GROWTH, ORGANIC NITROGEN FRACTIONS, AND BUFFER CAPACITY IN RELATION TO HARDINESS OF PLANTS

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Introduction

Since the evidence in the literature has become somewhat confused upon several points relating to winter hardiness in plants, it seemed desirable to investigate some of the points of controversy according to a newer technique and with the benefit of more specific information that has recently become available. The newer emphasis, that hardiness does not exist as such in a hardy variety, but that it must be developed, has been the result of the work of a number of investigators. Within the past year or two it has been shown that winter wheat plants of the most hardy varieties harden only poorly when placed at a low temperature in the dark (15, 3). The whole matter of increase in resistance to cold has been connected with opportunity for photosynthesis, storage of organic foods, low respiration, and slight vegetative growth (3, 7).

Experimentation

In the first experiment it seemed desirable to know whether winter wheat plants added materially to their dry-matter content if stored in a cold-room with artificial illumination. Minhardi plants were grown to an age of three weeks in the greenhouse, after which a part of the crop was moved to a room at 2° C.

In all experiments reported in this paper the plants were grown in quartz sand cultures with nutrient solutions. Six pots with 25 plants each were harvested prior to cold-room treatment, six were stored in the dark at 2° C., and six were stored in continuous light at 2° C. After two weeks the plants in the cold-room were harvested. The hardiness of the plants was determined before and after storage by the freezing-exosmosis method of DEXTER et al. (6). Table I shows the results of this experiment. Since the pots were very uniform, only totals are given.

From the table it would appear that the plants made marked growth, as seen by the more than doubling of the weight of the dry matter contained in them. It was not possible, however, to distinguish the two sets visually after removal from the cold-room. Increase in the length of the leaves was hardly more than the experimental error. The plants that were stored in the light in the cold-room were materially lower in total nitrogen per

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TABLE I
GROWTH OF WINTER WHEAT (MINHARDI) AT 2° C. SHOWING CHANGES IN HARDINESS WHEN ILLUMINATED AND WHEN STORED IN DARKNESS

<table>
<thead>
<tr>
<th>SAMPLE</th>
<th>TOPS</th>
<th>ROOTS</th>
<th>TOTAL INJURY SPECIFIC CONDUCTIVITY X 10^5 25° C.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DRY MATTER</td>
<td>GREEN WT.</td>
<td>DRY WT.</td>
</tr>
<tr>
<td>At the start</td>
<td>%</td>
<td>gm.</td>
<td>gm.</td>
</tr>
<tr>
<td>14 days 2° C.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>dark ............</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14 days 2° C.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>light ..........</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

gram dry matter than the other sets, although the total nitrogen per plant was much greater in them. It is evident that the plants which received illumination carried on active photosynthesis and took up nitrogen from the soil at the temperature of 2° C. These plants hardened materially, whereas the hardening in the plants stored in the dark was hardly perceptible. Analysis showed that the content of reducing sugars in the plants stored in the dark had dropped to a mere trace during the period in the cold-room.

In the next experiment, winter wheat plants were grown for two weeks in the greenhouse with a full nutrient solution. At the end of that time the nitrogen was washed from half the pots, and the plants were allowed to grow for two weeks without nitrogen in the nutrient medium. Calcium chloride was substituted for calcium nitrate. At the end of two weeks without nitrogen, extracts from the minus-nitrogen plants gave no test for nitrates with diphenylamine and the plants were presumed to be virtually free from inorganic nitrogen. They showed every evidence of nitrogen deficiency.

By means of this experiment it was hoped that several points might be clarified: (1) Does the commonly reported increase in soluble organic nitrogen result from uptake from the soil, or is it due to breakdown of proteins? (2) Does the winter wheat plant require nitrogen in the soil during hardening? (3) Will minus-nitrogen winter wheat plants harden at a low temperature in the dark?

