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Effect of Water Deficit and Domestic Storage on the Procyanidin Profile, Size, and Aggregation Process in Pear-Jujube (*Z. jujuba*) Fruits

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ABSTRACT: No information exists on the proanthocyanidin content of pear-jujube (*Ziziphus jujuba* Mill) fruit, their polymeric types and sizes, and their self-aggregation, or on the effect of different water deficit levels during the fruit maturation period on these compounds. Two trimers, two tetramers, and six B type procyanidin pentamers were identified and quantified for the first time. Water deficit increased the content of procyanidins of low molecular mass, improving their potential bioavailability and possible physiological effects on human health. The tendency of procyanidins to self-aggregate was similar in the edible portion and pit, and was not affected by water deficit. The procyanidin content of fruit from well watered trees increased during domestic cold storage, whereas the fruits from trees suffering severe water stress lost some of their procyanidin content.

KEYWORDS: *deficit irrigation, plant water relations, water deficit, procyanidins, LC-MS/MS*

INTRODUCTION

Pear-jujube (*Ziziphus jujuba* Mill), which belongs to the plant family Rhamnaceae, is a very interesting crop because it is able to withstand severe water deficits, while maintaining leaf turgor, which allows good gas exchange levels and, as a consequence, good leaf productivity.[1] Such leaf turgor maintenance is mainly due to two simultaneous and complementary mechanisms: stomata regulation and active osmotic adjustment. Cui et al.[2] indicated that the fruit maturation stage is the optimal stage for implementing water deficit in pear-jujube, because low, moderate, and severe water deficits have no effect on the fruit weight and volume, the fruits taste sweeter, and eating quality is improved. In addition, the fruit maturation period is shortened, increasing the market value of the fruit, while fruit firmness is enhanced and the percentage of rotten fruit after storage is reduced.

Jujubes are considered to be minor fruits and, from a research and development point of view, have not received any major emphasis. However, the fruits are an integral part of the culture and way of life of millions of diverse Asian peoples and have also become so for large regions of Africa after the major cultivated species were introduced. [3] A key characteristic of pear-jujube fruit is its health-promoting effects, and it is considered a "functional food", since it has nutritional as well as health-beneficial uses reported by *in vitro*, *in vivo*, and nutritional trials with humans.[4−11]

The presence of flavonoids may help explain the antioxidant, antitumor, antiproliferative, anti-inflammatory, and proapoptotic activities of *Z. jujuba* fruits, as well as its protective effect against cardiovascular diseases and type II diabetes.[12−16] In addition, the presence of phenolic acids provides potent antioxidant and anti-inflammatory effects, as well as a protective effect against the oxidative damage associated with diseases such as coronary heart disease, stroke, and cancers.[17−21]

Proanthocyanidins are of great interest in nutrition and medicine because of their potent antioxidant capacity and possible protective effects on human health.[22] In this sense, Carnésecchi et al.[23] were the first to describe the antiproliferative effect of procyanidins on human cancer cells. Mao et al.[24] and Saito et al.[25] indicated that the antiproliferative and antitumoral properties of flavanols and procyanidins are presumably related to their degree of polymerization. However, to the best of our knowledge, no information exists on the proanthocyanidin contents, their polymeric types and sizes, and their self-aggregation in *Z. jujuba* fruit.

Flavonoids and phenolic acids have been detected in cultivars of *Z. jujuba*. Hudina et al.[26] and Bekir San et al.[27] reported the following quantitative amounts (mg × 100 g^{-1} dry weight) of phenolics in *Z. jujuba*: rutin (0.89−5.87), (+)-catechin (0.74−1.37), (−)-epicatechin (0.48−5.13), chlorogenic acid (0.22−0.95), caffeic acid (0.09−0.37), ferulic acid (0.22), and *p*-hydroxybenzoic acid (0.08−0.18). Moreover, Choi et al.[10] indicated the presence (mg × 100 g^{-1} dry weight) of procyanidin B2 (14−30.7), epicatechin (258.8−352.), querce-

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tin-3-*O*-rutinoside (295.5−1147), quercetin-3-*O*-galactoside (1.4−15.7), kaempferol-glucosyl-rhamnoside (18.7−32.6) in pulp, and saponarin (133.0−170.4), spinosin (1303.8−2237.7), vitexin (44.7−134.0), swertish (10.9−14.8), 6″-hydroxybenzoylspinosin (54.0−84.6), 6″-feruloylspinosin (1237.3−1242.9) in seeds. Of note is the fact that phenolic compounds in the skin were 5−6 times higher than in the pulp.[9]

According to Mithofer et al.[28] the storage of indigenous fruits, such as those of *Ziziphus* genus, is one of the main strategies adopted by rural communities in Africa to reduce hunger, improve nutrition, and generate income. Although *Z. jujuba* fruit senesces rapidly at ambient temperature after harvest,[29] Tembo et al.[30] reported that the proportion of shriveled *Z. mauritiana* fruits increased as the storage temperature rose from 5 to 20 °C, and that fruits stored at low temperature (5 °C) had a very low proportion of shriveled fruits after 12 weeks of storage.

Information on the effect of plant water status on secondary metabolite contents is scarce, and many results could be considered contradictory, probably because of the fact that in most fruits it is not possible establish a linear correlation between these parameters.[31,32] Castellarin et al.[33] showed that water deficit has limited effects on proanthocyanidins in grape berries, and Tovar et al.[34] indicated that polyphenol contents of olive flesh increased as irrigation decreased. A decrease in phenolics during fruit development seems to be a general trend.[35,36] Conversely, there is some divergence with reference to changes in phenolics during fruit maturation and storage. In this sense, catechin and epicatechin are reduced during fruit storage at 25 °C, particularly at high relative humidity (75%). [37] Moreover, some authors indicated that the concentration of catechins has a tendency to decrease during fruit cold storage.[36,38] In contrast, Burda et al.[39] showed that procyanidins remained near constant from the maturation period to the end of cold storage.

The aim of this paper was to evaluate the profile of proanthocyanidin and their self-aggregation in pear-jujube fruits and to study the effect of different water deficit levels during fruit maturation on the proanthocyanidin content. In addition, we studied the changes in the proanthocyanidin content of the fruit after domestic cold storage for three months.

