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Selection of *Grifola frondosa* and *Fomitopsis pinicola* strains resistant to *Trichoderma viride* in teflon tubes confrontation method

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The aim of this study was to select fast growing strains able to resist *Trichoderma viride* in a short-time experiment to be realized with a concurrently performed fruiting experiment. The test was realized in Teflon® tubes. Five strains of *Grifola frondosa* (Dicks : Fr.) Gray and five strains of *Fomitopsis pinicola* (Swartz : Fr.) Karst. were used as test organisms. Different factors that can influencing substrate invasion rate and resistance to *T. viride* were analyzed: substrate composition, quantity of substrate included in the tubes and colony size of the tested strain at the time of the inoculation of the pest.

Results sometimes showed great variations in behavior between strains, but also for a given strain growing on different substrates, indicating the importance of trophic factors in the resistance capacity. We also showed that substrate quantity and colony size can modify the capacity to resist. However there was no correlation between growth rate on agar-malt medium, substrate invasion rate, resistance to *T. viride* and fruiting capacity, showing the importance to test these different factors to select the best strains which can be further recombined.

In mushroom culture, many pests and diseases can negatively affect the production. The major problems depend on the cultivated species, on the country and on the infrastructures. However among the pathogens, hyphomycetous fungi including *Trichoderma* are the most common in mushrooms cultures (GOLTAPEH & DANESH 2000, DELMAS 1989).

Mechanisms involved during competition between two fungi can be the competition for nutrients, chemical interference by production of antibiotics and extracellular enzymes (SAVOIE et al. 2000) or mycoparasitism (ELAD, CHET & HENIS 1982). Fast strains are important to compete *Trichoderma* spp. as this genus has a fast invasion rate with good capacities for nutrient use, together with a high reproductive rate. In the antagonism between *Lentinula edodes* and *Trichoderma* spp. for instance, it is in the first days that the competition takes place (SAVOIE et al. 2000). Moreover to stop the colonisation of an antagonist it is better to use natural abilities of fungi to produce antibiotics and extracellular fungal cell-wall degrading enzymes (SCHIRMBÖCK et al. 1994) which has already been shown to be variable between strains and in function of the substrate composition (MATA & SAVOIE 1998). Resistant strains should allow less loss during culture but also a better chance to find antibiotic substances like those demonstrated for the resistant strains of Shiitake for instance (TOKIMOTO & KOMATSU 1995). Indeed secondary metabolites might act as chemical weapons to defend a substrate against competitors (STERNER et al. 1985), but can also bring interesting new lead compounds for further studies on antimycotic agents. It is particularly interesting in the case of the pest *Trichoderma* spp. as there are only two existing fungicides, Benlate® and Thiophanate-methyl (Senator 70WP) against this pathogen in mushroom culture, and the first is no longer available on the market.

The worst mushrooms antagonist encountered during all culture development research in our laboratory was a fungus of the genus *Trichoderma*. It is very virulent and capable of attacking and killing the mycelium of certain cultured species in a few days. To our point or view, aptitude to competition should therefore be a parameter to select in screening for new strains to be cultivated, in addition to fruiting capacity.

Grifola frondosa (Dicks : Fr.) Gray and *Fomitopsis pinicola* (Swartz : Fr.) Karst are 2 species which culture has been developed in our laboratory and which mycelium is sensitive to *Trichoderma viride* in some conditions.

