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FULL PAPER

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Effects of sodium chloride on growth of ectomycorrhizal fungal isolates in culture

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Abstract We studied the tolerance of ectomycorrhizal (ECM) fungi to sodium chloride (NaCl) to find the best fungus to aid growth of *Pinus thunbergii*. Four ECM fungi, *Cenococcum geophilum*, *Pisolithus tinctorius*, *Rhizopogon rubescens*, and *Suillus luteus*, were grown in liquid MMN media with five different concentrations of NaCl for 30 days, and their mycelial weights were determined. Mycelial weights of *P. tinctorius* and *R. rubescens* were not significantly different between 0 mM and 200 mM, whereas those of *C. geophilum* and *S. luteus* decreased with increasing NaCl concentration, indicating that the former two species were more tolerant to higher NaCl concentrations than the latter species. We further studied the intraspecific differences in NaCl tolerance of nine *P. tinctorius* isolates. They were grown on MMN agar media with six different concentrations of NaCl for 21 days, and their radial growth was measured. In total, the hyphal growth at 25 mM NaCl was significantly higher than those at the other NaCl concentrations, and EC_{50} values were confirmed at between 50 mM and 200 mM. Among the isolates, Pt03 and Pt21 showed measurable growth at 200 mM; the growth of Pt03 was not significantly different between 0 mM and 200 mM. The results indicate that there are intraspecific variations in NaCl tolerance of *Pisolithus* species.

Key words Coastal forest · Intraspecific traits · NaCl · *Pisolithus* · Salinity

Introduction

In coastal areas, the Japanese black pine, *Pinus thunbergii* Parl., is mainly distributed along seashores from Honshu to Kyushu in Japan (Satake et al. 1989) and plays important roles in windbreak and tidewater-control forests as well as in enhancing coastal scenery (Murai et al. 1992). However, pine wilt, an infectious disease of *P. densiflora* Sieb. & Zucc. and *P. thunbergii* caused by the pinewood nematode *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle (Kiyohara and Tokushige 1971), has lead to a disastrous decline in coastal pine forests. To benefit their continuity and rehabilitation, these declining forests need to be rescued with pesticides or recruited with seedlings of *P. thunbergii* that are resistant to the nematode (Mamiya 2004; Nakamura and Yoshida 2004). However, coastal areas are generally harsh conditions for the growth of plants, especially seedlings, and effective techniques that promote colonization by, and growth of, seedlings are needed to reestablish healthy pine forests.

The fine roots of *P. thunbergii* are exclusively covered with fungal mantles of ectomycorrhizal (ECM) fungi (Ogawa 1979) that belong mainly to the Ascomycetes or the Basidiomycetes (Molina et al. 1992). Tree uptake of soil nutrients and water depends on these fungi (Smith and Read 1997), and ECM fungi have been used as inocula for tree planting programs (Castellano 1996). Among them, *Pisolithus* species are commonly used as fungal inocula because they have global distribution and grow faster than other ECM fungal species (Chambers and Cairney 1999). It is thus probable that application of the fungi to *P. thunbergii* seedlings might help restore declining pine forests.

Salt tolerance of terrestrial fungi has been studied (Tresner and Hayes 1971; Castillo and Demoulin 1997), and some arbuscular mycorrhizal fungi improve the growth of their associated plants under salt stress conditions (Giri and Mukerji 2004; Tian et al. 2004). However, a few studies have reported the growth of ECM fungi in saline conditions (Saleh-Rastin 1976; Chen et al. 2001; Kernaghan et al. 2002); several kinds of salt at concentrations up to 200 mM were used. Coastal pine trees can be established not only on sand dunes but also on rocky cliffs, where seawater, which has about 500 mM sodium chloride (NaCl), is sprayed both directly onto the needles and indirectly onto the root

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Table 1. Ectomycorrhizal fungal isolates used in experiments 1 and 2, and their sites, forest types, and putative host trees

