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USING " BIOLOG" MICROPLATE FOR THE IDENTIFICATION OF *Erwinia amylovora* THE CAUSAL AGENT OF PEAR FIRE BLIGHT IN EGYPT

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ABSTRACT

Fire blight disease of pear was first recorded in Egypt in 1962. The severe occurrence of the disease on pears took place at Behera Governorate during 1982 season. In April 1995,1996,1997 and 1998 symptoms characteristic of fire blight were observed in orchards at Behera, Menufia and Nubaria provinces. An extensive survey of most pear orchards revealed similar symptoms on trees in all locations.

Twenty seven isolates formed reddishorange colonies with deep orange centers on MS medium. They did not fluoresce under ultraviolet light when grown on King's medium B, bacterial ooze was produced on immature pear fruits.

Based on morphological, physiological and biochemical characteristics and also carbon source utilization as determined with the BIOLOG ™ system of the isolates studied all provided evidence to confirm the existence of fire blight in Egypt.

BIOLOG provides a very simple approach for identification of the bacterium *Erwinia amylovora* the causal agent of fire blight in Egypt.

***Key words** : biolog G-ve microplates, Erwinia amylovora, fire blight, pear . utilization of carbon sources .*

1. INTRODUCTION

Fire blight caused by *Erwinia amylovora* (Burrill) Winslow *et al.* was first recorded in Egypt in 1962 in a pear orchard at Mamal El-Kezaz in the region between Alexandria and Damanhur by El-Goorani, (1964), Abou-El-Dahab and El-Goorani, (1964), and El-Helaly *et al.*, (1964). However, subsequent investigations have indicated no case of fire blight was reported in the mentioned area as well as from other orchards during the period from 1966-1972 (El-Goorani 1973).

A severe outbreak of fire blight was observed on pears in the Nile Delta of Egypt in 1982 (Abou-El-Dahab *et al.*, 1983; Van der Zwet, 1986). Since then, the disease spread rapidly through different provinces and was responsible for serious economic losses to pear growers .

Monitoring populations of *E.amylovora* and *Pseudomonas syringae* on pear orchard were studied in 1984 and 1985 in Kumbaneit Abo-Keer, Behera Governorate by Abd El-Ghafar (1988). He found that the population of *E.amylovora* was higher than that of *Ps. syringae* for all tested samples from buds, flowers, leaves and fruits in the period from February to May . He also reported that high population of *E.amylovora* was found in cankers but no bacterial cells of *Ps. syringae* were found in cankers.

In 1994 the same author investigated the relationship between disease severity during autumn period and severity of the disease in the following spring . He found that high occurrence of cankers and infected clusters per tree reflected on the disease severity and decrease in yield in the following spring .

The disease, usually, causes gradual decrease in tree vigour and consequently in productivity to an extent which obliged some growers to remove the affected trees and sometimes the orchard. Pear plantation in Egypt is rapidly decreased. Its acreage of 7827 fed. in 1990 was reduced to 1124 fed. in 1998 at Behera Governorate. However at Nubaria province pear plantations decreased from 4545 fed. to 3418 in 1995 and 1998, respectively.

Contradictory reports revealed that media selective for fire blight failed to give rise to *E.amylovora* and the bacteriological

characteristics of the isolated pathogen were different from those known for *Pseudomonas syringae* van Hall but conform with those of *Pseudomonas syringae* pv. *phaseolicola* (Burkholder) Young *et al.*, (Farag, *et al.*, 1986).

In 1993, Farag endeavored to point out the reported spurious characteristics of the fire blight bacteruim that may help in discrimination of genuine ones based on bacteriological differences among cultures collected from different locations.

The objective of this study was to confirm the presence of *E.amylovora* in pear orchards in Egypt. Diagnostic and identification tests were performed on all isolates from different localities by using BIOLOG ™ system.

2. MATERIALS AND METHODS

2.1. General techniques

2.1.1. Collection of plant material and isolation of the pathogen

An extensive survey of pear orchards in Behera, Menufia Governorates and Nubaria province was conducted in the spring of 1995,1996,1997 and 1998. Samples of blossoms, leaves and cankerous branches with typical fire blight symptoms were collected. The pathogen was isolated from fresh ooze on blighted branches and petioles of leaves and blossoms by excising pieces and homogenizing in sterile distilled water. The resulting homogenates were plated onto MS plates (Miller and Schroth, 1972). Incubation for colony development was at 28 °C for 72-96 hr or until the reddish orange colored colonies, typical of *E.amylovora*, were observed. Transfers from such colonies were made onto King's B medium (King *et al.*, 1954) and all colonies which did not produce green fluorescence were used in subsequent pathogenicity tests .

