World Journal of Agricultural Sciences 3 (6): 757-763, 2007
ISSN 1817-3047

Virus Free Plantlets Production of Strawberry Through Meristem Culture

[1]*Manosh Kumar Biswas,* [2]*M. Hossain and* [2]*R. Islam*

[1]National Key Laboratory of Crop Genetic Improvement,
Huazhong Agricultural University, Wuhan, Hubei, 430070, China
[2]Department of Botany, University of Rajshahi, Bangladesh

Abstract: Virus free strawberry plantlets production were investigated through meristem culture of three strawberry clones. Meristem was isolated from field grown runner tip explants and cultured on different concentrations and combinations of growth regulators in Murashing and Skoog (MS) liquid medium. Among them 0.1 mg l^{-1} gibberellic acid (GA3) supplemented media showed effective for primary establishment of meristem. After four weeks of establishment meristems were transferred to growth regulators and growth regulator free semisolid MS medium. Among them Indole Butyric Acid (IBA) was effective for root development and GA_3 was effective for shoot elongation. After having DAS-ELISA test the *in vitro* grown plantlets were being used for massive micropropagation. The BA and Kin singly or in combination were used for multiple shoot regeneration. Low concentration of benzyladinine (BA) showed better performance in multiple shoot regeneration. Microshoots were isolated and cultured on half strength of MS medium for root development. Well developed rooted plants were successfully established in field. Visual evaluation of the morphological trails of the meristem culture derived plants showed normal and free from virus diseases. Substantial yield increase was obtained in meristem derived plants over their source plants.

Key words: Meristem culture · virus free plantlets · strawberry · BA

INTRODUCTION

Strawberry is an herbaceous perennial plant having a compressed, shortened stem and produces stolons. The fruit is an achene attached to a juicy, enlarged receptacle. It is one of the most popular fruits in the world and per capita consumption is increasing annually. Strawberry is the most popular yogurt flavour in many countries. Fruits are eaten raw or used in making juice, desserts, jam, syrup and wine.

Among different factors responsible for low yield of strawberry. Viral diseases are encounted one of them. Spoegel [1] reported that strawberry is susceptible to more than thirty viruses and phytoplasma. Single or multiple virus infection in strawberry plants often resulted low plant performances, i.e. reduced runner production, daughter plant production, mother plant root mass, reduction in fruit yield and diminished fruit quality.

So, viral disease is an important attributing factor to reduce yield of strawberry varieties. The yield reduction may be up to 80% caused by some viruses [2]. As such strawberry mottle virus (SMoV) alone may cause yield reduction of 30% [2]; Strawberry Mild Yellow Edge Virus (SMYEV), Strawberry Crinkle Virus (SCV) and Strawberry Ven Binding Virus (SVBV) losses can up to 80% production [3, 4].

In vegetative propagated strawberry crops once systematically infected with a viral disease. The pathogen is passed from one vegetative generation to next. So, to raise the disease free stock plant, virus elimination is a pre-requisite for successful strawberry production.

Meristem culture is a unique technique to free from various pathogens including viruses, viroides, mycoplasma, bacteria and fungi [5-8]. Benefit of using meristem culture as a means of regeneration is that the incipient shoot has already differentiated to establish a complete plant; only elongation and root differentiation are required. Many important plants contain systemic viruses, which substantially reduce their potential yield and quality. It is therefore, important to produce virus free stock plant, which can be possible for lack of vascular system and high metabolic activity on meristematic zone [9]. Strawberry is therefore another example of where tissue culture might be used to propagate the virus free

Corresponding Author: Dr. Manosh Kumar Biswas, National Key Laboratory of Crop Genetic Improvement, National Center of Crop Molecular Breeding, Huazhong Agricultural University, Wuhan, Hubei, 430070, China

plants [10]. Therefore, this investigation was under taken to develop a suitable protocol for producing virus free strawberry plantlets followed by healthy stock plant production under net house management for tropical, sub tropical and warm temperate environmental condition where viral disease are very frequent.

