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A diagnostic method for the differentiation of *Ophiostoma ulmi* and *O. novo-ulmi* using gallic acid

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Summary – Taking advantage of the differential production of extracellular laccase by *Ophiostoma ulmi* and *O. novo-ulmi,* a simple test for the identification of isolates from the field has been developed using the polyphenol gallic acid. In still culture, isolates of *O. novo-ulmi* develop a strong brownish colour after a few days of incubation whereas with strains of *O. ulmi,* the colour reaction is weak. This result was confirmed by measuring the absorbance of the culture fluid at 470 nm, *O. novo-ulmi* yielding a mean value of 0.54 which was five times higher than that reached by *O. ulmi.* Besides its determinative potential, this test may also be used for the screening and isolation of mutants lacking or overproducing extracellular laccase or other oxidoreductases oxidizing gallic acid.

Zusammenfassung – Unter Ausnützung der differentiellen Produktion von extrazellulärer Laccase durch *Ophiostoma ulmi* und *O. novo-ulmi* wurde ein einfacher Test zur Identifizierung von Wildisolaten entwickelt, der den Gebrauch des Polyphenols Gallussäure erfordert. In unbewegter Kultur bewirken Isolate von *O. novo-ulmi* eine starke Bräunung des Kulturfiltrats, während Stämme von *O. ulmi* nur eine schwache Verfärbung aufweisen. Dieses Resultat wurde durch Messung der optischen Dichte des Kulturüberstandes bei 470 nm bestätigt. *O. novo-ulmi* erreichte einen Durchschnittswert von 0.54 während *O. ulmi* einen fünfmal kleineren Wert aufwies. Neben des Potentials für die Identifizierung von Wildisolaten könnte dieser Test auch der Auffindung und Isolation von Mutanten, die extrazelluläre Laccase oder andere Gallussäure-oxidierende Oxidoreduktasen nicht mehr oder überproduzieren, dienen.

Introduction

The two Dutch elm disease pathogens, *Ophiostoma ulmi* (Buism.) Nannf. (formerly termed the non-aggressive form of *Ophiostoma ulmi* syn. *Ceratocystis ulmi* (Buism.) Moreau) and *O. novo-ulmi* Brasier (aggressive form) exhibit a very large number of distinct properties. *O. ulmi* is a weak pathogen on most European elm species whereas *O. novo-ulmi* is characterised by very high mortality rates among native European elms (Brasier, 1986). They are also strongly reproductively isolated and differ in a wide variety of morphological, physiological, molecular and genetical characteristics (Jeng, Bernier & Brasier, 1988; Brasier 1991 and references therein; Bates, Buck & Brasier 1993). *Ophiostoma novo-ulmi* is further divided into two subgroups, both being equally pathogenic. According to their respective geographical distribution, they have been named the Eurasian or EAN and North American or NAN race (Brasier, 1986). Up to now, the most important tool in routine strain identification is still the differential temperature optimum for growth between the two species (30 °C for *O. ulmi* and 20–22° for *O. novo-ulmi* as determined over an incubation period of seven days on malt extract agar; Brasier, Lea & Rawlings, 1981).

We recently (Binz & Canevascini, 1996) reported that isolates of *O. novo-ulmi* produce high amounts of extracellular laccase whereas isolates of *O. ulmi* do not. In addition, it has been shown that gallic acid can be oxidized by the purified laccase (Binz, 1996) and this oxidation leads to a brownish coloration of the assay mixture. These two facts were explored in order to design a diagnostic test for the differentiation of the two species based on mini liquid still cultures in the presence of gallic acid, a well suited substrate for laccase in general (Marbach, Harel and Mayer, 1984).

