

## Senescence of Rice Leaves XXXI. Changes of Chlorophyll, Protein, and Polyamine Contents and Ethylene Production During Senescence of a Chlorophyll-Deficient Mutant

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**Abstract.** Leaf senescence of a chlorophyll-deficient rice mutant (LT-8) was investigated. At 10 days after planting, the chlorophyll level in the third leaves of rice seedlings of the mutant was about one half that of normal leaves (Norin no. 8), whereas no difference in the protein level could be detected in the two genotypes. The protein level in leaves decreased with increasing age, and no significant difference could be detected during senescence in the two genotypes. Chlorophyll level in the normal leaves also decreased with increasing age. However, the chlorophyll level in the mutant leaves began to decrease only after more than 60% of the initial protein had been degraded. The pattern of ethylene production in the normal leaves was, in general, similar to that in the mutant leaves. Ethylene production first decreased with age, increased to a maximum at day 18, and decreased thereafter. Both spermidine and spermine levels in the leaves of the two genotypes decreased with increasing age. The pattern of the putrescine level in the normal leaves behaved somewhat similar to that in the mutant leaves. However, during the course of senescence, the putrescine level in the mutant leaves was always higher than that in the normal leaves. The possible relationship between endogenous polyamine levels and ethylene production is discussed.

derived from Norin no. 8. The mutation concerns a single recessive gene, which is inherited in a Mendelian fashion and thus located at the nucleus (unpublished data). Chlorophyll loss is commonly used as a prime indicator of leaf senescence. Thus, it would be of interest to investigate the senescence syndrome of rice leaves by using this mutant.

Polyamines have recently been implicated in several facets of plant development and are particularly associated with the retardation of senescence (Evans and Malmberg 1989). The mechanism that exogenous polyamines retard leaf senescence has been proposed to be related to their possible inhibition of ethylene biosynthesis (Fuhrer et al. 1982, Shih et al. 1982). Because of this, polyamine and ethylene physiologies may be linked during leaf senescence. Thus, this article also describes the changes in free polyamine levels and ethylene production in leaves during senescence of a chlorophyll-deficient mutant. As a comparison, leaves of normal genotype, Norin no. 8, were also included in the present investigation.

### Materials and Methods

Seedlings of *Oryza sativa* cv. Norin no. 8 (normal) and LT-8 (mutant) were grown in hydroponic culture as described previously (Chen et al. 1990). Leaf samples (3 cm from tip) were collected from the third leaves of seedlings at various times: 10, 12, 14, 16, 18, 20, and 23 days after planting. For the experiments with detached leaves, the apical 3-cm segments excised from the third leaves of 10-day-old seedlings were used. A group of 10 segments was floated in a Petri dish containing 10 ml of distilled water. Incubation was carried out at 27°C in darkness.

Chlorophyll content was extracted and determined according to the method of Arnon (1949). Protein was extracted and quantitated as described previously (Kao 1980). Both chlorophyll and protein were expressed as mg per g fresh weight.

Recently, plant mutants have been widely used in the study of plant development. Leaf senescence is a syndrome, comprising a number of biochemical and physiological changes. To date, the nonyellowing genotype of meadow fescue is the only mutant used to study leaf senescence (Emyr Davies et al. 1990). LT-8 is a chlorophyll-deficient mutant of rice

For ethylene determination, leaf segments were placed vertically in test tubes which were closed with rubber stoppers. The ethylene in the gas phase of the enclosed tubes was determined by analysis of a 1-ml sample withdrawn with a hypodermic syringe as described elsewhere (Kao and Yang 1983). Ethylene production was expressed as nl per g fresh weight per h.

