Turkish Journal of Biology

Volume 35 | Number 4 Article 7

1-1-2011

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Turk J Biol
35 (2011) 443-448

doi:10.3906/biy-1004-106

Effects of cold and salicylic acid treatments on nitrate reductase activity in spinach leaves

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Received: 14.04.2010

Abstract: The effects of long-term cold (5/3 °C) and salicylic acid (SA) (0.1 mM) treatments on the diurnal pattern of nitrate reductase (NR) activity in spinach (*Spinacia oleracea* L. cv. Gladiator) leaves were investigated. When the activities with cold treatment are compared to the control (20/15 °C), NR activity was decreased by 5 °C in the light period and by 3 °C in the dark period. The cold treatment also affected the timing of circadian rhythm in the diurnal pattern of NR activity. When the activities with SA treatments are compared to control and cold, it was determined that SA increases NR activity in the light period of control while SA decreases NR activity in the light period of cold.

Key words: Cold, nitrate reductase activity, salicylic acid, *Spinacia oleracea*

Soğuk ve salisilik asit uygulamalarının ıspanak yapraklarındaki nitrat redüktaz aktivitesine etkileri

Özet: Ispanak (*Spinacia oleracea* L. cv. Gladiator) yapraklarındaki nitrat redüktaz (NR) aktivitesinin gün içindeki değişimi üzerine uzun süreli soğuk (5/3 °C) ve salisilik asit (SA) (0.1 mM) uygulamalarının etkileri incelenmiştir. Soğuk uygulamalı aktiviteler kontrol (20/15 °C) ile karşılaştırıldığında, NR aktivitesi 5 °C ile ışık periyodunda ve 3 °C ile karanlık periyodunda azalmıştır. Soğuk uygulaması NR aktivitesinin gün içindeki sirkadian ritiminin zamanı üzerinde de etkili olmuştur. SA uygulamalı aktiviteler kontrol ve soğuk ile karşılaştırıldığında, SA'nın soğuğun ışık periyodunda NR aktivitesini azaltırken kontrolün ışık periyodunda NR aktivitesini arttırdığı belirlenmiştir.

Anahtar sözcükler: Soğuk, nitrat redüktaz aktivitesi, salisilik asit, *Spinacia oleracea*

Introduction

Nitrate reductase (NR) catalyses the reduction of nitrate to nitrite and plays a key role in the regulation of nitrate assimilation in higher plants (1). The NR exists in 3 forms: NADH-specific, NAD(P)H-bispecific, and NADPH-specific. Briefly, spinach leaf NR comprises two 100 kDa polypeptides; both contain 3 active redox cites: Mo, Heme, and FAD (2). More recently it has been shown that NR in fact also displays a nitrite reductase activity, catalysing NAD(P)H-dependent reduction of nitrite to nitrite oxide (NO). NR is one of the enzymes that are capable of producing NO in plants. This activity is different from the plastidic nitrite reducing activity catalysed by nitrite reductase (NiR), which reduces nitrite to ammonium. It is now clear that NR can produce NO

through its NR-NiR activity. NO is a diffusible, very reactive gas that is involved in the regulation of many processes in plants (3-5).

In plants, it is known that nitrate is the primary factor regulating NR induction (6). NR activity is also regulated by a number of other factors, including growth hormones, reduced nitrogen metabolites, drought, and light (7). In light/dark studies, a drop in the activity of NR is usually observed in plants when transferred from light to darkness. The activity rises when plants are transferred back to light (8). In these studies, the response could take between minutes and hours, but no decisive evidence exists so far clarifying the mechanism(s). However, it was observed that NR inactivation is not simply achieved by phosphorylation of the enzyme and an additional protein is required to inhibit the activity of the phosphorylated form of NR in dark-treated spinach leaves (9). In addition, it was shown that NR activity is also regulated by cold (10-19). In these studies at low temperature in the short term, it was observed that NR activity shows different responses in different plants. However, evidence for the effect of low temperature on the NR activity in plants is ambiguous.

