PN-ACK-037
106674

FINAL REPORT

Covering Period: [March 1995 – July 2000]

Submitted to the U.S. Agency for International Development; Bureau for Global Programs, Field Support and Research; Center for Economic Growth

Title of Project

APPLICATION OF MOLECULAR TECHNIQUES TO VIRUS CERTIFICATION IN FRUIT TREES

Principle Investigator: Dr. Sara Spiegel
Grantee Institution : Agricultural Research organization, The Volcani Center, Israel

Collaborator : Dr. Maria Kolber
Institution : Crop protection and Soil Conservation Service, Budapest, Hungary

Project Number: C12-127
USAID Grant Project Officer: Mr. William H. Crane

Project Duration: march 1995 – July 2000

3) Executive Summary

The general goal of this project was to facilitate production of virus-tested propagation material for the fruit-tree growing industry in Hungary and Israel. The targeted viruses were plum pox (PPV) and prunus necrotic ringspot (PNRSV). Both occur in many isolates (strains) and are a serious problem in stone fruit species.
The research activities in this project focused on:

- Application of target sequence amplification techniques for detection of virus/es in infected trees and evaluation of their suitability for large-scale testing.
- Attempts to establish a 'test calendar' for the targeted viruses.
- Studying virus strain differentiation by serological and molecular techniques.

PCR assays (RNA-RT-PCR; immuno-capture (IC)-PCR, sap-PCR and ELISA-PCR were evaluated for detection of PPV and PNRSV in infected trees / woody indicators. Multiplex – PCR, allowing detection of both viruses in a single reaction was also studied. Large-scale testing for both viruses was approached using three different PCR assays and compared to standard DAS-ELISA. A summary of these data and the results of monitoring the fluctuation in virus levels during the vegetation period allow making recommendations for certification schemes in fruit growing countries.
Differentiation among virus isolates was done by serotyping and by applying RFLP analysis (with specific restriction enzymes) to PCR products of PPV and PNRSV. It was concluded that most isolates collected in the 70s belonged to strain M (aphid transmitted) which caused severe damage to stone fruit crops in Hungary. The number of isolates of strain D increased in the last decade.
A different, molecular approach involving in vitro transcription of the targeted amplified viral products followed by gel electrophoresis was developed and applied in Israel to PNRSV isolates. This method, termed by us 'transcript conformation polymorphism' (TCP), differentiated among PNRSV isolates, however, questions regarding severity and mildness remain open. A further development involved formation of heterologous duplexes from RNA transcripts originating from several virus isolates and comparison of their electrophoretic mobility (homologous duplexes included) (double-stranded) (ds-TCP). We hypothesized that this polymorphism is granted by conformational variations among the transcript duplexes.
The results obtained in this project contributed to reliable detection and molecular differentiation of PPV and PNRSV and are relevant to other plant viruses.

4) Research Objectives

Production of virus-tested propagation material for the fruit-tree growing industry requires reliable, sensitive virus detection methods. The development of molecular methods based on target sequence amplification in the last decade opened up new possibilities for diagnosis and detection of viruses and also for virus strain differentiation. Application of the polymerase chain reaction technique (PCR)-based assays to given plant / virus systems required experimental studies in which various parameters (e.g. virus concentration and fluctuation, variability in virus isolates, PCR-interfering host compounds) were approached.

The general goal of this project was to facilitate production of virus-tested propagation material for the fruit-tree growing industries of Hungary and Israel. The research focused on the two most important viruses in stone fruit species: plum pox (PPV) and prunus necrotic ringspot (PNRSV). Both viruses occur in numerous isolates (or strains) and affect rosaceous fruit tree growing industries. The specific research objectives were:

- Application of target sequence amplification techniques for detection of virus in infected trees and evaluation of their suitability for large-scale testing.
- Establishing a 'test calendar' for both studied viruses.
- Studying virus strain differentiation.

Developing and validating PCR detection assays for fruit tree viruses were studied by research groups in Europe and the USA, including the Israeli group. Following the transfer of technology to Kolber's group in Budapest and establishing the PCR lab this group joined the effort and focused mostly on PPV (as planned in the proposal). Numerous research papers were published by others and by us (listed below in Project Outputs).

The use of PCR amplification for strain differentiation studies (nested PCR, RFLP of PCR products) was approached by several research groups for PPV and PNRSV. The Israeli group focused on transcript conformation polymorphism of PNRSV in addition to conventional (bioassays, serotyping, RFLP) typing. The single-stranded and double-stranded conformation polymorphism of homologous and heterologous RNA transcripts of PNRSV isolates developed in Israel by Rosner and co-workers added significantly to the body of knowledge on typing of PNRSV and in general to plant virus strain differentiation.

5) Methods and Results

The research work performed in the frame of this project can be divided into 4 major activities:

1. Adopting sensitive PCR assays for PNRSV and PPV.
2. Monitoring fluctuations in virus levels towards a 'test calendar'.
3. Bio-characterization of virus isolates.
4. Typing PNRSV isolates by serological and molecular assays.

5.1 Adapting sensitive PCR assays.

5.1.1 Evaluation of molecular detection methods for PPV and PNRSV

Determining the sensitivity and reliability of PCR assays in comparison to the standard ELISA is a pre-requisite for application of this new technique to virus detection in quarantines, certification schemes and large-scale tests. This issue was addressed throughout this project both for PNRSV and PPV. Results were reported in the annual reports, presented in meetings and compiled in publications.

PNRSV: A RT-PCR assay was designed for PNRSV (Spiegel et al. 1996) and later improved by amplifying small PCR products (Rosner et al. 1997). A PCR assay based on capturing virions by a specific antibody (immuno-capture, IC) was developed (Krizbai et al. 1998) and a comparison with amplifying viral RNA from sap was also done (Krizbai et al. 1998). Three PCR versions IC-PCR; RNA RT-PCR and direct sap PCR were evaluated for detection of PNRSV and were all found to be more sensitive than DAS-ELISA. The reliability of IC-PCR was limited in several cases, maybe due to inhibitors in the plant sap, typical of rosaceous hosts. A dilution series of plant sap and/or extracted RNA showed that 'sap' PCR was about 100 times more sensitive than ELISA. The amplified product was detected in a 1:50,000 dilution both by RT- and IC-PCR (Table 1).

Table 1. Comparison of the sensitivity of PCR detection assays for PNRSV.

Dilution	RT-PCR	IC-PCR	SAP-PCR	DAS-ELISA
1:1	+	+	+	+
1:120	+	+	+	+
1:6.250	+	+	+	-
1:12.500	+	+	+	-
1:25.000	+	+	+	-
1:50.000	+	+	-	-

PCR-ELISA, a recent, rapid diagnostic method (see scheme in Appendices), was adapted in Hungary for PPV and PNRSV. Results presented in Table 2 show the high OD values (over background readings) obtained in this assay for PNRSV infected samples. The sensitivity of PCR-ELISA was found to be about 100X higher in comparison to the analysis of PCR products in ethidium bromide stained gels. Among the advantages of this method three points should be mentioned:

- The high specificity of PCR-ELISA allows discriminating between specific and nonspecific PCR products due to the hybridization step with the capture probe.
- PCR-ELISA allows large-scale screening of samples in ELISA-plate formats.
- The DIG-labeled PCR products can be used as a probe in different non-radioactive hybridizations: Southern, Northern, and Dot-blotting.

The disadvantages of PCR-ELISA are the high cost of reagents, limiting its use for large scale testing and the amount of work involved. Using of DIG-labeled dNTPs, streptavidin-coated ELISA wells and the reagents for detection amounted in Hungary (in 1999) to about 8.0 USD for one PCR reaction.

Table 2. Detectability of PNRSV by RT-PCR and PCR-ELISA

PNRSV isolates	RT-PCR	PCR-ELISA / Extinction value
ÖK 28	+	+++ / 3,203
ÖK 44	+	+++ / 3,017
ÖK 47	-	- / 0,104
ÖK 63	+	+++ / 2,657
ÖK 66	+	+++ / 3,300
ÖK 67	+	+++ / 3,306
ÖK 95	+	+++ / 2,713
ÖK 100/1	+	+++ / 2,790
ÖK 133	+	+++ / 2,936
ÖK 138	+	+++ / 3,030
ÖK 147	+	+++ / 2,624
ÖK 152	-	+ / 0,237
ÖK 163	+	+++ / 2,736
ÖK165	+	+++ / 3,128
Healthy control	-	- / 0,100

PPV: PCR assays were adapted for PPV detection (Kolber et al. 1997). Developing a PCR detection assay for double infections (PNRSV and PPV) in a one-tube reaction (Multiplex RT-PCR, scheme in Appendices) was done in Hungary (Kolber et al. 1997; Krizbai et al. 1997). A summary of results obtained by comparing indexing on woody indicators, ELISA, RT-PCR assays and a multiplex RT-PCR assay for detection of PPV and PNRSV in one host is presented in Table 3. The RT PCR and Multiplex PCR results show that for these viruses, a one-tube multiplex reaction with 2 sets of specific primers (one pair for each virus) can be done reliably, thus reducing the cost and manipulations involved in two PCR tests. As can be seen from the colored gray cells in Table 3 ELISA is a reliable method for initial screening of plants while PCR is much more sensitive.

