Is hormonal analysis a predictive tool for grafting success in tomato?

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ABSTRACT: Anatomical, physiological, and biochemical analyses have been performed to predict graft compatibility. We analyzed if the concentrations of auxins, jasmonic acid, gibberellins, 1-aminocyclopropane-1-carboxylic acid, zeatin (cytokinin), salicylic acid, and abscisic acid could be used as predictors of compatibility between the rootstocks FOX1 and FOX4 (resistant to *Fusarium* wilt) and the scion of cherry tomatoes Sweet Heaven (SH). Self-grafted (SH/SH) and ungrafted SH plants (SH) were used as controls. Hormonal analyses were performed on leaves, at 20 and 70 days after grafting (DAG), and roots, at 20 DAG. No expressive concentrations of auxins, gibberellins, or jasmonic acid were detected. The concentrations of 1-aminocyclopropane-1-carboxylic acid, the immediate precursor of ethylene, and zeatin were altered at 20 DAG, but they stabilized at 70 DAG. Salicylic acid levels were reduced in the leaves of grafted plants at 70 DAG. The concentration of abscisic acid (ABA) in the leaves of SH grafted onto FOX1 was higher than in ungrafted and self-grafted plants at 70 DAG, suggesting some degree of incompatibility between these genotypes. The concentration of ABA in the combination FOX4/SH was similar to that in the self-grafted plants. Abscisic acid might be used as a reference phytohormone to predict graft compatibility among tomato genotypes.

Key words: abscisic acid, compatibility, phytohormones, *Solanum lycopersicum*, stress.

Phytohormones are essential to the growth and development of plants, and they also modulate the responses to biotic and abiotic stresses. However, little is known about the role of phytohormones in plants exposed to visible and invisible stresses of grafting (Nanda and Melnyk 2018). Thus, studying how grafting affects the synthesis of phytohormones may assist in the identification of stresses that influence vegetative and / or reproductive development in the future. If phytohormone evaluation works as a predictive tool for grafting success, the cost and time for development of new graft and rootstock combinations would be reduced.

To assess the hypothesis that phytohormone concentrations in grafted plants might be a predictive factor of compatibility in tomato, we explored here two hybrids of *Solanum lycopersicum* developed by our research group, called FOX1 and FOX4. These hybrids are resistant to *Fusarium oxysporum* f. sp. *lycopersici* (FOL) races 1, 2 and 3. However, the productivity and fruit quality of these genotypes were unfavorable for field cultivation. Then, we decided to use them as rootstocks for the commercial cherry tomato cultivar Sweet Heaven (SH), which is susceptible to FOL. When the commercial cherry tomato cultivar SH was grafted onto FOX4, there was no reduction in fruit yield or quality. In contrast, grafting SH onto FOX1 reduced the quality of the fruits. Although no visible incompatibility was found between SH and FOX1, we strongly believe that the undesirable traits of fruits were due to some degree of incompatibility between these genotypes.

The cherry tomato hybrid SH (Sakata Seed Sudamerica®) was used as scion. The hybrids FOX1 and FOX4, resistant to the three races of FOL, were used as rootstocks. The treatments consisted of grafting SH onto FOX1 (FOX1/SH) or FOX4 (FOX4/SH); self-grafting of SH (SH/SH), and ungrafted SH plants (SH).

Thirty-day-old plants of SH, FOX1, and FOX4 were grafted by whip grafting and kept in a growth chamber for four days under dark and high relative humidity (> 90%). The grafted plants were grown in polypropylene trays filled with organomineral substrate (Carolina Soil®, Pardinho, SP, Brazil). For acclimatization, plants were gradually placed for longer periods in a greenhouse under sunlight and room temperature, until the point in which they remained the full day. At this moment, 15 days after grafting, plants had full turgidity. Fifty seedlings were used in the experiments.

The experiments were carried out in greenhouse and field conditions. In greenhouse, the treatments FOX1/SH, FOX4/SH, SH/SH, and SH were arranged in a completely randomized design with five replications. Each plot consisted of a plant. The plants were sampled 20 days after grafting (DAG). The regions sampled were the roots, and the second expanded leaf counted from the apex. Sampling was carried out by removing the seedlings from the tray and separating the aerial part from the roots with a transversal cut using a steel blade. Then, the second leaf counted was collected, and the root system was quickly washed in running water to remove the adhered substrate. Then, the samples were placed in 15-mL conical tubes immersed in liquid nitrogen (-196°C) and used for hormonal analysis.

