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Nucleic Acid and Protein Changes in Relation to Cold Acclimation and Freezing Injury of Korean Boxwood Leaves[1,2]

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ABSTRACT

Quantitative and qualitative differences in nucleic acids of Korean boxwood (*Buxus microphylla* var. Koreana) leaves were determined by methylated albumin kieselguhr chromatography at different levels of cold hardiness. During cold acclimation there was an increase in RNA, mainly ribosomal RNA, with little or no change in DNA. The increase in ribosomal RNA was closely paralleled by an increase in water soluble and membrane bound proteins. As cold hardiness increased, ribonuclease activity declined.

Exposure of hardy boxwood plants to warm temperatures resulted in a rapid loss in cold resistance and a rapid synthesis of nucleic acids as judged by ^{32}P incorporation.

Following a killing frost to Korean boxwood leaves, there was a rapid decrease in all nucleic acid fractions which was attributed to nuclease activity. Within 5 hours there was no measurable soluble RNA and ribosomal RNA. Tenaciously bound RNA was somewhat more persistent.

Woody plant cells characteristically undergo a series of changes in the autumn which enable them to withstand freezing stresses. During this period, when growth has stopped, there are a surprising number of metabolic changes (12–14, 25). Siminovitch *et al.* (25) and Li and Weiser (13) have described the increases in proteins and RNA which occur in the living bark of deciduous, woody plants in the autumn during cold acclimation. These results have led to the suggestion that the proteins and RNA synthesized in the autumn play a causal role in cold acclimation (13, 14, 16, 25).

While this is an attractive idea, it is possible that observed autumnal increases in proteins and nucleic acids are due to reduced degradation by nucleases and proteases during sample preparation rather than increased synthesis rates. RNase activity has been shown to decrease in woody plant tissues during the autumn (8), and substantial nucleic acid degradation has been observed in boxwood leaves during lyophilization (5). Another possibility is that the nucleic acid and protein increases are real, but that these changes bear a coincidental rather than a causal relationship to cold acclimation. For example, proteins and nucleic acids may increase in the autumn as a result of a build up of precursor pools in the living bark of deciduous plants when leaves senesce and abscise.

This study was designed to investigate these possibilities by examining the changes in proteins and different classes of RNA during cold acclimation in Korean boxwood leaves. The fate of nucleic acids following lethal freezing was also examined. Korean boxwood is a broad-leaved evergreen shrub which retains its leaves throughout the autumn and winter when major changes in hardiness occur.

MATERIALS AND METHODS

The four general types of studies which were conducted involved: (*a*) characterization of cold acclimation of Korean boxwood leaves (*Buxus microphylla* var. Koreana) in the field with concurrent measurements of changes in nucleic acids, proteins, and RNase activity; (*b*) similar studies during acclimation at different photoperiods in controlled environment chambers; (*c*) examination of changes in the metabolism of nucleic acids during short term dehardening and rehardening when hardy plants were exposed to alternating periods of high and low temperatures; (*d*) a study of the fate of nucleic acids in leaves following exposure to a killing frost.

GENERAL METHODS

Leaf Hardiness Evaluations. The leaves of boxwood were the basic experimental unit for all analytical and hardiness evaluations in this study. The overwintering leaves are mature, differentiated organs which do not undergo cell division or other visible signs of growth and development during the autumn, winter, and spring when their cold resistance changes significantly. Since Korean boxwood is an evergreen species, this choice of material reduces the problem of a major build up in precursor pools such as that which occurs in stem tissues of deciduous plants at the time of leaf abscission.

