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ECOPHYSIOLOGY AND BREEDING OF MYCOPARASITIC *TRICHODERMA* STRAINS*

(A REVIEW)

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Losses due to plant diseases may be as high as 10–20% of the total worldwide food production every year, resulting in economic losses amounting to many billions of dollars and diminished food supplies. Chemical control involves the use of chemical pesticides to eradicate or reduce the populations of pathogens or to protect the plants from infection by pathogens. For some diseases chemical control is very effective, but it is often non-specific in its effects, killing beneficial organisms as well as pathogens, and it may have undesirable health, safety, and environmental risks.

Biological control involves the use of one or more biological organisms to control the pathogens or diseases. Biological control is more specialized and uses specific microorganisms that attack or interfere with the pathogens. The members of the genus *Trichoderma* are very promising against soil-born plant parasitic fungi. These filamentous fungi are very widespread in nature, with high population densities in soils and plant litters [1]. They are saprophytic, quickly growing and easy to culture and they can produce large amounts of conidia with long lifetime.

Keywords: *Trichoderma*, mycoparasitism, ecophysiology, mutagenesis, protoplast fusion, transformation

* Dedicated to the 70th birthday of Prof. Lajos Ferenczy

Biochemical background of antagonism by *Trichoderma* strains

The antagonistic properties of *Trichoderma* strains against plant pathogenic fungi are proposed to be the result of the synergistic effects of three basic mechanisms: antibiosis, competition and mycoparasitism. The biochemical basis of these mechanisms seems to be the production of antifungal compounds and extracellular enzymes.

Antifungal compounds

Comprehensive reviews are available on the antibiotic production of *Trichoderma* species [2, 3, 4 and references therein]. Dennis and Webster [2] described the production of trichodermin, a chloroform-soluble sesquiterpenoid metabolite, by *Trichoderma viride* and *T. polysporum*, and the production of other peptide antibiotics by *Trichoderma hamatum*.

Okuda et al. [5] showed that *Trichoderma* species exhibited their own production patterns of isonitrile antibiotics, isonitrile patterns correlated with the morphological and physiological characteristics of the species.

An isolate of *Trichoderma harzianum* was found to secrete 3-(2-hydroxypropyl)-4-(2-hexadienyl)-2(5H)-furanone, which inhibits microbial growth [6]. From this mycoparasitic species group two cyclonerodiols, an octaketide keto diol and three new octaketide-derived compounds with antibiotic activity towards the take-all fungus *Gauemannomyces graminis* var. *tritici* were also identified [4].

Trichorzins and trichorzianines are peptaibol antibiotics with antifungal activity. The parallel formation and synergistic effect of trichorzianines with hydrolytic enzymes in the inhibition of spore germination and hyphal elongation of *Botrytis cinerea* was shown by Schirmböck et al. [7]. Such synergisms may play an important role in the antagonism of *Trichoderma* species against plant pathogenic fungi.

Harzianum A, a trichothecene from *T. harzianum* showing modest antifungal activities, was characterized by Corley et al. [8].

Tricholin is a ribosome-inactivating protein isolated from *T. viride*, with fungicidal effects against *Rhizoctonia solani* [9]. The mode of action of this compound towards *R. solani* appeared to be attributed to the diminishing of polysome formation through damage to large ribosomal subunits.

The *T. harzianum* metabolite 6-pentyl-pyrone reduced the growth and spore germination of *R. solani* and *Fusarium oxysporum* f. sp. *lycopersici* in *in vitro* tests, suggesting a strong relationship between pyrone production and the *in vitro* antagonistic ability of *T. harzianum* [10].

Wilhite and Strancy [11] investigated the timing of the biosynthesis of gliotoxin (epidithiodiketopiperazine) in case of *Trichoderma virens* (formerly classified as *Gliocladium virens*), a species important for the biological control of *Pythium ultimum*. The examined strain synthesized gliotoxin only within a short 16-h period during replicative growth.

Viridiofungins are aminoacyl alkyl citrate compounds of *T. viride*, with a broad antifungal spectrum. However, these compounds are inhibitors of squalene synthase, an enzyme essential for ergosterol synthesis, Mandala et al. [12] showed that the broad antifungal activity of viridiofungins was due to the inhibition of serine palmitoyltransferase, the first committed enzyme of sphingolipid biosynthesis.

The broad spectrum of antifungal compounds produced by *Trichoderma* species should be discussed in a separate review. This summary will focus on the extracellular enzymes involved in the antagonistic processes of these fungi.