Ectomycorrhizal fungal species	Isolate no.	Site	Forest type	Putative host
Experiment 1				
Cenococcum geophilum	Cg01	Aichi, Japan	Naturally regenerated forest	*Abies firma*
Pisolithus tinctorius	Pt01	Mie, Japan	Coastal forest	*Pinus thunbergii*
Rhizopogon rubescens	Rr01	Ibaraki, Japan	Coastal forest	*P. thunbergii*
Suillus luteus	Sl01	Tokyo, Japan	Naturally regenerated forest	*P. densiflora*
Experiment 2				
P. tinctorius	Pt00	Mie, Japan	Coastal forest	*P. thunbergii*
P. tinctorius	Pt02	Mie, Japan	Coastal forest	*P. thunbergii*
P. tinctorius	Pt03	Mie, Japan	Coastal forest	*P. thunbergii*
P. tinctorius	Pt09	Mie, Japan	Coastal forest	*P. thunbergii*
P. tinctorius	Pt11	Mie, Japan	Coastal forest	*P. thunbergii*
P. tinctorius	Pt13	Mie, Japan	Coastal forest	*P. thunbergii*
P. tinctorius	Pt16	Mie, Japan	Coastal forest	*P. thunbergii*
P. tinctorius	Pt17	Mie, Japan	Coastal forest	*P. thunbergii*
P. tinctorius	Pt21	Mie, Japan	Coastal forest	*P. thunbergii*

systems of trees. We examined extremely high concentrations of NaCl, up to 1000 mM, to elucidate the potential tolerance of ECM fungi to NaCl.

Our aim was to clarify the salinity tolerance of ECM fungi to select appropriate fungal inocula to restore the declining coastal pine forests. For this purpose, we measured the growth of four ECM fungal species on nutrient media with different concentrations of NaCl, representing conditions in coastal areas. We then selected one species of ECM fungus and examined the growth of its isolates to detect intraspecific variations.

Materials and methods

Experiment 1

Four species of ECM fungi, *Cenococcum geophilum* Fr. (Cg01), *Pisolithus tinctorius* (Pers.) Coker & Couch (Pt01), *Rhizopogon rubescens* (Tul. & C. Tul.) Tul. & C. Tul. (Rr01), and *Suillus luteus* (L.) Gray (Sl01), were selected based on the growth experiment of fungi on nutrient media supplied with deep seawater (Table 1; Sai et al. 2002). The basal growth medium was a modified Merin–Norkans medium (MMN; Marx 1969) containing (l^{-1}): $(NH_4)_2HPO_4$, 250 mg; KH_2PO_4, 500 mg; glucose, 10 g; malt extract 3 g; NaCl, 25 mg; $MgSO_4 \cdot 7H_2O$, 150 mg; $CaCl_2$, 50 mg; 1% $FeCl_3$, 1.2 ml; and thiamine HCl, 0.1 mg. Liquid MMN amended with different concentrations of NaCl (0, 20, 50, 100, and 200 mM) was prepared, and the final pH was adjusted to 5.0–5.4 after autoclaving for 15 min at 121°C. Precultured fungal colonies on basal MMN agar plates were bored around the peripheries with a 10-mm cork borer to make fungal inocula and were transferred to 8.5-cm plastic culture plates, each containing 20 ml liquid medium. After inoculation, the plates were sealed with parafilm and incubated in dark conditions for 30 days at 25°C. There were seven replicate plates for each species, in five treatments. Following incubation, grown mycelium was filtered by a quantitative filter paper (Advantec no. 5C; Advantec Toyo Kaisha, Tokyo, Japan) and weighed after drying for 24 h at 105°C.

Experiment 2

Nine isolates of *P. tinctorius*, selected on the basis of the results obtained in experiment 1, were used in this experiment (see Table 1). They were isolated from sporocarps collected at a coastal *P. thunbergii* forest in Mie Prefecture, Japan (34°47′ N, 136°33′ E). NaCl was measured in soil samples collected at locations from the seashore to the forest using an ion meter (F-23; Horiba, Tokyo, Japan). NaCl concentrations ranged from 1.3 to 187 mM, and the mean concentration in samples from the place where the pine forest was established and the sporocarps were collected was about 10 mM NaCl.

