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Storage protein composition during germination and its association with physiological seed quality in common bean

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ABSTRACT. Seed reserve mobilization is considered a post-germination process; however, seed storage protein mobilization occurs during germination. Thus, the knowledge of seed protein composition is important to understand various processes during germination, and it be associated with seed vigor. Therefore, this study aimed to characterize landrace genotypes of common bean (*Phaseolus vulgaris*) through fractionation of seed storage proteins during germination and to verify the association between seed protein composition and seed vigor. Genotypes of the highest (55 and 81) and lowest (23 and 50) physiological quality were selected. Protein content analysis, profiling, and characterization were performed by combining different hydration times. Mass spectrometry involving in-gel digestion and MALDI-ToF analysis was employed for the qualitative identification of proteins. Glutelin extraction detected the enzyme lipoxygenase in genotypes 55 and 81 alone. In plants, this enzyme may be involved in diverse physiological processes, including growth and development, pest resistance, senescence, and response to wounding. In the process of germination, lipoxygenase removes reactive oxygen species during reserve mobilization; therefore, lipoxygenase may be a candidate biochemical marker for high-vigor genotypes.

Keywords: *Phaseolus vulgaris*; lipoxygenase; glutelin; 1D SDS-PAGE; mass spectrometry.

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Introduction

Germination involves various physiological processes that are sequentially initiated by water uptake (imbibition; D'Hooghe et al., 2019). Seed hydration increases respiration and metabolism, thus allowing the mobilization of carbon and nitrogen reserves necessary to support germination and post-germination growth until autotrophy is reached (Anzala, Paven, Fournier, Rondeau, & Limami, 2006). Therefore, seed germination is a physiological and biochemical process that involves different cascades of signal transduction and gene expression regulation.

The elucidation and subsequent availability of bean genome have enabled the rapid advancement of seed proteomics (Rossi, Valentim-Neto, Blank, Faria, & Arisi, 2017). Many studies of bean seed proteomics have demonstrated the efficiency of different protein extraction methods to optimize the quality of separation and subsequent identification of proteins (Martínez et al., 2012).

In 1924, Osborne developed the first classification of seed storage proteins (SSPs), in which SSPs are grouped according to their solubilities in water (albumins), dilute salt solutions (globulins), aqueous alcohols (prolamins), and weakly acidic or alkaline or dilute sodium dodecyl sulfate (SDS) solution (glutelins; Chéreau et al., 2016). Biochemical reactions during seed germination are carried out by different proteins, and the analysis of seed protein profile may be a highly precise approach to clarify this physiological process (Bojórquez-Velázquez, Barrera-Pacheco, Espitia-Rangel, Herrera-Estrella, & Rosa, 2019). With the availability of massive genomic data and development of mass spectrometric techniques, proteomics has enabled the analysis of dynamic and diverse biological processes (Gu et al., 2019). Most studies involving the characterization, bioavailability, and diversity of specific proteins in bean seeds have focused on phaseolin, a type of globulin (Martínez et al., 2012; Carović-Stanko et al., 2017).

According to Rajjou et al. (2012), further research into germination and seed vigor would greatly help in unraveling the key biochemical and molecular mechanisms underlying these complex traits, which are unique to plants. Characterization of biomarkers for seed vigor to improve seeds for breeding programs as

well as other biotechnological approaches will allow for the production of seeds of the highest possible quality, with the goal of improving crop yield, particularly under environmental stresses. Thus, studies including landrace genotypes can be advantageous, because these genotypes have an extensive genetic base and can adapt to different environmental conditions relatively easily, as opposed to commercial cultivars that have a restricted genetic base (Michels, Souza, Coelho, & Zilio, 2014).

To this end, the present study aimed to characterize the SSP composition of landrace genotypes of common bean (*Phaseolus vulgaris*) during germination and examine its association with physiological seed quality.

Material and methods

Seed material

This study was conducted using seeds of four landrace genotypes of common bean (23, 50, 55, and 81) acquired from the Bean Active Germplasm Bank of the Santa Catarina State University. These genotypes had undergone successive selfing selection that favored greater homogeneity and led to higher stability within the population, and they were previously characterized and studied during eight consecutive seasons. Seeds were produced through a field experiment conducted in a randomized complete block design during the agricultural season of 2012-2013 by conventional farming in Campos Novos, Santa Catarina. The harvested seeds were stored in a dry chamber at 10 ± 2ºC under 50 ± 5% relative humidity until further analysis. In a previous study, the physiological seed quality of these genotypes was determined using germination and vigor (accelerated aging) tests. Genotypes 55 and 81 showed the highest physiological quality (germination rate: 93 and 94%; vigor: 83 and 87%), whereas genotypes 23 and 50 showed the lowest physiological quality (germination rate: 85 and 83%; vigor: 79 and 78%) (Ehrhardt-Brocardo & Coelho, 2016).

