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A synthetic cytokinin primes photosynthetic and growth response in grapevine under ion-independent salinity stress

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ABSTRACT

Aiding optimal plant–environment interaction would favor plant resilience against environmental constrains including salt stress. We test the hypothesis that 6-Benzylaminopurine (BAP) primes grapevine's salt tolerance in vines (*Vitis vinifera*) received salt water (NaCl 100 mM) through the modulation of gene expression of BAP (*AHK4*, *AHP1*) and salt-stress (*CAT*, *APX*) inducible genes and morpho-physiological traits. A subgroup of vines had previously (48 h) been primed with BAP (80 mg/L) before salt stress. The gene expressions were 30% (*CAT*) and 56% (*APX*) lower in primed salt-stressed vines than that in un-primed. Salt treatment did not increase leaf Na^+ but it lowered stomatal conductance (g_s), photosynthesis (A), stem water potential (less negative) and photosystem-II efficiency (F_v/F_m). Chlorophyll-*a* concentrations were 30% higher in BAP-primed compared to un-primed. Adverse effects of salt were significantly reduced, maintaining high A/g_s, F_v/F_m and growth. After the relief of the stress, the BAP primed vines had a fast recovery.

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APX; *BAP*; *CAT*; gene activation; leaf gas exchanges; salt tolerance

1. Introduction

Climate change threatens agriculture through impaired plant growth resulting from changes in a large number of environmental factors (e.g. higher temperatures, extreme weather events, milder winters, reduced precipitation). These factors trigger extensive irrigation with deep (and salt) water contributing to increasing salt affected areas up to 20–30% of the irrigated land (Sahab et al. 2021). Central to increasing crop resilience to soil salinity will be an identification of the physiological, biochemical, molecular and genetic bases of salt tolerance (Zörb et al. 2019) to aid plant–environment interaction and develop 'next generation agriculture' (Guzmán et al. 2021).

The initial (stage 1) plant response to soil salinity mimics that to drought, it depends not on the accumulation of ions in the plant tissues but on the osmotic effects of the increased salt concentration in the rhizosphere (Isayenkov and Maathuis 2019). During stage 1 the plant suffers reduced carbon gain resulting from reduced stomatal opening, which is presumably targeted at reducing transpiration and the rate of uptake of water and thus of salt from the soil. Consequently, within a relatively short time (days or even minutes) shoot growth rates and leaf area expansion rates are reduced (Munns and Tester 2008). This ion-independent stage 1 of salt stress is attracting attention (Atieno et al. 2017) but it has not been much explored in perennials. Later, with persistent salt stress, plants enter the stage 2 response showing premature senescence and cell death due to the accumulation of toxic levels of ions.

The reduction of growth under the stage 1 response appears to be regulated by hormones (Munns and Tester 2008). In this respect, abscisic acid (ABA) is likely to be a key signaling molecule because of its link with stomatal aperture (Belfiore et al. 2021; Hsu et al. 2021) and its effect on lowering the levels of the other hormones involved in growth (e.g. of gibberellins and cytokinins) (Rulcová and Pospíšilová 2001; Munns and Tester 2008).

Cytokinins have been shown to lie behind a number of putative mechanisms (see Li et al. 2021 for review), emphasizing their central role in a range of plant growth responses to stress. Synthetic cytokinins are sometimes applied to mitigate certain environmental constraints (Oshchepkov et al. 2020).

The enhancement of plant tolerance to salinity embraces the use of mycorrhizal fungi, exogenous application of minerals and of analogue of brassinosteroids (Ahmad et al. 2018; Shi-chu et al. 2019) and the use of chemical 'priming' is attracting increased attention. Here, plants are treated with priming agents (including with hormones) prior to a stress exposure (Sako et al. 2020).

The 6-Benzylaminopurine (BAP) is among the synthetic cytokinins sometimes used to mitigate the negative effects of drought because of its effect of reducing stomatal aperture (Rulcová and Pospíšilová 2001; Nawaz et al. 2016; Geraci et al. 2018). Exogenous applications of BAP have also been reported to counteract salt-induced senescence in rice by limiting the salt-induced degradation of chlorophyll (Mitsuya et al. 2003). However, to our knowledge, how the application of exogenous cytokinins (such as BAP) changes the plant-salinity physiological interaction in grapevine has not been much explored.

Grapevine is vulnerable to salt stress which can be partly overcome by introducing genetically modified lines or by

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new rootstock-scion combinations (Marín et al. 2021). Recently, putrescine-functionalized nanoparticles have been examined as a chemical priming agent in grapevine (Gohari et al. 2021). Expanding knowledge on cytokinins impact on plant-salinity interaction might improve adaptation and should reduce the vulnerability of the global viticulture industry to salinity.

On this background, the first aim of this study was to test the hypothesis that BAP is a priming agent that activating the expression of cytokinin signaling genes in grapevine, primes it against later salt stress by better helping maintain stomatal opening and thus carbon assimilation. The study also examines the associated hypothesis that recovery from salt stress is accelerated by priming with BAP. The relative expressions of selected marker genes induced by salt stress and by external BAP treatments were measured to help confirm these hypotheses. It was also hoped to gain further insights into the effects of salt on grapevine and on the induction of salt tolerance at the level of gene expression.

2. Materials and methods

2.1. Plant material and experimental design

The experiment was conducted at the ALSIA-CRMA research Centre, southern Italy (40°23′31.4″N, 16° 47′10.9″E). We used grapevines (*Vitis vinifera* L) cv. Aleatico grafted on SO4 rootstocks (*Vitis berlandieri* Planch × *Vitis riparia* Michx) growing in a greenhouse. Vines were two years old, with each comprising a short vertical length (*c.* 0.22 m) of the previous season's wood, surmounted by a single vertical leafy shoot (*c.* 1 m, ∼15 nodes) of current season's growth with a leaf at each node. Vines were grown in 3.5 L PVC pots. The growth substrate was a 3:1 v/v mixture of sandy loam soil (82% sand, 7% silt and 11% clay) and peat. Plants were fertilized 15 days before the experiment using 3 g per pot of NPK fertilizer 14.7.14 (Slowenne 212, Valagro Spa, Atessa, Italy). Vines were irrigated daily (about 19 h) so as to restore 100% of daily water consumption and maintain soil moisture at field capacity (Figure S1).

For the various sampling, physiological measurements, the vine canopy was partitioned into lower (nodes 1–5 from the base of the current season's growth), middle (nodes 6–11) and upper (nodes >11) region of a plant canopy according to Briglia et al. (2020).

2.2. Treatments

A number of vines were prepared as above and a group of them ($n = 56$) were selected for uniformity. The selected vines were then assigned at random among four groups (G1, G2, G3 and G4). The experimental units (each unit comprising a pot plus a vine) were randomized spatially in the glasshouse to minimize potential spurious effects due to non-uniform conditions (Hurlbert 1984). The experiment was designed with two treatments (cytokinin, salt) which were imposed either individually, or in combination, and an untreated water control.

Cytokinin treatment: On 15 July 2019 (day 0) the whole canopies of two groups of vines, G1 ($n = 14$) and G2 ($n = 14$) were sprayed (primed) with an aqueous solution (80 mg/L) of the synthetic cytokinin 6-Benzylaminopurine (BAP) (Sigma-Aldrich; catalogue number B3408-5G; Castle Hill, NSW, Australia) using a portable backpack sprayer. The G1 vines were labeled *[BAP]*. These were later used for gene expression analysis of BAP-inducible genes (see below). The rationale for our choice of BAP at a concentration of 80 mg/L was based on published reports showing the positive effects of BAP in mitigating abiotic stress tolerance at similar concentrations (e.g. Nawaz et al. 2016). Meanwhile, higher BAP concentrations (200 and 400 mg/L) can reduce internode length and increase the emergence of lateral branches (Zhang et al. 2018).

