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DIPLOIDIZATION AND PATHOGENICITY IN *PROTOMYCES INUNDATUS*

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Abstract

It is shown that opposite mating type genuine haploid endospores of *Protomyces inundatus* do not cause infection to the host plant *Apium nodiflorum*. Usually infection is caused by sexually incompatible diploid endospores derived by fusion of opposite mating type haploid endospores. However, observations document that haploid strains could give rise to diploids, by self diploidization, and these sexually compatible diploids cause infection to the host plant. It is suggested that ability to infect is dependent on ploidy of the culture rather than on the presence of both opposite mating type genes.

Introduction

Valadon, Manners & Myers (1962) have made studies on the life history and taxonomic position of *Protomyces inundatus* Dangeard. The fungus is a parasite on *Apium nodiflorum*, however, the endospores grow readily on artificial medium. It is shown that haploid endospores only fuse if they are of opposite mating type. The estimation of DNA content has established that the haploid endospore cell fusion is accompanied by nuclear fusion and the resulting diploid endospore germinates on the host leaf surface, penetrates through epidermis and produces intercellular mycelium. The intercellular mycelium within the host cell has diploid nuclei and meiosis occurs in the chlamydospores, wherein haploid endospores are formed.

Valadon (1961) reported that some of the haploid mating type strains, established from single unfused endospore isolates, upon successive subculturing in the slant medium could infect the host plant and it was suggested that the occurrence of an opposite mating type mutation might be responsible for this infection. In this study the possible occurrence of an opposite mating type mutation has been checked by two methods. In the first method single cell isolates picked up from cultures of mating type strain, and maintained by subculturing technique, were scored for compatibility. In an other method the host plant was infected with *Protomyces inundatus* strains, established by mating type endospores and the mycelial cells of infective strains were tested for compatibility. Estimations of DNA contents per cell could tell the ploidy for any culture employed. It is shown that the host plant could be infected by mating type cultures because of the production of homozygous diploids by self diploidization rather than occurrence of opposite mating type mutation.

Materials and Methods

Isolation and maintenance of single cell cultures

A large collection of single cell endospore cultures, established from a germinating chlamydospore of *Protomyces inundatus*, were maintained over a period of one year by subculturing technique. The cultures established from unfused endospores were designated + or — sign, showing opposite mating compatibility, with arabic numerals. The cultures established from fused endospores were marked 2nA, 2nB, 2nC and so on. Five strain, 2—, 3—, 13+, 18+ and 2nB were chosen from the large

collection to test the production of mutants with changed compatibility or infectious behaviour. Twenty single cell isolates were made from each of the four strains and marked as 2-1, 2-2, 2-3, ————2-20; 3-1, 3-2, 3-3————3-20; 13+1, 13+2, 13+3, 13+20 and so on.

The single cell isolates were made with the aid of a Singer micromanipulator. A microscopic loop for the micromanipulator was fabricated from a needle of tungsten wire by the eletrolytic process (Hildebrand, 1950). A pair of short 18 swg tungsten wire pieces were connected in series with a 12 volt AC mains transformer and the free ends were dipped into a 1 M sodium hydroxide plus 1 M sodium thiosulphate solution in a beaker. When the current was passed the dipped ends of the wire were slowly etched away into microscopic fineness at the tips. The desired length and the fineness of the etched end were controlled by the length of the wire dipping into the solution and the time interval allowed for passing the current. A needle with 20 mm tapered etched end having a 3-4 u diameter tip was quite suitable for making the type of loop, a coiled hook, used for picking up *Protomyces inundatus* cells. The tip of the etched end transformed into a loop by mounting the needle on the micromanipulator and tapping the tip against the edge of a coverslip cemented to a microsocope slide, under direct microscopic observation. The needle was sterilized in a flame from an alcohol soaked cotton pad, the use of the hot flame produced by a bunsen burner was undesirable because it straightened the loop.

For making single cell isolations a thin suspension of cells in water was streaked by means of a suitable paint brush on the surface of a thin layer of 2% malt agar on a microscope slide and a single cell was picked up on the loop of the micromanipulator needle. The smear slide was then removed, and another slide containing about 10 x 10 mm agar blocks cut from 2% malt agar layer was put in its place on the microsocope stage and the cell was deposited on the surface of a marked agar block. This agar block was transferred to a 2% malt agar slant with a sterile scalpel and incubated at 10° C. The whole operation was carried out under aseptic conditions. The germination of single cell isolates were more than 99% for each strain. The colonies usually became visible after 5-7 days.

Isolation of mycelial cells

Mycelial cells of the infective strains were collected from infected leaves. Galls from the infected leaves were excised and the leaf parts were surface sterilized in 0.2% mercuric chloride for 10 min, washed with running water for 5 hr and then cut into pieces which were placed in 2% malt agar plates and incubated at 20°C to obtain colonies derived from mycelial cells.

