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# Cadmium-induced structural disturbances in *Pisum sativum* leaves are alleviated by nitric oxide

**Tuan Anh TRAN[1], Valya VASSILEVA[2], Petar PETROV[3], Losanka Petrova POPOVA[1,*]**

[1]Department of Photosynthesis, Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria
[2]Department of Plant Stress Molecular Biology, Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria
[3]Department of Mineral Nutrition and Water Relations, Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria

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**Abstract:** In the present study, the protective effect of nitric oxide (NO) against Cd-induced structural disturbances in pea (*Pisum sativum*) leaves was investigated. Cadmium treatment resulted in a decreased leaf size and thickness of the lamina, reduced intercellular spaces in the mesophyll, small pavement cells, and a high density of stomata. These abnormalities were partially or fully reversed by a simultaneous application of Cd and the NO donor, sodium nitroprusside (SNP). The concentration of 1000 μM SNP was very effective in counteracting the adverse effects of Cd and resulted in leaf structural parameters close to those of the control leaves. These findings suggest that exogenous NO can effectively facilitate structural adjustments in pea leaves under Cd stress, which could improve stress tolerance at the whole-plant level.

**Key words**: Cadmium, leaves, guard cells, nitric oxide, pavement cells, *Pisum sativum*

## 1. Introduction

Cadmium (Cd) is a highly toxic trace element, which enters the environment mainly from industrial processes and phosphate fertilisers. It can reach high levels in agricultural soils and is easily assimilated by plants. When taken up in excess by plants, Cd induces various visible symptoms of phytotoxicity, e.g., leaf roll, chlorosis and necrosis, growth retardation, browning of root tips, and finally death (Kahle, 1993; Namdjoyan et al., 2012; Tran & Popova, 2013). Cadmium disturbs mineral nutrition (Sandalio et al., 2001), water balance (Barceló & Poschenrieder, 1990), root morphology, root and leaf anatomy, and functionality and lipid composition of membranes (Ouariti et al., 1997; Popova et al., 2009). In addition, Cd causes a decrease in stomatal density and conductance to $CO_2$ (Khudsar et al., 2001) and reduces the number of open stomata (Barceló et al., 1988; Greger & Johansson, 1992), which would further affect the rate of photosynthesis. In contrast, Baryla et al. (2001) reported increased stomatal density in leaves exposed to Cd. The mechanisms of Cd-induced changes in stomatal parameters are not well understood but probably arise from a Cd-induced water deficiency [reviewed in Sanità di Toppi & Gabbrielli (1999) and Poschenrieder & Barceló (2004)]. The toxic level of Cd reduces photosynthetic rate, probably due to its detrimental effects on chlorophyll biosynthesis (Stobart et al., 1985; Padmaja et al., 1990; Gadallah, 1995) and the disturbed chloroplast development (Atal et al., 1991; Keshan & Mukherji, 1992; Šeršeň & Kráľová, 2001), water splitting apparatus of photosystem II (Baszynski et al., 1980; Yordanova et al., 2009), and photosynthetic electron transport (Mohanty & Mohanty, 1988; Atal et al., 1991). Cadmium also negatively affects activities of carboxylating enzymes (Krantev et al., 2008). It has been shown that Cd ions lower the activity of ribulose-1,5-bisphosphate carboxylase (RuBPC) and damage its structure by substituting for $Mg^{2+}$ ions, which are important cofactors for carboxylation reactions. Cadmium can shift RuBPC activity towards oxygenation reactions (Siedlecka et al., 1998) and can cause an irreversible dissociation of the large and small subunits of RuBPC, thus leading to the total inhibition of the enzyme (Stiborova, 1988; Malik et al., 1992).

Considerable evidence has been reported suggesting that Cd toxicity causes oxidative stress, induced by the stimulation of free oxygen radical production (Sanità di Toppi & Gabbrielli, 1999) and by the modified activity of various antioxidant enzymes (Hegedus et al., 2001). Under severe stress conditions, however, the antioxidant capacity may not be sufficient to reduce the harmful effect of oxidative injury. Survival in an adverse environment depends on the plant's ability to perceive the stimulus, generate and transmit signals, and induce biochemical

* Correspondence: lpopova@bio21.bas.bg

changes that adjust the metabolism accordingly. Therefore, the search for signal molecules that might mediate plant stress tolerance is an important step towards a better understanding of how plants acclimate to adverse environments.

Nitric oxide (NO) is one of the few known gaseous signalling molecules. The high reactivity and free diffusion across membranes make NO ideal for a transient signal molecule between adjacent cells and within cells. In recent years, significant progress has been made in elucidating the diverse biological activities of NO in plants. Various studies have reported its presence in the plant kingdom and involvement in different cell processes, such as growth and development, respiratory metabolism, senescence, and maturation, as well as plant response to abiotic and biotic stressors. Nitric oxide is classified as a phytohormone that might function as a gaseous endogenous plant growth regulator, as well as a nontraditional plant growth regulator [reviewed in Beligni & Lamattina (2001) and Popova & Tuan (2010)].

