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Heme oxygenase-independent endogenous production of carbon monoxide by soybean plants subjected to salt stress

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ABSTRACT

The exogenous application of carbon monoxide (CO) is a valuable strategy which enables study of the effects under different stress conditions. However, in this experimental model a true endogenous CO production by plants cannot be measured. In this work, so as to achieve an elevated sensitivity and to avoid invasive techniques, we quantify the endogenous CO production by tissues in salt-treated soybean plants through gas chromatography coupled to a reduction gas detector. This technique allows short and room temperature incubation of intact tissues and homogenates. We found that a 200 mM NaCl treatment induces total CO production in leaves and roots. The sensitivity of the technique offers no correlation between this increment and heme oxygenase (HO) activity measured as a function of CO production. We also found that untreated soybean plants continue to produce significant CO levels up to 7 days post planting, after which CO content decreases to a third and remains constant in the next days. However, HO activity does not change throughout these days. The data here reported shows that HO activity is not the main source of CO in soybean plants. We discuss alternative sources that could be implicated in this production. Taking our own results and data reported by other colleagues, we propose lipid peroxidation and ureide metabolism as potential sources of CO.

1. Introduction

Carbon monoxide (CO) is a small diatomic gas with low water solubility and a stable molecular structure which does not alternate between different redox species (Hartsfield, 2002). CO binds to iron in its reduced state (Fe^{2+}), and has little biochemical reactivity with non-iron compounds. The physiological importance of CO lies in its ability to bind to heme proteins.

The production of CO by algae and other plants has been studied since 1917 (Langdon, 1917; Chapelle and Krall, 1961; Delwiche, 1970). Some of these experiments were to identify sources for CO production. In this regard, in Wilks (1959) suggested that CO generation and liberation are related to photodegradation activity which involves chlorophyll system and requires both light and oxygen. However, Fischer and Lüttge (1978) argued that light-dependent CO production is a consequence of photorespiration. These authors also pointed out the difference in production by C_3 and C_4 plants (Lüttge and Fischer, 1980). In contrast, Tarr et al. (1995) have shown that direct CO photoproduction from plant matter is likely the result of direct photochemical transformations in the plant matrix, and not the result of gas phase photochemical decomposition of other organic material emitted by the plant. What is more, the results indicated that CO appears to be produced internal to living leaf matter and is subsequently released to the atmosphere through stoma.

While the origin of CO in plants remains still controversial, recently, and taking into account its similarity with nitric oxide (NO) (Hartsfield, 2002), many groups have been studying the regulatory role of CO.

Like NO and hydrogen sulfide (H_2S), CO has been proposed as an emerging gasotransmitter which operates on the guard cells which control the stomatal closure (studied in detail by García-Mata and Lamattina, 2013). The apparent role of CO in lateral root development in tomato and cucumber has been reported by Guo et al. (2009) and Xuan et al. (2008). Other authors have suggested

Abbreviations: CO, carbon monoxide; HO, heme oxygenase; TCO, total CO production; VeCO, total CO production by intact tissues; ZnPPIX:, Zn-protoporphyrin IX.

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that CO alleviates the oxidative damage induced by cadmium (Han et al., 2008), ROS donors (Sa et al., 2007) and salt stress (Liu et al., 2007).

Heme oxygenase (HO; EC 1.14.99.3) is an ubiquitous enzyme that catalyses to the oxidative cleavage of free heme to biliverdin with the release of Fe^{2+} and CO. In higher plants, HO plays a key role in phytochrome biosynthesis and in antioxidant defence (Shekhawat and Verma, 2010). In soybean plants, HO has been localised in the chloroplast and mitochondria of the leaf by Dixit et al. (2013) and in the peribacteroid membrane of root nodules by Zilli et al. (2011).

The current understanding of CO in plants resembles the historic beginning of similar studies regarding NO origin in plants. In those first publications it was thought that NO was only produced by a nitric oxide synthase (NOS), whereas today it is known that there are different systems (enzymatic and non-enzymatic) that can generate NO in plants (Del Rio et al., 2004; Corpas et al., 2004; Gupta et al., 2011).

