.88, 11.80, and 13.66 Units/mg protein, respectively. Compared with that in the control treatment, the response of APX in the scion cultivar PS grafted on different citrus genotypes significantly differed. The highest ascorbate peroxidase activity was detected in the PS grafted on CM (10.49, 10.52 and 12.63  $\mu$ mol H<sub>2</sub>O<sub>2</sub>/g) and X9 (9.65, 9.85 and 11.45  $\mu$ mol H<sub>2</sub>O<sub>2</sub>/g), while the lowest activity was detected in JK (5.82, 6.53, and 7.57  $\mu$ mol H<sub>2</sub>O<sub>2</sub>/g) under the control and 30 mM and 60 mM NaCl treatments, respectively. The CAT, GR and POD activities varied significantly among the PS-grafted plants on the different citrus rootstocks. Under 60 mM NaCl-induced stress, the maximum CAT, GR and POD contents were detected in PS grafted on CM and X9. Conversely, it was lowest in PS grafted on JK, C12 and TC.

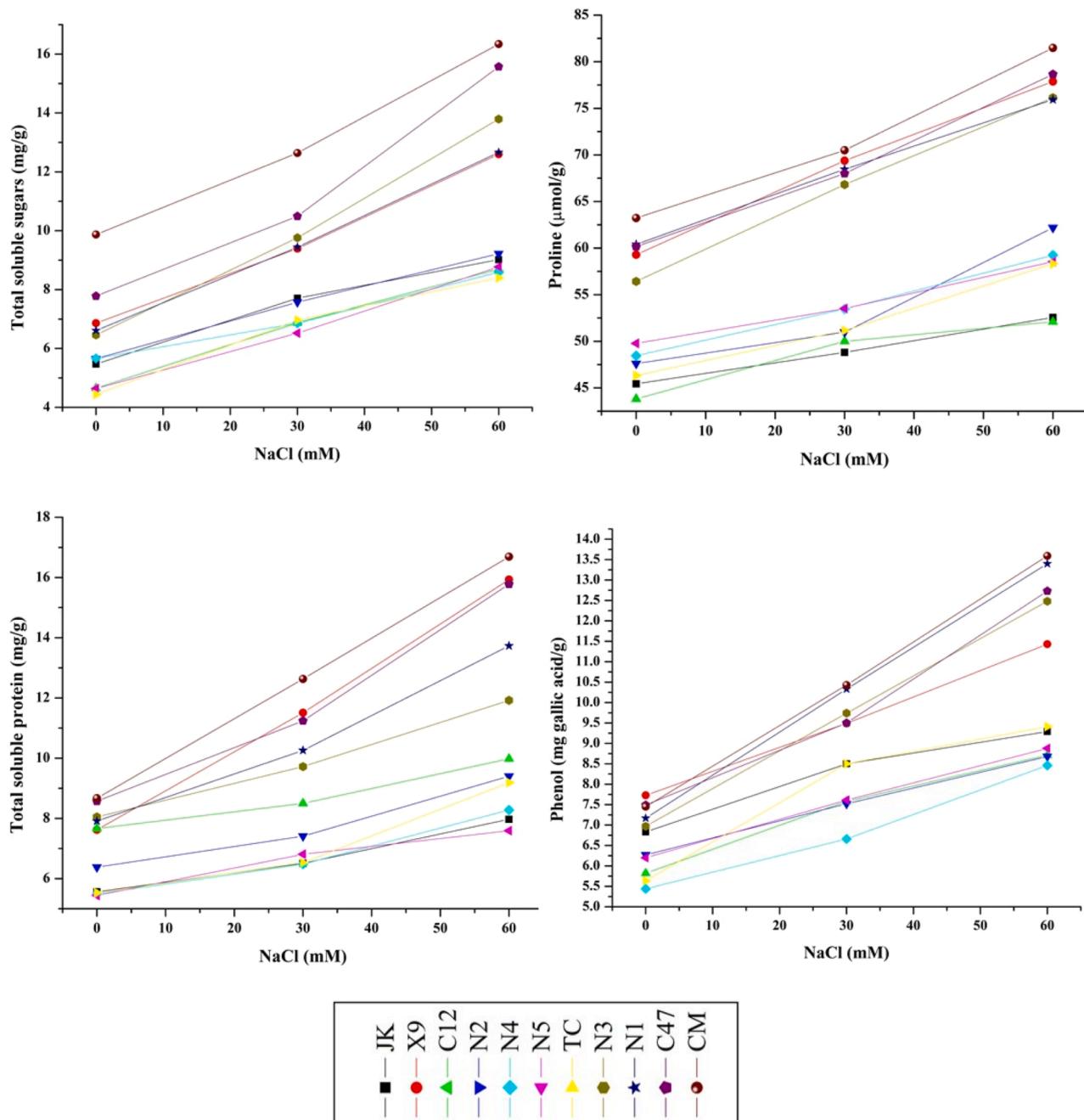
Compared with those of N2 (64.76  $\mu$ g/g), TC (69.14  $\mu$ g/g) and JK (69.81  $\mu$ g/g) under control conditions, the vitamin E content of the scions of the PS cultivar grafted onto genotypes X9 (95.43  $\mu$ g/g) and CM (92.84  $\mu$ g/g) reached a maximum. When the scions were subjected to 60 mM NaCl, CM (104.42  $\mu$ g/g) and X9 (101.71  $\mu$ g/g) exhibited the highest vitamin E contents, while N2 (66.86  $\mu$ g/g), JK (71.29  $\mu$ g/g) and TC (72.26  $\mu$ g/g) had the lowest vitamin E contents, indicating rootstock-dependent behavior. Under 30 mM and 60 mM NaCl stress, the maximum vitamin C content was noted in CM (14.5 mg/100 g; 19.62) and C47 (13.65 mg/100 g; 17.63), while it was lowest in PS on N2 (8.51 mg/100 g), N5 (8.66 mg/100 g) and TC (9.00 mg/100 g).



**Fig. 2.** Effect of NaCl stress on biochemical traits of Pusa Sharad grafted on different citrus rootstocks. APX: Ascorbate peroxidase; CAT: Catalase; GR: Glutathione reductase; POD: Peroxidase; Vitamin E and VC: Vitamin C.

The total soluble sugars varied significantly, with peak concentrations observed in PS when grafted onto CM ( $12.96 \text{ mg g}^{-1}$ ), C47 ( $11.28 \text{ mg g}^{-1}$ ) and N3 ( $10.00 \text{ mg g}^{-1}$ ). Conversely, the lowest concentrations were found for TC ( $6.6 \text{ mg g}^{-1}$ ), N5 ( $6.65 \text{ mg g}^{-1}$ ) and C12 ( $6.74 \text{ mg g}^{-1}$ ) under control conditions. When subjected to 30 mM and 60 mM NaCl, PS grafted on CM ( $12.65$ ;  $16.66 \text{ mg g}^{-1}$ ) or C47 ( $10.5$ ;  $15.57 \text{ mg g}^{-1}$ ) resulted in the maximum soluble sugar content, while PS grafted on TC ( $4.44 \text{ mg g}^{-1}$ ) or C12 ( $4.65 \text{ mg g}^{-1}$ ) had the lowest soluble sugar content. Overall, the leaf proline content tended to significantly increase in the scion cv.

Pusa Sharad on CM ( $71.7 \text{ }\mu\text{g/g}$ ), X9 ( $68.9 \text{ }\mu\text{g/g}$ ) and C47 ( $68.9 \text{ }\mu\text{g/g}$ ). Conversely, the lowest proline content was found in C12 ( $48.6 \text{ }\mu\text{g/g}$ ), JK ( $48.9 \text{ }\mu\text{g/g}$ ) and TC ( $51.9 \text{ }\mu\text{g/g}$ ) under control conditions. Conversely, JK ( $48.81 \text{ }\mu\text{g/g}$ ), C12 ( $49.99 \text{ }\mu\text{g/g}$ ) and N2 ( $50.98 \text{ }\mu\text{g/g}$ ) exhibited the lowest contents under 30 mM NaCl stress. When the NaCl concentration increased to 60 mM, PS grafted on CM ( $81.5 \text{ }\mu\text{g/g}$ ), C47 ( $78.66 \text{ }\mu\text{g/g}$ ), and X9 ( $77.88 \text{ }\mu\text{g/g}$ ) had the greatest proline content, while it had the lowest proline content on C12 ( $52.09 \text{ }\mu\text{g/g}$ ), JK ( $52.56 \text{ }\mu\text{g/g}$ ) and TC ( $58.31 \text{ }\mu\text{g/g}$ ).

