agronomy

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The Physiological Role of Inulin in Wild Cardoon (*Cynara cardunculus* L. var. *sylvestris* Lam.)

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Abstract: Wild cardoon (*Cynara cardunculus* L.) is a widespread Mediterranean plant that accumulates inulin in its roots. This study aimed to analyze the enzyme systems involved in inulin metabolism in the roots of one Sicilian wild cardoon population in relation to the plant's growth and development stages. During the winter season, the plant showed slow growth; its biomass was represented mainly by leaves and saccharides were mobilized into its roots. During the spring season, the plant doubled its growth rate and differentiated its reproduction organs as a consequence of the cold conditions. The maximum activities of the 1-SST were recorded in line with the high sucrose and inulin levels in roots, which increased quickly. The increase in the 1-FEH activity suggests that fructan-hydrolyzing activity is associated with the sprouting and elongation of plant stalks. The peak of the invertase activity occurred before the 1-FEH peak. The inulin accumulation in the wild cardoon roots was associated with the plant's reproduction. Sequential 1-SST and 1-FEH activities and the involvement of invertase and 1-FFT in carbohydrate mobilization, in response to the additional energy demand of the plant for stalk elongation before and for capitula development were observed, along with subsequent grain ripening.

Keywords: *Cynara* wild relative; fructans; abiotic stress; enzymes; sugars; roots

Citation: Branca, F.; Argento, S.; Paoletti, A.M.; Melilli, M.G. The Physiological Role of Inulin in Wild Cardoon (*Cynara cardunculus* L. var. *sylvestris* Lam.). *Agronomy* **2022**, *12*, 290. https://doi.org/10.3390/agronomy12020290

Academic Editor: Muhammad Shahid and Ali Sarkhosh

Received: 22 December 2021
Accepted: 20 January 2022
Published: 24 January 2022

1. Introduction

Fructans are linear or branched polymers of repeating fructose residues connected by β (2–1) and/or β (2–6) fructosylfructose linkages, optionally including one terminal glucosyl unit. Fructans are widely distributed in nature, occurring in bacteria, fungi, and over 40,000 higher plant species [1,2].

Inulin is a type of fructan constituted by fructose units that features mostly or exclusively the β (2 → 1) fructosyl-fructose linkage. It is essentially a linear polymer, but can branch with β (2 → 6) linkages at a low degree of polymerization. Inulin might also be useful in a number of food [3] and non-food applications [4], depending on its degree of polymerization [5,6], promoting health effects [7,8].

In dicot species belonging to the *Asteraceae*, including chicory (*Cichorium intybus* L.), Jerusalem artichoke (*Helianthus tuberosus* L.) [9], dandelion (*Taraxacum officinale*), dahlia (*Dalia variabilis*), yacon (*Polymnia sonchifolia*) [10], and *Cynara cardunculus taxa* [11–13], inulin is often stored in specialized organs, such as taproot, bulbs, and capitula. Inulin from these storage organs is used as a carbon source during regrowth and for sprouting in the spring. The breakdown of inulin often precedes the growth of plants. It is involved in the expansion of flowers and may be involved in the protection of plants during drought, salt, or cold stress [14]. Although the molecular mechanism explaining this protective role has not been

thoroughly elucidated, fructans have been shown to stabilize membranes during freezing and drying [14–16].

Fructan synthesis (Figure 1) begins in the vacuole, with sucrose as both the donor and substrate. The first transferase enzyme, sucrose-sucrose fructosyltransferase (1-SST), forms 1-kestose from two sucrose molecules (releasing glucose). An elongating enzyme, fructan-fructan fructosyltransferase (1-FFT), transfers a single terminal fructose residue from an oligosaccharide to the same carbon position on another molecule, thus producing the fructan inulin. This results in fructan chains with a wide range of lengths. Inulin degradation is catalyzed by fructan exohydrolase (1-FEH, EC 3.2.1.153), which releases free fructose from the terminal non-reducing fructosyl unit [17].

