Sampling for 3-Hydroxytyramine and Polyphenoloxidase in Sugar Beets[1]

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Further research work into the phenolic compounds occurring in the leaves of sugar beets is reported. This is a continuation of the work by Harrison et al. (1) describing the association of a certain phenolic compound with resistance to Cercospora leaf spot. The phenolic compound was later identified as 3-hydroxytyramine by Gardner (2). When this compound was oxidized in beet leaf extract, it was found to be highly toxic to *Cercospora beticola* Sacc. growing in pure culture. There is a naturally occurring polyphenoloxidase enzyme in the leaf that is capable of oxidizing the 3-hydroxytyramine.

Both of these compounds are currently being studied. However, preliminary results have indicated that data for both characters were quite variable when samples were taken from uniform populations. Local environmental conditions, weather and intensity of light appear to cause extreme differences in 3-hydroxytyramine content of the leaves.

The purpose of this report is to illustrate the difficulties involved in sampling plant parts for a chemical study, to present an organized approach to the problem and to describe some of the results obtained from these studies.

Methods

The concentration of 3-hydroxytyramine and polyphenoloxidase present at four stages of growth were determined on each of 10 populations which were known to differ in resistance to Cercospora leaf spot. These data show how the compounds build up in the leaves and indicate which stage to sample.

The 10 populations, covering a wide range of leaf spot resistance, were planted in a randomized block with four replicates

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on April 28, 1964. At each sampling, we selected leaves which were intermediate between the small young leaves and the largest old leaves which showed signs of senility. The leaves selected were not the largest leaves on the plant, but at that particular growth stage of the plant they represented the mature or most typical leaves. Usually four such leaves could be found on each plant. We took an equal number of leaves from each plant in a plot to make up the sample.

The four growth stages of the plant leaf when leaves were sampled for this study were taken on the dates noted below:

1. June 15, 1964. Taken as soon after thinning as a sufficient sample for analysis could be obtained.
2. July 13, 1964. Leaves about one-half fully expanded.
3. July 30, 1964. Leaves fully expanded.
4. August 5, 1964. Leaves mature.

Choice of leaves for the sample is difficult. Certainly some prejudice or bias occurs, and the impossibility of defining and identifying comparable, equally mature leaves should be recognized.

3-Hydroxytyramine determination

After harvest the leaves were carefully stacked, placed in a polyethylene bag and quick-frozen as soon as possible.

For analysis, a 7.5-gram sample was cut transversely from the middle section of the stacked, frozen leaves and placed in a blendor. Eighty ml of *n*-amyl alcohol-HCl solution (99.5 ml *n*-amyl alcohol, plus 0.5 ml concentrated HCl), were added and the sample was ground at high speed for 4-5 minutes. After scraping down any ground material on the sides of the jar, the mixture was filtered through Whatman No. 1 filter paper with the aid of suction. An additional 20 ml portion of the *n*-amyl alcohol-HCl mixture was used to rinse the jar, then poured through the filter.

The filtrate was transferred to a 250 ml Erlenmeyer flask. The suction flask was rinsed with exactly 15 ml distilled water which was added to the filtrate. This solution was shaken for 5 minutes on a shaker and then transferred to a 250 ml separatory funnel and allowed to stand until separation was complete. The aqueous layer was drained off and adjusted to pH 1.5 with about 6N HCl.

Two ml of the aqueous extract was pipetted into a 50-ml, calibrated test tube which contained about 10-15 ml distilled water. Five ml of Arnow's reagent, made by dissolving 10 g sodium molybdate and 10 g sodium nitrite in 100 ml of distilled water, was added. The mixture was shaken and allowed to stand for 3-4 minutes, to permit the initial reaction to take place. After the addition of 2.5 ml 10% NaOH the solution was made up

to exactly 50 ml with distilled water. The optical density was read at 500 mμ on a spectrophotometer. Determination of weight of 3-hydroxytyramine per volume of aqueous extract was made by comparison to a standard curve.

When analyzing a single whole leaf or various weights of leaf sample, the amount of *n*-amyl alcohol-HCl mixture and of the distilled water added before shaking was varied in direct proportion to the weight of the leaf sample used.

