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Full Length Research Paper

Effect of 6-benzyl aminopurine (BAP) on meristem culture for virus free seed production of some popular potato varieties in Bangladesh

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The present study was undertaken to fix the suitable concentration of 6-benzyl aminopurine on shoot development from meristem for producing virus free potato plantlet. The experiment consisted of five potato cultivars namely Diamant, Heera, Dheera, Granula and Cardinal for meristem culture and four 6-benzyl aminopurine levels namely 0, 1.0, 1.5, 2.0 mg/l. As a whole, twenty treatments were allotted in complete randomized design with three replications. Resulted *in vitro* regenerated plantlets were used as treatment for double antibody sandwich (DAS-), enzyme linked immunosorbent assay (ELISA) test, while, the infected plant parts were used as positive control. In virus elimination through meristem culture, Cardinal with 1 mg l^{-1} 6-benzyl aminopurine gave maximum number of shoots (2.43 cm) explant^{-1}, whereas, Dheera with 1.5 mg l^{-1} 6-benzyl aminopurine gave the tallest plantlet (5.23 cm). On the other hand, explants of Dheera and Cardinal with 1.5 mg l^{-1} 6-benzyl aminopurine and explants of Diamant, Heera, Granula and Cardinal with 2.0 mg l^{-1} 6-benzyl aminopurine produced no roots. Finally, after DAS-ELISA test, the infected field samples developed yellow color but *in vitro* regenerated plantlets of all the varieties under study showed 100% virus freeness.

Key words: Virus elimination, meristem culture, virus detection, seed potato.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is an important tuber crop and is mainly used as vegetable in Bangladesh (Rashid et al., 1993). In Bangladesh, about 8.5 million tons of potato were produced from nearly 0.472 million ha of land with an average yield of 18 t ha^{-1} (BBS, 2010). This yield is very low compared to many other countries like the Netherlands (45 t ha^{-1}), Germany (46 t ha^{-1}), Scandinavian countries (48 to 52 t ha^{-1}) and neighboring India (21 t ha^{-1})(Beukema and Vander Zaag, 1990; Rashid et al., 1993).

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Abbreviations: BAP, 6-Benzyl aminopurine; **DAS-ELISA,** double antibody sandwich enzyme linked immunosorbent assay; **CRD,** completely randomized design; **MS,** Murashige and Skoog; **PVX,** potato virus X; **PVY,** potato virus Y.

Lack of quality in seed potato is one of the most important reasons for this low yield. As potatoes are propagated vegetatively, the tissue borne pathogens of previous years can be perennated over the generation.Among the viruses, potato leaf roll virus (PLRV), potato virus X (PVX) and potato virus Y (PVY) are most prevalent (Khalid et al., 2000). All these viruses are tuber borne, which reduce plant vigour and yield potential of potato. The successful productions of potatoes demand the control of these viruses, which cannot be controlled by any physical or chemical agent. Eradication of virus diseases through meristem culture is a useful technique and is used in International Potato Centre (CIP), Peru, Lima (Ng and Dodds, 1989), and also in Scotland (Jeffries, 1986). But the technique is controlled by various physical (light, temperature, etc.) and chemical (growth regulators) factors. Meristem culture has been successfully applied in potato for

development of virus free plants. The *in vitro* technology combined with traditional practices have enhanced the commercial production of virus free seeds, which might be considered as an pre-requisite to maximize yield in potato (Faccioli and Colombarini, 1996). There are reports that meristem culture technique along with thermotherapy has become a powerful and successful tool for virus elimination from infected plants (Paet and Zamora, 1990) and among the serological procedures, double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) test required short time for inoculation and observation and hence, it was widely used for virus detection. Thus, the use of virus free potato seeds in Bangladesh not only increases production, but will also help in attaining food security. So, the present study was undertaken to find out the suitable concentration of 6-benzyl aminopurine (BAP) on shoot development from meristems of five potato cultivars and produced virus free potato and virus indexing through DAS-ELISA test.

