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Does water availability influence the abundance of species of the *Phialocephala fortinii* s.l. – *Acephala applanata* complex (PAC) in roots of pubescent oak (*Quercus pubescens*) and Scots pine (*Pinus sylvestris*)?

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ABSTRACT

The effects of irrigation on colonization of *Pinus sylvestris* and *Quercus pubescens* by members of the *Phialocephala fortinii* s.l. – *Acephala applanata* species complex (PAC) was assessed. Roots were collected from an irrigation experiment site. PAC species were identified based on 13 microsatellites. Irrigation and host species had a significant effect on the frequency of roots colonized by PAC. In oak (*Q. pubescens*) but not in pine (*P. sylvestris*), PAC were significantly more common on dry non-irrigated plots than on irrigated ones. Frequency of colonization of pine roots was twice as high as that of oak roots, and the mean number of PAC species per tree was significantly higher for pines. A hitherto unknown PAC species was found. The community structure was random except for the most frequently isolated *Phialocephala europaea* and *Phialocephala helvetica*, which inhibited one another in pine roots. The possible effects of PAC colonization on drought resistance of oak are discussed.

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1. Introduction

Root tissue represents a morphologically, physically and chemically complex microcosm that serves as a habitat for a large number of microorganisms (Sieber and Grünig, 2013). Dark septate endophytes (DSE) are amongst the most common of these microorganisms and form a group of often loosely related species (mostly ascomycetes) (Stoyke et al., 1992; Grünig et al., 2008b; Sieber and Grünig, 2013). They have been identified as root inhabitants on a large number of plants belonging to approximately 100 different plant families ranging from the tropics to the arctic (Jumpponen and Trappe, 1998). One common and thoroughly studied DSE fungal group is known as PAC (*Phialocephala fortinii* s.l. – *Acephala applanata* species complex). It consists of 21 closely-related, morphologically almost indistinguishable yet genetically unique species, i.e. cryptic species (CSP) (Grünig et al., 2001, 2002a, 2003,

2004; Queloz et al., 2010). PAC are very common root colonizers in conifers (Ahlich and Sieber, 1996; Ahlich-Schlegel, 1997; Addy et al., 2000; Grünig et al., 2002b, 2006). They also occur in the roots of deciduous trees, though less frequently (Halmschlager and Kowalski, 2004; Kwasna et al., 2008; Reininger et al., 2012; Santschi, 2015), and are even found in the roots of herbaceous plants and small shrubs (Stoyke and Currah, 1993; Harney et al., 1997; Addy et al., 2000). Their distribution comprises the majority of the northern hemisphere (Queloz et al., 2011). In contrast to mycorrhizal fungi, their occurrence is not limited to absorptive roots. PAC also colonize roots undergoing secondary growth and can therefore occur in the root cortex anywhere in a tree's root system. Consequently, they are among the most widespread of root inhabitants (Sieber and Grünig, 2013).

Despite their wide distribution and prevalence, their dispersal and behavioral biology have yet to be adequately explained. So far, no sexual state (teleomorph) has been uncovered. Non-germinating asexual spores (anamorph), are formed rarely after long incubation of cultures in the dark at 4 °C (Ahlich and Sieber, 1996; Grünig et al., 2008a). Locally, PAC spread via root contacts

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(Stroheker, 2017), but the extremely low growth rates of PAC mycelium observed in the soil also suggest that active proliferation through the soil is unlikely (Trüssel, 2011). It also remains unknown whether (and how) PAC spread over long distances. Thus far, PAC have not been detected in spore traps, practically ruling out airborne dispersal (Kausserud et al., 2005).

The geographic distribution of PAC species in the northern hemisphere cannot be explained through environmental factors. The rules that govern the population and community structure of PAC are not yet understood (Ahlich et al., 1998; Queloz, 2008; Queloz et al., 2011). The community dynamics of PAC appear to be relatively slow. Changes in population structure at a site on the Swiss Plateau revealed no changes after 3 y (Queloz et al., 2005). However, a reassessment of the same site revealed changes in the population structure after 10 y (Stroheker et al., 2016).

The PAC–host plant relationship has been investigated in several experiments and is a controversial topic. Effects ranging from “slightly positive” (Tellenbach and Sieber, 2012; Reininger and Sieber, 2013) to “pathogenic” (Wilcox and Wang, 1987; Tellenbach et al., 2011) have been noted. It was revealed that the pathogenicity of PAC depends on the inoculated genotypes. Pathogenicity and virulence are therefore genotype-specific (Tellenbach et al., 2011). Inoculation of several genotypes and species can have synergistic effects which are of benefit to the plant. PAC enhances defense against more pathogenic root inhabitants, in particular when combined with mycorrhizal fungi (Tellenbach and Sieber, 2012; Reininger and Sieber, 2013).

The fact that PAC can control root pathogens underlines the importance of understanding the ecology of this widespread species complex. The question of how PAC react to changing environmental conditions has been investigated in three experiments thus far. Reininger et al. (2012) demonstrated that the virulence of a pathogenic PAC strain was reduced at elevated temperatures (23 °C vs. 18 °C). At lower temperatures (19 °C vs. 25 °C), spruce (*Picea abies*) exhibited improved performance when its roots were colonized by both PAC and the mycorrhizal fungus *Laccaria bicolor* than if the roots were colonized by *L. bicolor* alone (Reininger and Sieber, 2012). Tellenbach and Sieber (2012) found that the protective effect of PAC against pathogenic oomycetes increased at higher temperatures (21.6 °C vs. 17.9 °C). The effect of temperature adjustment has therefore already been investigated in several studies under controlled laboratory conditions. In nature, however, the effects of global warming are not limited to an increase in temperature, but also major changes in the water balance are to be expected.

