NITROGENASE ACTIVITY (C₂H₂ REDUCTION) OF AZORHIZOBIUM IN 2,4-D-INDUCED ROOT STRUCTURES OF WHEAT

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Summary—The characteristics of C₂H₂ formation from C₂H₂ associated with 2,4-D-induced root structures on wheat seedlings inoculated with Azorhizobium caulinodans ORS571 have been investigated. Wheat seedlings treated with Azorhizobium plus 2,4-D were more tolerant in their C₂H₂ reduction activity to pO₂ 0.02 and 0.04 atm in the gas phase than wheat seedlings inoculated with Azorhizobium alone. C₂H₂ production was C₂H₂ dependent and was strongly inhibited by ammonia and nitrite. The nitrogenase activity of A. caulinodans associated with the 2,4-D-induced root structures of wheat seedlings was also confirmed by the fixation of ¹⁵N₂.

INTRODUCTION

Nie (1983) reported that nodule-like structures, induced on wheat by 2,4-D, can be inhabited by some microorganisms, including diazotrophs. Several attempts to achieve C₂H₂ reduction activity in wheat with 2,4-D induced root structures inoculated by rhizobia have failed (D. G. Yu, unpubl. M.Sc. thesis, CAAS, Beijing, 1988; Kennedy et al., 1990; Bender et al., 1990). C₂H₂ formation was reported in the wheat seedlings treated with 2,4-D and Azospirillum brasilense Sp7 (Tchan et al., 1991; Kennedy and Tchan, 1992; Zeman et al., 1992) and the C₂H₂ formation has been verified as nitrogenase activity performed by Azospirillum associated with the 2,4-D-induced root structures of the seedlings (Yu et al., 1993).

Azorhizobium caulinodans, capable of free-living N₂ fixation, is the microsymbiont in the stem nodules of the tropical legume Sesbania rostrata (Dreyfus et al., 1988). Chen et al., (1991, 1992) reported C₂H₂ reduction activity in 2,4-D-induced root structures of wheat inoculated with A. caulinodans, but the C₂H₂ reduction activity was small and only detectable after prolonged incubation (18–20 h). In our studies, the technique of inhibition by O₂ pressure (Tchan et al., 1991) was used to measure the activity of nitrogenase (C₂H₂ reduction) in 2,4-D-induced root structures of wheat with inoculation of A. caulinodans ORS571.

MATERIALS AND METHODS

Bacterial culture, plant growth and treatments of seedlings with 2,4-D and Azorhizobium

A. caulinodans ORS571 (purchased from LMG Culture-Collection, Gent, Belgium) was grown in a liquid N-free lactate medium (Dreyfus et al., 1983) with 0.01% (w/v) yeast extract for 24 h and a bacterial suspension used for inoculation of wheat seedlings. The method of Tchan et al. (1991) was used for surface sterilization of wheat seeds (cv. Miskle), bacterial inoculation, 2,4-D treatment and plant growth, except that the wheat seedlings at age 6–7 days were inoculated with 24 h old cultures of A. caulinodans ORS571 (ca. 10⁶–10⁷ cells per seeding tube) and treated with 2,4-D to make a final concentration of 0.6–0.7 pg ml⁻¹. C₂H₂ reduction and O₂ concentrations were assayed 14–16 days after the treatments.

C₂H₂ reduction and O₂ assay

A method designed to inhibit the nitrogenase activity of Azospirillum at the root surface of wheat (Tchan et al., 1991) was applied. The roots of wheat seedlings were immersed in Winogradsky's medium and shaken at a rate adequate to expose them thoroughly to O₂ in the gas phase of the flasks. For routine assays, 1 or 3 wheat seedlings aged 14–16 days after treatments with 2,4-D and the bacteria were transferred aseptically to 30 or 110 ml McCartney bottles containing 3 or 10 ml Winogradsky's N-free mineral solution. The gas phase in the bottles was replaced by evacuation and flushing 4 times with Ar or N₂. C₂H₂ and O₂ concentrations were assayed using direct injection sampling with a disposable hypodermic syringe (Yu et al., 1993).
Treatment with ammonia and nitrite

McCartney bottles containing seedlings showing positive C2H2 formation after 6-8 h incubation at 30°C were injected with differing amounts of (NH4)2SO4 or KNO3 solution and then incubated further. C2H2 reduction assays were performed at regular intervals.