MATERIALS AND METHODS

The experiment was carried out *in vitro* in Biotechnology Laboratory, Department of Biotechnology, Bangladesh Agricultural University, Bangladesh, during the period from August 2003 to February 2004. The experiment consisted of five potato cultivars namely Diamant, Heera, Dheera, Granula and Cardinal being used for meristem culture and four BAP levels namely 0, 1.0, 1.5, 2.0 mg l^{-1}. Consequently, the comprised treatments were twenty. The treatments were distributed in complete randomized design with three replications. Tested and infected tubers by PVX and PVY were collected from Tuber Crops Research Center (TCRC), Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh. Initially, the potato tubers were sprouted in the dark at room temperature (25°C) and 2 ppm GA_3 were sprayed for sprout initiation. After initiation, selected sprouts (approximately 1 cm long) were harvested for culture. Harvested sprouts were sterilized with 70% ethanol, 0.1% $HgCl_2$, 2 to 3 drops of Tween-20 and required amount of double distilled (dd) water. The sprouts were then transferred to culture medium (MS medium) and the culture was maintained at 25°C temperature under 16 h photoperiod. Microplants derived from cultured sprouts were ready for multiplication within 20 to 25 days. These microplants were repeatedly sub cultured on semisolid MS media for preparing sufficient materials required for *in vitro* experiments.

When the *in vitro* sprout cultured potato plantlets of all varieties grew to an adequate amount, apical buds were removed from the growing lateral buds, and then they produced more branches. After that these plantlets were moved to thermotherapy room and were kept under 7500 lux light intensity with 16 h photoperiod at 35°C and relative humidity of 60 to 65% (Islam and Chowdhury, 1998). Thermotherapy provided plantlets were used for meristem isolation after 4 to 5 weeks. Meristem parts were taken from leaf buds. As the top-shoot meristems are covered by numerous soft bristles; the petiole, immature leaves and leaf primordial were peeled off under binocular stereoscope microscope with forceps and mounted needles. Then meristems with two leaf primordial, about 0.2 to 0.5 mm long were excised by a single and accurate hit of the surgical blade. A single piece of isolated meristem was then transferred immediately to each test tube containing the semisolid MS media with α-napthalene acitic acid (NAA) 0.5 mg l^{-1} and different levels of BAP. Then the tubes were incubated at 25°C and photoperiod of 16 h. Data on number of shoots explant^{-1} and number of roots explant^{-1} were calculated on the basis of total explants cultured and plantlet height was calculated on the basis of total shoots regenerated. Data were analyzed by statistical package MSTAT (Version 1.41) and Microsoft Excel (Microsoft Office 2003) wherever applicable. The analysis of variance was performed and means were adjudged by the Duncan's Multiple Range Test (DMRT) by MSTAT.

Regenerated plantlets were tested for virus detection by DAS-ELISA test. Many certification authorities preferred visual detection of pathogens on the potato plants or seed tubers which did not produce detectable symptoms, as with certain strains of potato virus X (Banttari et al., 1993). Five meristem cultured potato cultivars were tested against the presence or absence of PVX and PVY by ELISA kits (BIOREBA, BIOREBAAG, Chr. Merian-Ring 7', CH-4153, Reinach BL 1, Switzerland). Infected samples of the same varieties were used as controls. Two microtiter plates, one for PVX and one for PVY were used. Each plate contained 96 wells. Against each virus, three samples from each meristem cultured cultivar and three samples from each sprout cultured, that is, controlled cultivar, were tested. Total treatments for each virus were 10 (meristem culture 5 + control 5) ×3 = 30. Data were recorded on the number of samples that developed yellow color for both PVX and PVY. Data were not analyzed statistically. Percentage of samples that developed yellow color was calculated on the basis of total samples tested for each cultivar.

RESULTS AND DISCUSSION

Number of shoots explant^{-1}

When varietal effect was considered from the analyzed data, it was found that Cardinal produced the highest number of shoots explant^{-1} (1.98) followed by Dheera (1.83), whereas the lowest number of shoots explant^{-1} (1.71) was obtained from Diamant (Table 1). Similar results also obtained by Nagib et al. (2003) where among the varieties; Cardinal was the best and Diamant was also found more responsive than Multa and Lal Pakri. So, comparing both studies, it could be recommended that Cardinal responded the best. The result is also supported by other findings of Millar et al. (1987) where differential responses of different potato varieties due to genetic makeup towards *in vitro* shoot multiplication and their development was reported.

Irrespective of variety, statistically significant differences were found at different BAP levels where 1.5 mg l^{-1} BAP (Figure 1) gave maximum number of shoots explant^{-1} (2.10) followed by 1.0 mg l^{-1} BAP (2.08). Minimum number of shoots explant^{-1} (1.35) was obtained without BAP (Table 1). The result is supported by Haque et al. (1996) where low concentration of BAP resulted in high number of shoot and node development in potato which declined at high concentration.