Treatment with sodium nitroprusside (SNP) as a donor of NO increases the rate of photosynthesis, chlorophyll content, transpiration rate, and stomatal conductance in cucumber seedlings (Fan et al., 2007). Lum et al. (2005) reported that SNP decreases the amount of RuBisCo activity and the β-subunits of the RuBisCo subunit-binding protein in mung bean (*Phaseolus aureus* L.). Nitric oxide has been found to enhance chlorophyll content in potato, lettuce, *Arabidopsis thaliana* (L.) Heynh, and maize leaves (Graziano et al., 2002). Some authors consider NO to be a stress-inducing agent (Leshem, 1996), while others have reported its protective role (Hsu & Kao, 2004) depending on its concentration, the plant tissue or age, and the type of stress.

An increase in NO production has been detected during both water and heat stress (Leshem & Haramaty, 1996). Plant responses to stressors, such as drought (García-Mata & Lamattina, 2001; Neill et al., 2002) and Cd (Hsu & Kao, 2004), are regulated by NO. Additionally, it has been found that exogenous NO protects potato plants against herbicides paraquat or diquat (Beligni & Lamattina, 1999). In our previous study, we showed that NO in combination with Cd alleviated the inhibitory effect of Cd on growth, photosynthesis, and transpiration and improved the membrane integrity, measured by electrolyte leakage (Tran et al., 2011). The observed protective effects of NO on photosynthesis and transpiration under Cd exposure might be attributed to: (1) protection of the leaf tissue during the early stages of leaf development, (2) increased antioxidant capacity of the cell, and (3) prevented penetration of Cd into the cell. To explore the first possibility, in this study we examined the impact of NO on morphological and anatomical leaf traits in Cd-treated pea plants in the presence or absence of the NO donor SNP.

## 2. Materials and methods

### 2.1. Plant growth and treatment with Cd

Seeds of pea (*Pisum sativum* L. 'Ran') were surface sterilised, soaked in tap water for 6 h, and allowed to germinate on moist filter paper in the dark for 3 days. Seedlings were then placed in polyethylene boxes filled with 1.8 L of modified Hoagland solution (0.3125 mM $KNO_3$, 0.45 mM $Ca(NO_3)_2$, 0.0625 mM $KH_2PO4$, 0.125 mM $MgSO_4.7H_2O$, 11.92 µM $H_3BO_3$, 4.57 µM $MnCl_2.4H_2O$, 0.191 µM $ZnSO_4.7H_2O$, 0.08 µM $CuSO_4.5H_2O$, 0.024 µM $(NH_4)_6Mo_7O_{24}.4H_2O$, 15.02 µM $FeSO_4.7H_2O$, 23.04 µM $Na_2EDTA.5H_2O$). $CdCl_2$ was added at a final concentration of 25 µM. Two concentrations of SNP, 500 µM and 1000 µM, were used. The nutrient solution was continuously aerated and changed every 3 days.

Plants were grown in a growth chamber at 22/18 °C day/night temperatures and a 16-h/8-h light/dark photoperiod, with relative humidity between 50% and 60%, and 120 µmol $m^{-2}$ $s^{-1}$ PAR.

Plants were harvested 15 days after planting and used for leaf analyses.

### 2.2. Microscopic observations

For epidermal studies, 15-day-old leaves were collected in absolute ethanol and cleared as described previously (Vassileva et al., 2010). Microphotographs were taken using an upright Olympus BX51 microscope coupled to an XC50 digital microscope camera (Olympus, Germany). Images were processed and analysed with ImageJ software (National Institutes of Health, USA). The size, perimeter, and density of stomata and pavement cells were calculated for both the upper and lower epidermal surfaces.

For anatomical studies, all leaf samples were taken at mid-lamina from the second leaf and fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.4). The thickness of the lamina between bundles was examined. Cross-sections were cut by hand. Microphotographs were taken using an Amplival microscope (Carl Zeiss Jena, Germany), and 10 morphometric measurements were made in triplicate and averaged.

### 2.3. Statistics

Microscopic images of the cross-sections of the leaves were captured and saved on a digital image processor (InternationalMicro-Vision Inc., USA). For the statistical analysis, leaf thickness was evaluated in cross-sections obtained from 4 leaves per treatment and was measured using 3D-Doctor software (Able Software Corp., USA). Three images of epidermis per plant were analysed. At least 5–10 pavement cells and 10 guard cells in the digital images were traced with a graphic tablet. The length,

width, perimeter of guard cells, and area and perimeter of pavement cells were measured.