The summary of the results obtained in this activity for both viruses allows a comprehensive discussion about the possible use of these molecular methods for routine detection, taking into consideration various factors (e.g. reliability, sensitivity, price of reagents) and making recommendations for certification schemes.

Table 3. Detection of PPV and PNRSV in hosts infected with both viruses by indexing, ELISA, RT-PCR and Multiplex-PCR.

	Indexing			ELISA		RT-PCR		Multiplex-PCR	
Sample	PPV		PNRSV	PPV	PNRSV	PPV	PNRSV	PPV	PNRSV
	field	GH	field						
Peach	+	+	+	0	0	+	+	+	+
Peach	+	+	0	(+)	0	+	0	+	0
Peach	+	+	+	+	(+)	+	+	+	+
Peach	0	0	+	0	(+)	0	+	0	+
Peach	+	+	0	+	0	+	0	+	0
Peach	+	+	0	(+)	0	+	0	+	0
Peach	+	+	0	+	0	+	0	+	0
Peach	+	+	0	+	0	+	0	+	0
Peach	0	0	0	0	+	0	+	+	+
Peach	+	+	0	+	0	+	0	+	0
S. cherry	/	0	+	0	+	0	+	0	+
S. cherry	/	0	+	0	+	0	+	0	+
S. cherry	/	0	+	0	+	0	+	0	+
S. cherry	/	0	0	(+)	0	+	0	+	0
S. cherry	/	0	0	+	0	+	0	+	0
S. cherry	/	0	0	(+)	0	+	0	+	0
S. cherry	/	0	+	0	+	0		0	+
S. cherry	/	0	+	0	+	0	+	0	+
S. cherry	/	+	0	(+)	0	+	0	+	0
S. cherry	/	0	+	0	+	0	+	0	+
H. peach	0	0	0	0	0	0	0	0	0
H. cherry	0	0	0	0	0	0	0	0	0
PNRSV +	0	0	+	0	+	0	+	0	+
PPV +	+	+	0	+	0	+	0	+	0

Note: /=not tested; 0=negative; +=positive

5.1.2 Large scale screening for PNRSV and PPV by ELISA and IC-PCR

Reliable large-scale testing for PPV and PNRSV is highly desired by advanced fruit tree growing industries and of major significance in disease outbreak situations. Following the adaptation of reliable PCR assays for the two targeted viruses, the Hungarian group undertook a comparative, large-scale study of 31 cultivars including scions and rootstocks of peach, apricot and plum (total 1,718 trees) of unknown health status. These trees were tested for PPV by IC-PCR and DAS-ELISA (known-infected controls were included). Results presented in Table 4 show that IC-PCR allowed detection of 2.2X more positives than DAS-ELISA (40 versus 18 trees). It should be noted that not all the known positives were detected by either method.

Table 4. Large scale testing for PPV by DAS-ELISA and IC-PCR

Plant species	No. of cultivars tested	Positive / tested plants	
		DAS-ELISA	IC-PCR
Peach			
scion	16	13/255	18/255
rootstock	3	1/585	10/585
Peach total	**19**	**14/840**	**28/840**
Apricot			
scion	8	2/741	6/741
Apricot total	**8**	**2/741**	**6/741**
Plum			
scion	3	1/4	1/4
rootstock	1	1/133	5/133
Plum total	**4**	**2/134**	**6/137**
total	**31**	**18/1715**	**40/1718**
PPV positive	Peach GF 305	47/53	50/53

5.2 Monitoring fluctuations in virus levels towards a 'test calendar'.

Fluctuation in virus levels of PPV in woody indicator species was studied during the vegetation period in the course of the project in Hungary and for PNRSV in Hungary and Israel. In order to follow the changes in virus level, the isolates were tested by ELISA (the measured extinction values parallel virus level in the tissue). For PNRSV, 8 isolates were maintained both on cherry (*P. avium)* and peach (*P. persica* GF 305) seedlings. For PPV, 31 selected isolates were maintained on GF 305. Results for PNRSV were similar in both countries. PNRSV level in cherry indicators was the highest in early spring and than continuously decreased until mid summer (August) and increased in the autumn. Some isolates could not be detected in the second half of the year. In cherry, much higher extinction values were measured in the spring months, than in peaches. This difference disappeared by May and the extinction values were about the same level in both species for the rest of the time In the case of PPV, after the high virus level in forced buds in March, virus titer decreased during the vigorous growing period of leaves in April then a second, but lower pick was measured in May. In the second half of the year the extinction values stayed at low level, and virus could not be reliably detected in several isolates. Summarized results and 2 figures were included in the 4th Annual report.
It can be concluded that the tendency of fluctuation in virus concentration was similar for the given virus in the successive years of observation, but there were higher differences in the extinction values measured in the same period of certain years. Very likely it was influenced by climatic conditions (mainly temperature) year by year. The most suitable period for testing of PPV and PNRSV by ELISA is early spring both for Hungary and Israel, but the optimal time of sampling may vary each year depending on weather conditions and therefore can not be generalized.

5.3 Bio-characterization of virus isolates

PNRSV: In the inoculation experiments altogether 25 PNRSV isolates were involved in Hungary. Those were isolated from different stone fruit species in distinct growing regions of Hungary and maintained on GF 305 peach seedling indicator (Table 5). During the four years of the project 8 woody indicators and 20 herbaceous indicator species were used for PNRSV and PPV (Table 6).

In the transmission studies conducted with PNRSV on woody indicators, there were differences in the symptoms provoked by the isolates identified between 1972 and 1978 only on 'Bing' cherry, GF 305 seedlings and Italian prune. On 'Bing' cherry, enations were formed using the isolates RS 28, RS 63, RS 66, RS 67, RS 133 and RS 138, while no enations were observed with the other isolates. The symptoms, induced on 'Bing' by the isolates introduced in 1998, can be read only in July-August 2000. Among the isolates collected until 1978, isolate RS 138 caused tip necrosis on GF 305 in every case, while with the other isolates, shoot necrosis did not or only seldom occurred. French and Israeli isolates provoked severe tip necrosis on GF 305. The isolate RS 180, 'Hermosa' peach from Israel caused the most severe symptoms on every indicator plant. Using the isolate RS 138, severe leaf necrosis and epinasty were observed on Italian prune. It was concluded that among the used *Prunus tomentosa* clones, IR.474 was the better one.

Table 5. PNRSV isolates involved in biological characterization experiments

Code of isolate	Maintaining indicator	Location of isolation	Source, species, cultivar	Year of isolation
Between 1995-1997				
RS 28	MF 12/1 GF 305	Erd (H)	Italian prune	1972
RS 63	MF 12/1 GF 305	Erd (H)	Grosse. rigorose	1972
RS 66	MF 12/1 GF 305	Erd (H)	Elberta	1972
RS 67	MF 12/1 GF 305	Siofok (H)	Champion	1972
RS 100/1	MF 12/1 GF 305	Cegled (H)	Sweet cherry	1986
RS 133	MF 12/1 GF 305	Cegled (H)	*Prunus mahaleb* C 982	1972
RS 138	MF 12/1 GF 305	Poloske (H)	Grand prize	1986
RS 142/1	MF 12/1 GF 305	Poloske (H)	Plum	1986
RS 152	MF 12/1 GF 305	Sasad (H)	Bergeron	1989
Between 1998-1999:				
RS 44	MF 12/1 GF 305	Erd (H)	Plum Kolhstock	1972
RS 95	MF 12/1 GF 305	Erd (H)	Apricot	1972
RS 147	MF 12/1 GF 305	Bak (H)	Plum Besztercei	1978
RS 163	MF 12/1 GF 305	Pecs (H)	*P.salicina*	1974
RS 165	MF 12/1 GF 305	Cegled (H)	Almond peach	1994
RS 168	MF 12/1 GF 305	Bordeaux (F)	PNRSV B 24	?
RS 169	MF 12/1 GF 305	Bordeaux (F)	PNRSV 42	?
RS 171	MF 12/1 GF 305	Bordeaux (F)	PNRSV 1844	?
RS 173	MF 12/1 GF 305	Bordeaux (F)	PNRSV PI 257	?
RS 174	MF 12/1 GF 305	(I)	*P. salicina* Ogden	1998
RS 180	MF 12/1 GF 305	(I)	Peach Hermosa	1998
RS181	MF 12/1 GF 305	(I)	Plum Ogden	1998
RS 182	MF 12/1 GF 305	Kecskemet (H)	Plum XII 10/5	1998
RS 183	MF 12/1 GF 305	Kecskemet (H)	Plum XII 10/7	1998
RS 184	MF 12/1 GF 305	Kecskemet (H)	Plum XII 13/1	1998
RS 185	MF 12/1 GF 305	Kecskemet (H)	Plum XII 1610	1998

