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Callus Induction and Plant Regeneration in *Solanum tuberosum* L. cultivars (Kufri Chipsona 3 and MP-97/644) via Leaf Explants

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Abstract

The aim of this study was to establish a protocol for rapid callus induction and plant regeneration of potato. The leaf explants of two potato (Solanum tuberosum L.) cultivars viz. (Kufri Chipsona 3 and MP-97/644) were cultured for callus induction and plant regeneration. Best callus growth from both the cultivars was observed on Murashige and Skoog (MS) (1962) media containing 3.0 mg/l of 2,4-D (2,4-dichlorophenoxy acetic acid) and1.0 mg/l of kinetin. MS medium supplemented with different concentrations and combinations of BA, Kinetin and $AdSO_4$ were employed for shoot regeneration. Best shoot regeneration from callus was observed on MS media containing 1.5 mg/l BA (6-benzyladenine) and 25.0 mg/l $AdSO_4$ (Adenine sulphate). The mean number of shoots/callus clump was 21.00 (Kufri Chipsona 3) and 18.67 (MP-97/644) after 60 days of inoculation. When the plants were transferred to green house, primary hardening was achieved within 10 days and about 99% plants survived.

Keywords: leaf explants, 2,4-dichlorophenoxyacetic acid (2,4-D), MS medium, *Solanum tuberosum* L.

Introduction

Potato (*Solanum tuberosum* L.) is the most important non-cereal food crop of the world. In monetary terms it ranks fourth in the world after wheat, rice and maize[1]. It produces the largest quantity of carbohydrates per day per unit area among the food crops. Potato recommended as food security crop, is consists of 80% water, 2-3% protein and 18% carbohydrate. Central Potato Research Institute, Shimla, India over the past 55 years has resulted in the development of 35 high yielding potato varieties for diverse agro climatic conditions and innovation of seed plot technique for augmenting the seed production. ICAR (Indian Council of Agricultural Research) has identified a new hybrid variety 'Kufri Chipsona-3'of potato for release. Kufri Chipsona-3 can be used both for processing (chips and flakes) as well as table purposes.

Tissue culture techniques are used worldwide to produce pre-basic, virus-free seed potatoes known as microtubers. Several tissue culture techniques have been used for huge number of potato cultivars[2]. Recently *in vitro* conservation protocol for potato is also confirmed[3]. Disease free and genetically uniform plantlets may be produced by callus culture[4]. The callus induction in potato was first observed by Steward and Caplin[5]. However, the past studies have shown that *in vitro* callus induction in *S. tuberosum* cultivars has been reported by few authors from different explants including nodal, intermodal and leaf explants[6], internodal and leaf explants[7], nodal explant[8], internodal explants[9, 10], stem segment[11, 12], and leaf discs[13, 14]. It is important to establish the protocol for plant regeneration through callus culture of two important indian potato cultivars (Kufri Chipsona 3 and MP-97/644). Regenerated plantlets through callus are genetically identical, disease free and produce a large number of plantlets in a very short period of time. Therefore, our objective is to introduce an efficient protocol for callus induction and regeneration from the leaf explants of potato cultivars (Kufri Chipsona 3 and MP-97/644).

Material and Methods

Plant material and callus induction: Two potato cultivars (Kufri Chipsona 3 and MP/97-644) were obtained from Central Potato Research Institute, Shimla. In our study leaf explants of both cultivars were chosen for callus induction. Leaf explants were washed thoroughly under tap water for 15 minutes, and then all the explants were treated with 0.2% bavistin for 10 minutes. Then all the explants were washed thoroughly with distilled water (4-5 times). The explants were further treated with 2-3 drops of teepol for 3-5 minutes followed by thorough washing with distilled water. The explants were then surface sterilized with 0.1 % (W/V) $HgCl_2$ for 5 minutes under laminar airflow. After that, explants were washed 4-5 times with sterile double distilled water to remove traces of mercuric chloride. The leaf explants, approximately 1.0 cm size, were excised and inoculated on MS[15] media containing different plant growth regulators (PGRs) (table 1). All culture bottles contained 25 ml of agarified medium. All media contained 30 g l^{-1} sucrose and pH was adjusted to 5.8 with 1.0 N HCl or 1.0 N NaOH before adding 0.8 % agar and autoclaving at 121 ºC for 20 min. Culture bottles were placed in a culture room at 25 ± 2 ºC and exposed to 40 µmol m^{-2} s^{-1} fluorescent light in a 16h photoperiod. Callus initiation was observed within 7-10 days of inoculation.

