SELECTED MILD STRAINS OF *Passion fruit woodiness virus* (PWV) FAIL TO PROTECT PRE-IMMUNIZED VINES IN BRAZIL

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ABSTRACT: The *Passion fruit woodiness virus* (PWV) is the most important virus affecting passion fruit (*Passiflora edulis* f. *flavicarpa* Deg.) crops in Brazil. The main purpose of this work was to select mild strains of PWV and to evaluate their protective effect against a severe strain of the virus. Three mild strains were selected from outstanding plants found in orchards severely affected by the virus (F-101, F-102 and F-103) and three others were obtained from blisters formed in passion fruit vine leaves showing mosaic (F-99, F-144 and F-145). The protective effect of the mild strains was evaluated for vines under greenhouse and field conditions. Plants pre-immunized with mild strains F-101, F-102 and F-144, in a greenhouse, had partial protection against the severe strain PWV-SP. In a first field experiment, all passion fruit vines pre-immunized with the six selected mild strains showed severe symptoms of the disease, approximately four months after the challenge inoculation with the PWV-SP strain. Results from a second field experiment, with vines pre-immunized with strains F-101 and F-144, followed by a quantitative evaluation of the mild strains in different leaves of the protected plants, indicated that breakdown in protection seems to be related to the low concentration and/or irregular distribution of the mild strains in leaves, which allows the existence of infection sites available for the establishment of the severe strain. Pre-immunization was not an appropriate alternative for the control of the passion fruit woodiness disease.
Key words: *Passiflora* sp., Potyvirus, cross protection

ESTIRPES FRACAS DO *Passion fruit woodiness virus* (PWV) NÃO PROTEGEM MARACUJAZEIROS PREMUNIZADOS

RESUMO: O endurecimento dos frutos do maracujazeiro (*Passiflora edulis* f. *flavicarpa* Deg.), causado pelo *Passion fruit woodiness virus* (PWV) é a virose mais importante da cultura dessa frutífera no Brasil. Este trabalho teve por objetivo selecionar estirpes fracas do PWV e avaliar o seu efeito protetor para o controle do endurecimento dos frutos por premunização. Foram selecionadas três estirpes fracas a partir de plantas de elite em pomares afetados pela doença (F-101, F-102 e F-103) e três a partir de bolhas que aparecem em folhas de maracujazeiro com mosaico (F-99, F-144 e F-145). O efeito protetor das estirpes fracas foi avaliado em maracujazeiros, em casa de vegetação e em campo. Plantas premunizadas com as estirpes F-101, F-102 e F-144, em casa de vegetação, ficaram parcialmente protegidas contra a estirpe severa PWV-SP. Em um primeiro experimento em campo, todos os maracujazeiros premunizados com as seis estirpes fracas selecionadas exibiram sintomas severos de mosaico, aproximadamente 4 meses após o desafio com a estirpe PWV-SP. Resultados de um segundo experimento de campo, com maracujazeiros premunizados com as estirpes F-101 e F-144, e estudos quantitativos dessas estirpes em diferentes folhas das plantas, indicaram que a quebra de proteção parece estar relacionada com a baixa concentração e/ou distribuição irregular das estirpes fracas nas folhas das plantas, que propiciam a existência de sítios de infecção para a estirpe severa posteriormente inoculada. A premunização não parece ser uma alternativa adequada para o controle do endurecimento dos frutos do maracujazeiro.
Palavras-chave: *Passiflora* sp., Potyvirus, controle

INTRODUCTION

The *Passion fruit woodiness virus* (PWV) is the most frequently found virus in passion fruit vines in Brazil. Incidences of 71.8% and 73.1 % have been found in commercial crops in the states of São Paulo and Ceará, respectively (Gioria et al., 2000; Lima et al., 1996). PWV causes serious damages in passion fruit vines in all producing regions, reducing leaf area, decreasing productivity, yielding fruit without commercial value and reducing the economical lifespan of the orchard (Rezende, 1994; Gioria et al., 2000).

PWV is a species of the genus *Potyvirus*, family *Potyviridae*, whith particles of 670 to 750 nm in length

and 12 to 15 nm in diameter, containing positive, single-stranded RNA and producing characteristic, pinwheel-shaped, lamellar inclusions in the cytoplasm of infected cells (Van Regenmortel et al., 2000; Taylor & Greber, 1973). Aphids are the vector of PWV in the field, especially the species *Myzus persicae* Sulz. and *Aphis gossypii* Glover (Chagas et al., 1981), with a non-persistent type of virus-vector relationship (Taylor & Greber, 1973). The virus is not transmitted through fruit seeds.

Although PWV was first found in Brazil at the end of the 1970's (Yamashiro & Chagas, 1979), no research effort has been carried out to develop permanent measures for fruit woodiness control. Several suggestions have been done, based on work developed abroad, but have not been effectively applied. Selection of resistant and/or tolerant plants, pre-immunization with mild strains of the virus and the adoption of cultural practices that could minimize the incidence and dissemination of the disease are some of these suggestions (Kitajima et al., 1986; Rezende, 1994; Gioria et al., 2000).

