Pathogenicity in *Heterobasidion annosum* s.l.

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Distribution and speciation

Root rot caused by the basidiomycete *Heterobasidion annosum* s.l. is one of the most destructive diseases of conifers in the northern boreal and temperate regions of the world. Economic losses attributable to *Heterobasidion* infection in Europe are estimated at 800 million Euros annually (Woodward *et al* 1998). The fungus has been classified into three European intersterile subspecies P (*H. annosum*), S (*H. parviporum*) and F (*H. abietinum*) based on their main host preferences, pine, spruce, and fir, respectively. In North America, two intersterile groups are present, P and S/F, but these have not yet been given scientific names. Detailed interaction studies on this pathosystem have been complicated by the fact that there are no known avirulent strains of the fungus and no host genotype in *Pinaceae* with total resistance against the pathogen.

Although separated on different continents for a long period of time (Johannesson & Stenlid 2003), the North American and European P groups are morphologically indistinguishable (Korhonen & Stenlid, 1998) and fully interfertile (Stenlid & Karlsson, 1991). Furthermore, they also share similar broad host preferences and are thus probably best regarded as two subpopulations of the same species. An interesting observation of intercontinental introduction of the American P group into Italy was recently reported (Gonthier *et al* 2004). Based on distinctive mitochondrial markers, the authors concluded that the fungus was probably introduced with woody material to a military camp during the Second World War, thereby creating an opportunity for geneflow between the two P group populations.

The phylogenetic relationship between the S- and F groups was studied by comparing DNA sequences of four nuclear gene fragments; calmodulin, glyceraldehyde 3-phosphate dehydrogenase, heat stress protein 80−1 and elongation factor 1-α, and one anonymous locus, from 29 fungal isolates originating from Europe, Asia and North America (Johannesson & Stenlid 2003). The phylogeny of each separate gene locus as well as the combined dataset consisted of three main clades: European F group isolates, Euroasian S group isolates and North American S group isolates, suggesting them to be separated into phylogenetic species. The results also support the hypothesis of an early separation between the S- and F groups, indicating that their distribution have followed their host tree species for a considerable time period.

The taxonomic status of the North American S group is less clear, it is partly interfertile with both the S and F groups from Europe, but has a distinct evolutionary history and in contrast to its European relatives, has a broad host range.

The intersterility in *H.annosum* s.l. is controlled by a genetic system consisting of at least 5 loci; P, S,V1,V2, and V3 (Chase & Ullrich 1990). Similar + alleles at any of the loci allow for mating between two homokaryotic strains. This system opens up for hybridisation between the intersterility groups (Garbelotto *et al* 1996; Olson & Stenlid 2001; 2002). Hybrid mycelia has been detected in the field and laboratory tests show that heterokaryons carrying nuclei of the American P and S type express the pathogenicity representative of the parent cytoplasm (Olson & Stenlid 2001). Although the genetic background for interfertility between species in Europe has not been formally sorted out, an interesting study on higher degree of intersterility was reported between the S and F group populations growing in sympatry in northern Italy as compared to Italian F populations and Finnish S populations, (Korhonen *et al* 1992). It would be of interest to study whether selection against hybrids has driven the alpine *H. parviporum* and *H. abietinum* into more distinctive intersterility gene genotypes as compared with the allopatric Northern European *H. parviporum* vs *H. abietinum*.

In addition to fascinating possibilities for reticulate evolution, the hybridisation also allows for genetic analysis of pathogenicity traits. The first steps have been taken for Quantitative Trait Loci (QTL) analysis of pathogenicity by analysing progeny of such hybrids (Lind *et al* 2005).

Pathogenicity

In angiosperm systems, the expression of virulence by a pathogen initiates at the point of attachment whereupon host-parasite recognition is concomitant with the onset of defence reactions and often presumed to be a determinant of host plant specificity (Albersheim & Anderson-Prouty 1975; Jones 1994). Using non-suberized roots as an experimental model, spore adhesion has been documented within 2 hours following inoculation of primary roots of juvenile conifer seedlings with conidiospores of *H. annosum* (Asiegbu 2000). Adhesion occurred mainly on the mucilaginous regions of the root but rarely on non-slimy regions and adhesion was significantly reduced by treatment of spores with potassium hydroxide, di-ethyl ether, Pronase E or periodic acid (Asiegbu 2000). By contrast to observations with fine roots, pre-treatment of wood discs, with di-ethyl ether had no effect on spore germination. Removal of soluble compounds from the wood disc by pre-treatment with periodic acid or KOH considerably reduced the ability of the spores to germinate and become established on the host material. The effect of periodic acid and

KOH suggests that the adhesive component and part of the nutrient source for the spores was a sugar or carbohydrate.

