Effect of Induced Nodule Senescence on Parameters Related to Dinitrogen Fixation, Bacteroid Size and Nucleic Acid Content

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Darkness and treatment with combined nitrogen (NH_4Cl or KNO_3) were used to induce nodule senescence in alfalfa and soybeans. Nodule senescence was assessed by determinations of the acetylene-reducing activity and leghaemoglobin and sugar contents of the nodules. Bacteroids from nodules of the treated plants were compared using flow microfluorimetry. Upon induced nodule senescence, alfalfa bacteroids decreased both in nucleic acid content and cell size while the soybean bacteroids were essentially unaffected.

INTRODUCTION

Dinitrogen fixation by *Rhizobium*-legume associations involves a complex physiological interplay between the symbiotic rhizobia (bacteroids) and the host plants. The reduction of dinitrogen occurs in the bacteroids while the required energy and reductants are supplied by the plant through photosynthesis. Ching *et al.* (1975) demonstrated in soybean nodules that if photosynthesis became limiting, acetylene reduction, ATP and sucrose contents and the energy charge of the nodules sharply decreased. In a separate study, Chen & Phillips (1977) have shown that combined nitrogen (NH_4Cl or KNO_3) also caused a sharp decrease in acetylene reduction in pea nodules. The reason for this effect is not known although various explanations have been suggested (Oghoghorie & Pate, 1971; Rigaud, 1976; Rigaud & Puppo, 1977).

Previous research from this laboratory (Paa *et al.*, 1978) has shown that the basal portion of the 6 week or older alfalfa nodule has very low acetylene reduction capability in comparison with the middle nodule region and that bacteroids in this basal region have considerably less nucleic acid than bacteroids of similar size in the middle nodule regions. The bacteroids in the basal region apparently have begun senescence. While the effects of limited light and combined nitrogen have been shown to reduce parameters related to dinitrogen fixation in the nodules, their specific effect on bacteroid senescence has not been reported. In this communication, we demonstrate the effect of darkness and treatment with combined nitrogen on the nucleic acid content of bacteroids from alfalfa and soybean nodules. Changes in the acetylene-reducing activity of the nodules and in the leghaemoglobin and sugar contents of the nodule cytosol are also reported.

METHODS

The maintenance and growth conditions of free-living *Rhizobium meliloti* F28 and *Rhizobium japonicum* 705 were as previously described (Cowles *et al.*, 1969). Alfalfa (*Medicago sativa* Buffalo) and soybean (*Glycine max* Chippewa) plants were inoculated 1 week after germination with cultures of *R. meliloti* F28 and *R. japonicum* 705, respectively. The soybean and alfalfa nodules were harvested 8 and 6 weeks after inoculation,

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respectively. The times at which treatments were initiated were chosen so that the control and treated plants were harvested on the same day. For dark treatments, the plants were covered with black plastic bags (NAPCO, 1×0.9 m) on the appropriate day before harvest. The control plants were covered with transparent plastic bags. The black bags transmitted 4, 6 and 7.5 % of incident light at 450, 550 and 680 nm, respectively; the transparent bags transmitted 62, 60 and 72 % of incident light at these wavelengths. For combined nitrogen treatments, 500 ml of 100 mM- NH_4Cl or KNO_3 was applied to the plants on the appropriate initial day and continued every other day until harvest.

The harvested nodules were first assayed for acetylene-reducing activity by gas chromatography (Hardy *et al.*, 1968). The gas phase consisted of 65 % helium, 24 % oxygen and 10 % acetylene. Afterwards, the nodules were homogenized and the bacteroids were harvested by differential centrifugation (Cowles & Evans, 1968; Cowles *et al.*, 1969). The supernatant (nodule cytosol) was used for determinations of leghaemoglobin, glucose and sucrose contents. Leghaemoglobin was determined spectrophotometrically, using human haemoglobin as a standard, by an assay modified from that of Johnson & Hume (1973): 2.5 ml of nodule cytosol was combined with 0.5 ml of 5 M-KOH containing 0.1 g sodium dithionite and A_{555} was determined. Glucose was determined using the 'glucose-stat' procedure as outlined by Worthington Biochemical Co. Sucrose was hydrolysed by invertase and the glucose formed was quantified by the 'glucose-stat' procedure. The nucleic acid content and cell size of the individual bacteroids were determined from ethidium bromide fluorescence and light-scattering properties, respectively, in a flow microfluorimeter (Paaü *et al.*, 1977*a, b*). The bacteroids were fixed overnight in 65 % (v/v) ethanol and stained with 0.005 % (w/v) ethidium bromide in 1.12 % (w/v) sodium citrate (Paaü *et al.*, 1977*a*). For DNA analysis, the fixed cells were digested with RNAase before staining (Paaü *et al.*, 1977*a*). The reported results are averages of duplicate experiments.

