Construction of new GFP-tagged fusants for *Trichoderma harzianum* with enhanced biocontrol activity

Mojegan Kowsari[1, 2], *Mostafa Motallebi*[1]*, *Reza Mohammad Zamani*[1]

[1] National Institute of Genetic Engineering and Biotechnology (NIGEB), Shahrak-e Pajoohesh, Tehran – Karaj Highway, P.O. Box 14965-161, Tehran, Iran

[2] Agricultural Biotechnology Research Institute of Iran, Mahdasht Road, P.O. Box 31535-1897, Karaj, Iran

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Abstract: *Trichoderma* is one of the most exploited biocontrol agents for the management of plant diseases**.** In biocontrol ecology, the critical factors are detection, and the monitoring and recovery of specific biocontrol agents either naturally present or deliberately released into the environment. Protoplast fusion is an appropriate tool for the improvement of biocontrol *Trichoderma* strains. Protoplast isolation from *Trichoderma harzianum* was achieved using 24 h culture age, 6.6 mg/ml Novazym L 1412 at 30°C which resulted the maximum protoplast yield of 5×10^8/ml. The self-fused protoplasts were regenerated and 12 fusants were selected based on their growth rate on 2% colloidal chitin medium. Next, a comparison was done for chitinase and antagonistic activity. Transcriptomic analysis based on quantitative real-time RT-PCR, demonstrated that T8-05 fusant expressed 1.5 fold of *chit42* transcript as compared with the parental line. This fusant with 7.02±0.15U chitinase activity showed a higher growth inhibition rate (100%) than the parent strain, against *Rhizoctonia solani*. To obtain a genetically marked fusant that can be used as a biomonitor, the fusant was cotransformed with the *gfp* and *amdS* genes. The morphology and viability of selected cotransformant (FT8-7MK-05-2) was similar to the parent. Green fluorescing conidia were observed within the first 2 days of incubation in the soil, and this was followed by the formation of chlamydopores after 60 days. The colonisation of the *gfp*-tagged fusant was also monitored visually on *R. solani* sclerotia by scanning electron microscopy. Production of *gfp*-tagged fusant of *Trichoderma* spp. provides a potentially useful tool for monitoring hyphal growth patterns and the population of biocontrol agent isolates introduced into environmental systems.

Key words: biomonitor, fusant, green fluorescent protein, real-time RT-PCR, *Trichoderma*

Introduction

Trichoderma spp. are one of the most importannt biological control agents against a wide range of phytopathogens. *Trichoderma* strains with: high reproductive capacity, strong aggressiveness against pathogens, an ability to survive under difficult conditions, a capacity to modify the rhizosphere and utilise nutrients, and the ability to efficiently promote plant growth and defense mechanisms, are ubiquitous biocontrol (Michereff *et al*. 1995; Gupta *et al*. 2010).

Protoplast fusion is an important strain improvement tool for developing hybrid strains and bringing together genetic material for recombination in filamentous fungi where the sexual cycle is difficult or impossible (Lalithakumari and Mathivanan 2003). Isolation, fusion and regeneration of protoplasts have been carried out in *Trichoderma* mainly for improving the biocontrol potential (Balasubramanian *et al*. 2012). Several methods have been used to study the distribution and occurrence of *Trichoderma* in soils, but few methods have allowed quantitative evaluation of population dynamics and survival. Genetic engineering of biocontrol agents with marker or reporter genes has provided useful tools for monitoring biocontrol agents in environments (Lo *et al*. 1998). For example, the *β*-glucuronidase (*GUS*) marker gene (Thrane *et al*. 1995) and the selectable hygromycin B phosphotransferase (*hygB*) gene (Migheli *et al*.1994), are promising tools for ecological studies of biocontrols. The green fluorescent protein (GFP) is a biomarker (Jansson 2003), which does not require additional cofactors or any substrate in order to fluoresce. The GFP could also facilitate studies of *Trichoderma harzianum* hyphal colonisation on the soil and surface of potato tubers diseased with *Rhizoctonia solani*. Black scurf is an economically important disease of potato, caused by the soilborne plant pathogen *R. solani*. The resulting malformed tubers and changes in tuber size and number may result in the reduction of a marketable yield (Banville 1989). Sclerotia are compact aggregates of fungal biomass. *R. solani* is passed over to the next growing season as sclerotia in soil and on tubers. No effective fungicides are available against *Rhizoctonia* diseases, although chemicals are sometimes recommended (Agric 2010). The use of chemicals is of growing concern to environmentalists. The main task would be to develop a biocontrol strategy. Biological control of *R. solani* by *Trichoderma* is well known (Harman 2006) and has been

*Corresponding address:
motalebi@nigeb.ac.ir

reported for infections of potato by *R. solani* (Brewer and Larkin 2005; Wilson *et al*. 2008).

