

Alterations in Chloroplast Ultrastructure and Chlorophyll Content in Rust-Infected Pinto Beans at Different Stages of Disease Development

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ABSTRACT

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An increase in chlorophyll level compared to healthy controls was detected from the time of the appearance of chlorotic flecks caused on bean leaves by *Uromyces phaseoli* until the end of symptom development. A similar pattern was found in the changes of carotenoid level. This may provide an explanation for the appearance of green islands. Seven days after inoculation, aggregation of cell organelles was observed around the haustoria at the center of infection sites. Starch accumulation was found in plastids of cells containing fungal haustoria as well as in the plastids of neighboring cells. The starch accumulation spread as the colonized area enlarged. By the 14th day, the starch grains had disintegrated in the center

of green islands and chloroplast degradation had begun in the cells around their margins. Twenty-three days after inoculation, chloroplasts of these cells were completely disrupted. Degradation of chloroplasts also was observed in the central zone, but even at that stage some thylakoids were seen near the center of the colony. Chloroplasts in the green islands of leaves kept under low light intensity appeared normal but contained abundant peripheral reticula. Comparison of Hill activity of chloroplasts isolated from infected and uninfected tissues revealed that the electron transport chain of chloroplasts is more stable in the infected tissue than in that of the controls.

Additional key words: *Phaseolus vulgaris*.

Tissue around the infection sites of different biotrophs shows increased metabolic activity in both compatible and incompatible combinations. It is well known that infections by rust fungi increase nutrient movement toward the infection sites, and it has been suggested that changes in cytokinin level might account for altered transport and accumulation of materials (7,10,26,32). Formation of green islands around the infection sites of obligate parasites is a generally observed phenomenon. Investigators dealing with rust-infected plants disagree upon the pattern of alterations in the level of chlorophyll (16,37), the integrity of chloroplasts (5,7,22,28,29) and the intensity of CO₂ fixation (19,38) as well as the mode of formation of green islands (2,9,13,26). It was also not clear whether the chlorophyll content decreases when the first symptoms (chlorotic flecks) appear and whether it increases at the green island stage.

The purpose of the present work was to monitor changes in chlorophyll level and ultrastructure of chloroplasts in tissues at and surrounding infection sites in bean plants infected by rust in relation to symptom development.

MATERIALS AND METHODS

Plant material. Bean plants (*Phaseolus vulgaris* L. 'Pinto') were grown in the greenhouse at about 24 C. In some experiments, the plants were kept under low light conditions. Primary leaves were inoculated with uredospores of *Uromyces phaseoli* (Pers.) Wint. when the plants were 2 wk old and incubated for 20 hr under a plastic film.

Electron microscopy. Samples were taken from the infected leaves 7, 9, 14, and 23 days after inoculation. Strips (2 × 6 mm) were cut along the diameter of infection sites. The samples were fixed in Karnovsky's solution (15), postfixed with OsO₄ and dehydrated. After dehydration the strips were embedded, parallel to the axis of capsule, in Araldite. At various distances from the infection center, thin transverse sections were cut using a Tesla BS 478 ultramicrotome, stained with uranyl acetate in 50% ethanol and

lead citrate (27), and examined with a JEOL 100 B electron microscope.

Pigment determination. For pigment determinations, 10 3-mm-diameter disks were taken from primary leaves of healthy and rust-infected plants. The leaf disks were ground in acetone with a pestle and mortar and then the pigments were transferred to peroxide-free ethyl ether. Chlorophyll content was spectrophotometrically determined using the multi-wavelength method (11). Carotenoid content of samples was determined from the optical density at 480 nm on the basis of the molar extinction coefficient of β-carotene (12) and corrected for the absorption of chlorophyll-*b* at this wavelength.

Hill-activity assay. Hill activity (36) was determined in chloroplasts isolated from the green area of rusted leaves 13 days

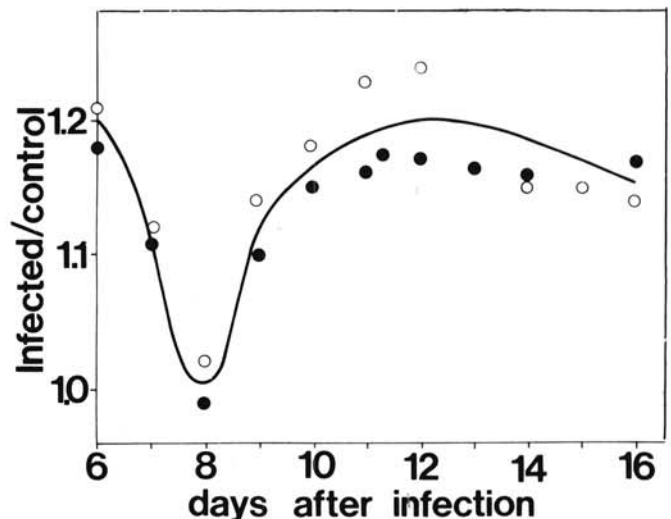


Fig. 1. Changes in the chlorophyll content of leaf tissue at various stages of the disease. Each value represents an average of three determinations. The open and closed circles represent the results of two separate experiments. The total chlorophyll content of control tissue on the 6th day after inoculation was 25.65 nmol/100 mm² in the first, and 28.40 nmol/100 mm² in the second experiment.