To evaluate cold resistance, foliated boxwood branches, 2 to 3 inches in length, were excised at random from uniform plants. Three branches were placed in each of a series of Dewar flasks in a freezer which was programmed to cool the samples at a rate of about 10 C/hr. A thermocouple in each Dewar flask monitored sample temperature. Sample flasks were removed from the freezer at successively lower test temperatures (at either 4 or 5 C intervals as noted) and then slowly rewarmed. Leaves were visually scored for injury after an in-

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cubation period of 1 week at 30 C in a humid chamber. Injured leaves darken and rapidly deteriorate under these conditions, while uninjured leaves remain green and healthy in appearance. The hardiness of leaves is expressed as lowest survival temperature, which was the lowest test temperature at which leaves were not killed by freezing. Triplicate samples at each test temperature consistently gave the same results. Changes in moisture content of leaves during acclimation were determined by weighing samples before and after drying at 70 C for 48 hr.

Nucleic Acid Extraction and MAK[4] Chromatography. In studies 1 and 2, nucleic acids were extracted from lyophilized boxwood leaves. The freeze-drier sample manifold was placed in a freezer at −20 C during lyophilization. It was necessary to carry out lyophilization at a low temperature to prevent nucleic acid degradation which occurred when samples were lyophilized at room temperature (5). Dry samples were ground in a Wiley mill (60 mesh screen) and then in a motorized mortar and pestle until the resulting fine powder would pass through a 149 μ sieve. Ground samples were then homogenized in an ice bath with a VirTis 45 homogenizer (45,000 rpm) for 2 min in 40 ml of 10 mM tris-HCl buffer, pH 7.7, 0.15 M NaCl, and 15 mM sodium citrate. Sodium lauryl sulfate was added to a concentration of 2%, and the homogenates were shaken for 10 min. An equal volume of buffer-saturated 88% phenol was added, and the homogenates were shaken for an additional 15 min. Homogenates were then centrifuged at 10,000g for 10 min, and the aqueous phase was collected and extracted with an equal volume of phenol. Nucleic acids were precipitated from the aqueous phase with 2 volumes of ethanol, collected by centrifugation, washed twice with ethanol (20 to 21 C) to remove residual phenol, and then dissolved in 40 to 50 ml of 50 mM sodium phosphate buffer at pH 6.7.

In studies 3 and 4, nucleic acids were extracted from fresh boxwood leaves after homogenation for 5 min in a VirTis 45 homogenizer (45,000 rpm) and then extracted as described in the preceding section. In all studies, nucleic acids were fractionated on MAK columns according to the method of Mandell and Hersey (15). The kieselguhr was washed with 1 N NaOH and 1 N HCl as suggested by Osawa and Sibatani (19). Elution was carried out at room temperature (20 to 21 C) employing a linear gradient of NaCl from 0.35 M (400 ml) to 1.25 M (400 ml) in 50 mM sodium phosphate buffer at pH 6.7. Tenaciously bound RNA was eluted with 100 ml of cold (0 to 4 C) 1.5 N NH_4OH and estimated by correcting the 260 nm reading against a blank as described by Dure *et al.* (4).

Ribonuclease Enzyme Assays. RNase assays of boxwood leaf samples were carried out at pH 5.5 according to the method of Johri and Maheshwari (10). The assay mixture contained 1.5 ml of dialyzed high molecular weight yeast RNA (5 mg/ml), 1.5 ml of aqueous leaf homogenate in 40 mM phosphate-citrate buffer and 5 mM $K_2S_2O_5$. Incubation was carried out at 25 C for 60 min. The aqueous homogenate was obtained by extracting 0.5 g of ground lyophilized boxwood leaves in 5 ml of 40 mM phosphate-citrate buffer and 5 mM $K_2S_2O_5$ at pH 5.5. $K_2S_2O_5$ was used to prevent protein-phenolic interactions (1). At the end of the digestion period, the undigested RNA was precipitated for 2 hr at −20 C with 6 ml of chilled 5% $HClO_4$ in ethanol. A 1-ml aliquot of the supernatant was diluted 16-fold and read against the enzyme blank at 260 nm. The results are expressed as enzyme units; one unit of RNase activity corresponding to the amount of enzyme which caused an increase of 1.0 in absorbancy over an enzyme blank.

