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Intraspecific Hybridization Between Color Mutants of *Trichoderma harzianum* by Protoplast Fusion

Chiradej Chamswarng[1], Kihashiro Ogawa[2], Wanwilai Gesnara[1] and Nuanwan Faroongsang[3]

ABSTRACT

Protoplasts of two color mutants of *Trichoderma harzianum* derived from ultraviolet treatment were prepared by using lytic enzyme system (10 mg Zymolyase, 150 mg Uzukizyme, 10 mg Chitinase and 150 mg Funcelase in 3 ml water, pH 5.0) containing 0.6 M NaCl as an osmotic stabilizer. Fusion between protoplasts of two color mutants was observed in the solution containing 0.05 M glycine-NaOH buffer, pH 7.5, 0.05 M $CaCl_2$. $2H_20$ and 35% polyethylene glycol (MW. 6,000). The fused protoplasts were successfully detected at the rate of 2 % as green colonies developed on a hypertonic solid culture medium (complete medium) overlaid with hypertonic soft medium (MM with 0.5% agar). Production of well defined white and yellow sectors on agar surface of minimal medium supplemented with 0.1% (W/V) d-camphor represented the heterokaryotic fusants. Green conidia were constantly obtained after transferring the green part from green-sectored colonies onto MM agar. This indicated that protoplast fusants were heterozygous diploid.

Keywords: protoplast fusion, *Trichoderma*, color mutant

INTRODUCTION

Trichoderma harzianum is a potential biocontrol agent against soil-borne plant pathogenic fungi (Cook and Baker, 1983). Available individual strains of *Trichoderma* could be obtained through selection or mutation (Papavizas, 1985). Genetic recombination is a more powerful method for developing superior biocontrol strains than either selection or mutation. Sexual stages are rare or lacking in most strains of *Trichoderma* species and conventional sexual crosses can not be used for genetic manipulation. Production of fungal hybrids by protoplast fusion is a standard method for improving strains of *Trichoderma* and has been successfully carried out using *T. reesei* (Ogawa *et al.*, 1987), *T. koningii* (Hong *et al.*, 1984) and *T. harzianum* (Stasz *et al.*, 1988).

In this study we attempted to develop appropriate techniques for detecting the successful fusion of protoplasts of *Trichoderma* by using color mutants.

MATERIALS AND METHODS

Organisms

Trichoderma harzianum (CB-Pin-01) [wild type - green conidia] was used as original strains. The white (whi) and yellow (yel) mutant conidia were obtained through the use of UV-irradiation, by exposing conidial suspension of *T. harzianum* (wild type) with UV-ray at a distance of 15 cm for 8 min.

1 Dept. of Plant Pathology, Faculty of Agriculture, Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand.
2 Dept. of Biological Resource Sciences, Faculty of Agriculture, Miyazaki University, Miyazaki 889-21, Japan.
3 Plant Pest Clinic and Quarantine Laboratory Unit, Central Laboratory and Greenhouse Complex, Kasetsart University Research and development Institute, Kamphaeng Saen, Nakhon Pathom 73140, Thailand.

Culture media

Inorganic salt medium supplemented with 2% glucose (w/v) was used as a liquid minimal medium (MM). The liquid MM supplemented with 0.5% casamino acid (or 0.5% yeast extract) was also prepared as a liquid complete medium (CM). For solid culture, agar was added to these media at a concentration of 2% (w/v). Sodium chloride (0.6 M) was served as an osmotic stabilizer during protoplast preparation and manipulation, and used as hypertonic media for protoplast regeneration.

Protoplast formation

A loopful of mycelia was taken from petri-dish, added to 20 ml of liquid CM in 100 ml Erlenmeyer flask, and incubated at 30 C on a reciprocating shaking culture apparatus. After incubation, the mycelium was collected on filter paper (Whatman No.1) and washed with distilled water under aseptic conditions. About 300 mg (fresh weight) of mycelium was then added to 3 ml of a lytic enzyme system (Zymolyase 10 mg, Uzukizyme 150 mg, Chitinase 10 mg and funcelase 150 mg in 3 ml distilled water, pH 5.0) containing 0.6 M NaCl as an osmotic stabilizer. The mycelium was incubated at 30 C for 1 hr on a reciprocating shaking water bath.

