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Senescence and protein remobilisation in leaves of maturing wheat plants grown on waterlogged soil

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Abstract

The influence of waterlogging on leaf senescence and protein remobilisation was investigated in winter wheat (*Triticum aestivum* L., cv.Arina), which was grown in large pots embedded in the field in spring. The soil of intact pots was flooded permanently from anthesis to maturity, while in perforated control pots the soil was well aerated throughout the maturation period. No major effects of waterlogging were observed in the third leaf from the top, which was already senescing when the treatment was started. The degradation of proteins and chlorophyll was accelerated slightly in the second leaf from the top and considerably in the flag leaf. The contents of free amino acids in the uppermost leaf blades were lower on waterlogged soil than on control pots. Furthermore, amino acids in the blades did not accumulate during the earlier protein remobilisation observed on flooded soil, indicating that the export of amino acids via the phloem was fully functional. The loss of leaf proteins and the changes in the pattern of peptide hydrolases during senescence were similar in treated and control plants. Endopeptidase activities increased in an early phase of senescence at pH 5 and later also at pH 9, while aminopeptidase activities decreased. Therefore, nitrogen remobilisation was accelerated on flooded soil, but the senescence-related changes were very similar to those observed in control plants.

Introduction

Nitrogen is redistributed in maturing cereals from senescing leaves to the maturing grains, allowing an efficient use of this frequently limiting macronutrient (Feller, 1990). In photosynthesising cells, nitrogen is mainly present in the form of proteins in the chloroplasts (Peoples and Dalling, 1988). Proteins must be degraded by peptide hydrolases prior to the export of this nitrogen via the phloem. Protein nitrogen is mainly remobilised at an early stage of senescence, when the leaf is still turgid (Feller and Keist, 1986). Characteristic changes in the activities of exo- and endopeptidases have been observed in naturally senescing leaves of field grown cereals (Feller, 1986; Huffaker, 1990; Peoples and Dalling, 1988). Aminopeptidase activities decrease during senescence, while endopeptidases reach in general maximal activities in this stage of development (Feller and Keist, 1986). A vacuolar endopeptidase with a pH optimum around 5 increases earlier than an extravacuolar endopeptidase with an alkaline pH optimum (Keist and Feller, 1985; Van der Valk and Van Loon, 1988; Wittenbach et al., 1982).

Various stresses, such as nutrient deprivation, drought or heat can influence senescence in plants (Dungey and Davies, 1982; Noodén, 1988; Roy-Macauley et al., 1992). Waterlogging accelerates senescence and nitrogen remobilisation in the oldest leaves of young wheat plants (Trought and Drew, 1980). Stomatal closure and foliar desiccation as a consequence of waterlogging have been observed by several groups (Bradford and Hsiao, 1982; Jackson et al., 1978; Zhang and Davies, 1986). Jackson and Kowalewska (1983) noticed in short-term experiments with waterlogged pea plants that stomatal closure (after 1 d) preceeded partial leaf desiccation (after 5–7 d). Influences of waterlogging on the hormonal balance (Bradford and Hsiao, 1982; Jackson et al., 1978, 1988; Neuman et al., 1990) as well as on the acquisition and redistribution of nutrients (Buwalda et al., 1988;

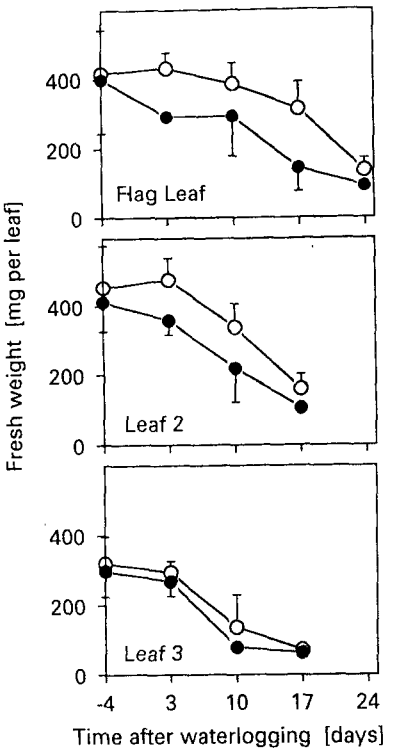

Fig. 1. Effect of waterlogging on the fresh weight of the three uppermost leaf laminas. Waterlogging started June 28 (day 0) and was maintained throughout the grain filling period. Means of 4 replicates are shown for control pots (○) and waterlogged pots (●). Standard errors are shown when exceeding the size of the symbol (on one side only for clarity).

