PHOTOSYNTHETICA 39 (4): 515-520, 2001

Photosynthetic response of barley plants to soil flooding

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Abstract

72 to 120 h of soil flooding of barley plants (*Hordeum vulgare* L. cv. Alfa) led to a noticeable decrease in the rates of CO_2 assimilation and transpiration, and in chlorophyll and dry mass contents. Stomatal conductance decreased following flooding without appreciable changes in the values of intercellular CO_2 concentrations. A drop in the activity of ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) and of the photorespiratory enzymes phosphoglycollate phosphatase (EC 3.1.3.18) and glycollate oxidase (EC 1.1.3.1) was observed, while the activity of phosphoenolpyruvate carboxylase (EC 4.1.1.31) increased in all flooded plants. Flooding of barley plants caused an increase in proline content and in leaf acidity.

Additional key words: carboxylases; *Hordeum vulgare*; leaf acidity; net photosynthetic rate; photorespiratory enzymes; proline; stress.

Introduction

Flooding leads to depletion of soil oxygen and anaerobic conditions. Most plant roots or shoots tolerate anoxia for only short periods of time before irreversible damage occurs but some species have developed strategies to avoid (Ricard *et al.* 1994, Crawford and Braendle 1996) or withstand (Anderson and Pezeshki 1999, Summers *et al.* 2000) anaerobiosis. In most cases oxygen shortage affects directly the roots and indirectly the shoots. When tissues are hypoxic or anoxic, the oxygen-dependent pathways, especially the energy-generating system, are suppressed, the functional relationships between roots and shoots are disturbed, and both carbon assimilation and photosynthate utilisation are suppressed (for review, see Vartapetian and Jackson 1997). Oxygen deprivation interferes with respiration at the level of electron transport. In the absence of oxygen the TCA cycle does not operate and ATP is synthesised only by alcoholic fermentation (Perata and Alpi 1993). The absence of an energy source in roots disturbs physiological processes in shoots. Effects include an inhibition of growth, decrease in the rate of photosynthesis, and altered distribution of photosynthates (Kozlowski and Pallardi 1984, Moog and Brüggemann 1993).

Stomata close in response to waterlogging both in the presence of water deficits (Coutts 1981) and in their absence (Jackson and Hall 1987). In tomato plants, stomata begin to close within 4 h of the start of soil flooding with a parallel decrease in transpiration (Else *et al.* 1995) and photosynthesis (Bradford 1983).

While much of the reported reduction of CO_2 assimilation can be attributed to stomata closure, part of it has been attributed to direct effect of flooding on the biochemical reactions of photosynthesis, *e.g.*, reduced activity of some Calvin cycle enzymes (Salcheva and Popova 1982), inhibition of photosynthetic electron transport and photosystem 2 (PS2) activity (Ladygin 1999, Titarenko 2000). However, details on the mechanisms by which oxygen deficiency affects barley CO_2 assimilation are not well understood.

In the present study, leaf gas exchange of barley plants was measured to distinguish the effect of flooding on photosynthesis caused by stomata closure and the direct (biochemical) effect of flooding on the photosynthetic apparatus.

Received 6 August 2001, *accepted* 15 October 2001.
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Abbreviations: C_a – ambient CO_2 concentration; C_i – intercelullar CO_2 concentration; Chl – chlorophyll; DTT – dithiothreitol; E – transpiration rate; g_s – stomatal conductance; PEP – phosphoenolpyruvate; PEPC – phosphoenolpyruvate carboxylase; P_N – net photosynthetic rate; RuBP – ribulose-1,5-bisphosphate; RuBPC – ribulose-1,5-bisphosphate carboxylase; TCA – tricarboxylic acid.
Acknowledgements: We thank Michael Jackson (IACR-Long Ashton Research Station, UK) for stimulating discussions and for critical reading of the manuscript.

