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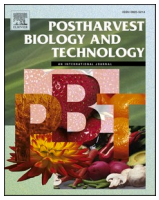

Hormone profiling and heat-induced tolerance to cold stress in citrus fruit

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ABSTRACT

The involvement of jasmonic acid (JA), different JA-related metabolites, salicylic acid (SA), indole-3-acetic acid (IAA) and abscisic acid (ABA) in the heat-induced cross-adaptation to chilling has been investigated in citrus fruit. The effects of heating 'Fortune' mandarins at 37 ºC, and storing them at a chilling temperature (2 ºC) after being exposed, or not, to a heat-conditioning treatment (3 d at 37 ºC) on the levels of these signalling molecules were examined. Jasmonate metabolism activation at 37 ºC was followed by that of SA, and then by a rise in IAA and a drop in ABA. Storage at 2 ºC transiently increased the contents of IAA, of the JA precursor, *cis*-(+)− 12-oxo-phytodienoic acid (OPDA), and of the JA-derivatives jasmonoyl-isoleucine (JAIle) and methyl jasmonate (MeJA), and decreased ABA in the non-conditioned fruit. The results also indicated that fruit were protected from developing chilling symptoms by virtue of the heat-conditioning treatment having higher JAIle levels than the non-conditioned fruit for very long cold storage periods, while the heat-induced rises in SA, OPDA and MeJA noted in the cold-stored fruit were transient.

1. Introduction

Cold storage is often used to extend the postharvest life of fruit and vegetables. Nevertheless, low temperature causes chilling injury (CI) and, hence, external quality deterioration, in many chilling-sensitive crops of tropical and subtropical origin, like citrus fruit (Lurie, 1998; Schirra et al., 2004; Lafuente et al., 2005; Sevillano et al., 2009). High-temperature conditioning prior to storage is an efficient approach to increase cold tolerance in citrus fruit (Rodov et al., 1995; Martínez-Téllez and Lafuente, 1997; Schirra et al., 2004; Mulas and Schirra, 2007). Different reports have shown the high efficacy of 3-day hot (37 ºC) humid air treatment in extending the postharvest life of citrus fruit at low temperature. Its efficacy is not influenced by either the fruit maturity stage or preharvest temperatures despite the variable susceptibility of citrus fruit to chilling during the season (Lafuente et al., 1997; González-Aguilar et al., 2000). So this treatment is a very useful tool for studying the mechanisms responsible for long-term heat-induced chilling tolerance in this fruit crop. Substantial work has been done to understand the global mechanisms induced by low-temperature storage in heat-conditioned citrus fruit (Sanchez-Ballesta et al., 2003; Perotti et al., 2011; Lafuente et al., 2017; Moreno et al., 2020). Physiological studies have focused mainly on the involvement of not only oxidative stress, lipid, carbohydrate and phenolic metabolism, but also of some hormones in citrus fruit susceptibility to CI, and how they are modified by different temperature-conditioning treatments that alleviate chilling symptoms (Lafuente et al., 2005).

Plant hormones are crucial for the adaptive response to exposure to biotic and abiotic stresses in plants and, despite their specific functions, they can interact cooperatively or antagonistically with one another by complex crosstalk. Plants specifically evolve hormone-mediated adaptive mechanisms in response to heat (Li et al., 2021) and cold (Peleg and Blumwald, 2011) stresses. Research aiming to know the effect of cold stress or high temperature-conditioning treatments, which alleviate CI in citrus fruit, on endogenous hormone levels have been limited to ethylene and abscisic acid (ABA) (Lafuente et al., 2005). Other hormones like salicylic acid (SA), jasmonic acid (JA), and the related jasmonate metabolites methyl jasmonate (MeJA) and jasmonoyl-isoleucine (JAIle), are also implicated in stress regulation in plants. Previous research has shown that applying JA, MeJA and SA may alleviate CI in citrus fruit (Droby et al., 1999; Habibi et al., 2019; Serna-Escolano et al., 2019; Siboza et al., 2014; Siboza et al., 2017). The involvement of indole-3-acetic acid (IAA), the commonest naturally-occurring auxin, in cold- and heat-induced responses, has also been demonstrated in model plants (Shibasaki et al., 2009; Li et al., 2021). Previous results also indicate that the expression levels of auxin responsive transcription factor (*CsARF19*) increase in response to cold stress in citrus fruit, and this effect is repressed if fruit are preconditioned for 3 d at 37 ºC (Lafuente et al., 2017). However, it is still

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unknown whether the low-temperature storage that causes CI in citrus fruit, or the high temperature-conditioning treatments that reduce the incidence of this postharvest disorder, actually affect the endogenous IAA levels in this fruit crop. From these findings, it can be hypothesized that JA and/or other JA-related metabolites (referred to as jasmonates), SA and IAA may act as signal molecules to cope with chilling stress in cold-stored citrus fruit by either orchestrating down-stream stress adaptive responses to cold stress or favouring the containment of chilling-induced lesion propagation. These metabolites may also participate in high-temperature conditioning-induced cross-adaptation to chilling in this fruit. To test this hypothesis, the present study compares the changes in the endogenous levels of the above-mentioned compounds while exposing citrus fruit to heat, cold, and the heat+cold combination, in the outer peel part (flavedo), and assesses fruit susceptibility to CI. Moreover, a comparative study was performed in the hormone profile of the non-symptomatic and symptomatic flavedo tissue of the same fruit. Among jasmonates, besides JA, we determined the levels of MeJA and of JAIle, which has been described as the molecularly active form of the hormone (Fonseca et al., 2009), as well as levels of the JA precursor (*cis*-(+)− 12-oxo-phytodienoic acid (OPDA)), which can also mediate in plant responses to biotic and abiotic stresses (Taki et al., 2005; Hazman et al., 2015). The effect of both cold stress and conditioning citrus fruit at 37 ºC for 3 d on endogenous ABA levels has been reported (Lafuente et al., 1997). Nevertheless, the ABA changes that occur at different time points while leaving fruit at 37 ºC, or whether there are differences in ABA between necrotic and healthy flavedo zones of chilled fruit, remain unknown. Considering this, and the fact that the impact of heat-conditioning treatment on modifying ABA levels may vary with fruit maturity and/or preharvest factors (Lafuente et al., 1997), any changes in ABA under our specific study conditions were also determined to further understand the possible relation among jasmonates, SA, IAA and ABA.