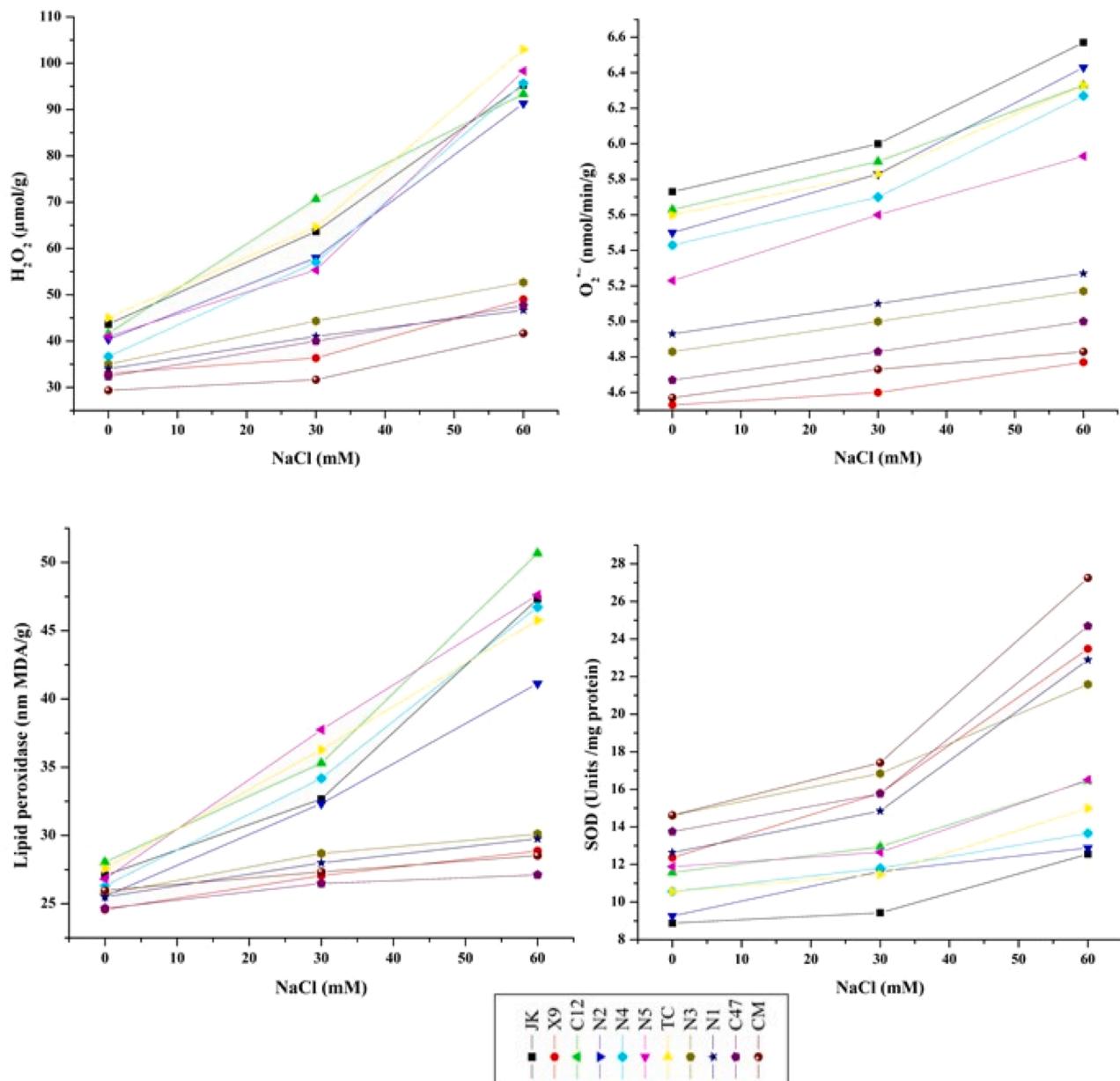


**Fig. 2a.** Effect of NaCl stress on total soluble sugars, proline, total soluble protein and phenol content in Pusa Sharad grafted on different citrus rootstocks.

The most significant interaction effect on protein content was observed for PS grafted on CM ( $16.70 \text{ mg g}^{-1}$ ) and X9 ( $15.93 \text{ mg g}^{-1}$ ) under  $60 \text{ mM}$  NaCl stress, while it was lowest for N5 ( $5.45 \text{ mg g}^{-1}$ ), N4 ( $5.49 \text{ mg g}^{-1}$ ) and TC ( $5.54 \text{ mg g}^{-1}$ ) under control conditions. The total phenol content in PS grafted on CM exhibited a maximum value ( $10.43 \text{ mg g}^{-1}$ ) in N1 ( $10.33 \text{ mg g}^{-1}$ ) and N3 ( $9.74 \text{ mg g}^{-1}$ ) under  $30 \text{ mM}$  NaCl stress. In contrast, the lowest total phenol content was detected in N4 ( $6.67 \text{ mg}$ ), followed by N2 ( $7.52 \text{ mg}$ ) and C12 ( $7.59 \text{ mg}$ ) under the same treatment. The most significant interaction effect for total phenol content was observed for PS grafted on CM ( $13.6 \text{ mg g}^{-1}$ ), N1 ( $13.41 \text{ mg}$ ) and C47 ( $12.7 \text{ mg}$ ) under  $60 \text{ mM}$  NaCl stress. In comparison, the lowest interaction effect was observed for PS on N4 ( $5.45 \text{ mg g}^{-1}$ ), TC ( $5.65 \text{ mg g}^{-1}$ ) and C12 ( $5.83 \text{ mg g}^{-1}$ ) under control conditions.

#### Correlation matrix

The correlation analysis of fourteen biochemical characteristics revealed significant positive and negative associations (Fig. 3). The total soluble sugars exhibited the strongest positive correlations with the vitamin C, catalase, phenol, total soluble protein, and proline contents. Conversely, it displayed the greatest negative correlations with superoxide and lipid peroxidation. The proline content, total soluble protein, and phenol content exhibited positive correlations with all variables except  $\text{H}_2\text{O}_2$ , lipid peroxidation, and superoxide, while  $\text{H}_2\text{O}_2$ , superoxide, and lipid peroxidation exhibited negative correlations. APX, CAT, GTR, peroxidase, and vitamin E showed positive correlations with all variables except  $\text{H}_2\text{O}_2$ , superoxide, and lipid peroxidation.



**Fig. 2b.** Effect of NaCl stress on  $\text{H}_2\text{O}_2$ ; Hydrogen peroxide,  $\text{O}_2^-$ ; Superoxide radical, Lipid peroxidase and SOD; Superoxide dismutase in Pusa Sharad grafted on different citrus rootstocks.

#### Principal component analysis

Principal component analysis was conducted to explore how variables related to tolerance to different scion-rootstock combinations under varying levels of salinity stress (Fig. 4). PC1 explained 65.16 % of the variation, with only vitamin A showing a positive correlation at 30 mM NaCl stress. Moreover, PC2 accounted for 19.02 % of the total variation, with superoxide and lipid peroxidation exhibiting positive correlations under 60 mM NaCl stress. The remaining variables in PC2 also exhibited positive correlations under 60 mM NaCl stress. This pattern holds for susceptible (JK, C12, TC) and tolerant (X9, CM, C47) rootstocks.

#### Metabolite changes

Metabolic profiling revealed 34 distinct metabolites, including organic acids, sugars, sugar alcohols, and fatty acids, within the leaf extracts of 33 citrus scion-rootstock combinations.

#### NaCl-responsive metabolites

In the analysis of metabolite data derived from both the control and NaCl-treated groups (with concentrations of 30 mM and 60 mM NaCl), PCA was employed for both visualizing sample groups and reducing data dimensionality, as illustrated in Fig. 5. The metabolites derived from the control and treated groups exhibited distinct differences in the first principal component (PC1), which accounted for 11.7 % of the total variation, and in the second principal component (PC2), which accounted for 12.2 % of the total variation. Most of the data points were within the 95 % confidence intervals. The PCA score plot revealed a clear difference in the identified metabolites between the control and treatment groups, revealing 23.9 % of the total variance. The PCA score plot particularly illustrates that the variation in the relative expression of metabolites associated with NaCl treatment predominantly influences PC1. In the loading plot of the PCA, metabolites pivotal to salinity tolerance were visually highlighted. Fifteen metabolites primarily influenced PC1, while 12 metabolites significantly impacted PC2. The

