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ORIGINAL ARTICLE

Impact of protein diversification on morphometric behavior of *Andrographis paniculata* Nees

Daryush Talei · Alireza Valdiani · Mohd Puad Abdullah

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Abstract Proteins are important biochemical parameters in genetic diversity and controlling morphological characteristics in plants. In this study, the proteomic and morphometric data of an important medicinal herb "Aci Paşa" (*Andrographis paniculata*) were combined together to illustrate their impacts on genetic variation of the plant's population and to realize the connection between protein patterns and phenotypic behavior of the species. We used three protein extraction buffers including Tris, potassium phosphate, and sodium citrate. The Tris buffer was significantly different ($p \leq 0.01$) than other two in terms of the quality and quantity of protein bands by producing 15 types of proteins ranged from 13 to 105 kDa of which two of them were polymorphic. Consequently, a total of 12 accessions of *A. paniculata* were subjected to morpho-proteomic analyses. The unweighted pair group method with arithmetic average cluster analysis of the accessions based on the protein data and morphological characteristics generated three and four clusters, respectively, at a Euclidean distance of 2.53 for the morphological traits. Moreover, seed proteins analysis revealed that the two polymorphic protein bands sized 20.5 (protein "b") and 30 kDa (protein "a") effectively diversified the morphological characteristics and phylogenetic relationships among the 12 accessions of *A. paniculata*. Interestingly, the protein "b" acted as an activator agent for the number of branches, leaves and total dry weight, while the protein "a" performed a suppressive role for the same traits. Additionally, the two high-weighted faint bands "c" (75 kDa) and "d" (100 kDa) with a very low expression in accession 11228 proved their suppressive role along with the "a" band, while these bands were strongly expressed in the rest of the accessions. These findings suggest that these four proteins should be sequenced and perfectly established for further proteomic analyses. Ultimately, the mentioned proteins can be developed for any prospective breeding program or gene identification.

Keywords Morpho-proteomic connection · Phylogenetic analysis · Proteome analysis · Seed protein

Introduction

Andrographis paniculata Nees known as Aci Paşa in Turkish and Nain-e Havandi in Persian is a precious medicinal plant species with a bright economic horizon (Valdiani et al. 2012a, b). Proteomic analysis is one of the crucial issues in biology and molecular investigations, while protein extraction is the most important step in this scope especially for plant material. The stability of proteins is an important factor during extraction process. Ionic environment (pH) and the buffering compound of the extraction buffer are essential points to keep the protein in a stable form. Generally, proteins are less soluble at their isoelectric point (PI) value, which is the pH at which the

D. Talei (✉)
Medicinal Plant Research Center, Shahed University, Tehran 3319118651, Iran
e-mail: d.talei1348@gmail.com

A. Valdiani (✉)
Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor DE, Malaysia
e-mail: alireza.valdiani@gmail.com

M. P. Abdullah
Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor DE, Malaysia

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protein has no net charge (Ries-Kautt and Ducruix 1997; Schmittschmitt and Scholtz 2009). The ability of extracting a large number of proteins in a single attempt necessitates employing new technologies for the rapid separation and identification of proteins of interest.

Biochemical markers and specifically proteins give the closest insight into the genetic makeup whereas they overcome most of the restrictions of phenotypic markers (Jehan and Lakhanpaul 2006). Nevertheless, even morphological data could become reliable tools in phylogenetic analyses when they are associated with molecular data (Huang et al. 2013). The seed protein patterns have been used in combination with morphometric markers to effectively characterize different plant species (Sheidai et al. 2000). The seed storage proteins are highly polymorphic with genetically and molecularly known structures. Most of these proteins are unique and adequate to be used as markers in genetic diversity analyses at inter- and intra-population levels. Protein markers are broadly used in plant breeding, genetic resources conservation as well as to demonstrate genome relationships especially in polyploid series (Asante et al. 2009; Gepts et al. 1990).