## 3. Results

Our previous experiments demonstrated that 500 and 1000 µM SNP are very relevant concentrations for physiological studies, because after long-term treatment (up to 15 days) they did not cause visible damage, such as wilting, growth retardation, or damage to photosynthesis and transpiration (Tran et al., 2011). The same work has also shown significant growth inhibition and decreased photosynthesis and transpiration in pea plants after treatment with 25 µM Cd for 15 days. These changes can be attributed to the fact that the plants were exposed to Cd at a very early developmental stage, and they have been under stress over the entire growth period.

### 3.1. Leaf size

Leaf size was examined as one of the important parameters in monitoring plant growth and development under the experimental conditions studied (Figure 1). The lowest value of this parameter, calculated as leaf area in $cm^2$, was recorded after treatment with 25 µM Cd. In contrast, treatment with 500 µM SNP slightly increased leaf area as compared to the control.

The negative effect of Cd was partly alleviated by treatment with SNP at a concentration of 1000 µM. There was no mitigation effect after supply of 500 µM SNP. Figure 1 shows sample pictures of the leaves used to determine leaf area.

### 3.2. Structural observations

The pea leaves are dorsiventral and amphistomatous in structure (Figure 2). Transverse sections of control plants displayed upper (adaxial) and lower (abaxial) epidermal layers with compactly arranged, large, barrel-shaped cells and mesophyll with dorsiventral organisation (Figure 2, control). Below the upper epidermis lay the palisade mesophyll, consisting of upright cells that are vertically oblong and densely packed. Spongy mesophyll, located above the lower epidermis, was represented by 5–6 cell layers, loosely arranged and enclosing large intercellular spaces. The cells were of varying shape and size. In the mesophyll, radially arranged groups of cells were seen around the bundles.

Distinct structural differences were observed in the leaves of Cd- and SNP-treated plants (Figure 2). The average thickness of the lamina declined from 250 µm in the control to 180 µm in the 25 µM Cd-grown plants (Figure 2). The mesophyll layer's thickness decreased under exposure to Cd. Intercellular spaces in the mesophyll area were nearly twice reduced as compared to the control (Figure 2). Plants treated with 25 µM Cd+1000 µM SNP showed similar structure to the control leaf (Figure 2). Leaf thickness, the volume of intercellular spaces, and cell layers reached the control level (Figures 2). Thus, simultaneous

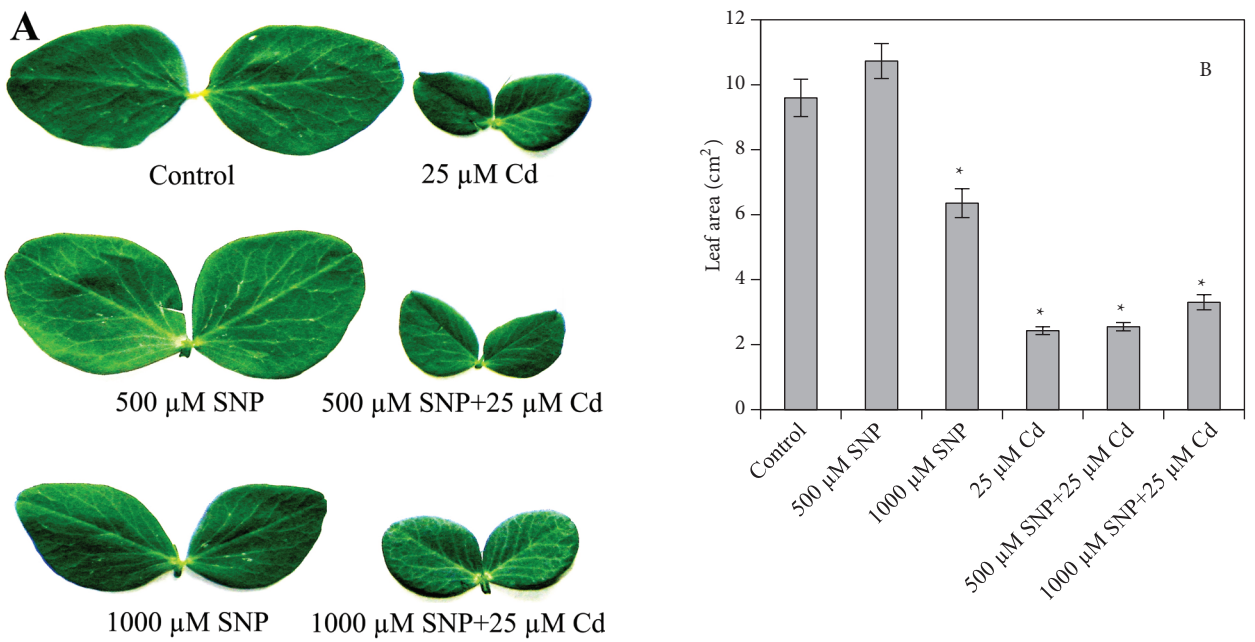


**Figure 1.** General view (A) and average size (B) of pea leaves exposed to 25 µM Cd in the presence or absence of 500 and 1000 µM SNP. Data are given as means ± SE (n = 4). Asterisk indicates significant differences when compared with the control (*: $P \leq 0.05$) by t-test. Scale bar: 5 cm.