The basal MMN medium was amended with 2% agar and six different concentrations of NaCl: 0, 25, 50, 200, 500, and 1000 mM. Fungal inocula were prepared as same as those of experiment 1. After inoculation, the plates were sealed with parafilm and incubated for 21 days in dark conditions at 25°C. There were five replicate plates for each isolate in each treatment. The longest and shortest diameters of each hyphal colony were measured and the hyphal growth was evaluated as follows:

$$\text{Hyphal growth (mm)} = (\text{longest diameter} + \text{the shortest diameter})/2 - 10$$

Statistical analysis

Data from each growth experiment were analyzed by one-way analysis of variance (ANOVA). However, Levene's equitability of deviation was not supported ($\alpha < 0.05$), and thus significant differences between means were determined at $\alpha < 0.05$ by the nonparametric multi comparison analysis of the Kruskal–Wallis test. All statistical analyses were performed using the SPSS computer program 11.5J (SPSS 2002).

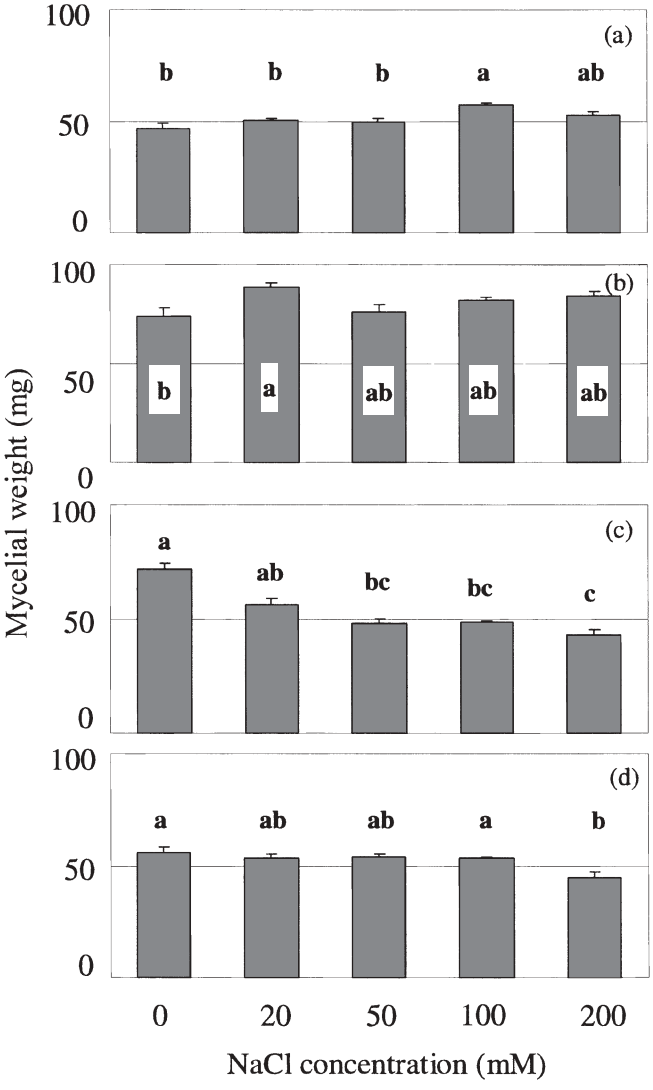

Fig. 1. Mycelial weights of four ectomycorrhizal fungi: *Pisolithus tinctorius* (**a**), *Rhizopogon rubescens* (**b**), *Suillus luteus* (**c**), and *Cenococcum geophilum* (**d**) in different concentrations of NaCl. Each value represents the mean + SE (n = 7). Values with different letters are significantly different (Kruskal–Wallis test, $\alpha < 0.05$)

