light $ET_{50\,Light}$ and the effective time for half the leaf disks to sink in the dark $ET_{50\,Dark}$ (see Figure 3).

In the dark, $1/ET_{50\,Dark}$ is proportional to the rate of respiration:

$$1/ET_{50\,Dark} = \text{rate of respiration} = 1/ET_{50RS}$$

for the example presented in Figure 3:

$$1/ET_{50RS} = 1/9.3 \text{ min} = 0.11 \text{ min}^{-1}$$

In the light, $1/ET_{50\,Light}$ is proportional to the rate of photosynthesis minus the rate of respiration since both processes occur in the light:

$$1/ET_{50\,Light} = \text{rate of photosynthesis} - \text{rate of respiration}$$

therefore,

$$\text{rate of photosynthesis} = 1/ET_{50\,Light} + 1/ET_{50\,Dark} = 1/ET_{50PS}$$

for the example presented in Figure 3,

$$1/ET_{50PS} = 1/7 \text{ min} + 1/9.3 \text{ min} = 0.25 \text{ min}^{-1}$$

The ratio of the rates of photosynthesis to respiration is:

$$\text{Photosynthesis/Respiration Ratio} = \frac{1/ET_{50PS}}{1/ET_{50RS}}$$

for the example presented in Figure 3:

$$\text{Photosynthesis/Respiration Ratio} = \frac{0.25}{0.11} = 2.5$$

Students may wish to study the influence of leaf maturity on the photosynthesis to respiration ratio.

Additional Studies

This laboratory exercise lends itself nicely to an open-ended investigatory approach. Often after students complete one of the exercises above, they are asked to design their own experiments to be conducted in subsequent laboratory periods. A great variety of topics can be explored; some suggestions follow:

1. Leaf disk size and geometry may influence the assay procedure. Using different cork-borers, one can vary the diameter of the disk. Some students have studied the effect of leaf thickness on the assay. A variety of paper punches which yield "cutouts" of varying geometry can be studied. Is one geometry more reliable than another with this assay procedure?
2. By placing the test tube rack in a water bath, the influence of temperature on photosynthesis can be investigated. The effect of a 10°C increase in temperature on the rate of a process is called Q_{10}. If a Q_{10} (Salisbury and Ross 1978) of 1.0 is found, the rate limiting factor for photosynthesis would be the photochemical portion of the reaction. Whereas, a Q_{10} of 2.0-3.0 would imply a biochemical rate limiting step.
3. Using buffers varying in pH permits students to study the influence of acidity and alkalinity on photosynthesis. Perhaps they could relate their findings to the acid rain problem.
4. In addition to placing bicarbonate in the assay solution, glucose may also be added. This permits students to study the influence of glucose on the process of photosynthesis. An osmotic agent which is not metabolized such as mannitol or sorbitol should be used as a control.
5. Inhibitors of respiration can be studied using floating leaf disks which are buoyed with O_2 produced by photosynthesis. In the dark, $1 \times 10^{-3}M$ potassium azide will greatly retard the sinking of leaf disks (South and Sung 1980); controls without azide will sink within 30 minutes.

Not only does the floating leaf disk assay for photosynthesis permit students to study the process of photosynthesis in the laboratory, it encourages them to design their own experiments and "do science." In the following article, we describe how this assay procedure may be employed to identify herbicide resistance in weeds when photosynthesis is the site of action of the agent. The evolution of ecotypes resistant to weed killers represents an international problem that could be resolved with the aid of studies by students.

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Photosynthesis

II. An Assay for Herbicide Resistance in Weeds

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Guy L. Steucek

During the last fifty years, the environment has been assaulted with a variety of chemical agents. Some of these have grown to be relatively innocuous, while others are potentially dangerous. Questions arise whether chemical agents in the environment act directly *(in situ)* as mutagens or indirectly as an artificial selective pressure. Yet, there now exists an impressive list of laboratory-confirmed physical and chemical mutagens entering the biosphere. Whether these are responsible for germinal mutations is still in dispute. Spontaneous somatic mutations, however, often are associated with environmental factors (Gardener 1972). When dominant mutant genes in germ cells are expressed, the progeny are immediately affected and obviously display the mutation, unlike somatic mutations which are not heritable. Selective pressures, on the other hand, would merely cause a shift (change) in the frequency of certain genes in the population and be reflected in the survival of tolerant individuals.