Table 6. Woody and herbaceous indicators used for bio-characterization of PNRSV and PPV isolates in Hungary

Woody indicators	Herbaceous indicators
PNRSV studies:	
Prunus avium seedling	*Ammi majus*
P. armeniaca seedling	*Antirrhinum majus*
P. persica GF 305 seedling	*Chenopodium album*
P. myrobalan hybrid GF 31	*C. amaranticolor*
P.avium cv. Bing	*C. botrys*
P. domestica cv. Italian prune	*C. foetidum*
P.tomentosa IR 43/1	*C. murale*
P.serrulata cv. Shirofugen	*C. quinoa*
	Cucumis sativus
	Cucurbita maxima
	C. pepo
	Gomphrena globosa
	Nicotiana benthamiana
	N. glutinosa
	N. tabacum cv. Samsun
	Petunia hybrida
	Tetragonia expansa
	Tithonia speciosa
	Vigna sinensis
	Zinnia elegans
PPV studies:	
P. persica GF 305 seedlings	*Chenopodium foetidum*
P. myrobalan hybrid GF 31	*C. quinoa*
P.tomentosa IR 43/1	*Nicandra physaloides*
	N. acuminata
	N. benthamiana
	N. bigelowii
	N. clevelandii
	N. debney
	N. quadrivalvis
	Pisum sativum
	P. arvense
	Verbenal officinalis

Among the 19 plant species used in indexing on herbaceous indicators, the virus provoked symptoms on *Chenopodium album, C. amaranthicolor, C. quinoa, C. botrys, Ammi majus, Antirrhinum majus, Cucumis sativus, Cucurbita maxima, C. pepo, Petunia hybrida, Vigna sinensis* and *Zinnia elegans.* There were only slight differences among the particular isolates in the severity of symptoms, the differentiating host plants characteristic of the certain strains were not found. Among the seven *C. quinoa* clones obtained from different countries, the one with large leaves obtained from France proved to be the most sensitive. Results of indexing on woody indicators were compiled in Table 7 and symptoms of mechanical inoculations on herbaceous in Table 8. These results were the basis for selection of isolates for typing PNRSV isolates described below.

Table 7. Symptoms of Hungarian PNRSV isolates on woody indicator plants* in field indexing

Isolates	*Prunus avium* seedlings**	*P. persica* seedling GF 305	*P. armeniaca* seedlings	*P. myrobalan* hybrid GF 31**	*P. mahaleb* Seedlings**	*P. avium cv.* Bing**	*P. domestica cv.* Italian prune	*P. tomentosa* IR 473/1	*P. tomentosa* IR 474/1	*P. serrulata* cv. Shirofugen
S 28	necrotic & chlorotic pattern	reduced growth, shoot necrosis (rare)	reduced growth	chlorotic pattern	chlorotic, necrotic spots & shot holes	enation on lower surface	—	necrotic & chlorotic patterns on leaves, reduced growth	necrotic & chlorotic patterns on leaves, reduced growth	gum flow & necrosis
S 63	necrotic & chlorotic pattern	reduced growth, shoot necrosis (rare)	reduced growth	chlorotic pattern	chlorotic, necrotic spots & shot holes	enation on lower surface	—	necrotic & chlorotic patterns on leaves, reduced growth	necrotic & chlorotic patterns on leaves, reduced growth	gum flow & necrosis
S 66	necrotic & chlorotic pattern	reduced growth, shoot necrosis (rare)	reduced growth	chlorotic pattern	chlorotic, necrotic spots & shot holes	enation on lower surface	—	necrotic & chlorotic patterns on leaves, reduced growth	necrotic & chlorotic patterns on leaves, reduced growth	gum flow & necrosis
S 67	necrotic & chlorotic pattern	reduced growth, shoot necrosis (rare)	reduced growth	chlorotic pattern	chlorotic, necrotic spots & shot holes	enation on lower surface	—	necrotic & chlorotic patterns on leaves, reduced growth	necrotic & chlorotic patterns on leaves, reduced growth	gum flow & necrosis
S 00/1	necrotic & chlorotic pattern	reduced growth, shoot necrosis (rare)	reduced growth	chlorotic pattern	chlorotic, necrotic spots & shot holes	—	—	necrotic & chlorotic patterns on leaves, reduced growth	necrotic & chlorotic patterns on leaves, reduced growth	gum flow & necrosis
S 133	necrotic & chlorotic pattern	reduced growth, shoot necrosis (rare)	reduced growth	chlorotic pattern	chlorotic, necrotic spots & shot holes	enation on lower surface	necrotic patterns on leaf & epinasty	necrotic & chlorotic patterns on leaves, reduced growth	necrotic & chlorotic patterns on leaves, reduced growth	gum flow & necrosis
S 138	necrotic & chlorotic pattern	**severe** shoot necrosis & reduced growth	reduced growth	chlorotic pattern	chlorotic, necrotic spots & shot holes	enation on lower surface	—	necrotic & chlorotic patterns on leaves, reduced growth	necrotic & chlorotic patterns on leaves, reduced growth	gum flow & necrosis insertion
S 42/1	necrotic & chlorotic pattern	reduced growth, shoot necrosis (rare)	reduced growth	chlorotic pattern	chlorotic, necrotic spots & shot holes	—	—	necrotic & chlorotic patterns on leaves, reduced growth	necrotic & chlorotic patterns on leaves, reduced growth	gum flow & necrosis
S 152	necrotic & chlorotic pattern	reduced growth, shoot necrosis (rare)	reduced growth	chlorotic pattern on leaves	chlorotic, necrotic spots & shot holes	—	—	necrotic & chlorotic patterns on leaves, reduced growth	necrotic & chlorotic patterns on leaves, reduced growth	gum flow & necrosis

Note: – = symptomless, * = 5 plants / indicator species, ** symptoms on leaves

Table 8. Symptoms of Hungarian PNRSV isolates on herbaceous indicator plants

	Herbaceous indicators			
Isolates	*Cucumis sativus*	*Chenopodium quinoa*	*C. foetidum*	*Cucurbita pepo*
RS 28	diffuse or ringlike chlorotic spots on cotyledons, tip.necrosis	grey, necrotic spots, chlorotic rings, deformation	chlorotic rings, necrotic spots	grey necrotic rings on cotyledons
RS 63	diffuse or ringlike chlorotic spots on cotyledons, tip necrosis	grey, necrotic spots, chlorotic rings, deformation	chlorotic rings, necrotic spots	grey necrotic rings on cotyledons
RS 66	diffuse or ringlike chlorotic spots on cotyledons, tip necrosis	grey, necrotic spots, chlorotic rings, deformation	chlorotic rings, necrotic spots	grey necrotic rings on cotyledons
RS 67	diffuse or ringlike chlorotic spots on cotyledons, tip necrosis	grey, necrotic spots, chlorotic rings, deformation	chlorotic rings, necrotic spots	grey necrotic rings on cotyledons
RS 100/1	diffuse or ringlike chlorotic spots on cotyledons, tip necrosis (rare)	grey, necrotic spots, chlorotic rings, deformation	chlorotic rings, necrotic spots	grey necrotic rings on cotyledons
RS 133	diffuse or ringlike chlorotic spots on cotyledons, tip necrosis	grey, necrotic spots, chlorotic rings, deformation	chlorotic rings, necrotic spots	grey necrotic rings on cotyledons
RS 138	diffuse or ringlike chlorotic spots on cotyledon tip necrosis (not in every case)	grey, necrotic spots, chlorotic rings, deformation	chlorotic rings, necrotic spots	grey necrotic rings on cotyledons
RS 142/1	diffuse or ringlike chlorotic spots on cotyledons, tip necrosis (rare)	grey, necrotic spots, chlorotic rings, deformation	chlorotic rings, necrotic spots	grey necrotic rings on cotyledons
RS 152	diffuse or ringlike chlorotic spots on cotyledons, tip necrosis (rare)	grey, necrotic spots, chlorotic rings, deformation	chlorotic rings, necrotic spots	grey necrotic rings on cotyledons

No symptoms were observed with *C. murale; Gomphrena globosa; Nicotiana glutinosa; N. benthamiana; N. tabacum cv. Samsun; Tetragonia expansa; Tithonia speciosa.*

PPV: In the inoculation experiments altogether 26 PPV isolates, (maintained on GF 305 indicators) were involved (Table 9). Transmissions were made on 3 woody and 11 herbaceous species (Table 6). Results were compiled and presented in Table 8 (due to its length, enclosed in Appendices). The comprehensive bio-characterization of these PPV isolates will allow further molecular approaches (not in the frame of this project).

