Tissue Culture Studies of West Indian Lime Infected with Vein-Enation Virus

P. R. DESJARDINS, CELIA FUERTES-POLO, and J. M. WALLACE

WALLACE AND DRAKE (8) reported that the vein-enation virus induced woody galls on Rough lemon (*Citrus jambhiri* Lush.) and West Indian lime [*C. aurantifolia* (Christm.) Swing.], that the galls were associated with natural wounds, and that artificial wounding of virus-infected Rough lemon and sour lime resulted in gall formation at the site of the wounds (9).

Greater knowledge of the virus and information leading to possible chemical control of the virus are the long range goals of tissue culture studies in our laboratory. In the preliminary studies reported here, our goals were more limited. For example, we wished to know if virus-infected citrus tissue would produce more abundant callus growth than does healthy citrus tissue, if the virus is present in the callus growth that develops, and if it persists in callus tissue grown in an isolated state.

Materials and Methods

VIRUS AND HOST.—We used the California strain of vein-enation virus. Young seedlings of West Indian lime were inoculated by bud and scion grafting. When vein-enation symptoms were evident and some growth of the seedlings had occurred, stem pieces were removed and prepared for culture. Comparable stem pieces from healthy lime seedlings were used as controls.

CULTURE MEDIA.—In the first experiments, virus-infected lime stem pieces were cultured on both solid and liquid media. More extensive and consistent callus growth occurred in the liquid medium, therefore this medium was chosen for later experiments.

Isolated callus tissue was cultured on both liquid and solid media. To minimize drying in the culture tubes, the solid medium used for isolated callus tissue was a mixture of 75 per cent solid medium plus 25 per cent liquid medium. The composition of both the solid and liquid media was the same except that the solid medium contained 1 per cent Kobe washed agar or Difco purified agar. All stock solutions as well as dilutions of media were made with glass-distilled water.

The composition of the medium was as follows: The inorganic components were qualitatively similar to those described by White (10) and

will not be described here; the carbon source was 2 per cent sucrose; amino acid and vitamin components were glycine (4×10^{-5} M), nicotinic acid (4×10^{-7} M), pyridoxine (4.8×10^{-7} M), thiamine (2.9×10^{-7} M), biotin (1×10^{-7} M), inositol (1.2×10^{-4} M), and calcium pantothenate (3.2×10^{-6} M); growth promoting substances were 2,4-dichlorophenoxyacetic acid (4×10^{-7} M) and coconut milk at a concentration of 15 per cent by volume. Both liquid and solid media contained coconut milk because in earlier studies it proved the most effective single substance for promoting callus tissue growth on citrus stem segments. Bové and Morel (1) reported similar results in their citrus tissue culture experiments. The pH of the mixed medium was adjusted to 6.0 with 0.1N NaOH or 0.1N HCl. The pH of agar media was adjusted while it was still melted.

CULTURE TECHNIQUE.—The stem segments were washed in detergent solution, rinsed, and surface sterilized in 15 per cent H_2O_2 after which they were aseptically introduced into the culture flask. When cultured in liquid medium, the glass support method of Lange and Desjardins (5) was used.

Callus tissue was aseptically removed from the stem segments so that no tissue from the original stem was included, and cultured on liquid medium using the filter paper support technique (2) and on solid agar medium in test tubes. All cultures were incubated at 28°C in the dark except as indicated below.

ASSAY METHOD FOR THE PRESENCE OF VIRUS.—The presence or absence of the virus in callus tissue was determined by placing thin slices of callus tissue under the bark of young West Indian lime seedlings and watching for the appearance of vein enations on the leaves.

Results

CALLUS TISSUE ON STEM PIECES.—Callus tissue developed on stem segments cultured on solid medium and continued to grow for about one month at which time further growth ceased. After callus growth stopped, small shoots with leaves developed on some of the segments. These cultures were removed from the incubator and kept in the light at room temperature. At first the leaves developed with good green color, but after a few weeks most of them became quite chlorotic. The development of callus tissue by stem segments was so inconsistent that this method was discontinued.

Callus tissue developed more consistently and extensively on stem seg-

ments cultured in liquid medium and in some cases continued to grow for four to five months (Fig. 1). However, some virus-free stem segments developed equally good callus growth. Although callus development from both the virus-infected and virus-free stem pieces varied considerably, growth from virus-infected stem pieces was not noticeably better than that from the healthy stem segments.

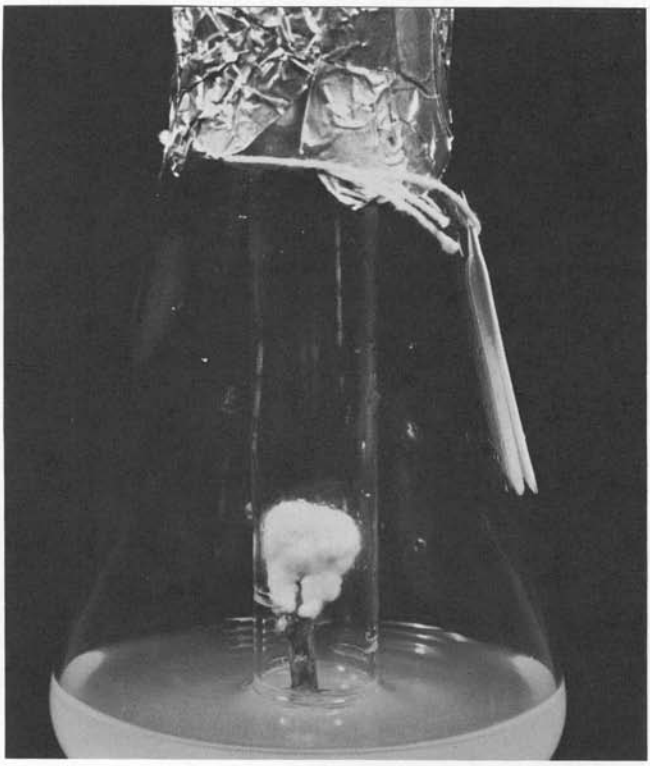

FIGURE 1. *Callus tissue growth on a vein-enation virus-infected lime stem segment growing on a liquid medium, three months after start of culture.*

