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AN EFFICIENT *AGROBACTERIUM*-MEDIATED TRANSFORMATION METHOD FOR POTATO C.V. KUFRI CHANDRAMUKHI

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ABSTRACT: An optimized regeneration and *Agrobacterium*-mediated transformation protocol based on internode explants was developed in potato cultivar 'Kufri Chandramukhi'. Potato internodes were transformed by cocultivation with *A. tumefaciens* strain EHA 105 harboring vector pRI101. MS medium with IAA 0.042mg L^{-1} + GA_3 3.0 mg L^{-1} + Zeatin 3.0 mg L^{-1} showed the maximum percentage of callus formation i.e. 76% with average number of shoots per explants was 7.00. This medium showed mimimal number of days for callus initiation as compared to other medium compositions The best combination for shoot regeneration was a medium of Murashige & Skoog salts with 0.042 mg L^{-1} IAA, 3.0 mg L^{-1} GA_3, 3.0 mg L^{-1} Zeatin and 0.008 mg L^{-1} NAA. Use of 50 mg L^{-1} Kanamycin, 250 mg L^{-1} Carbenicillin and 100 mg L^{-1} Cefotaxime in the callus induction medium minimized contamination. This method is useful for genetic transformation studies in KCM.

KEYWORDS: Potato, *Agrobacterium tumefaciens*, Transformation, Regeneration

INTRODUCTION

Globally potato is the third most important crop after rice and wheat It produces more food per unit area and time than any other crop, in the form of tuber with high dry matter, carbohydrates, proteins and vitamins. Potato is viewed to have potential to alleviate hunger and malnutrition in the developing world. Being autotetraploid and highly heterozygous, its improvement via conventional breeding has been slow. Therefore, various biotechnological tools like somatic hybridization, *in vitro* mutagenesis and genetic transformation can be exploited for potato improvement (Beaujean *et al*., 1998]. Two methods of genetic transformation in potato have been used; direct gene transfer, using particle gun bombardment, and, indirectly through *Agrobacterium*-mediated transformation. However, the cost of gene transfer using particle bombardment is high, and low regeneration is often observed. Apart from this, direct gene transfer methods lead to integration of high copy number of genes resulting in gene suppression and silencing. On the contrary, *Agrobacterium*-mediated transformation is an efficient protocol for single gene transfer. Single gene expression is often stable to subsequent generations (Chakravarty *et al*., 2007).

An efficient plant regeneration system is a prerequisite for developing an *Agrobacterium* mediated genetic transformation protocol. Successful genetic transformation and regeneration in potato is highly genotype specific. Both gene transfer and subsequent regeneration of plants is affected by several factors. These include the genotype of the plant, type of explant used, preculture time, co-cultivation time, antibiotics used for suppression of *Agrobacterium* overgrowth and the composition of the callus induction and

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regeneration medium. In potato regeneration has been reported to be strictly influenced by genotype. Significant differences in shoot formation efficiency in commercial cultivars have been observed (Martel *et al*., 1992 Kumlay and Ercisli; 2015). The choice of antibiotics and their concentration also influences potato regeneration. The phytotoxicity of the antibiotic varies with the genotype, its interaction with the medium components, explant type and age. Several investigations highlight the different effects of phytohormones on the regeneration ability in potato (Rawat *et al*., 2017, Dhaka *et al*., 2015).

Kufri Chandramukhi (KCM) is one of the earliest developed and a popular variety in India producing optimal yield, attractive tubers with good processing attributes. However, it is also highly susceptible to various diseases and many abiotic stresses. Genes conferring tolerance to these stresses can be introgressed in KCM to get transgenic potato with desirable characteristics (Singh *et al*., 2015) Thus, medium composition was optimized for transformation in KCM, using binary vector PRI10, harboring a potato annexin gene. Comparison of different phytohormones on callus induction, shoot and root initiation was done to establish an efficient regeneration protocol for *Agrobacterium*-mediated gene transfer in KCM. This protocol would be helpful to further study gene functions and transgenic development in potato.