The organic nitrogen fractions in the plants were determined in the following way. Fresh samples (20 plants) were treated with ether and
ground to a smooth paste with quartz sand. The sample was made to a
definite volume with tenth normal potassium sulphate, centrifuged, and the
residue washed and centrifuged three times. The resulting suspension was
coagulated with heat, after adding a few drops of acetic acid. The coagulated
material was removed by filtration. The three fractions were deter-
mined separately, i.e., the soluble or non-coagulable fraction, the coagulable
fraction, and the fraction thrown out by the centrifuge. It was not found
possible to obtain checks on duplicates for the latter two determinations,
although the ‘soluble’ fraction gave good checks. The ‘soluble’ nitrogen
is reported, then, as the percentage of the total nitrogen in the plants. On
this basis the duplicate samples checked well. Table II gives the results of
this experiment. The experiment was repeated, with the additional
analyses for amino nitrogen (Van Slyke), and for carbohydrates. Table II
includes these figures. According to a similar idea, samples of cabbage
leaves were prepared from plus-nitrogen plants, deficient in starch and
actively growing, and from minus-nitrogen plants high in starch. Samples
to be compared in any regard consisted of half-leaves, split at the midrib.
One set was analyzed as it came from the greenhouse, the other after stor-
age in a moist chamber at 2° C. in the dark for 7 days.

Table II appears to answer the first question as to the increase in
soluble organic nitrogen in plants stored at a low temperature. Several
workers have noted this increase and have attributed the increase in hardi-
ness partly to this cause (10, 6). In a recent paper Newton et al. (12) con-
cludes that the increase in soluble nitrogen, especially in alpha amino form,
is due to the occasional freezing of the plants during the early winter
season. The plants in this experiment were not frozen, however. That
the increase in soluble nitrogen comes from the breakdown of proteins
seems probable from the figures presented in the table. Plants which gave
no tests for nitrate nitrogen increased as much in soluble nitrogen as those
liberally supplied with the element in nutrient solutions, and in which
liberal amounts were present, according to the diphenylamine test.

Table II presents the data relating to hardening of these plants at 2° C.
both in light and in darkness. Since it was anticipated that the samples
might differ greatly in total soluble extractable electrolytes, samples were
killed by heating and extracted to give the total salts in the same volume of
water as was used for the freezing-exosmosis test (4). The samples of winter
wheat crowns were frozen at -8° C. for two hours; the cabbage at -6° C.
for two hours. The table shows that winter wheat plants high in carbo-
hydrates, due to nitrogen starvation, hardened well in the cold room.
Those in the light hardened more than those in the dark, but in either case
mineral nitrogen did not seem to be necessary for the hardening process.
High-carbohydrate plants of either wheat or cabbage hardened well in the
TABLE II
ORGANIC NITROGEN FRACTIONS IN PLUS- AND MINUS-NITROGEN PLANTS BEFORE AND AFTER EXPOSURE TO LOW TEMPERATURE (2°C.)

