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Near-lethal heat treatment induced metabolic changes associated with endodormancy release of Superior Seedless grapevine cv. (*Vitis vinifera* L.) buds

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Abstract

To assess the efficacy of near lethal heat stress for endodormancy release of Superior Seedless grapevine buds, single-node cuttings were soaked for 1 h in hot water (50°C) and then forced for one month. The effects of hot water treatment (HWT) on budburst, metabolic changes of carbohydrates, proline, free polyamines PAs (putrescine (Put), spermidine (Spd) and spermine (Spm)) and antioxidant enzymes' activity were investigated in bud under forcing conditions. Near-lethal heat stress caused a transient increase in starch hydrolysis, leading to an abrupt accumulation of soluble sugars, especially sucrose concentration during the first 3 days after treatment. This accumulation of soluble sugars coincided with a brief stimulation of the antioxidant system represented by ascorbate peroxidase (APX; EC 1.11.1.11), peroxidase (POD; EC 1.11.1.7) as well as an increase in the concentration of proline and free polyamines, especially putrescine (Put) and spermidine (Spd). These changes, which occurred immediately upon treatment, appear to be related with a process leading to endodormancy release. These results support the argument that a temporary and acute oxidative stress is involved in the mechanism leading to dormancy release and budbreak. Furthermore, it is possible that the stimulation of both peroxidases' activity and proline biosynthesis activated the pentose phosphate pathway (PPP) which helped the bud to overcome endodormancy. After this initial period and towards budbreak initiation, there was a rapid decline in the concentration of soluble sugars, proline and Put, while, Spm and Spd became abundant. Such post stress changes appear to be associated with the reactivation of growth leading to an earlier and more vigorous budbreak. The metabolic response to HWT was compared to those observed after bud chilling or the application of restbreaking agents such as hydrogen cyanamide (HC). The similarity in the response to these various stimuli suggests the presence of common regulatory pathways involved in bud dormancy release and subsequent sprouting.

Keywords: Grape bud dormancy; Hot water treatment; Carbohydrates; Antioxidant enzymes; Polyamines.
Abbreviations: APX_Ascorbate peroxidase, BSA_Bovine serum albumin, CAT_Catalase, Ctrl_Control, EC_Electrical conductivity, DMAB_3-dimethylamino benzoic acid, HC_Hydrogen cyanamide, HWT_Hot water treatment, MBTH_3-methyl-2-benzothiazolinone hydrozone hydrochloride monohydrate, OM_Organic matter, Pas_Polyamines, POD_Peroxidase, Put_Putrescine, Spd_Spermidine, Spm_Spermine.

Introduction

Perennial plants have the ability to suspend and resume growth recurrently in response to seasonal fluctuations in environmental conditions (Rohde and Bhalerao, 2007). This cessation of active growth and bud dormancy seen in most deciduous fruit trees and vines is an adaptative mechanism to adverse conditions. In the temperate zone, prolonged exposure to short days and/or low temperatures have been known to govern growth cessation and the onset of dormancy (Dokoozlian et al., 1995), while the release from endodormancy requires the accumulation of chilling during the winter period (Westwood, 1993). Hence, to resume growth, dormant buds must receive an amount of chilling called chilling requirement which is genetically controlled (Balandier et al., 1993). As for most temperate fruit trees, dormancy limits grapevines production in mild-winter climates where natural chilling requirement for budbreak is often not fulfilled. Such conditions may prolong dormancy and trigger uneven and abnormal patterns of budbreak and development leading to low commercial production (Fuchigami and Nee, 1987; Dokoozlian et al., 1995). To overcome this problem, horticulturists attempted to compensate for the lack of natural chilling needed to stimulate budbreak of deciduous fruit trees and vines by using artificial restbreaking agents such as potassium nitrate, paraffin mineral oil, thiourea and HC (Westwood, 1993). The latter is considered the most efficient on grapevines to achieve satisfactory budbreak and yield (Dokoozlian et al., 1995; Mohamed et al., 2010a). Near-lethal heat stress brought about by soaking in hot water was found effective in releasing buds of woody plants from dormancy (Shirazi and Fuchigami, 1995; Wisniewski et al., 1997; Halaly et al., 2011). However, the mechanism by which a short exposure to such stress overrides intrinsic locks on growth and development is still poorly understood. Bud growth regulation and the underlying physiological and biochemical responses have been intensively studied over the past decades

