**Project acronym: PROTEIN2FOOD**

**Project No.: 635727**

H2020-SFS-2014-2015/H2020-SFS-2014-2

Start date of project: March 2015

Duration: 5 years

Deliverable reference number and title

# D1.3 - Protocols for 3D imaging and analysis of internal structure of seeds for the enhancement of phenotypic characterization

Date: February 2018

Organisation name of lead for this deliverable:

## National Research Council (CNR)

| **Project co-funded by the European Commission within the Horizon 2020 Programme** | | |
|---|---|---|
| **Dissemination Level** | | |
| **PU** | Public | |
| **PP** | Restricted to other programme participants (including the Commission Services) | |
| **RE** | Restricted to a group specified by the consortium (including the Commission Services) | |
| **CO** | Confidential, only for members of the consortium (including the Commission Services) | |

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635727.

## 0. Summary

Non-destructive imaging of seed structure for high-resolution morphological phenotyping isn't a standardized procedure. It requires the definition of protocols for sample scanning settings and definition of image analysis algorithms to be used for obtaining proper quantitative information. Scanning protocols for bulk and single seed samples are defined and described in this report as well as examples of application of selected quantitative parameters useful for the characterization of novel traits for seed phenotyping. Outcomes from this report will allow to better address the screening of the studied cultivars and genotypes.

## 1. Introduction and Objectives

The role of imaging for characterization and quality evaluation of seeds is rapidly increasing due to the availability of cost effective imaging products, better computation power and increased interest in non-destructive examination of food items. Moreover, according to the European strategies on research infrastructures, Agri-Food is an "emerging field" for application of non-destructive imaging techniques to be considered in addition to the well-established Medical and Biological fields (e.g. in the ESFRI project: EuBi).

In such a framework, among the tasks of the WP1, was included the characterization of the studied protein rich seeds with X-ray microtomography imaging for both enhancing the screening of the cultivars and better analyze the main effects of environments and managements on seed quality characteristics. The main advantage of the X-ray μCT technique is the ability to perform non-destructive and non-invasive capturing of high-resolution three-dimensional (3-D) detail, thus allowing to directly measure three-dimensional parameters of the whole seed and its internal features, providing "high resolution" seed phenotyping. A series of "radiographs" are performed on a sample of material placed on a rotating support, obtaining projections, according to different rotation angles, of its capability to attenuate X-rays. From the "projection" images, the reconstructions of the cross-sectional images of the rotation axis are recalculated by re-calculating the inverse Radon transforms of the projections according to the different incidence angles of the X-rays. This procedure is also called "back projection" algorithm. The cross-sectional images are then "overlapped" by reconstructing three-dimensional geometry. As there are no standard procedures for seed micro CT scanning and analysis, above tasks require to set-up proper imaging protocols. The objectives in defining the protocols have been to obtain both the best possible statistical significance and high accuracy in seed measurements.

## 2. Activities for solving the task(s)

First activity carried out was to set up seed sample holders suitable for proper X-ray micro-CT scanning of both relatively large seeds, like those of legumes, and smaller ones like those of high quality protein crops quinoa and amaranth. Then, for all kinds of seed were selected scanning setting and defined parameters, provided by 3D image analysis procedures, capable to quantify and characterize both external and internal morphological traits for high-resolution phenotyping.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635727.

The X-ray microtomograph used to set-up the protocols was that available at CNR ISAFOM, namely the Bruker model Skyscan 1272 (Figure 1). It is a desktop microtomograph with a cone beam X-ray source adjustable in the 20-100kV energy range and maximum sample size a cylinder shaped volume of 6.5 cm in diameter and 7.2 cm height. Full technical specifications can be found at http://bruker-microct.com/products/1272.htm. Similar equipment is commercially available on the market from other companies.

