SOME PHYSIOLOGICAL STUDIES OF CROWN GALL AND CONTIGUOUS TISSUE [1]

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INTRODUCTION

As part of an investigation of the pathological plant growth induced by the crown gall organism it appeared desirable to study the nature and physiology of both diseased and contiguous tissue. Only a few comparisons of such tissues have been reported. Strohmer and Stift (*31*) [3] found that the galls of sugar beets were higher in ash, protein, and moisture but lower in sugar than the normal roots. Townsend (*34*) and others found that the galled beets were decidedly lower in sucrose than the normal ones. Klein and Keyssner (*15*) have made an extensive study of the forms of nitrogen found in galls and contiguous tissue of several hosts. In most instances the percentage of the various forms of nitrogen was higher in the galls than in the contiguous stems. Sylwester and Countryman (*32*) found both callus and gall tissue of apple to contain cellulose, pectin, lignin, and gum. The gall tissue also contained tannin, but the callus tissue did not. Berthelot and Amoureux (*4*) found similar differences in tannin content between galls and normal tissue of sugar beets. They (*3*) also compared gall tissues resulting from inoculation of sugar beets with two strains of *Phytomonas tumefaciens* (Smith and Town.) Bergey et al., but obtained no significant differences.

Glutathione and ascorbic acid have been considered to play an important role in the growth of plants. Hammett (*12*) in particular has emphasized the importance of glutathione. Through its constituent amino acids glutathione probably accelerates cell proliferation and protein reconstitution and differentiation. Virtanen (*36*) reported large increases in dry weight of plants grown in sterile nutrient solutions containing ascorbic acid. Binet and Magrou (*6*) and Berthelot and Amoureux (*4*) reported greater amounts of glutathione in crown gall than in the host plant. The latter investigators likewise found nearly twice as much ascorbic acid in the galls as in the beet root.

Several investigators have suggested a relation between the activities of oxidative enzymes and atypical growth. For example, Bristol (*7*) attributed cell stimulation to the unchecked action of locally concentrated, intercellular oxidizing enzymes. A disproportionate balance between the enzymes, especially an overabundance of peroxidase, he

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[3] Italic numbers in parentheses refer to Literature Cited, p. 553.

postulated, would result in hyperoxidation in the epithelial cells. Klein and Ziese (*16*) found the catalase activity in crown gall of beets to be greatly increased over that in healthy beets. Likewise (*17*) an increase in peroxidase in crown gall of horseradish paralleled the increase in catalase. The activity of diseased tissue was 80 to 100 percent greater than that of healthy tissue. Smith (*28*, *29*) and Robinson (*25*) were of the opinion that atypical growth may be caused by uncontrolled respiration but that the initial cell stimulation was caused by an "oxygen-hunger." In view of the strongly aerobic nature of *Phytomonas tumefaciens* (*26*), the oxygen may be greatly reduced in the intercellular spaces, thus modifying the enzyme relation and creating an environment for cell stimulation. According to Kluyver (*18*, *p. 529*), "* * * the conversion of a normal tissue cell into a cancer cell could ultimately depend on a quantitative change in property of one single catalytic agent which determines metabolism." In view of the importance of oxidative enzymes in cell metabolism it seemed desirable to compare the catalase, peroxidase, and oxidase content of normal and crown gall tissue of plants. A preliminary report on part of the work has already appeared (*21*). For a background to this work proximate and other analyses were also made.

EXPERIMENTAL WORK

Three kinds of plants were used to furnish material for analysis. Tomato (*Lycopersicum esculentum* Mill.) plants were grown in the greenhouse in the late fall and early winter of 1933 and in the field during the summers of 1934 and 1936. When the plants were 6 weeks old, a number of inoculations were made in the upper internodes with the crown gall organism, *Phytomonas tumefaciens*. A sufficient amount of gall tissue for analysis was produced in 6 weeks after inoculation. Simultaneously with the collection of the galls, sections of the stem immediately above and below the galls were taken to furnish samples of contiguous tissue. The samples from the greenhouse plants were placed in hot alcohol and stored until the following summer before being analyzed. Material from the plants grown in the field was analyzed immediately after harvesting.

