PHENOLOXIDASE AND PEROXIDASE ACTIVITIES IN ZONE LINES OF *PHELLINUS WEIRII*

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SUMMARY

Phenoloxidase and peroxidase were extracted from pigmented zones (zone lines) which were formed on agar media between two *Phellinus weirii* isolates or by one *P. weirii* isolate in response to the presence of an antagonist. Activity of enzymes from zone lines was compared with that of enzymes from mycelial tissues adjacent to the zone lines by isoelectric focusing on polyacrylamide gels. Zone lines produced stronger phenoloxidase and peroxidase reactions than did adjacent tissues. At least three phenoloxidases and as many as six peroxidase isozymes were detected in zone lines, some of which may have been induced or caused by the antagonists. Phenoloxidase and peroxidase were observed also in the zone lines formed by *P. weirii* in colonized Douglas-fir wood, but not in adjacent tissues.

Phellinus weirii (Murr.) Gilbertson is a destructive root pathogen of conifers in northwestern United States and southwestern Canada. In cultures, confronted isolates of this fungus from separate infection centers develop a darkly pigmented zone line at their interface. The zone line is a distinct narrow dark line, representing a sheet of fungal mycelium composed of characteristic swollen hyphal cells. Childs (**1963**), who first recorded the phenomenon in *P. weirii,* used the term 'line of demarcation' and called the paired isolates forming these lines "clones" of the species. Pigmented zone lines also can be formed by *P. weirii* on agar media or in *P. weirii*-colonized wood in response to some antagonistic microorganisms. Zone lines appear to enhance *P. weirii* survival in colonized wood by excluding antagonists (Nelson, **1964, 1975**).

Kuwana (**1958**) found that cell fusion between different strains of *Neurospora crassa* Shear and Dodge that normally do not produce phenoloxidases leads to induced phenoloxidase activity and pigment formation. Hiroth (**1965**) reported that interactions at the interface between different isolates of *Phellinus tremulae* (Bond.) Bond. et Boriss, resulted in induction of weak phenoloxidase activity and pigment

formation. Extracellular activities of phenoloxidases and peroxidases in *P. weirii* have been reported (Li *et al.*, **1968**; Koenigs, **1972**). The following study was undertaken to investigate the oxidase isozymes in zone lines formed by *P. weirii* in response to an antagonistic bacterium, an unidentified fungus, or another isolate of *P. weirii*.

MATERIALS AND METHODS

Microorganisms used in the study.—Phellinus weirii T-124, is a relatively fast-growing isolate from Douglas-fir [*Pseudotsuga menziesii* (Mirb.) Franco] near Quilcene, Washington; *P. weirii* T-55, is a darker and relatively slow-growing isolate from Douglas-fir near Randall, Washington. Both isolates have been maintained in culture on identical media for more than 5 yr. The *Bacillus* sp. was isolated from soil under red alder (*Alnus rubra* Bong.) near Otis, Oregon; the unidentified fungal antagonist was isolated from soil under a cottonwood plantation near Apiary, Oregon. Both organisms were antagonistic toward *P. weirii*, producing clear zones devoid of fungal growth between *P. weirii* and the antagonist. *Phellinus weirii* formed darkly pigmented zone lines at the edges of its colony in response to the antagonism.

Preparation of enzyme extracts.—Phellinus weirii T-124 and T-55 were each placed on 10% malt extract in water at opposite sides of 500 ml Erlenmeyer flasks or on malt agar (Difco)[1] in Petri dishes at room temperature (22–24 C) for 3–5 wk. After zone lines formed between the cultures, mycelia 1–2 mm away from the zone line and from zone lines themselves were separately homogenized by a Polytron with a saw-tooth generator, 165 × 12 mm, at maximum speed for 1 min in 50 ml of 0.1 M phosphate buffer, pH 6.5, containing 0.05% Triton X-100 and 1% Amberlite XAD-4 (Millinkrodt Chemical Works, St. Louis, Missouri). The resulting extract was filtered through a nylon mesh, 36.5 mesh count/cm, and centrifuged with a Sorvall RC-2 centrifuge at 30,000 × *g* at 2 C for 30 minutes. Four-tenths gram of moist Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, California) were added to the supernatant to remove Triton X-100 (Holloway, **1973**). The final supernatant was dialyzed against deionized water with continuous stirring overnight at 4 C. The enzyme solution was freeze-dried and resuspended in 2 ml of 25% sucrose solution.

