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THE EFFECT OF WATERLOGGING ON THE SYNTHESIS OF THE NITROGENASE COMPONENTS IN BACTEROIDS OF *RHIZOBIUM LEGUMINOSARUM* IN ROOT NODULES OF *PISUM SATIVUM*

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SUMMARY. The effect of waterlogging of root nodules on nitrogenase activity and synthesis was studied in *Pisum sativum* inoculated with *Rhizobium leguminosarum* (strain PRE). It was shown that: 1. nitrogenase activity of intact pea plants was decreased by waterlogging, 2. this decrease was paralleled by a decline of the amount of active nitrogenase determined in toluene EDTA treated bacteroids, 3. SDS-polyacrylamide gel electrophoresis revealed that the amount of nitrogenase component II (CII) decreased by waterlogging while the amount of component I (CI) was not markedly affected, and 4. analysis of bacteroid proteins after $^{35}SO_4^=$ labeling of pea plants showed that CII synthesis was repressed while CI synthesis continued indicating that the synthesis of CI and CII is regulated by independent mechanisms.

INTRODUCTION. In *Klebsiella pneumoniae* the nif-genes can be repressed in at least two different ways. A fixed nitrogen source (e.g. NH_4^+ or amino acids) will repress nitrogenase synthesis; glutamine synthetase (GS) probably plays a role in this repression (1). Secondly, oxygen can also repress nitrogenase synthesis in *Klebsiella*, which is a facultative anaerobic nitrogen fixing organism (2) and GS does not participate in the repression of nitrogenase synthesis by O_2 (2).

Regulation of nitrogenase synthesis in rhizobia probably differs from that in *K. pneumoniae*. In symbiotic as well as nitrogen fixing rhizobia *ex planta*, NH_4^+ decreases nitrogenase activity. In both cases, however, nitrogenase synthesis is probably not repressed as in *K. pneumoniae* and other nitrogen fixing organisms (3,4,5).

Oxygen is probably important in the regulation of nitrogenase synthesis in *Rhizobium*, a micro-aerobic nitrogen fixing organism. In nitrogen fixing *R. japonicum ex planta* 0.16% O_2 is the optimal concentration for nitrogen fixation (6). Higher as well as lower O_2 concentrations cause a decrease in

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nitrogenase activity. In root nodules of legumes, the O_2 concentration is important for nitrogenase activity. If the oxygen supply is reduced by waterlogging of the root nodules, nitrogenase activity is decreased (6,7). Whether nitrogenase synthesis is also influenced, however, was not determined. In this paper we report on the effect of waterlogging of the root system on nitrogenase activity and on the synthesis of the two nitrogenase components of *Pisum sativum* nodulated with *R. leguminosarum*.

MATERIALS AND METHODS. The growth of pea plants (*P. sativum*, var. Rondo) nodulated with *R. leguminosarum* (PRE), ^{35}S -sulfate labeling of pea plants, polyacrylamide gel electrophoresis and autoradiography were performed as described previously (5,9).

Waterlogging treatment. Plants of 20 days old were used for the waterlogging treatment. Waterlogging was effected by filling the trays, that contained the pea plants, with distilled water, so that all root nodules were submerged in the growth medium.

Preparation of soluble bacteroid proteins. Anaerobic isolation and lysis of bacteroids by osmotic shock after lysozyme treatment, were performed as described before (5,9). The bacteroids were isolated in a glove box flushed with N_2 (9) and buffers contained 20 mM dithionite and 4% polyvinylpyrrolidone.

Acetylene reduction. Nitrogenase activity of intact pea plants was measured by the acetylene reduction assay in 500 ml Erlenmeyer flasks containing 10% acetylene and 90% air. The waterlogged root nodules were blotted with absorbent paper prior to analysis. Acetylene reduction was followed for 15 min, after which the pea plants were placed back in the culture medium. Nitrogenase activity was expressed as nmole acetylene reduced per plant per hour.

Acetylene reduction of anaerobic bacteroid suspensions was measured after the bacteroids were treated with EDTA and toluene as described by Houwaard (4)

RESULTS.

