International Scholars Journals

African Journal of Virology Research ISSN 3421-7347 Vol. 13 (1), pp. 001-007, January, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

A survey of viral status on potatoes grown in Eritrea and *in vitro* virus elimination of a local variety '*Tsaeda embaba*'

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Accepted 20 December, 2018

Potato viruses are the major causes of yield loss and reduction in quality of seed tubers in Eritrea. A study was conducted to investigate the prevalence of viruses in potatoes (*Solanum tuberosum* L.) grown in Eritrea and to evaluate methods for their elimination. Leaf samples of two indigenous, *(Tsaeda embaba* and *Keyih embaba)* and three exotic varieties, (Ajiba, Spunta and Cosmos) were collected from fields growing potatoes in Maekel and Debub Administrative Zones and tested using the double antibody sandwich enzyme linked immunosorbent assay (DAS ELISA) technique. Five of the six most important potato viruses, PVX, PVY, PLRV, PVS and PVA, were detected in single and multiple infections. Virus elimination techniques were tested using *in vitro* plantlets of *T. embaba* established from field-grown tubers. Presence of PVX, PLRV and PVS was confirmed by ELISA test. The plantlets were then subjected to thermotherapy treatment for one and two weeks at 37^{o}C. The treatment was successful in eliminating only PLRV but failed to eliminate PVX and PVS. When meristem culture was combined with thermotherapy treatment for one week all three viruses PVX, PLRV and PVS were eliminated with a success rate of 86, 83 and 100%, respectively.

Key words: ELISA test, meristem culture, *Solanum tuberosum* L., thermotherapy, viruses.

INTRODUCTION

Potato is one of the most important crops in the world today. It produces more protein and calories per unit area per unit time and per unit of water than any other major plant food (Villamayor, 1984). It is considered to be the most important vegetable crop and is ranked fourth after rice, wheat and maize in terms of total production of fresh weight (Tadesse, 2000). According to the International Potato Center (CIP, 2005), potato production has increased at an annual average rate of 4.5% and area planted at 2.4% in the ten years to 2004. At present the production of potato worldwide stands at 328,000,936 Metric tonnes (FAOSTAT, 2005).

Potato is one of the four most important vegetable crops in Eritrea. The area under potato production is estimated to be 6,403 hectares with the bulk of it concentrated in the central highlands mainly in Debub and Maekel administrative regions (Tuku, 2000). The main constraints of potato production in Eritrea are low yields and poor quality seed tubers, both local and imported varieties.

Although documented studies are not available on the subject, it is a general assumption that there is a substantial potato yield loss due to virus degeneration in Eritrea. The locally available potato varieties produce only 5 tonnes per hectare (Tuku, 2000), but they are consistent in terms of maintaining that level of production. As such farmers are observed planting these local varieties alongside the imported high yielding varieties in case the latter fail or degenerate.

Conventional propagation of potato is done vegetatively using seed tubers and ensures uniformity of the crop in terms of growth and yield, but results in degeneration of the crop due to virus infection, the rate of degeneration

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varying from place to place and from cropping season to cropping season (Tadesse, 2000). The viruses are transmitted through different ways including through planting infected tubers. If the seed stock is not maintained well or frequently replaced with fresh ones, the virus infiltration can reach up to 100% in 3 - 4 successive crop seasons resulting in almost half or one third yields (Khurana et al., 2001). This is the major problem faced by seed producers.

Conventional seed multiplication methods take a long time and are prone to virus problems. Thermotherapy combined with micro-propagation using apical meristem culture is one of the techniques widely used nowadays to obtain clean potato planting materials (Khurana et al., 2001). It is, therefore, prudent to identify the high yielding local varieties which are adapted to the growing conditions of the country and study the types of viruses that are prevalent in them. Such varieties can be freed from pathogens and be used in seed cleaning and supply schemes instead of importing exotic varieties with poor adaptability to the local conditions.

This study, therefore, aims at identifying the types of potato viruses present in Eritrea and in establishing a mechanism of eliminating them using *in vitro* thermotherapy combined with meristem culture, thereby producing a collection of healthy potato plantlets of the most commonly grown varieties.

