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REGENERATION OF VIRUS-FREE POTATO PLANTLETS FROM INFECTED POTATO PLANTS AND TUBER SPROUTS THROUGH MERISTEM TIP CULTURE

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ABSTRACT: Meristem Tip Culture (MTC) technique was used to produce virus-free plantlets from potato plants that were naturally or mechanically infected with virus (es) as well as from tubers sprouts collected from infected plants. These viruses were *Alfalfa mosaic virus* (AMV), *Potato leaf roll virus* (PLRV), *Potato virus Y* (PVY), *Potato virus X* (PVX), *Potato virus A* (PVA), *Potato virus S* (PVS), *Tomato spotted wilt virus* (TSWV), and *Potato virus M* (PVM). MTC was successful in eliminating AMV, PVY and PVA from potato plants that were naturally infected or mechanically inoculated with the virus (es). Out of the 469 cultured shoot tips, 98 plantlets were regenerated, with 31 of which found to be free from virus infection when indexed with ELISA technique. All produced virus-free plantlets were from singly or doubly infected potato plants. MTC was successful in eliminating 1-4 viruses per plant from those found multiply virus-infected. With one exception, the different sizes of explants used, indicated that the smaller explants sizes gave higher percentages of virus-free plantlets. The explants obtained from apical sprouts yielded higher percentage of virus-free potato plantlets compared to those obtained from lateral sprouts of the same tuber.
Key words: ELISA, explants, Meristem tip culture, plantlet, Potato viruses, sprout

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INTRODUCTION

Potato is the fourth most cultivated food crop after wheat, rice, and Maize therefore, it is the most important dicotyledonous and tuber crop in the world. Potato is known to be a good and cheap source of carbohydrates, vitamins, minerals and proteins and provides most of the trace elements which can meet the energy requirements of the people living in the developing countries [1]. The average potato yield in US and Western Europe often reaches 40 tons per hectare [2].This number drops significantly to 1/3 of that value or even less in some developing countries due to the improper practices such as management of pests and diseases, including diseases caused by viruses [3].

Potato production which started in 1973 in Saudi Arabia has increased from an initial yield of 20 tons in 1976 and reached 390259 tons from an area of 15212 hectares in 2013 [4] as a result of the establishment of the Potato Development Programme in collaboration between the Saudi ministry of Agriculture and water and the Dutch ministry of Agriculture and Fishery to introduce potato in a large scale production [5].

Potato was reported to be affected with many diseases, of which virus diseases are considered worldwide [6, 7] to be one of the important factors attributed to the drop of potato yield which was reported to range between 15-80% [8,9, 10]. Eleven viruses were reported to infect potato in Saudi Arabia.

These included *Cucumber mosaic virus* (CMV), *Tobacco mosaic virus* (TMV), *Potato leaf roll virus* (PLRV), *Potato virus Y* (PVY), *Potato virus X* (PVX), *Potato virus A* (PVA), *Potato virus S* (PVS), *Tomato spotted wilt virus* (TSWV), *Alfalfa mosaic virus* (AMV), *Potato virus M* (PVM) and *Tobacco ring spot virus* (TRSV) (11, 8, 12) with AMV being the most spreading virus in the country. AMV was the most frequently detected virus in potato in a two year survey conducted in the six major potato-producing regions in Saudi Arabia with a percent of detection ranging between 44-78% and an average yield reduction of up to 46.1% [11, 8,12].

Today tissue culture applications have expanded to encompass much more techniques such as virus elimination in potato and other crops [13, 14,15,10,16, 17]. MTC was proved to be an efficient method for eradication of viruses from potato to produce virus-free potato plants [15, 10,18, 19,20, 21]. The objective of this study is to (a) produce virus-free potato plantlets through elimination of viruses from potato plants singly or multiply infected with viruses through MTC technique (b) eliminate viruses from sprouts of tubers collected from infected potato plants using MTC.

