Hypervalent Iodine Compounds as Potent Antibacterial Agents against Ice Nucleation Active (INA) *Pseudomonas syringae*

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Twenty-three hypervalent iodine compounds belonging to aryliodonium salts, **1**, aryliodonium ylides, **2**, and (diacyloxyiodo)arenes, **3**, were tested for their antibacterial activities against ice nucleation active (INA) *Pseudomonas syringae*, and the MIC and EC_{50} values were determined. All of the compounds examined caused a dose-dependent decrease in bacterial growth rates. Aryliodonium salts, especially those with electron-withdrawing groups, exhibit higher antibacterial activities with MIC = 8−16 ppm, whereas the nature of the anion does not seem to affect the activities of the diaryliodonium salts.

Keywords: *Antibacterial activity; hypervalent iodine; Pseudomonas syringae; ice nucleation active (INA) bacteria*

INTRODUCTION

Pseudomonas syringae is an important bacterial plant pathogen, which can reduce the productivity of many plant species including tree fruits such as citrus, pear, and almonds in several ways. Pathovars of these species cause a number of diseases including fruit spots, blossom and twig blast, and bacterial canker. This bacterial species can also cause frost injury to frost-sensitive plants by contributing ice nuclei active at temperatures above −5 °C (*1*−*3*). It has been shown that the frost sensitivity exhibited in the field by several plants is attributed to the presence of epiphytic bacterial ice nuclei that limit tissue supercooling to temperatures ≥−4 °C (*1*, *4*, *5*). These ice nucleation active (INA) bacteria are mainly strains of *P. syringae* and *Erwinia herbicola* (*2*). Frost injury to sensitive crop plants was shown to be a function of both the epiphytic population of INA bacteria on plant surfaces and the activity of bacterial cells to initiate ice formation (*2*, *6*).

Field application with a variety of chemical or biological agents that reduce the number and/or the ice nucleation activity of bacteria reduce also frost damage to frost-sensitive crops (*5*, *7*−*10*). Only a few chemicals are available for the control of bacterial diseases of plants, and among them sprays of copper compounds, mostly $Cu(OH)_2$, are mainly used for this purpose (*10*−*14*). Copper-containing compounds were the first chemicals used for plant disease control and, although their efficacy for bacterial disease control is low and variable among locations, they are still considered to be the main chemicals for the control of pathogenic bacteria (*3*, *10*−*12*, *14*, *15*). Although a number of metal ions, organic compounds, and antibiotics are toxic to INA bacteria, only copper compounds and streptomycin are registered for control of these species on crop plants (*10*).

Resistance to copper has been observed among phytopathogenic and saprophytic bacteria. Many strains of *P. syringae* isolated from tree fruits in California and Greece exhibit high levels of tolerance to copper in culture (*3*, *5*, *13*, *16*−*18*). Control of copper-tolerant strains was poor when $Cu(OH)_2$ or Bordeaux mixtures were applied either as eradicants (*3*, *11*, *19*) or as protectants (*3*, *15*, *18*) at the registered or higher rates. For this reason the need for new antibacterial agents for plant pathogenic bacteria is evident. Recent studies have shown the antibacterial effect of secondary metabolites derived from Labiatae aromatic plants against INA bacteria (*20*). More specifically, minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values have been estimated in the range of 0.2−2 and 0.4−2 mg/mL, respectively, for either the essential oils or their main constituents of these aromatic plants; the highest antibacterial activity was assigned in the fraction of oxygenated constituents, especially alcoholic and phenolic terpenoids (*20*, *21*). Consequently, the antibacterial effect of Labiatae plants may have applications in agriculture as a frost-control method for sensitive crops (*20*).

Organic compounds of hypervalent iodine comprise a very interesting class of compounds with a long history in organic chemistry. Study of the chemistry of these compounds has undergone a renaissance during the past two decades, which culminated with the publication of several review articles and two books (*22*, *23*). Hypervalent iodine compounds, besides the versatility and diversity in their chemical behavior, exhibit biocidal properties against a wide spectrum of microorganisms, such as bacteria, fungi, and yeast. Moreover, they are environmentally safe and for this reason tend to substitute other reagents with similar chemical properties, for example, (diacyloxyiodo)arenes for lead, thallium, and other heavy metal acetates.

