

Hybridity Test of Cucumbers *via* Ultrathin Layer Isoelectric Focusing Technique Using Water as the Extraction Buffer

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ABSTRACT

The ultrathin layer isoelectric focusing (UTLIEF) technique using water as the extraction buffer is a cheap, fast and reliable technique for varietal identification in cucumber. However, its application for hybridity testing has not been reported yet. Therefore, this study evaluated the possibility of the application of the UTLIEF technique for testing the hybridity of 10 F1 hybrid seeds and their respective parental lines. It was found that nine out of the ten pairs of the parental lines could be differentiated by the differences in the combination of male and female marker bands, while eight out of the ten F1 hybrid seed were verified using only male marker bands using this UTLIEF technique with water as the protein extract solvent and a gel pH range of 2–11. More male marker bands were found than female marker bands. Most of the polymorphic cucumber seed protein bands were focused in the pH range 6–10. The results indicated that the UTLIEF technique using water as the extraction buffer and a gel pH gradient of 2–11 is of value for hybridity testing in commercial cucumber hybrid seed production.

Keywords: cucumber, seed storage protein, electrophoresis, isoelectric point, water

INTRODUCTION

Cucumber is one of the economically important vegetable crops in the world (Huang *et al.*, 2009). Currently, new F1 hybrid cultivars are being continuously released to address the requirements of consumers which are changeable over short periods of time (George, 1999). Hence, cultivar verification and hybridity tests are inevitable in the quality control of breeding and seed production programs. The conventional

grow-out test takes time, labor intensive and costly. Moreover, it is influenced by uncontrollable environmental factors (Cooke, 1995, McDonald, 1998). The DNA fingerprint technique, which has the most potential for varietal differentiation, is too expensive for large-scale and routine commercial seed testing work, while the cheaper, isozyme analysis has many restrictions including the specificity of plant tissues and analytical environment sensitivity (Cooke, 1995, Dou *et al.*, 2012).

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Recently, the ultrathin-layer isoelectric focusing (UTLIEF) technique using a thin polyacrylamide gel of 0.15 mm was reported as a cheaper, faster and reliable technique for varietal identification and hybridity testing in several crops (Van den Berg, 1990, Yan, 2013). The technique is based on the fact that the seed storage proteins of different varieties are different in molecular weight, size, and electrical charge. Proteins with different electrical charges have different isoelectric point (pI) values and can be separated using isoelectric focusing gel electrophoresis. Using the appropriate protein extract buffer and gel pH gradient, the polymorphic protein band patterns among seed proteins from different varieties (electrophoretograms) can be found during protein separation in the isoelectric focusing gel and used to verify the varieties. The success of the UTLIEF technique for varietal identification was reported for tomato (Wang *et al.*, 2000) and cucumber (Onwimol *et al.*, 2010, Tu *et al.*, 2012). In hybridity testing, marker proteins from male and female parent lines are both present in the hybrid seed and can be used to verify the hybridity of their respective parents. According to the seed testing rules in International Seed Testing Association (2012), the UTLIEF technique is accepted as a hybridity test for maize and sunflower. The success of this technique for hybridity testing has also been reported in other crops, for example rice (Zhao *et al.*, 2005) and pumpkin (Yan, 2013a).

In cucumber hybridity testing, Tu *et al.* (2012) successfully found the male marker bands by using 2-chloroethanol as the extract solvent for seed proteins. This work indicated the polymorphic, cucumber-seed protein extracted was prolamin, an aqueous, alcohol-soluble protein. However, this extraction buffer is costly, toxic to humans, harmful to the environment and most of all, its import is restricted and not commercially available in Thailand (Ministry of Defence of Thailand, 2007). Onwimol *et al.* (2010) reported on polymorphic albumin proteins in F1 hybrid cucumber varieties using water as the seed protein

extract solvent and a gel pH gradient of 2–11. Water is a cheap, safe and environmentally friendly solvent. If water is also able to differentiate male and female marker proteins in cucumber hybridity testing, it would be a promising protein extract solvent for varietal identification and hybridity testing for cucumber *via* the UTLIEF technique. Hence, the objectives of this study were to investigate the possibility and efficiency of the UTLIEF technique using water as the seed protein extract solvent and a pH gradient of 2–11 as reported by Onwimol *et al.* (2010) for testing of hybridity in local cucumber hybrid varieties.