Shoot regeneration from Callus: Callus induced on MS media containing different combinations of 2,4-D and kinetin were subcultured on MS media incorporated with different concentrations of BA and kinetin as well as BA and $AdSO_4$ for shoot regeneration and their effects on the number of shoots regenerated were recorded at regular time intervals (table 2, 3, 4 and 5).

Sprouting from Microtubers: The shoot regeneration potential of microtubers formed on media supplemented with different hormones was verified *in vitro*. The media supplemented with different concentrations and combinations of BA and $AdSO_4$ showed varied response after 60 days of culture (table 6 and 7).

Root Induction: The plantlets of *S. tuberosum* cultivars (Kufri Chipsona 3 and MP-97/644) grown *in vitro* showed self rooting in the multiplication media if kept for a longer period. No different hormonal regime was provided to induce rooting.

Hardening of *in vitro* plantlets: The rooted plantlets were first transferred to protray containing 50% moist coco peat and 50% vermicompost as potting mix and kept in poly tunnel for 10 days in the green house to provide humidity. After 10 days, they were taken away from poly tunnel and found healthy. The plantlets were transferred to polybags containing soil: sand: FYM in1:1:1 proportion and kept in net house. About 99% plants survived in the green house and the plants were in healthy condition when transferred to the field also.

Statistical analysis: The callus induction and shoot regeneration experiments were conducted with a minimum of three replicates and all experiments were repeated three times. Data obtained from all experiments were presented as the mean ± SE of three replications. Statistically significant differences were determined by analysis of variance (ANOVA) and the Duncan multiple range test (DMRT) at a $P < 0.05$ level of significance.

Results and Discussion

In the present study complete regeneration was successfully achieved from *in vivo* leaf explants of *S. tuberosum* cultivars (Kufri Chipsona 3 and MP-97/644) through callus culture (figure 1 and 2). The sterilized leaves were inoculated on MS media containing different concentrations of 2,4-D (1.0-3.0 mg/l) and kinetin (1.0-3.0 mg/l). Callus induction from leaves of *S. tuberosum* on callusing media were observed and recorded periodically at 10 days interval (10 days, 20 days, 30 days and 40 days). Leaf explants induced callus on MS medium supplemented with different concentrations of 2,4-D and kinetin and their response was observed at different time period (Table 1). It is well known that various combination and concentration of auxins and cytokinins are effective for callus induction[8, 9]. Theoretically, equal amount of auxin and cytokinin promotes callusing, but in practice it differs to a good extend may be due to the variation in endogenous level of phytohormones. In the present study, curling as well as swelling was observed initially from the periphery and later on all the surface of leaf on MS medium containing 3.0 mg/l 2,4-D and 1.0 mg/l kinetin after 10 days of inoculation. The most effective media for Kufri Chipsona 3 for callus induction was found with high concentration of auxin, 2,4-D (3.0 mg/l) in combination with low concentration of cytokinin, kinetin (1.0 mg/l). Similarly, cultivar MP-97/644 showed better response in the same media. The calli were creamy and light yellowish in color. Similarly, best callus response were found in different potato cultivars *viz*. Diamant, Multa, Atlus and Lalpakri on MS medium supplemented with (3.0 mg/l) 2, 4-D[7]. The present findings were also similar to Gavinlertvatana and Li[13] who induced creamy white friable callus from *S. tuberosum* on media containing 3.0 mg/l 2,4-D and 0.3 mg/l kinetin. In several other plant species also like *Swertia angustifolia* and *Swertia mussotti* higher concentration of 2,4-D (3.0 mg/l) and Kinetin (2.5 mg/l) exhibited best callusing in leaf explants[16, 17]. Onamu et al[18] obtained highest callus induction from different explants in three potato cultivars (Alfa, Cambray Rosa Morelos and Atlantic) on MS medium supplemented with 4.0 mg/l BA and 1.0 mg/l NAA. Afrasiab and Iqbal[6] reported best callogenesis on MS medium supplemented with 1.0 mg/l NAA and 0.5mg/l BA in cv. Diamant. In contrast, Haque et al[19] reported 2, 4-D (1.0 mg/l) + Kinetin (0.25 mg/l) to be best for callus length and weight for cv. Diamant. The present results are also in accordance with the results of Omidi and Shahpiri[20] who obtained callus from leaf and internodes on MS media containing 5.0 mg/l 2, 4-D and 0.25 mg/l kinetin that is high concentration of 2,4-D and low concentration of 0.25 mg/l kinetin. The interaction of both hormones had significant result on callus induction but low concentration of 2, 4-D with high concentration of kinetin was found ineffective.

