



## Elevated CO<sub>2</sub> atmosphere enhances production of defense-related flavonoids in soybean elicited by NO and a fungal elicitor

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### ABSTRACT

Increased atmospheric pollutants including carbon dioxide (CO<sub>2</sub>) and nitric oxide (NO) have a large impact on vegetation, with detrimental or beneficial influences on plant growth and metabolism. Here, we evaluated the effect of an elevated CO<sub>2</sub> atmosphere on the production of soybean defensive secondary chemicals induced by NO and a fungal elicitor. We hypothesized that an excess of carbon may alter the production of specific flavonoids that were previously shown to be induced by NO in soybean cotyledons. Pots containing soybean seeds (*Glycine max* [L.] Merr.) were submitted to 380 and 760 μmol mol<sup>-1</sup> of atmospheric CO<sub>2</sub> in open-top chambers. After nine days, plantlets grown under these conditions were assessed for biochemical and physiological parameters. Defense-related flavonoids were evaluated in detached cotyledon diffusates elicited with two different NO donors and with the β-glucan elicitor from *Phytophthora sojae*. A CO<sub>2</sub>-enriched atmosphere stimulated initial growth, photosynthetic assimilation, and an altered C/N ratio in soybean plantlets resulting in allocation of precursors into different branches of the phenylpropanoid pathway in the cotyledons. Under elevated CO<sub>2</sub>, the biotic elicitor caused accumulation of phytoalexins (glyceollins) as the natural end products of the phenylpropanoid pathway. In contrast, elevated CO<sub>2</sub> combined with NO resulted in an increase of intermediates and diverted end products (daidzein—127%, coumestrol—93%, genistein—93%, luteolin—89% and apigenin—238%) with a concomitant increase of 1.5–3.0 times in the activity of enzymes related to their biosynthetic routes. These observations point to changes in the pool of defense-related flavonoids that are related to increased carbon availability in soybeans. This may alter the responsiveness of soybean plants to pathogens when they are grown in CO<sub>2</sub> atmospheric concentrations close to those predicted for the upcoming several decades.

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### 1. Introduction

Anthropogenic activities are the major source of the increase in atmospheric concentrations of gaseous, particulate, and photochemical pollutants, which are known to contribute to the greenhouse effect. Increased atmospheric pollutants have large impacts on vegetation, with a detrimental or beneficial influence on plant growth and metabolism depending on the contaminant, its concentration, and interactions with other pollutants and environmental factors (Vu et al., 2001; Gregg et al., 2003).

Elevated CO<sub>2</sub> atmospheric concentrations such as those predicted for the next few decades alter plant growth, biomass

allocation patterns, and accumulation of carbon-based secondary compounds (CBSC) (Koricheva et al., 1998; Mattson et al., 2005). CO<sub>2</sub>-enriched atmospheres under non-limiting nutrient conditions have been shown to enhance plant growth and productivity in many species, including soybean, mainly through stimulation of photosynthesis (Ainsworth et al., 2002, 2007). Changes in chemical composition are also common to many plants grown under elevated CO<sub>2</sub> conditions (Peñuelas and Estiarte, 1998; Kim et al., 2005).

A number of source–sink hypotheses that have been proposed over the last 20 years predict a larger accumulation of total non-structural carbohydrates (TNC) and CBSC in plant tissues when the available carbon and amounts of nutrients exceed the growth requirements (e.g. Bryant et al., 1983; Peñuelas and Estiarte, 1998; Mattson et al., 2005). Experimentally, studies with crop plants and trees have indicated that allocation to CBSC in response to increased CO<sub>2</sub> show enormous inter- and intraspecific variations as well as a dependence on other environmental variables (Peñuelas and Estiarte, 1998; Hartley et al., 2000 and references therein).

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Allocation of carbon to secondary metabolites in plants is linked to primary metabolism by the rates at which substrates are diverted from primary pathways and funnelled into secondary biosynthetic routes. Therefore, several factors affecting growth, photosynthesis, and other parts of primary metabolism such as increased CO<sub>2</sub> and other air pollutants will also affect the accumulation of CBSC (Lavola et al., 2000). Such changes can significantly affect plant performance in altered atmospheres in response to herbivores and microbial pathogens (Peñuelas and Estiarte, 1998).

Studies have shown that phenylpropanoid metabolism can be stimulated by air pollutants (Booker and Miller, 1998; Kim et al., 2005). Among distinct groups of CBSC, flavonoids are highly influenced by elevated CO<sub>2</sub> conditions and have been shown to increase in some species in response to a metabolic excess of carbon (Kim et al., 2005). According to Koricheva et al. (1998), elevated responsiveness of phenylpropanoids to an increase in the carbon supply could be due to the fact that these compounds and proteins share a common precursor, phenylalanine. Therefore, changes in growth and protein synthesis are expected to affect the availability of this amino acid for the phenylpropanoid biosynthetic pathway.

