

Phytotoxicity of microbial antibiotics helvolic and fusidic acids

ROBERT E. HOAGLAND

USDA, ARS, Southern Weed Science Research Unit
P.O. Box 350, Stoneville, MS 38776, USA
E. Mail: bob.hoagland@ars.usda.gov

(Received in revised form: August 28, 2008)

ABSTRACT

The phytotoxicity of high purity helvolic acid and a related compound, fusidic acid was assessed using multiple growth and biochemical endpoints performed on tissues of several plant species. Bioassay tests for phytotoxicity of helvolic (0.25 mM) and fusidic (0.50 mM) acids were conducted on weed and crop species including : germination/growth (seeds/seedlings) of hemp sesbania [*Sesbania exalta* (Raf.) Rydb.], sicklepod (*Cassia obtusifolia* L.), mung bean (*Vigna radiata* L.), wheat (*Triticum aestivum* L.), and cucumber (*Cucumis sativa* L.); leaf disk electrolyte leakage of corn (*Zea mays* L.) and giant ragweed (*Ambrosia trifida* L.) leaf tissues; and greening of etiolated tissues of hemp sesbania, sicklepod, wheat (*Triticum aestivum* L.), mung bean, and sorghum (*Sorghum vulgare* L.) tissues. Fusidic acid reduced the chlorophyll accumulation in hemp sesbania (95%), wheat (70%), mung bean (60%), sorghum (60%) and sicklepod (48%); helvolic acid at this low concentration had only weak effects, except in mung bean. Neither compound affected the germination at the concentrations used, but both inhibited the seedling growth of all test species except sicklepod and helvolic acid-treated hemp sesbania. Both chemicals increased the electrolyte leakage in corn and giant ragweed leaf disks after 48-h exposure. Overall these natural products have broad-range phytotoxicity and may affect the plants directly or indirectly via antibiotic effects on soil/rhizosphere organisms.

Key words: Allelochemical(s), bioherbicide, microbial compounds, natural product, phytotoxicity, plant bioassay, weeds

INTRODUCTION

The natural products, helvolic acid (a tetracyclic triterpene) and a related steroidal compound, fusidic acid have potent antibiotic activity against Gram-positive microorganisms. The structural similarity of these two fusidane class of compounds is shown in Figure 1. Helvolic acid, produced by *Aspergillus fumigatus* mut. *helvola* Yuill, have strong activity against Gram-positive microorganisms (3). It is also produced by other microorganisms [*Cephalosporium caeruleus*, *Emericellopsis terricola* (5)] and plant pathogenic fungus, *Sarocladium oryzae* (17,20). Its external application to rice seedlings produced lesions similar to those caused by *S. oryzae* (17). Fusidic acid is produced by *Fusidium coecineum* and several other fungi (9). Although its steroidal antibiotic mainly inhibits the Gram-positive microorganisms but it has also some activity against Gram-negative microbes (18). Resistance to this compound in microorganisms involves

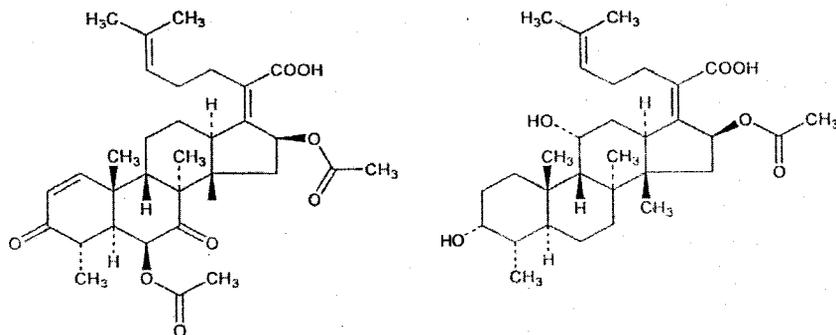


Figure 1. Chemical structural relationship of helvolic and fusidic acids.

mutation-alteration of protein synthesis elongation factor and resistance in *Rhodococcus erythropolis* has been attributed to an inducible extracellular fusidic acid inactivating enzyme (6).

