Sugar alcohol composition in leaves of *Cynara cardunculus* plants at different developmental stages under salinity conditions, via a metabolomics approach.

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Report of the results obtained by the research group, during the stay of Mônica T Veneziano Labate, for 10 days at IBAF-CNR, Porano (Tn) - Italy, started in October 14, 2013 and ended on October 25, 2013, as part of the Short Term Mobility Research Program - 2013

Abstract:

The species *Cynara cardunculus* is recognized as a promising species for biomass production in the Mediterranean environment. Little is known about key aspects of the *C. cardunculus* physiology, biochemistry and molecular control of biomass quality accumulation and also about the effect of environmental conditions on the same aspect.

It is known that *cardunculus* can accumulate large quantities of fructans, and preliminary results obtained at IBAF CNR indicate that the plant can also accumulate a number of sugar alcohols of possible relevance for biorefinary activities.

Albeit the pathways leading to the synthesis of fructans have been investigated in the past in grass and dicots species, including *C. cardunculus*, very little is known about the regulation of the synthesis and accumulation of sugar alcohols in general and particularly no reports of sugar alcohols in *C. cardunculus* have been found in the available literature.

The aim of this project is to study the synthesis and accumulation of sugar alcohols in *C. cardunculus* via a metabolomics approach, in order to investigate how the synthesis and accumulation of these compounds are controlled by the metabolism and the environment.

Introduction:

The production of energy crops is expected to benefit the development of new markets, to promote regional economic structures, to provide alternative sources of employment in rural areas, to promote the use of surplus and marginal lands, reducing CO_2 emissions, and decreasing dependence on short-term weather changes experienced by production of other forms of renewable energy (Zegada-Lizarazu et al., 2010).

Biomass is a renewable energy source with high potential and conversion versatility, i.e. by thermal, biological and mechanical or physical processes (Bridgwater, 2006). The utilization of biomass for energy production is encouraged in several countries by economical and geo-strategic reasons to reduce dependence on imported fossil fuels and by environmental considerations regarding CO_2 balance (Gominho et al, 2011).

Several species have been proposed in Europe as possible energy crops (Martínez-Lozano et al., 2009). However, in the Mediterranean region, it is well known that the soil water content is one of the most limiting factors for plant growth under rain-fed conditions. Water availability strongly affects the choice of bioenergy crops in Mediterranean environments (Fernández et al., 2006) where only few options are possible (Ledda et al, 2013). *Cynara cardunculus* is now considered the most important and promising source for solid biofuel. The reason is that cardoon is a perennial rosette plant grown as annual crop in some Mediterranean regions (Ruccia et al, 2010), well adapted to the xerothermic conditions of southern Europe, which can be utilized particularly for solid biofuel production. This is due to its minimum production cost, as this perennial weed may perform high biomass productivity on most soils with modest or without any inputs of irrigation and agrochemicals (Grammelis et al, 2008).

The crop is potentially useful in many ways: the stem as a source of lignocellulosic biomass for energy (Raccuia and Melilli, 2007) and paper pulp (Gominho et al., 2001), the seed as a source of protein and edible oil (Maccaroneet al., 1999) as well as a source of oil for producing biodiesel (Encinar et al., 2002), and the roots as a source of inulin (Raccuia and Melilli, 2010).

The cultivation of *C. cardunculus* and its main growth stages in the Mediterranean environment is described by Raccuia and Melilli (2007). Its growth strategy is based on a large supply of reserves in the form of storage organs and generally, carbohydrates are the major reserves within the perennating organs (Raccuia and Melilli, 2010).

In the current study, the aim is to analyze the content and composition of sugar alcohols in *C. cardunculus* leaves at different developmental stages of the plants in different conditions of salinity to investigate via a metabolomics approach how the synthesis and accumulation of these compounds are controlled by the metabolism and the environment.

Material and Methods:

Greenhouse trials:

Experiments were carried out in greenhouse in Viterbo at Tuscia University from April to June 2013. Cardoon plants (*C. cardunculus*) were grown in a floating system with full nutrient solution. After germination, the plants were submitted to four salt treatments (A = Control, B = NaCl 60mM, C = NaCl 30 mM, D = NaCl 30 mM + $CaCl_2$ 20 mM, E = NaCl 15 mM + $CaCl_2$ 10 mM).

The trial was assessed in a completely randomized block design (Figure 1) with three replicates for each treatment.

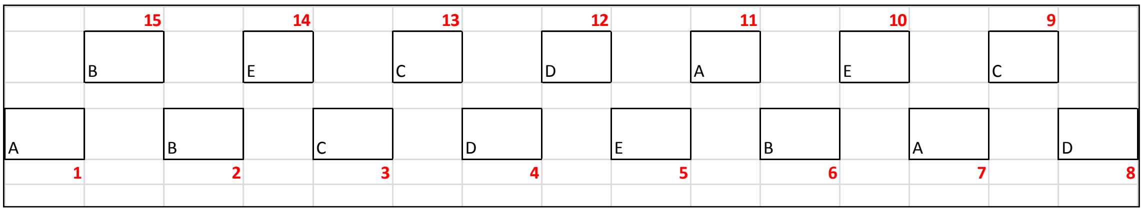

Figure 1. Randomized block design of the experimental trial.

Plant material and harvesting procedures:

Forty-five leaf samples of three plants each (3 replicates for each treatment) were harvested after 48, 70 and 95 DAS (days after sowing) and dried to a constant weight in a ventilated oven at 60◦C.

Sugar alcohol extraction and analysis:

The dried samples were ground to a fine powder in a ball mill. A representative sample of 10 mg of the powder was extracted with 2 mL of 50% ethanol at 70◦C for 30 min. Samples were centrifuged for 15 min at 16.000 *g*. After centrifugation, the supernatant was passed through a 0.2 µm filter.

