International Journal of Advanced Research in Biological Sciences
ISSN: 2348-8069
www.ijarbs.com
DOI: 10.22192/ijarbs Coden: IJARQG(USA) Volume 4, Issue 7 - 2017

Research Article

DOI: http://dx.doi.org/10.22192/ijarbs.2017.04.07.024

Universal and Specific 16S-23Sr RNA PCR Primers for Identification of Phytoplasma associated with sesame in Egypt.

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Abstract

Sesame (*Sesamum indicum* L.), family *Pedaliaceae* is one of the most ancient cultivated oilseed crops among the edible annual group in Egypt and all over the world. Phytoplasmas are pathogens of many plant species throughout the world including sesame causing a large variety of symptoms ranged from mild yellowing to death of infected plants. Symptomatic samples including green leaf-like floral organs, virescence, phyllody and proliferation were collected from infected sesame field in Giza Governorate. This research was undertaken to develop a diagnostic tool to identify phytoplasmas recently discovered infecting sesame in Egypt, and to classify them according to their phylogenetic group. Direct and nested PCR primers of 16S-23SrRNA gene, paired with phytoplasma universal primer followed by strain-specific PCR primers were used in this study. Spacer Region (SR) primers were employed for identification of Phytoplasma group associated with sesame symptoms. Results showed the presences of mixed infections of phytoplasma in the tested sesame samples which collected from the field with different type of Phytoplasma like symptoms. Results also indicated that polymerase chain reaction with primers from sequencing of 16S-32S rRNA and from SR opened new paths for research on phytoplasma identification and classification. Nested PCR has been applied to overcome problems related to sensitivity of phytoplasma detection, although this approach is more time consuming and subject to template. Unfortunately, nested-PCR also meets some difficulties: unspecific bands, false positives or negatives caused by DNA and contamination of single or nested PCR. Therefore, confirmation of PCR results by using different primer pair combinations (generic and group-specific) seems to be the way for correct phytoplasma identification in the examined sesame samples.

Keywords: Sesame, Symptoms, Phyllody, Phytoplasma detection, Universal primer, Specific primer, PCR amplification.

Introduction

Around alternate oilseed field crops, sesame (*Sesamum indicum* L), otherwise known as sesamum or benniseed, member of the family *Pedaliaceae*, is referred to a standout among the vital harvests in the reality to eatable oil production**.** It is one of the oldest oilseed crops known to mankind and grown in the countries where hand labor is inexpensive throughout the tropics and subtropics **(Cagirgan *et al*., 2013).** It is produced mainly in India, Myanmar, Tanzania, China, Sudan, Pakistan, Ethiopia, Paraguay, Uganda, Nigeria, Niger, Thailand, Furthermore Turkey (**Anonymous, 2010**). In Egypt, sesame is considered a food crop rather than oilseed crop because most of its seeds are consumed directly. It is grown in many

governorates and ranks first among the cultivated oil crops in Ismailia Governorate **(El-Bramawy, 2006).** Total area under sesame production in Egypt has increased from 11,264 ha in 1961 to 36,907 ha in 2010 and the productivity increased from 1,145.7 kg/ha in 2005 to 1,250.3 kg/ha in 2010 **(Faostat, 2012)**. Sesame oil is non-drying oil; highly stable rarely turning rancid in hot climates. It is very rich in protein, a polyunsaturated fat used in margarine production and cooking oils. Non-culinary uses include its use as an ingredient in soap, cosmetics, lubricants and medicines. Sesame is suitable for various cropping systems, but the major factor that limit its cultivation are seed shattering at maturity which requires manual harvest making it difficult to manage large areas and susceptibility to diseases **(Silme and Cagirgan 2010).**