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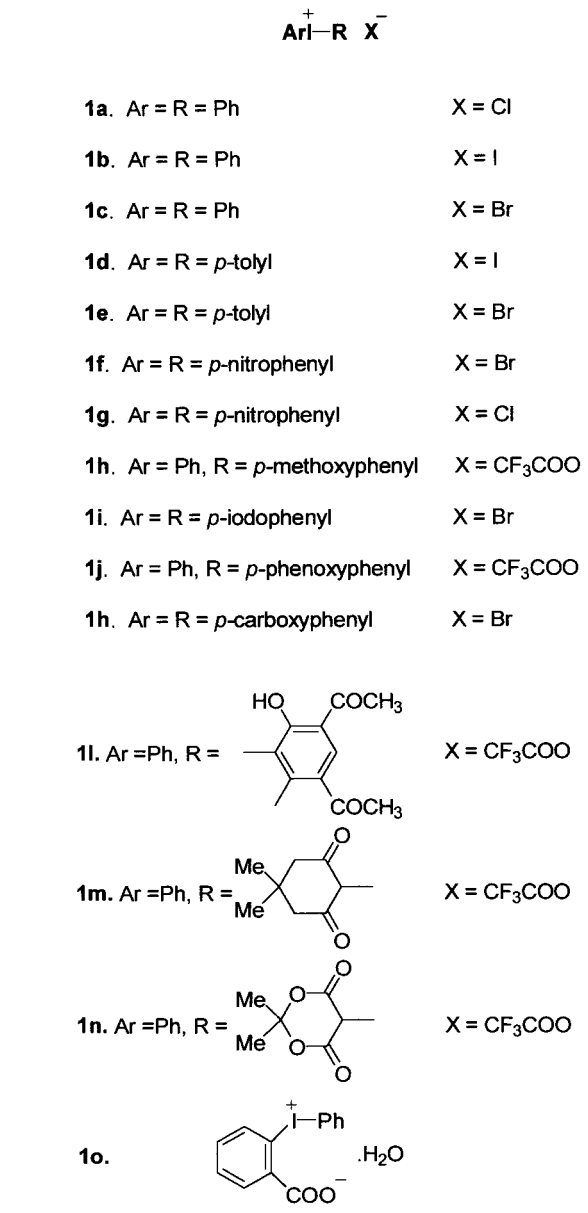

Figure 1. Structures of aryliodonium salts (**1a**−**n**) and the inner salt (**1o**).

Our continuing interest in the chemistry of iodonium compounds (*24*), combined with the reported biocidal properties of this class of compounds (*22*), prompted us to investigate their possible use for the control of INA *P. syringae*. A general survey on the antibacterial activity of some classes of hypervalent iodonium compounds has been conducted, and the results are presented in this paper.

MATERIALS AND METHODS

Hypervalent Iodine Compounds. The 23 iodonium compounds used for this study can be divided into three classes: aryliodonium salts, **1**, aryliodonium ylides, **2**, and (diacetoxyiodo)arenes, **3**, representing the three most important classes of hypervalent iodine compounds. Their structures are illustrated in Figures 1, 2, and 3, respectively.

Diaryliodonium salts **1a**−**k** are known compounds and were prepared according to standard literature methods (*25*). The salt **1l** was prepared from the corresponding ylide and trifluoroacetic acid (*26*). The inner salt **1o** was prepared from *o*-iodobenzoic acid according to an established method (*27*). Ylides **2a**−**c** are stable iodonium ylides and were prepared

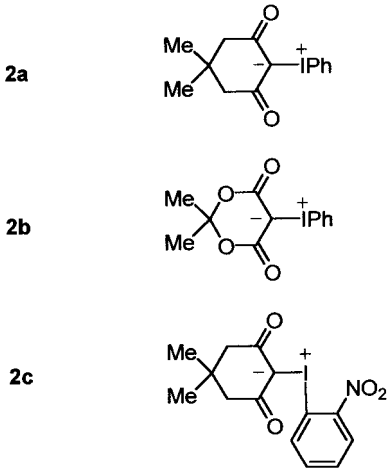

Figure 2. Structures of aryliodonium ylides (**2a**−**c**).