Salinity treatment: Two days later on 17 July 2019 (day 2) the primed G2 ($n = 14$) and un-primed G3 ($n = 14$) vines were irrigated with a salt solution comprising tap water and NaCl at concentrations of 50 mM (day 2), 75 mM (day 3) and 100 mM from day 4 to day 11. In the afternoon of day 11, the salt stress was relieved by over-irrigating with tap water. The G2 vines were labeled *[BAP + SALT]* and the G3 vines were labeled *[SALT]*. The remaining group (G4, $n = 14$) received neither salt nor BAP and was labeled *[CTRL]*. This group served as the controls for all determinations.

Throughout the experiment, air temperature (°C) and relative humidity (%) (HUMITER 50Y, Vaisala, Helsinki, Finland) and PAR (PPFD, μmol m^{-2} s^{-1}) (quantum sensor Model SKP 215, Skye Instruments LTD, Llandrindod, Wells, UK) were measured inside the greenhouse at 15 min intervals, and hourly averages were recorded (CR200, Campbell Scientific Inc., Utah, USA). The air vapor pressure deficit (*VPD*) was later calculated from the records of air temperature and relative humidity, according to Goudriaan and van Laar (1994).

2.3. Gas exchange, fluorescence, leaf water potential and soil electrical conductivity

For all four groups, net photosynthetic rate (A, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mol H_2O m^{-2} s^{-1}) and transpiration rate (E, mmol H_2O m^{-2} s^{-1}) per unit leaf area were measured around midday (from 11:30 to 12:30 h) on days 0, 2, 3, 4, 5, 8 and 11 using a portable photosynthesis system Li-Cor 6400 (Li-Cor, Inc., Lincoln, NE, USA). Gas exchanges were also measured on days 4 and 7 after salt stress relief (day 11). During gas exchange measurements the reference CO_2 concentration inside the cuvette was set to the prevailing environmental condition, PAR inside the cuvette was fixed at 900 μmol m^{-2} s^{-1} and the operating flow rate at 500 μmol s^{-1}. Gas exchange measurements were carried out on three vines per treatment using two fully-expanded leaves per vine, these were selected from the mid-region of the vine.

Concurrently with gas exchange measurements and on the same leaves, the photosystem II efficiency (F_v/F_m) was measured using a portable chlorophyll fluorometer (FluorPen FP 100 (Photon Systems Instruments, Drasov, Czech Republic)). A one-hour dark-adaptation was ensured before measurement using the FluorPen leaf clips.

Stem water potential (SWP, MPa) was measured using a Scholander type pressure chamber (Model 600, PMS Instruments, Corvallis, OR), pressurized with nitrogen, according to the protocol by Turner (1981) and Choné et al. (2001). Briefly, for SWP determination, one fully expanded leaf per vine (×3 vines per treatment) was tagged on the middle region of the main shoot. The still-attached leaf was then

covered with aluminum foil for at least 180 min before SWP measurement. The leaf was then sealed in a plastic bag, detached and promptly pressurized in the pressure chamber and the balance pressure recorded.

At the same time as the leaf gas exchange measurements, soil electrical conductivity (EC, dS m^{-1}) was measured in each pot using a single channel meter equipped with a TDR-based probe (W.E.T. sensor, Delta-T, Devices, Cambridge, UK).

2.4. Pigment and ion concentration analysis

After the SWP measurements, two disks per leaf (each 6.16 cm^2) were excised and promptly stored at −80°C pending chlorophyll (Chl) and total carotenoids (xanthophyll plus carotenes) determination. Each disk was separately crushed under liquid N_2, and the powder was extracted with 10 mL EtOH for 24 h at room temperature (*c*. 20°C). Absorbances of the supernatant at 470, 649 and 665 nm were determined spectrophotometrically (Varian 50-BIO, Varian Australia Pty Ltd, Victoria 3170 Australia) and the amounts of Chl-*a*, Chl-*b* and carotenoids were calculated using the formulae reported in Lichtenthaler and Wellburn (1983).

On the last day of salinity stress (day 11), leaves were sampled from the upper, middle and lower regions of the shoots of three vines per treatment. Each sample was separately dried to constant weight in a ventilated oven and analysed for potassium and sodium concentrations (ICP 4200 MP-AES, Agilent Technologies, 5301 Stevens Creek Blvd. Santa Clara, CA 95051, US) reported as mass per unit of dry weight.

2.5. Shoot length and root mass

At the beginning of the experiment d 0 (before treatments) and on the last day of salt stress (day 11), the mean vine height was determined on five vines per treatment measuring the length of the current season shoot. On the last day of salt stress treatment, the lengths of the eight distal internodes were also measured on the same vines. At the end of the salt stress (day 11) after careful removal of the substrate using water, three root subsamples were collected from each plant (×5 plants per treatment). Each root subsample consisted of a whole lateral permanent root of approx. 10 cm length branching directly off the main root (>10 mm diameter). For root dry matter measurement, the root subsample was then partitioned in its coarse (approx. >2 mm diameter) and fine root components (<2 mm diameter) and dried to constant weight at 70°C in a ventilated oven. The fine coarse root dry matter ratio was then recorded for each plant.

2.6. Gene expression analysis: total RNA extraction and quantitative RT–PCR

For the gene expression analysis, 3 leaves per vine were collected from 3 to 5 vines per treatment and immediately transferred into liquid nitrogen. Total RNA was extracted from 100 mg of frozen leaves from a total of 36 samples, using three biological replicates for each treatment.

Leaves for salt-inducible gene expression (see below) were collected from *[CTRL]*, *[BAP + SALT]* and *[SALT]* vines on day 5 and day 8. To test for BAP-inducible genes (see below) leaves were sampled from *[CTRL]* and *[BAP]* vines 3 h after BAP application and again on days 5 and 8 followed timings similar in range to those reported in Das and Majumder (2019).

The innuPrep Plant RNA Kit (Analytik Jena, Jena, Germany) was used for RNA extraction according to the manufacturer's instructions. Before extraction, leaves were manually ground under liquid nitrogen to a fine powder using mortar and pestle. Concentration, purity and integrity of RNA were monitored using an ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). RNA samples were checked to exclude residual genomic DNA contamination. First strand cDNA was prepared from the total RNA with oligo$(dT)_{16}$ using SuperScript® IV Reverse Transcriptase (Invitrogen/ Thermo Fisher Scientific, USA) as described by the manufacturer.

The quantitative RT–PCR (qRT–PCR) was carried out using an ABI Prism® 7900HT instrument (Applied Biosystems/Thermo Fisher Scientific, USA) and Platinum® SYBR-Green® qPCR SuperMix-UDG with ROX (Invitrogen/ Thermo Fisher Scientific, USA); three technical replicates for each RNA sample were used. PCR conditions were 5 min at 95°C followed by 40 cycles at 95°C for 15 s and at 58°C for 60 s.

Primers for the qRT-PCR analysis of Histidine kinase 4 (*AHK4*), and Histidine phosphotransfer protein 1 (AHP1) were designed using Primer 3 program (http://bioinfo.ut.ee/primer3-0.4.0/) while primers for Catalase (*CAT*), Ascorbate peroxidase (*APX*), and Actin reference gene (*ACT*) were available from Das and Majumder (2019). Oligos were synthesized by Invitrogen/Thermo Fisher Scientific and are listed in (Supplemental material Table S1). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of each gene examined.

2.7. Data analysis

Data processing and curve fitting employed OriginPro 9.1.0 (OriginLab Corporation, Northampton, MA 01060 USA). A one-way ANOVA was used to examine the differences between treatments and the Duncan test as a *post-hoc* test for multiple comparisons, p-values <0.05 were considered significant. The function to fit the EC vs Dark Green color band fraction and the threshold EC (EC*t*) were determined according to Martinez-Cob et al. (1987).

