Article Type: Full Paper

Selected Cardoon (*Cynara cardunculus* L.) Genotypes Suitable for PDO cheeses in Mediterranean Regions

Paulo Barracosa,*[a,b,c] Nuno Rosa,[d], Marlene Barros[d] and Euclides Pires[e]

[a]Escola Superior Agrária de Viseu – Instituto Politécnico de Viseu, Portugal; pbarracosa@esav.ipv.pt. [b]CI&DETS – Centro de Estudos em Educação, Tecnologias e Saúde, 3504-510 Viseu, Portugal. [c]Centro de Investigação e de Tecnologias Agroambientais e Biológicas (CITAB), Universidade de Trás-os-Montes e Alto Douro, Vila Real, Portugal. [d]Instituto de Ciências da Saúde, Universidade Católica Portuguesa- Pólo de Viseu, Portugal. [e]Departamento Ciências da Vida - FCTUC, Universidade de Coimbra, Portugal.

Abstract

Cardoon flower extract is a traditional and exclusive rennet used for some PDO cheeses in several Mediterranean regions, due its extremely high concentration in cardosins. In this preliminary study, six individual cardoon genotypes (1M-6M) were selected because they revealed a wide and consistent diversity of total and specific cardosin concentrations in flowers. During three growing seasons the stability of twelve biochemical characteristics of flower extracts and twenty-six plant morphological descriptors was confirmed. Surprisingly, the cardosin profiles of each genotype, based on four main groups A0, A1, A and B, were stable during the annual flower harvesting period and over all three years using ion-exchange chromatography and native-PAGE electrophoresis. This knowledge will allow an improvement in the quality and standardization of cardosin profiles from cardoon flowers used for cheese production and other innovative applications. The results obtained are promising for

development of a plant breeding program based on biochemical and morphological characteristics in order to obtain the most adapted plant architecture for combined purposes related to specific cardosins composition, flower and plant biomass production, and ease of harvesting.

Key words: *Cynara cardunculus* L., proteins, biological chemistry, "Serra da Estrela" PDO cheese, CARDOP.

Introduction

Cynara cardunculus L. belongs to the cardueae tribe Cass. (Cynarae Less.), family Asteraceae Dumortier and is native to the Mediterranean Basin. Being a cross-pollinated diploid species (2n=2x=34) with proterandrous and asynchronous sexual maturity, *C. cardunculus* harbours a highly heterozygous genetic background. [1][2] The wild cardoon [var. *sylvestris* (Lamk) Fiori] has been recognized as the ancestor of both the globe artichoke [var. *sativa* Moris, var. *scolymus* (L.), ssp. *scolymus* (L.) Hegi] and the leafy or cultivated cardoon (var. *altilis* DC), confirmed by isoenzyme[3] and molecular studies.[4] The domestication of these crops is not yet completely understood, however the molecular data suggest that the cultivated cardoon was domesticated in the Western Mediterranean and the artichoke in the East.[4] Hybridization experiments demonstrated that wild cardoon and cultivated species are genetically cohesive since they are completely interfertile and, therefore, they belong to the primary gene pool (GP1).[5]

Several studies have been conducted to evaluate the biodiversity of *C. cardunculus* L. based on morphological, biochemical and molecular analysis. Morphological studies showed differences for biomass production between cultivated and wild cardoon.[6][7] There have been some studies based exclusively on the characterization of protein diversity in wild cardoon flowers.[8][9] Lahoz *et al.*[10] by the combination of morphological and biochemical traits have

shown that the variability of individual bioactive compounds is dependent on specific genotypes, plant organs, environmental conditions and harvesting time.

Cardoon is actually a multipurpose and versatile crop with a wide spectrum of potential applications.[11][12] It helps to conserve the fragile agro-systems of the Mediterranean area with positive effects on the environment through water management and soil erosion control.[13] Cardoon flowers from the genus *Cynara* are traditionally used in the Mediterranean region, as a compulsory coagulant, for production of artisanal cheeses with protected designations of origin (PDO).[14][15] Moreover, consumer constraints on the use of animal and biotechnological rennets for religious reasons, diet (e.g., vegetarianism) or opposition to genetically engineered foods have prompted study of these enzymes as rennet substitutes, [14] with high potential to increase their uses in the future. However, previous studies reported heterogeneity of crude extracts obtained from cardoon flowers which could limit their use in applications requiring a standard quality.[16]

Cynara cardunculus L. produces two identified groups of typical plant APs, cardosins and cyprosins in mature flowers. Cardosins have been purified and characterized from fresh stigmas [17] and cyprosins from dried flower extracts.[18] Cardosins represent about 70% of the total protein content in cardoon flowers [19] and nine APs have been biochemically characterized in cardoon, which is one of the highest numbers of APs purified from a single organism.[20] The last four cardosins (E-H) purified and characterized, resemble cardosin A and were previously described as cardosin A0 group. At acidic pH between 75% and 90% of total extracted enzyme activity correspond to cardosin A, which in terms of specificity and kinetic parameters proved to be similar to chymosin, whereas cardosin B was similar to pepsin.[21]

The main objective of the present study, included in the CARDOP, a project designed to valorize cardoon genetic resources for PDO cheese production, was to evaluate six selected (1M-6M) genotypes of cardoon from the “Serra da Estrela” region, based on the diversity

and stability of cardosins from the flower, complemented with a morphological analysis, over three growing seasons. To increase the profitability of this multipurpose species, along with the cardosin characterization the plant architecture was also evaluated in order to use all these characteristics in future plant breeding programs.