2.1.2. Pathogenicity tests

The pathogenic ability of the isolates was tested on green immature pear fruitlets which were surface sterilized with ethanol 95% and inoculated by stabbing with a needle laden with (24hr. culture) of the bacteria. The inoculated fruitlets were placed in plastic boxes in which high humidity was maintained with wet cotton pieces

and incubated at 25-28 °C for 4 days. Control pear fruitlets were inoculated with sterile water. Pathogenicity assays were conducted twice with all isolates.

Induction of the hypersensitive reaction on leaves of young tobacco plants (*Nicotiana tabacum*) was tested with the infiltration method according to Schroth and Hildebrand, (1980). The plants were kept for 3-4 days in a greenhouse at 25 °C until clear necrotic lesions developed.

2.1.3. Cultural characteristics and biochemical tests

All isolates that showed pathogenicity to the host were subjected to complete identification. The cultural as well as the morphological, physiological and biochemical characteristics suggested by Dye (1968) and Schaad (1980) were considered.

2.2. Identification using BIOLOG micro-plates

BIOLOG ™ micro-plates (Biolog , Inc., 3938 Trust Way, Hayward, CA 94545, USA) test the ability of a micro-organism to utilize a preselected panel of different carbon sources. The test yields a characteristic pattern of purple wells which constitutes a "Metabolic Fingerprint" of the capacities of the inoculated organism (Bochner, 1989 ; Jones *et al.*, 1993; Harris-Baldurin and Gudmestad, 1996). Tetrazolium violet is used as a redox dye to colorimetrically indicate the utilization of the carbon sources.

To identify an unknown isolate, firstly perform a Gram stain. If the bacterium is G-ve, it is cultured on Tryptic soy agar (TSA) or Biolog universal growth medium (BUGM), (Bochner, 1991). A cell suspension is prepared at a specified density and is inoculated into the G-ve microplate. The microplates were incubated overnight to allow the purple color to settle to the bottom of the wells. The pattern of purple wells is then keyed into Biolog's Micrology computer program which automatically cross - references the pattern to an extensive library of species. If an adequate match is found, an identification of the isolate is made. Two dimentional clustering algorithms to recognize groups of related bacteria and quantify their relatedness were made.

3. RESULTS AND DISCUSSION

3.1. Field observations

Blossom infections usually are first noticed in April when the flowers withered and turn brown to black, the infection progresses into the spur leaving the entire cluster blighted. Bacterial exudate (ooze) was observed coming out on the petioles of the leaves and blossoms. The dead and blackened leaves and blossoms remained attached on the twigs. Later in June and July dead twigs and shoots were observed on the trees as a result of the progress of infection through the fruiting spurs to the shoots and twigs. The extended cankers down the branches and trunk were observed by discoloration of the inner bark tissues. The margins of the canker are indefinite at first, but late in the season the margin became more sharply defined and semetimes produced bacterial exudate (ooze) on the surface of "hold-over" cankers, . Such finding was approximately in the line with that reported by Hildebrand, (1936), Parker , (1936); Rosen, (1929) and Thomsen *et al.*, (1975) who noticed that the majority of cankers formed in one season did not become active hold - overs and produced visible ooze the following year.

Fire blight symptoms were observed in a large number of orchards surveyed in 1995, 1996, 1997 and 1998 throughout Behera, Menufia Governorates and Nubaria province. There was a loss in large branches and sometimes the entire trees. The loss of blossoms per tree varied from 50-100% of blossoms blighted. The weather during spring at such Governorates seemed to be favorable to outbreak and spreading of fire blight in 1995 and 1998. Therefore, the disease varies in severity from year to year and from location to location because of variation in environmental conditions and differences in amount of initial inoculum. The present results are in agreement with those obtained by (Eden- Green and Billing , 1974 Schroth *et al.*, 1974).

The principal sources of primary inoculum were the margins of cankers formed the previous seasons which were observed in most orchards surveyed from 1995 to 1998. Therefore, it is likely that fire blight became widespread in large areas of Behera, Nubaria and Menufia Governorates. In 1995 and 1998 trees bloomed considerably

later than usual, together with the extended rainfall, average temperatures from 7-28C and wind storms combined to initiate the prevalence of fire blight in these Governorates. On the other hand, it is important to notice that the level of precipitation at March and April helped in the activation of the margins of cankers and dissiminating the inocula to healthy shoots and blossoms .

