Wageningen Academic Publishers

Acetic acid urea-polyacrylamide gel electrophoresis: a rapid method for testing the genetic purity of sunflower seeds

J. Zheng[1#], D. Wen[1#], H. Zhao[2] and C. Zhang[1*]

[1]State Key Laboratory of Crop Biology, Agronomy College, Shandong Agricultural University, Tai'an, Shandong Province 271018, China P.R.; [2]Beijing Doneed Seeds Co., Ltd., A 6 Zhongguancun South Street, Beijing 100086, China P.R.; cqzhang@sdau.edu.cn; # these authors have contributed equally to this work

Received: 21 January 2015 / Accepted: 29 September 2015

RESEARCH ARTICLE

Abstract

Genetic purity testing is an important component of seed quality control. The objective of this study was to develop a rapid method for testing the genetic purity of sunflower seeds. We used six sunflower hybrids and their parental lines to study and improve a method based on the acetic acid urea-polyacrylamide gel electrophoresis (AU-PAGE) approach for testing the genetic purity of maize seeds. The results showed that the most abundant proteins in sunflower seeds were water-soluble proteins, followed by salt-soluble proteins, while there were relatively few other proteins. The AU-PAGE method was improved to increase the clarity of lanes backgrounds by adding an appropriate amount of sodium dodecyl sulfate to the protein extracting solution. The reduced background staining of the lanes improved the bands identification and increased the evidence of protein polymorphism. The method was validated as an efficient method for genetic purity testing of sunflower seeds. The improvements in this method could potentially be applied to other plants and other protein electrophoresis.

Keywords: genetic purity, protein electrophoresis, rapid testing, sunflower seeds

1. Introduction

Sunflower (*Helianthus annuus* L.) is an important crop source of edible oils (Tang *et al.*, 2002). With an increasing number of sunflower cultivars in production, it is increasingly difficult to distinguish them solely by morphological characteristics. In hybrid seed production, seed genetic purity is often reduced because of biological admixture and mechanical admixture. Therefore, there is an urgent need to develop a simple and rapid method to identify the genetic purity of sunflower seeds. Grow-out tests for genetic purity remain a classic and general method, but suffer from high cost and the requirement for a long test period (Kumar *et al.*, 2014; Naresh *et al.*, 2009; Sundaram *et al.* 2008). Seed protein electrophoresis has been used extensively for seed genetic purity identification in the International Rules for Seed Testing. Protein polymorphisms used as an effective biochemical marker play important roles in variety identification and germplasm studies.

In seed protein electrophoresis, proteins are separated by electrophoresis and displayed as bands on gels. The differences in band patterns of individual seeds are used to determine the seed genetic purity. The method is simple, rapid and low cost. Seed protein electrophoresis has been applied to detect the seed genetic purity in a number of crops, including wheat, barley, maize, rice and cotton. Non-coherent acetic acid urea-polyacrylamide gel electrophoresis was used to detect the genetic purity of maize seeds (Zhang *et al.*, 1995). Acetic acid urea glycine-polyacrylamide gel electrophoresis was implemented to study the polymorphism of cotton seed proteins and for genetic purity testing (Zhang *et al.*, 1998). Polyacrylamide gel electrophoresis (PAGE) was used for testing the genetic purity of cotton hybrids and parents (Murtaza *et al.*, 2005). Ultrathin-layer isoelectric focusing was used to test the genetic purity of two-line hybrid rice seeds (Zhao *et al.*, 2005) and for variety identification in *Cucurbita* (Tu *et al.*, 2012). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) has been used in varietal

identification of chilli (*Capsicum annuum* L.) (Litoriya *et al.*, 2010), diversity analysis in rice varieties (Ansari *et al.*, 2011), and variety identification of tomato (*Lycopersicon esculentum* Mill.) (Vishwanath *et al.*, 2011). Total seed proteins were analysed by SDS-PAGE in Ethiopian mustard (*Brassica carinataa*. Braun) germplasm (Zada *et al.*, 2013). However, because of a lower degree of band polymorphism in sunflower seed protein electrophoresis, very few sunflower cultivars can be distinguished by currently available electrophoresis methods and there is no robust protein electrophoresis method available for genetic purity testing of sunflower seeds.