G. frondosa is a prized cultivated edible polypore showing many biological activities and in particular antitumoral (KODAMA et al. 2002, MAYELL 2001, ADACHI et al. 1998, SUZUKI et al. 1985), immunostimulant (ATSUYUKI & NANBA 2002, NANBA et al. 2000, MAO et al. 1999), against hypertension (ADACHI et al. 1988), anticholesterolemic (KUBO & NANBA 1997) and in vitro inhibition of adipocytes conversion (NAKAI et al. 1999), antidiabetic (HIROYUKI & MASARU 2001, KURU-

Tab. 1: substrates composition, moisture and C/N coefficient

	Straw [% dry weight]	Sawdust [% dry weight]	Birdseeds* [% dry weight]	Wheat flour [% dry weight]	Industrial compost [% dry weight]	Moisture [% fresh weight]	C/N coefficient
Substrate A	40	40	15.7	3	1.3	73–76	75
Substrate B	80	20	0	0	0	73–76	90
Substrate C	40	40	10	0	10	68–71	125
Substrate D	10	60	10	0	20	68–71	265

*Seeds of the grass *Phalaris canariensis*

SHIMA, KODAMA & NANBA 2000), hepatoprotective (LEE et al. 2000), with positive impact in HIV patients in a long-term trial (NANBA et al. 2000). *F. pinicola* is a non edible polypore which only showed antibacterial activity against *B. subtilis* (KELLER, MAILLARD & HOSTETTMANN 1996), however its resistance potential toward bacteria and some hyphomycete fungi was already partially studied in our laboratory and showed interesting behaviours.

Thus we have chosen these two species to evaluate different strains' ability to invade a substrate quickly and to compete with the damaging pest *T. viride* on different substrates varying by their C/N coefficients in a Teflon tubes system in view to select fast and resistant strains.

Material and methods

Strains

Grifola frondosa.– Industrial strains: GfNE (Switzerland), GfSt (USA), GfJ (Japan). Strain from culture collection: MUCL 31544 (=ATCC 48141) (MUCL culture collection, Louvain-La-Neuve, Belgium), named GfB for our convenience. Recombinant strain between the industrial strain GfNE and a wild strain J1397: GfNE-J1397.

Fomitopsis pinicola.– Culture collection strains: MUCL 30677 and MUCL 30544 (MUCL culture collection, Louvain-La-Neuve, Belgium) and the wild strains FpK1, FpK2, FpK3, collected and identified by Dr. Jean Keller, and isolated during this work.

Trichoderma strain was isolated from *G. frondosa* strain GfNE contaminated substrate B bags and determined as *Trichoderma viride* Pers. by optic microscope characteristics.

Maintenance of the strains: they are all maintained at 4 °C in 2 % agar-malt (AM) medium in the mycotheca of the University of Neuchâtel, Switzerland, and transferred to a new medium once a year.

Somatic incompatibility test

Dual confrontation between the 5 strains of *G. frondosa* and the 5 strains of *F. pinicola* have been done by inoculating a mycelium plug two by two in a 2 % AM Petri dish and incubating the pairs at 25 °C until the contact of the colonies (4 replicates) according to WORRALL (1994).

Growth measurements on 2 % AM medium

For measuring mycelium front advance, a few days precultivated agar slant supporting the strain was inoculated in the center of the Petri dish containing 2 % AM medium. Mycelium was allowed to grow at 25° C in the dark. 25° C is the standard incubation temperature where most of *T. viride* infection problems occur in our research programs. Thus, temperature for all the experiments was fixed at 25°C. Mycelial elongation was measured (mm) between day 2 and day 5 and between day 5 and day 8 after inoculation, on 4 radial lines drawn before inoculation. Two replicates were realized.

Vegetative resistance on 2 % AM medium

For testing resistance of the strains, both tests on AM medium in Petri dish and on the four different substrates in tubes were realized. Resistance of the strains on 2 % AM medium was evaluated by inoculating a young conidiating mycelium plug from *T. viride* at the other end of the petri dish than the tested species colony with different diameters: 2, 4 and 6 cm and on the mycelium itself. Incubation was realized in the dark at 25° C and *T. viride* progression observed daily until no more changes happened. Resistance was determined by the time (days) the strains could resist *T. viride* when in contact.