3.2. Isolation and pathogenicity tests

The isolation of *E.amylovora* from different orchards at Behera, Menufia and Nubaria demonstrated that the pathogen has become established in northern and new land regions of Egypt.

Twenty two isolates from infected blossoms, leaves and blighted shoots produced on MS medium, reddish - orange colored colonies which were indicative of *E.amylovora* on the highly selective medium. All the isolates obtained did not fluoresce under ultraviolet light when grown on King's medium B. The main morphological , physiological and biochemical characteristics of the isolates studied , as well as various diagnostic tests are shown in Table (1). No variation between all isolates obtained was noted.

Results of pathogenicity tests demonstrated that all isolates which were identified by BIOLOG system were also pathogenic on immature pear fruitlets with no substantial difference in degree of oozing. Results of hypersensitive reaction on tobacco in Table (1) showed good correlatian with ooze formation on pear fruit qualitatively .

3.3. Identification with Biolog technique

The metabolic capabilities of all the isolates to utilize 95 different carbon sources were examined by the BIOLOG system. Most of *E. amylovora* isolates oxidized cyclodextrin, dextrin N-acetyl - D- glucosamine , L- arabinose, D- fructose, D- galactose, D-mannitol, B-methyl - D- glucoside, D- psicose, D- sorbitol, sucrose, D- trehalose, methyl- pyruvate, monomethylsuccinate, D- gluconic acid, succinic acid, bromosuccinic acid, L- alanine, L- glutamic acid, D- glucose, glycerol, glucose 1-phosphate and glucose 6 - phosphate under the test conditions used, (Fig 1,2) which showed that the rows from A to H and the columns from 1-12 represent 96 wells of the

Table (1) : The morphological characters, physiological and biochemical reactions of the isolated bacterium .

Character	Reaction
Gram staining	- ve
Sporulation	-
Reaction to various diagnostic tests :	
Colour of colony on MS medium [a]	+
Growth under UV on king`s medium B [b]	NF
Immature pear fruit [c]	O
Tobacco hypersensitivity	+
Hydrolysis of :	
gelatin	+
starch	-
aesculin	-
H_2S from cysteine	-
Acetoin	+
MR	-
Nitrate reduction	-
Reducing substances from sucrose	-
Production of :	
Levan	+
Indole	-
Urease production	-
Utilization of sugars and other carbon sources:	
L (+) arabinose	
fructose	+
lactose	+
cellobiose	-
maltose	-
glucose	-
mannitol	+
salicin	+
dulcitol	-
glycerol	-
methyl glucoside	-
	-

a Miller - Schroth medium: reddish orange colonies (+)

b Under ultraviolet light, no fluorescence (NF)

c Oozing (O)

+= positive reaction , - = negative reaction

Microplate, with different carbon sources. For example, the reference well (A-1) with no carbon source, (A-2) with cyclodextrin, (B-2) with D- fructose and (H-12) with glucose 6- phosphate . On the other hand,all wells scored as borderline <xxx> are positive reaction i.e. (B-4) showed that the organism oxidized D- galactose and the cells reduce the tetrazolium dye forming a purple color . These results are in line with those reported by Kim *et al.*, 1996.

The Biolog system regarded 10 bacterial species as closest species to the unknown bacterial strain no. 105 from Nubaria (Fig.2). These species were placed in three distinct clusters (Fig.3,4) which were constructed based on the distances shown in Fig.2. The smaller the distance , the more closely the species were. Thus each cluster included the closely related species . Evidently strain 105 was remotely related to the species in cluster (A), while it was closely related to those in cluster (B). For example , the distances between strain 105 and the closest species of cluster (A) ranged from 12.21 for *Actinobacillus seminis* to 8.861 for *Pasteurella anatis*. On the other hand, the distances between strain 105 and the closest species in cluster (B) ranged from 8.255 to 3.278 for *Vibrio Cholerae* and *Erwinia amylovora* A, respectively. It is also clear from such data that *E.amylovora* B was the only species included in cluster (C) and the distance between strain 105 and this species was 7.727. Therefore, it was concluded that *E.amylovora* was the closest species to the unknown bacterial strain 105 from Nubaria and also the similarity was 78% between them.

