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Fructan Metabolism in *A. tequilana* Weber Blue Variety along Its Developmental Cycle in the Field

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Supporting Information

ABSTRACT: Fructan, as reserve carbohydrate, supplies energy needs during vegetative development, thereby exhibiting variations in its content and composition. Fructan metabolism in *Agave tequilana* Blue variety from 2- to 7-year-old plants was analyzed in this work. Soluble carbohydrates were determined at all ages. Fructan (328−711 mg/g), sucrose (14−39 mg/g), fructose (11−20 mg/g), glucose (4−14 mg/g), and starch (0.58−4.98 mg/g) were the most abundant carbohydrates. Thin-layer chromatography exhibited that 2−5-year-old plants mainly stored fructooligosaccharides, while 6−7-year-old plants mainly contained long-chain fructans. The fructan degree of polymerization (DP) increased from 6 to 23 throughout plant development. The 7-year-old plants mainly stored highly branched agavins. Partially methylated alditol acetate analyzed by gas chromatography−mass spectrometry reveals that fructan molecular structures became more complex with plant age. For the first time, we report the presence of a large number of DP3 (seven forms), DP4 (eight forms), and DP5 (six forms) isomers for agave fructans. Overall, fructan metabolism in *A. tequilana* displays changes in its soluble carbohydrates, DP, type, and fructan structures stored, along its developmental cycle in the field.

KEYWORDS: *Agave tequilana Weber Blue variety, degree of polymerization, isomers, fructan metabolism, molecular structure, vegetative developmental*

INTRODUCTION

The Agavaceae family consists of eight genera and a total of 295 described species. Because all genera are found in Mexico, the country is considered the center of origin and diversity for this family. Within Agavaceae, the *Agave* genus is the most abundant, representing more than 56% of all reported species.[1,2] The plants within the genus perform CO_2 fixation by means of crassulacean acid metabolism (CAM),[3] from which fructans are the main photosynthetic product.[4] Fructans are fructose polymers, derived from sucrose and synthesized as reserve carbohydrates in approximately 15% of plants with flowers. According to their fructosyl linkage, they are classified as (1) inulins and (2) levans, with linear fructose residues linked by $\beta(2-1)$ and $\beta(2-6)$ bonds, respectively, (3) graminans, which have both types of linkages, $\beta(2-1)$ and $\beta(2-6)$, and (4) fructan neoseries, which are characterized by an internal glucose molecule that can be elongated by $\beta(2-1)$ and/or $\beta(2-6)$ linkages, producing inulin and/or levan neoseries, respectively.[5−7]

The *Agave* genus stores fructans that have wide structural diversity. *A. vera cruz* has mixtures of inulins and branched fructans.[8−10] Bathia and Nandra[11] reported inulin as the main storage carbohydrate (fructan) for *A. americana*; however, Ravenscroft et al.[12] more recently defined the fructan structure of *A. americana* as a neoseries class with $\beta(2-1)$ and $\beta(2-6)$ linkages and branches. Another genus, *Agave tequilana* Weber Blue variety, is an economically important crop in Mexico because it is the only raw material (usually from 6- to 7-year-old plants) used to produce tequila, a Mexican alcoholic beverage used worldwide.[13] Sánchez-Marroquín and Hope[14] described fructans of *A. tequilana* Weber Blue variety as inulins; however, López et al.[4] demonstrated that agave fructans are not inulins but rather complex mixtures of fructooligosaccharides (FOS) and fructans containing $\beta(2-1)$ and $\beta(2-6)$ linkages, with internal (neoseries fructans) and external (graminans fructans) glucose units. In 2006, Mancilla-Margalli and López[15] classified some of the most economically important agave species in Mexico. *A. tequilana* Weber Blue variety fructans were placed in group I with *Agave angustifolia* and *Agave potatorum*. Independent of group number, all agave species store two fructan types, graminans and agavins (neoseries), with agavins being the most abundant type. In this same study, both graminans and agavins showed a long degree of polymerization (LDP), with few branches. In general, fructans in *A. tequilana* constitute more than 60% of the reserve carbohydrates of these plants. *A. tequilana* fructans have recently increased in popularity because of their reported prebiotic capacity and overall health benefit effects to people, particularly for those who suffer from obesity, diabetes, and osteoporosis, among other.[16,17]

During the vegetative development of a plant, fructans as reserve material provide energy for plant growth.[7] Van den Ende et al.[18] found that chicory (*Cichorium intybus*) changed its carbohydrate and fructan compositions and concentrations in its roots throughout the growing season, in storage, and during forcing.[18] Fructans are also involved in the developmental flowering stages of *Campanula rapuncoides* by exhibiting

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Table 1. *A. tequilana* Weber Blue Variety Carbohydrate and Fructooligosaccharide Contents According to the Plant Age

A. tequilana age (years)	2	3	4	5	6	7
			Carbohydrate Contents			
fructans	328.7 ± 54	457.0 ± 59	533.8 ± 67	687.8 ± 26	679.4 ± 33	710.9 ± 18
sucrose	39.3 ± 12.4	32.2 ± 2.0	29.7 ± 4.6	22.0 ± 9.0	17.3 ± 7.6	14.1 ± 5.6
fructose	19.7 ± 1.4	20.8 ± 8.0	17.5 ± 6.6	14.0 ± 5.4	12.2 ± 5.3	11.7 ± 3.0
glucose	14.1 ± 1.7	11.7 ± 5.7	10.4 ± 1.9	4.2 ± 1.7	4.7 ± 1.3	4.3 ± 1.3
total starch	4.98 ± 0.8	2.46 ± 0.9	2.48 ± 0.9	1.57 ± 0.2	1.17 ± 0.07	0.58 ± 0.2
total WSC[a]	406.78 ± 70.3	524.16 ± 75.6	593.88 ± 81.0	729.57 ± 40.3	714.77 ± 47.3	741.58 ± 28.1
			Fructooligosaccharide Contents			
1-kestose (1K)	41.6	25.7	34.7	7.1	9.6	4.2
6-kestose (6K)	5.2	1.8	3.8	2.7	1.3	1.2
neokestose (NK)	43.1	45.7	29.2	16.6	8.5	9.2
1-nystose (1N)	21.6	19.4	18.1	6.8	3.2	3.4
1-^{F}fructofuranosylnystose (DP5)	8.9	7.0	7.1	4.4	1.5	1.4
total FOS[b]	120.4	99.5	92.9	37.7	24.1	19.4

[a]WSC = water-soluble carbohydrates (mg/g of mesontle dry weight). [b]FOS = fructooligosaccharides (mg/g of fructans dry weight).