Trought and Drew, 1980) have been frequently investigated at the seedling stage, while the reproductive stage has been far less considered in this context. Experiments performed by Watson et al. (1976) and by Cannell et al. (1980) have shown that the availability of nitrogen in the soil can prevent the yield loss and the premature yellowing of leaves observed in cereals after waterlogging. The nitrogen nutrition status of the shoot may influence degradation and remobilisation of proteins in leaves.

In the experiments reported here, waterlogging of field grown wheat plants started at anthesis, when the vegetative growth of the shoot was mostly completed and the grains represented the growing parts of the shoot. Changes in the pattern of peptide hydrolase activities and the catabolism of proteins and of chlorophyll were used to judge the effect of waterlogging on the time course of senescence in the three uppermost leaves.

Materials and methods

Winter wheat (*Triticum aestivum* L., cv. Arina) was grown in pots in a field near Bern, as described previously (Stieger and Feller, 1994). The three uppermost leaf laminas were collected weekly between anthesis and harvest from two different pots for each treatment. The fresh weight was determined and leaves were stored at -20°C.

Each lamina was homogenised in 4 mL extraction medium containing 20 m*M* sodium-phosphate buffer pH 7.5, 1 % (w/v) polyvinylpolypyrrolidone and 0.1 % (v/v) β-mercaptoethanol with a polytron mixer (Kinematica, Luzern), first for 10 s at medium speed and then for 5 s at full speed. The homogenates were filtered through Miracloth (Calbiochem, San Diego) and the filtrates were used for chlorophyll measurements according to Strain et al., 1971). For SDS-PAGE samples the filtrates were mixed with sample buffer and kept in a boiling water bath for 5 min. Gel electrophoresis was carried out in a Mini Protean II Dual Slab Cell (BIORAD, Richmond) according to Laemmli (1970) using 0.75 mm,thick slab gels (12.5 %). Proteins were visualised by staining with Coomassie Brillant Blue R-250.

The Miracloth filtrate was centrifuged at 12000 *g* for 10 min and the supernatant was used for the determination of soluble proteins by the method of Bradford (1976), using γ-globulin as the standard and for the determination of free amino groups with a ninhydrin reagent according to Cramer (1958). This reagent was prepared by mixing 50 mL 1 *N* NaOH, 50 mL 2 *N* acetic acid and 100 mL 2-methoxyethanol, then dissolving 1 g ninhydrin and finally adding 0.1 g $SnCl_2$ (after complete dissolution of ninhydrin). Sample or standard solutions (0.25 mL containing 0–0.15 μmol free amino groups) were mixed in reagent tubes with 0.5 mL ninhydrin reagent. These tubes were placed for 20 min in a boiling water bath. After cooling to room temperature, 0.5 mL 50 % (v/v) n-propanol were added. The solutions were mixed and 30 min later optical densities were read at 578 nm. Alanine (0–0.15 μmol per tube) was used as a standard.

The supernatant mentioned above was also used for the quantification of soluble carbohydrates with a method modified after Fluri (1959). Standard solution or supernatant (300 μL) were mixed with 1 mL anthron reagent (40 mL ethanol, 400 mg anthron, 200 mL H_2SO_4). After 10 min incubation in a boiling water bath the samples were cooled for 10–60 min and the

optical densities were read at 623 nm. Glucose (0–50 μg in 300 μL H_2O) was used for calibration.