MATERIALS AND METHODS

Experimental Conditions, Plant Material, and Treatments. The experiment was carried out in 2011, at a farm near the city of Albatera (Alicante, Spain) (38° 12′ N, 0° 51′ W), planted with 7 year-old pear-jujube trees (*Zizyphus jujuba* Mill.) of the autochthonous cultivar "Grande de Albatera" at 2 m × 6 m. The soil of the orchard is a Torrifluvent with sandy loam texture, very low electrical conductivity (109 μS/cm, 1:10 w:v), high lime content (57%), very low organic matter content (0.3%), low exchangeable potassium (40 mg/kg), and low available phosphorus (20 mg/kg) levels. The irrigation water had an electrical conductivity of between 1.7 and 2.2 dS/m and a Cl^- concentration ranging from 36 to 48 mg L^{-1}.

The climate of the area is strictly Mediterranean, with mild winters, low annual rainfall, and hot dry summers. During the experimental period, average daily maximum and minimum air temperatures were 32 and 22 °C, respectively, while mean daily air vapor pressure deficit (VPD_m)[40] ranged from 1.25 to 3.25 kPa, and reference crop evapotranspiration (ETo)[40] was 189 mm. Total rainfall was negligible (1.8 mm on day of the year (DOY) 221).

Three irrigation treatments were considered, in which irrigation was carried out daily and during the night using a drip irrigation system with one lateral pipe per tree row. Control plants (treatment T0) were irrigated in order to ensure nonlimiting soil water conditions (112% ETo), and T1 plants were irrigated according to the normal criteria used by the grower (64% ETo). T2 plants were irrigated as T0, but irrigation was withheld for 36 days (from DOY 202 to 238). Total water amounts applied during the measurement period were 213 and 122 mm for T0 and T1 treatments, respectively.

Pear-jujube fruits were harvested when T0 fruits reached commercial ripening state (S7 stage of growth, according to Choi et al.[4]) and immediately transported to the laboratory on 27 August (DOY 239). Harvested fruits of each replicate were divided into two groups. Fruits from one of these groups were divided into edible portion (peel and pulp) and pit (shell and seed) and directly frozen at −20 °C until analysis. The fruits of the other group were stored at 5 °C and 65% relative humidity for 12 weeks. All samples were freeze-dried before analyzing the proanthocyanidins and their self-aggregation by LC-MS/MS and light scattering, respectively.

Plant Water Status. Predawn (Ψ_{pd}) leaf water potential was measured in mature leaves located on the south facing side, from the middle third of the tree (two leaves per tree and four trees per treatment). Midday (12 h solar time) stem water potential (Ψ_{stem}) was measured in a similar number and type of leaves as used for Ψ_{pd}, enclosing leaves in a small black plastic bag covered with aluminum foil for at least 2 h before measurements in the pressure chamber (model 3005, Soil Moisture Equipment Co., Santa Barbara, CA).[41−43]

Midday gas exchange in attached leaves, leaf conductance (g_{lmd}), and net photosynthesis (P_{nmd}) were measured with a steady-state porometer (LI-1600, LI-COR Inc., Lincoln, NE) on the abaxial surface of the leaves in a similar type of leaf as used for the Ψ_l measurements. Two measurements were taken on four trees per treatment.

Reagents and Standards. Epigallocatechin, which was used as standard, was purchased from Phytoplan (Heidelberg, Germany). Acetonitrile and methanol, both of LC-MS grade, and acetone of HPLC grade were obtained from Panreac Química S.A. (Barcelona, Spain), and acetic acid of LC-MS grade was from Scharlau (Sentmenant, Spain).

Extraction of Proanthocyanidins. Proanthocyanidins were extracted as described by Buendía et al.[44] with some modifications. Briefly, 1.6 g of each freeze-dried and powdered portion was weighed and homogenized with 0.025 L of extraction solution (acetone/water/acetic acid; 70/29.5/0.5) by using an ultraturrax (Ika, Staufen, Germany) for 1 min. The samples were kept on ice before and during homogenization. The homogenates were then sonicated in an ultrasound bath for 15 min followed by centrifugation (JP Selected Centronic Centrifuge, Barcelona, Spain) for 10 min at 1765 *g* (3200 rpm) at room temperature. Supernatants were concentrated in a rotary evaporator at 35 °C, and the aqueous residue was filtered through a C18 Sep-Pak cartridge (Waters Associates, Milford, MA), previously activated with 0.010 L of methanol, water, and air sequentially. Retained phenolic compounds were eluted with 0.008 L of methanol. The methanol was evaporated in a rotary evaporator at 35 °C, the residue was dissolved in 0.001 L of acetonitrile/acetic acid (2%), which was filtered through a 0.22 μm PDVF filter (Millex HV13, Millipore, Bedford, MA), and 3 μL of the solution was directly injected into LC-MS/MS for identification and quantification of flavan-3-ols compounds.

Normal Phase LC-MS/MS. The analysis of proanthocyanidins was performed by normal phase analysis, as previously reported.[44] Chromatographic separation was carried out on a Develosil 100 Å normal phase column (250 mm × 0.5 mm, 5 μm particle size) (Phenomenex, Seto, Japan). Two types of eluents were used to separate the gradients: a mixture of acetonitrile−acetic acid (98/2 v/v) as solvent A and a mixture of methanol−water−acetic acid (95/3/2 v/v/v) as solvent B. The injection volume was 3 μL, and elution was performed at a flow rate of 10 μL min^{-1}. The linear gradient started with 0% B, reaching 40% B at 40 min and 80% at 50 min, and keeping isocratic conditions for 2 min, reaching 0% B at 55 min and finally 0% B at 70 min. Identification of the compounds was made in a 1200 series micro-HPLC-DAD system (Agilent Technologies, Waldbronn, Germany) equipped with a degasser (model G1379B), a thermostatted autosampler (model G1377A), a capillary pump (model G1376A), and photodiode array detector (model G1315D). HPLC

was coupled to an ion trap mass spectrometer (ultra HCT Bruker, Bremen, Germany) equipped with electrospray ionization (ESI) and operated in negative ion mode. Data acquisition and processing were performed using software B.01.03-SR2 [204] for ChemStation for LC 3D system from Agilent Technologies. The capillary and voltage were maintained at 350 °C and 4 kV, respectively. Mass scan (MS) and daughter (MS-MS) spectra were measured from m/z 100 to 1500. Collision-induced fragmentation experiments were realized in the ion trap using helium as the collision gas, with the collision energy set at 50%.

The different compounds were identified and quantified by their UV spectra, which were recorded at 280 nm, and their molecular mass and daughter ions acquired in the negative mode on the mass spectrometer.

The calibration curve was made using 4, 8, 15.6, 31.3, 62.5, 125, 250, and 500 μM of (−)-epigallocatechin standard solutions.