MATERIALS AND METHODS

The study consisted of two parts. The first part was the survey of viruses in potatoes grown in Eritrea and the second part dealt with testing techniques for eliminating the viruses.

Collection of leaf samples

Leaf samples were collected from potato producing fields from four villages (*Shiketi, Sheka wedi Bisrat, Adi Mongonti* and *Kudo Felassi*) in Debub Administrative Zone and five villages (*Adi Sheka, Quazen, Dekemhare, Geshnashim* and *Serejeka*) in Maekel Administrative Zone. Out of the 40 samples collected, 23 were from Debub and 17 from Maekel Administrative Zones. Two of the samples were taken from farms in Halhale involved in a seed multiplication pilot project initiated by the National Agricultural Research Institute (NARI). In all the cases information about the variety, source of the seeds, the number of generations the potato was being used for and other relevant information were collected from the farmers by conducting informal interviews. Five varieties including two local (*T. embaba* and *Keyih embaba),* and three exotic varieties (Ajiba, Cosmos and Spunta) were sampled.

Leaves of symptomatic and asymptomatic potato plants were collected, labeled and placed in plastic bags. A sample consisted of 10 to 14 leaves taken from five to seven plants in the same field, two leaves per plant, one from the upper and another from the lower part of the plant. The collected samples were kept in an ice box and placed in a refrigerator for overnight storage within the same afternoon and assayed for viruses the next day. The test for virus infection was done using the double antibody sandwich ELISA (DAS-ELISA) method described by Clark and Adams (1977). The samples were tested for presence of the six most important potato viruses PVX, PVY, PLRV, PVS, PVM and PVA using ELISA kits from Bioreba AG, Switzerland.

In vitro plant establishment

Sprouted tubers were used as sources of explants for *in vitro* establishment of the plantlets. The sprouts were cut into small pieces of approximately 3 - 4 cm containing three to four nodes and surface sterilized by washing under running tap water for 30 min followed by dipping in 70% ethanol for 5 s. The explants were then immers-ed in a 1% sodium hypochlorite solution with three drops of a wetting agent (Teepol) for 15 min in a glass jar. The bottle was shaken continuously to increase the contact between the explants and the sodium hypochlorite solution. After 15 min, the explants were rinsed with sterile distilled water (SDW) for 5 min and then soaked in a 3% kohrsolin solution (Glutaraldehyd, N,N'-bis-[hydroxymethyl Urea from Bode Chemie, Hamburg) for 15 min and shaken continuously. After the second wash, the explants were rinsed in sterile distilled water 3 times for 5 min each. The surface sterilized explants were then cut into single nodal segments and cultured in sterilized 25 x 150 mm culture tubes containing 12 ml solidified CMS medium (Lê and Collet, 1985) with 2% sucrose and 6.5% agar, one explant per tube. The culture tubes were covered with plastic caps and kept in a growth chamber for 3 to 4 weeks at 20/18^{o}C day/night temperatures and 16 h photoperiod supplied with Osram L fluorescent tubes with a photosynthetic photon flux of approximately 55 µmol/m^{2}/ s.

The established *in vitro* plantlets were then subcultured every 3 to 4 weeks using single node cuttings to build up the stock plants necessary for the experiment. Only nodes in the middle parts of the plantlets were used. The top parts of the subcultured plants were discarded, while the bottom parts were kept for ELISA testing.

Virus detection

Out of the total number of established *in vitro* plantlets, 32 mother plants were selected for sub culturing to increase the stock of plant material for the experiment. The mother plants, from which subcultures were obtained, were tested for the presence of viruses using the DAS-ELISA method mentioned above.

