Journal of Industrial Microbiology (1995) 15, 121–124

Multinucleation in the conidia of polyploids derived from *Trichoderma reesei* QM 9414 by colchicine treatment

H Toyama and N Toyama

Department of Food Technology, Faculty of Horticulture, Minamikyushu University, Miyazaki, Japan

Conidia of *Trichoderma reesei* QM 9414 were treated with colchicine in order to obtain polyploids (diploids; tetraploids). Cellulase production by diploids (mononucleate conidia) was almost twice as great as that of the original strain, but that of tetraploids (binucleate conidia) was not increased. When these latter conidia were re-treated with 2.0% (w/v) colchicine, multiple nuclei were produced in each conidium, and their diameter was almost the same as that of the original nucleus. Cellulase production of the diploid was almost the same in either mononucleate or multinucleate nature. However, cellulase production by the tetraploid which produced multinucleate conidia was greater than that of the binucleate tetraploid and that of the diploid. The multinucleation technique can contribute to enhancing cellulase production.

Keywords: *Trichoderma*; colchicine; cellulase

Introduction

We previously reported that minute nuclei are generated from the original nucleus in conidia of *Trichoderma reesei* QM 9414 by colchicine treatment. We called the minute nuclei the 'smaller nuclei' [13]. Colchicine produces micronuclei in animal cells and in plant cells [5,14]. Micronucleation occurred through abnormal nuclear division [7].

Colchicine is also known as a polyploidy inducer in animals, plants, and microbes [2,9,10]. Colchicine binds to tubulin and mitosis is arrested [4], inhibiting the normal distribution of chromosomes and yielding polyploid nuclei [3]. Polyploids are also formed by colchicine treatment in *T. reesei* QM 9414 [12]. We were interested in determining whether or not polyploids treated with colchicine promoted micronucleation. The formation of polyploids, their subsequent micronucleation with colchicine treatment, and comparative effects on cellulase production are reported.

Materials and methods

Microorganism and media

T. reesei QM 9414 was cultivated on potato glucose agar (PGA) at 28° C and preserved at 4° C. For colchicine treatment, Natick medium containing 1.0% (w/v) glucose, 0.5% (w/v) peptone, and colchicine was used (pH 6.0) [8]. For cellulase production, Natick medium additionally containing 1.0% (w/v) Avicel (Asahikasei) and 0.5% (w/v) peptone was used (pH 5.0).

Colchicine treatment

Green mature conidia of this fungus (10-day-old culture) were dried for at least 7 days in a desiccater in a 10-ml glass centrifuge tube. In the case of autopolyploidization, five loopfuls of these conidia (almost 2.0×10^8 conidia) were transferred to 3 ml of the medium for colchicine (0.001% or 0.01% w/v) treatment and incubated on a reciprocal shaker (120 strokes min^{-1}) for 24 h at 28° C.

In the case of multinucleation, five loopfuls of these conidia were transferred to 3 ml of the medium for colchicine (2.0% w/v) treatment and incubated on a reciprocal shaker for 10 days at 28° C. Every 24 h, conidia were collected by centrifugation at $1000 \times g$ for 10 min, and nuclear staining was carried out in order to observe nuclei.

Nuclear staining

Conidia were treated successively by 5, 3, and 1 N HCl at 60° C before nuclear staining. These pre-treated conidia were stained with Giemsa solution (Merck) or 4′,6-diamidino-2-phenylindole (DAPI) solution (Sigma) [16]. Photomicrographs were taken using an Olympus BH-1 (Olympus) microscope with UV-illumination (Olympus BH-2-RFL-T2) and an Olympus C35AD-4 camera. The nuclear diameter in 100 conidia was measured using digital calipers on enlarged photomicrographs. Nuclear number was also counted in 100 conidia in the enlarged photomicrographs.

Measurement of average DNA content of conidia

DNA was extracted by the method of Herbert *et al*, and measured by the method of Ceriotti [1,6]. Conidial number was counted using a haemocytometer.