<table>
<thead>
<tr>
<th>SAMPLE*</th>
<th>DAYS AT 2°C</th>
<th>ILLUMINATION</th>
<th>PERCENTAGE N IN SOLUBLE FORM</th>
<th>PERCENTAGE N IN AMINO FORM</th>
<th>CARBOHYDRATES AS PERCENTAGE DRY MATTER</th>
<th>SPECIFIC CONDUCTIVITY (× 10⁶, 25°C) AFTER FREEZING INJURY</th>
<th>HEATING (TOTAL SALT)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Winter wheat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plus-N</td>
<td>0</td>
<td>Light</td>
<td>25.3</td>
<td>9.94</td>
<td>100.0</td>
<td>22.2</td>
<td>67.4</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>Light</td>
<td>18.3</td>
<td>17.9</td>
<td>100.0</td>
<td>22.2</td>
<td>29.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Light</td>
<td>26.2</td>
<td>14.2</td>
<td>100.0</td>
<td>22.2</td>
<td>12.94</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dark</td>
<td>31.9</td>
<td>34.2</td>
<td>100.0</td>
<td>22.2</td>
<td>3.41</td>
</tr>
<tr>
<td>Minus-N</td>
<td>0</td>
<td>Light</td>
<td>21.1</td>
<td>10.9</td>
<td>100.0</td>
<td>22.2</td>
<td>25.6</td>
</tr>
<tr>
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<td>11.9</td>
<td>100.0</td>
<td>22.2</td>
<td>83.8</td>
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<td>24.3</td>
<td>9.8</td>
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<td>22.2</td>
<td>39.6</td>
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<tr>
<td></td>
<td></td>
<td>Dark</td>
<td>30.7</td>
<td>9.94</td>
<td>100.0</td>
<td>22.2</td>
<td>92.8</td>
</tr>
<tr>
<td>Repeated</td>
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<td></td>
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<tr>
<td>Plus-N</td>
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<td>Light</td>
<td>20.0</td>
<td>9.94</td>
<td>100.0</td>
<td>22.2</td>
<td>67.4</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>Light</td>
<td>29.1</td>
<td>17.9</td>
<td>100.0</td>
<td>22.2</td>
<td>29.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dark</td>
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<td>14.2</td>
<td>100.0</td>
<td>22.2</td>
<td>12.94</td>
</tr>
<tr>
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<td>Light</td>
<td>19.7</td>
<td>4.98</td>
<td>100.0</td>
<td>22.2</td>
<td>22.5</td>
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<tr>
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<td>22.5</td>
<td>10.60</td>
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<tr>
<td></td>
<td></td>
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<td>30.0</td>
<td>16.95</td>
<td>100.0</td>
<td>22.2</td>
<td>29.9</td>
</tr>
<tr>
<td>Cabbage</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Minus-N</td>
<td>0</td>
<td>Dark</td>
<td>8.80</td>
<td>22.2</td>
<td>100.0</td>
<td>22.2</td>
<td>181</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Dark</td>
<td>17.46</td>
<td>29.0</td>
<td>100.0</td>
<td>22.2</td>
<td>160</td>
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<tr>
<td>Plus-N</td>
<td>0</td>
<td>Light</td>
<td>13.96</td>
<td>No test for starch qualitatively</td>
<td>100.0</td>
<td>22.2</td>
<td>227</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>Dark</td>
<td>19.48</td>
<td></td>
<td>100.0</td>
<td>22.2</td>
<td>223</td>
</tr>
</tbody>
</table>

* Part of the winter wheat samples were stored at 20°C with continuous illumination, part in the dark. All cabbage samples were stored in the dark. Conductivity values for exosmosis from wheat crowns and from cabbage leaves following freezing injury and following heating are included.
dark; low carbohydrate plants little or not at all. If illuminated, plus-
nitrogen plants hardened well.

To summarize the results of this experiment it may be stated that:
(1) Proteins are split in the plants stored at this low temperature entirely
regardless of the hardening process. In every case the plants which
hardened least increased most in soluble nitrogen. (2) Cabbage and winter
wheat plants do not require nitrogen from outside the plant to carry on
the hardening process. (3) Minus-nitrogen plants, high in starch,
hardened well at 2° C. without illumination, in sharp contrast with those
low in carbohydrates.

Certain elements were not included in the nutrient solutions in the sand
cultures. The salts used were calcium nitrate, potassium acid phosphate,
and magnesium sulphate, with traces of iron as phosphate. Tests for
chlorides in the extracts from the crowns showed mere traces. Seemingly
chlorides are not necessary in the hardening process but did not prevent it,
since calcium chloride was used in the minus-nitrogen series. The apparent
loss of minerals from the samples of winter wheat which harden has been
described more fully in another paper (4).