At least, the perspective of the protein-based studies on *A. paniculata* is not "very bright" if we do not use the term "too dark", since the only conducted research in this regard refers to early 2000s, when Sabu et al. (2001) investigated the intraspecific variation of the herb using isozymes. Excluding this study, it is not an exaggeration to say that protein-based phylogenetic and physiologic studies have been totally disregarded in *A. paniculata*.

Hence, the present research aimed to consider the seed protein profiles and attempted to utilize them along with morphological characteristics, not only to illustrate the genetic variation, but also to discover a connection between protein patterns and morphometric behavior in the herb's population. In other word, we were wondering whether there is a possibility to detect any specific protein band or bands in relation with a particular morphological trait. Albeit, prior to shifting on the hierarchical clustering, the seed protein extraction was optimized by employing three different extraction buffers with varied pHs to find suitable protein extraction protocols for SDS-PAGE technique in terms of protein yield, number of protein bands, image quality, reproducibility.

Materials and methods

Genetic material and growth conditions

Seed samples of the 12 accessions of *A. paniculata* were provided from the Agro Gene Bank, University Putra Malaysia. The seeds were germinated using the method presented by Talei et al. (2012). The germinated seeds at two-initial-leaf stage were transported into the jiffy media. The 30-day seedlings were then transferred from jiffy pots into the pots filled with the mixture of top soil, peat, and sand in the ratio 2:1:1. The plants were arranged in a randomized complete block design (RCBD) with 12 treatments (accessions) and three replicates. During the experimental period, the plants were irrigated with the Hoagland nutrient solution. All plants at the stage 90-day old were harvested and data on morphological traits including; shoot and root length (SL and RL), number of leaves (NL), number of branches (NB), fresh and dry shoot weight (SFW and SDW), fresh and dry root weight (RFW and RDW), and total dry weight (TDW) after drying at 68 °C for 48 h were measured.

Protein sample preparation and quantification for SDS-PAGE

One gram of seed from each accession of *A. paniculata* was analyzed in three replicates (using three different 1-g seed sets of each accession). The seeds were collected from five different plants belonging to each accession. The samples were ground in liquid nitrogen using pre-cooled mortar and pestle to obtain a fine powder and then homogenized with three different extraction buffers as follows: the Tris buffer (50 mM Tris–HCl pH 7), the potassium phosphate buffer (50 mM phosphate pH 9), and the sodium citrate buffer (50 mM citrate pH 5) containing 10 % (v/v) glycerol, 2 % (w/v) SDS, and 1.5 % DDT. Each sample was vortexed for 10 min and centrifuged in Allegra 25 R model centrifuge (Beckman Coulter Inc., Germany) at 15,000 rpm for 20 min at 4 °C. The supernatants were collected and the total protein concentration was determined by the Bradford method (1976) employing bovine serum albumin (Sigma) as the standard at 595 nm, using a spectrophotometer (Perkin Elmer Lambda 25; UV/VS).

Protein separation using SDS-PAGE

In order to establish the extraction efficiencies, the protein samples were run on SDS-PAGE separation following the method described in Laemmli (1970). Different amount of protein (5, 10 and 15 μg) from each sample were solubilised with 2 × SDS/sample buffer [0.125 M Tris base, pH 6.8, 20 % (w/v) glycerol, 2 % (w/v) SDS, 2 % (v/v) 2-mercaptoethanol and 0.01 % (w/v) bromophenol blue] and loaded in each lane of the 12 % concentrated separating gel. Electrophoresis was accomplished at 100 V over 90 min using a Bio-Rad, Mini Protein electrophoresis system.

Staining of protein patterns on the SDS-PAGE gel

The gels were stained with 0.25 % Coomassie Brilliant Blue R-250 (Sigma) in 40 % (v/v) methanol and 7 % (v/v) acetic acid for 1 h and de-stained with 40 % (v/v) methanol and 7 % (v/v) acetic acid until the background was clear. The gel scanning and visualization were performed using a densitometer (GS-800, Bio-Rad, USA). The analysis was carried out using UVIDoc Analyser software.

