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Research article

Diffusions of sound frequencies designed to target dehydrins induce hydric stress tolerance in *Pisum sativum* seedlingsVictor Prévost^a, Karine David^b, Pedro Ferrandiz^a, Olivier Gallet^c, Mathilde Hindié^{c,*}^a Genodics, SAS, 23 Rue Jean-Jacques Rousseau, 75001 Paris, France^b CY Cergy Paris Université, Biology Department, F-95000, Cergy, France^c CY Cergy Paris Université, ERRMECE, F-95000, Cergy, France

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ABSTRACT

Among plant responses to environmentally induced stress modulating protein expression appears to be a key stage in inducible signaling. Our study was focused on an innovative strategy to stimulate plant stress resistance, namely, the use of targeted sequences of specific sound frequencies.

The influence of acoustic stimulation on plant protein synthesis was investigated. In our study green peas, *Pisum sativum*, were cultured under hydric stress conditions with targeted acoustic stimulation. Acoustic sequences targeting dehydrins (DHN) which accumulate in plants in response to dehydration were studied. We experimented on pea seedling with two different sequences of sounds: the first one corresponded to DHN cognate protein and the second one was aimed at the DHN consensus sequence. Shoot elongation after pea seed germination was estimated by fresh weight gain studied in the presence of various conditions of exposure to both sequences of sounds. DHN expression in peas was quantified via ELISA tests and Western-blotting by using specific antibodies.

A significant increase in fresh weight in peas grown under exposure to the DHN cognate sound sequence was observed, whereas the consensus sound sequence had no effect on growth. Moreover, the 37kDa DHN amount was increased in peas treated with the consensus acoustic sequence. These results suggest that the expression of DHN could be specifically modulated by a designed acoustic stimulus.

1. Introduction

In contrast to animals which may escape when they are exposed to environmental constraints, plants, which are rooted to the ground, have to commonly deal with a changing environment.

This is why plants develop more numerous and specific mechanisms to adapt to both biotic (pests, symbionts, etc.) and abiotic stresses (light, drought, temperature). Among abiotic stresses, it is now well established that plants are sensitive to physical and mechanical stimuli (e.g. wind, touch, vibrations, sounds).

Sound impact on plants has been investigated for decades, with an increase in interest attested by recent reviews on plant-sound interaction [1, 2]. Although plant perception of sounds has often been approached through a purely mechanical standpoint and associated with general mechanoperception [3, 4], some researchers have investigated plant sensitivity to specific acoustic stimulations.

Such studies on the influence of sounds on plants could be divided into two groups:

- Mainly effects of specific frequencies [5, 6, 7, 8, 9]
- More rarely, impact of musical sequences [10]

The influence of sound waves was mostly considered by researchers as a mechanical stimulation that modulates plant behavior. Sound amplitude also needs to be considered when sound waves are applied to plants. Different sound frequencies applied to the same plant type lead to different developmental and physiological adaptations including transcriptomic, proteomic, and hormonal changes used to increase plant growth and production [9, 11, 12].

This perception of sounds in plants is associated with specific metabolic reactions. A summary of these studies and their methods provided by A.A. Fernandez-Jaramillo et al [2] reveals that specific plant metabolisms can be up- or down-regulated with specific sounds between 20Hz and 500 kHz. Most of the reviewed studies show a significant effect of audible sounds (20Hz–20 kHz) on various functions such as root tropism, polyamine production, O₂ uptake, hormone regulation, fruit maturation, germination, ATP amount.

