AN ABSTRACT OF THE THESIS OF

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Title: Cytokinin Antagonists and Antibiotics Related to Nalidixic Acid: Effects in Phaseolus and Nicotiana Callus Tissue

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The effects of five cytokinin antagonists on the growth of cytokinin-autonomous lines of *Phaseolus lunatus* cv. Kingston, *Phaseolus vulgaris* cv. Great Northern, and *Nicotiana tabacum* cv. Wisconsin 38 callus tissues have been compared. The antagonists tested included four N^4-substituted 4-amino-2-methylthiopyrrolo[2,3-d]pyrimidines bearing the following N^4 side chains, 4-n-pentyl- (ms^2PnN^4Prp), 4-n-hexyl- (ms^2HxN^4Prp), 4-cyclopentyl- (ms^2cPnN^4Prp), and 4-cyclohexyl- (ms^2cHxN^4Prp) and one 7-amino-3-methylpyrazolo[4,3-d]pyrimidine with the N^7 side chain, 7-n-pentyl- (m^3PnN^7Pzp).

All antagonists tested inhibited *Nicotiana* and *Phaseolus* callus tissues growth. Inhibition of the *Phaseolus* callus tissues required higher antagonist

concentrations than required to inhibit *Nicotiana* callus tissue. With all three tissues, the most potent of the antagonists was m^3PnN^7Pzp. However, the antagonist structure/activity relationships observed in tests with the pyrrolopyrimidine derivatives were not identical for all three tissues. The inhibitory effects of ms^2PnN^4Prp and m^3PnN^7Pzp on the growth of cytokinin-autonomous *N. tabacum* and *P. lunatus* callus tissues were partially reversed by the addition of exogenous N^6-(Δ^2-isopentenyl)adenine (i^6Ade), as evidenced by the effect of i^6Ade in increasing the concentrations of antagonists required to produce 50% inhibition of callus growth (I_{50} values). The interactions of cytokinin antagonists and exogenous cytokinins in the cytokinin-autonomous *Nicotiana* callus tissue appear to be more complex than in the corresponding cytokinin-dependent *Nicotiana* callus tissue. The exogenously supplied cytokinins are themselves inhibitory to the growth of the cytokinin-autonomous line of *Nicotiana* callus tissue.

Nalidixic acid, a compound reported to inhibit mammalian cell cultures in a manner reversible by cytokinin treatment (Quesney-Huneeus *et al.*, (1980), Proc. Natl. Acad. Sci. USA., 77 5842.), was tested along with the related antibiotics, oxolinic acid and novobiocin, for effects on the growth of cytokinin-autonomous and cytokinin-dependent *P. lunatus* cv. Kingston and *N. tabacum* cv. Wisconsin 38 callus tissues. This investigation was undertaken to determine whether interactions of cytokinins and nalidixic

acid similar to those observed in mammalian cell cultures could be detected in plant cell cultures. All three antibiotics inhibited growth of the callus tissues. The *Nicotiana* callus tissues were more sensitive than the *Phaseolus* tissues. In all tissues, oxolinic acid was the most effective of the antibiotics in inhibiting callus growth, and novobiocin was the least effective of the three antibiotics.

The inhibitory effects of nalidixic acid and oxolinic acid on cytokinin-dependent *Phaseolus* callus tissue appeared to be partially alleviated by exogenous i^6Ade treatment as evidenced by the effect of i^6Ade in increasing the I_{50} values for nalidixic acid and oxolinic acid. By this criterion, the inhibitory effects of novobiocin on callus growth did not appear to be reversed by treatment with exogenous i^6Ade. Interestingly, in tests with the cytokinin-autonomous *Nicotiana* callus tissue, a low concentration (0.01 μM) of nalidixic acid appeared to reverse the inhibitory effects of high i^6Ade concentrations. A similar effect with this tissue was observed in tests of the antagonist m^3PnN^7Pzp. The results of this study raise the possibility that some part of the effects of nalidixic acid and oxolinic acid on plant tissues may be due to effects on processes specifically involving cytokinin metabolism or function. However, in no case was i^6Ade able to completely reverse the effects of antibiotic treatment.

Cytokinin Antagonists and Antibiotics Related to Nalidixic Acid: Effects on *Phaseolus* and *Nicotiana* Callus Tissues.

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CYTOKININ ANTAGONISTS AND ANTIBIOTICS RELATED TO NALIDIXIC ACID: EFFECTS ON *PHASEOLUS* AND *NICOTIANA* CALLUS TISSUES.

I. INTRODUCTION

Cytokinin antagonists are compounds that competitively inhibit the biological effects of cytokinins (Hecht *et al.*, 1975). Cytokinin antagonists were developed by Hecht and associates in collaboration with Skoog and co-workers (Hecht *et al.*, 1971, Skoog *et al.*, 1973 and Skoog *et al.*, 1975). Two major classes of antagonists have been synthesized: 4-substituted-pyrrolo[2,3-d]pyrimidines and 7-substituted-pyrazolo[4,3-d]pyrimidines. These antagonists are structural analogs of cytokinin-active adenine derivatives. Cytokinin antagonists have been studied extensively in the tobacco callus bioassay system (Hecht *et al.*, 1971; Hecht *et al.*,1975; Iwamura *et al.*, 1975; Iwamura *et al.*, 1979; Skoog *et al.*, 1975). The effects of these compounds on tissue culture systems other than those derived from *Nicotiana* have yet to be reported, and their effects in other types of plant systems are unpredictable (Iwamura *et al.*, 1976b; Iwamura *et al.*, 1979; Skoog and Ghani, 1981; Tanimoto and Harada, 1982).

Inhibitory effects of compounds which are structurally unrelated to cytokinins have been reported to be overcome by cytokinin treatment in animal cell cultures (Quesney-Huneeus, *et al.*, 1980). The effects of nalidixic acid, an

antibiotic inhibitor of DNA synthesis, were reversed in cultured mammalian cells by the addition of exogenous N^6-($\triangle^2$-isopentenyl)adenine (Quesney-Huneeus, et al., 1980). Nalidixic acid and related antibiotics have been extensively investigated in prokaryotic systems where they appear to function by inhibiting type II topoismerases (Gellert, 1981; Wang, 1985). The mechanism of action of these compounds in eukaryotic systems is still uncertain, and their effects on plant systems have received attention only recently (Heinhorst et al., 1985; Ciarrocchi et al., 1985).

The effects of cytokinin antagonists on Phaseolus and Nicotiana callus cultures were compared in the present study. In addition, the effects of nalidixic acid and related antibiotics on Phaseolus and Nicotiana callus tissues were examined in an attempt to determine whether interactions of cytokinins and nalidixic acid similar to those reported in animal cell cultures could be observed in plant tissue culture systems. Phaseolus callus tissues were selected for the studies reported here because these tissues have properties significantly different from those of Nicotiana callus cultures. Cytokinin-dependent Phaseolus callus cultures typically require higher concentrations of cytokinins for optimal growth than do Nicotiana callus tissues, and callus tissues derived from particular Phaseolus genotypes exhibit differences in cytokinin structure-activity relationships (Mok et al., 1978).

II. Literature Review

A. Structure of Cytokinins.

Cytokinin research began with the discovery and isolation of kinetin, 6-furfurylaminopurine, by Skoog and Miller in 1955 (Miller et al., 1955). They demonstrated that, in the presence of an exogenous auxin source, kinetin induced cell division and callus growth in tobacco pith cultures. Efforts were then made to synthesize compounds with kinetin-like biological activity and to isolate kinetin-like substances from plants. In 1963, Letham and co-workers (Letham et al., 1963) isolated the naturally occurring cytokinin, zeatin, from Zea mays. The name cytokinin was proposed for all compounds which promoted cell division and exhibited other growth regulatory functions similar to kinetin (Skoog et al., 1965). In subsequent years, more than twenty naturally occurring cytokinins were isolated from various sources and over a hundred cytokinins were synthesized (Skoog and Armstrong, 1970; Kende, 1971; Matsubara, 1980).

The structural requirements for cytokinin activity have been extensively investigated in the tobacco callus bioassay system (Skoog and Leonard, 1968; Skoog and Armstrong, 1970; Skoog, 1971). Two major classes of cytokinins are known: N^6-substituted adenine derivatives and phenylurea derivatives. Highly active cytokinins may be obtained with

either type of structure, but all naturally occuring cytokinins identified to date have been adenine derivatives.

Cytokinin activity of N^6-substituted adenine derivatives is strongly influenced by modifications of the purine ring and by the length, substitution and degree of saturation of the N^6-side chain (Skoog *et al.*, 1967; Skoog and Armstrong, 1970).

The urea and thiourea derivatives represent the other major class of cytokinins. Although Shantz and Steward reported the isolation of the weakly active cytokinin N,N'-diphenylurea from coconut milk in 1955 (Shantz and Steward, 1955), other investigators have been unsuccessful in attempts to repeat this observation (Letham, 1974; Van Staden and Drewes, 1974). However, over 500 synthetic urea derivatives have been tested for their cytokinin activity in a variety of cytokinin bioassay systems (Shantz and Steward, 1955; Bruce, *et al.*, 1965; Bruce and Zwar, 1966). The minimum structural requirements for cytokinin activity of urea derivatives appear to be an intact -NH-CO-NH- bridge and a phenyl ring attached to one side of the bridge. (Matsubara, 1980). In recent years, urea derivatives have been synthesized with cytokinin activity equivalent to or higher than that of the most active adenine derivative (Takahash, *et al.*, 1978; Matsubara, 1980)

The ability of both adenine and urea derivatives to exhibit cytokinin activity is puzzling in view of their very different chemical structures. It is still not certain

whether the two types of compound have similar or different modes of action. One hypothesis proposes that cytokinin-active urea derivatives act indirectly by promoting the synthesis of cytokinin-active adenine derivatives (Miller, 1961). However, it has also been argued that both classes of cytokinins must have a common action site (Kurosaki et al., 1981).

B. Cytokinin Antagonists

Cytokinin antagonists have been the subject of several reviews (Skoog, 1973; Iwamura, 1978; Hecht, 1979; Hecht, 1980). The development of cytokinin antagonists was based on the assumptions that 1) cytokinins bind to specific cellular receptor site(s) and 2) compounds lacking cytokinin activity but with structures sufficiently similar to cytokinins to be able to compete and bind to these receptor sites, could be synthesized.

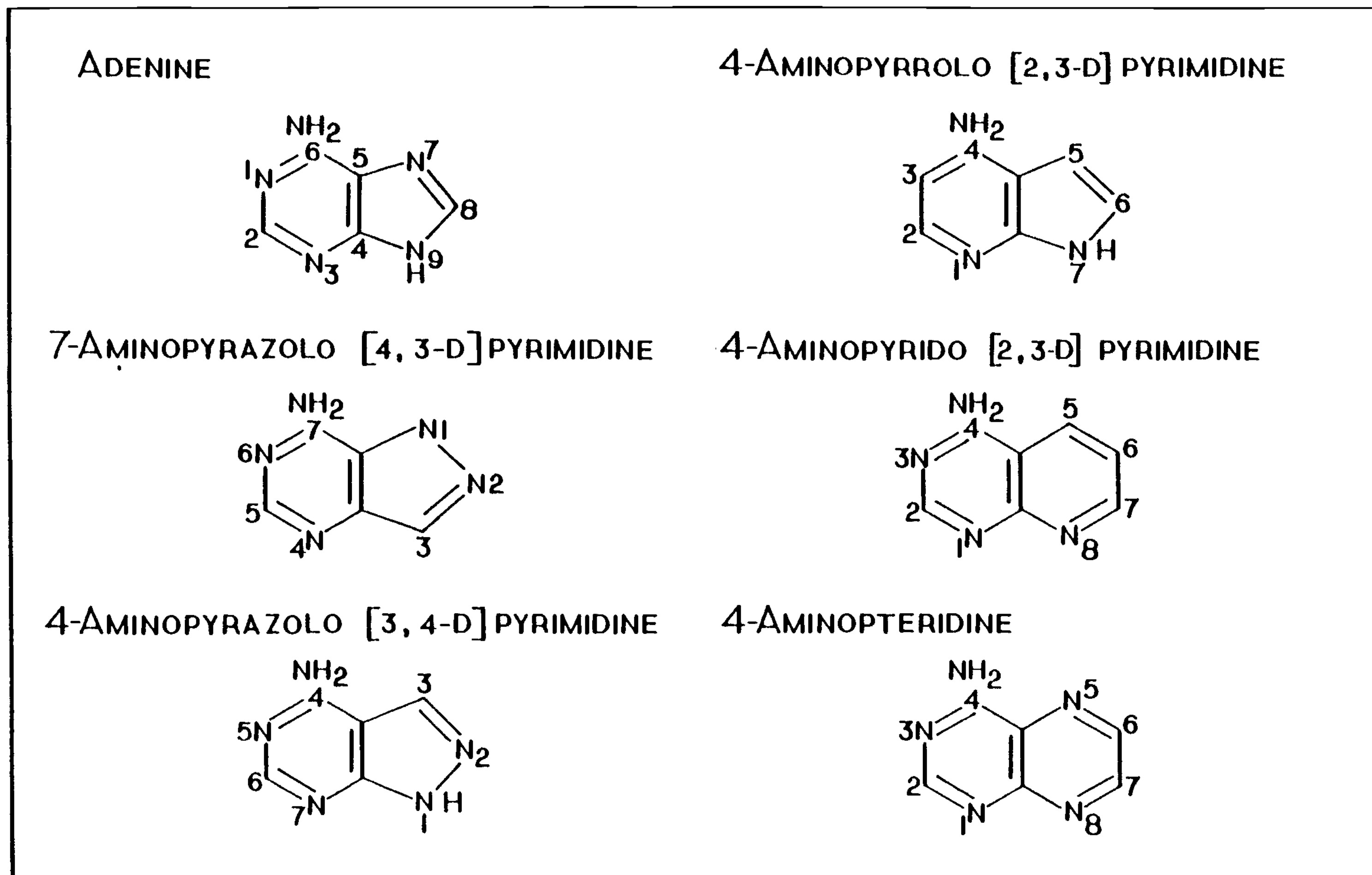
The first compounds tested for antagonist activity were the simple purine analogs, 8-azaadenine, 2,6-diaminopurine, and 8-azaguanine (Blaydes, 1966). These compounds exhibited weak inhibitory activity in the soybean callus bioassay, but no evidence of specific cytokinin antagonist activity was obtained.

The first synthesis of compounds exhibiting the behavior expected of specific cytokinin antagonists was achieved by Hecht and co-workers in 1971 (Hecht et al., 1971). The compounds synthesized by Hecht and co-workers

were a series of 7-substituted-3-methylpyrazolo[4,3-d]pyrimidine derivatives (Figure 1). These compounds were analogs of N^6-(Δ^2-isopentenyl)adenine (i^6Ade) in which the purine ring structure had been altered, the N6 side chain had been modified, and a methyl group had been added at the position equivalent to the 9-position of the purine ring. The introduction of a single structural modification or a combination of two modifications decreased cytokinin activity in the tobacco bioassay, but only compounds which contained all three structural alterations totally lacked cytokinin activity and were found to be effective as cytokinin antagonists.

The tobacco callus bioassay system was used in the tests that led to the development of cytokinin antagonists (Hecht et al., 1971). One of the more active cytokinin antagonists, 3-methyl-7-(3-methylbutylamino)pyrazolo[4,3-d]pyrimidine, was tested in several other biological systems (Skoog et al.,1973). The cytokinin antagonist showed little effect on the senescence of sweet corn and various dicotyedonous plants or on the germination and growth of wheat and radish seedlings. However, the growth of cytokinin-autonomous tobacco callus tissue was inhibited by the antagonist in the same manner as that of cytokinin-dependent tobacco callus tissue. The antagonist also inhibited bud formation in cytokinin-dependent tobacco tissue cultures.

Figure 1. Ring structures of adenine and cytokinin antagonists.



Questions concerning the specificity of the inhibitory effects of the cytokinin antagonists were raised by Helgeson and co-workers (Helgeson *et al.*, 1973). They examined the inhibitory effects of 3-methyl-7-(n-pentylamino)pyrazolo[4,3-d]pyrimidine on the growth rates of cytokinin-dependent tobacco callus cultures and tobacco cell suspension cultures. Although the inhibitory effects of the antagonist on tobacco callus cultures were reversed by cytokinins, the growth kinetics were not consistent with a simple competitive interaction affecting log phase growth rates. The effect of the antagonist appeared to be to limit the duration of log phase growth rather than to alter the growth rate. The effects of the antagonist on growth kinetics of tobacco cell suspension cultures were complex, but they appeared to include an increase in the duration of the lag phase. Although these results did not preclude the possibility that the pyrazolopyrimidine derivative was acting as a cytokinin antagonist, they did suggest that the interaction of cytokinin and antagonist was more complex than originally believed.

The effects of two pyrazolo[4,3-d]pyrimidine antagonists on cytokinin-dependent and cytokinin-autonomous suspension cultures of *Nicotiana tabacum* cells were studied by Gregorini and Laloue (1980). The two antagonists used were 7-(pentylamino)- and 7-(benzylamino)-3-methylpyrazolo[4,3-d]pyrimidine. Both compounds inhibited growth of cytokinin-autonomous and cytokinin-dependent

Nicotiana cell cultures. This growth inhibition was reversed by benzyladenine. The inhibitory effects of the antagonists on growth appeared to be related to their cytotoxicity. Inhibition of the growth of the tobacco cell suspension cultures was accompanied by cell death, which occurred earlier or later depending on inhibitor concentration and was antagonized by benzyladenine. Interestingly, nondividing cells (cells in the stationary phase) were insensitive to the antagonists. If stationary phase cells were induced to divide (by the addition of sucrose) while in the presence of the antagonist, then cell death occurred. This result was interpreted as indicating a possible effect of the antagonists on a specific cytokinin-requiring event(s) in the cell cycle.

The apparent activity of pyrazolo[4,3-d]pyrimidine derivatives as cytokinin antagonists led to attempts to synthesize other chemical classes of cytokinin antagonists. A series of pyrazolo[3,4-d]pyrimidine derivatives (Figure 1), which contain a carbon atom in the 3-position (equivalent to the 7-position in a purine ring) were synthesized by Hecht and co-workers (Hecht et al., 1975). These compounds inhibited tobacco callus growth. However, unlike the pyrazolo[4,3-d]pyrimidines, the pyrazolo[3,4-d]pyrimidines did not appear to be specific cytokinin antagonists since their inhibitory effects was not reversible by cytokinin treatment.

The 7-deaza analog of kinetin riboside, 4-furfurylamino-7-(B-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidine, was synthesized and tested for cytokinin antagonist activity by Iwamura and co-workers in 1974 (Iwamura et al., 1974). In this compound, a carbon atom is substituted for the nitrogen atom at the position corresponding to the 7-position of an adenine ring. This modification was designed to prevent glucosylation at the 7-position of the purine ring. Previous work by Letham and co-workers (Deleuze et al., 1972; Parker et al., 1972) and Fox and associates (Fox et al., 1973) had led these investigators to suggest that exogenously supplied cytokinins may be activated by conversion to 7-glucosyl derivatives (Deleuze et al., 1972; Parker et al., 1972; Fox et al., 1973). Although this interpretation does not appear to be correct (Laloue, 1977), modification of the 7-position of the purine ring of cytokinin ribonucleosides (to produce 4-substituted-7-(B-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidines) does produce compounds with cytokinin antagonist activity. In the following year, Iwamura and co-workers synthesized and tested a series of 4-substituted-7-(B-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidines on tobacco callus bioassay (Iwamura et al., 1975). In this series of compounds, high antagonist activity was found to be associated with side chain structures that conferred high cytokinin activity in N^6-substituted adenine derivatives.

The structural requirements for the antagonist activity of 4-substituted-pyrrolo[2,3-d]pyrimidines (Figure 1) were investigated by Skoog and his associates (Skoog *et al.*, 1975). They found that the free base forms of 4-substituted pyrrolo[2,3-d]pyrimidines were active as cytokinin. Addition of a methylthio group at the 2-position of the pyrrolo[2,3-d]pyrimidine ring or a ribosyl group at the 7-postion (equivalent to the 4 and 9 postions respectively of a purine ring) resulted in conversion of the 4-substituted pyrrolo[2,3-d]pyrimidines to antagonists. All N^4-substituted-2-methylthiopyrrolo[2,3-d]pyrimidines tested inhibited cytokinin-dependent tobacco callus growth, but the most active antagonists were the N^4-cyclopentyl and N^4-cyclohexyl derivatives. The inhibitory effects of these compounds were reversed by increasing concentrations of the cytokinin, N^6-(Δ^2-isopentenyl)adenine. Neither indole-3-acetic acid nor gibberellic acid was effective in overcoming the inhibition. Surprisingly, in the presence of high cytokinin concentrations, the 4-substituted-2-methylthiopyrrolo[2,3-d]pyrimidines enhanced (rather than inhibited) bud formation in tobacco callus cultures. This result suggested that the site involved in induction of budding might be different than that involved in cytokinin promotion of callus growth. On the other hand, Tanimoto and Harada (1982) have reported that 4-substituted-2-methylpyrrolo[2,3-d]pyrimidines inhibited cytokinin-induced

meristematic activity and adventitious bud formation in Torenia stem segments.

Iwamura and associates confirmed that the free base form of 4-substituted pyrrolo[2,3-d]pyrimidines were cytokinin active (Iwamura et al., 1976a) and investigated the possible use of 4-substituted-7-(B-D-ribofuranosyl)pyrrolo[2,3-d]pyrimidines as herbicides. (Iwamura et al., 1976b). The antagonists inhibited germination of rice, cucumber, and millet when applied prior to or immediately after radical emergence. The compounds were also inhibitory to seedling growth if applied as a soil drench. However, they were ineffective in inhibiting growth when applied as a foliar spray to rice, millet, cucumber and Brassica rapa seedlings.

The observation that under certain conditions cytokinin antagonists stimulated rather than inhibited cytokinin-induced bud formation in tobacco tissue cultures (Skoog et al.,1975) led Skoog and Ghani (1981) to examine the effects of a number of pyrrolo[2,3-d]pyrimidine, pyrazolo[3,4-d]pyrimidine and pyrazolo[4,3-d]pyrimidine derivatives on the release of lateral buds of Pisum sativum from apical dominance. The outgrowth of lateral buds is promoted by cytokinin-active compounds (Sachs and Thimann, 1964; Sachs and Thimann, 1966). Skoog and Ghani found that compounds which exhibited cytokinin antagonist activity in the tobacco callus bioassay also promoted (rather than inhibited) lateral bud outgrowth. The relative activity of these

compounds appeared to parallel their activity as antagonists in the tobacco callus bioassay. In particular, a substituent in the 2-position of the pyrrolo[2,3-d]pyrimidines (either a methyl group or a methylthio group) was essential for activity in releasing lateral buds from apical dominance.