2. Material and methods

2.1. Fruit material, experimental setup and temperature treatments

Fully mature 'Fortune' mandarins (*Citrus clementina* Hort. Ex Tanaka x *Citrus reticulata*, Blanco) were randomly harvested from adult trees grafted onto 'Cleopatra' mandarin (*Citrus unshui* L) grown in commercial orchards in Sagunto (Valencia, Spain). Those of uniform size and free of visual defects were immediately delivered to the laboratory and assigned to three different groups.

The fruit from the first and second groups were used to evaluate the effect of high temperature on endogenous phytohormone kinetics while holding fruit at 37 ºC, and to compare not only the cold-induced hormonal responses in previously conditioned 'Fortune' mandarins or not at 37 ºC for 3 d, but also their CI severity. Immediately after fruit harvesting, those in the first group were subjected to heat treatment, which consisted of leaving fruit in storage rooms at 37 ºC and 90–95 % relative humidity (RH) for 3 d. The fruit in this group were sorted into two subgroups. The first subgroup was used to examine hormone profiling during fruit exposure to 37 ºC and included three replicates, each with 10 fruit per sampling time, to determine the concentration of hormones at fruit harvest (0 h) and in the fruit left at 37 ºC for 12, 24 and 72 h. This totalled 120 fruit. After the heat-conditioning treatment, the fruit in the second subgroup were transferred to storage rooms at 2 ºC and 80–85 % RH for 60 d. To compare the cold storage effect on the hormone profiling of the heat-conditioned and non-conditioned fruit, the fruit in the second group were immediately stored at 2 ºC and 80–85 % RH for 60 d after fruit harvest. In all, 150 heat-conditioned and 150 non-conditioned mandarins were used to compare the cold stress effect. For each condition (heat-conditioning *vs.* non-conditioning), three replicates of 20 fruit each were included to periodically determine CI severity during fruit cold storage up to 60 d, plus three replicates of 10 fruit each per storage period to compare the cold-induced changes in hormones in the heat-conditioned and non-conditioned fruit. All the fruit were left in constant darkness during either the heat-conditioning treatment or the cold storage period.

The third subgroup was used to perform a comparative study of the symptomatic (necrotic) and non-symptomatic flavedo tissues from the same cold-stored fruit to, thus, understand whether the cold-induced changes in hormones were confined to necrotic areas. These fruit were not heat-conditioned to ensure chilling damage development, and were stored at 2 ºC and 80–85 % RH for 30 d in cold storage rooms. Three biological replicates with 60 fruit each were used to obtain enough flavedo tissue from the necrotic and non-necrotic zones to analyse the different hormones.

For the hormone analyses, flavedo samples were taken from the total surface of the fruit included in the first two groups, frozen in liquid nitrogen, homogenised by grinding them to a fine powder by a coffee mill in liquid nitrogen and left at −80 ºC. In the fruit from the third group, the necrotic and non-necrotic flavedo zones were carefully separated from the same cold-stored fruit, frozen, homogenised and left a −80 ºC for further analyses. Flavedo tissue was selected because CI only affected the outer peel part in this citrus fruit cultivar.

2.2. Determination of the CI index

CI disorder severity, which is manifested as brown-pit-like depressions on 'Fortune' mandarin fruit flavedo, was estimated as previously described by Martínez-Téllez and Lafuente (1997). A CI rating scale from 0 (no injury) to 3 (severe injury), established according to the necrotic surface area and browning intensity, was used to score each fruit. The results were expressed as the CI index. The CI index for a set of fruit was calculated by summing the product of the number of the fruit in each category, multiplied by the score of each category, and then dividing the resulting amount by the number of evaluated fruit. The same fruit were used on different evaluation dates. The results were the mean of three biological replicate samples of 20 fruit each ± standard error.

2.3. Determination of ABA

ABA was determined in the homogenised frozen flavedo (200 mg) following the method described in Romero and Lafuente (2020). Flavedo tissue was extracted with 1.5 mL of 80 % acetone containing 0.1 g L^{-1} of butylated hydroxytoluene and 0.5 g L^{-1} of citric acid in a Mini-Beadbeater-Plus Mini Cell Disruptor (Biospec Products, Inc., Bartlesville, OK, USA) for 1 min. Extracts were centrifuged at 13,000 × g and 4 ºC for 10 min. Supernatants were diluted in three serial dilutions with cold TBS (6.05 g L^{-1} Tris, 0.2 mg L^{-1} $MgCl_2$ and 8.8 g L^{-1} NaCl) at pH 7.8 to reach the final ABA concentrations, which fell within the linear range of the ABA standard curve. Two supernatant aliquots were employed to determine the hormone content in duplicate by the indirect ELISA method using the ABA-4′-BSA conjugate detailed in Lafuente et al. (1997). The results are the mean of three biological replicate samples ± standard error.

2.4. Determination of jasmonates, SA and IAA

The extraction of the jasmonate-related metabolites (OPDA, JA, MeJA and JAIle), IAA and SA was performed simultaneously from the ground/frozen flavedo samples. They were extracted twice from flavedo (100 mg) with 0.4 mL of 70 % methanol containing 1 % glacial acetic acid, both of Scharlau LC-MS grade (Sharlab, Barcelona, Spain), for 1 min using a Mini-Beadbeater-Plus Mini Cell Disruptor (Biospec Products, Inc.). Samples were extracted in 2 mL screw-cap microvials using a 2 mL vial adaptor for Mini-BeadBeater, which allowed 24 samples to be simultaneously extracted. Extracts were centrifuged at 13,000 × g for 10 min at 4 ºC and 450 μL of each combined supernatant were filtered through 0.2 μm Nylon Teknokroma (Barcelona, Spain) filters. All the

samples were extracted on the same day and the filtered supernatants were left overnight at −80 ºC prior to UHPLC determinations. Before extraction, 50 ng of prostaglandin B1 (Cayman Chemical Co, Ann Arbor, Michigan, USA) were added to each extract as an internal standard (Flors et al., 2008), and 10 μL of each supernatant were injected for hormone quantification by UHPLC at the SCSIE-UV Chromatography Facility (Valencia, Spain).

