

White Clover Background for Teachers:

White clover (*Trifolium repens*) can be used to study the genetic basis of plant diversity, as well as the concepts of coevolution and natural selection. White clover shows natural variation for cyanogenesis, which is the release of hydrogen cyanide (HCN) as a result of tissue damage. In natural populations, both cyanogenic and acyanogenic plants can be found. The cellular and biochemical basis of clover cyanogenesis has been known for nearly a century, and clover's cyanogenesis polymorphism has long been used as a model system for studying how and why natural selection maintains genetic variability in natural populations (reviewed by Hughes 1991, Olsen et al. 2007).

The production of poison is a common defense mechanism in plants, and cyanogenesis can be found in diverse plant species, including apples, almonds, lima beans, and flaxseed. In white clover, ecological studies have demonstrated that cyanogenesis is selectively favored as a defense against herbivores; cyanogenic plants are avoided by small herbivores, including slugs, snails, and voles. However, surveys of clover populations around the world have also revealed that the proportion of cyanogenic plants declines in colder climates. Most plants at higher elevations and latitudes are acyanogenic, while those in warmer climates are mostly cyanogenic. Two hypotheses have been proposed to explain why acyanogenic plants are more common in colder climates. First, since frost can result in cell rupture, it is possible that cyanogenic plants suffer from autotoxicity in areas of frequent freezing, caused by cyanide release within the plant tissue. Alternatively, there may be fewer clover herbivores in colder climates; since the production of the compounds required for cyanogenesis is energetically costly (requiring energy that would otherwise go into flowering and seed production), acyanogenic plants may be at a competitive advantage in low-herbivore environments. In either of these cases, both cyanogenic and acyanogenic plants are being selectively maintained within this species.

Two biochemical components must be present for a clover plant to be cyanogenic. The presence or absence of each of these components is controlled by a single gene. One gene, *Ac*, controls a plant's ability to produce cyanogenic glucosides (lotaustralin and linamarin), which are cyanide-containing sugars that release cyanide when acted on by the appropriate enzyme. The glucosides are stored in the vacuoles of leaf and stem cells. A plant must carry at least one dominant *Ac* allele to produce cyanogenic glucosides; a homozygous recessive plant (*acac* genotype) lacks cyanogenic glucosides. For the purposes of this investigation, we refer to the *Ac* gene as "C" for Cyanogenic glucosides.

The second gene, *Li*, determines the presence or absence of the enzyme linamarase, which is required for the hydrolysis of cyanogenic glucosides to release cyanide. Linamarase is stored in the cell wall, so it is only with tissue damage and cell rupture that cyanide release occurs. A plant that carries two recessive *Li* alleles (*lili* genotype) does not produce linamarase. At the molecular level, the *Ac/ac* and *Li/li* polymorphisms have been determined to arise through two unlinked gene deletion polymorphisms (Olsen et al. 2007, 2008). For the purposes of this investigation, we will refer to the linamarase gene *Li* as "E" for enzyme.

The presence of cyanide release can be detected in clover plants using leaf samples and Feigl-Anger test paper (Feigl and Anger 1966). The production of cyanide will cause a bright blue color change in the paper above a test well containing plant tissue that is positive for cyanide. Clover plants found to be acyanogenic can be subjected to further tests to determine if they are positive for the presence of cyanogenic glucosides (*Ac*) or the enzyme linamarase (*Li*), or neither (*acac*, *lili*).

Students will be using a modified Feigl-Anger assay developed by Dr. Kenneth Olsen of Washington University in Saint Louis. By gathering clover plants from their local areas, students will be able to determine the relative percentage of each genotype, compare samples to other locales, and make inferences about the selective forces affecting the clover population in their local climate.

References

- Feigl, F., & Anger, V. (1966) Replacement of benzidine by copperethylacetoacetate and tetra base as spot-test reagent for hydrogen cyanide and cyanogen. *Analyst*, 91, 282–284.
- Hughes MA (1991) The cyanogenic polymorphism in *Trifolium repens* L. (white clover). *Heredity*, 66, 105–115.
- Olsen, K.M., Sutherland, B.L., & Small, L.L. (2007). Molecular evolution of the *Li/li* chemical defence polymorphism in white clover (*Trifolium repens* L.). *Molecular Ecology*, 16, 4180–4193.
- Olsen, K.M., Hsu, S.-C., & Small, L.L. (2008). Evidence on the molecular basis of the *Ac/ac* adaptive cyanogenesis polymorphism in white clover (*Trifolium repens* L.). *Genetics*, 179, 517–526.