Although helvolic acid is produced by pathogenic and other soil fungi, but there are few reports on its phytotoxicity. Nearly 95% of about 250 strains of *A. fumigatus* (isolated from various soils and rhizospheres) were phytotoxic to corn (*Zea mays* L.) in root bioassay (2). It completely inhibited the root growth at 100-150 $\mu\text{g/ml}$. Helvolic acid from *Sarocladium oryzae* (rice sheath rot pathogen) is phytotoxic (growth inhibition and chlorosis) to various *Graminae* spp. and many dicotyledenous plant species (20). Reports on the phytotoxicity of fusidic acid are woefully lacking.

The biological control project in our laboratory is researching the development of bioherbicides (microbes and microbial products used as herbicides) useful to control weeds. We are also interested in aspects of phytopathogen modes and mechanisms of action including the production of phytotoxins by pathogens. Because of the structural similarities of helvolic and fusidic acids, the production of these compounds by pathogens and other microbes in soils and rhizospheres, and the relative lack of published information on their phytotoxicity prompted us to evaluate their phytotoxicity on 2 major weeds and 3 crops in bioassays.

MATERIALS AND METHODS

Helvolic acid (free acid) and fusidic acid (sodium salt) were high purity products obtained from Sigma Chemical Company, St. Louis, MO, USA. Weed seeds of hemp sesbania [*Sesbania exalta* (Raf.) Rydb.], sicklepod (*Senna obtusifolia* L.), and giant ragweed (*Ambrosia trifida* L.) were obtained from our field plots. Hemp sesbania and sicklepod seeds were mechanically scarified before use in bioassays. All species seeds possessed germination of 96 to 100%. Seeds of mung bean (*Vigna radiata* L.) and cucumber (*Cucumis sativa* L.) were obtained from W. Atlee Burpee Seed Company, Warminster, PA, USA, and wheat (*Triticum aestivum* L.) and corn (*Zea mays* L.) seeds were obtained from Farmers Feed & Supply Company, Leland, MS, USA.

Seedling Bioassays

Spray Application: Seeds of various test plants were planted in paper towel cylinders and grown in dark at 28 °C for 4 days as described (14). Uniform seedlings were then chosen, measured and sprayed (hand-held atomizer) with solutions of helvolic acid (0.25 mM), fusidic acid (0.50 mM), or water as the control treatment. The treated plants were grown in environmental chambers at 28 °C in dark for additional 72 h, and shoot elongation was measured. Each paper towel cylinder contained 4 to 6 seedlings and each treatment (including control) consisted of triplicate cylinders.

Root Feeding: Plants were grown as described above and the roots of 4-d-old seedlings were placed into test tubes containing solutions of helvolic acid, fusidic acid, or water. Shoot length was measured prior to treatment and again after 72 h additional dark growth, so that effects on growth could be assessed. Each tube contained 3 to 4 seedlings and each treatment was triplicated.

Plant Tissue Greening Protocol: Excised cotyledons or coleoptiles of 4-d-old dark-grown etiolated seedlings were placed in solutions of helvolic acid, fusidic acid, or water contained in well-plates for 2 h in dark at 28 °C. Then the plates were transferred to low light intensity ($\sim 100 \mu\text{E m}^{-2}\text{s}^{-1}$) at 28 °C for 72 h. After this treatment period, chlorophyll in the greening tissue segments was extracted with dimethyl sulfoxide (DMSO) and quantified spectrophotometrically (1,11). All treatments and controls were performed in triplicate.

Germination and Early Growth: Hemp sesbania, sicklepod, mung bean, wheat and cucumber seeds (10-20 each) were placed in 0.50 ml helvolic acid, fusidic acid, or water and allowed to imbibe for 24 h in dark. The germination (%) was then determined and the imbibed seeds were transferred to petri dishes for 48 h additional growth in dark at 28 °C. After the additional 48 h (total 72 h) period, total seedling length for each species was measured. All treatments and controls were set-up in triplicate.