A UHPLC ExionLC AD system (Sciex, Framingham, MA, USA), equipped with a refrigerated ExionLC AD Autosampler and connected to a Sciex Qtrap 6500 plus triple quadrupole mass spectrometer, was used. Analyses were performed in a XB-C18 Kinetex (2.1 × 100 mm, 1.7 μm) column from Phenomenex (Torrance, California, USA) and a binary gradient elution of water (solvent A) and acetonitrile (solvent B), both containing 0.1 % formic acid. After 0.7 min, the solvent composition changed from 30 % to 90 % solvent B during 3 min on a linear gradient. For the next 0.6 min, the solvent composition changed again to the 30 % solvent B before the next sample injection. The flow rate was 0.4 mL min^{-1}. The mass spectrometric detection of the analytical run was monitored in the positive and negative multiple reaction modes (MRM), and the parameters of the ion source were optimised to the following settings: temperature 400 ºC, ionisation spray voltage 5 kV, curtain gas 35 psi, heater gas 50 psi, turbo ion spray gas 50 psi and 10 V entrance potential. The declustering potential (DP), collision energy (CE) and collision exit potential (CXP) were optimised with the standards of each analyte under study. The Analyst® Software 1.5.0 (SCIEX) was used to process the quantitative data from the calibration standards and flavedo samples according to the retention times and accurate mass signals of the specific analytes. All the standards were purchased from Cayman Chemical Co., except MeJA, which was supplied by Sigma-Aldrich (Madrid, Spain). The results are the means of three biological replicate samples ± standard error.

2.5. Statistical analysis

Statistical analyses were performed using the Statgraphics Plus 4.0 Software (Manugistics, Inc.). A *t*-test ($p < 0.05$) was used when comparing the heat-conditioning treated and non-conditioned fruit during a specific cold storage period.

3. Results and discussion

3.1. Jasmonates in the heat-induced chilling tolerance of 'Fortune' mandarins

Jasmonates may participate in both early and long-term responses to stresses by acting as signalling messengers in plant and fruit defence and orchestrating responses, whose aim is content cell damage propagation (González-Aguilar et al., 2004; Wasternack, 2007; Kondo, 2022). 'Fortune' mandarin conditioned for 3 d at 37 ºC before storing them at 2 ºC did not develop CI, while CI symptoms were evident by day 30 and increased until 60 d in the non-conditioned fruit (Fig. 1). Therefore, we examined the kinetics of the selected hormones in the fruit response to high temperature (0–3 d at 37 ºC), and also their putative participation in the very short- (1 d) and long-term responses (30 and 60 d) induced by cold stress in the stored heat-conditioned and non-conditioned mandarin fruit.

Applying JA or MeJA to citrus fruit increases their resistance to CI (Droby et al., 1999; Siboza et al., 2014; Siboza et al., 2017; Rehman et al., 2018; Habibi et al., 2019; Serna-Escolano et al., 2019). The effect of cold and heat stress on jasmonates levels, or on JA signalling, has also been demonstrated in model plants (Sharma and Laxmi, 2016; Li et al., 2021). However, as far as we know, this is the first report to indicate that high temperature-conditioning (Fig. 2) and/or postharvest cold stress (Fig. 3) may strongly impact the levels of JA, of its precursor OPDA, and of its derivatives, MeJA and JAIle. Our results also highlight that conditioning citrus fruit at 37 ºC for 3 d altered JA metabolism in the cold-stored fruit (Fig. 3). A marked transient rise in OPDA (Fig. 2A) occurred by 12 h. This rise preceded the most important rises in JA (Fig. 2B) and JAIle (Fig. 2D), which peaked at 24 h. This result suggests

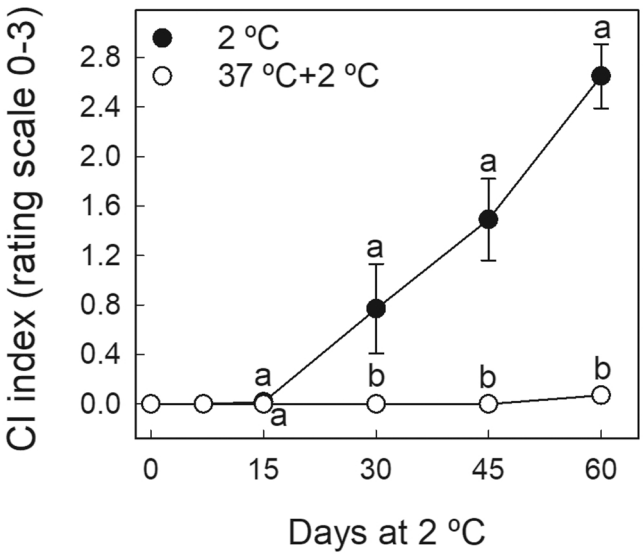

Fig. 1. Changes in the CI index in the 'Fortune' mandarins exposed to a 3-day heat-conditioning treatment at 37 ºC (○), or not (●), and then stored at 2 ºC and 80–85 % RH. Values are the means of three biological replicate samples. The error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the heat-conditioned and non-conditioned fruit for the same storage period.

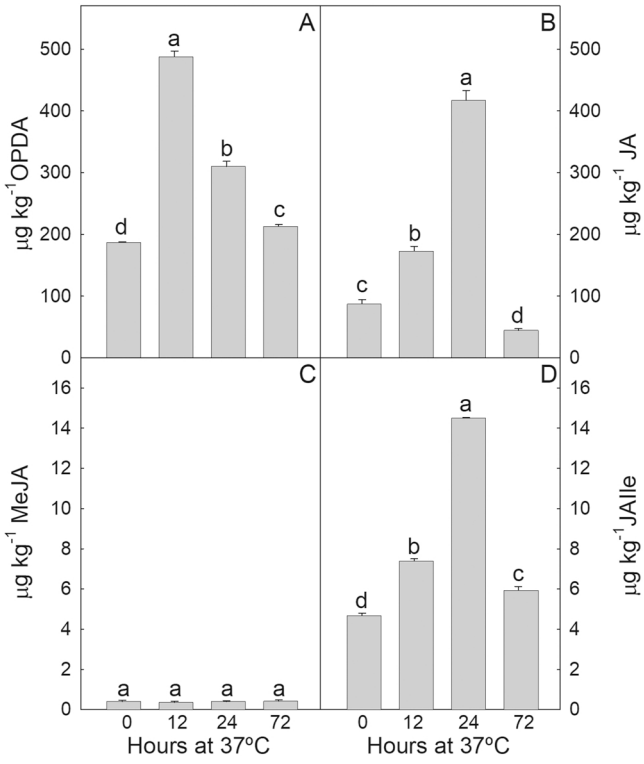

Fig. 2. Time course of the changes in OPDA (A), JA (B), MeJA (C) and JAIle (D) in the flavedo of the 'Fortune' mandarin fruit heated at 37 ºC and 90–95 % RH. Values are the means of three biological replicate samples and the error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) among the samples taken during distinct heat treatment periods.

