combination inhibit diatom growth. This complex contains at least one stimulatory and two inhibitory substances. (i) Inhibitor 1 is peach-colored, has a molecular weight (*12*) between 10,000 and 12,000, has a size between 30 and 50 Å (*13*), and is oily to the touch. (ii) Enhancer 1 is vivid mid-yellow, has a molecular weight between 1,000 and 10,000, has a size between 10 and 29 Å, and is possibly a steroid (*14*); it is masked by inhibitor 1 in full-strength filtrates but not when filtrates are diluted. (iii) Inhibitor 2 is colorless, has a molecular weight less than 500, and is less than 10 Å in size (*15*).

The inhibitory substance is nonvolatile and dialyzable. It retains its activity after extraction with ether, dialysis, or separation by ultrafiltration. It is partially eliminated by freezing. Twofold and fivefold concentrations produce increased inhibition and rapid death, respectively; dilutions to 0.5 times the natural concentration retain activity.

Although determination of the significance of blue-green algal allelopathy to bloom patterning in other lakes awaits further investigation, a minimal basis for generalization can be developed. Filtrates of Linsley blue-green algae were bioassayed against non-Linsley diatoms, and filtrates of axenic, non-Linsley *Nostoc* sp. were bioassayed against Linsley diatoms. In both cases the pattern of inhibition was incomplete. This finding contrasts sharply with the ubiquitous inhibition evidenced when only Linsley organisms were used (*16*). It should serve to caution investigators against using organisms isolated from different lakes because they are given the same taxonomic label as organisms in situ or because they are conveniently available from a culture collection (*17*). Such experiments, intended to parallel and illuminate events in situ, would produce results which only partially reflect events in situ. The degree of correlation of laboratory and in situ results would logically depend upon the degree of physiological, not taxonomic, similarity between organisms.

Allelopathy offers an explanation for the most offensive characteristic of accelerated eutrophication, the rapid takeover of the plankton by blue-green algae. It could also explain the contrasting responses of freshwater and marine ecosystems to excess nutrients. Finally, it suggests an opportunity to ameliorate the negative impact of heavy, prolonged, blue-green algal dominance in eutrophic lakes by intentionally eliminating winter blue-green algal blooms. In this way lake waters would purge themselves naturally of the allelopathic substances left behind by blue-green algae, thus permitting the return of diatoms to the spring bloom. Ultimately, such manipulation of natural populations can lead to biological control of eutrophication.