In Australia, control of passion fruit woodiness has succeeded especially with the use of purple and yellow passion fruit hybrids, which are tolerant to the disease (Taylor & Greber, 1973). In some cases, control has been achieved with the utilization of tolerant hybrids, pre-immunized with mild PWV strains (Peasley & Fitzell, 1981), after pioneer research conducted by Simmonds (1959). In Taiwan, the annual eradication of affected plantings and replanting with PWV-free seedlings is the usual procedure (Chang et al., 1992).

Pre-immunization with mild strains of a virus which do not significantly affect development and yield and protect plants against infection and/or manifestation of severe strains of the same virus, is an ecologically sound control alternative for plant viral diseases. In Brazil, this technology has been utilized quite successfully for over three decades, for the control of citrus tristeza (*Citrus tristeza virus* - CTV) (Müller & Costa, 1968; Müller & Carvalho, 2001). It has also been proved to be efficient for control of mosaic caused by the *Papaya ringspot virus* – type W (PRSV-W) in some cucurbit species (Rezende et al., 1994; Rezende & Pacheco, 1998; Rezende et al., 1999; Dias & Rezende, 2000).

More recently, a mild strain of the *Zucchini yellow mosaic virus* (ZYMV), selected by Rabelo (2002), efficiently protected zucchini squash plants against infection by severe strains of ZYMV. This author also demonstrated that the double pre-immunization was efficient for the control of PRSV-W and ZYMV in zucchini squash. Other examples of pre-immunization efficiency for plant viral disease control worldwide can be found in the review by Rezende & Müller (1995).

This research aimed to select mild PWV strains that could be utilized to control passion fruit woodiness virus by pre-immunization.

MATERIAL AND METHODS

Yellow passion fruit vines (*Passiflora edulis* f. *flavicarpa* Deg.) and *Phaseolus vulgaris* L. cvs. Jalo and Black Turtle 2 plants were obtained by sowing into 16 cm-tall aluminum pots 14 cm in diameter, containing fertilized substrate under greenhouse conditions.

A severe PWV strain PMV-SP, obtained from passion fruit vines at Vera Cruz, SP, Brazil (22º13'11"S, 49º19'10"W) was maintained on plants of the same species in the greenhouse. Mechanical transmission of the PWV was performed by an inoculum diluted at 1:20 (w/v), in a potassium phosphate buffer 0.02 M, pH 7.0, added of silicon carbide as abrasive. After inoculation, leaves were rinsed to remove excess inoculum and abrasive.

Myzus persicae Sulz., obtained from virus-free colonies on wild radish plants (*Raphanus raphanistrum* L.), was used for vector transmission. Aphids were removed from the radish leaves, placed into a plastic container, fasted for one hour, and then transferred to passion fruit vines systemically infected with PWV for virus acquisition (30 minutes). Groups of ten aphids were them transferred to the leader shoot of each test plant to transmit the virus. The aphids were confined within the leaves of the leader shoot by a sticky adhesive applied to the stalk, just below the leaves. Twenty four hours later the plants were sprayed with deltamethrin solution (0.2 mL L^{-1}) to eliminate the aphids.

The challenge inoculation, the process by which a severe strain is inoculated into plants already infected with a mild strain of the virus, was made by mechanical inoculations or by viruliferous aphids, as described above.

The search for mild PWV strains was carried out in passion fruit vine orchards severely affected by the virus, and in blisters of passion fruit vine leaves showing signals of PWV-SP mosaic. In the first case, five orchards in the region of Vera Cruz, SP were inspected and plants showing mild symptoms of the disease, good vegetative development and good fruit yield were selected. Apical branches from these outstanding plants were collected and grafted on to healthy passion fruit vines, maintained in the greenhouse for later evaluations.

For the search of mild strain from blisters, a modification of the procedure described by Rezende et al. (1994) was adopted: instead of flat ice cream spoons, plastic labels used for pot identification were utilized to extract the inocula. Extracts were individually inoculated on cotyledon leaves of passion fruit vines or *P. vulgaris* cv. Black Turtle 2 plants, selected because they present severe, systemic necrotic symptoms of the disease, resulting in death of plants. Plants which would not show severe symptoms could possibly bear mild strains, and were mechanically transferred to test passion fruit vines for later evaluation of the symptoms.

The inoculated passion fruit vines and bean plants were evaluated by a rating scale of manifestation and intensity of symptoms: 0 = no symptoms; 1 = mild mosaic without leaf deformations; 2 = severe mosaic without leaf deformations; and 3 = severe mosaic, blisters and leaf deformations. The scale utilized for bean plants was similar to that used for passion fruit vines, except for rating 3, which was attributed to plants with severe mosaic, blisters, leaf deformations and systemic necrosis followed by plant death.

Plant infection with PWV was confirmed by indirect DAS-ELISA test (Novaes & Rezende, 1999), with the use of polyclonal antisera produced in rabbit and chicken, respectively. Plants were considered as bearing a mild PWV strain when showed positive reaction to serological test and received ratings 0 or 1 for symptoms during the observation period.