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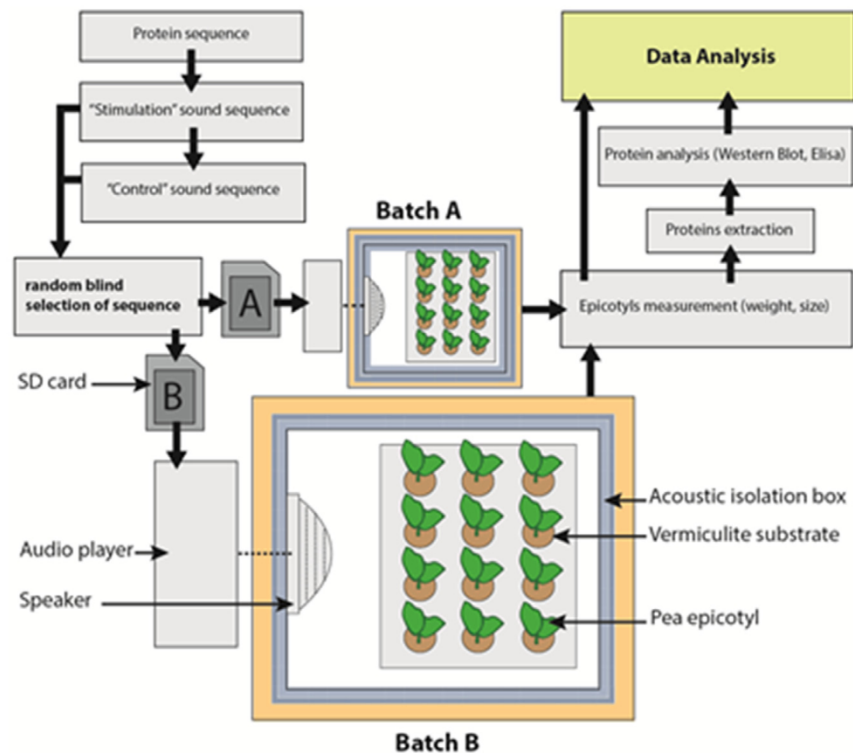


Figure 1. Schema describing the experiment procedure used.

Additionally, the idea of obtaining sounds and melodies from biological models has been explored through various attempts to identify and visualize musical patterns in living organisms. The search for musical patterns in genes [13, 14, 15, 16], biological functions [17], ecological microbiota [18] and protein sequences has provided a range of new tools for representing biological data [19, 20], as well as consistent confirmation of the underlying link between musical patterns and biological structures.

Those studies arrived at the same conclusion that studied plants are able to perceive various sound frequencies distinctively, and are also able to have a metabolic reaction to complex and informative rich sounds such as musical sequences [21].

Another less well studied physical aspect of the acoustic stimulation of organisms is the influence of the harmonic resonance of these sequences. Recently, the influence of such sequences composed of specific frequencies has been tested on human cancerous cells with significant results in reducing their proliferation [22].

Based on such previous reports, we decided to study the influence of specific acoustic sequences on plant behavior. Our experimental model has been designed to investigate the effect of specific sound frequency sequences on the common green pea *Pisum sativum*, by targeting a hydric stress resistance protein known as dehydrin (DHN) which is widely present in the plant kingdom. DHN are a family of drought resistance proteins present in various forms and proportions during the life cycles of *Pisum sativum* [23, 24]. The main role of DHN consists in water stress regulation, and DHN accumulation in plant tissues under different kinds of water stress is well established [25, 26, 27]. The exact role of the DHN protein family has still not been explained. DHN are known to minimize macromolecules unfolding during dehydration, ionic or osmotic stresses [28]. Saibi et al [29] also reported the regulation of protease activities in DHN transgenic *Arabidopsis thaliana* under salt stress as compared to the wild variety. It was also shown that a *Vitis riparia* DHN overexpressed

during abiotic stresses was able to protect DNA from damage caused by hydrogen peroxide - a reactive oxygen species (ROS) source - without interfering with any DNA functions [30]. Furthermore it was recently demonstrated that DHN could act on salt and osmotic stress signaling pathways as a positive regulator [31]. Recent data showed that a DHN inducible promoter is overexpressed under abiotic stresses such as high temperature, salt stress and drought [32]. All together, those data suggest that DHN overexpression is directly dependent on hydric stress and newly synthesized DHN will interact with macromolecules to protect them from abiotic stress damage caused by ROS and hydrogen peroxide.

Two acoustic sequences were designed to target two peptides characteristic of two different DHN. One note was associated with each amino acid of both peptides according to J. Sternheimer's patents [33, 34]. For instance an A0 note is associated with the amino acid glycine.

The germination and early growth of *Pisum sativum* epicotyls cultured under hydric stress conditions and exposed to these targeted sound sequences were investigated. The impact of this exposure of peas to DHN associated sound sequences was investigated on total pea lysates. The changes in DHN expression was quantified by Western-blotting.