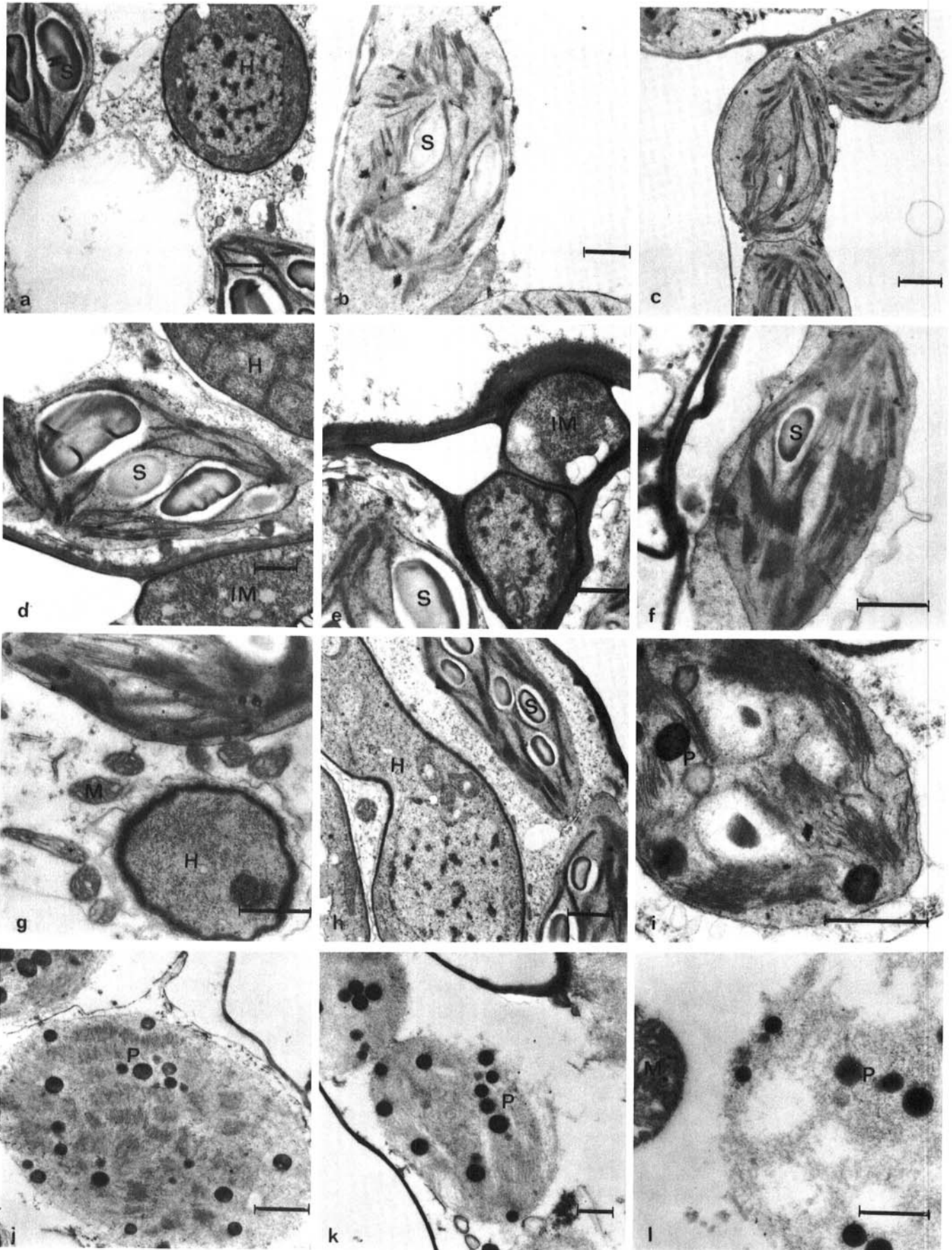


Fig. 2. Electron micrographs showing chloroplasts of rust-infected cells in various stages of the disease at various distance from the center of the infection site. First row (from the top) 7 days; second row, 9 days; third row, 14 days; fourth row, 23 days after inoculation; first column (from the left) at the center of infection site; second column at a distance of 1.5 mm; third column at a distance of 3.0 mm from the center of the infection site. Legend: S = starch, H = haustorium, IM = intercellular mycelium, M = mitochondrion, P = plastoglobule. In each case the bar represents 1.0 μ m.