Field observations (symptoms), colony morphology on highly selective medium and pathogenicity assays all provided evidence to confirm the existence of fire blight disease in Egypt. These observations are in agreement with others (El-Goorani, 1964; El-Helaly *et al.* 1964; and Abd-El-Ghafar, 1988 and 1994).

Based on traditional bacteriological tests as outlined by Dye (1968) and Schaad (1980) compared with carbon source utilization as determined with the BIOLOG^{TM} system (Bochner, 1989), it is evident that the causal agent of bacterial blossoms, leaves and shoot blight is the bacterium *Erwinia amylovora*.

MICROLOG (TM) 3 RELEASE 3.50 A

Date : 03 07/93
Hour : 24
Plate Type : GN
Media Type : TSA BUGM
Plate # : 2
Strain Name :
Strain # : 1098
Other Info : KAFR EL-DOWAR
Input Mode : Reader : BIOLOG MICROSTATION
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
XXX' = percent change in optical density versus A1 control well
<XXX> = positive, (XXX) = borderline, XXX = negative
-XXX = percent change negative
XXX+ = data negative or borderline, "=>" ID choice positive > 90% of time
XXX- = data positive or borderline, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	19	<183>	<132>	-2	-23	-8	<737>	-24	26	-17	2
B	-21	<421>	17	<421>	<225>	<768>	20	-4	-20	65+	<643>	12
C	3	<833>	<126>	11	-9	<506>	<842>	<665>	22	-25	53	<144>
D	2	13	-14	-22	10	-15	<288>	-23	-22	-24	-19	-21
E	0	-21	-36	51	-40	21	-25	4	-23	-21	-42	<258>
F	<185>	< 87>	6	0	-27	28	-8	37	<144>	<260>	-30	46
G	2	32	-3	-6	-25	-6	-27	-21	26	-31	-27	-20
H	28	<180>	43	27	-24	-18	-36	-8	<245>	-19	<803>	<527>

BIO-NUMBER : 1420-2702-3161-0040-0001-6014-0000-2013

SPECIES IDENTIFICATION : ERWINIA AMYLOVORA A

CLOSEST SPECIES :	SIM	DIST	AVG	MAX
1) ERWINIA AMYLOVORA A	0.777	3.360	1.063	5.165
2) PASTEURELLA ANATIS	0.000	7.982	0.800	1.413
3) VIBRIO CHOLERAE	0.000	8.228	2.094	3.869
4) ERWINIA AMYLOVORA B	0.000	8.722	0.563	1.106
5) VIBRIO MIMICUS	0.000	10.602	1.156	3.156
6) AEROMONAS SALMONICIDA SS MASOUCIDA	0.000	11.519	1.406	3.066
7) PASTEURELLA VOLANTIUM	0.000	11.745	0.344	2.706
8) TATUMELLA PTYSEOS	0.000	11.920	0.438	1.425
9) VIBRIO VULNIFICUS	0.000	12.005	1.750	5.375
10) XANTHOMONAS CAMPESTRIS PV VESICATORIA B	0.000	12.358	1.500	2.725
other :	-----	-----	-----	-----

Fig (1): Computer sheet showing species identification of *Erwinia amylovora* strain number 1098 isolated from Kafr-El-Dowar, tested with the Biolog GN Micro plate system.

MICROLOG (TM) 3 RELEASE 3.50 A

Date : 10/07/98
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 1
Strain Name : NUBARIA
Strain # : 105
Other Info : ?
Input Mode : Bio-Number
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
XXX = percent change in optical density versus A1 control well
<XXX> = positive, (XXX) = borderline, XXX = negative
-XXX = percent change negative
XXX+ = data negative or borderline, "=>" ID choice positive > 90% of time
XXX- = data positive or borderline, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	<100>	<100>	0	0	0	<100>	0	0	0	0
B	0	<100>	0	<100>	<100>	<100>	0	0	0	0+	<100>	0
C	0	<100>	<100>	0	0	<100>	<100>	<100>	0	0	0	<100>
D	0	0	0	0	0	0	<100>	0	0	0	0	0
E	0	0	0	0	0	0	0	0	0	0	0	<100>
F	<100>	0	0	0	0	0	0	0	<100>	<100>	0	0
G	0	0	0	0	0	0	0	0	0	0	0	0
H	0	<100>	0	0	0	0	0	0	<100>	0	<100>	<100>

