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Proceeding Paper

Chemical and Genetic Relationship of *Cynara cardunculus* L. (Cardoon) in Southern Portugal †

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Abstract: Southern Portugal has a high natural variability of *Cynara cardunculus* L. (Cc) at a biochemical and morphological levels, conducting to the necessity of genetic diversity studies. Cc represents a natural source of sesquiterpene lactones (SL), particularly cynaropicrin. Previously, 175 wild Cc individuals (generation F0) from Alentejo different geographical locations region were identified, collected and chemically and genetically characterized. To improve the biotechnological cardoon impact, based on SL chemical profile, a transcriptomic analysis is ongoing to select the best genotypes for cynaropicrin production. This knowledge is crucial to obtain molecular markers, related to characteristics of interest, for future cardoon breeding programs.

Keywords: *Cynara cardunculus* L.; genetic diversity; cynaropicrin; transcriptome

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1. Introduction

Cynara cardunculus L. (Cc) (2n = 2x = 34) is a perennial herbaceous specie well adapted to the Mediterranean climate, that belongs to the Asteraceae (Compositae) family. It includes artichoke (var. *scolymus (*L.*)* Fiori), wild cardoon (var. *sylvestris (*Lamk*)* Fiori) and the cultivated cardoon (var. *altilis* DC) [1], each one having distinct biological characteristics.

Cc has been described with a wide spectrum of applications. Its lignocellulosic fraction has great potential as a solid biofuel [2], it can also be used to produce biogas [3,4], and bioethanol [5,6]. Cc seed oil, with its fatty acid composition, also reveals a great potential to produce biodiesel [2,7]. Cc stalks can also be used to produce cellulose fibers [2,8,9]. The inflorescence pistils are a source of aspartic proteases, namely cardosines, used for cheesemaking [10]. Cc leaves are a valuable natural source of sesquiterpene lactones (SLs), in particularly cynaropicrin (Cyn), a secondary metabolite [11,12]. Cyn has a huge biological potential capable of valorization within different industries, being pharmaceutical and biotechnological the most favorable and with higher add-value [12–16].

Previous studies from our research group, revealed a high biochemical and genetic variation profiles, within the 25 Cc F0 naturally growing populations derived from the Alentejo region [13]. Cynaropicrin chemical variation within Cc plants belonging to a

same, or different, geographic populations, in consecutive years, has not been so far addressed. Moreover, the identification of genetic markers associated to Cc secondary metabolites of industrial interest, such as Cyn, is of great importance to plant selection. Current technologies of the next-generation sequencing and the release of globe artichoke genome sequence [14], lead to new possibilities of molecular studies of cyn biosynthetic pathways.

Our research group has installed two *Cynara cardunculus* experimental fields, (F1 generation with a total of 1061 individuals) with chemical and genetic characterization ongoing. It is our goal, to identify and select high added value cardoon plants, according to cynaropicrin production profiles along time, and to evaluate the differential expression of relevant transcripts involved in cyn biosynthetic pathways. A better understanding of the extent of genotypic groups is essential not only to assist plant breeders in selecting certain essential production, but also to provide a more rational basis for expanding the gene pool and identifying the materials which contain valuable alleles for Cc breeding.

2. Materials and Methods

2.1. F0 Populations and Individuals Collection

For a better understanding on Cyn variation within the years, F0 (naturally occurred) populations (pool of 7 individuals in a certain defined geographic area) and individuals leaves, were collected in June 2016 and June 2017. Samples were air dried at room temperature till dryness and then grounded with a domestic grounder (Moulinex). Dried samples were stored for prior cynaropicrin extraction and quantification.

2.2. F1 Individuals' Collection

Within the experimental fields installed (generation F1), 49 identified plants of Cc leaves were collected, during 4 consecutive months (March, April, May and June 2020). Samples were air dried at room temperature till dryness and then grounded with a domestic grounder (Moulinex).

2.3. Cynaropicrin Extraction and Quantification

Cynaropicrin extraction was performed according to previously described studies from our research group [15,16]. CC leaves-ethanolic extracts cynaropicrin content was quantified by High Performance Liquid Chromatography (HPLC) and results expressed in terms of mg/g dry weight (DW).

2.4. Transcriptome Analysis

Total RNA was extracted from leaves of the 49 identified Cc genotypes [(high (H) versus low (L) Cyn levels, from the experimental fields (F1)]. The concentration and integrity of RNA was evaluated by UV-Vis spectrophotometers, agarose gel electrophoresis (2%) and Agilant BioAnalyzer. cDNA libraries were prepared, for stranded paired-end sequencing through Illumina platform, and further bioinformatics analysis.

