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Detection of *Potato virus Y* in pepper by ELISA-RT-nested PCR

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Summary. We describe a reliable method to improve the detection of potato virus Y in pepper (*Capsicum annuum* L.), based on nested PCR performed after an ELISA-RT-PCR stage. Many problems were overcome by this approach, particularly those due to PCR inhibitory compounds which seem to be abundant in pepper.

Key words: *Capsicum annuum* L., potyvirus, inhibitors, sensitivity, specificity.

Introduction

Potato virus Y (PVY) is the type member of the potyvirus group, one of the largest plant virus groups (Riechmann *et al*., 1992). The PVY genome is a positive-sense single-stranded RNA of 10 Kb size, encapsidated into long flexuous rods of about 2000 capsid proteins (Dougherty and Carrington, 1988). PVY infects different Solanaceae such as potato, tomato, pepper and tobacco (Marchoux *et al*., 2000).

Prevention remains the most effective means to control this virus. Control consists in selecting healthy plants and seeds for cultivation, as well as in removing infected plants from the fields, thus reducing the transmission of the disease. However, prevention requires methods to detect PVY in test samples at an early stage. Such methods must be both selective and specific. Molecular diagnosis is a method that meets these requirements but it has certain problems, due to the unequal distribution of the virus throughout the plant (Helgera *et al*., 2001), and to the occurrence in plant tissue of phenolic and polysaccharidic compounds that strongly inhibit the reverse transcription-polymerase chain reaction (RT-PCR) (John, 1992; Singh and Singh, 1996). In this study we describe a reliable, sensitive and reproducible method for the detection of PVY in all conditions of infection that may be encountered in phytosanitary laboratories.

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Materials and methods

Plant material and inoculum source

Pepper samples (cv. Baklouti, D'hirat, Baker and Beldi) were collected from field-grown pepper fields mostly displaying the typical symptoms of PVY, and were used as infected plant material. The PVY^{O}-P21 isolate detected on pepper plants in Tunisia (Fakhfakh *et al.,* 1995) was propagated and maintained in tobacco (*Nicotiana tabacum* cv. Xanthi) under insect-proof conditions.

Indirect ELISA-RT-PCR

A modified protocol of ELISA-RT-PCR (Nolasco *et al*., 1993) was followed to detect PVY in pepper starting from the same sample material. The protocol consisted of three steps.

The first step was an indirect ELISA (as described by Clark and Adams, 1977) using plant extract to sensitize the microtitre plates and the anti-PVY^{O}-P21 mouse monoclonal antibody which had been prepared in the Laboratoire d'Immunogénétique, Faculté des Sciences de Tunis (Ounouna *et al*., 2002), to detect PVY. Immediately after reading the absorbance values, the wells were washed with PBS-Tween and submitted to the RT-PCR stage, in which a 15-mer oligo(dT) was used in reverse transcription for cDNA synthesis. For PCR we used the forward primer 1 (GCCTTCACTGAAATGATGG) and the reverse primer 2 (GCATGTAAATATACTTAT) complementary to the PVY capsid gene (Robaglia *et al*. 1989). Amplification was carried out in a thermocycler (Techgene). The amplified product was 916 bp in length.

Nested PCR and enzyme digestion

Nested PCR was performed using 1 μl of the ELISA-RT-PCR product as input DNA. The primers used were described by Nie and Singh (2001). The sense primer 3 (ACGTCCAAAATGAGAATGCC) and the antisense primer 4 (TGGTGTTCGTGATGTGACCT) amplified an internal 480 bp fragment. The *Rsa* I restriction endonuclease (BRL) was used to digest the nested PCR products.

Results and discussion

In Tunisia, infection of pepper with PVY is a problem. PVY infection has been studied biologically and serologically (Fakhfakh *et al*., 1994; Ounouna *et al*., 2002), and its molecular characterization has been carried out (Fakhfakh *et al*., 1995). All these studies show that the PVY^{O} pathotype is the most widespread strain in Tunisian pepper. However, there are some difficulties in PVY infection diagnosis since pepper veinal mottle virus (PVMV) produces the same symptoms as PVY (Gorsane *et al*., 1999).

