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**ARTICLE**

# Sodium nitroprusside affects the level of anthocyanin and flavonol glycosides in pea (*Pisum sativum* L. cv. Arkel) leaves

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**ABSTRACT** The effects of sodium nitroprusside (SNP), a nitric oxide (NO) donor were investigated on the levels of anthocyanin and flavonol glycoside in pea (*Pisum sativum* L.) cv. Arkel leaves. The study was conducted in leaf discs (ca. 20 mm$^2$) prepared from the youngest leaves. The anthocyanin and flavonol glycosides content diminish significantly (~ 21% of each) in leaf discs following 1 mM SNP (1 mM) treatment for 3 h under light (600 µmol $M^{-2}.s^{-1}$). However, a huge increase both in the levels of anthocyanin and flavonol glycosides, 72 and 53% respectively was recorded after 2 h of 1 mM SNP treatment. 0.5 mM SNP treatment of the leaf discs did not change the anthocyanin level but considerable declined (~13%) was observed in the level of flavonol glycosides as compared to the control. Surprisingly, the anthocyanin content in no SNP treated leaf discs after 3 h of incubation under light (600 µmol $M^{-2}.s^{-1}$) increased rapidly by 72% while flavonol glycosides content by 15% only. The photosynthetic capacities of SNP treated leaf discs were drastically inhibited. The study prelude that NO in combination of light influence the accumulation of anthocyanin and flavonol glycosides in pea leaves.
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**KEY WORDS**

anthocyanin
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*Pisum sativum* L. (cv. Arkel)
photosynthesis
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Sodium nitroprusside (SNP), which releases nitric oxide upon light irradiation, is widely used as nitric oxide (NO) donor in plants and animals to investigate the effects of NO. NO is an important signaling molecule in plants. NO has gained increasing interest as important intermediate and intracellular signaling molecule in plant systems which mediates various pathophysiological and developmental processes, including expression of defense-related genes and programmed cell death, stomatal closure, seed generation and root development (Lamattina et al. 2003; Neil et al. 2003; Deak et al. 2008; Kolbert et al. 2008a, b). In the past few years, a growing amount of research has provided evidence for the multiple physiological roles of this gaseous free radical in plants (Delledonne 2001; Wendehenne et al. 2004). See a recent review, nitric oxide as a potent signaling molecule in plants by Erdei and Kolbert (2008). Several previous studies showed inhibitory effects of NO on the net photosynthesis in plants such as in oat (*Avena sativa*) and alfalfa (*Medicago sativa*) leaves (Hill and Bennett 1970). In mung bean (*Phaseolus aureus*), SNP treatment of the leaves resulted in significant decrease in activities of several photosynthetic enzymes and glucose metabolism (Lum et al. 2005). SNP has also been reported to enhance the production of secondary metabolites *viz.,* catharanthine production in *Catharanthus roseus* suspension cells (Xu et al. 2004), secondary metabolism activities of *Taxus* Cells (Wang and Wu 2005). In *Artemisia annua,* NO potentiates oligosaccharide-induced artemisinin production in Hairy Roots (Zheng et al. 2008). Until now most of the studies with SNP were primarily aimed to investigate the effects of NO on photosynthesis and very little is known about the roles of NO in plant secondary metabolism. Therefore it would be interesting to know the roles of NO on secondary metabolites *viz.,* anthocyanin and flavonol glycosides while the photosynthetic activity of plant is halted by NO. Anthocyanins are a class of secondary metabolites that contribute to the red, blue, and purple colors in a range of flowers, fruits, in leaves particularly during senescence, stems, roots, and occasionally in fruit flesh and seeds (Feild et al. 2001; Regan et al. 2001; Schaefer et al. 2004). Anthocyanins and flavonols are synthesized *via* the flavonol pathway, a branch of the phenylpropanoid pathway. The pathway leads to synthesis of anthocyanins with branches for synthesis of flavonols. The anthocyanin and flavonol glycoside composition of the genus *Pisum* is described in detail in (Furuya and Galston 1965; Statham et al. 1972).

The aim of the present study is to investigate the effects of SNP *via* photosynthesis on accumulation of secondary metabolites in pea leaves. Here, we measured anthocyanins and flavonol glycosides in pea (*Pisum sativum* L. cv. Arkel) leaf discs following the SNP treatment.