[4] Abbreviations: MAK: methylated albumin kieselguhr: sRNA: soluble RNA; TB-RNA: tenaciously bound RNA.

Protein Extraction and Estimation. Water soluble protein was extracted in a 0.1 M potassium phosphate buffer and 10 mM $K_2S_2O_5$ at pH 7.0. Membrane-bound protein was extracted from the residues with a mixture of phenol-acetic acid-water (2:1:1, v/v) as described previously (5). Protein was determined as described by Potty (21).

Free Amino Nitrogen. Soluble amino nitrogen was extracted as described by Cruz *et al.* (2), and the amount was determined by using the ninhydrin method (17).

Nucleic acid, RNase, and protein assays at each sampling date were conducted on bulked leaf samples collected from uniform plants. The data in each table and figure are the means of three to six determinations. Agreement between replicates was good.

DESCRIPTION OF STUDIES

Field Studies 1967 and 1968. Boxwood leaf hardiness was evaluated weekly in 1967 and 1968 from late August to the end of November in St. Paul, Minnesota. Concurrent changes in leaf moisture content were evaluated in 1967. Six nucleic acid determinations and five protein and RNase determinations were made during acclimation in 1968.

Controlled Acclimation Studies. Boxwood plants were cold acclimated in controlled environment chambers. Twenty-four uniform plants growing in the greenhouse at a 16-hr photoperiod were divided into two equal groups and transferred to two controlled environment chambers with 16-hr and 8-hr photoperiods. The light in both chambers was supplied by a mixture of cool white fluorescent and incandescent bulbs with an intensity at pot height of 8.2×10^4 ergs/cm^2 sec at 15 C as measured by a YSI Kettering Model 65 radiometer. Plants in both chambers were exposed to the same day-night temperature regimes: 15 to 5 C for the first 2 weeks followed by three successive 2-week periods during which the temperature was lowered for 2 hr during the dark period to −2.0 C, −4.5 C and −6.5 C, respectively. The hardiness, moisture content of leaves, protein content, and RNase activity were determined at 2-week intervals throughout the study.

In a subsequent controlled acclimation study, 12 uniform plants, growing in the greenhouse at a 16-hr photoperiod, were transferred to a controlled environment chamber with a 10-hr photoperiod and a day-night temperature regime of 10 to 5 C. After 21 days, the plants were exposed to progressively lower temperatures (0, −3 and −6 C) for 2 hr during the dark period. The duration of these treatments was 21, 20, and 19 days, respectively. On days 1, 22, 43, and 63 of the dehardening cycle, leaf hardiness was tested and leaves collected for nucleic acid, protein, and soluble amino nitrogen analysis. The nucleic acid extraction method was slightly modified from that described previously in that an equal volume of aqueous phenol and 50 mg of bentonite were added to the buffer at the time of homogenization. Deproteinization with phenol was done three times, and the nucleic acids were dissolved in the presence of bentonite and fractionated on MAK columns as described previously.

Dehardening and Rehardening. Boxwood plants, in 1 gallon containers, were removed from the field in late February and transferred to the laboratory where they were subjected alternately to temperatures of 25 and −10 C at 24-hr intervals over a 8-day period. Plants were hardy to −25 C at the time they were brought in from the field. At the end of each 24-hr high or low temperature treatment, survival temperature was determined, 5 g (fresh weight) of leaves were collected, surface sterilized by washing in 1.0% sodium hypochlorite for 10 min, rinsed in sterile distilled water, and then incubated in a medium containing 250 μc of carrier-free ^{32}P disodium phosphate,

