



Determination of scanned virus-free potato planting materials by positive selection and screening of tubers from seed stores in the western highlands of Cameroon

A. P. NJUKENG^{1*}, M. G. CHEWACHONG¹, G. CHOFONG¹, P. DEMO², P. SAKWE³
and K. D. NJUALEM⁴

¹Department of Plant Biology, Faculty of Science, B.P. 67, University of Dschang, Cameroon.

²International Potato Centre (CIP) Lilongwe, PO Box 30258, Lilongwe 3, Malawi.

³Department of Crop Protection, Faculty of Agriculture and Agronomic Sciences,
University of Buea, Cameroon.

⁴Institute of Agricultural Research and Development (IRAD), Bambui, North West Region, Cameroon.

*Corresponding author, E-mail: pnjukeng@yahoo.com

ABSTRACT

Positive selection for the identification of virus-free potato planting material was evaluated in four locations in Cameroon. Leaves from asymptomatic plants were randomly collected, the plants marked and tubers collected four weeks later, and screened with DAS-ELISA for PLRV, PVY, PVA, PVX, PVS and PVM presence. Five viruses were prevalent in leaves while four prevalent in tubers. *Potato virus M* was highly prevalent in leaves and tubers while PVY was high only in leaves. *Potato virus A* was absent in both leaves and tubers while PVX was free only in tubers. A positive correlation was observed between virus prevalence in leaves and tubers ($r=0.806$). The prevalence of the six viruses in potato seed tubers from four seed stores was tested. *Potato virus M* was the most prevalent, while PLRV was the least. Small, medium and large tuber sizes were tested for the viruses, and infection rates decreased significantly the bigger the tuber size. Positive selection though not highly efficient can be recommended for resource-poor farmers, to control the economically important potato viruses. Tuber size can serve as a guide to identify healthy tubers, but must be combined with laboratory tests for effective use in selecting seeds for planting.

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INTRODUCTION

Pest and diseases constitute major constraints in the production of potato seed tubers (Dennis et al., 2010). Potato is particularly prone to attack by diseases caused by bacteria, fungi and viruses. These pathogens may infect the foliage, roots or tubers causing non-emergence, weakened plants, premature death and rotten or poor

quality tubers, (Wood and Jellis, 1984). Among the diseases, those caused by viruses are the least known and the most difficult to control (Salazar, 1996).

Potatoes are susceptible to more than 30 virus diseases (Salazar, 1996; Khurana 2004). The mode of transmission of a potato virus determines its spread in the crops and the kind of control measures that may be

effective against it (Salazar and Jayasinghe, 1997). Important viral potato diseases in Cameroon are *Potato leafroll virus* (PLRV), *Potato virus Y* (PVY), *Potato virus A* (PVA), *Potato virus X* (PVX), *Potato virus S* (PVS) and *Potato virus M* (PVM), (Struik and Wiersema, 1999). They are naturally transmitted in three ways mechanically by contact, by vectors and through infected vegetative planting material (Salazar and Jayasinghe, 1997; FERA, 2010). Actually, all potato viruses are transmitted and spread through tubers from infected plants (Salazar and Jayasinghe 1997). Aphids transmit thirteen potato viruses, *Myzus persicae* being the most important (Brunt, 2001; Ragsdale et al., 2001; Kotzampigikis et al., 2008). The quality of seed tubers planted and the virus disease prevalence and crop yield are therefore intricately linked (Struik and Wiersema, 1999; Dennis et al., 2010).

Since virus diseases cannot be cured as fungal and most bacterial diseases, one of the feasible ways of controlling them is by good quality seed production (Dennis et al., 2010). The traditional method of viral disease control is through the production of virus-free planting materials and the basic principle is to obtain in the shortest possible period, large amounts of quality seeds/seedlings in areas with low aphid pressure, which acts as vector for most potato viruses (Shepard and Claflin, 1975, Dennis et al., 2010).

It is possible to obtain virus-free seeds by field selection methods such as positive selection and pre-planting screening of sprouted tubers. Planting of virus-free seeds will likely reduce the number of virus source plants, reducing virus infection within and across the cropping season (Nienhaus, 1981).

