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Full Length Research Paper

In vivo and *in vitro* antibacterial activity of neomycin against plant pathogenic bacteria

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The purpose of this study was to evaluate the potential of neomycin to suppress the development of economically important plant pathogenic bacteria. The *in vitro* antibacterial activity against *Xanthomonas campestris* pv. *citri, Erwinia carotovora* subsp. *carotovora*, *Xanthomonas oryzae* pv. *oryzae* and *Ralstonia solanacearum* was evaluated. The minimal inhibitory concentration values for the four strains were 2, 0.5, 0.25 and 2 mg l^{-1}, respectively, and minimal bactericidal concentration values were 4, 2, 0.25 and 8 mg l^{-1}, respectively. Furthermore, this effect of neomycin on the cell morphology of *Xanthomonas oryzae* pv. *oryzae* was investigated by transmission electron microscope, and it indicated that neomycin caused damage to bacteria, resulting in the leakage of cytoplasmic contents. Finally, the effects of neomycin on disease development and spread were determined using potted plants in the greenhouse. *In vitro* studies indicated that post-infectional spraying with neomycin significantly inhibited the development of citrus bacterial canker caused by *X. campestris* pv. *citri*, cabbage soft rot caused by *E. carotovora* subsp. *carotovora*, ginger bacterial wilt caused by *R. solanacearum* and rice bacterial blight caused by X. oryzae pv.oryzae (80.51, 77.55, 77.54 and 69.07% disease reduction, respectively).

Key words: Neomycin, plant pathogenic bacteria, antibacterial activity.

INTRODUCTION

A wide range of crops are susceptible to plant diseases caused by bacteria. Bacterial diseases of plants are very difficult to manage and can lead to devastating losses to farmers (Strange and Scott, 2005). It has been well established that antibiotics can be used for combating certain bacterial diseases of many economically important fruit, vegetable, and ornamental plants (Vidaver, 2002). Antibiotics provide a protective barrier on the surface of plants to suppress the growth of the pathogens before infection. However, due to the emergence of antibiotic-resistant plant pathogens, the efficacy for control of plant diseases has been diminished in some regions (McManus et al., 2002). For streptomycin and terramycin, the two most commonly used antibiotics against bacterial plant diseases, the resistance is now extremely widespread (Rezzonico et al., 2009). Thus, they have given rise to an urgent need to develop effective and economic new antibiotics for the management of some of the most devastating bacterial plant diseases. Few new antibiotics are expected to be used in plant agriculture because of high costs of development, regulatory constraints, and environmental and human health concerns. However, they are being developed and marketed for their efficacy in controlling

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Abbreviations: HPLC, High performance liquid chromatography; **CGMCC,** China general microbiological culture collection center; **ACCC,** agricultural culture collection of China; **LB,** luria broth; **MIC,** minimum inhibitory concentration; **MBC,** bactericidal concentration.

