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Standardization of protocol for *in vitro* tuberization in potato (*Solanum tuberosum*) cultivar Kufri Sindhuri

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ABSTRACT

Potato (*Solanum tuberosum* L.) is an economically important dicotyledonous and tuber crop which is ranked as the fourth most cultivated food crop after wheat, rice and maize. Asexual propagation of potato is done through tubers which are prone to large number of fungal and viral diseases. Microtubers produced through tissue culture serve as an essential component for production of disease-free quality potato seed. The present study was carried out during 2018 and 2019 at School of Biotechnology, Sher-e-Kashmir University of Agriculture Science and Technology of Jammu, Jammu to standardize *in vitro* microtuber production protocol in potato variety Kufri Sindhuri using different explants. Nodal segments were the most suitable explants for culture establishment which resulted in maximum survival with least contamination and mortality. Murashige and Skoog medium supplemented with BAP (1.5 mg/litre) and NAA (0.1 mg/litre) resulted in 100% shoot regeneration with 3.75 shoots per explant. Vigorous shoot proliferation was achieved by fortification of calcium pentothenate (2 mg/litre) and gibberellic acid (0.25 mg/litre) in establishment medium. Pre-tuberization was done by incubating cultures for 28 days in liquid multiplication medium supplemented with NAA (0.5 mg/litre). Maximum microtubers (24) per culture flask were obtained in 10 days when tuberization medium was fortified with 80 g/litre of sucrose while maximum diameter of 0.9 cm was recorded in the presence of growth retardant chlorocholine chloride (500 mg/litre). Complete darkness was an essential factor for microtuber induction. The harvested microtubers (G_0) were stored at 4°C after treating them with fungicides.

Keywords: Chlorocholine chloride, *In vitro,* Microtubers*,* Photoperiod, Potato, Sucrose

Potato (*Solanum tuberosum* L.) belonging to the family Solanaceae is an important food and cash crop. It is native to South America and ranks first among non-cereal crops to ensure food security (Nikitin *et al.* 2018). It is source of more protein, calories, vitamins, minerals, carbohydrates and iron per unit area per unit time than any other major crop (Badoni and Chauhan 2010).

The conventional propagation of potato is done by planting the buds or eyes present on tubers (Gami *et al.* 2013) which is susceptible to several systemic fungi, bacteria and viral infections causing degeneration of the plants and finally result in huge yield and vigour losses (Nikitin *et al.* 2018). This susceptibility to pathogens also transmits diseases from one generation to another (Al-Hussaini *et al.* 2015). Therefore, use of virus-free planting material is necessary to ensure maximum yield potential of the plant. Plant tissue culture can serve as a potential alternate technology to propagate the plant material efficiently, maintain germplasm banks, facilitate genetic exchange, studying interactions of a species with biotic and abiotic factors and to produce disease-free seed without any limitations of seasonal constraints.

In vitro raised microtubers can be used as an alternative source for basic virus-free seed potatoes and serve as an important mode for rapid multiplication as well as germplasm exchange (Zakaria *et al.* 2008). Mass production of potato microtubers is likely to revolutionize the world potato production (Majid *et al.* 2014). Keeping in view the demand of quality planting material at commercial scale, the present study was undertaken to standardize a laboratory protocol for *in vitro* tuberization in potato cultivar Kufri Sindhuri by optimizing the media and culture conditions.

MATERIALS AND METHODS

Media and explant preparation: The present study was carried out during 2018 and 2019 at School of Biotechnology, Sher-e-Kashmir University of Agriculture Science and Technology of Jammu (SKUAST-J), Jammu. The apical buds with actively dividing meristematic zone, nodal segments and sprouting buds from tubers of healthy plants of potato

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variety Kufri Sindhuri were used as explants. They were kept under running tap water for 30 min followed by treatment with Tween-20 solution for 20 min with continuous shaking. These explants were then treated with 0.1% Bavistin (w/v) and streptomycin sulphate for another 30 min. The explants were finally surface sterilized with mercuric chloride (0.1%) for different durations followed by thorough washing with sterile distilled water.

The culture medium was prepared by adding appropriate amount of ready to use MS (Murashige and Skoog 1962) basal medium, 3% sucrose and different growth regulators in various combinations. All components of the medium were mixed and final volume was made by adding double distilled water and was solidified by adding 0.8% agar. The *pH* of the medium was adjusted between 5.6–5.8. The liquid medium was devoid of agar. The sterilization of medium was done at 1.1 kg/cm^2 pressure and 121°C temperature for 20 min.

