

Isolation and maintenance of normal leaf and mite-incited leaf gall tissues of *Cinnamomum tamala* in culture

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Received 24 July 1989; revised 20 November 1990

The growth regulator requirements of mite-incited leaf gall tissue of *C. tamala* did not differ fundamentally from that of the normal tissue. Optimum callusing of explants occurred on MS medium containing 2,4-D (10 mg/l), kinetin (0.1 mg/l) and β -mercaptoethanol (1-2 mM). Callus grew better in the medium fortified with 2,4-D (4 mg/l) and kinetin (0.4 mg/l). Gall tissue showed higher growth rate than the normal tissue. The two types of tissues showed differential growth response to auxins, kinetins and phenolics. While the normal tissue failed to grow in auxin-free medium, the gall tissue was able to do so after four to five passages on medium containing 2,4-D (4 mg/l) and kinetin (0.4 mg/l). However, in the auxin-free medium the growth was poor. One year old cultures of both normal and gall tissues could grow on medium devoid of cytokinin for a prolonged duration, presumably showing the phenomenon of habituation. Addition of caffeic acid and catechol enhanced the growth of both normal and gall tissues as compared to control. These phenolics did not exert any promoting effect in absence of auxin.

Plant tissue culture technique has been used as a tool to understand not only normal but also abnormal growth in plants under closely controlled conditions¹⁻². In a large number of tree species callus initiation and subsequent regeneration into whole plants have been reported³⁻⁵. However, certain genera are rather refractory to such handling⁶. One of the main problems in callus initiation of some woody tropical and subtropical plants is the accumulation of phenolics in culture medium which is toxic to the tissues^{6,7}. The ability of explants to survive, multiply and regenerate depends upon a number of factors both external and internal⁸.

It is widely accepted that plant tumors show abnormal growth hormone metabolism and possess the ability to grow axenically in defined culture media in absence of externally supplied growth hormones^{9,10}. The insect/mite-incited plant galls have received little attention in this regard as compared to bacterium-induced tumors. Conflicting reports are there on growth factor requirements of insect/mite-gall tissues. Added auxin is necessary to grow isolated insect gall tissues¹¹⁻¹³. However, the *Eriophyes* incited *Zizyphus* galls possess both transplantable and tumefacient properties¹⁴. The present communication deals with (i) successful isolation, and (ii) growth factor requirements of normal and mite-incited leaf gall tissue cultures of *Cinnamomum tamala*.

Materials and Methods

For isolation, young normal leaf and half-mature leaf galls (incited by unknown mite) of *Cinnamomum tamala* Fr. Nees were collected, cut into appropriate pieces presoaked in β -mercaptoethanol (β -ME; 1-2 mM) and sterilized with 0.1% HgCl₂ for 8-10 min followed by three rinses in sterilized double distilled water and transferred to modified Murashige and Skoog (MS)¹⁵ medium fortified with different concentrations of auxins (1-10 mg/l), such as 2, 4 dichlorophenoxyacetic acid (2, 4-D), and naphthaleneacetic acid (NAA), and kinetin (0.04-0.1 mg/l) in various combinations. The pH of the medium was adjusted to 5.7-5.8 before autoclaving it at 1.06 kg cm⁻² for 15 min.

Effect of antioxidants and other adjuvants on callus initiation To prevent browning of explants as well as to promote callus initiation, different concentrations of a number of antioxidants, viz. ascorbic acid (100-500 mg/l), ascorbic acid + citric acid (100 + 150 mg/l), dithiothreitol (10-100 mg/l), L-cysteine (100-500 mg/l), β -ME (1-5 mM) and other substances such as polyvinylpyrrolidone (1-2 g/l) activated charcoal (100-500 mg/l), casein hydrolysate (100-500 mg/l) and coconut water (50-200 ml/l) were incorporated separately into MS medium. Besides, presoaking of explants was done in ascorbic acid + citric acid and β -ME.

