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Full Length Research Paper

Comparison of *in vitro* regeneration efficiency of leaf explants in response to different cytokinins and assessment of genetic uniformity of regenerated plants of *Solanum surattense* Burm.f.

Sandeep K. Yadav, Sumita Kachhwaha and S. L. Kothari*

Experimental Morphogenesis and Tissue Culture Laboratory, Department of Botany, University of Rajasthan, Jaipur-302 004, India.

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An efficient, genetically stable and rapid regeneration system via multiple shoot formation was developed for an endangered medicinal herb *Solanum surattense*. Leaves were cultured on MS medium supplemented with different concentration of cytokinins; 6-benzylaminopurine (BAP), kinetin (Kn), thiadiazuron (TDZ), zeatin and inorganic phosphate (iP) alone or in combinations. Among five cytokinins used, TDZ (0.1 mg/l) gave the maximum regeneration response (35 shoot buds per explant), with 100% regeneration efficiency. Comparative regeneration efficiency of cytokinins was in the following order: TDZ > zeatin > iP > BAP > Kn and the comparative ratio was 6:4:3:2:1. Highest frequency of adventitious shoot bud induction (54 shoot buds per explant) was achieved on combination of TDZ (0.1 mg/l) and BAP (1 mg/l). Combinations of cytokinins were more effective for shoot bud regeneration as compared to cytokinin alone. Best root formation was observed on Murashige and Skoog (MS) medium supplemented with 1 mg/l indole-3-acetic acid (IBA). The rooted plantlets were hardened gradually and resulted in a production of more than 80% plantlets. The efficiency of auxins for root organogenesis was in the following order: IBA > IAA > NAA > PAA. Genetic stability of regenerated plants was analyzed using random amplified polymorphic DNA (RAPD) markers.

Key words: *Solanum surattense*, adventitious shoot buds, genetic fidelity, random amplified polymorphic DNA, thiadiazuron.

INTRODUCTION

Solanum surattense Burm.f. is a medicinal herb belonging to family Solanaceae distributed in arid and semiarid regions of the world, especially in Southeast Asia, Malay, tropical Australia and India. The plant is used as digestive, diuretic and astringent agent, and in bronchial asthma (Govindan et al., 2004). It is also valued for antispasmodic, antitumor, cardiotonic, hypotensive and anaphylactic activity. *S. surattense* produces glycol-alkaloids in all parts of the plant body which on hydrolysis and removal of sugar residues yield steroidal alkaloids solanine, solamargine and solasodine. Solasodine is considered as a potential alternative to diosgenin for commercial steroid drug synthesis like progesterone and cortisone (Galanes et al., 1984).

S. surattense is propagated only by seeds, but this

*Corresponding author. E-mail: Kothari-sl@uniraj.ernet.in. Tel/Fax: +91 141 2703439.

Abbreviations: TDZ, Thiadiazuron; **RAPD,** random amplified polymorphic DNA; **EtOH,** ethanol; **MS,** Murashige and Skoog; **BAP,** 6-benzylaminopurine; **Kn,** kinetin; **IAA,** indole-3-acetic acid; **IBA,** indole-3-butyric acid; **NAA,** naphthalene acetic acid; **PAA,** phenylacetic acid; **CTAB,** cetyl trimethyl ammonium bromide; **EDTA,** ethylenediaminetetraacetic acid; **iP,** inorganic phosphate; **GA_3,** gibberelin; **RFLP,** restriction fragment length polymorphism.

method is beset with difficulties such as: (i) The seeds show a low level of germination under normal conditions (ii)the seeds lose their viability on storage and (iii) seed derived progenies are not true-to-type due to cross-pollination. Due to over exploitation for high medicinal values and destruction of the habitat, this plant is becoming endan-gered (Khan and Frost, 2001), hence there is need for *ex situ* conservation through tissue culture technique. Sinha et al. (1979), Saxena et al. (1982) and Borgato et al. (2007) have attempted to regenerate *S. surattense* using leaf protoplast and anthers by callus cultures with limited success. In this study, we evaluated the use of thiadiazuron (TDZ) along with other cytokinins for plant regeneration. The genetic stability of the regenerated plants was also assayed using random amplified polymorphic DNA (RAPD) markers. We developed a high frequency plant regene-ration protocol via adventitious shoot bud induction which is cost effective and produces large number of clonal plantlets within a short period of time.