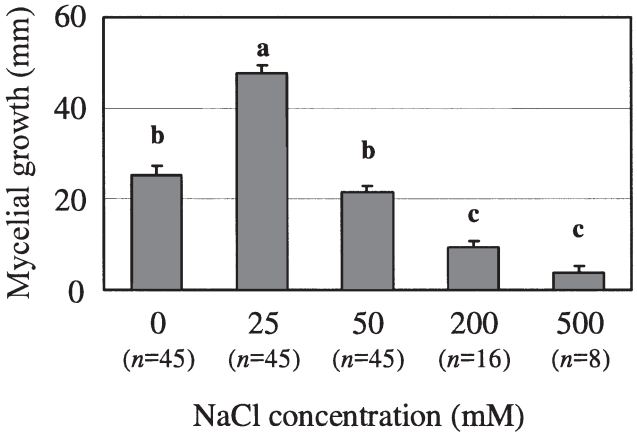

Fig. 2. Mycelial growth of *Pisolithus tinctorius* in different concentrations of NaCl. Each value represents the mean + SE. Values with different letters are significantly different (Kruskal–Wallis test, $\alpha < 0.05$). Nine isolates were pooled within each NaCl concentration. No growth was found at 1000 mM for all isolates

Results

Experiment 1

The mycelial dry weight of *P. tinctorius* at 100 mM NaCl was significantly higher than those at less than 50 mM, whereas the weight at 200 mM was not significantly different from that at 0 mM (Fig. 1a). For the mycelial weight of *R. rubescens*, although there was a significant difference between 0 and 20 mM, no significant difference was found between 0 and 200 mM (Fig. 1b).

On the other hand, the mycelial dry weight of *C. geophilum* and *S. luteus* decreased with increasing NaCl concentrations. A significant decrease in the weight of *S. luteus* was found at more than 50 mM compared with that of 0 mM, and the weight at 200 mM was 41% less than that at 0 mM (Fig. 1c). For *C. geophilum*, the trend of decreasing mycelial weight with different NaCl concentrations was less obvious than that found with *S. luteus*, and a significant difference was found only between 0 or 100 and 200 mM (Fig. 1d).

For all the species, no effective concentration inhibiting growth by 50% (EC_{50} value) was detected at the range of NaCl concentrations tested.

Experiment 2

When all isolates were pooled, mycelial growth of *P. tinctorius* was affected significantly by the addition of NaCl (Fig. 2; one-way ANOVA, $\alpha < 0.0001$). No growth was found at 1000 mM, irrespective of the fungal isolates (data not shown). Growth at 25 mM was significantly greater than that at the other NaCl concentrations ($\alpha < 0.001$), and growth at 50 mM was not significantly different from that at 0 mM. A significant decrease in growth was found at more than 200 mM as compared with 0 mM, and the EC_{50} value was found at concentrations between 50 and 200 mM.

All fungal isolates tended to show a similar growth pattern with increasing NaCl concentrations (Fig. 3). The growth at 25 mM, except for the isolates of Pt02 and Pt21, was significantly higher than that at 0 mM. However, fungal responses to NaCl varied depending on the concentration, and the isolates were accordingly divided into two groups. For all the isolates except Pt16, there were no significant differences in mycelial growth between 0 and 50 mM. Two isolates, Pt03 and Pt21, showed apparent growth at 200 mM, and the growth of the former isolate was not significantly different between 0 and 200 mM. EC_{50} values also differed among isolates. In the case of Pt03, the EC_{50} value was confirmed between 200 and 500 mM, whereas that of the remaining isolates was between 50 and 200 mM.