2. Material and methods

2.1. Experimental settings

Each experiment was conducted simultaneously on two separate pea batches. To avoid any phonic contamination, each batch was acoustically insulated from the other. Each batch consisted of germination trays in which *P. sativum* seeds Primavil (Vilmorin, France) were ground in a hydrated vermiculite substrate. Cultures of peas were performed for 8 days without any watering, providing a homogenous hydric stress on growing epicotyls.

Using frequencies calculated from the mass of each amino acid, a model of specific sounds designed to interact with living organisms has been developed and patented by J. Sternheimer [34]. All these sequences were obtained from a patented method and have been submitted for Author's right and Copyright. This method was designed to direct a sound sequence, correlated with the sequence of masses of each amino acid in a specific protein, in order to regulate the synthesis of the targeted protein. Those sound sequences were stored on identical memory cards (SanDisk 4GB) in MP3 audio format.

A blind protocol was established from this stage of the experiment: cards containing the sound sequences were randomly distributed amongst the two batches, i.e. "stimulation sequence" in one batch and "control sequence" in the other, which were only to be revealed after data analysis. Our technical approach is schematized in Figure 1.

Additionally, three different operators were in charge of setting the sequences, harvesting the data and analyzing the data respectively, which made up for a rigorous blind procedure [35].

The sequences were automatically played at the same volume (scattering volume of 15%) through speakers (Kenford-80 W maximum power) connected to a music player, for five minutes each night.

2.2. Protein sound sequences

The experiment of exposing *Pisum sativum* to protein sound sequences was split into two sub-experiments. First, in order to quantify the effect of the sound sequence on plant growth under hydric stress, we focused on a specific water stress resistance protein called DHN cognate [36]. Among the DHN cognate sequences we chose a 25 amino acid residue common to several Fabaceae DHN. Second, we targeted a 19 amino acid residue lysine-rich consensus sequence previously described in all plant DHN identified for which directed primary antibodies are commercially available.

- Growth and stress resistance

The sound sequences were obtained from the succession of amino acids in the selected part of the protein according to the above-mentioned method. The first 25 amino acids from the *Pisum sativum* DHN cognate were converted into the following note sequence (A3 = 440Hz):

DHN-cog amino acids: MAEENQNKYEEITTSATNSETIKDR.

Associated notes: A3 C3 A3 A3 G3 A3 G3 A3 C4 A3 A3 F3 F3 E3 C3 F3 G3 E3 A3 F3 A3 G3 A3 G3 C4.

- DHN stimulation

In order to positively regulate the *Pisum sativum* DHN pool synthesis, the consensual poly-lysine fragment (poly-K) common to all DHN was chosen [25].

The first 19 amino acids of this consensus fragment were converted into the following note sequence:

Poly-K amino acids: TGEKKGIMDKIKEKLPQGH.

Associated notes: F3 A2 A3 A3 A3 A2 G3 A3 G3 A3 G3 A3 A3 A3 G3 F3 A2 A3 B3b.

- Control sequences

"Control sequences" were deduced from the "DHN-cog sequence" and "DHN stimulation sequence". Those sequences were the same length, respectively, and contained the same notes but they were played in random order.

All these note sequences were then recorded in MIDI format, looped in order to reach a five-minute duration for each, and were then converted into MP3 format.

2.3. Harvest and measurement

Plants were grown for eight days before harvesting. During harvesting, each epicotyl was individually taken out of the substrate; vermiculite was washed off roots under running water. Each plant was gently dried with absorbent paper before being weighed. After measurements, plants were stored at -80 °C.

2.4. Protein extraction

Frozen whole plants were ground in a blade mill at +8 °C with a cold extraction buffer (TRIS 10mM pH 7.5 + 3mM PMSF dissolved in acetone; 0.2 ml/g protease inhibitor cocktail P9599 Sigma) at 1.5 ml extraction buffer per g of fresh germinations. After obtaining a homogenous solution, we filtered it on gauze (x2 layers). The filtrate was centrifuged (6,000g, 20min, 4 °C). The supernatant was recovered and centrifuged again under the same conditions. The final supernatant was recovered. We used the heat-shock resistance property of DHN to concentrate the final solution with targeted proteins. The previously recovered solution was placed in an agitated water bath (10min 70 °C) in order to precipitate non-heat-shock resistant proteins according to the purification method experimented by Ismail, A. M. et al [37]. The solution was then centrifuged (17,000g 1h 4 °C) and the supernatant carefully recovered and stored (-20 °C). Protein concentrations of each protein extracts were previously quantified through the Bradford assay.