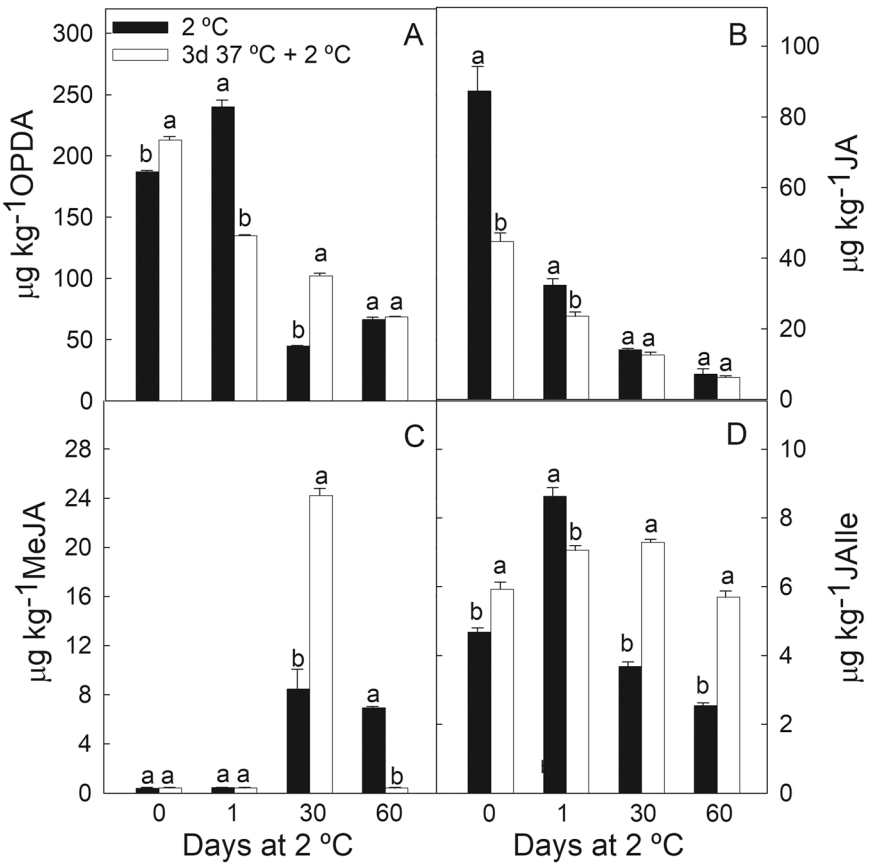

Fig. 3. Changes in OPDA (A), JA (B), MeJA (C) and JAIle (D) in the flavedo of the 'Fortune' mandarin fruit stored at 2 ºC and 80–85% RH immediately after harvest (•) or after 3 d and conditioning at 37 ºC and 90–95 % RH (○). Values are the means of three biological replicate samples and the error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the heat-conditioned and non-conditioned fruit for the same cold storage period.

that a high non-lethal temperature strongly influences the JA precursor and redirects the jasmonate metabolism towards the synthesis of JAIle rather than that of MeJA. As shown in Fig. 2C, MeJA levels were very low at fruit harvest and barely changed at 37 ºC. Based on these results, and the fact that OPDA, JA and JAIle may act as signal molecules to mediate stress-related responses (Peleg and Blumwald, 2011; Hazman et al., 2015), we cannot rule out the participation of any of them in high-temperature conditioning-induced cross-adaptation to chilling in citrus fruit.

The MeJA concentration rose during long-term low-temperature storage, while the heat-conditioning treatment promoted a sharp transient increase in the chilling-induced rise in MeJA by 30 d (Fig. 3C). Given these results, we cannot rule out the possibility of the participation of MeJA in the heat-induced chilling tolerance in citrus fruit. In the context of this work, it is worth mentioning that exogenous MeJA favours reactive oxygen species (ROS) detoxification in citrus fruit (Siboza and Bertling, 2013 and 2017; Serna-Escolano et al., 2019), as does the heat-conditioning treatment herein selected (Sala and Lafuente, 2000; Lafuente et al., 2017), while ROS may favour CI in citrus fruit (Sala and Lafuente, 2000) and play a central signalling role via their interactions with other key signalling components, including OPDA and jasmonate metabolites (Hazman et al., 2015; Sewelam et al., 2016). Likewise, MeJA activates phenylopropanoid metabolism in citrus fruit (Siboza et al., 2014; Serna-Escolano et al., 2019), which has been related to the containment of chilling-induced cell damage (Sánchez-Ballesta et al., 2000; Lafuente et al.; 2001), and to the effect of heat-conditioning treatment on reducing CI in this fruit (Sanchéz-Ballesta et al., 2003; Lafuente et al., 2017).

