Application of nested-PCR and RFLP analysis on grapevine Portuguese varieties and Scaphoideus titanus Ball for the detection of Flavescence dorée phytoplasma.

Summary

The vector of Flavescence Dorée Phytoplasma, Scaphoideus titanus Ball, was firstly identified in the North of Portugal in 2000. An official evaluation was started in order to assess for the presence of the disease in the country. To this end, a joint project was undertaken by the National Institute of Veterinarian Sanitary and Food Hygiene (INETI) and the Portuguese Grape and Wine Research Institute (IEN) for the evaluation of the grapevine Portuguese varieties for the presence of Flavescence dorée (FD) phytoplasma as a reflection of vector presence. In the current study, the RFLP analysis was used in the detection of FD phytoplasma from the grapevine Portuguese varieties. The additional nested PCR was carried out. Nested-PCR (Polymerase Chain Reaction) and RFLP (Restriction Fragment Length Polymorphism) were used to amplify several grapevine plants and vectors using universal primers P1/P7 and U3/U5 and resultant restriction enzyme AluI. The positive controls of Flavescence dorée were used. Two phytoplasma were detected using these techniques.

2. Materials and methods

2.1. DNA Extraction

Total nucleic acids were extracted from 0.5–1 g of symptomatic or asymptomatic grapevine leaves and tomato with polyvinylpyrrolidone in liquid nitrogen in a one-time extraction (19). The nucleic acid and polyvinylpyrrolidone were washed with 96% ethanol, air-dried, and resuspended in 200 μl of sterile deionized water. The DNA concentration and purity were determined by UV reading of the extracted samples at 260/280 nm using the spectrophotometer UV Atlas 180 (1).

2.2. Polymerase Chain Reaction (PCR)

Preliminary PCR experiments were carried out using the universal primer pair P1/P7 (Schneider et al. 1995). PCR mixes containing 2 μl of the DNA template, 10 μl MgCl2, 5 μl of a PCR buffer (pH 8.3) 0.25 μl and 2 μl dNTPs, 200 μl of ΔTaq polymerase in a total volume of 50 μl. Thirty-five cycles were conducted for each of the automated thermal cycles (M; BioRad PCR 2200, USA). The illumination parameters were used: 94°C for 1 min 30 s, 55°C (specific annealing) for 1 min 30 s and 72°C for 1 min 30 s. After this cycle, the product was observed on a 1% agarose gel followed by staining with ethidium bromide and visualisation of DNA bands using a UV transilluminator.

2.3. Nested PCR assays with two universal primer pairs

In nested-PCR, PCR products initially amplified using the universal primer pair P1/P7, were diluted 1/1000 with sterile deionized water. The DNA concentration and purity were determined by UV reading of the extracted samples at 260/280 nm using the spectrophotometer UV Atlas 180 (1).

2.4. Restriction fragment length polymorphism analysis (RFLP)

In nested-PCR, PCR products initially amplified using the universal primer pair P1/P7, were diluted 1/1000 with sterile deionized water and used as substrates for a subsequent series of 35 PCR cycles in which reaction mixtures contained the universal primers pair U3/U5 (Schneider, 1995).

2.5. Forward and reverse nested primers

In nested-PCR, PCR products initially amplified using the universal primer pair P1/P7, were diluted 1/1000 with sterile deionized water and used as substrates for a subsequent series of 35 PCR cycles in which reaction mixtures contained the universal primers pair U3/U5 (Schneider, 1995).

2.6. Restriction fragment length polymorphism analysis (RFLP) of PCR products

The DNA sequences amplified with primer pair U3/U5 were compared by RFLP analysis after mutation with enzyme AluI, for 2 h at 37°C and electrophoresis in 1% agarose.