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# BACTERIAL WILT OF POTATOES : II-SENSITIVITY OF THE PATHOGEN TO ANTIBIOTICS AND PATHOGENESIS BY STREPTOMYCIN-RESISTANT MUTANTS

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## *Abstract*

Sensitivity of *Pseudomonas solanacearum* to antibiotics was tested by the disk diffusion method. The eight isolates under investigation showed considerable variation against erythromycin and streptomycin, as well as low sensitivity to cephalosporin. No sensitivity could be detected against penicillin at any concentration used.

The least cidal concentration (LCC) of the afore-mentioned antibiotics and relative sensitivity (RS) of isolates were determined. Penicillin did not show any detrimental effect at the used concentrations, hence the recorded RS was zero. Cephalosporin showed low cidal effect as indicated by high LCC and low RS. Considerable variation in sensitivity was observed in case of erythromycin and streptomycin; however, both showed high deterimental effect. The mode of action of antibiotics either on protein synthesis or cell wall formation was considered.

Mutants resistant to streptomycin (100 ug/ml) were tested for virulence. Resistant mutants produced earlier onset of the disease and more severe wilt symptoms as compared with the parent isolates.

The use of streptomycin in bacterial wilt control is not recommended. Further investigations on other antimicrobial agents are needed.

## INTRODUCTION

The puzzling behaviour of *P. solanacearum* either in soil or in culture media has received great attention by plant pathologists. The organism exists in soil for long periods under complete bare fallow; however, rapid loss in virulence and viability in culture media was reported by many investigators (Kelman 1953). As a soil inhabitant, the pathogen is subject to antagonism by other soil organisms (Farag *et al.* 1980, Aspiras and Delacruz 1985). Therefore, trials with biological control were made by Aspiras and Delacruz (1985) who reported that a rapidly growing antagonistic bacteria were highly effective in disease control. In a preliminary work, Shetty and Rangaswam (1969) indicated the use of certain antibiotics in bacterial wilt control. Because of difficulties encountered in field application of biocontrol agents, the possible use of antibiotics was tried.

The purpose of this investigation is to study the sensitivity of the bacterial wilt pathogen to different antibiotics and the role of antibiotic-resistant mutants in pathogenesis.

## MATERIALS AND METHODS

### 1. Sensitivity of isolates to antibiotics:

Eight pathogenic isolates of *Pseudomonas solanacearum* were tested for sensitivity to antibiotics by the disk diffusion method (Gould and Bowie 1952). Streptomycin, erythromycin, cephalosporin and penicillin were tested at the rates of 50 , 100 and 200 ug/ml.

Flasks containing nutrient broth (100 ml/flask) were inoculated with standard inoculum ( 4 loops/flask ) and incubated for 3 days at 30°C. Turbidity was standardized to give the same optical density (0.46). Plates containing nutrient agar medium (10 ml / plate) were seeded with 0.1ml of the culture which was evenly spread on the surface by means of a glass rod spreader. Disks of filter paper (0.5 cm in diameter), impregnated with the desired antibiotic concentration were prepared.

Four disks of each concentration, were placed on the surface of each seeded agar plate and two plates were used in each treatment.

The mean diameters of inhibition zones were recorded after incubation at 30°C for 48 hr.

## 2 . Least bactricidal concentration (LCC):

The least cidal concentration (LCC) of streptomycin, erythromycin, cephalosporin and penicillin was detemined against the isolates under investigation. Stock solutions of the antibiotics were prepared in sterile distilled water, and definite volumes were transferred to tubes containing nutrient broth to give the desired concentrations. The tested concentrations were 5, 10, 15, 20, 25, 30, 40, 45 and 50 ug/ml.

Incubation was made with a standard inoculum of the organisms propagated on nutrient broth after correction of culture turbidity to give the same optical density (0.46). Growth was recorded after 5 days incubation at 30°C, then bacterial growth in different treatments was examined.

Tubes that showed no growth in each replicate were further inoculated in nutrient broth to confirm the absence of viable cells. The highest dilution of antibiotics that prevented growth in the three replicates was considered as the least bactericidal concentration.

