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Seed Water Absorption, Germination, Emergence and Seedling Phenotypic Characterization of the Common Bean Landraces Differing in Seed Size and Color

Monika Vidak [1], Boris Lazarević [1,2,*], Tomislav Javornik [1,3], Zlatko Šatović [1,3] and Klaudija Carović-Stanko [1,3]

1 Centre of Excellence for Biodiversity and Molecular Plant Breeding (CoE CroP-BioDiv), Svetošimunska cesta 25, 10000 Zagreb, Croatia
2 Department of Plant Nutrition, University of Zagreb Faculty of Agriculture, Svetošimunska cesta 25, 10000 Zagreb, Croatia
3 Department of Seed Science and Technology, University of Zagreb Faculty of Agriculture, Svetošimunska cesta 25, 10000 Zagreb, Croatia
* Correspondence: blazarevic@agr.hr; Tel.: +385-1-234-3961

Abstract: Common bean (*Phaseolus vulgaris* L.) is the most widespread legume in Croatia and its production is based on landraces of great morphological diversity. Landraces morphologically differ the most in the seed coat color and size. Because plant emergence and crop establishment represent the most sensitive stage in crop development, the aim of this study was to determine whether the seed coat color and seed size of Croatian common bean landraces affect the water absorption rate, seed germination, emergence and phenotypic characteristics of the seedlings. In this study seeds of four common bean landraces with different seed color and size, 'Biser' (white-colored, small-seeded), 'Bijeli' (white-colored, large-seeded), 'Kornjača' (dark-colored, small-seeded) and 'Trešnjevac' (dark-colored, large-seeded) were evaluated in three different experiments: (i) water uptake, (ii) seed germination and (iii) emergence and seedling phenotypic characterization. The results show that white-colored seeds have a higher absorption rate and release more electrolytes compared to dark-colored seeds of similar size (and weight). The germination results show that white-colored and smaller seeds germinate faster compared to dark-colored and large seeds. On the other hand, the white-colored landrace 'Bijeli' took the longest time to emerge, probably due to cell damage that occurred during the fast initial water absorption. Using multispectral imaging, chlorophyll fluorescence imaging and stomatal conductance analysis, the seedlings of the dark-colored and large-seeded landraces were found to contain more photosynthetic pigments and have higher light absorption. In contrast, seedlings of white-colored and large seeds have lower stomatal conductance and transpiration and higher photochemical efficiency (despite possible cell damage during water absorption and germination). Results suggest that dark-colored seeds could survive better under unfavorable soil conditions without absorbing water, swelling, emergence or molding than white-colored seeds. Despite all this, white-colored common bean landraces remain popular in human diets and are often grown on small-scale farms.

Keywords: chlorophyll fluorescence imaging; electrolyte leakage; multispectral imaging; seed coat color; stomatal conductance

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Citation: Vidak, M.; Lazarević, B.; Javornik, T.; Šatović, Z.; Carović-Stanko, K. Seed Water Absorption, Germination, Emergence and Seedling Phenotypic Characterization of the Common Bean Landraces Differing in Seed Size and Color. *Seeds* **2022**, *1*, 324–339. https://doi.org/10.3390/seeds1040027

Academic Editors: Elias Soltani, Carol Baskin and José Luis González Andújar

Received: 10 October 2022
Accepted: 22 November 2022
Published: 1 December 2022

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is the most widely consumed legume in the world and the most important legume produced for direct human consumption, with a commercial value that exceeds that of all other legumes combined [1]. Its nutritional value is also extremely important; it is rich in protein, fiber, fat, vitamins and minerals, contains almost all essential amino acids (except methionine and cysteine) and is a good substitute for meat when combined with cereals [2,3]. Common bean is the most widespread legume in Croatia and its production is based on landraces with great genetic and morphological diversity [4,5]. Croatian common bean landraces are known by their vernacular names, which are mostly based on the color and pattern of the seed coat [6].

Given the diversity of systems in which it is grown, common bean yields vary widely and are often below the genetic potential of the species [3]. The success of crop production highly depends on seed quality, as low-quality seed can lead to problems in germination and crop establishment, occurrence of diseases, uneven maturation, and harvest problems, as well as low yields [7]. Rapid and uniform seed germination and seedling emergence are necessary to ensure early plant establishment before strong weed competition occurs and to allow synchronous seed formation and maturation before harvest, which is the basic requirement for increasing crop yield and quality [7–10]. Germination begins with water absorption (imbibition) by the mature seed and ends with the emergence of the radicle [10,11]. In the first stage of germination, water is absorbed by the hilum, micropyle, and lens [10,12]. The water then diffuses through the parenchyma cells to the periphery of the cotyledon, allowing hydration of the entire embryo [10].

One of the factors which negatively affects legume establishment in the field is the damage caused by rapid water uptake by the seed, which leads to cell damage, electrolyte loss and reduced germination and growth [7]. White-seeded French bean [13] common bean [14], faba bean (*Vicia faba* L.) [15], chickpea (*Cicer arietinum* L.) [16] and rapeseed (*Brassica napus* L.) [17] have different effects on water uptake rate and seed germination compared to dark-colored seeds. White-colored seeds of grain legumes absorb water faster than dark-colored seeds, and it is precisely for this reason that white seeds are more damaged, resulting in weaker seedling growth and development [7,9,13], leading to poor plant traits and capabilities in the field or even in the food industry [18]. Despite this, white-colored common bean seeds are popular in the diet because their lower content of polyphenols and tannins increases digestibility, which also increases bioavailability of minerals, such as iron [19,20]. In addition, white-colored common bean seeds contain high levels of inhibitors of pancreatic a-amylase, which lower blood glucose levels in rats with type 2 diabetes [21]. Aside from seed color, seed size is also an important physical indicator of seed quality that affects emergence, plant growth and plant performance in the field, as sowing mixed seed of one species can result in uneven plant establishment, which, in turn, results in heterogeneity in plant vigor and size [22,23].

