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GROWTH PROMOTING ACTIVITY OF CERTAIN PENICILLINS ON CULTIVATED CELLS OF *BOUVARDIA TERNIFOLIA*

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Key Word Index—*Bouvardia ternifolia*, Rubiaceae; cell cultures, callus cultures, penicillin, antibiotics, growth promotion.

Abstract—A growth promoting effect similar to that of 2,4-D is induced in *Bouvardia ternifolia* calli and cell suspension cultures by some beta lactam antibiotics but not by others. It was found that this effect was exerted only by those penicillins that contain an auxin-like moiety associated with the 6-aminopenicillanic acid, such as carbenicillin or penicillin G. Phenylacetic acid and 6-aminopenicillanic acid were inactive on their own.

INTRODUCTION

The need to control microbial contamination of *in vitro* cultured plant tissues has stimulated studies on the effects produced by antimicrobial compounds (antibiotics) on plant cells. Although generally harmful to the plant cell [1, 2] some antibiotics are innocuous [3, 4], and there are reports of growth stimulation by very different types of antibiotics: penicillin, terramycin, streptomycin, thiolutin, bacitracin [5], isonicotinic hydrazide, oxytetracycline, chloromycetin, [6], and many penicillin derivatives [7]. Owens [8] reported that kanamycin very efficiently promoted organogenesis of shoots in calli derived from tobacco and carrot protoplasts. The basis for this stimulation, however, remains obscure.

While studying the transformation of *Bouvardia ternifolia* cells cultured *in vitro* in the presence of *Agrobacterium tumefaciens*, we observed an extremely high proportion of cell forming colonies in a medium lacking growth regulators. The frequency was so high that it could hardly be attributed to transformation. Reviewing this situation, we found that cells which had not been cultivated in the presence of *A. tumefaciens* also proliferated in the absence of growth regulators when carbenicillin, the antibiotic used to kill the bacteria, was present in the medium. Although the concentration of carbenicillin employed to disinfect the cultures (1 mg/ml) was very high (2.5 mM), the growth effect was so striking that we decided to investigate further

Here we report the growth promotion effect of some beta lactam antibiotics that appears to be dependent on the presence of an auxin component in the penicillin molecule whose activity is potentiated by the 6-aminopenicillanic acid (APA).

RESULTS

The growth of suspension cultures of *B. ternifolia* in the presence of carbenicellin (1 mg/ml) is illustrated in Fig. 1a, where it is compared in the absence and presence of 4.5 μM 2,4-D and 0.23 μM kinetin. To ensure that the content of the endogenous growth regulators was minimal at the initiation of the experiments, cultures in the stationary phase were used. After a long lag phase *Bouvardia* cell grew with a doubling time of 20 hr for seven days, after which time the growth rate declined. Carbenicillin (1 mg/ml) promoted growth at a slightly lower rate with a doubling time of 24 hr for seven days and then declined. In the absence of growth regulators the cells increased in size mainly due to water uptake, but they did not divide. When 2,4-D was reduced to half its original concentration, gowth was less s efficient than that with 1 mg/ml antibiotic Figure 1b shows that addition of either 2,4-D or kinetin alone did not modify the growth promoted by 1 mg/ml carbenicillin.

Higher concentrations of carbenicillin (1.5 and 2.0 mg/ml) did not enhance the growth response in suspension cultures (data not shown) nor in callus cultures (Fig. 2a). In spite of the slightly yellow colour of the tissues compared with the very white friable callus produced by 2,4-D and kinetin, carbenicillin did not seem to be toxic to the cells at the higher concentrations.

To see if other antibiotics promoted growth in our cultures, we looked at the effect of various penicillins and found dramatically different results; ampicillin (Fig. 2b) had no growth promoting effect, while penicillin G (Fig. 2c) provoked a very similar response to that of carbenicillin. The presence of optimal concentrations of growth regulator in these experiments indicated that the tissues employed were capable of responding to the stimulus and that the antibiotics were not exerting a deleterious effect. Although 2 mg/ml of the penicillins tested did reduce growth induced by 2,4-D and kinetin by 10–15%, cell viability was at least 95% (trypan blue exclusion) at the stationary phase in all the cultures.