Measurement of cellulose hydrolyzing activity

One loopful of conidia of *T. reesei* was grown in 50 ml of cellulase production medium in a 100-ml Erlenmeyer flask for 5 days on a rotary shaker (160 rpm) at 30° C. Mycelia were then removed on a 3G-3 glass filter and the filtrate was used as the source of cellulase.

Avicel-hydrolyzing activity, CMC-Na-hydrolyzing activity, and β-glucosidase activity were measured using 1% Avicel, 1% CMC-Na (carboxymethylcellulose sodium-salt) (D.S. 0.7), and 1% salicin (Wako), respectively, sus-

Correspondence: H Toyama, Department of Food Technology, Faculty of Horticulture, Takanabe-Cho, Hibarigaoka, Miyazaki 884, Japan
Received 25 October 1994; accepted 10 May 1995

pended in 0.1 M acetate buffer (pH 5.0) as substrates. Enzyme (2 ml) and substrate (4 ml) were mixed and incubated for 60 min at 40° C; the enzyme reaction was then stopped by addition of 0.1 N HCl. The amount of glucose generated was measured by 'Glucose-test Wako' (Wako). Enzyme activity was defined as the amount of enzyme that produces 1 μmol of glucose per min. Dry weights of mycelia were obtained after drying mycelium for 17 h at 80° C.

Results

Autopolyploidization

Green mature conidia were incubated in 0.001% or 0.01% (w/v) colchicine solution for 24 h at 28° C on a reciprocal shaker. After the incubation, the nuclear diameter was changed (Figure 1). The treated conidia were collected and spread on PGA medium containing 0.1% Triton X-100 followed by incubation for 4 days at 28° C. Triton X-100 limited the increase of the mycelia and promoted restricted colony formation.

Two colonies that produced a deeper yellow pigment were isolated. One yielded mononucleate conidia and the other binucleate conidia. These conidia contained larger nuclei than the native strain. The colony with spores containing mononucleate conidia via 0.001% colchicine treatment was termed strain A and the binucleate conidia colony resulting from 0.01% colchicine treatment was termed strain B.

Multinucleation

The conidia derived from A and B were re-treated with 2.0% (w/v) colchicine for up to 10 days. The treated conidia were collected by centrifugation and nuclear staining was carried out every 24 h.

As the incubation proceeded, nuclear diameter and nuclear number in the conidia changed (Figures 2 and 3). In the control conidia, the nuclear diameter ranged from 0.55 to 0.85 μm, and 'abnormal' nuclei were termed 'larger nuclei' (diameter greater than 0.85 μm) and 'smaller nuclei' (diameter less than 0.55 μm).

Colonies were isolated by plating treated conidia on PGA plates containing 0.1% Triton X-100 followed by incubation for 4 days at 28° C. Two colonies that generated multinucleate conidia were selected among 26 (strain A) and 32 (strain B) colonies, respectively, by nuclear staining. One derived from strain A was named strain C and the other derived from strain B was called strain D. The strains (native-mononucleate conidial strain; the 'larger nuclei'-conidial strain B; multiple nuclei in conidial strain D) were easily identified by the nuclear staining (Figure 4). The