MATERIALS AND METHODS

Plant material

Seeds of *S. surattense* collected from plants growing in the University Campus, Jaipur were used. The seeds were first washed with running tap water, then surface sterilized as follows: (i) Seeds were submerged in 70% (v/v) ethanol (EtOH) for 30 s, (ii) rinsed with sterile distilled water (iii) dipped in sodium hypochlorite (5% w/v) solution for 15 min, finally rinsed with sterile distilled water for three times. The seeds were then germinated on ½ strength Murashige and Skoog (1962) medium containing 1% (w/v) sucrose and 0.8% agar. For shoot regeneration, leaf (1 to 2 cm) excised from 4 week old sterile *in vitro* grown seedling were inoculated onto nutrient medium dispensed in 100 ml Erlenmeyer flasks. Leaves were cultured on MS medium containing different concentrations of cytokinins: 6-Benzylaminopurine (BAP), kinetin (Kn), TDZ, zeatin, 2-isopentenyl amino purine (0.001 to 3 mg/l) alone or in combination with other cytokinins incorporated with 3% sucrose.

Culture media and culture conditions

The pH of MS media used was adjusted to 5.8 using 1 N HCL or I N NaOH, before autoclaving at 120°C with 1.5 $kgcm^{-2}$ pressure for 20 min. The cultures were incubated in a growth chamber at 25 ± 1°C under 16/8 (light/dark) photoperiod with 25 µmol $m^{-2}s^{-1}$ illumination from cool fluorescent tubes (Philips, India).

Shoot bud regenerating cultures were kept on the regeneration medium for 5 weeks. The number of shoot buds was counted under a Nikon stereo microscope and number of elongated shoot buds was visually counted. Shoot buds were placed on the medium with different concentration of gibberellic acid (GA_3; 0.1 to 3 mg/l) for elongation.

Rooting and hardening

Elongated shoots (3 to 7 cm) were excised and placed on MS medium fortified with different concentration of indole-3-acetic acid (IAA) (0.1 to 2 mg/l), indole-3-butyric acid (IBA) (0.1 to 2 mg/l), naphthalene acetic acid (NAA) (0.1 to 2 mg/l), phenylacetic acid (PAA) (0.1 to 2 mg/l) alone for root induction. The plantlets (5 to 7 cm length) were washed with sterile water to remove traces of agar. For handening, he plantlets with fully developed root system were transferred to earthen pots containing soil and manure in 1:1 ratio.

Shoot regeneration experiments were arranged in a completely randomized design and repeated twice, each repeat using 3 explants in a flask (each repeat used 15 explants and a total of 30 explants per treatment for the whole experiment). Elongation and rooting experiments were also repeated twice, each rooting experiment was conducted in 80 ml tubes (Borosil) with one explant.

DNA isolation

DNA was extracted from fresh leaves by the cetyl trimethyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990) from eight randomly selected *in vitro* raised plantlets. The mother plant from which leaf explants were taken was selected as control. Approximately, 2 g of fresh leaves were ground to powder in liquid nitrogen using a mortar and pestle. The ground powder was transferred to a 25 cm^3 tube with 5 cm^3 of CTAB buffer: 10% (w/v) CTAB, 5 M NaCl, 50 mM ethylenediaminetetraacetic acid (EDTA), 1M sTris-HCL pH 8.0 and 0.2% (v/v) ß-mercaptoethanol. The homogenate was incubated at 60°C for 2 h, extracted with an equal volume of chloroform: iso-amyl alcohol (24:1). DNA concentration was estimated by using nanodrop spectrophotometer (ND 1000, Nano Drop Technologies, USA).

