EFFECT OF GLYCINE ON THE GROWTH IN ASEPTIC CULTURE OF HEALTHY AND AMPELAMUS-VIRUS-INFECTED TISSUE OF *NICOTIANA GLUTINOSA* L.

INEZ N. KAMP and H. H. THORNBERRY
Department of Plant Pathology, University of Illinois, Urbana, Illinois

Preliminary studies indicated that cultures of stem callus tissue from healthy *Nicotiana glutinosa* L. tolerated concentrations of glycine (amino acetic acid) greater than 3 mg/liter; the amount generally recommended (White, P. R., 1939 and 1943 and Hildebrandt, A. C. *et al.*, 1946). Since radioactive glycine affords a means of introducing tagged carbon and nitrogen or tritium into metabolites of healthy and virus-infected tissue, it seemed worthwhile to determine on chemically defined media the concentration of glycine for maximal growth rate of healthy and diseased callus tissue in aseptic culture. This paper reports the results of the experiments and compares some methods of measuring the growth rate of callus tissue in aseptic culture.

Materials and Methods

Culture Media. Two basic media were used: (W) White's medium (White, P. R., 1943) modified according to other media (Bonner, J., 1940, Gautheret, R., 1939, and Heller, R., 1953) and (H) Hildebrandt's medium (Hildebrandt, A. C. *et al.*, 1946) formulated for the culture of tobacco tissue. Composition of the two media depicted in Table 1 is given, when possible, in molarity to avoid confusion about water of crystallization in some chemicals.

Tissue Cultures. Cultures of callus tissue of *N. glutinosa* were derived from stems of healthy plants and plants infected with Ampelamus virus (an unidentified virus originally isolated from *Ampelamus albidus* (Nutt.) Britt. 1894 and presumably a strain of cucumber mosaic virus or of tobacco ring-spot virus). Tissue subcultures were made by transferring a "loopful," about 20 mg of "transferendum," of a typical culture into 125 ml screw cap Erlenmeyer flasks containing 40 ml of 0.6% agar (W) or (H) medium (see Table 1). "Transferendum," plural "transferenda," (a new term, coined in collaboration with J. L. Heller, Department of Classics, University of Illinois, meaning the material transferred from one container to another container) is used herein to avoid ambiguity of the terms "inoculum," "explant," or "transplant" in this particular frame of reference.

Cleaning of Glassware. Glassware was cleaned following a procedure involving four steps:

1. Soaking in aqua regia (3 parts HCl and 1 part HNO_3) at room temperature for at least 16 hours.
2. Rinsing with tap water to remove aqua regia.

TABLE 1.—Constituents and amounts in the two media. (W) modified White medium and (H) Hildebrandt *et al.* medium.

Constituent	Molarity of medium	
	(W) White:	(H) Hildebrandt:
Carbon source:		
Sucrose	6.0×10^{-2}	6.0×10^{-2}
Vitamins etc.:		
Cysteine HCl	6.3×10^{-5}	
Thiamine HCl	3.0×10^{-7}	
Nicotinic acid	4.0×10^{-6}	
Pyridoxine HCl	4.9×10^{-7}	
Minerals:		
$AlCl_3$	4.8×10^{-7}	
$Ca(NO_3)_2$	1.2×10^{-3}	2.5×10^{-3}
$CuSO_4$	1.2×10^{-7}	
$Fe_2(C_4H_4O_6)_3$	2.6×10^{-6}	6.1×10^{-5}
H_3BO_3	2.4×10^{-5}	5.0×10^{-6}
KCl	9.0×10^{-4}	9.0×10^{-4}
KI	4.5×10^{-6}	1.8×10^{-6}
KNO	8.0×10^{-4}	8.0×10^{-4}
$MgSO_4$	3.0×10^{-3}	7.4×10^{-4}
$MnSO_4$	3.0×10^{-5}	3.0×10^{-5}
NaH_2PO_4	1.4×10^{-4}	2.7×10^{-4}
Na_2SO_4	1.4×10^{-3}	5.6×10^{-3}
$ZnSO_4$	9.4×10^{-6}	2.0×10^{-5}
Other:		
Coconut milk	(15%)	(15%)
Agar	(0.6%)	(0.6%)
Water[a] to final volume	(1,000 ml)	(1,000 ml)
H-ion concentration	(pH5.6-5.8)	(pH5.6-5.8)
Total molarity, medium ($\times 10^{-3}$)	67.57889	70.944
Total molarity, vitamins ($\times 10^{-5}$)	6.827	
Total molarity, minerals ($\times 10^{-3}$)	7.51052	10.944

[a] Water, distilled and deionized.