MATERIAL AND METHODS

Plant material

Mother plant was obtained from germplasm lab of division of crop improvement, ICAR-CPRI Shimla. The tissue culture plant was sub-cultured routinely (Singh *et al.*, 2016) and maintained in MS medium (Murashige and Skoog, 1962). Four-week-old plantlets were selected for the experiment

Agrobacterium Strain and Plasmid

The construct was generated by introducing the full-length cDNA of potato annexin p34 gene (GenBank Accession no:**CAB92956)** into the binary vector pRI101 and driven by the cauliflower mosaic virus 35S promoter. The vector was transformed into the *Agrobacterium tumefaciens* strain EHA105. Cells were grown overnight in LB medium containing 50 mg L^{-1} kanamycin. About 20% of primary culture was inoculated into a flask containing 100 ml LB with 50 mg L^{-1} kanamycin and was allowed to grow overnight till OD_{600}- reading of the culture reached 0.8–1. *Agrobacterium* cells were harvested by centrifugation at 5000 rpm, 4°C and the pellets were resuspended in MS liquid media (MS salts +10 g glucose +20 g L^{-1} sucrose and pH5.8) so that OD600 of the culture was 0.6–0.8. *Agrobacterium* cells were activated by the addition of 100 mM acetosyringone in dark before cocultivation.

Pre treatment

Four-week-old tissue cultured potato plants was selected, internodes were excised (4-6 mm) and placed on plates containing preculture medium (Table 1). Plates were then incubated in tissue culture room for two days at 24±2°C with photoperiod of 16 h.

Co-cultivation and shoot induction

The internodes after two days of pretreatment were incubated in the *Agrobacterium* suspension in the petri plate for 15-20 minutes covered in foil paper. Gentle shaking was done 4-5 times. The explants were then blotted on autoclaved and sterilized tissue paper gently until they were dried completely. Internodes were then placed on Petri plate with preculture medium having

Table 1. Composition of media used in the present study.

S.No	Medium	Composition
1	Preculture medium	MS+20g L^{-1} sucrose+2g L^{-1}gelrite
2	**Selective medium**	
2a	Selective medium M1	MS + IAA 0.042mg L^{-1} + GA_3 3.0 mg L^{-1} + Zeatin 3.0 mg L^{-1} + Kanamycin 50 mg L^{-1} + Carbenicillin 250 mg L^{-1} + Cefotaxime 100 mg L^{-1} + NAA 0.008 mg L^{-1}
2 b	Selective medium M2	MS + NAA 0.4mg L^{-1} + BAP 1.2 mg L^{-1} + Kanamycin 50 mg L^{-1} + Carbenicillin 250 mg L^{-1} + Cefotaxime 100 mg L^{-1}
2c	Selective medium M3	MS + IAA 0.05mg L^{-1} + GA_3 2.5 mg L^{-1} + Zeatin 2.5 mg L^{-1} + Kanamycin 50 mg L^{-1} + Carbenicillin 250 mg L^{-1} + Cefotaxime 100 mg L^{-1}
3	**Rooting medium**	
3 a	Rooting medium R1	MS+ 50mg L^{-1} Kanamycin+ 0 IAA
3 b	Rooting medium R2	MS+ 50mg L^{-1} Kanamycin+ 0.2mg L^{-1} IAA
3 c	Rooting medium R3	MS+ 50mg L^{-1} Kanamycin+ 0.3mg L^{-1} IAA
3 d	Rooting medium R4	MS+ 50mg L^{-1} Kanamycin+ 0.4mg L^{-1} IAA

Whatman filter paper (grade 1; 110 mm) on it. Plates were then kept in tissue culture room for two days at 22°C with photoperiod of 16 h. After 2-3 days after incubation, co-cultivated explants were transferred to different selective media (Table 1).

Shoot induction

The explants were placed carefully on the plates with different compositions of phytohormones for selective medium. (Table 1) Internode explants were placed directly without any filter paper on the medium. Plates were watched regularly and growth was observed. Plates were discarded if any contamination occurred. After 20-30 days, explants were shifted to new plate. Shoots emerged on the calli developed which were then excised and transferred to tubes containing same medium composition for proliferation.

