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Full Length Research Paper

Viability assessment of *in vitro* produced synthetic seeds of cucumber

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Friable, embryogenic calli of F1 cucumber (*Cucumis sativus)* cultivar, Royal, were induced from the hypocotyl pieces cultured on solidified MS-basal media supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) and benzyl amino purine (BAP). Embryogenic calli were transferred to liquid Murashige and Skoog (MS)-basal media supplemented with 5 µM naphthaleneacetic acid (NAA) and 1 µM BAP. The mature somatic embryos were encapsulated in sodium alginate mixture in synthetic seeds. The encapsulation mixture containing 3% sodium alginate, 100 mM calcium chloride and one-fourth volume of the cell suspension nutrient mixture containing 5×10^{-4} somatic embryos per ml was found the best. Synthetic seeds remain viable up to 14 weeks when stored at 4°C. Germination efficiency of synthetic seeds was decreased to 57% after 10 weeks of storage followed by rapid decrease in survival rate to 0% after 15 weeks. Genetic diversity between mother plants and *in vitro* produced synthetic seeds showed resemblance as assessed by amplified fragment length polymorphism (AFLP) markers.

Key words: Artificial seed, *Cucumis sativus*, encapsulation, somatic embryogenesis, sodium-calcium alginate.

INTRODUCTION

Regeneration of cucumber (*Cucumis sativus* L.) through somatic embryogenesis by using solid and liquid media has been reported (Tabei et al., 1994; Chraibi et al., 1992). Somatic or asexual embryogenesis is the regeneration of embryo-like structures from somatic cells without gametic fusion. An artificial or synthetic seed is an excellent possibility available to the somatic embryogenesis technology in which somatic embryos can be encapsulated by hydrogel coating for the production of artificial seeds. Although commercially available, F1 hybrid seed of cucumber are genetically homogenous but heterozygous, hence they cannot be propagated for successive generations due to their segregation capability. However, if developing embryos obtained by somatic embryogenesis be used directly for propagation of plants or through encapsulation in a suitable material that promotes germination, plant propagules with the same vigor as the mother hybrid plant can be obtained. It simply avoids conventional laborious breeding techniques mandatory for F1 hybrid seed production. The modern technique provides rapid bulking up of material for the mass production of F1 hybrid and genetically engineered plants. Usually somatic embryogenesis occurs at low frequency and at different developmental stages in a given culture. A large number of embryos at specific stage are required as starting material for this purpose.

Production of synthetic seed has been reported in a number of cereals, millets, tuberous plants, vegetables, and other commercially important plants like soybean, mustards, coffee, tobacco and cotton (Datta et al., 1999). However, because of certain inherent obstacles, the production rate of the uniform and high quality embryo is lower. Consequently, the preparation of efficient and quality seed has been successful in only a few crops like carrot (Latif et al., 2007) and alfalfa (Datta and Potrykus

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Abbreviations: CH, Casein hydrolysate; **AFLP,** amplified fragment length polymorphism; **2, 4-D,** 2, 4 dichlorophenoxy acetic acid; **BAP,** benzyl amino purine; MS, Murashige and Skoog; NAA, naphthaleneacetic acid; **AFLP,** amplified fragment length polymorphism; **RFLP,** restriction fragment length polymorphism; **SSR,** simple sequence repeats; **RAPD,** random amplification of polymorphic DNA; **CH,** casein hydrolysate; **GA_3,** gibberellic acid; **PCR,** polymerase chain reaction; **PLBs,** protocorm like bodies.

1989). The primary goal of artificial seed system is to regenerate whole plantlets from artificial seeds.

Estimation of genetic relatedness is important in designing crop improvement programs for management of germplasm and evolving conservation strategies. Molecular markers are the best tools for determining genetic relationships. These include restriction fragment length polymorphism (RFLP), simple sequence repeats (SSR), and random amplification of polymorphic DNA (RAPD) (Karp et al., 1997) and amplified fragment length polymorphism (AFLP) (Vos et al., 1995). AFLP is currently the method of choice for analysis of germplasm, genetic diversity and phylogeny, gene tagging and molecular map construction (Breyne et al., 1997). AFLP has been applied successfully to crops such as rice (Mackill et al., 1996), tea (Paul et al., 1997), soybean (Maughan et al., 1996), wheat (Barrett and Kidnell, 1998) and cucumber (Witkowicz et al., 2003) etc.