A second source of material was the gall and normal tissue of sugar beets (*Beta vulgaris* L.) grown in the field and inoculated in the usual manner. A third source was the naturally occurring galls and contiguous bark from the roots of red raspberry (*Rubus strigosus* Michx.). The sugar-beet and raspberry samples were also analyzed immediately after harvesting. For this purpose 250-g samples were completely extracted with successive portions of hot 60-percent alcohol. The extracted residue was dried and ground to insure uniform sampling. It was employed for determinations of starch, pentosan, and uronic acid. The alcoholic extract was evaporated under reduced pressure to remove the alcohol, diluted with water to a definite volume, and then analyzed for sugar and for alpha amino, amide, ammonia, and nitrate nitrogen. Another portion of fresh tissue was dried at 100° C. for the determination of moisture, ash, ether extract, cellulose, and total nitrogen.

PROXIMATE AND OTHER ANALYSES

Analyses were made on the dried materials and solutions as follows: Ash, ether extract, total nitrogen, and pentosans by A. O. A. C. methods (*1*); ammonia, amide, and nitrate nitrogen by the method of Sessions and Shive (*27*); cellulose according to Kürschner and Hanak (*19*); starch by the method of Davis and Daish (*8*); uronic acid by the method of Dickson, Otterson, and Link (*10*); alpha amino nitrogen by the method of Van Slyke (*35*); and sugars according to Stiles, Peterson, and Fred (*30*). The results of the analyses are given in tables 1 and 2. Although considerable variability appears, there are certain conspicuous differences between crown gall, which grossly resembles embryonic tissue, and contiguous tissue. These differences are considered in the discussion.

TABLE 1.—*Chemical composition of normal stem and of gall tissues of tomato*

Analyses	Greenhouse material, 1933		Field material, 1934		Field [1] material, 1936	
	Stems	Galls	Stems	Galls	Stems	Galls
	Percent	*Percent*	*Percent*	*Percent*	*Percent*	*Percent*
Dry matter	10.4	10.3	14.0	11.4	16.8	10.1
Analyses on basis of dry matter:						
Ash	12.3	13.2	7.7	14.1	8.4	14.2
Ether extract	2.0	1.7	1.1	1.7	1.7	2.2
Total nitrogen	3.0	3.3	2.8	4.9	1.4	3.51
Alpha amino nitrogen	.1	.23	.40	.50	.25	.33
Amide nitrogen			.35	.26	.10	.08
Ammonia nitrogen			.15	.15	.06	.10
Nitrate nitrogen			.35	.24	.07	.12
Reducing sugars	4.5	1.9	4.6	2.3	3.9	5.1
Nonreducing sugars	3.0	2.3	.35	1.1	2.0	4.7
Starch	6.8	5.0	3.3	2.3	2.7	4.3
Cellulose	30.0	22.8	31.0	15.9	35.1	20.5
Pentosans	4.1	2.1	13.5	7.8	21.5	13.4
Uronic acids	8.9	10.9	10.4	9.1	3.7	13.4

[1] Produced during July and August 1936.

TABLE 2.—*Chemical composition of normal and of gall tissue of raspberry and sugar beets*

Analyses	Raspberry		Sugar beets [1]	
	Cortical tissue	Galls	Normal tissue	Galls
	Percent	*Percent*	*Percent*	*Percent*
Dry matter	40.7	17.9	20.0	16.1
Analyses on basis of dry matter:				
Ash	4.9	6.9	2.7	6.5
Ether extract	1.6	4.1	.4	1.0
Total nitrogen	1.4	3.1	.86	2.88
Alpha amino nitrogen	.33	.36	.2	.31
Amide nitrogen	.02	.05	.02	Trace
Ammonia nitrogen	.03	.23	.02	.02
Nitrate nitrogen	Trace	.0	.01	.03
Reducing sugars	5.7	8.0	.7	.8
Nonreducing sugars	1.9	.0	69.6	41.64
Starch	9.9	3.0	.1	.2
Cellulose	13.2	13.8	5.1	9.5
Pentosans	14.5	7.3	3.4	7.0
Uronic acids	10.1	8.2	4.5	4.1

[1] Produced during August and September 1937.