(1000 c/mmole, ICN Corp.) made up in 50 ml of 0.5 mM citric acid (pH adjusted to 6.0 with NH_4OH), 1% sucrose and 20 μg/ml of streptomycin sulfate. Leaves were vigorously shaken during the 195 min incubation period at 10 C. Following incubation, the leaves were rinsed several times with distilled water, and the nucleic acids were extracted by the fresh leaf extraction method and fractionated on MAK columns. After absorbance at 260 nm was determined for the 10-ml fractions, 1.5 mg of carrier bovine serum albumin was added to every second tube and the nucleic acids precipitated with 2.5 ml of 50% trichloroacetic acid at 0 to 4 C. The precipitates were collected on GF/C glass filter discs, washed three times with cold 5% trichloroacetic acid, two times with ethanol-ether (1:1, v/v), and then air dried in standard scintillation vials overnight. The samples were counted in 10 ml of a scintillation solution which consisted of 700 ml of toluene, 300 ml of 95% ethanol, 4 g of 2,5-PPO, and 0.1 g of POPOP.

Frost Injury Study. Boxwood plants, hardy to −10 C, were subjected to a killing frost (−20 C) for 24 hr and then incubated at 30 C for 24 hr. Nucleic acids were extracted and chromatographed on MAK columns from samples incubated at 0, 1, 2.5, 5, 10, and 25 hr after the killing frost.

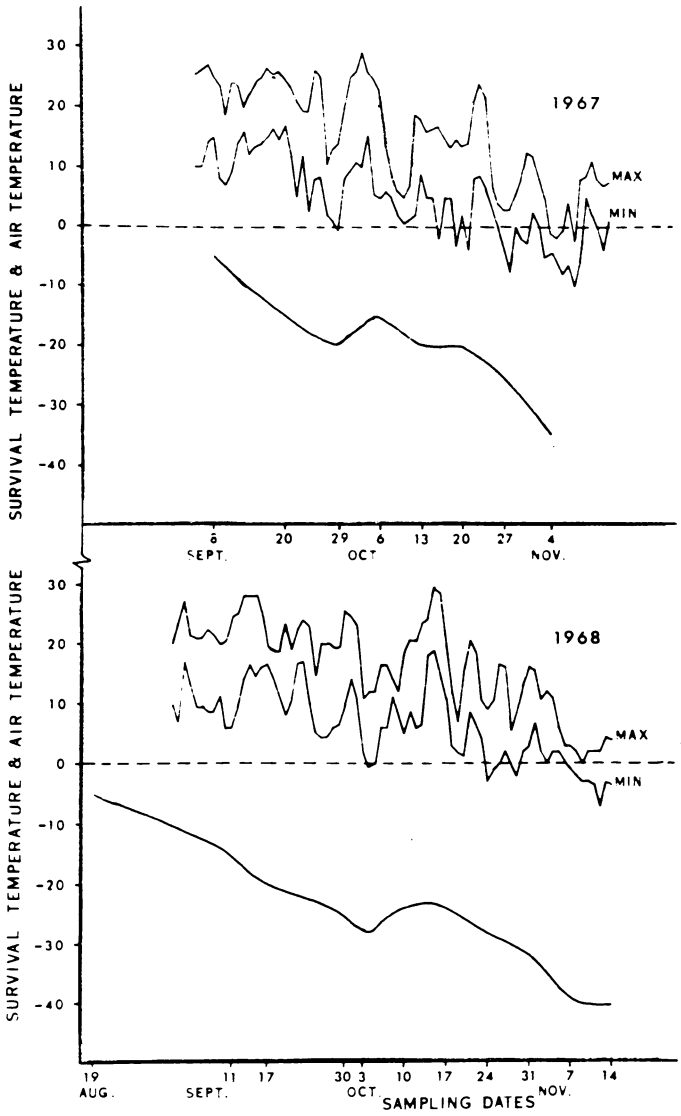

FIG. 1. Maximum-minimum air temperatures and seasonal cold acclimation patterns of Korean boxwood leaves in the autumns of 1967 and 1968 at St. Paul, Minnesota.