3. Results

3.1 Morpho-physiological effects of the priming chemical agent

The application of NaCl through the irrigation water *[SALT]* significantly reduced the elongation of the distal internodes of grapevine, with lengths being about 32% less than in the water controls *[CTRL]* at the end of the salt stress (Figure 1(A)). However, BAP significantly reduced this growth-inhibition due to salinity with shoot lengths in *[BAP + SALT]* being 15% less than in the water controls *[CTRL]* (Figure 1 (A)). Priming, reduced internode length in the *[BAP]* vines by approx. 20% compared to the *[CTRL]* vines. The growth reduction induced by salt and the beneficial effects of BAP are also evident in a comparison of vine heights. Throughout the experimental period, vine height increased by an average

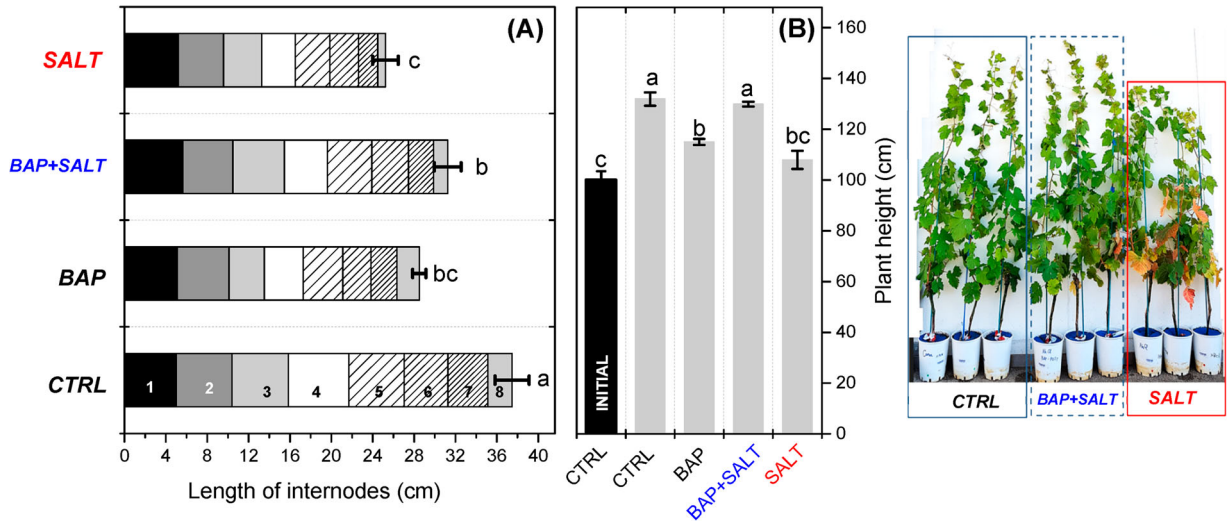

Figure 1. (A) Lengths of the distal eight internodes (single boxes in each horizontal bar) measured on the last day of stress on the main shoot of a vine under salt stress *[SALT]*, primed with 6-Benzylaminopurine *[BAP + SALT]*, control *[CTRL]* conditions and primed with the BAP *[BAP]*. Error bars and statistics refer to the total lengths of the measured nodes. (B) Average height of vines (from ground to apex) measured on the initial (black filled columns) and final (gray filled columns) day of stress. Comparing treatments different letters indicate statistically significant differences ($p < 0.05$). On the right, a view of vines (×3 per treatment, excepting the *[BAP]*) pictured on the last day of the salt stress.

about 30% in both the *[CTRL]* and the *[BAP + SALT]* vines, by about 15% in the *[BAP]* vines and by only about 7% in the *[SALT]* vines (Figure 1(B)).

The dry mass of fine roots (<1 mm) after 9 days of salt treatment in the *[SALT]* vines, was significantly lower than in the *[CTRL]* vines (results not shown). This led to about a 30% reduction in the ratio of fine/coarse roots in the *[SALT]* vines, than in the *[CTRL]* vines, while in the *[BAP]* vines, the ratio remained similar to that in the *[CTRL]* vines (Figure 2). After the 9 days of exposure to salt, potassium and sodium concentrations (and their ratio) in the leaf collected from the upper, middle and lower region of the canopy were not significantly influenced by exposure to salt or BAP (Supplemental material Figure S2).

During the experiment, the soil EC increased progressively and similarly in both *[SALT]* and *[BAP + SALT]* reaching a maximum of about 9 dS m^{-1}, while it remained stable

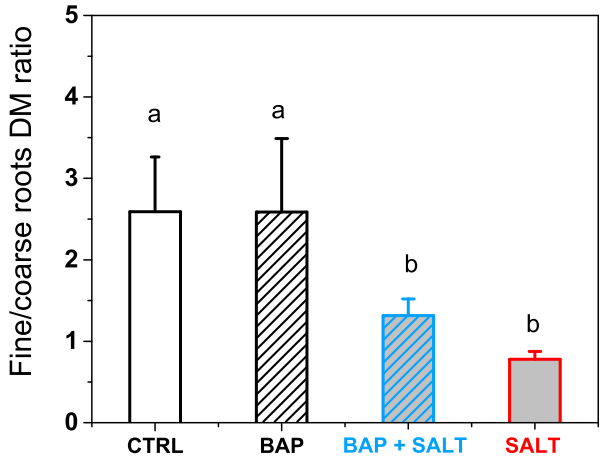

Figure 2. Fine/coarse root ratios in vines exposed to salt *[SALT]*, vines primed with the synthetic cytokinin BAP then exposed to salt *[BAP + SALT]*, in unprimed vines *[CTRL]* and vines primed with BAP *[BAP]* measured on the last day of exposure to salt (i.e. day 11 after priming was imposed). Data are the means (±SE) of 15 intersections of roots sampled from five pots per treatment. Different letters indicate statistically significant differences ($p < 0.05$).

at about 0.8 dS m^{-1} in *[CTRL]* and *[BAP]* vines (Figure 3 (A)).

Variations in SWP are reported in Figure 3(B) showing a prompt decline to about −0.6 MPa in the *[SALT]* vines, while in *[BAP + SALT]* vines SWP was similar to that in the *[CTRL]* vines for the first two days of salt treatment ranging from −0.3 to −0.45 MPa. Thereafter, as soil EC increased, SWP continued to decline with no statistically significant difference between the *[BAP + SALT]* vines and the *[SALT]* vines. By day 11 SWP approached about −0.6 MPa in the *[SALT]* vines independent of BAP treatment (Figure 3(B)). The SWP values of the *[BAP]* vines (ranging from −0.3 to −0.53 MPa) were similar to those of the *[SALT]* vines but with values about mid-way between those of the *[SALT]* vines and the *[CTRL]* vines.

One day following exposure to salt (day 3), stomatal conductance in the salt-stressed vines *[SALT]* had reduced to about 60% of that of water controls *[CTRL]* (Figure 4(A)). However, the following day the reduction in g_s was less pronounced (Figure 4(A)) likely due to a mild *VPD* (Figure S3). During the following days, the BAP-primed vines *[BAP + SALT]* decline in g_s was significantly slowed and this remained at a level close to 80% of that of the controls *[CTRL]*, however by day 8 the effect of BAP on maintaining the value of g_s vanished (Figure 4(A)). In the *[BAP]* vines, exposure to BAP in the absence of salt markedly decreased g_s to a lowest values at about day 4, but the g_s values then gradually recovered towards those in the *[CTRL]* vines, being transiently greater than in the *[CTRL]* vines at day 15 (Figure 4(A)).

