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RESEARCH ARTICLE

Role of salicylic and jasmonic acid in grapevine buds dormancy release induced by different dormancy-breaking agents

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ABSTRACT

Grapevine plants (*Vitis vinifera* L.) enter a dormant state during winter, and both exogenous and endogenous factors regulate their release. This study analyzed the effects of 4% hydrogen cyanamide (HC) and 10% garlic extract (GE) on the levels and signaling pathways of salicylic acid (SA) and jasmonic acid (JA) under forced dormancy-breaking conditions to break dormant buds of 'Flame seedless' grapevines. The buds treated with GE showed the highest bud break percentage at 64%, while the HC-treated and control buds showed 53% and 54%, respectively. The GE-treated buds exhibited the highest levels of SA at 10.3 g g^{-1} FW, while the HC-treated buds had the highest levels of JA at 44 mg g^{-1} FW. Increased expression of the signaling genes for both hormones confirmed their signaling pathways. Consequently, endogenous SA and JA play critical roles during the release of grapevine bud dormancy, and the dormancy-breaking agents can modulate their levels and signal differentially.

Key words: Defense hormones, garlic extract, hydrogen cyanamide.

INTRODUCTION

Grapevines (*Vitis vinifera* L.) are characterized by entry into a dormant state in the winter. During this period, there is a temporary cessation in the growth of meristematic buds, a reduction in metabolic activity, and abscission of leaves. The onset and dormancy release are regulated by environmental factors such as the photoperiod and temperature, as well as by endogenous factors such as hormones (Cai et al., 2024). Bud dormancy is an important process that influences plant productivity, due to the fact that the success of this process is reflected in the synchronicity of critical developmental stages such as bud break, flowering, and fruit quality (Pinto et al., 2007).

For dormancy release to occur, buds require the accumulation of chilling hours. The amount of cumulative chilling that grapevines require is specific to genotypes. The effective accumulation of chilling hours in the buds is achieved by prolonged and continuous exposure to temperatures on the range from 0 to 7 °C. However, when grapevines or other deciduous species are cultivated in subtropical regions where warm winters occur, bud dormancy release becomes a problem because of an insufficient accumulation of chilling hours (Salama et al., 2021). The lack of cold temperature causes prolonged dormancy, which consequently leads to an irregular bud break and delay in fruit ripening; this scenario leads to increases in production costs. Therefore, to address these problems, producers use exogenous dormancy-breaking compounds (Melke, 2015). Hydrogen cyanamide (HC) is the most widely used bud-breaking compound worldwide because of its ability to advance

and homogenize bud breaks; however, its use has been prohibited in some countries because it is highly toxic to human health (mainly for people who apply this compound in the field), environment, and grapevine buds (whereby it causes necrosis, depending on the applied cyanamide concentration) (Stover et al., 2016; El Mahdy and Kharoub, 2020). Garlic extract (GE) is another product that exerts positive effects on the release of bud grapevine dormancy and improves fruit yield without the drawbacks caused by HC (El-Senosy et al., 2021).

Even though HC is highly toxic, it provides a relatively uniform response of the bud population; therefore, it can be a useful tool to determine metabolic pathways that are involved in dormancy release. Changes in the profiles of nitrogenous compounds, amino acids, polyamines, and hormones have been identified during dormancy release caused by HC (Seif El-Yazal et al., 2014). In particular, with respect to hormones, most studies have evaluated their effects when they are exogenously applied to induce dormancy release; in several studies, their endogenous levels have been quantified. Moreover, studies addressing the pathways of hormone signaling occurring during this stage of grapevine development at the metabolic and gene expression levels are scarce.

Abscisic acid, ethylene, gibberellins, and indole acetic acid are the main phytohormones involved in bud dormancy release (El-Yazal, 2019). Although salicylic acid (SA) and jasmonic acid (JA) also play crucial roles in dormancy regulation, they have received very little attention in this respect because of their classical involvement in the plant defense or immunity system. In the case of SA, only its exogenous application to induce dormancy release in grapevine plants has been studied (Ahmadi et al., 2017), and in recent studies, we have reported (Orrantia-Araujo et al., 2021) that artificial application of cold temperatures to dormant grapevine buds stimulates the synthesis of SA. However, endogenous levels of JA have also been detected and quantified in dormant buds of sweet cherry plants (Ionescu et al., 2017). Therefore, we wondered whether the application of bud-breaking agents stimulates the synthesis of these two hormones in dormant grapevine buds. To address this, we analyzed the effects of the exogenous application of HC and GE on SA and JA contents and the expression of their signaling genes, with the objective of determining their involvement in the dormancy release of 'Flame seedless' grapevine buds.

