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Paclobutrazol-Induced Biochemical Changes in Pomegranate (*Punica granatum* L.) cv. 'Malas Saveh' under Freezing Stress

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Abstract
Freezing injury is an important limiting factor in the production of pomegranate in Iran.The aim of the present study was to evaluate the effect of paclobutrazol (PBZ) on cold hardiness of pomegranate (*Punica granatum* L.) cv. 'Malas Saveh'. Different concentrations of PBZ including 0 (control), 31, 62, 125 or 250 mg L^{-1} were sprayed on one-year-old plants in August and the electrolyte leakage of their stems was measured at three acclimation stages (November, January, and March). PBZ treatments, especially at 125 and 250 mg L^{-1} concentrations increased cold hardiness, and corresponding soluble carbohydrates and proline contents. The highest variation in freezing tolerance was observed between control and PBZ-treated plants in January, and the lowest found in November. Irrespective of PBZ treatment, correlations between cold hardiness and soluble carbohydrate concentrations were stronger, compared to proline. Soluble carbohydrates were higher in January, associated with deep dormancy, whereas the maximum proline content was detected in March, at deacclimation stage. Results suggest that PBZ application can reduce low temperature-induced dysfunction of cell membrane through increasing soluble carbohydrates and proline contents.

Keywords: electrolyte leakage, freezing injury, proline, soluble carbohydrates.

Abbreviations: PBZ, Paclobutrazol; **REL**, Relative electrolyte leakage; **DW**, Dry weight; **FW**, Fresh weight.

Introduction

Pomegranate (*Punica granatum* L.) is one of the oldest edible fruits widely grown in Iran (Sarkhosh *et al.,* 2009). Iran has an annual production of 670,000 tonnes (Anonymous, 2012), which is the first in the world. Freezing injury is one of the most important restrictions in commercial pomegranate production. Pomegranate is mostly grown in the margins of deserts in Central and Northeastern Iran where the night temperature during winter may drop to –20°C or lower. Plant growth regulators offer an approach to increase or prolong cold hardiness in fruit trees in hazardous areas. PBZ, a growth retardant from triazole group, is now commercially used in many tropical and subtropical regions to reduce growth (Srivastav *et al.,* 2003) and to increase tolerance of fruit crops to

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various environmental stresses (Fletcher *et al.,* 2000). PBZ is sold under the trade name 'Cultar,' for use on fruit trees and 'Bonsi' for use on ornamentals (Davis *et al.*, 1986). The retardant activity of PBZ is not accompanied by phytotoxicity or scorch even when applied at high rates (Dalziel and Lawrence, 1984). PBZ is readily taken up through the roots, stems, and leaves, but is almost exclusively translocated acropetally in the xylem (Anon, 1984). Many researchers stated that foliar-applied PBZ has no considerable impact on plant size (Ahmedullah *et al.*, 1986; Reynolds *et al.,* 1992). However, when being used to protect plants against environmental stresses such as low temperature stress, application by foliar spray ensures that the activity is confined to where it is required; the developing leaves (Fletcher *et al.,* 2000).

PBZ interferes with gibberellin biosynthesis by inhibiting oxidation of ent-kaurene to ent-kauronoic acid in endoplasmic reticulum through disabling cytochrome P 450-dependent oxygenase (Srivastav *et al.,* 2010). Based on PBZ's other functions, it has been used to protect plants against abiotic stresses such as chilling injury (Senaratna *et al.*, 1988; Lin *et al.,* 2006), water stress (Arzani and Yazdani, 2008), and salinity stress (Srivastav *et al.*, 2010).

Physiological effects of PBZ on plants including changes in growth (Arzani and Roosta, 2004; Arzani *et al.,* 2009) and morphology (Sugavanam, 1984), antioxidant activity (Kraus and Fletcher 1994), increasing levels of proline (Baninasab, 2009) and carbohydrates (Coleman and Estabrooks, 1992) play key roles in withstanding the low temperature stress. A number of researchers have investigated the influence of PBZ on cold resistance in woody plants such as apple (*Malus pumila*) (Coleman *et al.*, 1992), pecan (*Carya illinoinensis*) (Ali Khan, 1998), and hardy kiwi (*Actinidia arguta*) (Tafazoli and Beyl, 1993). Understanding the mechanisms by which growth regulators affect plant hardiness level would be important for further development of these techniques.