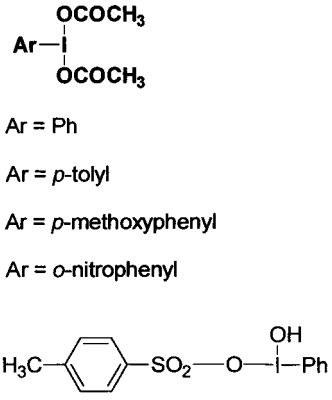

Figure 3. Structures of (diacyloxyiodo)arenes (**3a**−**d**) and [(hydroxy)(tosyloxy)iodo]benzene (**3e**).

from the corresponding dicarbonyl compounds and the proper (diacetoxyiodo)arene (*28*). Addition of trifluoroacetic acid to ylides **2a** and **2b** afforded the corresponding iodonium salts **1m** and **1n**. (Diacyloxyiodo)arenes **3a**−**d** were prepared by oxidation of the corresponding iodoarene with peracetic acid (*29*) or sodium perborate (*30*). Finally, [(hydroxy)(tosyloxy)-iodo]benzene, **3e** (Koser's reagent), was prepared from (diacetoxyiodo)benzene and *p*-toluenesulfonic acid (*31*).

Bacterial Strains and Media. Two strains of *P. syringae pv. syringae* bacteria Λ50 and Al 489 were used for the assays. The first, Λ50, was isolated from citrus trees in Greece; the other one, Al 489, was isolated from almond trees in California. Both strains were tested and found to be INA positive (*3*, *5*). These two strains had already been checked for their sensitivity to copper ions because copper compounds are the most commonly used bactericides to control INA bacrterial populations (*3*, *5*, *12*, *13*).

Culture of the bacteria stored on NAG (per liter, 24 g of Difco nutrient bacto agar and 20 mL of glycerol) slants at 4 °C was plated on KB [per liter, 20 g of Bacto peptone proteose 3, 1.5 g of K_2HPO_4, 1.5 g of $MgSO_4(H_2O)_7$, 15 g of Difco Bacto agar, 10 mL of glycerol, pH 7.2−7.5], medium plates and incubated for 48 h at 21 °C. Culture from KB plates was inoculated into NAG broth (per liter, 3.3 g of Difco Bacto peptone, 2.7 g of Difco nutrient broth, 2.0 g of Difco yeast extract, 25 mL of glycerol) and incubated for 18−24 h at 22 ± 1 °C on a wrist motion environmental shaker before the assay.

Determination of Bacterial Cell Growth, MICs, and MBCs. To evaluate a dose-dependent inhibition of growth and the concentration-dependent inhibition of the rate of growth, bacterial cell growth was assessed spectrophotometrically at 600 nm (1 $OD_{600} = \sim 10^9$ cells/mL). The assays were performed according to a 2-fold serial dilution method in broth. Test compounds were first dissolved in dimethyl sulfoxide (DMSO) to give a concentration of 10000 ppm and then diluted with