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2. D. Schwimmer and M. Schwimmer, in *Algae and Man*, D. Jackson, Ed. (Plenum, New York, 1964), p. 368.
3. Fragilariaceae may be less susceptible than other diatoms to blue-green algal allelopathy.
4. Numbers in parentheses after species names are identification numbers for culture collection.
5. Minimal in situ monitoring during years 4 and 5 of the study indicated bloom patterns similar to those of years 3 and 2, respectively.
6. Waters were passed through a 0.45-μm Millipore filter. Half of each sample was autoclaved. Approximately 1 week was required for reequilibration of dissolved CO_2. At inoculation, *p*H values were within 0.1 unit for each pair of filtered-only and filtered and autoclaved portions. Macronutrient (1½ percent ESI) was added to each test sample [L. Provasoli, "Cultures and collections of algae," *U.S.-Japanese Conference, Hakone, 1966* (Japanese Society of Plant Physiologists, Tokyo, 1968)].
7. Bioassayed diatoms included axenic *Nitschia frustulum* (224), axenic *Nitschia* sp. (352), axenic *Synedra famnilica* (202), axenic *Synedra* sp. (299), axenic *Cyclotella* sp. (211), bacterized *Asterionella formosa* (800), bacterized *Fragilaria* sp. (99), and bacterized *Tabellaria* sp. (764). For several tests a greater variety of test diatoms was used.
8. Review articles list varied examples of extracellular metabolite-based inhibitions between organisms [R. Hartman, *Pymatuning Symp. Ecol.* **2**, 38 (1960); R. Pourriot, *Année Biol.* **7–8**, 337 (1966)].
9. A common producer of nuisance blooms in freshwater, *Aphanizomenon flos-aquae* (766), was observed in quantity each July and August. It was never dominant.
10. For additional comment on the growth medium, see (*1*). A "filtrate" is a large-volume culture of a producer blue-green alga. It is harvested by passage through a 0.45-μm filter during early stationary growth.
11. Common marine diatoms, *Skeletonema costatum*, *Thalassiosira fluviatilis*, and *Cyclotella nana*, were bioassayed against filtrates of freshwater blue-green algae. The growth medium was a combination of half filtrate and half double-strength DC medium [L. Provasoli, J. McLaughlin, M. Droop, *Arch. Mikrobiol.* **25**, 392 (1957)]. All the diatoms evidenced a sensitivity to filtrates similar to that of the Linsley bioassay diatoms.
12. Molecular weight is the nominal retention weight, based on 90 percent retention by ultrafilters, of protein molecules of the listed molecular weights.
13. Size is based on the pore size of the ultrafilter or dialysis membrane.
14. Amicon informs users that their UM2 filter binds steroids selectively. Enhancer 1 is bound by UM2 filters.
15. Inhibitor 2 may be an artifact of the bioassay procedures; pending additional characterization or physical separation, it is suspect.
16. Of the four non-Linsley freshwater diatoms tested, none evidenced consistent inhibition in waters collected from Linsley or in filtrates of Linsley blue-green algae. Only three out of eight Linsley bioassay diatoms evidenced consistent inhibition in filtrates of the non-Linsley *Nostoc* sp.; five were neutral or produced results too subtle to be definitive.
17. This restriction is more important in freshwater studies than in marine studies. The surface/volume ratios of lakes far exceed those of the oceans; therefore, lake waters more closely reflect the unique chemistry and climate of their locales. Also, lake waters, and their plankton, tend to be physically isolated from each other.
18. I thank L. Provasoli and G. E. Hutchinson for advice; R. Patrick, S. Golubic, and F. Drouet for assistance with taxonomy; and Haskins Laboratories, Inc., Yale University, and the Department of Environmental Science, Rutgers University, for support. This work was supported in part by Environmental Protection Agency research grant RA 801387.

28 July 1977; revised 19 October 1977

Nitrogen Fixation and Delayed Leaf Senescence in Soybeans

Abstract. *Delayed leaf senescence has been found in a soybean population which maintains its chlorophyll and ribulosebisphosphate carboxylase activity in leaves and nitrogen fixation (acetylene reduction) activity in root nodules throughout seed maturation. Incorporation of delayed leaf senescence into an agronomically desirable genetic background may help to increase seed yield and symbiotic nitrogen fixation during seed development.*

Soybeans [*Glycine max* (L.) Merr.], one of the three major cash crops in the United States, must assimilate large quantities of nitrogen (6.0 kg of N per 100 kg of seed) to produce protein-rich seeds. A recent analysis suggests that soybeans are "self-destructive" in the sense that proteins required for photosynthesis are degraded in the leaf to supply amino acids to developing seeds (*1*). The role of photosynthesis in maintaining symbiotic nitrogen reduction by *Rhizobium* bacteria in legume root nodules has long been known (*2*). The enzyme ribulosebisphosphate carboxylase comprises about 25 percent of the protein in soybean leaves and is primarily responsible for photosynthetic carbon dioxide fixation in soybean leaves. Loss of ribulosebisphosphate carboxylase activity during senescence has been well documented in several plants (*3*). It has been suggested that an increase in nitrogen input during rapid seed development may be achieved by extending the exponential phase of nitrogen fixation (*4*) or by increasing the proportion of photosynthate allocated to support nitrogen fixation (*5*). Another way to increase nitrogen fixation would be to maintain photosynthetically active leaves throughout reproductive growth. This report provides data which demonstrate that certain soybean genotypes have the

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Table 1. Physiological parameters of six F_3 soybean plants produced from seed of an F_2 soybean plant which exhibited green leaves and acetylene-reducing nodules in the field at a time when pods were mature (*10*).

