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Photosynthesis, Leaf Resistances, and Ribulose-1,5-Bisphosphate Carboxylase Degradation in Senescing Barley Leaves[1]

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ABSTRACT

The relationship between loss of ribulose-1,5-bisphosphate carboxylase (RuBPCase) and the decline in photosynthesis during the senescence of barley primary leaves was assessed. Loss of RuBPCase accounted for about 85% of the decrease in soluble protein. RuBPCase was highly correlated with *in vitro* RuBPCase activity (r = 0.95) and gross photosynthesis (r = 0.96). However, the rate of photosynthesis per milligram RuBPCase increased during the early stages of leaf senescence. The concentration of nonreducing sugars was negatively correlated (1% level) with photosynthesis. Free α-amino N, in contrast to nonreducing sugars, declined markedly during senescence. A decrease in chlorophyll and an increase in *in vitro* protease activity was observed, but these changes did not appear to be closely related to the decline in photosynthesis and RuBPCase. Mesophyll resistance increased at the same rate that photosynthesis and RuBPCase declined. Stomatal resistance increased more rapidly than mesophyll resistance and accounted for about 24% of the total increase in resistance to CO_2 diffusion. The concentration of CO_2 in the intercellular air spaces decreased during the last stage of senescence. Although loss of RuBPCase probably is the primary event responsible for the decline in photosynthesis during leaf senescence, other factors such as *in vivo* regulation and stomatal aperture must also be considered.

The senescence of leaves is usually associated with loss of soluble protein, predominantly RuBPCase[2] (5, 12, 13, 21, 25, 26). Protein degradation and remobilization provide an important source of N and S for other parts of the developing plant (4, 21). The relationship between the function of RuBPCase as a storage protein (7, 12) and its catalytic function has not been well defined. Loss of RuBPCase during leaf senescence is usually accompanied by a loss in *in vitro* RuBPCase activity and a decline in CER (5, 12, 13, 21, 22, 25, 26). It is generally assumed that RuBPCase breakdown results in an increase in mesophyll resistance and a decline in gross photosynthesis, a component of CER. However, the concentration of RuBPCase catalytic sites in leaves can be as high as 3 mM (G. Lorimer, personal communication). Thus, RuBPCase concentration may not always limit *in vivo* RuBPCase activity and photosynthesis. The decline in CER is probably due not only to a decrease in gross photosynthesis but also to an increase in photorespiration (22). In addition, the decrease in CER may not be entirely a result of an increase in mesophyll resistance, as previously reported (26), but may be partially due to an increase in stomatal resistance (22, 24). In fact, stomatal aperture has been recently suggested as one of the main controlling factors in senescence (17). To date, no comprehensive study has been reported of the relationship between RuBPCase degradation and alterations in gross photosynthesis (as opposed to CER) and stomatal and mesophyll resistance. In the present study, all of these parameters were measured and were related to changes in RuBPCase activity, soluble protein, protease activity, total free amino acids, Chl, and carbohydrates. These measurements were made on intact leaves of barley (*Hordeum vulgare* L.). Although detached leaves are commonly used to study senescence, recent reports (9, 19) have demonstrated that detached leaves do not necessarily senesce in the same manner as intact leaves.

MATERIALS AND METHODS

Plant Culture. One hundred barley (*H. vulgare* L., var. Numar) seeds were planted at a depth of 2 cm in plastic pots (13.5 cm in diameter × 15.0 cm tall) containing Vermiculite. Each pot received 800 ml of nutrient solution at planting. Additional nutrient solution (100 ml/day) and distilled H_2O were supplied via cotton wicks extending from the bottom of each pot into a 1-liter glass jar. The nutrient solution contained, in mmol/l: $Ca(NO_3)_2$, 5; KNO_3, 1; K_2SO_4, 2; $MgSO_4$, 4; $NH_4H_2PO_4$, 2; and in μmol/l: $MnSO_4$, 18.3; H_3BO_3, 8.0; $ZnSO_4$, 3.8; $CuSO_4$, 1.5; $(NH_4)_6Mo_7O_{24}$, 0.1; NaCl, 28.2; and Fe as Fe-ethylenediamine di-(*O*-hydroxyphenylacetate), 110.4. Light intensity at pot height in the growth chamber was 550 μE m^{-2} s^{-1} as provided by a mixture of incandescent and metal halide lamps. Air temperature during the 15-h day was 24 ± 0.5 C, with a RH of 84 ± 2 %. Temperature during the 9-h night was 12.5 ± 0.5 with a RH of 84 ± 2 %.

