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Editor(s)	
Citation	Bulletin of the University of Osaka Prefecture. Ser. B, Agriculture and life sciences. 1995, 47, p.11-17
Issue Date	1995-03-31
URL	http://hdl.handle.net/10466/2783
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Bull. Univ. Osaka Pref., Ser. B, Vol. 47,
大阪府立大学紀要, 農学・生命科学, 1995

Fluctuations of *Pythium fluminum* var. *fluminum* in pond water

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(Received October 31, 1994)

Abstract

Population densities of *Pythium fluminum* Park var. *fluminum* (*P.fluminum*) in pond water are given for a year sampling period. Colony number per liter ranged from 0 to 260. A fluctuation in population density is clearly detectable being highest in winter and decreasing gradually until reached zero in summer. When the pond water temperature exceeded 25℃ the fungus was not detected. *P.fluminum* is different from any other species of *Pythium* in its capability for decomposing insoluble cellulose and reducing the filter paper weight by 20% in one month. A quantitative estimation of this fungus can be carried out by filtering pond water through filter paper circles using suction force. The filter papers were then seeded on CPV selective medium. After keeping for two weeks at 15℃, the pale salmon pink colonies could easily be counted.

Introduction

Pythium fluminum Park var. *fluminum* (*P.fluminum*) was first isolated in Ireland[1]. This fungus is unique in the genus decomposing insoluble cellulose and utilizing it as a sole source of carbon[2]. Park (1980)[3] studied seasonal fluctuations of this fungus in two rivers in Ireland for two years. During Park's investigation the taxon was recovered throughout the period studied. The colony number ranged from 2 to 1820 per liter with highest in winter and lowest in summer. The river water temperature ranged from 2 ℃ to 18℃. He postulated that the highly significant relationships were demonstrated with the preceding rainfall and river flow rate, but not with temperature or pH. Since that time there was no other record of this fungus outside Ireland. This fungus was, however, isolated from a pond in Osaka, the first record outside Ireland[4]. The still water in the pond with temperature ranged from 4 ℃ to 33℃ makes the ecological pattern different from that of the river.

The purpose of this study is to investigate the fluctuations of *P.fluminum* in pond water and to differentiate between the ecological patterns in the still pond water and running river water. Part of the work has been reported elsewhere[5].

Materials and Methods

Water samples have been collected from three sites at Tatsumi pond, Sakai, Osaka, from August, 1993, to July, 1994 (Figs. 1, 2). Bulk samples of pond water were collected, returned to the laboratory within 60 min, and mixed together. Each 100 ml pond water was filtered by suction through one 90 mm Toyo No.2 filter paper circle on Büchner funnel. The filter papers were then plated each on a selective agar medium with the following compositions per liter: $Ca(NO_3)_2 \cdot 4H_2O$, 0.4g; $MgSO_4 \cdot 7H_2O$, 0.15g: K_2HPO_4, 0.15g; KCl, 0.006g; 72-h-milled cellulose powder, 10g; agar, 15g. The basic medium was autoclaved for 15 min at 121℃. An antibiotic solution (100ml) was prepared by adding 0.5g pimaricin and 1g vancomycin (Sigma grade). This stock is prepared just before the addition and 10ml of the solution is added aseptically to each 100ml melted and cooled (50℃) agar before pouring. The medium is designated CPV (cellulose-pimaricin-vancomycin) agar[1]. The plates with filter papers were then incubated in the dark for two weeks at 15℃.

Total salinity and pH were measured in the laboratory using a Salinity-pH meter (PEC-3C; Sensoni X Co.Ltd., Tokyo). Transparency and COD were determined in the laboratory.