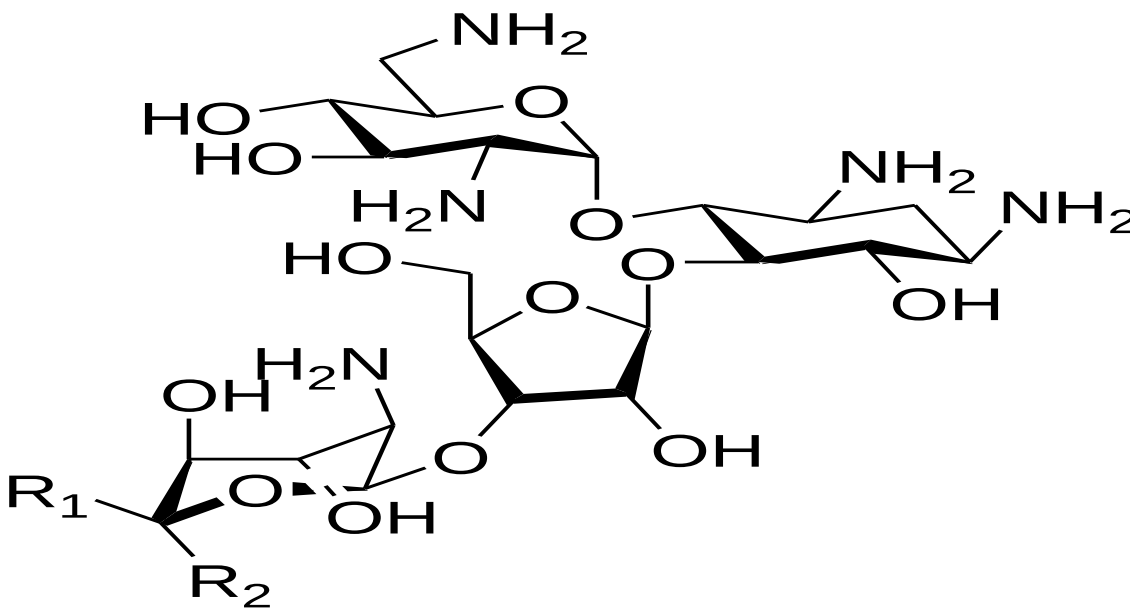

Figure 1. Chemical structure of neomycin isolated from *Streptomyces fradiae* strain HTP. Neomycin B: R_1=CH_2NH_2, R_2=H_2; chemical name: O-2,6-diamino-2,6-dideoxy-β-L- idopyranosyl (1→3)-O-β-D-ribofuranosyl-(1→5) O- [2,6-diamino-2,6-dideoxy-α-D- glucopyranosyl-(1→4)]-2-deoxy-D-streptamine; neomycin C: R_1=H_2, R_2=CH_2 NH_2,; chemical name:O-2,6-diamino-2,6-dideoxy-α-D- glucopyranosyl (1→3) -O-β-D- ribofuranosyl -(1→5)O-[2,6-diamino-2,6-dideoxy-α-D- glucopyranosyl- (1→4)]-2- deoxy-D-streptamine.

plant diseases. *Streptomyces* species generally synthesize a sizeable number of diverse natural secondary metabolites, many of which are used as agrochemical products (Copping and Duke, 2007; El-Naggar et al., 2006).

In the course of our screening for new bioactive secondary metabolites, neomycin (B and C compounds, Figure 1) was isolated from a liquid culture of the fungus *Streptomyces fradiae* strain HTP. Although neomycin is one of the clinical and veterinary important antibiotic for its antibacterial activity (Kitchen and Waksman, 1955; Waksman and Lechevalier, 1949). So far, there is no description that neomycin has antibacterial activity against plant pathogenic bacteria. Citrus bacterial canker, cabbage soft rot, rice bacterial blight and ginger bacterial wilt are the most destructive bacterial plant diseases in agriculture in China, and the problem of resistance is serious for long-time use of streptomycin and terramycin. The citrus bacterial canker does serious harm to most citrus species in China, and caused severe leaf drop, leaf chlorosis and poor growth of plant. All this significantly affects yield and quality of citrus (Deng et al., 2010). Chinese cabbage is widely planted in northeastern China. The losses caused by *Erwinia carotovora* subsp. *carotovora* account for 50% of total yield when the disease is severe (Qiu et al., 2011). Rice bacterial blight is one of the most destructive rice diseases in southern China and causes at least 10% yield loss of susceptible rice varieties under appropriate climate condition (Xu et al., 2010). Ginger bacterial wilt caused by *Ralstonia solanacearum* has become a severe problem on ginger in China, there are no effective measures to control this disease up to now (Yang and Guo, 2010). The objective of this paper is to develop an effective disease management strategy for the control of the above mentioned plant diseases, and simultaneously evaluate the potential of neomycin to suppress the development of economically important plant pathogenic bacteria. Thus, neomycin was studied for their disease suppression activity under *in vitro* and *in vivo* tests.

MATERIALS AND METHODS

Antibacterial compounds and source of strains

Neomycin used in this study was isolated from the culture broth of *S. fradiae* strain HTP and the composition and content of neomycin were determined by high performance liquid chromatography (HPLC) (neomycin B 88%, neomycin C 12%) (Taiping Hou, unpublished results). The commercial bactericide streptomycin was used as controls (Long March Pharmaceutical Company China). *X. campestris* pv. *citri* (ACCC CATAS EPPI2109), *E. carotovora* subsp. *carotovora* (CGMCC 1.1000), *X. oryzae* pv.*oryzae* (CGMCC 1.859) and *R. solanacearum* (ACCC 01474) were known as pathogens for citrus bacterial canker, cabbage soft rot, rice bacterial bligh and ginger bacterial wilt, respectively, in China. They were obtained from China general microbiological culture collection center (CGMCC) and Agricultural Culture Collection of China (ACCC), and were isolated from infected host plants in fields. The stock cultures of bacteria were maintained at 4°C on Luria broth (LB) agar medium.