RESULTS

Acetylene-reducing activity and leghaemoglobin, sucrose and glucose contents in nodules

The acetylene-reducing activity in alfalfa nodules had decreased by more than 50 % after 1 d in darkness and by more than 80 % after 3 d (Fig. 1*a*). About 70 % of the initial activity was retained in soybean nodules after 4 d in darkness (Fig. 1*d*). Acetylene-reducing activity was not detectable in nodules of either plant after 6 d. The decrease in acetylene-reducing activity in soybean nodules in the dark is consistent with that described by Ching *et al.* (1975). Treatment with KNO_3 caused a rapid and similar decrease in acetylene-reducing activity in both alfalfa and soybean nodules; more than 50 % of the initial activity was lost after 1 d (Fig. 1*b, e*). A similar response was caused by the NH_4Cl treatments except the rate of loss was somewhat slower than with KNO_3 (Fig. 1*c, f*).

The leghaemoglobin contents of alfalfa and soybean nodules also decreased in response to the dark and combined nitrogen treatments. After 11 d of treatment, the leghaemoglobin content remained above 50 % of that of the control nodules for both the alfalfa and soybean systems. The decrease in leghaemoglobin content during senescence probably influenced the rate of acetylene reduction (Swaraj & Garg, 1977).

The changes in sucrose and glucose contents caused by darkness or treatment with combined nitrogen are shown in Fig. 2. In both alfalfa and soybean nodules, the sucrose content had decreased by more than 30 % after 1 d in darkness and was less than 25 % of the content in control plants after 11 d (Fig. 2*a, c*). The decrease in glucose content in the dark was initially slower than that of sucrose but it then became more rapid. Treatment of alfalfa plants with KNO_3 caused the sucrose content to decrease initially (about 40 % in 8 d) and then stabilize (Fig. 2*b*). Sucrose contents in soybean nodules did not change appreciably (Fig. 2*d*). The glucose content in alfalfa nodules had increased twofold by 1 d after KNO_3 treatment and then decreased to below the control level by 11 d (Fig. 2*b*). In soybean nodules the increase in glucose content by 1 d after KNO_3 treatment was about fourfold. This concentration was maintained for up to 4 d, but by 7 d the glucose concentration was below that of the control (Fig. 2*d*). Results from plants treated with NH_4Cl were similar to those treated with KNO_3 (data not shown).

Fig. 1. Effect of darkness (*a, d*), KNO_3 (*b, e*) and NH_4Cl (*c, f*) treatments on the acetylene-reducing activity (●) and leghaemoglobin content (○) of alfalfa (*a, b, c*) and soybean (*d, e, f*) nodules. Values for the control experiments were: alfalfa, $3.16 \text{ mg leghaemoglobin (g nodule)}^{-1}$ and $4.5 \mu\text{mol C}_2\text{H}_2 \text{ reduced g}^{-1} \text{ h}^{-1}$; soybean, $2 \text{ mg leghaemoglobin (g nodule)}^{-1}$ and $2.6 \mu\text{mol C}_2\text{H}_2 \text{ reduced g}^{-1} \text{ h}^{-1}$.

Nucleic acid content of alfalfa bacteroids

The effect of darkness or treatment with combined nitrogen on the nucleic acid content of alfalfa nodule bacteroids was determined by flow microfluorimetry of isolated bacteroids. These analyses revealed a slight decrease in the average DNA content in bacteroids with increasing periods of darkness (Fig. 3*b* to *f*). The number of bacteroids with a high DNA content (100 units or higher) decreased in proportion to the period spent in darkness. The alfalfa bacteroid DNA content remained as high as that of the free-living rhizobia even after 10 d in the dark (Fig. 3*a, f*). If bacteroids from the control plants were not treated with RNAase before staining, the intensity of the fluorescence was generally greater in the untreated bacteroids (compare Fig. 3*b, h*). This difference is due presumably to the tertiary structures of the bacteroid RNA resulting in the binding of ethidium bromide. After 3 d in the dark, the RNA component of the ethidium bromide fluorescence from bacteroids had completely disappeared (Fig. 3*h* to *l*). This indicates that the dark treatment rapidly decreases the RNA content or changes its structure. There was no significant change in ethidium bromide fluorescence by RNA digestion in the free-living rhizobia (Fig. 3*a, g*).

Treatment of alfalfa plants with KNO_3 or NH_4Cl led to a decrease in the DNA and RNA contents of bacteroids similar to that caused by darkness (data not shown). The principal difference between the combined nitrogen and dark treatments was that the decrease in the RNA component of the ethidium bromide fluorescence was comparatively slower in the

Fig. 2. Effect of darkness (*a, c*) and KNO_3 (*b, d*) treatments on the sucrose (●) and glucose (○) contents of alfalfa (*a, b*) and soybean (*c, d*) nodules. (Left-hand scales refer to *a* and *c*, right-hand scales to *b* and *d*.) Values for the control experiments were: alfalfa, 3 mg sucrose and 0.1 mg glucose (g nodule)⁻¹; soybean, 5 mg sucrose and 0.2 mg glucose (g nodule)⁻¹.

nitrogen-treated plants; the decrease in the RNA component was not complete until about 7 d after the initiation of treatments.