In the present investigation BA in combination of $AdSO_4$ was essential for the regeneration of the calli. Callus induced from both cultivars (Kufri Chipsona 3 and MP-97/644) were subcultured on MS media supplemented with different concentrations of BA and Kinetin as well as BA and $AdSO_4$ for shoot regeneration and their effects on the number of shoots regenerated were recorded at regular time intervals (table 2, 3, 4 and 5). The different stages of shoot regeneration and multiplication from the callus clump of both cultivars are shown in (Figure-1 and 2). In the present study, best shoot regeneration per callus clump was observed on MS media containing BA (1.5 mg/l) and $AdSO_4$ (25.0 mg/l) where mean number was 21.00 (Kufri Chipsona 3) and 18.67 (MP-97/644) shoots/callus clump after 60 days of inoculation (Table 4 and 5). $AdSO_4$ (adenine sulphate) play a vital role for the mass multiplication as PGR. It is well known that cytokinins stimulate plant cell division and participate in the release of lateral bud dormancy, in the induction of adventitious bud formation, in the growth of lateral buds and in the cell cycle control[21, 22]. The benefits of adenine sulphate are often only noticed when it is associated together with cytokinins such as BAP or kinetin[23]. The simulative role of $AdSO_4$ in shoot multiplication has been emphasized from time

to time in various plants[24, 25]. Among all PGRs used, BA (1.5 mg/l) in combination with $AdSO_4$ (25.0mg/l) was the most effective for multiple shoot induction. A similar effect of BA was also observed by Bhuiyan[26]. Our results suggest that BA, in combination with Adenine sulphate, improves the process of organogenesis. The shoot regeneration gradually increased with time period. Although the media enriched with 2,4-D and kinetin also showed good response. Khatun et al[8] obtained 70 percent regeneration from callus on medium containing 5.0 mg/l BA, 1.0 mg/l IBA. But in our study reduced concentration of BA (1.5 mg/l) was found to be best for shoot multiplication. MS medium containing 4.0 mg/l Kinetin + 0.5 mg/l NAA was the best for maximum shoot regeneration from four potato cultivars[7]. Hamdi et al[27] reported regeneration from callus on MS media containing other hormones, 0.02 mg/l NAA, 2.0 mg/l Zeatin riboside and 0.02 mg/l gibberllic acid.

Table-1

Callus induction from leaves of *S. tuberosum* cvs. (Kufri Chipsona 3 and MP-97/ 644), In table columns with different letters are significantly different at *P*<0.05 according to Duncan's multiple range test

2,4-D + Kn (mg/l)	Induction of callus (%)							
	10 days		20 days		30 days		40 days	
	K.chip 3	MP-97/644	K.chip 3	MP-97/644	K.chip 3	MP-97/644	K.chip 3	MP-97/644
1.0+1.0	-	-	-	-	-	-	-	-
1.0+2.0	-	-	7.9c	2.9c	16.1c	11.6c	14.3c	9.8c
2.0+1.0	24.5b	22.9ab	38.3b	29.8b	54.9b	43.9b	54.6b	39.8b
2.0+2.0	31.7ab	27.3a	43.6b	39.5b	60.2b	48.6b	58.9b	41.1b
3.0+1.0	41.3a	36.1a	67.5a	58.6a	91.0a	80.6a	87.9a	78.1a
1.0+3.0	-	-	8.9c	-	12.9c	-	13.5c	-

