Interactions between Symbiotic Nitrogen Fixation, Combined-N Application, and Photosynthesis in *Pisum sativum*

By

GABOR J. BETHLENFALVAY and DONALD A. PHILLIPS

Department of Agronomy and Range Science, University of California, Davis, CA 95616, U.S.A.

(Received 20 June, 1977; revised 16 August, 1977)

Abstract

The effect of photosynthetic photon flux density (PPFD) on nitrogen utilization was determined in peas (*Pisum sativum* L. cv. Alaska) inoculated with *Rhizobium leguminosarum* and treated with nutrient solutions containing no combined nitrogen, 16 m*M* NO_3^-, or 16 m*M* NH_4^+. Plants were grown under controlled conditions at three PPFD values ranging from severely limiting to nearly saturating.

Carboxylation efficiencies and CO_2-exchange rates were highest in the N_2-fixing plants and lowest in plants supplied with NH_4^+, and they generally increased with increasing PPFD. Photoefficiencies increased with PPFD but did not differ appreciably with the form of nitrogen applied. Nitrogen fixation, calculated from C_2H_2-reduction and H_2-evolution data, was inhibited more by NH_4^+ than by NO_3^- application. Inhibition was counteracted by increasing PPFD. Percentage nitrogen decreased with increasing PPFD in plants treated with combined nitrogen and increased in the plants dependent on N_2 fixation.

The data reveal that photosynthetic efficiency and the capacity to fix N_2 in peas are functions of PPFD and the availability of combined nitrogen and that these two factors are interrelated.

Introduction

Inhibition or stimulation of N_2 fixation by high or low concentrations of combined-N in the soil solution is well documented (Gibson 1976, Munns 1977, Wilson 1935). The magnitude of these effects varies with the form of combined-N (Diener 1950, Vigue *et al.* 1977) and its time of application (Havelka and Hardy 1976). Photosynthesis also is influenced directly by the presence of combined nitrogen. The reduction of NO_3^- to NH_4^+ in leaves was shown to depend on light intensity (Beevers and Hageman 1972) and to compete with CO_2 fixation for reductant (Thomas 1976). Ammonia, in addition to its toxic effects on roots (Bennett 1971), also affects leaves (Puritch and Barker 1967). If present in excess of its assimilation, ammonia inhibits energy conservation by uncoupling ATP synthesis (Hewitt 1975, Jagendorf 1975). In view of the interdependence of photosynthesis, N_2 fixation, and combined-N application (Havelka and Hardy 1976), it is of interest to investigate the influence of each of these parameters on the others. This study was made to determine the effect of: (1) the availability of photosynthate on the inhibition of N_2 fixation by combined-N and on N-accumulation; (2) the form of N-source (N_2, or N applied as NO_3^- or NH_4^+) on carboxylation efficiency (Tregunna *et al.* 1966), CO_2 exchange rate (Shibles 1976), and photoefficiency (Bethlenfalvay and Phillips 1977b) and (3) the effects of both photosynthesis and N-nutrition on total plant productivity.

Abbreviations: μE, microeinstein $\cdot m^{-2} \cdot s^{-1}$; PPFD, photosynthetic photon flux density.

Materials and Methods

Pea (*Pisum sativum* L. cv. Alaska) plants were maintained in a growth chamber under a 16/8-h light/dark cycle at 21/15°C, 50/70% relative humidity, and PPFD of 100, 400, or 700 μE. PPFD (Shibles 1976) was measured with an LI-185 quantum sensor (Lambda Instruments) in the photosynthetically active range. Evenly spaced, alternating Sylvania MM400/BU-HOR metal halide and Norelco 160E23/SB/W mercury lamps were used as light sources. Plants were grown with vermiculite in 180-ml plastic pots and watered on alternate days with 100 ml of nutrient solution. Solutions used were N-free (4 m*M* $CaSO_4$, 2 m*M* K_2CO_3, 2 m*M* K_3PO_4, 1 m*M* $MgSO_4$), containing only ammonium-N (4 m*M* $CaSO_4$, 2 m*M* K_2CO_3, 2 m*M* K_3PO_4, 8 m*M* $(NH_4)_2CO_3$), or containing only nitrate-N (4 m*M* $Ca(NO_3)_2$, 2 m*M* KH_2PO_4, 8 m*M* KNO_3, 1 m*M* $MgSO_4$). The N-free and NH_4^+ solutions were adjusted with HCl to the pH of the NO_3^- solution (pH 5.6). Micronutrient