after infection. Two-gram samples from primary leaves of rust-infected and healthy plants were ground in a prechilled mortar in 0.05 M tris (hydroxymethyl)-aminomethane buffer (pH 7.8) containing 0.35 M NaCl and 10 mg of bovine serum albumin per liter. Homogenates were squeezed through two layers of cheesecloth and centrifuged at 3,500 *g* for 10 min. Chloroplasts were suspended in 0.05 M phosphate buffer (pH 6.4) containing 0.2 M sucrose. The reaction mixture contained chloroplasts corresponding to 6–10 μ M chlorophyll, and 10 mM 2,6-dichlorophenol indophenol, and (in some cases) 0.5 mM 1,5-diphenylcarbazine. Photoreduction of the dye was monitored at 578 nm by using an Aminco DW-2 spectrophotometer which was adapted for side-illumination. Actinic light was filtered by an Oriol G 772-6300 filter which allowed a red light of 7×10^4 erg \times cm² \times sec⁻¹ to fall on the cell.

RESULTS

Changes in chlorophyll content were monitored from the appearance of the first symptoms (flecking stage) until completion

TABLE 1. Hill activity of chloroplasts isolated from rust-infected and uninfected bean leaves

Experiments	Hill activity ^a		Ratio with DPC/ without DPC)
	With DPC ^b	Without DPC	
Immediately after chloroplast isolation			
Uninfected	102	81	1.3
Infected	130	130	1.0
One hour after chloroplast isolation			
Uninfected	128	76	1.7
Infected	72	51	1.4

^a Moles of 2,6-dichlorophenol indophenol reduced per mole of chlorophyll per hour.

^b Supplemented with 0.5 mM 1,5-diphenylcarbazine.



Fig. 3. Accumulation of starch grains in plastids at the infection site, 7 days after inoculation. S = starch, IM = intercellular mycelium. Magnification $\times 6,300$.

of symptom development when tissues surrounding the pustules plus the green islands became chlorotic. Six days after inoculation, at the flecking stage, chlorophyll content of infected tissues was 20% higher than that of healthy tissues (Fig. 1). As the disease developed, the chlorophyll level in tissues surrounding the infection sites remained above that of the healthy control even 16 days after infection, although the differences fluctuated. A similar pattern was found in the amount of carotenoids of infected leaf tissues.

Commencing 10–11 days postinoculation, leaf tissues under our conditions seemed to be deeper green around infection sites than in control leaves. The tissues appeared to be most green at about 12 days after inoculation. The difference in chlorophyll content between green islands and healthy leaves was the highest at this time. The areas surrounding the islands did not become senescent at this time, although disintegration of chloroplasts had started.

Hill activity of chloroplasts isolated from infected green and healthy green tissues was compared at this stage (12 days after inoculation) of disease development to elucidate whether the enhanced chlorophyll level and the characteristic ultrastructural changes of chloroplasts in infected tissues were accompanied by altered photosynthetic activity. In experiments performed immediately after preparation of chloroplasts, somewhat higher Hill activity was found in chloroplasts from infected leaves than in chloroplasts from uninfected ones. After a period of incubation (30–60 min), the Hill activity of chloroplasts from infected leaves declined below the activity of the control (Table 1). A common characteristic of chloroplasts isolated from infected leaves was the weak response to the artificial electron donor 1,5-diphenylcarbazine. This shows that the electron transport chain of chloroplasts in the infected tissue is more stable; the photosystems couple more intensely than those in the chloroplasts of uninfected leaves.

Electron microscopic investigations were performed at different stages of disease development to obtain a deeper insight into the