Among the diseases, phyllody is a destructive disease of sesame which causing significant economic losses by altering their floral part into leafy structures with no capsule and therefore no seeds consist **(Kolte,1985; Akhtar *et al.*, 2008)**. The disease caused by phytoplasmas discovered in 1967 and named mycoplasma-like organism (MLO). Phytoplasmas belong to class Mollicutes gram positive (low-G+C), lacks cell wall and instead is bound by a triple-layered membrane. The genome size of phytoplasmas ranges between 530 and 1350 kb **(Doi *et al.*, 1967)**. The typical phytoplasma exhibits a pleomorphic or filamentous shape and is less than 1 µm in diameter. As prokaryotes, phytoplasmas' DNA is found throughout the cytoplasm, rather than being concentrated in a nucleus. Phytoplasmas cause diseases in several 100 plant species including vegetable, cereal, fruit, ornamental and forest crops worldwide **(Lee *et al.*, 2000).**

A common symptom caused by phytoplasma infection is phyllody, the production of leaf-like structures in place of flowers. Evidence suggests the phytoplasma down-regulates a gene involved in petal formation (AP3 and its orthologous) and genes involved in the maintenance of the apical meristem (Wus and CLV1). Other symptoms, such as the yellowing of leaves, are thought to be caused by the phytoplasma's presence in the phloem, affecting its function and changing the transport of carbohydrates. During senior biotic constraints, phyllody was characterized by floral virescence and increase is major determine factor infecting sesame crop **(Tan. 2010)**. About 1% increase in disease thickness intensity reduces its output by 8.36 kg / ha **(Maiti *et al.*, 1988)**. Groups of phytoplasma 16SrI were identified in peach oncoming clarify rosette such symptoms at the CCG **(Zunnoon-Khan *et al.*, 2010)**, which perform a historic phytosanitary threat for other species considering that this is a phytoplasma group with a wide host range and a complex ecology **(Lee *et al.*, 2004)**. Within group 16SrI a number of subgroups have been identified. For accurate disease diagnosis it is important to identify phytoplasmas since the inability to culture it in vitro. PCR has proven to be a more versatile tool for detecting phytoplasmas in the host plant **(Ahrens and Seemuller, 1992; Schaff *et al* .,1992).** PCR primers have been developed from Phytoplasma-specific sequences within the 16SrRNA gene which is in some cases are very similar, thus identifying it is impossible, to design PCR primers that could identify a particular Phytoplasma **(Smart *et al.*, 1996)**.

The present study was carried out to characterize phytoplasmas at the 16S rRNA gene subgroup level and to use the spacer region SR primers as a specific diagnostic tool to identify Phytoplasma group manner.

Materials and Methods

Source of samples:

Sesame infected and healthy samples were collected from Giza Governorate, Egypt. Symptoms of phytoplasma like were recorded and all sampled applied directly for DNA extraction and molecular detection and identification.

DNA Extraction:

DNA was extracted from naturally infected and healthy sesame samples using a modified *Dellaporta* extraction method **(Dellaporta *et al.*, 1983)**. Approximately 1g of fresh tissue was used for each extraction. 500 µl of *Dellaporta* extraction buffer (100 mMTris pH 8.0, 50 mM EDTA, 500 mM sodium chloride and 10 mM β-mercaptoethanol), then vortex for 2 min. 33 microliters of 20% sodium dodecylsulphate (SDS) was added and vortex well, then incubated for 10 min at 65 C. 160 µl of 5 M potassium acetate was added and vortex, followed by centrifugation for 10 min at 10.000 rpm. The supernatant (450) was transferred carefully to new eppendorf tube. An equal volume of (PCI) phenol:chloroform:isoamyl alcohol, 25:24:1 (v:v:v) was added and vortexed for 5 min then centrifuged at 10,000 rpm for 5 min. The aqueous layer was transferred to new eppendorf tube. 0.5 Volume of isopropanol was added, centrifuged for 15 min at

15.000 rpm. 500 µl of 70% ethanol was added to wash the pellet and centrifuged for 5 min at 10,000 rpm. The pellet was exposed to air for 1 hr. then resuspended in 50 µl of dH_2O.