As expected, the reduced stomatal conductance reduced transpiration (Figure 4(B)) and photosynthetic rate (Figure 4(C)) which changed in proportion to the changes in g_s. However, photosynthetic rate in the *[BAP + SALT]* vines remained significantly higher than in the *[SALT]* vines, except for the initial value (Figure 4(C)). Notably, compared with in the *[CTRL]* vines, transpiration in the *[BAP]* vines was higher by 54% (day 11) and by 20% (day 15).

The variable photosystem II efficiency (F_v/F_m) in *[CTRL]* vines was on average at 0.76 ± 0.003 (SE) throughout the

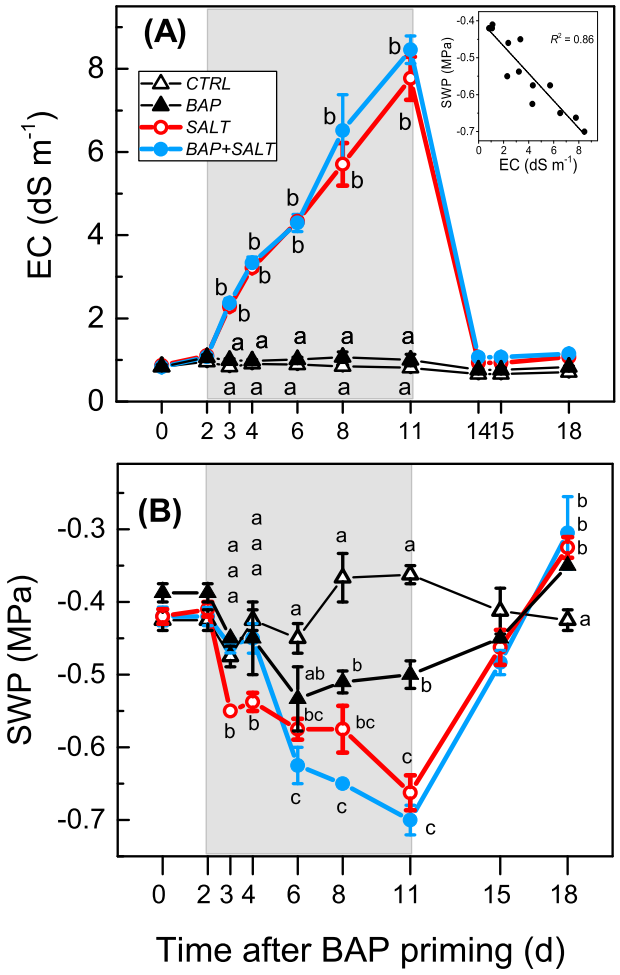

Figure 3. Mean values (±SE) of (A) soil electrical conductivity (EC) and (B) stem water potential (SWP) measured during the experiment in vines under salt stress (**o**, *[SALT]*), under salt stress primed with the BAP (●, *[BAP + SALT]*), control (Δ, *[CTRL]*) and vines primed with BAP (▲, *[BAP]*). Comparing treatments at the same time different letters indicate statistically significant differences ($p < 0.05$). Note that when differences among treatments were not statistically significant letters are not reported. SE bars are visible only when larger than the symbol; in the inset the correlation between soil EC and stem water potential (SWP) of vines under salt stress, note that data from BAP-primed *[BAP + SALT]* and un-primed *[SALT]* vines were pooled before linear fitting. The gray filled area indicates the salt-stress period.

experiment (Figure 5). During the salt stress period, F_v/F_m in *[BAP + SALT]* vines remained suboptimal and statistically comparable to that of the *[CTRL]* vines being on average 0.72 ± 0.01 (Figure 5), while the mean value of F_v/F_m in *[SALT]* vines was statistically significantly lower than *[CTRL]* being 0.68 ± 0.01. Exposure to BAP in the *[BAP]* vines gradually decreased F_v/F_m towards a minimum of 0.69 ± 0.004 at day 6 which was significantly lower (about 10%) than that in the *[CTRL]* vines. Thereafter, the fluorescence index recovered to values similar to those in the *[CTRL]* vines. At day 15 (4 days after the relief of salt stress) the F_v/F_m metabolic limitation of photosynthesis was fully recovered in both *[SALT]* and *[BAP + SALT]* vines i.e. independently of BAP application (Figure 5).

At the end of salt stress period (day 11 from priming) Chl-*a* concentration was significantly influenced by treatments application being 1.06 ± 0.05 (SE) mg g^{-1} DW (*[CTRL]*), 0.89 ± 0.04 mg g^{-1} DW (*[BAP + SALT]*) and 0.68 ± 0.05 mg g^{-1} DW (*[SALT]*) (Figure 6). Also *[BAP]* vines showed reduced values Chl-*a* compared with those in the *[CTRL]* vines (Figure 6).

At the 4th day after relieving exposure to salt (day 15 from BAP priming) the Chl-*a* concentration in the *[BAP + SALT]* vines recovered to values similar to those in the *[CTRL]* vines while in the *[SALT]* and *[BAP]* vines Chl-*a* remained significantly lower than in the *[CTRL]* vines.

At day 11 (and day 15) from BAP priming, the concentration of Chl-*b* was not significantly influenced by group treatments (if the *BAP]* vines at d 11 are excepted) averaging about 0.34 ± 0.02 mg g^{-1} DW across the treatments (Figure 6). At both day 11 and day 15 from BAP priming carotenoids in the *[BAP + SALT]* and *[CTRL]* vines were similar, at about 0.9 (day 11) and 1.1 (day 15). The carotenoids in the *[SALT]* and *[BAP]* vines were significantly lower than in the *[CTRL]* vines at both day 11 and day 15 (Figure 6).

3.2. Expression analysis of selected marker genes

The gene expressions of both *CAT* (Figure 7(A,B)) and *APX* (Figure 7(C,D)) determined at day 5 and 8 after BAP priming were significantly higher in salt-stressed vines *[SALT]* than in control *[CTRL]* ones. The relative expression levels of *CAT* and *APX* were significantly lower in salt-stressed vines primed with BAP *[BAP + SALT]* than that in un-primed ones *[SALT]*, with reductions close to 20 (day 5) to 30% (day 8) for *CAT* and from 24% (day 5) to 56% (day 8) for *APX* (Figure 7). Expression of *APX* at day 8 after priming was even lower than that in control vines (Figure 7(D)).

In our study, the expressions of *AHK4* and *AHP1* were measured in the BAP sprayed *[BAP]* vines and compared with the controls *[CTRL]*. As shown in Figure 8 both *AHK4* and *AHP1* gene expressions were significantly higher in BAP-primed vines *[BAP]* compared to control vines *[CTRL]*. For *AHP1* expression, the difference was observable just 3 h after BAP treatment and decreased progressively after day 5 and day 8 (Figure 8(A)). Conversely, for *AHK4* the difference in expression between treatments increased with time and was about 6-times higher in BAP-primed *[BAP]* than in un-primed control *[CTRL]* vines (Figure 8 (B)).

4. Discussion

This study reports on the ability of a single application of the synthetic cytokinin BAP to produce a 'primed' *status* in grapevines, so as to allow a faster response to later exposure to salt with both morphological and physiological traits superior to those in un-primed vines. The ability of BAP to alleviate salt-induced stress has been documented in model plants of *Arabidopsis* spp. (Golan et al. 2016) and in some annual crops including rice (Mitsuya et al. 2003), wheat (seed priming) (Bajwa et al. 2018) and sunflower (Mahto et al. 2021). Although BAP is now considered a promising priming agent for counteracting abiotic stresses (Mahto et al. 2021) the published results regarding its usefulness to mitigate salt stress in perennial species is limited.