- No callus

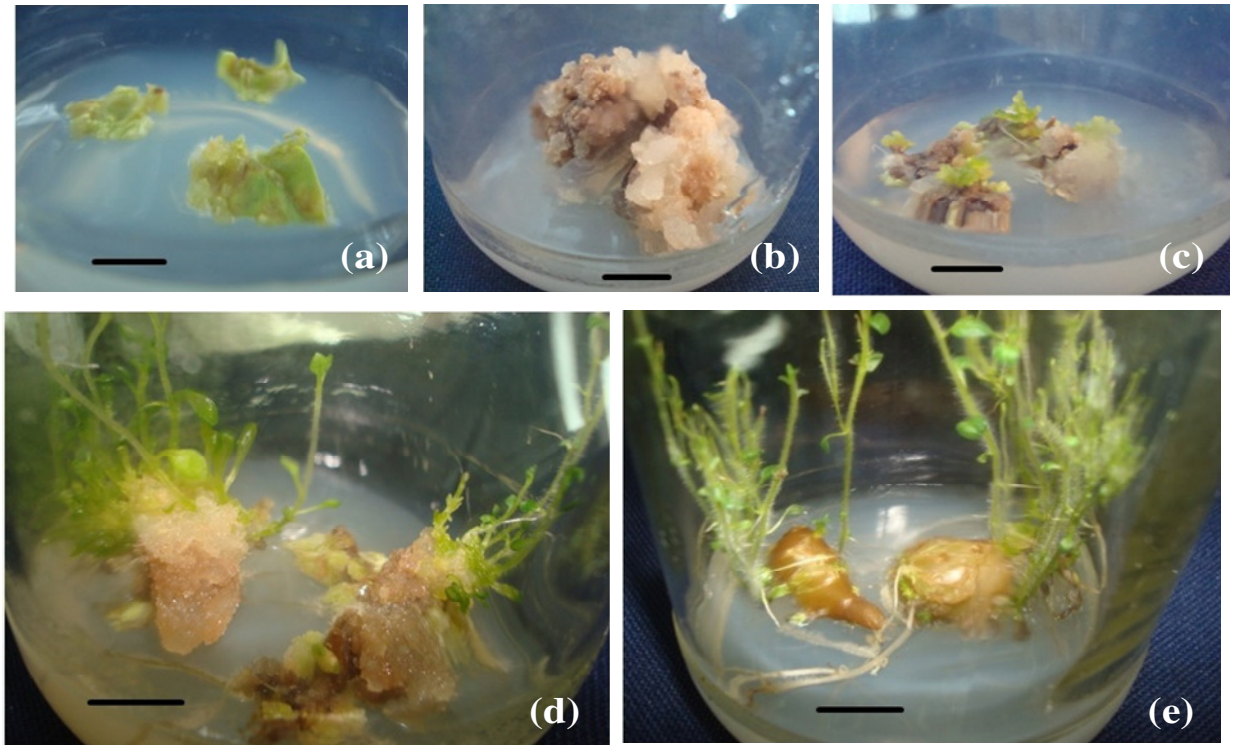

Figure-1

Different stages of callusing from the *in vitro* grown leaves of *S. tuberosum* (Cv. Kufri chipsona 3). (a) Curling and callus initiation from the periphery of leaves; (b) Callus induction after 30 days of inoculation (c) Shoot regeneration from callus after 20 days of inoculation; (d) Shoot regeneration from callus after 60 days of inoculation; (e) *In vitro* sprouting from microtubers of *S.tuberosum* L. (Kufri Chipsona 3) (Bars = 1.0 cm)

