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ISOLATION AND IDENTIFICATION OF *Spiroplasma citri* USING SOME SENSITIVE METHODS

[41]

Rasha M. Mahrous[1]; T.A. Moustafa[2]; Azza Galal Farag[1]; A.S. Sadik[3]; Sohair I. El-Afifi[3] and A.S. Gamal El-Din[1]

ABSTRACT

Spiroplasma citri, the causal agent of stubborn disease*,* was isolated from leaves and fruits of diseased citrus plants and cultured on solid and liquid C3G medium. On the basis of mode of transmission, symptomatology, shape on solid medium, staining of infected plant tissues with Dienes' stain and phase contrast microscopy, the isolated agent was identified as *Spiroplasma citri*. Identifiction was ensured by ELISA and PCR techniques. A fragment (1053 bp) from the spiralin gene of *S. citri* was amplified by PCR using two specific primers for the spiralin gene. The nucleic acid hybridization techniques (Southern and dot blots) were used for identification of the spiroplasmal genome using non-radioactive DNA probe specific for spiralin gene region. It was carried out as an alternative sensitive method for rapid detection of the Egyption isolate of *S. citri* using non-radioactive DNA probe specific for spiralin gene region.

Key words: *Spiroplasma citri,* Spiralin gene*,* PCR, DNA hybridization

INTRODUCTION

Citrus is one of the most important fruit crops in Egypt. The total cultivated area reached 344789 feddans.yielded about 2594853 tons according to the Minisitry of Agriculture data, 2002 **(Galal, Azza, 2004)** .

S. citri was first isolated from sweet orange (*Citrus sinensis*) trees affected by stubborn disease **(Doi *et al* 1967)**. It infects many plant species other than citrus, including periwinkle (*Catharanthus roseus*), in which it produce stunting, leaf yellows and wilting, *S. citri* has also been found in or transmitted to plants of 20 dicotyledonous

1- Virus and Phytoplasma Research Department, Plant Pathology Research Institute, Agricultural Research Center, (ARC), P.O. Box 12619, Giza, Egypt
2- Plant Pathology Dept., Faculty of Agriculture, Ain Shams University; P.O. Box 68 Hadayek Shobra, 1241, Shubra El-Kheima, Cairo, Egypt
3- Agric. Microbiology Department, Faculty of Agriculture, Ain Shams University; P.O. Box 68 Hadayek Shobra,1241, Shubra El-Kheima, Cairo, Egypt

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families and some monocots **(Doi *et al* 1967)**. *S. citri* is transmitted from plant to plant by the phloem-feeding leafhoppers (*Circulifer haematoceps* and *C. tenellus*) and by grafting **(Calavan and Bové, 1989)**.

In Egypt, *S. citri* was successfully isolated and cultured from diseased Navel orange fruits with typical symptoms of stubborn disease and cultured on a simple medium **(Abou-Zeid *et al* 1988 and Omar, 1999)**. Stubborn diseased lime trees.showed small abnormally upright leaves with symptoms of mottling and chlorosis. Punchy and rosetted foliage with numerous leafy branches were observed. Leaves almost drop and twigs die-back. The disease symptoms were obvious on one side of the tree which was stunted and excessively branched. Fruits formed on disased lime trees were small, hard, rough and showed irregular shape seeds **(EI-Banna, Om-Hashem, 1995)**.

Direct double antibody sandwich - ELISA technique demonstrated by **Clark *et al* (1978)** was used to detect *S.citri* by some investigators **(Bové *et al* 1984 and Azeri, 1992)**

In spiroplasmas, spiralin is such an immunodominant surface protein and accounts for up to 20 - 30% of the total protein mass of the organism. Several techniques for detecting *S. citri* are now available using ELISA **(Clark *et al* 1978)**, PCR **(Saillard *et al* 1993)**, dot-blot hybridization **(Saillard *et al* 1994)** and culturing **(Galal, Azza, 2004)**.

The present study was aimed to detect, isolate and identify *S. citri* using sensitive and diagnostic methods from different infected citrus trees grown at different governorates Egypt as well as using the most suitable gene for molecular detection. It is important to continu screening for the isolates found under the Egyptian conditions.