Results

Cardosin analysis

These six cardoon genotypes were selected for this study because they displayed a wide diversity of cardosins characteristics, as obtained from crude extracts of flowers (*Table 1, Figure 1*). In molecular exclusion chromatography the highest peak area of total cardosins was obtained in genotype 6M (18742 mAU*ml) with a protein concentration of 4000 µg/ml. The lowest peak area value and concentration of total cardosins were registered in genotype 2M (var. *sylvestris*) with 9644 mAU*ml and 1700 µg/ml, respectively (*Table 1*). The partially purified preparation of total cardosins obtained upon gel filtration was fractionated into four main peaks by ion-exchange chromatography in a HiPrepQ FF column (GE Healthcare) and all peaks revealed proteolytic and milk clotting activity (data not shown). The first peak, eluted at 40 ml, is probably related with non-processed forms of cardosins. The second peak, eluted at 75 ml, contained different forms of cardosin A0. The third peak, eluted at 110 ml, refers to cardosin A and the last peak, eluted at 150 ml, relates to cardosin B. All peaks, except the first, were previously biochemically identified and characterized in cardoon flowers. In this study, over the three-year experiment, specific flower profiles were consistently maintained from each selected genotype based on the presence/absence, relative proportion and concentration of each peak of cardosins (*Figure 1, 2*).

Figure 1. Profiles of ion-exchange chromatography on HiPrep Q FF (GE Healthcare) of cardosins peaks (A1, A0, A and B) obtained from the genotypes 1M-6M (A-F).

Table 1. Biochemical characteristics obtained in *C. cardunculus* L. with mean values, standard deviation and range in the six genotypes (1M-6M)(2012/3-2014/5).

Biochemical characteristics	Code	1M	2M	3M	4M	5M	6M	Total	Range
		Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	(Max- min)
1. Molecular exclusion area (mAU*ml)	MeA	15280±327	9644±872	14958±853	14938±842	13282±2319	18742±180	14404±2999	20340-8791
2. Molecular exclusion height (mAU)	MeH	925±182	576±85	856±38	844±35	771±176	1096±141	846±193	1223-506
3. Mol. Exclusion abs. top (280 nm)	MeAT	0.417±0.0	0.338±0.02	0.503±0.0	0.558±0.0	0.539±0.05	0.585±0.1	0.490±0.10	0.69-0.32
4. Mol. Exclusion abs. average (280 nm)	MeAM	0.171±0.0	0.168±0.02	0.222±0.0	0.340±0.0	0.310±0.04	0.370±0.0	0.263±0.09	0.46-0.15
5. Ion-exchange area A1 (mAU*ml)	IEArA1	782±92	836±330	898±70	420±127	271±89	282±112	582±304	1195-180
6. Ion-exchange height A1 (mAU)	IEHA1	45±2	51±14	51±6	30±5	17±3	18±6	35±16	67-11
6. Ion-exchange area A0 (mAU*ml)	IEArA0	280±38	288±51	1289±169	369±136	1037±65	273±134	589±435	1416-153
8. Ion-exchange height A0 (mAU)	IEHA0	21±6	19±1	64±12	27±15	56±5	20±9	35±20	75-13
9. Ion-exchange area A (mAU*ml)	IEArA	0±0	0±0	0±0	931±33	729±66	1969±187	605±740	2169-0
10. Ion-exchange height A (mAU*ml)	IEHA	0±0	0±0	0±0	58±1	52±7	98±11	35±39	109-0
11. Ion-exchange area B (mAU*ml)	IEArB	145±17	207±9	144±20	148±8	137±17	218±19	166±36	238-124
12. Ion-exchange height B (mAU*ml)	IEHB	11±3	14±2	10±2	12±1	11±1	16±2	12±2	18-8

(mAU – milli Absorbance Units)

All three genotypes (1M, 2M and 3M) revealed ion-exchange chromatographic profiles where the common characteristic was the total absence of the cardosin A peak in the regular retention volume. Genotypes 4M, 5M and 6M reveals ion-exchange chromatographic profiles which presented the typical peak of cardosin A. The genotype 6M showed a clear prevalence of cardosin A peak compared with the other peaks.

Figure 2. Absorbance values from the six genotypes (1M-6M). Total cardosins obtained by molecular exclusion chromatography from crude extracts of flowers by genotype (left); Proportion of specific cardosins peaks obtained by genotype in the ion-exchange on HiPrep Q FF (GE Healthcare) (right).

