horticulturae

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New Insights into the Role of Alternating Temperatures and Cyanide in the ROS-Mediated Cardoon Seed Dormancy Termination

Giuseppe Diego Puglia [1,*], Karina Balestrasse [2], José Santiago Bustos [3] and Héctor Roberto Huarte [4]

[1] Institute for Agricultural and Forestry Systems in the Mediterranean (ISAFoM), Department of Biology, Agriculture and Food Science (DiSBA), National Research Council (CNR), Via Empedocle, 58, 95128 Catania, Italy
[2] Cátedra de Bioquímica, Facultad de Agronomía, Universidad de Buenos Aires (UBA), Instituto de Investigaciones en Biociencias Agrícolas y Ambientales (INBA), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), San Martín 4453, Argentina
[3] Facultad de Ciencias Agrarias, Universidad Nacional de Lomas de Zamora, Ruta Provincial N° 4, Km 2, Llavallol CP 1836, Argentina
[4] CONICET/ Universidad Nacional de Lomas de Zamora, Facultad de Ciencias Agrarias., Ruta Provincial N° 4, Km 2, Llavallol CP 1836, Argentina
* Correspondence: giuseppediego.puglia@cnr.it

Abstract: Physiological dormancy in wild cardoon (*Cynara cardunculus* var. *sylvestris*) can be terminated by achenes exposure to alternating temperatures, likely with the participation of reactive oxygen species (ROS). Cyanide is a natural compound that mediates seed dormancy removal in some plant species in association with oxidative signalling exerted by ROS. To date, no study has been conducted on the cyanide effect on ROS homeostasis during the germination of cardoon. Here, we showed that the addition of cyanide at low concentrations in dormant cardoon achenes promotes dormancy breakage at a constant temperature, speeds up germination to alternating temperatures and promotes ROS accumulation in embryonic axes of dormant achenes. The in-silico transcriptome analysis showed that the expression levels of transcripts of genes associated with ROS signalling and production, calcium signalling, gibberellins biosynthesis and cell wall loosening were significantly up-regulated at the alternating temperatures imbibition condition. In contrast, the expression of gene transcripts associated with the inhibition of germination, ABA biosynthesis and signalling were up-regulated at the constant temperature imbibition. However, no significant difference in lipid peroxidation or protein carbonylation levels was observed when achenes were imbibed at constant or alternating temperature conditions. These results suggest that dormancy termination triggered by alternating temperatures or cyanide could be mediated by ROS production and signalling in the cardoon embryonic axis, but this does not determine extensive protein carbonylation.

Keywords: reactive oxygen species; fluctuating temperatures; seed germination; *Cynara cardunculus* var. *sylvestris*; transcriptome profiling; protein carbonylation; lipid peroxidation.

Citation: Puglia, G.D.; Balestrasse, K.; Bustos, J.S.; Huarte, H.R. New Insights into the Role of Alternating Temperatures and Cyanide in the ROS-Mediated Cardoon Seed Dormancy Termination. *Horticulturae* **2022**, *8*, 960. https://doi.org/10.3390/horticulturae8100960

Academic Editors: Ju-Sung Cho and Sergio Ruffo Roberto

Received: 19 September 2022
Accepted: 11 October 2022
Published: 17 October 2022

1. Introduction

Seed dormancy could be defined as the inability of a viable seed to germinate under favourable environmental conditions [1]. This trait allows a successful development of a new plant generation by preventing out-of-place or season germination [2]. The interaction of a seed with environmental factors, such as soil temperature and moisture, plays a crucial role in the regulation of dormancy and its progressive alleviation [3]. From mother plant dispersal to germination completion, the dormancy level is progressively reduced, and the seed population acquires a higher sensitivity to a second set of environmental

signals that allow dormancy termination [4]. Light and alternating temperatures are considered the principal signals for seed dormancy termination in many plant families since they can provide important information about soil burial depth [5,6]. Notwithstanding the ample literature on seed response to light, there are still many gaps in understanding the physiological and molecular regulation of seeds' response to alternating temperatures [7].

Cynara cardunculus L. is a Mediterranean species that includes the globe artichoke (subsp. *scolymus* (L.) Hegi), which has been proposed as a functional food [8], the cultivated cardoon (var. *altilis* DC.), also known as industrial cardoon for its uses as bioenergy crop [9,10], and the wild cardoon (var. *sylvestris* (Lamk) Fiori). Previous studies about the germination physiology of the wild cardoon demonstrated that alternating temperatures promoted the removal of seed dormancy [11–13], and this was shown to have a hormonal basis through a reduction of the gibberellins (GAs)/abscisic acid (ABA) ratio, possibly through a decrease in ABA sensitivity [14].