As soon as callusing was observed on the explants, they were transferred to a fresh medium. The cultures were maintained through regular subcultures every

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30 days on MS medium in dark at $22^{\circ} \pm 1^{\circ}\text{C}$ and 60-70% RH. For callus subculture approximately 100 mg of tissue was transferred to fresh nutrient medium in each flask.

Effect of growth regulators, mineral nutrients and phenolics on callus growth— Studies on the growth of callus tissues were carried out in their fifth and sixth passages. In order to establish hormonal and nutritional requirements of the callus tissues, the basal medium was variously supplemented with 2, 4-D, NAA, indole-3-butyric acid (IBA; 1-8 mg/l) kinetin (0.1-1.0 mg/l), benzylamino purine (BAP; 0.1, 0.4 mg/l), catechol and caffeic acid (O-dihydroxyphenols; 12.5-100 mg/l). Ionic strength of MS medium was also varied by doubling or reducing to half the strength of NH_4NO_3 , KNO_3 and CaCl_2 . In one experiment concentration of all constituents of MS medium were doubled or halved.

In each experiment the final weight of tissues was measured after 30 days of culture. Dry weight of tissues was determined by drying them in an oven at 60°C for 48 hr. For each experiment five replicates (except for callus initiation) were taken and experiments were repeated thrice.

Results and Discussion

Cinnamomum being rich in phenolics¹⁶ exhibited considerable problem in callusing as well as in further subculture. These phenolics or their oxidation products i.e. quinones, were responsible for (i) browning of explants as well as the medium, and (ii) the poor growth of the tissues. Of the various treatments tried to overcome the browning problem none proved to be satisfactory except lower concentrations of (1-2 mM) β -ME. Addition of β -ME prevented not only browning of the explants but also enhanced the frequency of callus formation. This could probably be due to inhibition of polyphenol oxidase catalysed oxidation of phenolics by β -ME as has been reported in *Medinella mangifica*¹⁷ and *Dioscorea bulbifera*¹⁸. Addition of polyvinylpyrrolidone and activated charcoal in the medium enhanced browning of the explants in *C. tamala*.

Callus initiation occurred within 20-25 days in both normal and gall tissue explants and was better in

the medium containing 2,4-D(10 mg/l) and kinetin (0.1 mg/l) as compared to the medium having NAA (10 mg/l) and kinetin (0.1 mg/l). Optimum callus induction occurred on MS medium containing 10 mg/l 2,4-D, 0.1 mg/l kinetin and 1-2 mM β -ME. For callus initiation and callus tissue growth 2,4-D has been reported as a superior auxin in many plant species¹⁹⁻²¹. In NAA and kinetin containing MS medium leaching of brown pigments from the gall tissue explants was more than that from normal tissue and also the former resulted in the lowering of the media pH (Table 1). Studies with pH indicators in the medium revealed that the tissues changed pH of the medium in the areas they occupied²².

Growth and colour of both normal and gall callus tissues were greatly influenced by auxin-cytokinin ratio. The tissues turned brown and subsequently failed to grow in media containing 2,4-D (1,6 and 8 mg/l), IBA (1-8 mg/l) in conjunction with kinetin (0.1-1 mg/l). The influence of 2,4-D(2-4 mg/l) and kinetin (0.1-1 mg/l) in MS medium on the growth of both normal and gall callus tissues are shown in Table 2. Optimum growth of both the tissues was rec-

Table 1 Changes in pH of NAA and kinetin containing MS media with normal and gall tissue explants cultured for 30 days

Medium	Initial pH	Final pH
With normal explant	5.8	5.5-5.0
With gall explant	5.8	4.5-3.8

