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Some Biological and Biochemical Activities of Resormycin, a Novel Herbicidal Antibiotic

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Biological and biochemical activities of resormycin were studied using unicellular green algae, *Selenastrum capricornutum* (abbreviated as *Selena.*), as a test organism. Resormycin inhibited the growth *in vitro* of *Selena.* more strongly in the dark than in the light. A weaker but more photo-stable derivative, (±)-2,3-dihydro-resormycin, showed more long-lasting activity against *Selena.* in the light. Resormycin started killing *Selena.* only after exposure for 2 days and longer, even at high concentrations. Resormycin at concentrations near IC_{50} rapidly inhibited incorporation of ^{3}H-leu, but not ^{3}H-UR or ^{3}H-TdR, into the TCA insoluble fraction of *Selena.* Herbicidal activity of resormycin was confirmed using some crops and weeds.

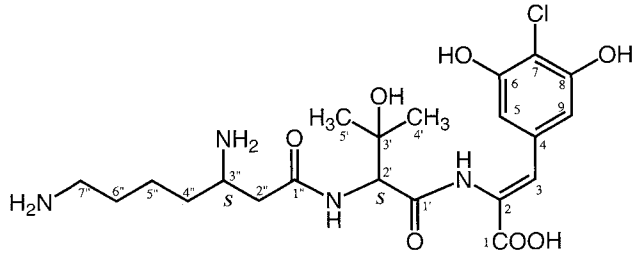
Resormycin (Fig. 1), a novel herbicidal and fungicidal antibiotic, has interested us to elucidate how it works in plant cells because of its unique structure and antibiotic spectrum[1,2]. As a test organism, we chose *Selenastrum capricornutum* ATCC22662, a unicellular green algae, because it showed a high sensitivity to resormycin and rapid growth under simple culture conditions. The present paper reports how resormycin acts in *Selena.* Supplementary data showing its herbicidal activity are included.

Methods

Growth of *Selena. In Vitro*

Growth inhibitory effect of resormycin and other compounds on *Selena.* was assayed using 24-well Tissue Culture Clusters (Costar 3254) as culture vessels, under sterilized conditions. As the culture medium, Hyponex (nitrogen/phosphorous/potassium: 5/10/5, Hyponex Co. Ltd.) was diluted 800-fold with distilled water and sterilized by filtration through 0.22 μm pore size filters and the filtrate was used for assays (starting with 2×10^{5} cells/2 ml/well) and for maintenance of the cell stock (starting with 2×10^{7} cells/40 ml/100 ml-vol. bottle). In most assays, cells were grown at a room temperature under 5000 lux (described as "in the light") or in the complete darkness except the time for various manipulations that were conducted under the room light (described as "in the dark"). Assay medium for "in the dark" experiments was supplemented with 0.1% (w/v) glucose. Since our *Selena.* stock was found to be contaminated by some bacteria that

Fig. 1. Structure of resormycin.



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grew better in poorly nourished media than in nutrient broth (although glucose was stimulatory), assays "in the dark" were conducted in the presence of 2~5 μg/ml of ofloxacin to suppress the bacteria. Attempts to remove the bacteria with different antibiotics have been so far unsuccessful. Stock of *Selena.* was maintained at the room temperature under the room light. Cell growth was monitored by counting the number of cells (particles of 2.7 to 9.5 μm in length) in a Coulter Counter, Model Z_2.

