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Full Length Research Paper

Detection and distribution of sweetpotato feathery mottle virus in sweetpotato using membrane immunobinding assay

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The serological technique called membrane immunobinding assay (MIBA) was used for the detection and distribution of sweetpotato feathery mottle virus (SPFMV) in clones of sweetpotato from China. The immuno-fingerprinting technique on nitrocellulose membrane is based on specific recognition between the viral antigens and a polyclonal antibody against them. The reaction on the membrane allows the observation of spots, which intensity depends on the viral concentration of the tested sample. These spots can be conserved for very long periods. With the dosage of the virus present in the tested leaf samples of clone Guangshu 62, the evolution as well as the distribution of the feathery mottle virus was followed. Thus, the individuals tested have a high viral concentration which, not only was age dependent, but also progressed toward the apex. Since the high multiplication of the feathery mottle virus in clone Guangshu 62 did not had an effect on the tubers' yield, the relationship between this clone and the virus is a tolerant one. The implications of this relationship are discussed by taking into account the cultural context and the yield component in rural area.

Key words: Sweetpotato, SPFMV, serological techniques, China, membrane immunobinding assay.

INTRODUCTION

Sweetpotato (*Ipomoea batatas* L. Lam) is a tropical plant cultivated for its tubers and leaves. This plant originates from Central America from where its spreads into Europe and Africa during the big explorations (Purseglove, 1988). *I. batatas* belongs to the Convolvulaceae. The plant is hexaploid, with a chromosome number of 90. It is a short-day cross-polinated plant, which fecondation is insured by bees. However, under natural conditions, few flowers are observed as well as incompatibility phenomena that are responsible for a weak seeds production. This plant is grown annually from divers vegetative parts. There are several varieties distinguishable by the tubers and flesh coloration, the form of the leaves and their responses to pathogens attack. Sweetpotato cultivation is widespread in several region of the world but is mostly cultivated under the tropics. In 2000, the world production was estimated at 122 millions tonnes and was ranked 4th in developing countries after rice, wheat and corn (Karyeija et al., 2000; Purseglove, 1968). The Asiatic continent is the biggest producer (90% of the world production). Sweetpotato is mainly used for human consumption, but in the developed countries, it is essentially used in industries. Sweetpotato is a very energetic food (113 cal per 100 g), with high carbohydrates content (starch and mostly soluble sugars that give a sweet taste unusual for starchy foods) and contains several mineral elements and vitamins. The use of sweetpotato for human consumption is constantly dropping. Actually, sweetpotato is generally associated with scarcity periods or is considered by certain groups of population as food for poor. The long-term storage of tubers is one of the major problems that limit the role of sweetpotato in the international commerce, causing seasonal fluctuations (Huang, 1982; Tsou and Villareal, 1982; Villareal, 1982).

Sweetpotato, like other tubers such as potato, are subject to attacks by numerous cellular and non cellular pathogens (Clark and Moyer, 1988; Moyer and Salazar, 1989). These pathogens have a negative impact on yield during the plantation and also on harvested tubers. Among the major pathogens of sweetpotato, there are

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the potyviruses, belonging to the potyviridae family. They measure between 650 and 900 nm long and 11 to 15 nm in diameter. They have a single nucleic acid (RNA) with 10 000 nucleotides. That RNA is translated into a polyprotein which is later cleaved into several functional proteins. The potyviruses are transmitted by aphids, mites or white flies (Sukla et al., 1994). There are several viruses in the potyvirus group. The most widespread and dangerous for sweetpotato is the sweetpotato feathery mottle virus (SPFMV). This virus is found in all production areas. It is transmitted by aphids in non-persistent manner and can also be transmitted mechanically. The feathery mottle virus has a wide spectrum of host plants beside sweetpotato. The infected plants show vein clearing, chlorosis of older leaves and feathery mottle on leaves. Depending on the virus strains, they cause internal cork or external cracks on the tubers, which can results in a total loss of the market quality (Clark and Moyer, 1988; Moyer and Salazar, 1989). A more than 80% homology is observed between different strains of the virus, especially the 3' end of the coat protein of viral particles (Sukla et al., 1991). There are two forms of the SPFMV in Africa where it has a negative effect on the plants (Karyeija et al., 1998a, 2001). The association of the SPFMV and another virus of the crinivirus family (SPCSV) cause the SPVD (sweet potato virus disease), the most disastrous viral disease actually known in Africa (Gibson and Aritua, 2000).