Recently several studies supported a major role of salicylic acid (SA), a natural and hormone-like signal molecule, in modulating the plant response to several abiotic such as ultraviolet light, drought, salt, heat, and chilling and biotic stresses (20-22). During chilling stress, it was observed that SA pretreatment could directly or indirectly change freezing tolerance and cellular antioxidant enzyme activities (23-29). There are also some studies that investigated the effect of short-term exogenous SA treatments on NR activity. Stimulation at low concentrations and inhibition at high concentrations of SA on NR activity have been demonstrated (30-33). In another study, it was shown that SA treatment protects NR activity under water stress (34). Results signified the role of SA in regulating the drought response of plants and suggested that SA could be used as a potential growth regulator, for improving plant growth under water stress. However, the precise mechanism of SA-regulated stress-responses is unclear. Under cold stress, no research about effect of SA treatment on NR activity has been found. Therefore, in this study, investigation of effects of cold and exogenous SA treatments in the long term on NR activity was aimed in both light and dark periods in spinach (*Spinacia oleracea* L. cv. Gladiator) leaves.

Materials and methods

Plant material

Spinach (*Spinacia oleracea* L. cv. Gladiator) seeds were grown in sand culture and supplied with standard Hoagland's solution. They were maintained in a growth chamber under control conditions (12 h light having intensity of 300 mmol m^{-2} s^{-1} at 20 °C + 12 h dark at 15 °C) for 45 days. After 45 days, salicylic acid solution (0.1 mM) (pH 5) was sprayed on the leaves of some of the control plants (29). Some of the control plants sprayed with and without 0.1 mM SA were transferred to cold condition (5/3 °C). The spinach leaves with and without SA were grown for an additional 1 day at both control and cold conditions. Spinach leaves growing in these different conditions for 46 days were harvested at 2 h intervals, frozen in liquid nitrogen and stored at –80 °C for nitrate reductase activity assays.

Determination of nitrate reductase activity

The frozen spinach leaves were ground with liquid nitrogen in a chilled mortar. Then extraction buffer (100 mM Tris-HCl pH 7.5, 10 mM cysteine, 1 mM EDTA, and 5 mM FAD) was added to the spinach powder (4 mL/g fresh leaf weight). The homogenate was centrifuged at 15,000 × *g* for 25 min and the resulting supernatant was used for determination of nitrate reductase activity. The whole extraction procedure was carried out at 4 °C. Nitrate reductase activity was estimated by measuring nitrite formed in an assay system containing 100 mM phosphate buffer (pH 7.5), 1 mM EDTA, 10 mM KNO_3, 0.3 mM NADH, and the enzyme preparation (0.2 mL), in a final volume of 1 mL of 1 M barium acetate and 1.9 mL of 96% (v/v) ethanol. After vigorous shaking in a mixer, the mixture was left to stand for 5 min at 4 °C and centrifuged at 2300 × *g* for 5 min. Then nitrite was measured on an aliquot from the clear supernatant solution by addition of 1 mL of sulphanilamide (1%, w/v, in 2 M HCl) followed by 1 mL of 0.02 (w/v) N-(1-naphthyl)ethylene-diamine. After 15 min, the absorbance of the sample was read at 540 nm (35). The determined values are the means

of 4 independent experiments. NR activity (EU/g leaf) was calculated as μg NO^-_2/ g fresh weight of the leaf. Average values are the means of the determined 7 values for both the light period (0-12 h) and dark period (12-24 h). The data were analysed by analysis of variance, and means were compared by Duncan's multiple range test.

Results and discussion

Effect of cold on nitrate reductase activity

In previous studies, it was reported that NR activity was increased by cold in black alder leaves and roots, *Pinus sylvestris* needles, and tomato and winter wheat leaves (10,11,13,14,17), and was decreased by cold in arctic plant leaves and roots, cryophilic algae species, marine phytoplankton, and pea leaves (12,15,16,18,19). In these short-term studies at low temperature, different effects were observed on NR activity. Since the NR activity also shows a circadian rhythm in normal diurnal pattern (8,36), in the present study the effect of cold in the long term on diurnal pattern of NR activity in spinach leaves was investigated by using samples harvested at 2 h intervals in the leaves growing in both light (0-12 h) and dark (12-24 h) periods. The determined activity values and calculated average values at cold (5/3 °C) and control (20/15 °C) conditions were shown in Table 1 A-B and Figure. In the light period, when NR activity values in cold (5 °C) are compared to the control (20 °C), it is seen that the activity was lower between 0 and 10 h and was decreased maximum 55% (10 h) by cold. In the dark period, when NR activity values in cold (3 °C) are compared to the control (15 °C), it is seen that the activity was lower between 16 and 24 h and was decreased maximum 72% (22 h) by cold. In order to see effects of the treatments during the period, average values of NR activities were also calculated in both light and dark periods. When averages of NR activity values in cold are compared to the control, it is seen that the average was decreased 25% in the light period and 42% in the dark period by cold. In the diurnal pattern of NR activity, it is also seen that cold (8 h) caused earlier decreasing than the control (12 h). As a result of this, cold (12 h) caused earlier increasing than the control (16 h).

