INFECTION OF *PINUS RADIATA* BY *DOTHISTROMA PINI*: EFFECT OF BUFFER CAPACITY OF NEEDLE HOMOGENATES

R. A. FRANICH and L. G. WELLS
Forest Research Institute, New Zealand Forest Service, Rotorua

(Received for publication 10 August 1976)

ABSTRACT

The buffer capacity measured at pH 6.2 of aqueous homogenates of 1-year-old needles from **Pinus radiata** D. Don trees aged 5, 10, 15, 20, and 40 years increased with tree maturity. **Dothistroma pini** Hulb. cultured on needle-extract agar did not show any tendency to optimise the pH of media with varying hydrogen ion concentration. The high buffer capacity of mature-tree needle homogenates does not appear to be a property directly related to mature-tree resistance to **D. pini.**

INTRODUCTION

Mature *Pinus radiata* D. Don trees growing in New Zealand show a significant degree of resistance to the needle-cast pathogen *Dothistroma pini* Hulb. While rainfall and other environmental factors can influence the disease intensity, it has been observed that the onset of resistance to *D. pini* occurs when trees are about 20 years old (Gilmour, 1967). The nature of this resistance is believed to be a property of the whole tree (Gibson, 1972) and, since completely resistant individuals have never been found, it appears that the resistance corresponds to the "horizontal" type as defined by Plank (1969). Trials have shown that the resistance character can be transferred by grafting (García and Kummerow, 1970), and by using cuttings taken from mature trees (Barnes, 1970).

Investigation into the mechanism of resistance of *Pinus sylvestris* L. to the needle-cast pathogen *Lophodermium pinastri* (Schrad. ex Hook.) Chev. showed that there was a high degree of correlation between the buffer capacity of the needle homogenate and the maintenance at a low pH during culture of the fungus on needle-extract agar. In a medium prepared using extracts of needles from susceptible trees, the pH of the medium close to the fungus colony increased to pH 5, the growth optimum for *L. pinastri*; thus the fungus was optimising its environment. On the other hand, the pH of the medium prepared from resistant-tree needle extracts remained low, about pH 4 (Scholz and Stephan, 1974).

Some studies have been made on the effect of hydrogen ion concentration on *D. pini* spore germination, mycelial growth, and spore production. Ivory (1967) found that conidia germinated over the range pH 2.2-5.5, with an optimum of pH 3.5, and

subsequent mycelial growth occurred over the range pH 2-7 with the same optimum. Rack and Butin (1973) varied the pH of malt-extract media by use of phosphate buffers to study rates of spore production. They found that mycelial growth and spore production occurred over the range pH 5.3-8.0, with a maximum at pH 6.2.

The possibility that the buffer capacity of *P. radiata* needle homogenates could be a property contributing to the overall resistance of mature trees to *D. pini* was raised and examined. This communication describes results of titration of *P. radiata* needle homogenates, and culture of *D. pini* on needle extract agar based on the methods used by Scholz and Stephan (1974) for the *P. sylvestris-L. pinastri* interaction.

METHODS

Green, healthy foliage which was exposed to full sun was collected during summer from trees aged 5, 10, 15, 20, and 40 years (5 trees/age) which were growing on the north-facing edge of compartments having similar soil types in Kaingaroa State Forest. Foliage was sampled from 5-year-old trees at heights 1-2 m above ground, from 10- and 15-year-old trees at 2-4 m, and from the 20- and 40-year-old trees at 4-6 m above ground. Fresh 1-year-old needles (10 g) were homogenised (Polytron) in 100 ml distilled water (with cooling) for 30 s. Duplicate homogenates were prepared, and the mixture was filtered with suction through a Whatman GFC filter. Samples (20 ml) of the clear solutions obtained were titrated with N/20 sodium hydroxide. The neutralisation curve was recorded on a Metrohm Potentiograph. A tangent to the curve was drawn at pH 6.2, the growth optimum for *D. pini* (Rack and Butin, 1973), and the slope, i.e., the angle subtended by the tangent and the line at pH 6.2 parallel to the abscissa, was measured with a protractor. The buffer capacity at pH 6.2 is given by the cotangent of this angle. The area bounded by the neutralisation curve, the ordinate, and the line at pH 6.2 was determined gravimetrically. This area is a measure of the buffer capacity of the solution expressed as integration units, described by Scholz and Stephan (1974).