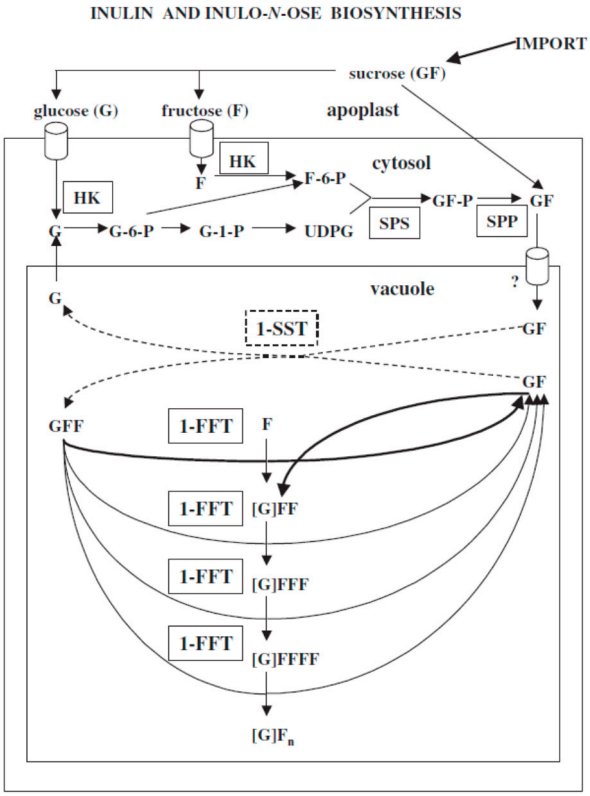

Figure 1. The model proposed by Van Laere and Van den Ende [10] for the biosynthesis of GF n and F n type inulins by 1-SST and 1-FFT in the vacuole.

Different researchers agreed that fructan synthesis and breakdown is more complex because different fructosyltransferase enzymes synthesize several different fructan molecules, depending on substrate availability (sucrose or inulin) or incubation conditions [10]. In open fields, inulin metabolism is also regulated by exogenous factors, such as low temperature, drought, CO_2 atmospheric concentration and climate, and growing conditions [18–21].

Wild cardoon (*Cynara cardunculus* L. var. *sylvestris* Lam.) is a widespread species in Sicily, a region characterized by the typically stressful conditions of the Mediterranean environment. This variety is considered the wild ancestor of globe artichoke [22,23], It is a robust thistle, with a characteristic rosette of large, spiny leaves and branched flowering stems. The plant sprouts in autumn; winter leaf rosettes appear in winter; stem elongation occur in April–May; full blossom occurs in June; ripe fruits appear in July; and the plant becomes fully dry aerial biomass in August. The plant then remains in a state of rest until the next growing season [12]. The particular climatic conditions of the Mediterranean basin, and arid and semi-arid regions in general, adversely affect the seedling establishment of the plant [24]. Its growth strategy is based on a large supply of reserve materials within the roots of the cardoon. In fact, it can accumulate inulin in its roots [25,26]. In a previous study,

focusing on sugar accumulation (inulin and water-soluble sugars) in the roots of globe artichoke, domestic cardoon and wild cardoon grown in a Mediterranean environment for two years, the storage of fructans in the underground organs of these plants was found to be an adaptive strategy to overcome the unfavorable Mediterranean conditions. In this view, the three taxa showed different biomass accumulation and partitioning, reflected in inulin accumulation and/or breakdown in the roots. These behaviors agree with the selection on this species for specific forms of production (the leaves of cultivated cardoon, and the heads of globe artichoke), suggesting that the wild form of *C. cardunculus* develops a large root apparatus to better escape the unfavorable conditions of the Mediterranean climate [13].

Previous studies focused on inulin metabolizing enzyme activities during the growth cycle of wild cardoon and their role in flower induction [27,28]. Except for these studies, no knowledge concerning correlations between the enzymes involved in inulin metabolism in roots and the plant's growth during its complete growth cycle under Mediterranean conditions is available. The present study aimed to discuss and correlate aspects of plant growth, and their relation to fructan accumulation and translocation, with the roots of wild cardoon.

2. Materials and Methods

2.1. Plant Material and Crop Management

The experiments were carried out in Sicily (South Italy), in Catania (37°27′ N, 15°4′ E, 10 m a.s.l.), during 2015/16. Heads of the genotype of wild cardoon were collected from native plants found in Ragusa, Sicily, South Italy, during the summer of 2015. Heads were threshed with a specific mini thresher to separate the achenes. The wild cardoon population is stored at the germplasm bank of National Council of Research—Institute of BioEconomy. Seeds were sown in pots (50 cm diameter), containing as substrate a mixture of black peats (50% v/v) and blondes (40% v/v) with bark humus and dried manure (10% v/v). The product is used in the cultivation of vegetables, usually in pots. The structure promotes excellent drainage and good ventilation for long periods. The natural richness of peats allows a growth period of several weeks without other nutrients. It is an environmentally friendly natural product without the addition of chemical correctives. Sowing was performed on 15 October 2015 and 100 pots, containing 1 plant each, were prepared. Pots were placed in open air in Catania, on the experimental farm of the University of Catania, Department of Agriculture, Food and Environment (37.52 °N, 15.07 °W). During the experiments, energy inputs for crop management were not applied. Crop water requirements were satisfied by rains.

2.2. Harvesting Procedures and Data Collection

Five plants (the experimental unit) were harvested from December 2015 to February to July 2016 for a total of ten samplings. Visual surveys were carried out to evaluate the number of plants at the same growth stage and the total number of pots and/or remaining pots at each harvest. When at least 75% of plants were uniform, the harvest was performed.

For each harvest's total biomass, roots included were immediately weighed to determine the fresh weight (FW).

