J. Agric. Sci. Mansoura Univ., 26 (11): 6813 - 6823, 2001.

ANTIMICROBIAL EFFECT OF CEPHALOSPORIN-C PRODUCED BY *Cephalosporium maydis*

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ABSTRACT

Cephalosporium maydis the causal organism of late wilt disease of maize is still considered one of the most important fungal diseases in the Egyptian corn fields. The fungus was internally and externally seed- borne in white and yellow cultivars (Michail *et al.,* 1999). Cephalosporins are products of the genus *Cephalosporium*. Such products are regarded as a group of B-lactam drugs has antimicrobial effect. Bioassay studies of Cephalosporin-C produced by *C. maydis* isolates as well as pure Cephalosporin-C and four semi-senthytic derivatives (Cephalexin, Cefatrexyl, Claforan, Curisafe) were carried out against many bacterial and fungal isolates . According to the aformentioned results, the antibacterial spectrum of such antibiotics was mainly specific to certain bacteria. Claforan (Cefotaxime sodium) showed the highest inhibitory effect of bacterial growth, especially the tested strains of *Erwinia amylovora*, (sensitive and resistant strains of streptomycin) and *Erwinia chrysanthemi* pathovar*. zeae* (Syn. *E. carotovora* var. *zeae*) the corn stalk rot incitant at the rate of 2μg/ml.

On the otherhand, all the used Cephalosporins products haven't any antifungal effect on corn stalk or ear rot even on the high dosage. Results also indicated that pure Cepanthalosporin-C and the same compound produced by *C. maydis* was less effective than other semi-synthetic derivatives of Cephalosporin-C.

INTRODUCTION

Corn (zea mays L.), is regarded next to wheat as source of bread and oil as well as source of food for farm animals and livestock (El-Khishen *et al*., 1992). Maize plant are susceptible to a number of diseases that reduce the yield and crop quality (Abou-El-Seoud, 1982). *C. maydis* was detected in a relatively higher percentage in different ear parts of corn.

Cephalosporins are B-Lactam antibiotics isolated from *Cephalosporium* species. There are several types of Cephalosporin: Cephalosporin-P_1 (a steroid with minimal antibacterial activity), Cephalosporin-N (isolated from *C. salmosynnematum*, identical as a Penicillin derivatives called now Penicillin-N) and Cephalosporin-C (produced by *C. acremonium*, mentioned as 7-aminocephalosporanic acid) (Delgado and Remers, 1990). Cephalosporin-C was first isolated from *C. acremonium* strains by Brotzu, 1948. It was also reported that it could be prepared into semi-synthetically for medical use as an antibiotic.

The culture filtrate could inhibit the growth of a wide variety of gram positive and gram negative bacteria. Cephalosporin-C possessed unique antibiotic properties by interfering with cell synthesis and was relatively nontoxic at high doses to human beings (Brotzu, 1948). Shirafuji *et al.*, 1979 classified the B-lactam negative mutants of *C. acremonium* into three groups : Penicillin-N (PCN) negative and Cephalosporin-C (CPC) positive mutants (the first group), (PCN) positive and (CPC) negative mutants (the second group), (PCN) negative and (CPC) negative mutants (the third group).

Ott *et al*., 1962 described Cephalosporin-C production process from *C. acremonium is* related to incubation period for about 72 hours at 25°C followed by 114 hours of fermentation process gave the maximum yield of such antibiotic.

Mahfouz, (1990) extracted *C. maydis* culture filtrate in petroleum ether. Four fractions of the same culture were tested and cephalosporin toxin was produced by both fractions I and II. It had an antagonistic effect on *Staphylococcus aureus* (6538p). *Bacillus subtilis* was also used for the biological detection and bioassay of Cephalosporin-C production (Refaat, 1979 and Talkan, 1990). It was found that Cephalosporin-C produced by *C. maydis* caused similar symptoms of late wilt to corn plant as the same caused by the fungus itself (Nour El-Din, 1995). Also, a new derivative Cephalosporin-C_X was found to be more active than Cephalosporin-C against the assay organism *B. subtilus* and *Escherichia coli* (W-208) (Demain *et al*., 1963).Strains of *Streptococci*, *Viridans*, *Pneumococci*, *Gonococci*, *Meningocci* and *Staphylococcus aureus* found to be moderately to highly susceptible to Cephalexin and Cephaloglycin (Cephalosporin-C analogues), whereas, *Haemophilus influenzae* and most of G^- bacilli were moderately to highly resistant (Braun *et al*., 1968).Shoeib, (1986) studied the effects of many antibiotics against the growth of *Erwinia amylovora*. Cefotaxime, and Cefotaxime-Na (10 μg/ml) were highly effective in inhibiting growth of the tested isolates, where Cephalexin and Cephalotin (50 μg/ml) were moderately effective in inhibiting growth of the same isolates (sensitive strains to streptomycin). Similar results has been reported by Levy and Novick., 1986, who found that Cephalothin (one of the derivatives of Cephalosporin-C and Streptomycin were selected for negative cross resistant of *E. coli*. Nasef and Shalaby, (1995) tested some substituted furocoumarins and their antimicrobial activity against *B. subtilis*, *S. aureus*, *P. putida*, *Serratia* spp., *Aspergillus sulphureus*, *C. maydis*, *Monilinia* spp. and *F. oxysporum*.

