Planta (1999) 207: 442–448

Planta

Increased nitrate reductase activity in leaf tissue after application of the fungicide Kresoxim-methyl

Johanna Glaab, Werner M. Kaiser

Julius-von-Sachs-Institut für Biowissenschaften, Universität Würzburg, Julius-von-Sachs-Platz 2, D-97082 Würzburg, Germany

Received: 17 June 1998 / Accepted: 2 September 1998

Abstract. Spinach (*Spinacia oleracea* L.) leaf discs floating on buffer solution were treated with Kresoxim-methyl (KROM), an inhibitor of respiratory electron transport. In the leaf tissue, actual and maximal nitrate reductase (NR) activities, nitrite content and ATP levels were determined. In darkened leaf discs incubated without KROM (control) actual NR activity decreased to 20% after 6 h in the dark. Treatment with 10 μg ml^{-1} (corresponding to 32 μM) KROM totally prevented inactivation of NR in the dark and also diminished NR-protein degradation during prolonged darkness. Due to restricted nitrite reduction in darkened leaf tissues, nitrite accumulated in KROM-treated discs. Inhibition of respiration decreased ATP and increased AMP levels in KROM-treated discs. In illuminated leaf discs, NR was highly activated to 65%. Nevertheless, KROM-treatment caused an additional activation of NR (activation state 76%) in the light. Possible side-effects of KROM on nitrite reduction and photosynthesis were also checked in the leaf-disc system. Neither nitrite reduction nor photosynthesis were altered in KROM-treated discs. The extent of KROM-induced activation of NR was dependent on the applied concentration and on the pH of the external medium. The highest activation of NR was achieved at an external pH of 4.8, confirming previous results (Kaiser and Brendle-Behnisch, 1995, Planta 196: 1–6) that cytosolic acidification might play an important role in the modulation of NR activity.

Key words: Nitrate reductase – Respiration inhibitors – *Spinacia* – Strobilurin

Abbreviations: AEC = adenylate energy charge; KROM = Kresoxim-methyl; NR = NADH-nitrate reductase; NRA = nitrate reductase activity

Correspondence to: J. Glaab;
E-mail: glaab@botanik.uni-wuerzburg.de;
Fax: 49 (931) 8886158

Introduction

Strobilurines are produced by several basidiomycetes, e.g. *Strobilurus tenacellus*, and belong to the E-beta-methoxyacrylate group of antibiotics (Becker et al. 1981; Weber et al. 1990). The cytostatic and antibiotic effects of strobilurines are based on their ability to inhibit cell respiration. They block mitochondrial electron transport specifically at the cytochrome *bc*1 complex (Becker et al. 1981). Due to this property, strobilurin derivatives like Kresoxim-methyl (KROM) are used as potent fungicides in agriculture.

Apart from its antifungal effect, KROM also causes long-term changes in the metabolism and growth of the treated plants. In many cases, biomass and yield increased after application of this fungicide. Another observation was the "greening effect" of older leaves of treated plants; they obviously contained more chlorophyll than untreated ones. In addition, the senescence of the plants was delayed (Gold et al. 1995). These findings indicate that a certain amount of KROM was taken up by the plant. A partial inhibition of respiration in the leaves may also explain the observed decrease of the CO_2-compensation point, eventually leading to more efficient photosynthesis (Retzlaff 1995). Strobilurin application also resulted in an altered composition of phytohormones in the treated plants (Grossmann and Retzlaff 1997), an effect which may also contribute to intensified growth.

However, an increase in biomass requires not only more carbon but also nitrogen. Vegetative growth is often N-limited. In consequence, nitrate assimilation has to be reinforced in order to match increased growth. Under nitrate nutrition, NADH-nitrate reductase (NR; EC 1.6.6.1) catalyses the rate-limiting step of the overall nitrate assimilation process (Beevers and Hageman 1969). The NR in higher plants is regulated at the level of transcription (Cheng et al. 1986; for review, see Crawford 1995) and translation (Somers et al. 1983; Deng et al. 1990; Pilgrim et al. 1993) and by posttranslational modulation (for review, see Kaiser and Huber 1994a). The direct NR-protein modification allows rapid

tuning of enzyme activity to changing environmental conditions. In leaves, NR is activated in the light, whereas darkening of the leaves leads to inactivation of the enzyme with a halftime of about 15 min (Kaiser et al. 1992; Riens and Heldt 1992). This regulation is based on a reversible phosphorylation mechanism (Huber et al. 1992; Kaiser et al. 1993; Spill and Kaiser 1994). A first step is the phosphorylation of NR, catalyzed by a Ca^{2+}-dependent protein kinase. Subsequently, an inhibitor protein (member of the 14-3-3-family) binds to phospho-NR and causes inactivation of the enzyme (Bachmann et al. 1995; Glaab and Kaiser 1995; MacKintosh et al. 1995). (Re-)Activation of NR is mediated by a protein phosphatase (Type 2A). After dephosphorylation of NR, the inhibitor protein is released and NR is active again.