Thermotherapy

Thermotherapy was conducted on the initial *in vitro* established stock plants at 37^{o}C and 16 h photoperiod (Lê and Collet, 1985) for one, two, three and four weeks. Twenty plantlets were used per treatment and the percentage of survival of the plants was calculated at the end of the treatment. The surviving plantlets were subcultured to obtain enough plant material for ELISA testing. One of the progeny of each plant was taken as a representative of the mother plant and used for ELISA test. The rate of success in removing the viruses that were present prior to the application of the heat treatment was determined by comparing the percentages of plants freed from viruses in each treatment duration and combination.

Meristem excision

Meristems were excised from *in vitro* plants, which had been exposed to thermotherapy for 1 week and 2 weeks as well as from the untreated plants kept as a control. Meristems were not isolated from plants treated for 3 and 4 weeks thermotherapy since none of the plants survived the treatment. Apical meristems, with one or two leaf primordia, were excised using hypodermic needles with a diameter of approximately 0.5 mm that had been supplied for insulin injections. The excised meristems were cultured in Petri dishes containing regeneration medium consisting of basal MS medium (Murashige and Skoog, 1962) with vitamins (2 mg litre^{-1}

Table 1. Potato virus incidence in Maekel and Debub Administrative zones.

Virus type	Infected samples			% of infected samples
	Maekel	Debub	Total	
PVX	13 (68 %)	6 (32%)	19	48
PVY	8 (40%)	12 (60%)	20	50
PLRV	14 (44%)	18 (56%)	32	80
PVS	13 (59%)	9 (41%)	22	55
PVM	0 (0%)	0 (0%)	0	0
PVA	7 (50%)	7 (50%)	14	35

glycine, 100 mg litre^{-1} myo-inositol, 0.50 mg litre^{-1} nicotinic acid, 0.50 mg litre^{-1} pyridoxine HCl and 0.10 mg litre^{-1} thiamine HCl), 2% sucrose and 6.5% agar supplemented with N^6 Benzyladenine (BA) at a rate of 0.01 mg litre^{-1}. The Petri dishes were sealed with para-film and placed in growth shelves at 27/20^{o}C day/night temperature regime in a 16 h photoperiod supplied with Philips TLD fluorescent tubes with a photosynthetic photon flux of approximately 55 µmol/m^2/ s.

The plant tips were used for meristem excision while the middle parts were used for subculturing as it was necessary to provide sufficient plant material for both ELISA testing and maintaining stock of plant material at the end of each treatment. Another advantage of this procedure is the possibility of comparing the effects of each treatment on the same plant.

Mericlones, (i.e. regenerated meristem derived plantlets) were subcultured by making micro-cuttings with at least 1 - 3 nodes per explant and transferring them to 25 x 150 mm culture tubes containing CMS medium without growth regulators. The subcultures were kept under a controlled environment in a growth chamber at 27/20^{o}C day/night temperature and a 16 h photoperiod supplied with Osram L fluorescent tubes with a photosynthetic photon flux of approximately 55 µmol/m^2/s. When the mericlone subcultures attained sufficient growth, one plantlet per line was assayed for virus by the DAS-ELISA protocol to determine the rate of success of virus eradication in the respective treatment combinations.

RESULTS AND DISCUSSION

Virus occurrence

Of all the 40 samples taken, 95% tested positive for one or multiple virus infections. Five of the six major potato viruses assayed for, PVX, PVY, PLRV, PVS and PVA were detected in both Maekel and Debub Administrative Zones (Table 1). With the exception of Halhale, where only PLRV was encountered, the other sites had at least three of the five viruses detected showing a wide distribution of the five potato viruses.

Only two fields, one in Shiketi and the other in Halhale, tested negative for all the assayed viruses. One of these two fields was planted with newly imported healthy seed tubers and the other with a second generation seed obtained from the National Agricultural Research Institute (NARI) seed multiplication project.

The most prevalent virus in the areas surveyed was potato leaf roll virus (PLRV), which was found as either single or multiple virus infection in 80% of the samples. It was followed by PVS, PVY, PVX and PVA. PVM was not detected in any of the samples. The high prevalence of PLRV in potato production is of great concern. PLRV is one of the three most important potato viruses in terms of yield reduction (Salazar, 2006).