Periods of drought are increasing in both frequency and intensity in Switzerland (Calanca, 2007; Scherrer et al., 2016). This can lead to drought stress for forest ecosystems (Dobbertin et al., 2007). For example, in Valais, a central-alpine valley in Switzerland, Scots pine (*Pinus sylvestris*) forests at low altitudes are undergoing change. Deciduous species, in particular pubescent oak (*Quercus pubescens*), are becoming more abundant while pine shows increasing mortality. It has been hypothesized that prolonged and more severe drought periods due to climate change and species-specific drought tolerance are key factors driving these trends (Eilmann et al., 2006; Rigling et al., 2010). However, the interaction of these tree species with other plants, arthropods, nematodes and microorganisms also plays a key role. There is a high incidence of mistletoes (*Viscum album* ssp. *austriacum*) on Scots pine in Valais (Rigling et al., 2010). Drought stress needs to be more severe to induce stomatal closure of mistletoe than of *P. sylvestris*. Thus, during drought periods water stress is felt more acutely by heavily infested *P. sylvestris* than by mistletoe-free trees. Consequently, mistletoes increase the risk of drought-induced mortality when its hosts grow in a xeric environment (Noetzli et al., 2003; Rigling et al., 2010). A meta-analysis of the effects of global change factors on plant growth indicated that

drought and nitrogen deposition have resulted in plant responses that are strongly influenced by fungi, highlighting that considering plant–fungal symbioses is critical in predicting ecosystem response to climate change (Kivlin et al., 2013).

Therefore, a near-natural study was initiated to examine the effects of drought stress on PAC–host symbioses. More specifically, the effects of drought stress on the colonization of Scots pine (*P. sylvestris*) and pubescent oak (*Q. pubescens*) roots by PAC were examined, as well as whether individual CSP perform better on dry sites and/or on one of the two host tree species. The Scots pine forest “Pfywald” in Valais, Switzerland, was used, where artificially irrigated areas alternate with corresponding, non-irrigated areas as controls (Herzog et al., 2014). Irrigation had no or only a moderate effect on the diversity and abundance of mycorrhizal fungi (Hutter, 2014; Hartmann et al., 2017), but nothing is known regarding the effect of irrigation on PAC in particular. The aims of this study were to examine the effects of irrigation on the PAC community in roots of Scots pine and pubescent oak, in particular on (i) the overall colonization density, (ii) the number of species, (iii) the frequency of occurrence of individual species, and (iv) the community structure.

2. Materials and methods

PAC communities were studied in 2016 in an experimental plot located in the forest “Pfywald” (canton of Valais, Switzerland, 46°18' N, 7°37' E, 615 m a.s.l.), where the effects of artificial irrigation on the forest ecosystem have been studied since 2003 (Dobbertin et al., 2010; Herzog et al., 2014). The vegetation is an Erico-Pinetum (Keller et al., 1998; Werner, 1985) with Scots pine (*P. sylvestris*) being the dominant canopy tree species. The mean stand age is 95 y. The top tree height is 10.8 m. The stand density is 730 stems per hectare (ha) with a breast height diameter (DBH) of ≥ 12 cm or a basal area of 27.3 m² ha⁻¹ (Dobbertin et al., 2010). *Quercus pubescens* is the most abundant woody plant species in the understorey and will probably substitute *P. sylvestris* in the long run, should the frequency and duration of drought periods further increase (Calanca, 2007; Scherrer et al., 2016).

The experimental plot has been subdivided into eight subplots measuring 25 × 40 m each (1000 m²). Four of the subplots were artificially irrigated on rainless nights during the vegetation period (May to October) from 2003 to 2012 with water from the nearby river (channel) (Dobbertin et al., 2010) (Fig. 1). The quantity of artificial rain amounts to 512 mm annually which corresponds to the local annual precipitation (Herzog et al., 2014), i.e. the irrigated plots receive twice as much water as they naturally would. The additional input of nutrients by the irrigation water can be considered negligible. The content of phosphorus in the irrigation water lies below the detection limit (PO₄ < 0.15 kg ha⁻¹ a⁻¹), and the input of nitrogen (2.4–3.3 kg ha⁻¹ a⁻¹) is less than that of the same amount of natural rainfall (N ≤ 3.5 kg ha⁻¹ a⁻¹) (Herzog et al., 2014). The pH of the top soil (0–5 cm) varies between 4.1 and 6.7, with the pH of the soil below measuring between 6.8 and 7.7 (Brunner et al., 2009). The pH of the irrigation water is high, leading to an increase in the pH of the top soil but not below (Dobbertin et al., 2010).

2.1. Sampling procedure

Roots were collected in the spring of 2016 from one half of each subplot; i.e. the halves closer to the river. The eight half-subplots were further subdivided into 20 squares measuring 5 × 5 m (Fig. 1). Living fine roots of the *P. sylvestris* and the *Q. pubescens* tree closest to the centre of the square were sampled, resulting in a total of 160 trees per tree species. The roots of the two tree species could be differentiated by the presence of resin ducts in *P. sylvestris* which

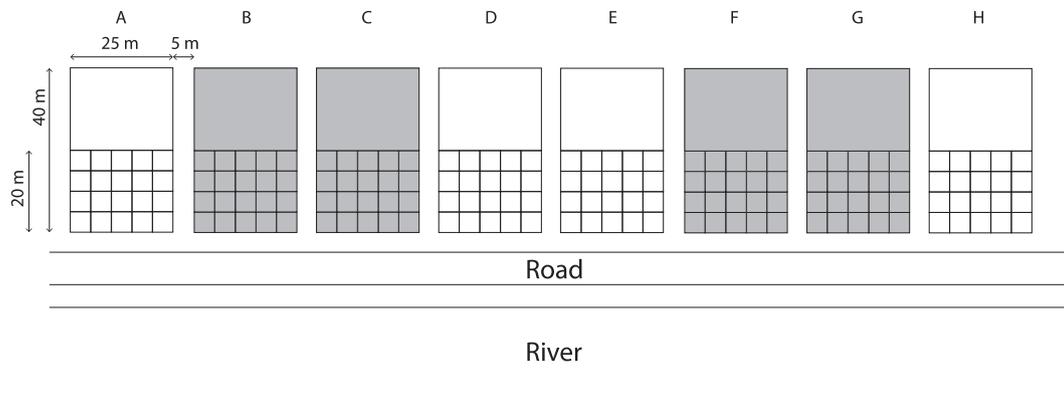


Fig. 1. Experimental setup of the irrigation experiment. Irrigated plots (grey) and non-irrigated control plots (white) with the nearby river which was used for irrigation (Herzog et al., 2014). In the present study, samples were only taken from the half of the plots closest to the river.

are absent in *Q. pubescens*. In contrast, *Q. pubescens* possesses comparatively thick medullary rays and vessels whereas the rays are very thin in *P. sylvestris* and vessels are absent. *Pinus sylvestris* trees tended to be older, whereas most of the *Q. pubescens* were young and shrub-like. The roots were carefully excavated and three root complexes of at least 10 cm in length per tree were collected at a soil depth of between 5 and 10 cm (in order to avoid top and sub soil). The root samples were stored in airtight plastic bags at 4 °C until further processing.