MATERIAL AND METHODS

Isolation

Samples from different parts of naturally diseased citrus trees [Navel orange, Mandarin, Balady (blood orange) and sweet orange] exhibiting symptoms, suspected to be due to *S. citri ,* were collected from some plantations at Giza , El-Qualubia and Sharkia governorates. Two methods of transmission i,e dodder and grafting were tried . C3G medium was used for primary isolation of the causal agent. A more complicated medium contains 780 mL d.H_2o, 21g PPLO broth, 1g fructose, 1g glucose 10g sucrose, 50g sorbitol, 10g trypton, 10 mL phenol red (1mg/ mL), 100 mL Foetal bovine serum (FBS) and 25mL Pencillin G (25 mg/ mL) was used for subculturing and maintaining the isolate agent. In all

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2- Plant Pathology Dept., Faculty of Agriculture, Ain Shams University; P.O. Box 68 Hadayek Shobra, 1241, Shubra El-Kheima, Cairo, Egypt
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cases the cultivation was taken place in screw capped tubes and incubated at 32 □C until a colour was changed from red to yellow, then 0.1 mL taken from the old medium was added to a new one. C3G soild medium was used for checking for the formation a fried-egg shape. The causal agent collected from samples was transmitted to healthy periwinkle plants using "dodder method" **(Moustafa, 1990)** .To ensure the transmissibility of the disease causing agent back inoculation to healthy citrus seedlings was carried out under green house conditions and the infected seedlings were used as a source of infection in the subsequent transmission.

Identification

***Transmission**

Two transmissibility of the causal agent from infected citrus seedlings to herbceous plants and vice virsa was studied using dodder and grafting methods as follow:

1- Dodder transmission

Dodder (*Cuscuta sp*.) Seeds were sown under greenhouse conditions as follows: clover seeds were sown in 30-50 cm diameter pots and after three-four weeks dodder seeds were sown in the same pots. Dodder seedlings were used to transmit the *S. citri* from naturally infected citrus plants to healthy periwinkle and citrus seedlings according to **Moustafa (1990)**.

2- Grafting

Sour Orange (*Citrus aurantium* L.) seedlings were used as root stocks and buds from healthy as well as from infected trees were inserted in T-shaped cuts **(Roistacher, 1991)**.

***Microscopical studies**

1- Phase contrast

A few drops of 3 days old liquid culture was examined by phase contrast microscope (Leitzortholux) as described by **El-Banna, Om-Hashem (1999).** Using Olympus Optoscient microscope Parasitic Acarinae Research Center P.A.R.C.).

2-Dienes'stain

Free hand sections from healthy and infected periwinkle and citrus plants were stained by Dienes'stain according to **Gamal El-Din *et al* (1983)** and Examined by light microscope **(x-100).**

***Serological tests**

-Enzyme linked immuonosorbent assay (ELISA)

ELISA test **(Clark and Adams, 1977)** was carried out to detect *S. citri* in pure culture (liquid and soild) as well as in infected citrus trees showing typical symptoms of stubborn disease**.** ELISA kits for *S.citri* detection were supplied by SANOFI, (Santa Animale, Paris,France).

Two gram of midrib or stem tissue was ground in 3-5 ml of extraction buffer (PBS) using a homogenize or a mortar and pestle, the homogenate was filtered through 2-4 layers of cheese clothe and centrifuged at 5.000 rpm for 5 min, the supernatant was collected and centrifuged at 24,000g for10min and added to the plates and all the steps of DAS- ELISA test to **CLark and adams (1977).**

Molecular detection

A- PCR detection

Total DNA was isolated from infected plants i.e., sweet orange and periwinkle plants as well as from pure culture liquid medium according to the methods of **Maixner *et al* (1995)** and **Duret *et al* (1999)** respectively.