Materials and methods

Growth and treatment of seedlings: Barley plants (*Hordeum vulgare* L. cv. Alfa) were grown for two weeks in soil in a growth chamber. The soil was classified as Eutric Fluvisol (Boiadjiev 1994), pH 5.75. The environmental conditions were: irradiance of 160 μmol m^{-2} s^{-1} PAR, 12 h-photoperiod, temperature 24±2 °C, and relative humidity of 60±5 %. When the plants were at the second- to third-leaf stage, half of the plants were flooded in the early morning by placing the pots inside larger glass containers filled with tap water to 25 mm above the level of the soil surface. Control plants remained well watered (60 % soil moisture) during the experiment. Samples were taken 72, 96, and 120 h after the start of flooding treatment. Each measurement was done independently.

Gas exchange measurements were performed by a portable photosynthesis system *Li-6000 (LI-Cor*, Lincoln, USA). All measurements were taken on attached, mature leaves. Leaves of 5-6 plants (the first well-expanded leaf) were placed in a 250 cm^3 chamber. Quantum flux density was 870 μmol m^{-2} s^{-1} PAR, provided by a 500-W incandescent lamp fitted with reflector. Flow rate through the cuvette was 18-20 cm^3 s^{-1}, boundary layer resistance (r_a) was 0.8 s cm^{-1}. Leaf temperature was 26±2 °C.

Enzyme extraction and assays: Leaf tissue without the major veins was ground in a mortar on ice at a ratio of 1 g fresh mass to 5 cm^3 cold extraction medium containing 0.33 M sorbitol, 0.05 M HEPES-NaOH, 2 mM KNO_3, 2 mM EDTA, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 0.5 mM K_2HPO_4, 0.02 M NaCl, and 0.2 M Na-isoascorbate, pH 7.6. The homogenate was quickly filtered through four layers of cheesecloth and centrifuged at 20 000×*g* for 15 min, and the supernatant used directly for enzyme assay.

RuBPC (EC 4.1.1.39) and PEPC (EC 4.1.1.31) activities were assayed from the activated crude preparation by following the incorporation of $NaH^{14}CO_3$ into acid stable products as described by Popova *et al.* (1996). The assay mixture for RuBPC contained in 50 mM HEPES-NaOH (pH 8.0): 20 μmol $MgCl_2$, 1 μmol dithiothreitol (DTT), 20 μmol $NaHCO_3$ (containing 1.48 MBq, specific radioactivity 0.38 MBq μmol^{-1}), and enzyme extract equivalent to 0.3-0.4 mg protein. The reaction volume was 1 cm^3. Reactions, at 25±1 °C, were initiated by addition of 2 μmol RuBP and stopped after 1 min reaction time with 6 M HCl. The assay mixture for PEPC activity contained in 50 mM HEPES-NaOH (pH 8.0): 20 μmol $MgCl_2$, 0.4 μmol NADH, 20 μmol $NaHCO_3$ (containing 1.48 MBq, specific radioactivity 0.38 MBq μmol^{-1}), 1 μmol DTT, and enzyme extract equivalent to 0.3-0.4 mg protein. Reaction volume was 1 cm^3. Reactions, at 30±1 °C, were initiated by addition of 3 μmol PEP. Reaction time was 1 min. The amount of fixed $^{14}CO_2$ was measured in a liquid scintillation spectrometer.

Activity of glycollate oxidase (EC 1.1.3.1) was measured as described by Popova *et al.* (1987). For enzyme extraction, 25 mg of leaves was ground in a pre-chilled mortar with purified sea sand and 20 cm^3 of 1/15 M K/Na phosphate buffer, pH 8.0. The homogenate was filtered through four layers of cheesecloth and centrifuged at 20 000×*g* for 15 min. To 5 cm^3 of extracts was added 0.5 cm^3 of 0.1 M Na-glycollate (H_2O for the controls). Reaction time was 10 min at 25±1 °C. At the end of the reaction, extracts were precipitated with trichloracetic acid (final concentration 3 %) and developed a colour reaction with 0.3 % phenylhydrazine hydrochloride and 1.5 % $K_3Fe(CN)_6$. The amount of glyoxylic acid was assayed spectrophotometrically at 530 nm (*Specol 10*, *Carl Zeiss*, Jena, Germany).