In combined effects, Cardinal gave maximum number of shoots explant^{-1} (2.43) with 1 mg l^{-1} BAP (Figure 2) followed by Granula with 1.5 mg l^{-1} BAP (2.40). The minimum number of shoots explant^{-1} (1.23) was obtained from Diamant without BAP (Table 2). Similar results were also obtained by Nagib et al. (2003) where Cardinal gave maximum number of shoots explant^{-1} with 0.5 mg l^{-1} BAP than with 2.0 mg l^{-1} and without BAP.

Table 1. Main effect of variety and BAP on shoots, roots and plantlets development *in vitro* at two months of explantation.

Variety / BAP	Number of shoots explant^{-1}	Number of roots explant^{-1}	Plantlet height (cm)
Variety			
Diamant	1.71^{a}	0.41^{b}	3.63^{b}
Heera	1.77^{a}	0.38^{b}	3.74^{b}
Dheera	1.83^{a}	0.76^{a}	4.00^{a}
Granula	1.82^{a}	0.70^{a}	3.68^{b}
Cardinal	1.98^{a}	0.50^{b}	3.28^{c}
BAP (mg l^{-1})			
0	1.35^{c}	1.77^{a}	2.49^{c}
1.0	2.08^{a}	0.38^{b}	3.73^{b}
1.5	2.10^{a}	0.05^{c}	4.69^{a}
2.0	1.74^{b}	0.01^{c}	3.77^{b}
CV (%)	17.54	31.88	8.31

Figures followed by same letter(s) are statistically similar as per DMRT.

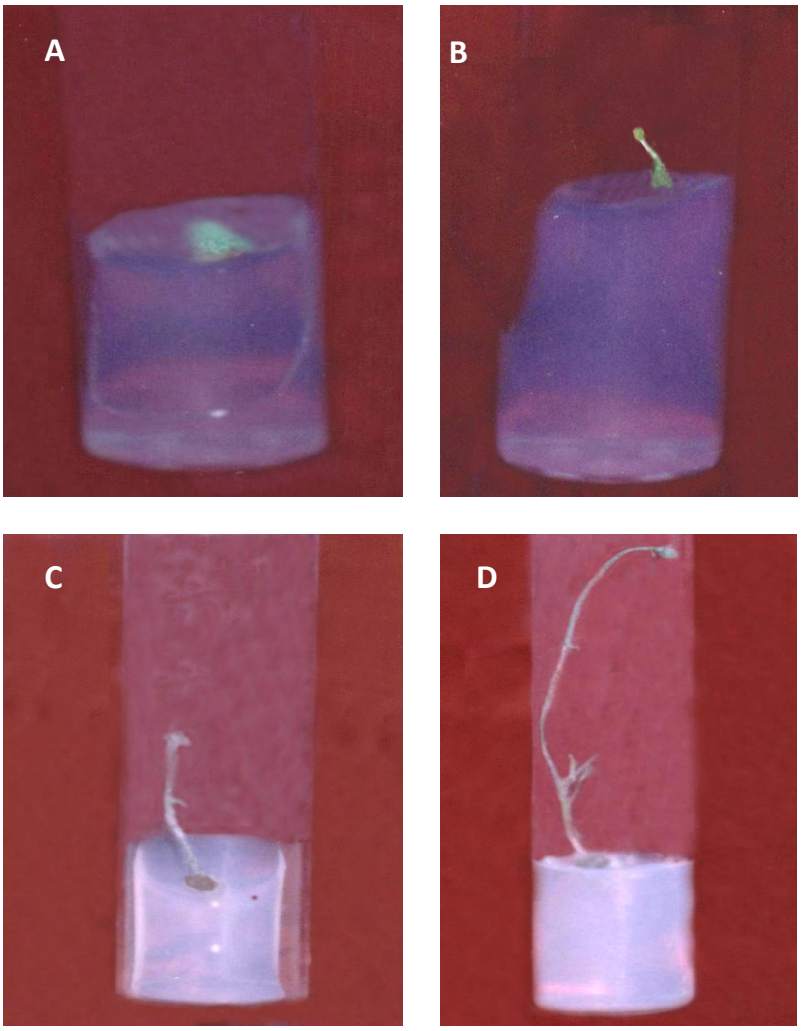

Figure 1. Development of plantlets during meristem culture of potato cv. Dheera; A) fifteen days after explantation; B) thirty days after explantation; C) forty five days after explantation and D) Sixty days after explantation.