For the field trial, the plants were transplanted to a greenhouse at 15 DAG. The soil of the greenhouse was not infested with *Fusarium oxysporum* f. sp. *lycopersici* and was corrected by a supply of lime and fertilizers based on Ribeiro et al. (1999). Plants were tutored with two stems up to 1.80 m and irrigated by dropping. The management of irrigation was based on the data collected by a meteorological station located in the greenhouse and the information provided by its software (Irriplus®, model E5000, Viçosa, MG, Brazil). The treatments were distributed in five randomized blocks, with six plants per plot. The four central plants were considered as useful plot. Central leaflets of the third expanded leaf from the apex were collected for hormonal analysis at 70 DAG. The third expanded leaf was chosen in an attempt to minimize the effect of the raindrops on the leaves and by representing the nutritional status of the plants.

Leaf and root hormones were extracted from the leaves and roots according to Müller and Munné-Bosch (2011), with modifications. Fresh tissue (110 mg) was ground in liquid nitrogen, and then extracted with 300 μL of methanol:isopropanol:acetic acid, 20:79:1 (v:v:v). The samples were vortexed for 20 seconds four times, ultrasounded for 5 min and kept on ice for 30 min. After centrifugation at 13,000 g for 10 min at 4°C, 250 μL of the supernatant was collected and transferred to a tube. The extraction process was repeated three times with the pellet. The supernatants were combined and centrifuged at 20,000 g for 5 min at 4°C.

We injected 5 μL of the obtained extract into the liquid chromatography with tandem mass spectrometry (LC-MS/MS) system (Agilent 1,200 Infinity Series, coupled with the triple quadrupole mass spectrometer – QqQ, model 6430 Agilent Technologies®). Chromatographic separation was performed using the Zorbax Eclipse Plus C18 column (1.8 μm, 2.1 × 50 mm, Agilent) in series with a 1.8 μm Zorbax SB-C18 guard column. The mobile phase consisted of: 0.02% acetic acid in water and 0.02% acetic acid in acetonitrile, in a time gradient/% B of 0/5; 11/60; 13/95; 17/95; 19/5; 5/20. The flow used was 0.3 mL / min at 23°C. The electrospray ionization source was used at gas temperature of 300°C, nitrogen flow of 10 L·min^{-1}, nebulizer pressure of 35 psi, and capillary voltage of 4,000 V.

The equipment was operated in the multiple reaction monitoring mode, in which the precursor/fragment ion masses were monitored by fragmentation tests of each molecule: cytokinin (zeatin) (220/136), ethylene via 1-carboxylic acid-1-aminocyclopropane (ACC) (102.1 / 56.2), abscisic acid (ABA) (263/153), auxins (indolacetic acid – AIA) (176/130), salicylic acid (SA) (137/93), gibberellins GA3 (345 / 142.9) and GA4 (331/21), jasmonates (JA) (209/59). Cytokinins, AIA and ACC were scanned in the positive mode, while ABA, SA, GA3, GA4, and JA were in the negative mode. A calibration curve (0.1 to 200 ng) was made to obtain the absolute quantification (Müller and Munné-Bosch 2011), using the standards for each hormone (Sigma-Aldrich). Data were submitted to analysis of variance ($p < 0.05$), and the means were compared by Tukey's test ($p < 0.05$) using the software R version 4.0.2.