Non-destructive early detection of phenotypic traits and selection of enhanced performance of plants using high-throughput phenotyping plant platforms (HTP) is often used for successful crop production [24]. The image-based approaches using digital image processing and computer vision technologies offer solutions to automatically measure a variety of different seed traits in a high-throughput way [25]. Two-dimensional- (2D) based systems are increasingly used. For example, Varga et al. [26] used computer vision to analyze seed coat color in common bean, whereas Baek et al. [27] designed an image acquisition device to quantitatively measure morphological and color parameters of soybean seeds.

In addition to seed analysis, HTPs can be used for the quantification of different morphological and physiological parameters in the seedling stage, which could be related to seed quality and the seed germination process.

The aim of this study was to determine whether seed coat color and seed size of Croatian common bean landraces affects the water absorption rate, seed germination and emergence, evaluated by high-throughput phenotyping techniques.

2. Materials and Methods

2.1. Plant Material and Experimental Design

In this study, the seeds of four Croatian common bean landraces grown on small scale farms in Croatia (Table 1) were collected in 2014 and multiplied in 2019 in nonreplicated field trials at the Maksimir experimental field, Department of Seed Science, University of Zagreb Faculty of Agriculture, were used. Two landraces with white seed coat 'Biser' and 'Bijeli' and two of dark-colored seed coat 'Kornjača' and 'Trešnjevac' were selected for this study. 'Biser', 'Bijeli' and 'Trešnjevac' are among the most widespread landraces in Croatia [6] and 'Kornjača' was selected because it is the only one that is small-seeded and black-colored. A total of 1400 seeds were used for the experiment, 50 seeds of each landrace for water uptake experiment (200 seeds in total) and 150 seeds of each landrace for germination and emergence experiment (1200 seeds in total). The weight of 100 seeds was determined following the protocol of Nadeem et al., [28] in which three replicates of 100 of randomly selected, fully mature and undamaged seeds were weighed, using an analytical balance (0.001 g).

Table 1. Croatian common bean landraces used in the study, with description of seed coat color, size, average 100-seed weight and location.

	Landrace	Color of Seed Coat	Seed Size	100-Seed Weight (g)	Location	Latitude	Longitude	
C1	'Biser'	white	small	18.58	Mali Bukovec	46.29 N	16.74 E	
C2	'Bijeli'	white	large	39.91	Kusać-Soljani	44.95 N	18.97 E	
C3	'Kornjača'	black	small	21.09	Ivanovec	46.37 N	16.48 E	
C4	'Trešnjevac'	dark red	large	40.80	Fužine	45.30 N	14.71 E	

2.2. Water Absorption Experiment and Electrical Conductivity Measurement

The general guidelines for conducting the electrical conductivity test (ISTA, 2015 modified by [29]) were followed, with the modifications described below.

First, the water absorption of dry seeds (50 seeds per landrace) was monitored over a 24-hour period. Each dry seed was first weighed on a precision analytical balance (0.001 g) and then placed in a plastic tube containing 15 mL of deionized and distilled water (ddH_2O). Plastic tubes containing ddH_2O with a reading of <0.05 µs cm^{-1} and seeds were placed in a chamber at a temperature of 25 ± 1 °C. Each seed was then removed from the water after 1.5, 3, 6, 9, 12, 20 and 24 h. Excess water was removed from the seeds by blotting with a paper towel. Seeds were weighed on a precision analytical balance (0.001 g) and placed back in ddH_2O.

After 24 h in ddH_2O water, the electrical conductivity (EC, ms m^{-1}), i.e., the electrolyte concentration in the water for each seed was measured using the Mettler Toledo™ FiveEasy™ Plus FP30 conductivity meter. The results were expressed as the mean of all seeds of each landrace separately. The EC test is considered one of the best tests to assess cell damage in seeds, i.e., to determine the concentration of electrolytes released from seeds during water uptake. A higher EC value indicates a higher degree of seed damage [30].

2.3. Seed Germination Experiment

After the experiment on water absorption, an experiment on seed germination of common bean landraces was conducted. The seeds used for the water uptake rate experiment could not be used for the germination experiment as they swelled after 24 h in ddH_2O, some cracked and some even germinated.

For each landrace, 150 dry seeds were used and distributed in three replicates in 5 Petri dishes with 10 seeds in each. A filter paper (Munktell 21/N, 580 × 580 mm, 80 g/qm) moistened with distilled water was placed on the bottom of the Petri dishes (Steriplan®, DURAN®, DWK Life Sciences GmbH, Mainz, Germany) and the seeds were placed on top. The Petri dishes were placed in a germination test chamber under controlled conditions according to the instructions of the International Seed Testing Association [31] (ISTA, 1993). The germination analysis was carried out at a temperature of 25 °C, 70% relative air humidity and a 16 h day and 8 h night regime. The number of germinated seeds (seeds with root size $\geq$ 2 mm) was determined every 24 h for seven days. At the end of the experiment the number of normal seedlings, abnormal seedlings and ungerminated seeds (dead seeds) were counted.

2.4. Emergence Experiment

For the emergence experiment, 150 dry seeds of each landrace were sown in three replicates in polystyrene seedling trays (52 cm × 32 cm) with 60 pots (4.5 cm in diameter each) in commercial seedling Substrat 1 (Klasmann-Deilmann GmbH, Geeste, Germany) in a growth chamber under 25 °C, 16 h day and 8 h night regime, 70% relative air humidity and 250 mmol m^{-2} s^{-1} of photosynthetic photon flux density (PPFD) provided by Valoya L35, NS12 spectrum LED lights (Valoya Oy, Helsinki, Finland). Seedlings were manually irrigated with 5 mL of water each. The number of emerging plants was determined every 24 h for seven days. At the end of the experiment, the number of normal seedlings, abnormal seedlings and ungerminated seeds (dead seeds) were counted.

