Chestnut Callus-Cultures: Tannin Content and Colonization By Endothia parasitica

F. V. Hebard and P. B. Kaufman

Department of Botany, University of Michigan, Ann Arbor, MI 48109. Present address of senior author: Department of Plant Pathology and Physiology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061

ABSTRACT.— Five clones of callus tissue cultures were obtained using scions of chestnuts that represented a gradient of blight resistance, from susceptible to resistant. These were, respectively, *Castanea dentata* (susceptible), C. *dentata* ("resistant"), an offspring of an open-pollinated Japanese X American, C. *crenata*, and *C. mollissima*. The growth rate (fresh weight) and time-course of tannin production (galloyl esters, ellagitannins, and condensed tannins) were determined in the five clones under two light regimes. Generally, the more blight-resistant the source of cultures, the more galloyl esters and ellagitannins the culture contained. Only cultures from the two most blight-susceptible sources contained significant amounts of condensed tannins. The differences among the hydrolyzable tannin levels of the callus tissue cultures of the five types of chestnut appear to be an excellent index of the blight resistance. The degree of colonization of callus of each clone by a hypovirulent *E. parasitica* was less than that by a virulent *E. parasitica*. However, the degree of colonization of the callus tissues was not correlated with the blight resistance of the parent trees. Possible reasons for this variation were differences in callus morphology and in the rate of senescence of the calli, which altered tannin levels.

[1] We would like to thank Michael J. Schneider, Alberto L. Manicinelli, Frank G. Lier, and, especially, Dominick V. Basile for their inspiration and support and Richard A. Jaynes for supplying chestnut scions and cultures of *E. parasitica*.

Tissue cultures have promise for greatly accelerating any type of program for control of chestnut blight by resistance. Assuming that it eventually will be possible to regenerate plants from chestnut tissue cultures, then the remaining problem is to determine the innate disease resistance of chestnut tissue cultures.

Nienstaedt (1953) presented evidence that the tannin content of chestnut bark may be an excellent index of blight resistance. Bazzigher (1957) reported that the tannins from the bark of blight-resistant *Castanea mollissima* Bl. and blight-susceptible *Castanea sativa* Mill. are both degraded by extracellular enzymes of *Endothia parasitica* (Murr.) P. J. and H. W. And. Bazzigher concluded that the differential tannin content of blight-resistant and blight-susceptible chestnuts was not a cause of their differing blight resistance. At low tannin concentrations (0.05 percent w/v), Nienstaedt's data indicate that tannins from Chinese chestnut bark stimulate the growth of *E. parasitica* on potato-dextrose agar more so than tannins from American chestnut bark. Barnett's

(1972) findings appear to substantiate this. However, at higher concentrations, tannins from the bark of Chinese chestnut inhibit the growth of *E. parasitica,* whereas tannins from American chestnut bark do not to as great an extent. This indicates that the tannin content of chestnut callus tissues may influence their colonization by *E. parasitica* (Grente and Sauret, 1961). Other factors influencing the outcome of such experiments include: the growth phase of the tissue when inoculated (Borrod, 1971); the light regime under which tissue is grown (Borrod, 1969); temperature; hormone regime; and callus morphology (Helgeson *et al.,* 1972). We attempted to control or account for these factors.

The callus tissue cultures used originated in the vascular cambium of chestnut stem segments, the same tissue from which callus originates on stems of Chinese chestnut colonized by *E. parasitica.* This callus can be invaded by the blight fungus (Headland *et al.,* 1976). This indicates that the interaction of *E. parasitica* with chestnut callus *in vitro* may mirror some parts of the *in vivo* host-parasite interaction.

MATERIALS AND METHODS

Cultures. Scions of *C. dentata* (Marsh.) Borkh. of Class V (least resistant) and C. *mollissima* of Class I (most resistant) were collected in Washtenaw County, near Ann Arbor, Michigan. Class V trees showed extensive canker growth with no callus formation and rapid death of colonized shoots. Class I trees showed little evidence of blight (Graves, 1950). Richard Jaynes supplied scions of a C. *dentata* from Scientists's Cliffs, Maryland, of Class IV resistance, an open-pollinated Japanese X American (HHR 4T7) of Class III, and a *C. crenata* Sieb. & Zucc. of Class II.

