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Interaction of Organic Solvents with the Green Alga *Chlorella pyrenoidosa*

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Solvents are often a component of bioassay systems when water-insoluble toxicants are being tested. These solvents must also be considered as xenobiotics and therefore, as potential toxicants in the bioassay. However, the effects of solvents on the organisms being tested and their possible interaction with the test compound are often overlooked by researchers. Although simple toxic effects can be corrected by the use of proper solvent controls, interactions between the solvent and test compound that lead to synergistic or antagonistic responses cannot be easily corrected for and can lead to erroneous toxicity data. Relatively few reports are available on the comparative toxicity of solvents towards test organisms, and these deal primarily with fish and aquatic invertebrates (Majewski et al. 1978; Bowman et al. 1981). Data for microbial systems are more limited (Stratton 1985), with some data available for algae (Rowe et al. 1982), and slightly more for blue-green algae (Stratton 1987) and fungi (Stratton 1985). Solvent-pesticide interactions in bioassays have been well documented (Stratton et al. 1982), and a procedure has been developed to identify and eliminate these effects from bioassays (Stratton et al. 1982). This method has been used to study the effects of various parameters on solvent-pesticide interactions in fungal bioassays (Stratton 1985), but detailed data are still limited for other microbial systems. The purpose of the present study was to compare the inhibitory effects of six solvents commonly used in pesticide bioassays towards growth of the common green alga Chlorella pyrenoidosa, and to examine the occurrence of solvent-pesticide interactions with this organism.

MATERIALS AND METHODS

The green alga Chlorella pyrenoidosa was used as the test organism and was obtained from the National Research Council of Canada, Atlantic Research Laboratory, Halifax, Nova Scotia, Canada. Axenic stock cultures were grown in 250-mL Erlenmyer flasks sealed with cotton bungs and containing 150 mL of an inorganic, nitrogen-free medium (Stratton 1987) supplemented with 1.5 g/L of $NaNO_3$. Flasks were incubated at 25±1°C and a light intensity of

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7000 lux on a 12 hour light-dark cycle.

The solvents employed included acetone, methanol, hexane (glass distilled, pesticide grade, Caledon Laboratories, Georgetown, Ontario, Canada), ethanol (absolute, Commercial Alcohols Ltd., Gatineau, Quebec, Canada), dimethyl sulfoxide (DMSO), and N,N-dimethylformamide (DMF) (both reagent grade, Fisher Scientific, Fair Lawn, New Jersey, U.S.A.). All solvent concentrations are given as percent (%) volume-volume. The herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine, technical grade, Ciba-Geigy Canada Ltd., Cambridge, Ontario, Canada; >95% pure) was used in solvent- pesticide interaction studies. Atrazine concentrations are given as ppm (ug/mL) of active ingredient.

During solvent bioassay experiments test organisms were cultured in test tubes (150 x 25 mm O.D.) in both the presence and absence of solvent, as outlined below. For solvent-pesticide interaction bioassays, atrazine was also added to test systems. Growth was monitored by following the increase in optical density over time for 10 to 14 days using a Turner model 330 spectrophotometer equipped with a universal test tube adapter and appropriate filters. All test tubes were optically standardized prior to inoculation and were incubated in racks inclined at a 45^{o} angle under the same environmental conditions as the stock cultures. The most suitable wavelength to use for monitoring culture growth was 660 nm, as determined using the method of Sorokin (1973). Cell concentrations were determined microscopically using a haemocytometer. Each solvent was assayed towards the growth of C. pyrenoidosa at five to ten concentrations ranging from 0.1 to 6.0% for acetone, 0.4 to 3.0% for ethanol, 2.0 to 6.0% for methanol, 1.0 to 8.0% for hexane, 0.1 to 4.0% for DMSO, and 0.02 to 1.5% for DMF. Each solvent concentration was replicated five to ten times. Appropriate control systems containing no solvent were included in each experiment. Bioassay systems contained 9.5 mL of growth medium, an appropriate volume of solvent, and 0.5 mL of inoculum standardized to yield an initial cell concentration of 6.5 to 7.8 x 10^{4} cells/mL. Previous studies have shown that inhibitory effects were due to the solvent and not the dilution of growth medium by the ranges of solvent levels employed (Stratton 1987). Incubation parameters were as outlined above. In each experiment, percent inhibition values, relative to growth in control systems, were calculated daily using spectrophotometric data. EC_{50} values (solvent concentration required to cause a 50% reduction in growth) were calculated using linear regression analysis of untransformed percent inhibition versus solvent concentration data. All correlation coefficients were >0.960. Significant differences between solvents were determined using an analysis of variance procedure followed by a Duncan's multiple range test at P = 0.05.

