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Effect of heat therapy to control pineapple wilt Virus in pineapple *(Ananas comosos)*

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Abstract

Pineapple wilt disease is a common occurrence in pineapple cultivations in Sri Lanka. Reddening of leaves, twisting of their tips and wilting of plants are the characteristic symptoms of this disorder. Clostero shaped virus particles measuring about 1400-1800 nm x 12 nm and bacilliform virus particles with dimensions of 133nm x 33 nm were observed in infected samples of wilted pineapple plants under immuno electron microscopy. Furthermore Australian made polyclonal antiserum gave a strong positive colour reaction when tested with wilted pineapple isolates from Sri Lanka.

Pineapple slip suckers known to be infected with clostero and bacilliform virus particles associated with mealy bug wilt of pineapple, were heat treated in hot air and water bath at 40^0,45^0,50^0,55^0 and 60^0 C temperatures for varying time durations. Plant survical was drastically reduced at 60^0 C. A significant observation was that in both methods of treatment, plants subjected to 55^0 C for 15 minutes, gave negative results during the first month after heat treatment showing it has temporarily suppreseed the virus in the tissues. However Enzyme linked immunosorbent assay, immunosero electron microscopy and polymerase chain reaction methods demonstrated that heat treatment does not elininate permanently, the two types of virus particles present in wilted pineapple plants in Sri Lanka.

Key words. Heat therapy, Virus control

1. Introduction

A wilt disease of Pineapple (Ananas comsus) described in Hawaii more than 75 years ago (Larson 1910) has remained a serious problem for the pineapple industry worldwide (Carter, 1932; 1933). The consistent association of mealy bugs with this disorder earned its name "Mealy bug wilt of pineapple:. It was postulated to have been caused by a toxin secreted by mealy bugs during their feeding on pineapple plants (Carter, 1932;1951).

In mealy bug wilt of pineapple, the above ground symptoms show a slight reddening of the leaves to about halfway, which later changes colour from red to pinka (Plate 1a). At the intermediate stage the symptoms show the characteristic colour without leaf curling or leaf tip death. In the advanced stage the leaf margins roll downwards with wilting of leaves but the plants remain alive (Plate 1b). The root system is also affected with limited root deaths but to a lower extent than with a fungal rot.

A long filamentous rod shaped virus (1200mm) has been isolated from pineapple (cv Smooth Cayenne) with symptoms of mealy bug wilt (Gunasinghe & German, 1989). Based on virus characterization this virus has been assigned tentatively to the type II closterovirus group by the same scientists. Furthermore sap of symptomatic mealy bug wilt affected pineapple plants of Smooth Cayenne group when negatively stained and examined under the electron microscope, showed clostero like virus particles. In addtion some bacilliform particles were also seen in partially purified preparations from both symptomatic and wilted pineapple leaves by Wakman et al (1995) in Australia.

Same authors reported that bacilliform particles measured about 133x33nm and they were trapped and decorated by the Queensland pineapple wilt virus antisurum and also with sugar cane badnavirus antiserum. Furthermore, they reported, the relationship of the clostero-like and bacilliform viruses to yield loss and mealy bug wilt of pineapple is un confirmed.

There are many methods to obtain virus free plants (Nyland and Coheen 1969, Rosetti *et al* 1965, Murashige *et al* 1972) and heat herapy is a common method practiced. Ullman *et al* 1989, reported that heat treatment at 50^0 C for 30 minutes of wilt virus infected pineapple crowns permitted 100% plant survival and rendered 100% of the plants free of the clostero like particles.

The purpose of this investigation was to confirm the virus etiology of pineapple wilt symptoms in Sri Lanka and investigate the possibility of eliminating viruses by heat therapy.

2. Materials and methods

Slip suckers of *Mauritius* cultivar pineapples were collected from market places at Miriswatte in the Gampaha district of Sri Lanka. The presence of viruses in these suckers were initially confirmed by indirect antibody sandwich enzyme linked immunosorbent assay at RARDC, Bombuwela, using an antiserum produced by Wakman *et al* 1995 (Table 1). The antiserum contained antibodies for both clostero and bacilliform virus particles. Further leaf samples of suckers were sent to Dept. of Primary Industries (DPI) Australia for electron microscopic investigations.