For culture of *D. pini* on needle extract agar, 500 g of 1-year-old needles from the same sample as above were homogenised in water (1 litre) and filtered through Whatman GFC; the solution was then freeze-dried to give a brown solid which was redissolved in distilled water to give a 10% w/v solution. This was sterilised by filtration through a 0.2 μm membrane filter. Petri dishes were poured by adding autoclaved agar to the sterile needle extract solution to give media containing 10, 5, 1, and 0.5% nutrient and 1% agar. Drops (0.05-ml) of a sterile *D. pini* spore suspension (*c.* 10^6 spores/ml) were placed on the medium, and the Petri dishes were incubated at 18°C for 20 days. The pH of the medium at the edge of the Petri dish and at the edge of the *D. pini* colony was determined using a single-probe electrode. The diameters of the fungus colonies were measured, and the weight of mycelium per colony was estimated by a spectrophotometric hexosamine analysis (Ralph, Franich, and Wells, 1976).

RESULTS

Buffer Capacity Measurement

When needle aqueous extract was titrated with N/20 sodium hydroxide, a sigmoid neutralisation curve was obtained. The point where the curves for the various-aged trees intercepted the line at pH 6.2 varied considerably, e.g., the "5" curves intercepted this

line after 3.0 to 6.2 ml of base had been added, while the "40" curves intercepted after 2.5 to 7.2 ml of base had been added. Similar variability for the other ages was found, and this is reflected in the areas measured under the curves. The mean area (sq. units of chart paper) and standard deviation for the various ages of tree are as follows: 5: 1029.0 ± 231.0; 10: 1040.5 ± 173.5; 15: 1040.5 ± 347.0; 20: 1011.5 ± 375.5; 40: 1185.0 ± 347.0.

The slope of the neutralisation curves at pH 6.2 did not show the large variation as did the above areas. The buffer capacity (with standard deviation) at pH 6.2, for the various tree ages, is shown in Fig. 1.

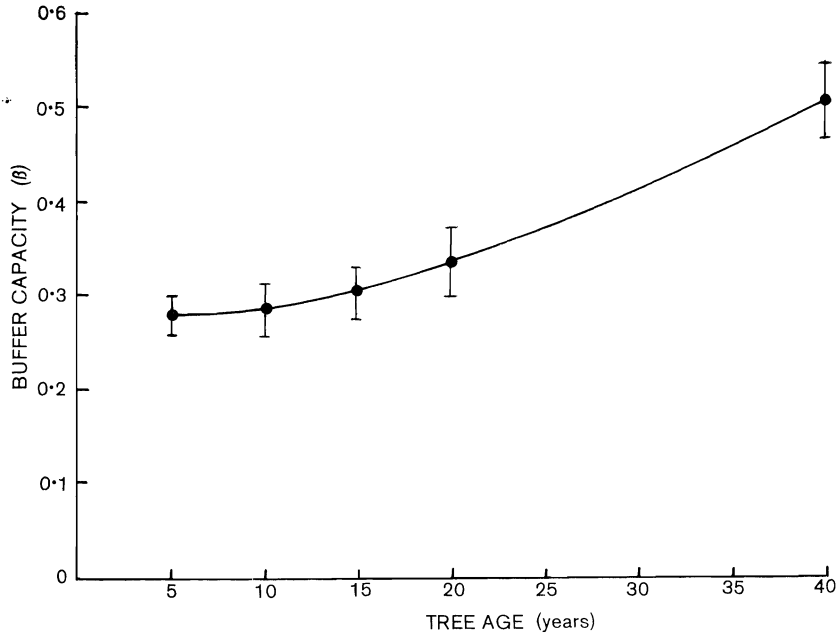

FIG. 1—Variation in needle homogenate buffer capacity, at pH 6.2, with tree age.

Dothistroma pini *Culture*

Attempts to prepare 10% needle-extract agar nutrient medium were vitiated since the medium failed to gel. *Dothistroma pini* grew slowly on 0.5% nutrient media to form a roughly circular colony (6-7 mm in diameter) having a dense black centre and a white halo of mycelium at the extremity. On water agar the colony formed a sparse white mat, sometimes with a small black dot in the centre, while on the 1% nutrient media the fungus grew to form a black mat several millimetres thick. A dark, dense convoluted mass was formed when *D. pini* grew on the 5% media. The pH values for the medium remote from and adjacent to the fungal colonies were identical, or at most showed only a 0.1 pH unit difference. Uninoculated media had identical pH values for each nutrient concentration. To compare *D. pini* growth rates on different concentrations of medium, whole colonies were separated from the agar and hydrolysed in sealed tubes

at 110°C for 15 h using 5.0 ml 4N HCl per colony. The hydrolysates were analysed for hexosamine content, the results for different tree ages and media (expressed as mg/colony) being as follows:

Tree age (years)	5	10	15	20	40
0.5% nutrient, av. pH 4.5	0.033	0.043	0.054	0.052	0.033
1.0% nutrient, av. pH 4.0	0.063	0.057	0.057	0.109	0.062
5.0% nutrient, av. pH 3.8	0.159	0.189	0.125	0.227	0.199

