Evaluation of Seed Dormancy, One of the Key Domestication Traits in Chickpea

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Abstract: Legume seed dormancy has been altered during the domestication process, resulting in non-dormant seeds with a testa that is readily permeable for water. Ultimately, this provides fast and uniform germination, in contrast to dormant seeds of the wild progenitor. To date, germination and seed dormancy were studied mostly in relation to two types of cultivated chickpea: kabuli and desi. We studied seed dormancy, from physiological and anatomical perspectives, in chickpea crops and compared cultivated chickpeas to the wild chickpea progenitor and set of recombinant inbred lines (RIL). There was significant difference in the macrosclereid length of parental genotypes. Cultivated chickpea (C. arietinum, ICC4958) had mean of 125 µm, while wild C. reticulatum (PI48977) had a mean of 165 µm. Histochemical staining of the seed coat also showed differences, mainly in terms of Sudan Red detection of lipidic substances. Imbibition and germination were tested and several germination coefficients were calculated. Cultivated chickpea seeds imbibed readily within 24 h, while the germination percentage of wild chickpea at various times was 36% (24 h), 46% (48 h), 60% (72 h) and reached 100% only after 20 days. RIL lines showed a broader distribution. This knowledge will ultimately lead to the identification of the underlying molecular mechanism of seed dormancy in chickpea, as well as allowing comparison to phylogenetically related legumes, such as pea, lentil and fava bean, and could be utilized in chickpea breeding programs.

Keywords: chickpea; domestication; dormancy; germination; legumes; macrosclereid; seed coat

1. Introduction

Legumes (Fabaceae) are a diverse family with worldwide distribution and a broad range of plant forms. They are the second most important crop plant family after Poaceae and grain legumes (pulses) account for 27% of global crop production [1], providing nutrition for millions of people. Legumes are considered to be a multipurpose crop, and can be used directly as food or in processed forms as a feed for livestock or as a rotation crop that improves soil health through nitrogen fixation [2].

Chickpea (Cicer arietinum L.) is an annual, self-pollinated legume crop [3] grown mainly in arid and semi-arid regions all over the world. It is the second most important legume crop and is a source of high quality nutrients. Chickpea seeds are rich in protein (20–30%), with a considerable amount of essential amino acids (e.g., lysine, tyrosine, glutamic acid and histidine), carbohydrates (up to 40%), minerals (e.g., phosphorus, calcium, magnesium, iron and zinc), vitamins (e.g., β-carotene) and fiber [4]. Chickpea, therefore, forms an essential part of a diet in developing countries. Moreover, farmers face climate change, a growing human population and demand for more high-quality crop food. Finding the solution for these issues is very challenging, especially in the context of increasingly limited land and water resources [2,5,6].
Chickpea was domesticated in the Fertile Crescent, which is also its place of origin. The ancestor of the cultivated *C. arietinum* is a wild annual plant, *Cicer reticulatum* Ladiz. [7], which is the only relative in the primary gene pool [8–10] that can be interbred with cultivated chickpea [9,10]. Cultivated chickpea differs from its wild relatives in its erect plant growth habit, reduced pod dehiscence, seed coat texture, larger plant size, reduced anthocyanin pigmentation and reduced seed dormancy. These traits are considered as part of a “domestication syndrome” [11–13]. Nevertheless, the key to chickpea domestication was the transformation from a winter habit with an autumn sowing to a spring habit, which evaded or reduced the threat of lethal infestation of the endemic ascochyta pathogen complex [10,14]. The main differentiation between wild and spring-sown cultivated chickpea for summer cropping is the loss of responsiveness to vernalization [10].

Cultivated chickpea can be further divided into two distinct types—desi and kabuli. The desi type has small and angular typically colored seeds, with a thick seed coat. The kabuli type has larger and smoother seeds, with a thin seed coat lacking in pigmentation [2]. The seed coat thickness of the kabuli and desi types exhibits monogenic inheritance, with the thin seed coat of kabuli being recessive [15]. Kabuli and desi types are clearly separated by molecular diversity analyses [10,16] and the large-seeded kabuli appears to have evolved from the smaller-seeded desi type [10,17]. The third type is sometimes referred to as the intermediate type of chickpea, with a pea shape, medium-to-small seed size and cream color [18,19].

Seed dormancy is one of the key domestication traits of legumes [20,21] which synchronizes seed germination with optimal environmental conditions and also distributes the germination in time. Germination determines the beginning of plant growth, and the transition from seed to seedling is a crucial event in the plant life cycle [22]. Wild plant seeds are usually dormant and germinate only after they are exposed to certain environmental conditions, while crop seeds germinate immediately when water is available [20]. In comparison with other legumes, a loss of seed dormancy and reduction of pod shattering is not considered as the main key domestication trait in chickpea [10,12], but rather the change from winter to summer cropping contributed most to the bottlenecks in genetic diversity of cultivated chickpea [10,14]. However, loss of dormancy has arguably played an important role in domestication process.