Cotyledons extraction and quantification

Protein content determination, profile analysis, and characterization were performed by combining different hydration times (0, 9, 12, 15, 18, and 21 hours) for each genotype to form a gene pool. Protein extraction followed the sequential extraction method modified by Landry, Delhaye, and Damerval (2000) using 1 mL of extraction buffer. Globulins were extracted in 500 mM sodium chloride (NaCl; pH 2.4) at 4ºC, and glutelins were extracted from the previous pellet in 0.5% SDS (pH 10; 25 mM sodium borate buffer) containing 0.6% 2-mercaptoethanol at room temperature.

The extraction procedure was repeated twice for each protein fraction. Three replicates of 0.1 g of fine cotyledons powder (lyophilized) were vortexed for 1 min. (AP56; Phoenix), shaken for 30 min. on an orbital shaker table (Oxy304; Oxylab), and then centrifuged at 1,500 × g for 7 min. (CE01-B1; Kacil); the supernatants were stored for further analysis. The soluble protein content was determined as described by Bradford (1976) using bovine serum albumin as a standard; absorbance was measure at 595 nm using a spectrophotometer (UV/VIS Spectro800S; Mars).

One-dimensional gel electrophoresis of proteins

Electrophoresis was performed under denaturing conditions (SDS-PAGE) in a mini-vertical apparatus (K33-10V; Kasvi) with 12 resolving gel and 4% stacking gel. Each previously extracted protein fraction was loaded onto the gel, as described by Ma and Bliss (1978). After quantification, soluble proteins samples (15 for globulins and 9 µg for glutelins) were dissolved in a sample buffer containing Tris-HCl (pH 6.7), glycerol, 10% SDS, 0.5% bromophenol blue and 2-mercaptoethanol at a ratio of 1:1, and distilled water. The electrophoresis system containing the samples was placed in a 10% buffer solution (25 mM Tris, 192 mM glycine, and 1% SDS; pH 8.3) at 400 mA for 3h at room temperature. After electrophoresis, the gels were washed with distilled water to completely remove the buffer and then stained for 3h with Coomassie Brilliant Blue R-250. The sample protein profiles were compared with a molecular range marker (Precision Plus Protein™, Bio-Rad, CA, USA). Reproducibility was confirmed by a minimum of two repetitions of each gel electrophoresis run under similar conditions and using samples from two independent protein extractions. Finally, digital documentation was performed in a white light transilluminator (DNR Bio-Imaging Systems Mini Bis Pro, Jerusalem - Israel) with Gel Capture.

In-gel digestion and matrix-assisted laser desorption/ionization (MALDI) time-of-flight (ToF) MS analysis of proteins

Bands of each fraction were selected based on differential intensity between high- (55 and 81) and low-vigor (23 and 50) genotypes using GelAnalyzer 2010a (one-dimensional gel electrophoresis image analysis freeware) with an automatic band detection tool. After image analysis, each intensely Coomassie-stained lane of the gel was divided into approximately 2.5 mm slices, and the slices were then subjected to in-gel digestion with trypsin (EC 3.4.21.4).

The stained protein bands were individually excised from the gel and subjected to destaining twice with a solution containing acetonitrile (50%) and 25 mM ammonium bicarbonate (NH_4HCO_3) for 30 min. at 25ºC. The solution was replaced by pure acetonitrile and dried under vacuum (10 min.). The excised bands were reduced and alkylated before digestion. The bands were incubated with 20 ng μL^{-1} trypsin (Trypsin Gold V5280 Promega) and 25 mM NH_4HCO_3 on ice for 30 min. and then at 37ºC for 14 hours. After digestion and extraction (Shevchenko, Tomas, Havli, Olsen, & Mann, 2006), peptides were mixed with the MALDI Matrix Solution (5 $\mu g \cdot mL^{-1}$ α-cyano-4-hydroxycinnamic acid, 50% acetonitrile, and 0.1% trifluoroacetic acid) at a 1:1 ratio, placed on an MTP 384 target plate (Bruker Daltonics, Germany), and dried at room temperature. MALDI-ToF MS was employed for protein identification using trypsin-digested bands on an Autoflex III MALDI-TOF Mass Spectrometer (Bruker Daltonics) with the full automatic mode using flexControl 3.3 (Bruker Daltonics), and the peak lists were created using flexAnalysis 3.0 (Bruker Daltonics).

Database search was performed with Mascot (Matrix Science; http://www.matrixscience.com) using the *Phaseolus vulgaris* Database (downloaded from Phytozome – phaseolus_20160609) and Viridiplantae Database (SwissProt 2016_06). The following search parameters were used: (1) type of search: peptide mass fingerprint; fixed modifications: carbamidomethylation of cysteine; variable modifications: methionine oxidation; mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ± 200 ppm; max missed cleavages: 1; and (2) type of search: MS/MS ion search; fixed modifications: carbamidomethylation of cysteine; variable modifications: methionine oxidation; mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ± 2 Da; fragment mass tolerance: ± 0.7 Da; max missed cleavages: 1. The Universal Protein Resource Knowledgebase (UniProt Consortium, 2017) was used to assess the relevant biological functions and descriptions of proteins.