Establishment and proliferation of in vitro cultures: The sterilized explants were inoculated on MS medium supplemented with different concentrations and combinations of growth regulators (BAP: Benzyl amino purine, KN: Kinetin and NAA: Naphthalene acetic acid) as shown in Table 1. The cultures were incubated at temperature of 22±2°C, photoperiod of 16:8 hours and light intensity of 3000 lux. *In vitro* raised shoots were cut into segments with 2–3 nodes per segment and subcultured for multiplication on shoot proliferation medium augmented with optimized concentration of BAP and NAA. Calcium pentothenate (0–3 mg/litre) and GA_3 (0.1–0.5 mg/litre) were added to establishment medium in order to observe their effect on shoot growth and proliferation. Shoot growth and multiplication rate was observed after 21 days interval.

Tuber induction of in vitro *raised shoots*: MS medium optimized for shoot multiplication was supplemented with calcium pentothenate, GA_3 and NAA and used for pre-tuberization of potato cultures. For this, 8–10 shoots from proliferated cultures were aseptically transferred into a culture flask containing 20 ml of liquid pre-tuberization medium and incubated in dark at temperature of 22±2°C with relative humidity of 75% for duration of 3 weeks. For microtuber induction, the pre-tuberization medium was replaced with liquid MS medium supplemented with different concentrations of sucrose (0–100 g/litre) followed by addition of chlorocholine chloride (100–500 mg/litre) and incubation at 22±2°C temperature for 60 days. It was followed by standardization of photoperiod requirement for tuber induction. For this, cultures were incubated under different photoperiod conditions ranging from complete darkness to 16:8 hr photoperiod.

Harvesting and storage of microtubers: The cultures kept in complete darkness were shifted to light for 10 days before harvesting the microtubers. These microtubers were washed carefully with tap water. The observations pertaining to number of microtubers, average weight of microtubers, diameter of largest microtuber and yield per flask were recorded after 72 days. The harvested microtubers were treated with Bavistin and Dithane M-45 (0.25% each) for 15 min and dried at room temperature before storage.

Data analysis: The experiments were independently performed in triplicates and the data was analyzed using two-way ANOVA (Analysis of variance) with $P \geq 0.05$ according to Gomez and Gomez (1984).

Table 1 Effect of mercuric chloride (0.1%) treatments on survival and establishment of aseptic cultures of potato

TD (min)	Bud eyes			Apical shoot			Nodal segment		
	C (%)	M (%)	R (%)	C (%)	M (%)	R (%)	C (%)	M (%)	R (%)
2	97.20 (81.95)	2.80 (8.01)	0.01 (0.40)	76.40 (60.93)	9.70 (18.05)	13.90 (21.83)	27.77 (31.73)	0.01 (0.04)	72.23 (58.22)
3	79.17 (62.9)	19.43 (25.93)	1.40 (4.21)	48.60 (44.17)	20.80 (27.04)	30.57 (33.47)	23.63 (28.93)	1.40 (4.21)	74.97 (60.123)
4	61.10 (51.4)	26.40 (30.83)	9.70 (18.05)	15.23 (22.92)	38.87 (38.51)	45.83 (42.58)	16.67 (23.90)	8.33 (16.41)	75.00 (60.18)
5	36.10 (36.89)	50.00 (44.98)	13.87 (21.80)	6.93 (15.09)	61.10 (51.39)	31.93 (34.38)	6.93 (15.09)	8.33 (16.41)	84.77 (67.04)
6	9.72 (18.07)	87.50 (69.44)	2.80 (8.01)	1.40 (4.21)	84.77 (67.04)	13.87 (21.80)	0.01 (0.40)	12.5 (20.51)	87.50 (69.44)
7	1.40 (4.21)	98.60 (85.93)	0.01 (0.40)	0.01 (0.40)	100.0 (89.83)	0.01 (0.40)	0.01 (0.40)	18.1 (25.11)	81.93 (64.84)
CD (P=0.05)									
Explant (E)	2.35	2.54	2.31	2.35	2.54	2.31	2.35	2.54	2.31
Duration (D)	3.32	3.59	3.28	3.32	3.59	3.28	3.32	3.59	3.28
E × D	5.76	6.23	5.68	5.76	6.23	5.68	5.76	6.23	5.68

TD, Treatment duration; C, contamination; M, Mortality; R, Regeneration. Figures within the parantheses are arcsine transformed values.