The concentrations of ABA, SA, ACC, and zeatin in leaves and roots varied at 20 (Fig. 1) and 70 DAG (Fig. 2). Auxins, gibberellins, and jasmonic acid were not detected during the experiment (data not shown).
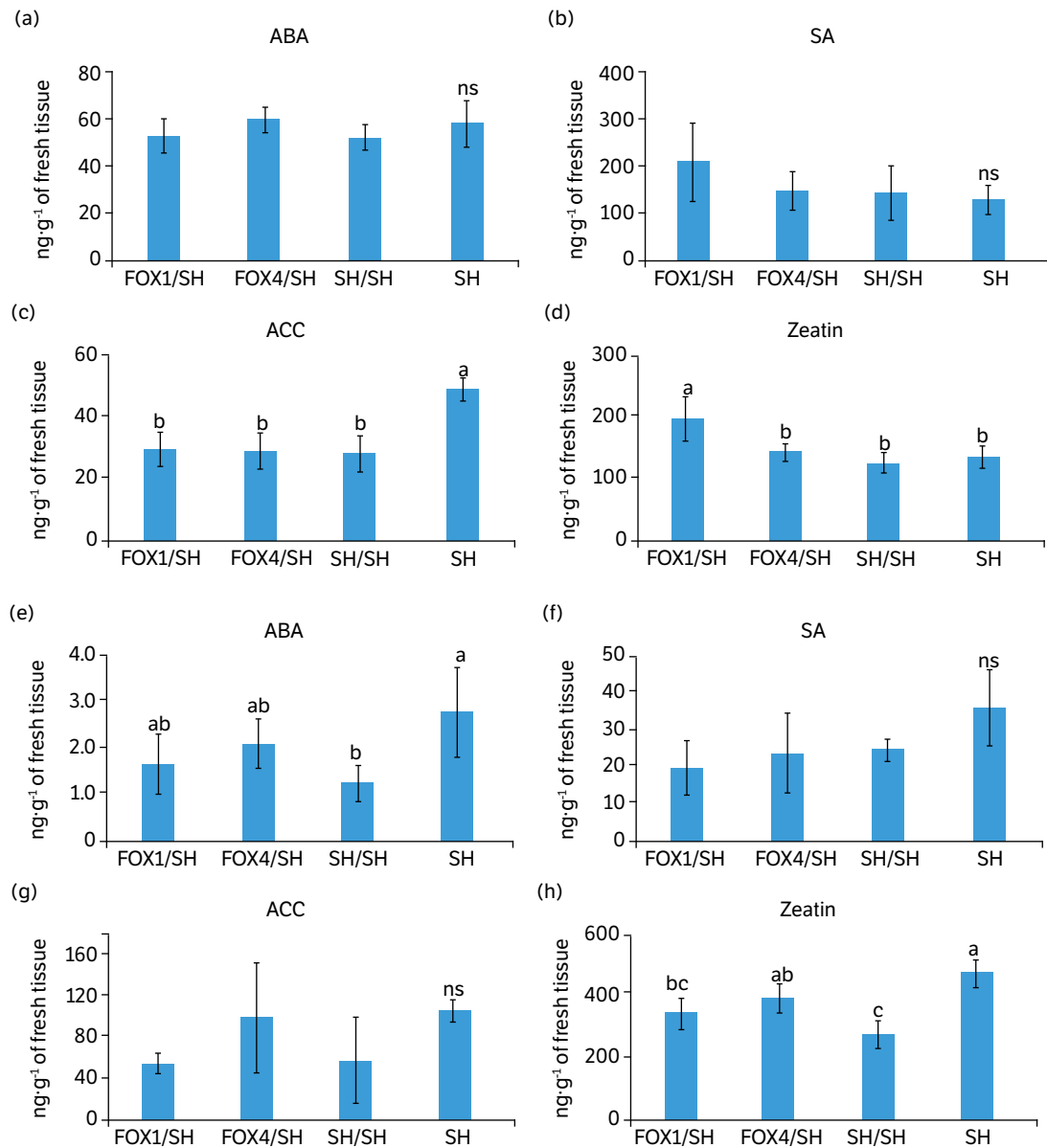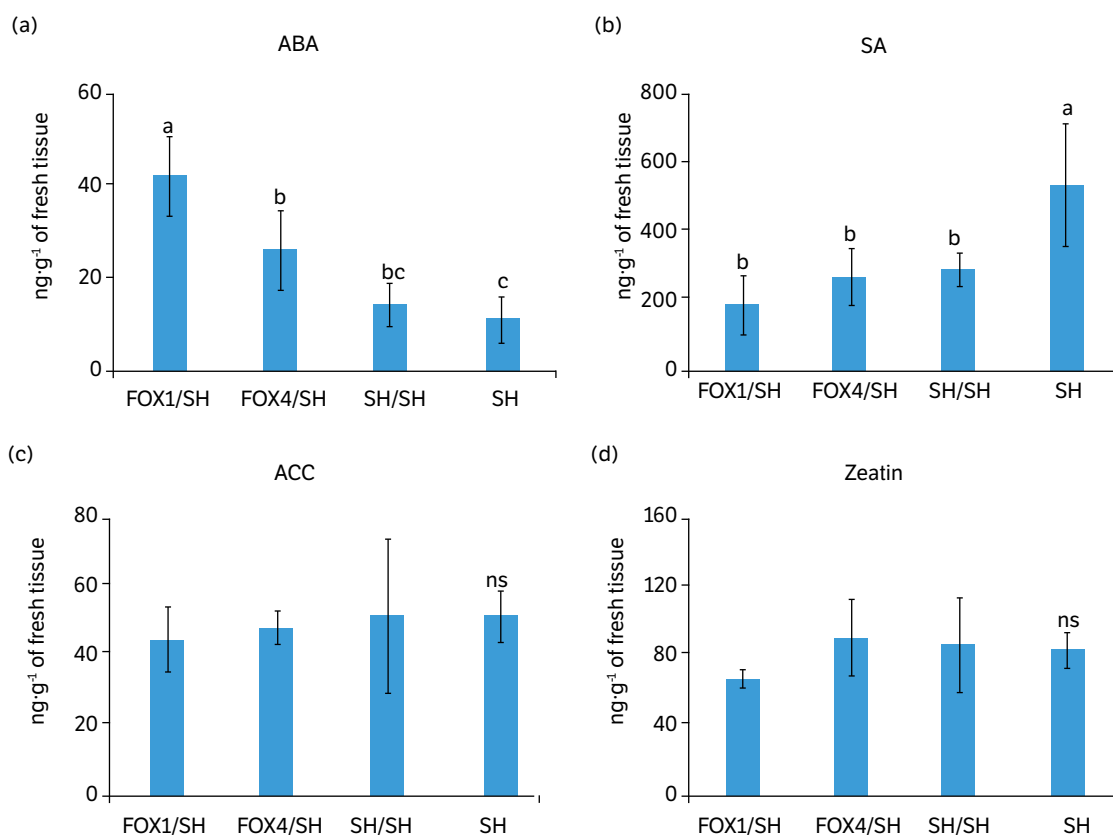
ABA: abscisic acid; SA: salicylic acid; ACC: 1-carboxylic acid-1-aminocyclopropane; SH: Sweet Heaven; ⁿˢnon-significant by F test (p < 0.05).