Dynamic Light Scattering. Self-aggregation of proanthocyanidins was analyzed using the dynamic light scattering technique (DLS).[45,46] After the proanthocyanidins had been extracted as described above, to ensure that no other insoluble components are present, and after dissolving the residue in acetonitrile/acetic acid (2% v/v), the samples were filtered through a 0.22 μm PDVF filter (Millex HV13, Millipore, Bedford, MA) to observe the degree of self-aggregation. After standing for a minimum of 24 h, the samples were filtered again through the same type of filter to remove macroaggregates. In some cases, these compounds were observed after the second filtration. These samples were allowed to stand for a new period of 24 h before a third filtration. In all cases, after the final filtration, DLS measurements were performed immediately (within 90 s).

DLS measurements were made in a Zetasizer Nano-ZS (Malvern Instruments, Malvern, U.K.) with a laser wavelength of 633 nm and a quartz cell of minimum volume ZEN2112. This system uses backscatter detections at 175°, and all measurements were performed at 293 K. The time-dependence of the scattered light was monitored, and the autocorrelation function of the particles was measured. The cumulant method was used to fit the autocorrelation curves. This method provides the z-averaged hydrodynamic radius, which is a measure of the average size of the aggregates. The results shown in this work are those of the first of 10 consecutive runs involving between 13 and 15 subruns each (a number chosen in the automatic mode of the apparatus). Final results are the average of 4 different experiments from 4 different samples.

Statistical Design and Analysis. The design of the experiment was randomized with four replications, each replication consisting of three adjacent tree rows, each with 11 trees. Physiological measurements were taken on the inner tree of the central row of each replicate, which were healthy, uniform, and very similar in appearance, while the other trees served as border trees. Data were processed using SPSS software version 19 for Windows (2010; SPSS Inc., Chicago). Two-way analysis of variance was carried out, and mean values were compared by Tukey's multiple range test. All means were compared at the 0.05 level of significance. Values for each replicate were averaged before the mean and the standard deviation of each treatment were calculated.

RESULTS

Plant Water Status. Midday leaf conductance (g_{lmd}) and net photosynthesis (P_{nmd}) values in T0 were high and nearly constant throughout the measurement period (Figure 1A,B). The g_{lmd} values of T1 plants were also almost constant and intermediate between T0 and T2 values. However, g_{lmd} and P_{nmd} values in T2 plants gradually decreased during the stress period, reaching minimum values of 111.00 mmol m^{-2} s^{-1} and 2.57 μmol m^{-2} s^{-1}, respectively, on DOY 238.

Ψ_{stem} values in T0 plants remained constant during the experimental period, whereas Ψ_{stem} values in T1 and T2 plants showed a tendency to gradually decrease, reaching minimum values of −2.28 and −3.14 MPa, respectively, on DOY 238

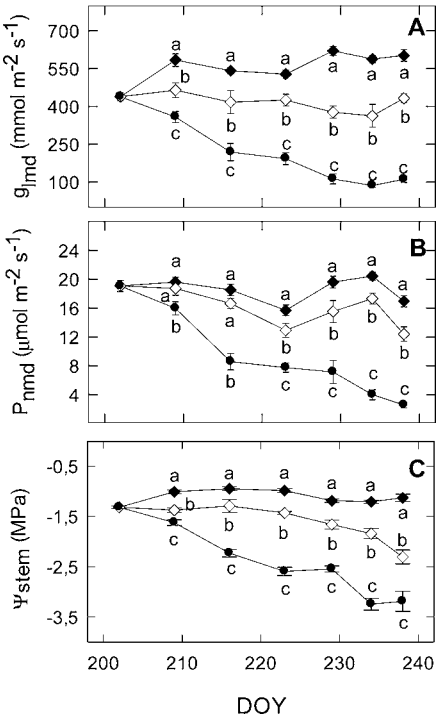

Figure 1. Midday leaf conductance (g_{lmd}, A), midday net photosynthesis (P_{nmd}, B), and midday stem water potential (Ψ_{stem}, C) values (mean ± SE, not shown when smaller than symbols, n = 4) for pear-jujube plants in T0 (◆), T1 (◇), and T2 (●) treatments during the experimental period (DOY, day of the year). Different letters on data points at each date indicate significant differences according to Tukey's test ($P \leq 0.05$).

(Figure 1C). Significant differences between treatments from DOY 209 to 238 were noted in Ψ_{stem} values, with the particular characteristic that T1 plants showed lower values than T0 plants and higher values than T2 plants (Figure 1C).

Effect of Water Deficit on the Proanthocyanidin Content, Their Self-Aggregation, and Size. The proanthocyanidin profile of pear-jujube fruits is shown in Figures 2 and 3 and Table 1, which illustrates the presence of one monomer of (epi)catechin and oligomers of this compound as a monomeric unit, which are known as procyanidins. Moreover, all of these procyanidins were type B because they contain only the single interflavan linkages (Figure 3). Procyanidins were tentatively identified on the basis of their mass spectra considering their mass (m/z 289, 577, 865, 1151, 1153, and 1441), their most characteristic fragmentations, and their elution order as previously described in Table 1.

Pear-jujube pits showed some essential differences in the proanthocyanidins content compared with observations made in the edible portion (Tables 2 and 3). These differences were due to the fact that the pentamers corresponding to peaks **9**–**12** were not detected in pits at harvest time.

The total procyanidins content in the edible portion of the fruits increased as a result of water deficit effect, although the differences between T1 and T2 contents were not significant (Table 2). However, the behavior observed in each procyanidin was not similar. In this sense, the (epi)catechin, the dimer (peak **2** in Figure 2), one trimer (peak **4** in Figure 2), and three pentamers (peaks **9** and **10** in Figure 2) showed a response to water deficit similar to that observed considering the total