Explants were one cm sections of one-year-old internodes. Scions were collected in the spring, after the buds had swollen, but before leaf expansion had occurred. The callus was initiated in 125-ml Erhlenmeyer flasks with 50 ml of medium, one explant per flask. The medium was composed of Murashige and Skoog (1962) salts, Linsmaier and Skoog (1965) organic constituents with 2.0 mg/1 IAA and 0.2 mg/1 kinetin as hormones. Callus (one per bottle) was maintained in the dark at 25 C on the same medium in 1 oz French-square prescription bottles containing a slant of 9 ml of medium (White and Risser, 1964). For subculturing, masses of callus were cut into 2 mm cubes and transferred to fresh medium every 5 weeks. Light-treated cultures were illuminated with 50 foot-candles (ft-c) of continuous, cool-white fluorescent light and kept at 25 C. Cultures to be light-treated were moved from continuous darkness to continuous light about three weeks after sub-culturing (Grente, 1961).

A highly pathogenic isolate of *E. parasitica* was obtained from a diseased C. *dentata* growing in a Chelsea, Michigan. An isolate of *E. parasitica* of reduced pathogenicity (hypovirulent) was obtained from Dr. Jaynes. This was a single conidium progeny of a culture isolated by Grente in France (Jaynes and Anagnostakis, 1973). The fungi were maintained in light at 23 or 25 C on chestnut callus cultures and transferred every five to ten weeks.

Collection of growth data. Each chestnut clone was routinely transferred into 65 bottles. The bottles containing the 15 largest cultures all contained cultures of approximately the same size. From these bottles, callus tissue was selected for inoculation with *E. parasitica* and for removal, weighing (growth data) and subsequent analysis of tannins. The four largest calli were used for subculturing.

Analysis of tannins. Immediately after weighing, a 0.2 g wedge of tissue was ground at room temperature with a pestle in a mortar containing five ml of 50 percent methanol. Calli weighing less than 0.2 g were placed whole into the mortar. The extract was diluted to ten ml in centrifuge tubes. The tubes were placed in a boiling water bath for five minutes, centrifuged at 1,000 x g for five minutes and stored overnight at 5 C. Aliquots of the supernatent were used in the following analyses: Galloyl esters (components of one of the two types of hydrolyzable tannins) were analysed after the method of Haslam (1966); ellagitannins (the second type of hydrolyzable tannins) after the method of Bate-Smith (1972); and condensed tannins after the method of Govindaraj an and Mathew (1965). The standard for galloyl esters was tannic acid MW 1,200 (Nutritional Biochemicals Corp., Cleveland, Ohio), which was calculated to contain seven galloyl esters per molecule. Ellagitannins are expressed as microequivalents (ueq.) of hexahydroxydiphenoyl glucose (HHDPG), using the molar absorption coefficient given by Bate-Smith (1972). Condensed tannins are expressed as ueg . of perlargonidin, assuming its molar absorption coefficient to be 30,000 for a path length of one cm (Sondheimer and Kertesz, 1950).

Inoculation of calli with *E. parasitica.* Callus was inoculated by placing one of a spore suspension (15 conidia/u1) on the surface of a callus tissue. At this time, five 1-/.41 aliquots of the suspension were also placed on PDA plates to check for contamination. The inoculated cultures were placed in different temperature environments as indicated in the Results. We considered that the difference in magnitude of colonization of a callus culture clone by the virulent and hypovirulent isolates would be an index of innate disease resistance.

RESULTS

Growth rates. Figure 1 depicts the change in fresh weight of chestnut callus cultures with time. The two American cultures and the Japanese cultures showed similar growth up to 28 days after transfer when the Japanese cultures began to grow more slowly. The two American cultures began to grow more slowly after 47 days. These three cultures