Statistical analyses

The SAS program version 9 (SAS Institute Inc. 2009a) was used for all statistical analyses including the raw data normality test and the main data analysis as well as for the Duncan's multiple range test ($p < 0.01$). The JMP 8 software (SAS Institute Inc. 2009b) was used to calculate the correlations and unweighted pair group method with arithmetic average (UPGMA) cluster analysis.

Results

Comparison of different protein extraction buffers

The three mentioned buffers for sample extraction were compared in terms of protein yield, number of protein bands, and protein bands resolution. Seed extracts obtained from each buffer were compared together for protein content using the Bradford assay. The results showed that the weight of the total protein obtained from 1 g of seed using different buffers was statistically significant ($p \leq 0.01$). A higher protein yield was obtained with the potassium phosphate buffer with pH 9 (0.815 ± 0.04 mg/g fresh weight), while the lowest was obtained with the sodium citrate buffer with pH 5 (0.349 ± 0.04) (Table 1). The SDS-PAGE analysis showed that the protein patterns of the three extraction buffers were different, and the quantity and quality of proteins in samples extracted using Tris buffer were higher (15 bands) compared to other extraction buffers. The sodium citrate buffer produced the lowest number of proteins (nine bands). Protein analysis showed that 15 different types of proteins were detectable in the seeds of *A. paniculata* (Fig. 1).

SDS-PAGE seed protein profiling and protein-based diversity

In practice, the seed protein analysis of the 12 accessions of *A. paniculata* unveiled the presence of 15 different types of proteins ranged from 13 to 105 kDa, yet, only two of them with low molecular weight were found polymorphic. The two polymorphic proteins were consisted of a 30 kDa protein band "a", which was absent only in accession 11228, and a 20.5 kDa protein band "b", which was present in accessions 11228, 11266, 11306, and 11348

Table 1 Comparison of different protein extraction buffers based on protein content and number of protein bands

Buffers	Protein content (μg/g seed)	Protein bands
Tris–HCl	0.479 ± 0.03[a]	15
Potassium phosphate	0.815 ± 0.04[b]	14
Sodium citrate	0.349 ± 0.04[a]	9

Data are mean values of three independent measurements and standard error of mean. Different letters indicate significant differences among the methods using Duncan's multiple comparison test at $p \leq 0.01$

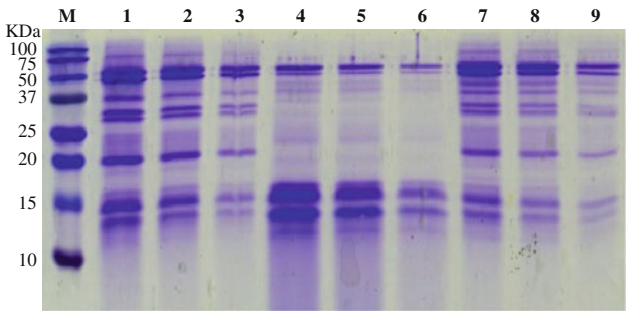

Fig. 1 SDS-PAGE polypeptide profile of the seed protein of *Andrographis paniculata* using three different protein extraction buffers. The *lane M* represents the protein marker; the *lanes 1*, *2,* and *3* represent 15, 10, and 5 μg protein samples using Tris buffer, the *lanes 4*, *5,* and *6* represent 15, 10, and 5 μg protein samples using citrate buffer and the *lanes 7*, *8,* and *9* represent 15, 10, and 5 μg protein samples using phosphate buffer

