Pak. J. Bot., 44(5): 1503-1508, 2012.

BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF SOMACLONAL VARIANTS AND INDUCED MUTANTS OF POTATO (*SOLANUM TUBEROSUM* L.) CV. DESIREE

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Abstract

In the present study, tissue culture and mutagenic techniques were applied to induce variations in potato (*Solanum* tuberosum L.) cultivar, Desiree. For obtaining somaclonal variants, internode-derived calli were incubated for 14-20 weeks in callus inducing medium. For mutation induction, ten week old, well proliferating calli were exposed to 5-50 Gy of gamma irradiation. Three variant lines (SV1, SV2 and SV3) and 6 gamma mutant lines (GM1, GM2, GM3, GM4 GM5 and GM6) were selected for further biochemical and molecular studies. In general an increase in total peroxidase activity in all the selected variants and mutants was observed. Same results were obtained when qualitative study was conducted on PAGE (polyacrylamide gel electrophoresis).Variation at isozymes and random amplified polymorphic DNA (RAPD) were used to detect variability and a total of 24 arbitrary sequence primers were evaluated. The RAPD primers produced 123 bands (88 polymorphic), whereas isozymes peroxidase produced 6 bands (5 polymorphic) showing genetic variation as compared to control. The current study demonstrates the production of useful variants both by tissue culture and gamma irradiation in potato and the use of isozymes and RAPD for assessing the genetic diversity among the variants and mutants.

Introduction

Over the years, potato has become an important crop for both farmers and consumers worldwide. It is the most important crop by volume of production, high yield, and high nutritive value and gives high returns to farmers (Ahmed & Bhutta, 2005). The narrow genetic base of potato hampers its improvement for disease resistance and agronomic characteristics by conventional breeding. The introduction of the resistance genes identified in different wild *Solanum* species into cultivated ones is limited by partial or total sexual incompatibility. This problem can be bypassed by using alternative methods like biotechnological techniques (Trabelsi *et al*., 2005). The recent advances in tissue culture and the flexibility of organ development in potato allows for alternative methods of propagation through *In vitro* techniques.

Genetic variability is commonly found in tissue culture raised plants. Larkin & Scowcroft (1981) defined this type of variation as *somaclonal* variation. The generation of *somaclonal* variation has been applied to crop improvement with the intention of including and exploiting useful and economically valuable characters that may not be readily available within other sources of germplasm (Jelenic *et al*., 2001). Induced mutations are another source of producing variations in plants. Naturally occurring mutation rate is too low for practical applications. Therefore, physical and chemical mutagens have proven useful for increasing the frequency of mutations and variations. In this regard, mutation breeding is considered complementary to the conventional cross method (Donnini & Sonnino, 1999). Gamma ray induced mutations in conjunction with *In vitro* culture of potato for disease resistance (Ahloowalia 1990; Sonnino *et al*., 1991; Love *et al*., 1993), heat tolerance (Das *et al*., 2000), microtuber production (Al-Safadi *et al*., 2000) have been reported. Isozymes and molecular markers have been applied for measuring the genetic variability produced in regeneration processes. During growth and development processes, many plants present dramatic changes in their enzymatic expression. The use of isozymes as markers is well documented and their genetically defined variants have evidenced their importance for evaluating genetic variability in economically important crops (Lara *et al*., 2003).

PCR based Random amplified polymorphic DNA (RAPD) analysis (Welsh & McClelland 1990; Williams *et al*., 1990) has previously been used in genetic studies of potato, for differentiation and identification of cultivars and clonal variants (Demeke *et al*., 1993; Ford & Taylor, 1997) and somatic hybrids (Baird *et al*., 1992; Rasmussen & Rasmussen, 1995) and in the assessment of genetic diversity and relationships of cultivated and wild potato species (Forapani, *et al*., 1999; Isenegger *et al*., 2001 and Moisan-Thiery *et al*., 2001). Yasmin *et al*., (2006) successfully used RAPD-PCR method to study the genetic diversity of 6 cultivars of potato. Aghaei *et al*., (2008) used this method to confirm distinct polymorphism between salt sensitive and salt tolerant cultivars of potato. Similarly Biswas *et al*., (2009) used RAPD technique as a tool for assessing genetic diversity and varietal relationships among ten varieties of *Solanum* sp. Similarly, Khan *et al*., (2011) used RAPD-PCR for the detection of genetic variations in micropropagated banana plants and Nasim *et al*., (2012) used this technique to determine the fingerprints of 10 isolates of *Alternaria alternata.*

