Some Properties of the False Broomrape Causal Agent and its Persistence in Soil and Refrigerated and Frozen Tumor Tissues

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ABSTRACT

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The false broomrape causal agent from ground tobacco root tumors suspended in a buffer solution sedimented from suspension at 48,000 *g* for 20 min, was retained on a 0.45 μm filter, was inactivated with chloroform, and was insensitive to streptomycin sulfate. These responses to the treatments are similar to those of some pathogenic bacteria. The agent persisted 5 yr in an artificially infested sand-soil mixture exposed to meteorological conditions. It was recovered from tumors refrigerated at approximately 6 C for 1 yr and tumors frozen at −20 C for 4.5 yr. The level of infection obtained on tobacco assay plants indicated the causal agent might persist more than 5 yr in a sand-soil mixture and 4.5 yr in frozen tumor tissues.

The causal agent of false broomrape (FBR) is unknown. It has been postulated that the hyperplastic growths (tumors) on tobacco roots might be caused by *Agrobacterium tumefaciens* (Smith and Townsend) Conn or *Corynebacterium fascians* (Tilford) Dowson (6), but typical tumors did not develop on tobacco roots inoculated with *C. fascians* (5). Nor have these or other organisms capable of causing the tumors been isolated from the hyperplastic growths (6). Apparently, the FBR causal agent is nonsystemic; when tumorous growths were grafted as scions to healthy tobacco plants, tumors did not develop on the stock roots (5). However, the disease agent can be transmitted by grinding the tumors into a slurry and inoculating the root systems of young plants with it (1), and the tumors are used as experimental inoculum. Observational evidence also indicates the causal agent is soilborne. The disease recurred annually in a Georgia tobacco plant bed (P. D. Dukes, *personal communication*), and developed in sweetpotato planted in one batch of potting soil in North Carolina (4). The agent was reported to persist in tumor tissue 15 days at −20 C (7).

Results reported here are from tests designed to investigate some properties of the causal agent and its persistence in an artificially infested soil-sand mixture and in refrigerated and frozen tumor tissues.

MATERIALS AND METHODS

The FBR inoculum used in all but one test initially was obtained from sweetpotato (4) and then maintained on tobacco. In the tests with refrigerated tissue, the 361-day tissue sample was tumors from naturally infected field tobacco. The FBR tumors were ground to a paste in neutral 0.1 M phosphate buffer with a sterile mortar and pestle and diluted at the ratio (w/v) of 1 g of tissue to 4 ml of buffer. These, or variously treated, suspensions were inoculated to roots of tobacco seedlings as described by Dukes et al. (1). The inoculated seedlings were planted in a sand-soil mixture in 5-cm pots and incubated in a sand bath at 35 C (2) for 7 days with irrigation supplied as needed. Then the soil and established tobacco plant were transferred to a 15-cm diameter pot of the sand-soil mixture. Each plant was fertilized weekly with 100 ml of nutrient solution and grew for 2.5 to 3.5 mo in a greenhouse maintained at 29 C minimum. At harvest, the soil was washed from the roots and they were examined for FBR tumors.

To obtain information on some properties of the FBR causal agent, suspensions of it, tobacco mosaic virus (TMV), and *Erwinia carotovora* var. *carotovora* (Jones) Dye were compared for sedimentation at 48,000 *g* for 20 min, passage through 0.8 and 0.45 μm filters, and inactivation by chloroform (4 ml/5ml of suspension) and streptomycin sulfate at 100 μg/ml. Tobacco leaves infected with TMV were ground in and diluted with buffer as for the FBR tumors. Both suspensions were blended 1 min, strained through four layers of cheesecloth, and passed through a 5-μm filter before treatment. *Erwinia carotovora* was grown on nutrient agar medium slants and suspended in 0.1 M buffer. The cell suspension was adjusted to 0.2 absorbance at 600 nm on a spectrophotometer. Five-ml aliquots of the suspensions received the physical and chemical treatments.

The treated suspensions or their fractions were assayed for infectivity. *Erwinia carotovora* suspensions were inoculated to fresh potato tuber slices, TMV suspensions were rubbed on Carborundum-dusted leaves of tobacco plants, and tobacco seedlings were inoculated with FBR suspensions as described above.

