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SHORT COMMUNICATION

Control of pathogenic PAC strains by non-pathogenic PAC strains *in planta* does not correlate with higher competitiveness of non-pathogenic PAC strains *ex planta*

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Abstract Ascomycetes of the *Phialocephala fortinii* s.l.—*Acephala applanata* species complex (PAC) are frequent root endophytes of forest trees. Roots are colonized by multiple PAC genotypes that interact, and recent findings indicate that adverse effects on plant performance caused by pathogenic PAC strains are attenuated by non-pathogenic PAC strains. However, it was not known if this "self-control" works only *in planta*, or also *ex planta*, i.e., prior to infection during saprotrophic life of the PAC. Interactions between PAC strains were therefore studied in a plant-free system on malt extract agar. The mycelia of two pathogenic (A and T1) and two non-pathogenic (B and C) PAC strains were mixed pairwise 5:1, 1:1 and 1:5 (fresh weight ratios) and incubated at 15 and 25 °C. Mycelial biomass of each strain was measured after 2 and 8 weeks. The combination of strains and the mixture ratio had a significant effect on strain biomass, whereas temperature influenced only the biomass of pathogenic strain T1. Biomass production of strain T1 was inhibited by all other strains, whereas biomass production of the other pathogenic strain A was significantly stimulated by the two non-pathogenic strains. This contrasts strongly with results from a previous experiment *in planta* using strains A, B and C, because the two non-pathogenic PAC strains successfully inhibited the pathogenic strain, probably by space occupation or the induction of host resistance. Therefore, it is impossible to predict the outcome of PAC-PAC interactions *in planta* based on the results gained from interactions *ex planta*.

Keywords Microsatellite-based quantification · Mycelial biomass · Competition · Root endophytes · Dark septate endophytes (DSE)

Introduction

Ascomycetes of the *Phialocephala fortinii* s.l.—*Acephala applanata* species complex (PAC) are ubiquitous tree-root endophytes and dominate the endophytic assemblages in roots of conifers and members of the Ericaceae in the Northern hemisphere (Addy et al. 2000; Queloz et al. 2011; Grünig et al. 2007, 2008a, b; Sieber 2002; Ahlich and Sieber 1996; Holdenrieder and Sieber 1992). PAC form multicellular sclerotia and chlamydospores in phellem cells of roots exhibiting secondary growth and in cortex cells of primary roots (Tellenbach et al. 2010; Sieber and Grünig 2013). PAC can behave as commensals, mutualists or opportunistic pathogens, depending on genetic traits and environment, and may affect plant performance (Grünig et al. 2008b). However, mutualistic behaviour in terms of plant growth stimulation was not exhibited by any of more than 30 PAC strains (Tellenbach et al. 2011). All strains reduced the host's growth increment, but some strains provided control of soil-borne pathogens and significantly reduced plant mortality (Tellenbach et al. 2011; Tellenbach and Sieber 2012).

PAC species and genotypes live spatially closely together in root cells, making competition for space and nutrients highly probable (Sieber and Grünig 2006; Grünig et al. 2008b; Queloz 2008; Queloz et al. 2005; Reininger et al. 2012). The mechanisms of competition may include the production of allopathic metabolites and toxins (antibiotics) inhibitory to other PAC species or genotypes, mycoparasitism,

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occupation of infection sites and the induction of systemic resistance (Harman et al. 2004; Pareja-Jaime et al. 2010). However, mycoparasitism or formation of barrier or inhibition zones between different PAC genotypes interacting on cellophane overlaid on water agar could never be observed (Sieber, unpublished). Colonies of different genotypes merge and hyphal growth is mutually limited only physically by the presence of hyphae, or biochemically by nutrient depletion. Thus, PAC species probably compete directly for space and nutrients on a first-come, first-served basis. Nevertheless, there seems to be a kind of "self control" among PAC genotypes, i.e., pathogenic strains are controlled by non-pathogenic strains as demonstrated recently by Reininger et al. (2012). The growth increment of plants singly inoculated with a pathogenic PAC strain was strongly reduced compared to that of plants dually inoculated with the same pathogenic strain and a non-pathogenic strain of PAC, indicating that non-pathogenic PAC strains successfully compete with pathogenic strains, probably by space occupation or the induction of systemic resistance. PAC "self control" *in planta* seems to work independently of temperature (Reininger et al. 2011a, 2012), an important finding particularly with regard to global warming. However, the contribution of the interaction between the PAC strains during the saprotrophic phase outside the host (*ex planta*) prior to the infection of the roots to this "self control" system could not be differentiated from the contribution of the interaction between the PAC strains during the biotrophic phase inside the host (*in planta*). Therefore, a plant-free experiment was designed to measure the pure effect of interactions between PAC strains *ex planta* on mycelial biomass production of PAC.

