RETARDATION OF SENESCENCE IN RICE (*ORYZA SATIVA* L.) LEAVES BY BENZIMIDAZOLE AND NICKEL CHLORIDE*

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The retardation of leaf senescence of rice variety IR-8 was studied with the application of benzimidazole, nickel chloride separately and in combination. Treatment with these two chemicals maintained a higher level of chlorophyll, soluble and insoluble protein and RNA, during the senescent period. Treatments also maintained the activity of ribonuclease at a higher level. The possible mechanism of action is discussed.

INTRODUCTION

Senescence is considered a genetically programmed phase of plant development. Changes in the cellular genome occur during this period (Atkin and Srivastava 1970). The genetic machinery takes over charge of synthesizing the proteins that act as the degrading enzymes. The loss, occasioned, not only by the reduction in the rate of synthesis but also by a 2-4-fold increase in the rate of degradation of cellular components (Trewaves 1970) terminating the life of the organism/organ.

In leaves, yellowing and loss of green colour (the manifestation of senescence) is accompanied by a decrease in the level of total protein and nucleic acids. Application of growth regulators can temporarily arrest these changes. In this investigation, two different compounds, i.e. benzimidazole (BZI) and nickel chloride were tested independently and in combination to assess their effect in the retardation of leaf senescence.

Both benzimidazole and nickel chloride are reported to retard leaf senescence. BZI has been successfully applied in retarding the loss of chlorophyll in wheat leaves (Person *et al.* 1957; Wang *et al.* 1961; Yoshida *et al.* 1969), Elodea (Yoshida 1970), groundnut and rice (Misra and Mishra 1968). It is also found to arrest the breakdown of protein and ribonucleic acid (RNA) in wheat leaves (King *et al.* 1963 *a, b;* Mishra 1963). Though much less work is done on the effect of nickel, yet it is reported to maintain chlorophyll level in wheat leaves (Bushnell 1966; Wang and Waygood 1959).

MATERIALS AND METHODS

The primary leaves of a high yielding variety of rice (*Oryza sativa,* var. IR-8) were used in the investigation. Leaves of approximately uniform size (5 cm) were detached, washed thoroughly in running tap water and then in distilled water and floated in groups of five, each on water or test solutions (BZI, 10^{-3} M; $NiCl_2$, 10^{-3} M;

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BZI + $NiCl_2$, 10^{-3} M) in Petri dishes. In order to prevent fungal and bacterial infection, chloramphenicol (20 μg) and penicillin (120 units) were added to each of the Petri dishes. The petri dishes were kept in complete darkness at room temperature (25°C). After the desired period (5, 10, 15 days, after floatation) the leaves were washed thoroughly and blotted and sampled for biochemical analysis.

(*i*) *Extraction and assay of chlorophyll, protein and nucleic acid*

The method of Osborne (1962) was followed with some modification as described below. The leaves were first kept in boiling 80 per cent ethanol and extracted three times. The chlorophyll thus extracted was made up to (10 ml). The optical density of chlorophyll extract was measured against 80 per cent ethanol blank in a spectrocolorimeter, at 665 nm.

The pigment-free leaf was macerated, homogenized in 80 per cent ethanol and centrifuged at 2500 g. Acid soluble substances were extracted four times from the solid residue with 10 per cent trichloroacetic acid (TCA) at 4°C. After TCA extraction the remaining solid residue was treated with ethanol (95 per cent, once), ethanol+ chloroform (3 : 1, twice) enthanol + ether (3 : 1, once) and finally with ether only. The remaining traces of ether were evaporated. Ten millilitres of 0.3 N KOH were added to the residue and then incubated for 20 hr at 37°C.

After incubation, 2 ml of the filtrate was kept aside for soluble protein estimation by the biuret method of Gornall *et al.* (1949) using bovine serum albumin as the standard.

The remaining 8 ml of the filtrate was acidified to *p*H 2.0 by adding cold perchloric acid, and was kept overnight at 4°C for precipitation of DNA and protein. RNA was analyzed in the supernatant after centrifugation and proper dilution, by pentose estimation (Markham 1955) using yeast RNA as a standard. The UV analysis was also carried out between 230 nm and 330 nm in Beckman DK-2A ratio recording spectrophotometer.

The residue was digested with 5 ml of 1 N NaOH at 100°C for 15 min, cooled, centrifuged and the filtrate was taken for estimation of insoluble protein as described before.

(ii) *Extraction and assay of ribonuclease*

For the extraction and assay of the enzyme ribonuclease, the procedure of Bagi and Fafkas (1967) was followed. The samples were homogenized in 10 ml of distilled water using chilled mortar and pestle, with quartz sand. The brei was filtered through four layers of cheese cloth. The supernatant was used for enzyme assay immediately.