Tobacco seedlings inoculated with an FBR suspension and incubated 1 wk at 35 C were transferred to 15-cm

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diameter pots and drenched with the antibiotics chlortetracycline hydrochloride and tetracycline hydrochloride which remit symptoms of some mycoplasma diseases (8) to see if they would prevent tumor development on the inoculated root systems. Five-hundred ml of solutions of the antibiotics at 100 μg/ml were applied twice a week for 7.5 wk.

The systemic nature of the FBR agent was tested as previously described (5). Five plants that emerged from tumors were excised and established in 10-cm pots of soil-sand mixture. After 2 mo of growth, terminal scions from the tumorous plants were cleft-grafted to healthy tobacco plants. The tumorous and grafted plants grew for 3 mo and then their root systems were examined for tumors.

A soil-sand mixture was infested with 160 g of tumor tissues on 25 September 1969. The tissues were ground with a meat chopper into 150 ml of 0.1 M phosphate buffer at pH 7. The suspension was made up to 500 ml with distilled water and mechanically blended for 3 min, the volume was increased to 1 liter, and the resulting suspension was used to infest 4 kg of sand. Eight kg of methyl bromide-fumigated potting soil was thoroughly mixed with the infested sand. Then about 250 g of the infested mixture was dispensed into 7.6-cm plastic pots. The filled pots were embedded in sand in wooden flats, and placed on the soil surface outside a greenhouse with a southern exposure. Weeds were removed as they developed in the pots of soil or sand.

Persistence of the causal agent was bioassayed by transplanting Hicks tobacco seedlings in the infested mixture of (usually) six pots. The transplants were incubated 7 days in a sand bath at 35 C (2), and then transferred to 15-cm diameter pots for further incubation and plant growth.

During the course of experiments, tumors were excised from tobacco root systems and refrigerated or frozen for future inoculum. They were placed in clip-top vials or screw-cap bottles and refrigerated at approximately 6 C or frozen to −20 C. Causal agent persistence in these temperature regimes was assayed periodically by inoculating tissue samples to (usually) six tobacco seedlings. Hicks tobacco seedlings in the three- to five-leaf stage were the bioassay plants in all but three assays in which susceptible Coker 187, NC-2326, and McNair 12 plants were substituted.

TABLE 1. Infectivity of suspensions of *Erwinia carotovora*, tobacco mosaic virus (TMV), and false broomrape agent (FBR) after centrifugation, filtration, and chemical treatments

	Infectivity of treated[a]					
	E. carotovora		TMV[b]		FBR[b]	
Suspension Treatment of	Test 1	Test 2	Test 1	Test 2	Test 1	Test 2
Centrifugation[c]:						
pellet	+	+	+	+	+	+
supernatant	−	−	+	+	−	−
Filtration:						
0.8 μm	+	+	+	+	+	+
0.45 μm	−	−	+	+	−	−
Chemical:						
chloroform	−	−	+	+	−	−
streptomycin	−	−	+	+	+	+

[a]Symbols in table: + = infection, − = no infection.
[b]The TMV and FBR suspension samples were introduced as inoculum to Coker 187 and McNair 12 tobacco plants in Tests 1 and 2, respectively.
[c]Centrifugation was at 48,000 *g* for 20 min.

RESULTS

The physical and chemical treatments of *E. carotovora*, TMV, and FBR suspensions were performed twice; the results are summarized in Table 1.

Suspensions of the FBR causal agent responded to the physical treatments the same as *E. carotovora* but they differed in infectivity following the chemical treatments. Chloroform rendered both suspensions noninfective, but only *E. carotovora* was noninfective with 100 μg/ml of streptomycin sulfate in the suspensions. None of the physical or chemical treatments removed TMV from suspension or rendered it noninfective.

TABLE 2. Persistence of the false broomrape causal agent in a sand-soil mixture infested 25 September 1969

Sampling interval (mo)	Infected tobacco plants from samples
0	3/6[a]
1	3/6
2	5/6
4	4/6
8	6/6
12	6/6
18	4/4
24	6/6
36	6/6
48	5/5
60	5/5

[a]Denominator represents number of seedlings planted in the infested mixture and the numerator the number that became infected.

