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Micropropagation and clonal fidelity testing in *Solanum Capsicoides* All

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Abstract
Solanum capsicoides All. Is a valuable medicinal plant from which the therapeutic agent 'kantakari' is extracted. This is used in the treatment of various ailments especially respiratory problems. The plant is diminishing in natural habitat due to over exploitation by pharmaceutical industry and this urges for protection and conservation of the plant. The objective of the study was to standardize a protocol for the direct organogenesis of *Solanum capsicoides* All. The explants such as cotyledon, shoot tip, and hypocotyl inoculated on MS medium supplemented with auxins and cytokinins in different concentrations and combinations initiated direct shoot buds after 15-30 days of inoculation. Shoot tips inoculated on MS medium augmented with 2.46µM 2iP produced maximum shoots with an average of 15.5. The initial cultures were subcultured on different hormonal regimes of which MS medium containing 6.97µM KIN produced an average of 57 shoots from a single cotyledon explant. The shoots thus obtained were rooted, acclimatized and transferred to field conditions. Clonal fidelity of regenerated plant was studied using ISSR marker. The polymorphism percentage ranged from 0 to 40% and the PIC value varied from 0 to 0.38 thus revealing low level of diversity. The protocol developed can thus be utilized for the mass multiplication and conservation of *S. capsicoides*.

Keywords: *solanum capsicoides*, direct organogenesis, cytokinins, auxins, issr

1. Introduction

Solanum capsicoides All. is an important medicinal plant of the family Solanaceae, which is commonly known as soda apple or cockroach berry. This plant is used as an alternate source of Kantakari (*Solanum xanthocarpum*), an important therapeutic agent which is used for the treatment of various respiratory problems [1]. The plant is also used for the treatment of ulcerated nose, toothache [2] and apoplexy [3].

Apprehension on health hazards and toxicity of synthetic drugs and antibiotics have increased the demand for herbal drugs [4] which in turn led to large scale collection of whole plants by uprooting. This over exploitation has resulted in the wiping out of many medicinally important plants from their natural habitats [5]. The medicinal plants that are diminishing and in danger of extinction due to ruthless exploitation are in need of protection and conservation as they are essential commodity of health care [6]. Conservation through ex situ method by growing the whole plants in botanical gardens or by seed storage is carried out globally [7]. Alternate method includes the application of biotechnological techniques such as tissue culture and cryopreservation [8]. Advantage of micropropagation over conventional propagation is the rapid propagation in limited time and space [9]. For mass propagation of any plant species through tissue culture technique, a protocol has to be primarily optimized since the culture requirements for each species vary.

Plant tissue culture can generate variations in regenerated plants and thus the micropropagated plants may show variations from parent plants [10]. Somaclonal variation is a phenomenon which occurs *in vitro* that leads to the development of genetically dissimilar plants [11]. Genetic variability in regenerated plants can be attributed to the chromosomal or gene level mutations [12]. Morphological, biochemical and cytological markers have been routinely used in the assessment of clonal variability among regenerated plants. Molecular markers are heritable, stable and reproducible and hence turned out to be the most reliable method for screening genetic stability among cultures [13].

Among the various molecular markers, Inter Simple Sequence Repeats (ISSR) markers are preferred over Random Amplification of Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP) etc. So ISSR markers are usually used to assess genetic stability of micro propagated plants [14] ISSR is genetically variable and generate multilocus data from the target genome in a single reaction. ISSR is highly reproducibility when compared to routine RAPD techniques and at the same time it is quick and less expensive than AFLP. The annealing temperature depends on the melting point of the primer used and is higher than that of RAPD [15]. In addition they are simple, highly polymorphic and do not require the use of radioactivity [16].

Thus the main objective of the present study was to standardize an efficient and reproducible regeneration protocol in *S. capsicoides* using different explants such as cotyledons, shoot tip and hypocotyl in MS medium supplemented with different concentrations and combinations of various auxins and cytokinins and to check the clonal fidelity of micropropagated plants in comparison with control plants (seed propagated) using ISSR markers.