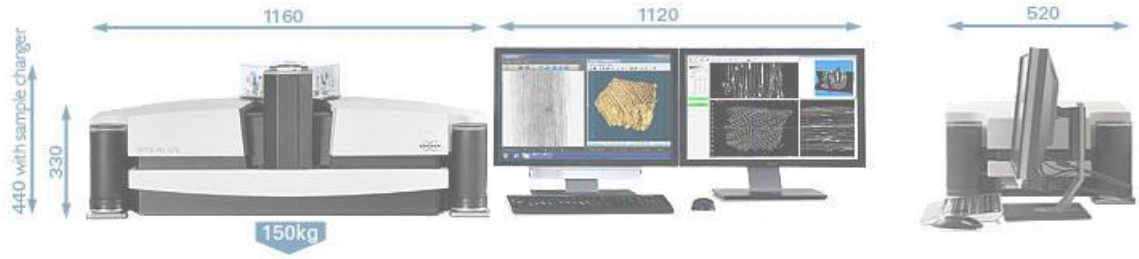



Figure 1. Desktop X-ray microtomograph used to set-up the protocols

An advanced workstation containing two Intel-XEON eight core processors, 128GB memory, 12TB disk space and two 24"UltraSharp LCD monitors was used for image processing and analysis.
Multiple scanning and image analysis tests were performed trying different settings and using different species of legumes and high quality protein crops before reaching the final setup for scanning and analysis protocols.

## 3. Results

Ultimate protocols found regard the sample scanning stage and the definition of the parameters for characterizing the morphological phenotypic traits.
The search of the best scanning settings was found considering that in X-ray microCT systems as the sample size increases the image resolution decreases, thus one has to find the best compromise between representativeness of the sample and accuracy of the details.
Choice of the parameters to quantify the morphometric traits has been driven by simplicity, unicity and, possibly, a clear physical meaning.

### *3.1 Bulk sample scanning*

This is the case of single scans for a relatively large number of seeds which are put on the rotating support of the microtomograph at one time. Such approach is useful to obtain statistically significant results for external morphometric measures of a given seed genotype with reasonable efforts in term of both, scanning time and image processing time Bulk sample scanning, especially in case of legumes, allows also to identify seed defects like insects infestation.

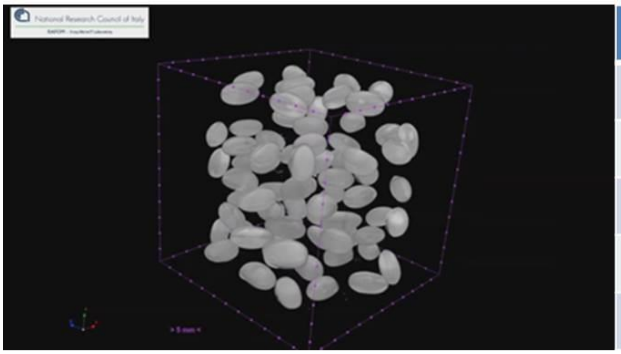
#### *3.1.1 Large seeds*

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635727.

For legume seeds like chickpeas, faba beans, grass peas etc…, the best sample holder arrangement has resulted that shown in figure 2. It consists of a PMMA cylinder of 6 cm diameter whose internal surface is coated with a polystyrene film. The cylinder is 6.5 cm height and contains five or six superimposed polystyrene circular grids containing twelve seeds each. Such arrangement allows the reconstruction and the analysis of 72 legume seeds from a single scan. PMMA material for the cylinder and the polystyrene for the grids were chosen because of their low X-ray attenuation capacity, allowing good contrast in the imaging of the seeds. The main function of the grids is to obtain an image where seeds don't touch each other, thus simplifying the following stage of image processing.


Figure 2. Sample holder set-up for bulk scanning of legume seeds.

In figure 3 is shown an example of bulk seed sample image reconstruction and the corresponding protocol describing the main scanning parameters set-up for this kind of scans. Scan duration for this configuration was 2h:15m as it required a double oversize offset scan. Ring artifact correction without beam hardening correction was used for the image reconstruction. The NRecon software, version 1.7.3 (www.bruker-microct.com) was used for image reconstruction.



| Scanning parameter | Value |
|---|---|
| Source Voltage (kV) | 80 |
| Source Current (uA) | 125 |
| Image Pixel Size (um) | 20 |
| Rotation Step (deg) | 0.6 |
| Filter | Al 1mm |

Figure 3. Image reconstruction of the bulk sample of legume seeds and scanning protocol.

### *3.1.2 Small seeds*

For small seeds, we refer here to those of quinoa and amaranth. The best sample holder arrangement for the seeds of these high quality crops has resulted that shown in figure 4.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635727.