3. Boiling in a solution of detergent in distilled water.
4. Rinsing with distilled water then with distilled-deionized water and drying in an inverted position.

Growth Rate Measurements. The growth was estimated by three types of measurements: wet weight, volume, and total nitrogen. *Wet weight* of callus tissue was determined by weighing the cultures separately in closed weighing bottles. *Volume* of the cultures was ascertained by measuring the volume of liquid displaced when the culture was submerged in a reservoir of fluid (25% aqueous ethanol) contained in a mi-

TABLE 2.—Effect of concentration of glycine on growth as measured by wet weight of healthy and Ameplamus-virus-infected cultures of *Nicotiana glutinosa* callus tissue in two basic media: (W) and (H).

Glycine, molarity	(W) Medium		(H) Medium	
	Healthy tissue	Diseased tissue	Healthy tissue	Diseased tissue
.00004	1.438[a] (100[b])	0.000	2.827(65)	1.116(71)
.0004	2.383(4)	0.000	2.331(3)	0.977(38)
.004	2.073(100)	0.000	2.376(37)	0.917(39)
.04	0.074(40)	0.000	0.060(39)	0.751(25)
046	1.015(82)	0.000	0.061(16)	0.352(71)

a Mean wet weight in grams of four cultures incubated for 35 days at 26° C in one experiment.
b Coefficient of variability of the mean, percentage.

crovolumeter to be described in a forthcoming paper. *Total nitrogen* in a culture was determined spectrophotometrically at 450mμ by the Hoffman and Osgood modification of the microKjeldahl method (Hoffman, W. S., and B. Osgood, 1940). A standard nitrogen curve was constructed from nitrogen determinations made on solutions of ammonium sulfate containing 0.025, 0.015, and 0.0075 mg N/ml.

Virus Assay. Ampelamus virus was assayed on primary leaves of *Vigna sinensis* Endl., Table Cowpea, California Blackeye, by mechanical inoculation with extract containing 7% abrasive powder (800 mesh silicon carbide).

EXPERIMENTS AND RESULTS

To determine the concentration of glycine necessary for maximal growth of healthy and of virus-infected *N. glutinosa* callus tissue in aseptic culture, transferendum of the two types of tissues were introduced into flasks containing (W) medium and (H) medium, respectively. Concentration of glycine in the culture flasks varied from 4 x 10^{-5}M to 4.6 x 10^{-2} molarity. The subcultures were incubated for 35 days at 26 ± 1° C and 60 ± 2% relative humidity. Four subcultures were used for each test.

The results given in Table 2 and Figure 1 indicate that healthy and virus-infected tissue differed in their response to the concentration of glycine contained in the two media. Growth rates of the healthy tissue on the two media were similar at all concentrations of glycine except 4 x 10^{-5}M. The significance of this difference is doubtful. Growth was retarded at 4 and 4.6 x 10^{-2}M glycine. One of the four cultures of healthy tissue on (W) medium containing 4.6 x 10^{-2}M glycine grew at an "explosive" rate of four times the mean growth rate indicated in Table 2

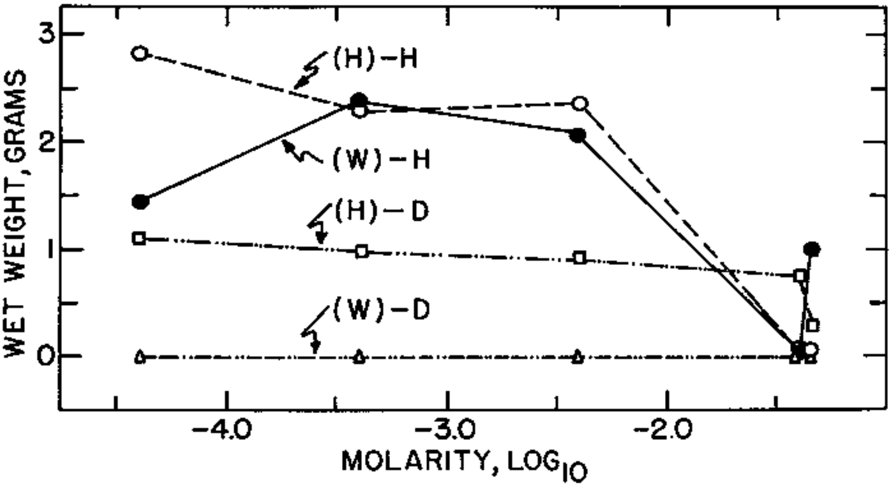

FIG. 1.—Effect of concentration of glycine (abscissa) as measured by wet weight of healthy and Ampelamus-virus-infected cultures of *Nicotiana glutinosa* callus tissue on two media for 35 days at 26° C. Symbols: (H), Hildebrandt medium; (W), White medium; -H, healthy tissue; -D, diseased tissue. (Data from table 2).