2. Methods

2.1. Collection and Identification of Plant Material

Mature, healthy plants of *Solanum capsicoides* All. were collected from Ernakulam District of Kerala, identified using referral herbaria and voucher specimens were deposited at Kerala Forest Research Institute (KFRI), Peechi, Kerala, with herbarium number KFRI 13056.

2.2. Inoculation of Explants

Ripened fruits were surface sterilized in 0.1% mercuric chloride ($HgCl_2$) solution for 15 min and were rinsed with sterile distilled water at least five times, 5 min for each wash. Seedlings at two cotyledonary leaf stage (3 - 4 cm long) were selected to collect the explants such as cotyledons, shoot tip and hypocotyl. The excised explants were inoculated and the tubes were incubated in the culture room. The optimum conditions such as 25 ± 2˚C temperature, 50% - 60% humidity and with a photoperiod of 16 hrs/day provided by cool white fluorescent lamps with an intensity of 2500-3000 Lx were maintained in the culture room.

2.3. Direct Shoot Regeneration

Cotyledon, shoot tip and hypocotyl explants of 0.5cm length were inoculated on semi solid MS (Murashige and Skoog) medium augmented with 0.1 – 1.0 mg/l of auxins such as 2,4-D (2,4-Dichlorophenoxyacetic acid), IAA (Indole 3-acetic acid), IBA (Indole 3-butyric acid) and NAA(α-Naphthyl acetic acid) and cytokinins such as 2iP (2 Isopentenyl adenine), BAP (Benzyl adenine), KIN (Kinetin) and ZEA (Zeatin) in different concentrations and combinations. After 45 days of incubation, the explants with shoot buds were subcultured on fresh semisolid MS medium fortified with 2.46 µM 2iP, 3.08 µM BAP, 6.97 µM KIN, 6.84 µM ZEA, 1.17 µM IAA or 2.46 µM IBA. Subculture was also done on MS medium supplemented with 0.57 µM IAA in combination with 2.46 µM 2iP, 2.64 µM BAP, 3.22 µM KIN or 2.28 µM ZEA. After 30 days of subculture the number and length of shoots produced were recorded. Twelve samples were used for every explant in each experiment and the experiment was repeated thrice.

2.4. Rooting, Hardening and Transplantation

The regenerated shoots with an average length of 4 cm were rooted in half strength liquid MS basal medium. The plantlets thus obtained were transferred to sterile cocopeat wetted with half strength MS liquid medium without sugar and were acclimatized. The acclimatized plantlets were transferred to pots filled with potting mixture containing soil and cow dung in the ratio 1:1 and were kept in the Botanical Garden, Union Christian College, Aluva for field evaluation and molecular characterization.

2.5. Histological Studies

Anatomical sections of explants with directly regenerated shoot buds were taken using microtome (Microm HM 325 model) and were photo micro graphed after proper staining using Olympus trinocular microscope attached with Magnus microscope camera.

2.6. Clonal Fidelity Analysis Using ISSR

Young leaves of both control and tissue cultured plants were collected and DNA was isolated following CTAB method [17]. Gel electrophoresis was used to determine the DNA integrity. Sixteen ISSR primer pairs were selected on the basis of previous literature on *Solanum* species of which only 5 primers gave clear and distinct bands, which were further used for the clonal fidelity analysis of micro propagated plants. The sequence and the annealing temperature of primers are given in table 1. The primers were diluted and the Tm was noted. Annealing temperature was taken as (Tm – 5). PCR was carried out with a total volume of 20µl reaction mixture. The PCR conditions consisted of an initial denaturation step at 94˚C for 3 min; 35 cycles of 1 min at 94˚C, 1min at respective annealing temperature, 2 min at 72˚C followed by a final elongation step at 72˚C for 10min.

