

SINTEZA TESTĂRILOR ÎN CÂMP A PRUNULUI TRANSGENIC ÎN ROMÂNIA OVERVIEW ON THE FIELD TRIALS OF TRANSGENIC PLUMS IN ROMANIA

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Abstract

Transgenic clones C2, C3, C4, C5, C6, PT3 and PT5 of *Prunus domestica* L. transformed with the Plum pox virus coat protein gene (PPV-CP) were evaluated for Sharka resistance under high infection pressure in field natural conditions in Romania. Transgenic clone C5, subsequently named "HoneySweet", showed high resistance to PPV. None of the C5 trees became naturally infected by aphids for more than ten years. Known to develop the post-transcriptional gene silencing (PTGS) mechanism, we assessed the effect of heterologous viruses on the efficacy and stability of PTGS displayed by the C5 plum against PPV. In this way, C5 trees were graft-inoculated with different combinations of *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV) and PPV-D strain. The engineered resistance to PPV in C5 transgenic plums was stable and was not suppressed by the presence of the assayed heterologous viruses over a three-year experimental period. Because the constitutive transcription of PPV-CP sequences naturally occurs in transgenic C5 plums, the environmental safety issues have been expressed on potential hazards concerning the emergence of PPV variants. In order to analyze this potential environmental effect was compared the serological and molecular variability of PPV detected in the transgenic trees versus those found in conventional plums. This risk assessment revealed a high similarity between PPV isolates from transgenic and conventional plums, and hence the transgenic plums utilized in this study do not affect the diversity of indigenous PPV populations.

Cuvinte cheie: virusul Plum pox, prun transgenic, gena CP, rezistenta, probleme de siguranta

Keywords: Plum pox virus, transgenic plum, CP gene, resistance, safety issue

1. Introduction

Plum pox virus (PPV) is the causal agent of the most devastating Sharka diseases of *Prunus* species, leading to important economic losses (Cambra *et al.*, 2006). Since its first description in Bulgaria (Atanasoff, 1932), the virus has spread to a large part of the European continent, around the Mediterranean basin and near Middle East, South and North America (Argentina, Canada, Chile, and USA) and Asia (Kazakhstan, China and Pakistan) (Capote *et al.*, 2006). Nevertheless, there are control measures against PPV based on two strategies: the reduction or elimination of the viral inoculum by quarantine and eradication programs. While measures such as quarantine and eradication of infected trees have appeared to be inefficient to stop the continuous spread of PPV, many countries are endemically affected. Due to the rapid spread of PPV by aphids and the presence of many potential hosts, Sharka disease is difficult to eradicate once it has become established in an area. Therefore, the use of resistant cultivars represents the alternate solution to control PPV infection.

Conventional breeding have exploited naturally occurring resistance. Unfortunately, the paucity of natural resistance genes has hampered the efforts to control Sharka disease. However, a multigenic hypersensitive reaction has been reported in *Prunus domestica* and a resistant hypersensitive cultivar 'Jojo' has been released (Hartman and Petruschke, 2002). The absolute resistance of this cultivar was oppugned by Polak *et al.* (2005) which showed that PPV can be present in the tissue of 'Jojo' cv. because the virus was transferred to the rootstock. The utilization of natural sources of resistance is important for the development of new varieties but it is difficult and long-term to incorporate such resistance into new stone fruits varieties through conventional breeding.

These offer the opportunity to utilize genetic engineering techniques to develop resistant plums by introducing a virus gene segment into the DNA of *Prunus* host plants. Sanford and Johnson (1985) reported the concept of Pathogen Derived Resistance (PDR) that could provide an useful alternative or complementary approach to conventional breeding for obtaining virus-resistant plants. Following this strategy, transgenic European plums (*Prunus domestica* L.) containing the CP gene of PPV were developed as an approach to obtain PPV resistant plums (Scorza *et al.*, 1994). One transgenic line, C5, subsequently named 'HoneySweet' (Scorza *et al.*, 2007) was found to be highly resistant to graft- and

aphid-mediated inoculation by PPV in greenhouse (Ravelonandro *et al.*, 1997, 2000; Hily *et al.*, 2004.). Field trials have been carried out in Poland, Spain (Malinowski *et al.*, 2006) and Romania (Zagrai *et al.*, 2008a, 2008b) to verify whether the PDR assayed in two different environments (continental and Mediterranean) was durable.

C5 viral resistance is based onto RNA silencing (Scorza *et al.*, 2001). The roles of RNA silencing include the developmental regulation of gene expression and protection from transposable elements and viruses. Virus infection in plants can trigger the PTGS pathway in which siRNAs are produced (Hamilton and Baulcome, 1999; Hily *et al.*, 2005). As a response to this defence mechanism, many viruses encode gene-silencing suppressor proteins acting at different points in the PTGS pathway (Anandalakshmi *et al.*, 1998; Voinnet, 2001). Viral PTGS suppressors have also been shown to suppress PTGS of non-viral transgenes (Beclin *et al.*, 1998). While viral suppression of gene silencing has been demonstrated in herbaceous species, it has not been reported in most crops including woody plant species or/and under field conditions.