differences in their content and type in vegetative parts, such petals, sepals, and ovaries, all as a result of carbohydrate redistribution throughout the flowering stages.[19] Variations in fructan contents and composition were also observed in the underground organs of *Vernonia herbacea*, along different phenological phases[20] and different growth and developmental phases of new plants regenerated from rhizophores.[21] Alternately, changes in the activities of fructan-metabolizing enzymes (GH32 enzymes) were found to occur throughout bulb development of three different cultivars of *Allium cepa*,[22] as well as in *V. herbacea* rhizophores, inducing sprouting from excision of aerial organs.[23] All of the above studies suggest that fructan profile variation, during the plant life cycle, occurs from differences in GH32 enzyme activities, as the need of the energy requirements for plant development. Mellado-Mojica et al.[24] found that *A. tequilana* fructans undergo changes during the biological cycle of the plant. Fluctuations in total reducing sugars and fructose content and the degree of polymerization (DP) of fructan were determined according to the plant age. To deepen the knowledge of fructan metabolism in *A. tequilana* Weber Blue variety, the aim of this work is to determine if changes in soluble carbohydrates, DP, and molecular structures of stored fructan occur in *A. tequilana*, along its developmental cycle in the field.

MATERIALS AND METHODS

Standards. Glucose, fructose, and sucrose were acquired from Sigma-Aldrich (St. Louis, MO). Inulin FOS, 1-kestose, 1-nystose, and 1-^{F}fructofuranosylnystose (DP3, DP4, and DP5, respectively), were obtained from Wako Pure Chemical Industries (Osaka, Japan). Raftilose (RSE) and raftiline (RNE), from Beneo-Orafti (Tienen, Belguim), were used as inulin standards for short and long degrees of polymerization. Neo-kestoses (DP3, DP4, and DP5) were kindly donated by Professor Norio Shiomi (Rakuno Gakuen University, Japan), and 6-kestose was provided by Professor Agustín López-Munguía (IBT-UNAM, Mexico).

Biological Material. *A. tequilana* Weber Blue variety of 2-, 3-, 4-, 5-, 6-, and 7-year-old plants (three each) were collected from the Amatitan region, Jalisco, Mexico. Plants were kindly donated by Brown-Forman R&D Casa Herradura. Plant age corresponded to its years in the field, starting from the plantation of the "*hijuelo*" (plant shoot). After plant collection, pines were cut into sections, and only the cores of the pines were used in all analyses. Agave tissue was lyophilized and stored in a desiccator until use.

Enzymatic Determination of Carbohydrate Contents in Lyophilized *A. tequilana* Mesontle. Fructan quantification was performed with the commercial kit, "Fructans" (K-FRUC) analytical test kit (Megazyme International Ireland, Ltd., Wicklow, Ireland) according to the instructions of the manufacturer. Glucose, fructose, and sucrose contents were simultaneously determined using the "Sucrose/D-Fructose/D-Glucose" (K-SUFRG) analytical test kit (Megazyme International Ireland, Ltd., Wicklow, Ireland) according to the instructions of the manufacturer. The total starch concentration was determined with the "Total Starch" (K-TSTA) analytical test kit (Megazyme International Ireland, Ltd., Wicklow, Ireland) according to the specification and instructions of the manufacturer.

Fructan Extraction. Fructan extractions were carried out according to the method established by López et al.,[4] with some modifications. In short, 30 g of lyophilized agave tissue (from plants of all ages) was extracted 3 times with 100 mL of distilled water. Fructans extractions were carried out at 60 °C for 1 h. The extracted fructans were combined and concentrated by evaporation on a rotary evaporator, lyophilized, and stored in a desiccator until analysis.

Thin-Layer Chromatography (TLC). A total of 1 μL of *A. tequilana* Weber Blue variety fructan solution (25 mg/mL) was applied to a silica gel TLC plate, with aluminum support. The TLC plate was developed in a system of butanol/propanol/water solvent,[25] and the TLC plates were sprayed with a reagent (aniline/diphenylamine/phosphoric acid in acetone) to achieve fructan visualization.[26]

High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC−PAD). Extracted agave fructans were analyzed, by HPAEC−PAD, in an ion chromatograph Dionex ICS-3000 (Sunnyvale, CA) with a guard-column CarboPac PA-100 (4 × 50 mm) and a CarboPac-PA100 (4 × 250 mm) column. The samples were diluted with deionized water (resistivity of 17 MΩ) to a concentration of 0.5 mg/mL and filtered through 0.45 μm nylon membranes before injection. A total of 25 μL of diluted sample was injected into the HPAEC. Fructan separation was achieved with a gradient of sodium acetate, in 0.15 M NaOH, at a flow of 0.8 mL/min, as follows: 0−5 min, 45 mM NaOH; 5−60 min, 0−375 mM sodium acetate; 60−65 min, 500 mM sodium acetate; and 65−75 min, 45 mM NaOH, at a column temperature of 25 °C. Applied potentials, for detection by the amperometric pulse, were as follows: E1 (400 ms), E2 (20 ms), E3 (20 ms), and E4 (60 ms) of +0.1, −2.0, +0.6, and −0.1 V, respectively.

Analysis of Glycosidic Linkages and Fructan Structures. The fructan extracts from all plants were derivatized to their partially methylated alditol acetate (PMAA) forms, according to the method described by Mancilla-Margalli and López,[15] with some modifications. A total of 10 mg of fructan was dissolved in 500 μL of dimethyl sulfoxide (DMSO), stirred and sonicated until total dilution occurred. Methylation was carried out by subsequent addition of powdered NaOH and CH_3I. The permethylated carbohydrates were extracted 3 times with CH_2Cl_2, washed with water, and evaporated to dryness under nitrogen flow. Hydrolysis of these derivatives was carried out

with 0.5 M trifluoroacetic acid (TFA), at 90 °C for 1 h. Hydrolyzed compounds were reduced with $NaBD_4$ and dissolved in 1.0 M NH_4OH, at 60 °C for 1 h. Excess $NaBD_4$ was destroyed by adding acetic acid, and the products were completely dry with a 15% methanolic solution of acetic acid. Acetylation was performed by adding 500 μL of acetic anhydride and 250 μL of pyridine as catalysts, at 90 °C for 2 h. The PMAAs were extracted 3 times with CH_2Cl_2, washed with water, and evaporated to dryness under nitrogen flow. The derivatized fructans were dissolved in 2 mL of CH_2Cl_2. For the gas chromatography coupled to mass spectrometry (GC−MS) analysis, 2 μL were injected, in a split-less mode, into a gas chromatograph (Hewlett-Packard 5890 Series II, Palo Alto, CA), which was coupled to a mass spectrometer detector (Hewlett-Packard 5972 Series, Palo Alto, CA) in an electron ionization mode. Separation was carried out in a 30 m × 0.250 mm × 0.25 μm HP5-MS capillary column (Agilent Technologies, Santa Clara, CA), at an initial temperature of 80 °C for 3 min, followed by the following temperature schedule: 5 °C/min, up to 160 °C for 1 min; 0.5 °C/min, up to 180 °C; and 14 °C/min, up to 280 °C for 19 min. The temperatures of the injector and detector were 270 and 300 °C, respectively. The carrier gas was helium, flowing at 2 mL/min, and the pressure was 8 psi. Result data are the average of independent measures of three *A. tequilana* plants from each of the 2−7-year-old plants.