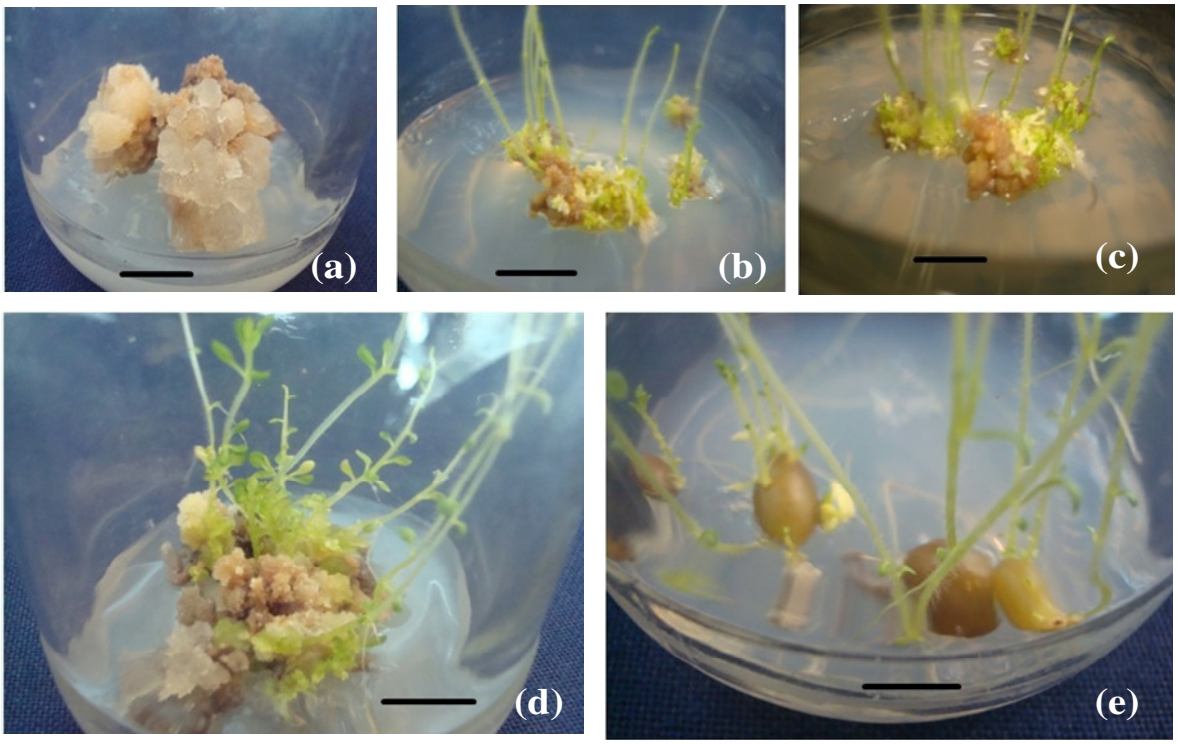

Figure-2
Different stages of callusing from the *in vitro* grown leaves of *S.tuberosum* (Cv. MP-97/644). (a) Callus induction after 30 days of inoculation; (b) Shoot regeneration from callus clump after 10 days of inoculation; (c) Shoot regeneration from callus after 20 days; (d) Shoot regeneration and multiplication; (e) *In vitro* sprouting from microtubers of *S.tuberosum* L. (MP-97/644) (Bars = 1.0 cm)

Table-2
Shoot regeneration from callus of Kufri Chipsona 3 *in vitro* induced by different concentrations of 2, 4-D and Kinetin. In table columns with different letters are significantly different at *P*<0.05 according to Duncan's multiple range test

2,4-D + Kn (mg/l)	Mean no. of shoots/ callus clump		
	20 Days	40 Days	60 Days
0.5 + 0.5	1.33 ± 0.58b	4.00 ± 0.33b	5.00 ± 0.88c
0.5 + 1.0	4.33 ± 0.67a	6.00 ± 0.58a	8.33 ± 0.33a
1.0 + 0.5	2.00 ± 0.88b	4.33 ± 0.67b	6.67 ± 1.00b

Table-3
Shoot regeneration from callus of MP-97/644 *in vitro* induced by different concentrations of 2, 4-D and Kinetin. In table columns with different letters are significantly different at *P*<0.05 according to Duncan's multiple range test

2,4-D + Kn (mg/l)	Mean no. of shoots/callus clump		
	20 Days	40 Days	60 Days
0.5 + 0.5	1.67 ± 0.67bc	2.33 ± 0.58c	3.67 ± 0.33c
0.5 + 1.0	3.00 ± 0.88a	4.33 ± 0.67a	7.33 ± 0.58a
1.0 + 0.5	2.00 ± 0.33ab	3.67 ± 0.33ab	5.00 ± 0.67b

Table-4
Effect of different concentrations of BAP and $AdSO_4$ on shoot regeneration from callus of Kufri Chipsona 3. In table columns with different letters are significantly different at *P*<0.05 according to Duncan's multiple range test