composition was according to Johnson *et al.* (1957). In addition, the solution was 4.2 n*M* in $CoCl_2$. All plants, regardless of N-treatment, were inoculated with *Rhizobium leguminosarum* 128C53 (obtained originally from Dr. J. C. Burton, Nitragin Co., Milwaukee, WI). CO_2 and N_2 fixation were assayed 24 days after planting. Dry weights of plant parts were determined after 24 h at 75°C. Initial seed weight was 0.22 to 0.26 g. Total reduced N/plant was determined by Kjeldahl analysis (Burris and Wilson 1957). Change in dry weight and N-content was computed by substracting average seed weight and N-content from the final plant values. Plants were harvested 24 days after planting, and had seven fully expanded leaves.

Assimilation of CO_2 by attached leaves was measured in a flow-through gas-exchange system with apparatus and data-handling procedures described by Augustine *et al.* (1976). One plant was selected from uniform stands of five replicates for photosynthesis measurements. The design of the CO_2 assimilation chamber permitted parts of the plant to be inserted in sequential steps without damaging the plant. Photosynthetic data on whole plants represent the sum of such sequential measurements. CO_2-exchange rate by the whole plant was determined at the growth PPFD with a CO_2 concentration of 300 μl/l. Photoefficiency measurements were made on the fifth leaf at the saturating PPFD of 1200 μE and the respective growth PPFD. Photoefficiency was calculated as 100× CO_2-exchange rate at growth-light intensity/CO_2-exchange rate at 1200 μE. CO_2-exchange rate was determined in the fifth leaf at four external CO_2 concentrations (50, 100, 150, and 300 μl/l) and a PPFD of 1200 μE regardless of growth PPFD. Internal leaf CO_2 concentrations were calculated from physical parameters (input CO_2 concentration, gas flow rate, leaf temperature, dew point of air entering and leaving assimilation chamber, leaf area) measured at each of the four external CO_2 concentrations (Augustine *et al.* 1976). A second-order regression line was then computer-plotted through the CO_2-exchange rates measured at each internal leaf CO_2 concentration (Augustine *et al.* 1976). The internal CO_2 compensation point (*x*-intercept) was determined by extrapolation. The slope of the regression line at the compensation point, an indication of the leaf's capacity to respond to changes in ambient CO_2 concentrations, was used as a measure of carboxylation efficiency (Tregunna *et al.* 1966). Leaf temperatures were maintained at 21°C in all cases. Leaf area was measured with an LI-3000 area meter (Lambda Instruments).

Acetylene-dependent C_2H_4 production by root nodules was used as a measure of the total flow of electrons through the nitrogenase complex. Hydrogen evolution by root nodules was determined in ambient air and could not be detected in the presence of C_2H_4. Roots were cut 5 mm above the cotyledonary node, freed from vermiculite by gentle shaking, and placed in jars containing an average of 200 ml of free space. Gas samples for H_2 determinations were taken from the jars 30 min after sealing. The jars were then opened, flushed with ambient air, sealed, and adjusted to contain 0.1 atm C_2H_2 in ambient air. Gas samples were taken after 5 and 35 min, and the hourly rate of C_2H_2 reduction was computed from the difference. Production of both H_2- and C_2H_2-dependent C_2H_4 was linear with time under the assay conditions. Controls in which two successive C_2H_2-reduction assays were conducted during the 75 min following root excision showed no significant difference in C_2H_2-dependent C_2H_4 production. It was concluded, therefore, that C_2H_2- and H^+-reduction assays were not affected by a changing physiological condition during the normal assay periods. Ethylene analyses were made with a Perkin-Elmer model 3920B gas chromatograph equipped with a hydrogen-flame-ionization detector. Acetylene and C_2H_4 were separated on a 0.3 × 122 cm column filled with Porapak R, 100–200 mesh. Hydrogen was measured with a thermal conductivity detector attached to the same instrument, after separation on a 0.3 × 254-cm column filled with Molecular Sieve 5A, 60–80 mesh. The carrier gas in both cases was nitrogen at a flow rate of 30 ml/min. Oven temperatures were 45°C for C_2H_4 and 100°C for H_2. Results represent the average of 4 replicates.