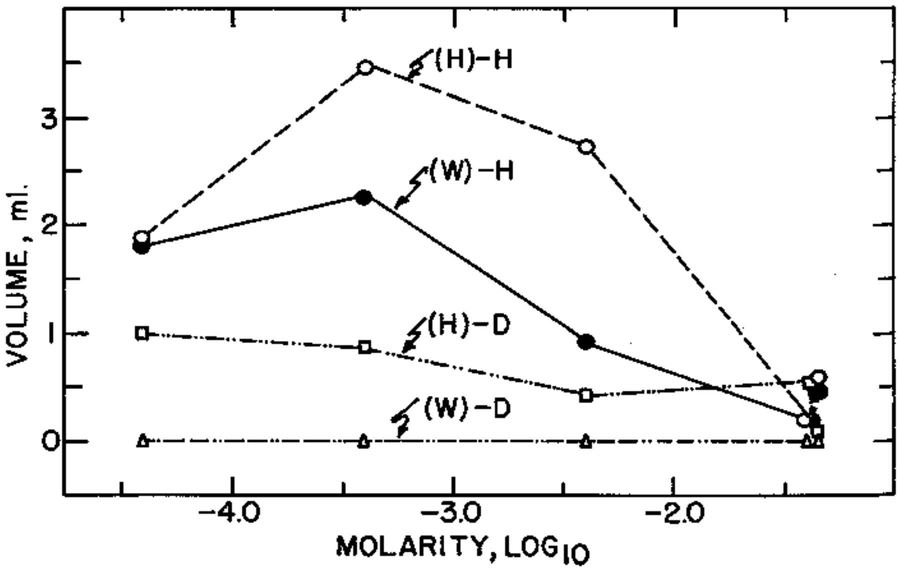

FIG. 2.—Effect of concentration of glycine on the volume in ml of healthy and Ampelamus-virus-infected cultures of *Nicotiana glutinosa* callus tissue on two media for 35 days at 26° C. Symbols: (H), Hildebrandt medium; (W), White medium; -H, healthy tissue; -D, diseased tissue. Coefficient of variability of means: from 6 to 73 for (H)-H, from 14 to 127 for (W)-H, from 18 to 100 for (H)-D.

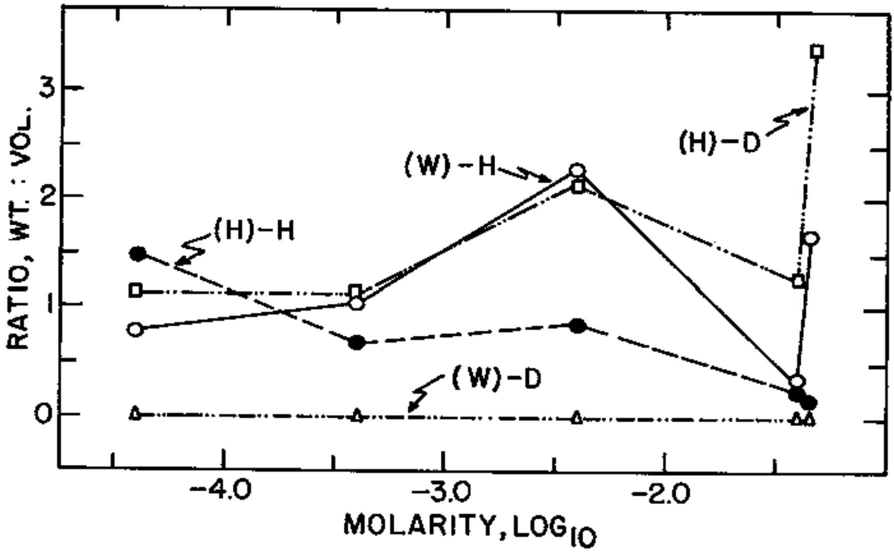

FIG. 3.—Effect of concentration of glycine on ratio of wet weight in grams to the volume in ml of healthy and Ampelamus-virus-infected cultures of *Nicotiana glutinosa* callus tissue on two media for 35 days at 26° C. Symbols: (H), Hildebrandt medium; (W), White medium; -H, healthy tissue; -D, diseased tissue.

and Figure 1. Diseased tissue on the (W) medium made little or no growth at any concentration of glycine tested. Diseased tissue on the (H) medium grew at all concentrations of glycine, but the rate of growth was slower than that of healthy tissue at all concentrations of glycine except 4 and 4.2 x 10^{-2}M.