The aims of this study were 1. to investigate the effect of PBZ on cold hardiness in 'Malas Saveh' pomegranate cultivar in three acclimation stages (November, January and March); and 2. to study changes in soluble carbohydrates and proline contents during acclimation and deacclimation as well as their relationships with cold hardiness.

Materials and Methods

Plant material and PBZ treatment

Rooted cuttings of pomegranate cultivar 'Malas Saveh' were obtained from a commercial nursery in Saveh, Iran, in July 2010 and transplanted to 15-L pots filled with orchard soil (silt loam). Plants were left outdoors, watered daily, and fed monthly with fertilizer 20N– 20P– 20K containing micronutrients (Delta Green South Co., Iran). Pomegranate plants were foliar sprayed with different concentrations of PBZ, formulated as 'Cultar' at 250 g L^{-1} suspension concentration (Syngenta, UK), including 0 (control), 31, 62, 125, or 250 mg L^{-1} on 25 August 2010. The experiment was conducted using a completely randomized design with 10 plants per treatment replicated three times for a total of 150 plants.

Freezing procedure

Shoots (20 cm in length) were collected at three acclimation stages (November, January, and March); transferred immediately to the laboratory for analysis. Shoots were washed with deionized water, cut into 1-cm long segments; five pieces were placed into 50-ml plastic tubes as one replicate. A measure of 2 ml of deionized water was added to each tube for immediate ice formation. Tubes were then transferred into a freezing chamber (Kimia Rahavard, Tehran, Iran) to expose them to

low temperatures (Ghasemi Soluklui *et al.*, 2012). Starting temperature was 5°C and cooling rate was 2°C h^{-1}. Treatment temperatures at the three acclimation stages were:

Stage one (30 November 2010): –6°C, –9°C, –12 °C, –15°C, –18 °C.

Stage two (20 January 2010): –12°C, –15°C, –18°C, –21°C, –24°C.

Stage three (25 March 2011): –6, –9°C, –12°C, –15°C, –18°C.

Samples were kept at the final temperature for 1 h and then removed from the freezing chamber.

Electrolyte leakage measurement

As much as 20 ml of deionized water was added to each tube, shaken for one hour (250 rpm) at 23°C and kept at room temperature for 24 h before the first electrical conductivity (EC1) measurement was carried out. Samples were then autoclaved at 120°C for 20 min to allow maximum leakage of ions, cooled at room temperature for 2 h, and then electrical conductivity (EC2) was measured again. REL was calculated using the formula: REL = (EC1 / EC2) × 100. Cold hardiness was expressed as LT_{50} (lethal temperature at which 50% of the total ion leakage occurs) by fitting response curves with the following logistic sigmoid function (Fiorino and Mancuso, 2000):

$$R = \frac{a}{1 + e^{b(x-c)}} + d \quad (1)$$

where R= REL, based on LT_{50} estimation method used; x= treatment temperature; b= slope of the function at the inflection point c; a and d determine the upper and lower asymptotes of the function, respectively.

Soluble carbohydrates

Soluble carbohydrates were determined based on the anthrone method (Yemm and Willis, 1954). Stem samples were powdered using liquid nitrogen. Soluble carbohydrates were extracted three times from 1 g of ground tissue with 5 ml of 80% ethanol and centrifuged for 15 min at 3000 gn. One ml of 0.2% anthrone reagent (2 g anthrone in 1 L of 72% sulfuric acid) was added to 100 µl of the ethanolic extract. The reaction mixture was heated in a boiling water bath for 10 min and then rapidly cooled on ice. Absorbance was measured using a spectrophotometer (Bel Engineering Srl, Monza, Italy) at 620 nm. Soluble carbohydrate concentration was finally calculated through a calibration curve and expressed as mg soluble carbohydrates g^{-1} DW.

