The evaluation of morphological and molecular techniques for discrimination among and verification of lucerne (*Medicago sativa*) cultivars

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ABSTRACT

The stability and usefulness of plant morphological characters for discrimination among lucerne (*Medicago sativa* L.) cultivars was investigated under three sets of field and glasshouse conditions, using two New Zealand cultivars and four Iranian ecotypic cultivars representing diverse geographical adaptations. Following the guidelines of the International Union for the Protection of New Varieties of Plants (UPOV) and the requirements of the Organization for Economic Co-operation and Development (OECD) seed certification scheme for testing distinctness, homogeneity and stability of lucerne cultivars in field test plots, morphological data were recorded from four replicates of 18 spaced plants per cultivar in the field in the establishment year (1993) and the following year. Flower colour was recorded from a further 150 one-and two-year old plants of each cultivar in the field. In a glasshouse, where the minimum and maximum temperatures were set at 16°C and 22°C respectively, morphological characters were recorded from three replicates of 22 one-year old plants of each cultivar. The majority of the 12 morphological characters recommended by UPOV and OECD for discrimination and verification of lucerne cultivars were not independent of the environment. Only three characters, number of plant stems immediately above the ground, plant recovery height, and leaflet width/length ratio were stable, and thus could be used as reliable morphological descriptors for lucerne cultivars. However, none of the individual characters, or any combination of these characters, were sufficient to differentiate all of the cultivars at $P<0.01$, the standard required by UPOV to detect differences among lucerne cultivars. Morphologically based methods are therefore not effective for discrimination among lucerne cultivars, and there is a need to find more precise and effective techniques for assessing whether cultivars are actually different (UPOV) or whether individual seed lots of a cultivar do not differ from the cultivar standard (OECD).

A digital image processing algorithm (VIPS) was used for image processing of 150 individual seeds of 17 seed lots of eight lucerne cultivars and also for image analysis of 66 leaflets from individual glasshouse grown plants of six cultivars. Of the 21 morphological characters and derived measurements recorded from individual seeds of

the cultivars, 10 were useful for cultivar verification and discrimination. Among individual characters, red colour/total intensity, and blue/total intensity of seed had the highest, while actual area of seed/convex area had the lowest ability (64 % vs 18%) to discriminate among cultivars. A better cultivar discrimination (86%) was obtained on the basis of a combined value from the 10 selected characters using Canonical Discriminant Analysis. This was further improved (to 94%) using the result from the 10 individual characters plus the combination of the selected seed characters. Using image analysis of leaflets of individual plants, only 67% of the cultivars could be discriminated.

Discontinuous SDS-polyacrylamide electrophoresis was used for protein analysis of both bulked and single seeds of 14 seed lots from six Iranian and two New Zealand cultivars. A series of preliminary experiments was first undertaken to determine the optimal protein loading volume for bulk, and also single seed samples of the cultivars, to enable production of clear, sharp and therefore easily evaluated bands. Of the many protein bands produced as a result of SDS-PAGE analysis of single seeds of the cultivars, the seventeen most distinctive bands were selected for analysis. Their intensities were assessed using a Vision Image Processing System (VIPS), and the data were used for discrimination among the cultivars. Electrophoresis of protein extracted from the bulked seed samples failed to differentiate the majority of the cultivars. However, 82% of the cultivars could be differentiated using the combined results from intensities of the 17 selected protein bands from single seeds of the cultivars. Since all eight cultivars could not be discriminated using SDS-PAGE of individual seeds, two other molecular techniques (Restriction Fragment Length Polymorphism (RFLP) and Random Amplified DNA Polymorphism (RAPD) were employed to determine if better discrimination among lucerne cultivars was possible using DNA techniques.

DNA samples were extracted from 4-5 frozen leaves (at -70° C) from 40 individual 45 day old seedlings of six Iranian, two New Zealand, and two internationally recognised cultivars. Following purification and quantification, bulked DNA samples of the cultivars, plus those from individual seedlings were digested with four common restriction endonucleases to cleave the DNA into smaller fragments. The digested DNA

fragments were separated by agarose gel electrophoresis and were then transferred to a nylon membrane. This membrane was hybridised with nine probes *i.e.* six lucerne cDNA probes (I013, 492, 281, 328, 473, 457), two apple probes (ADH, cDNA and ribosomal DNA (rDNA)) and one clover probe (ADH) to determine the best combination between restriction enzymes and probes to optimize the number of polymorphic bands. A non-radioactive method was used for probe labelling.

