PECTINASE ACTIVITY OF NECTRIA COCCINEA (PERS ex FRIES) FRIES IN RELATION TO BEECH BARK DISEASE[1]

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Abstract.--The pectinase activity of Nectria coccinea was studied in vitro and in vivo in relation to the pectinases of Cryptococcus fagisuga and the nature of the bark. Any pectinases necessary for degradation of pectic material were secreted by the fungus in vitro. Some pectinases produced by the insect are of great significance in the predisposition of the bark to alteration by the fungus. The assistance given by the scale insect and the pectinase pool of N. coccinea which is well adapted to the trunk bark, is a possible explanation of the specificity of the association C. fagisuga/N. coccinea.

INTRODUCTION

Recent outbreaks of beech bark disease especially in Europe have been of great economic importance and have promoted a renewal of interest and research on the disease complex. Recent investigations have brought some clarification of the aetiology of various bark necroses. Ehrlich's (1934) interpretation of disease development is widely accepted and demonstrated, and N. coccinea following Cryptococcus fagisuga attack is the main cause of the dieback of beech in Europe (Parker 1974; Perrin 1974, 1977, 1979). The insect provides more than just a means of entry for Nectria (Perrin 1979, 1980; Lonsdale 1980; Kunkel 1968). Predisposing factors are an important part of the disease. Nectria coccinea has been involved with beech bark necrosis without previous infestation by Cryptococcus fagisuga (Leibundgut and Frick 1943; Perrin 1977). Various factors like nutritional deficiency and abiotic stresses lead to predisposition of the tree, favourable to Nectria induced necrosis (Lonsdale 1980). The ability of N. coccinea to destroy the bark of beech has been frequently demonstrated but has always been related to previous predisposing factors, the most common of them being Cryptococcus fagisuga. This breakdown of bark tissue must depend not only on the presence of predisposing factors but also on the ability of N. coccinea to degrade cell wall materials.

Despite our imperfect knowledge of the nature of plant cell walls, pectic substances are regarded as of primary importance because of their abundance and above all as the principal constituent of the matrix of the cell wall. Alteration of pectic substances leads to a loss of tissue coherence. With respect to beech bark degradation, pectic enzymes may be prominent among the complex of factors involved in pathogenesis. Pectic enzymes produced by various pathogens have been the subject of numerous investigations but surprisingly very few of them have been related to bark necrosis fungi.

The aim of this study was to investigate the pectinase "weapons" of N. coccinea and their involvement in the beech bark disease process particularly in relation to C. fagisuga.

MATERIALS AND METHODS

The names of pectic enzymes conform to the classification proposed by Bateman and Millar (1966). Three isolates of N. coccinea were used. All of them are mycelial strains freshly isolated from typical beech bark diseased trees in three different regions of France: strain A, Forest of Lyons (west), strain I, Forest of Amance (east), strain W, Forest of Climbach (Alsace).

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A preliminary test of most of the media traditionally used by various authors has led to a choice of the best appropriate medium composed as follows:-

KH_2PO_4 0.1%, NH_4NO_3 0.1%, $MgSO_4$ 0.05% in distilled water, pectin (1%), pectic or polygalacturonic acids (0.25%), powdered bark (2%) or glucose (0.5%) for control, were used as carbohydrate sources.

The bark used in this study was of three different types collected on the same tree: bark of the trunk infested with C. fagisuga (score 5 Parker scoring system), bark of the trunk not infested with the beech scale (score 0) and bark of the twigs, corresponding to the juvenile state of the bark. Bark was stored as dry powder at 0°C for growth substrate use, or as water extracts by freezing (-24°C) for a study of the influence of bark contents on pectinase activity of N. coccinea. Twenty ml of medium were dispensed per 125 ml flask, autoclaved, then seeded with an 8 mm disk from an actively growing malt agar colony. Cultures were incubated without shaking at 23°C. After a few days, the liquid from the 5 replicate flasks was pooled and filtered on paper. Enzyme preparations were assayed immediately.