In the electrophoretic characterization of the six genotypes, five bands could be discriminated by native-PAGE electrophoresis (*Figure 3*). The two superior bands correspond to the first peak of ion-exchange chromatography and were more intense in the genotypes 1M, 2M, 3M and 4M. The three lower bands are relative to the peaks of cardosins A0, B and A, respectively. Genotypes 1M, 3M and 5M presented an intense band relative to cardosins A0. For cardosin A the genotypes 4M, 5M and 6M revealed the respective bands with higher intensity. It is also possible to see that genotypes 1M, 2M and 3M do not show the characteristic band of cardosin A.

Figure 3. A) Electrophoretic characterization of molecular exclusion chromatography from genotypes 1M-6M by native-PAGE: lane1–1M; lane 2–2M, lane 3–3M, lane 4-4M; lane 5-5M; lane 6-6M. B) Electrophoretic characterization of flowers extract (EX), molecular exclusion (EM) and ion-exchange chromatography from first peak (A1), cardosins A0, cardosin A and cardosin B.

Morphological analysis

The cardoon plants evaluated showed a wide biodiversity on morphological characteristics (*Table 2*). The main interest of this morphological analysis was related to plant characteristics that can directly influence the production of the number and dimensions

Table 2. Morphological characteristics obtained in *C. cardunculus* L. with mean values, standard deviation and range in the six genotypes (1M-6M)(2012/3-2014/5).

2014/5).Plant descriptor	Code	**1M**	**2M**	**3M**	**4M**	**5M**	**6M**	**Total**	**Total Range**
		Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	Mean ±SD	(Max- min)
1. Plant height (cm)	PH	243.3±30.64	148.3±25.7	200.0±40.0	170.0±40.0	226.7±23.1	208.3±27.5	199.4±43.3	270-120
2. Nº of stems	NS	6.0±1.0	1.7±0.6	5.0±0.0	5.0±0	2.0±1.0	2.0±1.7	3.6±2.4	9-1
3. Nº of primary ramifications	RpN	4.0±1.0	3.3±0.6	4.0±1.0	4.0±1.0	3.7±0.6	2.7±1.2	3.7±1.0	5-2
4. Nº of secondary ramifications	RsN	8.3±2.1	2.3±2.1	7.0±5.6	7.0±5.6	20.3±14.7	4.0±4.4	8.4±8.2	37-0
5. Total nº of inflorescences	TNI	57.3±2.3	6.7±3.8	36.0±26.9	38.0±15.7	45.7±23.4	22.0±30.3	34.3±24.9	81-4
6. Primary ramification height (cm)	PPrH	195.0±30.49	112.7±26.1	120.0±40.0	46.7±5.8	98.3±14.4	156.7±10.4	121.6±51.8	215-40
7. Diameter stem top (mm)	SDT	11.4±1.1	9.8±0.4	11.0±1.1	12.4±2.5	12.3±2.2	10.9±0.4	11.4±1.7	14.9-9.5
8. Diameter stem base (mm)	SDB	29.1±4.9	19.1±1.8	32.2±2.5	30.2±14.2	43.7±11.4	23.5±6.9	29.6±10.5	56.9-15.1
9. Spine length (mm)	SpL	2.0±0	21.7±4.9	9.3±0.6	2.0±1.0	2.0±1.0	2.0±0	6.5±7.7	25-1
10. Leaf length (cm)	LL	90.3±6.8	72.0±13.0	93.3±5.8	95.3±4.2	102.7±4.6	74.7±16.7	88.1±14.0	108-57
11. Leaf width (cm)	LW	42.0±2.6	33.0±5.2	46.0±3.5	50.0±4.4	55.3±2.5	40.3±8.1	44.4±8.4	58-27
12. Primary lobes length (cm)	LpL	21.8±2.6	15.5±3.0	25.8±2.5	23.3±5.5	30.7±1.5	20.7±4.5	23.0±5.6	32-13.5
13. Primary lobes width (cm)	LpW	9.0±2.0	5.3±1.2	10.3±0.3	7.2±2.8	13.7±4.0	7.8±1.9	8.9±3.4	16-4
14. Secondary lobules length (cm)	LsL	5.7±1.8	4.5±2.3	7.5±0.5	4.8±1.5	8.6±1.8	7.6±2.8	6.4±2.3	9.8-2.5
15. Secondary lobules width (cm)	LsW	1.7±0.3	1.0±0.1	1.9±0.1	1.6±0.6	2.6±0.6	2.2±1.1	1.8±0.7	3.2-0.9
16. Petiole width (mm)	LPtW	13.2±2.6	9.0±1.6	15.4±2.5	16.1±2.3	23.4±1.4	12.1±6.3	14.9±5.3	25-7.1
17. Petiole thickness (mm)	LPtT	17.4±1.2	9.9±1.6	18.2±4.6	18.6±3.0	23.4±2.8	11.7±2.6	16.5±5.2	25.7-8.2
18. Leaf weight (g)	LWg	75.8±10.9	26.7±5.7	85.8±34.2	53.9±13.5	119.9±5.9	45.2±18.9	67.9±34.6	126.6-22.4
19. Lobe weight (g)	LbWg	3.1±0.2	1.1±0.5	4.2±1.2	3.2±0.8	6.8±1.2	4.4±2.8	3.8±2.1	7.5-0.5
20. Petiole weight (g)	LPtWg	49.0±3.1	17.4±8.7	50.1±22.7	31.3±10.1	92.5±25.8	23.2±12.7	43.9±28.9	113.6-9.3
21. Inflorescence length (mm)	IpL	56.6±11.9	52.7±1.9	64.0±8.0	66.0±12.1	62.6±6.9	56.1±4.6	59.7±8.6	77.9-44.7
22. Inflorescence diameter (mm)	IpD	62.3±15.0	55.0±12.3	66.3±16.4	74.1±27.1	88.5±11.7	73.4±2.3	69.9±17.2	97.6-41.4