After incubation, the reaction mixture was filtered through a glass filter (15 AG 2) and the filtrate was refiltered through a glass filter (15 AG 3). The filtrate was centrifuged at 700 g for 5 min. Sedimented protoplasts were suspended in 10 ml of hypertonic liquid MM containing 0.6 M NaCl and centrifuged twice at 700 g for 5 min and finally the pellets were resuspended in 1 ml of hypertonic liquid MM.

Protoplast fusion and regeneration

Procedures for protoplast fusion were carried out by modifying the method of Anne and Peberdy (1976). Protoplasts of each mutant (10^8 ml^{-1}) were mixed and then centrifuged at 700 g for 5 min. Sedimented protoplasts were suspended in 1 ml of a solution of 35% (w/v) polyetylene glycol (PEG. MW. 6,000) and 0.05 M $CaCl_2.2H_20$ in 0.05 M glycine-NaOH buffer pH 7.5. After incubation at 25C for 10 min, the suspension was centrifuged at 700 g for 5 min. The sedimented protoplasts were resuspended in 1 ml of hypertonic liquid medium. For regeneration of protoplast fusants, the suspension was diluted appropriately and then aliquat (0.2ml) of suspension was spread over hypertonic solid medium before overlaid with 1 ml of hypertonic soft medium containing 0.5% agar (pre-dissolved at 45 C). These Petri dishes were incubated at 25 C for 2-5 days. After young mycelium was formed from fusion products, hyphal tip was transferred onto a Petri dish of MM agar containing 0.1% d-camphor, followed by incubation at 25 C for 6 days. Formation of colonies with white, yellow or green sectors indicated the successful fusion. The colonies were counted to estimate the fusion frequency.

RESULTS AND DISCUSSION

Characteristics of *T. harzianum* and mutants.

The two color mutants were similar to their parent (CB-Pin-01) in the ability to grow and sporulate. The reversion of both mutants back to their parental types (green conidia) was not determined, however, green conidia were never appeared following several sub-culturings of both color mutants on potato dextrose agar.

Protoplast formation

In preliminary test, protoplast could not be released from mycelia incubated at 30C for 1 hr in mycolytic enzyme solution that contained 0.1 M acetate buffer (pH 5.0), 5% Funcelase and 0.6 M NaCl. This result indicated that mycolytic enzymes including 1,3 β-glucanase, chitinase and protease in the solution must be well balanced qualitatively and quantitatively. Moreover, incubation period of mycelia in enzymes was related to the components and thickness of cell wall. Therefore, new enzyme system consisting of 10 mg Zymolyase, 150 mg Uzukizyme, 10 mg Chitinase and 150 mg Funcelase in 3 ml water, pH 5.0 was prepared. Protoplasts of 0.5 to 5.0 x 10^7 ml^{-1} were released from mycelia of approximately 300 mg (fresh weight) when young mycelial mass was suspended in adjusted enzyme solution and was incubated at 30 C for 1 hr. The average diameter of the protoplast was 4.0 μm. The protoplasts were readily obtained by using 0.6 M NaCl as an osmotic stabilizer (Fig. 1).

Protoplast fusion and regeneration

After fusion treatment, fused protoplasts of two or more protoplasts could be observed. In the early stage of regeneration of fusion product on hypertonic solid CM that overlaid with hypertonic soft agar (0.5%) MM, the fusant products became greatly swollen and formed in a chain of buds. (Fig.2A).