For the extraction of free polyamines, leaf samples weighing about 125 mg were homogenized in cold 5 ml of perchloric acid (5%, vol/vol), and the extracts were kept at 4°C for 24 h. The homogenate was centrifuged for 20 min at 17,000 g, and the clear supernatant was used for benzoylation following the method of Flores and Galston (1982). One milliliter of 2 N NaOH was mixed with the 1 ml supernatant. After addition of 15 µl benzoyl chloride, vortexing for 10 s, and incubation for 20 min at room temperature, 2.5 ml of saturated NaCl was added. Benzoyl-polyamines were extracted in 2.5 ml diethyl ether. After centrifugation at 3000 g for 15 min, 1.5 ml of the ether phase was collected, evaporated to dryness by vacuum concentrator, and redissolved in 100 µl methanol. Polyamine standards were benzoylated in a similar way. High-performance liquid chromatography (HPLC) analysis of benzoyl-polyamines was basically performed according to the method of Slocum et al. (1989). Ten-microliter aliquots of each redissolved sample were injected into a Waters M-6 UK Universal Liquid Chromatograph. They were eluted through a 4 × 250 mm, 5-µm particle size C18 reverse-phase column at a flow rate 1 ml/min using water (solvent A)/methanol (solvent B) stepped-gradient program (50–65% B in 7 min; 65–80% B in 10.5 min; 80–100% B in 2 min) followed by a column cleaning/regeneration cycle. Detection was accomplished by a Waters M481 absorbance detector at 254 nm. Results were recorded on a Waters M470 data module and plotted as nmol per g fresh weight.

## Results

Figure 1 shows the time courses of chlorophyll and protein levels in leaf segments excised from the third leaves of 10- to 23-day-old seedlings of normal and mutant genotypes. The protein level in leaves decreased with increasing age, and no significant difference could be detected during senescence in the two genotypes. At 10 days after planting, the chlorophyll level in the mutant leaves was about one half that of normal leaves. Chlorophyll level in the mutant leaves remained unchanged between days 10–20 and then declined with age. In contrast to the mutant leaves, the chlorophyll level in the normal leaves decreased progressively with age. When detached leaves of both the normal and mutant rice were incubated in darkness, the decline of chlorophyll and protein levels in the mutant leaves was found to be at the same rate as that in the normal ones (Fig. 2).

The pattern of ethylene production in the normal leaves was essentially similar to that in the mutant leaves (Fig. 3). Ethylene production first decreased with age, increased to a maximum at day 18, and then decreased again. The mutant leaves produced about 10% more ethylene than the normal ones during the period from days 10–18.

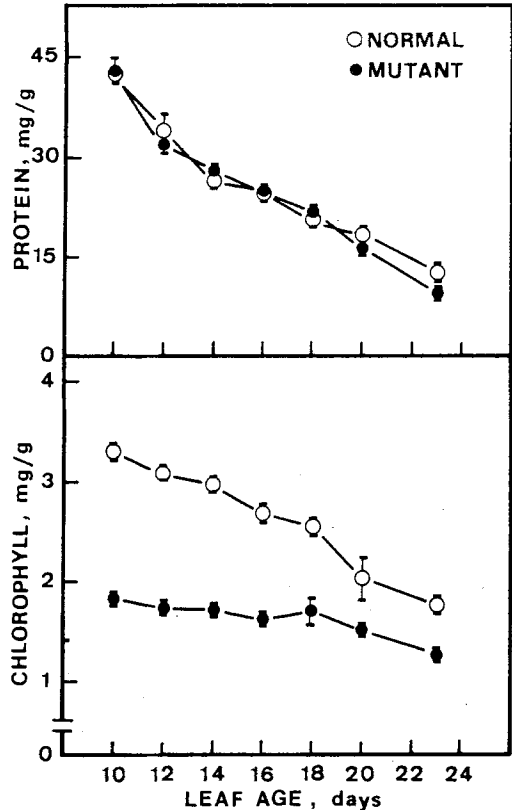


Fig. 1. Changes in chlorophyll and protein contents in normal and mutant leaves. Leaf samples were collected from the third leaves of seedlings at indicated times. Values are averages with standard errors ( $N = 3$ ).

Putrescine, spermidine, and spermine were present in both normal and mutant leaves. However, no detectable levels of cadaverine, a diamine usually found in the family *Leguminosae*, and diaminopropane, an oxidation product of naturally occurring polyamines, were observed in leaves of the two genotypes during senescence. Both spermidine and spermine levels in leaves decreased progressively with age in the two genotypes (Fig. 4). The change in the putrescine level in leaves during senescence showed a distinct pattern. Putrescine level in the mutant leaves increased up to day 16, decreased at day 18, and increased thereafter. The pattern of the putrescine level in the normal leaves behaved in a somewhat similar manner to that in mutant leaves. During the course of senescence, the putrescine level in the mutant leaves was always higher than that in the normal leaves; however, the spermidine level in the mutant leaves at days 16 and 18 was lower than that in the normal leaves.

