

Nitrite decreases ethanol production by intact soybean roots submitted to oxygen deficiency

A role for mitochondrial nitric oxide synthesis?

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Nitrate increases the tolerance of plants to hypoxia, although the mechanisms related to this beneficial effect are still unclear. Recently, we observed that cultivation of soybean plants with nitrate reduced hypoxic accumulation of fermentation end products by isolated root segments compared with the ammonium treatment. Interestingly, the same decrease in the intensity of fermentation was detected when ammonium-grown root segments were incubated with nitrite, suggesting the involvement of this anion in the nitrate-mediated modulation of fermentative metabolism. Here we extended these experiments to intact plants subjected to root hypoxia and observed similar effects of nitrate and nitrite in reducing root ethanol production, which indicates the physiological relevance of the *in vitro* results. In both experimental systems, nitrite stimulated nitric oxide emission by ammonium-grown roots to levels similar to that of nitrate-cultivated ones. The involvement of mitochondrial reduction of nitrite to nitric oxide in the root response to hypoxia is suggested.

Root hypoxia is a highly stressful condition which may occur as a consequence of soil flooding resulting from excessive rainfall or irrigation usually associated with poor soil drainage.¹ The deficiency of oxygen, the terminal acceptor of the mitochondrial respiratory chain, exerts negative effects on the growth and productivity of economically important species, as well as affecting the abundance and distribution of native species in natural environments.² At the biochemical level, oxygen deficiency leads to inhibition of oxidative phosphorylation followed by the induction of anaerobic processes of ATP production and NAD(P)⁺ regeneration, such as fermentation pathways.² Despite the transient induction of lactic acid fermentation which occurs in some plant species at the initial phase of the hypoxic response, ethanol is considered to be the main fermentative end product in nearly all plant roots subjected to hypoxia.³

There is strong experimental evidence, also supported by observations in the field, that the addition of nitrogen in the form of nitrate (NO_3^-), but not ammonium (NH_4^+), reduces the deleterious effects of oxygen deficiency in diverse plant species.⁴⁻⁸ A role for NO_3^- reduction by the nitrate reductase (NR) enzyme in proton consumption and NAD(P)⁺ regeneration during hypoxia has been suggested,^{9,10} although there is some controversy about this

hypothesis.^{11,12} Thus, the mechanisms by which NO_3^- improves plant tolerance to hypoxia are still under debate.

In a recent study,¹³ we showed that segments of soybean roots isolated from NO_3^- -cultivated plants produced lower amounts of fermentation end products under hypoxia in comparison with those of NH_4^+ -cultivated plants. Interestingly, the same decrease in the intensity of fermentation was detected when root segments from ammonium-grown plants were incubated with nitrite (NO_2^-), the product of NO_3^- reduction by NR. The effect of NO_2^- on fermentation occurred irrespective of the presence of NO_3^- and of the extremely low NR activity of ammonium-grown root segments. Therefore, the involvement of NO_2^- in the NO_3^- -mediated modulation of fermentative metabolism was suggested.¹³

It is noteworthy that our previous study,¹³ as well as most studies regarding the effect of NO_3^- and/or NO_2^- on hypoxic metabolism,^{11,12,14,15} was performed *in vitro*, using isolated root segments or coleoptiles as a model system. In order to verify whether our hypothesis of the involvement of NO_2^- in the NO_3^- -mediated response during hypoxia could have physiological relevance in a whole-plant context, here soybean plants were subjected to root hypoxia and the effect of distinct nitrogen regimes on ethanol production by intact roots was evaluated (Fig. 1A). In agreement

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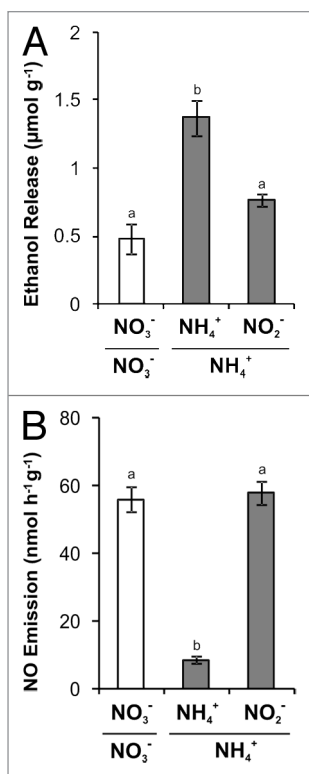