Because the constitutive expression of PPV-CP sequences naturally occur in transgenic plums, the environmental safety issues related to the release of virus-resistant transgenic crops need to be assessed. The major concern is the possible emergence of a PPV variant. As reported by Wintermantel and Schoelz, 1996, the virus transgene inserted in plant genome may generate new recombinant viruses with new biological properties. Interestingly, such scenario has been only reported with transgenic herbaceous plants in greenhouse conditions (Tepfer, 2002). So far, there is no report about the possible recombination with agronomical important virus-resistant transgenic crops actually grown in the field.

This paper is an overview of the results of three experiments performed at Fruit Research & Development Station Bistrita, Romania: the first was focused to field resistance trials of several transgenic plum clones, the second was addressed to the stability of RNA silencing displayed by the C5 clone under the coexistence of PPV and two heterologous ilarviruses, and the third was aimed to the environmental safety issue related to transgenic plums harbouring PPV-CP gene.

2. Material and Methods

Field resistance trials

Field experimental plots. The studies were carried out under conditions of high PPV inoculum pressure in three field experimental plots located at Fruit Research and Development Station Bistrita, Romania.

The first plot was set up in 1996 with 60 transgenic plum trees belonging to seven clones (C2, C3, C4, C5, C6, PT3 and PT5). Ten trees each of transgenic clones C2, C3, C4, C5 and C6, and five each of transgenic clones PT3 and PT5 were planted in the springtime. These plum trees were planted inside a 6 years old orchard, in a single row under high natural PPV infection pressure. At that time 23% of the conventional plums were infected. In this plot, a high number of transgenic plants died after planting, probably due to the inadaptation of *Prunus marianna* rootstock related to the heavy soil and climate in the Carpatian region. In order to correct this apparent inadaptation, two new experimental plots using Myrobolan as rootstock, were designed in 1998.

The second experimental plot included only the C5 transgenic clone (8 trees) planted in a single row in the vicinity of young conventional plums that are known to be tolerant to PPV. A large number of infected conventional plum trees which flanked the experimental plot provided a high infection pressure.

The third plot was set up in 1998 when 21 plum trees belonging to five transgenic clones (C2- three trees, C4- three, C5- ten, C6- two, and PT3- three trees) were planted in a new orchard within young trees (two years old) where PPV infection was rated to 4.5% of infected trees. In this plot the transgenic plums were randomly dispersed within the orchard.

Virus monitoring. The monitoring of disease based on visual observations of PPV symptoms on leaves and by serological and molecular diagnosis. Serological tests were performed by DAS-ELISA (Double Antibody Sandwich-Enzyme Linked Immunosorbent Assay) – Clark and Adams (1977), using polyclonal antibodies according to the manufacturer (Bioreba). Molecular detection was made by IC/RT-PCR (Immunocapture-Reverse Transcription-Polymerase Chain Reaction) using the P1/P2 polyvalent primer pairs (Wetzel *et al.*, 1991).

Field trials assessment of the stability of RNA silencing in C5 clone under mixed infections of PPV and heterologous viruses

Plant material and inoculated viruses. Two experiments were initiated in the field, in an experimental orchard and in a nursery respectively. Challenge heterologous viruses were *Prunus necrotic ring spot* virus (PNRSV) and *Prune dwarf* virus (PDV). The C5 transgenic clone was chip-bud inoculated with PPV (D strain) and with the combinations PPV + PDV or PPV + PNRSV. Buds infected with individual viruses were used for chip-bud inoculation. Conventional plums were similarly inoculated as controls.

Experimental orchard. Six C5 trees among the 10 plants grown in plot n° 3 were subjected to chip-bud graft inoculation in September 2003. Two C5 plum trees were used for each virus combination. Half

of the branches of each tree were graft inoculated and the other half not. On each inoculated branch 10 buds individually infected with the test viruses were alternately grafted.

Experimental nursery. Myroblan rootstocks were planted in an experimental nursery in April 2003 and grafted with buds from C5 or conventional plums on August 2003. The inoculation with the viruses was made as follow: buds of transgenic or conventional plums were inserted on the top of grafting area, buds with PPV were inserted in the middle and buds with heterologous viruses were inserted at the bottom. Ten plants were used for each virus combination.

Virus monitoring. Virus infection (PPV and heterologous viruses) was recorded by visual monitoring of symptom development and by serological and molecular methods. Serological virus detection was achieved by DAS-ELISA using polyclonal antibodies (PPV, PDV and PNRSV) according to the manufacturer (Bioreba, Switzerland). Molecular detection was performed by IC-RT-PCR using the PPV primer pair P1/P2 (Wetzel *et al.*, 1991), PNRSV-10F/PNRSV-10R for PNRSV (Marbot *et al.*, 2003) and PDV-17F/PDV-12R for PDV (Kummert *et al.*, 2001).

Environmental Impact Assessment of Transgenic Plums on the Diversity of Plum Pox Virus Populations

PPV Isolates. PPV isolates used in these studies were collected from the three field experimental plots afore mentioned. All PPV isolates identified in transgenic plums, 15 isolates respectively and 67 isolates selected from conventional plums surrounding the transgenic plums were analyzed. Sampling was initially based on typical PPV symptoms and virus infection was confirmed by serological and molecular testings.

Strain differentiation. Serological differentiation was made by TAS (Triple Antibody Sandwich) - ELISA with the PPV-D and PPV-M specific monoclonal antibodies provided by Durviz, Spain (Cambra *et al.*, 2004).

