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Stress causing dynamic changes of four phytohormones in tobacco and tomato: A GC-MS analysis

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ABSTRACT

Many analytical methods are in use to analyse plant hormones in different plants. Here this work provides a sensitive, accurate and quite accessible GC-MS (gas chromatography/mass spectrometry) method to quantify phytohormones indole-3-acetic acid (IAA), abscisic acid (ABA), jasmonic acid (JA) and salicylic acid (SA). These signaling molecules were analysed in two different plants, tomato and tobacco grown *in vitro*. The protocol designed to assess, how abiotic stress brings about changes in the level of endogenous hormones in the leaves of both the plants under study. A hormone profile of salt stressed leaves shows that different plant hormones are involved in diverse physiological processes. Crosstalk between these hormones result in synergetic or antagonic interactions which have important roles to play in abiotic stress response.

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INTRODUCTION

The Solanaceae include tobacco (*Nicotiana tabacum* L.) and tomato (*Solanum lycopersicum* L.). Tobacco and tomato are agriculturally important Solanaceae crops which are extensively studied [1-3]. Being sessile, plants as defense mechanism can rapidly modify in response to the changes in their surroundings such as drought, flood, salinity, shading or low/high temperature. Such stresses often found to be the major causes to affect plant production. Salinity stress is one of such leading causes for crop losses in the field. In order to give impetus to the stress-response-mechanism in tobacco and tomato, it is essential to understand their response to different abiotic stresses at multiple levels.

Flexibility in the growth patterns of plants is partly achieved by the action of phytohormones. They together form a signaling network which regulates plant response to different abiotic as well as biotic stresses. Several reviews in this regard add to the recent knowledge of hormonal cross-talk responsible for plant stress responses [4-6]. Plant hormones are the signaling chemicals which control almost all aspects of plant life.

Plants have successfully evolved through their developmental processes to face the challenges of environmental cues. Several hormones play major roles in abiotic (Figure 1) and biotic stress

responses, where ABA is the key player. This hormone has a major role in stress signaling causing an immediate response like hydraulic signal that triggers ABA biosynthesis in the system [7]. Phytohormones can mediate a wide range of plants' responses, from rapid (e.g. stomata closure) to long term adaptations by modulating the programs of plant growth and development. Cytokinins and auxins are predominantly positive regulators of cell division and growth. Abscisic acid as a growth inhibitor, acts in stress conditions like drought.

Plant hormones have an important role in the response mechanism against abiotic stress [8]. Stress ultimately may result into retarded growth thus the plant can focus its resources on combating the stress [9]. In nature, plants fight against stresses by modulating various physiological, biochemical and molecular actions. These counter-actions lead to alterations in gene expression, regulation of biogenesis, changes of cellular metabolite levels and changes in ion homeostasis. Regulation of gene at the transcription level is one of the major control points in biological system. These growth-regulators and transcription-factors are the key players in this process [10]. ABA as a stress-responsive signaling molecule is the most well studied hormone in the past decade. In the recent past, a lot of research has been done in the field of elucidation of the core ABA-signaling pathway and proper identification of ABA receptors.

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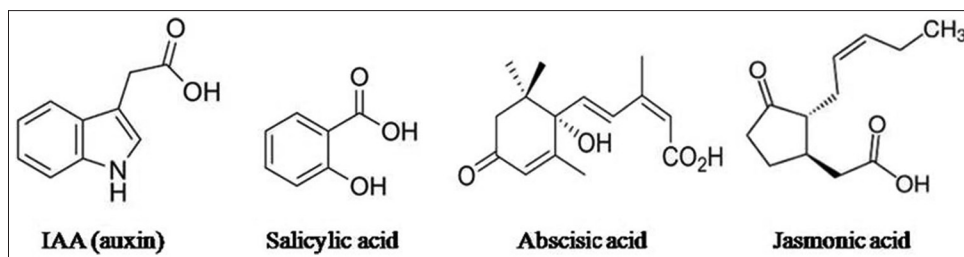