The objective of this study was to produce a *T. harzianum* fusant with improved antifungal activity and a stable biomonitor system.

Materials and Methods

Fungal strains and plasmids

T. harzianum (ABRIICC T8-7MK) and *R. solani* AG-3 (ABRIICC Rs201) were provided by the culture-type collection of the Agricultural Biotechnology Research Institute of Iran. The *amdS* plasmid p3SR2 was kindly provided by Dr. Michael Hynes from The University of Melbourne, Australia. The pDL2 plasmid carrying the *gfp* gene under the control of the constitutive promoter *pki1* of *T. reesei* was kindly donated by Dr. Rahim Mehrabi (Seed and Plant Improvement Institute of Iran).

Fungal growth media

T. harzianum T8-7MK was maintained on Potato Dextrose Agar (PDA) slants. Colloidal Chitin Agar (CCA) selective medium contained (g/l): colloidal chitin, 5.0; glucose, 1.0; $NaNO_3$, 2.0; K_2HPO_4, 1.0; KCl, 0.5; $MgSO_4$, 0.5; $FeSO_4$, 0.01; agar 15; distilled water 1,000 ml at a pH of 6.5. *Trichoderma* minimal medium (MM) contained (mg/ml): $(NH_4)_2SO_4$, 5; KH_2PO_4, 15; $MgSO_4$, 0.6; $CaCl_2$, 0.6; $CoCl_2$, 0.002; $FeSO_4 \cdot 7H_2O$, 0.005; $MnSO_4 \cdot H_2O$, 0.0016; $ZnSO_4 \cdot 7H_2O$, 0.0014. The selective medium for *amdS* expression was minimal medium containing acetamide (MMA) (minimal medium containing 10 mM acetamide as the sole nitrogen source and 12.5 mM CsCl).

Protoplast preparation

Trichoderma conidia was prepared by adding 5 ml of sterile distilled water to PDA culture medium at 2, 4, 5, 6, 8, and 16 days old. Aseptic conditions were maintained. The suspended conidia were centrifuged at 5,500 g for 8 min. The conidial suspension of 1×10^6 conidia/ml was transferred aseptically into 100 ml of rice straw water. The rice straw water was made up of ground rice and wheat straw (50 : 5 g) in 100 ml distilled water. The flasks were incubated for 27, 24, 17, 14, 12, and 72 h on a rotary shaker at 110 rpm. The young germ lines were harvested by filtration using sterilised cloth, and washed once with sterile distilled water followed by two washes with sterile osmotic stabiliser.

To release viable protoplasts from conidia, the lytic enzyme Novozym L1412 was used at different concentrations (2, 5, 6, 6.6, 7.3, and 8 mg/ml). As an osmotic stabiliser, 1 M sorbitol was used at a pH of 7.5. Flasks containing a mycelium and enzyme solution were incubated on a shaker at 110 rpm and in 25, 28, 30, and 32°C for different times (1.5, 2, 3, 3.5, and 4 h). The release of protoplasts was observed under light microscope. The number of protoplasts was counted using a haemocytometer using the current formula:

$$\text{Cells/ml} = \text{the average count per square} \times \text{the dilution factor} \times 10^4.$$

Fusant isolation and regeneration of protoplasts

The self-fusion of protoplasts was carried out by the method of Stasz *et al*. (1988) with slight modification. Polyethylene glycol (PEG) (MW 3500, Sigma) prepared in STC buffer (0.6 M sorbitol; 10 mM Tris-HCl; 10 mM $CaCl_2$, pH 6.5) was used as fusogen. One ml of protoplast suspension (1×10^6 protoplasts/ml) was mixed with an equal volume of 50% PEG solution. The fusion mixture was incubated at 28°C for 10 min. The mixture was diluted with 1 ml of STC buffer. The PEG in the fusion mixture was washed away using STC buffer, and the fused protoplasts were collected by centrifugation at 100 rpm for 10 min, suspended in STC buffer, and plated on 2% CCA selective medium. The protoplast regeneration and development of colonies were observed on plates that were incubated at room temperature.

