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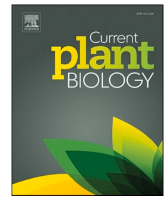

The proteome of the imbibition spillage in *Cicer arietinum* L.

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ABSTRACT

Being a preparative step for germination, seed imbibition is a hydration process that involves the release of seed molecules into the environment, an essential ecological aspect of this phase. On one side this leakage leads to unlocking the seed dormancy by removing abscisic acid and other pro-dormancy molecules, on the other side, it releases small molecules such as vitamins, amino acids, flavonoids, and proteins contributing to supporting germination by attracting symbionts, contrasting pathogens, and facilitating nutrients uptake. Here the proteome associated with the imbibition spillage of chickpea seeds emerges as a probe to understand the early events during germination and (pre-) symbiosis, providing a proxy to disclose the influence that the seed applies to the environment for optimal achievement of its eco-physiological needs. This proteome is clustered into two main groups that differ in chemical-physical properties and function. Most proteome entries belong to biochemical pathways that directly influence germination by enhancing nutrient uptake, protecting against stresses of various origins, and promoting symbiosis. A fraction of this proteome was found to be associated with accidental pathways due to the loss of proteins from teguments and fractured tissues. Here, germination, protection, and symbiosis emerge as a balanced proteomic triad aimed at enhancing and sustaining seedling emergence and plant growth.

1. Introduction

Seeds germination takes place through a complex sequence of events triggered by the phase of seed rehydration. During this phase, also known as imbibition, a significant exchange of substances with the environment occurs. While water represents the main substance that enters the seeds, a plethora of organic compounds, including proteins, leak from the seed. While the seed's leakage is only partially the result of an accidental spilling from teguments and tissues fractures, the rest of the substances released is related to three main functions: protection, symbiosis, and germination ([12,42]; Johansson and Bergman, 1992; [5,7,16,17,22,24,26,28,31]). This preparative stage of pre-germination is essential for driving seeds into their physiological maturity while shaping the neighbouring environment by dislocating molecules that are aimed at defending seeds against pathogens, attracting symbionts to promote symbiosis, and finally facilitating nutrients uptake. In this respect, it must be underlined the pivotal regulatory role that various phytohormones play by modulating both the end of dormancy (seed germination) and the plant-rhizobial symbiosis (nodule formation) [18].

Apart from studies where emerges the evident role of water during seeds imbibition [19], only a few studies highlighted the influences played by seed "leakage" and provided a measure of the extent to which this loss is essential in preparing the environmental conditions for promoting and enhancing germination [16,45,46]. By investigating the composition of the imbibing spillage from seeds, it is possible to gain hints about the early events of germination and (pre-)symbiosis, providing a probe to disclose the influence that the seed applies on the environment to satisfy its eco-physiological needs. One of the model organisms for this type of study is *Cicer arietinum* L., an annual legume widely known for its nutritional properties [43] and for a significant

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release of substances in the soil during the imbibition [7,20,35]. The complexity of the imbibing fluid in this species was already characterized in several reports providing important insights into its composition and the related functions of its components [35]. The seeds spillage was shown to unlock the dormancy by delivering abscisic acid and other pro-dormancy substances [3,21] as well as small molecules such as vitamins, amino acids, flavonoids, and proteins, to contribute to supporting germination by attracting symbionts, contrasting pathogens, and facilitating nutrients uptake [22,27,45]. It is worth mentioning that the relevant presence of amino acids, phytohormones, vitamins, and flavonoids in the imbibing solution [37] is essential in supporting germination and symbiosis with species-specific nitrogen-fixing bacteria such as *Mesorhizobium ciceri* [17,24]. Similarly, the presence of antioxidants, such as flavonoids and anthocyanins, provides important protection against environmental stress factors in Leguminosae [41]. Furthermore, promoting symbiosis since the very early stages of germination is very important because it contributes to rationalizing plant growth and development, by coping efficiently with the stress response [36]. In previous studies, the imbibing fluid from chickpeas was analysed by mass spectrometry showing it to be characterized by a consistent number of proteins and amino acids, with the latter able to induce chemotaxis hence promoting the early pre-symbiotic events [7]. Here, we deepen our investigation into the proteome of this solution to identify the key biochemical pathways and processes taking place during the imbibition phase. This approach allows exploiting the imbibing fluid as a molecular probe for unveiling the biochemical processes actuated by seeds to sustain germination. The results show a complex network of proteins and biochemical pathways important for promoting symbiosis and enhancing germination while a complex network of metabolic events takes place aimed at protecting the seed by preventing environmental stress and damage.

2. Results

2.1. The imbibition spillage proteome falls into two clusters of proteins

The imbibition spillage from mature-dry seeds of *C. arietinum* has been lyophilized obtaining 1.75 g of powder every 100 g of seeds, with 1 g of that amount being attributed to the protein content. This powder has been characterized by mass spectrometry resulting in a proteome of 812 unique Uniprot identifiers (entries). After identification, the amino acid sequence of each entry was used to obtain information such as molecular weight, isoelectric point (pI), and metabolic role. The descriptors' ranges and distribution analysed along the whole dataset were then applied to identify meaningful physiological patterns and indicators assuming the theoretical pI as meaningful when compared to its native counterpart [13] and considering the relevance of the pI for addressing protein compartmentalization [23,25,40].

