Tumorigenesis on Mineral-deficient Tomato Plants

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SUMMARY

Tumor morphogenesis on stems of Lycopersicon esculentum, variety Rutgers, initiated by Agrobacterium tumefaciens varies with the absence of certain elements from the nutrient solutions. Examinations of the tumors on plants grown in complete and incomplete nutrient solutions revealed different quantities and sizes of vessel elements as well as differences in the quantities of soluble protein. The absence of nitrogen, phosphorus, and calcium, in that order, was most effective in the reduction of tumor proliferation. The experimental findings reported in this study demonstrate that in tumors, growth rates, development, and internal tissue patterns are influenced by the absence or presence of certain minerals.

INTRODUCTION

Crown gall is a non-self-limiting disease of plants initiated by the activities of the bacterium Agrobacterium tumefaciens. Accompanying this transformation to tumor cells from normal cells are certain nutritional and metabolic modifications that distinguish the two tissue types.

Recent studies have shown several interesting facets concerning the metabolism of crown gall. Hussin and Deep (6) found that the optimum growth rate of tumor tissue is obtained when the nutrient supply is relatively low. Spurr et al. (16) distinguished normal from tumor tissue by high oxidase activity in the latter. Further studies by Lipetz and Garro (11) have shown that crown gall tissue releases peroxidase into the medium in response to the concentration of specific ions. Calcium, magnesium, and ammonium ions, in that order, are effective in the peroxidase release. These ions control the deposition of lignin on cell walls by affecting the peroxidase levels. These studies seem to indicate that the uptake of ions has some effect on the differentiation of the crown gall tissue.

The experiments described in this study deal with the roles of calcium, phosphorus, and nitrogen in the development and organization of the crown gall. If the metabolic pattern is indeed altered and the crown gall tissue can synthesize with the aid of mineral salts and sucrose the substances required for growth, the absence of these major elements should have an effect on the development and organization of the gall.

MATERIALS AND METHODS

Seeds of Lycopersicon esculentum, variety Rutgers, were germinated and grown in sterile soil for a period of four weeks. After this interval, the roots of the plants were excised, and the severed plants were washed in distilled water and transferred to nutrient solutions. Sets containing 20 plants each were grown on a complete nutrient solution (A), solution lacking calcium (B), solution lacking phosphorus (C), solution lacking nitrogen (D), and on distilled water (Table 1).

Following an adjustment period of one week in these culture solutions, the plants were inoculated with a 48-hour virulent culture of Agrobacterium tumefaciens, strain B-6, secured from The American Type Culture Collection at Rockville, Maryland. The organisms were cultured in nutrient broth and transferred to new media every three days in order to insure the virulence of the cultures. The inoculations were made with a sterile, 2-ml syringe, taking care to puncture the tissue near a node, thus allowing approximately 1 ml of the culture to enter the incision. The inoculated plants remained on a greenhouse bench at 25°C for six weeks. After the six week growth period, the tumors were excised for study.

Table 1

<table>
<thead>
<tr>
<th>Stock solutions</th>
<th>Control</th>
<th>-Ca</th>
<th>-N</th>
<th>-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 M Ca(NO₃)₂</td>
<td>5</td>
<td></td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>1 M KNO₃</td>
<td>5</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>1 M MgSO₄</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>1 M KH₂PO₄</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Micronutrients</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1 M CaCl₂</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 M KCl</td>
<td>5</td>
<td></td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>1 M NaNO₃</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe₃(C₂H₃O₂)₄</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Composition of the nutrient media.

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Growth and Development

Data on growth and development were obtained by determining the size and dry weight of the tumors. The tumors taken for these determinations were secured by cutting a 10-mm portion of the stem that contained the tumor and a 10-mm portion adjacent to and above that part containing a tumor; the difference in weights between these two portions wet or dry equals the tumor's weight. The determination of size was accomplished by measuring the diameter of the tumors and their growth perpendicular to the stem. The volume of the various tumors was calculated from the equation, \( V = \pi r^2h \), where the radius (r) is \( \frac{1}{2} \) the diameter and the growth perpendicular to the stem is h.

The vessel size determination was based on simple random sampling of 100 vessel elements from 10 slides in each of the several stained preparations per treatment. The two parameters upon which this determination is established are the mean values for the relative lengths and widths and their standard deviations.

Total Protein Determination

The protein method was a modified biuret technic (14). The tumors taken for this determination were cut into approximately 3-mm sections and boiled in 80% ethyl alcohol until chlorophyll was no longer detectable in the cells. Next, the tissue was placed in 10 volumes of 0.5 molar perchloric acid, incubated at 80°C for 90 minutes, and washed in triple-distilled water. The washed tissue was then suspended into an equal volume of biuret reagent and incubated at room temperature for one hour with occasional stirring. The debris was removed by centrifugation at 5,000 rpm before measuring the absorption. The absorption was read at 540 millimicrons and compared with a standard curve, which was run on the protein papain to determine the milligrams of protein in the tumors. A weight of stem proportional to that of the tumor was used for determination of stem protein.