Several control methods have been developed to fight against plants enemies. These methods are chemical, biological and genetic. Due to their non cellular nature, chemical and biological control methods are not effective against viral diseases. The control method used is the genetic method, by the development of varieties resistant to known viruses. This method consists of introducing resistance genes into cultivated varieties having an agronomic importance. The sources of resistance are searched for in primary gene pool of the specie to facilitate the genes transfer toward the cultivated variety. Under natural conditions, the epidemics in the production regions, allow the identification of resistant genotypes and their use in protocols of genetic improvement of susceptible genotypes. Under artificial conditions, the search for sources of resistance is done by creating an epidemic in order to determine the reaction of each individual. These artificial conditions, which favour the apparition and proliferation of the disease, put the tested genotypes under maximum stress. The following step is the precise identification of resistant individuals after the infection. Unlike cellular pathogens such as fungi, it is difficult to establish a progressive spectrum of symptoms apparition in virus infected plants. It is necessary to use diagnosis methods that are reliable and reproducible. They should be able to detect any viral presence and also estimate the virus concentration in different plant organs. The diagnosis methods should have two main objectives that are sensitivity and specificity. The sensitivity allows the identification of large number of viruses as in quarantine institutes, while the specificity allows the identification of a known virus (Lepoivre et al., 1994). The serological methods, based on the recognition between a specific antibody and the antigen, are widely used in the diagnosis of viral diseases (Portsmann and Kiessig, 1992). The antigen-antibody reactions have a big specificity, a short reaction time and a high sensitivity. The serological reaction observed is proportional to the viral concentration and the antibodies can be conserved for a long period (Matthews, 1991). With the use of microtiter plates in the ELISA (Enzyme Linked Immunosorbent Assay) test, it is possible to test several samples at once. This makes the method more practical in a large number of laboratories. However, interferences between undiluted plants saps and the final coloration constitute a handicap for the method. In order to bypass that difficulty, revelation techniques on nitrocellulose membrane have been developed with a few variants (MIBA, Membrane Immuno-Binding Assay; DIBA, Dot Immuno-Binding Assay). These tests are based on reactions of antigen-antibody recognition and the results are characterized by the appearance of dark bands on the membrane. The absence of coloration allows the use of undiluted plants saps with no interference problems (Alaux, 1994; Avrameas, 1992; Clauzel et al., 1992; Lepoivre et al., 1994; Portsman and Kiessig, 1992).

The objective of the present study is to identify sources of resistance to the sweetpotato feathery mottle virus in several clones of sweetpotato from China. Actually, several clones of sweetpotato purified by in vitro regeneration have shown, when planted, a complete absence of yield gain as compared with the susceptible clones. This behaviour implies a particular relationship between the clones and the feathery mottle virus. The ability of the methods on membrane-binding is used in the detection and distribution of the virus in artificially inoculated clones.