[1] Trade names used do not imply endorsement by the U.S. Department of Agriculture over similar products.

Similar extraction procedures were used with zone lines formed by *P. weirii* in colonized Douglas-fir wood collected near Corvallis, Oregon, and with those formed by *P. weirii* T-124 on malt agar or malt-peptone-yeast agar in response to an antagonistic fungus or *Bacillus* sp. The same procedures also were used for colonies of *P. weirii* T-124, T-55, fungal and bacterial antagonists growing alone.

Enzyme identification and assay in polyacrylamide gels.—Gels were prepared with Bio-Rad's Electrophoresis Purity monomers, 7.5% total monomer with 2.5% of this as methylene-*bis*-acrylamide. Each gel also contained 5% (w/v) glycerol and 2% (w/v) Bio-Lyte 3/10 ampholyte. The gels were photopolymerized using riboflavin 5′-phosphate, 8.4 micromolar (μM) and focused at 4 C (Righetti and Drysdale, **1974**).

The focused total proteins in the gels were stained by the procedures of Righetti and Drysdale (**1974**) and scanned with a Helena 'Auto Scanner'. The bands of phenoloxidase were identified by flooding the gels with 0.1 M acetate buffer, pH 5.0, containing one of the following substrates: 2.5 millimolar (mM) DL-β-3,4-dihydroxyphenylalanine (DOPA), 0.16 mM syringaldazine, 1.3 mM *o*-dianisidine, 2.5 mM *p*-cresol, or 2.5 mM L-tyrosine. Relative color intensity of the enzyme bands reacting with different substrates was graded on a 0–4 scale (Hiroth, **1965**; Yamamoto *et al.*, **1978**). Scale 4 denoted bands with the greatest color intensity; scale 0 denoted that no colored bands were observed. Scales 1, 2, and 3 indicated intermediate gradations of color intensity.

For peroxidase isozymes, the gels were placed for 10 min in 0.1 M acetate buffer, pH 5.0, containing 1 mM 3-amino-9-ethylcarbazole, and then for 10 min in the same substrate solution plus 0.01% H_2O_2 (Graham *et al.*, **1964**).

All experiments were repeated three to four times.

RESULTS AND DISCUSSION

T-124 and T-55 are two distinctly different *P. weirii* isolates as revealed by their mycelial protein patterns (Figs. 1, 2). Patterns were not altered with colony age over the 3- to 5-wk period tested. In the pH range of 3-10 carrier ampholytes, 15 protein bands were found on the gel with isoelectric points between 3.5 and 7.4. Bands of diminishing intensity were observed for *P. weirii* T-124 between pH 5.7 and 7, and no bands were obtained at pH 6.5. The entire protein patterns for T-55, however, were otherwise distributed over a range of 3.5–7.4. These differences between isolates indicate unlike genotypes (clones) which

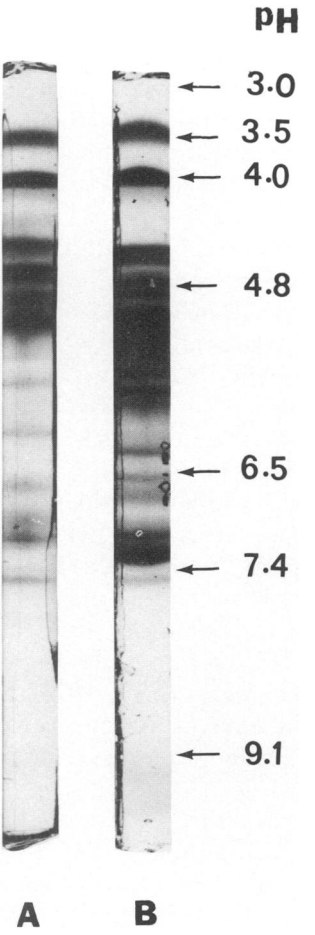

FIG. 1. Protein patterns of mycelial extracts of *P. weirii* isolates. A: *P. weirii* T-124; B: *P. weirii* T-55.

have been assumed to be responsible for the zone line reaction that occurs.