Table 9. PPV isolates involved in biological characterization experiments in Hungary

Isolate	Strain	Source of species	Location of isolation	Year of isolation
Studied in woody and herbaceous indicators (1995-1997)				
SK 6	M	apricot	Budateteny	1966
SK 16	M	*P. glandulosa*	Vacratot	1968
SK 40	M	peach	Balatonujhely	1982
SK 91	M	apricot	Cegled	1982
SK 49	M	green gage	Budapest	1974
SK 58	M	peach	Sasad	1976
SK 60	M	plum	Poloske	1986
SK 63	D/M	*myrobalan*	Cegled	1978
SK 70	M	plum	Cegled	1982
SK 208	M	apricot	Bekescsaba	1991
SK 209	D/M	peach	Bekescsaba	1991
SK 251	D	plum	Adand	1996
SK 252	D	plum	Adand	1996
PPV Jenzig	D	plum	Germany	?
PPV 40-2	M	peach	Bulgaria	?
PPV 6/283	D	?	Bulgaria	?
PPV M	M	?	Italy (Greece)	?
PPV D	D	?	Italy (France)	?
PPV EA		apricot	Italy (Egypt)	?
In 1998-1999:				
SK 6	M	apricot	Budateteny	1966
SK 76	M	plum	Erd	1974
SK 199	M	peach	Kecskemet	1991
SK 207	M	almond	Rakocifalva	1991
SK 243	D	peach	Siofok	1995
SK 246	D	plum	Debrecen	1995
SK 247	D	apricot	Debrecen	1995
SK 249	D	almond	Ortilos	1995

During the studies conducted with woody indicators it was concluded that there were no differences between the PPV M and D strains in the appearance and severity of the symptoms induced on the particular indicators. The M and D strains caused the below symptoms on *C. foetidum, Nicotiana clevelandii* and *N. benthamiana* among the used herbaceous species:

PPV strain	**Chenopodium foetidum**	**Nicotiana clevelandii**	***N. benthamiana***
M	ochre lesions	chlorotic lesions	Chlorotic lesions
D	ochre lesions with necrotic centre	chlorotic lesions	Chlorotic lesions

5.4 Typing PNRSV isolates by serological and molecular assays

Based on the bio-characterization data obtained for PNRSV (Tables 7,8) mainly in Hungary, isolates were selected for differentiation studies. Starting in the second year and till the end of the project, PNRSV differentiation was approached by serological and later by several molecular methods. Results were summarized in the annual reports (mainly 3rd and 4th annual reports), presented in meetings and published. Differentiation was studied by:

- Serotyping and restriction endonuclease analysis (Spiegel et al. 1999).
- Differential electrophoretic mobility of single-strand RNA transcripts copied from amplified virus-specific PCR products (transcript conformation polymorphism, TCP) (Rosner et al. 1998).
- Double-stranded conformation polymorphism of heterologous RNA transcripts (dsTCP) (Rosner et al. 1999, 1999a).

Restriction endonuclease analysis was applied to PCR products. TCP and dsTCP required a transcription step of the PCR products, This was done by attaching a T7 RNA polymerase promoter to one of the primers. The principles of single-stranded (transcript) and double-stranded conformation polymorphism are described schematically in Appendices.

It is interesting to note that the differentiation approaches used by us did not distinguish between the severe PNRSV isolate (RS 138), as determined by bioassays, and the other isolates. Isolate RS 152, one of the mild isolates, could be differentiated from the other studied isolates by serotyping, RFLP and by conformation polymorphism assays.

Serotyping and molecular characterization studies of PNRSV isolates results in new data added to the current body of knowledge on virus strain differentiation and may lead to better understanding of the disease, caused by this virus.

6) Impact Relevance and Technology Transfer

The establishment and putting into operation of the PCR lab in PHSCS, Budapest, with partial funding from this project, facilitated the addition of a new, much needed, molecular biology dimension to this diagnostic laboratory. The development of new PCR assays (one-tube, ELISA-PCR and multiplex RT-PCR) for detection of PNRSV and PPV provide highly sensitive diagnostic assays, now being performed in PHSCS and facilitates molecular studying of virus isolates.

The availability of a recently constructed, controlled greenhouse (from other funds) in Valence (about 50 km from Budapest) allows short-term indexing on woody indicators. Obtaining biological data within a short period of time contributes to shortening the testing process. It is anticipated that the sensitive methods developed in this project will be adopted for detection and characterization also for other viruses.

The addition of these capacities upgraded significantly the research of stone fruit viruses in Hungary. Sensitive, rapid detection assays and virus strain differentiation using molecular methods are now operating in the PHSCS lab. The possibility of introducing these methods in post-quarantine tests and in certification schemes for production of virus free plant materials is being discussed in various International bodies.

The transfer of the PCR technology from the Israeli group to the PHSCS lab was achieved by training an Hungarian scientist in the Volcani lab, sharing protocols, sequence information etc. and discussions by e-mail and meetings.

The individuals involved in this project included scientists from three generations.

- Dr. Maria Nemeth, a retiree from PHSCS, one of the founders of fruit tree virology internationally and publisher of a fundamental book in this area acted as a consultant in this project and led the biological work in the field and greenhouse. Dr. Nemeth shared her vast knowledge in symptomatology, virus isolates, virus transmission to herbaceous and woody hosts (bioassays and indexing) as well as her information on fruit tree growing with the Hungarian and Israeli scientists.
- Dr. Maria Kolber, head of the PHSCS lab, supervised the work on the Hungarian side and led the development of large scale NASH method and the ELISA tests. Dr. Gabor Tokes participated in ELISA and NASH experiments. Mr. Laszlo Krizbai, a Ph.D candidate, trained in Israel in the PCR technology was responsible for performing the PCR assays and developed the ELISA – PCR for PPV and PNRSV in Budapest. Mr. Krizbai trained Hungarian colleagues in PCR technology.
- Dr. Arie Rosner led the development and application of various PCR techniques and the development and characterization of PNRSV isolates by molecular methods.
- Dr. Sara Spiegel (P.I) coordinated the project, led the biological aspects of this project (identification and selection of infected trees, bio-characterization of isolates) and the serological (DAS-ELISA and MABs based assays) differentiation. Dr. Spiegel was in charge of annual reports and the final report.

7) Project Activities / Outputs

Meetings attended and held during the project

Hungarian and Israeli scientists cooperating in this project met at least once a year using funds from this project and other sources. The visits included - reviewing results, sharing and experiencing lab protocols, discussions and planning future work Field trips to commercial and experimental orchards, indexing plots, nurseries and

1995 - M. Kolber visited the VC (funds from other sources) on her way to the Far East. M. Kolber and S. Spiegel met during the Middle European Meeting on Plum Pox in Austria (funds from other sources).

1996 - S. Spiegel visited PHSCS (April)

1997 - M. Kolber, S. Spiegel and A. Rosner met (June) during the 17th International Symposium on Virus and Virus-Like Diseases of Temperate Fruit Crops in Bethesda MD, USA

1998 - S Spiegel and A. Rosner visited the PHSCS lab (July), using funds from this project (A. Rosner) and other funds (S. Spiegel). S. Spiegel and M. Kolber traveled together to attend the Middle European Meeting on Plum Pox in Slovakia.

1999 - S. Spiegel visited PHSCS (June)

2000 - S. Spiegel visited PHSCS (May) for final discussions of research results and preparing jointly the outline of the final report.

