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Effect of flooding on the activities of some enzymes of activated oxygen metabolism, the levels of antioxidants, and lipid peroxidation in senescing tobacco leaves

Weei Pirng Hurng & Ching Huei Kao*
Department of Agronomy, National Taiwan University, Taipei, Taiwan, Republic of China
**Author for correspondence*

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Abstract

Effects of flooding on the activities of some enzymes of activated oxygen metabolism, the levels of antioxidants, and lipid peroxidation in senescing leaves of tobacco were investigated. As judged by the decrease in chlorophyll and protein levels, flooding accelerated the senescence of tobacco leaves. Total peroxide and the lipid peroxidation product, malondialdehyde, increased in both control and flooding-treated leaves with increasing duration of the experiment. Throughout the duration of the experiment, flooded leaves had higher levels of total peroxide and malondialdehyde than did control leaves. Flooding resulted in an increase in peroxidase and ascorbate peroxidase activities and a reduction of superoxide dismutase activity in the senescing leaves. Glycolate oxidase, catalase, and glutathione reductase activities were not affected by flooding. Flooding increased the levels of total ascorbate and dehydroascorbate. Total glutathione, reduced form glutathione, or oxidized glutathione levels in flooded leaves were lower than in control leaves during the first two days of the experiment, but were higher than in control leaves at the later stage of the experiment. Our work suggests that senescence of tobacco induced by flooding may be a consequence of lipid peroxidation possibly controlled by superoxide dismutase activity. Our results also suggest that increased rates of hydrogen peroxide in leaves of flooded plants could lead to increased capacities of the scavenging system of hydrogen peroxide.

Abbreviations: GSH = reduced form glutathione; GSSG = oxidized form glutathione; GSSG reductase = glutathione reductase; MDA = malondialdehyde; SOD = superoxide dismutase.

1. Introduction

The senescence of leaves is characterized by a decrease in chlorophyll and protein levels [37]. Among the various ideas regarding senescence initiation in leaves, the free radical hypothesis has attracted considerable attention [4, 5, 14, 18]. Activated oxygen species, such as superoxide, hydrogen peroxide, and their interaction products, react with proteins, lipids, and nucleic acids [6, 18], and increased levels of these free radicals may initiate leaf senescence.

There are many reports of accelerated leaf senescence in flooded plants [16]. Some authors have viewed senescence as a gradual shifting of the metabolic status of the tissue from a reductive to an oxidative state [1, 26]. If such a shifting would also occur as a result of flooding, the activities of the enzymes of activated oxygen metabolism and lipid peroxidation would also be expected to rise during flooding, resulting in accelerated senescence. Hence it is of interest to study the activities of some enzymes of activated oxygen metabolism and lipid peroxidation in relation to

the pattern of senescence in senescing leaves of tobacco subjected to flooding. The present study was carried out with this objective in mind.

2. Materials and methods

2.1 Plant material and treatment conditions
Seeds of *Nicotiana tabacum* (cv. Speight G-70) were sown in plastic trays containing vermiculite and hydrocon (1:1). Seedlings were grown in a greenhouse with natural light at 30°C day/25°C night. At 4 weeks after sowing, seedlings were transplanted to pots ($2dm^2$) containing sandy loam. Each pot contained one seedling and received 15g of a compound fertilizer ($N\text{-}K_2O\text{-}P_2O_5$, 7-21-21). Pots were placed in the same greenhouse as described above. Flooding treatment started at 40 days after transplanting when tobacco plants had 7–8 leaves. Tobacco plants were flooded by maintaining the water level at 1cm above the soil surface. Unflooded control plants received the optimum amount of water. At the times indicated the lowest leaves of treated and control plants were collected for analysis of protein, chlorophyll, total phenolics, total peroxide, MDA, ascorbate, glutathione, and enzyme activities.

2.2 Determinations of chlorophyll and protein
Chlorophyll levels were determined according to Wintermans and De Mots [40] after extraction in 96% (v/v) ethanol. For protein extraction, leaf samples were homogenized in 25m*M* sodium phosphate buffer (pH 7.5). The extracts were centrifuged at 17,000×g for 20 min, and the supernatants were used in the spectrophotometric determination of protein by the method of Lowry et al. [19].