In some cultures the stem piece was positioned so that the basal end was up, to see if a reversal of polarity might enhance callus tissue development as suggested by Skoog (7). In this position of reversed polarity, callus growth seemed better, but the increase could not be measured quantitatively.

In all cultures, callus tissue developed first at the exposed cambium on the cut surface of the stem segment, but as growth continued, callus tissue often fully surrounded the segment (Fig. 1).

ISOLATED CALLUS TISSUE.—Growth occurred in 26 per cent of the cultures of isolated callus tissue on liquid medium. However, growth was not extensive and ceased as the liquid dried in the tubes. The problem of water loss by drying was not completely solved. In some cases where good growth had occurred the tissue was transferred to fresh tubes, but generally growth ceased.

In the cultures of isolated callus tissue on solid medium at least some growth occurred in 50 per cent of the cultures. The volume of growth varied, but was usually more extensive than on liquid medium (Fig. 2).

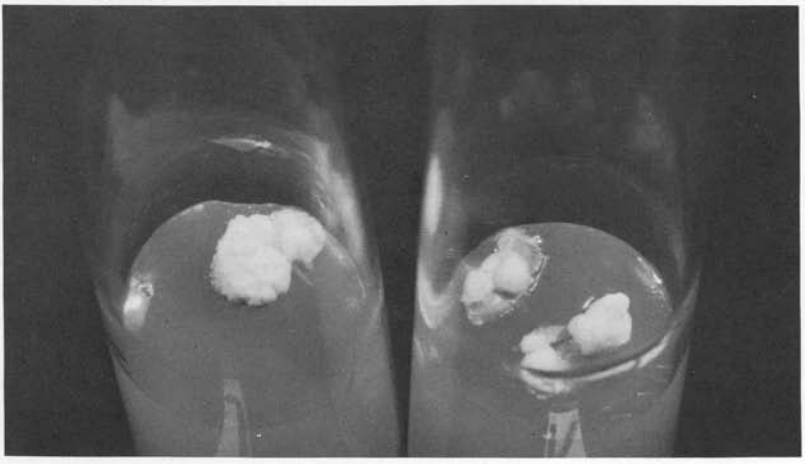

FIGURE 2. *Growth of isolated callus tissue on a solid medium. The two cultures shown were transferred two months previously from cultures of virus-infected lime stem segments.*

The best growth was not as extensive or rapid as that reported for isolated lemon callus tissue (1).

ASSAY FOR THE PRESENCE OF VIRUS IN CALLUS TISSUE.—Vein-enation virus was transmitted from 70 per cent of the callus tissue transplants from stem segments in one test and from 80 per cent in a second test. This indicates that the virus is translocated in the callus tissue to some extent.

Similar assays were made with callus tissue grown in an isolated state, but no virus was recovered.

Discussion

Non-tumorous stem tissue from vein-enation-infected limes developed no more callus than did tissue from healthy limes. The method of virus

assay used is only qualitative and makes no measure of the virus concentration in the tissue. However, transmission of the vein-enation virus from callus tissue of stem pieces proves that some virus moved from stem tissue into the callus tissue.

The failure to recover the virus from callus tissue grown in an isolated state suggests that the virus failed to increase and invade the newly formed tissue. Similarly, wound tumor virus could not be recovered from Rumex tumor tissue maintained in culture for a long period of time (6).

Several factors have been shown to affect the multiplication of tobacco mosaic virus in tissue culture, as follows: The pH of the culture medium (3); the rate of cell division and cellular synthesis (11); and the composition of the nutrient medium (4). It is not known if any of these factors affected multiplication and translocation of the vein-enation virus in tissue culture.

Citrus callus reportedly (Murashige, personal communication) does not differentiate vascular elements. Nevertheless, virus was shown to be present in the callus tissue attached to the stem segments. Failure to recover the virus from the isolated callus could result from insufficient virus in the tissue for successful inoculation of test plants, or simply from the absence of virus in the isolated callus. According to Murashige and Tucker (personal communication) citrus callus tissue cells rapidly become polyploid. Such a genetic change might alter the cells' ability to support virus multiplication. Thus, the virus in callus tissue attached to a stem piece may have originated in the stem tissues and not in the callus tissue. Callus tissue grew more consistently and extensively on stem pieces than when isolated. Because isolated callus grew poorly, the virus concentration could be too low to recover or it could be zero.

Much of the work in our laboratory has been directed towards obtaining better growth of isolated lime callus tissue by alteration of the culture medium. Until better growth of isolated tissue is obtained for use in graft inoculation of indicator host plants, it may not be possible to determine if vein-enation virus is maintained in isolated lime callus tissue. Future work with vein-enation virus-infected Rough lemon is planned, and this might be more useful since other workers have shown that tissue of this species of citrus grows well in an isolated state (1, 2, and Murashige and Tucker, personal communication).

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