Polyphenoloxidase determination

Polyphenoloxidase can be determined colorimetrically by using catechol as a substrate.

Ten grams of frozen sugar beet leaves were ground for 2 minutes in a blendor with 150 ml of ice water. The mixture was immediately filtered through fiber-bonded milk filters (cut to fit a Buchner funnel) with the aid of suction.

One ml of the cold filtrate was pipetted into 10 ml of dilute acetate buffer in a colorimetric tube. This dilute acetate buffer is prepared by a 1:5 dilution of a standard, pH 5, 0.5M acetate buffer. After shaking, the tube was placed in the spectrophotometer (set at 420 mμ wave length) and the optical density was adjusted to zero. One ml of fresh 0.25 M catechol solution was added, and the tube was shaken again and replaced in the spectrophotometer. Again the optical density reading was adjusted to zero. With the shutter remaining open, the reading was recorded at exactly 1-minute intervals for 5 minutes.

The optical density reading increases in direct proportion to the amount of polyphenoloxidase present due to the color reaction of polyphenoloxidase and catechol.

In this procedure, the filtrate should be kept as cold as possible to maintain the polyphenoloxidase activity and it should be read as quickly as possible after grinding.

Results and Discussion

The analysis of variance for concentration of 3-hydroxytyramine and polyphenoloxidase analyzed at various stages of leaf growth is listed in Table 1. The analyses indicate that the concentrations of 3-hydroxytyramine and polyphenoloxidase activity certainly differ when analyzed at these stages. There is also a stage by population interaction for 3-hydroxytyramine which suggests that some populations have a higher concentration of this compound at one stage and that other populations have a higher concentration at another stage. The F values due to population variations are highly significant, a factor which indicates there are differences in the concentrations of the phenolic compounds among populations.

Table 1.—Variance analysis of concentrations of 3-hydroxytyramine and polyphenoloxidase analyzed from leaf samples taken at four growth stages.

Source of variation	Degrees of freedom	3-hydroxytyramine		Polyphenoloxidase	
		MS	F	MS	F
Total	159				
Replication	3	191.5128	1.1434	0.0154	2.2319
Stage	3	15,512.7033	92.6134**	9.5587	138.5319**
Population	9	3,690.0512	22.0302**	0.0535	7.7536**
S × P	27	871.3069	5.2918**	0.0109	1.5797
Error	117	167.4995		0.0069	

** Denotes significance at 1% level

When looking at the concentration of 3-hydroxytyramine in Table 2 it may be noted, from the mean of all populations at each stage, that stages one and four do not differ significantly; nor do stages two and three; however, stages one and four have a lower concentration than do stages two and three. The concentration apparently builds up in the leaves as they grow and decreases after the leaves are fully expanded (see Figure 1), perhaps as a result of oxidation or translocation. Entries 1, 6 and 8 tend to have a higher concentration in stage two while entries 3 and 7 seem to be higher in stage three. Therefore, either stage two or three would be the best time to analyze for 3-hydroxytyramine because the concentrations are highest at that time.

The concentration of polyphenoloxidase apparently builds up in the leaves as they grow (as shown by the means in Table 2), much as the 3-hydroxytyramine does; but it does not decrease in older leaves (see Figure 2). There is less polyphenoloxidase present at the first stage than at the second stage and the second stage has less than either the third or fourth stages. Stages three and four do not differ significantly; hence the best time to obtain samples for maximum polyphenoloxidase activity would probably be any time after the leaves are fully expanded.

Since analyses for both 3-hydroxytyramine and polyphenoloxidase can be obtained from the same sample, time and labor can be saved if the samples are taken at one stage. The third stage when the leaves are fully expanded is probably the best time to sample for maximum quantities of both the phenolic compound and the enzyme in leaf samples.

Not only is there a change in the concentration of 3-hydroxytyramine with respect to the growing season, but there is also a difference in various parts of the leaves and with different aged leaves.