15N assay

15N2 was prepared by using (15NH4)2SO4 (Novachem Pty Ltd) by a method described by Burris (1976) and then preserved over saturated Na2SO4 solution in a 500 ml glass cylinder connected to the vacuum system. A method developed by Yu et al. (1993) was used for 15N2 exposure. The McCartney bottles (30 ml) each containing 2 seedlings were first evacuated and flushed with Ar 3 times. After the fourth evacuation, the bottles were exposed to a gas mixture of 75% Ar, 20% 15N(82 atom% excess)-enriched N2, 4% O2, and 1% C2H2 and then placed in a water bath and shaken (160 min -1) at 30°C for 24 h. Total N assay of the whole seedlings was carried out by distillation and titration with 36 mM HCl following Kjeldahl digestion (Bergersen, 1980). The 15N assay was performed on a Micromass 622 mass-spectrograph.

RESULTS AND DISCUSSION

The effect of different O2 concentrations on C2H2 reduction activity in the wheat seedlings treated with azorhizobia plus 2,4-D or azorhizobia alone was studied (Fig. 1). Uninoculated control seedlings either treated with 2,4-D or untreated failed to produce C2H2. Although the C2H2 reduction activity in the wheat seedlings treated with azorhizobia alone was high at low O2 concentration (1%), it decreased quickly with increased O2 concentration in the system, approaching zero at 4% of O2 concentration. However, the activity in the seedlings treated with azorhizobia plus 2,4-D was more tolerant to the increase of O2 concentration, retaining activity at 6% in the gas phase. This suggests that 2,4-D-treated seedlings may provide a niche in the root structures to protect azorhizobia from O2. This result is consistent with the results obtained using Azospirillum (Kennedy and Tchan, 1992; Christiansen-Weniger, 1992).

From the results shown in Fig. 1, an O2 concentration of 4% was selected to ensure inhibition of nitrogenase activity of azorhizobia in the rhizosphere of wheat. This O2 concentration is higher...
C2H2 reduction by 2,4-D/Azorhizobium-treated wheat root

Fig. 4. Time-course of C2H2 reduction by wheat seedlings treated with 2,4-D and A. caulinodans ORS571 in the presence of different concentrations of KNO3 (μM). Nitrite was added after 8 h. Data are mean values ± SE of 2 replicated flasks, each containing 3 seedlings. The gas compositions in the flask were 4% O2, 10% C2H2 and 86% Ar.

Colonization of A. caulinodans ORS571 in nodule-like structures of wheat roots induced with 2,4-D has been reported, showing that most azorhizobia were located in intercellular spaces and some within the cells in root structures (K. Han, unpubl. M.Sc. thesis, CAAS, Beijing, 1991). As A. caulinodans is a free-living N2-fixing organism, an approach to distinguish the nitrogenase activity of azorhizobia located within the root structures induced by 2,4-D from that of cells at the root surface of plants was required. Chen et al. (1991, 1992) have used Incidin (a commercial disinfectant composed of formaldehyde, glyoxal, glutaraldehyde and ethanol; Henkel, Germany) or 75% ethanol to sterilize the surface of wheat roots. However, that treatment may also reduce the nitrogenase activity of the azorhizobia not as well protected from such disinfectants in the root tissue of wheat as rhizobia would be in the nodules of legumes. In preliminary work, we tried several different methods, using altered pH or antibiotics, to distinguish the nitrogenase activity of azorhizobia located within the root structures induced by 2,4-D from that of cells at the root surface of plants, but failed to selectively inhibit the nitrogenase activity given by azorhizobia in the rhizosphere of treated seedlings (data not shown). The method designed to inhibit nitrogenase activity of azospirillia at the root surface of wheat with O2 concentration (Tchan et al., 1991) was superior.