Extracellular enzymes

Extracellular enzymes secreted by *Trichoderma* species play a basic role both in competition and mycoparasitism. In natural soil systems there is keen competition between the soil inhabitant microorganisms for the limited nutritional sources, such as carbon, nitrogen, iron and vitamins. A nutritional study of *Trichoderma* strains is available concerning these limiting factors [13]. The ability of 65 *Trichoderma* isolates to utilize 127 carbon sources was studied by Manczinger and Polner [14]. Besides the information on the competitive abilities of *Trichoderma* strains, such studies reveal useful information on the systematics of the genus. *Trichoderma* species are good competitors, with the ability to grow fast. Extracellular enzymes, such as cellulases and xylanases, enable them to colonize plant residues rapidly.

Mycoparasitism based on the degradation of fungal cell walls by lytic enzymes is suggested to be the main mechanism involved in the antagonism of *Trichoderma* strains against fungal plant pathogens [15]. After recognition, *Trichoderma* hyphae coil around the host hyphae, produce hooks and appresorium-like bodies, and penetrate the host cell wall. Inbar and Chet [16] showed that lectins play an important role in recognition: *T. harzianum* hyphae coiled around nylon fibers treated with concanavalin A or *Sclerotium rolfsii* lectin, producing hooks in a pattern similar to that observed with real host hyphae. Extracellular enzymes are important for the penetration and degradation of the host cell wall. The cell walls of fungi belonging to *Basidiomycota* and *Ascomycota* contain chitin and β-glucan (laminarin) fibrils embedded in a protein matrix, while the main cell wall components of *Oomycota* are β-glucan and cellulose. For the penetration of the cell wall of the host, mycoparasitic *Trichoderma* species need hydrolytic enzymes, which are able to degrade these cell-wall components.

Chitinases and β-1,3-glucanases were found to be the main enzymes involved in the degradation of the cell walls of *R. solani*, *S. rolfsii* and *Pythium aphanidermatum* [15, 17].

Chitin is a biopolymer composed of β-1,4-linked N-acetylglucosamine (GlcNac) residues. Based on the review of Sahai and Manocha [18], chitinolytic enzymes can be divided into three types: endochitinases, cleaving randomly at internal sites of the chitin microfibril producing chitotetraose, chitotriose and diacetylchitobiose; β-1,4-N-acetylglucosaminidases, splitting chitotetraose, chitotriose, and diacetylchitobiose into GlcNac monomers in an exo-type fashion; and exochitinases or chitobiosidases, releasing diacetylchitobiose in a stepwise fashion. Ulhoa and Peberdy purified and characterized an extracellular chitobiase belonging to the group of β-1,4-N-acetylglucosaminidases [19] and an extracellular chitinase [20] from *T. harzianum*. Later the chitinolytic system of this fungus was found to be more complex, consisting of six distinct enzymes: two β-1,4-N-acetylglucosaminidases and four endochitinases [21]. The induction of the chitinolytic system of *T. harzianum* seems to be an early event in mycoparasitism; it is triggered by recognition and is the first step in the antagonistic cascade [22]. Recent results clarified the role of an endochitinase of *T. virens* in the biocontrol of *R. solani* [23]: strains with the disrupted chitinase gene showed decreased biocontrol activities, while strains where the gene was over-expressed were more effective against the pathogen than the wild type strain.

β-1,3-Glucanases (laminarinases) can be divided into two classes: endo-type enzymes cleaving β-linkages at random sites along the polysaccharide chain and releasing short oligosaccharides, and exo-type enzymes releasing glucose residues from the non-reducing end of the substrate. The first *Trichoderma* β-1,3-glucanase, cleaving only glucans, was purified from *Trichoderma longibrachiatum* and characterized by Tangarone et al. [24]. The β-1,3-glucanolytic system of *T. harzianum* consists of at least seven extracellular enzymes upon induction with laminarin [25]. Glucose appeared to inhibit the formation of all of the detected enzymes. Based on the analysis of these enzymes, they might play different roles in the lysis of host cell walls.

In case of the mycoparasitic action against the oomycete *Pythium* species, the cellulases of *Trichoderma* may also play an important role in allowing the local penetration of the antagonist into the host hyphae [26]. However, the *Trichoderma* cellulases were studied extensively because of their industrial importance [27].

Other cell wall-degrading enzymes hydrolyzing minor polymers (proteins, β-1,6-glucans, etc.) may also be involved in the degradation of host cell walls. Flores et al. [28] showed that the over-expression of a proteinase-encoding gene of *T. harzianum* resulted in improved biocontrol activity, suggesting the importance of proteases in the degradation of the protein components of the host cell wall and in the lysis of whole

host cells. The role of *T. harzianum* proteases in the biocontrol of *B. cinerea* was recently investigated by Elad and Kapat [29]. An endo-β-1,6-glucanase related to the mycoparasitism of *T. harzianum* was also purified and characterized: the enzyme was found to act in cooperation with β-1,3-glucanases and chitinases in the hydrolysis of fungal cell walls [30].