Because CO is a by-product of the reaction catalysed by HO, various reports assert that HO is the sole enzymatic source of CO, and so the HO/CO system is involved in these regulatory processes (Cao et al., 2007; Xuan et al., 2008). However, it has been documented that in mammal tissue and cells there is a lipid peroxidation-induced, and heme-independent, CO production (Archakov et al., 2002; Vreman et al., 1998). Motivated by the contributions of Fischer and Lüttge (1978), who pointed out the breakdown of glyoxylate as a possible source of CO, we focused on other glyoxylate-producer pathways.

Glyoxylate could also derive from ureide catabolism. Some N_2-fixing legumes move fixed N from the nodules to the aerial portions of the plant in the form of ureides, allantoin and allantoate, as well as the oxidation products of purines that have been synthesised de novo in the nodule. Ureides are also products of purine turnover in senescing tissues, such as seedling cotyledons. In soybean plants, allantoin and allantoate degrade to ureidoglycolate, which in turn converts into glyoxylate (Todd et al., 2006).

In this work, the endogenous CO production by homogenates and intact plant tissues are studied by means of gas chromatography. We analyse and discuss CO as an indicator of HO activity in control and salt-treated plants.

2. Materials and methods

2.1. Plant material and growing conditions

Surface sterilised soybean (*Glycine max* L.) and alfalfa (*Medicago sativa*) seeds were germinated directly in plastic pots (containing vermiculite) in a controlled environmental chamber. There was a photoperiod of 16 h, photon flux density of 175 μmol/m^2/s, and a day/night regime of 26.5 °C with 75% humidity. After four days, the seedlings were transferred to a hydroponic system. The Hoagland solution was replaced each two days.

Arabidopsis thaliana seeds were germinated and kept in plastic pots in a greenhouse at 21 °C until harvest.

On the other hand, and just for CO assay control, fresh spinach (*Spinacia oleracea*) leaves were used.

2.2. Treatments

Salt treatment: Seven day old soybean plants were subjected to 100 or 200 mM NaCl for 24 or 48 h.

When the effect of Zn-protoporphyrin IX (ZnPPIX, 30 μM) on the *in vivo* system was investigated, it was added to Hoagland solution for 24 and 48 h. To test the *in vitro* effect, 0–100 μM ZnPPIX was added to the mix reaction in 1.5 ml vials. All determinations were performed in triplicate in three different experiments using six plants for each treatment.

2.3. Reagents

Methemalbumin 150 μM heme/0.15 μM albumin (MHA) and 4.5 mM NADPH were both freshly prepared daily. Hemin, albumin, ZnPPIX and NADPH were sourced from the Sigma Chemical Company (St. Louis, MO, USA).

2.4. CO production

2.4.1. HO activity assay

Fresh tissues were diced with scissors and 0.1 g was homogenised for 20 s using a biohomogenizer with a 1 ml 0.1 M KH_2PO_4 buffer. CO production activity was determined in the reaction mixture as previously described by Vreman and Stevenson (2001) and Vreman et al. (2011). Into five 1.5-ml amber GC vials (Alltech, Deerfield, IL), 20 μl MHA, 20 μl of tissue preparation, and 20 μl NADPH or 20 μl buffer were added. The vials were then sealed with septum caps and placed into a light shielded water bath at 37 °C. After five minutes, each vial was purged with CO-free air and the reaction left to continue for fifteen minutes. The reactions were ended by placing the vials into a rack placed in ice, and then by adding 5 μl of 30% (w/v) sulfosalicylic acid through the vial septum into the 60 μl reaction mixture.

CO in the vial headspace was quantified using a calibrated Reduction Gas Analyser RGA-1 system, consisting of a gas chromatograph with a mole sieve 5A column equipped with a sensitive reduction gas detector (Peak Laboratories, LLC, Mountain View, CA, USA). This analyser system has a sensitivity of 1 pmol CO.