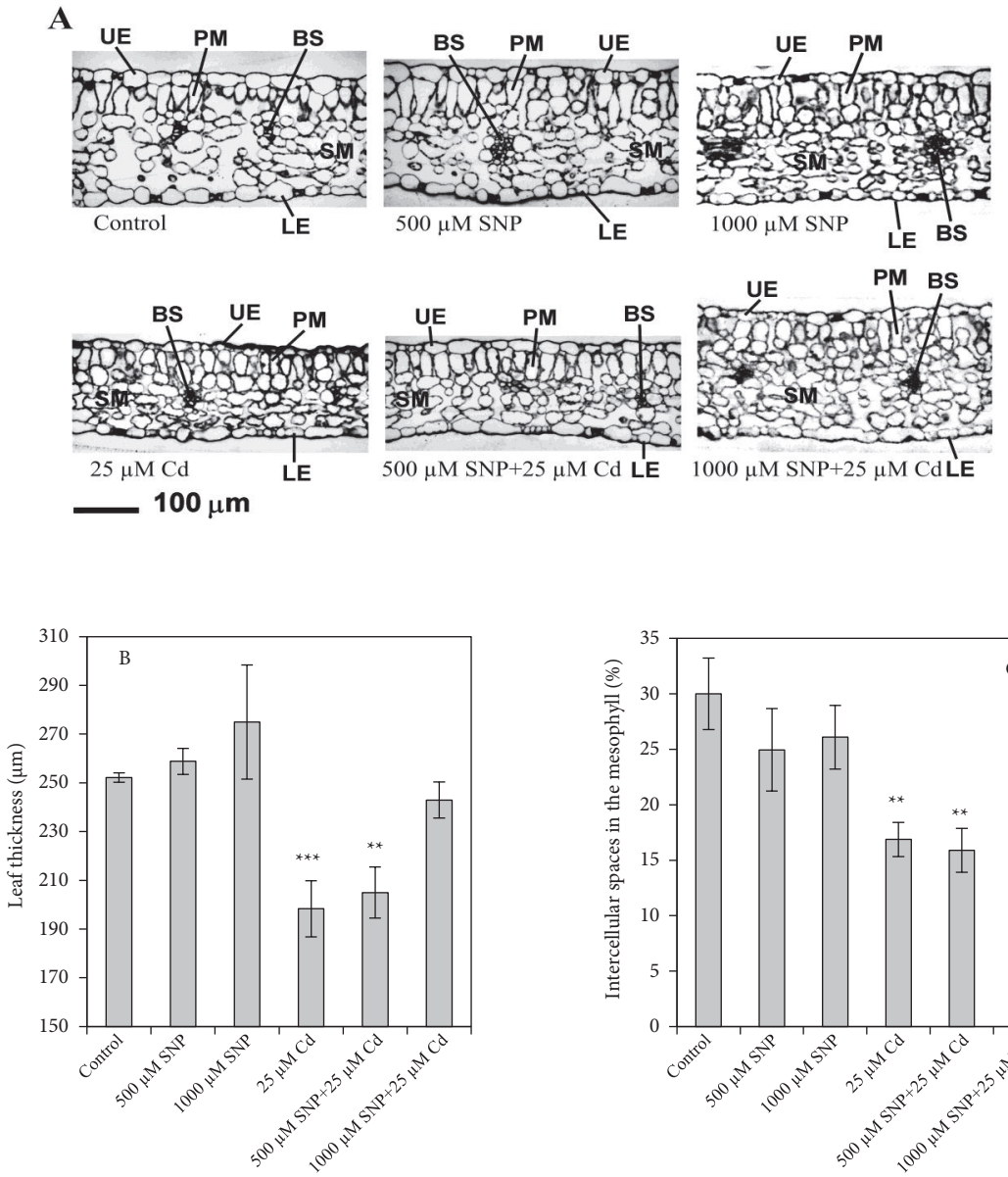


**Figure 2.** Anatomical features of leaves from pea plants exposed to 25 µM Cd in the presence or absence of 500 and 1000 µM SNP. (A) Leaf transverse sections under control conditions in the presence of 500 µM SNP, 1000 µM SNP, 25 µM Cd, 25 µM Cd+500 µM SNP, and 25 µM Cd+1000 µM SNP; (B) leaf thickness; (C) intercellular spaces in the mesophyll. Data are given as means ± SE (n = 4). Asterisks indicate significant differences (**, ***: $P \leq 0.01$ and $P \leq 0.001$, respectively) by one-way ANOVA followed by Dunnett's post-hoc test. UE, upper epidermis; LE, lower epidermis; PM, palisade mesophyll; SM, spongy mesophyll; BS, bundle sheaths. Scale bar: 100 µm.

treatment of pea plants with Cd and 1000 µM SNP led to almost complete restoration of leaf structure (Figure 2). The analogical treatment with 500 µM SNP could not completely alleviate the negative effect of Cd on pea leaf architecture.