There are few studies done to show evidence related to NR activity under cold stress. In a study, it was demonstrated that short-term low temperature causes a rapid activation of NR in winter wheat leaves resulting from NR protein dephosphorylation by protein phosphatase (14). In the other 2 studies,

Table 1. Nitrate reductase activities and average values of the activities in spinach leaves in control (20/15 °C), cold (5/3 °C), control + 0.1 mM SA and cold + 0.1 mM SA treatments. A) Light period, B) Dark period. Values in a column followed by the same letter are not statistically different at $P < 0.05$ level as determined by Duncan's multiple range test.

A) Light Period								
Treatment / Time (h)	0	2	4	6	8	10	12	Average
Control	91 a	94 a	82 b	78 b	73 b	71 a	35 b	74.9 b
Cold	72 b	72 b	65 c	66 c	36 c	32 b	48 a	55.9 c
Control + SA	97 a	99 a	111 a	113 a	100 a	72 a	36 b	89.7 a
Cold + SA	67 b	68 b	34 d	46 d	14 d	18 c	37 b	40.6 d
B) Dark Period								
Treatment / Time (h)	12	14	16	18	20	22	24	Average
Control	35 b	33 b	59 a	60 b	56 a	61 a	67 a	53.0 a
Cold	48 a	50 a	23 b	30 c	33 b	17 d	19 d	31.3 b
Control + SA	36 b	32 b	65 a	70 a	54 a	53 b	60 b	52.9 a
Cold + SA	37 b	34 b	21 b	24 d	35 b	28 c	30 c	29.9 b

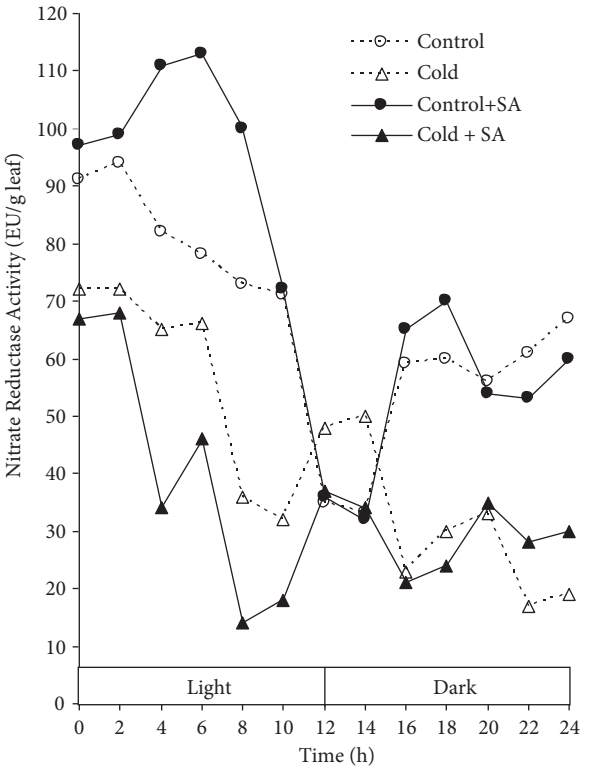

Figure. Nitrate reductase activities in spinach leaves in control (20/15 °C), cold (5/3 °C), control + 0.1 mM SA, and cold + 0.1 mM SA treatments.

temperature responses of NR were studied in the marine phytoplankton, psychrophilic alga, and mesophilic alga in which cold decreases NR activity and NR protein values (15,19). The results strongly suggested that structural modification(s) for cold adaptation affect thermodynamic properties of each of the functional domains within NR holoenzyme (19). It was also found that the FAD domain and electron transport among redox centres are sensitive to elevated temperatures (15). In addition to previous studies, for the first time, in the present study the obtained results showed that the long-term cold treatment decreases NR activity in the dark period more than in the light period and also affects the timing of circadian rhythm in the diurnal pattern of the activity.