Accordingly, the proteome was characterized for its mass and pI distribution. A basic cluster analysis (K-mean cluster; K=2) was performed to identify the clusters and the related centroids, finding coordinates 5.71; 4.5, and 9.12; 4.33 for Cluster 1 (C1 – acidic protein fraction) and Cluster 2 (C2 – basic protein fraction), respectively (Fig. 1a). The indication of the centroid serves to provide an averaged representation of the pI-to-mass ratio, offering insight into how the subgroups deviate from this average. Of the two groups, the C1 consisted of 515 entries with the pI centered in the 3.75–7.45 range, and the C2 consisted of 297 entries, centered in the 7.45–12.10 pI range (Fig. 1a and inset) with a C1/C2 ratio of 1.74.

The distribution of the pI values versus the charge was analysed in the pH range of 6–8, which is optimal for chickpeas' soil and growth. For the acidic fraction C1, the charge distribution was compact passing from prevalently positive at pH 6 to prevalently negative at pH 8 (Fig. 1b). Differently, the basic fraction C2 had a larger distribution and carried entries with an almost exclusively positive charge persisting without inversion at different pHs. Considering the pH range between 6 and 8 as optimal for chickpea growth, the low number of proteins (~3.7%) having a pI in this range, particularly around pH 7.5, could be explained

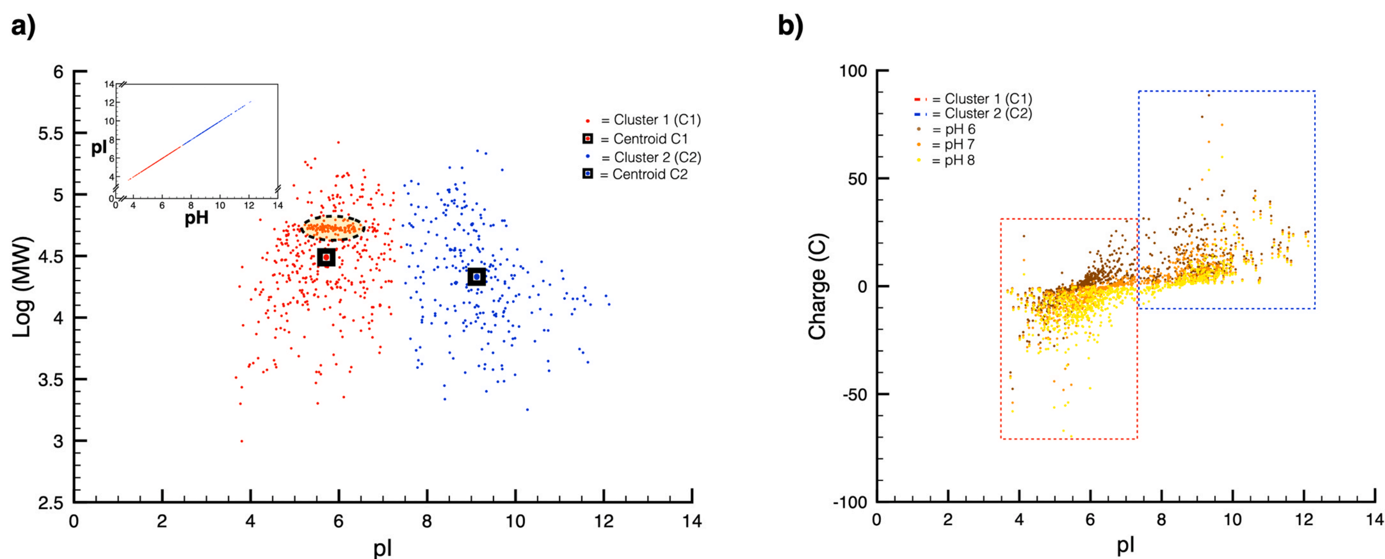

Fig. 1. a) Proteome distribution plotted as pI vs mass. When the pI of the entries is plotted versus their mass, the entries can be clearly separated into two groups, one in the 3.75–7.45 pI range (cluster 1, red dots) and the other in the 7.45–12.1 pI range (cluster 2, blue dots). Centroids for clusters 1 and 2 with coordinates 5.71; 4.5 (boxed-red dot) and 9.12; 4.33 (boxed-blue dot), respectively, are also indicated. A clear separation of the two groups can be observed when plotting pI versus pH (inset). It is also clear the presence of a subgroup of entries in cluster 1, with similar mass, represented by glycosyltransferases (molecular weight ~50 kDa and pI between 5.3 and 6.4; yellow, black-dashed ellipsoid). b) Proteome charge distribution at a physiological pH range. The two groups of entries behave differently at pH 6 (brown dots), 7 (orange dots), and 8 (yellow dots). In cluster 1 (red-dashed box), the charge distribution is compact and switches from prevalently positive at pH 6 to prevalently negative at pH 8; cluster 2 (blue-dashed box) shows a larger distribution and consists of entries with an almost exclusively positive charge persisting without inversion.

by the need to keep a net protein charge that enhances protein solubility and diffusion in the circulating solution of the soil's micropores. Interestingly, in C1 clearly emerges a subgroup of entries with similar mass (~50 kDa) and pI between 5.3 and 6.4 (Fig. 1a). This subgroup mainly consists of proteins that are correlated to the enzyme class of transferases, in particular glycosyltransferases, including UDP-glycosyltransferases.