The complex between phospho-NR and the 14-3-3-protein is only formed in the presence of divalent cations. Therefore, there are two possible ways of measuring the activity of extracted NR. The first assay contains Mg^{2+} which stabilizes the inactive complex phospho-NR plus inhibitor protein. Under these conditions only dephospho-NR is active. This assay gives the actual NR activity (NRA) which represents the NRA of the tissue at the time of harvest. In a second assay the extract is preincubated with an excess of EDTA (for 15 min) before the real reaction assay is started by adding the NR-substrates KNO_3 and NADH. Formation of NR-inhibitor protein complex is prevented by EDTA; PO_4^{3-} and AMP, which are also contained in the preincubation buffer, stimulate NR protein phosphatase. Thus, total extracted NR is fully activated in vitro and the measured activity is the maximal NRA, which also reflects the total amount of NR protein present in the extract. At present the signals which control the phosphorylation/dephosphorylation mechanism are not fully known, but metabolic and physical factors seem to be involved (Kaiser and Huber 1994a,b; Glaab and Kaiser 1996).

Cytosolic pH may play some role in modulation of NRA: artificial acidification of the cytosol causes an activation of NR (Kaiser and Brendle-Behnisch 1995). A decrease in the cytosolic pH occurs under natural conditions, for example in anaerobic roots (Botrel et al. 1996). During anaerobiosis the ATP-level decreases drastically. As a consequence, proton-ATPases at the tonoplast are assumed to be less active, and the pH-gradient between cytosol and vacuole can not be maintained. A similar situation develops when leaves or roots are treated with uncouplers of respiratory electron transport, such as 2,4-dinitrophenol (DNP) or carbonylcyanide *m*-chlorophenylhydrazone (CCCP), which also lead to a decrease in the ATP level and subsequently to a strong increase in NRA (Kaiser et al. 1992; Glaab and Kaiser 1993; Botrel et al. 1996). In analogy to these observations it was assumed that application of KROM, which partially inhibits electron transport in mitochondria of treated plants, may also activate NR. This might help to increase total nitrate assimilation of the plant.

In the present work the basic short-term effects of the strobilurin derivative KROM on NR were examined in a simple leaf-disc system. Spinach leaf discs were floated directly on buffer solutions containing KROM. In such an artificial system, uptake of KROM into the tissue is rapid and concentrations are well defined.

Materials and methods

Plant material. Spinach (*Spinacia oleracea* L. cv. Polka F1; seeds obtained from a local merchant) was grown in a greenhouse. The mean daylength was 11 h with supplementary illumination (HQi, 400 W; Schreder, Winterbach, Germany) at a total photon flux density of 250–400 μmol $m^{-2}s^{-1}$ photosynthetically active radiation. The plants were fed with a commercial nitrate/ammonium fertilizer. For experiments leaves of 7- to 9-week-old spinach plants were used.

Chemicals. Pure Kresoxim-methyl (BAS 490 F) was obtained from BASF AG (Limburgerhof, Germany) and directly used in the leaf-disc experiments. The stock solution contained 5 mg KROM per ml ethanol.

Leaf disc experiments. Spinach leaf discs (4 mm diameter) were carefully washed twice in buffer solution (50 mM Hepes, pH 7.6; 1 mM $CaCl_2$; 10 mM KNO_3) and subsequently transferred into fresh buffer medium (same as above; on average 40 discs per 10 ml buffer) with or without KROM at different concentrations (see figure legends). During the whole experiment the buffer solution was flushed with air to prevent anaerobiosis.

For determination of the nitrite-reduction capacity of the leaf discs, the experiment was carried out in the light and with a different buffer system: 50 mM Mes (pH 4.7) was used in order to promote the diffusion of nitrite as undissociated nitric acid into the tissue. The photon flux density was 300 μmol $m^{-2}s^{-1}$ photosynthetically active radiation.

Determination of NRA and nitrite content. Leaf discs were ground with liquid nitrogen and 2 ml of extraction buffer was added to 1 g FW. The extraction buffer for spinach leaves contained 50 mM Hepes (pH 7.6), 1 mM DTT, 10 μM FAD and 10 mM $MgCl_2$. After continuous grinding until thawing had occurred the suspension was centrifuged (14 500 *g*, 10 min, 4 °C). With 100 μl aliquots of the supernatant the following assays were carried out:

(i) *Determination of nitrite content.* Extract (100 μl) was added to a mixture of 900 μl buffer (50 mM Hepes, pH 7.6; 1 mM DTT; 10 μM FAD; 10 mM $MgCl_2$) and 125 μl zinc-acetate (0.5 M).

(ii) *Determination of actual NRA.* Extract (100 μl) was added to 900 μl reaction buffer (50 mM Hepes, pH 7.6; 1 mM DTT; 10 μM FAD; 10 mM $MgCl_2$; 5 mM KNO_3; 0.2 mM NADH). The reaction was carried out at 24 °C. After 3 min the reaction was stopped by adding 125 μl zinc-acetate.