2.5. Phenotyping Analyses

Phenotyping analyses were performed on eight-day-old seedlings. Measurements of the stomatal conductance (gsw), boundary layer conductance (gbw), total conductance to water vapor (gtw) and transpiration (T) were measured using LI-600 porometer (LI-COR Biosciences, Lincoln, NE, USA).

Chlorophyll fluorescence imaging and multispectral imaging were performed using CropReporter^{TM} (PhenoVation B. V., Wageningen, The Netherlands) and image analysis was performed using the DA^{TM} analysis software (version 5.6.1-64b) (PhenoVation B. V., Wageningen, The Netherlands).

A detailed description of the CropReporter and the imaging procedure is given in [32]. For the chlorophyll fluorescence imaging, plants were first dark-adapted for 30 min. On dark-adapted plants, photosynthesis was excited using 4000 µmol m^{-2} s^{-1} red LED light flash. The minimum chlorophyll fluorescence (F_0) was measured after ten (10) µs and maximum chlorophyll fluorescence (F_m) was measured after 800 ms. Following these measurements, plants were relaxed in the dark for 15 s and then were light-adapted for 5 min using actinic light of 250 µmol m^{-2} s^{-1}. Saturating pulse (4000 µmol m^{-2} s^{-1}) was applied for the photosynthesis excitation of the light-adapted plants. Steady-state fluorescence yield (F_s') was measured before the onset of the saturation pulse, and maximum chlorophyll fluorescence (F_m') of the light-adapted leaves was measured at saturation. After the measurement, actinic light was turned off, and in the presence of far-red light, minimal fluorescence yield of the illuminated plant (F_0') was estimated. These measured parameters were used for the calculation of different chlorophyll fluorescence parameters, which are shown in Table 2.

Table 2. List of calculated chlorophyll fluorescence traits with abbreviations, equation for calculation and the reference.

Abbrev.	Trait	Wavelength/Equation
F_v/F_m	Maximum Efficiency of Photosystem Two	$F_v/F_m = (F_m - F_0)/F_m$ [33]
F_q'/F_m'	Effective Quantum Yield of Photosystem Two	$F_q'/F_m' = (F_m' - F_s')/F_m'$ [34]
ETR	Electron Transport Rate	$ETR = F_q'/F_m' \times PPFD \times (0.5)$ [34]
NPQ	Non-Photochemical Quenching	$NPQ = (F_m - F_m')/F_m'$ [35]

Following the chlorophyll fluorescence imaging, actinic light 250 μmol m^{-2} s^{-1} was switched on again and multispectral imaging was performed. A list of all measured and multispectral parameters, as well as the calculated vegetation indices can be found in Table 3.

Table 3. List of analyzed multispectral traits with abbreviations, wavelength for measurement or equation for calculation and the reference if appropriate.

Abbrev.	Trait	Wavelength/Equation
R_{Red}	Reflectance in Red	640 nm
R_{Green}	Reflectance in Green	550 nm
R_{Blue}	Reflectance in Blue	475 nm
R_{SpcGrn}	Reflectance in Specific Green,	510–590 nm
R_{FarRed}	Reflectance in Far Red	710 nm
R_{NIR}	Reflectance in Near Infra-Red	769 nm
HUE	Hue (0–360°)	$HUE = 60 \times (0 + (R_{Green} - R_{Blue})/(max - min))$, if $max = R_{Red}$; $HUE = 60 \times (2 + (R_{Blue} - R_{Red})/(max - min))$, if $max = R_{Green}$; $HUE = 60 \times (4 + (R_{Red} - R_{Green})/(max - min))$ if $max = R_{Blue}$; 360 was added in case HUE < 0
SAT	Saturation (0–1)	$SAT = (max - min)/(max + min)$ if $VAL > 0.5$, or $SAT = (max - min)/(2.0 - max - min)$ if $VAL < 0.5$, where max and min are selected from the R_{Red}, RG_{reen}, R_{Blue}
VAL	Value (0–1)	$VAL = (max + min)/2$; where max and min are selected from the R_{Red}, R_{Green}, R_{Blue}
ARI	Anthocyanin Index	$ARI = (R_{550})^{-1} - (R_{700})^{-1}$ [36](Gitelson et al., 2001)
CHI	Chlorophyll Index	$CHI = (R_{700})^{-1} - (R_{769})^{-1}$ [37]
NDVI	Normalized Differential Vegetation Index	$NDVI = (R_{NIR} - R_{Red})/(R_{NIR} + R_{Red})$ [38]

2.6. Data Analysis

The germination and emergence characteristics were calculated at the end of the experiment. The same equations presented in the work of Vidak et al. [39] were used to calculate the germination and emergence characteristics.

2.7. Statistical Analysis

2.7.1. Water Absorption

The MIXED procedure in SAS v. 9.4 (SAS Institute Inc., Cary, NC, USA) was utilized for the analysis of variance (ANOVA) with repeated measures [40]. A completely randomized design (CRD) was used to examine effects of landrace, soaking time (used for repeated measures) and landrace × time interaction on water absorption (%). The covariance structure was chosen based on the Akaike information criterion with a correction for small sample sizes (AICc), including five covariance matrix structure types: unstructured (UN), variance components (VC), compound symmetric (CS), first-order autoregressive (AR(1)) and Toeplitz (TOEP). The significance of landraces within time was performed by Tukey's Honest Significant Difference post hoc test for partitioned F-tests (SLICE option). Additionally, the CONTRAST statements were used to compare the levels of water absorption between white- (C1 and C2) and dark-colored seeds (C2 and C4), as well as between small- (G1 and G3) and large-seeded (C2 and C4) landraces at each time point.