In the laboratory, the moisture content of a representative sample of biomass components (roots, shoots, leaves and heads) was measured by drying the plant material in a thermoventilated drying oven at 105 °C until a constant weight was reached [29].

2.3. Protein Extraction and Enzyme Assay

About 10 g of root tissue was homogenized using mortar and pestle in an equal amount (10 mL) of 50 mM Na-acetate buffer, pH 5, containing 1 mM phenylmethylsulfonyl fluoride, 10 mM $NaHSO_3$, 1 mM mercaptoethanol, 0.02% (w/v) Na-azide and 0.1% (w/v) Polyclar AT (Serva, Heidelberg, Germany). The analyses were performed according to the protocol of our previous studies [27,28], with some minor modifications. Briefly, the original

homogenate was centrifuged for 5 min at 10,000× *g*. Samples of the supernatant were mixed with 1.2 mL of saturated $(NH_4)_2SO_4$. After 0.5 h of incubation on ice, precipitates were collected by centrifugation at 10,000× *g* for 5 min. Subsequently, the precipitates were washed and dissolved in 300 µL of 50 mM Na-acetate buffer, pH 5, containing 0.02% Na-azide, and mixed. This protein extract was subsequently used for determining the enzymatic activities of 1-SST, invertase, 1-FFT and 1-FEH, and they are expressed as nkatal g^{-1} FW.

Both 1-SST and invertase activities were assayed in the same reaction mixture. Aliquots of the protein extract were incubated for 30, 60, and 120 min at 30 °C in 50 mM Na-acetate buffer pH 5 containing 50 mM Suc and 0.02% (*w*/*v*) Na-azide. The reaction was stopped by heating at 95 °C for 5 min. Samples were diluted threefold with 0.02% (*w*/*v*) Na-azide. From these, 25 µL was automatically injected onto a Dionex column (see carbohydrate analysis). For 1-FFT assay, aliquots of the protein extract were incubated for 30, 60, and 120 min at 0 °C in 100 mM MES-NaOH buffer, pH 6.25, containing 3% (*w*/*v*) commercial chicory root inulin (DP 10, Sigma) as a fructosyl donor, 10 mM Suc as a fructosyl acceptor, and 0.02% (*w*/*v*) Na-azide. The reaction was stopped by heating at 95 °C for 5 min. Samples were diluted threefold with 0.02% (*w*/*v*) Na-azide. From these, 25 µL was automatically injected onto a Dionex column (see carbohydrate analysis). The activity of 1-FEH was measured by incubation of aliquots of the protein extract together with 3% (*w*/*v*) commercial chicory root inulin (DP 10, Sigma) in 50 mM Na-acetate buffer, pH 5, containing 0.02% (*w*/*v*) Na-azide at 30 °C for 30, 60, and 120 min. The reaction was stopped by heating at 95 °C for 5 min. Samples were diluted threefold with 0.02% (*w*/*v*) Na-azide. From these, 25 µL was automatically injected onto a Dionex column (see carbohydrate analysis).

2.4. Carbohydrate Extraction and Analysis

A representative fresh root sample was washed with cold tap water, peeled, and ground to a fine powder by mortar and pestle before adding liquid nitrogen.

The original homogenate was diluted tenfold with water and placed in a boiling-water bath for 30 min. After cooling to room temperature, the extract was centrifuged at 3000× *g* for 5 min. A 5 mL sample of the supernatant (corresponding to 500 mg f. wt) was passed through a 1 mL bed volume of Dowex® 50 H+ and a 1 mL bed volume of Dowex®-1-acetate. The resins were rinsed twice with distilled water.

A part of this fraction was diluted 50 fold with distilled water to analyze free sugars (glucose, fructose and sucrose). Another fraction (500 µL) was hydrolyzed at 70 °C for 2 h using 5 µL of 3 N HCl for total fructose analysis. Both the fractions were analyzed using a HPAEC PAD, following the procedures described in previous studies [9,28,30].

Inulin content was calculated in accordance with Baert (1997) [31]: I = (F + G) − (f + g + s), Mean DP = (F − f − 0.525S)/(G − g − 0.525S), where F and G are the total fructose and glucose after acid-hydrolysis, and f, g and s are the reducing free sugars fructose, glucose, and sucrose before acid hydrolysis, respectively. All analyses were performed in duplicate for each sample and are reported on a dry matter (D.M.) basis. Inulin, sucrose, free glucose and free fructose content are reported in g kg^{-1} D.M.

2.5. Data Analysis

Data were submitted to Barlett's test for the homogeneity of variance and then analyzed using an analysis of variance (ANOVA). Means were statistically separated, based on the Student–Newmann–Kewls test, when the ANOVA 'F' test for treatment was significant at a probability level of at least 0.05. Significance was accepted at the $p < 0.05$ level. The percentage data were arcsin $\sqrt{\%}$-transformed before analysis [32]. Data without transformation are reported. Pearson product moment correlation coefficient was calculated between two independent variables using CoStat software, v. 6.400.

