Responses of Soybean to Oxygen Deficiency and Elevated Root-zone Carbon Dioxide Concentration

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INTRODUCTION

Field flooding causes premature senescence in soybean [Glycine max (L.) Merr.], which results in leaf chlorosis, necrosis, defoliation, cessation of growth and reduced seed yield (Stanley et al., 1980; Oosterhuis et al., 1990; VanToai et al., 1994; Linkemter et al., 1998). On-farm research indicated that flooding for as little as 3 d at the early vegetative growth stages can kill soybean (Sullivan et al., 2001). Damage and death of flooded plants have been attributed to the lack of oxygen to support root respiration (Huck, 1970; Jackson and Kowalewska 1983; Kozlowski, 1984; Setter and Belford, 1990; Crawford, 1992). Although soybean is injured in flooded fields, it can thrive in stagnant, oxygen-deficient water in the glasshouse (Boru et al., 1997). Therefore, soybean is much more tolerant to excess water and the lack of oxygen than previously expected (Grable, 1966; Sallam and Scott, 1987; Russell et al., 1990). Reasons underlying the dramatic differences between the responses to flooding in the glasshouse and flooding in the field are not known. In addition to the lack of O2, the concentration of CO2 in flooded soils may reach up to 50 % (v/v) of the total dissolved gases, and could be toxic to plants (Ponnamperuma, 1972). The actual concentration of soil CO2 depends on soil water content, soil type, amount of respirable substrate and activities of soil microorganisms (Duenas et al., 1995; Bouma et al., 1997b).

Conflicting results of the effects of elevated root-zone CO2 on plant and root growth have been reported in the literature. Stolwijk and Thimann (1957) showed that a 2 % (v/v) concentration of CO2 inhibited growth of pea (Pisum sativum L.) roots by 80 %, whereas 6-5 % (v/v) CO2 did not affect root growth of oat (Avena sativa L.) or barley (Hordeum vulgare L.). In 1962, Glinka and Reinhold reported that soil CO2 inhibited the uptake of water in sunflower (Helianthus annuus L. ‘Jupiter’) roots. Nodule biomass and activities in Leucaena leucocephala were reduced significantly by flooding or fumigation with different concentrations of CO2 (Zhang et al., 1995). Contrary to these observations, Bouma et al., (1997a) reported that 2 % CO2 had no effect on shoot and root growth of field beans (Phaseolus vulgaris L.) and citrus (Citrus volkameriana Tan. & Pasq). Geisler (1967) also reported that 10 % CO2 stimulated root development of pea. These contradictory results could be due to the differences in plant species, levels of CO2 or treatment duration.

The objectives of this study were to determine the responses of soybean in hydroponic culture to elevated root-zone CO2 and anaerobiosis similar to that found in flooded fields, and to compare the responses to those of rice (Oryza sativa L.), a flooding-tolerant species.

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Key words: Anoxia, Glycine max (L.) Merr., hydroponics, hypoxia, Oryza sativa (L.), rice.

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Hydroponic solution bubbled with 100% CO2. No air or gas respectively. Plants used for nutrient analysis were grown in conditions for 4 d before gas treatments were initiated. Gas treatment was imposed for 14 d by bubbling purified air (aeration), N2 gas (anaerobiosis), or 15, 30 or 50% (v/v) CO2 balanced with N2 gas through the hydroponic solution. Flow rates for air, N2 and CO2 were 250, 280 and 300 ml min−1, respectively. Plants used for nutrient analysis were grown in hydroponic solution bubbled with 100% CO2. No air or gas was bubbled through the solution in which plants in the non-aerated treatment were grown. Different CO2 concentrations were generated by regulating the CO2 flow rate through capillary tubes of different diameter and length. CO2 concentrations were monitored and adjusted every other day by sampling at the inlet port into the hydroponic solution and analysing by gas chromatography (model 436 equipped with a 50 × 0.6 cm Porapak T column and a thermal conductivity detector; Packard Company, Downers Grove, IL, USA).

