RESEARCH

CrossMark

Identification of discrepancies in grain quality and grain protein composition through avenin proteins of oat after an effort to increase protein content

Bindu A. Sunilkumar* and Eden Tareke

Abstract

Background: Oat prolamin (avenin) is a family of proteins that contain several polymorphic components. The high interspecific variability of avenin among cultivars, electrophoretic patterns have been used for grain protein comparison and species relationships. Therefore, the present study was carried out to evaluate the effect of environment on avenin constitution following an effort to increase protein content of oat using different approaches.

Results: The avenin electrophoretic patterns of 25 samples resulting from 20 oat cultivars and 5 mutated oat lines grown or developed in different fields and greenhouses were compared. Quantification of total proteins and SDS-PAGE analysis of avenin protein extracts were carried out. Different pattern of avenin allele in electrophoretic gel allowed the comparison within and between groups. This analysis shows that the cultivars obtained from Swedish fields had differences in the number of alleles compared with the parental cultivar, so were not stable across sites. The study revealed that oats treated with nitrogen and the selected mutated lines showed increase in protein concentration, with consistent avenin loci to the parental line, so these lines are now being used as potential candidates for developing high-protein oat lines in the future.

Conclusions: Our results provide useful information, on the stability of modifications, for breeders aiming to increasing the content and nutritional value of oat protein. The study will increase our knowledge in the stability of different modifications and thus to accordingly select oat lines with stable and improved nutritional values.

Keywords: Avenin, Mutation, Nitrogen, Geographical location, Electrophoresis

Background

Oat (*Avena sativa*) kernels contain proteins with good nutritional quality, high bioavailability, and high proportion of globular proteins and have been the target of an increasingly intense breeding campaign [1]. Oat lines with increased protein content would therefore be a very good complement to existing animal and plant protein sources. Soil fertility, genotype, geographical origin, and climatic and environmental factors such as altitude, humidity, moisture, temperature, photoperiod, and incident radiations strongly influence oat protein concentration [1–5]. Protein percent can vary by as much as 3–4 % within the cultivar when this is grown in different geographical regions [5]. Application of nitrogen fertilizers to soil increases protein concentration by up to 2 % [6–9]. Interaction of genetic and non-genetic factors with environmental factors, differences in breeding methods, and additive natural genes due to the double dose of maternal genes of seed endosperm can change the protein concentration in cultivars grown under the same agronomic conditions. This, in turn, can affect nutritional quality [10–13].

High protein content in cereals is a desirable character and goal of many cultivars. Various methods are

*Correspondence: bindu.sunilkumar@food-health-science.lu.se
Food for Health Science Centre, Lund University, P.O. Box 124,
221 00 Lund, Sweden

employed to achieve this, including conventional and mutational breeding, genetic engineering, marker-assisted selection, and genomic analysis. When developing cultivars with improved nutritional value, the improvements must be preserved and the cultivar must be as stable as possible [14]. It is therefore important to ensure that the protein quality is maintained and improvement attained in different environments and under different conditions.

Avenin is the collective name for alcohol-soluble prolamin proteins of oat that are highly heterogeneous in nature because of several polymorphic components [12, 15, 16]. Due to its polymorphic nature and environmental stability, compared with other cereal grain proteins, avenin is a potential candidate for inheritance studies, for identification of landraces, and for identification of genotype and germplasm [3, 17–20]. These identifications are very important for efficient handling of the breeding stocks for maintaining varietal purity and discriminatory markers for protected varieties [21]. In addition to their polymorphic nature, avenin proteins have charge and size heterogeneity that is absent in two other storage protein fractions, globulin and albumin [22]. Consequently, avenin fractions are more suitable for cultivar identification in cereal grains than globulin and albumin fractions [22, 23]. Separation of avenin using SDS-PAGE is recommended by the International Seed Testing Association (ISTA) for verifying species and cultivars [24].