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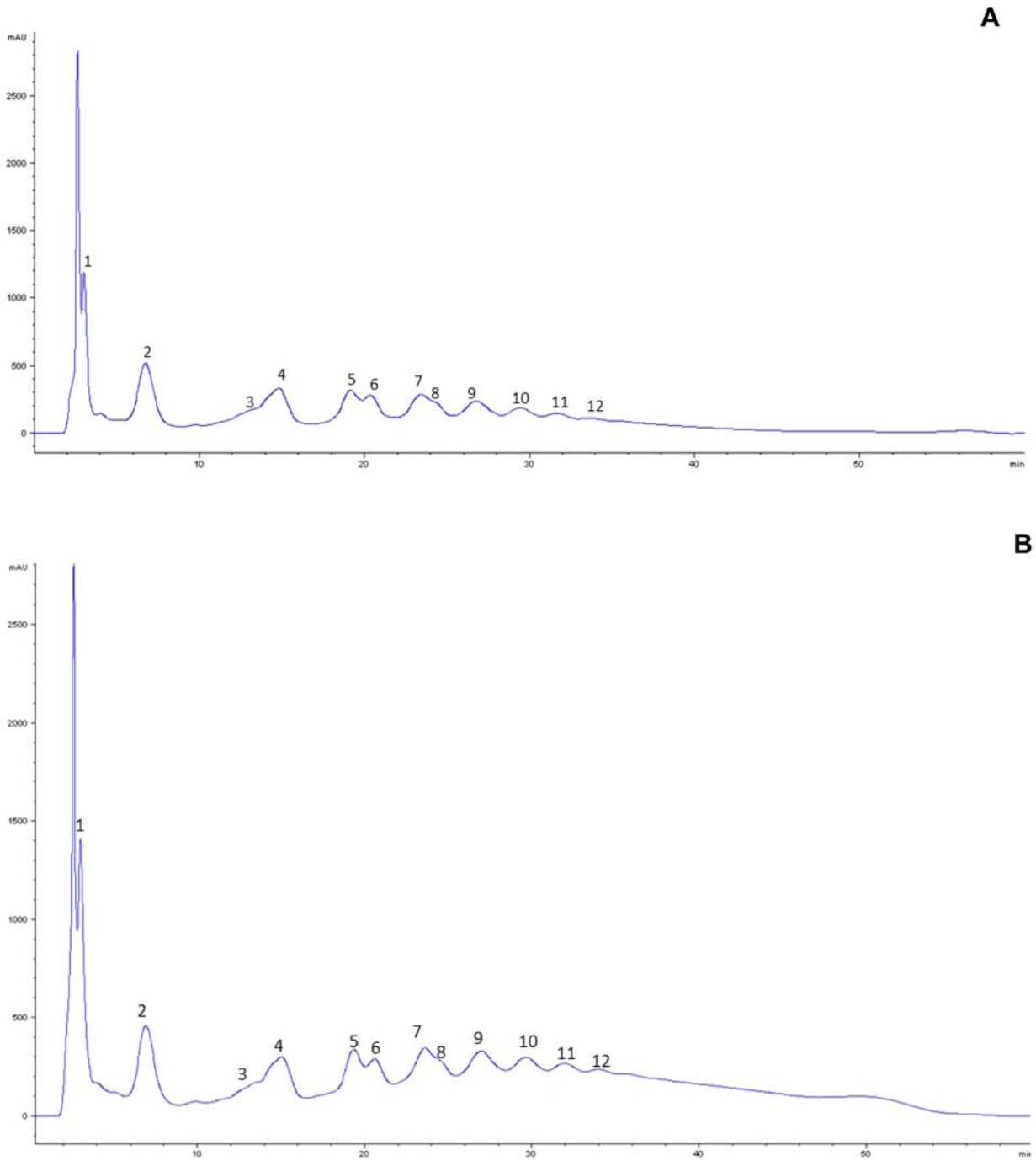

Figure 2. HPLC-DAD chromatogram (obtained at 280 nm) of edible portion (A) and pit (B) proanthocyanidins in pear-jujube fruits. Peak numbering as in Table 1.

proanthocyanidin contents, while the other proanthocyanidins did not change following water stress effect (Table 2).

The total procyanidin content in pits showed minimum values in T0 plants and maximum values in T2 plants. The behavior of each proanthocyanidins was not uniform (Table 3). In this respect, the dimer (peak **2** in Figure 2), the trimers (peaks **3** and **4** in Figure 2), and one tetramer (peak **6** in Figure 2) were more abundant under severe water stress (T2) conditions. The (epi)catechin and one pentamer (peak **8** in Figure 2) showed a progressive response to water deficit, the content in T2 pits being the highest and the contents in T1 intermediate between T0 and T2. An average behavior in response to water deficit was observed in one tetramer (peak **5** in Figure 2) and one pentamer (peak **7** in Figure 2), which increased in pits from T1 and T2 plants, although the differences between these treatments were not significant (Table 3).

Proanthocyanidin self-aggregates were considerably larger than oligomers, varying from 130 to 328 nm (Table 4). Self-aggregates in T1 and T2 fruit peel were larger than those from pit and flesh, respectively. Moreover, the effect of irrigation treatments on the self-agregate size from the different fruit portions was not significant (Table 4).