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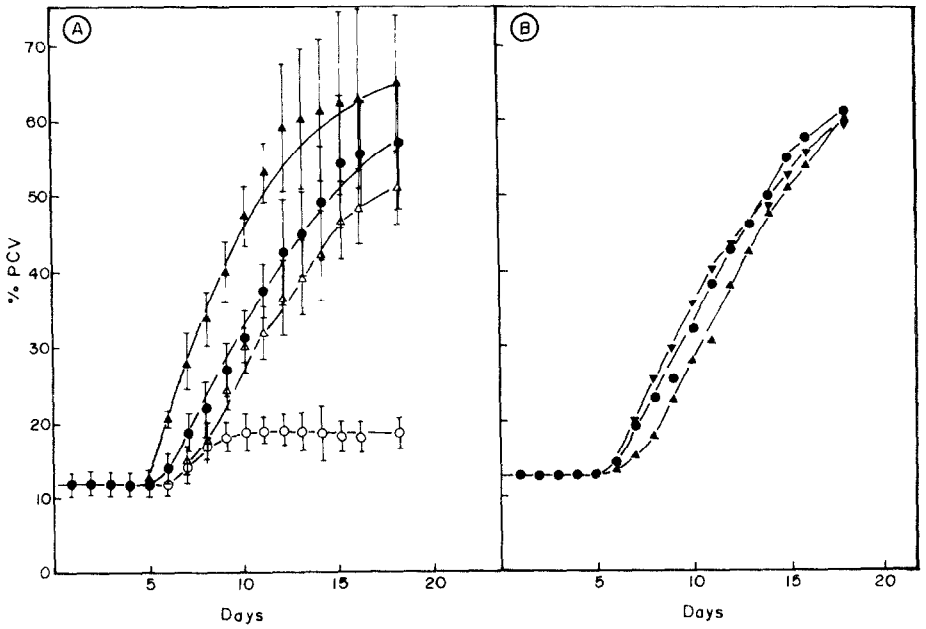

Fig. 1. (A) Growth of cell suspension cultures of *B ternifolia* in the MS medium in the absence of any growth regulators (○), or in the presence of 1 mg/ml carbenicillin (●), 1 mg/ml 2,4-D and 5 μg/l. kinetin (▲), 0 5 mg/l 2,4-D and 5μg/l kinetin (△). (B) Growth of cell suspension cultures of *B. ternifolia* in MS medium in the presence of 1 mg/ml carbenicillin (●) and carbenicillin plus 1 mg/l 2,4-D (▼) or 5 μg/1 kinetin (▲)

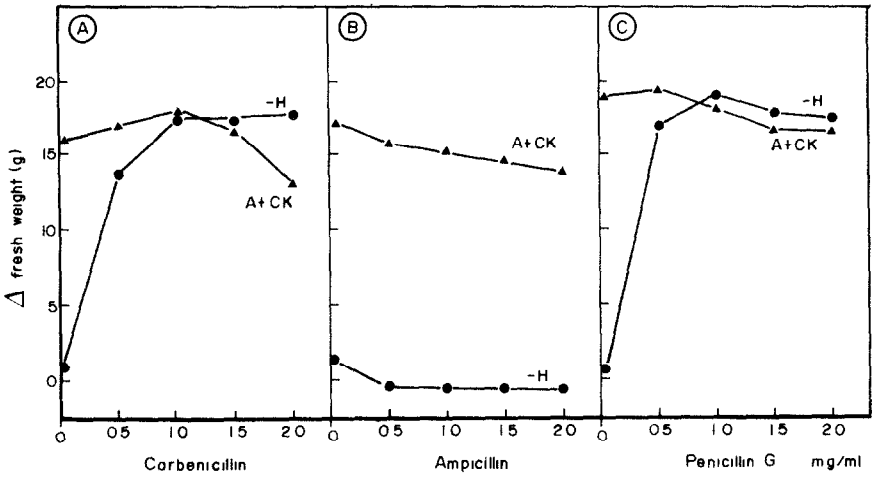

Fig 2 Growth of *B. ternifolia* calli in the presence of various concentrations of (A) carbenicillin, (B) ampicillin and (C) penicillin G with (▲) or without 1 mg/l 2,4-D and 5 μg/1 kinetin (●)