This is the first report of genetically uniform production of synthetic seeds of cucumber by standardizing culture conditions to induce somatic embryogenesis synchronously and at high frequency in cell suspension culture. Since the production of genetically stable and true-to-type plant is desired for production of synthetic seeds, AFLP analysis is linked to ensure the genetic integrity of mother plants.

MATERIALS AND METHODS

Plant materials and growth conditions

The seeds of cucumber cultivar, Royal F1 hybrid were surface sterilized by soaking in 0.1% (w/v) mercuric chloride solution for 5 - 10 min followed by thorough rinsing in sterilized distill water. Seeds were placed in sterile petriplates on moist filter paper in the dark at 22±2°C for germination.

Establishment of embryogenic callus

Light green, friable and embryogenic calli of Royal cultivar were initiated from its hypocotyl pieces on Murashige and Skoog (MS)-basal medium supplemented with 0.5 - 2 mg/L 2,4 dichlorophenoxy acetic acid (2,4-D); 0.5 mg/L benzyl amino purine (BAP); 30 - 90 mg/L sucrose, 1 mg/L casein hydrolysate (CH) under either continuous or 16 h photoperiod at 22±2°C. pH was adjusted to 5.7. All media were solidified with 2.5 mg/L phytagel. Developing calli were transferred to fresh medium after every 10 days.

Establishment of fast growing cell suspension

Cell suspension cultures were initiated by transferring 1 gm of friable and embryogenic calli to 25 mL MS-basal media containing 30 µM adenine sulphate, 3 µM thiamine HCl, 580 µM NaH_2PO_4, 0.1% CH, 5 µM naphthaleneacetic acid (NAA), 1 µM BAP in a 250 mL Erlenmeyer flask and placed on a gyratory shaker (80 rpm) at 22±2°C for 16 h photoperiod.

Somatic embryogenesis

Friable and fast growing cells present in Erlenmeyer flask were subcultured after every 10 days. To have synchronized cell suspension, cells were sieved in a stainless sieve of 150 µm (100mesh) size. After two successive subculturing, liquid media was modified by eliminating CH and NAA on the onset of somatic embryos.

Encapsulation of somatic seed

Mature cell suspension comprising of uniform cotyledonary-shaped somatic embryos was used to form synthetic/artificial seeds. One-fourth of mature cell suspension and three-fourth of sodium alginate solution (3%; w/v) was mixed. This mixture was then dispensed into 100 mM solution of calcium chloride ($CaCl_2.2H_2O$). Calcium alginate beads were formed as soon as the drop fell into calcium chloride solution, placed on a magnetic stirrer. Beads were left as such in the solution for about half an hour. Calcium chloride solution was decanted and beads were washed with 0.25% fungicide solution of Radomil Gold. It was kept for 1 h and followed by filtration to collect beads.

Germination of synthetic seeds

Synthetic seeds containing encapsulated somatic embryos in protective coating of calcium-alginate matrix were stored at 4°C for 15 weeks. Their germination percentage was checked after each week. Before letting the germination of synthetic seeds, the beads were immersed in a media containing MS-media, 3% sucrose, 14.4 µM gibberellic acid (GA_3), 4.8 µM BAP, 1 µM NAA and MS vitamins (30 µM adenine sulphate, 3 µM thiamine HCl, 580 µM NaH_2PO_4). The seeds were placed onto neutral phytagel matrix and kept for 16 h photoperiod at 22±2°C for germination.

DNA extraction and AFLP analysis

DNA extraction from control plants and from synthetic seed derived regenerants was carried out through “Phyto-Pure DNA Extraction kit” according to the manufacturer’s protocol.