to identify metabolites and pathway changes associated with bud dormancy release. These studies dealt with carbohydrates (Marquat et al., 1999; Mohamed et al. 2012; Ito et al., 2012), hormones, water content (Faust et al., 1997), polyamines (Rey et al., 1994), antioxidants (Or et al., 2002; Halaly et al., 2008) and respiration pathway (Young et al., 1987; Vergara et al., 2012). These approaches aimed to elucidate the mechanism of dormancy release and help to develop markers for adequate restbreaking practices. Although the numerous studies conducted, the mechanism underlying dormancy-breaking effect of restbreaking agents has not been fully revealed yet (Ophir et al., 2009; Vergara et al., 2012). However, it was suggested that the main cause of bud dormancy release is a transient disruption of respiration by hydrogen peroxide generated by restbreaking agents-induced oxidative stress (Or et al., 2002; Ophir et al., 2009; Pérez et al., 2009). Moreover, several reports indicate that dormancy release in buds coincides with the increase in activity of peroxide scavenging enzymes and the upregulation of other antioxidant systems (Or et al., 2002; Pacey-Miller et al., 2003; Pérez et al., 2008; Ophir et al., 2009; Walton et al., 2009). Polyamines (PAs) are a group of phytohormone-like aliphatic amine compounds. Putrescine (Put), spermidine (Spd) and spermine (Spm) are the major types of PAs found in plant. They are known to participate in plant tolerance to various kinds of stresses through their role as free radical scavengers (Gill and Tuteja, 2010). In addition to their protective properties in case of stress, they play a role in many plant development processes such as morphogenesis, cell division, differentiation, vascularization and shoot growth (Galston and Kaur-Sawhney, 1995). Among the major plant metabolites, carbohydrate reserves in the plant undergo seasonal fluctuations; they accumulate in perennial structures late during the growing season and they are used during bud growth resumption (Zapata et al., 2004). They are the main source of energy for the metabolic changes that occur during the dormancy period and for spring budburst. Recent research shows that sugars appear also to play key role in the release of buds from dormancy in many species such as peaches (Marquet et al., 1999), pear (Ito et al., 2012), grapevines (Mohamed et al., 2010b). The present study was undertaken to assess the effect of hot water treatment (HWT) on metabolic changes during dormancy release of *Vitis vinefera* L. cv. Superior Seedless. To this end, we evaluated the changes in antioxidant enzymes' activity and carbohydrates, proline and free PAs content and attempted to explain how these changes hasten budbreak.

Results

Dormancy release

Budbreak of single-node cuttings treated with hot water was significantly increased compared to controls (Fig. 1). Under forcing conditions, opening of hot water-treated bud started earlier by at least 4 days. Moreover, treated cuttings reached 50% budbreak or more after 18 days of forcing, while buds on control cuttings didn't reach this level even after one month of forcing. At the end of forcing period, treated cuttings achieved 94% budbreak, while control buds were only about 48% open.

Effect of heat treatment on carbohydrate concentrations

The dynamics of soluble sugars and starch concentrations in HWT and control buds during one month of forcing are given in Figure 2. In control buds, the concentration of starch

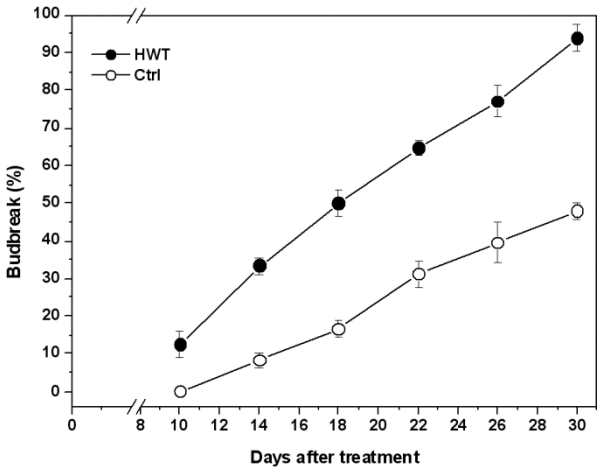

Fig 1. Effect of hot water treatment (HWT) on the budbreak response in single-node cuttings of cv. Superior Seedless grapevine forced for 30 days under 23 ± 2°C and 12 h d^{-1} photoperiod. Each point represents the mean ± SE of three replicates.

decreased by 20% during the first five days of forcing and then continued to decrease slowly to reach 2/3 of its initial concentration on day 20. In treated buds, starch concentration declined rapidly by 33% during the first 3 days after treatment (Fig. 2A). By the end of forcing period, starch was at its lower levels for both control and treated buds, but the latter were more depleted. So, starch hydrolysis was more rapid in heat-treated tissues. Concomitantly with starch degradation, soluble sugars sucrose, glucose and fructose accumulated considerably in treated bud to reach their maximum concentrations at the 3th day of forcing (Fig. 2). This increase was by about 76%, 54% and 47% for sucrose, glucose and fructose, respectively. Thereafter, with the initiation of budbreak, the main soluble sugars decreased rapidly to reach their lowest levels at the end of forcing period. However, the decrease in sucrose concentrations was slower compared with hexoses. Similarly, in untreated buds, we found about the same patterns of change in soluble sugars content, but the rate of changes was slower and the concentrations were lower. Indeed, maximum concentrations were about 140%, 124% and 121% of the initial levels of sucrose, glucose and fructose, respectively, at day 10 of forcing. Then, these concentrations declined to reach levels similar to those found in treated-buds towards the end of forcing period.

Effect of heat treatment on free proline concentration

Proline concentration in bud tissue was initially low then increased slightly in control buds to reach a maximum by day 10 of forcing (Fig. 3). In heat treated-cuttings, proline concentration rose sharply by more than 3 folds during the first 3 days of forcing then fell back to initial level.