Beyond the hormonal regulation of the ABA/GA ratio, reactive oxygen species (ROS) and cyanide (e.g., potassium cyanide, KCN) were both proposed to play essential roles in the regulation of seed germination in several plant species [15–17]. Our previous studies showed that the addition of ROS donors or ROS itself triggered germination completion of dormant cardoon achenes, while the presence of antioxidant compounds and ROS scavengers inhibits it [18]. On the other hand, higher antioxidant doses are needed if achenes are imbibed at alternating temperatures suggesting that the seed likely produces ROS compounds when it is imbibed at the dormancy-breaking condition [19]. A first transcriptome survey on dormant and non-dormant cardoon achenes showed that alternating temperatures activate ROS homeostasis regulation through the expression, among others, of catalases (*CcCAT2*), and an NADPH oxidase, a respiratory burst oxidase homolog (*CcRboh*) [20]. This suggests that ROS plays a role in dormancy termination at alternating temperatures, most probably within a fine-tuned mechanism for environmental sensing. In plant non-photosynthetic tissues, ROS are produced mainly in the mitochondrion at the respiratory chain or through the action of enzymes such as Rboh, peroxidase (*POX*), and amine oxidase (*AO1*) [21]. In addition, a recent study identified a wheat human-homolog of ROS modulator 1 (Romo1), which has been demonstrated to have a central role in the positive regulation of the ROS levels in the enhancement of anthers development [22]. ROS mediation in the alleviation of dormancy has been described throughout the germination process [23]. In the early stages, it interacts with cytoplasmic signalling pathways (Ca^{2+} and ABA pathways) through calmodulin (CaM) and protein Tyr phosphatase (PTP), as shown in sunflower [16]. In this regard, H_2O_2 was shown to activate ABA-8-hydroxylase, inducing ABA degradation [24,25]. ROS regulates gene expression in mid-germination stages through specific mRNA's oxidation in sunflower and wheat seeds [26,27]. Eventually, in later germination stages of endospermic seeds, it interacts with cell wall polysaccharides enhancing endosperm cap weakening and embryo elongation growth [28]. In these two processes, xyloglucan endotransglycolases/hydrolases (XTHs) and expansins, as non-enzymatic factors, are activated [29], but the link among ROS, expansins and XTHs remains largely unknown.

The cyanide effect in stimulating seed germination in non-dormant seeds was shown in the last decades [30,31], but its alleviation effect in primary dormant seeds is documented only for a few species [16,17]. It was ascertained that cyanide triggers specific protein carbonylation, a non-reversible oxidative modification, which elicits dormancy alleviation [15,32], and this effect is in common with ROS accumulation. Moreover, as for ROS, cyanide can play a dual role in plants; that is, it has a toxic effect at high concentrations and acts as a signal molecule at low concentrations [33–35].

The present study was a first attempt to investigate the cyanide effect on seed germination in cardoon achenes and analyse a possible mediation of ROS for the alternating temperature's effect. Thus, we used the cyanide compound in micromolar concentrations, and the cardoon seed germination was recorded. Moreover, an in-silico gene expression analysis was undertaken using available transcriptomic data [20] of achenes imbibed at

constant or alternating temperatures to identify the key role of genes associated with ROS, cyanide and dormancy termination pathways to get further insights into their expression modulation during this process.

2. Materials and Methods

2.1. Achenes Collection

We collected wild cardoon, *Cynara cardunculus* var. *sylvestris*, and mature achenes from 20 randomly selected plants exhibiting ripeness, which were growing in a plot located at Llavallol, Buenos Aires Province, Argentina. The collection of achenes was performed during the second half of January 2022. After harvesting, achenes from different plants were cleaned and exposed to airflow for 24 h to reach a moisture content of approximately 4–5%, which was measured using a humidity calculator (Rotronic, Ettlingen, Germany). The cleaned achenes were stored for three months at −18 °C in tightly closed jars filled to 50% with silica gel, which was replaced as soon as we observed colour turning. For all the tests performed in the present study, we used dry after-ripened achenes at 20 °C for 21 days, as reported in greater detail by Huarte et al. [20].

2.2. General Procedures for Germination Tests

Dry after-ripened achenes were sown on two layers of moist filter papers in 90-mm-diameter Petri dishes soaked in double-distilled water. Germination tests (three technical replicates of 30 achenes each) were performed in darkness by wrapping Petri dishes in two aluminium foil layers. Darkness was used to prevent the interference of light, which is a dormancy termination cue. Achenes were imbibed at 20 and 10 °C temperature (hereafter referred to as alternating temperatures) with a 12 h thermo-period, or a constant 15 °C (i.e., the mean temperature of the daily alternating cycle) in germination chambers in controlled temperature conditions (±1 °C). In all experiments, dishes were examined for germinated seeds daily, and we continued monitoring them for 14 days after the last achene germination (unless otherwise stated). Achenes with protrusion of the radicle > 1 mm were considered germinated and then removed. Data were statistically analysed using Infostat with the ANOVA test, and means were separated using Tukey's test at $p \leq 0.05$. Statistical elaborations were performed by comparing the cyanide effect for alternating and constant temperatures separately using, for each temperature regime, the direct imbibition in water as the control condition. The germination rate (GR) was calculated as in Puglia et al. [36].

2.3. Effect of Cyanide on Germination

Achenes were immersed for 0, 6, 16 and 24 h at room temperature in KCN solutions of 0, 5, 10, 50 and 100 μM to determine the effect of Potassium cyanide (CAS No: 151-50-8, Sigma Aldrich, Buenos Aires, Argentina) on germination. After the KCN pre-germination treatment, achenes were washed with running water, rinsed in distilled water three times, and transferred to the Petri dishes to be incubated at the corresponding temperatures.

2.4. Localization O_2 and H_2O_2

The ROS compound localisation was performed using surgically isolated embryos extracted from achenes incubated for 72 h at alternating and constant temperatures (i.e., previous to the radicle emergence for both treatments). The accumulation of O_2^- was detected using 0.5 mM NBT (nitro blue tetrazolium chloride); 2,2′-Di-p-nitrophenyl-5-5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-diphenylene)-ditetrazolium Chloride, PhytoTec Labs, Lenexa KS, USA) while H_2O_2 accumulation was determined by staining with DAB (3,3′-Diaminobenzidine tetrahydrochloride, Sigma Aldrich, Argentina). Embryos were incubated with 1 mM NBT in 10 mM Tris-HCl (pH 7.0) or 1 mg/mL DAB (pH 3.8) at room

temperature (20 °C) for 30 min, then washed with double-distilled water and photographed using the stereomicroscope (Labomed Pixel 4D, Los Angeles, CA, USA). For the detection of ROS triggered by cyanide treatment, we imbibed achenes in 100 μM KCN for 16 h, based on its most significant germination promotion effect obtained at a constant temperature (Table 1). Afterwards, the achenes were rinsed twice in distilled water and stained with NBT or DAB to visualize O_2^- or H_2O_2, respectively.

