



NITROGENASE ACTIVITY (C_2H_2 REDUCTION) OF *AZORHIZOBIUM* IN 2,4-D-INDUCED ROOT STRUCTURES OF WHEAT

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Summary—The characteristics of C_2H_4 formation from C_2H_2 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 C_2H_2 reduction activity to pO_2 0.02 and 0.04 atm in the gas phase than wheat seedlings inoculated with *Azorhizobium* alone. C_2H_4 production was C_2H_2 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 $^{15}N_2$.

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 C_2H_2 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). C_2H_4 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 C_2H_4 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 N_2 fixation, is the microsymbiont in the stem nodules of the tropical legume *Sesbania rostrata* (Dreyfus *et al.*, 1988). Chen *et al.*, (1991, 1992) reported C_2H_2 reduction activity in 2,4-D-induced root structures of wheat inoculated with *A. caulinodans*, but the C_2H_2 reduction activity was small and only detectable after prolonged incubation (18–20 h). In our studies, the technique of inhibition by O_2 pressure (Tchan *et al.*, 1991) was used to measure the activity of nitrogenase (C_2H_2 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. 10^6 – 10^7 cells per seedling tube) and treated with 2,4-D to make a final concentration of 0.6–0.7 $\mu g\ ml^{-1}$. C_2H_2 reduction and O_2 concentrations were assayed 14–16 days after the treatments.

C_2H_2 reduction and O_2 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 O_2 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 N_2 . O_2 and C_2H_2 were then injected to give the concentration (v/v) required for different experiments—routinely, 4 and 10%, respectively. The seedlings were then placed in a shaking waterbath (160 oscillations min^{-1}) at 30°C under light. C_2H_2 reduction assays and O_2 analysis of the gas space above the wheat seedlings were then carried out using direct injection sampling with a disposable hypodermic syringe (Yu *et al.*, 1993).

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Treatment with ammonia and nitrite

McCartney bottles containing seedlings showing positive C_2H_2 formation after 6–8 h incubation at 30°C were injected with differing amounts of $(NH_4)_2SO_4$ or KNO_2 solution and then incubated further. C_2H_2 reduction assays were performed at regular intervals.

^{15}N assay

$^{15}N_2$ was prepared by using $(^{15}NH_4)_2SO_4$ (Novachem Pty Ltd) by a method described by Burris (1976) and then preserved over saturated Na_2SO_4 solution in a 500 ml glass cylinder connected to the vacuum system. A method developed by Yu *et al.* (1993) was used for $^{15}N_2$ 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% ^{15}N (82 atom% excess)-enriched N_2 , 4% O_2 and 1% C_2H_2 and then placed in a waterbath 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 ^{15}N assay was performed on a Micromass 622 mass-spectrograph.

RESULTS AND DISCUSSION

The effect of different O_2 concentrations on C_2H_2 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 C_2H_4 . Although the C_2H_2 reduction activity in the wheat seedlings treated with azorhizobia alone was high at low O_2 concentration (1%), it decreased quickly with

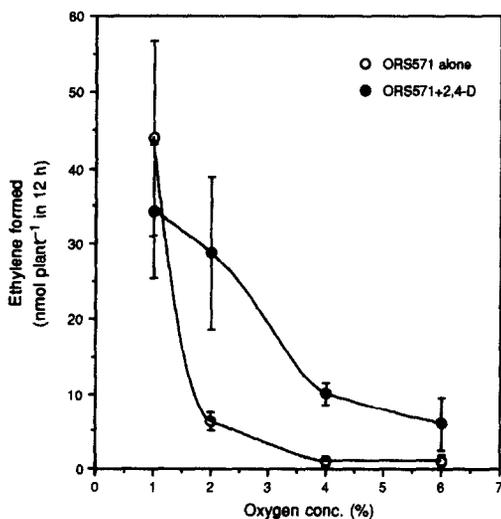


Fig. 1. The effect of O_2 concentration on C_2H_4 formation in wheat seedlings with inoculation of *A. caulinodans* ORS571. Data are mean values \pm SE for 10 replicated flasks, each containing 1 seedling. The residual gases in the flask were 10% C_2H_2 and N_2 .

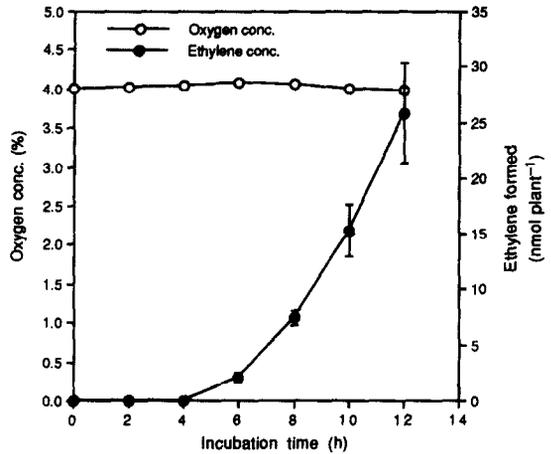


Fig. 2. Time-course of C_2H_4 formation and O_2 concentration in the flasks containing 2,4-D-treated wheat seedlings with inoculation of *A. caulinodans* ORS571. Data are mean values \pm SE for 6 flasks, each containing 1 seedling. The gas compositions in the flask were 4% O_2 , 10% C_2H_2 and 86% N_2 .

increased O_2 concentration in the system, approaching zero at 4% of O_2 concentration. However, the activity in the seedlings treated with azorhizobia plus 2,4-D was more tolerant to the increase of O_2 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 O_2 . 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 O_2 concentration of 4% was selected to ensure inhibition of nitrogenase activity of azorhizobia in the rhizosphere of wheat. This O_2 concentration is higher