MATERIALS AND METHODS

Plant material and bud break dynamics

This study was carried out in late winter 2018, and table grapevine (*Vitis vinifera* L.) 'Flame seedless' canes with four dormant compound buds were randomly selected from several 8-yr-old vines in a commercial vineyard located in Pesqueira (29°23′ N, 110°56′ W; 376 m a.s.l.), Sonora, Mexico, with an average monthly maximum temperature of 38.8 °C in August and an average monthly minimum temperature of 23.8 °C in January (CONAGUA, 2020). During cane collection in December 2018, the buds had accumulated an average of approximately 120 chilling units (CUs) of natural cooling according to the model of Utah (Melke, 2015), with data collected automatically at a weather station located approximately 12 km away from the vineyard where the canes were collected. The canes were covered with wet sawdust and immediately transported to the laboratory for treatment and analysis. Canes were randomly assigned to one of three experimental groups to address dormancy release: 4% Hydrogen cyanamide (HC), 10% garlic extract (GE), or distilled water as a control. Ten grapevine canes per treatment were grouped and soaked for 30 s in the abovementioned solutions. After the soaking treatment, the canes were drained, placed in 2 L plastic beakers with the basal ends (7 to 10 cm) submerged in distilled water, which was replaced twice a week, and transferred to a growth chamber at 25 °C with 75% relative humidity and a 16:8 h photoperiod to promote bud break. This experiment was performed in triplicate.

The bud break was monitored twice a week for 40 d, and bud break percentages were determined by comparing the number of buds in the green tip state to the total number of buds in the experimental group (Orrantia-Araujo et al., 2021).

Buds from treated canes were dissected at 3, 6, 9, 24, 48, 72, 96, 120, and 144 h after treatment application, frozen with liquid nitrogen, and stored at -80 °C for subsequent quantification of phytohormones and gene expression analysis.

Extraction and quantification of hormones in grapevine buds

For the extraction of salicylic acid (SA) and jasmonic acid (JA), samples of 2.5 g buds per replicate were frozen with liquid nitrogen and ground in a mortar until a fine powder was obtained. The fine powder was

homogenized with 10 mL ultrapure water with an Ultra Turrax T25 (Janke and Kunkel, IKA-Labortechnik, Wilmington, North Carolina, USA). The homogenate was then centrifuged at 10000 × g for 15 min at 4 °C in an RC 5C plus centrifuge (Sorvall, Waltham, Massachusetts, USA). The pH of the supernatant was adjusted to 2.8 with 15% glacial acetic acid, and a double liquid-liquid extraction was carried out using equal volumes of cold diethyl ether. The aqueous phase was discarded using 1PS phase separator filter paper (Whatman, GE Healthcare Life Sciences, Chicago, Illinois, USA), and the two organic phases were mixed and dried under a nitrogen gas stream. The dry residue was dissolved in 500 µL methanol and 0.2% glacial acetic acid (50:50 v/v). The remaining substance was referred to as the hormone extract, which was filtered through a 0.45 µm nylon syringe filter (Pall Gelman Science, Port Washington, New York, USA).

The detection and quantification of SA and JA were carried out via HPLC following the methods of Orrantia-Araujo et al. (2021). The HPLC device (Infinity 1260, Agilent Technologies, Palo Alto, California, USA) was equipped with a quaternary pump coupled to a diode array detector (DAD) (Agilent Technologies, Waldbronn, Germany). The hormone extract (100 µL) was injected into an Eclipse plus C-18 column with a length of 4.6 × 100 mm and a particle diameter of 3.5 µm. The elution system was operated at room temperature and at a flow rate of 0.3 mL min^{-1}. The mobile phases consisted of A (100% methanol) and B (0.2% glacial acetic acid in water), which were combined in a linear gradient. The gradient began with 10% phase A and 90% B for 10 min, followed by 5 min of phase A at 50% and another 5 min of 100% phase A; these conditions were maintained until minute 25 and subsequently returned to the original conditions. The SA and JA contents were monitored at a wavelength of 303 nm, and the absorption spectrum of a fraction coeluting with authentic SA and JA (purity 98%, Sigma-Aldrich, St. Louis, Missouri, USA) was obtained via DAD. To determine the endogenous concentrations of both hormones, standard calibration curves were generated using diluted solutions of authentic SA and JA over the range of 0.005 to 0.2 mg mL^{-1} JA and 0.02 to 1 mg mL^{-1} SA. The coefficient of determination (R^2) for the curves of both hormones was greater than 0.98.

Gene expression analysis

RNA extraction and cDNA synthesis. Extraction of total RNA was carried out following the method of García-Baldenegro et al. (2015). Twelve buds per sample were ground to a fine powder in a mortar with liquid nitrogen. The fine powder was extracted with a solution of 300 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2 M NaCl, 2% CTAB, 2% PVP40, 0.05% spermidine trihydrochloride, and 2% β-mercaptoethanol. The total RNA (5 µg) of each sample was treated with DNAse I (3 U 10 μg^{-1}) (QIAGEN, Germantown, Maryland, USA) to remove traces of DNA. The RNA quality and concentration were estimated from the A260/A280 and A260/A230 absorbance ratios using a NanoDrop 2000 (Thermo Scientific, Waltham, Massachusetts, USA). The RNA integrity was confirmed through electrophoresis on a 1% agarose-formaldehyde gel. Complimentary DNA was synthesized from 2 µg total RNA using the Superscript II First-Strand Synthesis System Kit (Invitrogen, Waltham, Massachusetts, USA).