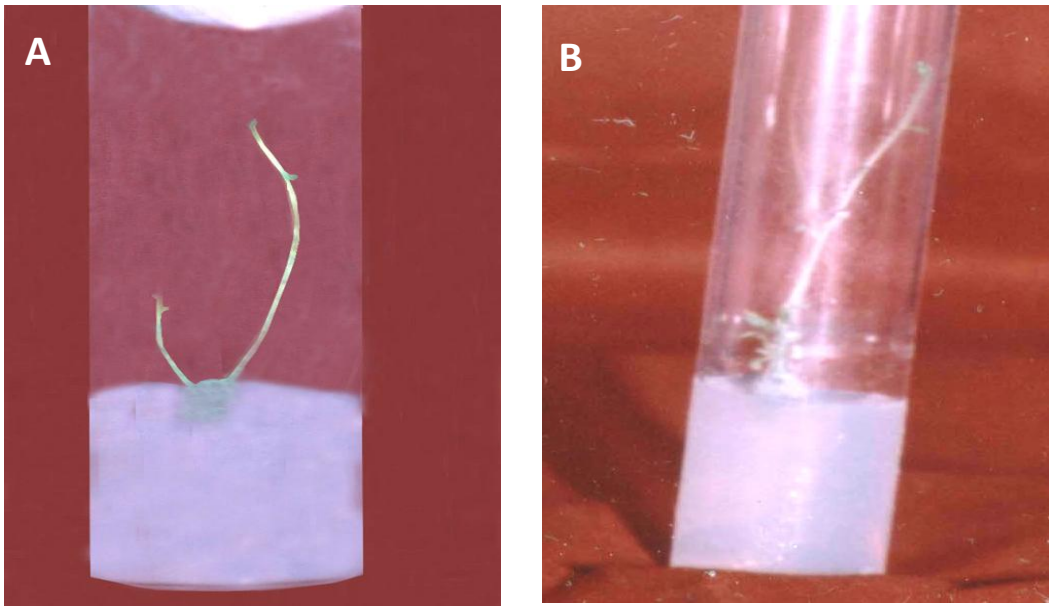

Figure 2. Number of shoots meristem cultured explant^{-1} under different treatments at sixty days of explantation; A) Cardinal with 1.0 mg l^{-1} BAP sixty days after explantation; B) Heera without BAP sixty days after explantation.

Table 2. Combined effect of variety and BAP on shoots, roots and plantlets development *in vitro* at two months of explantation.

Variety	BAP (mg l^{-1})	No. of shoots explant^{-1}	No. of roots explant^{-1}	Plantlet height (cm)
Diamant	0	1.23^{f}	1.33^{d}	2.27^{g}
	1.0	2.17^{a-d}	0.47^{ef}	3.63^{def}
	1.5	1.83^{a-f}	0.03^{h}	4.70^{ab}
	2.0	1.60^{def}	0.00^{h}	3.93^{cd}
Heera	0	1.27^{f}	1.03^{d}	2.53^{g}
	1.0	1.60^{def}	0.43^{efg}	3.80^{c-f}
	1.5	2.27^{abc}	0.07^{h}	4.77^{ab}
	2.0	1.93^{a-e}	0.00^{h}	3.87^{cde}
Dheera	0	1.47^{ef}	2.77^{a}	2.43^{g}
	1.0	2.10^{a-d}	0.23^{e-h}	4.27^{bc}
	1.5	1.77^{c-f}	0.00^{h}	5.23^{a}
	2.0	1.97^{a-e}	0.03^{h}	4.07^{cd}
Granula	0	1.37^{ef}	2.13^{b}	2.53^{g}
	1.0	2.10^{a-d}	0.54^{e}	3.63^{def}
	1.5	2.40^{ab}	0.13^{gh}	4.83^{a}
	2.0	1.40^{ef}	0.00^{h}	3.73^{c-f}
Cardinal	0	1.43^{ef}	1.80^{c}	2.67^{g}
	1.0	2.43^{a}	0.20^{fgh}	3.33^{ef}
	1.5	2.23^{abc}	0.00^{h}	3.90^{cde}
	2.0	1.80^{b-f}	0.00^{h}	3.23^{f}
CV (%)		17.54	31.88	8.31

Figures followed by same letter(s) are statistically similar as per DMRT.