Test of protective effect of mild PWV strains in passion fruit vines in the greenhouse

Each mild strain was inoculated on 18 healthy potted passion fruit vines, at the 4 to 6 leaves stage. Infection of plants was confirmed 15 days after inoculation by indirect DAS-ELISA. Soon after confirmation of infection, the severe strain PWV-SP, was mechanically inoculated into two expanded leaves from the leader shoot. For comparison purposes treatments were: a) plants pre-immunized with the mild strains, not challenged; b) plants pre-immunized with the mild strains, and challenged with PWV-SP; c) plants initially healthy, with the same age, and inoculated with PWV-SP at challenge; and d) healthy plants. All plants were maintained inside a greenhouse, and symptom readings were recorded every two weeks.

Field test of protective effect of mild PWV strains in passion fruit vines

Two independent experiments were conducted in Piracicaba, SP, Brazil (22º43'31"S and 47º38'57"W) to evaluate the protective effect of mild PWV strains on passion fruit vines. Seedlings previously potted in the greenhouse were transplanted to the field, at the four-to-six-leaves stage (Oct. 20, 2000), spaced 2 m between rows and 5 m between plants, and pre-immunized by mechanical inoculation of the two expanded leaves below the leader. Diagnose of infection was made 20 days later by indirect DAS-ELISA. The challenge inoculation was performed by mechanical inoculation of the severe PWV-SP strain into two expanded leaves below the leader, on Dec. 20, 2000. For comparison purpose, the following treatments were considered: a) plants pre-immunized with the mild strains, and challenged with PWV-SP, 30 days after pre-immunization; b) plants pre-immunized with two mild strains, not challenged; c) healthy plants inoculated with PWV-SP on the day of challenge; and d) healthy plants. Each treatment consisted of 5 plants randomly distributed in the field. Plants were evaluated with regard to the severity of symptoms during 4 months after challenge.

In the second experiment, seedlings were transplanted to the field on Jul. 18, 2001, under the same criteria adopted for the first assay, except that the experimental area was protected with 50 % shade cloth to minimize aphid access and the consequent natural transmission of PWV. Pre-immunization of plants was carried out on Aug. 02, 2001 and infection confirmation 50 days later by indirect DAS-ELISA. For comparison purpose the following treatments were considered: a) plants pre-immunized with the mild strains, and mechanically challenged with PWV-SP in the sixth and seventh leaves below the leader; b) plants pre-immunized with mild strains and challenged with 10 viruliferous aphids placed on the leader shoot of plants; c) plants pre-immunized with the mild strains, not challenged; and d) healthy plants. Treatments a and b consisted of 4 plants; c and d consisted of two and five plants, respectively; all plants were randomly distributed in the field. Two challenges were made by mechanical inoculation and two by viruliferous aphids. The first, 50 days after pre-immunization (Sep. 21, 2001) and the second occurred 110 days after pre-immunization (Nov. 21, 2001). Test plants were evaluated with regard to severity of symptoms during 4 months after the first challenge.

The efficiency of challenge inoculation performed mechanically and by viruliferous aphids was evaluated in healthy, pot-grown passion fruit vines, inoculated at the same day of challenge. These plants were maintained in the greenhouse for observation of symptom development.

The relative concentration of two mild PWV strains in different leaves of the plants was estimated based on absorbance values of indirect DAS-ELISA, because at certain dilutions, the concentration of extracts are directly proportional to the decimal logarithm of the virus concentration present in the sample (Novaes & Rezende, 1999). This test was run in all pre-immunized passion fruit vines in the second field assay, and samples were collected before plants were challenged. Initially, five expanded leaves near the leader shoot were selected. Three, 1-cm disks were collected from each leaf at different positions, separately macerated in PBS buffer containing Tween, at 1:50 (w/v) dilution and evaluated jointly by indirect DAS-ELISA. All samples, as well as the negative and positive controls (extract from healthy plant leaves and extract from passion fruit vines infected with the PWV-SP severe strain, maintained in the greenhouse respectively) were tested in duplicate wells. Mean absorbance values obtained at 405 nm higher than 3 times the mean absorbance of healthy samples were considered as positive, and utilized to compare the relative concentration of mild strains in leaf tissues.

RESULTS AND DISCUSSION

Selection of mild PWV strains

The passion fruit vine cultivation system on trellises made it difficult to locate individual plants, since they would intertwine, thus making it hard to visualize and separate plants that were asymptomatic or displayed mild symptoms of the disease. In despite of that, it was possible to identify five plants with mild mosaic symptoms, good development and good yield. Passion fruit vines grafted with branches from three out of the five outstanding plants that had been selected showed mild symptoms of the disease in tender shoots. The other two plants had severe symptoms of the disease and were discarded. The selected mild strains were called F-101, F-102 and F-103, respectively.

