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RESEARCH PAPER

PCR based detection of phytoplasma association in pot marigold (*Calendula officinalis* L.) and guldawari (*Dendranthema grandiflora* L.)

ANCHAL RANI[1], **PRAGATI MISRA**[1], JITENDRA SINGH[2], PANKAJ KUMAR[2], ROSY RANI[1] AND PRADEEP SHUKLA[1]

[1]Sam Higginbottom Institute of Agriculture, Technology and Sciences, ALLAHABAD (U.P.) INDIA
Email : pragati.misra@shiats.edu.in
[2]College of Biotechnology, Sardar Vallabhbhai Patel University of Agriculture and Technology, MEERUT (U.P.) INDIA

Flowering plants *Calendula officinalis* with phyllody and virescence and *Dendranthema grandiflora* with little leaf and formation of bladder like silique symptoms observed in Uttar Pradesh, India. The presence of phytoplasmas in diseased plants was detected by direct and nested polymerase chain reaction assays using phytoplasma-specific primer pairs P1/P7 and R16F2n/R2. In both flowering plants presence of phytoplasma was confirmed by amplification of 1200 bp product of phytoplasma 16S rRNA region with nested primer R16F2n/R2. This is the first report of phytoplasma associated with *Calendula officinalis* from India.

Key words : Phytoplasma, Virescence, Phyllody, Nested PCR

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INTRODUCTION

In 2012 chrysanthemum and pot marigold plants collected from Uttar Pradesh, India with severe symptoms of phytoplasma. Phytoplasmas are unculturable plant-pathogenic, wall-less bacteria (mollicutes) that cause diseases in several hundred plant species worldwide (McCoy *et al*., 1989). Similar symptoms in *chrysanthemum* were previously reported from New Delhi, India (Kumar *et al*., 2012). Chrysanthemum virescence caused by "*Ca.* P. aurantifolia" reported from Okinawa (Naito *et al*., 2007). Pot marigold plants phyllody and virescence symptoms were previously reported from Iran (Esmailzadeh-Hosseini *et al*., 2008, 2011). Phytoplasmas belonging to aster yellows (16SrI) group were identified in diseased pot marigold from Italy (Marcone *et al*., 1997) and Canada (Wang and Hiruki, 2001).

Little work has been done on occurrence and identification of phytoplasma in ornamental plants in India. Little leaf disease of *Portulaca grandiflora* reported by Ajaykumar *et al*. (2007) and Samad *et al*. (2008). Raj *et al*. (2007a, 2007b, 2009) observed phytoplasma disease in *Chrysanthemum morifolium, Adenium obesum*, and *Gladiolus* at Lucknow. Chaturvedi *et al*. (2009a, 2009b; 2010b) reported little leaf disease in *Rosa alba, Catharanthus roseus,* and *Hibiscus rosasinensis* in Gorakhpur. Aster yellows group of phytoplasma associated with *Alstroemeria hybrids, Duranta erecta, Steblus asper, Petunia hybrida* and *Zinnia elegans* from Uttrakhand and Gorakhpur district of Uttar Pradesh reported by Singh *et al.* (2011).

Dendranthema and *Calendula* are perennial plants of the family Asteraceae.*Calendula* is considered as among the easiest and most versatile flowers to grow in a garden, especially since they tolerate most soils. It is difficult to say with certainty when its culture began in India. In India, large flowered varieties of chrysanthemum are grown for exhibition purpose while small flowered varieties are grown for cut flower, making garland, wreaths, veni and religious offerings.

Calendula flowers were used in ancient Indian cultures as a medicinal herb as well as a dye for fabrics, foods and cosmetics. They are also used to make oil that protects the

skin. Plant pharmacological studies have suggested that *Calendula* extracts may have anti-viral, anti-genotoxic and anti-inflammatory properties*in vitro* (Jimenez-Medina *et al*., 2006). In an*in vitro* assay, the methanol extract of *C. officinalis* exhibited antibacterial activity and both the methanol and the ethanol extracts showed antifungal activities (Efstratiou *et al*., 2012).