Root induction

After the emergence of putative transgenic shoots, (2.5-3.0cm height) obtained on the best suitable selective shoot medium, these were excised and transferred to the four selective root regeneration media to select the best possible medium (Table 1). The whole plantlet was then sub-cultured on basal medium for further analysis

Analysis of potato transgenics

Genomic DNA was isolated from the transformed plants using DNeasy® Plant mini kit (Qiagen) PCR amplification was done using primers specific for nptII gene. The sequence of the forward and reverse primers was 5´GAGGCTATTCGGCTATGAGTG 3´ and 5´GCGATACCGTAAAGCACGAGG.3´ respectively. The PCR was carried out at the following conditions: 94 °C for 3 min, 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min, 35 cycles and followed by 1 cycle at 72 °C for 7 min. PCR products were visualized after electrophoresis in 1.2 % agarose gel photographed on gel documentation system (Bio Rad)

RESULTS AND DISCUSSION

Transformation and callus induction

In present study we optimized the *Agrobacterium* mediated transformation of potato cultivar Kufri Chandramukhi. using *Agrobacterium tumefaciens* strain EHA105, containing binary vector PRI101

with a potato annexin gene (GenBank Accession no: **CAB92956)** and *npt-II* (neomycin phosphotransferase-II) genes as a selectable marker. The gene construct was developed as a part of our ongoing studies in potato thermotolerance.

Three media for shoot regeneration were selected using prior studies to find the best one for KCM transformation (**Table 2**). M1 medium showed the maximum percentage of callus formation i.e. 76% with average number of shoots per explants was 7.00. This medium showed mimimal number of days for callus initiation as compared to other medium compositions (~ 53 and ~38 days respectively). The cells or tissues that were not transformed gradually turned brown after 45-50 days. Plates were monitored regularly for contamination. Approximately after three months, the emergence of putative transgenic shoots from callus was observed (**Fig. 1**). The best combination of growth regulators for shoot initiation in KCM was that of GA_3, zeatin and IAA. Using these growth regulators, a transformation rate of 28.97% and 24.37% has been reported in potato varieties "Asterix" and "Diamante" respectively (Molla *et al*., 2011). In potato a combination of BAP, GA_3 and NAA have also been used for successful shoot initiation

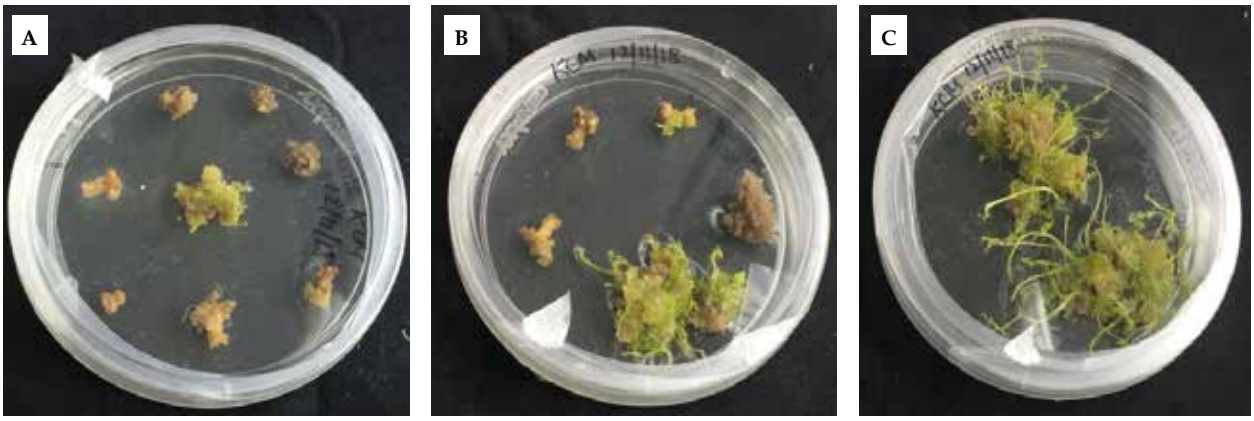

Fig. 1. Standardisation of callus and shoot regeneration medium for KCM transgenicsA; medium with composition MS + NAA 0.4mg L^{-1} + BAP 1.2 mg L^{-1} + Kanamycin 50 mg L^{-1} + Carbenicillin20 mg L^{-1} + Cefotaxime 40 mg L^{-1}; B: medium with composition MS + IAA 0.05mg L^{-1} + GA_3 2.5 mg L^{-1} + Zeatin 2.5 mg L^{-1} + Kanamycin 50 mg L^{-1} + Carbenicillin 250 mg L^{-1} + Cefo 100 mg L^{-1}; C: Best medium with composition MS + IAA 0.042mg L^{-1} + GA_3 3.0 mg L^{-1} + Zeatin 3.0 mg L^{-1} + Kanamycin 50 mg L^{-1} + Carbenicillin 250 mg L^{-1} + Cefotaxime 100 mg L^{-1} + NAA 0.008 mg L^{-1}

Table 2. Effect of medium composition on the callus and shoot initiation in potato cultivar Kufri Chandramukhi.