Phosphoglycollate phosphatase (EC 3.1.3.18) was extracted and assayed according to Randall *et al.* (1971). 3 g fresh plant material was ground in a chilled mortar with 6 cm^3 of medium containing 20 mM cacodylate buffer and 1 mM EDTA at pH 6.3. The homogenate was squeezed through cheesecloth and the extract was centrifuged at 10 000×*g* for 10 min. The enzyme activity was assayed in the supernatant for 15 min at 28±1 °C using 10 μM substrate, 100 μM cacodylate buffer (pH 6.3), and 1 mM $MgCl_2$ in a final volume of 1.5 cm^3. The reaction was terminated by the addition of 0.5 cm^3 10 % TCA. The precipitate was measured by centrifugation and release of phosphorus was measured by the method of Kondrashova *et al.* (1965).

Soluble protein content was determined by the method of Bradford (1976). Proline content was determined spectrophotometrically at 520 nm after Bates *et al.* (1973), and chlorophyll (Chl) was extracted by acetone and measured spectrophotometrically according to Arnon (1949).

Acidity: 0.5 g fresh material collected from different plants were ground in a pre-chilled mortar with 50 cm^3 of CO_2-free distilled water and filtered through four layers of cheesecloth. An aliquot (2-4 cm^3) of the homogenate was titrated with 0.01 M KOH (prepared fresh in CO_2-free distilled water) to an end point of pH 7.0 Acidity was expressed as meq kg^{-1}(d.m.).

Relative water content (RWC) was measured as described by Morgan (1986) except that saturated mass was obtained after soaking the leaves for 2 to 3 h in distilled water.

Results

Exposure of barley plants to flooding for 72, 96, and 120 h decreased RWC only slightly. Leaf RWC of control plants was about 91-92 %. The reduction in this parameter was only approximately 3 % for treated plants. No wilting was observed in flooded plants. Relative to controls, flooded plants exhibited a smaller leaf dry matter content. The average decrease in the values of this parameter was approximately 14 % of the controls (Table 1).

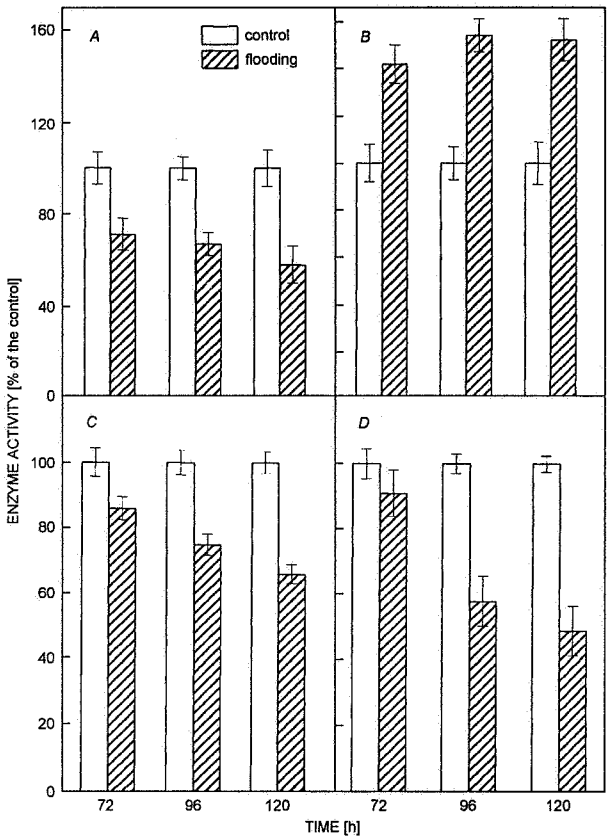

Fig. 1. Effect of flooding on the activities of RuBPC (*A*), PEPC (*B*), glycollate oxidase (*C*), and phosphoglycollate phosphatase (*D*). *Open and striped columns* represent controls and flooded plants, respectively. In controls the activity of RuBPC was 2.28 mmol(CO_2) kg^{-1}(protein) s^{-1}, PEPC 0.304 mmol(CO_2) kg^{-1}(protein) s^{-1}, glycollate oxidase 297.3 mg(glyoxylic acid) kg^{-1}(protein) s^{-1}, and phosphoglycollate phosphatase 407.3 mg(P_i) kg^{-1}(protein) s^{-1}. Means of 4 experiments ± SE.