Acetone and ethanol were also used in solvent-pesticide interaction experiments involving the herbicide atrazine. These data were analyzed using the solvent-pesticide interaction

analysis technique, which is fully documented elsewhere (Stratton et al. 1982; Stratton 1985). Here, the bioassay systems and incubation conditions were identical to those described above for solvent toxicity experiments, except that the solvent was replaced by a solvent-atrazine mixture. This was accomplished by first adding an appropriate volume of a stock solution containing atrazine dissolved in either acetone or ethanol, in order to obtain both an initial solvent concentration of 0.1% and an atrazine level of either 0.05, 0.08, 0.1, 0.2, or 0.3 ppm. Solvent concentrations greater than 0.1% were obtained by adding additional solvent directly to the test medium. Ethanol concentrations of 0.1, 0.5, 1.0, and 3.0%, or acetone levels of 0.1, 0.5, 1.0, 2.0, 4.0, and 5.0% were tested in each interaction experiment. Data for each atrazine concentration were analyzed separately, as follows. Net atrazine effects were determined by calculating the percent inhibition in each solvent-atrazine treatment with reference to activity in the proper solvent control (the same concentration of solvent, but without atrazine). Each net atrazine effect was then statistically compared (t-test at P=0.05) with that determined for the lowest solvent level used, which is the reference point for comparison purposes (see Stratton et al. 1982). Values significantly higher or lower than the reference point indicate synergism or antagonism, respectively (Stratton et al. 1982).

RESULTS AND DISCUSSION

Data for the effects of organic solvents towards the growth of C. pyrenoidosa are summarized in Table 1. DMF and ethanol were the most toxic solvents tested, followed by DMSO, hexane, and acetone. Methanol was the least toxic solvent used. These data indicate that DMF and ethanol would not be suitable solvents to use in toxicity bioassays involving algae because of their high toxicity. DMF and ethanol were also found to be the most toxic of six solvents tested towards blue-green algae (Stratton 1987) and fungi (Stratton 1985). However, DMF is often used in toxicity tests with aquatic animals because of its low toxicity towards these organisms (Hughes and Vilkas 1983). Ethanol is often employed in fungal bioassays, but it is not a very effective solvent for use in pesticide systems (Hess 1980). DMSO was less toxic than ethanol or DMF towards C. pyrenoidosa (Table 1), which is also similar to data reported previously for blue-green algae (Stratton 1987) and fungi (Stratton 1985). However, DMSO is equal in toxicity to ethanol when tested towards Chlamydomonas eugametos (Hess 1980), and more toxic than ethanol towards another strain of Chlorella pyrenoidosa (Rowe et al. 1982). Methanol and hexane caused intermediate effects when compared to the other solvents (Table 1), which is again consistent with data for other organisms (Stratton 1985, 1987). However, both methanol and hexane would be poor choices for use in toxicity bioassays, because of their inferior solvent capabilities when compared with DMSO or acetone.

