

**Short Communication**

# Nitrate Reductase in Barley Roots under Sterile, Low Oxygen Conditions<sup>1</sup>

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## ABSTRACT

Levels of nitrate reductase activity (EC 1.9.6.1.) as high as 11  $\mu$ moles nitrite produced/hour-gram fresh weight were found in barley (*Hordeum vulgare* cv. Compana) roots grown under low oxygen conditions. Roots of plants given identical treatment under sterile conditions did not develop the high levels of nitrate reductase activity. The results suggest that the buildup of particulate, reduced viologen-utilizing nitrate reductase reported in barley roots may be caused by bacterial contamination. The nitrate reductase activity in roots grown under low oxygen conditions was not specific for reduced nicotinamide adenine dinucleotide like the assimilatory nitrate reductase (EC 1.6.6.1.) normally found in aerated plant roots.

Nitrate reductase activity in plant roots is generally much lower than activity in leaf extracts (12). Boutard (2) and Coupe *et al.* (3) extracted nitrate reductase from barley roots and reported that roots were more efficient than leaves in nitrate assimilation. Mifflin (9) reported that the level of nitrate reductase activity in extracts of young barley roots was as high or higher than that in leaf tissue. Mifflin's data suggest two systems capable of reducing  $\text{NO}_3^-$  in barley root extracts: (a) a soluble component and (b) a particulate component. The particulate component utilized succinate more efficiently than NADH. Sanderson and Cocking (12) reported a similar succinate-stimulated reduction of  $\text{NO}_3^-$  by particulate fractions from roots and fruit of tomatoes. Further work by Mifflin (10) showed that cultures bubbled with  $\text{N}_2$  led to a buildup of a succinate-utilizing nitrate reductase in barley roots. In regard to Mifflin's work, Smith (14) indicated that nitrate reductase activity of comparable magnitude was never achieved in similar work with barley roots. Smith suggested that Mifflin's method of growing barley might cause one to suspect bacterial contamination. The dissimilatory or respiratory nitrate reductase (EC 1.9.6.1.) of bacteria allows the utilization of  $\text{NO}_3^-$  as a terminal electron acceptor when the  $\text{O}_2$  supply is low. This dissimilatory nitrate reductase unlike the assimilatory nitrate reductase (EC 1.6.6.1.) of plants cannot utilize NADH as a reductant.

Several low  $\text{O}_2$  experiments have been reported where barley and other plants have been used to study nitrogen metabolism,

and in some cases (7, 11),  $\text{NO}_2^-$  accumulation in the medium has been noted. The following experiments were designed to determine whether some of the nitrate reductase activity reported from plant root tissue resulted from microbial contamination, especially under low  $\text{O}_2$  conditions.

## MATERIALS AND METHODS

The intact tissue assay used by Ferrari and Varner (4) for barley aleurone layer nitrate reductase was adapted for use on whole barley (*Hordeum vulgare* cv. Compana) roots. The incubation mixture, totaling 4 ml, contained 0.25 mmole of potassium phosphate buffer (pH 7.5), 0.05 mmole of  $\text{KNO}_3$ , and 5% (v/v) ethyl alcohol, the optimum concentration of surfactant. Nitrogen gas was bubbled for 2 min through the incubation mixture containing three intact roots, and the tubes were tightly stoppered and placed in a water bath at 30 C. Reactions were stopped after 15 min by the addition of 2 ml of 1% sulfanilimide in 3 N HCl and the color was developed by addition of 2 ml of 0.02% N-1-naphthylethylenediamine dihydrochloride in 95% ethyl alcohol. The quantity of diazo-complex resulting from the nitrite produced was determined at 540 nm with a Gilford Spectrophotometer model 2000. All assays were replicated three times and results were averaged. Nitrite concentration of the culture media was determined directly by the preceding method.

The cell-free extract was prepared by grinding the barley roots with a mortar and pestle with acid-washed sand with an equal weight of the extraction buffer described by Mifflin (9). The extraction was conducted in a plastic bag under a stream of  $\text{N}_2$  after the bag had been evacuated and filled with  $\text{N}_2$  three times. The extract was centrifuged at 500g for 5 min and the cell-free nitrate reductase activity was determined by the method of Hageman and Flesher (5). Where specified, reduced methyl viologen (reduced by addition of 1:1 mixture of sodium dithionite and sodium bicarbonate) or formate was used instead of NADH as electron donors in the cell-free assay to ensure that endogenous levels of reductant would not be limiting.