Table 1. Mean final germination, t50 (expressed as days) and germination rate of wild cardoon (*C. cardunculus* var. *sylvestris*) achenes exposed to different KCN treatments and imbibed at constant or alternating temperatures. Statistical evaluation refers to final germination percentages. The alternating and constant temperature regimes were separately compared.

KCN Concentration	KNC exposure Time (h)	Imbibition Temperature	Final Germination (%)	t50	GR (1/t50)	Tukey Test $p \leq 0.05$
0	0	15 °C	45.56	4.06	0.24	A
	0	10/20 °C	78.89	4.11	0.24	BC
5 μM	6	15 °C	81.11	4.29	0.23	BC
	16	15 °C	71.11	3	0.33	ABC
	24	15 °C	72.22	3.61	0.27	ABC
	6	10/20 °C	83.33	4.06	0.24	-
	16	10/20 °C	82.22	3.5	0.28	-
	24	10/20 °C	80.00	3.36	0.29	-
10 μM	6	15 °C	64.44	3.78	0.26	ABC
	16	15 °C	84.44	3.75	0.26	C
	24	15 °C	68.59	4.24	0.23	ABC
	6	10/20 °C	94.44	3.85	0.25	-
	16	10/20 °C	92.22	3.50	0.28	-
	24	10/20 °C	90.00	2.30	0.43	-
50 μM	6	15 °C	55.56	3.67	0.27	AB
	16	15 °C	73.33	3.61	0.27	ABC
	24	15 °C	71.11	3.12	0.32	ABC
	6	10/20 °C	81.11	3.96	0.25	-
	16	10/20 °C	80.00	3.61	0.27	-
	24	10/20 °C	80.00	3.16	0.31	-
100 μM	6	15 °C	75.56	4.08	0.24	BC
	16	15 °C	85.56	3.54	0.28	C
	24	15 °C	78.89	3.38	0.29	BC
	6	10/20 °C	84.44	3.93	0.25	-
	16	10/20 °C	88.69	3.50	0.28	-
	24	10/20 °C	76.67	3.42	0.29	-

2.5. Determination of Carbonyl Content in Proteins

Carbonyl content was determined as an estimation of oxidatively modified proteins following the Sun and Leopold procedure [37]. In brief, embryos were powdered in liquid nitrogen, homogenised and centrifuged at 10,000× *g* for 20 min. Carbonyl content in the supernatant was detected by reaction with 2,4-dinitrophenylhydrazine for 1 h at room temperature. After incubation, proteins were precipitated with 10% trichloroacetic acid, and the pellet was washed three times with ethanol: ethyl acetate (1:1) and re-suspended with 6 M guanidine. The absorbance at 360–390 nm was measured, and carbonyl content was estimated ($\varepsilon = 22000 M^{-1}\ cm^{-1}$).

2.6. Determination of Thiobarbituric Acid Reactive Substances (TBARS)

Lipid peroxidation was measured on seeds imbibed at 15 °C and 10/20 °C as the amount of TBARS was determined by the thiobarbituric acid (TBA) reaction as described by Heath and Packer [38]. Embryos (0.3 g) were homogenised in 3 mL of 20% (*w*/*v*) trichloroacetic acid (TCA). The homogenate was centrifuged at 3500× *g* for 20 min. Then, 1 mL of 20% TCA containing 0.5% (*w*/*v*) TBA and 100 mL 4% butylated hydroxytoluene in ethanol was added to 1 mL of the supernatant. The mixture was heated at 95 °C for 30 min and then quickly cooled on ice. The contents were centrifuged at 10,000× *g* for 15 min, and the absorbance was measured at 532 nm. The value for non-specific absorption at 600 nm was subtracted. The concentration of TBARS was calculated using an extinction coefficient of 155 $mM^{-1}cm^{-1}$. The results are expressed as μmol g-1 DW of seeds and correspond to means of three measurements ± SE.

2.7. Transcriptome Analysis

For gene expression analysis, we used the RNA reads included in the NCBI SRA repository PRJNA627453 [20]. Illumina reads were mapped to the *C. cardunculus* genome (available at www.artichokegenome.unito.it, accessed on 31 November 2021) using Hisat2 aligner [39], and gene expression levels were calculated with featureCounts [40] considering the recently updated domestic cardoon gene annotation [41]. To functionally annotate the mapped transcripts, we aligned them to the NCBI non-redundant (nr) protein database (downloaded in May 2022), using a local BLASTX analysis with an *E* value cut-off of 10−25 and using InterProScan. HtSeq count [42] was used to evaluate gene expression, in terms of Transcripts per Millions (TPM), of cardoon transcripts using aligned pre-processed quality-trimmed reads. The DEseq2 R package [43] was used to assess differentially expressed genes (DEGs) with a criterion of $|\log2(\text{Ratio})| \geq 2$ and an FDR of ≤ 0.01.

