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MOLECULAR DETECTION OF PHYTOPLASMA ASSOCIATED WITH GREEN PÉTALO IN FRESA (Fragaria x ananassa Duchense) IN ZAMORA, MICHOACÁN

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Abstract: In millet plants (Fragaria x ananassa Duch.), which exhibited symptoms of early reddening in the nursery and cultivated fields, which were collected from fields in the Valle de Zamora, Michoacán, Mexico, the presence of phytoplasmas was detected using the reaction technique anidated polymerase chain (PCR). In a first reaction with the pair of P1/P7 strainers and a second amplification (anidated PCR), of the P1/Ptint strainers, where it was possible to amplify a product of 800-850 bp. In addition, high fluorescence signals of prokaryotic bodies were observed using the DAPI staining technique. Plants with virescent flowers and plants with fruits that exhibit symptoms of non-infectious phyllody were also subjected to PCR analysis, with negative results. The samples collected were submitted to PCR with the use of specific strainers fSTOL/rSTOL, for the detection of the Stolbur phytoplasma group in a mill where the Australian phytoplasma diseases (AUSGY), lethal yellowing (StrawLY) and belonging diseases are grouped al group 16Sr XIII, virescence of the Mexican vinca (MPV) group16SrXIII-A, green petal of the fresa (SGP) group16SrXIII-B. Prokaryotic DNA was not detected with the use of the PCR technique and specific strainers fSTOL/rSTOL and DAPI staining, therefore, the presence of phytoplasmas grouped in the Stolbur phytoplasma group in samples collected in vivarium and milling fields is ruled out.
Keywords: Phytoplasm, mill, green petal, phyllody.

INTRODUCTION

Mexico ranks sixth as a world producer of milling cutters, with 10,056 harvested hectares with a volume of 392,339 tons of fruit, and an important generator of employment with an average of 750 newspapers per hectare (FAO, 2010) and (SIAP, 2016).). The state of Michoacán occupies an important place as a producer of this fruit, currently it is a strategic crop in Bajío Michoacano, comprising a sowed area of 5,870 hectares mainly in the areas of (Maravatio, Jaconá, Tangancícuaro and Zamora) and an average yield of 43.86 t/ha, within the most outstanding municipalities is Zamora with an area of 1300 t/ha and yields of 56 t/ha (SIAP, 2016).

The root diseases caused by fungi and pseudofungals are the main phytosanitary limiting factors of the cultivation of the fresa (Fragaria x ananassa Duch.) Ceja et al. (2008). In recent years, a new type of alteration has been observed in milling plants, considering severe diseases, possibly caused by phytoplasmas (formerly called mycoplasma-like organisms MLOs). The phytoplasmas according to their specificity (they infect plants and insects), and are considered as the causal agents of yellowing, are limited to the phloem and are transmitted by insects, plant pathogenic bacteria that are responsible for disease scientes worldwide (Weintraub, 2007).

Plants infected by phytoplasmas present a wide variety of symptoms, these include virescence (development of green flowers and loss of normal flower pigments), phyllody (development of floral parts in leafy structures), sterility of flowers, proliferation of adventitious buds similar in appearance to those caused by "bruja brushes", abnormal elongations of the entrenudos and as a result slender shoots, a generalized reduction of "dwarfism" growth (flowers and small days and short entrenudos), discoloration of hojas and buds, curved hojas, or cup-shaped, decline (yellowing dwarfism or early reddening of las hojas) (Davis y Lee, 1982; McCoy et al., 1989; Bertaccini, 2007; Hogenhout et al., 2008).