When the leaves were divided transversely (into tip and basal halves), the bottom half of the leaves contained considerably

Table 2.—3-hydroxytyramine means in mg/100 ml of leaf extract and optical density reading for polyphenoloxidase at each of four growth stages.

Population	Entry number	Resistance index*	3-hydroxytyramine Growth stages 1	2	3	4	Polyphenoloxidase Growth stages 1	2	3	4
A56-3	1	MR	3.68	41.38	22.00	9.88	0.24	0.91	1.30	1.25
(52-305CMS × 52-408)F1	2	MR	6.52	57.75	59.88	12.75	0.24	0.95	1.40	1.29
SP 5481-0(2n)	3	R	2.00	15.50	27.00	9.00	0.32	0.86	1.32	1.32
SP 5481-0 (4n)	4	R	1.75	20.75	20.50	10.62	0.26	0.90	1.34	1.32
US 201	5	HR	2.22	41.75	43.38	14.00	0.25	0.99	1.31	1.39
(52-305CMS × 52-407)F1	6	MR	9.12	122.75	99.00	21.62	0.19	0.67	1.16	1.16
SP 5822-0	7	HR	2.42	20.50	28.38	7.38	0.24	0.96	1.54	1.29
CW 777-60A	8	MR	2.80	34.75	27.50	10.38	0.28	1.05	1.34	1.26
AC 62-4T22 (4n)	9	MR	2.98	48.00	43.75	17.38	0.34	1.03	1.26	1.34
HH 10	10	S	2.80	28.00	24.75	8.25	0.31	0.95	1.37	1.31
Mean of each stage			3.63	43.11	39.61	12.12	0.27	0.93	1.31	1.29
Standard Error				± 6.47				± 0.042		

* HR = high resistant, R = resistant, MR = moderately resistant, S = susceptible.

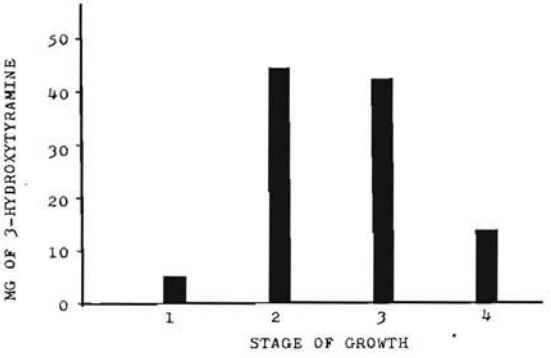

Figure 1.—Concentration of 3-hydroxytyramine in sugar beet leaves sampled at four growth stages.

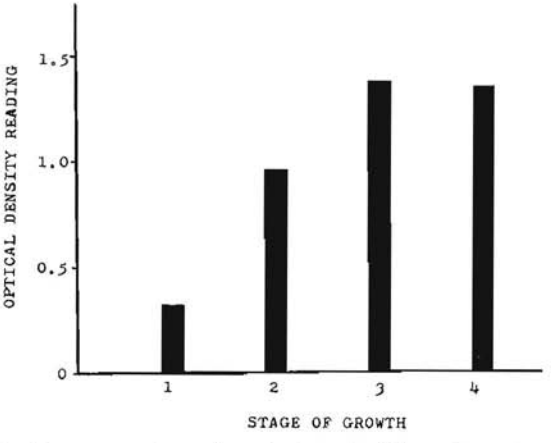

Figure 2.—Concentration of polyphenoloxidase in sugar beet leaves sampled at four growth stages.

larger amounts of 3-hydroxytyramine on a weight basis than the top half. But when the leaves were divided median longitudinally, down the mid-rib, the two halves contained nearly equal amounts of 3-hydroxytyramine on a weight basis. This was a consistent finding on leaves of different varieties and with different aged leaves. The petioles were much lower in 3-hydroxytyramine than the blades and only a trace was present in the roots.

The most reliable way to sample leaves for 3-hydroxytyramine was to cut a transverse section from the center of the leaves stacked one on top of another, discarding both the tip and the base of the leaves. This procedure also provided an adequate sample for polyphenoloxidase, since it was quite uniformly distributed throughout the leaf.