Purification of RuBPCase. RuBPCase was extracted from primary leaves of barley grown in continuous light for 7 days. A 10-g sample of leaf blades was ground (mortar and pestle) in 20 ml of 0.50% (w/v) BSA in 0.2 M Tris-SO_4 (pH 8.0). The homogenate was filtered through four layers of cheesecloth and made to 35% saturation with $(NH_4)_2SO_4$. After centrifugation at 27,000*g* for 15 min, the supernatant was made to 60% saturation, centrifuged again, and the precipitate resuspended in 2 ml of 0.08% (v/v) mercaptoethanol, 2 mM EDTA, and 100 mM Hepes (pH 7.8). The redissolved precipitate was then eluted through a 1.5- × 30-cm column of Sephadex G-200 with the Hepes buffer diluted to half-strength. A sample of 3–4 ml of the yellow solution that eluted with the void volume was collected and mixed with a 30-ml bed of preswollen DEAE cellulose equilibrated with half-strength Hepes buffer. Several bed volumes of half-strength Hepes buffer were added, and the DEAE-cellulose was allowed to settle. The supernatant was then drawn off, and the washing procedure was repeated at least twice. Finally, the DEAE-cellulose was washed twice with several bed volumes of half-strength Hepes buffer containing 0.2 M $(NH_4)_2SO_4$. The eluted protein was then precipitated in 60% $(NH_4)_2SO_4$ and stored at −15 C until use. All of the

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[2] Abbreviations: RuBPCase: ribulose-1,5-bisphosphate carboxylase; CER: carbon exchange rate.

foregoing operations were performed at 0–4 C. The protein (usually about 20 mg) isolated by this procedure was essentially pure RuBPCase as determined by polyacrylamide gel electrophoresis.

Preparation of RuBPCase-Specific Antibody. The $(NH_4)_2SO_4$-precipitated RuBPCase was pelleted (27,000*g*, 15 min) and resuspended in a small volume of 0.2 M Tris-SO_4 (pH 8.0). The RuBPCase was then mixed 1:1 with Freund's complete adjuvant (Difco, Detroit). A 1-ml sample containing at least 0.5 mg of RuBPCase was injected subcutaneously behind the shoulder blades of each of four New Zealand rabbits. After 1 month, a second injection was given using Freund's incomplete adjuvant. One week later, a sample of blood was collected from the marginal ear vein of each rabbit. The specificity of the antibody was verified using Ouchterlony diffusion plates and by polyacrylamide gel electrophoresis of the immunoprecipitates after SDS treatment. Additional serum was collected by terminal cardiac puncture and stored at −15 C. Each rabbit yielded enough serum for at least 300 assays of RuBPCase.

Measurement of Photosynthesis, Transpiration, and Leaf Resistances. The uptake of $^{14}CO_2$ and ^3H_2O was measured in the growth chamber with a dual-isotope diffusion porometer as described by Ting and Hanscom (23). Uptake was measured for a 23-mm^2 circular area on the lower surface of the primary leaf, about 6 cm from the tip. Exposure time was 20 s, after which a 12-mm^2 leaf punch was quickly collected and placed in a scintillation vial containing 1 ml of 6% (v/v) H_2O_2 and 80% (v/v) methanol. The vials were tightly capped and allowed to remain for three days at room temperature. The samples were counted in 10 ml of Bray's scintillation cocktail (3) with a scintillation spectrometer. Five leaf blades from each replication were assayed. All assays were conducted between 2.5 and 4.0 h after the start of the light period. Gross photosynthesis, transpiration, mesophyll resistance, and stomatal resistance (includes the relatively small boundary layer resistance) were calculated according to Ting and Hanscom (23). The temperature of the lower leaf surface was measured with a thermocouple (Vaisalla, Helsinki). All data were expressed on a leaf-area basis. The specific leaf weight varied little during the experiment. Stomatal frequency was nearly the same for both the upper and lower leaf surfaces.

Extraction and Assay of Enzymes and Chemical Constituents. Extraction of enzymes and other cell constituents was performed 3.5–5 h after the start of the light period. Enzyme extractions were performed at 0–4 C, whereas Chl, α-amino acids, and carbohydrates were extracted at room temperature.