### Materials and Methods

**Plant material and growth conditions**

Seeds of soybean genotype ‘Williams’ and rice breeding line ‘271’ were germinated in moist sand at 25 ± 2°C and 14 h light at a photosynthetically active radiation of 550 μmol photon m−2 s−1 for 10 d. Healthy, uniform seedlings were transferred to six 35 l plastic containers filled with nutrient solution as described by Imsande and Ralston (1981). The hydroponic containers were covered with 2-cm-thick styrofoam boards with 12 equally spaced holes through which six soybean and six rice seedlings were placed, one seedling per hole. Seedlings were fixed in an upright position using plugs of soft polyurethane and sealed with silicone grease. Styrofoam boards were sealed to the container using packing tape and made of silicone rubber tubing (Jacinthe and Dick, 1996) was placed in each hydroponic solution. Gas samples (1 ml) were withdrawn from the device every other day to analyse for CO2 concentration using gas chromatography as described above.

**Gas treatments**

Gas treatment was imposed for 14 d by bubbling purified air (aeration), N2 gas (anaerobiosis), or 15, 30 or 50% (v/v) CO2 balanced with N2 gas through the hydroponic solution. Flow rates for air, N2 and CO2 were 250, 280 and 300 ml min−1, respectively. Plants used for nutrient analysis were grown in hydroponic solution bubbled with 100% CO2. No air or gas was bubbled through the solution in which plants in the non-aerated treatment were grown. Different CO2 concentrations were generated by regulating the CO2 flow rate through capillary tubes of different diameter and length. CO2 concentrations were monitored and adjusted every other day by sampling at the inlet port into the hydroponic solution and analysing by gas chromatography (model 436 equipped with a 50 × 0.6 cm Porapak T column and a thermal conductivity detector; Packard Company, Downers Grove, IL, USA).

### Results

**Dissolved oxygen**

The equilibrium concentration of dissolved O2 was 8.0 mg l−1 in the aerated treatment and...
1·5 mg l⁻¹ in the non-aerated treatment. Therefore, plants in the non-aerated treatment experienced an oxygen-deficient environment around their roots. Since dissolved oxygen was not detectable in the N₂- and CO₂-bubbling treatments, these treatments were essentially anaerobic.

**pH.** Hydroponic solutions were buffered with 1 mM MES and had an initial pH of 6·5. The pH gradually decreased to 5·7 by the end of the experiment regardless of the treatment (CO₂, aeration or stagnation).

**Dissolved CO₂.** CO₂ equilibrium concentrations of the aerated and non-aerated solutions were 0·04 and 4 %, respectively. CO₂ concentrations in the hydroponic solution collected by the gas-sampling devices were consistent, with the supplied CO₂ concentrations of 15, 30 and 50 % in the gas mixtures being passed through the hydroponic solution.

**Effects of elevated root-zone CO₂ on soybean plants**

*Plant survival.* Survival of soybean plants was not affected by the anaerobic N₂ gas treatment or by oxygen deficiency (1·5 mg l⁻¹) in the non-aerated treatment (Table 1). The 4 % CO₂ of the non-aerated treatment and the 15 % and 30 % CO₂ in N₂ treatments also had no detrimental effects on soybean survival. In the 50 % CO₂ in N₂ treatment, 25 % of soybean plants died.

*Plant height, leaf area and number of branches.* At the end of the 2-week experiment, the average height of plants in the aerated treatment (39·7 cm) was not significantly different from that of plants in the non-aerated treatments (41·3 cm) (Table 1). While the average height of plants in the N₂ treatment was reduced to 31·2 cm, that of plants in the 15, 30 and 50 % CO₂ in N₂ treatments was only 24·1, 16·7 and 10·2 cm, respectively. Similarly, the leaf area of plants in the aerated and non-aerated treatments was also the largest (124 and 122 cm², respectively), followed by that of plants in the N₂ treatment (99 cm²). As CO₂ concentrations in the anaerobic root-zone increased from 15 to 30 and 50 %, leaf area was reduced to 46, 35 and 18 cm², respectively.

Plants in the aerated treatment had the most branches (3-4 branches per plant). Plants in the non-aerated, N₂ gas and 15 % CO₂ in N₂ treatments had a similar number of branches (2·0-2·3 branches per plant). The number of branches was reduced to 1·2 per plant in plants in the 30 % CO₂ in N₂ treatment, while no branches formed on plants given 50 % CO₂ in N₂.