Relative sensitivity (RS) of isolates to antibiotics under investigation was determined according to Farag *et al.* (1982). RS to a given antibiotic was extrapolated from the determined LCC by using the following numerical grades:

| Numerical Grade | Range of LCC, ug / ml |
|---|---|
| 1 | 1-10 |
| 2 | 11-20 |
| 3 | 21-30 |
| 4 | 31-40 |
| 5 | 41-50 |

The number of isolates in each category is multiplied by the corresponding numerical grade. The latter is divided by the maximum numerical grade multiplied by the total number of isolates according to the following formula :

$$RS = 100 - \frac{\text{( number of isolates x numelical grade)}}{\text{Total NO. of isolates x Maximum Numerical grade}} \times 100$$

### 3. Effect of streptomycin-resistant mutants on wilt severity :

Small colonies developed in haloes of 200 ppm streptomycin sulphate, in the disk diffusion test were selected and propagated on glucose nutrient broth containing 100 ppm of the antibiotic. Incubation was made at 30°C for 4 days. The parent isolates were propagated on glucose nutrient broth and incubation under the same condition. Three parent isolates, and three antibiotic-resistant clones selected from the parent isolates were used. Turbidity of broth cultures was standardized to give an optical density of 0.46, and the cultures were used for plant inoculation.

Inoculation was made through soil infestation of potato plants cv King Edward after severing the root system, at one side of the plants with sharp scalpel. Control plants were severed in the same way and tap water (250 ml/pot) was added instead of bacterial inoculum. Plants were kept under green house conditions. Disease readings were taken according to Winstead and Kelman (1952).

## RESULTS

### 1. Sensitivity of *P. solancearum* to antibiotics:

Data in Table (1) show the sensitivity of brown rot bacteria to erythromycin, cephalosporin, penicillin and streptomycin.

Disks containing either 50 ug/ml erythromycin or streptomycin were almost similar in their effect on isolates under investigation and the mean diameters of inhibition zones were 33.8 and 34.1 mm., respectively. It was observed that the iso-

Table 1. Sensitivity of *P. solanacearum* isolates to antibiotics.

| Isolates / Antibiotic (Conc.) | Mean diameter of inhibition zones ( mm ) | | | | | | | | Mean |
|---|---|---|---|---|---|---|---|---|---|
| | $Br_1$ | $Br_2$ | $Br_3$ | $Br_4$ | $Br_5$ | $Br_6$ | $Br_7$ | $Br_8$ | |
| Erythromycin | | | | | | | | | |
| 50 | 27.3 | 35.4 | 33.1 | 33.1 | 31.0 | 31.8 | 40.0 | 38.0 | 33.8 |
| 100 | 29.8 | 42.0 | 26.3 | 27.5 | 29.9 | 31.0 | 40.0 | 38.0 | 33.1 |
| 200 | 38.0 | 42.0 | 36.0 | 37.0 | 30.8 | 36.5 | 40.0 | 38.0 | 37.3 |
| | | | | | | | | | 34.7 |
| Cephalosporin | | | | | | | | | |
| 50 | 22.0 | 24.0 | 26.0 | 18.0 | 20.0 | 21.0 | 22.0 | 21.0 | 21.7 |
| 100 | 24.0 | 24.0 | 26.0 | 18.0 | 18.0 | 22.0 | 22.0 | 22.0 | 22.0 |
| 200 | 25.0 | 26.0 | 27.0 | 18.0 | 22.0 | 23.0 | 22.0 | 22.0 | 23.1 |
| | | | | | | | | | 22.3 |
| Penicillin | | | | | | | | | |
| 50 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 100 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 200 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | | | | | | | | | 0.0 |
| Streptomycin | | | | | | | | | |
| 50 | 29.5 | 34.0 | 29.3 | 28.9 | 40.0 | 30.8 | 40.0 | 40.0 | 34.1 |
| 100 | 34.5 | 40.0 | 31.5 | 40.0 | 40.0 | 32.8 | 40.0 | 40.0 | 37.4 |
| 200 | 40.0 | 40.0 | 31.5 | 40.0 | 40.0 | 40.0 | 40.0 | 40.0 | 38.9 |
| | | | | | | | | | 36.8 |

late Br 5 showed lower sensitivity to erythromycin (31.0 mm) as compared with streptomycin (40.0 mm); however, the isolate Br 7 showed equal sensitivity to these antibiotics (40.0 mm). The same effect could be observed in case of higher concentrations of erythromycin and streptomycin when tested against isolates under investigation.

Cephalosporin, on the other hand, showed slight inhibitory effect. The mean diameters of inhibition zones around disks containing 50 ug/ml were 21.7mm. Also , higher concentrations of cephalosporin gave mean diameter of inhibition of 22.3mm. So, it was not effective against *P. solanacearum*. Moreover, penicillin gave negative results at all concentrations tested.

Generally, it could be concluded that the tested isolates of *P. solanacearum* showed considerable variations with regard to their sensitivity to antibiotics. This may be attributed to a possible genetic differences between isolates of the same species.