MATERIALS AND METHODS

Production of plantlets from AMV - infected potato plants

Potato plants produced from certified tubers of Ajax cultivar were mechanically inoculated with AMV inoculum in the green house at the age of three weeks, after dusting with carborundum. The inoculum was prepared by grinding of AMV -infected potato leaves in 0 .1 M potassium phosphate Buffer, pH 7 .01, in a ratio of 1g of plant tissues/4 ml buffer. Six weeks later, shoot apices 2-3 cm long were excised from plants showing obvious AMV symptoms and reacted positively with ELISA kits of AMV. These shoot apices were surface sterilized by immersing in 10% Chlorex (5.25% Sodium hypochlorite) containing a few drops of tween 20. The disinfested shoot apices were then rinsed three times with distilled deionized water under sterile conditions. Apices of 0.2, 0.3, 0.5, 0.6, 0.8, 1.0 and10.0 mm in size were surgically isolated with the aid of a dissecting microscope and a special graduated slide to accurately measure the required size. These apices were cultured on the establishment medium which contains MS salt mixture, White's organics and (in mg/l): 100 inositol, 80 adenine sulphate, 0.3, 1A, 0. 1 kinetin, 30000 sucrose and 6000 agar [22]. Similarly, shoot tips of identical sizes were excised from shoot apices of field grown potato plants collected from Gassim region, that were found to be infected with AMV alone. Shoot apices excised from these plants were cultured on a nutrient medium similar to that used with the apices excised from green house grown plants as mentioned earlier. Twenty shoot apices for the last three sizes and 40 shoot apices for each of the remaining sizes were cultured in each of the two experiments.The produced Plantlets were propagated, some were kept as stock and the rest were tested by ELISA, 18 weeks after culture.

Trials were also conducted to determine the effects of the position of the sprouts on the potato tuber on freedom of shoot apices from AMV. Shoot apices, 0.5 mm were isolated from apical and lateral sprouts of tubers that were collected from infected potato plants. The apices were surface sterilized separately and culture on the establishment medium. Virus indexing was conducted using ELISA 18 weeks after culturing.

Production of plantlets from potato plants known to be infected with more than one virus

Potato plants showing symptoms of virus infection were collected from potato fields in Alkharj area, Riyadh region. Both the plants and their tubers were brought to Riyadh and tested by ELISA. Two or more of the following viruses: AMV, PVA, PVY, PVM, PVS, PLRV, and TSWV were detected in these naturally infected potato plants. The techniques suitable for culture of isolated shoot apex, its growth, development and subsequent propagation were established to the standard and the shoots apices excised from these plants were cultured on the establishment medium [22]. Tubers of each plant were also sprouted and shoot apices were excised from apically and laterally produced sprouts were cultured on the establishment medium as well. Eighty shoot tips were cultured, and 27 plantlets were produced. Virus indexing of the produced plantlets, with ELISA, preceded mass multiplication or conservation of *in vitro* produced plantlets. The virus indexing of in *vitro* produced plantlets consisted of two parts: the first one was concerned with production of virus-free potato plantlets from plants that were singly infected with AMV, the most frequently detected virus in potato fields in the kingdom [11, 8]. The Second Part was concerned with production of virus-free potato plantlets from plants that were found multiply infected with more than one virus.

RESULTS AND DISCUSSION

Production of plantlets from AMV - infected potato plants:

Out of the 220 cultured potato shoot tips from potato plants that were infected through mechanical inoculation with AMV, 35 *in vitro* plantlets were successfully regenerated and 12 of which were found to be free from AMV (Table 1).

The greatest percentage of eradication was achieved from explant size of 1 mm, followed by 0.2 mm (75%), then 0.3, 0.6, 0.8 and the lowest was from explant size 10 mm (9.1%). The 220 shoot tips cultured from potato plants that were found naturally infected with AMV alone in potato fields in Gassim, yielded 36 potato plantlets (Table 2). Sixteen of the 36 plantlets were found to be free from AMV. All the explant sizes tested, produced AMV- free *in vitro* plantlets, however, the smaller the explant size used, the greater the percentage of AMV-free *in vitro* plantlets achieved. Each of the smallest explant sizes (0.2 and 0.3 mm) produced 75% AMV-free plantlets compared to the largest explants sizes used (1 and 10 mm) that each of which produced 20% AMV-free *in vitro* plantlets. The percentage of AMV-free plantlets produced from explants sizes of 0.5, 0.6 and 0.8 fell in between the highest and lowest percentages indicated above (Table 2).