*Root length, plant biomass and R : S ratio.* Roots were longest (71·5 cm) in the aerated treatment and were reduced to 30·0 cm in plants in the non-aerated treatment and 28·4 cm in those treated with N₂ (Table 1; Fig. 1). Root growth was even more severely inhibited in plants treated with elevated CO₂. As compared with the N₂ gas treatment, root length of plants treated with 15, 30, and 50 % CO₂ in N₂ was reduced by 46, 73 and 86 %, respectively. The majority of roots in the non-aerated, N₂ and elevated CO₂ in N₂ treatments are newly formed adventitious roots that grew from the base of the stem (Figs 1 and 2).

Significant differences (P < 0·05) in root and shoot biomass were observed among the treatments (Table 1). Plants in the aerated treatment had the heaviest shoot (3·9 g) and root biomass (1·1 g), followed by plants in the non-aerated treatment (3·5 g shoot, 0·9 g root biomass). As the concentration of anaerobic root-zone CO₂ increased from 15 to 30 and 50 %, shoot and root biomasses were reduced substantially. Plants treated with 15 % CO₂ in N₂ produced 1·9 g shoot tissue and 0·4 g root tissue, while those treated with 30 % CO₂ in N₂ produced 1·1 g shoot tissue and 0·2 g root tissue. Plants subjected to 50 % CO₂ in N₂ were the smallest, with a shoot biomass of 1·0 g and a root biomass of 0·1 g.

The R : S ratio of 0·28 in the aerated treatment was reduced to 0·26 in plants in the non-aerated and N₂ gas treatments. Elevated root-zone CO₂ depressed root growth much more severely than shoot growth, resulting in further reductions of R : S ratio to 0·21, 0·18 and 0·10 in plants treated with 15, 30 and 50 % CO₂ in N₂, respectively.

*Leaf greenness.* The time course of the changes in leaf greenness during the 2-week experiment, as determined by SPAD readings, indicated that leaf chlorophyll content declined gradually in plants in the aerated as well as in the non-aerated and N₂ gas treatments (Table 2). While soybean plants in the non-aerated and the N₂ gas treatments had no symptoms of chlorosis during the entire experimental period, leaves of plants treated with elevated CO₂ were yellower after only 2 d. By 12 d, leaves of plants treated with 30 % CO₂ in N₂ were more yellow than those of plants treated with 15 % CO₂ in N₂. Leaves of plants treated with 50 % CO₂ in N₂ were the most chlorotic after 6 d.
However, plants surviving this treatment were able to produce adventitious roots that grew into the soft polyurethane plugs. As a result, the leaves re-greened by day 10 (15-1 SPAD units). Nevertheless, at the end of the experiment, leaves of plants treated with 50 % CO₂ in N₂ still contained the least chlorophyll (19-3 SPAD units) compared with those of the other treatments.

**Nutrient uptake.** The concentration of minerals (mg g⁻¹ d. wt) in shoots and roots was analysed to evaluate the effect of gas treatments on nutrient uptake. Plants treated with CO₂ contained higher levels of P, Ca, Fe, Mg, Mn, Na and Zn than plants in other treatments. Levels of K, Cu and B did not differ among plants of different treatments (Table 3).