2.7. Gel Electrophoresis

2.5% gel was prepared for the better separation of bands. 1µl of loading dye was mixed with the total volume of PCR product and were loaded into the wells along with a 100 bp ladder. The gels were marked and documented using Gel Doc for band scoring.

2.8. Statistical Analysis

The data obtained after six replicates of experiments were analyzed using Analysis of Varianace (ANOVA) with post hoc-Tukey test using IBM SPSS Statistics version 21. Data were presented as mean and standard error and significance difference were accepted at $p \leq 0.05$. For ISSR analysis presence of band was scored as 1 and absence was scored as 0. Percentage polymorphism and polymorphism information content were calculated. The polymorphism information content (PIC) value was calculated using the formula $PIC = 1 - \left[(p)^2 + (q)^2\right]$ [18], where, p= frequency of allele bands present, q= Frequency of allele bands absent. Percentage polymorphism was calculated by the formula.

$$\frac{No.of\ polymorphic\ bands}{Total\ no.of\ bands} \times 100$$

3. Results & Discussion

The explants inoculated on MS medium supplemented with different concentrations and combinations of auxins such as IAA or IBA and cytokinins such as 2iP, BAP, KIN or ZEA showed an initial enlargement after three days followed by bulging of the cut ends after 7 days. After 15-30 days of culture all explants at their cut ends enlarged profusely and initiated minute buds (Fig. 1 a). The micro shoot buds later enlarged and produced fully developed shoots. The direct development of shoots from the explants was confirmed by microtome sections (Fig. 1b).

3.1. Organogenesis

Among the three explants inoculated on MS medium fortified with various cytokinins, maximum number of shoots was developed from shoot tip explants within 29 days after inoculation on MS medium augmented with 2.46µM 2iP with an average of 15.5 ± 2.12 ($p < 0.05$) with 0.65 ± 0.7 cm mean length (Table 2; Fig. 1 c). The different explants inoculated on MS medium complemented with IAA, NAA, IBA and 2, 4-D responded differently. When NAA and 2, 4-D were used only callus development was observed. When IAA was incorporated to the culture medium hypocotyl explants produced a maximum of 8.5 ± 0.5 shoots ($p < 0.05$) after 45 days of incubation with an average length of 0.55 ± 0.05cm (Fig. 1 d). In the case of IBA, hypocotyl explants responded well with an average of 2.66 ± 0.8 shoots per explant with an average length of 3.33 ± 0.6cm. When IAA in combination with cytokinins was used direct organogenesis was observed in all explants among which shoot tip produced maximum plantlets. Shoot tip explant inoculated on MS medium supplemented with 0.57 µM IAA in combination with 2.28µM ZEA produced a

maximum shoots of 10 shoots ($p > 0.05$) with a mean length of 1cm. (Fig. 1e).

The explants with regenerated shoots were subcultured on the medium that produced maximum shoots during the initial cultures. Among the various explants, cotyledons produced maximum direct shoots after subculture in 6.97 µM KIN with an average of 57 shoots (Table 3; Fig. 1f). Effect of auxins on shoot multiplication was found to be less when compared to cytokinins. Subculture of regenerated shoots on medium supplemented with1.17 µM IAA produced a maximum of 6 shoots and the subculture medium augmented with IBA (2.46 µM) also produced 6 shoots. Various cytokinins in combination with IAA were also found to be effective for shoot multiplication through subculture. Both cotyledon and shoot tip explants inoculated on medium supplemented with 3.22 µM KIN and 0.57 µM IAA developed a maximum of 22 ($p < 0.05$).

3.2. Rooting, Hardening and Transplantation

Shoots inoculated in half strength MS medium produced roots within 7 days of culture (Fig. 1g) and the number of roots developed in regenerated plants was almost similar in plantlets derived from different hormonal media. The regenerated plantlets after field transfer showed a survival rate of 91.6% (Fig. 1h).

