Nitric oxide emission from barley seedlings and detached leaves and roots treated with nitrate and nitrite

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ABSTRACT

Nitric oxide (NO) emission from detached barley leaves, roots and whole plants treated with various nitrate or nitrite concentrations under light/dark and aerobic/anaerobic conditions was quantified by using a chemiluminescence detector. NO emission from detached tissues and whole plants treated with moderate nitrate concentration (60mM) was relatively higher under anaerobic condition, and was positively correlated with nitrite concentration. Darkness and anaerobic condition remarkably induced NO emission from detached barley leaves**.** On the contrary, NO emission from detached roots and whole plants was relatively higher in light. As for whole plants treated with 60mM nitrate and 12mM nitrite, the pattern of NO emission in normal environment was broken by light-dark and aerobic-anaerobic transition. Light and anaerobic condition induced NO emission significantly in the whole plant. The whole barley plant emitted significantly higher amount of NO than detached leaves or roots.

Keywords: *Hordeum vulgare*; chemiluminescence; light; dark; aerobic; anaerobic

The use of fertilizers to improve food production conduced to the increase of nitrogen input to intensively managed agricultural ecosystems (Abdalla 2009). Recently, numerous studies were carried out on the nitrogen cycle of the agricultural ecosystems, especially involving nitrogen oxides emission by microbial nitrification and denitrification, such as nitric oxide (NO), nitrogen dioxide (NO_2), nitrous oxide (N_2O) and so on (Fang and Mu 2007). Some researchers paid more attention to NO emission from the soils which was enhanced by nitrogen fertilizer application due to increasing soil available nitrogen content (Li and Wang 2007). It is noteworthy that NO, which is a byproduct of plant nitrogen metabolism, can also be released from plant (Wildt et al. 1997).

Furthermore, studies conducted during the past decades have shown that NO is an exceptional molecule due to the versatility of its actions in plant growth and development such as seed germination (Beligni and Lamattina 2000), stomatal closure (Neill et al. 2002), repression of flowering (He et al. 2004), etc. Based on the multiple physiological roles of NO in plant, it is important to clarify how much NO is produced and how NO production is controlled by environmental factors.

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NO can be produced in plants by enzymatic and non-enzymatic systems. A well established NO producing system in plants is assimilatory nitrate reductase (NR). Under favorable conditions, nitrite is produced by the NR reaction, and then nitrite reductase (NiR) converts the nitrite to ammonium ion which is assimilated into amino acids (Li and Oaks 1995). Under some abnormal conditions such as photo-inhibition, NR can catalyze nitrite which is accumulated in cells to produce NO at the expense of NAD(P)H (Rockel et al. 2002). A recent study has shown that NOS-like protein may function as an NO source in plants (Guo et al. 2003). Besides enzymatic pathway, a non-enzymatic chemical reaction of nitrite to NO was suggested to occur in the acidic apoplast of some tissue types such as aleurone layers (Bethke et al. 2004).

NO produced by plant tissues was first observed by Klepper (1979) in soybean plants treated with photosynthetic inhibitor herbicides or other chemicals as well as dark and anaerobic conditions (Klepper 1987, 1990, 1991). It was reported that this NO emission was involved in chemical reactions of accumulated nitrite with salicylate derivatives and other plant metabolites or the chemical decomposition of HNO_2. Further research found that soybean and winged bean produced NO during the *in vivo* nitrate reductase assay (Dean and Harper 1986). Moreover, Rockel et al. (1996) found that various nitrate-nourished plant species had a compensation point for NO uptake from the atmosphere showing a net NO emission at low atmosphere NO concentration (Rockel et al. 1996, Wildt et al. 1997). Recently, some works on detecting NO in plant tissues or cell cultures were published (Lea et al. 2004, Gupta et al. 2005, Planchet et al. 2005), whereas knowledge on quantifying NO production in whole plants and the impact factors on NO emission rates are comparatively limited, and the comparison of NO emission from detached leaves or roots with whole plants is rarely reported. Here we measured NO in the gas phase to quantify NO emission from detached barley leaves, roots and whole plants which were fed with different nitrogen (nitrate or nitrite) by using the chemiluminescence detector at light/dark and air/nitrogen conditions. We first showed the difference of NO emission from detached tissues and whole plants, and how NO emission from whole plants was modulated by the external environmental factors. These data can contribute to a better understanding of the physiology function of NO in plant growth and development.