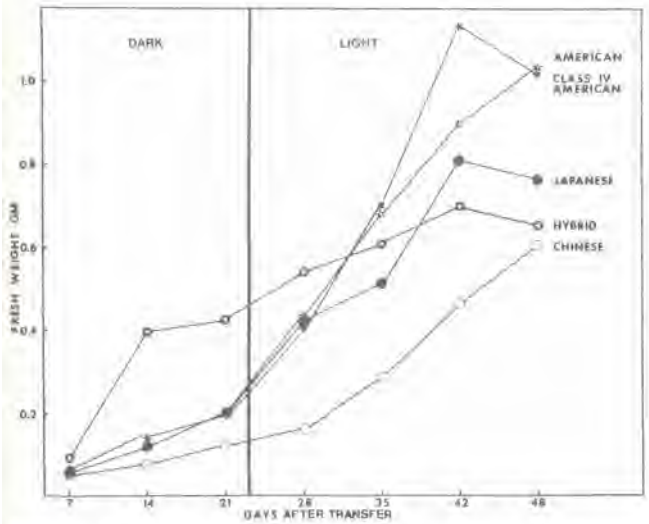

Figure 1. The time-course of the increase in fresh weight of the five chestnut callus cultures. The cultures were continuously illuminated with 50 ft-c of cool-white fluorescent light after 23 days incubation in continuous darkness. These are the same cultures used to generate the data presented in Fig. 6.

entered the linear phase of sigmoid growth near day 21.

In the data depicted in Figure 1, the hybrid cultures were already in the linear phase of sigmoid growth at day 7. By day 21, they were well into the plateau phase. During earlier experiments, the hybrid cultures remained in linear phase growth until approximately day 35, and attained fresh weights of approximately 1.5 g at 35 days after transfer. The alteration in growth rate of the hybrid cultures occurred when they were transferred after they had entered the plateau phase of growth (about 32 days after the previous transfer).

The Chinese cultures did not enter the linear phase of sigmoid growth until after day 28. They had just begun to enter the plateau phase of growth at day 48, when the experiment was terminated. If any cultures but the Chinese were moved to the light before they had entered the linear phase of sigmoid growth, their growth was reduced by a factor of four. It was desirable to have the larger calli for the colonization experiments described below.

The net outcome of these experiments was that cultures could not be moved to the light until 21 days after subculture, and were not large enough to be inoculated with *E. parasitica* until 35 days after subculture. Around this time, they were beginning to leave the linear phase of sigmoid growth.

Callus morphology. Clones of Chinese chestnut from three different trees were initially a friable callus composed of aggregates of brown spheres of tissue one mm in diameter. By selecting for white tissue subculturing, it was possible to obtain callus partially composed of a white, compact tissue. The callus cultures of the other chestnut species were a white compact tissue (at least up to plateau phase of growth). Their cellular arrangement resembled that of a cambium-derived tissue in that the cells (isodiametric) were closely packed and arrayed in rows and columns (Jacquiot, 1973).

The two American chestnut clones were similar in morphology. Initially, they grew out flat on the agar surface with radial symmetry. About 45 days after transfer, the upper surface of the callus became meristematic, and the disk of tissue enlarged to resemble a cylinder. In the American chestnut cultures, the phase of growth after the linear phase was therefore not a plateau. Rather, there was a steady increase in fresh weight for two or three months.

The Japanese chestnut cultures grew upward to resemble a column, with minimal lateral growth. The top of the column turned brown (became necrotic) about 42 days after transfer. Soon after, the entire callus became necrotic. American chestnut callus cultures grown on a medium containing gibberellic acid (one mg/1) displayed similar morphology and necrosis.

The hybrid cultures were entirely covered by a surface layer of white, friable cells. They expanded uniformly in all directions, displaying spherical symmetry.

Galloyl esters. Figure 2 depicts the time course of galloyl ester production. Cultures incubated in continuous darkness for periods of up to 32 days showed levels of galloyl esters similar to those shown at days 14 and 21 in Figure 2. At day 35, the correlation between the blight resistance of the source of a dark-grown culture and its content of galloyl esters no longer existed. When the hybrid cultures remained in linear phase of sigmoid growth until 35 days after transfer, they contained approximately 100 A eq. of galloyl esters per gram fresh weight through day 23. The galloyl ester content of later hybrid cultures (Fig. 2) dropped 40 percent between 7 and 14 days after transfer, concomitant with the end of the linear phase of growth in these cultures (Fig. 1). The Japanese chestnut cultures