Effect of heat treatment on antioxidant enzymes' activity

Hot water treatment had a significant effect on antioxidant enzyme activities of grapevine buds (Fig. 4). The activity of CAT declined by 38% during the first three days of forcing. Thereafter, it increased to attain its maximum on day 10, and then it leveled off for the rest forcing period. However, the maximum activity attained on day 10 was lower than the

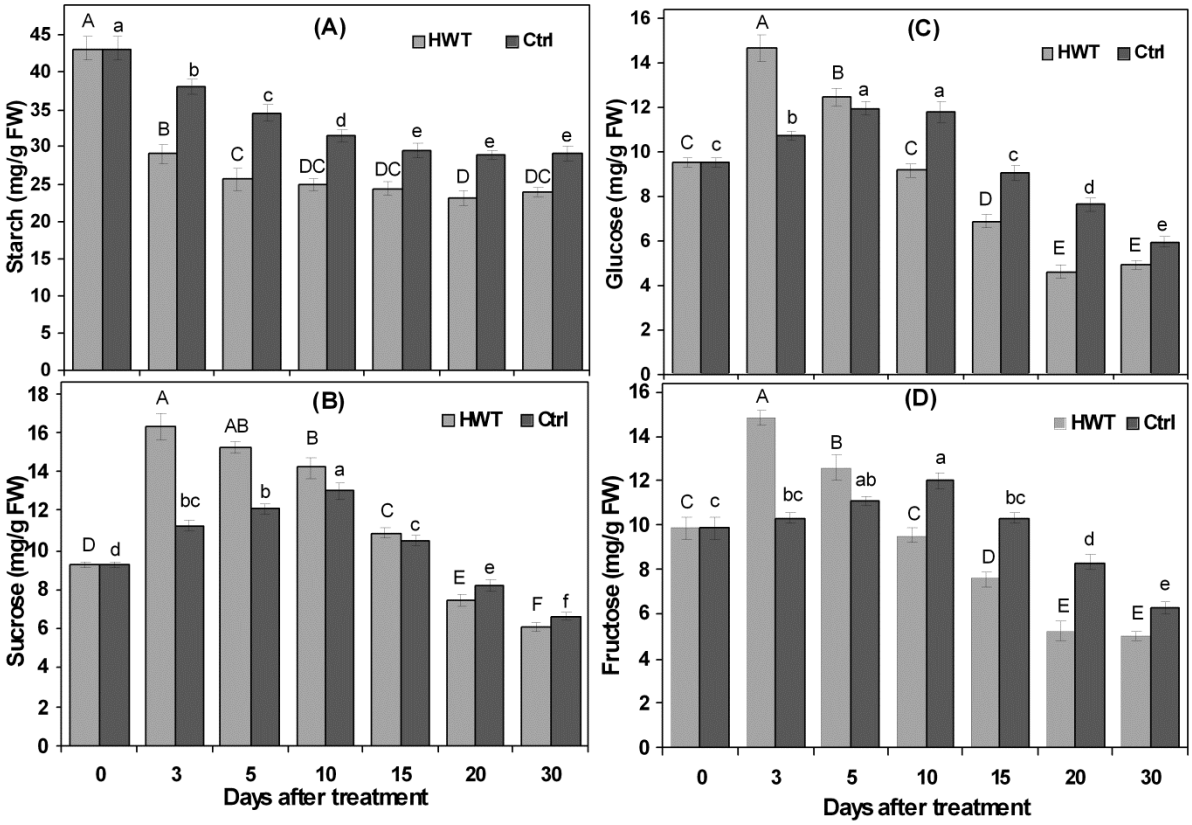

Fig 2. Changes in starch (A), sucrose (B), glucose (C) and fructose (D) levels in the buds of cv. Superior Seedless grapevine cuttings immersed in hot (HWT) or warm water (Ctrl) and forced during one month under 23 ± 2°C and 12 h d^{-1} photoperiod. Values are the mean ± SE of three replicates. Different letters indicate significant differences in carbohydrate levels between different forcing periods according to Duncan's Multiple Range Test at $P \leq 0.05$.

initial activity prior to immersion in hot water. In control buds, CAT activity remained unchanged until day 10 then decreased slightly for the remaining forcing period (Fig. 4A). Unlike CAT activity, both POD and APX activities were strongly induced by hot water treatment (Figs. 4B, C); they increased by 44% and 28% for POD and APX, respectively, by day 3 of forcing. Thereafter, they declined gradually. However, in control buds, both APX and POD activities showed similar trends. They fell slightly at the beginning of forcing then remained steady. It can be inferred that bud endodormacy release and hastened budbreak caused by heat treatment appear to be correlated with a rapid inhibition of CAT activity and stimulation of both APX and POD activities. Although these enzymes respond differently to hot water treatment, the changes were temporary and recovery occurred with the onset of budbreak.

Change in free polyamine concentration in response to heat treatment

The effect of hot water treatment on the concentration of free total PAs, Put, Spd and Spm was investigated. Heat treatment by immersion of dormant buds in hot water increased considerably the concentration of free PAs (Fig. 5). During the first 3 days of forcing, free PAs concentration increased to twice its initial level before decreasing gradually after day 5. In control buds, there was only a slight and delayed increase by 11% during the first 10 days of forcing and then a gradual decline to a level similar to what was measured in treated buds. Hence, hot water treatment enhanced considerably and transiently the amount of free polyamines.