Filter pond water through three layers of cheesecloth

- Standardize N/40 $KMnO_4$ by pouring 100 ml distilled water into 300 ml Erlenmeyer flask, add 10 ml H_2SO_4 [1 part H_2SO_4 + 2 parts dist. H_2O (v/v)] solution, add 10 ml N/40 $Na_2C_2O_4$, keep the mixture warm between 60-80°C and then titrate against N/40 $KMnO_4$. Designate the volume of $KMnO_4$ as X`. Obtain X`` by using the same procedure as described above but without 10 ml $Na_2C_2O_4$. Calculate factor (f) by the following equation.
  f = 10/X , whereas X = X` - X``
- Take appropriate amount of water sample into 300 ml Erlenmeyer flask, add dist. H_2O to make 100 ml (depending on the rate of pollution expected), add 10 ml H_2SO_4 sol., and 1 g Ag_2SO_4 and mix vigorously. Keep the reaction mixture to stand for several min, add 10 ml N/40 $KMnO_4$ and then boil for 30 min in water-bath.
- Add 10 ml N/40 $Na_2C_2O_4$ until the pink color disappear. Keep the reaction mixture at a temperature between 60-80°C and then titrate against N/40 $KMnO_4$. The end point is pale pink color. Perform blank test following the same procedure by using 100 ml dist. H_2O.
- Calculate the oxygen consumption according to the following equation:
  $COD_{Mn} = (b-a) \times f \times 1000/V \times 0.2$
  ,whereas
  COD_{Mn} = mg O / l consumed by $KMnO_4$,
  b= ml N/40 $KMnO_4$ titrated for the sample,
  a= ml N/40 $KMnO_4$ titrated for the experimental blank,
  f= factor of N/40 $KMnO_4$,
  V= ml of the water sample. Sample should be taken so as to remain more than one half volume of N/40 $KMnO_4$ after reacting for 30 min in boiling water.
  0.2 = each 1 ml of N/40 $Na_2C_2O_4$ equal to 0.2 mg oxygen.

Fig. 9. Measurement of COD_{Mn}

Transparency was determined by a Transparency meter cylinder (35mm diam, 100cm long and 780ml water capacity, TO-100, Kagaku Kyoeisha Co. Ltd., Osaka) at 25℃. COD_{Mn} was measured according to the method described by Nippon Kikaku Kyoukai (Japan Standardization Association)[6] (Fig. 9). A preliminary experiment has been done to differentiate between the number of colonies appeared with or without suction, and there was no difference in the number derived by the two ways.

When the direct colony counts could be made, each colony was transferred to CPV agar medium and incubated at 20℃. Sexual reproduction was observed by putting grass (*Paspalum thunbergii* Kunth) leaf blades on the actively growing colony at 20℃ for 3 days and then by transferring the blade to a Petri-dish (70mm diam) containing 10ml sterilized deionized water and followed by incubating at 20℃ for 14h[7]. Sexual structures can be examined on CPV agar medium after 4-6 weeks. Sometimes species of *Rhizophlyctis rosea* Karling can grow on the filter paper circles which also give pinkish colonies. Therefore, care should be taken to confirm the identification of each colony.

A baiting method was also carried out in which water samples of 30ml were poured out into sterilized Petri-dishes and baited with 8 sterilized filter paper discs (6mm diam). After 7-10 days at 25℃ a pink colored mycelial growth became visible on the filter paper discs. To confirm identification and to maintain the fungi, the colonized baits were washed thoroughly in sterile distilled water and excessive water was removed between sterile filter papers. The baits were seeded on CPV agar medium[1]. After about two weeks *P.fluminum* could be recognized by the pink coloration growth (arrow) which accompanied a cleared zone of the cellulose medium (Fig. 3). The fungi can be maintained in basal salt solution plus filter paper at 17℃ and renewed every year (Fig. 4).

Table 1. Physical and chemical data of pond water surveyed.

Year & month sampled	Detection by baiting method	pH	Transparency (ml)	Salinity (ms/ml)	COD (mgO/l)
Aug., '93	− [a]	9.7 ± 0.06 [b]	25.0 ± 0.33	0.30 ± 0.000	20.2 ± 0.21
Sep., '93	+	9.0 ± 0.12	20.0 ± 0.58	0.30 ± 0.000	18.2 ± 0.23
Oct., '93	+	7.8 ± 0.03	19.0 ± 0.33	0.29 ± 0.000	17.1 ± 0.23
Nov., '93	+	8.0 ± 0.06	23.0 ± 0.67	0.38 ± 0.000	15.5 ± 0.87
Dec., '93	+	7.9 ± 0.03	33.0 ± 0.44	0.49 ± 0.003	29.6 ± 1.35
Jan., '94	+	8.9 ± 0.10	15.0 ± 0.88	0.49 ± 0.000	19.1 ± 0.66
Feb., '94	+	8.7 ± 0.03	23.0 ± 0.58	0.47 ± 0.000	23.4 ± 0.75
Mar., '94	+	9.2 ± 0.12	30.0 ± 0.58	0.41 ± 0.000	22.1 ± 0.82
Apr., '94	+	7.5 ± 0.12	35.0 ± 0.67	0.40 ± 0.000	21.4 ± 1.01
May, '94	−	9.6 ± 0.09	17.0 ± 0.44	0.36 ± 0.000	26.5 ± 0.50
Jun., '94	−	8.1 ± 0.10	44.0 ± 0.33	0.34 ± 0.000	19.4 ± 0.45
Jul., '94	−	10.8 ± 0.15	7.5 ± 0.29	0.34 ± 0.000	33.7 ± 0.55