The enzyme assay mixture contained 0.5 ml of leaf extract, 0.5 ml of 7.5 mg/ml yeast RNA solution (in *p*H 6.0 citrate buffer), and 0.5 ml of 0.1 M acetate buffer at *p*H 5.0 and was incubated at 37°C for 30 min. The reaction was stopped by adding Mc Fadyen's reagent (0.25 per cent Uranyl acetate in 2.5 per cent TCA). The mixture was kept overnight at 4°C, centrifuged and was suitably diluted. The absorptivity of the supernatant was measured at 260 nm in a Beckman DK-2A ratio recording spectorphotometer. The results were expressed in enzyme units corresponding to an increase in absorptivity of 0.010 over the zero time control.

Bacterial and fungal infection

There was no bacterial or fungal contamination up to 6 days of treatment. After that period the solutions of $NiCl_2$ alone and of $NiCl_2$ in combination with BZI, were free from contamination till the end of the experimental period. The control solution was heavily contaminated after 10 days of the treatment. The leaves floated on BZI were also susceptible to heavy contamination after 10 days.

EXPERIMENTAL RESULTS

As more than 50 per cent of the leaves showed the visible sign of senescence after 4–5 days of detachment, the samples were assayed for the changes in the chlorophyll, protein and RNA content on the 5th day after treatment.

The results shown in the Table I are typical of three (for Chlorophyll) or at least two separate experiments (for protein and RNA). BZI reduced the rate of net loss of chlorophyll, protein and RNA. However, $NiCl_2$ was more effective than BZI in reducing the loss of these vital constituents. BZI in combination with $NiCl_2$ was by far the best to have the protective action on the degradation of these compounds.

More than 50 per cent of leaves floated on water, were chlorotic by the 5th day and by the 7th day all of them lost greenness. Therefore, the data obtained for chlorophyll, protein, RNA, and ribonuclease for the leaves floated on water are designated as the 'terminal level' at day 5, since further analysis after day 5 might have given erratic results, due to bacterial and fungal contamination. The data presented for water-floated leaves, at the days 10 and 15, should be considered with caution and reservation. However, the leaves floated on BZI (for 10 days), Nickel (for 15 days) independently or in combination of both (for 15 days) remained considerably green and turgid and therefore the validity of the data cannot be questioned.

(i) *Chlorophyll*

The loss in the chlorophyll content of detached leaves was retarded by the addition of BZI, $NiCl_2$ and their combination (Table I).

Up to 15th day of detachment the chlorophyll-maintaining effect of both the compounds in combination was significant, there being only 3 per cent loss of chlorophyll.

$NiCl_2$ maintained the chlorophyll content till the 5th day of treatment, but gradually the chlrophyll-maintaining effect of $NiCl_2$ was markedly decreased and at the end of the experimental period, the total loss of chlorophyll was 23 per cent of the initial content.

BZI alone was capable of maintaining the chlorophyll level for a period of 5 days, after which the loss was sharp, resulting in 84 per cent loss of chlorophyll at the end of the experimental period.

In the leaves floated on water, the loss of chlorophyll was about 10 per cent per day, during the first 5 days of detachment. Thereafter there was a distinct lag phase during which total chlorophyll decreased at the rate of 5 per cent per day. Hence, by the 10th day more than 85 per cent of total chlorophyll was lost from the leaves floated on water.

TABLE I

Drift in the chlorophyll, protein and RNA content

(Value expressed as per cent of initial chlorophyll, protein and RNA content)

	Days floated	Water	BZI	$NiCl_2$	BZI+ $NiCl_2$
Chlorophyll	5	42.3	97.9	103.3	103.4
Protein	5	62.2	91.3	95.6	99.2
RNA	5	74.2	111.2*	88.8*	99.6
Chlorophyll	10	12.3	59.5*	88.1*	98.4
Protein	10	74.2	95.6	104	106.0
RNA	10	—	119.6*	87.1*	95.6
Chlorophyll	15	6.1	16.8*	77.1*	97.1
Protein	15	88.4	108.3	110*	119.5*
RNA	15	67.1	88.6*	96.0	107.6

*Significantly different from the initial content at 5% level by student's "t" test

(ii) Protein

Protein decomposition followed a pattern generally similar to that of chlorophyll, up to 5 days of detachment (Table I). However, after this period, the leaves floated on water became vulnerable to bacterial and fungal contamination, which perhaps resulted in an increased protein content. The protein loss was not as extensive as that of chlorophyll. However, it was about half of the chlorophyll loss for the first 5 days of floatation on water.