F_3 plant	Total dry wt. (g)	Pod dry wt. (g)	Pod dry matter (%)	Chlorophyll (mg/g, fresh wt.)	Leaf protein (mg/g, fresh wt.)	Ribulosebisphosphate carboxylase: Activity (μmole CO_2 per hour per gram fresh wt.)	Ribulosebisphosphate carboxylase: Protein (mg/g, fresh wt.)	Acetylene reduction (μmole C_2H_4 per hour per gram nodule, dry wt.)
1	74.4	30.8	32.8	1.3	11.7	36.1	2.1	1.4
2	67.4	28.0	32.6	1.6	16.1	54.4	3.3	0.7
3	63.5	25.6	31.6	1.4	14.0	45.8	1.9	1.0
4	84.1	27.5	32.1	0.5	6.1	20.6	0.9	0.7
5	60.0	20.9	28.7	0.5	5.1	16.9	0.7	0.1
6	52.4	19.8	29.1	0.4	4.9	12.0	0.5	0.1

genetic potential to maintain active ribulosebisphosphate carboxylase activity in the leaves and apparent nitrogen fixing activity in root nodules throughout seed development.

Soybeans in symbiotic association with *Rhizobium* bacteria can obtain nitrogen from both the soil and the atmosphere. It was reported that nitrate reductase activity in soybeans, an indicator of soil nitrate assimilation by the plant, was maximum at full-bloom stage and declined rapidly soon thereafter (*6*). Reduction of atmospheric nitrogen by *Rhizobium* within the root nodules, however, complemented nitrate utilization by becoming most active during flowering and early pod-filling and declining during the later stages of seed development. Assuming the existence of a genetic mechanism which regulates enzymatic activity during vegetative and reproductive growth in soybeans, one can infer that alterations in the control of the developmental sequence will occur occasionally. Apparent uncoupling of vegetative and reproductive growth has been reported in peas (*7*), and delayed leaf abscission has been noted in soybeans (*8*).

The possibility that soybeans with delayed leaf senescence might be found which continue to photosynthesize and to reduce nitrogen throughout seed development led us to examine several thousand plants in the F_2 generation of various soybean crosses in the field at Davis, California, in October 1976. Five plants were observed which had brown mature pods at a time when the leaves subtending those pods were completely green. The plant which showed the highest nitrogen fixation activity among the five plants possessing green leaves was selected for further study. At the time of selection this F_2 plant, derived from a cross between the soybean lines Lee 68 and L63-1097, had root nodules which exhibited no detectable hydrogen evolution under ambient conditions and had an acetylene-dependent ethylene production rate of 1.5 μmole/hour per gram of dry nodule.

Seeds of the selected F_2 plant were used to produce 14 F_3 plants under a controlled environment (*9*). Nine plants showed normal senescence and five showed delayed senescence. Three plants of each senescing group were assayed; the results are reported in Table 1. A positive relationship was found between chlorophyll content, total leaf protein, ribulosebisphosphate carboxylase protein, and ribulosebisphosphate carboxylase activity in the leaves and nitrogenase activity in the root nodules, as measured by acetylene reduction at the yellow pod stage. The F_3 plants 1, 2, and 3, which showed delayed senescence, had leaves with high ribulosebisphosphate carboxylase activities and acetylene reduction in the root nodules. Conversely, F_3 plants 5 and 6, which showed normal senescence as it occurs in most soybean cultivars, had low ribulosebisphosphate carboxylase activities in the leaves and little nitrogenase activity in the nodules. Plants showing delayed leaf senescence flowered an average of 1 week earlier and maintained their green leaves 3 weeks later than those showing normal leaf senescence. Delay in leaf senescence did not reduce total dry matter or pod dry weight accumulation.

Seeds from F_3 plants 1 and 5, representing the delayed and normal senescence traits, respectively, were selected to produce F_4 plants. Seven plants of each group were grown under controlled conditions (*9*) until flowering, when a random selection of three progeny from each plant was made. Also, two plants of each original parent, Lee 68 and L63-1097, were grown concurrently. Although the fourth-generation progeny would not be expected to be genetically homogeneous, they were not markedly different and have been averaged together in Table 2 for simplification. The results for the progeny of F_3 plants 1 and 5 (Table 2) were comparable to those for the F_3 plants reported in Table 1.