Ecophysiological properties of *Trichoderma* strains

The antagonistic ability of *Trichoderma* species makes them potential candidates for biocontrol against plant pathogenic fungi as a part of complex integrated pest management. Ecophysiological studies of the genus are of great importance: biocontrol strains should be thoroughly characterized before application. Environmental stress factors, such as competitive soil microorganisms, pesticide or heavy metal contamination, low temperature and low water potential affect *Trichoderma* species significantly. Therefore, studies on the influence of these parameters on the mycelial growth, sporulation, competitive ability, enzyme secretion and enzyme activities of biocontrol strains should be carried out in the laboratory before field experiments.

Studies are available on the effects of temperature on the spore germination and germ-tube growth [31], the growth and competitive abilities [32], the saprophytic ability [33] and the *in vitro* extracellular enzyme activities [34] of *Trichoderma* strains. The influence of environmental factors, such as temperature and the inhibitory effect of bacteria, on the competitive saprophytic ability of *Trichoderma* species was investigated by Naár and Kecskés [35], and the competitive success of *Trichoderma* was suggested to be attributable mainly to its tolerance to the inhibitory effect of bacteria.

One of the most important limitations of the use of *Trichoderma* strains as biofungicides is their low osmotolerance level. The water conditions in soils are limiting parameters affecting fungal activities. Dry conditions may occur even in normally less dry soils as a result of normal drying between rains. On the other hand, biocontrol agents may be needed against plant pathogens in dry soils. The mycelial growth of *Trichoderma* is significantly affected by low water potential values. However, the *in vitro* enzyme activities of the examined strains were not influenced by the lowering of the water potential to such a considerable extent [36].

Studies on the effects of pesticides on biocontrol *Trichoderma* strains are of great importance for planning the combined application of biofungicides with chemicals. The effects of the herbicide propyzamide and five fungicides (benomyl, quintozene, vinclozolin, thiram and prothiocarb) on the colonization of substrates by *T.*

harzianum was investigated and discussed with a view to practical applications [37]. The selective effects of two systemic fungicides on soil fungi, including *T. viride*, was examined by Abdel-Fattah et al. [38]. The sensitivity of the fungal antagonists (*Chaetomium globosum* and *Trichoderma* species) of onion white rot, caused by *Sclerotium cepivorum*, to captan, mancozeb, thiram, benomyl and two dicarboximides was also evaluated and dicarboximide-resistant biotypes were selected [39].

Although several heavy metals (e.g. copper, zinc, nickel, cobalt, etc.) are necessary trace elements for the growth of fungi, they are toxic at high concentrations. The sorption of toxic metals by fungi (*Rhizopus arrhizus* and *T. viride*) and clay minerals was examined by Morley and Gadd [40]. Studies on the accumulation of zinc, cadmium and mercury by *T. harzianum* are also available [41]. When subjected to 1-millimolar concentrations of different heavy metals, the activity of the mycoparasitic enzymes produced by *Trichoderma* strains could tolerate high doses of these toxic compounds with the exception of mercury [Kredics et al. unpublished results]. Extracellular enzymes seem to remain active even under environmental conditions where mycelial growth has already ceased.

The breeding of *Trichoderma* for cold-tolerance, osmotolerance, heavy metal- and pesticide-resistance and constitutive extracellular enzyme production may result in effective mycoparasitic strains for biocontrol application against fungal plant pathogens under a wide range of environmental conditions.

Breeding by mutagenesis and protoplast fusion

Many biocontrol *Trichoderma* strains could be originally resistant to a wide variety of chemical fungicides frequently used for controlling phytopathogenic fungi. Combining fungicide resistant biocontrol strains with chemical fungicides could result in a decrease in the fungicide level in soils, which should be maintained for effective pest control. This is the basis of integrated pest management, which could be very effective and less dangerous to the ecosystem and human health than chemical control alone [42]. In some cases biocontrol strains are also sensitive to the fungicide to be applied (e.g. to the frequently used fungicide benomyl). In such cases resistant strains could be isolated by induced mutagenesis [43]. Effective pest control may require 10^5–10^6 propagula to be present in one gram of soil in case of *Trichoderma* and *Gliocladium* [44]. The presence of such high amounts of conidia is not desirable from the point of view of ecology, economy and public health. To solve this problem, the biocontrol strains of *Trichoderma* and *Gliocladium* should be bred for increased effectiveness. Strains, which do not require a long inductive process for the production of degrading enzymes, could be more effective as biocontrol agents. Generally,

biocontrol strains produce β-1,3-glucanases constitutively, some elements of the chitinase system require induction and the most effective proteases are also produced inductively [45, 46].