2.7.2. Germinability/Emergence/Phenotyping

A completely randomized design (CRD) with three replications (50 seeds per landrace/replication) was used to examine effects of landrace (C1: 'Biser', C2: 'Bijeli', C3: 'Kornjača', C4: 'Trešnjevac') on the seven seed germinability, the seven analogous emergence traits and the 21 seedling phenotypic traits. The univariate analysis of variance (ANOVA) using PROC GLM in SAS v. 9.4 (SAS Institute Inc., Cary, NC, USA) and a post hoc Tukey test ($p < 0.05$) were carried out to compare the values among landraces. Additionally, the CONTRAST statements were used to compare the trait values between white- (C1 and C2) and dark-colored seeds (C2 and C4), as well as between small- (C1 and C3) and large-seeded (C2 and C4) landraces. The trait values expressed as percentages [germinability (G); coefficient of variation of the germination time (CV_{gt}); emergence (E); coefficient of variation of the emergence time (CV_{et})] were normalized by arcsine transformation prior to analysis.

3. Results

In this study, the relationship between water uptake and leaching of electrolytes from seeds, seed germination, emergence, multispectral imaging, chlorophyll fluorescence imaging and stomatal conductance analysis considering seed coat color and seed size of four Croatian common bean landraces was investigated.

3.1. Water Absorption and Electrical Conductivity

Table 4 shows the average water uptake rate of four Croatian common bean landraces for 50 seeds monitored over a 24-hour period. It was found that all four landraces differed significantly in terms of the time they took to absorb water. Both seed color and seed size had a significant effect on the water absorption rate. On average, the white-colored landraces had a higher water absorption rate (absorbed more water at an early measurement time) than the dark-colored landraces of similar mass. In addition, small-seeded landraces had a higher water absorption rate than large-seeded landraces up to 9 h from imbibition. Thus, the small-seeded white-colored landrace 'Biser' (C1) absorbed water faster compared to the small-seeded dark-colored landrace 'Kornjača' (C3). The large-seeded white-colored landrace 'Bijeli' (C2) also absorbed water faster on average than the large-seeded dark-colored landrace 'Trešnjevac' (C4) (Table 4).

Table 4. Differences between Croatian common bean landraces in average amount of water absorbed (g) per seed and landrace and electrical conductivity (EC) after 24 h per seed and landrace.

Time (h)	1.5		3		6		9		12		20		24		EC24 (ms m^{-1})	
P(F)	***		***		***		**		*		*		ns		***	
C1	61.25	A	88.02	A	115.62	A	116.87	AB	120.85	AB	122.33	AB	127.05	A	82.12	C
C2	22.96	C	60.41	B	107.20	B	120.06	A	124.52	A	127.86	A	128.75	A	177.87	A
C3	40.88	B	86.80	A	111.40	AB	117.58	A	120.11	AB	123.14	AB	125.08	A	56.45	D
C4	8.45	D	38.74	C	95.41	C	110.33	B	115.57	B	119.25	B	121.76	A	114.57	B
Contrast W vs. C P(F)	***		**		**		*		*		*		*		***	
Contrast S vs. L P(F)	***		***		***		ns		ns		ns		ns		***	

C1—'Biser', C2—'Bijeli', C3—'Kornjača', C4—'Trešnjevac', EC24—electrical conductivity after 24 h, W—white, C—colored, S—small, L—large. Values followed by the same letter in each column are not significantly different based on the Tukey test at 0.05 probability. ns—not statistically significant $p > 0.05$, * $0.05 > p > 0.01$, ** $0.01 > p > 0.001$, *** $p < 0.001$.

Significantly higher EC values were found for larger seeds and for white-colored seeds compared to small and dark-colored seeds (Table 4). The highest average electrolyte concentration (177.87 ms m^{-1}) was measured for the large-seeded white-colored landrace 'Bijeli' (C2) compared to all other landraces, and a higher electrolyte concentration (82.12 ms m^{-1}) was measured in the small-seeded white-colored landrace 'Biser' (C1) than the black small-seeded landrace 'Kornjača' (C3), which had the lowest electrolyte concentration (56.45 ms m^{-1}).

3.2. Seed Germination

Croatian common bean landraces have a very good germination capacity; the small-seeded white-colored landrace 'Biser' (C1) and the large-seeded dark-colored 'Trešnjevac' (C4) had a germination capacity of 100%, and that of the large-seeded white-colored landrace 'Bijeli' (C2) and the small-seeded dark-colored 'Kornjača' (C3) was 98%. All germinated seeds were normal seedlings; there were no abnormal seedling and the rest of seeds (2%) of landraces 'Bijeli' and 'Kornjača' were dead. White-colored and smaller seeds germinate faster than dark-colored large seeds (Table 5). The small-seeded white landrace 'Biser' (C1; 1.83 days) had a significantly shorter mean germination time (*MGT*) than the large-seeded white-colored 'Bijeli' (C2; 2.32 days) and large-seeded dark-colored 'Trešnjevac' (C4; 2.71 days), while the small-seeded dark-colored 'Kornjača' (C3; 2.13 days) was not significantly different from 'Biser' (C1) and 'Bijeli' (C2). The small-seeded dark-colored 'Kornjača' (C3) differs significantly in coefficient of variation of the germination time (CV_{gt}), uncertainty of the germination process (U) and synchrony of the germination process (*Z*) from other landraces. The small-seeded white-colored landrace 'Biser' (C1) differs significantly in mean germination rate (*MGR*) from other landraces, and the large-seeded dark-colored 'Trešnjevac' (C4) differed significantly from the small-seeded landraces 'Biser' and 'Kornjača' (C1 and C3). In germination index (GI), the small-seeded landraces 'Biser' and 'Kornjača' (C1 and C3) and large-seeded dark-colored 'Trešnjevac' (C4) were significantly different, while the large-seeded landrace 'Bijeli' (C2) differed significantly from 'Biser' (C1) and 'Trešnjevac' (C4) (Table 5).

Table 5. Differences between four Croatian common bean landraces in germination characteristics considering seed size and seed coat color.