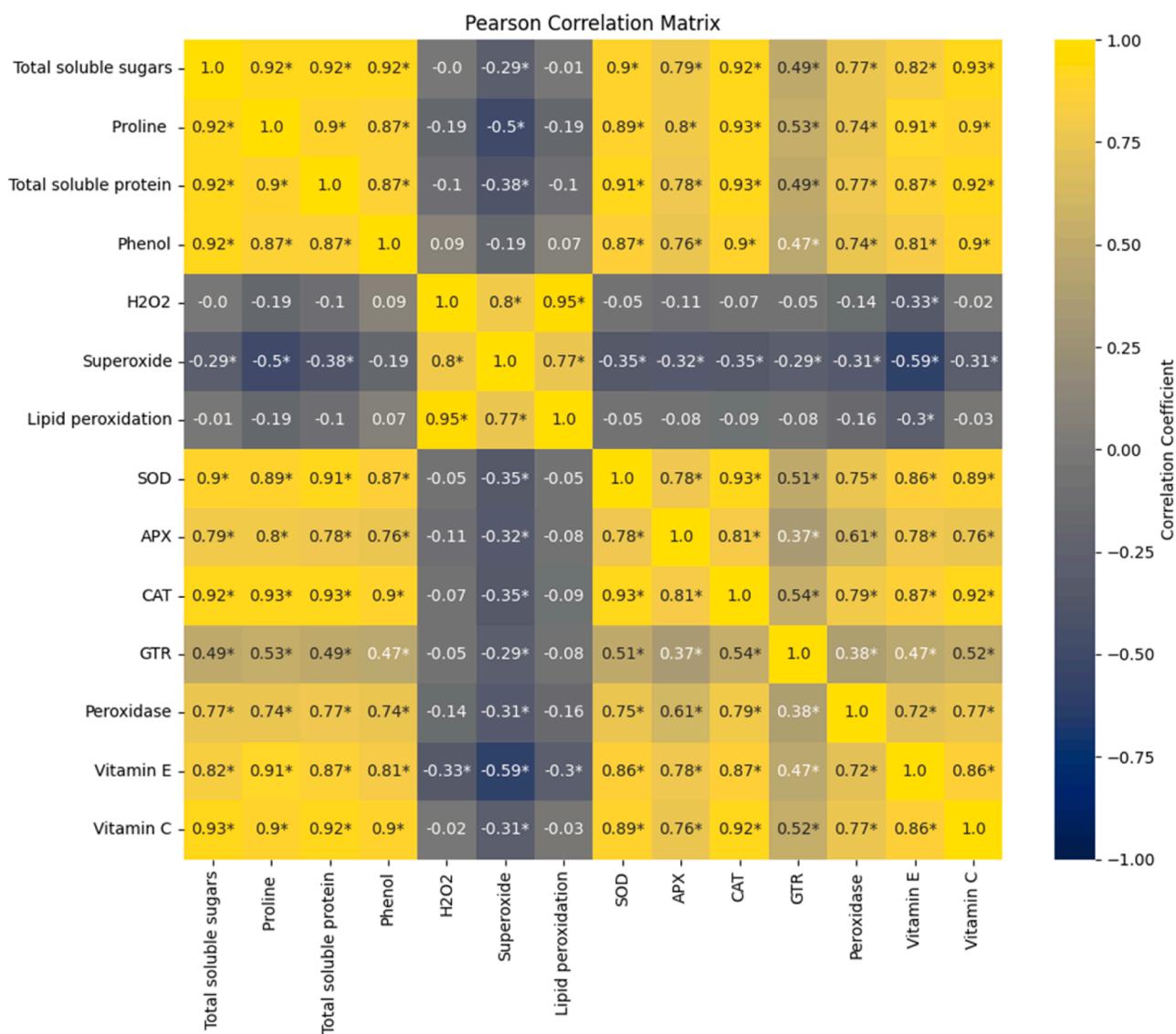


Fig. 3. Pearson's correlation matrix among biochemical characteristics.

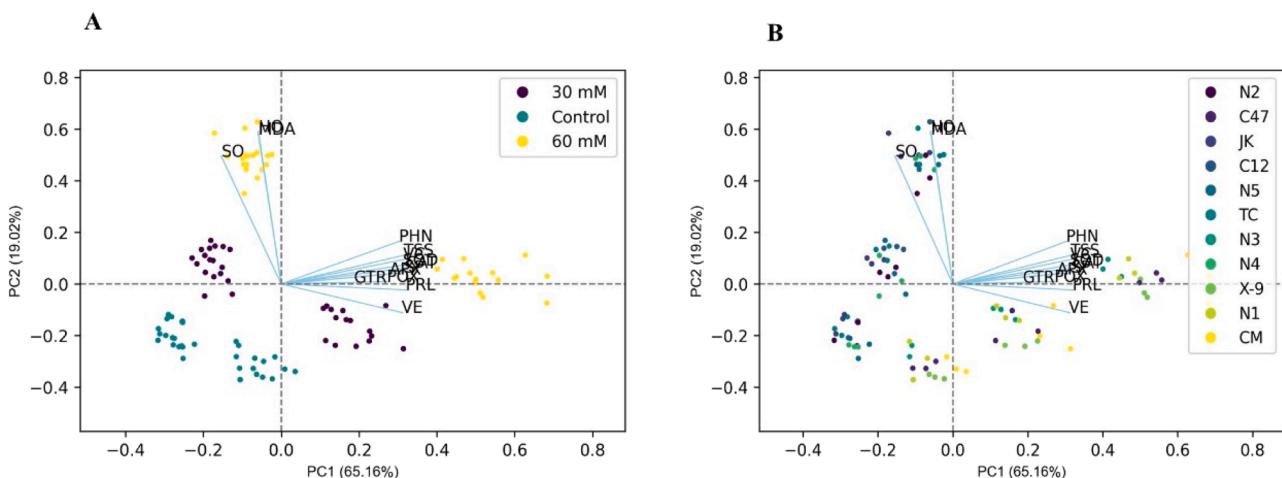
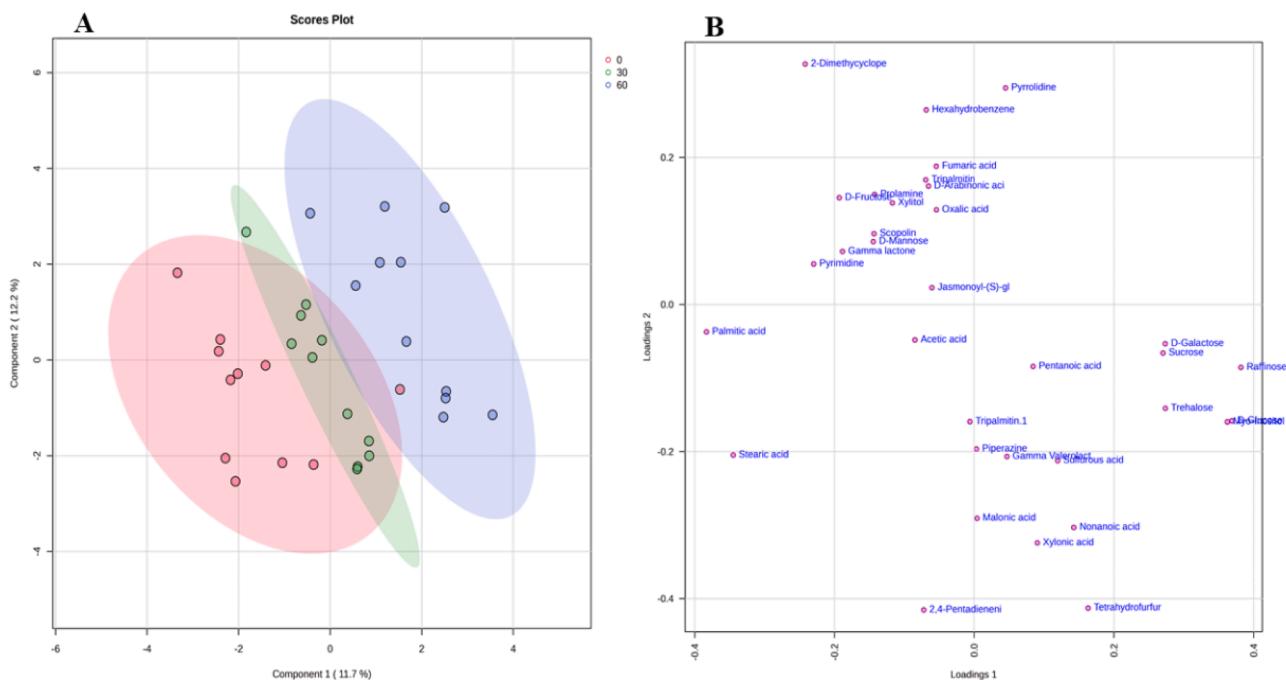


Fig. 4. Principal component analysis (PCA) of biochemical characteristics. A. Plots representing the control (green), 30 mM (purple) and 60 mM (yellow) salinity treatments. B. Plot indicating different scion/rootstock combinations.