Of the six lucerne probes screened with the restriction endonucleases (EcoR *I*, Bam H*I*, Hind *III* and Xba *I*) all except I013 produced extremely faint bands from the bulked DNA samples of the cultivars. Although a combination of I013 and Hind *III* gave the highest number of RFLP fragments in initial experiment, the result was not reproducible.

No distinct RFLPs were detected using the clover ADH probe from the bulked DNA samples of the cultivars, and the apple rDNA probe detected polymorphism between only some of the cultivars. This result suggested that DNA fingerprinting of lucerne cultivars may not be feasible on the basis of the RFLPs from bulk DNA samples of the cultivars. It was therefore hypothesised that better discrimination among the cultivars might be possible on a population basis *i.e.* by examining the percentage of plants within each cultivar containing particular fragments. This hypothesis was tested by analysing scoring data based on the presence (1) and absence (0) of the RFLP fragments from 40 individual seedlings of each cultivar using Canonical Discriminant Analysis. RFLP analysis of individual seedlings of the cultivar using each of the rDNA and the ADH probes produced distinct but highly polymorphic RFLPs among the seedlings which was an indication of great genetic diversity within each of the cultivars. Seventy percent of cultivar pairs could be discriminated using the RFLPs detected by apple rDNA compared with 56% for the clover ADH probe at $P<0.05$. Some pairs of cultivars which could not be discriminated using the rDNA, were discriminated on the basis of RFLPs detected by the ADH probe. When the results from these two probes were combined, 91% of the cultivars could be discriminated.

Using the PCR based RAPD technique, bulk DNA extracts from 10 lucerne cultivars plus DNA from 40 individual 45 day old seedlings of each cultivar were analyzed.

Twenty-six 10-base arbitrary primers were screened using bulk DNA samples of the cultivars to select those which were able to generate clear RAPD bands. Of these, four (*i.e.* OPA08, OPB13 OPO19 and OPC10) produced sharp and distinctive RAPD bands. OPB19 produced the highest number of distinct RAPD fragments and all of the cultivars, even those which were closely related, could be discriminated. Although all of the cultivars could not be discriminated using the RAPD profiles generated by OPA08, OPB13 and OPC10 individually, a combination of the results from these primers provided sufficient information for discrimination among all of the 10 cultivars. The number of distinct RAPD fragments generated by a combination of the primers OPB19 and OPC10 was less than those produced by individual primers alone. To check the reproducibility of the RAPD results, replicated reactions were carried out using two primers (OPB19 and OPC10) alone and in combination with the standard reaction mixture using different batches of enzyme from the same manufacturer. This demonstrated that a high degree of reproducibility of results obtained by the RAPD technique is possible.

To assess genetic variation within and among the cultivars, nine distinct RAPD bands generated by primer OPO8 were scored. The RAPD products from 40 individual seedlings of the each cultivars produced distinct but very diverse profiles, indicating genetic diversity within each individual population. Pairwise comparison of the LSMEANS of the RAPD profiles illustrated that 86% of the cultivars were significantly different.

These molecular techniques and seed image analysis were also assessed for their ability to test uniformity, and detect genetic relationships among the cultivars. For seed protein and DNA analysis, uniformity was assessed using similarity of protein and DNA banding profiles from individual seeds and seedlings from each of the seed lots of the lucerne cultivars. For seed image analysis, uniformity of the cultivars was assessed on the basis of the proportion of uniform seeds in each of the seed lots. Genetic relatedness among the cultivars was detected using the squared Mahalanobis distances (D^2) of the cultivars.

The genetic relatedness established among the cultivars was almost the same irrespective of whether the three molecular or the seed image analysis techniques were used, despite the different nature of each analysis. The results were consistent with the genetic background, autumn-dormancy and geographical adaptation of the cultivars examined. However, these techniques did not produced the same results for testing of uniformity. Analysis of a larger sample size from a number of cultivars with known genetic background would be needed before drawing any final conclusion as to the relative merits of these techniques for estimation of relatedness and uniformity of lucerne cultivars.

Comparisons between protein banding profiles and also seed morphological characters of seed lots from the same cultivar illustrated significant differences between the seed lots of some of the cultivars. This suggested that genetic shift had occurred during seed multiplication. As all of the seed lots studied had been certified under the OECD scheme, the scheme is apparently not capable of detecting this degree of genetic shift.

Of all the techniques investigated, image analysis is regarded as the most suitable alterative to plot testing for discrimination among, and verification of lucerne cultivars, and for detection of genetic shift. The reasons for this are the speed of analysis, lower running costs, lack of need for chemicals and ease of operation of the equipment. However, this will be dependent on access to image analysis facilities. For genetic analysis of lucerne cultivars the RAPD technique was considered the best among the molecular techniques used.

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