Substrates preparation Any new species to be cultivated in our laboratory is first tested for fruiting capacity on four substrates varying in their C/N coefficient from 75 to 265 (Tab 1): substrates A, B, C and D, in the order of increasing values (or decreasing nitrogen content). They were therefore chosen to be the substrates tested during these selection tests. The four substrates were prepared according to JOB, SCHIFF & GIOVANNINI (2004). It was packed into polypropylene bags with incorporated filter (bottom area 12 x 20 cm, height 35 cm). Bags of substrates A, B and C weighted about 1.8 kg while bags of the more compact substrate D weighted about 2.2 kg. The substrates were then pasteurised 10 hours at 90 °C. Substrates were either used to fill up the tubes for invasion and resistance analysis, or used directly for fruiting experiments.

Tab. 2: Time of inoculation of *T. viride* in days after the start of the experiment

Species and strains	days				
	Tubes a-b	Tubes c-d	Tubes e-f	Tubes g-h	Tubes i-j
***G. frondosa* GfSt**	6	20	35	58	58
***G. frondosa* GfJ**	6	20	35	65	65
***F. pinicola* FpK1**	5	7	10	20	25
***F. pinicola* FpK2**	5	7	10	16	21

Tubes preparation and substrate invasion rate

Translucide, autoclavable Teflon® FEP tubes (Fisher Scientific, Wohlen, Switzerland) were used. The Teflon tubes, internal diameter 14,4 mm, were cut at a length of approximately 15 cm. Metallic caps for 16 mm test tubes were used to close the two opened sides after filling with the different pasteurised substrates. The tubes were sterilised in an autoclave at 121 °C for 1/2 hour. The strain to be tested was inoculated sterilely with a 12 mm diameter disk of mycelium on 2 % AM medium at one end of the tube and allowed to grow in the dark at 25 °C.

Influence of substrate quantity on resistance

Tubes were filled with 3 g, 5 g, 7 g or 9 g B substrate by the maximum hand compressed substrate at the and then allowed to extend naturally. Length after autoclaving: 35 mm, 55 mm, 75 mm, 90 mm respectively. Three lines were drawn along the tubes to determine place of measures of substrate invasion rate and *T. viride* penetration, before the tested strain inoculation. Substrate invasion rate was reported to a value per 24 hours. *T. viride* was inoculated 41 days after the start of the experiment with each of the tested strain. *T. viride* penetration in the tested strain colony was measured along the lines by observation of the green conidiation 52 and 103 days after the start of the experiments for *F. pinicola* and *G. frondosa* respectively. Two replicates were realized.

Influence of the colony size on resistance

For this experiment, two strains of each species were tested. Tubes were filled with 7 g of B substrate with the same density than in the culture bags. Six lines were drawn along the tubes. Twelve tubes (each one noted by the letters “a” to “l”) were inoculated with each of the strains at opposite ends of the substrate colonisation (Tab 2). Tubes “k” and “l” were used as blanks.

Influence of substrate type on resistance

In each tube, 9 g of substrate A, C or D were filled at the same density as in the culture bags. Six lines were drawn along the tubes. Each tested strains was inoculated and substrate invasion rate was measured two times until day 13. *T. viride* was inoculated on day 30 after total invasion of the substrates by all the strains. *T. viride* penetration was measured in the same manner as previously described.

Statistical test

The results having been interpreted employing the Student’s-T test (Job & Rajchemberg, 1988) and the ANOVA test (Diorio et al., 2003).

Fruiting ability

The 5 strains of each species were inoculated sterilely in bags of substrates A, B, C and D by adding about 2 % of the substrate weight sterile birdseeds totally invaded by the strain’s mycelium on the top of the substrates without mixing. Incubation was usually performed at 25 °C ± 1 °C in the dark and bags transferred to the fructification room (Cenviron, Canada) at 18–19 °C with high humidity level (91–100 %), constant air exchange and 6 h light cycles per 24 hours, when primordia appeared. The filters were removed when primordia started to differentiate into a fruit body.