Infected pineapple suckers were subjected to heat treatment by two different methods. In the first evaluation method, suckers were exposed to hot air treatment by using a hot air oven. Temperatures of 40^0-,50^0 55^0 and 60^0 C were given for varying time durations (Fig. 1a). The time period of 1 month was applied for the 40^0 C Treatment only. In this treatment the plants were allowed to stand at room temperature (30-33^0 C) for 6 hours every day.

In the second evaluation method, a series of heat treatments were applied using a water bath. Suckers were subjjected to different temperature regimes of 45^0,50^0,55^0 and 60^0 C for different periods of time (Fig 1b). A Thermo couple probe inserted inside the core of a leaf sample in the tank, monitored the temperatures.

After the heat duration treatments, the suckers were kept at room temperature for 6 hours before being planted in pots containing sterile soil. After 4 weeks of heat treatments, the suckers were field planted at the Agricultural Research Unit Gabadawatte, Homagama. Field planting was done in double rows. This consisted of 20 suckers per row and 40 suckers per treatment. One metre spacing was given between rows and 30 cm between suckers The percentage survival was recorded. (Figs 1a & 1b). One and three months after heat treatment, plants were indexed by indirect ELISA for PWV.

Out of the two methods of heat treatment tests only the water bath treatment was found to be feasible for further investigations. Several water bath treatments and one air treatment were selected for further evaluations.

Enzyme linked immunosorbent assay

Heat treated suckers were tested for the presence of viruses with an antiserum produced by Wakman et al 1995 by indirect ELISA using a technique previously described (Dassanayake & Wickremasingha, 1994). In this

evaluation leaf samples were collected from the inner whirl of the sucker. Since the frinding was done using a mortar and pestle only, succulent bases of leaves were used for sap extractions.

Plants were first sampled for the virus, one month after treatment when new growth appeared and the ELISA tests were repeated 3 months after heat treatment. Results are given in Tables 2a & 2b.

Immune electron Microscopy

Pineapple leaf samples before heat treatment and 3 months after heat treatment were also sent to Department of Primary Industries, (DPI), Australia for Immune Electron Microscopy (IEM) to confirm the presence of PWV. In these investigations Wakman et al (1995) antiserum was used. For observation of clostero virus particle by IEM, Gunasinghe & German (1989) method was used, while bacilliform particles were viewed by Lockhart (1986) method. Results are given in Table3. (Plates 2a&2b)

Polymerase chain reaction (PCR)

All samples were tested 3 months after heat treatment by PCR for bacilliform virus only, according to Thomson et al (1996). The upstream primes 5' GTT TAC ACA AAG GAG TGG AAG AGC ACC 3' and downstream primer 5' TGA AAT CTG GTA AGG CAA GTC AGG GGC 3' Were used for this amplification. This series of tests were also done at DPI Queensland Australia. Results are shown in Table 3 & Plate 3.

Disease index

All the heat treated plants were indexed 3 months and 18 months after field planting by using 5 unit disease index scale denoted by

Mottling 0-5
Reddening 0-5
Tip twisting 0-5

Where 0=no symptoms;5=severe symptoms

Results are given in Table 4:

3. Results and Discussion.

Initial indexing of test samples using electron microscopy have shown that planting material contained both clostero (particle size 1400-1800 nm x 12 nm) and bacilliform virus particles (particle size 133 x 33nm)

Indirect ELISA using Australian made antiserum detected virus in collected sapmles. Some results are given in Table 1. Results further confirmed the presence of virus in initial pineapple suckers used for this experiment.

Table 1 : ELISA absorbance values for the tested samples

Sample No.	405 Absorbance
1	.218+
2	.243+
3	.118+
4	.148+
5	.312+
6	.293+
7	.426+
8	.394+
9	.219+
10	.314+
11	.394+
12	.282+
13	.362+
Disease control	.321+
Healthy control	.023

+= Positive for the test

Threshold value = value greater than twice the healthy control was considered as positive

Plate was read 2 hours after substrate addition at room temperature.