3.3. ISSR Analysis

A total of 24 bands were generated by 5 primers. The banding pattern was compared between seed propagated and directly regenerated plants (Fig. 2). The polymorphism percentage ranged from 0 to 40% and the PIC value varied from 0 to 0.38 (Table 4). This low PIC value is suggestive of low genetic diversity which in turn is a proof for clonal identity of regenerated plants.

The regeneration of plantlets from the explants directly without any callus stage is direct organogenesis. In the present study direct organogenesis was observed from all the explants inoculated on MS medium supplemented with auxins and cytokinins in different concentrations and combinations. When cytokinins alone were used 2iP produced higher number of plantlets when compared to BAP, KIN and ZEA. Plant regeneration from embryogenic calli was reported in *Allium porrum* L. by the application of 2iP [19]. 2iP has promoted shoot proliferation in *Rhododendron* [20], *Sophora tonkinensis* [21], and *Santalum album* [22] etc. In Indian ginseng (*Withania somnifera* L.) also 2iP induced shoot regeneration via direct organogenesis [23]. In our experiments, shoot tip explants produced best results in direct regeneration. Shoot tip explants accumulates more amounts of cytokinins since it is the growing point. Hence the increased regeneration of direct shoots can be attributed to the additive effect of already present cytokinins in combination with the exogenously supplied growth regulators. Similar effects of cytokinins were reviewed by Hill and Schaller (2013) during organogenesis [24].

When the explants were inoculated on MS medium augmented with IAA or IBA, direct shoot multiplication was observed. In the present study hypocotyl explants produced considerable number of plantlets when cultured *in vitro* in presence of auxins. MS basal medium fortified with IAA, NAA or IBA induced shoot regeneration in *Metabriggsia ovalifolia* W. T. Wang has been reported by Ouyang *et al*. (2016) [25].

Mutasim *et al.* (2010) reported that when explants were inoculated on MS medium supplemented with ZEA in combination with IAA, maximum shoot regeneration was observed from shoot tip explants.[26] Similar results were observed in *S. lycopersicum Diospyros kaki* [27, 28]. IAA and ZEA are naturally occurring auxins and cytokinins respectively in higher plants [29, 30]. According to Skoog and Miller (1957), the differentiation of a plant tissue into either root or shoot depends upon the ratio of auxin to cytokinin *in vitro* [31]. High auxin / cytokinin ratio led to root formation whereas low auxin / cytokinin ratio resulted in shoot formation and intermediate levels resulted in the development of undifferentiated callus from plant tissues. But in the present study contrary results were obtained. Though shoot formation was observed in all cultures, root development was found to be negligible. This may be due to the inhibitory effect of endogenous cytokinins on root growth or mutation in auxin signal transduction pathways [29, 30].

After studying the initial response of cultures by observing for 45 days, they were subcultured in shoot multiplication medium. After 30 days of subculture on medium supplemented with auxins such as IAA and IBA, maximum multiplication of shoots was observed in explant inoculated on IAA containing medium as that observed during the initial cultures. But among various cytokinins tested, maximum shoot regeneration was observed from explants inoculated on MS medium containing KIN on the contrary to 2iP that gave maximum shoots in the initial phase. When the explants were treated with cytokinins in combination with IAA, maximum shoot proliferation was observed on medium containing both KIN and IAA. But during initial cultures ZEA and IAA combinations produced best results. The effect of KIN alone or in combination with IAA in maximum shoot regeneration was reported in *Populus ciliate*, [32] *Matthiola incana*, [33] *Mentha piperata*,[34] *S. melongena* [35, 36] and *Cucumis sativus* [37]. These results support the direct shoot regeneration in *S. capsicoides* mediated by the action of IAA in combination with KIN.