The effect of waterlogging on nitrogenase activity. The effect of waterlogging on nitrogenase activity was studied on whole pea plants. Two groups of 6 nodulated pea plants of 20 days old with about equal acetylene reducing capacity were selected out of 10 groups. One group was placed under waterlogging conditions while the other was used as a control. Nitrogenase activity was followed over a period of 4 days. Fig. 1 shows that the activity of the control plants increased slightly during the first 3 days. At the third day nitrogenase activity reached its maximal value after which it decreased

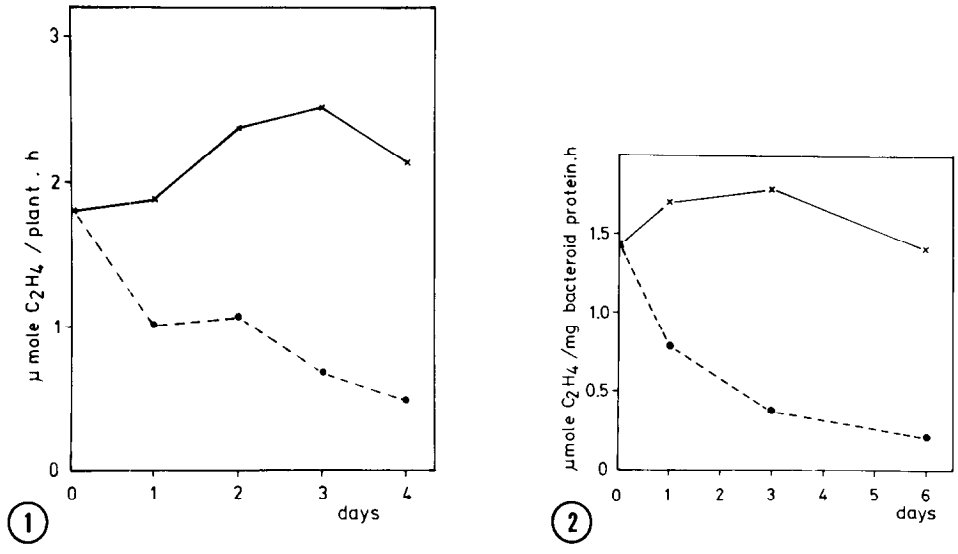

Fig. 1. Acetylene reducing activity of whole pea plants. The same plants were used throughout the experiments. x—x, control; o--o, waterlogging.

Fig. 2. Acetylene reducing activity of toluene EDTA treated bacteroids, isolated from control and waterlogged root nodules. x—x, control; o--o, waterlogging.

slightly at the fourth day. Nitrogenase activity of the waterlogged plants decreased rapidly after the onset of waterlogging. After 4 days of waterlogging nitrogenase activity of the waterlogged plants was 25% of that in control plants.

Nitrogenase activity depends on several factors, e.g. amount of nitrogenase and supply of energy, and reduction equivalents. To determine if the amount of active nitrogenase is limiting in waterlogged root nodules we determined the amount of active nitrogenase in bacteroids isolated from these nodules. Anaerobic nitrogenase activity of bacteroid suspensions, treated with toluene and EDTA, supplied with energy (ATP) and reduction equivalents (dithionite) was determined. Nitrogenase activity of waterlogged bacteroids decreased parallel to the activity of whole plants while nitrogenase activity of control bacteroids remained about constant over a period of 5 days (Fig. 2). After 5 days waterlogging, only 15% of the control activity was left. This result strongly indicated that by waterlogging the amount of active

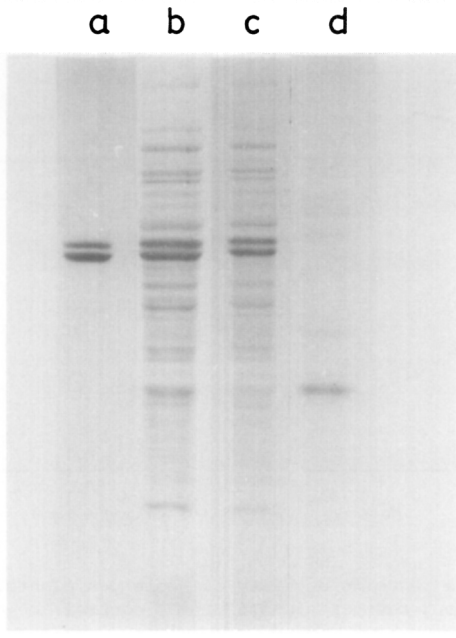

Fig. 3. Polyacrylamide gel electrophoresis of bacteroid proteins isolated from control and waterlogged root nodules. a. Component I, b. Control bacteroid proteins, c. Bacteroid proteins from waterlogged root nodules, d. Component II.

nitrogenase in bacteroids decreases, as in toluene EDTA treated bacteroids the amount of active nitrogenase limits activity.

Nitrogenase synthesis during waterlogging. The amounts of the separate nitrogenase components in bacteroids can be estimated by polyacrylamide gel electrophoresis of bacteroid proteins. Fig. 3 shows an electropherogram of proteins stained with Coomassie brilliant blue, prepared from bacteroids isolated from control plants (Fig. 3b) and from pea plants placed for 4 days under waterlogging conditions (Fig. 3c). The nitrogenase components were identified with purified nitrogenase components (Fig. 3a, d). This figure shows that the amounts of the two subunits of component I (CI) are not markedly affected but the amount of component II (CII) is reduced in comparison to the other protein bands.