There are several dormancy classes. Morphological dormancy occurs when an embryo is not completely developed and requires more time to grow and germinate. Hormone mediated dormancy is the predominant form, and is referred to as physiological dormancy. It comprises of abscisic acid (ABA) and gibberellins (GAs) metabolism. Moreover, there exist morphophysiological and combinational dormancies [20,23]. In most legume species, another class of dormancy exists: physical dormancy (often called hardseededness) which is characterized by a water-impermeable seed coat. This class exists in 17 plant families, including legumes [23], and is caused by layer of water-impermeable palisade cells. Testa thickness is substantial, but is not the sole basis of impermeability [24,25]; it is also affected by the composition of the seed coat [25–27]. Abundant accumulation of phenolic and polyphenolic compounds in palisade cells was confirmed in pea and *Medicago truncatula* Gaertn. Upon oxidation, these compounds seem to change the seed permeability [27–29]. Hard and impermeable seeds become permeable to water (but also gases) after mechanical abrasion, the microbial effect of the digestive tract of animals, or by cracks in the seed coat. Physical dormancy can be also broken by thermal or humidity changes in the environment. Temperature oscillation is considered to be the best option in terms of a physical dormancy-breaking mechanism, and this has been confirmed for several legumes [20,30,31]. Two to three loci have been identified as involved in seed dormancy in pea through seed coat structure and seed coat thickness [27,32]. Two genes were identified in soybean as directly involved in seed coat water impermeability [33]. However, the genetic basis and genes related to seed dormancy in chickpea have not yet been identified.

The legume seed is typically composed of an embryo, in some cases of nutritive tissue (endosperm) and a protective seed coat (testa) (Figure 1). The seed coat of legumes is
developed from inner and outer integuments. The inner integument vanishes during development, while the outer integument is responsible for a typical legume seed coat structure. The layer of tightly packed palisade elongated cells (sclerenchyma cells or sclereid) is formed by the epidermis of the outer integument. Bottle-like shaped sclereid cells have thickened cell walls and are big in size, and are therefore called macrosclereids. Within macrosclereid cells exists a region that separates the terminal caps of cells from their basal parts. This region forms a line that runs across the macrosclereid layer and is referred as to as a light line. The sub-epidermal layer is differentiated into a single layer of osteosclereid cells (hourglass cells). The osteosclereid cells layer has significant intercellular spaces and thick cell walls. The macrosclereid and osteosclereid layers both contribute to the mechanical strength of the seed coat. The underlying (hypodermal) layer is formed by irregularly shaped parenchyma cells with intercellular spaces. The parenchyma cells layer serves as a nutritional tissue [20,25,34].

Figure 1. Anatomical structure of chickpea seed coat. Transversal section of *C. reticulatum* seed coat stained with Sudan Red (left). Scale bar = 50 μm. Schematic drawing (right, modified from Smykal et al. [20]). Abbreviations: ms—macrosclereid cells, ost—osteosclereids, par—parenchyma, ll—light line.

The aim of this study was to analyze the germination and dormancy status of cultivated (*C. arietinum*) and wild chickpea (*C. reticulatum*) and recombinant inbred lines derived from the cross of these two. Due to the assumption of a division of the RIL population into non-dormant and dormant categories, we were able to analyze the probable physical dormancy-related traits of chickpea seed coats in terms of physiology, anatomy and histochemistry. To date, a comparative study of the chickpea crop and its wild progenitor, with additional analysis of RIL population in the matter of germination, has not been carried out. Similarly, analysis of macrosclereid cell length has not been conducted with contrasting chickpea parental genotypes and the associated RIL population. We performed anatomical and physiological analysis of the chickpea seed coat as part of a comprehensive chickpea seed dormancy study that will comprise anatomical, physiological, genetic and transcriptomic approaches.
2. Materials and Methods

2.1. Germination Testing

To estimate the dormancy status of seeds, seed imbibition and germination testing was performed according to Hradilová et al. [27]. Seeds of two parental genotypes—wild dormant *Cicer reticulatum* (PI48977) and cultivated non-dormant *Cicer arietinum* (ICC4958)—and 129 recombinant inbred lines (RILs) of the F8 generation were received from Dr. C. Coyne, USDA Pullman, USA. The seeds originated from field grown (Central Ferry Farm, Pullman, WA, USA) plants. Twenty five intact dry seeds of each line were placed into 90 mm Petri dishes with filter paper (Whatman Grade 1, Sigma-Aldrich Ltd., St. Louis, MO, USA) and moistened with 3 mL of tap water and subjected to analysis of seed imbibition and germination. A fungicide (Maxim XL035 FS, Syngenta, Basil, Switzerland) was added to prevent fungal growth. The petri dishes with seeds were incubated at 25 °C in a temperature-controlled chamber in the dark (Incubator I, Memmert, Büchenbach, Germany) and the seeds were monitored at 24 h intervals for 30 days. Water was added continuously when needed. Germination testing was performed twice (2020, 2021) and the arithmetical mean and standard deviation were counted.

2.2. Germination Data Analysis

The number of imbibed seeds (i.e., swollen seeds) was scored daily for the period of 30 days for each RIL/parental genotype. The number of germinated seeds (i.e., radicle breaking through seed coat/radicle protrusion) was also scored. The primary interest of this study is physical dormancy (PY) realized by the barrier of the seed coat for water entry, and therefore the number of imbibed seeds was used as a measure of germinated seeds in all calculations. The data contain both imbibed and fully germinated seeds.

Several mathematical measurements were used to describe the germination process. The final germination percentage (FGP), mean germination time (MGT), coefficient of velocity of germination (CVG), first day of germination (FDG), last day of germination (LDG) and time spread of germination (TSG) were calculated over a period of 30 days, according do Kader [35]. In addition, the Timson germination index (TGI) was calculated according to Ranal and Santana [36].