2.2. The secretomic properties of the imbibition spillage proteome

Next, we defined the intracellular origin of each entry and the distribution of the entries' cellular origin in both, C1 and C2. A pI value smaller or greater than 7.45 was used as a discriminant to cluster the entries either in the acidic fraction C1 (pI < 7.45) or in the basic fraction C2 (pI >7.45) (Fig. 1a inset). The dataset localized the entries into 15 possible cell compartments with prevalent contributions from the cytoplasm, plastid, endoplasmic reticulum, nucleus, and cell membrane (Fig. 2a). Interestingly, among the most representative compartments, the contribution of the plastid, together with the cytoplasm, equally represented almost half of the proteome origin (~47.8%). The contribution of each compartment into each cluster shows a prevalence of C1 with respect to C2, a trend which is consistent with C1 greater than C2 when referring to the total proteome (Fig. 1a).

For each cell compartment origin, we investigated the possible causes/mechanisms that lead to the release from the seed, also gaining information about the accidental fraction (entries strictly unrelated to extracellular functions) with respect to the non-accidental ones (entries potentially related to extracellular functions). An important fraction of

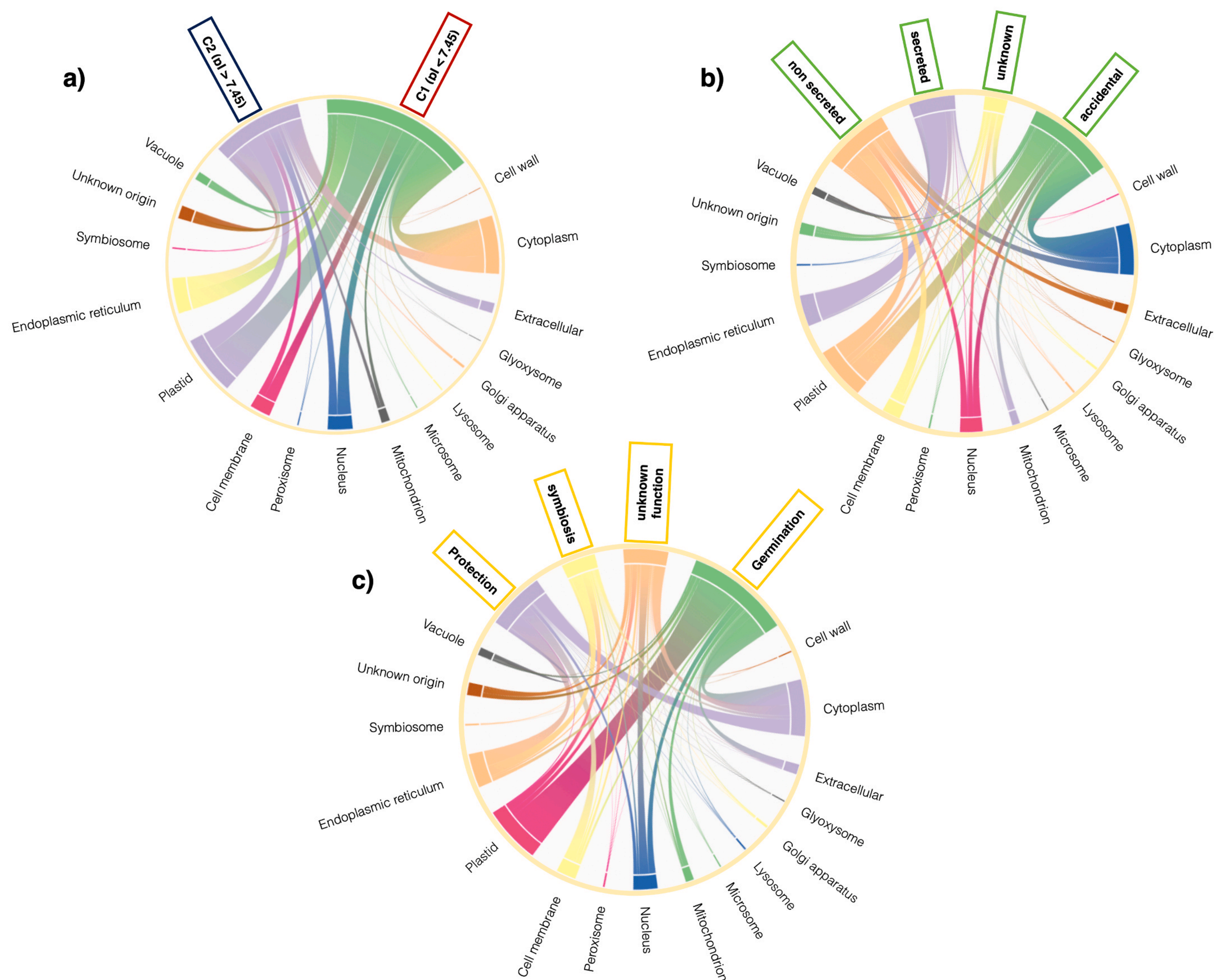

Fig. 2. a) Connectivity between the proteome origin and the clusters. Almost half of the proteome (~47.8%) accounts for proteins from the cytosol and the plastids (mainly chloroplast) followed by entries from the endoplasmic reticulum and the nucleus. In general, from each compartment, the contribution to C1 is greater or equal to the one to C2. b) Connectivity between the proteome origin and the way of release in the spillage. A main part of the proteome is of accidental origin with a significant contribution from cytoplasm and plastids (accidental, ~39.8%). The non-secreted fraction is the second most representative part of the proteome with contributions from plastids, cell membranes, and nucleus (non-secreted, ~30.3%). As expected, the secreted fraction mainly originates from the endoplasmic reticulum (secreted, ~20.2%). A fraction of entries that cannot be grouped in any of the previous categories (unknown, ~9.7%) is also indicated. c) Connectivity between the entries' origin and their function. The main contribution to the proteome is provided by entries involved in germination with a significant part of them originating from the cytosol and the plastid (germination). The second more representative group shows functions of protection and symbiosis, with a significant part of the entries originating from the endoplasmic reticulum. The rest of the entries, constituting the third most representative group, are proteins with unknown functions.

the proteome, ~39.8%, was identified as accidentally present while the remaining categories, in order of representativity, consisted of non-secreted (~30.3%), secreted (~20.2%), and unknown entries (~9.7%) also providing an overview of their cellular origin (Fig. 2b). Interestingly, the main part of the accidental fraction consisted of cytoplasmic and plastidial entries suggesting a direct release of the cell content during tissues fracture.

While some accidental entries can certainly be assigned to this category (e.g., membrane proteins like photosynthetic and respiratory complexes subunits), many others cannot be excluded a priori. For this reason, the accidental group was also retained for the overall analysis.

2.3. The metabolomic properties of the imbibition spillage proteome