It consists again of a PMMA cylinder but of 2.5 cm diameter whose internal surface is coated with a polystyrene film. The cylinder is 2.8 cm height and contains from five to eight superimposed polystyrene thin discs having small depressions containing about thirty seeds each. Such arrangement allows the reconstruction and the analysis till about 250 small seeds from a single scan. PMMA material for the cylinder and the polystyrene for the discs were chosen because of their low X-ray attenuation capacity, allowing good contrast in the imaging of the seeds. The main function of the discs with the housings for the seeds is to obtain an image where seeds don't touch each other, thus simplifying the following stage of image processing.

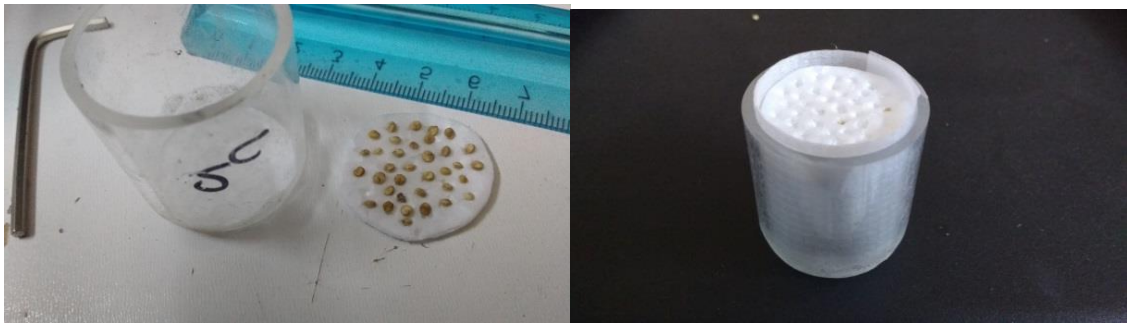
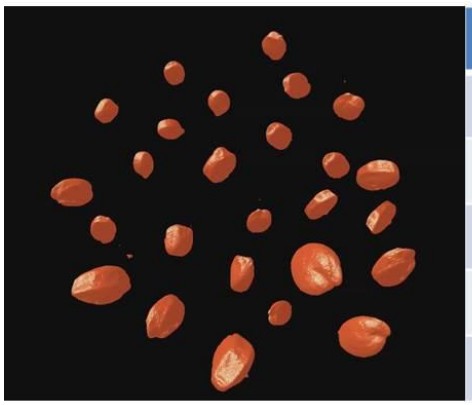


Figure 4. Sample holder set-up for bulk scanning of pseudocereal seeds.

In figure 5 is shown an example of bulk quinoa seed sample image reconstruction and the corresponding protocol describing the main scanning parameters set-up for this kind of scans. Scan duration for this configuration was 9m:56s, much shorter than for the legumes because of the smaller size of the cylinder which is completely included in the field of view of the CCD detector of the microtomograph without the need of offset scans. Ring artifact correction with beam hardening correction of 30% was used for the image reconstruction. As in the case of the legume seeds the NRecon software, version 1.7.3 (www.bruker-microct.com) was used for image reconstruction.



| Scanning parameter | Value |
|---|---|
| Source Voltage (kV) | 50 |
| Source Current (uA) | 200 |
| Image Pixel Size (um) | 15 |
| Rotation Step (deg) | 0.6 |
| Filter | No |

Figure 5. Image reconstruction of the bulk sample of pseudocereal seeds and scanning protocol.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635727.

## *3.2 Single seed scanning*

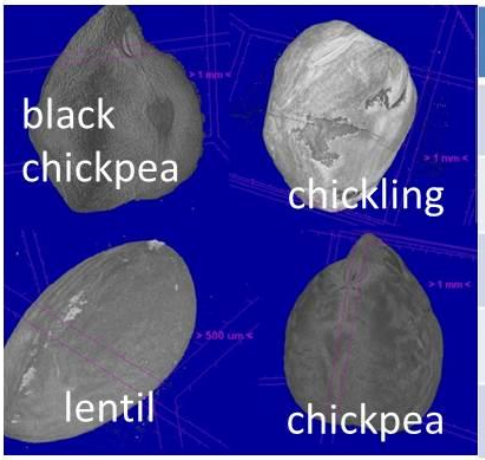
When analyses have to be deepened, a single scan for each seed is performed. Such approach is mandatory if very small external or internal traits must be distinguished. In particular it is needed when measurements on seed components or specific seed tissues have to be done. For both, large and small case, the seeds are directly put on the rotating stage using a very thin support to which they are held using dental wax (see figure 6). Such dental wax is suitable for the purpose because of its very low x-ray attenuation coefficient.