Preparation of 3-H Anomeric (±)-2,3-Dihydro-resormycin

In 3 ml of 50%(v/v) aqueous dioxane, 69 mg (0.13 mmol) of resormycin mono-HCl salt, 100 mg of di-*t*-butyl dicarbonate (0.43 mmol) and 40 mg of triethylamine were dissolved and the solution was stirred for 4 hours at room temperature to allow the reaction to proceed. The solution was mixed with 50 ml of ethyl acetate to extract the reaction product into it. The ethyl acetate layer was taken, washed with 10 ml of 0.2 M citric acid and concentrated *in vacuo*, leaving 58.7 mg of *N*-diBoc-resormycin (66% yield). A 54.7 mg portion (0.074 mmol) of *N*-diBoc-resormycin was dissolved in 2 ml of 50%(v/v) aqueous ethanol and the solution was stirred with 10 mg of 10% Pd-C in the hydrogen atmosphere for 24 hours. The solution was filtered and the filtrate was concentrated *in vacuo* to give 46.1 mg of (±)-*N*-diBoc-2,3-dihydro-resormycin (84% yield), which was dissolved in 1 ml of 2 M HCl-50%(v/v) aqueous THF and left standing at a room temperature for 2 hours. The solution was neutralized with 1 M NaOH and concentrated *in vacuo*. From the residue, 25.4 mg of pure (±)-2,3-dihydro-resormycin was obtained (43% overall yield) by Sephadex LH20 column chromatography. Physicochemical data on (±)-2,3-dihydro-resormycin were as follows: FABMS *m/z*: 489 $(M+H)^+$; $C_{21}H_{34}ClN_4O_9$; ^1H NMR (400 MHz, D_2O): mixture of 3-H anomer δ 6.37 (2H, s, 5 and 9), 6.35 (2H, s, 5 and 9), 4.63 (2H, m, 2), 4.39 (1H, s, 2'), 4.36 (1H, s, 2'), 3.52 (2H, m, 3''), 3.09 (2H, dd, J=8 and 24 Hz, 3), 3.05 (1H, dd, J=8 and 24 Hz, 3), 2.93 (6H, m, 2'' and 7''), 2.85~2.6 (4H, m, 2 and 2''), 1.68 (8H, m, 4'' and 6''), 1.48 (4H, m, 5''), 1.25 (3H, s, 4'-CH_3 or 5'-CH_3), 1.24 (3H, s, 4'-CH_3 or 5'-CH_3), 1.13 (3H, s, 4'-CH_3 or 5'-CH_3), 1.09 (3H, s, 4'-CH_3 or 5'-CH_3).

Fractionation of Yeast Extract

Ten gram of yeast extract (Difco) was dissolved in 50 ml of distilled water and charged on an Amberlite Dowex 50 w (H^+, 75 ml) column. The column was washed with distilled water and eluted with 1 M NH_4OH. Active fraction was collected and concentrated *in vacuo* yielding 23.3 gm dark brown syrup, which was submitted to chromatography with Amberlite CG-50 (NH_4^+, 150 ml), developed with distilled water and 1 M NH_4OH. Active material(s) was further purified with a cellulose column (Avicel, Asahikasei Co., 150 ml) developed with $CHCl_3$: MeOH : conc.NH_4OH : H_2O in the ratio of 1 : 4 : 2 : 1. Active fraction was collected and concentrated *in vacuo* yielding 30.3 mg light brown syrup, whose main components were lysylleucine, lysylisoleucine and lysylvaline detected as ninhydrin-positive spots on a cellulose PTLC (Avicel, Asahikasei Co.) developed with PrOH : pyridine : AcOH : H_2O=15 : 10 : 3 : 12. The Rf values and weights (parenthesized) of lysylleucine, lysylisoleucine and lysylvaline were 0.67 (5.3 mg), 0.6 (3.0 mg) and 0.53 (2.0 mg), respectively.

Determination of DNA, RNA and Protein Syntheses in *Selena.*

Selena. cells were inoculated at 4×10^5 cells/2 ml/well. Resormycin was added and the cells were left standing in the light for a day. The cell number was counted with a small aliquot of the cell suspension and ^{3}H-TdR, ^{3}H-UR, or ^{3}H-Leu was added to the cultures at 3 μC/ml. After 1-hour culture, cell-associated radioactivity in 10%TCA-insoluble fractions was counted using LSC.

