Potency of Pseudomonas fluorescens a Biotic Inducer Inhibitors Against Cucummber mosaic Cucumovirus.

[1]El-Dougdoug, Kh. A.; [2]Shamy, M.M.M. and [3]Heba, M.A. Khalil

[1]Microbiol. Dept., Fac. Agric., Ain Shams Univ., [2] Botany Dept., Fac. Sci., El-Monofia Univ., [3] Microbiol. Dept., Soil, Water and Environment Inst. Agric., Res. Center, Giza, Egypt.

Abstract: An antiviral producing Ps. Fluorescens when designated a EG isolates from potato rhizosphere soil in Egypt were identified based on morphological; biochemical tests and protein polymorphism. The strains were grown in King's B broth medium and the culture supernatent obtained was filtered through a 0.45 µl filter. It was further boiled at 100ºC for 10 min and tested to induce LAR and SAR for its ability to control a Satellite cucumber mosaic virus (st. CMV-EG). In Local acquire resistance (LAR) boiled culture filtrate (BCF) was treated on one half of the leaves of Chenopodium amaranticolor followed by st. CMV. EG inoculated on both halves. In the systemic acquire resistance (SAR), BCF was treated on the lower leaves of Nicotiana glutinosa, and st CMV-EC mechanically inoculated onto the untreated upper leaves. In LAR. BCF treatment was able to considerably reduce the number of viral lesion and in SAR plants treated with BCF shown no visible and mild mosaic viral symptoms, compared to the King's B media and remained throughout the study period. Thus, Ps. Fluorescens was able to produce an antiviral component in the culture filtrate, which was found to be heat stable, non-phytotoxic and effective in local as well as systemic host of CMV. [Journal of American Science 2010;6(8):88-93]. (ISSN: 1545-1003).

Key words: Boiled culture filtrate (BCF), cucumber mosaic virus (CMV), local lesion, Pseudomonas fluorescens.

1. Introduction

Cucumber mosaic virus (CMV) belong to the genus cucumovirus (family bromoviridae), is one of the economically important viruses, which causes enormous losses by infecting more than 1.000 species of plants shrubs and trees world-wide. It is transmitted non-persistently into healthy plants by aphids, which acquire the virus during their brief probes on infected hosts or the symptomless carrier woods in the field (Zehnder et al, 2000). Various strategies, based on the avoidance of sources of infection, control of vectors, modification of cultural practices, and the use of resistant varieties and transgenic plants have been conventionally employed to minimize the losses caused by CMV. These strategies, however, have not been effective as control measures. Many screening studies have been conducted on antiviral agents from different sources. Most of these come from plants sources, with some showing systemic control ability against a range of viruses that infect plants (Kubo et al., 1990). Comparatively, antivirals from microbial sources have been little studied. Recently, Raupach et al. (1996); El-Badry et al. (2006); Ipper et al. (2005) and Megahed (2008) showed the systemic control of CMV in cucumbers and tomato employing rhizosphere colonization of some bacteria by a ISR mechanism. Kim, et al. (2004); Ipper et al. (2005) and Megahed (2008) used culture filtrate of Acenetobacter, species KTB3, Ps. Fluorescens Gpfol and Trichoderma sp. Respectively, to systemically control some viruses.

This study was described the antiviral activity from the heat stable culture filtrate of Ps. Fluorescens EG against st-CMV EG which produces local lesions in the hypersensitive host and systemically infected many important plants.

2. Material and Methods

Bacterial strains and growth conditions: Soil-adhered cucumber roots were obtained from a cucumber field. The roots were homogenized using demineralized water and the homogenate was serially diluted and plated onto King's B media (King et al., 1954) followed by 24 hr incubation at 28ºC. Numerous colonies with different morphologies were picked from the dilution plates. Each of these was assayed for antiviral activity using half leaf method, as described by Noordam (1973). One colony that showing maximum antiviral activity was selected. This colony was stored on nutrient broth medium containing 20% glycerol. Other bacterial strain Ps. Fluorescens ATCC, 10325 was obtained from Cairo microbiological resources center (MIRCEN) , faculty of agriculture , Ain Shams university. This strain was used for identification and comparative study of the morphological, physiological, biochemical and protein patterns of Ps. Fluorescens EG.

In order to identify the Ps. fluorescens EG strain, morphological, physiological and biochemical

tested (Table 1) were carried out as described by Sehaad et al. (1988).

Maintenance of virus: St.CMV-EG was obtained from Virology Lab., Fac. Agric., Ain Shams Univ. (Megahed, 2008). The virus was inoculated into N. glutinosa and maintained on the same host throughout the period of this study. The inoculum consisted St-CMV-EG systematically infected leaves ground in 0.01M sodium phosphate buffer (SPB) pH 7.0.