## Discussion

In senescence studies, it is commonly assumed that

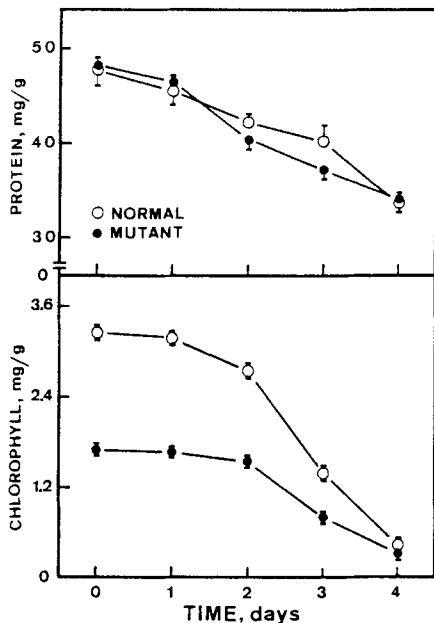


Fig. 2. Changes in chlorophyll and protein contents in detached leaves of normal and mutant rice. Detached leaves were incubated in darkness. Values are averages with standard errors (N = 4).

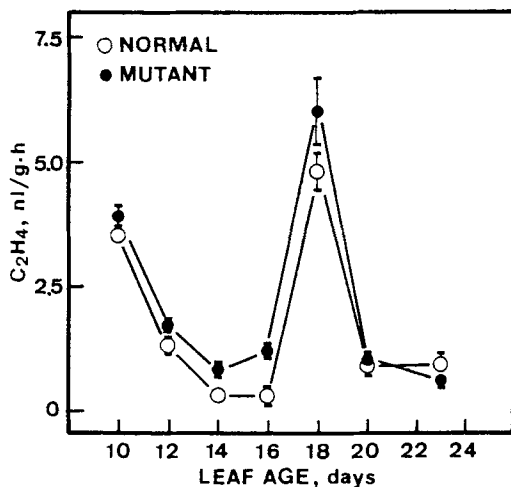


Fig. 3. Changes in ethylene production in the normal and mutant leaves. Values are averages with standard errors (N = 4).

chlorophyll loss is linked to protein degradation. However, the present investigation shows that the chlorophyll level in the mutant leaves began to decrease only after more than 60% of the initial protein had been degraded, suggesting that the leaf chlorophyll level is not always a reliable measure of senescence. If chlorophyll is used as the prime indicator of senescence, the leaves of the mutant

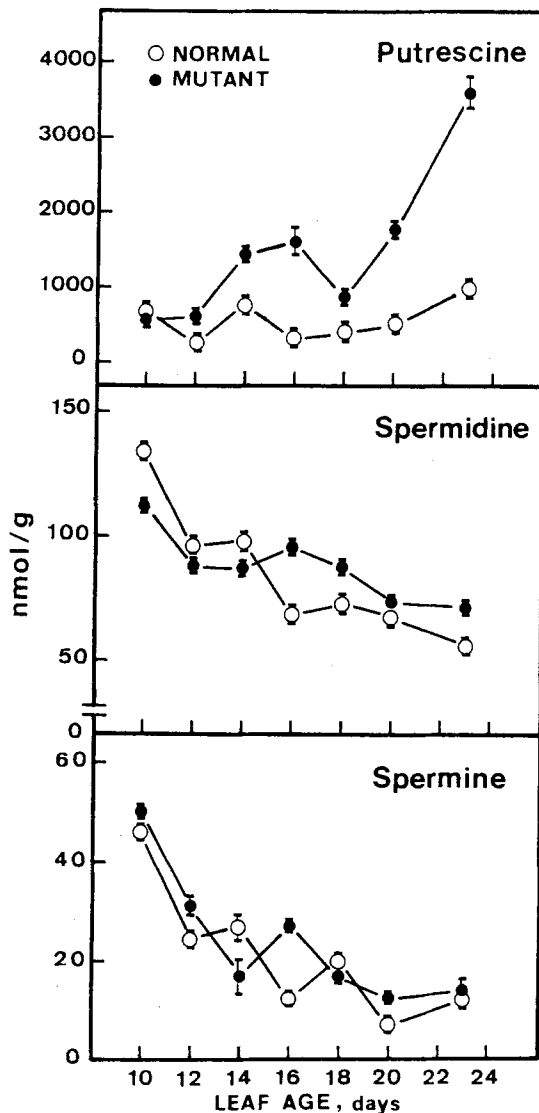


Fig. 4. Changes in polyamine contents in the normal and mutant leaves. Values are averages with standard errors (N = 3).