2.3 Determinations of total phenolics, total peroxide, and MDA
In determining of total phenolic levels, leaf tissues were extracted in 50% methanol and estimated by the method of Price and Butler [30]. Total peroxide (H_2O_2 plus lipid peroxide) levels were estimated according to Sagisaka after extraction in 5% (w/v) trichloroacetic acid. MDA was extracted with 5% (w/v) trichloroacetic acid and determined according to Heath and Packer [11].

2.4 Determinations of ascorbate and glutathione
Ascorbate in 5% trichloroacetic acid and glutathione in 3% sulfosalicylic acid extract were determined as described by Laws et al. [17] and Smith [35], respectively.

2.5 Enzyme assays
For extraction of enzymes, leaf tissues were homogenized with 0.1*M* sodium phosphate buffer (pH 6.8, containing 0.1% Triton X-100 and 0.05g Polycar AT in a homogenizer and centrifuged (12,000×g, 20min). The supernatants were used for enzyme assays. Peroxidase activity was measured using a modification of the procedure of Curtis [3]. The assay medium contained 0.1*M* sodium phosphate buffer (pH 5.8), 7.2mM guaiacol, 11.8m*M* H_2O_2 and 0.1ml enzyme extract in a final assay volume of 3.0ml. The reaction was initiated by the addition of H_2O_2 and the change in optical density at 470nm was measured. Activity was calculated using the extinction coefficient (26.6m$M^{-1}cm^{-1}$ at 470nm) for tetraguaiacol. Catalase activity was assayed by measuring the initial rate of disappearance of H_2O_2 [14]. Three ml catalase assay reaction mixture contained 0.1*M* sodium phosphate buffer (pH 7.0), 2m*M* H_2O_2 and 0.1ml enzyme extract. The decrease in H_2O_2 was followed as the decline in optical density at 240nm, and activity was calculated using the extinction coefficient (40m$M^{-1}cm^{-1}$ at 240nm) for H_2O_2 [14]. Glycolate oxidase activity was determined according to Brennan et al. [1], and SOD activity by the method of Foster and Hess [7]. Ascorbate peroxidase activity was determined according to Nakano and Asada [25]. The decrease in ascorbate concentration was followed as a decline in optical density at 290nm and activity was calculated using the extinction coefficient (2.8m$M^{-1}cm^{-1}$ at 290nm) for ascorbate. GSSG reductase activity was determined according to the method of Foster and Hess [7].

3. Results

3.1 Chlorophyll and protein
Senescence of tobacco leaves was followed by measuring the decrease of chlorophyll and protein. Figure 1 shows the changes in chlorophyll and protein in leaves of tobacco with or without flooding. The decrease of protein in control leaves

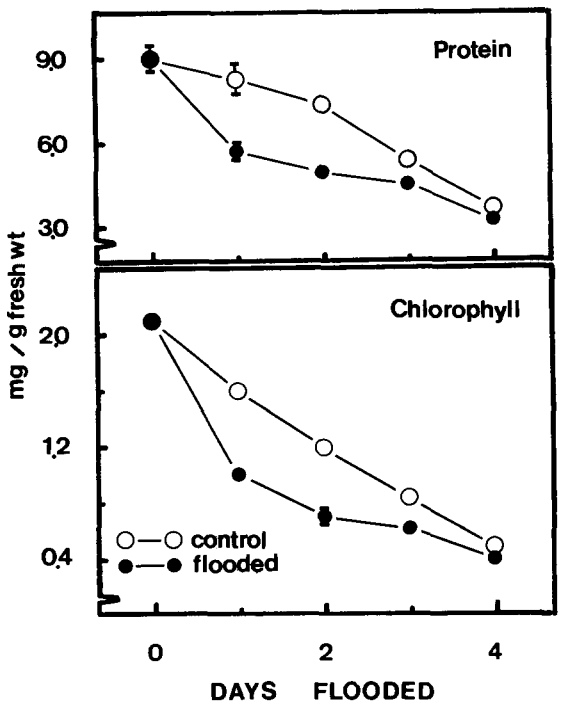

Fig. 1. Effects of flooding on protein and chlorophyll levels of senescing tobacco leaves. Vertical bars represent standard errors (n = 4). Only those standard errors larger than the symbol size are shown.