For peptide hydrolase assays the supernatants were desalted bycentrifugation through Sephadex G-25 columns equilibrated with 20 m*M* sodium phosphate buffer pH 7.5, as described previously (Feller et al., 1977). For the endopeptidase assay, azocasein (1 % w/v) was dissolved in 200 m*M* sodium acetate buffer pH 5 or in 200 m*M* bis-Tris-propane buffer adjusted with HCl to pH 9. Desalted extract (100 μL) was mixed with 100 μL azocasein solution and incubated in a microtitration plate with U-shaped wells for 3 h at 37°C. The reaction was stopped with 40 μL TCA (30 %). The plates were kept for 15 min at 0–4°C and then centrifuged at 750 *g* for 25 min. The supernatant was mixed with 1 *N* NaOH (1:1) in a microtitration plate with flat wells and the optical densities were read at 450 nm with a multichannel photometer (modified after Salgo and Feller, 1987). The activities of aminopeptidases were measured in microtitration plates with amino acid p-nitroanilides (leucine, glycine and alanine as substrates) according to Blättler and Feller (1988).

For the analyses of nutrient contents, leaf laminas of the flag leaf were collected weekly, dried at 105 °C, weighted and heated in glass tubes for 8 h at 550 °C. After cooling 0.2 mL HCl (10 *N*) and 7.8 mL H_2O were added to each tube. The solutions were mixed and diluted with 1.267 g L^{-1} CsCl suprapur in 0.1 *N* HCl (for potassium), with 13.37 g L^{-1} $LaCl_3$. $7H_2O$ in 0.1 *N* HCl (for calcium) and with 0.1 N HCl (for iron and manganese) prior to measuring the elements by atomic absorption spectrometry.

All results are expressed per leaf lamina. This basis is not subject to changes during senescence and is therefore more suitable for such experiments than the fresh weight or the leaf area. The three uppermost leaves were fully expanded at the beginning of permanent waterlogging (Fig. 1). The dry matter per leaf lamina was analysed on August 5 (final harvest) in a separate set of plants, since this value cannot be determined in the samples used for the extraction of enzymes. The means±SD of 4 replicates were computed for the flag leaf (control: 73±17 mg; flooded: 69±24 mg), for the second (control: 67±8 mg; flooded: 67±17 mg) and for the third leaf (control: 39±9 mg; flooded: 48±10 mg). These results indicate that the dry matter per lamina was similar for control and flooded plants throughout the maturation period.

Results

A sequential loss of turgidity was observed in the three uppermost leaves of control plants (Fig. 1). The fresh weight per lamina decreased initially in the third leaf from the top, while the flag leaf remained turgid longer. The sequential senescence of the three uppermost leaves also became evident from the net degradation of chlorophyll and soluble proteins (Fig. 2). Waterlogging after anthesis caused no major changes in the time course of senescence in the third leaf from the top. Senescence was in the second leaf slightly, and in the flag leaf considerably, accelerated by this treatment, as jugded by the decreases in protein and chlorophyll contents. From these results it can be concluded that the two uppermost leaves were affected soon after the beginning of flooding.

Iron and manganese are solubilised in waterlogged soil (Ponnamperuma, 1984) and larger quantities of these micronutrients may reach the shoots of crop plants. Although increased contents of iron and manganese have been detected in whole wheat shoots (Stieger and Feller, 1994), the levels in the flag leaf lamina were not or only slightly influenced (Table 1). It appears, therefore, unlikely that increased iron or manganese fluxes into this leaf blade affected its senescence behaviour.

Proteins in the extracts of leaf blades were separated by SDS-PAGE and quantities as well as the patterns of treated plants and controls were very similar 4 days before the beginning of volontary flooding. As reported previously (Stieger and Feller, 1994), a minor hypoxia was detected in intact pots after heavy rain prior to the onset of permanent waterlogging. The initial protein contents (Fig. 2) and the initial protein patterns identified by SDS-PAGE (data not shown) were similar in perforated and intact pots and indicate that this minor hypoxia caused no major changes in protein metabolism. However, already after three days of waterlogging the band intensities on the stained gel were decreased in the flag leaf, but the pattern was still comparable to the control. The accelerated loss of all major bands was more pronounced after waterlogging for 10 and 17 days. Flooding caused only minor differences in the second, and essentially no differences in the third leaf from the top. The SDS-PAGE analyses suggest that the changes in the protein pattern during senescence were similar for waterlogged and control plants, but flooding accelerated senescence and net protein degradation in the two uppermost leaves.