The very different response elicited by structurally similar molecules, which have similar known antimicrobial effect, indicates a different structural requirement for the two biological activities. An examination of the chemical structure of these three compounds (Fig. 3) immediately pointed to the presence of a moiety with weak auxin-like activity in penicillin G which is absent in ampicillin. The radical, bound to the APA moiety which could be released by acid hydrolysis or by the enzyme penicillin amidase, corresponds to phenylacetic acid in the case of penicillin G. while in the case of ampicillins it is an amino acid Phenylacetic acid is the only other natural auxin known, apart from IAA and its derivatives, and its effects are well documented. Amino acids do not have auxin-like activity The radical joined to APA in carbenicillin is phenylpropionic acid, but this could be decarboxylated to phenylacetic acid

We then decided to test the activity of the other penicillins illustrated in Fig 3, hypothesizing that none of them should promote growth in *B. ternifolia* cells as they did not contain an auxin-like moiety nor were able to produce an auxin upon hydrolysis The results are presented in Fig 4. We are now preparing new penicillin derivatives that contain an auxin-like moiety, e.g. it

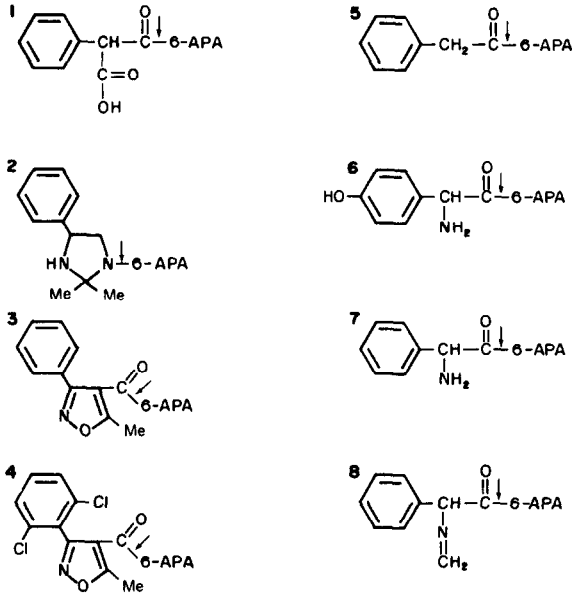

Fig. 3 Chemical structures of the penicillin derivatives used in this study Only the group attached to the beta lactam ring of 6-aminopenicillanic acid is represented

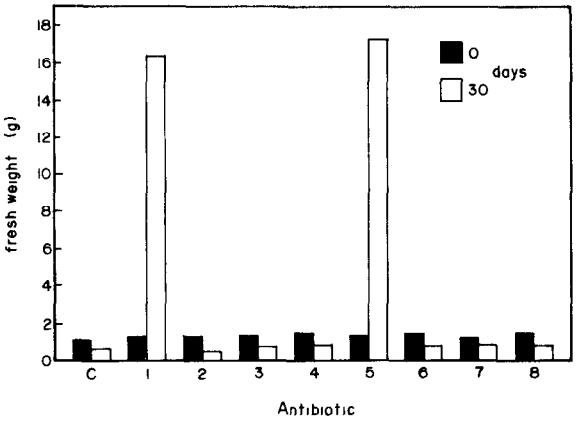

Fig 4. Growth promotion induced by several antibiotics (1 mg/ml) on callus cultures of *B ternifolia*. C=control, (1) carbenicillin, (2) hetacillin, (3) oxacillin, (4) dichloxacillin, (5) penicillin G, (6) amoxicillin, (7) ampicillin, (8) metampicillin.

would be expected that penicillin V and its chlorinated analogues would yield 2,4-D type-molecules and thus be more active in stimulating growth than penicillin G.