MATERIALS AND METHODS

Plant material and growth

Selected barley seeds (*Hordeum vulgare* L. cv. Kunlun 12) from Qinghai Academy of Agricultural Forestry Sciences, Xining, China were surface-sterilized with 0.5% hypochlorite for 30 min, washed thoroughly with distilled water, soaked in water overnight. Subsequently, the seeds were sown on nylon flat mesh and cultivated in a growth chamber after germination with a temperature regime of 22/19°C (day/night) and a 12 h photoperiod at 120 μmol/m^2 s Photosynthetically Active Radiation (PAR), 40–60% relative humidity. The seedlings were irrigated with full Hoagland solution containing 15mM nitrate three times a week. All investigations were carried out at day 7 after germination when the second leaves were fully expanded.

Experimental set-up for gaseous NO measurements

Detached leaves experiments. Gas phase NO was measured by a chemiluminescence technique as described by Rockel et al. (2002) and Planchet et al. (2005) with some modifications. The NO measurement equipment is shown in Figure 1. The principle of NO measurement method is the detection of chemiluminescence by NO analyzer, which occurs when nitrogen oxide (NO) reacts with ozone (O_3). For detached barley leaves experiments, the leaves were cut off from the plants, placed in nutrient solution, where the leaves were

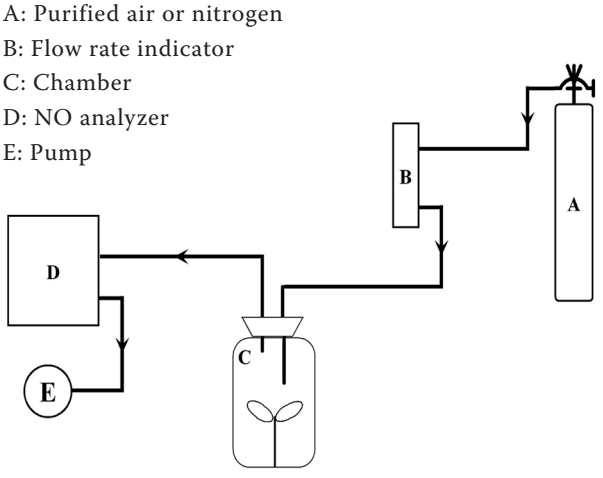


Figure 1. Schematic diagram of the NO measurement equipment

cut off a second time from the vaginas below the solution surface. The leaves were then placed in a transparent glass chamber (0.25 l volume) with 0.02 l KNO_3 (0, 15, 30, 60, 90, 120mM) or KNO_2 (0, 1, 2, 4, 8, 12mM) solution. As for a closed measurement system, the glass chamber was filled with measuring gas (purified air or nitrogen), and then closed about 1 h by a stopple with an inlet and an outlet. The NO accumulated in the chamber was pulled from the outlet to a chemiluminescence NO detector (200EU, Advanced Pollution Instrumentation Inc., USA, detectable limit 0.4 ppb) with a constant flow of 1 l/min, and the outside air was passed by the inlet. The NO concentration of the inflowing air was measured before and after each experiment, whereas the outflowing air was measured continuously during the experiments. NO emission fluxes were calculated from the concentration differences between the inlet and outlet of the chamber.

Detached roots experiments. Roots were washed three times by distilled water, and then cut into 1 cm (approx.) segments. Three grams (3 g) of root segments were immersed in a transparent glass (0.5 l volume) with 0.2 l KNO_3 (0, 15, 30, 60, 90, 120mM) or KNO_2 (0, 1, 2, 4, 8, 12mM) solution, closed about 1 h with purified air or nitrogen, whisked constantly with a magnetic stirrer (150 rpm) in course of closure. NO concentration was measured by the chemiluminescence detector as described above.

NO measurement of whole plants. The barley seedlings, which were pretreated with 0.25 l KNO_3 (0, 15, 30, 60, 90, 120mM) for 3 days, were placed in a transparent lid chamber of 6 l volume for NO measurement. As for KNO_2 treatment, the roots of barley seedlings were immersed in KNO_2 (0, 1, 2, 4, 8, 12mM) solution, and whole plants were transferred to the experiment setup for NO measurements immediately. The NO emission from whole plants was measured by two methods: (1) a closed system as described as the method of detached leaves and roots; (2) an open system with a constant flow by measuring gas (purified air or nitrogen). The gas was pulled through the chamber in flow rate with 1 l/min and subsequently through the chemiluminescence detector for measuring momentarily. Both gas concentrations of the inflowing and outflowing air were measured alternately at 0.5 h intervals.