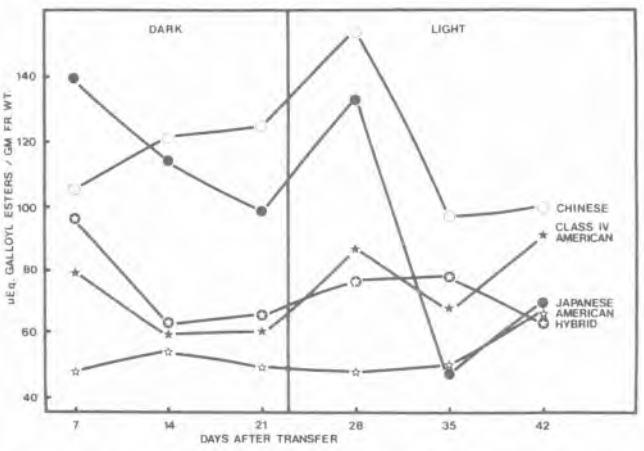

Figure 2. The time course of the galloyl ester content per gram fresh weight of the five chestnut callus cultures.

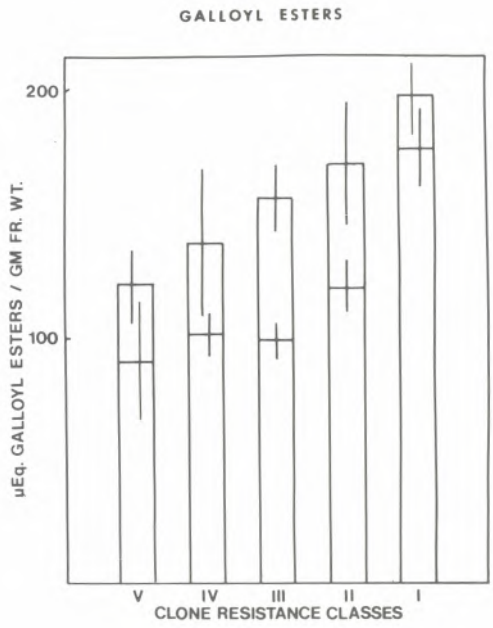

Figure 3. The mean value of the galloyl ester content per gram fresh weight of five replicate cultures each of the five chestnut callus cultures. The vertical lines are standard error *(S)* bars. The lower horizontal lines are the values for cultures incubated for 36 days after transfer in continuous darkness. The upper horizontal lines are the values for cultures incubated for 17 days in continuous darkness followed by 19 days incubation under cool-white fluorescent light of an illuminance of 50 ft-c. The Roman numerals depict the resistance class (Graves, 1950) of the clone: 1 = Chinese chestnut; II = Japanese chestnut; III = an open-pollinated Japanese x American chestnut; IV = an American chestnut from Scientist's Cliffs, Md.; V = an American chestnut from Dexter, Mich. The same extracts were used to generate the data in Figures 5 and 7. Cultures from the light-treated group were employed for the colonization experiment depicted in Figure 8.

showed a dramatic decrease in their galloyl ester content between 28 and 35 days after transfer which also paralleled the transition from the linear to the plateau phase of sigmoid growth (Fig. 1). The Japanese cultures turned brown (became necrotic) between 40 and 55 days after transfer whereas the hybrid cultures did not to as great an extent. This is probably the reason for the large decrease in galloyl ester content of the Japanese cultures at plateau phase of growth.

Figure 3 depicts the mean value of the galloyl ester content of two sets of five replicate cultures each of the five clones. All the light-treated clones (upper lines) contained more galloyl esters than their dark-grown counterparts (lower lines). In the light-grown cultures, the more blight-resistant the source of a culture, the more galloyl esters the culture contained. There is no correlation between the galloyl ester content of dark-grown cultures at or near plateau phase of growth and the blight resistance of the source of the culture (Fig. 3). In this experiment, the galloyl ester content of the light-grown Japanese chestnut cultures had not yet dropped as occurred at day 36 in the experiment depicted in Figure 2. However, the beginning of this drop is evident by the large standard deviation (Fig. 3). The large standard deviation in the galloyl ester level of the light-grown, Class IV American chestnut cultures reflects the increase in the galloyl ester levels of these cultures by day 43 of the time-course experiment (Fig. 2).

In summary, light treatment increased galloyl ester concentration. Among the clones, galloyl ester content increased with increasing blight resistance of the source of a culture. When the hybrid and Japanese cultures entered plateau phase of growth, their galloyl ester levels declined whereas those of the two American chestnut cultures increased at this point.