The rate of CO production, measured in terms of pmol CO/15 min/20 μl homogenate and subsequently normalised to nmol CO/h/g fresh weight (FW), was then calculated for the total reaction. The rate observed for the blank reaction was subtracted.

2.4.2. Quantification of CO production by intact tissues

When total CO was quantified in intact fresh tissues (VeCO), 8-mm-leaf disc or 15-mm-root pieces were placed in amber vials with 60 μl of 0.1 M KH_2PO_4 buffer. Then, sealed vials were purged with CO-free air and incubated at room temperature for different periods of time. CO was determined using the same reduction gas analyser system described above. The content of CO was normalised by one gram of fresh weight.

2.5. Statistics

Data in the text and tables indicate mean values while their error bars refer to one standard deviation. Differences among treatments and species were analysed by one-way ANOVA, taking $p < 0.05$ as significant according to Tukey's multiple range test.

3. Results and discussion

3.1. Total CO production by soybean leaf homogenates is augmented by salt treatment at the expense of blank increase

In vitro homogenate HO activity was determined by gas chromatography in the absence of light in leaves and roots subjected to salt stress.

In Fig. 1a we present the results for the total CO production (TCO) in leaves as a function of the treatment. The upper bars represent the net CO (total CO minus blank), which is proportional to HO activity (see Section 2.4.1). As it can be seen in the figure, for 100 mM-NaCl-treated plants there was no significant increment in TCO in respect to untreated plants after 24 h, but a 30%

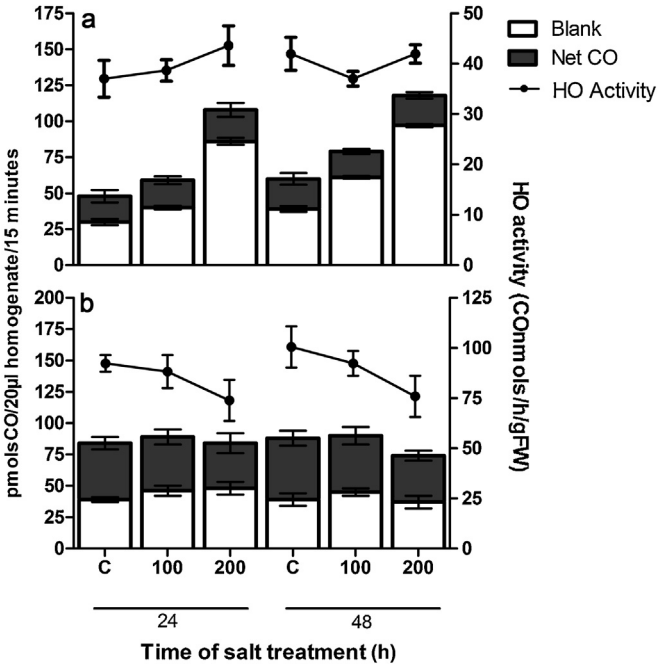

Fig. 1. CO production and HO activity in leaves (a) and roots (b) subjected to 100 and 200 mM NaCl at 24 and 48 h. The lower bars correspond to blank CO content of the *in vitro* reaction in vials. The upper bars represent net CO level which is proportional to HO activity. Values are the mean of three independent experiments. Error bars refer to one standard deviation.

increment was present after 48 h. On the other hand, for 200-mM-NaCl-treated plants there was an increment in TCO of 100% after 24 and 48 h. The sensitivity of the technique employed allows to visualize that this pattern of increments in TCO is at expenses of the increment in the blank (lower bars in Fig. 2).

In Fig. 1b we present the same previous analysis but for roots. As it can be seen in the figure, in this case neither HO activity or TCO values were affected by salt treatment. In any case, it is worth noticing that again the sensitivity of the technique allows to recognize that half of the TCO is due to the blanks.

From these results, it would be logical to put forward the following hypothesis: there is CO production from other sources besides HO and, in leaves, these sources are considerably induced by salinity.