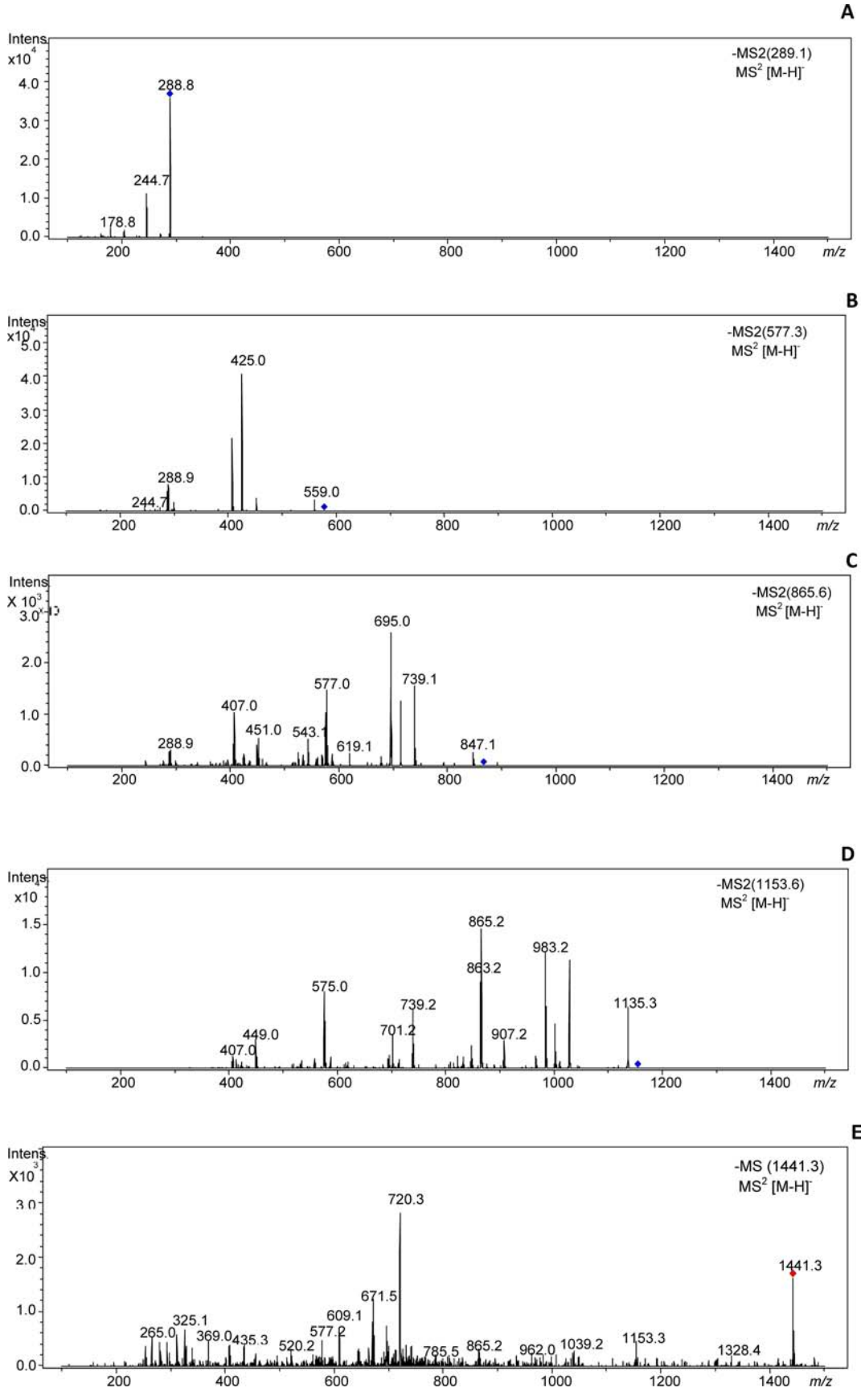

Figure 3. HPLC-MS-MS spectra in negative ionization mode of proanthocyanidins in edible portion of pear-jujube fruit: (epi)catechin (A), dimer B type (B), trimer B type (C), tetramer B type (D), and pentamer (E) (peaks 1, 2, 3, 5, and 9, respectively, in Figure 2).

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Table 1. Tentative Proanthocyanidin Oligomer Compound Identification in Pear-Jujube Fruits Identified by LC-MS/MS[a]

ID	proanthocyanidin	$[M-H]^-$	product ions (m/z)	t_R (min)	refs
1	(epi)catechin	289	245, 179	2.9	Buendia et al., (2010); Vallejo et al., (2012); Gu et al., (2003)(1); Gu et al., (2003)(2); Sarnoski et al., (2012)
2	dimer B type [(E)C−B−(E)C]	577	425, 407, 289	6.7	Buendia et al.,(2010); Vallejo et al., (2012); Gu et al.,(2003)(1); Gu et al., (2003)(2); Sarnoski et al., (2012)
3	trimer B type [(E)C−B−(E)C−B−(E)C]	865	739, 695, 577, 575, 451, 407, 289	11.3	Buendia et al.,(2010); Vallejo et al., (2012); Gu et al.,(2003)(1); Gu et al., (2003)(2); Sarnoski et al., (2012)
4	trimer B type [(E)C−B−(E)C−B−(E)C]	865	739, 695, 577, 575, 451, 425, 407, 287	13.3	Buendia et al.,(2010); Vallejo et al., (2012); Gu et al., (2003)(1); Gu et al., (2003)(2); Sarnoski et al., (2012)
5	tetramer B type [(E)C−B−(E)C− B−(E)C−B−(E)C]	1153	1135, 1027, 865, 863, 739, 575, 449, 407	19.3	Gu et al., (2003)(1); Gu et al., (2003)(2); Sarnoski et al., (2012)
6	tetramer B type [(E)C−B−(E)C− B−(E)C−B−(E)C]	1153	1137, 865, 695, 577, 476, 407	20.6	Gu et al., (2003)(1); Gu et al., (2003)(2); Sarnoski et al., (2012)
7	pentamer B type [(E)C−B−(E)C− B−(E)C−B−(E)C−B−(E)C]	1441	1152, 983, 865, 739, 577, 407	23.4	Gu et al., (2003)(1); Gu et al., (2003)(2)
8	pentamer B type [(E)C−B−(E)C− B−(E)C−B−(E)C−B−(E)C]	1441	1152, 865, 577, 407	24.6	Gu et al., (2003)(1); Gu et al., (2003)(2)
9	pentamer B type [(E)C−B−(E)C− B−(E)C−B−(E)C−B−(E)C]	1441	1153, 865, 720, 695, 577	26.8	Gu et al., (2003)(1); Gu et al., (2003)(2)
10	pentamer B type [(E)C−B−(E)C−B−(E)C−B−(E)C−B−(E)C]	1441	1153, 1135, 863, 575, 407, 285	29.5	Gu et al., (2003)(1); Gu et al., (2003)(2)
11	pentamer B type [(E)C−B−(E)C− B−(E)C−B−(E)C−B−(E)C]	1441	1153, 1135, 863, 577	31.7	Gu et al, (2003)(1); Gu et al, (2003)(2)
12	pentamer B type [(E)C−B−(E)C− B−(E)C−B−(E)C−B−(E)C]	1441	1153, 1135, 863, 577	33.8	Gu et al., (2003)(1); Gu et al., (2003)(2)

[a]Abbreviations used: identification (ID), molecular ion ($[M-H]^-$), retention time (t_R), (epi)catechin ((E)C), B type linkage (B). Peaks 3−12 have been described for the first time in pear-jujube fruit. References indicate previous MS^n spectra of procyanidins described in other fruits or fruit products coincident with those found in pear-jujube fruit.

Effect of Domestic Cold Storage in Normal and Water Stressed Fruits. The total proanthocyanidin content of the edible portion of the fruits increased with cold storage in T0 fruits and decreased in T2 fruits, while the storage effect in T1 fruits was not significant (Table 2). The behavior observed for each proanthocyanidin in T0 fruits reflected a significant increase as a result of cold storage, except (epi)catechin, whose content remained constant during storage (Table 2). The content of each proanthocyanidin in T1 fruit tended to remain constant during storage except the (epi)catechin (peak **1** in Figure 2) and the dimer (peak **2** in Figure 2), which decreased, and one pentamer (peak **11** in Figure 2), which increased. Although the total proanthocyanidin content decreased significantly during storage, the observed behavior of each proanthocyanidin differed. The (epi)catechin (peak **1** in Figure 2), the dimer (peak **2** in Figure 2), one trimer (peak **4** in Figure 2), one tetramer (peak **5** in Figure 2), and one pentamer (peak **12** in Figure 2) contents decreased; the content of the others proanthocyanidins did not change during storage (Table 2).