Biological differences in response to cytokinin antagonists were investigated by Iwamura and co-workers (Iwamura et al., 1979). They compared the cytokinin antagonist activity of sixteen 4-substituted-2-methylpyrrolo[2,3-d]pyrimidines in three cytokinin bioassay systems: the tobacco callus bioassay, the lettuce seed germination test and the Amaranthus betacyanin synthesis assay (Iwamura et al., 1979). The effectiveness of particular compounds varied depending on the bioassay system. For example, the 4-phenyl derivative showed little activity in the betacyanin synthesis test, was active as an antagonist in the tobacco callus bioassay, and exhibited cytokinin activity in the lettuce seed germination test. In a recent study, Bianco-Colomas (1984) observed that the antagonist 3-methyl-7-n-pentylaminopyrazolo[4,3-d]pyrimidine inhibited betacyanin synthesis in the Amaranthus bioassay system, and this inhibition was reversed by cytokinin treatment or by light treatment. In general, the results obtained in tests of antagonists using various bioassay systems suggest that it may be possible to synthesize

antagonists having high selectivity for particular biological systems.

The variations in response to cytokinin antagonists observed in comparison of cytokinin bioassay systems have been interpreted as providing evidence for multiple sites of cytokinin action (Hecht, 1980). Interactions of antagonists with the active sites of enzymes involved in cytokinin metabolism might also be expected to occur in plant systems, and some of the differences in responses of plant tissues to antagonists may be due to differences in cytokinin metabolism. Letham and associates (Entsch _et al_., 1980; Parker _et al_., 1986) have reported that the antagonist 3-methyl-7-n-pentylaminopyrazolo[4,3-d]pyrimidine competitively inhibits the enzyme cytokinin 7-glucosyltransferase, and interactions of antagonists with other enzymes involved in cytokinin metabolism appear likely.

Iwamura and associates did a detailed analysis of the effects of structural modification on the biological activity of 4-substituted-2-methylpyrrolo[2,3-d]pyrimidines (Iwamura _et al_.,1979; Iwamura _et al_., 1983). They derived an equation in which binding to the cytokinin receptor site could be predicted from the steric properties, hydrophobicity, and electon withdrawing properties of the N^4-substituent (Iwamura _et al_., 1983). Electron withdrawing properties and hydrophobicity were side chain characteristics that increased binding. Activity was

influenced by all three properties, but steric dimensions determined the type of activity (cytokinin activity or antagonist activity). The critical steric parameter appeared to be the maximum width of the N^4-substituent. Iwamura defines Wmax as "the maximum width of substituents from the bond axis between the exocyclic nitrogen atom and its α-carbon atom" (Iwamura _et al_., 1979; Iwamura _et al_., 1983). The Wmax values for cytokinin-active compounds in this series fell between 4.7 and 6 Å, while values greater or smaller were associated with cytokinin antagonists.

Wilcox and Wain (1982) found that the cytokinin antagonist activity of 4-substituted-2-methylthiopyrrolo[2,3-d]pyrimidines was little affected by a change of the side chain bridging group from an -NH- linkage to an -O-linkage. They synthesized a series of 4-alkoxy-2-methylthiopyrrolo[2,3-d]pyrimidines and tested the activity of these compounds in the tobacco callus bioassay system. None of the compounds tested showed cytokinin activity. However, three compounds showed strong cytokinin antagonist activity, two compounds were weak cytokinin antagonists, and two were inactive.

Cytokinin antagonists belonging to the pyrrolopyrimidine or the pyrazolopyrimidine series contain a fused 6-5 member ring. In 1979, Iwamura and associates synthesized a series of cytokinin analogs having fused 6-6 membered ring structures (Iwamura _et al_., 1979). These compounds were 4-substituted-2-methylthiopyrido[2,3-

d]pyrimidines (Figure 1). Of the ten compounds tested in the tobacco bioassay, only one had cytokinin activity. The other compounds exhibited antagonist activity, although weaker than that of the corresponding pyrrolo[2,3-d]pyrimidine analogs. As in the case of the pyrrolo[2,3-d]pyrimidines, antagonist activity was strongly influnced by the Wmax value and by the hydrophobicity of the cytokinin side chain. As tested in the tobacco bioassay system, the most active antagonist of this series was n-4-butyl-2-methylthiopyrido{2,3-d}pyrimidine. The compounds with the least antagonist activity were the benzyl- and phenyl-derivatives.

The results from the pyrido[2,3-d]pyrimidine study inspired Iwamura and co-workers (Iwamura et al., 1980) to synthesize a class of azanaphthalene analogs, the 4-alkylaminopteridines (Figure 1). These compounds were tested in the tobacco callus bioassay. One compound (4-cyclohexylaminopteridine) appeared biologically inactive; one compound (4-benzyl-aminopteridine) exhibited weak cytokinin activity; and the third compound (4-cyclopentylaminopteridine) was a weak cytokinin antagonist.

The development of cytokinin antagonists from urea derivatives was first reported by Kefford and co-workers in 1968 (Kefford et al., 1968). In tests using detached radish leaves, the substitution of a benzyl group for a phenyl group in certain N,N′-diphenylurea derivatives was reported to yield compounds that antagonized the effects of both

cytokinin-active phenylurea derivatives and cytokinin-active adenine derivatives in retarding senescence. Although only those compounds that contained both a benzyl group and a phenyl group were effective as antagonists, not all such N-benzyl-N'-phenylurea derivatives exhibited antagonist activity. N-benzyl-N'(4-bromophenyl)urea, one of the compounds exhibiting antagonist activity in the radish leaf senscence bioassay, was tested in the tobacco callus bioassay by Kuriosak and co-workers (Kuriosak _et_ _al_., 1981). This compound inhibited the cell division promoting activities of N^6-benzyladenine and of the cytokinin-active urea derivative, N-(4-pyridyl)-N'-phenylurea, in a fully reversible manner. Similarly, the cytokinin antagonist 4-cyclobutylamino-2-methylpyrrolo[2,3-d]pyrimidine (a purine analog) exhibited competitive interaction with the cytokinin-active urea derivative as well as with N^6-benzyladenine. These results suggest that N^6-substituted adenine derivatives and phenylurea derivatives may have a common receptor site.

The effects of cytokinin antagonists on mammalian cells have been investigated by Hecht and associates (Hecht _et_ _al_., 1974; Hecht, 1979). The systems studied included mouse fibroblast cultures (Hecht, 1979) and human lymphocytes transformed by phytohemagglutinin (PHA) treatment (Gallo _et_ _al_, 1972). In PHA-treated human lymphocytes, the cytokinin-active ribonucleoside, N^6-(Δ^2-isopentenyl)adenosine either stimulated or inhibited cell multiplication depending on the

concentration applied and time of treatment (Gallo *et al.*, 1969). In mouse fibroblast cultures, adenosine and cytokinin-active adenosine derivatives inhibited cell multiplication (Hecht *et al.*, 1976). Hecht and co-workers (Hecht *et al.*, 1974) found that the biological response elicated by treating these systems with cytokinin antagonists were similar to those observed following treatment with N^6-(Δ^2-isopentenyl)adenosine. It has been suggested (Hecht, 1979) that cytokinin ribonucleosides may affect the regulation of cyclic AMP metabolism in animals cells, and both N^6-(Δ^2-isopentenyl)adenosine and a number of cytokinin antagonists were found to inhibit phosphodiesterase activity (Gallo *et al.*, 1972). However, it is still unclear whether the biological effects of these compounds in animal systems may be attributed to their effects on cyclic AMP metabolism.

In summary, structure-activity studies of cytokinin antagonists indicate that specific cytokinin antagonists may be derived from several types of chemical structures. Although, cytokinin antagonists are potentially useful tools in investigating cytokinin metabolism and function in plant systems, much work remains to be done to determine their specificity and effectiveness in different plant tissues. The abilities of particular cytokinin analogs to function as antagonists appear to vary in different test systems. Whether this is due to differences in cytokinin metabolism

or to differences in the specificity of cytokinin receptor sites is still unclear.

C. Compactin and Nalidixic Acid: Reversal of Inhibitory Effects by Mevalonate and Cytokinin Treatment.

Exogenously supplied cytokinins have been reported to exert regulatory effects on some mammalian cell systems including cultured fibroblasts (Faust et al., 1980; Ishii and Green, 1973)) and phytohemagglutenin stimulated lymphocytes (Gallo et al., 1972; Perkins et al., 1982). The significance and biochemical basis of these effects are not certain. However, some evidence suggests that cytokinins may be capable of regulating events linking mevalonate metabolism and DNA synthesis in mammalian cells (Quesney-Huneeus et al., 1980).

Mevalonate (MVA) is a key intermediate in sterol biosynthesis in all living cells. It is also the precursor of a variety of other naturally occurring isoprenoid compounds. In mammalian cell cultures, the known nonsterol products of mevalonate metabolism include 1) the long-chain polyisoprenyl alcohol, dolichol, 2) the polyisoprene side chain of ubiquinone, 3) and the N^6 side chain of N^6-(2-isopentenyl)adenosine residues in transfer RNA (Brown and Goldstein, 1980).

Mevalonate is synthesized by the enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase). The fungal metabolite compactin structurally resembles the

HMG substrate of the enzyme and competitively inhibits HMG CoA reductase (Endo, 1981). Because of this competetive inhibition, compactin has been used as a tool to study the regulatory properties of HMG CoA reductase and the MVA pathway in cultured mammalian cells (Brown and Goldstein, 1980).

An apparent relationship between HMG CoA reductase activity and DNA synthesis in cultured Chinese hamster cells was observed by Quesney-Huneeus, Wiley and Siperstein (1979). When compactin was added to cultured hamster cells, DNA synthesis was inhibited. DNA synthesis could be restored by the addition of MVA to the cultures but not by the addition of cholesterol, indicating that MVA plays a role in DNA synthesis that is independent of its role in cholesterol synthesis. In tests of various isoprenoid compounds, the cytokinin N^6-(Δ^2-isopentenyl)adenine (i^6Ade) was found to be 100 to 200 times more effective than MVA in overcoming compactin inhibition in cultured mammalian cells (Quesney-Huneeus _et al_., 1980; Quesney-Huneeus _et al_., 1983). When cells blocked by compactin in the early G1 phase were given cholesterol followed by either MVA or i^6Ade during the late G1 phase, the cells proceeded into the S phase (Quesney-Huneeus _et al_., 1983). If the order of additions were reversed, DNA synthesis was not restored. It appears from these studies that MVA has two roles in the cell cycle. First of all, MVA is a key intermediate in the synthesis of cholesterol which is necessary during early to

mid-G1 phase of the cell cycle if cells are to enter the growth phase. Secondly, MVA or a MVA derivative is required during the late G1 to S phase if these cells are to initiate DNA replication.

The extent to which these results may be extrapolated to other animal systems is not certain. Perkins and associates (Perkins _et_ _al_., 1982) examined the relation between the isoprenoid pathway and induction of DNA synthesis in cultured lymphocytes. DNA synthesis induced by concanavalin A treatment of mouse spleen lymphocytes was found to be inhibited by compactin. The inhibition was reversible with the addition of MVA but not by the addition of either exogenous cholestrol or i^6Ade. However, it should be noted that only the nucleoside forms of cytokinins appear to be effective in either promoting or inhibiting lymphocyte activation (Gallo _et_ _al_., 1969). Therefore, it may not be surprising that the free base i^6Ade was ineffective in this case.

The ability of i^6Ade to reverse the inhibitory effects of compactin on DNA synthesis led Quesney-Huneeus and co-workers to examine the interaction of cytokinins with other inhibitors of DNA synthesis (Quesney-Huneeus _et_ _al_., 1980). The inhibition of DNA synthesis in cultured mammalian cells resulting from treatment with the antibiotic nalidixic acid was also observed to be reversed by MVA and by i^6Ade. In contrast to compactin, nalidixic acid is presumed to function by inhibiting DNA topoisomerase II. These results

raise the possiblilty that nalidixic acid and i^6Ade may act upon a common or similar site involved in DNA replication.

The regulatory properties of HMG CoA reductase have only recently been investigated in plants (Ceccarelli and Lorenzi, 1984; Ito et al, 1979; Suzuki and Uritani, 1977; Wong et al, 1982) and there has been only one study of the effects of compactin on plant cells. Ceccarelli and Lorenzi (1984) have examined the effect of compactin on the growth of Helianthus tuberosus tissue cultures. They reported that compactin inhibits the growth of Helianthus tuberosus cultures at a concentration of 2.5×10^{-5} M. Addition of 2mM MVA to the compactin treated cultures reversed compactin inhibition, indicating that compactin was inhibiting MVA synthesis. Addition of farnesol or squalene (sterol intermediates and products of MVA metabolism) only partially reversed the compactin inhibition. However, when low concentration of MVA (0.2 mM) was added in addition to farnesol or squalene, total reversal was achieved. This suggests that compactin prevents the synthesis of MVA and MVA derived compounds which is consistent with the evidence obtained from mammalian studies (Brown and Goldstein, 1980) and the known roles of the mevalonate pathway in plants. However, various nonsterol MVA derived compounds, such as ABA, GA_3, dolichol monophosphate, and Co Q along with squalene or farnesol did not replace the requirement for a small quantity of MVA. Furthermore, when a variety of cytokinins were added along with squalene or farnesol to

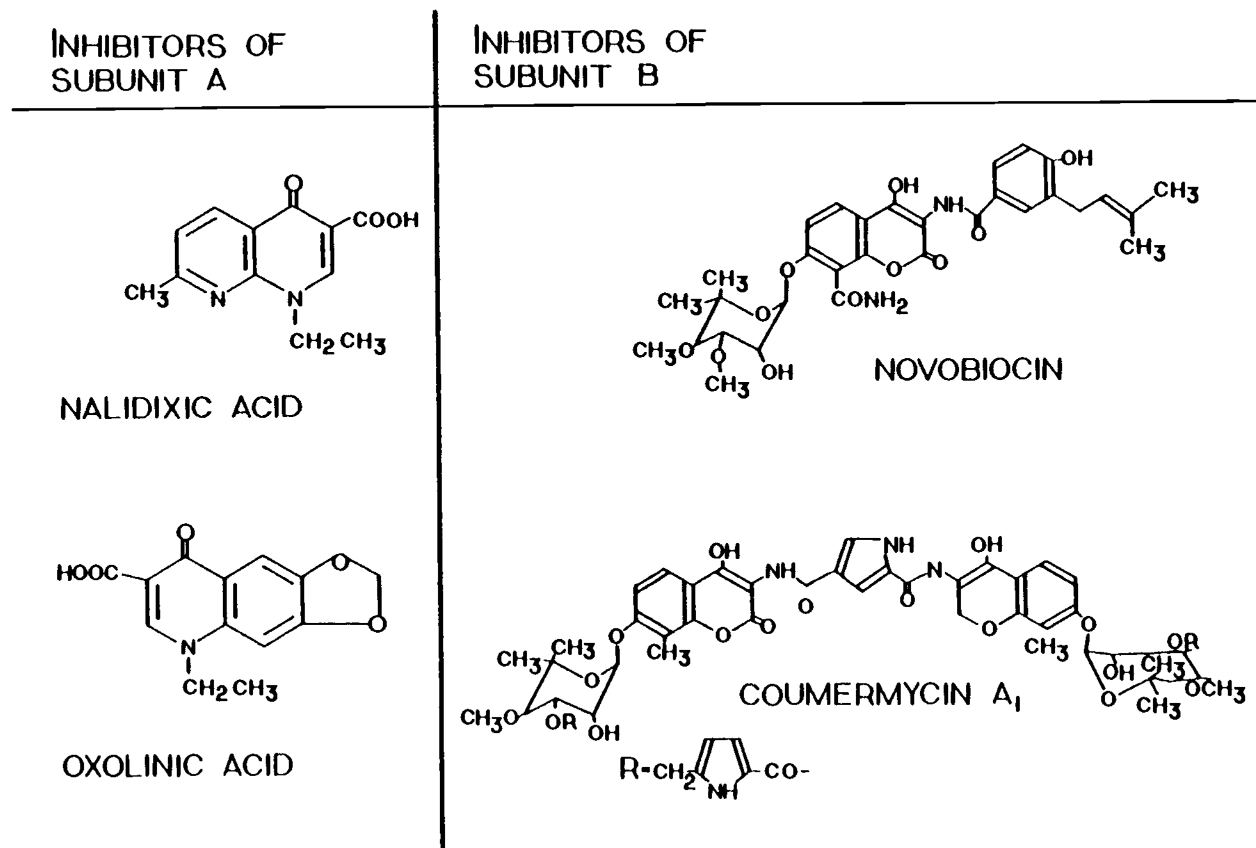
compactin inhibited cultures, none of the cytokinins reversed the requirement for MVA.

D. Structure and Properties of Nalidixic Acid and Related Antibiotics.

Nalidixic acid, oxolinic acid, novobiocin, and coumermycin A_1 (Figure 2) are antibiotics that inhibit type II topoisomerases (Gellert, 1981; Wang, 1985). Topoisomerases are enzymes that convert DNA from one topological form to another. They are found in both prokarotic and eukaryotic cells. The exact functions of topoisomerases are not completely understood, but these enzymes may play important roles in DNA replication and in transcription and recombination. DNA topoisomerases break DNA strand(s), pass a segment of DNA through the break and rejoin the cut ends. This process can result in; 1) catenation and decatenation of DNA circles 2) supercoiling or relaxation of circular DNA 3) knotting and unknotting of circular DNA and 4) intertwining of single strand rings of complementary sequences.

The structure and function of topoisomerases have been the subject of several reviews (Gellert, 1981; Wang, 1981; Wang, 1985). Topoisomerases change the linking numbers of circular DNA. Linking number is defined as the number of times one strand of DNA goes completely around the other strand. Two types of topoisomerases are recognized, based on the types of reactions the enzymes catalyze. Type I

Figure 2. Structures of inhibitors of type II topoisomerases.



topoisomerases cut single strands of a DNA double helix and change the linking number in steps of one. Type I topoisomerases do not require ATP as a cofactor. Type II topoisomerases cut both strands of the DNA double helix and change the linking number in steps of two. Negative supercoiling of closed circular DNA, by the action of type II topoisomerases, may require ATP as a cofactor (Gellert, 1981). Type II topoisomerases are composed of two types of subunits (A_2B_2) (Gellert, 1981; Wang, 1985).

Nalidixic acid, oxolinic acid, novobiocin, and coumermycin A_1 have been used extensively in studies of DNA gyrase, a type II topoisomerase activity present in _Escherichia coli_. Nalidixic acid and oxolinic acid inhibit subunit A of the enzyme and interfere with the nicking-closing steps. Novobiocin and coumermycin A_1 inhibit the covalent binding of ATP to subunit B which inhibits ATP hydrolysis (Gellert _et al_., 1977; Sugino _et al_., 1977; Gellert 1981; Fairweather _et al_., 1980; Filutowicz, 1980; Mizuuchi _et al_., 1978)

The function of topoisomerases in eukaryotes has been investigated by examining the effects of mutation in the gene encoding topoisomerases, the localization of topoisomerases in nuclei and on chromosomes, and the effects of the antibiotic inhibitors of topoisomerase II. Yeast has type I and type II topoisomerases which are encoded by single genes, _TOP_ 1 and _TOP_ 2. Initial studies indicated that topoisomerase I was nonessential during any stage of

the cell cycle (Thrash et al., 1984) while topoisomerase II appeared to be nonessential except during mitosis (Holm et al., 1985). Through the use of two mutants, a top 1 mutant and a temperature-sensitive top 2 mutant, Uemura and Yanagida (1984) found that cells could survive with a single mutation in one of these genes, but a combination of top 1 and temperature-sensitive top 2 mutation at a non-permissive temperature resulted in cell death. These results indicate that topoisomerase activity is required throughout the yeast cell cycle but this requirement can be met by either type I or type II topoisomerase.

Type II topoisomerases are among the chromosome scaffold proteins (Earnshaw et al., 1985). Chromosome scaffold proteins are structural proteins which remain after DNA and histones have been removed from the chromosome. The remaining structure is composed of nonhistone proteins and resembles the size and shape of the chromosome. Topoisomerase II appears to be bound at the base of the "radial loop domains" of mitotic chromosomes (Earnshaw and Heck, 1985).

The effects of inhibitors of topoisomerases have been examined in several eukaryotic systems. In vitro studies have shown that type II topoisomerase in yeast is inhibited by coumermycin and (less sensitively) to novobiocin. However, the enzyme appears to be insensitive to nalidixic acid and oxolinic acid. Nalidixic acid, oxolinic acid, and novobiocin have been reported to inhibit the in vitro

activity of yeast glycyl- and leucyl-tRNA synthetase although at concentrations (1-2 mM) which are several folds higher than those that inhibit bacterial gyrases (Wright *et al*., 1981).

Han and co-workers reported that novobiocin prevents the heat shock response in *Drosophila* (Han *et al*., 1985). The specific changes in gene activity resulting from heat shock were not observed if novobiocin was added prior to the heat treatment. If novobiocin was added after the heat treatment, the transcription of the heat shock genes was rapidly turned off. It would appear from this study that topoisomerase II in eukaryotic systems may be involved in gene activation and may be required continuously throughout the cell cycle.

Nalidixic acid and the related antibiotics have been used to study the involvement of topoisomerases in DNA replication in mammalian mitochondria (Castora *et al*., 1979; Castora *et al*.,1983). Mammalian mitochondria DNA is double stranded and occurs predominantly in a closed, circular, negatively supercoiled configuration. This configuration resembles bacterial DNA structure. Novobiocin and coumermycin A_1 appear to act on the ATPase subunit of the mitochondrial topoisomerase, while nalidixic acid and oxolinic acid inhibit the nicking-closing subunit of the enzyme. From these studies, it appears that the mammalian mitochondria DNA topoisomerase is similar to the bacterial gyrases.

Topoisomerases have not been extensively investigated in plant systems. Topoisomerase type I has been reported to occur in wheat germ (Dynan *et al*., 1981), in cauliflower inflorescence (Fukata and Fukasawa, 1983) and in spinach chloroplasts (Siedleck *et al*., 1983). Topoisomerase type II activity has been reported in cauliflower inflorescences (Fukata and Fukasawa, 1983; Fukata and Fukasawa, 1984; Fukata *et al*., 1986). Type II topoisomerase isolated from cauliflower inflorescences is able to relax supercoiled DNA. In addition, the enzyme catalyzes the formation of DNA catenanes. Catenation and relaxation both require ATP as a cofactor.

Only recently has nalidixic acid been tested in higher plant systems. Heinhorst, Cannon and Weissbach (1985) studied the effect of nalidixic acid on chloroplast and nuclear DNA replication in suspension cultures of *Nicotiana tabacum* cells. *Nicotiana* cells were exposed to nalidixic acid for 19 hours. At low concentrations (5-20 µg/ml), nalidixic acid exerted a greater effect on chloroplast DNA replication than on nuclear DNA replication. Higher concentrations (50 µg/ml) of nalidixic acid inhibited both nuclear and chloroplast DNA synthesis. DNA synthesis was restored when nalidixic acid was removed from the medium. In earlier work on *Euglena gracilis*, nalidixic acid had no effect on cell division; but it did inhibit chloroplast DNA replication which caused irreversible bleaching of the cells (Pienkos *et al*, 1974).