Quantitative real-time PCR (qPCR). The genes selected to study the regulation of the SA signaling pathway were *nonexpressor of PR gene 1* (*NPR1*), pathogenesis-related protein 1 (*PR1*), and the gene encoding the transcription factor WRKY70 (*WRKY70*), whereas those selected for the JA signaling pathway were coronatine insensitive 1 (*COI1*), JASMONATE ZIM-domain (*JAZ1*), and vegetative storage protein 2 (*VSP2*). The representative sequences of each gene were taken from the genoscope database Grape Genome Browser (http://www.genoscope.cns.fr/externe/GenomeBrowser/Vitis/). These sequences were used for the design of specific primers (Table 1) using the online interface Primer3 plus software. Transcript quantification was performed on three biological replicates and three technical replicates. Amplification was carried out in a final volume of 20 µL, containing 5 µL cDNA (20 ng RNA equivalents), 10 µL iTaq Universal SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, California, USA), 1 µL 5 µM forward and reverse primers, and 3 µL ultrapure water (Milli-Q system). The amplification conditions were 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min in a StepOne Real-Time PCR System with a 48-well plate (Applied Biosystems, Foster, California, USA). For the calculation of the relative expression levels of each gene, the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used. For this purpose, the cycle threshold (Ct) values of each gene of interest were normalized to the Ct values of the housekeeping gene actin (*ACT*), and the Ct values at time zero were used as calibrators. Previously, dynamic range validation experiments were performed for each gene of interest, with serial dilutions of cDNA.

Table 1. List of specific primers designed for quantitative real-time PCR amplification of salicylic acid (SA) and jasmonic acid (JA) signaling genes. The codes in parentheses indicate the identifier of the genoscope annotation region of each gene.

Gene	Forward primer (5'-3')	Reverse primer (3'-5')
VvNPR1 (GSVIVT01015181001)	GTGGCGGTTTTGGGGTATTTGT	GCACCTCCACCATGAAATCCAC
VvWRKY70 (GSVIVT01032661001)	CCAATGAACTGGGGAGCCTTG	GCACGAGGAAGCATGAGCAAA
VvPR1 (GSVIVT01037005001)	CTCATGTGTTGGTGGGCAATGTG	GCACCCAAGACGCACTGATTTG
VvCOI1 (GSVIVT01016368001)	TACCTGCAAGACCGACCCACAT	CGTGTGCTCCTGGTGCTT
VvJAZ1 (GSVIVT01015042001)	TTCACCGGTTCTTGGAGAAG	GTGTCCTCTTCAGGCTTTGG
VvVSP2 (GSVIVT01015277001)	TGTGACATTCCTCTGGGGTTC	GAATGGGTGAATTTGGCTGAGG
VvACT (GSVIVT01026580001)	GCTGAGAGATTCCGTTGTCC	GCCACCACCTTGATCTTCAT

Statistical analysis

The bud break percentage was analyzed via ANOVA in a randomized complete block design (RCBD), in which the sampling time was the blocking factor. The hormone content and gene expression data were analyzed via one-way ANOVA at each sampling time with a completely randomized experimental design. The comparisons of means were performed via the Tukey-Kramer test ($P < 0.05$). The statistical analyses were carried out via NCSS software version 2007 (Number Cruncher Statistical System, Kaysville, Utah, USA).

RESULTS

Bud break percentage

The bud break response of both the chemical-treated plants and the control plants followed a similar pattern but to different extents (Figure 1). The GE-treated buds reached the highest bud break percentage (64%) and were consistently greater from 20 d onward, showing the greatest ability to induce bud break ($P < 0.05$) among the three treatments. The HC-treated buds presented a similar bud break percentage ($P < 0.05$) to that of the control buds, with 53% and 54%, respectively. Although HC is recognized as the most effective bud-breaking inducer and is, therefore, the most widely used worldwide, this study did not show the best results, reaching only 53% of bud breaks.

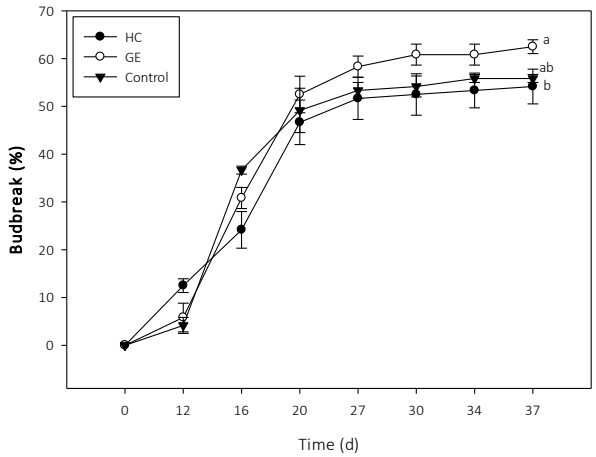

Figure 1. Effects of dormancy release agents on budbreak percentages in dormant 'Flame seedless' grapevine buds. The budbreak percentages were determined by forcing conditions in a growth chamber. HC: Hydrogen cyanamide; GE: garlic extract; Control: distilled water. Each value represents the mean of three replicates ± SE ($n = 3$). Different letters represent significant differences between treatments, determined by Tukey's test ($p < 0.05$).

Endogenous contents of SA and JA

The SA concentrations were analyzed under growth conditions after the plants were treated with hydrogen cyanamide, garlic extract, or control. We observed (Figure 2) that the three treatments had a similar trend, with successive increases and decreases in endogenous levels, but the pattern of HC was opposite to that of GE and control. Compared with the control, HC caused increases in SA levels at 9, 120, and 144 h, whereas GE had the highest levels at 3, 6, 9, 24, 48, and 96 h.

Like those of SA, endogenous JA levels were quantified in buds. Figure 3 shows that HC caused a significant increase in the endogenous content of this hormone throughout the analyzed period. The treatment with the highest levels of JA was followed by the buds treated with GE, which also caused an increase in JA. Moreover, no detectable levels of this hormone were detected in the control buds.