2.2. Isolation and single-hyphal tip culture (SHT)

Five approximately 5-cm-long fine root pieces were excised from each of the three root complexes per tree and surface-sterilized according to Ahlich and Sieber (1996). The roots were washed under running tap water and sterilized according to the following protocol: immersion in 99% (v:v) ethanol for 1 min, 5 min in 35% (v:v) hydrogen peroxide and finally 30 s in 99% (v:v) ethanol. Subsequently, 0.5-cm-long segments were cut from the middle of the root pieces and incubated at 20 ± 2 °C in the dark on terramycin malt agar (TMA: 20 g/l malt extract, 16 g/l agar, 50 mg/l terramycin (active ingredient: oxytetracycline, Pfizer Ltd., Hyderabad, Telangana, India)). Per 5×5 m sampling unit and tree species, 15 root segments (five segments in each of three Petri dishes) were incubated, i.e. a total of 4800 root segments were plated out. The agar plates were checked periodically during incubation. Which fungi grew from the root segments was recorded. Rapidly growing species (PAC growth is relatively slow) were removed from the TMA plates to prevent contamination of the remaining colonies. In cases where it was possible to assign a fungal colony to PAC (see Ahlich and Sieber (1996) for colony characteristics), a single-hyphal tip culture (SHT) was prepared. For such purposes, an actively growing culture was placed under a binocular microscope equipped with transmitted light and, at maximum magnification, a single hypha is cut out from the edge of the culture with a flamed scalpel and transferred to a new malt agar plate (20 g/l malt extract, 16 g/l agar) followed by incubation at 20 ± 2 °C in the dark for 2–3 weeks. The SHT cultures were grouped into morphotypes and two to four SHT cultures per tree were subjected to microsatellite analysis. The number of segments from which non-PAC mycelia emerged was also surveyed, but the mycelia were not identified.

2.3. DNA-extraction, microsatellite analysis and multigene characterization

Approx. 40 mg of mycelium per culture were transferred in tube

strips (2 ml) and freeze-dried for 24 h. The DNA was then extracted according to the NucleoSpin® 96 Plant II Kit protocol (Macherey-Nagel AG, Oensingen, Switzerland) and amplified at 13 loci using the multiplex method developed by Queloz et al. (2010). The PCR products were stored at 4 °C until further processing (maximum two days). The microsatellite data were analyzed using GeneMapper® v. 4.0 (Applied Biosystems, Foster City, USA) software and compared to the data reported in Queloz et al. (2010) using the software GeneClass2 (Piry et al., 2004). Since GeneClass2 only uses a subset of our microsatellite data, assignments with an accuracy of over 95% were assumed to be safe. Samples with lower accuracy levels were compared manually to all entries in our PAC microsatellite database (Queloz et al., 2010, 2011) and, where possible, assigned to CSPs. One group of PAC isolates with identical microsatellite alleles could not be assigned unequivocally to an existing CSP, but it appeared to consist of a hybrid of *Phialocephalalhelvetica* (CSP 4) and CSP 10 according to the allele combination at the microsatellite loci. Thus, these isolates were interpreted as belonging to a hitherto unknown CSP and are referred to as CSP22. CSP22 was further characterized by means of the five sequence loci proposed by Grünig et al. (2007) to define CSPs of PAC. Ten strains of each of the CSPs 4, 10 and 22 were selected and subjected to multigene analysis as described in Grünig et al. (2007). Unrooted neighbour-joining trees were constructed for each locus and the concatenated sequence of the β -tubulin, the EF1-alpha and the pPF-061 locus using the Tamura-Nei distance as implemented in Geneious® 9.1.8 (Biomatters Ltd., Auckland, New Zealand). Bootstrapping to generate 1000 pseudosamples was used for accuracy estimation. Microsatellites are ideally suited to PAC species (CSP) identification, but they do not possess adequate discriminatory power for reliably differentiating between genotypes.

2.4. Statistical analysis

The “colonization density” responses (= the number of root segments colonized divided by the number of root segments examined) of both PAC and non-PAC, “the mean number of PAC species per tree” and “the proportion of trees colonized by a given CSP” as influenced by the fixed effects “tree species”, as well as “irrigation” were all modelled using analysis of variance (ANOVA). Normal distribution of the residuals was examined using residual analysis. Chi-square tests served to investigate the independence of the two most frequently isolated PAC species and the independence of colonization of the two hosts in the same 5×5 m square by a given PAC species. A p-value of ≤ 0.05 was considered to indicate a significant outcome (Stahel, 2002). All statistical calculations were

performed using the software R (R for Windows, Version 3.2.1, R Foundation for statistical computing, Vienna, Austria).

3. Results

3.1. Density of colonization

From the 4800 incubated root pieces 2205 PAC cultures were obtained, corresponding to an overall colonization density of 0.46 (or 46%). PAC were isolated from 1472 pine root segments (61%) and 733 oak root segments (31%). All trees were colonized by PAC except for 13 of the 160 oak trees. Seven of these oaks were from irrigated plots and six from non-irrigated plots. The factors “tree species” and “irrigation”, but also the interaction between the two factors had a statistically significant influence on colonization density (Table 1). A significant interaction requires that unifactorial ANOVAs are calculated for each of the factors separately. Again, the tree species played a crucial role, i.e. the colonization density of PAC on *P. sylvestris* was almost twice as high as on *Q. pubescens*. In contrast, irrigation had a significant influence on colonization density of *Q. pubescens* only, and not that of *P. sylvestris* (Table 1; Fig. 2A). Colonization density of PAC in roots of *Q. pubescens* was 40% higher in non-irrigated than in irrigated plots. Non-PAC were isolated from 660 pine root segments (28%) and 1252 oak root segments (52%). The difference in the colonization density between the two tree species was statistically significant. However, irrigation had no effect on this for either tree species.