Protease and RuBPCase were extracted by grinding (mortar and pestle) a 1.0-g sample of the apical 12 cm of primary leaves in 3.0 ml of buffer (0.04% [v/v] mercaptoethanol in 0.2 M Tris-SO_4 [pH 8.0]). Insoluble PVP (0.1 g) was added to each leaf sample prior to homogenization. Each homogenate was centrifuged at 27,000*g* for 10 min. Triplicate 0.1-ml aliquots of the supernatant were mixed with 5 ml of 5% trichloroacetic acid and stored at 4 C until assayed for soluble protein (8). Another aliquot of the supernatant was diluted 1:5 with Tris-mercaptoethanol buffer for determination of RuBPCase concentration and activity. A third aliquot was diluted 1:15 with 0.1 M Tris-malate (pH 5.5) for assay of protease activity.

RuBPCase activity was measured as prescribed by Jensen and Bahr (6). Duplicate 0.1-ml aliquots of the diluted extract were incubated (30 C, 10 min) with 0.1 ml of 44 mM $MgCl_2$, 22 mM $NaH^{14}CO_3$ (0.5 μCi/μmol), and 55 mM Tris-SO_4 (final pH 8.0). The reaction was initiated by the addition of 0.02 ml of 10 mM RuBP and terminated after 2 min by addition of 0.05 ml of 2 N HCl. Duplicate 0.05-ml aliquots from each assay mixture were counted in 10 ml of Aquasol (New England Nuclear, Boston) with a scintillation spectrometer.

RuBPCase concentration was determined for triplicate 0.05-ml aliquots of the diluted extract. Before use, thawed rabbit serum was incubated (56 C, 1 h) to inactivate complement. Serum (0.3 ml) was then added to each aliquot of RuBPCase extract or to a Tris-mercaptoethanol buffer blank. After incubation (37 C, 2 h), 1 ml of saline solution (0.85% w/v NaCl) was added and the mixtures stored overnight at 4 C. The immuno-precipitates were centrifuged (1,100*g*, 15 min), the saline carefully removed, and another 1 ml of saline added. After four washings, the precipitates were assayed for protein using BSA as a standard (8). A standard curve was prepared using purified RuBPCase. This standard curve was similar to that reported previously (7).

Protease activity was assayed using azocasein (Sigma) as a substrate. A 0.6-ml aliquot of the diluted extract was incubated (37 C, 2 h) with 0.4 ml of azocasein (10 mg/ml). Blanks were prepared by adding 1.0 ml of 14% $HClO_4$ immediately after the azocasein. After termination of the reaction with $HClO_4$, the samples and blanks were placed on ice for 10 min, centrifuged (1,100*g*, 10 min), and the *A* of the supernatant measured at 340 nm. When extracted and measured under the foregoing optimum conditions, barley proteases hydrolyze a wide variety of substrates (*e.g.* casein, azocasein, BSA, hemoglobin) with no apparent specificity for the natural substrate, RuBPCase (B. Miller, unpublished data). Azocasein hydrolysis was measured because of the ease, sensitivity, and reproducibility of this assay.

Chl, free amino acids, and soluble sugars were extracted by grinding (mortar and pestle) 1 g of leaves (apical 12 cm) in 5 ml of 80% (v/v) ethanol. The extract was filtered through Whatman No. 1 filter paper. The filters were washed with 80% ethanol to obtain a final volume of 50 ml. The Chl concentration was determined by dividing the *A* (660 nm) by 1.52 to obtain the *A* (652) in 80% acetone and then using the equation of Arnon (1). Free α-amino N was determined by the method of Moore (10). The remainder of the extract (44 ml) was heated to evaporate the ethanol, filtered through Whatman No. 1 filter paper, and made to a 100-ml volume. Reducing sugars and total soluble sugars (after acid hydrolysis, [14]) were assayed (11). Acid hydrolysis yielded the same quantity of nonreducing sugars as did treatment with baker's yeast invertase (Sigma). However, this does not preclude the existence of an invertase-labile fructosan (16).

Statistical Analysis. The experiment was set up in a completely randomized design with three replications, each replication being a single pot of barley. Treatments consisted of eight samplings arranged at 2-day intervals from 8 to 22 days after planting. A LSD (0.05 level) was calculated for each data set. The F-ratio was always significant. Changes in the measured parameters were described by regression analysis and usually followed apparent first-order kinetics. Observations made in this experiment have been substantiated in separate experiments.