The proteome was further investigated for its functional traits. The main part of the entries (~81.2 %) was linked to 3 functions, among which germination was the most represented one, immediately followed by protection and symbiosis. The remaining entries, which could not be assigned to one of the 3 previous categories, were pooled in a fourth group of entries (~18.8 %) with mostly unknown functions (Fig. 2c). Interestingly, for the two groups with the function of protection and symbiosis, a significant contribution is given by entries from the endoplasmic reticulum, while a consistent fraction of proteins involved in germination originated from plastids. The 812 entries were analysed using KEGG [15] and divided into four metabolic domains: i) Primary Metabolism Pathways (PMP; ~43.5%) generally involved in development and differentiation (carbohydrates metabolism, proteins, and amino acids metabolism, cofactors metabolism, nucleotides metabolism, transport, lipids, fatty acids metabolism, and plant hormones); ii) Secondary Metabolism Pathways (SMP; ~37.7%) including oxidative stress and plant-pathogen or plant-symbiont interactions; iii) Interface Metabolism Pathways (IMP; ~3.8%) considering metabolic pathways localized halfway between PMP and SMP hence including enzymes that are in common with different metabolisms; iv) Unknown Metabolism Pathways (UMP; ~15.0%) including unknown entries to which an univocal localization cannot be assigned. For each entry, the correlation between the release mechanism and the metabolic pathway was analysed (Fig. 3), revealing that almost all the secreted proteins belong to the SMP and the majority of accidentally released proteins are in the PMP, while the rest of the entries are spread between all the pathways. This result further highlights the active role of the secreted entries that, as extensively reported for the subsequent germination phases [1,32,39], also here are pivotal in establishing the plant-symbiont interactions while protecting the seeds from environmental and pathogenic stress.

2.4. The physiological properties of the imbibition spillage

Considering the functional clusters identified in the previous sections, the imbibition spillage was further investigated for physiological effects on the seeds and the symbiont. Previous findings showed that the imbibition spillage is important for inducing the chemotaxis of the symbiont towards the seed in early stages of germination [7]. Considering that the proteome profile of the imbibition spillage is primarily related to protection, germination, and symbiosis (Fig. 2c), specific assays were aimed at investigating these possible effects by enhancing differential properties associated to the imbibition spillage composition. Therefore, germination experiments were performed in water and in imbibition spillage monitoring the rates of emission and growth of the embryonal root. Differential effects could be observed, in particular, the imbibition spillage initially slowed down the germination when compared to controls, while an inversion of this tendency was recorded after 5/6 days (Fig. 4a and b). These findings are consistent with the presence of pro-dormancy molecules (including abscisic acid) released in the spillage fraction during imbibition. For seeds germinated in the imbibition spillage, the presence of these substances may cause a decrease in the osmotic rates of the pro-dormancy substances' excretion and in the water uptake. On the contrary, the increase in growth rate after 5/6 days with respect to the controls might be explained by a cumulative effect due to both the released as well as the previously present pro-germination molecules such as pro-hormones and proteins. This is consistent with a main part of the imbibition spillage proteome being involved in the germination (Fig. 2c).