Training

Mr. Laszlo Krizbai spent six months (Nov. 96-May 97) in the Israeli lab with Drs. Rosner and Spiegel training mostly in PCR technology and related methods. During the project Mr. Krizbai spent several short periods of training in Hungary (Ray Zoltan Biotechnology Institute, Szeged and Biotechnology Institute, Godollo).

List of Publications:

1. Spiegel, S., Scott, S.W., Bowman-Vance, V., Tam, Y., Galiakparov, N.N., Rosner, A. 1996. Improved detection of prunus necrotic ringspot virus by the polymerase chain reaction. Eur. J. Plant Pathology 102:681-685.
2. Kolber, M., Nemeth, M., Krizbai, L., Szemes, M., Kiss-Toth, E., Dorgai, L. and Kalman, M. 1997: Detection of mixed infections caused by *Prunus* necrotic ringspot and plum pox viruses using multiplex RT-PCR method. Novenyvedelmi Tudomanyos Napok ' 97. Budapest, Abstract of Papers are in Hungarian and therefore not attached.
3. Kolber, M, Nemeth, M., Krizbai, L., Kiss-Toth, E., Kalman, M. 1997. Detection of plum pox virus by different methods. In: Diagnosis and Identification of Plant Pathogens. Proceedings of the 4th International Symposium of the European Foundation for Plant Pathology 1996, Germany, Kluwer Academic Publishers p.317-319.
4. Krizbai, L., Kiss-Toth, E., Kolber, M., Nemeth, M., Dorgai, L., Kalman, M., Spiegel, S., Rosner, A., Levy, L. 1997: A one-tube multiplex RT-PCR for detection of mixed infections caused by *Prunus* necrotic ringspot and plum pox viruses using polymerase chain reaction. EU-Cost 823 New Technologies to Improve Phytodiagnostics. pp. 49-55.
5. Rosner, A., Maslanin, L., Spiegel, S. 1997. The use of short and long PCR products for improved detection of prunus necrotic ringspot virus in woody plants. J. Virol. Methods 67:135-141.
6. Krizbai, L., Kolber, M., Nemeth, M., Rosner, A., Spiegel, S. 1998. Detection of prunus necrotic ringspot virus by immunecapture PC (IC-PCR) assay (in Hungarian). Scientific Plant Protection Days'98, Budapest, Abstracts of Papers p. 114.
7. Rosner, A., Maslenin, L., Spiegel, S. 1998. Differentiation among isolates of prunus necrotic ringspot virus by transcript conformation polymorphism. J. Virol. Methods 74:109-115.
8. Rosner, A., Shiboleth, Y., Spiegel, S., Krizbai, L., Kolber, M, 1998. Evaluating the use of immunocapture and sap-dilution PCR for the detection of prunus necrotic ringspot virus. Acta Hortic. 472:227-233.
9. Spiegel, S., Tam, Y., Maslenin, L., Kolber, M., Nemeth, M., Rosner, A. 1999. Typing prunus necrotic ringspot virus by serology and restriction endonuclease analysis of PCR products. Ann. Appl. Biol. 135:395-400.
10. Rosner, A., Maslenin, L., Spiegel, S. 1999a. Conformation polymorphism of RNA transcripts derived from various PNRSV isolates is affected by the temperature of electrophoresis. Eur. J. Plant Pathology 105:413-415
11. Rosner, A., Maslenin, L., Spiegel, S. 1999. Double-stranded conformation polymorphism of heterologous RNA transcripts and its use for virus strain differentiation. Plant Pathology 48:235-239.
12. Ulubas, C., Rosner, A., Spiegel, S. 2000 Sequence comparison of full-length RNA 3 clones of two distinct prunus necrotic ringspot virus (PNRSV) isolates. (Abstract). 18th International Symposium on Virus & Virus-like Diseases of Temperate Fruit Crops, England.

8) Project Productivity

Both the Hungarian and Israeli scientists involved in this project concluded that the project was very successful and contributed to improving fruit tree growing in the two countries and to plant virus detection and differentiation. Several decisions were made during the 4 years, regarding the planned research work, mainly due to developments and availability of new information and technologies relevant to this project's objectives. In retrospect, the project benefited from these decisions.

9) Future Work

Study of Israeli stone fruit cultivars for susceptibility to PPV (Hungary)
Although PPV does not occur in Israel, its presence in neighboring countries (Egypt, Syria, Lebanon and recently Jordan) and other Mediterranean countries (Turkey, Greece, Italy, Spain etc.) pose a constant threat to the Israeli stone fruit growing industry.
The interaction between the Hungarian and Israeli scientists in this CDR project led to an initiative in which the main Israeli stone fruit cultivars are evaluated in Hungary for their susceptibility to PPV, the most devastating among the fruit trees viruses. The experiment was started in Hungary with budwood from selected stone fruit cultivars imported from Israel and local PPV isolates. Funding for this experiment did not come out of this project.

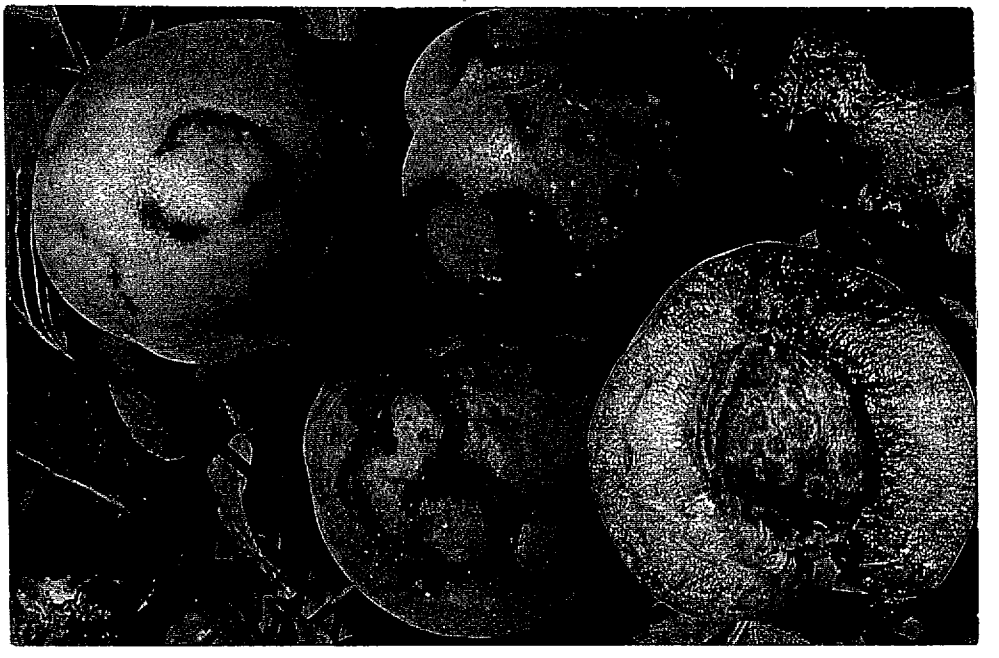
APPENDICES

- Symptoms caused by PPV on plum fruit – photographs
- Fruit symptoms caused by PPV on apricot - photographs
- Fruit symptoms caused by PPV on peach – photographs
- Symptoms caused by PPV on leaves of indicator GF305 - photograph
- Symptoms caused by PNRSV on leaves of *Prunus avium* seedlings - photograph
- The scheme of PCR-ELISA
- Detection of PPV and PNRSV in a Multiplex RT-PCR assay
- Table 10: Biological characterization of Hungarian and foreign PPV isolates on woody and herbaceous indicator species
- Transcription of PCR products
- Formation of double-stranded duplexes of RNA transcripts
- Single-strand transcript conformation polymorphism (ss-TCP)
- Double-strand transcript conformation polymorphism (ds-TCP)
- Visits and discussions of Hungarian and Israeli scientists – photographs