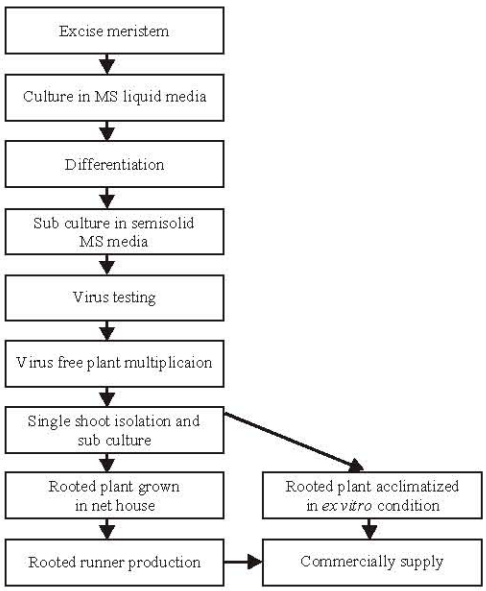
MATERIALS AND METHODS

The research work was envisaged at Plant Breeding and Biotechnology laboratory, Department of Botany, University of Rajshahi, Bangladesh. The meristems of three strawberry clones viz., pbgel-01, pbgel-23 and RU-31 were used as explants. Runner tips were collected from 40-45 days old field grown plant. Excised runner tips were washed under running tap water for 20 min and subsequently sterilized by 0.1% $HgCl_2$ solution with gently shaking for few minutes followed by 3-5 times washing by sterilized distil water. All these operations were made inside the running laminar airflow. The sterilized runner tips, immature leaves and leaf primordial were snapped off and then meristems were prepared (0.3-0.5 mm). The meriatem was quickly transferred on to the filter paper bridge in test tubes containing liquid MS [11] medium having GA_3, BA and Kin either singly or in combination and medium having no growth regulators for primary establishment. (Fig. A). The meristems those were responded after 3-4 weeks of inoculation, they were sub cultured in MS medium with (GA_3, Kin and IBA singly) or without growth regulators for shoot and root induction. Three concentrations were tested for each growth regulators treatment. Data were recorded on number of root development/explants, shoot length and root formation.

Before shoot multiplication, a serological identification was done in the cultured plants and to detected virus. In this detection the double antibody sandwich enzyme-linked immunesrbent assay (DAS-ELISA) methods were followed. Virus free plantlets were used for mass propagation in MS semi-solid medium with different levels of BA and Kin either singly or in combinations for shoot multiplication. After 4-5 weeks it was sub cultured on MS semisolid medium containing GA_3 and IBA for root and shoot development.

All the cultures were incubated at 25±2°C temperature under 16h photoperiod. Sucrose was used as 30 g l^{-1} in both MS liquid and MS semi-solid media and the pH was adjusted to5.7-5.8. The mass propagation was evaluated by number of shoot and root formation, shoot length and root formation frequency.

When the plantlets attained in 3-5cm heights and folded few leaves and roots were taken out from the test tube and washed the roots by water to remove medium and the plantlets were planted in sterile soil containing plastic pot. After that plantlets were kept under shade 7-10 days for hardening and then it is ready for field transplantation. Before transplanting the fields were treated with 1% formaldehyde solution to prevent the soil borne pathogen. After 3 days of soil treatment the plantlets were transplanted to the field in rows with 20 cm apart keeping 12 cm space between two plantlets and were covered with polythene sheets for 3-5 days. The entire field was covered with nylon nets in order to prevent viral vectors. Then the plantlets were observed up to fruit harvest. The field performance of meristem derived plantlets was evaluated on morphological characters (plant height, number of leaves/plant, canopy, number of fruit/plant and average fruit weight/fruit) and viral diseases index from randomly selected 10 plants. The entire protocol for commercial production of diseases free plantlets is presented as in flow chart below.



RESULTS AND DISCUSSION

Primary establishment of meristem: Runner tips were used as explants for meristem isolation. It was collected from field grown plant and surface sterilization was carried out with 0.1% $HgCl_2$ solution at different time duration. Among the different time period used for sterilization five min. was found best for survived of explants. About 90% of explants were found healthy and free of contamination. Less than 5 min explants were found contaminated and

Table 1: Effect of different types and concentration of growth regulators on growth response of apical meristem cultured in MS liquid medium

Growth regulators	Morphogenic response of isolated meristem	Clone		
		pbgel-01	pbgel-23	RU-31
Control	Days to response	8-11	7-10	7-9
	Growth Response (%)	41	43	44
GA_3(0.1 mg l^{-1})	Days to response	5-8	5-9	6-9
	Growth Response (%)	75	74	74
GA_3(0.5 mg l^{-1})	Days to response	5-9	5-10	5-9
	Growth Response (%)	72	73	72
BA (0.1 mg l^{-1})	Days to response	7-9	7-9	7-9
	Growth Response (%)	69	68	69
BA (0.5 mg l^{-1})	Days to response	8-10	7-10	6-10
	Growth Response (%)	70	67	72
Kin (0.1 mg l^{-1})	Days to response	8-12	9-12	8-13
	Growth Response (%)	51	53	50
Kin (0.5 mg l^{-1})	Days to response	8-13	8-12	8-12
	Growth Response (%)	50	47	45