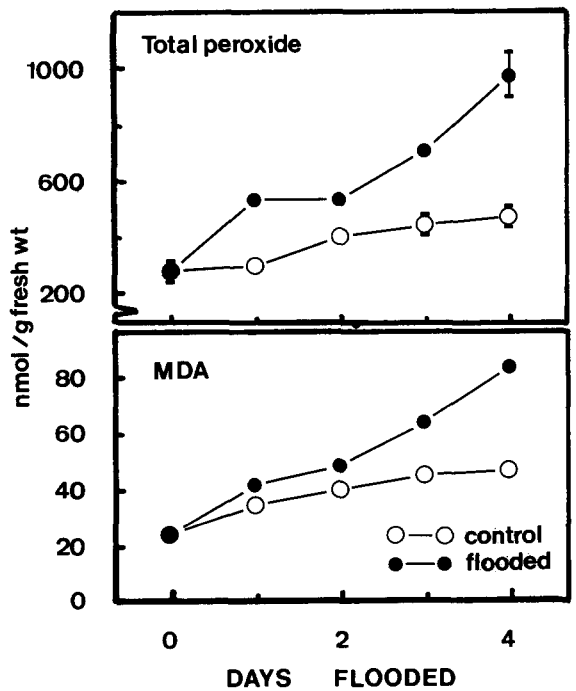

Fig. 2. Effects of flooding on total peroxide and malondialdehyde (MDA) levels of senescing tobacco leaves. Vertical bars represent standard errors (n = 4). Only those standard errors larger than the symbol size are shown.

was evident on day 2, and chlorophyll loss in control leaves became significant after 1 day. Flooding significantly accelerated the loss of both protein and chlorophyll.

3.2 Total peroxide, lipid peroxidation, and total phenolics

Changes in the levels of total peroxide and MDA during flooding of tobacco leaves are shown in Figure 2. There was no increase in total peroxide in control leaves on day 1 of the experiment. Thereafter there was a clear increase of total peroxide levels. In the control leaves, the increase in the level of MDA was evident on day 1. Flooding induced increases in MDA and total peroxide levels during entire duration of experiments. Changes in total phenolics (Figure 3) were similar to changes in the level of lipid peroxidation. Flooding treatment strongly promoted the rise in total phenolics.

3.3 Ascorbate and glutathione

Figure 4 shows the changes in levels of total

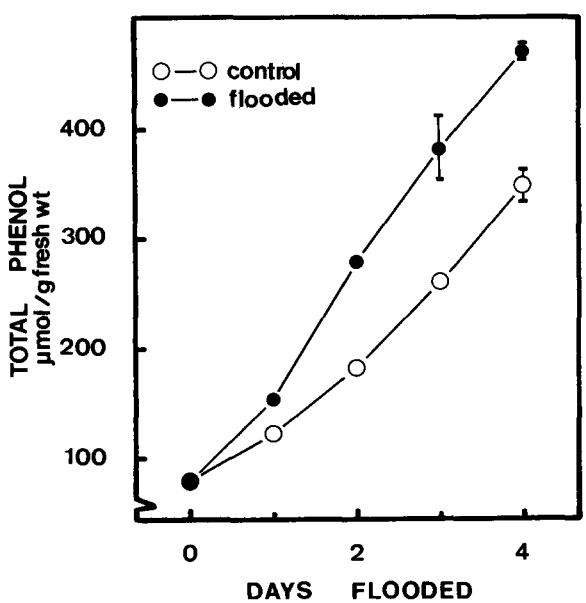

Fig. 3. Effects of flooding on total phenolic contents of senescing tobacco leaves. Vertical bars represent standard errors (n = 4). Only those standard errors larger than the symbol size are shown.