In this study, acetic acid urea-polyacrylamide gel electrophoresis (AU-PAGE) was modified to detect the genetic purity of sunflower seeds. Initially, AU-PAGE was developed in our laboratory for genetic purity testing of maize seeds, which showed high polymorphism and therefore efficiency of the method. However, AU-PAGE could not be used directly for testing the genetic purity of sunflower seeds because of high oil and protein content in the seeds. Thus, we optimised the extraction buffer for sunflower seed proteins and established a novel scoring system for the protein profile so that AU-PAGE can be applied for testing the genetic purity of sunflower seeds.

2. Materials and methods

Materials

Sunflower hybrids and their parental lines (Table 1) were provided by the Beijing Doneed Seeds Co., Ltd. (Beijing, China), and represent the prevailing sunflower cultivars planted in China. Their female parents and male parents were male sterile and restorer lines, respectively. Hybrid SC89 is an edible cultivar and the others are oil cultivars. Seeds of hybrid SC89 are significantly bigger than the other cultivars.

Table 1. Sunflower hybrids and their parental lines used in this study.

No.	Hybrid	Female parent	Male parent
1	S31	P14337	P34019
2	S18	P14366	P34019
3	MGS	P13630	P32450
4	S510	P13630	P34354
5	S65	P14271	P32450
6	SC89	PAC855	P65101
7	HySun39	N/A	N/A

Acetic acid urea-polyacrylamide gel electrophoresis

Gel solution was prepared by adding 65-80 µl catalyst solution (3.5% ammonium persulfate) to 40 ml gel solution (10% acrylamide, 10% urea, 0.2% methylene bisacrylamide, 3% acetic acid and 0.02% ferrous sulfate). The mixture was swirled quickly with a glass rod then poured into the gap between the glass gel plates. A sample comb was inserted into the solution and removed after 2-3 min.

Sunflower seeds were individually ground using a home-made single seed miller, extracted with protein extracting solution (18% urea, 12% acetic acid, 3% tetramethylethylenediamine, and 0.05% methyl green) for 30 min at 25 °C, and centrifuged for 4 min at 4,000×*g* (Eppendorf centrifuge 5418R; Eppendorf, Hamburg, Germany). The supernatant (30 µl) was pipetted into the sample well for each sample, and then the electrode buffer (2% glacial acetic acid) was added to the electrophoresis tank. Electrophoresis was run at 85 mA for 2 h (Bio-Rad Power Pac3000; Bio-Rad, Hercules, CA, USA), and gels were stained with the staining solution (0.08% Coomassie Brilliant Blue R-250, 5% ethanol and 10%-12% trichloroacetic acid) for 12 h. The stained gels were washed briefly and viewed on a light box to observe band patterns.

Analysis of protein content and composition

The gel image was opened in Adobe Photoshop (Adobe, San Francisco, CA, USA) and viewed in greyscale mode, then inverted. In this way, the light parts of the image became dark, and the dark parts became light. In Adobe Photoshop, the values of light points were near 255, and the values of dark points were near zero. The grey values of protein bands were measured directly by histograms that cover the protein bands, and used to calculate the protein content.

3. Results

Composition of sunflower seed proteins

The above protein extracting solution can extract water-soluble proteins, salt-soluble proteins and other proteins. For analysing the composition of sunflower seed proteins, water-soluble proteins, salt-soluble proteins and other proteins were extracted step-by-step from sunflower seeds using deionised water, saline solution (1 M NaCl), and protein extracting solution (18% urea, 12% acetic acid, 3% tetramethylethylenediamine, and 0.05% methyl green), respectively. Extraction of water-soluble proteins and salt-soluble proteins was different from the extraction of other proteins only in the nature of the extracting solution used and the addition of an equal volume of gel loading buffer

(36% urea, 24% acetic acid, 6% tetramethylethylenediamine, and 0.1% methyl green) to the former two extracts. The band patterns and contents in different hybrids were analysed. As shown in Figure 1, water-soluble proteins were most abundant proteins in sunflower seeds producing about 25 bands, followed by salt-soluble proteins (about 15 bands), with fewer other proteins (about 10 bands). Polymorphism of these low molecular weight proteins was low, and not very useful in genetic purity testing of the sunflower seeds. When the images were viewed in greyscale mode and then inverted, the average grey values were used to measure the protein content, that is, the higher the grey value, the higher the protein content. As shown in Table 2, most of the total detectable protein content of sunflower seeds was water-soluble proteins, followed by salt-soluble proteins, with fewer other proteins. Therefore, for genetic purity testing of sunflower seeds, the polymorphism of water-soluble and salt-soluble proteins should be used. For this purpose, we modified the composition of the protein extracting solution to increase the number of bands that were readily readable from the gels.