The digestion of plant cell wall polymers provides nutrients and aids the penetration of cells, allowing survival and spread through woody tissues. However, few of the enzymes (amylase, catalase, cellulase, esterase, glucosidase, hemicellulase, manganese peroxidase, laccase, pectinase, phosphatase, proteases) secreted by *H. annosum* have been thoroughly studied (Johansson 1988; Karlson & Stenlid 1991; Korhonen & Stenlid 1998; Maijala *et al* 1995, 2003; Asiegbu *et al* 2004) and little is known about their role in pathogenesis. *H. annosum* s.l. secretes a range of polysaccharide-degrading enzymes. Cellulase, mannanase, xylanase, aryl-β-glucosidase and β-glucosidase have been identified although their role in pathogenesis is still not thoroughly investigated. Beta-glucosidase enables *H. annosum* s.l. to use the energy in the glucosidic bond of cellobiose, an enzyme system that appears to be rare in white-rot fungi. A higher number of polygalacturonase and pectin esterase isozymes are present in *H. annosum* s.s. than in *H. parviporum* (Karlsson & Stenlid 1991). Additionally, the total pectin-degrading capabilities of *H. annosum* s.s. are higher than in *H. parviporum*, which has been hypothesised to account for the greater host range of *H. annosum* s.s. (Johansson 1988).

Several low molecular weight toxins are secreted by *H. annosum*, including fomannoxin, fomannosin, fomannoxin acid, oosponol and oospoglycol (Basset *et al* 1967; Sonnenbichler *et al* 1989). Application of fomannosin to stem wounds provoked systemic response leading to accumulation of pinosylvin (Basset *et al* 1967). Another toxin produced by *H. annosum* s.l. is fomannoxin, which have a 100-fold greater toxicity to *Chlorella pyrenoidosa* than fomannosin (Hirotani 1977). This toxin has been isolated from *H. annosum* s.l. infected Sitka spruce stem wood (Heslin 1983). Uptake of fomannoxin by Sitka spruce seedlings resulted in rapid browning of the roots accompanied by chlorosis and progressive browning of needles. This, and the production of fomannoxin by actively growing hyphae, suggests a role for fomannoxin during pathogenesis.

One factor that has limited the research about *H. annosum* pathogenesis is the lack of coding sequence information. Therefore, a project on producing sequence data from *H. annosum* by generating ESTs was initiated (Karlsson *et al* 2003). The collection of sequence data will assist future research on *H. annosum* together with the high-density cDNA arrays that were also constructed in this work. It is interesting that 30 % of the genes identified did not have any similarity to any known proteins and 16 % had similarity only with proteins with unknown functions. This is a typical number of unknown unigenes for other fungal EST sequencing projects and highlights a lack of sequence information on fungi.

The next step was to identify individual genes that encode putative pathogenicity factors (Karlsson 2005). This was done by identifying genes that have high transcript levels during infection stages as compared to other treatments, and by studying sequence similarities with proteins that have a characterised role in pathogenesis in other systems. The transcriptional responses of several genes were studied with realtime-PCR during fungal infection of conifer material. Genes with a putative involvement in secondary metabolism, protection against oxidative stress and degradation of host material were shown to be differentially expressed. A cytochrome P450 gene displayed sequence similarities towards genes encoding proteins involved in toxin biosynthesis and was highly expressed during growth in Norway spruce bark. Transcript profiles of a superoxide dismutase gene and two glutathione-S-transferase genes suggest that oxidative stress is involved in the interaction. An arabinase gene was exclusively expressed during infection of Scots pine seedlings. An increase of the transcription rate of a laccase and a cellulase gene was detected during a time-coarse experiment of fungal infection of Norway spruce tissue cultures.

Recently, progress has been made in work on mapping the pathogenicity factors in *Heterobasidion* using a hybrid between North American P and S homokaryons. Based on AFLP markers, a genetic linkage map was established that allowed for mapping QTLs for pathogenic growth towards seedling roots and pine innerbark (Lind *et al* 2005). The next step underway is to verify the identity of candidate genes located within the established region of the genome. Future functional analysis of both QTL and EST-derived candidate genes should be aided by the recently established Agrobacterium-mediated transformation system in *Heterobasidion* (Samils *et al* 2006).

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