It is common to find symptoms similar to those caused by phytoplasmas corresponding to Phylodias virescence of flowers, transformation of floral to foliar structures in the totality of commercial varieties, but there

are different symptoms to the latter. They differ and are very likely their association with one or more organisms, in the case of the viruses which have caused symptoms never seen before, as well as new viruses recently reported in California, USA (Tzanetakis et al., 2003). Obtaining plants in a vivarium is obtained from the importation of registered mother plants from California USA, and from direct refrigerated plants from nurseries in Argentina and Chile. In recent years, it has been frequent to observe symptoms similar to those caused by phytoplasmas and viruses in vivariums and fields of milling. In some cases they can be more serious than the most common diseases that are normally caused by fungi and bacteria in plants that are imported/exported, and it is for these reasons that the objective of the following work is to detect the presence of phytoplasmas through the use of nucleic acid staining techniques (DAPI) and polymerase chain reaction (PCR) on miller plants (Fragaria x ananassa Duchense).

MATERIALS AND METHODS

COLLECTION OF SICK AND ASYMPTOMATIC VEGETATIVE MATERIAL

Samples of plants with characteristic symptoms of phytoplasmas were collected (early decay, which is characteristic at the reach of the maturity of the vivarium plants), yellowing of the aster, green petal, phyllody, non-infectious phyllody and asymptomatic plants. In the field, the symptoms are manifested in February to March in commercial crops and asymptomatic plants in nurseries in the month of August in the Ejido de Atecucario municipality of Zamora. Plants of the Camarosa and Aromas varieties were collected, and they were collected and transported in plastic bags or in a dry cell. Samples were collected from plants with vivarium symptoms, four plants from the Camarosa y Aromas varieties (Figure 1).

The DNA samples were amplified by PCR with specific oligonucleotides rStol/fStol and universal oligonucleotides PI/P7, P1/Tint. The PCR products were sequenced for phytoplasmal DNA amplification. The DAPI and PCR analyzes were carried out in the Biotechnology laboratories of the Graduate School in Montecillo, State of Mexico.

PHYTOPLASMA DETECTION BY MEANS OF DAPI STAINING

Millet plants with symptoms of curling, early reddening, aster yellowing, green petal, phyllody and non-infectious phyllody, as well as asymptomatic plants collected in the field, were disinfected with 1% sodium hypochlorite for 10 min and then rinsed three times in sterile distilled water. 20 pieces of 10 mm of root and stem were cut with symptoms, longitudinal cuts were generated with a freezing microtome, of a thickness of 30 µ. The sections were dyed with 4,6-diamidino-2-phenylindole (DAPI Sigma Co., USA) in a 0.1 M phosphate buffer pH 7.0 (1mg/mL), and observed under a Zeiss flourescence microscope, with a mercury lamp of high pressure steam (HBO 200 w w/4), with a B1 excitation filter (IF of 450-490 nm of wavelength) and an LP 520 nm barrier filter. The presence of phytoplasma was evaluated indirectly by the intensity of the flourescence in the phloem.

DETECTION BY THE MOLECULAR METHOD OF PCR

The total extraction of deoxyribonucleic acid (DNA) was performed from leaflets of plants with symptoms as well as asymptomatic plants. The DNA of the milling plants of the Camarosa y Aromas variety is extracted using the methodology (Lee et al., 1993). The DNA obtained was quantified by means of a spectrophotometer, it was also diluted in sterile deionized water to obtain a concentration of

Figure 1. Symptoms in milling plants, caused by phytoplasmas associated with the green petal.

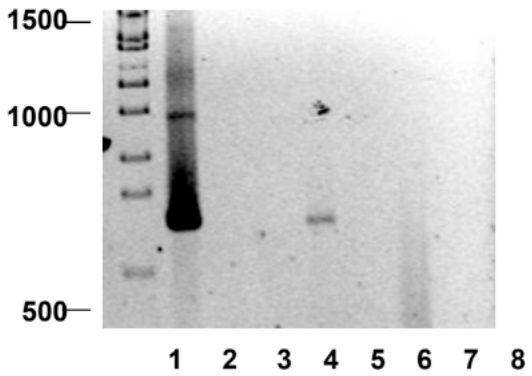

Figure 2. Electrophoresis of agarose gel of DNA amplified by chain reaction of the polymerase (PCR), and the amplification of the sequences 16 Sr DNA, with the pair of primers P1/Tint, Line 1, 1 kb Ladder, molecular marker; Line 2, positive testigo vinca plant Catharanthus roseus with viriscencia; Line 3, asymptomatic plant; Line 4, aster yellowing; Line 5, early reddening; Line 6, fruit plants with non-infectious phyllody; Line 7, plant with virescent flowers; Line 8, negative water control