Figure 1: Chemical structures of important plant hormones involved in abiotic stress response and tolerance



Figure 2: Tobacco and tomato plants in laboratory condition

For example, less water availability is first confronted by the plant roots which results in stomatal closure of leaf and thereby resulting into reduced transpiration to a great extent by the action of the stress hormone ABA [11]. Many recent experiments on plant hormones have shown that some hormones (such as ABA, auxins, cytokinins, SA, JAs, brassinosteroids etc.) have the potential to elevate the abiotic stress tolerance in various plant species [12]. Such hormonal responses are fundamental to the plant growth and development. In addition to their regulatory functions in development they have also key roles to play in coordinating different signal transduction pathways in environmental stress responses [13].

The analysis of plants/mutants with an altered phytohormone profile has uncovered a high degree of interactions between auxins and cytokinins, abscisic acid and ethylene [14], jasmonates and ethylene [15], brassinolides and jasmonates [16], auxins and ethylene [17, 18], ethylene and cytokinin [19] or gibberellins and auxin [20].

Generally, the use of mass spectrometry (MS) is often coupled with an appropriate separation method like HPLC or GC [21, 22]. Both the techniques are powerful enough to detect trace amounts of organic compounds even at the level of picogram (10^{-12} g) or femtogram (10^{-15} g).

This study describes a simple method with extensive potential applicability. An objective of GC-MS based analysis of biochemical signals is to uncover the complex interaction and

intensive crosstalk between ABA, IAA, SA and JA during salinity stress that can modulate the levels of these growth factors in the aerial parts of plants.

MATERIALS AND METHODS

Plants and Growth Condition

Two plant species (Figure 2) were used for the experiment. Tobacco (*Nicotiana tabacum*) cv SR1, Tomato (*Solanum lycopersicum*) cv Punjab Keshari. Tomato, and tobacco seeds were surface sterilized with 0.1% (w/v) HgCl_2 for 10 minutes then rinsed thoroughly and imbibed in sterile water for 6 to 8 hours and finally spread over sterile wet blotting papers in petriplates. Plates were kept in dark at 26°C. Seeds were germinated in aseptic condition in the tissue culture room within 3 to 7 days. Seedlings were transferred in 0.25X Murashige & Skoog [23] liquid medium (Hi-media) and grown for another 15 days (16 h dark and 8 h light period, 25-26°C). The 15 day old plantlets were cultured in 0.5X Murashige & Skoog liquid medium with or without salt (150mM NaCl solution) and kept on observation for a month. The media were replaced weekly.

Harvest and Extraction of Plant Material

We evaluated four growth factors in terms of reproducibility, carryover and linearity. Our attempt was to develop a simple but near accurate method as much as possible for the detection of selected non-polar compounds in leaf tissues of two different plants. To achieve the goal, we adopted the extraction procedure routinely used in our lab (Muller's method with modification) [21] for GC-MS analysis which has been found reliable method for the simultaneous monitoring of different plant hormones during physiological processes.

For the experiment, 200mg of leaf tissue were harvested from 30 days' culture of two plant species in Murashige and Skoog medium. Healthy leaf tissues of plants were cut off with a scissor which were found of typical stature and absolutely free from any signs of senescence and were immediately weighed on a Sartorius weighing tool. The desired parts immediately were ground in porcelain mortar-pestle using solvent (hot methanol). After that the homogenized tissue was incubated in the solvent for 1 hour at room temperature and the resulting fluid centrifuged at 10,000 rpm to remove cell debris. After centrifugation, the debris-free supernatant was decanted and transferred into an eppendorf tube. 1ml of ethylacetate was

added in each tube. All samples were analysed immediately to prevent any degradation of facile phytohormones.

All samples were performed in triplicate. We always determine the optimum level of standard by a preliminary analysis of representative samples in a first run before the actual experiment for quantitation. Four standard solutions were prepared.

Stock solutions as standard of original phytohormones were prepared at 1 mg/ml in methanol. For calibration and comparison, working solutions of standards were prepared diluting stock solutions in methanol: water (7:3), at different concentration for each phytohormone depending on the range of the calibration curve.