Positive selection depends on the materials that are removed from a seed production field, where healthy plants are marked and tubers collected at harvest to be used as planting materials for next cropping season (Salazar, 1996; Leisa Magazine, 2007). It is necessary to evaluate the efficiency of this technique.

There is paucity of information on pre-planting detection of potato viruses in sprouted tubers. In 2001, a DAS-ELISA test for detection of potato viruses in 10 different seed tuber lots produced in six different environments in North-West and West provinces of Cameroon was conducted (Demo and Njuaem, 2001). Results of the test showed that the overall incidence of all viruses tested (PLRV, PVY, PVX-AS) ranged from 2.08% to 47.92%. Five years later, this work addresses the detection of viruses in potato tuber sprouts, from stored potato seed tubers by DAS-ELISA, and determines the prevalence of the six seed-borne potato viruses in potato seed tubers in the North West Province of Cameroon.

MATERIALS AND METHODS

Sample collection

Potato leaf samples were randomly collected from Bambui, Banson, Dschang, and Mberenka all in the Western Highlands of Cameroon. Sixty asymptomatic plants per location, 15 per plot were marked with pegs. Leaf samples were also collected from some symptomatic plants per locality. Three leaves (top, middle and bottom) from each plant were harvested directly into special labelled plastic bags. The samples were kept overnight in a fridge at 4 °C.

Three tubers from each marked plant were collected at harvest, 4 weeks after collecting leaf samples. The leaf samples and tuber were tested by DAS-ELISA for the presence of viruses.

Four potato seed-stores located at Upper Farms Bambui, Ruhvitangtah in Banson Rock Farm and Wara Farm in Santa, all in the North-West Region of Cameroon, were visited and a total of 1175 potato tubers of the variety CIPERA were collected. From each store tubers were already classified into three sizes, large (diameter > 8 cm), medium (diameter about 5.5 cm) and small (diameter about 3 cm). 10 out of every 100 sprouting potato tuber seeds were randomly picked from each lot. The sample size from the seed stores was as indicated in Table 1. Labelled plastic

bags were used to detach 3-5 sprouts from the potato tubers for the detection of PVX, PVY, PVS, PVA, PVM and PLRV.

Virus detection

Detection of the viruses was done using the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) described by Clark and Adams (1977) with the same composition of buffers.

A coating solution was prepared by dilution (6x) of buffer 1 (0.2 g of Na₂CO₃, 0.44 g NaHCO₃, 0.03 g NaN₃ and 30 ml distilled water, pH 9.6) with distilled water. This solution was then dispensed into six dark bottles, each labelled with one of the six viruses to be detected. Antibody solution was prepared by diluting the different antibodies (PVS-IgG, PVY-IgG, PVX-IgG, PVA-IgG, PVM-IgG and PLRV-IgG) from (CIP, Lot No. 2004) at a 1:1000 (v/v) in coating solution. Subsequently, 100 µl of the solution were dispensed into each microtitre plate well, excluding the outer wells. The latter wells were coated only with coating buffer. After incubating the plates at 37 °C for 3 h, they were removed and washed three times with Phosphate buffer saline pH 7.4, with 0.1% Tween 20 (PBS-T), incubating them for 3 min between each washing.

Approximately 0.75 g of the tuber sprouts were macerated by rolling a wooden pestle over each sample bag, and 1.5 ml of extraction buffer were added to the sap and

homogenised. The sap was then dispensed into the microtitre plate (100 µl/well) excluding the external wells and four other wells to serve as a negative control check. Aliquots of each sample were loaded in two microtitre plate wells in each of the six plates labelled with one of the six viruses and left overnight at 4 °C. The sap was then discarded and washed thrice with PBS-T as in the earlier step. Each corresponding antibody conjugate was then diluted 1/300 in conjugate buffer and 100 µl dispensed into each microtitre plate well. Two of the four wells not loaded with the sample were loaded with the conjugate to serve as positive control. This was followed by incubation of the plates at 37 °C for 3 h, after which they were emptied and washed thrice once more with PBS-T.