Tests of extracts from *P. weirii* isolates growing alone, from zone lines formed between these two isolates, and from mycelial tissue adjacent to the zone lines with each one of five phenolic substrates revealed two common phenoloxidase bands. One band on the gel at pH 3.5 was made visible with either *o*-dianisidine or syringaldazine, indicating two different enzymes; another band on the gel at pH 4 was made visible only with DOPA. Bands at pH 3.5 or 4 did not react with

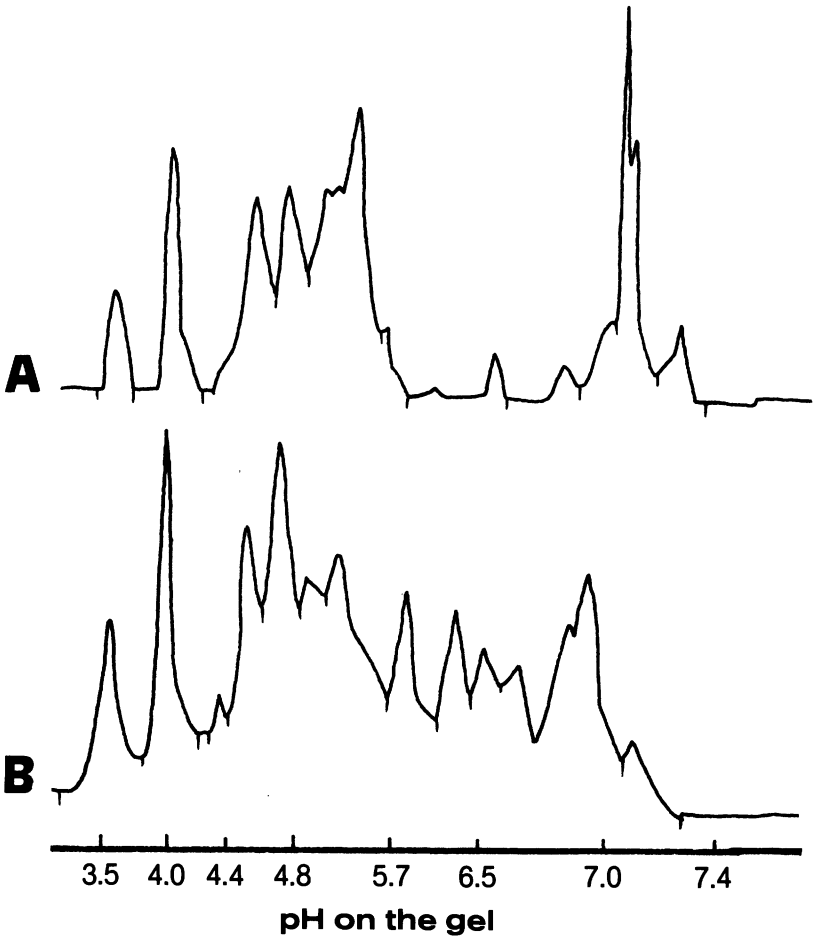

FIG. 2. Densitometric traces of coomassie brilliant blue R-250-stained protein patterns from two isolates of *P. weirii*. A: *P. weirii* T-124; B: *P. weirii* T-55. Tracings were made with a Helena 'Auto Scanner', and position of each band was marked with two neighboring vertical lines.

tyrosine or *p*-cresol (TABLE I). Thus, a minimum of three phenoloxidases, occurring on gels at pH 3.5 and 4 was detected in zone lines formed between two *P. weirii* isolates and in mycelial tissues of either isolate adjacent to the zone lines. The dark zone lines, however, always showed stronger phenoloxidase reactions with *o*-dianisidine and DOPA than the adjacent mycelial tissues or mycelial tissues of *P. weirii* isolates growing alone.

TABLE I

PHENOLOXIDASE ACTIVITIES OF ZONE LINES FORMED BETWEEN *Phellinus weirii* T-124 AND *P. weirii* T-55 ON MALT EXTRACT IN WATER

Culture	Relative staining intensity of bands reacting with[a]			
	p-cresol	*o*-dianisidine	DOPA	Syringaldazine
Zone lines	0	4	4	2
P. weirii T-55 growing alone or adjacent to zone lines	0	2	2	2
P. weirii T-124 growing alone or adjacent to zone lines	0	1	1	1

[a] 0 = no band, 1 = faint intensity, 2 = light intensity, 3 = moderate intensity, 4 = highest intensity.