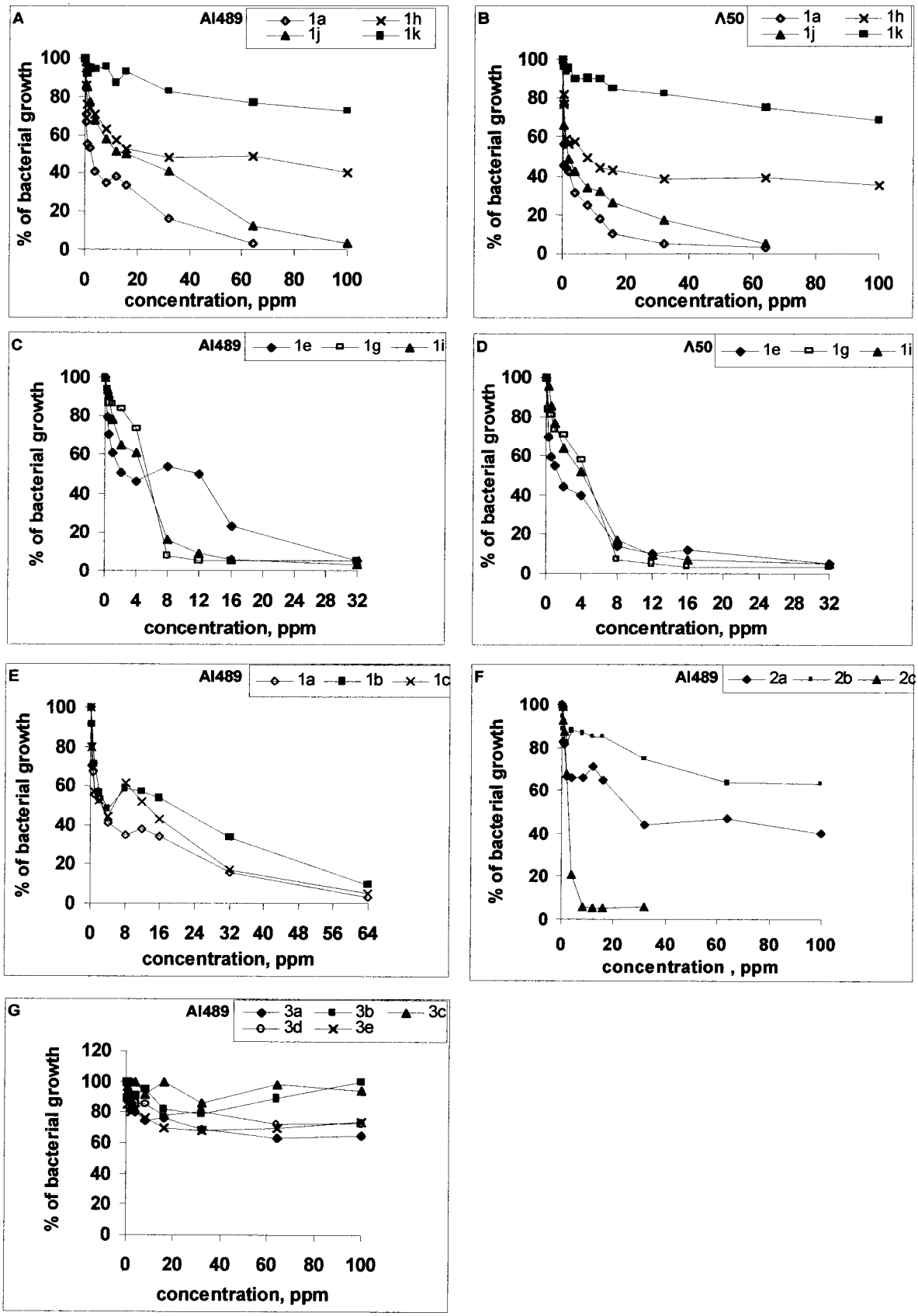

Figure 4. Concentration-dependent effect of hypervalent iodine compounds on the growth of *P. syringae* strains Al 489 (A, C, E−G) and Λ50 (B, D) cells in broth culture after 24 h of incubation. The experiments were repeated twice with three replications for each concentration, and each point represents the mean value.

double-distilled sterile water, to give the desired concentration of the working solution. One hundred microliters of the working solution was added to 5 mL of NAG broth to obtain the final appropriate for the assay concentration of 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16, 32, 64, 100, or 200 ppm. Finally 100 μL of a 24 h culture (logarithmic to early stationary phase) of the test strain was inoculated, and the tubes were then incubated in an environmental shaker at 22 ± 1 °C. The initial inoculum

Table 1. Antibacterial Activities of Hypervalent Iodine Compounds against INA *P. syringae* Strains Al 489 and Λ50

	P. syringae Al 489				*P. syringae* Λ50			
compd	MIC, ppm	MBC, ppm	EC_{50}, ppm	equation, *r*	MIC, ppm	MBC, ppm	EC_{50}, ppm	equation, *r*
1a	64	>200	2.3	$y = 5.228 - 0.622 \log x$ $r = 0.92326$	64	>200	0.5	$y = 4.829 - 0.6068 \log x$ $r = 0.98678$
1b	64	>200	4.4	$y = 5.605 - 0.946 \log x$ $r = 0.9687$	64	>200	0.9	$y = 4.977 - 0.709 \log x$ $r = 0.9858$
1c	64	>200	4.5	$y = 5.324 - 0.498 \log x$ $r = 0.9333$	64	>200	0.4	$y = 4.831 - 0.419 \log x$ $r = 0.9630$
1d	64	>200	5.0	$y = 5.716 - 1.029 \log x$ $r = 0.9516$	32	>200	2.1	$y = 5.285 - 0.874 \log x$ $r = 0.9959$
1e	32	>200	2.1	$y = 5.302 - 0.915 \log x$ $r = 0.9959$	32	>200	1.4	$y = 5.099 - 0.647 \log x$ $r = 09913$
1f	8	16	5.1	$y = 6.063 - 1.499 \log x$ $r = 0.9417$	8	16	1.2	$y = 5.144 - 1.580 \log x$ $r = 0.9578$
1g	8	16	4.8	$y = 6.081 - 1.585 \log x$ $r = 0.9547$	8	16	8.8	$y = 6.101 - 1.165 \log x$ $r = 0.9279$
1h	>200	>200	27.6		>200	>200	12.1	
1j	64	>200	11.1	$y = 6.119 - 1.069 \log x$ $r = 0.9681$	64	>200	3.7	$y = 5.313 - 0.5554 \log x$ $r = 0.9788$
1i	16	64	3.5	$y = 5.812 - 1.481 \log x$ $r = 0.9409$	8	64	3.1	$y = 5.8104 - 1.644 \log x$ $r = 0.9663$
1k	200				200			
1l	>200				>200			
1m	>200				>200			
1n	>200				>200			
1o	>200				>200			
2a	>200				>200			
2b	>200				>200			
2c	8	16	2.6	$y = 5.884 - 2.097 \log x$ $r = 0.9685$	8	16	1.9	$y = 5.505 - 1.776 \log x$ $r = 0.9864$
3a	>200				>200			
3b	>200				>200			
3c	>200				>200			
3d	>200				>200			
3e	>200				>200			