Table 1.--Enzyme assay methods

Enzyme Substrate	Assay Method	
	Buffer	
	Sodium citrate 0.02 M pH 4.5	Tris HCl 0.05 M pH 8.5
1% Pectin sodium salt	Polymethylgalacturonase.PMG endo-PMG: viscosimetry (a)	Polymethyltranseliminase.PMTE endo-PMTE: viscosimetry (a)
	exo-PMG: D.N.S. test (optical density 575 mμ)	exo-PMTE: T.B.A. test (optical density 547 mμ)
1.2% Sodium polypectate	Polygalacturonase.PG endo-PG: viscosimetry (b)	Polygalacturonatetranseliminase.PGTE endo-PGTE: viscosimetry (b)
0.25% Poly-galacturonic acid	exo-PG: D.N.S. test (optical density 575 mμ) T.B.A. test (optical density 515 mμ)	exo-PGTE: T.B.A. test (optical density 547 mμ)

Viscosimetry with Feuske-Oswald viscosimeter for liquid viscosity.

(a) viscosity between 63-100 centipoises (b) viscosity between 16-25 centipoises

Enzyme assay methods are summarised in Table 1. Quantitative enzymatic data are expressed by the number of units of enzymatic activity per ml culture filtrate or water extract. Three assay methods were used:
1. Viscosimetric method for endo-polymethylgalacturonase (endo-PMG), endopolymethyltranseliminase (endo-PMTE), endopolygalacturonase (endo-PG) and endo-polygalacturonatetranseliminase (endo-PGTE). Units of enzymatic activity were defined by the quantity necessary for a 50% decrease of reaction mixture viscosity in 100 mins.
2. Dinitrosalicylic acid test (DNS) (Bompeix 1972) for exopolymethylgalacturonase (exo-PMG) and exopolygalacturonase (exo-PG). Units of enzymatic activity were the quantity of enzyme necessary for liberation of 1 micro-mole of galacturonic acid per minute and per ml of culture filtrate. 3. Thiobarbituric acid test (TBA) (Bugbee 1975) for exopolymethyl-transeliminase (exo-PMTE) and exopolygalact-uronatetranseliminase (exo-PGTE). Units of enzymatic activity were equal to the quantity of enzyme arbitrarily corresponding to 0.1 optical density variation for 30 minutes.

Preparation of bark extracts for in vivo measurements

In vivo assay of pectinase activity was made for different types of bark, and from liquid produced by homogenising many insects. Bark was collected during spring and autumn from:- trunk heavily infested by C. fagisuga, trunk with typical beech bark disease syndrome, young trees artificially inoculated with strain A of N. coccinea alone and from a "healthy" trunk. The tissue was homogenised in a Waring blender and extracted with water (1/1 volume) with or without added NaCl (0.5 M). After centrifugation (2000 g) the supernatant was dialysed against distilled water at +5°C for 24 hours. If not assayed immediately enzyme preparations were stored at -5°C and tested within 2 days.

RESULTS

1. In vitro expression of pectinase activities

The results are presented in Tables 2-10 and Figs 1-2. In vitro results reveal that all strains of N. coccinea can be induced to secrete any pectinases necessary for degradation of

Tables 2 to 5.--Pectinase activity of the three strains of N. coccinea. Cultures on various substrates were 14 days old (one hour incubation). For method of assessment and units of enzymatic activity see text.

Table 2 endo-PG / Table 3 exo-PG

Strains	A	I	W	A	I	W
Culture substrate						
Twig bark	2.43	1.99	0.80	3.05	3.39	2.66
Trunk bark	2.66	2.33	2.36	2.90	2.72	2.98
C. fagisuga infested trunk bark	2.93	2.30	2.36	2.99	3.13	3.11
Pectic acids	0.47	0.63	0.37	1.05	1.54	0.09
Control (glucose)	0.02	0.00	0.00	0.00	0.03	0.01

Table 4 endo-PMG / Table 5 endo-PMTE

Twig bark	1.53	1.86	1.23	2.00	1.93	1.37
Trunk bark	2.63	1.27	2.57	2.80	1.70	2.67
C. fagisuga infested trunk bark	2.43	2.16	2.43	2.60	2.70	2.67
Pectin	0.87	1.90	0.40	2.90	1.60	1.00
Control (glucose)	0.07	0.01	0.03	0.04	0.02	0.00

pectic substances. Extensive investigations of the last 15 years have demonstrated that many

fungal pathogens when growing saprophytically have these abilities.