It seemed desirable to investigate the hardening capacity of other minus-
nitrogen plants. Winter rye, winter barley, and winter oats were grown
as previously described. As in the case of winter wheat, minus-nitrogen
plants of rye, barley, and oats were found to harden in the dark at 2° C.,
whereas plus-nitrogen plants hardened very slightly. To a few pots of
plus-nitrogen plants of these four species, a 2 per cent. sucrose solution
was added, and the plants set at 2° C. in the dark. With all four cereals
hardening was somewhat better than the corresponding plants which did
not receive sugar, but not so complete as the minus-nitrogen plants in the
dark (2). According to a similar idea, the freshly severed stems of cabbage
plants were placed in water, in 2.5 and 10 per cent. sucrose solutions, and
kept in the greenhouse for 40 hours. The samples were then analyzed for
sugars, for freezing injury at –6° C., and for ice formed, by the calorimetric
method. The results were perfectly regular and orderly. More water was
left unfrozen in the plants placed in 10 per cent. sugar than in 2.5 per cent.,
which in turn showed more unfrozen water than the plants kept in water
alone. Average water unfrozen in four samples of each was 9.95, 8.70,
and 6.94 gm. in the 10 per cent. sucrose, the 2.5 per cent. sucrose, and the
water only, respectively. Injuries, according to specific conductivities
taken, were 44.5, 59.5, and 75.1 for the samples as just given. The amount
of total sugars in the plants kept in 10 per cent. sugar was more than double
that in the others. Peculiarly enough the reducing sugars were about
twice as great in amount as in the normal plants, although sucrose only
was supplied. Evidently the plant has a capacity to hydrolyze rapidly
sugars furnished in this way. The marked protection against freezing injury might well be explained by the decrease in the ice formed. The increase in hardiness, however, was far less than can readily be accomplished by simple exposure to low temperatures for a week, during which time the increase in sugar is far less than was found in these cases. It seems improbable that increase in hardiness can usually be explained in such simple terms.

In another experiment, the buffer capacity of samples of cabbage leaves before and after hardening was investigated. Half-leaf samples were again prepared from plants high and low in carbohydrates respectively. One set of half-leaves was ground with a pinch of quartz sand before exposure to cold; the other half-leaves were put to harden in the cold room for seven days. They were stored in a moist chamber in the dark. After grinding to a smooth paste, the samples were made to a definite volume, and the hydrogen ion concentration determined with a hydrogen electrode. Definite volumes of tenth normal hydrochloric acid or sodium hydroxide were added with a pipette to half the sample, and the hydrogen ion concentration again determined, until five additions of acid or base had been made. A total of eight samples of each type of sample was run, on two occasions. The voluminous data obtained will not be presented in full. In seven out of eight samples of cabbage leaves which actually hardened at 2° C., the ground leaves were found to be slightly more alkaline after exposure to cold than before; in six out of eight samples of low carbohydrate leaves, which did not actually harden at 2° C., the leaves were found to be slightly more alkaline after exposure to cold than before. Thus whether the plants hardened or failed to harden, this response appears to be more or less identical. The changes were practically the same in each case and amounted to about 0.2 pH unit. In all cases, however, the plants that were high in carbohydrates were distinctly more acid than those low in carbohydrates. Those high in nitrogen were found to be better buffers, as well, either before or after exposure to cold. Exposure to cold did not appear to change the buffer capacity in either case.

The increase in hardiness has been attributed to an increase in soluble nitrogen and an increase in buffer capacity. An increase in soluble nitrogen unquestionably occurs on exposure to low temperatures, but it occurs to a greater degree in plants that have not hardened than in those which have. Newton (11) failed to find a correlation between buffer capacity and hardiness in winter wheat. Harvey (8), who reported a definite increase in buffer capacity in cabbage which was hardened, used the expressed juice from the plants. This expressed juice, after hardening, is usually higher in total solids than before hardening, and might well show more buffer capacity since it contains more dry matter. In the technique
used in this experiment, however, virtually the same amount of dry matter was used in each case. No change in buffer capacity was found in a given number of cells or a given amount of material. The increase in soluble nitrogen in high carbohydrate plants during exposure to cold is not particularly great, since the quantities present are small. In the samples analyzed, less than 0.3 mg. of soluble nitrogen was present per gram of fresh leaves. Even though this amount almost doubles during exposure to cold, it must still be a very small factor in the buffering of the plant juice. The amount of soluble nitrogen present in the vegetatively active cabbage plants was about four times this amount.