was 10^8-10^9 colony-forming units (cfu)/mL, resulting in final concentration of the test solutions of $\sim 2 \times 10^6$ cfu/mL. The growth of bacteria was measured as turbidity (OD at 600 nm, Spectronic 20D, MiltonRoy Co.) at various time intervals for up to 24 h. The highest concentration used for the assay was 200 ppm, because of solubility difficulties in the water-based media at higher concentration. The final concentration of DMSO in the broth was <0.5% v/v, and the same amount was added to the controls. The controls included inoculated growth medium without test compounds. The presence of DMSO at this concentration does not affect the growth of the bacteria tested. Sample blanks contained uninoculated medium only. The lowest concentration of the test compound that totally inhibited the visible bacterial growth after 24 h of incubation at 22−24 °C was defined as the MIC. All assays were repeated at least two times, and three samples were used for each concentration. For a positive control the bactericide flumequine (Firestop 15FL) was used.

Bactericidal Assays. Growth curves of *P. syringae* Al 489 and Λ50 using a viable cell count assay were obtained to verify the bacteriostatic or bactericidal activity of the most potent compounds **1g** and **2c**. The assays were performed in KB broth [per liter, 20 g of Bacto peptone proteose 3, 1.5 g of K_2HPO_4, 1.5 g of $MgSO_4(H_2O)_7$, 15 g of Difco Bacto agar, 10 mL of glycerol, pH 7.2−7.5] containing 0.5, 8, and 16 ppm of test compounds and initial inoculum of 2×10^6 cfu/ml. Samples were removed at 0, 2, 4, 8, 11, and 22 h of incubation, and the number of cfu per milliliter was determined by serial 10-fold dilution plating onto KB plates after 48 h of incubation at 22−24 °C. The MBC was the lowest concentration of antibacterial compound that produced ≥99.9% killing of the initial inoculums.

RESULTS AND DISCUSSION

The antibacterial activity of the hypervalent compounds was tested against two strains of *P. syringae* species. The choice of these bacterial strains as a model system for study was based on their significance as plant pathogens (*12*, *13*, *21*). To determine whether treatment of *P. syringae* strains with hypervalent iodine compounds affects bacterial cell growth, bacterial cells of *P. syringae* strains were incubated separately with various concentrations (0.25−200 ppm) of the iodine compounds. The bactericide flumequine was also used as a chemical control. Results obtained from these studies show that all 23 hypervalent iodine compounds cause a dose-dependent inhibition of growth for both *P. syringae* strains. Compared to the control, the rate of bacterial growth was reduced during the 24-h incubation period. The responses of both strains to the tested compounds were similar. Figure 4 illustrates representative graphs of the concentration-dependent effect of the tested compounds on the growth of *P. syringae* strains. The results obtained from the experiments with *P. syringae* Al 489 are shown in Figure 4A,C,E−G. The growth patterns of *P. syringae* Λ50 showed trends similar to those of *P. syringae* Al 489 in the presence of the 23 tested compounds, and some representative graphs of these experiments are shown in Figure 4B, D. Compounds **1f**, **1g**, **1i**, and **2c** caused >50% inhibition of cell growth in either Al 489 or Λ50 strain at a concentration as low as 2 ppm and abrogated bacteria replication at 8 ppm. The minimum concentration that completely inhibits bacterial growth (MIC) over a 24-h incubation period was determined, and the results are presented in Table 1. These results show that all compounds exhibited a variable degree of antibacterial activity, and their MICs ranged from 8 to >200 ppm. Compounds **1f**, **1g**, **2c** with MICs = 8, and **1i** with MIC