Ciarrocchi and co-workers studied the effect of nalidixic acid and novobiocin on Daucus carota suspension cultures (Ciarrocchi et al., 1985). Daucus suspension cultures are very sensitive to these antibiotics. At concentrations of less than 0.1 mM, nalidixic acid and novobiocin inhibited DNA, RNA and protein synthesis. Cell growth was inhibited by 0.3 mM concentrations of either antibiotic. Cell growth resumed upon removal of nalidixic acid; however, inhibition due to novobiocin appeared to be irreversible. Examination of the intercellular ATP pool showed that the ATP pool decreased with time of exposure to either drug. This effect was particularly striking and rapid in the case of novobiocin treatments. This suggested that the observed inhibition of cellular metabolism may have resulted primarily from an inhibition of ATP synthesis rather than from effects of the drugs on topoisomerase function. However, the results obtained to date are too limited to be certain of the mechanism of action of these antibiotics in plant systems.

III. MATERIALS AND METHODS.

A. Chemicals.

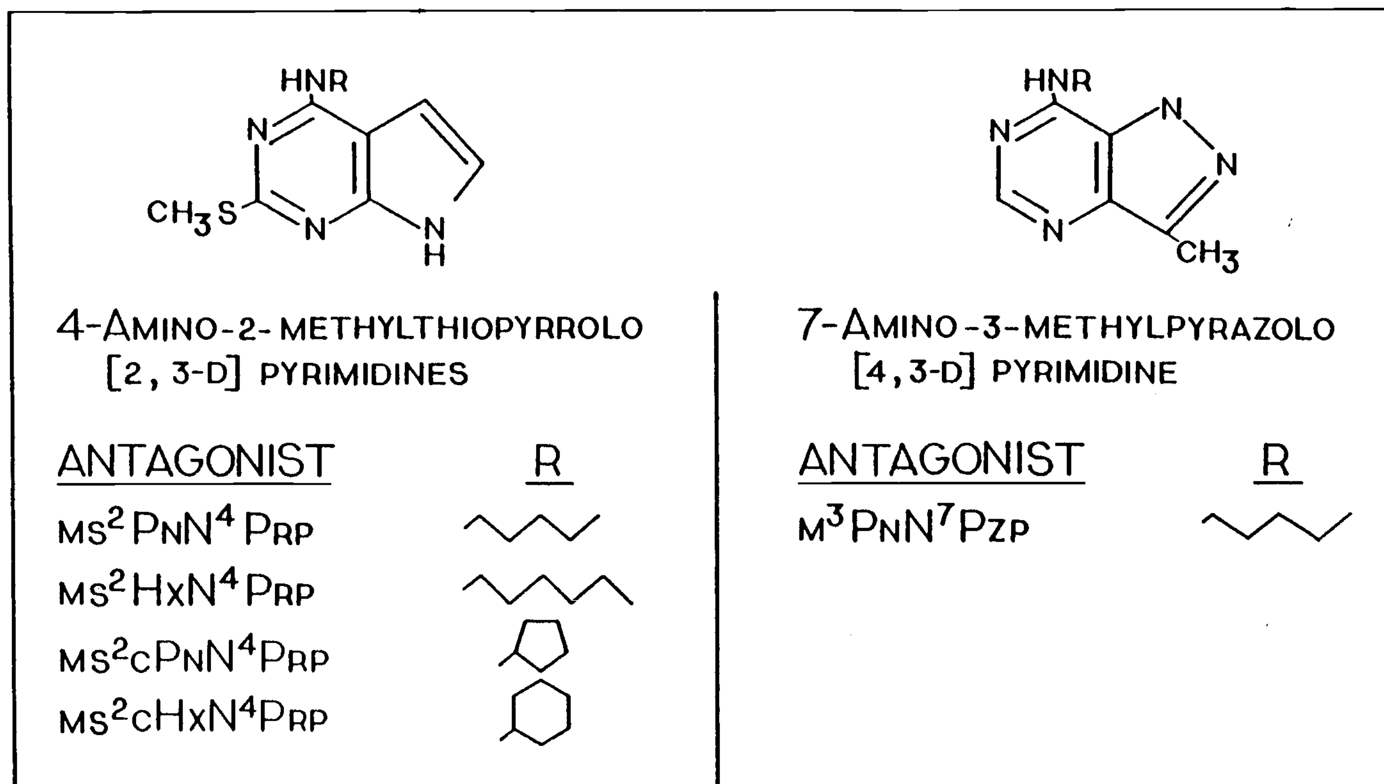
Cytokinin antagonists were a gift from Dr. Sidney Hecht (University of Virginia, Charlottesville, VA). The five antagonists tested were: 4-(n-pentylamino)-2-methylthiopyrrolo[2,3-d]pyrimidine, 4-(n-hexylamino)-2-methylthiopyrrolo[2,3-d]pyrimidine, 4-cyclohexylamino-2-methylthiopyrrolo[2,3-d]pyrimidine, 4-(cyclopentylamino)-2-methylthiopyrrolo[2,3-d]pyrimidine, and 7-(n-pentylamino)-3-methylpyrazolo[4,3-d]pyrimidine. The structures of these compounds and their abbreviations are shown in Figure 3.

Nalidixic acid, oxolinic acid, novobiocin (sodium salt), coumarin, kinetin, and N^6-(Δ^2-isopentenyl)adenine were obtained from Sigma.

B. Plant Material.

Seeds of *Phaseolus lunatus* L. cv. Kingston and *Phaseolus vulgaris* L. cv. Great Northern were obtained from Burpee's Seed Company. The seeds were surface sterilized for 5 minutes in 50% Chlorox containing 0.1% Tween 20, rinsed three times in sterile water, and germinated on moist paper toweling under aseptic conditions. Seeds were germinated in a Percival growth chamber using a 16 hour day (640 foot candles, 27°C) and an 8 hour night (20°C). Callus cultures were established from hypocotyl tissue of 5-7 day old seedlings. The callus cultures obtained in this manner

Figure 3. Structures of cytokinin antagonist test compounds.



were cytokinin-dependent. Cytokinin-autonomous callus lines of these genotypes were obtained by selection from originally cytokinin-dependent lines (Armstrong, unpublished). To select for autonomous growth, the cytokinin-dependent tissues were grown for one passage on sub-optimal concentrations of kinetin and then transferred to cytokinin-free media for selection. The cytokinin-autonomous lines used here were obtained in 1982 and have been maintained on cytokinin-free media since that time.

The cytokinin-dependent line of _Nicotiana_ _tabacum_ cv. Wisconsin 38 were established from pith tissue in 1980. The cytokinin-autonomous line of tobacco callus tissue originated spontaneously as rapidly growing tissue in cytokinin-dependent Wisconsin 38 cultures maintained at the University of Wisconsin (Schmitz and Skoog, unpublished). The cytokinin-autonomous line has been in culture since 1972. Einset and Skoog (1973) have reported some of the properties of the autonomous line.

C. _Tissue Culture Media._

The basal medium used for _Phaseolus_ tissue culture consisted of the inorganic nutrients described by Murashige and Skoog (1962) with the following organic substances added: sucrose (30 g/l), _myo_-inositol (100 mg/l), thiamine-HCl (1 mg/l), nicotinic acid (5 mg/l), pyridoxin-HCl (0.5 mg/l), and picloram (2.5 μM). Kinetin (5 μM) was included

in medium used for the initiation and maintenance of the cytokinin-dependent Phaseolus tissue cultures.

The Nicotiana basal medium contained the same inorganic nutrients (Murashige and Skoog, 1962) with sucrose (30 g/l), myo-inositol (100 mg/l), thiamine-HCl (0.4 mg/l), and indole-3-acetic acid (2 mg/l). Kinetin (0.15 μM) was included in medium used for the maintenance of cytokinin-dependent Nicotiana tissue cultures.

The pH values of all tissue culture media were adjusted to 5.7. Difco Bacto-agar (10 g/l) was added, and the media was dispensed into 125 ml Erlenmeyer flasks (50 ml/flask) and autoclaved at 120°C for 15 minutes.

Cytokinin antagonists, coumarin, and thidiazuron were dissolved in dimethyl sulfoxide (DMSO)(Schmitz and Skoog, 1970) and added to the autoclaved media prior to solidification. Kinetin and N^6-(Δ^2-isopentenyl)adenine were dissolved in DMSO and added to the media prior to autoclaving. In all cases where DMSO was used, the total amount of DMSO added per flask was 50 μl DMSO/ 50 ml media.

Nalidixic acid and oxolinic acid were neutralized and dissolved in NaOH. The final pH values of the antibiotic stock solutions were carefully adjusted to 11. Appropriate dilutions were prepared using double distilled water adjusted to pH 11. The resulting solutions were cold sterilized by Millipore filtration. Novobiocin (sodium salt) was dissolved in double distilled water and also cold sterilized by Millipore filtration. Aliquots (0.5 ml) of

the filter-sterilized antibiotic solutions were added to each flask containing 50 ml of autoclaved media prior to solidification. The pH of the medium was not detectably altered by the addition of the antibiotic solutions.

D. Growth and Harvest of Cultures.

Stock cultures of cytokinin-dependent and cytokinin-autonomous *Phaseolus* and *Nicotiana* callus tissue were maintained on the media described above and grown in the dark at 28°C. Three pieces of callus tissue (ca. 25 mg each) from three- to four-week old stock cultures were planted per experimental flask. In each experiment, four flasks were planted for each concentration of each treatment. All *Phaseolus* tissue cultures and the cytokinin-autonomous *Nicotiana* tissue cultures were harvested after 28 days of growth in the dark at 28°C. Cytokinin-dependent *Nicotiana* tissue cultures,grown under the same enivironmental conditions, were harvested after a growth period of 35 days. All data are the average of two experiments unless otherwise indicated.

IV. RESULTS

A. Biological Responses of *Phaseolus* and *Nicotiana* Callus Tissues to Cytokinin Antagonists.

1. Responses of Cytokinin-Autonomous Callus Tissues.

Cytokinin-autonomous callus tissues from *P. lunatus* cv. Kingston, *P. vulgaris* cv. Great Northern, and *N. tabacum* cv. Wisconsin 38 were tested for their responses to five cytokinin antagonists. The antagonists tested included four pyrrolo[2,3-d]pyrimidine derivatives and one pyrazolo[4,3-d]pyrimidine derivative (Figure 3). The effects of the antagonists on the three lines of callus tissues are compared in Figures 4 to 8. The antagonist concentrations required to obtain 50% inhibition of growth (I_{50} values) are summarized in Table I.

The growth of the cytokinin-autonomous *Nicotiana* callus tissue was completely, or nearly completely, inhibited by all of the antagonists at concentrations less than or equal to 10 µM. Compared to the *Nicotiana* callus tissue, both of the cytokinin-autonomous *Phaseolus* callus tissues were less sensitive to antagonist inhibition. The I_{50} values for the pyrrolopyrimidine antagonists varied from 1.2 µM to 3.2 µM in tests on the *Nicotiana* tissue. The corresponding I_{50} values for the *Phaseolus* callus tissues varied from 3.6 µM to greater than 10 µM. The differences in sensitivity to

Figure 4. Effects of 4-(n-pentylamino)-2-methylthiopyrrolo[2,3-d]pyrimidine (ms^2PnN^4Prp) on the growth of cytokinin-autonomous _Phaseolus_ and _Nicotiana_ callus tissues.

Cytokinin-autonomous lines of _P. lunatus_ cv. Kingston, _P. vulgaris_ cv. Great Northern, and _N. tabacum_ cv. Wisconsin 38 callus tissues were grown as described in "Materials and Methods". The cytokinin antagonist was dissolved in DMSO and added to the autoclaved tissue culture media (50 µl DMSO/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) averaged ±11.5% of the means and did not exceed ±15%.

Figure 4

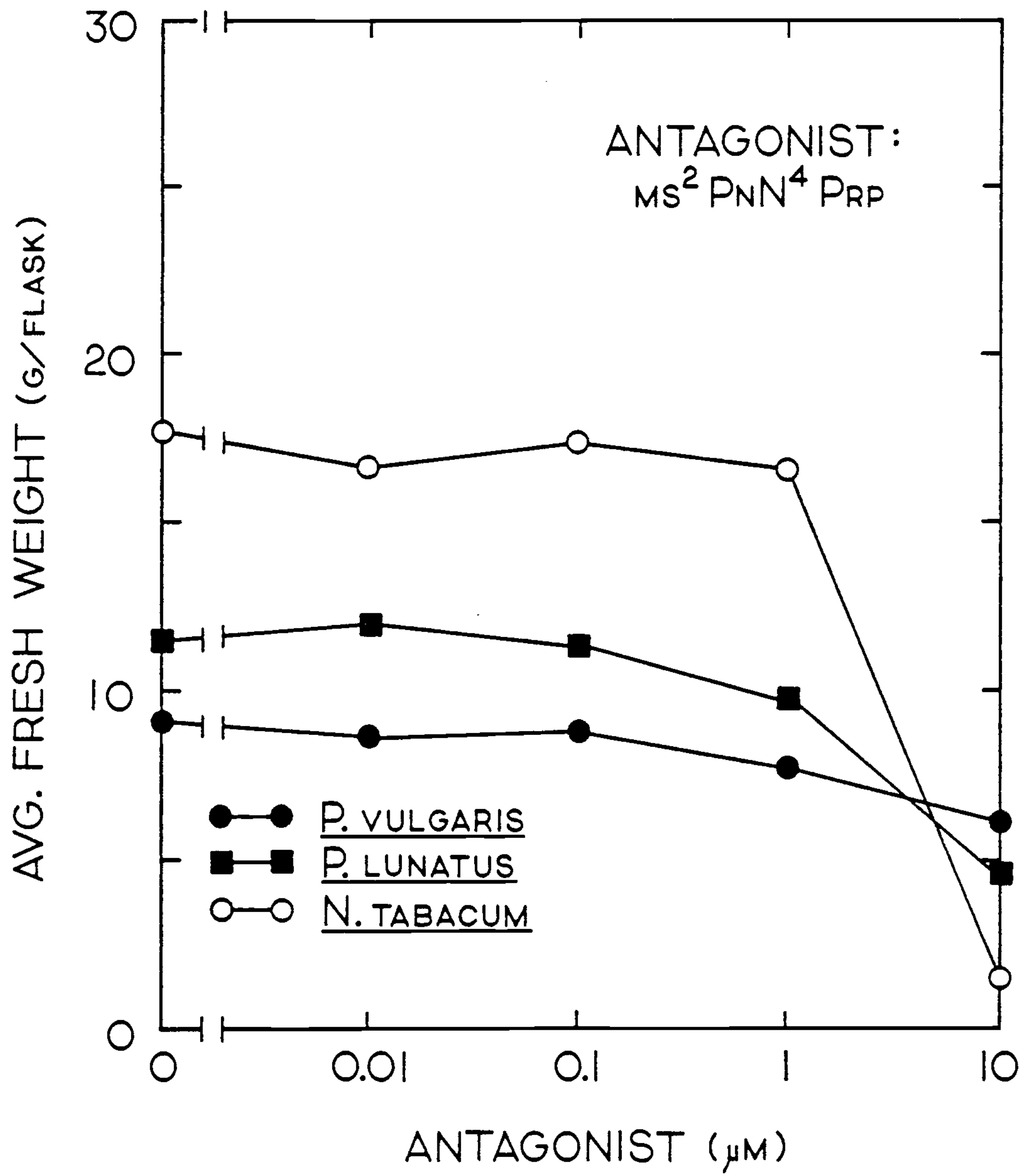


Figure 5. Effects of 4-(n-hexylamino)-2-methylthiopyrrolo[2,3-d]pyrimidine (ms^2HxN^4Prp) on the growth of cytokinin-autonomous Phaseolus and Nicotiana callus tissues.

Cytokinin-autonomous P. lunatus cv. Kingston, P. vulgaris cv. Great Northern, and N. tabacum cv. Wisconsin 38 callus tissue were grown as described in "Materials and Methods". The cytokinin antagonist was dissolved in DMSO and added to the autoclaved tissue culture medium (50 µl DMSO/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) averaged ± 8% of the means and did not exceed ±14%.

Figure 5

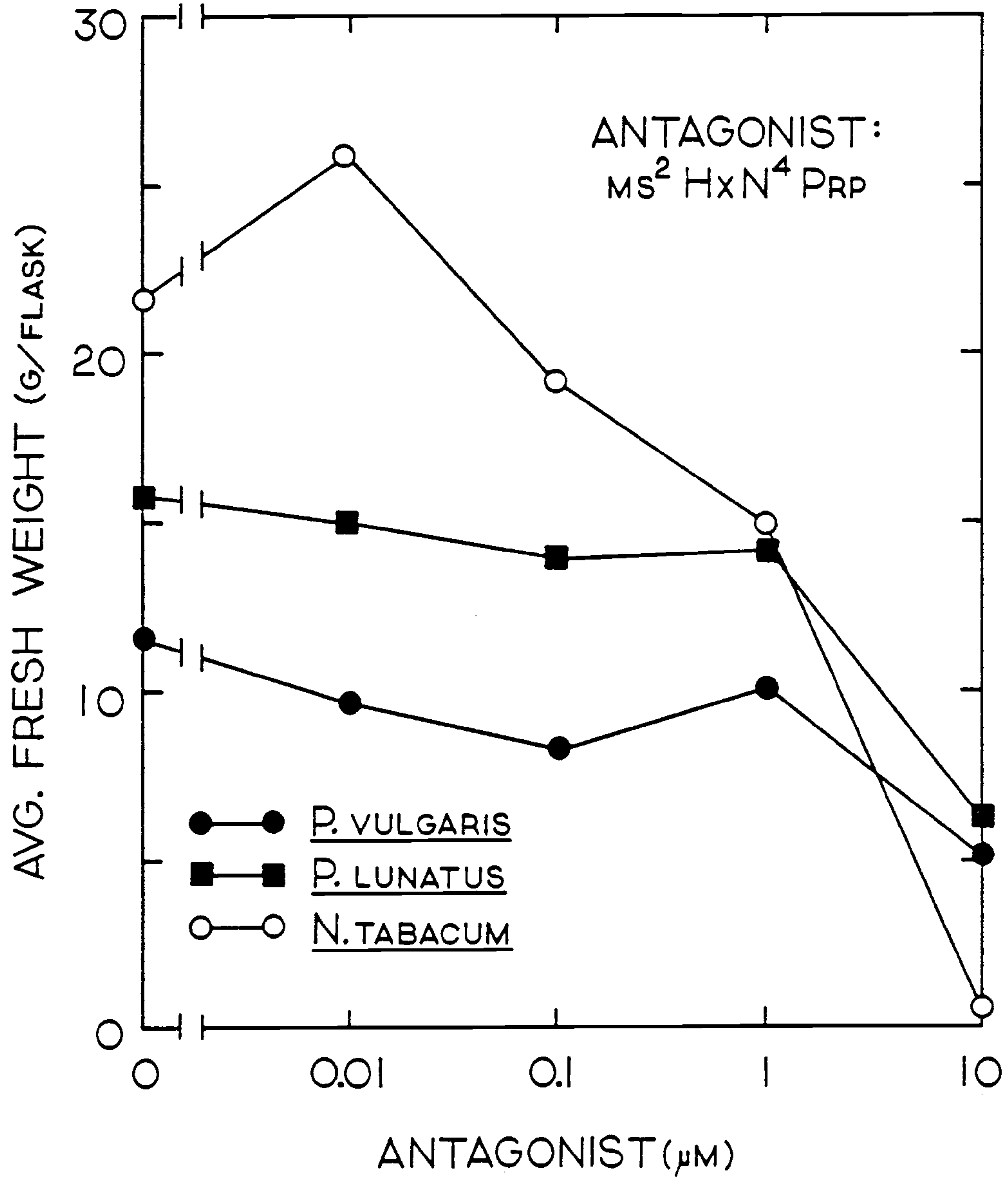


Figure 6. Effects of 4-cyclopentylamino-2-methylthiopyrrolo[2,3-d]pyrimidine (ms^2cPnN^4Prp) on the growth of cytokinin-autonomous Phaseolus and Nicotiana callus tissues.

Cytokinin-autonomous P. lunatus cv. Kingston, P. vulgaris cv. Great Northern, and N. tabacum cv. Wisconsin 38 callus tissues were grown as described in "Materials and Methods". The cytokinin antagonist was dissolved in DMSO and added to the autoclaved tissue culture medium (50 µl DMSO/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark.. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) averaged ±8% of the means and did not exceed ±14%.

Figure 6

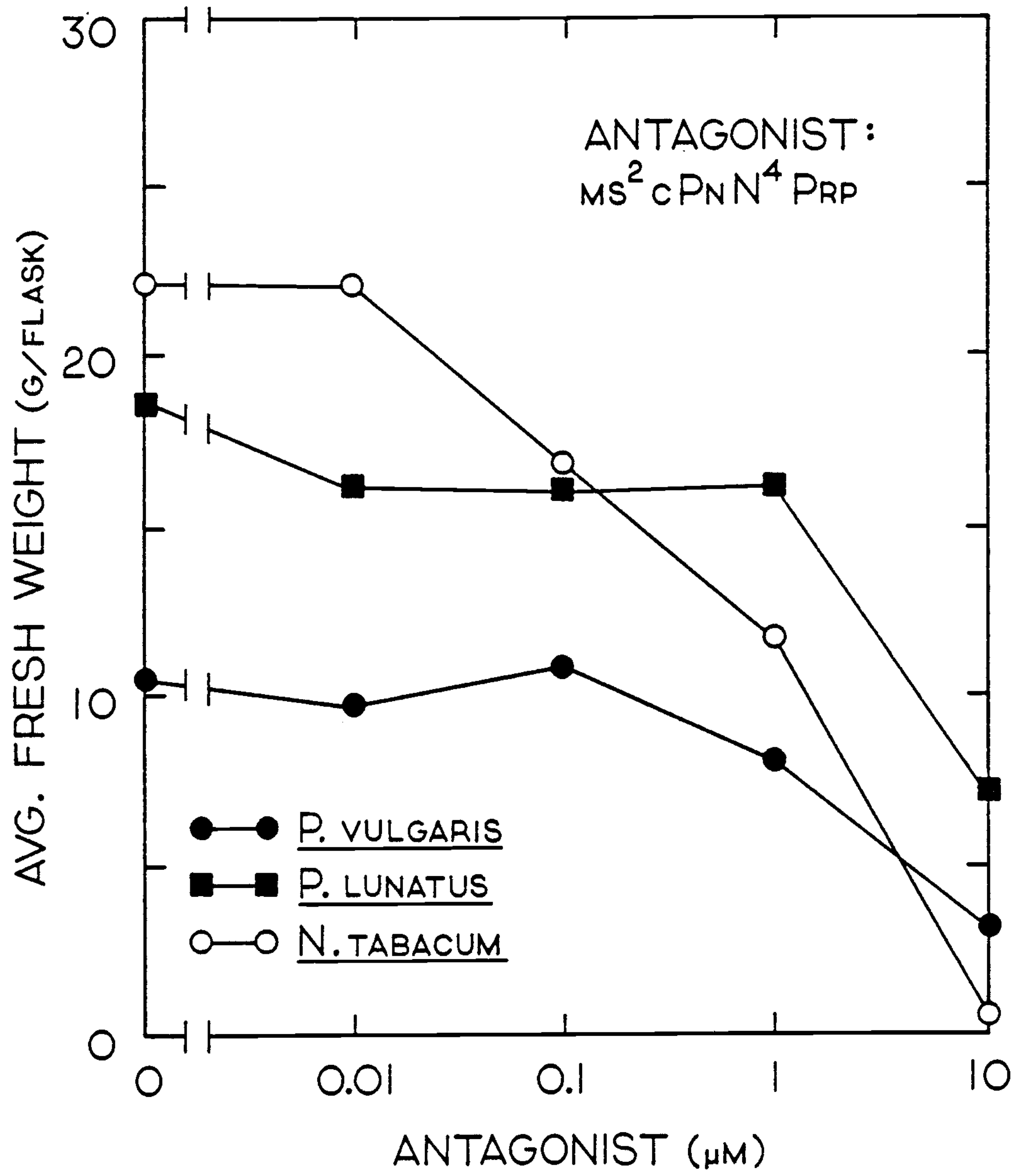


Figure 7. Effects of 4-cyclohexylamino-2-methylthiopyrrolo[2,3-d]pyrimidine (ms^2cHxN^4Prp) on the growth of cytokinin-autonomous Phaseolus and Nicotiana callus tissues.

