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Developmental Variation in *Agave tequilana* Weber var. Azul Stem Carbohydrates

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ABSTRACT

Phenology studies the effect of seasonal and climatic variations on plant life cycles; and these variations are mainly due to temperature, light and precipitation changes among others. The content and type of carbohydrate are often used to establish seasonal, varietal, phenological and developmental stages of plants. The *Agave* genus is very appreciated due to its high adaptability under extreme conditions, such as water deficit and high and low temperatures, but nowadays it is more important due to its high fructan content. The purpose of this work was to establish the carbohydrate fluctuation in *Agave tequilana* Weber var. Azul of different ages or different stages of development. Fructans were extracted and separated into long- and short-DP fractions, and characterized by TLC and MALDI-TOF-MS. All Agaves presented glucose, fructose and sucrose like most plants, fructans and fructooligosaccharides (FOS) were always present. Qualitative and quantitative carbohydrate differences were observed at all ages. TLC results showed large differences between and within long- and short-DP fractions. The presence of neotype fructans was also observed by TLC in all samples, this FOS has always been found in all Agave plants. MALDI-TOF-MS analyses allowed the establishment that DPs range between 3 and 28, and the highest DP was found in the 8 years old plants. Fructan accumulation in *A. tequilana* presented a consecutive increment except for the 4 and 10 year-old. During these stages, *A. tequilana* Weber var. Azul reached two relevant physiological reproductive stages, shoots and inflorescence (seeds), for these reasons their overall carbohydrate content and structure might have changed. The main differences found for reducing carbohydrates and fructans are good indexes to evaluate the changes in the developmental stages of *A. tequilana* Weber var. Azul.

Keywords: degree of polymerization, long- and short-DP fructans, MALDI-TOF-MS, phenology
Abbreviations: CAM, crassulacean acid metabolism; **DP**, degree of polymerization; **FOS**, fructooligosaccharides; **MALDI-TOF-MS**, matrix assisted laser desorption/ionization-time of flight-mass spectrometry; **LDP**, long degree of polymerization; **SDP**, short degree of polymerization; **TLC**, thin layer chromatography

INTRODUCTION

Mexico has been considered the point of origin, biodiversity and evolution of the *Agave* genus, and from the 166 species reported worldwide, 125 can be found in Mexico (Eguiarte *et al.* 2001). Agave plants belong to the crassulacean acid metabolism (CAM) category and store fructans as reserve carbohydrates (López *et al.* 2003).

According to Mancilla-Margalli and López (2006) fructans in *A. tequilana* Weber var. Azul represent more than 60% of total water soluble carbohydrates, with an estimated degree of polymerization of 18 and belong to the group I among the *Agave* fructans species classification. *A. tequilana* store two types of fructans: graminans and highly branched neofructans (agavins) in a ratio of 1: 4 (**Fig. 1**). However, López *et al.* (2003) reported a degree of polymerization of 28, as the highest one, for this same species, but using a MALDI-TOF-MS analysis.

Fructans of *A. tequilana* are the raw material allowed for tequila production, which is a unique Mexican beverages and one of the most consumed alcoholic beverage worldwide (Norma Oficial Mexicana NOM006SCF1-2005).

As reserve carbohydrates, fructans supplies the necessary energy to the plant during the different phenological stages like: sprouting, flowering, dormancy and vegetative growth (Machado de Carvalho and Dietrich 1993; Roten

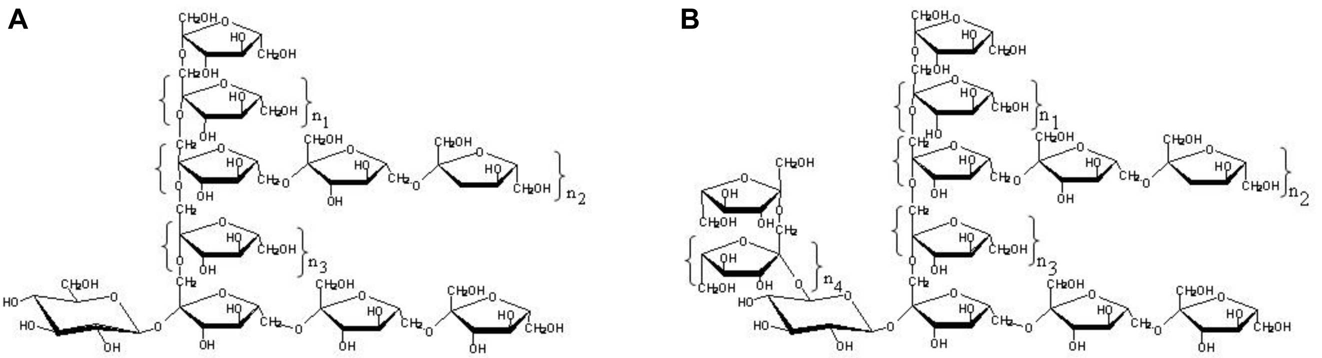

Fig. 1 Molecular structures of Agave fructans group I. (**A**) Graminans and (**B**) Agavins.