3.2. Number of PAC species (CSP) and frequency of colonization

For PAC species identification, 948 SHT cultures were prepared from the 2205 cultures and subjected to microsatellite analysis. 877 of the SHTs could be assigned unambiguously to a CSP. The remaining 71 SHTs analyzed could not be definitively assigned to a CSP, as they were either contaminated or not a member of the PAC. A total of seven different CSPs were found. The number of PAC species was significantly higher for *P. sylvestris* than for *Q. pubescens* (Table 2; Fig. 2B). However, irrigation had no notable impact. The interaction between “tree species” and “irrigation” was statistically insignificant. The number of trees colonized varied widely among CSPs: from 1 to 246 (Table 3). Due to their rare occurrence, CSP 6 and CSP 13 were excluded from further statistical analyses. Significant host preference for *P. sylvestris* was found for *Phialocephala europaea* (CSP 3), *P. helvetica* (CSP 4) and CSP 22 (Fig. 3). In addition, *P. europaea* occurred preferentially in plots with no irrigation (Table 3; Fig. 3A). Six of the seven plots possessing more than ten trees colonized by *P. europaea* were non-irrigated. *Phialocephala helvetica* was most frequent and reached a colonization density above 0.75 (i.e. 75% of all trees colonized). It was significantly more common on *P. sylvestris* than on *Q. pubescens* (Table 3; Fig. 3B). CSP 10 was the only species (except CSP 13) more frequent on *Q. pubescens* than on *P. sylvestris*. However, this was not statistically significant (Table 3; Fig. 3C). In contrast, CSP 22, closely related to CSPs 4 and 10, showed a pronounced preference for *P. sylvestris* as

host (Table 3; Fig. 3D).

3.3. Distributional pattern of PAC species

No pattern was discernible in the distribution of the PAC species in the plots (Fig. 4). However, for pine, a dependency between the two most common species (*P. europaea* and *P. helvetica*) was observed. The two species were significantly less common in the same 5×5 m square than would have been expected by coincidence, regardless of the irrigation method (non-irrigated, $p = 0.03$; irrigated, $p = 0.01$). In contrast, *P. europaea* and *P. helvetica* were independent regarding colonization of oak roots (non-irrigated, $p = 0.44$; irrigated, $p = 0.96$).

Colonization of one host by either *P. europaea* or *P. helvetica* did not depend on whether the other host in the same 5×5 m square was also colonized regardless of the irrigation method (*P. europaea*, non-irrigated, $p = 0.32$; *P. europaea*, irrigated, $p = 0.07$; *P. helvetica*, non-irrigated, $p = 0.32$; *P. helvetica*, irrigated, $p = 0.95$). Although statistically not significant ($p = 0.07$), there was a tendency for *P. europaea* to occur more frequently than randomly expected in both hosts in the same 5×5 m square of the treatment with higher water availability.

3.4. Newly discovered CSP 22

Of the 875 successfully analyzed SHTs, 74 SHTs could not be unambiguously assigned to one known CSP. These 74 SHTs were most closely related to CSP 10 and *P. helvetica* in terms of the number of identical alleles but represent a new CSP, i.e. CSP 22. PAC encompassed 21 CSPs until now, of which eight species have been formally described (Grünig and Sieber, 2005; Grünig et al., 2008a; Queloz et al., 2011). CSP 22 possesses the same allele as *P. helvetica* (CSP 4) and CSP 10 at three of the 13 microsatellite loci (Table 4). Moreover, CSP 22 has the same allele as *P. helvetica* at 5 additional loci and the same allele as CSP 10 at another 4 loci. Consequently, CSP 22 has a balanced mix of alleles of *P. helvetica* and CSP 10, which suggests that CSP 22 might be a hybrid of *P. helvetica* and CSP 10. Concerning sequence loci, CSP 22 is more closely related to *P. helvetica* than CSP 10 at two of the five tested loci (β -tubulin and pPF-061) and more closely related to CSP 10 at another two loci (EF1-alpha and pPF-018) (Fig. 5). Locus pPF-076 was not suitable for proper separation of *P. helvetica* and CSP 10 but CSP 22 clearly differed from these two CSPs. Considering the 1887 bp-long concatenated sequence of the three loci β -tubulin, EF1-alpha and pPF-061, the difference among the three CSPs is expressed by single nucleotide polymorphism (SNP) at 55 (3%) of the nucleotide positions (Table 5). CSP 22 possesses the same nucleotide as *P. helvetica* at 33 of these positions but the same nucleotide as CSP 10 at maximally 14 of the positions (depending on the CSP 10 strain, nucleotides vary at 9 of the SNP positions). Thus, it cannot be excluded that CSP 22 represents the result of a recombination between *P. helvetica* and CSP 10. However, compared to the total genome size of 70 Mb of PAC (Schlegel et al., 2016), the section of the genome examined here is too small to prove hybridization with

Table 1
Influence of the factors “tree species” and “irrigation” on the average colonization density. Overview of the mean values of colonization density and the P values of the bifactorial and unifactorial ANOVAs. Level of significance: *0.05, **0.01, ***0.001.

