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Virus Free Potato Tuber Seed Production Through Meristem Culture In Tropical Asia

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Abstract: In order to produce virus free potato tuber seeds *in vitro* culture was established using apical meristem of four widely cultivated varieties of Bangladesh namely Diamant, Cardinal, Multa and Lalpakri. Meristem of these varieties of potato were cultured for shoot proliferation and root induction in MS and MS_0 medium supplemented with different types and concentration of phytohormones. Among the different growth regulator formulations, 0.5 mg l^{-1} GA_3+0.04 mg l^{-1} KIN was found to be the best medium for the primary establishment of meristem. The primary established meristems were subcultured on to MS_0 medium and MS medium containing BA and IBA singly or in combinations. Considering all the treatments singly use of IBA (0.5 mg l^{-1}) is recommended for proper shoot and root development from primary meristem. After DAS-ELISSA test the virus free in vitro grown potato plantlets were using for massive micro propagation. GA_3 and KIN singly or in combinations are used for shoot and root multiplication, among all these combinations 2.0 mg l^{-1} GA_3 was found to be the best media for shoot induction for the studied varities. For high frequency of root formation, combination of GA_3 (0.1 mg l^{-1})+KIN (0.1 mg l^{-1}) was most effective. Rooted plantlet were gradually acclimatized and successfully established in the field. Visual evolution of the morphological trials of the tissue culture derived plants showed all plants were found normal and free from virus diseases. Substantial yield increase was observed in meristem derived plants over their source plants.

Key words: Meristem, diseases elimination, tuber seeds, potato

Introduction

Now a days potato (*Solanum tuberosum* L.) is a vegetable crop of major economic importance of Asian countries including Bangladesh. It is most productive, common and multiuse horticultural vegetable crop. It is a short duration crop that produces a large amount of calories in a short period of time (Vrolijk, 1994). Potato produces more protein and calories per unit area per unit time and per unit of water than any other major plant food (Villamayor, 1984). Today, almost one quarter of the world's potatoes are grown in Asia. Over the last three decades, potato production in Asia has tripled to exceed 60 million tones. While productivity per hectare has increased from an average of 5.85 tones (1961) to 13.29 tones (1991) (van der Zaag, 1992). But the average yield of potato crops in Asia remains low, for in contrast, the average yield of a potato crop in Western Australia is 39.5 t ha^{-1} (Batt, 1994).

Being one of the important cash crops, potato yields higher net return although it requires more labour input than any other crops (Sabur, 1988). But commercial potato production is a complex and highly specialized commercial enterprise that demands a high degree of technical skills and practical experience on the plant of the producer. In many developing countries, yields of root and tuber crops are significantly reduced below their potential due to seed-borne diseases and pests (Clark and Moyer, 1988). In vegetative propagated potato crops, once systematically infected with a viral disease, the pathogen is passed from one vegetative generation to next. There are approximately 23 virus and virus like organisms that cause disease in potato. Khan (1981) reported that a single plant of potato variety has been infected with four to five viruses. The presence of viral disease is an important reason attributed to low yield of potato varieties (Ahmed, 1981; Siddique and Hussaain, 1988). The yield reduction may be up to 75% caused by the infection of some viruses (Rashid, 1987).As such PVX alone may cause yield reduction of 15-30% (Mellor and Stace-Smith, 1987), PLRV and strains of potato virus Y (PVY) frequently reduce tuber yield by 50-80% (deBokx, 1972) and severe strains of potato spindle tuber viroid (PSTV) have been reported to reduce yields by 64% (Shing *et al*., 1971). As a result Asian farmers do not get sufficient virus free seed potato as they need for their cultivation. The major constraint to potato production in Asia is the inadequate supply of reasonably priced, good quality seed tubers of the desired varieties (Crissman, 1987; van der Zaag, 1990; van der Zaag, 1992; Rasco and Aromin, 1994**)**. For this purpose to meet the demand, many Asian countries including Bangladesh are importing a large quality of foundation and certified virus free seed potato from broad and for this they are lossing a great amount of foreign currency.