After the addition of the substrate the microtitre-plate wells were visually observed 30 -90 min at room temperature (stopping the reaction with 3M NaOH) and recorded as positive (+) or negative (-) for wells with a yellow colouration or without any colour change, respectively.

Statistical analysis

The Generalized Linear Module (GLM) of the Statistical Analysis System (SAS) was used for the analysis of data. From ANOVA results Duncan's Multiple Range Test (DMRT) at a probability level of P<0.001 separated means.

Table 1: Number of potato seed tubers (1/10th of the total number per seed lot per store) randomly picked from the seed stores according to the three tuber sizes.

Potato seed stores	No of small tubers	No of medium tubers	No of Large tubers	Total number of tubers sampled
Upper Farm	130	125	120	375
Ruhvitangtah	100	100	100	300
Wara	70	70	60	200
Rock Farm	100	100	100	300
Total per seed size	400	395	380	1175

RESULTS

Prevalence of Potato Viruses in Leaves and Tubers collected by Positive selection

Attempts to select virus-free potato plants through asymptomatic leaves tested showed that all the plants were infected with PVM and PVY in Bambui and Banzo and Dschang as well as in most samples from Mberenka. Also PLRV was detected in all samples from Banzo. On the other hand, PVA and PVS were not detected in leaf samples from Bambui and Mberenka, as well as PVX and PVA were absent in samples from Dschang and Banzo (Figure 1).

The prevalence of viruses in sampled tubers by positive selection followed the same trend as for the leaf samples. Tubers from Bambui, and Dschang were all infected with PVY, while PVM was detected in all tubers from the different locations. Tubers from Banzo and Mberenka were negative for PLRV, PVX and PVA (Figure 1).

Prevalence of potato viruses in potato seed tubers

The relative prevalence of the viruses in all potato tubers in Upper Farm was (82%), significantly ($P < 0.001$) higher than that of the other three seed stores (Figure 2). Rock Farm had the lowest virus prevalence (55%) compared with the others.

The trend observed with the individual viruses presents PVM as the most prevalent

virus in three of the four seed stores, highest in Upper farm store (100%) and lowest (85%) in Rock farm and Rutvitangtah stores (Figure 2). The most prevalent virus in potato tubers from Ruhvitangtah was PVA with 88% while PLRV (32%) was the least prevalent compared with all the other viruses tested (Figure 1). The latter was generally the least prevalent virus.

Generally, PVX (90%) and PVS (89%) are the most prevalent viruses among seed potato tubers in the sampled area, while PLRV (35%) is the least prevalent (Figure 3).

Significant differences ($P < 0.001$) were observed in the prevalence of potato viruses in different sizes of potato seed tubers tested (Figure 4). Small sized tubers had the highest prevalence of the viruses (71%), and medium and large sized tubers recorded 68% and 63%, respectively.

PVX (90%) and PVS (89%) were the most prevalent and PLRV (26%) the least in all small potato tubers from the different seed stores (Figure 4). The trend was different with the medium and large sized tubers, where PVY and PVX were the most prevalent.

The trend of general virus prevalence with respect to tuber sizes, with all the tubers grouped together in Figure 5 is different with regards to seed stores, except for Ruhvitangtah. Virus prevalence was highest in medium size tubers in Upper farm and Rock farm, and in large size tubers from Wara.

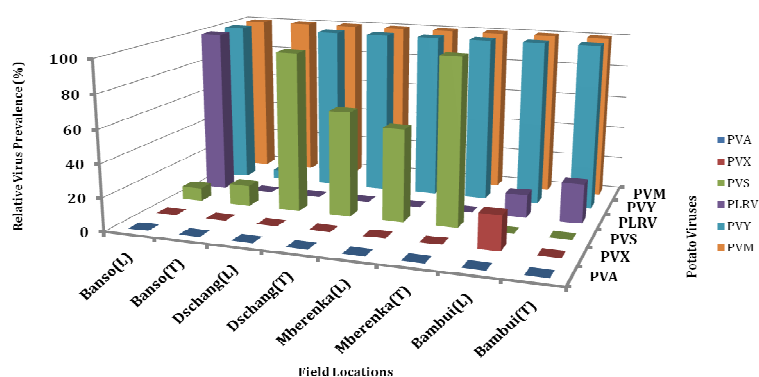


Figure 1: The relative prevalence of potato viruses in leaf and tuber samples collected by positive selection from four locations. L= leaf samples, T = Tuber samples.