Treatment with Cd affected the morphology of both the adaxial and abaxial leaf surfaces (Figures 3–5). Morphometric analysis showed that in Cd-treated plants, the average length and width of guard cells and the guard cell perimeter were smaller as compared to the control (Figure 4).

When plants were grown on solutions with Cd and SNP, these features nearly reached control levels. It should be noted that the most variable parameter was stomatal density, referring to the number of stomata/mm$^2$ (Figure 4). In Cd-treated pea plants, this parameter was more

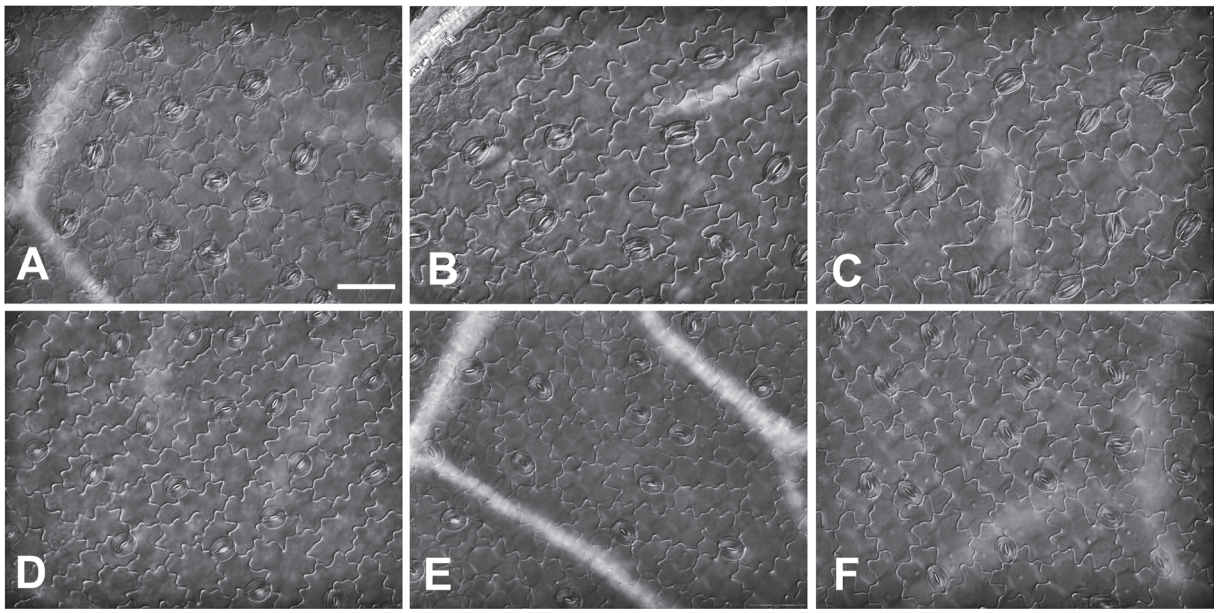


**Figure 3.** Representative digital images of lower leaf epidermal surface of pea plants exposed to 25 μM Cd in the presence or absence of 500 and 1000 μM SNP. (A) control, (B) 500 μM SNP, (C) 1000 μM SNP, (D) 25 μM Cd, (E) 25 μM Cd+500 μM SNP, (F) 25 μM Cd+1000 μM SNP. Scale bar: 50 μm.

than 2 times higher than in the control. A smaller increase in stomatal density was observed after simultaneous application of Cd and SNP.

In addition, as a consequence of Cd treatment, a reduction in the mean area and perimeter of leaf pavement cells was observed (Figure 5). The application of SNP together with Cd tended to increase the area of pavement cells and their perimeter, but these characteristics could not reach the control values.

## 4. Discussion

The plant resistance to heavy metals is usually associated with the activation of a wide variety of defence responses that prevent cell injuries and plant growth and development.

During exposure to biotic or abiotic stress, plants produce increased amounts of hormones that may interact with one another in regulating stress signalling and plant stress tolerance. There is rapidly increasing evidence that NO plays an important role in cell protection by regulating the level and toxicity of reactive oxygen species (ROS) and by inducing transcriptional changes of genes involved in different functional processes. Nitric oxide contributes to structural and functional plasticity and stress habituation (Mazid et al., 2011).