In all experiments involved in light condition, a 400W hydrargyrum quartz iodide-lamp(Hqi) above the chamber provided a steady light (about 250 μmol/m^2 s PAR). Air temperature in the chamber was continuously monitored by a KTP-01 thermocouple temperature probe, and was usually about 22°C in dark and 23–25°C in light.

Statistical analysis. All data in the figures were analyzed by one-way ANOVA, using SPSS software 11.0 (SPSS Inc., Chicago, IL, USA). Values are means of four independent experiments. Means were tested by using the Least Significant Difference (LSD) at a significant level of $P < 0.05$. Standard errors were indicated by error bars.

RESULTS AND DISCUSSION

NO emission from detached barley leaves. Detached leaves treated with nitrate showed no significant change in the NO emission, either in light or dark under aerobic condition (Figure 2a); yet, it was higher in anaerobic condition, especially in dark ($P < 0.05$). Under anaerobic and dark condition, NO emission from detached barley leaves treated with 60mM nitrate reached 0.697 nmol/g FW/h, which is significantly higher (approx. 8 times) than that in light (0.088 nmol/g FW/h) ($P < 0.01$). However, higher nitrate concentration (90 and 120mM) may affect normal metabolism of barley seedlings and may finally result in the decrease of NO emission.

Nitrite, as the first product of NR enzymatic reaction, is the main limiting factor of NO production (Planchet et al. 2005). Furthermore, the excessive nitrite accumulation in cells is toxic to plant (Gupta et al. 2005). So plants may reduce too high internal concentration of nitrite by means of releasing high level of NO. Under aerobic or anaerobic condition, detached barley leaves treated with various nitrite concentrations released NO, and the NO release intensity was positively correlated with nitrite concentration (Figure 2b). The effects of light/dark on NO emission from nitrite-fed leaves were indistinctive under aerobic condition. Consistent with nitrate treatments, NO emission from nitrite-fed leaves increased strongly under anaerobic condition. Furthermore, NO emission in dark was markedly higher (4-fold) than that in light with the treatment of 8mM nitrite ($P < 0.05$) (Figure 2b). This was in accordance with the 'light-off peak' or 'light-off burst' that was found in sunflower, spinach and tobacco leaves (Rockel et al. 2002, Planchet et al. 2005). It was usually considered to be correlated with a more transient nitrite accumulation in dark (Riens and Heldt 1992).

NO emission from detached barley roots. NO emission from detached barley roots treated with nitrate was below 0.1 nmol/g FW/h. Under anaero-

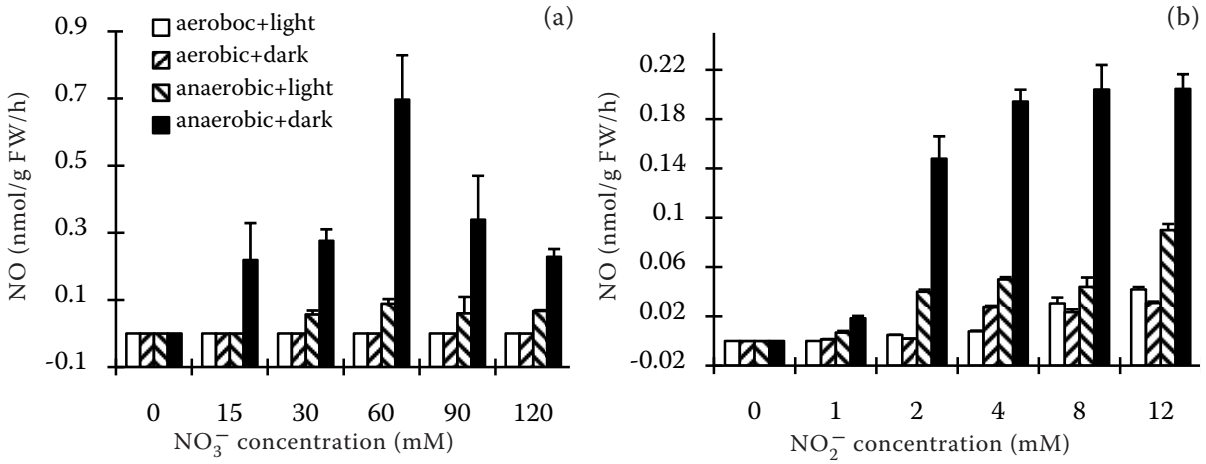


Figure 2. NO emission from detached barley leaves treated with 0–120mM nitrate (a) and 0 to 12mM nitrite (b) under dark/light or aerobic/anaerobic conditions. Light intensity was 250 μmol/m^2 s PAR. Results are means of samples from at least four different experiments