Sugars were separated on a Dionex CarboPac MA1 analytical column (250 mm x 4 mm) connected to a CarboPac MA1 guard column (50 mm x 4 mm) from Thermo Fisher Scientific. The mobile phase was 480 mM NaOH with the flow rate of 0.4 mL/min, maintained at 30◦C. Detection setting was: time = 0.00 s (E = + 0.1 V), time = 0.41 s (E = - 2.00 V), time = 0.43 s (E = + 0.60 V), time = 0.44 s (E = - 0.1 V).

Metabolite extraction for LC and GC-TOF-MS

Metabolites were extracted from 25 mg of ground *C. cardunculus* leaves, considering three biological replicates per treatment. The aim is to identify a large number of metabolites from different chemical classes. For this purpose we shall use two different protocols for extraction; one, according to Gullberg et al. (2004), for analysis of primary metabolites by GC-MS. The other protocol, proposed by De Vos et al. (2007) is for the analysis of secondary metabolites *via* LC-MS.

The extracted samples were injected, in the positive and negative mode, into two different Mass Spectrometers: a Q-TOF-Ultima API Waters (LC-ESI-MS) and a Pegasus 4D LECO (GC/GC-TOF-MS), available at the “Laboratório Max Feffer de Genética de Plantas”, from the Departament of Genetics/Esalq, of the University of São Paulo, Brazil . The parameters used for chromatography and injection of the samples, will be the ones proposed by De Vos et al. (2007) and Gullberg et al. (2004), respectively, for LC-MS and GC-MS. In the case of the differentially expressed metabolites, identified by LC-MS, an additional analysis by MS/MS will be done for fragmentation of the molecules for elucidation of their structure.

Identification of the metabolites and statistical analysis:

The processing and interpretation of the data obtained by Mass Spectrometry will be done after the correction of the base lines, exclusion of noises, deconvolution of the spectra, detection and integration of pikes and alignment of the chromatograms. The data generated by LC-MS and GC-MS will be analyzed using the MassLynx (Waters) and ChromaTOF (LECO) softwares, respectively. The identification of the molecular mass and the statistical analysis will be done using the following programs: MassLynx, MetaboAnalyst, MetNetDB, R.

Results and Discussion:

HPLC analysis:

The polyol composition of the leaf samples of *C. cardunculum* harvested after 48 DAS, 70 DAS and 95 DAS, detected by HPLC are shown in Tables 1, 2 and 3, as mean values recorded for the three replicates of each sample, expressed as percentage of dry matter. The results showed that the sucrose content did not change significantly due to the salt stress, for samples collected after 48 DAS and 70 DAS. However, for the samples collected after 95 DAS, the sucrose content increased significantly with the salt stress, probably to support the later stage of development of the plants.

Interestingly, as highlighted in Tables 1, 2 and 3, we can observe an increase of the xylitol content, associated with a decrease in the glucose content in some samples subjected to the salt stress, relative to the control ones, particularly in the initial stages of development (48 DAS and 70 DAS). For the case of samples collected after 95 DAS, only the treatment with 60 mM NaCl had an effect on the total content of glucose. However, the xylitol/glucose ratio were higher for all samples subjected to salt stress in relation to the control ones for all stages of development. It seems that there was a mobilization of xylitol from leaves to prevent salt stress.

This observation is very interesting, once xylitol is a very important sugar alcohol (alditol) which has applications in hygiene and nutraceutical formulations and products. It is used as a diabetic sweetener which is roughly as sweet as sucrose with 33% fewer calories. Unlike other natural or synthetic sweeteners, xylitol is actively beneficial for dental health by reducing caries to a third in regular use and helpful to re-mineralization (Steinberg et al, 1992).

Xylitol is naturally found in low concentrations in the fibers of many fruits and vegetables, and can be extracted from various berries, oats, and mushrooms, as well as fibrous material such as corn husks and sugar cane bagasse (Rao et al, 2006). However, industrial production starts from xylan (a hemicellulose) extracted from hardwood (Converti et al, 1999), which is hydrolyzed into xylose and catalytically hydrogenated into xylitol.

Table 1: Polyol composition of *C. cardunculus* leaves harvested after 48 DAS. Values

Polyol	Control		NaCl 60 mM		NaCl 30mM		NaCl 30mM Ca Cl_2 20 mM		NaCl 15mM Ca Cl_2 10 mM	
	mean	s.e.	mean	s.e.	mean	s.e.	mean	s.e.	mean	s.e.
Inosytol	0.428	0.014	0.592	0.026	0.629	0.032	0.631	0.084	0.480	0.016
Glycerol	0.864	0.016	1.244	0.094	1.211	0.022	1.252	0.128	1.092	0.130
Xylitol	0.163	0.027	1.151	0.181	0.800	0.156	0.964	0.084	0.622	0.068
Mannitol	0.558	0.122	0.562	0.094	0.562	0.105	0.543	0.097	0.456	0.090
Tot.Polyol	2.013	0.117	3.549	0.341	3.202	0.123	3.391	0.305	2.651	0.302
Glucose	6.695	0.932	2.112	0.335	4.711	0.358	6.334	2.316	5.141	0.822
Fructose	0.267	0.029	0.133	0.009	0.193	0.015	0.217	0.061	0.197	0.023
Sucrose	2.256	0.327	2.057	0.459	2.626	0.401	2.980	0.574	2.713	0.872
Tot Soluble	9.218	1.111	3.599	0.689	7.531	0.680	9.531	2.607	8.050	0.642
Starch	0.203	0.028	0.185	0.041	0.136	0.009	0.163	0.014	0.204	0.011
Tot Carbohydrate	9.421	1.087	3.784	0.657	7.667	0.678	9.694	2.611	8.254	0.646
Xylitol/Glucose ratio	0.026	0.006	0.588	0.010	0.173	0.041	0.183	0.043	0.132	0.035

are means of three replicates, expressed as % dry matter.