Glutathione determinations were made on galls, stems, and actively growing tips of tomato plants by the method of Okuda and Ogawa (*23*). This method gives both the oxidized and reduced forms of the sulphydryl group. The data (table 3) show that the tips of greenhouse plants contained much more glutathione than either the galls or stems. The galls from field plants had more than the stems.

Ascorbic acid determinations, also, were made on galls and on the tomato tissues by the method of Bessey and King (*5*). The results are given in table 4. As with glutathione, actively growing tips contained much more ascorbic acid than stems or galls in which relatively little was found.

TABLE 3.—*Glutathione content of crown gall and of contiguous tomato stems*

Material	Trials	Glutathione per 100 g of dry tissue		Material	Trials	Glutathione per 100 g of dry tissue	
		Reduced form	Oxidized form			Reduced form	Oxidized form
Grown in the greenhouse:	*Number*	*Milligrams*	*Milligrams*	Grown in the field:	*Number*	*Milligrams*	*Milligrams*
Galls	8	1.6	1.6	Galls	10	5.0	5.5
Stems	7	1.1	1.4	Stems	8	2.9	2.0
Active growing tips	4	7.5	3.7				

TABLE 4.—*Ascorbic acid content of crown gall and of contiguous tomato tissue*

Material	Trials	Ascorbic acid per 100 g of dry tissue	Material	Trials	Ascorbic acid per 100 g of dry tissue
Grown in the greenhouse:	*Number*	*Milligrams*	Grown in the field:	*Number*	*Milligrams*
Young galls	3	0.7	Galls	12	.1
Old galls	3	.8	Stems	12	.6
Young stems	3	.3			
Old stems	3	.6			
Active growing tips	3	2.2			

CATALASE

Catalase activity was determined by means of Appleman's apparatus as modified by Davis (*9*). The method involves the measurement in a gas burette of the oxygen liberated from hydrogen peroxide by the enzyme. Two-gram and 10-g samples of gall and contiguous stem tissue, respectively, were ground with a small amount of water, an excess of calcium carbonate, and a small amount of sand. After 2 minutes of grinding the macerated tissue was put through a fine wire gauze, thoroughly washed, and made up to 100 ml. Two milliliters of the gall extract and 10 ml of the stem extract were placed in flasks containing 10 ml of phosphate buffer (pH 7.0) and the flasks attached to a mechanical shaker in a 25° C. constant-temperature bath. When the contents of the flasks reached the desired temperature, 5 ml of 3-percent neutral hydrogen peroxide was added to the flasks, the shaker was started, and the volume of the evolved gas was measured in a gas burette at regular intervals. The readings were converted to standard pressures and temperatures. The galls liberated 160 percent more gas than the same weight of

contiguous stem tissue. The data in figure 1 are based on the average of 14 determinations on galls and an equal number of corresponding stems.

PEROXIDASE

Peroxidase activity was determined according to the method of Guthrie (*11*), based on the formation by the enzyme of phenol indophenol from *p*-phenylenediamine hydrochloride and alpha-naphthol, in the presence of hydrogen peroxide. One milliliter of a 10-percent plant extract prepared by grinding 10 g of tissue and diluting to 100 ml was used for each determination. As seen from figure 2, the galls were approximately 120 percent more active in the formation of indophenol than an equal weight of the contiguous stem. The data