Preparation of boiled culture filtrate (BCF): Each of 5 isolates of Ps. fluorescens were inoculated onto 100 ml King's broth and grown at 28ºC for 48 hr. with shaking at 175 rpm. The cultures were centrifugated at 12.000 rpm for 10 min.The cultures supernatant were then filtered through a 0.45 µm filter. The filtrates obtained were boiled at 100ºC for 10 min and used for the antiviral assay.

Antiviral bioassay: The antiviral activity of the BCF of 5 Ps. fluorescens isolates were assayed via induced local acquired (LAR) and systemic acquired resistance (SAR), LAR was assayed on a hypersensitive host of CMV, Ch. amaranticolor using the half leaf assay. The upper right halves of the leaves were treated with the BCF, using paintbrush, and the upper left halves were left untreated. As a control treatment, the upper right halves of another leaves were treated with sterilized water, with the left halves of the leaves for both treatments. Each treatment was performed in duplicate with 3 leaves each. The local lesions were counted after 7-10 days intreated and control and calculated formula: (I-T/C) x 100 where, C is the number of LL on the control half of the leaves and T the number of LL on the treated half on the leaves.

In SAR, 5 to 6 leaved N. glutinosa plants were used under SAR. BCF was treated onto the three basal leaves, after 24 h. CMV isolate inoculated onto one upper untreated leaf. As a control treatment, the lower leaves were treated with King's B media and the CMV isolated was inoculated the upper untreated leaves. Each treatment was replicated five times. The plants were kept in a greenhouse, with 12-14 h daylight and on temperature of 28-30ºC.

Determination of protein: Protein content of Pseudomonas isolates were determined by Bradford (1976) using bovine serum albumin as a standard.

Protein analysis : Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) under reducing conditions in discontinuous electrode buffer system. Protein samples were denatured and reduced completely by mixing the protein with in equal vol. Of 2x sample buffer (Lucine et al., 1987) and heating mixture at 92ºC in a water bath for 5 min and chilled on ice buffer. Treated protein were centrifuged at 12000 rpm for 10 min. Electrophoresis was carried out at room temperature at a constant current at 25 mA for 1 hr followed by 30 mA for 4 hr. At end of the run the gel was stained with comassie blue and distained in the stain solvent and photographed.

3. Results and Discussion

Isolation of Pseudomonas spp.: The bacteria were isolated from rhizosphere potato soil. Different morphological colonies were developed on King's B plates. Each one isolated colony was inoculated on slant medium. The bacterial isolates Pseudomonas sp. were subjected to an identification program to the genera level was performed as shown in Table (1). Pseudomonas appears fluorescence pigment on nutrient agar, short rod shape, gram negative and no spore formers. As well as, physiological and biochemical tests on Ps. fluorescens isolates showed, oxidase, proteolytic and lipolytic activities as most Ps. fluorescens isolates. As well as utilization of mannitol glucose, galactose and sobritol as most Ps. fluorescens isolates. However, no ethanol of utilization was observed. The growths at higher temperatures tests were negative. The growth of Pseudomonas isolates on butryrate, galactose as most of isolates. However no growth of isolate on Nicotiana were observed, which is usually shown by Ps. fluorescens isolate No. 1. Plotted on the basis of specific media and other test results, as shown in Table (1), indicates that these isolates are closely placed with the other Ps. fluorescens strains (Table 1). It is suggested to belong to the species Fluorscens according to criteria described by consulting Bergy's Manual of Systemic Bacteriology (Sneath, 1986). It could be given the tentative name Pseudomonas fluorescens EG isolates. The purified Pseudomonas isolates were subjected to screening against program of antiviral activities. All isolates exhibited antiviral activities against Cucumber mosaic virus (Ipper et al., 2005).

The most potent Psedomonas isolates belonging to Ps. fluorescens EG were selected for the biosynthesis of the active metabolite having antiviral activity. For this reason Ps. fluorescens EG was inoculated in nutrient broth media for antiviral production.

Antiviral, effect: The antiviral culture filtrate from Ps. fluorescens isolates (namely ATCC, 1,2,3,4 and 5) showed inhibitory inactivity against CMV with variability among isolates. The BCF treatment due to LAR whereas the half leaves of the hypersensitive host; Ch. Amaranticolor showed 85.0, 182.5, 72.9, 75.0, 68.5 and 85.4% inhibition of the production of LL respectively. Compared to the untreated the half leaves. The control plants were unable to show inhibitions of CMV-EG which is

shown 95 L.L. The average numbers of L.L. in the case of the BCF were much lower, 11.8, 18.5, 28.6, 26.4, 33.2 and 30.7 L.L than those of the sterilized water treated half leaves. The BCF due to elucidate

Table 1. Morphological, physiological and biochemical characteristics of Pseudomonas spp. isolates.