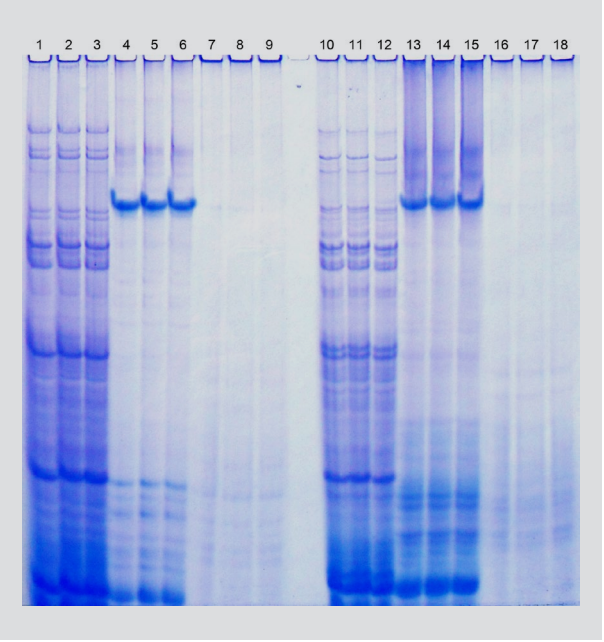

Figure 1. Acetic acid urea-polyacrylamide gel electrophoresis of the composition of sunflower seed proteins. Lanes 1-3: water-soluble proteins from S65; lanes 4-6: salt-soluble proteins from S65; lanes 7-9: other proteins from S65; lanes 10-12: water-soluble proteins from SC89; lanes 13-15: salt-soluble proteins from SC89; lanes 16-18: other proteins from SC89.

Table 2. Composition of sunflower seed proteins.

Lane	Hybrid	Type of protein	Content (%)
1-3	S65	Water-soluble proteins	60.45
4-6	S65	Salt-soluble proteins	26.83
7-9	S65	Other proteins	12.72
10-12	SC89	Water-soluble proteins	41.45
13-15	SC89	Salt-soluble proteins	41.35
16-18	SC89	Other proteins	17.20

Ratio of seed weight to volume of protein extracting solution

Seed proteins of hybrid SC89 were extracted with different volumes of protein extracting solution. The ratios of seed weight to volume of protein extracting solution were 1:6, 1:9, 1:12, 1:15 and 1:18, and extraction was repeated three times at each ratio. Representative results of electrophoresis are shown in Figure 2. When the ratio was 1:6, the lanes had strong background staining, making the bands of interest blurred and unreadable. With increasing volumes of protein extracting solution, the brightness of the protein bands weakened gradually and the background staining of lanes became gradually weaker. However, one-third of the lanes (representing the higher molecular weights) remained heavily background-stained, and some of the bands were difficult to identify. While increasing the volume of the protein extracting solution helped reduce background staining in the lower two-thirds of the lanes, it might diminish the intensity of bands from low-content proteins. Therefore, variation of the volume of protein extraction solution was not a successful method overall, and other strategies were explored to obtain clear gel backgrounds with lower loss of band intensity.