The present study expands on our previously reported findings concerning the putative physiological roles of NO in plants and emphasises the potential ability of NO to enhance plant tolerance to environmental constraints, like Cd toxicity. The toxic effect of Cd in pea plants is manifested by a severe and rapid inhibition of both shoot and root growth, as well as a strong inhibition of photosynthesis and transpiration processes. Cadmium significantly decreases the surface area of the root for absorption. Roots in Cd-treated plants undergo visible alterations, such as root thickening, no lateral root development, or browning of root tissue (Tran et al., 2011). These changes mean that the potential of roots to absorb water and mineral elements is very limited, leading to a disturbance of the main physiological processes of photosynthesis and transpiration in leaves.

Our study showed that in Cd-treated pea plants, leaf emergence was delayed, and mature leaf blades were less developed and remained smaller in size (Figure 1). The Cd-induced reduction of leaf expansion is a common stress effect that tends to reduce the total transpiration area. These responses to Cd stress were also observed by Poschenrieder et al. (1989); similar responses to salinity stress (Miteva & Vaklinova, 1991) and nutrient stress (Chiera et al., 2002) have also been reported. Structural changes that occur in leaves upon Cd exposure included a reduction in lamina thickness and shrinkage of epidermal, palisade, and spongy parenchyma cells (Figure 2).

These findings are in agreement with the reports by other authors (Barceló et al., 1988; Fiskesjö, 1988; Liu et al., 1992) and can serve as a basis to assume that the observed loss of one mesophyll cell layer is due to the inhibitory effect of Cd on cell division during leaf primordium development. Sobkowiak and Deckert (2003) have shown that an exposure of soybean to Cd impairs cell division by causing earlier cell entry into the S phase and by decreasing the rate of DNA synthesis.