Materials and methods

Strain information and setup of experiment to test mycelial interactions

Four PAC strains originating from roots collected at the same location (Bödmerenwald, Switzerland, 46°58′57.13″ N/8°49′ 23.90″ E) but differing in pathogenicity were used in this experiment (Table 1). The strains were incubated on 2 % (*w*/*v*) malt extract agar (MEA; 20 g l^{-1} malt extract (Hefe Schweiz AG, Stettfurt, Switzerland), 15 g l^{-1} agar) at 20 °C in the dark. After 2 weeks, one colonized plug of agar (diameter 4 mm) from the margin of the growing colony was transferred to 50 ml of 2 % malt broth (20 g l^{-1} malt extract) in a 100-ml Erlenmeyer flask per strain and incubated at 20 °C in the dark on a rotary shaker at 90 r.p.m. After 20 days, the mycelium was harvested under sterile conditions by pouring the content of the Erlenmeyer flask into a sieve. The retained mycelium was washed twice with sterile water and blotted dry. Mycelia of two strains were mixed at ratios of 5:1, 1:1 and 1:5 (*w*/*w*) for each of the six possible combinations of the four strains. The total mycelial fresh weight of each mixture amounted to 0.6 g. Correspondingly, 0.6 g mycelium of a pure culture of each of the four strains served as controls. The mycelia were suspended in 10 ml sterile water and homogenized with a blender for 10 s. Then, another 30 ml of sterile water was added to obtain a final mycelium concentration of 15 g l^{-1}, and 1.4 ml of this suspension was distributed homogenously on the surface of 20 ml MEA in each of four Petri dishes per strain, strain combination and mixture ratio. Two of the four Petri dishes were incubated at 15 °C and the other two at 25 °C. Mycelium was harvested after 2 and 8 weeks of incubation as follows. Per Petri dish, three rectangular colonized pieces of MEA measuring 10×20 mm in surface view were cut with a scalpel and served as replicates. The uppermost colonized 1 mm was then removed from the rectangular MEA pieces, frozen at −80 °C, freeze-dried and DNA extracted as described previously (Grünig et al. 2003). Amplification of microsatellites and microsatellite fragment analysis occurred according to Queloz et al. (2010), and mycelial weight estimation according to Reininger et al. (2011b) and Reininger and Sieber (2012, 2013) (see below).

Calibration of the microsatellite-based quantification method

Multiplex and singleplex PCR for the amplification of microsatellites of PAC has been developed recently, and allele data are available for more than 5,000 PAC strains (Queloz et al. 2010). The suitability of four microsatellite loci (mPF_043, mPF_049, mPF_142B, and mPF_860B) for DNA quantification was tested by Reininger et al. (2011b), and locus mPF_142B showed to be most suitable because this locus produced fewest stutter bands. The same locus was also used in this study and calibration of DNA amounts followed the protocol of Reininger et al. (2011b). The four strains were cultivated in malt broth as described above. The mycelium was harvested and lyophilized. The freeze-dried mycelia of the strain to quantify (target strain 1) and the admixed strain (additional strain 2) were mixed according to the weight ratios (m_1/m_2) 1:14, 3:12, 5:10, 7:8, 9:6, 11:4, 13:2, and 14:1 amounting to a total weight of 15 mg, and a constant amount of 15 mg mycelium (m_r) of a third strain (reference strain) was added. Mycelial mixtures were homogenized, DNA extracted as described previously (Grünig et al. 2003), locus mPF_142B amplified by PCR, fragment lengths and peak areas determined (see below). The coefficients (slopes) (β_1 and β_2) of the calibration curves were determined by linear regression exploiting the proportionality of the peak and mycelial ratio according to Reininger et al. (2011b): $(p_r/p_1)\sim(m_r/m_1)$, $(p_r/p_2)\sim(m_r/m_2)$, respectively (p_r=peak area of the reference strain; p_1, p_2 peak areas of the target strain 1 and the additional strain 2). The resulting equations were used to estimate the mycelial weight of m_1 and m_2 in the colonized pieces of MEA.

