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The Effects of Nitric Oxide on Some Early Germination Parameters and Mitotic Activity In Lentil (*Lens Culunaris* Medik.)

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Abstract

Germination of seeds is a crucial stage in plant life cycle. It is known that NO also plays an important role in seed dormancy and germination. Lentil is one of the most important crops in Turkey. Hence we chose this plant as an experimental material. The aim of the study is to examine effects of four different concentrations of SNP (0,01 μM, 100 μM, 600 μM and 700 μM), a NO donor, on lentil seeds. We established germination rate, primary root length, protein amount, activities of enzymes such as amylase, acid phosphatase, protease as well as mitotic index at 0, 24, 48 and 72 hours. We found that 100 μM SNP induces germination of lentil seeds, whereas 700 μM SNP clearly inhibits it. It was also established that 100 μM SNP was highly effective in primary root length and acid protease activity. Total soluble protein amounts were found to be higher than the control group in all SNP applications depending on the concentrations, and the most obvious increase was seen in the application of 700 μM SNP which also enhanced amylase activity. All SNP applications had the same effect on acid phosphatase activity, whereas acid and neutral protease activities changed depending on time and concentration. In addition, it was established that 700 μM SNP caused a reduction in mitotic activity. In the light of the findings obtained in this study, it can be claimed that depending on the concentration, As a result, NO has a noteworthy effect as a plant growth regulator on the germination of lentil seeds.

Key words: Nitric oxide, germination, protein, germination enzymes, mitotic index

INTRODUCTION

Nitric oxide (NO) is an ubiquitous signaling molecule. This most simplest molecule can easily diffuse through cell membranes due to its low molecular weight and lipophilic qualification. NO has been prven as only gaseous signal transmitting molecule, especially in mammalian cells, and in plants most recently [1, 2]. As a pollutant, NO is produced by both car engines and power stations [3]. NO was at first studied in plants with an emphasize on its role in atmospheric pollution. The presence of NO in plants and its effects on plant growth were defined in the 1970 for the first time [4, 5]. Even though studies on NO in plants are not as plenty as in animals, recent investigations have considerably revealed presence and function of NO in the plant kingdom [6, 2], including comprehensive studies examining the biological role of NO in many physiological processes such as growth, programmed cell death and defense responses [7, 8, 9, 10]. Interest in NO as an endogenous and potent regulator of plant development and physiology has grown exponentially in the last few years [2, 11, 12, 13]. As a developmental regulator, NO stimulates leaf expansion, seed germination and de-etiolation, inhibits hypocotyl and internode growth, leaf maturation and senescence [1, 14]. Moreover, it functions both as an antioxidant and as an anti-stress agent against various biotic and abiotic stresses, such as wounds, infections, drought, salinity, extreme temperatures, ultra-violet radiation and ozone exposure in plants [3, 14, 15, 16, 17, 18, 19].

Germination of seeds is a crucial stage in plant life cycle. There are several factors affecting seed germination. Of these, some endogenous compounds reduce seeed dormancy, but the action mechanism of any of these compounds is not known yet. As an endogenous factors, plant growth regulators play important roles in seed germination. NO, which was identified as an environmental pollutant also affects seed germination [20]. Especially, it was paid attention, concerning its role in seed dormancy. In addition, numerous reports have shown that NO plays an important role both in seed dormancy and germination [11, 21, 22, 23, 24, 25]. Two NO donors, sodium nitroprusside (SNP) and S-nitroso-N-acetylpenicillamine (SNAP), induce germination of lettuce (*Lactuca sativa* L. cv. Grand Rapids) seeds in a light-dependent manner (e.g. 26 degrees C). This indaction is a dose-dependent response and arrested by addition of an NO scavenger, carboxy-PTIO [1, 11]. Upon treatment with active SNP, a widely used NO donor, the germination of seeds and development of seedlings are significantly less delayed in transgenic lines compared with the retard of non-transformed seedlings [24]. Kopyra and Gwóźdź [26] have shown that SNP stimulates seed germination and root growth of lupin (*Lupinus luteus* L. Cv. Ventus), and germination process is promoted at concentrations between 0,1 μM and 800 μM SNP in a dose dependent manner. In addition, Ling et al. [23] have revealed that exogenously applied SNP could significantly promote the germination index and the early germination rate of rice seed under salt stress. Furthermore, it was raported that NO reduces seed dormancy in Arabidopsis [22].