Similar experiments were performed to assess the pro-symbiosis properties of the imbibition spillage. Previous experiments showed that this solution is a complete media for the *C. arietinum* symbiont *M. ciceri* [7]. Considering that symbiosis is strictly based on the balance between plant and symbiont and the number of bacterial cells above or below a threshold is a critical aspect, here we analysed the effect on the

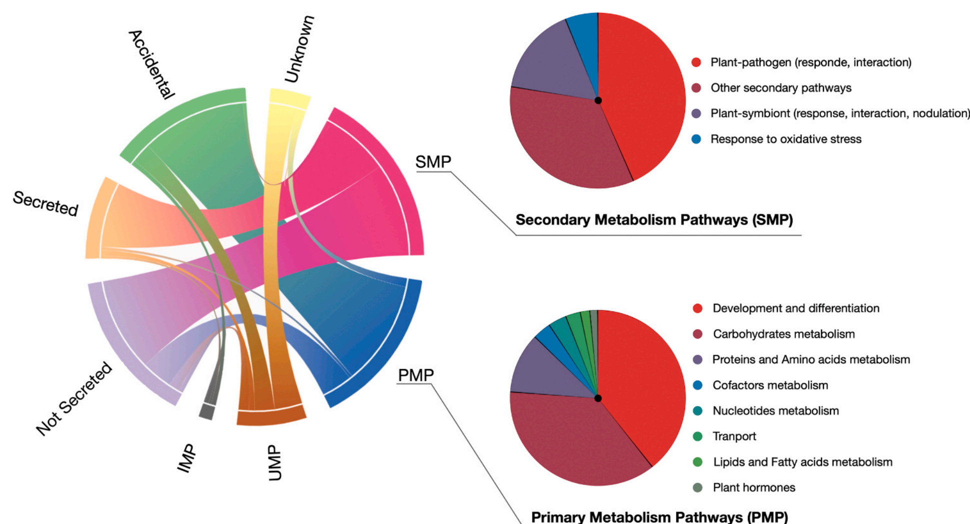

Fig. 3. Connectivity between the mechanism of entries' release in the spillage and their metabolic role. The chord diagram shows the connections between the mechanisms of release of the entries and their role in different metabolic pathways. The entries that are either secreted or non-secreted are mainly involved in Secondary Metabolism Pathways (SMP, ~37.7%) related to protection and symbiosis (top-right inset). A consistent part of the non-secreted entries and the majority of the accidental entries are instead involved in Primary Metabolism Pathways (PMP, ~43.5%) related to germination and development (down-right inset). A small part of the secreted entries together with the unknown and the accidental appear to be involved in Unknown Metabolism Pathways (UMP, ~15.0%) and the accidental are also involved in Interface Metabolism Pathways (IMP, ~3.8%) with general metabolic functions.