An AFLP depiction was obtained by utilizing the AFLP analysis system from Applied Biosystem. Restriction digestion, adaptor ligation and preselective amplification were performed according to the manufacturer’s instructions. Modifications were connected with selective amplification in which PCR products of preselective amplification were used as template, using five EcoR1 and Mse1 primer combinations available in the kit. The reaction mixture for selective amplification contained 10X polymerase chain reaction (PCR) buffer (100 mM Tris HCl, pH 8.3; 500 mM KCl; 25 mM $MgCl_2$), 1 mM dNTPs, 5 µM Mse1 primer (Primer-Cxx), 1 µM EcoR1 primer (Dye-primer-Ax), 1 unit Taq polymerase, 3 µl preselective amplification product, and an ultra pure autoclaved water in a final volume of 15 µl. The reactions were subjected to 30 cycles, after an initial denaturation at 94°C for 2 min. Each cycle was optimized at 94°C for 20 s, 56°C for 30 s and 72°C for 2 min with a final 72°C for 10 min. Control DNA samples included in the AFLP kit were run in parallel with cucumber samples during the initial stages of this study.

PCR products of different sizes were pooled by adding 1.5 - 2.0 µl of the PCR product in 12 µl of de-ionized formamide containing 0.3 µl of ROX size standard. The samples were denatured at 94°C for 5 min and then subjected to quick chill by keeping it in ice for 5 - 6 min before running in the ABI Prism 3100 genetic analyzer.

RESULTS AND DISCUSSION

Callus induction

Friable, nodular and embryogenic callus is considered

Figure 1. Friable, nodular callus of cucumber cv. Royal obtained from hypocotyl.

pre-requisite to establish fast growing cell suspension culture and subsequent regeneration. The hypocotyl pieces of Royal cultivar developed into calli when cultured on MS-media containing 2.0 µM 2,4-D, and 0.5 µM BAP with 3% sucrose (Figure 1). The direct formation of somatic embryos on the callus cultures at low frequency was also observed. These somatic embryos developed into plantlets when placed onto regeneration media. It was confirmed that an appropriate concentration of 2,4-D in combination with BAP was necessary for the initiation of friable callus from cucumber hypocotyl, however its requirements were varied from cultivar to cultivar as revealed by Tabei et al. (1995).

Cell suspension

Following callus induction, cell suspension of cv. Royal was established in its respective callus induction media. Best suspension culture was obtained from MS-media containing 3% sucrose, 30 µM adenine sulphate, 3 µM thiamine HCl, 580 µM NaH_2PO_4, 0.1% CH, 5 µM NAA and 1 µM BAP. Cultures were placed on a gyratory shaker with rotation at 80 rpm at 22±2ºC for 16 h photoperiod. Freshly established cell suspension was heterogeneous with respect to cell morphology. At initial stages, two types of cells were observed. The first type consisted of elongated, vacuolated, large and with no starch contents. The number of elongated cells in suspension cultures was 5 - 10%. The elongated cells were eliminated by sieving through 150 µm mesh. The second type of cells was round in shape and small in size. They were found up to 90 - 95% of the total viable cells. These cells contained dense cytoplasm, apparent nucleolus and were rich in starch and plastid. A similar type of cell morphology in suspension cultures was reported in carrot (Fujii et al., 1989) and in cucumber (Tabei et al., 1995). Our findings suggest a clear difference between monocot and dicot cell suspension culture. In case of monocots, elongated cells disappear after 3 to 4 weeks and never reappear (Nasir and Riazzuddin, 2007) but in case of dicots, the elongated cells start emerging again after few days of sieving. Schwendiman et al. (1988) observed that the presence of starch is often related to embryogenic cells and is generally considered as an indication of development leading to somatic embryogenesis.

It was observed that the suspended cells of cucumber in the cell suspension cultures passed through four stages of somatic embryogenesis. i) Normal globular shaped cells developed into ii) heart shape, iii) torpedo shape and iv) somatic embryos. We found that if cell suspension cultures were sieved after long interval than normal ten days, the percentage of the non-embrygenic elongated cells increased. Clump formation in the suspension culture was also found associated with delayed sieving which consequently decreased regeneration significantly. Contrary to the above, subculture interval of 8 - 10 days proved best for maintaining embrogenic potential of the cells. Tabei et al. (1995) also observed different stages of somatic embryogenesis in cucumber cell suspension cultures. Binarova and Dolezel (1988) demonstrated that long subculture intervals without sieving kept the cells in a stationary phase and increase their ploidy level which is referred to as somaclonal variation.

