PATHOLOGY

Identification of *Ceratocystis ulmi*, Based on Production of Coremia in Vials.—Culturing large numbers of samples suspected of containing *Ceratocystis ulmi* (Buisman) C. Moreau, the causal fungus of Dutch elm disease, is time-consuming. The surface sterilized twig sections are pealed, then small wood chips are aseptically removed and transferred to petri dishes containing potato dextrose agar. Plant protection instructions (unpublished, late 1940's) call for the incubation of 15 chips from each of six twigs in a sample. About 10 years ago, at our laboratory, we reduced the number of chips to five per twig but even with this reduction it takes 20 to 30 minutes to culture each sample, depending on how easily the bark can be removed. Also, some training in aseptic techniques is required because this work is usually performed by summer assistants and they change almost yearly.

C. ulmi produces coremia in moist chambers on infected pieces of wood (R. Campana, University of Maine, Orono, Maine, personal communications) and we found this to be true also, on infected, debarked pieces of twigs. After some experimentation the following method was chosen and tested for reliability before being adopted as a routine laboratory technique for determining *C. ulmi* from samples suspected of Dutch elm disease.

A twig selected from a sample is surface sterilized by dipping in 70% alcohol which is removed by flaming. After the aseptic removal of the bark, the end 2–3 cm section of the twig is cut off to remove the portion of the twig into which alcohol may have penetrated. The cut is made at about 45° to give a larger cut surface. The next cut is made about 3–4 cm from the first and the section is put into a screw-cap vial (about 20 ml capacity, 20 x 50 mm) about half-filled with sterile distilled water. The closed vial is shaken vigorously for about 30 seconds, then the water is aseptically decanted, the cap loosely replaced, and the "moisture chamber" incubated at room temperature either in light or darkness. The average time for sample preparation is 3 to 5 minutes.

After 4 to 5 days incubation, without removing the twig from the vial, it is examined under a dissecting microscope for the presence of coremia of *C. ulmi*, and is checked periodically. If, after 2 weeks incubation there are no coremia found, the sample which has been kept in "cold storage" during this period, is cultured by the conventional method. Sometimes I find that even when coremia are absent there is frequently enough mycelia growing on the surface of the twig to ascertain the presence and identity of *C. ulmi* from its *Cephalosporium* stage. However, by plating the "negative" vial samples, the "misses" can be picked up, true negative samples confirmed, and other fungi causing symptoms on the tree, can be identified.

The Table shows the results obtained with two groups of samples received for identification in two different years.

Only 29 of the 243 samples required culturing on artificial medium, and in both years over 95% of the samples positive for *C. ulmi* were identified by the vital technique. In the first year, over 60% of the positive samples produced coremia within the first week of incubation.

TABLE 1
Results obtained with the vial technique for identifying Dutch elm disease

	Number of samples	
	1973	1974
Total samples tested by vial method	114	129
Coremia produced within 1 week	67	not recorded
Coremia produced within 2 weeks	106	108
C. ulmi identified by plating	5	3
Sterile, bacteria, other fungi	3	18
Efficiency of coremia determination	95.5%	97.3%

The advantages of the described technique include: (1) reduction in culturing time, resulting in up to ten-fold increase in the number of samples that can be handled by persons with minimum training in culture techniques; (2) reduction in media preparation, saving both time and culture medium; (3) reduction in the number of contaminated samples because no media other than the natural wood section is involved; (4) reduction in examination time, due to the reduction in the need for microscopic mount preparation; and (5) maintenance of the reliability of the chip plating technique.

The vial technique of *C. ulmi* determination could be carried one step further by supplying persons collecting suspect-samples with a small bottle of alcohol and tightly closed vials containing sterile water, and requesting that a vial preparation of the twig be made in the field and included with the sample. Assuming no more than 4 days in transit, the vials would reach the laboratory at the time when coremia development usually starts, thereby shortening the necessary in-laboratory incubation period and also shortening the interval between collection and identification. The latter has practical implications when control measures against *C. ulmi* are anticipated.—Laszlo P. Magasi, Maritimes Forest Research Centre, Fredericton, N.B.