Materials and Methods

All test isolates (27 in total: nine *O. ulmi*, nine *O. novo-ulmi* EAN and nine *O. novo-ulmi* NAN) were from the Dutch elm disease culture collection of the Forest Research Station, Alice Holt Lodge, Farnham, Surrey GU 10 4LH, UK (for details cf Binz & Canevascini, 1996). Geographical origin and subgroup distribution are given in Table 1. Blind tests were performed with selected strains isolated by P. Högger (isolates were: for *O. ulmi:* M849, M850; for *O. novo-ulmi:* ch100, ch119, ch123, ch126, M869; Högger, Binz & Heiniger, 1996). The isolates were initially grown on malt extract agar 2 % (Oxoid) at 20 °C, and then stored as dense conidiospore suspensions in 15 % (v/v) glycerol at –70° (Bernier, 1993). Starter cultures generating the inoculum (yeast cells) were prepared by inoculating 50 ml of the defined liquid medium (2 % glucose) of Brun-

ton & Gadd (1991) modified with phosphate buffer to pH 6.0 with a few drops (ca. 50 µl) of conidia suspension from the glycerol stock solution. The starter cultures were then incubated at 25 °C on a rotary shaker (150 cycles min^{-1}) for 6–7 days. Cultures were carried out in 12 ml (contained in a 30 ml glass test tube, diam. 2 cm) of defined liquid medium supplemented with 0.2 % of yeast extract to trigger the mycelial form (Binz & Canevascini, 1996) and 1 mM gallic acid. The medium was inoculated with cells of late stationary phase starter cultures to an initial cell density of approx. 3 x 106 ml^{-1} (i.e. to an A_{400nm} = 0.2) and the tubes were then inclined and incubated for 7 days at 25 °C. To determine culture kinetics, the whole content of one glass tube was harvested each day during 1 week. The sample was centrifuged (1900 g, 10 min, r_{av} = 10 cm) and the extent of oxidized gallic acid determined by measuring the optical density of the supernatant at 470 nm. For biomass (dry weight) determination, the mycelial pellet was resuspended in 4 ml of distilled water and filtered through tared membrane filters (0.2 µm pore size, 3 cm diam., Schleicher & Schuell). The filters were dried to constant weight at 90°C.

Results and Discussion

Fig. 1 shows cultures of different isolates of *Ophiostoma ulmi* and *O. novo-ulmi* (six isolates each) when grown in a gallic acid supplemented medium. All strains of *O. novo-ulmi* developed a strong brown colour after seven days of incubation whereas culture media of *O. ulmi* isolates remained almost colourless. In addition, a blind test including various isolates of previously unknown origin (cf Materials and Methods) was perfectly able to identify all strains in an unambiguous way. Therefore, a clear distinction between the two species is possible by simple optical evaluation. Furthermore, when measuring the A_{470} of the culture supernatants, the mean value obtained with *O. novo-ulmi* was five fold higher than that of *O. ulmi* (Table 1).

As indicated by the amount of final biomass (dry weight), this difference was not due to impaired growth of *O. ulmi* isolates. Both species were also cultivated in control experiments without gallic acid and in these cases, A_{470} remained zero during the whole incubation period. In order to improve the colour reaction, different carbon sources including pectin (an inducer for the laccase of *Botrytis cinerea;* Marbach, Harel & Mayer, 1985), elm sapwood and birchwood xylan at various concentrations (0.5–3%) were also tested. Although equally suited for the diagnostic purpose as the medium containing glucose, none of these substrates enhanced or accelerated the colour development. The effect of temperature was not tested since the secretion of laccase by *O. novo-ulmi* is not affected by this parameter (Binz & Canevascini, 1996).

Table 1: Geographic origin, subgroup distribution, development of color and growth (dry weight) in still cultures with basal medium (defined liquid medium supplemented with 0.2 % yeast extract) containing 1 mM gallic acid after 7 days of incubation at 25 °C.