In view of the findings of these previous experiments, it seemed desirable to reinvestigate the matter of the hardening of plants in an alternating temperature. Harvey (9), Tysdal (16), and others in unpublished work, have observed increased hardening in plants that were subjected to alternating temperatures. Frequently these plants were given continuous illumination, whether at a constant or an alternating temperature. Dexter (3) and Tysdal (16) have used various lengths of day and night during hardening. Dexter has presented data which seem to indicate that the increased hardening under such conditions is due to increased photosynthesis and greater net storage of organic foods. In the experiment now reported, half-leaf samples were stored at 2°C continuously. The other halves were alternated between 2°C and 20°C at approximately 12-hour intervals. A third sample, as nearly like the duplicate half-leaf samples
as possible, was stored at a constant temperature of 15° C. It was estimated that the respiration of this sample would be approximately that of the sample alternated between 2° and 20° C. The samples were contained in moist chambers, in the dark, and no wilting was evident at the end of the experiment although those at the higher temperatures were somewhat yellowed. Five samples were stored under each temperature condition for examination of hardiness. Samples were also prepared for examination of change in soluble nitrogen and for sugar analysis. Table III presents the data from this experiment.

From the data given in table III, it can be seen that the samples stored at 2° C. hardened a great deal more than duplicate half-leaf samples alternated from 2° to 20° C. Each set was in the dark. The samples alternated appear to be almost precisely as hardy as the samples kept constantly at 15° C. These were not duplicate half-leaf samples but were as nearly identical as possible. The soluble nitrogen in the leaves kept at 2° C. continuously is less than half that of the alternated leaves. The increase in soluble nitrogen in the alternated leaves evidently did not give them greater hardiness. The sugars, and especially sucrose, were higher in the sample kept continuously at 2° C. than in either of the other two samples. The other two samples agree perhaps as well as could be expected, since they were not duplicate half-leaves. These data would seem to support the idea previously put forth (3) that if greater hardening is found under conditions of alternating temperature, above the freezing point, it is due to the greater photosynthesis under those conditions. Alternating temperatures, with the plants in the dark, or in the light without carbon dioxide, do not seem to stimulate the hardening reaction.

Summary and conclusions

1. A reexamination of some of the theories of winter hardiness of plants has been made. It should be recognized in any work dealing with the hardening reaction, that plants which are usually thought of as hardy need not necessarily harden if placed at a low temperature. This reaction seems to be largely dependent upon the organic nutrition of the plant, if opportunity for photosynthesis is denied at the low temperature.

2. Plants of a hardy variety of winter wheat were grown with excess and with minimum nitrogen supplies in the nutrient medium. When placed at 2° C. without illumination, it was found that those high in nitrogen did not harden, although there was a considerable increase in soluble organic nitrogen in the plants during the period at the low temperature. The minus-nitrogen, high-carbohydrate plants hardened well in the dark with an increase in soluble organic nitrogen. Sugars increased in the high-carbohydrate plants and decreased in the plus-nitrogen plants during
such storage. Although the behavior in regard to organic nitrogen was almost indistinguishable, the difference in hardening was marked. With both sets of plants in the light at a low temperature, hardening was efficiently carried out, and the increases in soluble organic nitrogen were less than with corresponding plants in the dark, where hardening was in all cases less. Thus increase in soluble organic nitrogen is no indication of increase in hardiness.

3. Minus-nitrogen, high-carbohydrate plants of winter rye, winter barley, winter oats, and cabbage were found to harden at 2°C in the dark, while corresponding plants low in carbohydrates due to surplus nitrogen in the nutrient medium did not harden under those conditions.

4. Cabbage leaves, low in carbohydrates and high in nitrogen, although they hardened but little at a low temperature were found to be more alkaline and better buffers, both before and after exposure to cold, than leaves high in carbohydrates and low in nitrogen. While the samples of both sets were generally slightly more alkaline after exposure to cold for a week, they were not distinguishably better in buffering capacity than before exposure to cold.

5. Constant low temperature in the dark was more effective in causing hardening in cabbage leaves than alternating temperatures in the dark. Higher sugar content was found in the samples kept constantly at 2°C than in samples alternated between 2°C and 20°C or stored at a constant temperature of 15°C. Soluble nitrogen was higher in the alternated leaves than in those held at 2°C.

6. There appears to be a series of reactions which proceed in a plant at a low temperature entirely regardless of increase in hardiness. There is an increase in soluble organic nitrogen. There is a decrease in respiratory rate which is not correlated with increase or decrease in sugars or enzyme activity (5, 1). There is, usually at least, an increase in sugars, which may or may not be accompanied by decidedly increased hardiness. A continued and critical examination of the theories of winter hardiness is needed.

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