3. Results

3.1. Cyanide Treatment

Regardless of the imbibition temperature conditions and duration of the treatment, the cardoon achenes pre-treated with cyanide showed a significantly higher final germination percentage and an enhanced germination rate (Figure 1 and Table 1). This was more evident in achenes imbibed at the constant temperature with an almost doubled germination in the presence of KCN, especially after longer treatments. Amongst the conditions tested, the highest germination percentage was attained with 10 μM KCN at alternating temperatures for 24 h. Whereas, for constant temperature (the dormancy-maintaining condition), the most effective cyanide treatments were 10 or 100 μM for 16 h, while the 24 h treatment resulted in a less promoting effect for achenes imbibed at 15 °C. We also observed a significant promotion of seedling elongation with a prevalence of radicle length (data not shown).

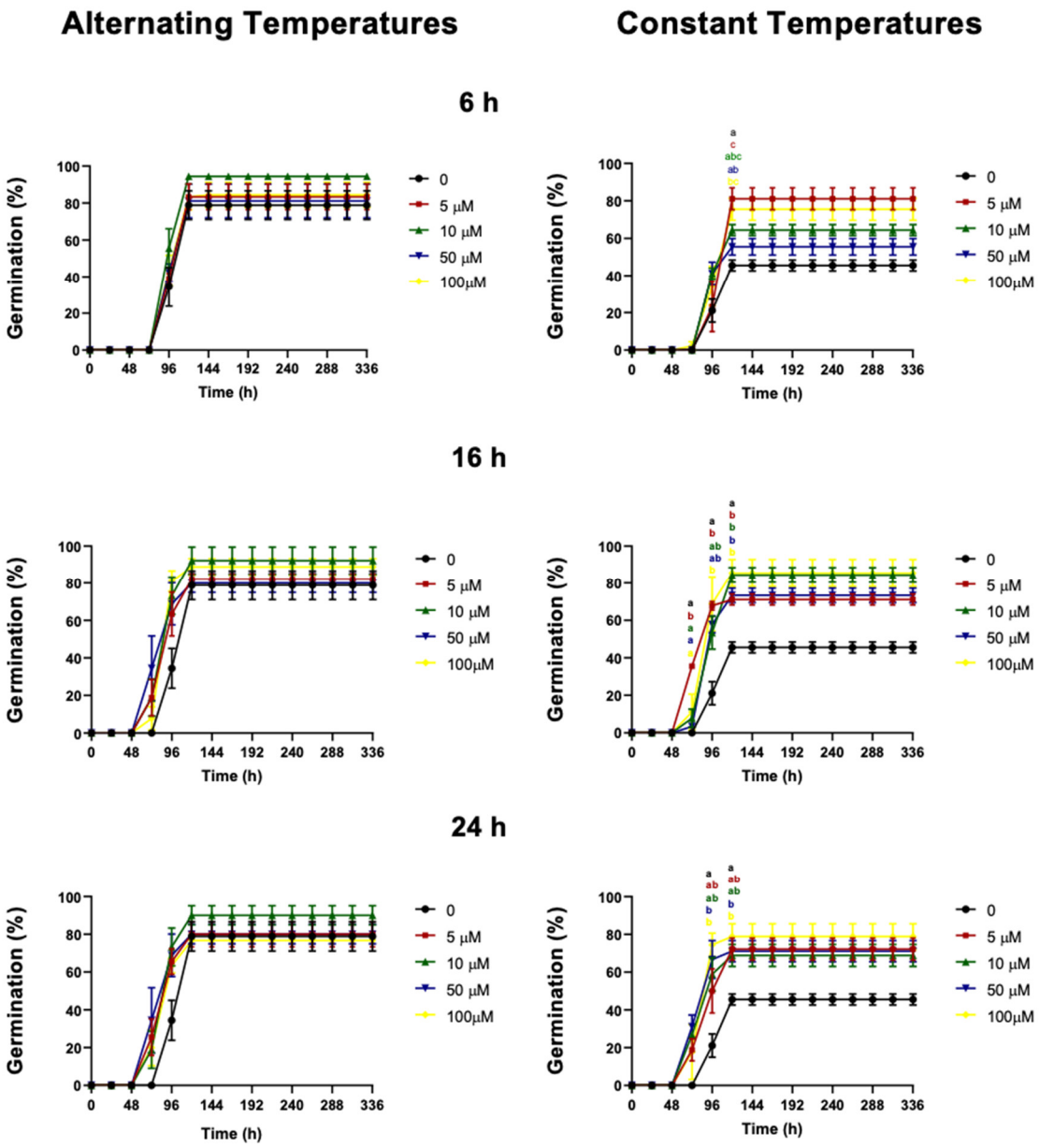

Figure 1. Effects of cyanide on wild cardoon (*C. cardunculus* var. *sylvestris*) achene germination. Pre-treatments with KCN at different concentrations (5, 10, 50 or 100 µM) were carried out for 6, 16 or 24 h and then the achenes were imbibed at alternating (10/20 °C) or constant (15 °C) temperatures. For control treatment (0), achenes were imbibed in water. Different coloured letters represent the significant differences according to one-way ANOVA and Tukey test ($p < 0.05$) between cyanide-pre-treated samples (KCN) and the control (H_2O). For alternating temperatures, there was no significant difference.