Table 2: Effect of different concentration of growth regulators in MS medium on shoot and root development from developed primary meristem after 21 days of cultivation

Growth regulators	Morphogenic traits	Clone		
		pbgel-01	pbgel-23	RU-31
Control	Shoot length	3.41±0.12	4.03±0.21	3.96±0.03
	Number of root	4-6	4-6	4-6
	Root formation %	48	45	46
GA_3 (0.1 mg l^{-1})	Shoot length	7.87±0.51	7.39±0.42	8.47±0.28
	Number of root	3-6	4-6	4-5
	Root formation %	52	53	50
GA_3 (0.5 mg l^{-1})	Shoot length	7.53±0.24	8.92±0.25	7.33±0.65
	Number of root	4-7	4-6	4-6
	Root formation %	53	54	52
GA_3 (1.0 mg l^{-1})	Shoot length	9.35±0.21	10.21±0.52	9.73±0.42
	Number of root	4-5	4-5	4-6
	Root formation %	55	52	55
Kin (0.1 mg l^{-1})	Shoot length	5.12±0.21	6.04±0.34	5.39±0.62
	Number of root	5-8	5-7	5-8
	Root formation %	53	53	54
Kin (0.5 mg l^{-1})	Shoot length	5.43±0.34	5.93±0.21	6.21±0.51
	Number of root	5-7	5-8	5-8
	Root formation %	50	51	50
Kin (1.0 mg l^{-1})	Shoot length	5.77±0.02	5.23±0.33	5.95±0.55
	Number of root	5-6	5-6	5-6
	Root formation %	48	50	51
IBA (0.1 mg l^{-1})	Shoot length	4.31±0.29	4.59±0.51	4.97±0.54
	Number of root	8-10	8-12	8-10
	Root formation %	100	100	100
IBA (0.5 mg l^{-1})	Shoot length	3.97±0.24	4.01±0.56	3.65±0.92
	Number of root	8-12	8-10	8-12
	Root formation %	100	100	100
IBA (1.0 mg l^{-1})	Shoot length	4.07±0.52	4.29±0.27	3.78±0.32
	Number of root	8-13	8-12	8-13
	Root formation %	100	100	100

Table 3: Effect of different concentration of BA and KIN in MS semi-solid medium for massive micropropagation of three strawberry clones at 35 days after inoculation

Growth regulators	Morphogenic traits	Clone		
		pbgel-01	pbgel-23	RU-31
Control	No of micro shoots/explants	1	1	1
	Root number	2-4	2-5	2-4
	Multiple shoot formation %	0	0	0
BA (0.1 mg l^{-1})	No of micro shoots/explants	33-37	35-38	32-40
	Root number	0-1	0-1	1-2
	Multiple shoot formation %	86	84	88
BA (0.5 mg l^{-1})	No of micro shoots/explants	40-48	42-48	45-50
	Root number	1-2	0-1	0-1
	Multiple shoot formation %	93	90	91
BA (1.0 mg l^{-1})	No of micro shoots/explants	55-62	57-63	54-59
	Root number	1-2	0-1	0-1
	Multiple shoot formation %	95	97	94
BA (1.5 mg l^{-1})	No of micro shoots/explants	53-60	52-62	55-63
	Root number	1-2	0-1	0-1
	Multiple shoot formation %	94	96	95
BA (2.5 mg l^{-1})	No of micro shoots/explants	45-51	43-53	41-50
	Root number	1-2	0-1	0-1
	Multiple shoot formation %	83	81	82
Kin (0.1 mg l^{-1})	No of micro shoots/explants	1	1	1
	Root number	2-3	1-2	1-2
	Multiple shoot formation %	0	0	0
Kin (0.5 mg l^{-1})	No of micro shoots/explants	1	1	1
	Root number	2-3	1-2	1-2
	Multiple shoot formation %	0	0	0
0.1BA+0.15Kin	No of micro shoots/explants	27-32	28-35	27-35
	Root number	0-1	0-1	0-1
	Multiple shoot formation %	92	89	91
1.0BA+0.15Kin	No of micro shoots/explants	29-37	30-37	30-39
	Root number	0-1	0-1	0-1
	Multiple shoot formation %	93	90	93
1.0BA+0.5Kin	No of micro shoots/explants	30-39	32-41	34-42
	Root number	0-1	0-1	0-1
	Multiple shoot formation %	92	90	92