The search for outstanding plants in fields severely affected by the disease should be prioritized in pre-immunization projects, since the protective effect of the mild strain is already being naturally tested in the field (Müller & Costa,1987). In Australia, the selection of mild PWV strains was performed by selection of outstanding plant in the field (Simmonds, 1959) and so far this seems to be the only case of pre-immunization success for the control of passion fruit woodiness around the world. A classical example of success with the use of this methodology was the selection of a mild strain for the *Citrus tristeza virus* in Brazil (Müller & Costa, 1968), which has been utilized for its control over three decades (Müller & Carvalho, 2001). Other examples of selection of mild strains from outstanding plants in the field can be found for *Cocoa swoolen shoot virus* in Africa (Posnette & Todd, 1955), for *Papaya ringspot virus* – type P (PRSV-P) in Brazil (Rezende, 1985), and for *Vanilla necrosis virus* in Tonga (Liefting et al., 1992).

To search for mild strains from blisters, extracts from 406 blisters obtained from passion flower leaves having mosaic caused by the PWV-SP strain were inoculated individually. Extracts from 180 blisters were inoculated into passion fruit vines, and the remaining 226 were inoculated into *P. vulgaris* cv. Black Turtle 2 plants (Table 1). All passion fruit vines were indexed by indirect DAS-ELISA. Extracts from all plants with symptoms reacted positively to the antisera against the PWV in the serological test. Four plants displayed mild mosaic, and were rated 1. From the 127 plants without evident symptoms, which received rating 0, four were infected, since they produced a positive reaction in the indirect DAS-ELISA. Eight strains were consequently selected and three were confirmed as mild PWV strains, after being transferred to new passion fruit plants. The other five strains induced severe symptoms and were discarded. The selected mild strains were called F-99, F-144 and F-145.

Infection of *P. vulgaris* cv. Black Turtle 2 plants was also verified by the indirect DAS-ELISA. Among the 159 plants with symptoms and testing as positive in the serological test, three showed mild mosaic symptoms (Table 1). Extracts of leaves from those plants were separately inoculated into passion fruit vines. Fifteen days after inoculation, the passion fruit vines presented severe mosaic symptoms and were discarded. The prior passage of isolates obtained from blister extracts through *P. vulgaris* cv. Black Turtle 2, which is hypersensitive to PWV and could enable the selection of mild strains, did not prove adequate in the present work.

The utilization of blister extracts as a form of obtaining mild strains was first reported by Rezende et al. (1982) for PRSV-P, for papaya trees. These authors selected three PRSV-P mild strains from extracts of 76 blisters inoculated into papaya seedlings. Years later, Rezende et al. (1994) were also successful in the selection of mild strains of PRSV-W from blisters of zucchini squash leaves showing mosaic. From 87 blisters tested, three stable mild strains were selected, which protected zucchini squash plants against infection by the severe PRSV-W.

Protective effect of mild PWV strains in passion fruit vines

Initially a protection test was performed in the greenhouse to evaluate the protective effect of two mild strains obtained from outstanding plants in the field (F-101 and F-103) and a strain obtained from blisters of passion fruit vine leaves showing mosaic (F-144). Fifteen days after challenging, plants that were initially healthy and that were inoculated with the PWV-SP strain displayed infection, showed severe mosaic and foliar deformations (Table 2). During the same period, 29% of the

Table 1 - Reaction of passion fruit vines and *P. vulgaris* cv. Black Turtle 2 inoculated with extracts of leaf blisters from passion fruit vine showing mosaic caused by the *Passion fruit woodiness virus*.

Test plants	No. of inoculated plants	No. of plants according to the severity of symptoms*			
		0	1	2	3
Passion vine**	180	127	4	0	49
P. vulgaris cv. Black Turtle 2***	226	67	3	0	156

*Symptom rating scale utilized for each species; ** Evaluation performed 30 days after inoculation; *** Evaluation performed 15 days after inoculation.

48 plants that were pre-immunized with the three mild strains and challenged with strain PWV-SP, also exhibited severe symptoms of the disease. Forty five days after challenging, approximately 63% of those pre-immunized plants exhibited severe mosaic symptoms and foliar deformations. Thirty seven per cent of the pre-immunized and challenged plants remained symptom-free or, in a few cases, displayed mild mosaic symptoms, receiving ratings 0 and 1 for symptoms, respectively. Plants pre-immunized and not challenged remained symptom-free or showed mild symptoms of the disease, during the evaluation period.

For the first field experiment the protective effect of the six selected mild strains was evaluated. All healthy plants inoculated with strain PWV-SP at the time of challenge inoculation of pre-immunized plants showed severe symptoms of the disease 19 days after inoculation (Table 3). Fruits of these plants were totally deformed and displayed irregular-shaped corticous spots, which sometimes extended throughout the entire surface of the fruit. Approximately 73% of the pre-immunized and challenged plants also received the maximum rating for symptoms, 19 days after challenging (08 Jan. 2001). The remainder of the pre-immunized and challenged plants continued showing mild symptoms (rating 1), and even yielded fruit with no symptoms of the disease. However, from the first month after challenging, disease symptoms in those plants gradually intensified and 64 days after challenging (22 Feb. 2001), only two plants pre-immunized and challenged did not exhibit severe symptoms of the disease. By the end of the experiment, 110 days after challenging (08 Apr. 2001), all plants that had been pre-immunized with the six mild strains and challenged with the severe strain exhibited maximum rating for symptoms. Fruits from these plants were also affected by the disease. Plants pre-immunized and not challenged, and healthy plants exposed to natural infection, were infected by the severe strain, since all of them exhibited severe symptoms of the disease, six months after installation of the experiment in the field. This infection occurred naturally, by transmission via aphids.