PCR was performed in plant tissues and culture in 50 µl total volume reaction containing 10 pmole of each amplification primer. **D: 5`- CCC TTG TGA ATC ACC ACC -3 `**and **D´: 5`- CCC TTG TGA ATC ACC ACC3-3`** that constructed as conserved sequences in the spiralin gene of *S. citri* strains R8A2HP for spiralin gene, 3µl of total (200ng) DNA from citrus, periwinkle tissues and culture following by mixture of 5µl untis of 10X PCR buffer, 2 mM $MgCl_2$, 5 µl 2 mM dNTPs with 2.5 of the *Taq* DNApolymerase (Promega) and d.H_2O to a volume of 50 µl. The amplification was carried out using the DNA Thermal Cycler 480 (Perkin-Elmer Cetus). The primers D, D´ were submitted to 40 repeated cycles as recomended by **Foissac *et al* (1996)** Theamplification of DNA was carried out for 1 min at 94 ºC, 1 min at 57ºC, 2 min at 72ºC, followed by an final extension step for 10 min at 72ºC. The amplified PCR products were electrophoresed in agarose gel. Ten µl of the reaction mixtures each containing 8µl of the PCR product for spiralin gene and 2µl of loading dye (methylene blue)were analyzed on 1% agarose gel dissolved in 1X TAE buffer **(Sambrook *et al* 1989)** , in the presence of 0.5 mg/ml ethidium bromide and electrophresis at 100V. The gel was photographed with MP4 polaroid camera under UV transilluminator (wavelength 245nm).

B- Southern blotting hybridization

Southern blotting technique was performed on the amplified *S. citri* PCR fragments of spiralin gene that analyzed on agarose gel as described by **Southern (1975)**. Membrane was cross-linked for the next procedure and used with a non-radioactive hybridization technique.

Probe preparation

Probe for Southern blot and dot blot hybridizations were generated by PCR in the presence of digoxigenin-11-dUTP (Boehringer Mannheim, Indianapolis, IN). PCR prduct from *S. citri* using primer D,D´ generally probe spiralin gene, was used as the template to synthesize a specific *S. citri* DNA probe. Digoxigenin labelled nucleotides was incorporated into the newly synthesized DNA during 40 cycles for D,D´. PCR were performed in thin-walled PCR tubes containing the following reaction mixture. 1µl PCR product for primers (D,D´), 0.5µl *Taq* DNA polymearse, 5µl of 10XPCR buffer,1µl dNTPs labelled mixture (Promega), 1µl of 10 pmol of primer (D, D´) or and d.H_2O up to volume of 50 µl. The mixtures were over laid with 50 µl of mineral oil and hot started 2 min at 94°C in a using DNA Thermal Cycler 480 (Perkin-Elmer Cetus) then PCR reaction was amplified with the cycling parameters as described by **Foissac *et al* (1996)**.

C- Dot blot hybridization

A weight of 0.3 gm of *S. citri* plant tissues (Navel orange and Mandarin) were homogenized in 100 µl of extraction buffer (0.2 M potassium phosphate, 5 mM Dithiothreitol, 0.1% Triton X-100,10 mM, 2-mercaptoethanol, pH 8.3) and then ground using knots pestle. Equal volume of denaturation solution, 1XSSC (15 Mm NaCl, 15 Mm Sodium citrate, pH 7.0, 50 % Formaldhyde) was added and heated at 60°C for 15 min, then centrifuged in microfuge tubes at 10,000 rpm for 5 min. 5 µl of each sample was spotted onto the membrane using the protocol according to **Loebenstein *et al* (1997)**. Membranes were air dried and the nucleic acids were cross-linked to the membrane by exposure to UV irradiation from a transilluminator for 3 min. Prehybridization and hybridization with single digoxigenin labeled probe was done. Colour detection with nitro blue tetrazolium chloride (**NBT)** and 5-bromo-4- chloro- 3- indolyl phosphate (**BCIP)** reagents as a substrate was carried out using the Genius II DNA Labelling and Detection Kit (Boehringer Mannheim, IN System).

RESULTS

Symptoms of citrus stubborn disease

Leaf and young shoot symptoms of naturally infected citrus trees were characterized with mottling, multiple buds and chlorosis.Whereas the fruits of infected trees were small in size and deformed (lopsided or a corn shaped) (Fig.1**a, b**).

Isolation and culturing of *S.citri*

Periwinkle plants inoculated by "dodder method" showed symptoms after 3 weeks from inoculation .The growing leaves were small and turned yellow, also abnormal growth of axillary buds were observed. As a result of back inoculation, typical symptoms were appeared on citrus seedlings, the growing leaves were small and yellow were appeared on Periwinkle. The coulour of inoculated medium was changed from red to yellow after 7-10 days from inculation at 32°C. In subculture liquid media, colour was changed after 3 days from inoculation at 32°C. Typical fired egg shape granular colonies were developed on the surface of C3Gsolid medium withen 10-15 days after incubation at 32°C. Colonies were circular, opaque center surrounded by a lighter coloured when examined by light microscope (Figure, 1[**C**]).