Polymerase chain reaction (PCR) amplification

Twenty seven arbitrary 10-base primers (Operon Technologies Inc., Alameda, California) were used for PCR. Amplification reactions were performed in 25 µl reaction volume containing 2.0 µl of 1.25 mM each of dNTP's, 20 pmol of the primer, 1× Taq polymerase buffer, 0.5 U of Taq DNA polymerase (Genei, India) and 40 ng of genomic DNA. DNA amplification was performed in a DNA thermocycler (Corbett Research, Australia) programmed for 40 cycles: 1st cycle of 5 min at 95°C, 1 min at 37°C and 2 min at 72°C; then 39 cycles each of 1 min at 95°C, 1 min at 37°C, 2 min at 72°C followed by one final extension cycle of 7 min at 72°C. Amplified products were electrophoresed in a 1.2% (w/v) agarose (Sigma, USA) gels with 1× TBE buffer, stained with ethidium bromide and photographed under ultraviolet (UV) light. The size of the amplification products was estimated for a 1 kb ladder (M.B I. Fermentas Inc.). All the reactions were repeated at least thrice.

Amplified DNA markers were scored as present or absent both in the regenerated and the mother plants. Electrophoretic DNA bands of low visual intensity that could not be readily distinguished as present or absent were considered ambiguous markers and not scored.

Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) by Fischer's least significant difference at P = 0.05 (Gomez and Gomez, 1984).

RESULTS

Effect of cytokinins used alone and with combinations

Leaves inoculated on media with different types of

Table 1. Effect of cytokinins on shoot bud induction from cultured leaf explants taken from *in vitro* seedling of *S. surattense.*

Cytokinin (mg/l)	Percentage response (%)	No. of shoot buds/explant mean ± S.D	Shoot bud length range (cm)
BAP			
1.0	86	7.6±0.5[a]	0.7 - 0.9
4.0	93	11.8±0.9[b]	0.9 - 1.3
5.0	86	9.6±0.7[c]	0.5 - 0.7
Kn			
1.0	66	4.2±0.8[d]	0.8 - 1.0
3.0	93	6.9±9.9[e]	1.2 - 1.5
5.0	86	4.8±0.6[f]	0.8 - 1.0
TDZ			
0.001	93	12.2±0.8[g]	0.3 - 0.5
0.01	100	20.5±1.2[h]	0.4 - 0.6
0.1	100	36.5±1.1[i]	0.8 - 1.0
Zeatin			
0.01	80	6.2±0.8[j]	0.2 - 0.4
0.1	86	13.3±0.9[k]	0.2 - 0.4
1.0	93	27.1±1.4[l]	0.5 - 0.7
iP			
0.01	73	5.9±0.8[m]	0.3 - 0.5
0.1	86	10.4±0.8[n]	0.4 - 0.6
1.0	94	18.3±1.1[o]	0.5 - 0.8

Culture period: 5 weeks. Means in the same column followed by different letters are significantly different at P = 0.05.

cytokinins responded to form shoot buds at varied intensity. Among the different concentrations of Kn used, best shoot bud induction was observed along petiolar end of explant on 3.0 mg/l Kn in the medium. BAP in comparison with Kn responded better but the shoot bud length was less (Table 1). MS medium with inorganic phosphate (iP) (1 mg/l) was more effective than BAP. Zeatin (1 mg/l) could induce higher number of shoot buds (Table 1). Low concentration of TDZ (0.001 mg/l) was effective and could also produce shoot buds along whole surface of explant and increase in the concentration of TDZ increased shoot bud number. Highest number of shoot bud induction was achieved on MS medium with TDZ (0.1 mg/l) without any formation of callus. Increase of TDZ beyond this level led to decrease in number of shoot buds and any further increase enhanced only green dense callus in large amount from the whole surface of leaf. Effectiveness of the cytokinin tested for shoot buds regeneration was TDZ > zeatin > iP > BAP > Kn (Table 1) and the ratio of shoot bud induction was 6:4:3:2:1 (Table 1).

In a second set of experiment, the effect of combinations of cytokinins BAP, Kn (0.1 to 5 mg/l), iP, zeatin (0.01 to 2 mg/l) and TDZ (0.001 to 3 mg/l) was tested. High frequency shoot bud induction was achieved on all the combinations tried (Table 2) but a remarkable increase in number of shoot buds was seen on MS medium and a combination of TDZ (0.1 mg/l) + BAP (1 mg/l) with up to 54 shoot buds per explants were produced (Figure 1). Shoot buds were separated and cultured on medium with gibberelin (GA_3) for elongation.