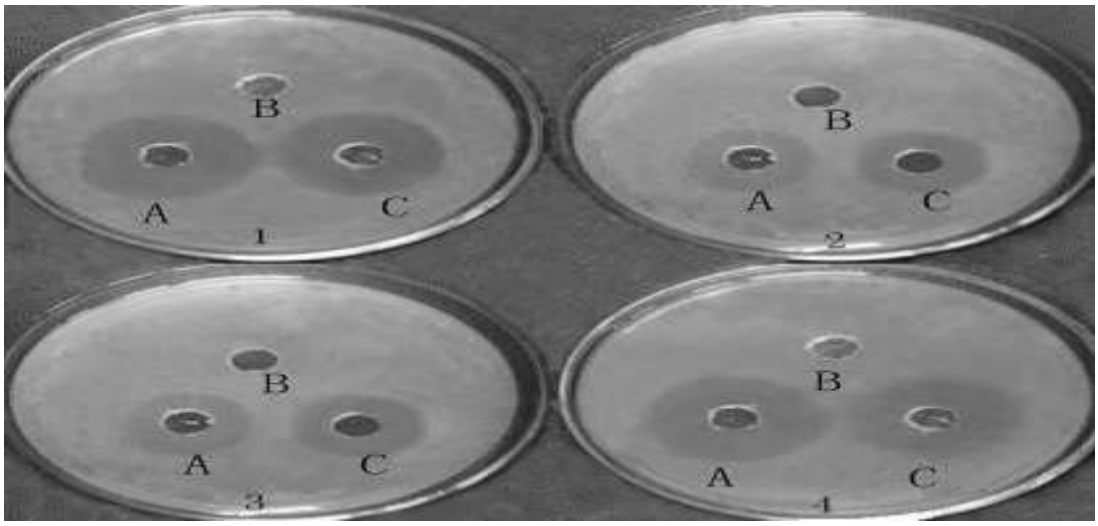

Figure 2. Inhibition zone of neomycin against the test strains. 1. *X. campestris* pv*. citri* , 2. *E. carotovora* subsp*. carotovora* , 3. *X. oryzae* pv.*oryzae, 4. R. Solanacearum*; **A** neomycin; **B** sterile distilled water; **C** streptomycin.

In vitro antibacterial activity

Disc diffusion method was used to detect the antibacterial activity of neomycin (Derbel et al., 2010). The four bacteria suspensions containing 10^7 cfu ml^{-1} were separately poured on LB agar media. The discs (6 mm diameter) were impregnated with neomycin solution (100 mg l^{-1}, 20µl) and placed on the inoculated agar and incubated at 35°C for 24 h. Antibacterial activity was evaluated by measuring diameter of inhibition zones in mm. Sterile distilled water served as negative control and streptomycin (100 mg l^{-1}, 20 µl) was used as positive control. Furthermore, the minimum inhibitory and bactericidal concentration (MIC and MBC) values were determined using the two-fold serial dilution technique (Kim et al., 2010). Neomycin was dissolved in sterile distilled water to achieve final concentrations of 0.25, 0.5, 1, 2, 4, 8, 16 and 32 mg l^{-1}, and then was added in 0.1 ml freshly prepared bacterial suspensions, respectively (10^7 cfu ml^{-1}) and incubated at 35°C. 24 h later, the MIC was observed. The minimum concentrations of neomycin at which no visible growth was observed were defined as MICs. Then, the tube contents were subcultured in fresh nutrient agar medium separately for 24 h, and MBCs were determined as that concentration of neomycin which killed 99.9 of the initial bacterial inoculum.

Transmission electron microscope analysis

After treatment with neomycin at the MIC (0.25 mg l^{-1}), *X. oryzae* pv.*oryzae* cells were prefixed in 0.5% glutaraldehyde-phosphate buffer (pH 7.0) for 2 h at 4°C, and then washed three times with the same buffer. The cells were further fixed in 1% osmium tetroxide-phosphate buffer (pH 7.2) overnight at 4°C, dehydrated in an acetone series, and then embedded in Epon812. Thin section was performed and stained with uranyl acetate and lead citrate**.** Viewing and photography were performed by a H-600IV TEM (Hitachi, Japan).

In vivo antibacterial activity

Neomycin was evaluated *in vivo* for antibacterial activity against the following diseases: citrus bacterial canker (*X. campestris* pv. *citri*), cabbage soft rot (*E. carotovora* subsp*. carotovora*), rice bacterial blight (*X. oryzae* pv.*oryzae*) and ginger bacterial wilt (*R. solanacearum*). Citrus, cabbage, ginger, and rice plants were grown in a greenhouse, and the temperature was set at 25°C, the relative humidity was set at 80%. Forty-eight hours after inoculation with its corresponding pathogen, the plants were sprayed with neomycin at a concentration of 200 mg ml^{-1} with three replicates. Streptomycin with the same concentration was used as positive control, and unsprayed plants were served as negative control. Observations on disease severity were recorded 10 days after inoculation. Disease severity were assayed for extent of infection by visual estimation of the percentage damage, browning or necrotic symptoms on the inoculated seedlings, and graded on a 0 to 4 scale as follows: 0 = no leaf symptoms; 1 = 25% of the leaf surface with symptoms; 2 = 26 to 50% of the leaf surface with symptoms; 3 = 50 to 75% of the leaf surface with symptoms; 4 = 76 to 100% of the leaf surface with symptoms. Disease index was calculated using the formula: Disease index = (Total grade points×100)/ (Number of leaves observed × Maximum grade).

