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BRIEF COMMUNICATION

Okadaic acid did not change the nitrate reductase activation state in tomato seedlings

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Abstract

In this study, the total and actual nitrate reductase (NR) activity, and NR activation state, in tomato seedlings (*Solanum lycopersicum* cvs. Kmicic and Faworyt) treated with okadaic acid (OA) was evaluated. Seedlings were grown in a half-strength Murashige and Skoog (MS) medium in a growth chamber at day/night temperatures of 22/20 °C, a photon flux density of 150 µmol m^{-2} s^{-1}, and a 16-h photoperiod. After 10 days, plants were transferred into MS medium with 0 (control), 0.01, 0.05, 0.1, 0.5, 1.0 µM OA. It was found that the total and actual NR activity increased in Kmicic leaves treated with 0.1, 0.5, and 1.0 µM OA compared to control. However, the NR activation state did not change in both roots and leaves of OA-treated tomato seedlings.

Keywords: nitrate, okadaic acid, protein phosphorylation, protein phosphatase inhibitor, *Solanum lycopersicum*.

In plants, nitrogen is the most crucial mineral nutrient involved in synthesising proteins, amino acids, chlorophyll, and various N-containing metabolites (Kusano *et al.* 2011). As plants require a high quantity of N, its deficiency is a limiting factor for plant growth, development, and biomass accumulation (Miller *et al.* 2007, Schachtman and Shin 2007). Plants can use various chemical N forms, ranging from simple inorganic N compounds such as NH_4^+ and NO_3^- to polymeric N forms such as proteins (Paungfoo-Lonhienne *et al.* 2008). Nitrate ions are the primary nitrogen source for plants, as organic nitrogen uptake is limited. The reduction of NO_3^- is catalysed by cytosolic enzyme nitrate reductase (NR), which conducted the two-electron reduction of NO_3^- to nitrite (Wang *et al.* 2014). The NR activity is regulated by NO_3^- in the ambient substrate. The reduction of NO_3^- can take place in both the shoots and roots but is spatially separated between the cytoplasm (for NO_3^- reduction) and plastids/chloroplasts (for NO_2^- reduction) (Tischner 2000, Orsel *et al.* 2002). Amino acid N is in many cases used as a synonym for organic N. Many plant species form symbioses with fungi. The capacity of mycorrhizal fungi to degrade polymeric N compounds is well established, as is the function of amino acid absorption (Smith and Read 2008, Näsholm *et al.* 2009). To prevent the accumulation of toxic to plant cells NO_2^-, NR activity is regulated by several mechanisms, including enzyme synthesis, reversible inactivation, degradation, effector regulation, and substrate concentration (Lillo 2008, Marschner 2011).

The irradiance of plants is one of the environmental

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Abbreviations: BSA - bovine serum albumin; DTT - dithiothreitol; EDTA - ethylenediaminetetraacetic acid; HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MS - Murashige and Skoog; NADH - nicotinamide adenine dinucleotide; NADPH - nicotinamide adenine dinucleotide phosphate; NEDA - N-(1-naphthyl)ethylenediamine dihydrochloride; NR - nitrate reductase; OA - okadaic acid; PMSF - phenylmethyl sulphonyl fluoride; PP2A - protein phosphatase 2A; PSP - protein serine/threonine phosphatase; PVP - polyvinylpolypyrrolidone.
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factors regulating nitrate reductase; it can increase the content of NR-mRNA, NR protein, and the activity of the already formed enzyme (Lillo 2004). The effect of radiation on NR can be considered as a direct response to a signal (perceived by phytochrome photoreceptors) or an indirect one as by-products of photosynthesis as sugars, NAD(P)H, or reduced ferredoxin (Yanagisawa 2014).

The NR gene is one of many genes whose expression is up-regulated by sugars (Maas *et al.* 1990). The availability of sugars regulates the formation and activity of NR in the leaves, the organs that conduct photosynthesis (Kaiser *et al.* 1999, Iglesias-Bartolomé *et al.* 2004). However, it has also been shown that NR formation in roots is dependent on sugar availability (Botrel and Kaiser 1997). Also, NR activity in roots can be regulated by the availability of sugars (Reda 2015). Interestingly, the author showed that the effect of sucrose depends on a different signalling pathway in *Arabidopsis* roots than the effect of hexoses (Reda 2015). The effect of radiation and sugars on NR is interestingly and thoroughly presented in Yanagisawa (2014).