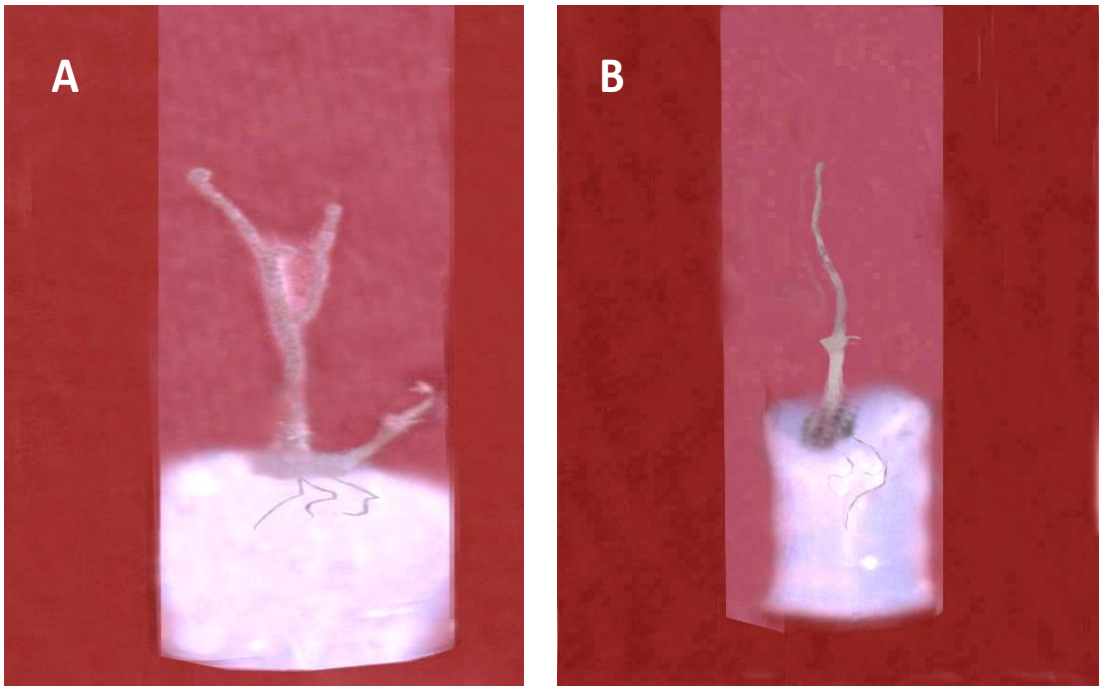

Figure 3. Number of roots meristem cultured explant^{-1} under different treatments at sixty days of explantation; A) Dheera without BAP sixty days after explantation; B) Granola with 1.5 mg L^{-1} BAP sixty days after explantation.

Number of roots explant^{-1}

The differences among the varieties was found statistically significant where maximum number of roots explant^{-1} (0.76) was obtained from Dheera followed by Granula (0.70) and minimum (0.38) from Heera (Table 1). Similar results were also obtained by Sanavy and Moeini (2003) where meristems of cvs. Agria and Marfona were varied significantly in respect of root number and root length. In another study (Nagib et al., 2003), comparing the different varieties, Diamant and Cardinal were found more responsive than Multa and Lal Pakri in respect of root number.

The differences among the number of roots explants^{-1} also varied significantly with different BAP levels. The maximum number of roots explant^{-1} (1.77) was obtained without BAP and minimum (0.01) was found with 2.0 mg l^{-1} BAP (Table 1). The results were supported by Sanavy and Moeini (2003) where medium without NAA and BAP was found to be the best for the formation of roots whereas addition of BAP and NAA in the medium decreased rooting.

In case of combined effects, the highest number of roots (2.27) was obtained from Dheera (Figure 3) followed by Granula (2.13) without BAP. Explants of Dheera and Cardinal with 1.5 mg l^{-1} BAP and Diamant, Heera, Granula and Cardinal with 2.0 mg l^{-1} BAP produced no roots (Table 2). Interaction effects between cultivar and hormone (BAP and NAA) were also significant at 1% level for root number and greater root length (Sanavy and Moeini, 2003).