3. Results

3.1. Meteorological Data

The meteorological course during the experiment was that of a typically Mediterranean environment (Figure 2). The season was typical in relation to the long-term climate, experiencing similar maximum and minimum temperatures during the growing period. The rainfall in the relevant 30 year period (1965–1994) was 681 mm, which was higher than that recorded in our experiment (278.6 mm). In particular, the maximum temperature measured during the crop growing season ranged from 15.5 °C (mid-January) to 31.1 °C (mid-July), while the minimum ranged from 4.3 °C (late January) to 20.1 °C (July).

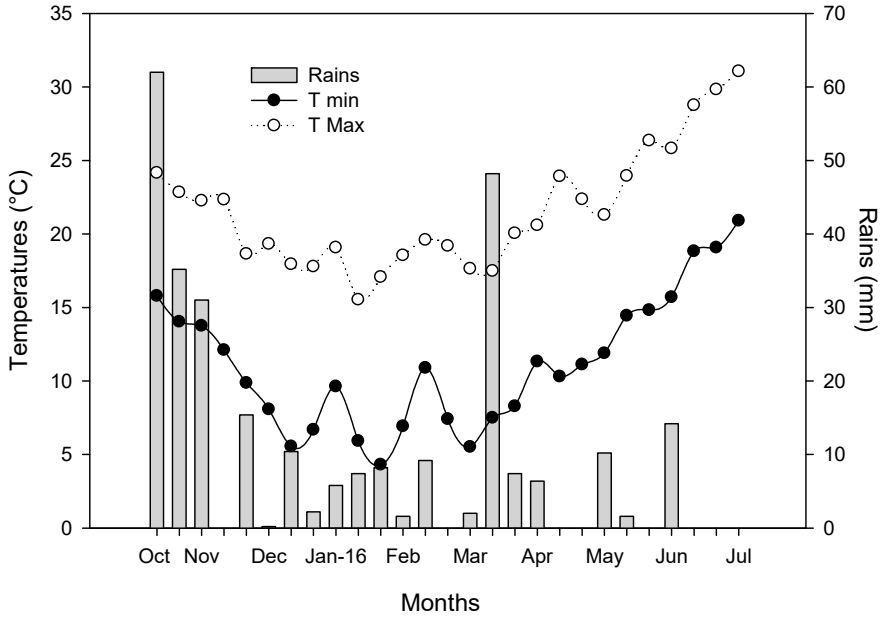

Figure 2. Air temperatures (as mean value over three decades) and rains (as sum value of three decades) during the growing period at the experimental site.

3.2. Plant Growth

The total biomass and its partitioning are shown in Figure 3 and Table 1. It was possible to determine distinct phases. From 60 to 116 days after sowing, the rate of biomass accumulation, which consisted of leaves and roots was slow, at 0.18 g plant^{-1} day^{-1} DM. From 144 to 187 days after sowing, the plant's DM accumulation was characterized by a faster period of growth (0.55 g plant^{-1} day^{-1}). Until 208 days after sowing, it consisted of leaves and roots. After this period, the plant's development was directed more toward reproduction. From 228 to 249 days after sowing, the rate of biomass accumulation was 1.3 g plant^{-1} day^{-1}. From the end of this period up to 270 days after sowing, when the above-ground biomass dried up, the total biomass rate decreased from 365.53 g to 319.32 g plant^{-1} (1.18 g plant^{-1} day^{-1}).