The aim of this study was to determine the possible association of the phytoplasmas with symptoms of phyllody and virescence with *Calendula officinalis* and little leaf and formation of bladder like silique symptoms with *Dendranthema grandiflora* in India.

RESEARCH METHODOLOGY

Survey of infected plants :

A survey was carried out in Uttar Pradesh to collect the phytoplasma infected plants. Infected and healthy plants of pot marigold collected on April, 2012 from Meerut and chrysanthemum on May, 2012 from Allahabad, India.

DNA extraction :

Total DNA was isolated from fresh leaf, midrib and flower petals using the plant genomic DNA extraction kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions and the DNA was stored in sterile distilled water at -20°C. The concentration of DNA in different plant samples were estimated using UV- spectrophotometer (UV-1800, Shimadzu) and DNA rich sample was used in PCR reaction.

Polymerase chain reaction :

Three phytoplasma universal primer pairs designed from rRNA operon region P1/P7 (Deng and Hiruki, 1991), P1/Tint (Smart *et al*., 1996) and R16F2n/R2 (Gundersen and Lee, 1996) were used to prime phytoplasma. Direct hot start PCR amplifications were carried out with universal primer pair P1/P7 (5'AAG AGT TTG ATC CTG GCT CAG GATT3'/ 5'CGT CCT TCA TCG GCT CTT3') or P1/Tint (5'AAG AGT TTG ATC CTG GCT CAG GAT T3'/ 5'TCA GGC GTG TGC TCT AAC CAG C3') separately in a thermocycler (Biometra, T Personal 48). Each 50 µl reaction containing 1X PCR buffer, 2mM $MgCl_2$, 200 µM of each dNTP (MBI, Fermentas), 100mM P1/P7 or P1/Tint primers and 1U of Taq DNA polymerase (MBI, Fermentas) consisting of denaturation at 94°C for 10 min, 35 cycles at 94°C for 30 sec, annealing at 55°C for 60 sec, extension at 72°C for 90 sec and a final extension step at 72°C for 10 min.

To increase the sensitivity of the PCR the universal primer pair R16F2n/R16R2 (5'GAA ACG ACT GCT AAG ACT TGG3'/5'TGA CGG GCG GTG TGT ACA AAC CCC3') was used in the nested-PCR, to amplify a portion of 16S rDNA gene. In the nested-PCR assay, DNA amplified by direct PCR with primer pair P1/P7 was used as template, which primes a segment of the 16S rDNA gene approximately 1200 bp in size. For nested PCR reaction initial denaturation performed for 10 min at 94°C and than performed thirty five PCR cycles with the following parameters; 30 sec at 94°C, 60 sec at 50°C, 90 sec at 72°C and a final extension at 72°C for 10 min. The 50 µl reaction mix contained 10X PCR buffer, 25 mM $MgCl_2$, 2 mM each dNTP, 100 ng/µl R16F2n/R2 and 1U of Taq DNA polymerase. Amplified DNA was separated by electrophoresis in 0.8 per cent agarose gel.

RESEARCH FINDINGS AND ANALYSIS

The results obtained from the present investigation as well as relevant discussion have been summarized under the following heads :

Survey of infected plants :

During survey phytoplasma like symptoms were observed in infected pot marigold and chrysanthemum plants with symptoms including phyllody and virescence (green petals on the place of yellow) symptoms in pot marigold and little leaf and formation of bladder like siliques in chrysanthemum (Fig. 1). Disease incidence was recorded 60-65 per cent and 16-18 per cent in pot marigold and chrysanthemum plant, respectively. Healthy plants showed vigorous growth and were free from the above mentioned symptoms.

DNA extraction :

Total DNA of chrysanthemum and pot marigold symptomatic and asymptomatic plants from leaf, midrib and flower petals was extracted. Estimation showed that chrysanthemum and pot marigold DNA quality was good and average concentration was 0.400µg/µl and 0.405µg/µl, respectively.