Var	G		MGT		CV_{gt}		MGR		U		Z		GI	
Landrace—P(F)	ns		**		***		***		*		**		***	
Repetition—P(F)	ns		ns		ns		ns		ns		ns		ns	
C1	1.57	A	1.83	C	0.35	B	0.55	A	0.93	A	0.61	B	29.89	A
C2	1.46	A	2.32	AB	0.43	B	0.43	BC	0.95	A	0.54	B	22.00	BC
C3	1.48	A	2.13	BC	0.59	A	0.47	B	0.62	B	0.80	A	24.63	B
C4	1.57	A	2.71	A	0.38	B	0.37	C	0.97	A	0.60	B	19.33	C
Contrast W vs. C P(F)	ns		**		**		**		*		**		***	
Contrast S vs. L P(F)	ns		***		*		***		*		**		***	

C1—'Biser', C2—'Bijeli', C3—'Kornjača', C4—'Trešnjevac', G—Germinability (%), MGT—mean germination time (day), CV_{gt}—coefficient of variation of the germination time, MGR—mean germination rate, U—uncertainty of the germination process, Z—synchrony of the germination process, GI—germination index, W—white, C—colored, S—small, L—large. Values followed by the same letter in each column are not significantly different based on the Tukey test at 0.05 probability. ns—not statistically significant $p > 0.05$, * $0.05 > p > 0.01$, ** $0.01 > p > 0.001$, *** $p < 0.001$.

3.3. Emergence

The Croatian common bean landraces have very good emergence, with 98% of seedlings emerging in 'Biser' and 'Bijeli' and 99% in 'Trešnjevac' and 97% in 'Kornjača'. All sprouted seeds were normal seedlings, there were no abnormal seedlings and the rest of seedlings were dead (2% of 'Biser' and 'Bijeli', 1% of 'Trešnjevac' and 3% of 'Kornjača'. Seed color did not have a significant effect on emergence, whereas seed size had a significant effect on mean emergence time (MET), coefficient of variation of the emergence time (CV_{et}), mean emergence rate (MER), synchrony of the emergence process (ZE) and emergence index (EI) (Table 6). Namely, small-seeded landraces had higher CV_{et}, MER and EI and lower MET and ZE compared to large-seeded landraces (Table 6), which means that small-seeded landraces have faster emergence and higher variation in the emergence time. The landraces differ significantly in mean emergence time (MET). The white-colored and small-seeded landrace 'Biser' (C1) emerged significantly faster (3.63 days) than the large-seeded white landrace 'Bijeli' (C2; 4.23 days) and the large-seeded dark-colored 'Trešnjevac' (C4; 4.13 days), while the small-seeded dark-colored landrace 'Kornjača' (C3) was not significantly different from the other landraces (Table 6). The small-seeded and dark-colored landrace 'Kornjača' (C3) differed significantly from others (C1, C2 and C4) in coefficient of variation of the emergence time (CV_{et}). The white-colored and small-seeded landrace 'Biser' (C1) and large-seeded white landrace 'Bijeli' (C2) differed significantly in mean emergence rate (MER), while the small-seeded dark-colored landrace 'Kornjača' (C3) and large-seeded dark-colored landrace 'Trešnjevac' (C4) differed significantly in synchrony of the emergence process (ZE). In emergence index (EI), the small-seeded landraces 'Biser' (C1) and 'Kornjača' (C3) differed significantly from the large-seeded landraces 'Bijeli' (C2) and 'Trešnjevac' (C4).

Table 6. Differences between four Croatian common bean landraces in emergence characteristics considering seed size and seed coat color.

Var	E		MET		CV_{et}		MER		UE		ZE		EI	
Landrace—P(F)	ns		*		*		*		ns		*		**	
Repetition—P(F)	ns		ns		ns		ns		ns		ns		ns	
C1	1.43	A	3.63	B	0.46	AB	0.28	A	1.13	A	0.51	AB	13.95	A
C2	1.43	A	4.23	A	0.37	AB	0.24	B	1.13	A	0.55	AB	11.77	B
C3	1.53	A	3.83	AB	0.50	A	0.26	AB	1.30	A	0.46	B	13.52	A
C4	1.41	A	4.13	A	0.35	B	0.24	AB	0.95	A	0.64	A	11.94	B
Contrast W vs. C P(F)	ns		ns		ns		ns		ns		ns		ns	
Contrast S vs. L P(F)	ns		**		**		**		ns		*		***	

C1—'Biser', C2—'Bijeli', C3—'Kornjača', C4—'Trešnjevac'. E—emergence, MET—mean emergence time, CV_{et}—coefficient of variation of the emergence time, MER—mean emergence rate, UE—uncertainty of the emergence process, ZE—synchrony of the emergence process, EI—emergence index. W—white, C—colored, S—small, L—large. Values followed by the same letter in each column are not significantly different based on the Tukey test at 0.05 probability. ns—not statistically significant $p > 0.05$, * $0.05 > p > 0.01$, ** $0.01 > p > 0.001$, *** $p < 0.001$.

3.4. Phenotypic Traits

Emergence assessment and phenotypic characterization were carried out in a growth chamber using multispectral imaging, chlorophyll fluorescence imaging and stomatal conductance analysis. Seed size and color significantly affected chlorophyll fluorescence parameters, with small-seeded landraces having higher F_v/F_m and NPQ and lower F_q'/F_m' and ETR than large-seeded landraces. In addition, seedlings of white-colored landraces had, on average, lower F_v/F_m and higher F_q'/F_m' and ETR than dark-colored landraces. However, these results were primarily caused by the significantly lower F_q'/F_m' and ETR and higher NPQ found in 'Kornjača', a small sized- and colored-seed landrace (C3) (Table 7).