Spraying with BAP likely affected the leaf cytokinin level as supported by the induction of the corresponding signaling genes (Figure 8) suggesting the establishment of a 'primed' status. In addition, the induced primed status was also detected through the analysis of the *[BAP]* vines across the morpho-physiological variables measured. The extent of the changed cytokinin level and its value relative to the naturally occurring hormone remain to be elucidated.

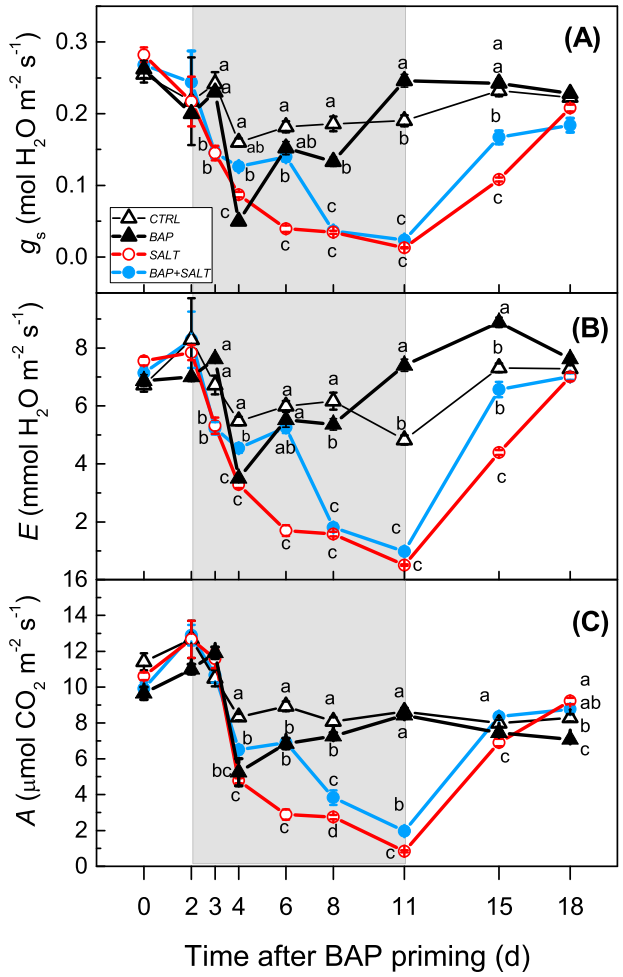

Figure 4. Variations in (A) stomatal conductance, (B) transpiration and (C) photosynthetic rate measured in vines under salt stress (**o**, *[SALT]*), under salt stress primed with the BAP (●, *[BAP + SALT]*), control (Δ, *[CTRL]*) and vines primed with BAP (▲, *[BAP]*) during the salt stress (gray filled area) and recovery periods. Comparing treatments at the same time different letters indicate statistically significant differences ($p < 0.05$). Note that when differences among treatments were not statistically significant letters are not reported. Bars are SE and are visible only when larger than the symbol.

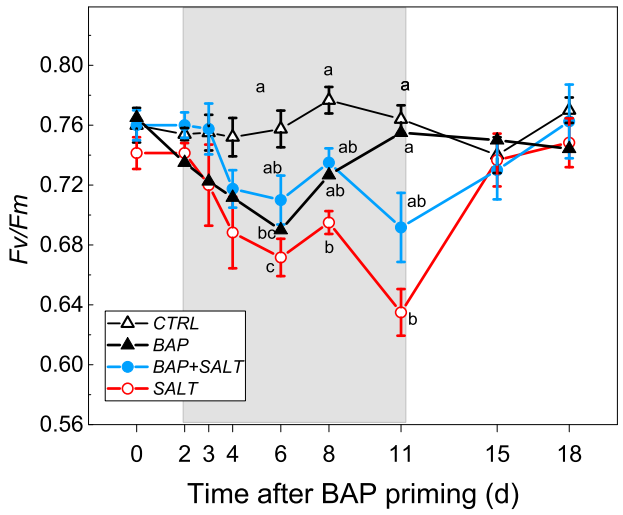

Figure 5. Variation in chlorophyll fluorescence measured in vines under salt stress (**o**, *[SALT]*), under salt stress primed with BAP (●, *[BAP + SALT]*), control (Δ, *[CTRL]*) and vines primed with BAP (▲, *[BAP]*) during the application of salt (gray filled area) and recovery phases. Comparing treatments at the same time different letters indicate statistically significant differences ($p < 0.05$). Note that when differences among treatments were not statistically significant letters are not reported. Bars are SE and are visible only when larger than the symbol.

Early effects of salt stress are associated with the osmotic effects of the salt in the soil, rather than with the toxic effects of the salt in the tissues (Munns and Tester 2008). Here, the salt treatment (over a 9-day period) was not long enough to significantly increase the Na^+ concentrations (or lower the K/Na ratio) in the leaf tissues, compared with those in the controls *[CTRL]* (Figure S2) in contrast with much longer-term, salt-stress experiments (e.g. 30–40 days, Baneh et al. 2014; Gohari et al. 2021).

Considering the similar leaf Na^+ (and leaf K^+) concentrations among our treatments (Figure S2), in the face of significant morpho-physiological impairments (see below) it might be inferred that we were observing a first stage (osmotic) stress response to salt *in sensu* Munns and Tester (2008), however further evidences such as genes involved in osmolytes synthesis or proline quantification remain to be specifically tested.

4.1. Morphological effects of the priming chemical agent

Cytokinins are essential for plant development, to the extent that growth in cytokinin-deficient plants is inhibited and internodes are short (Werner et al. 2001). Hence, exogenous cytokinin applications or reductions in its enzyme-based degradation are expected to overcome growth limitations due to environmental constraints including salinity.

Application of BAP effectively antagonized the plant growth reduction induced by salt stress as in the *[BAP + SALT]* vines plant height and the lengths of the distal internodes were significantly greater than in the *[SALT]* vines.

In our study, comparing vine height in *[CTRL]* and *[BAP + SALT]* showed similar final heights which conceivably masked the difference of the examined distal internodes. As the carbon gain is essential for plant growth, the comparable heights of *[CTRL]* and *[BAP + SALT]* was sustained by the relatively high photosynthetic rate induced by BAP in salt-exposed vines compared with the *[SALT]* vines (see Figure 1(B)).

The significant beneficial effects of the early application of the BAP on the observed morphological traits (i.e. length of internodes and vine height) is in line with the participation of cytokinins in the regulation of plant growth processes (Sosnowski et al. 2019; Li et al. 2021). This is supported by the increase in expressions induced by BAP of genes involved in cytokinin signaling (see discussion on *expression of selected marker genes*) (Kieber and Schaller 2018).

Although cytokinins have essential roles in plant growth, the supply of exogenous cytokinins, such as BAP may induce shorter internodes and so reduce overall elongation rates while also increasing the number of lateral branches (Zhang et al. 2018). In our study, we consistently observed reductions in the growth of both the terminal internodes and in the overall vine height in the *[BAP]* vines which provide evidence of a 'primed' status.