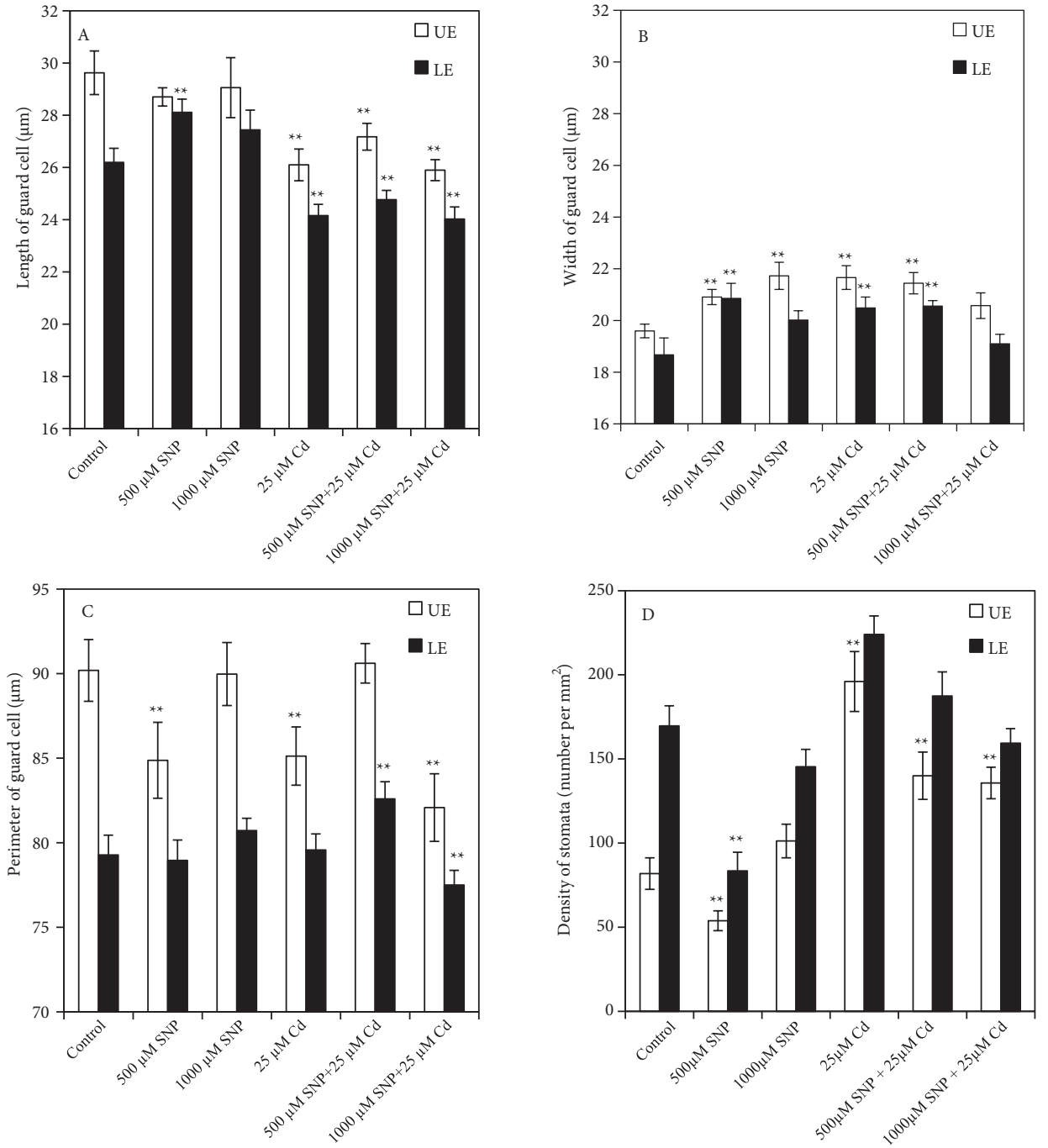


**Figure 4.** Average size of guard cells and stomatal density on the upper and lower leaf epidermal surfaces of pea plants exposed to 25 µM Cd in the presence or absence of 500 and 1000 µM SNP. (A) Length of guard cells, (B) width of guard cells, (C) perimeter of guard cells, (D) stomatal density. Data are given as means ± SE (n = 10). Asterisks indicate significant differences when compared with the control (**: $P \leq 0.01$) by t-test for the same leaf surface. UE, upper epidermis; LE, lower epidermis.

Leaf development is driven by 2 fundamental growth processes: cell division and cell expansion. During leaf development of dicotyledonous plants, an initial cell proliferation phase, characterised by actively dividing cells, is followed by a cell expansion phase, characterised by cell growth and differentiation (Asl et al., 2011). The final leaf size is determined by the total number of cells and the average cell size. In *Elodea canadensis* plants, Cd interferes with normal morphogenetic processes by inhibiting cell division and affecting cell enlargement, which results in less-expanded leaves (Dalla Vecchia et al., 2005). Changes in stomatal density were

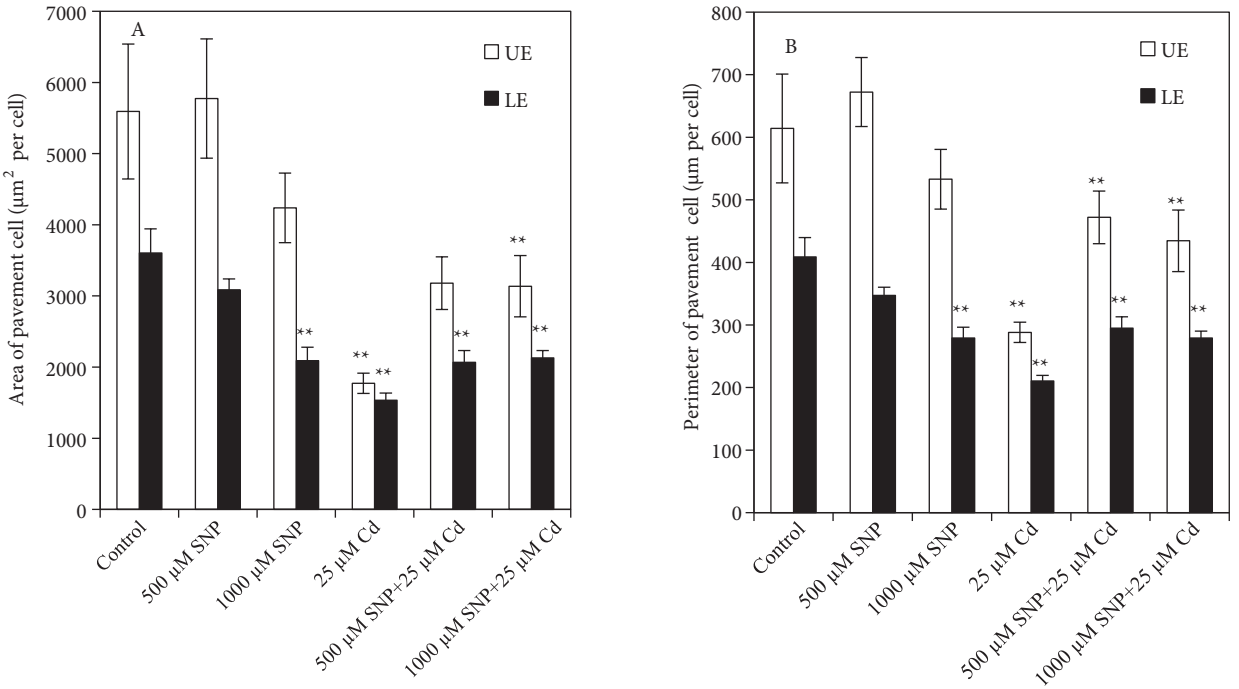


**Figure 5.** Average size (A) and perimeter (B) of pavement cells on the upper and lower leaf epidermal surfaces of pea plants exposed to 25 μM Cd in the presence or absence of 500 and 1000 μM SNP. Data are given as means ± SE (n = 5–10). Asterisks indicate significant differences when compared with the control (**: P ≤ 0.01) by t-test for the same leaf surface. UE, upper epidermis; LE, lower epidermis.

also observed in *Trichosanthes cucumerina* L. after salinity exposure (Adebooye et al., 2012).