The present study was concerned with the impact of Cephalosporin-C as antimicrobial against *Erwinia amylovora* and different bacterial and fungal species.

MATERIALS AND METHODS

1. Extraction Of Cephalosporin-C*: -*

Disks, 10mm diameter, taken from the margins of *C. maydis* culture previously grHxn on the selected medium Richard's medium (Booth, 1971) or complete medium (Nuesch *et al*., 1973) were transferred to 250ml. conical flasks, each containing 50ml. of the complex medium No. 2 (20g corn meal, 15g soya flour, 1g $(NH_4)_2$ So_4, 3g Ca Co_3, 16ml methyl oleate and tap water up to 1liter) then adjusted to ph6 (Ott *et al*., 1962). Flasks were placed on a rotary shaker (250rpm) at 25-28°C for 72 hours. Three replicates were made for such treatment. Five ml of the shacked liquid medium of that isolate were retransferred to 250ml conical flasks containing 50ml of corn steep liquor medium (30g lactose, 30g cerelose, 20g soya flour, 2g Ca Co_3, 1g $(NH_4)_2$ So_4, 4g Dl. Methionine, 1g L-cysteine HCl, 30 ml corn steep liquor and tap water up to 1liter) for

fermentation process. The pH was adjusted to pH6. Three replicates were made. Flasks were placed on rotary shaker (250rpm) at 25-28°C for 114 hours (Ott *et al*., 1962). Culture filtrate was then centrifuged at 4000 rpm, for 10 minutes by using (Laboratory centrifuge, Model 800, China). The clear supernatent of *C. maydis* isolate was transferred to 10 ml glass vial and kept in the freezer for the following tests.

2-The Bioassay Of Cephalosporin-C:-

Three streptomycin sensitive strains (Str^s) of *E. amylovora* namely (Ea_6, Ea_9 and Ea_{10}) and three streptomycin resistant strains (Str^r) namely (Ea_1, Ea_3 and Ea_8) as well as other eight isolates of different genera of bacteria, *Fusarium moniliforme* and other five isolates of corn stalk and ear rot fungi were tested . Bacterial and fungal cultures were obtained from stock culture collection, Department of Plant Pathology, Faculty of Agriculture, Alexandria University, Egypt.

A- *In vitro* tests: Cephalosporin-C produced by *Cephalosporium maydis* isolates, standard Cephalosporin-C (CPC)(SIGMA Chemical Co.) as well as four semisenthytic derivatives namely :Claforan (cefotaxime) (HOECHST ORIENT Co.), Cefatrexyl (cephapirin sodium) (BRISTOL – MYERS SQUIBB Co.), Cephalaxin (cefadroxil monohydrate) (ADCO Co.), and Curisafe (cefadroxil monohydrate) (PHARCO PHARMACEUTICALS Co.) were tested against the tested of bacteria and fungi .

1- The minimal inhibitory concentration (MIC). The MIC was determined according to the Agar Dilution Method (Lennett *et al*., 1980).Fresh stock suspension of each antibiotics at concentration of (2, 6, 10, 30, 50, 100 and 200 μg/ml) were prepared in steril distilled water. Aliquots of the desired final concentration of the active ingredient were added aseptically to sterile liquified sucrose nutrient agar medium (Billing *et al*., 1960) after cooling to about 48°C to avoid heat inactivation of the antibiotics. Plates of solidified medium were inoculated with a 48 hours old culture of bacterial suspension (Ca.10^8 CFU/mL), then incubated at 27°C for 3 days and with 2 weeks old culture of tested fungi, then incubated at 27°C for 7-15 days.Three replicates were used for each concentration. Plates were examined for the developed growth of the fungus and bacteria. Plates which didn't receive any antibiotic served as check.