(iii) *Determination of maximal NRA.* Extract (100 μl) was preincubated at 24 °C for 15 min with 15 mM EDTA (final concentration 5 mM), 5 mM AMP and 5 mM PO_4^{3-}. After 15 min the reaction was started by adding reaction buffer containing the substrates KNO_3 and NADH to a final volume of 1 ml. In this assay the reaction buffer contained 15 mM EDTA (final concentration 5 mM) in order to totally complex Mg^{2+}. After 3 min at 24 °C the reaction was stopped by adding 125 μl zinc-acetate.

The colorimetrical determination of nitrite either built in vivo before harvesting or formed additionally by NR during the reaction assay was carried out as described by Hageman and Reed (1980).

Determination of adenine nucleotides. Leaf discs (0.1–0.3 g FW) were ground in liquid nitrogen. Three milliliters of perchloric acid (4.5%) was added to the plant material and mixed until thawing. Seventy-five microliters Tris (2 M, pH not adjusted) was added. For complete extraction, samples were kept on ice for 1 h. After

that, the suspension was cleared by centrifugation (4000 *g*; 10 min; 4 °C) and 2.7 ml of the supernatant was adjusted to pH 7.8 with 5 M K_2CO_3. After another centrifugation step the supernatant was kept frozen at −80 °C. Adenine-nucleotide levels were determined with a commercially available luciferine-luciferase assay (Boehringer, Mannheim, Germany) in combination with the phosphoenol-pyruvate/pyruvate kinase (EC 2.7.1.40)/myokinase (EC 2.7.4.3.) system in a lumat LB 9501 (Berthold, Wildbad, Germany).

Determination of the $^{14}CO_2$-fixation rate. First, leaf discs (10 per 10 ml of 50 mM Hepes, pH 7.6; 1 mM $CaCl_2$; 10 mM KNO_3) were incubated with or without KROM in the dark to allow uptake of KROM into the tissue. After 1 h, the discs were transferred to new buffer solution (5 ml) which contained additionally 20 mM $KHCO_3$. To each assay radioactive labelled $H^{14}CO_3^-$ was added. The specific activity was 3.7×10^8 Bq μmol^{-1}. The tubes were kept in the light with 320 μmol $m^{-2}s^{-1}$ photosynthetically active radiation and gently shaken for 1 h. Then the incubation medium was removed and the discs were covered with liquid nitrogen to stop the reaction. To the discs, 2 ml of acetic acid was added in order to remove the non-incorporated $H^{14}CO_3^-$. After 20 h, 0.75 ml of the leaf-disc extract was added to the same volume of acetic acid and dried with aeration. Addition of acetic acid (0.75 ml) was repeated in order to completely remove all traces of $H^{14}CO_3^-$. After drying, 200 μl H_2O was added to resolve the compounds. Scintillation cocktail (4 ml) was added and incorporated ^{14}C was determined in a scintillation counter.

Results and discussion

Effect of KROM on NRA in darkened leaf discs. In all cases, spinach leaves were harvested 3–4 h after the beginning of the light phase, when NR is highly active. Preparation of the leaf discs took 20–30 min. During this period actual NRA in the leaf material decreased somewhat. Nevertheless, the NR-activation state at the beginning of the experiments (initial values) was always high enough to observe a pronounced dark-inactivation of NR. For the indicated time, leaf discs were floating on buffer solution (see figure legends) which was continuously flushed with air. A probable NR-activating effect of KROM should be more obvious under conditions causing inactivation of the enzyme. Therefore the discs were kept in the dark for several hours. Figure 1 shows the rapid decline in actual NRA (measured in the presence of 10 mM Mg^{2+}) in the darkened control discs. After 2 h in the dark, only 35% of the initial activity was detectable. In contrast, the addition of KROM to the external medium diminished the inactivation of NR in the dark (with 1 μg KROM ml^{-1}) or even prevented it completely (with 10 μg KROM ml^{-1} and more; Fig. 1).

In darkened leaves or leaf tissues, nitrite reduction is drastically diminished (Canvin and Atkins 1974; Wyn Jones and Sheard 1978; Reed and Canvin 1982). When NR is artificially activated in darkened leaves, e.g. by anaerobiosis, nitrate reduction and nitrite consumption are unbalanced resulting in an accumulation of nitrite in the tissue. This was also observed in KROM-treated leaf discs. Whereas control discs contained only a very small amount of nitrite up to 23 nmol (g FW)$^{-1}$ (Fig. 2), even the lowest KROM concentration (1 μg ml^{-1}) caused a clear accumulation of nitrite in the tissue, which increased with time during the experiment. With higher