To test this hypothesis, we carried out another experiment in presence of ZnPPIX, a strong specific HO inhibitor. *In vivo* pretreatment by adding 30 μM ZnPPIX to hydroponic nutrient solution did not modify CO levels (data not shown). Moreover, when the inhibitor was directly added to the vials, in order to test *in vitro* effect, also no difference was found within the statistical uncertainty (see Fig. 2).

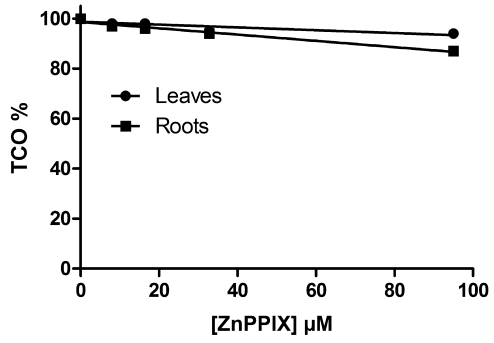

Fig. 2. Effects of ZnPPIX on relative total CO production (TCO) in leaves and roots. Values are the mean of three independent experiments. Error bars refer to one standard deviation.

We consider as a possible explanation of the produced CO the lipid peroxidation. We have previously reported that 100 and 200 mM NaCl treatments triggered the formation rate of malonyldialdehyde (MDA) (Balestrasse et al., 2008; Zilli et al., 2008), an early parameter of lipid peroxidation. This concurs with the CO production reported in this paper. Archakov et al. (2002) investigated the CO production from rabbit liver microsomes during iron-dependent lipid peroxidation. This CO production was NADPH dependent and required Fe^{2+} but inhibition of HO activity with ZnPPIX did not alter the CO production. After the microsomes were treated with antioxidants, CO production decreased, accompanied by a decline in MDA. These data demonstrated lipid peroxidation induced, but not heme-dependent, CO production.

At last, there are two observations concerning the results in this section which should be mentioned.

The first one is that in previous works, we have reported that 100 mM NaCl treatment induces HO activity and its expression in leaves and nodules after 24 h (Balestrasse et al., 2008; Zilli et al., 2008). Whereas for 200 mM NaCl treatment there is a decrease in both HO activity and its transcript in leaves (Zilli et al., 2009), nodules (Zilli et al., 2008) and roots (unpublished data). The observed differences with the present article is that in the mentioned works the HO activity was determined through the traditional technique by quantification of biliverdin (Muramoto et al., 2002). In particular, in the previous works the blanks usually account for not more than 15% of the total HO activity, whereas in the present technique of CO quantification the blanks are considerably higher and they may be interfering with the CO produced by HO activity.

The second observation is that previous reports showing pretreatments with HO inhibitors or CO scavengers enhancing the effects induced by different stress conditions, are not in contradiction with our results as we focused on CO sources.

3.2. Total CO production by soybean intact tissues augmented by salt treatment

In vitro CO production by intact tissue (VeCO) was quantified in leaf discs and root pieces.

VeCO analysis of leaf discs revealed a correlation with TCO in leaf homogenates. While VeCO value in the plants treated with the lowest salt concentration did not show differences respect to untreated plants, the highest concentration increased 3-fold VeCO in leaf discs (Figs. 3a and 4a).

Interestingly, when the roots were analysed, no correlation was found between TCO and VeCO (Figs. 1b and 3b). While TCO in roots remained in control values, VeCO was induced twofold by exposure to 200 mM NaCl (Figs. 3b and 4b). This could be due to a significant loss of CO by some root matter component during the homogenisation process.

In addition, VeCO kept linearity in all the incubation times assayed for treated and control plants in both tissues. As can be seen in Fig. 4, to shorten experimental time, VeCO could be measured at 25 min instead of 90 min because the statistical difference in CO levels can be detected within this short time.