2- *E. amylovora* (Str^s & Str^r) and *E. carotovora* var. *zeae* were furtherly evaluated by the diffusion test procedure (The Disk – Diffusion Method) (Lennette *et al*.,1985) against the inhibitory effect of tested antibiotics Filter paper disks (12mm diameter) were impregnated with 100μg/ml suspension of each antibiotics, placed on sucrose nutrient agar. Plates previously seeded with 100μL of bacterial cell suspension (Ca.10^8 CFU/ml) of tested strains and with 0.5 ml of fungal spore suspension (for 25 ml of PDA medium). Three replicates were used for each treatment. The inhibition zone was measured after incubation at 27°C for 3-7 days.

B- *In vivo* tests: for controlling the growth of *E. amylovora*, selected pear fruits cv. Leconte were washed under running tap water, dipped in a detergent solution for 10 minutes, rinsed in water, then kept to air dry. Bacterial suspension in amount of 0.1ml (10^6 – 10^7 cfu / ml) of *E. amylovora* isolate (Str^r) (24 – 48 hours) was injected singly using a syringe into nick cut (Lelliot and Stead, 1987). The required concentrations of the used antibiotics (streptomycin and claforan) were freshly prepared. Fruits artificially inoculated with *E. amylovora* served as check. Each treatment comprised four repticates and kept at humid chamber. The length of the rotted portion was estimated.

RESULTS AND DISCUSSION

Cephalosporins, standard Cephalosporin-C (CPC) and four manufactured derivatives were tested against pathogenic fungal isolates and different pathogenic & non-pathogenic genera of plant bacteria. According to the obtained results, it appeared that the antibacterial spectrum of such antibiotics was mainly specific to certain individual bacteria. Furthermore, the cross-resistance between cephalosporins and streptomycin was also detected in case of *E. amylovora* (streptomycin resistant strains (Str^r)
Data in Table (1) indicated the following:

1. The standard Cephalosporin-C was effective in inhibiting the bacterial growth at different concentrations. The MIC was < 2 μg/ml in case of *Bacillus megaterium* and *Erwinia carotovora* var. *atroseptica*, > 6-10 μg/ml in case of *Bacillus subtilus*, > 10-30 μg/ml in case of *E. carotovora* var. *zeae* and *Staphylococcus aureus*, > 30-50 μg/ml in case of *Rhodococcus fascines* (*corynebacterium fascines*) and *E. carotovora* var. *carotovora* and > 100-200 μg/ml in case of *Erwinia amylovora* (Str^s and Str^r).

2. Cefatraxyl derivative inhibited the growth of all tested bacteria at relatively low concentrations. *B. subtilus*, *B. megaterium*, *S. aureus* and *E. carotovora* var. *atroseptica* were sensitive to this antibiotic since they didn't grow at the concentration of 2 μg/ml. The MIC ranged from > 2-6 μg/ml in case of *Agrobacterium tumefaciens*, > 30-50 μg/ml in case of *E. amylovora* strains (Str^s and Str^r) as well as *R. fasciens* and both *E. carotovora* var. *carotovora* and *E. carotovora* var. *zeae*.

3. Claforan derivative inhibited the growth of *E. amylovora* strains (Str^s and Str^r), *B. megaterium*, *S. aureus*, *E. carotovora* var. *carotovora*, *E. carotovora* var. *atroseptica* and *E. carotovora* var. *zeae* at relatively low concentration (2 μg/ml) (Fig. 1). However the

MIC ranged from > 6-10 μg/ml in case of *R. fasciens* and > 10-30 μg/ml in case of *A. tumefaciens* and *B. subtilus*.

4. Cephalexin derivative inhibited the growth of *B. subtilus*, *B. megaterium* and *S. aureus* at 2 μg/ml *E. carotovora* var. *atroseptica* was inhibited at > 2-6 μg/ml, while *R. fasciens* was sensitive to this antibiotic at > 6-10 μg/ml.

Table1

Table1cont

Fig (1): The effect of CEPHALOSPORIN-C fungal product,(cont)., CLAFORAN (1) and CEPHALOSPORIN-C (2) on the growth of *E. amylovora* (Str^r).

Fig (2): Show the effect of a mixture of Claforan and Streptomycin (1), streptomycin only (2) on protecting pear fruits against fire blight (3).

5. The MIC ranged from > 30 – 50 μg/ml in case of (Str^s) strains of *E. amylovora*, *E. carotovora* var. *zeae* and *A. tumefaciens*. However it ranged from 100-200 μg/ml in case of (Str^r) strains of *E. amylovora* as well as *E. carotovora* var. *carotovora*.

