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Responses of the root rot fungus *Collybia fusipes* to soil waterlogging and oxygen availability

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SUMMARY

Collybia fusipes is a common root rot fungus in mature pedunculate oak forest, that causes drastic destruction of the tree root systems, especially in dry or mildly waterlogged soils. We wanted to check, under controlled conditions or in forest ecosystems, whether reduced O₂ during saturation of the soil by water could interact with disease evolution. Susceptibility of waterlogged oak seedlings to *C. fusipes* was tested in a greenhouse and the survival of the pathogen in woody substrates was assessed in hydromorphic soils in a forest. A direct and detrimental effect of soil waterlogging on *C. fusipes* survival was evidenced both under controlled conditions and in forest stands. Growth of *C. fusipes* mycelium on agar media was monitored under low O₂ mole fraction and compared to that of *A. mellea* and *H. annosum*. A drastic reduction in mycelial growth was evidenced in *C. fusipes* and *H. annosum* but not in *A. mellea*.

INTRODUCTION

Collybia fusipes (Bull.: Fr.) Quél is a primary parasite that causes slowly evolving root rots in various species of broadleaved trees (Delatour & Guillaumin, 1984). In France, *C. fusipes* is common in oak forests and develops mainly at the base of mature pedunculate oaks (*Quercus robur* L.) (Marçais, Caël & Delatour, 1998; Piou, Delatour & Marçais, 2002). The distribution of the parasite in forest ecosystems and the severity of root damage induced during infection strongly depend on soil factors. Indeed, *C. fusipes* is preferentially distributed in oak forests growing on coarse textured soils (Camy et al., 2003b). This root rot pathogen was also reported to occur preferentially in dry soils (Guillaumin et al., 1985; Nanta & Vellinga, 1995). At the stand level, temporary waterlogging in the soil is an important limiting factor for the severity of *C. fusipes* natural infection on *Q. robur* and *Q. rubra* L. because trees on waterlogged soils are much less prone to disease (Piou et al., 2002; Camy & Marçais, 2002; Camy, Delatour & Marçais, 2002a). Furthermore, the success of direct inoculation of mature oak trees was strongly reduced in waterlogged soils (Camy et al., 2003c).

Excess of water in soils results in gradual oxygen depletion. As a consequence, chemical and biological soil properties are severely affected with important consequences for the activity of soil micro-organisms and plant roots (Drew & Lynch, 1980). Micro-organism communities shift to anaerobes and potentially toxic chemicals such as phenolic and aromatic acids or hydrogen sulfides may accumulate (Drew & Lynch, 1980). Denitrification occurs at high rates as a consequence of soil water saturation (Grundmann & Rolston, 1987). Thus, under field conditions, many factors related to waterlogging could interact with

C. fusipes, and affect mycelium survival, infection and disease development. However, we hypothesised that the low frequency of presence of the pathogen in waterlogged soils, as compared to the very high frequency in coarse textured soils, could primarily result from its high susceptibility to hypoxia. An impact of oxygen availability on the survival and distribution of root rot fungi has already been mentioned by several authors. Blenis, Mulaga & Hiratsuka (1989) observed that the survival of *Armillaria ostoyae* (Romagn.) Herink. on woody inocula was higher in sandy soils and related this observation to a larger oxygen availability in such soils. Production of *Armillaria* spp. rhizomorphs is reduced under hypoxia and their abundance decreases with the depth of soil or soil water saturation in relation to oxygen availability (Morrison, 1976; Rishbeth, 1978; Singh, 1981; Pearce et Malajczuk, 1990). Damage induced by *Heterobasidion annosum* also tends to be greater in soils with a high sand content and adequate aeration (Alexander, Skelly & Morris, 1975; Froelich, Dell & Walkinshaw, 1966). *H. annosum* is able to grow in the heartwood of trees, where the oxygen availability is low (Stenlid & Redfern, 1998). However, heartwood and soil are very different environments which may explain the difference. Conflicting results have been published about the direct effect of oxygen availability on *H. annosum* growth *in vitro*, from no effects of low oxygen (Gundersen, 1961; Lindberg, 1992) to a decrease of growth in other cases (Courtois, 1973). Decreasing O₂ availability was detrimental to the decay activities of several other species of heartwood and sapwood fungi (Highley *et al.*, 1982).