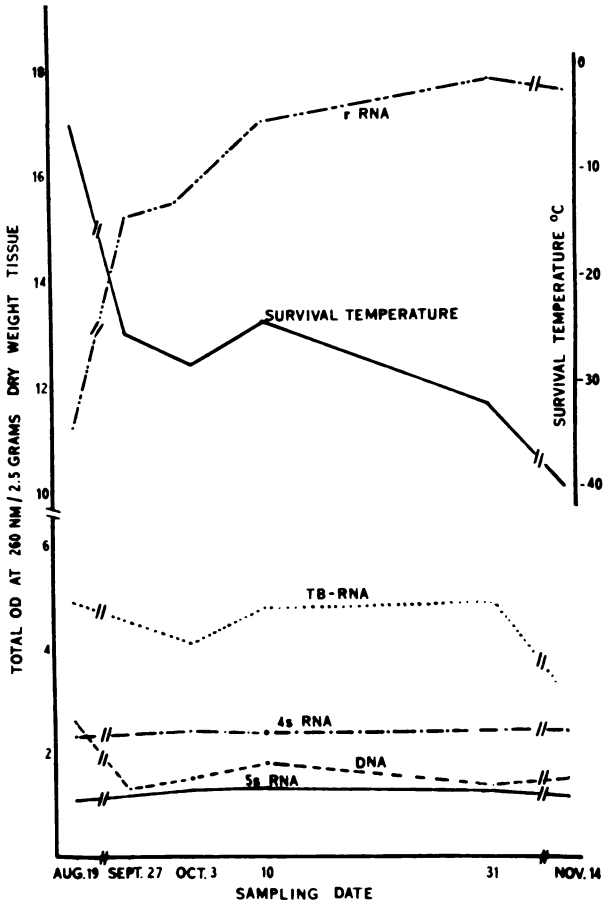

FIG. 2. Changes in the nucleic acid fractions of Korean boxwood leaves during natural cold acclimation in 1968 at St. Paul, Minnesota.

RESULTS

Field Studies 1967 and 1968. The natural seasonal patterns of cold acclimation for boxwood leaves during the autumns of 1967 and 1968 are shown in Figure 1. The cold resistance of leaves increased from −5 to −35 C in 1967 and from −5 to −40 C in 1968. Leaf hardiness appeared to be closely related to environmental temperature. This relationship would probably have been more apparent with frequent sampling. There was about a 10% reduction in moisture content as the leaves acclimated from −5 to −35 C in 1967. The greatest reduction in leaf hydration appeared to occur during periods when hardiness was increasing rapidly, but the moisture content remained relatively constant during periods when hardiness fluctuated.

Nucleic acid patterns during acclimation in the autumn of 1968 are shown in Figure 2. The greatest change was observed in rRNA which increased 50% during the acclimation cycle with the major portion of this increase occurring during the early phase of cold acclimation. The 4 sRNA, 5 sRNA and DNA classes remained relatively constant throughout acclimation while TB-RNA, which decreased slightly during the initial phases, remained relatively constant for a time and then decreased approximately 60% as ultimate hardiness was attained in early winter.

Water soluble protein increased approximately 2-fold as the leaves increased in cold resistance from −5 to −40 C (Table

Table I. *Water Soluble Protein Content and RNase Activity in Boxwood Leaves Collected From the Field at Different Stages of Cold Resistance*

Date	Lowest Survival Temperature	Soluble Protein	RNase Enzyme Units/g Dry Wt	RNase Enzyme Units/μg Protein
		μg/g dry wt		
08/19/68	−5	5,300	296	0.056
09/19/68	−20	6,200	242	0.039
10/13/68	−28	7,300	234	0.032
10/17/68	−24	10,600	212	0.020
11/07/68	−40	10,700	214	0.020

Table II. *Water Soluble Protein and Moisture Content, RNase Activity, and Hardiness Levels of Boxwood Leaves Cold Acclimated in a Growth Chamber at a 16- or 8-Hr Photoperiod*