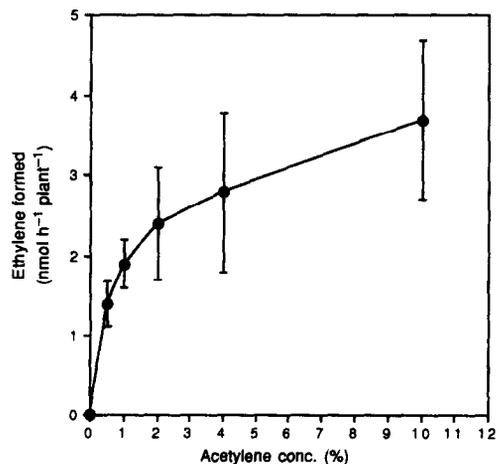


Fig. 3. The effect of C_2H_2 concentration on C_2H_4 formation in the 2,4-D-treated wheat seedlings with inoculation of *A. caulinodans* ORS571. Data are mean values \pm SE for 5 flasks for each C_2H_2 concentration, each flask containing 1 seedling. The residual gases in the flask were 4% O_2 and Ar.

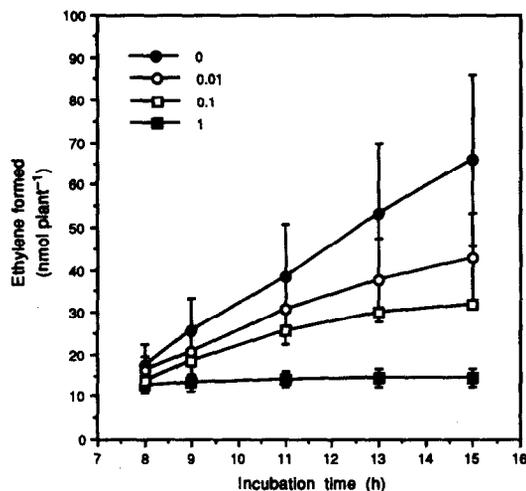


Fig. 4. Time-course of C₂H₂ reduction by wheat seedlings treated with 2,4-D and *A. caulinodans* ORS571 in the presence of different concentrations of KNO₂ (mM). Nitrite was added after 8 h. Data are mean values \pm SE of 2 replicated flasks, each containing 3 seedlings. The gas compositions in the flask were 4% O₂, 10% C₂H₂ and 86% Ar.

than the 2–2.5% used in the 2,4-D-treated seedlings inoculated with *Azospirillum* (Kennedy *et al.*, 1990; Tchan *et al.*, 1991; Zeman *et al.*, 1992; Yu *et al.*, 1993), consistent with the ability of *A. caulinodans* ORS571 to express N₂ fixation in free-living conditions at a higher O₂ concentration of 3% (Dreyfus *et al.*, 1983).

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

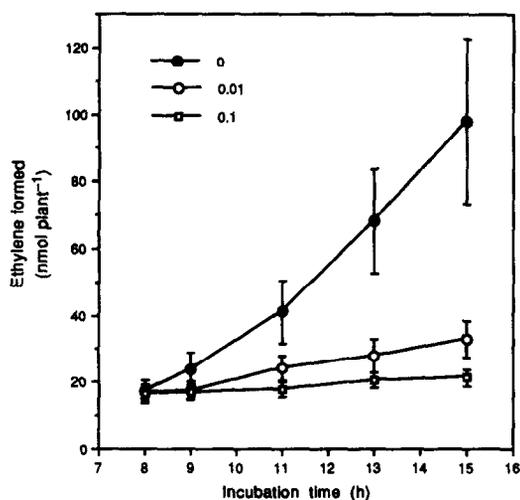


Fig. 5. Time-course of C₂H₂ reduction by wheat seedlings treated with 2,4-D and *A. caulinodans* ORS571 in the presence of different concentrations of (NH₄)₂SO₄ (mM). Ammonia was added after 8 h. Data are mean values \pm SE of 2 replicated flasks, each containing 3 seedlings. The gas compositions in the flask were 4% O₂, 10% C₂H₂ and 86% Ar.

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 C₂H₄ 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 C₂H₄ 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; Sriskandara-jah *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 Chen *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

Table 1. C₂H₄ formation and ¹⁵N incorporation by 2,4-D-treated wheat seedlings inoculated with *A. caulinodans* ORS571

Wheat seedlings treatment	Total N (mg)	¹⁵ N enrichment (atom%)	¹⁵ N fixed (ng)	C ₂ H ₄ formed (nmol)
Control seedlings	1.60 ± 0.09	0	0	0
2,4-D alone	1.37 ± 0.12	0	0	0
ORS571 alone	1.57 ± 0.17	0.0015 ± 0.0011	11.3 ± 6.5	5.1 ± 1.7
ORS571 + 2,4-D	1.59 ± 0.05	0.0333 ± 0.0071	168.0 ± 27.9	94.6 ± 12.3

Data are means of 3 replicates, each containing 2 seedlings in a McCartney bottle (30 ml). After exposure to the gas mixture of 75% Ar, 20% enriched N₂, 4% O₂ and 1% C₂H₂, the bottles were placed in shaking waterbath (160 min⁻¹) at 30° for 24 h. Standard errors of assays (±SE) are shown. Expression of activities relative to seedling N content (ca. 1.5 mg N/2 seedlings) does not significantly affect the relative activity of the treatments.

with 1 mM KNO₂ 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, KNO₂ 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 C₂H₂ 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 1). It should be noted that the data in Table 1 cannot be used to calculate the rate of C₂H₂ reduction and N₂ fixation for the system, since the relative rates of C₂H₂ reduction and N₂ reduction are not known. This would be expected to be controlled by the concentration of each substrate, relative to its respective *K_m* 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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