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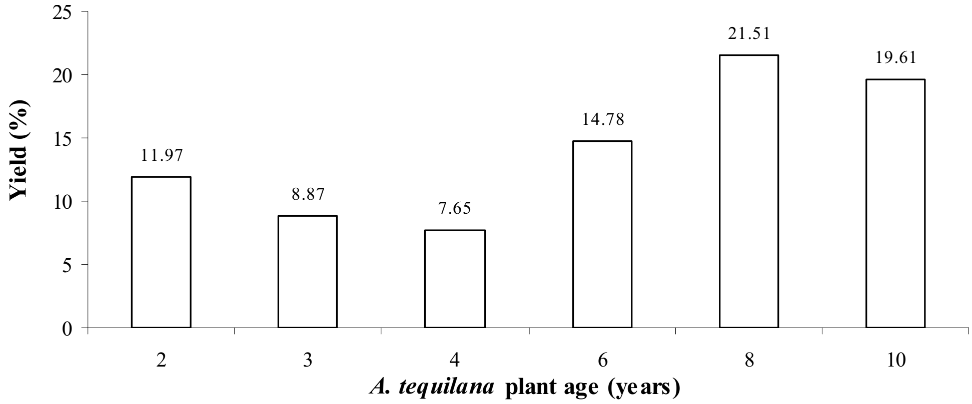


Fig. 2 Fructan yield percentage of *A. tequilana* Weber var. Azul plants of different ages.

and Wehrmeyer 2004). In addition, the fructan content is directly related to the biosynthetic and physiological development of the plant. *A. tequilana* is a perennial plant and has long life cycle of about 12 years. Our interest was to establish fructan differences among 2, 3, 4, 6, 8 and 10 year-old *A. tequilana* plants.

MATERIALS AND METHODS

Agave tequilana Weber var. Azul plants

A. tequilana Weber var. Azul plants of 2, 3, 4, 6, 8 and 10 years-old were kindly donated by Brown-Forman Global R&D.

Fructan extraction

Agave's fructans were extracted according the method of López *et al.* (2003). In brief, 100 g of freeze-dried stem (mesontle) were treated with 80% ethanolic solution followed by an aqueous extraction. Soluble carbohydrates were deionized and fractionated by addition of absolute ethanol, LDP fructans were precipitated and SDP fructans were concentrated by evaporation in a rotavapor. Both fructans samples, LDP and SDP fractions, were freeze-dried and stored in a humidity-free container previous to their lyofilization.

°Brix or total soluble carbohydrates

Standard curves for glucose, fructose and sucrose were done using °Brix. Fifty microliters of each fructan extraction were also analyzed in the same manner. A Sugar/Brix Sper Scientific Refractometer was used.

Thin Layer Chromatography (TLC)

One microliter of 10 mg/mL fructan solutions was applied to silica gel TLC plates on aluminum support. TLC plates were developed in a butanol/propanol/water system (Kanaya *et al.* 1978), and fructan spots were visualized with aniline/diphenylamine/phosphoric acid reagent in acetone base (Anderson *et al*. 2000).

Determination of carbohydrates

Total reducing carbohydrates were determined according to the method of Dubois *et al.* (1956). Direct reducing carbohydrates were also performed according to the method of Somogyi (1945) to determine the free fructose *in planta* as a result of enzymatic hydrolysis. Fructose determination was done following the method of ATS (antrone-tryptophan-sulfuric acid), established by Somani *et al.* (1987).

Matrix assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS)

MALDI-TOF-MS measurements were performed using a Hewlett-Packard (Cupertino, CA) LDI AOOXP MS in the positive ion mode. The instrument was operated at an accelerating voltage of 30 kV and an extractor voltage of 9 kV. The pressure was 2.1 × 10^{-6} Torr. The samples were ionized with a nitrogen laser (λ 337 nm) with a pulse width of 3 ns and a 4-7.5 J pulse. The samples were dissolved in water and the matrix was 25-dihydroxybenzoic acid.

RESULTS AND DISCUSSION

Fructans are the main storage carbohydrate present in Agave stems. According to the age of Agave plants, fructan extractions presented different yield percentage (**Fig. 2**), the highest fructan concentration was found in 8 year-old plants, while the lowest was found in 4 year-old. In addition, a declining concentration was found in 10 year-old plants. These findings are important because at these ages, Agave plants undergo a relevant physiological and morphological changes, like developed of outsprings (*hijuelo*) at 4 year-old, large abundance of fructans at 8 year-old, and finally, the inflorescence at 10 year-old. Therefore, the Agave fructan content is directly related to the age and physiological stage of the plant.