The nature of the substrate is one of the many factors affecting the level of pectinase activity in culture filtrates. For these isolates of N. coccinea, the inducing power for hydrolase activity of natural substrates e.g. bark powder was many times greater than those of artificial substrates (Tables 2-4). This is especially true for the W strain hydrolase activity which was very low on artificial substrates and became equivalent to those of other strains on trunk bark only. Bark of the trunk enhances endohydrolase activity while the bark of the branch allows the greater exo-PG activity of some strains.

Table 6.--exo-PMTE and exo-PMG activities of the three strains of Nectria coccinea. Cultures were 14 days old on pectin as substrate. For method of assessment and units of enzymatic activity see text.

Strains	A	I	W	A	I	W
Enzyme	exo-PMTE			exo-PMG		
Substrate						
Pectin	2.47	1.25	0.10	4.99	7.14	1.06
Control (glucose)	0.01	0.00	0.00	0.00	0.00	0.00

Transeliminase activities were not affected to the same extent by the nature of the substrate (Tables 5 and 7). The natural substrate elicited a greater transeliminase activity than did artificial substrates only in the case of strain W alone (Tables 5 and 7). Exo-PGTE activity was greatest when N. coccinea grew on bark from an infested trunk and the twig bark as substrate reduced endo-PMTE activity (Table 5).

Table 7.--exo-PGTE activities of three strains of Nectria coccinea. Cultures were 14 days old on various substrates. For method of assessment and units of enzymatic activity see text.

Strains	A	I	W
Substrates			
Twig bark	0.942	0.754	0.825
Trunk bark	0.554	0.292	0.308
Infested trunk bark	1.375	1.125	1.133
Pectic acids	1.300	1.470	0.000
Na Polypectate	0.070	0.090	0.040
Control (glucose)	0.010	0.030	0.000

2. In vivo evidence of pectinase activities

The results are shown in Table 8. In vivo evidence of transeliminase activity except endo-PMTE was lacking whatever the type of bark and the sampling season. Endo-PMTE activity could be revealed as well as hydrolase activity other than exo-PMG in the bark at various stages of disease development but only at spring time.

Table 8.--In vivo evidence of pectinase activities in beech bark, or in C. fagisuga larvae at spring time. For method of assessment and units of enzymatic activity see text.

Enzyme activity	endo-PG	exo-PG	endo-PMG	endo-PMTE
Extract				
Diseased bark	0.033	trace	trace	0.024
Bark with N. coccinea necrosis	0.089	0.280	0.070	0.124
C. fagisuga infested bark	0.057	0.200	0.052	0.045
C. fagisuga larvae	0.067	0.000	0.047	0.042
"Healthy" bark	0.000	0.020	0.000	0.000
Control (Healthy bark autoclaved)	0.000	0.000	0.000	0.000

Cryptococcus fagisuga showed clear endohydrolase and endo-PMTE activities at spring time.

3. Influence of bark additives on pectinase activities of N. coccinea

One ml of healthy bark or C. fagisuga infested bark of beech was added to the enzyme substrate reaction mixture at the beginning of the reaction. The results are summarised in Table 9. The different exoenzyme activities were slightly reduced by C. fagisuga infested bark. Endo-PMG was the only endoenzyme influenced by bark extracts. C. fagisuga infested bark induced an increase of that activity.

Table 9.--Influence of bark additives on some pectinase activities of N. coccinea culture filtrates. Cultures were 14 days old on bark substrates. For method of assessment and units of enzymatic activity see test.

Strains	A			I		
Additive	Water	Healthy bark	Infested bark	Water	Healthy bark	Infested bark
Enzyme activity						
exo-PMTE	2.467	2.208	1.921	1.250	1.196	0.987
exo-PGTE	1.300	1.563	1.396	1.470	1.671	1.533
endo-PMG	0.800	0.870	1.470	1.600	1.660	2.100

4. Sequential occurrence of pectinases

These investigations were conducted with the most efficient strain A. Data are presented in Table 10 and Fig. 2.

The development of endoenzyme activities with culture age followed the same rate except for endo-PGTE which was delayed. In 8 day old

Table 10.--Development of exo-PG and exo-PGTE activity in culture filtrates of strain A of N. coccinea growing on healthy bark of C. fagisuga infested bark. For methods of assessment and units of enzymatic activity see text. Figures underlined for emphasis.