The objective of this study was to test a sensitive method, PT-PCR, for the detection of PVY in infected field-grown pepper. When operating on mechanically inoculated tobacco, we showed that RT-PCR is 25 times more sensitive than DAS-ELISA. This sensitivity is in agreement with other reports (Vunsh *et al*., 1990; Olmos *et al*., 1997). When attempts were made to use this method to detect PVY in pepper samples from the field, DAS-ELISA detected PVY in the tested samples, but surprisingly the RT-PCR test on the same samples was consistently negative (data not shown). If it is assumed that the ELISA used here was specific to PVY, this unexpected result with RT-PCR might be explained in different ways, leading to further optimization of the method.

The failure of RT-PCT to detect PVY in pepper samples from the field raised the problem of reproducing test results under laboratory and field conditions, and in a variety of species, without preliminary optimization. The need for optimization depends on the epidemiological conditions in a given country. In addition, optimization techniques must be adapted to the capacity of local laboratories, particularly in developing countries where for economical and technological reasons it is difficult to use molecular techniques in routine testing.

In order to overcome these constraints, we combined different data and techniques to devise a reliable, sensitive and reproducible method for the detection of PVY in pepper. We modified the protocol previously described by Nolasco *et al*. (1993), which combined ELISA, reverse transcription and PCR stages. In our protocol we replaced the DAS-ELISA by indirect ELISA, allowing the virions on the polystyrene microplate to be trapped directly without an immunocapture stage. Moreover, this approach as it has been said, avoided RNA extraction for further molecular amplification.

For optimization of the ELISA-RT-PCR we used four pepper plants showing PVY symptoms (Marchoux *et al*., 2000). The plants were first tested with simple indirect ELISA. The absorbance values with this test demonstrated high levels of PVY in three samples (Fig. 1A, lanes 7 to 15), while no infection was detected in the fourth (Fig. 1A, lanes 4 to 6). The reason for this last sample testing negative, however, was that the sensitivity of this ELISA technique was too low. We therefore performed a further molecular amplification of a PVY capsid gene fragment starting from the same well. The combination of ELISA and RT-PCR had the advantage of avoiding the anomalous effect caused by the unequal distribution of the virus in the plant and of

limiting the variability due to the experiment. After the ELISA step, the solution in the well was eliminated by washing it out with PBS-Tween, and then the RT-PCR step was carried out. Figure 1B shows that no amplification of the 916 bp expected fragment was obtained in any of the four samples (Fig. 1B, lanes 4 to 15). By contrast, we obtained a specific amplification of PVY in the infected tobacco samples tested in the same way (data not shown). This result may be due to higher levels of polyphenolic compounds in the pepper samples, reducing the sensitivity of PVY detection by interfering with reverse transcriptase, Taq polymerase, or both. The concentration of polyphenolic compounds varies between plant species and cultivars (John, 1992; Singh and Singh, 1996).

In order to assess the inhibitory effect of pepper leaf compounds on Taq enzymatic activity, we started from an already amplified PVY DNA fragment of 916 bp size used at different dilutions, which were mixed with different concentrations of crude pepper extract (1/10 to 1/5000), and submitted to a PCR reaction using primers 3 and 4. The inhibitory effect of the pepper compounds was