Factor	Colonization density		P-value bifactorial ANOVA		P-value unifactorial ANOVA
Tree species	<i>P. sylvestris</i>	0.61	<i>Q. pubescens</i>	0.31	<0.0001***
	irrigated	0.43	non-irrigated	0.49	0.00824**
Irrigation	<i>P. sylvestris</i> irrigated	0.61	<i>P. sylvestris</i> non-irrigated	0.62	0.618
	<i>Q. pubescens</i> irrigated	0.26	<i>Q. pubescens</i> non-irrigated	0.36	0.001433**

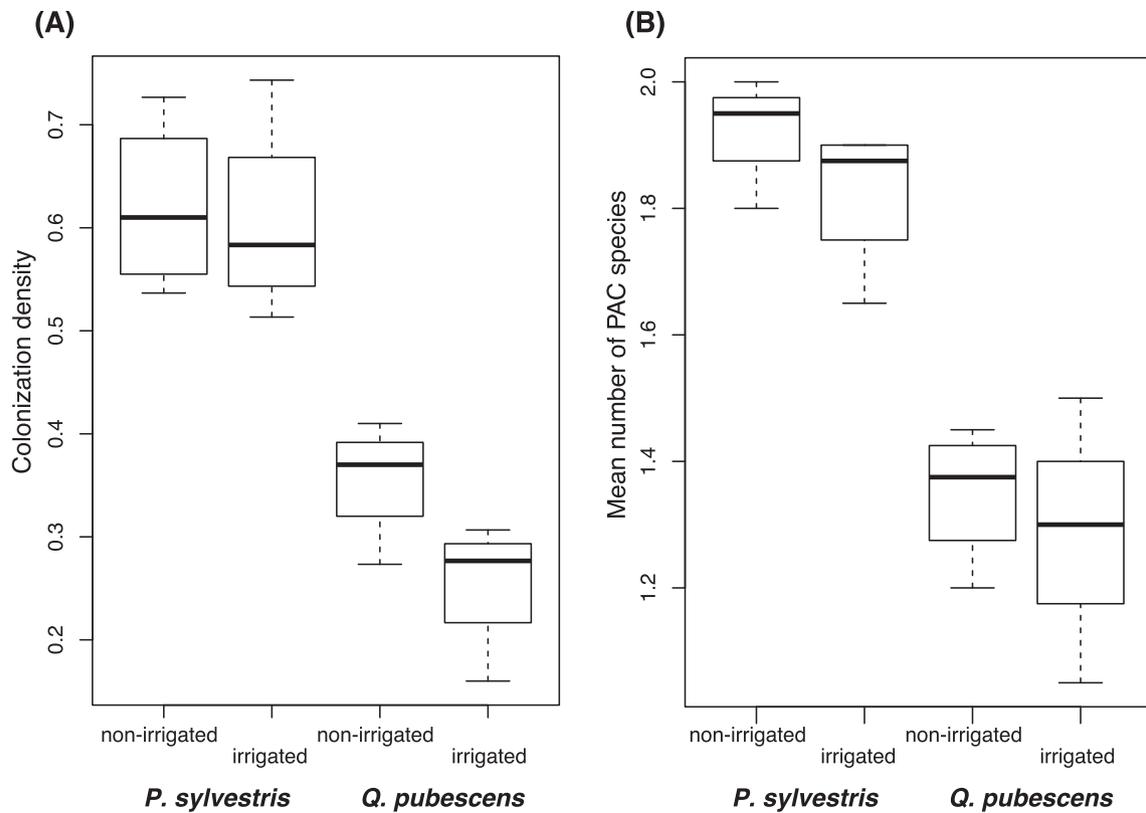


Fig. 2. The effect of tree species and irrigation on (A) the density of colonization by PAC and (B) the mean number of PAC species per tree.

Table 2

Influence of the factors “tree species” and “irrigation” on the mean number of species. Overview of the mean number of PAC species per tree and the P values of the bifactorial ANOVA. Level of significance: *0.05, **0.01, ***0.001.

Factor	Mean number of PAC species per tree				P-value bifactorial ANOVA
Tree species	<i>P. sylvestris</i>		<i>Q. pubescens</i>		<0.0001***
	1.88	1.32			
Irrigation	irrigated		non-irrigated		0.322
	1.56	1.64			
Tree species x Irrigation	<i>P. sylvestris</i> irrigated		<i>P. sylvestris</i> non-irrigated		0.819
	1.83	1.93			
	<i>Q. pubescens</i> irrigated		<i>Q. pubescens</i> non-irrigated		
	1.29	1.35			

Table 3

Influence of the factors “tree species” and “irrigation” on the number of trees colonized by each CSP modelled using bifactorial ANOVA.^a

CSP ^b	Number of trees colonized (n = 320)	Tree species			Irrigation		
		<i>P. sylvestris</i> (n = 160)	<i>Q. pubescens</i> (n = 160)	P-value	irrigated (n = 160)	non-irrigated (n = 160)	P-value
2	12	6	6	1.00	9	3	0.079
3	161	95	66	0.001**	69	92	0.009**
4	246	136	110	0.00055**	123	123	1.00
6	3	2	1	na	1	2	na
10	21	8	13	0.26	12	9	0.50
13	1	0	1	na	0	1	na
22	67	53	14	<0.0001***	35	32	0.67
Total	511	300	211		249	262	

^a The interaction “tree species” x “irrigation” was not significant for any CSP.

^b CSP 2 is *Phialocephala letzii*, CSP 3 *P. europaea*, CSP 4 *P. helvetica* and CSP 6 *P. subalpina* according to Grünig et al. (2008a); CSPs 10, 13 and 22 have not been formally described.

sufficient certainty.