S.No.	Treatment	Medium Composition	Percent Callus Formation	Average number of shoots per explants	Average number of days for callus initiation
1	M1	MS + IAA 0.042mg L^{-1} + GA_3 3.0 mg L^{-1} + Zeatin 3.0 mg L^{-1} + Kanamycin 50 mg L^{-1} + Carbenicillin 250 mg L^{-1} + Cefotaxime 100 mg L^{-1} + NAA 0.008 mg L^{-1}	76%	7.00 ± 1.15	22.33± 1.45
2	M2	MS + NAA 0.4mg L^{-1} + BAP 1.2 mg L^{-1} + Kanamycin 50 mg L^{-1} + Carbenicillin 250 mg L^{-1} + Cefotaxime 100 mg L^{-1}	20%	2.33 ± 0.33	53.66 ± 2.18
3	M3	MS + IAA 0.05mg L^{-1} + GA_3 2.5 mg L^{-1} + Zeatin 2.5 mg L^{-1} + Kanamycin 50 mg L^{-1} + Carbenicillin 250 mg L^{-1} + Cefotaxime 100 mg L^{-1}	45%	3.66 ± 0.33	38.66 ± 2.33
	CD ±SE(m)			0.48 ± 0.14	0.56 ± 0.16

(Rawat *et al*., 2017, Khatun *et al*., 2012 and Beaujean *et al*., 1998 Farhatullah and Abbas; 2007). Optimum Kanamycin concentration is crucial for the selection of transformants. Previous studies have reported use of kanamycin concentration of 20 mgL^{-1}, 25 mgL^{-1}, 50 mgL^{-1} and 80 mgL^{-1}. (Onamu *et al*., 2012 and Saker *et al*., 2012) We used 50 mgL^{-1} kanamycin to select the growth of transformed shoots in all the selective media. The use of higher concentration of the antibiotics allows less escapes but lowers the regeneration potential o (Wenzler *et al*., 1989).

Root induction

The *in vitro* developed putative transgenic shoots were then transferred on the tubes containing same selective shoot regeneration medium having kanamycin 50 mg/Cefotaxime 100 mg L^{-1} and carbenicillin 250 mg L^{-1} for multiplication. After obtaining 2-3 cm height, the shoots were transferred to the selective root regeneration medium. The putative transgenic shoots (2.5-3.0 cm height) obtained on the selective shoot multiplication medium were excised and transferred to the selective root regeneration medium. All four different rooting media were significantly different from each other in terms of number of roots and root length (Table 3). The roots initiated after 12-15 days of inoculation on the selective medium and within 25-28 days well developed roots were observed (**Fig. 2**). However, in medium, with no hormone i.e. R1, no rooting was observed till 3 months. Medium R3 with MS+ 50 mg L^{-1} Kanamycin+ 0.3mg L^{-1} IAA showed maximum rooting within minimal number of days as compared to other medium. Average number of roots were also observed maximum in same rooting medium. (**Fig. 3**) The best rooting was observed in medium composition with MS+ 50mg L^{-1} Kanamycin+ 0.3mg L^{-1} IAA. Alternative to IAA many researchers have used IBA for the rooting of potato plants with concentration varying from 0.5mg L^{-1} 1.0mg L^{-1} IBA *In vitro* root induction of callus-regenerated shoots was studied in potato cultivars "Pasinler", "Granola" and "Caspar" by Kumlay and Ercisli (2015) with the addition of various concentrations of NAA and IBA with GA_3 in MS medium. Best rooting was obtained with 0.1 mg L^{-1} GA_3 + 1.0 mg L^{-1} IBA in Pasinler suggesting