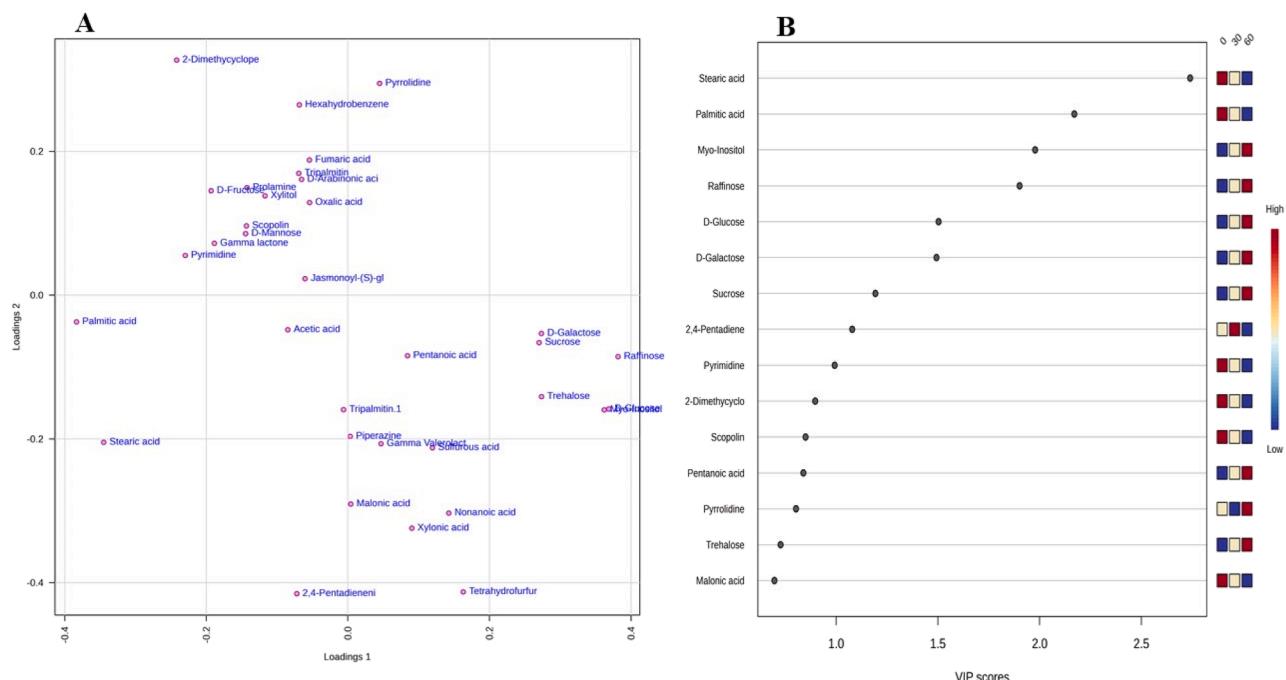


**Fig. 5.** Exploring salinity-induced alterations in citrus leaf metabolites: insights from (A) PCA score and (B) loading plots. Red dots indicate the control, green dots indicate the 30 mM NaCl treatment, and blue dots indicate the 60 mM NaCl treatment.

loading plot highlighted that under NaCl treatment, trehalose, raffinose, sucrose, D-galactose, myo-inositol, piperazine, acetic acid, malonic acid, palmitic acid, stearic acid, and pentanoic acid were major contributors to PC1, exhibiting increased levels in treated plants compared to the control.

On the other hand, hexahydrobenzene, gamma lactone, pyrrolidine, D-fructose, prolamine, oxalic acid, fumaric acid, D-arabinonic acid, and jasmonoyl-(S)-glutamic acid primarily influenced PC2. This suggests decreased levels under 30 and 60 mM NaCl stress in the scion compared

to those in the control. Partial least squares discriminant analysis (PLS-DA) is a supervised model that contrasts with principal component analysis (PCA). PLS-DA employs multivariate techniques to derive linear combinations of initial variables, enabling the prediction of class membership based on the most influential indicator variable. The determination of modified metabolites was accomplished by examining the loading plots associated with the first and second components of the PLS-DA pairwise comparison models. The loading plot generated by PLS-DA revealed 19 metabolites influencing component 1 and 15



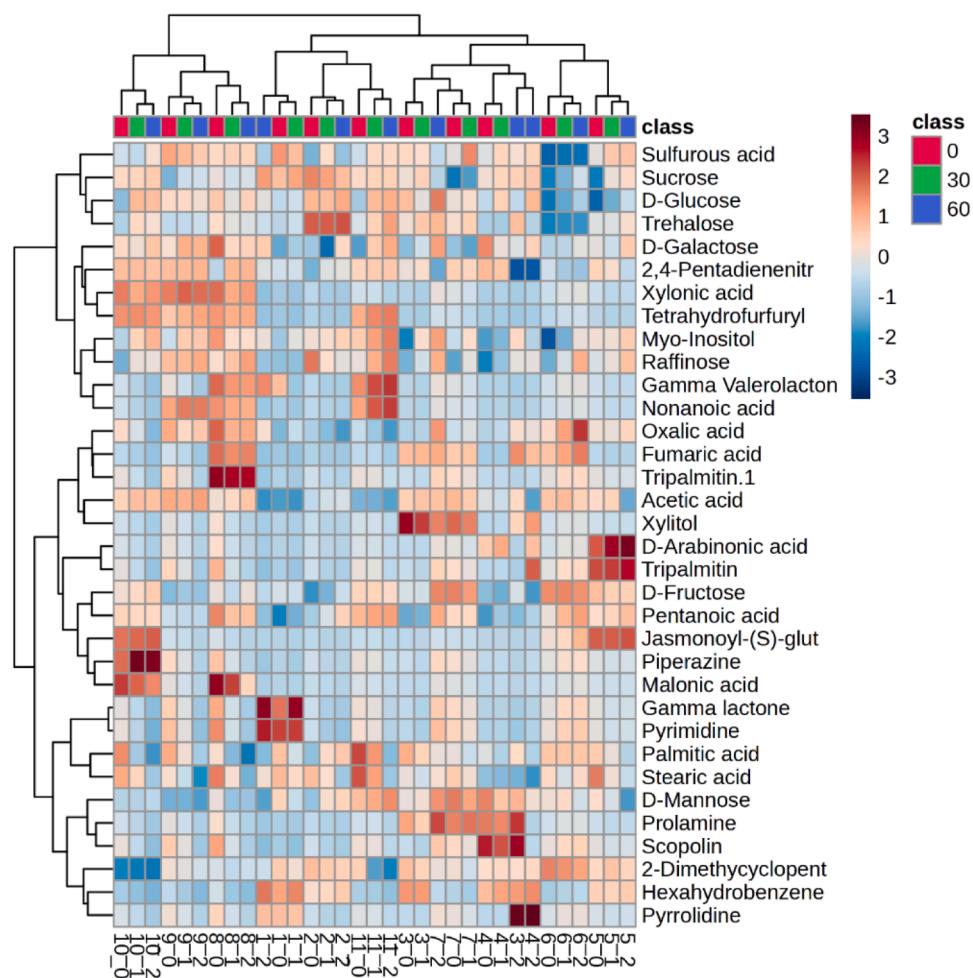
**Fig. 6.** Discriminating salinity-induced metabolite alterations revealed by partial least squares discriminant analysis (PLS-DA). (A) Loading plot and (B) VIP score plot. Significantly up- and downregulated metabolites in response to NaCl treatment (30 and 60 mM) with VIP scores  $> 1$ .

metabolites influencing component 2, indicating their potential role in salt tolerance. Notably, trehalose, pentanoic acid, raffinose, sucrose, D-galactose, and *myo*-inositol emerged as pivotal metabolites significantly contributing to the first component, with their expression notably elevated in plants subjected to NaCl treatment. Metabolites with variable importance in projection (VIP) values exceeding one were deemed noteworthy in the context of salt tolerance. The analysis revealed substantial alterations, particularly in sugars and sugar alcohols, organic acids, and intermediates of the tricarboxylic acid (TCA) cycle (Fig. 6).