Results

Photosynthetic activity, as measured by carboxylation efficiency (Figure 1), CO_2-exchange rate (Figure 2), and photoefficiency (Table 1) in individual leaves at the same stage of development, were generally greater with increasing PPFD. The form of N available to the plant had a marked effect on carboxylation efficiency and CO_2-exchange rate: both were greatest with N_2 as the source of N. Photo-

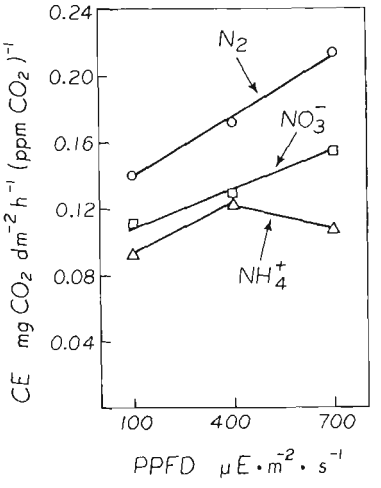

Figure 1. *Carboxylation efficiency* (CE) *in pea plants grown at different photosynthetic photon flux densities* (PPFD) *with* NO_3^- *or* NH_4^+ *or without combined-N* (N_2). Each datum represents the slope (at the CO_2-compensation point) of a regression line through data points determined by four internal CO_2 concentrations and the corresponding CO_2-exchange rates. The fifth leaf in each plant was used in these measurements.

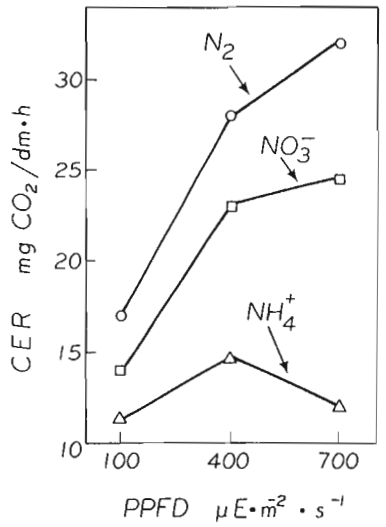

Figure 2. *CO_2-exchange rates* (CER) *in pea plants grown at different photosynthetic photon flux densities* (PPFD) *with NO_3^- or NH_4^+ or without combined-N* (N_2). The fifth leaf in each plant was used in these measurements.

Table 1. *Photoefficiency* (PE) *of pea plants grown at different photosynthetic photon flux densities* (PPFD). PE was computed as 100 × (CER at growth PPFD/CER at saturating PPFD). CER: CO_2-exchange rate. Values in %.

	PPFD, $\mu E \cdot m^{-2} \cdot s^{-1}$		
Nitrogen source	100	400	700
N_2	56	88	93
NO_3^-	54	90	96
NH_4^+	61	87	89

efficiency varied only slightly with the source of N. Carboxylation efficiency and CO_2-exchange rate declined in the NH_4^+-treated plants at the highest PPFD used. The growth-light response of CO_2-exchange rate in whole plants (Figure 3) was similar to that for individual leaves (Figure 2), but the effect of the N-source changed: plants dependent on N_2 fixation had less net CO_2 reduction than plants supplied combined N at 100 and 400 μE. At the highest PPFD, however, NH_4^+-grown plants were again least efficient.

Nitrogen fixation rates (Figure 4) were approximated from C_2H_2-dependent C_2H_4 production and H_2 evolution data (Table 2) by the formula

$$\frac{C_2H_2 \text{ reduced} - H_2 \text{ evolved}}{3}$$

which takes into account the recent finding of H_2 as a significant end-product of nitrogenase activity (Schubert and Evans 1976). Nitrogen fixation increased with PPFD, and