Materials and Methods

Callogenesis and plant regeneration: Callus was initiated from inter-nodal explants of potato (cv. Desiree), in MS (Murashige & Skoog, 1962) medium supplemented with NAA (1.0 mg/l) and BAP (0.5 mg/l). For obtaining *somaclonal* variants, well-proliferated calli were incubated for 14 to 20 weeks in the same medium. For plant regeneration, the calli were transferred to regeneration medium comprising MS salts supplemented with NAA (0.5 mg/l) and BAP (2.0 mg/l).

Gamma irradiation: Ten weeks old, well proliferating calli with an average weight of 1.35g was used for irradiation at different exposures of gamma-rays (5, 10, 15, 20, 25, 30, 40 and 50 Gy). The process of irradiation was carried out at Mark-IV Irradiator at NIAB, Faisalabad. After irradiation, the cultures were shifted immediately on fresh hormone free MS medium, incubated for 5 days and then sub-cultured in callus inducing medium for further proliferation of callus. After two weeks, the calli were transferred to regeneration medium. The plantlets thus formed were used to get R1 and R2 generation for further analysis.

Biochemical investigation: For extraction of isoenzyme peroxidase, homogenate was prepared from 0.5 g fresh leaves from each selected sample crushed in liquid nitrogen in an ice chilled mortar and pestle with 1.0 ml of 0.1 M phosphate buffer at pH 7.2. The extract was centrifuged at 14,000 rpm for 20 minutes at 4ºC and the supernatant was separated. A discontinuous SDS system of polyacrylamide gel electrophoresis (PAGE) described by Laemmli (1970) was used for qualitative analysis of enzyme. The staining procedure of Siegel & Galston (1967) was applied for staining peroxidases. Two ml of Guaiacol was added to 98 ml of 0.1 M phosphate buffer (pH 5.8) followed by 0.2ml of H_2O_2. The gel was stained for half an hour. The visual bands were recorded and the gel was photographed.

DNA isolation: DNA was extracted from fresh potato leaves of control plants and selected *somaclonal* variants and induced mutants by the modified CTAB procedure (Doyle & Doyle, 1990). Fresh leaf tissue (0.1 g) was ground in liquid nitrogen, mixed with 2.5ml CTAB extraction buffer and incubated at 65ºC for 30 minutes. The sample was extracted with 2.5ml of chloroform / isoamyl alcohol (24:1 v/v) and the aqueous phase was mixed with 2/3 volume of cold iso-propanol. Nucleic acid was removed by centrifugation, washed with 0.1ml cold wash buffer, dried and resuspended in 0.1ml TE or sterile water. Single strand RNA was digested with 1.0µg/µl RNase A for 30 minutes at 37ºC and the DNA was quantified by gel electrophoresis.

Polymerase chain reaction (PCR): To analyze diversity among the variants/mutants RAPD analysis was carried out. The amplification reactions were performed in volumes of 25µl containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 50µM each of dATP, dTTP, dGTP, dCTP (Fermentas Inc. 7520 Connelley Drive, Maryland 21076, USA), 0.2µM primer, 0.5µg of template DNA and 2.5 units of Taq DNA polymerase (Fermentas). Twenty-four oligonucleotide decamer primers were employed (Lambda Biotech Inc. 3830 Washington Ave. St Louis MO 63108 USA). Amplification was performed in a GenAmp-2700 thermocycler (Applied Biosystems) programmed for a first denaturation step of 4 minutes at 92ºC and 1.30 minutes at 72ºC followed by 40 cycles of 45 seconds at 92ºC and 1.30 minutes annealing temperature of 28ºC and 1.30 minutes extension temperature at 72ºC. The final reactions were kept at extension temperature of 72ºC for 5 minutes and then held at 4ºC until the tubes were removed. All reactions were repeated with each DNA sample, DNA was not added to negative controls and reproducible amplified products were scored for data analysis. Amplification products were analyzed by electrophoresis and viewed under UV transilluminator and photographed (Dolphin-Doc with ID software, Wealtec Corp. USA).

