Bulletin of Insectology **60** (2): 257-258, 2007
ISSN 1721-8861

Developing a method for phytoplasma identification in cactus pear samples from California

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Abstract

Cactus pear plants showing proliferation and stunting of cladodes in Californian cultivations were tested in order to define a molecular methodology for reliable phytoplasma detection. After several unsuccessful trials a simple extraction method was developed to reduce the mucilage content in nucleic acid preparations that was seriously affecting pathogen detection. Nested PCR on 16S ribosomal gene and RFLP analyses together with sequencing of obtained amplicons allow to verify the presence in symptomatic plants of 16SrV-A and 16SrI-B phytoplasmas respectively related to '*Candidatus* Phytoplasma ulmi' and '*Ca*. P. asteris'.

Key words: Cactus pear, nucleic acid extraction, phytoplasma identification, PCR/RFLP, sequencing.

Introduction

Cactus pear [*Opuntia ficus-indica* (L.) Mill.] production is of economic importance in many parts of the world, and in these areas the species shows often symptoms such as stunted growth, cladode enlargement and loss of the green colour that were recently associated with 16SrII-C phytoplasma presence; the disease was also proved to be graft transmissible (Granata *et al*., 2006). Cladode enlargement was first reported in Mexico but it is also present in Chile, Argentina, South Africa and Italy. Flower proliferation on the flat part of cladodes, premature abscission of flowers and deformation and yellow discoloration of young cladodes have been described in Mexico, South Africa and Italy (Pimienta, 1990; Granata, 1995).

Similar symptoms affect some cactus pears in Californian cultivations where severe stunting and cladode proliferation are present. However, in some cases, there is a tendency of recovery in some cladodes of symptomatic plants. But none of the recovered (symptomless) plants bear cladodes of normal size. Thus far, diverse protocols employed for phytoplasma detection in samples collected from these plants have failed. (P. Felker, unpublished data). In the present study a number of different nucleic acid techniques were compared in order to verify presence and identity of phytoplasmas in such 'fastidious' plant material.

Materials and methods

Phloem tissues dissected from cactus pear samples showing stunting and cladode proliferation symptoms and from similar tissues of asymptomatic plants were employed for nucleic acid extraction following four protocols: chloroform/phenol, CTAB (Angelini *et al*., 2001; de la Cruz *et al*., 1997) and "DNaesy plant Mini kit Qiagen". Baby cladodes (2-3 days old) were also collected from the same plants after rooting in greenhouse and nucleic acid was extracted with the same 4 methods described above. Nucleic acid was then maintained at 4 °C and employed at the concentration of 20 ng in direct PCR with universal primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995), followed by nested PCR with primers F1/B6 (Davis and Lee, 1993; Padovan *et al*., 1995), and by a second nested PCR with R16F2/R2 (Lee *et al.*, 1995). RFLP analyses with selected restriction enzymes, and comparison with phytoplasma reference strains (Bertaccini *et al*., 2000) was then carried out for identification of associated phytoplasmas.

Another extraction procedure was developed to reduce the mucilage content in the preparations by using baby cladodes from greenhouse (see above) and on young fruits from naturally infected plants growing under field conditions. For these experiments 1 g of fresh tissue from phloematic areas of cladodes and of small fruits was ground with liquid nitrogen in sterile mortars with a grinding buffer as described by Lee *et al*., (1991); after centrifugation at 11,400 rpm for 30 minutes in a Beckman "Allegra 45" centrifuge the supernatant was employed in PCR amplification with the protocol described above after 1 : 30 - 1 : 60 dilution in sterile distilled water.

Nested PCR yielded two amplicons, one amplified with nested primer pairs F1/B6 and the other with R16F2/R2. RFLP analyses of the two amplicons were performed. Selected amplicons were purified with "QUIAquick" PCR purification kit and sequenced both directions. Sequences were aligned with those of phytoplasmas available in GenBank and percentage of homology was verified.

Results

All the four protocols for nucleic acid extraction failed to yield nucleic acid preparations from symptomatic or asymptomatic cactus pear materials that were suitable for PCR amplification.

Using the diluted supernatants in PCR, amplicons (putative phytoplasmas 16S rDNA fragments) with ex-

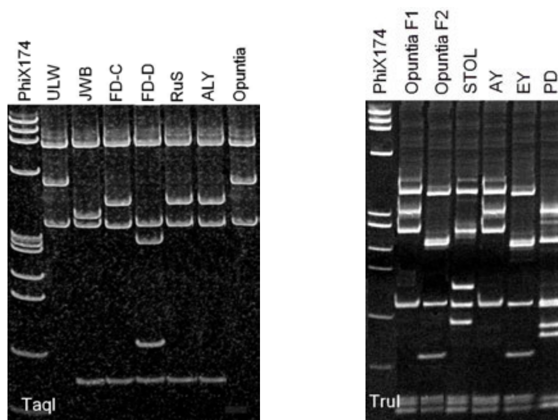

Figure 1. RFLP digestion of F1/B6 (left) and R16F2/R2 (right) amplicons from Californian cactus pear.

pected length bands were obtained in first nested PCR with F1/B6 primers (about 1,700 bp) and in second nested PCR with R16F2/R2 primer pairs (about 1,200 bp). RFLP analyses with *Taq*I, *Rsa*I, *Bfa*I and *Tru*I restriction enzymes on F1/B6 (figure 1) and on R16F2/R2 amplicons allow to identify phytoplasmas belonging to ribosomal subgroup 16SrV-A in naturally infected baby cladodes growing in greenhouse and in one of the fruits from the same plant collected in the field. The same phytoplasmas were identified in a fruit collected in field from a different plant showing partial stunting symptoms. A phytoplasma belonging to ribosomal subgroup 16SrI-B was also detected in another fruit collected from naturally infected plants.

Sequencing of F1/B6 and of R16F2/R2 amplicons from naturally infected plants and from one of its fruits allow to verify that both sequences share 99% homology with phytoplasmas belonging to ribosomal group 16SrV and share 100% homology between them. Virtual RFLP confirmed the presence of 16SrV-A phytoplasmas i.e. phytoplasmas related to '*Candidatus* Phytoplasma ulmi' (Lee *et al*., 2004).

Discussion

Detection of phytoplasmas in symptomatic cactus pear plants from California confirms that proliferation and stunting symptoms are associated with the presence of phytoplasma. The finding of phytoplasmas distinct from 16SrII-C previously reported to be associated with this disease of cactus pears, indicates that diverse phytoplasmas can be associated in cactus pear with similar symptoms. Moreover, this is the first report of identification of an elm yellows phytoplasma in cactus pear, while aster yellows phytoplasmas (16SrI-B) were recently described in one symptomatic plant of *Opuntia* sp. from a village of Yunnan region in China (Wei *et al*., 2007). Further improvement of the extraction methodology will allow to extensive survey in Californian fields to reduce the disease impact in cactus pear cultivations.

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