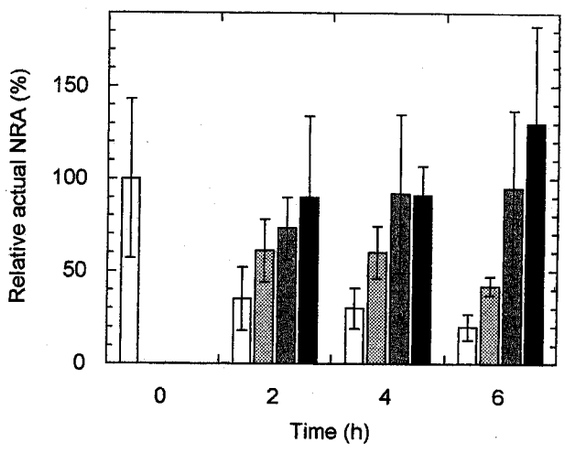

Fig. 1. Actual NRA in darkened spinach leaf discs treated with different KROM concentrations. Spinach leaves were harvested 3–4 h after the onset of light, when the NR-activation state is high. During preparation of the discs (20–30 min) the actual NRA decreased slightly. Nevertheless, at the beginning of the experiment (time 0) the NR-activation state was still about 53%. Discs were floating on buffer solution containing KROM and darkened at time 0. Applied KROM concentrations: □, control; □, 1 μg ml^{-1} KROM; ▨, 10 μg ml^{-1} KROM; ■, 100 μg ml^{-1} KROM. The medium was continuously flushed with air. Leaf discs were harvested at the indicated time points and actual NRA was determined in the extracts in the presence of 10 mM Mg^{2+}. Mean values ± SD from three (data for 2 h and 6 h) or eight (data for time 0 and 4 h) separate experiments. The NRA at time 0 corresponds to 100% and was about 12.7 ± 5.5 μmol NO_2^- (g FW)$^{-1}$h^{-1}

KROM concentrations the effect was more pronounced; on average up to 800 nmol NO_2^- (g FW)$^{-1}$ was accumulated with 100 μg KROM ml^{-1} after 6 h incubation (Fig. 2). The maximum value achieved was 1552 nmol NO_2^- (g FW)$^{-1}$. Leaf discs with high nitrite contents excreted about 70% of the total nitrite found to

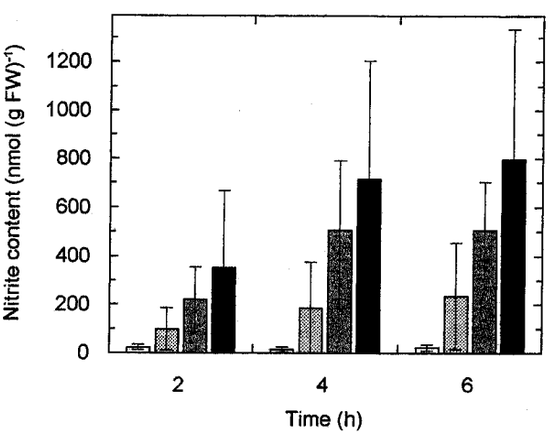

Fig. 2. Nitrite accumulation in darkened spinach leaf discs treated with different KROM concentrations: □, control; □, 1 μg ml^{-1} KROM; ▨, 10 μg ml^{-1} KROM; ■, 100 μg ml^{-1} KROM. Discs were treated as described in Fig. 1. The nitrite content of the extracts was colorimetrically determined. Mean values from three to eight separate experiments ± SD (as in Fig. 1)

the medium (data not shown). This might explain the relatively small increase in tissue nitrite content after 6 h.

In the same samples, maximal NRA, representing approximately the level of NR protein, was also determined (after preincubation with EDTA, AMP and PO_4^{3-} and assayed in excess EDTA). In control leaf discs, maximal NRA decreased slowly to 46% after 6 h (Fig. 3), indicating considerable NR-protein degradation during prolonged darkness. In KROM-treated discs the decline in maximal NRA was reduced; after 6 h still almost 80% of the initial activity was detectable. Obviously KROM had a stabilizing effect on maximal NRA. This could be either a consequence of a KROM-induced reduction of protein degradation in general (Gold et al. 1995) or due to a specific inhibition of NR degradation. From other experiments (data not shown) it seems to be likely that the phosphorylated form of NR is the favoured target for degradation, whereas activation of NR – as in this case with KROM – always prevented a decrease in maximal NR activity.