Table 1 Characterization of the PAC strains included in this study

Strain label	Culture collection accession numbers		PAC species	Pathogenicity[c]	Original host	Allele length of locus mPF_142B [bp][d]	Mycelial biomass [mg mycelium per g substrate] of strain in the first column after 8 weeks of incubation in peat: vermiculite (1:1) at 20 °C in dual culture with strain...[e]			Stimulation/inhibition of endophytic mycelial growth of the strain in the first column in living spruce roots at 18 °C and 23 °C dually noculated with strain...[f]					
										18 °C			23 °C		
	ETH[a]	CBS[b]					A	B	C	A	B	C	A	B	C
A	6_2_7v	134503	*Phialocephala subalpina*	pathogenic	*Vaccinium myrtillus*	174	–	6.2	1.9	–	inhibition	slight inhibition	–	slight inhibition	slight inhibition
B	6_37_6v	134504	*Phialocephala subalpina*	not pathogenic	*Vaccinium myrtillus*	162	1.1	–	2.8	no effect	–	inhibition	no effect	–	inhibition
C	7_45_5	119281	*Phialocephala fortinii* s.s.	not pathogenic	*Picea abies*	154	0.7	1.2	–	no effect	no effect	–	no effect	no effect	–
T1	T1_52_2	134898	*Acephala applanata*	pathogenic	*Picea abies*	141	n.a.[g]	n.a.	n.a.	n.a.[g]	n.a.	n.a.	n.a.[g]	n.a.	n.a.

[a] ETH Zurich, Zurich, Switzerland

[b] CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands

[c] Pathogenicity on spruce seedlings according to Tellenbach et al. (2011)

[d] according to Queloz et al. (2010)

[e] according to Reininger (unpublished)

[f] according to Reininger et al. (2012)

[g] not available

Amplification of microsatellites by competitive PCR

Locus mPF_142B was amplified by PCR according to Queloz et al. (2010) in 15 μl volumes containing 2 μl 1:50 or 1:500 diluted DNA, 50 mM KCl, 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 200 μM dNTPs (Amersham Pharmacia Biotech), 0.4 μM of each primer (F, GCTTTCACATCACCATCCAG; R, GGTG AGTTGGTTGCGAGTTT) and 0.3 U Taq polymerase (Amersham Pharmacia Biotech). The running conditions were 2 min at 94 °C followed by 36 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 53 °C, and extension for 30 s at 72 °C (followed by a final extension step of 10 min at 72 °C).

Microsatellite fragment analysis

Fifteen-fold diluted triplicates were prepared from each PCR reaction: 4 μl of the dilutions were mixed with 9.05 μl Hi-Di™ formamide and 0.25 μl GeneScan™ 500 LIZ™ (Applied Biosystems). Fragment lengths and the peak area, i.e., the amount of PCR product, of each fragment, were measured using an ABI 3730xl DNA analyser (Applied Biosystems) and analysed using the GeneMapper v. 4.0 software (Applied Biosystems) (Queloz et al. 2010).

Statistical analyses

The effects of strain combination, mixture ratio, temperature and duration of incubation on mycelial dry weight [used synonymously: 'mycelial density', 'mycelial biomass'; mg freeze-dried mycelium per 200 mm^3 (10×20×1 mm) of MEA] of each strain was determined using ANOVA:

$$y_{ijklm} = \mu + \alpha_i + \beta_j + \gamma_k + \delta_l + e_{ijklm} \quad (1)$$

i	1, 2, 3, 4 (strain combination, e.g. A, A & B, A & C, A & D)
j	1, 2, 3 (mixture ratio: 1:5, 1:1, 5:1)
k	1, 2 (temperature: 15 °C, 25 °C)
l	1, 2 (duration of incubation: 2 weeks, 8 weeks)
m	1, 2, 3, 4, 5, 6 (six replicates)
y_{ijklm}	mycelial dry weight of the mth replicate of the ith combination of strains mixed at the jth ratio incubated for the lth duration at the kth temperature
μ	overall mean mycelial dry weight
α_i	effect of the strain combination
β_j	effect of the mixture ratio
γ_k	effect of temperature
δ_l	effect of the duration of incubation
e_{ijklm}	random error.

Interactions not shown in formula (1).