RESULTS AND DISCUSSION

The experiments performed for standardization of sterilization protocol in the present investigation gave promising results. Amongst the types of explants used for initiating axenic potato cultures, nodal segments were observed to be the most suitable explants. Mercuric chloride has been extensively used for surface sterilization of potato explants (Joseph *et al.* 2015, Kaur *et al.* 2015, Mohapatra *et al.* 2016). The nodal segments responded well when treated with 0.1% mercuric chloride for 6 min resulting into maximum regeneration of 87.50% with least contamination. It was observed that with gradual increase in duration of $HgCl_2$ treatment of explants from 2–6 min, there was decrease in infection and simultaneously the regeneration percentage increased (Table 1). On the contrary, Dessoky *et al.* (2016) obtained highest per cent survival of nodal segments when sterilized with 0.2% $HgCl_2$. Better *in vitro* potato regeneration with stem segments have also been reported by Kaur *et al.* (2017) and Silva Filho *et al.* (2018).

MS medium is the most appropriate medium for propagating the plantlets and shoot growth (Mohapatra *et al.* 2016, Emaraa *et al.* 2017, Samant *et al.* 2018). The sterilized explants were cultured on MS medium supplemented with growth regulators. Maximum shoot regeneration of 100% along with an average number of 3.75 shoots per explant were obtained in MS medium augmented with BAP (1.5 mg/litre) and NAA (0.1 mg/litre) which was at par with MS medium supplemented with BAP (2.0 mg/litre) where average number of shoots was 3.55 per explant as shown in Table 2. The nodal explants showed signs of bud break within one week of culture. Replacing BAP with KN showed decline in regeneration per cent to 88.83 indicating that BAP is a preferred cytokinin for regeneration. Contarary to this, medium containing Kinetin individually or with auxins (NAA or IAA) have been found to promote multiplication and growth of shoots (Hoque 2010, Mohapatra and Batra 2017) while Badoni and Chauhan (2009) observed lower concentration of NAA (0.01 mg/litre) with Gibberellic Acid (0.25 mg/litre) to be the best combination for regeneration of complete plantlets from meristem tips. The established cultures after 3 weeks were subcultured on same medium and developed into plantlets within 6 weeks of culture. Liquid establishment medium fortified with GA_3 (0.25 mg/litre) and calcium pentothenate (2 mg/litre) was also used which resulted in formation of healthy and vigorous shoots.

In vitro induction of micro tuber is a two step method which includes pre-tuberization followed by tuberization. Pre-tuberization of proliferated shoots was done in MS medium augmented with calcium pentothenate (2 mg/litre), GA_3 (0.25 mg/litre) and NAA (0.5 mg/litre) for 28 days which was replaced by tuberization medium (Fig 1). This was followed by replacing this medium with tuberization medium which was modified by increasing sucrose concentration and adding growth retardants. Moeinil *et al.* (2011), Choudhary and Mittal (2014) and Samant *et al.* (2018) had similar observations about effectiveness of GA_3 and calcium pentothenate along with other growth regulators for successful micropropagation and *in vitro* tuberization in potato.

Carbohydrates are the most important source of energy for growth of *in vitro* plants and sucrose provides the most critical stimulus for tuber formation. As evident from Table 3, maximum number (24) and yield of microtubers (2.46 g) was obtained within 10 days in liquid medium supplemented with sucrose at concentration of 80 g/litre which can be attributed to the fact that with increase in sucrose level, the osmolarity of medium increases leading to stress condition which shift the plant behavior towards maturity resulting in tuber induction (Elaleem *et al.* 2015). Absence or low concentration of sucrose failed to produce microtubers but developed healthy shoots indicating that low levels of sucrose were responsible for vegetative growth of shoots. Many workers (Liljana *et al.* 2012, Saha

Table 2 Effect of growth regulators on the establishment of *in vitro* culture of potato cv. Kufri Sindhuri