bic condition, NO emission was relatively higher in light, reached the maximum in 60mM nitrate (about 0.06 nmol/g FW/h) (Figure 3a). NO emission from detached barley roots was positively correlated with nitrite concentration (Figure 3b), and increased significantly under anaerobic condition. Similar to the roots treated with nitrate, NO emission from anaerobic roots in light was higher than that in dark, up to 0.87 nmol/g FW/h in 12mM nitrite. Interestingly, in the same nitrite concentration, NO emission from roots was much higher (over 10-fold) than that from detached leaves, especially under light and anaerobic condition (Figures 2b and 3b).

Roots, in contrast to leaves, may be frequently exposed to oxygen deficiency under water-logged soil. Anaerobic plant tissues reduced nitrate to nitrite at a high rate, however they were unable to further reduce nitrite to ammonia (Stoimenova et al. 2003). Consequently, anaerobic roots accumulate excessive nitrite. Moreover, under anaerobic condition, NR is not the only pathway to produce NO, other pathways just like mitochondrial electron transport also reduce nitrite to NO (Tischner et al. 2004). Gupta et al. (2005) revealed that mitochondria purified from roots, but not those from leaves, reduced nitrite to NO at the expense of NADH. In our research, NO emission from detached roots was

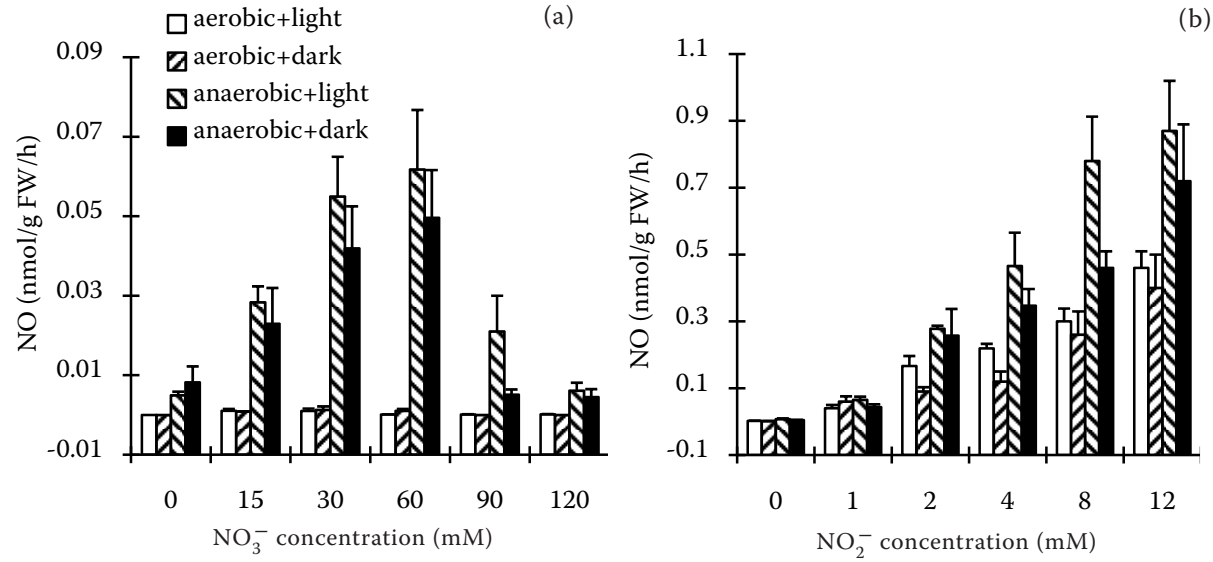


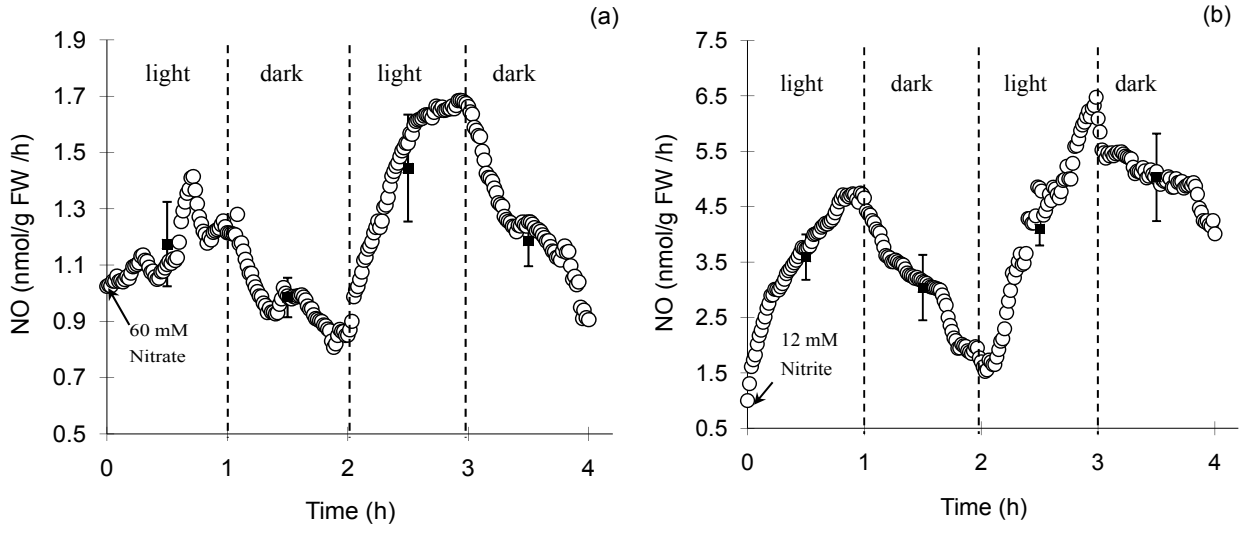
Figure 3. NO emission from detached barley root segments immersed in 0–120mM nitrate (a) and 0 to 12mM nitrite, (b). Light intensity was 250 μmol/m^2 s PAR. Each value is the mean of samples from at least four different experiments

remarkably much higher than from leaves under anaerobic condition; this result maybe related to the different capacity of mitochondria electron transport in roots and leaves.