2.5. Enzyme linked immunosorbant assay (ELISA)

The amount of DHN in each sample was quantified via indirect ELISA. 100µl of protein extracts diluted in carbonate buffer (pH 9.4) at 20 µg/ml concentration were applied to Maxisorp® 96-well plates (NUNC) and were incubated overnight at 4 °C according to the method developed by Hnasko R. et al [38]. Plates were washed four times with Tris-buffered saline with 0.1% Tween-20 (TBST) at room temperature. Wells were blocked for 1h at 25 °C with TBST supplemented with 10% of skimmed-milk powder. The wells were then aspirated and 100µl of anti-DHN primary antibody (ADI-PLA-100 Enzo Scientific) 1/1000 diluted in TBST supplemented with 0.1% skimmed-milk powder were applied. After 1h incubation at 25 °C the wells were washed four times with TBST. Then horseradish peroxidase-coupled secondary antibody (NA934V GE) 1/5000 diluted in 0.1% non-fat milk added to TBST was incubated 1h at 25 °C. Four washes with TBST were done and antibodies were revealed with a tetramethylbenzidine (T0440 Sigma) substrate for 30 min at 37 °C, the reaction was stopped with 100µl 1N HCl and measurements were taken at 450nm.

2.6. Western blot

The DHN enriched solution of pea proteins was mixed with a Laemli buffer (4X) and loaded onto a 12% polyacrylamide gel at 20µg per well. After the SDS-PAGE, proteins were transferred onto a nitrocellulose membrane using a trans-blot cell (Bio-Rad). Ponceau Red staining was then performed to check protein transfer onto the nitrocellulose membrane. An image of each membrane was acquired using an imager (Image Quant LAS 500 GE) to obtain total loaded protein quantification. The membrane surface was then saturated in a solution of 5% w/v skimmed-milk powder, 0.1% v/v PBS + Tween-20 (2h). The membrane was washed with PBS + Tween-20 (0.1% v/v) and incubated (overnight, 8 °C) with polyclonal anti-DHN rabbit antibody (ADI-PLA-100 Enzo Scientific) at 1/1000 dilution in 5% w/v defatted-milk powder, 0.1% v/v PBS + Tween-20. After incubation, the membrane was washed with PBS + Tween-20 (0.1% v/v) and then incubated to reveal specific antibody bindings with phosphatase alkaline conjugated anti-rabbit antibody

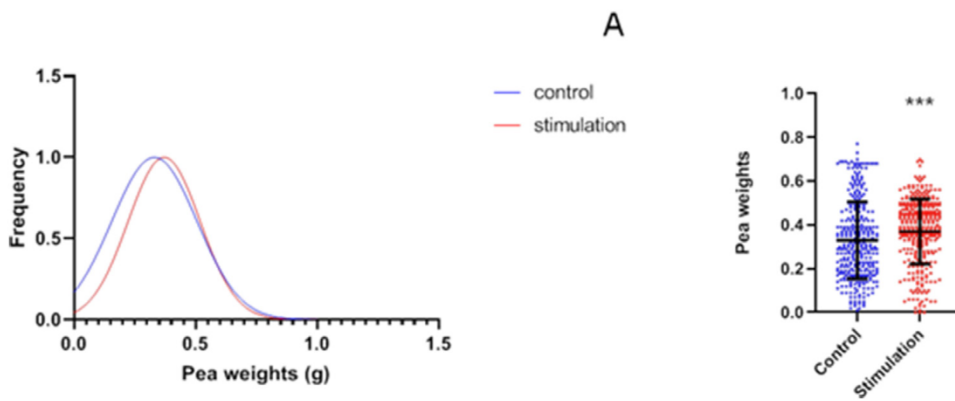


Figure 2. Effect of acoustic treatment on peas subjected to hydric stress and grown for 8 days in darkness. Acoustic sequence corresponds to DHN-cognate. A: Weight frequency distribution, red and blue colors indicate “stimulation” and “control” sequence respectively. B: Weight of peas was measured for each culture condition; analyses were performed on 3 biological replicates. Means of data with standard deviations are presented. Stars show significant differences ($p < 0.01$) as determined by Student's t-test.