Isolate and species	Country	Source*	Dry weight (mg ml^{-1})	OD_{470}
O. ulmi				
PG401	Portugal	CMB	3.74	0.157
H173	USA	JNG	3.32	0.112
H200	Ireland	AM	3.81	0.123
PG386	Portugal	CMB	3.27	0.060
H830	USA	DH	3.62	0.069
TR117	Turkey	CMB	3.44	0.181
ES255	Spain	CMB	3.58	0.071
H815	USA	DH	3.22	0.112
H365	Netherlands	HH	3.53	0.123
Mean			**3.50**	**0.112**
O. novo-ulmi				
EAN race				
P114	Poland	CMB	3.89	0.587
Yu16	Yugoslavia	CMB	4.12	0.521
CKT-11	Iran	CMB	3.09	0.449
P127	Poland	CMB	3.53	0.458
H219	Ireland	AM	3.35	0.612
CA4	Tashkent	CMB	3.05	0.505
R64	Romania	CMB	3.11	0.691
H224	Denmark	HJ	3.48	0.603
H327	Czechoslovakia	JJ	3.50	0.515
Mean			**3.46**	**0.552**
NAN race				
H965	Canada	GBO	3.14	0.565
H172	USA	JNG	3.06	0.690
PG402	Portugal	CMB	3.31	0.618
H363	North Ireland	DS	3.11	0.390
PG64	Portugal	CMB	3.09	0.491
H351	Belgium	MM	3.36	0.587
ES391	Spain	CMB	3.26	0.523
W2	UK	JNG	3.06	0.521
H975	Canada	GBO	3.14	0.451
Mean			**3.17**	**0.534**

*CMB, C.M. Brasier; JNG, J.N. Gibbs; AM, A. Mangan; DH, D. Houston; JJ, J. Jamnicky; HJ, H. Jorgensen; GBO, G.B. Ouellette; DS, D. Seaby; MM, M. Meulemans

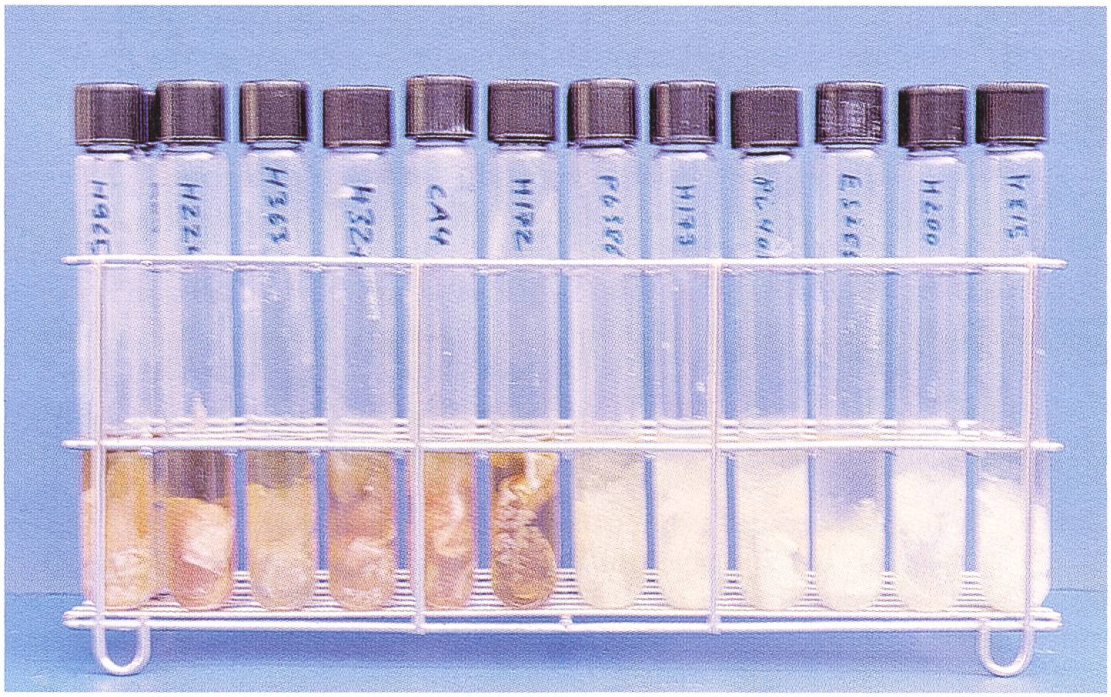

Fig.1: Colour development of 6 different isolates of O. ulmi *(right) and* O. novo-ulmi *(left) when grown in still culture on basal medium supplemented with 1 mM gallic acid.*