Effect of Resormycin on Lettuce Seedling

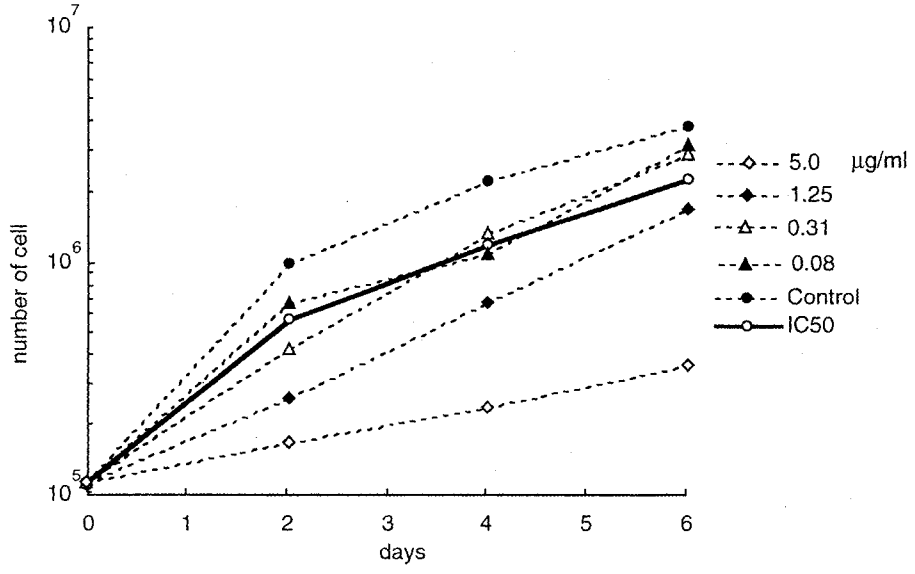
Aqueous solutions of test compounds, including resormycin, were neutralized with HCl, appropriately diluted with distilled water, and a 2 ml aliquot was added to each 5 g of sea sand (Koso Chem. Co.) that had been placed in a test tube (27 mm×55 mm). Ten to twenty seeds of lettuce were put on the wet sand in a tube, kept standing at 25°C under 5000 lux or in the complete darkness for 7 days. Growth inhibitory effects, if any, were visually inspected and photographed.

Results and Discussion

Growth Inhibition of *Selena.* by Resormycin

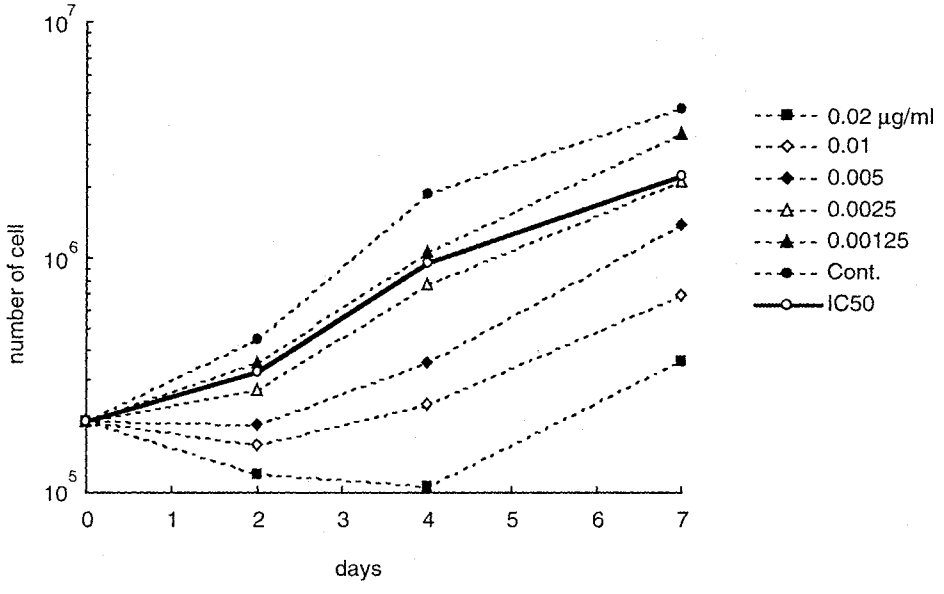
Selena. grows in an inorganic medium (Hyponex medium, see Methods) in the light, while grows also in the dark if the medium is supplemented with 0.1%(w/v) glucose. Glucose was rather growth-inhibitory in the light. Growth inhibition of *Selena.* by resormycin was much stronger in the dark than in the light as shown in Fig. 2a and 2b. Apparent IC50% (based on initial concentrations) on day 4, for example, were 0.31 μg/ml and 0.002 μg/ml in the light and in the dark, respectively. It must be taken into account, however, that resormycin in aqueous solutions degraded about 3-times as fast in the light as in the dark

Fig. 2a. Growth inhibition by resormycin in the light.