Isoflavonoids constitute a group of natural products derived from the phenylpropanoid pathway, which is abundant in soybeans. These compounds have diverse biological activities and play numerous roles in the interactions that occur between the plant and the environment. The inducible accumulation of low molecular weight antimicrobial pterocarpan phytoalexins, the glyceollins, is one of the major defense mechanisms implicated in soybean resistance. Rapid and extensive accumulation of glyceollins has been observed in incompatible interactions between soybean and *Phytophthora sojae* (Abbasi et al., 2001). The expression and activity of phenylalanine ammonia-lyase (PAL), the first enzyme of the phenylpropanoid pathway, have also shown to increase in response to pathogen attack (Delledonne et al., 1998). The isoflavone daidzein is the first committed metabolite in the biosynthetic pathway that gives rise to the phytoalexin glyceollin. Genistein is also an antifungal isoflavone involved in the pathway leading to the phytoalexin response of soybean cells. Both daidzein and genistein are present as large pools of pre-formed conjugates in soybean tissues (e.g. daidizin and genistin), being released by the hydrolyzing activity of  $\beta$ -glucosidases in response to incompatible interactions with pathogens or treatment with fungal or plant elicitors (Hsieh and Graham, 2001). Apigenin and luteolin are flavones that are also found in soybean tissues (Modolo et al., 2002), but their role in plant defense against pathogen remains to be established.

In a previous study, we observed remarkable differences in phytoalexin production between soybean cultivars susceptible and resistant to stem canker disease in response to a biotic elicitor when the plants were previously exposed to a CO<sub>2</sub>-enriched atmosphere. High levels of CO<sub>2</sub> caused significant improvement of glyceollin production only in the resistant soybean cultivar (IAC-18). This increase was not observed for daidzein content after 20 h of elicitation, suggesting that the increased carbon supply specifically affected the biosynthetic steps leading to conversion of this precursor into glyceollins rather than equally stimulating the whole phenylpropanoid pathway (Braga et al., 2006).

Recently, convincing evidence has demonstrated the involvement of nitric oxide (NO) in the induction of phytoalexins in soybeans. We have demonstrated that NO is implicated in the activation of phenylpropanoid biosynthesis as part of the defense mechanism of soybean plants against attack by the fungus *Diaportha phaseolorum* f. sp. *meridionalis* (Dpm), the causal agent of stem canker disease (Modolo et al., 2002).

NO gas is a common air pollutant generated by traffic and industrial combustion and it can exert a number of diverse effects on plant metabolism, being toxic or beneficial depending on its con-

centration. Substantial amounts of NO can also be emitted by plants into the atmosphere as a result of different mechanisms of endogenous biosynthesis related to nitrate assimilation and reduction of nitrite (Salgado et al., 2006).

Whether it is produced endogenously or is present in the atmosphere, there is compelling evidence that NO exerts a strong influence on plant metabolism. As a developmental regulator, it promotes root growth and seed germination, controls stomata movement and delays flowering and senescence (Lamattina et al., 2003). As a modulator of disease resistance, NO induces localized cell death in host tissue and the expression of several defense genes, resulting in the activation of biosynthetic pathways leading to accumulation of antimicrobial compounds, such as phenylpropanoids in soybeans, preventing pathogen spread (Delledonne et al., 1998; Modolo et al., 2002).

In the present work, we studied the responsiveness of soybeans grown under an increased CO<sub>2</sub> concentration to induce defensive responses when activated by exogenous NO. We hypothesized that an excess of carbon may alter the production of specific flavonoids, which were previously shown to be induced by NO, in soybeans. A very active fungal elicitor was used for comparison. Beyond the physiological parameters, we assessed flavonoid metabolism related to the defensive response of soybeans, examining changes in the production of phytoalexins and some of their precursors and in the activity of three enzymes related to their biosynthetic pathways.

## 2. Materials and methods

### 2.1. Species

Soybean (*Glycine max* [L.] Merr.) seeds of the cultivar IAC-18, which is resistant to the fungus *D. phaseolorum* (Cooke & Ellis) Sacc. f. sp. *meridionalis* Morgan-Jones (Dpm), were provided by the Instituto Agronômico de Campinas, Campinas, São Paulo, Brazil. Seeds were sown in a bed of soil and vermiculite (1 layer of vermiculite at the bottom, followed by 1 layer of soil on top of which the seeds were placed, and 1 layer of vermiculite on top) in large pots (6 L) and were watered daily with tap water. Water tension in the soil was monitored using a tensiometer and kept above  $-10$  kPa. Leaves and/or cotyledons of nine-day-old plantlets, corresponding to the VC cotyledon stage, were used for all determinations described below.

### 2.2. Open-top chamber experiments

Seven pots containing 50 seeds each were kept in open-top chambers—OTCs (1.53 m<sup>3</sup> each) located inside the glasshouse at the Seção de Fisiologia e Bioquímica de Plantas, Instituto de Botânica, São Paulo, SP, Brazil. Details of the chamber construction and CO<sub>2</sub> injection and monitoring were as described by Braga et al. (2006). Two OTCs were maintained under  $760 \pm 11 \mu\text{mol mol}^{-1}$  of atmospheric CO<sub>2</sub> and another two were maintained under a normal CO<sub>2</sub> atmospheric concentration ( $380 \pm 9 \mu\text{mol mol}^{-1}$ ). Six independent experiments were conducted during the summer of 2005–2007 (December–February), with a natural photoperiod (13.5 h) and a photosynthetic flux density at the pot surface of  $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Air temperature in the chambers varied from 32 to 18 °C (day to night) and the relative humidity varied from 51 to 69%.

### 2.3. Gas exchange measurements

The net carbon assimilation rate ( $A$ ), stomatal conductance ( $g_s$ ) and transpiration rate ( $E$ ) under saturating photosynthetic photon

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