4. Discussion

Colonization by PAC was significantly denser in pubescent oak (*Q. pubescens*) roots from drier, non-irrigated plots than irrigated ones. A similar observation was made by Stroheker et al. (2018) for

naturally regenerating Norway spruce (*P. abies*) seedlings, the roots of which were significantly more frequently colonized by an artificially introduced PAC strain in dry rather than in wet plots. Occurrence of higher colonization densities under drier conditions is rather counterintuitive and raises the questions of which mechanisms caused PAC to be more abundant in roots of non-irrigated pubescent oaks than in those of irrigated ones. There are

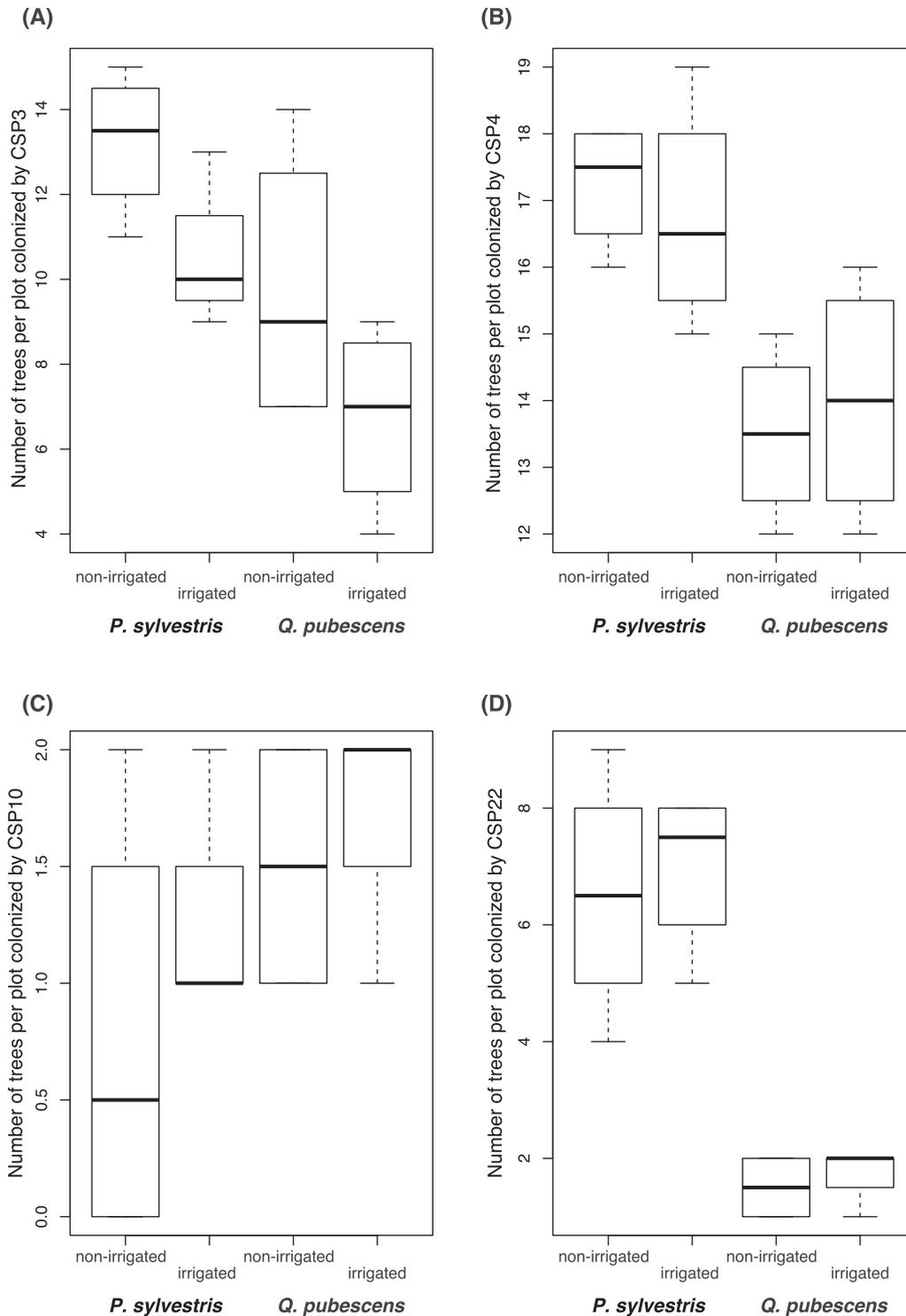


Fig. 3. The effect of tree species and irrigation on the number of trees ($n = 20$) colonized by (A) *Phialocephala europaea* (CSP 3), (B) *P. helvetica* (CSP 4), (C) CSP 10 and (D) CSP 22.

at least two possible explanations:

- (i) PAC are known to form extended layers of microsclerotia in culture and in the root cortex (pseudosclerotia) (Grünig et al., 2008b; Sieber and Grünig, 2013). These microsclerotia are resistant to drought, as well as repeated freezing and thawing (Ahlich-Schlegel, 1997). The walls of the fungal cells forming the microsclerotia contain melanin, making them

waterproof. Thus, the microsclerotia are able to protect roots against desiccation. Whether *Q. pubescens* forms more microsclerotia than *P. sylvestris* remains to be tested. However, if formation of microsclerotia increases under drought stress the ratio of isolation of PAC is expected to increase in roots under non-irrigated conditions.

- (ii) Drought stress was more severe in the non-irrigated plots (Dobbertin et al., 2007, 2010), and could have made roots

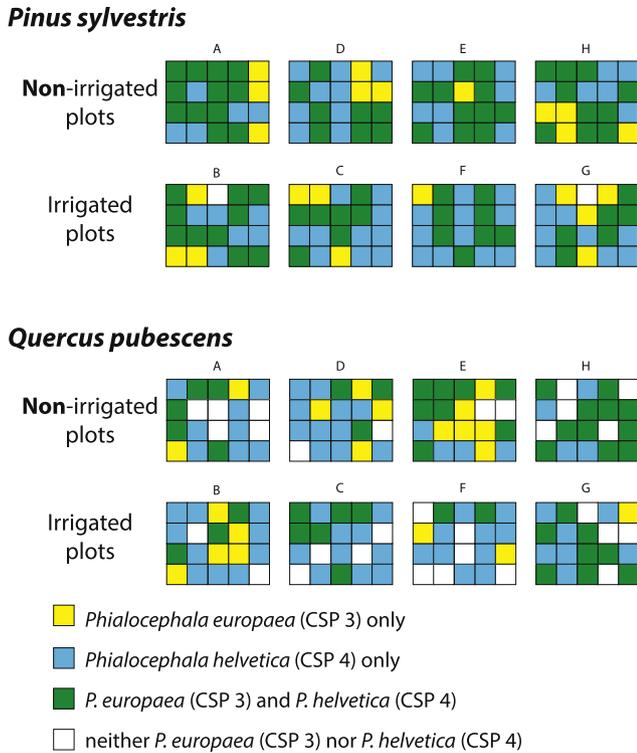


Fig. 4. Distribution of the two most frequently isolated PAC species. The plots are arranged according to the irrigation variants, i.e. there is no correspondence with the position of the plots in the field. However, the plots are marked with the same letters as in Fig. 1, allowing their positions to be determined.

more susceptible to fungal infections, including PAC (Desprez-Loustau et al., 2006). Since the roots of *P. sylvestris* are heavily colonized by PAC even without water stress, significant additional colonization is hardly possible under water stress. In contrast, roots of *Q. pubescens* are far less densely colonized by PAC, and susceptibility to PAC may increase if its roots are exposed to drought.