When the genetic stability of regenerated plants in present study was analysed, polymorphism was detected. The average percentage of polymorphism was 15.2% which is considered as a low value for polymorphism. This suggests the low level of genetic diversity among the regenerated plants and the genetic uniformity among them. Similar results were reported by Olatunji and Afolayan (2019) among the varieties of *Capsicum annuum* L. and *Capsicum frutescens* L [38].

The clonal similarity obtained among the regenerated plants of *S. capsicoides* was consistent with the data obtained in other plants such as *Solanum tuberosum*, [39] *Saccharum officinarum* etc [40].

Table 1: List of primers with sequence, size (bp) and annealing temperature

Sl. No.	Primer names	Primer sequence 5'-3'	Length (bp)	Annealing Temperature (°C)
1	ISSR 808	AGAGAGAGAGAGAGAGC	17	47
2	ISSR 809	AGAGAGAGAGAGAGAGG	17	47
3	ISSR 810	GAGAGAGAGAGAGAGAT	17	45

4	ISSR 814	CTCTCTCTCTCTCTCTA	17	45
5	UBC 812	GAGAGAGAGAGAGAGAA	17	45
6	ISSR 816	CACACACACACACACAA	17	45
7	ISSR 817	CACACACACACACACAA	17	45
8	ISSR 818	CACACACACACACACAG	17	47
9	UBC 825	ACACACACACACACACT	17	45
10	UBC 827	ACACACACACACACACG	17	47
11	UBC 840	GAGAGAGAGAGAGAGAT	17	47
12	UBC 820	GTGTGTGTGTGTGTGTC	17	47
13	ISSR 815	CTCTCTCTCTCTCTCTG	17	45
14	UBC 819	GTGTGTGTGTGTGTGTA	17	45
15	UBC 830	TGTGTGTGTGTGTGTGC	17	45
16	UBC 847	CACACACACACACACAC	17	47