The results indicated that MeJA should be less relevant than the other JA-derivative JAIle for heat-induced chilling tolerance. JAIle is considered the endogenous bioactive jasmonate given its relevance for JA signalling (Fonseca et al., 2009). However, the involvement of JAIle in the susceptibility of horticultural crops to CI remains almost unknown (Lee et al., 2020). Besides high temperature-conditioning treatment increased JAIle levels while fruit were exposed to 37 ºC (Fig. 2D), its decline was avoided for up to 60 d in the cold-stored fruit (Fig. 3D). As a result, the JAIle concentration was at least 2-fold higher in the heat-conditioned than in the non-conditioned fruit after very long-term cold storage. Our findings also indicate that, albeit transient, the JAIle increase was a very early (1 d) response to cold stress in the non-conditioned 'Fortune' mandarins. Taken together, these results highlight the relevance of JAIle as a signal molecule for protecting citrus fruit against chilling, *versus* the other JA-related metabolites, and suggest the participation of JAIle in both the early perception of citrus fruit to cold stress and long-term responses by participating in the containment of cell damage propagation in the heat-conditioned fruit stored at low temperature. In this regard, we should bear in mind that JA levels decreased in 'Fortune' mandarins at 2 ºC and were similar in both the heat-conditioned and non-conditioned fruit after 1 d (Fig. 3B). Likewise, although OPDA was higher in the heat-conditioned mandarins by 30 d, this effect did not last for up to 60 d (Fig. 3A). The overall results indicated that the heat-conditioning treatment favoured the synthesis of JA derivatives MeJA and JAIle in the cold-stored fruit, and JAIle synthesis prevailed after very long-term storage (60 d) (Fig. 3). In view of these results, it would be interesting to determine whether JAIle mediates in the efficacy of exogenous MeJA by reducing CI in citrus fruit (Siboza et al., 2014; Serna-Escolano et al., 2019) and in other chilling-sensitive fruit (González-Aguilar et al., 2004), or in the efficacy of prohydrojasmon (PDJ), an analogue of JA, which may increase chilling tolerance in mango (Kumar et al., 2020). Results of the present paper also encourage future research studying the effect of applying PDJ or other compounds like L-isoleucine, which may promote the synthesis of JAIle in apple fruit (Kondo et al., 2021; Kondo, 2022), on reducing CI in citrus fruit. Gaining such an understanding might help to design more

efficient methods to reduce CI in chilling-sensitive crops.

In order to investigate whether jasmonates changes were directly related to fruit exposure to cold stress or, alternatively, to the tissue damage associated with it, jasmonate contents were compared in healthy tissue and in tissue with chilling-induced necrotic lesions, which were taken from the same non-conditioned fruit held for 30 d at 2 ºC. All the jasmonates were higher in the tissue zone that developed necrotic lesions (Fig. 4), which agrees with the results found when examining changes in the activity of the enzyme phenylalanine ammonia-lyase (PAL) (Sanchez-Ballesta et al., 2000). This enzyme is at the entry point of the phenylpropanoid metabolism and plays a role in reducing chilling damage in citrus fruit (Lafuente et al., 2001). Special attention should be paid to the large JA differences found between necrotic and non-necrotic flavedo (Fig. 4), together with the fact that JA decreased at 2 ºC (Fig. 3B). Both findings suggest that the rise in JA in necrotic tissue is related to a citrus fruit defence response to contain cell damage propagation once it has started because of chilling stress, which agrees with the proposed role of JA in chilled guava fruit (González-Aguilar et al., 2004).

3.2. SA, IAA and ABA levels in 'Fortune' mandarin's heat-induced chilling tolerance

The SA content underwent an early (24 h) transient increase during the high temperature-conditioning treatment (Fig. 5A), which coincides with the effect of heat increasing SA in citrus leaves (Zandalinas et al., 2016). The SA levels were higher in the heat-conditioned than in the non-conditioned fruit during both short- (1 d) and long-term (30 d) cold storages in spite of SA not increasing in the non-conditioned 'Fortune' mandarin to cope with cold stress (Fig. 6A). According to this result, it would appear that 'Fortune' mandarin's ability to increase SA to protecting itself from CI is very poor. In fact no difference was found in the SA content between the necrotic and non-necrotic tissues in the same fruit (Fig. 4). These results indicate that the heat-conditioning treatment may improve the ability of citrus fruit to increase endogenous SA in response to cold stress (Fig. 6A). Therefore, we cannot rule out the participation of SA in regulating the downstream responses associated with heat-induced resistance to chilling. These responses might still

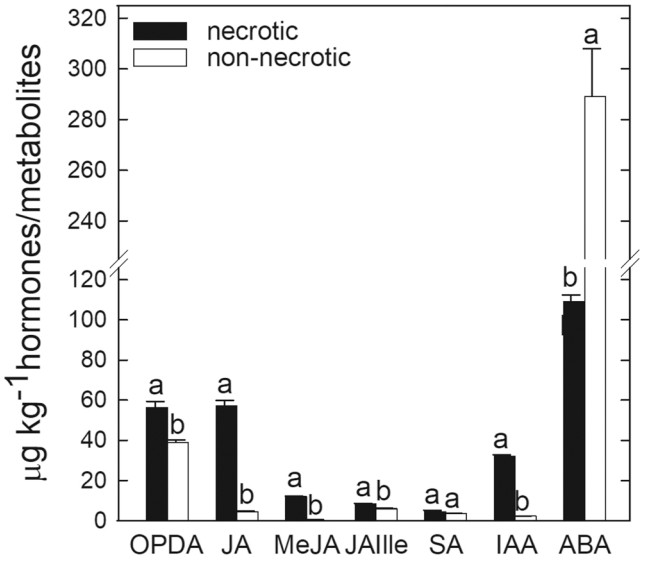

Fig. 4. The OPDA, JA, MeJA, JAIle, SA and ABA levels in the non-necrotic (white bar) and necrotic (black bar) tissues of the 'Fortune' mandarins stored for 30 d at 2ºC and 80–85% RH. Values are the means of three replicates samples and the error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the necrotic and non-necrotic tissues for the same metabolite.