3.2. Comparison of ROS Localization in Embryos Isolated from Seeds Exposed to Cyanide or Alternating Temperatures

To investigate the possible role of ROS in the cyanide germination promotion effect, we treated cardoon achenes with 100 µM of KCN for 16 h (the most effective treatment at constant temperature). Then we stained imbibed achenes with NBT or DAB to observe the accumulation of two ROS, superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), respectively (Figure 2). Embryos imbibed in water at constant temperatures did not show any colouration indicating ROS presence. Whereas, in embryos imbibed at 10 and 20 °C and stained spots were observed at embryo axe level. The pre-treatment with 100 µM of KCN resulted

in evident stained areas for achenes imbibed at constant and alternating temperatures. The ROS measurements presented in the present study could not be used for a quantitative assessment of embryos. However, they provide a first view on ROS accumulation association with alternating temperatures and cyanide treatment.

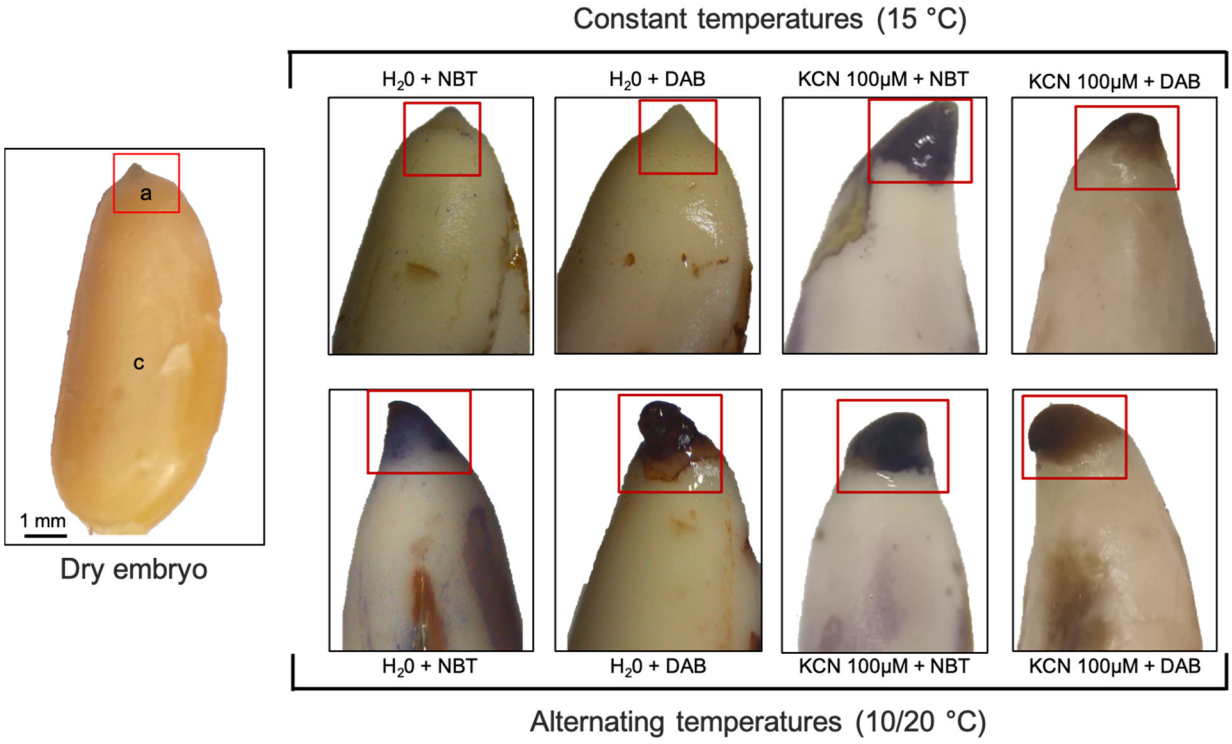

Figure 2. Analysis of $O_2^{\cdot-}$ and H_2O_2 in wild cardoon (*C. cardunculus* var. *sylvestris*) embryos obtained with NBT and DAB staining, respectively. On the left, dry embryo showing axe (**a**) and cotyledon (**c**); On the right, staining of the embryonic axis by NBT or DAB imbibed at constant temperatures (15 °C), upper part, or alternating temperatures (10/20 °C) treated with 100 µM of KCN for 16 h or in H_2O (control). Photographs are representatives of at least 10 replicates.

3.3. Effects of Alternating Temperatures on Protein and Lipid Oxidation

To examine the impact of ROS accumulated in the relief of dormancy over the protein and lipid oxidation, we measured the presence of oxidatively modified proteins and lipids peroxidation in achenes incubated at 15 °C and 20/10 °C throughout imbibition (Figure 3). We observed a slight delay in the germination dynamics with respect to the cyanide experiment (Figure 3C). No harmful levels of oxidative damage were observed for the two analysed macromolecules, suggesting that no ROS damage was achieved during imbibition at alternating temperatures. As for the protein carbonylation, non-dormant and dormant achenes showed different accumulation patterns, with the first exhibiting higher carbonylation levels in the early stages of imbibition (72 h), while the latter higher values were observed after 84–96 h. A similar trend was observed in lipid oxidation (Figure 3A). The accumulation of TBARS did not differ significantly between seeds exposed to alternating or constant temperatures (Figure 3B), indicating the lack of differential lipid peroxidation in seeds exposed to this stimulus.