2.3. Anatomical and Histochemical Analysis

Samples of testa were dissected from dry seeds of both parental genotypes and selected RILs of dormant and non-dormant groups. Testa segments were saturated with a 2% sucrose solution under vacuum for 1 h, according to Janská et al. [25]. Subsequently, these segments were placed into cryo-gel (Cryo-gel Leica, Leica Biosystems, Nussloch, Germany) on the cryostat alum chuck, frozen down to −20 °C and cut with a cryotome (Leica CM1950, Leica Microsystems Europe, Breckland, UK) into 20 µm transversal sections. These were stained according the protocol of Soukup [37] with Ruthenium Red (0.05%, w/v, Sigma-Aldrich Ltd., St. Louis, MO, USA), Toluidine Blue O (0.01%, w/v, Sigma-Aldrich Ltd., St. Louis, MO, USA) and Sudan Red 7B (0.1%, w/v, Sigma-Aldrich Ltd., St. Louis, MO, USA) and observed with an Olympus IC70 microscope (Olympus Corporation., Tokyo, Japan) in a bright field. The figures were documented with a Canon EOS camera (Canon Inc., Tokyo, Japan).

The ImageJ program [38] was used to analyze five sections of seed coats of parental genotypes and selected dormant and non-dormant RILs. Each section was measured at three points in triplicate in order to quantitative evaluation of macrosclereid cells length as a measure of seed coat thickness. Statistical analyses of obtained data were performed with program Statistica 12 (StatSoft CZ, Inc., Tulsa, OK, USA, 2016).
caused by a water-impermeable seed coat. The seeds of cultivated ICC4958 imbibed easily and germinated within 24 h, while the seeds of wild PI48977 displayed certain level of dormancy that were mediated by the seed coat permeability. The germination percentage (FGP) of wild PI48977 was recorded at several time intervals of 24 h (36.1%), 48 h (46.1%), 72 h (60.2%), 5 days (67.8%), 10 days (90.9%), 20 days (100%) and 30 days (100%). Figure 2 shows the FGP comparison for parental genotypes ICC4958 and PI48977 at seven time intervals. Cultivated ICC4958 reached 100% FGP immediately after 24 h, while wild dormant PI48977 reached FGP 100% between the 10th and 20th day.

![Figure 2](image-url)  
Figure 2. Comparison of final germination percentage of chickpea parental genotypes—cultivated *C. arietinum* (ICC4958) and wild *C. reticulatum* (PI48977) at seven time intervals.

The mean germination coefficient (MGT) represented the speed of germination, and thus the lowest MGT value indicated that seed germination was fast. MGT after 30 days was for cultivated ICC4958 as one day and for wild PI48977 as five days. The first day of germination (FDG) was the same for both parental genotypes, at day 1, which indicated a fast initiation of germination. However, the last day of germination (LDG) differed, for cultivated ICC4958 where all seeds imbibed on the first day, the value was day 1, while for wild PI48977, the value was day 14. The time spread of germination (TSG) represented as the time in days between the first and last germination event. TSG for cultivated ICC4958 was 0 and for wild PI48977 it was 13 days. The coefficient of velocity indicated the rapidity of germination and differed significantly between parental genotypes. CVG after 30 days for cultivated ICC4958 was 100 and for wild PI48977 was 20. Cultivated ICC4958 had the highest possible value, and this state occurred when all seeds germinated on the first day. The Timson germination index (TGI) differed in parental genotypes after 30 days. For cultivated ICC4958 the value was 30 and for wild PI48977 the value was 26.

RILs population displayed even more variability in each of these measurements. FGP after 24 h ranged from 2–100% (Figure S1), and after 30 days ranged from 24–100% (Figure S2); MGT ranged from 1.0–23.98 days (Figure S3); CVG ranged from 4.17–100 (Figure S4); and TGI ranged from 17.57–30 (Figure S5).

Representative non-dormant RILs were chosen based on FGP data of 24 h, when 10 RIL lines had 100% germination and, in an additional eight RILs, germination ranged from 88–99%. Dormant RILs were chosen primarily based on FGP data of 24 h with the lowest FGP values, but also with consideration of the data obtained after 30 days and the overall development of FGP data during testing. Five lines had very strong dormancy,
with FGP range from 0–20% at 24 h and 10 lines were chosen as dormant, with the FGP range from 26–80%. Figure 3 shows the germination of 15 selected dormant RILs at four time intervals. Evaluation after 30 days showed that only two lines (CRIL2-14, CRIL2-115) reached 100% FGP. Table S1 summarizes data for parental genotypes and selected RILs in the categories of dormant (15 lines) and non-dormant (18 lines). The average FGP at 30 days within the dormant group of 15 RILs was 70.5% and for the non-dormant group of 18 RILs was 99.3%. Within selected lines, the MGT ranged from 1, which was the lowest possible value and meant that the population germinated on the first day, to 23.98 (CRIL2-42), with very slow germination. The dormant group of 15 RILs had an average MGT of 7.26 days while non-dormant group of 18 RILs had an average MGT of 1.2 days. The CVG value determined the rapidity of germination. Within selected RILs, the CVG ranged from 4.17 to 100. The average CVG after 30 days was for the non-dormant group of 18 RILs at 87.0 and for the dormant group of 15 RILs was 22.17. Ten lines of the non-dormant group had the highest possible CVG value of 100, which indicated that germination occurred on the first day. Within selected lines, the TGI ranged from 17.57 to 30. The average TGI after 30 days was, for the non-dormant group of 18 RILs, 29.8 and for dormant group of 15 RILs was 25.9.

![Comparison of final germination percentage for selected dormant chickpea RILs at four time intervals. Error lines represent standard deviation of data.](image)

**Figure 3.** Comparison of final germination percentage for selected dormant chickpea RILs at four time intervals. Error lines represent standard deviation of data.