Statistical analysis

The experiment was conducted using a randomized complete block design, and analysis of variance (*F* test) was performed. Averages were compared using Tukey test with Assistat (Silva & Azevedo, 2016). Cotyledons proteins of landrace genotypes of common bean identified by MS were converted into a binary matrix by grouping the genotypes into hierarchical clustering, generated through the Paleontological Statistics Software Package for Education and Data Analysis (PAST) software, using the Jaccard similarity coefficient (Hammer, Harper, & Ryan, 2001).

Results and discussion

Cotyledons protein analysis

Majority of the proteins were salt-soluble (globulins). Genotypes 23 and 55 showed higher protein content throughout germination (pool) than the other genotypes (Table 1).

Table 1. Soluble cotyledons protein content of landrace genotypes of common bean during germination.

Genotype	Globulins	Glutelins
	mg 100 mg^{-1}	mg 100 mg^{-1}
23	4.41Ab	0.40b
50	4.33B	0.51a
55	4.82A	0.25c
81	4.35b	0.40b
CV (%)	3.89	4.76

Data are expressed as mg·100 mg^{-1} of cotyledons dry matter. The values are means of three repetitions. Mean values followed by the same letter do not differ significantly (Tukey test, 5%).

One-dimensional gel electrophoresis

In electrophoretic profiles of globulin, the tested landrace genotypes showed bands ranging from 15 to 150 kDa, with higher band intensity in molecular masses between 25 and 50 kDa (Figure 1). In electrophoretic profiles of glutelins, the tested landrace genotypes showed bands ranging from 25 to 150 kDa, with higher band intensity in molecular masses between 37 and 75 kDa (Figure 2). Globulins and glutelins showed differences in band intensities in protein profile analysis using one-dimensional SDS-PAGE (Figures 3 and 4); this difference may be attributed to the inhibition or overexpression of specific proteins.

Cotyledons protein identification

Proteins from landrace genotypes of common bean were extracted and separated via one-dimensional SDS-PAGE (Figure 1 and 2), followed by identification using MALDI-ToF MS.

The conversion of proteins identified by MS into a binary matrix and subsequent hierarchical clustering allowed the grouping of genotypes according to the similarities in proteins in the pool profile formed at different hydration times (0, 9, 12, 15, 18, and 21 hours).

For the globulin group (Figure 5), high-vigor genotypes (55 and 81) showed a greater similarity in the protein pool present in three excised bands, whereas low-vigor genotypes (23 and 50) did not show any similarity in the detected proteins, specifically between bands 1 and 3. This technique of band characterization achieved a greater separation and better detection sensitivity of the expressed proteins in each genotype during germination. However, due to certain technical limitations, some proteins were detected in multiple excised bands. PHAE, V5YN37, and I0J8I4 were detected in band 3 alone in high-vigor genotypes (55 and 81) and in band 1 alone in a low-vigor genotype (50; Figure 5). Other proteins including Q8RVH3, Q8RVY0, and V7C654 were detected in band 1 alone in a low-vigor genotype (50) and in band 3 alone in high-vigor genotypes (55 and 81).

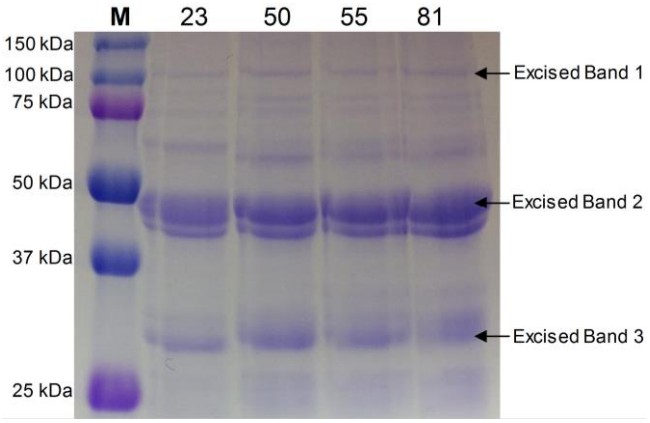

Figure 1. One-dimensional SDS-PAGE of cotyledons globulins in landrace genotypes of common bean during germination. Excised bands are shown (#1-3). M: molecular range marker.