All the shoot buds inoculated on MS medium containing different concentration of GA_3 (0.1 to 3 mg/l) showed significant improvement in elongation. Maximum elongation of shoot (6.8 cm) was observed on MS medium with 0.5 mg/l of GA_3 (Table 3, Figure 1). It was also observed that the increased concentration of GA_3 (above 1.0 mg/l) reduced the shoot bud proliferation and elongation.

Effect of auxins in root induction

Elongated shoots were cultured on MS medium containing different concentration (0.1 to 3 mg/l) of IAA, IBA, NAA and PAA for rooting. In terms of rooting percentage, IBA was the more effective auxin. This treatment was also effective in terms of the mean number of roots per shoot (14.1 roots) and the mean number of length of growing roots (12.6 cm) compared with other auxins (Table 4) (Figure 1). Increasing concentration of IBA, however, resulted in steady decrease in all the parameters recorded. Of all the auxins tried for rooting,

Table 2. Effect of combination of cytokinins on shoot bud induction from cultured leaf explants taken from *in vitro* derived seedling of *S. surattense*.

Cytokinin (mg/l)		Percentage response (%)	No. of shoot buds/explants mean ± S.D.	Shoot bud length range (cm)
TDZ	**Zeatin**			
0.05	0.5	86	26.4 ± 0.9[a]	0.2 - 0.4
0.05	1.0	80	21.4 ± 1.1[b]	0.2 - 0.4
0.1	0.1	73	13.6 ± 1.3[c]	0.1 - 0.3
0.1	1.0	66	11.8 ± 1.1[d]	0.1 - 0.3
TDZ	**BAP**			
0.05	1	100	38.0 ± 1.2[e]	0.5 - 0.9
0.05	4	100	40.8 ± 0.9[f]	1.0 - 1.6
0.1	1	100	54.2 ± 1.1[g]	1.2 - 2.2
0.1	4	100	47.2 ± 1.3[h]	0.6 - 1.4
TDZ	**Kn**			
0.05	1.0	73	21.2 ± 1.3[i]	0.4 - 0.8
0.05	3.0	80	26.8 ± 1.2[j]	0.6 - 1.0
0.1	1.0	93	30.8 ± 0.8[k]	0.9 - 1.5
0.1	3.0	86	25.2 ± 1.6[l]	0.7 - 1.3
TDZ	**iP**			
0.05	0.5	86	20.2 ± 1.8[m]	0.1 - 0.3
0.05	1.0	73	24.2 ± 1.1[q]	0.2 - 0.4
0.1	0.5	66	20.2 ± 1.5[m]	0.3 - 0.5
0.1	1.0	66	13.4 ± 0.8[n]	0.2 - 0.4
Zeatin	**BAP**			
0.5	1.0	86	17.6 ± 1.3[o]	0.2 - 0.4
0.5	4.0	93	30.2 ± 0.9[p]	0.3 - 0.7
1.0	1.0	80	24.4 ± 0.8[q]	0.3 - 0.5
1.0	4.0	73	22.2 ± 1.1[r]	0.1 - 0.3
Zeatin	**Kn**			
0.5	1.0	53	16.0 ± 1.3[s]	0.3 - 0.5
0.5	3.0	53	17.0 ± 1.8[t]	0.5 - 0.9
1.0	1.0	46	19.4 ± 1.9[u]	0.2 - 0.4
1.0	3.0	46	13.8 ± 1.4[v]	0.2 - 0.4

Culture period: 5 weeks. Means in the column followed by different letters are significantly different at P = 0.05.

Table 3. Elongation response of shoot buds obtained from leaf explants (taken from *in vitro* derived seedlings) of *S. surattense* on MS medium supplemented with various concentrations of GA_3.

GA_3 (mg/l)	Percentage response (%)	Shoot length (cm) mean ± S.D.
0.1	33	2.4 ± 0.5[a]
0.3	53	4.2 ± 0.8[b]
0.5	86	6.9 ± 0.9[c]
1	73	6.1 ± 1.1 [d]
2	66	5.9 ± 0.8[e]

Culture period: 4 weeks. Means in the column followed by different letters are significantly different at P = 0.05.