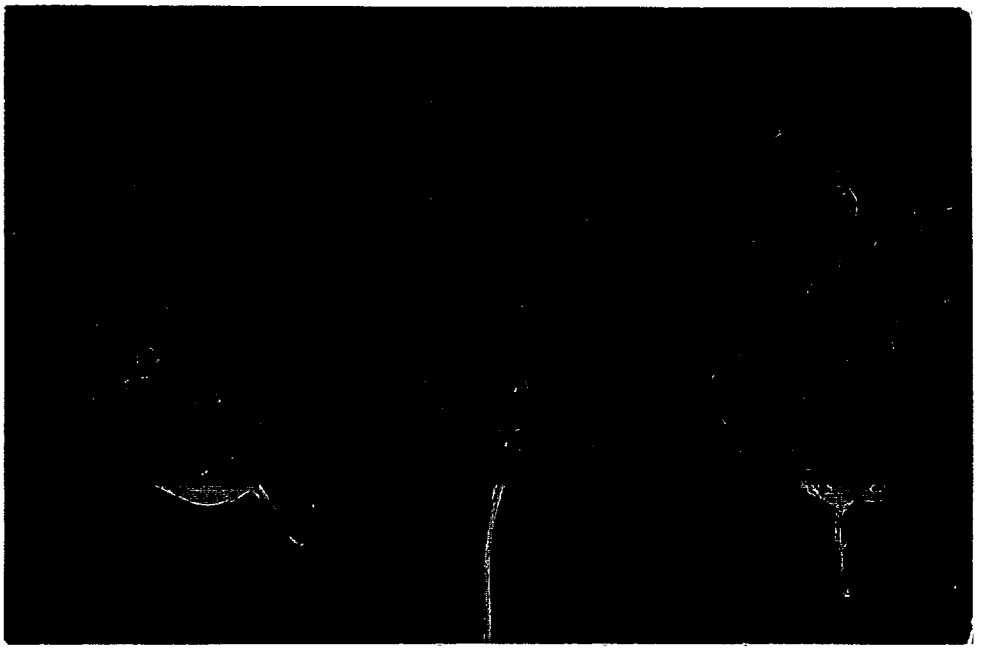
Symptoms caused by plum pox virus on plum

Severe fruit drop caused by plum pox virus at suspectible cultivar Besztercei

Fruit symptoms caused by plum pox virus on apricot



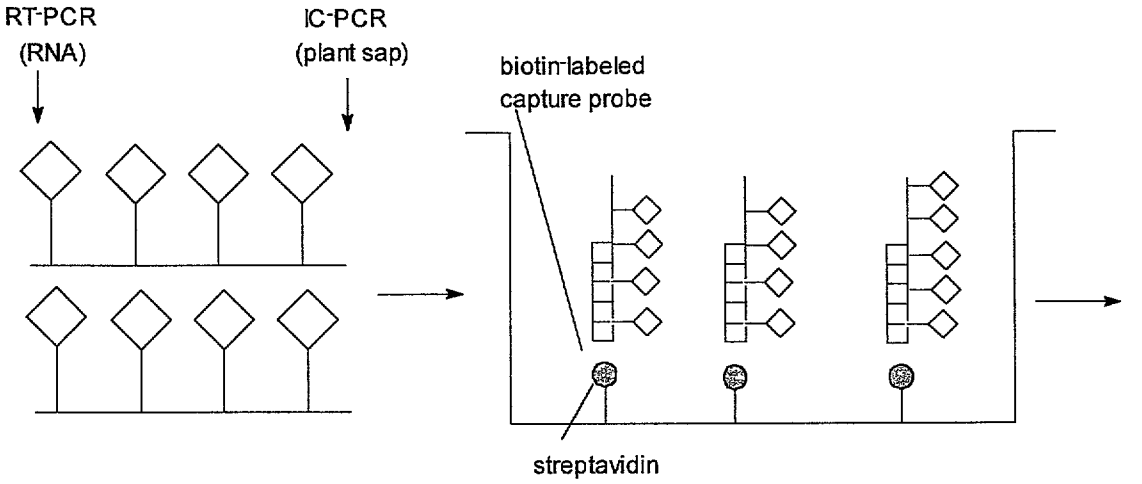
Fruit symptoms caused by plum pox virus on peach



Symptoms caused by PNRSV on leaves of *Prunus avium* seedling

Symptoms caused by PPV on leaves of indicator GF 305

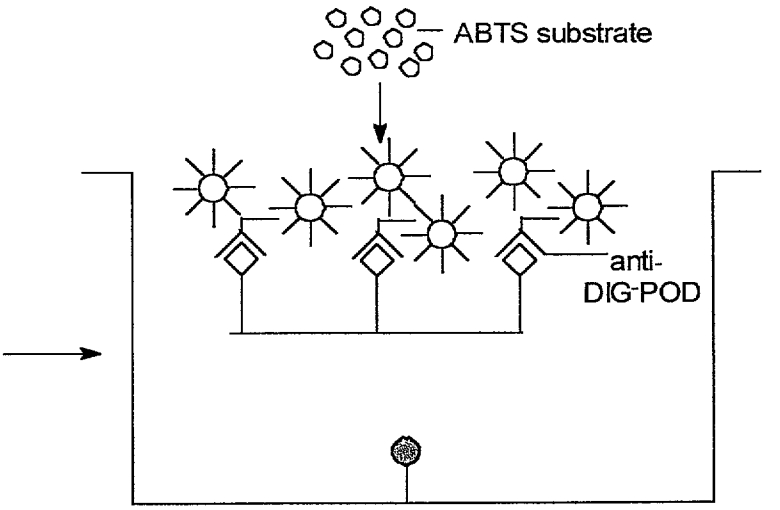
The scheme of PCR-ELISA



Incorporation of DIG-d UTP
During PCR using virus
specific primers

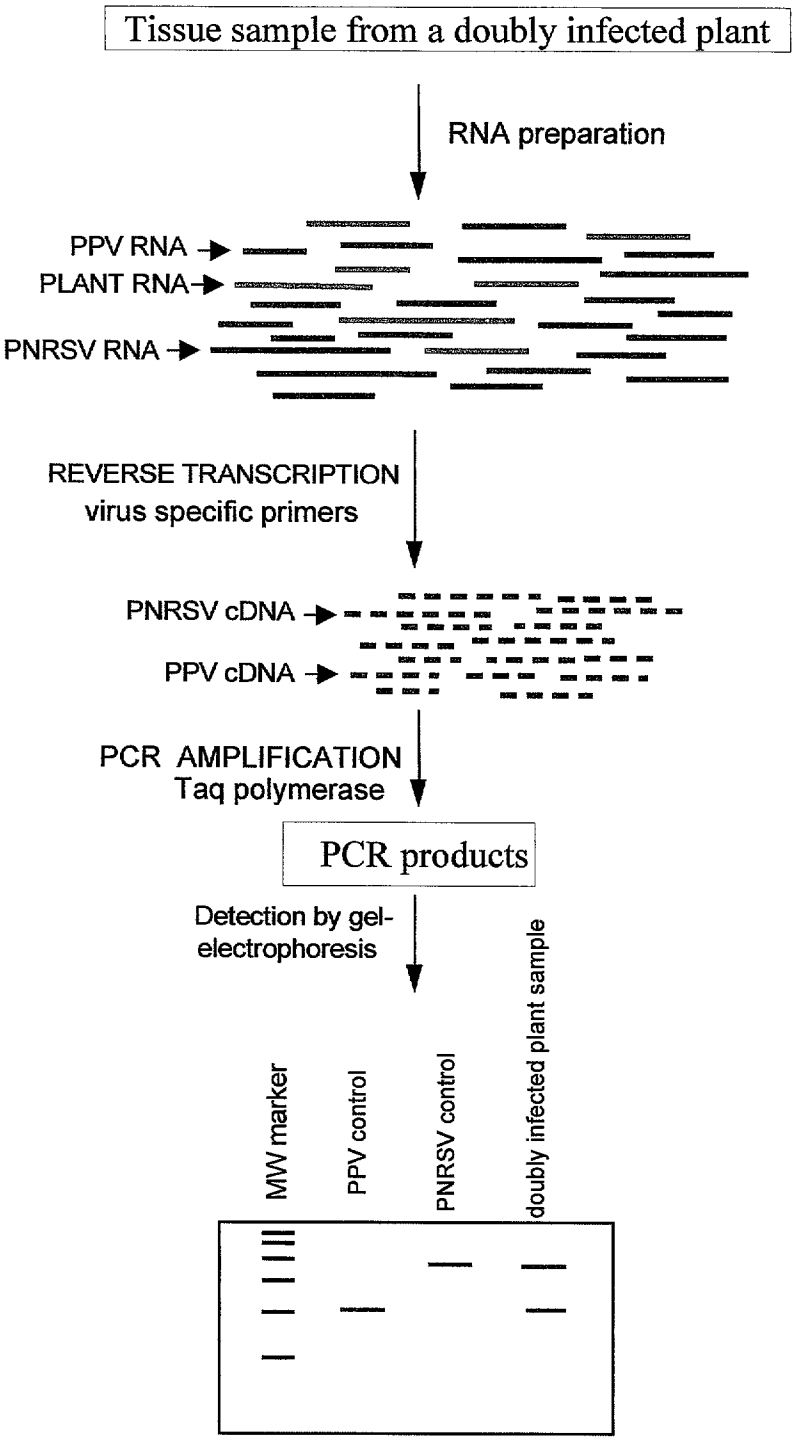
Capturing the denatured DIG-PCR products
with immobilized biotin-labeled capture probe
onto streptavidin-coated ELISA plate