Nitrate reductase is rapidly inactivated/activated by phosphorylation/dephosphorylation and further reaction with 14-3-3 protein in response to environmental stimuli and various treatments (Kaiser and Huber 2001, Lillo *et al.* 2004). Protein phosphorylation and dephosphorylation are, among others, the key events by which plants regulate the cell cycle and are catalysed by phosphotransferases - protein kinases and phosphatases (Ardito *et al.* 2017). *In vivo*, kinases always catalyse phosphorylation, whereas dephosphorylation is driven by phosphatases (Bononi *et al.* 2011). Their expression and activity are tightly controlled by extracellular and intracellular signals (Polit and Kaźmierczak 2007). Post-translational regulation of NR is essential for avoiding the accumulation of NO_2^- under certain growth conditions (Lillo *et al.* 2003). NR is inactivated through a two-step process that involves phosphorylation of Ser-543 followed by a magnesium-dependent binding of an inhibitory 14-3-3 protein (Bachmann *et al.* 1996, Moorhead *et al.* 1999). NR in roots is rapidly modulated by a reversible protein-phosphorylation mechanism like that in leaves (Kaiser and Huber 1994, Glaab and Kaiser 1995). NR can also be reactivated by protein serine/threonine phosphatase (PSP) family heteromeric protein phosphatase 2A (PP2A), directly affecting and dephosphorylating the enzyme (MacKintosh 1992, Lillo 2008, Heidari *et al.* 2011). Divalent cations are required to bring the phosphorylated NR and 14-3-3 complex into an inactive form. After complete chelation of divalent cations, even after NR phosphorylation, NR remains active, irrespective of its phosphorylation state. Phosphorylated NR is dephosphorylated by PP2A (MacKintosh 1992, Heidari *et al.* 2011). The marine sponge toxin okadaic acid (OA), a specific PSP inhibitor, has greatly facilitated the study of enzymes *in vitro* because each enzyme from this family shows different sensitivity to this drug. For instance, the IC50 for OA is around 0.1 - 1.0 nmol concerning PP2A activity (Schonthal 1998).

The influence of OA (50 and 500 nmol) on the induced accumulation of NR and NiR (nitrite reductase) mRNAs in excised barley leaves was demonstrated (Sueyoshi *et al.* 1999), which confirms the participation of OA-sensitive protein phosphatases in the NR gene expression. Furthermore, the OA largely prevented the acid-induced activation of NR in spinach *in vivo* (Kaiser and Brendle-Behnisch 1995).

Also, the activity of NR can be regulated by the addition of kinases or proteases inhibitors to the reaction medium (Reda and Kłobus 2006). However, reactivation of NR by ethylenediaminetetraacetic acid (EDTA) is not affected by protein phosphatase inhibitor (Microcystin LR). This inhibitor decreased the stimulation of NR by adenosine monophosphate (AMP) only.

Bearing in mind the above reports and the search for a method of inactivation of NR with the use of chemical inhibitors, the authors hypothesised that treating plants with OA would block the action of PP2A, which would increase the share of phosphorylated NR (because dephosphorylation would be blocked) and accelerate its deactivation. In this study, the total and actual NR activity, and NR activation state, in two tomato cultivars treated with 0 - 1.0 µM OA were evaluated.

Tomato (*Solanum lycopersicum* L. cvs. Faworyt and Kmicic) were obtained from *W. Legutko Breeding Company* (Jutrosin, Poland). The seeds were sterilised using 1 % (m/v) NaClO in ethanol for 5 min and washed with deionised water twice. The sterilised seeds were sawn in Petri dishes (12 × 12 cm) containing half-strength Murashige and Skoog (1962; MS) medium with 1 % (m/v) sucrose and 0.4 % (m/v) phytagel (pH 5.8). The plates were placed vertically in a versatile environmental growth chamber (*MLR-351H, SANYO Electric Co.*, Osaka, Japan) at day/night temperatures of 22/20 °C, a photon flux density of 150 µmol m^{-2} s^{-1}, a 16-h photoperiod, and relative air humidity of 50 %. Experiments were repeated three times.

After ten days from sowing, tomato seedlings were transferred to fresh Petri plates with half-strength MS medium, 1 % sucrose (pH 5.8), 0.4 % phytagel, and 1 cm^3 of OA at different concentrations spread over the surface [0 (control), 0.01, 0.05, 0.1, 0.5, 1.0 µM) (10 plants per plate)]. The plates were placed horizontally under the same growth conditions for 18 h.