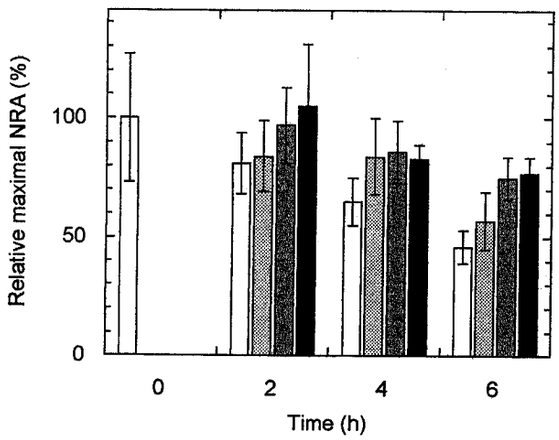

Fig. 3. Maximal NRA in darkened spinach leaf discs treated with different KROM concentrations. Discs were treated as described in Fig. 1. Applied KROM concentrations: □, control; ▨, 1 μg ml^{-1} KROM; ▩, 10 μg ml^{-1} KROM; ■, 100 μg ml^{-1} KROM. For determination of maximal NRA, extracts were preincubated with EDTA (in excess), AMP and PO_4^{3-} for 15 min. Then the reaction was started by adding KNO_3 and NADH and carried out in the presence of EDTA (final concentration 5 mM). Mean values ± SD from three (data for 2 h and 6 h) or eight (data for time 0 and 4 h) separate experiments. The NRA at time 0 corresponds to 100% and was about 24.2 ± 6.4 μmol NO_2^- $(g\ FW)^{-1}h^{-1}$

Effect of KROM on adenine nucleotide levels. Concerning NR activation and nitrite accumulation in darkened leaves, KROM effects were consistent with effects of uncouplers of respiratory electron transport, e.g. DNP (Kaiser et al. 1992) or CCCP (Glaab and Kaiser 1993; Botrel et al. 1996). The primary effect of these substances is a drastic decrease in ATP levels in the tissue. In order to find out to what extent respiratory ATP production was affected by KROM, adenine nucleotide levels were measured in the leaf discs. Discs treated with different KROM concentrations were harvested after 4 h incubation time. In all KROM treatments the ATP content of the discs and, in consequence, the adenylate energy charge (AEC) of the tissue was drastically lowered (Table 1). The intensity of the effect was concentration dependent up to 10 μg ml^{-1}; above that KROM concentration no additional effect could be observed. With 10 μg KROM ml^{-1}, ATP dropped from 81 nmol $(g\ FW)^{-1}$ in controls to 30 nmol $(g\ FW)^{-1}$ in KROM-treated discs, whereas the AMP level increased from 5 to 30 nmol $(g\ FW)^{-1}$. The AEC of the KROM-treated discs was only 0.50 compared with 0.77 in controls. Probably the low ATP content caused a decrease in the activity of the proton-ATPases which have K_M-values around 0.2 mM (Hedrich et al. 1986, 1989). Therefore, due to leakage of protons from the vacuole to the cytoplasm, the cytosol becomes more acidic and in consequence NR is activated. Whether this activation in vivo is due to inhibition of the NR-kinase or to stimulation of the NR-protein phosphatase or both, is presently unclear. In vitro data indicated that NR-kinase activity dropped below pH 7.0 (Bachmann et al. 1995; Kaiser and Brendle-Behnisch 1995), whereas NR-protein phosphatase showed a slight optimum at pH values between 6.5 and 7.0 (Kaiser and Huber 1994b).

Effect of KROM at different external pH values. In order to check the causal connection between KROM-induced activation of NR and acidification of the cytosol, leaf discs were incubated in buffer solutions with different pH values (see legend of Table 2). At pH 8.5 the NR-activating effect of KROM was indeed only small (Table 2). The actual NRA was twofold higher than in controls and the decrease in maximal NRA during

Table 1. Levels of adenine nucleotides and AEC in KROM-treated leaf discs. Discs from illuminated spinach leaves were incubated in the dark for 4 h with or without (control) the indicated KROM concentrations. For more details of the measurement see *Materials and methods*. Data are mean values ± SD from four separate experiments. The AEC ratios are mean values of the AEC calculated for each replicate

Treatment	ATP [nmol $(g\ FW)^{-1}$]	ADP [nmol $(g\ FW)^{-1}$]	AMP [nmol $(g\ FW)^{-1}$]	AEC
Control	81 ± 14	51 ± 4	5 ± 6	0.77 ± 0.04
1 μg ml^{-1} KROM	44 ± 9	53 ± 6	12 ± 6	0.64 ± 0.03
10 μg ml^{-1} KROM	30 ± 10	52 ± 8	30 ± 13	0.50 ± 0.10
100 μg ml^{-1} KROM	30 ± 11	50 ± 6	27 ± 14	0.52 ± 0.05

Table 2. Effect of KROM on NRA and ATP levels in spinach leaf discs incubated at different pH values. Discs were incubated for 4 h in the dark either on Tris, pH 8.5 or Mes, pH 4.8. Propionic acid (5 mM) was additionally added in one treatment. Actual and maximal NRA, NR-activation state and ATP levels were determined. Data are mean values ± SD of three separate experiments