Light microscopy

A few drops of the inoculated liquid medium were examined by phase contrast light microscope. The isolated organism has a motile short helical filaments and small round bodies (Figure, 1[**d**]), No organism were also seen in the un inoculated medium colour control .

Use Dienes'stain

Free hand sections taken from infected citrus plants showed that the phloem was stained blue while that of healthy ones remained unstained (Figure, 2).

Dodder transmission

Dodder plant showed a high ability to transmit the causal agent from infected periwinkle or citrus plants to healthy

ones. Symptoms were appeared 2-3 weeks or 3-4 weeks post inoculation of citrus and periwinkle plants, respectively (Figure, 2).

Grafting

T- Grafting was used to transmit the isolate from naturally infected citrus plants to healthy citrus seedlings ones. Typical symptoms were observed after 2 months.

ELISA detection

All the samples taken from different parts of naturally infected citrus trees of Navel orange, Mandarin, Balady (blood orange) and sweet orange were reacted positively with ELISA test. Data represented in **Table (1)** showed ELISA

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Table 1. Detection of *S. citri* in the different citrus species (Navel orange, Balady (blood orange), sweet orange and Mandarin) by ELISA

Samples of tested citrus plants	ELISA values at A405 nm	
Parts	Infected	Healthy
Navel orange		
	0.967	0.050
Midrib	0.526	0.045
	1.304	0.061
Stem		
Fruit		
Balady (blood orange)		
	0.501	0.051
Midrib	0.406	0.044
	0.587	0.063
Stem		
Fruit		
Sweet oranges		
	0.336	0.049

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	0.578	0.055
Midrib	0.495	0.049
	0.301	0.065
Stem		
Fruit		

Figure 1. **a-** Chlorotic small seedlings from a stubborn–diseased (right) and healthy trees (Left).

b- Small lopsided half fruit from Navel orange infected with stubborn disease (right) and half fruit from healthy tree (Left).

c- Typical fried egg shape colonies of *S. citri* on C_3G solid medium 10-14 days post incubation at 32 □C (x -100) (Right) , fried egg shape granular colonies (x -1000) (Left)**.**

d- Phase contrast microscopy after 3 days from inoculation (short helical and small round bodies (x3300).

Figure 2. (Upper) Dodder transmission.
Healthy periwinkle, (Right) and inoculated periwinkle (Left).
(Lower) Cross section of stem Navel orange infected by stubborn disease (Right) showing phloem tissues stained with dark blue colour after treatment with Dien's stain and uninfected (Left)

values of different parts of each citrus species. In general, citrus fruits contain the highest concentration of *S. citri* except in case of mandarin, while the citrus stems contain the lowest concentration. As compared with fruits and leaves. Navel orange admit a high multiplication of *S. citri* in comparison with other citrus species while sweet orange is the lowest .Samples with an absorbance reading equal to or greater than twice that of healthy controls were considered positive results.

Molecular detection

PCR detection

Spiroplasma citri was detected in plant samples of citrus sweet orange (*Citrus sinensis*), preiwinkle and in its

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liquid culture by technique. PCR .Spiralin gene was amplified using the extracted DNA with the D, D´ pairs of primer. The expected fragment 1053 bp were obtained with genomic DNA extracted from *S. citri* culture and from infected plant tissues (Figure, 3[**A**])**.**

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Figure 3. **A- Agarose gel electrophoresis of amplified spiralin gene fragment using primers D, D´**

Lanes 1 – 3 show PCR products DNA of citrus plant, culture and periwinkle of a size1053 bp amplified from D, D´ the correct.

Lanes (4 , 5) represent healthy citrus navel orange sample, culture and periwinkle.

M: PCR marker (Promage) (2645,1193,676,517,350,222).

B- Southern blot hybridization

Analysis using D,D´ for spiralin gene DNA Dig- labeled probe of the same samples as for the agarose gel in Fig. (A).

Positive reaction was shown to lanes (1,2,3).

C- Dot blots hybridization

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Assay using spiralin gene region DNA probe labeled with **dig.** The membrane hybridized with specific non-radioactive *S. citri* probe with dot blots.

Track (2 an4) strong signal with infected leaves of (Navel orange and Mandarin) respectively. No reaction with leaves (negative control) with track (3).

PCR positive control spiralin gene in track (1).