NO emission from barley seedlings treated with light-dark and air-nitrogen transition. In order to assess the effect of light on NO emission from whole plants, the whole barley seedlings treated with 60mM nitrate or 12mM nitrite were exposed continuously under air condition (aerobic). And then, the NO emission was detected during the light-dark transition. For eliminating the influences of plant internal factors (e.g. biological rhythm) during the real-time dynamic measurement of NO emission, we observed the characteristics of NO emission from nitrate or nitrite-fed whole barley seedlings under normal conditions (air, 25°C, 120 μmol/m^2 s). NO emission from whole barley seedlings was maximum after 1.5 h in 60mM nitrate (up to 0.866 nmol/g FW/h) and then dropped to a lower level (0.536 nmol/g FW/h, data not shown). With the treatment of 12mM nitrite, the NO emission from whole barley seedlings increased continually during the first 1 h, and then followed by a stable higher NO release level (about 2.2 nmol/g FW/h, data not shown).

When barley seedlings were treated with light-dark transition, the average NO emission rate from barley seedlings induced by 60mM nitrate for 3 days in light was much higher than that in dark (Figure 4a). During double light treatments, NO emission increased up to 1.483 and 1.730 nmol/g FW/h, and then dropped to 0.831 and 0.914 nmol/g FW/h in dark, respectively. Similarly, NO emission from whole barley seedlings treated with 12mM nitrite reached to 4.592 nmol/g FW/h at the first hour in light and 6.475 nmol/g FW/h at the third hour in light, respectively. Then it decreased to 1.701 nmol/g FW/h at the second hour in dark and 4.140 nmol/g FW/h at the fourth hour in dark, respectively (Figure 4b). Consistent with the results of detached roots, the NO emission from whole seedlings can be remarkably stimulated by light (Figures 3 and 4), due to the increase of the substrates related to NR pathway and NR activity in light (Li and Oaks 1995, Xu and Zhao 2003).