Early effects of salt stress are attributable to roots somehow sensing the salt (Sinclair and Hoffmann 2003). In the present study, a significant deterioration of the fine (absorbing) roots was recorded in salt-exposed vines (Figure 2) according to (Shani et al. 1993) and to an *in-vitro* experiment (Haider et al. 2019). Although cytokinins can be transported from shoot-to-root inhibiting root growth (Kieber and Schaller 2018), in our study the foliar application of BAP

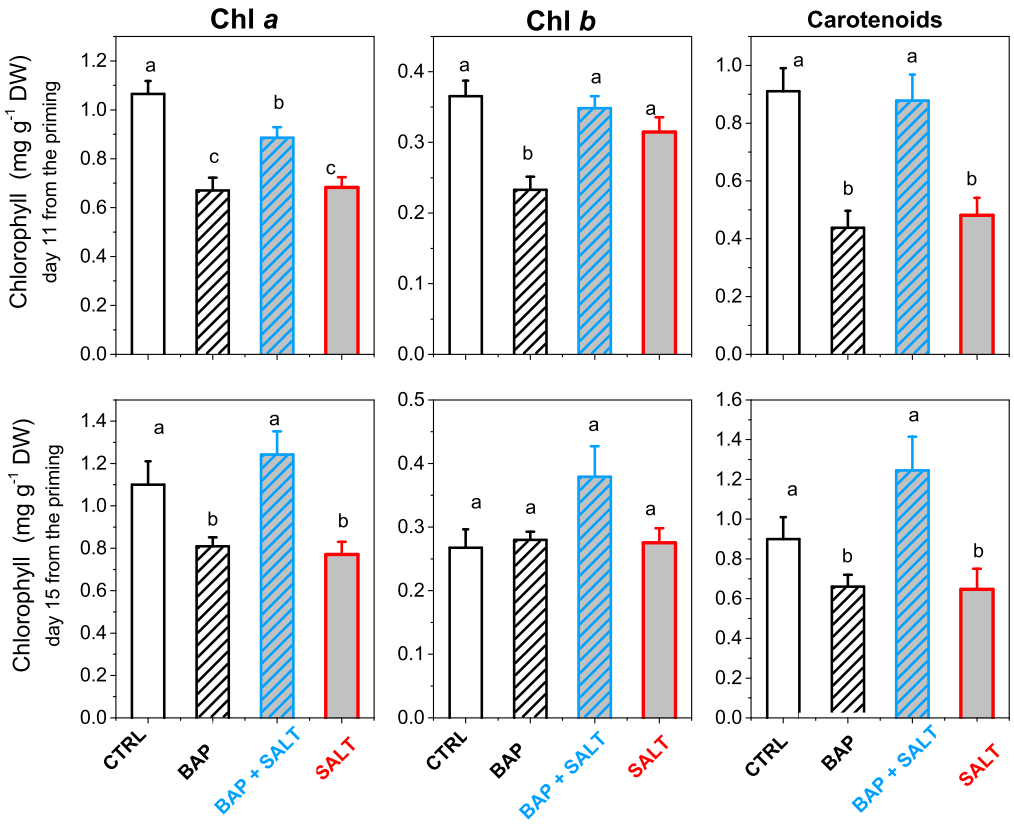

Figure 6. Concentrations (±SE) of chlorophyll *a* (left panels), chlorophyll *b* (center panels) and carotenoids (right panels) measured on the last day of salt stress (upper row) and on after recovery (bottom row) in vines irrigated with tap water (*[CTRL]*) and vines primed with BAP (*[BAP]*), receiving salt *[SALT]* and primed with the BAP (*[BAP + SALT]*). Gray filled bar = salt exposure, dashed bar = primed with BAP). Comparing treatments in each panel different letters indicate statistically significant differences ($p < 0.05$).

did not significantly affect root growth depression as can be inferred from the similar fine/coarse root dry weight ratios in the *[CTRL]* and *[SALT]* vines, compared with the *[BAP]* and *[BAP + SALT]* vines (Figure 2).

4.2. Water relations and leaf gas exchanges

The initial response of plants to salt stress is generally similar to that to drought, including a reduction in SWP (Munns and Tester 2008). Exogenous BAP treatments increase the accumulation of osmoprotectants (e.g. proline) so contributing to a reduced leaf water potential (Thomas et al. 1992; Fozouni et al. 2012). In our study, the values of stem water potential recorded in the *[BAP]* vines were significantly more negative than in the *[CTRL]* vines at day 8 and day 11 supporting the idea that the vines had, indeed, been primed (Figure 3).

Values of soil EC promptly responded to salt treatment and were similar to that reported in a field experiment on grapevine (Ben-Asher et al. 2006), then EC lowered the SWP confirming their close similarity (see the inset in Figure 3(A)) (Yu and Kurtural 2020). In our study values of EC progressively increased because a progressive salt stress was imposed instead of a sudden one (see M&M section) in order to mimic the progressive increase of EC which would occur in open field in a short temporal horizon (Ahmed et al. 2007). In addition, the progressive imposition of the salt stress was also intended to avoid a sudden increase of leaf ion concentrations (Almansouri et al. 1999).

Soon after salt application SWP in vines receiving BAP the SWP remained similar to that of *[CTRL]* and significantly differed from that of *[SALT]* likely because of different plant hydraulics due to different stomatal conductance (g_s) (see below) and a putative slow accumulation of osmoprotectants (Fozouni et al. 2012; Haider et al. 2019). However, from day 6 onward SWP in the *[BAP + SALT]* vines reached the lowest values, suggesting BAP augmented the salt-induced accumulation of osmoprotectants, as reported in Thomas et al. (1992).

Appropriate regulation of leaf gas exchange is central to the tolerance of abiotic stresses, including that induced by exposure to salt (Chaves et al. 2009). Increasing cytokinins level usually decreases stomata aperture and/or stomatal density (Dodd 2003; Martins et al. 2018), even though a leaf-age-dependent effect may occur (Farber et al. 2016). Taking this paradigm together with the physiological responses (e.g. gas exchanges and fluorescence) observed in our *[BAP]* vines supports the view that these vines had been effectively primed.

Cytokinins exert an antagonistic effect against ABA (which accumulates during osmotic stress) through the facilitation of degradation of the ABA signaling component transcription factor (Cortleven et al. 2019). This supports the retardation of g_s decline (stomatal closure) in the salt-treated and BAP-primed vines *[BAP + SALT]*.

In addition, considering the effect of SWP on stomatal conductance (Buckley 2019) higher SWP in the *[BAP + SALT]* vines compared to *[SALT]* vines at the beginning of salt stress (Figure 3(B)) might contribute to explain the higher g_s recorded in BAP-primed compared to un-primed vines.

As a result of increased stomatal conductance, values of transpiration and photosynthetic rate in the *[SALT]* and *[BAP + SALT]* vines, relative to the *[CTRL]* vines, reveal the benefit of priming for that functional traits due to their

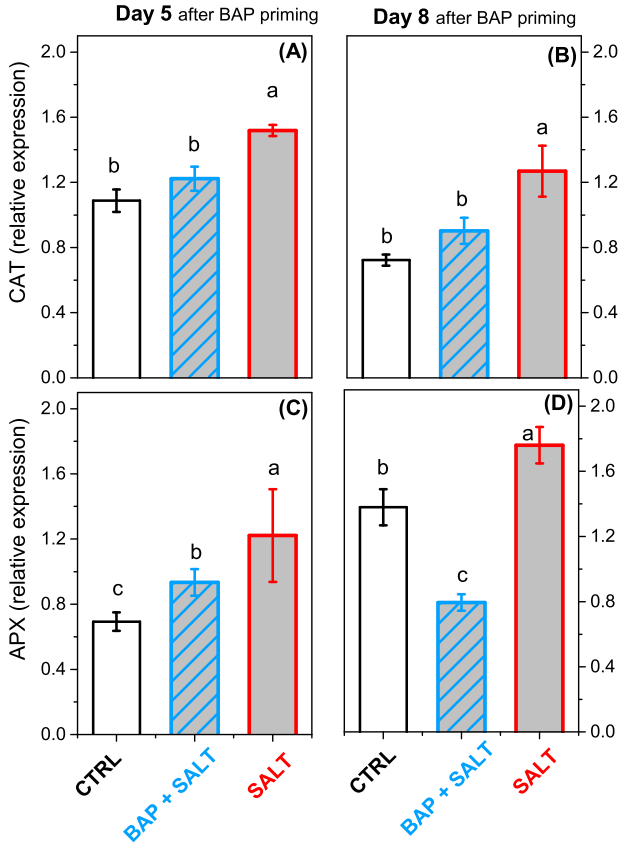

Figure 7. Relative expression (±SE) of the *CAT* and *APX* genes in *Vitis vinifera* leaves under salt stress (*[SALT]*), primed with the exogenous cytokinin application (*[BAP + SALT]*) and under control (*[CTRL]*) determined on day 5 and 8 after priming. Transcript levels of the genes analysed were measured by qRT-PCR and were normalized to the expression of the reference Actin gene. Comparing treatments in each panel different letters indicate statistically significant differences ($p < 0.05$).