To the best of our knowledge, no study has investigated the stability of avenin in a single oat cultivar exposed to chemicals or grown under different geographical conditions. Thus, the purpose of this study was to evaluate the effect of nitrogen fertilizer, mutation, greenhouse, and geographical location on the stability of avenin protein fractions of Belinda oat cultivars (BOCs) so that the cultivar with stably inherited avenin could be selected to assure the original gene pool, resulting in increased protein content and in improved cultivars.

Methods

Grain material

Grains of 20 oat cultivars and 5 mutated lines were evaluated in this study and were divided into five groups (Table 1). The parental Belinda cultivar was obtained from the SW collection (Svalöf Weibull AB, Landskrona, Sweden). The high-protein oat lines were selected after screening 1050 different mutated lines from a tilling population constructed from the spring oat cultivar SW Belinda (Sunil Kumar et al. in preparation). Control plants and plants treated with nitrogen at different concentration [60–240 N (kg/ha)] were grown in a greenhouse under halogen lamps, giving a photon flux density of 240 μmol/m^2/s with a photoperiod of 18 h. Day and night temperatures were 25 and 16 °C, respectively. Plants were grown in 5-L pots in standard soil for oat growth. The treatments were arranged in completely randomized pots with three replications. The amount of soil in each pot was quantified to optimize fertilizer amount to mimic or reflect the actual field conditions. Split application of nitrogen fertilizer was applied for two members in group V in which the first fertilization was given at sowing and the second was at the tillering stage. Fertilizer was sprayed in the growing crop. Seeds were harvested for one season and analyzed.

Chemicals

PageRuler™ Unstained Protein Ladder (Thermo Scientific), sodium dodecyl sulfate (SDS), and acetone were purchased from Sigma Aldrich. Nacl (1 mol/L), 2-chloroethanol, tris(hydroxymethyl)aminomethane, and glycine were obtained from Merck. All chemicals were of analytical grade.

Stock solutions

Five stock solutions were made, using 25 % 2-chloroethanol and Milli-Q water. All stock solutions were prepared at room temperature.

Table 1 Sampling location, year of sampling, mutated oat lines, and fertilizer application rates of Belinda oat cultivars

Swedish field (in different years)—group I	Green house (in different years)—group II	Provinces from China—group III	Mutated oat lines—group IV	Fertilizer used N (kg/ha)—group V
Oat cultivars of Belinda collected from				
2009	2011	Baicheng	M#1	60
2010	2012	Inner Mongolia	M#2	100
2011	2013	Qinghai	M#3	120
2012	2014	Hebei	M#4	120 + 60[a]
2013	2015	Shanxi	M#5	120 + 120[a]

M: mutated, followed by number indicates mutant line

[a] Split application of nitrogen—dividing total nitrogen application into two or more treatments

Milling and defatting of oat flour

Grain samples were ground using a benchtop ball miller (Minimill Pulversett 23, Fritsch, Germany) at 50 Hz for 5 min one day before analysis and kept in a desiccator to equalize the water content in all samples. The milled oat was defatted with acetone by dispersing 10 wt% of oat flour in acetone, and the mixture was stirred using a magnetic stirrer for 1 h at room temperature. The mixture was centrifuged at 14,000 rpm for 20 min at 20° C using a Beckman Optima LE-80K Ultracentrifuge (Palo Alto, California). The precipitate was collected and kept open overnight at room temperature to remove the remaining acetone present in the precipitate.

Analysis of total protein content

Total and soluble protein contents of the ground oat seeds were determined by measuring nitrogen content with elemental Flash EA 1112 N (Thermo Fisher Scientific, Delft, Netherlands) and using a modified Dumas method [25]. Total protein percentage was calculated by measuring the total nitrogen content in seed flour using a thermal conductivity detector. Aspartic acid was used as the standard. A nitrogen-to-protein conversion factor of 6.25 was used in the calculations. A total protein content of 11.7 % for non-mutated parental oat cultivar Belinda was used as control.