Of perhaps greater significance was the comparison of the concentration of 3-hydroxytyramine from different aged leaves of the same plant. If the first fully mature leaves (usually four) were taken, the 3-hydroxytyramine content in each of these leaves was found to be nearly equal; but when several leaves were taken of different ages, the concentration of 3-hydroxytyramine varied over a wide range. Samples were taken from individual plants. In one experiment four leaves, very nearly the same age, were collected from four plants. From four other plants, we collected leaves, starting with the oldest living leaf and working in toward the center of the crown and young leaves (Tables 3 and 4).

Table 3.—3-hydroxytyramine content in leaves of same age:

Genotype number	Weight of leaf (grams)	mg 3-hydroxytyramine per 100 ml extract
1	16.0 mature leaf	5.0
	9.7 mature leaf	11.0
	12.0 mature leaf	6.5
	13.0 mature leaf	5.5
2	6.3 mature leaf	31.5
	10.0 mature leaf	17.0
	6.8 mature leaf	21.5
	7.0 mature leaf	21.5
3	8.9 mature leaf	18.0
	9.6 mature leaf	14.0
	10.7 mature leaf	13.5
	7.7 mature leaf	12.0
4	11.0 mature leaf	3.0
	8.8 mature leaf	4.5
	11.0 mature leaf	2.5
	11.2 mature leaf	2.0

Table 4.—3-hydroxytyramine content in leaves of varying ages:

Genotype number	Weight of leaf (grams)	mg 3-hydroxytyramine per 100 ml extract
5	3.5 young leaf	25.0
	4.7 mature leaf	17.5
	15.5 old leaf	4.0
6	3.3 very young leaf	143.5
	5.2 young leaf	145.0
	12.0 mature leaf	37.0
	20.0 old leaf	8.0
7	3.7 very young leaf	90.5
	4.9 young leaf	56.5
	17.0 mature leaf	8.5
	34.0 old leaf	2.5
8	3.0 young leaf	99.5
	5.8 mature leaf	26.0
	18.0 old leaf	2.5

Mature leaves from the same plant contain similar amounts of 3-hydroxytyramine (Table 3), however the concentration of 3-hydroxytyramine decreases in the older leaves (Table 4).

In a similar, later experiment, we took data on both individual plants and plots. We collected leaves from individual plants as before and took small, medium and large leaves from 10-plant plots. We recorded the average leaf weight for both the plot and the single plant samples. The 3-hydroxytyramine content and the activity of the polyphenoloxidase enzyme were determined (Tables 5, 6). Figure 3 is a graph showing a typical decrease in 3-hydroxytyramine and increase in activity of polyphenoloxidase with increasing leaf weight.

Table 5.—3-hydroxytyramine content and the polyphenoloxidase activity in leaves from individual plants:

Plant number	Average leaf weight (grams)	mg 3-hydroxytyramine per 100 ml extract	5 min. reading of polyphenoloxidase
1	3.5	265.0	..... *
	8.4	123.0	0.45
	15.9	40.0	0.78
2	2.5	370.0	0.13
	7.9	214.0	0.17
	17.5	40.0	0.63
3	2.8	120.0	..... *
	5.9	64.0	0.52
	10.9	29.0	0.68
	23.3	9.0	1.16

* insufficient sample

Table 6.—3-hydroxytyramine content and the polyphenoloxidase activity in leaves from plots:

Plot number	Average leaf weight (grams)	mg 3-hydroxytyramine per 100 ml extract	5 min. reading of polyphenoloxidase
1	3.2	220.0	0.26
	8.4	54.0	0.41
	14.2	23.0	0.62
2	2.6	169.0	0.25
	6.1	72.0	0.80
	9.9	38.0	0.87
3	3.1	153.0	0.30
	6.2	50.0	0.48
	10.4	16.0	0.86

Results in Tables 5 and 6 support results in Tables 3 and 4, and show that polyphenoloxidase has a negative correlation with 3-hydroxytyramine. At a given growth stage for a plant, the youngest leaves contain much more 3-hydroxytyramine and lower levels of polyphenoloxidase, whereas the more mature leaves contain less 3-hydroxytyramine and more polyphenoloxidase.