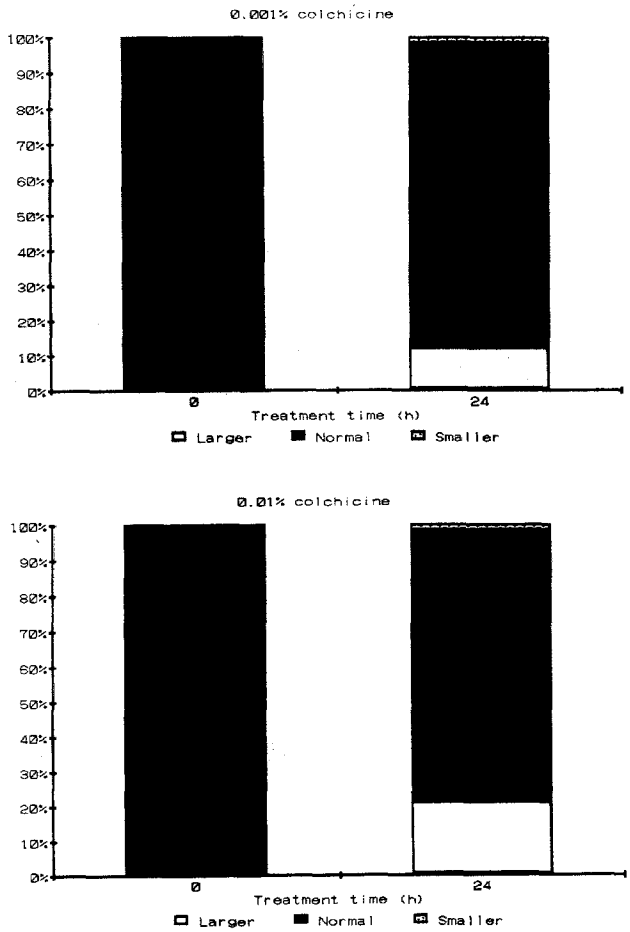

Figure 1 Polyploidization in conidia. Conidia of *T. reesei* were treated with 0.001% or 0.01% (w/v) colchicine solution (pH 6.0) for 24 h at 28° C on a reciprocal shaker

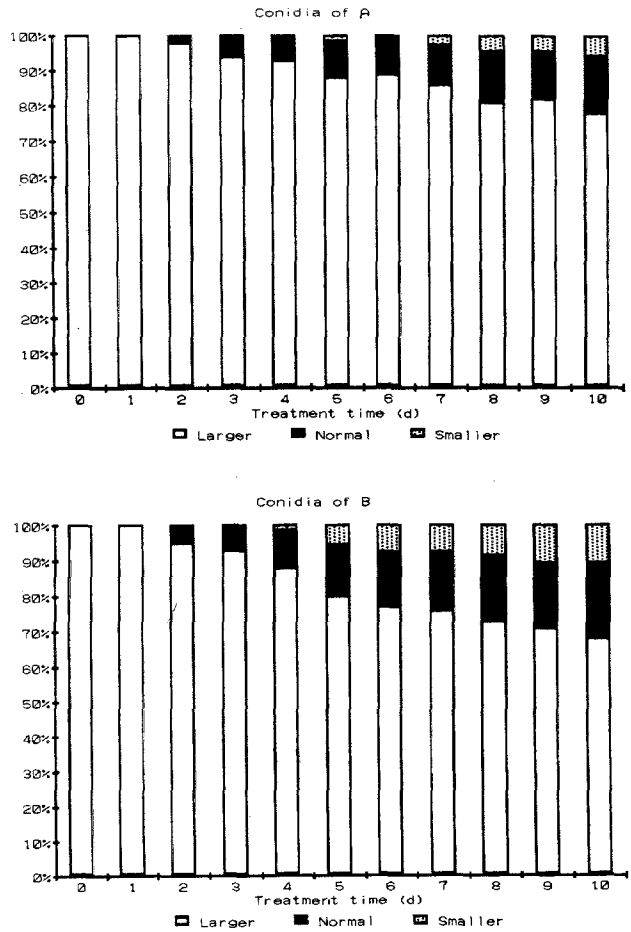

Figure 2 Change in nuclear diameter in conidia of strains A and B during multinucleation. Conidia of strains A and B were incubated in a 2.0% colchicine solution (pH 6.0) for 10 days at 28° C