20 ng/µl. 80 ng of the dilution obtained was taken and then subjected to the polymerase chain reaction (PCR) for DNA amplification using a pair of specific starters fStol/RStol, and universal starters P1/P7, after which some reaction of the product was diluted (1:30) and then added again to a second reaction with the pair of P1/Tint feeders, using 25 ng in a final volume of 25 µl. Direct PCR and anidated PCR were performed using an automatic thermocycler (Perkin-Elmer Cetus, Norwalk, model., 2400) under the following conditions: first initial denaturalization cycle at 94 °C for 2 min, 35 later cycles at 94 ° C for 1 min of denaturalization, 2 min of alignment at 55 °C, a first extension for 3 min at 72 °C and a final extension of 7 min at 72 °C. DNA from asymptomatic plants (R) and DNA from vinca plants Catharanthus roseus with symptoms of virescence (P) were used as a negative test. 7 µl of the DNA products obtained by direct and anidated PCR were loaded, run in a 1% agarose gel at a load of 85 volts, after which the agarose gel was placed in a tray with ethidium bromide and subsequently The detection of the DNA bands corresponding to phytoplasmas was carried out using the photo documentation system (Gel Doc 2000, Bio Rad), taking as reference the molecular weight of the 1Kb marker (GIBCO BRL).

The separation of amplified PCR products is performed by electrophoresis on high resolution agarose gels. For the resolution in agarose gels, 20 cm wide x 24 cm wide, an apparatus was used (e.g. BRL H4 horizontal gel apparatus Gibco BRL #1025RD).

Preparation at 1.5% agarose gel using TAE dampener. (Tris M, pH 8.0, Tris base 242 g per litre, 57.1 ml of glacial acetic acid and 18.61 g of Na2EDTA).

The nervaduras de la today and petiolos were sectioned (15 fragments of each plant, each 2 mm in length) from a total of 12 plants of the 2 varieties grown in Zamora (Aromas and Camarosa) all of them showing the symptoms of reddening and radical necrosis, fixed in darkness in 4% glutaraldehyde in 0.1 M cocodylate buffer (pH 7.5), fixed in 1% ammonium tetraoxide, dehydrated in a series of ethanol (25-100%), and soaked in epoxy resin (Epon 812) according to the method described by (Luft, 1961).

RESULTS AND DISCUSSION

RESULTS BY MEANS OF DAPI COLORANT

The staining technique (DAPI) allowed the diagnosis of phytoplasmas in millet plants collected in fruit production fields with symptoms of reddening, yellowing of the aster, green petal, phyllody, non-infectious phyllody and asymptomatic plants. Plants with early reddening symptoms showed a high fluorescence of phytoplasmal DNA in the conductive tissues in the phloem, this was not observed in samples of asymptomatic plants, and with symptoms of common phyllody, non-infectious phyllody, yellowing of aster, green petal. On the other hand, the uneven distribution or low concentrations of phytoplasmas in plants, as well as the time of collection are factors that determine the success of this technique (Nourrisseau et al., 1993). In recent years, the DAPI or fluorochrome technique has been considered an uncommon and reliable technique for the detection of Mollicutes (Lee et al., 1993) and (Sinclair et al., 1989) considers that this technique has some specificity. DAPI is a low cost technique and a rapid method to detect bacterial bodies in different plant tissues, but it is not a specific tool that can detect the DNA of other microorganisms or organelles such as mitochondria and chloroplasts (Franova et al., 2007).