Polymerase chain reaction :

Presence of phytoplasma was detected by PCR. Plants DNA was preceded initially with P1/P7 and P1/Tint universal primers. In leaf and midrib samples of diseased plants, shearing was observed on the place of 1800 bp with only primer pair P1/P7 in both plants, and no amplification found with P1/Tint primer pair. These shearing PCR products amplified with P1/P7 used as a template in nested PCR and a ~1200 bp fragment was amplified (Fig. 2) in both plants, but no amplification found in healthy plants and flower petals sample of diseased plants. Pot marigold and chrysanthemum R16F2n/R2 amplified, 16S rDNA product (~1200bp) were gel eluted and send for sequencing. But due to low concentration of PCR products sequencing was not successful. Results revealed that all symptomatic plants selected for phytoplasma disease detection tested positive.

Fig. 1 : (a) Little leaf and formation of bladder like siliques in diseased plant of *D. grandiflora* collected from Allahabad (b) Infected plant of *Calendula offcinalis* resembles symptoms of virescence, green petals of flower on the place of yellow petals collected from Meerut.

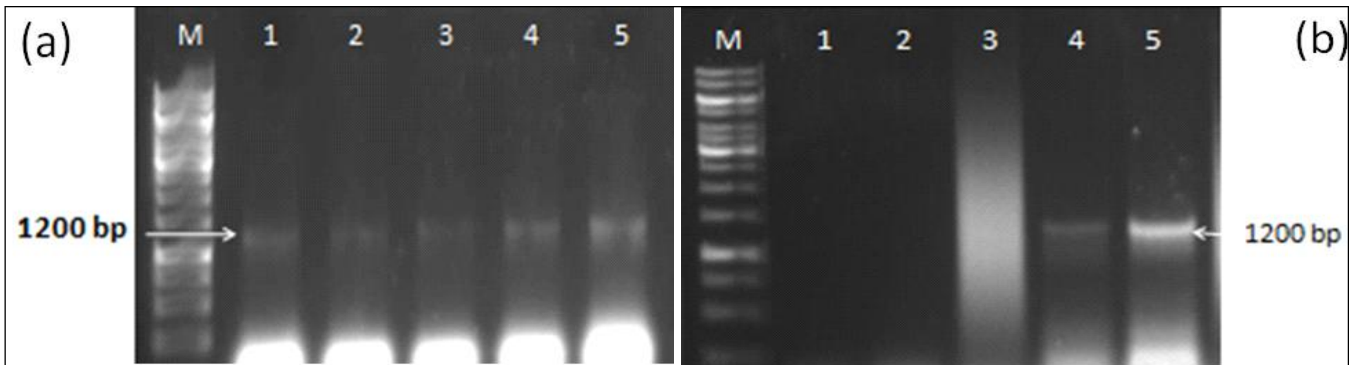

Fig. 2 : Agarose gel electrophoresis of 16S rDNA PCR products obtained with primers R16F2n/R2 in *D. grandiflora* and *C. officinalis*. (a) Lane M: 1kb DNA ladder (fermantas), Lane 1, 2, 3, 4 & 5: ~1200 bp amplified product in all five infected *D. grandiflora* plants. (b) Lane M: 1 kb DNA ladder (fermentas), Lane 1, 2 & 3: no amplification in pot marigold, Lane 4 & 5: ~1200 bp amplicon confirm the phytoplasma presence in *C. officinalis*.

Nested PCR assay, designed to increase both sensitivity and specificity, is indispensable for the amplification of phytoplasmas from samples in which unusually low titers are present that may interfere the PCR efficacy (Garcia-Chapa *et al*., 2004). Phytoplasma is a problematic issue due to its high mobility through insect vectors involved in the transmission of this disease. Interactions between phytoplasma, vector and plants have serious implication because infected plant can serve as reservoir for phytoplasma and sources of infection to other economically important crop plants. Observation of phytoplasma like symptoms in *C. officinalis* and *D. grandiflora* ornamental plants marked them as hosts for phytoplasma. Only a low titer of phytoplasma was observed in both infected plants, therefore, sequencing was unsuccessful of amplified 16SrDNA sequence of chrysanthemum and pot marigold. Symptoms reported in *D. grandiflora* were previously reported from India, but this is the first report of phytoplasma infection in *C. officinalis* from India.

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