The objectives of this study were to determine, under controlled conditions and in forest ecosystems, whether the absence of *C. fusipes* in waterlogged soil could be explained by reduced O₂ during soil water saturation. Susceptibility of waterlogged oak seedlings to *C. fusipes* was tested in a greenhouse and the survival of the pathogen in woody substrates was assessed in waterlogged soils in a forest. In a final step the growth of *C. fusipes* mycelium on agar media under low O₂ atmospheres was measured and compared with that of *A. mellea* (Vahl.: Fr.) Kumm. and *H. annosum* (Fr.) Bref.

MATERIAL AND METHODS

C. fusipes inoculum production

Five isolates were used in the three following experiments. *C. fusipes*, C41, C49, C62 were isolated from *Q. robur* (south-west France, 1994), *Q. rubra* (central France, 1992) and *Q. petraea* (Matt) Liebl. (north-east France, 1994), respectively. *A. mellea*, M2 was isolated by J. J. Guillaumin, from *Prunus persicæ* (South-east France, 1969). Isolate 47-R1-6 (P group) of *H. annosum* was from *Pseudotsuga menziesii* (north-east France, 2000).

C. fusipes inoculum was produced according to Marçais & Delatour (1996). Briefly, stems of hazel, *Corylus avellana* L., 1.5-2.5 cm in diameter, were cut into 3 cm segments, placed in plastic jars and inoculated with blocks from a *C. fusipes* agar culture. Care was taken to maximise the aeration of the jars by drilling a hole in the jar top and plugging it with cotton wool. The jars were incubated at 23°C for 9-11 months. These hazel segments, colonised by *C. fusipes*, were used for inoculations in experiment 1 and to assess the effects of waterlogging under field conditions in experiment 2.

C. fusipes, *H. annosum* and *A. mellea* isolates were used to test their mycelium growth on malt-agar under low O₂ mole fraction, in experiment 3.

Experiment 1: inoculation by *C. fusipes* and water-logging under controlled conditions

Seventy-six one-year-old seedlings of pedunculate oak (*Quercus robur* L., north-eastern France) were planted in 5 litre pots. Half of the pots were filled with a 1/1 v/v mix of sand and of forest soil with a clay loam texture (from Amance forest, Meurthe-et-Moselle).

Final texture was determined in the laboratory and was 13% clay, 22% silt, 65% sand. The other half was filled with a peat / sand 1/2 v/v substrate. Potted seedlings were grown in a greenhouse at Champenoux, N-E France. The temperature was kept above 5°C during winter. Seedlings were inoculated with *C. fusipes* nine months later, in December 2000. Top soil was removed at the base of the seedlings, and the root collar was cleaned. A hazel stem segment was fastened directly against the bark with an elastic link, 2-5 cm below soil surface and soil was replaced. Fourteen seedlings were inoculated with isolate C41 and 22-26 with isolate C49 in each substrate. Half of the seedlings of each of the 4 categories were completely water-logged with tap water one month after inoculation (Table 1). Waterlogging was maintained during three months from January to March, and the level of the water table controlled daily, in order to induce conditions close to those encountered in forest soils. One year after inoculation, seedlings were removed from the pots and root collars and inocula were examined. Survival rate of inoculum was recorded. *C. fusipes* was assumed to have survived in the hazel stem segments whenever black crusts covered the inoculum and white mycelium was present underneath and/or when the wood had a bright orange colour. Otherwise *C. fusipes* was assumed to have disappeared from the hazel stem segment. Width and height of necrotic bark were recorded. Surface area of induced lesions was estimated from the geometric mean of these two diameters ($\pi \times (\text{height} \times \text{width})/4$). Isolations of the fungus were attempted both from inoculum and host tissues. Tissues were washed under water, surface sterilised for 1 min. in sodium hypochlorite (3.75% active chlorine) and rinsed three times in sterile water. Chips of dead root collar bark or decayed wood were plated on MAT medium (10 g.l⁻¹ of malt Difco, 15g.l⁻¹ agar, 100 mg.l⁻¹ penicillin, 100 mg.l⁻¹ streptomycin and 250 mg.l⁻¹ thiabendazole).