Cellulolytic Enzymes Enhance Wound Response in Lodgepole Pine.—The bark beetle, *Dendroctonus ponderosae* Hopk., carries spores of blue stain fungi into the stems of lodgepole pine [*Pinus contorta* Dougl. var. *latifolia* Englm.] where it colonizes the living cells of the inner bark and sapwood. The tree responds by synthesizing resins and aromatic compounds that impregnate the tissues around the wound (Shrimpton, Can. J. Bot. 57: 527, 1973). Visible symptoms and changes at the cellular level were described by Reid *et al.* (Can. J. Bot. 45:1115, 1967). Although triggered by a physical wound, the response is enhanced by the blue stain fungi. This note presents evidence to suggest that a similar response may be triggered by enzymes of the type secreted by sapwood invading fungi.

Six lodgepole pin logs, about 25x180 cm, were cut on the Kananaskis Forest Experiment Station, Alberta, and their cut surfaces sealed immediately with wax. Loose bark scales were brushed off but living tissues were not exposed. Commercial enzyme preparations, to be injected into the logs, were pectinase – 40 mg/ml dissolved in 1/10 N phosphate buffer, pH 40; – cellulase 40 mg/ml dissolved in 1/10 N phosphate buffer, pH 4.0; hemicellulase – 40 mg/ml dissolved in 1/10 N phosphate buffer, pH 4.5. Enzyme solutions were sterilized by filtration through a 0.3 mμ millipore filter. Prior to inoculation the bark was rinsed with methanol. Holes 1.5 cm deep were punched about 30 cm from the basal end of the log with a sterilized nail. A sterilized, 1 ml syringe fitted with a needle was filled, positioned in the hole, and sealed in place with wax before the contents were injected. Each log received five treatments spaced equally around the circumference: one each of the three enzymes, one of sterilized water and one control, the nail hole alone.

Wounds were examined after 18 days. A chip of wood, removed asceptically from sapwood adjacent to the hole, was placed on water agar to check for microorganisms. The appearance of wood and phloem affected by the wound and the extent of resin soaking was noted. Criteria of Reid *et al.* (*op. cit.*) were used. Treatments in which microorganisms were found were not included in results.

The changes around each wound were of the same shape and general appearance as those observed in wounded, living trees (Reid *et al.*, *op. cit.*). A white streak extended vertically

for the length of the log, and resin had accumulated in all holes. Each hole was surrounded by an elliptical zone of resin-soaked tissue that extended through the phloem to a few annual rings into the sapwood. The size of the resin-soaked zone, measured in the sapwood, varied within each treatment from log to log, but was consistently larger for enzyme treatments than for controls. Sizes are given in Table 1. There was no difference in appearance and little in the size of the resin-soaked zone for the pectinase, cellulase or hemicellulase treatments.

TABLE 1
Sizes of the resin soaked area from a physical wound and from cellulolytic enzymes free of microorganisms

Treatment	Hole only	Water	Pectinase	Cellulase	Hemicellulase
Number	3	2	2	4	3
Sizes of resin-soaked area	9x6 5x4 4x5	8x5 5x5	79x5 31x5	41x7 48x6 36x7 52x9	78x6 70x5 35x8
Average Size*	6x5	7x5	55x5	44x7	44x6

* Length x width in mm.

The enzymes used in this investigation have at least two effects upon the sapwood. First, the walls of the ray cells are dissolved and, because protoplasts of these cellss are interconnected, this damage will be sensed by neighboring cells. Second, the bordered pits are damaged by the enzymes (Meyer, Wood Sci. 6:220, 1974), which alters the local moisture regime. Previous work on the response of lodgepole pine to wounding has shown that the resin-soaked zone arising from wounds involving blue stain fungi is much larger than that from a mechanically inflicted wound from which fungi were excluded. Also, a zone of dry sapwood usually develops behind open wounds and resin soaking occurs within this zone (Reid *et al.*, *op. cit.*). These results suggest that the development of resin soaking in lodgepole pine in response to a wound is promoted either by the action of fungi as they invade ray cells or by a changing moisture regime. Because moisture movement is minimal in a log, disruption of ray cells seems more probable.—D. M. Shirmpton, Pacific Forest Research Centre, Victoria, B.C.

SILVICULTURE

Influence of Bacterial Inoculations on Growth of Containerized Douglas-fir Seedlings.—Early work with bacterial inoculations of *Azotobacter* sp. or *Bacillus megaterium* var. *phosphaticum* to stimulate growth of agricultural and vegetable crops in field and pot experiments has had limited success (Brown *et al.*, Plant Soil, 20:194-214, 1964). With arboreal plants, there is only one report of a stimulatory influence, *i.e.* when oak and ash grown in sand culture were inoculated with *Azotobacter chroococcum* (Akhromeiko and Shestakova, International Conference on the Peaceful Uses of Atomic Energy, 2nd Geneva. 1958. pp 193-199).