Synchronization of embryogenesis

The procedure adopted by Giuliano et al. (1983) to synchronize the cucumber cell suspension was followed. All cells were subcultured and passed through 150 µm sieve (100 mesh) after every 10 days subculturing and then incubated on gyratory shaker at 80 rpm at 22±2℃. After two successive subculturing, cells at globular stage were shifted onto modified suspension media containing CH and NAA only. Cells turned into heart and torpedo shapes after 2 - 3 days. They were then subcultured on MS basal media, devoid of growth regulators which helped in uniform maturation of somatic embryo.

Synthetic seed/ encapsulation

Mixture of mature cell suspension and sodium alginate at a ratio of 1:4 were dropped into 100 mM calcium chloride solution to form synthetic seeds. These somatic seeds were hardened by rinsing in double distilled water followed by immersing seeds in MS-media containing 3% sucrose, 14.4 µM GA_3, 4.8 µM BAP, 1 µM NAA and MS vitamins (30 µM adenine sulphate, 3 µM thiamine HCl, 580 µM NaH_2PO_4) and kept at gyratory shaker for 20 - 30 minutes (Figure 2).

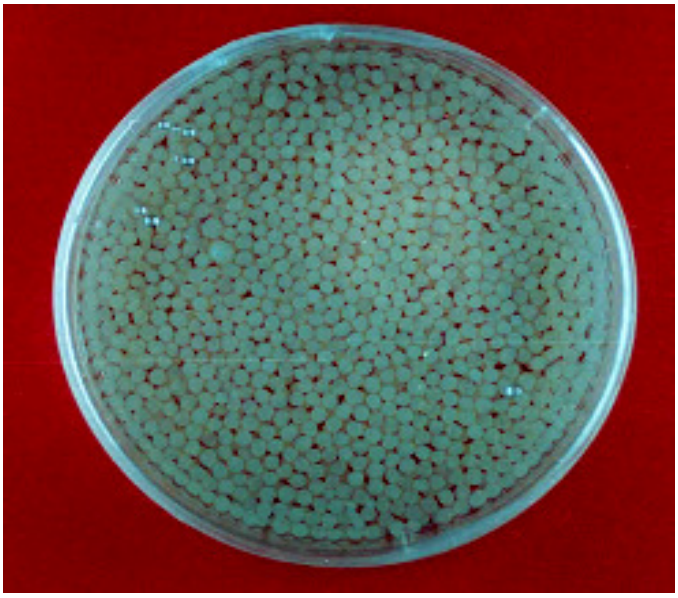

Figure 2. Synthetic seeds, mature somatic embryos encapsulated with sodium alginate.

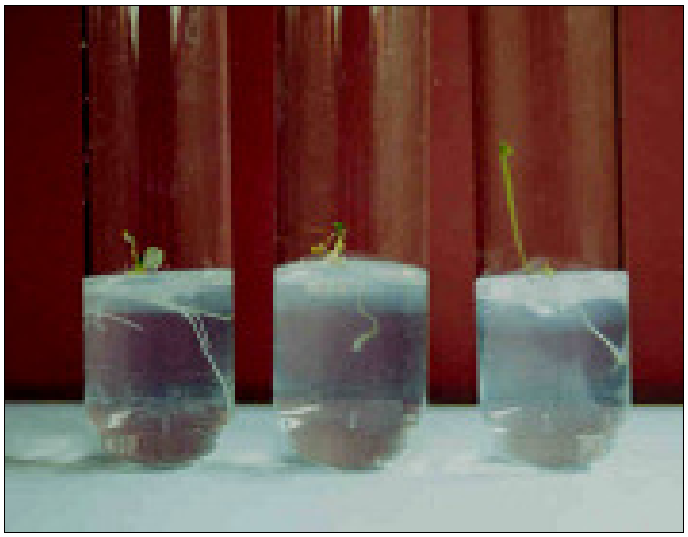

Figure 3. Germination of cucumber synthetic seeds on neutral gel.