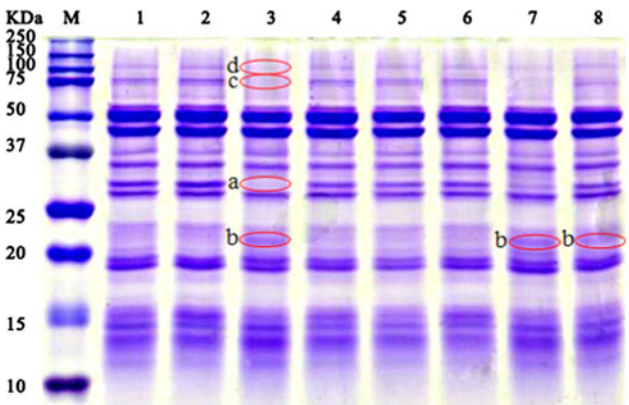

Fig. 2 SDS-PAGE polypeptide profile of the seed protein of *Andrographis paniculata* on 12 % polyacrylamide gel. The *lane M* represents the protein marker, (*1*) 11179, (*2*) 11216, (*3*) 11228, (*4*) 11249, (*5*) 11264, (*6*) 11265, (*7*) 11266, and (*8*) 11306. Protein samples were loaded with equal amount of 15 μg. The protein band "*a*" was not found in accession 11228, and the protein band "*b*" was found in accessions 11228, 11266, and 11306

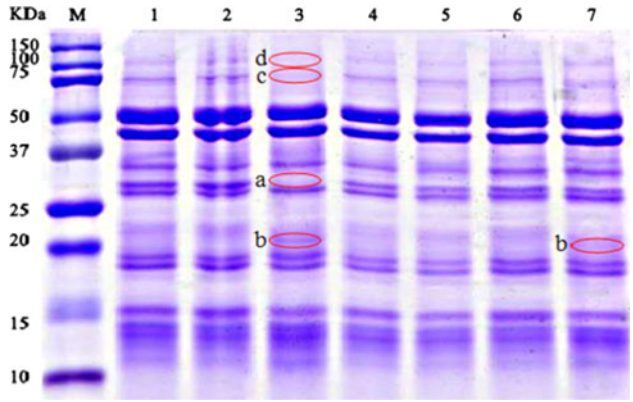

Fig. 3 SDS-PAGE polypeptide profile of the seeds of *Andrographis paniculata*. The *lane M* represents the protein marker, (*1*) 11179*, (*2*) 11216*, (*3*) 11228*, (*4*) 11314, (*5*) 11329, (*6*) 11339, and (*7*) 11348. Protein samples were loaded with equal amount of 15 µg. The protein band "*a*" was not found in accession 11228 and the band "*b*" was only found in accession 11348. *To confirm the reproducibility of the protein bands, some of the protein samples have been run twice on the SDS-PAGE

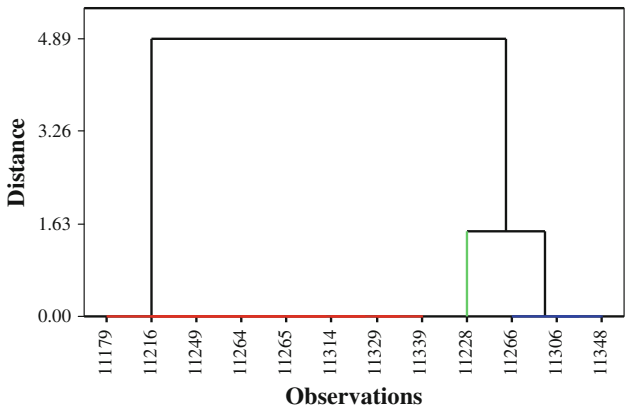

Fig. 4 Dendrogram generated by UPGMA clustering method based on seed protein profiles showing the phylogenetic relationships of the 12 accessions of *Andrographis paniculata*

(Figs. 2, 3). Additionally, two high-weighted bands "c" (75 kDa) and "d" (100 kDa) appeared extremely faint in accession 11228 while these two bands were strongly expressed in the rest of the accessions (Figs. 2, 3). The results indicated that most of the bands were similar in all accessions. Potentially, these protein bands can also serve as useful markers to hybridization and breeding programs in future studies.