MATERIALS AND METHODS

Two plant species were used in this study: the cultivated specie *Ipomoea batatas* L. Lam and a wild species *I. nil*. This wild specie is very susceptible to the feathery mottle virus and constitutes the most susceptible indicator plant from which the inoculations are done. Several clones of the cultivated specie were used for a different purpose. Clones 209M, 865M and 90MT, purified beforehand, were used as susceptible controls in graft-inoculation tests. The infected clone GN1 Yanzhishu was used as grafting material for the different clones of the cultivated species. The clones Guangshu 62 were the plants which behavior in the presence of the virus needed to be determined. The antibody used in antibody-antigen complex formation was a rabbit polyclonal antibody against the sweetpotato feathery mottle virus (SPFMV). The revelation of the obtained complex was realised with a goat anti-rabbit antiglobulin antibody coupled with alkaline phosphatase. Seeds of the wild species were sown and the obtained plantlets were mechanically inoculated with undiluted sap from an infected *I. nil* plant. The leaf samples were ground in a pH 7.2 grinding buffer (0.05 M potassium dihydrogenophosphate + 0.01 M sodium

diethyldithiocarbamate). Plants were dusted with carborundum. The inoculation was done at the cotyledon stage with no light to avoid burns on the plants. Excess carborundum was removed by abundant rinsing. Clones of the cultivated specie were inoculated at the three leaf stage by an infected grafting material of clone GN1 Yanzhishu. Grafting was carried out by making one incision in plants just above the collar. The graft and the recipient plant were tight together with parafilm. All the experiments were carried out in a tropical greenhouse at a temperature varying between 30 and 35°C and under abundant watering in order to maintain a high humidity. Leaf samples were taken on plants to be tested 4 to 5 weeks after grafting. The plants had between 12 and 15 leaves that were all tested for the presence of virus. All the tests were done on five plants and repeated three times. For the virus detection tests, leaf samples taken for each repetition were different from the preceding ones in order to test the maximum leaf samples per plant. For virus detection and distribution on grafted plants, all the leaves were used in the indexing and were numbered starting from the oldest leaf located near the grafting point at the collar level.

The protocol for serological revelation of the feathery mottle virus on nylon membrane has three main stages: the fixing of the antibody-antigen complex, the enzymatic revelation of this complex and the visualization of the spots. The leaf samples were put in Eppendorf tubes, grounded directly with 100 ml of PBS/tween solution (10 mM NaH_2PO_4, 10 mM KOH, 150 mM NaCl, 0.2% Tween-20), centrifuged for 5 min at 13 000 rpm and conserved on ice. 1 µl of the supernatant was put on the membrane and allowed to dry for 10 min at room temperature. In order to avoid the mixing of the samples, the membranes were cut into 100 cm^2 and cross-ruled. The membranes were incubated for 30 min at room temperature with 50 ml of the fixing solution (Tris 0.1 M pH 7.5 ; NaCl 0.15 M, Boerhinger stopping agent 1%). This step allowed a complete saturation of the membrane by the antigens. The membrane was incubated the entire night at 4°C with 20 ml of the fixing solution containing the antibody anti-SPFMV at the dose of 1 µl of antibody per ml of buffer. After the antigen-antibody complex formation, the membrane was washed three times for 10 min with a washing solution (0.1 M Tris pH 7.5; 0.15 M NaCl) + 0.3% tween-20 to eliminate all the molecules not fixed to the membrane. The enzymatic revelation of the complex was carried out by incubating the membrane for 1 h at room temperature in 20 ml of fixing solution containing 20 µg of the goat anti-rabbit anti-antibody conjugated with alkaline phosphatase (GAR-PA). The membrane was again rinse three times for 10 min . The visualization started with a short incubation of the membrane in a solution containing 0.1 M Tris-Hcl pH 9.5; 0.1 M NaCl and 50 mM $MgCl_2$. Then followed a 5 min incubation at room temperature in 10 ml of substrate solution (100 µl of chemi-luminescent substrate CSPD (disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro) tricyclo [3.3.1.$1^{3,7}$] decan}-4-yl) phenylphosphate). The membrane was dried for 15 min at 37°C then transferred in a revelation chamber and covered with a film. The film was developed with two successive treatments in the substrate solution and then rinsed with water.