Proline content

Free proline concentration was determined as described by Bates *et al.* (1973). Stem samples were ground in liquid nitrogen and 0.5 g of ground tissue homogenized in 10 ml of 3% (w/v) aqueous sulfosalicyclic acid. The homogenate was then filtered through a Whatman No.1 filter paper. Afterward, 2 ml of filtered extract was taken for the analysis to which 2 ml ninhydrin and 2 ml glacial acetic acid were added. The reaction mixture was incubated in a boiling water bath for one hour and the reaction was finished in an ice bath. Toluene (4 ml) was added to the mixture and the organic phase was extracted. Absorbance of the extract was read at 520 nm using a spectrophotometer, while toluene was used as a blank. Proline concentration was finally calculated through a calibration curve and expressed as µM proline g^{-1} FW.

Statistical analysis

LT_{50} values, soluble carbohydrates, and proline concentrations of stem samples at each acclimation stage were analyzed using a one-way analysis of variance (PROC GLM, SAS Institute, Cary, NC). Means were separated using Duncan's multiple range tests ($P \leq 0.05$). Moreover, polynomial linear (L), quadratic (Q), and cubic (C) contrasts were used to test the effect of levels of PBZ on LT_{50} values.

Correlation analysis between LT_{50} levels, and soluble carbohydrates and proline concentrations were performed using Pearson's correlation coefficient (PROC CORR). To explore the relationship between biochemical changes and cold hardiness in greater details, correlation between soluble carbohydrates and proline concentrations with REL of stem samples in January (full dormancy) at –12 and –21°C, treatment temperatures lower and higher than LT_{50} values at this stage, were also analyzed.

Results

Cold hardiness

PBZ treatment increased cold hardiness of pomegranate plants in November, January, and March. In November (early hardening), LT_{50} values were affected by PBZ in a predominantly quadratic manner. Plants treated with PBZ at 125 and 250 mg L^{-1} showed the highest cold hardiness in November, whereas no difference in freezing tolerance was observed among other treatments (Table 1). In January (full hardening), freezing tolerance of all PBZ-treated plants increased by 1.5 to 3.5°C compared to November. However, in untreated plants, cold tolerance enhancement was <1°C.

In January, PBZ increased freezing tolerance of pomegranates in a predominantly linear manner, with plants treated with PBZ at 250 mg L^{-1} and controls reached LT_{50} at –17.8 and –14.2 °C, respectively. Freezing tolerance of pomegranate plants, regardless of PBZ treatments, decreased in March and PBZ treatments at 31 and 62 mg L^{-1} did not maintain their positive efficacy on cold hardiness at this stage. However, freezing tolerance of pomegranates in January increased linearly by increasing PBZ concentration; plants treated with 250 mg L^{-1} PBZ showed the highest cold tolerance compared to the other PBZ treatments (Table 1).

Table 1. Effect of PBZ on LT_{50} values estimated by electrolyte leakage measurement of pomegranate cv. 'Malas Saveh' at different acclimation stages.

PBZ (mg L^{-1})	LT_{50} (°C)		
	November	January	March
0	-13.33±0.04b*	-14.20±0.07c	-10.72±0.06c
31	-13.65±0.05b	-16.43±0.1b	-10.58±0.02c
62	-14.23±0.23b	-16.74±0.28ab	-11.48±0.02bc
125	-15.56±0.06a	-16.90±0.60ab	-11.96±0.29b
250	-14.43±0.26ab	-17.83±0.07a	-13.62±0.07a
P-value	0.012	≤0.001	≤0.001
P-value of polynomial contrasts			
Linear	0.022	≤0.001	≤0.001
Quadratic	0.005	0.026	0.768
Cubic	0.153	0.016	0.841

* Data represents the mean of three replicates ± SE. Similar letters in each column indicate non-significant differences among treatments at $P \leq 0.05$.

Soluble carbohydrates

The amount of soluble carbohydrates increased during cold acclimation from November to January and decreased in March. The overall means were 2.48, 10.55, and 1.82 mg g^{-1} DW, respectively (Fig. 1).

In November, higher levels of soluble carbohydrates were obtained from 125 and 250 mg L^{-1} PBZ treatments, whereas the remaining treatments were not different. Regardless of the PBZ treatment, the amount of soluble carbohydrates increased from November to January. However, increase in soluble carbohydrates was concentration-dependent, and plants treated with 250 mg g^{-1} PBZ and untreated ones showed 3.8- and 5-fold increases in soluble carbohydrates, respectively.