IAA and ABA (100µg/ml), SA and JA (200µg/ml).

Gas chromatography – mass spectrometry

The compounds under study were determined using Mass spectrometer (Model: POLARIS Q; Serial no: MS 211912) coupled with trace GC ultra gas chromatograph (Thermo Fisher Scientific India Pvt. Ltd., Model: Trace GC Ultra 320080111). A DB-5MS column (30 m x 0.25 mm ID x 0.25 µm film thicknesses with stationary phase 5% Phenyl polysilphenylene siloxane was used. Helium gas of 99.999% purity was used as a carrier gas at a flow rate of 1 ml/min with a linear velocity of 10ml/s. 1 µl extracted sample was injected with autosampler (Model no: AI3000) into the column in a split mode. Initially the temperature of GC oven was programmed at 50°C with a hold time of 1 min and gradually raised to 300°C at the rate of rising temperature 80°C/min with a hold time of 5 min and was finally raised to 320°C at the rate of rising temperature 10°C/min with a hold time of 10 min. The MS (mass spectra) was carried out in the electron impact mode (EI) at 70 eV. Keeping the temperature at 250°C, the detector was set at 40-600 D. Mass spectrum of GC-MS was interpreted using the database of National Institute Standard and Technology (NIST) harbouring 1,50,000 patterns. With the aid of database information and the data store software XCALIBUR, the name of the compound of the experimental materials, molecular weight and structure were determined.

Validation of the method

Standard solutions of all four selected compounds were run before analysis of each sample to assess the linearity of the profiling method. A standard solution containing a mixture of JA, IAA, SA and ABA (Sigma-Aldrich, USA) was prepared. The

calibration graph was obtained in the concentration range of the standard phytohormones (Table. 1).

For reproducibility, all samples were run on the same day because sample preparation procedure and instrumental analysis can contribute to the variability of the method adopted.

For recovery, the chemical stability of the metabolites/derivatives and the extraction method were assessed. In this recovery test, double amounts of standard compounds were mixed in the leaf-extract at the initial stage of the extraction procedure. Both tissue mixes and unspiked extracts from the same test samples were then run to compare and calculate the recovery rates. The eluted compounds were characterized on the basis of their molecular formula, structure, retention time, and percent relative peak area. Processed data were subjected to statistical analysis (the term significant is used only when $p < 0.05$ according to the t-test embedded in Microsoft Office Excel).

RESULTS AND DISCUSSION

GC-MS based metabolite study is still considered the most versatile platform with many advantages over other analytical methods in respect to high separation power and reproducibility [12,13]. The method adopted here to detect the most abundant phytohormones in leaf sample of tobacco and tomato. Several studies have shown that between different phytohormones, there are synergistic as well as antagonistic actions in plants [24].

Enough reports are available authenticating that in the regulation process of plant growth and development, there are signaling crosstalks between several plant hormones besides their individual actions.

Hormonal changes in response to salt stress

Based on GC-MS results (Figures 3 and 4), salt stress has been found to have elevated levels of IAA, ABA, SA and JA in leaves of tobacco and tomato compared to the control. Endogenous level of all signaling molecules under study was found higher in tomato than in tobacco.

ABA is a well documented phytohormone for stress responses. Its signaling pathway is the core of salt stress response in plant system. ABA-mediated signaling has a vital role to play in plant responses to different adverse environmental conditions (abiotic constraints) and also biotic stresses like plant pathogens [25]. producing more ABA than tomato under stress (Figures 3-5).