Somatic embryos encapsulation seems to be one of the promising methods for sowing embryos, because encapsulation of nutrient mixture of somatic embryos and calcium alginate containing essential nutritional elements, carbon source, plant hormones and antimicrobial agents are coated completely to protect the embryos from mechanical damages during handling. It gives birth to true to type production as gauged by AFLP analysis.

Rederbangh et al. (1993) tried several agents for encapsulation and reported that sodium alginate complexing with calcium chloride is most suitable because of its moderate viscosity, low toxicity and quick gelation.

Figure 4. Synthetic seed derived cucumber plantlet.

Latif et al. (2007) reported that 2% sodium alginate solution and 75 mM solution of calcium chloride is best for coating of somatic embryos to form synthetic seeds in carrot.

Regeneration efficiency of synthetic seed

Shoot formation was observed when synthetic seeds were placed on neutral gel formed by dissolving phytagel in autoclaved tap water. The test tube containing neutral gel plus synthetic seeds were kept for 16 h photoperiod at 22±2°C. Shoots enlarged up to 10 days in neutral gel media when appropriate temperature and humidity were provided. Tissue culture derived synthetic seeds of cucumber cv. Royal were encapsulated in calcium alginate gelling agent. These calcium alginate beads were regenerated into complete plantlets (Figures 3 and 4). Figure 5 depicts the percentage germination of synthetic seeds. It is clear from this figure that with the increase in storage time, germination efficiency was considerably reduced. Germination efficiency was fine (57%) up to 10 weeks of storage but as the storage time was prolonged to the 15th week, germination percentage was reduced to zero after 14 weeks of storage at 4°C. Fuiji et al. (1989) also encapsulated cell suspension derived somatic embryos and observed regeneration. Similarly, Datta et al. (1999) used encapsulation technique for geodoruman orchid by encapsulating its protocorm like bodies (PLBs) in sodium alginate and noticed 88% germination of artificial seeds. In our results plantlet regeneration from artificial seeds was achieved in the presence of NAA, BAP and GA_3.

AFLP analysis

In order to detect the level of variation between cucumber

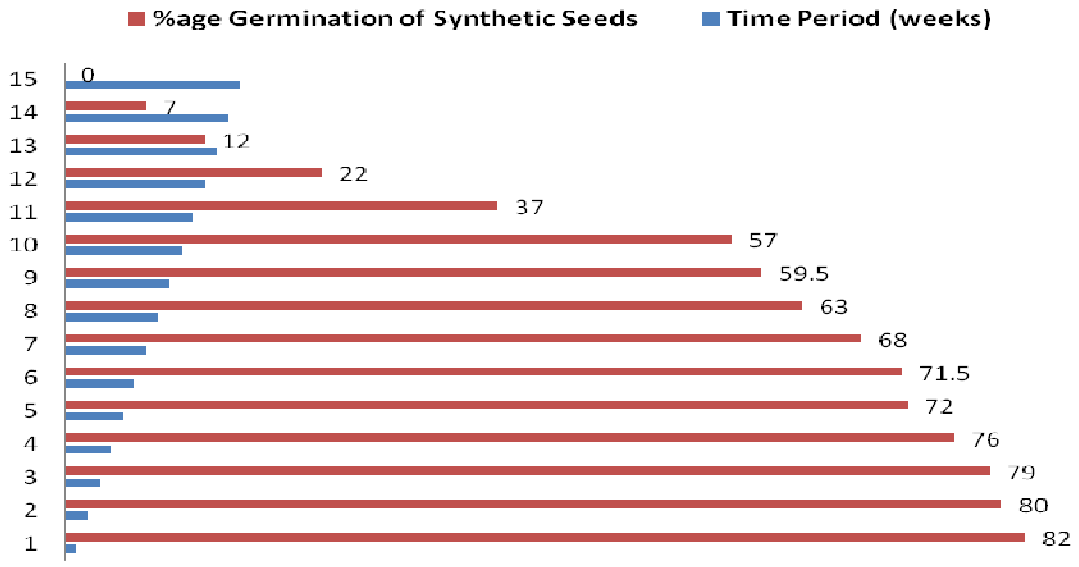

Figure 5. Germination percentage of cucumber cv. Royal synthetic seeds.