Table 2 - Number of passion fruit plants pre-immunized, pre-immunized and challenged with the severe strain of *Passion fruit woodiness virus* (PWV-SP), and healthy plants inoculated with strain PWV-SP, which showed severe symptoms of the disease (rating 3) on two evaluation dates, in the greenhouse.

Treatment	No. of tested plants	No. of plants with maximum degree of symptoms*	
		15 d.a.c.**	45 d.a.c.
Pre-immun. F-101	2	0	0
Pre-immun. F-102	2	0	0
Pre-immun. F-144	2	0	0
Pre-immun. F-101 + PWV-SP***	16	4	12
Pre-immun. F-102 + PWV-SP	16	6	10
Pre-immun. F-144 + PWV-SP	16	4	8
Healthy, inoculated w/ PWV-SP	6	6	6
Healthy	6	0	0

*Rating 3 = severe mosaic, blisters and leaf deformations; **d.a.c. = days after challenging; ***Pre-immunized with mild strain and challenged with severe PWV strain, 15 days later.

Table 3 - Number of passion fruit plants pre-immunized, pre-immunized and challenged with the severe strain of *Passion fruit woodiness virus* (PWV-SP), and healthy plants inoculated with strain PWV-SP, which showed severe symptoms of the disease (rating 3) on three evaluation dates, in the field.

Treatment	No. of tested plants	No. of plants with maximum degree of symptoms*		
		19 d.a.c.**	64 d.a.c.	110 d.a.c.
Pre-immun. F-101	5	0	0	5
Pre-immun. F-103	5	0	4	5
Pre-immun. F-99 + PWV-SP***	5	5	5	5
Pre-immun. F-101 + PWV-SP	5	2	4	5
Pre-immun. F-102 + PWV-SP	5	4	5	5
Pre-immun. F-103 + PWV-SP	5	5	5	5
Pre-immun. F-144 + PWV-SP	5	3	4	5
Pre-immun. F-145 + PWV-SP	5	3	5	5
Healthy, inoculated w/ PWV-SP	5	5	5	5
Healthy, non-inoculated	5	0	3	5

*Rating 3 = severe mosaic, blisters and leaf deformations; **d.a.c. = days after challenging; ***Pre-immunized with mild strain and challenged with severe PWV strain, 30 days later.

In view of the partial protection provided by mild strains F-101, F-102 and F-144, in the greenhouse tests (Table 2) and in the complete absence of protection in the field experiment, utilizing the six mild strains that had been selected (Table 3), two hypotheses were presented to explain the intensification of symptoms in pre-immunized passion fruit vines challenged with the severe strain of the virus: a) the selected mild strains belong to a different Potyvirus species, serologically related to PWV, and do not provide protection against the severe strain of the later; and b) the low concentration and/or irregular distribution of the mild strains in the foliar tissues of pre-immunized plants allows the infection and establishment of the severe strain inoculated later on.

The hypothesis that the mild strains belong to a different species of PWV was discarded based on results of Novaes (2002), who studied the protection provided by mild strains F-101 and F-144 to *Crotalaria juncea* L., in greenhouse and field tests. The author verified that 100% of the pre-immunized plants became protected against infection by the mechanically inoculated PWV-SP severe strain. The protection verified in this species was an indication that we were dealing with strains of the same virus, because protection is a common phenomenon between strains of a viral species (Dodds, 1982). In addition, it has been considered as one of the taxonomic criteria for Potyvirus species identification (Van Regenmortel et al., 2000). The confirmation that the mild strains belong to the same species as the severe strain came from the comparative analyses of nucleotide sequence of the coat protein gene (CP) and of the 3'-non-translated region (NTR) from mild strains F-101 and F-103 and severe strain PWV-SP (Novaes, 2002). In this analysis, strains F-101 and F-103 presented 99.7 and 100% identity for the CP gene and for the 3'-NTR, respectively. When compared to strain PWV-SP, the identity values for CP and the 3'-NTR were 97.5 and 95.5%, respectively. The Potyvirus taxonomic criteria establish that identity values greater than or equal to 85 and 75%, for the CP gene and for the 3'-NTR, respectively, indicate strains of the same viral species (Van Regenmortel et al., 2000).