23. Achene length (mm)	AcL	7.76±0.32	6.39±0.17	7.55±0.42	8.05±0.47	7.37±0.26	7.56±0.34	7.51±0.58	9.30-6.10
24. Achene width (mm)	AcW	4.35±0.24	3.98±0.29	3.68±0.32	3.49±0.22	4.07±0.23	4.40±0.21	3.95±0.44	4.98-2.71
25. Achene thickness (mm)	AcT	3.04±0.20	2.71±0.16	2.65±0.27	2.73±0.23	2.84±0.25	2.81±0.19	2.80±0.28	3.74-2.02
26. Achene weight (g)	AcWg	0.060±0.006	0.046±0.005	0.049±0.009	0.050±0.006	0.054±0.006	0.061±0.007	0.054±0.01	0.075-0.028

of inflorescences and the harvesting procedure. Genotype 1M presented the highest plant height (PH) (243.3 mm) with highest number of stems (NS) (6) and number of inflorescences (TNI) (57.3), while the genotype 2M (var. *sylvestris*) presented, simultaneously, the lowest PH (148.3 mm), NS (1.7) and TNI (6.7).

A wide variation was registered on the stem base diameter (SDB) from 19.1 mm (genotype 2M) to 43.7mm (5M). The number of secondary ramifications (RsN) also presented a wide range between 2 (genotype 2M) and 20 (5M). The genotypes 4M and 5M showed the largest inflorescences. Three classes of spine length (SpL) were observed. The genotype 2M, which represents a typical wild cardoon, showed long spines with 21.7 mm, genotype 3M had medium spines of 9.3 mm and the remaining genotypes presented short spines of 2 mm. The longest, widest and heaviest leaves were registered in the genotype 5M, as well the longest and heaviest petioles. Achenes (seeds) presented a wide variation in length, width, thickness and weight between genotypes. The genotype 4M presented the longest (8.05 mm) and thinnest (3.49 mm) achenes. The thickest achenes were achieved in genotype 1M (3.04 mm). The heaviest achenes were observed in 6M (0.061g) and the lightest in 2M (0.046g).

NTSYS analysis

Principal component analysis (PCA) based on 12 biochemical flower extracts and 26 morphological characteristics on the planes defined by the components 1 and 2 tends to group the three growing seasons (2012/3-2014/5) of each genotype (1M-6M) *(Figure 4*). The eigen values indicated that three components provided a good summary of the data accounting for 67% of the variance (*Table 3*).

The first component (PC1), with 36%, was dominated positively by plant characteristics, namely, plant height (PH), secondary ramifications (RsN), total number of inflorescences (TNI), stalk diameter at the base (SDB), leaf length (LL), leaf width (LW), primary lobe length (LpL) and width (LpW), secondary lobules length (LsL) and width (LsW), petiole width (LPtW) and thickness (LPtT), weights of leaf (LWg), lobe (LbWg) and pet-

iole (LPtWg), inflorescence diameter (IpD) and negatively by spine length (SpL) and stalk diameter at the top (SDT).

The second component (PC2), accounting for 21% of the total variance, is dominated by cardosin characteristics, positively by ion-exchange area of the peak A1 (IEA1) and negatively by exclusion molecular area (MeA) and height of total cardosin peak (MeH), ion-exchange area (IEArA) and height of cardosins A (IEHA) and B peaks (IEHB).

All accessions of genotype 5M (2013-15), presented the highest plant biomass at stem, leaf and inflorescence levels. The accessions of genotype 2M (2013-15), placed on the left upper quadrant presented the lowest plant biomass and longest spines. All accessions of genotype 6M (2013-15) are placed in the lower quadrants and presented the highest content of total cardosins, especially of cardosin A, followed by the accessions 4M. Genotypes 1M, 2M and 3M are placed in an upper position of the projection due the absence of cardosin A and higher content of cardosins from the first ion-exchange peak (A1).

Table 3. Factor loadings for each variable on the components of PCA analysis.