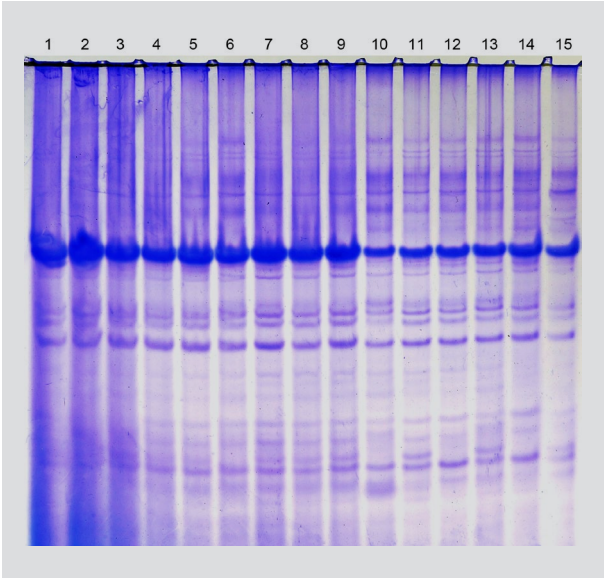

Figure 2. Effect of mass/volume ratios of sunflower seed weight and volume of protein extracting solution on acetic acid urea-polyacrylamide gel electrophoresis. Lanes 1-3: 1:6; lanes 4-6: 1:9; lanes 7-9: 1:12; lanes 10-12: 1:15; lanes 13-15: 1:18.

Optimisation of sodium dodecyl sulfate concentration in protein extracting solution

After a series of optimisation assays, it was found that the background staining could be reduced significantly by adding SDS to the protein extracting solution. SDS concentrations were tested from 0 to 0.6% at an interval of 0.1%, at 1:6 ratio of seed weight to volume of protein extracting solution in three replicates. A larger ratio of seed weight to volume of protein extracting solution might result in the loss of the bands of low-content proteins (see above). The results obtained are shown in Figure 3A. Clear lanes with low background staining were obtained when 0.2% SDS was added to protein extracting solution. A further increase in SDS content did not improve the clarity but reduced the number of bands.

To further optimise the SDS content, SDS concentrations were compared between 0 and 0.3% at an interval of 0.05% with three replicates (Figure 3B). When SDS content was less than 0.15%, there was considerable background staining. At 0.15% SDS content, the lanes were clear with little background staining. Although the brightness of bands was slightly weaker for low molecular weight proteins, it was similar for high molecular weight proteins. When compared with 0.1% SDS. At 0.2% SDS content, the background staining and protein bands were similar to those with 0.15% SDS. More than 0.2% SDS content, lack of protein bands increased gradually. Based on these results, 0.15% SDS in the protein extracting solution was considered to be optimal for low background staining with a high number of readable protein bands from sunflower seeds.

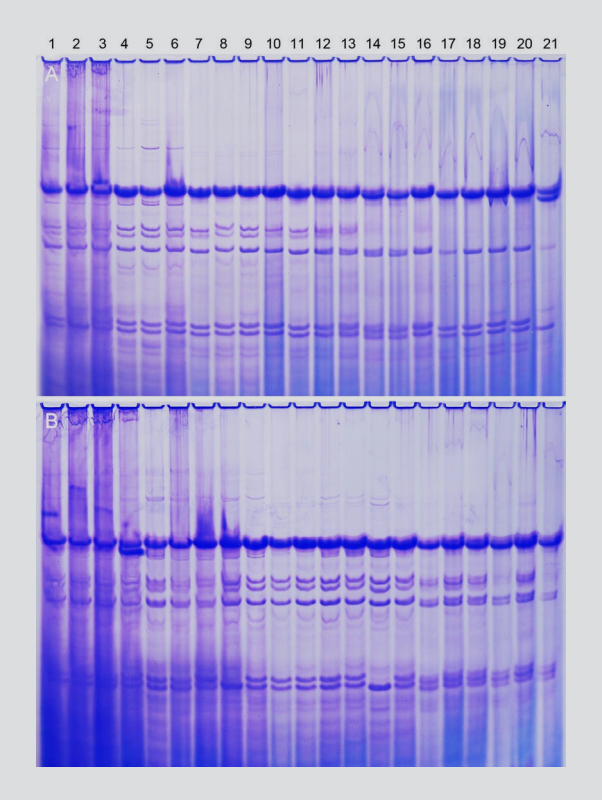

Figure 3. Effect of sodium dodecyl sulfate (SDS) content in protein extracting solution on acetic acid urea-polyacrylamide gel electrophoresis of sunflower seed (SC89) proteins. (A) Lanes 1-3: 0% SDS; lanes 4-6: 0.1% SDS; lanes 7-9: 0.2% SDS; lanes 10-12: 0.3% SDS; lanes 13-15: 0.4% SDS; lanes 16-18: 0.5% SDS; lanes 19-21: 0.6% SDS. (B) Lanes 1-3: 0% SDS; lanes 4-6: 0.05% SDS; lanes 7-9: 0.1% SDS; lanes 10-12: 0.15% SDS; lanes 13-15: 0.2% SDS; lanes 16-18: 0.25% SDS; lanes 19-21: 0.3% SDS.