Ellagitannins. The time-course of ellagitannin levels (Fig. 4) was essentially similar to that of galloyl esters (Fig. 2). The levels of ellagitannins in the cultures of Japanese chestnut dropped between days 28 and 35, when these cultures were entering plateau phase of growth (Fig. 1). The low levels of ellagitannins in all cultures at day 7 were due to the low weight of the cultures at that time combined with the generally low amount of ellagitannins in the cultures and the low sensitivity of the assay procedure.

The mean value of ellagitannins in five replicates is shown in Figure 5. The Japanese chestnut culture has low levels of ellagitannins at the time of this assay (36 days after transfer). The drop in the hydrolysable tannin content of the Japanese cheEtnut cultures was detectable by the ellagitannin assay (Fig. 5) at a point when it only caused a large standard deviation in the galloyl ester assay (Fig. 3). Both assays were run on the same extracts. The light treatment increased ellagitannin levels (Fig. 5)

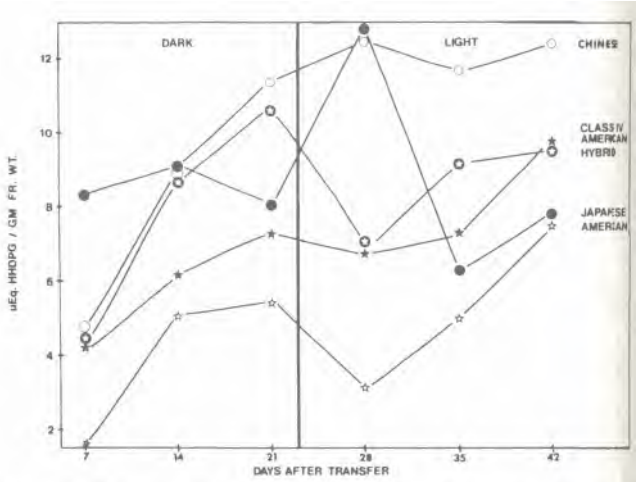

Figure 4. The time-course of the ellagitannin content per gram fresh weight of the five chestnut callus cultures.

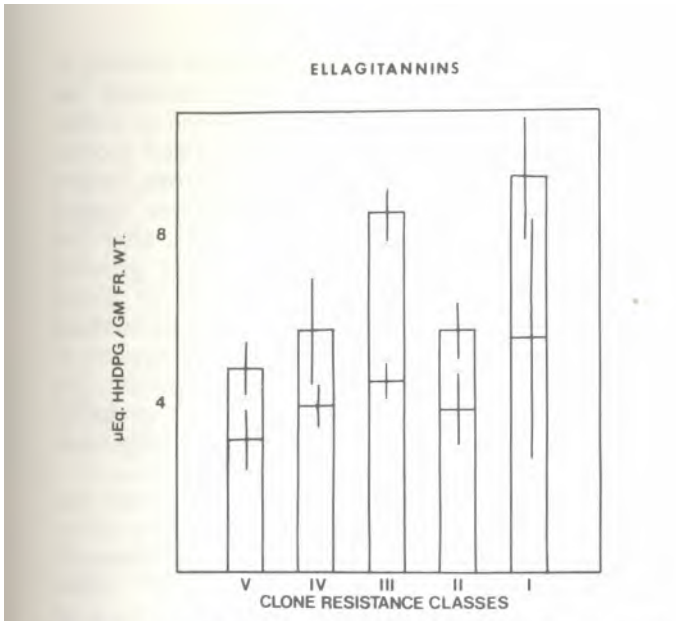

Figure 5. (left) The mean-value of the ellagitannin content per gram fresh weight of five replicate cultures each of the five chestnut callus cultures. See the caption of Figure 3 for further information.

as it has increased galloyl ester levels (Fig. 3). With the exception of the Japanese chestnut cultures, the ellagitannin content of both dark- and light-grown chestnut cultures correlated with the blight resistance of their source (Fig. 5).

Condensed tannins. Only the two American chestnut cultures contained significant levels of leucoanthocyanins (Figs. 6, 7). The light treatment increased the condensed tannin content of the American chestnut cultures ‹ Figs. 6, 7) as it had galloyl esters (Fig. 3) and ellagitannins (Fig. 5). The levels of condensed tannins in American chestnut cultures incubated in constant darkness remained constant during logarithmic and linear phases of growth. Only the condensed tannin assay worked well on crude extracts of chestnut bark. In this assay, the Japanese chestnut bark extract, as well as the American chestnut bark extracts, contained leucoanthocyanins. Data are not presented because other compounds in the Chinese and hybrid extracts were reacting with the reagents to give brown-colored substances which absorbed light of a wavelength of 500 nm. However, this color was easily distinguished from the distinctive red of anthocyanins.