more than that results tissue killing. Isolated meristems of three clones viz. pbgel-01, pbgel-23 and RU-31 were cultured on filter paper bridge containing test tubes in liquid MS medium supplemented with different concentrations of growth regulators and their response are presented in (Fig. A and Table 1). The cultured meristem commenced their initial growth by increasing in size and gradually changed to light green colure within 7-13 days (Fig. A and B). The results on establishing primary meristem further showed that for quick developed high percentage of growth response of meristem, use of growth regulators was found essential. Most quick response was obtained when the meristems were cultured in MS medium supplemented with GA_3. MS medium supplemented with 0.1 mg l^{-1} GA_3 showed better response for all cultivars and highest of 75% responded was found in clone pbgel-01 within 5-8 days.

The results further demonstrated that GA_3 (0.1 mg l^{-1}) in MS liquid medium showed quick establishment of meristem in culture media. In liquid culture method for tissue culture has also been reported [12-14]. Effective role of GA_3 for establishing meristem culture was also reported by others [9,14-17].

Shoot and root development from established meristem: The meristem in liquid medium developed tiny shoots after four weeks (Fig. C). Those tiny shoots were transferred on semisolid MS media with GA_3, Kin and IBA individually at different concentrations for shoot and root development and the results are presented in Table 2.

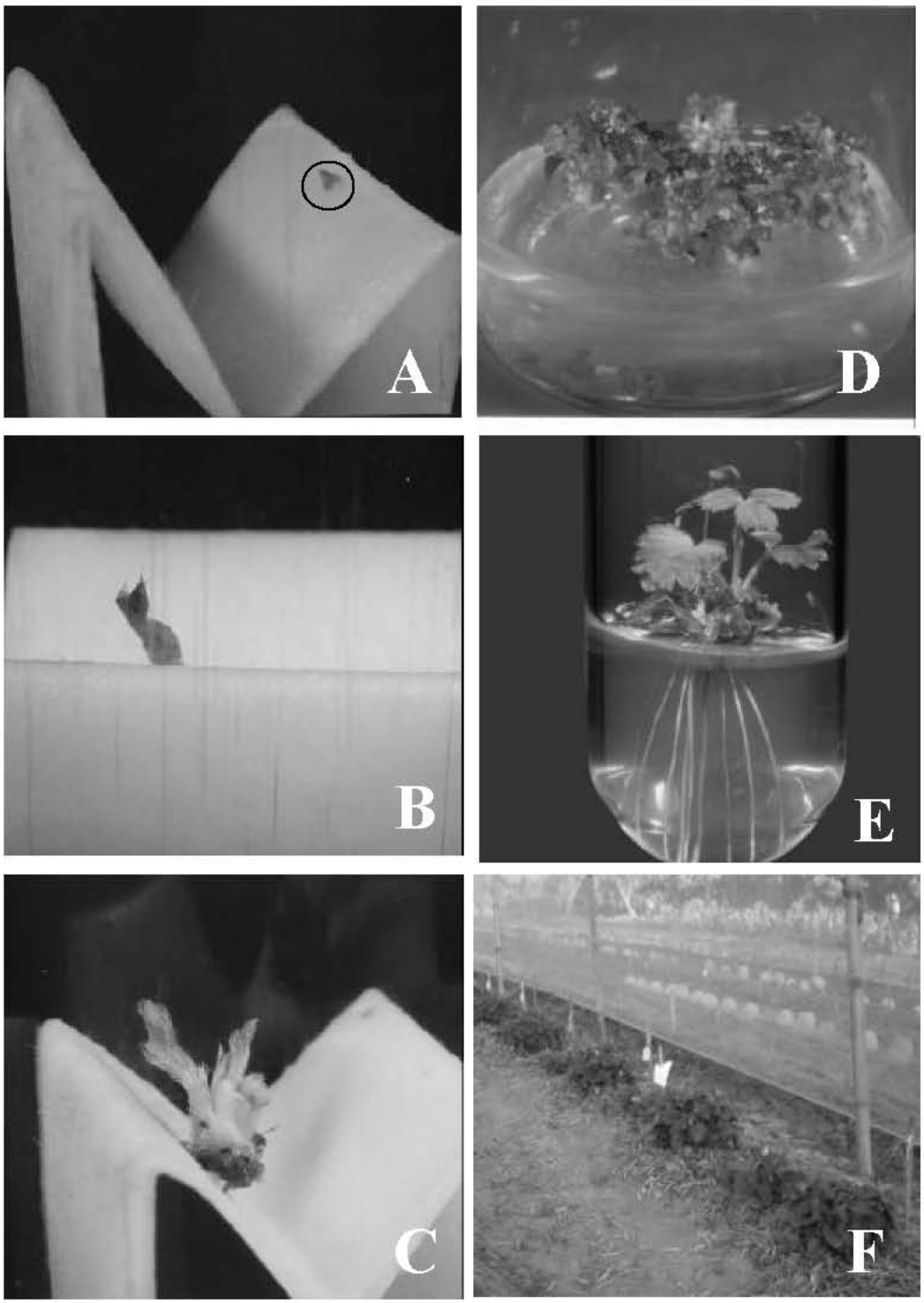


Fig. (A): Development of meristem (3-days old) on filter paper bridge in liquid medium, (B.) Shoot initiation (21 days old) from the isolated meristem in liquid medium, (C.) Development of shoot with leaves from meristem at 28 days of culture in liquid medium, (D.) Massive multiplication of plantlets using shoot proliferation from meristem derived plantlets in semisolid medium. (E.) Root initiation in isolated micro shoot, (F.) Soil establishment of meristem derived plantlets in net house

Table 4: Field performance of meristem derived plantlets. Morphological and yield data were recorded at 90 days after transplantation of plantlets in net house

Clone	Plant height (cm)†	No.of leaves/plant†	Canopy/Plant†	Average fruit weight/fruit.(g)†
pbgel-01	10.73±0.52	19.53±0.89	26.06±0.70	12.90±0.60
pbgel-23	12.86±0.78	24.40±1.23	30.46±0.66	15.93±2.67