Internal Organization

The tumors taken for histologic studies were killed and fixed in formalin:acetic acid:alcohol and embedded in paraffin. The sections were cut at 12 microns and stained with lacmoid to show xylem and/or phloem (3).

RESULTS

Gross Development

The data in Table 2 reveal the effects of the absence of certain elements on tumor developments on tomato plants. Determination of size is made by measuring the extension (tumor growth perpendicular or at right angles to the stem) and diameter of the tumors. Averages of ten tumors from each treatment are given.

The data indicate that there is a direct relationship between tumor size and the nutrients available to the plants. The nutrient solutions lacking nitrogen have the greatest effect on the size of the tumors, in which case they were found to be small in comparison to the other growths. The tumors proliferated and grew were similar on nutrient solutions lacking calcium or phosphorus. Although some of the plants grown on the triple-distilled water did survive the duration of the experiment, the injections made on these plants did not initiate any detectable tumorigenesis. The inability of the tumors to develop or initiate on these plants indicates that tumorigenesis requires minerals.

Internal Organization

The tumors are composed of comparatively thick-walled parenchymatous cells that are larger than those found in the normal stem tissue, meristematic regions, and lignified elements. The lignified elements are not associated with phloem, which is absent in all tumors, but with meristematic tissues or cambial regions that give rise to these elements. The lignified elements are scattered randomly throughout the tumors with no evidence of a pattern and with various sizes (Table 3).

Although the general texture of the galls is similar, there are specific differences in certain structures, i.e., vessel elements. The greatest concentration of lignified elements is in the tumors of the control group. In solutions lacking either calcium, phosphorus, or nitrogen, the tumors are second, third, and fourth respectively in regard to the quantity of lignified elements found.

### Table 2

<table>
<thead>
<tr>
<th>Determinations</th>
<th>Control</th>
<th>-Ca</th>
<th>-P</th>
<th>-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wet weight (gm)</td>
<td>2.47</td>
<td>1.60</td>
<td>0.87</td>
<td>0.56</td>
</tr>
<tr>
<td>Dry weight (gm)</td>
<td>0.3490</td>
<td>0.1074</td>
<td>0.1180</td>
<td>0.0190</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>12.60</td>
<td>8.14</td>
<td>8.70</td>
<td>7.16</td>
</tr>
<tr>
<td>Extension (mm)(^a)</td>
<td>8.66</td>
<td>5.57</td>
<td>5.61</td>
<td>5.14</td>
</tr>
<tr>
<td>Dry weight per 0.56 gm wet weight</td>
<td>0.070</td>
<td>0.081</td>
<td>0.072</td>
<td>0.0190</td>
</tr>
<tr>
<td>Micrograms of protein per tumor</td>
<td>9.10</td>
<td>3.05</td>
<td>2.86</td>
<td>1.50</td>
</tr>
<tr>
<td>Micrograms of protein per 0.56 gm wet weight of tumor tissue</td>
<td>1.85</td>
<td>2.29</td>
<td>1.76</td>
<td>1.50</td>
</tr>
<tr>
<td>Micrograms of protein in a wet weight of stem proportional to tumor's wet weight</td>
<td>2.56</td>
<td>1.28</td>
<td>1.20</td>
<td>0.94</td>
</tr>
<tr>
<td>Ratio of tumor to stem protein per equal wet weight</td>
<td>3.5:1</td>
<td>2.3:1</td>
<td>2.3:1</td>
<td>1.8:1</td>
</tr>
<tr>
<td>Volume (cumm)</td>
<td>1016</td>
<td>273</td>
<td>335</td>
<td>206</td>
</tr>
</tbody>
</table>

Data from chemical and morphologic determinations.

\(^a\)Growth perpendicular to the stem.
solutions lacking nitrogen have the least dry weight. The values for those lacking nitrogen indicate that those tumors on stems in solutions lacking calcium have the largest dry weight per volume, indicating that other processes besides gross proliferation are occurring, such as differences in the anatomic structures and levels of protein content.

The data presented in this paper have divulged several interesting facts concerning the development of tumors on nutrient deficient tomato plants. The results show that nitrate ions, phosphate ions, and calcium ions, together, are essential for maximum gross proliferation of crown galls on tomato stems. Gadgil and Roy (4), working with tissue cultures, found that hollyhock gall growth stopped totally in the absence of sodium phosphate, calcium nitrate, and magnesium sulphate. Link et al. (9) showed that by varying the levels of the nitrate-nitrogen concentrations, tumor growth on tomato plants grew at characteristic rates. Although there is a decrease in size of the tumors with the removal of one of these elements, the cessation of tumorous growth is not as drastic as in tissue culture.