3.2. Anatomical and Histochemical Analysis

The continuity of the seed coat surface was interrupted by minor (ICC4958 and non-dormant lines; Figures 4B, 5F and 6E) or major (PI48977 and dormant lines; Figures 4A, 5C and 6F) fissures. The surface was covered with a thin cuticle. The cytological arrangement of the analyzed seed coats varied, especially in macrosclereids, and seed coat thickness was primarily managed by their length.
5C and 6F) fissures. The surface was covered with a thin cuticle. The cytological arrangement of the analyzed seed coats varied, especially in macrosclereids, and seed coat thickness was primarily managed by their length.

**Figure 4.** Histochemical Sudan Red staining of chickpea parental genotypes and RIL lines (A)—PI48977, (B)—ICC4958, (C)—CRIL2-60 (dormant group), (D)—CRIL2-80 (non-dormant group), (E)—CRIL2-106 (dormant group), (F)—CRIL2-51 (non-dormant group), (G)—CRIL2-114 (dormant group), (H)—CRIL2-50 (non-dormant group), (I)—CRIL2-6 (dormant group), (J)—CRIL2-89 (non-dormant group). Scale bars = 50 µm. Abbreviations: ms—macrosclereid cells, ost—osteosclereids, par—parenchyma, ll—light line.
Figure 4. Histochemical Sudan Red staining of chickpea parental genotypes and RIL lines (A)—PI48977, (B)—ICC4958, (C)—CRIL2-60 (dormant group), (D)—CRIL2-110 (non-dormant group), (E)—CRIL2-129 (dormant group), (F)—CRIL2-65 (non-dormant group), (G)—CRIL2-115 (dormant group), (H)—CRIL2-89 (non-dormant group). Scale bars = 50 µm. Abbreviations: ms—macrosclereid cells, ost—osteosclereids, par—parenchyma, ll—light line.

Figure 5. Histochemical Ruthenium Red staining of chickpea parental genotypes and RIL lines (A)—PI48977, (B)—ICC4958, (C)—CRIL2-60 (dormant group), (D)—CRIL2-110 (non-dormant group), (E)—CRIL2-129 (dormant group), (F)—CRIL2-65 (non-dormant group), (G)—CRIL2-115 (dormant group), (H)—CRIL2-89 (non-dormant group). Scale bars = 50 µm. Abbreviations: ms—macrosclereid cells, ost—osteosclereids, par—parenchyma, ll—light line.
Figure 5. Histochemical Ruthenium Red staining of chickpea parental genotypes and RIL lines (A)—PI48977, (B)—ICC4958, (C)—CRIL2-60 (dormant group), (D)—CRIL2-110 (non-dormant group), (E)—CRIL2-129 (dormant group), (F)—CRIL2-65 (non-dormant group), (G)—CRIL2-115 (dormant group), (H)—CRIL2-89 (non-dormant group). Scale bars = 50 µm. Abbreviations: ms—macrosclereid cells, ost—osteosclereids, par—parenchyma, ll—light line.

Figure 6. Toluidine Blue staining of chickpea parental genotypes and RIL lines (A)—ICC4958, (B)—PI48977, (C)—ICC4958, (D)—PI48977, (E)—CRIL2-47 (non-dormant group), (F)—CRIL2-60 (dormant group), (G)—CRIL2-80 (non-dormant group), (H)—CRIL2-129 (dormant group). Scale bars = 50 µm (A,B,E,F), 200 µm (C,D), 100 µm (G,H). Abbreviations: ms—macrosclereid cells, ost—osteosclereids, par—parenchyma, ll—light line.
The lipidic character of the cuticle covering the surface can be stained by Sudan Red. Lipidic substances (Figure 4) were detected at the cuticle, but there was also a signal below the light line of the macrosclereid as a continuous strip in wild PI48977 (Figure 4A) and several lines, mostly in the dormant group (Figure 4C,E) but also in the non-dormant group (Figure 4D). In the middle part of the macrosclereid, closer to the basal part of cells, lipidic content was visible with strong staining (Figure 4G,I).

Pectin substances were revealed with Ruthenium Red (Figure 5). The seed coats had a strong staining signal in the upper parts of the macrosclereid cells (Figure 5A–H). Significant staining was observed below (Figure 5A,C,E,G) and above (Figure 5F,H) the light line. Similarly to the Sudan Red staining, Ruthenium Red showed a visible stained line in the middle of the macrosclereid cells (Figure 5C,D). A light line was clearly visible in all of the analyzed RIL lines and in both parents.

Metachromatic staining with Toluidine Blue (Figure 6) showed the difference between the cultivated chickpea and the wild parent. The seed coat surface of the cultivated ICC4958 (Figure 6A,C) had a more compact character, while the wild PI48977 (Figure 6B,D) seed coat surface had frequent protrusion and extensions of macrosclereids. Similarity between the cultivated parent and non-dormant RIL lines and the wild parent and dormant RIL lines was observed. The seed coats of the non-dormant lines (Figure 6E,G) were more compact, while the seed coats of dormant lines (Figure 6F,H) were gritty, with many macrosclereid extensions and fissures. The Toluidine Blue staining also clearly visualized the osteosclereids and the underlying parenchyma cell layer (Figure 6E,F). A light line was also present, but not clearly visible. A strong staining signal in macrosclereid tips below the cuticle (Figure 6A,F) was determined as the polyanionic cell wall components. This signal was present in both cultivated and wild parents and non-dormant and dormant lines (Figure 6A–H).