Cytokinin-autonomous P. lunatus cv. Kingston, P. vulgaris cv. Great Northern, and N. tabacum cv. Wisconsin 38 callus tissue were grown as described in "Materials and Methods". The cytokinin antagonist was dissolved in DMSO and added to the autoclaved tissue culture medium (50 µl DMSO/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. The average data of two replicate experiments are plotted. The standard error (n=4 for each experiment) averaged ±8% of the means and did not exceed ±14%.

Figure 7

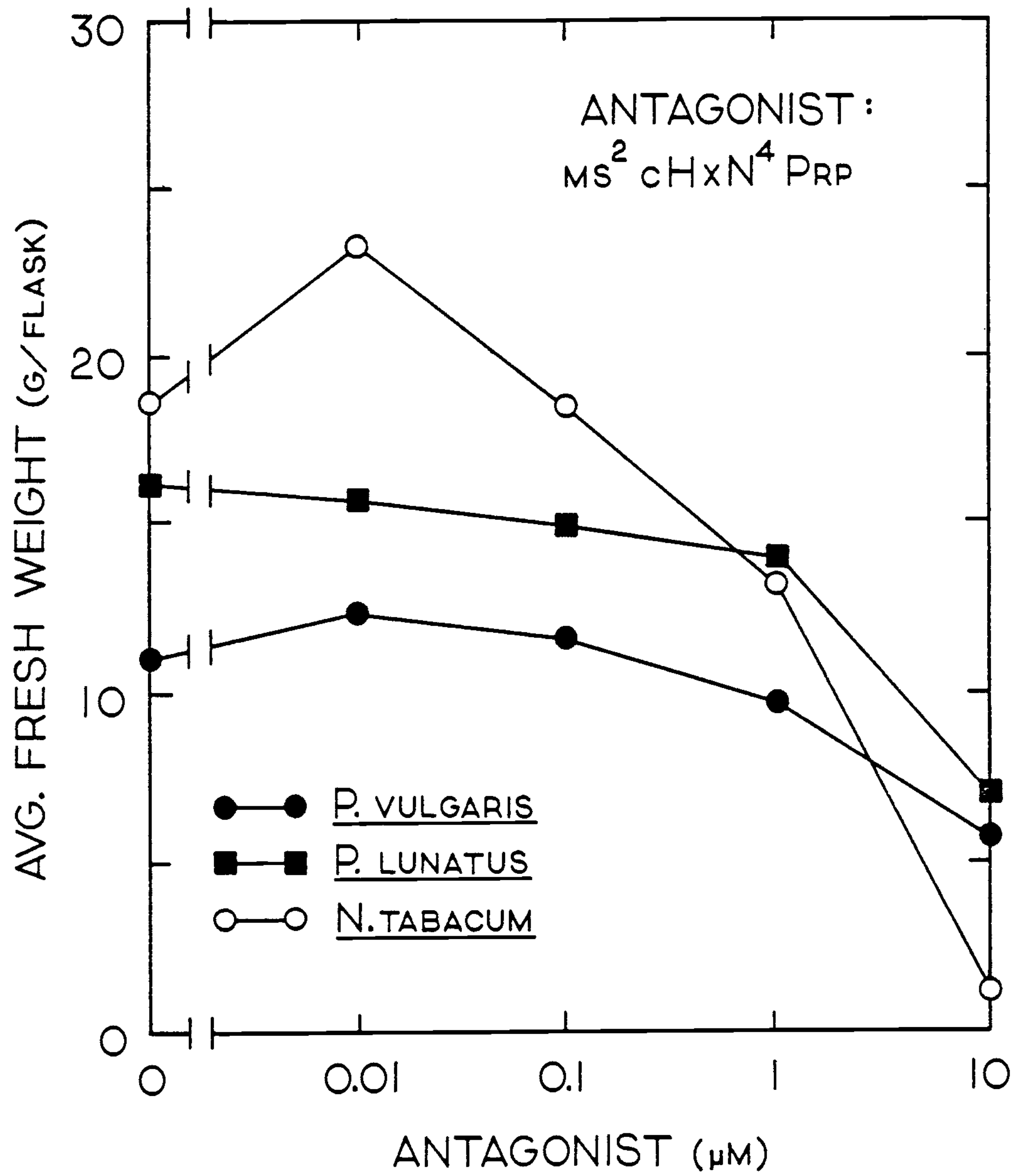


Figure 8. Effects of 7-(n-pentylamino)-3-methylpyrazolo[4,3-d]pyrimidine (m^3PnN^7Pzp) on the growth of cytokinin-autonomous *Phaseolus* and *Nicotiana* callus tissues.

Cytokinin-autonomous line of *P. lunatus* cv. Kingston, *P. vulgaris* cv. Great Northern, and *N. tabacum* cv. Wisconsin 38 callus tissues were grown as described in "Materials and Methods". The cytokinin antagonist was dissolved in DMSO and added to the autoclaved tissue culture medium (50 µl DMSO/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. The average data of two replicate experiments is plotted. The standard errors (n=4 for each experiment) averaged ±9% of the means and did not exceed ±15%.

Figure 8

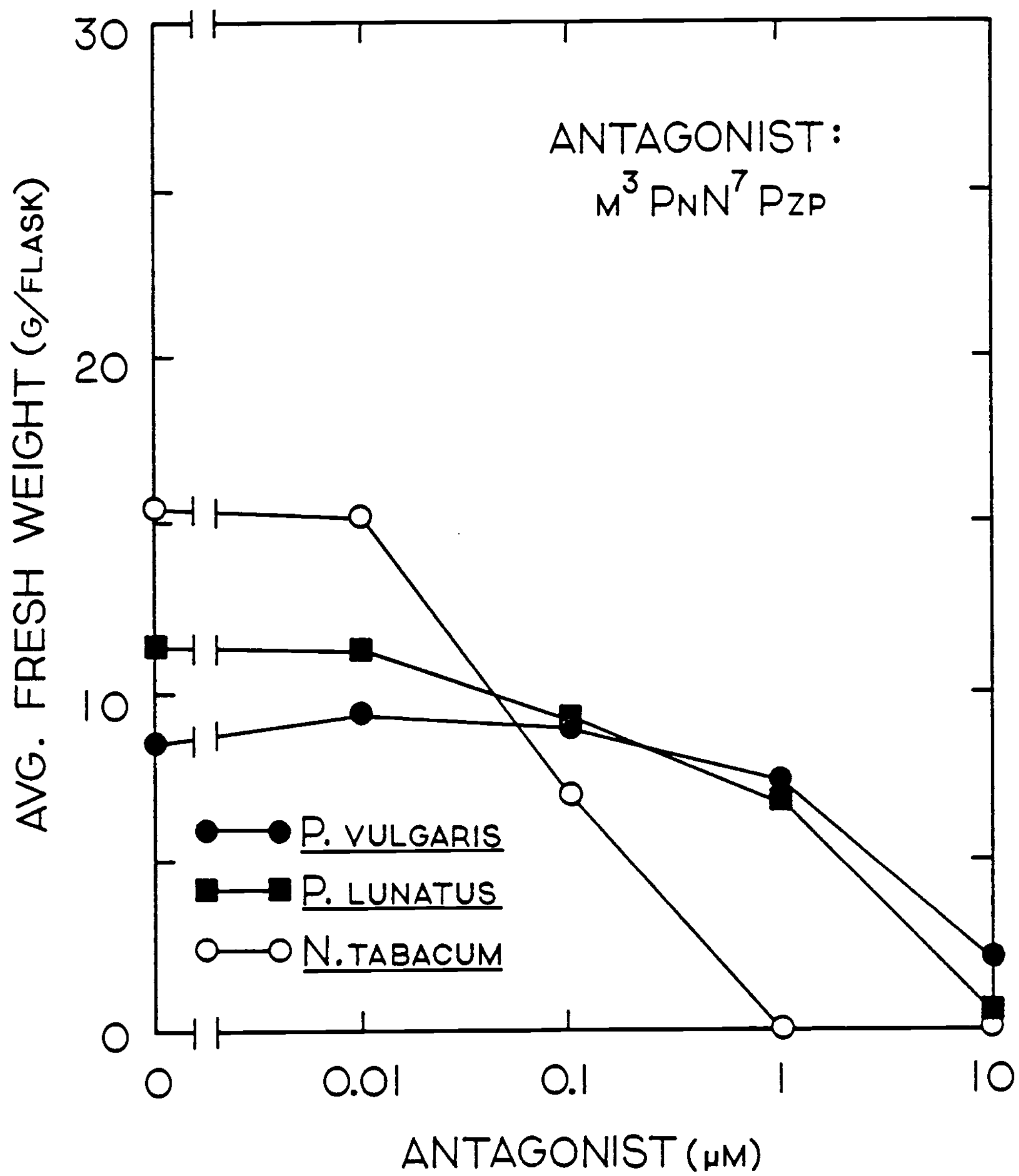


Table I. Inhibitory Effects of Cytokinin Antagonists on Cytokinin-Autonomous *Phaseolus* and *Nicotiana* Callus Cultures.

Antagonist[b]	I50 Values (μM)[a]		
	P. lunatus cv. Kingston	*P. vulgaris* cv.G.Northern	*N. tabacum* cv. Wis. 38
ms^2PnN^4Prp	5.6	>10.0	3.2
ms^2HxN^4Prp	4.8	6.8	1.9
ms^2cPnN^4Prp	6.0	3.6	1.2
ms^2cHxN^4Prp	7.1	>10.0	2.4
m^3PnN^7Pzp	1.6	2.4	0.08

a) I_{50} values are defined as the antagonist concentrations required to obtain 50% inhibition of growth.

b) Abbreviations:

ms^2PnN^4Prp
4-n-pentylamino-2-methylthiopyrrolo[2,3-d]pyrimidine

ms^2HxN^4Prp
4-n-hexylamino-2-methylthiopyrrolo[2,3-d]pyrimidine

ms^2cPnN^4Prp
4-cyclopentylamino-2-methylthiopyrrolo[2,3-d]pyrimidine

ms^2cHxN^4Prp
4-cyclohexylamino-2-methylthiopyrrolo[2,3-]pyrimidine

m^3PnN^7Pzp
7-n-pentylamino-3-methylpryazolo[4,3-d]pyrimidine.

antagonist inhibition were even more marked in the case of the pyrazolopyrimidine derivative. The *Nicotiana* callus tissue was approximately 20 to 30 times more sensitive to this antagonist than were the *Phaseolus* callus tissues.

The I_{50} values observed with the two *Phaseolus* callus tissues were rather similar. However, the cytokinin-autonomous callus tissue derived from *P. lunatus* cv. Kingston appeared to be slightly more sensitive to the antagonists than the corresponding callus derived from *P. vulgaris* cv. Great Northern, except in the case of the antagonist ms^2cPnN^4Prp.

The pyrazolopyrimidine derivative m^3PnN^7Pzp was significantly more effective in inhibiting growth of all three callus lines than the pyrrolopyrimidine derivative bearing the corresponding side chain substitution (ms^2PnN^4Prp). Among the four pyrrolo[2,3-d]pyrimidine derivatives tested, ms^2cPnN^4Prp was the most effective inhibitor of the cytokinin-autonomous *Nicotiana* and *P. vulgaris* tissues, and ms^2HxN^4Prp was the most effective inhibitor of the cytokinin-autonomous *P. lunatus* callus tissue.

2. Responses of Cytokinin-Dependent Callus Tissues.

The effects of cytokinin antagonists on cytokinin-dependent callus tissues were not extensively investigated in the present study. However, cytokinin-dependent P. *lunatus* cv. Kingston callus tissue, grown on media

containing varying concentrations of the cytokinin N^6-(Δ^2-isopentenyl)adenine (i^6Ade) was not sensitive to ms^2cHxN^4Prp at antagonist concentrations up to 10 µM (Figure 9). (In tests using the cytokinin-autonomous line of callus tissue from this genotype, the I_{50} value for ms^2cHxN^4Prp was 7.1 µM.)

3. Reversal of the Inhibitory Effects of Cytokinin Antagonists.

The ability of the cytokinin N^6-(Δ^2-isopentenyl)adenine (i^6Ade) to reverse the inhibitory effects of antagonists on the growth of cytokinin-autonomous N. tabacum callus tissue was tested using two antagonists, ms^2PnN^4Prp and m^3PnN^7Pzp (Figures 10 and 11). Interpretation of the results is complicated by the fact that exogenously supplied i^6Ade, at concentrations greater than 0.01 µM, was itself inhibitory to the growth of the cytokinin-autonomous Nicotiana callus tissue. However, at concentrations of ms^2PnN^4Prp and m^3PnN^7Pzp that partially inhibited growth of the Nicotiana callus tissue (10 µM and 0.1 µM, respectively), the addition of exogenous i^6Ade to the culture media resulted in partial reversals of antagonist inhibition. The effects of the exogenous cytokinin are reflected in changes in the I_{50} values for antagonist inhibition (Table II). The I_{50} value for ms^2PnN^4Prp was 4.0 µM in the absence of exogenous cytokinin and increased to greater than 10 µM in the presence of 0.1 µM i^6Ade. The corresponding I_{50} values for

Figure 9. Effects of 4-cyclohexylamino-2-methylthiopyrrolo[2,3-d]pyrimidine (ms^2cHxN^4Prp) on the growth of cytokinin-dependent <u>Phaseolus</u> callus tissue.

Cytokinin-dependent line of <u>P</u>. <u>lunatus</u> cv. Kingston callus tissue was grown on various concentrations of N^6-(Δ^2-isopentenyl)adenine (i^6Ade) The i^6Ade was dissolved in DMSO and added to the unautoclaved tissue culture medium (25 µl DMSO/50 ml medium). The cytokinin antagonist was dissolved in DMSO and added to the autoclaved tissue culture medium (25 µl DMSO/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. The average data of one experiment are plotted. The standard errors (n=4 for each experiment) averaged ±6% of the means and did not exceed ±16%.

Figure 9

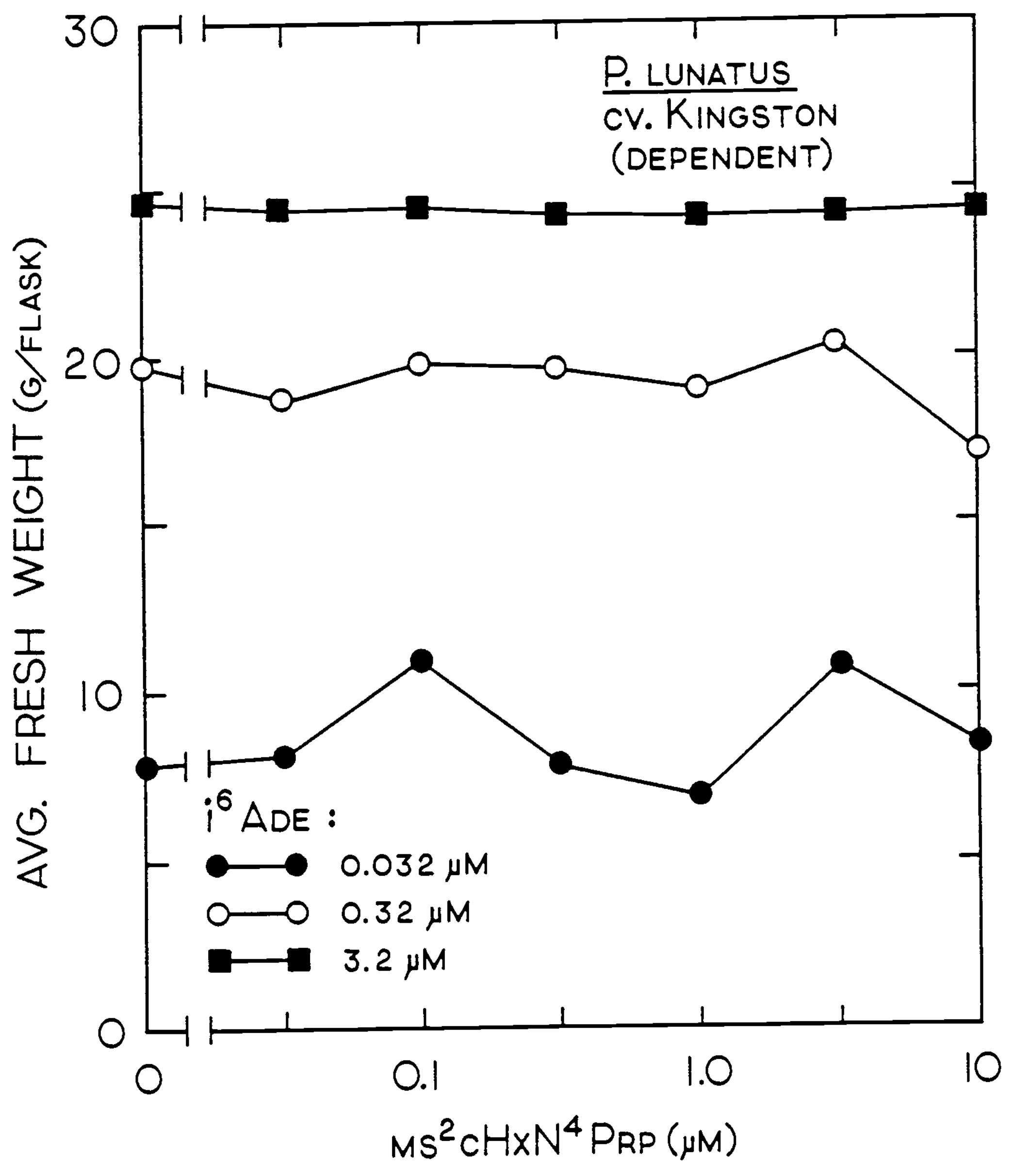


Figure 10. Interactions of 4-(n-pentylamino)-2-methylthiopyrrolo[2,3-d]primidine (ms^2PnN^4Prp) and N^6-(Δ^2-isopentenyl)adenine (i^6Ade) in cytokinin-autonomous *Nicotiana* callus cultures.

Cytokinin-autonomous line of *N. tabacum* cv. Wisconsin 38 callus tissue was grown as described in "Materials and Methods". The i^6Ade was dissolved in dimethyl sulfoxide (DMSO) and added to the unautoclaved tissue culture media (25 µl DMSO/50 ml medium). The cytokinin antagonist was dissolved in DMSO and added to the autoclaved tissue culture media (25 µl DMSO/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. Data are from one experiment. The standard errors (n=4 for each experiment) averaged ±9.5% of the means and did not exceed ±15%.

Figure 10

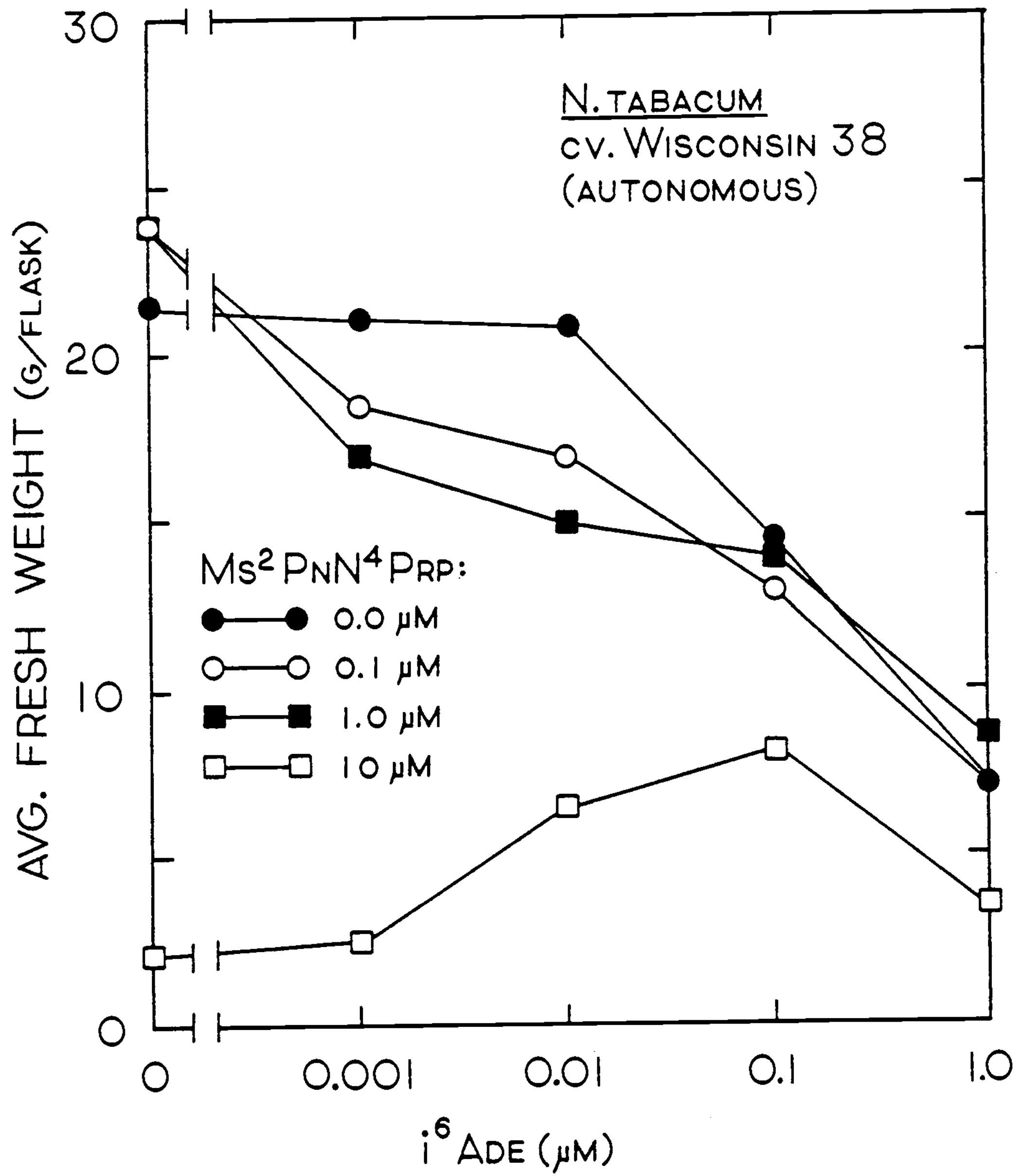


Figure 11. Interactions of 7-n-pentylamino-3-methylpyrazolo[4,3-d]pyrimidine (m^3PnN^7Pzp) and N^6-(Δ^2-isopentenyl)adenine (i^6Ade) in cytokinin-autonomous <u>Nicotiana</u> callus cultures.