Electrolyte Leakage Studies: The effects of these two compounds on electrolyte leakage was examined in corn (*Zea mays* L.) and giant ragweed (*Ambrosia trifida* L.). Ten disks (4 mm) were cut from greenhouse-grown corn and giant ragweed plants and placed in petri dishes with MES buffer (pH 6.8) containing helvolic acid, fusidic acid, or water; incubated 28 °C in continuous light ($\sim 150 \mu\text{E m}^{-2}\text{s}^{-1}$). Electrolyte leakage was determined via conductivity measurements of the bathing solutions at 0, 48, and 72 h after treatment. All treatments and controls were set-up in triplicate.

Statistical Treatment: All tests were performed in triplicate and were repeated. Differences between treatment means were compared at the 5% level of significance using Fisher's

RESULTS AND DISCUSSION

When helvolic acid and fusidic acid solutions were applied via spraying onto the foliage of five plant species [(hemp sesbania [*Sesbania exalta* (Raf.) Rydb.], sicklepod (*Cassia obtusifolia* L.), mung bean (*Vigna radiata* L.), and wheat (*Triticum aestivum* L.)], fusidic acid exhibited the most phytotoxicity (Figure 2A.), fusidic acid exhibited the most phytotoxicity (Figure 2A). This compound caused significant shoot inhibition in hemp sesbania, wheat and sorghum. Helvolic acid at a lower concentration, only significantly inhibited the sicklepod shoot elongation. Inhibition of shoot elongation by these two chemicals ranged from 6 to 19 % at 72 h after treatment. Root-feeding tests of these compounds on seedlings of these five species also demonstrated phytotoxicity (reduction of seedling growth) of these compounds (Figure 2B). Inhibition of shoot growth under these conditions occurred in fusidic acid-treated hemp sesbania, sicklepod, mung bean and sorghum (Figure 2B). The helvolic acid and fusidic acid did not reduce the growth of wheat. Other steroidal compounds causes growth inhibition in many plants seedlings, when applied to roots and foliage (13).

Seed germination and early seedling growth

Neither helvolic acid nor fusidic acid significantly affected the seeds germination of these five species, 24 to 48 h after initiation of imbibition (data not shown). However, when these seeds that had imbibed solutions of helvolic and fusidic acids were allowed to grow under dark for 72 h following the imbibition treatment, both compounds caused significant growth inhibition in all species (Figure 2C). Overall, fusidic acid caused the most severe growth reduction in shoot elongation (30 to 80 % reduction) compared to control. Helvolic acid reduced growth from 5 to 50 % as before, it should be pointed out that helvolic acid concentrations used were lower than fusidic acid.

Greening tissues of test species

When excised, etiolated tissues of five plant species were immersed in solutions of these two steroidal compounds and then exposed to low light levels, substantial deleterious effects on greening of tissues occurred. Chlorophyll analysis of these treated tissues showed that helvolic acid caused significant reduction in total chlorophyll levels only in mung bean, but fusidic acid significantly reduced the synthesis and accumulation of this pigment in tissues of all five species (Figure 3). Helvolic acid interferes with chlorophyll biosynthesis (2). Saponins and related steroids also caused similar effects on greening plant tissues (13).

Electrolyte leakage

Because steroidal compounds can reduce the growth and the greening of plant tissues (13) and some of these compounds also disrupts membrane synthesis and function in microorganisms (7,16), the effects on membranes were assessed using two plant species (Figure 4). Fusidic acid exhibited more phytotoxicity and caused substantial increases in electrolyte leakage (as measured by increased conductivity) in both corn and giant ragweed leaf disks. Helvolic acid caused increased electrolyte leakage in giant ragweed,