Nevertheless, neither APA (Fig. 5a) nor phenylacetic acid (Fig. 5b), nor tyrosine (data not shown) had any effect when applied alone or together in equimolar concentrations. The addition of kinetin (0.23 μM) did not change this situation At concentrations of 2 mg/ml, neither APA nor phenylacetic acid had any significant effect on growth promoted by 2,4-D + kinetin

DISCUSSION

Nickell [5] was the first to report the stimulation of plant growth by antibiotics in virus-induced tumour

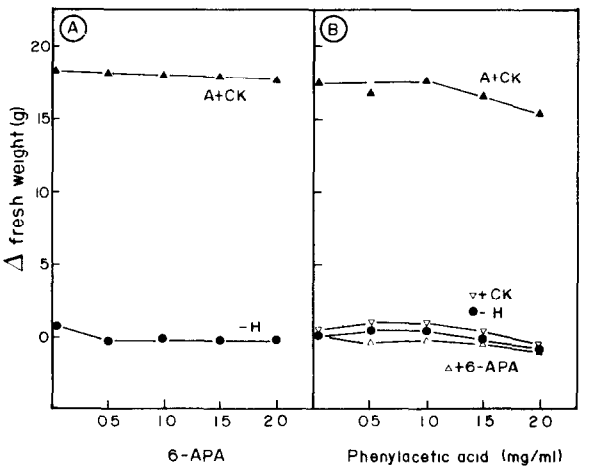

Fig 5 Growth of calli of *B ternifolia* in the presence of various concentrations of 6-aminopenicillanic acid (A) and phenylacetic acid (B) These were supplemented with 1 mg/l 2,4-D and 5 μg/l kinetin (▲); 5 μg/l kinetin alone (▽), or without any hormone (●).

tissues of *Rumex acetosa*. In this system, all antibiotics tested: penicillin, terramycin, streptomycin, bacitracin and thiolutin, stimulated growth (fresh weigh gains) but surprisingly, penicillin was the least active. Nickell and Finlay [6] further characterized the growth promoting activity of a broader range of antibiotics using the duckweed (*Lemna minor*) test and found penicillin G to be by far the most active compound producing a 530% gain in fresh weight at concentrations of 20 mg/l followed by bacitracin (270%) after eight weeks of culture. Although thiolutin produced a 600% increase in fresh weight at very low concentrations (1 mg/l) it was highly inhibitory at high concentrations.

We cannot explain this generalized effect of all kinds of antibiotics, as other antibiotics such as kanamycin and streptomycin were not active in our cultures (results not shown). On the other hand, the results we present here suggest that the promotion effect of antibiotics is not related to their antimicrobial activity and is dependent on very specific structural characteristics: penicillin G and carbenicillin are very active in promoting growth while all the others are completely inactive.

It also appears that there are very definite structural requirements for activity in the group linked to the beta lactam ring of the APA. The only difference between penicillin G (active) and ampicillin (inactive) being a hydrogen atom substituted by an amino group in the latter. Nickell and Celmer [9] analysed the activity of a wide range of synthetic penicillins and screened more than 100 APA derivatives in the duckweed test. Only seven showed some degree of growth promotion but even in the best case, the compounds only doubled the growth rate of the controls Of particular relevance to the results we report here is the fact that all seven compounds have an auxin-like moiety attached to the beta lactam ring of APA; the most active of the seven (220%) contained phenylacetic acid as the auxin-like moiety It is interesting that they also found phenylacetic acid inactive on its own; in fact, all the free acids were inactive and had a growth promoting capacity only when they were bound to the APA. However, contrary to our finding that APA on its

own has no effect on the growth of *B ternifolia* calli, Nickell and Celmer [7] found APA to be more active than all the penicillins tested with the exception of penicillin G