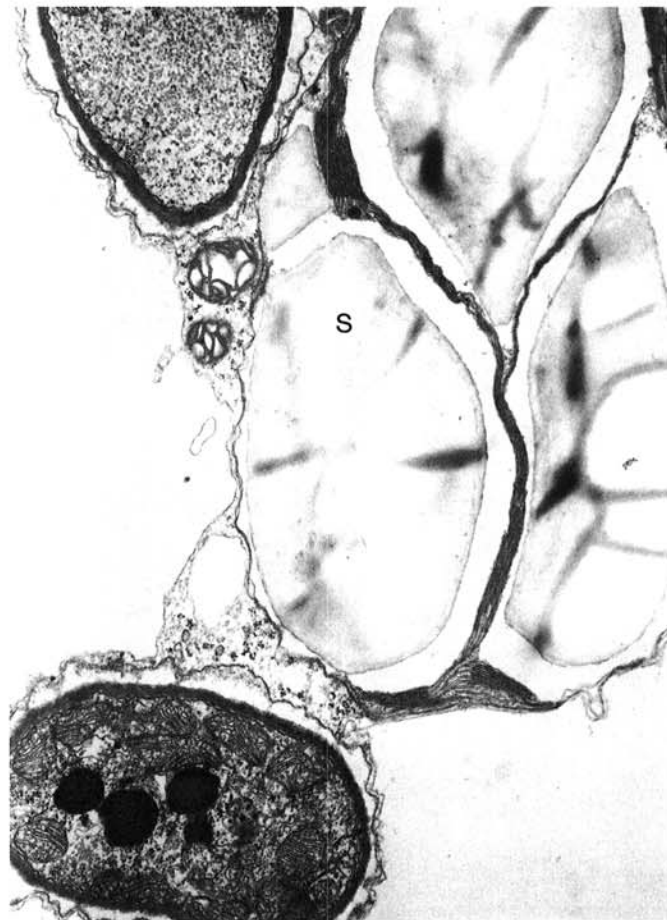


Fig. 4. Starch grains in plastids at a distance of 1.5 mm from the center, 14 days after inoculation. S = starch. Magnification $\times 13,000$.

ultrastructural changes taking place during pathogenesis. Seven days after inoculation, when flecking was still visible, haustoria were observed at the center of the infection sites. The cell organelles were clumped around the haustoria of the fungus. The chloroplasts contained several large starch grains (Figs. 2a and 3). In the immediate environment of the flecks, where the fungus was not present, chloroplasts showed starch accumulation (Fig. 2b). Nine days after inoculation, starch accumulation advanced from the center of the infection sites (Fig. 2d) as the growth of the intercellular mycelia of the fungus advanced (Fig. 2e). At 3 mm from the center of pustules there was a slight starch accumulation (Fig. 2f).

By the 14th day, the starch grains were disintegrated in the center of colonized areas, but the lamellar system of the chloroplasts remained intact (Fig. 2g). The adjacent cells contained numerous haustoria, and the chloroplasts kept the starch grains and their lamellar system as well (Fig. 2h, Fig. 4). In the more distant cells around the green islands the starch grains and the thylakoids were destroyed and a large number of plastoglobuli could be observed (Fig. 2i).

At 23 days after inoculation, the degradation of chloroplasts was observed also in the central zone, but even at this stage some thylakoids were seen near the center of the colony (Fig. 2j,k). In the cells surrounding the green islands, however, the chloroplasts were completely disrupted, and the membrane system and the envelope were lost. The place of the formerly existing chloroplasts was indicated only by the groups of plastoglobuli (Fig. 2l).

On plants kept under low light conditions (Fig. 5), the formation of green islands was conspicuous. The chloroplasts seemed to be in a relatively integrated state (Fig. 6a) although they were slightly damaged with abundant peripheral reticula (Fig. 6b). The latter characteristic indicated stress (14). On the other hand, the relatively integrated structure of chloroplasts indicated the influence of a cytokinin-type effect.

DISCUSSION

Formation of green islands can be observed around infection sites of several biotrophs (3,4). Despite this there are few data on the chlorophyll content of plants infected by obligate parasites. Wang (37) reported that infected bean leaves contained higher amounts of chlorophyll-*a* and chlorophyll-*b* in the sporulation stage than the control leaves of healthy plants. He also demonstrated an increased chlorophyll level in the early stages of disease development. However, in his experiments the opposite leaves served as controls. Király (16) reported an increase in the total chlorophyll content and a higher value of red absorption *in vivo* in rust-infected leaves in the sporulation stage. Our results are in agreement with these findings.

In contrast to the results obtained by Wang (37), we found that the difference between the chlorophyll content of infected and healthy tissues decreased 7–8 days after inoculation (Fig. 1). However, a later increase in chlorophyll content was detected which coincided with the development of pustules and attained a maximum 11–13 days after inoculation when the green islands became visible. During this period, the chloroplasts isolated from infected leaves responded weakly to an artificial electron donor, suggesting that the electron transport chain of chloroplasts in infected tissues was more stable than in the uninfected tissues.