Colonization of the cultures of *E. parasitica*. Figure 8 depicts the degree of colonization of the five clones (light-grown) by virulent and hypovirulent *E. parasitica*. In every clone, the hypovirulent isolate colonized the surface of the callus tissues less than the highly pathogenic isolate. Both isolates colonized dark-grown cultures equally. However, the degree of colonization by both isolates did not correlate well with the blight-resistance of the source of the clones. Transforming the data to account for the differential colonization of the clones by the hypovirulent isolate improved the correlation, but it was still imperfect.

The differences in magnitude of colonization between the two fungus strains and between the five callus cultures were much less pronounced when pieces of mycelium or spore suspensions containing more than 15 or 20 conidia were inoculated onto the calli or when the inoculated cultures were incubated at 25 C instead of 23 C.

In these colonizations, the mycelia of both isolates grew over the surface of the callus, penetrating only two or three cell layers deep into the callus. *In vivo, E. parasitica* commonly colonizes the interior of chestnut bark. When a conidial suspension was injected into the callus with a 36 G hypodermic needle, in an attempt to duplicate this con-

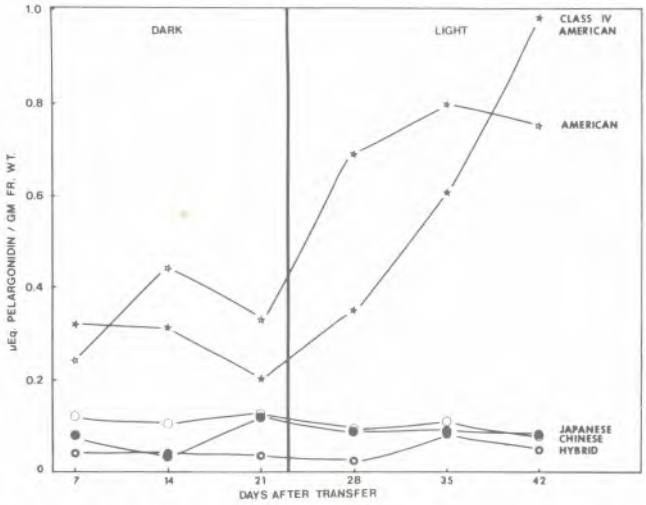

Figure 6. The time-course of the leucoanthyocyanin content per gram fresh weight of the five chestnut callus cultures.

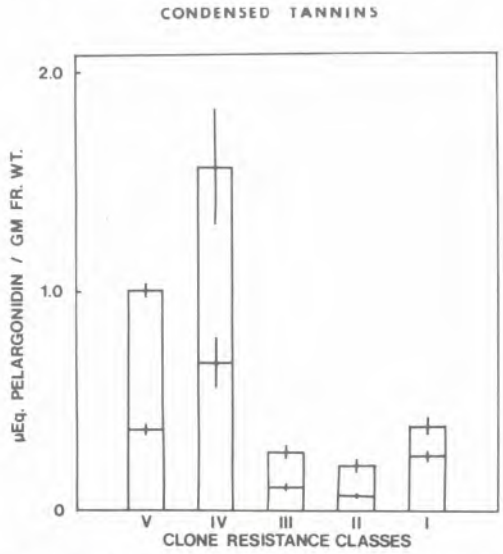

Figure 7. (right) The mean-value of the leucoanthycyanin content per gram fresh weight of five replicate cultures each of the five chestnut callus cultures. See caption of Figure 3 for further information.

dition, the hyphae grew out of the resultant hole and still colonized the surface of the callus first.