Table 2. Polyol composition of *C. cardunculus* leaves harvested after 70 DAS. Values are means of three replicates, expressed as % of dry matter.

Polyol	Control		NaCl 60 mM		NaCl 30mM		NaCl 30mM $CaCl_2$ 20 mM		NaCl 15mM $CaCl_2$ 10 mM	
	mean	s.e.	mean	s.e.	mean	s.e.	mean	s.e.	mean	s.e.
Inosytol	0.413	0.085	0.316	0.002	0.453		0.343	0.020	0.333	0.020
Glycerol	0.503	0.117	0.472	0.037	0.708		0.524	0.073	0.443	0.015
Xylitol	0.264	0.070	1.428	0.178	1.171		1.206	0.217	0.864	0.155
Mannitol	0.345	0.161	0.374	0.042	0.163		0.368	0.050	0.218	0.018
Tot.Polyol	1.525	0.289	2.591	0.097	2.495		2.441	0.169	1.858	0.178
Glucose	10.125	2.459	2.165	0.462	4.932		5.448	1.080	7.975	2.210
Fructose	0.377	0.130	0.070	0.016	0.160		0.120	0.025	0.193	0.058
Sucrose	2.787	0.567		3.144	2.584		3.238	0.503	3.119	0.348
Tot Soluble	13.288	2.614	2.538	1.778	7.676		8.805	1.576	11.287	1.925
Starch	0.123	0.006	0.083	0.008	0.109		0.103	0.003	0.138	0.008
Tot Carbohydrate	13.412	2.619	2.621	1.786	7.785		8.908	1.576	11.426	1.931
Xylitol/Glucose ratio	0.034	0.016	0.680	0.063	0.237		0.240	0.055	0.132	0.044

Table 3. Polyol composition of *C. cardunculus* leaves harvested after 95 DAS.Values are means of three replicates, expressed as % of dry matter.

Polyol	Control		NaCl 60 mM		NaCl 30mM		NaCl 30mM $CaCl_2$ 20 mM		NaCl 15mM $CaCl_2$ 10 mM	
	mean	s.e.	mean	s.e.	mean	s.e.	mean	s.e.	mean	s.e.
Inosytol	0.227		0.442	0.075	0.413	0.027	0.426	0.028	0.455	0.091
Glycerol	0.278		0.364	0.006	0.394	0.063	0.359	0.025	0.562	0.053
Xylitol	0.140		1.453	0.601	0.779	0.300	0.943	0.246	0.844	0.257
Mannitol	0.129		0.227	0.009	0.164	0.038	0.187	0.051	0.247	0.130
Tot.Polyol	0.774		2.485	0.529	1.750	0.261	1.914	0.192	2.108	0.503
Glucose	3.170		0.768	0.465	3.835	1.339	5.661	1.963	4.691	1.036
Fructose	0.130		0.070	0.030	0.167	0.038	0.210	0.080	0.167	0.023
Sucrose	1.708		3.809	0.181	2.440	0.412	4.159	0.737	3.785	1.188
Tot Soluble	5.009		4.647	0.675	6.441	1.629	10.030	2.780	8.643	2.099
Starch	0.003		0.222	0.085	0.225	0.047	0.243	0.041	0.169	0.035
Tot Carbohydrate	5.165		4.869	0.760	6.666	1.621	10.272	2.739	8.812	2.110
Xylitol/Glucose ratio	0.044		3.725	3.034	0.212	0.037	0.207	0.115	0.175	0.035

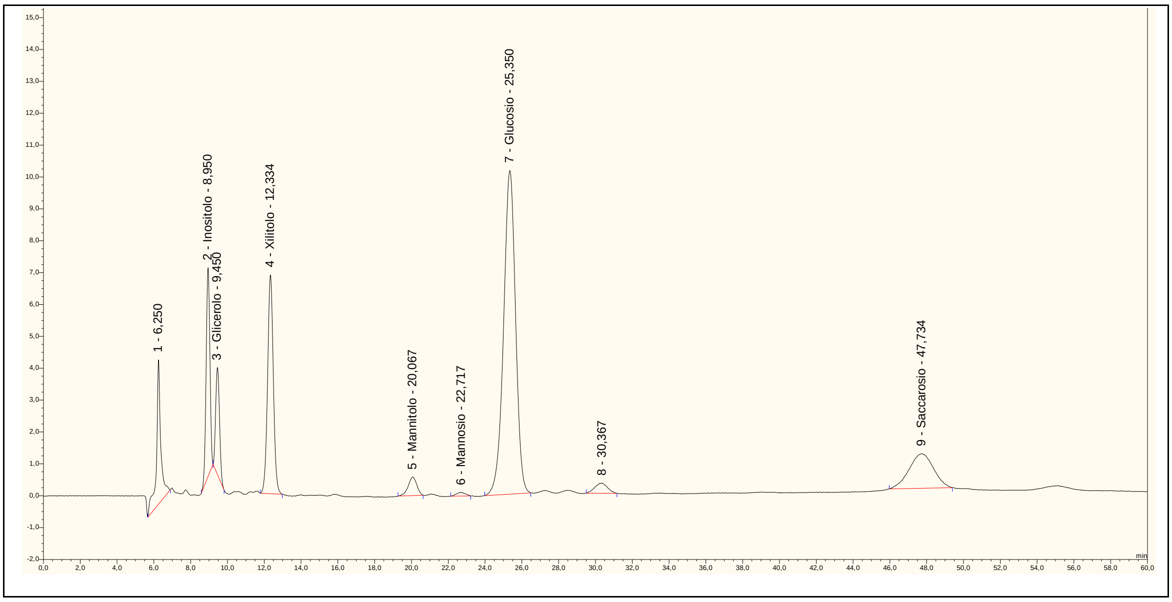

Figure 2: A representative spectrum of the composition of the polyols in the leaves of *c. carduncul*um samples analysed by HPLC.