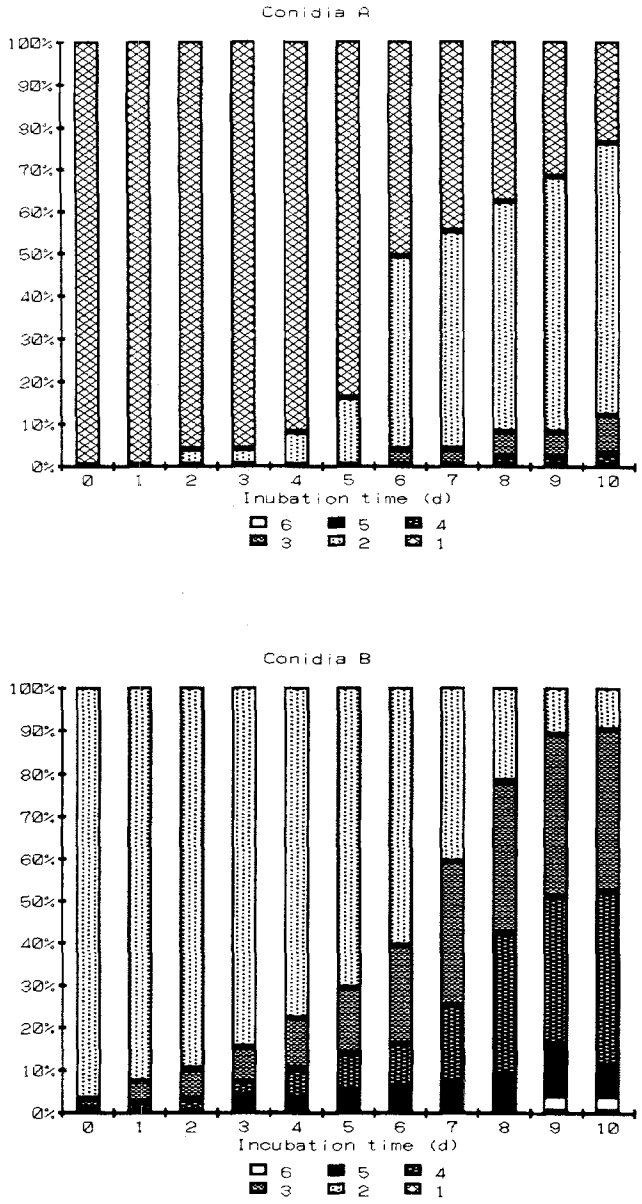

Figure 3 Change in nuclear number in conidia of strains A and B during multinucleation. Conidia of strains A and B were incubated in a 2.0% colchicine solution (pH 6.0) for 10 days at 28° C