(A3687 Sigma) (dilution 1/5000 in saturating buffer for 1h). Phosphatase activity was detected using a Bio-Rad AP color reagent kit and quantified with the Image Studio® software. The normalized signal was calculated using the total protein quantification previously done with Ponceau Red staining.

2.7. Signal detection and analysis

Results are presented as means of three independent experiments with standard deviations (SD). Each experiment was repeated at least three times. Statistical analyses were performed using unpaired two-tailed Student's t-tests. All statistical tests and graphs were generated using Prism8 (GraphPad).

3. Results and discussion

3.1. Effect of acoustic sequences on pea growth

The effects of acoustic stimulations on pea growth were initially tested with cultures in darkness. The analysis of the effect of either “stimulation” or “control” sequences on the germination of peas grown for 8 days under hydric stress highlighted a significant difference in epicotyl weight frequency distribution as presented in Figure 2. Peas receiving the acoustic sequences corresponding to the DHN-cog “stimulation” sequence presented higher weight values as compared to those grown under the “control” acoustic sequence.

Mean epicotyl weights were significantly higher for “DHN-cognate stimulation” (population ($0.37g \pm 0.15g$) versus “control” population ($0.33g \pm 0.18g$)). The data presented are the results of 3 different experiments with no less than 340 epicotyls for each condition.

In a second set of experiments, peas were grown for 8 days in daylight under hydric stress in the presence of either “DHN-cognate stimulation” or the “control” acoustic sequence (Figure 3). Weights of whole peas

were measured and differences were observed: peas cultured with DHN-cognate acoustic stimulation were significantly heavier ($1.13g \pm 0.39g$, $n = 167$) compared to the control ones ($0.84g \pm 0.37g$, $n = 167$).

In both experiments, a gain in the pea epicotyls' weight was observed in the presence of DHN-cog acoustic stimulation. Pea growth was not impacted by potent luminous input but by the acoustic stimulation.

The peas were then cultured under drought conditions in darkness for 8 days, and the DHN consensus sequence was used for acoustic stimulation. Control cultures were done in the absence of the DHN consensus “stimulation” sequence and were exposed to the corresponding “control” sequence. Unlike previous results with the DHN-cognate “stimulation” sequence, no significant difference was observed on the epicotyls' growth. Mean weight for the pea population cultured with the DHN consensus “stimulation” sequence ($1.03g \pm 0.26g$, $n = 476$) was comparable to the control one ($1.04g \pm 0.23g$, $n = 478$). The data shown are the results of 4 different experiments with no less than 476 epicotyls for each condition. The DHN consensus acoustic stimulation didn't produce significant weight change in peas grown in darkness in hydric stress conditions as observed in Figure 4.

Hence the same hydric stress was applied in all sets of experiments with only changes in the acoustic sequence applied. Thus the gain in weight observed in peas cultured with the DHN-cognate “stimulation” sequence was due specifically to the acoustic stimulation sequence applied. Since the same notes played in random order did not influence pea weight as observed in control samples, we conclude that the gain in weight presented in Figures 2 and 3 was induced by the specific acoustic stimulation sequence of the DHN cognate played during experiments.

Furthermore, the DHN cognate sequence used in this study that is common to some Fabaceae contained both the specific Fabaceae 25 amino acid residue (DHN-cog amino acids) and the plant consensus poly-K DHN. We hypothesize that the gain in weight observed was induced by all the DHN expressed in peas.