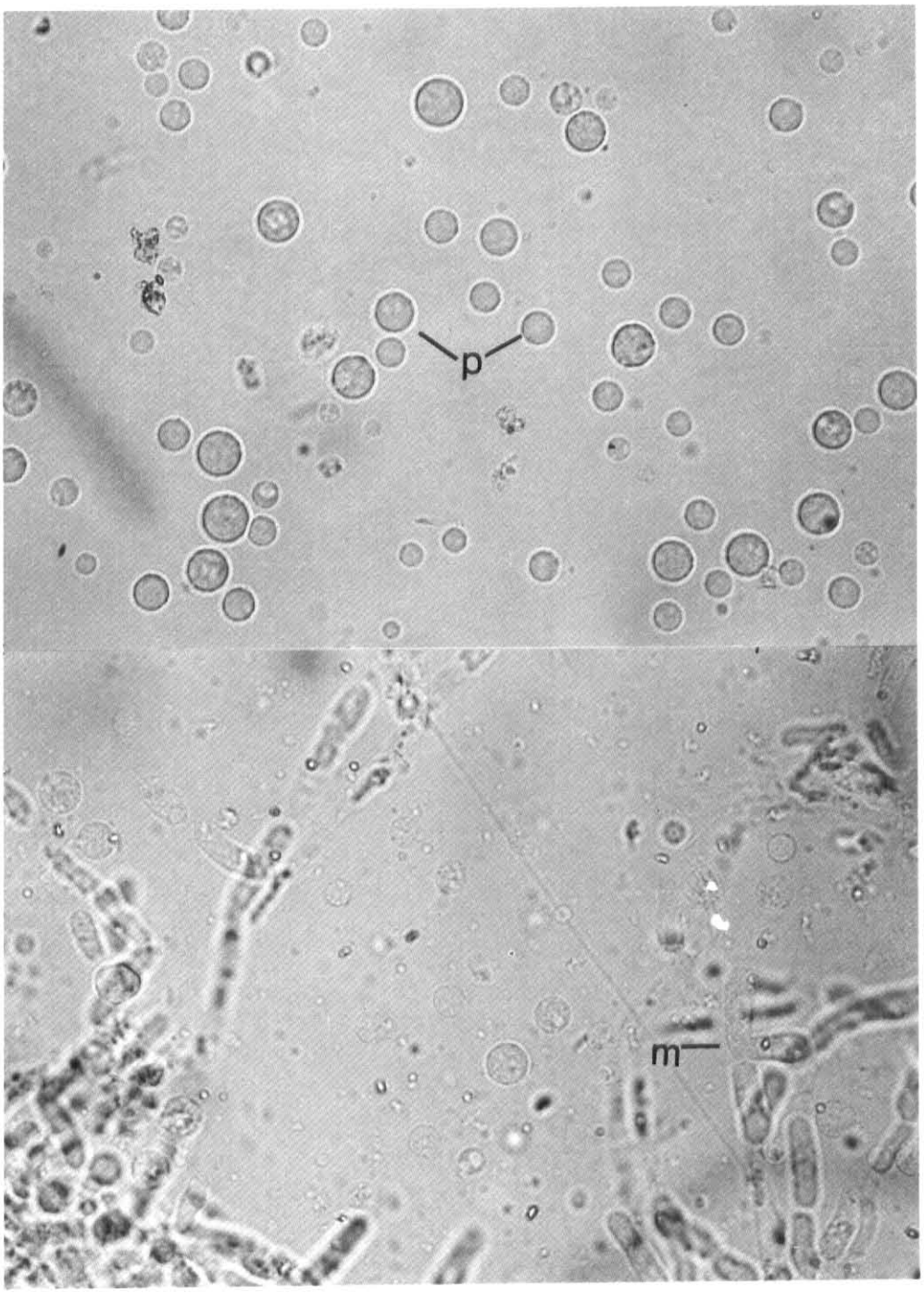

Figure 1 The release of protoplasts (P) from mycelia (m) of *Trichoderma harzianum* after incubation in enzyme solution for 1 hr.