But there are reports that meristem culture technique for

elimination of virus (Morel and Martin, 1955). Production of virus free through meristem culture technique has been reported in many crops (Bhojwani and Razdan, 1983). These *in vitro* meristem culture has appeared a new venture in obliging virus free potato tuber seeds. Zhang (1995) reported 40% yield increase in potato using virus free tuber seeds. Similar techniques can be applied to a large number of important potato varieties in agriculture in Bangladesh and also in other countries of tropical Asia. Therefore, this present investigation was under taken to develop reproducible protocol for producing of virus free potato tuber seeds applicable for tropical asian countries. The success of this experiment will led to additional financial support from respective government to government and non-government entrepreneurs for producing diseases free tuber seeds of potato.

Materials and Methods

The shoot tips of the commercial cultivars viz, (Diamant, Cardinal, Multa and Lalpakri) collected from 25-35 days old field grown seedlings were used as explants for meristem culture. The experiment was carried out in the Biotechnology and Plant breeding laboratory of Department of Botany, Rajshahi University, Rajshahi, Bangladesh during 1999-2002. At first the excised clean shoot tips were sterilized in savlon and tween-20[polyoxuethelen (20) sorbitan, oleate] flowed by 2-3 times washing with sterilized distil water. Then the explants were washed with 0.1% $HgCl_2$ solution with gently shaking for 2-8 minutes (3 min. is best) flowed by 3-5 times washing with sterilized distil water. From sterilized shoot tips, immature leaves and leaf primordia were snapped off. Then the isolated meristems (0.3 mm) were quickly transferred on the filter paper bridge in test tubes containing liquid MS (Murashig and Sookge, 1962) medium supplemented with BA, GA_3 and KIN either singly or in combination and MS_0 (hormone free medium) for primary establishment (Fig. A). In this level data were recorded on quick responses (days) on the establishment of primary meristem of the studied genotypes. After 3 to 4 weeks of inoculation of meristem, the developed meristems were sub cultured on semi-solid MS_0 and MS medium with different levels of BA and IBA either singly or in combinations for 3-5 weeks for shoot elongation and root formation. Here data were recorded on number of shoot and root formation, shoot length and root formation frequency of four studied genotypes. All the surface sterilization operations were done in side the running laminar airflow cabinet.

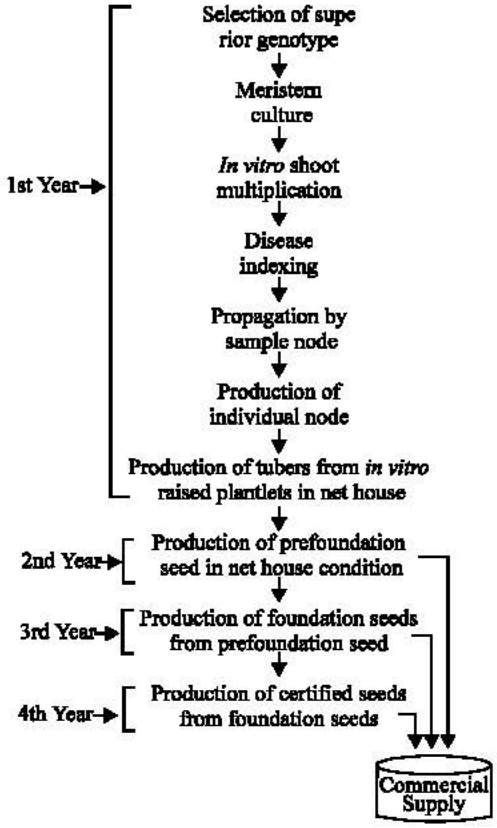
Before massive shoot multiplication, a serological identification/ serodiagnosis was done of the developed plantlets for virus detection. Here the double antibody

Fig. A: Development of meristem (2-days old) on filter paper bridge in liquid medium

sandwitch enzyme-linked immunosorbent assay (DAS-ELISA) was done followed by Clark and Bar-Joseph (1984). The plantlets, which were free from virus, showed no colour development. The single node with auxiliary bud of virus free plantlets were used for massive propagation in MS semi-solid medium with different levels of GA_3 and KIN either singly or in combinations for shoot and root multiplication. All the cultures were incubated at 25 ± 2^0C temperature under 16 photoperiod. The rate of massive propagation was evaluated by the traits viz, number of shoot and root formation, shoot length and root formation frequency of four studied genotypes. For carbon source sucrose was used as 30 g l^{-1} in both MS liquid and MS semi-solid condition and the pH was adjusted to 5.7-5.8.