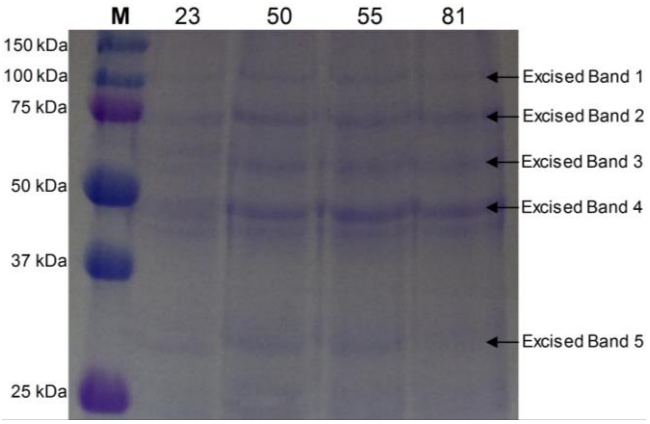

Figure 2. One-dimensional SDS-PAGE of cotyledons glutelins in landrace genotypes of common bean during germination. Excised bands are shown (#1-5). M: molecular range marker.

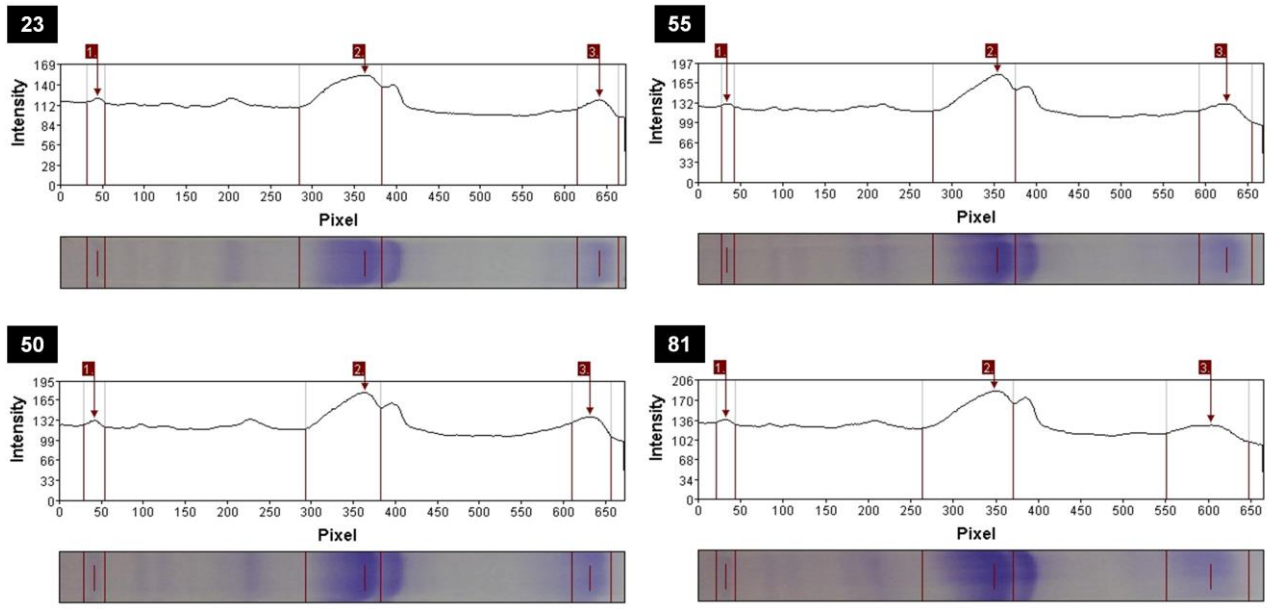

Figure 3. Differential intensity of cotyledons globulins between low- (23 and 50) and high-vigor (55 and 81) genotypes using GelAnalyzer. Excised bands are shown (#1-3).

