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Proceeding Paper

Exploring the Involvement of the *Alternative Respiratory Pathway* in *Pisum sativum* L. Seed Germination †

Lénia Rodrigues [1], Ricardo Claudino [2], Steven P.C. Groot [3], Pierre Hohmann [4], Amaia Nogales [5], Lee Hansen [6] and Hélia Cardoso [1,*]

[1] MED (Instituto Mediterrâneo para a Agricultura, Ambiente e Desenvolvimento), IIFA (Instituto de Investigação e Formação Avançada), Universidade de Évora, Pólo da Mitra, Ap. 94, 7002-554 Évora, Portugal; email1@gmail.com
[2] Escola de Ciências e Tecnologia, Universidade de Évora, Colégio Luís António Verney, Rua Romão Ramalho, 7000-671 Évora, Portugal; email2@gmail.com
[3] Wageningen Plant Research, Wageningen University & Research, Wageningen, The Netherlands; email3@gmail.com
[4] IRTA Institute of Agrifood Research and Technology, Sustainable Plant Protection Programme, Fruitcentre, 25003 Lleida, Spain; email4@gmail.com
[5] LEAF (Linking Landscape, Environment, Agriculture and Food) Instituto Superior de Agronomia, Universidade de Lisboa, Tapada da Ajuda, 1349-017 Lisboa, Portugal; email5@gmail.com
[6] Department of Chemistry and Biochemistry, Brigham Young University, Provo, UT 84602, USA; email6@gmail.com
* Correspondence: liar@uevora.pt or hcardoso@uevora.pt

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Abstract: Organic agriculture, recognized as a more sustainable agricultural system, strongly depends on the use of highly resilient genotypes. Resilient seeds, with increased tolerance to germinate and provide vigorous seedlings under environmental stresses, currently represent one of the most important agronomical traits. Seed germination involves the activation of several metabolic pathways, including cellular respiration. Alternative oxidase (AOX), a key enzyme in the alternative respiratory pathway, plays a crucial role in regulating cell reprogramming by controlling metabolic transitions related to the cellular redox state and the variable carbon balance. The involvement of the alternative respiratory pathway during germination was explored by analysis of *PsAOX* gene/protein expression. Seeds of four *Pisum sativum* L. cultivars ('Respect-1', 'S134', 'G78' and 'S91') were imbibed in sterile tap water for 16 h and metabolic parameters measured by calorespirometry (heat and CO_2 emission rates) in a Multi-Cell Differential Scanning Calorimeter in isothermal mode at 25 °C. The involvement of PsAOX was evaluated by transcript quantification (PsAOX1, PsAOX2a, and PsAOX2b) through RT-qPCR, and by of analysing the PsAOX expression through Western blot. The results demonstrate that the cv. 'S91', characterized by a low germination rate, exhibited the lowest metabolic heat and CO_2 emission rate. However, contrary to expectations, *PsAOX* transcript accumulation and PsAOX protein expression were significantly higher for 'S91' than for the other cultivars. These results indicate that higher levels of AOX (transcript and protein) could be linked to lower metabolic rates for embryo growth when seed germination is compromised.

Keywords: LIVESEED; pea; AOX; alternative respiratory pathway; seed germination

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1. Introduction

Legumes have been referred to as the basis of a healthy diet, representing the most prominent source of food protein [1]. Among the cultured legumes, pea (*Pisum sativum* L.) is one of the most widely spread crops in Europe, playing a very important role in human nutrition and animal feed [2]. Considering the importance of this grain legume together with the selection of organic agriculture as a more sustainable agricultural system, the development of more resilient cultivars has been recognized as a key strategy. Seed germination and seedling establishment are among the most critical stage and key factors for successful crop production [3]. In order to support the imposed needs, several breeding programs have been implemented aiming to develop new cultivars characterized by seeds with greater plasticity toward environmental pressures, allowing them to more efficiently germinate. The European project LIVEESED [4] which integrates efforts by seed production companies and research/breeding Institutes, is one example of the high interest in seed research.