Molecular strain typing was done by IC-RT-PCR targeting three genomic regions corresponding to: the first (Cter) CP, using specific primers PD and PM that distinguish the two major PPV strains D and M (Olmos *et al.*, 1997); the second (Cter) N1b – (Nter)CP, using the pair primer mD5/mM3 (Subr *et al.*, 2004) that detect directly a natural recombinant Plum pox virus (PPV-Rec) between D and M previously reported (Glasa *et al.*, 2002, 2004); the third CI, using Clf/ CID or CIM primer sets (Glasa *et al.*, 2002) to confirm the presence of PPV-Rec. Aliquots of PCR products corresponding to (Cter) CP were subjected to RFLP (Restriction Fragment Length Polymorphism) analysis in order to distinguish the D and M strains based on Rsa I polymorphism located in this genomic section.

To confirm the molecular variability of the sampled PPV, amplified DNAs were purified by Wizard SV Gel and PCR Clean-Up System (Promega). Then they were sequenced by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The samples were run on the ABI Prism 310 Genetic Analyzer (Applied Biosystems). By using the program package BioEdit version 5.0.9 (Hall, T.A. 1999), alignment of nucleotides from 44 PCR products corresponding to (Cter)CP (15 PPV isolates which infected transgenic plums and 29 PPV isolates selected from conventional plums) and five amplified fragments spanning (Cter) N1b – (Nter)CP region (two isolates from transgenic plums and three isolates from conventional plums) was performed. Subsequently, the sequences were compared with those available in NCBI Data Base and Gene Bank.

3. Results and Discussion

Field resistance trials

PPV in transgenic and conventional plums

In the first plot, after 10 years of field testing, 11 transgenic plum trees belonging to C2, C3, C4, C6 and PT3 clones were found infected (44% of trees) while the percentage of conventional plums infected increased from 23% in 1996 to about 76% in 2006. Although the presence of aphid vectors was often observed on C5, this transgenic clone remained uninfected exhibiting a high and durable resistance to PPV. The temporal spread of PPV in the first plot showed a continual increase in infection of conventional plums and a delay of infection in C2, C3, C4 (Figure 1). It is possible that the design of the first plot was not appropriate to stimulate virus spread, because, generally, PPV infection in transgenic plums was delayed.

In the second plot none of the C5 transgenic trees were infected by PPV during eight years of field testing while most of the tolerant conventional plums surrounding C5 become infected. The temporal spread of PPV in the second plot revealed also a continual evolution of infection in conventional plums while C5 exhibited durable resistance to PPV (Figure 2).

In the third plot, after five years of field testing only C5 remained uninfected. The infection in conventional plums increased from 4.5% in 1998 to 39.8% in 2003. This demonstrated an exposure to high infection pressure mediated by aphid vectors. Using DAS-ELISA and IC-RT-PCR detection we confirmed that clone C5 was the only plum that remained PPV free. The temporal spread of PPV in the third plot showed that, after five years of field testing the infection rate in conventional and transgenic plums (excepted C5) was similar (Figure 3). This demonstrated that the design of experimental plots can

play an important role in virus spread. Moreover, aphid vectors did not make any difference between transgenic and conventional plums (Zagrai *et al.*, 2007, 2008c). Overall, no C5 trees became infected. Under these conditions, this clone shows a high and durable resistance to natural PPV infection.

Field trials assessment of the stability of RNA silencing in C5 clone under mixed infections of PPV and heterologous viruses

Experimental orchard. PPV, PDV and PNRSV could not be detected by symptom observation in the inoculated C5 trees or by DAS-ELISA tests at one year post-inoculation. Low concentrations of PPV and PDV were detected in the inoculated conventional plums.

Two years post-inoculation DAS-ELISA tests revealed that the heterologous viruses (PDV and PNRSV) were translocated from the inoculum to the C5 trees. PPV could be detected with a very low titer in C5 trees only on a few discrete symptomatic leaves from the graft inoculated branches. Conversely, very severe PPV symptoms and high viral concentration appeared on graft inoculated branches from conventional plums. In addition, the PPV symptoms observed on the non-inoculated branches indicated that the virus invaded a large part of the canopies of conventional plums.

The evaluation performed at three years post-inoculation showed no spread of PPV infection in C5 trees. In all cases (both singular and mixed infections on C5), PPV could be observed (discrete diffuse spots and sporadic symptoms) and detected by DAS-ELISA and IC-RT-PCR only near the inoculum points. No PPV symptoms were observed in the non-inoculated part of the C5 canopy. The absence of the virus was confirmed by molecular testing. Although PDV and PNRSV showed no clear symptoms in the plum canopy, these viruses were detected both in the inoculated canopy and in the non inoculated part of the C5 trees.

Interestingly, no difference in PPV spread was observed in the C5 trees when PPV was inoculated alone or in combination with heterologous viruses.

Experimental nursery. PDV readily invaded whole C5 plants inoculated with the PPV + PDV combination. PPV also translocated from the inoculum bud to C5 but the virus could only be detected at the basal half of the plants. Although PPV was locally detected at one year post-inoculation, the infection did not systemically spread in the following two years (2005 and 2006).

In C5 plants inoculated with PPV + PNRSV, diffuse spots of PPV symptoms sporadically appeared on a few leaves at the basal part of the plants. DAS-ELISA (2004 and 2005) and IC-RT-PCR (2006) confirmed the presence of PPV in this part of the plants. However, PPV could not be detected by ELISA and IC-RT-PCR on the top half part of the inoculated plants. Although PNRSV produced no symptoms the virus was detected with high titer in the whole plant since the first year post-inoculation.