Scoring and analysis of RAPD data: Data matrices were created from photographs of gels by assigning 1 to visible bands and 0 to absent bands. Estimates of genetic similarity were calculated according to Jaccard (1908). The data was processed by cluster analysis using the unweighted pair group average method (UPGMA) and plotted in the form of dendrogram using MSVP Version 3.1 Software.

Results

Isoenzyme: The SDS-PAGE used for qualitative analysis of peroxidase of variants and mutants of cv. Desiree, showed maximum number of bands (6) in SV3 and GM6 and minimum number of bands (3) in EMS3, while the rest of the variants and mutants showed 4-5 bands. A band of R_f value 0.423 which was present in control was also observed in all the variants and mutants of cv. Desiree. This band is the characteristic of this cultivar. The bands present in different variants and mutants showed different band intensities among themselves and in comparison with control (Fig. 1).

Similarity matrix of Jaccard's coefficients was created from isozymes data. UPGMA analysis identified the variants and mutants as subsets with Jaccard's coefficient ranging from 1.0 to 1.98 (Fig. 3A). Two main clusters were identified, one cluster containing 2 sub-clusters, one containing the entire gamma irradiated mutants except GM5, which was present in the other sub-cluster along with all the somaclonal variants. The other cluster contained only the Desiree control, showing dissimilarity from both the variants and mutants (Fig. 3A).

RAPD amplification: A total of 24 random oligonucleotide primers were evaluated for their ability to prime PCR amplification of potato genomic DNA of cultivar desiree along with its somaclonal variants (SV) and induced mutants {Gamma irradiated mutants (GM)}. The primers revealed a total of 123 clear and easily scorable bands. The size of the bands that were produced in the PCR reactions ranged from 200-3,000 bp, but most of the bands were between 300 and 2,000 bp. The primers were able to amplify the genomic DNA giving both monomorphic and polymorphic bands. About 63% of the bands were polymorphic among the variants and mutants of cv. Desiree. The level of polymorphism was different with different primers among different cultivars and mutants (Fig. 2).

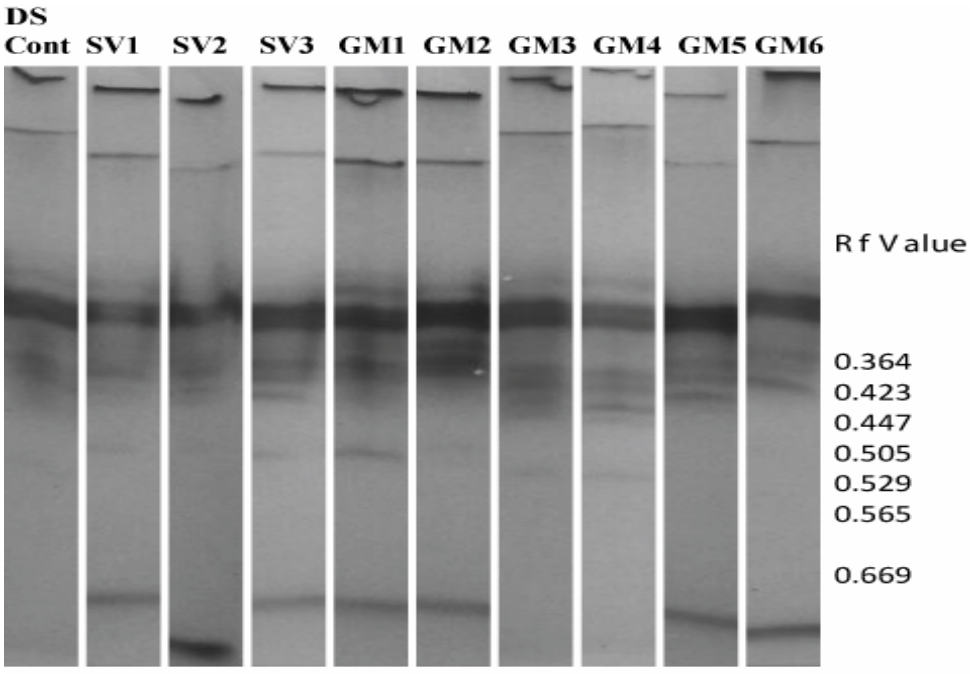

Fig. 1. The isozymic pattern of peroxidases of *Solanum tuberosum* cv. Desiree control and its somaclonal variants and induced mutants by PAGE.