All these data point to the fundamentally important role that the APA molecule plays, either by helping the uptake of the auxin-like moiety into the cells prior to hydrolysis that would release a free auxin, or by potentiating the activity of the auxin moiety The latter could be achieved in one of several ways (i) by preventing its inactivation through decarboxylation or conjungation and producing a slow release of free auxin, or (ii) by increasing the affinity of the auxin for specific receptor sites Enzymatic hydrolysis is a distinct possibility since we have detected a penicillin amidase activity in the walls of *B ternifolia* cells, and hydrolysis of the antibiotics does not occur spontaneously in the culture medium

Other studies analysing the effect of various antibiotics on plant cell cultures show penicillins without or with only very mild growth promoting activity Pollock *et al.* [4] found penicillin G, carbenicillin and ampicillin without effect or mildly inhibitory on the relative plating efficiency of protoplasts of *Nicotiana plumbaginifolia* Although they mentioned that the three of them and the cephalosporines tested all potentiated the growth of the calli derived from these protoplastes Phillips *et al* [3] found that benzylpenicillin and phosphomycin at 10 μg/ml slightly increased the number of cells in cultures of *Helianthus tuberosus*, however, at higher concentrations there was no effect

Penicillins did not promote growth in cultures of *Nicotiana glutinosa* [9], *N tabacum* var xanthi, [10], and *Silene alba* [11] This could be due to the fact that the investigations had the aim of controlling microbial contamination and the cells were growing in the presence of optimal concentrations of growth regulators or that the concentrations used were simply not high enough to manifest the activity

We believe that the growth response observed in the culture of *B ternifolia* constitutes a good experimental system to elucidate the mechanism by which penicillins promote growth

EXPERIMENTAL

Culture media All culture media were prepared with Murashige and Skoog's [12] basic salts and vitamins and were supplemented with growth regulator and/or antibiotics as specified in the text Agar (1%) was added to gel the media for callus culture

Kinetin, 2 4-D and phenylacetic acid were purchased from Sigma, carbenicillin was obtained from Sanfer (carbecin) and APA and all other antibiotics were kindly provided by Laboratorios Quinona de Mexico, S A All of these were dissolved as Na salts and sterilized by Millipore filtration before they were added as concentrated aliquots to the culture media Antibiotic solns (1 ml) were added to the surface of the solid media at least 24 hr before the initiation of the experiments to allow for diffusion

Cell culture Cell cultures of *Bouvardia ternifolia* were induced and maintained as described in ref [13] by germinating the previously sterilized seeds on MS supplemented with 1 mg/l 2,4-D and 5 μg/l kinetin The white and friable calli formed from the radicular meristems were transferred to fresh MS medium with increased kinetin (50 μg/l) for rapid growth

To eliminate any residual effect of auxins or cytokinins, the calli were kept on MS medium without growth regulators for 48 hr before testing the growth promoting activity of the antibiotics Samples of *ca* 1 g of callus tissue were placed in baby food jars (7 cm high × 6 cm diam) containing 30 ml of MS medium solidified with 1% agar and supplemented with 2,4-D, kinetin and/or antibiotics as specified

Suspension cultures were initiated by shaking white friable calli in liquid MS with 1 mg l 2,4-D and 50 μg kinetin Aliquots (5 ml) from cultures in stationary phases containing *ca* 2×10^5 cells/ml were transferred to 250 ml conical flasks containing 50 ml of fresh medium supplemented with growth regulators and/or antibiotics as specified and incubated with shaking at 25 rpm

All cultures were incubated in a growth room under continuous light (8 W/m^2) at 27 $\pm 1^\circ$ The growth of calli was measured by determining the gain in fresh wt after 20 days The growth curves in liquid media were determinated by taking 10 ml aliquots every 2 days and measuring the cell vol of the pellet

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