Plantlet height

The differences of the plantlet height were found statistically significant where the tallest plantlets (4.00 cm) were obtained from Dheera followed by Heera (3.74 cm), whereas Cardinal (3.28 cm) found to be the shortest (Table 1). The results were supported by Sanavy and Moeini (2003) where plantlet length (3.62 cm) in Agria cultivar was more than Marfona cultivar (3.32 cm), from where it could be concluded that varieties might have significant effect on plantlet height.

In respect of different BAP levels, the highest result (4.69 cm) was obtained from 1.5 mg l^{-1} BAP and the lowest (2.49 cm) was obtained in the absence of BAP and the differences were found statistically significant (Table 1). The results were inversely related with the study of Sanavy and Moeini (2003) where treatments without BAP produced taller (5.80 cm) and 1.5 mg l^{-1} BAP shorter (2.48 cm) plantlets. Based on the present results, it might be concluded that BAP does not have uniform responses on varieties studied. It may be because of different genetic make up .

For combined effects, differences were also found significant where the highest result (5.23 cm) was obtained from Dheera (Figure 4) with 1.5 mg l^{-1} BAP and the lowest (2.27 cm) from Diamant (Figure 5) without

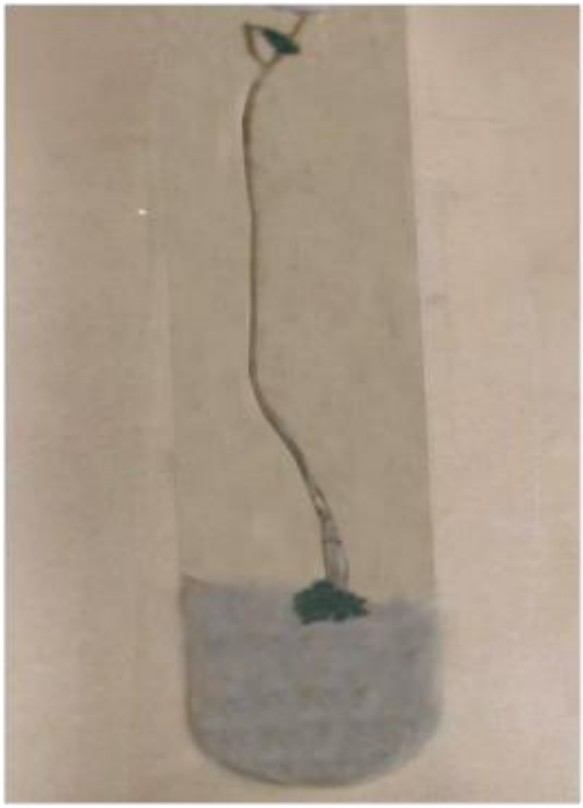

Figure 4. Height of meristem derived plantlet of Dheera with 1.5 mg l^{-1} BAP at two months of explantation.

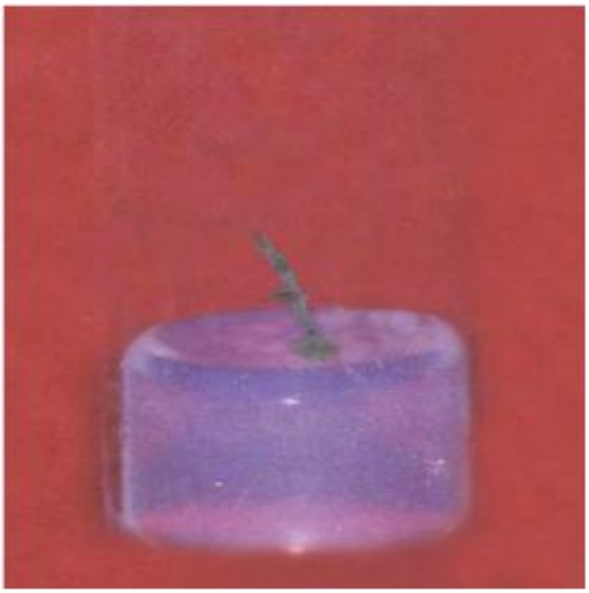

Figure 5. Height of meristem derived plantlet of Diamant without BAP at two months of explantation.