Investigators dealing with different host-parasite combinations have disagreed upon the pattern of the formation of green islands. It would appear from Allen's (2) studies on wheat infected by powdery mildew that the formation of green islands is due to resynthesis following decomposition of chlorophyll. On the contrary, Wang (37) concluded that the green island induced by bean rust is due to the retention of chlorophyll. Harding et al (13) studying green island formation on cotyledons of *Brassica juncea* came to the same conclusion. Our results show that there is an increased synthesis of chlorophyll at the infection sites

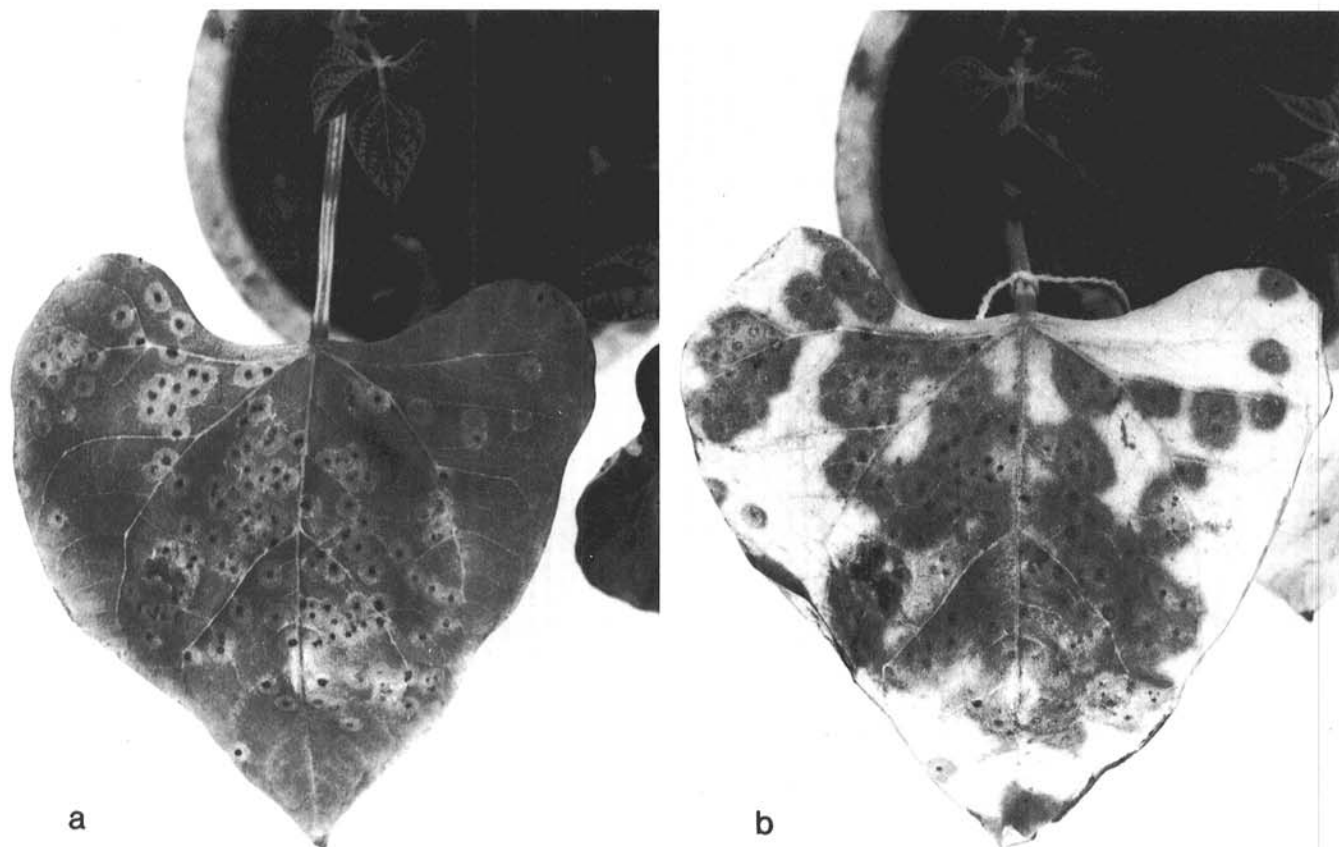


Fig. 5. Characteristic symptoms of rust development in bean leaves kept under low light conditions. **a**, Early sporulation stage with flecks, 9 days after inoculation. **b**, Green island stage, 15 days after inoculation.



Fig. 6a. Chloroplasts from the green island of a leaf kept under low light conditions, 15 days after inoculation. Magnification $\times 22,000$.