Table 2: Initial effect of various cytokinins on direct shoot regeneration

PGR (µM)				No. of shoots			Shoot length (cm)		
2iP	BAP	KIN	ZEA	C	S	H	C	S	H
0.49				0	0	4.5±0.70^{a*}	-	-	0.65±0.49^{a}
1.47				2.5±0.7^{a}	0	1.5±0.7^{a}	0.25±0.07^{a}	-	0.2±0^{a}
2.46				0	15.5±2.12^{c}	0	-	0.65±0.7^{b}	-
4.92				0	6±2.6^{ab}	7.5±9.19^{a}	-	0.5±0.2^{ab}	0.4±0.14^{a}
7.38				5±1.41^{a}	11.6±4.16^{bc}	2.75±3.5^{a}	0.5±0^{b}	0.36±0.15^{ab}	.37±0.41^{a}
9.84				0	0	1±0^{a}	-	-	0.25±0.07^{a}
12.3				1.5±0.7^{a}	9±1.73^{bc}	1.5±0.70^{a}	0.25±0.07^{a}	0.83±0.28^{b}	0.15±0.07^{a}
	2.22			5±0.6^{cd}	4.6±1.4^{ab}	3.8±1.39^{a}	1.7±0.5^{b}	0.88±0.53^{a}	0.48±0.1^{a}
	2.64			4.5±0.78^{bcd}	5.83±0.9^{ab}	4.6±1^{a}	1±0.39^{ab}	1.06±0.39^{a}	1.61±0.72^{a}
	3.08			4.9±0.56^{cd}	7.4±1.1^{b}	3.33±0.7^{a}	0.67±0.15^{a}	0.54±0.36^{a}	0.7±0.12^{a}
	3.52			5.75±0.57^{d}	5.66±0.76^{ab}	5±1.2^{a}	0.45±0.08^{a}	1.01±0.4^{a}	1±0.27^{a}
	3.96			4.36±0.36^{bcd}	6.16±0.74^{ab}	3.2±08^{a}	0.4±0.09^{a}	1.28±0.7^{a}	0.42±0.15^{a}
	4.44			3.16±0.34^{abc}	5±0.68^{ab}	4±1^{a}	0.65±0.1^{a}	0.51±0.2^{a}	0.2±0^{a}
	6.66			4.09±0.53^{bcd}	4.5±1.04^{ab}	2.8±0.37^{a}	0.3±0.04^{a}	0.25±0.08^{a}	0.6±0.23^{a}
	8.88			3.25±0.61^{abcd}	4.66±0.8^{ab}	3±1^{a}	0.36±0.08^{a}	0.16±0.03^{a}	1±0^{a}
	11.1			2±0.33^{ab}	0	2.66±0.3^{a}	0.28±0.08^{a}	-	0.6±0.13^{a}
	13.32			1.55±0.17^{a}	2±0.7^{a}	1.5±0.5^{a}	0.2±0^{a}	0.17±0.02^{a}	0.4±0.1^{a}
		2.32		6.5±0.5^{b}	4±1^{a}	6±1.7^{a}	1.33±0.3^{b}	1.9±0.1^{b}	1.83±1.09^{a}
		3.22		1±.0^{a}	6±2^{a}	7.5±3.5^{a}	0.5±.0^{ab}	0.25±0.05^{a}	0.25±0.05^{a}
		4.14		-	3±0^{a}	3.5±0.5^{a}	-	0.35±0.05^{a}	0.35±0.05^{a}
		4.65		7±1^{b}	5.33±1.5^{a}	9.5±3.5^{a}	0.35±0.15^{a}	0.3±0.2^{a}	1.5±1^{a}
		5.57		1±.0^{a}	-	2±1^{a}	0.3±0^{a}	-	0.35±0.15^{a}
		6.03		-	-	5.5±0.5^{a}	-	-	0.4±0.1^{a}
		6.49		-	-	8±.0^{a}	-	-	0.25±0.05^{a}
		6.97		10±.0^{c}	-	5±.0^{a}	0.6±0.1^{ab}	-	0.35±0.15^{a}
		9.3		2±.0^{a}	-	-	0.2±0^{a}	-	-
			0.46	0	0	4±.0^{a}	-	-	0.3±0.1^{a}
			1.38	-	-	-	-	-	-
			2.28	5.14±1^{a}	6.66±1^{a}	5.4±1.5^{a}	0.64±0.17^{a}	0.46±0.1^{a}	0.82±0.23^{a}
			4.56	7±1.5^{a}	8.5±1.1^{a}	4.4±1.4^{a}	0.6±0.16^{a}	0.95±0.16^{a}	0.74±0.22^{a}
			6.84	6.66±2.5^{a}	11±9.8^{a}	5±1^{a}	0.78±0.09^{a}	0.35±0.15^{a}	0.65±0.15^{a}
			9.12	3.25±1.3^{a}	4.5±0.5^{a}	3±0^{a}	0.35±0.1^{a}	1±.0^{a}	1±0^{a}
			11.4	0	9.33±1.4^{a}	0	-	0.45±0.12^{a}	-

C-Cotyledon, S-Shoot tip, H-Hypocotyl *Means in each column followed by same letters were not significantly different according to Tukey test at $p \le 0.05$

Table 3: Effect of cytokinins on direct shoot multiplication through subculture after 30 days