Subsequently, we detected NO emission from whole barley seedlings treated with aerobic-anaerobic switch, i.e. air-nitrogen transition. The results showed that barley seedlings induced by the presence of 60mM nitrate for 3 days usually led to a little NO release in air (less than 2.0 nmol/g FW/h). On the contrary, NO emission increased significantly after switching to nitrogen from air; it reached to ca 8.0 nmol/g FW h within 20 min. When the barley seedlings were returned to air, NO emission dropped back to 0.52 nmol/g FW/h drastically (Figure 5a). The average NO emission rate in nitrogen was up to about 4.0 nmol/g FW/h which was remarkably higher than that in air (1.2 nmol/g FW/h). The characteristics of NO emission from barley seedlings treated with 12mM nitrite during the air-nitrogen transition were similar to nitrate treatment. In this case, NO emission



Figures 4. NO emission from whole barley seedlings induced for 3 days by 60mM nitrate (a) and in 12mM nitrite (b) in the chamber during the light-dark transition. Light intensity was 250 μmol/ms PAR. The curves of NO instantaneous emission rate (○) are representative experiments out of four separate experiments which produced almost identical curves. Average NO emission rate (■) is the mean of four different experiments

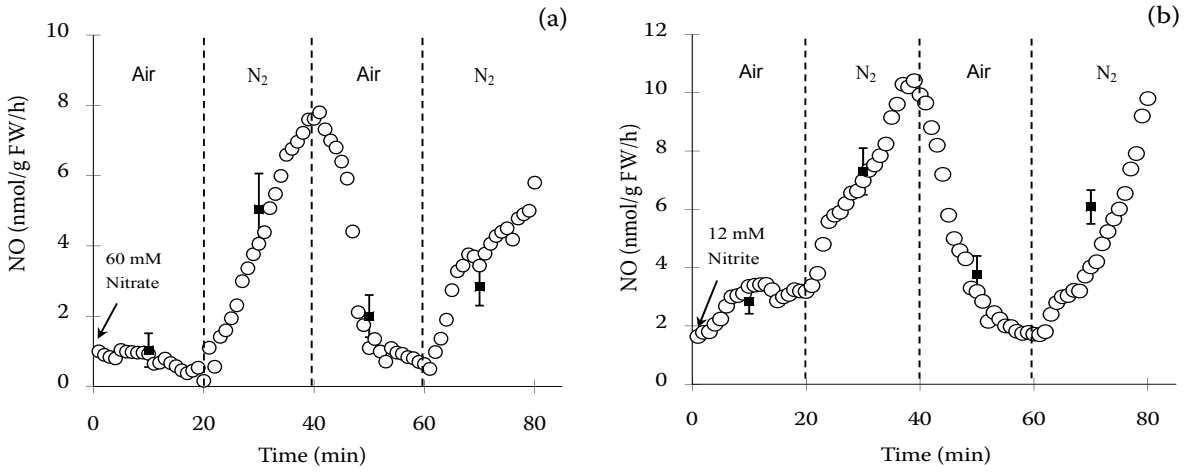


Figure 5. NO emission from whole barley seedlings induced for 3 days in 60mM nitrate (a) or 12mM nitrite (b) during the air-nitrogen transition with a continuous light intensity (120 μmol/m^{2}s PAR). The curves of NO instantaneous emission rate (○) are representative recordings out of at least four similar independent experiments. Each value of average NO emission rate (■) is the mean of four different experiments

increased sharply under nitrogen condition by up to 10.05 nmol/g FW/h in maximum and the average NO emission rate in nitrogen was two times higher than that in air (Figure 5b).

Under anaerobic condition, both whole plants (Figure 5) and detached barley tissues (Figures 2 and 3) emitted much more NO than that of aerobic condition, this is consistent with the results of tobacco root segments, leaves and suspension cells (Gupta et al. 2005, Planchet et al. 2005). The activation of NR induced by cellular acidification and a decreased rate of plastidic nitrite reduction may bring anaerobic nitrite accumulation (Kaiser and Brendle-Behnisch 1995, Botrel et al. 1996, Lea et al. 2004), and then the accumulative nitrite further led to abundant NO emission.