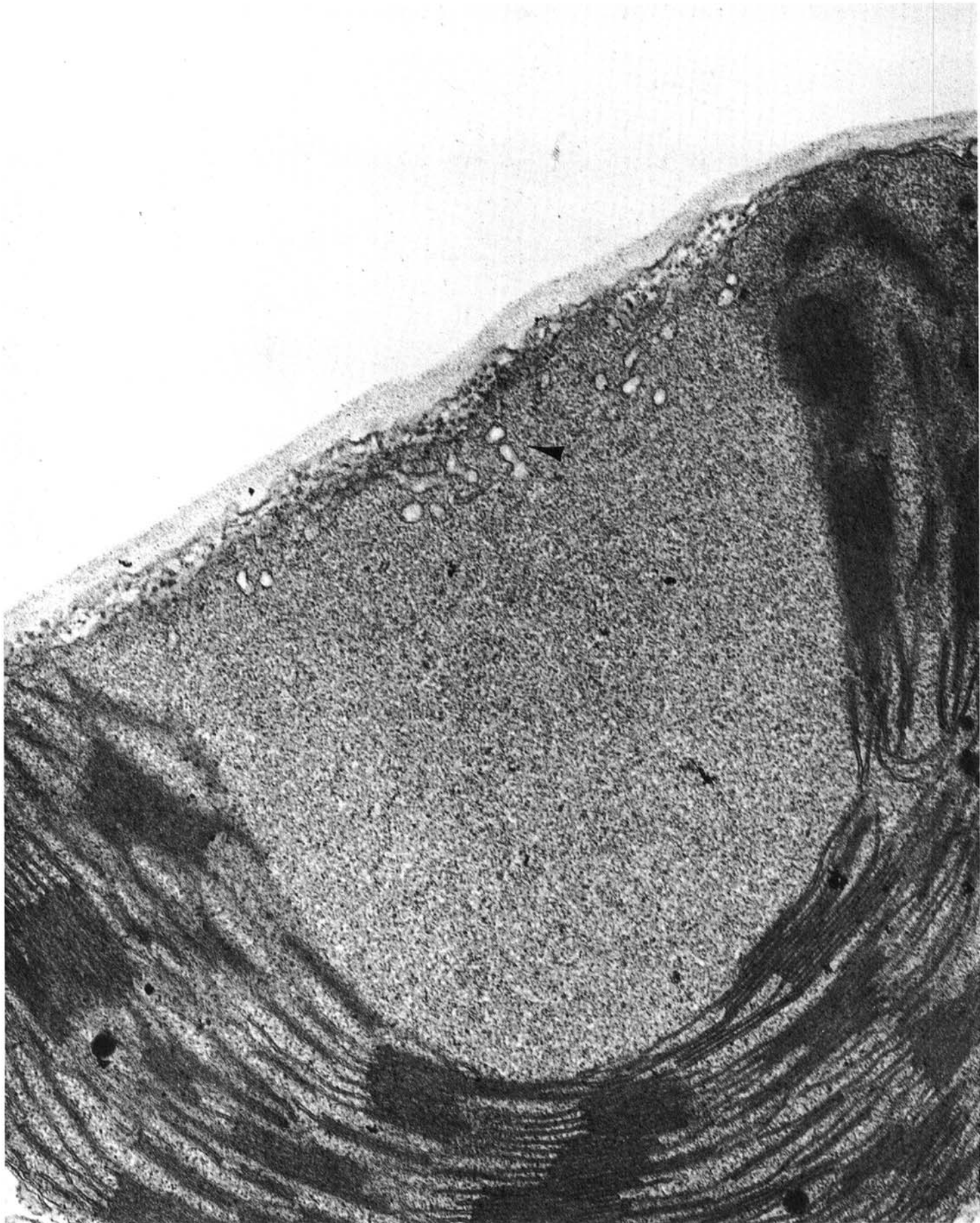


Fig. 6b. Peripheral reticulum (arrowhead) induced by infection stress in a chloroplast of a green island developed under low light conditions, 15 days after inoculation. Magnification $\times 56,000$.

commencing with the flecking stage. The increased level of chlorophyll at the infection sites during the whole process of symptom development may provide an explanation for the appearance of green islands.

Green island formation can be induced by extracts of mildew and rust spores (3,8) and certain cytokinins (23). Other changes caused by rust infection, such as an increased leaf growth and altered phloem transport can also be simulated in Pinto beans by application of cytokinins (25,26). Furthermore, an enhanced cytokinin level has been demonstrated in rust-infected Pinto bean leaves (8,17) as well as in plants infected by different obligate parasites (1,18,31,33-35). Therefore, the increased cytokinin level of infected tissues might be involved in inducing characteristic changes caused by rust infection, including formation of green islands.

Structural changes in chloroplasts of rust-infected plants have been described in different host-parasite combinations (5,14,20-22,28,29). In the early stage of disease development the cell organelles were found to be aggregated around the haustoria, and starch accumulation in chloroplasts could be observed. In the sporulation stage, the starch grains in some plastids of rust-infected flax and sunflower plants were disintegrated and transformed into glycogenlike granules. Extended thylakoids and plastoglobuli were also observed in affected tissues (5).