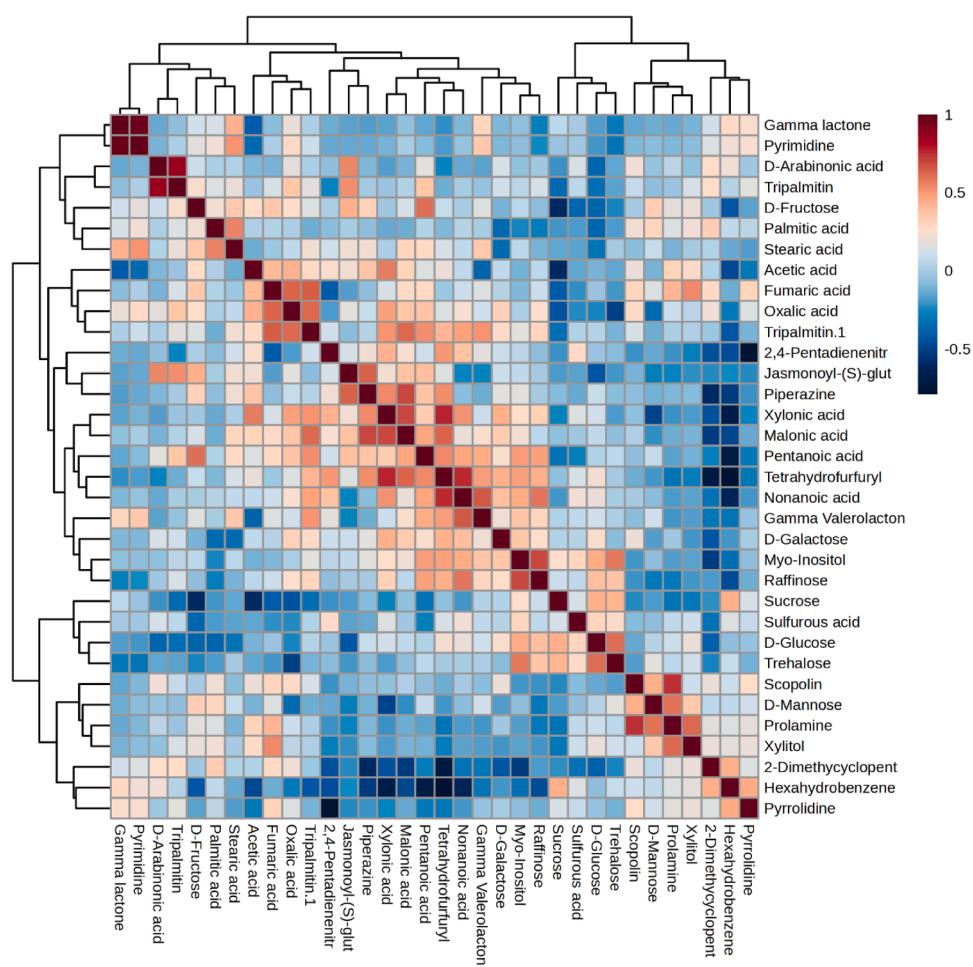
#### Cluster analysis approach

Using cluster analysis, we explored how metabolites from citrus scions and rootstocks interact. A heatmap showing their abundance was generated, and their relationships were clarified using a correlation matrix. The heatmap revealed an increase in sugars and sugar alcohols such as D-galactose, D-glucose, *myo*-inositol, and trehalose in the scion cultivar PS when grafted onto rootstocks X9, C47, and CM under 30 and 60 mM NaCl stress compared to those in the control (see Fig. 7). When grafted onto X9, the scion PS showed an increase in palmitic acid, hexahydrobenzene, and pentanoic acid; piperazine in C47; acetic acid in N1; 2,4-pentadienenitrile, acetic acid and raffinose in N3; and hexahydrobenzene, D-mannose, pentanoic acid, nonanoic acid, gamma valerolactone, raffinose, tetrahydrofurfuryl, and sulphurous acid in CM with increasing levels of salinity stress. An increasing trend in TC was observed for oxalic acid at 60 mM NaCl. In contrast, stearic acid, gamma lactone, malonic acid, pentanoic acid, and D-glucose in C12 decreased as

the level of NaCl stress increased. A correlation matrix was created using Pearson's correlation test to explore the connections between these metabolites (Fig. 8). This allowed us to calculate correlation coefficients, providing insights into the relationships between these metabolites. Gamma valerolacton was positively correlated with D-galactose, *myo*-Inositol, raffinose, sucrose, sulphurous acid, D-glucose, trehalose and D-mannose but negatively correlated with prolamine, xylitol, 2-dimethylcyclopent, hexahydrobenzene, pyrrolidine and scopolin. Palmitic and stearic acid were positively correlated with oxalic acid, jasmonoyl-(S)-glutamic acid, pentanoic acid, nonanoic acid, and 2-dimethylcyclopent but negatively correlated with most sugars and sugar alcohols. Fumaric acid and oxalic acid were positively correlated with tripalmitin, xylonic acid, malonic acid, pentanoic acid, nonanoic acid, D-galactose, raffinose, scopolin, prolamine, xylitol, and 2-dimethylcyclopent but negatively correlated with sucrose, sulfuric acid, D-glucose and trehalose. Moreover, D-galactose was positively correlated with *myo*-inositol, raffinose, sucrose, sulfuric acid, D-glucose, scopolin, and pyrrolidine and negatively correlated with trehalose, D-mannose, prolamine, xylitol, 2-dimethylcyclopent and hexahydrobenzene. *Myo*-inositol was positively correlated with raffinose, sucrose, sulfuric acid, D-glucose, and scopolin but negatively correlated with scopolin, D-mannose, prolamine, xylitol, 2-dimethylcyclopent, hexahydrobenzene and pyrrolidine. D-mannose and prolamin were positively correlated with xylitol, 2-dimethylcyclopent, hexahydrobenzene, and pyrrolidine.



**Fig. 7.** Visualization of citrus scion rootstock metabolites under salinity treatment: heatmap analysis of control and NaCl treatment (30 mM and 60 mM) with  $\log_{10}$  transformation and Pearson's correlation for hierarchical clustering (0; Control, 1; 30 mM and 2; 60 mM; 1 to 11 as series of rootstock given in Table 1).

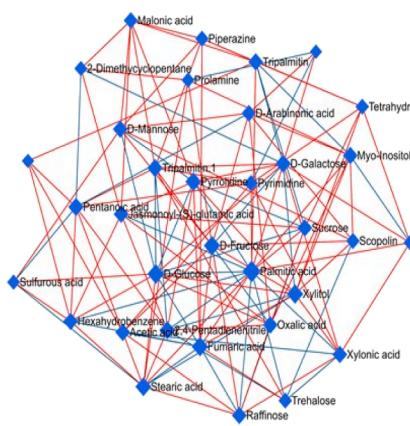
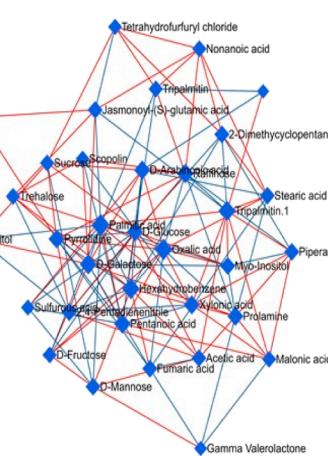
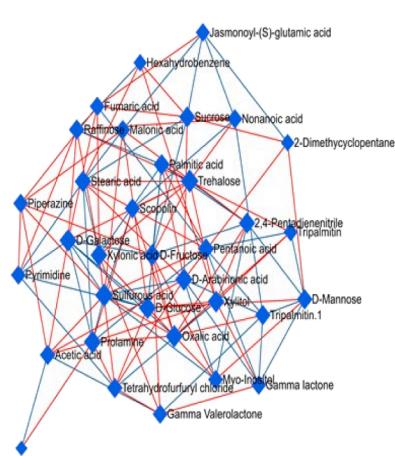


**Fig. 8.** Exploring metabolite correlations in citrus scion rootstock under salinity stress: Pearson's control and NaCl treatment effects analysis.

*Network analysis of correlations revealed stress-induced metabolic perturbations and intermetabolite relationships in response to salinity stress*

A network was built using correlation analysis to understand the connections among different metabolites under varying levels of salinity

stress (Fig. 9). Three separate networks were created using correlation-based modeling to represent control conditions, 30 mM NaCl treatment, and 60 mM NaCl treatment on scion-rootstock systems. Initially, a *p* value threshold ( $\leq 0.011$ ) and a *q* value of 0.05 were set to construct the correlation network. Then, various network properties, such as the

**A****B****C**

**Fig. 9.** Correlation network analysis of scion rootstock metabolites under control conditions (A) and in response to (B) 30 mM and (C) 60 mM NaCl treatment. Nodes denote metabolites, and edges indicate correlations between metabolites, with red edges indicating positive correlations and blue edges representing negative correlations.

average node degree and clustering coefficient, were examined across different p values to find the most suitable correlation coefficient. This study determined that a correlation coefficient equal to or greater than 0.3 was the appropriate threshold. When considering a *p* value <0.01 and a correlation coefficient exceeding 0.7, exposure to NaCl stress led to a decrease in both the number of edges (from 135 to 125) and the average node degree (from 16 to 11) within the nonsalinated treatment (control) compared to the 60 mM NaCl treatment. The control plant network had 135 edges at *P* < 0.05, whereas the networks under 30 mM and 60 mM NaCl stress had 128 and 125 edges, respectively. These edges represent the intricate associations among metabolites and the variations in their interactions crucial for imparting salt tolerance. Analysis of the networks revealed that each metabolite in the NaCl-treated scion-rootstock network was linked to an average of 5 or 6 neighbors. In contrast, the control network showed an average of 8 neighbors per metabolite. The diversity within the correlation network increased from 0.39 in the control network to 0.52 in the network subjected to NaCl treatment. The metabolites that showed significant alterations were also associated with the *Arabidopsis thaliana* KEGG reference pathway. This correlation analysis aimed to link the identified metabolites with specific biological pathways relevant to the salt tolerance mechanism in sweet orange cv. Pusa Sharad.

#### Pathway enrichment and metabolic pathways involved in the response to salinity stress

A KEGG pathway enrichment test was conducted for the salinized treatments to evaluate the activation of biological processes at the metabolic pathway level, and the findings were compared with those of the control. As shown in Table 4, and Fig. 10 pathway enrichment analysis revealed that 25 biological pathways significantly influenced the salinity tolerance of the fruits of the sweet orange cultivar Pusa Sharad grafted onto eleven scion rootstocks. The identified metabolites were mapped onto the KEGG pathway using the model plant *Arabidopsis thaliana* to demonstrate the involvement of distinct metabolites in diverse biochemical pathways. Our research revealed pronounced adverse impacts of salt stress on glycolysis, as indicated by elevated levels of D-galactose, D-glucose, myo-inositol, and trehalose in the scion cultivar grafted on rootstocks X9, C47, and CM in comparison to those in the scion cultivar grafted on rootstocks JK, TC, and C12. These effects were more prominent under 30 and 60 mM NaCl stress conditions than under the control conditions. Salt stress induced the accumulation of sugar alcohols such as myo-inositol and xylitol, whereas D-mannose decreased under salinity. In addition, the PS grafted onto X9 showed