Table 1: Fragmented parameters and obtained values in GC-MS method for quantification of phytohormones

Analyte	Standard concentration (ng/ml) (n=3)		Tobacco (ng/g FW) (n=3)		T omato (ng/g FW) (n=3)		Retention time (min)	ion m/z
	Range of conc	Linearity	Control	Treated	Control	Treated		
IAA	1-50	0.9887	3	2.1	15.2	18	2.04	141
SA	1-50	0.9878	8.8	25.5	101	138	5.45	120
ABA	5-100	0.9954	80	150	421	440.4	12.06	200
JA	5-100	0.9988	3	5.7	25	28	22.71	183

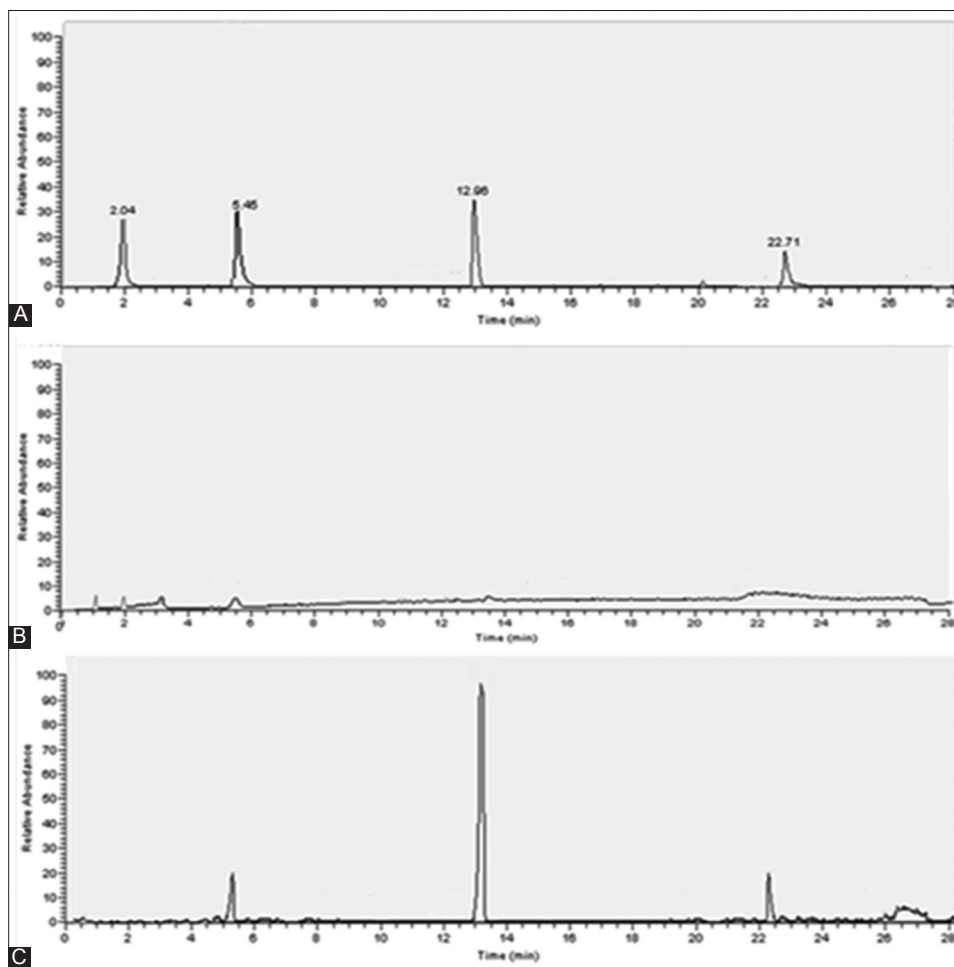


Figure 3: Representative GC chromatograms of selected plant hormones. This study with leaf sample shows that tobacco is detection and quantification of plant hormones by using GC-MS. A) Standards of phytohormones showing the peaks of IAA (indole acetic acid), ABA (abscisic acid), SA (salicylic acid) and JA (jasmonic acid); B) ethyl acetate extract of untreated leaf sample of tobacco; C) ethyl acetate extract of 150 mM NaCl treated leaf sample of tobacco displaying the chromatographic peaks in the range as compared with the standard.