RESULTS AND DISCUSSION

Total soluble protein (per g fresh weight or per leaf) in the primary leaf of barley declined with increasing leaf age, with the greatest loss occurring 12–18 days after planting (Fig. 1A). During this period, 85% of the loss was due to the loss of RuBPCase. Non-RuBPCase protein did not decline at the same rate as RuBPCase; consequently, the proportion of soluble protein constituted by RuBPCase was highest (74–81%) 10–14 days after planting (Fig. 1A). RuBPCase reportedly comprises about half of the soluble protein in the barley primary leaf (2, 12). The particular growth conditions (saturating light, greater nutrient availability, etc.) employed in this experiment may explain the unusually high initial RuBPCase proportion. High light intensity will increase the total amount of RuBPCase and the proportion of soluble protein as RuBPCase (2). Furthermore, we have observed (unpublished data) that RuBCPase comprises about half of the soluble leaf protein of barley grown in continuous light of a lower intensity (400 $\mu E\ m^{-2}\ s^{-1}$) than employed here (550 $\mu E\ m^{-2}\ s^{-1}$).

The diminution in RuBPCase (Fig. 1A) was accompanied by a

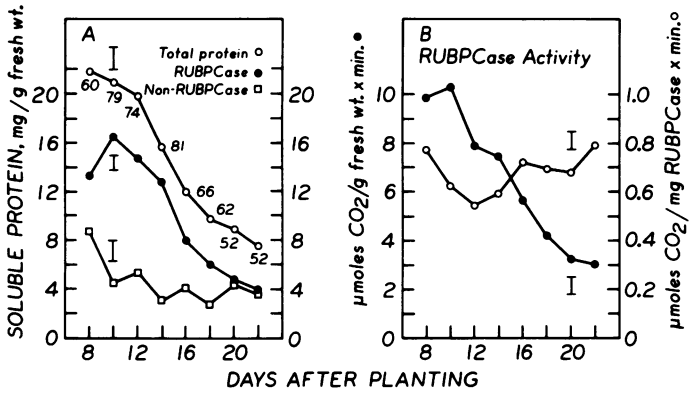

FIG. 1. Total protein (A), RuBPCase (A), non-RuBPCase protein (A), and activity of RuBPCase (B) in senescing barley primary leaves. Proportion of total protein constituted by RuBPCase is depicted for each total protein value in A. Bars represent LSD (0.05 level) for each parameter. Leaf fresh weight was constant (0.14 g/leaf) throughout the experiment.

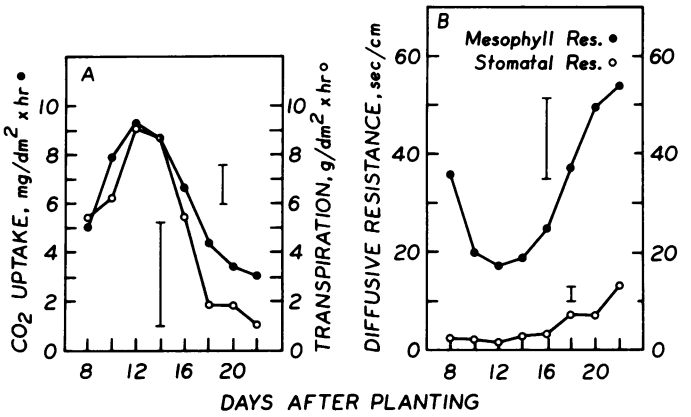

FIG. 2. Gross photosynthesis or CO_2 uptake (A), transpiration (A), and mesophyll and stomatal resistance to CO_2 diffusion (B) in senescing barley primary leaves. Bars represent LSD (0.05 level) for each parameter.

loss in RuBPCase activity expressed on a fresh weight basis (Fig. 1B). Although RuBPCase and RuBCPase activity were highly correlated (r = 0.95), RuBPCase specific activity increased significantly after day 12 (Fig. 1B). Wittenbach (25) observed a decline in the specific activity of RuBPCase when wheat (*Triticum aestivum* L.) primary leaves were detached and allowed to senesce in darkness. Likewise, RuBPCase specific activity in senescing barley primary leaves decreases when the leaves are detached (12) but increases when the leaves are left intact (13). These and other observations (9, 19) demonstrate that the senescence of detached leaves is not always analogous to the senescence of intact leaves.