TLC of fructan fractions (**Fig. 3**) showed a good separation of SDP and LDP. The LDP fractions of 4 year-old plants showed the lowest concentration with respect to all others fractions and the largest concentration was observed in 6 and 8 years-old fractions. SDP fractions showed fructans of DP 3-6 inulin and inulin neoserie types. On the other hand, the 10 years-old fractions presented glucose, fructose and sucrose as the principal carbohydrates, therefore, at this age, the LDP fructans are hydrolyzed to fructose, to supply the energetic demand of the inflorescence stage.

The standard curve of soluble carbohydrates in °Brix (**Fig. 4**) shows a good correlation between fructan concentration and °Brix at all ages. Water-soluble carbohydrates of

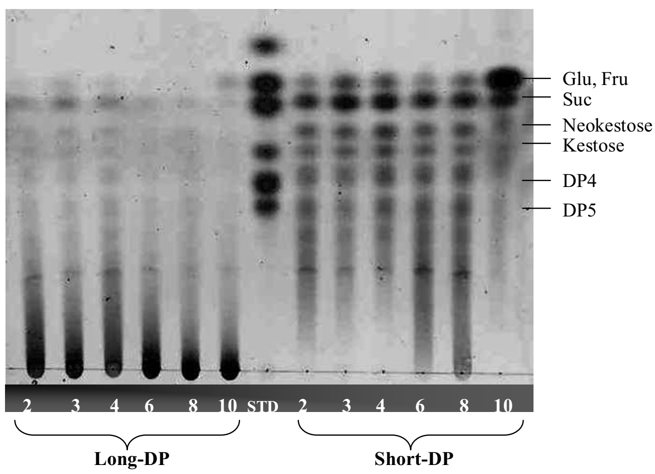


Fig. 3 Thin layer chromatography of short- and long-DP fructans from *A. tequilana* Weber var. Azul plants of different ages.

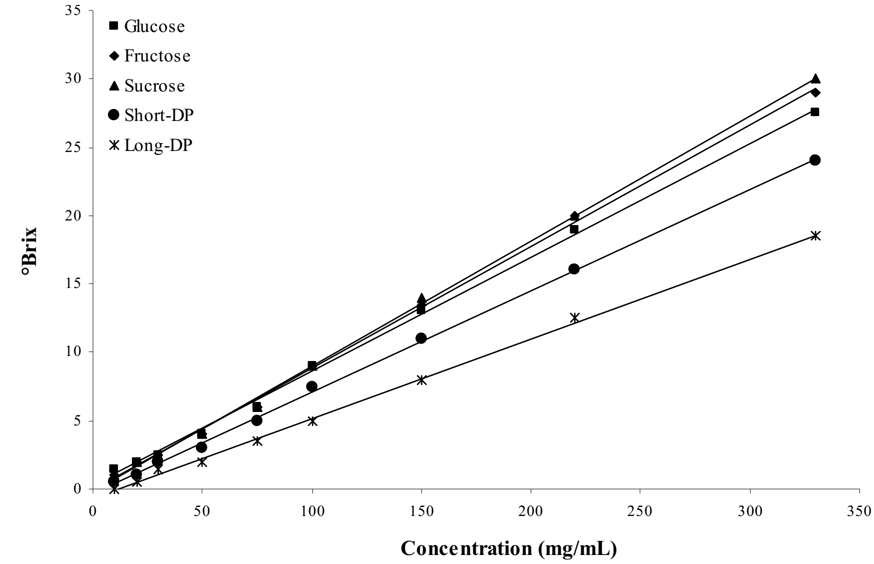


Fig. 4 Standard curves of soluble carbohydrates based on °Brix.