Treatment	Actual NRA [μmol NO_2^- (g FW)$^{-1}h^{-1}$]	Maximal NRA [μmol NO_2^- (g FW)$^{-1}h^{-1}$]	NR activation state (%)	ATP [nmol (g FW)$^{-1}$]
Initial value	8.6 ± 0.5	26.4 ± 1.5	33 ± 1	76 ± 6
Control (pH 8.5)	1.8 ± 0.1	13.3 ± 0.5	14 ± 1	74 ± 6
10 μg ml^{-1} KROM (pH 8.5)	4.1 ± 0.7	17.3 ± 1.2	24 ± 4	41 ± 7
Control (pH 4.8)	3.0 ± 0.9	16.1 ± 1.1	18 ± 5	76 ± 12
10 μg ml^{-1} KROM (pH 4.8)	13.2 ± 2.0	22.0 ± 1.8	60 ± 5	38 ± 10
Control (pH 4.8 + 5 mM propionic acid)	12.8 ± 1.6	23.0 ± 0.6	56 ± 6	56 ± 7
10 μg ml^{-1} KROM (pH 4.8 + 5 mM propionic acid)	19.8 ± 0.4	24.2 ± 0.5	82 ± 2	17 ± 3

darkness was around 35% compared to 50% in the control discs. When incubation of the leaf discs was carried out at an external pH of 4.8, even the control NRA was slightly higher than at pH 8.5, indicating acidification-induced activation of NR. The KROM treatment at the acidic external pH caused a drastic increase of actual NRA to 13.2 μmol NO_2^- (g FW)$^{-1}h^{-1}$. Here, NR was activated to 60%, whereas in the control discs NR had an activation state of less than 20%. The NR-stimulating effect could be increased by adding permeating propionic acid to the incubation medium (Table 2), in order to intensify cellular acidification. Under these conditions actual NR, even in controls, increased up to 12.8 μmol NO_2^- (g FW)$^{-1}$ h^{-1} and the activation state reached 56%. But despite this fact KROM-treated discs showed an additional activation of NR up to 19.8 μmol NO_2^- (g FW)$^{-1}$ h^{-1} and the NR-activation state was around 80%. Thus, the KROM-induced activation of NR was clearly pronounced at the acidic external pH. One might argue that this was due to lower KROM uptake at alkaline pH. The KROM uptake was not measured, but ATP levels were similarly affected at both pH values (Table 2). Thus, pH effects on KROM uptake appear improbable.

Effect of KROM on nitrite reduction and photosynthesis in illuminated leaf discs. So far the NR-activating effect of KROM was clearly confirmed in the leaf-disc system. In order to assess the specifity of KROM as an inhibitor of mitochondrial electron transport and to exclude side-effects on photosynthesis or on nitrite reduction both aspects were investigated. In order to measure nitrite reduction, leaf discs were again kept in aerated buffer solution, but the experiments were carried out in the light to promote nitrite reduction. One millimolar KNO_2 was added to the buffer medium and the time-dependent decrease in external nitrite was measured. The rate of nitrite disappearing from the medium was taken as an estimation of net-nitrite consumption in the leaf discs. It should be noted that NR was activated in the light and therefore actual nitrite reduction in the tissue was probably higher than the rather low rates observed. In control discs a nitrite consumption rate of 1.8 ± 0.6 μmol NO_2^- (g FW)$^{-1}h^{-1}$ was determined (±SD; $n = 8$). The KROM-treated (10 μg ml^{-1}) discs showed no significant difference, the consumption rate was here 1.7 ± 0.4 μmol NO_2^- (g FW)$^{-1}h^{-1}$ (±SD; $n = 9$). Obviously, nitrite reduction was not inhibited by KROM.

In order to check for a possible effect of KROM on photosynthesis, the CO_2-fixation capacity of the leaf discs was monitored. First, the leaf discs were incubated with or without 10 μg ml^{-1} KROM in the dark for 1 h to allow uptake of KROM. Then, discs were transferred to fresh medium which contained additionally 20 mM $KHCO_3$. Radioactively labelled $H^{14}CO_3^-$ was added to the external buffer medium and after 1 h in the light (320 μmol m^{-2}s^{-1}) the amount of incorporated ^{14}C was determined. The CO_2-fixation rate of controls was on average 45.1 μmol CO_2 (g FW)$^{-1}h^{-1}$ [$n = 2$; 48.8 and

Table 3. Effect of KROM on NRA and ATP levels in spinach leaf discs incubated in the dark and then in the light. Discs were incubated for 1 h in the dark in order to allow uptake of KROM. Then one part of the discs remained in the dark whereas another part was transferred to fresh medium containing additionally 20 mM $KHCO_3$ and incubated in the light for 1 h (300 μmol m^{-2} s^{-1}). At the indicated time points, discs were harvested and NRA and ATP levels were determined. Data are mean values ± SD of three separate experiments