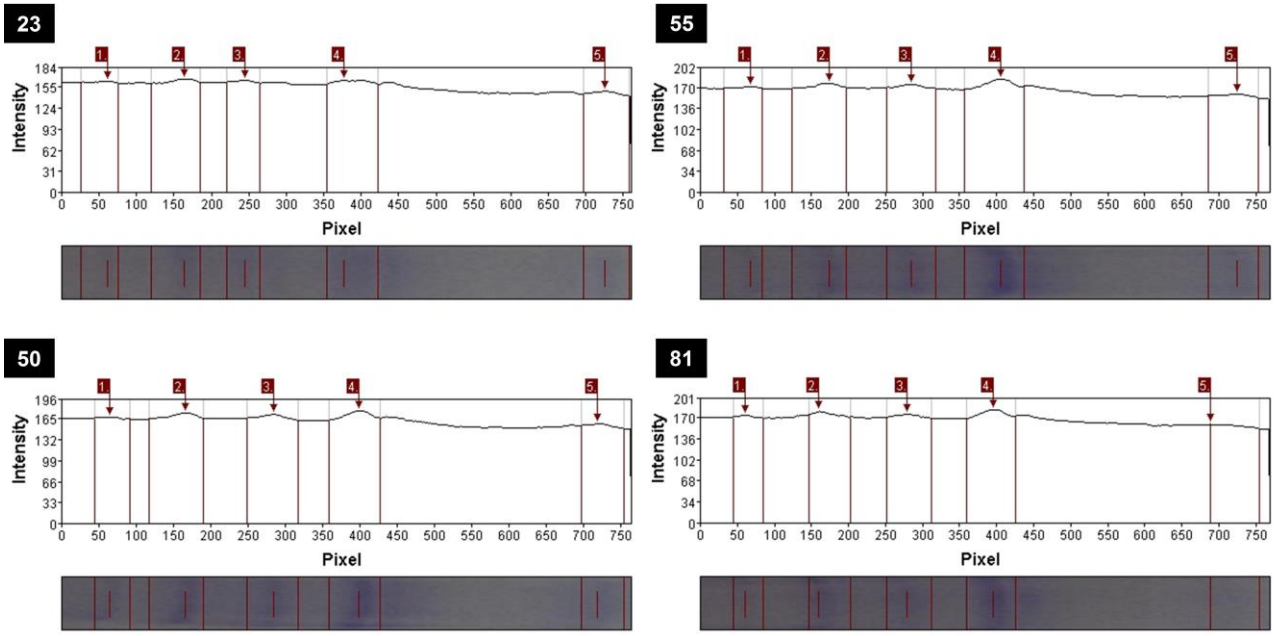

Figure 4. Differential intensity of cotyledons glutelins between low- (23 and 50) and high-vigor (55 and 81) genotypes using GelAnalyzer. Excised bands are shown (#1-5).

Based on the clusters formed within the glutelin group, high- and low-vigor genotypes did not correspond to one another in the five excised bands (Figure 6). Although the proteins corresponding to bands 2, 4, and 5 did not allow for the separation of genotypes based on vigor, band 1 corresponded to a protein detected only in high-vigor genotypes (55 and 81), lipoxygenase (LOX; Table 2).

Functional classification of the identified cotyledons proteins

The results generated lists of abundant proteins per extract, and numerous proteins were identified per band excised from different proteins extracts. The proteins identified by one-dimensional SDS-PAGE and MALDI-ToF MS analyses were grouped into categories according to their possible biological functions (molecular or biological) using the UniProtKB and SwissProt databases.

In electrophoretic profiles of globulins (Figure 1 and 3), genotype 81 showed different intensity of the excised bands compared with the other genotypes. Some identified proteins, such as alpha-phaseolin and phaseolin, serve the molecular function of nutrient reserve activity. However, they may show differences in

amino acid number or diverse post-translational modifications. Phaseolin (alpha-type) is 436-amino acid long, while phaseolin (beta-type) is 421-amino acid long, and both proteins are glycosylated. The excised band 2 showed a higher intensity, which was consistent with phaseolin. Phaseolin exhibited a high conversion efficiency of nutrient reserves in all genotypes, because some cotyledons proteins were not completely hydrolyzed and mobilized to the embryonic axis due to different hydration times.

In electrophoretic profiles of glutelins (Figures 2 and 4), high-vigor genotypes (55 and 81) showed different intensities of the five excised bands compared with low-vigor genotypes. For both high-vigor genotypes, band 1 corresponded to the enzyme LOX. This enzyme is 860-amino acid long, and it may be involved in diverse physiological processes in plants.

LOX was described in 1932 as a fatty acid-oxidizing enzyme; however, studies on the molecular and functional characteristics of this enzyme started only in the mid-90s when the role of jasmonic acid, which is one of the basic products of the LOX pathway, was discovered and extensively studied (Babenko, Shcherbatiuk, Skaterna, & Kosakivska, 2017). Among various described LOX-derived plant lipid mediators, jasmonic acid has received the most attention due to its important roles in defense against pathogens and insect pests via the activation of specific genes in plants (Bannenberg, Martínez, Hamberg, & Castresana, 2009). In general, lipid oxidation is a common metabolic reaction in all biological systems, and it occurs as a developmentally regulated process as well as in response to abiotic and biotic stresses (Andreou & Feussner, 2009).