RESULTS AND DISCUSSION

Soluble Carbohydrate Contents According to the Plant Age in *A. tequilana* Weber Blue Variety.

Table 2. TLC and HPAEC−PAD Profiles of Carbohydrates in Fructans Analyzed

R_f[a]	R_t[b]	nomenclature	carbohydrate	DP[c]
0.72	nd[d]	X	xylose	1
0.62	6.83	G	glucose	1
0.62	7.92	F	fructose	1
0.56	12.68	S	sucrose	2
0.44	16.05	1K	1-kestose	3
0.52	16.73	F3	inulotriose	3
nd	17.70	6K	6-kestose	3
0.49	18.53	NK	neokestose	3
0.38	19.07	1N	1-nystose	4
nd	19.68	B	bifurcose	4
nd	19.92	4c	neonystose *4c*	4
0.35	20.23	F4	inulotetraose	4
nd	21.03	6N	6-nystose	4
nd	21.87	4b	neonystose *4b*	4
0.32	22.20	DP5	1-^{F}fructofuranosylnystose	5
nd	23.02	5c	neopentaose *5c*	5
0.30	23.97	F5	inulopentaose	5
0.26	25.37	DP6	DP6	6

[a]R_f = retention factor in TLC. [b]R_t = retention time (min) in HPAEC−PAD. [c]DP = degree of polymerization. [d]nd = not determined.

Fructan Content. Fructans are the main storage carbohydrates in the *Agave* genus, constituting more than 60% of total soluble carbohydrates.[15] To evaluate the fructan contents along the developmental cycle of *A. tequilana* in the field, 2−7-year-old plants were collected and three plants of each age were analyzed. Their fructan concentrations fell within 328−711 mg/g of pine (30−70% in fructan dry weight). The lowest fructan concentration occurred in 2-year-old plants (328.7 mg/g; Table 1). After that age, a linear relationship between the increment in the fructan content and the plant age occurred up to 5-year-old plants (Table 1). The drop in carbohydrates between 3- and 4-year-old plants, reported by Mellado-Mojica et al.,[24] was not observed in this work (that difference might be due to the new agronomic handling of this cultivar that is used to keep the plant in an optimal nutrimental state). For 5−7-year-old plants, we found no significant linear changes in fructan concentrations (688−711 mg/g); therefore, *A. tequilana* likely reaches its highest fructan content, in the field, at 5-year-old plants. This result agrees with the data published by Mancilla-Margalli and López,[15] who found fructan concentrations around 700 mg/g for *A. tequilana* Weber Blue variety pines from 6- and 8-year-old plants. More recently, Arrizón et al.[27] observed a fructan amount of 123.8 mg/g of pine (fresh weight) for 6.5-year-old *A. tequilana*, whereas Ávila-Fernández et al.[28] found concentrations in a range of 129−174 mg/g (fresh weight) of pine for 7- and 8-year-old plants. Considering that the water content in *A. tequilana* ranges between 65 and 75%, the fructan content in the above 6-year-old plants studied in this work would be around 203 mg/g of pine. Despite small differences among the fructan contents of all three studies, it can be pointed out that a linear increment in the fructan content between 2-, 4-, and 6-year-old plants is comparable to that described by Arrizón et al.[27] In this study, however, we were limited to 7-year-old plants as a result of the difficulty in finding older cultivations because (1) *A. tequilana* is a semelparous plant (monocarpic) that produces one single inflorescence in its life and then dies[1] and (2) its fructans stored as reserve carbohydrates are used to supply the energy demand of the plant during its flowering phenomenon at around a 7-year-old plant, with an expected drop in the fructan content.[24] Furthermore, this is the age (6−7-year-old plant) at which tequila industries harvest plants, because they are at their highest sugar levels. In this study, the fructan content in *A. tequilana* increased from 2- to 5-year-old plants and remained unchanged at levels between 5- and 7-year-old plants in the field.

Glucose, Fructose, Sucrose, and Total Starch. Although fructans are the main storage carbohydrates in *A. tequilana*, soluble carbohydrates, such as glucose, fructose, and sucrose, are also present.[17] We found that carbohydrates can differ according to the plant age (Table 1). Sucrose and total starch were the most and least abundant, respectively, at all ages. For 2−7-year-old plants, the glucose content ranged from 14.1 to 4.2 mg/g, the fructose content ranged from 20.8 to 11.7 mg/g, and the sucrose content ranged from 39.3 to 14.1 mg/g. Our results also showed a linear decrement in the content of these carbohydrates as the plants become older. This behavior is similar to that reported by Arrizón et al.,[27] who found that free sugar content decreased in 2-, 4-, and 6-year-old plants. Our results, however, show that 2−5-year-old plants contained higher sucrose concentrations that lead to fructan synthesis, correlating with the linear fructan increment observed in plants of that ages (Table 1). A similar behavior was observed during the developmental cycle of *Polimnia sonchifolia*, in which TLC revealed a decrement in glucose, fructose, and sucrose contents in developmental stages with high fructan content.[29] Starch was the least abundant carbohydrate (4.98−0.58 mg/g) at all ages studied. It decreased with age to almost undetectable levels in the oldest plants.