Growth rate of fusants

Twelve regenerated self-fusants of *T. harzianum* T8-7MK were selected based on their rate of growth on selective medium. One mycelial disc (5 mm) of each self-fusant was inoculated on 0.5% CCA and PDA media and incubated at 28°C for 4 days.

Screening of fusants against *R. solani* in dual culture

In vitro tests were conducted to evaluate the antagonistic effect of fusants against the *R. solani* pathogen on PDA medium using the dual culture technique. Mycelial discs were cut from fusant cultures of *T. harzianum* (5 mm) and *R. solani* (5 mm). The discs were placed on opposite sides of a PDA Petri dish and incubated at 26°C. The plates were incubated at 28°C. Three plates (replications) were used for each fusant and test pathogen, based on a completely randomized design. The plates that received only the mycelial disc of pathogens served as the control. The colony interaction was assayed as the percentage of inhibition on the PDA plate after 4 days of incubation following the formula:

$$\text{Inhibition of growth} = (X - Y/X) \times 100\ (\%),$$

where: X – mycelial growth of pathogen in the absence of *Trichoderma* (the control), Y – mycelial growth of pathogen in the presence of fusants, the formula suggested by Sundar *et al*. 1995.

Transcriptomic analysis by quantitative real-time RT-PCR

Chit42 transcripts were quantified by quantitative real-time RT-PCR. Total RNA from 100 mg of freeze-dried mycelial powder (derived from a single spore) of the fusant and parent was isolated using the RNeasy Plant Mini Kit (Qiagen). The cDNA were synthesised from 1 μg of total RNA using the cDNA synthesis kit with an oligo (dT) primer. One μl of the cDNA was used in the PCR. Real-time PCR was performed using an ABI system with a SYBR green master mix. All PCRs were performed in triplicate in a total volume of 10 μl for 40 cycles under

the following conditions: denaturation, 95°C, 45 sec; annealing, 60°C, 1 min; extension, 72°C, 1 min. The number of cDNA transcripts was normalized against the expression of the housekeeping *β*-tubulin gene (Glass and Donaldson 1995). Data were expressed as 2-ΔΔCT (Livak and Schmittgen 2001). The following specific primers were used: chiF 5'TGCCTACGCCGATTATCAGAAGCA3' and chiR 5'CTTCAAGTTGCGGTTGGCCTTCTT3' and *β*-tubulinF 5'TTCTTGCATTGGTACACTAGCG3' and *β*-tubulinR 5'ATCGTTCATGTTGGACTCAGCC3'.

Chitinase activity

T. harzianum was grown in a flask of 50 ml of colloidal chitin broth (CCB). Each flask was inoculated with 1 ml of conidial suspension (10^6 conidia/ml) and incubated at 28°C for 120 rpm, with triplicates for each strain. After 5 days, the cell free culture filtrates were used as enzyme sources for chitinase assay and the mycelia were freeze-dried for RNA extraction. The released *N*-acetylglucosamine in the mixture was calculated by the method of Reissig *et al*. (1955). The protein content in the culture filtrates was estimated by the method of Bradford (1976).

Protoplast cotransformation and regeneration

For monitoring of the fusant in the soil, its protoplasts were transformed with *gfp* marker gene. The transformation procedures were based on the methods of Penttila (Penttila *et al*. 1987) with modification. The transforming DNA, pDL2, and p3SR2 plasmid (10 µg of each plasmid) was mixed with 320 µl of protoplast suspension. The mixture was incubated for 20 min on ice. For regeneration, transformed protoplasts were plated on selective MMA for *amdS* expression. Added onto MMA, was 0.75% selective top agar containing 1 M sorbitol as osmotic stabilizer. Individual colonies were randomly chosen for *amds* in selective medium incubated at 28°C after 5 days.