Figure 1. Concentrations of abscisic acid, salicylic acid, 1-carboxylic acid-1-aminocyclopropane, and zeatin in FOX1 (FOX1/SH), FOX 4 (FOX4/SH), and SH (self-grafted – SH/SH; ungrafted – SH) in (a-d) leaves and (e-h) roots of tomato at 20 days after grafting. Error bars represent standard deviation from the mean (n = 4). Coefficient of variation (%) = (a) 13.23, (b) 36.96, (c) 16.17, (d) 14.87, (e) 35.46, (f) 33.32, (g) 44.5, and (h) 12.82. Bars with equal letters within each graph do not differ statistically by Tukey's test (p < 0.05).

Foliar concentrations of ABA and SA were similar in all treatments at 20 DAG (Figs. 1a-1b), ranging from 52.6 to 60.8 ng of ABA per g of fresh tissue and from 129 to 211.4 ng of SA per g of fresh tissue of tomato. The levels of ACC were higher in leaves of ungrafted plants than in grafted plants at 20 DAG (Fig. 1c). Leaves of SH grafted onto FOX1 had higher concentrations of zeatin than those of the other treatments (Fig. 1d).

Low concentrations of ABA and SA were detected in the roots in all treatments at 20 DAG (> 40 ng·g⁻¹ of fresh tissue – Figs. 1e-1f). The concentrations of ABA in the roots of FOX1 and FOX4 were similar to those observed in the roots of SH (Fig. 1e). No treatment significantly influenced root concentrations of SA and ACC at 20 DAG (Figs. 1f-1g). The concentration of zeatin in roots of FOX4 was similar to those found in roots of ungrafted SH (Fig. 1h). However, the levels of this hormone were reduced in roots of FOX1 and self-grafted SH (Fig. 1h).

At 70 DAG, foliar concentrations of ABA were higher in plants grafted onto FOX1 (FOX1/SH) and FOX4 (FOX4/SH) in comparison to the ungrafted SH plants (Fig. 2a). The levels of this hormone in FOX1/SH leaves were higher than in those of self-grafted plants (SH/SH) (Fig. 2a). The concentration of SA in ungrafted plants was higher than in the other treatments (Fig. 2b). No significant difference on ACC and zeatin levels was observed between grafted and ungrafted plants at 70 DAG (Figs. 2c-2d).

ABA: abscisic acid; SA: salicylic acid; ACC: 1-carboxylic acid-1-aminocyclopropane; SH: Sweet Heaven; [ns]non-significant by F test ($p < 0.05$).

Figure 2. Leaf concentrations of (a) abscisic acid, (b) salicylic acid, (c)1-carboxylic acid-1-aminocyclopropane, and (d) zeatin in ungrafted (SH) and grafted tomato SH (self-grafted – SH/SH; grafted on FOX1 – FOX1/SH; grafted on FOX4 – FOX4/SH) at 70 days after grafting. Error bars represent standard deviation from the mean (n = 4). Coefficient of variation (%) = (a) 28.22, (b) 31.90, (c) 29.10, and (d) 24.45. Bars with equal letters within each graph do not differ statistically by Tukey's test ($p < 0.05$).