PCR reaction conditions for phytoplasma identification

PCR reaction mixture of 25 µl contained (3 µl) extracted DNA, 0.5 µl of each primer and 15.8 µl ddH_2O, 5 µl 5x MyTaq Reaction Buffer and 0.2 µl Paq5000^{tm}DNA polymerase, (STRATAGENE). First PCR using P1/ P7 universal primer was; Initial denaturation for 3 min at 94°, 35 cycles of the following steps: denaturation for 1 min at 94°C, annealing for 2 min at 53°C, extension for 2 min at 72°C and final extension for 5 min at 72°C. Second (nested and heminested) amplification consisted of 30 cycles of the following steps: denaturation for 1 min at 94^{o}C; annealing for 1 min at 48^{o}C (P1/BLTVAint and P1/WXint), or 56^{o}C (P1/AYint); and extension for 2 min at 72^{o}C. PCR-amplified DNA fragments were separated by gel electrophoreses in 1% agarose in 0.5x TBE buffer (Trisborate-EDTA, 90 mM trisa-cetate, 90 mM boric acid, 2 mM EDTA) and visualized with UV trans-eliminator after staining for 15 min with ethidium bromide **(Sambrook *et al.*, 1989)** and photographed using a Bio-Rad Gel documentation system.

Table (1). Sequences of the oligonucleotide primers used for PCR amplification.

Primer	Location [a]	Oligo nucleotide sequence	References
P1	16S	5'AAGAGTTTGATCCTGGCTCAGGATT3'	**Deng and Hiruki. 1991**
P7	23S	5'CGTCCTTCATCGGCTCTT3'	**Schneider *et al.*, 1995**
AYint	SR	5'TACAATTTGCAAGCAAGTTAC3'	**Smart *et al.*, 1996**
WXint	SR	5'GACAGTGCTTATAACTTTTA3'	
BLTVAint	SR	5'GATGATTTTAGTATATATAGTCC3'	

[a] Location of primer within the rRNA, 16SrRNA gene, 23SrRNA gene, SR spacer region

Results

Symptoms of Phytoplasma on Sesame plants:

Different type of phytoplasma symptoms were noticed on sesame plants. Phyllody disease symptoms observed in the samples collected from the field (Fig. 1), the most characteristic symptoms of the disease are transformation of floral parts into green leaf-like structures, followed by abundant vein clearing in different floral parts. The ovary is replaced by elongated structures, almost resembling a shoot. The calyx becomes polysepalar, and the sepals become leaf-like and remain smaller in size. Phyllody flowers become actio morphic in symmetry, and the corolla becomes polypetalous and deep green. The veins of the flower become thick and quite conspicuous. The stamens retain their shape, but become flattened, showing a tendency to be leaf-like. The anthers become green and contain abnormal pollen grains. The carpals are transformed into a leaf fusion at the margins, and this false ovary enlarges and flattens, exhibiting a soft texture and a wrinkled surface due to the thickening of capillary wall veins. Instead of ovules inside the ovary, there are small petiole-like outgrowths, which later grow and burst through the walls of the false ovary, providing small shoots (Fig.1).These shoots continue to grow and produce more leaves and phyllody flowers.

Fig.(1): Different symptoms of phyllody observed on sesame plants, naturally infected at different locations on the field: (A) Healthy sesame plant, (B) Phyllody ,(C)Phyllody with virescence, (D) Virescence, (E) Late Phyllody symptoms, (F) Floral Proliferation,(G) Shoots showing internodes shortening with dense leaves, (H) Phyllody and (I) Shoots showing green leaf like floral organs with virescence.

Sesame plants also showed symptoms like witches broom (Fig.2) gain bushy appearance, yellowing and apical dominance and this may due to the stress placed on the plant by infection, rather than specific pathogencity of the phytoplasma leading to changes in their normal growth patterns. Phytoplasma strain symptoms vary by timing of infection, plant species, and age of the plant and environmental conditions which may lead to increase severity of the symptoms. Many adventitious shoots with short internodes looking like aster yellow symptoms also observed (Fig.3).