BA + $AdSO_4$ (mg/l)	Mean no. of shoots/callus clump		
	20 Days	40 Days	60 Days
1.0 + 15.0	2.67 ± 0.67bc	4.33 ± 0.88d	8.00 ± 0.58d
1.0 + 25.0	3.33 ± 0.58b	7.00 ± 1.00c	14.33 ± 0.88c
1.5 + 15.0	4.00 ± 0.33b	9.67 ± 0.67b	16.67 ± 0.33b
1.5 + 25.0	6.67 ± 0.67a	11.33 ± 0.88a	21.00 ± 0.58a

Table-5
Effect of different concentrations of BAP and $AdSO_4$ on shoot regeneration from callus of MP-97/644, In table columns with different letters are significantly different at *P*<0.05 according to Duncan's multiple range test

BA + $AdSO_4$ (mg/l)	Mean no. of shoots/callus clump		
	20 Days	40 Days	60 Days
1.0 + 15.0	2.00 ± 0.58cd	3.67 ± 0.88d	6.33 ± 1.00d
1.0 + 25.0	3.33 ± 0.67c	7.33 ± 1.00c	10.00 ± 0.33c
1.5 + 15.0	4.67 ± 0.88b	10.00 ± 0.33b	14.33 ± 0.67b
1.5 + 25.0	7.00 ± 0.33a	11.67 ± 0.88a	18.67 ± 0.58a

Table-6
Role of different concentrations of BA and $AdSO_4$ on shoot regeneration potential of microtubers of Kufri Chipsona 3, In table columns with different letters are significantly different at *P*<0.05 according to Duncan's multiple range test

BA + $AdSO_4$ (mg/l)	Mean no. of shoots/microtuber		
	20 Days	40 Days	60 Days
1.0 + 20.0	1.33 ± 0.33bc	2.67 ± 0.67c	4.33 ± 0.88c
1.0 + 25.0	4.00 ± 0.58a	7.33 ± 0.88a	9.67 ± 0.58a
1.5 + 20.0	2.33 ± 0.88b	4.33 ± 0.33b	6.00 ± 1.00b
1.5 + 25.0	1.67 ± 0.58bc	4.00 ± 1.00b	5.00 ± 0.67bc

Table-7
Role of different concentrations of BAP and $AdSO_4$ on shoot regeneration potential of microtubers of MP-97/644. In table columns with different letters are significantly different at *P*<0.05 according to Duncan's multiple range test

BA + $AdSO_4$ (mg/l)	Mean no. of shoots/microtuber		
	20 Days	40 Days	60 Days
1.0 + 20.0	1.00 ± 0.33bc	1.67 ± 0.58bc	2.67 ± 0.67cd
1.0 + 25.0	2.33 ± 0.67a	4.00 ± 0.88a	8.33 ± 0.67a
1.5 + 20.0	1.67 ± 0.88ab	2.33 ± 0.33b	5.00 ± 0.58b
1.5 + 25.0	1.33 ± 0.33bc	2.00 ± 0.88bc	3.67 ± 0.33c

The shoot regeneration potential of microtubers formed on media supplemented with different PGRs was verified *in vitro*. The MS medium supplemented with different concentrations and combinations of BA and $AdSO_4$ showed varied response after 60 days (table 6 and 7). The best response was on media with 1.0 mg/l BAP and 25.0 mg/l $AdSO_4$ with mean number of shoots 9.67 and 8.33 of Kufri Chipsona 3 and MP-97/644 respectively (figure-1e, 2e). The media supplemented with 1.5 mg/l BA and 25.0 mg/l $AdSO_4$ also showed good response. The plantlets of *S. tuberosum* cultivars (Kufri Chipsona 3 and MP-97/644) grown *in vitro* showed self rooting in the multiplication media if kept for a maximum two months of period (figure-3a). The rooted plantlets were first transferred to portray containing 50% moist coco peat and 50% vermicompost as potting mix and kept in poly tunnel for 10 days in the green house to provide humidity (figure-3b). After 10 days, they were taken away from poly tunnel and found healthy. The plantlets were transferred to soil: sand: FYM in1:1:1 proportion and kept in net house. About 99% plants survived in the green house and the plants were in healthy condition when transferred to the field also (figure-3c). After 120 days tubers formed in Kufri Chipsona 3 and in MP-97/644 (figure 3d, 3e).