A decrease in tumor weight is associated with a decrease in size when one of these elements is absent from the nutrient solution. The dry weight from equal wet weight of tissue indicates that those tumors on stems in solutions lacking calcium have the largest dry weight per volume, indicating that other processes besides gross proliferation are occurring, such as differences in the anatomic structures and levels of protein content.

The fact that these tumors have differences in size and weight points out that the absence of these elements may have some influence on the anatomy of the tumors. Besides the similarity in texture of the galls, the quantities of lignified elements vary. In the absence of calcium, larger quantities of lignified elements are produced. These results coincide with those findings established by other investigators. Nightingale et al. (13) revealed that calcium deficient media result in somewhat stiff and woody tomato plants and that microscopic examinations of these tomato stems show a high percentage of very thick-walled mechanical and conductive tissue. Lipetz (10), using in vitro technics reported that media with different concentrations of calcium had marked influences on the quantity of lignification in sunflower crown gall tumor tissue. It was also shown that lignification was promoted at low levels and inhibited at high levels of calcium. These independent studies when combined are parallel to the results of the tumors grown on stem lacking calcium. The tumors on nutrients lacking in calcium are highly lignified.

Aside from the quantities of lignification, the absence of these elements may produce other effects. The sizes of the xylem elements vary with the nutrient solutions. The largest are found in the control tissue grown in the complete solution. The vessel elements in the other nutrients vary in size and shape—long to short, and slender to broad. These variations in the gall structures could be due to physicochemical stresses which may be compensated only by alterations in its potential for development.

These studies emphasize further the role of the absence of the several elements on the quantity of protein. It has been established by Seitz and Hochester (15) that the level of proline in primary tobacco and tomato crown gall stem is larger than that in normal, uninfected stem tissue. The results in this experiment reveal that the protein content of the crown gall and the normal, uninfected stems are not equivalent. These studies further show that the absence of these elements tested does not induce the same proportional reduction in protein content in every case. The tumors on stems in solutions lacking calcium have the largest protein content per tumor, which indicate possibly that these tumors take up more minerals per volume that are essential for protein synthesis. Similar findings have been reported by other investigators. Neish and Hibbert (12) found the protein content to be six times that present in the normal tissue, and the tumors maintained 64% of their Kjeldal nitrogen in the form of protein compared with only 39% for normal tissue, thus reflecting a greater tendency for the tumors to synthesize protein.
The influence of phosphorus on the development of the gall results in a decrease in size, indicating that some metabolic system is being blocked. Klein (8) reported increases in phosphorus compounds in tomato tumor tissue as early as five days after inoculation. The increased concentration was found to be due to increased amounts of orthophosphate and acid-soluble organic compounds containing phosphorus. Other studies have shown that increases in acid-soluble phosphorus, RNA, and DNA result in tumorous tissue growths. It can be inferred that the absence of phosphorus from the nutrient solutions decreases the availability of this element for synthesis of phosphorus-containing compounds that are essential for maximum proliferation of tumor tissue.

As shown in this study and others (1, 2, 17), the autonomy of the neoplastic growth is not a fixed and unvarying character but has a wide range of expressions both chemically and morphologically. At one extreme is found slowly growing benign tumors that remain localized in the host. At the other extreme are the most malignant cancers that grow rapidly, infiltrate neighboring tissues, and metastasize. Theoretically, such autonomy of tumor cells requires something newly activated and distinctive, something that induces such cells to continue abnormal and unregulated growth. The effectiveness of such agents in eliciting tumor formation appears to be determined in large part by the hereditary constitution of the host (7).

From what has thus been learned of the metabolism of normal and crown gall tumor cells, one may gain insight into problems of the physiology of cell types. Although tumor cells that develop in a host are commonly considered to be less differentiated and less specialized than the normal cells from which the tumor cells were derived, a strong case could be made for the statement that the tumor cells are also specialized cells. Such cells can synthesize continuously and with high efficiency the specialized metabolites that are required for their proliferation.

ACKNOWLEDGMENTS

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REFERENCES

FIGS. 1–4. Typical crown galls, Fig. 1, control; Fig. 2, minus calcium; Fig. 3, minus phosphorus; and Fig. 4, minus nitrogen.

FIGS. 5–8. Photographs depicting the anatomical studies of the tumors. In Fig. 5, control, and Fig. 6, minus calcium, note the profusion of the xylary elements. The quantity and compactness of the xylary elements have been reduced in Fig. 7, minus phosphorus, and in Fig. 8, minus nitrogen. × 100.
Figs. 9–12. Photographs showing the xylary tissue from each treatment. The sections are from tumors of the control (Fig. 10), minus phosphorus (Fig. 9), minus calcium (Fig. 11), and minus nitrogen (Fig. 12). Note the vessel size differences indicated by the arrows. × 800.
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