Although this approach is not the optimal one to obtain largely representative results, the repetition of single seed scans on a number of casually selected individuals can however allow statistical evaluations of the results referred to a given crop.

Figure 6: Black chickpea and amaranth seeds prepared for single seed scanning.

Figure 7 shows an example of single seed reconstruction and the relative protocol defined to fit for legumes external traits analysis. In this case, duration of the scans was 31 minutes and both, ring artifact and beam-hardening corrections were applied for reconstruction of the images at 30% and 50 % level, respectively.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635727.

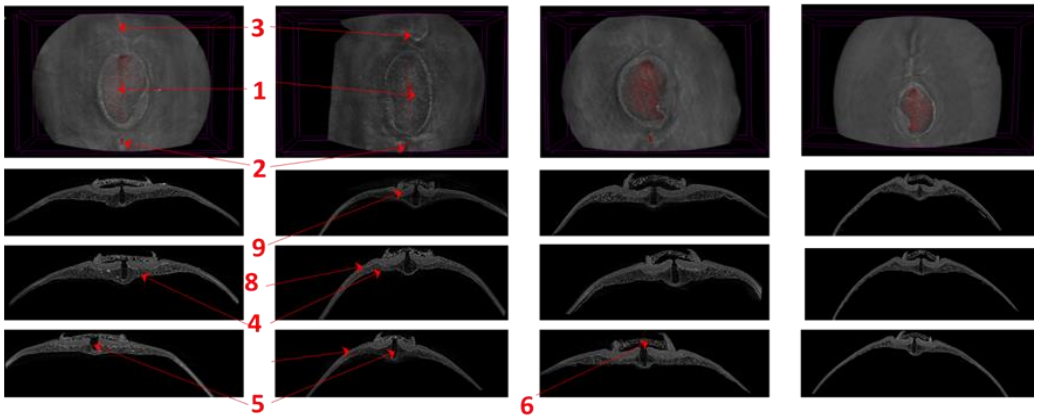



| Scanning parameter | Value |
|---|---|
| Source Voltage (kV) | 50 |
| Source Current (uA) | 200 |
| Image Pixel Size (um) | 5 |
| Rotation Step (deg) | 0.2 |
| Filter | No |

Figure 7: Image reconstruction of external traits of legumel seeds and scanning protocol.

In figure 8 is shown an example of reconstruction of internal traits of four legume seeds and, below, the corresponding scanning protocol. More specifically internal tissues of the seed hilum region have been reconstructed. Duration of scanning in that configuration was 1h:40m and no ring artifact or beam hardening corrections were applied for image reconstruction.

Finally, in figure 9 are shown examples of image reconstruction of small seeds internal traits from single seed scans with the relative scanning protocol. Duration for such kind of scans was 12h:30m with moderate (10%) ring artifact and beam hardening corrections applied to obtain a proper reconstruction.