Put and Spd were the main PAs present in both treated and control buds (Fig. 6). Following hot water treatment, Put concentration significantly increased by more than 2.6 folds by the 3rd day of forcing; then it decreased gradually to reach its lowest level towards the end of forcing. As with total free PAs, Put in control buds increased slightly (14%) during the 10 days of forcing, then decreased gradually (Fig. 6A). Spd concentration increased by more than 57% at the third day after treatment, then remained high until day 15 before decreasing thereafter. By comparison, in control buds, Spd level remained relatively constant throughout the forcing period (Fig. 6B). Spm concentration remained somewhat unchanged until day 5 in control buds, but it increased in hot water-treated ones. Afterwards, it increased but much more so in treated buds (Fig. 6C). This increase could be the result of Put degradation (Fig. 6A).

Discussion

In mild winter climates, tree bud chilling requirement is generally only partially met. Therefore, restbreaking agents have been used effectively to supplement cold temperature to achieve satisfactory budbreak. Other sub-lethal stresses such as heat (45-50°C) can break dormancy (Fuchigami and Nee, 1987). Moist heat has been found to be more effective than dry heat (Wisniewski et al., 1997). Our results show that immersion in hot water improved bud sprouting by up to 94% after 30 days of forcing. The percentage of budbreak in cuttings which have been immersed in 20 °C–water (control) was lower than that of hot water-treated cuttings throughout the period of forcing (Fig. 1). Moreover, heat treatment gave

a higher percentage budbreak that HC as reported by Mohamed et al. (2012). Like HC, hot water hastened budbreak too. The above effects of heat treatment were accompanied with changes in the dynamics of carbohydrate, free PAs and proline concentrations and antioxidant enzyme activities. Often, nutrients, especially carbohydrates, lead to growth. In this study, the breaking of endodormancy by hot water treatment and the subsequent bud development are associated with a rapid sugar mobilization. Changes in carbohydrate reserve in the treated buds reflect a quick inter-conversion of starch into soluble sugars as was reported in response to natural chilling (Sauter, 1988; Ito el al., 2012). This mobilization induced a brief accumulation of the main soluble sugars found in buds, especially sucrose. Such accumulation is well correlated with bud endodormancy release (Marquat et al., 1999), whereas, the subsequent sugar consumption as budbreak approached indicates that these nutrients were used to sustain metabolic activity. The early and brief accumulation of sugars in response to immersion in hot water may reflect the transient stress that heat treatment exerts and then the subsequent recovery. Such accumulation, resulting from starch soluble sugar conversion, may be doubly beneficial for buds. Indeed, soluble sugars play a pivotal role as compatible osmolytes which protect the cells against potentially destructive effects of abiotic stresses. Sugar reserves are also the main source of carbon and energy for new growth after dormancy release. Moreover, their accumulation in the cytosol lowers its water potential and attracts water thus creating cell turgor necessary for cell expansion. Furthermore, sugars not only fuel cellular carbon and energy metabolism but may also play pivotal roles as signaling molecules throughout all stages of the plant's life cycle including meristem development (Rolland et al., 2006). Accordingly, it appears that grape endodormancy release is tightly related to starch conversion into soluble sugars in response to chilling (Mohamed et al., 2010b), hydrogen cyanamide (Mohamed et al., 2012) and heat in the present case. It is worth mentioning that these changes were faster and budbreak was more frequent when buds were subjected to heat stress (Fig. 1). Hot water treatment led to a transient stimulation of both APX and POD while CAT was inhibited (Fig. 4). These changes, which were absent in untreated buds, were not permanent and recovery was detected 3-5 days later. Similar results were reported in grape buds following the application of HC which induced both gene expression and activity of APX and POD, while gene expression and activity were repressed for CAT (Halaly et al., 2008; Pérez et al., 2008) with the concomitant increase in the level of H_2O_2 (Halaly et al., 2008). In general, heat stress, like other abiotic stresses, leads to the production of more reactive oxygen species (ROS), forcing the activation of antioxidant systems. CAT inhibition in response to high temperature was also reported in herbaceous plant species such as bean (Babu and Devaraj, 2008) and wheat (Dash and Monhanty, 2002). It was suggested that CAT activity is associated with cell survival under non-lethal heat stress, but it is inactivated when the stress level is high and becomes sub-lethal for cells (Dash and Monhanty, 2002). Thus, our results further indicate the effectiveness of sub-lethal thermal stress to trigger changes which lead to dormancy release. It appeared therefore that the increase of the POD and APX activities, observed in the present study, may be partly be explained by the higher H_2O_2 levels in buds cells resulting from the low catalase activity, known to have a high affinity for hydrogen peroxide. In addition, our results show that hot water treatment led to a quick and transient accumulation of free proline (Fig. 3), a common stress response plants which was also reported in

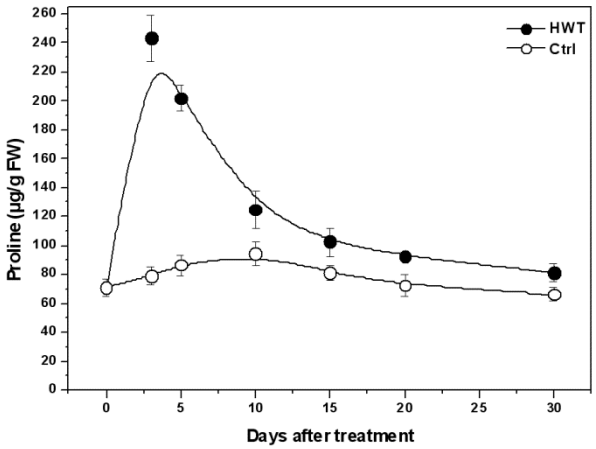

Fig 3. Changes in proline level (mg g^{-1} FW) in the buds of Superior Seedless grapevine cuttings immersed in hot (HWT) or warm water (Ctrl) and forced during one month. Each point represents the mean ± SE of three replicates.