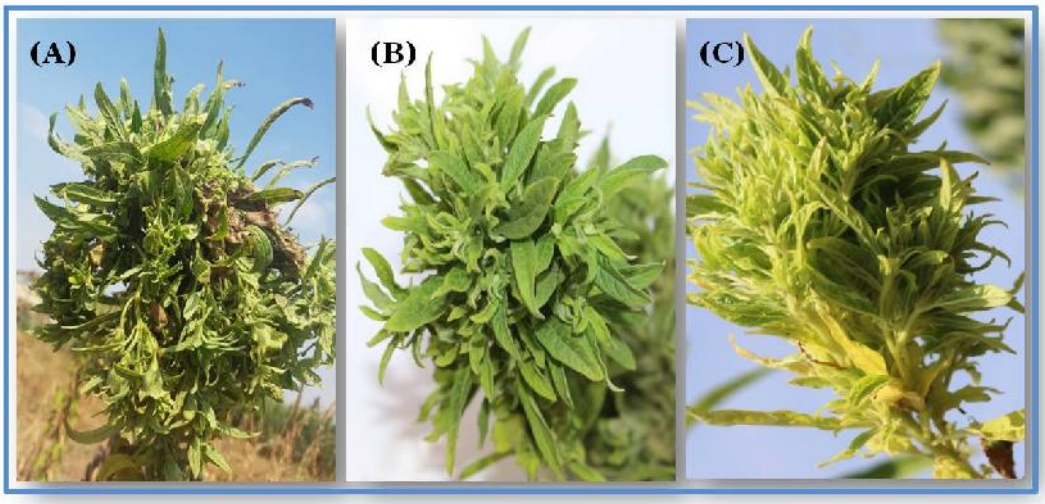

Fig.(2): Witches broom symptoms and short internodes with yellow, reduced, twisted leaves on sesame plants

Fig.(3): (A) and (B) Aster Yellow Phytoplasma group symptoms on sesame plants, (C) Cracked capsules with no seeds or germinated seeds.

Detection and identification of Phytoplasma:

Nucleic acid amplification method Detection and identification of phytoplasmas is necessary for accurate disease diagnosis. Sensitive methods need to be implemented in order to monitor the presence and spread of phytoplasma infections. Hence, it is necessary to devise a rapid, effective and efficient mechanism for detecting and identifying these microorganisms. Molecular diagnostic techniques for the detection of phytoplasma introduced during the last two decades have proven to be more accurate and reliable than biological criteria long used for phytoplasma identification. Polymerase Chain Reaction (PCR) is the most versatile tool for detecting phytoplasmas in their plant and insect hosts. One of the most utilized protocols for phytoplasma detection and characterization encompasses nested-PCR. Universal phytoplasma primer P1/P7 were used as first PCR detection (Fig.4) this primer amplified about 1800bp from all phytoplasma infected plants.

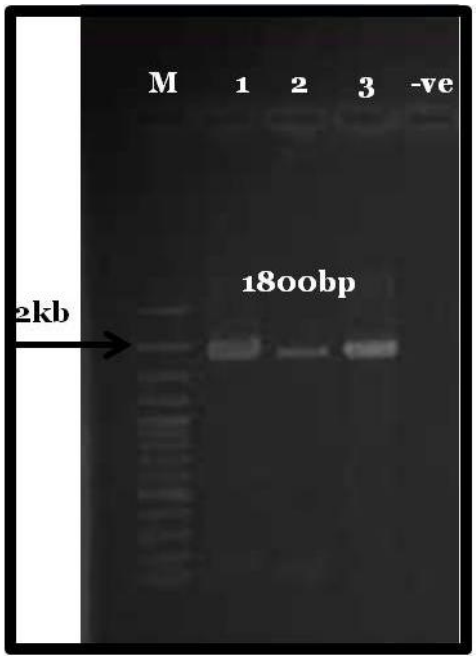

Fig.(4): Agarose gel electrophoreses analysis of PCR using the universal phytoplasma primer pair P1/P7, derived from highly conserved ribosomal sequences amplify a DNA fragment of approximately 18000 bp in length from all phytoplasma infected sesame plants.