PAA was less effective in terms of percentage root responds per shoot, number of roots per shoot and mean number of root length per shoot. Effectiveness of the auxins tested for root induction was IBA > IAA > NAA > PAA (Table 4). Rooted shoots were finally transferred to pots containing soil and manure in 1:1 ratio and kept under growth room conditions for two weeks before moving to room conditions at low humidity.

Table 4. Effect of various auxins on rooting of shoots obtained from leaf explants (taken from in vitro derived seedling) of *S. surattense*.

Auxin's (mg/l)	Percentage response (%)	No. of roots/explants	Roots length (cm) mean ± S.D.
IAA			
0.5	73	8.4 ± 1.1^{a}	6.3 ± 0.9^{al}
1.0	86	11.0 ± 0.8^{b}	10.1 ± 0.7^{b}
2.0	66	9.2 ± 1.3^{c}	6.8 ± 0.8^{c}
IBA			
0.5	80	11.8 ± 0.8^{d}	10.8 ± 0.9^{d}
1.0	93	14.1 ± 1.1^{e}	12.9 ± 1.2^{e}
2.0	66	12.6 ± 0.9^{f}	9.8 ± 0.7^{fx}
NAA			
0.5	53	4.8 ± 1.3^{g}	4.2 ± 0.8^{g}
1.0	66	8.2 ± 1.1^{a}	6.1 ± 0.8^{al}
2.0	60	5.6 ± 0.7^{h}	5.0 ± 0.9^{h}
PAA			
0.5	40	2.2 ± 0.9^{il}	2.2 ± 0.8^{im}
1.0	46	4.0 ± 0.5^{j}	3.8 ± 0.7^{j}
2.0	46	2.5 ± 0.8^{kl}	2.3 ± 0.6^{km}

Culture period: 4 weeks. Means in the column followed by different letters are significantly different at P = 0.05.

Approximately 80% of the shoots survived.

RAPD analysis

Genetic stability of regenerated plants was evaluated to ascertain their true clonal status utilizing RAPD markers. In all, 27 primers were used. 14 out of 27 primers showed reproducible patterns. The size range of bands was 300 – 1400 bp across the mother plant and its 9 randomly selected tissue culture progenies. Primer OPR-02, OPK-19, OPA-09, OPA-13, OPA-16, OPA-08, OPA-10 to OPA-15, OPB-04 and OPP-12 generated monomorphic banding patters in selected tissue culture progenies. The results confirmed that micropropagated plant were genetically stable (Figure 1).

DISCUSSION

An efficient and rapid *in vitro* plant regeneration system through adventitious shoot bud formation has been developed in *S. surattense*. The present protocol made it possible to reduce the time span required to regenerate *S. surattense* plants to as short as 5 weeks of culture, with a 100% of leaf explants producing as many as 54 shoot buds per leaf explants when cultured on medium containing combination of lower TDZ (0.1 mg/l) with BAP (1.0 mg/l). This frequency of *in vitro* regeneration is remarkably higher than those reported for *S. surattense* (Gupta and Chandra, 1982). To our knowledge, the effect of TDZ on *in vitro* plant regeneration of *S. surattense* and genetic uniformity in regenerated shoot was tested for the first time. We were able to regenerate shoot buds at very high frequencies, so that up to 260 - 310 shoots were obtained from a single leaf explants within a period of 12 weeks through subcultures. Park and Lim (1999) also found that leaf were more productive for shoot formation for both callus and shoot development than other explants. This system therefore, can provide not only a rapid propagation method but also an excellent platform for gene transformation studies.

In the first set of experiment where effectiveness of cytokinins on shoot bud induction on leaf culture of *S. surattense* was studied, it indicated that BAP was nearly twice more effective than Kn in number of shoot bud per explants. Many workers have observed superiority of BAP over Kn (Kothari and Chandra, 1984; Dhaka and Kothari, 2005) although Kn supported increase in length of shoots rather than BAP (Table 1). It was also observed that the shoot length reduced with increase in BAP concentration and similar results were observed by Ramage and Williams (2004). Results indicated that iP was more effective than BAP for shoot bud induction and zeatin was more effective than iP. Remarkable improvement in results was observed when TDZ was utilized for shoot initiation. TDZ which is a substituted phenyl urea compound was developed for mechanized harvesting of cotton bolls and has now emerged as a highly efficacious bioregulant of morphogenesis in the tissue culture of many plant species (Peddaboina et al., 2006; Yucesan et al., 2007). TDZ is relatively stable in regeneration systems