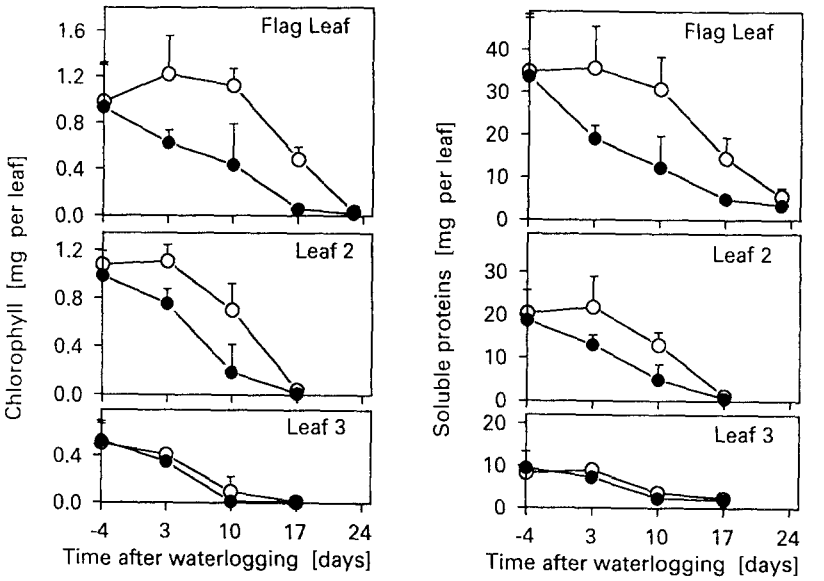

Fig. 2. Effect of waterlogging on chlorophyll and soluble protein contents in the three uppermost leaf laminas. Waterlogging started June 28 (day 0) and was maintained throughout the grain filling period. Means of 4 replicates are shown for control pots (○) and waterlogged pots (●). Standard errors are shown when exceeding the size of the symbol (on one side only for clarity).

Table 1. Effect of waterlogging on nutrient contents in the flag leaf lamina. Waterlogging started June 28 (day 0) and was maintained throughout the grain filling period. Means±SD of 4 replicates are shown. Significant differences (t-test) at the 5% level (*) are indicated for each harvest date (ns: non significant)

Nutrient	Treatment	Days after waterlogging				
		-4	3	10	17	24
Calcium	Control	1307±314	1170±270	1331±556	1526±537	1189±553
(μg per leaf)	Flooded	1493±439 ns	1439±559 ns	1169±98 ns	1009±126 ns	1062±187 ns
Potassium	Control	1791±883	2056±427	1161±605	2060±716	958±306
(μg per leaf)	Flooded	2508±655ns	1851±622ns	1717±331 ns	741±324*	549±406ns
Iron	Control	11± 5	9± 2	10± 2	11± 1	9± 3
(μg per leaf)	Flooded	13± 5 ns	12± 1 *	14± 3 *	19± 7 ns	16± 8 ns
Manganese	Control	10± 5	11 ± 9	9± 1	9± 7	8± 5
(μg per leaf)	Flooded	12± 5 ns	11 ± 0 ns	10± 4 ns	7± 3 ns	10± 6 ns