Apparent IC 50% (based on the initial concentration; μg/ml) increased with culture time; those on day 2, 4, and 6 were 0.125, about 0.31 and 0.31~1.25, respectively.

Fig. 2b. Growth inhibition by resormycin in the dark.



Experiment was conducted as the one shown in Fig. 2a except that glucose and ofloxacin were added in the medium and cells were grown in the dark (see Methods). Apparent IC50% stayed almost unchanged with culture time about 0.002 μg /ml.

(see below). Real difference in the effectiveness of resormycin between the two conditions, therefore, must be smaller. Real-time concentrations of resormycin through culture periods were not monitored.

Among existing herbicidal compounds, bifenox and DCMU (diuron), both of which are inhibitors of photosynthesis[3,4], inhibited the growth of *Selena.* about 30 times more strongly in the light than in the dark, in contrast to resormycin; IC50% for bifenox were 0.004 μg /ml in the light and 0.1 μg /ml in the dark and those for DCMU were 0.03 μg/ml in the light and 1.0 μg/ml in the dark. On the other hand, butachlor, an inhibitor of protein synthesis[5], inhibited the growth of *Selena.* almost equally in the light and in the dark; *i.e.*, IC50% were 0.008 μg/ml in the light and 0.004 μg/ml in the dark. These results suggested that resormycin inhibited some biological process not involved in photosynthesis.

Degradation of Resormycin in Aqueous Solutions

Relative extent of growth inhibition by resormycin

Fig. 3. Degradation of resormycin and dihydroresormycin in aqueous solutions.

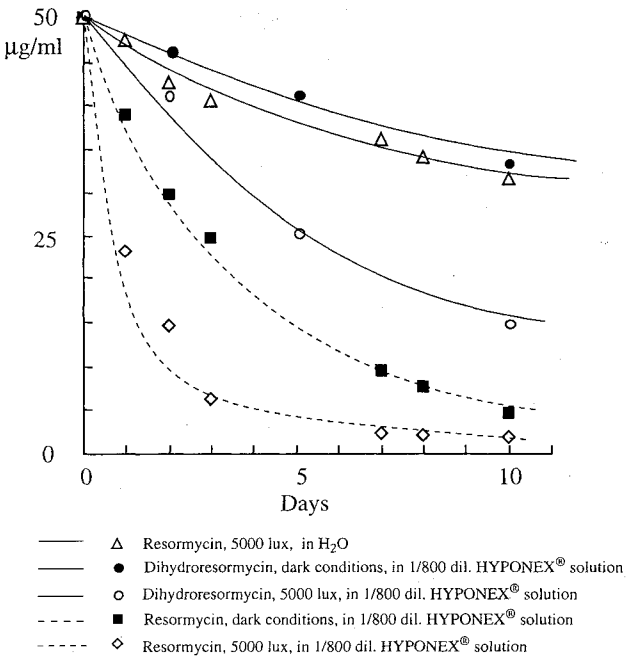
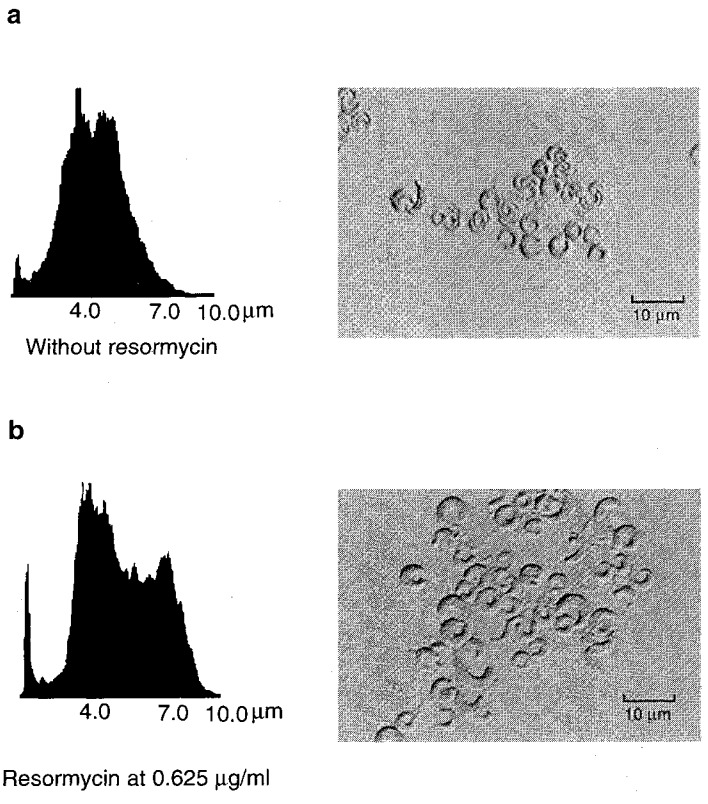