The UPGMA cluster analysis of the accessions based on the protein profiles using the Ward method generated three clusters (Fig. 4). The first cluster with *red color* contained eight accessions, indicating the close similarity among most accessions, the second cluster (the *green cluster*) contained only one accession and the third cluster (the *blue one*) comprised of three accessions. Accession 11228, which was lacking the protein "a", was located separately in the second cluster. Although, the cluster analysis showed that the accessions possessing the protein "b" are much more related together than those accessions, which were lacking this protein, and were located in the *blue cluster* (Fig. 4).

Morphometric and phylogenetic analyses of the accessions

The variance analysis of the investigated characteristics revealed that there were significant differences among accessions in terms of NB, NL, SFW, SDW, and TDW traits. In addition, the results showed that there were no significant differences on SL, RL, RFW, and RDW of the 12 accessions (Table 2). This outcome, in fact evidenced that the selected accessions for this experiment were significantly different from each other based on morphological traits. The NB ranged from 19.7 to 24 with a mean of 21.6. The NL ranged from 370.3 to 528 with a mean of 446.5. The TDW ranged from 28 to 36.6 with a mean of 32.3. Accession 11228 indicated the highest amounts of NB, NL, SFW, SDW, and TDW, while accession 11329 showed the lowest amounts on most of the studied morphological traits. Table 3 shows the comparison of mean using multiple test for the studied qualitatives at $p \leq 0.01$.

The UPGMA cluster analysis of the 12 accessions of *A. paniculata* based on the measured morphological characteristics using the minimum variances (ward method) produced four clusters at a Euclidean distance of 2.53. The first cluster (*red cluster*) included five accessions, the second cluster (*orange cluster*) contained one accession, and the third cluster (*green cluster*) indicates four accessions, and the fourth cluster (*blue cluster*) comprised of two accessions (Fig. 5). The traits that contributed for clustering of *A. paniculata* accessions were NB, NL, SFW, SDW, and TDW. The third cluster was associated with the highest mean of NB (22.3), NL (496), and TDW (35.3 g), while the second cluster had the lowest mean of NB (19.7), NL (402.9), and TDW (29.8 g). Accession 11228 showed the highest significance in almost all morphological characters except SL (47 ± 0.58) and RL (22 ± 0.00) when compared to the others.

Morpho-proteomic association of the accessions

The cluster analysis based on both morphological and protein data provided evidences to prove the existence of differences among the accessions. Grouping the accessions based on the presence or absence of these protein bands and comparing the groups in terms of the NB, NL, and TDW data using one-way ANOVA indicated that there were significant differences between groups (Table 4). The

Table 2 Variance analysis of morphological characteristics of the 12 accessions of *Andrographis paniculata*

Source	*df*	Mean square								
		SL	NB	NL	SFW	SDW	RL	RFW	RDW	TDW
R	2	21*	4.694^{ns}	642.86^{ns}	681.94^{ns}	28.30*	24.11*	126.74**	1.43**	28.30**
Accession	11	6.92^{ns}	4.29*	7,881.66**	562.06*	21.52**	6.76^{ns}	19.57^{ns}	0.55^{ns}	24.41**
Error	22	3.85	1.82	511.77	237.27	5.05	4.96	10.29	0.25	4.93

ns no significant, *SL* shoot length, *NB* number of branches, *NL* number of leaf, *SFW* shoot fresh weight, *SDW* shoot dry weight, *RL* root length, *RFW* root fresh weight, *RDW* root dry weight, *TDW* total dry weight

Statistical significance is indicated by ** $p \leq 0.01$, * $p \leq 0.05$

Table 3 Comparison of mean according Duncan's multiple tests in the 12 accessions of *Andrographis paniculata*