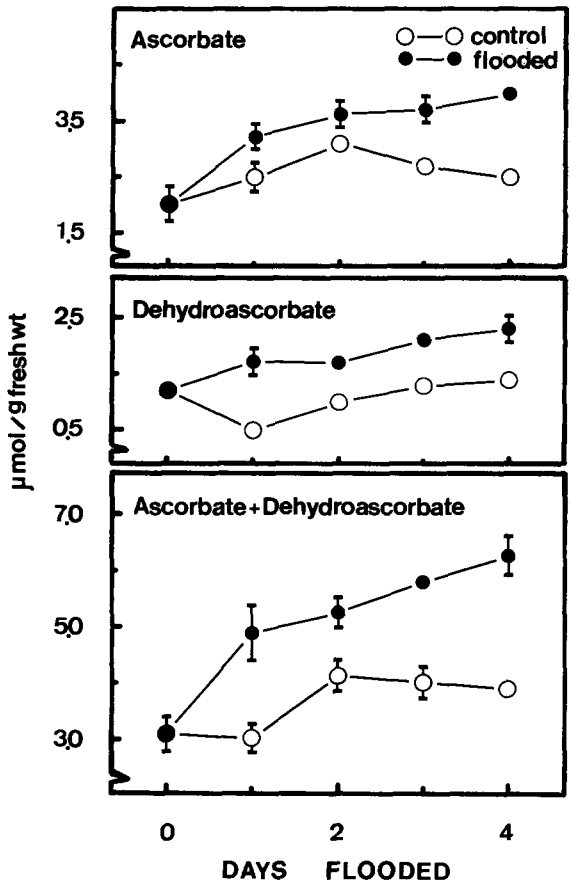

Fig. 4. Effects of flooding on total ascorbate (ascorbate+dehydroascorbate), ascorbate, and dehydroascorbate levels of senescing tobacco leaves. Vertical bars represent standard errors (n = 4). Only those standard errors larger than the symbol size are shown.

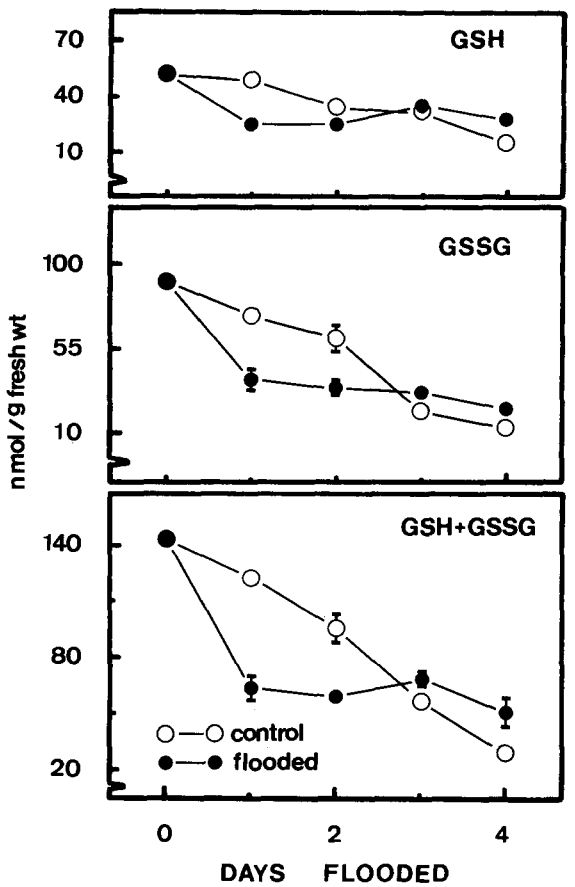

Fig. 5. Effects of flooding on total glutathione (GSH+GSSG), GSH, and GSSG levels of senescing tobacco leaves. Vertical bars represent standard errors (n = 4). Only those standard errors larger than the symbol size are shown.

ascorbate (ascorbate + dehydroascorbate), ascorbate, and dehydroascorbate in leaves of tobacco with or without flooding. Total ascorbate level in flooded leaves was found to be higher than the controls during the entire duration of the experiment. Flooded leaves also had higher ascorbate or dehydroascorbate levels than did control leaves. The changes of total glutathione (GSH+GSSG), GSH, and GSSG in flooded leaves was lower than that in control leaves during the first two days of the experiment (Figure 5). However, total glutathione level in flooded leaves was higher than that in control leaves at days 3 and 4 (Figure 5). The effects of flooding on GSH and GSSG levels were similar to that on total glutathione level.

