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The Toxicity of Antibiotics to Plant Cell Cultures

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Abstract

We have examined the toxicity of over twenty antibiotics to protoplast-derived cells of *Nicotiana plumbaginifolia.* The least toxic antibiotics are the betalactams: ampicillin, carbenicillin and the cephalosporins can be used to provide broad spectrum antimicrobial activity without significant toxicity to plant cells. Similar broad spectrum activity can also be obtained by combining rifampicin and trimethoprim. Other antibiotics which may be useful are erythromycin and colistin. The aminoglycosides are not recommended.

Introduction

Despite their widespread use in the culture of animal cells antibiotics have not found favour with those working with plant cells. While there is no doubt that plant tissue culture medium is less conducive to bacterial growth, infections can be a problem and it would be useful to have means of eradicating them.

To be effective, the ideal antibiotic should be bactericidal in plant tissue culture medium, non-toxic to plant cells and have a broad spectrum of microbiological activity. This paper reports an examination of the relative toxicities of a range of antibiotics to cells derived from *Nicotiana plumbaginifolia* protoplasts. This method was chosen as: (i) slight toxicity of antibiotics to plant cells may be overlooked in short term cultures but will be revealed in longer term experiments; (ii) the plating efficiency of *N. plumbaginifolia* is reproducibly high and constant over a range of plating densities so quantitative comparisons between antibiotics can be made; (iii) cells derived from protoplasts are very sensitive to phytotoxic agents and could reveal toxic effects not apparent in callus or in seedlings (Zilkah and Gressel 1977).

Although we have used *N. plumbaginifolia,* comparison of our results with the limited amount published by others shows that compounds found to be toxic with these cells are likely to be toxic to other plant cell types. We hope that our work will stimulate others to explore the use of antibiotics for the sterilization of tissue and the elimination of contamination in plant cultures.

Materials and Methods

Mesophyll protoplasts were prepared from haploid plants of *N.plumbaginfolia* grown in controlled environment rooms (21-23°C, 16 hour day, 50-60% RH, 7-8000 lux). Briefly, the undersurfaces of leaves were abraded and leaf pieces floated on pre-plasmolysis medium (culture medium supplemented with 12% sucrose) for one hour. Culture medium was B5 supplemented with 750mg/l $CaCl_2$, 2mg/l NAA 0.5mg/l BAP, pH 5.8. Protoplasts were prepared by overnight digestion in the above medium containing 2% cellulase R10, 0.5% macerozyme R10 and after washing were cultured for 4-5 days at 2×10^4 protoplasts/ml in culture medium supplemented with 10% sucrose. Protoplast derived cells were plated at 100 units/ml in 2mls of 'A' Medium (Caboche 1980) in 5cm tight-lidded Petri dishes (Falcon) and appropriate amounts of filter-sterilised antibiotic solution added. After approximately 3-4 weeks the protoplast-derived colonies were 1-2mm in diameter and plating efficiency was scored by eye. Figure 1 shows that plating efficiency of *N. plumbaginifolia* protoplast-derived cells is constant over a wide range of plating densities, providing a quantitative method for assaying drug toxicities.

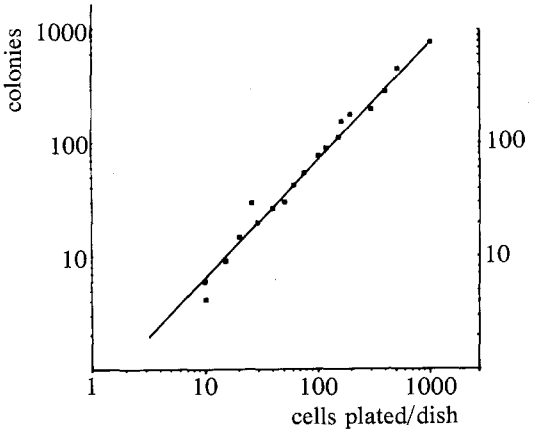

Figure 1
Plating efficiencies at variable cell densities. Protoplast-derived cells were plated at 4-5 days into medium A, and the resulting colonies counted 3-4 weeks later.

Results

Antibiotics that interfere with bacterial cell wall synthesis

Betalactam antibiotics

1) *The Penicillins:* We have examined the toxicity of Penicillin G (Benzyl Penicillin) and two derivatives, ampicillin and carbenicillin. These antibiotics are relatively non toxic to plant cells (figure 2a). The lack of phytotoxicity of these antibiotics has been noted by others (Bancroft *et al* 1975, Phillips *et al* 1981). Penicillin G is very active against Gram-positive bacteria but much less effective against Gram-negative organisms; it is also rather acid-labile. Ampicillin has a broader spectrum of activity and is acid-stable but like penicillin is sensitive to betalactamases. Carbenicillin has an even broader spectrum of activity, has resistance to some betalactamases but is less acid-stable than ampicillin (Garrod *et al* 1981).