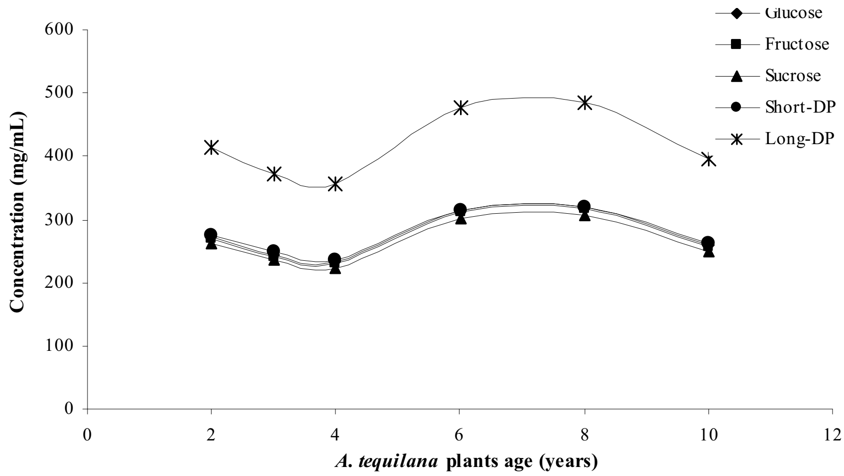


Fig. 5 Water-soluble carbohydrates of *A. tequilana* Weber var. Azul from 2, 3, 4, 6, 8 and 10 year-old plants. The same pattern was observed in all determinations. Four year-old plants always presented the lowest values.

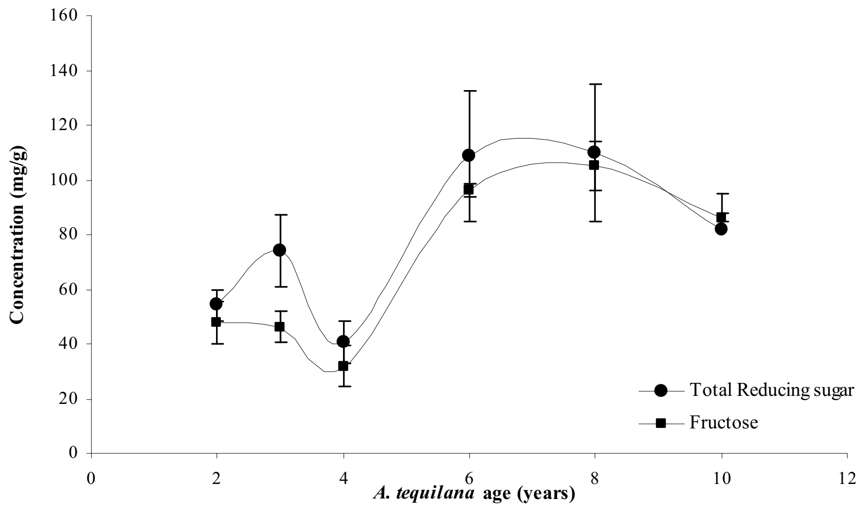
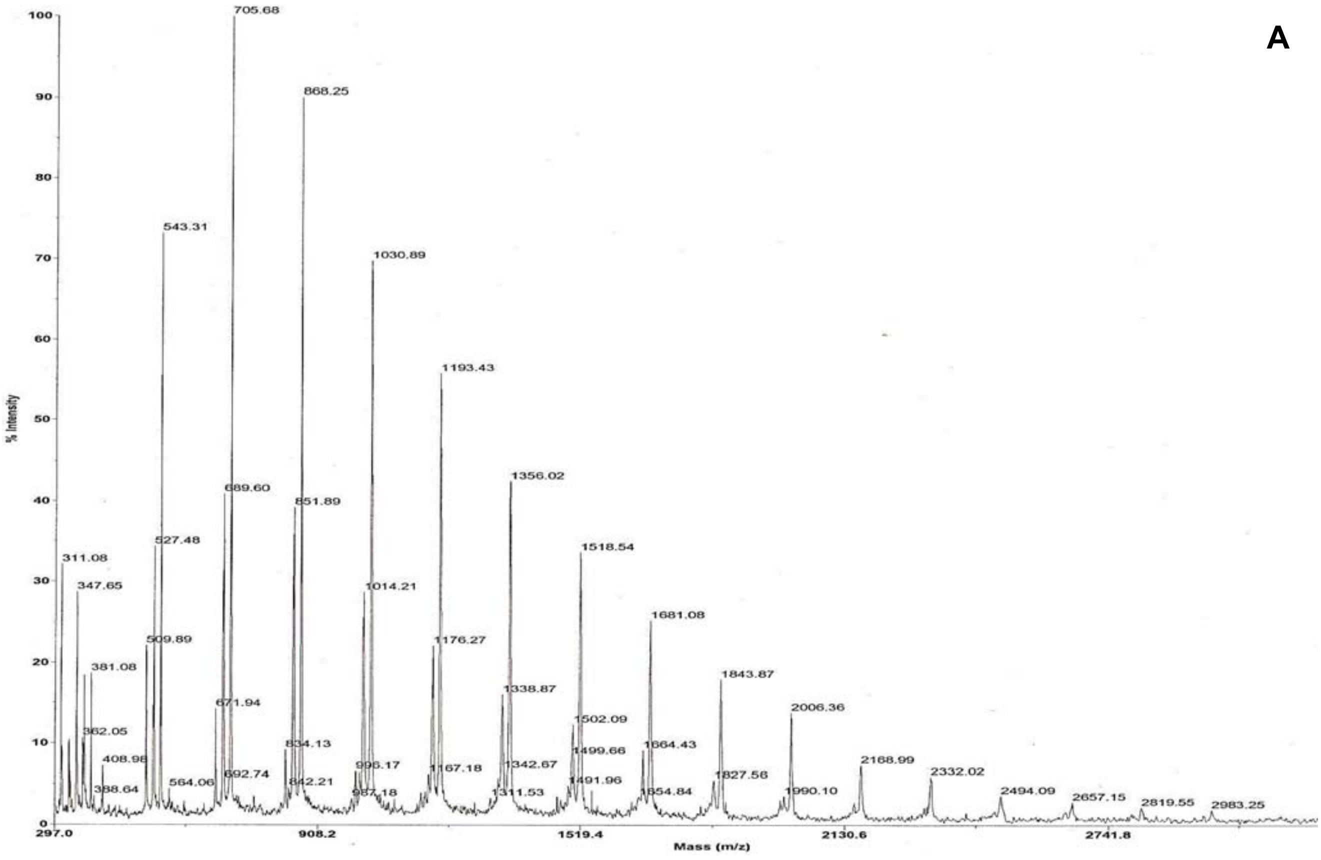