Compatibility in single cell isolates

The cultures were grown on 2% malt agar. The crosses were made by mixing cells of any two cultures, for testing compatibility, on the surface of 2% malt agar slants and incubating at 20°C. After 24 hr incubation the cells were suspended in water and examined for criss-cross bodies under the microsope and the presence of such bodies was considered as a positive test for compatibility.

Infection of *Apium nodiflorum*

The presence of diploid cells in haploid cultures of + or — type would be detected by their being infective to the host plant because haploid cultures do not infect. The cells of any strain under test were harvested from a 3-4 day old growth on 2% malt agar, washed twice with water and made into thick suspension. Freshly repotted, vigorously growing *Apium* plants were inoculated (being aquatic their pots were stood in saucers of water, which ensured vigorous growth) by painting thick cell suspension on leaves. Inoculated plants were kept at 25-30°C in a greenhouse under artificial light provided for 16 hr each day from a bank of three 5 ft 80 W white flourescent reflectorized tubes fixed 2 ft above the greenhouse bench. Humidity and stray infection were controlled by keeping the plants under polythene bags with muslin tops.

Estimation of DNA per cell

The cells were grown in 100 ml liquid cultures using 350ml Erlenmeyer flasks on reciprocating shaker at 50 strokes per minute. The cells were grown at 20°C and harvested while in log phase. The cells were harvested in graduated tubes by centrifugation, washed with ice cold water, suspended in known volume of water, and 0.1ml of suspension was taken and stored at 0°C for cell counts. Then the suspension was centrifuged and the cell pellet was used for DNA estimation. The volume of each cell pellet was 0.4-0.7 ml.

The 0.1 ml cell suspension was diluted suitably with water containing 0.001% methylene blue which helped in differentiating living and dead cells. The cells were counted with a Fuchs-Rosenthal haemocytometer. In practice there were very few dead cells. The buds were counted as separate cells.

Twenty volumes of boiling spectroscopic ethanol were poured on a water washed cell pellet and the suspension was stirred with a glass rod and then allowed to boil for 3-5 min at 85°C, centrifuged and washed twice with ethanol. The ethanol treated cells were white and could be stored at 0°C for one month, but were used within a week in the present study.

The ethanol extracted cells were treated with 10 ml of 0.2% perchloric acid at 0°C for 1 hr and centrifuged. Then the DNA was extracted in 10 ml of 10% perchloric acid by heating at 65°C for 25 min and the DNA was estimated by the diphenylamine method (Giles & Myers, 1965).

Results and Discussion

A set of five single cell isolate cultures, 2—1, 3—1, 13+1, 18+1 and 2nB, was tested for compatibility in all possible crosses. These cultures had compatibility pattern comparable to parent strains (Table 1A). The compatibility pattern in the other nineteen set of cultures did not differ from that of parent strains. These results indicate that the opposite mating type mutation occur rarely, if at all.

However, it was surprising to see that strains 3— and 13+ that were found, at the time of isolation noninfective to the host plant, had infected *Apium* plants after subculturing (Table 1B). Mycelial cells of the infective strains 3— and 13+ and

TABLE 1. Cross fusion and infective behaviour of some strains of *Protomyces inundatus*.

A		2—	3—	13+	18+	2nB
	2—	0	0	x	x	0
	3—	0	0	x	x	0
	13+	x	x	0	0	0
	18+	x	x	0	0	0
	2nB	0	0	0	0	0
B		2—	3—	13+	18+	2nB
	Infection	I—	I+	I+	I—	I+
C				M3—	M13+	M2nB
			2—	0	x	0
			18+	x	0	0

A. Compatibility in various strains, cross fused: X, no fusion: O.

B. Infection caused by various strains, infection: I+, no infection: I—.

C. Compatibility in mycelial cells of infecting strain and noninfecting strains, cross fused: X, no fusion: O.

TABLE 2. Infective behaviour and deoxyribonucleic acid contents of various strains of *Protomyces inundatus;* DNA contents are expressed per cell as ug x 10^{-9}.

Strain	DNA contents	Ploidy	Infection
2—	23.02	1n	I—
M3—	39.32	2n	I+
M13+	35.97	2n	I+
18+	21.62	1n	I—
M2nB	39.79	2n	I+

2nB were collected, and their compatibility pattern is shown in Table 1C. Mycelial cells cultures M3— and M13+ were sexually compatible and the compatibility pattern was like the parent cells, while M2nB culture was sexually incompatible.

DNA estimation showed cultures M3— and M13+ and M2nB were diploids (Table 2). These results indicate that culture M3— and M13+, although diploid, were sexually compatible and thus these would have originated by self diploidization. It shows that in *Protomyces inundatus* the ability to infect the *Apium* plant is dependent on the ploidy of the culture rather than on presence of both + or — mating type genes.

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