Accession	Mean values ± standard error				
	NB	NL	SFW	SDW	TDW
11179	23.0 ± 0.6^{ab}	420.7 ± 10.2^{bc}	137.2 ± 16.1^{abcd}	28.0 ± 1.5^{bcde}	31.7 ± 1.5^{bcdef}
11216	22.3 ± 0.9^{abc}	515.7 ± 8.4^{a}	152.0 ± 14.9^{abcd}	31.4 ± 1.6^{ab}	35.7 ± 1.4^{ab}
11228	24.0 ± 0.0^{a}	528.0 ± 2.0^{a}	161.5 ± 3.9^{a}	32.4 ± 0.3^{a}	36.6 ± 0.5^{a}
11249	22.0 ± 1.2^{abcd}	488.0 ± 5.2^{a}	158.1 ± 3.6^{ab}	29.9 ± 1.7^{abc}	33.4 ± 2.0^{abcde}
11264	21.0 ± 0.6^{bcd}	370.3 ± 7.5^{d}	128.1 ± 10.3^{bcd}	26.6 ± 1.0^{cde}	29.8 ± 1.2^{ef}
11265	22.0 ± 1.2^{abcd}	436.7 ± 21.1^{b}	131.6 ± 9.6^{bcd}	30.0 ± 1.7^{abc}	34.0 ± 1.4^{abcde}
11266	21.0 ± 0.6^{bcd}	506.0 ± 18.5^{a}	148.1 ± 12.6^{abcd}	29.9 ± 2.1^{abcd}	34.1 ± 2.2^{abcd}
11306	21.7 ± 0.3^{abcd}	434.3 ± 19.6^{b}	157.2 ± 6.9^{abc}	30.4 ± 1.1^{abc}	34.8 ± 1.1^{abc}
11314	21.3 ± 0.7^{bcd}	406.0 ± 9.2^{bcd}	123.1 ± 9.2^{d}	24.2 ± 1.6^{d}	28.0 ± 1.0^{f}
11329	19.7 ± 0.7^{d}	434.7 ± 11.6^{b}	129.3 ± 6.9^{bcd}	25.7 ± 2.5^{de}	30.2 ± 2.6^{def}
11339	21.3 ± 1.3^{bcd}	388.0 ± 16.2^{cd}	127.3 ± 4.4^{cd}	24.7 ± 0.8^{d}	28.3 ± 0.8^{f}
11348	20.0 ± 1.0^{cd}	429.3 ± 13.4^{bc}	142.6 ± 6.0^{abcd}	27.8 ± 1.0^{bcde}	31.4 ± 1.1^{cdef}

NB number of branches, *NL* number of leaf, *SFW* shoot fresh weight, *SDW* shoot dry weight, *TDW* total dry weight

Different letters indicate a significant difference between the values of pairs of treatment within columns (mean values ± standard error) at $p \leq 0.01$

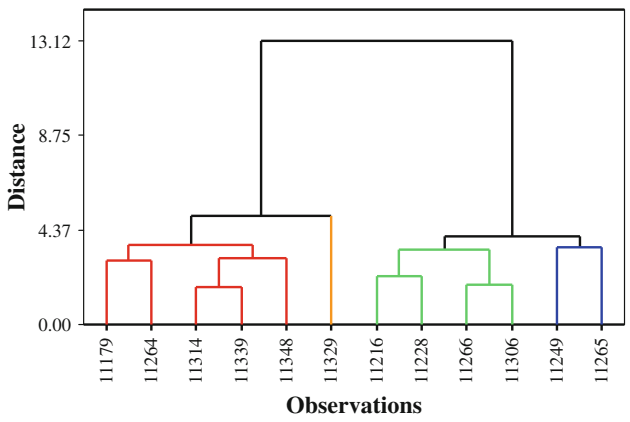

Fig. 5 Dendrogram generated by UPGMA clustering method of the 12 accessions of *Andrographis paniculata* based on the studied morphological traits

results revealed that the presence or absence of these proteins might be related to the morphological characteristics. The mean of NB, NL, and TDW in accessions containing the protein "a" were lower than accessions that did not have this protein, but the mean of NB, NL, and TDW in accessions containing the protein "b" were higher than accessions without that.