Age of culture (days)	2		4		6		8		14	
Pectinase activity	exo-PG	exo-PGTE	exo-PG	exo-PGTE	exo-PG	exo-PGTE	exo-PG	exo-PGTE	exo-PG	exo-PGTE
Substrate										
Healthy bark	0.005	0.003	0.024	0.000	0.389	0.133	0.330	0.170	2.900	0.550
C. fagisuga infested bark	0.000	0.000	0.250	0.000	1.514	0.092	1.380	0.210	3.000	1.380

culture filtrates endo-PG activity was slightly greater than that of endo-PMG and endo-PMTE.

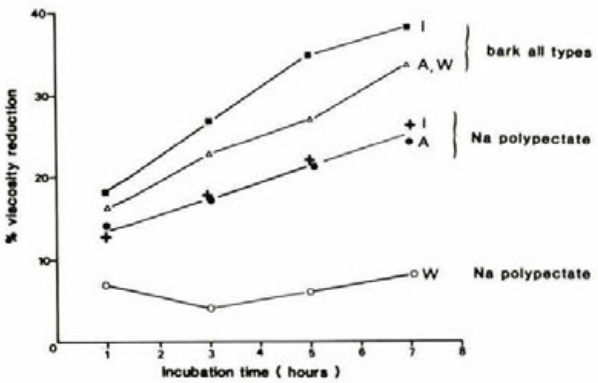

Figure 1.--endo-PGTE activity of three different strains of Nectria coccinea. Cultures were 14 days old on various substrates.

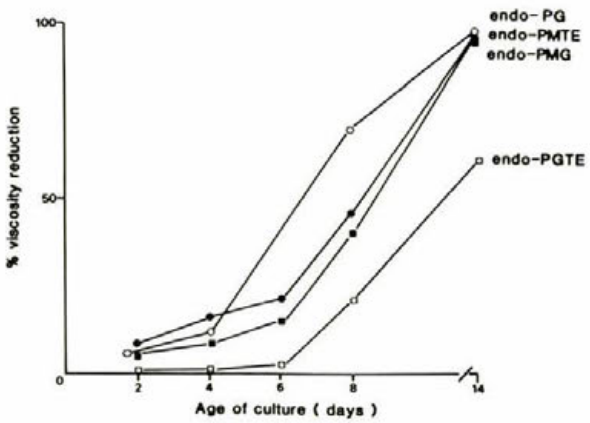

Figure 2.--Sequential appearance of endopectinases in culture filtrates of strain A of Nectria coccinea.

Exo-PG activity appeared very early in culture filtrates. The level of exo-PGTE activity was very low even after 8 days of growth of the fungus. C. fagisuga infested bark allowed a better exo-PG activity in culture as young as 6 days of age and led to a greater level of exo-PGTE activity.

DISCUSSION AND CONCLUSIONS

In vitro, it was possible to induce N. coccinea to secrete any pectinase necessary for degradation of pectic material in the cell wall. In vivo evidence of some pectinase, hydrolase and endo-PMTE activities gave a satisfactory proof of their implication in the process of necrosis. With respect to these considerations production of endohydrolase and endo-PMTE by the insect may be of great significance in the predisposition of the bark to alteration by the fungus. The beech scale seems to be capable of initiating the first steps of the degradation of pectic substances. In addition the presence of C. fagisuga and its influence on bark contents enhance some pectinase activities of the fungus. In vitro studies indicated that N. coccinea secretes first polygalacturonases, while pH is in the acid range (4.3-4.5). These activities were strongly increased when N. coccinea grew on infested bark as the substrate. Transeliminase activity appeared a few days later in culture filtrate, when the pH had reached 5-5.5. Moreover C. fagisuga infested bark induced an increase of exo-PGTE activity of N. coccinea. Bark pH measured in nature changes from 4.5 in healthy bark to 6.5-7.5 in typical beech bark disease necrosis (R. Perrin, unpublished data). The pH of C. fagisuga infested bark is 0.5 units greater than that of healthy bark.

According to Skou (1979) calcium strongly inhibited polygalacturonase activity, whereas this cation weakly stimulated transeliminase activity. The calcium concentration of trunk bark is almost 5 times greater than that of the twig (Kreutzer 1976). It seems that pectinase weapons of N. coccinea and the help given by pectinase of the insect are well adapted to the bark environment of the beech trunk, a possible cause of the specificity of this association.

No relation can be found between the potential enzymatic pool and the virulence of these different strains of N. coccinea (unpublished data). Pectic enzymes are only one of the weapons of the pathogen, acting in concert, to destroy plant tissues and the results reported here indicate a complete pectinase potential of the organisms involved in beech bark disease which seems not to be a limiting factor in pathogenesis.