Metabolomic analysis via LC-MS

Preliminary results were obtained for the analysis of the metabolic composition of leaf samples of *C. cardunculus* collected after 48 DAS and 70 DAS for all treatments (A = Control, B = NaCl 60mM, C = NaCl 30 mM, D = NaCl 30 mM + $CaCl_2$ 20 mM, E = NaCl 15 mM + $CaCl_2$ 10 mM). Figures 3 and 4 show the representative LC-MS chromatograms from leaf samples of *C. cardunculus* collected after 48 and 70DAS.

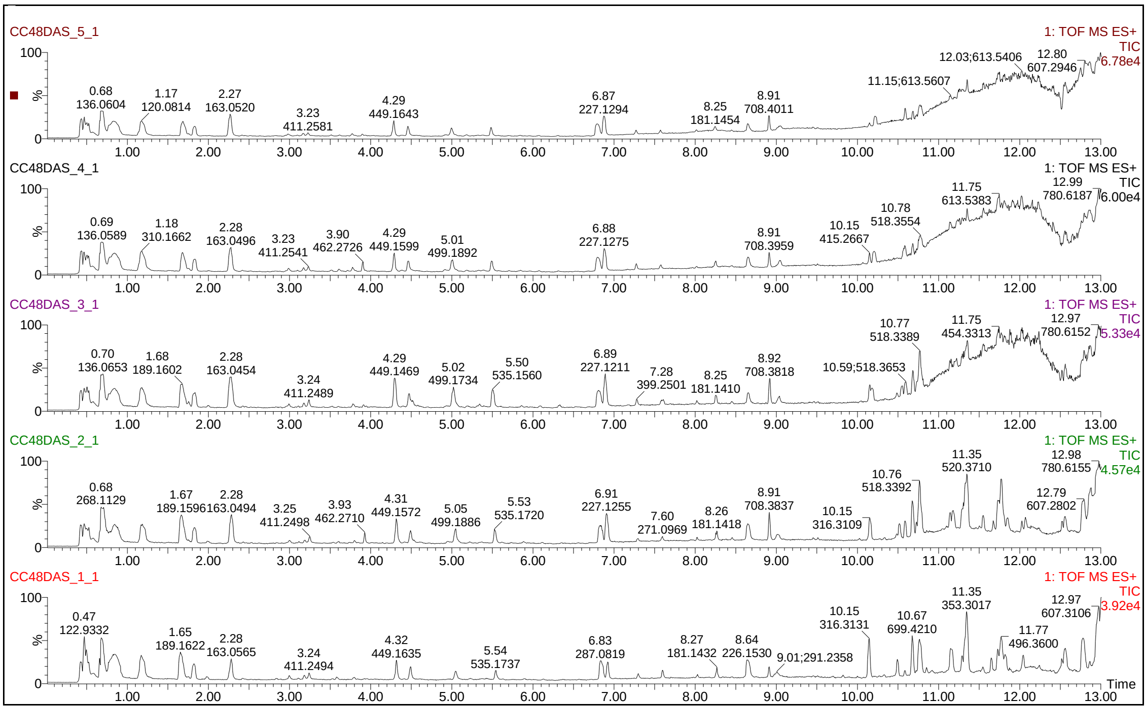

Figure 3: Representative LC-MS chromatograms from leaf samples of *C. cardunculus* collected after 48 DAS. Samples CC48DAS_1_1 = Control, CC48DAS_2_1 = NaCl 60mM, CC48DAS_3_1 = NaCl 30 mM, CC48DAS_4_1 = NaCl 30 mM + $CaCl_2$ 20 mM, CC48DAS_5_1 = NaCl 15 mM + $CaCl_2$ 10 mM).