Cytokinin-autonomous line of <u>N</u>. <u>tabacum</u> cv. Wisconsin 38 callus tissue was grown as described in "Materials and Methods". The i^6Ade was dissolved in dimethyl sulfoxide (DMSO) and added to the autoclaved tissue culture media (25 µl DMSO/50 ml medium). The cytokinin antagonist was dissolved in DMSO and added to the autoclaved tissue culture media (25 µl DMSO/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. Data are from one experiment. The standard errors (n=4 for each experiment) averaged ±7% of the means and did not exceed ±16%.

Figure 11

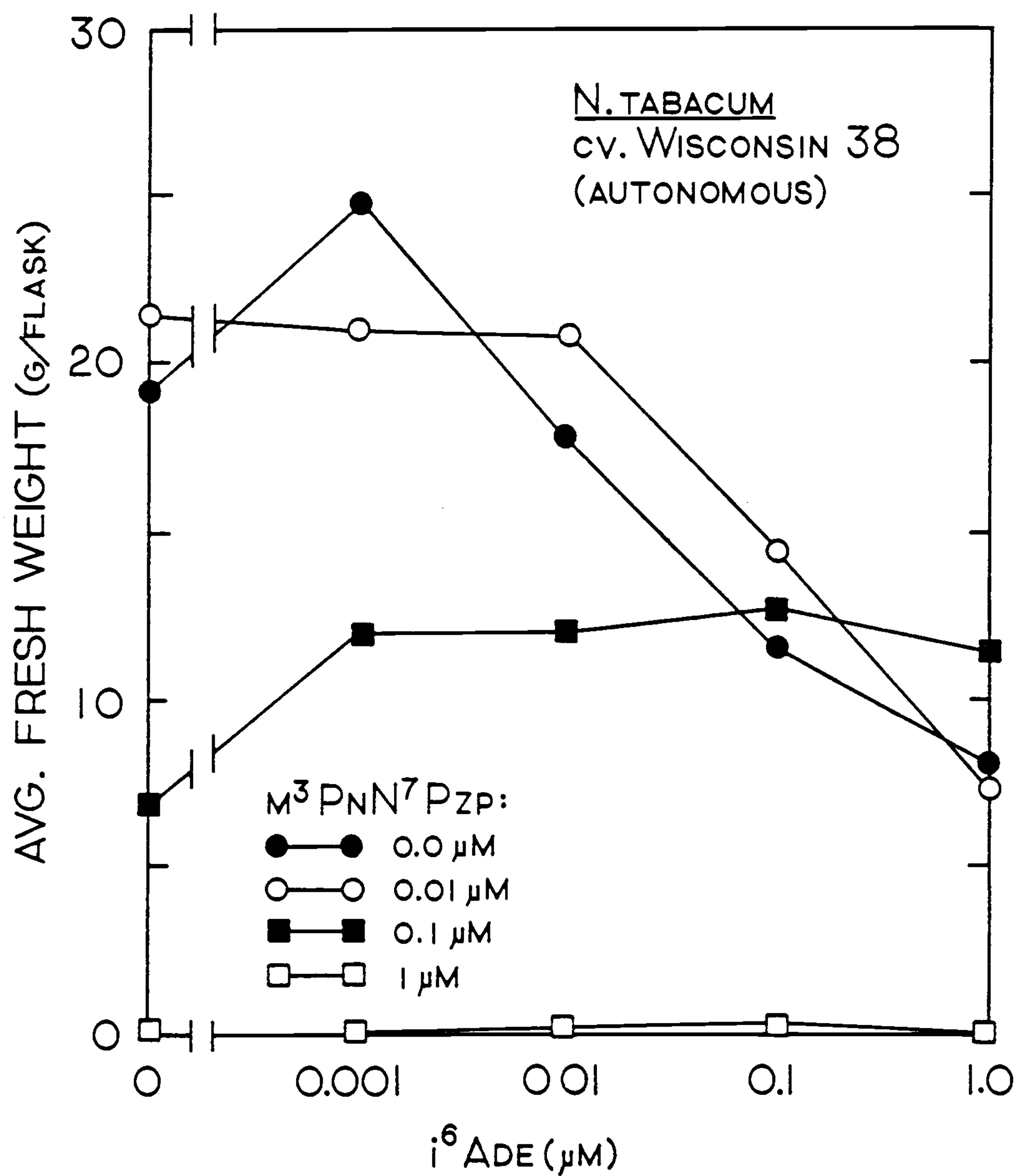


Table II. Interaction of Antagonists and N^6-(Δ^2-Isopentenyl)Adenine (i^{6}Ade) in Cytokinin-Autonomous *Phaseolus* and *Nicotiana* Callus Cultures.[a]

	Antagonists I50 Values (μM)[b]		
	N. tabacum cv. Wisconsin 38		*P. lunatus* cv. Kingston
i^{6}Ade concentration (μM)	ms^{2}PnN^{4}Prp	m^{3}PnN^{7}Pzp	m^{3}PnN^{7}Pzp
0.00	4.0	0.045	1.4
0.01	4.1	0.145	1.9
0.1	>10.0	0.27	3.2
1.0	9.0	0.47	3.0

a) Abbreviations:

ms^{2}PnN^{4}Prp, 4-n-pentylamino-2-methylthiopyrrolo[2,3-d]pyrimidine.

m^{3}PnN^{7}Pzp, 7-n-pentylamino-3-methylpyrazolo[4,3-d]pyrimidine.

ms^{2}cPnN^{4}Prp, 4-cyclopentyl-2-methylthiopyrrolo[2,3-d]pyrimidine.

b) I_{50} values are defined as the antagonist concentrations required to obtain 50% inhibition of growth.

m^3PnN^7Pzp were 0.05 µM in the absence of exogenous cytokinin and 0.27 µM in the presence of 0.1 µM i^6Ade. Concentrations of m^3PnN^7Pzp greater than 1.0 µM completely inhibited growth of the tissue, and the effects of these concentrations were not reversed by cytokinin treatment.

The ability of exogenous i^6Ade to reverse inhibitory effects of the antagonist m^3PnN^7Pzp was also tested using the cytokinin-autonomous line of P. lunatus cv. Kingston callus tissue. The results are shown in Figure 12. As in the case of the cytokinin-autonomous Nicotiana tissue, the effects of m^3PnN^7Pzp on the cytokinin-autonomous P. lunatus callus tissue were partially reversed by the addition of i^6Ade to the media. The I_{50} value for m^3PnN^7Pzp increased from 1.4 µM in the absence of exogenous cytokinin to 3.2 µM in the presence of 0.1 µM i^6Ade (Table II). In a separate experiment in which the concentration of i^6Ade was extended to 10 µM (data not shown), reversal was not significantly enhanced by the additional increase in cytokinin concentration.

The inhibitory effects of ms^2cPnN^4Prp on cytokinin-dependent Nicotiana callus tissue were reversed by increases in the cytokinin (kinetin) concentration in the media (Figure 13). The changes in antagonist I_{50} values associated with changes in kinetin concentration are summarized in Table III. The I_{50} value for ms^2cPnN^4Prp increased from 0.02 µM (at 0.1 µM kinetin) to 0.42 µM (at 1.0 µM kinetin). However, the latter kinetin concentration

Figure 12. Interactions of 7-n-pentylamino-3-methylpyrazolo[4,3-d]pyrimidine (m^3PnN^7Pzp) and N^6-(Δ^2-isopentenyl)adenine (i^6Ade) in cytokinin-autonomous Phaseolus callus cultures.

Cytokinin-autonomous line of P. lunatus cv. Kingston callus tissue was grown as described in "Materials and Methods". The i^6Ade was dissolved in dimethyl sulfoxide (DMSO) and added to the autoclaved tissue culture media (25 µl DMSO/50 ml medium). The cytokinin antagonist was dissolved in DMSO and added to the autoclaved tissue culture media (25 µl DMSO/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissue was harvested and weighed after 28 days of growth at 28°C in the dark. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) averaged ±10% of the means and did not exceed ±16%.

Figure 12

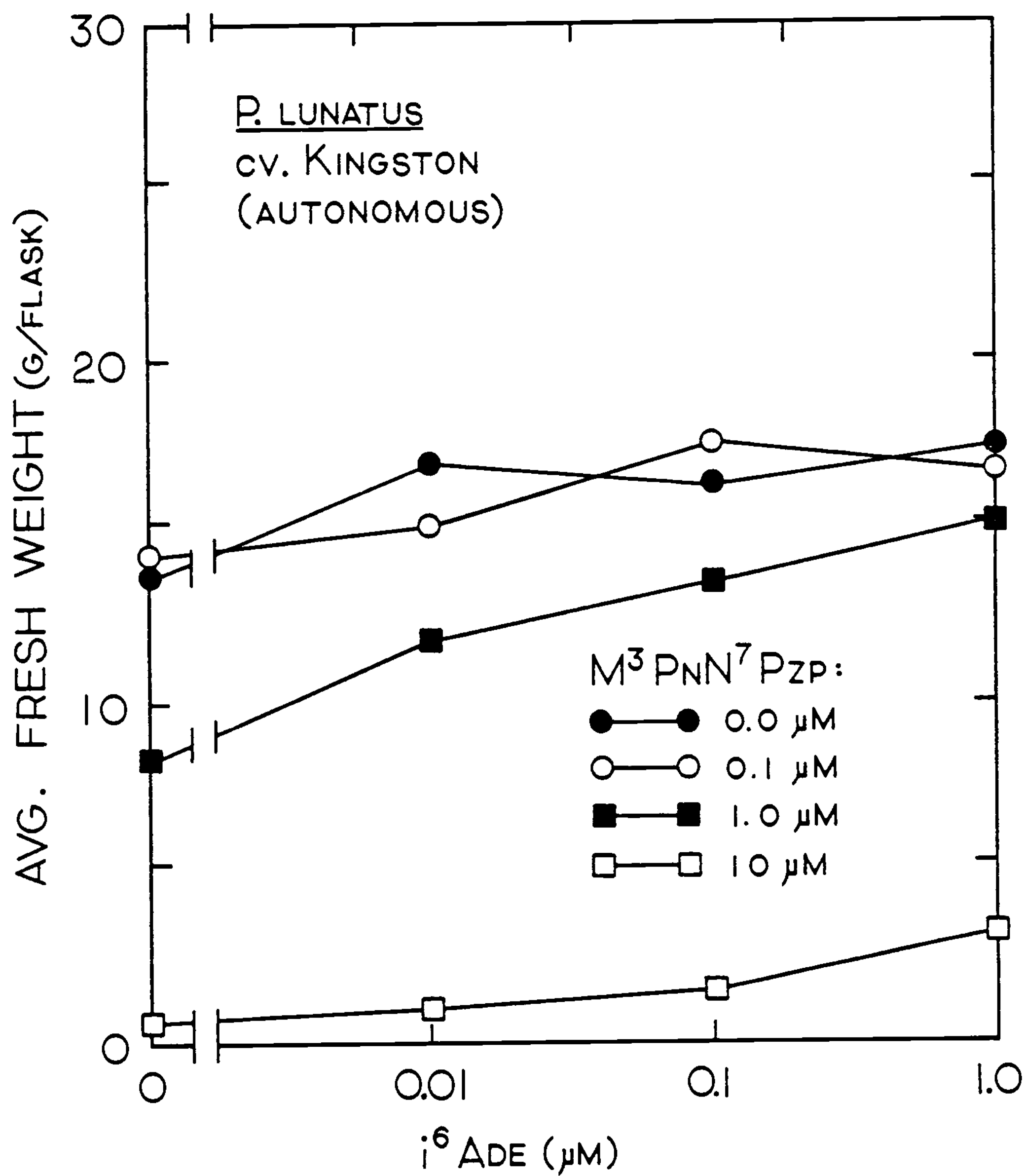


Figure 13. Interaction of 4-cyclopentylamino-2-methylthiopyrrolo[2,3-d]pyrimidine (ms^2cPnN^4Prp) and kinetin in cytokinin-dependent Nicotiana callus cultures.

Cytokinin-dependent line of N. tabacum cv. Wisconsin 38 callus tissue was grown as described in "Materials and Methods". Kinetin was dissolved in DMSO and added to the unautoclaved tissue culture medium (25 µl DMSO/50 ml medium). The cytokinin antagonist was dissolved in DMSO and added to the autoclaved tissue culture medium (25 µl DMSO/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 35 days of growth at 28°C in the dark. The average data of one experiment are plotted. The standard error (n=4 for each experiment) averaged 7±% of the means and did not exceed 15±%.

Figure 13

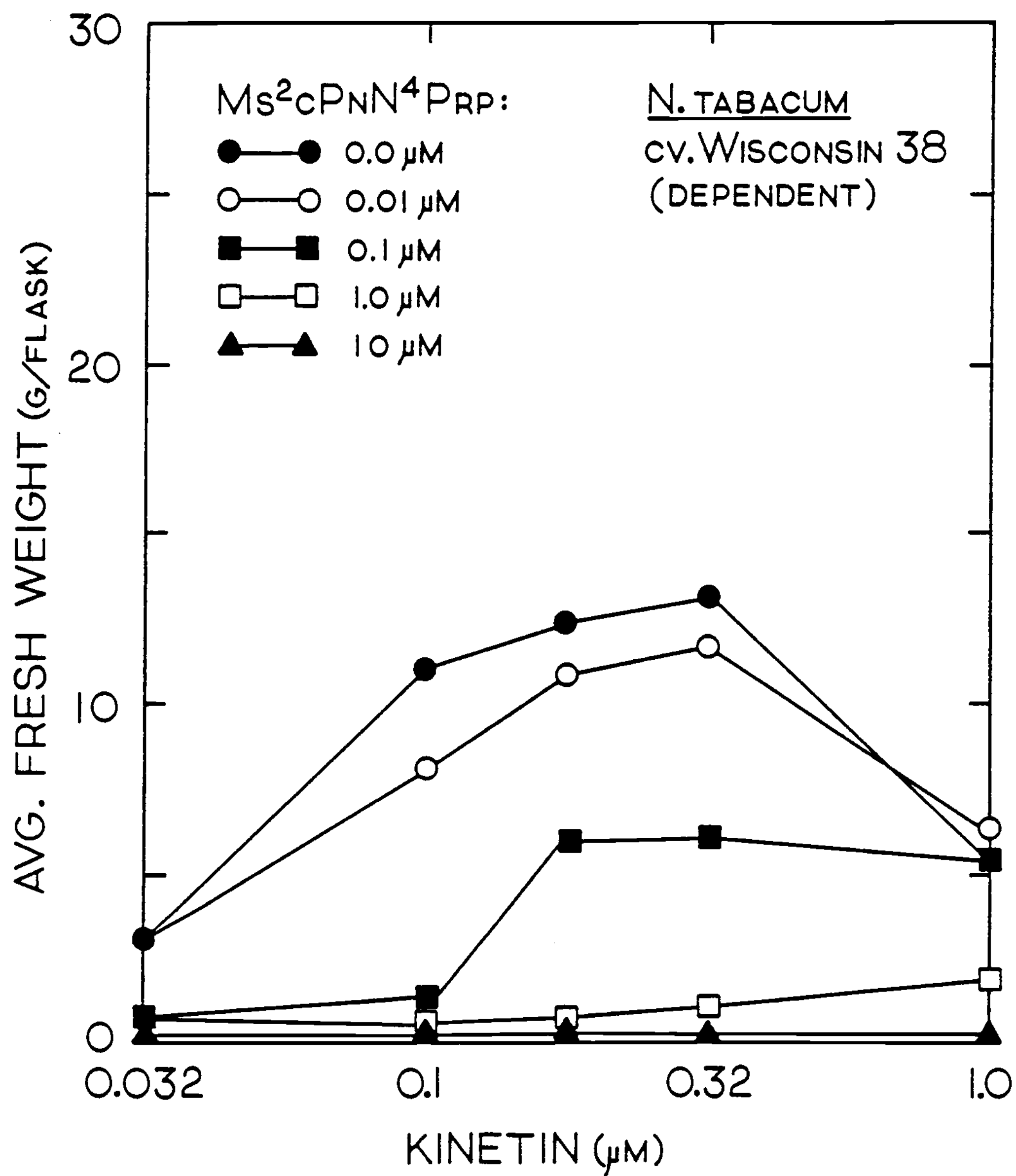


Table III. Interaction of 4-Cyclopentylamino-2-methylthiopyrrolo[2,3-d]pyrimidine (ms^2cPnN^4Prp) and Kinetin in Cytokinin-Dependent Callus Cultures of *Nicotiana tabacum* cv. Wisconsin 38.

Kinetin concentration (µM)	ms^2cPnN^4Prp I50 Values (µM)[a]
0.1	0.02
0.32	0.09
1.0	0.42

a) I_{50} values are defined as the antagonist concentrations required to obtain 50% inhibition of growth.

somewhat suppressed the growth of the Nicotiana callus tissue.

B. Biological Responses of Phaseolus and Nicotiana Callus Tissues to Nalidixic Acid and Related Antibiotics.

1. Responses of Cytokinin-Autonomous Callus Tissues.

The effects of nalidixic acid and oxolinic acid (antibiotics that inhibit subunit A of type II topoiomerases) on Phaseolus and Nicotiana callus tissues were tested and compared with the effects of novobiocin (an antibiotic that inhibits subunit B of type II topoisomerases). Antibiotic structures are shown in Figure 2.

All of the antibiotics tested inhibited growth of the cytokinin-autonomous lines of P. lunatus cv. Kingston and N. tabacum cv. Wisconsin 38 callus tissues (Figures 14 and 15, respectively). The I_{50} values are summarized in Table IV. The cytokinin-autonomous Nicotiana callus tissue was more sensitive to the inhibitory effects of the antibiotics than was the cytokinin-autonomous Phaseolus callus tissue. The I_{50} values observed in tests with the cytokinin-autonomous Nicotiana tissue ranged from 0.22 µM to 22.2 µM. The corresponding I_{50} values for cytokinin-autonomous Phaseolus callus tissue ranged from 4.9 µM to 210 µM. In both types of callus cultures, oxolinic acid was the most effective of the antibiotics in inhibiting callus growth,

Figure 14. Effects of nalidixic acid, oxolinic acid, and novobiocin on the growth of cytokinin-autonomous Phaseolus callus tissue.

Cytokinin-autonomous line of P. lunatus cv. Kingston callus tissue was grown as described in "Materials and Methods". Nalidixic acid and oxolinic acid were dissolved in 0.1 N NaOH (final pH=11) and novobiocin was dissolved in ddH_2O. Solutions were cold sterilized by Millipore filtration and aliquots were added to the autoclaved tissue culture media (0.5 ml/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) averaged ±9% of the means and did not exceed ±15%.

Figure 14

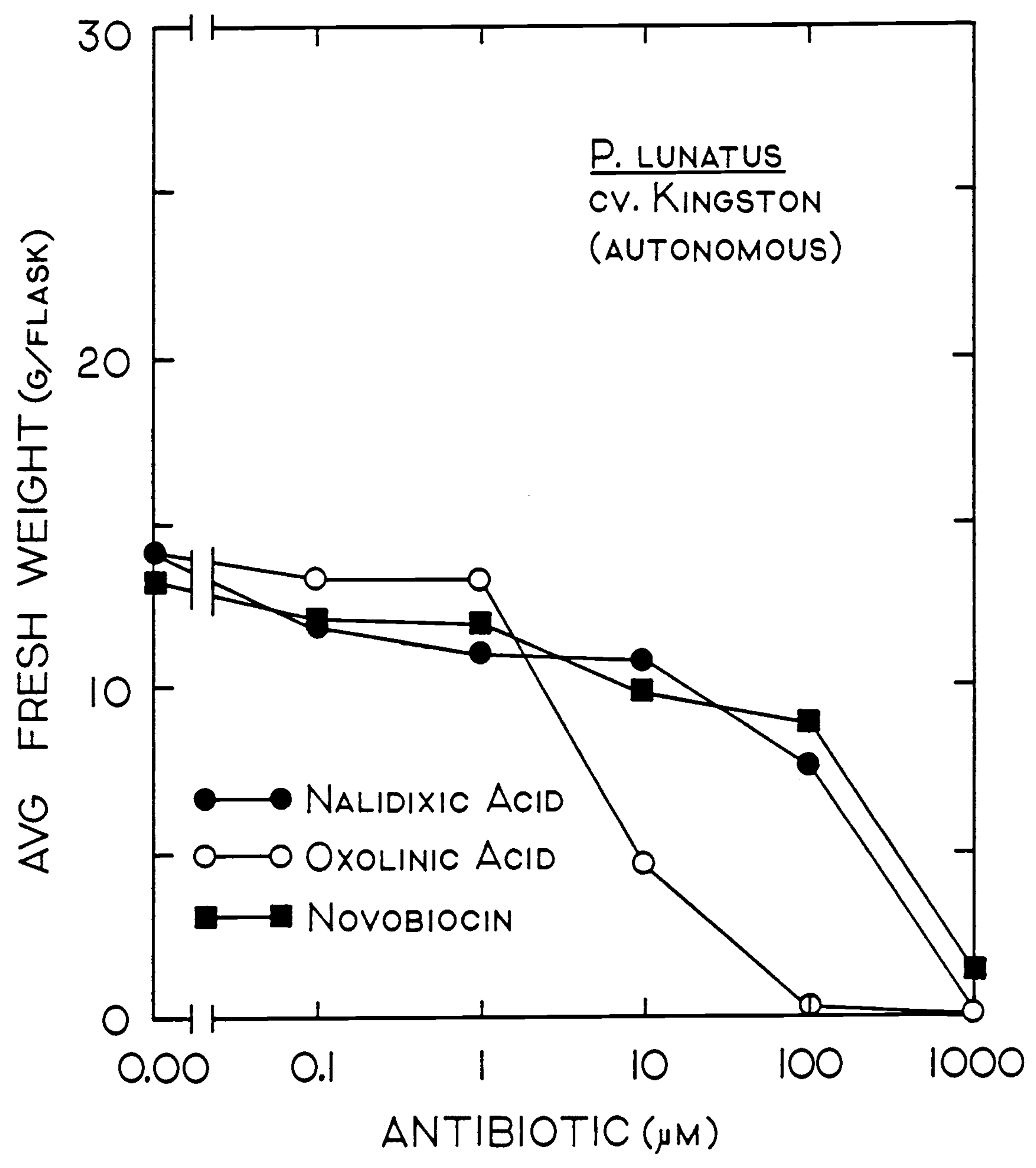


Figure 15. Effects of nalidixic acid, oxolinic acid, and novobiocin on the growth of cytokinin-autonomous *Nicotiana* callus tissue.