No difference in symptom development or PPV spread was observed on C5 grown in the nursery when PPV was inoculated alone or in combination with heterologous viruses.

Regardless of singular (PPV) or mixed (PPV+PDV, PPV+PNRSV) infection, C5 transgenic plums revealed a similar behaviour in regard to PPV infection: PPV could produce a mild and limited infection in C5 independently of the presence of the heterologous viruses. PPV symptoms were extremely mild, sporadic or absent, indicating an effective inhibition of the virus multiplication. Across both trials experiments (orchard and nursery), the infection remained close to the inoculation site and did not systemically spread. Malinowski *et al.* (2006) showed the same mild infection phenotype in C5 trees graft-inoculated with PPV in an experimental open-field trial in Poland. These results show that there is no evidence that heterologous viruses can affect the stability of the engineered protection in transgenic C5 plums.

Environmental Impact Assessment of Transgenic Plums on the Diversity of Plum Pox Virus Populations

The differentiation of PPV isolates from transgenic plums established by TAS-ELISA using D and M monoclonal antibodies and by IC-RT-PCR using PD and PM specific primers was identical. All isolates reacted positively to PPV-D or PPV-M monoclonal antibodies. Thus, from 15 isolates detected in transgenic plums, 8 were identified as PPV-D and 7 as PPV-M. Molecular strain typing by IC-RT-PCR using specific primers PD and PM confirmed the serological results. RFLP analysis permitted to distinguish the two strains by the presence of the *RsaI* sites in PPV-D strain (Table 1).

All PPV isolates selected from conventional plums surrounding the transgenic plums also reacted positively to at least one of the two monoclonal antibody as well as PPV-D or/and PPV-M specific primers (Table 2). A slight difference could be observed between the results obtained by serological and molecular tests. Thus, 38/67 isolates were identified as PPV-D by TAS-ELISA, 27/67 as PPV-M and 2 as a mixed infection (PPV-D + PPV-M). IC-RT-PCR analysis revealed the presence of PPV-D in 38 isolates while the PPV-M was identified in 26 isolates. Three cases reflect a mixed infection involving D and M strains. These results were confirmed by RFLP analysis using *RsaI* digestion. Detection of PPV-D strain and its presence in a mixed infection were thus recorded.

Using the pair of primers mD5/mM3 was observed that all PPV isolates typed as PPV-M in (Cter)CP region were identified as PPV-Rec in (Cter)N1b – (Nter)CP region, both in transgenic (Table 3) and conventional plums (Table 4). Using specific primers to distinguish the two strains D and M in CI region were detected only fragments belonging to PPV-D. That confirmed the presence of PPV-Rec.

The phylogenetic grouping of PPV isolates based on nucleotide sequences corresponding to C-terminus of PPV coat protein confirmed the similarity of PPV isolates from transgenic (Fig. 4a) and conventional plums (Fig. 4b).

To check if the recombination breakpoint position suspected to occur in (C-ter)N1b - (N-ter)CP region correspond with those PPV-Rec previously reported by Glasa *et al.* (2002, 2004), five PCR products spanning this genomic section (two belonging to isolate samples from transgenic plums and three belonging to isolate samples from conventional plums), were sequenced (Fig. 5). The multiple alignment indicated that the recombination breakpoint is located in C terminus of the N1b gene at the nucleotide position 8450. The sequences of our PPV-Rec are similar in transgenic and conventional plums. The DAG motif that is considered as essential for potyvirus aphid transmission is also present in the PPV-Rec isolates. Expectedly, this site is located at downstream of the recombination breakpoint. Based on the comparative alignment, the sequencing results revealed a high similarity (98-99%) with different sequences of PPV -Rec previously reported and available in Gene Bank. All these recombinant isolates share the same recombination breakpoint.

The ratio of PPV strains in transgenic (Fig. 6a) and conventional (Fig. 6b) plums surrounding the transgenic plums was approximately similar. This can suggest that aphid vectors do not make any differentiation between plums tested. The difference is that the mixed infections were detected only in conventional plums. The transgenic resistant C-5 plum could not be sampled in this study since this clone cannot be infected by natural aphid transmission due to its high and durable resistance to natural PPV infection. It is therefore considered to be particularly safe in the sense of not affecting PPV strain variability or recombination.

4. Conclusions

1. Regardless of the different conditions of the field testing, the C5 transgenic clone could not be infected by natural aphid transmission exhibiting a high and durable resistance to natural PPV infection.
2. Graft-inoculation of transgenic C5 plums with the mixed infection comprising PPV and *Prunus necrotic ringspot* or *Prune dwarf* ilarviruses in the field did not affect the efficacy and stability of PTGS over a three-year period.
3. The rate of different PPV strains in transgenic and conventional plums surrounding the transgenic plums was approximately similar. This can suggest that aphid vectors do not make any differentiation between the two types of plums. Serological and molecular variability of PPV populations in transgenic and conventional plums confirmed that the transgenic plums expressing PPV-CP gene do not represent an environmental risk for any emerging PPV strain.
4. Based on the characteristics of C5 plums, the emergence of recombinant PPV in these transgenic trees should be less likely than in conventional plums.