Nested PCR:

The use of nested-PCR has been reported for diagnostic purposes particularly in plants when phytoplasmas occur in low titer in the phloem vessels of their host-plants and their concentration may be subjected to seasonal fluctuation**.** Three nested specific primers were used to identify phtyoplamas groups. Primer pair P1/AYint (**Smart *et al*., 1996**) is specific for phytoplasma inducing virescnce and phyllody, and this specific for members of aster yellows group (Fig. 5). The primer pair P1/WXint amplified rDNA (Fig. 6) from western X-disease group, walnut witches' broom, and vaccinium witches broom. Primer pair P1/BLTV Aint (Fig.7) is specific for members of the beet-leafhopper-transmitted virescnce group (**Smart *et al*., 1996**).

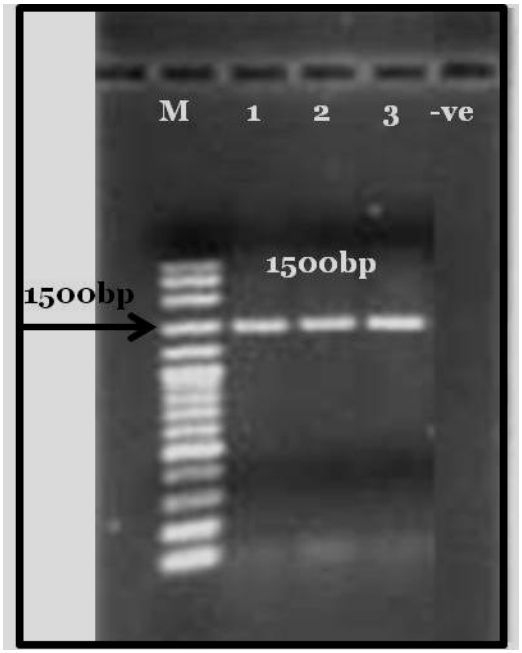

Fig.(5): Agarose gel electrophoreses analysis of PCR using primer pair P1/AYint Specifically detected members of the aster yellow group. Amplified fragment about 1500bp isolated from naturally infected sesame plants.

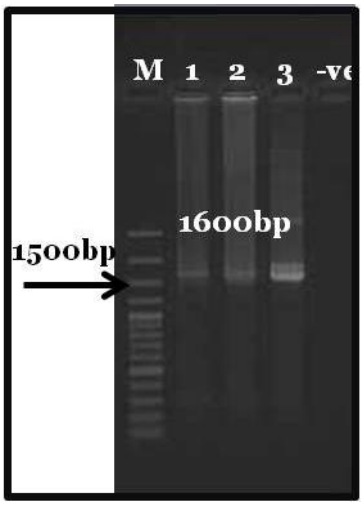

Fig.(6): Agarose gel electrophoreses analysis of PCR using primer pair P1/ WXint specifically detected members of the western X-disease group. Amplified fragment about 1600bp isolated from naturally infected sesame plants.

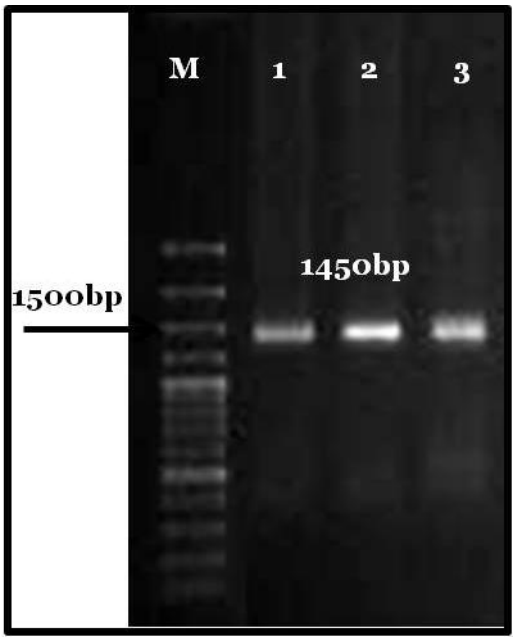

Fig.(7): Agarose gel electrophoreses analysis of PCR using primer P1 / BLTVAint specifically detected members of the beet leafhopper transmitted virescence group. Amplified fragment about 1450 bp isolated from naturally infected sesame plants.