The results in Figure 2 on growth rate measured by volume are not in close agreement with the results from measurements by wet weight. According to volumetric measurements, 4 x 10^{-4}M glycine is optimal for healthy tissue on either (W) medium or (H) medium. The volume of growth of diseased tissue on (H) medium was not greatly influenced by the concentration of glycine except at 4.6 x 10^{-2} molarity which retarded growth strongly. The diseased tissue on the (W) medium was too small for reliable measurements by volume.

The density of the cultures at varied concentrations of glycine was estimated by calculating the ratio of tissue wet weight in grams to tissue volume in milliliters. These ratios plotted in Figure 3 indicate that the density of healthy tissue on (H) medium correlated inversely with the concentration of glycine. The density of healthy tissue on the (W) medium and also of the diseased tissue on (H) medium was greatest at 4 x 10^{-3}M glycine. Density of growth at 4.6 x 10^{-2}M glycine is not significant since the amount of growth was too small for reliable measurements by either wet weight or by volume.

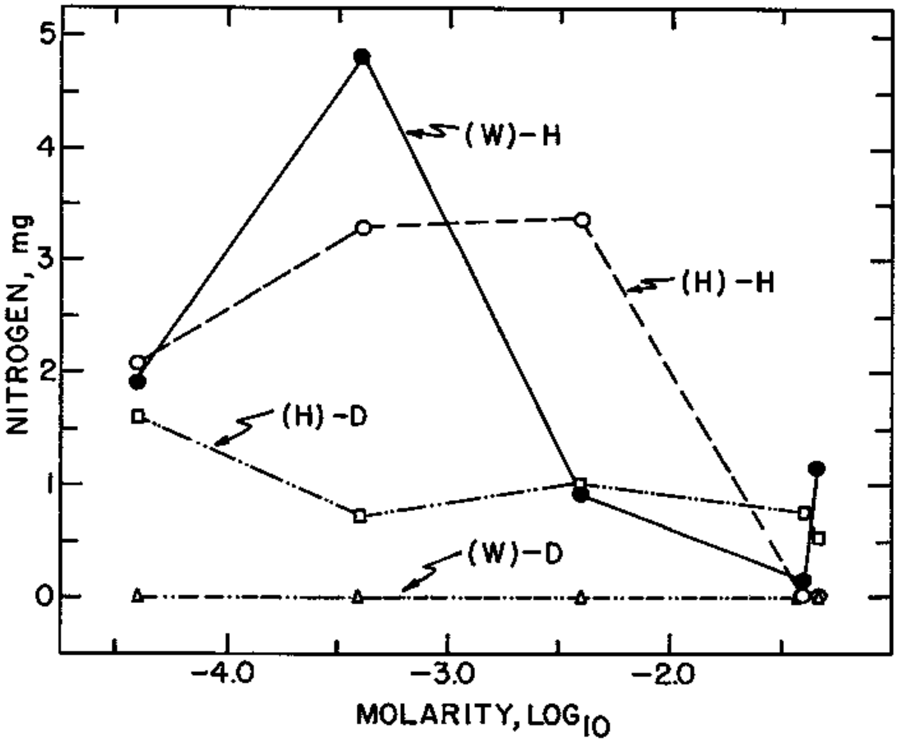

FIG. 4.—Effect of concentration of glycine on total nitrogen of healthy and Ampelamus-virus-infected cultures of *Nicotiana glutinosa* callus tissue on two media for 35 days at 26° C. Symbols: (H), Hildebrandt medium; (W), White medium; -H, healthy tissue; -D, diseased tissue. Coefficient of variability of means: from 2 to 88 for (H)-H, from 16 to 148 for (W)-H, from 18 to 100 for (H)-D.

The magnitude of growth of healthy and diseased tissue on the various media was estimated by determining the amount of total nitrogen in the various cultures. Protein nitrogen was not determined since some cultures were too small even for reliable determination of total nitrogen. Total nitrogen per wet weight of culture as shown in Figure 4 is not in agreement with the results obtained by wet weight measurements. According to nitrogen analyses, maximal growth of healthy tissue occurred at 4 x (10^{-4} and 10^{-3})M glycine in the (H) medium and at 4 x 10^{-4}M glycine in the (W) medium. The growth curve of diseased tissue on (H) medium was somewhat similar to the growth curves determined by volume and wet weight.