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Tables and Figures

Table 1. Serological and molecular differentiation of PPV isolates identified in transgenic plums

| Isolate | DAS / TAS-ELISA | | | | IC-RT-PCR (P1/P2 and P1/PD or PM) | | | | RFLP <i>Rsa</i> I |
|--------------|-----------------|-------------|-------------|------------|-----------------------------------|-------------|-------------|------------|----------------------|
| | PPV poly | PPV-D | PPV-M | PPV-D+M | PPV poly | PPV-D | PPV-M | PPV-D+M | |
| C6-2 (1/21) | + | + | - | - | + | + | - | - | + |
| C6-2 (1/23) | + | + | - | - | + | + | - | - | + |
| PT3-2 (1/25) | + | + | - | - | + | + | - | - | + |
| PT3-2 (9/11) | + | + | - | - | + | + | - | - | + |
| PT3-2 (9/12) | + | + | - | - | + | + | - | - | + |
| C4-6 (9/21) | + | - | + | - | + | - | + | - | - |
| C6-2 (15/20) | + | - | + | - | + | - | + | - | - |
| C2-10 16/36) | + | - | + | - | + | - | + | - | - |
| C6-2 (1/22) | + | + | - | - | + | + | - | - | + |
| C3-9 (1/6) | + | - | + | - | + | - | + | - | - |
| C3-9 (1/8) | + | - | + | - | + | - | + | - | - |
| C3-9 (1/9) | + | - | + | - | + | - | + | - | - |
| C3-9 (1/10) | + | + | - | - | + | + | - | - | + |
| C4-6 (1/15) | + | + | - | - | + | + | - | - | + |
| C2-10 (1/2) | + | - | + | - | + | - | + | - | - |
| TOTAL (%) | 15 (100.0) | 8 (53.3) | 7 (46.7) | 0 (0.0) | 15 (100.0) | 8 (53.3) | 7 (46.7) | 0 (0.0) | 8 (53.3) |

Table 2. Serological and molecular differentiation of PPV isolates selected from conventional plums surrounding the transgenic plums