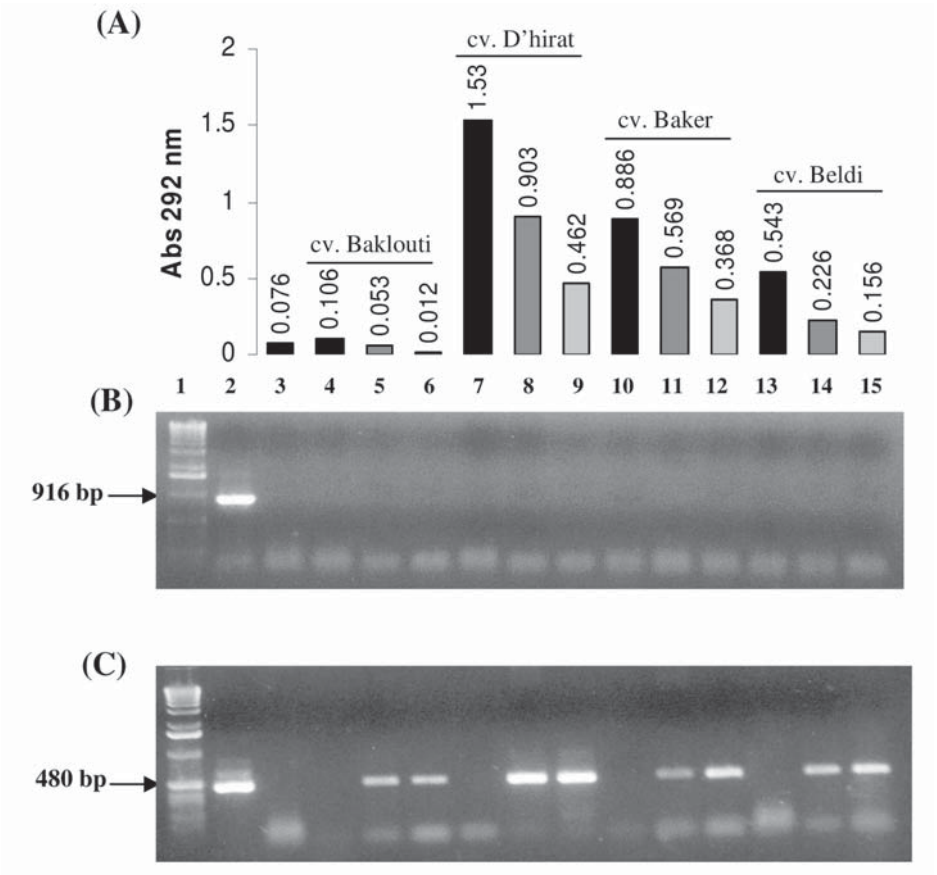

Fig. 1. Detection of PVY in pepper leaves by indirect ELISA-RT-PCR-nested PCR. Panel A, indirect ELISA absorbance values. Panel B, agarose gel electrophoretic analysis after indirect ELISA-RT-PCR. Panel C, agarose gel electrophoretic analysis after nested PCR. Each number indicates the same sample at each stage. Lane 1, 1 kb marker (Gibco-BRL). Lane 2, PCR positive control (the pCR®2.1 vector with capsid sequence, as input). Lane 3, PCR negative control (tobacco leaf extract from healthy plant). Lane 4, crude leaf extract from pepper cv. Baklouti. Lanes 5 and 6, 1/5 and 1/10 (v:w) dilutions respectively of leaf sap from pepper cv. Baklouti. Lane 7, crude leaf extract from pepper cv. D'hirat. Lanes 8 and 9, 1/5 and 1/10 (v:w) dilutions respectively of leaf sap from pepper cv. D'hirat. Lane 10, crude leaf extract from pepper cv. Baker. Lanes 11 and 12, 1/5 and 1/10 (v:w) dilutions respectively of leaf sap from pepper cv. Baker. Lane 13, crude leaf extract from pepper cv. Beldi. Lanes 14 and 15, 1/5 and 1/10 (v:w) dilutions respectively of leaf sap from pepper cv. Beldi.

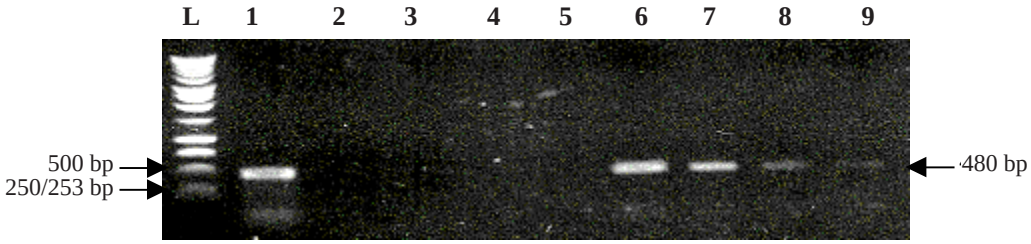

Fig. 2. Analysis by ELISA-RT-PCR of a serial dilution of the reverse transcription product starting from a crude extract of PVY infected pepper. L, 1 kb Ladder (Promega, Madison, WI, USA). Lane 1, PCR positive control (the pCR®2.1 vector with capsid sequence, as input). Lane 2, PCR negative control (tobacco leaf extract from healthy plant). Lane 3, crude leaf extract. Lanes 4 to 10: 1/10, 1/50, 1/100, 1/500, 1/1000 and 1/5000 (v:w) dilutions respectively in PBS of the RT product from the crude leaf extract sample.

avoided only at a dilution of 1/5000 (data not shown). This result clearly showed that some inhibitory molecules occurring in pepper interfered with Taq polymerase activity and acted at the PCR step; their concentration in pepper was so high that a dilution of up to 1/5000 of the pepper extract was required to abolish their effect.