3.3. CO production as a function of age for untreated plants

We have found that the *in vitro* reaction of HO activity in spinach leaves, when used as a control in tuning up of this technique, blanks were around 10% of TCO, while soybean blanks accounted for a 60–80% of TCO. This is another indication of existence of more relevant alternative sources than HO activity. In order to explore in detail this conclusion, a serial analysis of HO activity as a function of the plant age was carried out (Fig. 5).

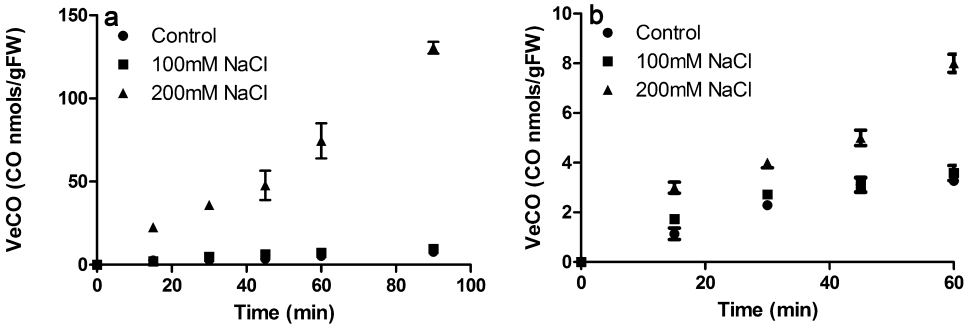

Fig. 3. Time-course salt-induced CO release by intact leaf discs (a) and root pieces (b). Values are the mean of three independent experiments. Error bars refer to one standard deviation.

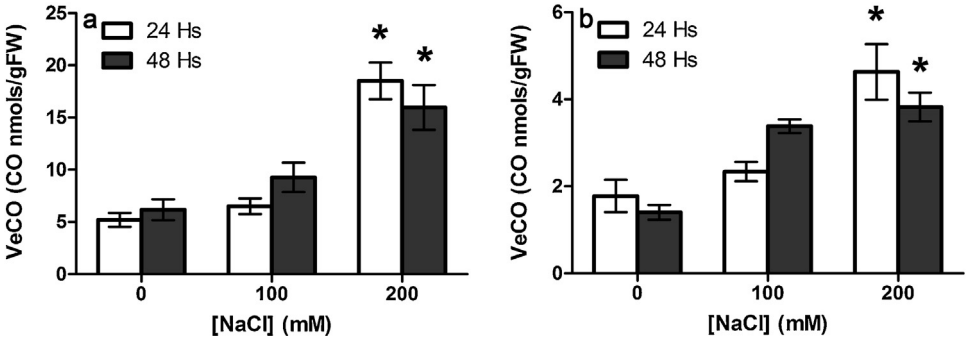

Fig. 4. VeCO at 25 min in leaves (a) and roots (b) subjected to 100 and 200 mM NaCl at 24 and 48 h. Values are the mean of three independent experiments. Error bars refer to one standard deviation. $^{*}p < 0.01$ compared with control according to impaired *t* test.

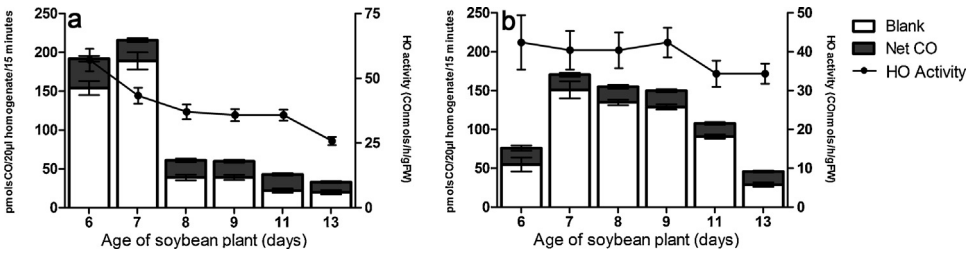

Fig. 5. CO production and HO activity in leaves (a) and cotyledons (b) over the study period. Values are the mean of three independent experiments. Error bars refer to one standard deviation.