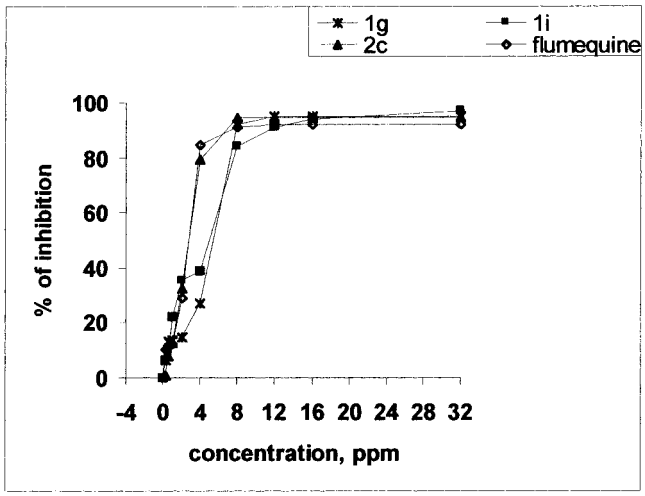

Figure 5. Concentration-dependent inhibition of the rate of growth of *P. syringae* Al 489 by **1g**, **1i**, **2c**, and a chemical control, the bactericide flumequine.

16 ppm, were the most potent antibacterial agents and almost equally effective as flumequine (MIC = 4 ppm), a registered bactericide used for the control of fireblight (Figure 5). Lower activity, with MIC = 32 and 64 ppm, was measured for the iodonium salts **1a**−**e** and **1j**. Minimum concentrations with bactericidal activity (MBC) were also determined for these compounds. For all other compounds MICs > 200 ppm were determined, but in all cases a dose-dependent inhibition of growth was observed (Table 1; Figure 4). In Figure 6 the time- and concentration-dependent inhibition of the growth rate of *P. syringae* Al 489 by iodine compounds **1a**, **1g**, **1i**, and **2c** is illustrated representatively. Similar results were obtained for *P. syringae* Λ50. The results showed that 8 ppm of **1g** and **2c** or 64 ppm of **1a** abrogates bacterial replication and that the lower concentrations caused a considerable decrease in the growth rate of *P. syringae*. The presence of 8 ppm of compound **1i** inhibits the bacterial growth for the first 11 h of incubation; however, the surviving cells start growing after that time. Generally, all compounds that exhibited antibacterial activity showed the same pattern of dose-dependent inhibition of growth. In the first hours of incubation no bacterial growth was observed at the lower concentrations, but after that period, a slight growth was measured. Although only two strains of INA *P. syringae* were tested, our preliminary results showed that compounds active against these strains are expected to retain a similar order of activity against a variety of INA *P. syringae* strains.

It is obvious that the presence in the molecule of electron acceptors, for example, $-NO_2$ for **1f**, **1g**, and **2c** or −I for **1i**, increases the antibacterial activity of the iodonium compounds. The nature of the anion X^- does not seem to affect the activity of the diaryliodonium salts **1a**−**n** (Figure 4E). (Diacyloxyiodo)arenes **3a**−**d** and the hydroxytosyloxy derivative **3e** were the most ineffective agents in the antibacterial assays (Figure 4G). This can be explained due to the instability of these