gradual BAP-induced decline when EC >3 dS m^{-1} (Figure 9). We noted an increase in transpiration similarly to that reported after seven applications of BAP in tomato plants where the increase was attributed to increased stomatal density (Farber et al. 2016). Here, gas exchange was measured on mature leaves where changes in stomatal density would not be expected suggesting that regulation of gas exchange by BAP was due mainly to changes in stomatal aperture.

The faster recovery of gas exchanges induced by BAP recorded after stress relief (see day 15 in Figures 4 and 9) is consistent with that observed in bean plants during recovery from drought stress (Rulcová and Pospíšilová 2001). It might be attributable to its beneficial effects on maintaining the levels of Chl-*a* and carotenoids at concentrations significantly higher than those in the *[SALT]* vines at the end of the stress period likely through maintenance of chloroplast structure and of chlorophyll synthesis (Cortleven and Schmülling 2015).

It is widely accepted in plants not experiencing a stress that the value of maximal quantum yield of PSII photochemistry (F_v/F_m) is about 0.8. This is believed the threshold of photoinhibition (Krause and Weis 1991). In our study, the non-stressed *[CTRL]* vines had the highest F_v/F_m which lay close to that threshold. In tomato plants, increases in cytokinin level may trigger oxidative stress and so have inhibitory effects on the metabolism of photosynthesis (e.g. inhibitions of the carboxylation efficiency of Rubisco and electron transport rates, increases in non-photochemical-quenching) (Novák et al. 2013). In line with the detrimental effects of high cytokinin, the BAP priming vines induced values of F_v/F_m in the *[BAP]* vines to gradually decline, reaching a value significantly lower (10%) than that in the *[CTRL]* vines at day 6.

Metabolic limitations of photosynthesis start soon after salt stress is imposed (Chaves et al. 2009). Accordingly, values of F_v/F_m declined progressively in the *[SALT]* vines soon after the salt treatment, suggesting an inhibition of photochemistry including the electron transport of PSII which may be associated with oxidative damage in the leaf tissue (lipid peroxidation) caused by increased ROS (Aremu et al. 2014). The exogenous cytokinin did not exert a significant effect on F_v/F_m in the *[BAP + SALT]* vines, likely because PSII may not have been the target during the first (ion-independent) stage of salt stress as discussed for tomato plants in Aremu et al. (2014).

However, considering that the increasing concentrations of the putrescine used as priming agent by Gohari et al. (2021) progressively reduced the salt-induced photoinhibition, a higher BAP concentration would seem worthwhile to try, to further mitigate salt-induced metabolic impairment of photosynthesis.

Both stomatal and non-stomatal co-regulation of leaf carbon gain limitation have been reported in field-grown grapevines under water stress (Escalona et al. 1999). The results presented here on the decline of g_s induced by salt stress (Figure 4(A)) confirms the behavioral similarity between drought stress and the initial (osmotic) stage of salt stress (Munns and Tester 2008).

Plotting the intrinsic water use efficiency (the ratio between photosynthetic rate, A, and stomatal conductance to water vapor i.e. A/g_s) (Figure 10) the advantage offered by BAP in minimizing both stomatal and metabolic impairment of photosynthesis under salt stress is confirmed. That is, for g_s values below 0.1 mol H_2O m^{-2} s^{-1} which identify moderate to severe water-stress conditions (Cifre et al. 2005), the value of A/g_s in the *[BAP + SALT]* vines continued to increase even at very low values of g_s, suggesting that photosynthesis was predominantly limited by stomatal closure (Escalona et al. 1999). By contrast, in the *[SALT]* vines within the same g_s range, A/g_s tended to drop sharply highlighting that the stomata were not the only limiting factor and that a metabolic impairment of photosynthesis (e.g. a reduction in F_v/F_m) occurred.

4.3. Effect of salt stress and BAP priming on expression of selected marker genes

Exposure to salt stress causes increased formation of reactive oxygen species (ROS) and thus increased oxidative stress in plants (Yang and Guo 2018). Therefore, a complex array of detoxification mechanisms has evolved to maintain ROS homeostasis. These include non-enzymatic components (e.g. antioxidant ascorbate, glutathione) as well as enzymatic scavengers. The major antioxidant enzymes include superoxide dismutase (*SOD*), ascorbate peroxidase (*APX*), catalase (*CAT*) and glutathione peroxidase (*GPX*) (Jiang et al. 2007; Yang and Guo 2018). In grapevine, these antioxidant enzymes are strongly upregulated by salt stress and so limit the excessive accumulation of ROS and in turn limit cell damage (Das and Majumder 2019).

In our study, *CAT* and *APX* gene expressions were used as qRT-PCR markers for salt stress in grapevine. The

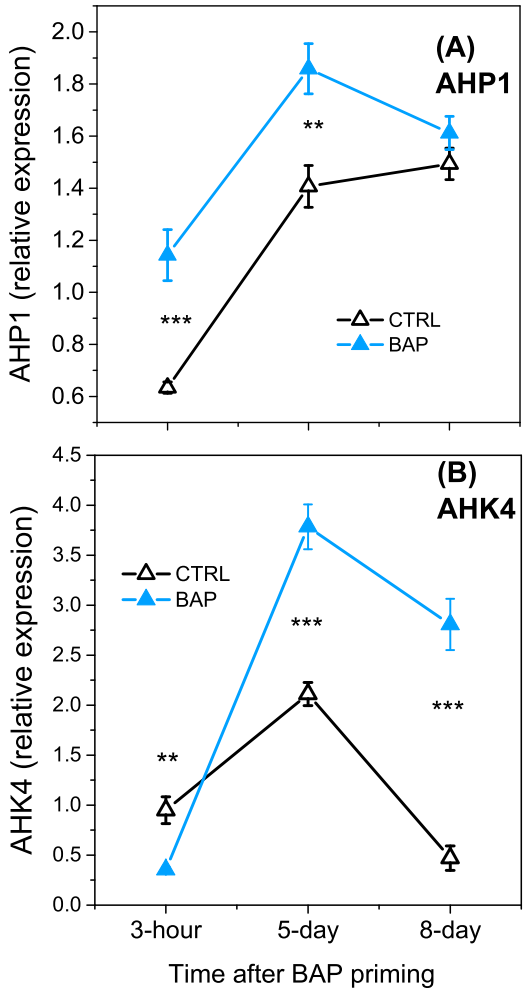

Figure 8. Relative expressions (±SE) of (A) *AHP1* and (B) *AHK4* genes in leaves of *Vitis vinifera* irrigated with water (Δ, ▲) and subjected to cytokinin priming treatment (▲, *[BAP]*) measured at 3 h, 5 and 8 days after BAP spraying. Transcript levels of the genes analysed were measured by qRT-PCR and were normalized to the expression of the reference actin gene. * indicates statistically significant differences (**$p < 0.01$, *** $p < 0.001$; Student's *t*-test).

expressions of these genes have been normalized to actin because of its stability under salt stress as shown in other grapevine qRT-PCR studies (Das and Majumder 2019).