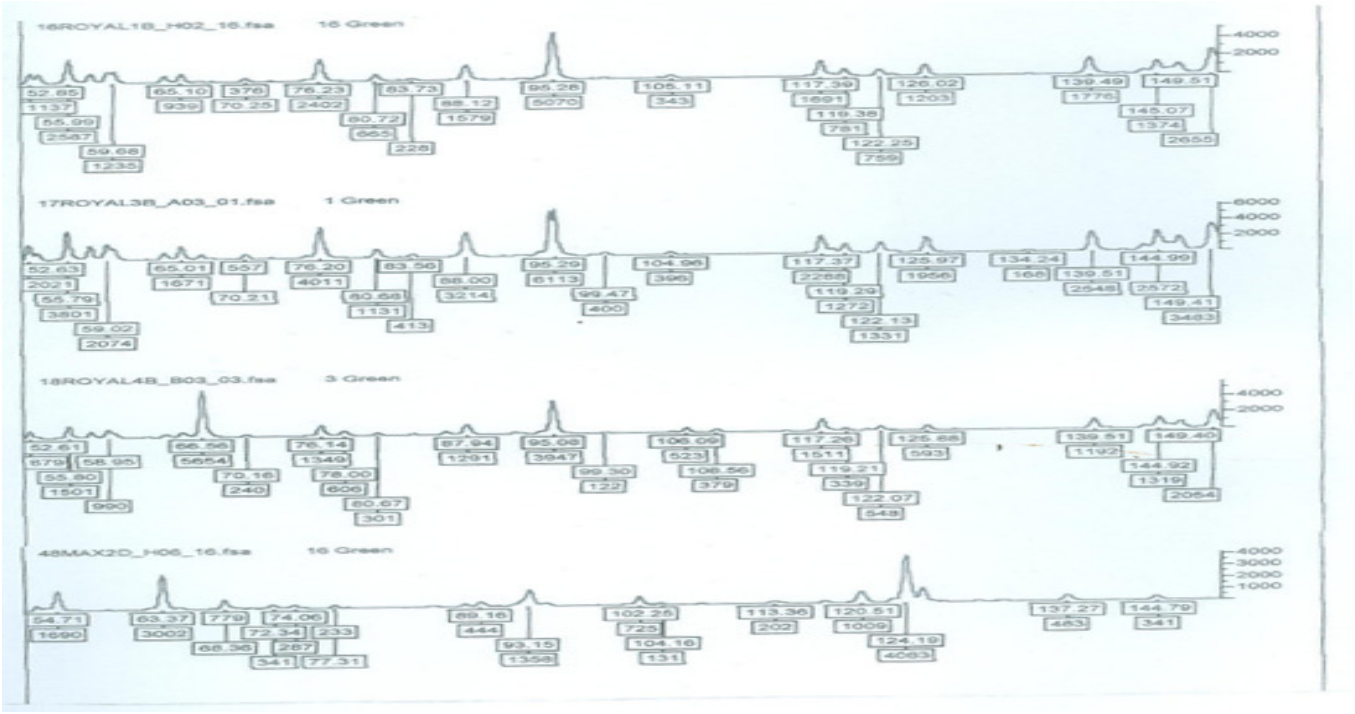

Figure 6. Multi-lane electropherograms comparing the DNA fingerprint patterns between cucumber cvs. and their synthetic seeds derived regenerants obtained with E-AG+M-CAG primer combination within the 50 -150 bp range. ROX-labelled DNA size marker (red) was used. Peak size denotes intensity.