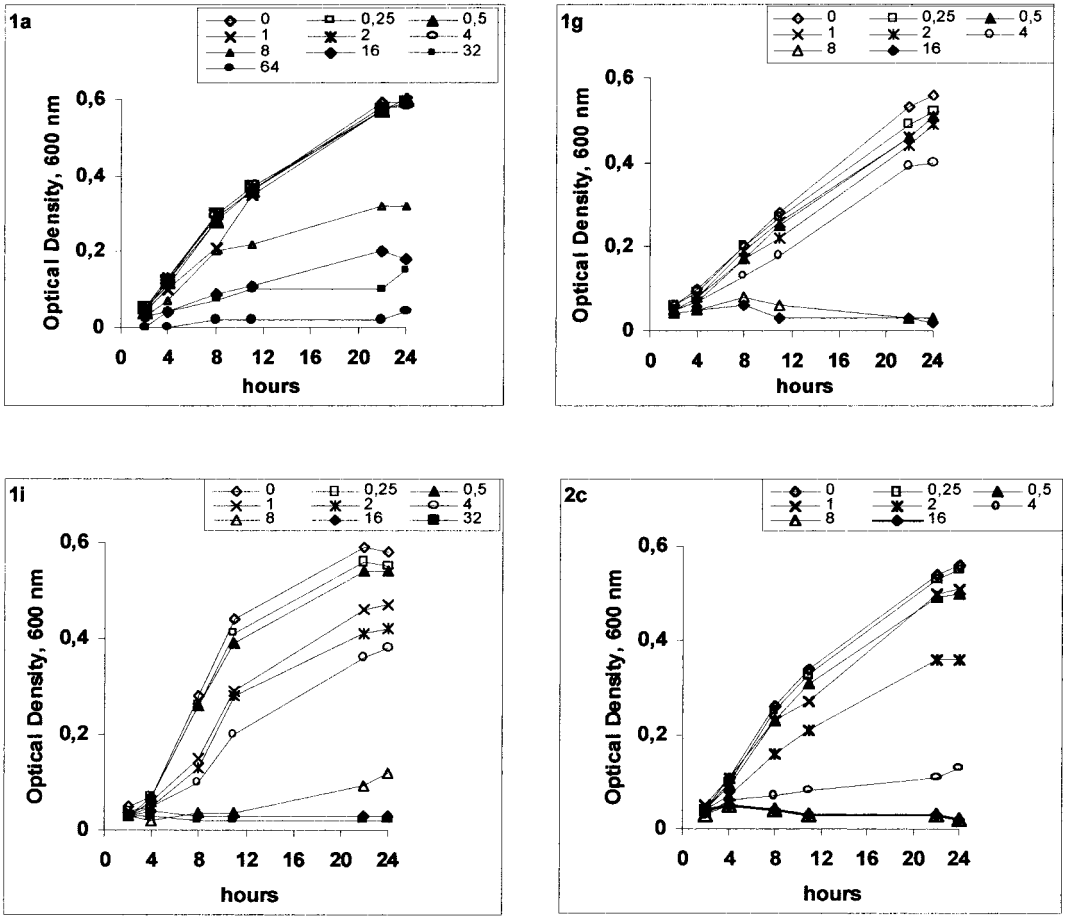

Figure 6. Time- and concentration-dependent inhibition of the growth rate of *P. syringae* Al 489 by iodine compounds **1a**, **1g**, **1i**, and **2c**. The experiments were repeated twice with three replications for each concentration, and each point represents the mean value.