Considering that germination involves the activation of several metabolic pathways, including cellular respiration to provide the required energy, the involvement of the alternative respiratory pathway during germination was explored. Alternative oxidase (AOX) is a mitochondria inner membrane enzyme with a key role in the alternative respiratory pathway, in which this enzyme introduces a branch into the electron transport chain (ETC) at the ubiquinone pool. In this branch, AOX allows the transport of electrons to oxygen directly from the ubiquinone, preventing excessive reduction of the downstream complexes [5]. The involvement of this pathway in plant response to a diversity of environmental stresses and cell reprogramming events has been demonstrated [6,7], mainly associated with the ability to contribute to the control of reactive oxygen species (ROS), thereby reducing oxidative damage [8]. In addition, a link between AOX gene expression and metabolic and respiratory parameters was established in different plant species under biological systems/stress conditions [9]. Considering that seed germination involves the activation of several metabolic pathways, metabolic and respiratory changes were monitored by using calorespirometry. Calorespirometry simultaneously measures metabolic heat rates (R_q) and CO_2 emission rates (R_{CO2}) of biological samples and has been used as a screening tool to assess metabolic and respiratory changes associated with cell reprogramming events [10].

The present research investigates the involvement of PsAOX on efficient seed germination, and explores the link between AOX and metabolic/respiratory changes.

2. Methods

Seeds of four *P. sativum* L. cultivars ('Respect-1', 'S134', 'G78' and 'S91') provided by LIVESEED partners were selected for calorespirometry measurements. Previously, seeds were imbibed in sterile tap water for 16 h. Calorespirometric parameters (heat and CO_2 emission rates) were measured at 25 °C using a Multi-Cell Differential Scanning Calorimeter in isothermal mode. A total of 16 biological replicates (16 seeds) were considered per cultivar. Heat rate (R_q) and CO_2 emission rate (R_{CO2}), were determined according to Rodrigues et al. [11]. Germination rates were recorded 6 days after calorespirometric measurements.

The involvement of PsAOX in seed germination was evaluated by gene expression analysis of the three *P. sativum AOX* genes (*PsAOX1*, *PsAOX2a*, and *PsAOX2b*), and by the analysis of AOX protein levels through Western blot. Samples of the four pea cultivars were collected at 16 h post imbibition and further homogenized using liquid nitrogen. A total of 12 samples, each one consisting of a pull of 4 seeds, were used per cultivar.

For *PsAOX* gene expression studies, the cv. 'Respect-1' and cv. 'S91' were selected based on the results obtained by calorespirometry. Total RNA was extracted from the harvested material using the Maxwell® 16 LEV simplyRNA Cells Kit (Promega) and integrity evaluated through 1.2% agarose gel electrophoresis. 1 µg of total RNA was used for cDNA synthesis using the SensiFAST™ cDNA Synthesis Kit (Bioline). Transcript level accumulation of the three pea *AOX* genes (*PsAOX1*, *PsAOX2a* and *PsAOX2b*) was assessed in an ABI 7500 system using SensiFAST SYBR Lo-ROX kit (Bioline). *PsPOB* and *PsSAR1* were used as reference genes for data normalization. Primer specificity was evaluated by visualization of a single peak at the dissociation curve and efficiency (E) was calculated by the equation: E (%) = $10^{-(1/slope) - 1)}$ × 100 [12]. To obtain the slope value a standard curve of a 4-fold dilution series was generated for each primer pair.