Since growth is restricted by both cell division and elongation, NO may be involved either in inducing dedifferentiation and division in pericycle cells or in increasing the elongation rate of cells in LR (lateral root) primordia. Recently, NO was

demonstrated to be required as a part of the molecular events involved in root growth and development including adventitious root development induced by indole acetic acid (IAA) [27, 28, 29], and in LR formation [30, 31]. Correa-Aragunde et al. [31] have reported that NO plays a central role in determination of lateral root development in tomato, and suggested a novel role for NO in the regulation of LR development, probably acting in the auxin signaling transduction pathway. NO is also modulate expression of cell cycle regulatory genes during lateral root formation in tomato. NO modulation of cell cycle regulatory genes occurs in G_1-S phase transition in tomato roots during LR initiation [30].

In this study, we examined the effects of SNP on germination rate, primary root length, total protein amount, activities of germination enzymes as well as mitotic index at early stages of germination in lentil.

MATERIAL AND METHOD

Seeds of Lentil (*Lens culunaris* Medik. "Sultan 1") were kindly supplied by "Anatolian Agricultural research Institute"(Eskişehir, Turkey). SNP [Na2Fe(CN)5·NO] was purchased from Sigma. The seeds were pre-treated with distilled water (control) and experimental solutions (0.01 µM, 100 µM, 600 µM and 700 µM SNP) overnight (12 h). Then, they were placed on to petri dishes (15 cm in diameter) containing filter paper moistened with 12 ml of distilled water and 12 ml of experimental solution, and allowed to germinate at 25±2°C for 3 days (72 h) with a photoperiod of 12 h light (about 6000 lux, cool-white fluorescent lamps) and 12 h dark in the climate room. 60 seeds for each experiment were used to estimate germination rate and primary root length. Certain germination parameters such as total soluble protein amount, amylase, acid phosphatase and acid and neutral protease activity, 72. hours were spectrophotometrically measured by excising plumula and radical of germinated seeds at hours 0, 24, 48 and 72. The effects SNP on mitotic index were also examinedl.

Germination Rate

Seeds with radical of 1 mm or longer were accepted as germinated. Germination rate was estimated by counting germinated seeds daily for 3 days after pre-treated with SNP concentration of 0,01 µM, 100 µM, 600 µM ve 700 µM SNP overnight (12 h).

Primary Root Elongation

Primary root elongation was measured daily at 24, 48 and 72 hours.

Total Protein Determination

Total protein was measured by Dye-binding method of Bradford [32] in which Bovine Serum Albumin (BSA) was used as standard.

Amylase Activity Assay

Amylase activity was spectrophotometrically measured according to Jennings and Duffus' [33] method, and defined quantitatively as α_{608} / 5 min/g.

Acid Phosphatase Activity Assay

Acid phosphatase activity was determined by using method of Tanaka et al. [34], and defined as unit/ml.min.

Protease Activity Assay

50 mM citrate phosphate buffer at two different pH (pH 4,2 for acidic protease, pH 6,6 for neutral protease) was employed with Azocoll (Calbiochem) as substrate to determine protease activity [35]. Absorbance of supernatants was measured at 520 nm, and protease activity was defined as α520/g F.W.

Mitotic Index

3 days after germination, root tips of 0.5 cm were cut and hydrolyzed with 1 N HCl at 60°C for 10 min. Then they were transferred into basic fuchsin for 1.5-2 hr in dark. Squash preparations were made in 2% aceto-orsein. For cytological analysis, mitotic index and number of aberrant cells were established. Concerning mitotic index, more than 5000 cells from well spread slides were counted for each treatment. Mitotic index was calculated as the percent ratio of dividing cells and total number of cells counted.

Statistical Analysis

All experiments were repeated three times and the vertical bars show the standard error.

RESULTS

Effect of NO on Seed Germination

The effect of various concentrations of SNP 24 hours after imbibition on the germination of lentil seeds is summarized in Figure 1. In comparison with the control group, promoting effects of 11% and 35% were seen at 0.01 µM SNP and 100 µM SNP, respectively. Whereas inhibiting effects of 12% and 95% were observed for 600 and 700 µM SNP, respectively. Maximum germination rate was found at 100 µM SNP.