In view of this result, we added a dilution step of 1/5000 after the RT reaction and before performing the PCR. No amplification was obtained with any sample in the weak dilutions, but amplified fragments were observed starting from dilution 1/100, though only for the ELISA positive samples (Fig. 2). From this result it appeared that the dilution introduced after the RT step increased PCR sensitivity as compared to figure 1B, but did not give a real advantage compared to ELISA. Since dilution also decreased the concentration of cDNA used as template in PCR, the negative samples may have been PVY free, or may have carried only a weak concentration of the virus.

To increase the sensitivity of the ELISA-RT-PCR we added an extra cycle of PCR with internal primers that would give a 480 bp size product. This step, called "nested PCR" (Barbezange and Jestin, 2002) improved sensitivity 100-fold (Helgera *et al*., 2001). When 1 μl of the supposed amplified 916 bp viral DNA fragment was used as input in nested PCR, the expected 480 bp band was observed in lanes 5, 6, 8, 9, 11, 12, 14 and 15 corresponding to diluted crude sap samples at 1/5 and 1/10 v:w (Fig. 1C). Moreover, the previous negative samples with ELISA and RT-PCR now became positive, indicating that the nested PCR step was important to increase the sensitivity of the method but was not enough to avoid the dilution stage, since the reaction was still negative at weak dilutions, confirming the high levels of inhibitory compounds in pepper samples.

In order to confirm the specificity of the nested PCR, the amplified DNA fragments were digested with *Rsa* I endonuclease. This enzyme was selected on the basis of the chart of possible restriction sites described in the PVY coat protein reference sequence. According to the DNA strider™ 1.1 program, this enzyme should generate two DNA fragments of 275 bp and 205 bp if applied to the 480 bp amplified fragment. Figure 3 illustrates the results obtained after digestion with *Rsa*I, and shows that two fragments of the expected size were generated by cutting the nested PCR products (lanes 3 to 6), confirming the specificity of the PVY amplification.

Use of ELISA-RT-dilution-nested PCR therefore

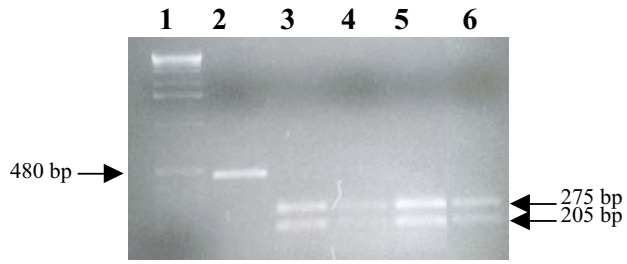

Fig. 3. Analysis of *Rsa*I digestion products on 1.4% agarose gel. Lane 1, 1 kb marker (Gibco-BRL). Lane 2, nested PCR product 480 bp (before digestion by *Rsa*I). Lanes 3, 4, 5 and 6, digestion products from pepper cv. Baker, cv. Baklouti, cv. D'hirat and cv. Beldi respectively.

significantly increased the sensitivity of detection. Out of 30 pepper samples with symptoms of PVY, ELISA detected 6 and our method 28.

In conclusion, we consider any sample PVY infected if it is positive by either ELISA or RT-dilution-nested PCR, and any sample PVY free if it tests negative with both these methods. In itself, each of these methods is not new. What is new, however, is their combination (ELISA, RT-dilution-PCR, Nested PCR-RFLP) under different experimental conditions (low PVY distribution in the plant, unequal PVY distribution in the plant, presence of PCR inhibitors in plant extract) and taking into account the time and cost of detection when testing large numbers of samples.

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