Explanations (i) and (ii) are equally probable. Dark septate endophytic fungi (DSE), which include PAC, were shown to have positive effects on plant performance under water-limited conditions in several studies. Mandyam and Jumpponen (2005) assume that the main role of DSE is to improve water balance and drought tolerance. Furthermore, DSE enhance the drought resistance of several plant species (Zhang et al., 2010; dos Santos et al., 2017; Valli and Muthukumar, 2018). In a meta-analysis of the effects of global change factors on plant responses, DSE consistently exhibited a positive effect on plant biomass production (Kivlin et al., 2013). Similarly, plants colonized by non-DSE endophytes are also better-adapted to drought and show increased biomass compared to non-symbiotic plants (Bailey et al., 2006; Waqas et al., 2012; Azad and Kaminskyj, 2016; Lata et al., 2018; Pan et al., 2018).

Each CSP was found at least once on both examined hosts (except for CSP 13, which was only detected once on oak). No pronounced host specificity could be determined for any of the CSPs but three of the seven CSPs (*P. europaea*, *P. helvetica* and CSP 22) showed a preference for *P. sylvestris*. Antecedent studies on host preference had differing results, though on other host species. In an experiment including European ash (*Fraxinus excelsior*), sycamore maple (*Acer pseudoplatanus*) and Norway spruce (*P. abies*) at two locations near Zurich, *P. helvetica* showed pronounced host preference for *P. abies*, whereas *P. europaea* occurred equally frequently on all three host species (Santschi, 2015). Kennedy et al. (2003) found 39 of a total of 56 ectomycorrhizal fungi behaving host-specific on either *Pseudotsuga menziesii* or *Lithocarpus densiflora*. In a study by Ishida et al. (2007), however, only eight of the 205 ectomycorrhizal species were strictly host-specific for one of eight tree species. Dickie (2007) concluded that ectomycorrhizas are not definitively host-specific, i.e. there are no physiological or anatomical obstacles, and consequently, the apparent host preference reflects, rather, the influence of environmental factors.

Consequences of irrigation could only be shown for *P. europaea*, which was 50% more common in non-irrigated than irrigated plots, suggesting that it might be a species adapted to drought. Whether *P. europaea* also occurs more frequently at other water-limited sites and becomes more competitive with other CSPs due to drought is a matter for further research.

The colonization density of PAC was significantly higher on the conifer (*P. sylvestris*) than on the deciduous tree (*Q. pubescens*) under the same conditions. It is also widely reported that PAC are more abundant on conifers (Ahlich-Schlegel, 1997; Addy et al., 2000; Grünig et al., 2006; Queloz et al., 2011) than on deciduous trees, e.g. on *Quercus* species (Halmschlagler and Kowalski, 2004; Kwasna et al., 2008). While colonization densities of over 90% were found on pine roots (Queloz et al., 2011), PAC was found only on a maximum of 7.5% of the oak roots (Halmschlagler and Kowalski, 2004). PAC colonization densities of only 1–14% were found in roots of the deciduous tree species *A. pseudoplatanus* and *F. excelsior* during another field study (Santschi, 2015). Reininger et al. (2012) found quadruple the PAC biomass in roots of Norway spruce roots (*P. abies*) than those of birch (*Betula pendula*) in an in vitro experiment using artificial inoculation. A colonization density of more than 30% on *Q. pubescens* under field conditions is therefore considered to be above average. The reason may be the high infection pressure caused by the abundance of PAC in neighboring pine roots.

The irrigation experiment in the Pfywald in Valais was designed to clarify the role of drought in the increasing mortality of *P. sylvestris*. Other studies have revealed various reasons for the increasing weakness of this species. The most important being mistletoe, bark beetles and nematodes (Dobbertin and Rigling, 2006; Polomski et al., 2006; Dobbertin et al., 2007; Wermelinger et al., 2008; Rigling et al., 2013). The microsclerotia formed by PAC probably have a waterproof outer layer like the pseudo-sclerotial plates formed by *Armillaria* species or by members of the Xylariales in decaying wood (Rayner and Boddy, 1988; Garraway et al., 1991; Grünig et al., 2008b). These pseudosclerotia were

Table 4

Allele lengths of CSP 22 at the 13 microsatellite loci. X indicates that CSP 4 and/or CSP 10 possess the same allele as CSP 22, empty cells indicate different allele.

Locus	mPF 011	mPF 022	mPF 043	mPF 0644	mPF 0860A	mPF 138B	mPF 008	mPF 035A	mPF 0672	mPF 068	mPF 0860B	mPF 088	mPF 142B
CSP22	228	177	116	219	156	122	134	149	248	117	Null	Null	145
CSP4	X		X		X	X		X	X	X		X	
CSP10	X	X		X			X	X		X	X		