Variable	Code	PC1	PC2	PC3
1. Exclusion molecular area (mAU*ml)	MeA	0.31	**-0.74**	0.25
2. Exclusion molecular height (mAU)	MeH	0.17	**-0.67**	0.23
3. Excl molecular abs. top (280 nm)	MeAT	**0.65**	-0.59	-0.27
4. Excl molecular abs. average (280 nm)	MeAM	0.46	**-0.73**	-0.40
5. Ion-exchange area A1 (mAU*ml)	IEArA1	-0.40	0.58	0.26
6. Ion-exchange heigh A1 (mAU)	IEHA1	-0.47	**0.63**	0.17
7. Ion-exchange area A0 (mAU* ml)	IEArA0	0.53	0.45	-0.34
8. Ion-exchange heigh A0 (mAU)	IEHA0	0.57	0.40	-0.32
9. Ion-exchange area A (mAU*l)	IEArA	0.18	**-0.94**	-0.16
10. Ion-exchange heigh A (mAU*ml)	IEHA	0.28	**-0.89**	-0.23
11. Ion-exchange area B (mAU*ml)	IEArB	**-0.66**	-0.50	-0.05
12. Ion-exchange heigh B (mAU*ml)	IEHB	-0.49	**-0.66**	-0.13
1. Plant height (cm)	PH	**0.60**	0.01	**0.64**
2. Nº of stems	NS	0.17	0.20	0.38
3. Nº of primary ramifications	RpN	0.33	0.48	0.18
4. Nº of secondary ramifications	RsN	**0.76**	0.26	0.03
5. Total nº of inflorescences	TNI	**0.65**	0.19	0.43
6. Primary ramification height (cm)	PrH	-0.15	-0.06	**0.85**
7. Diameter stem top (mm)	SDT	**-0.67**	0.52	-0.19
8. Diameter stem base (mm)	SDB	**0.89**	0.24	0.07
9. Spine length (mm)	SpL	**-0.67**	0.52	-0.19
10. Leaf length (cm)	LL	**0.82**	0.27	-0.14
11. Leaf width (cm)	LW	**0.93**	0.03	-0.21
12. Primary lobes length (mm)	LpL	**0.94**	0.12	-0.06
13. Primary lobes width (mm)	LpW	**0.86**	0.24	0.09
14. Secondary lobules length (mm)	LsL	**0.67**	-0.10	0.00
15. Secondary lobules width (mm)	LsW	**0.76**	-0.22	0.08
16. Petiole width (mm)	LPtW	**0.91**	0.12	-0.24
17. Petiole thickness (mm)	LPtT	**0.87**	0.12	-0.24
18. Leaf weight (g)	LWg	**0.84**	0.27	0.00
19. Lobe weight (g)	LbWg	**0.80**	-0.16	-0.05
20. Petiole weight (g)	LPtWg	**0.80**	0.29	-0.04
21. Inflorescence length (mm)	IpL	0.58	0.05	-0.16
22. Inflorescence diameter (mm)	IpD	**0.68**	-0.11	0.08
23. Achene length (mm)	AcL	0.56	-0.39	0.19
24. Achene width (mm)	AcW	-0.05	-0.27	**0.71**
25. Achene thickness (mm)	AcT	0.19	-0.06	**0.77**
26. Achene weight (g)	AcWg	0.23	-0.46	**0.64**
Eigen values		**36**	**21**	**10**
% of variance		**11**	**8**	**7**
Cumulative %		**36**	**57**	**67**

Factor loadings ≥ |0.6| are in bold

Figure 4. Projection of the six genotypes (1M-6M) from the years of 2012/3-2014/5 (13-15) based on twelve cardosins characteristics and twenty-six morphological in the plan defined by the principal components 1-2.

Discussion

This study evaluated and characterized specially the diversity of cardosins as a basis for the selection of cardoon genotypes well adapted for traditional and innovative applications in the Mediterranean region. Despite all previous studies finding that cardosin A is clearly dominant over the others, [19] [21] [22] in our study we obtained, four main groups of cardosins with a wide diversity in concentration and proportion between them. Three of the genotypes, namely 1M, 2M and 3M do not reveal the peak of cardosin A at the normal retention volume in ion-exchange chromatography. The first peak of ion-exchange chromatography (A1), which presents one or two upper electrophoretic bands in native conditions, probably refers to non-processed forms of cardosin with Plant Specific Insert (PSI) and prosegment. These forms were not previously characterized in cardoon flowers,[23] [24] but only in seeds and leaves.[25] In these six genotypes, a reduction on the intensity of the upper bands, was observed along with an increase in the intensity of the lower bands corresponding to active forms of cardosins, namely A0, A and B, which seems to confirm the conversion of the precursor forms into activated cardosins. In our study each genotype, surprisingly, maintained a similar cardosins profile in its flowers throughout the harvest period, between May and July, and all over the three growing seasons revealing great stability.