a) − : Undetected, + : detected
b) Standard error

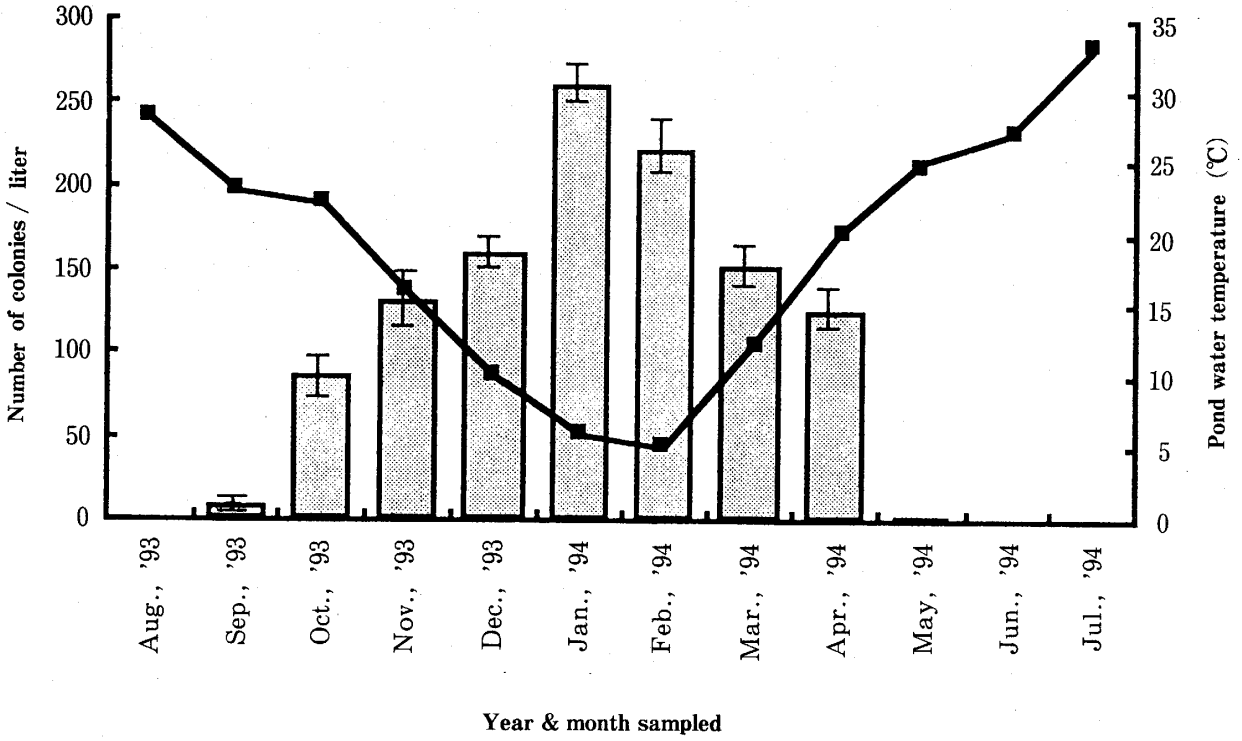

Fig. 10. Counts of propagule populations (□) of *P. fluminum* in pond with relation to water temperature (▬), from Aug., 1993, to Jul., 1994. Bars indicate means and standard errors for three measurements. There was no significant difference in temperature values in each measurement.

Results

Table 1 shows the physical and chemical data of the pond water studied. Generally, the hydrogen ion concentration of the pond water increases in summer due to the consumption of carbon dioxide by algae[8,9]. Total soluble salts (salinity or conductivity) were also influenced by the occurrence of algae which utilize some salts for their multiplication. Transparency is affected by algal occurrence as well as the amount of organic matter in the water. No direct relationship has been found between transparency and COD, and the fungal presence.