Several factors may have contributed to the wides-pread occurrence of these viruses in Eritrea. Most prominent among them is the continuous recycling of old seeds without replenishment. In the absence of well established healthy seed multiplication and distribution systems, the farmers resort to selecting seeds from current season crops of ware potato or using left over tubers from the market. These seeds are the smallest tubers with little or no market value as ware potato (Tuku, 2000). This practice of using small sized tubers left over from the ware potato crop, in addition to providing the means of easy spread for viruses from one region to the other, may also be contributing to the high prevalence of PLRV, since PLRV was reported to cause production of a high proportion of small sized tubers (Mih and Atiri, 2006).

Multiple infections

The incidence of multiple virus infections was high. Different combinations of two, three, four and five viruses were found in 72.5% of all the samples and 25% of the samples involved multiple infections of four and five viruses. With the exception of one sample of third generation Ajiba, all the samples that had combinations of multiple virus infections, (3 to 5 viruses) were of local varieties. These local varieties have been cultivated for many successive generations which create conditions favourable for virus build up (Khurana et al., 2001). Hence it is not surprising to find high level of virus incidence in the local varieties. Given this high incidence of multiple infections in the fields planted with the local varieties, new infections on the first generation seeds can be expected. That is in fact what was observed in the survey. The implication is that the old degenerated seed stocks may represent a massive reservoir of viruses that act as constant sources of inoculum to new healthy seed stocks.

Table 2. Summary of serological test (DAS-ELISA) of the established *in vitro* plantlets.

Viruses	Number of Plantlets tested	Number of plantlets that tested positive	% of plantlets that tested positive
PVX	32	32	100
PLRV	32	30	94
PVS	32	7	22
PVX + PLRV	32	25	78
PVX + PVS	32	2	6
PVX + PLRV + PVS	32	5	16

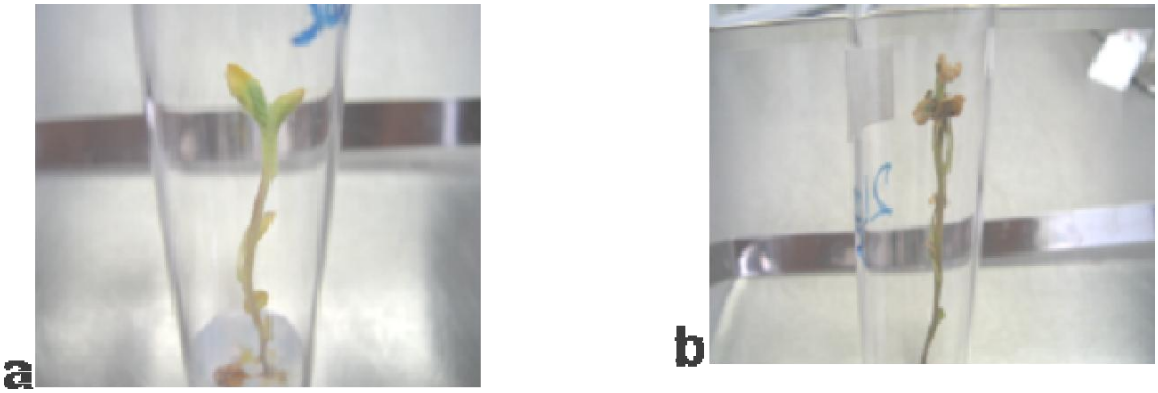

Figure 1. *In vitro* potato plants (cv *Tsaeda embaba*) at the end of thermotherapy of 1 week (a) and 2 weeks (b)

Initial virus detection results

Results of the ELISA test on the initial *in vitro* plants used for the experiment showed that all the plants were infected with a combination of either two or three viruses (Table 2).

Thermotherapy

Results of the heat treatment revealed that, plant survival rate decreased from 90% at the end of the first week to 55% after two weeks and to 0% at the end of the third week. Even at one week, the high temperature treatment had a severe effect on the development of the *in vitro* plants (Figure 1a). Rapid proliferation of branches was observed in some of the plants. All the branches and leaves turned upwards and most of the plants assumed a compact stance with leaves chlorotic at the edges. At the end of two weeks, most of the leaves were completely dehydrated and were growing almost vertically and closely hugging the stems (Figure 1b). The leaves may have acted as protective covers over the emerging buds, protecting them from excessive moisture loss.