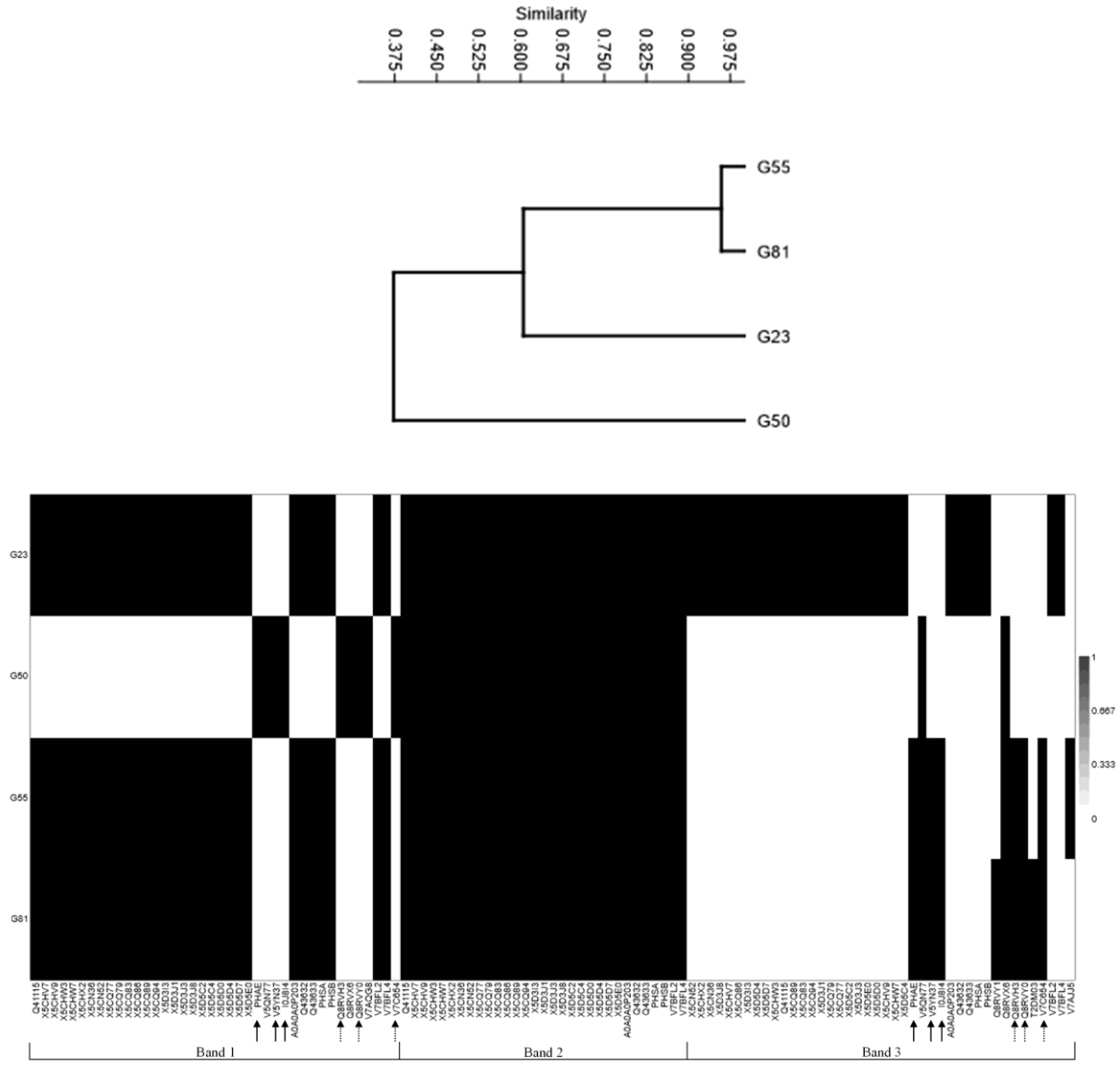

Figure 5. Hierarchical clustering of cotyledons globulins between low- (23 and 50) and high-vigor (55 and 81) genotypes from the matrix plot generated with PAST using the Jaccard similarity coefficient. Excised bands are shown (#1-3). Arrows indicate proteins detected in multiple bands.