Lowest Survival Temperature	Moisture Content	Soluble Protein	RNase Enzyme Units/g Dry Wt	RNase Enzyme Units/μg Protein
C	%	*μg/g dry wt*		
16-Hr photoperiod				
−5	55	3,500	416	0.119
−20	53	4,625	370	0.080
−30	52	5,750	379	0.066
−30	52	9,000	387	0.043
−35	52	7,325	380	0.052
8-Hr photoperiod				
−5	55	3,525	391	0.111
−10	53	5,250	346	0.066
−30	51	5,825	343	0.059
−30	51	6,875	268	0.039
−35	51	5,750	247	0.043

I). During this period, RNase activity decreased about 27%. A decrease in RNase activity with increasing cold resistance has also been reported to occur in Mimosa seedlings (8).

Controlled Acclimation Studies. The growth chamber studies showed that it is possible to acclimate boxwood effectively under controlled conditions. Low temperature appeared to be the primary hardiness inducing factor. In the first study leaves acclimated to −35 C regardless of the photoperiod (Table II). In deciduous species, short photoperiods have been shown to promote acclimation (27).

In this study, acclimation was slightly more rapid under the 16-hr photoperiod. It is probably unwarranted to conclude that long photoperiods enhance acclimation, however, because plants in the 16 hr treatment received twice as much total radiation as those grown at the 8-hr photoperiod.

Water soluble proteins increased, whereas RNase activity decreased with increasing hardiness under both photoperiod treatments (Table II). Water soluble protein content was higher in leaves from the long daylength treatment, but this is not unexpected since plants under the 16-hr photoperiod had a photosynthetic advantage over those grown at 8 hr. RNase activity decreased about 9% under the long day treatment and 47% under the short day treatment as plants acclimated from −5 to −35 C, and leaf moisture content decreased about 1%.

In the second controlled acclimation study, the changes in nucleic acids and proteins were similar to those observed in the field. Again, rRNA was the most responsive species of RNA to changes in cold hardiness. As the hardiness increased from −8 to −28 C, the total yields of sRNA, rRNA and DNA increased approximately 16%, 19%, and 9% respectively (Table III). There was a slight reduction in RNA as maximum hardiness was reached.

Water-soluble and membrane-bound proteins increased in leaves during acclimation until the final sampling date when there was a decrease in the membrane fraction (Table IV). The amount of soluble amino nitrogen did increase slightly with maximal accumulation of protein (Table IV). This would appear to indicate that a higher level of amino acids will contribute to a greater accumulation of protein. Craker *et al.* (1) have shown that new protein bands appear while others disappear from electrophoretic patterns during the cold acclimation of bark from hardy apple trees.

Controlled Dehardening and Rehardening. The data in Figure 3 shows the influence of alternating high and low temperature on short term changes in hardiness and ^{32}P incorporation into nucleic acids. During the first dehardening (25 C) and subsequent rehardening (−10 C) treatment, leaf hardiness decreased and then increased 15 C within 24 hr. Similar rates of dehardening and rehardening have been observed in apple bark tissues (9). As in the case of apple, the dehardening-rehardening cycle was not fully reversible and leaves rehardened little by the 5th day of alternating temperature treatments. Following the initial dehardening treatment, ^{32}P incorporation was high in all fractions of nucleic acids, but incorporation diminished in successive treatments. Incorporation following the warm treatments was always higher than that following cool treatments.

Frost Injury Study. RNA was rapidly degraded in leaves following a killing frost (Fig. 4). Within 5 hr, TB-RNA was the only RNA recovered by MAK chromatography from samples incubated at 30 C. The loss of DNA was less rapid: 80% in 25 hr. Some recent research has indicated that cryoinjury in plants (3) and animal cells (7, 20) may result from rapid enzymatic degradation of cell constituents following disrup-

Table III. *Changes in the Nucleic Acid Fractions at Different Levels of Hardiness in Boxwood Leaves Acclimated in a Controlled Environment Chamber*