MATERIALS AND METHODS

Seed samples

Ten hybrid cucumber (*Cucumis sativus* L.) varieties and their parental lines (denoted CS01 to CS10), obtained from Thai Seed and Agriculture Co. Ltd., Bangkok, Thailand, were used. The seed production of the parental lines and the hybrids followed the description of Robinson (2000) with hand emasculation and pollination techniques. The experimental seed field was located at 14° 45'N, 100° 04'E. After harvesting from a commercial seed production field, the seeds had been stored at 5 ± 1 °C in darkness for 2 yr, until the seed protein extraction was conducted.

Protein extraction

The extraction of seed storage proteins was conducted as described in Onwimol *et al.* (2010). Distilled water was used as the protein extraction solvent. Seed samples of 1 g of F1 hybrid and the parental lines were randomly selected by a seed divider. Each sample was crushed in a 1.5 mL microcentrifuge tube using sterilized metal beads and a grinding mill. Distilled water at a ratio of 5:2 (volume per weight) was then added and left at 25 ± 2 °C for 2 hr before centrifuging at 10,000 round per minute (rpm) for 5 min. The supernatant was transferred to a new tube and again centrifuged at 10,000 rpm for 5 min

and then the supernatant was used for UTLIEF.

Ultrathin layer isoelectric focusing

The UTLIEF gels were prepared according to the flap technique (International Seed Testing Association, 2012). The polymerization solution master mixed for the 10 gels was prepared by mixing 50.0 mL acrylamide (6.8% total acrylamide) with 1.60 g taurine, 50.0 μ L N N N'-N'-tetramethylethylenediamine (TEMED) and 350 μ L of 20% (weight per volume, w/v) ammonium peroxydisulphate. A gel pH gradient was generated by adding 4.40 mL of 2-11 ampholytes (SinuLyte™) into the polymerization solution. An aliquot of 6.5 mL polymerization solution was dropped on the Gel-Grip™ film (240 × 180 × 0.12 mm, Sinus, Heidelberg, Germany). The cover glass plate with two strips of adhesive tape (0.15 mm thick) stuck on both long sides as spacers was gently placed on top to evenly spread the aliquot over the film and allow it to polymerize for 1 hr at 25 ± 2 °C.

The iso-electro focusing (IEF) was carried out on an IEF-SYST™ horizontal electrophoresis unit (Scie-Plus™, Cambridge, UK). The unit was connected to a cooling apparatus and the gel surface was cooled to 8°C. The anodal electrode was placed on the top and the cathodal electrode on the bottom of gel. The anode solution contained 0.33% (w/v) L-aspartic acid and 0.37% (w/v) L-glutamic acid while the cathode solution contained 0.47% (w/v) L-arginine, 0.36% (w/v) L-lysine and 12% (volume per volume, v/v) ethylenediamine. An application strip was placed on top of the gel surface about 5 mm away from the anode strip. An aliquot of 20 μ L of each seed protein sample after being diluted with distilled water to a dilution of 8:12 (v/v) was loaded into the respective wells of an application strip. The IEF was carried out for 90 min at $150 \text{ V} \cdot \text{cm}^{-1}$. A power unit (Consort E833, Turnhout, Belgium) supplied 5 mA, 15W and 1,500 V.

After focusing, the gel that had stuck on the surface of the Gel-Grip™ was fixed in 12% (w/v) trichloroacetic acid for 20 min, then stained

in Coomassie Brilliant Blue solution (0.015% (w/v) Coomassie R250, 0.045% (w/v), Coomassie G250, 11% (v/v) acetic acid, 18% (v/v) ethanol and 71% (w/v) water) for 15 min and destained in the destaining solution (30% (v/v) ethanol, 5% acetic acid and 65% (v/v) water) for 10 min. After rinsing with water, the gel was air dried overnight at room temperature. The dried gel that stuck on the surface of the Gel-Grip™ was then covered with transparent adhesive film before evaluation and interpretation of the electrophoretograms of the seed protein following International Seed Testing Association, (2012).

RESULTS

The UTLIEF electrophoretograms were analyzed to evaluate the hybridity test. At least one unique protein band must be found in the male protein band pattern (male marker band; MMB) and appear in the protein band pattern of the respective hybrid seed, so that its hybridity can be verified. The self-pollinated seed is verified for any individual hybrid seed that shares the common protein band pattern with its respective female parental line without any MMB. The out-crossing seed is verified for any individual hybrid seed that contains any alien protein-band which does not belong to either the respective male and female parental lines.