Effect of Heat treatment

Pineapple suckers readily wihstood heat treatments of 40^0,45^0,50^0C with 100% of the plants surviving all treatment durations (Figs: 1a & 1B). Plant survival decreased dreastically when suckers were subjected to 55^0C for more than 30 mnutes. According to the results presented in Figs. 1a & 1b, plant survival was very low (O-15%) in heat treatment of 60^0C.

Pineapple Wilt Virus Detection

Table 2a: Pineapple wilt virus indexed by ELISA after hot air treatment

Temp in dg C	Duration in minutes	%PWV detection by ELISA 1m	3m
40	180	100	100
	360	100	100
	540	100	100
	30 days	46	100
50	15	100	100
	30	100	100
	45	100	100
	60	100	100
55	15	50	100
	45	100	100

1m = 1 month after heat treatment
3m = 3 months after heat

Each treatment represents mean values for 40 suckers

Table 2b : Pineapple wilt virus indexed by ELISA after hot water treatment

Temp in deg C	Duration in minutes	%PWV detection by ELISA 1m		3m
40	60	100		100
	90	-	do	-
	180	-	do	-
	240	-	do	-
50	15	100		100
	30	-	do	-
	45	-	do	-
	60	-	do	-
55	15	60		100
	30	100		100

1m = 1 month after heat treatment
3m = 3 months after heat treatment

*Each treatment represents mean values for 40 suckers

Fig 1a: Effect of HOT AIR treatment on the survival of pineapple suckers

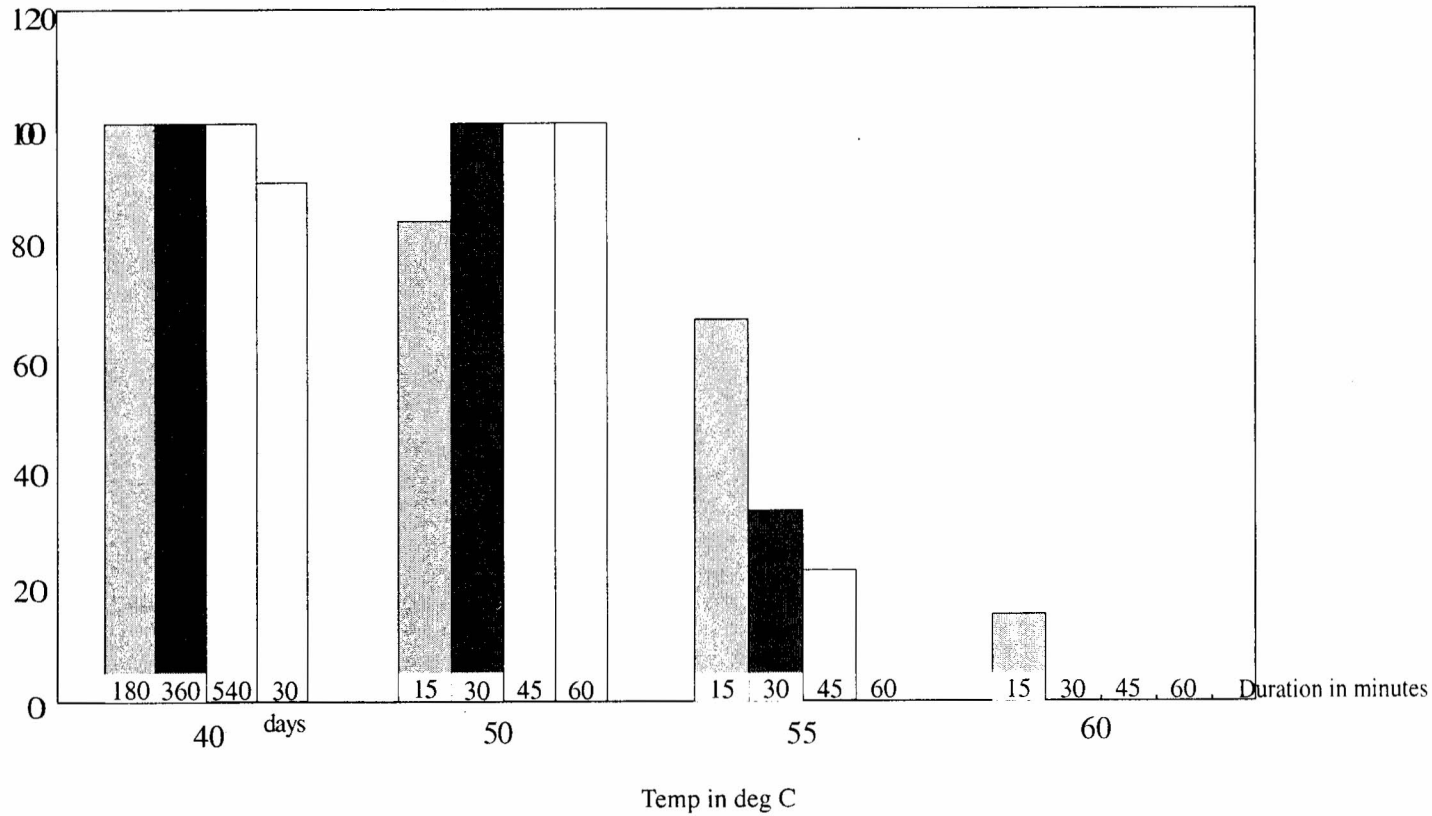
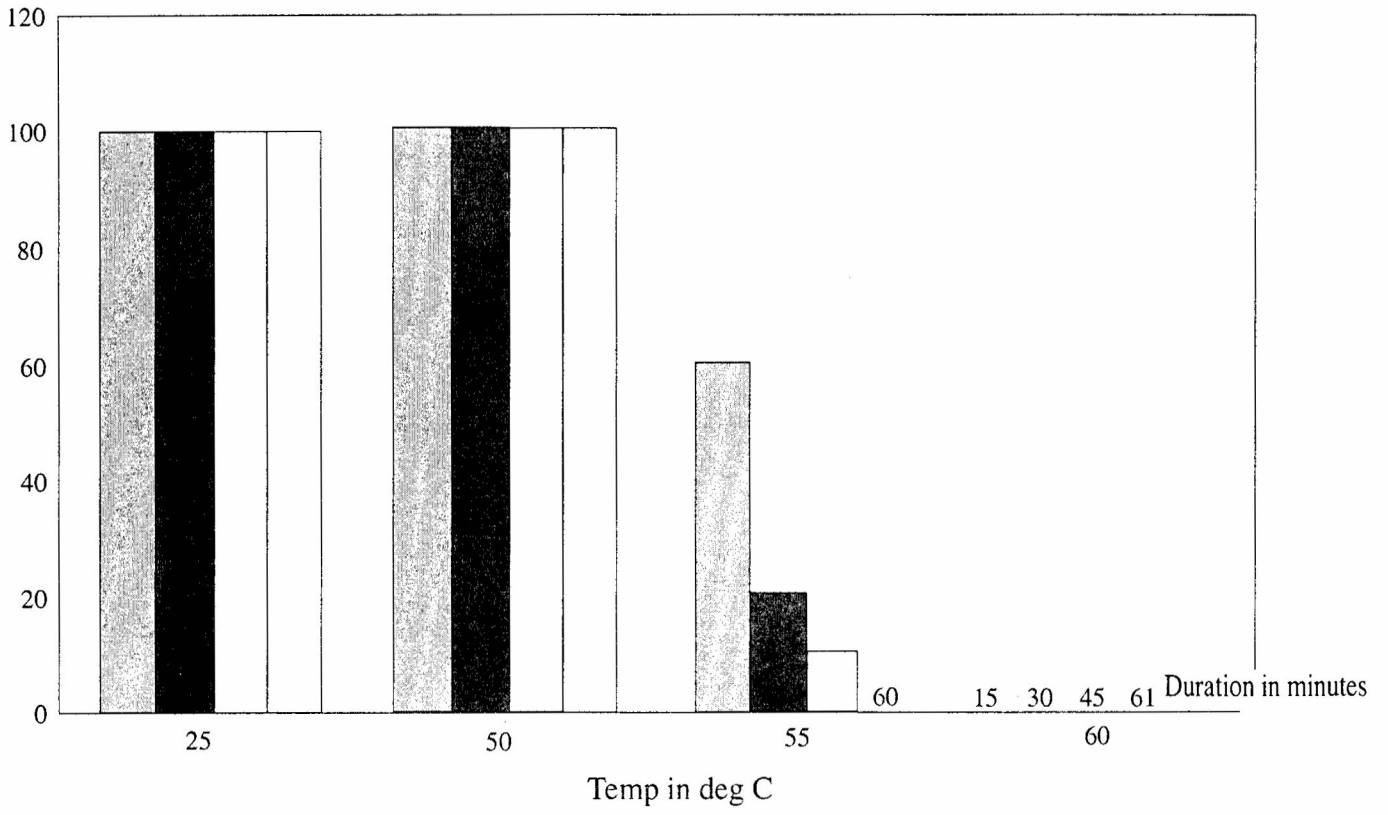