Extraction of avenins

Albumins and globulins were extracted with 200 μL of 1 mol/L Nacl in 0.1 mol/L sodium phosphate buffer, pH 7 and temperature 4 °C. The crude extract was centrifuged (15 min, 6000 rpm). After centrifugation, 120 μL of supernatant was pipetted into another microcentrifuge tube and lyophilized for 6 h. After separation of albumins and globulins, the lyophilized material was extracted with 25 % 2-chloroethanol (200 μL per grain) for 4 h at the room temperature. The samples were then centrifuged (15 min, 6000 rpm). After centrifugation, 120 μL of supernatant was pipetted into a fresh microcentrifuge tube and lyophilized for 6 h. This has been shown to be the optimum concentration of 2-chloroethanol for extraction of avenin. After centrifugation, the clear supernatant was diluted with two volumes of pre-cooled 1.5 % NaCl solution and the precipitate separated by centrifugation at 6000 rpm for 30 min. The sample was then dried under vacuum in a desiccator.

Preparation of samples for SDS-PAGE electrophoresis

The avenin protein fractions in 25 samples under study were separated by SDS-PAGE under reducing conditions using 8–25 % gradient Phast® gels (GE HealthCare) and a GE PhastGel system (GE Healthcare, Uppsala, Sweden). The SDS-PAGE conditions were 250 V, 10.0 mA, 3.0 W, 15 °C, and 65 Vh. The gels were developed at room temperature using PlusOne Silver Staining Kit (GE Healthcare, Uppsala, Sweden) and according to the provided instructions. After staining, gels were routinely air-dried at room temperature between two sheets of wetted, untreated cellophane and clamped in an acrylic plastic frame.

Statistics

GraphPad Prism Program (GraphPad Softwares, SanDiego, CA) was applied for the analysis of unpaired t test. Mean and standard error values were calculated using Excel software [26, 27].

Results

Protein content in the wild BOCs ranged from 11.6 ± 0.01 to 12.9 ± 0.07 and was similar when the BOCs were grown in the field (in Sweden 2009–2013) or in green house (2011–2015) (Table 2). The same oat cultivar grown in China exhibited significantly higher protein levels, 12.01 ± 0.1 to 14.1 ± 0.05, compared to those grown in Sweden, thereby showing the effect of geographical location on protein content. The variation in protein content in the BOCs grown in greenhouses, where growing parameters are controlled, was less than field-grown BOCs. Mutated BOLs, or those cultivars

Table 2 Total protein percentage of all Belinda oat lines (BOLs) analyzed using particle elemental analyzer

Groups	From field	From green house	From China	From mutated oat lines	From nitrogen-treated oat lines
Total protein percentage of Belinda oat cultivars obtained					
Belinda (control)	11.7 ± 0.05	11.7 ± 0.05	11.7 ± 0.05	11.7 ± 0.05	11.7 ± 0.05
I	12.9 ± 0.07^{b}	12 ± 0.03	12.19 ± 0.2^{c}	20.2 ± 0.7^{a}	17.8 ± 0.8^{a}
II	13.4 ± 0.3^{c}	12.7 ± 0.01^{c}	13.7 ± 0.3^{b}	17.4 ± 1.8^{a}	24 ± 0.01^{a}
III	11.6 ± 0.01	12 ± 0.02	14.1 ± 0.1^{b}	18.1 ± 0.4^{a}	17.7 ± 0.07^{a}
IV	12.1 ± 0.11^{c}	12.2 ± 0.07^{c}	14.1 ± 0.05^{b}	17.3 ± 0.03^{a}	12.8 ± 0.03^{b}
V	11.9 ± 0.03	11.9 ± 0.07	12.01 ± 0.1	19.7 ± 0.03^{a}	15.1 ± 0.03^{a}

Results expressed as mean value ± standard deviation. Mean values followed by different letters on the same line are significantly different. c = p < 0.05, b = p < 0.005, a = p < 0.0001

treated with N_2, had significantly higher protein levels, clearly showing the effect of treatment on protein levels (Sunilkumar B et al., manuscript under preparation). In the mutated lines, the protein content ranged from 17.3 to 20.2 %. Similarly, BOLs treated with N_2 fertilizers showed higher protein levels and highest variation in protein content, ranging from 11.7 to 24 %.