RESULTS BY PCR

Direct PCR

Of the 10 samples that were subjected to direct PCR, with the game of specific strainers fSTOL/rSTOL and universals in direct P1/P7 PCR, none of them managed to amplify phytoplasmas in samples with early reddening of today, plants with fruits with phyllody, green petal, non-infectious phyllody, amarillamientos del aster and positive testigo vinca mexicana.

nested PCR

The products that had been amplified by PCR in a first reaction with the game of universal staplers P1/P7, from these a second re-amplification of the products of the direct PCR with the game of universal staplers P1/Tint was carried out, and this time managed to evidence the presence of phytoplasmas in plants with symptoms of reddening cv. Camarosa and positive testigo vinca mexicana, to amplify a DNA band with a molecular weight of 800 bp to 850 bp (Figure 2).

The presence of different phytoplasmas in green petal, and a second re-amplification with P1/P7 strainers in the region 16Sr prokaryotic DNA, is confirmed by the use of the P1/Tint universal strainers using molecular tests by PCR and DAPI fluorescence. molecular weight product (800-850 bp) and its presence in samples of diseased plants in cultivations of the Camarosa variety with symptoms of premature reddening, root necrosis, flower abortion.

It was not possible to amplify specific phytoplasmas in samples of asymptomatic plants in production fields, the flowers of these plants especially in the petals, in the manifestations of virescence symptoms, which are common due to the infection of phytoplasmas such as the green petal and the yellowing aster, without embargoing the fruits near its maturation if symptoms of phyllody were manifested. The symptoms in sick plants and the PCR results in this work coincide with reports in other countries where the illness has been detected. Lethal decline in miller or disease-X, affecting plants in the nursery and mill production fields in regions of California USA, and has also been seen to cause damage to other hosts belonging to the Rosaceae family such as cherry and durazno (Schwartze et al, 1987). Harju et al. (2007) reported the presence of disease-X, in plants of (Delphinium sp) where they obtained a PCR product of (834bp), identifying the pathogen as a possible member of the disease-X, of the group (16SrIII) 'Candidatus Fitoplasma pruni '. There are reports of other species of phytoplasmas which can be confused with the X-illness, such is the case of the fasciación del stalk de la fresa, known as Phormium yellow leaf or flat stalk, more than one pathogen with certain similarities but taxonomically were found distinct and through the use of R16F2-R16R2 and NGF-NGR starters (Andersen et al., 1998). Symptoms similar to Lethal Decline have been reported in Francia and España Nourrisseau et al. (1993), the only causal agent is Phlomobacter fragariae.

1. The presence of phytoplasmas was detected in plants of the Camarosa variety nursery with symptoms of reddening and root necrosis, by means of DNA re-amplification by annidated PCR, with the games of universal cebadores P1/P7 and P1/Tint, if obtuve a product of 800-850 base pairs.

2. The protobacterial organism amplified by PCR nested in this work, does not correspond to the groups of virescence of the Mexican vinca (MPV) group16SrXIII-A, green petal of the fresa (SGP) group16SrXIII-B, as well as the groups belonging to them aster yellowing (AY), group 16SrI, clover phyllody 16SrI-C and the STOL group, 16SrXII C. Phytoplasma australiense (AUSGY), lethal

yellowing (StrawLY), in samples collected where specific strainers fSTOL/rSTOL were used for su detection.

3. It was not possible to amplify DNA of phytoplasma-like organisms in plants that exhibited symptoms of non-infectious phyllody, green petal, with the use of universal starters, P1/Ptint P1/P7 and specific fSTOL/rSTOL.

4. It is suggested to carry out more work to group to this species of different phytoplasmas the virescence of the Mexican vinca (MPV) and the green petal of the fresa (SGP).

CONCLUSIONS

1. The presence of phytoplasmas was detected in plants of the Camarosa variety nursery with symptoms of reddening and root necrosis, by means of DNA re-amplification by annidated PCR, with the games of universal cebadores P1/P7 and P1/Tint, if obtuve a product of 800-850 base pairs.

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