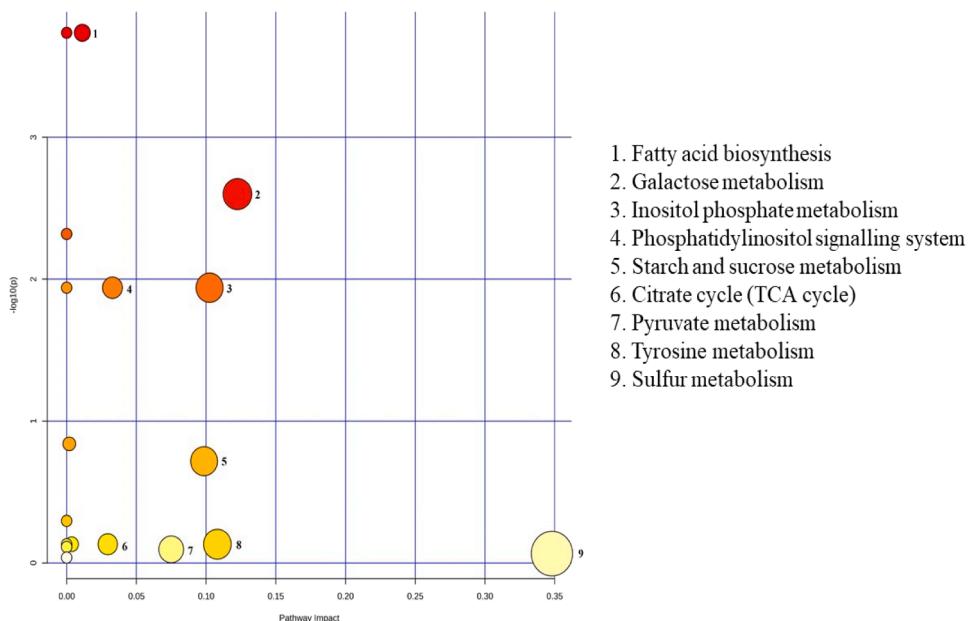
upregulation of palmitic acid, hexahydrobenzene, and pentanoic acid; piperazine in C47; acetic acid in N1; 2,4-pentadienonitrile, acetic acid and raffinose in N3; and hexahydrobenzene, D-mannose, pentanoic acid, nonanoic acid, gamma valerolactone, raffinose, tetrahydrofurfuryl and sulphurous acid in CM with increasing levels of salinity stress. However, the levels of metabolites such as gamma valerolactone, raffinose, proline, scopolin in JK and stearic acid, gamma lactone, malonic acid, pentanoic acid, and D-glucose in C12 decreased with increasing NaCl stress. In general, the findings indicate that alterations in metabolites are linked to various metabolic pathways, including galactose metabolism, starch and sucrose metabolism, amino sugar and nucleotide sugar metabolism, fructose and mannose metabolism, pyruvate metabolism, unsaturated fatty acid biosynthesis, fatty acid biosynthesis, ascorbate and alternate metabolism, the citrate cycle (TCA cycle), glycolysis/glycogenesis, the phosphatidylinositol signaling system, and inositol phosphate metabolism.

#### Discussion

The varied response of the antioxidant and metabolites were recorded in sweet orange scion cultivars Pusa Sharad (PS) grafted on Jatti Khatti (JK), X-639 (X9), CRH-12 (C12), NRCC-1 (N1), NRCC-2 (N2), NRCC-3 (N3), NRCC-4 (N4), NRCC-5 (N5), Troyer citrange (TC), CRH-47 (C47), and Cleopatra mandarin (CM) rootstocks subjected to different salinity stress levels (0, 30 and 60 mM). The accumulation of H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup> and malondialdehyde (MDA) was most affected in Pusa Sharad grafted on the TC, JK, N2 and C12 rootstocks, while the CAT, SOD, APX, vitamin C and vitamin E contents were notably high in PS grafted onto CM, X9, and C47 under 60 mM NaCl stress. Gas chromatography-mass spectrometry (GC-MS) revealed that trehalose, raffinose, sucrose, D-galactose, myo-inositol, piperazine, acetic acid, malonic acid, palmitic acid, stearic acid and pentanoic acid played vital roles in metabolic adjustment under increasing NaCl stress. Previous work indicated that citrus is glycophyte and can only tolerate NaCl upto 25–30 mM (Ziogas et al., 2021). The sensitivity of citrus scion toward NaCl stress due to changes in cell turgor, osmotic potential, accumulation of compatible metabolites, ions especially Na<sup>+</sup> and Cl<sup>-</sup> in scion and rootstock (Turner 2018). However, this can be overcome through scion-rootstock combination because rootstock play an important role in imparting tolerance in scion varieties/cultivars though, ion exclusion (as cleopatra mandarin for Cl<sup>-</sup>; Alam et al., 2020), osmotic adjustment (Fino 49 limon grafted on *Citrus microphylla*; Perez-Perez et al., 2009) and overexpression of candidate Na<sup>+</sup> transporter genes (*SOS1*, *NHX1*, *HKT1*) as well as tonoplast proton pumps (*V-ATPase*, *V-PPiase*) in the

**Table 4**  
KEGG pathway enrichment of metabolites within the scion of Pusa Sharad grafted onto eleven distinct citrus genotypes under NaCl-induced stress conditions.

Metabolic pathway	Total compound	Hits	Raw p	-log (p)	Holm adjust	FDR	Impact
Fatty acid biosynthesis	56	2	0.000184	3.7362	0.003671	0.001836	0.01123
Biosynthesis of unsaturated fatty acids	22	2	0.000184	3.7362	0.003671	0.001836	0
Galactose metabolism	27	4	0.002516	2.5994	0.045281	0.016014	0.1225
Fatty acid elongation	23	1	0.004804	2.3184	0.08167	0.016014	0
Fatty acid degradation	37	1	0.004804	2.3184	0.08167	0.016014	0
Cutin, suberin and wax biosynthesis	18	1	0.004804	2.3184	0.08167	0.016014	0
Inositol phosphate metabolism	28	1	0.011488	1.9398	0.16083	0.025529	0.10251
Phosphatidylinositol signaling system	26	1	0.011488	1.9398	0.16083	0.025529	0.03285
Ascorbate and aldarate metabolism	18	1	0.011488	1.9398	0.16083	0.025529	0
Glycolysis/Gluconeogenesis	26	2	0.1447	0.83954	1	0.28939	0.00189
Starch and sucrose metabolism	22	2	0.19174	0.71729	1	0.34862	0.09857
Pentose and glucuronate interconversions	16	1	0.50532	0.29644	1	0.84219	0
Tyrosine metabolism	16	1	0.73796	0.13197	1	0.89164	0.10811
Citrate cycle (TCA cycle)	20	1	0.73796	0.13197	1	0.89164	0.0295
Alanine, aspartate and glutamate metabolism	22	1	0.73796	0.13197	1	0.89164	0.0036
Arginine biosynthesis	18	1	0.73796	0.13197	1	0.89164	0
Glyoxylate and dicarboxylate metabolism	29	2	0.77103	0.11293	1	0.89164	0
Pyruvate metabolism	22	2	0.80247	0.095569	1	0.89164	0.075
Sulfur metabolism	15	2	0.85946	0.065775	1	0.90469	0.34806
Amino sugar and nucleotide sugar metabolism	50	1	0.91603	0.038092	1	0.91603	0



**Fig. 10.** Pathway enrichment illustrates the metabolome perspective of salinity-induced alterations, revealing various metabolic pathways (explained in Table 4). The color gradient, ranging from yellow to red, signifies metabolites with varying degrees of significance. Specifically, red denotes heightened significance, while yellow denotes comparatively lower significance.

roots of Cleopatra mandarin (Xie et al. 2018). In our study, sweet orange scion cultivar Pusa Sharad (PS) grafted onto various rootstocks has demonstrated differences in their ability to NaCl stress. In this experiment, we explored how different citrus genotypes, each with varying levels of salinity tolerance, respond to NaCl stress. This approach enhanced our understanding of the adaptive mechanisms in citrus plants under saline conditions. Our findings reveal that salinity-tolerant rootstocks exhibit superior adaptation of PS scion by leveraging their antioxidant systems and metabolites.