Treatment	Actual NRA [μmol NO_2^-(g FW)$^{-1}h^{-1}$]	Maximal NRA [μmol NO_2^-(g FW)$^{-1}h^{-1}$]	NR activation state (%)	ATP [nmol (g FW)$^{-1}$]
Initial value	8.7 ± 1.9	24.3 ± 1.7	35 ± 7	62 ± 2
Control				
1 h darkness	3.9 ± 1.8	20.3 ± 4.3	18 ± 6	76 ± 4
1 h darkness, then 1 h light	15.6 ± 0.8	24.0 ± 1.0	65 ± 2	65 ± 9
10 μg ml^{-1} KROM				
1 h darkness	6.5 ± 1.2	23.6 ± 1.6	27 ± 4	38 ± 3
1 h darkness, then 1 h light	19.9 ± 2.7	26.3 ± 2.7	76 ± 3	26 ± 2

41.4 μmol CO_2 (g FW)$^{-1}$h^{-1}], which is below the rate observed by gas-exchange measurements in intact leaves. In KROM-treated discs (10 μg ml^{-1}) the CO_2-assimilation rate was unaltered at 45.7 μmol CO_2 (g FW)$^{-1}$h^{-1} [$n = 2$; 41.5 and 49.9 μmol CO_2 (g FW)$^{-1}$h^{-1}]. Obviously, KROM had no effect on net photosynthesis under the applied condition. This conclusion was also confirmed by P_{700}-fluorescence measurements with isolated chloroplasts treated with the same KROM concentration (data not shown).

Effect of KROM on NRA in illuminated leaf discs. Under natural day/night cycles the actual NRA in leaves decreases not only after the beginning of the dark period. NRA shows a diurnal course with rising activity in the morning hours, highest activity in the middle of the day and – depending on species and on nitrate supply – a more or less marked decline during the afternoon. Thus, it would have important consequences for the plant, if KROM also prevented – at least in part – the decrease in actual NRA during daytime.

Therefore the effect of KROM on NR was checked with leaf discs that were first kept in the dark for 1 h to allow the uptake of KROM and then transferred into the light. To support photosynthesis, $KHCO_3$ (20 mM) was added to the external medium. After 1 h darkness, control discs showed a decrease in actual NRA to 45% of the initial activity and also the maximal activity was lower than at the beginning of the experiment (Table 3). After 1 h in the light, the actual NRA rose again and the NR activation state increased from 18 % up to 65%. The ATP content in control discs was stable around 70 nmol (g FW)$^{-1}$ throughout the experiment, whereas KROM-treated discs (10 μg ml^{-1}) showed a drastic decline in ATP levels. After 1 h in the dark only 38 nmol (g FW)$^{-1}$ of ATP was detectable and during 1 h in the light the ATP content further decreased to 26 nmol (g FW)$^{-1}$. In the context of unaltered photosynthesis in KROM-treated leaf discs this result suggests that cytosolic ATP may drop without significant effects on net photosynthesis. As in the previous described experiments, KROM diminished the dark-inactivation of actual NRA and kept maximal NRA almost stable, whereas controls showed a slight decrease in maximal NRA (Table 3). It was remarkable that KROM also had an effect on NR in the light. After 1 h light, the actual NRA in KROM-treated discs increased to 19.9 μmol NO_2^- (g FW)$^{-1}$h^{-1} compared to 15.6 μmol NO_2^- (g FW)$^{-1}$h^{-1} in controls. In consequence, the NR-activation state in KROM-treated discs increased to 76% compared with 65% in controls. This indicated that KROM was in fact able to activate NR above its normal activation state not only in the dark, but also in the light.

In conclusion, the basic hypothesis was confirmed in the leaf-disc system: even under light conditions, KROM caused an additional activation of NR which was probably mediated via acidification of the cytoplasm. The fungicide also prevented in part the degradation of the NR protein. With respect to the agricultural application of KROM on intact plants it is important that nitrite reduction was not inhibited by KROM and that photosynthesis was undisturbed.

Altogether the data suggest that KROM may indeed enhance nitrate reduction, at least in short-term experiments. Further investigations are needed in order to determine the long-term effects of KROM on NR under more realistic field conditions.

This work was supported by BASF AG, Limburgerhof, Germany and SFB 251.

References

Bachmann M, McMichael RW Jr, Huber JL, Kaiser WM, Huber SC (1995) Partial purification and characterization of a calcium-dependent protein kinase and an inhibitor protein required for inactivation of spinach leaf nitrate reductase. Plant Physiol 108: 1083–1091

Becker WF, von Jagow G, Anke T, Steglich W (1981) Oudemansin, strobilurin A, strobilurin B and myxothiazol: new inhibitors of the bc1 segment of the respiratory chain with an E-beta-methoxyacrylate system as common structural element. FEBS Lett 132: 329–333

Beevers L, Hageman RH (1969) Nitrate reduction in higher plants. Annu Rev Plant Physiol 20: 495–522

Botrel A, Magné C, Kaiser WM (1996) Nitrate reduction, nitrite reduction and ammonium assimilation in barley roots in response to anoxia. Plant Physiol Biochem 34: 645–652

Canvin DT, Atkins CA (1974) Nitrate, nitrite and ammonia assimilation by leaves: Effect of light, carbon dioxide and oxygen. Planta 116: 207–224