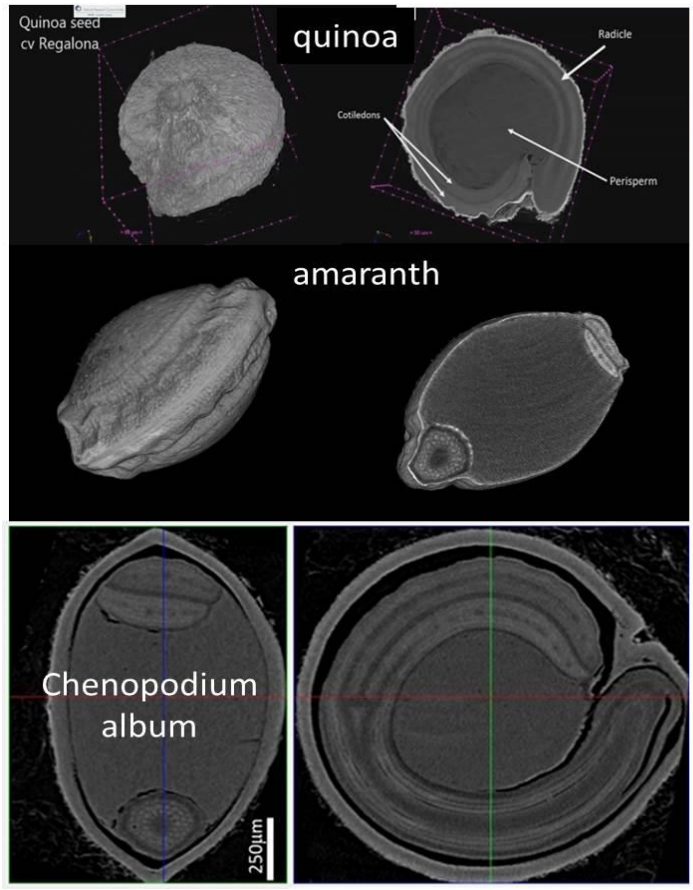



1. Hilum area 2. Micropyle hole 3.Strophiole area 4.Vascular tissue 5.Tracheid bars
6.Funiculi residue honeycomb cells 8. Seed coat (single palizade cells) 9. Double palizade cell tissue

| Scanning parameter | Source Voltage (kV) | Source Current (uA) | Image Pixel Size (um) | Rotation Step (deg) | Filter |
|---|---|---|---|---|---|
| Value | 40 | 250 | 2 | 0.2 | NO |

Figure 8: Image reconstruction of legume seed testa internal traits and scanning protocol.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635727.




| Scanning parameter | Value |
|---|---|
| Source Voltage (kV) | 50 |
| Source Current (uA) | 200 |
| Image Pixel Size (um) | 1 |
| Rotation Step (deg) | 0.2 |
| Filter | No |

Figure 9: Image reconstruction of quinoa, amaranth and chenopodium seed internal traits and scanning protocol.

## *3.3 Image analysis*

Quantification of morphological traits for "high resolution" seed phenotyping is performed on the reconstructed images by applying different approaches of image analysis. In particular the so-called "objects analysis" approach is applied for determination of morphometry of specific parts (isolated objects) above the image background while the "mathematical morphology" is applied for determine size distributions inside a single part (object) of the images. The first approach was applied here using the software "Image Pro Premiere 3D" (www.mediacy.com), the second one using the software "Conmorph" written in Matlab at CNR ISAFOM. Both image analysis approaches share the same preliminary stage which is the "image segmentation" that has been performed using the CTAn software (www.bruker.com). Good image segmentation is the base for any good traits quantification and strongly depends from the determination of good scanning protocols. All applied image analysis procedures can be performed with other commercial software available on the market and also using open platforms like "Image J" (https://imagej.net). In the following are described the main parameters used to quantify the morphological traits of the seeds.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635727.

### 3.3.1 *External morphometry*

In figure 10 are reported the basic measures selected to be performed on the seeds. They are useful for characterizing external traits regarding the size and shape of the seeds. Of course they or their combinations can be also used to characterize seed components or tissues.

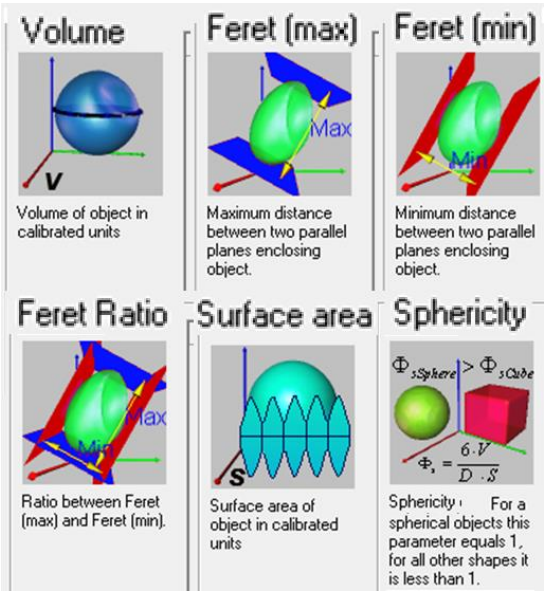



Figure 10: Definition of the 3D basic measures selected to describe the external traits of the seed and/or the seed components.