3.4 Peroxidase, SOD, catalase, and glycolated oxidase

Figure 6 shows the time courses of peroxidase activities in leaves of tobacco treated with or without flooding, an increase in peroxidase activity in control leaves was evident by 2 days after the start of the experiment. Both activity and specific activity of peroxidase was higher in flooded leaves than untreated leaves throughout the entire duration of the experiment.

In control leaves, SOD activity decreased markedly during the first 3 days (Figure 7). Flooding strongly accelerated this decrease in SOD activity. The specific activity of SOD was also lower in flooded leaves than untreated leaves. In contrast to SOD, catalase and glycolate

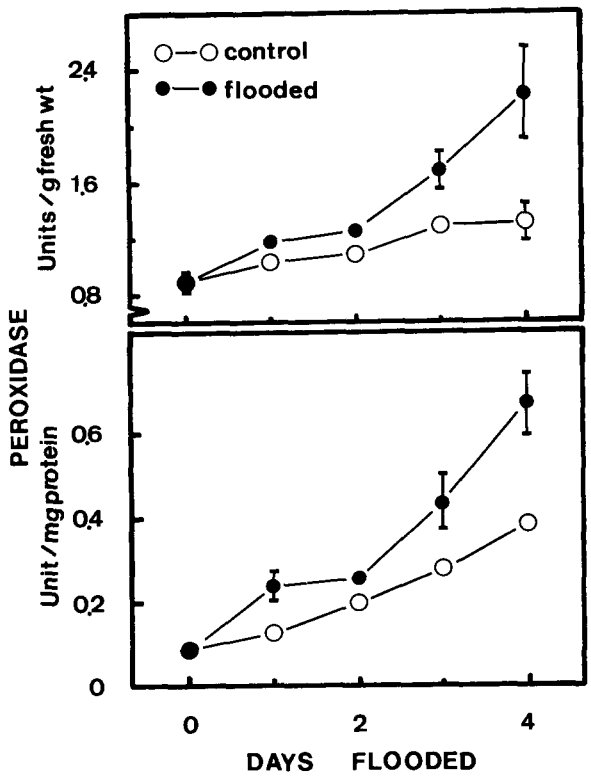

Fig. 6. Effects of flooding on peroxidase activity of tobacco senescing leaves. Vertical bars represent standard errors (n = 4). Only those standard errors larger than the symbol size are shown.

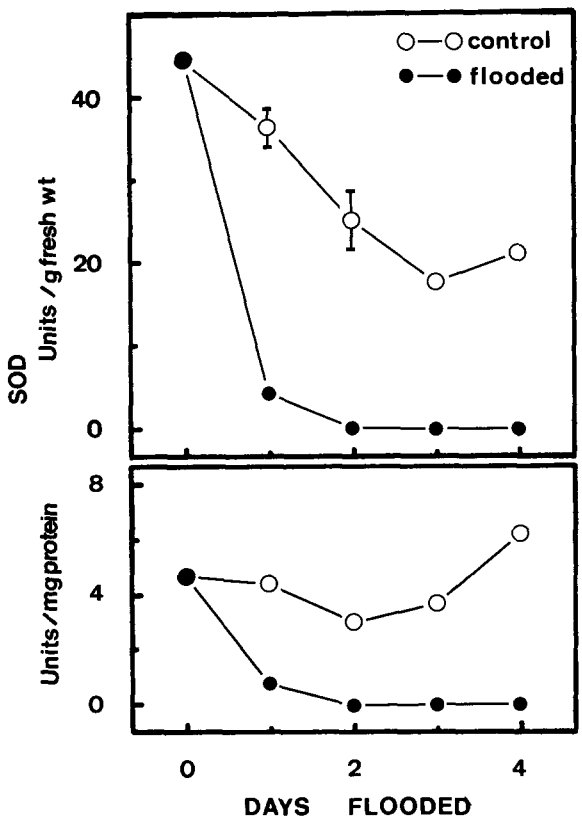

Fig. 7. Effects of flooding on superoxide dismutase (SOD) activity of senescing tobacco leaves. Vertical bars represent standard errors (n = 4). Only those standard errors larger than the symbol size are shown.

oxidase were not affected by flooding (data not shown).