The result may be because tomato is moderately salt tolerant. Previous study [26] with wheat also showed lesser enhancement of ABA under stress. So in different plant species ABA synthesis and catabolism are regulated differently. ABA is also produced in the roots where the plant may be under stress. ABA is then translocated from roots to the aerial parts/leaves and there it rapidly alters the osmotic potential of the guard cells of the stomata, resulting into stomatal shrinkage and closure. The rate of transpiration is reduced by ABA-induced stomatal closure. This phenomenon prevents further water loss from the leaves at the time of water scarcity [27]. As also described by He and Cramer [28], salt withstanding plants produce low amount of ABA than sensitive ones and can perform normally during moderate salinity [29].

IAA level of occurrence in our findings is of quite variable in nature (Figure 5). IAA is known to be involved in response to salinity in crop plants. According to the GC-MS result (Figures 3 and 4) NaCl treatments resulted in a marginal increase of IAA in tomato but decreased in tobacco. The difference between two plant species can be perceived by noting the ability of the plants in resisting the rise of IAA level in leaves under salinity [30], and a higher level of IAA also has been correlated

with retarded growth [31]. A study shows 75% reduction in IAA level in tomato while under salinity stress [32]. A report suggests significant reduction of this regulator in crop plants such as rice and tomato [33]. Another report shows that salinity caused reduction in IAA levels in maize plants but SA application could enhance them effectively [34]. According to an another study, elevation of IAA (auxin) level by overexpression of auxin biosynthetic related YUCCA3 caused hypersensitivity to salt stress [35]. Several investigations have shown that auxins have the ability to increase stress tolerance in various plant species.

SA is considered as the stress hormone and also has been recognised as a contributor to improved plant abiotic stress tolerance [36]. The result demonstrates (Figure 5) that SA increased 3-fold in tobacco whereas tomato shows 1.5-fold enhancement. So under stress, elevation is lesser in tomato in comparison to tobacco leaves. SA has been identified for its involvement in plant response to abiotic stress (e.g. drought, salinity cold, and heat) [37,38]. Previous study [39] has shown that several proteins were induced by SA in cucumber. The expression of these proteins were found to be involved in cell defence, carbohydrate metabolism, photosynthesis,