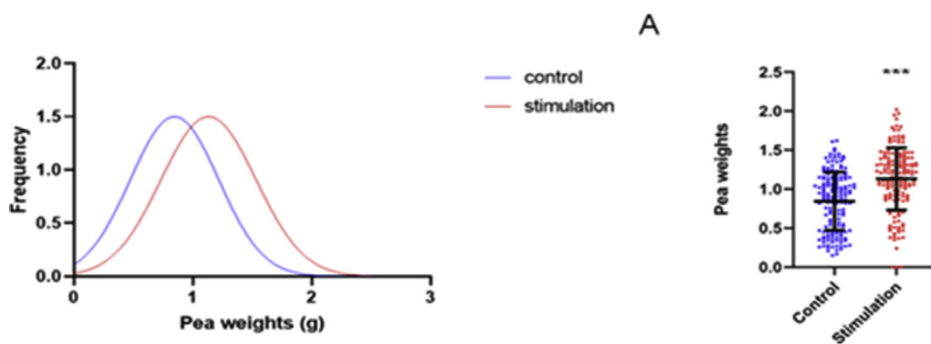


Figure 3. Effect of acoustic treatment on peas subjected to hydric stress and grown for 8 days in daylight. DHN-cognate sequence was used for acoustic stimulation. A: Weight frequency distribution, red and blue colors indicate stimulation and control sequence respectively. B: Weight of peas was determined for each culture condition; analyses were performed on 3 biological replicates. Means of data with standard deviations are presented. Stars show significant differences ($p < 0.01$) as determined by Student's t-test.

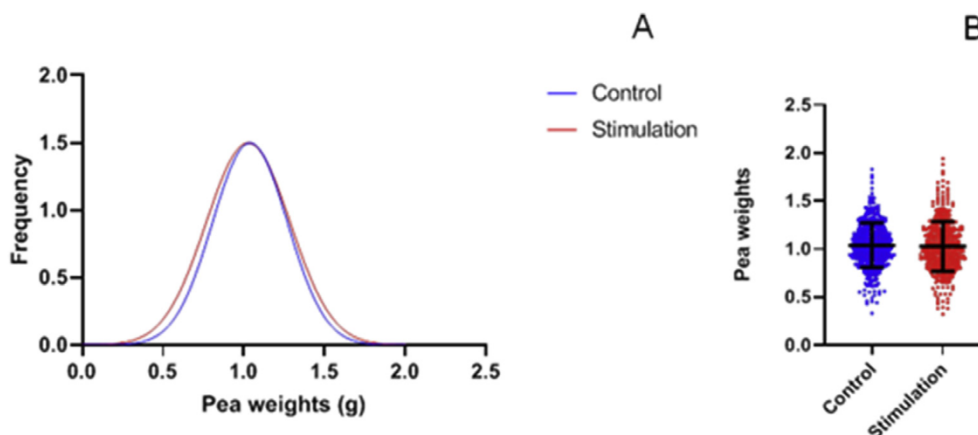


Figure 4. Effect of acoustic treatment on peas subjected to hydric stress and grown for 8 days in darkness. DHN consensus sequence was used for acoustic stimulation. A: Weight frequency distribution, red and blue colors indicate stimulation and control sequence respectively. B: Weight of peas was determined for each culture condition; analyses were performed on 4 biological replicates. Means of data with standard deviations are shown.

3.2. Analysis of DHN expressed in peas under sound treatment

Our results demonstrated an effect of the DHN-cognate acoustic sequence on pea fresh weight. The expression of DHN in the peas grown was then studied to determine if it was directly modified by the DHN consensus acoustic stimulation. First, ELISA tests were performed to quantify the DHN expressed in peas after acoustic stimulation. Available anti-dehydrin antibodies were directed towards the consensus poly-K segment of dehydrins. Therefore, only ELISAs performed on peas