DISCUSSION

Correlation of tannin content with resistance. The galloyl ester contents of the light-grown chestnut cultures at 36 days after transfer correlate well with the blight resistance of their sources (Fig. 3). There is a one in 120 chance (5!) of occurrence of the pattern observed in Figure 3. The mean values for clones, two resistance classes apart are separated by two standard errors of the mean. The galloyl ester content of dark-grown cultures is also correlated with blight resistance up to 32 days after transfer (Fig. 2). The ellagitannin content of chestnut cultures correlates well with blight-resistance, but the lack of sensitivity of the assay and the rapid decrease in the ellagitannin content of cultures upon beginning the plateau phase of growth (Figs. 1, 4, 5) makes this assay a less reliable index of the innate disease resistance of these cultures. Thus, there appears to be a strong relationship between blight-resistance and the hydrolyzable tannin content of chestnut tissues. This result agrees with and extends Nienstaedt's (1953) findings. In addition, Nienstaedt found that tannin from Chinese chestnut bark, which is composed solely of hydrolyzable tannins, is strongly inhibitory to the growth of *E. parasitica in vitro.* This, in combination with our findings, suggests that the resistance of Chinese chestnut bark to colonization by *E. parasitica* is caused by the high content of hydrolyzable tannins in the bark. In contrast, Bazzigher's (1957) findings imply that such a cause and effect relationship does not exist. This discrepancy will have to be clarified by further research.

The condensed tannin contents of chestnut callus cultures are negatively correlated with the blight resistance of their sources (Figs. 6, 7). This concept is reinforced by our findings that Japanese chestnut callus cultures contain no leucoanthocyanins whereas bark does. Nienstaedt (1953) also found that Japanese chestnut bark contains condensed (catechol) tannins. This suggests that condensed tannins may not be a constituent of new wound callus and phloem parenchyma which are produced in Japanese chestnut bark in response to colonization of the bark byE. *parasitica.*

Effects of growth phases on tannin content. We generally found that the tannin levels in chestnut callus cultures remained constant during the log and linear phases of sigmoid growth and then decreased if the cultures became necrotic or increased if growth persisted past the first linear phase of sigmoid growth. In *Haplopappus* callus cultures Strickland and Sunderland (1972) found that anthocyanins per gram fresh weight rose rapidly then decreased with growth but that chlorogenic acid per gram fresh weight remained constant well into plateau phase of growth. The *Haplopappus* results concerning chlorogenic acid agree with our results. The increase and decline in anthocyanin content in the *Haplopappus* cultures may be mirroring the commonly observed increase and decline in anthocyanin content of elongating seedlings and shoots. In suspension cultures of Paul's Scarlet rose, Davies (1972) found that polyphenol synthesis lagged behind growth and reached a maximum after the cultures had entered plateau phase of growth. Davies' results compare with a report by Swain (1965) that tannin concentrations in tissues increase as the tissues age. We observed such an increase in tissues which did not suddenly become necrotic. The tannins of the necrotic tissues were probably oxidized to quinones by peroxidase and polyphenol oxidase, thus their sudden decline.

The light-stimulated increase in tannin levels was probably another example of general light-stimulated increase in phenolics, a commonly observed process in tissue cultures (Schopfer, 1977). This process is always accompanied by an increase in phenylalanine ammonia lyase activity, which could increase both condensed and hydrolyzable tannin levels.

Relationship of growth phase, morphology and tannin content of callus to colonization by E. *parasitica*. Twelve days after the calli were inoculated, most of the Japanese cultures were beginning to turn brown (become necrotic). When the expanding brown area reached an area occupied by the fungus, the entire callus was immediately colonized (Fig. 8). Less frequently, the same thing happened in the hybrid cultures. This necrosis, which was accompanied by low hydrolyzable tannin levels at 36 days after transfer (the day of inoculation) (Figs. 2 and 4), is probably the reason for the high incidence and rate of colonization of these cultures by *E. parasitica.* When the hybrid cultures maintained vigorous growth for long periods, they were not colonized as rapidly by either isolate of *E. parasitica.* The American cultures were meristematic in the regions which became colonized. This meristematic tissue contained high amounts of tannins (Figs. 2, 4, 6), that inhibited colonization, and low levels of the brown, nontannin substances, which probably stimulated colonization of the hybrid and Japanese cultures. When the upper layer of meristematic tissue was not formed, cultures were more rapidly colonized. Thus, changes in tannin levels may have resulted in some of the anomalous results of the colonization experiments.