3.3. Length of Macrosclereid

The length of the macrosclereid in cultivated ICC4958 ranged from 97.81 µm to 166.05 µm (Figure 7, Table S2), with the arithmetical mean of all sections being 125.49 µm and the standard deviation value of 21.94 (Table 1). While for wild PI48977 the upper value of the range was much higher, with the length ranging from 95.48 µm to 213.75 µm (Figure 7, Table S2), and the arithmetical mean of all sections with a value of 164.73 µm and standard deviation value of 35.30 (Table 1). There was a significant difference (p = 0.001) between the parental genotypes. The macrosclereid length of five representative dormant lines measured on five independent sections in triplicate ranged from 72.27 µm to 308.28 µm (Figure 7, Table S2). The means of the length for the dormant group (Table 2) were CRIL2-60 (150.97 µm), CRIL2-106 (168.45 µm), CRIL2-114 (159.94 µm), CRIL2-21 (157.33 µm), and CRIL2-115 (176.85 µm). The macrosclereid length of five representative non-dormant lines measured on five independent sections in triplicate ranged from 96.16 µm to 172.47 µm (Figure 7, Table S2). The means of length for the non-dormant group (Table 2) were CRIL2-6 (120.56 µm), CRIL2-81 (130.83 µm), CRIL2-43 (137.71 µm), CRIL2-110 (127.45 µm), and CRIL2-65 (138.28 µm). There was a significant difference (p = 0.0001) between the lengths of the non-dormant and dormant lines. The wild dormant parent PI48977 and dormant group of RILs had values usually above 150 µm, while the cultivated non-dormant parent and non-dormant group of RILs had values usually under 140 µm. Representation of all measured macrosclereid lengths for each parent or RIL lines with means and SD values is shown in the box-plot (Figure 7). Figure 8 shows the differences in the macrosclereid cells within the single seed of CRIL2-60 line.
Figure 7. Box-plot of macrosclereid cells length of chickpea parental genotypes (cultivated ICC4958, wild PI48977), dormant lines (CRIL2-60, CRIL2-106, CRIL2-114, CRIL2-21, CRIL2-115) and non-dormant lines (CRIL2-6, CRIL2-81, CRIL2-43, CRIL2-110, CRIL2-65).

Table 1. Macrosclereid cells length of chickpea parental genotypes—cultivated ICC4958 and wild PI48977.

|            | ICC4958 |           | PI48977 |           |
|------------|---------|-----------|---------|-----------|
|            | Length [µm] | Mean | SD      | Length [µm] | Mean | SD      |
| 1          | 143.57  | 22.41    | 1       | 177.45  | 41.25 |
| 2          | 114.78  | 14.81    | 2       | 142.88  | 55.14 |
| 3          | 113.12  | 19.00    | 3       | 155.39  | 15.04 |
| 4          | 134.59  | 31.41    | 4       | 169.49  | 36.84 |
| 5          | 121.40  | 16.10    | 5       | 178.47  | 31.48 |
| Mean       | 125.49  |          | Mean    | 164.73  |        |
| SD         | 21.94   |          | SD      | 35.30   |        |

Table 2. Macrosclereid cells length of 5 selected chickpea dormant RILs (CRIL2-60, CRIL2-106, CRIL2-114, CRIL2-21, CRIL2-115).

|            | CRIL2-60 |           | CRIL2-106 |           | CRIL2-114 |           | CRIL2-21 |           | CRIL2-115 |           |
|------------|---------|-----------|-----------|-----------|-----------|-----------|---------|-----------|-----------|-----------|
|            | Length [µm] | Mean | SD      | Mean | SD      | Mean | SD      | Mean | SD      | Mean | SD      |
| 1          | 99.45   | 23.56    | 162.82   | 37.65  | 149.47   | 13.06  | 143.18  | 8.71  | 158.73  | 7.89 |
| 2          | 230.50  | 82.61    | 184.61   | 90.85  | 158.44   | 34.61  | 172.62  | 44.74  | 210.90  | 17.58  |
| 3          | 124.70  | 17.28    | 160.84   | 41.91  | 155.68   | 15.23  | 191.57  | 28.57  | 141.56  | 3.42  |
| 4          | 129.33  | 26.52    | 151.54   | 19.06  | 174.75   | 42.70  | 148.85  | 6.63  | 167.34  | 16.26  |
| 5          | 183.94  | 67.08    | 182.44   | 75.41  | 161.39   | 44.80  | 130.45  | 3.54  | 205.71  | 19.45  |
| Mean       | 150.97  |          | 168.45   |          | 159.94   |          | 157.33  |          | 176.85   |          |
| SD         | 65.55   |          | 51.72    |          | 29.17    |          | 30.59   |          | 50.50    |          |

Table 3. Macrosclereid cells length of five selected chickpea non-dormant RILs (CRIL2-6, CRIL2-81, CRIL2-43, CRIL2-110, CRIL2-65).

|            | CRIL2-6 |           | CRIL2-81 |           | CRIL2-43 |           | CRIL2-110 |           | CRIL2-65 |           |
|------------|---------|-----------|---------|-----------|---------|-----------|---------|-----------|---------|-----------|
|            | Length [µm] | Mean | SD      | Mean | SD      | Mean | SD      | Mean | SD      | Mean | SD      |
| 1          | 120.22  | 15.45    | 120.49  | 14.33   | 142.62  | 11.63   | 128.93  | 9.82   | 137.26  | 7.23   |
Figure 7. Box-plot of macrosclereid cells length of chickpea parental genotypes (cultivated ICC4958, wild PI48977), dormant lines (CRIL2-60, CRIL2-106, CRIL2-114, CRIL2-21, CRIL2-115) and non-dormant lines (CRIL2-6, CRIL2-81, CRIL2-43, CRIL2-110, CRIL2-65).