Characteristic	Ps. fluorescences ATCC 10325	Pseudomonas isolates				
		Ps1	Ps2	Ps3	Ps4	Ps5
Morphological						
Cell shape	Rod	Rod	Rod	Rod	Rod	Rod
Size (nm)	2x12	3x1.1	3x1.9	2.5x1.0	2x1.7	1x1.5
Gram reaction	SR	SR	SR	SR	SR	ST
Physiological	Diffusible	Diffusible	Diffusible	Diffusible	Diffusible	Non-diffusible
Fluorescent pigment						
Oxidase	+	+	+	+	+	+
Pectolytic activity	+	+	+	+	+	+
Lipolytic activity	+	+	+	+	+	-
Proteolytic activity	+	+	+	+	+	+
Amylolytic activity	-	+	-	-	-	+
Urease	-	V	-	-	V	-
Utilization						
Mannitol	+	+	+	+	+	+
Benzoate	-	-	-	-	-	+
Cellobiase	-	-	-	+	-	-
Starch	-	-	-	+	-	-
Serbitol	+	+	+	+	+	+
Sucrose	-	-	-	+	-	-
Glucose	+	+	+	+	+	-
Glycerol	+	+	+	+	+	+
Galactose	+	+	+	+	+	+
Xylose	-	-	-	-	+	-
Fructose	-	-	+	-	+	-
Growth on:						
Butyrate	+	+	+	+	+	+
Ethanol	-	-	-	-	-	-
Nicotinate	+	+	-	-	+	-
Growth at						
40ºC	-	-	-	-	+	-
35ºC	+	-	+	-	+	-
5ºC	+	+	+	+	+	+

V = Variable - Negative result + = Positive result

SAR, it was found that, the plants treated with BCF showed variation in viral symptom due to Ps. fluorescens isolates, ATCC and P1 revealed symptomless (0.0 L.L), P2,P3,P4 and P5 revealed mild mosaic (8.5, 10.2, 12.9 and 9.5 L.L respectively throughout the study period. The plants treated with both media (as a control) showed severe symptoms (Fig. 1(A). This results reveals the antiviral activity of BCF from Ps. fluorescens isolates was due to involvement of plant defense mechanism. In both the above LAR and SAR, no damage to the host plant was observed due to BCF treatment. BCF, thus can be characterized as a non-tioxic antiviral agent, which could give the necessary efficiency in combating CMV-EG. The activity of the inhibitory agent percent in the BCF obtained from Ps. fluorescne isolates was not destroyed by heating at 100ºC for 10 min, indicating that the antiviral agent percent in BCF is a heat stable BCF induces protection against CMV-EG in both local as well as a systemic hosts.

Virus variability and concentration were determined as local lesion morphology and mean number on Ch. amaranticolor as indicator plant for CMV ,Fig.1(B). The virus variability produced by Pseudomonas isolates considered as indication of LAR and SAR. The obtained results showed that, all isolates treatments were able to vary number and similarly (size, center, halo or without halo) produced by CMV infection. Ps. fluorescens isolates has the

lowest mean number of L.L compared to control. The same results were obtained by Zhong et al. (2002); Ipper et al. (2005); Zhao and Wu (2007) and Megahed (2008).

Protein content were determined in Pseudomonas isolates related to BSA as standard protein; the results revealed that, all isolates differed in protein content (1.305; 1.596; 1.770, 1.581, 1.602 and 1.575 mg/g fresh weight cells for ATCC, P1, P2, P3, P4 and P5 respectively.

Quantitative of antiviral protein were determined on the basis of the number, intensity, molecular weight and reproducibility of SDS-PAGE five biotic inducers bands. The total number of protein bands were 13 bands include of 10, 10, 9, 7, 7 and 7 for ATCC, P1, P2, P3, P4 and P5 respectively (Table 4 and Fig.2). Sex bands with the same mobility were treated as identical fragment for Pseudomonas isolates (monomorphic) or common fragment with 46%. Weak bands with negligible intensity and smear band were both excluded from final analysis. The numbers of scored new bands varied among isolates were 28.4, 30.5, 105.5 and 95.0 KDa-bands of ATCC, P1, P2 and P3 respectively, were treated as unique 9genetic marker) with 31%. The polymorphic (specific bands) for each isolate 80.2, 33.4 and 14.3 KDa bands.