The storage effect on the total proanthocyanidin content in fruit pits from the three irrigation treatments was similar to that observed in the edible portion (Tables 2 and 3). In T0 fruit pits the content of each proanthocyanidin increased with storage except two pentamers (peaks **7** and **8** in Figure 2), which decreased. In contrast, the behavior of each proanthocyanidin contents in fruit pits from T1 and T2 was not so uniform. In T1 pits, the (epi)catechin (peak **1** in Figure 2), the dimer (peaks **2** in Figure 2), the trimers (peaks **3** and **4** in Figure 2), and the tetramers (peaks **5** and **6** in Figure 2) did not change, whereas two pentamers (peaks **7** and **8** in Figure 2) decreased and four pentamers (peaks **9**−**12** in Figure.2) increased (Table 3). In T2 pits, the contents of the dimer (peak **2** in Figure 2), one trimer (peak **4** in Figure 2), and two pentamers (peaks **11** and **12** in Figure 2) were constant during storage. However, the (epi)catechin, (peak **1** in Figure 2), one trimer (peak **3** in Figure 2), one tetramer (peak **5** in Figure 2), and two pentamers (peaks **9** and **10** in Figure 2) increased. Also, one tetramer (peak **6** in Figure 2) and two pentamers (peaks **7** and **8** in Figure 2) did not change, but showed a similar behavior to that observed for the total proanthocyanidins content (Table 3).

DISCUSSION

The fact that Ψ_{stem}, g_{lmd}, and P_{nmd} values in T0 plants were very high and almost constant during the measurement period (Figure 1) suggested that the irrigation applied to this treatment was sufficient to avoid any water deficit during the measurement period. The differences in Ψ_{stem}, g_{lmd}, and P_{nmd} values between T0, T1, and T2 plants clearly indicated a water deficit situation in T1 and T2 plants. However, the fact that at maximum stress Ψ_{stem} values in T2 plants were very low (−3.14 MPa) and that a strong degree of stomatal regulation was observed in the plants of this treatment (Figure 1A and B) indicated that T2 represented a severe water deficit situation. The decrease in Ψ_{stem} values resulting from the T1 deficit irrigation treatment led to low Ψ_{stem} values (−2.28 MPa). However, g_{lmd} and P_{nmd} values, despite being lower than those in T0 (Figure 1A,B), were still very high and nearly constant, indicating that water deficit in T1 can be considered as moderate.

The fact that the proanthocyanidins in pear-jujube fruits consisted exclusively of B type procyanidins, is in agreement with Gu et al.[47] who also indicated that fruits are the major source of these compounds in the diet. Previously, the presence of (+)catechin, procyanidin B2, and (epi)catechin has been demonstrated in in pear-jujube fruits.[10,26,27] However, the

Table 2. Effect of Irrigation Treatments (T0, T1, and T2) at Different Times (0, at Harvest; 1, after 12 Weeks at Domestic Cold Storage) on the Proanthocyanidin Oligomeric Species Content (mg/kg DW) in the Edible Part (Peel and Flesh) of Pear-Jujube Fruits[a]

proanthocyanidin	time	treatment					
		T0		T1		T2	
(epi)catechin	0	252.8 ± 43.2	bA	739.2 ± 51.5	aA	718.7 ± 41.2	aA
	1	435.7 ± 6.7	aA	374.7 ± 2.9	bB	247.0 ± 14.2	cB
dimer B type [(E)C−B−(E)C]	0	385.2 ± 81.8	bB	1074.3 ± 77.7	aA	1143.6 ± 141.5	aA
	1	758.7 ± 17.6	aA	672.1 ± 20.9	bB	246.5 ±9.85	cB
trimer B type [(E)C−B−(E)C−B−(E)C]	0	274.2 ± 50.9	aB	465.6 ± 43.5	aA	442.1 ± 40.8	aA
	1	539.9 ± 12.7	aA	497.6 ± 37.2	aA	260.8 ± 25.1	bA
trimer B type [(E)C−B−(E)C−B−(E)C]	0	182.5 ± 47.5	bB	669.3 ± 47.9	aA	658.3 ± 34.7	aA
	1	790.5 ± 27.2	aA	512.0 ± 29.2	bA	389.9 ±10.7	bB
tetramer B type [(E)C−B−(E)C−B−(E)C−B−(E)C]	0	411.3 ± 84.8	aB	682.5 ±46.9	aA	805.3 ± 87.2	aA
	1	885.6 ±10.5	aA	678.5 ± 22.6	bA	472.3 ± 13.5	cB
tetramer B type [(E)C−B−(E)C−B−(E)C−B−(E)C]	0	282.1 ± 57.3	aB	476.0 ± 40.2	aA	461.9 ± 18.9	aA
	1	673.3 ± 11.8	aA	592.3 ± 12.8	aA	287.6 ± 27.9	bA
pentamer B type [(E)C−B−(E)C−B−(E)C−B−(E)C−B−(E)C]	0	541.0 ± 105.3	aB	751.5 ± 107.9	aA	874.2 ± 103.4	aA
	1	1339.5 ± 33.2	aA	1124.9 ± 36.4	aA	499.3 ± 75.9	bB
pentamer B type [(E)C−B−(E)C−B−(E)C−B−(E)C−B−(E)C]	0	250.3 ± 80.6	aB	393.8 ±41.3	aA	376.3 ± 22.7	aA
	1	649.0 ± 37.2	aA	511.0 ± 16.0	abA	377.4 ± 36.8	bA
pentamer B type [(E)C−B−(E)C−B−(E)C−B−(E)C−B−(E)C]	0	288.4 ± 71.0	bB	820.2 ± 118.9	abA	1018.2 ± 134.8	aA
	1	1333.3 ± 18.3	aA	1224.3 ± 15.4	aA	755.4 ± 63.9	bA
pentamer B type [(E)C−B−(E)C−B−(E)C−B−(E)C−B−(E)C]	0	80.8 ± 23.1	bB	606.9 ± 99.4	aA	828.4 ± 126.2	aA
	1	1162.8 ± 46.0	aA	942.7 ± 102.7	abA	666.5 ± 65.7	bA
pentamer B type [(E)C−B−(E)C−B−(E)C−B−(E)C−B−(E)C]	0	nd		404.6 ± 150.0	aB	693.4 ± 191.1	aA
	1	915.0 ± 29.9	a	899.9 ± 23.3	aA	486.1 ± 29.0	bA
pentamer B type [(E)C−B−(E)C−B−(E)C−B−(E)C−B−(E)C]	0	nd		378.6 ± 86.4	aA	551.4 ± 95.4	aA
	1	565.2 ± 13.5	a	472.2 ± 11.8	bA	419.0 ± 11.9	bB
total	0	2948.6 ± 644.6	bB	7547.6 ± 911.6	aA	8571.7 ± 961.6	aA
	1	10048.4 ± 104.1	aA	8502.2 ± 92.7	bA	5107.7 ± 160.3	cB

[a]Means within a row for each proanthocyanidin and storage time followed by different small letter, and within a column for each proanthocyanidin and treatment followed by different capital letter, are significantly different at $P = 0.05$ by Tukeys test. Abbreviations used: (epi)catechin ((E)C), not detected (nd).

occurrence of the other procyanidins (Figures 2 and 3, Table 1) has not been reported, and two trimers, two tetramers, and six pentamers are described here in pear-jujube fruit for first time to our knowledge.