Fresh roots and leaves were separately homogenised with the 2 cm^3 of extraction buffer containing 0.05 M HEPES-KOH (pH 7.5), 1 mM phenylmethyl sulphonyl fluoride (PMSF), 1 % (m/v) polyvinylpolypyrrolidone (PVP), and 1 mM dithiothreitol (DTT). Samples were centrifuged at 10 845 *g* and 4 °C for 15 min (*ROTINA 380RM*, Hettich, Germany). The supernatant was used for the determination of total protein content and measurements of NR activity (NRA). Total protein content was determined according to the Bradford (1976) spectrophotometric method. The 0.05 cm^3 of extracted proteins were mixed with 1 cm^3 of Bradford reagent. Absorbance was read at 595 nm (*U-2900*, *Hitachi*, Tokyo, Japan). Protein content in the plant extract of tomato roots and leaves was calculated according to the bovine serum albumin calibration curve.

The NRA was determined in the presence (actual

enzyme activity) or absence (total enzyme activity) of $MgCl_2$, according to Kaiser and Huber (1997) and Nemie-Feyissa *et al.* (2013) with some modifications. The plant protein extract was incubated for 10 min with 10 mM EDTA or 10 mM $MgCl_2$ solution at room temperature, and then the 10 mM KNO_3 was added. The reaction was started with 0.2 mM NADH (in the case of roots with the addition of NADPH), and incubation was conducted at 27 °C for 30 min. The amount of NO_2^- produced by NR was determined spectrophotometrically with sulphanilamide and N-(1-naphthyl)ethylenediamine dihydrochloride (NEDA) (Hageman and Reed 1980) at 540 nm (*U-2900, Hitachi*). The results are presented as the specific activity of an enzyme. NR activation state was calculated by dividing actual NR activity by total NR activity and expressed as a percentage.

All results are presented as means ± standard errors (SEs). Data were statistically analysed with a one-way analysis of variance (*ANOVA*) and Fisher's LSD test with the assumption of $\alpha = 0.05$. All data analyses were made using *STATISTICA 13* (*TIBCO Software* 2017) from *Statistica* (data analysis software system, *version 13*. http://statistica.io)).

The total and actual NR activity, and NR activation state, in two cultivars of tomato seedlings treated with different concentrations of OA, were determined. In cv. Kmicic leaves, no significant differences were found in total NR activity among treatments with 0.01 and 0.05 µM OA and control (0 µM) (Fig. 1*A*). However, the total NR activity was higher in Kmicic leaves treated with 0.1, 0.5, and 1.0 µM OA (by 52, 52, and 49 %, respectively). A similar tendency was determined on actual NR activity in Kmicic leaves (Fig. 1*C*). No significant differences were found in total and actual NR activity in cv. Faworyt leaves treated with various concentrations of OA (Fig. 1*B,D*). The NR activation state did not change significantly in both tomato cultivars treated with OA.