We carried out detailed morphometric studies of leaf epidermal cells in order to determine the pattern of variation in pavement and stomatal guard cells under the treatment conditions investigated (Figures 4 and 5). Generally, leaf area enlargement is strongly correlated with an increase in the size of pavement cells (Asl et al., 2011). The qualitative evaluation of leaf growth rate in pea plants showed that external treatments stimulating leaf growth also stimulate the expansion of epidermal cells (den Os et al., 2007). It is tempting to explain the reduced leaf size in Cd-exposed plants by the decreased size of pavement cells (Figure 5). It could be expected that the Cd effect on leaf disc expansion would equally reflect the pavement cell expansion. However, the inhibitory effect of Cd on the size of pavement cells (Figure 5) was less pronounced than that on the leaf size (Figure 1), suggesting that the smaller leaf size was related not only to cell expansion but to cell divisions as well. The size of stomatal guard cells was only slightly affected by the Cd exposure (Figure 4); however, the number of stomata increased more than 2 times (Figure 4). In general, leaves with many small stomata can increase water use efficiency (Poulos et al., 2007). Compared with guard cell length, stomatal density is relatively plastic and potentially adaptive to environmental changes (Sekiya & Yano, 2008).

The modification of the frequencies and size of stomata as a response to environmental stress is an important way to control the absorption of pollutants by plants, as well. The high stomatal density generally correlates with a small stomatal size, which ensures fast response to external stimuli. The increased stomatal density could also be a compensatory measure to the reduced transpiration area, ensuring the maintenance of $CO_2$ flow without major harm to photosynthesis (Mazid et al., 2011). According to de Melo (2007), the increase in stomatal density, coupled with the decrease in stomatal size, would be an alternative to adequate supply of $CO_2$ for photosynthesis, without excessive water loss due to stomata with smaller pores. The observed reduction in stomatal aperture may also tend to conserve water and restrict $CO_2$ entry into the leaves.

The higher SNP concentration used in this study could partially offset the negative effect of Cd on leaf development and blade expansion (Figure 1). Depending on the concentration applied, NO appears to have either a stress-protecting effect or an inhibitory effect on leaf growth in pea plants. The promoting effect could be partly due to the marked deceleration of stress ethylene, mediated by the NO ability to counteract emission of ethylene. At higher NO concentrations however, leaf expansion is effectively inhibited (Leshem & Haramaty, 1996). Ötvös et al. (2005) have shown that low (1–10 μM) concentrations of SNP promote cell division and

embryogenic cell formation in leaf protoplast-derived cells of alfalfa, whereas high concentrations (100 μM) inhibit DNA replication. A similar dual effect of the NO donor SNP is noted in drought-treated wheat seedlings (Tian & Lei, 2006).

In our study, the concentration of 500 μM SNP had rather a stimulating effect on pea leaf development, as compared to the control (Figure 1). The 1000 μM SNP level showed a slightly negative impact on leaf growth, but in the condition of Cd stress, this concentration was more effective in alleviating the deleterious effects of Cd. The observed changes in internal leaf structure, particularly in the volume of intercellular spaces in the leaf mesophyll, could partly underlie these differences (Figure 2). An important factor that may contribute to proper leaf function is the presence of a well-developed system of air spaces in the mesophyll tissue that would facilitate rapid gas exchange (Bondada et al., 1994). The decreased intercellular spaces in Cd-treated leaves may restrict carbon flow toward the chloroplasts, decreasing the rate of photosynthesis (Tran et al., 2011). The observed recovery of mesophyll structure in Cd-treated leaves after supply of 1000 μM SNP (Figure 2) may be indicative of the rearrangement in mesophyll intercellular spaces, thus increasing tissue permeability and favouring carbon acquisition. There were no statistically significant differences in the leaf thickness of control plants and Cd-treated plants after supply of 1000 μM SNP (Figure 2), which may be due to the fact that the number of mesophyll cell layers was restored to the control level. These results suggest that NO may participate in the control of cell division during leaf development. So far, there are very few data on either the direct or indirect involvement of NO in signal transduction events related to the cell cycle or cell division in plants cells. Pasternak et al. (2007) reported auxin-dependent parallel activation of cell division and oxidative stress defence against the oxidising agent paraquat in alfalfa leaf protoplast-derived cells.

In summary, this study demonstrated that NO had a beneficial effect on leaf structure and guard cell morphology, partially alleviating the negative effect of Cd on pea plants. Treatment with the NO donor SNP alongside Cd mitigated Cd-induced abnormalities in leaf development and structure; additionally, it protected guard cell morphology, which could lead to improved leaf functioning. The results indicated that exogenous NO could effectively facilitate structural adjustments in pea leaves under Cd stress, thus improving stress tolerance at the whole plant level.

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