cv. Royal and its regenerants obtained from synthetic seeds (encapsulated somatic embryos), five EcoR1+ Mse1 primer combinations were used (in each case). Amplification products were generated in the size range of 50 –150 bp (Figure 6). Using five primer combinations in Royal, a total of 109 bands were scored (an average of 21.8 primer or 35.77%). The number of bands scored with each primer ranged between 13 (E-AG+M-CTC) to 28 (E-AA+M-CTC). The polymorphism ranged between 7.14% with primer E-AA+M-CTC to15.38% with primer E AG+M-CTC (Table 1). The percentage polymorphism obtained in our study is almost negligible, depicting genetic

Table 1. Analysis of the polymorphism obtained with AFLP primer combinations among regenerants of cucumber cv. Royal.

Primer	Total no. of bands (a)	No. of polymorphic bands (b)	No. of monomorphic bands (b)	% Polymorphism (b/a ×100)
E-AA+M-CTC	28	2	26	7.14
E-AG+M-CAG	22	3	19	13.6
E-AA+M-CTT	25	3	5	12
E-AG+M-CTC	13	2	11	15.38
E-TA+M-CTA	26	2	24	7.69
TOTAL	89	17	85	11.16

integrity of tissue culture derived *in-vitro* synthetic seeds.

AFLP has a higher marker index, an overall measure of marker efficiency (Nakajima et al., 1998). AFLP has also been reported to be of highly reproducible low error rates (Jones et al., 1997), which provides a definite advantage over RAPD. It is clear from our findings that polymorphism among cucumber cv. Royal and its regenerants obtained via synthetic seed germination was very low. Williams et al. (1993) reported that polymorphism in amplified fragments might result from changes either in the sequence of the primer binding site (e.g. point mutation) or change which alter the size or prevent successful amplification of target DNA (e.g. insertion, deletions, inversions). Lima et al. (2002) used AFLP to investigate the genetic relationship in a group of 79 cultivars of sugarcane. They employed 21 primer combinations and generated a total of 2331 bands with a polymorphic rate, on an average of 50% per primer combination. AFLP markers are widely used for the evaluation of genetic variation between forms with a differentiated degree of relatedness, especially between genotypes with a small genetic distance, such as between a cv. and the mutants derived from it, between recombinant lines and between isogenic lines (Agrama et al., 2002).

CONCLUSION

This is the first report of synthetic seed production of cucumber and assessment of germination viability up to 15 weeks. Main purpose of the present work was to develop a simple inexpensive method of clonal synthetic seed production system that will enable the vegetative propagules to be stored for long duration and subsequent germination of viable plants. Synthetic seeds can reduce the cost of transportation and increase the rate of uniformity. It can help to provide large-scale delivery of elite genotypes selected from hand pollinated hybrids or genetically engineered plants.

REFERENCES

Agrama HA, Houssin SF, Track MA (2002). Cloning of AFLP markers linked to resistance to *Peronosclerospora sorghi* in maiz. Mol. Gen. Genet. 267: 814-819.

Barrett BA, Kidnell KK (1998). AFLP-based genetic diversity assessment among wheat cultivars from the pacific northwest. Crop Sci. 38: 1261-1271.

Binarova P, Dolezel J (1988). Alfalfa embryogenic cell suspension culture: growth and ploidy level stability. J. Plant Physiol. 133: 561-566.

Breyne P, Boerjan W, Gerats T, Van Montagu M, Van Gysel A (1997). Applications of AFLP in plant breeding, molecular biology and genetics; Belg. J. Bot. 129: 107-117.

Chraibi BMK, Castelle JC, Latche A, Rostan JP, Fallot J (1992). High frequency of plant regeneration in sunflower from cotyledons via somatic embryogenesis. Plant Cell Rep. 10: 617-620.

Datta KB, Kanjilaz B, Sarker DDE (1999). Artificial seed technology, development of a protocol in *Geodorum densiflorum*(Lam)Schltr.-an endangered orchid. Morel GM., Am. Orchid Soc. Bull. 1960(29): 495-497.