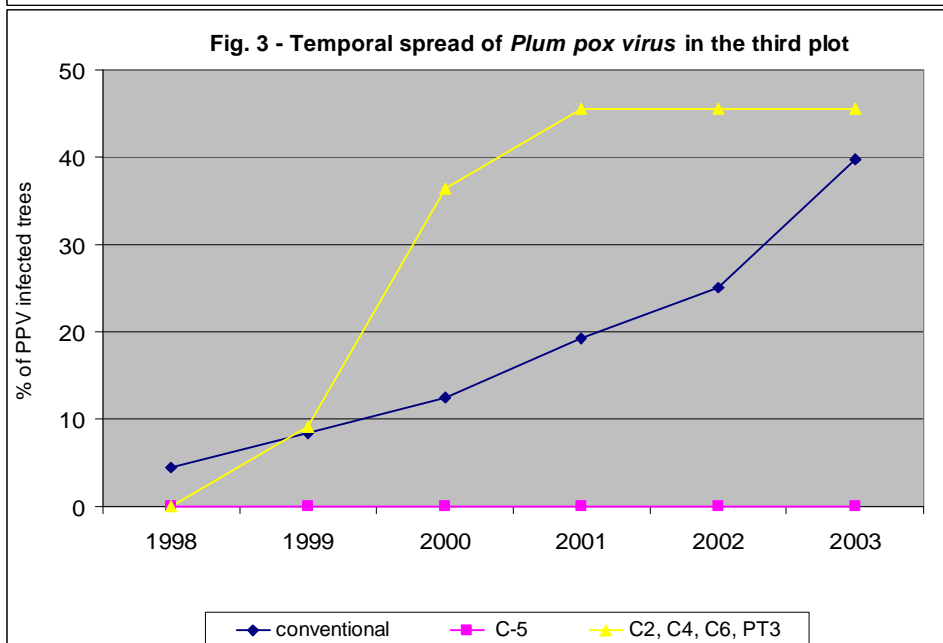
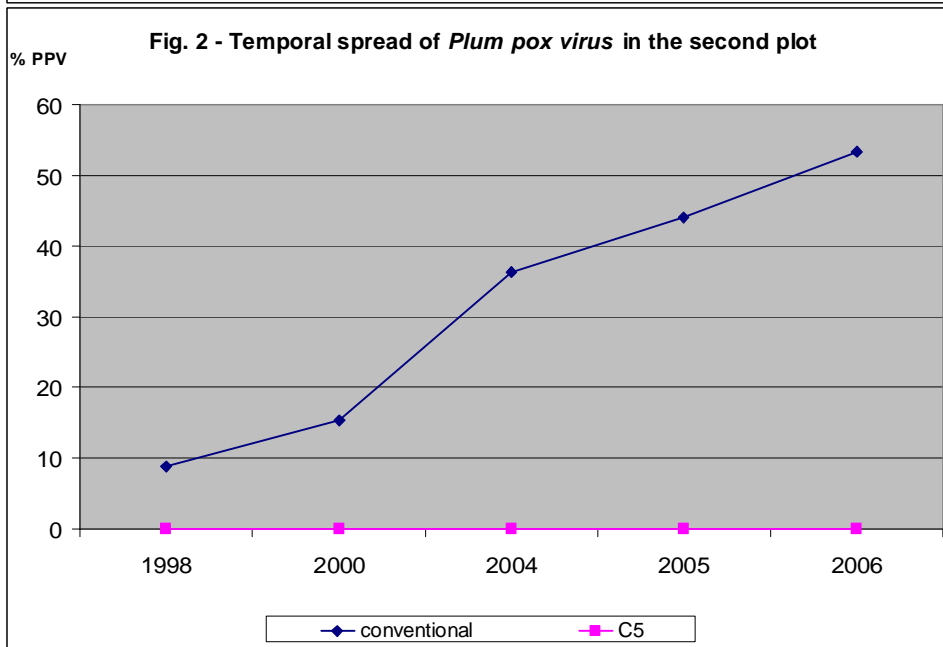
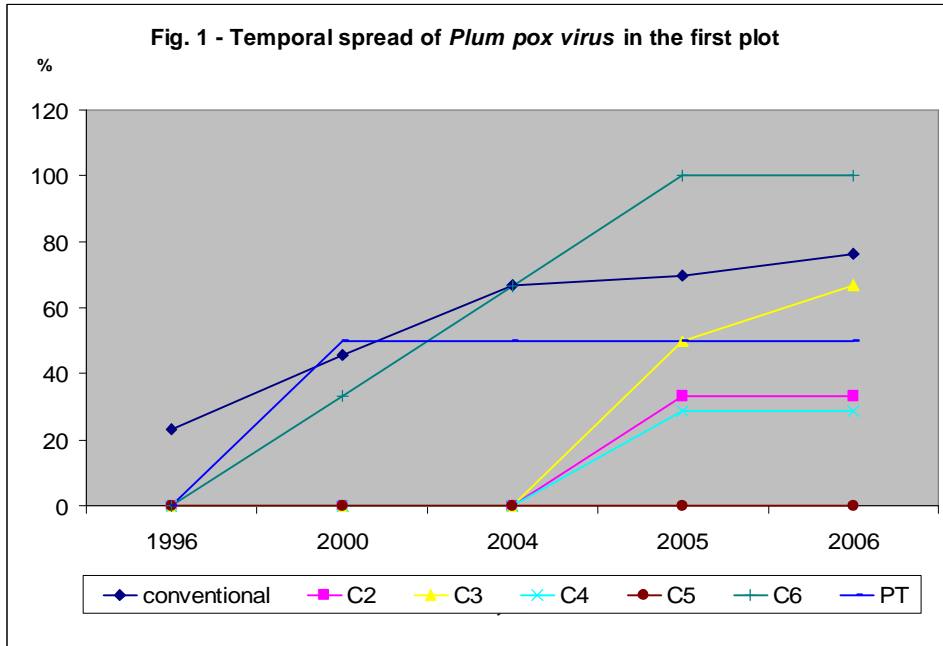
| Plot no. | No. of isolates | DAS / TAS-ELISA test (OD=405nm) | | | | IC-RT-PCR (P1/P2 and P1/PD or PM) | | | | RFLP <i>Rsa</i> I | | |
|-----------|-----------------|---------------------------------|--------------|--------------|------------|-----------------------------------|--------------|--------------|------------|-------------------|------------|------------|
| | | PPV poly | PPV-D | PPV-M | PPV D+M | PPV poly | PPV-D | PPV-M | PPVD +M | PPV-D | PPV-M | PPV-D+M |
| 1 | 37 | 37 | 25 | 11 | 1 | 37 | 24 | 10 | 3 | 24 | 0 | 3 |
| 2 | 13 | 13 | 10 | 3 | 0 | 13 | 10 | 3 | 0 | 10 | 0 | 0 |
| 3 | 17 | 17 | 3 | 13 | 1 | 17 | 4 | 13 | 0 | 4 | 0 | 0 |
| Total (%) | 67 (100) | 67 (100) | 38 (56.7) | 27 (40.3) | 2 (3.0) | 67 (100) | 38 (56.7) | 26 (38.8) | 3 (4.5) | 38 (56.7) | 0 (0.0) | 3 (4.5) |

Table 3. Results of molecular typing based on different target region of PPV isolates from transgenic plums

| Isolates | Target region | | |
|---------------|---------------|--------------------------|----|
| | (C-ter) CP | C-ter (Nlb) - (N ter) CP | CI |
| C6-2 (1/21) | D | - | D |
| C6-2 (1/23) | D | - | D |
| PT3-2 (1/25) | D | - | D |
| PT3-2 (9/11) | D | - | D |
| PT3-2 (9/12) | D | - | D |
| C4-6 (9/21) | M | REC | D |
| C6-2 (15/20) | M | REC | D |
| C2-10 (16/36) | M | REC | D |
| C6-2 (P22) | D | - | D |
| C3-9 (P6) | M | REC | D |
| C3-9 (P8) | M | REC | D |
| C3-9 (P9) | M | REC | D |
| C3-9 (P10) | D | - | D |
| C4-6 (P15) | D | - | D |
| C2-10 (P2) | M | REC | D |

Table 4 . Results of molecular typing based on different target region of PPV isolates from conventional plums

| No. plot | No. Isol. | Target region | | |
|----------|-----------|---------------|-------------------------|----|
| | | (C-ter) CP | C-ter (Nlb)– (N ter) CP | CI |
| 1 | 24 | D | - | D |
| | 10 | M | REC | D |
| | 3 | D+M | REC | D |
| 2 | 10 | D | - | D |
| | 3 | M | REC | D |
| | 0 | D+M | REC | D |
| 3 | 4 | D | - | D |
| | 13 | M | REC | D |
| | 0 | D+M | REC | D |
| total | 38 | D | - | D |
| | 26 | M | REC | D |
| | 3 | D+M | -REC | D |