All the surviving plants produced meristems. Meristem excision was much more difficult with the plants treated for two weeks because the degree of dehydration was so high that the meristems were small in size. In addition they tended to stick to the needles during the excision procedure.

The duration of treatment in this study was shorter when compared to work done by others. But the method of application of the heat treatment was different. These workers used either alternating high and lower temperatures (Mellor and Stace-Smith, 1970); or maintained a lower constant temperature (Brown et al., 1988; Faccioli and Colombarini, 1996).

Meristem regeneration

The proportion of mericlones that regenerated to plantlets was different for the three groups of plants (Table 3). The proportion of meristems that regenerate to give plantlets is mostly low (Griffiths, et al., 1990). The size of the explant, culture condition, amount of damage inflicted upon the meristem are factors that determine the regeneration of meristems, and that was the case in this experiment as well.

Development of the healthy mericlones was rapid. The time from the meristem excision to transferring the plantlets capable to grow on CMS medium was on average 38 days (5.4 weeks). The time it took for the meristems to attain a size large enough for microcutting and transferring to regular CMS medium varied from plant to plant. Seven out of the nine mericlones derived from plants heat treated for one week were transferred to

Table 3. The proportion of meristems of *"Tsaeda embaba"* regenerated in the different thermotherapy treatments.

Treatment	No. of meristems excised	No. of meristems regenerated	% of meristems regenerated
Untreated	20	7	35
Thermotherapy for one week	20	9	45
Thermotherapy for two weeks	11	4	36

Table 4. Number of virus-free *Tsaeda embaba* plants produced at different treatments for the various viruses (numbers in brackets indicate percentages of virus free plants).

Treatments	PVX	PVS	PLRV
1 week HT	0/15 (0%)	0/5 (0%)	8/15 (53%)
2 week HT	0/11 (0%)	0/3 (0%)	10/11 (91%)
1 week HT + MC	6/7 (86%)	2/2 (100%)	5/6 (83%)
2 week HT + MC	1/4 (25%)	1/1 (100%)	4/4 (100%)
MC	0/5 (0%)	1/3 (33%)	6/6 (100%)

HT = Heat treatment; MC = Meristem culture.

CMS medium in five weeks. This is a rather fast rate of growth when compared to other works reported in literature (Salazar and Jayasinghe, 2006). The rapid rate of development of the mericlones may be due to the high temperature at which they were cultured. Pennazio and Vecchiati (1978) reported that meristems cultured at 30^{o}C developed to rooted plantlets in 50 days, whereas meristems kept at 24^{o}C required 90 days to reach that stage of development. They attributed the rapid development of the meristems to the high temperature at which they were cultured. In this experiment the meristems were cultured at a 16 hour photoperiod and 27/20^{o}C (day/night) temperature regime.

Virus eradication

The plantlets were tested for the presence of viruses and the results are given in Table 4. PLRV was eradicated with heat treatment alone. The proportion of eradication of PLRV increased with the increase in the treatment period. The effects of the one-week and two-week heat treatments on PLRV observed in this experiment are in agreement with results of other authors. Fernow et al. (1962) eliminated PLRV by heat treatment of potato tubers prior to planting. The success rate increased with the increase in duration of treatment. Thirumalachar (1954) reported natural elimination of PLRV in potatoes stored in rural stores in summer at temperatures that can reach 36^{o}C.

Heat treatment alone did not remove PVX and PVS from any of the plants subjected to one and two week duration of thermotherapy. The results confirm the findings of Stace-Smith and Mellor (1968) who found both PVX and PVS to be very stable and difficult to remove from potato plants by thermotherapy alone.