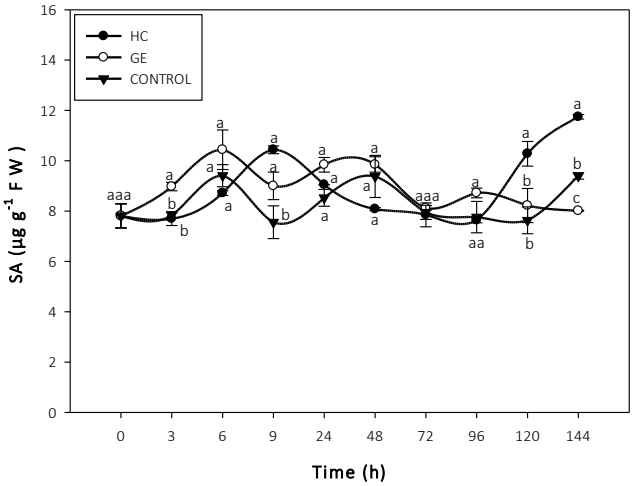

Figure 2. Endogenous salicylic acid (SA) levels in dormant 'Flame seedless' grapevine buds induced by dormancy release agents: Hydrogen cyanamide (HC), garlic extract (GE), distilled water (Control). Each value represents the mean of three biological replicates ± SE (n = 3). Different letters represent mean values with significant differences between treatments, determined by Tukey's test ($p < 0.05$).

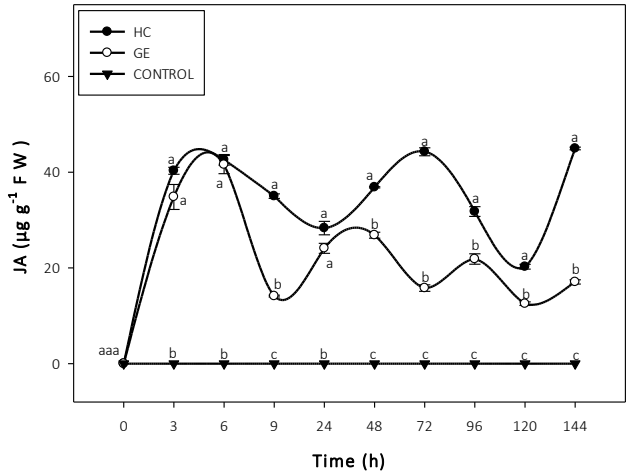

Figure 3. Endogenous jasmonic acid (JA) levels in dormant 'Flame seedless' grapevine buds induced by dormancy release agents: Hydrogen cyanamide (HC), garlic extract (GE), distilled water (Control). Each value represents the mean of three biological replicates ± SE (n = 3). Different letters represent mean values with significant differences between treatments, determined by Tukey's test ($p < 0.05$).

Relative expression of SA signaling genes

The relative fold changes in the expression of the *NPR1* and *PR1* genes of the SA signaling pathway were determined. The GE caused a slight increase in the expression of *NPR1* at 9, 72, and 120 h, whereas HC did not affect the expression of this gene. All the treatments showed a decreasing trend in the first hours. Interestingly, the control buds presented the highest fold-change levels of the three treatments at 6 and 24 h (Figure 4).

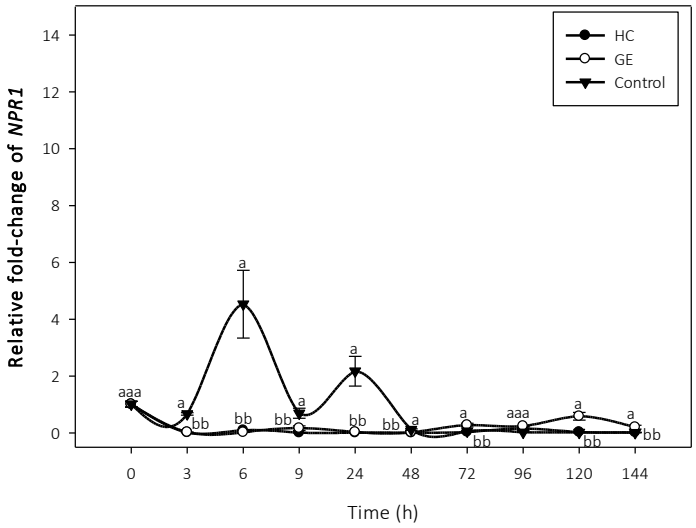

Figure 4. Relative fold-change expression of *NPR1* gene in dormant 'Flame seedless' grapevine buds after applying dormancy release agents and by forcing conditions: Hydrogen cyanamide (HC), garlic extract (GE), distilled water (Control). Values were normalized to the mean cycle threshold of time zero. Each value represents the mean of three technical replicates that come from three biological replicates ± SE (n = 3). Different letters represent mean values with significant differences between treatments, determined by Tukey's test (P < 0.05).