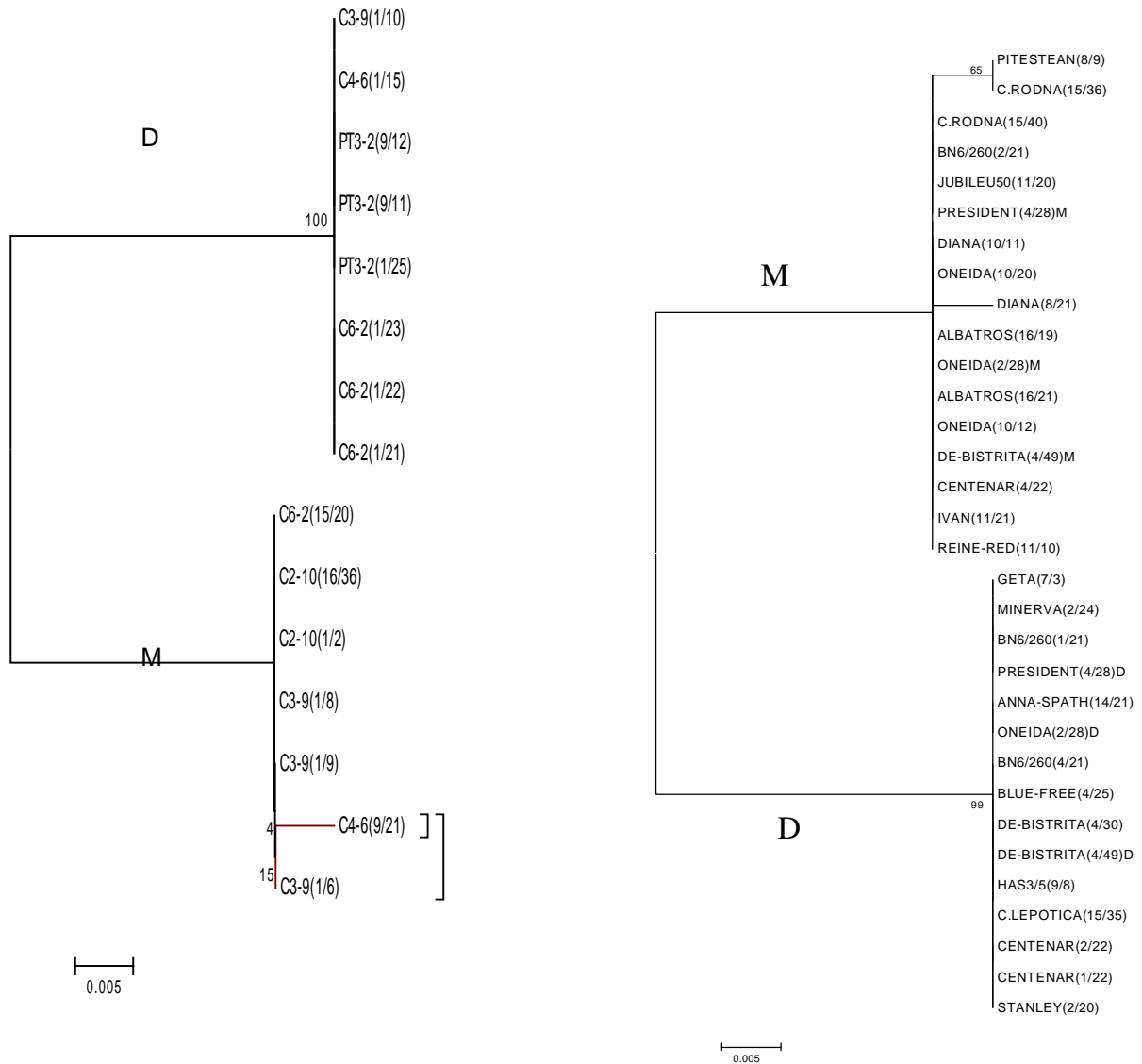


Fig. 4. Phylogenetic grouping of PPV isolates based on nucleotide sequences corresponding to C-terminus of PPV coat protein: a – transgenic plums; b-conventional plums. The isolates from conventional plums mark with D respectively M represent mixed infection and they were sequenced both for PPV-D and PPV-M.