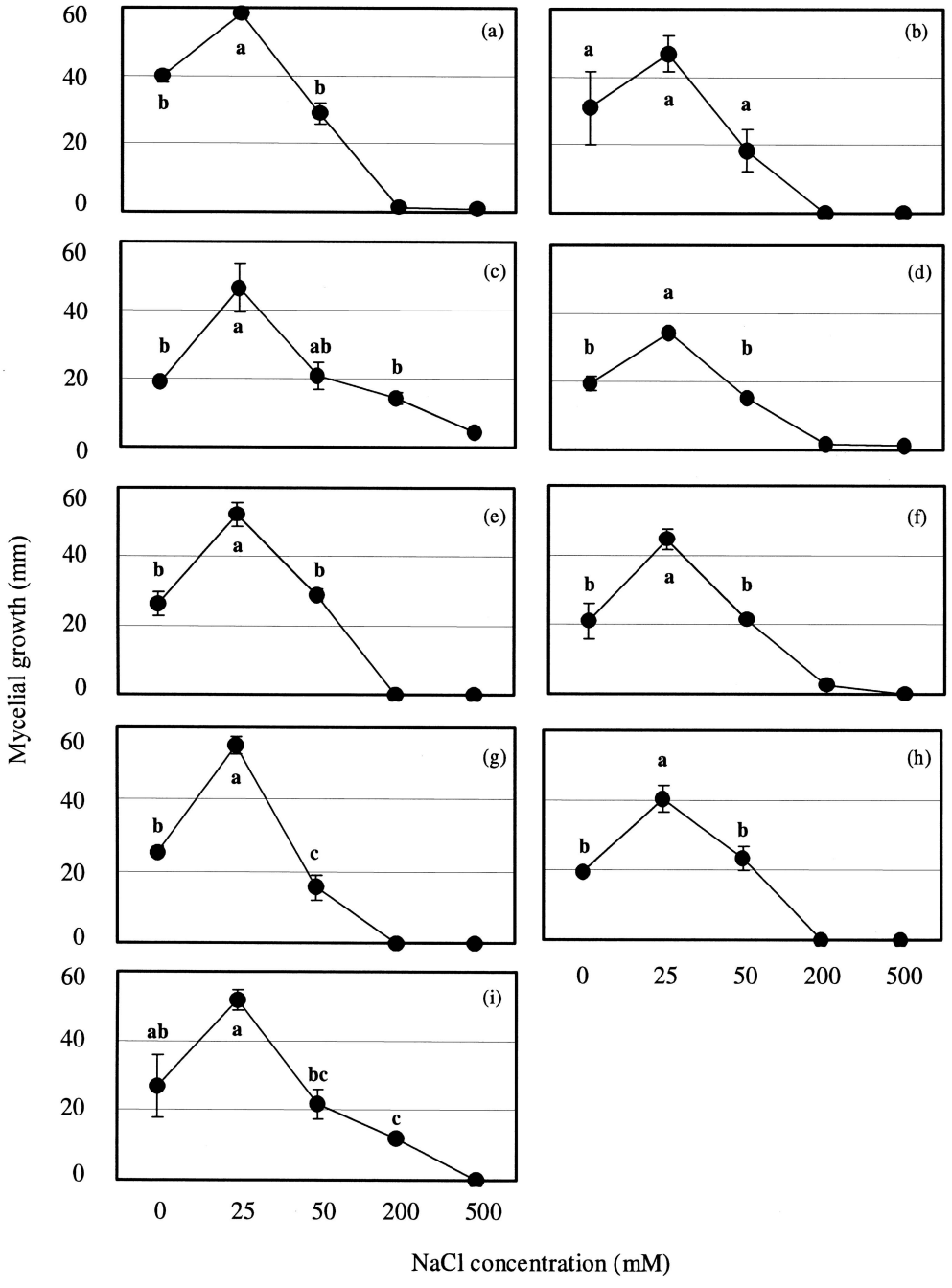

Fig. 3. Mycelial growth of nine isolates of *Pisolithus tinctorius* in different concentrations of NaCl. Each value represents the mean ± SE ($n = 5$): **a** Pt00; **b** Pt02; **c** Pt03; **d** Pt09; **e** Pt11; **f** Pt13; **g** Pt16; **h** Pt17; **i** Pt21. Values with different letters are significantly different (Kruskal–Wallis test, $\alpha < 0.05$)

Discussion

The response to NaCl varied among the species tested, which we divided into two groups. One group comprised *P. tinctorius* and *R. rubescens*; the biomass of both these species did not seem to be affected by increasing NaCl concentrations. In fact, the mycelial weight of each species at 200 mM was not significantly different from that at 0 mM. The other group comprised *C. geophilum* and *S. luteus*; the biomass of these species tended to decrease with increasing NaCl concentrations. At 200 mM NaCl, the mycelial weight of each species was significantly lower than that at 0 mM. These results suggest that the effect of NaCl on the growth of ECM fungi varies among fungal species and that *P. tinctorius* and *R. rubescens* are relatively NaCl-tolerant species.