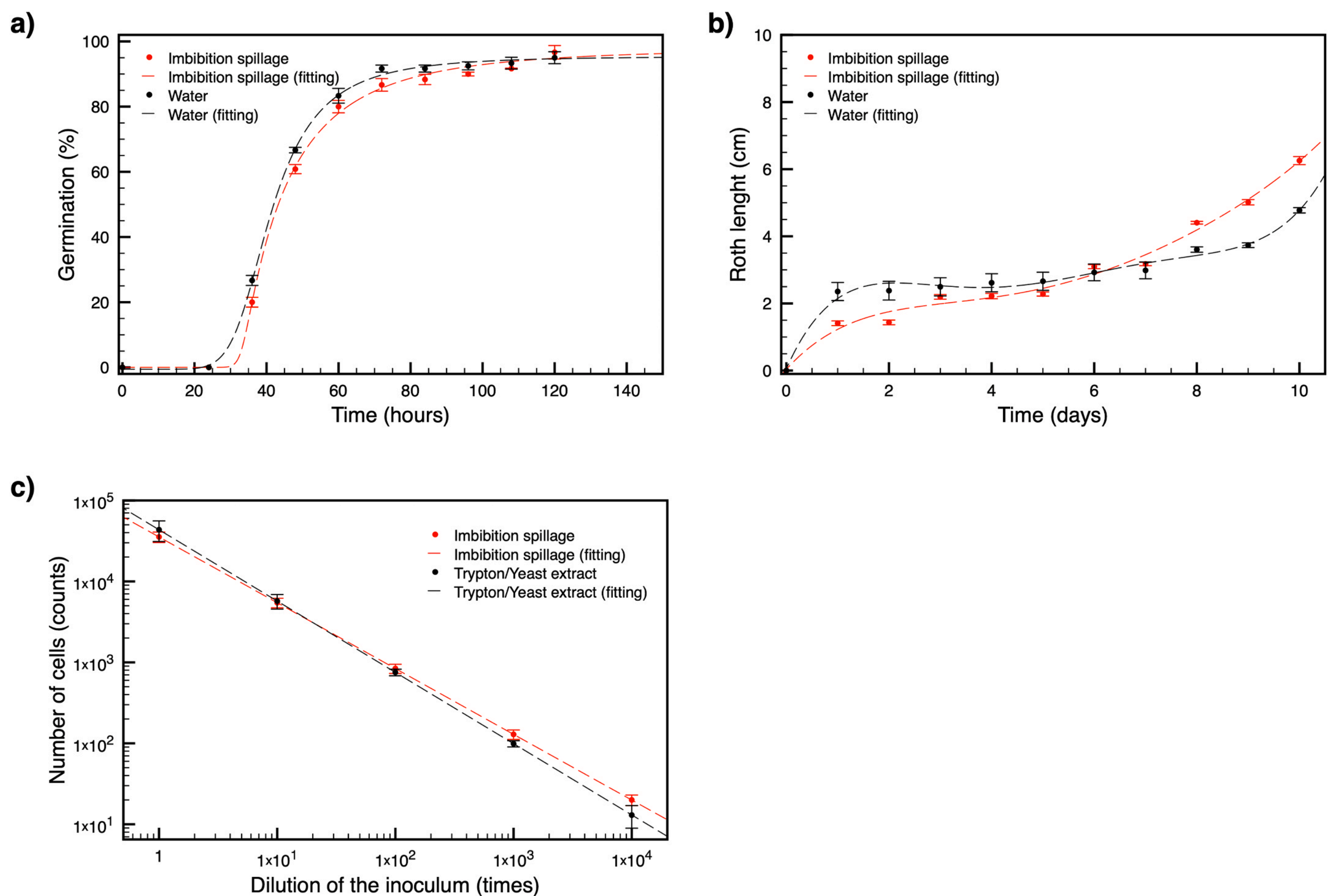

Fig. 4. Profiles of seeds germination (a) and root growth (b). Plots show the timing of germination and root growth for *C. arietinum* seeds incubated either with sterile spring water (black dots and black-dashed curves fitting) or sterile imbibition spillage (red dots and red-dashed curves fitting). In (a) an inversion of the rate takes place after 5 days while in (b) after 6 days. c) Profiles of growth at different dilutions for the symbiont *M. ciceri*. The plot shows the growth either **on** sterile imbibition spillage (red dots) or Trypton/Yeast extract (black dots) at four different dilutions. An inversion in growth can be observed at the intermediate dilution (1×10^2). Data were expressed as mean ± SD of three replicates.

bacterial growth. Accordingly, the *M. ciceri* CC1192 was used to understand the effect of different inoculum's concentrations on cultures grown either on the imbibition spillage or on the election media Trypton/Yeast Extract (TY). Also in this case differential effects could be observed, in particular at higher densities (1x, 10x, 100x dilutions) the cell growth in the imbibition spillage appeared slower with respect to the controls, while at lower densities (1000x, 10000x) the cell growth appeared to be faster (Fig. 4c). This aspect is important because it supports the pro-symbiosis role of the imbibition spillage also suggesting that it promotes the growth and, more importantly, it functions as a bacteriostatic media able to tone the load for establishing symbiosis optimally. Again, being proteins the main part of the imbibition spillage, is likely that these properties are also related with the proteome fraction related to symbiosis (Fig. 2c). These results are consistent with previous findings on the role of the imbibing spillage in promoting germination, chemotaxis of symbionts and resulting symbiosis [7].

3. Discussion

Communication and probing are two interconnected factors essential for ensuring the survival and fitness of organisms. While environmental perception is something trivial for organisms in their active metabolic state, all the biological stages which require organism (re)activation face the critical step of perceiving the environment correctly. This is particularly true for plants where sensing takes place already during the pre-activation of seeds or stems and does not allow for mistakes. The activation timing requires "to read" the right moment for starting and enhancing the metabolic events leading to germination and growth while protecting the seed and promoting symbiosis. As for stems in dormant plants during the winter-spring transition, seed germination requires fine mechanisms able to provide exhaustive information about the chemical, physical, and biological descriptors of the environment. Moreover, the perception must work continuously, reading the environmental variations in time finally allowing the sensing of the related trends. It implies that organisms, by a "time-resolved reading", increase the reliability of the acquired environmental information, reducing the probability of errors, especially in critical stages such as during seed germination. It is well known that during the early germination stages, the seed must actively interact with the environment gaining as much information as possible while possibly modifying the surrounding environment to optimize the conditions for safe and optimal germination. In the present work, the imbibition spillage released during seed activation was used as a probe to gain insights into the metabolic events and related pathways that are activated during this early stage and was found to be linked to the three main aspects of seed activation: germination, protection, and symbiosis. On the contrary, no entries strictly related to dormancy were found, while many others were connected i) with dormancy rupture and in particular with mobilization of reserves such as amylases, known for their activity in the sub-tegumental regions (aleurone layer) in the early stages [33]; ii) with ethylene production and its mediated tegument rupture inducing robust and banded embryonal stems during germination [2]; and, finally, iii) with the

pre-dormancy stages when the final phases of embryo development take place (e.g., Group 4 Late Embryogenesis Abundant proteins – LEA) [29].

In the present study, the pI was considered a driving descriptor for the analysis and interpretation of the spillage proteome. This agrees with previous observations showing a relatively small divergence in values (not relevant for the qualitative aims of the present study) between the theoretical pI (or pI measured in denaturing conditions) and the native pI [13], and a correlation between the theoretical pI of a given entry and its subcellular localization [23,25,40]. Both these aspects were essential to provide a discriminative interpretation of the data.