An second test was set up to study survival of *C. fusipes* in hazel stem segments during waterlogging. Eight 3-litre pots were filled with the peat/sand substrate and 8 others with the soil/sand substrate. During April 2001, 2-3 hazel stem segments colonized by *C. fusipes* isolate C41 were buried in each pot 2 to 5 cm below soil surface (total of 47 segments). The pots were all completely flooded one month later with tap water and kept flooded in a glasshouse for 53 days. The level of the water table was checked daily. One hazel stem segment was removed from each pot at three dates: 21, 35 and 53 days after flooding. Survival of the inoculum in the stem segments was recorded based on the previously described method.

Experiment 2: Influence of hydromorpha on *C. fusipes* survival under field conditions

Six oak stands located in north-eastern France, in which *C. fusipes* had been detected, were selected based on contrasting soil textures and large within-stand heterogeneity in the level of hydromorpha. In each plot a sample of soil from a depth of 30 cm was analysed in the laboratory for texture (%sand). The assessment of hydromorpha severity was based on the depth of reddish traces of insoluble oxydized iron and/or discoloration indicating iron depletion, which reflects the severity of waterlogging. During January 2001, 10 hazel stem segments colonized by *C. fusipes* isolate C41 were buried in the soil of the severely hydromorphic area and 10 others in the mildly hydromorphic area of each stand. The inoculum was inserted at about 1 m from stem base on both sides of five trees in each area. The segments were buried below the hydromorphic soil layer in severely hydromorphic areas and above this layer in mildly hydromorphic areas. The hazel stem segments were excavated one year later and brought back to the laboratory. As a few segments could not be recovered at the end of experiment, only 8 segments were assessed per individual area in Vitrimont and 9 in the well drained area of Amance. Survival rates of *C. fusipes* inoculum were recorded as in to experiment 1.

Experiment 3: growth of *C. fusipes*, *A. mellea* and *H. annosum* under reduced oxygen mole fraction

In vitro growth of three isolates of *C. fusipes*, one of *A. mellea* and one of *H. annosum*, was measured under different O₂ mole fractions. For each isolate, a 4 mm malt agar disk was cut from the margin of a young colony and placed on the surface of 20 ml solid malt-agar plates. Venting ribs were present on the lower side of the Petri dishes lids in order to provide adequate O₂ supply to the cultures. After one week for *C. fusipes* and *A. mellea* and 3 days for *H. annosum*, cultures were placed in airtight plastic jars of 50 X 50 X 50 cm filled with air mixtures differing in O₂ mole fraction: 21% (normal atmospheric concentration), 8.0%, 5.0%, 3.0%, 0.5%, 0.3% and 0.1%. Target O₂ mole fractions were reached by injecting N₂ into the jars. O₂ mole fractions were measured with a medical O₂ monitor (Toptronic, model 74223, with a resolution of 0.1%). A little fan homogenised the atmosphere within each jar and avoided CO₂ accumulation in the plates. Eight experiments were carried out. In each experiment, 2 to 3 different oxygen concentrations plus 21% oxygen were tested (3-4 jars). Each concentration was tested 3 to 4 times. At first, 25 cultures (5 repetitions per isolate) per jar were preincubated for acclimation during 24 hours under the target O₂ mole fraction. Then, cultures were removed and their perimeter was marked as starting point for the measurement of hyphal growth. Cultures were replaced into jars for seven days, O₂ mole fractions were readjusted and controlled twice a day. After this period, cultures were removed again from jars and hyphal growth during the preceding seven days was measured.

Statistical analyses

The survival of *C. fusipes* in hazel stem segments (experiments 1 and 2) was analysed by logistic regression, using the procedure 'genmod' of SAS. The model validity was checked using the deviance/degree of freedom ratio, by plotting deviance residuals against the linear predictor and with a half-normal plot (Collett, 1991). In experiment 1, since few inocula had survived in waterlogged pots, infection success was taken into account only for control seedlings with surviving inoculum. In experiment 3, the growth of isolates under reduced atmospheres was divided by the mean isolate growth under ambient atmosphere during the same experiment. This proportion was subjected to an analysis of variance (SAS, 1989). In the model, the isolate effect was tested as independent variable nested in the species effect.