Cheng C-L, Dewdney J, Kleinhofs A, Goodman HM (1986) Cloning and nitrate induction of nitrate reductase mRNA. Proc Natl Acad Sci USA 83: 6825–6828

Crawford NM (1995) Nitrate: nutrient and signal for plant growth. Plant Cell 7: 859–868

Deng M-D, Moureaux T, Leydecker M-T, Caboche M (1990) Nitrate-reductase expression is under the control of a circadian rhythm and is light inducible in *Nicotiana tabacum* leaves. Planta 180: 257–261

Glaab J, Kaiser WM (1993) Rapid modulation of nitrate reductase in pea roots. Planta 191: 173–179

Glaab J, Kaiser WM (1995) Inactivation of nitrate reductase involves NR-protein phosphorylation and subsequent "binding" of an inhibitor protein. Planta 195: 514–518

Glaab J, Kaiser WM (1996) The protein kinase, protein phosphatase and inhibitor protein of nitate reductase are ubiquitous in higher plants and independent of nitrate reductase turnover. Planta 199: 57–63

Gold RE, Köhle H, Gall A, Steiniger M, Wiede K, Leinhos G, Schelberger K (1995) Kresoxim-methyl. Der neue Wirkstoff nach dem Vorbild der Natur. BASF AG, Limburgerhof, Germany

Grossmann K, Retzlaff G (1997) Bioregulatory effects of the fungicidal strobilurin Kresoxim-methyl in wheat (*Triticum aestivum*). Pestic Sci 50: 11–20

Hageman RH, Reed AJ (1980) Nitrate reductase from higher plants. Methods Enzymol 69: 270–280

Hedrich R, Flügge U-I, Fernandez JM (1986) Patch-clamp studies of ion transport in isolated plant vacuoles. FEBS Lett 204: 228–232

Hedrich R, Kurkdjian A, Guern J, Flügge U-I (1989) Comparative studies on the electrical properties of the H^+ translocating ATPase and pyrophosphatase of the vacuolar-lysosomal compartment. EMBO J 8: 2835–2841

Huber JL, Huber SC, Campbell WH, Redinbaugh M (1992) Reversible light/dark modulation of spinach leaf nitrate reductase activity involves protein phosphorylation. Arch Biochem Biophys 296: 58–65

Kaiser WM, Brendle-Behnisch E (1995) Acid-base-modulation of nitrate reductase in leaf tissues. Planta 196: 1–6

Kaiser WM, Huber SC (1994a) Posttranslational regulation of nitrate reductase in higher plants. Plant Physiol 106: 817–821

Kaiser WM, Huber SC (1994b) Modulation of nitrate reductase in vivo and in vitro: effects of phosphoprotein phosphatase inhibitors, free Mg^{2+} and 5′AMP. Planta 193: 358–364

Kaiser WM, Spill D, Brendle-Behnisch E (1992) Adenine nucleotides are apparently involved in the light-dark modulation of spinach-leaf nitrate reductase. Planta 186: 236–249

Kaiser WM, Spill D, Glaab J (1993) Rapid modulation of nitrate reductase in leaves and roots: indirect evidence for the involvement of protein phosphorylation/dephosphorylation. Physiol Plant 89: 557–562

MacKintosh C, Douglas P, Lillo C (1995) Identification of a protein that inhibits the phosphorylated form of nitrate reductase from spinach (*Spinacia oleracea*) leaves. Plant Physiol 107: 451–457

Pilgrim ML, Caspar T, Quail PH, McClung CR (1993) Circadian and light-regulated expression of nitrate reductase in *Arabidopsis*. Plant Mol Biol 23: 349–364

Reed AJ, Canvin DT (1982) Light and dark controls of nitrate reduction in wheat (*Triticum aestivum* L.) protoplasts. Plant Physiol 69: 508–513

Retzlaff G (1995) Untersuchungen zur Strobilurinwirkung in Gramineen. Phytomedizin 25: 45

Riens B, Heldt HW (1992) Decrease of nitrate reductase activity in spinach leaves during a light-dark transition. Plant Physiol 98: 573–577

Somers DA, Kuo T-M, Kleinhofs A, Warner BL, Oaks A (1983) Synthesis and degradation of barley nitrate reductase. Plant Physiol 72: 949–952

Spill D, Kaiser WM (1994) Partial purification of two proteins (100 kDa and 67 kDa) cooperating in the ATP-dependent inactivation mechanism of spinach leaf nitrate reductase. Planta 192: 183–188

Weber W, Anke T, Steffan B, Steglich W (1990) Antibiotics from basidiomycetes. XXXII. Strobilurin E: a new cytostatic and antifungal (E)-beta-methoxyacrylate antibiotic from Crepidotus fulvomentosus Peck. J Antibiot 43: 207–212

Wyn Jones R, Sheard RW (1978) Accumulation and stability of nitrite in intact aerial leaves. Plant Sci Lett 11: 285–291