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Non-radioactive hybridization technique

Non-radioactive DNA hybridization technique was used for detection of the spiralin gene . DNA probe was generated by PCR amplification the spiralin region using primers D D´. The amplified products successfully labeled by digoxigenin. PCR product was used as a DNA labelling probe in southern and dot blot hybridization techniques.

Southern blot and Dot blot

Southern blot hybridization process was valuable for successful detection of *S. citri* in infected tissue. The results showed that the DNA hybridization technique was as specific as the PCR (Figure, 3[**B**]). A single band of the observed size (1035bp) was amplified using *S. citri* spiralin gene specific primers. No reaction was observed with healthy sample (negative control). The membrane hybridized with specific non-radioactive *S. citri* probe with dot blots. The reaction between dig -labeled DNA specific for *S.citri* spiralin gene region using dot blot and PCR blot was demonstrated in (Figure, 3**[C**]) . Positive strong reaction of dot blot hybridization was observed with extract and PCR product of infected Navel orange and Mandarin. No signals were observed in healthy control.

DISCUSSION

A survey was carried out in this work to study the spread of the stubborn disease on citrus in different Governorates, 300 trees were inspected visually in every region. The study showed that the incidence of stubborn in citrus fields varies from zero to 30.3% in Kafr El-Sheikh, from 4.3 to 31.7% in EI-Gharbia and from 25.0 to 40.0% in EI-Qualubia Governorates. The highest percentage of infection was recorded in EI□Qualubia (32.2%), followed by EI-Gharbia (20.76%) and Kafr El-Sheikh (16.43%) Governorate **(Omar, 1999).**

Symptoms of citrus stubborn disease caused by *Spiroplasma citri* were observed under natural Egyptian conditions on Navel orange, Sweet orange, Balady (blood orange) and Mandarin. Stubborninfected trees showed stunting and lopsided fruits. These observations are in accordance with the early description of citrus stubborn disease symptoms by **Abou-Zeid *et al* (1988), Omar (1999) and Sidaros *et al* (2000).** The causal organism, *S.citri* was isolated during this inevestigation from young leaves, stem and fruit samples of citrus trees showing typical symptoms of citrus stubborn disease. These results

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agree with the results of **Abou-Zeid *et al* (1988); Omar (1999) and Galal, Azza (2004).**

The presence of citrus stubborn agent (*S. citri*) causes changing in the colour of inoculated C3G medium from red to yellow after 7 days of inocubation at 32ºC. In subculture liquid media, colour was changed after 3 days from inoculation at 32°C. This cuolor change due to the assimilation in the sugar and induced acids, i.e., pyrofic acid 3-4 days post incubation at 32ºC. This in agreement with that found by **Abou-Zeid *et al* (1988), Omar (1999), El-Banna *et al* (2000) and Galal, Azza (2004).**

Typical fired-egg shape colonies were developed on the surface of C3G solid medium 10-15 days after incubation at 32□C. Colonies were circular, opaque center surrounded by a lighter colour, granular when examined by light microscope. On plate, the colonies were clear, very small as head pin and their growth was observed inside the soild medium. These results are in line with those obtained by **Fudl-Allah *et al* (1972), Abou-Zeid *et al* (1988), Omar (1999), El-Banna *et al* (2000) and Galal, Azza (2004).** Using phase contrast light microscopy, the isolated organism has a motile, short, or helical filaments structure. These result come in agreement with **Abou-Zeid *et al* (1988), Omar (1999) and Galal, Azza (2004).**

Dienes' stain was used for detecetion of the aggregates of MLOs including spiroplasma in the phloem tissues of diseased plants **(Gamal El-Din *et al* 1983 and Sidaros *et al* 2000).**

The enzyme linked-immunosorbent assay (ELISA) is a sensitive serological procedure used for the identifiation of the cultivable plant spiroplasmas **(Najar *et al* 1998 and Sidaros *et al* 2000).** ELISA test were carried and for detection of *S. citri* in leaves and fruits of symptomatic infected citrus trees. The obtained results showed that the concentration of *S. citri* was higher in fruits and midribs than in stems. Navel orange fruits contained high concentration of *S. citri* than fruits of Mandarin, Balady (blood orange) and sweet orange.This result confirm the results of isolation.this was also stated by **Abou-Zeid *et al* (1988); El-Banna, Om-Hashem (1995); Najar *et al* (1998) and Galal, Azza (2004).**

Utilization of PCR technology for detection and identification of plant viruses and mycoplasma appears to be promising tool because of its great sensitivity. The PCR provides a good alternative to other diagnostic methods and can speed diagnosis, reduce the sample size and often eliminates the need for radioactive probes. The use of PCR has facilitated the production and cloning of cDNA. Amplified DNA was detected by staining the agarose gel with ethidium bromide **(Hadidi and Yang, 1990)**. The first step required for the successful detection of any pathogen by PCR based on the method of DNA extraction from the samples in the optimal time.