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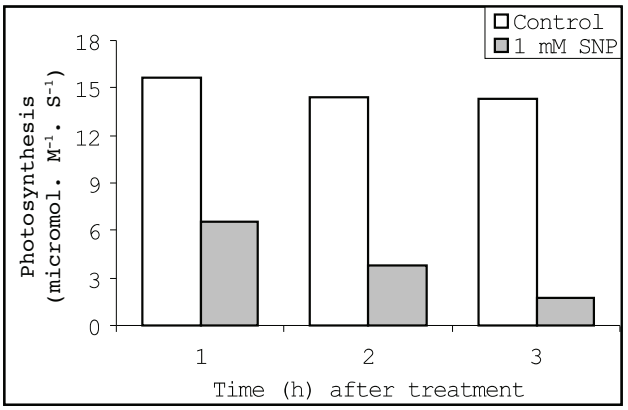


**Figure 1.** Changes in photosynthetic capacity of pea leaf discs following 1 mM SNP treatment. 15 leaf discs were kept in Petri dish containing 6 ml of distilled water as control, or 6 ml of different dilutions of sodium nitroprusside and incubated under light (600 µmol.$M^{-2}$.$S^{-1}$) for 3 h. Data presented in the figure are the average of the three independent experiments.

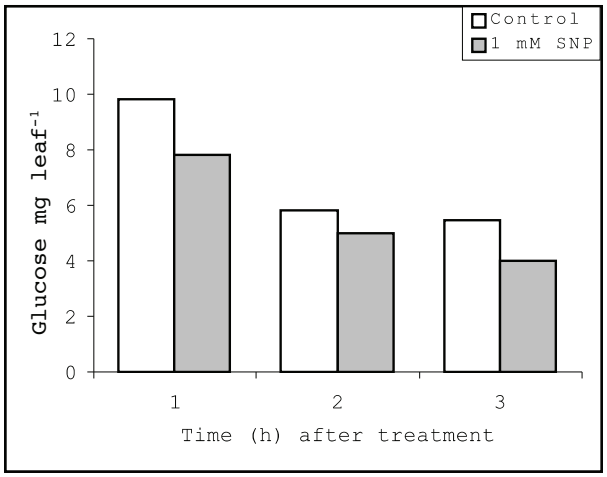


**Figure 2.** Changes in the glucose concentration in pea (*Pisum sativum* L.) cv. Arkel leaves after SNP treatment. Pea leaf discs were treated with 1 mM sodium nitroprusside and water (as control) under light (600 µmol.$M^{-2}$.$S^{-1}$) for 3 h. Data presented in the figure are the average of the three independent experiments.

## Materials and Methods

### Plant Material

Sterilized seeds of pea (*Pisum sativum* L. cv. Arkel) were grown in plastic trays filled with soil and farmyard manure (in a ratio of 1:3). Plants were grown outdoors for two weeks under 12-h-light/dark cycle and temperature of 40º/ 27ºC day/ night (in summer).

### Preparation of leaf discs and SNP treatment

Leaf discs (ca. 20 $mm^2$) of the youngest fully expanded leaves were prepared by a 15 mm diameter leaf punch and used for each measurement. To investigate the effects of SNP two sets of experiment were conducted. In the first set, 15 leaf discs were kept in Petri dish containing 6 ml of distilled water alone as control and incubated under light regime 600 µmol.$M^{-2}$.$S^{-1}$ for 3 h. In the second set, 15 leaf discs were kept in Petri dish containing 6 ml of sodium nitroprusside (0.5 to 5.0 mM) and incubated for 3 h. During the incubation, leaf discs were floating in the covered but not sealed Petri dishes. SNP solutions were prepared fresh.

### Measurement of photosynthetic oxygen evolution

Photosynthetic capacity of pea leaf discs was measured in terms of $O_2$ evolution on illumination using Clark type oxygen electrode (Hansatech Instruments Ltd., Kings lynn, UK). After SNP treatment, the leaf discs (15 in number) were quickly blotted dry and transferred into the leaf disc oxygen electrode. The components in the chamber were arranged as per the instructions of the manufacture. The topmost capillary matting was moistened with 200 µL of 1 M bicarbonate buffer (pH 9.0). The leaf discs were arranged on this matting symmetrically. Oxygen in the chamber was calibrated for every sample as per the instructions of the manufacturer. Photosynthetic oxygen evolution was measured at a constant temperature of 25ºC by a computerized leaf disc oxygen electrode system (LDp2, Hansatech Instruments Ltd. UK). Light at the required intensity was provided by an array of light emitting diodes.