RU-31	11.28±0.49	21.42±0.85	25.21±0.12	11.21±1.24
Control	12.36±0.77	20.22±1.59	29.36±0.25	13.54±0.97

† = Mean±Standard Error

Among three growth regulators GA_3 was found most effective for shoot development. On the other hand IBA was found effective for root development. The highest length of shoot was found in pbgel-23 in 1.0mg l^{-1} GA_3 containing medium. Low concentration of IBA was enough for maximum number of roots and the frequency of root formation. The IBA used for root development in MS medium from meristem drive plantlets was also reported in different crops [14,18-20] IBA was effective for root and GA_3 for shoot development from isolated meristem.

Confirmation of virus elimination in meristem derive plant sample by DAS-ELISA test: Before mass micropropagation meristems derive plantlets were tested for virus detection by DAS-ELISA technique. Virus free plantlets were used for mass micro propagation.

Mass micro propagation of virus free plantlets: Based on the results of ELISA test, virus free plantlets were cultures on MS medium containing either singly or in combination in BA and Kin. The results are presented in Table 3. Multiple shoots were formed when the explants were cultured in MS medium supplemented with singly BA (0.1mg l^{-1}-2.5 mg l^{-1}) and in combination with BA and Kin (1.0mg l^{-1}+ 0.15-0.5 mg l^{-1}). On the other hand no multiple shoots were found in control and MS medium supplemented with Kin (0.1-0.5mg l^{-1}). Among the low concentration of BA (0.1-2.5 mg l^{-1}) containing MS medium 1.0 mg l^{-1} of BA was found to be best for multiple shoot formation (Fig. D). Khanam *et al.* [10] reported that BA was found to be most effective for induction of multiple shoots. No distinct variation was observed in *in vitro* shoot multiplication for the studied three cultivars. Micro shoots were isolated and cultured on half strength of MS for root induction. Well developed rooted plantlets were planted in net house.

Field performance of meristem derived plantlets: The results on field performance of meristem derived plants are presented in Table 4. Morphological characters of merisetem derived plants were found normal (Fig. F) varietals stability was also reported among the meristem derived regenerated plants [21]. As per visual observation of the plants, no symptoms of viral disease were noticed. Fruit yield of meristem derived plants was more than that of runner derived plants.