Cytokinin-autonomous line of *N. tabacum* cv. Wisconsin 38 callus tissues were grown as described in "Materials and Methods". Nalidixic acid and oxolinic acid were dissolved in 0.1 N NaOH (final pH=11) and novobiocin was dissolved in ddH_2O. Solutions were cold sterilized by Millipore filtration and aliquots were added to the autoclaved tissue culture media (0.5 ml/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) averaged ±12% of the mean and did not exceed ±23%.

Figure 15

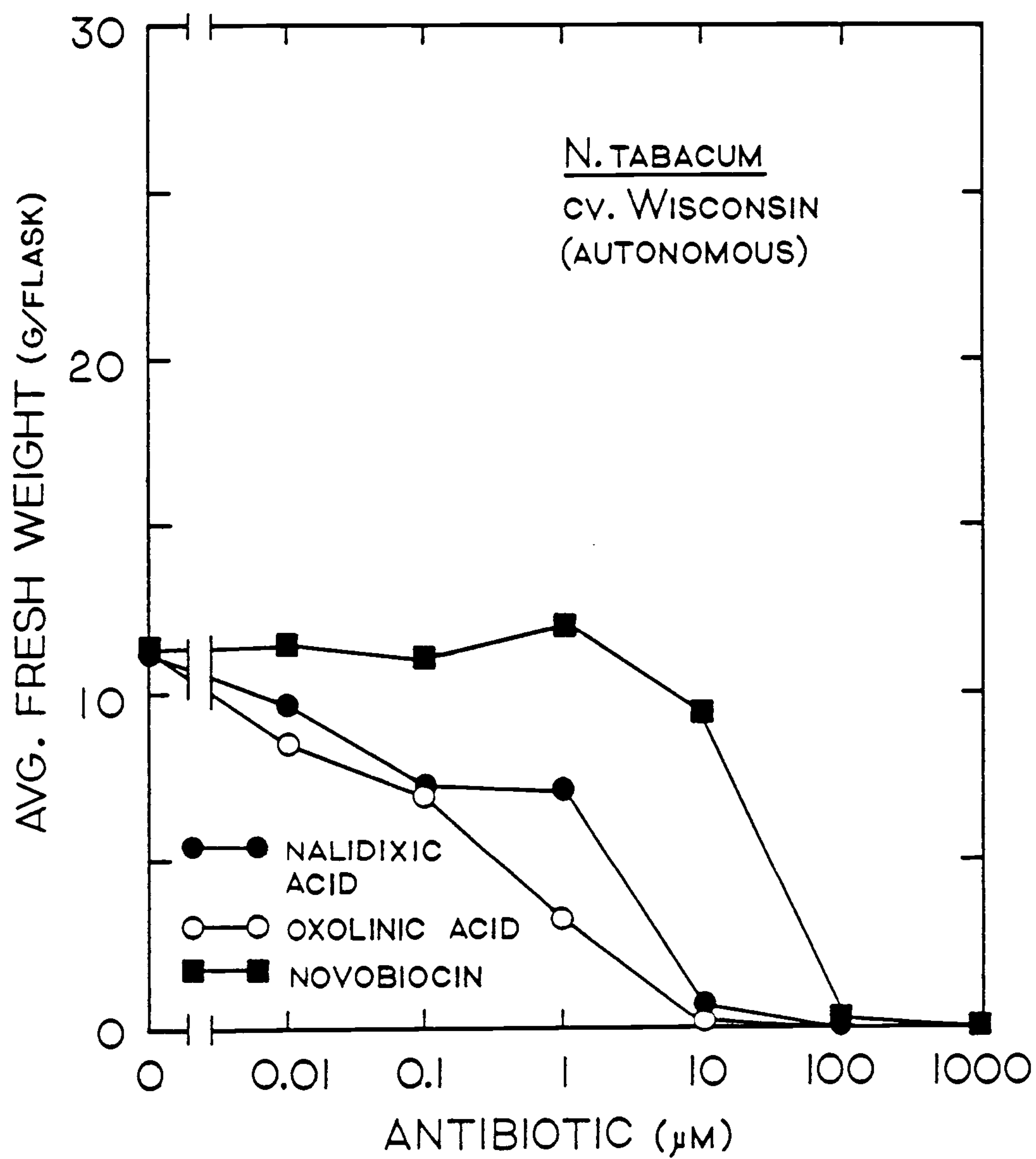


Table IV. Inhibitory Effects of Nalidixic Acid and Related Antibiotics on *Phaseolus* and *Nicotiana* Callus Cultures.

Tissue	I_{50} values (μM)[a]			
	Nalidixic Acid	Oxolinic Acid	Novobiocin	Coumarin
P. lunatus cv. Kingston (autonomous)	110	4.9	210	40
P. lunatus cv. Kingston (dependent)[b]	200	12	270	340
N. tabacum cv. Wisconsin 38 (autonomous)	1.7	0.2	22	12
N. tabacum cv. Wisconsin 38 (dependent)[c]	28	2.7	120	230

a) I_{50} Values are defined as the antibiotic concentrations required to obtain 50% inhibition of growth.

b) The cytokinin-dependent *P. lunatus* tissue was grown on media containing 5 μM kinetin.

c) The cytokinin-dependent *N. tabacum* tissue was grown on media containing 0.15 μM kinetin.

and novobiocin was the least effective of the three compounds.

The antibiotic coumermycin A_1, like novobiocin, binds to subunit B of type II topiomerases (Figure 2). Results obtained in tests of the effects of coumermycin A_1 on cytokinin-autonomous lines of P. lunatus cv. Kingston, P. vulgaris cv. Great Northern, and N. tabacum cv. Wisconsin 38 callus indicated that these tissues were not sensitive to coumermycin A_1 at concentrations less than or equal to 10 uM (Figure 16).

Novobiocin and coumermycin A_1, are both complex coumarin derivatives. Coumarin itself was more effective than either novobiocin or coumermycin A_1 in inhibiting growth of cytokinin-autonomous Phaseolus and Nicotiana callus tissues, and it was only slightly less effective than novobiocin in inhibiting the correponding cytokinin-dependent lines (Figure 17 and Table IV).

2. Responses of Cytokinin-Dependent Callus Tissues.

Cytokinin-dependent lines of P. lunatus cv. Kingston and N. tabacum cv. Wisconsin 38 callus tissues, grown on media containing 5 μM and 0.15 μM kinetin, respectively, were tested for their responses to nalidixic acid, oxolinic acid, and novobiocin. The results are shown in Figures 18 and 19. The I_{50} values observed here, together with the values obtained in tests with the corresponding cytokinin-autonomous callus lines, are summarized in Table IV.

Figure 16. Effects of coumermycin A_1 on the growth of cytokinin-autonomous *Phaseolus* and *Nicotiana* callus tissues.

Cytokinin-autonomous lines of *P. lunatus* cv. Kingston, *P. vulgaris* cv. Great Northern, and *N. tabacum* cv. Wisconsin 38 callus tissues were grown as described in "Materials and Methods". Coumermycin A_1 was dissolved in DMSO and added to the autoclaved tissue culture medium (50 µl DMSO/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) averaged ±11% of the means and did not exceed ±16%.

Figure 16

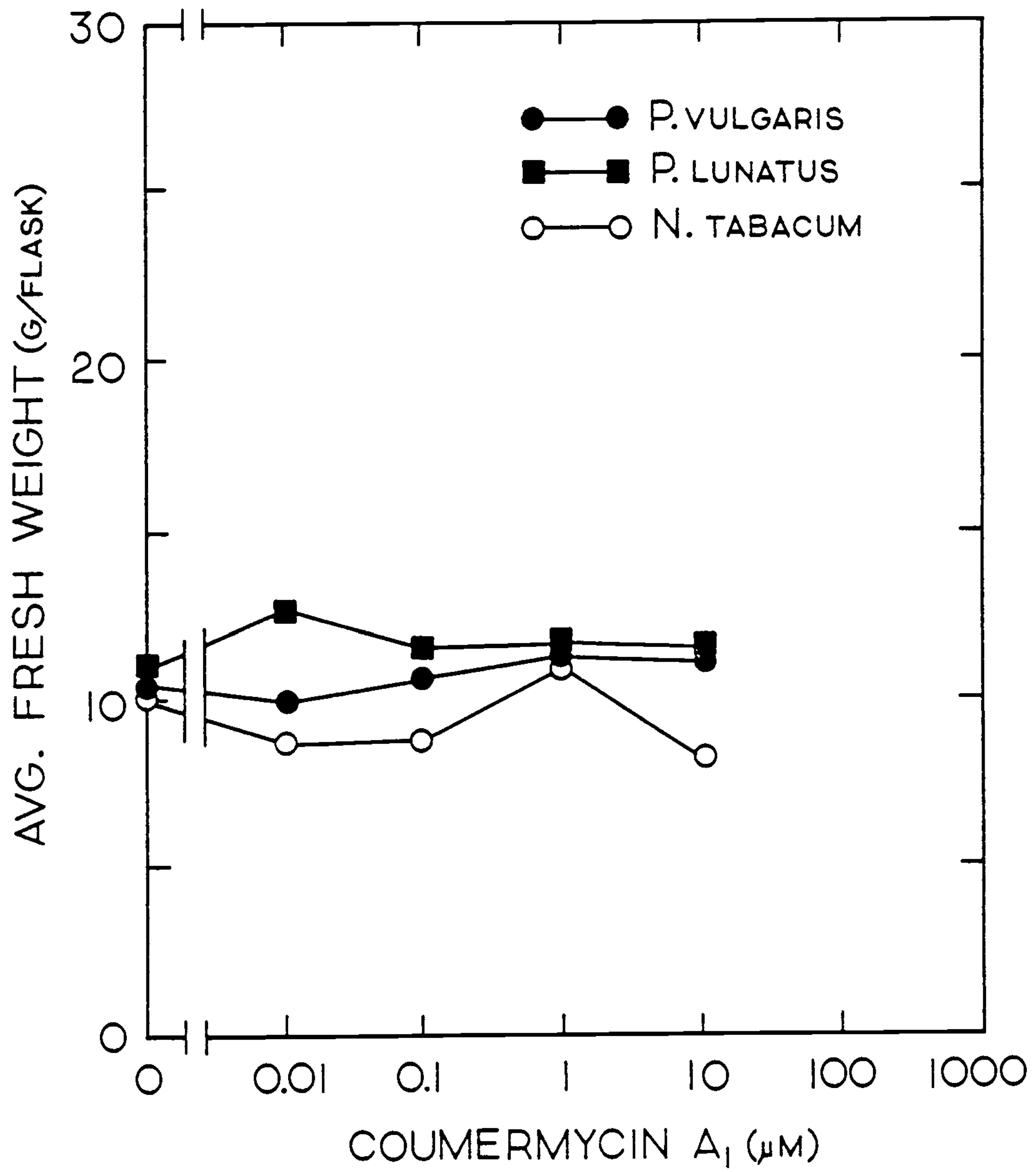


Figure 17. Effects of coumarin on the growth of cytokinin-dependent and cytokinin-autonomous *Phaseolus* and *Nicotiana* callus tissues.

Cytokinin-autonomous and cytokinin-dependent lines of *P. lunatus* cv. Kingston and *N. tabacum* cv. Wisconsin 38 callus tissues were grown as described in "Materials and Methods". Coumarin was dissolved in DMSO and added to the autoclaved tissue culture medium (50 µl DMSO/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. *Phaseolus* and cytokinin-autonomous *Nicotiana* tissues were harvested and weighed after 28 days of growth at 28°C in the dark. Cytokinin-dependent *Nicotiana* tissue was harvested after 35 days of growth. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) averaged ±7% of the means and did not exceed ±19%.

Figure 17

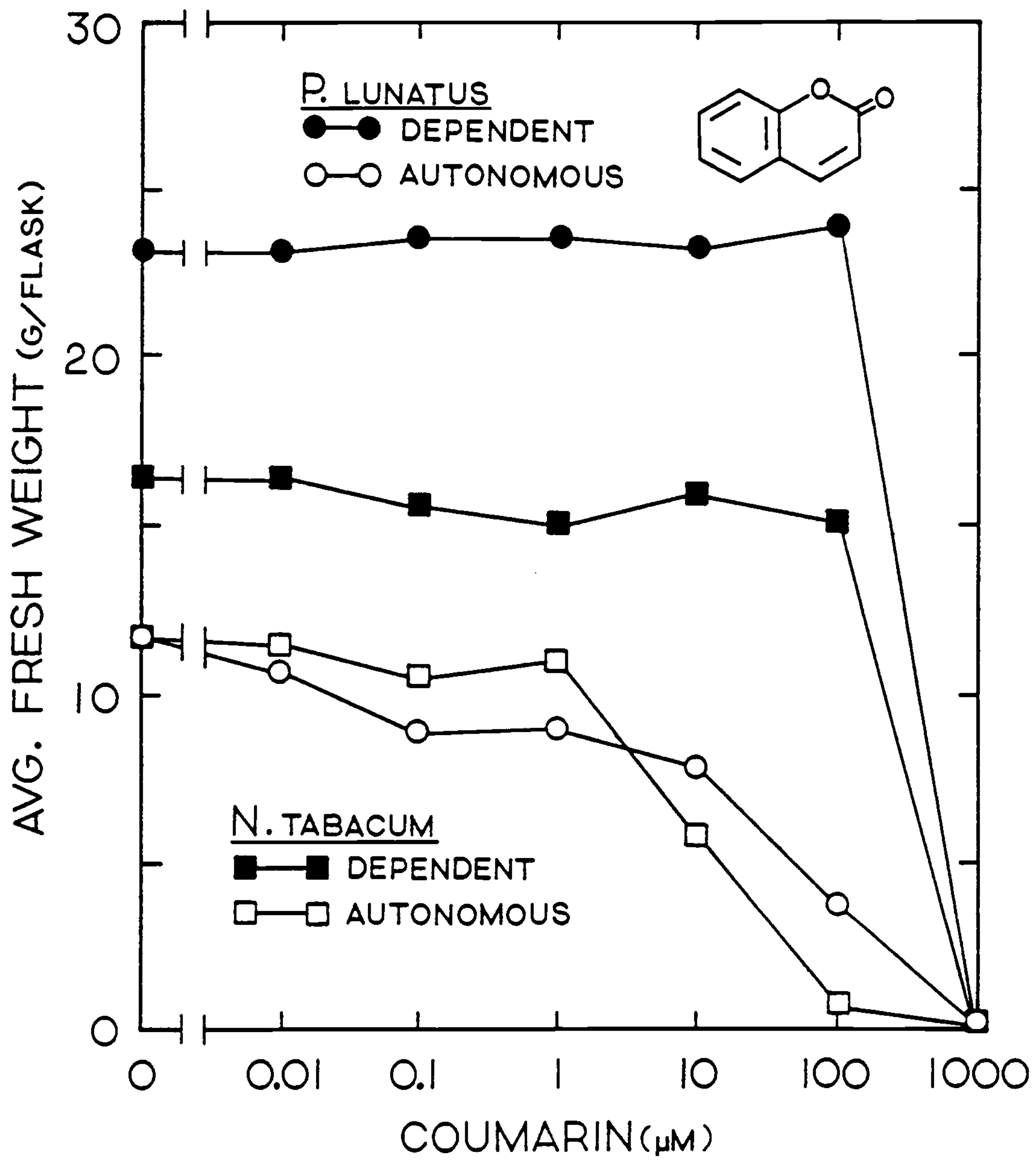


Figure 18. Effects of nalidixic acid, oxolinic acid, and novobiocin on the growth of cytokinin-dependent Phaseolus callus tissue.

The cytokinin-dependent line of P. lunatus cv. Kingston callus tissue was grown as described in "Materials and Methods". Nalidixic acid and oxolinic acid were dissolved in 0.1 N NaOH (final pH=11) and novobiocin was dissolved in ddH_2O. Solutions were cold sterilized by Millipore filtration and aliquots were added to the autoclaved tissue culture media (0.5 ml/ 50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicated flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) average ±3% for the means and did not exceed ±12%.

Figure 18

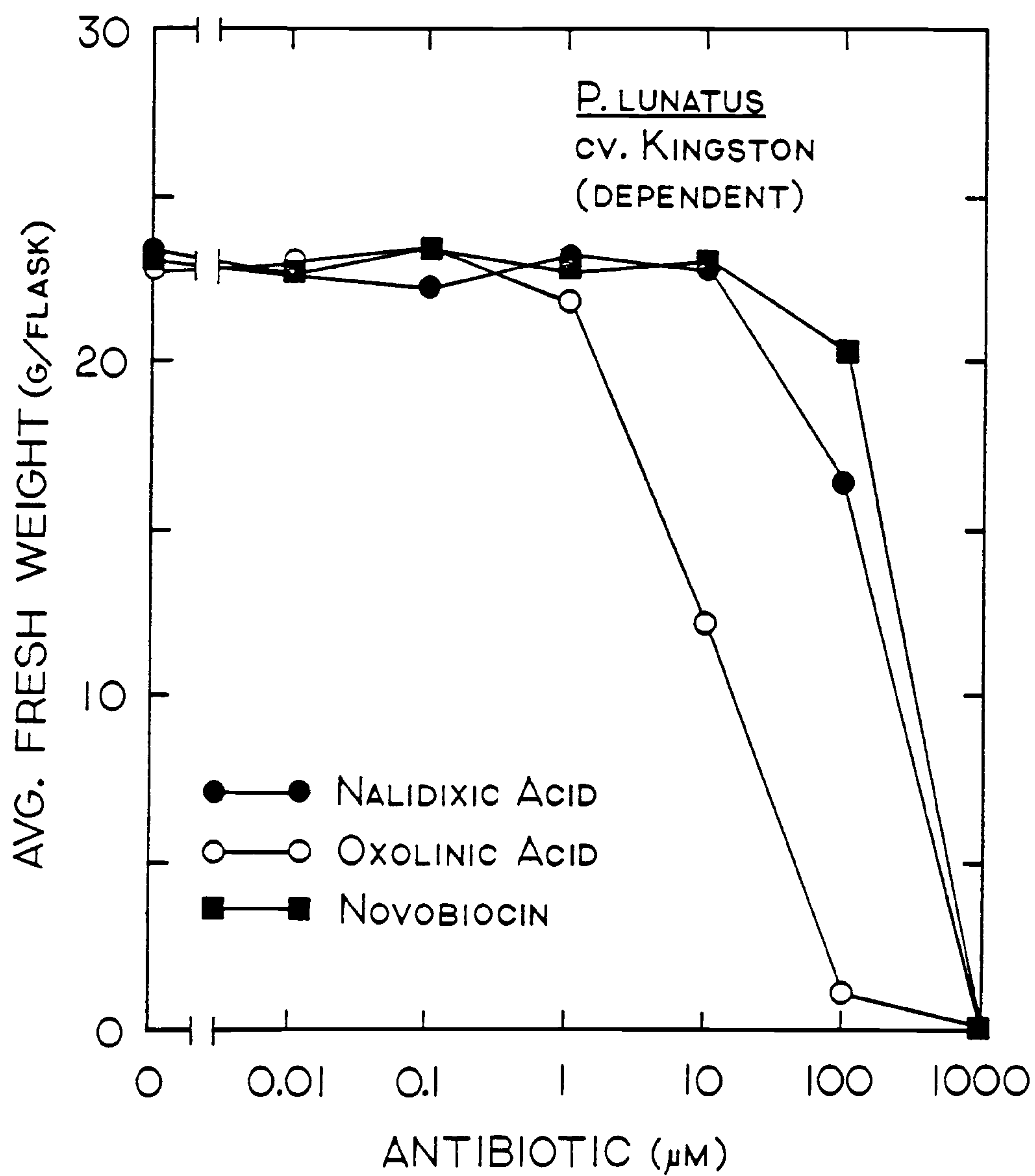
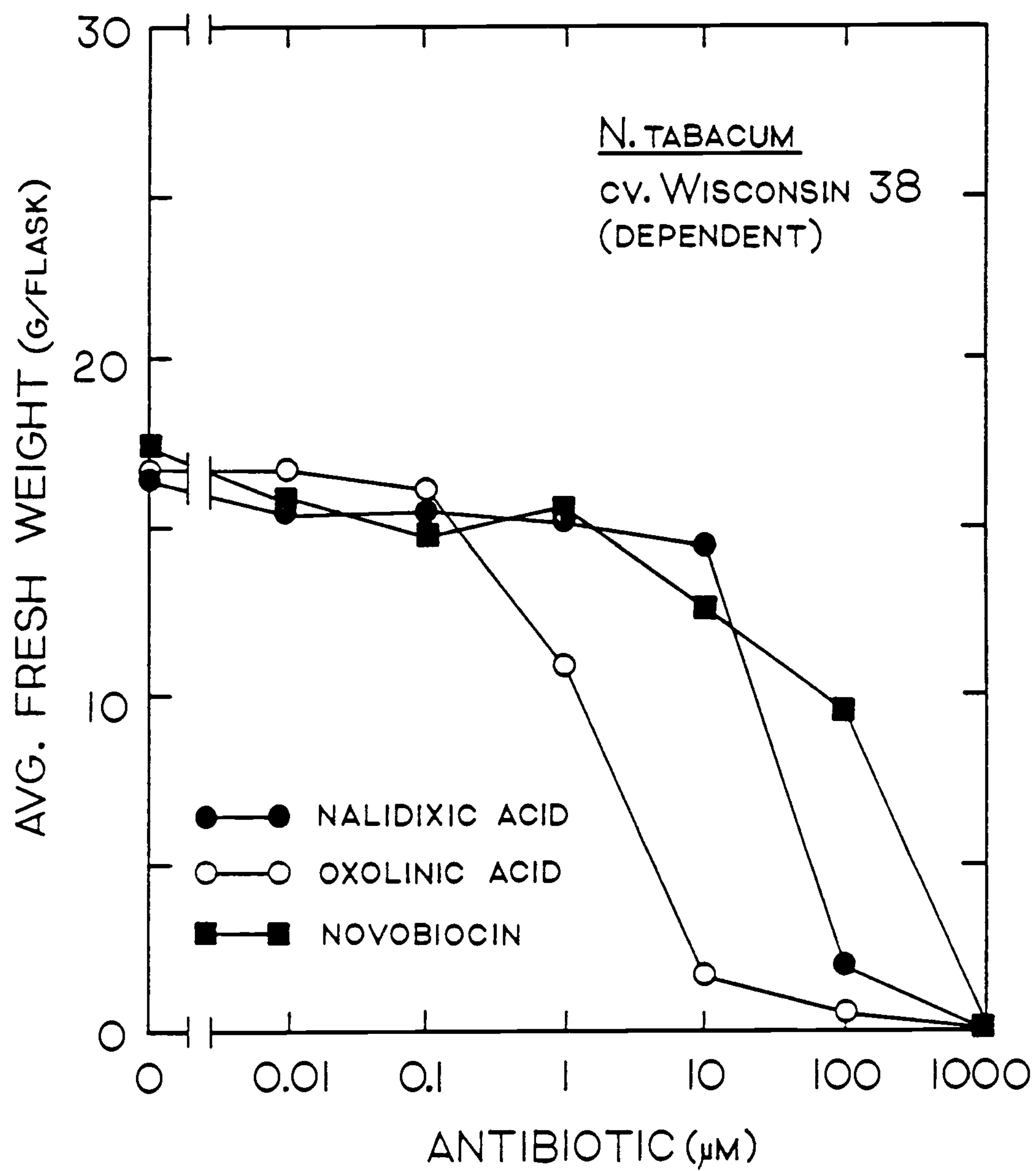


Figure 19. Effects of nalidixic acid, oxolinic acid, and novobiocin on the growth of cytokinin-dependent Nicotiana callus tissue.