Maintaining photosynthesis and nitrogen assimilation during a long reproductive period may be crucial for increasing soybean yields. The data reported here demonstrate that certain early-flowering soybeans which possess green leaves during seed maturation have a heritable component which maintains carboxylation activity in the leaves and continued nitrogen fixation throughout the reproductive period of the plant. Incorporation of these characteristics into a favorable genetic background may help to increase seed yield and symbiotic nitrogen fixation during seed development.

Table 2. Physiological parameters of progeny of F_3 plants 1 and 5 (represented in Table 1) and the original parents, Lee 68 and L63-1097 (*10*).

Plant	Chlorophyll (mg/g, fresh wt.)	Leaf protein (mg/g, fresh wt.)	Ribulosebisphosphate carboxylase: Activity (μmole CO_2 per hour per gram, fresh wt.)	Ribulosebisphosphate carboxylase: Protein (mg/g, fresh wt.)	Acetylene reduction (μmole C_2H_4 per hour per gram nodule, dry wt.)
Lee 68	2.5	27.6	19.2	10.2	6.4
L63-1097	1.6	24.3	14.2	7.3	0.0
F_3 plant 1 progeny	3.0 ± 0.2	32.3 ± 1.3	19.3 ± 0.6	8.9 ± 0.4	1.0 ± 0.5
F_3 plant 5 progeny	1.3 ± 0.2	20.7 ± 1.2	6.3 ± 1.3	4.5 ± 0.8	0.0 ± 0.0

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9. Plants were maintained in a growth chamber on a 16/8-hour light/dark cycle for the first 5 weeks and a 9/15-hour cycle thereafter, at temperatures of 27° and 19°C, relative humidities of 60 and 80 percent, and a photosynthetic photon flux density of 800 μE m^{-2} sec^{-1} measured in the photosynthetically active range. Plants were inoculated with *Rhizobium japonicum* strain 61A96 (obtained originally from J. Burton, Nitragen Co., Milwaukee, Wisc.) and grown in vermiculite in 6-liter pots. Plants were watered with a nutrient solution containing 4 m*M* $CoCl_2$, major elements as recommended by E. G. Bollard and reported by E. J. Hewitt [in *Sand and Water Culture* (Technical Communication 22, Commonwealth Agricultural Bureaux, Farnham Royal, Bucks, England)], and trace elements according to C. M. Johnson, P. R. Stout, T. C. Broyer, and A. B. Carlton [*Plant Soil* **8**, 337 (1957)].
10. Ribulosebisphosphate carboxylase activity was determined as described by G. E. Kleinkopf, R. C. Huffaker, and A. Matheson [*Plant Physiol.* **46**, 204 (1970)]. Ribulosebisphosphate carboxylase protein was determined after acrylamide gel electrophoresis and scanning the band in a spectrophotometer as described by B. J. Davis [*Ann. N.Y. Acad. Sci.* **121**, 404 (1964)]. Nitrogen fixation was estimated by acetylene-dependent ethylene production as outlined by G. J. Bethlenfalvay and D. A. Phillips [*Plant Physiol.* **60**, 419 (1977)]. Evolution of hydrogen by root nodules was not detected in any of the F_3 and F_4 plants tested. The parent Lee 68 showed delayed senescence compared to L63-1097, which exhibited normal senescence.
11. This material is based upon research supported by National Science Foundation grant AER 77-07301. Any opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the NSF. This work was performed while S.S.A.S. was a visiting scientist supported by an award from the International Development Research Center, Ottawa, Canada. The authors express appreciation to B. L. Miller for technical help and to B. H. Beard, who allowed us to use plants from his F_2 soybean nursery.
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31 October 1977; revised 30 December 1977

Epidermal Patterns of the Lemma in Some Fossil and Living Grasses and Their Phylogenetic Significance

Abstract. *Morphological study of fossil grass anthoecia of* Berriochloa *and* Nassella *collected from Miocene-Pliocene strata in Kansas has revealed well-preserved epidermal structure. This seems to be the first micromorphological information known from fossil grass floral bracts. The epidermal pattern on the lemma in the fossils and their living counterparts are evidence in support of the view that the North American species of* Stipa *of the section* Hesperostipa *Elias and species of* Piptochaetium *have a common ancestry in* Berriochloa, *and that species of both taxa have been distinct from species of the* Nassella, Oryzopsis, *and other* Stipa *since at least the Miocene or Pliocene.*