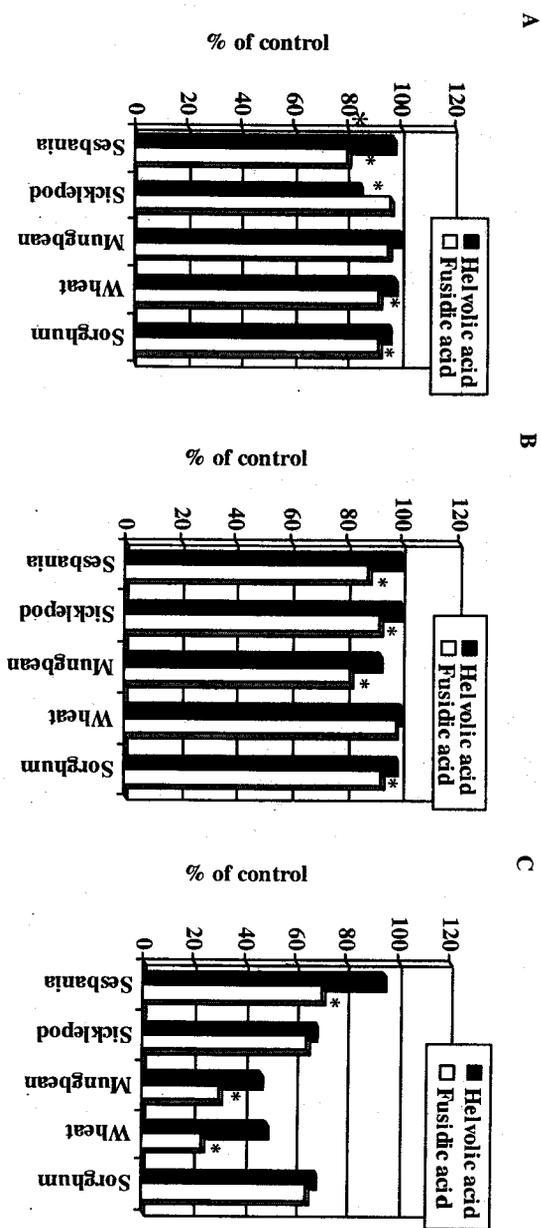


Figure 2. Effects of helvolic acid and fusidic acid applications on the growth of several plant species. (A) Effects of spray applications of helvolic acid (0.25 mM) and fusidic acid (0.50 mM) on shoot elongation of test species grown in dark 72 h after treatment. (B) Effects of root-feeding applications of helvolic acid (0.25 mM) and fusidic acid (0.50 mM) on shoot elongation of test plants grown in the dark, 72 h after treatment. (C) Effects of seed imbibition applications of helvolic acid (0.25 mM) and fusidic acid (0.50 mM) on shoot elongation of test plants grown in dark, 72 h after imbibition. An asterisk (*) above a histogram bar indicates value is significantly different than control (Fisher's LSD test, 5% level).

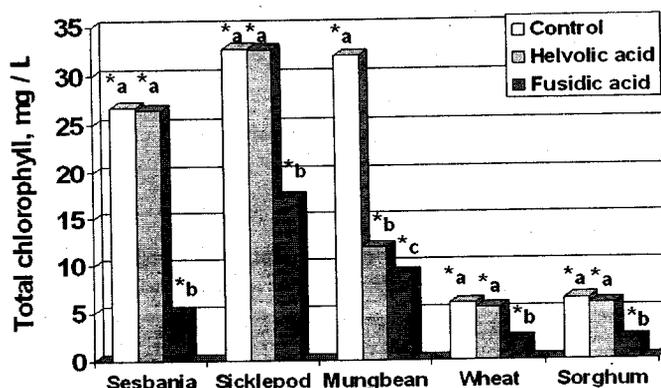


Figure 3. Total chlorophyll content in greening excised etiolated cotyledon or leaf tissues of test plant spp. after treatment with helvolic acid (0.25 mM) and fusidic acid (0.50 mM). An asterisk (*) above histogram bar indicates value is significantly different than control (Fisher's LSD test, 5% level). Different letters above histogram bars for each species, indicate statistically significant differences as determined according to Fisher's protected LSD test at the 5% level.