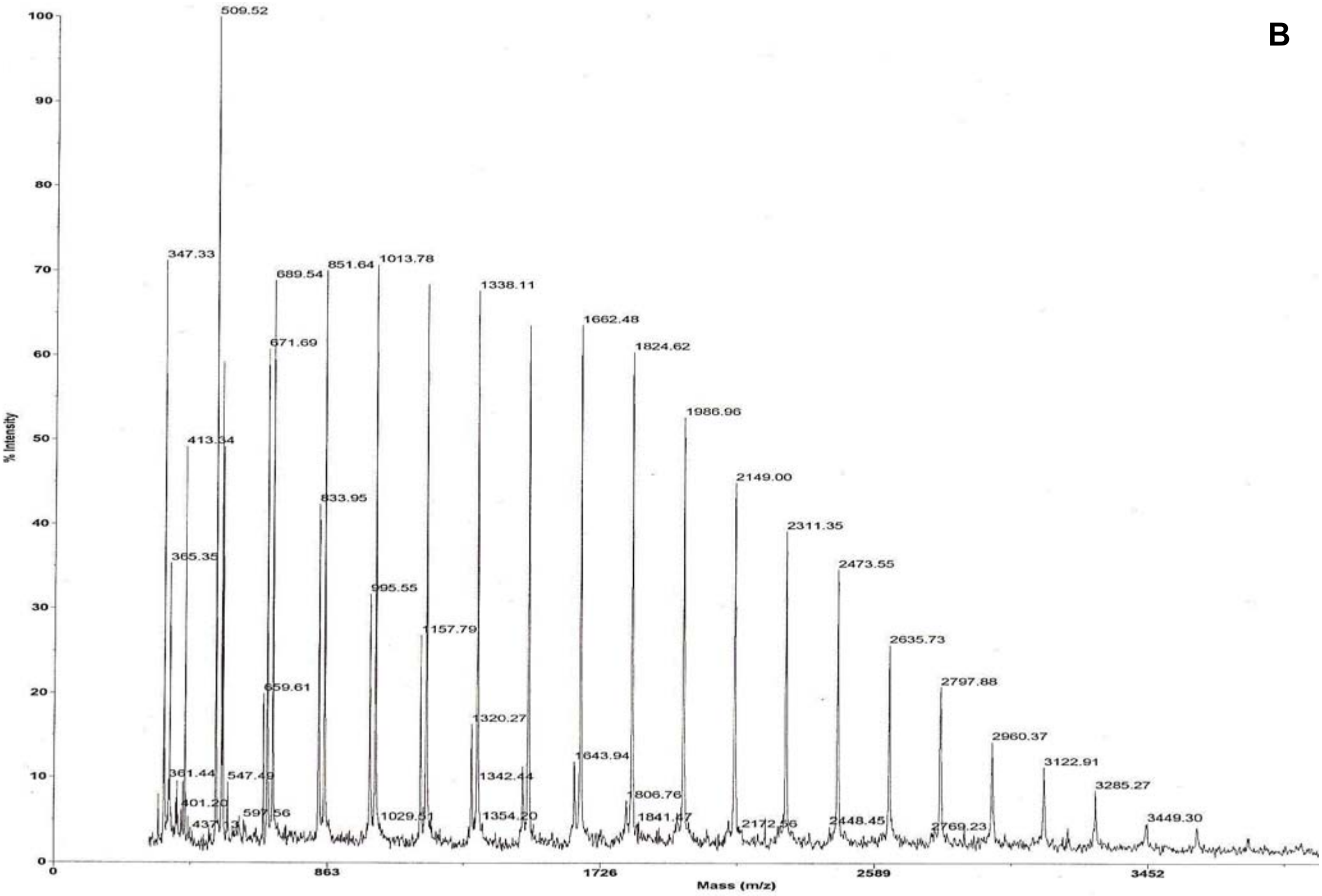
Fig. 6 Total reducing sugar and fructose in *A. tequilana* of all ages.