Fig 1b: Effect HOT WATER treatment on the survival of pineapple suckers



Pineapple Wilt Virus Detection

Except for a few treatments; hot air 40 deg C for 1 month, 55 deg C hot air for 15 minutes and water bath heat treatment at 55 deg C for 15 minutes, all other heat treated suckers showed 100% positive results indicating the presence of pineapple wilt virus when tested by ELISA after 1 month heat treatment (Tables 2a & 2b). However in both treatments of 55 deg C hot air for 45 minutes and 55 deg C water bath for 30 minutes positive results were given after one month from heat treatment. Moreover the heat treated plants at 55 ^{0}C for 15 minutes in either methods and at 40^{0}C for 1 month gave 100% positive results when tested by ELISA 3 months after treatment.

Walkey 1976, reported that high temperature treatment at longer periods stops virus synthesis and also inactivates the resistance factor in the host plant. It has been postulated that within an infected plant, virus synthesis and virus degradation occur simultaneously and at higher temperatures virus synthesis ceases but degradation continues (Kassanis 1957). Furthermore treatments which gave negative results initially also showed positive results for PWV when tested 3 months after heat treatment.

Table 3: Presence of different virus particles detected by immuno electron microscopy (IEM) and polymerase chain reaction (PCR).

Hot water heat treatment		Viruses detected	
		IEM & PCR	IEM
500 C	-15 minutes	PBV	PCV
500 C	30	PBV	PCV
500 C	45	PBV	PCV
500 C	01 hour	PBV	PCV
550C	15 minutes	PBV	PCV
550C	30	PBV	PCV
400 C	1month (hot air)	PBV	PCV

Electron microscopic observations and PCR analysis further confirmed the presence of clostero and bacillifrom virus particles in heat treated samples (Table 3)

DISEASE INDEX

Table 4: Disease severity evaluated on a 5 unit Disease Index scale, 6-18 months after planting.

TREATMENT	MEAN DISEASE INDEX	
	3MAP	18 MAP
50° C - 15 minutes	0.5	5
50° C 30	0.5	5
50° C 45	0.5	5
50° C 01 hour	0.5	5
55° C 15 minutes	0.5	5
55° C 30	0.5	5
55° C 45	0	5
40°C 1month (hot air)	1.0	6
Control	1.0	10

Rating for symptom expression

A=leaf reddening 0-5 0=no symptoms; 5=severe symptoms
B=Wilting 0-5
C=Leaf tip wilting 0-5 Disease Index=A+B+C

Disease Index was computed from mean of 40 suckers

At the early stages (3MAP), all heat treated plants and non-treated plants gave very low disease index values, However after about 18 months in the field, some plants showed typical wilt symptoms. Symptoms were more prominent during the dry period and disappeare with the rains.