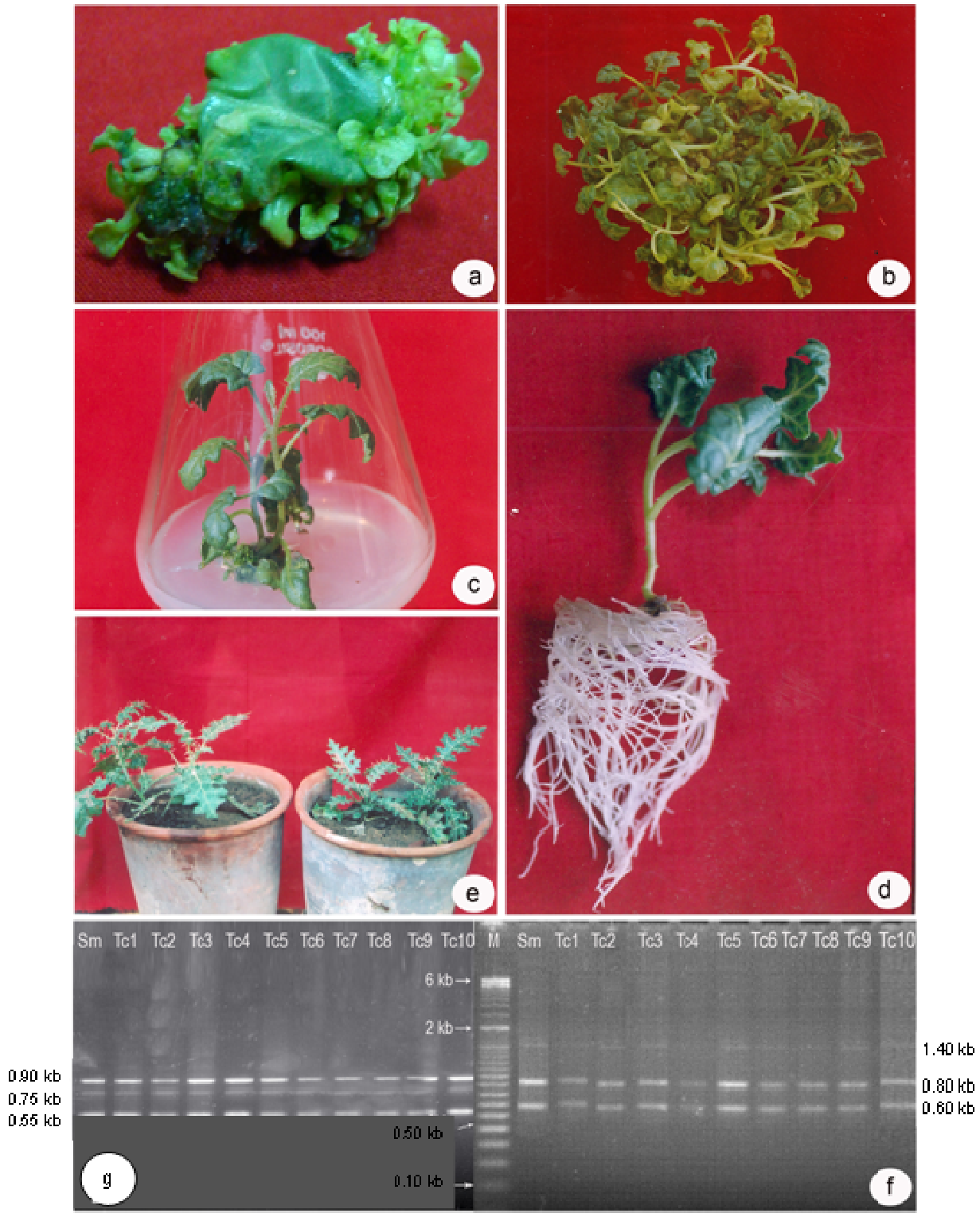

Figure 1. Morphogenetic response of leaf explants of *S. surattense* on MS medium supplemented with various plant growth regulators. (a) Induction of shoot buds on MS + TDZ (0.1mg/l) + BAP (1 mg/l); (b) proliferation of shoot buds on MS + BAP (1 mg/l); (c) elongation of shoot on MS + GA_3 (0.5 mg/l); (d) *in vitro* regenerated plantlet; (e) field transferred plant after 2 months; (f and g) agarose gel electrophoresis of RAPD fragments obtained from primer OPK-19, OPA – 09; (f) primer OPK-19 showing monomorphic bands; Lane 1 shows bands from a field grown mother plant of *S. surattense* (Sm); Lane 2 to 11 shows bands from tissue culture raised plantlets; (g) Primer OPA-09 showing monomorphic bands; Lane 1 shows the molecular marker (M); Lane 2 shows bands from a field grown mother plant of *S. surattense* (Sm); Lane 3 to 11 shows bands from tissue culture raised plantlets.

and induced a wide array of response with a high degree of efficacy.

In the second set of experiment, we observed that synergistic effect of combination of cytokinins was more efficient for shoot buds regeneration than cytokinins used alone. Cytokinin combinations enhanced multiple shoots in several plant species (Franklin et al., 2004; Baskaran and Jayabalan, 2008). Among all the cytokinins used in the study, MS medium with BAP (1 mg/l) allowed best proliferation of shoot buds. Shoot buds when treated for elongation on MS medium containing different concentration of GA_3 (0.1-3 mg/l), showed increase in length, but significant elongation of shoot was observed on GA_3 (0.5 mg/l).

IBA was found to be more effective for root induction when compared to IAA, NAA and PAA; similar observation was reported in *Echinacea purpure* (Choffe et al., 2000). The efficiency of auxins for root organogenesis was in the following order: IBA > IAA > NAA > PAA.