Figure 8. Seed coat of chickpea RIL line CRIL2-60—comparison of dormant group. Seed coat differences within one seed. (A)—slightly elongated macrosclereids, (B)—extensively elongated macrosclereids. Scale bars = 100 µm. Abbreviations: ms—macrosclereid cells, ost—osteosclereids, par—parenchyma, ll—light line.

4. Discussion

Most of the information about physical dormancy is based on anatomical and morphological structure studies, phenolic content determination and cuticle composition [20]. The use of domesticated crop and wild progenitors offers the advantage of comparative analysis, which can lead to identification of the underlying mechanism. It has been shown that reduction of seed coat thickness during domestication led to the reduction of seed coat impermeability [20], which we demonstrated with cultivated non-dormant and wild dormant chickpea parental genotypes. In addition to thickness, the structure of the palisade and cuticular layer [27,39] and presence or absence of cracks [27,40,41] was proposed to be associated with seed hardness [27,39], which we also noticed in the wild parent and dormant lines. Seeds of the wild chickpea parent had a thicker macrosclereid palisade layer, which supports the finding in dormant pea seed genotypes, which probably contributes to the water impermeability of seed coats [42]. However, the structural differences of seed coats are not the only and major factor [24,25,27]. Rather, the chemical composition of the testa seems to be the most important factor. It was shown that content of polysaccharides, hydroxylated fatty acids or phenol compounds is related to testa properties [26,27,43–46]. We have found substantial structural differences in seed coat properties between the wild and domesticated chickpea. Seed coats differed remarkably in terms of the presence of protrusions and discontinuity in wild parent and dormant lines. On the contrary, the surface of the seed coat of different cultivars of soybean was found to have only small discontinuities [47], which is in accordance with our cultivated chickpea parental genotype.

The use of recombinant inbred lines derived from the cross of two contrasting parental lines provides the advantage of decoupling the effect of the indirect relationship, such as pigmentation, seed content, etc., which are the result of a thousand years of selection. The
testing of such RIL lines allows the dissection of traits directly related to the phenotype, such as the seed dormancy level. The evaluation of germination and seed dormancy caused by a water impermeable seed coat based on imbibed seeds data divided RILs into non-dormant and dormant groups. This strategy was previously used for pea [48] and led to the identification of differentially expressed genes [27]. In our study, we observed germination differences in 2020 and 2021 testing mostly in wild chickpea and in several lines, while the cultivated parent imbibed immediately within 24 h in both cases. In general, it cannot be stated that germination in 2020 was strictly faster or slower than in 2021; some RIL lines imbibed almost in same period of time, but some RIL lines showed substantial differences between two years of testing. The final germination percentage after 30 days in the chickpea RIL population ranged from 24–100%, which means that at least a few seeds of dormant lines imbibed. In contrast, pea accessions tested over a period of 28 days had a final germination percentage from 0–100% [48]; this means that some accessions were completely dormant and did not imbibe at all. Beside the species differences, this might also be attributed to the environment of cultivation, since pea were cultivated in glasshouses while chickpea from field grown plants. The effect of the maternal environment on seed dormancy has been shown [49] at least in Arabidopsis and other plants with physiological dormancy types, but it is likely expected also in physical dormancy, in which it was found that the release of physical dormancy of Medicago truncatula accessions from arid regions occurred to a greater extent under higher (35/15 °C) rather than lower (25/15 °C) temperature alternations [31]. In addition, the position of seed development on the parental plant predominantly influences the physiological dormancy in Brassicaceae [50] and can be expected to affect dormancy in many other plant families, including legumes.

The effect of seed coat pigmentation on seed dormancy has been extensively documented by transparent testa mutants in Arabidopsis [49] and also pigmented legume seeds generally imbibe slower than non-pigmented ones and germination is also delayed [48,51–53]. This was observed also in our study, where chickpea RIL lines with bigger, non-pigmented and smoother seeds imbibed earlier than smaller, pigmented and notched seeds. This was also confirmed with the seed coat browning of common bean [54,55] and chickpea [56].Moreover, there is a difference between kabuli and desi chickpea types. Non-pigmented kabuli seeds imbibed quickly, within 4–8 h, whereas pigmented desi genotypes reached maximum water imbibing capacity at up to 16 h [57]. In our study, cultivated chickpea seeds were representative of the desi type and were usually imbibed within a few hours, maximally up to 24 h. We did not conduct our study with the cultivated kabuli type, but we assume that it would imbibe even earlier. Moreover, the thicker macrosclereid cells of desi type are responsible for the seed coat color and its polyphenolic content [34,58]. There also exists a connection with the seed coat thickness, with the thicker seed coat desi type (comprising 14% of total seed weight) and thinner seed coat kabuli type (comprising 5% of total seed weight) [34,59]. Since we studied the cultivated parent of the desi type, with a generally thicker seed coat and wild chickpea with a former seed coat not affected by domestication, it can be assumed to have RIL population with thicker seed coats. Interestingly, the thick seed coat is believed to be responsible for resistance to bruchid beetle infestation [19].