A total of 5–8 protein bands were resolved among the five groups numbered I, II, III, IV, and V (Table 2) in the SDS-PAGE gels stained with silver staining procedure, as shown in Fig. 1a. Only one group of cultivars, obtained from the Swedish field, possessed all eight bands, which is shown in the diagrammatic representation of respective alleles based on SDS-PAGE gel, shown in Fig. 1b. Cultivars from the greenhouse, mutated as well as cultivars grown under excess N_2, had four bands. The cultivar obtained from China had five bands. As shown in Fig. 1b, four distinct loci were present in all groups and four additional loci (one dense and three mild bands) in group I, which represent the cultivars grown in Swedish fields. Molecular weight of all major four polypeptides ranged from 15 to 33 kDa (numbered D to I in Fig. 1b). The molecular weight in the three additional bands in the cultivar from the Swedish fields ranged from 40 to 66 kDa (Fig. 1a, group I). Three major bands and one minor band consistently stained heavily (mild line region), appearing in all oat lines examined. The cultivars grown in China showed an additional minor band with a molecular weight of 25 kDa, shown by an arrow in the gel picture (Fig. 1b, lane 3).

Discussion

Although the cultivars and mutated lines originated from a Swedish spring oat Belinda cultivar, it is not surprising to find considerable similarities in electrophoretic pattern as well as total protein percentage. However, total protein determination showed that the cultivars obtained from Sweden in different years (group I), greenhouse (group II) and from different provinces of China (group III) showed change in protein percentage that was either higher or lower than the 12 % seen in the original SW Belinda. However, the SDS-PAGE electrophoresis of avenin protein fraction showed eight allelic loci for cultivar grown in Sweden compared with five loci for groups II and III, so the Swedish cultivars were markedly different from the other groups. A small increase in protein percent was observed in the Chinese cultivars, but the variation between lines was not great (12–14 %) compared with cultivars from the greenhouse. Both have the same avenin loci, despite a minor band being found in one of the Chinese lines obtained from the province of Shanghai. According to the previous reports, some of the avenin alleles are associated with geographical origin of the cultivars, and SDS-PAGE showed alleles with

alternative protein bands with different mobility [3]; such minor bands may occur in cultivars from Sweden as well as from China.

A significant increase in protein percentage was found in cultivars grown under different treatments. However, both showed nearly identical nature of electrophoretic patterns for avenin loci, indicating no such differences in avenin protein fraction obtained after mutation and fertilizer application. Evidence from the previous experiments showed that fertilization with high amounts of N_2 increased globulin relative to other fractions of oat protein [28]. Consequently, an increase in protein content here may be due to the capacity for maximum absorption of nitrogen in the container-grown plants in the greenhouse due to less percolation compared with soil in the field, which allows maximum permeation or percolation [29]. This could probably increase the globulin fractions that eventually gave a high protein percentage. Similarly, in mutated BOLs, the possibility of alteration in protein encoding genes that modulate certain biosynthetic pathways could overproduce protein [30]. These results indicated that the effect of mutation and nitrogen fertilizers on protein content was more pronounced than the effect from geographical factors. However, another discriminatory method must be developed to resolve avenin gene families for variety identification in oats.

Production of genetically pure seed is an important criterion for any seed certification program. Identification of oat cultivars using electrophoresis offers many advantages over some of the current field testing procedures, which are purely based on morphological characteristics such as color and size of the plant, and the presence or absence of awns [31]. Even so, ISTA 1999 and other authors [22, 32, 33] reported that SDS-PAGE patterns can be useful to differentiate oat species, only requiring simple laboratory equipment and minimum laboratory supplies. Development of such tests can prove beneficial for developing and protecting high-quality cereal grains, especially for self-fertilizing species [34]. Developing an appropriate oat cultivar and its further acceptability in the market depends solely on the yield, maturity, disease resistance, and nutritional value for food, feed, and functionality [35]. Our results from this study will help us develop new high-protein oat lines that are stable and environmentally sound, with reasonable grain quality, by using those genotypes showing consistency in protein content with distinct banding pattern through a crossing program.