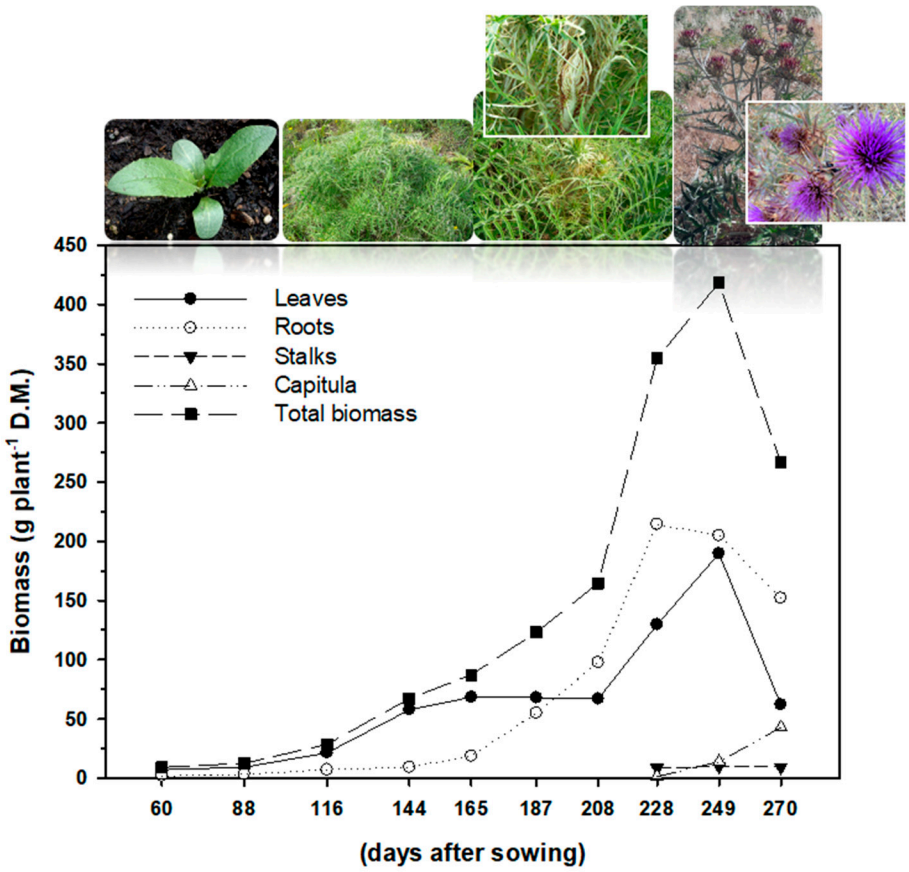

Figure 3. Biomass production per plant (g plant^{-1} D.M.) variations during the whole growth cycle of wild cardoon.

Table 1. Plant growth rate (g plant^{-1} day^{-1} D.M.) and biomass partitioning (% of total biomass) variations during the whole growth cycle of wild cardoon.

Days after Sowing	Plant Growth Rate	Leaves	Roots	Stalks	Heads
(d)	(g plant^{-1} day^{-1} D.M.)	(% of Total Biomass)			
60	0.15 g	79.7	20.3		
88	0.14 g	74.5	25.5		
116	0.25 f	74.7	25.3		
144	0.47 e	86.2	13.8		
165	0.53 d	78.5	21.5		
187	0.66 cd	55.3	44.7		
208	0.79 c	40.7	59.3		
228	1.55 a	36.6	60.6	2.43	0.39
249	1.47 a	45.3	49.0	2.41	3.29
270	1.18 b	23.3	57.1	3.52	16.1

Different letters within the same column indicate statistical differences at $p < 0.05$ among sampling.

From the beginning of the experiment up to 208 days after sowing, the biomass accumulation in the leaves and roots was proportionate to their growth. Until 165 days after sowing, leaves contributed 78% of the total biomass and roots contributed 22% of the total biomass. In the two following harvests, the roots' contribution to the total biomass was 56%. Once the reproductive phase began, with the formation of stalks and flowers, dry matter accumulated mainly in the roots (60% of the total biomass; 208 days after sowing). This phase lasted for 15 days; the stalks, on average, contributed 2.7% of the total biomass, and the heads 5.9%. The leaves then dried up, representing only 19% of the total

biomass. These results are in accordance with our previous work on *Cynara* genus cropped in Mediterranean environments [33].

3.3. Changes in Carbohydrates Content in Roots

The seasonal dynamics of free glucose, free fructose, and sucrose, as detected by HPAEC, are shown in Table 2. Averaged for the ten harvests, glucose resulted in 25.5, fructose 18.8, and sucrose 7.7 g kg^{-1} DM. The profile of the soluble carbohydrates reflects the assimilation activities during the vegetative and reproductive development phases. The free glucose contents at the first sampling were 24.9 g kg^{-1} D.M. and did not undergo any significant changes until 144 days after sowing, when they reached a peak of 44.5 g kg^{-1} D.M. From 165 to 208 days after sowing, they remained greater than 30 g kg^{-1} D.M. and then declined steadily until the last harvest, reaching values close to 10 g kg^{-1} D.M. The free fructose content ranged from values of 6 g kg^{-1} D.M. in the earliest stages of the biological cycle to values above 30 g kg^{-1} D.M., recorded in March, during the vegetative growth of the plant.

Table 2. Glucose fructose and sucrose (g kg^{-1} D.M.) variations during the growth cycle of wild cardoon.

Days after Sowing	Glucose	Fructose	Sucrose
(d)		**(g kg^{-1} D.M.)**	
60	24.9 ± 1.10 e *	6.11 ± 1.12 f	0.98 ± 0.65 f
88	26.8 ± 0.77 de	6.30 ± 1.12 f	1.35 ± 1.16 f
116	21.7 ± 2.55 f	29.3 ± 0.61 b	4.21 ± 0.24 e
144	44.5 ± 2.62 a	30.8 ± 0.33 ab	3.73 ± 0.48 e
165	38.7 ± 1.37 b	32.3 ± 0.92 a	5.62 ± 1.19 d
187	29.5 ± 1.61 d	12.7 ± 2.00 e	16.1 ± 1.39 a
208	34.3 ± 1.39 c	15.6 ± 0.79 d	9.46 ± 1.95 c
228	15.1 ± 1.10 g	12.3 ± 0.70 e	9.86 ± 1.41 c
249	10.3 ± 2.12 h	22.4 ± 0.85 c	12.8 ± 2.12 b
270	9.13 ± 2.12 h	20.7 ± 1.27 c	13.3 ± 0.71 b

* Different letters within the same column indicate statistical differences at $p < 0.05$ among samples. All values are mean ± SD of three independent measurements of each sampling.