Discussion

Prior to conducting any proteomic experiment, a performant extraction method should be optimized and developed. The current study met this matter perfectly as it introduced the Tris buffer (at pH 7) as the best candidate to carry out any proteomic analysis using seed samples in *A. paniculata*. This may be due to the stability of Tris at the adjusted pH in comparison with the other extraction buffers. Therefore, changing the pH to optimize the conditions that keep the protein in solution is important (Durst and Staples 1972). Despite observing the satisfying results of Tris buffer in the present experiment, developing any new protein extraction protocol in this plant using different tissues requires a

Table 4 Variance analysis of morphological traits based on the seed protein band in the 12 accessions of *Andrographis paniculata*

S·O.V	*df*	Protein (a)			Protein (b)		
		MS (NB)	MS (NL)	MS (TDW)	MS (NB)	MS (NL)	MS (TDW)
Groups	1	18.68**	21,753.09*	60.02*	0.06 ^{ns}	14,056.06*	64.36*
Error	34	2.91	2,279.11	10.99	2.84	2,505.5	10.86

ns no significant, *MS* mean square, *NB* number of branches, *NL* number of leaf, *TDW* total dry weight

Statistical significance is indicated by ** $p \leq 0.01$, * $p \leq 0.05$

modified extraction buffer and may even lead to better results. In addition, the gel analysis resulted in detection of two polymorphic protein bands sized 30 kDa approximately (band "a"), that was absent only in accession 11228, and 20.5 kDa (band "b"), that was present in accessions 11228, 11266, 11306, and 11348. This situation caused that accession 11228 to be located in a separate cluster. Furthermore, grouping the accessions based on the presence or absence of these protein bands and comparing the groups in terms of the NB, NL, and TDW data using one-way ANOVA indicated significant differences between groups (Table 4). Our results presented the unequivocal impact of seed proteins in improving (the protein b) or decreasing (the protein a) the morphological traits such as SL, NL, NB, SFW, SDW, and TDW. For instance, it was confirmed that the absence of protein "a" and presence of the protein "b" in accession 11228 caused the highest NB, NL, and TDW in this accession compared with the others (Table 3). It is evident from the results that the morphometric variation observed in the accession 11228 might be due to the specific protein profiling. As a matter of fact, there seems to be little published information on the relationship between banding patterns produced by seed storage protein electrophoresis and morphological characteristics. In line with this priority, Andrews et al. (2006) without emphasizing on a specific protein, claim that generally, results in the literature are compatible with the hypothesis that macronutrients, water, irradiance, and CO_2 affect shoot: root dry weight ratio (S:R) through changes in shoot protein concentration. On the other hand, some references have proposed that day length could act as an abiotic factor for alteration of protein patterns and these changes can lead to a different morphological expression in plants (Chang et al. 1998; Victor et al. 2010). Obviously, this issue cannot be an important factor in the present research, because this experiment was conducted in a tropical area, where the day and night lengths are almost the same. However, protein bands or spots have rarely been connected to morphological traits in plants, but it is clear that the modern studies have tended to make a precise connection between specific proteins (or protein divergence) and morphological diversification (Hanada et al. 2009). In accordance with this new trend, a relevant investigation confers that the protein expression (number of protein spots) in rice seedlings was changed by inducing jasmonic acid (JA) on them, consequently, the growth of shoots and roots in the JA-induced rice seedlings were reduced (Cho et al. 2007). Recently, protein bands have specifically been employed as markers to describe the morphological variation between the wild and cultivated taxa of Sesamum as it is noticed that a specific protein with a band size of 17 kDa was the unique feature of the cultivated species, whereas two protein bands with molecular weight 88 and 23 kDa were characteristics of the wild taxa "*S. occidentale*" (Akhila and Beevy 2011). This report also is in agreement with our results except we have not used the protein bands for identifying the cultivated taxa from the wild ones, because all the accessions we used were wild anyway. Interestingly, sometimes, proteins seem to represent the repressor and activator role in plants' architecture, simultaneously. The best example for this case is the impact of MADS-domain protein MPF1 of *Physalis floridana* in regulating plant height, seed size, and flowering time (He et al. 2010). The protein MPF1 seems to act as a repressor for main stem growth and as an activator of lateral shoots. As a remarkable point, these tasks were enforced by separate proteins in our exploration, so that the suppressive role was performed by the "a" protein for the traits NB, NL, and TDW in some accessions, while the "b" protein played an activator role for the same traits, but in different accessions. Moreover, the strong expression of the protein bands "c" and "d" in all the accessions (except for accession 11228) caused a reduction in the morphological potential of the accessions carrying these proteins. Hence, the feeble presence of the mentioned bands (c and d) in accession 11228 and morphological superiority of this accession could be considered as the suppressive role of these proteins along with the protein "a". The result also showed that the concurrent presence of the activator and suppressive proteins in an accession could lead to a fluctuating increase in different morphological traits. Such a fragile situation could be observed in accessions 11266, 11306, 11348 where the presence of the activator protein (b) was affected by the presence of suppressive proteins (a, c and d). Therefore, despite the presence of protein "b" the enhancement of morphological characteristics was not happened decisively. These outcomes highlight the importance of gene knockout and knockdown techniques as the alternative strategies to overcome the