Lowest Survival Temperature	Time	Nucleic Acids (A_{260}/4 g dry wt)		
		sRNA	DNA	rRNA
C	*days*			
−8	1	1.94	2.34	8.72
−28	22	2.26	2.54	10.35
−36	44	2.02	2.07	9.50
−32	65	1.76	2.00	8.60

Table IV. *Changes in Water Soluble and Membrane Bound Proteins and Soluble Amino Nitrogen at Different Levels of Hardiness in Boxwood Leaves Acclimated in a Controlled Environment Chamber*

Lowest Survival Temperature	Time	Water-soluble Protein mg/g Dry Wt	Membrane-bound Protein, mg g Dry Wt[1]	Soluble Amino Nitrogen
C	*days*			*μmoles/g dry wt*
−8	1	4.16	26.40	22.6
−28	22	5.36	31.34	31.8
−36	44	5.44	29.04	31.8
−32	65	5.52	24.72	28.4

[1] Phenol-acetic acid-water extractable.

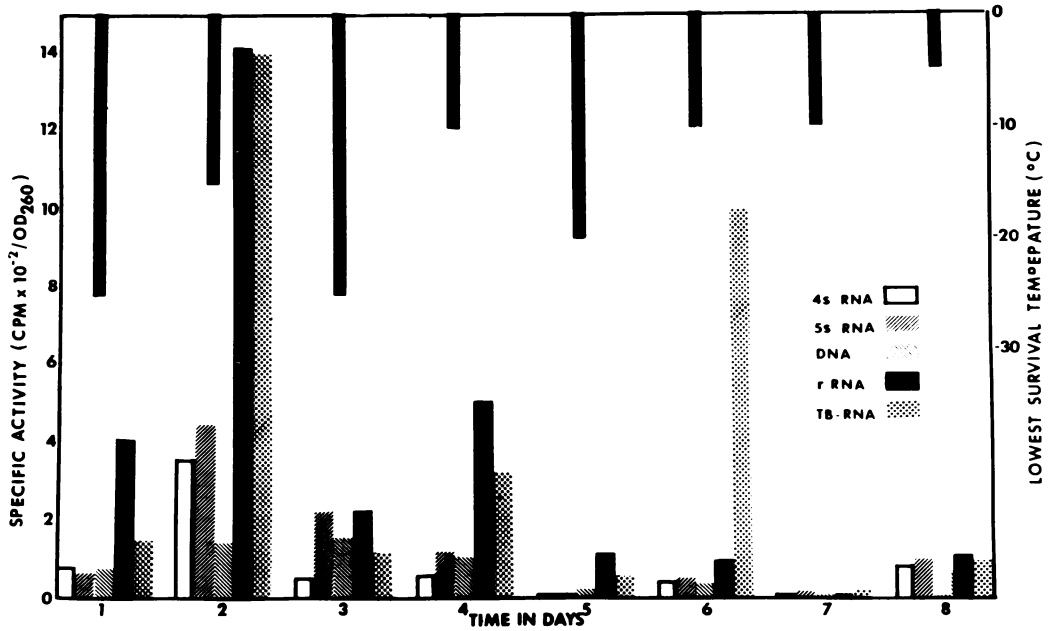

FIG. 3. Nucleic acid synthesis patterns in boxwood leaves as related to short-term dehardening and rehardening responses induced by alternating exposure to low (−10 C) and high (25 C) temperature. Plants were exposed to −10 C on odd numbered days and 25 C on even numbered days.

tion of intracellular compartmentalization. While this study does not permit evaluation of that hypothesis, the destruction of certain nucleic acid fractions was extremely rapid.

DISCUSSION

In many organisms, growth cessation is generally associated with a decline in the synthesis of protein and RNA (particularly rRNA), and an increase in RNase activity (11, 18, 24). However, in woody plants which cold acclimate effectively, the reverse is true, as indicated by this and previous studies on deciduous species (13, 14, 25). These studies indicate that there are substantial increases in protein and RNA (particularly rRNA) at times when visible growth has stopped and the plant is becoming more cold resistant.