A great number of observations have demonstrated the occurrence of genetic variation in cultured cells and plants originated *in vitro* (Larkin and Scowcroft, 1981; Isabel et al., 1996). These variations are often heritable and unwanted in somatic clones. It is important to detect these variations early in the life of a plant to avoid economic disasters later. Molecular markers, restriction fragment length polymorphism (RFLP) (Atchison et al., 1976; Vedel et al., 1976), RAPD (Williams et al., 1990) and amplified fragment length polymorphism (AFLP) (Vos et al., 1995) are powerful tools in genetic identification. However, RAPD markers are more advantageous than RFLP and AFLP markers, as these are easy to perform and large number of sample can be analyzed quickly and economically (William et al., 1990). There are many reports on molecular characterization of micropropagated plants by the RAPD technique, e.g. *Populus deltoides* (Rani et al., 1995), *Prunus dulcis* (Martins et al., 2004), *Gypsophila paniculata* (Rady, 2006), *Withania somnifera* (Sinha et al., 2010) and *Capparis decidua* (Tyagi et al., 2010).

REFERENCES

Atchison BA, Whitefield PR, Bottomley W (1976). Comparison of chloroplast DNAs by specific fragmentation with *Eco*RI endonuclease. Mol. Gen. Genet. 148: 263-269.

Baskaran P, Jayabalan N (2008). Effect of growth regulators on rapid micropropagation and psoralen production in *Psoralea corylifolia* L. Acta. Physiol. Plant. 30: 345-351.

Borgato L, Pisani F, Furini A (2007). Plant regeneration from leaf protoplast of *Solanum virginianum* L. (*Solanaceae*). Plant. Cell. Tiss. Org. Cult. 88: 247-252.

Choffe KL, Murch SJ, Saxena PK (2000). Regeneration of *Echinacea purpure*: induction of root organogenesis from hypocotyls and cotyledon explants. Plant. Cell. Tiss. Org. Cult. 62: 227-234.

Dhaka N, Kothari SL (2005). Micropropagation of *Eclipta alba* (L.) Hassk – An important medicinal plant. *In vitro* Cell. Dev. Biol. Plant. 41: 658-661.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus, 12: 13-15.

Franklin G, Carpenter L, Devis E, Reddy CS, Al-Abed D, Alaiwi WA, Parani M, Smith B, Goldman SL, Sairam RV (2004). Factors influencing regeneration of soybean from mature and immature cotyledons. Plant Growth Regul. 43: 73-79.