In the context of inulin metabolism during periods of high photosynthesis, sucrose is immediately metabolized in the cells of roots by the enzyme 1-SST to 1-kestose, and subsequently transformed by 1-FFT to higher polymers of inulin. In this context, the level of sucrose, unlike the contents of glucose and fructose, resulted in less than 5 g kg^{-1} D.M. in the period before the month of March (vegetative growth), when the contents of glucose and fructose were higher; values higher than 10 g kg^{-1} D.M., with a peak of 31.5 g kg^{-1} D.M. (between 144 and 165 days after sowing), during the reproductive phase were recorded.

The sucrose content was higher during the vegetative phase. This can be attributed to the mobilization of inulin from the roots to support the formation of stalks and heads. During the vegetative phase, this molecule was highly correlated with inulin (r^2 = 0.727 **), 1-SST (r^2 = 0.920 ***) and 1-FEH activities (r^2 = 0.731 **), while it was inversely correlated with 1-FFT (r^2 = −0.843 ***). During the reproductive phase, the signal molecule was inversely correlated with FEH, which was mainly governed by glucose amount (r^2 = 0.986 ***), probably for mobilizing glucose for grain ripening (Table S1).

The inulin content was consistently the most important component of the root total sugars, with significant changes in the content, varying in different phases of the cycle between values close to 67 (60 days after sowing) to over 290 g kg^{-1} D.M. (249 days after sowing). The accumulation of inulin followed the same root growth rates. From the month of February, steady increases in the inulin content, up to 187 days after sowing (176 g kg^{-1} D.M.), were observed, followed by a sudden increase up to 249 days after sowing. In the first phase, the inulin amount was mainly correlated with 1-SST (r^2 = 0.727 **) enzyme activity and 1-FFT (r^2 = 0.785 **), while during the reproductive phase, the enzymes 1-SST and 1-FEH were inversely correlated, meaning that the mobilization of inulin in free

sugars for plant growth occurred. At the end of the cycle, in relation to free sucrose, when the plants were dry and the maturation of the achenes occurred, the content of inulin in the roots was 278 g kg^{-1} D.M. (Figure 2).

The chain length variations in the inulin during the growth cycle of the wild cardoon plants are presented in Figure 4. The DP of inulin shows about the same variation tendency in the inulin content until 165 days after sowing. We observed about 30 units of fructose during all the vegetative phase; this number increased quickly at the beginning of the reproductive phase until 228 days after sowing, at which point it peaked (73 fructose units); it then decreased at a higher speed, reaching 42 fructose units.

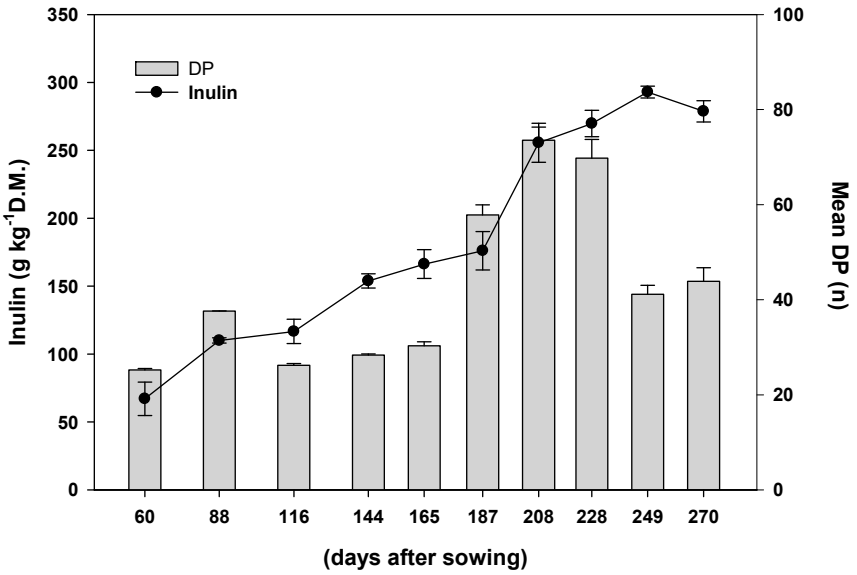

Figure 4. Inulin accumulation in roots and its relative degree of polymerization (mean DP) variations during the growth cycle of wild cardoon. Vertical bars indicate standard deviation values.