Pesticides represent only one class of chemical agents. With intensive and extensive use, some organisms formerly controlled by pesticides are now resistant. It will be helpful to define briefly some of the responses an

organism can have to these agents. *Susceptible* organisms are those that are detrimentally affected by pesticides (Lebaron, H. M. & Gressel J., 1982). *Tolerance* is generally defined as ". . . the natural and normal variability [in response to] pesticides and other agents which exists within a species. . . .". Although tolerance refers to the variability within a species, it may also be used to make comparisons between species. *Resistance*, defined by the same authors, is ". . . a decreased response of a population . . . to a pesticide or control agent as a result of their application." A pesticide used to control plants is called an herbicide. Some plants have evolved tolerance or resistance to herbicides. Tolerant plants metabolize herbicides to nontoxic biochemicals, whereas resistant plants do not. This paper describes a method for identifying herbicide resistant weeds which may be used as research tools to teach some principles of photosynthesis and evolution.

Populations of the same species may respond to herbicides differently. The dissimilar responses are of two types. First, biotype or species variations in *tolerance* can be due to differential root uptake, foliar absorption, translocation, or metabolism (detoxification). Second, plants can be *resistant* due to mutation or artificial selective pressure. Triazine resistant biotypes (R-biotypes) photosynthesize in the presence of this chemical, while susceptible biotypes (S-biotypes) are sensitive to it. Research indicates that this resistance is at the plastid (chloroplast) level (Radosevich 1977). R-biotype plants were not present prior to the introduction of herbicides.

Regardless of whether mutation or natural selection is responsible, a significant number of organisms are now resistant to previously effective control chemicals. The following resistances are known: 428 species of arthropods to insecticides, 81 cases of fungicidal resistance to benzimidazole, 8 cases of resistance to the bactericide streptomycin, and the medical literature is replete with examples of pathogens resistant to former drugs including penicillin (Lebaron and Gressel 1982).

To date, 30 common annual weed species in 18 genera, including 23 dicots and seven monocots previously susceptible to the triazane herbicides have been found to be resistant. All of the above cases coincide with the appearance of organic herbicides in the last 40 years. Since their initial use more than 200 chemical compounds have been introduced to agriculture alone as pesticides.

Triazine Resistant Weeds

Triazine resistant weeds have appeared world wide. Several plants from the United States include: common groundsel *Senecio vulgaris* L. (California, Oregon, Washington); smooth pigweed, *Amaranthus hybridus* L. (Connecticut, Delaware, Maryland, Massachusetts, New York, Pennsylvania, and Virginia) (Figures 1, 2); and common lambsquarter, *Chenopodium album* L. *sensu lato* (Michigan, New York, Pennsylvania, Virginia, Washington, and Wisconsin). It should be noted that the plants from Pennsylvania are distinguishable as *C. album* ssp. *missouriense*, recognized by many authorities as *Chenopodium missouriense* Aellen (Wahl 1954).

Triazine resistant plants can serve as valuable research tools for teaching principles of photosynthesis and genetics. They will undoubtedly be used to elucidate problems in plant biology: inheritance and gene studies (by geneticists); the role of artificial selective pressure (by evolutionists); mechanisms of photosynthesis (by physiologists); possible phenotypic expression (by taxonomists); and transferring resistance characteristics from weeds to crops via protoplast fusion or DNA biotechnology (by genetic engineers). We detail the practical use of triazine resistant plants (R-biotype) in classroom studies at both the high school and undergraduate college levels.

FIGURE 1. Spring seedlings of pigweed *(Amaranthus hybridus)*, R-biotype, in corn seedlings (early June) from a southeastern Pennsylvania farm.

Mechanisms of Triazine Resistance

The s-triazine herbicides are a class of chemicals used for industrial and agricultural weed control. Most are

FIGURE 2. Mature pigweed *(Amaranthus hybridus)*, R-biotype, in dry corn (late September) from a central Pennsylvania farm.

applied to the soil and absorbed by plants through the roots. The triazines are marketed under the names[1]: Simazine (Princep), atrazine (AAtrex), and propazine (Milogard) in the United States. The first triazines were synthesized and screened in the laboratories of J. R. Geigy S. A., Basel, Switzerland in 1952. Currently in the U.S., herbicides account for over 65 percent of all pesticide sales (CAST, Council for Agricultural Science and Technology 1982); triazines constitute a significant portion.