In tissues of rust-infected bean plants the arrangement of the accumulated and disintegrated starch grains was the most characteristic symptom. The accumulation of starch in chloroplasts precedes the colonization of tissues. The degradation of starch occurred at first far from the center of infection, ie, in the cells just outside the green islands that had formed later in the center of infection sites. Transformation of starch grains into glycogenlike particles was not observed. The disintegration of chloroplasts also occurred at first in cells surrounding the green islands and spread to the center of infection. Disintegration of chloroplasts was accompanied by the appearance of plastoglobuli which are characteristic for degenerating chloroplasts (cf 24).

On bean plants kept under low light, some of the chloroplasts in the green islands exhibited peculiar features. On one hand, the appearance of the abundant peripheral reticula referred to some stress. This is believed by Heath (14) to indicate a chromoplastlike development of the chloroplasts that is associated with increased senescence (increased ethylene production). On the other hand, we found that the chloroplast integrity was maintained. One can suppose that in the area of green islands both damaging and (perhaps cytokinin-mediated) repairing processes affect the chloroplasts simultaneously.

Wang (37) suggested that the accumulation of starch in chloroplasts is due to de novo synthesis at the infection sites. Starch accumulation may serve as a source for processes of high energy requirement during advanced stages of pathogenesis (cf. 6). Starch accumulation, similar to the formation of green islands, can be induced by extracts of spores of obligate parasites (cf. 7). It is interesting in this context that starch grains can be observed in chloroplasts of tobacco callus in the presence of relatively high amounts of kinetin. The necessity of kinetin for chloroplast maturation in cultured tissue was also demonstrated (30).

Summarizing, we have found evidence that in rust-infected Pinto beans the level of chlorophyll increased in the leaves from the beginning of the appearance of symptoms until late in the infection period and that this was accompanied by starch accumulation and the maintenance of chloroplast integrity in the green islands. On the basis of our earlier investigations and the results of other authors, one can suppose that the enhanced chlorophyll content and the accumulation of starch might be due to an increase in the cytokinin level in the infected leaves.

LITERATURE CITED

1. Abou-Mandour, A. A., and Volk, O. H. 1971. Nachweis von Cytokinin-Aktivität in rost-infizierten *Pelargonium*-Blättern. Z. Pflanzenphysiol. 65:240-247.
2. Allen, P. J. 1942. Changes in the metabolism of wheat leaves induced by infection with powdery mildew. Am. J. Bot. 29:425-435.
3. Bushnell, W. R. 1967. Symptom development of mildewed and rusted tissues. Pages 21-40 in: The Dynamic Role of Molecular Constituents in Plant-Parasite Interaction. C. J. Mirocha and I. Uritani, eds. Bruce Publishing Co., St. Paul, MN.
4. Bushnell, W. R., and Allen, P. J. 1962. Induction of disease symptoms in barley by powdery mildew. Plant Physiol. 37:50-59.
5. Coffey, M. D., Polevitz, B. A., and Allen, P. J. 1972. Ultrastructural changes in rust infected tissues of flax and sunflower. Can. J. Bot. 50:1485-1492.
6. Daly, J. M. 1976. The carbon balance of diseased plants: changes in respiration, photosynthesis and translocation. Pages 450-479 in: Physiological Plant Pathology. R. Heitefuss and P. H. Williams, eds. Springer-Verlag, Berlin, Heidelberg, and New York.
7. Dekhuijzen, H. M. 1976. Endogenous cytokinins in healthy and diseased plants. Pages 526-559 in: Physiological Plant Pathology. R. Heitefuss and P. H. Williams, eds. Springer-Verlag, Berlin, Heidelberg and New York.
8. Dekhuijzen, H. M., and Staples, R. C. 1968. Mobilization factors in uredospores and bean leaves infected with bean rust fungus. Contrib. Boyce Thompson Inst. 24:39-52.
9. El Hammady, M., Pozsár, B. I., and Király, Z. 1968. Increased leaf growth regulated by rust infections, cytokinins, and removal of the terminal bud. Acta Phytopathol., Acad. Sci. Hung. 3:157-164.
10. Favali, M. A., and Marte, M. 1973. Electron microscope autoradiography of rust-affected bean leaves labelled with tritiated glycine. Phytopathol. Z. 76:343-347.
11. French, C. S. 1960. Chlorophylls *in vivo* and *in vitro*. Pages 259-271 in: Encyclopedia of Plant Physiology. Vol. 5/1. W. Ruhland, ed. Springer-Verlag, Berlin, Göttingen, and Heidelberg.
12. Goodwin, T. W. 1955. Carotenoids. Pages 272-312 in: Moderne Methoden der Pflanzenanalyse, Vol. 3. K. Paech and M. V. Tracey, eds. Springer-Verlag, Berlin, Göttingen, and Heidelberg.
13. Harding, H., Williams, P. H., and McNabola, S. S. 1968. Chlorophyll changes, photosynthesis and ultrastructure of chloroplasts in *Albugo candida*-induced "green islands" on detached *Brassica juncea* cotyledons. Can. J. Bot. 46:1229-1234.
14. Heath, M. C. 1974. Chloroplast ultrastructure and ethylene production of senescing and rust-infected cowpea leaves. Can. J. Bot. 52:2591-2597.
15. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. 27:137A-138A.
16. Király, Z. 1968. Role of cytokinins in biochemical and morphogenetic changes induced by rust infections. (Abstr.) Page 105 in: Proc. 1st Int. Cong. Plant Pathology, 14-26 July 1968. London.
17. Király, Z., El Hammady, M., and Pozsár, B. I. 1967. Increased cytokinin activity of rust-infected bean and broad bean leaves. Phytopathology 57:93-94.
18. Király, Z., Pozsár, B. I., and El Hammady, M. 1966. Cytokinin activity in rust-infected plants: Juvenility and senescence in diseased leaf tissues. Acta Phytopathol., Acad. Sci. Hung. 1:29-37.
19. Livne, A. 1964. Photosynthesis in healthy and rust-affected plants. Plant Physiol. 39:614-621.
20. MacDonald, P. W., and Strobel, G. A. 1970. Adenosine diphosphate glucose pyrophosphorylase control of starch accumulation in rust-infected wheat leaves. Plant Physiol. 46:126-135.
21. Mirocha, C. J., and Zaki, A. I. 1966. Fluctuation in amount of starch in host plants invaded by rust and mildew fungi. Phytopathology 56:1220-1224.
22. Młodzianowski, F., and Siwecki, R. 1975. Ultrastructural changes in chloroplasts of *Populus tremula* L. leaves affected by the fungus *Melampsora pinitorqua* Braun. Rost. Physiol. Plant Pathol. 6:1-3.
23. Mothes, K., and Engelbrecht, L. 1961. Kinetin-induced transport of substances in excised leaves in the dark. Phytochemistry 1:58-62.
24. Mühlthaler, K. 1971. The ultrastructure of plastids. Pages 7-29 in: Structure and Function of Chloroplasts. M. Gibbs, ed. Springer-Verlag, Berlin, Heidelberg, and New York.
25. Pozsár, B. I., and Király, Z. 1964. Cytokinin-like effect of rust infections in the regulation of phloem-transport and senescence. Pages 190-210 in: Proceedings of the Symposium of Host-Parasite Relations in Plant Pathology. Z. Király and G. Ubrizsy, eds. Research Inst. for Plant Protection, Budapest.
26. Pozsár, B. I., and Király, Z. 1966. Phloem-transport in rust-infected plants and the cytokinin-directed long-distance movement of nutrients. Phytopathol. Z. 56:297-306.
27. Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17:208-213.
28. Shaw, M., and Manocha, M. S. 1965. Fine structure in detached senescing wheat leaves. Can. J. Bot. 43:747-755.