RESULTS

The indexing tests of the sanitary state of clones inoculated by grafting showed that the serological technique on membrane (MIBA) was efficient. In individual sweetpotato plants, 4 to 5 weeks after inoculation by grafting, except for the older leaves with moderate symptoms, the other leaves did not present symptoms of an attack by SPFMV. Nevertheless, the

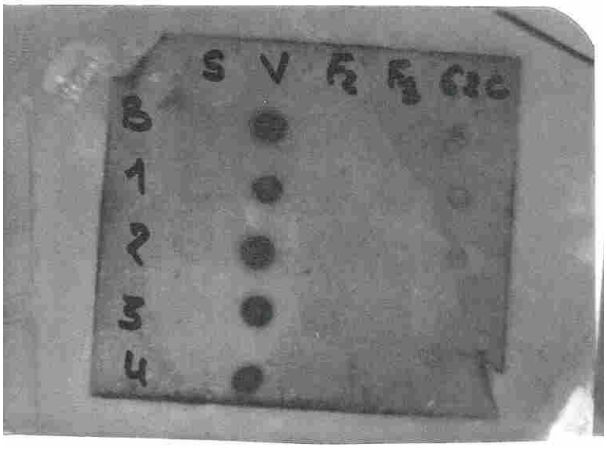

Figure 1. Sensitivity of the serological reaction of the feathery mottle virus on nitrocellulose membrane. S, negative control (non infected *I. nil*); V, positive control (infected *I. nil*); F2, F3, 62C, healthy sweetpotato clones (*Ipomoea batatas* L. Lam); B, undiluted sap; and 1-4, dilution of sap 3X, 9X, 27X and 81X.

MIBA technique was able to detect the presence of virus in leaf tissues. This technique was also able to clearly distinguish between the healthy control (negative control) and the infected control (positive control). Symptoms on the latest were characterized by spots of very high intensity when no spots were observed on healthy plants. Likewise, the dilutions made (3x, 9x, 27x and 81x), also showed spots with high intensity, indicating the high sensitivity of the method used (Figure 1). The identification tests of grafts and susceptible controls were done on 4 and 5 clones, respectively. The results obtained are shown in Table 1. Concerning the grafts, clone GN1 reacted positively with a high intensity, clone GN78 gave a weak intensity, while clones YuB and 90 did not react to the feathery mottle virus detection test. The clone GN78 was therefore used as graft materials in the indexing tests. For the susceptible controls, the virus was detected in the clones 207MT and 1023M (Table 1). The clones 90MT and 209M were exempt of virus and were therefore used as susceptible controls in the infection tests (Table 1).

The results of the feathery mottle virus indexing test in the susceptible clones 209M and 90MT, 4 to 5 weeks after inoculation by grafting are shown in Figure 2. The virus was present in almost all the leaves and virus multiplication occurred in susceptible individuals after grafting. Viral infection during the growth period progressed from the collar which was the grafting point. The leaves, located between the grafting point and the apex, have the highest virus concentrations. Some leaves had spots with the same intensity as the infected control, meaning maximum virus propagation in these tissues. The results of the indexing of the clone Guangshu 62 are shown in Figure 3. Spots with very high intensity similar to the reaction of the undiluted virus in

Table 1. Results of the clones indexing tests.

Dilutions	Sweetpotato clones									
	T	V	GN1	YuB	90	GN78	207MT	1023M	90MT	209M
B	-	+++	++	-	-	+	+	+	-	-
1	-	+++	++	-	-	+	+	+	-	-
2	-	+++	++	-	-	+	+	+	-	-
3	-	+++	++	-	-	+	+	+	-	-

V : positive control (*Ipomoea nil*).
GN1, YuB, 90, GN78, 209M, 1023M, 90MT, 865M: Sweetpotato clones (*Ipomoea batatas* L. Lam).
B: undiluted sap.
1-3: dilution of sap 3X, 9X and 27X.
-: no reaction
+ : mildly infected clone.
++: averagely infected clone .
+++: highly infected clone.
T: negative control (healthy *I. batatas*)