#### ROS

Abiotic stress such as salinity impacts photosynthetic and respiratory processes, frequently causing the generation and excessive buildup of reactive oxygen species (ROS) within cells, which leads to oxidative stress. These highly reactive and damaging ROS attack nucleic acids, lipids, and proteins, ultimately resulting in cell death (Sachdev et al., 2023). Hydrogen peroxide and superoxide plays a critical role in plant defense mechanisms and is often used as an indicator of oxidative stress (Smirnoff and Arnaud 2019). When PS were subjected to 60 mM NaCl, the highest levels of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> were observed in the TC. Conversely, the lowest levels of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> were found in PS scion grafted on CM, followed by N1 and C47. The PS grafted onto TC, N5, or N4 exhibited increased ROS production in response to stress. NaCl induced more severe oxidative damage in PS scions grafted onto JK, TC, and C12 rootstocks compared to those grafted onto CM, X9, and C47, as evidenced by higher concentrations of H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup>. This suggests that the extent of salinity-induced oxidative damage is associated with the specific genotypes of the rootstocks. This finding of the study is in accordance with Shahid et al. (2019) who reported similar result in Kinnow mandarin grafted on susceptible (Sancton or Carrizo citrange) than tolerant rootstock (Rubidoux and Rangpur lime). Furthermore, Niu et al. (2018) hypothesized that an increase in H<sub>2</sub>O<sub>2</sub> can result from the activation of NADPH oxidases and the disruption of the cellular redox balance. The mechanism behind the tolerance of PS grafted on CM and X9 can be linked through the RNA-seq results of Xie et al. (2018) who identified the overexpression of candidate Na<sup>+</sup> transporter genes (*SOS1*, *NHX1*, *HKT1*) as well as tonoplast proton pumps (*V-ATPase*, *V-PPiase*) in the roots of

1. Fatty acid biosynthesis
2. Galactose metabolism
3. Inositol phosphate metabolism
4. Phosphatidylinositol signalling system
5. Starch and sucrose metabolism
6. Citrate cycle (TCA cycle)
7. Pyruvate metabolism
8. Tyrosine metabolism
9. Sulfur metabolism

Cleopatra mandarin having Na<sup>+</sup> exclusion capacity under salt stress. Lipid peroxidation, a critical cellular process, involves the oxidative degradation of lipids, resulting in the generation of reactive oxygen species (ROS) and ultimately causing damage to cellular membranes. Malondialdehyde (MDA) is a widely accepted biomarker for assessing the extent of lipid peroxidation, and its concentration is an indicator of oxidative stress-induced damage (Dayal et al., 2014). The maximum lipid peroxidation content was detected in C12, followed by N5 and N4 under 60 mM NaCl stress. The lowest levels were detected in C47, followed by CM and X9. These findings indicate that different genotypes or cultivars have varying susceptibilities to oxidative stress induced by high NaCl concentrations. Khalid et al. (2022) reported that when Volkamer lemon tetraploid rootstock was grafted with diploid Kinnow mandarin, imparted salt tolerance through a robust antioxidant defense mechanism and was highly effective in osmotic adjustment, which is evident from the findings of the present study. The tolerance mechanism was achieved through overexpression of Cl<sup>-</sup> tonoplast transporter genes (*CsDTX35.1* and *CsDTX35.2*) in *Oronules* mandarin grafted on *Citrus microphylla* suggesting that these transporters involved in Cl<sup>-</sup> compartmentalization as compared to Navelina orange grafted onto salt sensitive Carrizo citrange rootstock (Vives-Peris et al., 2023).

#### Osmolytes, enzymatic and nonenzymatic antioxidants

Superoxide dismutase (SOD) is a crucial antioxidant enzyme that plays a significant role in removing O<sub>2</sub><sup>-</sup> from plant tissues (Dumanovic et al., 2021). SOD as antioxidant enzyme rapidly scavenges oxygen radicals through oxidation-reduction cycles involving transition metal ions at its active site and catalyzes the decomposition of O<sub>2</sub><sup>-</sup> into H<sub>2</sub>O<sub>2</sub> and molecular oxygen (O<sub>2</sub>) (Zheng et al., 2023). CAT enzymes break-down hydrogen peroxide into water and oxygen, hence reducing oxidative stress, upregulating catalase activity and helping plants detoxify ROS and prevent oxidative damage at the cellular level (Ibrahim et al., 2018). GRs maintain the pool of reduced glutathione (GSH) by converting oxidized glutathione (GSSG) back to its reduced form (GSH) (Csiszar et al., 2016). This regeneration of GSH is crucial because GSH acts as a powerful antioxidant that can directly scavenge ROS or serve as a cofactor for other antioxidant enzymes (Gill et al.,

2013). It has been reported that citrus plants typically upregulate GR activity and expression to ensure reduced glutathione (GSH) regeneration and enhance their antioxidant defense system (Neill et al., 2002). The specific response may vary among citrus genotypes and depends on the duration and severity of the stress. Under salinity stress, citrus plants often respond by increasing the activity of GR. Peroxidase helps maintain cell membrane integrity by reducing lipid peroxidation and preventing cell membrane damage by triggering signaling mechanisms that adjust peroxidase activity in the leaves as a part of a systemic stress response (Farooq et al., 2009). As the salinity stress level increased to 60 mM NaCl, CM, C47 and X9 had significantly greater SOD and CAT activity in PS than those on JK N2 and N4. The upregulated and down-regulated of these activities suggested that PS grafted on various genotypes responded differently to varying levels of salinity stress, with CM exhibiting the most robust defense mechanism. Our findings are in line with the findings of Tsabarducas et al. (2015), who observed a similar effect on the salt stress of *Citrus limon*, with higher SOD activity as a means of better coping ability. The impact of salinity on ascorbate peroxidase (APX) activity in PS grafted on different citrus genotypes under NaCl stress can be explained by the fact that genotypes experiencing greater oxidative stress may require more significant APX activity to maintain the cellular redox balance, as suggested by Snoussi et al. (2022). This increased APX activity helps to detoxify excess H<sub>2</sub>O<sub>2</sub> generated under salinity stress, reducing oxidative damage to plant cells. Our results are consistent with those of Seday et al. (2014), who studied the impact of 0, 45, 90 and 135 mM NaCl on Carrizo citrange, Cleopatra mandarin, Volkamer lemon, sour orange, rough lemon and trifoliata orange and reported that APX increased with increasing salt levels. Tanou et al. (2009) reported the upregulation of glutathione reductase in sour orange under 150 mM NaCl stress. This finding of the study thus clearly suggests that the antioxidant systems of these rootstocks are crucial for their adaptation mechanisms, functioning independently of the toxic ion accumulation in PS leaves. Essentially, the rootstocks enhance the scion's antioxidant activities, demonstrating their ability to induce greater antioxidant responses.

Vitamin E is a fat-soluble antioxidant that protects cells from oxidative stress (Fryer 1992). PS grafted on different citrus genotypes had higher vitamin E contents in CM, X9 and N1. The lowest values were noted in N2 JK and TC. Since vitamin E is an antioxidant, enhanced vitamin E in the tolerant genotypes suggests its role in nullifying ROS free radicals. Kostopoulou et al. (2014) reported its depletion under high levels of salinity stress. The reduced vitamin E in the susceptible genotypes may be attributed to the imbalance in the production of ROS and the plant's ability to detoxify them. Jabar and Ethbeab (2023) studied the response of *citrus limonia* to graded levels of salinity stress and reported a similar effect. The maximum vitamin C content at the highest level of salinity stress, i.e., 60 mM NaCl stress, was detected in CM (19.62 mg), followed by C47 (17.63 mg) and X9 (17.58 mg). Conversely, the lowest levels were observed in N2 (9.53 mg), followed by N5 (10.56 mg) and C12 (10.58 mg). The reduced ascorbic acid in these genotypes resulted from the disruption of normal metabolic processes in plants. Kostopoulou et al. (2014) reported that ascorbic acid plays a vital role in the salt adaptation response of *Citrus aurantium*. When faced with stress, plants might divert their resources from synthesizing vitamin C to prioritize stress responses and the generation of osmoprotectants such as proline and glycine betaine. These osmoprotectants assist plants in managing salt stress (Singh et al., 2022). Our study followed that of Naz et al. (2022), who reported an increase in the level of vitamin C with increasing salinity in tolerant genotypes of other crops. Different osmolytes, such as sugars, proline, glycine betaine, and phenols, are responsible for maintaining membrane integrity, osmotic adjustment, alleviating ion toxicity and scavenging ROS under NaCl stress (Arif et al., 2020). The CM rootstock had the greatest total soluble sugar content in the leaf tissue of the PS treatment, followed closely by the C47 and N3 treatments, and the lower total sugar content in the TC treatment suggested a differential genotypic response of the rootstocks. The

differences in sugar accumulation observed among various rootstocks indicate their varying abilities to cope with stress and maintain metabolic processes (Rosa et al., 2009). Howie and Lloyd (1989) recorded a similar response to saline irrigation in Washington Navel Oanges, in which the soluble sugar content was consistently lower in the leaves of trees irrigated with high-salinity water, indicating that production rather than the utilization of carbohydrates is the major criterion that limits citrus productivity under saline conditions. A greater proline concentration was detected in the scion leaves of PS on CM, C47 and X9 under 60 mM NaCl stress conditions. Conversely, C12 had the lowest proline content, followed by JK and TC. The proline content in tolerant genotypes must increase to mitigate the damaging effect of salinity stress by maintaining the cellular osmotic potential, which acts as an antioxidant and ROS scavenger, thereby protecting citrus scion plants from oxidation (Khoshbakht et al., 2018). Proline can stabilize enzymes and cellular structures, which can be disrupted by high salt concentrations. This stabilizing effect helps citrus scion plants maintain metabolic processes even under high salt stress (Kishor et al., 2005). The mechanism behind increased level of proline synthesis grafted on tolerant rootstock is through overexpression of the *D-1-pyrroline-5-carboxylate synthetase* (*P5CS*) gene (Snoussi et al., 2022). Our results align with the findings of Yang and Guo (2018), who reported enhanced proline concentrations in roots and leaves with increasing salinity stress. It was clear that the grafting of PS onto the CM genotype led to a substantial increase in the mean total soluble protein content, followed by C47 and X9. These findings suggest that this combination is highly effective at coping with salt stress and maintaining protein levels due to enhanced metabolic activities. Conversely, the lowest protein levels recorded in N5, JK and N4 may be attributed to damage in the protein synthesis mechanism due to zinc deficiency, which correlates well with our data showing considerably lower levels of Zn in these genotypes. Hasegawa et al. (2000) reported that ROS can damage or denature proteins, affecting their functionality. This study correlates well with the findings of Sharma et al. (2011), who reported a similar effect in the salt-sensitive citrus rootstock Karna khatta. Phenol plays a role in scavenging and protecting ROS from oxidative stress caused by salt-induced damage (Mahmoud et al., 2021). At the highest 60 mM NaCl stress level, enhanced phenol accumulation was noted in the scion cultivar PS grafted on CM. In contrast, N4 had the lowest total phenol content. This explains the upregulation of the *P5CS* gene, which catalyzes the rate-limiting step in proline biosynthesis from glutamate which helps stabilize proteins and membranes, acts as an osmoprotectant, and scavenges reactive oxygen species ROS, thus contributing towards salinity stress tolerance (Snoussi et al., 2022). Hussain et al. (2012) reported the upregulation of proline in Cleopatra mandarin after 85 days of salinity treatment.