BZI or $NiCl_2^-$ induced senescence retardation tested for protein loss showed that all these treatments resulted in a maintenance of the protein content. The loss in the protein content was to the extent of 5 per cent only in the treated leaves.

(iii) Soluble protein

In the leaves floated on water, (Table II-A) soluble fraction declined at a rate of 6 per cent per day, for the period of first 5 days of detachment, after which it increased at a rate of 2 per cent per day. The leaves floated on BZI showed a slight decrease in the soluble protein content, till the 5th day of detachment after which surprisingly the treatment induced rapid increase, at a rate of 5 per cent per day. In the leaves floated on $NiCl_2$, there was constantly an increase (contrary to the initial decrease in water-floated leaves), in the soluble protein content at the rate of 4 per cent per day for the period of first 10 days, after which this level was maintained till the 15th day. Leaves floated on the combined solution of BZI and $NiCl_2$, showed a very slight increase at a rate of 2 per cent per day in the soluble protein content for the first 5 days, after which the rate of soluble protein increase was 5 per cent per day till the 15th day of detachment.

TABLE II-A

Drift in the soluble protein content

(Value expressed as per cent of initial soluble protein content)
(Mean of two independent experiments)

Days floated	Water	BZI	$NiCl_2$	BZI+$NiCl_2$
5	63.6	94.2	121.7	110.0
10	80.0	111.7	134.5	126.5
15	101.3	143.3	138.3	153.3

Standard error ± 4 % n = 8

(iv) Insoluble protein

The pattern of insoluble protein change in the leaves floated on water showed the same trend as noted for the changes in the soluble protein content (Table II-B). In these leaves, the rate of protein loss was the same for both soluble and insoluble protein fraction till the 5th day, after which the curve showed an upward increase which is obviously due to contamination.

TABLE II-B

Drift in the insoluble protein content

(Value expressed as per cent of initial insoluble protein content)
(Mean of two independent experiments)

Days floated	Water	BZI	$NiCl_2$	BZI+$NiCl_2$
5	61.3	88.3	69.5	88.3
10	68.3	79.5	73.5	85.5
15	75.4	73.3	81.7	85.8

Standard error ± 4.3% ; n=8

In the treated leaves the decrease in insoluble protein was gradual in the BZI treatment but this decline was at a slower rate in BZI + $NiCl_2$ till the 15th day of detachment.

(v) RNA

The drift in the total RNA level determined by pentose analysis (Table I), and UV analysis (Table III) clearly indicate the decline in RNA content in the water-floated leaves, whereas this decline was considerably arrested and indeed a net increase in RNA content (as determined by pentose analysis) was noted till the 10th day of detachment. The UV analysis also indicates, that although RNA content increased for the first 5 days, yet the upward surge declined, as the experiment was continued till the 15th day.

TABLE III

Drift in RNA content (By UV analysis)

(Value expressed as per cent of initial RNA content)
(Mean of two independent experiments)

Days floated	Water	BZI	$NiCl_2$	BZI+$NiCl_2$
5	92.5	127	135	129
10	92	109	115	86
15	97.5	120	112	101

(vi) *Ribonuclease*

Ribonuclease activity increased with detachment. This increase was the greatest for the leaves floated on water on the 5th day, after which the ribonuclease activity declined (Table IV). The ribonuclease activity in the treated leaves increased till the end of the experimental period, except in BZI, treated leaves, where enzyme activity declined after 10th day of detachment.

TABLE IV

Drift in ribonuclease activity

(Value expressed as enzyme units)
(mean of two independent experiments)

Days floated	ID	Water	BZI	$NiCl_2$	BZI+$NiCl_2$
0	1900	—	—	—	—
5	—	3250	2825	2555	2910
10	—	2850	4560	3480	3700
15	—	2680	3785	4725	4760

DISCUSSION

The breakdown of chlorophyll, protein and RNA in detached leaves can be considerably arrested by treatment with BZI and Nickel ions, either separately or in combinations. There was a similarity in the pattern of chlorophyll, protein and RNA loss. This is consistent with the view that senescence may be controlled through regulation of RNA metabolism (Atkin and Srivastava 1970). It is possible that either BZI and/or nickel ions exert their effect upon a specific fraction of RNA. However, no close correlation between RNA content and senescence was noted by Beevers (1966) and Srivastava and Ware (1965). In the present experiment also there was an actual increase in RNA content above the zero time level and hence it is probable that BZI not only maintains the initial rate of synthesis but also increases it till the 5th day of treatment. Whether BZI may also lessen the rate of degradation of RNA is yet to be established?