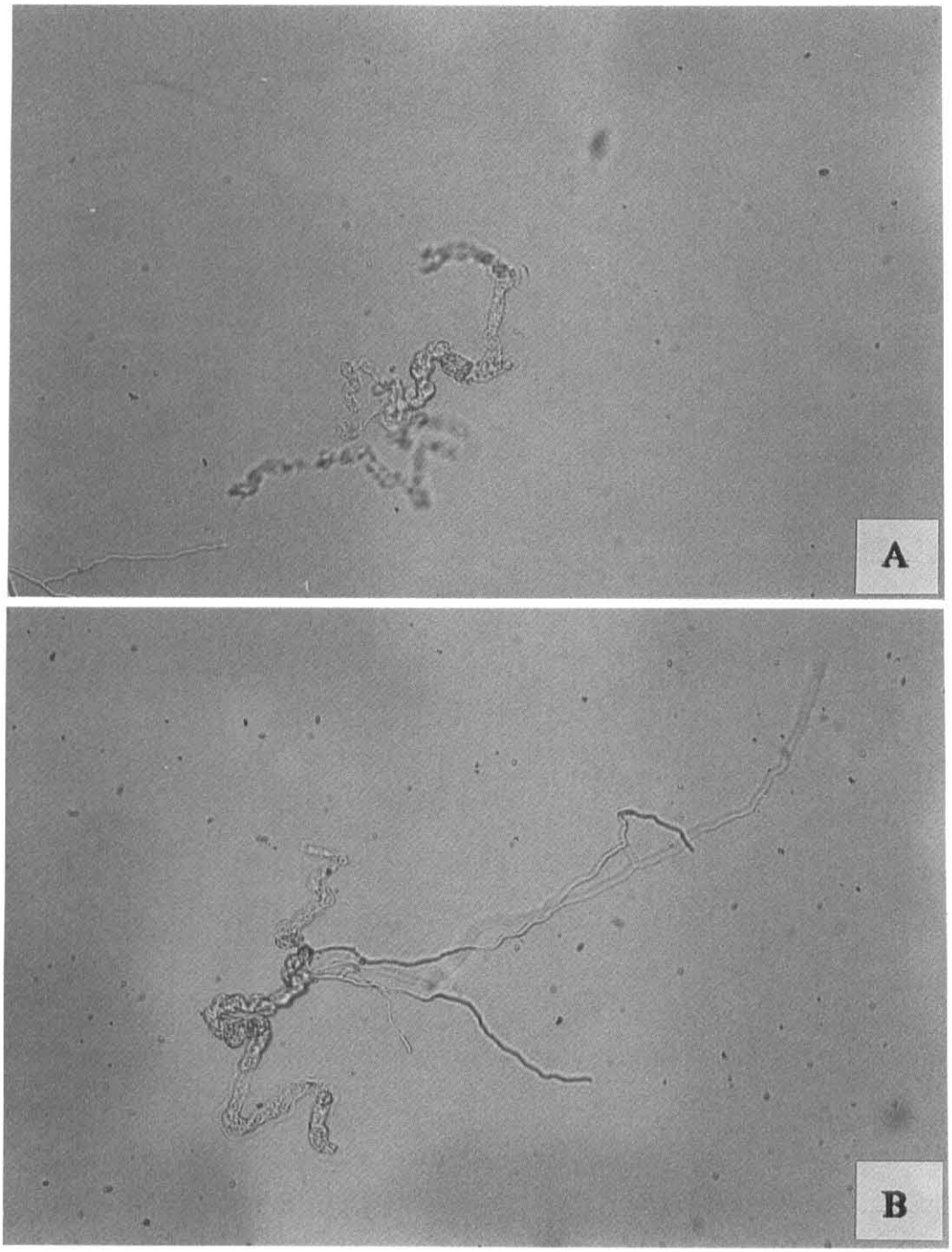

Figure 2 Microphotographs of protoplast regeneration in *T. harzianum* (CB-Pin-01) color mutant. (A. 24-48 hrs, B. over 48 hrs.) Protoplast suspension was spread on solid CM medium and then overlaid with hypertonic MM containing 0.5% agar. Incubation temperature was 25°C.