Figure 1. Effect of distinct nitrogen treatments on ethanol production and nitric oxide emission by hypoxic soybean roots. Non-nodulated soybean plants [*Glycine max* (L.) Merrill cv IAC-23] were hydroponically cultivated in a greenhouse under natural conditions of light and temperature (mean min/max: 19°C/36°C). Hoagland and Arnon's N-free nutrient solution at 1/3 strength was supplemented with 5 mM NO₃⁻ or 5 mM NH₄⁺ as the nitrogen source.¹³ Plants at the V4 stage were transferred to a N₂-purged nutrient solution which was then covered with mineral oil in order to maintain root hypoxia. Measurements using an O₂ electrode demonstrated that O₂ levels in the solution were maintained at less than 5% of saturation. In the case of NO₃⁻-cultivated plants (white bars), the hypoxic nutrient solution always contained 5 mM NO₃⁻, whereas NH₄⁺-cultivated plants (gray bars) were treated with 5 mM NH₄⁺ or 1 mM NO₂⁻. **(A)** Ethanol release in nutrient solution was analyzed enzymatically after 24 h of root hypoxia treatment. **(B)** Nitric oxide emission by roots submitted to hypoxia for 3 h was determined using the fluorescent probe DAF-2, as described before.¹³ Data represent mean ± SE (n = 6). Different letters indicate significantly different values according to one-way ANOVA followed by the Tukey test (p < 0.05).

with our previous *in vitro* results, roots from NO₃⁻-cultivated plants released significantly lower amounts of ethanol in the nutrient solution under hypoxia when compared with NH₄⁺-cultivated roots. Moreover, the addition of NO₂⁻ significantly reduced the intense hypoxic ethanol production by NH₄⁺-cultivated roots. Thus, the role of NO₃⁻ in decreasing the alcoholic fermentative metabolism of intact roots and the involvement of NO₂⁻ in this mechanism can be suggested. It remains to be established what

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impact different nitrogen treatments might have on the hypoxic levels of other carbon metabolites, such as sugars and organic acids, not only in roots but also in leaves.

During both *in vitro*¹³ and *in vivo* (Fig. 1B) experimental approaches, it was also determined that hypoxic roots from NO₃⁻-cultivated plants emitted much higher levels of nitric oxide (NO) than those from NH₄⁺-cultivated ones, which is consistent with recent data showing that a considerable amount of root NO₃⁻ is directed to NO synthesis during oxygen deficiency.¹⁶ Furthermore, a clear effect of NO₂⁻ in stimulating NO emission by NH₄⁺-grown roots was observed *in vitro*¹³ and *in vivo* (Fig. 1B). Interestingly, NO₂⁻-induced NO emission by root segments was prevented in the presence of potassium cyanide, suggesting the involvement of mitochondrial respiratory chain in this mechanism.¹³ These results are in accordance with the growing evidence concerning the importance of mitochondrial reduction of NO₂⁻ in NO synthesis under oxygen deficiency.¹⁷

Overall, results shown in Figure 1 indicate a negative correlation between NO emission and ethanol production in intact roots, similar to that observed in our *in vitro* study.¹³ Indeed, NO₂⁻ reduction to NO by the mitochondrial respiratory chain has been associated with anaerobic ATP production and NAD(P)⁺ regeneration,¹⁸ which may constitute an alternative to fermentation, thus reducing the intensity of this process.¹³ The relevance of the mechanism of NO synthesis from NO₂⁻ in maintaining mitochondrial functionality during this stress condition has been suggested.^{17,18} Therefore, a striking link between NO₃⁻ nutrition, mitochondrial reduction of NO₂⁻ and plant response to hypoxia arises from these observations.

Another interesting possibility is a signaling function of the NO produced by roots during hypoxia. Although we showed that NO *per se* did not directly modulate the fermentative metabolism of soybean root segments,¹³ it cannot be excluded that NO may act as a mediator of other aspects of plant response to hypoxia, such oxygen sensing¹⁹ and hormone signaling.^{16,20} Given the broad effect of NO on gene regulation,²¹ the involvement of this radical in the hypoxia-induced transcriptional reprogramming in plant tissues is promising. A NO-mediated control at the post-translational level requires further investigation, since hypoxia has been recently shown to increase the level of S-nitrosylated compounds in *Arabidopsis thaliana* roots.¹⁶

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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