This early onset and high incidence and rate of colonization of the Chinese chestnut cultures was probably due to their friable nature, as Helgeson *et al.* (1972) found in the tobacco -*Phytophthora parasitica* system. The large surface area and small internal volume of friable cultures presents an ideal substrate to parasites which tend to colonize the surface of cultures. The results of inoculating callus by injecting conidia show that *E. parasitica* tends to colonize the surface of cultures. There are two reasons which might explain this tendency. First, the air surrounding a callus is at 100 percent relative

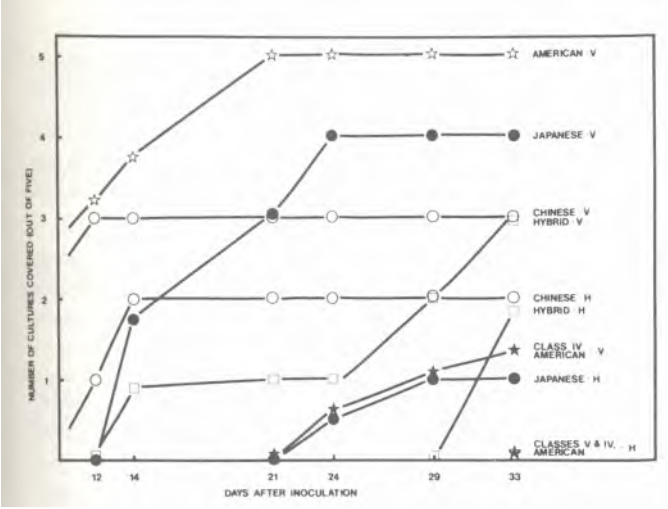

Figure 8. The time-course of the extent of colonization of five cultures each of the five chestnut callus cultures by virulent and hypovirulent *E. parasitica.* The value of each point was determined by measuring the fraction of the surface area of each culture covered by fungal mycelium and summing the measurements of the five replicate cultures. The letters V or H following the name of each callus culture clone denote whether those cultures were inoculated with virulent (V) or hypovirulent (**H)** *E. parasitica.* The cultures were incubated at 23 C after inoculation. The cultures were inoculated 38 days after transfer, after 21 days incubation in light.

humidity, so hyphae are not dessicated. Second, a fungus colonizing the surface of callus culture encounters no barriers (epidermis or periderm) to repeated infections of the upper cell layers of the callus. Since the callus is grown in atmospheres of 100 percent relative humidity, it is doubtful that protective cell layers (epidermis or periderm) would be found.

Thus there were three main problems with the colonization experiments. First was the lack of invasive colonization. Second was the friable nature of the Chinese chestnut cultures. Third, it was not possible to have all five cultures in a state where they would continue growing as compact tissues for 30 days or so after inoculation. It took 35 days to obtain tissue large enough to work with. Increasing tannin levels by illuminating the cultures could not be done until all clones but the Chinese were in the linear phase of sigmoid growth. The second two problems could be solved by employing larger culture bottles, which permit longer growth periods, by transferring cultures every month and by substituting Class I resistance Japanese chestnut cultures for the Chinese.

Hypovirulence. We found that the hypovirulent isolate did not colonize as well as the virulent isolate even when the clones were not responding to the virulent isolate in a manner reflective of their *in vitro* blight resistance (Fig. 8). This suggests that the hypovirulence factor operates generally to reduce the vigor of the pathogen, and that it does not interfere specifically with mechanisms of pathogenicity.

CONCLUSIONS

The tannin assays described in this paper could serve to categorize the blight resistance of products of *in vitro* genetic manipulations. Additional chemical indices of resistance might be based on the work of McCarron (1979) and Samman (1979). It will be necessary to have detailed knowledge of the histopathology of chestnut blight cankers before any *in vitro* host-pathogen interaction can be evaluated for its resemblance to the *in vivo* interaction. Even if close resemblances are found, it will still be necessary to categorize the blight resistance of whole plants produced in a tissue culture breeding program. Depending on the procedures employed for genetic manipulations, this might obviate a need for detailed study of the host-parasite interaction *in vitro.*

Regarding tannins, it would be desirable to know if they play an integral role in blight resistance. This would give one confidence that cultures of American chestnuts of high blight resistance (Classes I and II) would display tannin contents similar to those of Oriental chestnuts. One approach to this problem might be obtained with tissue cultures, namely, varying the tannin content of chestnut tissue cultures and observing the effects of this on colonization. Preliminary observations indicate that addition of gibberellic acid increases tannin content and delays colonization.

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