Table 1. Results of infections after artificial inoculations by two *C. fusipes* isolates of potted *Q. robur* seedlings submitted or not to soil water logging (experiment 1)

Substrate	Isolate	Treatment (seedling number)	Number of surviving inocula (%)	Number of seedlings with bark infections	Infected bark area (cm ²)
1/2 forest soil 1/2 sand	C41	Control (7)	6 (85.7)	3	2.4
		Waterlogged (7)	1 (14.3)	0	-
	C49	Control (13)	4 (30.8)	0	-
		Waterlogged (13)	0 (0.0)	0	-
2/3 sand 1/3 peat ^b	C41	Control (7)	5 (71.4)	3	4.39
		Waterlogged (7)	1 (14.3)	1	9.28
	C49	Control (11)	9 (81.8)	5	3.14
		Waterlogged (11)	0 (0.0)	0	-

RESULTS

Experiment 1: inoculations and waterlogging under controlled conditions

C. fusipes was isolated from all surviving inocula and all lesions. There was a severe and negative effect of waterlogging on inoculum survival independent of the growth substrate and of the *C. fusipes* isolate (Table 1). Survival was slightly higher for isolate C41 (Tables 1 and 2). After 3 months waterlogging, only one inoculum piece had survived in the soil/sand substrate and one in the peat/sand substrate. A tendency towards more seedlings with infected bark in peat/sand than in soil/sand substrate was visible (57% and 30% respectively, Table 1). But the low number of replicates did not allow any statistical test of this observation.

Table 2. Logistic regression analysis of the relationship between inoculum survival, type of substrate (peat/sand or soil/sand), waterlogging during 3 months and fungal isolate (C41 or C49 ; Experiment 1).

Source	deviance	df	Chi-square	Pr>Chi-square
Intercept	45.6	-	-	-
Substrate	43.9	1	1.69	0.193
Waterlogging	10.9	1	33.01	<0.001
Isolate	6.0	1	4.85	0.028
Waterlogging x Substrate	5.4	1	0.58	0.447
Waterlogging x Isolate	3.9	1	1.58	0.208

During the second test, set up to follow the dynamic of *C. fusipes* survival during waterlogging, the fungus had completely disappeared from hazel stem segments after 21 d of water-logging since it could never be isolated from the segments after 21, 35 and 53 days of flooding, whatever the substrate.

Experiment 2: influence of hydromorpha on *C. fusipes* under field conditions

The survival of *C. fusipes* after one year in hazel stem segments was independent of the sand content of soils but differed among plots (Tables 3 and 4). Survival was lower in a majority of severely hydromorphic areas in comparison with mildly hydromorphic ones. However, the interaction 'hydromorpha x plot' was significant. Indeed, the survival of *C. fusipes* was high in the mildly waterlogged area in each stand. Conversely, its survival in the severely waterlogged areas was high only in Filain and Adelans (Table 2).

Experiment 3: growth of *C. fusipes*, *A. mellea* and *H. annosum* under reduced oxygen mole fractions

Under ambient O₂, *A. mellea*, *C. fusipes* and *H. annosum* displayed a daily growth of 0.75 ± 0.03 mm, 1.81 ± 0.04 mm and 7.18 ± 0.12 mm, respectively. There was a significant effect of O₂ mole fraction, species and isolates on root rot fungi growth under hypoxia in comparison with their growth under ambient atmospheres (Table 5, Fig. 1). *C. fusipes* growth gradually decreased when O₂ mole fraction dropped below 8% and was slightly more affected than that of *H. annosum*. The significant interaction between O₂ mole fraction and species is mainly due to the smaller influence of hypoxia on *A. mellea* growth compared to *C. fusipes* and *H. annosum* (Table 5, Fig. 1). There was also a significant interaction between oxygen concentration and isolates, which could be explained by the slightly lower growth of the *C. fusipes* isolate C49 under oxygen concentrations higher than 5%, in

comparison with the two other *C. fusipes* isolates and by its slightly greater growth under concentrations lower than 0.5%. When cultures of *C. fusipes*, *H. annosum* and *A. mellea* were returned to under ambient atmosphere after exposure to hypoxia the mycelium growth resumed at initial rates (results not shown).

Table 3. Main soil properties in the selected plots in *Q. robur* stands in different forests of North Eastern France and survival of *C. fusipes* on woody inocula after one year burial (experiment 2).