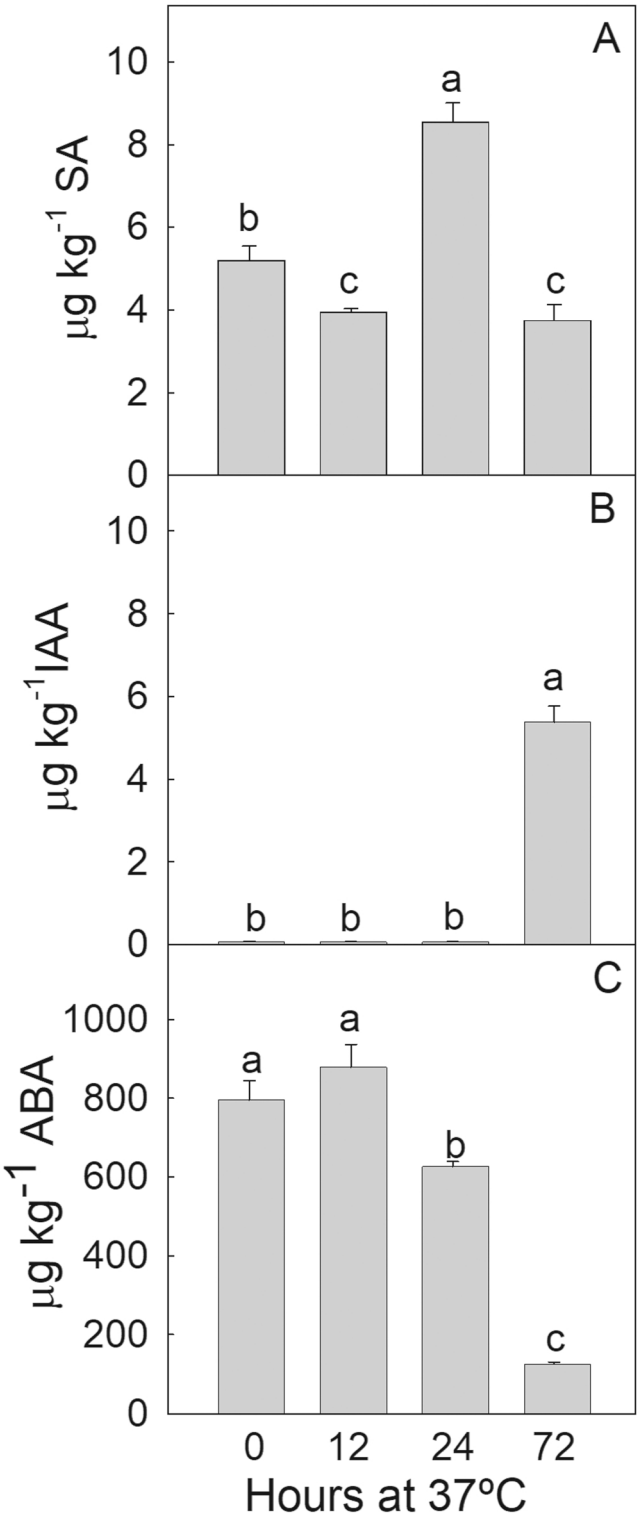

Fig. 5. Time course of the changes in SA (A), IAA (B) and ABA (C) levels in the flavedo of the 'Fortune' mandarin fruit heated at 37 ºC and 90–95 % RH. Values are the means of three biological replicate samples and the error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) among the samples taken during the distinct heat treatment periods.

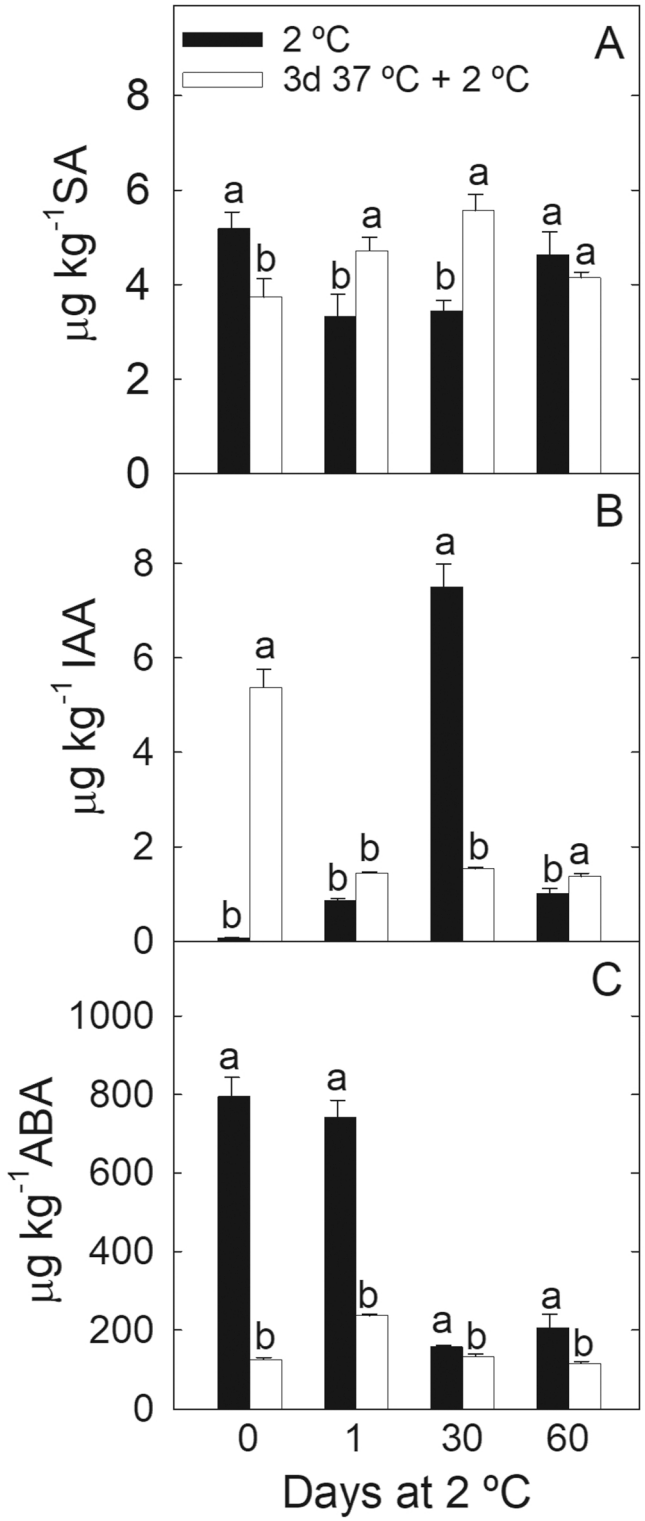

Fig. 6. Changes in SA (A), IAA (B) and ABA (C) in the flavedo of the 'Fortune' mandarin fruit stored at 2 ºC and 80–85 % RH immediately after harvest (•) or after 3 d conditioning at 37 ºC and 90–95 % RH (○). Values are the means of three biological replicate samples and the error interval indicates the standard error of the estimated mean value. Different letters mean significant differences ($p \leq 0.05$) between the heat-conditioned and non-conditioned fruit for the same cold storage period.

have operated after 30 d in the heat-conditioned fruit despite the drop in SA and there being no differences between the conditioned and non-conditioned fruit by 60 d. The effect of the heat-conditioning treatment on the SA levels in the cold-stored 'Fortune' mandarins was similar to that found for some enzymes belonging to the antioxidant enzymatic system (Sala and Lafuente, 2000) and, interestingly, exogenous SA may protect citrus fruit from oxidative stress caused by chilling (Siboza and Bertling, 2013; Habibi et al., 2019).