Fig. 4. Enlargement of the cell size by resormycin.



a: Control: Hyponex medium for 72 hours in the light.
b: Resormycin at 0.625 μg/ml, otherwise the same as control. The effect was also observed in the dark.

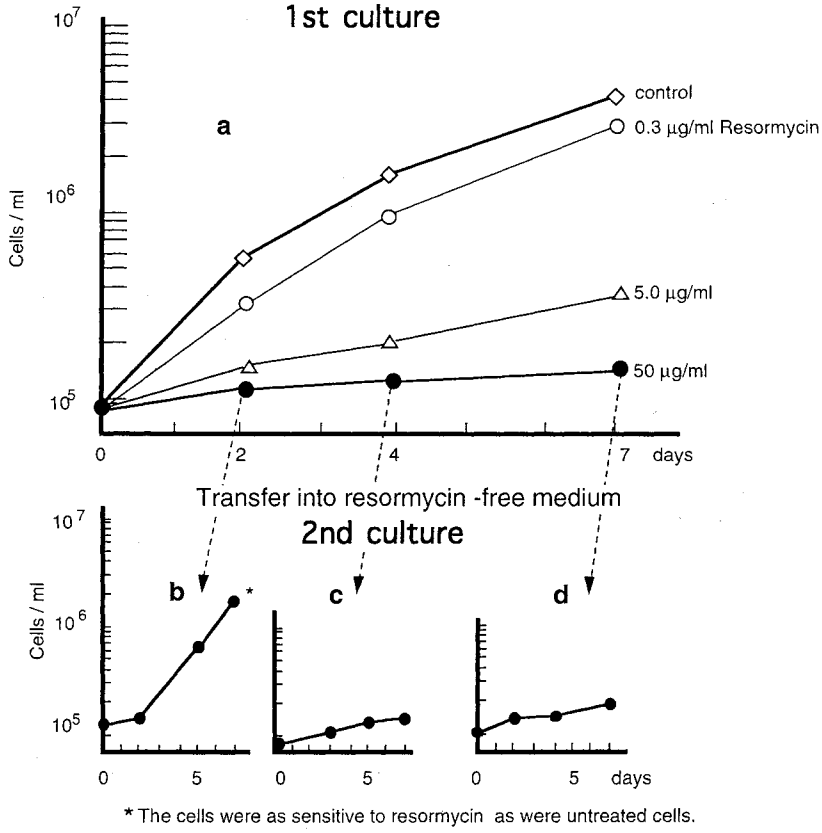
became smaller with culture time (*i.e.*, apparent IC50% increased with culture time; see the legend to Fig. 2a), especially under the conditions of "in the light", suggesting that resormycin degraded in the culture medium especially "in the light". This possibility was tested and the results are shown in Fig. 3. Aqueous solutions of resormycin gradually turned brown in the light in parallel with decrease in the amount of the antibiotic in the solutions, suggesting that some oxidative polymerization should progress more rapidly in the light. This hypothesis was supported by the fact that (±)-2,3-dihydro-resormycin (abbreviated as dihydroresormycin) was more stable than resormycin under the same conditions (Fig. 3). Since EDTA slowed down the degradation of resormycin in the light (data not shown), some cooperative role between metallic ions and the light was suspected. The biological activity of dihydro-resormycin against *Selena.* was weaker than that of resormycin; apparent IC50% of dihydroresormycin on day 4 in the light and in the dark were 1.6 μg /ml and 0.4 μg/ml, respectively. No polymerized or inactivated products derived from resormycin or dihydroresormycin have so far been identified.