These measures are obtained applying the above mentioned "objects analysis" image processing approach.

In figure 11 is reported an example of external traits measurements from bulk seed scanning. For each seed of the sample (beans in this example) the parameters defined in figure 10 are automatically calculated and reported by the used software on the table visible on the right in figure 11. Average values are also provided capable to morphologically characterize the seed phenotype of the examined crop.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635727.

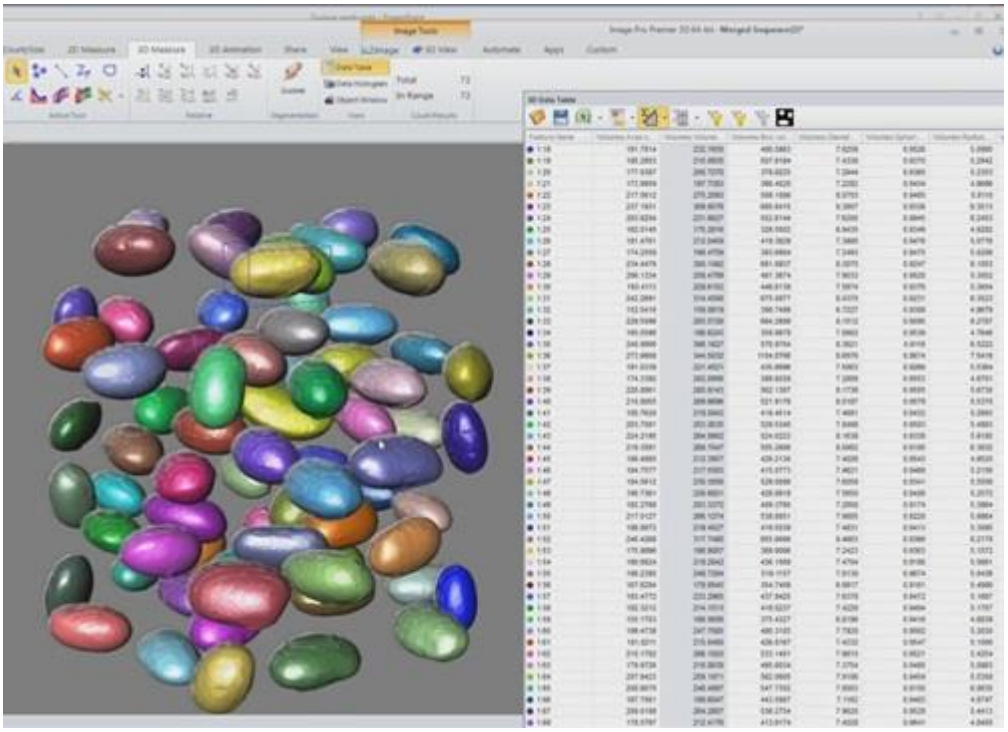


Figure 11: Example of measurements of external morphological traits of legume seeds from bulk scanning. Each seed is automatically identified with different color and results of measurements are listed on the table visible on the right.

### 3.3.2 Internal traits

They regard seed components and/or specific seed tissues. In figure 12 are reported examples of internal traits measurements used for characterizing a quinoa genotype. Some of them can be provided from bulk sample scanning (Figure 12a) while other can be provided only from single seed scans (Figure 12b).

In figure 13 is an example of internal traits measurements regarding specific tissues of the seed, namely those around the hilum region of a bean seed. Volumetric proportions of those tissues have been calculated in that case.

All the above examples of internal traits measurements of seeds are based on calculation of the basic parameters defined in figure 10 while calculation of pericarp thickness distribution of quinoa and vascular diameter distribution shown in figure 14 are examples of measurements based on mathematical morphology approach, namely iteratively applying the algorithm of “opening”.

This project has received funding from the European Union’s Horizon 2020 research and innovation programme under grant agreement No 635727.