2.5. Statistical Analysis

F0 generation were analyzed using the PROC GLM option of SAS (SAS Institute Inc., Cary, NC, USA). Least square means and standard error of the mean (SEM) are present in tables. When significant effects ($p < 0.05$) were detected multiple comparisons ofmeans were conducted following Tukey's method.

F1 generation were first submitted to the evaluations of normality (Shapiro-Wilk) and homogeneity (Bartlett). The data considered normal and demonstrating homogeneity of variance were submitted to analysis of variance (ANOVA). Interactions between treatments (months variation) and populations were included in the model. The model was used within each time of evaluation. When differences were detected by ANOVA, the

means were compared by the tukey test ($p < 0.05$), using R software (R Core Team, 2014) and the ExpeDes, Lattice, and ggplot packages.

3. Results and Discussion

According to the characterization of different Cc geographic locations (generation F0), in Alentejo region, southern Portugal, using SSR markers, the highest proportion of Cc genetic variation was identified within a geographic group, while the variation was smaller between groups [13]. Geographical areas with greater genetic diversity were identified in populations from the North of Alentejo compared to the regions of South Alentejo [13].

On what regards to Cyn variation among the different geographic locations a remarkable variation between 27 and 103 mg/g DW of Cyn extracts content, was observed on 2016 (Figure 1). When comparing the results obtained on 2017, it was observed that some populations presented a lower Cyn content (1-CH, 11-MC, 13-HR and 16-JRB), in contracts to other population which presented high Cyn contents (3-QS and 14-HSR) and the great majority presented statistically equal Cyn contents, with differences explanation still needing to be target of research.

The results underlie a great variability of chemical profiles within populations and demonstrates a great variability within the same populations, in different years.

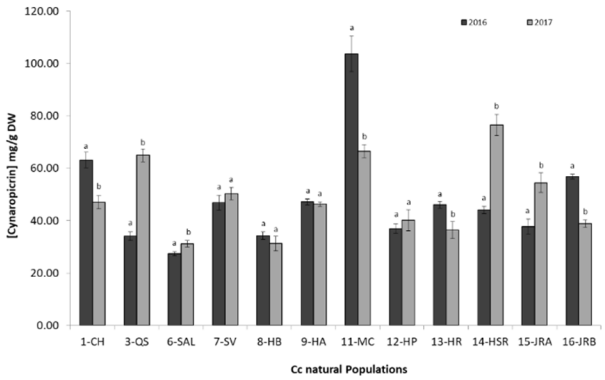

Figure 1. Graphical representation of the results from Cynaropicrin content (mg/g DW) of populations F0 (natural populations of Cc, represented by different letters and numbers: 1-CH; 3-QS; 6-SAL; 7-SV; 8-HB; 9-HA; 11-MC; 12-HP; 13-HR; 14-HSR; 15-JRA and 16-JRB) in two consecutive years (2016 and 2017). Columns with different letters (a, b) represent means significantly different only between populations in the two different years (one-factor ANOVA analysis, Duncan's test, $p < 0.05$).

Seven individuals from the Monte da Chaminé population (CM) were analysed, due to the greatest variation on Cyn content. As can be observed in Figure 2, all plants presented higher values than 40 mg/g DW of Cyn. Besides, in 2017 a clearly decrease on Cyn content was observed for the same individuals. (F0 2016 vs. F0 2017).

In order to understand the Cyn concentration profile during the same season and to further explore the production capacities as well as the chemical stability in daughter plants, seeds of all individuals were germinated, and experimental fields were installed in different areas of Alentejo (F1 generation).

On the different experimental fields, 49 plants (F1 generation) were selected and the results obtained for the Cyn content in the different plants over the 4 months collection showed a great variability, concerning the genotype and collection period. Different chemical profiles were identified, *Cynara cardunculus* leaves ethanolic extracts presented a remarkable range between 12.7 (low Cyn content) and 80.7 (high Cyn content) mg/g DW of cynaropicrin (Figure 3).

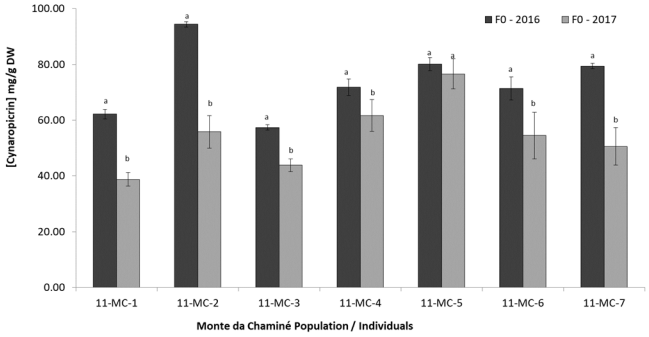

Figure 2. Graphical representation of the results from Cynaropicrin content (mg/g DW) of the population F0 (natural population of Cc—Monte da Chaminé) in two consecutive years. Columns with different letters (a, b) represent means significantly different only between individuals in two different years (one-factor ANOVA analysis, Duncan's test, $p < 0.05$).