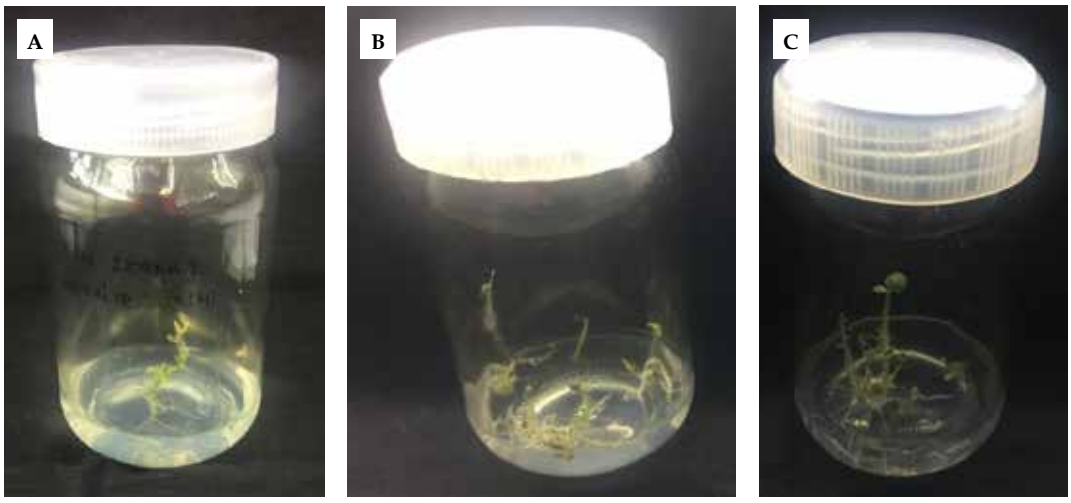

Fig.2. Standardisation of rooting medium for KCM transgenics; A: medium with composition (MS+ 50mg L-1 Kanamycin+ 0.2mg L^{-1} IAA); B: medium with composition MS+ 50mg L^{-1}Kanamycin+ 0.4mg L^{-1} IAA; C: The best rooting medium with concentration MS+ 50mg L^{-1}Kanamycin+ 0.3mg L^{-1} IAA

Table 3. Effect of medium composition for the root initiation and full-grown plantlets forpotato cultivar Kufri Chandramukhi.

S. No.	Treatment	Medium Composition	Number of days for rooting	Average Number of roots	Average root length after 15 days of initiation
1	RI	MS+ 50mg L^{-1} Kanamycin+ 0 IAA	-	0	0
2	R2	MS+ 50mg L^{-1} Kanamycin+ 0.2mg L^{-1} IAA	60	6.66 ±0.33	0.67 ±0.05
3	R3	MS+ 50mg L^{-1} Kanamycin+ 0.3mg L^{-1} IAA	15	31.33 ±2.02	2.62 ±0.10
4	R4	MS+ 50mg L^{-1} Kanamycin+ 0.4mg L^{-1} IAA	48	18.66 ±3.84	1.29 ± 0.03
		CD ±SE(m)		0.82 ± 0.24	0.06 ± 0.01

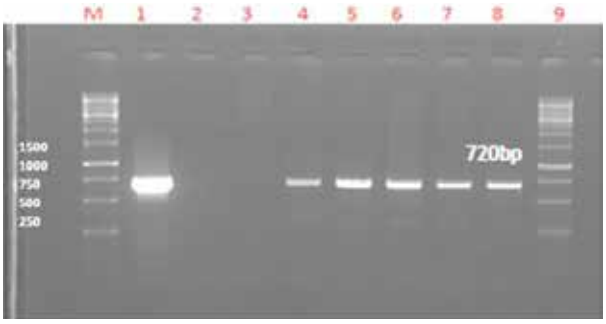

Fig. 3. Confirmation of the putative transgenics for presence of npt II gene; M 1kb ladder; 1: positive control as plasmid; 2: KCM negative control plant DNA; 3: water; 4-8: putative transgenic KCM lines showing integration of NptII gene; 9: ladder

efficacy of IBA in root induction. Proper root formation and root length in regenerated plants is crucial for the plants for their acclimatization and hardening. (Sanavy and Moeini; 2003)

Confirmation of transgene integration

To verify successful transformation, DNA was isolated from 52 transformants and was subjected to PCR analysis using *nptII* gene specific primers. Out of 52, 15 lines exhibited bands of expected size of 720 bp, corresponding to the *nptII* gene. There was no amplification in the non-transformed KCM plants used as control. A PCR reaction mix containing no DNA was also used as negative control whereas plasmid PRI 101 with full length annex in gene was used as a positive control. Thus successful integration of the gene was confirmed in potato lines The overall transformation efficiency was 28.8%

CONCLUSION

Kufri Chandramukhi (KCM) is a very popular and one of the earliest developed variety of potato. Though method for direct *in vitro* propagation of KCM is available to our knowledge, Agrobacterium mediated transformation and regeneration in KCM has not been reported previously. The callus induction and regeneration reponse in potato is highly genotype specific Efficient transformation and regeneration protocol is critical for developing transgenics gene editing and function studies. protocol the present study will help in the further improvement of KCM through genetic transformation ogin shortest period of time with minimum contamination.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest

ETHICAL STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors

LITERATURE CITED

Lecardonnel A and Sangwan-Norreel BS (1998) *Agrobacterium*-mediated transformation of three economically important potato cultivars using sliced internodal explants: an efficient protocol of transformation. *J. Expt. Botany***49**: 1589-1595.