BIO-NUMBER : 1420-2702-3151-0040-0001-4014-0000-2013

SPECIES IDENTIFICATION : ERWINIA AMYLOVORA A

	CLOSEST SPECIES	SIM	DIST	AVG	MAX
=> 1)	ERWINIA AMYLOVORA A	0.782	3.278	1.063	5.166
2)	ERWINIA AMYLOVORA B	0.000	7.722	0.563	1.106
3)	VIBRIO CHOLERAE	0.000	8.255	2.094	3.869
4)	PASTEURELLA ANATIS	0.000	8.861	0.800	1.413
5)	VIBRIO MIMICUS	0.000	10.298	1.156	3.156
6)	PASTEURELLA VOLANTIUM	0.000	11.128	0.344	2.706
7)	PASTEURELLA CABALLI	0.000	11.781	0.656	3.256
8)	CARDIOBACTERIUM HOMINIS	0.000	11.818	0.563	4.075
9)	PASTEURELLA GALLINARUM	0.000	12.186	0.616	2.656
10)	ACTINOBACILLUS SEMINIS	0.000	12.210	0.604	4.519
	other :	-----	-----	-----	-----

Fig (2): Computer sheet showing species identification of *Erwinia amylovora* strain number 105 isolated from Nubaria, tested with the Biolog GN Micro plate system.