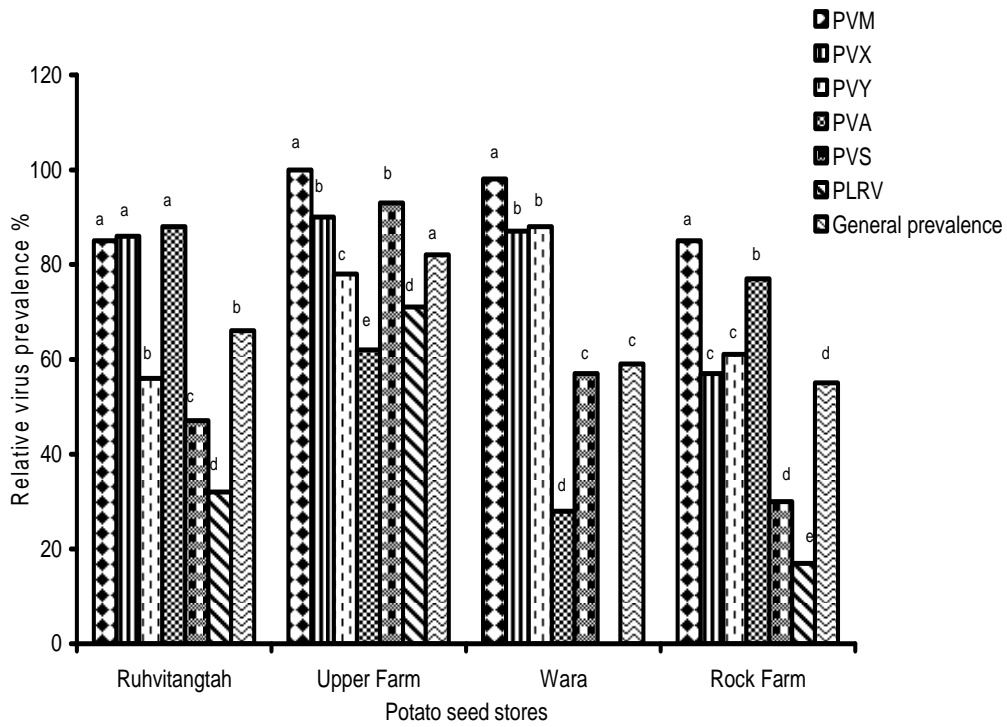


Figure 2: General virus prevalence and the six individual potato viruses in seed tubers tested from each store sampled. Bars with the same letter (a, b, c, d, and e) are not significantly different ($P < 0.001$) according to Duncan's Multiple Range Test. The individual viruses are compared within each tuber size, and all the tuber sizes compared for general prevalence.

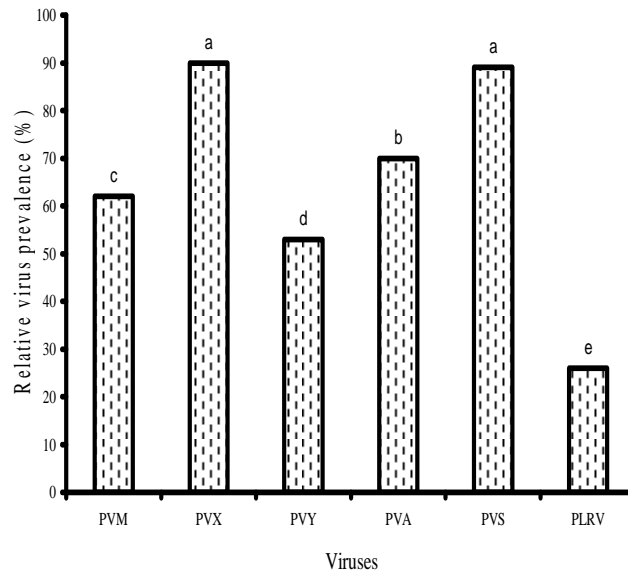


Figure 3: Relative prevalence of the six potato viruses in all potato tubers tested. Bars with the same letter (a, b, c, d, and e) are not significantly different ($P < 0.001$) according to Duncan Multiple Range Test.