A. tequilana Weber var. Azul from 2, 3, 4, 6, 8 and 10 year-old plants (**Fig. 5**) show again a bimodal pattern with two carbohydrate drops, one in 4 and another one in 10 year-old plants. Four years-old plants always present the lowest fructan values among all others. The LDP fructans always presented the largest concentrations at all ages, being the most abundant carbohydrate in agave plants.

Total reducing sugar and fructose in *A. tequilana* of all ages (**Fig. 6**) show the same pattern observed in all determinations. Again, four years-old plants had the lowest concentration and 6-8 year-old presented the largest concentrations, 10 years-old plants showed a sudden decline in their carbohydrates levels.

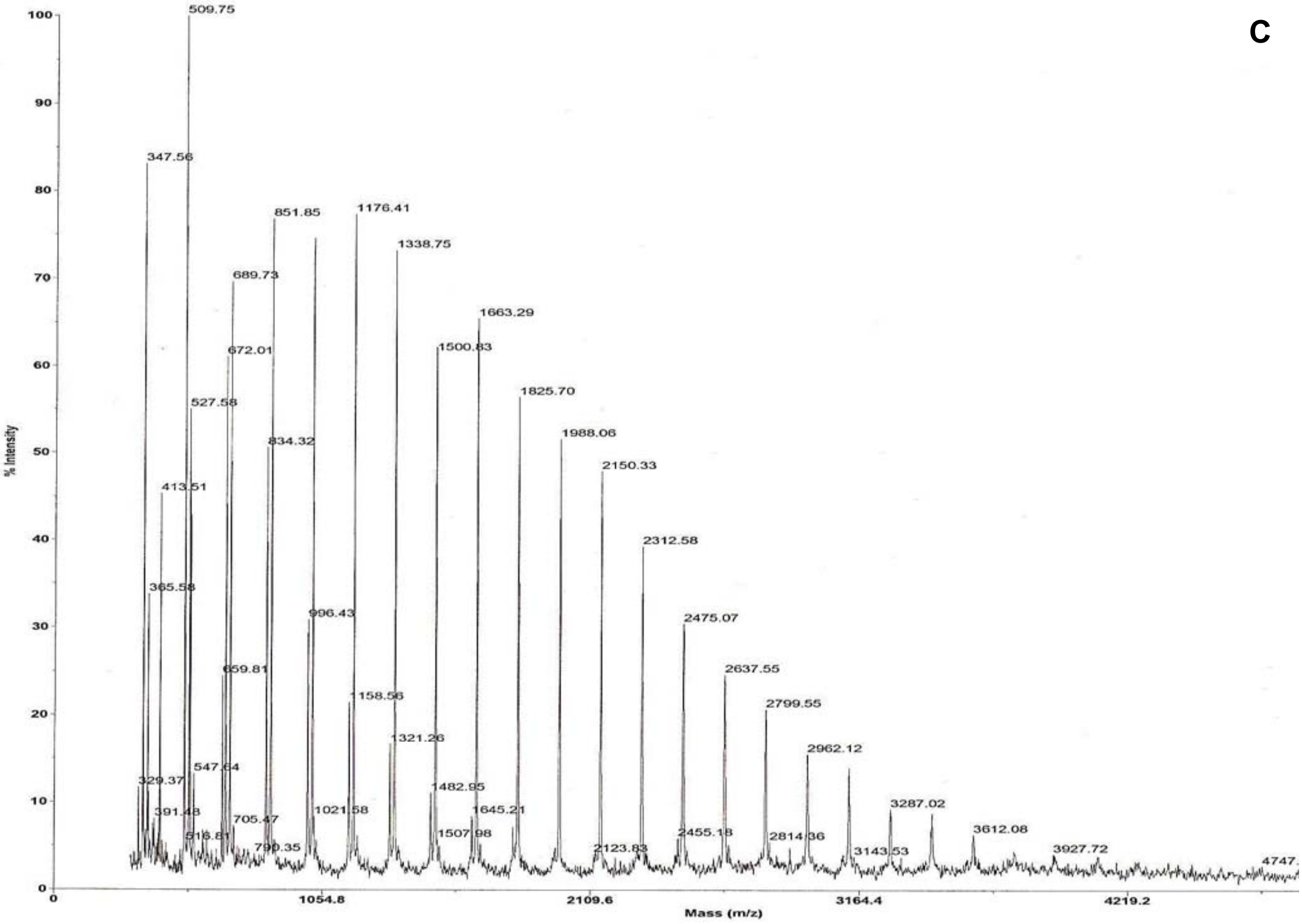
MALDI-TOF-MS spectra of fructans extracted from *A. tequilana* plants of different ages (**Fig. 7**) presented different ion patterns. 4 year-old plants (**Fig. 7A**) show the