Conclusions

Five different groups studied here showed a distinct banding pattern for avenin. The difference in cultivars obtained from the field related to other four groups
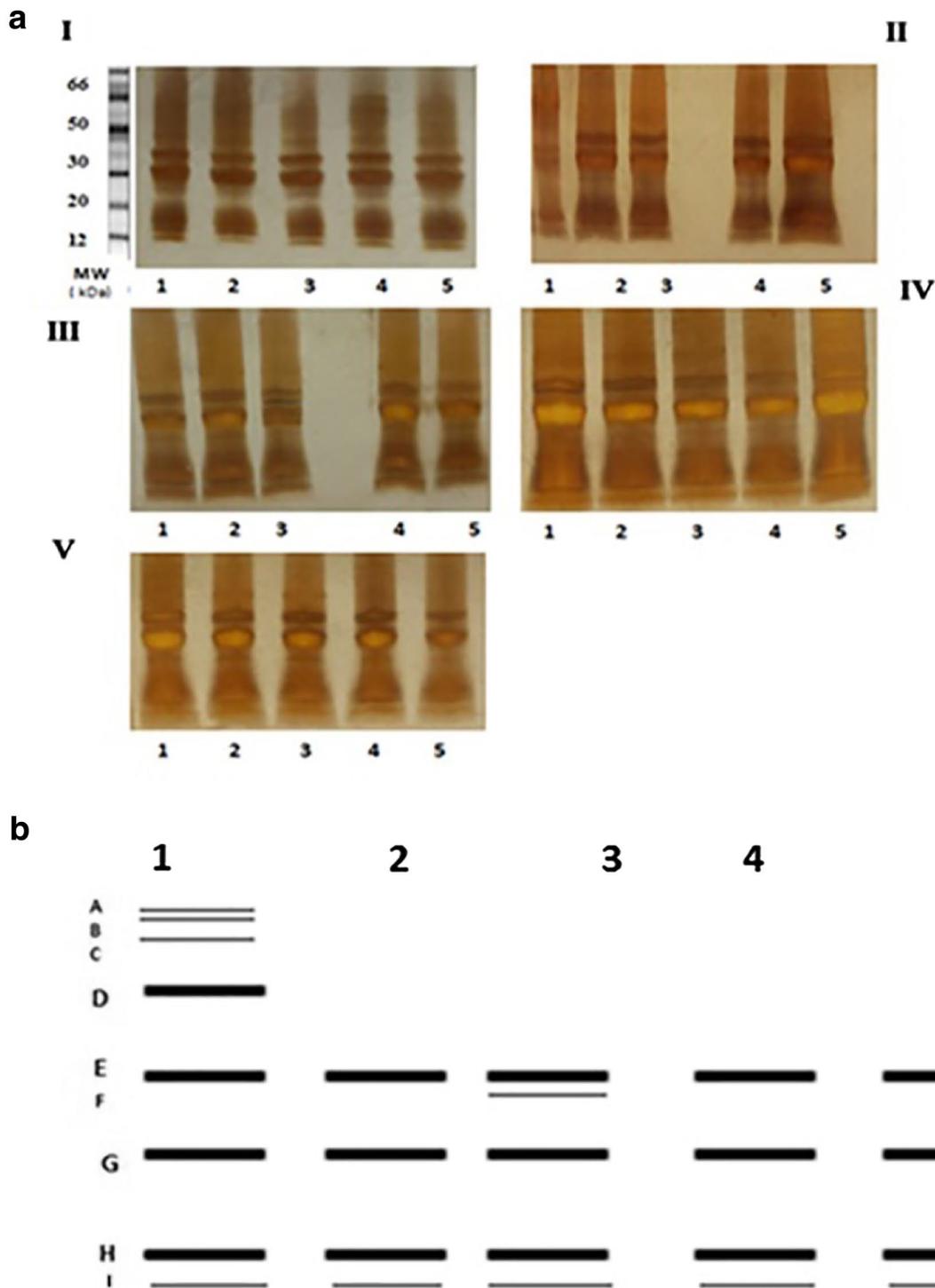
Fig. 1 **a** Avenin polyacrylamide gel electrophoresis pattern of (I). Belinda oat cultivars from Swedish field from the year 2009–2013 (II). Five different Belinda cultivars grown in green house (III). Five different Belinda cultivars grown in different location from China (IV). Mutated Belinda oat lines (V). Five Belinda plants treated with nitrogen fertilizers. An *arrow* showed in group III third lines is the nonallelic loci. **b** Diagrammatic representations of SDS-PAGE banding patterns of the respective allelic blocks which indicated (by *arrows*) in the figure of SDS-PAGE gel image. The *lines* numbered *1*, *2*, *3*, *4*, and *5* represent groups I, II, III, IV, and V, respectively, shown in (**a**). Each protein bands were numbered from A to I. Intensely stained bands are numbered using alphabet with high font size and mildly stained bands with alphabet having small font size