Multispectral traits and calculated vegetation indices were affected by seed size and color, and there were also significant differences among the studied landraces (Table 8). Seedlings of white-colored landraces showed higher values of reflectance (R_{Red}, R_{Green}, R_{Blue}, R_{FarRed} and SpcGrn), higher SAT and VAL and lower values of traits related to pigment content (CHI, ARI and NDVI). Hence, seedlings of the small seed sized landraces had higher reflection in R_{Red}, R_{Green}, R_{Blue}, R_{FarRed} and lower CHI and SAT compared to large-seeded landraces. The highest values of R_{Red}, R_{Green}, R_{FarRed} and SpcGrn lowest ARI, CHI and HUE were found in 'Bijeli' (C2) (Table 8). The seedlings of the small-seeded landraces and those of the dark-colored landraces had significantly higher stomatal conductance, total conductance and transpiration (Table 9).

Table 7. Differences between four Croatian common bean landraces in chlorophyll fluorescence traits considering seed size and seed coat color.

Abbr.	F_v/F_m		F_q'/F_m'		ETR		NPQ	
Landrace—P(F)	***		***		***		***	
Repetition—P(F)	**		ns		ns		**	
C1	0.80	A	0.30	B	7556	C	1.18	A
C2	0.79	C	0.38	A	10058	A	1.03	B
C3	0.81	A	0.23	C	6093	D	1.20	A
C4	0.80	B	0.39	A	8936	B	0.94	B
Contrast W vs. C P(F)	***		***		***		ns	
Contrast S vs. L P(F)	***		***		***		***	

C1—'Biser', C2—'Bijeli', C3—'Kornjača', C4—'Trešnjevac'. F_v/F_m—maximum quantum yield of PSII, $F_{q'}/F_{m'}$—effective quantum yield of PSII, ETR—electron transport rate, NPQ—non-photochemical quenching. W—white, C—colored, S—small, L—large. Values followed by the same letter in each column are not significantly different based on the Tukey test at 0.05 probability. ns—not statistically significant $p > 0.05$, ** $0.01 > p > 0.001$, *** $p < 0.001$.

Table 8. Differences between four Croatian common bean landraces in multispectral traits considering seed size and seed coat color.

Abbr.	R_{Red}	R_{Green}	R_{Blue}	HUE	SAT	VAL	SpcGrn	R_{FarRed}	R_{NIR}	CHI	ARI	NDVI
Landrace—P(F)	***	***	***	***	***	***	***	***	**	***	***	***
Repetition—P(F)	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
C1	913 AB	1542 B	708 A	105.0 B	0.54 B	0.02 B	1795 B	3575 B	14564 B	3.0B	4.13 C	0.88 C
C2	942 A	1714 A	631 B	103.0 C	0.63 A	0.03 A	2020 A	3844 A	14747 B	2.8C	3.61 D	0.88 C
C3	865 B	1473 B	664 AB	105.4 B	0.55 B	0.02 B	1719 B	3672 B	15511 A	3.2B	4.92 B	0.89 B
C4	733 C	1254 C	578 C	106.8 A	0.54 B	0.02 C	1456 C	3103 C	14538 B	3.7A	5.53 A	0.90 A
Contrast W vs. C P(F)	***	***	***	***	***	***	***	***	ns	***	***	***
Contrast S vs. L P(F)	**	ns	***	ns	***	ns	ns	***	ns	*	ns	ns

C1—'Biser', C2—'Bijeli', C3—'Kornjača', C4—'Trešnjevac'. R_{Red}—red reflectance, R_{Green}—green reflectance, R_{Blue}—blue reflectance, HUE—hue, SAT—saturation, VAL—value, SpcGrn—reflectance in specific green region, R_{FarRed}—far-red reflectance, R_{NIR}—near-infrared reflectance, CHI—chlorophyll index, ARI—anthocyanin index, NDVI—normalized digital vegetation index. W—white, C—colored, S—small, L—large. Values followed by the same letter in each column are not significantly different based on the Tukey test at 0.05 probability. ns—not statistically significant $p > 0.05$, * $0.05 > p > 0.01$, ** $0.01 > p > 0.001$, *** $p < 0.001$.

Table 9. Differences between four Croatian common bean landraces in stomatal conductance traits considering seed size and seed coat color.

Abbr.	gsw		gbw		gtw		T	
Landrace—P(F)	***		ns		***		***	
Repetition—P(F)	ns		ns		ns		ns	
C1	0.18	B	2.92	A	0.17	B	1.87	B
C2	0.091	C	2.92	A	0.09	C	1.07	C
C3	0.232	A	2.92	A	0.21	A	2.55	A
C4	0.088	C	2.92	A	0.09	C	0.99	C
Contrast W vs. C P(F)	***		ns		***		***	
Contrast S vs. L P(F)	***		ns		***		***	

C1—'Biser', C2—'Bijeli', C3—'Kornjača', C4—'Trešnjevac'. gsw—stomatal conductance, gbw—one-sided boundary layer conductance, gtw—total conductance, T—transpiration. W—white, C—colored, S—small, L—large. Values followed by the same letter in each column are not significantly different based on the Tukey test at 0.05 probability. ns—not statistically significant $p > 0.05$, *** $p < 0.001$.

4. Discussion

4.1. Water Absorption and Electrical Conductivity

It is well known that seed quality is of paramount importance for crop production and food security, and germination tests are widely used to assess seed quality [41,42]. Seed germination is a complex physiological process that begins with water uptake by the dry seed and ends with the emergence of the radicle [7,43]. During water uptake by the seeds, the expansion of embryonic cells leads to the emergence of the embryo and marks the end of germination [43].

Water uptake in legumes begins as soon as the seed is immersed in water, and the rate of water uptake is higher at the beginning and decreases during the soaking period [44]. This is in agreement with our findings, which showed that the seeds absorbed the greatest amount of water in the first hours (1.5 and 3 h), during which the greatest differences in the amount of water absorbed between landraces were found.