TABLE 3. Persistence of false broomrape causal agent in refrigerated and frozen tumor tissues

Recovery of causal agent from:			
Refrigerated tissue		Frozen tissue	
Days	Infected tobacco plants	Months	Infected tobacco plants
12	6/6[a]	1.0	6/6[a]
32	4/6	2.0	6/6
59	3/4[b]	3.5	6/6
258	6/6	12.0	5/5
283	5/5	18.0	6/6
361	4/5[c]	42.0	5/5
460	2/6	54.0	6/6
713	0/6		

[a]Denominator, bioassay plants inoculated; numerator, plants infected.
[b]Bioassay plants were tobacco cultivar NC-2326.
[c]Bioassay plants were tobacco cultivar McNair 12. The inoculum was from naturally infected tobacco.

The tobacco plants inoculated with FBR and drenched twice a week with solutions of chlortetracycline hydrochloride and tetracycline hydrochloride developed normal tumors on the roots of 7 of 8 and 6 of 8 treated plants, respectively.

The scions from tumorous plants grafted to healthy plants grew into normal plants with inflorescences as did the tumorous plants from which they were excised. None of the root systems of grafted plants developed tumors, but tumors were present on the roots of the 5 plants derived from tumors. As was previously demonstrated (5) the FBR agent was not transmitted from the scion.

The FBR causal agent persisted in the soil-sand mixture 60 mo (Table 2). Only three to five of the six inoculated plants became infected during the first 4 mo of testing and the number of tumors per root system ranged from one to six. All plants became infected at subsequent test intervals, the infections from the 12 to 36-mo samples were numerous, and individual infections were difficult to distinguish. The infections per root system were more numerous on plants inoculated from the 48- and 60-mo samples than from samples taken the first 4 mo. The 60-mo sample exhausted the supply of infested sand-soil mixture, but the amount of infection obtained from this sample indicated that the causal agent would have persisted longer under the conditions of this test.

The persistence of the FBR causal agent in refrigerated tissue exceeded 1 yr and good infection was obtained with inoculum prepared from tissues stored 361 days (Table 3). With inocula prepared from tissues after 460 days of refrigeration, two of six plants became infected and there were only a few infections on these plants. No infection was obtained from tumors refrigerated 713 days. The loss of infectivity was associated with the natural decomposition of the tumors. After several months of storage the normally white tumors changed to shades of brown and ultimately black with a progressive accumulation of liquid in the container.

Persistence of the causal agent in frozen tissue was at a high level over the 54-mo period (Table 3). The level of infection obtained from tumors frozen 54 mo indicated that the agent might survive much longer at −20 C.

DISCUSSION

The removal of the FBR causal agent from suspension by a 0.45 μm filter, its failure to be transmitted by grafting, and its sedimentation by a time-gravity force insufficient to sediment TMV indicates that the agent is not a virus or mycoplasma. The failure to prevent tumor (symptom) development with chlortetracycline hydrochloride and tetracycline hydrochloride also suggests it is not a mycoplasma sensitive to these antibiotics. The responses of the FBR agent and *E. carotovora* to the physical and chemical treatments support the hypothesis that the FBR causal agent is a microorganism or associated with a microorganism similar to some bacteria.

The persistence of the FBR agent in the infested sand-soil mixture for 5 yr confirms the observation on its soil-borne nature. The decomposition of tumors formed on tobacco roots in the field would renew soil infesting inoculum for the following and some succeeding years.

Evidence has been presented that the development of tumors on tobacco root systems is associated with an imbalance of the cytokinin-auxin ratio within the plant (3). It is doubtful that cytokinins from the original tumor tissues used to infest the sand-soil mixture would have persisted for 5 yr and provided the imbalance of the cytokinin-auxin ratio associated with false broomrape plants (3). Owning to the physical and chemical properties associated with the FBR causal agent, it seems more plausible that microorganism propagules capable of synthesizing, or stimulating the plant to synthesize cytokinins, survived in the sand-soil mixture.

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