A significantly HO-independent CO production was determined in six and seven day old untreated plants, and the blank values were 72 and 88% of TCO, respectively. After seven days, CO release drastically fell and remained constant after two weeks. More sporadic determinations were done at different times, and the values were roughly constant. This behaviour is clearly not light-dependent because all experiments were carried out in dark vials.

Interestingly, we found that nine day old etiolated plants produce more CO than those grown in normal conditions. Fig. 6 shows that TCO in etiolated leaves was increased 4.25-fold respect to control plants. This result resembles those obtained by She and Song (2008), who have shown that darkness-induced stomatal closure is mediated by CO in *Vicia Faba*. In addition to CO, NO and H_2S have been proposed as new guard cell regulators, contributing to tolerance mechanisms to drought stress. These issues have been reviewed by García-Mata and Lamattina (2013). H_2O_2 and ABA seem to be implicated in this process (Cao et al., 2007). Besides being a potent HO inductor, H_2O_2 triggers lipid peroxidation. Endogenous production of CO from lipid peroxidation is prevalent in many types of mammal cells, including brain, kidney, lung, spleen and the bloodstream, and these inductions are also heme-independent (Vreman et al., 1998).

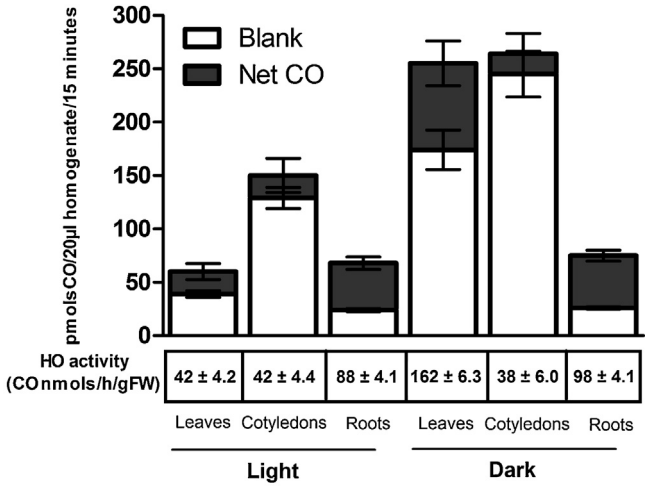

Fig. 6. CO production and HO activity in leaves, cotyledons and roots of plants grown at different light conditions. The lower bars correspond to blank CO content of the *in vitro* reaction in vials. The upper bars represent net CO level which is proportional to HO activity. Values are the mean of three independent experiments. Error bars refer to one standard deviation.

Table 1
CO production and HO activity in different plant species.

Species	Blank	Total CO	Net CO	HO activity
	(CO pmol 20 μl^{-1} 15 min^{-1})			(CO nmol h^{-1} g^{-1} FW)
S. Oleracea[a]	15 ± 3	109 ± 12	93 ± 11	186 ± 22
A. Thaliana[a]	25 ± 2	77 ± 6	52 ± 6	104 ± 12
M. Sativa (7 days)	53 ± 6	120 ± 10	67 ± 11	134 ± 22
M. Sativa (8 days)	36 ± 3	83 ± 5	47 ± 6	93 ± 12
M. Sativa (10 days)	29 ± 2	73 ± 4	44 ± 4	88 ± 8

[a] Did not show significative differences at different times. Data are mean values of three independent experiments ± SD.

Furthermore, HO activity was measured in cotyledons. As well as in leaves, blank cotyledons were the main contribution to TCO. These values were between 88% and 63% of TCO at days seven and thirteen, respectively (Fig. 5). Moreover, TCO in etiolated cotyledons was 176% higher than those growing in normal conditions. It is interesting to note that any difference in TCO and HO activity in dark-growing plants is also due to the existence of increased blanks.