As can be seen from Figure 2, SNP has a marked effect on primary root lengths when compared with the control. At hours 24 and 48, the promoting effect was seen at 0.01 µM SNP and 100 µM SNP, while inhibiting effect was observed at 600 and 700 µM SNP. Especially at the 72nd hour a promoting effect of 43% was seen at 100 µM SNP compared to the control, while an inhibition of 37% was determined at 700 µM SNP. The primary root lengths at 0.01 and 600 µM SNP were almost similar to the control group.

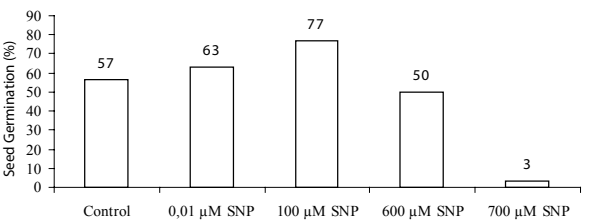

Figure 1. The effect of NO on germination rate in 24h.

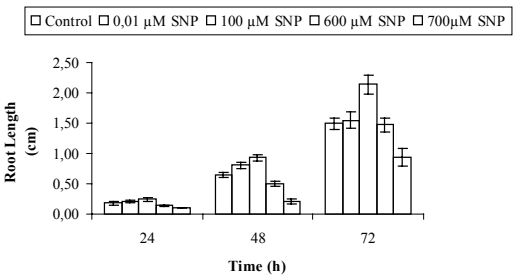

Figure 2. The effect of NO on the primary root length of Lentil in 24, 48 and 72 hours.

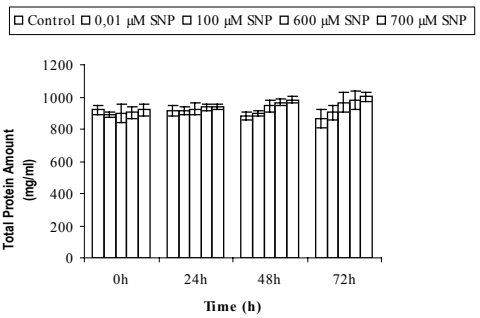

Figure 3. The effect of NO total protein amount of Lentil in 0, 24, 48 and 72 hours.

Total Soluble Protein Amount

As seen in Figure 3, while total protein amount remains almost the same in the control group depending on time, increased values were observed in all control groups depending on both time and SNP concentrations. At 0 hour following imbibition, total protein amount was found to be lower than the control at 0.1, 100 and 600 µM SNP, and a value which slightlyt exceeded the control group was observed at 700 µM SNP.

Amylase Activity

Effects of NO on amylase activity during the germination were examined at 24, 48, and 72 hours (Figure 4). Amylase activity of the control seeds was established at 0 h and gradually reached maximum level at 24 h, while control amylase activiy remained at more or less constant level at 48 h. At 0 hour of germination, amylase activity was close to the control at 0.01 µM SNP, and was slightly above the control at 100 µM SNP. NO activity was observed at 600 and 700 µM SNP. At 24 h of germination, a marked activity was seen in all experimental groups except for 100 µM SNP, while amylase activity of seeds decreased by 30% compared to 0 h. While maximum activity at 24 h was seen in 700 µM SNP application, the activity gradually decreased by reaching a minimum at 48 h. Amylase activity at 48 h decreased at the concentrations 0.01, 600 and 700 µM SNP by 50%, 38% and 91%, respectively, while at 100 µM SNP an increase of 92% was observed, which even exceeded the value at 0 h. NO activity was not seen at 72 h of germination in all experimental groups.

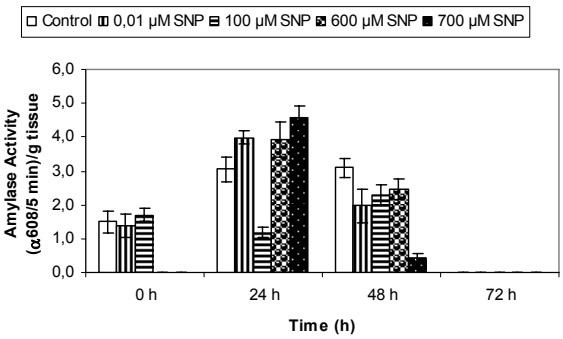

Figure 4. The effect of NO amylase activity of Lentil in 0, 24, 48 and 72 hours.