29. Shaw, M., and Manocha, M. S. 1965. The physiology of host-parasite relations. XV. Fine structure in rust infected wheat leaves. *Can. J. Bot.* 43:1285-1292.
30. Stetler, D. A., and Laetsch, W. M. 1965. Kinetin-induced chloroplast maturation in cultures of tobacco tissue. *Science* 149:1387-1388.
31. Sziráki, I., Barna, B., El Waziri, S., and Király, Z. 1978. Effect of rust infection on the cytokinin level of wheat cultivars susceptible and resistant to *Puccinia graminis* f. sp. *tritici*. *Acta Phytopathol., Acad. Sci. Hung.* 11:155-160.
32. Thrower, L. B. 1965. On the host-parasite relationship of *Trifolium subterraneum* and *Uromyces trifolii*. *Phytopathol. Z.* 52:269-294.
33. Vizárová, G. 1974. Level of free cytokinins in susceptible and resistant cultivars of barley infected by powdery mildew. *Phytopathol. Z.* 79:310-314.
34. Vizárová, G. 1975. Effect of powdery mildew on the level of endogenous cytokinins in barley with regard to senescence. *Phytopathol. Z.* 84:105-114.
35. Vizárová, G., and Minarcic, P. 1974. The influence of powdery mildew upon the cytokinins and morphology of barley roots. *Phytopathol. Z.* 81:49-55.
36. Vernon, L. P., and Shaw, E. R. 1969. Oxidation of 1,5-diphenylcarbazine as a measure of photosystem 2 activity in subchloroplast fragments. *Biochem. Biophys. Res. Commun.* 36:878.
37. Wang, D. 1961. The nature of starch accumulation at the rust infection site in leaves of Pinto bean plants. *Can. J. Bot.* 39:1595-1604.
38. Zaki, A. I., and Mirocha, C. J. 1965. Carbon dioxide fixation by rust-infected bean plants in the dark. *Phytopathology* 55:1303-1308.