Soil flooding for 72 h or more had marked effects on both stomata behaviour and photosynthetic capacity (Table 1). Exposure of barley plants to flooding caused a gradual decrease in P_N, the effect being greater after 120 h of flooding. Transpiration rate was also decreased following the time of exposure of plants to flooding.

Plant exposure to the described soil flooding resulted in an almost 2.7-fold decrease in g_s as compared with the controls. A small increase in C_i was observed in plants flooded for 72 h.

The activities of both carboxylating enzymes (RuBPC and PEPC) were differently affected by root flooding. RuBPC activity was reduced when plants were subjected to flooding (Fig. 1*A*). A significant increase in the activity of PEPC was found in all flooded plants (Fig. 1*B*). As a result of the inverse effect of flooding on the activity of both carboxylases, the RuBPC/PEPC ratio declined in all treated plants. Flooding caused a gradual decrease in the activities of phosphoglycollate phosphatase and glycollate oxidase, with a more pronounced effect on phosphoglycollate phosphatase (Fig. 1*C,D*).

A progressive decrease in Chl (*a*+*b*) content in barley leaves was observed after 96 and 120 h of flooding. No changes in leaf protein content were found when the values were calculated per dry mass. Proline content in leaves of barley increased in plants flooded for 72, 96, and 120 h. The highest effect was found after 120 h of flooding—over 90 % of the control.

Relative to control, flooded plants also exhibited a larger accumulation of leaf titratable acidity. The increases in the values of this parameter occurred much faster than those observed for proline content. The average increase in the values was more than 2.9-fold of the controls (Table 2).

Discussion

Long-term exposure (up to 120 h) of barley plants to soil flooding led to a noticeable decrease in photosynthesis and the activity of RuBPC. The activity of the photorespiratory enzymes phosphoglycollate phosphatase and glycollate oxidase was also reduced, suggesting that the rate of photorespiration might be suppressed. Flooding also reduced dry mass and Chl content.

The response of photosynthesis to soil flooding resembled that produced by other stresses (osmotic shock and drought). The common events of this response are the slowed rate of carbon assimilation, inhibition of RuBPC activity, and changes in photorespiratory carbon metabolism (Kicheva *et al.* 1994, Popova *et al.* 1996, Tsonev *et al.* 1998).

The observed changes in P_N after flooding might be a result of: (*a*) an indirect effect, mediated by stomata closure, causing a reduction in CO_2 supply, or (*b*) effects of flooding on the capacity of plants for CO_2 fixation, independent of increased limitation to inward diffusion.

Flooding treatment caused a substantial decrease in g_s,

Table 1. Effect of soil flooding on the relative water content (RWC), dry matter content and gas exchange characteristics in barley leaves. P_N, net CO_2 assimilation at 450 mg m^{-3} ambient CO_2; C_i, intercellular CO_2 concentration at 450 mg m^{-3} ambient CO_2; g_s, stomatal conductance; E, transpiration rate. Means ± SE, $n = 4$ (RWC and dry matter) or 6 (other characteristics).

Treatment	RWC [%]	Dry matter content [g kg^{-1}(f. m.)]	P_N [mg(CO_2) m^{-2} s^{-1}]	E [mg(H_2O) m^{-2} s^{-1}]	g_s [cm s^{-1}]	C_i [mg m^{-3}]
control	91.58±0.44	94.56±4.12	0.240±0.007	47.17±4.3	0.21±0.04	329.2±27.6
72 h flooding	91.17±0.37	76.56±3.80	0.141±0.003	37.67±3.7	0.14±0.02	377.1±16.4
control	92.07±0.26	94.67±4.86	0.227±0.009	49.27±5.9	0.25±0.01	359.4±25.4
96 h flooding	90.72±0.68	82.51±2.47	0.119±0.017	37.73±4.7	0.14±0.02	366.3±15.0
control	92.14±0.38	93.55±4.53	0.207±0.018	39.77±4.3	0.24±0.03	338.5±33.2
120 h flooding	89.74±1.01	80.55±2.94	0.110±0.017	27.37±5.7	0.09±0.02	372.0±15.2

Table 2. Effect of soil flooding on chlorophyll (Chl) and leaf protein contents, proline accumulation, and titratable acidity. Means ± SE, $n = 6$.