The typical growth pattern and the kinetics of brown colour development in the culture supernatant of representative isolate of *O. ulmi* (H200) and *O. novo-ulmi* (CKT-11) are shown in Fig. 2. Biomass accumulation was identical for both isolates. In contrast, for *O. novo-ulmi,* the A_{470} of the culture supernatant increased very rapidly and reached a final value of 0.82 whereas with *O. ulmi,* A_{470} remained low during the whole growth period. This result strikingly resembles the kinetics of extracellular laccase production in shaken cultures (carried out in the same medium, but in a time interval of approximately 60 hours) as reported previously (Binz & Canevascini, 1996). It is still possible, however, that other enzymes (i.e. peroxidases and polyphenol oxidases other than laccase) are involved in the colour development. This aspect certainly requests further investigation.

We also attempted to distinguish *O. ulmi* from *O. novo-ulmi* by the method of Bavendamm (1928) using solid agar media supplemented with 0.3% tannic acid. *O. ulmi* isolates produced a weak browning of the substrate compared to the rapid discolouration observed with most isolates of *O. novo-ulmi.* Growth of both organisms, however, was strongly impaired, probably due to the strong toxicity of tannic acid. In addition, since in a few cases the results were ambigous, this test may not be suitable for a diagnostic purpose.

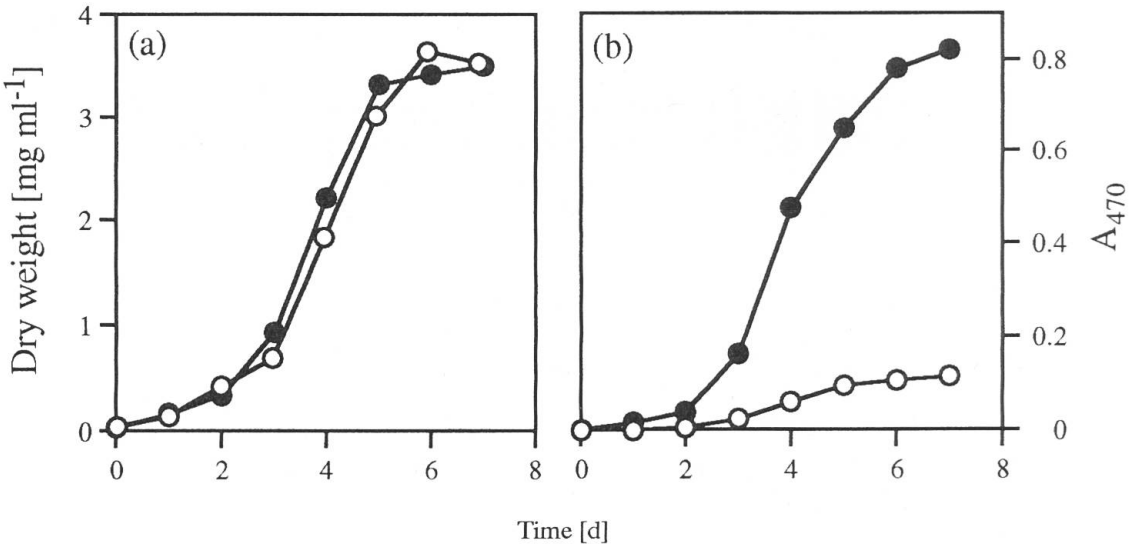

Fig.2: Typical growth pattern (a, dry weight accumulation) and increase of A_{470} (brown colour development, b) in the culture supernatant of O. ulmi *H200 (○) and* O. novo-ulmi *CKT-11 (●).*

Our results illustrate the potential use of gallic acid for the identification of *O. ulmi* and *O. novo-ulmi* isolates from the field. Furthermore, this method may well be miniaturized and thus become less laborious and time consuming. For instance, the use of only 0.2 ml of medium contained in a well of a microtiter plaque and inoculated with 1 µl of glycerol solution containing conidia (as described in Materials and Methods) gave positive results within 3 days of incubation at 25 °C. Finally, it may be suitable for the screening and isolation of mutants lacking or overproducing extracellular laccase or other oxidoreductases oxidizing gallic acid.

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