response to both chilling (Mohamed et al., 2010b) and HC (Walton et al., 1998) treatments. This transient increase in proline concentration, which peaked at day 3 before it declined, may be closely associated with the stimulation of POD and APX activities. Indeed, a strong positive correlation was found between proline concentration and both POD (r = +0.97) and APX (r = +0.95) activities (Fig. 7). Likewise, Ozden et al. (2009) reported an increase in POD and APX activities but not CAT activity and an accumulation of proline in grapevine leaves exposed to oxidative stress. The increase in proline biosynthesis and the high activity of peroxidases may lead to an increase in $NADP^+$/NADPH ratio. Such situation is most likely to activate the oxidative pentose phosphate pathway (PPP), a major source of NADPH in non-photosynthetic tissues (Tian et al., 1998), which is important in antioxidative defence (Hare and Cress, 1997). Thus, we speculate that hot treatment led to the development of a transient oxidative stress which likely results in an activation of PPP could be crucial steps in the breakage of endodormancy. These findings were consistent with the response of grape and other dormant plants to chilling and artificial treatments (Panneerselvam et al., 2007; Pérez et al., 2009; Walton et al., 2009; Tan et al., 2013). The changes mentioned above were transient. In fact, by the 5^{th} day of forcing, there was a decline in both APX and POD activities and proline level; this may indicate a recovery from stress. Accordingly, it appears that proline is a key factor here: when it accumulates, it helps with stress survival and upregulates PPP inducing a chain of events leading to dormancy release; upon recovery from stress, proline degradation activates anabolic pathways responsible for budbreak initiation. Indeed, it was reported that mitochondrial proline oxidation provides ATP, phosphorylated sugars and nitrogen during flower development (Walton et al., 1998). The relative levels of metabolites during dormancy release and bud outgrowth are consistent with their known physiological functions. PAs have been shown to affect plant growth and modulate the response to various stress factors (Kusano et al., 2008). As for proline, we recorded a transient but large accumulation of total free PAs in heat-treated buds (Fig. 5). Similarly, the breaking of apple bud dormancy by thidiazuron (Wang et al., 1986) or HC (El-Yazal and Rady, 2012) is correlated with greater polyamine biosynthesis. This rapid increase in polyamine content, attributed mainly to Put and Spd, may indicate the end of endodormancy as was suggested by Wang

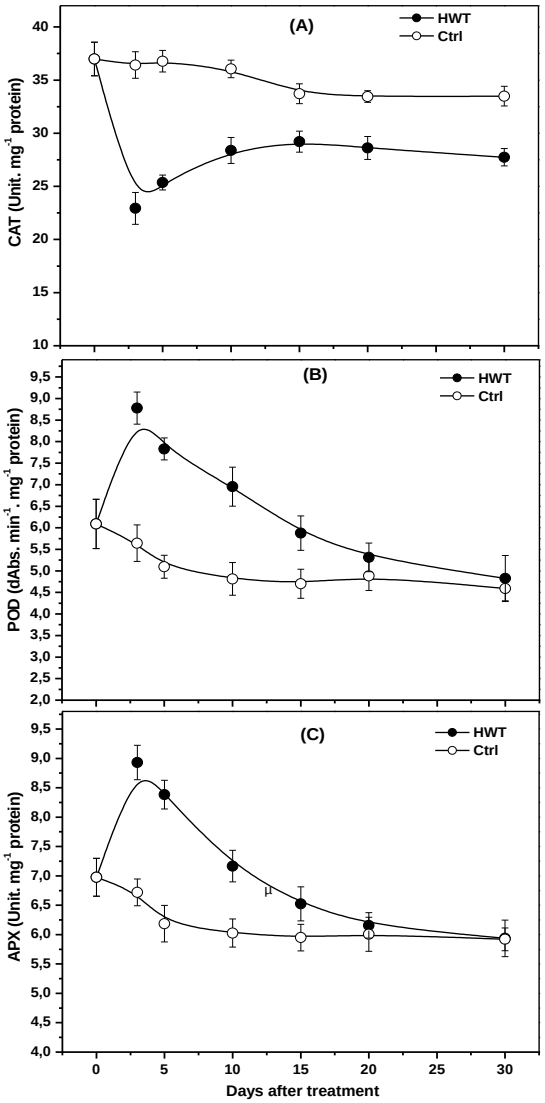

Fig 4. Effect of hot water treatment (HWT) on the activities of three antioxidant enzymes in the buds of Superior Seedless grapevine cuttings during one month of forcing. The enzymes are catalase (CAT) (A), peroxidase (POD) (B) and ascorbate peroxidase (APX) (C). Each point represents the mean ± SE of three replicates.