In January, stem samples treated with PBZ at 125, 250, and 62 mg L^{-1} contained the highest soluble carbohydrates. The values were considerably dropped in March. Maximum and minimum amounts of soluble carbohydrates were obtained from 250 mg L^{-1} PBZ and control, respectively, while the remaining treatments showed intermediate effects.

Higher correlations were revealed between LT_{50} values and soluble carbohydrates at all stages (Table 2). When three stages were pooled, cold hardiness was considerably associated with the amounts of soluble carbohydrates. Moreover, correlation coefficients between soluble carbohydrate concentrations and REL at –12 and –21°C in January were r = –0.67, $P \leq 0.01$ and r = –0.73, $P \leq 0.01$, respectively.

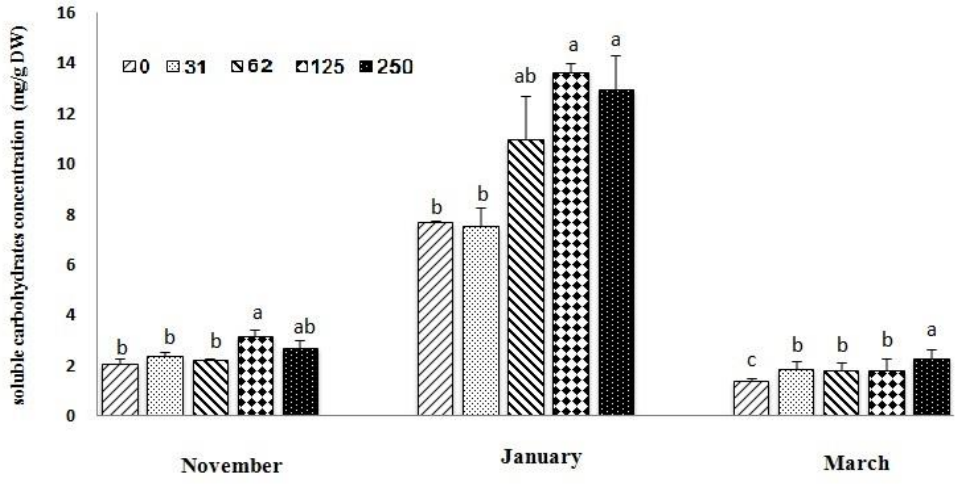

Fig. 1. Effect of PBZ (0, 31, 62, 125, and 250 mg l^{-1}) on soluble carbohydrates in stem samples of pomegranate cv. 'Malas Saveh' at three acclimation stages. Values are means ± SE of three replicates. Similar letters at the top of the columns indicate non-significant differences among PBZ treatments at each stage at $P \leq 0.05$.

Table 2. Pearson correlation coefficients between the concentrations of soluble carbohydrates and proline in stems of pomegranate cv. 'Malas Saveh', and LT_{50} values, estimated at different acclimation stages †.

Variables	LT_{50}			
	November	January	March	All three stages
Soluble carbohydrates	-0.78***	-0.79***	-0.71**	-0.72**
Proline	-0.58*	-0.74**	-0.69**	-0.34*

† Number of observations per each stage and all three stages together were 15 and 45, respectively. *, **, ***Significant at $P \leq$ 0.05, 0.01 or 0.001, respectively.

Proline

In November, all PBZ treatments, except 31 mg L^{-1}, resulted in increase in proline, compared to the control. The highest proline content was obtained from PBZ treatment at 125 mg L^{-1} concentration (3.88 µM g^{-1} FW), while the least amount (0.67 µM g^{-1} FW) was found in untreated plants. In January, increased amounts of proline were still observed with PBZ treatments compared to the control. However, proline concentrations showed a narrow range of variation at this stage from 1.37 to 2.83 µM g^{-1} FW (Fig. 2).

In March, irrespective of PBZ treatment, proline content of stem samples was higher than those measured in January and the maximum amounts were observed with PBZ at 250, 125, and 62 mg L^{-1}, respectively. There were positive correlations between proline contents and LT_{50} values at each acclimation stage (Table 2). However, when three stages were pooled, correlation coefficient between cold hardiness and proline content was not relatively high. Furthermore, there was a relatively slight relationship between proline concentrations and REL evaluated at lower treatment temperatures. Correlation coefficients between proline values and REL at –12 and –21°C in January were $r = –0.78$, $P \leq 0.001$; and $r = –0.42$, $P \leq 0.05$, respectively.