Hudina et al.[26] studied the catechin and (epi)catechin contents in seven Chinese pear-jujube fruit varieties (Bianhesuanzao, Yuanlingzao, Fupingdazao (Syn. Pozao), Zanhuangdazao, Zizao, Huizao, and Jinsixiaozao) and a wild ancestor of pear-jujube (Acid jujube), showing that these compounds varied from 0.01 to 0.02 and from 0.01 to 0.05 g/kg DW, respectively. Also, Choi et al.[10] reported that the (epi)catechin and one dimer contents in the pulp of three Korean varieties (Boeun-deachu, Mechu, and Sanzoin) of pear-jujube fruits ranged between 2.6 and 3.5 and 0.1 and 0.3 g/kg DW, respectively. In our case, the (epi)catechin content in the fruits from the three irrigation treatments (0.3−0.7 g/kg DW, Table 2) was higher than those reported in the Chinese varieties and lower than those reported in Korean varieties. Also, the dimer contents in the Korean fruit varieties were lower than those found in the fruits from T0, T1, and T2 treatments (0.4−1.1 g/kg DW, Table 2).

Taking into consideration that in our experiment the pear-jujube fruit moisture levels varied between 65% (T2) and 84% (T0) (data not shown), the procyanidin content would correspond to a range of 0.5 to 3.0 g/kg FW. These values could be considered as intermediate according to the concentrations of proanthocyanidins in 21 different fruits (from 0.04 g/kg FW (kiwi) to 6.64 g/kg FW (choke berries)) reported by Gu et al.[48] However, our results point to a higher procyanidin content than those observed in other stone fruits like apricots, peaches, and plums.[48,49]

The increase in procyanidins in pear jujube fruits following water deficit effect (Tables 2 and 3) agrees with observations of Sun et al.[11] who found that pear-jujube fruits from semiarid regions had the highest antioxidant activity, and with Guo et al.[50] who also indicated that flavonoid levels increased under harsh growing conditions, even though flavonoid levels were more dependent on plant material than growing conditions. In addition, when the procyanidin content increased in the fruit edible portion in response to water deficit (Tables 2 and 3), no significant differences were observed in its content between T1 and T2 fruits. This fact could be related with the gas exchange levels observed in T1 and T2 plants despite the water stress (Figure 1). In this sense, it is important to take into account that plant growth begins to decline at a water deficit level lower than that at which stomatal closure takes place. Therefore, in plants under water deficit (T1 and T2 plants), when carbohydrates exceed the amount used for growth concentrations, the considerable CO_2 assimilation levels observed could increase the biosynthesis of carbon-based secondary metabolites.[32] Moreover, the increase in the procyanidin content through a water stress effect could also be related with the fact that water deficit can lead to an increase in the levels of free phenylalanine,[51] a precursor in the procyanidin

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Table 3. Effect of Irrigation Treatments (T0, T1, and T2) at Different Times (0, at Harvest; 1, after 12 Weeks at Domestic Cold Storage) in Proanthocyanidin Oligomeric Species Content (mg/kg DW) in Pits (Shell + Seed) of Pear-Jujube Fruits[a]

		treatment					
proanthocyanidin	time	T0		T1		T2	
(epi)catechin	0	350.7 ± 9.2	cB	436.4 ± 7.0	bA	541.5 ± 9.9	aA
	1	564.4 ± 69.7	aA	345.8 ± 11.4	abA	290.2 ± 10.0 b	bB
dimer B type [(E)C−B−(E)C]	0	219.5 ± 1.2	bB	219.8 ± 14.8	bA	280.5 ± 2.5	aA
	1	979.4 ± 131.2	aA	365.1 ± 6.6	bA	228.1 ± 7.5	bA
trimer B type [(E)C−B−(E)C−B−(E)C]	0	277.2 ± 6.2	bB	310.6 ± 10.2	bA	400.8 ± 11.0	aA
	1	665.5 ± 69.4	aA	363.4 ± 22.4	abA	216.3 ± 14. 8	bB
trimer B type [(E)C−B−(E)C−B−(E)C]	0	277.6 ± 6.7	bB	329.5 ± 9.1	bA	493.1 ± 14.1	aA
	1	1061.5 ± 196.3	aA	466.6 ± 23.6	bA	164.3 ± 10. 5	bA
tetramer B type [(E)C−B−(E)C−B−(E)C−B−(E)C]	0	573.4 ± 12.5	bB	801.9 ± 10.2	aA	894.9 ± 27.1	aA
	1	1020.6 ±167.7	aA	488.5 ± 7.5	bA	252.4 ± 11.1	bB
tetramer B type [(E)C−B−(E)C−B−(E)C−B−(E)C]	0	378.0 ± 14.3	bB	453.8 ± 22.6	bA	1004.6 ± 61.1	aA
	1	674.5 ± 37.5	aA	464.4 ± 8.1	bA	221.3 ± 20.7	cB
pentamer B type [(E)C−B−(E)C−B−(E)C−B−(E)C−B−(E)C]	0	1281.7 ± 24.0	bA	1429.2 ± 10.7	abA	1586.1 ± 80.4	aA
	1	798.3 ± 52.7	aB	465.1 ± 19.5	bB	229.0 ± 14.3	cB
pentamer B type [(E)C−B−(E)C−B−(E)C−B−(E)C−B−(E)C]	0	1121.5 ± 39.3	cA	1324.3 ± 18.2	aA	1508.2 ± 12.7	aA
	1	613.7 ± 26.1	aB	430.2 ± 16.2	bB	227.6 ± 43.9	cB
pentamer B type [(E)C−B−(E)C−B−(E)C−B−(E)C−B−(E)C]	0	nd		nd		nd	
	1	1592.7 ± 108.1	a	1055.5 ± 31.7	b	444.8 ± 30.3	c
pentamer B type [(E)C−B−(E)C−B−(E)C−B−(E)C−B−(E)C]	0	nd		nd		nd	
	1	1098.9 ± 109.9	a	826.9 ± 36.0	a	306.9 ± 57.9	b
pentamer B type [(E)C−B−(E)C−B−(E)C−B−(E)C−B−(E)C]	0	nd		nd		nd	
	1	987.4 ± 18.9	a	650.9 ± 12.6	b	nd	
pentamer B type [(E)C−B−(E)C−B−(E)C−B−(E)C−B−(E)C]	0	nd		nd		nd	
	1	1082.0 ± 32.8	a	660.6 ± 56.8	b	nd	
total	0	4479.6 ± 74.0	cB	5305.5 ± 46.2	bA	6709.8 ± 165.9	aA
	1	10010.5 ± 535.6	aA	6229.9 ± 148.9	bA	2504.8 ± 222.5	cB