and Faust (1994) for apple buds. In addition, these two compounds, Put and Spd, are largely believed to play a pivotal role in promoting antioxidant responses by activating enzymatic and non-enzymatic antioxidants (Zhang et al., 2009). The synchronous accumulation of proline and PAs could be considered as a physiological trait associated with plant cell survival under stress conditions (Tonon et al., 2004) including heat stress (Cvikrová et al., 2012). These findings lend more support to the contention that temporary oxidative stress may be a part of the mechanism leading to endodormancy release following hot water treatment or HC application (Ophir et al., 2009). Towards budbreak initiation, Put level dropped leading to a great decrease in the total PAs level, while the percent composition of Spm and Spd in treated buds became most prominent (Fig. 6). It is generally believed that Spd and Spm act as growth regulators due to their ability to stimulate protein syntheses required for new cell division and differentiation (Igarashi and Kashiwagi, 2000). Thus, their concentrations were positively correlated with spring sprouting of hazelnut buds (Rey et al., 1994) and bulbs of tuberose (Sood and Nagar, 2005). Hence, as reported above, the role of Spm and Spd could be more important after dormancy release during active growth. Based on the above, the changes observed in free PAs in response to restbreaking agents may reflect their involvement in the control and regulation of dormancy release and bud outgrowth phenomena.

Materials and Methods

Plant material and experimental design

Eight-year old vines of the early ripening table grape *V. vinifera* cv. Superior Seedless were used in this trial. They were located in a commercial vineyard near the town of Medenine, Tunisia (33°19' N; 10°23' E; altitude 117 m) where winter is generally mild. The vines were grafted on R110 rootstock, planted with 2 m × 3 m spacing on a sandy soil (OM = 0.97%; EC = 1.6 ms/cm; K = 19 ppm; P = 37.4 ppm; N = 0.09%; pH = 7.5) and trained onto a two-wire trellis. We estimated the variety's chilling requirement to be about 440 chill units (Mohamed et al., 2010a). The vines were pruned to four spurs and four long canes with an average of 16 buds each at mid-November. Mature, lignified shoots of comparable caliper (about 1 cm in diameter) were collected from the median part (between the 4th and 11th buds) of one-year-old canes of several vines on 15 November (late leaf fall). These shoots were cut into single-bud cuttings (~7 cm long) and were exposed to continuous low temperature (5°C) in a cold room to simulate the accumulation of 300 chilling hours, the equivalent of 2/3 of the variety's chilling requirement (Mohamed et al., 2010b) which is considered to be the adequate time for restbreaking agents (Faust et al., 1997; Mohamed et al., 2010a). After chilling treatments, the cuttings were pulled out of the cold room and divided into two sets; one set was subjected to dormancy breaking hot water treatment (HWT) by immersing them in 50 ± 0.1°C distilled water for 1 h as described by (Halaly et al., 2008). The second set was immersed in distilled water at room temperature (20 ± 1°C) for 1 h to serve as a control. After immersion in water, the cuttings were placed under forcing conditions (23 ± 2°C; 12 h light: 12 h d^{-1} photoperiod) for one month (Or et al., 2002); fluorescent white light having a flux density of 100 µmol m^{-2} s^{-1} was used. During forcing, the basal ends of the cuttings were immersed in water and cut (Balandier et al., 1993) every five days to prevent xylem vessel clogging by algal growth. Water was replaced daily. Bud tissues were sampled after 0, 3, 5, 10, 15, 20 and 30 days of forcing. The samples were immediately frozen in liquid nitrogen and stored at -80°C until analyzed. Three replicates of 16 single-node cuttings each were prepared from control and HWT-treated cuttings and were used to identify the effect of hot water treatment (HWT) on dormancy release. Buds that reached green tip stage were considered open and budbreak percentages were determined at 10, 14, 18, 22, 26 and 30 days of forcing.

Determination of sugar and starch concentrations

Sugars were extracted as described by Bonhomme et al. (2005) with some modifications. Samples were ground in liquid nitrogen. Then 100 mg of tissue were extracted in 1 mL of 80% (v/v) ethanol at 80 °C for 15 min in a water

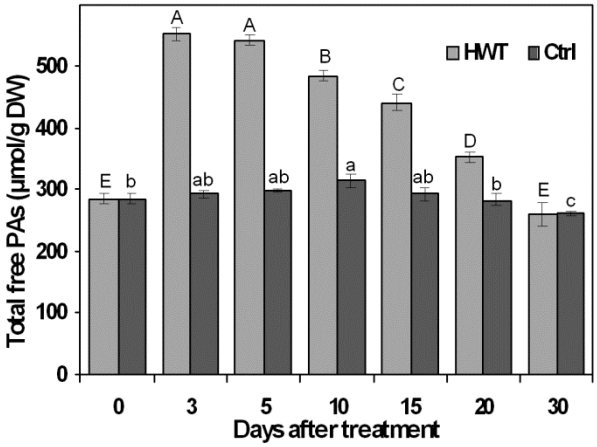

Fig 5. Changes in total free polyamines (µmol g^{-1} DW) in the buds of Superior Seedless grapevine cuttings immersed in hot (HWT) or warm water (Ctrl) and forced during one month. Different letters indicate significant differences in total free PA levels between different forcing periods according to Duncan's Multiple Range Test at $P \leq 0.05$.