Enlargement of the Cell Size Caused by Resormycin and Dihydroresormycin

Resormycin and dihydroresormycin at partially growth-inhibitory concentrations, in the light as well as in the dark, enlarged the cell size from 4 μm in length (normal average size) to 7 μm. An example is shown in Fig. 4a and 4b. It should be noticed that only a fraction of total cells was enlarged to the 7 μm size. At high concentrations of resormycin, cells became rather smaller.

Among other herbicides tested, methyl viologen, an oxygen radical inducer[6], also enlarged the cell size, but not in the same manner as did resormycin. The average size of

Fig. 5. Slowly progressing cytocidal effect of resormycin.



Cell culture was initiated with an initial concentration of resormycin at 50 μg/ml. On each of days 2, 4, and 7, cell suspension was collected from 6 wells (12 ml in total for each day, a portion of which was used for cell counting for Fig. 5a, 50 μg/ml) and centrifuged at 1200 rpm (r=15 cm) for 20 minutes. Cells (precipitate) were suspended in 8 ml of the fresh medium (without resormycin) and centrifuged. The washed cells (precipitate) were suspended in the fresh medium at a cell density of 10^5/ml. A secondary culture (Fig. 5b, c or d) was initiated with 3 wells, each containing 2 ml of the washed-cell suspension.

the enlarged subpopulation with methyl viologen was 5.2 μm, in contrast to the 7-μm-long subpopulation with resormycin. The mechanism of cell enlargement caused by these compounds has remained unknown.

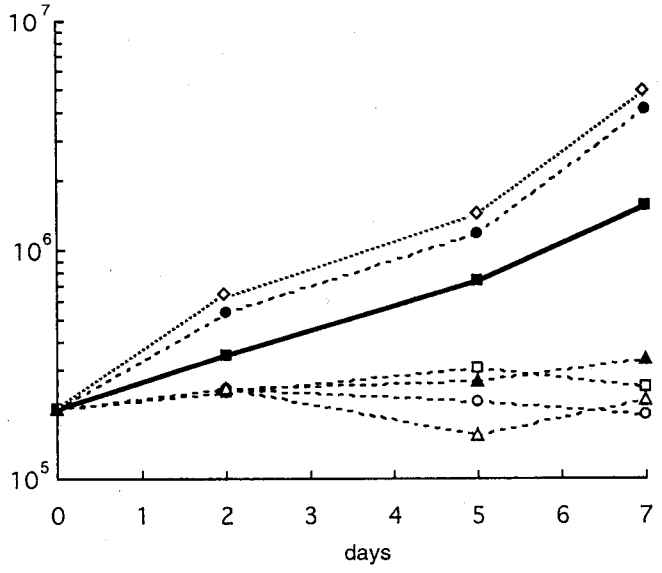
Slowly Progressing Cytocidal Effect of Resormycin

Resormycin even at high concentrations did not kill *Selena.*, if the exposure time was short. Cells survived the exposure to an initial concentration of 50 μg/ml for 2 days in the light; the cells resumed growth after being washed free of resormycin and suspended in the drug-free medium (Fig. 5b). However, cells that were maintained in the resormycin-containing medium for 4 days or longer were found dead, no cell growth resumed after transfer into the drug-free medium (Fig. 5c and 5d). The small increase in the ordinates of the secondary cultures (Fig. 5c and 5d) was not due to growth of *Selena.* but to growth of some contaminating bacteria (see Methods). *Selena.* cells that survived the exposure to resormycin for the first 2 days and resumed growth in the fresh medium (Fig. 5b) were as sensitive to resormycin as untreated cells (data not shown), indicating that an incomplete treatment with resormycin did not select or induce any resistant cells.

Yeast Extract Protected *Selena.* from Resormycin

Yeast extract at 0.1%(w/v) completely blocked the growth inhibitory activity of resormycin at 5 μg/ml in the

Fig. 6. Lysylleucine protected *Selena.* from resormycin.