lowest fructan content and SDP fructans as the most abundant. 6-8 year-old plants (**Fig. 7B, 7C**) showed the largest abundance of both fructan fractions, SDP and LDP, and their DP range was between 3 and 28. By this technique, the 10 year-old plants (**Fig. 7D**) did not show the presence on any type of fructans (inflorescence stage), but fructose and sucrose were mainly present.

Fructan accumulation in *A. tequilana* presented a consecutive increment except for 4 and 10 years-old plants. During these stages, *A. tequilana* Weber var. Azul plants reach two highly relevant physiological reproductive stages, shoots and inflorescence (seeds), for these reasons their overall carbohydrate content and structure might have changed. The differences in enzyme activities could be cor-



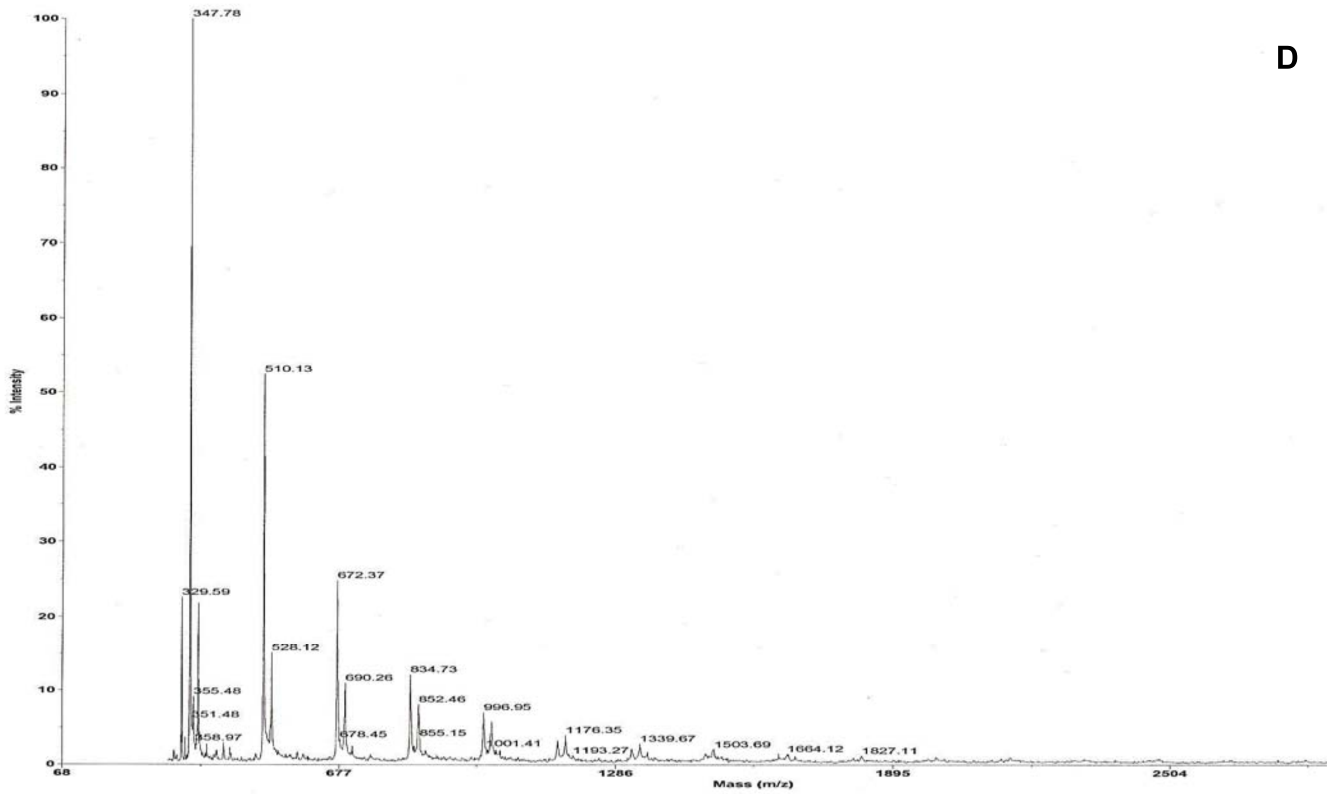


Fig. 7 MALDI-TOF-MS of *A. tequilana* Weber var. Azul extracted from plants of different ages. m/z corresponds to the Na-adduct. (**A**) 4 year-old, (**B**) 6 year-old, (**C**) 8 year-old, and (**D**) 10 year-old.

related to the differences in fructan contents, we propose a model to explain the phenological stages for *A. tequilana* Weber var. Azul and fructosyltransferases involved in all of them (**Fig. 8**). The main differences found for reducing carbohydrates and fructans are good indexes to evaluate carbohydrate changes in the developmental stages of *Agave tequilana* Weber var. Azul.