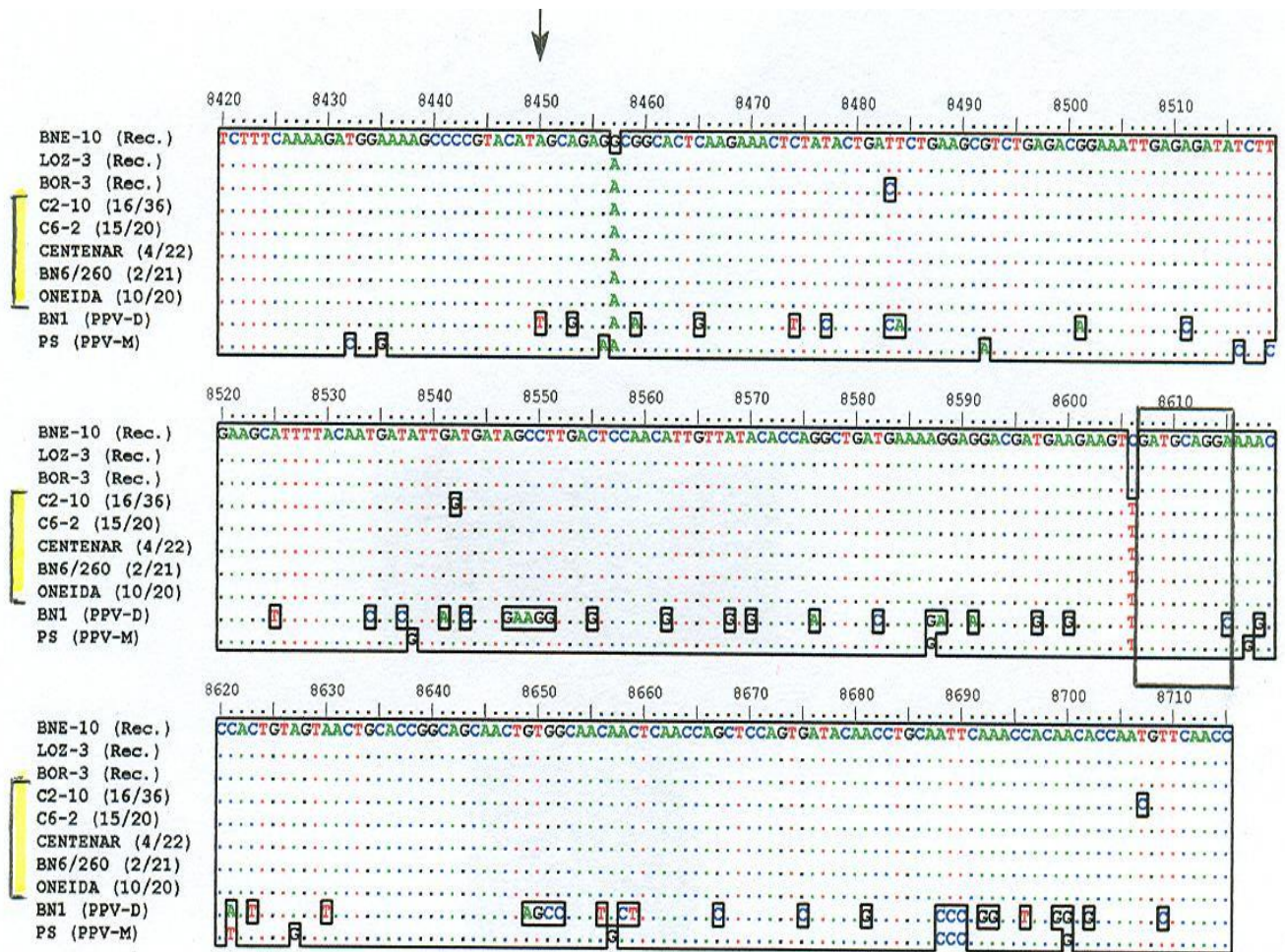


Fig. 5. Multiple alignment of recombinant sequences (Nlb/CP) of five Romanian isolates [C2-10 (16/36), C6-2 (15/20) – transgenic plums; Centenar (4/22), BN 6/260 (2/21), Oneida (10/20)’ – conventional plums] and three isolates (BNE-10, LOZ-3, BOR – 3) previously reported.

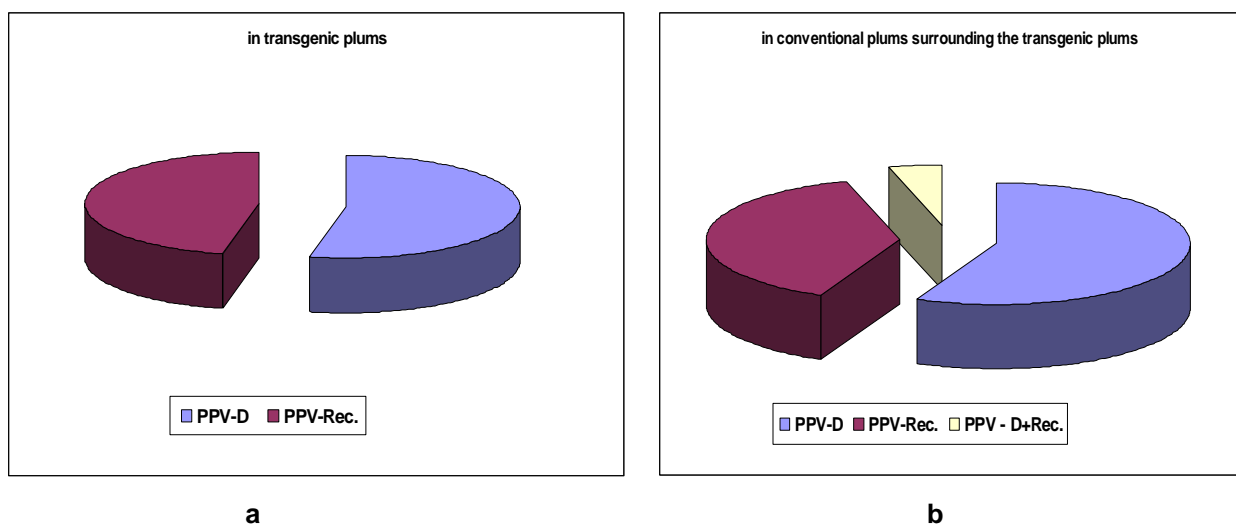


Fig. 6. Ratio of PPV strains in transgenic (a) and conventional (b) plums