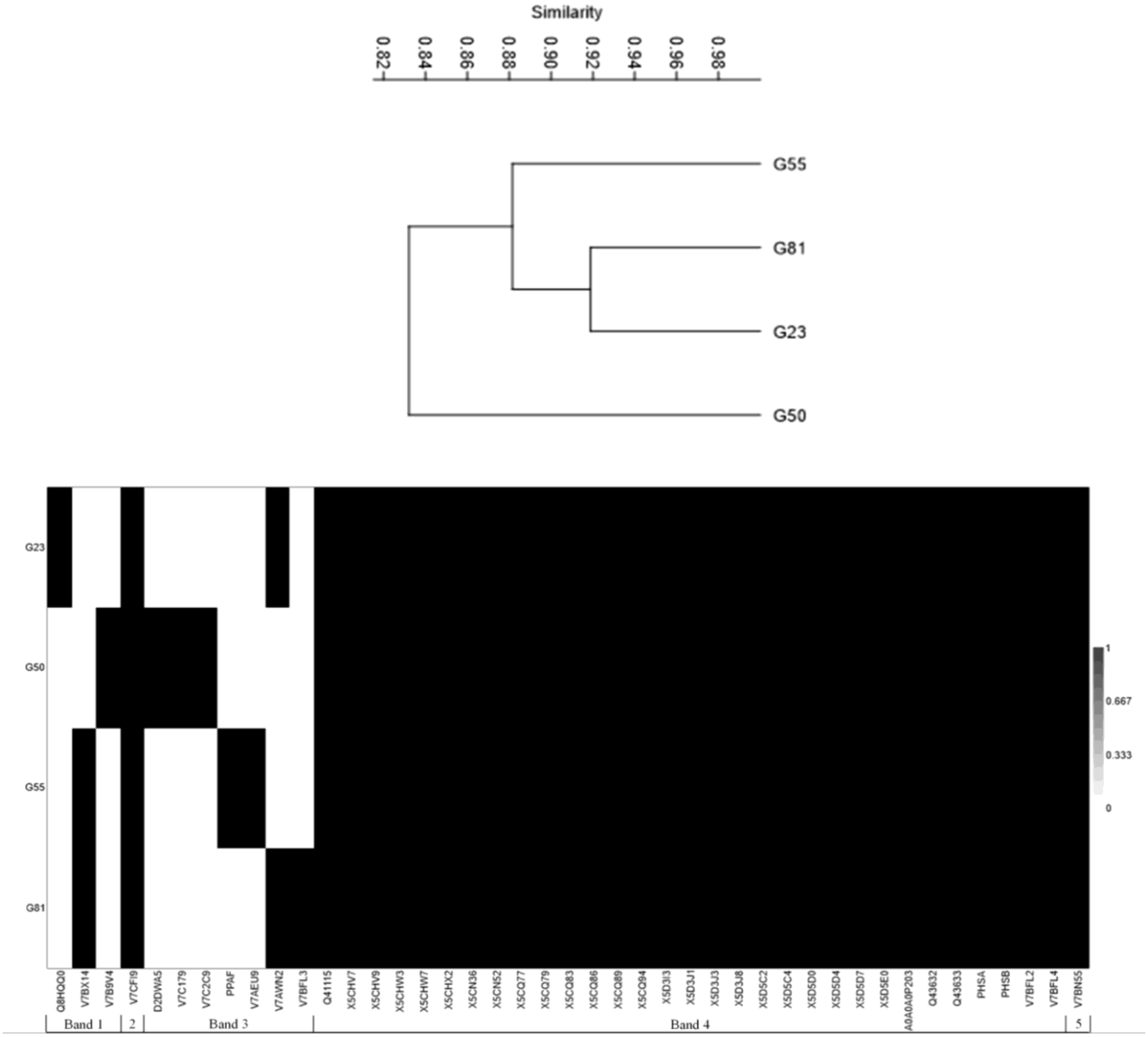

Figure 6. Hierarchical clustering of cotyledons glutelins between low- (23 and 50) and high-vigor (55 and 81) genotypes from the matrix plot generated with PAST using the Jaccard similarity coefficient. Excised bands are shown (#1-5).

Biochemically, LOXs catalyze polyunsaturated fatty acid oxygenation, the first step in the biosynthesis of a large group of biologically active fatty acid metabolites, collectively named oxylipins (Bannenberg et al., 2009). Phyto-oxylipins likely play pivotal roles in immune defense, because they act as signal molecules, protective compounds (antibacterial and wound-healing agents), or cutin constituents (Blée, 2002). According to Yang, Jiang, and Yu (2012), LOXs may be involved in stress responses through the formation of growth regulators, signal molecules, and volatile compounds in cucumber (*Cucumis sativus* L.). In soybean vegetative tissues and seeds, LOXs were considered to be storage proteins and might be associated with immune response (Porta & Rocha-Sosa, 2002). In *Phaseolus vulgaris* seedlings, LOX accumulation in response to phytohormone, cold, and desiccation stress was detected (Porta & Rocha-Sosa, 2002). During seed germination, LOXs are essential enzymes involved in the mobilization of carbon reserves (Aanangi et al., 2016). Another possibility is the effect of genetic diversity among the genotypes tested in this study. Differences in seed vigor may be observed regardless of similar germination indices (Michels et al., 2014).

In the process of germination, LOXs removed reactive oxygen species (ROS) during reserve mobilization in soybean seeds. These enzymes use molecular oxygen during their function in lipids degradation and may therefore help relieve oxidative stress; this likely explains the negligible abundance of proteins involved in redox regulation in soybean seeds (Han, Yin, He, & Yang, 2013). Similarly, Naguib (2019) reported that LOX activity was increased during germination in cotton seeds. Although the results of this study generated an extensive list of proteins identified per band excised from different proteins extracts, as described earlier,

LOX was detected in the glutelin profiles of genotypes 55 and 81 alone. As demonstrated in soybean and cotton studies, LOX may help remove ROS during reserve mobilization in common bean seed germination. Lack of LOX detection in the excised bands of low-vigor genotypes does not rule out the possibility that this enzyme plays a key role in antioxidant mechanisms of these genotypes; it may be present at a trace amount and may thus remain undetected.

Table 2. Cotyledons glutelins of landrace genotypes of common bean identified by mass spectrometry.