Statistical analyses

All statistical analyses were performed by SPSS 11.0.1 statistical software (SPSS Inc., USA) statistical package.

RESULTS

In vitro antibacterial activity assay

The *in vitro* antibacterial activity of neomycin was qualitatively and quantitatively assessed by the presence or absence of inhibition zones, and MICs and MBCs. As shown in Figure 2, neomycin showed great potential of antibacterial activity against four plant pathogenic bacteria, and the diameters of the inhibition zones of

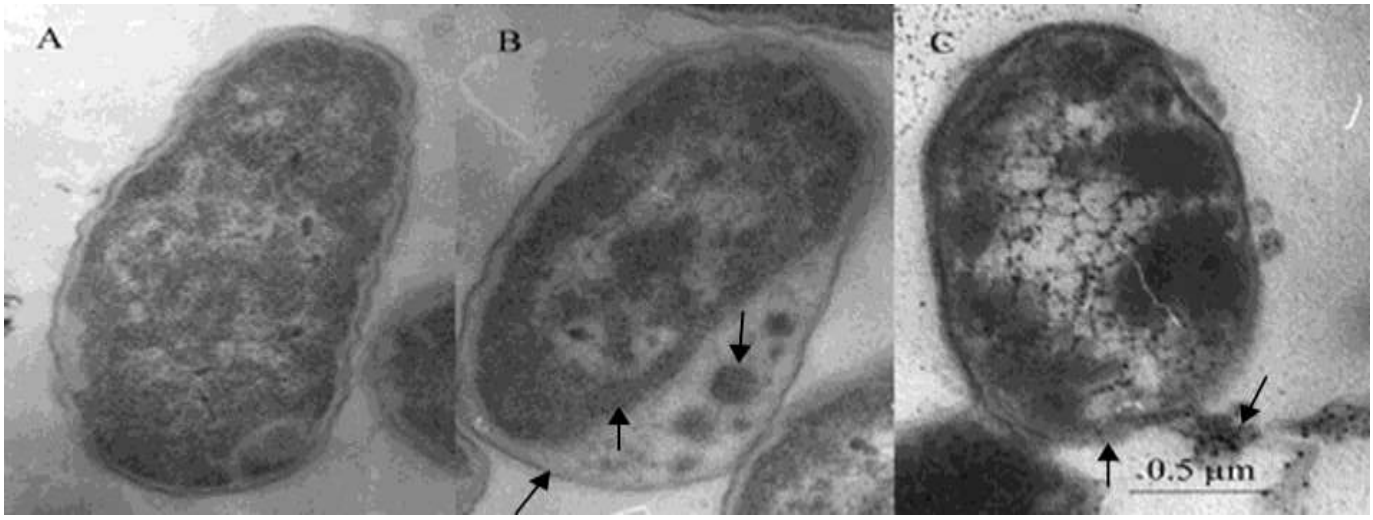

Figure 3. Transmission electron microscope image of *X. oryzae* pv. *oryzae*. *X. oryzae* pv. *oryzae* cells were incubated with 0.25 mg l^{-1} of neomycin at 35°C for 60 (**B**), 120 (**C**) min. Control was not treated with neomycin (**A**). Scale bar = 0.5 µm. The morphological changes of *X. oryzae* pv. *oryzae* cells were indicated by the arrow.

neomycin were in the range of 21.6 to 24.0 mm. The inhibition zones of neomycin compared favorably with that of streptomycin against the tested plant pathogenic bacteria. In addition, MICs for the four strains were 0.5, 2, 2 and 0.25 mg l^{-1}, respectively, and MBCs were 2, 4, 8 and 0.25 mg l^{-1}, respectively. It also indicated that neomycin not only reduced the growth rate of pathogens but also killed existing populations. In order to evaluate the application potential of neomycin, there *in vitro* antibacterial activities were compared to that of standard commercially available agricultural antibiotics streptomycin. Thus, the decision to direct continuing efforts to evaluation of *in vivo* control efficacy was made.