No self-pollinated seed or out-crossing seed was found in any F1 hybrid seed electrophoretogram. The numbers of MMBs and female marker bands (FMBs) found in the 10 pairs of male and female parental lines (CS01–CS10) tested are shown in Table 1.

Six of the pairs contained both MMBs and FMBs and three contained either MMBs or FMBs. It can be concluded for these nine pairs that that polymorphic protein bands between male and female parental lines were found in all pairs except CS10. MMBs existed in eight male parental lines namely, CS01, CS02, CS04, CS05, CS06, CS07, CS08, and CS09 and five of them (CS01,

Table 1 Number of male and female marker bands found in each male and female parental line (CS 01–CS10) with cucumber hybridity test *via* ultrathin layer isoelectric focusing using water as protein extraction buffer.

Line	CS01	CS02	CS03	CS04	CS05	CS06	CS07	CS08	CS09	CS10
Male marker bands	1	2	0	1	1	2	1	1	2	0
Female marker bands	0	1	1	4	1	1	1	1	0	0

CS04, CS05, CS07, and CS08) contained only one MMB (Figure 1) while three (CS02, CS06 and CS09) contained two MMBs (Figure 2). Only the CS03 and CS10 parental lines showed protein band patterns containing no MMB when water was used as the protein extraction solution. However, the hybridity test of CS03 was successfully accomplished in a later retest of UTLIEF using a phosphate buffer solution as the protein extract solvent (data not shown). In addition, the most polymorphic bands were found in the high pH range (around 6–10). Examples of UTLIEF electrophoretograms are shown in Figures 1 and 2.

DISCUSSION

The major protein type in cucumber seed is salt-soluble protein globulins (Vickery *et al.*, 1941). Interestingly, the results from the current study showed that the polymorphic albumin proteins (the minor and water-soluble storage protein in cucumber seed) of the male and female parent lines could be found in nine crosses or 90% of the tested crosses. Albumin is mostly found in the food-reserving tissue parts in seed such as the endosperm in monocotyledons or in cotyledons in dicotyledonous species (Shimada *et al.*, 2003). Degtyarenko *et al.* (1986) reported success in the varietal identification of 20 cucumber varieties using electrophoresis to separate the albumin fractions. The current results confirm the high efficiency of water as the protein extract solvent to discriminate cucumber seed proteins as reported by Onwimol *et al.* (2010). Only one cross (CS10) showed a difference in the albumins between the

parental lines indicating that genetically different proteins, if there are any, could belong to other protein groups and thus, different extract solvents are required as reported by Tu *et al.* (2012) and Yan (2013b). Eight pairs of crosses of the F1 hybrid seed and their parent lines, or 80% of samples in the current study, were successfully identified using this technique with water as the protein extract solvent, which shows the possibility and efficiency of water as the protein extract solvent for a cucumber hybridity test *via* the UTLIEF technique. Water is the most convenient, safest and cheapest solvent compared with other solvents. Therefore, it should be the first choice of protein extract solvent when the cucumber hybridity test is conducted using the UTLIEF technique.

In the current study, the marker proteins were found to be focused in the gel pH range 6–10. This gel pH range was in agreement with Tu *et al.* (2012) who reported that polymorphic protein bands of cucurbit seeds occurred at gel pH 6–9 in varietal identification using the UTLIEF technique. The porous size of the polyacrylamide gels and the position of pI in these polymorphic seed proteins in cucumber should be 24–205 kD, indicating acidic proteins (Biosciences, 1999). The successful attempt to verify the hybridity of CS03 with their parental lines by changing the solvent from water to a phosphate buffer solution as the protein extract solvent before carrying out UTLIEF in this study indicated that the polymorphic proteins of this cross might be salt-soluble protein globulins. This reveals that the UTLIEF technique is still efficient for the hybridity test of this cross providing a suitable extraction buffer is chosen.