Acetone was relatively low in toxicity towards C. pyrenoidosa (Table 1), which is similar to results obtained with other micro-

Table 1. Comparison of solvent effects towards growth of Chlorella pyrenoidosa

Solvent[a]	Calculated EC_{50}[b]	95% limits[c]	
		lower	upper
acetone	3.02 [d]	2.72	3.32
ethanol	1.18 [e]	0.97	1.39
methanol	3.60 [d,f]	3.46	3.74
hexane	2.66 [f]	1.97	3.35
DMSO	2.01 [e]	1.76	2.26
DMF	0.94 [e]	0.84	1.04

[a] DMSO = dimethyl sulfoxide; DMF = dimethylformamide
[b] Solvent concentration (% v/v) causing a 50% reduction in growth
[c] 95% limits for the calculated EC_{50} (% v/v)
[d,e,f] Those EC_{50} values that are followed by the same letter do not differ significantly at P = 0.05

bial systems (Stratton 1985, 1987). Acetone is the solvent of choice in many bioassay systems (Majewski et al. 1978), primarily because of its superior solvent properties. The EC_{50} of acetone towards growth in the blue-green algae Anabaena and Nostoc ranges from 0.4 to 4.4% (Stratton 1987), while levels <0.1 to 0.4% stimulate photosynthesis, nitrogen fixation, and heterocyst formation in Anabaena (Stratton and Corke 1981a). Acetone concentrations >1.0% are usually required for severe inhibition of these processes in Anabaena (Stratton and Corke 1981a). Acetone levels <0.2% usually have no effect on the growth of algae such as Chlamydomonas eugametos (Hess 1980) and Skeletonema costatum (Kleppel and McLaughlin 1980). Acetone concentrations of 3.33% cause severe cytological damage in Chlorella pyrenoidosa (Parasher et al. 1978), but levels up to 1.0% have no effect on photosynthesis in C. pyrenoidosa and Scenedesmus quadricauda (Stratton and Corke 1981b). With fungi, EC_{50} values for acetone range from 2.0 to 12.0% (Stratton 1985). Based upon the data presented here, acetone would be a suitable solvent for use in bioassays employing algae as test organisms.

Acetone and ethanol were also used in solvent-pesticide interaction studies utilizing atrazine as the test pesticide. Atrazine is a photosynthetic inhibitor, and is therefore a useful compound to use in solvent-pesticide interaction studies involving algae. Data for the effects of ethanol-atrazine combinations towards growth in C. pyrenoidosa are summarized in Table 2. Ethanol and atrazine interacted antagonistically at solvent levels greater than 0.1% for all pesticide concentrations assayed, except 0.1 ppm atrazine, where ethanol interacted antagonistically above 0.5%. The magnitude of interaction (the difference in net pesticide effect between the additive solvent range and the highest solvent level used) ranged from 15 to 60%, with the level of magnitude increasing as the atrazine concentration went from 0.05 to 0.30 ppm. Similar results have also been obtained using

Table 2. Effect of ethanol-atrazine combinations on the growth of Chlorella pyrenoidosa.[a]

ethanol concn (%v/v)	atrazine concentration (ppm) 0.05	0.08	0.10	0.20	0.30
0.1	45.5(2.9)[b]	59.4(3.0)[b]	60.2(4.2)[b]	77.1(3.9)[b]	87.8(2.8)[b]
0.5	31.5(2.9)[c]	48.1(3.8)[c]	54.9(3.5)[b]	66.6(3.7)[c]	78.9(4.2)[c]
1.0	28.2(2.9)[c]	28.8(2.8)[c]	35.7(3.0)[c]	52.0(3.8)[c]	71.9(3.8)[c]
3.0	31.3(5.2)[c]	29.1(3.4)[c]	22.9(4.6)[c]	26.5(1.8)[c]	25.7(7.5)[c]

[a] Table entries (net pesticide effect) are mean % inhibition values, followed by the standard deviation in brackets, calculated from growth in control systems containing only the appropriate level of solvent.

[b] These values do not differ significantly (P=0.05) from that calculated for the lowest concentration of solvent and are indicative of an additive interaction response. Each atrazine concentration is considered separately.