Morphological changes of *X. oryzae* pv. *oryzae* cells induced by neomycin

The influence of neomycin on the cell morphology of *X. oryzae* pv. *oryzae* cells was demonstrated by transmission electron microscope. The inner membranes and outer membranes of the untreated cells were clearly visible, and the cytoplasm was occupied by free ribosomes and fibrous electron dense material (Figure 3a). As compared to the untreated control, membrane blisters were derived from the outer membrane and the cellular contents leaked out from the inner membrane of the cells after treatment with neomycin for 60 min (Figure 3b). Moreover, 120 min neomycin-treated cells were damaged significantly and the cellular contents leaked into the medium (Figure 3c). Transmission electron microscopy findings suggested that neomycin exerted its bactericidal effect by acting on the bacterial cell surface initially and then on the cytoplasmic contents, and finally caused damage to bacteria, resulting in the leakage of cytoplasmic contents.

Evaluation of *in vivo* efficacy control

The promising neomycin was tested against the four pathogens in pot culture under glasshouse condition. Neomycin, like streptomycin, was phytotoxic to plants (data not shown) at high concentrations. Thus it was applied to the surface of plants and not injected. As shown in Table 1, post-inoculation spraying with neomycin significantly most effectively inhibited the development of citrus bacterial canker, and reduced the intensity of brown spot of citrus by disease index 11.47 (80.51% disease reduction) compared to the unsprayed plants. It was also active *in vivo* against *E. carotovora* subsp. *carotovora, R. solanacearum and X. oryzae* pv. *oryzae*, and their disease index were 11.76 (77.55% disease reduction), 15.90 (77.54% disease reduction) and 4.07 (69.07% disease reduction) compared to the unsprayed plants, respectively. It was also noted that neomycin had significant disease reduction compared to positive control streptomycin.

DISCUSSION

It was interesting to note that neomycin had potential antibacterial activity *in vitro* and *in vivo* conditions to control plant pathogenic bacteria. At a concentration of 200 mg l^{-1}, the disease reduction for neomycin treatment ranged from 69.07 to 80.51%, better than that for streptomycin control (50.00 to 72.56%). Neomycin has

Table 1. *In vivo* antibacterial activity of neomycin against the test strain.

Treatment [A]	Disease index (± SE) [B]	Disease reduction (%)
Cabbage (*E. carotovora* subsp. *carotovora)*		
neomycin	11.76 ±0.29 [a]	77.55
streptomycin	24.44±1.24 [b]	53.34
unsprayed	52.38±1.64 [c]	0
Citrus (*X. campestris* pv. *citri)*		
neomycin	11.47 ±0.76 [a]	80.51
streptomycin	16.15 ±3.62 [a]	72.56
unsprayed	58.86 ±5.01[b]	0
Ginger (*R. solanacearum)*		
neomycin	15.90 ±2.08[a]	77.54
streptomycin	19.60 ±1.45[a]	72.32
unsprayed	70.80±2.46 [b]	0
Rice (*X. oryzae* pv. *oryzae)*		
neomycin	4.07 ±0.21 [a]	69.07
streptomycin	6.58 ±0.60 [a]	50.00
unsprayed	13.16 ±0.66 [b]	0

[A] Neomycin concentration was 200 mg l^{-1}. Streptomycin with the same concentration was used as positive control, and unsprayed plants were served as negative control.[B] Values followed by the same letter are not significantly different at the 0.05 level according to Fisher's least significant difference test. [a,b,c] Numbers in a column with superscript letters are significantly different (P<0.05).

potential to suppress the development of economically important plant pathogenic bacteria, and provide an alternative therapy against bacterial infections in plants caused by resistant strains. Neomycin was natural, with a wide range of action over several pathogens, easy to produce. Meanwhile, the acute toxicity, the acute skin toxicity and eye irritation test of neomycin were studied and the results indicated that neomycin met the standard of GB15670-1995 "Toxicological Test Methods for Pesticides Registration"(data not shown). The priority for the next decades should be focused in the development of the alternative, neomycin. For streptomycin, surveillance for antibiotic-resistant plant pathogens has been sporadic and usually undertaken only after an antibiotic failed to control the disease (McManus et al., 2002). There are also two aminoglycoside resistance genes found in the neomycin gene cluster (Huang et al., 2005). Thus, it was thought that enhanced disease resistance in the present study could also take part. One approach to this problem focuses on the generation of new antibiotic agents by modification of existing antibiotic structures. In our other study, neomycin analogues started to be synthesized in an effort to examine their structure activity relationships. A field experiment was being conducted during September 2008 to December 2009 in the farm at key laboratory of bio-resource and eco-environment, ministry of education, sichuan university, chengdu (China) to assess the efficacy of neomycin against the four test organisms in a randomized block design.

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