No accumulation of amino acids was detected during protein remobilisation (Fig. 3). The contents of free amino acids and of soluble carbohydrates suggest that in plants grown on waterlogged soil, as well as in control plants, low molecular weight compounds were rapidly exported from the leaf laminas. The levels of amino acids remained lower in treated plants than in control plants even during the phase of accelerated protein remobilisation. Individual amino acids were not quantified in this study, since the total content of amino acids reacting with the ninhydrin reagent remained low throughout senescence. However, it must be considered that the nitrogen in some amino acids (e.g. proline, asparagine, glutamine) is underestimated by the procedure used and the amino acid levels reported here allow therefore only a rough comparison. The low contents of the phloem-mobile macronutrient potassium in the flag leaf of treated plants after waterlogging for 17

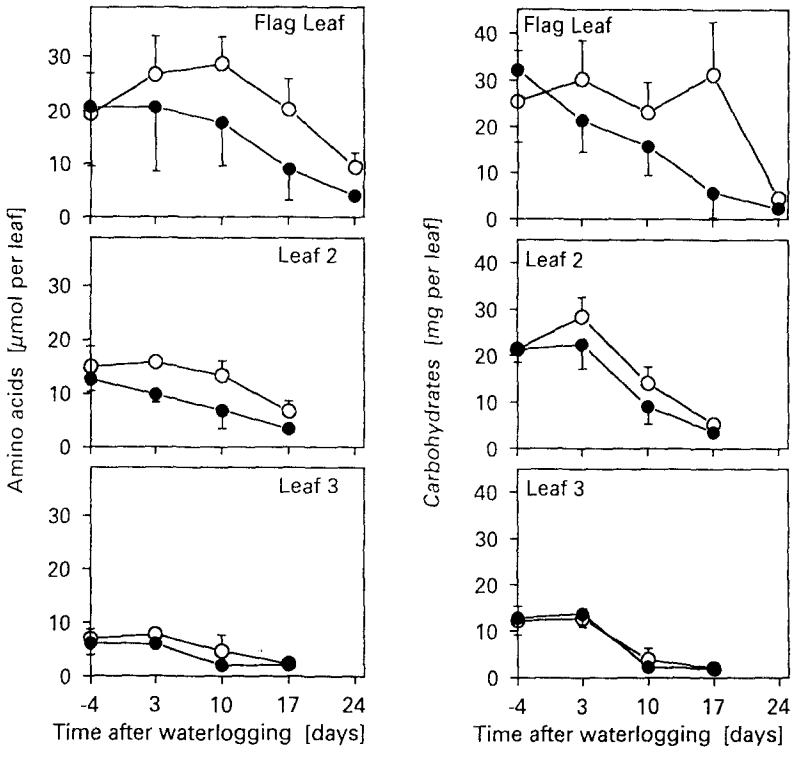

Fig. 3. Effect of waterlogging on contents of soluble carbohydrates and amino acids in the three uppermost leaf laminas. Waterlogging started June 28 (day 0) and was maintained throughout the grain filling period. Means of 4 replicates are shown for control pots (○) and waterlogged pots (●). Standard errors are shown when exceeding the size of the symbol (on one side only for clarity).