According K. Eljounaidi et al. [17], there is an accumulation of cynaropicrin content in the leaves at different stages of development of the artichoke (*Cynara cardunculus* L. var. *scolymus*). The same is not visible according to the results obtained in our study with *Cynara cardunculus* L. var. *sylvestris*. According to results, there is an upward trend between the months of March, April and May. In May we verified the highest Cyn contents, followed by June, with a decreasing trend.

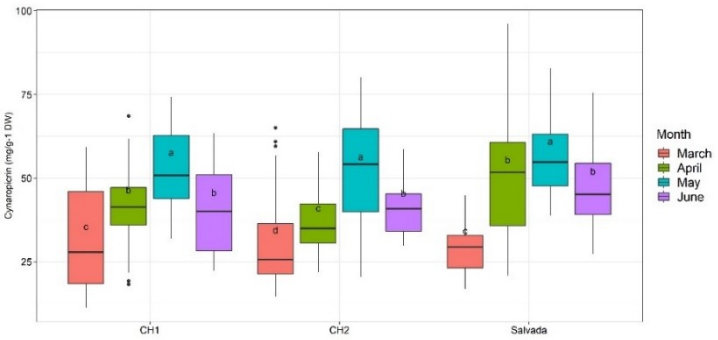

Figure 3. Graphical representation of the results from Cynaropicrin content (mg/g DW) of the population F1 in 4 consecutive months (march, April, May and June 2020). Box plot with different letters represent means significantly different between individuals in the different months sampled and in the different installed fields over the time (ANOVA analysis, Tukey's test, $p < 0.05$).

For transcriptome analysis, total RNA was successfully extracted from biological samples with high and low level of cynaropicrin content. Total RNA amount of 1 µg and RIN (RNA Integrity Number) higher than 8 were confirmed by highly accurate and precise electrophoresis (Figure 4b).

After cDNA libraries preparation, stranded paired-end sequencing will be performed on Illumina Sequencers. The next step will be the bioinformatics analysis of the transcriptome data.

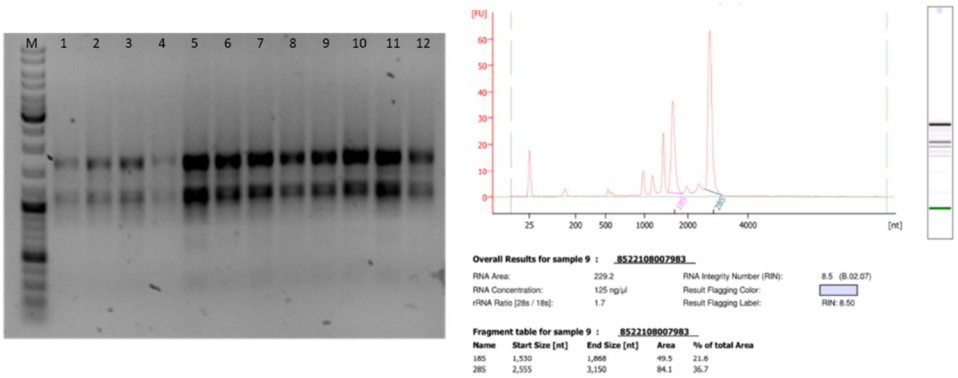

Figure 4. (**a**) Agarose gel electrophoresis (2%) of total RNA from different Cc leaves samples; (**b**) Electropherograms (Agilent technologies) of total RNA from cardoon leaves.

4. Conclusions

According to results from F0 populations, there was a great chemical and genetic variability in natural Cc populations. The natural variability of chemical and genetic profiles carries an enormous agronomic potential, which ultimately requires the selection of the best producers and the guarantee of maintenance of production throughout the plant's development cycle. The best plants must be selected due to their great heterogeneity, for a sustainable economic exploitation of *Cynara cardunculus*.

Installation of the experimental Cc fields, allows the accessibility of different Cc plants with different genetic, morphological and chemical profiles, turning it more appropriate for Cc genes conservation as well as the contributor of feedstock for preceding studies and applications.

In F1 plants, we can state that there is an increasing trend in cynaropicrin content in all experimental fields sampled over the months March to May, and that after that month the content decreases. We can also conclude that there is great chemical variability in different individuals.

This research is an important step for a superior conservation of the wild cardoon gene pool and for a more efficient use for future reproduction programs of *Cynara cardunculus*.

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