No significant differences were determined in the total

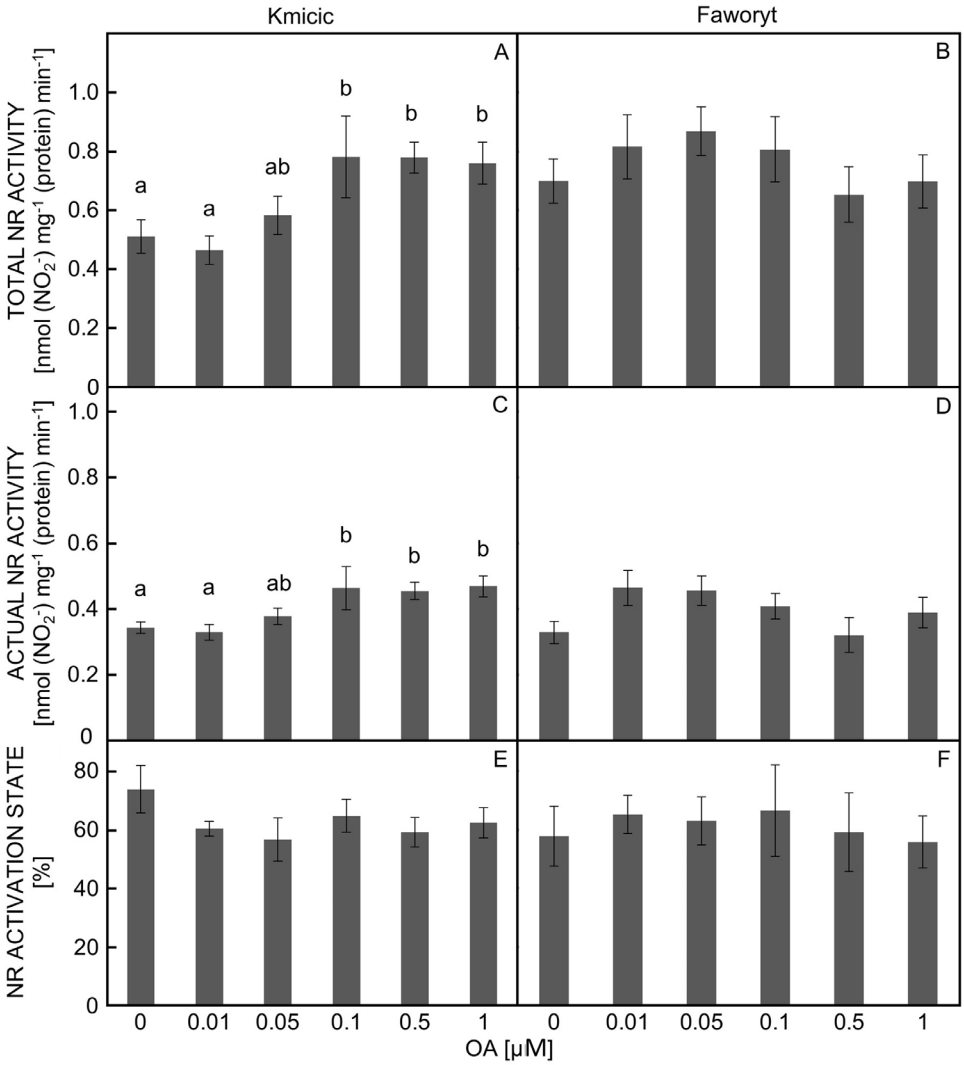

Fig. 1. The total and actual nitrate reductase (NR) activity, and NR activation state in tomato seedlings (10 d old) of cvs. Kmicic (*A,C,E*) and Faworyt (*B,D,F*) leaves treated with different concentrations of okadaic acid (OA). Means ± SEs, $n = 12$; letters indicate homogenous groups according to statistical analysis and $\alpha = 0.05$, lack of letters informs about lack of differences.

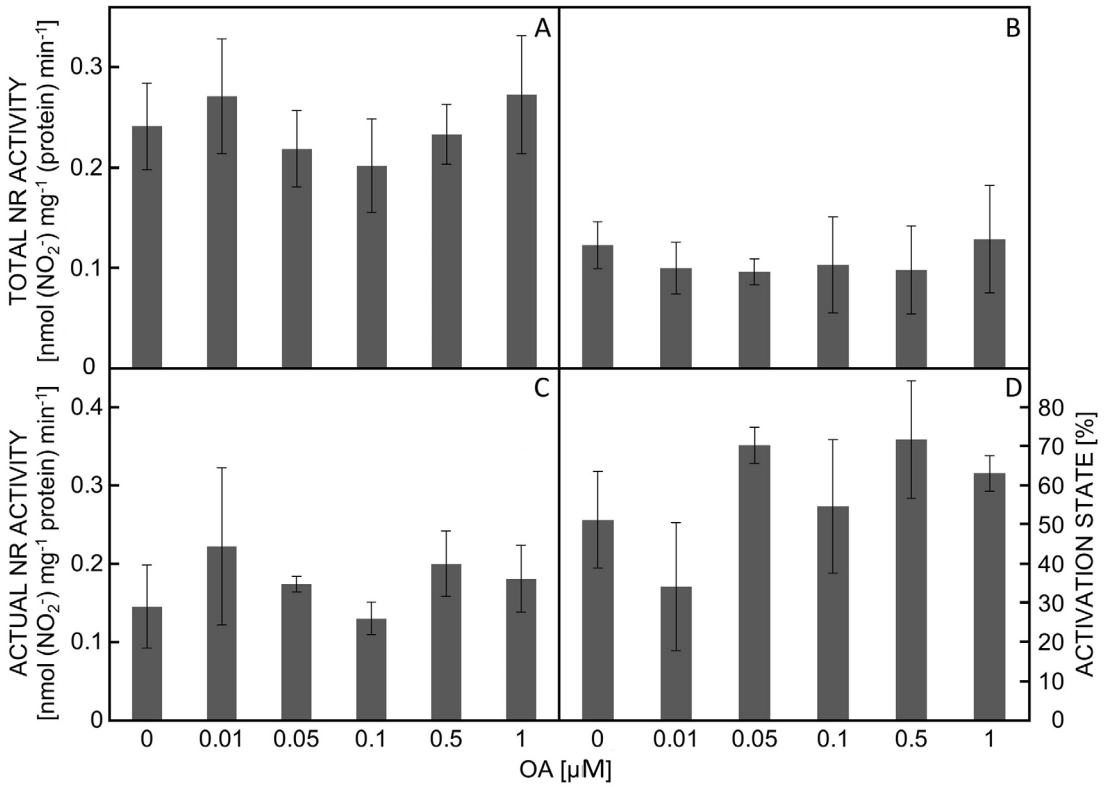

Fig. 2. The total and actual nitrate reductase (NR) activity, and NR activation state in tomato seedlings (10 d old) of cvs. Kmicic (*A,C,D*) and Faworyt (*B*) roots treated with different concentrations of okadaic acid (OA). Means ± SEs, $n = 6$; letters indicate homogenous groups according to statistical analysis and $\alpha = 0.05$, lack of letters informs about lack of differences.