bath with gentle shaking. The extract was centrifuged at 14,000 × g for 10 min. This extraction was repeated three times and the supernatants were collected and pooled. To the pellet, we added 0.5 mL of 80% (v/v) ethanol and kept it at -20 °C for starch determination. To remove phenolic compounds, supernatants were filtered on activated charcoal and insoluble polyvinylpolypyrrolidone (PVPP). The aliquots were dried by vacuum centrifugation with a speed-vac for 5 h at room temperature. The samples were later dissolved in ultra-pure water prior to analysis. A 20 µL-aliquot was filtered through a membrane (pore size, 0.45µm) and injected into a HPLC equipped with an Aminex column HPX-87C (BioRad, USA). Column temperature was kept at 80 °C and ultra-pure water was used as a solvent at a flow rate of 0.3 mL min^{-1}. External standards of glucose, fructose and sucrose were used to identify soluble sugars. Starch content was determined using the perchloric acid method described by Robin et al. (1991). The pellet, left after sugar extraction, was further extracted three times with 5 mL of 35% (v/v) perchloric acid with continuous shaking at low speed for 15 min. The extracts were pooled and centrifuged for 5 min at 10,000 × g. The supernatants were collected in graduated tubes and brought to 20 mL with distilled water. For colorimetric determination, a 1-mL aliquot of extract was mixed with 5 mL of anthrone reagent (0.175% w/v in 75% cold sulfuric acid) in a test tube and mixed briefly. The mixture was placed into boiling water for 12 min and then set on ice. The mixture's absorbance was read at 620 nm using a spectrophotometer (Shimadzu, Japan). Glucose standards from 0 to 100 µg ml^{-1} were used for calibration.

Determination of free proline concentrations

Free proline was determined by ninhydrin method modified method by Bates et al. (1973) and described by Mohamed et al. (2010b). Briefly, 200 mg (FW) of plant material was extracted with 5 mL of 40% (v/v) methanol heated to 80ºC for 30 min. Then 1 mL of supernatant was mixed in a reaction test tube with 2 mL glacial acetic acid, 1 mL ninhydrin solution (25 $mgmL^{-1}$) and 2 mL of a mixture consisting of 24% (v/v) distilled water, 60% (v/v) glacial acetic acid and 16% (v/v) orthophosphoric acid. In test tube, the mixture was boiled in a water bath at 100ºC for 30 min to develop the colours. The reaction was arrested in an iced bath

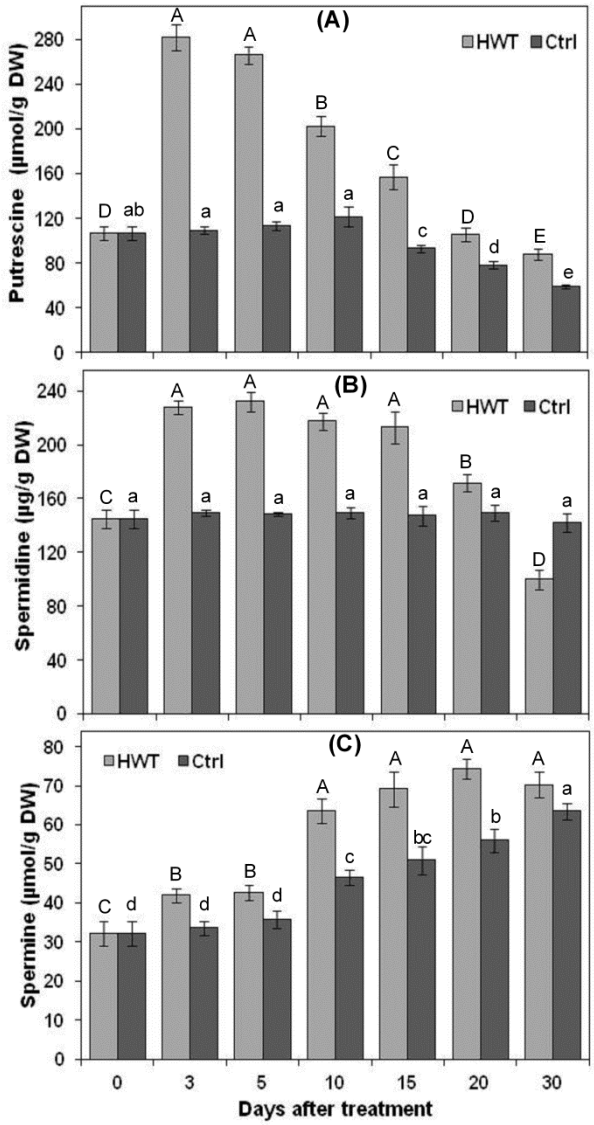

Fig 6. Changes in free Put (A), Spd (B) and Spm (C) levels (µmol g^{-1} DW) in the buds of Superior Seedless grapevine cuttings immersed in hot (HWT) or warm water (Ctrl) and forced during one month. Different letters indicate significant differences in total free PA levels between different forcing periods according to Duncan'sMultiple Range Test at $P \leq 0.05$.

and the chromophore obtained was extracted with 3 mL of toluene by vigorous shaking for 20 sec. The absorbance was measured at 528 nm and the proline concentration was determined using a standard curve.