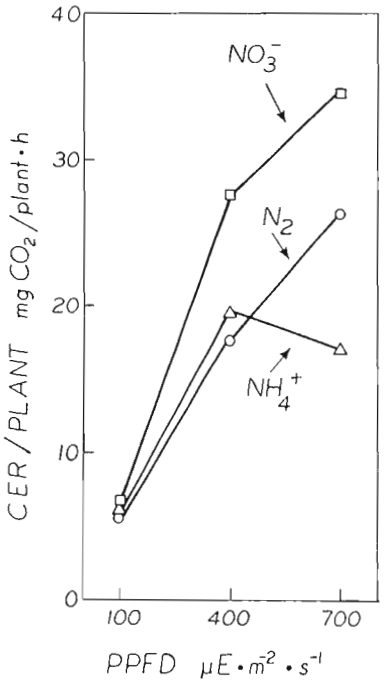

Figure 3. *CO_2-exchange rates* (CER) *in pea plants grown at different photosynthetic photon flux densities* (PPFD) *with NO_3^- and NH_4^+ or without combined-N* (N_2). CER was determined in the entire plant by sequential insertion of plant segments into the assay chamber, and summing the data from all segments.

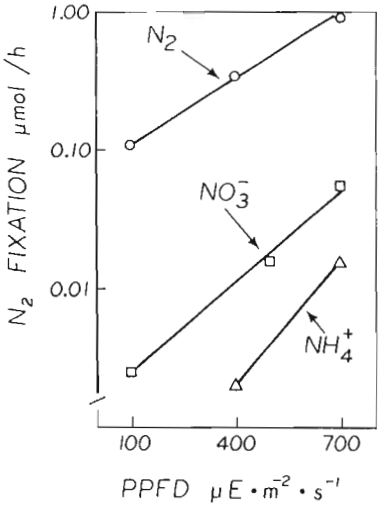

Figure 4. *Nitrogen fixation in pea plants grown at different photosynthetic photon flux densities* (PPFD) *with NO_3^- or NH_4^+ or without combined-N* (N_2). N_2 fixation values were computed from C_2H_2-dependent C_2H_4 and H_2 evolution data by the formula:

$$\frac{C_2H_2 \text{ reduced} - H_2 \text{ evolved}}{3}.$$

Table 2. *Acetylene-dependent ethylene and hydrogen evolution in pea plants grown at different photosynthetic photon flux densities* (PPFD) *with or without combined-N.* Data reflect averages ± SE of 4 replicates. Values in nmol/h.

N-Source	PPFD, $\mu E \cdot m^{-2} \cdot s^{-1}$ 100	400	700
		C_2H_2 reduction	
N_2	355 ± 92	1697 ± 177	3285 ± 245
NO_3^-	11 ± 6	89 ± 18	177 ± 55
NH_4^+	0.0	8 ± 5	85 ± 24
		H_2 evolution	
N_2	0.0	117 ± 61	312 ± 78
NO_3^-	0.0	0.0	17 ± 2
NH_4^+	0.0	0.0	6 ± 1

Table 3. *Dry weight* (mg), *nitrogen content* (mg) *and ΔN/Δ dry weight ratios of pea plants grown at different photosynthetic photon flux densities* (PPFD). Average dry weight and N-content of oven dried seeds were 200 ± 5 mg and 7.8 ± 0.4 mg. Changes in N and dry weight were computed by subtracting seed data from final plant dry weight and N-content. Data reflect averages ± SE of 5 replicates.

N-Source	PPFD, $\mu E \cdot m^{-2} \cdot s^{-1}$ 100	400	700
		Dry weight/plant	
N_2	331 ± 21	587 ± 33	889 ± 38
NO_3^-	353 ± 22	1215 ± 53	1721 ± 93
NH_4^+	324 ± 14	856 ± 93	1116 ± 45
		Reduced nitrogen/plant	
N_2	10.9 ± 0.6	21.7 ± 0.6	33.1 ± 1.9
NO_3^-	14.9 ± 0.7	43.2 ± 1.2	54.8 ± 2.6
NH_4^+	15.8 ± 0.9	35.6 ± 3.6	43.8 ± 2.2
		Δ Nitrogen/Δ dry weight (×100)	
N_2	2.3	3.4	3.7
NO_3^-	4.7	3.5	3.1
NH_4^+	6.2	4.2	3.9

Table 4. *Nodule dry weight* (mg) *in pea plants grown at different photosynthetic photon flux densities* (PPFD) *with or without combined-N.*