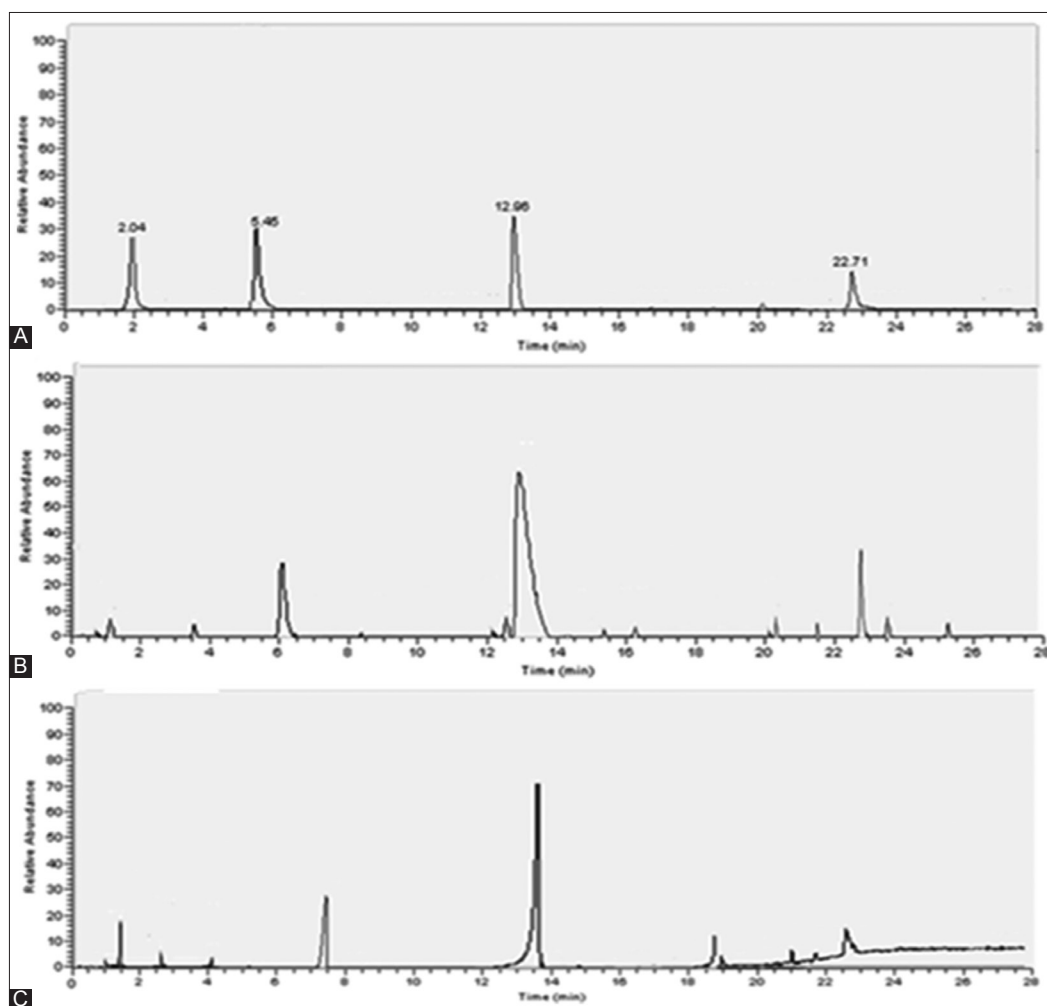


Figure 4: Representative GC chromatograms of selected plant hormones. Detection and quantification of plant hormones by using GC-MS. A) Standards of phytohormones showing the peaks of IAA (indole acetic acid), ABA (abscisic acid), SA(salicylic acid) and JA (jasmonic acid); B) ethyl acetate extract of untreated leaf sample of tomato; C) ethyl acetate extract of 150 mM NaCl treated leaf sample of tobacco displaying the chromatographic peaks in the range as compared with the standard.

antioxidative reactions, respiration and energy homeostasis, protein folding and biogenesis. Alteration of SA in two species under study indicates that tomato here is being less affected by the stress imposed. Endogenous level of SA increased significantly in tobacco. SA in general, involved in the process of defence mechanism [40]. A report suggests that endogenous SA increased salt stress tolerance in wheat seedlings [41]. SA induction could ameliorate abiotic stress such as water deficit on cell membrane by upregulating ABA and proline [42].

JAs have major function in the abiotic and biotic stresses as well as [43,44]. This plant growth regulation includes senescence, reduced growth, tendril coiling, flower development and leaf abscission. Based on our GC-MS analysis (Figures 1 and 2), it was noted that JA level was significantly increased, reaching to more than 1.5-fold in salt treated tobacco compared to the control (Figures 3 and 5). Here in treated tomato leaves, no significant elevation of JA was detected (Figures 4 and 5). The results indicate that abiotic stress like salinity differentially modulates the endogenous level of JA. There are sporadic reports of evaluation that plant's responses under stress in this regard [45].

Comparatively lesser is known about JA's role in abiotic stresses in relation to biotic stress. According to a study done, JA signaling is also active in the response to different abiotic stresses [46]. JA signaling research performed in wheat plant suggests that JA has an important role in getting rid of potentially harmful ROSs [47-49]. Zhao *et al.* [50] demonstrated salinity tolerance in wheat via the JA signaling pathway. Other studies demonstrated that jasmonate levels were enhanced under stresses [51,52]. A study on rice brought out that, both low availability of water and high salinity stresses resulted in an induced and enhanced jasmonate levels in the leaves and roots [53,54]. Another research in rice reported overexpression of OsJAZ6 improved salt and mannitol stresses [55]. Ismail *et al.* [45] suggested that salt stress response may be modulated by jasmonate. The present study is in accordance with several other experiments cites that JA can differentially regulate the plants' responses and adaptation to various kinds of abiotic stresses.