The trend can be explained by the fact that wild cardoon inulin is accumulated first in the roots, and then in the flowers. At the beginning, the low DP of inulin may have been attributable to the high free sugar content. Flowering started 228 days after sowing, and it was supported by inulin stored in the roots. Consequently, during flowering, wild cardoon roots start translocating assimilates from the roots to the flowers, which causes the inulin content and DP to decrease. As leaves and stalks dry up, the content and DP of inulin start decreasing after reaching their peak.

3.4. Enzyme Activities in Roots

As described above, the growth of the plant and the concentration of sugars were confirmed by the enzymatic activity that governs the metabolism of the sugars in wild cardoon roots. Across the entire growth cycle of the wild cardoon, all the enzymes studied were present and were isolated and quantified, showing activity with substantial differences among the different periods.

Trisaccharide from enzyme 1-SST, which catalyzes the union of one unit of fructose and one of sucrose to synthesize 1-Kestose, was the part of the synthesis of inulin that showed activity from the first to the seventh sampling, with strong fluctuations during the growth cycle of the plant (Table 3). The 1-SST enzyme showed a first peak (25.2 nkat g^{-1} f.w.) 88 days after sowing. The simultaneous activity of this enzyme and 1-FFT in this period was related to the increase in biomass in the roots, which was mainly due to the accumulation of inulin. The maximum activity of 1-SST was recorded at 187 days after sowing (70.4 nkat g^{-1} f.w.), when the plants passed from the vegetative to the reproductive phase. The high activity observed in this period was attributable to the high sucrose content (r^2 = 0.920 ***) (Table S1).

Table 3. 1-SST, 1-FFT, 1-FEH, and acid invertase (nkat g^{-1} f.w.) variation during the growth cycle of wild cardoon.

Days after Sowing	1-SST	1-FFT	1-FEH	Invertase
(d)	(nkat g^{-1} f.w.)			
60	5.48 f *	10.4 a	30.3 c	2.46 e
88	24.8 c	8.89 c	11.7 f	3.43 c
116	8.81 e	9.46 b	8.19 g	3.03 d
144	18.4 d	9.71 b	31.7 c	10.5 a
165	30.2 b	4.94 e	32.3 c	3.34 c
187	69.2 a	3.59 f	50.8 b	2.33 e
208	2.74 g	0.90 h	57.8 a	3.90 b
228	0.30 h	5.00 e	19.7 d	0.57 g
249	0 h	7.17 d	13.0 f	1.61 f
270	0 h	2.29 g	15.8 e	0.60 g

* Different letters within the same column indicate statistical differences at $p < 0.05$ among samples. 1-SST means sucrose-sucrose fructosyltransferase; 1-FFT means fructan-fructan fructosyltransferase; 1-FEH means fructan exohydrolase.

From this date, the content of inulin in the roots increased by 29%, passing from a value of 165 to 232 g kg^{-1} DM in a fortnight (r^2 = 0.727 **). After this period, the 1-SST activity disappeared. The 1-FFT enzyme activity was, on average, 9.7 nkat g^{-1} f.w. (first four harvests), and then decreased to 1.0 nkat g^{-1} f.w. (208 days after sowing) when the plants were in the reproductive phase, reflecting the increase in inulin degradation due to 1-FEH enzyme activity. However, the amount of fructose liberated from the chains of inulin did not impact on the accumulation of the polymer. Another peak (7.17 nkat g^{-1} f.w.) of 1-FFT enzyme was found 249 days after sowing, when the heads were already developed and the maturation of the grain was ongoing.

The activity of the degradation of inulin, mainly linked to 1-FEH enzyme, was found throughout the biological cycle of the plant, with average values from across the ten harvests of 27.4 nkat g^{-1} f.w. (Table 3). In particular, high activity was found in April, when the activity of the 1-SST and 1-FFT enzymes decreased. The invertase activity was noted throughout the biological cycle of the plant, with an average value of 3.2 nkat g^{-1} f.w.. At 144 days after sowing, a peak of activity was detected (10.45 nkat g^{-1} f.w.), reflecting an increase in the level of free glucose in the roots. According to Hellwege et al. [34], the mean DP and the distribution pattern of the fructan chain lengths accumulated in a given species are mainly defined by the characteristics of the FFT, although the involvement of the FEH cannot be ignored. The continuous activity of 1-FFT detected in the trial suggested that this enzyme acts by decreasing the fructan chain size during mobilization, thus favouring subsequent 1-FEH activity. The variation in free sugars and inulin content during the whole growth cycle of wild cardoon gives support to the role of fructans as reserve compounds in these plants.