### Determination of anthocyanin and flavonol glycosides

Anthocyanin and flavonol glycosides were extracted according to procedure described by Hrazdina et al. (1982). 15 leaf discs were extracted with 1.0 ml methanol-0.1 N HCl for 30 min and the extract decanted. 20 µL of the extract was added to 980 µL methanol-0. 1 N HCl and the absorption spectrum recorded in a spectrophotometer. The concentration of anthocyanin was determined from the A at 530 nm using a molar extinction coefficient (ε) of 38,000 L x $mol^{-1}$ x $cm^{-1}$ that of the flavonol glycosides at 360 nm (ε = 20000 L x $mol^{-1}$ x $cm^{-1}$, determined from a pure sample of quercetin 3-glucoside).

### Pea leaf viability tests

Following the SNP treatment, viability of plant cell in epidermal strips of leaf was assessed by Evans blue (EVB). Lower epidermal layer of pea leaf was peeled of and incubated for 10 min at room temperature in EVB solution (400 mg/ml 0.65 M mannitol). After incubation, epidermal strips were viewed and photographed under bright-field transmitted light for EVB.

**Table 1.** Changes in the photosynthetic capacity of the pea leaf discs in response to varying concentrations of sodium nitroprusside (SNP). Data presented in the table are the average of the three independent experiments.

| Treatment | Photosynthesis ($\mu mol.M^{-2}.s^{-1}$) | Photosynthesis % inhibition |
|---|---|---|
| Control (no SNP) | 14.0 | 00.0 |
| 0.5 mM SNP | 9.2 | 34.0 |
| 1 mM SNP | 5.0 | 64.0 |
| 5 mM SNP | 2.5 | 82.0 |

15 leaf discs were kept in Petri dish containing 6 ml of distilled water as control, or 6 ml of different dilutions of sodium nitroprusside and incubated under light (600 $\mu mol.M^{-2}.S^{-1}$) for 1 h.

## Results

### Effects of SNP on photosynthesis and glucose level

To investigate the effect of NO on photosynthesis, pea leaf discs were treated with varying sodium nitroprusside concentration (0.5 to 5 mM) under light 600 µmol $m^{-2}$. $s^{-1}$ for 3 h. Data presented in Table 1 shows that the photosynthetic capacity of the pea leaf discs rapidly diminish in accordance to the varying concentrations of the SNP. SNP affect the photosynthesis in dose dependent manner. Figure 1 depicts the changes in the net photosynthesis of the leaf discs following 1 mM SNP treatment. Photosynthesis in leaf discs was inhibited 88% as compared to the control after 3 h of 1 mM SNP treatment. Although the glucose level in non treated leaf discs after 1 h of incubation under light (600 µmol $m^{-2}$. $s^{-1}$) decreases substantially thereafter maintained at constant level, the glucose level of 1 mM SNP treated leaf discs were always found to be slightly (24-27%) lower than the glucose level of non treated leaf discs (Fig. 2). In the present study only 0.5 and 1.0 mM SNP concentrations were used because these concentrations of SNP was found to be non effective in terms of inducing cell death in leaf discs during chase period 3 h. Cell viability was tested by Evans blue staining of the pea leaf epidermis (Fig. 3).

### Effects of SNP on anthocyanin and flavonol glycosides

The levels of anthocyanin and flavonol glycoside measured in non treated pea leaf discs were 13.2 and 175.0 $nmolleaf^{-1}$ respectively. As shown in Figure 4 A and B, the levels of both anthocyanin and flavonol glycosides (nmol $leaf^{-1}$) reached at peak after 2 h of 1 mM SNP treatment then immediately declined below the control (13.2 and 175.0 $nmolleaf^{-1}$) level at 3 h. During treatment period, the level of anthocyanin increased by 72% while that of flavonol glycosides by 53% from 1 to 2 h and then declined approximately by 21% after 3 h as compared to the control. The anthocyanin content (nmol $leaf^{-1}$) of the non treated leaf discs (incubated under light 600 µmol $M^{-2}.S^{-1}$ alone) used as control increases from 18.4 to 31.6 (representing 72% increase) while flavonol glycosides from 222.5 to 257 (representing only 15% increase) during the period 1 to 3 h. Therefore, light (600 µmol $M^{-2}.S^{-1}$) alone caused marked increase in the level of anthocyanin, however it adds only 15% to the level of flavonol glycosides. SNP (0.5 mM), however, not at all affected the anthocyanin content but caused a reasonable decrease (~20%) in the amount of flavonol glycoside as compared to the control during 3 h incubation period.