For analysis of AOX protein levels, all four cvs. were considered: 'Respect-1', 'S134', 'G78' and 'S91'. Total protein content was extracted by phenol precipitation from 50 mg of plant material (adapted from [13]) and total protein quantification of 12 replicates of each cultivar were performed using the Pierce 660 nm Protein Assay Reagent (Thermo Scientific™). Western blotting was used for comparison of the levels of PsAOX protein between the cultivars. Briefly, after protein separation by SDS PAGE (25 µg total protein from each sample) in 14% polyacrylamide gels, using Laemmli buffer system [14], proteins were transferred to a PDVF membrane by electroblotting using a *Semi-Dry Trans-Blot Turbo Transfer* (Bio-Rad) system. After transferring, blocking was performed with 5% non-fat milk powder in TBS-Tween 20 and the membrane was incubated with primary antibody Anti-AOX1/2 (Agrisera AS04054; dilution: 1:1000), overnight at 4 °C. AOX bands were detected with an alkaline phosphatase-linked secondary antibody (anti-rabbit, Agrisera AS09607, 1:10,000 dilution), using a chemifluorescent substrate (ECF Plus Western Blotting Detection Reagents, GE, Healthcare). Membranes were placed in a transilluminator (Bio-Rad Gel-doc system) and a semi-quantitative analysis of band intensity was carried out using the software Bio-Rad Image Lab 5.2.1. For Western blot analysis, six replicates of each cultivar were used.

Statistical analyses were performed by SPSS version 22.0. Normality and homoscedasticity were checked in all data. One-way ANOVA followed by a Tukey HSD test was used for the comparison of calorespirometric parameters, germination rate and AOX band volume between the four cultivars. The comparison of transcript level of *PsAOX1, PsAOX2a and PsAOX2d* between the two cultivars was made using the T-Student. When the data did not meet the assumptions to perform parametric tests, the Kruskal-Wallis or Mann-Whitney nonparametric tests were conducted. Statistical significance was considered at $p < 0.05$.

3. Results and Discussion

At 25 °C, calorespirometric parameters Rq and R_{CO_2} (Figure 1) were significantly lower in cvs. 'S91' and 'G78', compared to cvs. 'Respect-1' and 'S134'. Later, at 6 days post imbibition, seeds of cvs. 'G78', 'S134' and 'Respect-1' presented significantly higher germination rate, compared to the cv. 'S91'. These results show that higher Rq and R_{CO_2} could be related with higher germination rates, as previously reported by Edelstein et al. [15], who observed that higher values of metabolic activity were associated with higher germination rates of melon seeds. With seed lots from onion and several Brassica species a positive correlation has been shown between single seed respiration and seed quality [16].

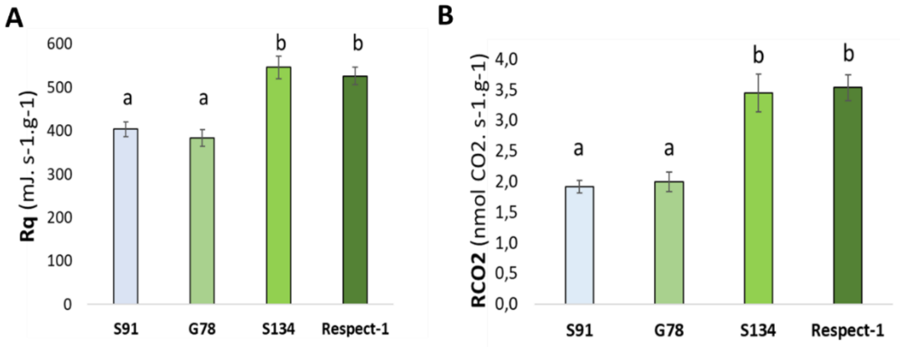

Figure 1. Calorespirometric parameters for the four pea (*Pisum sativum* L.) cultivars ('S91', 'G78', 'Respect-1' and 'S134') measured at 25 °C, 16 h post imbibition. (**A**) Respiratory heat rate—Rq, (**B**) CO_2 production rate—R_{CO_2}. Data are the mean value of 16 measurements ± standard error. Different letters indicate significant differences between the cultivars. Statistical significance was considered for $p < 0.05$.