The relative fold change in expression of the *WRKY70* gene (Figure 5) revealed that both the HC and the GE caused a decrease in expression in the first 3 h and maintained low levels, but for the 144 h, a slight increase was detected in the buds treated with GE. These slight increases in the GE-treated buds could indicate that *NPR1* activated the SA signaling pathway with the participation of the transcription factor *WRKY70*. In the control buds, an increase in the expression of *WRKY70* was observed at 24 h. Figure 6 shows the relative fold change in the expression of the *PR1* gene, in which GE caused a significant increase in expression between 72 and 144 h, whereas HC caused a decrease in expression at all times analyzed. In the control buds, a significant increase in the fold change in the expression of *PR1* was observed from 48 to 96 h. Together, the results obtained from the relative fold change in the expression of *NPR1* and *PR1* revealed that, in the GE-treated and control buds, SA signaling was activated.

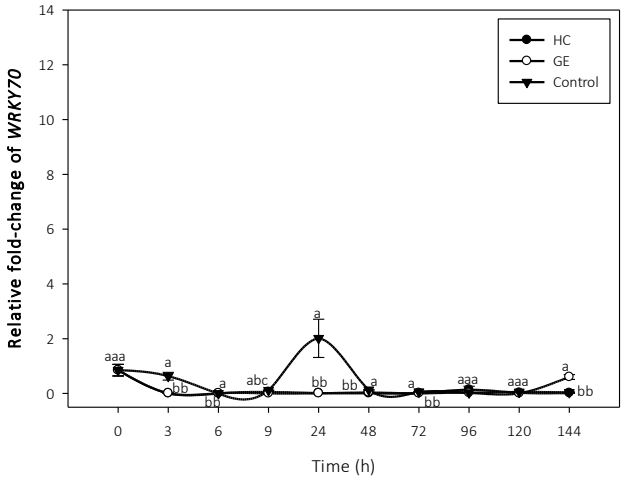

Figure 5. Relative fold-change expression of the *WRKY70* gene in dormant 'Flame seedless' grapevine buds after applying dormancy release agents and by forcing conditions: Hydrogen cyanamide (HC), garlic extract (GE), distilled water (Control). Values were normalized to the mean cycle threshold of time zero. Each value represents the mean of three technical replicates that come from three biological replicates ± SE (n = 3). Different letters represent mean values with significant differences between treatments, determined by Tukey's test $P < 0.05$).

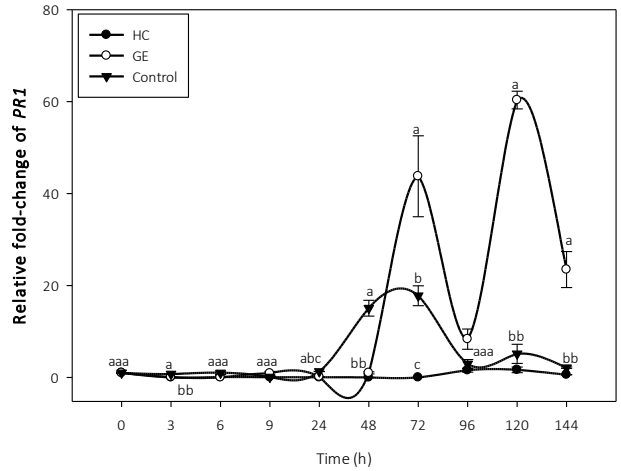

Figure 6. Relative fold-change expression of *PR1* gene in dormant 'Flame seedless' grapevine buds after applying dormancy release agents and by forcing conditions: Hydrogen cyanamide (HC), garlic extract (GE), distilled water (Control). Values were normalized to the mean cycle threshold of time zero. Each value represents the mean of three technical replicates that come from three biological replicates ± SE (n = 3). Different letters represent mean values with significant differences between treatments, determined by Tukey's test ($P < 0.05$).

Relative expression of JA signaling genes

According to the results of the relative fold change expression of the *COI1* gene (Figure 7), a pronounced increase in the fold change was observed in the control buds at 6 and 24 h. The HC-treated buds presented a significant increase in expression from 72 to 144 h, and GE slightly increased at 120 h, with a value that did not exceed the initial value. The expression of the *VSP2* gene was similar to that of the *COI1* gene, where the control buds presented an increase in expression at 6 and 24 h, and the HC-treated buds presented a marked increase

from 72 to 120 h. Meanwhile, the GE-treated buds presented a slight increase in expression at 120 h, with a value that did not exceed the initial value (Figure 8). A significant increase in the expression of the *JAZ1* gene (Figure 9) was detected in the control buds; there was an increase in the fold change at 9, 72, and 120 h. In the HC-treated buds, there was no effect on the fold change in the expression of this gene. By combining these results with the patterns in the expression of the *COI1* and *VSP2* genes, we can infer the activation of the JA signaling pathway by HC.

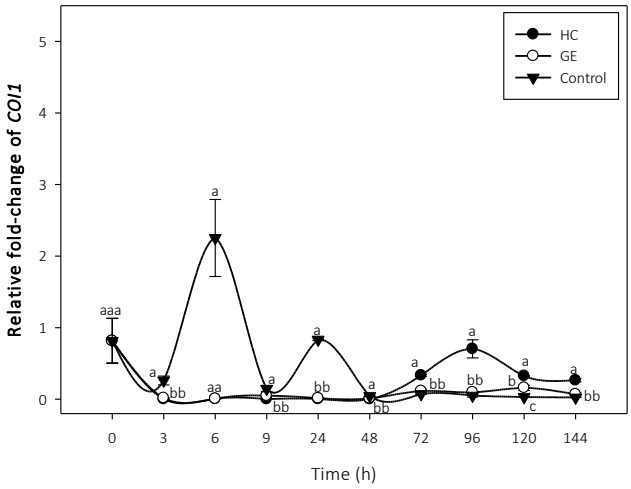

Figure 7. Relative fold-change expression of *COI1* gene in dormant 'Flame seedless' grapevine buds after applying dormancy release agents and by forcing conditions: Hydrogen cyanamide (HC), garlic extract (GE), distilled water (Control). Values were normalized to the mean cycle threshold of time zero. Each value represents the mean of three technical replicates that come from three biological replicates ± SE (n = 3). Different letters represent mean values with significant differences between treatments, determined by Tukey's test (P < 0.05).