The physical-chemical analysis of the proteome allowed the identification of a basic and an acid group of proteins (Fig. 1a). Meaningfully, the sub-basic pH region (~ pH 7.5), which is separating the two groups, is the optimal one for the substrates and growth of *C. arietinum*. This aspect is essential and indicates that at optimal pHs only a small part of the released proteome, ~3.7%, is characterized by a near-to-zero or an absent net charge while the rest is fully soluble and able to diffuse as part of the soil's dynamic phase. This small fraction of entries consist of proteins involved with protection such as subtilases, proteases, NAC transcription factors, and ubiquitination-related proteins [9,10,47], most likely representing a forefront for seeds' defence. Of the two groups of proteins, the acidic one also includes an interesting subgroup of transferases with similar masses (~ 50 kDa) ranging between 5.2 and 6.4 pI (Fig. 1a). Transferases are important enzymes known for their primary role during germination being essential in stepping the progress of this physiological process and in promoting symbiosis and protection [4,14,38,44,48]. The two groups of proteins also show different behaviour with respect to their charge properties at different pHs. The acidic group has a spread charge passing from positive to negative values with an increase in the pH, while the basic group has a less spread charge, mostly limited to positive values at different physiologic pHs (Fig. 1b). This different behaviour supports a possible functional distinction between groups and might reflect a different relationship with the soil components affecting protein diffusibility. Diffusibility, in fact, represents an important factor in this stage when considering that a main role of the proteome's components is to "explore the environment" to induce the root's adaptation, symbionts attraction, and protection. Here, the acidic group has a more pronounced negative charge hence it tends to be in solution as part of the soil's liquid fraction associated with the micropores, finally depending on the hydraulic conductivity of the soil; on the opposite, the basic group has an exclusive positive charge and it easily tends to interact with the solid fraction of the soil with an extent depending to the specific cation-exchange capacity at the surface of the soil particles. This implies that the acidic group could easily diffuse reaching longer distances than the second group of entries, which would stay nearer to their zone of release. This observation is congruent with the acidic group being associated with functions of protection and symbiosis for which is important an easy diffusion away from the seed and with the basic group being associated with proteins involved directly in the germination process. Similar results were shown by preliminary tests on other legumes, such as *Vicia faba*, *Pisum sativum*, *Lens culinaris*, and of non-legume species such as *Nicotiana tabacum* (data not shown) suggesting that the observation made on *C. arietinum* can be extended, at least qualitatively, to other species.

Considering the physiological insights either on the seed (Fig. 4a and b) or on the symbiont (Fig. 4c) and given the extensively reported properties of the secretome during germination [1,32,39], the present results and more in general any hint about the very early preparative and pre-germinative stages can contribute to adapting the cultivation techniques for improving crops production. Accordingly, the peculiar distribution of two protein pools separated by a pH region with almost no entries (Fig. 1a) provides direct suggestions for the optimal range of pH into which the crop should fall at least during the stages of seed imbibition. This fact further suggests that the use of specific soil conditioners aimed at optimizing the chemical-physical conditions of the soil could optimize the pre-germination stages making more efficient the subsequent ones.

In conclusion, the present findings show a framework where specific metabolic pathways involved with protection, symbiosis, and germination drive the seed's metabolism during its transition to seedling while the environment is actively shaped to provide protection and comfort for the roots and their symbionts.

4. Materials and methods

4.1. Plant material and Imbibition

Seeds (200 gr) of dry *Cicer arietinum cv. Principe* were incubated in 500 mL of sterile-spring water, in the dark, at 4 °C for 8 h, allowing the process of diffusion to reach completion which was evaluated by UV-Vis spectroscopy at 280 nm. After incubation, seeds were removed, and the imbibition spillage was filtered with a 0.22 μm cut-off nitrocellulose filter. This solution was then lyophilized, and the obtained powder has been used for all the following studies. The data here presented result from an optimized procedure of imbibition spillage standardization aimed at promoting reproducibility. Optimization was performed by a careful evaluation of the ratio between seeds' weight, the volume of water used for the imbibition step, and the time duration of the imbibition step. Further standardization and reproducibility were achieved by using spring water (Acqua Federica – https://acquafederica.it/), avoiding microbiological contamination while maintaining chemical-physical stability. The reproducibility of the imbibition spillage was assessed by UV-Vis absorption spectroscopy using the ratio of the main maxima in the measuring range. The procedure of standardization was performed for more than 50 independent tests over four years and on four consequent crops. A typical representative sample obtained in this way was subjected to MS analysis.