## 2- Least bactericidal concentration (LCC) and relative sensitivity (RS) to antibiotics :

Data in Table (2) show the least bactericidal concentration of antibiotics against the eight isolates under investigation. Penicillin did not show any detrimental effect at the used concentrations, hence the recorded RS was zero. Meanwhile, cephalosporin showed very low cidal effect as indicated by the recorded high figures of LCC, hence the calculated RS value was 2.5%.

The isolates used in this investigation showed considerable variation in sensitivity to either erythromycin or streptomycin. The recorded LCC in case of erythromycin ranged from 10ug/ml to 40ug/ml and the calculated RS value was 50%. The corresponding figures of streptomycin were 10-25 ug/ml and 60%. This indicates higher sensitivity of isolates to streptomycin than to erythromycin. Similar effect was previously concluded in case of testing sensitivity by the disk diffusion method. However, it is worthy to note that the latter may be confusing in certain circumstances, as in case of cephalosporin, where haloes may be produced at high concentrations because of slow diffusion and/or osmotic tension.

## 3 - Efffect of streptomycin-resistant-mutants on wilt severity:

Data in Table (3) show the effect of inoculation with streptomycin-resistant

Table 2. Least bactericidal concentration and relative sensitvity of *P. solancearum* isolates to antibiotics (ug/ml broth culture).

| Isolates / Antibiotics | $Br_1$ | $Br_2$ | $Br_3$ | $Br_4$ | $Br_5$ | $Br_6$ | $Br_7$ | $Br_8$ | * Relative sensitivity ( RS ) |
|---|---|---|---|---|---|---|---|---|---|
| Erythromycin | - ( 40 ) | - ( 25 ) | - ( 25 ) | - ( 20 ) | - ( 20 ) | - ( 15 ) | - ( 10 ) | - ( 25 ) | 50.0 % |
| Cephalosporin | - ( 45 ) | - ( 40 ) | - ( 50 ) | - ( 50 ) | - ( 50 ) | - ( 50 ) | - ( 45 ) | - ( 45 ) | 2.5 % |
| Penicillin | - ( 50 ) | + ( 50 ) | + ( 50 ) | + ( 50 ) | + ( 50 ) | + ( 50 ) | + ( 50 ) | + ( 50 ) | 0.0 % |
| Streptomycin | - ( 20 ) | - ( 10 ) | - ( 25 ) | - ( 10 ) | - ( 15 ) | - ( 15 ) | - ( 25 ) | - ( 20 ) | 60.0 % |

$$* RS = 100 - \frac{\text{No. of isolates x Numerlical grade}}{\text{Total NO. of x Maximum Numerical}} \times 100$$

+ denoting growth.

- denoting absence of growth.

mutants of *P. solanacearum* on wilt development in cv. King Edward. Mutants resistant to streptomycin (100 ug/ml) caused earlier onset of the disease, 35 days after planting , as compared with the parent isolates. The recorded severities were 38.2%, 30.4 % and 32.5 % for $Br_1R$, $Br_3R$ and $Br_4R$, respectively. No wilt syndrome could be observed on plants inoculated with parent isolates 35 days after planting followed by a pronounced wilt incidence at the second period. The recorded severities were 28.2%, 25.0% and 28.4 % for the parent isolates $Br_1$ , $Br_3$ and $Br_4$, respectively after 45 days.

The corresponding figures for resistant mutants were higher than those recorded for parent isolates, being 44.2%, 45.5%, and 42.3%, respectively. The same trend could be observed 55 and 65 day after planting.

The indicates that streptomycin-resisitant mutants are more virulent than the parent isolates under investigation.

The percentage of increase in wilt severity, however, was different between selected mutants and ranged from 12.2% to 30.9 % at the end of the experimental period.

## DISCUSSION

To study the effect of antibiotics on virulent isolates of *P. solanacearum*, the disk diffusion mehtod was used. Antibiotics known to inhibit protein synthesis in bacterial cells such as streptomycin and erythromycin (amino-glycosides and macrolides, respectively) were compared with those which interfere with cell wall formation such as cephalosporin and penicillin (Jawetz *et al.*, 1976). The isolates used in this work showed considerable variation in their sensitivity to either streptomycin or erythromycin as indicated by the recorded diameters of inhibition zones. However, it seems probable that streptomycin is more detrimental than erythromycin and the recorded variation in sensitivity may be attributed to a possible genetic differences among isolates of the same species. Cephalosporin and Penicillin G, acting essentially through inhibiton of cell wall synthesis, showed very weak or no detrimental effect on the isolates as indicated by the disk diffusion method. In fact, the