Datta SK, Potrykus I (1989). Artificial seed in barley: encapsulation of microspore derived embryos.Theor. Appl. Genet. 77: 820-824.

Fujii J, Slade A, Redenbaugh K (1989). Maturation and greenhouse planting of alfalfa artificial seeds. *In vitro* Cell Dev. Biol. 25: 1179.

Giuliano GD, Rosellini D, Terzi M (1983). A new method for the purification of the different stages of carrot embryoids. Plant Cell Tissue Organ. Cult. 17: 191-218.

Jones CJ, Edwards KJ, Castaglione S, Winfield MO, Sala F, Van De Wiel C, Bredemeijer G, Vosman B, Matthes M, Daly A, Brettscneider R, Bettini P, Buiatti M, Maestri E, Malcevschi A, Marmiroli N, Aert R, Volckaert G, Rueda J, Linacero R, Vazquez A, Karp A (1997). Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of european laboratories; Mol. Breed. 3: 381-390.

Karp A, Kresovich S, Bhat KV, Ayad WG, Hodgkin T (1997). Molecular tools in plant genetic resources conservation: a guide to the technologies; in IPGRI technical Bulletin No. 2. International Plant Genetic Resources Institute, Rome, Italy.

Latif Z, Nasir IA, Riazuddin S (2007). Indigenous production of synthetic seeds in *Daucus carota*. Pak. J. Bot. 39(3): 849-855.

Lima MLA, Garcia AAF, Oliveira KM, Matsuoka S, Arizono H, De Souza CL, De Souza AP (2002). Analysis of genetic similarity detected by AFLP and coefficient of parentage among genotypes of sugarcane (*Saccharum* spp.). Theor. Appl. Genet. 104(1): 30-38.

Mackill DJ, Zhang E, Redona ED, Colowit PM (1996). Level of polymorphism and genetic mapping of AFLP markers in Rice. Genome, 39: 969-977.

Nakajima Y, Oeda K, Yamamoto T (1998). Characterization of genetic diversity of nuclear and mitochondrial genomes in *Daucus* varieties by RAPD and AFLP; Plant Cell Rep. 17: 848-853.

Nasir IA, Riazuddin S (2007). New approaches to generate disease-resistant Gladiolus. Published online in WJMB.

Paul S, Wachira FN, Powell W, Waugh R (1997). Diversity and genetic differentiation among populations of India and Kenyan tea (*Camellia sinensis* (L.) O.kuntz) revealed by AFLP markers. Theor. Appl. Genet. 94: 255-263.

Rederbangh K, Fujii JA, Slade D (1993). Hydrated coatings of synthetic seeds. In: Synseeds (Ed.): K. Redenbaugh. CRC Press, Boca Raton. pp. 35-46.

Schwendiman J, Pannetier C, Michaux-Ferrriere N (1988). Histology of somatic embryogenesis from leaf explant of the oil palm *Elaeis*

guineensis. Ann. Bot. 62: 43-52.
Tabei Y, Nishio K, Kurihara K, Kanno T (1994). Liquid culture and short induction period enhanced enhanced somatic embryogenesis in cucumber (*Cucumis sativus* L). Breed. Sci. 44: 47-52.
Tabei Y, Seino E, Nishimura S (1995). Liquid culture and short induction period enhanced somatic embryogenesis in cucumber (*Cucumis sativus*). Plant Tissue Cult. Lett. 12(1): 79-83.
Vos P, Hogers R, Bleeker M, Reijans M, Van De Lee T, Hornes M, Freijters A, Pot J, Peleman J, Kuipe M, Zabeau M (1995). AFLP a new technique for DNA fingerprinting. Nucleic Acids Res. 23: 4407-4414
Williams JKG, Hanafey MK, Rafalski JA, Tingey SV (1993). Genetic analyses using random amplified polymorphic DNA markers. Methods Enzymol. 218: 704-740.
Witkowicz J, Urbanczyk-Wochniak E, Przybecki Z (2003). AFLP marker polymorphism in cucumber (*Cucumis sativus* L.) near isogenic lines differing in sex expression. Cell Mol. Biol. Lett. 8: 375-381.