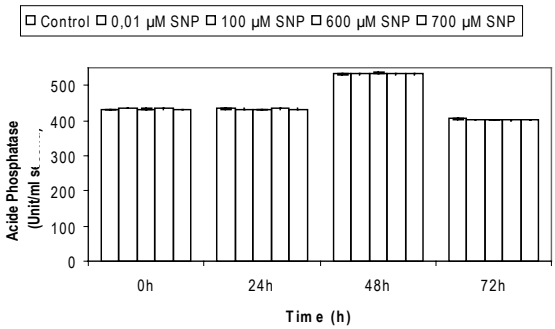

Figure 5. The effect of NO acid phosphatase of Lentil in 0, 24, 48 and 72 hours.

Acid Phosphatase Activity

With regard to NO effect, a marked difference between acid phosphatase activities of the control and experimental groups treated with SNP was observed (Figure 5). As can be seen from the accompanying figure, the acid phosphatase activities of all control groups at 0, 24 and 72 hours were found to be more or less the same. An increase at 48 h in all experimental groups was found to be the same as the control.

Acid Protease Activity

At 0 h, an increase, in proportion with the concentration, was observed in acid protease activity of experimental groups compared to the control, while at 24 h, there was a decrease by 5% and 13% at 600 and 700 µM SNP, respectively. However, increment of 86%, 73% and 23% were observed at control, 0.01 and 100 µM SNP concentrations, respectively. At 48 h values were more or less the same as the group 0 h. At 72 h a marked decrease occurred at 700 µM SNP, while an increase was observed in other experimental groups (Figure 6).

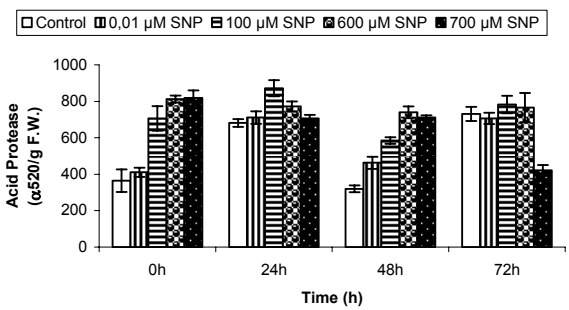

Figure 6. The effect of NO on acid protease of Lentil in 0, 24, 48 and 72 hours.

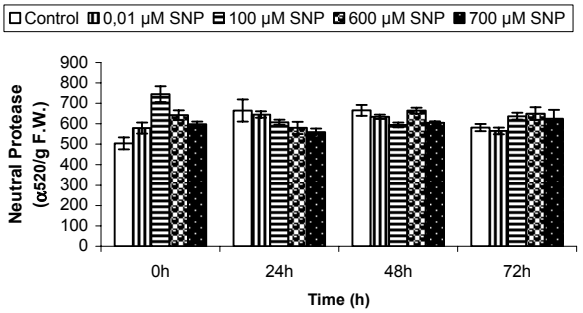

Figure 7. The effect of NO on neutral protease of Lentil in 0, 24, 48 and 72 hours.

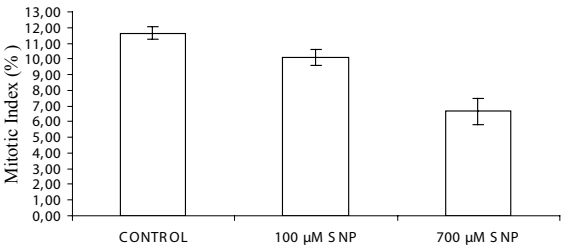

Figure 8. The effect of NO mitotic index of Lentil in 72 hours.

Neutral Protease Activity

As can be seen from Figure 7, at 100 µM SNP, a maximum activity of 47% was observed at 0 h compared to the control. Values close to or below the control were determined at 24 and 48 hours. At 72 h values remained unchanged.

Mitotic Index

Concerning the effect of different concentrations of SNP on mitotic index, the results were provided in Figure 8. As compared to the control, 100 µM SNP caused a decrease (13%) in mitotic index. This decrease was higher at 700 µM SNP. Cells underwent plasmolysis and marked impairment took place at 700 µM SNP. With 100 µM SNP treatment, cells were damaged, not being as much as with 700 µM SNP.