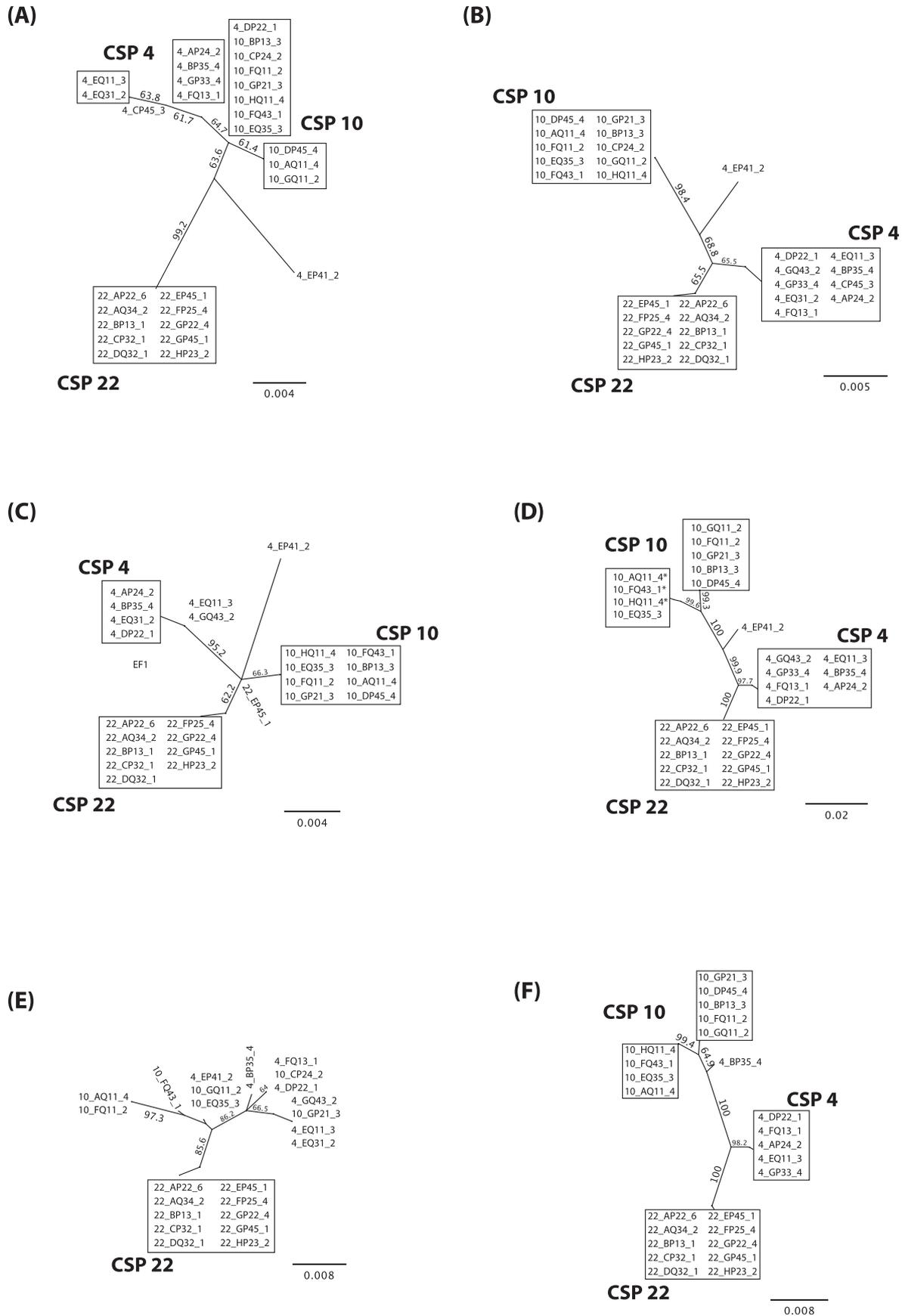


Fig. 5. Unrooted neighbour joining trees depicting the relationship of the newly discovered CSP22 with its closest relatives *Phialocephala helvetica* (CSP 4) and CSP 10 based on five DNA-sequence loci: (A) elongation factor EF1-alpha, (B) β -tubulin, (C) pPF-018, (D) pPF-061 and (E) pPF-076. (F) A tree of the concatenated sequences of EF1-alpha, β -tubulin and pPF-061. Strains with an asterisk in (D) possess a 280-bp-long insertion. The scale bars show the number of changes per nucleotide position, and bootstrap support values of >50% from 1000 replicates are shown above or below branches. See Table S1 for GenBank accession numbers of each strain.

Table 5

Single nucleotide polymorphism (SNP) at nucleotide positions of the concatenated sequences of EF1-alpha, beta-tubulin and pPF-061 of the three closely related CSPs 4, 10 and 22.

Position:	EF1-alpha						beta-tubulin						pPF-061																																														
	43	73	83	243	251	359	604	646	724	840	853	875	1039	1150	1165	1252	1254	1282	1293	1305	1306	1333	1354	1356	1377	1413	1431	1437	1438	1445	1447	1448	1452	1455	1456	1463	1495	1497	1572	1581	1590	1591	1622	1628	1645	1699	1701	1731	1733	1749	1767	1792	1845	1852					
CSP																																																											
22	C	Gap	T	A	A	T	T	C	A	G	C	T	C	T	C	T	A	C	G	G	T	G	G	C	T	G	A	G	Gap	A	G	G	G	A	A	C	G	Gap	A	T	T	A	A	A	G	A	A	A	C	T	C	A	G	G					
4	T	Gap	C	G	G	C	C	T	A	C	C	T	C	T	T	T	G	C	G	G	T	G	G	T	C	G	A	G	Gap	A	G	A	A	G	A	C	G	C	A	A	T	A	A	A	G	G	A	A	A	C	T	G	A	T	G				
10	T	Gap	C	G	G	C	C	C	G	C	T	C	T	A	C	C	T	A	G	A	A	C	A	G	T	T	A	T	A	G	A	A	A	G	G	A	T	C	A	C	G	A	C	G	T	G	A	G	A	T	T	G	T	C	C	C	T	A	G

shown to protect the decaying wood and the fungus against desiccation or to prevent a too high moisture content as in the case of some xylariaceous fungi (Rayner and Boddy, 1988). If it can be confirmed in future experiments that the higher frequency of PAC in oak roots in non-irrigated plots is due to the higher formation of waterproof microsclerotia, then PAC could protect oak roots against desiccation and improve drought resistance of *Q. pubescens*.

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Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.funeco.2019.100904>.

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