Previous work (5) showed that the −20 C lyophilization and the dry grinding procedures used in this study reduced the possibility of enzymatic RNA degradation during sample preparation. Also, the observation that bentonite, a nuclease inhibitor, did not improve RNA yields from boxwood leaf extracts (5), further supports the view that nuclease activity was inhibited during this phase of analysis.

There was a close agreement between the results of this study and those on the living bark of deciduous species (14, 25) regarding the characteristic autumnal increase in rRNA and protein during acclimation. Since this study was conducted on evergreen leaves, the possibility that these increases are merely due to a build up of precursor pools during leaf senescence and abscission is unlikely.

In this, as in previous work (13, 14, 25), rRNA was the most responsive class of nucleic acid to the environmental stimuli which induce acclimation. It has been suggested that the status of ribosomes in the cell (free or bound) may control what kinds of proteins are synthesized (26). This was recently confirmed in rat liver where membrane bound ribosomes were shown to be solely responsible for the *in vivo* synthesis of glycoprotein apopolypeptides (6) and serum proteins (23).

The rapid loss of cold resistance following exposure to warm temperatures was also associated with a rapid synthesis of nucleic acids as judged by ^{32}P incorporation. This suggests

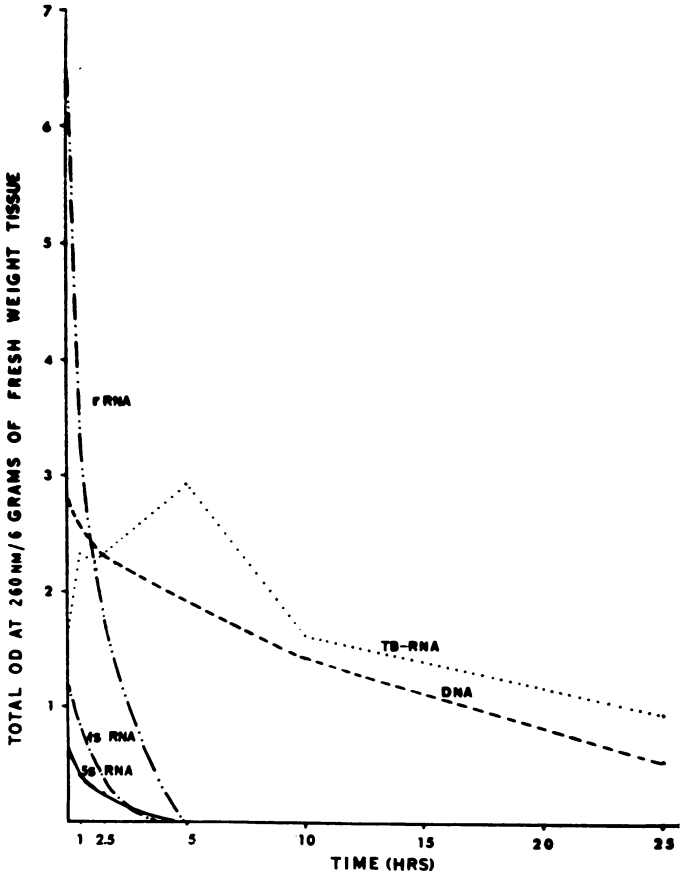

FIG. 4. Degradation of boxwood leaf nucleic acids incubated at 30 C after exposure to a killing frost.

that the dehardening of plant tissues in nature following warm days during the winter and early spring may not be due strictly to physical changes. Attempts to attenuate dehardening by suppressing metabolic activity might prove to be a practical

avenue for research on tree crops where hardiness is lost rapidly during warm days and regained more slowly during cold weather (9, 22).

Work is presently being conducted to determine the relationship between changes in nucleic acid and protein metabolism in the autumn and the environmentally triggered physiological responses which enable hardy plants to resist freezing stress.

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