Growth regulators (mg/litre)			% shoot regeneration	Average no. of shoots per explant	Average no. of nodes per explant
BAP	KN	NAA			
-	-	-	38.93 (38.57)	1.00	1.75
0.5	-	0.1	66.73 (54.86)	1.25	2.75
1.0	-	0.1	86.06 (68.27)	1.75	3.75
1.5	-	0.1	100 (90.00)	3.75	5.25
2.0	-	0.1	97.2 (84.37)	3.55	4.25
-	0.5	0.1	53.03 (46.57)	1.00	2.50
-	1.0	0.1	63.83 (53.03)	1.25	3.50
-	1.5	0.1	88.83 (70.70)	3.25	4.25
-	2.0	0.1	86.06 (68.27)	2.75	4.50
CD (P=0.05)				8.071	

Figures within the parentheses are arcsine transformed values.



Fig 1 Initiation of *in vitro* tuberization of potato cv. Kufri Sindhuri.

Table 3 Effect of different sucrose and chlorocholine choloride concentrations and photoperiods on microtuber induction in potato cv. Kufri Sindhuri

Sucrose					Chlorocholine chloride (CCC)					Photoperiod				
Conc. (g/ litre)	Days to micro tuber induction	No. of micro tubers per culture flask	Yield per culture flask (g)	Diameter of largest micro tuber (cm)	Conc. (mg/litre)	Days to micro tuber induction	No. of micro tubers per culture flask	Yield per culture flask (g)	Diameter of largest micro tuber (cm)	Exposure (hrs)	Days to micro tuber induction	No. of micro tubers per culture flask	Yield per culture flask (g)	Diameter of largest micro tuber (cm)
0	-	-	-	-	0	-	-	-	-	16:8	20	2	0.36	0.4
20	-	-	-	-	100	18	4	0.4	0.4	8:16	16	9	1.45	0.6
40	13	4	0.45	0.6	200	13	8	0.96	0.4	Complete dark	6	24	2.50	0.8
60	10	10	1.0	0.6	300	11	13	1.2	0.6					
80	10	24	2.46	0.7	400	10	16	1.43	0.6					
100	8	20	1.82	0.5	500	8	20	2.56	0.9					
					600	8	23	2.24	0.7					

et al. 2013, Hossain *et al.* 2017, Islam *et al.* 2017) have observed the effects of different sucrose concentrations on microtuberization of potato.

Chlorocholine chloride (CCC) is a growth retardant that plays a vital role in tuber induction. Reduction in shoot growth with increase in CCC concentration was recorded. Maximum of 20 microtubers with a total yield of 2.56 g/flask and largest diameter of 0.9 cm were obtained in MS medium supplemented with CCC at a concentration of 500 mg/litre (Table 3). It was observed that with the increase in number of microtubers, the size of the tubers decreased, indicating that total storage of starch remained same. As the concentration of CCC decreased below optimum, the number of tubers decreased, indicating that CCC played a vital role in tuber induction. No microtuber was produced in control, i.e. MS medium without CCC. Vecchio *et al.* (1994) reported that the presence of CCC in the medium reduced GA_3 biosynthesis and increased tuberonic acid synthesis, which enhances the tuber formation.

Photoperiod has an influence on microtuber induction. It was observed that complete darkness was an essential factor in tuber induction and maximum number of tubers (24 per flask) were obtained after six days with a significantly higher total yield per flask (2.50 g) and largest tuber diameter (0.8 cm) (Table 3). The cultures kept under 16 hrs light produced only two microtubers. Incubation of cultures under light leads to GA_3 synthesis, which inhibits tuber induction while darkness enhances tabernacle acid synthesis, which plays an important role in tuber formation. Donnelly *et al.* (2003) have also demonstrated that microtuberization efficiency increased by short day's exposure or continuous darkness during culture conditions.

After incubating the flasks for 60 days in complete darkness for tuber induction, the culture flasks containing microtubers were shifted to 16:8 hr photoperiod for 10–12 days which changed the color of microtubers to green. The harvested microtubers depicted in Fig 2 were treated with Bavistin (2.0 g/litre) and Dithane M-45 (1.0 g/litre) solution and stored in refrigerator at 4ºC. An efficient protocol for



Fig 2 Harvested *in vitro* produced microtubers of potato.

microtuber induction in potato cv. Kufri Sindhuri has been developed which with minor modifications may be useful for micropropopagation and mass multiplication of other varities of potato as well.

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