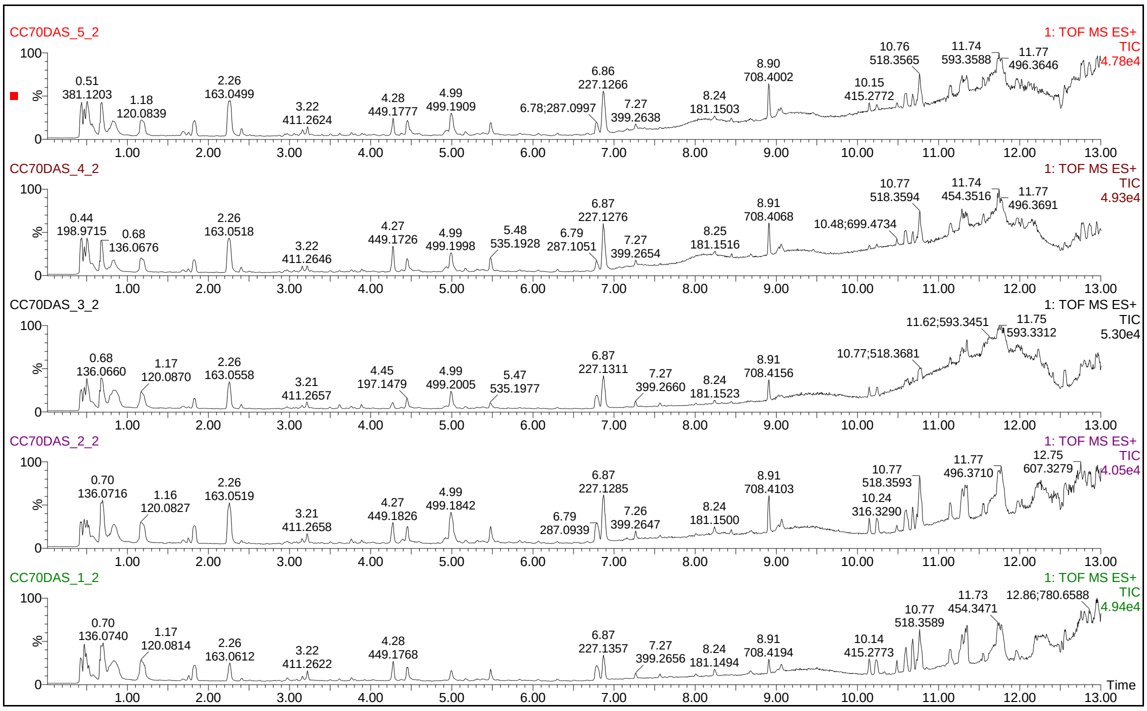

Figure 4: Representative LC-MS chromatograms from leaf samples of *C. cardunculus* collected after 70 DAS. Samples CC70DAS_1_2 = Control, CC70DAS_2_2 = NaCl 60mM, CC70DAS_3_2 = NaCl 30 mM, CC70DAS_4_2 = NaCl 30 mM + $CaCl_2$ 20 mM, CC70DAS_5_2 = NaCl 15 mM + $CaCl_2$ 10 mM).

The LC-MS fingerprints were collected for all control samples and the ones subjected to the salt stress after 48 and 70 DAS, as shown in Tables 2 and 3. The mass spectra were found to be very distinctive, showing sets of differential diagnostic ions, for each type of sample.

When applied to the LC-MS data, the chemometric method (Cazar, 2003) of principal component analysis split the samples into well-defined groups (Figure 6). Moreover the two developmental stages (48 DAS and 70 DAS) of the plants were also split into two distinct groups (Figure 5). In both cases, the two PC (principal components) explaining nearly 90% (Figure 5) and 60% (Figure 6) of total variance, respectively.

Table 2: *m/*z values accquired from the LC-MS spectra of *C. cardunculum* leaf samples. The chromatographic peak intensity values are means of 2 technical replicates.

m/z	CC48DAS_1	CC48DAS_2	CC48DAS_3	CC48DAS_4	CC48DAS_5
102.063	191.5523	204.4799	161.91045	140.2053	115.53345
120.0822	518.477	361.58895	296.0703	354.17645	404.00795
121.1	20.6348	20.6786	18.7241	21.26705	19.55375
136.082	47.61555	35.73705	82.49515	47.2549	38.6574
144.1149	402.1468	356.99145	295.2363	315.14775	247.02795
144.1226	37.3276	26.3972	20.47905	0	0
146.0821	49.61675	46.59515	55.95385	56.6281	41.80755
147.0671	82.51255	36.79035	46.8183	54.65085	47.4192
158.1804	41.3486	22.40445	18.85635	22.06565	26.51695
163.0527	567.46875	516.75165	439.7948	517.2671	570.30945
163.0669	0	36.2331	59.63285	38.71005	33.2304
164.0701	0	5.07035	18.56635	7.82445	15.52245
166.1085	115.80985	94.6099	89.3343	101.6076	89.1184
179.137	39.1361	43.1574	41.37955	40.41425	48.42855
181.1308	19.19045	110.6845	152.6556	90.5764	110.8339
181.147	424.79685	324.7978	285.60165	280.43285	240.1209
188.0887	613.41355	447.4216	379.2243	450.10235	481.7653
189.1678	628.2432	438.21275	358.569	430.03955	490.36735
190.187	40.9166	38.2189	32.4345	30.10435	30.7797
193.1213	0.8976	0	0.3911	0	3.08785
197.1434	511.4028	389.7223	337.9717	377.54745	438.4388
199.1256	33.4386	102.057	133.2749	85.1533	105.3544
205.1834	109.8487	63.51645	52.2125	27.57715	28.8656
209.137	0	0	3.67915	0	6.1548
209.1882	65.8281	53.45305	57.69975	49.1064	35.6197
212.1522	128.4268	65.65745	29.08325	107.20115	55.39595
215.2006	0	0	28.2358	33.47005	23.20305
226.1587	750.72075	510.09605	327.7905	422.26835	428.2558
227.1301	523.7133	527.7589	441.6935	510.9397	583.84755
227.171	45.1383	31.7589	13.4807	18.0909	15.9237
228.1496	0	33.2455	38.18725	29.28745	33.8643
229.1611	0	0	21.96285	33.5708	22.7339
229.1621	42.3882	11.0659	33.6449	37.4254	30.72905
229.163	0	0	0	30.7115	25.1754
245.1591	0	58.2568	68.4843	55.50685	59.95915
245.224	7.62105	29.4366	14.45795	21.66175	10.8338
247.1751	0	0	26.9888	43.88955	31.9018
256.0988	8.40215	25.3591	3.92575	0	0.8636
257.2309	20.7538	19.9239	15.8779	17.25065	17.02405
271.1018	194.1432	144.0726	145.18155	185.6179	179.6108
275.2451	64.79835	57.9647	63.2227	43.6961	49.029
287.0898	748.03895	527.60505	443.6279	522.15845	592.2733
287.0977	190.4756	322.637	377.1238	292.01965	291.4288
287.0978	5.97255	31.2049	56.0493	11.62755	0
288.1014	18.50995	27.8278	32.91615	29.78285	33.1092
291.2428	78.61955	27.8496	32.45525	27.2329	34.738
298.1447	30.6221	25.1554	53.37155	30.39185	26.1763
301.1719	0	0	0	0	0
310.1715	283.6079	112.61145	202.5977	331.1212	297.19095
325.2704	163.4653	5.9715	143.85175	0	0
355.1542	0	0	20.45375	16.84175	17.12345
364.2324	0	64.8811	67.02105	57.17655	56.45785
371.195	0	0	20.4174	24.39695	22.3387
377.1342	136.6029	333.44785	375.88865	369.6786	443.33385
377.202	34.81975	35.9405	27.35775	28.2492	26.2683
378.1526	4.6656	0	6.28765	0	6.32885
395.1918	53.1537	39.79215	38.223	45.97465	43.76855
399.2582	94.7187	39.67545	118.7383	145.1487	118.49505
409.2489	16.487	11.5601	27.0357	27.5507	28.0984

Cont. Table 2: *m/*z values accquired from the LC-MS spectra of *C. cardunculum* leaf samples.