Detection of stable cotransformants

To study the stability of cotransformants, each transformant was incubated on MMA for 5 days at 28°C. Conidia of each transformant were then collected and plated on MMA medium. After 4 days, several distinct colonies were transferred to new MMA slants. Zeiss Fluorescence Axiophot microscopy at 380–480 nm detected GFP activity. After testing for GFP activity, subculturing of *gpf*-tagged isolates was repeated three times in selective medium. To test the stability of the expression of cotransformants, they were subcultured continuously on PDA six times, without selection pressure. A mycelial plug of each cotransformant was transferred to a PDA plate and the plate was incubated at 28°C for 4 days.

Monitoring of cotransformants in soil

Cotransformed fusant was grown on PDA for 6 days, and five 0.5 cm discs from the culture were placed in 300 ml of rice straw water, in a 500 ml flask. The flasks were incubated at 28°C on 110 rpm, with 14 h of light per day, for 2 days. The biomass of each flask was collected, rinsed with sterile distilled water, and added to 20 g of moist rice straw powder (50 g rice straw, 5 g wheat straw in 30 ml of distilled water as carrier). This powder was then mixed with agricultural soil (pH 5.5 to 6.5). The pots containing the mixed soil were incubated at 25°C, with 12 h light per day. After 1, 4, 10, 20, and 60 days of incubation, three random soil samples of each pot were diluted in sterile distilled water and cultured on selective MMA plates. Each soil sample was examined using fluorescence microscopy.

Monitoring of cotransformants on potato tubers with black scurf

The potato tubers with black scurf were planted in the centre of three pots containing *gfp*-tagged fusant with inoculated agricultural soil, at a depth of 5 cm. The pots were incubated at 25°C, with 12 h of light per day for 20 days. The colonisation of *T. harzianum* on tuber and sclerotia was investigated after 4, 10, and 20 days with the use of fluorescence microscopy.

***In vivo* study by scanning electron microscopy (SEM)**

The antagonism and colonisation of *gfp*-tagged fusant on hyphal cells and sclerotia of *R. solani* were studied in detail using scanning electron microscopy. The soil was autoclaved three times at 121°C for 30 min and mixed with the fusant. The infected potato tubers with sclerotia were plated in each pot and incubated for 20 days at 25°C. Three discs (1 cm^2) from each potato tuber were fixed with vacuum, on holder and sputter coated with gold (Leser *et al*. 2009).

***R. solani* sclerotia germination**

The viability of sclerotia *R. solani* was investigated after inoculation with *gfp*-tagged fusant. After 2, 4, 6, 8, and 10 days of incubation at 25°C, sclerotia from each potato tuber were recovered and placed on PDA plates at 25°C, and their germination and growth rate were evaluated. Sclerotia on potato tuber in untreated pots were used as the control.

Results

Protoplast fusion

For optimisation of protoplast fusion, different parameters were evaluated. The prepared conidia were grown in an inexpensive medium (rice bran water). The maximum numbers of protoplast were released after 24 h of conidia incubation (Fig. 1A). The effect of different concentrations of Novozym L1412 on protoplast production was investigated when the fungal mycelia were digested in an osmotic stabiliser. The tested lytic enzyme increased the number of protoplasts while increasing the enzyme concentration, yielding the maximum number of protoplast (5×10^8/ml) with 6.6 mg/ml (Fig. 1B).