Diseased cultures at the completion of the experiments contained infective virus. The amount of virus in the various cultures was not assessed.

Discussion and Conclusions

The data indicate that healthy tissue and Ampelamus-virus-infected tissue of *N. glutinosa* differ in nutritional requirements.

The similarity of growth rates of healthy tissue on the two media suggests that the nutritional requirements of the healthy tissue was supplied equally well by both media. The (W) medium contained four vitamins, $CuSO_4$ and $AlCl_3$ not included in the (H) medium, therefore it appears that these particular substances had little or no effect on the growth rate of healthy tissue. Even though the concentration of micronutrients common to the two media differed by 0.0034 total molarity, this difference likewise had little or no influence on the growth rate of healthy tissue.

The dissimilarity of growth rates of virus infected tissue on the two media indicate that the nutritional requirements of the diseased tissue differs markedly from that of healthy tissue. Although the growth of diseased tissue on the (H) medium was not as great as that of the healthy tissue on this medium, the nutritional requirements of diseased tissue is partially supplied by the constituents of the (H) medium. The inability of the diseased tissue to grow on (W) medium was not due to lack of essential nutrients because it contained all the nutrients that were present in the (H) medium. The defectiveness of the (W) medium for diseased tissue, therefore, must have been due to (a) one or more of the vitamins, $CuSO_4$, or $AlCl_3$ retarding growth, (b) imbalance of nutrients, or (c) a deficiency in total concentration of nutrients in the medium.

According to the results of these tests, the concentration of glycine in the two media can be increased from $4 \times 10^{-5}M$ (3 mg/L, the amount in each original medium) to $4 \times 10^{-3}M$ without retarding the growth rate of healthy tissue. Even a greater concentration of glycine ($4 \times 10^{-2}M$ or 3 g/L) in the (H) medium is tolerated by diseased tissue. Thus, radioactive glycine at a wide range of concentration may be incorporated into the (H) medium for tracer studies on healthy callus tissue or tissue infected with Ampelamus virus.

The data do not suggest a reason for the "explosive" growth of one culture of healthy tissue in the (W) medium containing $4.6 \times 10^{-2}M$ glycine.

Growth rates of cultures determined by wet weight seem reliable. Volume measurements which seem reasonably reliable have promise since the estimate can be made quickly without destroying the sample for other use. Total nitrogen assessments of growth are unreliable and difficult to obtain especially on small samples of tissues. Density of the tissue cultures appears to be reliable and may be useful in detecting characteristics of cultures that are not ascertainable by wet weight or by volume singly.

Studies are being conducted to ascertain the influence of ionic strength of the (H) medium on the growth of healthy and virus-infected tissues in aseptic culture.

The data support the following conclusions: (a) healthy and Ampelamus-virus-infected tissue of *N. glutinosa* differ in their tolerance and response to concentrations of glycine, (b) the virus-infected and healthy tissue differ in their nutritional requirements, and (c) the failure of the virus-infected tissue to grow on the (W) medium was not due to lack of nutrients. The data suggest that the concentration of glycine in the medium influences the density of growth of healthy and of virus-infected tissue.

Summary

The effect of glycine on the growth rate of healthy and virus-infected tissue was determined for two media: (W) medium, a modification of White's formulation and (H) medium developed by Hildebrandt et al. for healthy tobacco tissue. The (W) medium contained all the constituents of the (H) medium plus four vitamins, $CuSO_4$, and $AlCl_3$. The two media differed by 3.46511×10^{-3} total molarity (70.044×10^{-3}M (H) medium 67.57889×10^{-3}M (W) medium). Concentration of glycine varied from 4×10^{-5}M to 4.6×10^{-2}. Each test included four cultures. Growth rates of healthy tissue on the two media were similar at glycine concentrations from 4×10^{-5}M to 4×10^{-3}M; glycine at 4 and 4.6×10^{-2}M retarded growth. One culture at 4.6×10^{-2}M glycine in the (W) medium grew at an "explosive" rate. Diseased tissue failed to grow on the (W) medium. On the (H) medium, the diseased tissues grew at a moderate rate at glycine concentrations from 4×10^{-5} to 4×10^{-2}, but at 4.6×10^{-2}M glycine, growth was retarded. Measurement of growth rates by wet weight was reliable, reasonably reliable by volume, and unreliable by total nitrogen. Density of cultures calculated as the ratio of wet weight in grams to volume in ml is reasonably reliable.

Acknowledgment

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