Discussion

Based on the amplification of 16S-23SrRNA, using the universal primer pairs P1/P7 phytoplasma was consistently detected in symptomatic sesame plant samples. The presence of phytoplasma was demonstrated by amplification of a fragment characteristic 1.8kb DNA, visualized in agarose gel. The DNA bands were typical for phytoplasmas, when the universal primer pairs are used to amplify 16S-23SrRNA (**Gundersen and Lee, 1996**). No amplification was observed in PCR containing DNA obtained from asymptomatic plant and water as template. Repeated PCR assays confirmed the same results. These findings demonstrated the expected association of a phytoplasma with sesame exhibiting different type of symptoms such as phyllody, shoot proliferation, internodes shortening, and small leaves and flowers.

The choice of primer sets for phytoplasma diagnosis by nested PCR mostly depends on the phytoplasma we are looking for. Nested-PCR with a combination of different universal primers can improve the diagnosis of unknown phytoplasmas present with low titter in the symptomatic host. Universal ribosomal primers followed with nested with group-specific primers are extremely useful when the phytoplasma to be diagnosed belongs to a well-defined taxonomic group (**Marzachi 2004**). PCR products are usually visualized on 1% agarose gel prepared in 1xTAE buffer, stained with ethidium bromide (**Delić *et al*., 2007**).

Phytoplasma diagnostics has been routinely based on phytoplasma-specific universal (generic) or phytoplasma group specific Polymerase Chain Reaction (PCR) primers designed on the basis of the highly conserved 16S ribosomal RNA (rRNA) gene sequences (**Ahrens and Seemüller. 1992, Davis and Lee. 1993, Deng and Hiruki. 1991, Harrison *et al*., 1996, Jomantiene *et al*., 1998, Schaff *et al*., 1992, Smart *et al*., 1996**). Nevertheless, to detect phytoplasmas in DNA samples universal phytoplasma primers designed on sequences of the 16S-23S rRNA spacer region (SR) (**Smart *et al*., 1996**) are generally using. Nested-PCR is performing by preliminary amplification using a universal primers pair followed by second amplification using a second universal primer pair. By using a universal primer pair followed by PCR using a group specific primer pair, nested-PCR is capable of detection of dual or multiple phytoplasmas present in the infected tissues in case of mixed infection (**Lee *et al*., 1994**).

Many PCR assays have been developed to identify phytoplasmas (**Deng and Hiruki 1991**, **Firrao *et al*., 1994**, **Jarausch *et al*.,1994** , **Lee *et a*l., 1993, Lorenz *et al* 1995** and **Schaff *et al* ., 1992**), SR primers will be exceptionally useful for three reasons. First, the majority of the SR primers can be used in conjunction with primer P1 (**Deng and Hiruki 1991**), so the total number of primers necessary for group-specific phytoplasma identification is reduced. Second, because the SR region is more variable than the 16S rRNA gene, it will be comparatively easier to identify SR sequences from which primers can be designed for the detection of other phytoplasma groups. Finally, because the SR is much shorter than the full-length 16S rRNA gene, it is easier to sequence (**Smart *et al*., 1996**). A database of more than 60 phytoplasma SR sequences now exists (**Kirkpatrick *et al*., 1994**), which should facilitate the identification of other phytoplasma clade-specific PCR primers.

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DOI:10.22192/ijarbs.2017.04.07.024	

How to cite this article:
Sahar A.Youssef, Yasmin Sayed, Osama S. Hassan, Gehan Safwat and A. A. Shalaby. (2017). Universal and Specific 16S-23Sr RNA PCR Primers for Identification of Phytoplasma associated with sesame in Egypt. Int. J. Adv. Res. Biol. Sci. 4(7): 191-200.
DOI: http://dx.doi.org/10.22192/ijarbs.2017.04.07.024