Galanas IT, Webb DT, Rosario O (1984). Steroid production by callus and cell suspension cultures of *Solanum aviculare* Forst. J. Nat. Prod. 47: 373-376.

Gomez KA, Gomez AA (1984). Statistical procedures for agriculture research. John Wiley and Sons, New York, p. 680.

Govindan S, Viswanathan S, Vijayasekaran V, Alagappan R (2004). Further studies on the clinical efficacy of *Solanum xanthocarpum* and *Solanum trilobatum* in bronchial asthma. Phytother. Res. 18: 805-809.

Gupta SC, Chandra N (1982). Control of Organogenesis of Leaf, Multilayered Strips & Segments of Stem and Root in *Solanum surattemse* Burm.f. Indian J. Exp. Bio. 20: 126-131.

Isabel N, Boivin R, Levasseur C, Charest PM, Bousquet J, Trmblay FM (1996). Occurrence of somaclonal variation among somatic embryo-derived white spruces (*Picea glauca*, Pinaceae). Am. J. Bot. 83: 1121-1130.

Khan TI, Frost S (2001). Floral biodiversity: a question of survival in the Indian thar desert. The Environmentalist, 21: 231-236.

Kothari SL, Chandra N (1984). *In vitro* propagation of African marigold. Hort. Sci. 19: 703-705.

Larkin PJ, Scowcroft WR (1981). Somaclonal variation-a novel source of variability from cell cultures for plant improvement. Theor. Appl. Genet. 60: 197-214.

Martins M, Sarmento D, Oliveira MM (2004). Genetic stability of micropropagated almond plantlets, as assessed by RAPD and ISSR markers. Plant Cell Rep. 23: 492-496.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures Physiol. Plant. 15: 473-497.

Park EJ, Lim HT (1999). Establishment of an efficient *in vitro* plant regeneration system in chicory (*Cichorium intybus* L. var *sativus*). Acta. Hort. 483: 367-370.

Peddaboina V, Thamidala C, Karampuri S (2006). *In vitro* shoot multiplication and plant regeneration in four Capsicum species using Thidiazuron. Sci. Horti. 107: 117-122.

Rady MR (2006). *In vitro* culture of *Gypsophila paniculata* L. and random amplified polymorphic DNA analysis of the propagated plants. Biol. Plant. 50: 507-513.

Ramage CM, Williams RR (2004). Cytokinin- induced abnormal shoot organogenesis is associated with elevated *Knotted1*-type homeobox gene expression in tobacco. Plant Cell Rep. 22: 919-924.

Rani V, Parida A, Raina SN (1995). Random amplified polymorphic DNA (RAPD) markers for genetic analysis in micropropagated plants of *Populus dltoides* Marsh. Plant Cell Rep. 14: 459-462.

Saxena PK, Gill R, Rashid A, Maheshwari SC (1982). Plantlets from Mesophyll protoplast of *Solanum surattense*. Plant Cell Rep. 1: 219-220.

Sinha A, Jain R, Kachhwaha S, Kothari SL (2010). Optimization of the level of micronutrient copper in the culture medium imporves shoot bud regeneration in Indian Ginseng [*Withania somnifera* (L.) Dunal.]. Nat. acad. Sci. let. (In Press). 33:11-16.

Sinha S, Roy RP, Jha KK (1979). Callus formation and shoot bud differentiation in anther culture of *Solanum surattense*. Can. J. Bot. 57: 2524-2527.

Tyagi P, Khanduja S, Kothari SL (2010). Micropropagation of *Capparis decidua* and assessment of clonal fidelity of the regenerated plants. Biol. Plant. 54: 126-130.

Vedel F, Quetier F, Bayen M (1976). Specific cleavage of chloroplast DNA from higher plants by *Eco*RI restriction nuclease. Nature, 263: 440-442.

Vos P, Hogers R, Bleeker M, Reijans M, Lee TVD, Hornes M, Frijters A, Potential J, Peleman J, Kuiper M, Zabeau M (1995). AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res. 23: 4407-4414.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990). DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18: 6531-6535.

Yucesan B, Turker AU, Gurel E (2007). TDZ-induced high frequency plant regeneration through multiple shoot formation in witloof chicory (*Cichorium intybus* L.). Plant. Cell. Tiss. Org. Cult. 77: 23-27.