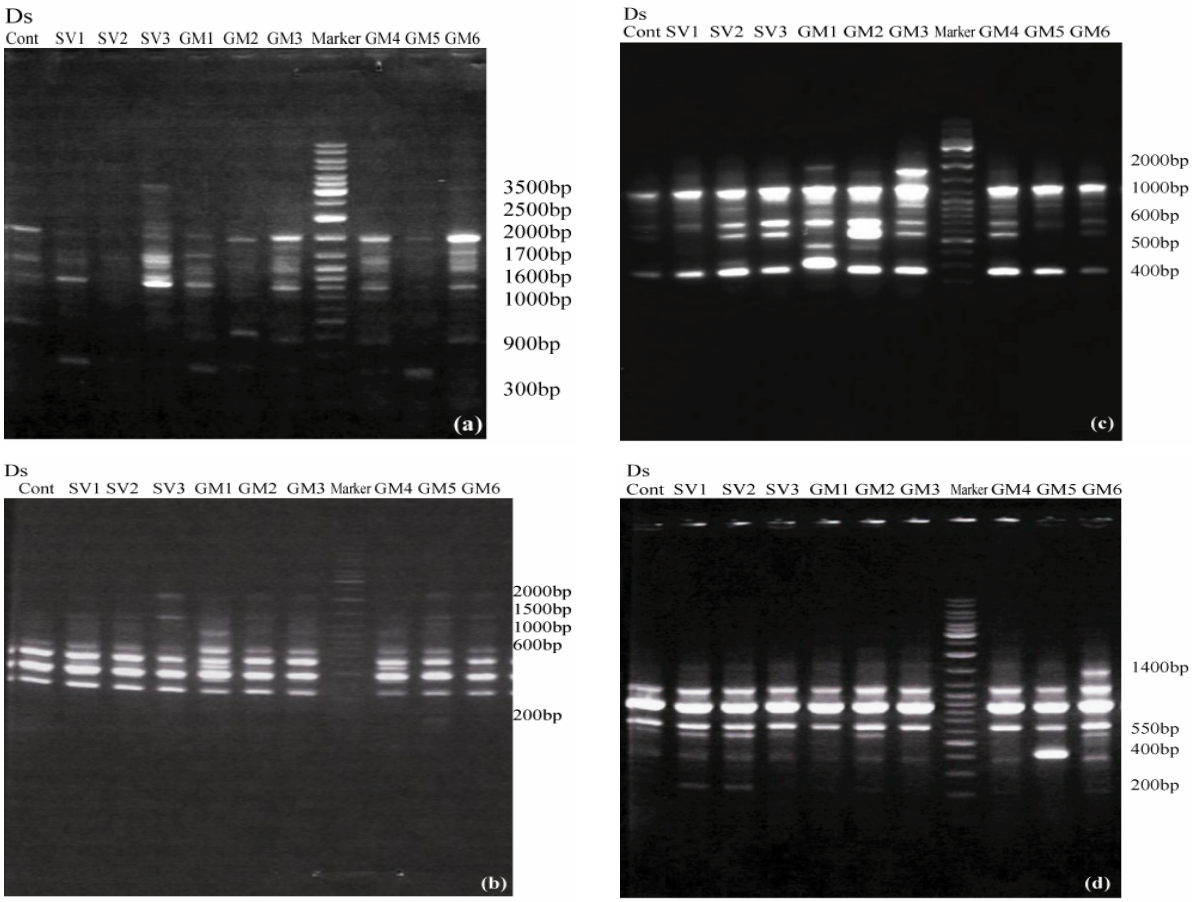

Fig. 2. RAPD loci amplified in *Solanum tuberosum* cv Desiree with decamer primers S-10 (a), S-114 (b), S-118 (c) and S-77 (d) showing monomorphism and polymorphism.