Gross photosynthesis or CO_2 uptake (Fig. 2A) reached a maximum after RuBPCase and *in vitro* RuBPCase activity (fresh weight basis) had begun to decline (Fig. 1). Furthermore, gross photosynthesis per mg RuBPCase (Table I) was not constant, indicating that RuBPCase concentration was not the sole determinant of photosynthetic rate. Photosynthesis per mg RuBPCase was highest on day 16 and did not vary significantly thereafter. Hall *et al.* (5) observed a decline in net photosynthesis as wheat leaves senesced that was not associated with loss of RuBPCase protein. As discussed by Jensen and Bahr (6), both RuBPCase protein concentration and the *in vivo* regulation of RuBPCase probably influence leaf photosynthesis.

Mesophyll and stomatal resistance (Fig. 2B) increased 3- and 9-fold, respectively. Transpiration and true photosynthesis (Fig. 2A) decreased simultaneously during the period when the total leaf resistance to CO_2 diffusion was increasing. Stomatal resistance

Table I. *Photosynthesis per mg RuBPCase, the ratio of Photosynthesis to Transpiration, and the Concentration of CO_2 in the Intercellular Air Spaces [CO_2^{IAS}] in Senescing Barley Leaves*

External CO_2 concentration [CO_2^{Ext}] was 268 μl/l. [CO_2^{IAS}] = [CO_2^{Ext}] minus (photosynthesis × stomatal resistance).

Time after Planting	Photosynthesis per mg RuBPCase	Photosynthesis to Transpiration	CO_2^{IAS}
days	*nmol mg^{-1} min^{-1}*	*mg/g*	*μl/l*
8	88	1.08	250
10	110	1.46	244
12	144	1.20	248
14	156	1.30	245
16	193	1.66	237
18	166	2.61	222
20	168	2.02	232
22	178	3.12	210
LSD, 0.05	34	1.07	20

accounted for 24% of the total increase in resistance to CO_2 diffusion. The increase in stomatal resistance affected transpiration more than photosynthesis, as evidenced by the increase in the ratio of photosynthesis to transpiration (Table I). The intercellular CO_2 concentration (Table I), however, declined, suggesting that stomatal aperture was imposing some limitation on photosynthesis. During senescence, the conservation of water and maintenance of turgor may be more crucial to the leaf than photosynthesis.

In spite of the decline in photosynthesis, the concentration of soluble sugars, particularly nonreducing sugars, increased dramatically with increasing leaf age (Fig. 3A). Starch does accumulate in primary leaves of barley (unpublished data) or oats (16). A basipetal translocation of amino acids, but not soluble sugars, occurs when oat leaves senesce (19). Similarly, in this experiment, total soluble protein (Fig. 1A) and free α-amino acids (Fig. 3B) declined. These observations suggest that a selective inhibition of sucrose transport occurs during senescence. Photosynthesis (leaf area basis) and the concentration of nonreducing sugars were negatively correlated (1% level). The greatest decrease in photosynthesis (Fig. 2A) and the greatest increase in nonreducing sugars (Fig. 3A) occurred after day 16 when photosynthesis per mg RuBPCase had peaked (Table I). It is possible that the accumulation of nonreducing sugars results in a feedback inhibition of photosynthesis. Dark respiration increases during leaf senescence (16, 18, 19, 21, 26). The increase in soluble sugars and dark respiration during leaf senescence indicates that C is not conserved to the same extent as N.

An increase in protease activity is often associated with loss of soluble leaf protein during senescence (12, 21, 25). In this experiment, most of the increase in protease activity against azocasein occurred before day 12 (Fig. 3C) before any major loss of soluble protein (Fig. 1A). An increase in general protease activity is not prerequisite for loss of soluble protein during the senescence of *Pisum sativum* leaves (15) or barley leaves (9). Thus, it seems that some other metabolic event(s) such as a change in cell compartmentation or the action of a RuBPCase-specific protease might initiate the degradation of RuBPCase during senescence.

A visible yellowing and loss of Chl is probably the most well recognized symptom of leaf senescence. In this experiment, however, Chl loss (Fig. 3D) did not begin until day 14, 4 days after the initial loss of RuBPCase (Fig. 1A) and 2 days after the initial decline in photosynthesis (Fig. 2A). This suggests that the mechanism responsible for loss of Chl is not the same as that for RuBPCase and photosynthetic ability. Alternatively, Chl may be temporarily protected from hydrolysis because of its association