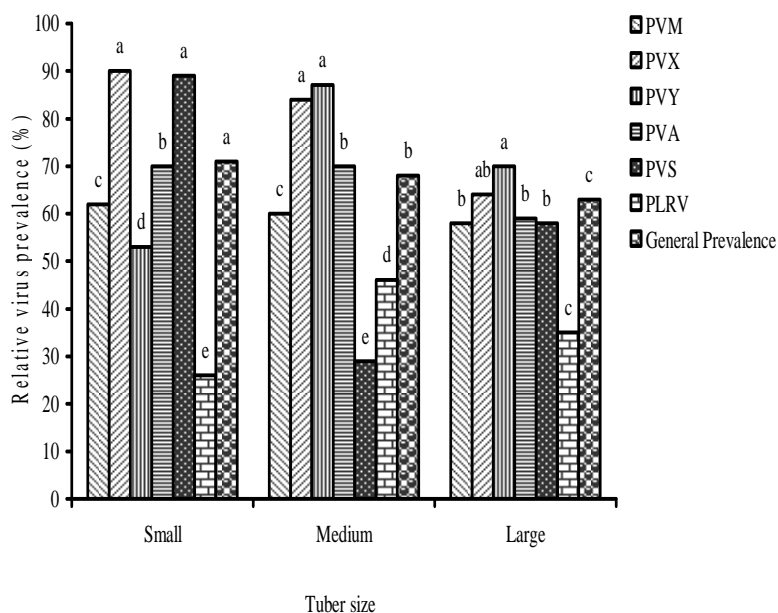


Figure 4: General virus prevalence and the six individual viruses in small (diameter about 3 cm), medium (diameter about 5.5 cm), and large (diameter \geq 8 cm) size potato seed tubers from the four seed stores sampled. Bars with the same letter (a, b, and c) are not significantly different ($P < 0.001$) according to Duncan's Multiple Range Test. The individual viruses are compared within each tuber size, and all the tuber sizes compared for general prevalence.

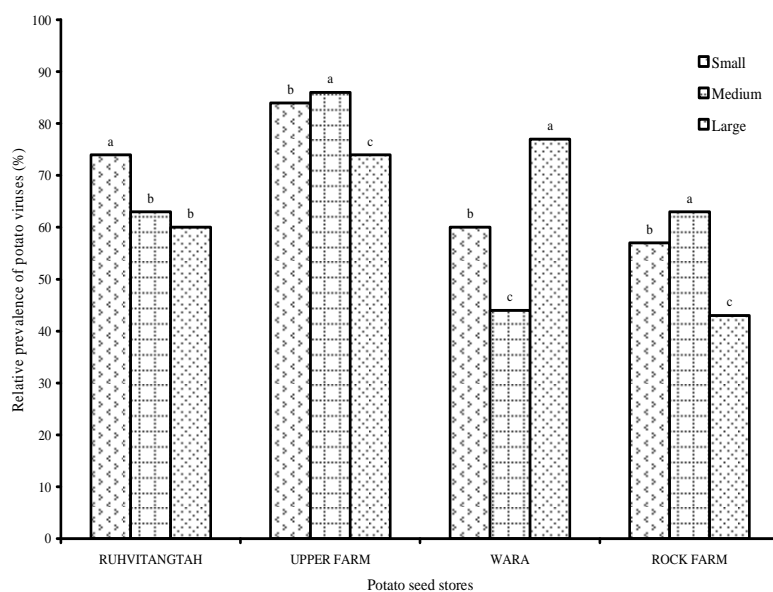


Figure 5: Relative prevalence of potato viruses in small (diameter about 3 cm), medium (diameter about 5.5 cm), and large (diameter \geq 8 cm) size potato tubers from each seed store sampled. Bars with the same letter (a, b, and c) are not significantly different ($P < 0.001$) according to Duncan's Multiple Range Test.