To determine whether this decrease of the amount of CII is caused by a repression of the synthesis of CII, bacteroid protein synthesis was followed

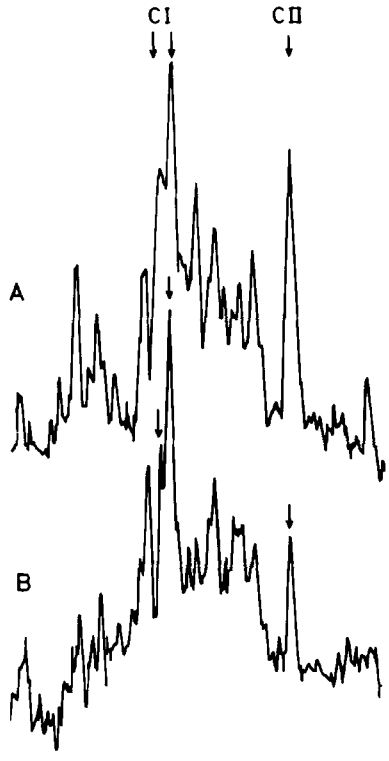

Fig. 4. Densitograms of bacteroid proteins isolated from $^{35}SO_4^=$ labeled control and waterlogged root nodules. A. Control bacteroid proteins, B. Bacteroid proteins of waterlogged root nodules. The arrows indicate the position of the two subunits of CI and CII.

by $^{35}SO_4^=$ labeling. Two groups of pea plants were labeled with $^{35}SO_4^=$ for 24 h. Bacteroid proteins were analysed by polyacrylamide gel electrophoresis and an autoradiograph was made. Figs. 4a and b show densitograms of autoradiographs of the bacteroid proteins shown in Fig. 3b and c. Total bacteroid protein synthesis is not markedly affected by waterlogging: control, 166 cpm/µg protein and waterlogging 181 cpm/µg protein. Fig. 4 shows that the incorporation of ^{35}S into CII is markedly reduced, since the peak corresponding with CII is reduced in comparison to the other proteins. The incorporation of ^{35}S into the two subunits of CI is hardly affected by waterlogging however. Besides the repression of CII, proteins with molecular weights of about 80, 48 and 22,000 were also repressed.

The decrease of ^{35}S incorporation into CII can be caused by an increased degradation or a repressed synthesis of CII. Analysis of ^{35}S labeled bacteroid proteins isolated from 1 day waterlogged root nodules, revealed that the amount of CII on polyacrylamide gels was not significantly decreased, while the incorporation of ^{35}S into CII was repressed (result not shown). This result indicates that the decrease of ^{35}S incorporation into CII is caused by a repressed synthesis rather than increased degradation of CII.

DISCUSSION. Sprent (8) proposed that a reduced oxygen supply to the root nodules, causing a decrease in the energy supply to the bacteroids, is responsible for a decreased nitrogenase activity under waterlogging. If only this reduced energy supply to the root nodules was responsible for the decrease of nitrogenase activity, we would expect an immediate decrease of nitrogenase activity at the beginning of the waterlogging treatment after which it should remain constant. Our results confirm those of e.g. Minchin *et al.* (10), who showed that nitrogenase activity decreases continually during the waterlogging treatment.

It was shown in experiments with toluene EDTA treated bacteroids that waterlogging of the root nodules caused a decrease in the amount of nitrogenase enzyme in parallel with the decrease of nitrogenase activity. $^{35}SO_4^=$ labeling showed that CII synthesis was repressed. Therefore, besides the reduced O_2 supply to the root nodules, a decreased amount of active nitrogenase is probably responsible for the continued decrease of nitrogenase activity during waterlogging. It appears that the decrease in the amount of nitrogenase is parallelled by a decreased CII synthesis, while CI synthesis seems rather unaffected.

Since only the synthesis of CII of nitrogenase seems to be repressed by waterlogging, the synthesis of CI and CII of nitrogenase probably can be regulated independently. This is in accordance with results we reported in previous papers on nodule development (9,11).

The mechanism by which waterlogging causes a repression of CII synthesis is yet unclear. We speculate that the decrease of the O_2 supply to the root nodules plays a role in the repression of CII synthesis.

The decrease of nitrogenase activity of toluene EDTA treated bacteroids is caused by a repression of CII synthesis. If nitrogenase activity is proportional to the amount of CII, with CI present in excess, the turnover rate of CII during waterlogging can be estimated. Fig. 2 shows that after 3-5 days of waterlogging acetylene reduction has decreased to 15% of the original value. This indicates that $t_{\frac{1}{2}}$ of CII is between 1 and 2 days. This value is consistent with the turnover rates determined in other ways (Bisseling, unpublished results).

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