The combination of thermotherapy and meristem culture gave the best result by eliminating all three viruses at the same time (Table 4). The results of the ELISA test of mericlones derived from plants subjected to one week thermotherapy showed 86% and 83% success rate in eliminating PVX and PLRV respectively and 100% eradication of PVS (Table 4). When the treatment duration was extended, the resulting number of virus free plants decreased due to the low survival rate of the plants.

The rate of PVX elimination was lower from plants treated for two weeks prior to meristem excision than from plants treated for only one week. This may be due to the difficulty in excising the meristems after prolonged exposure to the high temperature. The extreme dehydration made the meristem excision very difficult and larger parts of the surrounding tissue containing viral particles may have been included. Consequently the percentage of virus free plants subjected to two weeks thermotherapy before meristem excision was lower. None of the mericlones derived from control plants which were not subjected to thermotherapy were free of all three viruses. Although there was a100% eradication of PLRV, there was only a 33% success rate of removing PVS while PVX was still present in all of the mericlones.

These results support previous works which have found a combination of thermotherapy and meristem culture to give the best results (Sip, 1972: Brown et al., 1988). In most reports the success rate in eliminating PVX and PVS from infected plants has been low. Meristem culture alone has yielded very low results because the two viruses have the capacity to invade the meristem tissue

itself (Pennazio and Redolfi, 1974; Faccioli and Rubies-Autonell, 1982). Thermotherapy alone has failed for the most part because of the heat stable nature of different strains of the viruses (Mellor and Stace-Smith, 1970). The method that is reported to give the highest rate of success in the elimination of PVX and PVS is a combination of the two methods.

Although the duration of heat treatment in this experiment was short, the success rate in eliminating PLRV, PVX and PVS was high. The results confirm earlier work by Lê and Collet (1985) which showed that treating plants under constant high temperature for short periods is effective in eliminating even the most difficult viruses PVX and PVS. The temperature used in the present experiment (37^{O}C) was slightly lower than that used by Lê and Collet (1985). But the results obtained were comparable in terms of the total number of virus-free plants obtained. These initial results confirm the effectiveness of the combined treatment of thermotherapy and meristem culture on the local variety *T. embaba.*

Conclusion

The results of the survey give an insight on the occurrence of five of the six major potato viruses in the main potato producing parts of Eritrea. The study has also shown the prevalence of PLRV, which was detected in all the sites where samples were taken. The detection of primary PLRV infections in fields planted with new certified seeds probably indicates that the primary vectors of the virus, aphids, are active in the potato producing areas. Studies should be conducted to confirm this and to identify these aphid species and their migratory patterns. Their population dynamics should also be carefully studied since it has serious implications to seed potato multiplication, in particular and to potato production in the country, in general. The high incidence of viruses in all the sampled sites indicates a reservoir of potato viruses on the surveyed areas. The very high proportion of multiple infections by different combinations of three to five viruses implicates the local varieties as the reservoir in which the viruses are surviving. The fact that even new certified seeds planted for the first time were found to be infected with viruses is confirmation of this fact. Therefore, it is necessary to implement a system of regularly cleaning and supplying healthy seeds of varieties with a high yield potential to farmers on a regular basis.

The results of the testing techniques for eliminating the viruses show that this could be successfully done. The initial results obtained in this study have shown that the virus elimination protocol was effective with the local variety used, *T. embaba*. However, the experiment should be repeated with different local varieties, including *K. embaba* and with different temperature levels to find the level of temperature and duration of treatment best suited for the respective varieties before any recommendation can be made. However, the protocol used in this study gave very clear and positive results and can be used as a starting point for future work.

ACKNOWLEDGEMENTS

The financial assistance of the University of Asmara (UOA), the Syngenta Foundation for Sustainable Agriculture (SFSA), the Center for Development and Environment (CDE) of the University of Bern, Switzerland and Sustainable Land Management (SLM) – Eritrea and Eastern and Southern Africa Partnership Programme (ESAPP) is gratefully acknowledged. The authors would also like to thank Dr. Cong-Linh Lê of Agroscope, Changins-Wadenswil Research Station ACW, Switzerland, for his technical support.

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