The grafting of SH onto FOX1 and FOX4 changes the concentrations of stress-related phytohormones, including ethylene, cytokinin, SA, and ABA. ACC is the immediate precursor of the ethylene, responsible for regulating various plant processes, including biotic and abiotic stress responses (Nanda and Melnyk 2018). ACC is transported from the roots to the aerial part, in which it is converted into ethylene, in the presence of oxygen (Lin et al. 2009). The injuries caused by cutting the stems trigger the synthesis and accumulation of ethylene around the grafting point (Yin et al. 2012). It is possible that the lower concentrations of ACC in the leaves of grafted plants at 20 DAG are due to an increase in ethylene synthesis and transport. However, ethylene concentration in the leaves tends to stabilize at 70 DAG. Probably, FOX1 and FOX4 metabolize the ACC to the point of overcoming the stress caused by the grafting.

The concentration of zeatin, a hormone in the group of cytokinins, varied in roots and leaves of grafted and ungrafted plants at 20 DAG. Cytokinins and auxin are involved in the formation of completely functional vascular connections between scion and rootstock (Nanda and Melnyk 2018). It explains why the highest concentrations of zeatin were observed at 20 DAG, especially in leaves of SH grafted on FOX1, with a consequent reduction in the levels of this hormone at 70 DAG.

Salicylic acid (SA) is a phenolic compound that modulates biological and metabolic responses of plants under attack by pathogens, acting as a signaling agent for systemic defense (Janda et al. 2014). In addition, SA can enhance photosynthesis

and attenuate water deficit. However, little is known about the role of SA in grafting, either in the process of vessel formation or in plants with the union already established (Nanda and Melnyk 2018). In general, the foliar concentration of SA increased after grafting, being higher in ungrafted than in grafted plants. However, it is not known yet how grafting can influence the synthesis of this hormone, and this aspect deserves further investigation.

Abscisic acid (ABA) is associated with plant responses to water stress (deficit or excess), acts on seed maturation and germination, regulation of stomatal opening and in response to biotic stresses (Vishwakarma et al. 2017). Similar foliar levels of ABA in all plant combinations at 20 DAG could suggest the absence of incompatibility between the materials. However, higher concentrations of ABA were observed in SH leaves grafted on FOX1 under more severe conditions in field at 70 DAG. Therefore, some type of stress has occurred in this case; possibly water stress, even with the availability of water supplied by irrigation. Water deficit causes rapid redistribution and accumulation of ABA in the most sensitive plant tissues. Then, this hormone triggers mechanisms that reduce water loss, activating stomatal closure and inhibiting leaf expansion (Wilkinson et al. 2012).

The availability of water for the different parts of the plant can be altered for reasons intrinsic to its root development, healing, vascularization of the grafting region, osmotic regulation, and transport (Nanda and Melnyk 2018). In our unpublished study, SH fruits had reductions of 14.3% in firmness, 1.2% in the pH of the pulp, and 6.75% in total soluble solids when FOX1 was used as a rootstock. Based on these findings and the results of the present study, we believe that there is some degree of incompatibility between the scion cultivar SH and the rootstock FOX1. In the case of the hybrid FOX4, there is no evidence of incompatibility, based on the ABA concentrations observed in this study, corroborating the fact that the productivity and quality of SH fruits grafted on FOX4 are similar to those of ungrafted plants in our unpublished study. Abscisic acid might be a reference phytohormone to be evaluated in grafted plants to verify compatibility between tomato materials. To validate this approach, further studies must be carried out, including different genotypes, grafting techniques, and field conditions.

AUTHORS' CONTRIBUTION

Conceptualization: Fernandes, R. H. and da Silva, D. J. H.; **Methodology:** Fernandes, R. H., Vital, C. E., Lopes, E. A. and da Silva, D. J. H.; **Investigation:** Fernandes R. H., Delazari, F. T. and Vital, C. E.; **Data curation:** Fernandes R. H. and da Silva D. J. H.; **Formal analysis:** Fernandes R. H. and da Silva D. J. H.; **Validation:** Fernandes R. H.; **Writing – Original Draft:** Fernandes, R. H., Lopes, E. A. and da Silva, D. J. H.; **Writing – Review and Editing:** Lopes, E. A. and da Silva, D. J. H.; **Funding Acquisition:** da Silva, D. J. H.; **Resources:** da Silva, D. J. H.; **Supervision:** da Silva, D. J. H.; **Project Administration:** da Silva, D. J. H..

DATA AVAILABILITY STATEMENT

All dataset were generated and analyzed in the current study. Data will be available upon request.

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