Fischer and Lüttge (1978) proposed glyoxylate as a source of CO because its oxidation and decarboxylation leads to formic acid, which degrades to CO. However, glyoxylate also could derive from ureide catabolism. This pathway is active in both fixing and non-fixing N_2 legumes and is related to purine turnover in senescing tissues. Stebbins and Polacco (1995) reported that purine degradation leads to ureide accumulation in senescing cotyledons of soybeans.

To our knowledge there are no published articles supporting a correlation between CO and ureide catabolism, but we consider that as a glyoxylate producer pathway this could be a potential CO source, and should be investigated.

3.4. Endogenous CO levels vary in different plant species

In view of high endogenous levels of TCO in control soybean plants, we decided to extend the CO quantification to other species. To this aim, we choose *A. thaliana* and *M. sativa*, two model plant species employed to study tolerance and adaptive response to stress. Table 1 summarises all the data. As it can be seen, *Arabidopsis* plants did not change their CO content as it grew. In contrast, alfalfa plants had a similar behaviour to soybean plants: that is, at an early stage of leaf development, blank content was 53 pmols, but decreased to 36 and 29 CO pmols at eight and ten days, respectively. Although the absolute values are lower than soybean plants, its relative variation within the timeframe is similar. The rate of CO release by soybean leaves was 156 pmol/min/g FW, 2.5 and 8-fold higher than alfalfa and *Arabidopsis*, respectively (Fig. 7).

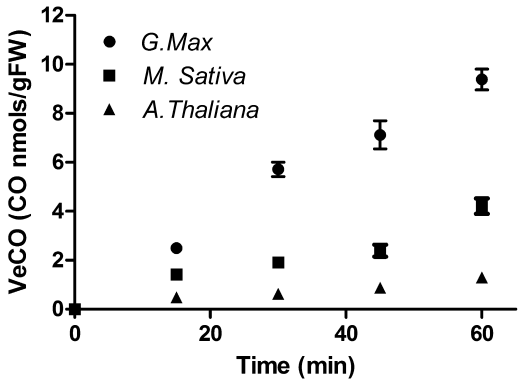

Fig. 7. Time-course VeCO in different plant species. Values are the mean of three independent experiments. Error bars refer to one standard deviation.

In his paper, Wilks measured CO content in several species. Among these species, alfalfa was the largest producer of CO (2.1 mg/100 g FW), while the rest produced less than 0.7 mg/100 g FW). Soybean data were not available in Wilks's report (1959).

In an attempt to explain the differences observed between these species, we found results of other papers that support a potential CO-ureide correlation. Allantoate amidohydrolases (AAHs) hydrolise the ureide allantoate to ureidoglycolate, CO_2, and two molecules of ammonium. AAHs from *Arabidopsis* (AtAAH) and from soybean (GmAAH) have been identified, characterised and localised by Werner et al. (2008). The leaf allantoate content was several times lower in *Arabidopsis* than in soybeans. In *Arabidopsis*, values between 34 and 400 mmol/kg FW was measured by Werner et al. (2008), and Todd and Polacco (2006), respectively. For soybean, leaf ureide content has been reported between 500 and 1.500 mmol/kg FW (King and Purcell, 2005; Ladrera et al., 2007). According to Werner et al. (2008), the lower Km of AtAAH compared to GmAAH may reflect the lower abundance of allantoate in *Arabidopsis*.

4. Conclusions

While it is true that the effects of CO are independent of origin, a large difference can be seen because of its availability under different conditions, its tissue distribution patterns, its enzymatic kinetics and affinities.

We have investigated the endogenous production of CO by salt-stressed plants rather than its exogenous application. We have found a significant variation on endogenous TCO between days in seedlings that must to be considered before investigate the regulatory role of CO.

According to the results presented in this work, we conclude that HO activity is not the main source of CO in soybean plants.

A more detailed and broad future investigation should be directed towards identifying the alternative significant sources of CO in soybean and also other legumes.

From the results in this work, we consider that quantification of CO production is not the best parameter to measure HO activity in plants with high basal level of CO.

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