ZUSSAMENFASSUNG

Die Pektinaseaktivität von Nectria coccinea wurde in vitro und in vivo in Abhängigkeit von den Pektinasen von Cryptococcus fagisuga und der Art der Rinde untersucht.

In vitro wurden von dem Pilz alle Pektinasen gebildet, die für den Abbau entsprechenden Materials benötigt werden. Einige von der Buchenwollaus gebildete Pektinasen sind im Zusammenhang mit der Prädisposition der Rinde für Veränderungen durch den Pilz von grosser Bedeutung. Das gut auf die Stammrinde abgestimmte Pektinasebesteck von N. coccinea und die Unterstützung durch Cryptococcus fagisuga sind eine mögliche Erklärung für das Besondere dieses Laus-Pilz-Komplexes.

RÉSUMÉ

L'activité pectinolytique du Nectria coccinea fut étudiée in vitro et in vivo en relation avec les pectinases du Cryptococcus fagisuga et la nature de l'écorce. Toutes les pectinases nécessaires à la dégradation du matériel pectique étaient sécrétées par le champignon in vitro. Certaines pectinases produites par l'insecte sont très importantes dans la prédisposition de l'écorce à l'altération par le champignon. La situation d'une production de pectinases bien adaptées à l'écorce du tronc de N. coccinea et de l'assistance donnée par la cochenille peut expliquer l'association spécifique C. fagisuga/N. coccinea.

LITERATURE CITED

Bateman. D.F., and Millar, R.L. 1966. Pectic enzymes in tissue degradation. Ann. rev. Phytopath. 4: 119-146.

Bompeix. 1972. Recherches sur la biologie du Pezicula alba Guthrie. Thèse Doctorat es Sciences, Université de Paris VI, Mai 1972, 232 p.

Bugbee, W.M. 1975. Peroxidase, polyphenoloxidase and endopolygalacturonate-transeliminase in different tissues of sugar beet infected with Phoma betae. Can. J. Bot. 53: (14) 1347-1351.

Ehrlich, J. 1934. The beech bark disease, a Nectria disease of Fagus following Cryptococcus fagi (Baer). Can. J. Res. 10: 593-692.

Kreutzer. 1976. Effect on growth in next rotation (regeneration). In Symposium on the harvesting of a larger part of the forest biomass FAO/CEE/011. IUFRO 14-16 June 1976. Hyuinkaa (Finland).

Kunkel, H. 1968. Untersuchungen über die Buchenwollschildlaus Cryptococcus fagi Bär (Insecta, Coccina) einer Vertreter der Rindenparenchymsauger. Z. angew. Ent. 61: (4) 373-380.

Leibundgut, H., Frick, L. 1943. Eine Buchenkrankheit im schweizerischen Mitteland. Schweiz. Z. Fortwes. 94: (10) 297-306.

Lonsdale, D. 1980. L'infection de l'écorce du Hêtre par Nectria coccinea: variations de la maladie en relation avec des facteurs prédisposants. Ann. Sci. Forest. 37: (4) 307-318.

Parker, E.J. 1974. Beech bark disease. Ph.D. Thesis. Univ. Surrey. 161 pp.

Perrin, R. 1974. Le dépérissement du Hêtre en France. Eur. J. For. Path. 4: 249-251.

Perrin, R. 1977. Le dépérissement du Hêtre. Revue for. fr. 29: (2) 101-126.

Perrin, R. 1979. Contribution à la connaissance de l'étiologie de la maladie de l'écorce du Hetre: I - Etat sanitaire des hêtraies. Rôle du Nectria coccinea (Pers ex Fries) Fries. Eur. J. For. Path. 9: 148-166.

Perrin, R. 1980. Contribution à la connaissance de l'étiologie de la maladie de l'écorce du Hêtre: II - Etude expérimentale de l'association Cryptococcus fagisuga Lind. Nectria coccinea (Pers ex Fries) Fries. Rôle respectif des deux organismes. Ann. Sci. Forest. 37: (4) 319-332.

Skou, J.P. 1979. Radiation induced sprout and growth inhibition in vegetables with special reference to the susceptibility to microbial attacks and the effect of calcium. RIJO National Laboratory publication, 265 p.