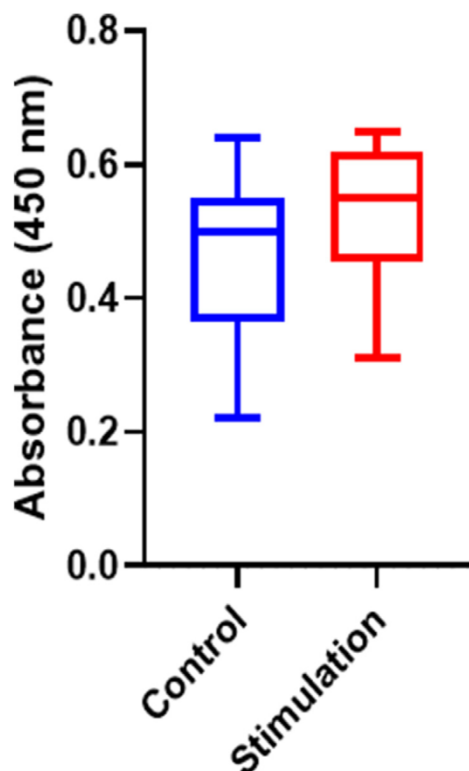


Figure 5. Effect of sound treatment on DHN synthesis in peas exposed to hydric stress and grown for 8 days in darkness. DHN consensus sequence was used for acoustic stimulation. DHN level in pea extracts was quantified via ELISA. Expression of DHN was quantified for each culture condition, 4 replicates were performed for each condition. For each plot the line within the box represents the median, as presented by Novitsky et al [41]. The lower and upper lines of the box represent the 25th and 75th percentiles, and the lower and upper adjacent lines (whiskers) the 10th and 90th percentiles respectively.

stimulated by the acoustic stimulation corresponding to the consensus sequence are presented. In contrast, even though the DHN cognate presents a poly-K consensus sequence, it was weakly recognized by commercial antibodies; no exploitable data were obtained with extract from DHN cognate stimulated peas (data not shown).

Since polyclonal antibodies against the consensus poly-K segment of DHN used for ELISA tests are able to link to all forms of DHN present in the pea extract, every DHN could be detected, including those not directly correlated to the hydric stress induced in our model. More than 3 different forms of DHN were detected in *Pisum sativum* and their synthesis was not co-occurring [24]. Nevertheless, a slight increase in the DHN expressed in the pea extract after consensus sequence acoustic stimulation was observed demonstrating an increase in DHN in epicotyls as presented in Figure 5.

To determine what type of DHN could be influenced by the acoustic stimulation, Western blots were performed on heat-stable protein lysates from peas with the same polyclonal antibody raised against the DHN consensus poly-K segment (Figure 6).

Among the pea epicotyl proteins collected, three protein bands were recognized by the anti-DHN antibody. These bands were identified with a molecular weight of 37 kDa, 27–30 kDa (as observed by Garnzarska et al [23]) and 15 kDa respectively (Figure 6). Since the band doublet 27–30 kDa appears weakly (however enhanced under stimulated conditions), it was not investigated any further. The level of expression of 37 kDa and 15 kDa proteins was measured through densitometry and signals were normalized using total protein stains. The 37 kDa DHN band revealed a larger amount of DHN in the “stimulation sequence” samples versus the “control sequence” samples, whereas the 15 kDa bands were identical for both culture conditions. The normalized values of band signals were directly correlated with the amount of DHN extracted from the epicotyls.

Our results also suggest an increase in the band located around 37 kDa in the case of the “stimulation sequence”, compared to “control” peas under the same hydric stress. This suggests that the “DHN stimulation acoustic sequence” promotes an early expression of one type of DHN despite the hydric stress already applied.

A 16 kDa band was well observed in each assay. The densitometric analysis of this band showed no significant difference between both culture conditions. A previous study on pine DHN has identified a constant 16 kDa DHN concentration under hydric stress conditions in pine needles [39]. Similar results were also observed on Bermudagrass, where Suk et al. [40] demonstrated that 16 kDa DHN contributes to drought tolerance. Although such a mechanism has not been investigated in *Pisum sativum* yet, we hypothesize that 16 kDa DHN is expressed at a constant rate in response to hydric stress but does not seem to be influenced by the DHN poly-K segment acoustic stimulation.