Difference of NO emission between detached tissues and whole plants. Although the NO emission from detached leaves and roots by using closed measurement system at the variable conditions of dark and light, aerobic and anaerobic condition with a series of treatments with nitrate and nitrite were reported in Figures 2 and 3, the differences of NO emission among detached leaves, roots and whole seedlings under a stable normal physiological condition remain unclear. To further elucidate this question, a controlled experiment was conducted. Firstly, we set up the same nitrate and nitrite concentration series as already described previously with the normal physiological conditions, i.e. 25°C in temperature, 120 μmol/m^{2}s in PAR and ambient air with normal oxygen. And then the NO emis-

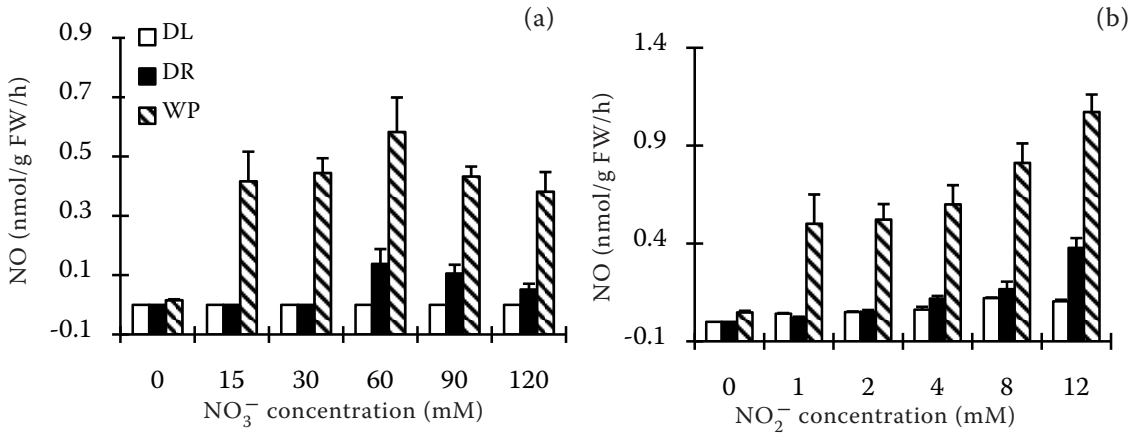


Figure 6. NO emission from detached barley leaves, roots and whole plants grown with 0–120mM nitrate (a) and 0–12mM nitrite (b). All data are obtained at a light flux of 120 μmol/m^{2}s and a temperature of 25°C. DL – detached leaves; DR – detached roots; WP – whole plants

sion was measured by using a closed measurement system as described in 'Materials and Methods'. The results of nitrate treatments showed that detached leaves and roots emitted very little NO (below 0.138 nmol/g FW/h), whereas whole plants emitted a large amount of NO, especially in moderate nitrate concentration, up to 0.583 nmol/g FW/h in 60mM nitrate (Figure 6a). Similar to nitrate treatments, NO emission from whole plants under the treatment of nitrite was significantly higher than that from detached leaves or roots ($P < 0.01$) (Figure 6b). For example, at 12mM nitrite treatment, NO emission from whole plants was up to 1.074 nmol/g FW/h, but only 0.105 and 0.374 nmol/g FW/h for detached leaves and roots, respectively.

We searched for the reason why the NO emission from whole plants was always so high under nitrate and nitrite treatments. We speculated that whole plant was an integrative and continuous living system, and thus the result of higher NO emission from whole plants was attributed to the production of NO by NR pathway which consumed lots of NAD(P)H that were ultimately offered by photosynthesis in light (Rockel and Kaiser 2002). However, in detached tissues, the NAD(P)H was exhausted in a short time, resulting inevitably in the absence of NAD(P)H which is important substrate in NO production by NR pathway, although another substrate (nitrate or nitrite) is abundant by excessive addition.

In conclusion, the main objective of this study was to explore the characteristics of NO emission from detached barley leaves, roots and whole plants treated with various nitrate or nitrite concentrations, under light/dark and aerobic/anaerobic conditions. These data indicated that NO emission had some dose-dependent manner with nitrate or nitrite concentration. Anaerobic condition and light remarkably stimulated NO production from detached roots and whole plants, while as for detached barley leaves, NO emission was relatively higher in dark. A new and interesting finding was that NO emission from whole barley plants was much higher than from detached tissues. Based on the results mentioned above, further research is required to find out the reasons that refer to the difference of plant NO emission in detail, to study other factors affecting NO emission, and to further quest for both the molecular mechanism and the regulation of NO production.

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