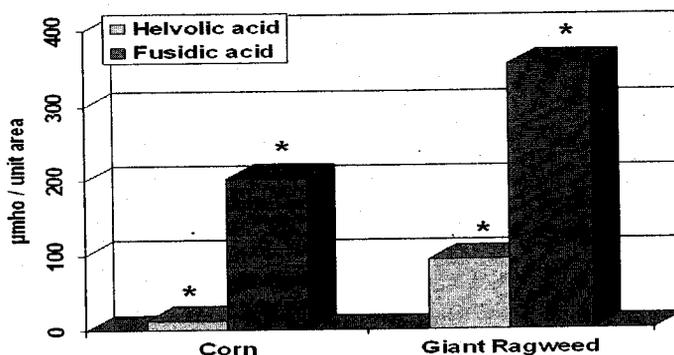


Figure 4. Effects of helvolic acid (0.25 mM) and fusidic acid (0.50 mM) on electrolyte leakage of corn and giant ragweed 72 h after treatment under low light. An asterisk (*) above histogram bar indicates value is significantly different than control (Fisher's LSD test, 5% level).

but not in corn. Helvolic acid increases the electrolyte leakage in a susceptible rice cultivar, but caused lower leakage in a moderately resistant cultivar (17). Various other steroidal-type compounds increases the electrolyte leakage in several weed and crop plant tissues (13).

The overall findings indicate that in addition to their antibiotic properties, both of these steroidal-type compounds possess phytotoxic activity on different plant species using a variety of bioassays. Fusidic acid exhibited more phytotoxicity in these bioassays, however, one reason for this is that the concentration of fusidic acid was twice than

helvolic acid. Different concentrations were used because helvolic acid (free acid) has lower water solubility than the sodium salt of fusidic acid. Differences in phytotoxicity of these chemicals among the various plant species could be due to differential uptake of the compounds, or possibly due to metabolic or detoxification of the compounds in plants. The basis for resistance in prokaryotic and eukaryotic organisms has been attributed to modification of target site, i.e., elongation factors (4,19) and to metabolic detoxification via de-acetylase or esterase enzyme activity (10). Several other steroidal-type compounds are phytotoxic against several plants species (13).

The phytotoxicity of such compounds may allow them to act as virulence factors in plant pathogens that contain such biosynthetic pathways. This is important, especially for pathogens useful as bioherbicides (fungi and bacteria used as weed control agents) because the mechanism of infectivity of such bioherbicides could then be explained and perhaps certain bioherbicide formulations could then be more easily optimized. Fungi that produce these compounds have inherent resistance to these chemicals, thus there is potential to increase fungal weed biocontrol agent efficacy by supplementation of propagule formulations with these compounds. There is a need to improve the efficacy of formulations of weed specific microbial weed control agents and to find and identify allelochemical compounds useful for weed control (12). Other steroidal compounds have also been shown to play a regulatory role in the pathogenesis of root disease in cereal crops (15) and to deleteriously alter the activity and growth of certain rhizobacteria (21). Recently an antibacterial hydroxyl-fusidic acid analogue was discovered and isolated from *Acremonium crotocinigenum*, a phytopathogen of certain trees species (8).

These bioassay results demonstrate some phytotoxic effects in various weed and crop species. These findings are also important in understanding the pathogen action and the impact of these secondary allelochemical metabolites in soils, rhizospheres and the phylloplane.