	Plot	Soil sand content (%)	Depth of first traces of hydromorpha (cm)	Depth of hazel stem segments (cm)	Hazel segments with surviving <i>C. fusipes</i> (%)
Severely waterlogged areas	Filain	6.9	25	45	80
	Amance	8.3	4	23	0
	Adelans	17.4	10	23	70
	Mersuay	30.4	5	27	0
	Ainvelle	38.6	6	40	30
	Vitrimont	41.3	6	27	0
Mildly waterlogged areas	Filain	8.3	44	21	90
	Amance	5.4	48	23	89
	Adelans	24.2	48	20	90
	Mersuay	34	none until 100	24	90
	Ainvelle	64.1	60	21	70
	Vitrimont	67.5	58	20	100

NOTE : N = 10 inocula per plot

Table 4. Logistic regression analysis of the relationship between survival of *C. fusipes* in hazel stem segments in forest plots and soil factors (experiment 2).

Source	deviance	df	Chi-square	Pr>Chi-square
intercept	155.6	-	-	-
Sand content	155.6	1	0.00	0.949
Plot	120.6	5	34.94	< 0.001
waterlogging	90.9	1	29.67	< 0.001
Waterlogging x plot	72.4	4	18.50	0.001

Table 5. Analysis of variance of the relationship between mycelium growth under low O₂ and the species of root rot fungi, *C. fusipes*, *A. mellea* and *H. annosum* (experiment 3).

Source	df	F	p
O ₂ mole fraction	1	406.37	< 0.001
Species	2	149.88	< 0.001
Isolate (Species)	2	3.68	0.026
O ₂ mole fraction x species	2	34.10	<0.001
O ₂ mole fraction x isolate (Species)	2	2.88	0.057

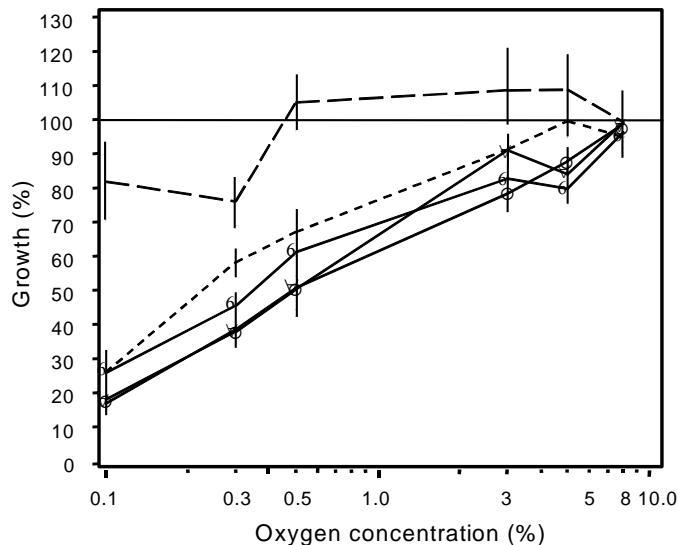


Fig. 1. growth of *A. mellea* (— — —), *H. annosum* (-----) and *C. fusipes* isolates, (—*—), C41, (—6—) C49 and (—▽—) C62, under reduced oxygen mole fraction (8, 5, 3, 0.5, 0.3 and 0.1 %) in comparison with growth of isolates at ambient atmosphere (20.9%). The bars represent the confidential interval of the mean.

DISCUSSION

We have shown a direct and detrimental effect of soil waterlogging on *C. fusipes* survival both under controlled conditions and in forest stands. We have also demonstrated that hypoxia induced a drastic reduction of *C. fusipes* and *H. annosum* mycelial growth whereas *A. mellea* was almost insensitive.

Earlier work had shown that *C. fusipes* survival increased with soil sand content (Camy et al., 2003c). In this study, we expected the survival of *C. fusipes* in stem segments buried in forest soils to confirm this result. This was not the case, possibly due to the lower number of investigated stands (6 vs. 10) and the shorter duration of the experiment compared with the previous work (1 vs. 2.5 years). In these forest soils, the survival of *C. fusipes* strongly differed between severely hydromorphic areas in the different stands. *C. fusipes* survival in the hydromorphic areas of Filain and Adelans was high despite a relatively low soil sand content. This high survival rate might be explained by the difference of depth in hydromorphic traces which appeared slightly deeper in these 2 stands. In all studied stands, the waterlogging was mainly winter waterlogging and was induced either because the drainage of rain water was impeded by an impervious soil layer in Amance, Adelans or Filain or because of the fluctuation in the level of the alluvial groundwater in Mersuay, Ainvelle or Vitrimont. Waterlogging may be very irregular in place where it is induced by impeded drainage and was may not have been severe during the year of the experiment in Filain and Adelans, even in the so-called severely waterlogged area.