Chakravarty B Wang-Pruski, G Flinn B Gustafson V and Regan S (2007) Genetic transformation in potato: Approaches and strategies. American Journal of Potato Research. **84:** 301-311.

Dhaka M and Nailwal TK (2015) High efficiency macropropagation of potato (*Solanum tuberosum* L.) cv. Kufri Jyoti in Kumaun Hills. *J. Plant Breed. Crop Sci***7**: 203-210.

Ullah I Jadoon M Rehman A Zeb T and Khan K (2012) Effect of different GA3 concentration on in vitro propagation of potato variety Desiree. *Asian J. Agric. Sci***4**(2):108-109

Khatun A, Hasan MM, Bachchu MAA, Moniruzzaman M and Nasiruddin KM (2012) *Agrobacterium*-mediated Genetic Transformation of Potato (*Solanum tuberosum* L.) var. Cardinal and Hera. *The Agriculturists* **10** (1): *81-86*

Kumlay AM & Ercisli S (2015). Callus induction, shoot proliferation and root regeneration of potato (Solanum tuberosum L.) stem node and leaf explants under long-day conditions. *Biotechnology & Biotechnological Equipment*, *29*(6), 1075-1084.

Martel and E Carcia (1992) In vitro formation of adventitious shoots on discs of potato (*Solanum tuberosum* L. cv. Sebago) tubers. *Phyton Buenos Aires***53**: 57-64

Miller PR, Amirouche L, Stuchbury T and Mathews S (1985) The use of plant growth regulators in micropropagation of slow-growing potato cultivars. *Potato Res***28**: 479-486

Molla MMH, Nasiruddin KM, Al-Amin M, Haque MS and Maniruzzaman (2011) *Agrobacterium*-mediated Transformation in Potato. *Thai J. of Agri. Sci* **44**(2)*: 93-102*

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

Onamu R, Juan PL, Jaime CS, Jose LR and Joel NP (2012) *In vitro* regeneration and *Agrobacterium*-mediated transformation of potato (Solanum tuberosum L.) cultivars grown in Mexico. *Plant Tissue Cult. & Biotech***22**(2): 93-105

Rawat TS, Krishnaprasad BT and Anil VS (2017) Direct and Indirect Regeneration of Potato Cultivar Kufri Jyoti. *J. of Biotech. and Biochem***3**(4): 31-34

Saker MM Tarek AA. Moussa NZH Amany HA Abo E and Abdel-Rahman RMH. (2012) Selection of an efficient in vitro micro-propagation and regeneration system for potato (*Solanum tuberosum* L.) cultivar Desiree. *Afr. J. Biotech***11**(98): 16388-16404

Sarker RH and Mustafa BM. (2002) Regeneration and *Agrobacterium*-mediated genetic transformation of two indigenous potato varieties of Bangladesh. *Plant Tiss. Cult* **12**(1): 69-77

Singh A Siddappa S Bhardwaj V Singh B Kumar D and Singh BP (2015) Expression profiling of potato cultivars with contrasting tuberization at elevated temperature using microarray analysis. *Plant Physiology and Biochemistry*. **97:** 108– 116.

Singh A, Kaushal N, Sharma R, Bhardwaj V, Singh B, et al. (2016) Effect of Elevated Temperature on In Vitro Microtuberization of Potato Genotypes with Different Thermotolerance Levels. Vegetos**29**(3): 6-9

Sanavy SAMM Moeini MJ (2003) Effects of different hormone combinations and planting beds on growth and plantlets from potato meristem culture. Plant Tissue Cult. 2003; 13:145–150.

Farhatullah ZA and Abbas JS (2007) In vitro effects of gibberellic acid on morphogenesis of potato explant. *Int. J. Agric. Biol***9**: 200

Wenzler H, Mignery G, May G, Park W. (1989) A rapid and efficient transformation method for the production of large numbers of transgenic potato plants. Plant Science. Jan 1;63(1):79-85.

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