Effect of salicylic acid on nitrate reductase activity

SA is recognised as the endogenous regulator of plant metabolism. However, exogenous application to plants generates diverse physiological effects (20-22,26,29). There are also some studies that investigated the effect of SA in the short term on NR activity in maize leaves and roots, black gram, and mustard (30-33). In these plants, stimulation at low concentrations of 0.01, 0.1, and 0.5 mM SA and inhibition at high concentrations of 1, 5, and 10 mM SA on NR activity have been demonstrated. It is seen that these concentrations of SA might play an active role in regulation of NR activity. Since NR activity shows a circadian rhythm in normal diurnal pattern (8,36), in the present study the effects of 0.1 mM SA in the long term on diurnal pattern of NR activity were investigated at 2 h intervals in spinach leaves growing in both light (0-12 h) and dark (12-24 h) periods at control temperature. The determined activity values and calculated average values in control (20/15 °C) and control + SA conditions are shown in Table 1 A-B and the Figure. In the light period, when NR activity values in control + SA are compared to the control, it is seen that the activity was higher between 0 and 10 h and was increased maximum 45% (6 h) by SA. When averages of NR activity values in control + SA are compared to the control, it is seen that the average was increased 20% in the light period by SA.

There is only one study that interprets effect of SA on NR activity in mustard (33). According to the interpretation, the increased activity of NR by the dilute concentration (10^{-5} M) of SA could have been an expression of the interaction of the acid with NR specific inhibitors and/or through the mediation of the other hormone(s). For the first time, in the present study the obtained results showed that the SA treatment in the long term increases the activity in the light period and does not change the activity in the dark period in the diurnal pattern of the NR activity.

Effect of salicylic acid on nitrate reductase activity under cold stress

Until now, only a recent study was reported that SA treatment protects NR activity under water stress (34). No research about the effects of SA treatment on NR activity under cold stress has been found. In the present study, the effect of 0.1 mM SA in the long term on diurnal pattern of NR activity under cold (5/3 °C) in the long term was investigated at 2

h intervals in spinach leaves growing in both light (0-12 h) and dark (12-24 h) periods. The determined activity values and calculated average values in cold and cold + SA conditions were shown in Table 1 A-B and Figure. At the light period, when NR activity values in cold + SA are compared to cold, it is seen that the activity was lower between 0 and 12 h and was decreased maximum 61% (8 h) by SA. When averages of NR activity values at cold + SA are compared to cold, it is seen that the average was decreased 27% at the light period by SA.

In a previous study, it was supposed that the decreased activity of NR at freezing stress could be attributed to the lower stability of the enzyme, resulting in a disturbed electron transfer throughout the NR channel at the interdomain site between heme and MoCo domains (13). In that study, molybdenum supplied to winter wheat prevented the frost-induced decline of NR activity. It was assumed that Mo is important for stabilizing the assembly of the NR domains into an active enzyme under freezing conditions (13). In a recent study, it was found that short-term low temperature causes a rapid activation of NR in winter wheat leaves resulting from NR protein dephosphorylation (14). The study demonstrated that the cold-induced NR activation is dependent neither on cold-triggered calcium influx nor on high endogenous abscisic acid levels. It was also shown that reduced rate of nitrate uptake at low temperature does not limit the cold-induced activation of leaf NR (14). For the first time the results obtained in the present study showed that the SA treatment in the long term decreases the activity in the light period and does not change the activity in the dark period in the diurnal pattern of the NR activity under cold stress.

In the present study, the results obtained by using cold and exogenous SA treatments in the long term can be important for regulation in the diurnal pattern of NR activity since there is no previous report. In the present study, NR activity was observed to be differently affected by the cold, SA, and cold + SA treatments. It was also shown that the timing of circadian rhythm in the diurnal pattern of NR activity is affected by the cold treatment. In addition to these effects, it was seen that light, dark, and time are also important factors on NR activity in both cold and SA treatments. In view of complex regulation of NR, it was not surprising that different effects were established in the present study. In the light of the results, it is suggested that endogenous SA produced in the leaves may be involved in regulation of NR activity at both control and low temperatures. However, the molecular events involved in SA signalling on NR activity are not yet fully understood. In addition, it is now becoming clear that SA interacts both negatively and positively with other major signalling pathways including those regulated by jasmonic acid and ethylene (37). Recently, it has been found that NR can produce NO through its NR-NiR activity and several lines of evidence point to an inter-relationship between NO and SA in plant defence (3-5). The role of endogenous SA with NO and other major signalling pathways is still unknown and needs to be studied further in order to understand dephosphorylation, structural modification, and electron transport mechanism(s) in the regulation of NR activity at different temperatures.

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