4. Conclusions

Virus Indexing by different methods has confirmed the presence of clostero and bacilliform viruses in Sri Lankan pineapples infected with pine-

apple wilt virus. The survival heat treatment temperature of pineapple slip suckers has been established by the investigations and reported to be 40^0 C for 1 month (hot air), 50^0C for one hour duration and 55^0 C has also been withstood by pineapple for a duration of 45 minutes. Temporary suspension of virus synthesis was observed in both hot air and water - bath treatments of 55^0C for 15 minutes. Asimilar observation was made for suckers treated at 400C for 1 month in hot air. The possibility that these observations are also due to factors other than the heat treatment cannot be ruled out as the treated plants were not raised under growth cabinet conditions due to unavailability of the facility.

However it has not been established that heat treatment is a satisfactory method of control of clostero and bacilliform viruses associated with wilted pineapple plants.

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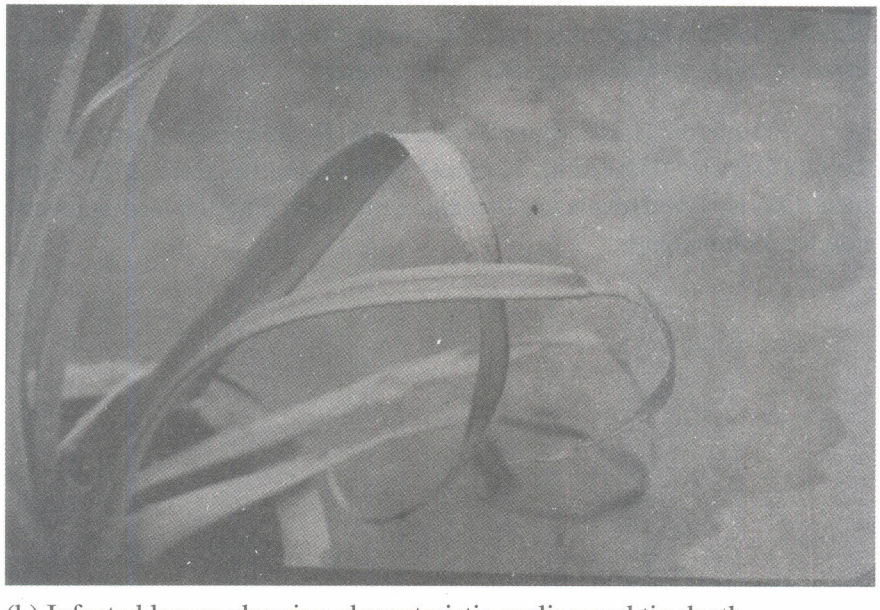
Plate 1

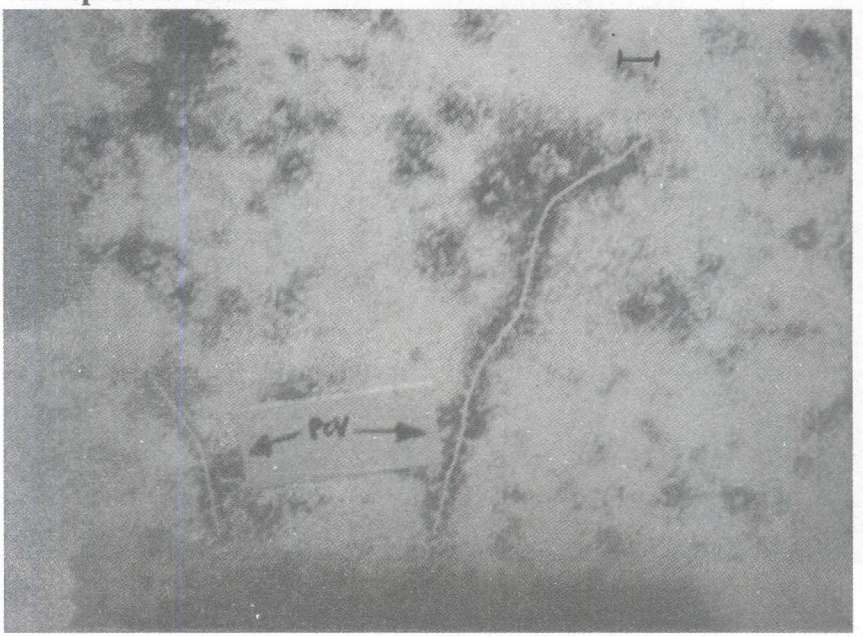
(a) Initial symptoms of Pineapple Wilt Virus (PWV)