Goodness of fit and normal distribution of residuals were tested using residual analyses. If necessary, the response variable 'Mycelial dry weight' (y_{ijklm}) was transformed. The full models were reduced deleting non-significant factors. Full and reduced models were compared using chi-square tests to avoid loss of fit. Tukey's HSD post-hoc test was used for pairwise comparisons. All statistical analyses were performed using R (R_Development_Core_Team 2010).

Results

Macroscopically, all cultures looked uniform, and there was barely a difference between dual and single cultures, indicating that the mycelia had been homogeneously distributed all over the surface of the medium during inoculation. No demarcation lines or barrier zones could be recognized to differentiate genotypically different thalli.

Calibration of the microsatellite-based quantification method was successful for all strain mixtures, since the additional strain (strain 2) did not have a disturbing effect on the estimation of the biomass of the target strain (strain 1) in any of the strain mixtures. None of the β_2 coefficients deviated significantly from zero, i.e., the bivariate regressions could be reduced to univariate regressions. All β_1 coefficients differed significantly from zero and assumed values close to 1. Thus, the resulting calibration curves allowed estimating the mycelial weight of any strain (m_1 and m_2) in any of the colonized pieces of MEA (Reininger et al. 2011b).

The factors 'strain combination', 'mixture ratio' and the interaction between these two factors had a significant effect on the biomass of all strains (Table 2). Biomass was highest if strains were cultured singly, except for strain A, which grew better in combination with strain B or C (Fig. 1a; see below). In addition, 8 weeks compared to 2 weeks of incubation led to greater biomass of strains A, B and T1, but not of strain C. T1 was the only strain exhibiting a significant temperature effect. It accumulated more biomass at 25 °C than at 15 °C. However, although not significant, temperature had to be retained in the models for strains A and B to avoid loss of fit. Strain B was influenced by an intricate network of interactions between all four factors.

Strains significantly differed in competitiveness. Strain A was most competitive and accumulated more biomass per volume of substrate (biomass density) than any other strain independently on the strain combination, mixture ratio, duration of incubation and temperature (Fig. 1). In contrast to single culture, growth of strain A was statistically significantly stimulated in dual culture with strain B or C at a mixture ratio of A:B=A:C=5:1 (Fig. 1a). Even at a mixture ratio of 1:5, biomass of A was always greater than that of the other strain. In contrast, presence of strain T1 did not lead to stimulation of A (Fig. 1a). Strain B was significantly inhibited by A and C if

Table 2 Results of the analyses of variance showing the best fitting, reduced model for the mycelial dry weight of each strain

Strains and factors	Df	Sum of Squares	Mean Square	*F* value	*p*	Significance level[a]
Strain A						
Strain combination (1)	2	7.078	3.5388	12.8257	<0.00001	***
Mixture ratio (2)	2	19.312	9.6559	34.9961	<0.00001	***
Duration of incubation (3)	1	6.8	6.7996	24.6439	<0.00001	***
Temperature (4)	1	0.036	0.0361	0.1307	0.7181	
Interaction (1)×(2)	4	7.402	1.8504	6.7065	0.00005	***
Residuals	188	51.872	0.2759			
Strain B						
Strain combination (1)	2	26.3227	13.1614	290.2111	<0.00001	***
Mixture ratio (2)	2	18.2748	9.1374	201.4818	<0.00001	***
Duration of incubation (3)	1	0.2727	0.2727	6.0122	0.01527	*
Temperature (4)	1	0.0222	0.0222	0.4899	0.48499	
Interaction (1)×(2)×(3)×(4)	29	10.9865	0.3788	8.3536	<0.00001	***
Residuals	162	7.3469	0.0454			
Strain C						
Strain combination (1)	2	0.8404	0.4202	5.8547	0.00342	**
Mixture ratio (2)	2	8.101	4.0505	6.4372	<0.00001	***
Interaction (1)×(2)	4	1.9392	0.4848	6.7549	0.00005	***
Residuals	187	13.421	0.0718			
Strain T1						
Strain combination (1)	2	6.1635	3.08173	71.1574	<0.00001	***
Mixture ratio (2)	2	4.7556	2.37778	54.903	<0.00001	***
Duration of incubation (3)	1	0.3276	0.32761	7.5645	0.00655	**
Temperature (4)	1	0.2137	0.21373	4.9351	0.02755	*
Interaction (1)×(2)	4	1.0706	0.26766	6.1803	0.00011	***
Residuals	182	7.8822	0.4331			

[a] significance levels: * $0.05 \geq p > 0.01$; ** $0.01 \geq p > 0.001$; *** $0.001 \geq p$

mixed at B:A=1:5, B:C=1:5 or B:C=5:1 (Fig. 1b). Strain C was significantly inhibited by all three strains if mixed at C:A=C:B=C:T1=1:5, but not at a mixture ratio of 5:1 (Fig. 1c). T1 was inhibited by all other strains independently on the mixture ratio (Fig. 1d).