It seems that the success in obtaining virus-free plantlets through shoot tip culture of AMV-infected potato plants depends on the original explant size excised from the shoot tip. It was generally found that the smaller the explant size the more likely is obtaining greater percentages of virus-free plantlets and vice versa. However, exceptions of this assumption can also sometimes occur as reported by Mellor and stace-Smith, 1977 [23], and Murashige, 1974 [22]. This is also clear in table 1, where the percent of virus eradication was greater at the shoot tip size of 1 mm (100%) compared to the shoot tip size of 0.2 mm (75%). Definite interpretation for these results is not known yet, but these results suggest the influence of the interaction of several factors between the host plant, the virus and may also be due to the surrounding environmental conditions of the host plant and the nature of the virus infection which may be implicated in the results obtained here [23, 22]. It was proved that the cultured shoot tips are usually affected by several factors for instance, size and rate of growth of cultured shoot tip [23], host plant [24, 25] and the virus [26, 27], nutrient media components, [28, 29], and site or location of shoot tip on the infected plant [30, 31]. It was suggested by Mellor and stace-Smith, 1977 [23] that virus eradication may result from a metabolic malfunction of the shoot tip cells as a result of excision.

Table-1. Number of *In Vitro* regenerated potato Plantlets from AMV-infected potato plants in the greenhouse after 18 weeks of Shoot-tip culture and percent of those found free from AMV

Shoot tip		No. of plantlets produced and tested	No. of virus-free plantlets	Percent of AMV-free plantlets
Size (mm)	Number			
10.0	20	11	1	9.1
1.0	20	4	4	100
0.8	20	7	1	14
0.6	40	3	1	33
0.5	40	3	0	00.0
0.3	40	3	2	67
0.2	40	4	3	75

Table-2. Number of *in Vitro* regenerated potato Plantlets from potato plants naturally infected with AMV in Gassim region, after 18 weeks of Shoot-tip culture and percent of those found free from AMV

Shoot tip		No. of plantlets produced and tested	No. of virus-free plantlets	Percent of AMV-free plantlets
Size (mm)	Number			
10.0	20	5	1	20
1.0	20	5	1	20
0.8	20	4	1	25
0.6	40	5	2	40
0.5	40	5	2	40
0.3	40	4	3	75
0.2	40	8	6	75

The trials concerned with explant source (apical or lateral Sprouts) in this study showed that apices obtained from apical sprouts produced higher percentage of virus - free plantlets compared to apices obtained from lateral sprouts of the same sprouted tuber. These results agree with findings previously reported on a similar study [32]. Apically produced sprouts grow much faster than laterally produced sprouts because of apical dominance exerted on the lateral buds in general. Shoot apices excised from apical sprouts are thus expected to contain lower virus titer since virus concentration in plant cells, tissues or organs are related to cell activity [33]. Actively growing organs contain lower virus concentration compared to dormant organs. Differences in type and amount of growth regulators in the cultured shoot apices of apical and lateral sprouts could also be implicated in the high frequency of virus elimination by culturing shoot apices from apically produced sprouts as reported earlier [30, 31]. From the experiments that were carried out for production of AMV – free potato plantlets it can generally be concluded that the smaller the size of explant, the greater the number or percentage of AMV - free plantlets produced (Table 1and 2).