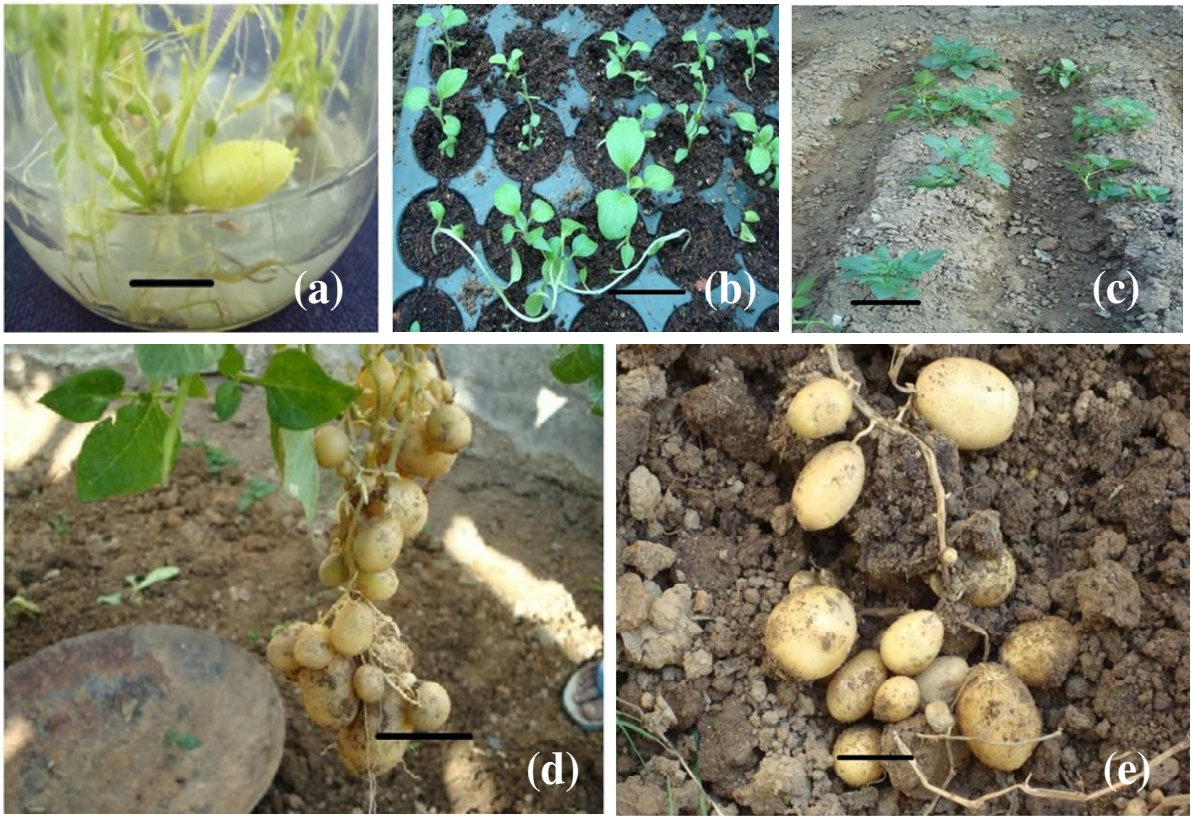

Figure-3
(a) Multiplication, microtuberization and rooting of shoot tips of *S.tuberosum* (Cvs.) (bar = 1.0 cm); (b) Tissue cultured plants transferred in protray after rooting. Primary hardening was achieved within 10 days of transfer (bar = 1.2 cm); (c) Plantlets of *S.tuberosum* (Cv. Kufri Chipsona 3 and MP-97/644) transferred into soil (bar = 1.5 cm); (d) Minituber formation on tissue cultured plants of *S.tuberosum* (Kufri Chipsona 3) (bar = 4 cm); (e) Minituber formation on tissue cultured plants of *S.tuberosum* (MP-97/644) (bar = 4 cm)

Conclusion

The present study was undertaken with a view to optimizing *in vitro* propagation through callus culture in two potato varieties of India with disease free and could be propagated for large-scale production and also can be conserved as virus free germplasm. . In addition, *S. tuberosum* can be supplied year round for commercial cultivation without any geographical and seasonal constraints.

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