REFERENCE

1. Spiegel, S., 1998. Virus certification of strawberries. Plant virus disease control APS press, st Paul Minn., pp: 320-324.
2. Thompson, J.R. and W. Jelkman, 2003. The detection and variation of strawberry mottle virus. Plant Disease, 87: 385-390.
3. Horn, N.L. and R.G. Carver, 1962. Effect of three viruses on plant production and yields of strawberries. Plant Disease Report, 46: 762-765.
4. Mellor, F.C. and H. Krczal, 1987. Strawberry mottle. pp. 10-16. In: Virus Diseases of Small Fruits. Ed. R.H. Converse. United States Department of Agriculture, Agriculture Handbook No. 631, Washington, D.C.
5. Morel, G. and C. Matin, 1955. Gguerison de pommes de terre atteintics de maladies a virus. Compt. Rend, 41: 472-474.
6. Walkey, D.G.A., 1978. *In vitro* methods for virus elimination. In T.A. Thorpe (ed) Frontiers in plant tissue culture. University Calgary press. Calgary, Canada, pp: 245-254.
7. Bhojwani, S.S. and M.K. Razdan, 1983. Plant tissue culture. Theory and practice Elsevier science publisher Amsterdam. pp: 1-502.
8. Pierik, P.L.M., 1989. *In vitro* culture of higher plants. Martinus Nijhoff Publisher. Dordrecht. The Netherlands. pp: 1-344.
9. Ahmed, I., M. Hossain, M.A. Bari and M.F. Alam, 2000. Plant regeneration of two tomato (*Lycopersicon esculentum* Mill.) varieties through meristem culture. Bangladesh Journal Genet Biotechnology, 1: 109-113.
10. Khanam, D., M.M. Khatun, S.M. Faisal and M.A. Hoque, 1998. *In vitro* Propagation of Strawberry through Meristem Tip Culture. Plant Tissue Culture, 8 (1): 35-39.

11. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Plant Physiology, 15: 473-497.
12. Goodwin, P.B., 1966. An improve medium for the rapid of isolated potato buds. Journal Experimental Botany, 17: 590-595.
13. White, P.R., 1934. Potential unlimited growth of excised tomato root tips in a liquid medium. Plant Physiology, 9: 585-600.
14. Nagib, A., S.A. Hossain, M.F. Alam, M.M. Hossain, R. Islam and R.S. Sultana, 2003. Virus free potato tuber seed production through meristem culture in tropical Asia. Asian journal of Plant sciences, 2: 616-622.
15. Foxe, M.J., U.E. Wilson and S.M. Macken, 1986. Rapid *in vitro* propagation of virus indexed potatoes. Research peport 1984-1985. Faculty of general agriculture. University college Dublin, pp: 170.
16. Poleyava, V.S., S.G. Yashina and G.A. Oleinlkova, 1988. Production and propagation *in vitro* of tomato regenerates from isolated meristems. Biologya Kul tiviruemykh kletok I biotekhnologiya, 1: 157-158.
17. Vasanthi, V.J., V. Shanmugam and M. Ramiah, 2001. Elimation of Indian cassava mosaic virus in cassava (*Manihot esculenta* Crantz) through meristem tip culture and specific detection by serological means. Orissa journal of Horticulture, 29(2): 69-74.
18. Marani, F. and A. Pisi, 1977. Meristem-Tip Culture and Vegetative Propagation in Potato.ISHS Horticulturae 78: Symposium on Tissue Culture for Horticultural Purposes, 56.
19. Zaman, S.M., A. Quraishi, G. Hassan, S. Raziuddin, A. Ali, Khabir and N. Gul, 2001. Meristem Culture of Potato (*Solanum tuberosum* L.) for Production of Virus-Free Plantlets. OnLine J. Biol. Sci., 1: 898-899.
20. Alam, M.F., M.L.A. Banu, A.M. Swaraz, S. Parvez, M. Hossain, M. Khalekuzzaman and N. Ahsan, 2004. Production of virus free seed using meristem culture in tomato plant under tropical conditions. Jornal of plant Biotechnology, 6: 221-227.
21. Merja, D. and A. Stasa, 1997. *In vitro* Regeneration and Propagation of Potato and Its Genetic Homogeneity Determination By Means of Protein Polymorphism of Tubers. ISHS Acta Horticulturae, 462: I Balken Symposium On Vegetables and Potatoes, 153.