Production of *in vitro* plantlets from potato plants known to be infected with more than one virus

Three of the potato plantlets that were regenerated from apically produced sprouts of tubers collected from naturally infected plants were successfully freed from virus infection. Two of which were regenerated from potato plants that were singly infected with PVY (number 2 and 3), whereas the third was from a potato plant doubly infected with PVY and PVA (number 6) (Table 3). Three plantlets regenerated from laterally produced sprouts of tubers collected from naturally infected plants (one was singly infected with PVY and two were doubly infected each, either with PVY and PVA or with PVY and AMV) were all found to be infected with the same viruses before and after applying MTC, i.e. not freed from virus infection (Table 3). TSWV was successfully eliminated from the multiply infected plant (sample number 12). No virus elimination occurred in the rest of the plantlets that were regenerated from tuber sprouts and that the number of viruses detected in these potato plantlets was the same as that detected in the infected field plants (Table 3).

Table-3. Viruses detected in sprouts of potato tubers before and after the meristem shoot tip culture using ELISA

Sample Number	Viruses detected in sprouts of tubers collected from field plants	Viruses detected in tissue culture plantlets[1]
1	PVY	PVY[3]
2	PVY	None[2]
3	PVY	None[2]
4	PVY + AMV	PVY + AMV[3]
5	PVY + PVA	PVY + PVA[3]
6	PVY + PVA	None[2]
7	AMV+ PVM + TSWV	AMV+ PVM + TSWV
8	AMV + PVM + TSWV	AMV + PVM + TSWV
9	AMV + PVM + TSWV	AMV + PVM + TSWV
10	AMV+ PVM + TSWV	AMV+ PVM + TSWV
11	AMV + PVM + TSWV	AMV + PVM + TSWV
12	AMV + PVM + TSWV	AMV + PVM +
13	AMV+ PVM + TSWV	AMV+ PVM + TSWV
14	AMV + PVM + PVA + PVS + PVY	AMV+ PVM + PVA + PVS + PVY

[1]All the plantlets were produced from sprouts of potato tubers collected from naturally infected plants in Alkharj area, Riyadh region.
[2]Plantlets produced from apically produced sprouts.
[3]Plantlets produced from laterally produced sprouts.

The potato plantlets in table 4 were produce from meristematic tips of naturally infected field potato plants that were found to be multiply infected with five viruses each, using ELISA. Although MTC did not produce virus-free plantlets from the plants infected with five viruses, it was successful in reducing the number of viruses detected in the naturally infected potato plants by 1-4 viruses. Hence the produced plantlets, contained 1, 2, 3, or 4 viruses of the five initially encountered in the naturally field infected potato plants (Table 4).

When the results for regeneration of virus-free plantlets from sprouts of tubers collected from infected plants (table 3) and those produced from infected plants (Table 4) are compared, one would observe that the three virus-free plantlets produced were from those produced from apically produced sprouts of tubers of infected plants, whereas the plantlets produced from tips of infected plants were not completely virus - free, though the number of initially infecting viruses was greatly reduced. This is not because the technique is not capable of producing virus-free plantlets from plants multiply infected with viruses as this was previously achieved [34], but because the viruses that were eliminated from sprouts were PVY and/or PVA which were reported to be easier to eliminate [30] compared to other viruses. This is confirmed by the eradication of these same viruses from some of the infected plants in this study as well (Table 4). Therefore it can be concluded from this investigation that the source of explants might play an important role in virus elimination from the infected potato materials. The results obtained in this study for eradication of viruses from infected plants agree with what was achieved earlier [10, 18, 19, 21, 35, 36]. What was achieved was the production of virus - free plantlets from plants that were singly infected with PVY or doubly infected with PVY and PVA (Table 3). Eradication of PLRV was also relatively easier and this virus was not detected along with PVY and PVA in the last two plantlets in table 4, which were produced from multiply infected plants. However, PVS was relatively difficult to eradicate and was neither eliminated from either of these two plantlets nor from 10 other plantlets from which removal of these viruses was attempted (Table 4). Although these results may suggest ease in eradication of viruses from potato in single and double infection through meristem culture compared to multiple infection with more than two viruses, this does not seem to be the case as some viruses were found to be easily eliminated compared to others regardless of single or multiple virus infection. PVY, PVA and PLRV which were easily eliminated in this study were found to be easy to eliminate in similar studies such as the one conducted by Quak, 1977 [30] where the percentage of plants freed from PLRV, PVY & PVA was greater than 70% compared to 10% of PVX - free plants. This agrees with what was achieved in this investigation.