The rate and extent of seed water uptake are controlled by seed composition, seed coat chemical composition and structural characteristics (e.g., seed coat thickness and water permeability) and the availability of water in the medium [9,10]. In our study, white landraces ('Biser' and 'Bijeli') had higher water absorption rate and absorbed more water compared to dark-colored landraces ('Kornjača' and 'Trešnjevac') of similar mass, especially at early measurements (1.5–9 h). Previous studies have shown that white seeds have a thinner seed coat, greater seed coat permeability, lower content of lignans, phenols, tannins and unsaturated fatty acids and lower seed coat adhesion to cotyledons compared to colored seeds, which leads to faster water uptake and increased risk of imbibition damage and faster seed germination [9,45]. Imbibition damage is also widely implicated in poor emergence and low vigor of crop seeds [46]. On the other hand, thick seed coats in colored seeds lead to slower water uptake and more uniform cotyledon swelling, reducing cracking in the seed coat and cotyledons, both detrimental factors for bean germination and early seedling growth [47,48]. Mandizvo and Odindo [49] found that dark seeds had the thickest seed coats and light seeds of Bambara groundnut had the thinnest seed coats. Liu et al. [50] found that dark-colored soybean seeds had better storability than light-colored seeds and had higher germination parameters compared to light-colored soybean seeds. Khan et al. [46] suggest that the thick and dark-colored seed coats of persistent proso millet (*Panicum miliaceum* L.) biotypes slow down water uptake and possibly reduce imbibition damage and solute leakage. Thick, highly pigmented seed coats may also provide a chemical barrier to pathogens.

In addition to white-seeded landraces, the small-seeded landraces ('Biser' and 'Kornjača') had a higher water absorption rate compared to the large-seeded landraces ('Bijeli' and 'Trešnjevac'). The possible explanation could be that small seeds have an overall bigger seed surface area in relation to seed mass, and thus greater contact between the seed surface and water than large seeds [51]. In addition, white-colored seeds ('Biser' and 'Bijeli') released more electrolytes than dark-colored seeds of similar mass ('Kornjača' and 'Trešnjevac'); while large-seeded landraces released more electrolytes, probably due to their larger seed storage reserves [52]. This probably resulted in greater damage inside the seeds, e.g., the seed coats detached from the seeds of the white landraces and the white landraces started to mold during germination. For a better understanding of this process, the biochemical test for viability (tetrazolium test) is also desirable, as it plays an important role in assessing the physiological quality of the seed (e.g., viability and vigor index) and in diagnosing possible quality problems of the seed, such as mechanical damage, insect damage, weathering before harvest and deterioration during storage [53].

Measuring electrical conductivity provides information on the extent of electrolyte leakage from the seed and is one of the most common tests used to assess seed quality and vigor in seed laboratories [42,54]. When dried seeds are soaked in water, the leaching of electrolytes from the cellular components indicates the degree of disorganization of the seed's cell [42]. Poor seed vigor is often associated with transverse cracks in the cotyledon and seed coat, an open hilum, damage due to rapid uptake of water and increased leakage of solutes from the seeds [16].

4.2. Seed Germination and Emergence

Rapid and uniform seed germination and seedling emergence under different environmental conditions are desirable traits for crops, as they represent a rapid transition to the growth phase in a plant's life cycle [7]. Rapid establishment and uniform plant population are prerequisites for high yields in agroecological regions, which, in turn, depend on high seed vigor [16]. Moreover, common bean seeds that germinate slowly are likely to grow slowly [45].

Seed size has been identified as a factor influencing common bean germination, playing an important role in early seedling establishment and probably related to water uptake, a key process in seedling emergence [45,55]. In our study it was found that white-colored and smaller seeds germinate faster than dark-colored large seeds. That is, the small-seeded white-colored landrace 'Biser' germinated the fastest, followed by the small-seeded dark-colored landrace 'Kornjača' and the large-seeded white-colored landrace 'Bijeli', and the slowest was the large-seeded dark-colored 'Trešnjevac'. This can be explained by the fact that large colored seeds need more time to absorb enough water to germinate, so they germinate more slowly compared to small and white seeds [3,48,56]. Crawford and Williams (2019) found that the seed size of soybean (*Glycine max* x (L.) Merr.) had no effect on overall emergence, but small seeds emerged 10% faster than large seeds. On the other hand, large seeds have a greater food supply for embryo growth and development, resulting in vigorous seedling growth that competes with the small seeds for light and soil factors, leading to higher yield [48,55].

Our results indicate that white seeds absorb water faster and germinate faster; however, there were large differences between emergence rates for the white-colored landrace 'Bijeli', which took the longest time to emerge (highest MET, lowest MER and EI). Although in this experiment we did not measure the oxygen consumption and seed respiration, Fatokun et al. [57] found that imbibition damage reduced seed respiratory activities and germination and lowered seedling emergence and growth. Seed germination is an energy-consuming process that requires functioning mitochondria immediately after water absorption and one of the earliest events of seed germination is the progressive proliferation and differentiation of mitochondria, termed mitochondrial biogenesis [58]. That is, at the beginning of germination, reactivation of mitochondrial bioenergetics is followed by the fusion of all mitochondria in a cell to form the chondriome, and at the end of germination, there is fragmentation of the chondriome and heterogeneous redistribution of nucleoids among the mitochondria, resulting in a mitochondrial population tailored to the growth of the seedling [59]. Mitochondria can form dynamic, interconnected networks regulated by a dynamic balance between fusion and fission processes, which, in turn, determine their number, size, shape and functionality [60]. However, little is known about the biogenesis and function of mitochondria during common bean germination, so it would be useful to investigate this. This could explain by the damage that occurred inside the seed during water uptake, which is evident in the highest EC in this seed type. Thus, the possible explanation for the slower and least synchronous emergence in the landrace 'Bijeli' could be the cell damage that occurred during the fast water absorption, which then decreased the respiration rate and the energy needed for emergence. This was also confirmed by Ozden et al. [56] who concluded that excessive irrigation after sowing led to imbibition damage in seed lots with white coat of French bean (*Phaseolus vulgaris* L.) and reduced the percentage of emergence. Khan et al. [46] show that darker proso millet (*Panicum miliaceum* L.) seeds have a heavier seed coat, imbibe and germinate more slowly and have less imbibition damage (measured as electrolyte loss), which contributes to the increased persistence of the dark seeded plants in the soil. Zhang et al. [61] reported that darker seed of *Brassica napus* resulted in a higher percentage of seedling emergence, vigor, shoot length and root length. Rather than laboratory germination, electrical conductivity may be used as an indicator for determining field emergence [16] because the white ones have the most electrolytes in the water, i.e., the greatest damage, but also the weakest emergence, which is the most similar (in this research) to field conditions.