showed the clear indication of adaptive strategy and appeared to be evidence of an ecological pattern of variation in avenin. This needs to be considered when different breeding strategies are applied to produce cultivars adapted to one or more locations for food and feed.

Abbreviations
SDS-PAGE: sodium dodecylsulfate polyacrylamide gel electrophoresis; ISTA: International Seed Testing Association; BOCs: Belinda oat cultivars; BOLs: Belinda oat lines; SW: Svalöf Weibull AB; N (kg/ha): kilograms of nitrogen/hectare; EA: elemental analyzer.

Authors' contributions
BS and ET conceived the study. BS collected and analysed the samples. BS and ET together interpreted the data, which is going to be a part of BS's PhD studies in Food Chemistry at Lund University, Sweden. Both authors read and approved the final manuscript.

Authors' information
BS is a doctoral candidate in Food for Health Science Centre, Lund University, Sweden. ET is permanent senior researcher in the same department. ET received her PhD degree in Toxicology and Nutrition, Stockholm University, Sweden, and conducted her postdoctoral studies in nutrition science. ET is expert in food analytical chemistry.

Competing interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References
1. Peterson DM. Protein concentration, concentration of protein fractions, and amino acid balance in oats. Crop Sci. 1976;16(5):663–6.
2. Rayapati PJ, Gregory JW, Lee M, Wise RP. A linkage map of diploid Avena based on RFLP loci and a locus conferring resistance to nine isolates of *Puccinia coronata* var. 'avenae'. Theoret Appl Genet. 1994;89(7–8):831–7.
3. Souza E, Sorrells ME. Prediction of progeny variation in oat from parental genetic relationships. Theor Appl Genet. 1991;82(2):233–41.
4. Forsberg RA, Reeves DL. Breeding oat cultivars for improved grain quality, vol. 33. Madison: American Society of Agronomy: Crop Science Society of America; 1992.
5. Peterson DM, Wesenberg DM, Burrup DE, Erickson CA. Relationships among agronomic traits and grain composition in oat genotypes grown in different environments. Crop Sci. 2005;45(4):1249–55.
6. Portch S, MacKenzie AF, Steppler HA. Effect of fertilizers, soil drainage class and year upon protein yield and content of oats. Agron J. 1968;60:672–4.
7. Ohm H. Response of 21 oat cultivars to nitrogen fertilization. Agron J. 1976;68(5):773–5.
8. Welch RW, Yong YY, Hayward MV. The distribution of protein and non-structural carbohydrate in five oat varieties during plant growth and grain development. J Exp Bot. 1980;31(4):1131–7.
9. Eggum BO. Current methods of nutritional protein evaluation. Improving plant protein by nuclear techniques. Vienna: Int. Atomic Energy Agency; 1969. p. 289–302.
10. Shewry P. Avenins: the prolamins of oats. In: Shewry P, Casey R, editors. Seed proteins. Dordrecht: Springer; 1999. p. 79–92.
11. Portyanko VA, Sharopova NR, Sozinov AA. Characterisation of European oat germ plasm: allelic variation at complex avenin loci detected by acid polyacrylamide gel electrophoresis. Euphytica. 1998;102(1):15–27.
12. Kim SI, Mossé J. Electrophoretic patterns of oat prolamines and species relationships in Avena. Can J Genet Cytol. 1979;21(3):309–18.
13. Polisenska I, Nedomova L, Vyhnanek T. Characterisation of oat genetic resources using electrophoresis of avenins. Czech J Genet Plant Breed. 2011;47(4):166–71.