Results

Somatic incompatibility between all the *G. frondosa* and *F. pinicola* strains have been proved in dual confrontation by the presence of a thick line at the meeting of the two colonies often with orange or brown discoloration.

In all experiments on 2 % agar-malt medium with the 5 *G. frondosa* strains, *T. viride* was able to continue its development over *G. frondosa* mycelium without any growth rate decrease. The 5 *F. pinicola* strains however were able to stop the progression of *T. viride* during several days in the mycelium contact zone before to be overgrown, and the resistance duration were inversely proportionaby to the *Fomitopsis* colony diameter (significantly different P = 0.03) as show in Tab. 3.

To evaluate the influence of substrate quantity in the tubes for testing resistance, the five strains of the two studied species were inoculated on various quantities of substrate B. Fig 1a shows substrate invasion rate of all the strains but the data obtained for the different substrate quantities are not significantly different (P = 0.03) in both species. There is no an strong influence of aeration and substrate drying in small substrates quantities to produce differences in invasion rate values. Fig 1b shows *T. viride* penetration in the substrates previously fully colonised by the same strains. It clearly shows that *F. pinicola* strains become resistant on that substrate only when

Tab. 3: Time of resistance (in days) of the *F. pinicola* strains at the contact zone with *T. viride* with different colony diameters. The resistance of the 3 colony diameter groups were significantly different (P = 0.03)

Strains	Resistance duration (days) at 2 cm diameter	Resistance duration (days) at 4 cm diameter	Resistance duration (days) at 6 cm diameter
Fpk1	17	13	9
Fpk2	22	8	4
Fpk3	8	4	0
Fpk 30544	12	11	3
Fpk 30677	11	6	2

Tab. 4: Mean colony length (mm) in the substrate of *G. frondosa* GfJ and GfSt strains and *F. pinicola* FpK1 and FpK2 strains at the inoculation of *T. viride* .The total length of the substrate was about 67 mm

	Tubes a-b	Tubes c-d	Tubes e-f	Tubes g-h	Tubes i-j
***G. frondosa* GfJ**	0	32	60.6	67	67
***G. frondosa* GfSt**	0	26.5	52.3	67	67
***F. pinicola* FpK1**	17.5	25	36	57.8	67
***F. pinicola* FpK2**	19	27.8	42.1	65.9	67

Tab. 5: fructifying ability of 4 strains of *G. frondosa* and 3 of *F. pinicola*. - = no primordia formation, P = primordia not differentiating into fruit bodies, F= well formed fruit bodies

	Substrate A	Substrate B	Substrate C	Substrate D
***G. frondosa* GfSt**	F	F	F	F
***G. frondosa* GfJ**	–	–	F	–
***G. frondosa* GfNE**	–	F	–	–
***G. frondosa* GfB**	P	–	F	–
***F. pinicola* FpK2**	P	–	P	P
***F. pinicola* Fp30544**	P	P	P	F
***F. pinicola* Fp30677**	–	–	F	F

at least 5 g substrate has been colonised. Results obtained with *G. frondosa* strains however are not explained and are not statistical significantly.

To evaluate the influence of colony size at the time of inoculation of *T. viride*, two strains of each species were inoculated on 7 g B substrate and mycelium was allowed to grow to different extends before the pathogen inoculation (Tab 4). Final *T. viride* penetration in the four strains colonies is shown in Fig 2. For the four tested strains, colony size at the moment of confrontation with *T. viride* had an influence on their resistance capacity. FpK1 strain and FpK2 strain were shown to enhance their invasion ability the day following *T. viride* inoculation and then lower it drastically even before being in contact with the pathogen. This might show an early detection of *T. viride* at a distance by the *F. pinicola* tested strains and then an inhibition probably caused by antibiotic production by *T. viride* also at a distance. This was not observed with *G. frondosa* strains.