### Table 2. Time course of the changes in leaf greenness of soybean plants in response to anaerobiosis, oxygen deficiency and elevated root-zone CO₂ treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SPAD unit</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeration with air</td>
<td>38-2 ± 4-0*</td>
<td>37-3 ± 4-4</td>
<td>36-0 ± 4-2</td>
<td>36-9 ± 4-6</td>
<td>35-8 ± 4-9</td>
<td>33-4 ± 4-9</td>
<td>32-7 ± 5-3</td>
<td></td>
</tr>
<tr>
<td>Non-aerated</td>
<td>38-9 ± 3-8</td>
<td>36-0 ± 4-0</td>
<td>35-1 ± 3-9</td>
<td>35-6 ± 4-1</td>
<td>35-6 ± 4-8</td>
<td>33-7 ± 5-1</td>
<td>31-8 ± 4-8</td>
<td></td>
</tr>
<tr>
<td>100 % N₂ gas</td>
<td>34-1 ± 3-6</td>
<td>33-1 ± 3-8</td>
<td>35-7 ± 4-0</td>
<td>36-8 ± 4-5</td>
<td>36-0 ± 5-2</td>
<td>32-7 ± 4-6</td>
<td>30-5 ± 4-0</td>
<td></td>
</tr>
<tr>
<td>15 % CO₂ gas + 85 % N₂ gas</td>
<td>32-3 ± 3-5</td>
<td>30-2 ± 3-0</td>
<td>29-5 ± 3-5</td>
<td>28-0 ± 3-7</td>
<td>26-2 ± 4-1</td>
<td>26-5 ± 4-5</td>
<td>27-2 ± 3-6</td>
<td></td>
</tr>
<tr>
<td>30 % CO₂ gas + 70 % N₂ gas</td>
<td>30-8 ± 3-2</td>
<td>28-8 ± 3-2</td>
<td>27-3 ± 3-1</td>
<td>25-7 ± 3-0</td>
<td>25-5 ± 3-7</td>
<td>24-0 ± 4-2</td>
<td>23-4 ± 3-3</td>
<td></td>
</tr>
<tr>
<td>50 % CO₂ gas + 50 % N₂ gas</td>
<td>28-6 ± 3-0</td>
<td>25-0 ± 3-2</td>
<td>12-2 ± 2-2</td>
<td>13-3 ± 2-9</td>
<td>15-1 ± 3-0</td>
<td>18-2 ± 3-5</td>
<td>19-3 ± 3-1</td>
<td></td>
</tr>
</tbody>
</table>

Three measurements were taken on each of the three uppermost fully expanded leaves and averaged to give individual plant readings. Mean values of each experiment were calculated from six plants. Values presented are means ± s.d. of two experiments on a total of 12 plants.

* Three measurements were taken on each of the three uppermost fully expanded leaves and averaged to give the plant reading.

### Table 3. Nutrient content in soybean root and shoot tissues after treatments lasting 14 d

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nutrient content (µg g⁻¹ dry mass)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
</tr>
<tr>
<td>Aeration with air</td>
<td>1062 ± 150</td>
</tr>
<tr>
<td>Non-aerated</td>
<td>1981 ± 310</td>
</tr>
<tr>
<td>100 % N₂ gas</td>
<td>2289 ± 280</td>
</tr>
<tr>
<td>100 % CO₂ gas</td>
<td>4287 ± 450</td>
</tr>
</tbody>
</table>

All values are expressed as means ± s.d. of two experiments, each with combined samples of six plants.

(12-2 SPAD units). However, plants surviving this treatment were able to produce adventitious roots that grew into the soft polyurethane plugs. As a result, the leaves re-greened by day 10 (15-1 SPAD units). Nevertheless, at the end of the experiment, leaves of plants treated with 50 % CO₂ in N₂ still contained the least chlorophyll (19-3 SPAD units) compared with those of the other treatments.

**Effects of elevated root-zone CO₂ on rice plants.**

Contrary to the results obtained for soybean plants, rice plants in the non-aerated, 100 % N₂ gas, and 15 and 30 % CO₂ in N₂ treatments grew as well as aerated plants, as determined by leaf greenness, plant height, number of tillers, shoot biomass and R : S ratio (Table 4). Differences (P < 0.05) in root length, however, were detected among the treatments. Root length was greatest in the aerated treatment (17-8 cm), followed by the non-aerated (15-9 cm) and N₂ gas (15-8 cm) treatments. Treatment with 15 and 30 % CO₂ in N₂ reduced root length by 13-3 % as compared with N₂ gas alone, while root length of plants exposed to 50 % CO₂ in N₂ was reduced by 25 %. Since the elevated CO₂ treatments did not lead to a reduction in root mass, plants of these
treatments actually produced a larger number of adventitious roots that were shorter and closer to the base of the stem than roots of the N₂ treatment (Fig. 3).