The salt stress increased the gene expressions of both *CAT* and *APX* (compare *[CTRL]* and *[SALT]* in Figure 7) according to Das and Majumder (2019) who found similar inductions of *CAT* and *APX* in grapevine under a comparable salt stress (i.e. 150 mM NaCl).

In our study, BAP priming significantly reduced the expressions of salt-stress-related genes (Figure 7) according to results in *Arabidopsis thaliana* (Golan et al. 2016) and in perennial ryegrass (Ma et al. 2016). It has been reported that in *Agrostis stolonifera* an increased cytokinin level mediated by overexpression of the *IPT* gene, involved in cytokinin synthesis, may suppress ROS accumulation and mitigate cell membrane damage (Xu et al. 2016). A possible mechanism for this effect is that cytokinins may counteract osmotic-stress-induced premature senescence by redistributing soluble sugars and inhibiting the expressions of senescence-associated genes (Worakan et al. 2017). However, the positive effect of cytokinin-base priming on salt tolerance is still debated. In some cases, the increase in cytokinins and the consequent reduced expressions of antioxidant genes can be associated with an increase in ROS and a reduced tolerance to stress (Yu et al. 2021). This may be explicable as due to differences among experimental plant species or among treatment/conditions. Our research dealt with ion-independent salt stress, under these conditions cytokinins may have alleviated the stress-induced growth inhibition, increasing tolerance as observed in *Arabidopsis thaliana* (Golan et al. 2016).

To assess the effectiveness of our exogenous BAP treatment, the Histidine kinase 4 (*AHK4*) and Histidine phosphotransfer protein 1 (*AHP1*) genes were selected as the BAP-inducible genes. These genes are among the major components involved in cytokinin signal perception and transduction pathways (Gujjar and Supaibulwatana 2019). In *Malus x domestica AHK4* and *AHP1* genes are both significantly upregulated in BAP-treated buds (Li et al. 2019). Figure 8 shows the statistically significant high expressions of the *AHK4* and *AHP1* genes in control vines treated with the BAP (*[BAP]*). Hence, in addition to the above physiological traits, the molecular analyses confirm the effectiveness of BAP as a primer of salt tolerance suggesting a possible role for the cytokinin-signaling genes in the observed increase in tolerance to salt stress when BAP was applied externally. Hence, *AHK4* and *AHP1* expressions in the *[SALT]* and *[BAP + SALT]* vines were measured. Under salt stress, *AHK4* and *AHP1* genes were downregulated compared to the *[CTRL]* vines with no significant difference appearing between the BAP-primed *[BAP + SALT]* and the un-primed *[SALT]* vines (data not shown).

Downregulation of *AHK4* under salt stress is in accordance with previous evidence in *Arabidopsis thaliana*

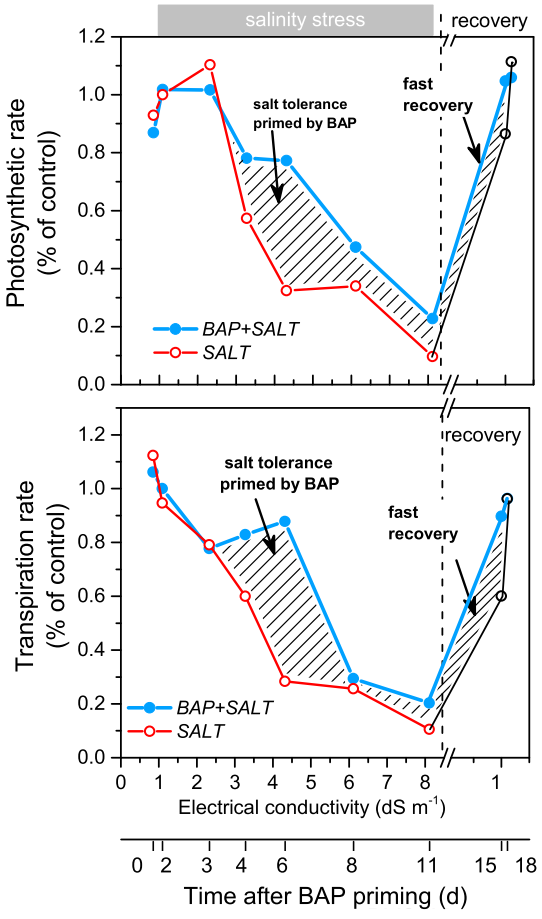

Figure 9. Values (relative to that of control) of photosynthetic rate (upper panel) and transpiration rate (lower panel) in vines under salt stress primed with the cytokinin (●, *[BAP + SALT]*) and un-primed (**o**, *[SALT]*) plotted against soil electrical conductivity during the salt-stress and recovery periods. The horizontal gray filled band indicates the salt stress period and vertical dashed line the beginning of recovery.

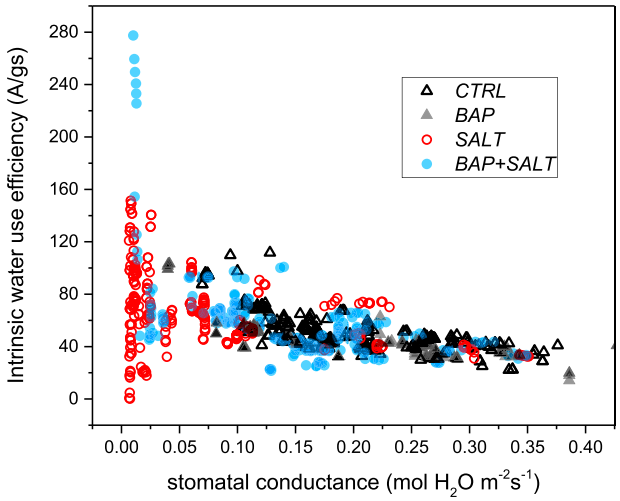

Figure 10. Correlation between intrinsic water use efficiency (A/ g_s) and stomatal conductance (g_s) values detected in vines under salt stress (**o**, *[SALT]*), under salt stress primed with BAP (●, *[BAP + SALT]*) control (Δ, *[CTRL]*) and vines primed with BAP (▲, *[BAP]*). Note that the for the symbols ● and ▲ a 70% transparency has been used.

(Tran et al. 2007). In the grape cv. Thompson seedless, *AHK4* and *AHP1* did not differ between salt-stressed and control plants (Das and Majumder 2019). Considering the other members of the *AHK* and *AHP* gene families, only *VIT_11s0016g03170* (similar to *AHP4*) appeared to be strongly upregulated under salt stress (Das and Majumder 2019). Hence, it can be inferred that *AHK4* and *AHP1* may have roles in the activation of cytokinin signaling in the priming phase between the exposure to BAP and the occurrence of salt stress.

5. Conclusions

A single earlier application of a synthetic CK reduced the morpho-physiological impairments triggered by a salt stress during the early (ion-independent) stage. The primed response of grapevine was triggered at molecular scale through the reduction of the expression of those salt-inducible genes we have examined.

This priming agent BAP induced a stomatal and non-stomatal co-regulation of photosynthesis as it slowed the reduction in g_s induced by salt and reduced the metabolic limitations of photosynthesis. The effect on carbon gain was efficiently translated into a sustained rate of shoot growth in BAP-primed vines under salt stress comparable to that of the controls.

In conclusion, the successful application of BAP as priming agent expand the portofolio of innovative strategy to improve the performance of grapevine under saline stress, and open future research perspectives to test the effect of repeated applications of BAP and its efficacy in longer (ion-dependent) salinity stress.

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Data availability

The data supporting the findings of this study are available from the corresponding author (G. Montanaro), upon request.

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