DISCUSSION

Recent evidence shows that NO serves as an important signaling molecule that regulates physiological processes such as plant growth and development, stomatal movement and responses to biotic and abiotic stresses [2, 11, 14, 16, 18, 19, 22, 36, 37]. In the present study, exogenous NO in the form of NO-releasing compound, especially 100 µM SNP, markedly stimulated seed germination at 24 h, while it was strongly inhibited by 700 µM SNP, meaning that SNP concentration is of vital importance in seed germination. Our data are consistent with those of others in that NO donor compounds promoted germination of Arabidopsis [38] and *Lactuca sativa* [1]. Kopyra and Gwozdz [26] also found that SNP between 0.1 and 800 µM stimulated seed germination and root growth of Lupin dose-dependently. In addition, Ling et al. [23] revealed that there existed a positive effect of application of glucose plus SNP on rice seed germination. Sarath et al. [39] stated that SNP also promoted seed germination in two warm-season grasses. Moreover, SNP reduced seed dormancy in Arabidopsis, of which seeds showed 90-95% germination rate when imbibed in the presence of SNP [21, 22]. Data of Libourel et al. [40] also support hypothesis that NO disappears seed dormancy in Arabidopsis, they showed that for some dormant seeds, exposure to exogenous NO was sufficient to trigger germination. Their study emphasized that NO either promotes or inhibites germination of lentil seeds depending on concentration.One can think that germination is significantly inhibited since seeds can not receive necessary water from medium at higher SNP concentrations. On the other hand, we can put forward that SNP could show its promoting effect by acting on endogenous gibberellin which plays an important part in germination. However, further studies are needed to support this claim. Furthermore, NO is said to regulate initiation of germination between 0 and 24 h of lentil germination after imbibition.

With this study,we showed that the most effective concentration is 100 µM SNP for root growth, and that 700 µM SNP causes remarkable inhibition, with an emphasis on the importance of concentration in primary root growth. These results are consistent with the finding of Zhang et al. [25] who reported that SNP markedly promotes the germination of wheat seeds and radicle growth depending on concentration. Inhibitory effect of SNP concentration of 700 µM was most pronounced at 48 h and 72 h and ceased after 72 h of imbibition. This result is in agreement with Parani et al. [41] is study in which 100 µM SNP inhibites root growth by increasing gene expression of two enzymes (ACC synthase and ACC oxidase) responsible for the biosynthesis of ethylene. In addition, Tian et al. [42] found that SNP markedly inhibites root elongation at concentrations >50 µM in *H. moscheutos*. A similar inhibitory effect of SNP on elongation of primary roots and hypocotyls has been reported [1, 31]. Although the mechanism underlying the inhibitory effect of SNP at relatively high concentrations on root elongation is unclear, there are several suggestions to account for this inhibitory effect. One of these is that cytotoxic peroxynitrite (ONOO-) changes some physiological responses related with root elongation in the presence of excessive NO [7, 43] Another possibility is that SNP promotes ethylene biosynthesis [41]. Pagnussat et al. [29] indicated that NO plays a role in auxin response in cucumber (*Cucumis sativus*) during apical root formation. Root elongation in *C. tora* was found to be insensitive to SNP up to a concentration of 800 µM [44]. Recently, it was demonstrated that NO is involved in auxin signalling cascade during root growth and development including adventitious root development [29] and LR formation [31]. From the results of this, it can be said that exogenously applied NO may have a direct or indirect role in the regulation of primary root lengths of lentil seeds. NO may be thought to act along with auxin in marked promoting effect caused by 100 µM SNP, and with ethylene in inhibition at 700 µM SNP. Additionally, one can suggest that the response formed against NO in the primary root elongation may change according to plant type.