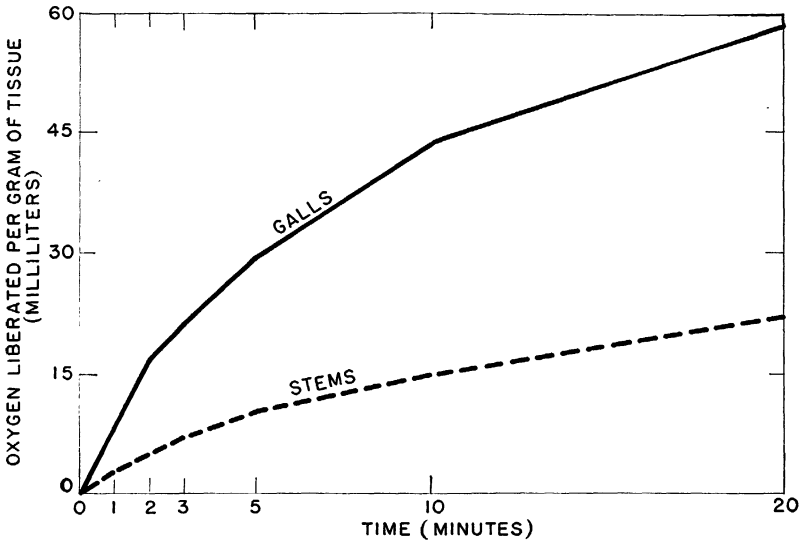

FIGURE 1.—Catalase activity of crown gall and of contiguous stem tissue of tomato.

are based on the average of 19 determinations on galls and an equal number of corresponding stems.

OXIDASE

Oxidase activity was measured by the Bunzel apparatus as modified by Harvey (*13*), based on the absorption of oxygen by pyrogallol. Twelve milliliters of a 1-percent solution of pyrogallol was placed in the larger of the two arms of the apparatus, and in the other, 5 ml of gall or stem juice together with 1 ml of phosphate buffer (pH 6.5). In the vial was placed 1 ml of concentrated sodium hydroxide. The apparatus was immersed in a 25° C. constant-temperature bath and agitated for 2 hours by means of a mechanical shaker. The amount of oxygen absorbed was measured by the difference in the mercury column. The galls showed approximately 130 percent more oxidase activity than the contiguous stem (fig. 3). The data are based on the average of nine determinations on galls and an equal number of corresponding stems.

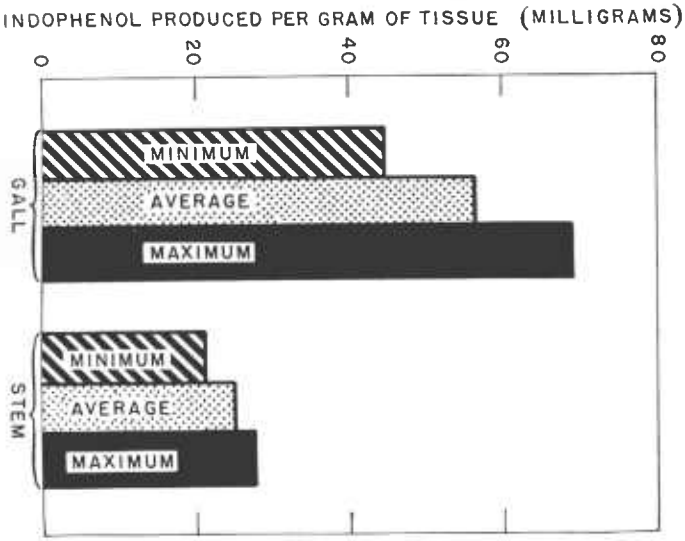

FIGURE 2.—Peroxidase activity of crown gall and of contiguous stem tissue of tomato.

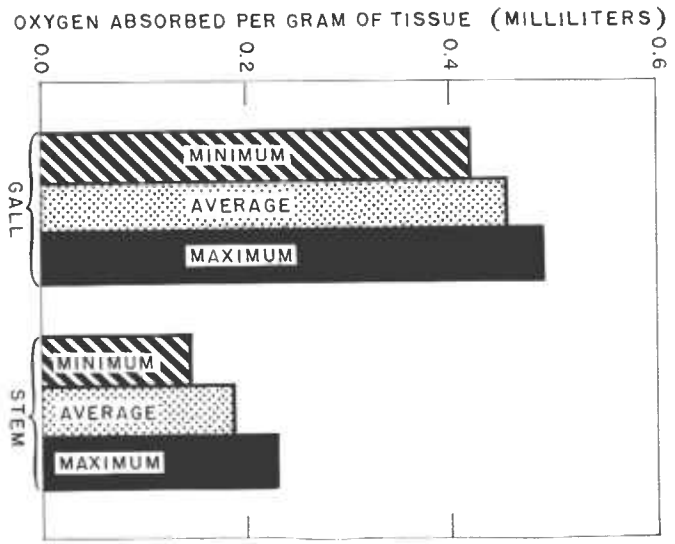

FIGURE 3.—Oxidase activity of crown gall and of contiguous stem tissue of tomato.