When the plantlets attained in 3-5 cm heights with few leaves and well developed roots system, were taken out from the controlled environment of growth chamber and were kept in room temperature for 3-5 days to bring them in contact with normal temperature. After 3-5 days of hardening the plantlets were taken out from the test tube and washed the roots under running tap water to remove medium. Then the plantlets were ready for field. Before transplanting the plantlets, the fields were treated with 1% formaldehyde solution to prevent the soil borne pathogen. After 3 days 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 final tuber harvest. The field performance of meristem derived plantlets was evaluated on morphological characters (plant height,

number of leaves plant^{-1}, number of branches plant^{-1} and plant and tuber yield plant^{-1}) and viral diseases index from randomly selected 10 plants. The entire protocol for commercial production of disesses free potato tuber seeds is presented as in flow chart below:



Results and Discussion

For the primary establishment of *in vitro* meristem culture from field grown plant, surface sterilization of shoot tips was carried out with 0.1% $HgCl_2$ solution at different time duration. About 90% of explants were found healthy and free of contamination when the shoot tips were treated for 3 min. Less than 3 min., explants were found contaminated and more than that results tissue killing. Results on establishing primary meristem of four studied genotypes of potato are presented in Table 1. The cultured isolated meristem (0.3 mm) commenced their initial growth by increasing in size and gradually changed to light green colure within 7-21 days (Fig. A and B). According to Stone (1963) only shoot tips between 0.2 and 0.5 mm most frequently produce virus free carnation plants. The explants smaller than 0.2 mm cannot survive, and those larger than 0.7 produce plants that still contain mottle virus. The results on establishing primary meristem further showed that for quick developed and high percentage of growth response of meristem, use of growth regulators was found essential. Most quick

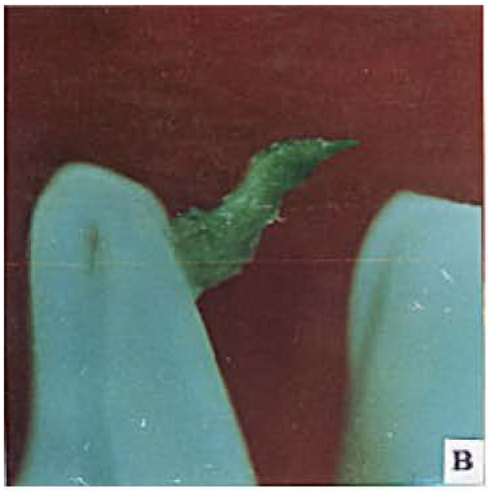


Fig. B: Shoot initiation (14-days old) from the isolated meristem in liquid medium

Fig. C: Development of shoot with leaves from meristem at 21 days of culture in liquid medium

response was obtained when the meristems were cultured in MS medium supplemented with GA_3 along with kin. Better results for plantlet regeneration from meristem have been reported using NAA, IAA and kin (Merja and Stasa, 1997). Regarding concentration 0.5 mg l^{-1} of GA_3 + 0.04 mg l^{-1} of KIN was found most effective. But days for quick response was found variable in different genotypes. For Diamant, Cardinal, Multa and Lalpakri, it was 8-21, 7-21, 9-21 and 10-21 days, respectively. In MS_0 (control) medium growth response (%) of apical meristem was ranged from 10 to 30%, where as in MS+GA_3 and kin the same was ranged from 60 to 80%. Use of liquid culture method for potato tissue culture has also been reported earlier (Goodwin, 1966).