In addition, small-seeded landraces have faster emergence in this research than large-seeded landraces which is in agreement with research of Souza and Fagundes [52], who found that small seeds forage of annual forage legumes had a higher percentage of germination and emerged faster compared to large seeds. Nevertheless, seedlings from larger seeds have a longer development time, resulting in more vigorous seedlings. De Ron et al. [7] came to similar conclusions: small seeds of common bean genotypes emerge faster than large seeds, and large seeds showed lower emergence in the field under real growing conditions.

4.3. Phenotypic Traits

Seed size and color significantly affected chlorophyll fluorescence and multispectral parameters, as well as stomatal conductance and transpiration. Small-seeded landraces and white-colored landraces had higher Fv/Fm and NPQ and lower Fq′/Fm′ and ETR compared to large-seeded and dark-colored landraces. Values of Fv/Fm, as one of the most widely used parameter for stress assessment [62], indicate an overall good condition of the studied plants. Also, these results indicate that small-seeded and white-seeded landraces could have more open PSII. However, lower Fq′/Fm′ and ETR combined with higher NPQ indicate a lower ability to utilize light in photochemistry and increased energy dissipation for these landraces, especially found in 'Kornjača' (small sized- and colored seed landrace). There was limited literature that associated seed color and seed size with the phenotypic characterization of developing seedlings. However, Gomes et al. [63] found that plants from dark-colored seeds of cowpea (*Vigna unguiculata* (L.) Walp.) maintained higher photochemical use of energy and a lower need of photoprotective thermal dissipation mechanisms under drought conditions.

In our study, chlorophyll fluorescence traits were accompanied by stomatal conductance and transpiration analysis. The seedlings of the small-seeded landraces and those of the dark-colored landraces had significantly higher stomatal conductance, total conductance and transpiration. Higher stomatal resistance to water vapor would lead to higher resistance to CO_2 diffusion and vice versa. In addition, electron transport rate (ETR) is correlated with CO_2 assimilation [62,64]. Thus, the values of gsw, gbw, gtw and T found in the small-seeded and dark-colored landrace 'Kornjača' indicates that stomatal limitation is not the reason for the lower photochemistry, which is indicated by the lower ETR and Fq′/Fm′, and higher NPQ found in this landrace.

In contrast to our results, Norsazwan et al. [49] found that net photosynthesis, stomatal conductance and transpiration rate were similar in seedlings grown from seeds of different colors. However, Lima et al. [3] found that large seeds of common bean increased the leaf area index and shoot and root biomass of bean landraces, particularly at the beginning of the growth cycle, while plants originating from small seeds had a higher relative growth rate and net assimilation rate than plants from large seeds.

Multispectral traits show that seedlings from small-seeded, compared to large-seeded, landraces and white-seeded, compared to dark-colored, landraces had higher reflectance in red, green, blue, and far-red, and lower pigment content, as indicated by the CHI, ARI and NDVI. The CHI and NDVI are strongly correlated with chlorophyll content [37,65], whereas ARI is a good indicator of leaf anthocyanin content [37]. A lower pigment content will cause an increase in reflectance in the visible and far-red spectrum [65]. Similar results related to chlorophyll content were published by Ayeh et al. [66], who found that the red variety of cowpea had the highest chlorophyll content, whilst the lowest chlorophyll content was found in the cream variety.

5. Conclusions

The results show that the color of the common bean seed coat and seed size can be good indicators of seed quality and initial seedling growth. White-colored landraces had a higher water absorption rate than dark-colored landraces of similar mass at the same time. White-colored landraces release more electrolytes, resulting in greater damage within the seeds. White-colored and small-seeded landraces germinate faster than dark-colored large- and small-seeded landraces, while large-seeded white-colored landraces emerged more slowly, probably because of the cell damage that occurs during the fast initial water absorption. Despite this cell damage, slower emergence, lower chlorophyll content and lower stomatal conductance and transpiration, the large-seeded, white-colored landrace 'Bijeli' retains the highest ETR and Fq′/Fm′, indicating high photochemical efficiency. In addition, white-colored common bean landraces are still very popular in human diets and are often grown on small-scale farms. However, our results indicate that dark-colored seeds can survive longer under unfavorable soil conditions without absorbing water, swelling, emergence or molding. As both white and dark-colored common beans have been shown to have both advantages and disadvantages, further NPR research is needed in unfavorable conditions and by applying a biochemical test for viability to determine which landraces cope better with the environment.

Future studies should investigate the genetics of seed coat color of Croatian common bean landraces and its variations if this trait is to be used for future crop improvement.

Author Contributions: Conceptualization, B.L., K.C.-S., Z.Š. and M.V.; formal analysis, Z.Š. and B.L.; investigation, M.V. and T.J.; resources, M.V. and B.L.; writing—original draft preparation, M.V.; writing—review and editing, B.L. and K.C.-S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the project KK.01.1.1.01.0005 Biodiversity and Molecular Plant Breeding, Centre of Excellence for Biodiversity and Molecular Plant Breeding (CoE CroPBioDiv), Zagreb, Croatia.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

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

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