There are few publications regarding the breeding of mycoparasitic strains by mutagenesis. Some of these investigations report only on tests of random isolates following mutagenic treatments, others write about obtaining fungicide resistant mutants, which could be used in integrated pest management and only some of them deal with the mutagenic improvement of the enzyme secretion abilities of the strains [47, 48]. More attention should be paid to the mutagenic methods of breeding, because strains bred by mutagenesis can get registration for on field use from environmental protection agencies more easily than strains produced by protoplast fusion, transformation or via gene cloning.

Protoplast fusion is a quick and easy method for combining the advantageous properties of distinct promising strains. It was successfully applied in the breeding of *T. harzianum* biocontrol strains [49, 50]. The parasexual cycle of *Trichoderma* has some interesting phenomena, which were first published for *Trichoderma reesei* [51]. The parameiotic behavior was later also published for *T. harzianum* and *T. viride* [52]. Protoplasts could be produced easily from biocontrol *Trichoderma* strains and their induced fusion resulted in genetic recombinants with elevated biocontrol abilities in many instances [53].

A progeny from the fusion between *T. harzianum* strains T95 lys- and T12 his- showed improved growth, rhizosphere competence and control activity as compared with the parental strains and has recently been registered as a microbial pesticide (F-Stop R) [49].

In order to improve the properties of antagonistic *T. harzianum* isolates by genetical means protoplast fusion was attempted between 3 strains isolated from soil samples in Italy and Israel, using sclerotia of *Sclerotinia minor* as baits [54]. Protoplasts from *T. harzianum* isolates and from their auxotrophic mutants obtained after N-methyl-N'-nitro-N-nitrosoguanidine treatment could be produced efficiently by incubating the mycelium in a crude cell wall lytic enzyme solution containing NovoZym 234 and Lytic enzyme No. 2 from *Cytophaga*. The fused protoplasts of complementary auxotrophic strains could be selected as heterokaryons on selective medium. They produced prototrophic mycelium and the conidia of both parents were produced during sporulation.

When two different auxotrophs of the same *Trichoderma* strain (*T. harzianum*, *T. hamatum*, *Trichoderma koningii* and *T. viride*) were fused, rapidly growing prototrophic progenies were obtained at high frequency [52]. Progenies from these intrastrain fusions appeared to be balanced heterokaryons and no evidence of

recombination between the two parental strains was obtained. Following interstrain fusions presumptive somatic hybrids developed very slowly and in low numbers as compared with hybrids from intrastrain fusions. The authors concluded from their results that protoplast fusion in the genus *Trichoderma* gave rise to great variability, but the classical parasexual cycle was not required for variation to occur.

Protoplasts of high purity could be obtained from the young thalli of strains T12 and T95 of *T. harzianum* by the digestion of cell walls with NovoZym 234. Protoplasts fused by polyethylene glycol regenerated readily. On minimal medium the fusion between the lysine-requiring and histidine-requiring auxotrophs of strain T95 gave rise to about 10% of the number of colonies produced on minimal medium supplemented with histidine and lysine. Nearly all conidia from these prototrophic colonies were auxotrophic. The number of those requiring histidine and those requiring lysine was approximately equal. These results indicate that the prototrophic progenies of this fusion were balanced heterokaryons, with the parental nuclei present in equal numbers.

Conversely, fusion between T12 his- and T95 lys- strains gave rise to unbalanced heterokaryons with T12 predominating. The frequency of prototrophic formation was less than 0.01% and the original isolates grew very slowly, but a progeny showed improved growth, rhizosphere competence and control activity as compared with the parental strains [55].

Protoplast fusion techniques were used for the production of new antagonistic strains from two British and two Italian strains of *Trichoderma* with different biocontrol potentials against *B. cinerea, Sclerotinia sclerotiorum* and *P. ultimum* [56]. The strains were treated with UV radiation and mutants tolerant to hygromycin B or propiconazole were selected. Hybrids were obtained in 13 crosses, isolated on fungicide-amended media based on the inheritance of resistance markers from both parental strains. Selected fast-growing, stable mutants were tested in biocontrol trials against *P. ultimum* on lettuce seedlings and *B. cinerea*. No significant increase was achieved in the activity after fusion; the hybrids were generally less active than their parental strains.