would be regarded as nonsenescent or slowly senescent. However, under dark conditions, the chlorophyll level in detached leaves of the mutant decreased at a similar rate as that of detached leaves of the normal. It has been recognized for some time that excised leaves display some very substantial differences in their senescence compared with their intact counterparts (Lewington et al. 1967, Simon 1967). In view of the importance of correlative controls in senescence, it is not surprising that excision produced some big changes. Thomas and Stoddart (1975) reported that chlorophyll in detached leaves of a mutant genotype of meadow fescue remained near the initial level during a period of 6 days. They provided evidence to show that this phenomenon

was not merely an artifact of excision, since leaves attached to the plant also retained their green color to an advanced stage of maturity.

The present investigation demonstrated that putrescine, spermidine, and spermine were present in both normal and mutant rice leaves throughout senescence. However, cadaverine and diaminopropane could not be identified in leaves of the two genotypes. Our unpublished data and previous reports showed that exogenous application of polyamines was effective in retarding chlorophyll loss in detached rice leaves (Cheng and Kao 1984, Cheng et al. 1984). If polyamines do play an important role in regulating leaf senescence of the two genotypes, then endogenous polyamine levels would be expected to decrease with increasing age. This is indeed the case with spermidine and spermine, but not putrescine. Kaur-Sawhney et al. (1982) and Srivastava et al. (1981) also found that endogenous levels of polyamines decreased with advancing leaf age.

The effect of exogenous polyamines in retarding leaf senescence is generally thought to be mediated through inhibition of ethylene production (Evans and Malmberg 1989). If this is indeed the case, then the pattern of ethylene production during senescence would be expected to be inversely correlated with that of polyamine levels. However, this is not the case in both normal and mutant leaves. Recently, we found that polyamines promoted, rather than inhibited, biosynthesis of ethylene in rice leaves (Chen et al. 1991). All these data raise the possibility that the mechanism by which polyamines regulate leaf senescence in intact rice plants may not be mediated through inhibition of ethylene production.

A dramatic decrease in ethylene production was observed at the early stage of senescence in both mutant and normal leaves. The increase of ethylene production only occurs in an advanced stage of senescence, which is too late to play a regulatory role in leaf senescence, and is probably caused by earlier events taking place in the course of senescence. Thus, the earlier indication, that senescence of detached rice leaves is directly linked to ethylene production (Kao and Yang 1983), does not hold for senescence of intact rice leaves of the two genotypes.

Of particular interest is the finding that the mutant leaves accumulated significantly higher amount of putrescine than the normal leaves during senescence. Several mutants exhibiting abnormal flower morphologies have been shown to have elevated polyamine levels (Gerats et al. 1988, Malmberg and McIndo 1983, Rastogi and Sawhney 1990). Polyamine levels in the flower have also been shown to

be related to ploidy level in *Nicotiana plumbaginifolia* (Figueras et al. 1990). Martin-Tanguy et al. (1990) recently demonstrated an inverse relationship between polyamine levels and the degree of phenotypic alteration induced by root-inducing, left hand-transferred DNA from *Agrobacterium rhizogenes*. We believe that our observations provide the first evidence showing that genotypic alteration results in an elevated level of putrescine in leaf tissue.

It appears that the common precursor, S-adenosylmethionine, limits both ethylene and polyamine synthesis. At about day 18, an increase in S-adenosylmethionine permits formation of more ethylene, the preferred pathway. In the mutant, but not wild type, the extra S-adenosylmethionine is totally consumed in making the extra ethylene; this leads to the accumulation of putrescine, which requires S-adenosylmethionine to be converted to spermidine. However, the possibility of decreased putrescine metabolism and/or increased putrescine synthesis in the mutant leaves cannot be excluded. Further studies should provide valuable information on the mechanism of putrescine accumulation in the mutant leaves during senescence.

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