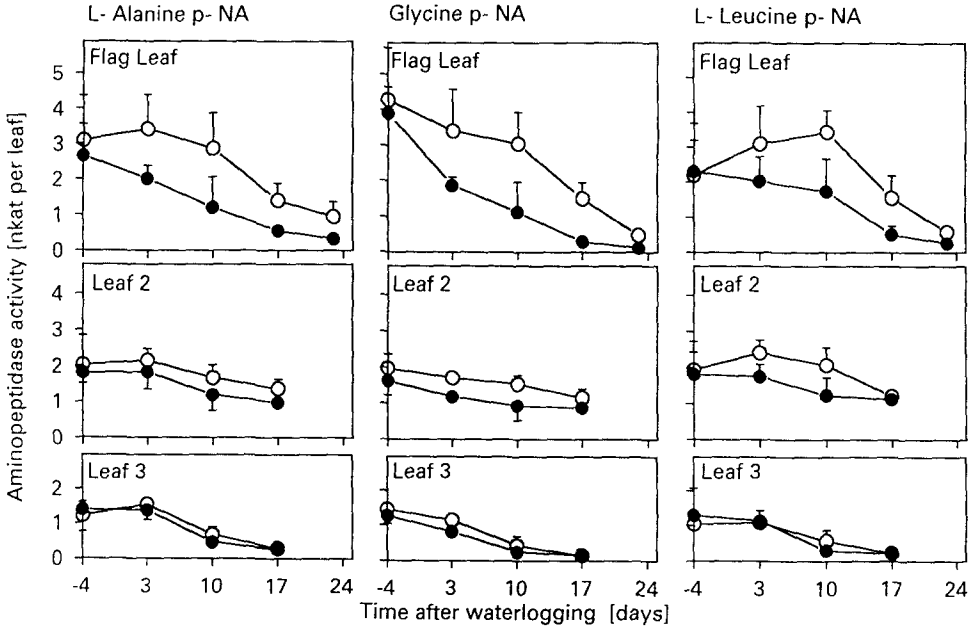

Fig. 4. Effect of waterlogging on aminopeptidase activities in the three uppermost leaf laminas. Waterlogging started June 28 (day 0) and was maintained throughout the grain filling period. Three different amino acid p-nitroanilides (p-NA) were used as substrates. Means of 4 replicates are shown for control pots (○) and waterlogged pots (●). Standard errors are shown when exceeding the size of the symbol (on one side only for clarity).

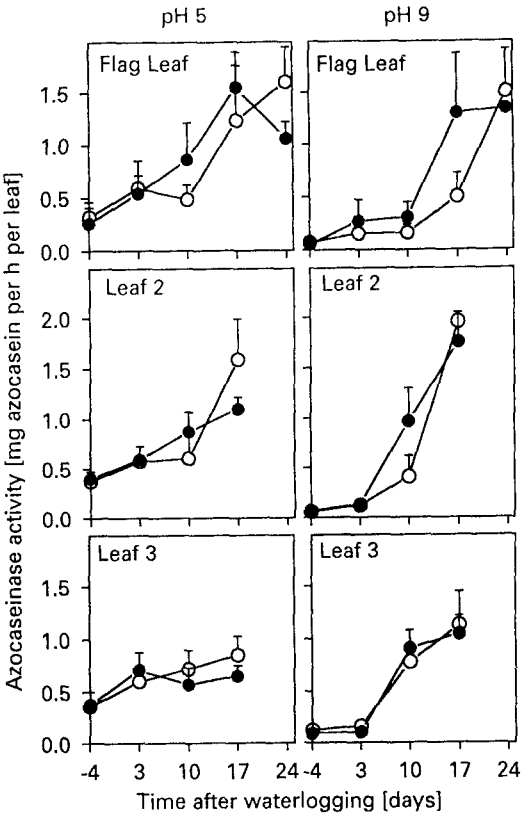

Fig. 5. Effect of waterlogging on azocaseinase activities in the three uppermost leaf laminas. Waterlogging started June 28 (day 0) and was maintained throughout the grain filling period. Means of 4 replicates are shown for control pots (○) and waterlogged pots (●). Standard errors are shown when exceeding the size of the symbol (on one side only for clarity).

days supports the hypothesis that export via the phloem was fully functional (Table 1). No major differences were observed for the phloem-immobile macronutrient calcium.

Aminopeptidase activities analysed with various substrates decreased earlier in the flag leaf lamina on waterlogged soil than on control pots (Fig. 4). Endopeptidases reached highest activities during senescence (Fig. 5). The activity at pH 5 peaked earlier than the activity in the alkaline pH-range. The activities at both pH-values were maximal at a late phase of senescence (Fig. 5), when a considerable percentage of the proteins was already degraded (Fig. 2). The endopeptidase activities increased in the flag leaf of flooded plants earlier than in controls, while the second leaf from the top was less affected by the treatment, and essentially no effect was detected in the third leaf.

Discussion

Symptoms of accelerated leaf senescence were already detected three days after the beginning of permanent waterlogging. The flag leaf and the second leaf from the top were affected simultaneously, whereas the third leaf was already in an advanced stage of senescence and was not influenced by the treatment. Waterlogging synchronised senescence in the two uppermost leaves, which senesced sequentially in control plants.