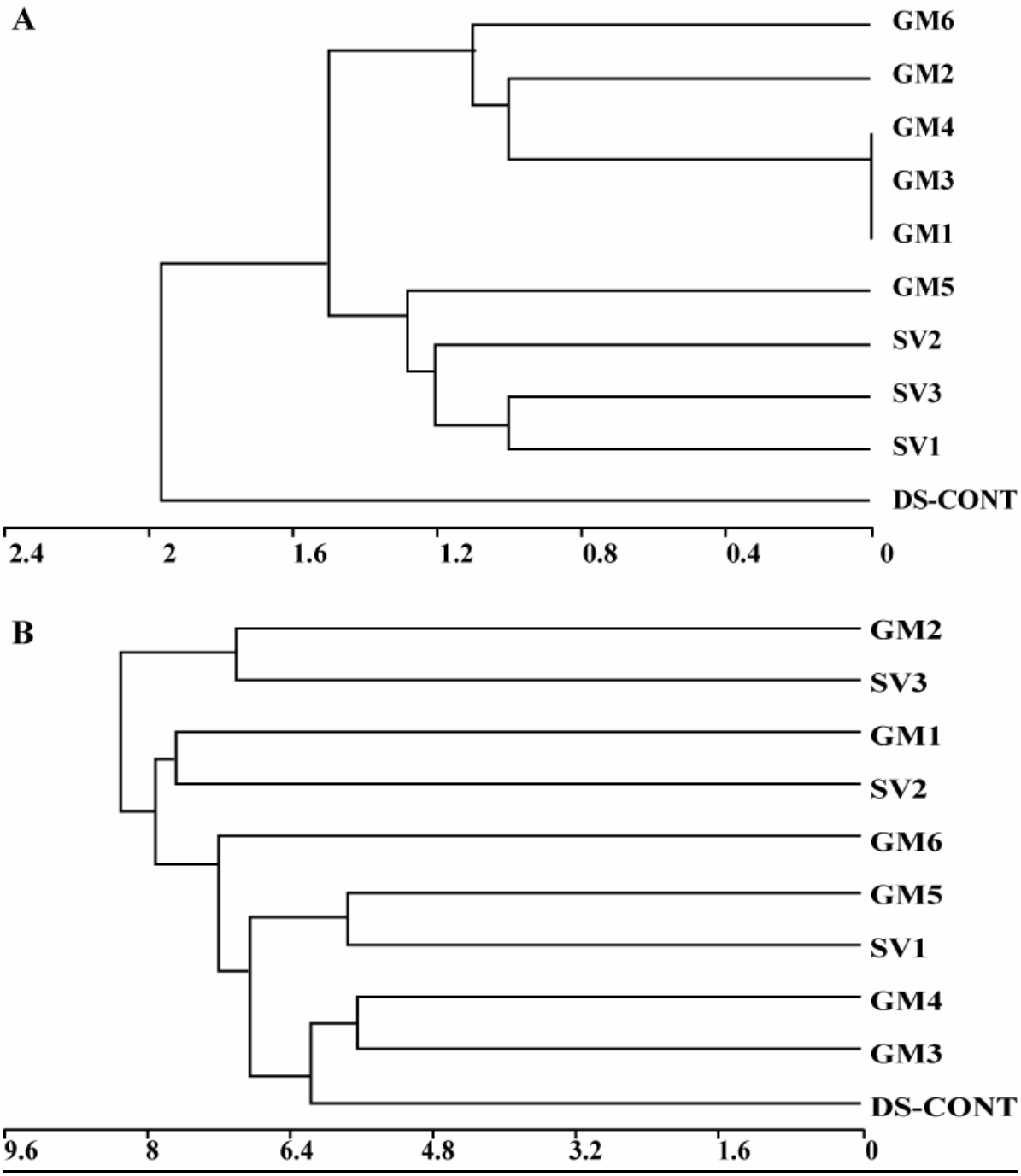

Fig. 3. Dendrogram based on Jaccard's coefficients. (A) Dendrogram generated from isoenzyme data (B) Dendrogram generated from RAPD data.