411.259	107.4591	89.54515	95.63025	77.0781	71.3611
432.2734	0	0	25.74345	27.00835	9.89385
443.2987	101.9442	0	0.3911	0	0
444.2738	0	42.35565	6.433	42.4935	15.0902
447.1618	0	28.95475	57.84895	21.66915	11.0477
449.1659	31.82845	58.6311	125.21115	39.8027	0
449.1687	499.23585	443.90805	434.06165	428.38265	484.23455
449.2409	0	0	17.7761	21.3276	20.22905
450.1791	0	26.2328	41.08795	26.03365	28.6748
455.2581	0	26.41225	23.98605	18.69265	6.692
462.2767	0	332.01535	50.6701	353.97845	60.6214
463.1551	83.92625	176.8724	297.9822	101.82535	112.9942
479.268	0	0	30.7537	8.75415	20.465
484.2621	1.06975	25.4272	9.34895	32.7802	0
499.1929	201.93055	349.1433	402.75595	359.4072	302.70605
500.2063	0	11.11965	38.6002	23.16085	19.6491
519.196	15.8191	30.46855	91.55755	36.3143	39.0682
533.1653	0	0	22.9929	6.63395	0
535.1834	310.76855	415.40265	434.89125	385.70085	411.3393
536.1949	0	27.4331	58.3057	25.7023	26.12635
539.2026	0	11.62995	49.98595	29.74405	27.425
543.2769	0	0	0	0	0
561.2891	0	0	0	0	0
563.3079	0	0	0	0	2.2754
568.3913	15.96305	0	0	19.43985	15.5322
571.3269	7.2866	12.1442	20.54305	12.47995	18.11285
573.3471	0	0	18.5347	4.8997	10.5366
589.3396	5.59145	26.9916	28.54495	8.0434	11.70755
593.3519	37.89035	22.77305	0	0	19.7649
593.3588	40.389	22.77305	31.6112	58.3365	24.02335
593.3627	0	18.92175	27.9986	20.62615	24.92565
593.3725	0	0	0	0	16.31475
594.3578	20.64905	11.708	0	0.1083	0.0824
595.2646	0	0.14905	0.8424	1.72565	4.21
656.3568	0	0	0	23.9243	13.7642
675.3993	0	0	0	0	0
689.3795	3.82745	16.4151	0	3.17305	6.8534
691.3858	0	87.5012	87.9629	53.7702	72.6685
692.3979	2.8443	12.7121	0	4.9801	12.27705
708.4041	169.969	439.17315	382.8501	355.7386	437.2985
709.4125	13.87875	71.19025	71.07525	46.6266	61.28185
711.3651	6.3097	10.26775	2.4802	2.2323	11.1604
713.3682	98.99095	194.5882	183.22675	114.8461	171.27555
714.3755	14.93985	26.2107	23.16755	20.01905	26.4614
715.3881	0	34.603	36.08195	28.46945	32.7629
715.3966	0	0	3.89495	0	4.73685
1099.5257	0	0	2.3983	7.8957	13.1059
1099.7682	0	0	4.3737	28.32045	19.7078
1100.0199	0	0	2.67475	23.01335	16.86755
1100.2791	0	0	1.1245	7.042	10.67235

Table 3: *m/*z values accquired from the LC-MS spectra of *C. cardunculum* leaf samples. The chromatographic peak intensity values are means of 2 technical replicates.