The morphological analysis was based on plant characteristics that directly influence the production of inflorescences (number, dimensions and consequently the weight of the flowers), those that could influence the ease of harvesting of the flowers (plant height, dimension of spines in the stem, leaves and inflorescence), and those related to the general architecture of the plant (the number of ramifications and the height of the lower branching in the main stem). Genotypes 1M and 5M were the tallest plants with the highest number of inflorescences, however both the plant architecture as well the cardosins ion-exchange profiles differed. The genotype 1M lacked the presence of the cardosin A peak and showed a plant architecture defined by high number of stalks of medium diameter which produced a high total number of inflorescences. Genotype 5M

had the cardosin A peak and showed precocious flowering with a reduced number of stems with high diameter and the largest inflorescences. Genotype 2M, a typical wild cardoon, produced the plants with the lowest height and low number of small inflorescences with the longest spines. To combine the flower production with biomass production the best plant architecture should have a height of two meters and a number of primary and secondary ramifications that would give a total of fifty inflorescences in three or four stems per plant. The stem diameter at the base relates to the number of ramifications and inflorescences. The plant should present reduced spine or total absence to facilitate the harvesting procedure.

In the PCA projection the accessions 4M, 5M and 6M, placed in the right lower quadrant, presented the highest biomass production and cardosin concentration, especially cardosins A and B. On the contrary, in the upper left quadrant, accession 2M (var. *sylvestris*) has low biomass production, longer spines with low total cardosin concentrations, and a higher quantity of non-processed forms.

Conclusions

This is to our knowledge the first study to evaluate comparatively the diversity of cardosins in cardoon flowers from specific individual plants and genotypes, over three growing seasons. It seems essential to characterize the germplasm that has been conserved in our region through time for PDO “Serra da Estrela” cheese production. These preliminary results reveal no relation between morphological and cardosin composition in flowers, which could mean that it will be possible to find genotypes with similar morphological characteristics but distinct cardosin composition and concentrations in the same edapho-climatic conditions. The stability of distinct cardosin patterns permits to obtain crude or purified extracts with specific compositions and concentrations of cardosins with the aim of reducing the heterogeneity of the vegetable coagulant from cardoon flowers used in the cheese industry and prospective other potential applications related to health promotion, pharmaceutics, cosmetics and food nutrition. It is now

possible to characterize biochemically flowers collected by farmers from wild plants or cultivated plants to create standardize lots of flowers.

The use of achenes from plants of specific genotypes does not assure the same genotypic pattern. So *in vitro* propagation or vegetative propagation, which we are developing must be used in order to produce, on a large scale, selected cardoon genotypes with specific biochemical composition and morphological characteristics. In addition it should be possible to develop a plant breeding program through mass selection to produce achenes of cardoon genotypes with the characteristics of interest for flower production.

Experimental Section

Plant Material

All six genotypes (1M-6M) selected for this study belong to cardoon (*C. cardunculus* L. var. *altilis*) with exception of 2M which is a wild cardoon (*C. cardunculus* L. var. *sylvestris*). All accessions used for biochemical and morphological characterization over three growing seasons (2012/13-2014/15) were situated at Viseu (Portugal) (40°42'17.5"N, 7°54'45.8"W) under identical soil and climatic conditions.

Cardosin Analysis

To evaluate the diversity of cardosin profiles related to composition and concentration, fresh cardoon flowers were picked from inflorescences in each of six selected genotypes (1M-6M) and immediately frozen at -80ºC until analysed. Flowers were collected in the three growing seasons between May and July during the flower maturation and twelve characteristics from flower extracts were recorded. Using molecular exclusion chromatography the area (MeA) and the height (MeH) of the total cardosins peak was measured. Two samples obtained from the top (MeAT) of this peak and from the total volume (MeAM), respectively, were measured at 280 nm. On the subsequent ion-

exchange chromatography the area (IEAr) and the height (IEH) from all four peaks specific for each cardosin groups (A1, A0, A and B) were measured (*Table 1*).

Extraction of cardosins

Cardosins were extracted and purified using a methodology based on the one described earlier [21] with minor modifications. For extraction of each cardoon accession 2 g of fresh flowers were used, homogenized in a mortar and pestle with 12 ml of sodium citrate 100 mM buffer, pH 3.0, and centrifuged for 10 minutes (13,000g). The filtered supernatant was applied to a Hiload Superdex 75 semi prep (GE Healthcare) equilibrated and eluted with 25 mM Tris–HCl buffer, pH 7.6, at a flow rate of 3 ml/min. The active fraction was further purified on a Q-Sepharose HiPrep Q FF 16/10 column (GE Healthcare), equilibrated with 25 mM Tris–HCl buffer, pH 7.6. The proteins were eluted with a gradient of NaCl (0.2–1 M) at a flow rate of 3 ml/ min.

Polyacrilamide gel electrophoresis

The protein profiles analyzed by Native-PAGE gel (12.5% separating gel) was performed in a Bio-Rad Mini Protean II electrophoresis apparatus as in Laemmli (1970), [26] and stained with Coomassie Brilliant Blue R-250 (Sigma).

Quantification of protein

Protein concentration was determined by the Micro BCA™ protein assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. A NanoView (GE) spectrophotometer was also used to determine protein A280 concentration.