Chloroplasts are plant cellular organelles that compartmentalize enzymes and pigments which function in photosynthesis. Isolated chloroplasts are at least partially autonomous, possessing extrachromosomal nucleic acid (DNA) and are the only location for all molecular events involving capture of solar energy. Photosynthesis consists of two general processes: light dependent production of ATP and NADPH by membrane-bound molecules (pigments, proteins, etc.); and the light independent, "dark," reaction of the stroma (chloroplast ground solution) which utilizes the NADPH and ATP for CO_2 fixation into sugars (Calvin-Benson Cycle). Membrane bound reactions comprise the "Z-scheme" of physiology. They consist of two photosystems (I and II) with two photoreaction centers (Salisbury and Ross 1978).

Chloroplast membranes in resistant weed biotypes have lost the ability to bind S-triazines, which explains their resistance. The mode of action of triazines is to block the photosynthetic process; they inhibit photosystem II (Radosevich, Steinback, & Arntzen 1979). The alleles for triazine resistance are now generally distributed in various species of weeds. A global geographical distribution of resistant weeds makes material accessible to many teachers and researchers.

Choice of Populations: Site and Taxa Selection

Areas where triazine herbicides are used extensively, intensively, and continually are excellent places to begin examining plants for resistant biotypes. Species to select for screening would include those that have the following characteristics: at least partial self-fertility; herbaceous, annual life cycle; rapid development to maturity; and the biotype which normally is sensitive (susceptible) to triazines. A selection of some plant species susceptible to triazines can be found on the herbicide product label. The following is a nonexhaustive list of places to look for R-biotype weeds. (See Table 1.) (1) *No-tillage or minimum-till corn or sorghum fields.* Farmers generally apply the triazines as a preemergence weed killer in early to mid-spring. The appearance of weed seedlings despite the herbicide application may indicate resistance. Variations in control of a particular weed with the same herbicide could also be due to differences in herbicide application, differences in formulation (mixing), age of the herbicide, soil type differences, rate of herbicide breakdown or metabolism (biotic and abiotic factors), depth and time of seed germination, climate etc. Some resistant weeds of corn fields include lambsquarter, smooth pigweed, ragweed (*Ambrosia artemisiifolia* L.) and mustard (*Brassica campestris* L.). (2) *General nurseries, conifer nurseries, orchards, and perennial woody crops.* All are communities where triazines are the traditional herbicides used for weed control. Resistant *Senecio* is common in these situations. (3) *Commercial asparagus patches.* Triazines are the preferred herbicide for asparagus culture. (4) *Total vegetation control.* This approach is used along railroads, roadside maintenance projects, and highways. Areas like these have had few confirmed cases of herbicide resistance, however, R-biotype Summer-Cypress (*Kochia scoparia* L. (Roth)) and Cheatgrass brome (*Bromus tectorum* L.) have been found. (5) *Chemical-fallow.* This practice is common for control of weeds in wheat fields.

Collection of historical data such as previous cropping practice, tillage, land use patterns, herbicide use patterns, etc. may yield information which will assist in designing strategies for preventing future occurrences of resistance.

TABLE 1. Possible sources of triazine herbicide resistant weeds

1. No-till or minimum-till corn or sorghum fields
2. General nurseries, conifer nurseries and perennial woody crops.
3. Commercial asparagus patches.
4. Total vegetation control.
5. Chemical-follow fields.

[1]Disclaimer. No discrimination is intended and no endorsement is implied by authors or their affiliate institutions.

Collection of Material

Several methods have been successfully used to collect material. After choosing a population one can gather seeds as they ripen. These can be stored and later sown in flats in the greenhouse. The plants, including cotyledons, can be used for assessing herbicide resistance. If living material is available, entire plants with a ball of soil containing undisturbed roots can be removed from the field. These can be transported from the site to the greenhouse in a styrofoam ice chest. Flaccid, wilted leaves with low turgor pressure are unsuitable for testing. Field transplanted material can be maintained in the greenhouse until all signs of wilt disappear. Only photosynthetically active tissue will yield reliable results.

A Simple Method of Testing Plants for Herbicide Resistance

A variety of field, laboratory, and greenhouse techniques (Truelove and Hensley 1982) have been devised to recognize herbicide resistant plants. They vary in complexity from laboratory isolation of photosynthetic membranes to direct application of herbicides in field situations. Most involve challenging the plants, tissues, chloroplasts, or organelle components with toxic levels of herbicide while simultaneously recording effects on plant growth, survival, or metabolic functions. A suitable, easy method is the floating leaf-disk technique, (Hensley 1981) described in Part I.

Procedure

1. *Buffer, Infiltration and Bicarbonate Solutions:*

These solutions are made according to the directions provided in Part I.