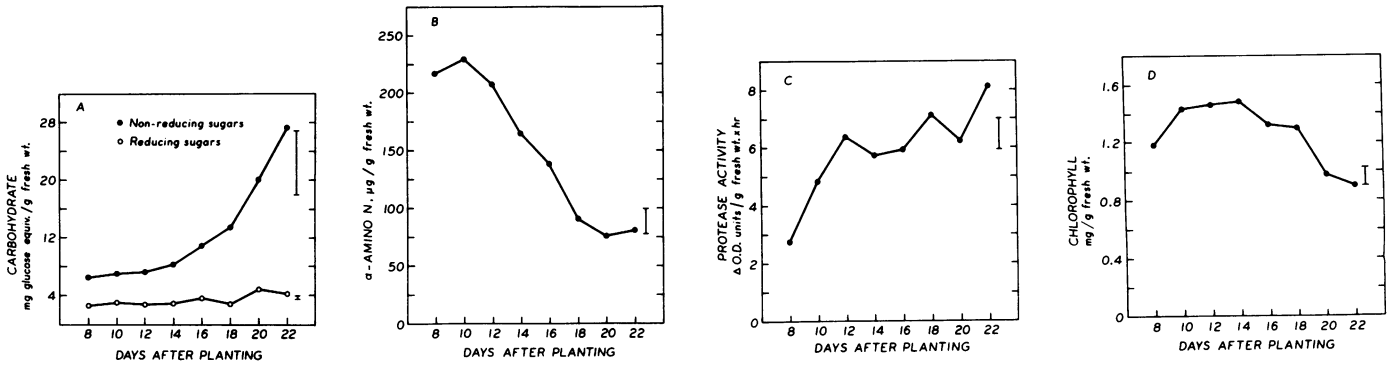

FIG. 3. Carbohydrates (A), α-amino N (B), protease activity (C), and Chl (D) in senescing barley primary leaves. Bars represent LSD (0.05 level) for each parameter. Leaf fresh weight was constant (0.14 g/leaf) throughout the experiment.

with the chloroplast thylakoid membrane. From day 12 to day 22 the decline in photosynthesis is significantly correlated with loss of Chl (r = 0.63) but is correlated even more highly with loss of RuBPCase (r = 0.96). Thomas and Stoddart (20) concluded that Chl loss is not an obligatory part of the senescence process.

The physiological parameters measured during leaf senescence fall into three classes based on rate of change (Table II). Transpiration and stomatal resistance (class I phenomena) changed more dramatically than any other parameter. The rapidity of the stomatal response suggests that hormonal influences may be important here. Application of hormones to senescing leaves has a pronounced effect on stomatal resistance (17) and phenomena intimately associated with CO_2 assimilation or evolution, such as RuBPCase (12, 25), RuBPCase activity (12), and dark respiration (16, 18). Class II phenomena include RuBPCase protein, gross photosynthesis, mesophyll resistance, RuBPCase activity, α-amino N, nonreducing sugars, and total soluble protein. These phenomena have Δ_{50}'s of 5–8 days and have great significance for general plant growth and metabolism. Importantly, the Δ_{50}'s for gross photosynthesis, mesophyll resistance, and RuBPCase were nearly the same indicating a coordinate relationship. Class III phenomena were Chl, protease activity, and reducing sugars. Because of their slow rate of change (Δ_{50}'s greater than 10 days), it seems that class III phenomena are secondary alterations in metabolism during leaf senescence.

Table II. *Regression Coefficients and Δ_{50}'s for Alterations in Physiological Parameters during Senescence of Barley Primary Leaves*

Class of Phenomenon	Parameter[a]	Period Used for Regression	Regression Coefficient[b]	Δ_{50}[c]
		days after planting	r^2	*days*
Class I	Transpiration (−)	12–22	0.80	3.0
	Stomatal resistance (+)	12–22	0.81	3.0
Class II	RuBPCase (−)	10–22	0.96	5.2
	True photosynthesis (−)	12–22	0.88	5.4
	Mesophyll resistance (+)	12–22	0.85	5.5
	RuBPCase activity (−)	10–22	0.96	6.4
	α-Amino N (−)	10–22	0.91	6.7
	Nonreducing sugars (+)	8–22	0.75	6.9
	Total soluble protein (−)	8–22	0.95	8.2
Class III	Chlorophyll (−)	14–22	0.84	10.4
	Protease activity (+)	8–22	0.59	12.3
	Reducing sugars (+)	8–22	0.49	19.8

[a] Sign indicates whether the parameter increased (+) or decreased (−) during senescence.

[b] All regressions were significant at the 1% level.

[c] Δ_{50} is the time required for the parameter to increase two-fold or decrease 50%.

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