The cytokinin-dependent line of N. tabacum cv. Wisconsin 38 callus tissue was grown as described in "Materials and Methods". Nalidixic acid and oxolinic acid were dissolved in 0.1 N NaOH (final pH=11) and novobiocin was dissolved in ddH_2O. Solutions were cold sterilized by Millipore filtration and aliquots were added to the autoclaved tissue culture media (0.5 ml/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 35 days of growth at 28°C in the dark. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) averaged ±6% of the means and did not exceed ±15%.

Figure 19



The cytokinin-dependent lines of Phaseolus and Nicotiana callus tissues were less sensitive to the inhibitory effects of the antibiotics than were the corresponding cytokinin-autonomous lines. This difference in sensitivity was particularly striking in the case of the Nicotiana callus tissues (Table IV). In general, however, the results obtained with the cytokinin-dependent callus lines paralleled those observed with the cytokinin-autonomous tissues. Thus, oxolinic acid was the most effective of the three antibiotics in inhibiting growth of the dependent callus lines, and the cytokinin-dependent Nicotiana callus tissue was more sensitive to all three antibiotics than was the cytokinin-dependent Phaseolus callus tissue. The antibiotic I_{50} values ranged from 2.7 µM to 117 µM in tests on the cytokinin-dependent Nicotiana callus line and from 12 µM to 271 µM in test on the cytokinin-dependent Phaseolus callus tissue.

3. Interactions of Nalidixic Acid, Oxolinic Acid and Novobiocin with Cytokinins.

The ability of N^6-(Δ^2-isopentenyl)adenine (i^6Ade) to reverse the inhibitory effects of nalidixic acid, oxolinic acid, and novobiocin on callus growth was tested using the cytokinin-dependent line of P. lunatus cv. Kingston callus tissue. The results are shown in Figures 20, 21, and 22, and I_{50} values are summarized in Table V. The I_{50} values for nalidixic acid and oxolinic acid increased

Figure 20. Interaction of nalidixic acid and N^6-(Δ^2-isopentenyl)adenine (i^6Ade) in cytokinin-dependent Phaseolus callus cultures.

The cytokinin-dependent line of P. lunatus cv. Kingston callus tissue was grown as described in "Materials and Methods". The i^6Ade was dissolved in dimethyl sulfoxide (DMSO) and added to the unautoclaved tissue culture media (0.05 ml DMSO/50 ml medium). Nalidixic acid was dissolved in 0.1 N NaOH (final pH=11) and cold sterilized by Millipore filtration. The sterilized nalidixic acid solutions were added to the autoclaved tissue culture media (0.5 ml/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) averaged ±9% of the means and did not exceed ±17% .

Figure 20

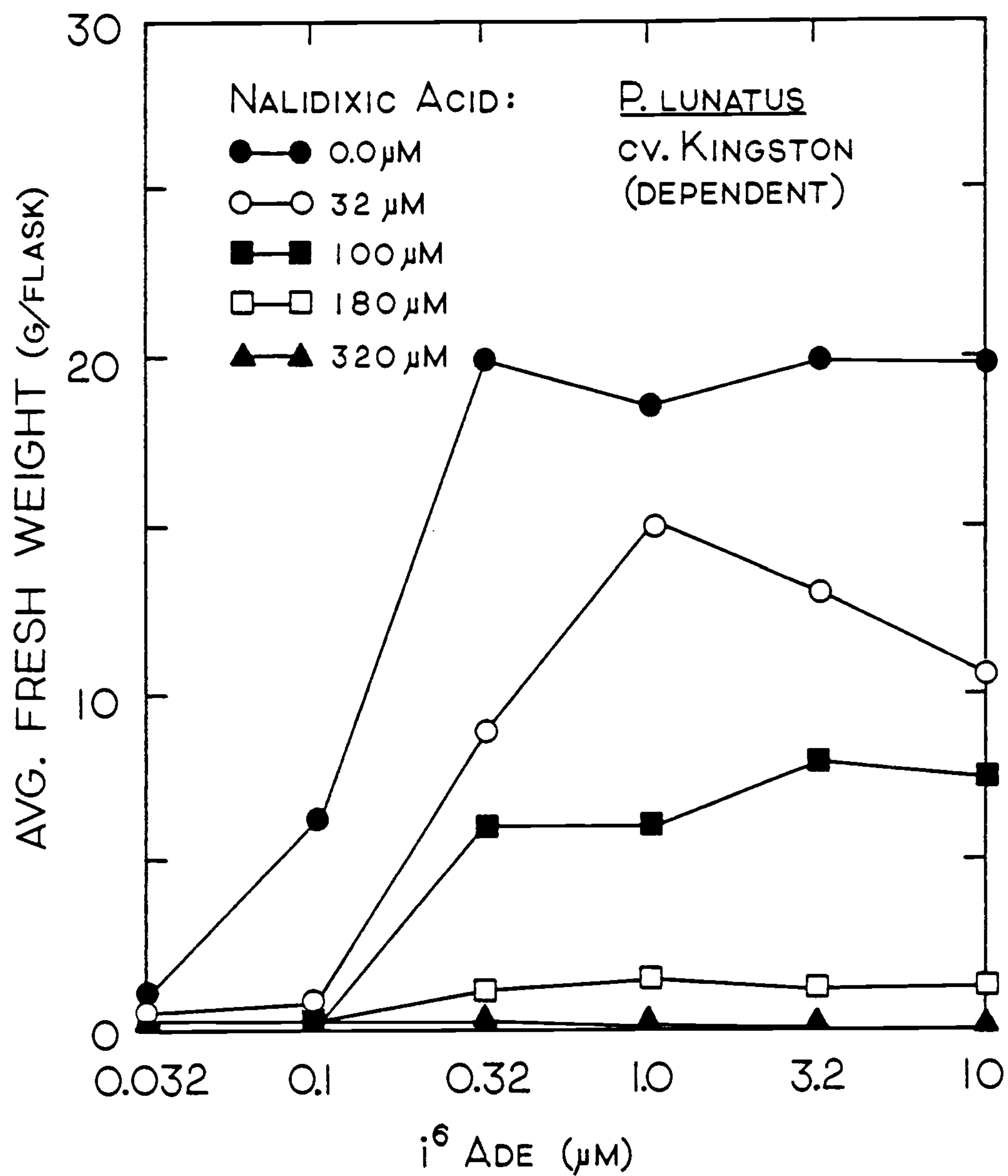


Figure 21. Interaction of oxolinic acid and N^6-(Δ^2-isopentenyl)adenine (i^6Ade) in cytokinin-dependent Phaseolus callus cultures.

The cytokinin-dependent line of P. lunatus cv. Kingston callus tissue was grown as described in "Materials and Methods". The i^6Ade was dissolved in dimethyl sulfoxide (DMSO) and added to the unautoclaved tissue culture media (0.05 ml DMSO/50 ml medium). Oxolinic acid was dissolved in 0.1 N NaOH (final pH=11) and cold sterilized by Millipore filtration. The sterilized oxolinic acid solutions were added to the autoclaved tissue culture media (0.5 ml/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) averaged ±10% of the means and did not exceed ±19% .

Figure 21

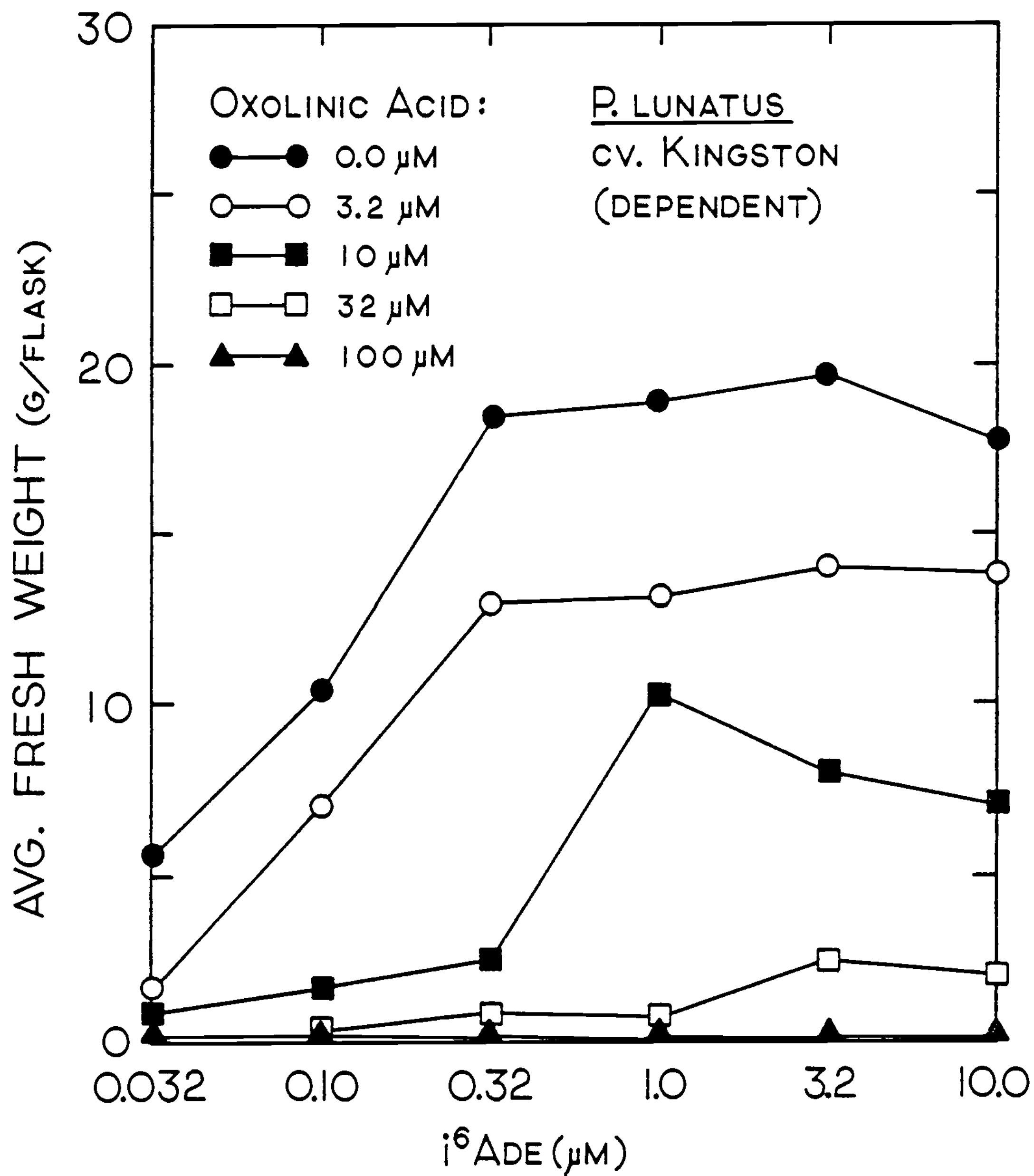


Figure 22. Interaction of novobiocin with N^6-(Δ^2-isopentenyl)adenine (i^6Ade) on cytokinin-dependent *Phaseolus* callus cultures.

The cytokinin-dependent line of *P. lunatus* cv. Kingston callus tissue was grown as described in "Materials and Methods". The i^6Ade was dissolved in dimethyl sulfoxide (DMSO) and added to the autoclaved tissue culture media (50 ul DMSO/50 ml medium). Novobiocin was dissolved in double distilled water and cold sterilized by Millipore filtration. Sterilized novobiocin solutions were added to the autoclaved tissue culture media (0.5 ml/flask). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) averaged $\pm$7% of the means and did not exceed $\pm$16%.

Figure 22

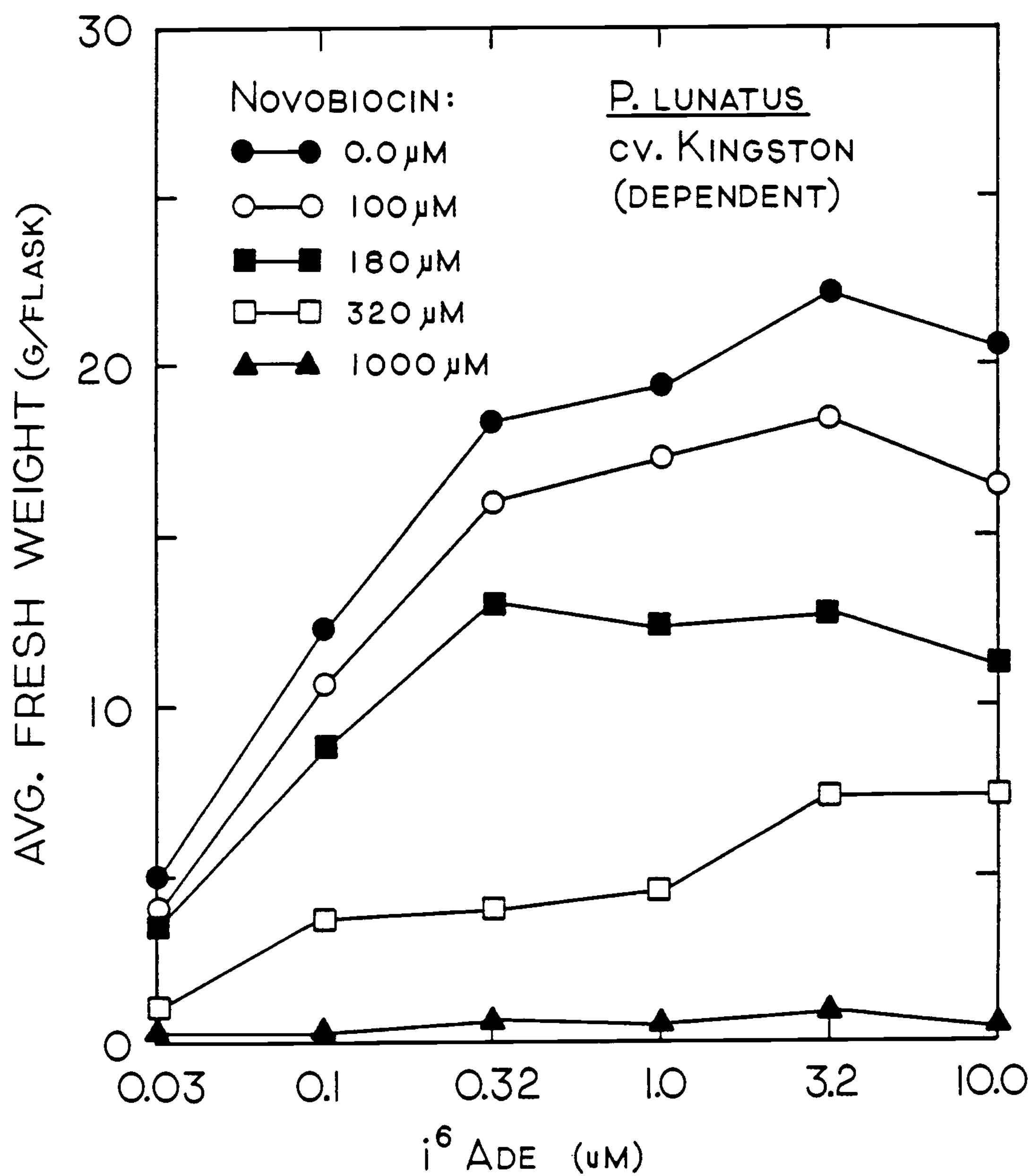


Table V. Interaction of Antibiotics and N^6-(Δ^2-Isopentenyl)adenine (i^6Ade) in Cytokinin-Dependent Cultures of <u>Phaseolus</u> <u>lunatus</u> cv. Kingston Callus Tissue.

	Antibiotic I50 Values (μM)[a]		
i^6Ade concentration (μM)	Nalidixic Acid	Oxolinic Acid	Novobiocin
0.1	33	6.0	230
0.32	49	4.7	225
1.0	67	9.0	210
3.2	70	6.7	205

a) I_{50} values are defined as the antibiotic concentrations required to obtain 50% inhibition of growth.

(approximately doubling) with increasing concentration of i^6Ade. In contrast to these results, the I_{50} values for novobiocin exhibited little change with i^6Ade concentration. However, it should be noted that the inhibitory effects of nalidixic acid and oxolinic acid were not fully reversed by the addition of supraoptimal concentrations of i^6Ade. Furthermore, in a separate experiment (data not shown), increasing the concentration of i6Ade to 100 μM, did not enhance the reversal of antibiotic inhibition.

The ability of i^6Ade to reverse the inhibitory effects of nalidixic acid was further tested using the cytokinin-autonomous lines of N. tabacum cv. Wisconsin 38 and P. lunatus cv. Kingston callus tissues (Figure 23 and 24, respectively). Although there appeared to be a slight reversal of the effects of nalidixic acid on the cytokinin-autonomous P. lunatus tissue (Table VI), exogenous i^6Ade did not significantly alleviate the inhibitory effects of the antibiotic on the cytokinin-autonomous Nicotiana callus tissue. However, low concentrations (0.01 μM) of nalidixic acid had the interesting effect of reversing the inhibition of callus growth resulting from treatments of the cytokinin-autonomous Nicotiana callus tissue with high concentrations of i^6Ade. It should be noted that a somewhat similar effect was observed with the antagonist m^3PnN^7Pzp (Figure 11). At a low concentration (0.1 μM), m^3PnN^7Pzp appeared to partially alleviate the inhibitory effects of i^6Ade on this tissue.

Figure 23. Interaction of nalidixic acid and N^6-(Δ^2-isopentenyl)adenine (i^6Ade) in cytokinin-autonomous *Nicotiana* callus cultures.

The cytokinin-autonomous line of *N. tabacum* cv. Wisconsin 38 callus tissue was grown as described in "Materials and Methods". The i^6Ade was dissolved in dimethyl sulfoxide (DMSO) and added to the unautoclaved tissue culture media (0.05 ml DMSO/50 ml medium). Nalidixic acid was dissolved in 0.1 N NaOH (final pH=11) and cold sterilized by Millipore filtration. Sterilized nalidixic acid solutions were added to the autoclaved tissue culture media (0.5 ml/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) averaged $\pm$9% of the means and did not exceed $\pm$14%.

Figure 23

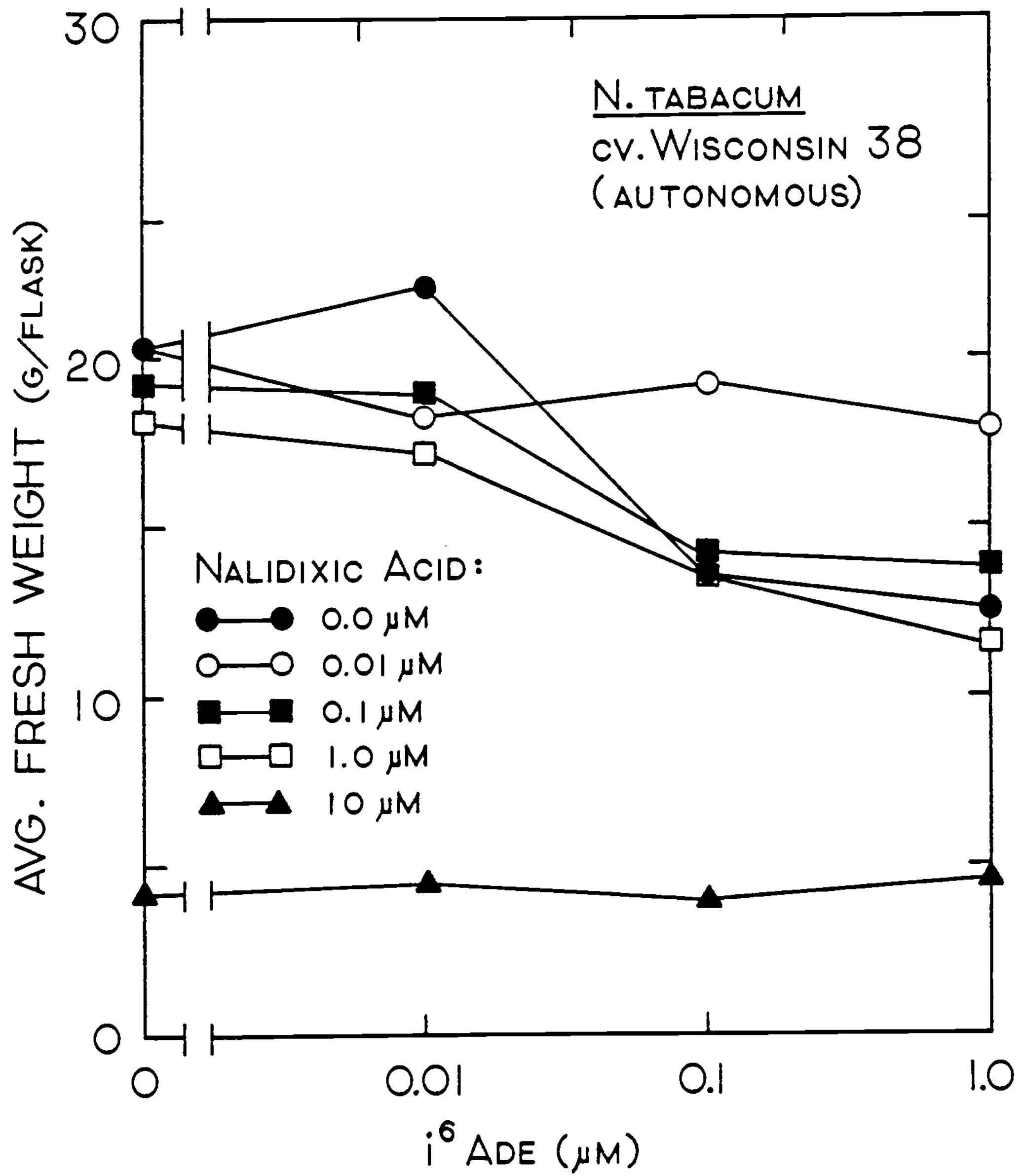


Figure 24. Interactions of nalidixic acid and N^6-(Δ^2-isopentenyl)adenine (i^6Ade) in cytokinin-autonomous *Phaseolus* callus cultures.