▲ Resormycin (5 μg/ml), ○ resormycin (5 μg/ml) +lysine (100 μg/ml), □ resormycin (5 μg/ml) +leucine (100 μg/ml), △ resormycin (5 μg/ml)+lysine (100 μg/ml)+leucine (100 μg/ml), ■ resormycin (5 μg/ml)+lysylleucine (100 μg/ml), ● control, ◇ resormycin (5 μg/ml)+0.1% (w/v) yeast extract.

Fig. 7. Effect of resormycin on DNA, RNA and protein syntheses in *Selena.*

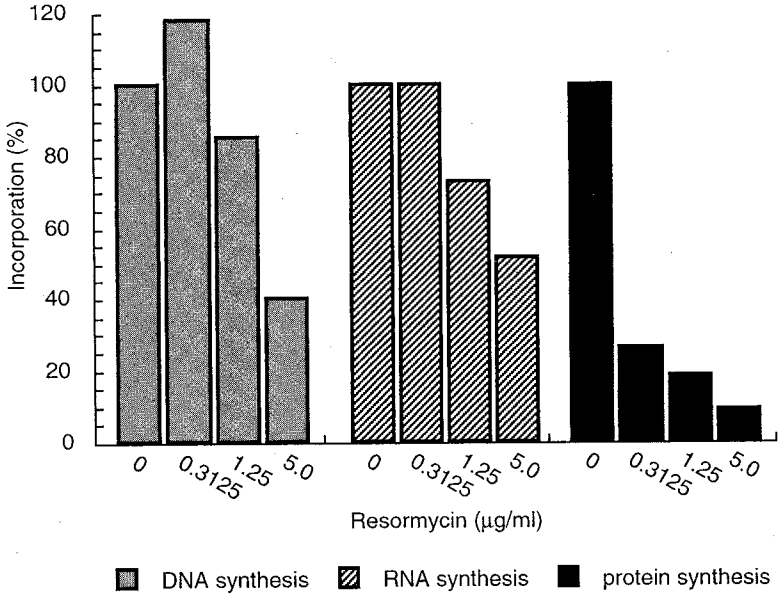
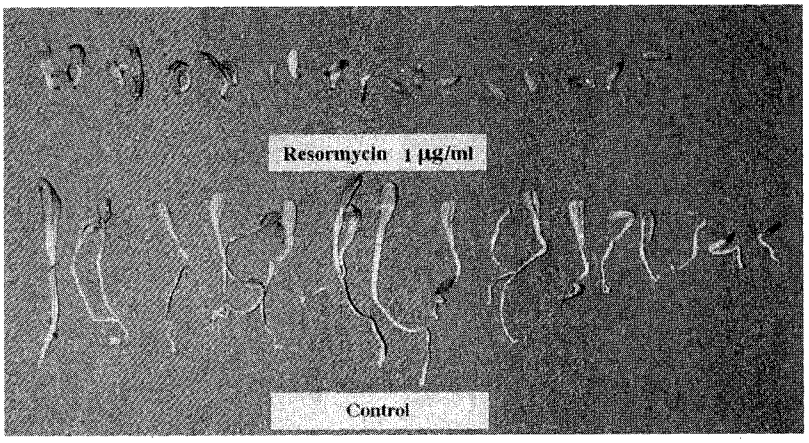
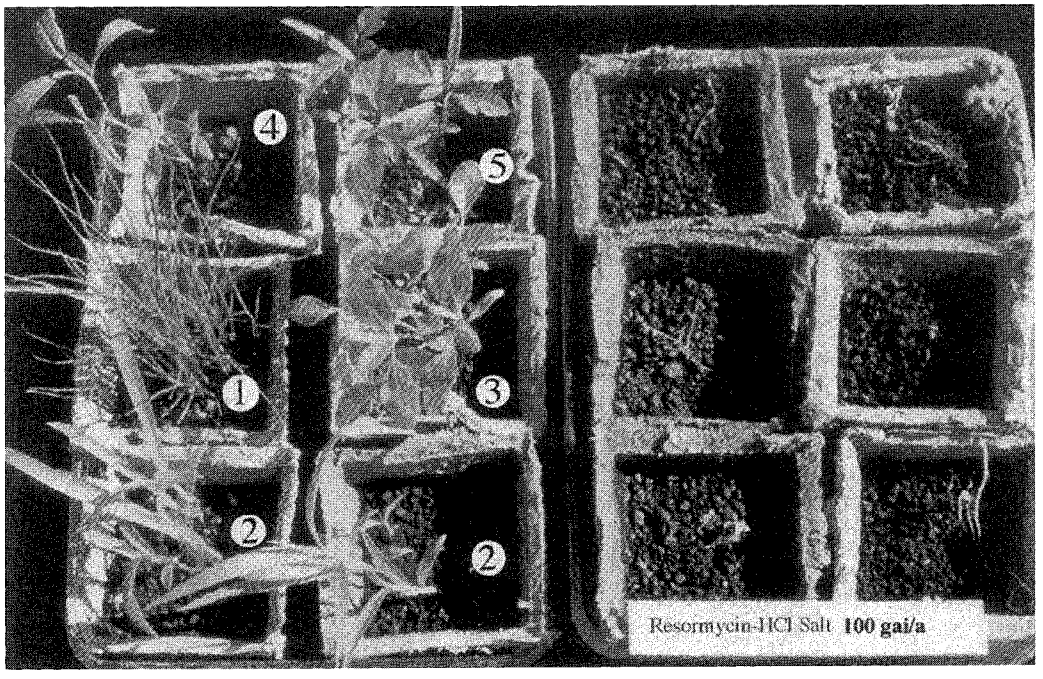