Drought stress caused lower protein contents and increased total endopeptidase activities in the leaves of *Phaseolus vulgaris* (Roy-Macauley et al., 1992). In the experiments reported here, the decreasing water content in the flag leaf and the second leaf after waterlogging suggest water stress, which could have led to an earlier protein and chlorophyll degradation. An increase in endopeptidase activities first in the slightly acid and later in the alkaline pH-range have been observed after the decrease of the fresh weight. The question as to which extent the net protein degradation and the increase in these endopeptidase activities are linked remains open.

After anthesis the developing grains represent strong sinks within the shoot. It appears therefore unlikely that a reduced sink capacity in the roots, and as a consequence the accumulation of metabolites in the leaf blades, caused senescence. Oxygen deficiency in the soil may directly influence the nutrient availability in the soil solution, the energy metabolism in the roots and the acquisition of nutrients by the plants. In particular, the macronutrient nitrogen may become less available (Cannell et al., 1980; Stieger and Feller, 1994; Watson et al., 1976). Flooding can indirectly influence leaf senescence by an altered delivery of solutes from the roots to the shoot via the xylem. The nature of the senescence-inducing signal reaching the leaf laminas has not yet been identified in our system. Nutrient and hormone fluxes from the roots to the leaves have been considered as regulatory factors at the whole plant level, but the network of causes and symptoms remains to be elucidated (Jackson, 1991; Neuman et al., 1990; Zhang and Davies, 1986, 1987).

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References

Blättler R and Feller U 1988 Identification and stability of aminopeptidases in extracts from bean seeds. Aust. J. Plant Physiol. 15, 613–619.

Bradford K J and Hsiao T 1982 Stomatal behavior and water relations of waterlogged tomato plants. Plant Physiol. 70, 1508–1513.

Bradford M M 1976 A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein dye binding. Anal. Biochem. 72, 248–254.

Buwalda F, Barrett-Lennard E G L, Greenway H and Davies B A 1988 Effects of growing wheat in hypoxic nutrient solution and of subsequent transfer to aerated solutions. II. Concentrations and uptake of nutrients and sodium in shoots and roots. Aust. J. Plant Physiol. 15, 599–612.

Cannel R Q, Belford R K, Gales K, Dennis C W and Prew R D 1980 Effects of waterlogging at different stages of development on the growth and yield of winter wheat. J. Sci. Food Agric. 317, 117–132.

Cramer F 1958 Papierchromatographie. Verlag Chemie, Weinheim. 102 p.

Dungey N O and Davies D D 1982 Protein turnover in isolated barley leaf segments and the effect of stress. J. Exp. Bot. 33, 12–20.

Feller U 1986 Proteolytic enzymes in relation to leaf senescence. *In* Plant Proteolytic Enzymes Volume II. Ed. M J Dalling. pp 49–68. CRC Press Inc., Boca Raton, Florida, USA.

Feller U 1990 Nitrogen remobilisation and protein degradation during senescence. *In* Nitrogen in Higher Plants. Ed. Y P Abrol. pp 195–222. Research Studies Press Ltd., Somerset, England.

Feller U and Keist M 1986 Senscence and nitrogen metabolism in annual plants. *In* Fundamental, Ecological and Agricultural Aspects of Nitrogen Metabolism in Higher Plants. Eds. H Lambers, J J Neeteson and I Stulen. pp 219–234. Martinus Nijhoff Publishers Dordrecht, the Netherlands.

Feller U K, Soong T-S T and Hageman R H 1977 Leaf proteolytic activities and senescence during grain development of field-grown corn (*Zea mays* L.). Plant Physiol. 59, 290–294.

Fluri R 1959 Der Kohlenhydrathaushalt eines Schleimpilzes (*Phycomyces blakesleanus*) in Abhängigkeit von den Kulturbedingungen. Arch. Microbiol. 33, 195–222.