The cytokinin-autonomous line of *P. lunatus* cv. Kingston callus tissue was grown as described in "Materials and Methods". The i^6Ade was dissolved in dimethyl sulfoxide (DMSO) and added to the unautoclaved tissue culture media (0.05 ml DMSO/50 ml medium). Nalidixic acid was dissolved in 0.1 N NaOH (final pH=11) and cold sterilized by Millipore filtration. The sterilized nalidixic acid solutions were added to the autoclaved tissue culture media (0.5 ml/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 28 days of growth at 28°C in the dark. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) averaged ±7.5% of the means and did not exceed ±16%.

Figure 24

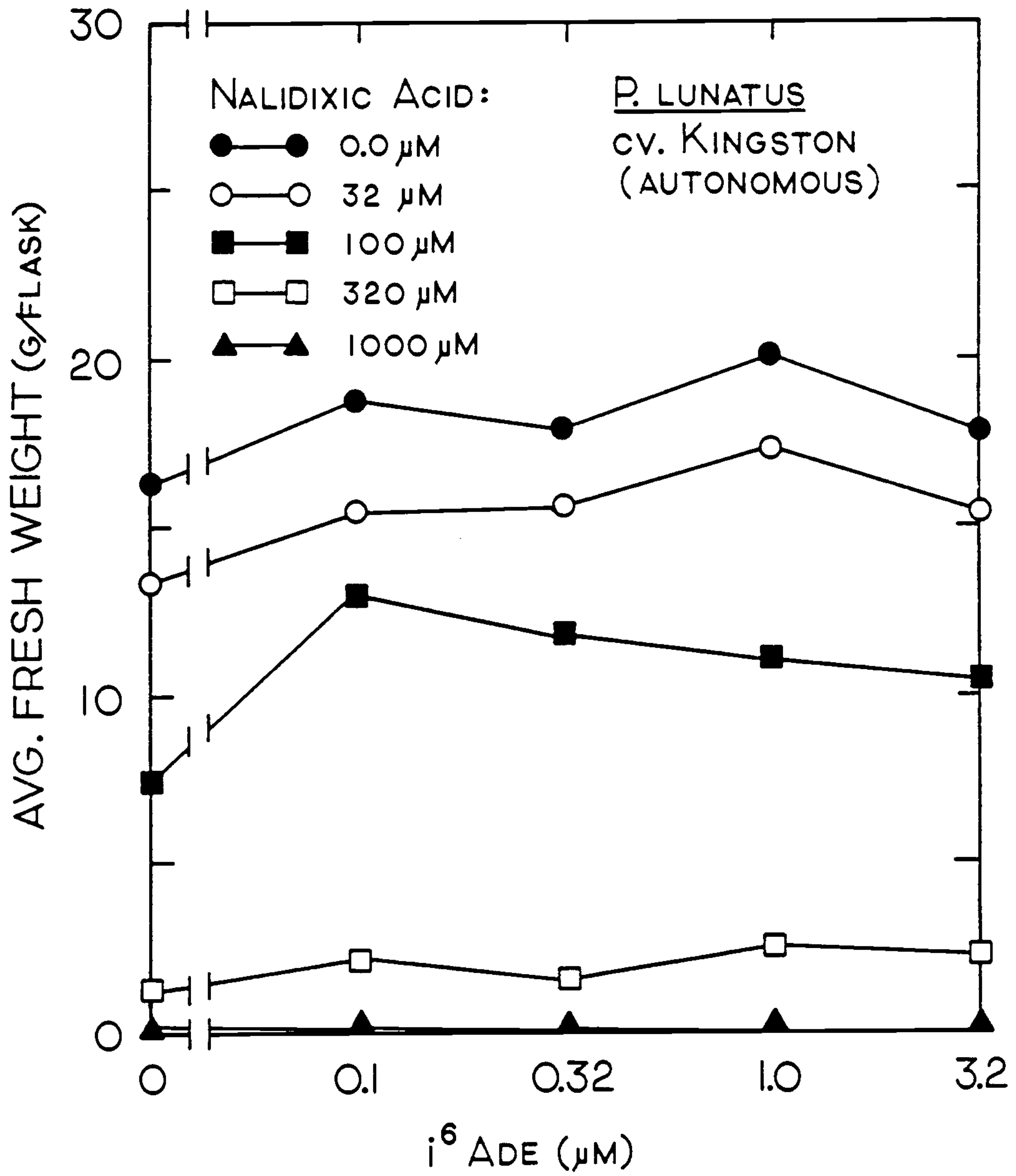


Table VI. Interaction of Nalidixic Acid and N^6-(Δ^2-Isopentenyl)Adenine (i^6Ade) in Cytokinin-Autonomous *Phaseolus* and *Nicotiana* Callus Cultures.

i^6Ade concentration (µM)	Nalidixic Acid I50 Values (µM)[a]	
	N. tabacum cv. Wisconsin 38	*P. lunatus* cv. Kingston
0.00	3.8	85
0.1	4.5	115
1.0	5.3	110

a) I_{50} values are defined as the antibiotic concentrations required to obtain 50% inhibition of growth.

Reversal of the inhibitory effects of oxolinic acid on cytokinin-dependent _N. tabacum_ cv. Wisconsin 38 callus tissue was attempted using kinetin as a source of cytokinin activity (Figure 25). In contrast to the results obtained with the cytokinin-dependent _Phaseolus_ callus tissue, the inhibitory effects of oxolinic acid on Nicotiana callus tissue did not appear to be significantly reversed by increasing concentrations of kinetin. Only a very slight increase in the I_{50} value for oxolinic acid was observed with increasing kinetin concentration (Table VII).

Figure 25. Interactions of oxolinic acid and kinetin in cytokinin-dependent Nicotiana callus cultures.

The cytokinin-dependent line of N. tabacum cv. Wisconsin 38 callus tissue was grown as described in "Materials and Methods". Kinetin was dissolved in dimethyl sulfoxide (DMSO) and added to the unautoclaved tissue culture media (0.05 ml/50 ml medium). Oxolinic acid was dissolved in 0.1 N NaOH (final pH=11) and cold sterilized by Millipore filtration. Sterilized oxolinic acid solutions were added to the autoclaved tissue culture media (0.5 ml/50 ml medium). Three callus pieces weighing about 25 mg each were planted per flask. Four replicate flasks were planted per treatment. Tissues were harvested and weighed after 35 days of growth at 28°C in the dark. The average data of two replicate experiments are plotted. The standard errors (n=4 for each experiment) averaged ±8.5% of the means and did not exceed ±18%.

Figure 25

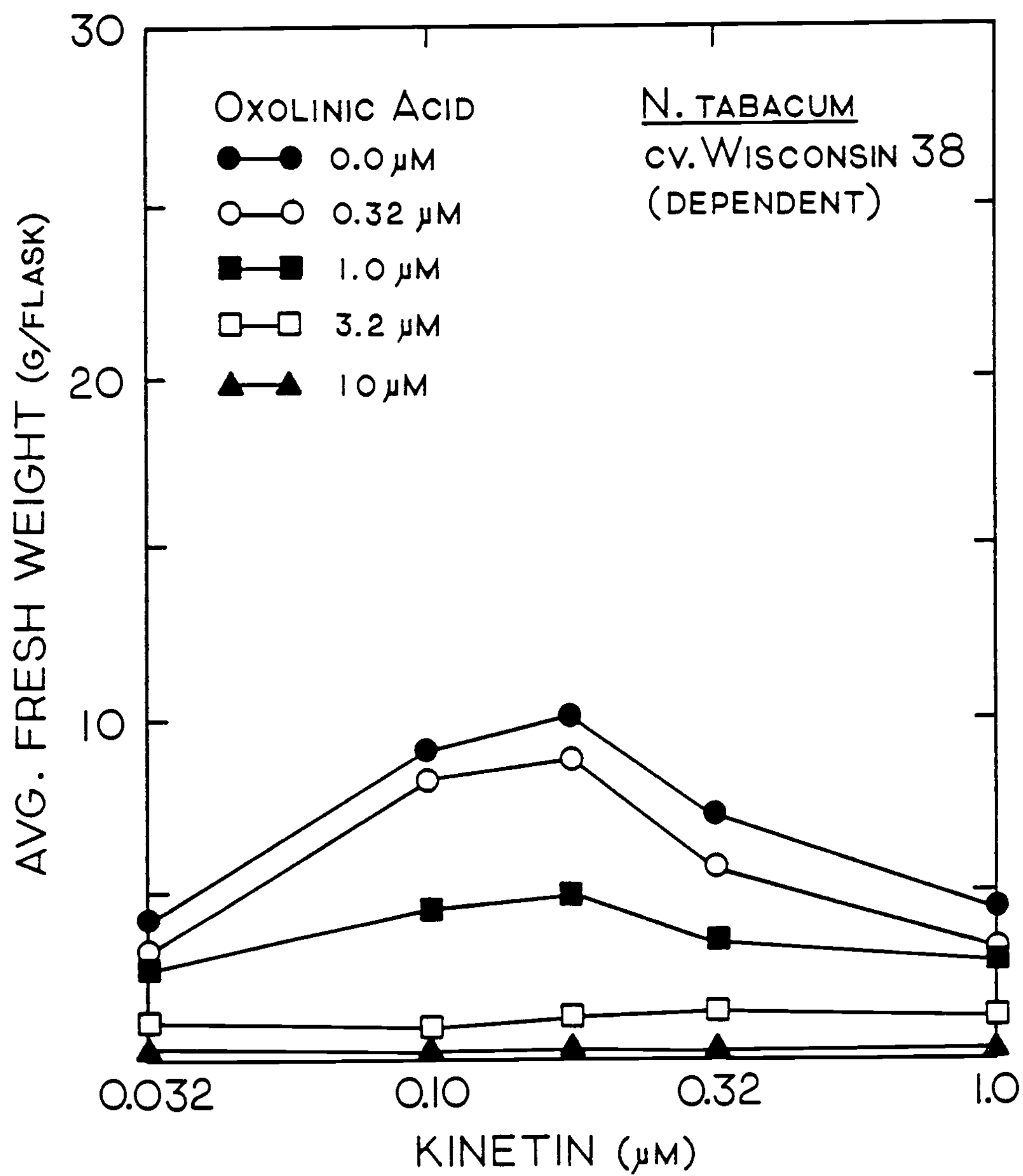


Table VII. Interaction of Oxolinic Acid and Kinetin in Cytokinin-Dependent Cultures of *Nicotiana tabacum* cv. Wisconsin 38 Callus Tissue.

Kinetin concentration (µM)	Oxolinic Acid I50 Values (µM)[a]
0.1	1.0
0.32	1.1
1.0	1.5

a) I_{50} values are defined as the antibiotic concentrations required to obtain 50% inhibition of growth.

V. DISCUSSION

A. Responses of Phaseolus and Nicotiana Callus Tissues to Cytokinin Antagonists.

The effects of five cytokinin antagonists on the growth of cytokinin-autonomous lines of P. lunatus cv. Kingston, P. vulgaris cv. Great Northern, and N. tabacum cv. Wisconsin 38 callus tissues were tested and compared in the work reported here. The antagonists tested included four pyrrolo[2,3-d]pyrimidine derivatives and a pyrazolo[4,3-d]pyrimidine derivative. The current study appears to be the first investigation in which the effects of cytokinin antagonists on tissue culture systems other than those derived from Nicotiana have been examined. The results of this study also provide the most extensive information available to date concerning the effects of antagonists on cytokinin-autonomous (as contrasted to cytokinin-dependent) lines of callus tissues.

The cytokinin-autonomous lines of Phaseolus and Nicotiana callus tissues tested here exhibited marked differences in sensitivity to the inhibitory effects of the cytokinin antagonists. Compared to the Nicotiana callus tissue, both lines of Phaseolus callus tissue were considerably less sensitive to all of the antagonists tested. The differences in antagonist sensitivity of the cytokinin-autonomous lines of Phaseolus and Nicotiana callus tissues parallel differences in cytokinin sensitivity

exhibited by cytokinin-dependent lines of callus tissues derived from the same genotypes. The cytokinin concentrations required for optimal growth of cytokinin-dependent *Phaseolus* callus tissues are typically several fold higher than those required for optimal growth of cytokinin-dependent *Nicotiana* callus tissue (Skoog and Armstrong, 1970; Mok *et al.*, 1978). Similarly, the antagonist concentrations required to achieve 50% inhibition of callus growth (I_{50} values) in cultures of the cytokinin-autonomous *Phaseolus* callus tissue were several fold higher than those required to produce 50% inhibition of the cytokinin-autonomous *Nicotiana* callus tissue.

Differences in antagonist structure/activity relationships are evident in comparing the effects of the five antagonists on the three callus tissues used in this investigation. Although m^3PnN^7Pzp was the most potent of the antagonists in all three systems, the pyrrolopyrimidine derivatives differed in their relative activities depending on the callus tissue tested. Thus, ms^2cPnN^4Prp was the most active of the pyrrolopyrimidine derivatives in test using the *P. vulgaris* and *N. tabacum* callus tissue but ms^2HxN^4Prp was the most effective of the pyrrolopyrimidine antagonists in tests using the P. *lunatus* callus tissue.

The cytokinin-dependent and cytokinin-autonomous lines of *N. tabacum* cv. Wisconsin callus tissue used in this investigation appear to differ in their resistance to the inhibitory effects of antagonists. The cytokinin-dependent

Nicotiana callus tissue grown on suboptimal or optimal kinetin concentrations exhibited I_{50} values for the antagonist ms^2cPn^4Prp in the concentration range of 0.02 µM to 0.4 µM (Table III). In a similar experiment using the cytokinin-autonomous line of *Nicotiana* callus tissue, the I_{50} value for ms^2cPnN^4Prp was 1.2 µM (Table I). Skoog and co-workers (Skoog *et al*., 1973) have reported that cytokinin-dependent *N. tabacum* cv. Wisconsin 38 callus tissue grown on a suboptimal N^6-(Δ^2-isopentenyl)adenine (i^6Ade) concentration (0.003 µM) was completely inhibited at 0.05 µM ms^2cPnN^4Prp, a value which is in good agreement with the results obtained here. In their tests with the cytokinin-dependent Wisconsin 38 tissue, Skoog and co-workers (Skoog *et al*..1973; Skoog *et al*., 1975) also found, in separate experiments, that the pyrrolopyrimidine antagonist ms^2cPnN^4Prp was effective in inhibiting callus growth at concentrations that were several fold lower than those required to obtain the same inhibition with the pyrazolopyrimidine derivative m^3PnN^7Pzp. On the basis of the results obtained in the present study, the cytokinin-autonomous line of *N. tabacum* callus tissue appears to reverse this order of sensitivity. The I_{50} values for ms^2cPnN^4Prp and m^3PnN^7Pzp were 1.2 µM and 0.08 µM, respectively, in tests with the cytokinin-autonomous line of *Nicotiana* callus tissue

The effects of cytokinin antagonists on cytokinin-autonomous callus cultures have not previously been examined

in any detail. Most studies of cytokinin antagonists have utilized cytokinin-dependent Nicotiana callus cultures as a test system (Skoog et al., 1973; Skoog et al., 1975; Iwamura et al., 1975; Iwamura et al., 1979). Although Skoog et al. (1973) demonstrated that the antagonist 3-methyl-7-(3-methylbutylamino)pyrazolo[4,3-d]pyrimidine inhibited growth of the cytokinin-autonomous line of N.tabacum cv. Wisconsin 38 callus tissue used in the present study, it is not clear from the published data whether this inhibition could be reversed by treatment with exogenous cytokinins. The results obtained here indicate that interactions of antagonists and exogenously supplied cytokinins in cytokinin-autonomous cultures of N. tabacum cv. Wisconsin 38 are complicated by the inhibitory effects of the cytokinins themselves. Nevertheless, exogenous i^6Ade was effective in inducing at least partial reversals of the inhibitory effects of two cytokinin antagonists (ms^2PnN^4Prp and m^3PnN^7Pzp) on this callus line. Reversals were evidenced by increases in the I_{50} values for antagonist inhibitions.

The limited supplies of antagonist available to this project have not permitted extensive investigation of the interactions of antagonists and cytokinins in Phaseolus callus cultures. However, the inhibitory effects of the antagonist m^3PnN^7Pzp on cytokinin-autonomous callus tissue of P. lunatus cv. Kingston were partially reversed by exogenously supplied i^6Ade.

The interactions of antagonists and exogenous cytokinins appear to be more complex in the cytokinin-autonomous tissues used here than has been observed in tests with the cytokinin-dependent line of N. tabacum cv. Wisconsin 38 callus tissue. However, the published reports concerning the effects of antagonists on the cytokinin-dependent Wiconsin 38 callus tissue (e.g. Skoog et al., 1975) indicate that even with this tissue there is a relatively narrow concentration range where complete reversal of antagonist inhibition is acheived. Both antagonists and cytokinins may interact with several sites, which have somewhat different specificities and properties.

B. Reponses of Phaseolus and Nicotiana Callus Tissues to Nalidixic Acid and Related Antibiotics.

The effects of nalidixic acid and related antibiotics on callus tissues of P. lunatus cv. Kingston and N. tabacum cv. Wisconsin 38 were examined in the work reported here and compared with the effects of cytokinin antagonists. This investigation was motivated by the report of Quesney-Huneeus and associates (1980) that the inhibitory effects of nalidixic acid on DNA synthesis in Chinese hamster kidney cells were reversed by treatment with i^6Ade. The effects of nalidixic acid on plant systems have received only limited attention to date (Ciarrocchi et al., 1985; Heinhorst et al., 1985). The present study has examined

these effects and attempted to determine whether interactions of cytokinins and nalidixic acid similar to those observed in animal cell cultures could be detected in plant cell cultures.

All of the antibiotics tested (nalidixic acid, oxolinic acid, and novobiocin) inhibited growth of both cytokinin-autonomous and cytokinin-dependent lines of _P_. _lunatus_ cv. Kingston and _N_. _tabacum_ cv. Wisconsin 38 callus tissues. The _Nicotiana_ callus tissues were more sensitive to the inhibitory effects of these antibiotics than were the _Phaseolus_ callus tissues, and the cytokinin-autonomous lines of callus tissues appeared to be more sensitive to the antibiotic treatments than were the corresponding cytokinin-dependent lines. In all of the callus systems tested here, oxolinic acid was the most effective inhibitor of callus growth. Nalidixic acid was somewhat less effective than oxolinic acid, and novobiocin was the least active of the three antibiotics.

Nalidixic acid and oxolinic acid are structurally related (Figure 2) and both antibiotics are known to inhibit type II topoisomerases by binding to the A subunit (Gellert, 1981). Novobiocin and the structurally related compound coumermycin A_1 inhibit type II topoisomerases by binding to the B subunit (Gellert, 1981). However, there is no certainty that the inhibitory effects of these antibiotics on callus growth are due to effects on type II topoisomerases. In fact, Ciarrocchi _et al_. (1985) suggested

that novobiocin and nalidixic acid inhibit carrot (Daucus) cell suspension cultures by interfering with ATP synthesis.

The concentrations of nalidixic acid required to inhibit the growth of the *P. lunatus* callus tissues used here were similar to those reported by Ciarrocchi *et al.* (1985) to inhibit carrot cell suspension cultures. Nalidixic acid at 0.3 mM completely inhibited the carrot cultures (Ciarrocchi *et al.*, 1985), and the I_{50} values for nalidixic acid inhibition of *P. lunatus* callus cultures were in the range of 0.1 mM to 0.2 mM. The concentrations of nalidixic acid required to inhibit growth of the *Nicotiana* callus tissue (I_{50} values of 0.002 µM to 0.03 µM) were considerably less than those required to inhibit the *Phaseolus* callus tissues, but they were in the range of concentrations reported by Heinhorst *et al* (1985) to inhibit plastid and nuclear DNA synthesis in *Nicotiana* suspension cultures.

The mechanisms by which nalidixic acid, oxolinic cid, and novobiocin inhibit growth of the callus tissues used here are not certain, but the present study has provided some evidence of specific interactions between cytokinins and the antibiotics nalidixic acid and oxolinic acid. The inhibitory effects of nalidixic acid and oxolinic acid on a cytokinin-dependent line of *P. lunatus* cv. Kingston callus tissue were partially reversed by the addition of exogenous i^6Ade, as evidenced by the effect of i^6Ade in increasing the I_{50} values for nalidixic acid and oxolinic acid. In

contrast to the results with these two antibiotics, the I_{50} values for novobiocin did not appear to be affected by increases in the concentration of exogenous i6Ade supplied to the cytokinin-dependent P. lunatus callus tissue. It is of interest in this connection that Ciarrocchi et al. (1985) observed that the inhibitory effects of novobiocin on carrot cell suspension cultures appeared to occur by a somewhat different mechanism than the inhibition produced by nalidixic acid.

Additional evidence of specificity in the interactions between nalidixic acid and i^6Ade is provided by the results obtained with in the cytokinin-autonomous line of Nicotiana callus tissue. In this system, low concentrations of nalidixic acid appeared to alleviate the inhibitory effects exerted by high concentrations of i^6Ade on Nicotiana callus tissue. This effect of nalidixic acid treatment resembles the effects of low concentration of the antagonist m^3PnN^7Pzp in the same system.

The ability of exogenous i^6Ade to partially reverse the inhibitory effects of nalidixic acid and oxolinic acid on cytokinin-dependent line of P. lunatus callus tissue is interesting, particularly in view of the results with animal cell cultures (Quesney-Huneeus et al., 1980). However it should be noted that complete reversal of the inhibitory effects of these antibiotics was not achieved in the present study. Furthermore, in tests using the cytokinin-dependent line of N. tabacum cv. Wisconsin 38 callus tissue, the

inhibitory effects of oxolinic acid appeared to be relatively unaffected by increasing concentrations of kinetin. This result suggests that not all cytokinins may be equally effective in alleviating the inhibitory effects of the antibiotics or that these effects are tissue specific. Further work will be needed to clarify this and other points concerning the nature of the interaction of cytokinins with these antibiotics, but the results reported here do raise the possiblity that some part of the effects of nalidixic acid and oxolinic acid on plant tissues may be due to effects on processes specifically involving cytokinin metabolism or function.

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