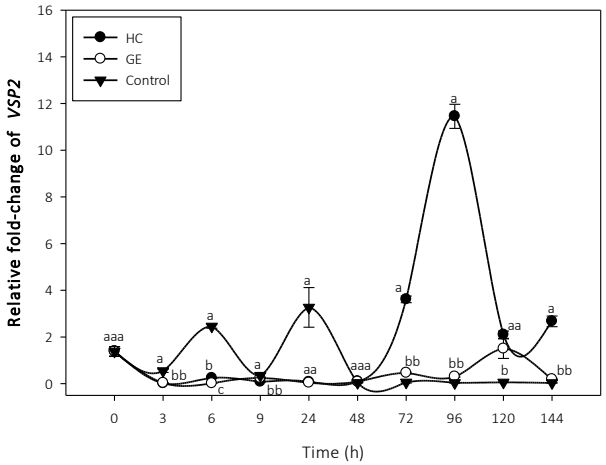

Figure 8. Relative fold-change expression of *VSP2* gene in dormant 'Flame seedless' grapevine buds after applying dormancy release agents and by forcing conditions: Hydrogen cyanamide (HC), garlic extract (GE), distilled water (Control). Values were normalized to the mean cycle threshold of time zero. Each value represents the mean of three technical replicates that come from three biological replicates ± SE (n = 3). Different letters represent mean values with significant differences between treatments, determined by Tukey's test (P < 0.05).

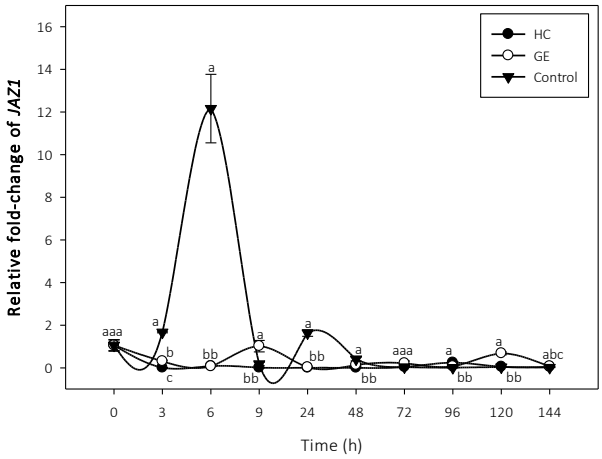

Figure 9. Relative fold-change expression of *JAZ1* gene in dormant 'Flame seedless' grapevine buds after applying dormancy release agents and by forcing conditions: Hydrogen cyanamide (HC), garlic extract (GE), distilled water (Control). Values were normalized to the mean cycle threshold of time zero. Each value represents the mean of three technical replicates that come from three biological replicates ± SE (n = 3). Different letters represent mean values with significant differences between treatments, determined by Tukey's test ($P < 0.05$).

DISCUSSION

Garlic extract (GE) was the dormancy-breaking agent that had the most significant effect on the induction of bud break (64%), which coincides with reports in other studies regarding grapevines, kiwi, and apricots. These studies indicate that GE has a positive effect on bud break and improves plant performance and fruit quality (Salama et al., 2021). This effect is due to GE being rich in S compounds, which are assimilated as amino acids such as cysteine and methionine. These amino acids serve as precursors for synthesizing proteins, metabolites, and growth regulators (El-Senosy et al., 2021). In this study, the low effectiveness of hydrogen cyanamide (HC) in inducing bud break may be due to the grapevine buds having accumulated only 120 chilling units (CUs). In studies regarding kiwi has been reported that the best time to apply dormancy-breaking agents such as HC is when the buds have accumulated ~ 75% of their cold requirements (Powell et al., 2000). In this study, the amount of cold accumulated by the buds did not reach ~ 50% of the cold requirements for 'Flame seedless' grapes, which has been calculated as an average of 350 CUs (Siller-Cepeda et al., 1994).

These results support the concept of bud break and its relationship with jasmonic acid (JA) and salicylic acid (SA) content (Orrantia-Araujo et al., 2021). The GE-treated buds showed the highest SA levels over time (10.43 $\mu g\ g^{-1}$ FW); despite the similar pattern demonstrated by the three treatments in terms of SA content (Figure 2), due to its S compounds, GE promotes the activation of signaling pathways of phytohormones such as SA (El-Senosy et al., 2021). The exact mechanism by which S compounds stimulate SA synthesis is not known. However, these compounds are assimilated as cysteine, which stimulates the synthesis of glutathione (GSH) (De Bont et al., 2022), which is a molecule that has been reported to stimulate SA synthesis (Han et al., 2013a). The SA content observed in the HC-treated buds can be considered a secondary response due to the increase in reactive oxygen species (ROS) generated by the stress caused by this chemical (Sudawan et al., 2016). To control ROS levels, GSH is synthesized (Kovacs et al., 2015), which has already been reported to increase in grapevine buds with the application of HC (Orrantia-Araujo et al., 2019); this GSH triggers SA synthesis (Han et al., 2013a). Nevertheless, the control buds showed a similar pattern to those treated with the dormancy-breaking agents; however, they demonstrated the lowest SA contents.