TYROSINASE

The activity of tyrosinase was determined by a modified method of Raper and Wormall (*24*), which involves the estimation of the unchanged tyrosine by means of phenol reagent at various intervals. Fifty milliliters of expressed crown gall juice were placed in a 500-ml flask containing 200 ml of 0.05-percent tyrosine solution buffered with borate to pH 8.0. The flasks were immersed in a 25° C. constant-temperature bath and rapidly aerated. At regular intervals 10-ml samples were withdrawn and pipetted into 0.5 ml of 10-percent acetic acid and the mixture was brought to boiling. The solution was filtered into 100-ml volumetric flasks and the precipitate washed well with hot water. The filtrate was diluted to about 50 ml; 5 ml

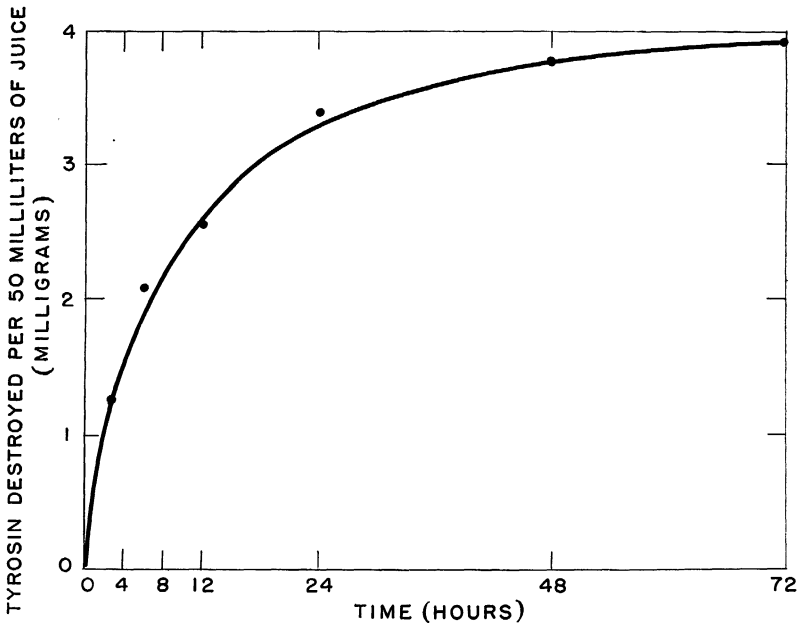

FIGURE 4.—The rate of destruction of tyrosine by the tyrosinase of crown gall from tomato.

of phenol reagent and 25 ml of saturated sodium carbonate were added. After standing for one-half hour the flasks were made up to 100 ml and compared colorimetrically with a standard containing 1 to 4 mg of tyrosine in 100 ml prepared at the same time as the unknown. In 8 hours 50 ml of crown gall juice destroyed one-half of the tyrosine, whereas no loss was detected from a similar preparation of stem tissue (fig. 4). The data are based on the average of six determinations on galls and an equal number of corresponding stems.

HYDROGEN-ION CONCENTRATION

Hydrogen-ion concentration of galls and of contiguous stem both above and below the galls was determined with a glass electrode. About 3 g of tissue was mascerated in 10 ml of water, and the determinations on the liquid were made within 3 minutes. Nineteen

representative determinations gave the following averages: Gall tissue, pH 5.72; stem tissue pH 5.79. This indicates that no significant difference existed between the tissues, when large masses were thus examined. It is possible, however, that significant differences exist between different portions of a given tissue, as suggested by Berridge (*2*).