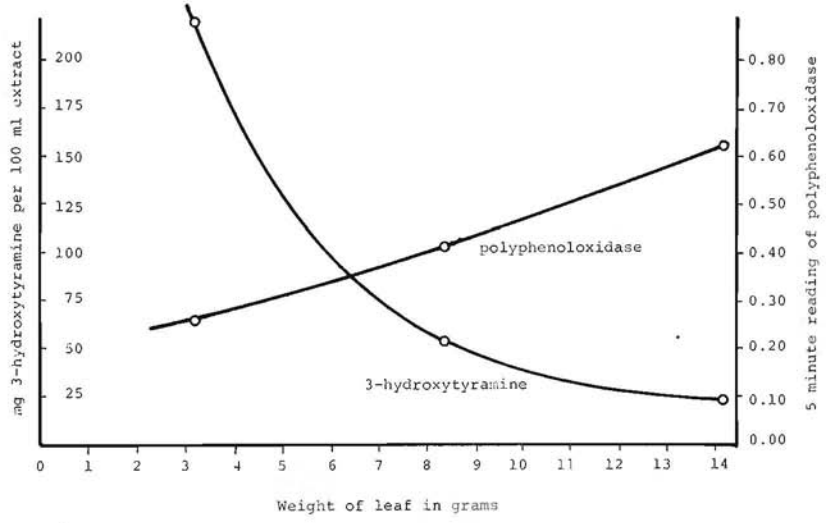

Figure 3.—Concentration of 3-hydroxytyramine and polyphenoloxidase activity as related to leaf weight.

An attempt was made to determine the effect of injury upon the concentration of 3-hydroxytyramine in the leaves. Fresh leaves were placed in moist chambers for up to 24 hours. One longitudinal half of the leaf was used as a control and the other half as a treatment, but the leaves were kept intact until analysis.

When one-half of the leaf surface was injured by scratching with very fine emery cloth, 3-hydroxytyramine always increased in the injured half of the leaf as compared to the adjacent, uninjured half. Leaves were divided down the mid-rib for analysis. Injury by scratching and applying a solution of ground *Cercospora beticola* fungus may have increased the 3-hydroxytyramine more than scratching alone. However, due to the unequal injury by scratching, we could draw no conclusions.

The amount of increase in 3-hydroxytyramine in injured tissue did not appear to have any relation to the amount of 3-hydroxytyramine in the uninjured portion of the leaf. This again may have been due more to variable injury than to any basic property of the leaves. However, the resistance of a plant may depend more on its ability to provide 3-hydroxytyramine upon injury or other stimuli than on any original concentration present.

These results may have significance when the biochemical nature of Cercospora leaf spot resistance is better understood. They do serve to illustrate the difficulty of sampling for a chemical compound which responds readily to environmental conditions.

Summary

1. We have presented the sequence of steps followed in determining a reliable sampling procedure for 3-hydroxytyramine and polphenoloxidase analysis of sugar beet leaves along with various problems encountered. All observations were made under normal field conditions at the experimental site near Fort Collins, Colorado.

2. The content of 3-hydroxytyramine in beet leaves reached a peak when the leaves are one-half to fully expanded and then decreases. Time of maximum content apparently depends on the genotype.

3. The content of polyphenoloxidase reaches a maximum when the leaves are fully expanded and does not decrease significantly in mature leaves from older plants.

4. The optimum time for sampling leaves to determine both 3-hydroxytyramine and polyphenoloxidase activity is when the leaves are fully expanded.

5. The most reliable portion of the leaf for sampling is a transverse section from the center of the leaf.

6. Leaves from seedlings contain less 3-hydroxytyramine than leaves from older plants; and the younger leaves on a given plant contain more 3-hydroxytyramine than do senile leaves from the same plant.

7. On a given plant younger leaves contain less polyphenoloxidase activity than do older leaves.

8. Injury, by scratching the leaf surface, causes an increase in 3-hydroxytyramine content.

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