Table- 4. Viruses detected by ELISA in field-infected potato plants and in plantlets produced from them through meristem tip culture technique

Sample Number	Viruses detected in field plants showing virus-like symptoms before meristem shoot tip culture	Viruses detected in plantlets[1] produced through meristem tip culture from potato plants known to be naturally infected with more than one virus
1	AMV+ PVM+ PVY + PVA + PVS	AMV+ PVM+ PVY + PVS
2	AMV+ PVM+ PVY + PVA + PVS	AMV+ PVM+ PVS
3	AMV+ PVM+ PVY + PVA + PVS	AMV+ PVS
4	AMV+ PVM+ PVY + PVA + PVS	AMV+ PVS
5	AMV+ PVM+ PVY + PVA + PVS	AMV
6	AMV+ PVM+ PVY + PVA + PVS	AMV+ PVS
7	AMV+ PVM+ PVY + PVA + PVS	AMV+ PVS
8	AMV+ PVM+ PVY + PVA + PVS	AMV+ PVM + PVS
9	AMV+ PVM+ PVY + PVA + PVS	AMV+ PVS
10	AMV+ PVM+ PVY + PVA + PVS	AMV+ PVM + PVS
11	AMV+ PVM+ PVY + PVA + PVS	AMV+ PVM+ PVA + PVS
12	PLRV+ PVM+ PVY + PVA + PVS	PVM + PVS
13	PLRV+ PVM+ PVY + PVA + PVS	PVM + PVS

[1]All the plantlets were produced from meristematic tips of naturally infected plants.

Although none of the potato plants that were multiply infected with more than two viruses was completely freed from the infecting viruses through meristem culture, the technique was successful in eliminating 1-4 viruses from plants that were multiply infected with five viruses, AMV, PVA, PVY, PLRV and TSWV (Table-4). However MTC was also capableof producing virus-free plants from plants that were multiply infected with five viruses [34]. Additional treatments such as chemotherapy and thermotherapy are generally applied to increase the number of virus-free plantlets produced from potato plants infected with viruses [13, 21, 37] as well as from other infected plants [38, 39] besides MTC.

Reason for the ease of eradication of PVY, PVA, and PLRV and the difficulty of eradication of PVS by MTC has been attributed to the size and shape of the virus i.e viruses such as PVY and PVA have larger sizes than PVS and were therefore not easily translocated to the shoot tips because of lack of fully differentiated cells, narrow protoplasmic connections between the cell and lack of fully developed vascular system in that area. Hence, their elimination is relatively easier compared to PVS which is relatively smaller and can probably be translocated to these tissues easily. However, PVS could also be eliminated if additional treatments are employed. Such treatments include chemical or thermal treatments, which are normally used for that purpose [36,13, 21, 35,40, 41, 42].

CONCLUSION

It can be concluded from the results of this study that meristem shoot tip culture technique alone was successful in elimination of AMV, PVY and PVA from naturally virus-infected or mechanically inoculated plants (Table 1, 2 and 3). It also helped to reduce the number of viruses per plantlets produced from potato plants and tubers that were multiply infected with up to five viruses, to the minimum without any additional treatments (Table 4). However, if production of additional virus - free plantlets is intended then these plantlets could be manipulated by further treatments such as thermal or chemical therapies [43,13,15,21,37, 40] which proved to be very efficient in potato and other crops to attain that objective. In this study, it was also observed that the percentage of virus-free plantlets produced from infected tuber sprouts seem to depend on sprout position on the tuber, as virus-free plantlets were successfully regenerated from apically produced sprouts but not from the laterally produced ones.

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