The second hypothesis was investigated by repeating the protection test of pre-immunized passion fruit vines with mild strains F-101 and F-144, challenged with strain PWV-SP, inoculated either mechanically or through viruliferous aphids, which is the mode of transmission of the virus in the field. Plants were protected by a shade-netting structure. The mechanical challenge inoculation of plants pre-immunized with each mild strain was performed on the sixth and seventh leaves below the leader shoot in a group of four plants. Challenge inoculation with viruliferous aphids was made by confining the insects to the leader shoots of another group of four plants. Before challenging, the relative concentration of the mild strains was estimated in five leaves of one branch, by indirect DAS-ELISA (Table 4). Thirty days after the first challenge (Oct. 21, 2001), 0 and 25% of the plants pre-immunized with mild strains F-101 and F-144, respectively, mechanically challenged with strain PWV-SP, showed mosaic symptoms, foliar deformations and blisters. The same occurred with 50% of the plants pre-immunized and challenged by means of viruliferous aphids, regardless of the mild strain utilized at pre-immunization. At 60 days after the first challenge (Nov. 21, 2001), 75% of the plants pre-immunized with the two mild strains and mechanically challenged exhibited severe symptoms of the disease. However, for plants pre-immunized and challenged by means of aphids, the number of plants exhibiting severe symptoms remained at the same level as in the previous evaluation (Oct. 21, 2001). The healthy, non-inoculated plants did not display symptoms of the disease at that occasion.

Table 4 - Number of passion fruit plants pre-immunized, pre-immunized and challenged with the severe strain of *Passion fruit woodiness virus* (PWV-SP) either mechanically or by aphids, and healthy plants, which showed severe symptoms of the disease (rating 3) on four evaluation dates, in the field.

Treatment	Challenge inoculation	No. of tested plants	No. of plants with maximum degree of symptoms*			
			1st challenge**		2nd challenge	
			30 d.a.c.	60 d.a.c.	30 d.a.c.	60 d.a.c.
Pre-immun. F-101		2	0	0	1	2
Pre-immun. F-144		2	0	0	1	2
Pre-immun. F-101 + PWV-SP***	Mechanically	4	0	3	4	4
Pre-immun. F-144 + PWV-SP	Mechanically	4	1	3	3	4
Pre-immun. F-101 + PWV-SP	Aphids	4	2	2	2	4
Pre-immun. F-144 + PWV-SP	Aphids	4	2	2	3	4
Healthy		5	0	0	2	5

*Rating 3 = severe mosaic, blisters and leaf deformations; **d.a.c. = days after challenging; ***Pre-immunized with mild strain and challenged with severe PWV strain, 60 and 120 days later.

On the evaluation of Dec. 21, 2001, thirty days after the second challenge, an increase in the number of plants showing severe symptoms was observed. By the end of the second month (Jan. 21, 2002), after the second challenge, all plants pre-immunized with the two mild strains and challenged with the severe strain, either by mechanical inoculation or inoculated through aphids, were at maximum rating for symptoms. Plants that had been pre-immunized but not challenged, as well as healthy plants, even though protected by the shade-netting structure, were infected by the severe strain, since all of them exhibited severe symptoms of the disease, six months after installation of the experiment in the field. This infection must have been carried out by aphids which entered the netting structure.

All initially healthy plants, maintained in the greenhouse and inoculated with the severe PWV strain, either mechanically or by viruliferous aphids, on the occasion of both challenges of plants in the field, presented severe symptoms of the disease, 15 and 30 days after inoculations, respectively. Therefore, both mechanical challenge inoculation and the challenge inoculation through viruliferous aphids were effective.

Estimates of mild strain relative concentrations (F-101 and F-144) in passion fruit vines utilized in the second protection experiment are presented in Figures 1