The pigmented zone lines formed by *P. weirii* isolate T-124 on agar medium in response to the presence of an antagonistic fungus also produced stronger phenoloxidase reactions with *o*-dianisidine and DOPA than did adjacent tissues (TABLE II). Similarly, phenoloxidases were observed in zone lines formed in colonized wood, while the surrounding tissues adjacent to them lacked detectable enzyme activity (TABLE III). Stronger phenoloxidase reactions with *p*-cresol and *o*-dianisidine, in addition to reactions with DOPA and syringaldazine, occurred in zone lines of *P. weirii* T-124 formed on malt-pep-

TABLE II

PHENOLOXIDASE ACTIVITIES OF ZONE LINES FORMED BY *Phellinus weirii* T-124 IN THE PRESENCE OF AN ANTAGONISTIC FUNGUS ON MALT AGAR

Culture	Relative staining intensity of bands reacting with[a]			
	p-cresol	*o*-dianisidine	DOPA	Syringaldazine
Zone lines of *P. weirii* T-124	0	4	2	1
P. weirii T-124 growing alone or adjacent to zone lines	0	1	0	0
Antagonistic fungus growing alone or adjacent to zone lines	0	1	1	1

[a] 0 = no band, 1 = faint intensity, 2 = light intensity, 3 = moderate intensity, 4 = highest intensity.

TABLE III

PHENOLOXIDASE ACTIVITIES OF ZONE LINES FORMED BY *Phellinus weirii* IN DOUGLAS-FIR STEM WOOD

Culture	Relative staining intensity of bands reacting with[a]			
	p-cresol	*o*-dianisidine	DOPA	Syringaldazine
Zone line tissues	0	2	1	1
Tissues adjacent to the wild zone lines	0	0	0	0

[a] 0 = no band, 1 = faint intensity, 2 = light intensity.

tone-yeast agar in response to the presence of the *Bacillus* isolate (TABLE IV). The *Bacillus* isolate growing alone or opposite to zone lines of *P. weirii* on this agar medium did not contain detectable phenoloxidases as tested.

The same enzyme extracts used in the procedure for phenoloxidases were tested for peroxidase activity. No fewer than six different peroxidase bands were identified (Figs. 3, 4). Two were apparently stimulated by the bacterium, and one by the antagonistic fungus. Peroxidase isozymes from zone lines, in most cases, had stronger enzyme activity than those from adjacent tissues of *P. weirii* or from the antagonists. A peroxidase with an isoelectric point between pH 6.5–7.4 was common to all extracts except those from wood tissues adjacent to

TABLE IV

EFFECT OF THE PRESENCE OF ANTAGONISTIC *Bacillus* SP. ON THE PHENOLOXIDASE ACTIVITIES IN ZONE LINES FORMED BY *Phellinus weirii* T-124 ON MALT-PEPTONE-YEAST AGAR

Culture	Relative staining intensity of bands reacting with[a]			
	p-cresol	*o*-dianisidine	DOPA	Syringaldazine
Zone lines of *P. weirii* T-124	3	4	1	2
P. weirii T-124 growing alone or adjacent to zone lines	1	2	1	2
Bacillus growing alone or opposite zone lines	0	0	0	0

[a] 0 = no band, 1 = faint intensity, 2 = light intensity, 3 = moderate intensity, 4 = highest intensity.