The role of IAA in plants' tolerance to chilling conditions leading to tissue damage remains practically unknown (Du et al., 2012). IAA considerably increased and peaked by day 30 in response to chilling in the non-conditioned 'Fortune' mandarin fruit (Fig. 6B). Moreover, IAA sharply increased at 37 ºC by 3 d (Fig. 5B). The IAA content was much lower in the heat-conditioned than in the non-conditioned fruit by day 30 (Fig. 6B), when the non-conditioned fruit developed CI symptoms (Fig. 1). Considering these results, and previous findings indicating that rice mutants with reduced free IAA contents show alleviated oxidative damage and increased cold tolerance (Du et al., 2012), we can speculate that the beneficial effect of the heat-conditioning treatment might be related to its ability to avoid the chilling-induced rise in IAA. This situation is not simple because IAA increased at 37 ºC (Fig. 5B). However, we should bear in mind that such rise took place after long heat exposure, and IAA has been related to heat tolerance in plants (Li et al.; 2021). So we cannot rule out the possibility of the rise in IAA that occurred by 3 d at 37 ºC being a protective mandarin mechanism to avoid heat damage. In line with this, it is worth considering that, for the same storage period, IAA was much higher in necrotic zones than in healthy ones in the same fruit held at the chilling temperature (Fig. 4), because auxin is produced around dying cells as a result of the high tryptophan levels released from proteins (Sheldrake, 2021). Consequently, our results might also suggest that during the 3-day conditioning treatment at 37 ºC, some defence mechanisms against chilling began, including the accumulation of IAA and/or SA and jasmonate-related compounds, which would protect mandarins from developing cell damage. In this situation, the cold-induced rise in IAA noted in the chilled non-conditioned mandarins, and putatively associated with the development of many zones containing dying cells, would not occur in the heat-conditioned cold-stored fruit. IAA content lowered from 30 d to 60 d cold storage in the non-conditioned fruit (Fig. 6B) despite chilling symptoms still increasing (Fig. 1). However, we should bear in mind that cells' ability to degrade tryptophan, and the conjugation and destruction of IAA in older cells, may outweigh such production (Sheldrake, 2021).

ABA content lowered from 12 h to 72 h at 37 ºC (Fig. 5C), which agrees with the response of other plant species to heat (Asensi-Fabado et al., 2013), and remained almost constant after transferring fruit from 37 ºC to 2 ºC (Fig. 6C). ABA levels lowered while exposing the non-conditioned mandarins to chilling, but were always significantly higher in the non-conditioned vs. the heat-conditioned fruit (Fig. 6C). This trend in the ABA changes confirm our previous observations, which suggested that ABA does not play a protective role against chilling and is not relevant in high-temperature conditioning-induced cross-adaptation to chilling in citrus fruit (Lafuente et al., 1997; Alférez et al., 2005). According to these observations, it is not surprising that the ABA concentration was much lower in the symptomatic than in the non-symptomatic zones (Fig. 4).

3.3. Comparison of the trend of hormonal changes in 'Fortune' mandarins' response to heat, cold, and the heat+cold combination

The crosstalk among hormones may result in synergistic or antagonistic interactions that are crucial in the plant protection against stresses. The characterisation of such crosstalk is simplified by using model plants in which hormone-specific mutants can be easily generated, but the availability of artificial mutants is not common in fruit from woody plants. This interaction might be affected in model plants by

the translocation of hormones and/or their signalling-related molecules between plant organs, as commonly occurring between the root and the upper parts of the plants (Shibasaki et al., 2009; Li et al., 2021). Fortunately, we can disregard these facts when considering harvested fruit. Nevertheless, our results provide valuable information about the putative relation among jasmonates, SA, IAA and ABA in response to the specific or combined environmental temperature stresses included in this study in citrus fruit (summarised in Fig. 7). They indicate that the kinetics of jasmonates, IAA and SA differed when fruit were left at 37 °C (Figs. 2 and 5). Jasmonate metabolism activation was an early heat-induced response starting by 12 h (Fig. 2). The rises in SA and IAA respectively occurred by 24 h and 72 h (Fig. 5). These results encourage further research to decipher whether interplay among jasmonates, SA and IAA exists in citrus fruit in response to heat. In this case, jasmonate action should take place upstream of SA activation in the heat-conditioned citrus fruit, while IAA would occur at the end of the downstream hormone response cascade. This would agree with the results reported in studies that have employed the model plant *Arabidopsis thaliana*, which propose that IAA operates downstream of SA in response to heat (Li et al., 2021). In this model plant, jasmonate and ethylene antagonistically regulate the plant heat response (Li et al., 2021). This would not be the case of citrus fruit because the results of this work and our previous research results (Holland et al., 2012) indicate that both jasmonates and ethylene transiently increase during 'Fortune' mandarin exposure to 37 °C. The trend of the ABA changes in response to heat treatment was the opposite to that of IAA (Fig. 4). Therefore, future research that determines whether both hormones have an antagonistic effect on citrus fruit in response to heat stress also deserves further attention.

Cold exposure, as well as heat, induced a decrease in ABA and an increase in IAA, which further support a putative antagonistic effect between both hormones in citrus fruit thermotolerance (Figs. 5 and 6). This contrasting effect was also observed when comparing the necrotic and non-necrotic tissues (Fig. 4). A similar trend in the response of this mandarin cultivar to both high- and low-temperature (non-conditioned) fruit was found when examining the changes in OPDA and JAIle because both temperature regimes induced very early transitory shifts in these metabolites. A contrasting effect of heat and cold stress was observed when comparing the changes induced in JA, MeJA and SA contents because MeJA increased after long-term cold storage, JA continuously decreased, and the changes in SA were barely relevant in the chilled non-conditioned fruit (Figs. 3 and 6). According to this comparative effect of both chilling and hormone kinetics, OPDA and/or JAIle action might lay upstream of IAA in chilled citrus fruit. The rise in IAA in the cold-stored fruit was concomitant with chilling development. Since chilling also induces an increase in ethylene production in 'Fortune' mandarin (Martínez-Téllez and Lafuente, 1997), it would be interesting to know if an interplay between IAA and ethylene exists in the cold-stored 'Fortune' mandarin. In this regard it is worth mentioning that *ARF19* is regulated by ethylene in plants (Peleg and Blumwald, 2011), and a *CsARF19* is up-regulated by cold stress in this mandarin cultivar (Lafuente et al., 2017). Another noteworthy point is that both IAA and ethylene (Holland et al., 2012) increase at 37 °C in 'Fortune' mandarin and, therefore, a connection between ethylene and IAA in this mandarin cultivar's heat response may exist.