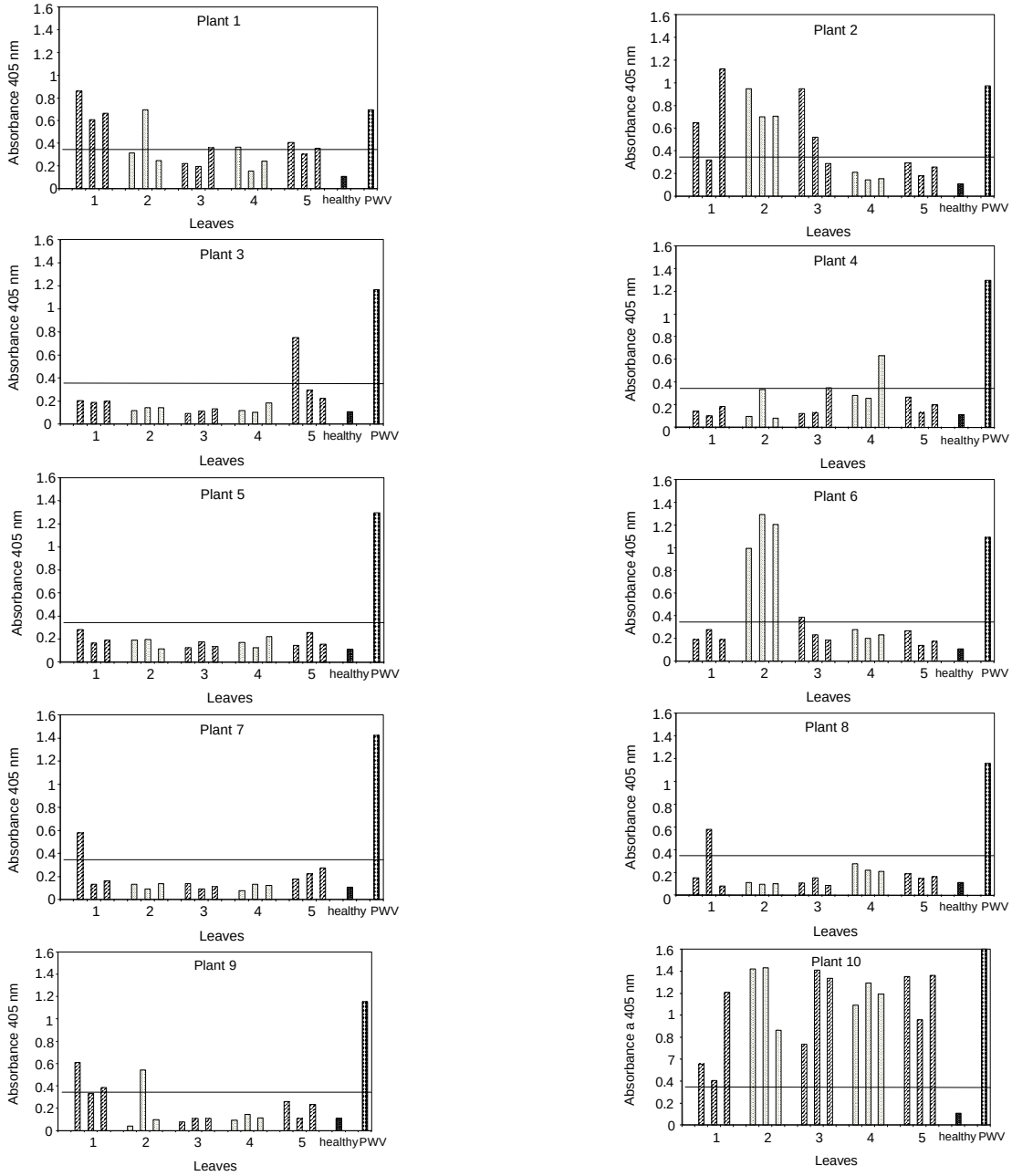

Figure 1 - Mean absorbance of extracts of five different leaves, at three distinct points of each leaf (set of three equal bars), collected from 10 passion fruit vines pre-immunized with mild strain F-101, by the indirect DAS-ELISA test (continuous horizontal line indicates the value that is equal to three times the mean absorbance of the healthy plant extract = 0.33). PWV = positive control.

and 2, respectively. It was not possible to detect the presence of the virus in 108 and 97 foliar disks from plants infected with mild strains F-101 and F-144, respectively, by the criteria adopted for the indirect DAS-ELISA. Mild strain F-101 was detected in 42 out of the 150 foliar disks analyzed, at various concentrations, since the absorbance values oscillated from 0.35 (plant 4, leaf 3) to 1.43 (plant 10, leaf 2). The same was verified in 53 of the 150 foliar disks analyzed from plants infected with strain F-144, where the absorbance varied from 0.34 (plant 8, leaf 1) to 0.96 (plant 9, leaf 2). Low concentration of mild strains, in several regions of the foliar tissues might represent viable areas for infection by any severe strain of the virus, inoculated at a later time. After establishing at the inoculation point, the severe strain, moves systemically and express itself in the leaves of the leader shoots in the branches.

One of the requirements for protection between strains of the same virus is the presence of the first strain in all virus replication sites in the cell, preventing the establishment of another related strain (Kunkel, 1934). Lack of protection related to low concentration of virus in the