m/z	CC70DAS_1	CC70DAS_2	CC70DAS_3	CC70DAS_4	CC70DAS_5
102.063	0	2.3115	2.72395	0	3.39835
120.0822	500.05915	363.86215	438.56755	367.699	366.3532
121.1	32.5844	21.73765	16.17645	15.0096	16.8075
136.082	147.6555	96.56415	45.29285	82.31435	72.7255
144.1149	0	4.83545	5.4742	0	0
144.1226	0	0	0.4967	0.3035	0
146.0821	87.9476	55.8212	30.64425	37.579	54.1625
147.0671	62.5831	28.10755	39.5789	37.02225	28.88335
158.1804	50.7715	31.44505	48.13045	32.1392	27.972
163.0527	543.76285	538.07465	644.55405	560.27505	553.15055
163.0669	41.43175	112.1941	79.03975	57.3365	79.87605
164.0701	11.69365	22.92635	19.7973	25.4715	27.93695
166.1085	139.7113	96.89745	65.5561	64.3329	73.60445
179.137	60.15285	49.23085	45.39875	46.5584	46.40755
181.1308	105.1931	233.80955	264.33155	335.00005	299.8948
181.147	353.1802	243.21335	202.1542	145.4204	163.54285
188.0887	637.38185	458.6162	464.9956	436.37325	460.0286
189.1678	64.25415	95.76015	97.3799	83.41835	158.60425
190.187	0.7276	1.60375	1.6707	1.44165	3.7602
193.1213	11.5086	7.35615	17.27755	17.6668	26.81325
197.1434	543.2494	409.07445	454.0034	406.4435	412.14025
199.1256	119.67835	212.73945	284.7529	337.3018	276.3173
205.1834	1.4622	1.40045	0.37755	1.48765	0.22005
209.137	16.5159	23.47415	30.44165	31.0467	29.33265
209.1882	96.54375	68.395	66.49045	46.0477	64.0363
212.1522	89.3549	38.7177	226.27375	52.43405	72.22455
215.2006	21.328	21.02045	25.0628	20.2027	14.39375
226.1587	11.00425	3.8483	5.9166	4.49055	5.2328
227.1301	686.96225	550.02695	685.1824	570.84045	558.6946
227.171	0	0.6699	1.0545	1.56345	0.78235
228.1496	35.1195	61.14785	61.3776	68.2117	67.56825
229.1611	13.35275	23.19505	0	6.69815	13.8875
229.1621	43.40045	30.7323	33.74395	47.53895	49.8187
229.163	13.3262	22.9368	25.5348	21.7534	18.95455
245.1591	58.05075	112.0205	117.38275	128.8937	126.6682
245.224	6.6545	5.6756	6.21155	2.5925	1.4669
247.1751	38.4225	30.92755	10.1841	8.67015	18.45855
256.0988	0.4994	0	0	0	0
257.2309	23.92405	14.2056	13.0109	8.1015	9.47695
271.1018	156.55605	86.656	154.13495	64.19865	55.3276
275.2451	81.3278	47.8509	48.92105	18.12945	18.8533
287.0898	676.5748	514.8069	599.40325	345.07505	388.93845
287.0977	325.70395	168.991	80.99815	374.17115	205.7967
287.0978	0	0	0	14.17445	4.3074
288.1014	0	25.3665	0	8.34795	2.8115
291.2428	68.2463	28.56455	20.50535	9.31555	11.75615
298.1447	98.06625	64.96675	28.05435	53.2556	50.92825
301.1719	5.0261	17.07895	3.68185	7.73845	30.71325
310.1715	250.6668	87.5064	227.74195	44.2646	64.14945
325.2704	0	0	0	0	0
355.1542	9.3794	17.0139	14.30155	21.41445	24.2395
364.2324	46.6338	90.21705	86.91415	91.4525	110.2196
371.195	32.70425	27.02335	33.57705	34.1388	32.951
377.1342	171.04585	487.4428	498.95915	529.773	521.92185
377.202	44.429	27.75815	0	0	8.9466
378.1526	8.0313	19.6659	17.8987	26.8412	26.8909
395.1918	105.7186	83.0287	177.76345	167.5328	231.4473
399.2582	137.0724	128.35905	158.4349	117.55475	74.68395
409.2489	44.74755	41.66065	38.2952	38.96265	42.70245

Cont. Table 3: *m/z* values accquired from the LC-MS spectra of *C. cardunculum* leaf samples.

411.259	202.05615	126.7361	130.98175	83.95705	132.1941
432.2734	12.12045	48.80345	47.98655	40.47475	50.26285
443.2987	0	0	0	0	74.2596
444.2738	3.631	29.2467	34.07315	26.8348	25.23605
447.1618	44.38635	37.0974	0	33.048	22.1969
449.1659	0	0	0	0	0
449.1687	558.82005	349.899	152.9792	483.9955	396.9814
449.2409	0	0	0	0	0
450.1791	31.69865	0	2.77005	30.87245	10.69265
455.2581	0	33.05145	0	11.3522	29.989
462.2767	4.02205	50.15815	135.98885	54.4626	47.38505
463.1551	166.9956	313.4937	83.6885	134.3794	107.75775
479.268	31.99235	31.8043	17.1575	26.92955	28.89415
484.2621	0	0	0	0	0
499.1929	282.51295	541.2237	555.01005	475.7175	495.2702
500.2063	22.7723	84.07095	43.0199	38.5512	47.8159
519.196	69.8023	40.69045	58.1764	74.23705	70.4706
533.1653	0	0	1.0854	0	0
535.1834	446.08055	403.35625	229.50425	423.26675	373.3125
536.1949	12.9	28.14735	0	30.1931	24.27545
539.2026	24.8555	97.0038	67.7899	57.07385	65.92875
543.2769	16.7281	15.7387	23.6398	36.49895	31.8717
561.2891	0	34.5338	16.1491	46.01175	57.5743
563.3079	7.87005	12.4699	19.66285	23.43015	21.94875
568.3913	14.58475	11.41885	48.0123	6.47565	12.8478
571.3269	19.9156	37.01715	29.33915	43.8504	40.776
573.3471	10.6997	35.55035	31.46995	36.7472	38.15015
589.3396	23.05305	55.72755	43.84375	63.0753	56.4679
593.3519	0	0	0	0	0
593.3588	32.9383	14.25615	43.1928	0	0
593.3627	15.9892	12.47435	46.9014	0	0
593.3725	44.4333	16.9795	65.72585	1.7387	0
594.3578	0	6.8395	0	1.55545	0
595.2646	2.425	54.4421	4.2471	3.784	1.95015
656.3568	30.92305	18.93735	15.82845	19.74905	33.5172
675.3993	6.8949	11.91805	18.37045	25.1738	26.4687
689.3795	13.6833	22.53845	20.43005	35.79855	36.22605
691.3858	59.3305	132.5866	122.5042	161.1364	168.00685
692.3979	10.55695	19.035	21.095	24.13895	25.434
708.4041	387.89085	488.34745	582.40865	519.8173	515.6354
709.4125	49.65055	101.1102	97.8735	114.54125	120.5826
711.3651	11.43615	13.6915	9.81215	29.2511	27.87865
713.3682	185.2444	340.1226	285.70395	386.0283	377.2128
714.3755	29.7351	38.3747	38.47435	47.8347	45.7809
715.3881	28.5279	71.0695	54.67835	83.9039	85.2501
715.3966	3.5179	19.4235	17.34885	27.14745	25.6503
1099.5257	31.25265	32.7949	5.98955	37.7153	30.849
1099.7682	51.6218	47.06695	0	54.69705	44.31775
1100.0199	40.8271	39.29105	7.4759	44.27735	39.01535
1100.2791	24.2453	22.92885	3.7839	24.25455	22.48325