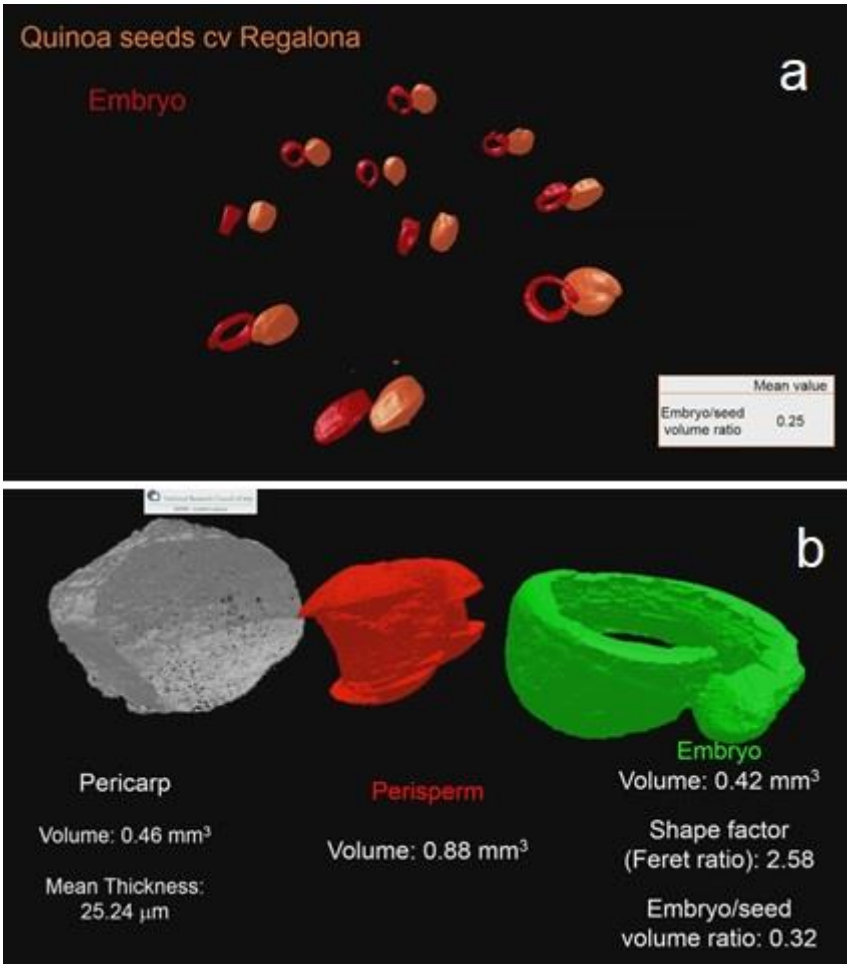



Figure 12: Example of measurements of internal morphological traits (seed components) of quinoa seeds from both, bulk (a) and single seed (b) scanning.

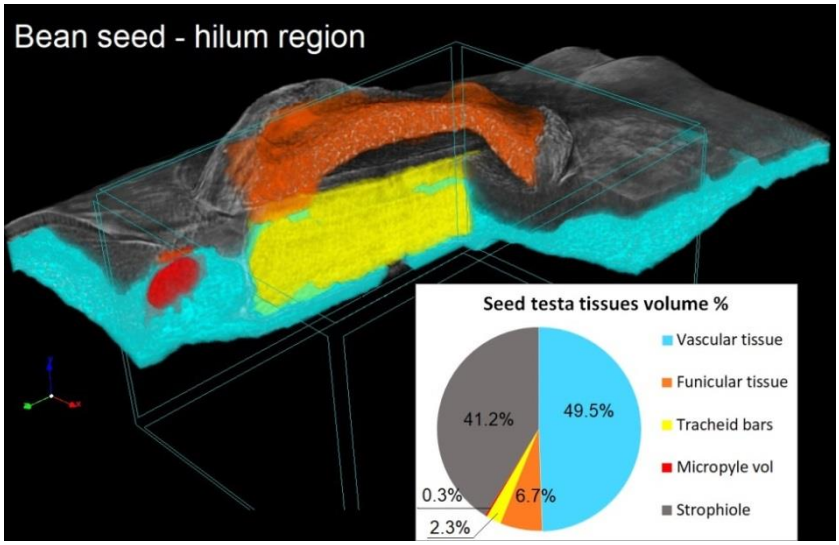



Figure 13: Example of measurements of internal morphological traits (seed hilum region tissues) of a bean seed from single seed scanning.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635727.