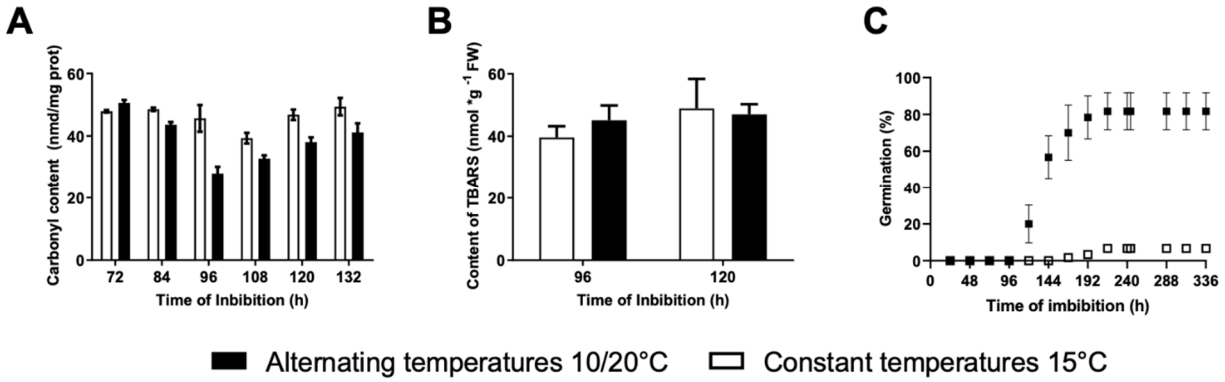

Figure 3. Protein and lipid peroxidation in wild cardoon (*C. cardunculus* var. *sylvestris*) embryos imbibed at constant (15 °C) (open bars) or alternating temperatures (20/10 °C) (filled bars). (**A**) Protein carbonyl content during imbibition of achenes. (**B**) Lipid peroxidation was measured as TBARS content. (**C**) Germination dynamics at alternating (circles) and constant temperatures (squares).

3.4. In Silico Expression Pattern Analysis of Genes Associated with Alternating or Constant Temperatures

To shed light on the possible ROS mediation into seed dormancy termination in cardoon achenes, we analysed the available transcriptome dataset [20], including dry, imbibed at 15 °C and imbibed at 10/20 °C for 48 h cardoon achenes. Novel gene annotation is provided for transcripts identified in this study (see Supplemental Material, Table S1). Expression patterns were evaluated for each gene as Transcripts per Millions (TPM) and normalised among samples focussing on ROS production and signalling and the most widely known regulating factors of seed dormancy (Figure 4).

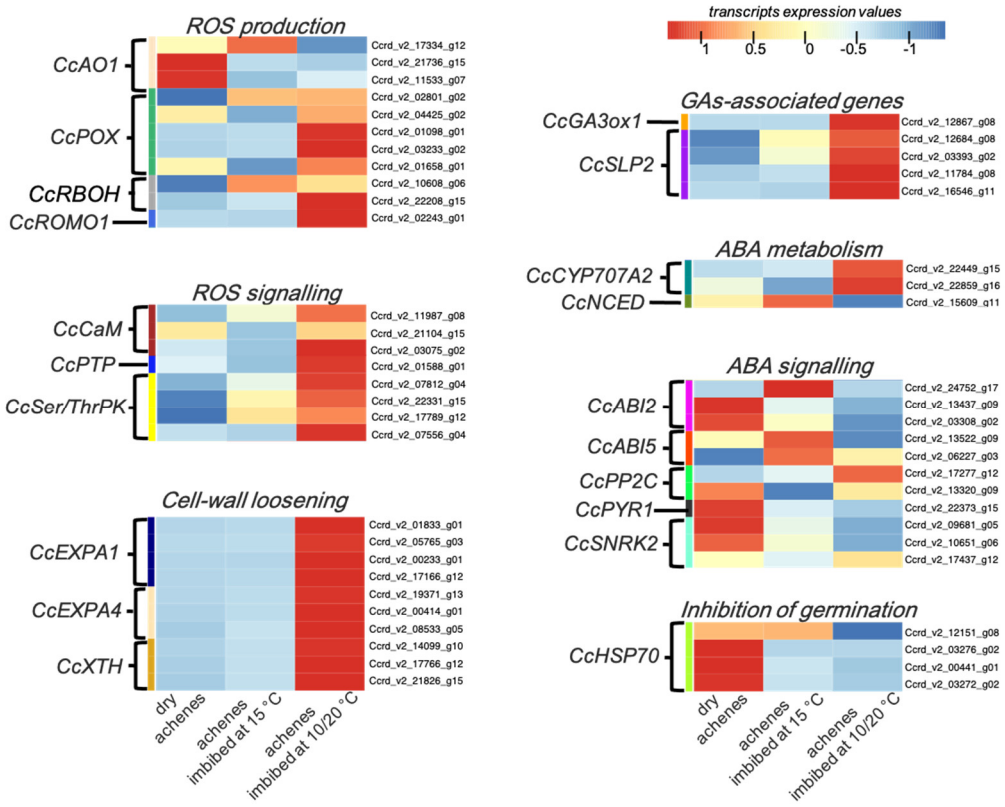

Figure 4. Heat map of ROS signalling, ROS production and seed germination control-associated transcripts in dry, imbibed at constant (15 °C) and imbibed at alternating temperatures (10/20 °C)

wild cardoon (*C. cardunculus* var. *sylvestris*) achenes for 48h. The colour scale represents the log_2-transformed TPM value.

3.4.1. ROS Production and Signalling

Analysis of available transcriptome showed that different imbibition temperatures were associated with a clearly different gene expression pattern in ROS production and signalling-related genes. Among the formers, peroxidases (*CcPOXs*) and *CcRbohs* were mainly up-regulated when achenes were imbibed at alternating temperatures. At the same time, their expression was down-regulated at the constant imbibition condition and in dry achenes. High accumulation of amine oxidases (*CcAO1*) transcripts in dry seeds and their decay in both the constant and alternating imbibition conditions suggest that they are not involved in ROS production in cardoons during seed germination, even if a direct amine oxidase protein activity should be undertaken to confirm this hypothesis. In addition, we identified a cardoon human-homolog of ROS modulator 1 (*CcRomo1*) that exhibited a strong up-regulation at alternating temperatures, while it was down-regulated at the other conditions.