The optimised AU-PAGE identification effect

In optimised AU-PAGE, sunflower seeds were ground after de-husking, and extracted in a 1.5 ml centrifuge tube with protein extracting solution containing 0.15% SDS at 1:6 seed weight per volume of protein extracting solution, for 30 min at 25 °C. The extracts were centrifuged for 4 min at 4,000×*g*, and then 30 µl supernatants were loaded onto gels for electrophoresis at a constant current of 85 mA for 2 h. To validate the effect of optimised AU-PAGE in genetic purity testing, six sunflower hybrids and their parents (some of them have the same parents) were tested. The results, shown in Figure 4A, indicated that the electrophoresis was effective in resolving the proteins of the sunflower hybrids and parental seeds. The background of the lanes was clear and protein bands were easy to read and analyse. The nine parental lines and six hybrids could be readily distinguished from each other. In Figure 4B, the results from the six sunflower hybrids are shown after optimised AU-PAGE analysis of the seed proteins with three replicates; the six sunflower hybrids could be easily identified from their band patterns. Some lanes showed bands other than those from the hybrids under test, indicating that contamination had occurred in the seed samples.

Protein fingerprint database

For genetic purity testing of sunflower seeds, we compiled a protein fingerprint database based on the electrophoresis results. Gels were standardised by a novel method. The position of the bottom of the sample well was defined as 'zero' and a very strong band present in each sample in the middle of the gel lanes was defined as '50' (Supplementary Figure S1). The whole gel was then subdivided into equal distances with positions 0 and 50 as defined. The positions of all protein bands were read using this scale to create a protein fingerprint database of the hybrids and their parents (Supplementary Table S1).

The database (Supplementary Table S1) may be described as follows: position 50 is marked with grey dotted shading. A red frame indicates female parent data, a green frame hybrid data, and a blue frame male parent data. Bands with yellow