BAP (Table 2). The results were supported by Sanavy and Moeini (2003) where BAP responded differentially with cultivars. BAP decreased plantlet height in both potato cultivars Agria and Marfona but the effect on

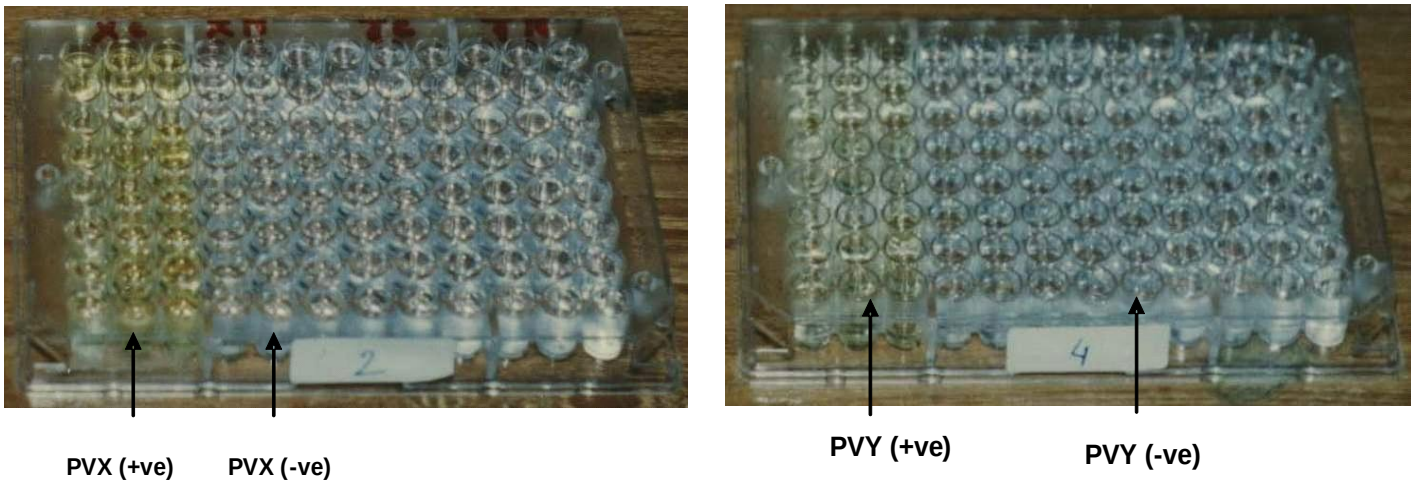

Figure 6. Colour development in microtiter wells after ELISA test for; A) potato virus X (PVX); B) potato virus Y (PVY).

Table 3. Observation of colour development during ELISA test.

Virus	Variety	Number of samples tested		Number of samples developed colour		% virus freeness
		Meristem cultured	Controlled	Meristem cultured	Controlled	
PVX	Diamant	3	3	0	3	100
	Heera	3	3	0	3	100
	Dheera	3	3	0	3	100
	Granula	3	3	0	3	100
	Cardinal	3	3	0	3	100
PVY	Diamant	3	3	0	3	100
	Heera	3	3	0	3	100
	Dheera	3	3	0	3	100
	Granula	3	3	0	3	100
	Cardinal	3	3	0	3	100

Marfona was more pronounced.

Virus detection through ELISA tests from meristem cultured plantlets

PVX

None of the three samples for each of the *in vitro* regenerated varieties showed any colour, whereas, all the infected field samples (symptoms showing) developed yellow colour after DAS-ELISA test (Figure 6A and Table 3). This indicated that the antiserum reacted strongly with all infected plants but the *in vitro* regenerated plantlets did not react. This may be due to the regenerated plantlets becoming 100% free from virus through meristem culture. This result is supported by Khan et al. (2003) who observed similar results for the detection of potato viruses through DAS-ELISA test.

PVY

No sample out of three for each variety showed any colour, whereas all the three infected varieties showing positive symptoms from the field for each variety developed yellow colour after DAS-ELISA test (Plate 6B). This result indicates that the *in vitro* regenerated plantlets became 100% free from virus through meristem culture. After DAS-ELISA test, it was confirmed by Jeffries (1998) that the plants that showed no symptom of virus infection were not virus affected.

Conclusion

We can say that this protocol has the potential to produce large number of virus free plantlets in a very short time and play an important role in the rapid multiplication of virus free seed potato for food security.

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