Enzyme extraction and assays

Enzymes were extracted according to Wu et al. (2008) with slight modifications. One hundred milligrams (0.1 g) of frozen tissue were crushed into 1 mL of ice-cold potassium phosphate buffer (50 mM, pH 7.5) containing EDTA (1 mM) and 2% (w/v) of polyvinylpyrrolidone. For APX activity, 5 mM ascorbic acid was included in the grinding medium. The extracts were centrifuged at 4°C and 10,000×g for 30 min, and the supernatants were collected and immediately used for protein and enzyme assays. Catalase (CAT) (EC 1.11.1.6) activity was measured using spectrophotometric method of Aebi (1984), in which the disappearance of H_2O_2 was monitored

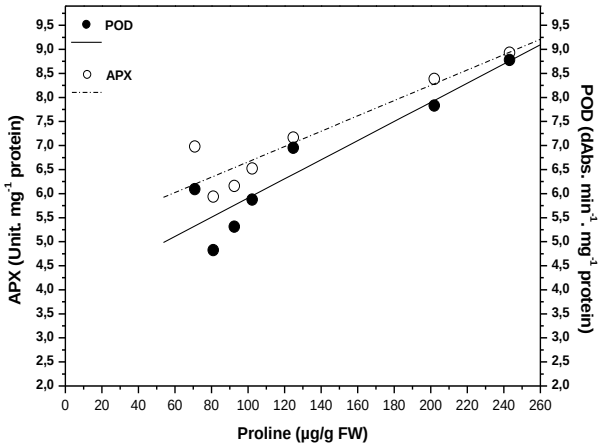

Fig 7. Regression analysis of relationship between peroxidase (POD) and ascorbate peroxidise (APX) activities and proline content in hot water treated grapevine buds.

at 240 nm using an extinction coefficient of 39.4 $mM^{-1}cm^{-1}$. Ascorbate peroxidase (APX) (EC 1.11.1.11) activity was assayed according to the modified method of Vyas and Kumar (2005). The reaction mixture, having a final volume of 3 mL, contained: 50 mM potassium phosphate buffer (pH = 7), 0.4 mM ascorbate, 10 mM EDTA, 10 mM H_2O_2 and 0.1 mL enzyme extract. The reaction was initiated by adding H_2O_2. The activity of APX was determined spectrophotometrically by assessing the decrease of ascorbate content at 290 nm after 3 min at 25°C using an extinction coefficient of 2.8 mM^{-1} cm^{-1}. Both CAT and APX activity were expressed in enzyme unit mg^{-1} protein, where one enzyme unit was defined as a change by 0.01 in absorbance between the blank and the sample per minute of reaction time (Zhang and Kirkham, 1994). Total POD (EC 1.11.1.7) activity was determined spectrophotometrically by monitoring the formation of a coloured complex from 3-dimethylamino benzoic acid (DMAB) and 3-methyl-2-benzothiazolinone hydrozone hydrochloride monohydrate (MBTH) at 590 nm in the presence of H_2O_2, according to the method described by Ngo and Lenhoff (1980). The specific enzyme activity was expressed as the change in absorbance at 590 nm per minute and per milligram of protein. The soluble protein content was determined according to the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

Polyamine analysis

Free polyamines (Put, Spd, Spm) were quantified according to the method of Geuns et al. (2006). In brief, 10-mg sample of freeze-dried powdered tissue was homogenized in 1mL 4% (v/v) $HClO_4$ containing 1,7-diaminoheptane–2HCl as internal standard. After extraction at 4 °C for 1h, the homogenate was centrifuged. To 50 µL of the supernatant, 100 µL 0.4M borate:NaOH buffer (pH 11) and 100 µL of dansyl chloride solution (7 mg mL^{-1} acetone) were added. After heating at 70°C (in the dark) for 15 min, the dansylated polyamines were extracted with 0.6 mL of toluene. The extract was mixed with 0.6 mL hexane and loaded on a 0.25 g silica gel column and washed with 250 µL toluene and then with 250 µL toluene–triethylamine (10:0.3, v/v). The column was sucked dry and the dansylated polyamines were eluted twice with 0.3 mL ethyl acetate which was later evaporated in a vacuum centrifuge. Then, the residue was dissolved in 250 µL methanol, then 10 µL of the solution were injected in a fully automated HPLC system (Knauer, Germany).

Statistical analysis

Data were subjected to analysis of variance for a completely randomized design with three replicates using SAS statistical software version 6.12 (SAS Institute, Cary, NC, USA). Where applicable, means were separated by Duncan's Multiple Range Test ($P \leq 0.05$).

Conclusion

Immersion in hot water was effective in overcoming endodormancy of grapevine buds as indicated by the large percentage of budbreak after one month of forcing. Similar to what was reported following application of HC or chilling, several biochemical changes took place nearly simultaneously shortly after moving the treated buds to forcing conditions. By the third day of forcing, CAT activity was at its minimum while that of APX and POD at their maximum. Concomitantly, starch level declined while sucrose, glucose, fructose, proline, Put and Spd accumulated. These changes hint at the possibility that similar mechanisms lead to endodormancy release independent of the rest-breaking agent used. However, these changes appear more rapid in response to hot water treatment as indicated by the more precocious budbreak. The use of hot water to induce a near-lethal heat stress promises to be a useful technique to study the changes associated with dormancy release; it also raises the hope to develop safer cultural practices to overcome dormancy in horticultural crops. A more careful analysis over shorter intervals of time of metabolites and transcription products is needed to establish the chain of events leading to endodormancy release and active growth resumption.

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