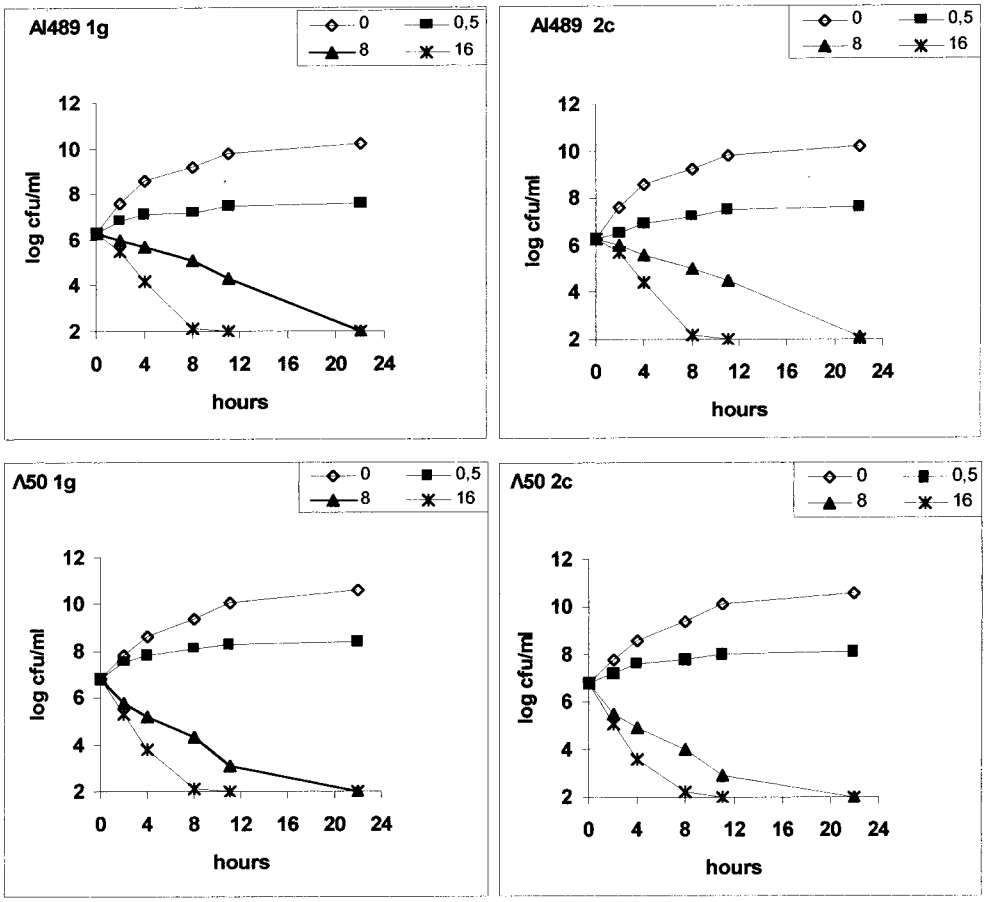

Figure 7. Bactericidal effects of **1g** and **2c** on *P. syringae* Al 489 and Λ50. The number of viable cells was determined in KB broth in the presence of 0.0, 0.5, 8, and 16 ppm of **1g** and **2c**.

compounds in solution, as they react easily with almost all classes of organic compounds. This reactivity has as a result the reduction of I^{3+} species to I^- species, which do not exhibit any special activity. The I^{3+} species seem to be the factors of antibacterial activity. O'Donnell et al. (*32*) suggest that phenyl radicals, derived from iodonium compounds, are the reactive species which cause inhibition of flavoenzymes. Our findings suggest that a similar involvement of aryl radicals is most likely because the presence of NO_2 groups, which cause stabilization of aryl radicals, also increases antibacterial activity. Of interest is the finding reported by Gallop (*33*) that another class of iodonium compounds, alkynyliodonium salts, cause highly effective inhibition of PQQ (methaxatin), an organic cofactor, in an increasing number of biological redox processes.

To determine the mean effective concentration (EC_{50}) of the iodine compounds, for which MICs had been determined in the range of 8−64 ppm, the probit of percentage of inhibition was regressed against the logarithm of their concentration, and the EC_{50} value for each compound was calculated by interpolation to probit = 5.00. The results are presented in Table 1. In all cases a linear correlation was found, and the values of regression coefficient *r* ranged from 0.9233 to 0.9959. EC_{50} values ranged from 2.3 to 27.6 ppm and from 0.4 to 12.1 ppm for Al 489 and Λ50 strains, respectively. For all other cases for which MICs were >200 ppm, EC_{50} values were not calculated. It is interesting enough that the inhibition of cell growth caused by all of these iodine compounds was found in the range of 40−55% at the highest tested concentration of 200 ppm (Figure 4). Although the antibacterial activities of the iodine compounds against the two *P. syringae* strains are similar, the EC_{50} values suggest that the Λ50 strain seems to be more sensitive than the Al 489 strain. In all cases the percentage of growth inhibition caused to *P. syringae* Al 489 was greater than that to *P. syringae* Λ50. It is known (*3*, *5*, *12*) that the two strains exhibit different sensitivities against copper ions and copper bactericides. Strain Al 489 is considered to be a copper-tolerant bacterial strain with EC_{50} = 40.6 ppb of Cu^{2+}, whereas Λ50 is considered to be a copper-sensitive one with EC_{50} = 7.5 ppb of Cu^{2+}.

The use of the liquid dilution method and the measurement of the bacterial growth as turbitity cannot differentiate between the bacteriostatic and bactericidal effects of the tested compounds. MIC values measured as turbitity were found to be lower than MBC values, indicating that a number of cells are still viable at these concentrations or that a bacteriostatic rather than a bactericidal activity occurs. To verify this point, the bactericidal activities of the most potent compounds **1g** and **2c** were estimated using the viable count method to analyze the growth curve of bacteria cells in the presence of both compounds. The growth curves of *P. syringae* Al 489 and Λ50 in the presence of **1g** and **2c** are shown in Figure 7. Both compounds exhibited bactericidal activity against *P. syringae* Al 489 at concentrations of 8 ppm. Bacterial cells were killed after 22 h of exposure to 8 ppm of **1g** and **2c**. Moreover, these compounds at 16 ppm rapidly killed this bacterium within 8 h of incubation.

In conclusion, some hypervalent iodine compounds show antibacterial activity against INA bacteria, and this property makes them good candidates as anti-

microbial agents. Their potency is comparable with that of either known bactericides or some other promising agents such as essential oils and their constituents. Although these compounds are generally considered to be environmentally safe, more detailed studies are needed to verify their suitability for agricultural uses.

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