Protoplasts were fused from two auxotrophic mutants of *T. harzianum* (ATCC 32173), obtained from young thalli following cell wall digestion by NovoZym 234 [53]. The antagonistic abilities of the prototrophic strains were found to vary with each pathogenic fungus. Strain A2 controlled effectively the *Rhizoctonia* damping-off of cotton seedlings in the greenhouse, when compared with the parental strains. It was concluded that protoplast fusion appeared to be a useful tool for combining desirable traits from parental strains to produce improved biocontrol strains.

The level and pattern of the rhizosphere competence of *T. harzianum* strain 1295-22, derived from fusing protoplasts of auxotrophic mutants of the prototrophic

strains T12 and T95, were studied and compared with those of the original strains [49]. Both strains T12 and T95 colonized the entire length of maize roots, but strain 1295-22 was more effective in colonizing the middle sections of the roots than either parental strain. So in this experiment a hybrid with improved rhizosphere competence was obtained by protoplast fusion between the benomyl tolerant *T. harzianum* strain T95 and *T. harzianum* strain T12.

A variety of auxotrophic mutants were prepared from several species and strains of *Trichoderma* by nitrosoguanidine. Following intrastrain fusions, $2–10\times10^{-2}$ of the viable colony forming units grew under selective conditions regardless of the auxotrophs involved, indicating that induced heterofusions were frequent and nutritional complementation was functional. In interstrain fusions, however, only $1–20\times10^{-5}$ of the viable colony forming units produced colonies under selective conditions, indicating a low level of compatibility [57]. The restricted growth of these somatic hybrid colonies, which were not heterokaryotic, appears to result from the fusion of heterologous protoplasts.

Limited compatibility may reduce the likelihood of parasexual recombination, but does not preclude the possibility of the genetic manipulation of *Trichoderma* strains by protoplast fusion.

Intergeneric protoplast fusions were attempted between *G. virens* and *T. harzianum* to obtain viable fusion products [58]. Auxotrophic mutants of *G. virens* G88 and the benomyl resistant *T. harzianum* T95 were used to isolate fusants. Fusants obtained from the intergeneric protoplast fusion segregated spontaneously into various strains during continuous culturing on complete medium. The several intergeneric hybrids were classified into three types: parent-like hybrids, segregants, and recombinants.

Breeding by cloning and transformation

Breeding by introducing the target genes directly into the cells can be more effective than breeding by mutagenesis or protoplast fusion. The realization of this transfer of genes requires the availability of efficient transformation systems and cloned genes.

Many publications deal with the transformation methodology of *Trichoderma* strains. In general, transformation is performed by using protoplasts, because these wall-less cells are easy to produce from most strains, but there are some alternative technologies, such as biolistic [59, 60] and *Agrobacterium tumefaciens* mediated [61] procedures. The most frequently used methods for the selection of transformants are based on dominant markers, such as fungicide resistance genes (beta-tubulin gene [62],

hygromycin B phosphotransferase gene [62, 63, 64, 65]), auxotrophy-complementing genes (arginin auxotrophy [66], uracil auxotrophy [67, 68]) and acetamid-catabolitic genes [69], while others are based on the activities of reporter genes (green fluorescent protein [70], β-galactosidase gene [66, 69], β-glucuronidase [59]). As the frequency of transformation was under the desirable level in many cases, further research was performed to increase the frequency by using homologous genes [71, 72, 73], homologous expression signals [74] or nuclease inhibitors [64]. Mitotic instability can also reduce the number of transformation progenies. There is evidence for a correlation between the stability of transformants and the integration of the vector into the genome, whereas when extrachromosomal plasmid molecules occur, unstable transformants are obtained.

In order to improve the biocontrol efficiency of the mycoparasitic *Trichoderma* strains by transformation, genes of enzymes important in the mycoparasitic process were cloned and used for gene transfer. Most frequently chitinase genes [23, 75, 76], in some cases protease [28] and β-1,3-glucanase genes [77] were used to construct transformant strains with an increased copy number of the genes. In some cases strains with increased biocontrol efficiency could be found among the derivates. Another benefit of transformation comes up in the monitoring of strains, where the specific *Trichoderma* strains released into the soil are genetically marked. The reporter genes of β-glucuronidase and green fluorescent protein could provide a valuable tool for this purpose [70, 78, 79].

Although each of the three methods of breeding is suitable to obtain biocontrol strains with better ecophysiological properties, all of them have both advantages and disadvantages, and until the *Trichoderma* strains bred by protoplast fusion and transformation can hardly get registration for on field use from environmental protection agencies, mutagenesis remains the main tool of breeding for the practice.

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