Glutathione has been related to the activation of SA and *NPR1.* Therefore, the slight increase in the fold change in *NPR1* observed in the buds treated with GE may be due to the increase in this antioxidant metabolite. Higher GSH concentrations have been reported to cause a reduced environment in the cell, which can stimulate

SA synthesis. By increasing the levels of this hormone, redox changes cause *NPR1* to monomerize and enter into the nucleus, thus activating the signaling pathway (Han et al., 2013a). Therefore, *NPR1* functions as a cotranscription factor of SA response genes, such as PR genes (Backer et al., 2019).

The increase in the expression of *NPR1* that was observed in the control buds (4.53-fold increase) (Figure 4) is similar to the findings of other studies performed with grapevines, with one study conducted by Le Henanff et al. (2009) and other conducted in our laboratory, in which we report the constitutive expression of *NPR1* independent of SA in buds without treatment (Orrantia-Araujo et al., 2021).

The expression of *WRKY70* in buds treated with GE has not yet been reported; however, in grapevine buds treated with HC, an increase in the expression of other *WRKY* genes (but not that of *WRKY70* specifically) has been reported (Sudawan et al., 2016). The SA has been reported to induce the expression of a family of *WRKY* genes, which participate in various developmental processes, such as germination, dormancy, and senescence. These genes encode WRKY proteins that function as transcription factors that induce the expression of defense genes such as *PR1* under biotic and abiotic stress conditions (Wang et al., 2023). A slight increase in the fold change in *WRKY70* was observed in both the control and the GE-treated buds. This may indicate that in the buds treated with GE, the activation of the SA signaling pathway was dependent on *NPR1* with the possible participation of *WRKY70*. In contrast, in the control buds, the observed *PR1* expression was mediated by *WRKY70* but was independent of SA (Kumar et al., 2025).

The increase in the fold-change of *PR1* (60.33-fold change) (Figure 6) that was observed in the GE-treated buds confirms the activation of the signaling of the SA pathway due to the fact that it has been reported that by activating the SA signaling pathway, the expression of the *PR1*, *PR2,* and *PR5* genes can be induced (Ali et al., 2017). The PR genes encode a group of proteins related to pathogenesis, which are important aspects of plant immunity and trigger the development of resistance. The PR proteins can have antifungal, antimicrobial, and antiviral properties and can tolerate abiotic stress (Ali et al., 2018). There are currently no studies conducted with dormant grapevine buds treated with a dormancy-breaking agent that have reported the expression of *PR1*. However, it was previously reported that GSH can stimulate the expression of *PR1* in a manner dependent or independent of SA (Kovacs et al., 2015). The increases in the expression of *PR1* in the GE-treated buds were dependent on SA mediated by *NPR1* and *WRKY70*. In contrast, the increases in the control buds were dependent on *NPR1* but independent of SA, thus confirming the increase in the fold change in *NPR1* and the low levels of AS. These results suggest that the application of GE, in addition to providing the buds with a pool of S that is beneficial for their growth, can induce systemic resistance through the expression of *PR1* in response to the activation of the SA signaling pathway. This protects buds against possible adverse conditions that may arise during bud break (Li et al., 2024).

The outstanding effect of HC on the increase in JA (44.94 $\mu g\ g^{-1}$ FW) (Figure 3) coincides with that reported in studies performed with dormant buds of sweet cherry that were treated with this same chemical (Ionescu et al., 2017). This result occurred because HC is a compound that provides N (Jamshidian et al., 2024), which causes an increase in endogenous levels of JA when supplied, as reported in a study performed with rice plants (Jang et al., 2008). An increase in this hormone benefits the plant because, in addition to participating in defense mechanisms, it regulates various processes, such as seed development and germination. The JA has also been reported to be essential in inducing bud-breaking flower development and flower opening (Ionescu et al., 2017).

Although GE caused an increase in JA levels, these levels were lower than those induced by HC. To date, no studies have reported the influence of GE on JA contents in dormant buds. However, our laboratory previously reported that GE in dormant grapevine buds stimulates increased GSH content (Orrantia-Araujo et al., 2019). In *Arabidopsis*, GSH has been reported to stimulate the expression of JA synthesis and signaling genes (Han et al., 2013b). Consequently, an increase in the endogenous content of this hormone, which was observed in this study, can be expected. However, the levels of JA in the control buds were so low that they could not be detected via HPLC.

The increase in the expression of *COI1* in the HC-treated buds (0.70-fold change) (Figure 7) indicates that part of the existing JA can be activated by *JAR1* to be detected by *COI1*, thus leading to the initiation of signaling. The *COI1* gene codes for a JA receptor protein, through which the signaling of this hormone begins, thereby inducing the expression of response genes such as *VSP2* (Ulrich et al., 2021). Therefore, we can theorize that HC caused the activation of the JA signaling pathway. In *Arabidopsis*, increased GSH content stimulates the synthesis and expression of JA signaling genes (Han et al., 2013b). In a previous study that was performed in

our laboratory by Orrantia-Araujo et al. (2019) performed with dormant grapevine buds, it was reported that HC causes an increase in GSH levels. Therefore, HC may activate the JA signaling pathway through an increase in GSH levels due to the oxidative stress caused by H_2O_2 and nitric oxide-derived radical (ON), which this chemical temporarily generates.