The capture probe is specific to target PCR products

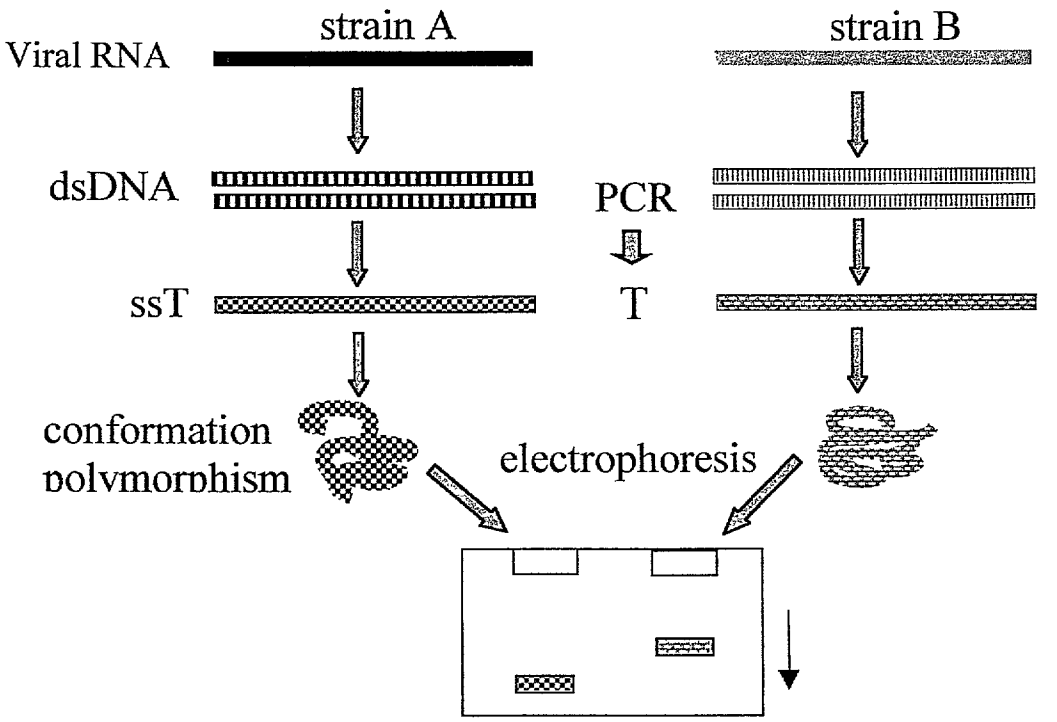


Colorimetric detection of DIG-PCR products
with anti-DIG-POD conjugate and ABTS substrate

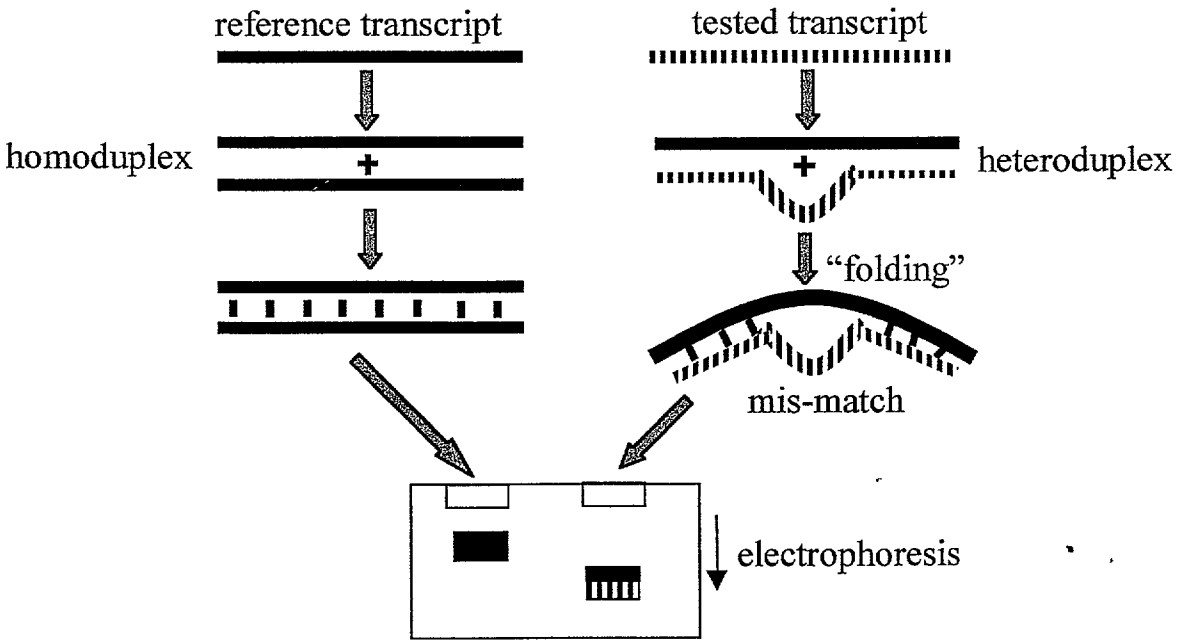
DETECTION OF PPV AND PNRSV IN A MULTIPLEX RT-PCR ASSAY



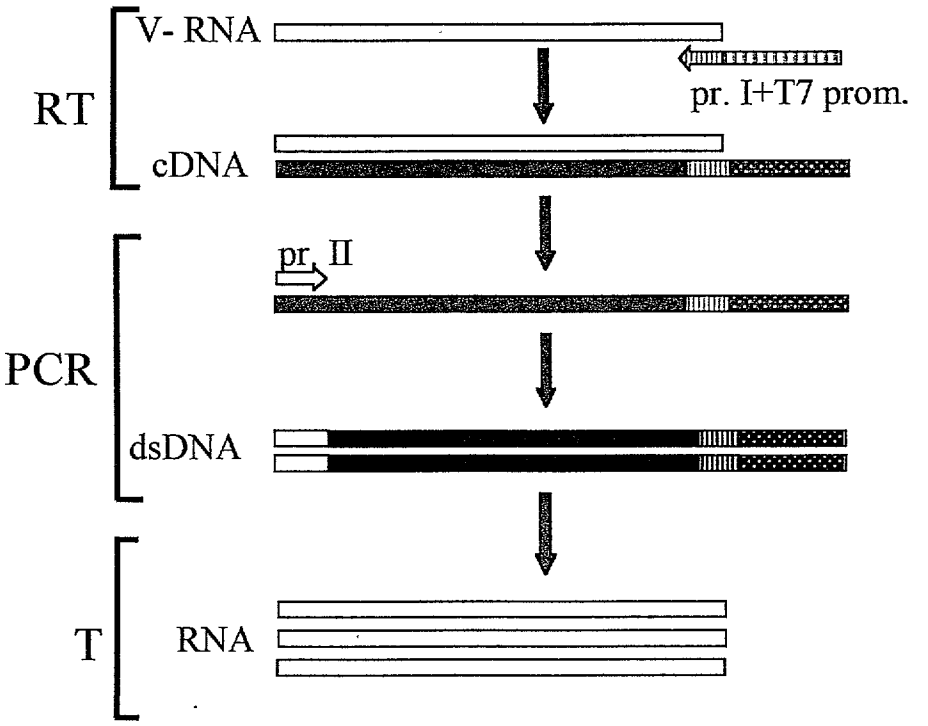
Single-strand transcript conformation polymorphism (ss-TCP)



Double-strand transcript conformation polymorphism (ds-TCP)



Transcription of PCR products



RT-reverse transcription; pr.–primer; prom.-T7 RNA promoter; T - transcription.