TLC Profiles of Fructans Stored in A. tequilana 2−7-Year-Old Plants. Using TLC, we analyzed fructan extracts from *A. tequilana* Weber Blue variety plants (2−7-year-old plants) along with a standard mixture (STD; X, xylose; G, glucose; F, fructose; S, sucrose; 1K, 1-kestose; 1N, nystose; and DP5, 1-^{F}fructofuranosylnystose), commercial inulin of short (SDP)

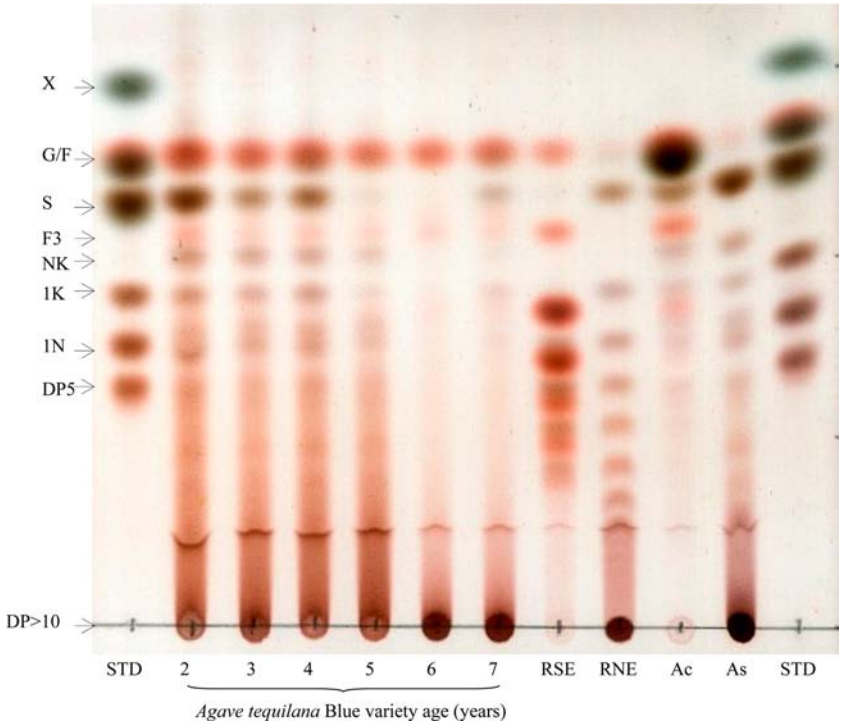

Figure 1. TLC of fructans stored in *A. tequilana* Weber Blue variety plants at different ages of their life cycle in the field. The carbohydrate nomenclature is listed in Table 2. STD, standards; RSE, raftilose; RNE, raftiline; Ac, *A. cepa* (onion); and As, *A. sativum* (garlic).

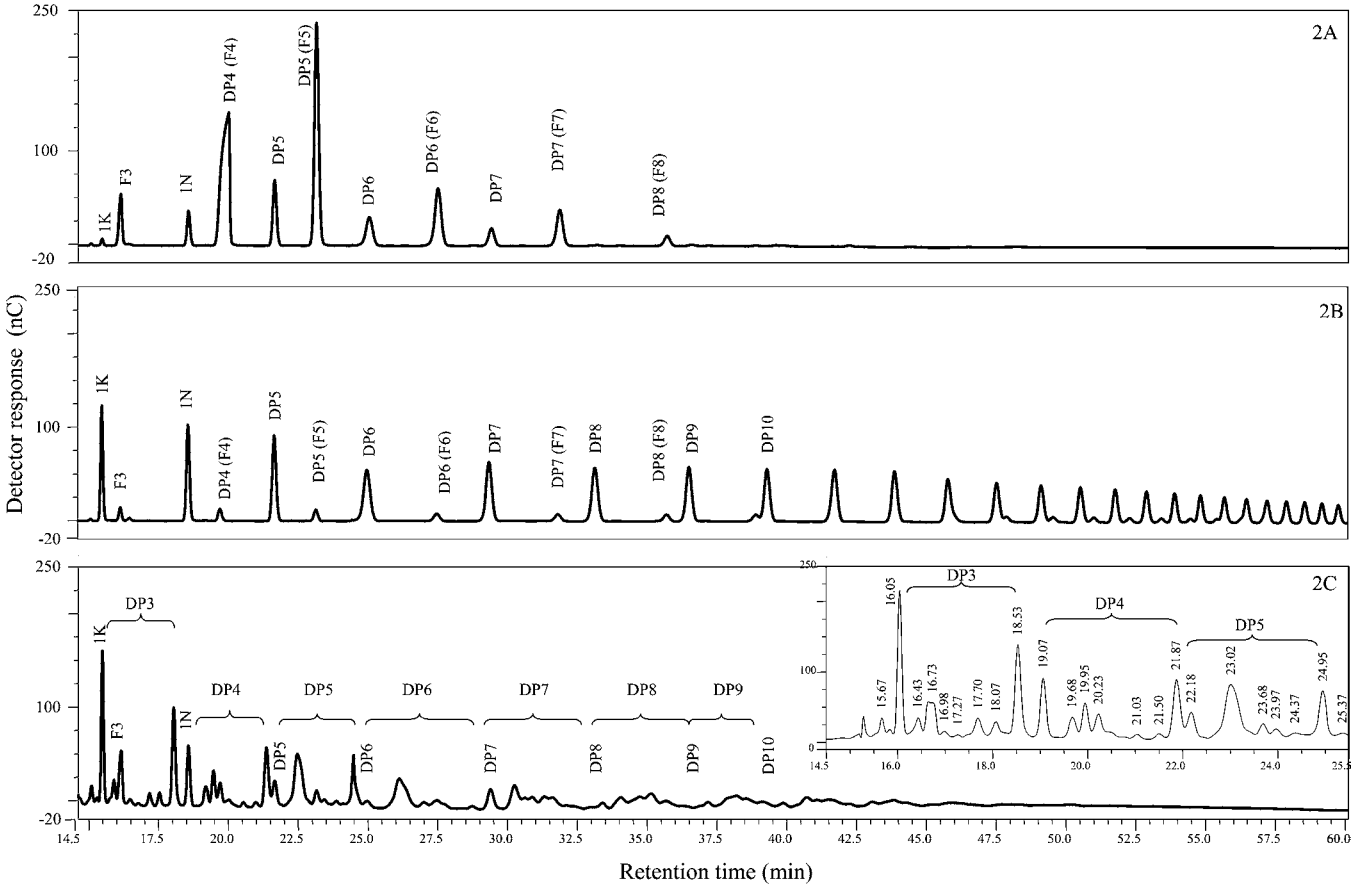

Figure 2. HPAEC–PAD profiles of fructans from raftilose (RSE; panel 2A), raftiline (RNE; panel 2B), and *A. tequilana* Weber Blue variety of 2 year-old plants (panel 2C). Panel 2C shows a group of fructans with the same DP. (Inset) DP3–DP5 isomers.