Based on data from all primers, the similarity matrix of Jaccard's coefficient was calculated. The UPGMA analysis did not divide the variants and mutants into strictly separate clusters. In the dendrogram two main groups of clusters were identified and Jaccard's coefficient ranged from 5.6 to 8.3. First cluster comprised of GM2 and SV3 and the rest of the variants and mutants including control were clustered in the second group (Fig. 3B). The most related appeared to be pairs of GM3-GM4, SV1-GM5, SV2-GM1 and SV3-GM2 showing more genetic similarity. GM3-GM4 and GM5-SV1 were more similar to control. GM1 and SV2 were closer to GM6, which formed a distinct cluster (Fig. 3B).

Discussion

Somaclonal variations and induced mutations are considered to be a good supplement to conventional crop improvement. Both result in the production of new genotypes with a limited change in the original genome.

In recent years biochemical and molecular markers have been widely used as a tool to provide an estimate of germplasm similarity (or diversity) and the data derived from banding patterns were judged as informative as those coming from pedigree analysis.

In the present work isozymes like peroxidases and molecular marker like RAPD-PCR was used to detect genetic variability among the selected somaclonal variants and gamma radiation induced mutants of potato cv. Desiree. In our work, an increase in peroxidase content was observed in all the selected somaclonal variants and induced mutants as compared to control (data not given). Similarly banding pattern of peroxidases in qualitative analysis also reflected a similar increase as compared to control. Similar results were obtained by Lara *et al.,* (2003) using peroxidases as markers for monitoring genetic stability and/or variability, caused by *In vitro* cultures in potato. They also observed a high enzymatic activity in the analyzed material. Iglesias *et al*., (1995) reported the presence of biochemical polymorphism as well as better resolution and repeatability in peroxidase of leaf tissue than that observed in shoot and root samples of potato cultivars.

The stress reaction in plants often results in the alteration of protein metabolism. Phillips *et al.*, (1994) emphasized that somaclonal variation most likely occurs by a stress – response mechanism. Similarly increase in protein content under radiation exposure has been reported in barley (Khebbnyi *et al*., 1980), soy bean (Afify *et al.,* 1988) and *Oryza sativa* (Khanna & Meherchandani, 1985).

Peroxidase isoenzyme has been widely used as genetic marker, since it presents different isoforms in most vegetative tissues (Lara *et al.,* 2003). Peroxidase isoenzymes also play an important role in the biosynthesis of cell wall components, as well as cellular differentiation (Christensen *et al*., 1998) and their relationship with resistance to adverse biotic and abiotic factors (Dalisay & Kuc, 1995). Other workers also reported changes in plant peroxidases due to treatment with radiation with a marked increase in peroxidase activity of leaves from plants originated by irradiated nodal segments of sweet potato (Lage *et al*., 2002). Sah *et al*., (1996) reported enhanced peroxidase activity in barley after irradiation of seeds with gamma-irradiation. Enhancement in peroxidase activity by radiation has also been reported by Erdem & Oldacay (2004) in sunflower.

In the present study twenty two out of twenty four primers employed presented polymorphic bands in the DNA amplification process used to evaluate genetic variation among the somaclonal variants and induced mutants. Many workers (Forapani *et al.,* 1999; Polzerova & Ptacek 2000; Isenegger *et al*., 2001) considered RAPD markers as a useful tool for characterizing and identifying cultivars and for differentiating and estimating genetic relationship besides being a fast technique. Molecular analysis using RAPD technique is also being used to detect somaclonal variation in various crops. Similar work was reported by Bordallo *et al*., (2004) using RAPD to detect somaclonal variation in 5 commercial potato cultivars, Achat, Baraka, Baronesa, Bintje and Contenda using twenty arbitrary sequence primers.

Ford & Taylor (1997) used RAPD technique to differentiate closely related potato cultivars which was not possible by morphological techniques. Wendt *et al.,* (2001) studied the influence of gamma irradiation on the regeneration rate of potato explants of cv. Macaca and molecularly characterized the regenerated plants using RAPD markers. Ehsanpour *et al.,* (2007) developed a method based on RAPD-PCR as a molecular marker for detection of somaclonal variation caused by UV-C radiation of potato cultivar cosima.

Conclusion

It can be concluded that that both isoenzymes and DNA banding pattern using RAPD analysis can be used to detect genetic variation caused by somaclonal variation and induced mutation in potato cv. Desiree.

Acknowledgement

The work reported in this manuscript is a part of Ph.D. thesis of the first author.

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(Received for publication 18 March 2010)