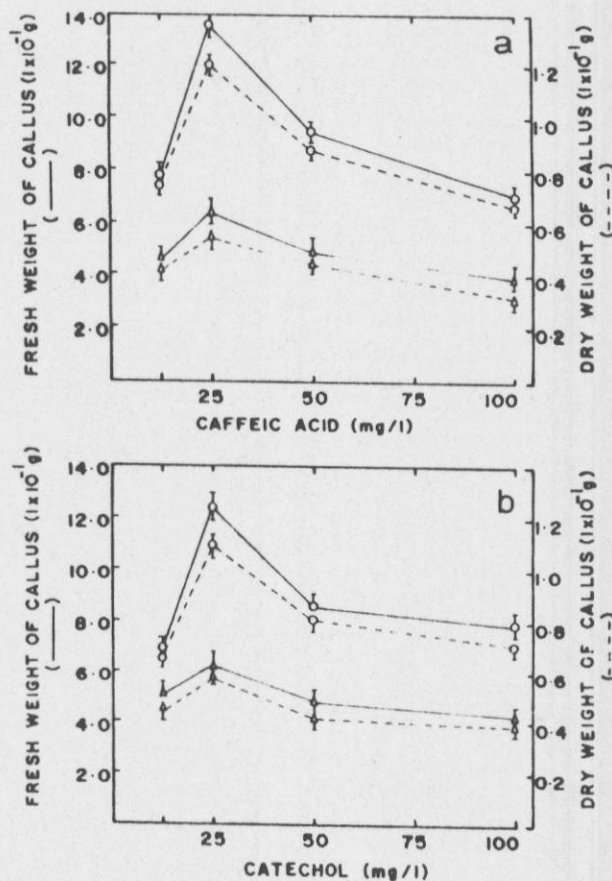


Fig. 1 Growth of normal (Δ) and gall (\circ) callus tissues in response to different concentrations of, (a) caffeic acid and (b) catechol in MS medium containing 4 mg/l 2, 4-D + 0.4 mg/l kinetin

orded in presence of 4 mg/l of 2,4-D and 0.4 mg/l kinetin and it was regarded as control for subsequent experiments. Normal and gall callus tissues became greyish and showed poor growth in media containing 4 mg/l of 2,4-D in conjunction with 0.1 or 0.4 mg/l of BAP. However, normal callus tissue showed better growth as compared to gall in both the combinations (Table 2). No appreciable changes in growth of normal and gall callus tissues were noticed when mineral nutrients and the strengths of MS medium were chan-

ged. This indicates that the proper balance of growth regulators and the concentrations of both macro- and micro-nutrients in the media is critical for optimum growth of tissues.

Addition of caffeic acid and catechol enhanced the growth of both normal and gall callus tissues as compared to control. The growth of gall callus tissue was markedly higher as compared to normal (Figs 1a and b). This difference in growth clearly reflects the differential responses of these tissues towards phenolics.

Table 2—Growth of normal and gall callus tissues subjected to different growth regulators [Values are mean ± SE]

Treatment		Normal		Gall	
2,4-D (mg/l)	Cytokinin + Phenol (mg/l) Kinetin	Fresh wt (g)	Dry wt (g)	Fresh wt (g)	Dry wt (g)
2	0.1	0.210 ± 0.06	0.018 ± 0.003	0.220 ± 0.04	0.018 ± 0.003
	0.2	0.238 ± 0.04	0.023 ± 0.002	0.248 ± 0.03	0.027 ± 0.004
	0.4	0.359 ± 0.03	0.033 ± 0.004	0.375 ± 0.06	0.035 ± 0.005
	0.8	0.309 ± 0.05	0.030 ± 0.002	0.290 ± 0.04	0.032 ± 0.004
	1	0.214 ± 0.03	0.019 ± 0.002	0.190 ± 0.05	0.016 ± 0.002
3	0.1	0.268 ± 0.06	0.025 ± 0.002	0.288 ± 0.04	0.027 ± 0.004
	0.2	0.310 ± 0.06	0.030 ± 0.004	0.460 ± 0.08	0.042 ± 0.004
	0.4	0.370 ± 0.08	0.033 ± 0.003	0.510 ± 0.06	0.047 ± 0.003
	0.8	0.350 ± 0.06	0.032 ± 0.004	0.400 ± 0.04	0.034 ± 0.004
	1	0.290 ± 0.06	0.026 ± 0.004	0.310 ± 0.04	0.029 ± 0.005
4	0.1	0.237 ± 0.06	0.026 ± 0.002	0.285 ± 0.06	0.030 ± 0.004
	0.2	0.320 ± 0.06	0.030 ± 0.003	0.410 ± 0.04	0.039 ± 0.004
	0.4	0.485 ± 0.08	0.046 ± 0.004	0.715 ± 0.06	0.072 ± 0.008
	0.8	0.335 ± 0.08	0.036 ± 0.005	0.435 ± 0.08	0.040 ± 0.006
	1	0.280 ± 0.10	0.024 ± 0.003	0.300 ± 0.08	0.026 ± 0.004
4	0	0.265 ± 0.02*	0.029 ± 0.002	0.490 ± 0.02*	0.047 ± 0.004
0	0.4	—	—	0.285 ± 0.08	0.030 ± 0.003
0	0	—	—	—	—
0	0.4	—	—	—	—
	+				
	caffeic acid (25)	0.137 ± 0.08	0.012 ± 0.002	0.283 ± 0.04	0.026 ± 0.002
0	0.4	—	—	—	—
	+				
	catechol(25)	0.125 ± 0.04	0.010 ± 0.001	0.267 ± 0.02	0.026 ± 0.003
	BAP				
4	0.1	0.210 ± 0.04	0.018 ± 0.002	0.160 ± 0.06	0.014 ± 0.001
4	0.4	0.465 ± 0.12	0.052 ± 0.002	0.220 ± 0.10	0.018 ± 0.002