Total protein amounts of lentil seeds remained at higher levels in the experimental groups compared to the control. In other words, values of protein amount were in direct proportion with increasing concentration. The decrease in total protein amount of the control group can be explained as hydrolysis of proteins by rapidly activated enzymes responsible for protein

destruction after imbibition. At increasing SNP concentrations the enzymes that hydrolyze stored proteins may not be active, and consequently protein destruction may not have taken place. That is why germination rate is lower and root elongation is slower at 700 µM SNP. However, another possibility is that excessive NO may cause stress for the plant by turning into other products of cytotoxic characteristics. As a result of this, synthesis of enzymes related with antioxidant system increases, and higher values of protein amount may be possible at 700 µM SNP compared to lower SNP concentrations. Liul et al. [45] found that NO accelerates germination by inducing expression of intrinsic protein (aquaporins) and water intake. Although germination rate is very low at 700 µM SNP, the total protein amount is higher than both control group and other experimental groups. Unfortunately, discussion of this is quite difficult due to absence of literature about direct relationship of NO with total protein amount in germination. Therefore, further studies should be conducted on this matter.

Amylase is one of the most important enzymes which act in germination. It is activated during germination, and provides glucose necessary for nutrition and hence the growth of embryo, by breaking down starch of endosperm [46]. There are numerous studies inspecting the effect of various plant growth regulators on amylase activity [47, 48], but only a few reveal the relation between amylase and NO. In this study there is a discordance between the high rate of germination of lentil seeds at 100 µM SNP at 24 h and amylase activity. The maximum increase in amylase activity may have taken place between 0 h and 24 h following imbibition. However, It is necessary to determine the changes in enzyme activity by employing more detailed molecular methods. Absence of activity at 72 h in all experimental groups shows cessation of germination.

Wheat seeds were sensitive to NO treatment rather than gibberellin in the early phases of germination. Depending on concentration, NO caused a rapid increase in β-amylase activity without affecting α-amylase activity [25]. Here it was shown that SNP both played an interesting role in the dissociation of free β-amylase and induced the change of bound β-amylase into free form directly. In this study, parallel to the maximum germination rate at 24 h, a high amylase activity would be expected. However, our opinion that amylase acted in an earlier phase of germination is confirmed by studies of Zhang et al. [25]. Further, NO may have directly stimulated amylase synthesis or its transformation into free form or its release.

Acid phosphatases (E.C. 3.1.3.2) are commonly encountered in plants. To date, activities of plant acid phosphatases in seeds, cotyledons, leaves, roots, tubers, bulbs and root nodules were studied [34, 49]. Acid phosphatases are known to play a role in the metabolic process of germination. Expression of acid phosphatases takes place in the seed, and there is an increase in their activity during germination. Thus stored materials are formed for the growing embryo [50]. In this phase of rapid growth and division, the need for phosphorus increases. Acid phosphatases act on activity of phosphorus stores during germination [51]. Since acid phosphatase activity is the same in all experimental groups in this study, increase in the activity formed only at 48 h may not be related with SNP treatment. It can be thought that NO does not have any effect on acid phosphatase activity, or at least there is not a significant relation between NO and acid phosphatase activity during the germination of lentil seeds.

The changes in protease activity, another enzyme which has a part in germination, are of utmost importance. As it is known, stored proteins undergo a proteolytic break down during seed germination [52, 53]. Acid protease activity in lentil seeds had a marked increase in the first 24 h, and neutral protease activity had a marked increase in the 0 h following imbibition. This is concordant with the notion that changes in protease activity occur in earlier phases of germination. This finding is consistent with the results of Zhang et al. [25]who reported that SNP causes a slight increase in protease activity during early germination phase of wheat seeds, and NO is not responsible for this effect.

Mitotic index was found to be almost the same as the control at 72 h for 0.01 and 600 µM SNP applications. At 100 µM SNP although primary root length was quite above the control, mitotic index was determined to be lower than the control. Decrease root growth in 700 µM SNP treated group may be due to low mitotic activity. Correa-Aragunde et al. [30] reported that SNP-mediated LR promotion could be prevented by the cell cycle inhibitor olomoucine, suggesting that NO is involved in cell cycle regulation. In addition, auxin-dependent cell cycle gene regulation is dependent on NO. We may suggest that inhibition caused by high SNP treatment occurs with the formation of cytotoxic products, and that NO has a direct or indirect effect on it.

In conclusion, it is now clear that NO is an important component in the germination of lentil seeds and root growth even if it does not play a key role. Thus, for elucidation of NO signalling in lentil germination and root growth at biochemical, cellular and molecular levels, further studies are needed.

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