In a nutritional trial with container-grown Douglas-fir in 1972, we superimposed a mixed bacterial inoculation upon the nutrient treatments. A coastal Douglas-fir seedlot (B.C. Forest Service #315), from 460 m elevation that had been stratified prior to seeding, was sown in "Styroblock 2" containers (Matthews, Can. For. Serv. Info. Rep. BC-X-58, 1971) on 25 April 1972. The soil was a 3:1 (v/v) unsterilized peat vermiculite mix adjusted to pH 4.8 with 3 kg dolomite lime per m^3. Nutrient solutions of 28:14:14 (Plant Products Ltd., Port Credit, Ont.), at a concentration of 156 mg/liter, were applied to field capacity twice weekly throughout the experimental period, with extra watering as required. Half the plants were leached with additional twice-weekly watering to field capacity and then rewatering until water flowed freely through the soil volume.

The bacterial inoculum was prepared by collected 10 5-cm soil cores from a 28-year-old Douglas-fir stand (elevation 330 m) and thoroughly mixing them before removing a 5 g subsample and placing it in 1 liter of medium. The medium had the following composition: K_2HPO_4, glucose, avicel (TG104), and soluble starch at 0.1% concentration; $MgSO_4 \cdot 7H_2O$, 0.22%; $CaCl_2$ and NaCl, 0.01%; $FeCl_3$, 0.001% and actidione at 60 ppm. The actidione was incorporated to prevent fungal development in the inoculum. This solution was incubated on an orbital shaker (200 rpm) for 4 days at 13-15°C, and 10 ml was removed and inoculated to fresh medium. The subsampling and re-inoculation was repeated three times before 2 ml of the mixture was inoculated to each seedling cavity on 18 May 1972. Uninoculated blocks were similarly treated with 2 ml of the heat sterilized mixture. The plants were kept in a shadehouse until sampled in March 1973. Measurements of a 20-plant sample from each quarter block included stem diameter (at cotyledons); height (from cotyledons to tip +2.5 cm); total dry weight (120 hr at 70°C), and concentration of nitrogen, phosphorous, potassium, iron, copper, zinc, boron, manganese, calcium and magnesium in the whole plant.

Table 1 demonstrates the difference in plant size between control and leached treatments. As expected, the leached treatment, which provided a lower nutrient supply, produced smaller plants. This difference in nutrient supply is, however, not reflected in the plant analysis, where the concentration of all elements monitored is greater in leached than in control treatments. This would suggest that an element, not measured but very mobile in solution, may be the limiting factor (*i.e.* sulfur). Another possibility is that the higher copper content in the leached plants have lead to a copper-induced iron deficiency (Dykeman and de Sousa, Can. J. Bot. 44: 971-978, 1966), thus reducing growth.

TABLE 1
Modifying influences of bacterial inoculation of seedling growth and nutrient content

Factors	Control		Leached	
	Uninoculated	Inoculated[1]	Uninoculated	Inoculated[1]
Stem Diameter (mm)	2.5	104	1.9	110*
Height (cm)	12.6	102	7.3	122*
Whole Plant Dry Weight (g)	1.349	111	0.721	127
Nutrient Concentrations				
Nitrogen %	1.10	116*	1.37	70*
Potassium %	.44	98	.48	116*
Magnesium %	.18	95	.24	110*
Copper (ppm)	17.3	110*	35.2	109*
Iron (ppm)	61.9	110	67.5	132*
Zinc (ppm)	26.2	69*	44.5	69*
Manganese (ppm)	83.8	112	125	65*
Boron (ppm)	23.8	106	29.0	83*
Calcium (%)	.28	104	.40	69*
Phosphorous (%)	.35	95	.34	76*

* Significantly different from the uninoculated ($P = 0.05$)
[1] Expressed as a % of uninoculated plants.

Visual examination of plants, 5 weeks after bacterial inoculation, indicated that inoculated plants were slightly smaller and weakly chlorotic. This was gradually overcome and, in 13 weeks, the inoculated plants were visibly larger. Table 1 indicates the positive bacterial inoculation influence on growth. There is a significant increase in stem diameter of plants grown under both nutritional regimes and a significant increase in height with the leached treatment. In addition, there were increases of 11 and 27% in the dry weight of plants of the two treatments, respectively, as a result of bacterial inoculation. This difference in growth is not mycor-