Huffaker R C 1990 Proteolytic activity during senescence of plants. New Phytol. 116, 199–231.

Jackson M B 1991 Regulation of water relationships in flooded plants by ABA from leaves, roots and xylem sap. *In* Abscisic Acid; Physiology and Biochemistry. Eds. W J Davis and H G Jones. pp 217–226. Bios. Scientific, Oxford, England.

Jackson M B, Gales K and Campbell D J 1978 Effect of waterlogged soil conditions on the production of ethylene and on water relationships in tomato plants. J. Exp. Bot. 29, 183–193.

Jackson M B and Kowalewska A K B 1983 Positive and negative messages from roots induce foliar desiccation and stomatal closure in flooded pea plants. J. Exp. Bot. 34, 493–506.

Jackson M B, Young S F and Hall K C 1988 Are roots a source of abscisic acid for the shoots of flooded pea plants? J. Exp. Bot. 39, 1631–1637.

Keist M and Feller U 1985 Changes in the endopeptidase pattern of wheat leaves during senescence. Botanica Helvetica 95, 73–80.

Laemmli U 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Neuman D S, Rood S B and Smit B A 1990 Does cytokinin transport from root-to-shoot in the xylem sap regulate leaf responses to root hypoxia? J. Exp. Bot. 41, 1325–1333.

Noodén L D 1988 The phenomena of senescence and aging. *In* Senescence and Aging in Plants. Eds. L D Noodén and A C Leopold. pp 1–50. Academic Press Inc., San Diego, USA.

Peoples M B and Dalling M J 1988 The interplay between proteolysis and amino acid metabolism during senescence and nitrogen reallocation. *In* Senescence and Aging in Plants. Eds. D L Noodén and A C Leopold. pp 181–239. Academic Press Inc., San Diego, USA.

Ponnamperuma F N 1984 Effects of Flooding on Soil. *In* Flooding and Plant Growth. Ed. T T Kozlowski. pp 9–45. Academic Press, London, Great Britain.

Roy-Macauley H, Zuily-Fodil Y, Kidric M, Pham Thi A T and Vieira da Silva J 1992 Effect of drought stress on proteolytic activities in *Phaseolus* and *Vigna* leaves from sensitive and resistant plants. Physiol. Plant. 85, 90–96.

Salgo A and Feller U 1987 Rapid micro methods for the determination of exo- and endopeptidase activities in plant extracts. Microchem. J. 35, 12–21.

Stieger P A and Feller U 1994 Nutrient accumulation and translocation in maturing wheat plants grown on waterlogged soil. Plant and Soil 160, 87–95.

Strain H H, Cope B T and Svec W A 1971 Analytical procedures for the identification, estimation and investigation of the chlorophylls. *In* Methods of Enzymology 23. Ed. A San Pietro. pp 452–487. Academic Press Inc, New York, USA.

Trought M C T and Drew M C 1980 The development of waterlogging damage in wheat seedlings (*Triticum aestivum* L.) II. Accumulation and redistribution of nutrients by the shoot. Plant and Soil 56, 187–199.

Van der Valk H C P M and Van Loon L C 1988 Subcellular localization of proteases in developing leaves of oats (*Avena sativa* L.). Plant Physiol. 87, 536–541.

Watson E R, Lapins P and Barron R J W 1976 Effect of waterlogging on the growth, grain and straw yield of wheat, barley and oats. Aust. J. Exp. Agric. Anim. Husb. 16, 114–122.

Wittenbach V A, Lin W and Hebert R R 1982 Vacuolar localization of proteases and degradation of chloroplasts in mesophyll protoplasts from senescing primary wheat leaves. Plant Physiol. 69, 98–102.

Zhang J and Davies W J 1986 Chemical and hydraulic influences on the stomata of flooded plants. J. Exp. Bot. 37, 1479–1491.

Zhang J and Davies W J 1987 ABA in roots and leaves of flooded pea plants. J. Exp. Bot. 38, 649–659.

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