DISCUSSION

The ionic compounds present in aqueous extracts of *P. radiata* needles and which affect pH were shown by simple chemical tests to be carboxylic acids, phenols, amino acids, and protein. Metal ions and carbohydrates were also present in the extract, which was initially a pale amber colour. During titration of the extract with N/20 sodium hydroxide, the extract colour darkened to brown when the solution became alkaline (pH 8-9). Calculation of the buffer capacity of the needle extracts at pH 6.2 showed this to increase as the tree age increased (Fig. 1). Although the buffer capacity can be calculated for any point on the neutralisation curve, the point at pH 6.2 was chosen because this pH value gave the maximum rate of growth and spore production of *D. pini* (Rack and Butin, 1973), and it coincided fortuitously with the inflexion in the neutralisation curve. Since none of the extracts had a pH below *ca. 3.7,* the pH value (3.5) for maximum growth determined by Ivory (1967) could not be used. The buffer capacity, expressed in integration units (Scholz and Stephan, 1974), for the whole of the neutralisation curve up to pH 6.2 showed high variability, and therefore this index appeared of little value for correlation with tree age.

For the culture of *L. pinastri* on *P. sylvestris* needle-extract agar, Scholz and Stephan (1974) autoclaved a mixture of the extract with agar before pouring Petri dishes. They record a number of pH changes that occurred during the autoclaving step. When the same procedure was carried out for preparing *P. radiata* needle extract for *D. pini* culture, none of the media gelled despite the use of several grades of agar. Consequently, the media had to be prepared by the addition of autoclaved agar solution to membrane-filter sterilised needle-extract. The medium composed of 10% needle extract and 1% agar failed to gel when prepared in this manner. The lowest concentration of needle extract on which *D. pini* grew at a satisfactory rate was 0.5%. As the concentration of the extract, and therefore total acids, was increased, the pH of the medium was lowered (Results, *above*). Increase in the concentration of needle extract in the medium also resulted, as expected, in an increased rate of growth of the fungus. In no instance was there found any significant change in the pH of the medium in the vicinity of a *D. pini* colony. Although *D. pini* shows a growth response to changes in pH of the culture medium (Rack and Butin, 1973), the fungus does not appear to optimise the pH of media having varying hydrogen ion concentration, as does *L. pinastri* (Scholz and Stephan, 1974).

Since no correlation of the measured buffer capacity of *P. radiata* needle homogenates and the behaviour of *D. pini* in needle-extract agar culture was obtained, there is no simple interpretation of the role of high buffer-capacity in resistance of mature *P. radiata*

trees to *D. pini.* Since, however, the disease resistance property is believed to be associated with maturation (Gibson, 1972), the measurement of buffer capacity of needle homogenates may be able to be used empirically as a measure of tree maturity, provided that variation in other parameters such as soil type, growing site, and climate are minimal.

ACKNOWLEDGMENTS

Dr J. M. Uprichard is thanked for the use of the Metrohm Recording Potentiograph, and the technical assistance of Mr Robert Carr is acknowledged.

REFERENCES

BARNES, R. D. 1970: The prospects for re-establishing **Pinus radiata** as a commercially important species in Rhodesia. **S. Afr. Forest J. 72:** 17-9.

GARCIA, J. and KUMMEROW, J. 1970: Infection of Monterey pine graftings with **Dothistroma pini. Plant Dis. Reptr. 54:** 403-04.

GIBSON, I. A. S. 1972: **Dothistroma** blight of **Pinus radiata. Ann. rev. Phytopathol. 10:** 51-72.

GILMOUR, J. W. 1967: Host list for **Dothistroma pini** in New Zealand. **N.Z. For. Serv., For. Res. Inst., Research Leaflet No. 16.**

IVORY, M. H. 1967: Spore germination and growth in culture of **Dothistroma pini** var **keniensis. Trans. Brit. Mycol. Soc. 50:** 563-72.

PLANK, J. E. van der 1969: Pathogenic races, host resistance, and an analysis of pathogenicity. **Neth. J. Plant Pathol. 75:** 45-52.

RACK, K. and BUTIN, H. 1973: A quick method for the production of **Dothistroma pini** spores in culture. **Eur. J. For. Path. 3:** 201-9.

RALPH, J., FRANICH, R. A. and WELLS, L. G. 1976: Quantitative estimation of fungal cell wall material by spectrophotometric hexosamine analysis. **N.Z. For. Serv., For. Res. Inst., For. Path. Int. Rep. No. 43** (unpubl.).

SCHOLZ, F. and STEPHAN, B. R. 1974: Physiologische Untersuchungen über die unterschiedliche Resistenz von **Pinus sylvestris** gegen **Lophodermium pinastri.** 1. Die Pufferkapazität in Nadeln. **Eur. J. For. Path. 4:** 118-26.