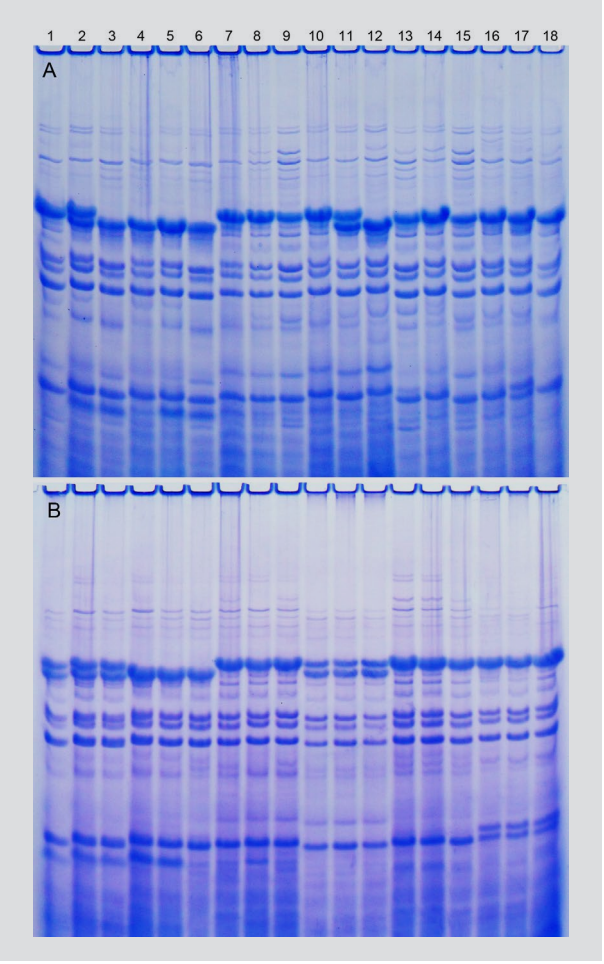

Figure 4. Genetic purity testing of sunflower parental lines and hybrids by improved acetic acid urea-polyacrylamide gel electrophoresis. (A) Lane 1: line 14337, female parent of hybrid S31; lane 2: hybrid S31; lane 3: line 34019, male parent of hybrid S31; lane 4: line 14366, female parent of hybrid S18; lane 5: hybrid S18; lane 6: line 34019, male parent of hybrid S18; lane 7: line 13630, female parent of hybrid MGS; lane 8: hybrid MGS; lane 9: line 32450, male parent of hybrid MGS; lane 10: line 13630, female parent of hybrid S510; lane 11: hybrid S510; lane 12: line 34354, male parent of hybrid S510; lane 13: line 14271, female parent of hybrid S65; lane 14: hybrid S65; lane 15: line 32450, male parent of hybrid S65; lane 16: line AC855, female parent of hybrid SC89; lane 17: hybrid SC89; lane 18: line 65101, male parent of hybrid SC89. (B) Lanes 1-3: hybrid S31; lanes 4-6: hybrid S18; lanes 7-9: hybrid MGS; lanes 10-12: hybrid S510; lanes 13-15: hybrid S65; and lanes 16-18: hybrid SC89.

background were complementary bands, bands with a red background were shared between hybrid and female parent, and bands with a blue background were shared between hybrid and male parent. Bands with a grey background had the lowest protein content among the three bands from the hybrid, male and female parents.

The yellow complementary bands and male-specific blue bands were the major bands useful to determine the genetic purity of hybrid seeds. For example, positions 70 and 72 in the fingerprint of hybrid SC89 were complementary bands; position 35 in hybrid S31 was a shared band with its female parent; position 76 of hybrid S31 was a band shared with its male parent; position 37 of female parental line 14337 of hybrid S31 had lower protein content relative to its hybrid S31 and male parent 34019, and this band could be used to distinguish it from hybrid S31 and its male parent 34019.

Testing genetic purity of sunflower seeds with optimised AU-PAGE

Two hundred seeds of hybrid HySun39 were tested for genetic purity using the optimised AU-PAGE method and specimen results are shown in Figure 5. High resolution of seed proteins was obtained with clear gels and readily readable bands for the hybrid. Among the 200 seeds tested, 195 seeds were identified as hybrid HySun39 and five seeds were identified as off-type seeds. Therefore, the genetic purity of the sunflower seed lot was 97.5%.

4. Discussion

In sunflower seed proteins, water-soluble proteins are the most abundant, followed by salt-soluble proteins, while there are fewer other proteins. Electrophoresis of sunflower seeds indicates fewer bands and lower polymorphism than for maize. When analysed with the AU-PAGE method developed for maize seeds, the gels of sunflower seed proteins had a very strong background staining and bands were hard to read. This heavy background might be due to the higher protein and oil content of sunflower seeds compared with maize. Defatting the crushed seed powder before extraction of proteins may reduce the background, but the defatting process is time consuming and increases

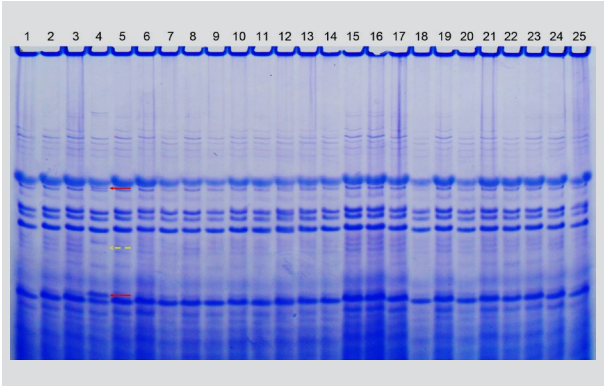

Figure 5. Genetic purity testing of hybrid HySun39 by improved acetic acid urea-polyacrylamide gel electrophoresis. This is a representative gel showing results for 25 seeds. The red (solid) and yellow (dotted) arrows indicate additional and absent bands from off-type seeds, respectively.

the testing procedure. A sunflower seed lot needs to detect at least 200 seeds in genetic purity testing. Increasing the defatting process will extend the time when several sunflower seed lots need to be tested. Thus, the defatting process is not suitable for a rapid method for testing the genetic purity of sunflower seeds.

By increasing the volume of protein extracting solution, the background staining was reduced but was still heavy in around one-third of each gel lane. The observed reduction in background staining may simply be due to reduced protein content in the extract as a result of using an increased volume of protein extracting solution. However, there is a higher content of high molecular weight than medium molecular weight proteins in sunflower seeds. Our results showed that the background staining could be dramatically reduced by adding SDS to the protein extracting solution. The reason for this remains unclear and needs further research.