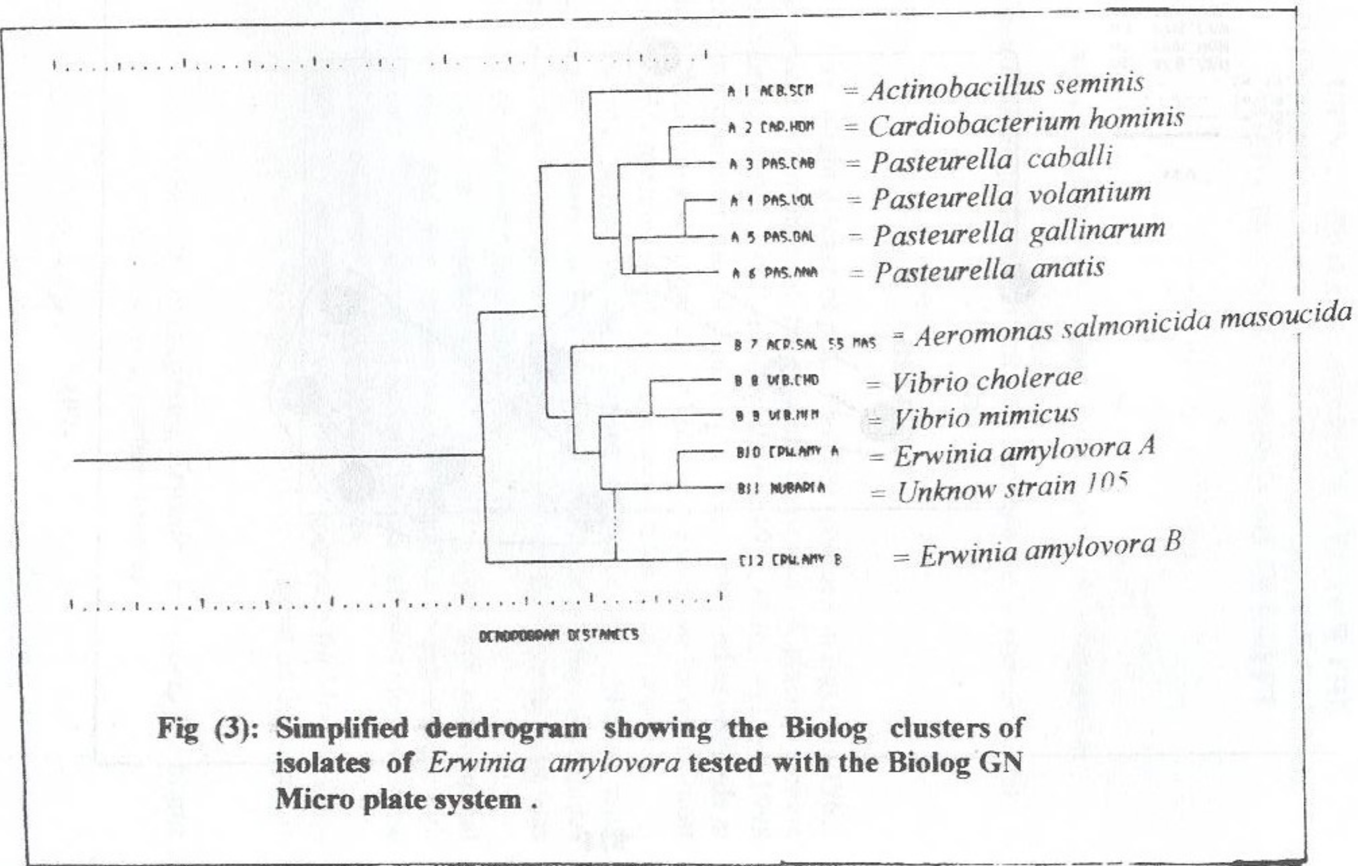

Fig (3): Simplified dendrogram showing the Biolog clusters of isolates of *Erwinia amylovora* tested with the Biolog GN Micro plate system .

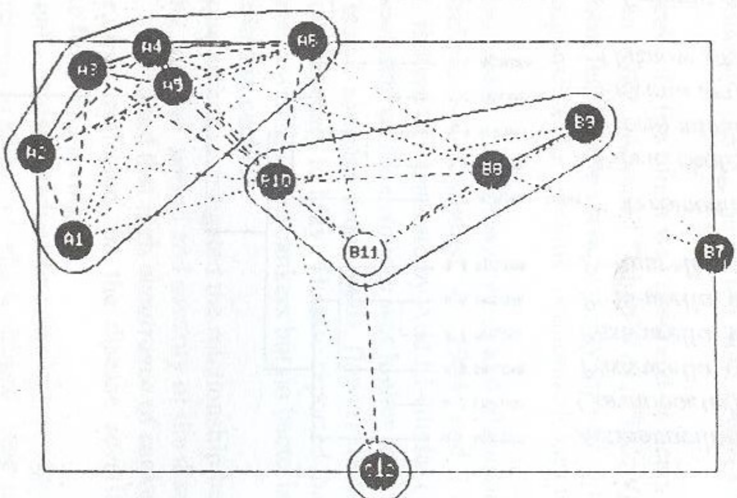

Fig (4): Simplified dendrogram showing the Biolog clusters of isolates of *Erwinia amylovora* tested with the Biolog GN Micro plate system.

It can be concluded that the identification of the bacteria is not a trivial matter. Biolog provides a very simple and practical approach to deal with this problem.

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استخدام نظام البيولوج في تعريف البكتريا " إيرونيا أميلوفورا " كمسبب لمرض اللفحة النارية فى مصر

على السيد توفيق – ناجى محمد أبو زيد – أحمد محمد حسنين

معهد بحوث أمراض النباتات – مركز البحوث الزراعية– الجيزة

ملخص

سجلت أعراض مرض اللفحة النارية لأول مرة فى مصر عام 1962 . إنتشر المرض في عام 1982 في كثير من زراعات الكمثرى بمحافظة البحيرة واتخذت مظاهر الإصابة شكلاً وبائيا في ذلك الوقت . فى ربيع أعوام 1995 – 1998 في شهر إبريل لوحظت الأعراض النموذجية لمرض اللفحة النارية فى زراعات الكمثرى بمحافظات البحيرة والمنوفية والنوبارية وإتضح نتيجة للحصر المكثف الذي تم في أغلب مزارع الكمثرى تشابه في أعراض الإصابة على الأشجار في جميع المواقع .

تم تجميع 27 عزلة أظهرت جميعها المستعمرات البكتيرية المميزة على بيئة MSولم تظهر إي منها المظهر الفلورسنتى عند تنميتها على بيئة King's medium B وتعريضها للأشعة فوق البنفسجية – كذلك أعطت نتائج إيجابية عند اختبار القدرة المرضية لها على ثمار كمثرى غير ناضجة . وقد تم تعريف البكتريا المعزولة على أساس الطرق الميكروبيولوجية المتبعة عالميا على أنها بكتريا " إيرونيا أميلوفورا " وأخيرا تم تأكيد التعريف باستخدام نظام BIOLOG TMوهو أحد الطرق الحديثة التي تعتمد على استخدام 95 مصدر كربونى مختلف للبكتريا المعزولة والذي يعتبر طريقة سريعة لتعريف المسببات المرضية وقد أكدت النتائج المتحصل عليها من الأبحاث السابقة ومن الدراسة المعملية لنفس العزلات .

المجلة العلمية لكلية الزراعة – جامعة القاهرة – المجلد (51) العدد الأول يناير (2000):107–122.