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Studies on the Physiological Plant Pathology of Witches' Broom Disease.

Improving our knowledge of the biology and life cycle of Moniliophthora perniciosa

Witches' Broom Disease (WBD) of cocoa is a significant factor affecting productivity of cocoa in South America. The causal organism, the basidiomycete fungus *M. perniciosa* (formerly known as *Crinipellis perniciosa*) has a complex and unusual lifecycle which involves initial biotrophic infection within living host tissues, stimulating the production of characteristic green "brooms", followed by a saprotrophic phase synchronous with the death of the plant tissues. The rate of development and final size of the green brooms, and the subsequent production of spore-producing mushrooms on the dead broom tissues are important factors in the epidemiology of the disease. This research project set out to study the complex developmental changes at the level of gene expression during the switch from biotrophy to saprotrophy in order to increase our understanding of the host-pathogen interaction.

Experiments using artificially killed brooms, showed that the switch from biotroph to saprotroph is concurrent with broom death however this may occur. Since other saprophytic fungi quickly colonized the dying broom tissues and suppressed the quantity of *M. perniciosa* mycelium, it seems likely that *M. perniciosa* is a poor competitor and that the biotrophic phase of its lifecycle has evolved to give it a head start in colonising its own carbon source before plant defences are breached by competitive saprophytic fungi.

All three of the main pathotypes of *M. perniciosa* were studied and model systems were developed using the S-type which infects several solanaceous plants, due to the ease of spore production of this type in culture and the availability of various mutant varieties of tomato and aubergine which are useful for studies of gene expression during infection. Changes in extracellular fungal enzymes, typically associated with saprotrophy such as laccase, and pathogenesis-related proteins that appeared before the switch from biotropy to saprotrophy were studied in these model systems using proteiomic techniques.

The protein profiles of *M.perniciosa* mycelia cultured *in vitro* were also studied and several proteins which appeared to be developmentally regulated were noted though it was not possible to identify them using the fungal protein databases available at the time of this study. However, a non-specific oxidase was identified which also appeared to be developmentally expressed in the saprotrophic phase. This was tentatively identified as a laccase using specific antibodies and it is postulated that laccase production is a feature of saprotrophy and a possible precursor to basidiocarp production in the saprotrophic stage of growth.

Infection of tomato by S-biotype of *M. perniciosa*. (R. Birch)

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ABSTRACT
Studies on the Physiological Plant Pathology of Witches' Broom Disease.

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Witches' Broom Disease (WBD) of cocoa is a significant factor affecting productivity of cocoa in South America. The causal organism, the basidiomycete fungus Crinipellis perniciosa (Stahel) Singer has a novel lifecycle which involves initial biotrophic infection of the host (during which the characteristic brooms are formed), followed by a morphological switch to a saprotrophic mode of growth synchronous with the death of the host broom.

Research into the relationship between C. perniciosa and the host has been hampered by the difficulties of producing fungal spores (the only infective agent) and culturing the biotrophic phase in vitro. C. perniciosa cultures collected in the field or reactivated from the culture collection maintained at Aberystwyth were tested for ease of culture, vigour of growth and sporulation ability. Individual cultures from C-, S- and L-type C. perniciosa pathotypes (the 3 major pathotypes characterised by their host species) were tested on a range of plant species from the Solanaceae Bignoniaceae and Sterculiaceae to identify a potential model system. The S-type isolate of C. perniciosa, coded as WMA5, was found to be very amenable to in vitro culture and produced basidiocarps far more reliably than any other pathotype, thus providing a regular spore supply. However, S-type spores did not produce brooms on cocoa seedlings and were only effective on plants within the Solanaceae. Tomato (Lycopersicon esculentum var. 'Ailsa Craig') and aubergine (Solanum melongena var. 'Long Purple') were together found to be very susceptible to WBD. Both were therefore used as model systems because aubergine has a determined mode of growth similar to cocoa, and tomato, although it has non-determined growth, is a well-researched organism with a number of important molecular tools associated with it, which would aid study of WBD.

The chosen method of research involved the study of protein activity in infected plants using 2-dimensional polyacrylamide gel electrophoresis (2-D PAGE or 2-DE). Samples of infected tomato brooms were extracted and their protein profiles observed by separation according to their charge (1st dimension PAGE) and then their mass (2nd dimension PAGE). The resulting gels produced using this procedure enabled comparison between infected and uninfected plants, and any proteins that may be specific to either category identified. Proteins were eluted from the gels and analysed by matrix assisted laser desorption and ionisation – time of flight mass spectroscopy (MALDI-Tof MS) which fragments proteins one peptide at a time and produces a peptide mass fingerprint (PMF) which is viewed as a mass-to-charge spectrum. Peaks in this spectrum were entered into a database search engine (PeptIdent) which searches molecular databases (SWISS-PROT and TrEMBL) and matches the target sample with proteins which have been sequenced. 12 proteins were identified with a high degree of confidence using this method, by comparison of repeated samples. Proteins of particular interest included pathogenesis-related proteins (PRP's) and proteins associated with growth and respiration. Novel proteins were further sequenced using microsequencing, which fragments peptides into individual amino acids and produces sequence data. This allows comparison of the target sequence with the sequence in any comparable proteins – a more accurate method of identification for unknown proteins. Further confirmation of the identity of proteins was obtained with the use of western blotting; a number of antibodies to tomato proteins being available. These techniques

confirmed that in tomato, PR proteins are activated during the biotrophic, broom-forming phase of infection, before broom necrosis and the switch to saprotrophy occurs.

Electrospray ionisation mass spectroscopy (ESI-MS) was used in conjunction with 2-DE and MALDI to identify changes in the chemistry of small molecular weight compounds in the plant apoplast. A number of common compounds were identified, including the pigment naringenin, which allowed separation of aubergine control and broom samples by principal component analysis.

Similar proteomic techniques were applied to C. perniciosa mycelium cultured in vitro. 2-DE protein profiles of all pathotypes were made, and proteins observed in C-, S- and L-type dikaryons were not observed in unmated single spore isolates (SSI's) of the outcrossing L-type, suggesting that they may be developmentally regulated. The proteins, although effectively sequenced, could not be identified using PeptIdent searches due to the smaller number of fungal proteins in databases. Only 2 fungal proteins were identified using microsequencing methods. However, activity PAGE gels identified a non-specific oxidase which also appeared to be developmentally expressed, and antibodies to laccase from the basidiomycete Trametes versicolor worked equally effectively on C. perniciosa. This laccase was found to be developmentally expressed in the saprotrophic stage of broom colonisation.