[c] These values are significantly lower (P=0.05) than that calculated for the lowest concentration of solvent and are indicative of an antagonistic interaction response. Each atrazine concentration is considered separately.

Table 3. Effect of acetone-atrazine combinations on the growth of Chlorella pyrenoidosa.[a]

acetone concn (%v/v)	atrazine concentration (ppm) 0.05	0.08	0.10	0.20	0.30
0.1	31.7(6.9)[b]	54.0(3.8)[b]	59.7(6.0)[b]	72.9(7.6)[b]	80.6(8.8)[b]
0.5	35.1(3.0)[b]	50.0(3.3)[b]	62.2(2.7)[b]	79.2(1.9)[b]	83.6(2.5)[b]
1.0	37.3(3.9)[b]	47.9(4.4)[b]	59.4(2.7)[b]	78.1(4.1)[b]	82.5(5.1)[b]
2.0	35.6(4.1)[b]	42.9(5.1)[c]	51.8(4.9)[b]	72.6(5.4)[b]	81.4(5.9)[b]
4.0	33.3(6.4)[b]	41.9(5.4)[c]	43.7(7.4)[c]	70.1(4.1)[b]	78.7(7.0)[b]
5.0	29.9(5.0)[b]	42.5(5.6)[c]	31.1(7.4)[c]	57.0(9.6)[c]	63.9(8.1)[c]

[a,b,c] refer to footnotes in Table 2

photosynthesis as a test criterion, in that ethanol and atrazine interact antagonistically above 0.5% ethanol with interaction magnitudes of around 30% (T.M. Smith, unpublished results). Acetone and atrazine also interacted antagonistically towards growth of C. pyrenoidosa (Table 3), but only at solvent levels greater than 4.0 or 5.0%. The only exceptions occurred at atrazine concentrations of 0.05 ppm, where acetone interacted additively at all solvent levels assayed, and 0.08 ppm, where acetone interacted antagonistically above 1.0%. With acetone, the magnitude of interaction ranged from 10 to 30%. When photosynthesis is used as the test criterion, antagonism occurs above 0.5 to 1.0% acetone (T. M. Smith, unpublished results). With both solvents, the EC_{50} for atrazine (calculated using growth data in the additive solvent

concentration range only) was in the range of 0.05 to 0.08 ppm. Therefore, the solvent used in bioassay systems appears to be relatively unimportant, as long as it interacts additively with the compound being tested.

Few other data are available on solvent-pesticide interactions in algae. It has been well established that a solvent and pesticide can interact additively, synergistically, or antagonistically depending upon the solvent, solvent concentration, and pesticide chosen (Stratton et al. 1982; Stratton and Corke 1981 a,b). Any non-additive interactions can adversely affect the toxicity of test compounds. For example, the toxicity of chlordane towards a fresh water teleost can vary up to 7-fold depending upon the solvent used (Dalela et al. 1979). The toxicity of dieldrin and parathion towards aquatic arthropods can be enhanced up to 190-fold by increasing the concentration of methanol or DMSO from 0.4 or 0.5% up to 2.0% (Bowman et al. 1981). Similarly, the EC_{50} of captan towards soil fungi can be modified up to 6-fold and 20-fold by increasing the acetone concentration in the test medium from 0.1 to 1.0% (Stratton et al. 1982). Although it has been recommended that solvent levels in bioassays not exceed 0.1 to 0.5% (USEPA 1975), such guidelines are often inappropriate for microbial bioassays which employ solvent concentrations up to 1.0%, due to problems associated with the use of small test volumes, toxicant solubility, and other technical limitations. However, more data are required on solvent-pesticide interactions in algae before the results outlined here can be adequately explained.

Whenever a solvent must be used in toxicity bioassays, a screening experiment should first be performed in order to choose a solvent with low inherent toxicity towards the organisms being tested. Then an interaction procedure similar to that performed here should be employed to choose a solvent concentration which interacts additively with the toxicant used. Only then can the toxicity data obtained be considered accurate.

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