DISCUSSION

The idea of obtaining virus free tubers for the beginning of a seed production scheme was the objective of this work. Apparently, this can be achieved only to a limited extent using the positive selection technique, due to rampant latent infections of potato viruses. From this study, it is possible to select clean potato tubers against PVA, PVX and PLRV with a high degree of certainty. However, PVM and PVY-free tubers can only be selected through laboratory testing, due to their presence in all or most of the samples tested. *Potato leaf roll virus* was highly prevalent on leaves only in one location while generally low in tubers. PLRV and PVY are the most economically important potato viruses (CIP, 1990; Wales et al., 2008). Low prevalence of PLRV was responsible for about 90% yield reduction, but latent infection of the virus can produce as much as healthy plants (Jayasinghe, 1988). Also PVS, PVM and PVX are known to cause less significant yield losses (CIP, 1990). Clean potato seeds cannot be selected against PVY and PVM by positive selection, based on the results of this study.

Although virus prevalence in leaves and tubers was closely similar, it was relatively higher in leaves than in tubers. Differences between the two samples types in the same location were not expected since both were collected from the same plants. Although it has been reported that after viruliferous aphids have fed on the foliage a virus need some 8-10 days to reach the tubers (Salazar and Jayasinghe, 1997), this was taken care of by collecting tubers samples four weeks after the leaf samples. Apparently, the ELISA test needed to be confirmed with another more sensitive test like PCR.

In positive selection leaf symptoms guide in the selection of virus free tubers, hence both sample types are significant in the process. Although this technique is not very efficient, it can be applied by resource-poor farmers who buy clean seeds from time to time to avoid primarily infected plants being used for next planting season. In this way, the

probability of getting more virus-free seeds is high and will reduce their need to frequency go for new stocks of clean seeds.

It will be necessary to evaluate the seed degeneration rate of virus-free potato seeds in the different main potato growing regions in Cameroon. This will determine the frequency at which farmers need to get new stocks.

The screening of potato seeds from seed stores showed the prevalence of all the 6 viruses. This agrees with earlier reports on the occurrence of the six viruses in all areas where potato is grown (Wood and Jellis 1984; Struik and Wiersema, 1999). Each sample tested was infected with 1-6 viruses except two tubers, which were negative for all the viruses, tested. An earlier report on the prevalence of these viruses in 2,905 seed potato samples in Peru showed only 142 uninfected samples (CIP 1995). Corroborating the latter, this study suggests that potato seed tubers are often infected with one or more viruses wherever potato is cultivated.

Variation in the relative prevalence of viruses in potato seed tubers from the different seed stores is probably due to factors determining virus infection and spread in the locations where the tubers were harvested. Also, the method of handling by storekeepers and farmers as well as vector population might have been of significance importance. A report by Wood and Jellis (1984) describes the incidence of viruliferous aphid vectors early in the growing season as the most significant of all interacting factors that enhance virus spread. *Potato leafroll virus* is transmitted only by colonizing aphids in a persistent manner and thus requires extended feeding periods (Nienhaus, 1981; CIP, 1990; Struik and Wiersema, 1999). Furthermore, the high incidence of PVM, PVX, PVY, PVA and PVS could be explained by the fact that they are transmitted mechanically and also by aphids. This transmission method increases virus spread as aphids move from virus source plants or tubers to healthy plants or tubers. Separate reports by Nienhaus (1981) and

Struik and Wiersema (1999) also linked high infections to the feeding habits of aphids.

Seed transmission is important for the survival and dissemination of these viruses (Allen et al., 1982; Radcliffe and Ragsdale, 2002). The high relative prevalence of these viruses in the seeds is probably linked to the presence of aphids, which were seen on potato tubers in the seed stores, moving from one tuber to the other. Salazar and Jayasinghe (1997) had reported that seed transmission is the most serious when a vector is present.