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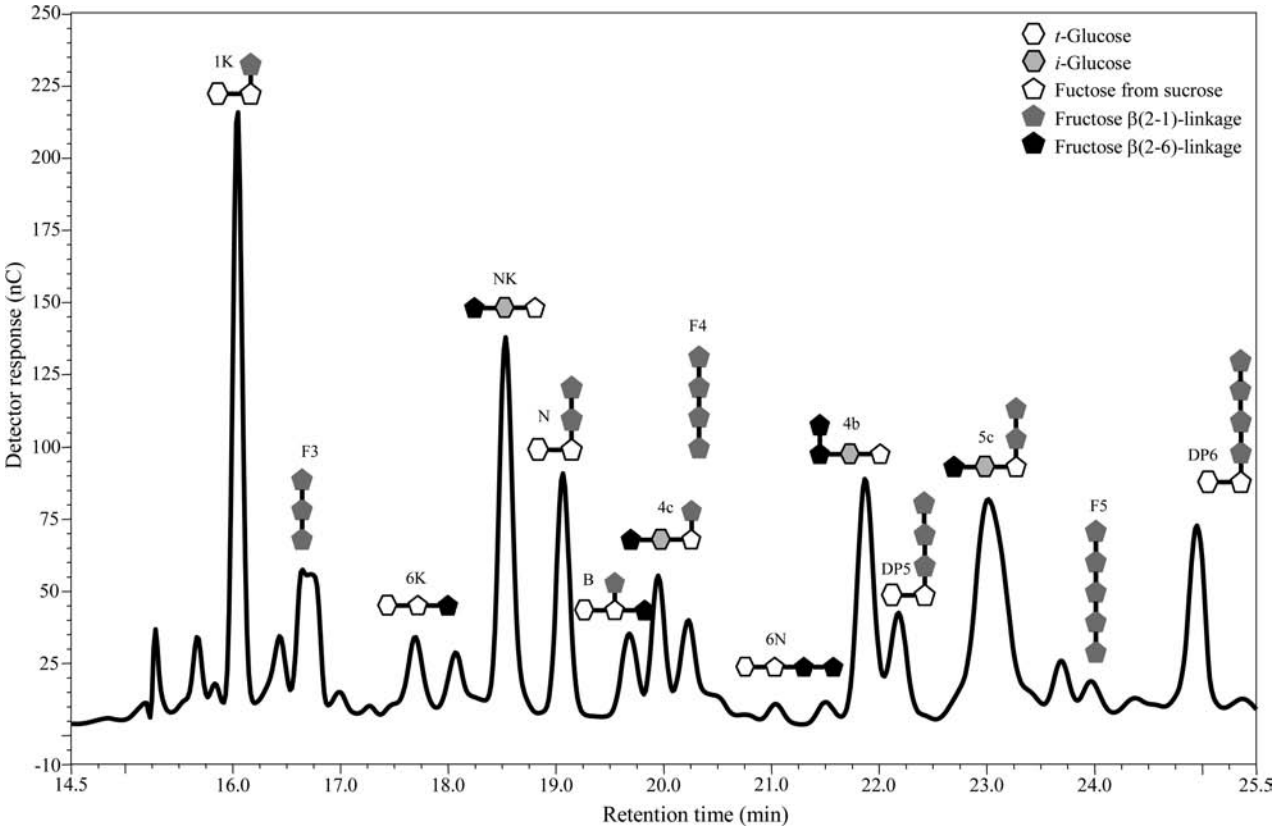

Figure 3. HPAEC−PAD fructan isomers profile, with DP from 3 to 5, for 2-year-old *A. tequilana* Weber Blue variety. The specific names and retention times for each isomer are listed in Table 2.

and long (LDP) degrees of polymerization (RSE, raftilose; RNE, raftiline, respectively), and fructan-producing crops, such as onion (*Allium cepa*, Ac) and garlic (*Allium sativum*, As), with SDP and LDP, respectively (Table 2).

TLC profile of carbohydrates of *A. tequilana* fructan extracts were identified by their retention factors (R_f values) and spot colors (Figure 1). In all cases, color intensity was related to carbohydrate type and abundance: glucose (R_f = 0.62, bluish color), fructose (R_f = 0.62, reddish), and sucrose (R_f = 0.56, brown) (Figure 1). All of these carbohydrates were present in all samples, independent of the plant origin and/or source. All of the spots observed below the sucrose spot corresponded to fructans from DP = 2 to DP > 10. TLC showed that RSE is composed of FOS (Figure 1), which have been shown to be conforming to mainly an inulin Fn fructan series (fructans without a glucose molecule[30]), while RNE was mostly a GFn inulin fructan series. The TLC profiles of agave fructans (lines 2−7) displayed a different profile when compared to RSE and RNE; however, lines 2−4 show similarity to As (garlic fructans) (Figure 1). The agave profiles (lines 2−7) consisted of the FOS: inulotriose (DP3), neokestose (DP3), 1-kestose (DP3), 1-nystose (DP4), and 1-^{F}fructofuranosylnystose (DP5), which were identified by comparative analysis to the pure standard and fructan extracted from onion (Ac) and garlic (As).

TLC also showed that 2−4-year-old agave plants mainly stored SDP fructans, while the most abundant carbohydrates in 5−7-year-old plants were LDP fructans, in agreement with Mellado-Mojica et al.,[24] who specifically found SDP fructans in 2- and 4-year-old plants and primarily found LDP fructans in 6- and 8-year-old plants. Similarly, during the developmental cycle of rhizomes in *Polimnia sonchifolia*, TLC analysis showed that, at early stages of development, SDP fructans are more abundant, while in mature plants, those fructans are replaced by LDP fructans.[29] This might indicate that *A. tequilana* stores SDP fructans at the beginning of its vegetative development and uses them for the LDP fructan synthesis during its biological cycle; therefore, a relationship can be observed between the increment in DP of agave fructan and the plant age. On the other hand, the capacity of *A. tequilana* to synthesize fructans with different DPs at different ages can be an alternative for the agave fructans to be used in specific food industry niches.