In the present study DNA was extracted using CTAB extraction buffer (hexadecyltrimethylammonium bromide) with tissue homogenization system **(Maixner *et al* 1995).** The CTAB buffer should be more amenable to plant material containing polysaccharides **(Fang *et al* 1992 and Andersen *et al* 1998).**

Spiralin is a major protein of *S. citri* and the most abundant polypeptide in the *S. citri* membrane **(Bové *et al* 1993; Garnier *et al* 1993; Bové *et al* 2003 and**

Duret *et al* 1999). Its amphiphilic and antigenic properties and its membrane location and arrangement have been extensively studied **(Saillard *et al* 1993; Fahmy, 2000 and Bové *et al* 2003)**. Using the specific primers of spiralin gene (D,D´) a PCR product of 1053 bp was a mplified by agarose gel electrophoresis.

(Osorio, 2000) indicated that the PCR detection should be superior to culture in both sensitivity and reliability. It was added that the PCR method is also very specific with virtually no false positives because of the unique nature of the spirilin primers.

The procedure of DNA hybridization with specific probes as mentioned by **Degorce-Dumas *et al* (1983); Hull (1984 and 1986)** and **Razin *et al* (1987)** was used to detect *S. citri* in field samples showing symptoms suspected to be caused by spiroplasma **(Bove *et al* 1993 and Saillard *et al* 1993)**.

In the present study a simple, specific and rapid method for the detection of *S. citri* in new leaves of citrus plants Navel orange**,** and Mandarin on the dot and Southern blottings in samples of infected and healthy trees onto a nitrocellulose membrane, followed by hybridization with a *S. citri* using DNA probe of spiralin gene (1053bp). The same result was obtained by **Bove *et al* (1993) and Galal, Azza (2004)**

It was also showed the Southern blot hybridization was an effective method to confirm the validation of PCR product spiralin gene (1053bp) for stubborn disease throught the nucleic acid hybridization with *S. citri*-DNA probe. Similar results were obtained by **Foissac *et al* (1997) and Bové & Garnier (2002).**

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1- قسم بحوث الفيروس والفيتوبلازما – معهد بحوث أمراض النباتات – مركز البحوث الزراعية – الجيزة مصر

2- قسم أمراض النبات – كلية الزراعة – جامعة عين شمس – شبرا الخيمة – القاهرة مصر

3- قسم الميكروبيولوجيا الزراعية – كلية الزراعة – جامعة عين شمس – شبرا الخيمة – القاهرة مصر

[illegible] (C3G) [illegible] ELISA . [illegible] 1053 [illegible]

1- Virus and Phytoplasma Research Department, Plant Pathology Research Institute, Agricultural Research Center, (ARC), P.O. Box 12619, Giza, Egypt

2- Plant Pathology Dept., Faculty of Agriculture, Ain Shams University; P.O. Box 68 Hadayek Shobra, 1241, Shubra El-Kheima, Cairo, Egypt

3- Agric. Microbiology Department, Faculty of Agriculture, Ain Shams University; P.O. Box 68 Hadayek Shobra,1241, Shubra El-Kheima, Cairo, Egypt

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في الكشف الدقيق للمسبب المرضي تحت

تحكيم: أ.د مصطفى محمد مصطفى
أ.د مصطفى محمد القاضي

1- Virus and Phytoplasma Research Department, Plant Pathology Research Institute, Agricultural Research Center, (ARC), P.O. Box 12619, Giza, Egypt
2- Plant Pathology Dept., Faculty of Agriculture, Ain Shams University; P.O. Box 68 Hadayek Shobra, 1241, Shubra El-Kheima, Cairo, Egypt
3- Agric. Microbiology Department, Faculty of Agriculture, Ain Shams University; P.O. Box 68 Hadayek Shobra,1241, Shubra El-Kheima, Cairo, Egypt

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