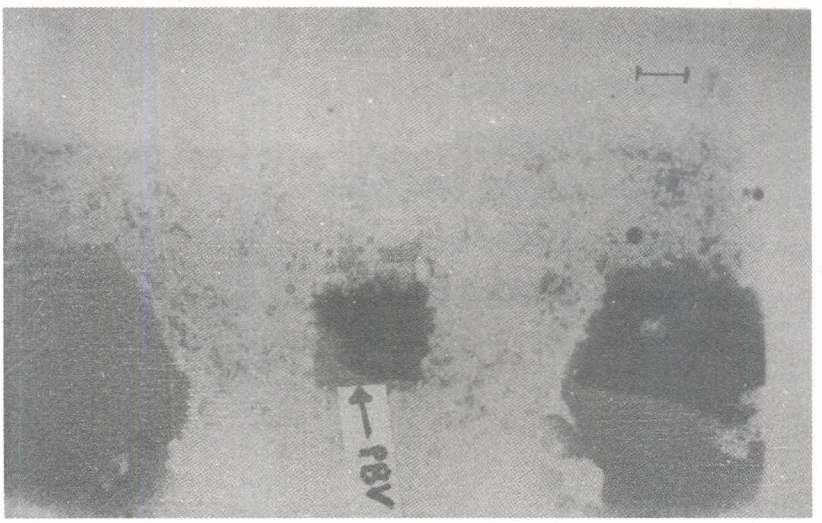
(b) Infected leaves showing characteristic curling and tip death

Plate 2

bax represents 100nm.

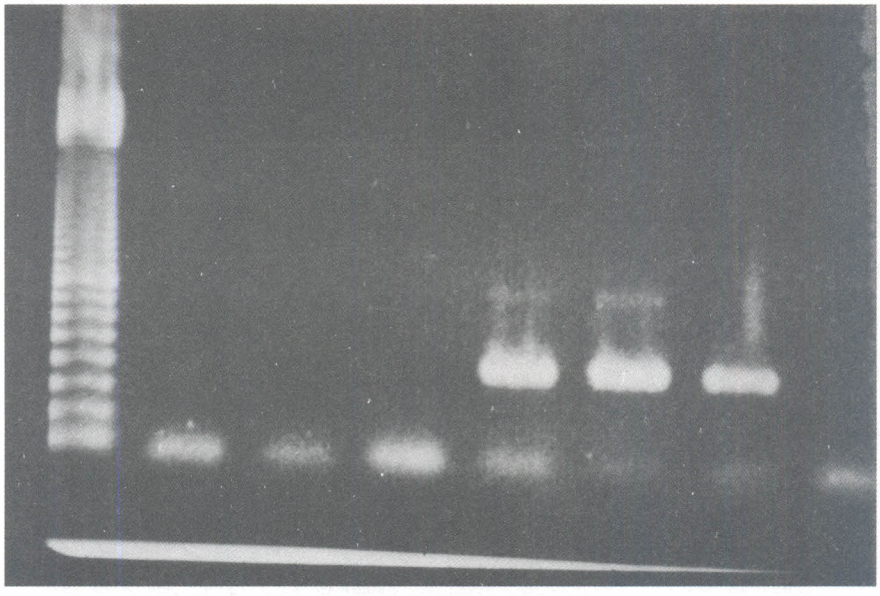


(a) Electron micrograph showing clostero virus particles in a heat treated sample from Sri Lanka.



(b) Electron micrograph showing bacilliform virus particles from similar samples

Plate 3



Polymerase Chain Reaction (PCR) amplification using specific primers.

M=Marker (100bp apart)
Lane 1 - Negative control

2 - Buffer control

3 - Bromilades (virus free)

4 - PBV infected Pineapple

5 - Heat treated pineapple 500C-30min

6 - -do- 550C-45 min

7 - No template (PCR mix only)

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