Morphological traits

Twenty-six morphological characteristics were recorded at harvest time on six genotypes. The selection of traits for this specific morphological characterization was done by adapting the UPOV (International Union for the Protection of New Varieties of Plant) descriptors edited for cardoon (*Table 2*). These genotypes were characterized and

evaluated over three growing seasons (2012/13 - 2014/15) and the analysis began in the second year after planting. Plant height (PH) was measured from the soil to the highest point of the plant and the total number of offshoots per plant (SN) was obtained. The tallest stalk was selected to count the number of primary ramifications (RpN), which derive directly from it, and the secondary ramifications (RsN) considered all the others. The total number of inflorescences (TNI) was accounted from all offshoots of the plant. The stalk diameter at the base (SDB) was measured at 30 cm from the soil and the top diameter (SDT) at 15 cm below the main capitula. Leaves used for morphological characterization were collected at 40 cm from the soil. The principal inflorescences were selected to measure the largest diameter (IpD), and the length (IpL). For fruit characterization, 20 achenes, commonly known as “seeds” were selected from the main capitula in each plant.

NTSYS analysis

Mean values of thirty-eight continuous characteristics were used in the multivariate analysis in order to differentiate the genotypes (OTUs). The characteristics were standardized before computation of correlation and average distance coefficients among OTUs. A principal component analysis (PCA) which employed the DCENTER and EIGEN procedures was performed. The first two principal coordinates were used to produce a two-dimensional scatter plot to understand how each axis influenced the variation among genotypes and which characteristics are determinants for their discrimination. All computations for multivariate analysis were carried out using the NTSYS-pc version 2.02k software. [27]

Acknowledgments

This work was supported by the project PRODER-18648-02009975 (1/01/2011-31/03/2015). This work is supported by: European Investment Funds by FEDER/COMPETE/POCI– Operacional Competitiveness and Internacionalization Programme, under Project POCI-01-0145-FEDER-006958 and National Funds by FCT -

Portuguese Foundation for Science and Technology, under the project UID/AGR/04033/2013. The authors are grateful to Jimmy Shadbolt for the final linguistic revision and to Paulo Medeiros for artwork.

Compliance with ethical standards Conflict of interest. The authors declare that they have no conflict of interest.

Author Contribution Statement

P.B., M.A. and E.P. designed the work, P.B. and N.R. performed the experiments and analysed the data, P.B. and E.P. wrote the manuscript. All the authors read and approved the manuscript.

References

[1] E. Portis, L. Barchi, A. Acquadro, J.I. Macua, S. Lanteri, `Genetic diversity assessment in cultivated cardoon by AFLP (amplified fragment length polymorphism) and microsatellite markers´ *Plant Breeding* **2005**,*124*, 299 – 304.

[2] D. Scaglione, A. Acquadro, E. Portis, C. Taylor, S. Lanteri, S. Knapp,`Ontology and diversity of transcript-associated microsatellites mined from a globe artichoke EST database´ *BMC Genomics* **2009**,*10*, 454.

[3] A. Rottenberg, D. Zohary, E. Nevo,`Isozyme relationships between cultivated artichoke and the wild relatives´, *Genet. Res. Crop. Evol.* **1996**, *43*, 59 – 62.

[4] G. Sonnante, D. Pignone, K. Hammer,`The Domestication of Artichoke and Cardoon: From Roman Times to the Genomic Age´, *Annals of Botany* **2007**, *100*, 1095 – 1100.

[5] A. Rottenberg, D. Zohary,`Wild genetic resources of cultivated artichoke´ *Acta Horticulturae* **2005**, *681*, 307 – 311.

[6] S. A. Raccuia, M. G. Melilli,`*Cynara cardunculus* L. a potential source of inulin in the Mediterranean environment: screening of genetic variability´ *Aust. J. Agr. Res.* **2004**, *55*, 693 – 698.

[7] P. Barracosa, J. Oliveira, M. Barros, E. Pires,`Morphological evaluation of cardoon (*Cynara cardunculus* L.): assessing biodiversity for applications based on tradition, innovation and sustainability´ *Genet. Res. Crop. Evol.* **2018**, *65*, 17 – 28.

[8] E. Ordiales, A. Martin, M. J. Benito, A. Hérnandez, S. Ruiz-Moyano,`Technological characterization by free zone capillary electrophoresis (FCZE) of the vegetable rennet (*Cynara cardunculus*) used in "Torta del Casar"´ *Food Chem.* **2012**, 133, 227 – 235.

[9] A. Ben Amira, J. Bauwens, E. De Pauw, S. Besbes, H. Attia, F. Francis, C. Blecker, `Identification of proteins from wild cardoon flowers (*Cynara cardunculus* L.) by a proteomic approach´ *J. Chem. Biol.* **2017**, *10*, 25 – 33.

[10] I. Lahoz , J. A. Fernández , D. Migliaro, J. I. Macua, C. Egea-Gilabert, `Using molecular markers, nutritional traits and field performance data to characterize cultivated cardoon germplasm resources´ *Sci. Hortic.* **2011**, *127*, 188 – 197.