2) *The Cephalosporins:* We have tested cephalothin, cefoxitin and cefotaxime which are broad spectrum antibiotics; none of which appear to be toxic up to levels of 100μg/ml or higher (figure 2b). Another cephalosporin, cephaloridine has been shown by others to be non-toxic in short term protoplast cultures (Bancroft *et al* 1975). Cephaloridine and cephalothin are degraded by a number of betalactamases; however cefoxitin and cefotaxime are resistant (Garrod *et al* 1981).

We have noticed that both the penicillins and the cephalosporins potentiated colony growth. The ability of penicillin to act as a plant growth hormone has been noted by others (Nickell 1952). The reason for this stimulation remains to be elucidated.

Both the penicillins and cephalosporins are bactericidal in activity. The betalactam antibiotics are also non-toxic to fresh protoplasts and protoplast-derived cells (results not shown). Their lack of effect on plant cells is probably due to their specific action on components of the bacterial cell wall; targets which do not exist in plant cells.

Because of their low toxicity, the penicillins or cephalosporins should be the antibiotics of choice with plant cell cultures. Their efficiency is sometimes limited by the presence of batalactamase producing bacteria, although the use of betalactamase resistant antibiotics such as cefoxitin and cefotaxime can overcome this problem.

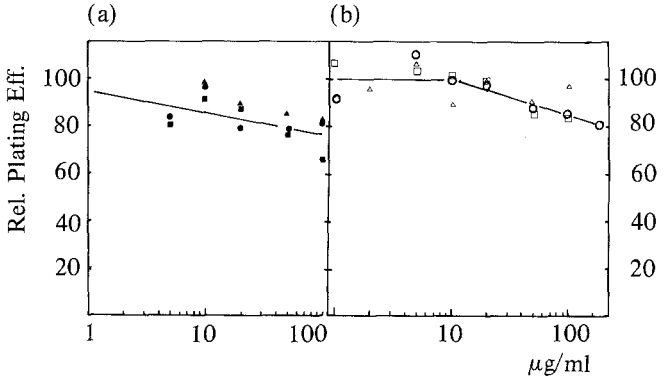

Figure 2
(a) Relative plating efficiency of cells in the presence of ● penicillin G, ■ ampicillin, ▲ carbenicillin.
(b) ○ cephalothin, □ cefoxitin, Δ cefotaxime.

Vancomycin

This antibiotic has a relatively narrow spectrum of activity and is bactericidal against some Gram-positive bacteria. All Gram-negative bacteria are resistant. The antibiotic is not toxic to plant cells at levels up to 100μg/ml (table 1), neither is it toxic to fresh protoplasts or calli (not shown) or to *C. aurantiaca* seedlings (Thurston *et al* 1979). Is relatively narrow spectrum (covered by the betalactam antibiotics) means that vancomycin has limited utility with plant cells.

Polymixin B (Aerosporin) and Polymixin E (Colisin)

These cyclic peptide antibiotics were examined as they are more active against Gram-negative than Gram-positive organisms. Strictly speaking these drugs do not interfere with the bacterial cell wall but with the outer membrane of Gram-negative bacteria. Polymixin B has been reported to inhibit protein synthesis in plant protoplasts (Watts and King 1973) and inhibit development of *Xenopus* embryos (Laskey 1970). However, polymixin E is non-toxic to animal cells and we find it the least toxic of the polymixins in plant cell culture (table 1), and its use at 5μg/ml should be considered against Gram-negative bacteria that are resistant to carbenicillin or the cephalosporins.

Antibiotics affecting bacterial protein synthesis

The aminoglycosides

This group of antibiotics is bactericidal against a wide range of Gram-positive and Gram-negative bacteria. We have tested a range of aminoglycoside antibiotics (streptomycin, neomycin, kanamycin, gentamicin, G418, amikacin and tobramycin).

All these antibiotics are toxic to varying degrees to *N. plumbaginifolia* cells (figure 3). These results confirm and extend the results of others (Watts and King 1973, Dix *et al* 1977, Eichholtz *et al* 1982, Ursic *et al* 1981). This toxicity is probably due to the action of the aminoglycosides on the 'prokaryotic-like' ribosomes of chloroplasts and mitochondria. Streptomycin is the least toxic of the aminoglycosides but is toxic at levels (100μg/ml) used in animal cell culture medium.