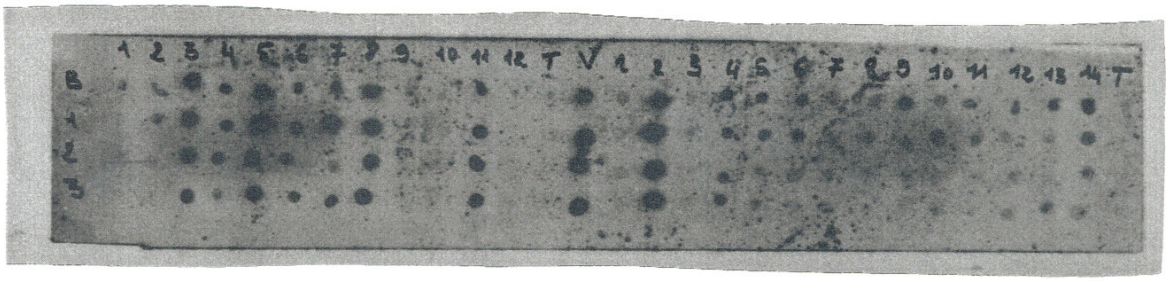

Figure 2. Serological detection on nitrocellulose membrane (MIBA) and distribution of the feathery mottle virus in susceptible clones. T, negative control (healthy *I. batatas* L. Lam); V, positive control (infected *I. nil*); 1-12 and 1-14, leaf position in clones 90MT and 209M respectively (1: oldest leaf near grafting point; 12-13: young leaf located at the apex); B, undiluted sap; and 1-3, dilution of sap 3X, 9X and 27X.

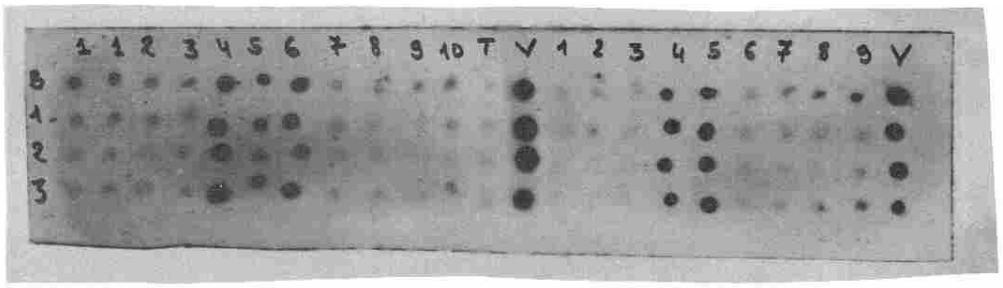

Figure 3. Serological detection on nitrocellulose membrane (MIBA) and distribution of the feathery mottle virus in clone Guangshu 62 inoculated by grafting. T, negative control (Healthy *Ipomoea batatas* L. Lam); V, positive control (Infected *Ipomoea nil*); 1-10 and 1-9, leaf position on clone Guangshu 62 (1: oldest leaf near grafting point; 9-10, young leaf located at the apex); B, undiluted sap; and 1-3, dilution of sap 3X, 9X and 27X.

some plant leaves were observed. These leaves were located between the grafting point above the collar and the apex. The presence of spots with moderate and weak intensity was observed in other leaves. In some plants, all the leaves reacted positively to the test of detection of the feathery mottle virus with a moderate intensity near the collar, a high intensity in the middle region and weak close to the apex. In other plants of clone Guangshu 62, leaves were either exempt of virus or presented extremely weak doses. The spots with higher intensity were observed in leaves located in the middle region as in susceptible clones. The viral distribution and intensity in leaves of susceptible clones and clone Guangshu 62 are presented in Figures 4 and 5. Leaves of susceptible clones, located in the middle region, were severely infected. With the exception of the leaf before the last, the youngest leaves are exempt from the virus. It seemed as if, in the susceptible clones, SPFMV colonize a zone and greatly multiplied itself there before moving to a higher level zone (Figure 4). In clone Guangshu 62, the virus rapidly propagated in the entire plant but was present in weak quantity close to the grafting point and the apex. In