From the combined heat+cold effect, first it is worth highlighting the effect of the prior heat treatment on favouring higher JAIle levels by the long-term cold storage periods (30 and 60 d), and second the higher transient levels (30 d) of MeJA and SA, which may reflect a connection among these signal molecules in heat-induced cross-adaptation to chilling (Figs. 3 and 6). The trend of the IAA changes in response to the heat+cold combination resembles that of ethylene (Martínez-Téllez and Lafuente, 1997) because the heat-conditioning treatment avoided the chilling-induced rises in IAA and ethylene. As the heat-conditioning treatment barely affected the JA levels in the cold-stored fruit, the combined effect of heat and cold on JA would appear to be independent of other hormones.

On view of the overall results, it should be interesting to determine in future studies whether the integration treatment of different hormones would improve the effect of individual hormones on reducing CI in citrus fruit. Likewise, results of this work highlighting the relevance of JAIle on the heat-induced chilling tolerance, also encourage to examine the effect

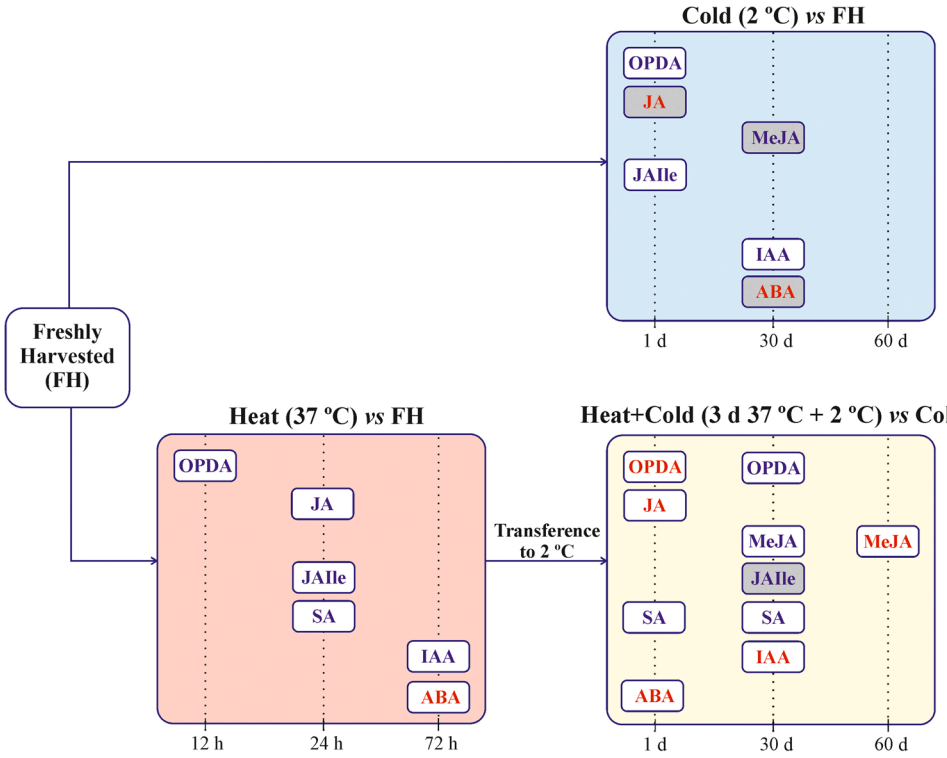

Fig. 7. Schematic integration of the sequential changes in the IAA, SA, ABA and JA, and JA-related metabolite contents in response to heat, cold, and the heat+cold combination. Only the most drastic changes are highlighted in the schematic diagram. Those hormones not showing relevant changes in response to a specific regime temperature are not included. Increases and decreases in hormone levels are indicated by blue and red letters, respectively. Shaded boxes denote changes that persisted from the indicated time until the end of the cold storage period. White boxes correspond to transient changes and are allocated at the temperature exposure period when the hormone/metabolite showed the maximum increase/decrease.

of compounds like PDJ and isoleucine, which increases JAIle content in other fruit (Kondo et al., 2021) on the tolerance of citrus fruit to low temperature storage.

4. Conclusions

Our results unravel the notion that low (2 ºC) and high (37 ºC) temperatures, and the heat+cold combination, strongly impact endogenous hormones levels in flavedo, which may contribute to heat-induced cross-adaptation to chilling in citrus fruit. Cold stress induces early transient increases in OPDA and JAIle in the non-conditioned citrus fruit, which could reflect cell perception of cold stress. Prior conditioning treatment may provide fruit with the ability to increase SA at low temperature. The comparison of the necrotic and healthy areas of the non-conditioned cold-stored fruit also highlights the relevance of jasmonates and IAA in chilling-induced cell damage. Holding fruit at 37 ºC lowers ABA, but transiently increases OPDA, JA and JAIle, which precede rises in SA and, thereafter, in IAA. From these results, it seems plausible that these heat-induced molecules may trigger downstream adaptive responses against subsequent cold stress. The results also highlight the relevance of JA-Ile in the defence of citrus fruit against temperature stress. JAIle participates in the early perception of citrus fruit to heat and cold, and the concentration of this signalling molecule is still higher in the heat-conditioned than in the non-conditioned fruit by day 60. Moreover in the cold-stored fruit, heat-conditioning treatment favours a transient increase in OPDA and MeJA that peak by day 30, and barely modifies JA.

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CRediT authorship contribution statement

M.T. Lafuente: Conceptualization, experimental design, Methodology, analyses, Validation, Resources, Writing – original draft, Writing – review and editing, Project administration, Funding acquisition, Formal analysis. **P. Romero**: Methodology, Writing – review and improving of the original draft. Software, editing. All authors have read and agreed to the published version of the manuscript.

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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