REFERENCES

1. Barnes, J.D., Balaguer, L., Manriques, E., Elvira, S. and Davison, A.W. (1992). A reappraisal of the use of DMSO for the extraction and determination of chlorophylls *a* and *b* in lichens and higher plants. *Environmental and Experimental Botany* **32**: 85-100.
2. Berestetsky, O.A., Patyka, V.F. and Nadkernichnyi, S.P. (1974). Phytotoxic properties of *Aspergillus fumigatus*. *Mikrobiologicheskii Zhurnal (Kiev)* **36**: 581-568.
3. Chain, E., Florey, H.W., Jennings, M.A. and Williams, T.I. (1943). Helvolic acid, an antibiotic produced by *Aspergillus fumigatus*, mut. *helvola* Yuill. *British Journal of Experimental Pathology* **24**: 108-119.
4. Chopra, I. (1976). Mechanisms of resistance to fusidic acid in *Staphylococcus aureus*. *Journal of General Microbiology* **96**: 229-238.
5. Cole, R.J. and Cox, R.H. (1981). *Handbook of Toxic Fungal Metabolites*. Academic Press, New York, USA. Pp. 806-809.
6. Dabbs, E.R. (1987). Fusidic acid resistance in *Rhodococcus erythropolis* due to an inducible extracellular inactivating enzyme. *FEMS Microbiology Letters* **40**: 135-138.
7. Defago, G. and Kern, H. (1983). Induction of *Fusarium solani* mutants insensitive to tomatine: their pathogenicity and aggressiveness to tomato fruits and pea plants. *Physiological Plant Pathology* **22**: 29-37.
8. Evans, L., Hedger, J.N., Brayford, D., Stavri, S., Smith, E., O'Donnell, G., Gray, A.I., Griffin, G.W. and Gibbons, S. (2006). An antibacterial hydroxy fusidic acid analogue from *Acremonium crotocinigenum*. *Phytochemistry* **67**: 2110-2114.

9. Godtfredsen, W.O., Johnson, S., Lerck, H., Roholt, K., Lybring, L. (1982). Fusidic acid: a new antibiotic. *Nature* **193**: 987-991.
10. von der Haar, B., Walter, S., Schwäpenheuer, S., and Schrempf, H. (1997). A novel fusidic acid resistance gene from *Streptomyces lividans* 66 encodes a highly specific esterase. *Microbiology* **143**: 867-874.
11. Hiscox, J.D. and Israelstam, G.F. (1979). A method for extraction of chlorophyll from leaf tissue without maceration. *Canadian Journal of Botany* **57**: 1332-1334.
12. Hoagland, R.E. (2001). Microbial allelochemicals and pathogens as bioherbicidal agents. *Weed Technology* **15**: 835-857.
13. Hoagland, R.E., Zablotowicz, R.M. and Reddy, K.N. (1996). Studies of the phytotoxicity of saponins on weed and crop plants. In: *Saponins Used in Food and Agriculture*. (Eds., G.R.Waller and K. Yamaski) pp. 57-73. Plenum Press, New York, USA.
14. Hoagland, R.E. (1995). A hydroponic seedling bioassay for the bioherbicides *Colletotrichum truncatum* and *Alternaria cassiae*. *Biocontrol Science and Technology* **5**: 251-259.
15. Kintia, P.K., and Lupashku, G.A. (1996). Regulatory effects of saponins in the pathogenesis of root rots in cereal crops. In: *Saponins Used in Food and Agriculture* (Eds., G.R.Waller and K. Yamaski) pp. 75-82. Plenum Press, New York, USA.
16. Nisius, A. (1988). The stromacentre in *Avena* plastids: An aggregation of β -glucosidase responsible for the activation of oat-leaf saponins. *Planta* **173**: 474-481.
17. Sakthivel, N., Amududha, R. and Muthukrishnan, S. (2002). Production of phytotoxic metabolites by *Sarocladium oryzae*. *Mycological Research* **106**: 609-614.
18. Steinkraus, G.E. and McCarthy, L.R. (1979). *In vitro* activity of sodium fusidate against anaerobic bacteria. *Antimicrobial Agents and Chemotherapy* **16**: 120-122.
19. Tanaka, N., Kawano, G. and Kinoshita, T. (1971). Chromosomal location of a fusidic acid resistance marker in *Escherichia coli*. *Biochemical and Biophysical Research Communications* **42**: 564-567.
20. Tschén, J.S.-M., Chin, L.-L., Hsieh, S.-T. and Wu, T.-S. (1997). Isolation and phytotoxic effects of helvolic acid from plant pathogenic fungus *Sarocladium oryzae*. *Botanical Bulletin of Academia Sinica* **38**: 251-256.
21. Zablotowicz, R.M., Hoagland, R.E. and Locke, M.A. (1996). Effects of saponins on the growth and activity of rhizosphere bacteria. In: *Saponins Used in Food and Agriculture*. (Eds., G.R.Waller and K. Yamaski) pp. 83-95. Plenum Press, New York, USA.