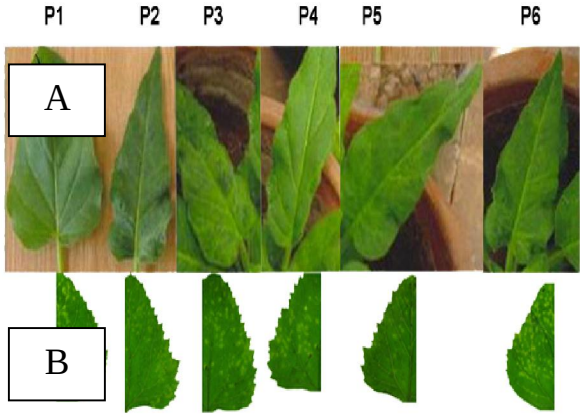

Fig.1 (A): Single local lesion diversity of CMV inoculated on Ch. Amaranticolor treated with BCF of Ps. fluorescens isolates (ATCC 13223; O1, P2, P3, P4 and P5). (B): N. glutinosa treated with BCF of Pseudomonas isolates and mechanically inoculated with CMV-EG. showing different of symptoms on leaves. Ps. ATCC, Ps.1, Ps.2, Ps. 3, Ps. 4 and Ps. 5, C = untreated with BCF.

Table (2): Effect of BCF on CMV-EG infection in Ch. amaranticolor using local lesion assay.

Ps. fluorescens	Control	LAR*			SAR*		
		BCF treated	BCF untreated	Inhibition (%)	BCF treated	BCF untreated	Inhibition (%)
ATCC	105.5**	11.8	22.5	85.0	0.0	0.0	0.0
P 1	105.5	18.5	36.1	82.5	0.0	6.5	0.0
P 2	105.5	28.6	36.7	72.9	8.5	16.3	91.0
P 3	105.5	26.4	35.8	75.0	10.2	17.5	89.2
P 4	105.5	33.2	44.2	58.5	12.9	18.5	86.4
P 5	105.5	30.7	41.5	70.9	9.5	15.1	89.9

LAR = Local acquire resistance. SAR = Systemic acquire resistance. Control = Leaf treated with sterilized water Treated -= Half or leaf treated with BCF Untreated = upper half or leaf untreated with BCF
Determinated as local lesion

The BCF of Ps. fluorescens isolates treated Ch. amaranticolor and N. glutinosa due to induce increase in the accumulation of host proteins. The induction of additional proteins may merely a disturbance in host physiology and have related to the primary mechanism responsible for the reduction of infection. It has been suggested that, the induced protein may help to limit virus spread or multiplication (Gianinazzi and Kassanis, 1974 and Chen, et al., 2006). Lochenstein (1972) considers an protein inhibitor can be an inhibite of virus replication of its effective when applied 5 to 8 hours past-virus inoculation.

Four types of antiviral protein activities have been characterized and some antiviral proteins have been capable of more than one activity. The activities were; 1- Aggregation; 2-inhibition of establishment; 3-induction of a systemic viral resistant state and 4-inhibition of replication by inactivation of protein synthesis, cited from Chessin et al. (1995).

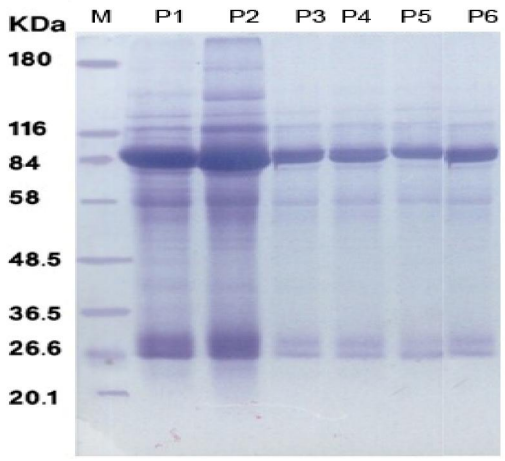

Fig. (2): SDS-PAGE 12% of protein fractions extracted from 6 Ps. fluorescens isolates (ATCC, P1, P2, P3, P4 and P5). M = marker protein (KDa).

Table (4): Protein fractions of Ps. fluorescens isolates using SDS-PAGE.

Isolates	Ps. fluorescens isolates						Polymorphism
MW (KDa)	ATCC	P4	P2	P3	P4	P5	
1058.5	-	-	+++	-	-	-	Unique
95.0	-	-	-	++	-	-	Unique
85.5	++	++	+++	+	+	+	Monomorphic
80.2	++	++	++	-	-	-	Polymorphic
75.0	++	++	+++	+	+	+	Monomorphic
54.7	+++	++++	++++	+++	+++	+++	Monomorphic
70.5	+++	+++	+++	++	++	++	Monomorphic
33.4	++	++	++	-	+	+	Polymorphic
30.5	-	++	-	-	-	-	Unique
28.4	++	-	-	-	-	-	Unique
14.3	+	+	-	-	-	-	Polymorphic
13.7	++	++	++	++	++	++	Monomorphic
12.0	++	+++	+++	++	++	++	Monomorphic
No. bands	10	10	9	7	7	7	-

Ps, fkyirescebs (ATCC 1`3223) Unique = polypeptide marker (genetic marker)
Monomorphic = or common polypeptides on all isolates Polymorphic = specific polypeptides on some isolates

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5/3/2010