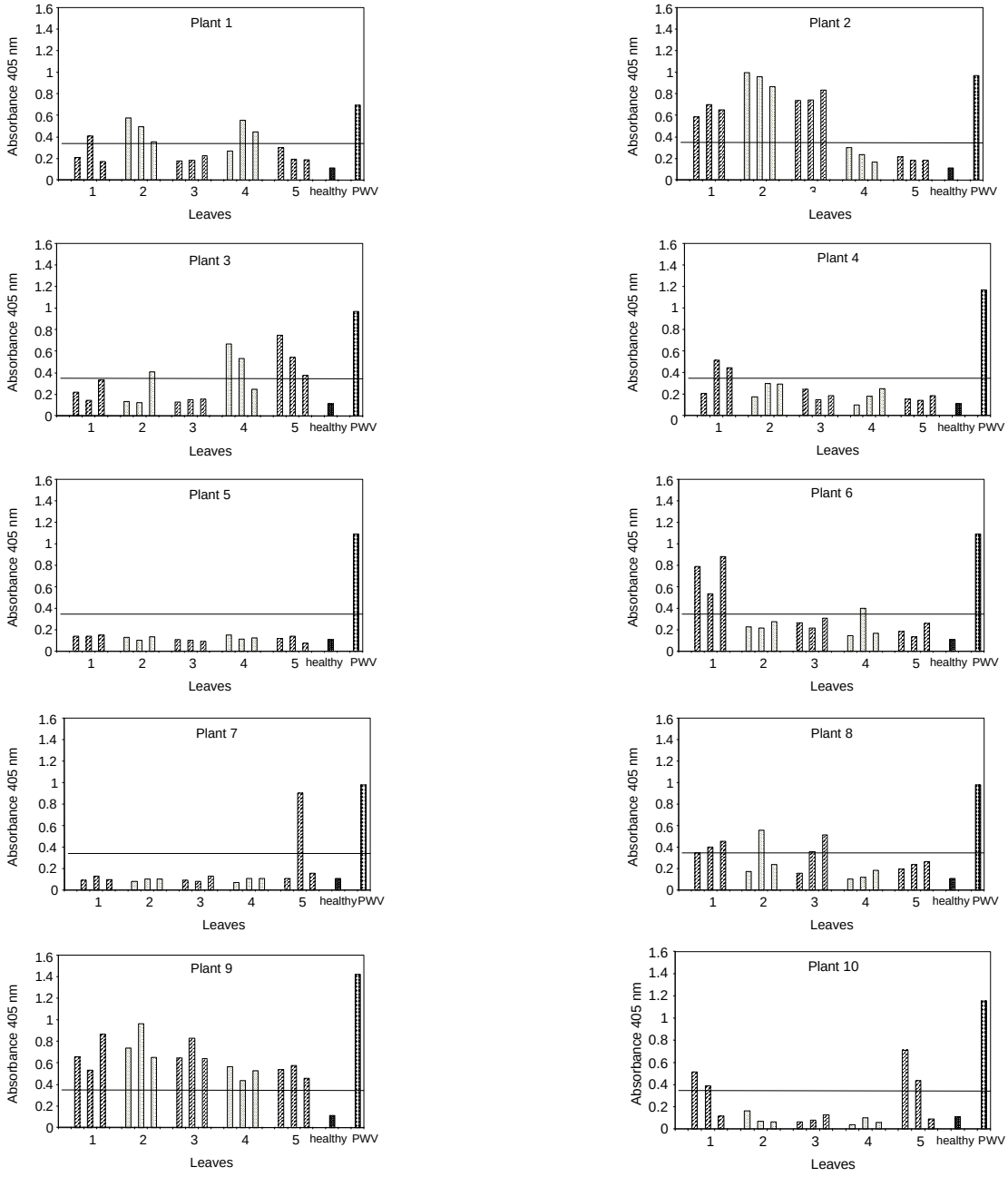

Figure 2 - Mean absorbance of extracts of five different leaves, at three distinct points of each leaf (set of three equal bars), collected from 10 passion fruit vines pre-immunized with mild strain F-144, by the indirect DAS-ELISA test (continuous horizontal line indicates the value that is equal to three times the mean absorbance of the healthy plant extract = 0.33). PWV = positive control.

tissues was observed by Sherwood & Fulton (1982) in *Nicotiana sylvestris* Speg. infected by a *Tobacco mosaic virus* (TMV) strain which causes mosaic. When the plants were challenged with a strain which causes necrotic local lesions, the symptoms of the challenging strain were observed only in dark-green areas of the leaves, where the concentration of the protective strain was very low. There was no lack of protection in light-green areas of the same mosaic-bearing leaves, where the concentration of the first strain was high. Rezende & Sherwood (1991), while working with *N. tabacum* L. cvs. Samsun and Xanthi, also demonstrated that the protection failure between TMV strains was associated with the lower concentration of the protective strain in dark-green areas of the mosaic-bearing leaves. They also showed that after establishment of the challenging strain in the inoculated leaf, the virus moved systemically and was detected in the leader shoot leaves of the plants.

Even though the pre-immunization was not efficient to control passion fruit woodiness, this kind of approach toward the problem must not be abandoned yet. Absence of protection seems to be associated with the irregular concentration of mild strains in the foliar tissues, favoring the existence of areas that allow infection by other strains of the virus. Therefore, the selection of passion fruit vine clones that allow a better and more uniform multiplication of the mild strains, without intensifying the symptoms, could make pre-immunization viable to control the disease in the field. Plants 10 (Figure 1) and 9 (Figure 2), seem to be examples that reinforce this suggestion, since the respective mild strains were detected in all foliar disks analyzed, suggesting a more homogeneous distribution of the virus. Some thought may also be given to the possibility of selecting other mild strains bearing greater invasive power, with the consequent protective effect. Concurrently, new research lines for the development of passion fruit woodiness control methods should be investigated. Among these are cultural practices that might minimize the incidence and dissemination of the disease, as suggested by Gioria et al. (2000), the selection of plants that are tolerant to the disease and the development of transgenic plants that would provide resistance to infection by severe PWV strains.

ACKNOWLEDGMENTS

To FAPESP and CNPq for fellowship to the first and second authors, respectively.

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Received September 27, 2002
Accepted June 10, 2003