Waterlogging of inoculated seedlings allowed us to confirm under controlled conditions the direct negative effect of soil water saturation on *C. fusipes* survival in conditions close to those encountered in forest. This low ability of *C. fusipes* to survive an episode of waterlogging in the soil probably explains why natural infections are more developed in dry or mildly hydromorphic soils (Piou et al., 2002; Camy et al., 2003a). Chemical and biological properties of forest soil could not be the same than those of our pot substrates, especially in

the peat/sand substrate. However, these difference in soil properties did not interact with waterlogging effects on *C. fusipes* survival, which was very low in the both types of substrates. This might indicate that soil water saturation is unfavorable to the pathogen mainly because it induces hypoxic conditions, although we cannot rule out other possible mechanisms such as production of toxic compounds generated during the soil waterlogging.

A high susceptibility of *C. fusipes* to hypoxia was evidenced by reduced growth of mycelium on malt-agar under hypoxia. The hypoxia induced growth reductions were completely reversible as *C. fusipes* recovered a normal growth once replaced under ambient atmosphere. Effects of soil water saturation on *C. fusipes* were more drastic, since the fungus did not survive in waterlogged soil after 21 days. This could be explained by the shorter exposure to reduced oxygen availability in the *in vitro* experiment (7 days versus 21-90 days) or the extremely low oxygen availability in soil water. It could also be due to the fact that *C. fusipes* in soil was possibly submitted to toxic compounds and/or competitors, much more adapted to hypoxic conditions.

The growth of *A. mellea* *in vitro* was slightly affected by hypoxia. This could explain the fact that *A. mellea* appears preferentially distributed in heavy soils (Termorshuizen & Arnolds, 1994). By contrast *H. annosum* growth was strongly affected by hypoxia. Courtois (1973) found similar decreases in *H. annosum* growth under low O₂. Other reports also indicate little or no influence of high hypoxia on its growth in comparable conditions (Gundersen, 1961; Lindberg, 1992).

Unfavourable effects of wood moisture content on growth of *H. annosum* had also been reported under controlled conditions (Kuhlman, 1980; Lindberg & Johansson, 1992). Johansson & Stenlid (1985) studied interactions between host and *H. annosum* during the initial stages of infection process in living sapwood of excised spruce roots, in water-saturated roots as well as in nitrogen atmosphere. They demonstrated a reduction in *H. annosum* mycelium growth linked with modifications of enzyme activities, dependent on oxygen supply. Cwielong & Hüttermann (1987) demonstrated that a minimum concentration of oxygen was required for oxidation of toxic phenolic compounds, and growth of *H. annosum* was inhibited at lower O₂ levels.

Furthermore, Redfern (1998) found after artificial field inoculations, a low level of infection induced by *H. annosum* in peat soils and hypothesised a direct and negative effect of soil moisture and pH to the transfer of infection from a tree to its neighbours. Negative impact of soil water saturation under field conditions was also reported on *Inonotus tomentosus* (Fr.) Teng. and on *A. ostoyae* which incidence decreased in soils with wet moisture regimes (Bernier & Lewis, 1999; Whitney, 1995).

The direct and negative effect of hypoxia induced by water saturation of soil on *C. fusipes*, demonstrated in this study, is certainly a major explanation of earlier results showing that oak trees were less frequently infected in such soil conditions (Piou *et al.*, 2002; Camy & Marçais, 2002; Camy *et al.*, 2003a). Waterlogging might also influence the pathogen survival through other mechanisms such as changes in the soil micro-organism community or generation of toxic compounds. The strong susceptibility of *C. fusipes* mycelium to hypoxia might be one of the reasons why this parasite is more frequently present in coarse textured soils where oxygen availability is expected to be higher.

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