2. *Plant Material: Cutting Leaf Disks:*

Healthy, photosynthetically active, turgid leaves are selected. Leaves should be of the same age or plastochron. Two sets of leaf disks are excised. Leaf disks are randomly cut from one side of the mid-vein. One set (A) is always from the laminar area left of the midvein, the second set (B) is a "mirror image" punched from the opposite side.

3. *Herbicide Resistance Assay:*

Ten leaf disks from each side, A (left) and B (right), are transferred to

each of two tubes: one, A (control), contains 3 ml infiltration solution (buffer plus surfactant). The other, B (experimental), is like tube 1, but also contains herbicide (see Part I). Simazine ($10^{-4}M$) is made by dissolving 24.2 mg of 80 percent active Princep in 1 L of buffer. Since simazine is soluble in alcohol, it is dissolved first in 2 ml of spectral grade methanol which is subsequently added to the buffer solution. Princep can be purchased from farm supply stores. Some carrier will remain as particulate matter in the solution with a small amount of simazine attached.

Both sets are rapidly infiltrated using a vacuum as described in Part I. Rapid infiltration is essential.

After the release of the vacuum the disks sink to the bottom of the solution. Any disks still floating are removed using a cotton swab. Be certain to use separate swabs for retrieving the floating disks in each tube, to avoid contamination; the tissue can be very sensitive to the herbicide.

Add 15 ml fresh control bicarbonate solution (set A) and experimental bicarbonate solution containing herbicide (set B) to the appropriate tubes. For best results, high light intensities should be used. We illuminate tubes at 28°C with a fluorescent light source at an intensity of three kilolux.

Photosynthetically generated oxygen within the tissues will increase the buoyancy of the leaf disks. Disks float to the surface in the control (set A). If plant material is from an R-biotype plant the experimental tube (set B) will also have floating disks. If the tissue is from an S-biotype plant, the herbicide will inhibit photosynthesis, thereby retarding oxygen evolution; the infiltrated disks remain submerged.

Photosynthesis and Triazine Concentration

The degree of resistance of pigweed to simazine is illustrated in Figure 3 where $1/ET_{50}$, which is used as an index of photosynthesis (see Part I), is plotted as a function of simazine concentrations. Clearly the photosynthesis in the R-biotype is not inhibited by simazine up to $10^{-4}M$ concentrations. Conversely, photosynthesis in the S-biotype is severely inhibited in $10^{-4}M$ solutions.

Additional Studies

Experimental variations might include:

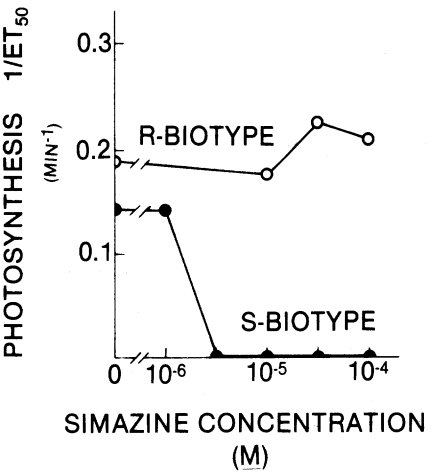

FIGURE 3. Photosynthesis as a function of simazine concentration for resistant (R-BIOTYPE) and sensitive (S-BIOTYPE) pigweed *(Amaranthus hybridus)* plants.

(1) Select species presumed susceptible to triazine from an intensely sprayed area (nursery, roadside, no-till corn field) and determine if some resistant biotypes exist.

(2) Determine differential resistance due to the age of a plant. Using leaf disks from leaves attached to a living plant will allow the plant to continue growing. Samples can therefore be taken over time.

(3) Determine if all plants in a population are resistant.

(4) Determine if all the seedlings from a self-fertile R-biotype also are resistant.

(5) Determine the inheritance of a partially (or facultatively) out-crossing taxon. It has been suggested that resistance is inherited maternally (not by pollen-transfer) or partially maternally with nuclear modifiers (Souza-Machado and Randeen 1982).

(6) Determine cross-resistance to other herbicides of the same (and other) chemical groups.

(7) Determine the ED_{50} (effective dose) of each sensitive biotype. The ED_{50} is the concentration of herbicide at which 50 percent of the disks float.

(8) The authors' population studies indicate that resistant plants first appear as single plants or a few plants scattered in a field. Single, self-fertile plants produce stable duplicates of readily adaptable genotypes. Study of population dynamics of resistance in weed populations is urgent; to date none have been published.

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