To evaluate influence of substrate type on resistance of the strains and validity of the method, 9 g of 3 different substrates types were inoculated with each of the tested strain. Substrate invasion rates are shown in Fig 3a, where results obtained on 9 g B substrates of the first experiment were added for comparison. All *G. frondosa* strains grew better on A substrate which contains the higher Nitrogen content, while *F. pinicola* strains all invaded the C substrates faster than the others. After total invasion of the substrate, *T. viride* was inoculated. Final penetration of *T. viride* in the colony of the tested strains is shown in Fig 3b. These results clearly show that resistance ability can depends of the strain (in *G. frondosa*) and also greatly from substrate composition (in the two studied species).

Tab 5 shows whether strains were able to induce fruiting cycle or not, and in the first case either they aborted to primordia stage or completed their reproduction cycle. *F. pinicola* fruit bodies were only obtained on substrates with the lower Nitrogen content.

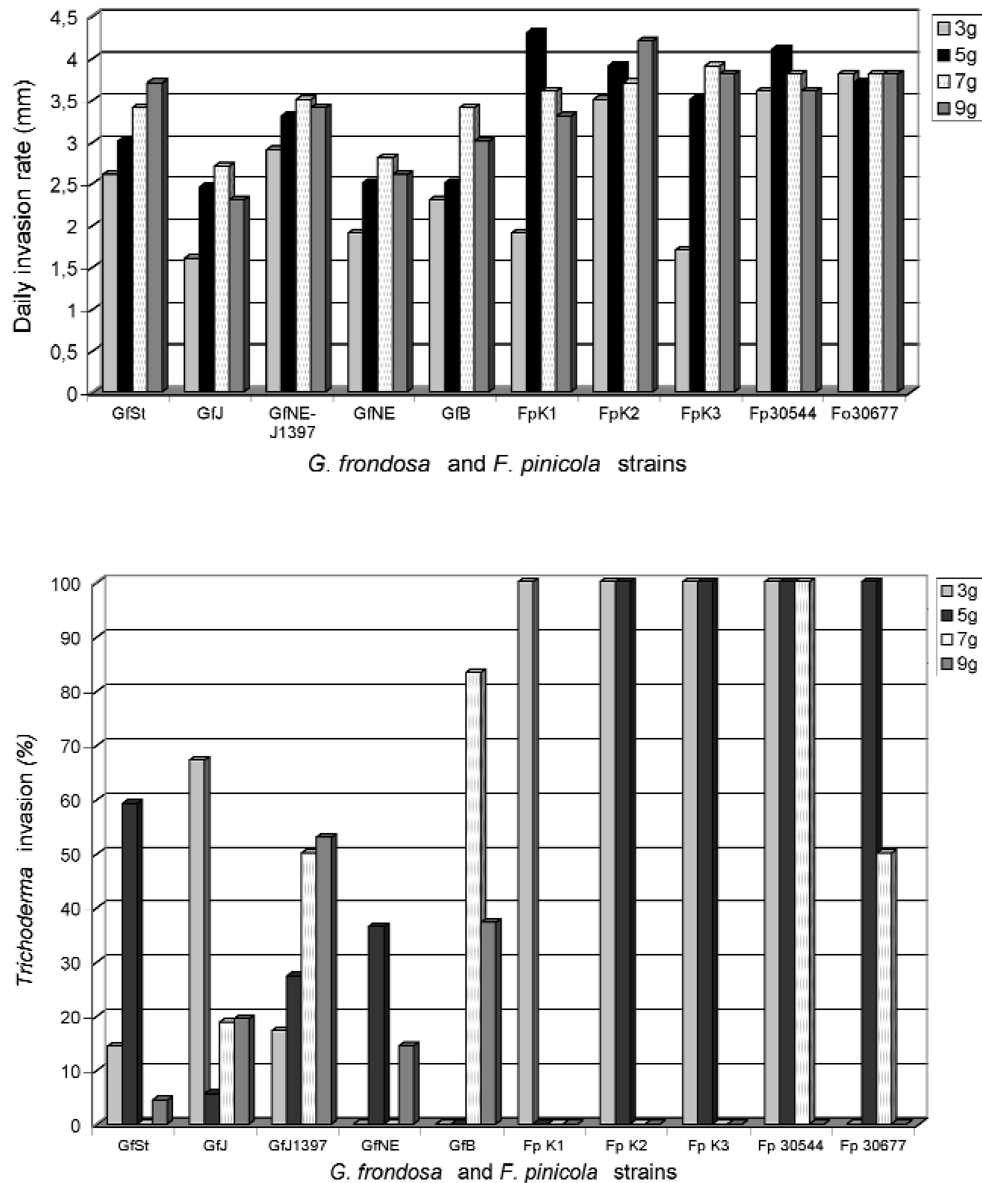

Fig. 1: a. Daily invasion rate (mm) in various B substrate quantities by *G. frondosa* and *F. pinicola* strains. The invasion rate data obtained for the different substrate quantities are not significantly different (P = 0.03) in both species. – **b**. Final *T. viride* penetration (%) in different quantities of substrate B invaded by the strains of *G. frondosa* and *F. pinicola*.