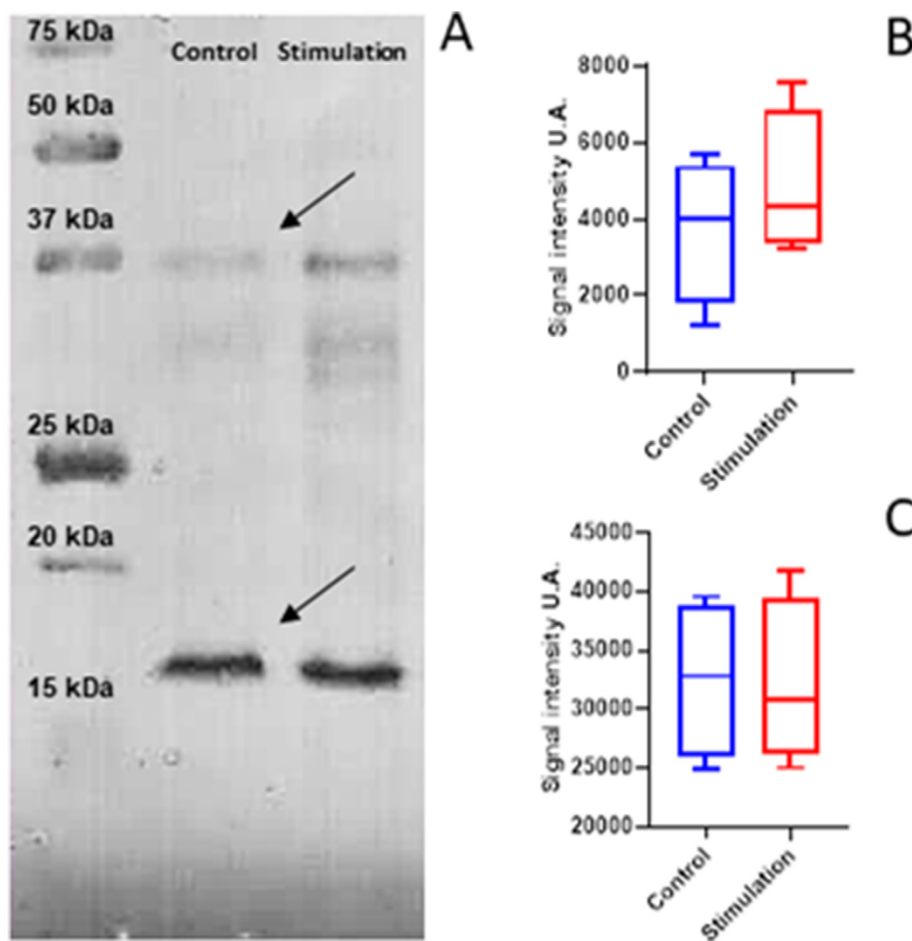


Figure 6. Effect of acoustic stimulation on DHN expression in peas subjected to hydric stress and cultured for 8 days in darkness. DHN consensus sequence was used for acoustic stimulation. (A) Representative Western-blot, (B) densitometric analysis of 37 kDa DHN bands, normalized by the total amount of proteins and (C) densitometric analysis of normalized 15 kDa bands. Vertical box plots of the normalized signal ($n = 4$). For each plot, the line within the box represents the median. The lower and upper lines of the box represent the 25th and 75th percentiles, and the lower and upper adjacent lines (whiskers) the 10th and 90th percentiles respectively.

Those results suggest a specific increase in the 37 kDa DHN amount in peas exposed to the “DHN consensus stimulation sequence”, compared to peas exposed to the “control” sequence.

4. Conclusions

Taken together, our results suggest that exposure to an acoustic frequency sequence correlated with a specific amino acid sequence of DHN could act as a positive modulation factor in the adaptation of *Pisum sativum* to hydric stress. Using two specific sequences correlated with DHN cognate on the one hand, and with DHN consensus on the other hand, we obtained two specific responses depending on the acoustic stimulation.

An increase in the fresh weight of pea epicotyls was induced when they were exposed to acoustic stimulation targeting the 25 amino acids DHN cognate peptide described in several Fabaceae DHN.

The expression of 37 kDa and 29–30 kDa DHN in pea epicotyls under water stress was augmented when exposed to the proteodies correlated with the 19 amino acid lysine-rich DHN consensus protein fragment. Given, on the one hand, recent data showing that a DHN inducible promoter is overexpressed under abiotic stress [32] and, on the other hand, our results demonstrating the increased amount of DHN under specific acoustic stimulations (proteodies), we could hypothesize that proteodies diffusion acts in a specific way in synergy with hydric stress signaling pathways. Those preliminary results could be further investigated with

accurate protein and gene expression monitoring in living organisms with a well-defined metabolism such as bacteria, fungi, mammalian cells, etc.

Declarations

Author contribution statement

V. Prévost, P. Ferrandiz: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

K. David: Performed the experiments.

O. Gallet, M. Hindié: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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