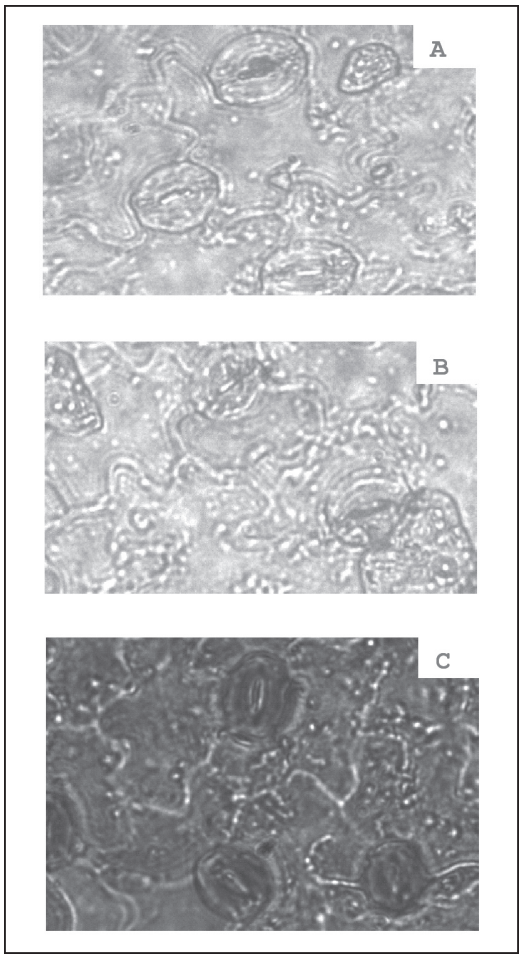


**Figure 3.** Cell viability Test by Evans blue (EVB) staining. None of the cell was stained when the pea epidermal strips were treated in water (as control). Also, no cell death was visible in 1 mM SNP treated leaves [A], however, the number of EVB stained cell (dead cells) significantly increased after treatment with 5 mM SNP for 3 h [C].

## Discussion

Here in the present study effects SNP has been investigated on anthocyanin and flavonol glycosides accumulation in pea (*Pisum sativum* L.) cv. Arkel leaf discs to reveal the role of

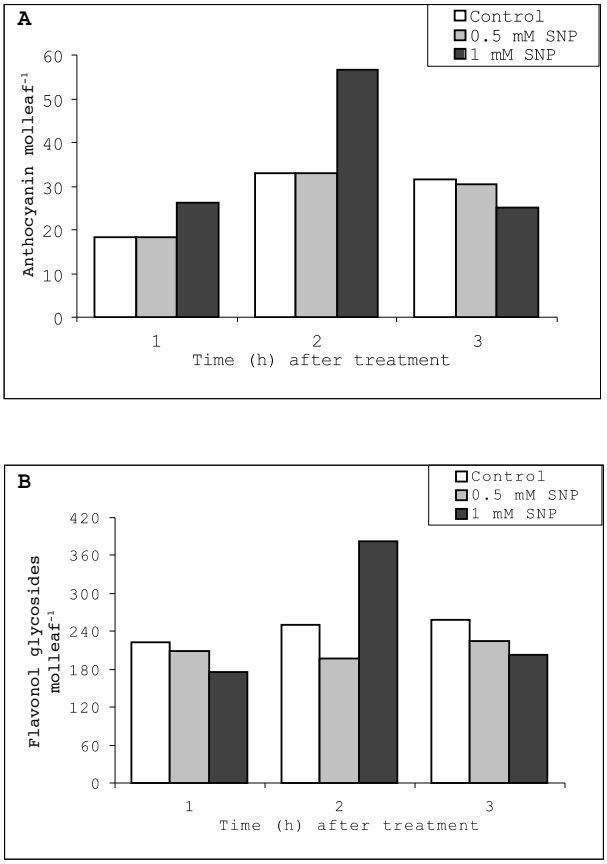


**Figure 4.** Changes in the anthocyanin [A] and flavonol glycosides [B] content of pea (*Pisum sativum* L.) cv. Arkel leaves after 1 mM SNP treatment. 15 leaf discs were kept in Petri dish containing 6 ml of distilled water as control (no SNP), or 6 ml of 1 mM sodium nitroprusside and incubated under light (600 µmol $m^{-2}$. $s^{-1}$) for 3 h. Data presented in the figure are the average of the three independent experiments.