4.2. Mass spectrometry

The lyophilized imbibition spillage was subjected to Mass Spectrometry analysis (MS). Briefly, samples were resuspended in sodium deoxycholate (SDC) lysis buffer (1% SDC in 100 mM HEPES/50 mM DTT), reduced (30'/56 °C), alkylated (iodoacetamide - IAA - added to final concentration of 15 mM in the dark, for 30' at room temperature) and then trichloroacetic acid (TCA) precipitation was performed (one part ice cold 100% TCA added to four parts of protein sample) and left on ice for 20–30' before centrifugation at 20800 x *g*, 20', 4 °C). The supernatant was removed, and the pellet was washed 3 times with 1 mL chilled solution of 10% TCA and pure acetone in a 1:2 ratio for each washing steps, after resuspension the sample was centrifuged, and supernatants removed. Resulting protein pellet was allowed to air-dry, resuspended in LysC digestion buffer (3 M Urea, 100 mM HEPES) and added with LysC (1:100 ratio enzyme: protein). The mix was incubated for 4 h at 37 °C. For the trypsin digestion, the digestion buffer was diluted 1:1 with water before adding trypsin (1:100 ratio enzyme:protein). The mix was incubated overnight (16 h) at 37 °C. Digested peptides were then processed (desalted and data acquired) as reported by Farci et al. [6]. Raw data were processed in MaxQuant version 1.5.3.28 as the search engine against the Uniprot *C. arietinum* database with a list of common contaminants appended. For the search 2 missed cleavages, with trypsin/P specificity defined were allowed. Oxidation (methionine) and acetylation (N-term) were considered as variable modifications, while carbamidomethyl (cysteine) was fixed. A 1% False Discovery Rate (FDR) was applied at peptide and protein group levels. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [30] partner repository with the dataset identifier PXD038374.

4.3. Proteome analysis and software

The 812 entries obtained by mass spectrometry were subjected to

bioinformatic analysis and sorted by different means. The subcellular localization of each entry was assigned using the computational framework of Plant-mSubP [34]. First, the hybrid protocol based on amino acid (total sequence, N-terminal, Center, and C-terminal), dipeptide composition, and polarity (hydrophobicity and hydrophilicity values of the amino acids in the total sequence - PseAACNCCDipep) was applied. Next, the protocol based on similarity search (Protein Blast-based) was used to refine the results obtained with the previous step. The identification of the biochemical networks into which each entry is involved was performed using Kyoto Encyclopedia of Genes and Genomes [15] against *C. arietinum*. Theoretical net charge with respect to the pH was calculated with Protein Calculator 3.4 in a range of pH 4–10 and a pH step of 0.5 (http://protcalc.sourceforge.net/). The data were visualised by using Plot2 (https://plotdoc.micw.org/), while the DataSmith server (www.datasmith.org) and Circa (https://omgenomics.com/circa/) were used to facilitate a connective visualization. The pI versus log (MW) of the entries was subject to a K-mean cluster analysis with K= 2 using Origin (https://www.originlab.com/).

4.4. Physiological assays on the imbibition spillage

Germination tests were performed according to Farci et al., [8] but in a sterile glass jar. Briefly, first the seeds were subjected to topic sterilization for 30 min in a solution of 1% sodium hypochlorite for 30' at room temperature (RT) and then washed three times in sterile-spring water. After imbibition, done accordingly to the first paragraph (Plant material and Imbibition), *Cicer arietinum* seeds were placed on a sterile cotton layer wet with either sterile-spring water or sterile imbibition spillage. The growth was monitored on 120 seeds (3 groups of 40 seeds) for up to 12 days under a constant temperature of 25 °C, 50% relative humidity in the dark. This setup allowed the visualization of the early evasion of the primary root as well the overture of the emitted cotyledon leaves. Measurements were done twice a day for the entire duration of the experiment. Data were expressed as mean ± standard deviation (SD) of three replicates.

Tests of symbiont growth were performed on *M. ciceri* strain CC1192 [11]. The growth, repeated in triplicate, was performed in both sterilized imbibition spillage (IS) and TY (Tryptone 0.5%, Yeast Extract 0.3%, 6 mM $CaCl_2$). A 10 µL volume of cultures grown overnight on TY (O.D .590 nm = 0.5) was inoculated according to a series of four dilutions in IS equivalent to 1, 10, 10^2, 10^3, 10^4 times, respectively. After 24 h, for each dilution10 µL of cultures was further diluted 1, 10, 10^2, 10^3, 10^4 times and spotted either on IS or TY Agar 1.5% plates. After incubation for 24 h, the number of colonies was counted at the minimal dilution sufficient to not allow confluence. The quality of the data was estimated by calculating mean and SD from a set of three independent measurements (experiments).

Ethics approval and consent to participate

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CRediT authorship contribution statement

Conceptualization: DP, DF. Methodology: DP, DF, JK. Validation: DP, DF, JK. Formal analysis: DP, DF, FT, SFF, JK. Investigation: DP, DF, FT, SFF. Resources: DP, DF, JK. Data curation: DP, DF. Writing – Original Draft: DP, DF, FT, SFF, JK. Visualization: DP, DF. Supervision: DP, DF. Project Administration: DP, DF.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2022) partner repository with the dataset identifier PXD038374.

Acknowledgment

Proteomic data are available via ProteomeXchange with the identifier PXD038374. Pure lines of *C. arietinium* cv *Principe* were purchased from the company Fratelli Ingegnoli (https://www.ingegnoli.it). The study on *C. arietinium* cv *Principe* complied with the local and national regulations. The lines used in the present manuscript are common wild type strains available in the market.

Consent for publication

Not applicable.

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