Figure 2 shows the time course of C2H2 formation and O2 concentration in the gas phase above the seedlings treated with azorhizobia plus 2,4-D. Typically, there was a lag of 4–6 h before C2H2 formation was observed, followed by an extended period of approximately linear activity. A similar lag period was also reported in the seedlings treated with 2,4-D plus Azospirillum (Yu et al., 1993; Sriskandarajah et al., 1993). Such lags in the expression of nitrogenase activity are characteristic of diazotrophs associated with grasses, apparently sometimes a result of a need to deplete fixed N [van Berkum (1978), quoted in Giller and Wilson (1991)]. Compared with the 18 h lag reported by Cleu et al. (1991, 1992) in their system, the lag time at 4–6 h presented here was much shorter. There was no significant change of O2 concentration in the incubation flasks containing the seedlings during the assay period of 12 h. A similar result was also found of no change in pO2 over 17 h in the 2,4-D-treated seedlings with Azospirillum (Yu et al., 1993). Thus any imbalance between respiration and photosynthesis was too small to significantly affect the O2 concentration in a vial, with about 30 ml of gas space per seedling with 4% of O2 added initially.

Figure 3 shows the effect of different C2H2 concentrations on the rate of C2H4 formation by seedlings treated with azorhizobia plus 2,4-D at 4% of O2. The rate of C2H4 formation (nmol plant⁻¹ h⁻¹) was taken from the period of linear activity after the lag from 6 to 12 h. The relationship between C2H2 concentration and C2H4 production was hyperbolic, indicating a dependence of C2H4 formation on C2H2.

The time-courses shown in Figs 4 and 5 illustrate the inhibitory effects of nitrite and ammonia, respectively, on C2H4 formation in the seedling system. Treatment...
with 1 mM KNO₃ or with 0.1 mM (NH₄)₂SO₄ nearly eliminated C₂H₄ production during the assay period of 7 h. This is consistent with our work with seedlings treated with 2,4-D and Azospirillum (Yu et al., 1993). However, KNO₃ at smaller concentrations of 0.01 or 0.1 mM was less inhibitory (Fig. 4), while (NH₄)₂SO₄, with 0.01 mM, still was strongly inhibitory (Fig. 5). This differs from the Azospirillum system, which was less tolerant of nitrite but more tolerant of ammonia. However, these results provide corroborative evidence that the C₂H₄ formation in the seedling system is nitrogenase-related, because nitrogenase activity in vitro is known to be inhibited by nitrite and ammonium.

To provide further evidence that C₂H₄ formation obtained from the 2,4-D-treated wheat seedlings with A. caulinodans ORS571 represented genuine nitrogenase, an approach developed by Yu et al. (1993), based on the simultaneous measurement of nitrogenase activity for C₂H₄ reduction and N₂ reduction in the same flask, was used to investigate the correlation between C₂H₄ formation and 1⁵N enrichment in seedlings treated or not treated with 2,4-D. The result showed a good correlation between C₂H₄ formation and 1⁵N enrichment (1⁵N fixed) in the same seedlings, with 2,4-D treatment leading to about a 15-fold increase of the total 1⁵N fixed, similar to the increase in C₂H₄ formation (Table I). It should be noted that the data in Table I cannot be used to calculate the rate of C₂H₄ reduction and N₂ fixation for the system, since the relative rates of C₂H₄ reduction and N₂ reduction are not known. This would be expected to be controlled by the concentration of each substrate, relative to its respective Km in the absence of inhibiting effects. This result, together with the inhibition by nitrite and ammonia, support the claim that the 2,4-D-treated wheat seedlings inoculated with A. caulinodans ORS571 are capable of elevated N₂ fixation.

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