N-source	PPFD, $\mu E \cdot m^{-2} \cdot s^{-1}$ 100	400	700
N_2	7.6 ± 1.4	26.7 ± 0.6	36.4 ± 3.0
NO_3^-	0.2 ± 0.0	3.7 ± 0.8	7.0 ± 1.4
NH_4^+	0.2 ± 0.0	1.0 ± 0.2	17.4 ± 2.1

was severely inhibited in plants grown on combined N (Figure 4). This inhibition was partially relieved by increased PPFD. Plant dry weights and reduced N accumulated increased with greater PPFD (Table 3) and showed a response to applied N which was similar to that of whole-plant CO_2-exchange rate (Figure 3) except at the highest PPFD. The $\Delta N/\Delta$ dry weight ratios decreased with increasing PPFD in plants grown on combined N, and increased in N_2-fixing plants (Table 3). Nodule mass increased with PPFD irrespective of N source, but combined-N application reduced the total nodule mass below that of plants dependent on N_2 (Table 4). Nodule numbers counted at the highest PPFD were 246 ± 12, 163 ± 14, and 301 ± 15 in plants with N_2, NO_3^-, or NH_4^+ as N source.

Discussion

The interdependence of photosynthetic activity and the form of available N was demonstrated by the following observations: (1) Higher rates of photosynthesis induced by greater PPFD counteracted the inhibition of N_2 fixation by combined-N (Figure 4) and decreased the $\Delta N/\Delta$ dry weight ratios in combined-N-treated plants (Table 3) but increased N_2 fixation (Figure 4) and $\Delta N/\Delta$ dry weight ratios in plants grown free of combined-N. (2) The form of available N affected photosynthesis by producing markedly different values of carboxylation efficiency and CO_2-exchange rate (Figures 1 and 2). The primary effect of increasing levels of PPFD was an increase in plant dry weight accompanied by a concomitant increase in the amount of N assimilated (Table 3). The decreasing $\Delta N/\Delta$ dry weight ratios (Table 3) in plants treated with combined-N indicate a more rapid C-assimilation relative to N-uptake at higher levels of PPFD. The development of N_2 fixation in plants treated with combined-N and grown at higher PPFD (Figure 4) suggests that the larger and relatively more N-deficient biomass (Table 3) of these plants represents a stronger sink for nitrogenous products than that of plants grown in dim light, and that this sink strength is a factor in controlling N_2 fixation as a source of additional N. Under the conditions of this experiment nodule function rather than nodule formation was affected by the N-source, as evidenced by the large numbers of nodules with very low nitrogenase activity in combined-N-treated plants. Sink limitation may be one of the factors influencing this low level of activity.

In the absence of combined-N the seedlings must rely on seed reserves for N until the onset of N_2 fixation, during the second week after planting (Bethlenfalvay and Phillips 1977a). Early plant development is delayed accordingly (Gibson 1976), as shown also by lower CO_2-exchange rate/plant in the absence of combined-N application (Figure 3). Limiting photosynthate availability at low PPFD results in lower levels of N_2 fixation (Bethlenfalvay and Phillips 1977b) and thus lower $\Delta N/\Delta$ dry weight ratios (Table 3). The light-dependent changes in $\Delta N/\Delta$ dry weight ratio thus appear to be an indication of different mechanisms for plants

grown with or without combined N. For the former it may be regarded as a measure of sink-strength for nitrogen with low (PPFD-dependent) values indicating stimulation of N_2 fixation as an additional source of N-uptake; for the latter low values indicate photosynthate limitation on N_2 fixation.

Lower values of carboxylation efficiency and CO_2-exchange rate (Figures 1 and 2) in single leaves of plants treated with NO_3^- as compared to leaves on plants relying solely on N_2-fixing symbionts may be ascribed to competition (Thomas *et al.* 1976) for electrons between the CO_2 and NO_2^- reduction mechanisms in the chloroplast. The difference in carboxylation efficiency and CO_2-exchange rate values between the NH_4^+-treated and combined N-free plants was probably due to NH_4^+ inhibition at the relatively high levels of NH_4^+ used. This effect was most severe at the highest PPFD, as shown by the declines in carboxylation and CO_2-exchange rate (Figures 1 and 2). The higher leaf temperature and transpiration rate under this condition probably increased NH_4^+ influx and accumulation to toxic levels in the leaves. Photoefficiency, a measure of the capacity of the light-capture mechanism to respond to different PPFD, increased markedly with PPFD but varied little with the source of N (Table 1), indicating that, in contrast to the carboxylation mechanisms, the photosynthetic pigment system and its associated proteins are functionally independent of the source of N available to the plant.