Fig. 8a. Herbicidal effect on lettuce seeding and growth (6 days).



Herbicidal effect was also observed in the dark.

Fig. 8b. Eighteen-day-old seedlings of the weeds were treated with resormycin by foliage application.



Eighteen-day-old seedlings of the weeds were treated with resormycin by foliage application. Herbicidal effects were inspected on the 21st day of the treatment. Weeds used for the herbicidal test were as follows; 1: *Digitaria adscendens* (Henry crabgarss), 2: *Setaria viridis* (Green foxtail), 3: *Alopecurus aequalis*, 4: *Polygonum lapathifolium* (Pale smartweed), and 5: *Bidens pilosa* (Hairy beggarticks).

light, as shown in Fig 6. Similar antagonism was observed also in the dark, although the concentrations of resormycin tested were far lower. Yeast extract was fractionated (see Methods) to identify a component(s) that would antagonize resormycin. An active fraction included lysylleucine, lysylisoleucine and lysylvaline as main components. L-Lysylleucine of a commercial source antagonized resormycin, but none of lysine, leucine or lysine plus leucine did, as shown in Fig. 6. None of other essential amino acids, alone or in combination, antagonized resormycin (data not shown). It should be noticed that lysylleucine resembles a partial structure of resormycin. Antagonism of lysylleucine against resormycin may be the result of competition at the membrane transport system and/or at the active site of the target molecule (unidentified yet) of resormycin. In this respect, peptide transporters with low substrate-specificity have been reported in some plant cells[7]. We wondered if other dipeptides, structurally unrelated to resormycin, could also antagonize resormycin. The answer was negative, however; lysylleucine but not alanylalanine nor glycylglycine was active (data not shown).

Effect of Resormycin on DNA, RNA and Protein Syntheses in *Selena*.

Resormycin inhibited protein synthesis in cells that had been exposed to a partially growth-inhibitory concentration (0.31 μg/ml) for one day as shown in Fig. 7. DNA and RNA syntheses were not influenced under the conditions. Given the slowly progressing cytocidal effect of this antibiotic (Fig. 5a, b, c and d), inhibition of synthesis of some crucial protein(s) that turns over slowly should be a cause for cell death. High specificity of resormycin to plant cells suggests that the protein synthesizing system in plant cells may include an essential component (possibly a protein) that is vulnerable to resormycin.

Herbicidal Activity of Resormycin

Resormycin at low concentrations inhibited growth of lettuce seedling, as shown in Fig. 8a. Herbicidal activity against mono- and dicotyledonous weeds was demonstrated by foliar application at a dosage of 100 gai/a, as shown in Fig. 8b.

Acknowledgement

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