Table 3. Effect of Streptomycin-resistant mutants on wilt severity (after planting).

| Treatments | Wilt severity % | | | | % of increase severity |
|---|---|---|---|---|---|
| | 35 days | 45 days | 55 days | 65 days | |
| $Br_1$ | 00.0 | 28.2 | 46.5 | 56.0 | 00.0 |
| $Br_3$ | 00.0 | 25.0 | 39.0 | 48.0 | 00.0 |
| $Br_4$ | 00.0 | 28.4 | 41.1 | 58.9 | 00.0 |
| Mean | 00.0 | 27.0 | 42.2 | 53.6 | 00.0 |
| $Br_1$ R | 38.2 | 44.2 | 67.5 | 78.0 | 28.2 |
| $Br_3$ R | 30.4 | 45.5 | 60.0 | 69.5 | 30.9 |
| $Br_4$ R | 32.5 | 42.3 | 52.5 | 67.1 | 12.2 |
| Mean | 33.7 | 44.0 | 60.0 | 71.5 | 23.8 |

% Increase in severity at the end of the experimental period

$$= \frac{\text{Severity produced by resistant} - \text{Severity produded by parent}}{\text{Severity produced by resistant}} \times 100$$

width of inhibition zones depends upon variables that influence the diffusion of the antibiotics such as the pH, depth and concentration of the agar medium, number and rate of growth of the inoculated bacteria as well as the conditions of incubation (Cruickshank *et al.* 1975). Therefore, the least bactericidal concenrtation (LCC) and relative sensitivity (RS) of isolates might be more reliable criteria for evaluation of antibiotics. According to the recorded figures of LCC and calculated values of RS,

Penicillin did not show any cidal effect at the used concentrations. Cephalosporin showed very low cidal effect. It is well know that cephalosporins are active in concentrations of 10 ug/ml or less against gram-positive bacteria; and *Pseudomonas* and some strains of coliform are resistant to that conentration and may be inhibited by levels reaching 300-500 ug/ml, (Jawetz *et al.* 1976). Generally, it could be concluded that antibiotics acting essentially as inhibitors of cell wall formation, such as Penicillin and Cephalosporin, have no or very weak bactericidal effect on *P. solanacearum*, as compared with those interfering with protein synthesis, i.e. Streptomycin and Erythromycin. The sensitivity of brown rot pathogen to antibiotics was also reported by Shetty and Rangaswami (1969), Seif El Yazal (1980) and Farag *et al.* (1986).

Although the reported high detrimental effect of streptomycin on *P. solanacearum*, the resistant mutants proved to be more pathogenic than the parent isolates. This indicates that the parent isolates contained a proportion of streptomycin resistant clones having higher pathogenic potential. In a preliminary work, on the effect of streptomycin sulphate on brown rot control, Farag *et al.* (1986) reported higher disease incidence after streptomycin application to seed-pieces and attributed the cause to higher root exudation, that was used by the bacterium to establish greater population, thus increasing inoculum density in the rhizosphere. Therefore, the increase in wilt severity by resistant clones may be another justification on the failure of using streptomycin as a control measure for brown rot of potatoes. Further investigations are needed to study the interrelation between resistance to antibiotics and disease severity.

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# الذبول البكتيرى فى البطاطس II- حساسية البكتريا المرضية للمضادات الحيوية وقدرة الطفرات المقاومة للستربتومايسين على إحداث المرض

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أختبرت حساسية البكتريا *بسيدوموناس سولاناسيوم* للمضادات الحيوية باستعمال طريقة أقراص ورق الترشيح وقد أظهرت ثمانى عزلات من البكتريا حساسيتها المختلفة للأرثروميسين والإستربتوميسين وحساسية ضعيفة لسيفالوسبورين . ولا توجد حساسية ضد البنسلين .

وقد قدر أقل تركيز قاتل والحساسية النسبية للعزلات بالنسبة للمضادات الحيوية السابقة ولم يظهر أي تأثير قاتل للبنسلين فى التركيزات المستعملة وبالتالى فقد كانت الحساسية النسبية صفر. وقد أظهر السيفالوسبورين تأثيرا قاتلا ضعيفا وذلك بناء على التركيزات العالمية من أقل تركيز قاتل والأرقام المنخفضة للحساسية النسبية.

وقد ظهر بعض الاختلافات فى الحساسية بالنسبة للأرثروميسين والإستربتوميسين بالرغم من التأثير القاتل العالى لكليهما.