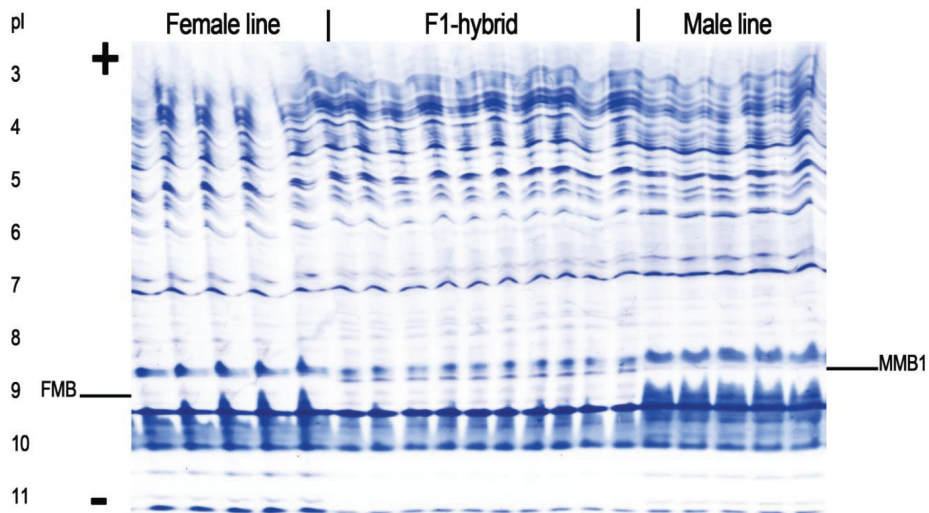


Figure 1 Ultrathin layer isoelectric focusing electrophoretogram of water extracted seed proteins of CS05 cross carried out on gel with pH gradient of 2–11 (FMB = female marker band; MMB = male marker band; + = anode; - = cathode).

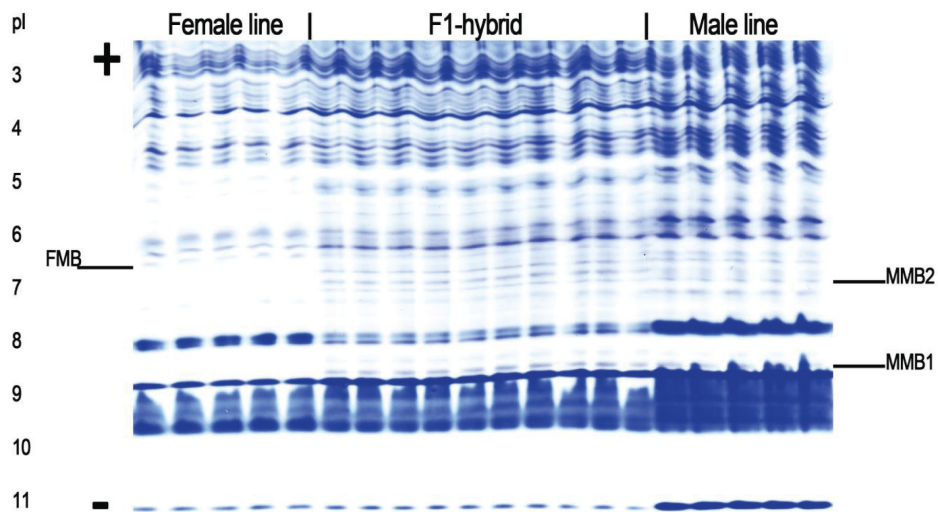


Figure 2 Ultrathin layer isoelectric focusing electrophoretogram of water extracted seed proteins of CS02 cross carried out on gel with pH gradient of 2–11 (FMB = female marker band; MMB = male marker band; + = anode; - = cathode).

Thailand exported approximately 105 tonnes of cucumber seeds in 2011, of which more than 90% were hybrid varieties worth around USD 9.3 million (FAOSTAT, 2014). Hence, an effective hybridity test is essential for the Thai seed industry.

Furthermore, the current study has shown that the UTLIEF technique using water as the protein extract solvent is cost effective and reliable for cucumber hybridity testing.

CONCLUSION

The results revealed that the UTLIEF technique using water as the protein extract solvent was successful for verifying F1 hybrid seed and their parents in 8 pairs out of the 10 tested. This is because the polymorphistic seed proteins of the other two cucumber crosses were not belong to albumin group and is not soluble in water. Nonetheless, the results showed that water is a promising seed protein extract for carrying out UTLIEF for cucumber seed hybridity testing. This application can help breeders and seed producers to verify the hybrid purity more efficiently in terms of cost and safety.

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