In this study, several ROS signalling-associated genes were identified in wild cardoon, ranging from the Ca^{2+} signalling pathway, with calmodulin (*CcCaMs*) and protein Tyr phosphatase (*CcPTP*), to ROS signal transduction with *Ser/Thr kinases* (*CcSer/ThrPKs*). They were all univocally up-regulated at the alternating imbibition condition (dormancy-breaking condition), and this also applies to the different isoforms identified for each gene. While in dry and imbibed achenes at constant temperature ROS signalling-associated genes were always down-regulated or weakly expressed.

3.4.2. Cell-Wall Loosening

As regards the genes encoding proteins involved in cell wall loosening, we identified in the annotated achene cardoon transcriptome the presence of **α**-expansins (*CcEXPA1* and *CcEXPA4*), xyloglucan endotransglycolases/hydrolases (*CcXTHs*), but no endo-(1,4)-β-d-glucanases were found. Their absence could unlikely be a signal of their unrelatedness to this process in cardoon, while, more likely, it could be a consequence of the limited *C. cardunculus* genome annotation level coverage. *CcEXPAs* and *CcXTHs* were up-regulated at the alternating temperatures imbibition condition, while very low transcripts levels were detected for dry and imbibed at 15 °C achenes.

3.4.3. GAs-Associated Genes

In this study, a gene transcript of a *Gibberellin 3 beta-hydroxylase* (*CcGA3ox1*), a GA biosynthetic gene, was identified in wild cardoon. Its expression was highly up-regulated at the dormancy-breaking condition, while no expression was observed at other conditions. This suggests that only specific imbibition conditions activate GA biosynthetic pathway. Moreover, as expected, the *Subtilisin-Like Serine Protease 2* (*CcSLP2*) transcript follows the *CcGA3ox1* expression pattern.

3.4.4. ABA Metabolism and Signalling

In the present study, a new transcript was identified associated with *ABA-8'-hydroxylase 2* (*CcCYP707A2*) in addition to what was reported in Huarte et al. [19]. The expression pattern of the two *CcCYP707A2s* suggests that ABA degradation was activated at the alternating temperatures imbibition condition. At the same time, the upregulation of *CcNCED* at 15 °C indicated that the biosynthesis of ABA was promoted at this regime temperature. Moreover, a weak transcript level expression was observed in dry achenes suggesting a preliminary *NCED* mRNAs synthesis during seed maturation to maintain the dormancy status upon imbibition.

Analyses of ABA signalling-associated genes showed the presence of novel identified for *ABI2*, *ABI5*, *PP2c*, *PYR1* and *SnRK2* transcripts. The *CcABI5s* and one *ABI2*-associated

transcript were up-regulated at the constant temperature imbibition condition promoting ABA signalling and maintaining dormancy status. Whereas the other two ABI2-associated transcripts showed higher up-regulation in dry achenes. This pattern is common with *CcPYR1* and *CcSNRK2s*, although at 10/20 °C, the transcripts level was always lower than at 15 °C. On the other hand, the two *CcPP2C* transcripts were up-regulated at the alternating temperature and inhibited at the constant condition.

3.4.5. Inhibition of Germination

We identified several transcripts of genes encoding for heat shock proteins 70 (*CcHSP70s*), which are usually associated with the inhibition of germination. They were up-regulated in dry achenes and down-regulated under other conditions. The observed expression pattern was related to the inhibition of germination and suggests that chaperone activity can play a role in maintaining dormancy. The in-silico analysis conducted in the present study did not allow us to identify any gene transcripts encoding for small heat shock proteins, such as *HSP40s* or *HPS20s*, which regulates seed germination in response to the dynamics of temperatures.

4. Discussion.

The rising agronomic interest in *C. cardunculus* in most temperate regions for its food properties (e.g., globe artichoke) or as a bioenergy crop (e.g., cultivated cardoon) contributes to the gaining popularity of this Mediterranean plant. Since propagation by achenes is still largely used, shedding light on germination control remains crucial. The wild cardoon is the most seed-dormant variety within the *C. cardunculus* botanical species, and dormancy termination is promoted by imbibition at alternating temperatures [18]. Recently, we showed that the addition, at constant temperatures, of ROS elicitors or donors similarly triggered germination to alternating temperatures [19,20]. Notwithstanding the well-known interactions between ROS and cyanide, no investigations have been conducted about its effect on cardoon germination, and the present study first investigated this process. It provided evidence that the transduction of KCN and alternating temperatures share a common ROS mediation for triggering dormancy termination. Here, we show that pre-treatments with 100 and 10 μM KCN for 16 h promoted the highest germination of dormant cardoon achenes. However, even at lower concentrations, significant enhancement of germination was observed. Previous studies [15,44] showed similar promotion of germination in dormant sunflower and apple embryos using higher cyanide concentrations, i.e., 1 mM, although a toxic effect of cyanide at high concentrations is likely to occur [33,34].