Cross-talk of IAA, SA, ABA and JA

The plant hormones such as auxins, cytokinins (CKs), gibberellins (GAs), abscisic acid (ABA), salicylic acid (SA), jasmonic acid (JA),

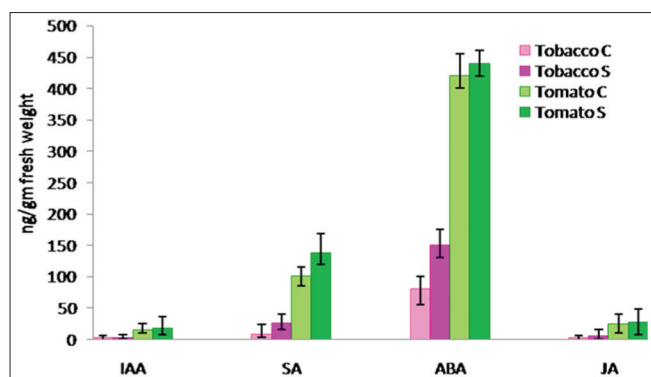


Figure 5: Salt stress alters the hormonal balance of leaves. Comparison of the phytohormones in control(C) and stressed(S) leaf tissue. The abundance of the endogenous phytohormones IAA, ABA, JA and SA. Determination of the level of phytohormones present in the control and salt-stressed tissue of Tobacco and Tomato, demonstrates that GC-MS method is highly helpful in obtaining the dynamic range of phytohormones with small amounts of starting material (200 mg). Values are means \pm SD ($n=3$ independent samples).

ethylene (ET), and brassinosteroids (BRs) respond to diverse stresses through synergistic and antagonistic ways. This interaction between various phytohormones often referred to as signaling cross talk. In fact, the response mechanism to different stresses not solely restricted to these hormones only. Recent researches provide clues that the interactions of these hormones with each other and with other hormones are increasingly more complex than previously thought in various crop species. In plants, abiotic stress response regulated by phytohormone-signaling depends upon several factors like stress type, duration of exposure and intensity. ABA is known to be occupying the key position as a hormone regulator in stresses. The cross-talk between SA and JA was initially observed in tomato responding to wound [56]. SA, JA take active roles in plant abiotic stress tolerance, whereas IAAs are involved in stress tolerance of both kinds [57, 58, 38]. SA and JA are biochemically linked signaling molecules. Their induction can be triggered by stresses and become integral part of plant defence responses [59]. JA, SA also interact with ABA to trigger stomatal closure and thereby restricting water loss during osmotic stresses [60, 61, 62]. In an experiment on Arabidopsis, Miao and Zentgraf [63] demonstrated that two pathways of SA and JA interact during senescence and regulate a senescence-responsive TF, WRKY53 antagonistically. A previous research found that in spite of being antagonistic, both SA and JA signaling pathways simultaneously become active in certain conditions [64]. Our findings propose that in the process of stress responses, phytohormone signaling is critical for homeostasis and for maintaining a fine balance between ABA, IAA, SA and JA. In this experiment tomato seems more efficient in keeping hormonal balance in the shoot parts. On the contrary tobacco responds with higher alteration in the hormonal accumulation in leaves against salinity stress.

CONCLUSIONS

With the great advantage of sensitivity and simplicity of the powerful tool GC-MS, the individual hormones IAA, ABA, JA or SA targeted here for quantitative measurement in two different

plant species. Differences in the accumulation of chemical signals (IAA, ABA, JA and SA) in tobacco and tomato, is possibly due to the differences in their ability to stress resistance. Though in minor amount but these molecules have significant roles in balancing plants behaviour under salinity stress. The same method should be applicable to a broad range of other regulating factors or metabolites of interest. The versatility of the technique is important. Moreover, analysis of altered levels of signaling molecules at different experimental conditions in our study is providing more precise insight into the dynamics of regulatory processes. Present study may be helpful in pronouncing the fate of selected phytohormones. It also may assist in evaluating the capacity of detoxification in various plant species. The underlying molecular mechanism of signaling networks between ABA and other hormones is complex and more comprehensive study is needed in future. In this direction, phytohormone engineering seems promising for plant biologists.

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