HPAEC−PAD Profiles of A. tequilana Weber Blue Variety Fructans from 2- to 7-Year-Old Plants. Fructan profiles of *A. tequilana* plants at different ages were analyzed by HPAEC−PAD. Although this work focused on fructan metabolism in *A. tequilana* along its life cycle in the field, this work was also the first attempt to characterize agave fructan isomers by HPAEC−PAD. The same agave fructan extracts analyzed by TLC were analyzed by HPAEC−PAD (Figure 2). We used the chromatographic profiles of RSE (Figure 2A) and RNE (Figure 2B) to identify the presence of inulin-type GFn and Fn series in agave samples (Figure 2C). A typical agave fructan chromatogram profile exhibits more complexity when compared to RSE and/or RNE. We identified eight isomers with DP3, seven with DP4, and six with DP5 (inset of Figure 2C). From those, only 14 forms were identified, confirming the complexity of agave fructans with respect to other fructans. The HPAEC−PAD of agave fructans, found between 14.5 and 25.5 min, showed the presence and molecular structures of DP3, DP4, and DP5 isomers (Figure 3), which is supported by assignments to pure standards. We identified the following isomers (and their

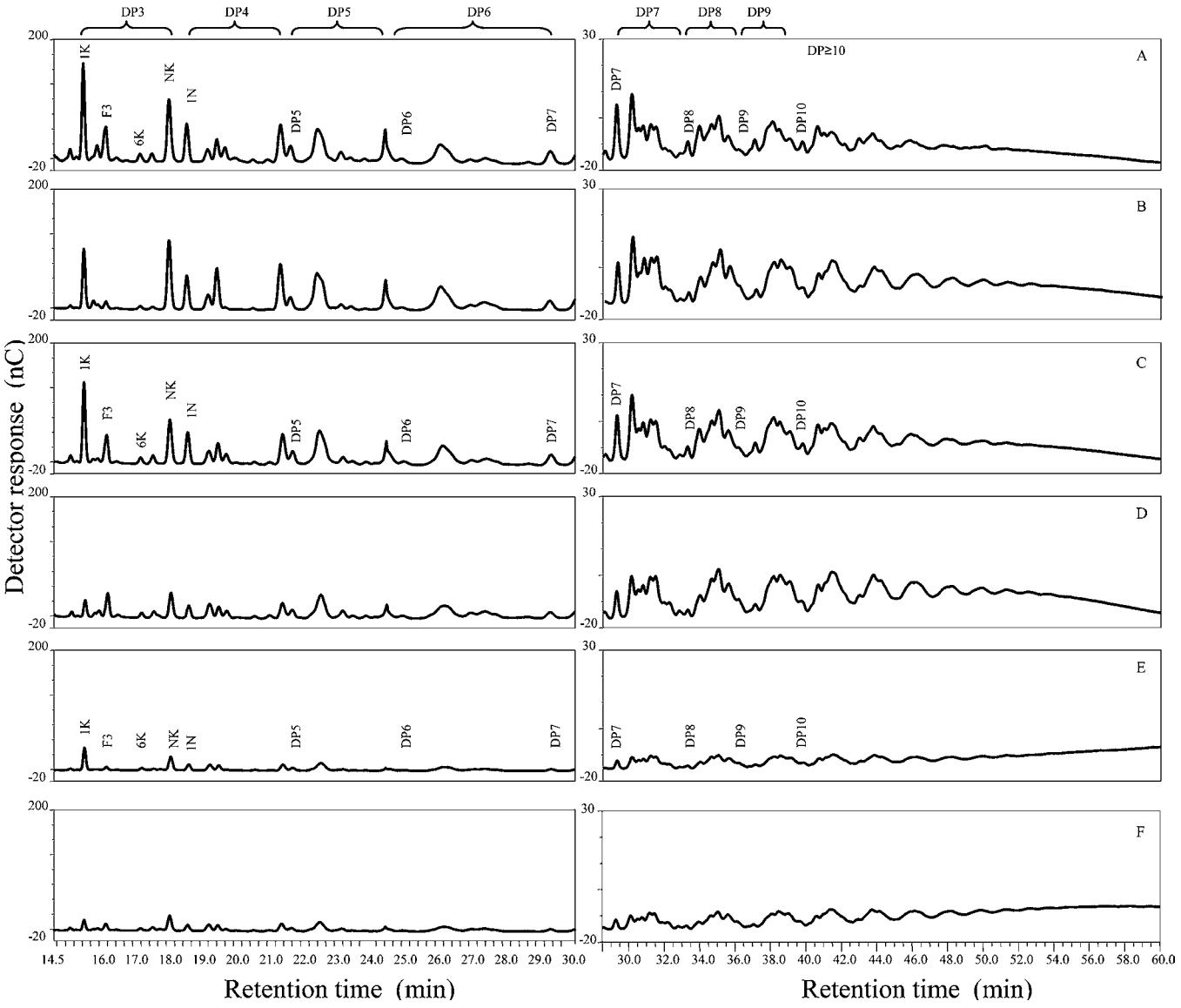

Figure 4. Differential HPAEC–PAD profiles of fructans from 2- to 7-year-old *A. tequilana* Weber Blue variety plants (panels A–F, respectively). The specific names and retention times for each isomer are listed in Table 2. Brackets identify fructans with the same DP.

retention times) (Table 2): 1-kestose (16.05 min), inulotriose (16.73 min), 6-kestose (17.70 min), and neokestose (18.53 min), all from the eight DP3 isomers found with DP3 (four more need to be identified); 1-nystose (19.07 min), neonystose *4c* (19.92 min), inulotetraose (20.23 min), neonystose *4b* (21.87 min), bifurcose (19.68 min), and 6-nystose (21.03 min), all from the seven DP4 isomers (the last two forms were identified only by comparison to published data,[31−34] leaving only one form to be established); and only 1-^{F}fructofuranosylnystose (22.20 min), neopentaose *5c* (23.02 min), and inulopentaose (23.97 min) were identified from the six DP5 isomers. Some isomers remain unidentified, but the identification of isomers with a longer DP is very complex, hence no attempt was performed in this study.

HPAEC profiles for all *A. tequilana* Weber Blue variety fructan extracts were performed (panels A–F of Figure 4). We clearly found very similar profiles in younger plants (2–4-year-old plants), where SDP fructans were characteristic, while 5–7-year-old plants displayed more poorly defined profiles (this might be probably due to their large number of isomers). Independent of agave fructan complexity, we observed similar HPAEC profile characteristics, such as the presence of small peaks from DP7 to DP12, which suggests that many isomeric forms exist. When the synthesis of *A. tequilana* fructans is compared to other fructan-producing crops, one could conclude that more complex biosynthetic pathways occur in *A. tequilana* than in species such as *C. intybus*, *A. cepa*, and *A. sativum*. Abundant isomeric forms in *A. tequilana* suggest that it may have more than a sucrose:sucrose 1-fructosyltransferase (1-SST)/fructan:fructan 1-fructosyltransferase (1-FFT) system, similar to chicory, such as a 1-SST/1-FFT/fructan:fructan 6G-fructosyltransferase (6G-FFT) system, similar to onion.[5−7] The different isomeric products observed in *A. tequilana* fructans of this study suggest that at least five enzyme systems are necessary for this crop.