6. Curisafe was effective at concentration of 2 μg/ml in inhibiting the growth of *B. megaterium* and *S. aureus*, while *A. tumefaciens* was sensitive at > 2-6 μg/ml. The MIC ranged from > 10-30 μg/ml in case of *R. fasciens*, *E. carotovora* var. *atroseptica*, *E. carotovora* var. *carotovora* and *E. carotovora* var. *zeae*. *E. amylovora* strains (Str^s and Str^r) were inhibited at > 100-200 μg/ml.

It is worthy to note that Cephalosporin-C produced by *C. maydis* was less effective than its derivatives, the growth of *E. amylovora* didn't inhibit at a concentration of 100 μg/ml (Fig. 1). Otherwise, the semisynthetic Cephalosporin-C derivative namely Claforan strongly inhibited the growth of tested strains of *E. amylovora* at low dosage (Fig 1) as well as other bacteria except *A. tumefaciens*, *R. fasciens* and *B. subtilus*. This finding was in line with Demain *et al*., (1963) who found that the new derivative Cephalosporin-C_x was more active than the fresh Cephalosporin-C solution against the gram-negative cultures, *Proteus vulgaris*, *Klebsielle pneumoniae*, *Salmonella schottmuelleri* and *Escherichia coli*. The present study also indicated that both types of *E. amylovora* either (Str^s or Str^r) strains revealed similar response towards the tested antibiotics called Cephalosporins. This action explained the bacterisidal effect of the compound .

The tested strains of *E. amylovora* that are resistant to Streptomycin didn't show cross-resistant to Claforan. The obtained results suggest that using mixture of Claforan and Streptomycin may be better than using streptomycin alone in controlling fire blight disease (Fig. 2). Such treatment might also reduce population level of streptomycin resistant strains of *E. amylovora* which are increasing year after year since 1986 in Egypt (El-Goorani and El-Kasheir 1989, El-Goorani *et al*., 1989 and Shoeib and Hassanein, 1994). Such results supported the control of fire blight of apples and pears in Egypt with Noroxine, Micyclin and Ampicilline (Ahmed. 1997).

Data in Table 2 show that all tested Cephalosporin-C derivatives or Cephalosporin-C produced by *C . maydis* isolates as well as standard Cephalosporin-C have not any antifungal effect on the tested Fungi.

Table2

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التأثير الميكروبي للسيفالوسبورين-ج المنتج بواسطة الفطر سيفالوسبوريم مايبدز المصاحب لحبوب الذرة

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لأيزال فطر سيفالوسبوريم مايبدز المسبب لمرض الذبول المتأخر على الذرة واحدا من أهم الأمراض الفطرية فحقول الذرة في مصر حيث يعتبر أحد الفطريات المصاحبة لحبوب الذرة داخليا وخارجيا للأصناف البيضاء والصفراء ٠ أجريت دراسة لاختبار التأثير الحيوي لمركب سيفالوسبورين-ج المنتج بواسطة فطر سيفالوسبوريم مايبدز بالإضافة آلي أربعة مشتقات أخرى شبة تخليقية هي: (سيفالاكسين – سيفاتراكسيل – كلافوران – كيورسيف) على العديد من العزلات البكتيرية والفطرية ٠ وقد اظهرت النتائج آن هناك تخصص لمثل هذه المركبات في تأثيرها التثبيطي للبكتريا المختبرة حيث اظهر مركب الاكلافوران أعلي معدل تثبيطي لنمو البكتريا المسببة لمرض اللفحة النارية للكمثرى (اروينيا اميلوفورا) ومسبب عفن سيقان الذرة (اروينيا كاروتوفورا) على معدل منخفض ٢ ميكروجرام/مللي. كما اظهرت النتائج مستويات متفاوتة للتأثير التثبيطي للمركبات المستخدمة ضد باقي العزلات المختبرة٠ وعلى النقيض اظهرت النتائج المتحصل عليها ان السيفالوسبورينات المختبرة لم يكن لها آي تأثير مضاد لنمو الفطريات المسببة لعفن ساق وكيزان الذرة مع الجرعات العالية ٠ أشارت النتائج أيضا ان مركب سيفالوسبورين –ج النقي والمركب المنتج بواسطة الفطر سيفالوسبوريم مايبدز ذات تأثير تثبيطي اقل من مثيلاتها من المشتقات المخلقة ٠

Table (1): Effect of Cephalosporin-C and its Derivatives at Different Concentrations (μG/ML A-I) ON *E. Amylovora* StRAINS and other Tested Bacteria.