Nucleic acid content of soybean bacteroids

Bacteroids isolated from soybean nodules have a similar DNA content to free-living *R. japonicum*. Soybean plants subjected to darkness or treated with combined nitrogen for 11 d did not show any significant change (< 4%) in the bacteroid DNA content. The results of experiments involving NH_4Cl treatments are shown in Fig. 4. The RNA contribution to the ethidium bromide fluorescence in the soybean bacteroids was less than 2% and did not vary significantly with either treatment.

Bacteroid size in alfalfa and soybean bacteroids

The bacteroid populations isolated from soybean and alfalfa nodules were analysed by flow microfluorimetry using the light-scattering signals (Paau *et al.*, 1977*a*). Soybean bacteroids were similar in size to free-living *R. japonicum*, and treatment of soybean plants with either darkness (Fig. 5*b, c*) or combined nitrogen (results not shown) did not significantly alter the bacteroid size. On the other hand, the majority of the bacteroids in alfalfa nodules are larger than the free-living rhizobia. Darkness (Fig. 5*e, f*) or treatment with combined nitrogen (results not shown) greatly decreased the number of enlarged bacteroids. The decrease in alfalfa bacteroid size, however, was not apparent until after 3 d of treatment. The disappearance of large bacteroids in the alfalfa nodules occurred after the substantial decrease in acetylene-reducing activity and bacteroid nucleic acid content.

Fig. 3. Effect of darkness on the distribution of alfalfa bacteroids based on nucleic acid content: (a to f) distribution according to DNA content; (g to l) distribution according to ethidium bromide fluorescence without RNAase digestion (see text). (a, g) Free-living *R. meliloti*, at early stationary phase; (b, h) untreated control; (c, i) 1 d in the dark; (d, j) 3 d in the dark; (e, k) 7 d in the dark; (f, l) 10 d in the dark. The channel number is directly proportional to the intensity of ethidium bromide fluorescence which is positively correlated to the DNA and total nucleic acid content of bacteroids.

Fig. 4

Fig. 5

Fig. 4. Effect of NH_4Cl treatment on the distribution of soybean bacteroids based on total nucleic acid content: (a) free-living *R. japonicum*, at early stationary phase; (b) bacteroids from untreated plants; (c) bacteroids from plant treated for 11 d. The samples were not digested with RNAase.

Fig. 5. Effect of darkness on the distributions of soybean (a, b, c) and alfalfa (d, e, f) bacteroids based on cell size: (a, d) untreated control; (b, e) 3 d in the dark; (c) 14 d in the dark; (f) 10 d in the dark. The channel number is directly proportional to the intensity of the light-scattering signal which is positively correlated to the cell size.

DISCUSSION

The responses of soybean and alfalfa nodules to darkness and treatment with combined nitrogen are similar in many respects but there are significant differences. Both treatments caused a rapid (detectable in 1 d) loss in nucleic acid content in the bacteroids of alfalfa plants and a subsequent reduction in bacteroid size. These changes were not seen in bacteroids of soybean nodules. Thus our observations support earlier suggestions that the two *Rhizobium* species (one species being involved with each plant species) are not closely related (Dixon, 1969; Gibbins & Gregory, 1972).

The loss of acetylene-reducing activity and leghaemoglobin content in the dark and the decrease in sucrose and glucose contents was initially more rapid in the alfalfa nodules than in the soybean nodules. The rapid loss of acetylene-reducing activity in alfalfa nodules in the dark may be related to the amount of energy reserve in alfalfa nodules as well as the metabolic activity of the infecting organism. The concentration of glucose and sucrose in alfalfa nodules was only about 50% of that in soybean nodules. In addition, cultures of free-living *R. meliloti* grow three to four times as fast as cultures of *R. japonicum*. If similar differences in metabolic activity occur in the symbiotic micro-organisms, then the already small energy reserve in alfalfa nodules would be depleted faster and result in a rapid loss of acetylene-reducing activity in alfalfa nodules. Interestingly, the most rapid decrease in acetylene-reducing activity in the dark-treated soybean nodules begins at about the same time as the glucose content rapidly decreases. In the alfalfa nodules the glucose content begins to decline rapidly after 1 d in the dark.

Although the decrease in acetylene-reducing activity in the dark has been attributed to the limited availability of energy and reductants from photosynthesis (Ching *et al.*, 1975), the decrease caused by treatment with combined nitrogen has drawn varied explanations (Oghoghorie & Pate, 1971; Rigaud, 1976; Chen & Phillips, 1977; Rigaud & Puppo, 1977). Bisseling *et al.* (1978) concluded that decreased nitrogenase activity in nodules of peas treated with NH_4NO_3 resulted from a decreased leghaemoglobin content. The concomitant decrease in the number of large bacteroids with a high nucleic acid content in the alfalfa nodule system with the loss in acetylene-reducing activity is also perplexing. It seems unlikely that the decrease in numbers of large bacteroids is due solely to photosynthetic limitations since the nodules of plants treated with NH_4Cl and KNO_3 had similar sucrose and glucose contents to the control plants up to 6 d after initiation of treatment. While the mechanism which regulates the degradation of large alfalfa bacteroids is unknown, it is interesting that bacteroids responded similarly in size and nucleic acid content to darkness and to treatment with combined nitrogen.

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