DISCUSSION

The analyses made of the galls and contiguous tissue indicate a condition in the galls similar to that found in young plant tissues. The ash, total nitrogen, and simple forms of nitrogen in tomato are generally higher, whereas nonmetabolically active materials, such as cellulose and pentosans, are lower in the galls than the host plant. The cellulose and pentosans of sugar beets are higher in galls than in contiguous uninfected tissue. The difference between the two tissues in all three host plants is particularly noteworthy with respect to the more highly organized nitrogen. This conclusion is apparent if the sum of the nitrogen fractions is subtracted from the total. The difference in nitrogen thus obtained, probably polypeptide or protein in character, is from two to four times as high in the gall as in normal tissue. So far as the analyses reported are concerned the papers cited in the introduction are in general confirmed by the present studies.

Glutathione is found in greater abundance in the galls than in contiguous stems, but it is even more abundant in the actively growing tips. This situation is in accordance with Hammett's views on the role of glutathione in the organization of protein.

The activity of the oxidizing enzymes may be considered a measure of the metabolic status of the tissue, and hence may serve as an indicator of the physiological response of plants to various treatments. Since the galls are in a highly active vegetative state, it is not surprising to find the concentration of catalase, peroxidase, and oxidase greater in the galls than in the stems. It is doubtful that the increase in catalase is owing to the hydrogen-ion concentration as suggested by Harvey (*13*), since no significant difference was found in the pH value of the galls and neighboring tissue used. Bristol (*6*) and Lantz (*20*) reported an inhibitory action of catalase on the oxidation processes within the cell. Unpublished determinations in the writers' laboratories on the rate of respiration, as measured by the Barcroft apparatus, indicated a great increase in the uptake of oxygen in the galls over that of the contiguous uninoculated tissue. It appears that under these conditions an environment would be created in which there would be an insufficient amount of oxygen for some of the cells. This, according to Smith (*28*), and Smith, Brown, and Townsend (*29*), would compel these cells to divide if they were to live.

The tyrosinase activity of the gall tissue deserves special comment. Although traces of this enzyme were observed both in a culture of *Phytomonas tumefaciens* and in the tomato plant, the tyrosinase of the galls is so great that the amounts cannot be quantitatively compared. Alpha amino nitrogen and tyrosinase activity were, respectively, 20 and 200 percent greater in the galls than in the contiguous stem. Tottingham, Nagy, and Ross (*33*) found a condition similar to this in abnormal potatoes. Rapidity of oxidation, indicated by

the increase in oxidative enzymes, and presence of a large amount of amino acids may favor both production and activity of tyrosinase in the galls. Nobutani (*22*) found a marked stimulation of tyrosinase activity on a *p*-cresol substrate by a number of amino acids.

All of the enzyme determinations are calculated on the basis of whole tissue and, therefore, may not be on a comparable basis. The contiguous tissue contains larger proportions of inert material, such as cellulose and pentosans, than is found in the galls. Since the metabolically active protoplasm is largely composed of nitrogenous material with only a small amount of carbohydrates, the nitrogen content instead of whole tissue may be used as another basis for calculating enzyme activity. But even on the basis of total nitrogen the galls were higher in catalase, oxidase, and peroxidase, by 86, 73, and 57 percent, respectively.[4]

SUMMARY

A number of analyses have been made on the galls and contiguous tissue from tomatoes, raspberry, and sugar beets. In general the composition of gall tissue resembled that of young plants, being high in nitrogen and low in fibrous material. In sugar beets, however, the galls were more fibrous than the succulent host plant. The composition of the galls and contiguous tissues varied greatly depending upon the time of harvest and the species of plant.

The glutathione content of the tomato galls was greater than that of the contiguous stems, but was much lower than that of the growing tips.

The more metabolically active tissue produced the greater amount of ascorbic acid.

The hydrogen-ion concentration of stems and galls of tomato tissue were approximately the same.

Catalase, oxidase, and peroxidase activity, on the wet-weight basis, were 160, 130, and 120 percent greater, respectively, in the tomato galls than in the contiguous tomato stem tissues. Calculated on the basis of total nitrogen instead of wet weight, the figures for galls were 86, 73, and 57 percent greater, respectively, than those for stems.

Extracts from tomato galls rapidly destroyed tyrosine but a similar preparation from stems showed no tyrosinase activity.

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[4] In making these calculations the 1934 data were used as all enzyme analyses were made on that year's samples.

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