Discussion

The microsatellite-based method developed by Reininger et al. (2011b) proved to be reliable to measure fungal biomass of each strain in strain mixtures, because both the correlation coefficients (R^2) and the slopes (coefficient β_1) of the regression lines were close to 1, indicating excellent fit of measured values and model. Strain A produced denser mycelia on malt extract agar than any other PAC strain, and it was the only strain showing significant growth stimulation in the presence of other strains (Fig. 1). Similarly, growth of strain A was stimulated on 1:1 (*v*/*v*) peat:vermiculite by the presence of strain B or C in another study (Table 1) (Reininger, unpublished). Under saprotrophic conditions, growth of A seems to be stimulated by the presence of other PAC strains. This contrasts with the performance of A under biotrophic conditions in roots. Growth of the endophytic mycelium of A is inhibited if the roots are simultaneously colonized by other PAC strains (Table 1) (Reininger et al. 2012).

In single culture, formation of denser mycelia was positively correlated with pathogenicity, i.e., the two pathogenic strains A and T1 produced denser mycelia than the non-pathogenic strains B or C (Fig. 1). However, in dual culture there was no such correlation since growth of A was stimulated, whereas growth of T1 was inhibited by the presence of any other PAC strain (Fig. 1a and d).

Predictability of the endophytic biomass accumulating in dually inoculated plants by the mycelial density developing in plant-free dual culture is strain-dependent. Prediction was possible for dual cultures of strains B and C, but not for those including strain A. For example, presence of non-pathogenic strain B stimulated growth of pathogenic strain A on malt extract agar in the current experiment and in peat:vermiculite, but inhibited growth in living spruce roots (Fig. 1a, Table 1) (Reininger et al. 2012), indicating that strain A is a strong