**DISCUSSION**

Soybean, one of the world’s major crops, is considered to be sensitive to flooding stress (Stanley et al., 1980; Oosterhuis et al., 1990; VanToai et al., 1994; Linkemer et al., 1998). Field flooding of soybean for as little as 3 d often results in leaf chlorosis, defoliation, cessation of growth and plant death (Sullivan et al., 2001). A lack of oxygen has been proposed to be the main problem associated with flooding (Armstrong, 1979; Jackson and Drew, 1984; Kozlowski, 1984). However, in the present hydroponic study, treatments involving 2 weeks without oxygen (100 % N₂ gas) or 80 % oxygen reduction (non-aerated) had no effect on soybean survival (Table 1). Plants adapted to the prolonged low oxygen and no oxygen conditions by producing adventitious roots, undergoing stem hypertrophy and developing aerenchyma for transportation of oxygen to the roots (Fig. 1A). Similar results were reported in soybean flooded for 21 d (Bacanamwo and Purcell, 1999). These adaptive mechanisms allow the plants to produce functional nodules (Fig. 1A) and probably explain the absence of leaf chlorosis (Table 2).

In addition to oxygen deficiency, the concentration of CO₂ in flooded soils can reach up to 50 % (v/v) of the total dissolved gases and could be toxic to plants (Ponnamparum, 1972). Actual CO₂ concentrations depend upon many factors including soil water content, soil type, organic matter content and microbial activity (Duenas et al., 1995; Bouma et al., 1997b). To determine soil CO₂ concentrations in flooded soybean fields in Ohio, USA, silicone rubber gas sampling devices were placed 25 cm deep in the field. Gas samples were taken every other day during flooding and these were analysed by gas chromatography. The soil CO₂ concentration of non-flooded soybean fields was about 1 % (v/v); this increased to 30–35 % (v/v) after 2 weeks of flooding.

In the hydroponic study, soybean plants did not die after 2 weeks exposure to 30 % CO₂ in N₂, a concentration similar to that of flooded fields. However, the plants were chlorotic, stunted, and produced fewer shoots and roots than plants in the no oxygen (nitrogen gas) or low oxygen (non-aerated) treatments (Tables 1 and 2; Fig. 2D). When anaerobic root-zone CO₂ was increased to 50 %, a quarter of the plants died. Those plants that survived showed severe symptoms of chlorosis, necrosis and root death. In contrast, 50 % root-zone CO₂ did not affect survival of rice plants (Table 4), nor did the plants become chlorotic.

The cellular toxicity of CO₂ is well documented (Fox, 1932). Sea water saturated with CO₂ halts protoplasmic streaming and increases its viscosity in *Nitella clavata* (Fox, 1933). Kramer and Jackson (1954) reported that field soil saturated with CO₂ inhibited water uptake by reducing the permeability of tobacco cell membranes. Treatments consisting of bubbling with 100 % CO₂ for 10 min h⁻¹ for 36 h

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**TABLE 4.** Responses of rice plants to 14 d of anaerobiosis, oxygen deficiency and elevated root-zone CO₂ treatments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Leaf greenness (SPAD unit)</th>
<th>Plant height (cm)</th>
<th>Number of tillers per plant</th>
<th>Root length* (cm)</th>
<th>Dry weight (g per plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aeration with air</td>
<td>48.5 ± 3.3</td>
<td>39.5 ± 4.2</td>
<td>5.2 ± 0.5</td>
<td>17.8 ± 2.8</td>
<td>0.45 ± 0.18</td>
</tr>
<tr>
<td>Non-aerated</td>
<td>48.1 ± 5.7</td>
<td>37.9 ± 3.8</td>
<td>5.0 ± 0.4</td>
<td>15.9 ± 2.2</td>
<td>0.49 ± 0.15</td>
</tr>
<tr>
<td>100 % N₂ gas</td>
<td>46.5 ± 5.2</td>
<td>38.2 ± 4.0</td>
<td>5.0 ± 0.4</td>
<td>15.8 ± 2.4</td>
<td>0.48 ± 0.18</td>
</tr>
<tr>
<td>15 % CO₂ gas + 85 % N₂ gas</td>
<td>48.8 ± 5.0</td>
<td>38.4 ± 4.3</td>
<td>5.0 ± 0.5</td>
<td>13.7 ± 1.7</td>
<td>0.50 ± 0.21</td>
</tr>
<tr>
<td>30 % CO₂ gas + 70 % N₂ gas</td>
<td>47.1 ± 4.7</td>
<td>40.5 ± 5.1</td>
<td>4.9 ± 0.5</td>
<td>13.8 ± 1.5</td>
<td>0.49 ± 0.20</td>
</tr>
<tr>
<td>50 % CO₂ gas + 50 % N₂ gas</td>
<td>47.6 ± 4.9</td>
<td>37.2 ± 3.2</td>
<td>4.8 ± 0.5</td>
<td>11.8 ± 1.3</td>
<td>0.47 ± 0.17</td>
</tr>
</tbody>
</table>