The results on shoot and root development from primary

Table1: Primary establishment of apical meristem

Treatment		Varietiy			
		Diamant	Cardinal	Multa	Lalpakri
MS_0 (control)	Days to response	9-21	10-21	13-21	10-21
	Growth Response(%)	30	30	20	10
GA_3(0.5mg l^{-1})	Days to response	8-21	8-21	9-21	10-21
	Growth Response(%)	50	60	40	40
GA_3+KIN (0.5+0.04) mg l^{-1}	Days to response	8-21	7-21	9-21	10-21
	Growth Response(%)	80	80	60	60
KIN (0.04mg l^{-1})	Days to response	8-21	7-21	12-21	10-21
	Growth Response(%)	40	50	20	30
BA (0.5mg l^{-1})	Days to response	8-21	7-21	12-21	10-21
	Growth Response(%)	70	70	50	50

Table 2: Effect of different concentration of hormone on shoot and root development: number and length (cm) of shoot, and root number and formation (%) from developed primary meristem. Data were recorded at 21 days after inoculation

Treatment			Variety			
			Diamant	Cardinal	Multa	Lalpakri
MS0 (control)		Number of shoot	1.10 ±0.10	1.13±0.10	1.10 ±0.10	1.22± 0.15
		Number of root	1.90±0.38	1.88±0.34	5.11±0.82	2.22±0.61
		Shoot length	2.32±0.47	2.49±0.08	2.49± 0.47	2.70± 0.14
		Root formation	40	50	50	44
BA	0.5 mg l^{-1}	Number of shoot	2.60 ±0.22	2.82 ±0.15	2.22±0.22	1.20 ±0.20
		Number of root	0.80±0.22	--	1.00±0.18	--
		Shoot length	3.75±0.31	3.34±0.15	2.87± 0.30	2.37± 0.25
		Root formation	20	--	20	--
	2.0 mg l^{-1}	Number of shoot	1.80 ±0.49	2.20 ±0.13	1.30 ±0.21	2.40 ±0.27
		Number of root	-	-	0.80±0.37	-
		Shoot length	3.75±0.19	3.86±0.26	3.75±0.38	3.80± 0.37
		Root formation	-	-	10	-
IBA	0.5 mg l^{-1}	Number of shoot	1.20 ±0.49	1.30 ±0.21	2.50 ±0.17	2.40 ±0.22
		Number of root	14.20±2.44	18.70±2.52	14.50±1.82	16.80±1.41
		Shoot length	4.86±0.37	6.94±0.34	6.64±0.32	4.46±0.88
		Root formation	100	100	100	100
	1.0 mg l^{-1}	Number of shoot	1.20 ±0.10	2.10 ±0.10	2.10 ±0.40	1.50 ±0.25
		Number of root	12.30 ±2.31	16.10±1.66	13.10 ±2.46	7.40±1.31
		Shoot length	6.22±0.71	7.07±0.63	4.43±0.80	3.51±0.24
		Root formation	100	100	100	100
BA+IBA	0.5+0.5 (mg l^{-1})	Number of shoot	2.20 ±0.15	2.30 ±0.15	2.50 ±0.22	1.20 ±0.20
		Number of root	14.00 ±2.38	14.00±1.98	15.80 ±2.55	15.30 ±1.70
		Shoot length	3.22±0.55	4.61±0.44	5.43± 0.82	3.61± 0.47
		Root formation	100	100	100	100
	1.0+1.0 (mg l^{-1})	Number of shoot	2.20 ±0.15	2.20 ±0.15	1.40 ±0.13	2.20 ±0.13
		Number of root	9.30±1.59	10.90±0.95	8.70±1.70	7.80±1.74
		Shoot length	4.36±0.73	4.10±0.44	4.05± 0.76	3.42± 0.72
		Root formation	100	100	100	100

meristem are presented in Table 2. Among the different combination of BA and IBA either singly or in combinations, singly use of BA (0.5 mg l^{-1}), was found effective for high number shoot formation. Single use of low concentration of BAP (2 mg l^{-1}) results high number of shoot and node development and decrease at higher concentration in potato (Hoque *et al*., 1996). On the other hand singly use of IBA (0.5 mg l^{-1}) was found enough for high number and parentage root formation as well as longest shoot formation. Combination of BA (0.5 mg l^{-1}) + IBA (0.5 mg l^{-1}) also found good for high parentage root and shoot formation. Singly use of BA (1.0 mg l^{-1}) in modified MS medium was reported good for plantlets development from meristem-tip culture is potato (Marani and Pisi, 1977). For proper root development from plantlets derived from meristem-tip, use of IBA (1 mg l^{-1}) was reported effective (Marani and Pisi, 1977 and Zaman *et al*., 2001). Comparing the different varieties, primary meristem of Diamant and Cardinal were found more response than Multa and Lalpakri. Considering the all treatments singly use of IBA (0.5 mg l^{-1}) is recommended for proper root and shoot development from primary meristem (Fig. C and D). Here also the results in control medium uses not encouraging.