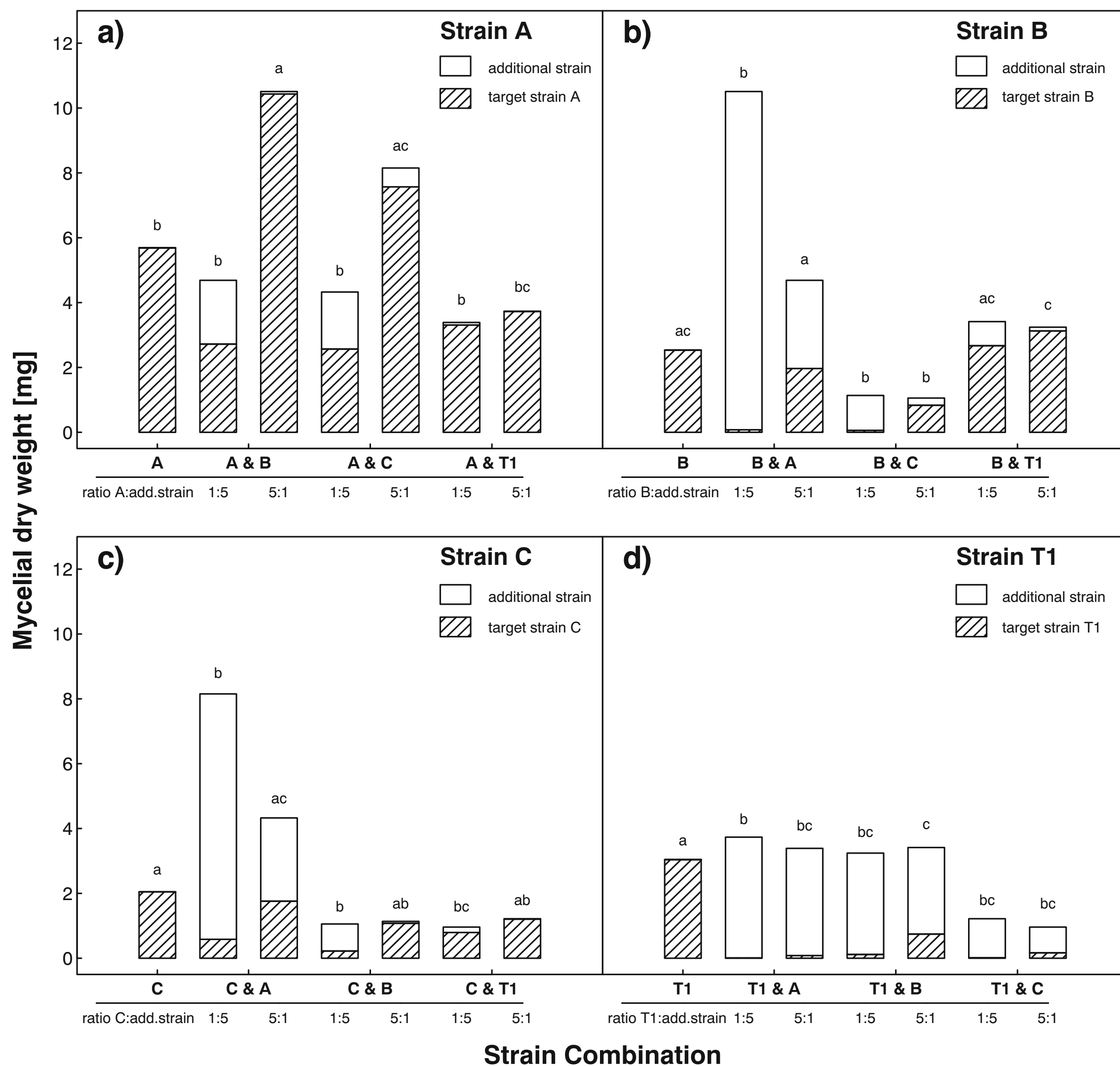

Fig. 1 Bar charts depicting the effects of the factors '*Strain combination*' and '*Mycelial mixture ratio*' on the *mycelial dry weight* [*mg*] of the target (*hatched areas*) and the additional (*blank areas*) strain after 2 weeks of incubation on MEA at 25 °C. (**a**) *target strain A*, (**b**) *target strain B*, (**c**) *target strain C*, (**d**) *target strain T1*. Different letters above the bars indicate significantly different effects of the treatments on mycelial dry weight of the target strain (Tukey's HSD)

competitor on dead organic matter but not in living plant tissues. This is probably the reason why this strain behaves as a necrotrophic organism, i.e., it must kill the plant tissue to exploit it. However, strain A is only able to kill if no other PAC strain is present (Reininger et al. 2012; Tellenbach et al. 2011). *In planta*, non-pathogenic PAC strains B and C successfully compete with pathogenic strain A, probably by space occupation or the induction of host resistance, thereby keeping endophytic biomass of pathogenic strains below the threshold above which plant growth is inhibited significantly (Sieber 2007). In contrast to strain A, mycelial densities resulting from interactions between the non-pathogenic strains B and C on malt extract agar were mostly congruent with interactions between the two strains *in planta*. Growth of B was significantly inhibited by the presence of C, both on malt extract agar in the current experiment and *in planta* (Fig. 1b) (Reininger et al. 2012). Pathogenic strain T1 behaved rather like non-pathogenic strains B and C on malt extract agar, i.e., completely different than pathogenic strain A. Biomass accumulation of T1 was strongly reduced by the presence of any of the other three strains (Fig. 1). Performance of T1 in plants dually inoculated with T1 and another PAC strain has never been tested, and thus, a comparison *ex planta* and *in planta* is not possible for this strain.

In summary, predictability of the mycelial densities resulting from a tripartite PAC-PAC-plant interaction by the outcome of a dipartite interaction between two PAC strains in a plant-free system depends on the strains involved. Consequently, the outcomes of interactions in dual PAC-PAC systems are not always additive, i.e., the results of a tripartite interaction is not simply the sum of the results of all dual interactions.

Interacting PAC genotypes do not show any signs of inhibition (barrier zone formation, antibiosis), stimulation or mycoparasitism when grown on artificial media *ex planta*. Growth seems only to be limited by nutrient depletion of the medium, and exploitation of the substrate occurs on a first-come, first-served basis. Hyphae intermingle in the contact zone and grow as far into the others' "territory" as nutrients support growth. Thus, the mechanisms responsible for the attenuation of adverse effects on plant growth caused by pathogenic PAC by non-pathogenic ones remain enigmatic and require further studies.

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