In the protein profile, separated individual protein bands were scored by establishing a novel method. We did not use the molecular weight and Rm (Relative mobility values) to score protein bands because these two methods were time consuming. The novel scoring system can be used to rapidly analyse of protein bands and seems more intuitive.

5. Conclusions

In conclusion, AU-PAGE was improved and optimised for the genetic purity testing of sunflower seeds. The optimised AU-PAGE used SDS in the protein extracting solution to reduce background staining to produce more clearly readable bands and increase evidence of polymorphism. The improvements enabled the establishment of a validated and efficient test technology for genetic purity of sunflower seeds, and could potentially be applied in other plants and other protein electrophoresis.

Acknowledgements

This work was supported by Beijing Doneed Seeds Co., Ltd., and we thank them for providing the sunflower seeds.

Supplementary material

Supplementary material can be found online at http://dx.doi.org/10.3920/QAS2015.0593.

Figure S1. Standardised gel.

Table S1. Protein fingerprint database of sunflower hybrids and their parents.

References

Ansari, I.T., Sahito, M.A., Ghanghro, A.B., Memon, A.N. and Khan, S., 2011. Electrophoretic analysis of seed proteins from different varieties of rice cultivated in Sindh. Pakistan Journal of Nutrition 10: 663-666.

Kumar, M.B.A., Dadlani, M., Kumar, R. and Jacob, S.R., 2014. Identification and validation of informative SSR markers suitable for ensuring the genetic purity of brinjal *Solanum melongena* L. hybrid seeds. Scientia Horticulturae 171: 95-100.

Litoriya, N., Kaur, D., Patel, N.J. and Talati, J.G., 2010. Varietal identification of chilli *Capsicum annuum* L. by electrophoretic technique. Indian Journal of Agricultural Biochemistry 23: 36-40.

Murtaza, N., Kitaoka, M. and Ali, G.M., 2005. Genetic differentiation of cotton cultivars by polyacrylamide gel electrophoresis. Journal of Central European Agriculture 6: 69-76.

Naresh, V., Yamini, K.N., Rajendrakumar, P. and Dinesh Kumar, V., 2009. EST-SSR marker-based assay for the genetic purity assessment of safflower hybrids. Euphytica 170: 347-353.

Sundaram, R.M., Naveenkumar, B., Biradar, S.K., Balachandran, S.M., Mishra, B., Ilyas Ahmed, M., Viraktamath, B.C., Ramesha, M.S. and Sarma, N.P., 2008. Identification of informative SSR markers capable of distinguishing hybrid rice parental lines and their utilization in seed purity assessment. Euphytica 163: 215-224.

Tang, S., Yu, J.K., Slabaugh, M., Shintani, D. and Knapp, S., 2002. Simple sequence repeat map of the sunflower genome. Theoretical and Applied Genetics 105: 1124-1136.

Tu, H., Zhang, J.Q. and Wang, X.F., 2012. Variation in seed protein within and among species of *Cucurbita* by ultrathin-layer isoelectric focusing. Seed Science and Technology 40: 113-117.

Vishwanath, K., Prasanna, K., Pallvi, H.M., Prasad, S. and Ramegowda, D.P., 2011. Identification of tomato *Lycopersicon esculentum* varieties through total soluble seed proteins. Research Journal of Agricultural Sciences 2: 8-12.

Zada, M., Shinwari, Z.K., Zakir, N. and Rabbani, M.A., 2013. Study of total seed storage proteins in Ethiopian mustard *Brassica carinata* A. Braun germplasm. Pakistan Journal of Botany 45: 443-448.

Zhang, C., Jin, X., Zheng, C. and Deng, Y., 1995. NAU-PAGE technology and purity test of maize seed. Scientia Agricultura Sinica 28: 20-24.

Zhang, C., Yin, Y., Gao, R. and Jia, J., 1998. Polymorphism of seed protein and identification of cotton cultivar. Scientia Agricultura Sinica 31: 16-19.

Zhao, T., Yan, M., Lu, Y.P., Yang, F., Huang, J. and Wang, X.F., 2005. Genetic purity testing of two-line hybrid rice seeds by ultrathin-layer isoelectric focusing of proteins. Seed Science and Technology 33: 45-52.