**Sterile Barley Root Culture.** Seeds were sterilized by soaking in absolute ethyl alcohol for 5 min, then the ethyl alcohol was decanted and 40% Clorox solution was vacuum-infiltrated three times for 10 sec each time during a 5-min period. The Clorox solution was decanted and the seeds were used without rinsing. The seeds were placed in Petri dishes containing sterile water and allowed to germinate overnight in the dark at 22 C. Twelve embryos were excised and transferred to Petri dishes containing a nutrient medium consisting of 0.3% (w/v) peptone, 0.2% (w/v) yeast extract, 0.5% (w/v) agar, and 1% (w/v) glucose in a 0.2 mM  $\text{CaSO}_4$  solution. The seeds were placed in the dark for 3 days at 22 C where approximately 50% of the embryos grew. Usually about 20% of the Petri dishes were found to be contaminated at this point. Seedlings from sterile Petri dishes were then

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transferred to 1000-ml tall form beakers containing 400 ml of a sterile nutrient medium and covered with Petri dishes. The medium contained 0.5% (w/v) agar, 0.3% (w/v) yeast extract, and 1% (w/v) glucose in full strength Hoagland's solution (6) with micronutrients and 1 mg/l Fe (Sequestrene 330). Plants (usually about 6) from one Petri dish were transferred to each beaker and the beakers were placed in the growth chamber. The seedlings were given 16 hr of light (27 C) and 8 hr of darkness (21 C). The light intensity was 1800 ft-c at the plant level. After 4 days, plants from beakers remaining sterile were transferred to sterile half-strength Hoagland's solution in 1000-ml flasks and were supported by sterile cotton plugs. Solutions were bubbled with N<sub>2</sub> or air coming through a sterile cotton plug and then through a sterile 0.22  $\mu$ m Millipore filter. Nitrate reductase was assayed after the specified treatment period and some roots were plated on the previously mentioned peptone-yeast extract nutrient agar to check for contamination.

## RESULTS AND DISCUSSION

**Effect of Aeration of Nitrate Reductase Activity.** Non-sterile barley plants were germinated and grown according to the procedure of Blevins *et al.* (1) for 2 weeks in half-strength Hoagland's solution and then transferred for 4 days to aerated half-strength Hoagland's solution or to a similar solution bubbled with N<sub>2</sub>. The nitrate reductase activity of roots from plants grown in aerated solutions was constant over the 4-day period; however, root nitrate reductase activity of plants grown in solutions under N<sub>2</sub> increased markedly (Fig. 1).

In some experiments, low O<sub>2</sub> barley roots developed a nitrate reductase activity up to 20-fold greater than the NADH-nitrate reductase activity found in aerated roots. These high levels of activity required viologen dyes or formate as electron donors and could not be achieved with NADH, NADPH, or succinate.

In low O<sub>2</sub> roots ground in a N<sub>2</sub> atmosphere and fractionated by differential centrifugation in the extraction buffer, the nitrate reductase activity was located primarily in the particulate fraction (Table I). The formate-linked nitrate reductase activity was lost during maceration of the tissue, possibly as a result of the

Table I. *Differential Centrifugation Study of Barley Root Nitrate Reductase from Plants under N<sub>2</sub> and Nonsterile Conditions*

Electron Donor	Activity of Nitrate Reductase			
	500g (5 min)		150,000g (2 hr)	
	Crude	Supernatant	Supernatant	Pellet
	$\mu$ moles NO <sub>2</sub> <sup>-</sup> /hr-g fresh wt			
NADH	2.6	2.2	2.3	0.3
Methyl viologen	50.5	38.1	2.1	48.9

ability of formate dehydrogenase. The particulate fraction was also effective in chlorate reduction.

Since many facultative anaerobes possess extremely high levels of nitrate reductase (8, 15), an experiment was conducted to determine whether nitrate reductase activity increased in low O<sub>2</sub> barley plants grown under sterile conditions. Sterile plants failed to attain the high levels of nitrate reductase activity reported for non-sterile conditions, even after 7 days in low O<sub>2</sub> culture. The non-sterile roots produced a nitrate reductase activity 10-fold higher than roots from the sterile plants. The actual values for the nitrate reductase activity for roots from non-sterile and sterile seedlings were 11.3 and 1.2  $\mu$ moles of NO<sub>2</sub><sup>-</sup> produced/hr-g fresh weight, respectively.

Shive (13) found greater NO<sub>3</sub><sup>-</sup> uptake and reduction in plants grown with decreasing O<sub>2</sub> content of the culture medium, while Mifflin (10) in recent work reported that low O<sub>2</sub> barley roots have higher nitrate reductase activity than roots from aerated cultures. We have found high levels of nitrate reductase activity in barley roots given the anaerobic treatment (Fig. 1), but observed low levels of the enzyme in sterile roots given the same treatment. These results suggest that the buildup of the particulate viologen-utilizing nitrate reductase reported by Mifflin (10) for barley roots may be bacterial contamination. The nitrate reductase in the nonsterile barley root studies reported here differs from that of Mifflin (10) in that succinate would not serve as an electron donor.

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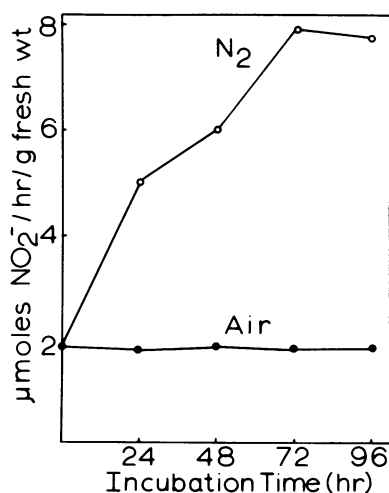


FIG. 1. Nitrate reductase activity in barley roots from plants grown in nonsterile solutions and treated with either N<sub>2</sub> or air. The intact tissue assay was used for the determination of nitrate reductase activity shown in this experiment with methyl viologen as supplemental electron donor when reduced with sodium dithionite.