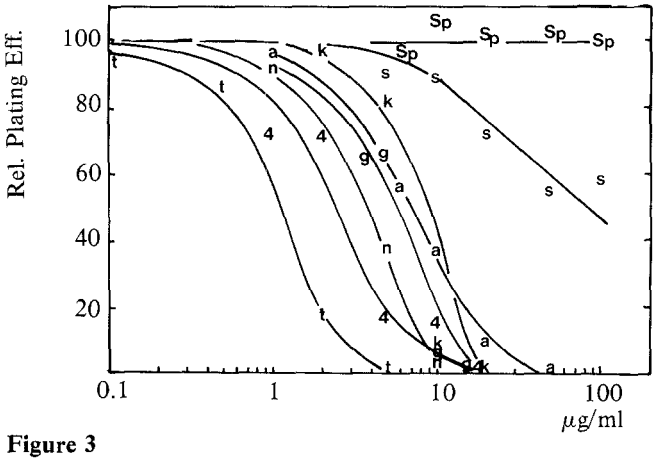

Figure 3
Relative plating efficiencies of cells in the presence of aminoglycoside antibiotics. t tobramycin; 4 G418; n neomycin; k kanamycin; s streptomycin; sp spectinomycin; g gentamicin; a amikacin.

One great disadvantage of the aminoglycosides, as far as plant tissue culture is concerned, is that their activity is sharply dependent on pH. For instance, streptomycin is only 0.2% as active at pH 5.5 as at pH 8.5. The other aminoglycosides are affected similarly but to a lesser degree (Garrod *et al* 1981). This means that the levels of these antibiotics needed to kill bacteria in plant tissue culture medium (pH 5.5 - 6) will almost certainly be lethal to plant cells. We have also examined the effect of spectinomycin an aminocyclitol antibiotic which is bacteristatic to a wide range of Gram-positive and Gram-negative bacteria. In contrast to the aminoglycosides, this antibiotic is not toxic in the range tested (figure 3). However, it has been reported to be toxic to *Euglena* (Nicolas 1981) and to *C. aurantiaca* seedlings at 100μg/ml (Thurston *et al* 1979).

Table 1
Toxicity of various antibiotics to protoplast-derived cells of *N. plumbaginifolia*

Antibiotic	Concentration (a)
Vancomycin	10 - 200
Polymixin B	0.6 - 0.7
Polymixin E	5 - 10
Erythromycin	30 - 80
Tetracycline	5 - 10
Chloramphenicol	2 - 5
Nalidixic Acid	<5
Rifampicin	25 - 30
Trimethoprim	8 - 90

(a) First figure is the concentration (μg/ml) at which plating efficiency is reduced by 20%; second figure is the concentration giving 50% survival.

The expermiments were performed as described in the materials and methods and in the legends to figures 2 and 3.

Erythromycin
Erythromycin is a member of the macrolide group of antibiotics and is generally more active against Gram-positive than Gram-negative organisms. The antibiotic is bacteristatic at low concentrations but at higher concentrations is slowly bactericidal. The minimal inhibitory dose for Gram-positive organisms is less than 1μg/ml; for Gram-negative organisms, doses of 50-100μg/ml and higher are sometimes required. The activity of the antibiotic is affected by pH; the antibiotic is unstable below pH 5, and its antibiotic activity increases with increasing pH up to pH 8.5 (Garrod *et al* 1981). The drug is relatively non-toxic to plant cells at up to 20 μg/ml (table 1). Whether this tolerance is due to breakdown of the antibiotic is not clear. It may be useful against Gram-positive organisms where penicillins cannot be used because of the presence of betalactamases.

Tetracyclines
These are a broad spectrum, bacteristatic group of antibiotics with little difference in activity.

We find that tetracycline is very inhibitory in long term toxicity tests at concentrations greater than 5μg/ml (table 1) It had also marked toxicity on fresh protoplasts (not shown) and in view of the bacteristatic, rather than the bactericidal action of the antibiotic, it is not to be recommended.

Continuous exposure to tetracycline at 5μg/ml has been reported to have little effect on the growth of carrot suspension cultures (Lesley and Behki 1977). Continuous exposure to higher concentrations is toxic although high doses for short periods are tolerated.

Chlorotetracycline (aureomycin, biomycin) inhibits germination of peas and lentils at 5μg/ml and Jerusalem artichokes are inhibited at 10μg/ml and higher (Nētien and Bertrand 1959).

Numerous workers use tetracycline as part of an antibiotic cocktail with ampicillin and gentamicin during the preparation of plant protoplasts. Since the action of penicillin is antagonized by the simultaneous presence of a bacteristatic antibiotics (such as tetracycline) we recommend that the use of such cocktails be abandoned.