Singly use of GA_3 or kin and joined use of GA_3+kin for massive micro propagation of virus free plantlets using nodal segments derived from meristem culture plantlets are presented in Table 3. Based on earlier results (Table 2) MS_0 (control) medium was not tested here. The data reveal that use of GA_3 was found useful for massive micro

Table 3: Effect of different concentration of GA_3 and KIN, and GA_3 + KIN in MS semi-solid medium for massive micropropagation of four varieties of potato using nodal segment derived from meristem culture plantlets. Data were recorded on shoot and root number, shoot length (cm) and root formation frequency at 30 days after inoculation

			Varieties			
Treatments			Diamant (X ±SE)	Cardinal (X ±SE)	Multa (X ±SE)	Lalpakri (X ±SE)
GA_3 (mg l^{-1})	0.1	Shoot Number	1.50±0.25	1.20±0.20	1.00±0.00	1.75±0.43
		Root Number	2.00±0.76	2.40±0.63	1.00±0.00	2.00±0.00
		Shoot length	2.70±0.77	2.86±0.47	2.67±0.83	2.28±.066
		Root formation frequency	75	75	50	50
	0.5	Shoot Number	1.50±0.29	1.25±0.25	1.40±0.24	1.25±0.25
		Root Number	1.20±0.20	1.25±0.35	1.40±0.24	0.25±0.00
		Shoot length	4.88±0.26	4.25±0.13	3.12±0.28	3.48±0.95
		Root formation frequency	--	50	75	25
	1.0	Shoot Number	1.80±0.20	2.20±0.37	1.20±0.20	1.10±0.10
		Root Number	3.40±0.27	4.00±0.68	2.40±0.52	2.75±0.07
		Shoot length	5.36±0.56	6.66±1.21	5.08±0.29	3.55±0.55
		Root formation frequency	20	60	60	25
	2.0	Shoot Number	2.40±0.24	2.60±0.60	2.00±0.00	1.20±0.40
		Root Number	3.80±1.34	3.20±0.44	1.00±0.29	3.60±0.06
		Shoot length	6.32±0.12	5.70±0.44	4.45±0.23	4.62±0.63
		Root formation frequency	100	60	75	80
KIN (mg l^{-1})	0.1	Shoot Number	1.75±0.48	1.60±0.45	1.10±0.10	1.10±0.10
		Root Number	2.00±0.67	2.20±0.97	1.80±0.48	2.50±0.09
		Shoot length	3.63±0.87	3.52±0.36	2.20±0.16	2.52±0.48
		Root formation frequency	75	100	80	75
	0.5	Shoot Number	1.10±0.10	2.00±0.00	2.00±0.45	1.40±0.24
		Root Number	1.80±0.56	1.60±0.32	1.40±0.22	1.80±0.00
		Shoot length	3.96±0.87	3.88±0.54	3.06±0.71	3.18±0.53
		Root formation frequency	80	50	80	100
	1.0	Shoot Number	2.25±0.25	1.60±0.24	1.25±0.25	1.25±0.25
		Root Number	1.75±0.27	1.80±0.13	--	1.75±0.08
		Shoot length	3.97±0.41	3.04±0.67	2.30±1.21	2.40±0.44
		Root formation frequency	75	40	--	100
	2.0	Shoot Number	1.25±0.25	1.20±0.20	1.50±0.37	1.20±0.20
		Root Number	0.75±0.35	1.20±0.20	--	--
		Shoot length	3.33±0.67	4.24±0.71	3.48±0.67	3.70±0.31
		Root formation frequency	50	80	--	--
GA_3 (mg l^{-1})+KIN (mg l^{-1})	0.1+0.1	Shoot Number	2.00±0.00	2.00±0.53	1.40±0.40	1.50±0.50
		Root Number	2.00±0.32	2.50±1.26	1.60±0.24	2.25±1.06
		Shoot length	4.22±0.27	3.25±0.45	3.48±0.67	3.70±0.31
		Root formation frequency	100	75	100	75
	0.5+0.5	Shoot Number	2.20±0.20	1.35±0.29	1.75±0.48	1.50±0.29
		Root Number	1.20±0.30	0.75±0.35	0.80±0.26	0.75±0.05
		Shoot length	2.96±0.53	3.48±0.75	2.18±0.41	2.53±0.41
		Root formation frequency	50	50	75	25
	1.0+1.0	Shoot Number	1.25±0.25	2.00±0.50	1.00±0.00	1.25±0.25
		Root Number	--	1.25±0.87	--	0.75±0.00
		Shoot length	5.98±0.90	6.00±1.67	2.50±0.59	3.80±0.67
		Root formation frequency	--	75	--	75
	2.0+2.0	Shoot Number	1.50±0.29	1.25±0.25	1.60±0.40	1.40±0.24
		Root Number	2.00±1.26	2.20±0.18	0.60±0.32	0.80±0.03
		Shoot length	5.10±1.05	5.28±0.43	3.41±0.41	4.58±0.97
		Root formation frequency	50	50	75	40