It is difficult to obtain a quantitative ecological data using the baiting method, although the fungus could be detected (Fig. 5). Failure of fungal detection in May, 1994, by baiting method and the obtaining of only one colony (arrow on Fig. 7) by filtration method in the same month may be due to the low numbers of the fungal propagules in the water. Figure 10 shows the time-course of the numbers of *P.fluminum* in Tatsumi pond per liter. Examination of the bars shows that the maximum numbers are in January and February, and then decrease gradually until reach zero in the summer (see Figs. 6,7).

To evaluate the ability of the fungi isolated to decompose filter paper, they were grown on CPV liquid medium, supplemented with filter paper circle instead of cellulose and without antibiotics, for two months at 25℃. Twenty per cent of the filter paper weight has been utilized under these conditions (Fig. 8).

Discussion

Spring is the most favorable season for growth and drastic seasonal variation for the great majority of Saprolegniaceae[10]. Later, winter is the most suitable season of growth of aquatic fungi, while two maxima, one in early spring and the other in late autumn were observed[11,12,13]. Low numbers of aquatic fungi were recorded during warm season, while fungal species began to build up in autumn and reached maximum in the spring[14]. Recently, Elnaghy *et al.*[15] demonstrated that the occurrence of aquatic zoosporic fungi was inversely correlated with temperature.

Park[3] studied the ecology of *P.fluminum* in two rivers in Ireland during a period of two years. He pointed out that the fungus was consistently present in the two rivers and the numbers in one river were somewhat lower in summer than in the other seasons, and highest numbers occurred in the period from November to February. While the numbers in the second river did not show distinct fluctuations. During Park's investigation the river water temperature ranged from 2 ℃ to 18℃ and within this range it is difficult to obtain a clear peaks of the numbers of colonies derived in each season. The pond water temperature recorded here ranged from 4 ℃ to 33 ℃ and this range represents a suitable environment for studying the seasonal fluctuation of this fungus in such habitat. Because of the still water the temperature reaches its extremes in winter and summer. Failure of detection of *P.fluminum* in the summer may be attributed to the suppression of zoospore formation. Abdelzaher *et al.*[7] in their study concerning the effect of temperature, hydrogen ion concentration and osmotic potential on zoospore formation by three aquatic *Pythium* spp. concluded that the optimum temperature for zoospore production of *P.fluminum* was 15℃. However, the low temperature such as 4 ℃, 7 ℃ and 10℃ supported the production of zoospore over a longer period. Therefore, cooler ambient water temperatures are more favorable for the fungal detection. *P.fluminum* had two pH optima, i.e., one in the acidic side (pH 5.5) and the other in the basic side (pH 9.5)[7]. The pond water studied here had a pH ranged from 7.5 to 9.6 and within this range the fungus can produce zoospores. We can assume therefore that occurrence of *P.fluminum* is strongly related to temperature rather than pH, salinity or COD. However, shifts of pH values, high concentration of total salinity and high osmotic potential may retard the zoospore production. During the summer period, the fungus survives as oospores either in the bottom soil of the pond or in plant debris, and when then temperature becomes favorable the asexual reproduction takes place.

Since the statement which represents *P.fluminum* as a type locality of North Ireland became invalid, this fungus might be occurred in other countries. Studies on isolation and ecology of *P.fluminum* in countries with moderate and cold weather should be investigated.

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Explanation of Plate

Fig. 1. Tatsumi pond in summer, surrounding by reed plants (*Fragmitis communis*).

Fig. 2. Tatsumi pond in winter.

Fig. 3. Pinkish colony (arrow) of *P.fluminum* on CPV plate after two weeks at 20℃.

Fig. 4. Maintainance of *P.fluminum* in Park's mineral medium at 17℃. The right flask contains one year old culture whereas the left one contains six-months old culture.

Fig. 5. Colonized filter paper disc baits by *P.fluminum* (arrow) after 10 days at 25℃.

Fig. 6. Quantitative isolation of *P.fluminum* in winter by filtration method after two weeks at 15 ℃. Each filter paper circle was used for filtering 100ml pond water.

Fig. 7. Only one colony (arrow) detected in May, 1994, under the same condition described in Fig. 6.

Fig. 8. *P.fluminum* growing on three types of culture media at 25℃ after two months, the right medium is Czapeks (initial pH: 6.5), the middle one is Park's medium adjusted to pH 6.5 by 0.025M MES buffer and the left flask contains Park's medium without buffer (initial pH: 7.6).

Plate I