Some of the most extraordinary plant fossils are the remains of fossil grass floral bracts (*1*) reported from the Late Tertiary High Plains strata of central North America (*2, 3*). Genera previously described include *Berriochloa* (*3–5*), *Nassella*, and *Paleoeriocoma* of the tribe Stipeae (*6*), and *Panicum* and *Setaria* of the tribe Paniceae (*7*). Elias was one of the first to describe and compare the High Plains fossils with their modern counterparts (*3*). He developed preliminary evolutionary schemes on the basis of features of the floral bracts observable at low magnification (from ×10 to ×33) with a binocular microscope, for example, shape, size, and surface texture.

The classification of the grasses has been revolutionized by the introduction of microscopic characters from the internal and external anatomy and morphology of both vegetative and reproductive organs (*8*). The use of these characteristics, along with some traditional ones of gross morphology, has made it possible to produce a system of classification with high phylogenetic and predictive value. Among the micromorphological features utilized in the "new" systematics have been the features of the floral bracts of grasses (*9*). Collections of fossil grass anthoecia in Kansas have provided specimens that are well enough preserved to allow detailed study of the lemma epidermises. The epidermal features of the lemma can be used in determining relationships between some fossil and living grasses of the tribe Paniceae (*10*). I now present evidence that the epidermal patterns of the lemma, in several fossil and modern grasses of the tribe Stipeae, reflect systematic relationships and evolutionary trends dating from at least the Miocene or Pliocene epochs.

A diverse assemblage of three-dimensional silicified grass anthoecia, as well as borage nutlets, sedge achenes, and hackberry nutlets, have been collected from Tertiary strata in Ellis and Rooks counties, Kansas (*11*). The fossils were found in sands and silts of the Ogallala Formation. I studied the fossil-bearing strata (*5*) and dated them as Miocene to Pliocene on the basis of vertebrates associated with the fossil grasses.

Fossils were picked by hand to free them from their enclosing matrix. Specimens of several described and undescribed fossil species of the grass genera *Berriochloa* and *Nassella* (Fig. 1, A to C) were coated with carbon and gold and examined with a scanning electron microscope (JEOL-35). A similar procedure was unsuccessful with modern counterparts of the fossils because the thick cuticle obscured the epidermal patterns and no entirely successful method was discovered for removing it for an examination of the underlying epidermal pattern. Lemmas of modern species of *Piptochaetium, Stipa, Oryzopsis*, and *Nassella* were cleared with 5 percent NaOH, treated with chlorine bleach, and stained with chlorozol black E. The prepared lemmas were then studied with light microscopy. Epidermal patterns of modern taxa revealed by light microscopy are presented in the form of line drawings rather than photographs so that tissues underlying the lemmatal epidermises do not obscure the essentials of the patterns (Fig. 2).

Among the fossil Stipeae examined were 15 species of *Berriochloa* and 4 species of *Nassella*. The basic lemma pattern of *Berriochloa* is one of long cells (*12*) with strongly sinuous, well-developed lobes (Fig. 1, E to G). Short cells are entirely absent. The observed lemma pattern of fossil *Nassella* contrasts sharply with that of *Berriochloa*. Short cells (silica or suberin cells) are abundant and the basic long cells have been reduced in length (Fig. 1D). Long cells have sinuous walls but not to the extent found in *Berriochloa*.

Included in the modern Stipeae examined were 21 species of *Piptochaetium*, 11 species of *Nassella*, 13 species of *Oryzopsis*, and 38 species of *Stipa*. Examination of the lemmas under a binocular microscope reveals that many extant species of *Piptochaetium* possess ridges and grooves. Higher magnification revealed that the lateral walls of the long cells are strongly developed and project above the central area of the cell (Fig. 2C). The exact mechanism for this modification is unknown. Modern species of *Nassella*, like their fossil counterparts, have an epidermal pattern consisting of greatly reduced long cells and an abundance of short cells (Fig. 2D). Species of *Stipa* examined could be assigned to a number of distinctive types. The most significant to this discussion is the pattern observed on *S. comata* Trin. and Rupr., *S. spartea* Trin., and *S. neomexicana* (Thurb.) Scribn., which com-