[a]Means within a row for each proanthocyanidin and storage time followed by different small letter, and within a column for each proanthocyanidin and treatment followed by different capital letter, are significantly different at $P = 0.05$ by Tukeys test. Abbreviations used: (epi)catechin ((E)C), not detected (nd).

Table 4. Effect of Irrigation Treatments (T0, T1, and T2) on the Average Molecular Radius (nm) of Procyanidin Self-Aggregates in Peel, Flesh, and Pit of Pear-Jujube Fruits[a]

treatment	peel	flesh	pit
T0	222 ± 85 aA	130 ± 41 aA	149 ± 56 aA
T1	262 ± 78 aA	193 ± 75 aAB	140 ± 42 aB
T2	328 ± 61 aA	171 ± 78 aB	190 ± 82 aAB

[a]Means within a column for each fruit portion or within a row for each treatment followed by different small letter or capital letter, respectively, are significantly different at $P = 0.05$ by Tukeys test.

synthesis, and an increase in L-phenylalanine ammonia lyase (PAL) activity[52] and, probably, PAL synthesis.[34,53]

It is important to emphasize that it has been suggested that the majority of proanthocyanidins transit into the small intestine intact and are degraded mainly by colonic microflora in the cecum and large intestine.[54,55] According to Santos-Buelga and Scalbert,[22] low molecular mass proanthocyanidins can be absorbed in the human gastrointestinal tract. Déprez et al.[56] showed that proanthocyanidins with a polymerization degree higher than three appear not to be absorbed directly from the gastrointestinal lumen. Furthermore, Holt et al.[57] detected dimers in blood after human subjects consumed a proanthocyanidin-rich diet, and trimers have been shown to be absorbed through the human intestinal cell line Caco-2.[56] In our study, the increase in total procyanidin content in the edible portion of the fruits was based mainly on an increase in the low molecular mass compounds (Table 2), which allows concluding that pear-jujube fruits from trees under water deficit produce procyanidins of higher potential bioavailability and with greater potential physiological effects for human health.

The self-aggregation is a new parameter that we underline at this point since it influences the bioaccesibility of the gut microbiota to the procyanidins to metabolize them. Therefore, a lower self-aggregation of the procyanidins could favor the absorption of them, and it could affect the total bioavailability of these compounds. Poncet-Legrand et al.,[58] using a hydroalcoholic solvent system, detected aggregation of procyanidins in apple and pear parenchyma and grape seeds. In this sense, self-aggregation is due to the preferred interaction of molecules with others of the same nature but not with those of the solvent. However, kinetics of the process and final size of aggregates can be affected by the nature of the solvent. For this, the fact that proanthocyanidin self-aggregates in pear-jujube fruits (Table 4) showed similar size to those found by Poncet-Legrand et al.[58] in other fruits could indicate that self-aggregate conformation and interaction with both hydroalcoholic and acetonitrile/acetic acid solvents were similar.

To explain why procyanidin content changes take place in the edible portion and pit of pear-jujube fruits during domestic cold storage (Tables 2 and 3), it is important to consider that water deficit accelerates the onset of ripening.[33,59] Despite the nonclimateric pattern of *Z. jujuba*,[60] other authors such as Abbas and Fandi[61] demonstrated that evergreen species of the genus *Ziziphus* (*Z. mauritiana*) showed changes in respiration

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rates and ethylene production during fruit development that were typical of climacteric fruits, while Wang et al.[29] demonstrated that *Z. jujuba* fruits senesce rapidly at room temperature due to their climacteric character. In this respect, the fact that the proanthocyanidin content decreased in T2 fruits during cold storage, whereas the storage effect in T1 fruits was not significant and induced a proanthocyanidin content increase in T0 fruits (Tables 2 and 3), can be explained if we consider that at harvest time the ripening degree would be proportional to the water deficit achieved (Figure 1). In this sense, the changes observed in procyanidins in T2 fruits (Tables 2 and 3) would agree with the decrease in phenolics observed in grape berries during overripening by Nadal[62] and in maoluang fruits by Butkhup and Samappito.[63] Therefore, at harvest time, T0 fruits would be less ripe than T1 and T2 fruits. So, during domestic cold storage, these fruits would ripen more, increasing phenolics due to an increase in PAL activity, as indicated by Tovar et al.[34] and Nadal.[62] However, it is difficult to explain why the procyanidin content of T1 fruits did not change during cold storage, although an intermediate ripening degree at harvest time would have led to constant level of procyanidins.

The current work demonstrates the occurrence of novel procyanidins in pear-jujube. To date, only two procyanidins [(epi)catechin and its dimer] have been described. In the present study, two trimers, two tetramers, and six procyanidin pentamers have been tentatively identified and quantified for the first time in pear-jujube. The results confirm that proanthocyanidins in pear-jujube fruits consist exclusively of B type procyanidins, whose levels are increased by water deficit during the fruit maturation stage. The fact that the total procyanidin content of the edible portion of fruits under water deficit is based mainly on an increase in the low molecular mass compounds leads us to conclude that pear-jujube fruits from trees exposed to water deficit increase procyanidin bioavailability and enhance the potential physiological effects on human health. The tendency of these molecules to self-aggregate does not change with the portion of the fruit or the irrigation treatment and is similar to that observed in other fruits. Additionally, fruits from well watered trees may increase their procyanidin content during fruit cold storage, whereas fruits from trees that were exposed to severe water stress (T2) decrease their procyanidins content during cold storage.

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Notes

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