The results of the ANOVA statistical test evidence the influence of the substrate quantities in *T. viride* resistence for *F. pinicola* (F: 19.5, probability of null hypothesis 0.003) but not in *G. frondosa* (F:1.325¸ probability of null hypothesis 0.233).

Discussion

The system in Teflon® tubes seems to be adequate for testing both substrate invasion and competition ability of strains on different substrate in a relatively short term experiment. It allows a good visibility of the interactions because of their transparency. This kind of system was previously used to determine *Lentinula edodes* competition ability against *Trichoderma* spp. by strains selection in glass tubes (Tokimoto & Komatsu 1995) and by environmental and cultural conditions analysis in drinking straw (Badham 1991). If in our experiments only substrate composition and genetic variations between strains were considered, other factors like temperature could also be analyzed. Indeed the competition mechanism clearly depends, among other factors, of the temperature, which has been shown to influence *Trichoderma* mycelial growth (Samuels 1996) and competitive ability (Badham 1991).

For both species and all strains tested, it is clear that not only there are different behavior between strains, but also for a given strain grown on different substrates. Our results agree with those obtained by Savoie et al. (2000) in the study of the

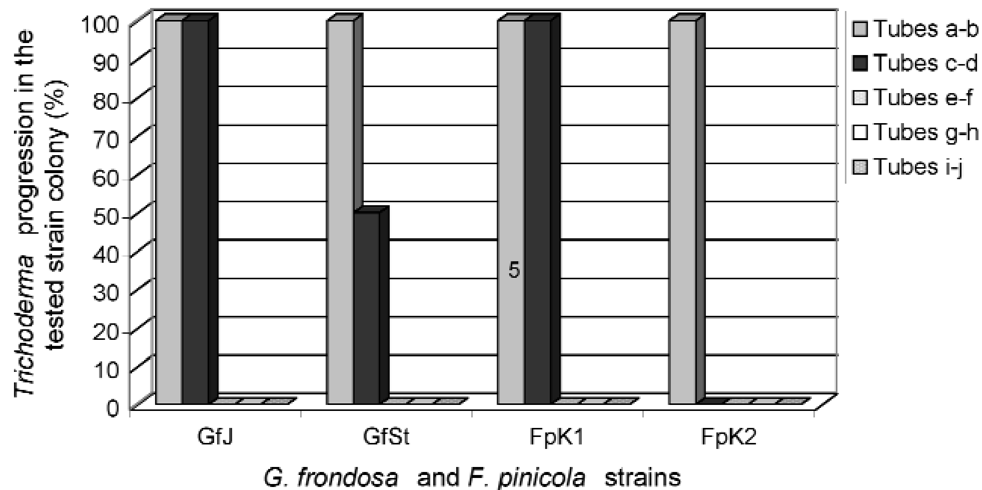

Fig. 2: *T. viride* penetration (%) in the substrate previously invaded with different colony sizes of G. *frondosa* GfJ and GfSt strains and with *F. pinicola* FpK1 and FpK2 strains.