*One year old cultures were used; — No growth.

Addition of phenolic compounds in the medium enhanced the growth of tissues in some cases^{11,23,24}. The striking feature of the present study was that the phenolics influenced growth only in presence of auxin and kinetin. In absence of auxin, they did not exert any promoting effect. This envisages the synergistic role of phenolics with auxin in growth which is different from the findings of Shah and Mehta²⁵ who reported better growth of *Crotalaria* callus in the medium free of auxin but containing phenolics.

In the present investigation, the gall callus tissue could be grown in auxin-free medium after four or five passages on the medium containing 2,4-D (4 mg/l) and kinetin (0.4 mg/l). One year old cultures of normal and gall tissues of *C. tamala* also showed cytokinin autonomy. There could be two possibilities for the acquisition of growth hormone autonomy by normal and gall tissue cultures: (i) an increase in active pool size of growth hormone, and (ii) the tissue may acquire an increasing ability to respond to growth substances¹⁰. The latter does not seem to be the case as the growth of callus tissue in the auxin or kinetin free media (in case of gall tissue only) was poor compared to control (Table 2). Horgan¹⁰ reported that the second possibility could not occur in case of tissue which already responded to externally applied growth substances and became autonomous by losing the requirement for the particular substance. Studies on the levels of IAA catabolic enzymes (peroxidase, IAA-oxidase and polyphenol oxidase) and inhibitors of IAA oxidation (O-dihydroxyphenols) showed that gall callus tissues grown on auxin-free media contained low peroxidase and polyphenol oxidase activities and high O-dihydroxyphenol contents¹⁶. This suggests that the capacity for autotrophic growth of habituated cells of *C. tamala* may be caused by changes in the metabolism of the compound concerned. One year old cultures of normal and gall tissue grown on kinetin-free-media also showed similar pattern for IAA catabolism as that of the gall tissue grown on auxin-free medium¹⁶. It was also found that in absence of growth regulators (both auxin and kinetin) the gall and normal callus tissues of *C. tamala* did not grow and instead became necrotic. There are evidences for complex interactions of auxin and cytokinin in regulating cell proliferation and growth factor production^{26,27}. Thus studies of the enzymes related to both auxin and cytokinin metabolisms in such cultures (habituated ones) would be helpful to establish the relationship between the capacity for autotrophic growth and growth factor production.

In conclusion, the mite-incited leaf gall tissue of *C. tamala* do not differ fundamentally in growth factor requirements from its normal counterpart. The conti-

nuous presence of growth hormones (auxin and/or kinetin) is essential for its better growth. This is in sharp contrast with the behaviour of crown gall and *Zizyphus* gall tissues.

Acknowledgement

Financial support from CSIR, New Delhi is acknowledged.

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