All values are expressed as means ± s.d. of two experiments on a total of 12 plants.

* Determined as the length of the longest root from the base of the shoot.
reduced water uptake of wheat (*Triticum aestivum*), maize (*Zea mays*) and rice roots by 14 to 50 % (Chang and Loomis, 1945). In this hydroponic study, soybean plants that died in the 50 % CO₂ in N₂ treatment showed chlorosis, necrosis and wilting prior to death. No wilting symptoms were observed in rice plants. In addition to the reduction of water absorption and transport, CO₂ treatments reduced absorption of K, N, P, Ca and Mg by roots of wheat, maize and rice and also led to Fe and P deficiency in sorghum plants (Chang and Loomis, 1945; Matocha and Mostaghim, 1988). In the present hydroponic study, soybean plants exposed to elevated CO₂ treatments contained similar levels of K, Mn, Cu and B to those in the aerated treatment, while concentrations of P, Ca, Fe, Mg, Na and Zn were 50 to 300 % higher (Table 3). Nutrient deficiency (except for N, which was not measured) was unlikely to be the cause of injuries observed in soybean exposed to elevated root-zone CO₂.

Concentrations of root-zone CO₂ >2 % were shown to inhibit ethylene binding and action in sunflower and potato plants (Govindarajan and Poovaiyah, 1982; Finlayson and Reid, 1996; Scott and David, 1996). Ethylene is known to be necessary for the formation of adventitious roots and aerenchyma in the acclimation responses to flooding (Jackson et al., 1985; Justin and Armstrong, 1991). Soybean plants treated with elevated CO₂ produced fewer adventitious roots than did plants in the N₂ bubbling and non-aerated treatments (Fig. 2). Thus, some of the injurious effects of high root-zone CO₂ in soybean might be caused indirectly by the lack of ethylene action. In rice, treatment with 30 % CO₂ in N₂ had no adverse effect on the number of adventitious roots (Table 3; Fig. 3). In many plant species, CO₂ absorbed by the root is transported to the shoot through the transpiration stream at a very low concentration (Enoch and Olesen, 1993). A second transport route is present in rice plants: in this species, CO₂ can be transported in the gaseous phase via an extensive network of lysigenous gas spaces at concentrations three- to four-fold higher than in other species (Higuchi et al., 1984).

Due to the buffering capacity of 1 mM MES in the hydroponic solution, the elevated CO₂ treatments in this study did not acidify the hydroponic solution more than did the aerated, non-aerated and N₂ bubbling treatments. However, CO₂ is known to penetrate the cytoplasmic membrane readily, resulting in toxic cellular acidification (Jacobs, 1920). While the extent to which the internal pH of rice and soybean root cells was changed by CO₂ is not known, it is conceivable that the tolerance of rice to elevated root-zone CO₂ is attributable to its ability to regulate cellular acidosis.

**CONCLUSIONS**

Soybean plants are very tolerant of excess water and anaerobiosis, but are injured under anaerobic conditions when root-zone CO₂ concentrations increase to those found in flooded soybean fields (30 %). Rice, a flooding-tolerant species, is much more tolerant of elevated root-zone CO₂ levels than is soybean. Results suggest that CO₂ toxicity is a factor affecting soybean tolerance to flooded soils.

**ACKNOWLEDGEMENTS**

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