The results of the ANOVA statistical test evidence the influence of the colony size in *G. frondosa* (F: 46, probability of null hypothesis 0.000) and *F. pinicola* (F: 8.5¸ probability of null hypothesis 0.003) *T. viride* resistence.

interaction between *L. edodes* and *Trichoderma* spp, where it was observed that there are great differences between two modified substrates for each strain. In the same manner TOKIMOTO & KOMATSU (1979) have observed that a C rich medium favours *L. edodes* while a N rich medium favours *Trichoderma* and that damage could be reduced by controlling nutritional conditions. In our experiment it seems that the opposite is observed for *F. pinicola*: the substrate with the lower Nitrogen content was favourable to *Trichoderma*. GOLTAPEH & DANESH (2000), TOKIMOTO & KOMATSU (1995), BADHAM (1991) have also demonstrated that substrate composition has an influence in the interaction between two fungi.

Results showed that growth rate on AM medium was not extrapolable to substrate invasion capacity of the different strains. Thus these tests in tubes allow better one to select a fast colonising strain able to compete with *T. viride* for nutrient uses on chosen substrates. Pattern of hyphae branching is, however, not taken into account in the mycelial elongation measures so that the final fungal biomass and substrate utilisation could differ between substrates for a given strain. Invasion rate, competition ability and fructification capacities were not correlated either. For instance *F. pinicola* strain MUCL 30677 fructified on C and D substrates on which it could not resist at all against *T. viride* contrary to the other two substrates where it did not even form primordia. GfSt was the slowest strain on 2 % AM medium and it is also the only strain which was never totally colonized by *T. viride*, except with small substrate quantities or small colony size. This is contrary to what was observed by TOKIMOTO, KOMATSU & FUKUMASA-NAKAI (1994) who showed by multiple regression analysis that *L. edodes* damage by *T. harzianum* in the substrate was predicted by damage level in the sawdust medium and growth level of the mycelium on Agar medium. Our results show the importance of testing 3 characteristics, that's to say growth rapidity, *Trichoderma* resistance ability and fruiting ability of different strains, to select the best ones. The best strains could be recombined to obtain the desired final characteristics.

Interaction between the studied species and *T. viride* varied between strains and substrate formulation. However selection of fast growing strains resistant on given substrates is possible by using Teflon® tubes. As colony size of the tested strains has shown to also influence resistance ability, *T. viride* should be inoculated a few days after the tested strains in a range of tubes and when the entire substrate is invaded by the tested strain in another range of tubes, in view to select strains able to resist already early in the invasion stage.

In a further trail, this method will be adapted by testing tens of species. Then the invaded substrate of resistant strains should be extracted to be tested in a bioassay against *T. viride* Antifungal compounds should be isolated.