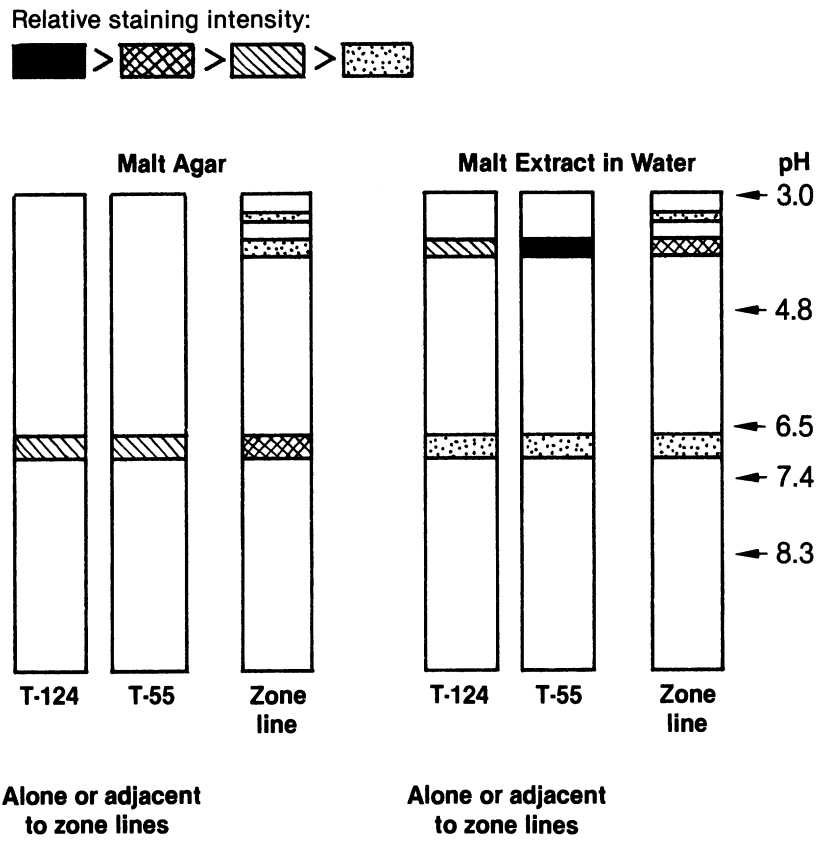

FIG. 3. Peroxidase isozymes of zone lines formed between *P. weirii* T-124 and *P. weirii* T-55.

zone lines. The results indicate that interactions between *P. weirii* isolates or between *P. weirii* and antagonistic microorganisms elevated peroxidase activity at the zone lines. These studies also showed that *P. weirii* isolates or the fungal antagonist growing alone had relatively the same levels of phenoloxidase and peroxidase activities as those of mycelial tissues adjacent to zone lines. *Bacillus* growing alone or opposite to zone lines of *P. weirii* T-124 contained no phenoloxidases that reacted with one of the five phenolic substrates tested (TABLE III).

Browning of fungal tissues resulting from enzymatic oxidation of phenolic substrates is commonly observed (Collins *et al.*, **1963**). The enzymes catalyzing the oxidation are phenoloxidase and peroxidase. The initial products of oxidation are usually *o*-quinones. Being

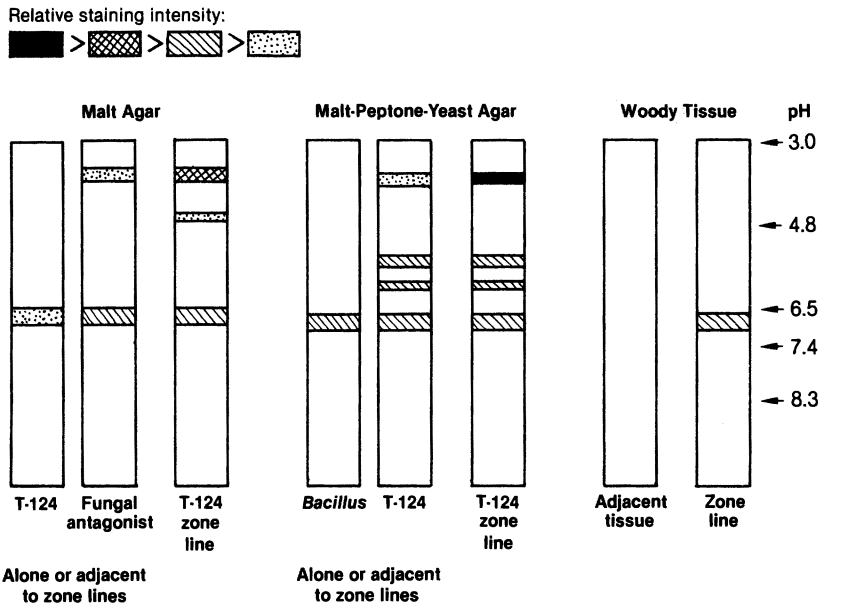

FIG. 4. Peroxidase isozymes of zone lines formed by *P. weirii* T-124 in response to antagonistic fungus or *Bacillus* sp. and of zone lines in *P. weirii*-colonized stemwood of Douglas-fir.

highly unstable, they undergo polymerization to yield brown or black melanin pigments of high molecular weight. Lockwood (**1960**), Linderman and Toussoun (**1966**), Potgieter and Alexander (**1966**), Bloomfield and Alexander (**1967**), Kuo and Alexander (**1967**), and Bull (**1970**) have demonstrated the relationship between the presence of melanin, or melanin-like pigments in fungi and resistance to microbial lysis. Sclerotia of *Sclerotium rolfsii* Sacc. and some other soil-borne fungi are known to persist outside a host for long periods in the presence of a heterogeneous microflora. The resistance of the fungal structures is correlated with the presence in the external surface of melanin or melanin-like materials that resist microbial attack. Bull (**1970**) reported that melanins can function as a physical barrier, preventing access by cell wall-degrading enzymes of other organisms.