PGR (µM)					No. of shoots			Shoot length (cm)		
2iP	BAP	KIN	ZEA	Subculture medium	C	S	H	C	S	H
2.46				2.46 2iP	-	21.33±2.51^{a}	-	-	4.65±0.4^{b}	-
4.92					-		18.66±2.08	-		3.46±0.47
7.38					-	19±2.64^{a*}	-	-	2.33±0.28^{a}	-
12.3					-	20.33±2.54^{a}	-	-	2.56±0.37^{a}	-
	2.22			3.08BAP	15.57±4.1^{b}	10.6±6.1^{ab}	4±1^{a}	5.14±0.73^{d}	2.17±0.33^{a}	1.1±0.4^{a}
	2.64				9±2.2^{ab}	7.33±1.4^{ab}	18.6±4.6^{b}	3±1.0.8^{bcd}	4±2^{a}	3.5±0.5^{a}
	3.08				9.87±1.4^{ab}	11±5^{ab}	4.75±0.8^{a}	2.15±0.34^{abc}	3.12±0.65^{a}	1.87±0.8^{a}
	3.52				15.5±4.1^{b}	10±2.3^{ab}	8±1^{a}	3.87±0.52^{cd}	3.62±1^{a}	3.76±2.2^{a}
	3.96				4.75±0.67^{a}	9.75±2.1^{ab}	5.75±1.5^{a}	1.65±0.58^{abc}	3.25±0.5^{a}	0.85±0.23^{a}
	4.44				6.08±1^{ab}	9.5±1.8^{ab}	4.6±1.6^{a}	1.57±0.34^{abc}	4.8±2.6^{a}	0.85±0.35^{a}
	6.66				9.55±1.5^{ab}	18±5^{ab}	5.4±1^{a}	2.17±0.46^{abc}	2.7±0.41^{a}	1.88±0.47^{a}

	8.88				11.25±2.7^{ab}	12.25±3^{ab}	5±0.5^{a}	2.25±0.23^{abc}	3.4±0.76^{a}	1.56±0.23^{a}
	11.1				3.6±0.71^{a}	24.5±5^{b}	6.3±2^{a}	1.05±0.26^{abc}	3.41±0.5^{a}	2.33±0.7^{a}
	13.32				2.66±0.61^{a}	3±0.4^{a}	2.5±0.5^{a}	0.51±0.08^{a}	2.4±0.5^{a}	1.5±.0^{a}
		2.32		6.97KIN	-	15±1.7^{a}	14±2^{a}	-	3.16±0.4^{a}	2.72±1.2^{a}
		3.22			-	42±4.6^{b}	-	-	10±2^{a}	-
		4.65			-	25±2.3^{a}	20±7^{a}	-	7.3±1^{a}	3.8±1^{a}
		6.97			36±2.3	-	29±7^{a}	2.33±0.16	-	3.18±0.3^{a}
		9.3			**57±4.6**	-	-	**5.33±1**	-	-
			2.28	6.84ZEA	12±1	15.33±1.7^{a}	16±2.3^{a}	3.26±0.25	2.33±0.6^{a}	1.83±0.44^{a}
			4.56		-	16±1.1^{a}	13±2.8^{a}	-	2.16±0.16^{a}	1.6±0.1^{a}
			6.84		-	15±1^{a}	11±0.5^{a}	-	2.16±0.16^{a}	1±0.11^{a}

C-Cotyledon, S-Shoot tip, H-Hypocotyl *Means in each column followed by same letters were not significantly different according to Tukey test at $p \leq 0.05$

Table 4: Results of clonal fidelity test

Sl. No.	Primer name	Total no. of bands	No. of polymorphic bands	Polymorphism (%)	PIC Value
1	ISSR 808	5	0	0	0
2	ISSR 818	4	0	0	0
3	UBC 812	5	2	40	0.38
4	UBC 820	5	1	20	0.19
5	UBC 827	5	1	20	0.19
	Total	24	4		

Fig 1: Various stages of *in vitro* propagation of *Solanum capsicoides* through direct organogenesis: a) Shoot bud initiation from hypocotyl explant, b) Microtome sections of hypocotyl explant showing direct origin of shoot buds, c) Shoots developed in cytokinin containing medium from shoot tip, d) Shoot regeneration from Hypocotyl explants on MS medium with IAA, e) Shoots regenerated through combined effect of IAA and ZEA from cotyledon, f) Shoot multiplication in 1.5 mg/l KIN from cotyledon after subculture, g) Rooting in half strength MS basal liquid medium, h) Field transferred mature plants