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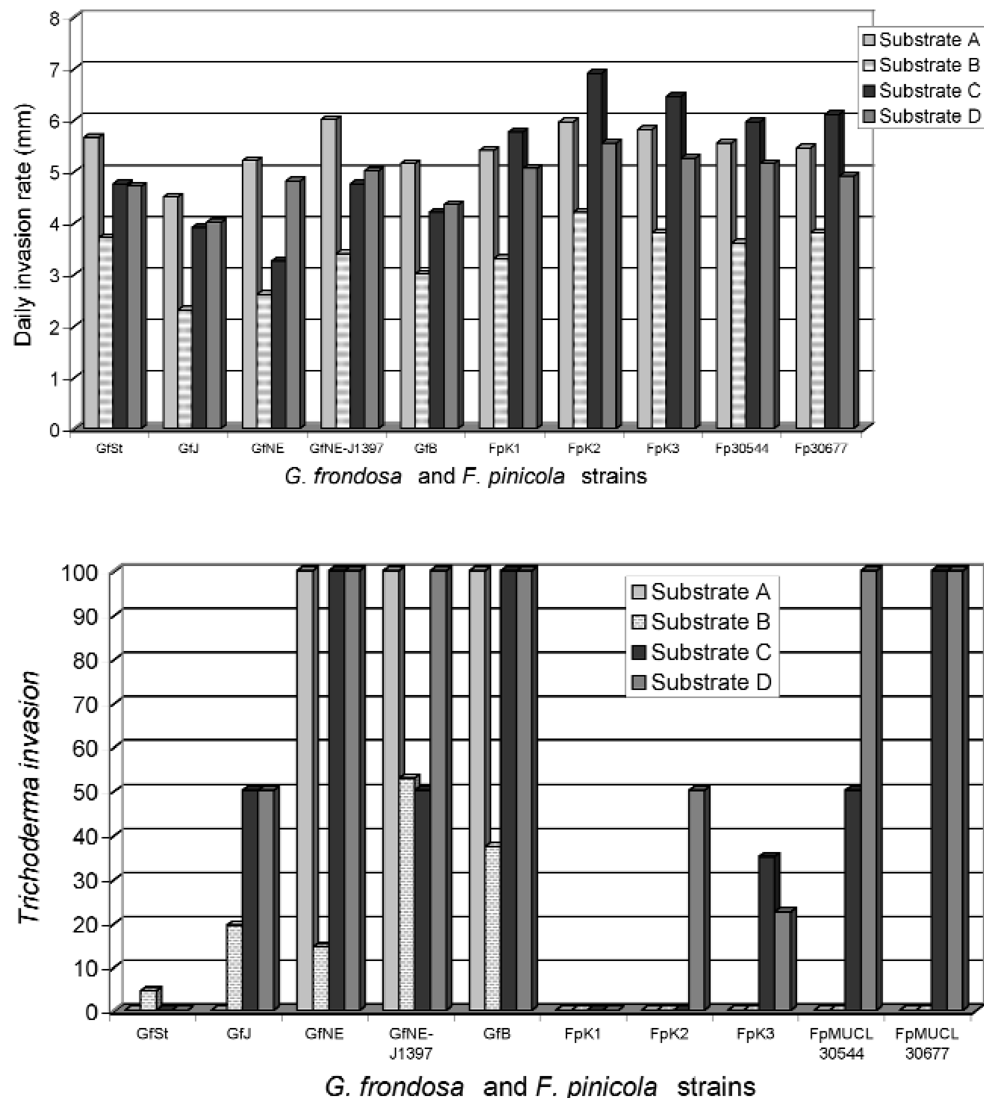

Fig. 3: a. Daily invasion rate (mm) of the five strains of *G. frondosa* and *F. pinicola* in substrates A, B, C and D. The results of the ANOVA statistical test evidence the influence of the substrate type in the invasion rate of *G. frondosa* (F: 23.73, probability of null hypothesis 0.000) and *F. pinicola* (F: 53.27¸ probability of null hypothesis 0.000). – **b**. Percentage of *T. viride* penetration (%) in A, B, C and D substrates previously colonised by *G. frondosa* or *F. pinicola* strains.

The results of the ANOVA statistical test evidence for *G. frondosa* the influence of the strain in the resistence to *T. viride* (F: 13.39, probability of null hypothesis 0.000) but not for *F. pinicola* (F: 1.272, probability of null hypothesis 0.324). The Student's-T test show for *G. frondosa* that in the b substrate the resistence was significantly different (P = 0.03) compare with the others and for *F. pinicola* the resistence were significantly different (P = 0.03) in the a and b substrate compared to the c and d.

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