Quantification of most of the above compounds from standards (Table 1) confirms our findings from TLC, demonstrating that plants in an early developmental stage (2–4-year-old plants) mainly synthesize SDP fructans that might be used as substrates for fructosyltransferases and by

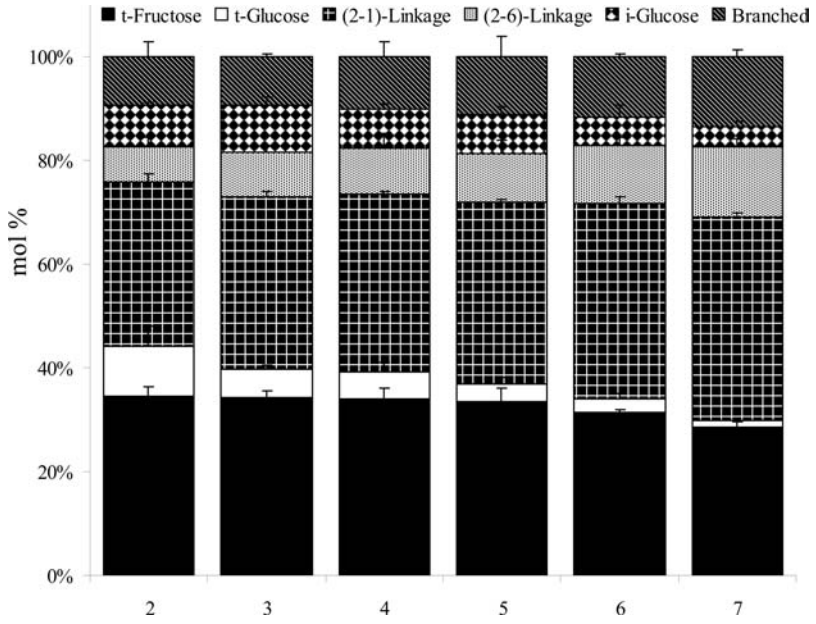

Figure 5. Glycosyl linkage composition in molar percentages of fructans for *A. tequilana* Weber Blue variety along its life cycle in the field.

Table 3. Quantitative Contribution (Molar Percent) of Each Fructan Derivative in *A. tequilana* Weber Blue from 2- to 7-Year-Old Plants

age (years)	DP[a]	α-D-Glucp	*i*-α-D-Glucp	*t*-β-D-Fructf	(2−6)-β-D-Fructf	(2−1)-β-D-Fructf	1,6-bi-β-D-Fructf
				A. tequilana			
2	5.9 ± 1.2	0.5 ± 0.1	0.5 ± 0.1	2.0 ± 0.3	0.4 ± 0.2	1.9 ± 0.5	0.6 ± 0.2
3	7.0 ± 1.0	0.4 ± 0.1	0.6 ± 0.1	2.4 ± 0.5	0.6 ± 0.2	2.4 ± 0.4	0.7 ± 0.1
4	8.2 ± 1.4	0.4 ± 0.1	0.6 ± 0.1	2.7 ± 0.2	0.8 ± 0.2	2.9 ± 0.5	0.9 ± 0.5
5	9.9 ± 2.4	0.2 ± 0.1	0.7 ± 0.1	3.1 ± 0.5	1.0 ± 0.5	3.5 ± 1.0	1.2 ± 0.6
6	16.2 ± 5.9	0.3 ± 0.1	0.7 ± 0.1	5.2 ± 2.2	1.9 ± 0.9	6.3 ± 2.4	1.8 ± 0.6
7	23.2 ± 2.4	0.2 ± 0.1	0.8 ± 0.1	7.4 ± 1.2	3.3 ± 0.6	8.9 ± 1.1	2.6 ± 0.5
				Standard			
FOS[b] (*Ci*)[c]	14.8	1.0	nd	1.7	nd	11.8	0.3

[a]DP = estimated degree of polymerization of each species based on the sum of the relative abundances of both internal (*i*-α-D-Glucp) and terminal (α-D-Glucp) α-D-glucopyranosides considered as one unit. [b]FOS = fructooligosaccharides. [c]*Ci* = *C. intybus*.

Table 4. Ratio Correlations between Fructan Residues in *A. tequilana* Weber Blue Variety along Its Life Cycle in the Field

A. tequilana age (years)	agavins/graminans	β(2−1)/β(2−6)	branched frequency
2	0.9 ± 0.3	4.6 ± 1.3	3.9 ± 1.2
3	1.7 ± 0.6	3.9 ± 0.8	3.6 ± 0.3
4	1.7 ± 0.6	3.7 ± 1.5	3.8 ± 1.4
5	3.0 ± 1.1	3.4 ± 0.8	3.4 ± 1.0
6	2.3 ± 0.6	3.3 ± 0.5	3.4 ± 0.5
7	3.6 ± 1.3	2.7 ± 0.4	2.9 ± 1.2

transfructosylation reactions giving place to LDP molecules as final products, which are the main fructans stored in plants during mature stages and correlated with greater carbohydrate content. Our quantification of DP3 FOS showed that 1-kestose in a 2-year-old plant was 10 times more concentrated versus 7-year-old plants, 6-kestose and neokestose were around 5 times more abundant in 2- versus 7-year-old plants, and DP4 and DP5 FOS were 6 times more concentrated in 2- versus 7-year-old plants.

Glycosyl Linkage Analysis of A. tequilana Fructans Stored at Different Ages. To establish the structural diversity of fructans in *A. tequilana*, along its life cycle in the field, fructans extracted from 2- to 7-year-old plants were derivatized to their PMAA forms. We identified and quantified each detected compound in GC−MS by comparative analysis of fragmentation patterns generated by electron impact and using the criteria defined by Mancilla-Margalli and López.[15] The chromatographic profiles of the derivatization products of agave fructans, at different ages, were similar, displaying only qualitative differences (see Table S1 and Figure S1 of the Supporting Information). The molar proportions of PMAAs showed different tendencies according to plant age (Figure 5): with PMAA corresponding to branches, linkages β(2−1) and β(2−6) showed a linear increment for plants from 2- to 7-year-old plants, while a different tendency was observed in the glucose units (internal and external). Once the molar contribution of each derivated product was analyzed (Table 3), and knowing each fructan molecule had at least one glucose unit (an internal or external α-D-glucopyranose), we were able to identify tangible differences in the composition of glycosidic linkages of *A. tequilana* fructans according to the plant age. An increment in the DP of fructans stored in plants from 2- to 7-year-old plants in range from DP = 6 to DP = 23 was determine, with the latter coinciding with the DP = 18−28