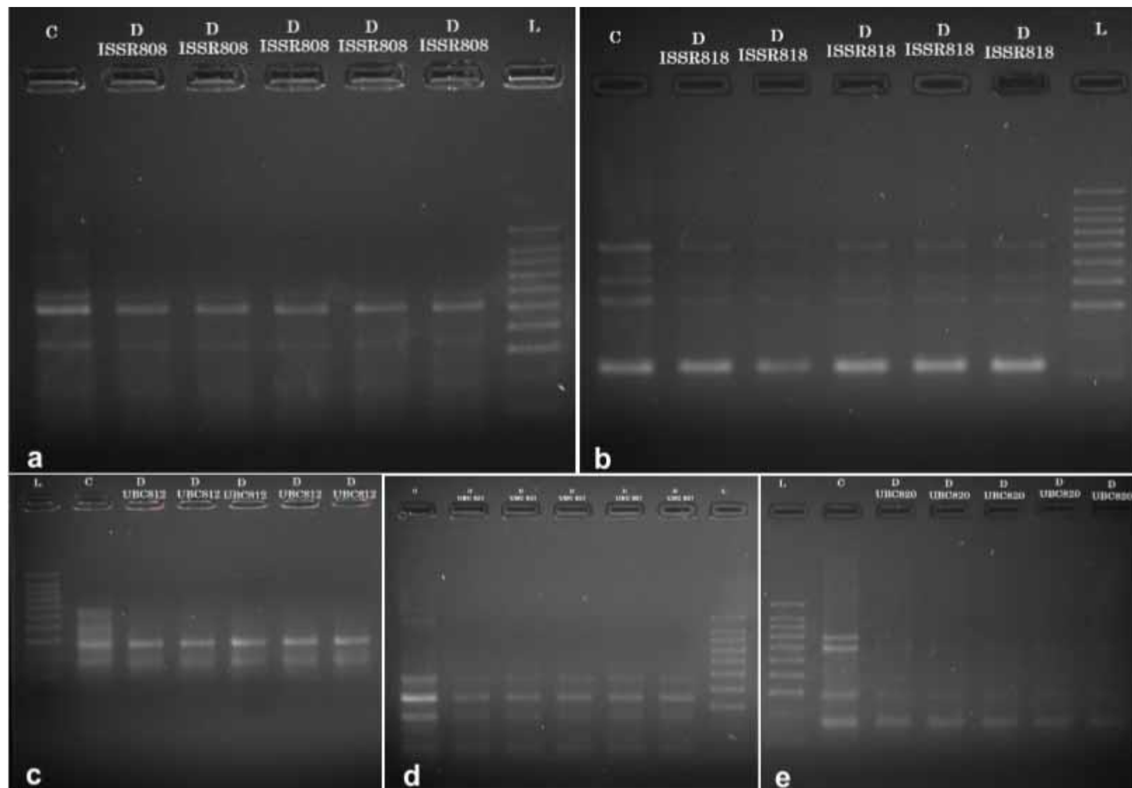

Fig 2: ISSR profiles of directly regenerated plants; C-Control, D-directly regenerated plants, L-100bp ladder; a) ISSR 808, b) ISSR 818, c) UBC 812, d) UBC 827, e) UBC 820

4. Conclusions

The *in vitro* propagation system via direct organogenesis has resulted in the large scale multiplication of genetically identical plants in *S. capsicoides* as envisaged in the objective of the study. MS medium supplemented with 2.46µM 2iP gave best results in the initial cultures within one month from shoot tip explants. However, cotyledon explants with initial shoot buds on subculture in MS medium with 6.66 µM KIN produced a maximum of 57 shoot buds from cotyledon explants. The regenerated shoots were rooted in half strength MS basal liquid medium and on field transfer 91.6% survival rate were observed. Thus the protocol developed can be used for the mass multiplication and conservation of *S. capsicoides.*

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