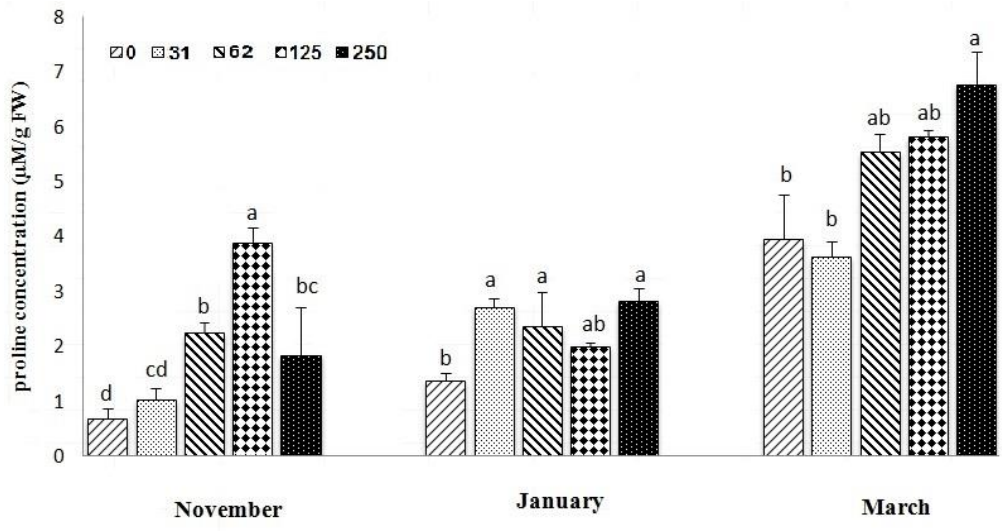

Fig. 2. Effect of PBZ (0, 31, 62, 125, and 250 mg L^{-1}) on proline content in stem samples of pomegranate cv. 'Malas Saveh' at three acclimation stages. Values are means ± SE of three replicates. Similar letters at the top of the columns indicate non-significant differences among PBZ treatments at each stage at $P \leq 0.05$.

Discussion

PBZ treatment substantially enhanced cold hardiness in plants at all acclimation stages, but the efficacy of treatments was different at each stage. The highest variation in freezing tolerance was observed between control and PBZ-treated plants in January and the lowest was found in November. Several research groups reported the positive effect of PBZ on freezing tolerance of woody plants such as kiwi fruit (Tafazoli and Beyl, 1993), apple (Coleman *et al.*, 1992; Coleman and Estabrooks, 1992), and citrus rootstocks (*Citrus sp.*) (Yelenosky *et al.*, 1995).

However, a few researchers reported no enhancement in freezing tolerance of PBZ-treated peach (*Prunus persica*) (Walser and Davis, 1986) and Concord grape (*Vitis labrusca*) (Ahmedullah *et al.*, 1986). On the other hand, the use of PBZ apparently

does not diminish the inherent tolerance to severe freezes (Yelenosky *et al.,* 1995).

In addition, a number of studies indicated the useful effect of PBZ on the induction of low temperature tolerance in herbaceous plants such as watermelon (*Citrullus vulgaris*) (Baninasab, 2009) and cucumber (*Cucumis sativus*) (Feng *et al.,* 2003). The effect of growth retardants may vary between plant species and even among cultivars (Palonen and Buszard, 1997), so different plants may have different mechanisms regulating cold hardiness in response to triazole treatment (Tafazoli and Beyl, 1993).

Increasing freezing tolerance in woody plants treated with PBZ may simply be attributed to the growth-controlling effect of this compound, which consequently led to higher carbohydrate reserves. Pomegranate plants treated with 125 and 250 mg L^{-1} had an earlier growth cessation and leaf abscission by 10 days, compared to the control, resulting in early acclimation. However, foliar spray of PBZ on raspberries (*Rubus* spp.) (Maage, 1986) and soil application of this compound on citrus rootstocks (Yelenosky *et al.,* 1995) resulted in a decrease in plant growth without increasing cold hardiness. Plants treated with PBZ had increased amounts of soluble carbohydrates at all acclimation stages compared to control. PBZ treatment increased soluble carbohydrates in apple (Steffens and Wang, 1986; Coleman *et al.,* 1992), Kiwi fruit (Tafazoli and Beyl, 1993), sweet cherry (*Prunus avium*) (Vu and Yelenosky, 1992), and carrot (*Daucus carota*) (Gopi *et al.,* 2007). Increasing the amount of carbohydrates in PBZ-treated plants may be due to a direct effect of this compound on carbohydrate metabolism rather than due to reduced growth (Wang *et al.,* 1986).