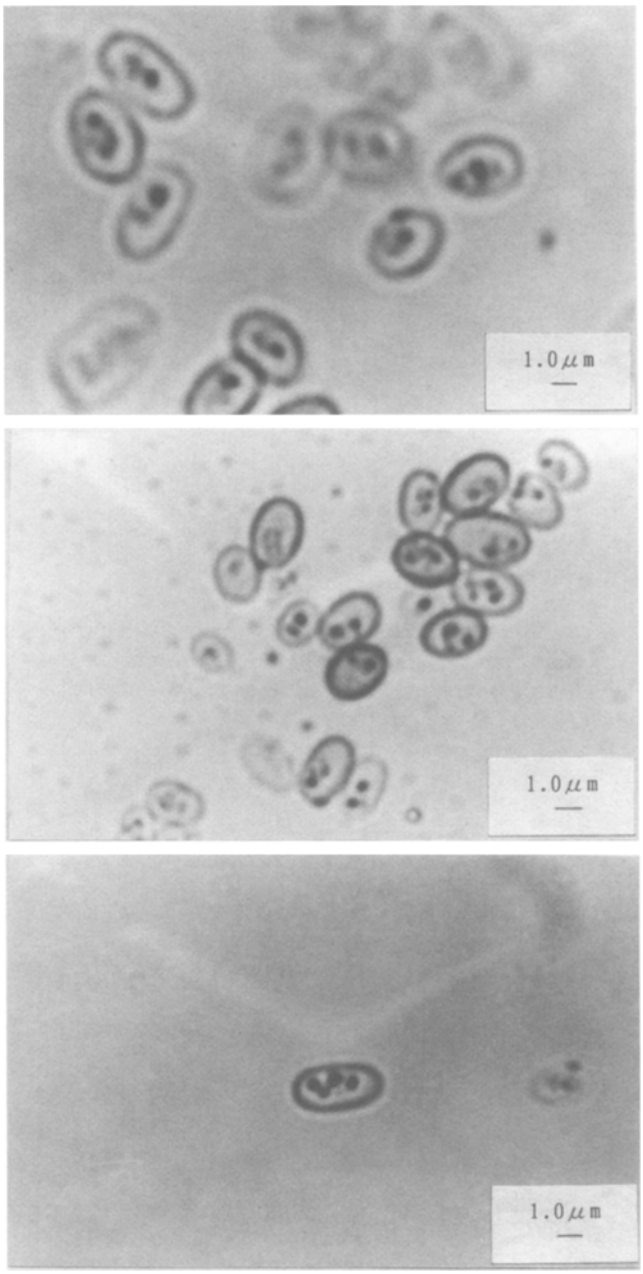

Figure 4 Original conidia, the binucleate conidia of strain B, and the multinucleate conidium of strain D. Original conidia (Top) were derived from the original strain. The binucleate conidia (Center) were derived from strain B. The multinucleate conidium of strain D (Bottom) was derived from the conidia of strain B which were treated by 2.0% (w/v) colchicine solution for 10 days at 28° C

average nuclear number per conidium of strains A and B was 1.0 and 2.1, respectively, while that of strains C and D was 2.2 and 3.3, respectively. The nuclear number or diameter did not change after Giemsa or DAPI staining.

Measurement of average DNA content of conidia

Average DNA content per conidium of the original strain and conidial strains A–D, showed about a two-fold increase in strains A and C, while that of strains B and D increased about four-fold (Table 1).

Measurement of cellulose hydrolyzing activity

Avicelase, carboxymethylcellulase (CMCase), and β-glucosidase activity were compared among the conidial variants (conidia A, B, C, and D). Cellulase (all three components) production of conidial strains A increased (Table 2). And the cellulase production decreased in strain B but increased in strain D.

Discussion

In the autopolyploidization, the 'larger nuclei' were regarded as polyploid nuclei and the conidia of strain A were then diploids and those of strain B were regarded as tetraploids. The formation of 'smaller nuclei' occurred later after the incubation was induced by colchicine

Table 1 Average DNA content of conidia

Conidia	Average DNA content ($\times 10^{-8}$ μg conidium^{-1})
Original conidia	8.08
Conidia of strain A	17.24
Conidia of strain B	33.29
Conidia of strain C	16.87
Conidia of strain D	32.46

DNA was extracted by the method of Herbert *et al*, and measured by the method of Ceriotti [1,6]

Table 2 Cellulase activity in conidia

Conidia	Avicelase (IU ml^{-1})	CMCase (IU ml^{-1})	β-glucosidase (IU ml^{-1})
Original conidia	5.92	4.44	2.22
Conidia A	7.40	10.36	9.99
Conidia B	4.44	2.22	9.62
Conidia C	14.57	12.34	11.55
Conidia D	18.87	18.80	12.21

One loopful of conidia was added to cellulose medium, and cellulase was measured after 5 days at 30° C (see Methods)

(micronucleation). The ploidy may vary later in the autopolyploidization owing to this.

We considered the mechanism of the change in nuclear number in conidia. After the second colchicine treatment, multiple nuclei whose diameter was similar to that of the haploid nuclei appeared in the conidia. One larger nucleus apparently yielded multiple small nuclei/conidium. We called this phenomenon multinucleation, and named strains C and D the multinucleate bodies. We suggest that this is caused by a mechanism similar to micronucleation [15]. In the original strain, one nucleus was distributed to one conidium correctly [11], while approximately equivalent numbers of nuclei were distributed to conidia in the multinucleation strains. Further investigation is needed to determine the mechanism of nuclear distribution.