3.5 Ascorbate peroxidase and GSSG reductase

On the basis of fresh weight, ascorbic peroxidase activity in control leaves remained unchanged during the first days and decreased subsequently (Figure 8). The activity of ascorbate peroxidase in flooded leaves was higher than in the controls. Specific activity of ascorbate peroxidase in flooded leaves was also found to be higher than that in the controls (Figure 8).

GSSG reductase activity in control leaves decreased significantly at the first day of the experiment and remained unchanged thereafter (Figure 9). In contrast to ascorbate peroxidase, GSSG reductase activity was not affected by flooding. Higher specific activity of GSSG reductase in flooded leaves was observed (Figure 9); this was entirely the consequence of its lower protein level.

4. Discussion

The present investigation showed that flooding accelerated leaf senescence, as judged by the decreases in chlorophyll and/or protein, of tobacco plants. This result is in agreement with earlier reports [16]. Our results showed that both total peroxide (H_2O_2 and lipid peroxide) and lipid peroxidation product (MDA) increased with increasing the duration of the experiment in both control and flooding-treated leaves. We also demonstrated that flooded leaves had higher levels of total peroxide and MDA than control leaves throughout the entire duration of the experiment. These results suggest that H_2O_2 levels in flooded leaves are higher than those in control leaves. The increases in H_2O_2, total phenolic levels, and peroxidase activity in tobacco leaves after flooding suggest that flooding-enhanced

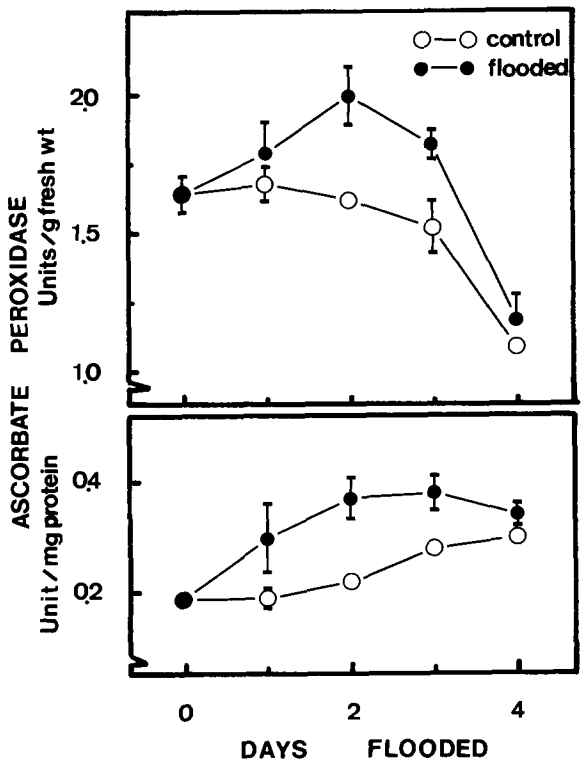

Fig. 8. Effect of flooding on ascorbate peroxidase activity of senescing tobacco leaves. Vertical bars represent standard errors (n = 4). Only those standard errors larger than the symbol size are shown.