prohibition act of the suppressive genes in the future studies on *A. paniculata*. The findings of the present study indicated significant differences among the accessions based on both morphological and protein data. The results also matched up well with the previous findings that show the absence of one specific protein could relatively be inferred as a resistance source to bacterial stripe in rice (Talei and Fotokian 2008).

Although, the importance of morphological variation in *A. paniculata* accessions was verified previously (Valdiani et al. 2012a, b), but from the results of the present study it is evident that the protein variation among the *A. paniculata* populations can be seriously taken into account in molecular studies of this herb, henceforth. Furthermore, the electrophoresis of seed proteins can be utilized as an effective strategy in the programs involved with *A. paniculata* conservation. The differences observed among the accessions would be of immediate importance for development of the *A. paniculata* gene bank and may be used in hybridization and breeding programs.

As a statistical principle, replication is the best way to reduce the experiments' errors and to confirm the obtained results. This rule has perfectly managed our experiment's procedure by using different accessions with three replicates. So, from this point of view, we are almost sure that the protein "b" somehow causes enhancement in NB, NL, and TDW of those accessions carrying this protein. Nevertheless, upon this, several entangled questions arise, whether such an increase under which mechanism could possibly happen? Is there any environmental factor caused this protein to be synthesized? What is the biochemical combination of this protein? Does it include a single amino acid or this single band is an amalgamation of multiple peptides? Does this protein encode a specific gene or it referrers to a couple of genes? Could this result be possibly connected to epigenetic mechanisms? And finally a critical question comes up that, since *A. paniculata* as a cryptic species is famed because of its anticancer compounds (Valdiani et al. 2013), are these proteins possibly linked to the phytochemical content of the plant? A supplementary identifier step could be taken over the direct QTL mapping or even physical mapping of these two proteins "a" and "b" to find their exact locations on the chromosomes, or using them indirectly in marker-assisted selection of morphological and agronomic traits. This procedure seems to be feasible as it has been achieved by other scientists using seed proteins, in the past (Campa et al. 2011).

Conclusion

Even though seed protein and phenotypic characterization were used for providing data to study the diversity and interrelationships of *A. paniculata* populations, our main purpose was indeed to improve the quantity and quality of extractable proteins from this species. The highest and lowest protein yields were obtained with the potassium phosphate and sodium citrate buffer, respectively. The best quality and the highest number of proteins (15 bands) were produced by Tris buffer, whereas the sodium citrate buffer produced the lowest number of proteins (nine bands). A specific protein band with 20 kDa molecular weight was detected in three accessions of *A. paniculata* including 11266, 11306, and 11348. This protein not only resulted in a close interrelationship among the mentioned three accessions, but also it caused the highest increasing records of some morphological traits including NB, NL, and TDW in these accessions. Three protein bands with 30, 75, and 100 kDa molecular weights led to the lower ranges of NB, NL, and TDW in those accessions carrying this protein. These two proteins can be sequenced for further analyses and after identifying their exact type, they can be developed for the potential breeding programs in the future.

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