Cellulase production may be repressed normally in the tetraploid (strain B) but multinucleation occurring in the tetraploid may overcome the repression, with an increase in cellulase production. The mechanism of this repression is now under investigation. When conidia of this fungus were immersed in 2.0% colchicine solution lacking nutrients for up to 10 days at 28° C, no increase of cellulase production and no multinucleation were seen (data not shown). These results can be inferred to indicate that the increase of cellulase production in strain D is not caused by mutation.

Based on the above results, it is suggested that the multinucleate body is a natural attribute of polyploids. It was concluded that multinucleation of polyploids can contribute to enhancing cellulose production and perhaps the use of polyploids in general.

Acknowledgements

We wish to express our gratitude to Dr ET Reese and Dr MG Mandels.

References

1 Ceriotti G. 1955. Determination of nucleic acids in animal tissues. J Biol Chem 214: 59–70.
2 Derman H. 1940. Colchicine polyploidy and technique. Bot Rev 6: 599–635.
3 Eigsti OJ. 1971. Comparison of effects of colchicine, colcemid, and vinblastine upon mitosis in pollen. Genetics 68: 17–22.
4 Garland D and D Teller. 1973. Mechanism of colchicine binding. J Cell Biol 59: 107–112.
5 Geraud G, F Laquerriere, C Masson, J Arnoult, B Labidi and D Hernandez-Verdun. 1989. Three-dimensional organization of micronuclei induced by colchicine in PtK1 cells. Exp Cell Res 181: 27–39.
6 Herbert D, PJ Phipps and SE Strange. 1971. Chemical analysis of microbial cells. In: Methods in Microbiology 5b (Norris *et al*, eds), pp 324–334, Academic Press, New York.
7 Majone F, S Tonetto, C Soligo and M Panozzo. 1992. Identification of kinetochores and DNA synthesis in micronuclei induced by Mitomycin C and colchicine in Chinese hamster ovary cells. Teratogen Carcinog Mut 12: 155–166.
8 Mandels M and D Sternberg. 1976. Recent advances in cellulase technology. J Ferment Technol 54: 267–286.
9 Oenfelt A and I Klasterska. 1983. Spindle disturbances in mammalian cells. II. Induction of viable aneuploid/polyploid cells and multiple chromatid exchanges after treatment of V79 Chinese hamster cells with carbaryl. Mutat Res 119: 319–330.
10 Povova LS. 1966. Production of polyploids of various species of *Candida* by the action of mitotic poisons. Mikrobiologiya 35: 271–275.
11 Rosen D, M Edelman, E Galun and D Danon. 1974. Biogenesis of mitochondria in *Trichoderma viride*: structural changes in mitochondria and other spore constituents during conidium maturation and germination. J Gen Microbiol 83: 31–49.
12 Toyama H and N Toyama. 1990. Autopolyploid formation of *Trichoderma reesei* QM9414 by colchicine treatment. J Ferment Bioeng 69: 51–53.
13 Toyama H and N Toyama. 1991. Nuclear abnormality of conidia induced by colchicine in *Trichoderma reesei* QM 9414. Agric Biol Chem 55: 2393–2394.
14 Verhoeven HA, KS Ramulu and P Dijkhuis. 1990. A comparison of the effects of various spindle toxins on metaphase arrest and formation of micronuclei in cell-suspension cultures of *Nicotiana plumbaginigolia*. Planta 182: 408–414.
15 Wakata A and MS Sasaki. 1987. Measurement of micronuclei by cytokinesis-block method in cultured Chinese hamster cells: comparison with types and rates of chromosome aberrations. Mutat Res 190: 51–57.
16 Yamada M, Y Matsumoto, S Hamada, S Fujita and Y Yoshida. 1986. Demonstration and determination of DNA in *Pneumocystis cariniii* by fluorescence microscopy with 4′,6-diamidino-2-phenylindole (DAPI). Zbl Bakt Hyg 86: 6503–6507.