The *AOX* gene family is nucleus encoded, composed of one to six members distributed in two subfamilies (*AOX1* and *AOX2*) [17]. In pea, the AOX gene family is composed by one AOX1-subfamilymember (*PsAOX1*) and two AOX2-subfamily members (*PsAOX2a* and *PsAOX2b*) [18]. Based on calorespirometric results, for *PsAOX* gene expression studies, the cv. 'Respect-1' and cv. 'S91' were selected. Regarding the analysis of *PsAOX* gene expression performed 16 h post water imbibition, a higher transcript accumulation of *PsAOX2b* was observed in comparison with *PsAOX1* and *PsAOX2a*. Additionally, transcript accumulation was significantly higher in cvs. 'S91' when compared with cv. 'Respect-1 (data not shown).

Previous studies with tobacco and Arabidopsis have demonstrated the importance of *AOX1* in photosynthesis-related pathways and respiration under light conditions [19]. Members of the AOX1-subfamily have also been associated with plant responses to abiotic stress factors [6,7]. At 16 h post imbibition, the photosynthetic apparatus is still not activated which could explain the lower levels of *PsAOX1* transcript. Additionally, besides AOX involvement in ROS control, at the timepoint considered, the ROS levels were already reduced through the enzymatic ROS scavenging system and the action of antioxidant metabolites.

On the other hand, members of the AOX2-subfamily have been mainly associated with developmental processes [6]. The higher level of transcript accumulation achieved for *PsAOX2a* lead us to suggest a higher involvement of this gene in the germination process, particularly in case of cv. 'S91'.

The analysis of total protein concentration in the four *P. sativum* cultivars showed significantly higher protein concentration in cv. 'Respect-1' compared to cvs. 'S134', 'G78' and 'S91'. No statistically significant differences in total protein concentration were observed among cvs. 'S134', 'G78' and 'S91' (data not shown). The PsAOX protein levels were evaluated 16 h post water imbibition by Western blot. The band corresponding to the PsAOX protein was detected in all cultivars and higher levels of protein were observed in cv. 'S91' when compared to cvs. 'Respect-1' (RS), 'S134' and 'G78'. Although we were not able to analyze the different isoforms of the AOX protein through the Western Blot analysis, the results at protein level confirm the results observed by transcript analysis, i.e., a higher AOX protein expression in the cultivar that presents the highest level of AOX gene expression.

The absence of a direct relationship between the total protein concentration and the level of the AOX protein could be expected, since the AOX is only a part of the total protein concentration [20]. In fact, the values of total protein concentration reflect essentially the concentration of storage proteins which is variable among cultivars [21]. Moreover, studies focused on the protein composition of pea seeds showed that a large percentage of the identified proteins correspond to storage proteins (mainly albumin, legumin and vicilin), with the rest being involved in the response to biotic and abiotic stress, energy production, metabolism and storage of essential non-protein compounds [21].

Overall, seeds from the cultivar characterized by lower R_q and R_{CO2} (cv. 'S91'), coincident with lower vigour, exhibited significantly higher expression of the *PsAOX* genes (*PsAOX1, PsAOX2a* and *PsAOX2d*) and PsAOX protein. From these results, we hypothesize that higher levels of AOX (transcript and protein) in germinating seeds are linked to lower metabolic rates and germination. Further studies will be required to validate this hypothesis.

4. Conclusions

To the best of our knowledge, the study presented hereby represents the first approach aiming to establish a link between respiratory parameters, monitored by calorespirometry, and *PsAOX* transcript accumulation/PsAOX protein expression during germination of pea seed. The present work suggests the involvement of AOX and the alternative respiratory pathway during seed germination and the applicability of calorespirometry to assess seed vigor, pointing to this method as a useful non-destructive phenotyping tool for selecting genotypes with superior germination capacity.

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Conflicts of Interest:

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