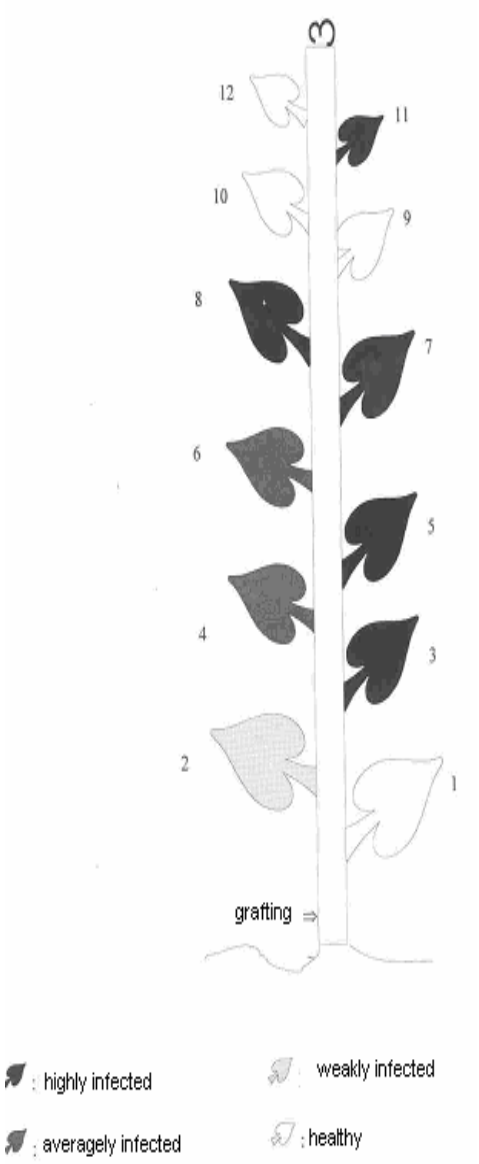

Figure 4. Concentration and distribution of the feathery mottle virus in sweetpotato control clones.

the leaves of the middle region, virus multiplication was high (Figure 5). The visualization of the viral distribution and proliferation in susceptible clones as well as in clone Guangshu 62 showed that the two types of clones were neither able to stop nor slow down SPFMV.

DISCUSSION

Symptoms apparition is generally the first tangible signal in plants infection. When the infection causes damages on the harvested parts, the consequences are highly felt by the producers. Preventive treatments can have an advantage, especially concerning weed control and