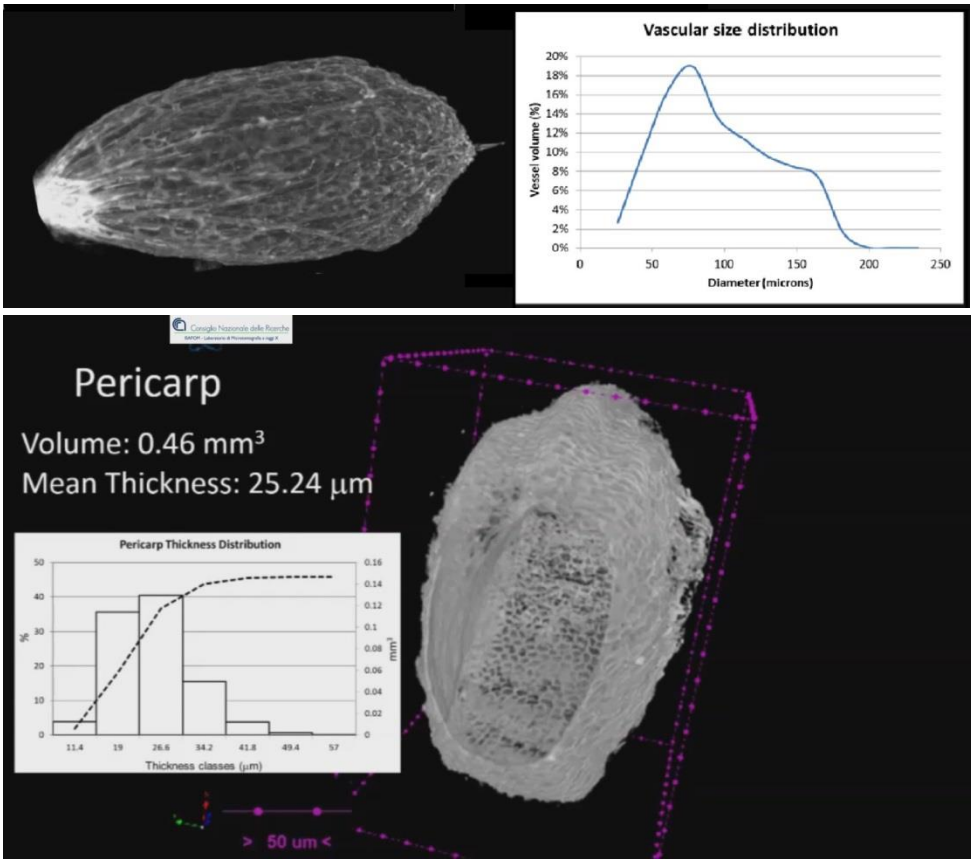



Figure 14: Example of measurements of internal morphological traits of seeds based on ”mathematical morphology” image analysis approach.

## 4. Conclusion and next steps

Definition of the X-ray microCT scanning protocols reported here is a general result from different research activities that CNR ISAFOM is carrying out in the framework of the WP1 also in collaboration with other partners of the P2F project. It represents an important practical outcome towards the filling of the gap between genotype and phenotype information for protein-rich seeds. Indeed, the collection of geometrical information on novel morphological traits, impossible to be observed non-destructively, allows to better address the screening of the considered cultivars for all the purposes of the project. Next steps will be the application of the scanning protocols and the selected 3D trait measurements described here, focusing on selected study cases that exhibit high potential in terms of novelty of research and general impact on society.

## 5. Delays and difficulties

Most of the delays that affected the production of this deliverable were related to the initial uncertainties about the choice of cultivars/genotypes which to focus on. It surely was not a trivial task due to the need of coordination of the activities of many experimental fields in

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635727.

different European countries, with many protein-crops and several cv/varieties for each crop.
Overall, “high resolution” phenotyping is in itself still a hard task to carry out extensively and in short time.

## 6. Impact and dissemination activities

To establish a definitive protocol for X-ray microCT seed scanning and measurements will have an immediate impact on the ongoing activities of the WP1, especially after the recent definition of the cultivars on which field experiments have to continue until the end of the project. Moreover, as microtomography imaging of seed is not still a standard procedure for seed phenotyping, the obtained results may provide a useful reference for further studies in this field.
The results coming from the application of the defined protocols will be summarized and disseminated through open-access scientific publication and, possibly, stakeholder interaction.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 635727.