Band	Protein	UniProtKB Accession	M_r (Da)	23	50	55	81
	Aspartate aminotransferase	Q8HQQ0_PHAVU	50560	+	-	-	-
1	Lipoxygenase	V7BX14_PHAVU	97527	-	-	+	+
	Uncharacterized protein	V7B9V4_PHAVU	94545	-	+	-	-
2	Uncharacterized protein	V7CFI9_PHAVU	85178	+	+	+	+
	Formate dehydrogenase (FDH), mitochondrial	D2DWA5_PHAVU	41524	-	+	-	-
	Formate dehydrogenase (FDH), mitochondrial	V7C179_PHAVU	42578	-	+	-	-
	Formate dehydrogenase (FDH), mitochondrial	V7C2C9_PHAVU	44388	-	+	-	-
3	Purple acid phosphatase	PPAF_PHAVU	52880	-	-	+	-
	Purple acid phosphatase	V7AEU9_PHAVU	45278	-	-	+	-
	Uncharacterized protein	V7AWN2_PHAVU	59095	+	-	-	+
	Uncharacterized protein	V7BFL3_PHAVU	57523	-	-	-	+
	Alpha-phaseolin	Q41115_PHAVU	48533	+	+	+	+
	Alpha-phaseolin	X5CHV7_PHAVU	48511	+	+	+	+
	Alpha-phaseolin	X5CHV9_PHAVU	48686	+	+	+	+
	Alpha-phaseolin	X5CHW3_PHAVU	48531	+	+	+	+
	Alpha-phaseolin	X5CHW7_PHAVU	48714	+	+	+	+
	Alpha-phaseolin	X5CHX2_PHAVU	48501	+	+	+	+
	Alpha-phaseolin	X5CN36_PHAVU	48501	+	+	+	+
	Alpha-phaseolin	X5CN52_PHAVU	48487	+	+	+	+
	Alpha-phaseolin	X5CQ77_PHAVU	48577	+	+	+	+
	Alpha-phaseolin	X5CQ79_PHAVU	48570	+	+	+	+
	Alpha-phaseolin	X5CQ83_PHAVU	48543	+	+	+	+
	Alpha-phaseolin	X5CQ86_PHAVU	48514	+	+	+	+
	Alpha-phaseolin	X5CQ89_PHAVU	48541	+	+	+	+
	Alpha-phaseolin	X5CQ94_PHAVU	48547	+	+	+	+
	Alpha-phaseolin	X5D3I3_PHAVU	48516	+	+	+	+
4	Alpha-phaseolin	X5D3J1_PHAVU	48550	+	+	+	+
	Alpha-phaseolin	X5D3J3_PHAVU	48591	+	+	+	+
	Alpha-phaseolin	X5D3J8_PHAVU	48502	+	+	+	+
	Alpha-phaseolin	X5D5C2_PHAVU	48586	+	+	+	+
	Alpha-phaseolin	X5D5C4_PHAVU	48759	+	+	+	+
	Alpha-phaseolin	X5D5D0_PHAVU	48684	+	+	+	+
	Alpha-phaseolin	X5D5D4_PHAVU	48516	+	+	+	+
	Alpha-phaseolin	X5D5D7_PHAVU	48517	+	+	+	+
	Alpha-phaseolin	X5D5E0_PHAVU	48633	+	+	+	+
	Phaseolin	A0A0A0P203_PHAVU	47449	+	+	+	+
	Phaseolin	Q43632_PHAVU	47525	+	+	+	+
	Phaseolin	Q43633_PHAVU	48448	+	+	+	+
	Phaseolin, alpha-type	PHSA_PHAVU	49241	+	+	+	+
	Phaseolin, beta-type	PHSB_PHAVU	47536	+	+	+	+
	Uncharacterized protein	V7BFL2_PHAVU	41272	+	+	+	+
	Uncharacterized protein	V7BFL4_PHAVU	41678	+	+	+	+
5	Uncharacterized protein	V7BN55_PHAVU	33677	+	+	+	+

'+' indicates the presence of a protein and '-' the absence of a protein in a band.

Future studies are warranted to investigate the association between antioxidant enzyme activity and seed vigor in landrace genotypes of common bean under various stresses. In future studies, the biochemical and physiological responses of these genotypes to oxidative stress through the antioxidant defense system as well as the mechanisms underlying ROS removal and oxidative damage relief in the presence of specific biotic or abiotic stresses will be evaluated.

Conclusion

The quantification of soluble protein fractions during bean seed germination associated with mass spectrometry (MS) technique proved to be an efficient tool in these genotypes characterization. Although

globulins are the most abundant fraction, the high-vigor genotypes differed from those of low-vigor in not abundant fractions, the glutelins. In the tested landrace genotypes of common bean, LOX was present at a concentration within the detection limit only in high-vigor genotypes. Thus, further studies with additional common bean genotypes with contrasting vigor characteristics are recommended. Assessment of additional genotypes will allow for the verification any association between seed storage protein composition and seed vigor, and also, if LOX may be a possible biochemical marker candidate protein for high-vigor genotypes of common bean.

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