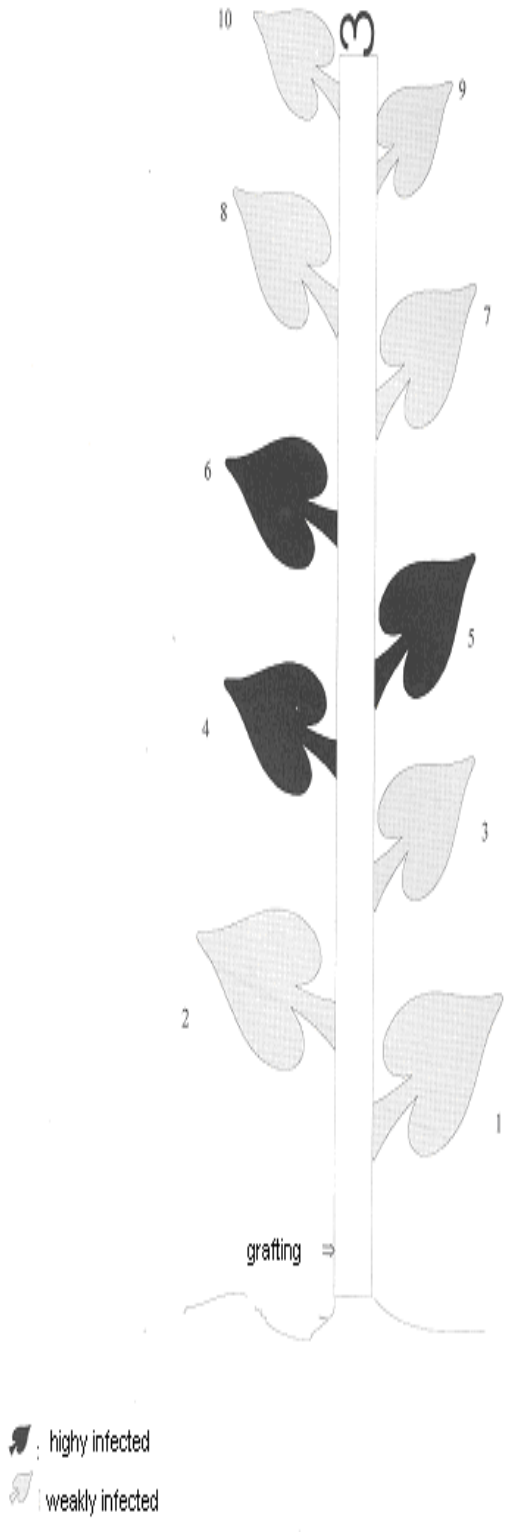

Figure 5. Concentration and distribution of the feathery mottle virus in the Guangshu 62 clone.

ecllular pathogens. However, it is totally inefficient for viral diseases. Biological tests are longs and therefore

inadequate when the infection starts on a plant. The molecular diagnostic has the great advantage since the target is the genetic making of an individual. In a study on the transmission of wheat yellow stunt virus, Lapierre and Maroquin (1986) showed that the biological tests needed 30 days in order to establish a sure diagnosis, while the ELISA gave reliable results 4 days after artificial inoculation. The early detection of the pathogens in a plant before symptoms apparition allows the anticipation of the disease and therefore make the better choices in terms of control methods to use. This early detection is especially important in the case of viral diseases since viruses are internal and the symptoms they produce are short-lived and unreliable diagnosis value (Comeau, 1986). Among the many techniques for diseases detection and diagnosis, the serological methods are more efficient and cost-effective (Lepoivre et al., 1994). In fact, there is a multitude of antibodies against viral antigens. Their use can be automated and large number of samples can be tested simultaneously. In addition, with the use of degenerated primers it was, in some cases, possible to detect the presence of different types of viruses as in the case of sweetpotato potyviruses (Colinet et al., 1993). Thus, the immuno-fingerprinting technique on membrane was successfully used in the early diagnosis of sugar cane red root disease (Hiremath and Naik, 2004). Moreover, this method on membrane is economic in the management of antibodies and reaction solutions as compared to the ELISA method (Wang et al., 1998).