4. Discussion

In the stressful Mediterranean environment, which is characterized by prolonged drought, the storage of inulin in underground organs, the state of dormancy in summer and the sprouting in autumn, when rains naturally occur, is the strategy for wild cardoon to overcome these unfavourable conditions. In this context, the role played by sugars in roots related to inulin-metabolizing enzymes and plant growth rate in *C. cardunculus* var. *sylvestris* allows a better understanding of the involvement of fructans in the tolerance of plants to these abiotic stresses. In fact, as long as temperature is low, growth is slow and, due to the low temperatures recorded in the winter period (on average 7 °C), the biomass consisted mainly of leaves (about 80% of the total biomass). The high amount of monosaccharides recorded in the roots was probably due to the low temperatures recorded in this period, as the saccharides were mobilized from roots to prevent cold stress. Additionally, sucrose could be transferred to the cell for metabolism as well as for stabilizing

the living processes of roots (e.g., supporting processes of lignification to strengthen the skin, thus protecting the roots). In stressful environments, moreover, fructans are thought to stabilize cellular membranes or to contribute to the whole cellular homeostasis through reactive oxygen species-scavenging mechanisms, especially small fructans. In this way, fructans are able to protect plants against abiotic stresses [14,16,35–37]. It is well known that fructan accumulation depends on a concentration of sucrose that must exceed a threshold typical for each species, activating a sucrose-specific signaling pathway [35,36,38]. In wild cardoon, t marked increases in glucose, fructose, and sucrose concentration were induced for a very short period immediately following the cold month of January and during dormancy. According to Livingston et al. [14], because fructans can be considered as a source of hexose sugars, plants can use sugars, synthesized during cold acclimation or from hydrolyzed fructan, to resist plasmolysis by increasing osmotic pressure within cells. This is in accordance with the high level of glucose and fructose recorded during the vegetative phase in the wild cardoon and with the 1-FEH activity recorded in the same phase.

Hence, the increase in biomass of the underground tissues could be correlated with inulin accumulation and occurred between February to May, suggesting that in this species, during the vegetative phase of aerial growth, sucrose is synthesized in excess and translocated to the underground organs, where it is metabolized into inulin.

Moreover, the 1-SST enzyme peak recorded 88 days after sowing and the simultaneous activity of this enzyme and 1-FFT in this period could be related to the increase in biomass in the roots, which was mainly due to the accumulation of inulin.

When the mean air temperatures increased, from the month of March, the plant growth rate begun doubling and the plant's development was directed more toward reproducing and the levels of free sugars reached their highest value. The maximum activity of 1-SST was recorded when the sucrose content was high and the inulin amount increased quickly. In the same period, the 1-FEH enzyme activity increased the amount of the fructose liberated from the chains of inulin, but it did not exert any impact on the accumulation of the polymer.

Starting from the month of March, the DP increased, even though the physiological benefit of accumulating higher DP inulin fructans in some Asteracean species remains unclear [21]. In *Viguiera discolor*, an Asteracean species from the Brazilian cerrado, the accumulation of its high DP inulin-type fructans is linked to its high DP 1-FFT. The authors suggested that the capacity to accumulate high DP inulin-type fructans may represent an adaptation to the special conditions of drought stress and burning [39].

Considering that the growth of aerial organs and the subsequent sprouting of buds from underground reserve organs are processes that demand high amounts of energy, the increase in 1-FEH activity suggests that fructan-hydrolyzing activity is associated not only with sprouting, but also with the growth and elongation of new shoots in plants. On the other hand, the decrease in 1-SST activity indicates that the activity of this enzyme depends on a constant flow of photo-assimilates to the main site of fructan synthesis. The role played by invertase in the process of carbohydrate mobilization during the vegetative phase of *C. cardunculus* seems to be related to the use of sucrose prior to the use of fructans, since the peak activity of this enzyme occurred before the peak of the 1-FEH activity. These results are in accordance with the hypothesis presented by Asega and de Carvalho [20] about the temporal control of fructan-metabolizing enzymes present in the same cell compartment, the vacuole.

5. Conclusions

Inulin accumulation in the roots of wild cardoon is associated with plant development. We observed the sequential activities of the 1-SST and 1-FEH enzymatic systems and the involvement of the invertase and 1-FFT enzymatic systems in carbohydrate mobilization in response to the increases in energy that the plant requested for stalk elongation before and for capitula development, as well as subsequent grain ripening.

Cold conditions play an important role in carbohydrate biochemical processes, induced by enzymatic systems, for storing sucrose and inulin in the root system during the cold season, and using these sugars in the roots during the spring season for the vegetative stage.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12020290/s1, Table S1: Pearson product moment correlation coefficient (r^2) among sugars and enzymes in roots of wild cardoon. ***; ** and * indicate significant at $p < 0.001$, 0.05 and 0.01, respectively, and ns not significant.

Author Contributions: Conceptualization, M.G.M. and F.B.; methodology, M.G.M. and S.A.; validation, M.G.M. and F.B., formal analysis, S.A.; investigation, M.G.M. and S.A.; resources, M.G.M. and F.B.; data curation, M.G.M., S.A. and F.B.; writing—original draft preparation, M.G.M. and A.M.P.; writing—review and editing, F.B., S.A., A.M.P. and M.G.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data for this study has been included in the tables, figures and Supplemental Material.

Conflicts of Interest: The authors declare no conflict of interest.

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