There were significant differences in the prevalence of viruses in the different tuber sizes for all four seed stores sampled. Relative prevalence of viruses was inversely related to tuber size, which agrees with the idea that virus infections lead to low yield and reduction in tuber size. However, Salazar (1996) only linked the high proportion of viruses in small tubers to the fact that they are often the last to be formed and have a greater chance of carrying late primary infections. The small sized tubers may be the result of late formation and virus infections. Farmers in Sub Saharan Africa often obtain planting materials by saving small size tubers from the harvest of table potato crops, and selling larger size tubers for consumption. This practice leads to an unconscious selection bias for infected potato tubers for seed, since viruses like PLRV cause a high proportion of disease in small size tubers (Mih and Attiri, 2003). This may be the most likely reason for high prevalence of viruses, though the lack of good seed production practices, including virus-testing methodologies may aggravate the situation. Farmers could therefore select large tubers for use as planting material since such tubers would likely be a less important source of primary inoculum than medium and small size tubers. Rapid clean seed development programs in conjunction with positive selection may offer a good alternative.

Most of the seeds tested showed high mixed virus infections, which is in line with earlier reports (CIP, 1990). This is probably due to the transmission of the viruses by

Myzus persicae, through planting of infected tubers, mechanically by tuber/ plant contact (CIP, 1990, Struik and Wiersema, 1999; Kotzampigikis et al., 2008). The most important aphid pest of potato is green peach (peach-potato) aphid, *Myzus* (*Nectarosiphon*) *persicae* (Sulzer), which transmits PLRV, PVY, PVS, and PVA (Hille Ris Lambers, 1972, 1982; Dennis et al., 2010). This has significant epidemiological implications as synergistic interactions have been reported between/among some of these viruses leading to severe yield losses (CIP, 1990; Salazar, 1996, Burrows and Zitter, 2005). Kotzampigikis et al. (2008) had described PLRV and PVY as the most damaging potato viruses. The presence of mixed infections of PLRV and PVY calls for the need for urgent control measures if the health status of potato planting materials is to be improved.

PVM, PVX and PVS were the most prevalent viruses in the potato from seed stores sampled. It would be useful to find out if this correlates with field reports from the area of study. A report from CIP (1990) indicated that PVM was the least prevalent of the six viruses in Peru. The high prevalence rate of PVM in potato is in line with the report of Parker et al. (1983), that the transmission of PVM in potato is important in seed stores in developing countries with diffused light but less important in developed countries, because potatoes are stored in the dark and at low temperatures. The relatively low prevalence of PLRV in this study is likely due to the fact that PLRV is not naturally mechanically transmitted (Nienhaus, 1981; Boquel et al., 2011), hence handling tubers or storing them together.

The prevalence of the six potato viruses (PVA, PVM, PVS, PVY, PVX and PLRV) is high in potato in the western highlands of Cameroon. A similar study conducted by Demo and Njuaem (2001) on 10 seed potato tuber stocks from the same region revealed lower virus infection rates that ranged across seed stocks from 0.00% to 43.75% (with a mean of 7.08%), 0.00% to 4.17% (mean of 0.83%), 0.00% to 27.08% (mean of 4.37%),

for PLRV, PVY and PVX, A and S, respectively. The present study conducted five years later reveals higher virus incidence levels. This can be explained by the quasi absence of virus control measure especially in farmers' fields since virus symptoms cannot be recognized by most farmers.

This study suggests the urgent need to improve the quality of potato seed either by replacing present seed stocks or improving the sanitary status of the seeds by a systematic seed improvement program. Control of virus spread, however, remains the most effective method of reducing virus diseases in potato seed production. The reliability of detecting potato viruses in tuber sprouts from stored potato seeds using the DAS-ELISA method is also confirmed. It is, therefore, possible to select virus-free potato seeds and establish virus-free stocks. From such seed stocks, foundation seeds can be produced at field locations isolated from virus inoculum sources. This method of detection of viruses in stored potato seeds could therefore lead to the production of virus-free planting material that will allow farmers to obtain higher yields and reduce degeneration of cultivars.

From this study, it is recommended that (i) standards for potato seed quality monitoring and assurance be developed and utilized, at least for the basic seed production. (ii) Existing informal seed growers as well as other farmers who save seed tubers from ware potato crops be trained on the use of existing simple techniques like positive and negative selection, to control virus diseases in planting materials. (iii) Larger potato tubers that are less likely virus source be used as planting materials for the next planting season if seed tubers have to be obtained from a potato crop with high virus incidence in which no seed selection method was used during the growing season.

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