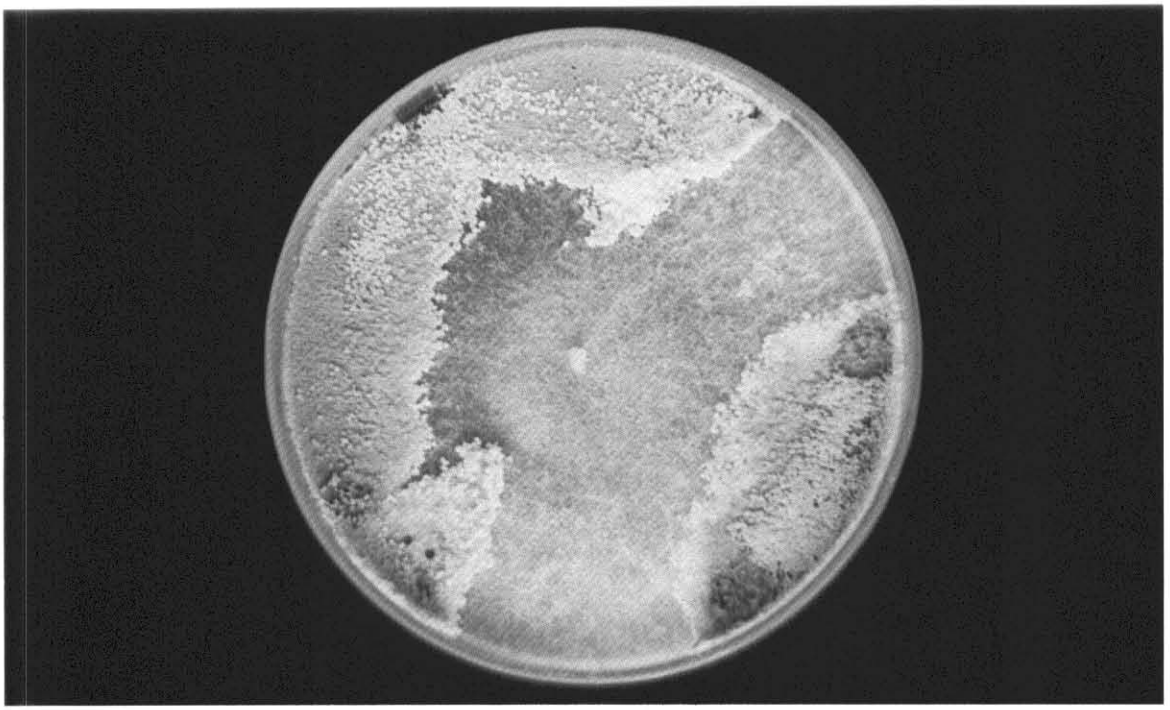

Figure 3 Sectors obtained from fusants of white and yellow color mutants of *T. harzianum* cultured on PDA supplemented with 0.1% d-camphor.

After prolonged incubation (48 hr), septate hyphae were produced from budding-like chain (Fig. 2B). Colonies with colored conidia and sector were developed after 5-day-incubation on CM medium. Determination on the ratio of green sectored colonies (parental colonies) revealed that fusion frequency of the protoplasts was 2% (Table 1).

Properties of the fusants

Diploidization among fusants was induced by transferring the fusants appeared on the hypertonic CM overlaid with hypertonic soft agar medium to MM agar containing 0.1% (w/v) d-camphor. Incubation of those transferred fusants at 25C for 6 days, good mycelial growth and sporulation as well as some sectors were evidently produced. Conidial heads produced on the sectors were green, white or yellow. (Table 2, Fig.3). These results were similar to the reports of Ogawa *et al.* (1987) which color mutants of *T. reesei* QM 9414 were fused and produced white, brown and green conidia sectors. In this study, the expression of wild type (green) conidia presented the formation of heterozygous diploids, whereas production of white and yellow conidia suggested the heterokaryotic nuclear condition of the fusants.

Investigation on diploidization of fusants by transferning green conidia from green sectors onto MM agar containing 0.1% (w/v) d-camphor and incubated at 28C for 14 days revealed that the conidia produced from colonies which developed from protoplast fusants were green. Multisectored colonies were also observed, however there was no complete segregation among those sectors and the colors of the conidia were not obviously changed. From Ogawa et al. (1987)'s study, multisectored colonies with white, brown and green conidia developed from green-sectored conidia. They strongly suggested that the green sector was deploid. The aneuploid break-down products were likely to be parental and /or new recombinant strains. The DNA content of green conidia was found to be approximately 1.9 times higher than that of the parental strain. After series of transferring of green conidia from green-sectored colonies to MM agar, green conidia were constantly obtained without

Table 1 The frequency of fusion of protoplasts from white and yellow mutant strains of *Trichoderma harzianum*.

Strain	Total number of non-sectored colony	Total number of of green-sectored colony	Fusion frequency (%)
White	1×10^6	1×10^5	2
Yellow	1×10^6		

Fusion frequency = The percentage of green-sectored colonies growing on complete medium, as compared to the total number of regenerated colonies.

Table2 Characteristics of fusion products (118 colonies) after being transferred to minimal medium (MM) agar supplemented with 0.1% d-camphor for 5 days.

Color of conidia	No. of isolates	Percent[1]
Non-sectored colony		
yellow	106	89.8
white	6	5.1
Sectored colony		
yellow-white	4	3.4
yellow-white-green	2	1.7

1 Percents of isolates as compared to the total numbers of fusion products (118 colonies).

any phenotypic segregation. This expression indicated that the tested protoplast fusants or new recombinant strains were heterozygous diploid. It is concluded that attempts to develop the techniques for protoplast isolation, fusion, and regeneration of the fusants of *T. harzianum* color mutants were successful. Moreover, diploidization technique to produe the diploid fusants by using d-camphor was satisfactorily obtained. Knowledges from this study will be very useful for future investigations on the creation of new superior strains of *T. harzianum* which can enhance the disease protection capability and sustenance in soil environments. However, more researches are still needed to demonstrate the stability of new protoplast fusants both in laboratory and in nature.

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