The maintenance of chlorophyll level due to various treatments suggests strongly that treatments might block the degradation of (i) the chlorophyll molecule, (ii) chlorophyll-protein complex, or (iii) chloroplast integrity. Chlorophyll metabolism

is dependant upon protein and RNA metabolism (Evans and Smillie 1962). In the present experiment both BZI and Nickel ions have the same protective effect on RNA and protein as on the chlorophyll content. It is tempting, therefore, to speculate that the regulation of senescence operates by a direct effect of BZI and Nickel ions upon DNA dependent RNA synthesis as suggested for other growth regulators (Fletcher and Osborne 1965).

Ribonuclease activity increased in detached leaves at a rapid rate. During a 5 day period the leaves floated on water showed considerable yellowing and RNase activity increased by more than 50 per cent. Perhaps the ribonuclease of this investigation is only the destructive ribonuclease. The rapid increase in the ribonuclease activity till the 5th day of detachment and the yellowing of water-floated leaves could be due to the destructive ribonuclease as suggested by Kessler and Englerberg (1962).

REFERENCES

Atkin, R. K., and Srivastava, B. I. S. (1970). Studies on protein synthesis by senescing and Kinetin treated barley leaves. *Physiologia Pl.*, **23**, 304-316.

Bagi, A., and Farkas, G. L. (1967). On the nature of increase in ribonuclease activity in mechanically damaged tobacco leaf tissues. *Phytochemistry*. **6**, 161–169.

Beevers, L. (1966). Effect of gibberellic acid on the senescence of leaf discs of nasturtium (*Tropeolum majus*) *Pl. Physiol.*, **41**, 1074–1076.

Bushnell, W. R. (1966). Delay of senescence in wheat leaves by cytokinins nickel and other substances. *Can. J. Bot.*, **44**, 1485–1493.

Evans, W. R., and Smillie, R. M. (1962). Role of nucleic acid in the formation of chloroplasts. *Pl. Physiol.*, **37**, 38.

Fletcher, R. A., and Osborne, D. J. (1966). Gibberellin as a regulator of protein and ribonucleic acid synthesis during senescence in leaf cells of *Taraxacum officinale*. *Can. J. Bot.*, **44**, 739–745.

Gornall, A. G., Bardwill, C. S., and David, M. M. (1949). Determination of serum protein by means of Biuret reaction. *J. biol. Chem.*, **177**, 751–761.

Kessler, B., and Englerberg, N. (1962). Ribonucleic acid and ribonuclease in developing leaves. *Biochem. biophys Acta*, **55**, 70.

King, C. C., and Hsu, T. Y. (1963). Effect of benzimidazole and kinetin on leaves. *Acta Biol. expl. Sinica.*, **8**, 155–162.

King, C. C., Hsu, T. Y., and Jing, J. A. (1963). Action of benzimidazole on protein synthesis in detached leaves. *Acta Biol. expl. Sinica*, **8**, 163–169.

Markham, R. (1955). Nucleic acids, their components and related compounds. *In* : Modern Methods of Plant Analysis, Vol. IV, K. Paech and M. V. Tracey (Eds.). Springer Verlag, Berlin.

Mishra, D. (1963). A study of the effect of benzimidazole and kinetin on the metabolism of detached primary leaves of wheat variety Khapli. Ph. D. Thesis, University of Monitoba, Winnipeg.

Mishra, D., and Misra, B. (1968). Effect of growth regulating chemicals on degradation of chlorophyll and starch in detached leaves of crop plants. *Z. Pflanzenphysiol*, **58**, 207-211.

Osborne, D. J. (1962). Effect of kinetin on protein and nucleic acid metabolism in Xanthium during senescence. *Pl. Physiol.*, **37**, 595–602.

Person, C., Samborski, D. J., and Forsyth, F. R. (1957). Effect of benzimidazole on detached wheat leaves. *Nature, Lond*, **180**, 1294–1295.

Srivastava, B. I. S., and Ware, G. (1965). The effect of kinetin on nucleic acid and nucleases of excised barley leaves. *Pl. Physiol.*, **40**, 62–64.

Trewaves, A. (1970) The turnover of nucleic acids in *Lemna minor*. *Pl. Physiol.*, **45**, 742–751.

Wang, D., and Waygood, E. R. (1959). Effect of benzimidazole and nickel on the Chlorophyll metabolism of detached leaves of Khapli wheat. *Can. J. Bot.*, **37**, 743–749.

Wang D, Hao, M. S. H., and Waygood, E. R. (1961). Effect of benzimidazole anologues on stem rust and chlorophyll metabolism. *Can. J. Bot.*, **39**, 1029–1036.

Yoshida, Y. (1970). Control of chloroplast senescence of detached *Elodea* leaves. *Bot. Mag. Tokyo*, **83**, 137–143.