Tested Bacterium	CHECK	Pure Cephalosporin-C							Cefatraxyl							CLAFORAN						
		2	6	10	30	50	100	200	2	6	10	30	50	100	200	2	6	10	30	50	100	200
Phytopathogenic BACTERIA:																						
Erwinia amylovora																						
(Str^s)																						
Ea6	+	+	+	+	+	+	+	-	+	+	+	+	-			-						
Ea9	+	+	+	+	+	+	+	-	+	+	+	+	-			-						
Ea10	+	+	+	+	+	+	+	-	+	+	+	+	-			-						
(Str^r)																						
Ea1	+	+	+	+	+	+	+	-	+	+	+	+	-			-						
Ea3	+	+	+	+	+	+	+	-	+	+	+	+	-			-						
Ea8	+	+	+	+	+	+	+	-	+	+	+	+	-			-						
Agrobacterium tumefaciens	+	+	+	+	+	+	+	-	+	-						+	+	+	-			
Rhodococcus fasciens	+	+	+	+	+	-			+	+	+	+	-			+	+	-				
Bacillus subtilus	+	+	+	-					-							+	+	+	-			
Erwinia carotovora var. atroseptica	+	-							-							-						
Erwinia carotovora var. carotovora	+	+	+	+	+	-			+	+	+	+	-			-						
Erwinia carotovora var. zeae	+	+	+	+	-				+	+	+	+	-			-						
OTHER BACTERIA																						
Bacillus megaterium	+	-							-							-						
Staphylococcus aureus	+	+	+	+	-				-							-						

*** Data average of three replicates . + (Growth) - (Inhibition).**

Cont. Table (1): Effect OF cephalosporin-C and its derivatives at different concentrations (μg/ml A-I) on *E. amylovora* strains and other tested bacteria.

Tested bacterium	check	cephalexin							curisafe							extracted Cephalosporin-C						
		2	6	10	30	50	100	200	2	6	10	30	50	100	200	2	6	10	30	50	100	200
Phytopathogenic bacteria:																						
Erwinia amylovora																						
(Str^s)																						
Ea6	+	+	+	+	+	-			+	+	+	+	+	+	-	+	+	+	+	+	+	-
Ea9	+	+	+	+	+	-			+	+	+	+	+	+	-	+	+	+	+	+	+	-
Ea10	+	+	+	+	+	-			+	+	+	+	+	+	-	+	+	+	+	+	+	-
(Str^r)																						
Ea1	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-
Ea3	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-
Ea8	+	+	+	+	+	+	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	-
Agrobacterium tumefaciens	+	+	+	+	+	-			+	-						+	+	+	+	+	+	-
Rhodococcus fasciens	+	+	+	-					+	+	+	-				+	+	+	+	-		
Bacillus subtilus	+	-							+	+	+	+	-			+	+	-				
Erwinia carotovora var. atroseptica	+	+	-						+	+	+	-				-						
Erwinia carotovora var. carotovora	+	+	+	+	+	+	+	-	+	+	+	-				+	+	+	+	-		
Erwinia carotovora var. zeae	+	+	+	+	+	-			+	+	+	-				+	+	+	-			
OTHER BACTERIA																						
Bacillus megaterium	+	-							-							-						
Staphylococcus aureus	+	-							-							+	+	+	-			

*** Data average of three replicates . + (Growth) - (Inhibition)**

Table (2): Effect OF Cephalosporin-C and Its Derivatives at Different Concentrations (μG/ML A-I) ON CORN Stalk and Ear ROT FUNGI.

Tested Fungus	Check	Pure Cephalosporin-C							CEFATRAXYL							CLAFORAN						
		2	6	10	30	50	100	200	2	6	10	30	50	100	200	2	6	10	30	50	100	200
Fusarium moniliforme	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. oxysporum	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aspergillus flavus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Alternaria alternata	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Curvularia lunata	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sclerotium bataticola	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Cont. Table (2)

Tested fungus	CHECK	Cephalexin							Curisafe							Extracted Cephalosporin-C						
		2	6	10	30	50	100	200	2	6	10	30	50	100	200	2	6	10	30	50	100	200
Fusarium moniliforme	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
F. oxysporum	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aspergillus flavus	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Alternaria alternata	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Curvularia lunata	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sclerotium bataticola	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

2. Data are average of three replicates, (growth) , - (Inhibition) , (CPC) standard cephalosporin-C .

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