Formation of double-stranded duplexes of RNA transcripts

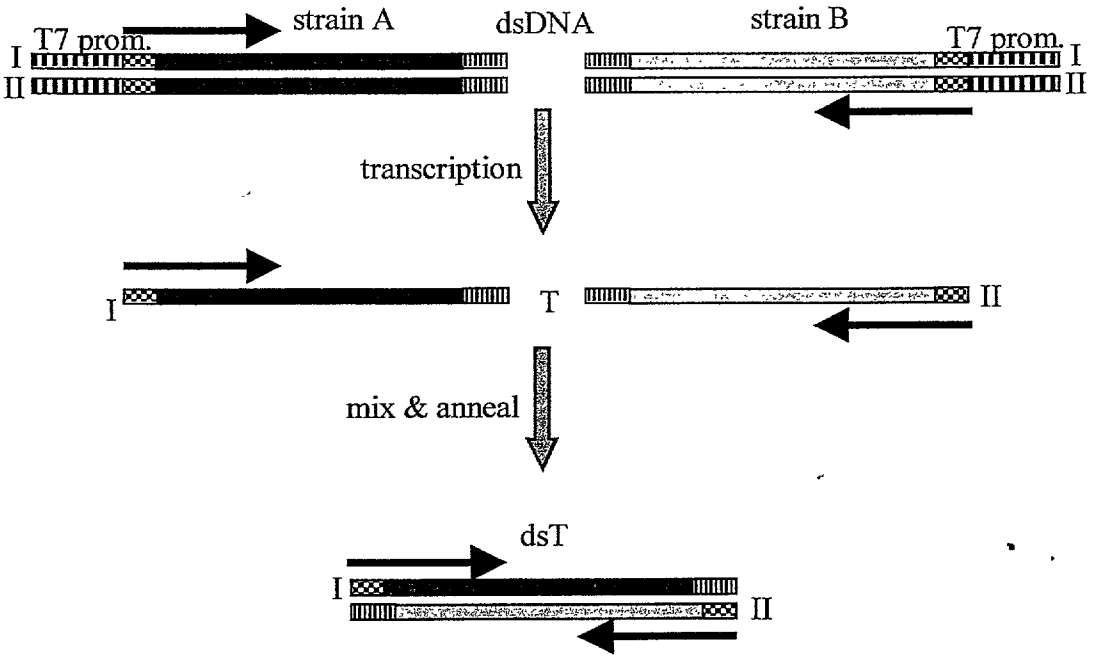


Table 10: Biological characterization of Hungarian and foreign PPV isolates on woody and herbaceous indicator species

ode of isolate, ear of isolation	Woody indicator species			Herbaceous indicator species					
etween 1995-97	GF 305	GF 31	*P. tomentosa*	*C. foetidum*	*C. amaranticolor*	*C. murale*	*C. quinoa*	*N. clevelandi*	*N. benthamiana*
K 91 apricot ungary, 1982	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions on inoculated leaves	0	0	0	chlorotic spots, rings	0
K 6 apricot, ungary, 1966	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions on inoculated leaves	0	0	0	chlorotic spots, rings	—
K 40 peach ungary, 1973	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions on inoculated leaves	0	0	0	chlorotic spots, rings	—
K 49 green ge, ungary, 1974	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions on inoculated leaves	0	0	0	chlorotic spots, rings	0
K 58 peach, ungary, 1976	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions on inoculated leaves	0	0	0	chlorotic spots, rings	—
K 60 plum, ungary, 1986	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions on inoculated leaves	0	0	0	chlorotic spots, rings	—
K 63 *P. yrobalan*, ungary, 1978	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions with necrosis on inoculated leaves	0	0	0	chlorotic spots, rings	—
K 70 plum, ungary, 1982	chlorotic spots, vein chlorose,	leaves: chlorotic spots, one year	leaves: chlorotic-necrotic	yellow lesions on inoculated	0	0	0	chlorotic spots, rings	—

	leaf distortion	shoots: bark necrosis	rings, bands	leaves					
: 208 apricot, ıngary, 1991	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions with necrosis on inoculated leaves	0	0	0	chlorotic spots, rings	chlorotic spots
; 209 peach ıngary, 1991	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions with necrosis on inoculated leaves	0	0	0	chlorotic spots, rings	chlorotic spots
' 18 *P. andulosa,* ıngary, 1970	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions on inoculated leaves	0	0	0	chlorotic spots, rings	—

ıde of isolate, ear of isolation	**woody indicator species**			**Herbaceous indicator species**					
	GF 305	**GF 31**	***P. tomentosa***	***C. foetidum***	***C. amaranticolor***	***C. murale***	***C. quinoa***	***N. clevelandi***	***N. benthamian***
; 251 Bluefre Hungary, 96	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions with necrosis on inoculated leaves	small chlorotic lesions	inoculated leaves: small chlorotic spots, upper leaves: chlorotic spots, deformation	0	chlorotic spots, rings	chlorotic spot:
; 252 than green ge 38 ıngary, 1996	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions with necrosis on inoculated leaves	small chlorotic lesions	inoculated leaves: small chlorotic spots, upper leaves: chlorotic spots, deformation	—	chlorotic spots, rings	chlorotic spot:
PV Jenzig, ermany	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions with necrosis on inoculated leaves	necrotic lesions	0	necrotic lesions	chlorotic spots, rings	chlorotic spot:
PV 40-2 cv yette peach, ılgaria	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark	eaves: chlorotic-ne bands nekrotikus mintáza	yellow lesions on inoculated leaves	0	—	—	Chlorotic spots, rings	chlorotic spot:

		necrosis							
V 6/283 mania	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions with necrosis on inoculated leaves	0	—	—	Chlorotic spots, rings	chlorotic spot:
V M Italy	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions on inoculated leaves	0	—	—	Chlorotic spots, rings	—
V D Italy	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions with necrosis on inoculated leaves	small chlorotic lesions	inoculated leaves: small chlorotic spots, upper leaves: chlorotic spots, deformation		Chlorotic spots, rings	chlorotic spot:
V El-Amar y	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions with necrosis on inoculated leaves	0	—	—	Chlorotic spots, rings	chlorotic spot:
ween 1998-99									
6 ngary, 1966	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions with necrosis on inoculated leaves	small chlorotic lesions	inoculated leaves: small chlorotic spots, upper leaves: chlorotic spots, deformation	—	chlorotic spots, rings	chlorotic spot:
76 ngary, 1974	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necrotic rings, bands	yellow lesions with necrosis on inoculated leaves	necrotic lesions	0	necrotic lesions	chlorotic spots, rings	chlorotic spot:
de of isolate, ar of isolation	**Woody indicator species**			**Herbaceous indicator species**					
	GF 305	**GF 31**	***P. tomentosa***	***C. foetidum***	***C. amaranticolor***	***C. murale***	***C. quinoa***	***N. clevelandi***	***N. benthamian***
199 ngary, 1974	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark	eaves: chlorotic rings, bands nekrotikus mint	yellow lesions on inoculated leaves	0	—	—	Chlorotic spots, rings	chlorotic spo

		necrosis							
‹ 207 ıngary, 1991	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necro tic rings, bands	yellow lesions with necrosis on inoculated leaves	0	—	—	Chlorotic spots, rings	chlorotic spo
‹ 243 ıngary, 1991	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necr otic rings, bands	yellow lesions with necrosis on inoculated leaves	small chlorotic lesions	inoculated leaves: small chlorotic spots, upper leaves: chlorotic spots, deformation	—	chlorotic spots, rings	chlorotic spo
‹ 246 ıngary, 1955	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necr otic rings, bands	yellow lesions with necrosis on inoculated leaves	small chlorotic lesions	inoculated leaves: small chlorotic spots, upper leaves: chlorotic spots, deformation		Chlorotic spots, rings	chlorotic spo
‹ 247 ıngary, 1955	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necr otic rings, bands	yellow lesions with necrosis on inoculated leaves	small chlorotic lesions	inoculated leaves: small chlorotic spots, upper leaves: chlorotic spots, deformation		Chlorotic spots, rings	chlorotic spo
‹ 249 ıngary, 1955	chlorotic spots, vein chlorose, leaf distortion	leaves: chlorotic spots, one year shoots: bark necrosis	leaves: chlorotic-necr otic rings, bands	yellow lesions with necrosis on inoculated leaves	small chlorotic lesions	inoculated leaves: small chlorotic spots, upper leaves: chlorotic spots, deformation		Chlorotic spots, rings	chlorotic spo

Visit of open field indexing in Valence, Hungary (June 1998). Symptoms on young GF305 plants. From left to right: Sara Spiegel, Arie Rosner, Maria Nemeth, Rozalia Hangyal

Group discussion in the PHSC Budapest lab (June 1998). From left to right: Sara Spiegel, Maria Kolber, Lazlo Krizbai, Arie Rosner.