Macrosclereid lengths as a measure of seed coat thickness have been already performed in several legume species, including cultivated chickpea. While in the study of Dattatreya et al. [60] macrosclereid cells were found to be elongated, bended at one end and narrowed at both ends with lengths ranging from 37.6–155.6 µm with a mean of 94.9 µm, in our study we observed longer lengths of macrosclereid cells, with a mean of 125.49 µm. This difference is likely due to variation present in cultivated chickpea, since we used in our study the RIL lines population derived from cultivated chickpea with a slightly thicker seed coat and wild chickpea parents. Similarly, two desi genotypes’ macrosclereid cells had 141 µm and 144 µm, respectively, where substantial similarity can be noticed with our cultivated desi chickpea with the macrosclereid cell length mean of 125.49 µm, while kabuli type had a macrosclereid cells length of 110 µm [34,59]. The thin kabuli seed coat
is apparently due to a thinner layer of palisade and parenchyma cells with fewer pectic polysaccharides and less proteins [34].

CROSSES OF DESI AND KABULI TYPES WERE USED TO ESTIMATE THE GENETIC BASIS OF THE TESTA THICKNESS [15]. ALTHOUGH THE DATA FOR LENGTH ARE NOT PROVIDED, THEY CONSIDERED A SEED COAT AS THICK WITH A LENGTH OVER 70 µM AND THIN WHEN UNDER 70 µM. IN OUR STUDY, WE USED A SIGNIFICANTLY HIGHER VALUE (150 µM) AS THE BOUNDARY OF SEED COAT THICKNESS BECAUSE WE USED POPULATION DERIVED FROM THE CROSSING OF CULTIVATED AND WILD CHICKPEA, UNLIKE IN THE LATTER STUDY. MEASUREMENTS FOR WILD CHICKPEA (C. RETICULATUM) WERE NOT CONDUCTED UNTIL THIS STUDY, AND THEREFORE NO COMPARISON IS FEASIBLE. OUR RESULTS REGARDING SEED COAT THICKNESS ARE IN AGREEMENT WITH THE SEED COAT DIFFERENCES BETWEEN DORMANT AND NON-DORMANT PEA SEEDS, WHERE DORMANT PEA ACCESSIONS HAD A THICKER SEED COAT (MEAN 139 µM) THAN NON-DORMANT PEA ACCESSIONS (MEAN 97 µM) [27,48], COMPARABLE TO CHICKPEA. HOWEVER, NON-DORMANT PEA ACCESSIONS HAD A VARIABLE MACROSCLEREID CELL LENGTH AND VALUES OFTEN REACHED THE HIGH VALUES TYPICAL FOR DORMANT ACCESSIONS (ABOVE 80 µM) [48] WHICH WAS IN ACCORDANCE WITH NON-DORMANT CHICKPEA, WHERE SEVERAL MACROSCLEREID MEASUREMENTS EXCEEDED THE BOUNDARY OF 150 µM. HOWEVER, MACROSCLEREID CELLS OF DORMANT PEA ACCESSIONS DID NOT FALL BELOW 80 µM, WHICH WAS DISSIMILAR TO CHICKPEA WHERE SEVERAL LENGTH MEASUREMENTS FALL BELOW 150 µM.

LIPIDIC COMPOUNDS STAINING WITH SUDAN RED MOSTLY OCCUR IN THE CUTICLE ON THE SURFACE OF THE SEED COAT OF CHICKPEA, WHICH IS SIMILAR TO PEA [25,61], WITH A STRONG SIGNAL OBSERVED IN MIDDLE AND BASAL PARTS OF THE MACROSCLEREID CELLS. IN ADDITION, NEARLY MATURE PEA SEEDS HAD SIGNAL ABOVE THE LIGHT LINE [61] THAT CAN BE OBSERVED IN SOME CHICKPEA LINES. THE PRESENCE OF NON-CUTICULAR WAXY EXTRACELLULAR COMPOUNDS WAS OBSERVED IN THE TIPS OF THE MACROSCLEREIDS ABOVE THE LIGHT LINE IN ANOTHER PEA STUDY [25] AND WAS CONFIRMED BY AUTOFLUORESCENCE. IN OUR STUDY OF CHICKPEA SEEDS WE OBSERVED THE LIGHT LINE, THE PRESENCE OF WHICH WAS CONFIRMED ALSO IN CULTIVATED AND WILD PEA GENOTYPES. HOWEVER, THE LIGHT LINE OF WILD PEA GENOTYPES WAS MORE PRONOUNCED [61], WHICH CANNOT BE UNAMBIGUOUSLY STATED FOR WILD CHICKPEA. A LIGHT LINE THAT LAYS WITHIN THE MACROSCLEREIDS WAS INTERPRETED VARIOUSLY, BUT ITS MAIN PURPOSE IS NOT YET CLARIFIED [61–66]. IT IS BELIEVED THAT THE LIGHT LINE IS THE RESULT OF AN ALTERED ORIENTATION OF CELLULOSE MICROFIBRILS OR STRUCTURAL DISCONTINUITY BETWEEN THE LIGNIFIED AND NON-LIGNIFIED PARTS OF THE CELL WALL [61,67,68].