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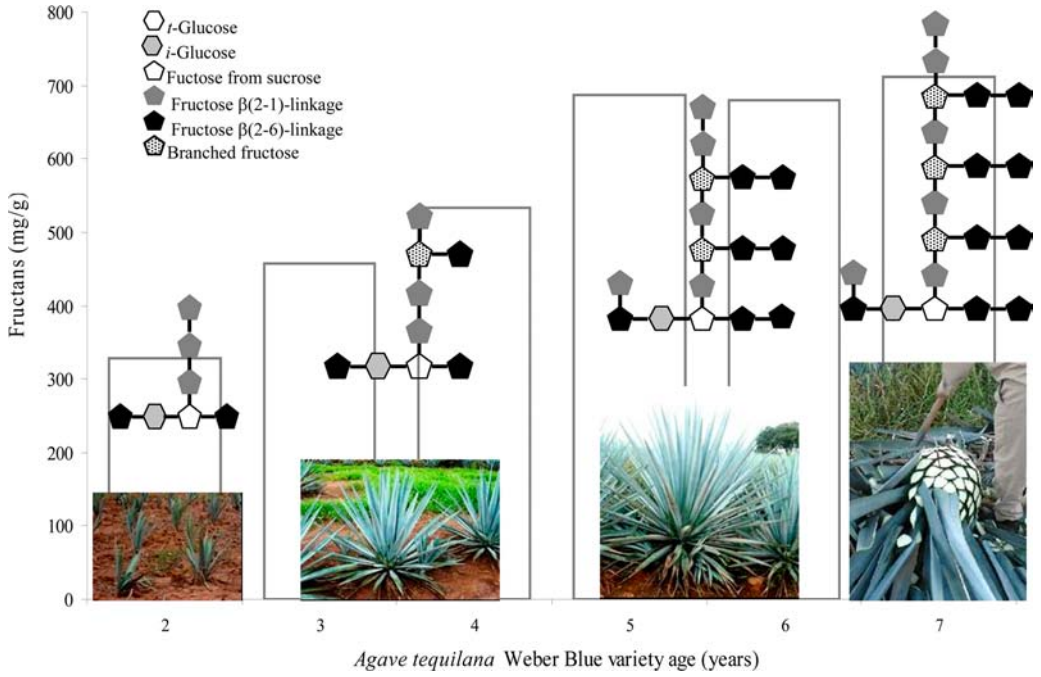

Figure 6. Fructan contents and their proposed molecular structure relationships in *A. tequilana* Weber Blue variety along its life cycle in the field.

range previously reported for 6−7-year-old plants of this species.[4,15,24,27] Branches were absent in 2-year-old plants, emerging at 4-year-old plants, and reaching the highest abundance in fructans from 7-year-old plants. Graminans and agavins were present at all ages; however, their proportions diverged according to the plant age.

The generated information established important relationships between the glycosidic linkages of *A. tequilana* fructans and the plant age (Table 4). In summary, 2-year-old plants stored the lowest DP fructans, with no branches, the highest ratio of $\beta(2-1)/\beta(2-6)$ linkages, and graminans and agavins in equal proportion. Similar features were observed in fructans from 3- and 4-year-old plants. They had SDP fructans with short branches and two agavins for each graminan fructan. The 5-year-old plant fructans were very similar to those from previous ages; however, the agavins/graminans correlation increased to 3, with branched molecules becoming more abundant and more frequent. LDP fructans were observed in 6- and 7-year-old plants, mainly consisting of highly branched agavins with the lowest proportion of $\beta(2-1)/\beta(2-6)$ linkages. The above data fall together with the results described by Mancilla-Margalli and López,[15] for *A. tequilana* fructans from 6- to 8-year-old plants classified into group I. We, therefore, conclude that *A. tequilana* plants, in their early stages of maturation, store almost the same proportions of graminan and agavin, which are SDP, while plants in mature stages (with greater fructan content) mainly store LDP branched agavins.

Molecular Structures of Fructans Stored by A. tequilana Weber Blue Variety along Its Developmental Cycle in the Field. Agave fructans are complex mixtures of fructans (graminans and agavins) that contain $\beta(2-1)$ and $\beta(2-6)$ linkages and branches that vary according to the plant age. Here, we propose new possible molecular structures for agave fructans (Figure 6). Along its life cycle in the field, the *A. tequilana* fructan content increased up to 5-year-old plant and remained constant up to 7-year-old plant. Fructan synthesis starts with equal proportions of agavins/graminans but moves toward a higher abundance of agavins and large DP as plants age. Molecules become more branched, thereby producing more isomeric forms that are more complex and difficult to identify. The molecular structure of agave fructans is converted to highly complex structures by the end of plant development. In summary, fructan metabolism in *A. tequilana* Weber Blue variety exhibits changes in carbohydrate and fructan contents, fructan DP, type, and molecular structure. Young plant fructan biosynthesis starts with less complex SDP molecules that might be used as substrates for fructosyltransferases in the synthesis of highly branched complex molecules with LDP.

Finally, the presence of a large number of isomeric forms suggests the possible presence of a large fructosyltransferase system in the metabolism of *A. tequilana* fructans: 1-SST synthesizes 1-kestose; 1-FFT synthesizes inulin-type fructans with DP > 3, both previously identified in this species;[35,36] 6G-FFT synthesizes neokestose (the base molecule of neo-fructans); and sucrose:fructan 6-fructosyltransferase (6-SFT) synthesizes 6-kestose and the graminan fructan series. Future studies may, therefore, study the role of fructosyltransferases in fructan metabolism of *A. tequilana* Weber Blue variety along its developmental cycle in the field.

■ ASSOCIATED CONTENT

Supporting Information

PMAA identified in *A. tequilana* fructans (Table S1) and chromatographic profile of derivatization products of *A. tequilana* Weber Blue variety fructans along its developmental cycle in the field: (A) 2-, (B) 3-, (C) 4-, (D) 5-, (E) 6-, and (F) 7-year-old plants (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS USED

1-FFT, fructan:fructan 1-fructosyltransferase; 1-SST, sucrose:-sucrose 1-fructosyltransferase; 6G-FFT, fructan:fructan 6G-fructosyltransferase; 6-SFT, sucrose:fructan 6-fructosyltransferase; Ac, *A. cepa* (onion); Ac, *A. sativum* (garlic); CAM, crassulacean acid metabolism; DMSO, dimethyl sulfoxide; DP, degree of polymerization (S, short; L, long); FOS, fructooligosaccharides; GC−MS, gas chromatography coupled to mass spectrometry; HPAEC−PAD, high-performance anion-exchange chromatography with pulsed amperometric detection; PMAA, partially methylated alditol acetate; RNE, raftiline; RSE, raftilose; TFA, trifluoroacetic acid; TLC, thin-layer chromatography; WSC, water-soluble carbohydrates

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