14. Wenefrida I, Utomo HS, Linscombe SD. Mutational breeding and genetic engineering in the development of high grain protein content. J Agric Food Chem. 2013;61(48):11702–10.
15. Peterson DM, Smith D. Changes in nitrogen and carbohydrate fractions in developing oat groats. Crop Sci. 1976;16(1):67–71.
16. Kim SI, Charbonnier L, Mosse J. Heterogeneity of avenin, the oat prolamin. Fractionation, molecular weight and amino acid composition. Biochim Biophys Acta. 1978;537(1):22–30.
17. Li Gregová E, Žák I, Kraic J. Electrophoretic distinguishing of cultivated oats (*Avena sativa* L.) by seed storage proteins. Rostl Výr. 1996;42:169–72.
18. Murphy JP, Phillips TD. Isozyme variation in cultivated oat and its progenitor species *Avena sterilis* L. Crop Sci. 1993;33(6):1366–72.
19. Dvořáček V, Čurn V, Moudrý J. Suitability of oat-seed storage protein markers in grain and mixed flour samples. Plant Soil Environ. 2003;49(11):486–91.
20. Jussila M, Sontag-Strohm T, Ulvinen O. The identification of Finnish oat cultivars (*Avena sativa* L.) by the use of SDS-PAGE of the avenins in homogeneous and gradient. Acta Agriculturae Scandinavica, Section B — Soil & Plant Science. 1992;42(2):106–10.
21. Peterson DM. Subunit structure and composition of oat seed globulin. Plant Physiol. 1978;62(4):506–9.
22. Robert LS, Nozzolillo C, Altosaar I. Molecular weight and charge heterogeneity of prolamins (avenins) from nine oat (*Avena sativa* L.) cultivars of different protein content and from developing seeds. Cereal Chem. 1983;60(6):438–42.
23. Králová M. Vybrané metody chemické analýzy půd a rostlin. Praha: Academia; 1991.
24. International Seed Testing Association. Verification of species and cultivar. Chapter VIII. In: International rules for seed testing, vol. 41–43. ISTA, Bassersdorf; 1999. p. 252–54.
25. Cunniff P. Association of official analytical C: official methods of analysis of AOAC international. Washington, DC: Association of Official Analytical Chemists; 1995.
26. Excel Î. Microsoft Excel 2007. 2013.
27. Motulsky HJ. Analysing data with GraphPad prism. SanDiego, CA: Graph-Pad Software; 1999.
28. Peterson DM, Smith D. Changes in nitrogen and carbohydrate fractions in developing oat groats. Crop Sci. 1976;16(1):67–71.
29. Han J, Shi J, Zeng L, Xu J, Wu L. Effects of nitrogen fertilization on the acidity and salinity of greenhouse soils. Environ Sci Pollut Res Int. 2015;22(4):2976–86.
30. Galili G. New insights into the regulation and functional significance of lysine metabolism in plants. Annu Rev Plant Biol. 2002;53:27–43.
31. Chaugule AA, Mail SN. Evaluation of shape and color features for classification of four paddy varieties. Int J Image Graph Signal Process. 2014;6(12):32–8.
32. Burgess SR, Shewry PR, Matlashewski GJ, Altosaar I, Miflin BJ. Characteristics of oat (*Avena sativa* L.) seed globulins. J Exp Bot. 1983;34(147):1320–32.
33. Lauriere M, Mosse J. Polyacrylamide gel-urea electrophoresis of cereal prolamins at acidic pH. Anal Biochem. 1982;122(1):20–5.
34. Shewry PR. Improving the protein content and composition of cereal grain. J Cereal Sci. 2007;46(3):239–50.
35. Girardet N, Webster F, Wood P. Oat milling: specifications, storage, and processing. Oats Chem Technol. 2011;Ed. 2:301–19.