Moreover, here we showed evidence that the pre-treatment of dormant achenes with KCN generates ROS production and this effect was analogue to the imbibition at alternating temperatures (Figure 2). Future investigations are necessary to determine the enzymatic antioxidant machinery, i.e., super-oxide dismutase (SOD), CAT and Glutathione Reductase (GR), activity in each condition. Moreover, only limited protein carbonylation was observed. It was higher in non-dormant embryos at the early stage of imbibition, while it increased in dormant achenes after 84 h. This contrast with what was detected in sunflower embryos, for which two distinct carbonylation patterns were observed in dormant and non-dormant embryos [15]. Hence, this process may not occur in cardoon with the same strength. However, further studies are needed to shed light on this low protein oxidation level registered and on its possible association with the enzymatic antioxidant activation, i.e., *CcCAT*, at alternating temperatures, in agreement with our previous findings [20]. Also, the produced ROS could only have a signalling role without directly regulating the protein's function. In this view, the high transcript expression level observed for *CcHSPs* in dry achenes can be regarded as ready mRNAs to maintain ROS homeostasis and stabilise membranes upon imbibition.

Cyanide interaction with ROS-producing pathways is documented in other species through the upregulation of NADPH oxidase [15,44]. Similarly, in cardoon, we observed

an up-regulation of (*CcPOXs*) and NADPH oxidase (*CcRbohs*) at the alternating temperatures imbibition condition, corroborating the hypothesis that cyanide and alternating temperatures cues share common molecular signalling routes. In the present study, we identified, for the first time, a cardoon human-homolog of ROS modulator 1 (*CcRomo1*), which was significantly up-regulated at alternating temperatures. Recently a wheat ROS modulator 1 was demonstrated to promote ROS production, and it can be regulated by the male-sterile gene (*Ms2*) through oligomerisation [22]. This provides new evidence for novel players in ROS production in plants that can modulate its generation and signalling.

The alternating temperatures effect was probably involved in the activation of the Ca^{2+} signalling pathway as we detected an upregulation of calmodulin (*CcCaMs*) and protein Tyr phosphatase (*CcPTP*), which were described also interacting with the H_2O_2 signalling pathway [45]. A slightly higher expression was also documented for *HaPTP* in sunflower embryos exposed to ROS donors [16]. Ser/Thr protein kinases (Ser/Thr PKs) also participate in ROS signal transduction to *AtMPK6* [46,47]. The *CcSer/ThrPKs* transcripts identified in this study showed higher expression at the dormancy-breaking condition.

Expansins, xyloglucan endotransglycolases/hydrolases and endo-(1,4)-β-d-glucanases are likely responsible for the cell wall loosening and remodelling process [29,48]. Overrepresentation of cell wall-loosening-related genes in the transcriptome was shown in *Lepidium sativum* and Arabidopsis germinating seeds [49] and in the translatome of sunflower seeds [50]. We identified **α**-expansins (*CcEXPA1* and *CcEXPA4*) and xyloglucan endotransglycolases/hydrolases (*CcXTHs*) in the present study. Still, no endo-(1,4)-β-d-glucanases were found, which can likely be a consequence of the limited *C. cardunculus* genome annotation level coverage. All the cell wall loosening-related transcripts were up-regulated at alternating temperatures, presumably, indicating turgor decrease through stress relaxation in the cell wall, which precedes germination completion.

Hormonal regulation was affected by imbibition at different temperature regimes. At 10/20 °C, we observed a marked activation of *CcCYP707A2* and *CcPP2C*, involved in ABA degradation and inhibiting its signalling, respectively. Interestingly *SlPPC* expression was also shown to be promoted in the presence of cyanide in tomato seed germination [17]. Moreover, at the dormancy-breaking condition temperature conditions, the *CcGA3ox1*, a GA biosynthetic gene, and *SLP2*, a gene transcript encoding for a protease quickly induced by gibberellins [51,52], were highly up-regulated. On the other hand, at the alternating temperature condition, we observed a down-regulation of an ABA biosynthetic gene, *CcNCED*, as well as *CcSnRK*, *CcPYR*, and *ABI2* and *ABI5*, which encode for main ABA signalling factors and this pattern are similar to what was reported in tomato seed pre-treated with cyanide [17].

5. Conclusions

In summary, our study demonstrated that cyanide promotes the germination of dormant cardoon achenes, and its action involves ROS mediation. The most effective condition for germination triggering was 100 μM of KCN for 16 h, but significant promotion was observed even at the lowest KCN concentration. This is possibly associated with the signalling role of this small molecule interacting with ROS. The imbibition of achenes at alternating temperatures provides similar germination and ROS generation, as observed for cyanide pre-treatment. In silico analyses confirmed that gene transcripts encoding for proteins associated with ROS production and signalling pathways were up-regulated in non-dormant achenes. Our research provides new insights into understanding the mechanisms regulating dormancy termination in the presence of cyanide and alternating temperatures in the wild cardoon.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8100960/s1, Table S1: Transcript annotation of novel identified sequences.

Author Contributions: G.D.P. and H.R.H. conceived the research; H.R.H., K.B. and J.S.B. performed the laboratory experiments; G.D.P. elaborated the data; G.D.P. and H.R.H. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was partly founded by LOMASCyT IV/Universidad Nacional de Lomas de Zamora.

Data Availability Statement: Not applicable.

Acknowledgments: We thank Carla Zilli and Pablo Vallecorsa for all their help with MDA and protein carbonylation measurements. We acknowledge the special issue editors and the two anonymous reviewers for their insightful comments that improved the manuscript. HRH would like to express all his gratitude to Marcela Simontacchi for her crucial mentoring in ROS experiments and interpretation of alternating temperatures' effect on seed physiology. Hence, we dedicate this paper to Marcela Simontacchi's memory.

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

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