POLYSACCHARIDES PRESENT IN CHICKPEA COTYLEDON CELL WALLS CONSIST OF CELLULOSE, XYLOGLU-CAN, ARABINAN AND OF PECTIN [69], THAT CAN BE STAINED WITH RUTHENIUM RED. A STRONG STAINING SIGNAL WAS OBSERVED IN THE UPPER PARTS OF MACROSCLEREIDS, BELOW THE LIGHT LINE FOR SEVERAL DORMANT LINES AND ABOVE THE LIGHT LINE FOR SEVERAL NON-DORMANT LINES. IN TERMS OF THE LIGHT LINE, IT WAS DISTINCT IN THE MAJORITY OF LINES. PECTIN IS ONE OF THE CHEMICAL COMPOUNDS THAT MAY SIGNIFICANTLY INFLUENCE THE PHYSICOCHEMICAL PROPERTIES OF DIFFERENT CELL TYPES DURING ITS DEVELOPMENT AND THE FURTHER PROCESSING OF SEEDS [68]. THIS CLASS OF POLYSACCHARIDES WAS CONFIRMED ALSO IN THE CELL WALLS OF PEA SEED COATS WHERE ITS CONCENTRATION VARIED BY LOCATION AND DEVELOPMENTAL STAGE [34,70] AND WAS DIFFERENTLY LOCALIZED BETWEEN WILD AND CULTIVATED PEA SEED COATS [25,61]. NEVERTHELESS, WE DID NOT CONDUCT ANALYSIS OF DEVELOPMENTAL STAGES, WHICH WOULD PROBABLY SHOW US CERTAIN CHANGES IN PECTIN SEED COAT DISTRIBUTION.

THE HYPODERMAL LAYER, COMPRISING OF A SINGLE LAYER OF OSTEOSCLEREIDS, IS UNIFORMLY THICKENED IN CHICKPEA AND WAS OBVIOUS IN ALL CHICKPEA LINES. AS MENTIONED EARLIER IN THE PEA STUDY [20], OSTEOSCLEREIDS IN CHICKPEA ALSO CONTRIBUTE TO THE MECHANICAL STRENGTH OF THE SEED COAT. INTERESTINGLY, THE SEED COAT OF THE INTERSPECIFIC BREEDING LINE DERIVED FROM ANOTHER WILD CHICKPEA (CICER ECHINOSPERMUM P.H.DAVIS) CLOSELY RELATED TO C. RETICULATUM, DIFFERED FROM THE OTHER DESI GENOTYPES, EXCEPT FOR ITS HETEROGENEITY IN TERMS OF OSTEOSCLEREID CELL SHAPE. THIS HETEROGENEITY IS CONSIDERED AS A SIGNATURE TRAIT OF C. ECHINOSPERMUM THAT HAS BEEN INHERITED BY THE INTERSPECIFIC LINE. ANY POSSIBLE ROLE OF THIS TRAIT IN SEED DORMANCY OR GENERALLY IN SEED PROTECTION IS INDISTINCT [34].

IN RECENT YEARS, TWO TYPES OF CULTIVATED CHICKPEA, KABULI AND DESI, STAND IN THE FOREFRONT OF THE INTEREST OF RESEARCHERS. THEREFORE, THIS STUDY FOCUSED ALSO ON A WILD CHICKPEA PROGENITOR AND SUBSEQUENT EVALUATION OF THE RIL POPULATION WAS NEEDED. TAKEN TOGETHER,
this study showed the anatomical and histochemical differences related to the level of seed dormancy in chickpea. This knowledge, in combination with genetic mapping and transcriptomic analysis of the seed coat, will ultimately lead to the identification of the underlying molecular mechanism of seed dormancy in chickpea, as well as allow comparison to phylogenetically related legumes, such as pea, lentil and faba bean, domesticated in the same region of the Fertile Crescent. In addition, information about the chemical composition of chickpea seed coats in relation to seed dormancy needs to be further determined and, along with comprehensive examination of mentioned genetic, transcriptomic and chemical analysis could be utilized in further chickpea breeding programs, particularly in relation to regulation of seed germination which is essential for successful crop establishment and final yield.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agronomy11112292/s1, Figure S1: Final germination percentage after 24 h for chickpea RIL lines population. Representatives of dormant and non-dormant lines are highlighted, Figure S2: Final germination percentage after 30 days for chickpea RIL lines population. Representatives of dormant and non-dormant lines are highlighted, Figure S3: Mean germination time for chickpea RIL lines population. Representatives of dormant and non-dormant lines are highlighted, Figure S4: Coefficient of velocity of germination for chickpea RIL lines population. Representatives of dormant and non-dormant lines are highlighted, Figure S5: Timson germination index for chickpea RIL lines population. Representatives of dormant and non-dormant lines are highlighted, Table S1: Germination data for chickpea parental genotypes (cultivated ICC4958, wild PI48977) and representative dormant and non-dormant chickpea RIL lines, Table S2: Macrosclereid cells length of chickpea parental genotypes (cultivated ICC4958, wild PI48977), dormant lines (CRIL2-60, CRIL2-106, CRIL2-114, CRIL2-21, CRIL2-115) and non-dormant lines (CRIL2-6, CRIL2-81, CRIL2-43, CRIL2-110, CRIL2-65).

Author Contributions: Conceptualization, P.S.; methodology, V.S., L.Z. and P.S.; formal analysis, V.S., P.H., M.G. and P.S.; investigation, V.S. and P.H.; resources, P.S.; data curation, V.S. and P.S.; writing—original draft preparation, V.S., P.H. and P.S.; writing—review and editing, V.S., P.H. and P.S.; project administration, P.H. and P.S.; funding acquisition, P.H. and P.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Internal Grant Agency of Mendel University in Brno, grant number AF-IGA2020-IP011.

Acknowledgments: We thank Clarice Coyne from USDA-ARS, WSU and Eric Bishop von Wettberg from UVM for providing plant material.

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

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