There are two main distinctive plant reactions to pathogens: susceptibility and resistance which is separated into vertical and horizontal resistance (Van der Planck, 1968). Susceptibility is characterized by a total absence of reaction from the plant face to the large number of rapidly multiplying pathogens in all organs and tissues of attacked plants (Semal et al., 1994). A dosage of virus concentration indicates an important concentration of the pathogen in the tissues of the susceptible plant. This can result in important yield loss. Resistance is characterized by an important reaction of the plant to the attack of the parasite. In the case of vertical resistance, the plant reaction is fast and vigorous at the beginning of the infection and results in a total resistance to the pathogen. The yield of an individual showing this type of resistance to a pathogen stays the same in the presence or absence of an attack. The presence of the virus cannot be detected or only traces were found, resulting from the inhibition of the pathogen's multiplication as it penetrates the tissues of the vertical resistant plant. The horizontal resistance is less specific with the attacked individual giving a good yield . The dosage of the virus concentration indicates a low level in plant tissues. Resistance implies a monogenic and specific relationship in vertical resistance and polygenic with additive effect but with less specific in horizontal resistance. The dosage in leaf samples of clone Guangshu 62 showed a high virus concentration similar to that found in leaf samples of susceptible controls. This high presence of the virus in the plant organs excluded a resistance type relationship between the clone Guangshu 62 and the feathery mottle virus. The invasion of different infected plant tissues started from the grafting point toward the apex. The older leaves had more virus than the young ones. This distribution of the feathery mottle virus observed in the clone Guangshu 62 was similar to that observed in the wild species *I. nil* (Abad and Moyer, 1992). For the leaves located in the middle region, the spots observed showed the same intensity as those obtained with the crude virus extract. Such a virus proliferation in the plant seemed to indicate a susceptible relationship between clone Guangshu 62 and SPFMV. However, comparative studies conducted on the yield of purified and not purified clones Guangshu 62 in the Guangdong's laboratory in China did not show any yield loss in the clones not purified. This result implies that the proliferation of the feathery mottle virus in the plant of the clone Guangshu 62 did not have any negative effect on the yield, excluding a susceptibility type relationship. The clone Guangshu 62 did slow down virus multiplication, but there was no significant impact on the yield. This type of relationship between the virus and the plant indicates a tolerance. According to Semal et al. (1989), tolerance between a plant and a pathogen can be defined as the capacity of the plant to withstand a pathogen colonization without showing important disease signs. The main difference between resistance and tolerance according to Nicks et al. (1993) is about the evolution of the infection. The resistant clones considerably slow down the multiplication of the pathogen as opposed to the tolerant clones which support quite well a high concentration of the pathogen. This tolerance of a plant in the presence of a pathogen can have many variability levels. McLaren (1992) showed that a variety of South African sorghum could be considered tolerant to fungi up to an infection level of 28.20% since it supported that concentration with not much yield loss. The tolerant varieties can constitute an alternative in the management of the impact of pathogens on plants in rural area. In most tropical zones of sweetpotato cultivation, there is a high parasitic pressure favoured by conditions of high temperature and humidity. Resistant varieties especially those having vertical resistance to pathogens have their resistance broken down due to that high pathogens pressure. Such varieties which have been proven effective in temperate zones under intensive agriculture were not always adapted in the tropics. The clone Guangshu 62 could be an alternative choice in order to insure a subsistence production in a context where the production is only for local consumption. However, the high virus concentration in infected tolerant plants could be a source of inoculum. The presence of the vector in the plantation could also contribute to the transmission of the virus to other plants. The tolerant plant becomes a source

of aggravation and propagation of the pathogen and can therefore be disastrous for sweetpotato production. The use of tolerant clones should be at short-term replaced by resistant varieties. Sources of resistance to SPFMV had been found in African wild sweetpotato (Karyeija et al., 1998b) and could be a starting point for a programme on genetic improvement of cultivated species. This is a long-term goal since the development of a resistant variety by classical genetic improvement is time consuming (Comeau, 1986).

In conclusion, the immuno-binding assay technique on nitrocellulose membrane (MIBA) was able to efficiently detect the feathery mottle virus in leaves and gave a quantification of the virus level in tested tissues. This technique precisely detected the presence of the virus before symptoms apparition and could therefore be used as a mean to prevent disease propagation in a given culture. With the membrane-binding technique, the results of the plants indexing can be saved for several years. This is an advantage when compared with colorimetric reaction used in the ELISA technique. There was a tolerance relationship between the clone Guangshu 62 and the feathery mottle virus. This clone was not able to stop virus distribution in the plant tissues but maintained the same production level as in the absence of the virus. This tolerance to SPFMV could be an advantage in the tropical context of sweetpotato cultivation because tropical regions are under very high parasitic pressure, which generally, ends up breaking down resistances. The tolerant clones, like clone Guangshu 62 could be an alternative to balance the traditional level of production in the absence of resistant varieties.

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