Table 4: Field performance of meristem derived plantlets. Morphological and tuber yield data were recorded at 60 and at 90 days, respectively after transplantation of plantlets in net house

				Tuber yield $plant^{-1}$ (g)		Viral diseases index	
Variety	Plant hight (X±SE)cm.	No. of leaves $plant^{-1}$ (X±SE).	No. of branch $Plant^{-1}$ (X±SE).	Meristerm plant	Tuber plant	Meristem plant.	Tuber plant
Diament	51.42±3.60	66.58±1.69	3.50±0.25	419	220	-	40
Cardinal	58.42±1.91	70.39±2.20	4.11±0.43	124	61	-	25
Multa	47.22±3.08	56.34±7.22	2.67±0.51	475	236	-	20
Lalpakri	42.92±4.28	53.58±6.17	3.00±0.38	364	122	-	-

propagation of plantlets (Fig. E). Regarding the trait shoot length, use of 2.0 mg l^{-1} of GA_3 was found most effective for the studied varieties. Similar observations are also reported by others (Goodwin *et al*., 1980; Hoque *et al*.,

Fig. D: Development of shoots and root after transferring in semisolid medium

Fig. E: Massive multiplication of plantlets using node from meristem derived plantlet in semisolid medium

1996; Novak and Zadina,1987 and Sarker and Mustafa, 2002). Use of GA_3+BA for many adventitious shoots development has been reported (Marani and Pisi, 1977 and Evans *et al*., 1984). However Zaman *et al*. (2001) recommended use of combination of NAA and IBA for shoot development with high number of leaves. For high frequency of root formation combination of GA_3 (0.1 mg l^{-1}) + kin (0.1 mg l^{-1}) was most useful. Same as Table 2, the varieties Diamant and Cardinal were more responsive for massive micro propagation of plantlets than Multa and Lalpakri. Differential responses of different potato varieties due to genetic make up towards *in vitro* shoot multiplication and their development were also reported

Fig. F: Soil establishment of meristem derived plantlets in net house

by earlier workers (Hussey and Stacey, 1981; Millar *et al*., 1985 and Bajaj, 1981). Considering the all results , for massive micropropagation of plantlets from nodal segments of meristem derived plant, 2 mg l^{-1} of GA_3 in MS mediums is recommended.

The results on field performance of meristem derived plantlets are presented in Table 4. Morphological characters of the meristem derived plantlets were found normal (Fig. F). Varietal stability was also reported among the meristem derived regenerated plants (Merja and Stasa, 1997). From visual observation of the plants no symptoms of viral diseases were noticed. Tuber yield of meristem derived plants was twice or more than that than tuber derived plants.

Considering the all experimental results, it is strongly recommended of using meristem culture techniques for production of virus free potato tuber seed stock and their commercial use for potato production in tropical Asian countries.

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