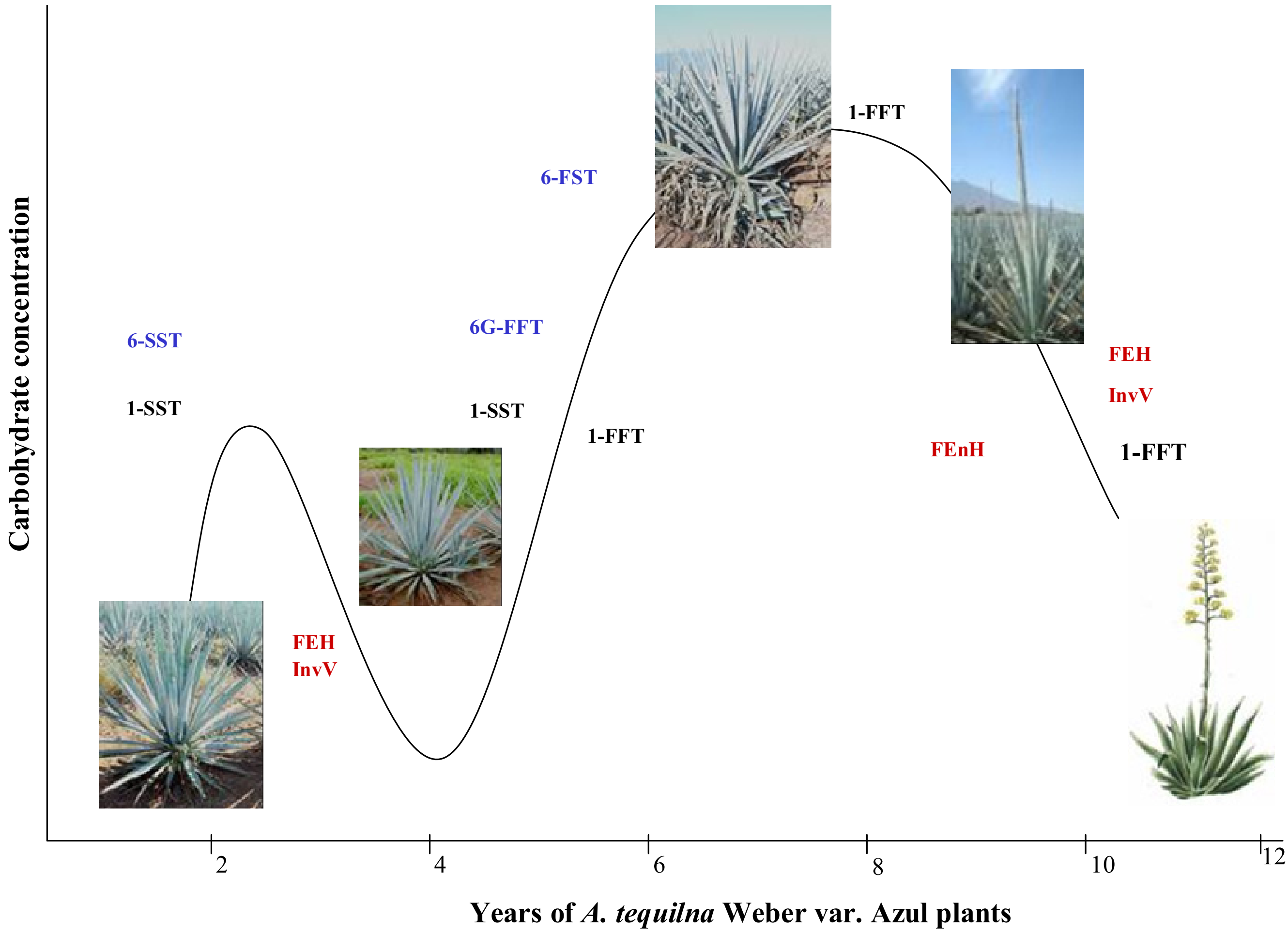


Fig. 8 Propose phenological stages for *A. tequilana* Weber var. Azul, carbohydrates and fructosyltransferases.

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