This material is based upon research supported by the National Science Foundation under Grant No. AER 77-07301 and PCM 76-23472. 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 National Science Foundation.

References

Augustine, J. J., Stevens, M. A., Breidenbach, R. W. & Paige, D. F. 1976. Genotypic variation in carboxylation of tomatoes. — Plant Physiol. 57: 325–333.

Beevers, L. & Hageman, R. H. 1972. The role of light in nitrate metabolism in higher plants. — Photophysiology 7: 85–113.

Bennett, A. C. 1971. Toxic effects of aqueous ammonia, copper, zinc, lead, boron, and manganese on root growth. — *In* The Plant Root and its Environment (E. W. Carson, ed.), pp. 669–683. Charlottesville, Virginia. The Univ. Press of Virginia.

Bethlenfalvay, G. J. & Phillips, D. A. 1977a. Ontogenetic interactions between photosynthesis and symbiotic nitrogen fixation in legumes. — Plant Physiol. 60: 419–421.

— — 1977b. Effect of light intensity on the efficiency of CO_2 and N_2 reduction in Pisum sativum L. — *Ibid.* In press.

Burris, R. H. & Wilson, P. W. 1957. Methods for measurement of nitrogen fixation. — *In* Methods in Enzymology (S. P. Colowick and N. O. Kaplan, eds.), Vol. 4, pp. 355–366.

Diener, T. 1950. Über die Bedingungen der Wurzelknöllchenbildung bei Pisum sativum L. — Phytopathol. Z. 16: 129–170.

Gibson, A. H. 1976. Recovery and compensation by nodulation legumes to environmental stress. — *In* Symbiotic Nitrogen Fixation in Plants, Int. Biol. Programme, Vol. 7 (P. S. Nutman, ed.), pp. 385–403. Cambridge University Press.

Havelka, U. D. & Hardy, R. W. F. 1976. Legume N_2 fixation as a problem in carbon nutrition. — *In* Proc. 1st Int. Symp. on Nitrogen Fixation, Vol. 2 (W. E. Newton and C. J. Nyman, eds.), pp. 456–475. Washington State University Press.

Hewitt, E. J. 1975. Assimilatory nitrate-nitrite reduction. — Annu. Rev. Plant Physiol. 26: 73–100.

Jagendorf, A. T. 1975. Mechanism of photophosphorylation. — *In* Bioenergetics of Photosynthesis (Govindjee, ed.), pp. 413–492. Academic Press, N.Y.

Johnson, C. M., Stout, P. R., Broyer, T. C. & Carlton, A. B. 1957. Comparative chlorine requirements of different plant species. — Plant Soil 8: 337–353.

Munns, D. N. 1977. Mineral nutrition and the legume symbiosis. — *In* A Treatise on Nitrogen Fixation, Vol. IV. (R. F. W. Hardy and A. H. Gibson, eds.), pp. 353–391. John Wiley and Sons, N.Y.

Puritch, G. S. & Barker, A. V. 1967. Structure and function of tomato leaf chloroplasts during ammonium toxicity. — Plant Physiol. 42: 1229–1238.

Schubert, K. R. & Evans, H. J. 1976. Hydrogen evolution: A major factor affecting the efficiency of nitrogen fixation in nodulated symbionts. — Proc. Natl. Acad. Sci. U.S.A. 73: 1207–1211.

Shibles, R. 1976. Terminology pertaining to photosynthesis. — Crop Sci. 16: 437–439.

Thomas, R. J., Hipkin, C. R. & Syrett, P. J. 1976. The interaction of nitrogen assimilation with photosynthesis in nitrogen deficient cells of Chlorella. — Planta 133: 9–13.

Tregunna, E. B., Krotkov, G. & Nelson, C. D. 1966. Effect of oxygen on the rate of photorespiration in detached tobacco leaves. — Physiol. Plant. 19: 723–733.

Vigue, J. T., Harper, J. E., Hageman, R. H & Peters, D. B. 1977. Nodulation of soybeans grown hydroponically on urea. — Crop Sci. 17: 169–172.

Wilson, P. W. & Wagner, F. C. 1935. Combined nitrogen and the nitrogen fixation process in plants. — Trans. Wis. Acad. Sci. Arts Lett. 30: 43–50.