Although a slight increase in the expression of *COI1* was observed in the GE-treated buds, this treatment elicited the lowest expression levels, which may not be enough to trigger signaling. In the control buds, despite JA being undetectable, an unexpected increase in the expression of *COI1* was observed (2.25-fold change), which may be due to the presence of a molecule other than JA that also binds to *COI1*, such as 12-oxo-phytodienoic acid (OPDA), which has been reported in studies performed with *Arabidopsis* (Liu and Park, 2021). However, more studies are needed to ensure this result's validity.

The high levels of expression of *VSP2* (11.45-fold change) that were demonstrated in the HC-treated buds may be due to the *VSP* genes being regulated both by the supply of N and by the increase in JA (Xiong et al., 2025), which are stimulated by this chemical. These results are similar to those reported by Horvath et al. (2008) in a study performed with dormant buds of *Euphorbia esula* L., which reported that high expression of *VSP2* indicates the presence of storage proteins that prepare the buds for breaking dormancy. This effect is because the proteins that are encoded by this gene temporarily store amino acids and participate in the redistribution of N (Xiong et al., 2025).

The slight increase in the expression of *VSP2* observed in the GE-treated buds may be due to the supply of nutrients provided by this extract, which is rich in S compounds (El-Senosy et al., 2021). The exact mechanism by which S and its derivatives affect the metabolism of JA is unknown; this effect could involve GSH. However, more research is needed concerning this possible mechanism. Furthermore, the increase in the expression of *VSP2* observed in the control buds (3.26-fold change) is similar to that reported in studies performed with soybean plants, in which the expression of *VSP2* tends to increase in developing organs or in response to some type of stress, such as mechanical injuries or injuries caused by insects (Utsugi et al., 1998). However, it is not easy to establish a clear reason without further studies.

The pronounced increase in the expression of *JAZ1* (12.16-fold change) observed in the control buds may indicate a possible abundance of JASMONATE ZIM-domain proteins (JAZ) due to the low levels of endogenous JA (Ghorbel et al., 2021). Therefore, the abundance of JAZ proteins can lead to the repression of JA signaling, thus affecting the expression of response genes such as *PDF1.2* and *VSP2* by binding *JAZ1* with MYC transcription factors (Ghorbel et al., 2021). However, the slight fold change increase in *VSP2* observed in these buds is somewhat contradictory. It has been reported that there may be different target molecules of JAZ proteins in specific tissues and certain stages of tissue or plant development (Fernández Barbero, 2015). Therefore, *JAZ1* can repress the expression of other target genes. The slight increase in the fold change of *JAZ1* observed in the GE-treated buds was not high enough to ensure its repressive function on JA signaling. In addition, a slight increase in the fold change of the *VSP2* gene was also observed in these buds. Therefore, more studies are needed to clarify or identify the types of transcription factors involved in the expression of *VSP2* in dormant grapevine buds dependent on and independent of both *COI1* and JA.

In contrast, HC did not significantly affect the induction of *JAZ1* expression. This result is similar to that reported in a study performed with dormant sweet cherry buds treated with HC, wherein the authors noted that this inducing agent did not affect the expression of this gene (Ionescu et al., 2017). Therefore, an increase in the expression of the *VSP2* response gene was observed in this study. This is because the repressive nature of *JAZ1* does not affect the expression of *VSP2* because JAZ proteins function as coreceptors and transcriptional repressors in the JA signaling pathway (Ghorbel et al., 2021). These results suggest that HC (through the generation of oxidative stress) stimulates an increase in JA concentrations and the activation of the signaling pathway of this hormone. In the case of GE, it is also influenced by the rise in the endogenous content of JA, although it does not activate signaling.

CONCLUSIONS

In this study, different dormancy-breaking agents (hydrogen cyanamide and garlic extract) were shown to stimulate the metabolism of defense hormones such as salicylic acid and jasmonic acid in grapevine buds, thus activating their signaling. Garlic extract positively affected bud break and salicylic acid signaling; conversely,

hydrogen cyanamide did not have a significant effect on bud break but stimulated the jasmonic acid signaling. With the increase of both hormones, the bud defense system is strengthened and favors bud breaking and flowering. Therefore, it can be inferred that the signaling pathways of both hormones may significantly participate in the dormancy release of grapevine buds, which is critical to understand to directly influence the hormonal systems of the buds in the future and to generate more efficient production systems for different deciduous fruit trees.

Author contributions

Conceptualization: M.A.O-A. Methodology: C.C-M., E.A-H. Software: Validation: I.V-A. Formal analysis: M.A.M-T. Investigation: M.A.O-A. Resources: I.V-A. Data curation: M.A.O-A. Writing-original draft: M.A.O-A. Writing-review & editing: I.V-A. Visualization: M.R-D. Supervision: I.V-A. Project administration: I.V-A. Funding acquisition: M.A.M-T. All co-authors reviewed the final version and approved the manuscript before submission.

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