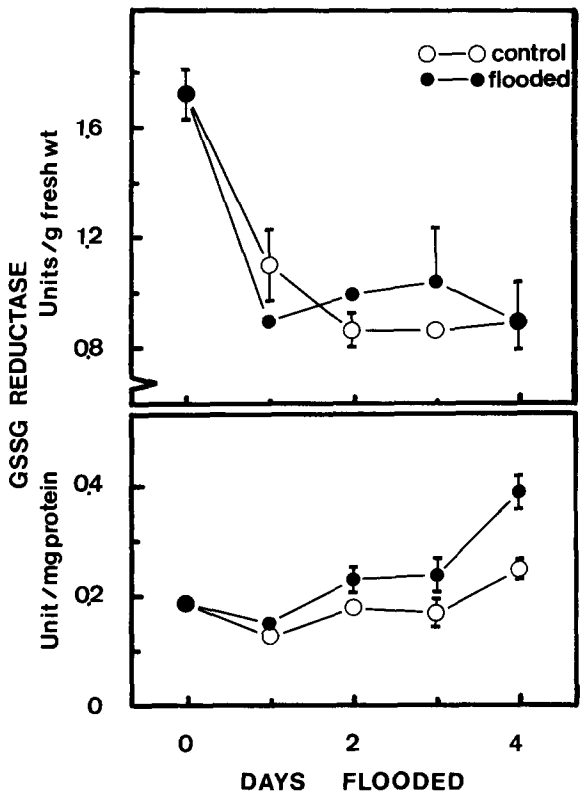

Fig. 9. Effect of flooding on GSSG reductase activity of senescing tobacco leaves. Vertical bars represent standard errors (n = 4). Only those standard errors larger than the symbol size are shown.

chlorophyll degradation of tobacco leaves is due to a phenolic–peroxidase–H_2O_2 system, as proposed by Kato and Shimizu [13]. It has been reported that H_2O_2 is involved in regulating leaf senescence of a variety of plants [20, 21, 22, 26]. Thus, the possible involvement of H_2O_2 per se in promoting leaf senescence of tobacco by flooding can not be excluded.

In C_3 plants, glycolate oxidation is a major source of H_2O_2 production. Catalase is known to catalyze the breakdown of H_2O_2. Simultaneous increases in glycolate oxidase activity and decreases in catalase activity have been offered as reasons for accumulation of H_2O_2 in some plant species under water stress [2, 9, 24]. However, neither glycolate oxidase activity nor catalase activity in tobacco leaves was affected by flooding. Thus, it is unlikely that accumulation of H_2O_2 in flooded tobacco leaves is regulated by glycolate oxidase and catalase.

Plant tissues generate superoxide radicals [10] which are removed by SOD. Accumulation of free radicals can bring about lipid peroxidation with consequent membrane damage [4, 15, 28]. SOD activity decreases as leaves senesce [4, 27, 29]. Post-anoxic injuries of soybean roots and in rhizomes of the anoxia-sensitive species, *Iris germanica*, after anoxic stress were associated with increased superoxide radical production capacity and lipid peroxidation coupled with a reduced SOD activity [12, 23, 39]. Although anoxic stress is a major factor in a flooded environment, it is not known whether flooding affects similarly SOD activity and lipid peroxidation in leaf tissues. Here we provide evidence that flooding resulted in increased lipid peroxidation and reduced SOD activity in senescing tobacco leaves. In conclusion, our results suggest that leaf senescence of tobacco induced by flooding may be a consequence of lipid peroxidation possibly controlled by, among other factors, SOD activity.

The present investigation also showed that flooding resulted in an increase in extractable activity of ascorbate peroxidase in senescing tobacco leaves even though there was a decline in leaf protein level. However, GSSG reductase does not seem to be affected by flooding treatment.

It has been shown that severe leaf water deficit resulted in an increase in ascorbate peroxidase activity in *Eragrostis tef* [34]. Since no significant decrease in water content in tobacco leaves during flooding was observed (data not shown), it is unlikely that the increase in ascorbic peroxidase in tobacco leaves by flooding is attributable to leaf water deficit. An increase in ascorbate peroxidase activity has also been observed in ozone- and paraquat-treated plants [8, 36].

Ascorbate level has been reported to be increased by illumination, fumigation with ozone and a shift from anaerobic to aerobic conditions [32, 33, 36, 38]. However, the effect of flooding on the level of ascorbate in plants has not been reported before. Here we showed that ascorbate level in senescing leaves of tobacco was increased by flooding.

Using growth analysis techniques, we were able to show that flooding resulted in decreased net assimilation rate in leaves of tobacco [13]. Our unpublished data also showed that stomatal conductance of tobacco plants was drastically reduced by flooding. It appears that flooding, while inhibiting photosynthesis, could alter the balance between electron transport and carbon fixation. The results of the present investigation suggest that increased rates of hydrogen peroxide production in leaves of flooded plants could lead to increased capacity of the scavenging system of hydrogen peroxide.

Acknowledgement

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