NO in secondary metabolism. Pea leaves being of relatively simple construction, consisting of mono-cellular epidermal layers bounding a mono-cellular layer of tightly packed palisade cells and a 3 to 5 cell layer deep spongy mesophyll were chosen for the study (Hrazdina et al. 1982).

Several previous studies has proven the role of NO as signaling molecule in several physiological processes though could not expose exact mechanism of NO action. Although nitric oxide (NO) has crucial role in fundamental processes such as growth and development, its role in secondary metabolic processes is still poorly understood. In general, sodium nitroprusside (SNP) is used to investigate the effects of NO in plants (Delledonne et al. 2001; dePinto et al. 2002; Graziano et al. 2002; Correa-Aragunde et al. 2004). SNP releases NO in the form of nitrosonium cation ($NO^+$) rather than its free radical (Murgia et al. 2004) has bee described as potent inhibitors photosynthesis in different plants previously (Yamasaki, 2000; Takahashi and Yamasaki, 2002; Lum et al. 2005). Therefore, inhibition in photosynthesis observed in SNP treated pea leaf disc was expected due to release of NO from SNP. However, still the mechanism of action of NO remained poorly understood. Even if so, direct effects of NO on photosynthetic enzymes, on the photosynthetic electron transport chain and on chloroplast may result in inhibition of photosynthesis (Lum et al. 2005) or might be due to its action on photosystem-II (PSII) (Sanakis et al. 1997). Previously, Lum et al. (2005) have studied the effects of SNP on glucose metabolism in mung bean leaves. In mung bean leaves, glucose metabolism was highly affected after SNP treatment and the glucose level was almost depleted after 6 h of treatment. In the present study, no significant alteration in glucose metabolism was observed in SNP treated pea leaf discs. Hence the role of NO in glucose metabolism could not become very clear as it was reported in mung bean leaves (Lum et al. 2005).

As shown in Figure 4 (A and B), SNP treatment of pea leaf discs resulted in an overall (21%) decrease in anthocyanin and flavonol glycoside content after 3h. The depletion in amount of these two plant natural products in SNP treated leaf discs could be *via* photosynthesis which has been completely inhibited by NO released from SNP thereby stopped the energy supply for biosynthesis of anthocyanin and flavonol glycosides. However, a substantial increase in their levels at 2 h is surprising and it seems to be due to rapid metabolism of glucose providing metabolic precursor and energy for their rapid biosynthesis or may be due stimulatory effects of NO most probably via its effect on the expression phenylalanine ammonia lyase (PAL), a key enzyme in anthocyanin biosynthetic pathway.

On the other hand, anthocyanin content of pea leaf discs remarkably (73%) increased compared to a reasonable (15%) increase in flavonol glycosides following their incubation under light (600 µmol $m^{-2}$. $s^{-1}$) alone. The results suggest that in pea leaves, light would have induced the anthocyanin biosynthetic genes that resulted in higher accumulation of anthocyanins. Very recently, Takaos et al. (2006) have studied the light induced expression of *MYB* genes that regulate anthocyanin biosynthesis in red apple. Previously, Hrazdina et al. (1982) studied the distribution of biosynthetic enzymes of anthocyanins and flavonol glycosides in pea leaves. There study revealed that anthocyanins and flavonol glycosides in pea leaves are accumulated in epidermal vacuoles, while their biosynthetic enzymes are present in both epidermal and parenchyma tissues suggesting that there is close coordination between cells and tissues at the level of the precursors and end products.

In conclusion, the study revealed that SNP affects the level of anthocyanin and flavonol glycosides most probably *via* its inhibitory effects on photosynthesis in pea leaves. Also the study has revealed the role of NO in glucose metabolism in pea leaves. However, study of the effects of NO on glucose metabolism is under progress.

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