Chloramphenicol
This antibiotic is a broad spectrum, bacteristatic antibiotic and is toxic to plant tissues at low concentrations, 5-10μg/ml inhibiting *Nicotiana* suspension cultures (Maliga *et al* 1980, Lurquin and Kleinhofs 1982). Other plant tissues are also inhibited by the drug (Nētien and Bertrand 1959, Phillips *et al* 1981). Our own results (table 1) show that chloramphenicol is toxic to *N plumbaginifolia* cells at levels above 1μg/ml. Its use is not recommended because it is both toxic and bacteristatic in action.

Other Antibiotics
Rifampicin
This antibiotic is bactericidal and more active against Gram-positive than Gram-negative bacteria (Garrod 1981).

Rifampicin has been used successfully at levels of up to 50μg/ml for short periods to clear Jerusalem artichoke tuber explants of contaminating bacteria (Philips *et al* 1981). Its use at above 20μg/ml for long periods is not recommended, although plating efficiency is high (table 1), there is some inhibition of growth as the colonies appear smaller. Neither should the drug be added to freshly isolated protoplasts; however, after 3 days the protoplasts have become reasonably resistant (results not shown).

Rifampicin is somewhat unusual for a bactericidal antibiotic in that combination with another bactericidal antibiotic may be antagonistic and with one that is bacteristatic the effects may be synergic (Garrod *et al* 1981). Rifampicin can be combined with trimethoprim to produce a broad spectrum bactericidal cocktail (Kerry *et al* 1975).

Trimethoprim
This is one of a group of folate antagonists which owe their importance as antimicrobial agents to an inhibitory effect which is much greater on the target enzyme (dihydrofolate reductase) in protozoa or bacteria than on the corresponding eukaryotic enzyme.

We find that plant cells are rather resistant to trimethoprim, doses as high as 10^{-4}M (30μg/ml) - which is in the antibacterial range - being well tolerated (table 1). Similar results have been reported by others. The toxic dose for *N. debneyi* protoplasts being 1.5×10^{-3}M. (Scowcroft and Larkin 1980). However, the drug is rather toxic to freshly isolated protoplasts (results not shown).

As mentioned above trimethoprim may act synergistically with rifampicin to produce a broad spectrum bactericidal cocktail. We have tested combinations of rifampicin and trimethoprim at up to 20μg/ml of each drug and find no effect on plating efficiency (not shown). This combination may prove useful in plant tissue culture.

Discussion

Although there have been a number of isolated reports on the effects of antibiotics on different plant tissues there have been only a few attempts to test an array of antibiotics on a single tissue so that comparative toxicities can be assessed.

We have examined the effect of over twenty antibiotics on the plating efficiency of protoplast-derived cells of *N. plumbaginifolia.* This species was chosen since the plating efficiencies are high and independent of cell density which enables quantitative comparisons between antibiotics to be made. Protoplast derived cells were used as they are probably more sensitive than calli or seedlings to phytotoxic agents and so can reveal toxic compounds of low potency.

The least toxic antibiotics are the betalactams. Either ampicillin or carbenicillin provides a broad spectrum of bactericidal activity. Carbenicillin is rather more resistant to betalactamases but less stable in acid. Cephalosporins such as cefoxitin and cefotaxime can also be recommended. Doses of up to 100μg/ml of each of these antibiotics can be used without apparent ill effect. Broad spectrum bactericidal activity non-toxic to plant cells may also be produced by combining rifampicin with trimethoprim (20μg/ml of each).

Other antibiotics which could be used with caution are erythromycin at less than 20μg/ml for Gram-positive organisms or colistin at less than 5μg/ml for Gram-negative organisms. These latter antibiotics should only be considered where other less toxic broad spectrum preparations have failed.

The aminoglycosides cannot be considered as they are toxic at levels that would be active in plant media. Spectinomycin (strictly an aminocyclitol) is an exception as it is non toxic in our system but is only bacteristatic in action.

It should be stressed that antibiotics are no substitute for good sterile technique but their use should be considered in the disinfection of hard-to-sterilize tissues or in ridding irreplaceable cultures from contamination.

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Abbreviations

RH = Relative Humidity, NAA = 1- Naphthylacetic Acid, BAP = 6- Benzylamino Purine.

B5 Medium is Gamborgs B5 (Gamborg, OL (1970) Plant Physiol. *45* 372-375) and purchased from Flow Laboratories.

G418 was a kind gift of Dr Daniels, Schering Corporation, Bloomfield, New Jersey, USA.