Soluble carbohydrate reserves contribute to an increase in cryostability of cell membranes, which is a precondition of cold hardiness (Shao *et al.,* 2008). They are involved in maintaining membrane integrity resulting in less electrolyte leakage under freezing stress (Pagter *et al.,* 2008). Considering all three acclimation stages, a strong correlation was noticed between soluble carbohydrates and cold hardiness. Moreover, maximum concentrations of soluble carbohydrates and maximum cold hardiness were observed simultaneously in January. These results conform to studies on walnut (*Juglans regia*) (Ameglio *et al.,* 2004), European oak (*Quercus robur*) (Morin *et al.,* 2007), and pomegranate (Ghasemi Soluklui *et al.,* 2012). Results indicated a considerable relationship between concentration of soluble carbohydrates and freezing tolerance of plants subjected to severe freezing stress at –21°C. Coleman *et al*. (1992) stated that changes in both carbohydrates and cold hardiness are probably induced by weather changes, and coincidence between high concentrations of carbohydrates and cold hardiness does not necessarily prove a casual relationship between them (Palonen, 1999). Although cold hardiness has been found to be associated with the concentration of soluble carbohydrates in many woody plants (Pagter *et al*., 2008; Ghasemi Soluklui *et al*., 2012), divergent results obtained from a number of studies imply that the role of soluble carbohydrates in the regulation of frost injury in fruit trees is still inconclusive.

Stem samples of plants treated with PBZ contained more proline compared to untreated plants. Our results were consistent with previous reports on carrot (Gopi *et al.,* 2007) and mango (*Mangifera indica*) (Srivastav *et al.,* 2010).

Triazoles induce a transient rise in the ABA concentration and this increase could be due to an increased proline content (Gopi *et al.,* 2007). Proline has many roles in cold tolerance, including cellular osmotic adjustment and membrane stabilization (Delauney and Verma, 1993), but it is less efficient under severe cold stress (Santarius, 1992). Although stem proline contents and cold hardiness had a positive correlation at

each acclimation stage, soluble carbohydrates were found to be a better indicator of cold hardiness than proline, since proline contents were still high after deacclimation and reducing freezing tolerance in March, phenomena that were previously reported in pomegranate (Ghasemi Soluklui *et al.,* 2012). Proline accumulates slowly in plants after onset of stress and typically after acquisition of frost tolerance (Wanner and Junttila, 1999). However, accumulation of proline in cytoplasm is only beneficial when membranes remain intact (Chen and Li, 2002).

Perhaps of greatest importance are the residue levels found in the fruits. Studies on the influence of PBZ mode of application on soil residue levels and vegetative growth retardation of 10-year-old 'Starkrimson Delicious' apple trees showed that the greatest carry-over effect occurred in the soil drench application which resulted in the highest soil residue rates throughout a three-year period (Mauk *et al.,* 1990). Studies using the seedlings of apple show that there is no movement of PBZ from the site of application on mature leaves and that foliar-applied PBZ is not transported to stems or roots (Davis *et al.,* 1988). However, foliar application of 1000 mg l^{-1} PBZ on grape cv. 'Riesling' exceeded tolerance levels (0.1 ppm) for unregistered chemicals, established by Health and Welfare Canada (Reinolds *et al.,* 1992).

In conclusion, results showed that foliar spray of PBZ moderately reduced freezing injury in pomegranate plants with evidence of less electrolyte leakage through upregulating soluble carbohydrates and proline contents. However, there were no remarkable differences in cold hardiness of PBZ-treated and untreated plants. Further studies on optimizing the time of application and dosage of PBZ may be needed. Although pomegranate has a thick and inedible skin, it is worth measuring the residue levels of PBZ in fruits following foliar application.

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