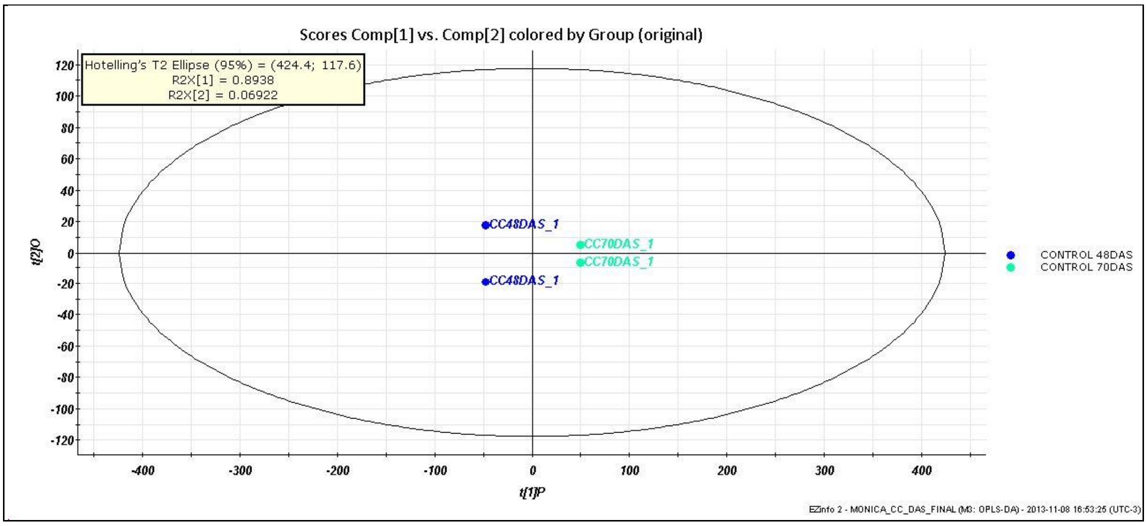

Figure 5: PCA generated by the MassLinx software for the LC-MS analysis of *C. cardunculum* leaf control samples collected after 48 DAS (*CC48DAS_1*) and 70 DAS (*CC70DAS_1*).

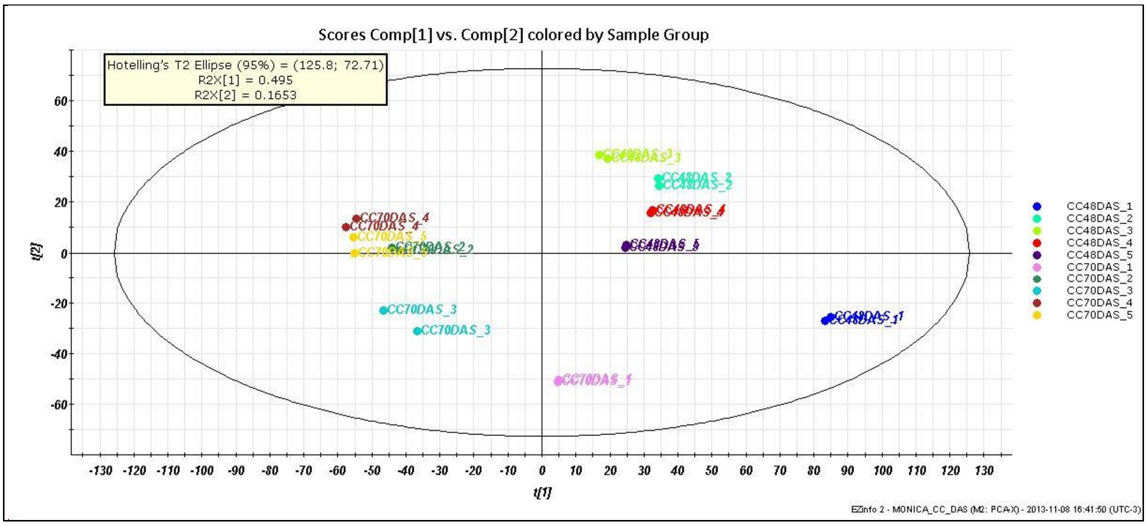

Figure 6: PCA generated by the MassLinx software for the LC-MS analysis of *C. cardunculum* leaf samples collected after 48 DAS and 70 DAS. (*CC48DAS_1, CC70DAS_1= control samples for 48DAS and 70 DAS; CC48DAS_2, CC70DAS_2 = NaCl 60mM; CC48DAS_3, CC70DAS_3= NaCl 30 mM; CC48DAS_4, CC70DAS_4= NaCl 30 mM +* $CaCl_2$ *20 mM; CC48DAS_5, CC70DAS_5 = NaCl 15 mM +* $CaCl_2$ *10 mM).*

Conclusions:

This study, in addition to contributing to the knowledge of the polyol composition of *Cynara cardunculum* plants, as a potential source for production of biomass for biofouels, provide useful agronomic information about the right time to harvest the plants in order to maximize the quality and quantity of xylitol for both food and non-food applications. Moreover, the metabolomic approach can be potentially applied to the rapid and accurate analysis of the metabolomic composition of *C. cardunculum* as a promissing source of rich biomass for biofuels.

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