#### GC-MS

Numerous studies in citrus plants have underscored the genotypic or rootstock-dependent nature of citrus plants (Vives-Peris et al., 2023), which are affected by sodium chloride (Shankar et al., 2023) due to ionic and osmotic stress (Alam et al., 2020). In a recent study on the morphophysiological and photosynthetic responses of eleven citrus genotypes grafted with the sweet orange cultivar Pusa Sharad to understand sodium chloride stress tolerance (Shankar et al., 2023), Pusa Sharad grafted on CM, X9 and C47 was more tolerant than C12, JK, and TC at 30 mM and 60 mM sodium chloride stress, with improved transpiration and photosynthetic rates (Shankar et al., 2023), and Pusa Sharad adapted to environmental stresses through metabolic modifications (Ziogas et al., 2015). The accumulation of sugar alcohols such as myo-inositol and xylitol triggered, while a decrease in D-mannose levels has been reported in response to salinity stress by Gomez-Gonzalez et al. (2010). Organic acids serve as crucial osmolytes within plant vacuoles, and managing their metabolic processes is pivotal for conferring resilience to salt-induced stress in plants (Guo et al., 2022). Notably, alterations in

the abundance of sugars, sugar alcohols, organic acids, and intermediates of the tricarboxylic acid (TCA) cycle were detected in the leaf extracts of plants treated with different combinations of citrus rootstock scions. The findings of the study elucidate the negative impact of salt stress on glycolysis, as evidenced by the elevated levels of D-galactose, D-glucose, myo-inositol, and trehalose in the scion cultivar Pusa Sharad grafted onto X9, C47, and CM, in comparison to JK, TC, and C12, at both 30 and 60 mM NaCl stress levels in contrast to those in the control group. Myo-inositol, a sugar alcohol, has been proposed to enhance salt tolerance by preserving osmotic balance and safeguarding cellular structures against damage from reactive oxygen species (Sengupta et al., 2012). Our findings align with the observations of Kumari and Parida (2018), who reported the upregulation of xylose, glucose, galactose, rhamnose, glycerol, and myo-inositol in *Salvadora persica* leaves under conditions of elevated salinity. The metabolites gamma valerolactone, raffinose, prolamine, and scopolin in PS on JK, as well as stearic acid, gamma lactone, malonic acid, pentanoic acid, and D-glucose in PS on C12, exhibited downregulation with increasing levels of NaCl stress. Under 60 mM NaCl stress, specific metabolites involved in the TCA cycle, such as fumarate and citrate, resulted in the grafting of PS to X9 and CM. Glycolysis, the TCA cycle, and the mitochondrial chain yield the vital ATP NADH, which is crucial for energy and physiology (Fernie et al., 2004). PS grafted onto CM exhibited a significant accumulation of glycolytic intermediates under the same NaCl stress conditions. This finding aligns with that of Melgarejo et al. (2022), who identified 19 metabolites (5 organic acids, three sugars, two metabolites and nine amino acids) in the leaves of the lemon cultivar Verna on three different rootstocks and culture media. These metabolites are often key intermediaries in various metabolic pathways. The response of PS grafted onto X9 to palmitic acid, hexahydrobenzene, pentanoic acid, and piperazine in C47; acetic acid in N1; 2,4-pentadienonitrile, acetic acid, and raffinose in N3; and hexahydrobenzene, D-mannose, pentanoic acid, nonanoic acid, gamma-valerolactone, raffinose, tetrahydrofurfuryl, and sulforus acid in CM increased with increasing levels of salinity stress. These findings indicate that when Pusa Sharad is grafted onto X9, C47, and N1, it mitigates the impact of salt stress by reconfiguring the fatty acid composition. Under salt stress conditions, there is a usual disruption in the fatty acids of the plasma membrane, which is crucial for maintaining membrane integrity and ion balance (Lopez-Perez et al., 2009). Increase in oxalic acid levels in PS grafted onto TC under 60 mM NaCl stress. Oxalic acid plays a role in defense mechanisms. The levels of metabolites such as gamma-valerolactone (GVL), raffinose, prolamine, and scopolin in JK and stearic acid, gamma lactone, malonic acid, pentanoic acid, and D-glucose in C12 decreased with increasing levels of NaCl stress. Raffinose contributes to the tolerance of plants to drought stress, as evidenced by positive associations between the accumulation of raffinose, piperazine and scopolin in leaves and the occurrence of drought stress (Zhang et al., 2022; Siwinska et al., 2014; Downie et al., 2003). Most sugars were found at relatively high levels in PS grafted on X9, C47 and CM. These results agree with the findings of Killiny et al. (2017) in Sugar Belle mandarin on metabolite profiling of scion-rootstock under stress.

The increased resistance of PS grafted onto Cleopatra mandarin, CRH-47, and X-639 to address oxidative damage induced by sodium chloride stress has been observed (Arbona et al., 2008). Additionally, these graft combinations exhibit a distinctive adaptation in regulating transpiration rates. Our previous study (Shankar et al., 2023) showed increased tolerance of the above rootstocks compared to PS grafted onto CRH-12, Jatti Khatti and Troyer citrange. In addition, the ability of Cleopatra, CRH-47, and X-639 to deal with high sodium chloride concentrations could prevent further modification of their metabolism. In contrast, the heightened sensitivity of CRH-12, Jatti Khatti, and Troyer citrange necessitates profound modifications in both primary and secondary metabolism to address the physiological and biochemical imbalances induced by salt stress.

## Conclusions

Our study revealed that the levels of specific metabolites, including trehalose, raffinose, sucrose, D-galactose, myo-inositol, piperazine, acetic acid, malonic acid, palmitic acid, stearic acid, and pentanoic acid, increase under salinity stress. These metabolites could serve as markers for salinity stress in citrus plants. Conversely, hexahydrobenzene, gamma lactone, pyrrolidine, D-fructose, prolamine, oxalic acid, fumaric acid, D-arabinonic acid, and jasmonoyl-(S)-glutamic acid decreased under both 30 and 60 mM NaCl stress in the scion cultivars compared to those in the control. This study highlights the significant negative impact of salt stress on glycolysis, as evidenced by elevated levels of D-galactose, D-glucose, myo-inositol, and trehalose in the scion cultivar Pusa Sharad grafted onto the X9, C47, and CM rootstocks compared to those in the JK, TC, and C12 rootstocks under both 30 and 60 mM NaCl stress. The metabolite profiles indicate the roles of trehalose, raffinose, sucrose, D-galactose, myo-inositol, piperazine, acetic acid, malonic acid, palmitic acid, stearic acid, and pentanoic acid in metabolic adjustments to varying levels of NaCl stress. The increased concentrations of essential sugars, sugar alcohols, and fatty acids observed in Pusa Sharad when grafted onto the X9, CM, C47, and N1 rootstocks, as opposed to those in the JK, C12, TC, and N5 rootstocks, suggest an enhanced accumulation of these metabolites. Developing new chemicals which could upregulates reported secondary metabolites in our finding under varying salt conditions in different citrus scion-rootstock combination that imparts tolerance will have significant industrial application. This accumulation is crucial for maintaining osmotic and ionic balance in response to NaCl treatment and could also serve as a building block for osmoprotectants. Understanding these salt tolerance mechanisms in citrus scions grafted onto various salt-sensitive, moderate, and salt-tolerant rootstocks paves way for future studies and aid in screening different citrus scion-rootstock combinations. Further application of nanomaterials can be explored for studying the upregulation of metabolites in the study which imparts tolerance in citrus scion cultivars under salinity stress.

## CRediT authorship contribution statement

**Kripa Shankar:** Writing – original draft, Investigation. **Om Prakash Awasthi:** Writing – original draft, Validation, Supervision, Resources, Data curation, Conceptualization. **Supradip Saha:** Software, Formal analysis, Conceptualization. **Jai Prakash:** Writing – review & editing, Methodology. **Renu Pandey:** Validation, Resources. **Theivanai Murugan:** Formal analysis. **Aria Dolatabadian:** Writing – review & editing, Data analysis.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

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## Supplementary materials

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