NR activity in the roots of both tomato cultivars (Fig. 2*A,B*). Also, the increment of actual NR activity and decrement of the activation state of Kmicic roots was observed, but the results were not significant (Fig. 2*C,D*). These parameters were not evaluated on Faworyt roots.

It is thought that roots do not need to change the nitrate or nitrite reduction rate as rapidly as leaves (Kaiser and Huber 2001). However, our results demonstrated that the NR activation state in leaves was like that in roots.

OA can be used as an inhibitor of protein phosphatases for experiments in four different ways: *1*) as an addition to the medium in which the plants are grown (Rojo *et al.* 1998); *2*) as an addition to the medium in which parts of plant tissue are incubated (Kuo *et al.* 1996); *3*) as an addition to the reaction medium (Reda and Kłobus 2006); and *4*) applied directly to investigated tissue (Jones and Ort 1997).

In the presented work, the method of application on the medium on which the plants are placed was selected - modification of method 1. The concentrations of OA applied in our study were within the range of that being shown effective in other *in vivo* systems (5 to 50 μM) (Yaneva *et al.* 2002). *In vitro*, OA inhibits protein PP2A at 1 nM concentration (Cohen *et al.* 1990). The changes in NR activity measured *in vitro* are not always associated with changes in nitrate reduction rates *in vivo*, suggesting that NR can be under solid substrate limitation (Kaiser and Huber 2001). Plants - both leaves and roots - were in contact with the solution containing OA. Perhaps the incubation period was too short of ensuring good diffusion. However, it is worth emphasising that the incubation in the presented experiment was 18 h, and in another research (Rojo *et al.* 1998), only 1 h.

Yaneva *et al.* (2002) demonstrated that the inhibitory effect of OA on NRA in winter wheat leaves decreased gradually when OA concentration in the nutrient solution was lowered below 1 μM and tended to disappear when leaves were fed with 10 nM OA. The increased NR activity in cold-treated winter wheat leaves was also correlated with a higher content of NR mRNA but not with an increased NR protein content. It means that feeding OA to the leaves significantly reduced NR transcript accumulation but did not affect the content of NR protein in these leaves. A similar response to OA treatment has previously been shown on radiation-regulated NR expression in maize leaves (Redinbaugh *et al.* 1996). OA is also known to affect the expression of other plant genes. For example, OA caused increased accumulation of an auxin-regulated mRNA in tobacco (Dominov *et al.* 1992).

Actual NR activity was unaffected after adding the protein phosphatases type 1 and 2A inhibitors (microcystin-LR and cantharidin) into the incubation medium (Reda *et al.* 2011). In the cited studies, the inhibitors acted directly on the extracted enzyme. In our research, we also observed no effect of OA on the actual activity of NR, but the whole plant was treated with the inhibitor.

Excised and segmented cucumber roots were incubated for 2 h with or without 40 μM OA in buffer (pH 5.2). After

this incubation, the NR activation state was decreased from 63 to 32 % by OA treatment (De la Haba *et al.* 2001). After incubation in OA, the total NR activity was like in the control; however, the actual NR activity was much lower. In the presented experiment, roots' actual activity and activation state were unaffected by OA treatment, but the whole, undamaged plants were treated with the inhibitor, explaining the different reactions.

OA is a relatively stable compound among the inhibitors of protein phosphatases. Thanks to its reasonably hydrophobic property, it can easily penetrate the cell membrane and the cell interior. However, it should be remembered about its poor solubility in water (Swingle *et al.* 2007). At first glance, it appears to be a good candidate for the inhibition of protein phosphatases, which may affect the degree of phosphorylation and the activity of NR. Ultimately, however, when analysing the obtained results and the discrepancies that can be found in the literature, we do not recommend using OA to treat whole, undamaged plants.

In conclusion, our brief study showed that OA did not have an inhibitory effect on the NR activation state in tomato seedlings. Moreover, one tomato cultivar showed increased activity of total NR after the treatment with OA. Our results contradict those published in previous studies; more detailed investigations are required to find a selective inhibitor of NR activity.

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