In experiment 1, the four ECM fungal species showed various growth patterns in response to NaCl concentration. However, no species was suppressed completely at 200 mM, which is about 40% of the NaCl concentration in seawater (about 500 mM). At 200 mM NaCl, the mycelial weight of *S. luteus* was the most strongly affected among the four species: it produced about 60% biomass compared with that at 0 mM. Chen et al. (2001) considered ECM fungal isolates to be salinity tolerant when they maintained more than 50% of

their mycelial weights at certain salinities compared with nonsaline conditions. Furthermore, soils containing more than 40 mM NaCl are defined as saline (Marshner 1995). Taking these definitions into account, all the species tested may potentially be resistant to NaCl stress. In fact, Saleh-Rastin (1976) showed that an isolate of *C. graniforme* (Sowerby) Ferd. & Winge (=*geophilum*) could grow in concentrations of NaCl as high as 188 mM. In addition, the growth of some *Pisolithus* spp. isolates also suggested saline tolerance and was not suppressed in 200 mM NaCl (Chen et al. 2001).

Although the biomass of *C. geophilum* and *S. luteus* decreased with increasing NaCl concentration, indicating some susceptibility to NaCl, they originated from naturally regenerated forests where NaCl concentrations in the soil would be relatively low. The origins of fungal isolates can affect the functional diversity or ecological plasticity of the fungi (Colpaert et al. 2004) and thus may affect their range of salt tolerance (Tian et al. 2004). Further studies should include a wide variety of ECM species associated with coastal pine trees to determine NaCl-tolerant fungi.

In experiment 2, mycelial growth of the isolates responded differently to various NaCl concentrations, and the isolates could be divided into two groups. Pt03 and Pt21 were able to grow at 200 mM, but the other seven isolates (Pt00, Pt02, Pt09, Pt11, Pt13, Pt16, and Pt17) showed little or no growth at 200 mM (see Fig. 3). These results suggest that there are intraspecific variations among the isolates in the response to NaCl. Our results were consistent with those of Chen et al. (2001), who found intraspecific growth variations among 18 strains of *Pisolithus* species at various salinities. On the other hand, Kernaghan et al. (2002), who examined the ECM fungal growth of 6 strains of *Hebeloma crustuliniforme* (Bull.) Quél. and 3 strains of *Laccaria laccata* (Scop.) Fr. at various salinities, found no intraspecific growth differences, despite using several kinds of salinity (NaCl, Na_2SO_4, and composite tailings). These contradictory results among studies may be explained partly by variations in the number or origins of fungal isolates.

In our study, Pt03 growth was not significantly different between 0 mM and 200 mM. Moreover, this isolate exhibited a trace of mycelial growth at 500 mM. These observations indicate that the mycelial growth of the Pt03 isolate might increase, even at high salinity, and that this isolate may be a good candidate as an ECM inoculum for outplanting of *P. thunbergii* seedlings in coastal forests. Although salinity imposes both ionic and osmotic stresses (Tester and Davenport 2003), few studies of ECM fungi have unambiguously discriminated between them. To understand the mechanism of NaCl tolerance, we need to focus on the effect of ionic stresses, such as those imposed by Na^+ and Cl^-, on the growth of ECM fungi.

Recent morphological and molecular studies have suggested that the genus *Pisolithus*, originally recognized as a monotypic genus, comprises more than one different species (Martin et al. 2002; Moyersoen et al. 2003). However, our random fragment length polymorphism (RFLP) analyses of internal transcribed spacer (ITS) regions within rDNA showed that the nine isolates produced a single RFLP pattern, indicating that they belonged to one *Pisolithus* species (data not shown). Because physiological and metabolic traits can vary not only among species but also within species (Cairney 1999), it is highly probable that salt tolerance differs among isolates, even when they originate in a limited habitat area, as in this study.

In conclusion, we demonstrated that the species *P. tinctorius* and *R. rubescens* were more NaCl tolerant than the other species tested. Moreover, the tolerance varied among isolates within *P. tinctorius*, and effective isolates seemed to be present even in a locally restricted pine forest. Further work is required to determine whether an NaCl-tolerant isolate can alleviate salinity stress in the associated *P. thunbergii*. Future work must also evaluate the ECM fungal communities and/or population structures in coastal pine forests to clarify the spatiotemporal distribution of tolerant ECM species in these forests.

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