Treatment	Chl ($a+b$) [g kg^{-1}(d.m.)]	Leaf soluble protein [g kg^{-1}(d.m.)]	Proline [mmol kg^{-1}(d.m.)]	Titrable acidity [meq kg^{-1}(d.m.)]
control	13.74±0.55	96.86±3.16	8.35±0.74	62.71±4.23
72 h flooding	13.79±0.13	110.89±2.16	13.06±1.04	160.92±4.25
control	14.25±0.55	98.55±1.05	10.14±1.06	57.78±6.30
96 h flooding	11.03±0.45	97.44±2.42	13.33±1.58	169.68±3.64
control	14.33±0.21	90.64±1.07	8.98±1.39	69.70±4.23
120 h flooding	10.06±0.74	89.63±2.48	17.01±2.48	196.15±6.22

but the C_i values were not changed or even were a little higher than in the controls. This implies that stomata closure did not restrict CO_2 entry into the leaf enough to reduce C_i. Because mesophyll cells were equally well supplied with CO_2 both in the control and flooded plants, reductions in photosynthesis probably were mostly non-stomatal.

According to the model of Caemmerer and Farquhar (1981) and Farquhar and Caemmerer (1982), greater inhibition of photosynthesis at higher C_i than at a low C_i would suggest that flooding primarily affected RuBP regeneration, which includes photosynthetic electron transport, NADPH and ATP syntheses, and the reductive pentose phosphate cycle. Recent data indicate that long-term (96 h) exposure of mango to soil flooding led to decrease in root respiration rate and P_N (Zude-Sasse and Ludders 2000). This corroborates the results of Titarenko (2000) who showed that prolonged flooding of flooding-sensitive apricot caused considerable declines in P_N, E, and photorespiration rate. In a more tolerant species, apple, such changes were not observed. Non-stomatal limitation to CO_2 under soil flooding might be related not only to a decline in the activity of RuBPC but also to alterations in primary photochemistry. This suggestion is in agreement with recent investigations that show that flooding inhibits electron transport activity of PS2 (Ladygin 1999). The decrease in generation of ATP and reducing equivalents is important in the regulation of RuBPC. Partially these results could be explained with the observed chloroplast destruction of *Amaranthus* plants grown under oxygen deficiency (Knacker *et al.* 1984, Ladygin 1999).

Under our experimental conditions we observed changes in the RuBPC/PEPC ratio, due to opposite effect of flooding on the activity of these major carboxylases (Fig. 1*A,B*). We presented similar results for wheat and rye plants grown in waterlogged soil (Salcheva and Popova 1982) and for barley plants exposed to drought or salinity (Popova *et al.* 1996, Tsonev *et al.* 1998). One possible explanation might be a decrease in mesophyll conductance, which may cause a decrease in the chloroplast CO_2 concentration in the vicinity of RuBPC. Hence the enhanced activity of PEPC after different stress conditions might work as an adaptive photosynthetic mechanism for improving CO_2 assimilation.

The regulation of cytosolic pH is now considered to be the major determinant of anoxic or hypoxic plant tissue (Roberts *et al.* 1984, 1985; for review see Ricard *et al.* 1994). The acidification of cytosol is an important factor in regulation of photosynthesis (Flügge *et al.* 1980). Hence the observed alterations in the values of proline content and leaf acidity might be regarded as an adaptive response of barley plants to flooding. Further study of the changes in contents of NAD(P)H, ATP, and organic acids after exposure of plants to flooding is needed.

In summary, we found that flooding of barley plants for 72, 96, and 120 h decreased P_N and the activity of RuBPC. The activities of phosphoglycollate phosphatase and glycollate oxidase were also suppressed. Although partial stomata closure also occurred, it did not appear to be the only cause for decreased photosynthesis. At present, we can not explain the enhanced activity of PEPC after flooding, but our suggestion is that this is part of the biochemical adaptation of photosynthesis to environmental stresses.

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