[11] J. Fernández, M. D. Curt, P. L. Aguado, `Industrial application of *Cynara cardunculus* L. for energy and other uses´, *Ind. Crop Prod.* **2006**, *24*, 222 – 229.

[12] S. Lanteri, E. Portis, `Globe Artichoke and Cardoon´ in `Vegetables I´ Ed. Springer, New York, 2008, pp. 49 – 74.

[13] P. Grammelis, A. Malliopoulou, P. Basinas, N. G. Danalatos, `Cultivation and Characterization of *Cynara cardunculus* for Solid Biofuels Production in the Mediterranean Region´ *Int. J. Mol. Sci.* **2008**, *9*, 1241 – 1258.

[14] L. B. Roseiro, M. Barbosa, J. M. Ames, R. A. Wilbey, `Cheesemaking with vegetable coagulants-the use of *Cynara* L. for the production of ovine milk cheeses´ *Int. J. Dairy Technol.* **2003**, *56*, 76 – 85.

[15] S. V. Silva, F. X. Malcata, `Studies pertaining to coagulant and proteolytic activities of plant proteases from *Cynara cardunculus´ Food Chem.* **2005**, *89*, 19 – 26.

[16] M. Sales-Gomes, M. Cavaco, M.E. Lima-Costa,`Allocation of proteolytic activity in the seedlings of Cynara cardunculus L. in the initial growth stages´ *J. Plant. Physiol*. **2008**, *165*, 1041 – 1048.

[17] P. Veríssimo, C. Esteves, C. Faro, E. Pires, `The vegetable rennet of *Cynara cardunculus* L. contains two proteinases with chymosin and pepsin-like specificities´ *Biotechnol. Lett.* **1995**, 17, 621 – 626.

[18] M. C. Cordeiro, Z. T. Xue, M. Pietrzak, M. S. Pais, P. E. Brodelius, `Isolation and characterization of a cDNA from flowers of *Cynara cardunculus* encoding cyprosin (an aspartic proteinase) and its use to study the organ-specific expression of cyprosin´ *Plant Mol. Biol.* **1994**, 24, 733 – 741.

[19] A. Oliveira, C. Pereira, D. Soares da Costa, J. Teixeira, F. Fidalgo, S. Pereira, J. Pissarra, `Characterization of aspartic proteinases in *C. cardunculus* L. callus tissue for its prospective transformation´ *Plant Science* **2010**, *178*, 140 – 146.

[20] A. C. Sarmento, H. Lopes, C. S. Oliveira, R. Vitorino, B. Samyn, K. Sergeant, G. Debyser,J. V. Beeumen, P. Domingues, F. Amado, E. Pires, M. R. M. Domingues, M. T. Barros, `Multiplicity of aspartic proteinases from *Cynara cardunculus* L.´ *Planta* **2009**, *230*, 429 – 439.

[21] P. Veríssimo, C. Faro, A. J. Moir, Y. Lin, J. Tang, E. Pires, `Purification, characterization and partial amino acid sequencing of two new aspartic proteinases from fresh flowers of *Cynara cardunculus* L.´ *Eur. J. Biochem.* **1996**, *235*, 762 – 768.

[22] M. Ramalho-Santos, P. Veríssimo, C. J. Faro, E. V. Pires, `Action on bovine αs1-casein of cardosins A and B, aspartic proteinases from the flowers of the cardoon *Cynara cardunculus* L.´, *Biochem. Biophys. Acta* **1996**, 1297, 204 – 212.

[23] M. Ramalho-Santos, J. Pissarra, P. Veríssimo, S. Pereira, R. Salema, E. Pires, C. J. Faro, `Cardosin A, an abundant aspartic proteinase, accumulates in protein storage vacuoles in the stigmatic papillae of *Cynara cardunculus* L.´, *Planta* **1997**, *203*, 204-212.

[24] M. Vieira, J. Pissarra, P. Veríssimo, P. Castanheira, Y. Costa, E. Pires, C. Faro, `Molecular cloning and characterization of the cDNA encoding cardosin B, an aspartic proteinase accumulating extracellularly in the transmitting tissue of Cynara cardunculus L.´ *Plant Mol. Biol.* 2001, 45, 529 – 539.

[25] C. S. Pereira, D. da Costa, S. Pereira, F. M. Nogueira, P. M. Albuquerque, J. Teixeira, C. Faro, J. Pissarra, `Cardosins in postembryonic development of cardoon: towards an elucidation of the biological function of plant aspartic proteinases´ *Protoplasma* **2008**, *232*, 203 – 213.

[26] U. K. Laemmli, `Cleavage of structural proteins during the assembly of the head of bacteriophage T4´ *Nature* **1970**, *227*, 680 – 685.

[27] F. J. Rohlf, NTSYS-pc, Numerical taxonomy and multivariate analysis system, Exeter Software version 2.02k, 2000.