Similarly, pigmented zone lines formed by *P. weirii* in response to unfavorable environmental conditions may be responsible for the prolonged survial of the fungus in roots and stumps. The extracted pigments gave positive reactions for phenols by producing greenish-blue color with $FeCl_3/Fe(CN)_3$ (Barton *et al.*, **1952**). Although the evidence indicates a correlation between the presence of melanin-like pigment

and resistance to advance by antagonists, no direct test of the hypothesis has been made. This study, however, has shown that increased phenoloxidase and peroxidase activities, which can lead to formation of melanin-like pigment, is stimulated by the presence of antagonists, and that these activities are generally higher in zone lines than in adjacent mycelium. Studies are in progress on the chemical and biological nature of melanin-like pigments produced in zone lines by *P. weirii*.

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LITERATURE CITED

Barton, G. M., R. S. Evans and J. A. F. Gardner. 1952. Paper chromatography of phenolic substances. *Nature* **170**: 249-251.

Bloomfield, B. J., and M. Alexander. 1967. Melanins and resistance of fungi to lysis. *J. Bacteriol.* **93**: 1276-1280.

Bull, A. T. 1970. Inhibition of polysaccharases by melanin: Enzyme inhibition in relation to mycolysis. *Arch. Biochem. Biophys.* **137**: 345-356.

Childs, T. W. 1963. *Poria weirii* root rot. *Phytopathology* **53**: 1124-1127.

Collins, R. P., L. B. Warner, and L. Paige. 1963. The tyrosinase activity of *Strobilomyces strobilaceus*. *Mycologia* **55**: 764-774.

Graham, R. C., U. Ludholm, and M. J. Karnovsky. 1964. Cytochemical demonstration of peroxidase activity with 3-amino-9-ethylcarbazole. *J. Histochem. & Cytochem.* **13**: 150-152.

Hiroth, J. 1965. The phenoloxidase and peroxidase activities of two culture types of *Phellinus tremulae* (Bond.) Bond. et Boriss. *Norwegian Forest Res. Inst.* **20**: 255-272.

Holloway, P. W. 1973. A simple procedure for removal of Triton X-100 from protein samples. *Analytical Biochem.* **53**: 304-308.

Koenigs, J. W. 1972. *Poria weirii* as a possible commercial source of peroxidase. *Appl. Microbiol.* **23**: 835-836.

Kuo, M. J., and M. Alexander. 1967. Inhibition of the lysis of fungi by melanins. *J. Bacteriol.* **94**: 624-629.

Kuwana, J. 1958. Melanization in the mycelium due to the interaction of two strains of *Neurospora crassa*. *Bot. Mag. (Tokyo)* **71**: 270-274.

Li, C. Y., K. C. Lu, J. M. Trappe, and W. B. Bollen. 1968. Enzyme systems of red alder and Douglas-fir in relation to infection by *Poria weirii*. Pp. 241-250. *In: Biology of Alder*. Eds., J. M. Trappe, J. F. Franklin, R. F. Tarrant, and G. M. Hansen. Pacific Northwest Forest and Range Exp. Station. Portland, Oregon.

Linderman, R. G., and T. A. Toussoun. 1966. Behavior of albino chlamydospores of *Thielaviopsis basicola*. *Phytopathology* **56**: 887 (Abstract)

Lockwood, J. L. 1960. Lysis of mycelium of plant pathogenic fungi by natural soil. *Phytopathology* **50**: 787-789.

Nelson, E. E. 1964. Some probable relationships of soil fungi and zone lines to survival of *Poria weirii* in buried wood blocks. *Phytopathology* **54**: 120–121.

——. 1975. Survival of *Poria weirii* in wood buried in urea-amended forest soil. *Phytopathology* **65**: 501–502.

Potgieter, H. J., and M. Alexander. 1966. Susceptibility and resistance of several fungi to microbial lysis. *J. Bacteriol.* **91**: 1526–1532.

Righetti, P. G., and J. W. Drysdale. 1974. Isoelectric focusing in gels. *J. Chromatogr.* **98**: 272–321.

Yamamoto, H., H. Hokin, and T. Tani. 1978. Peroxidase and polyphenoloxidase in relation to the crown rust resistance of oat leaves. *Phytopathol. Z.* **91**: 193–202.

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