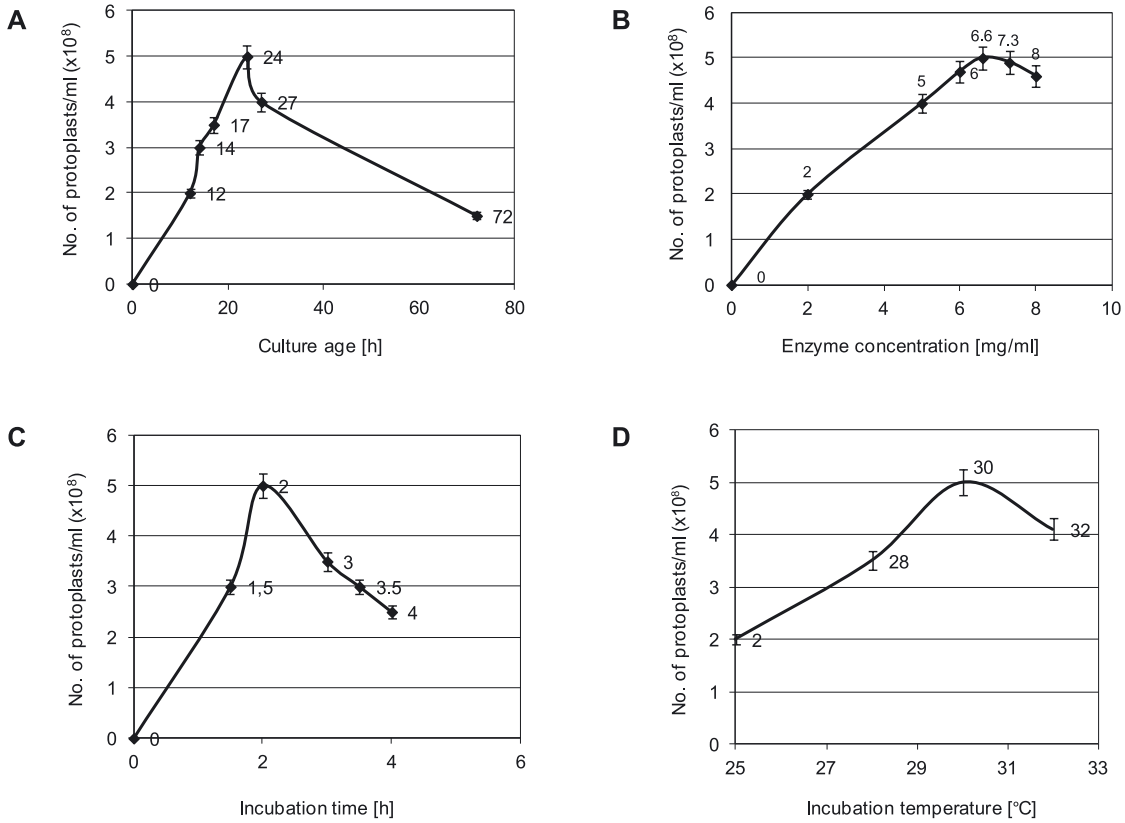

Fig. 1. Effect of different factors on release of protoplasts: A – effect of the culture age on release of protoplasts; B – effect of enzyme concentrations on the release of protoplasts; C – effect of incubation time in enzyme solution on the release of protoplasts; D – effect of incubation temperature on the release of protoplasts

Temperature is one of the most important factors for the isolation of protoplasts. Among the temperatures tested for the effective protoplast release, 30°C for 2 h was found to be suitable for the isolation of maximum protoplasts. Any increase or decrease in the temperature and time, reduced the number of released protoplasts (Fig. 1C, D).

Growth rate and antagonistic activity of fusants

Based on the mycelial growth of fusant on 2% CCA selective medium, 12 fast growing self-fusant colonies were selected and designated as FT8-7MK-01 to 12.

The growth rate of these selected fusants was examined on non-selective media (0.5% CCA and PDA). The fusant FT8-7MK-05 showed the highest mycelial growth of 5.1 and 7.7 cm on these two media, respectively (Table 1). Also this fusant (FT8-7MK-05) exhibited the highest growth inhibition (95%) on phytopathogenic fungus, *R. solani* compared to the parent isolate (T8-7MK) with 34.2% inhibition (Table 1). Most of the fusants grew faster on PDA and CCA medium than the parent strain grown on PDA and CCA medium (Table 1). Antagonistic activities exhibited by the fusants were better than the antagonistic activities of the parent (Table 1).

Transcriptomic analysis

To test the expression level of *chit42*, the cDNA of self-fusant FT8-7MK-05 and parent T8-7MK grown in colloidal chitin was performed and analysed by quantitative real-time RT-PCR. Differential expression of *chi42* was detected in fusant and parent. The *chi42* transcript level in FT8-7MK-05 which was calculated by 2-ΔΔCT equation and *β*-tubulin as an internal reference gene, was 2.83 fold that of the parent.

Table 1. Growth rate and antagonistic activity of self fusants. Results and standard error are the average of three replicates

Row	Strain	Mycelia	Growth [cm]	Inhibition mean [%]
		CCA (0.5%)	PDA	
1	T8-7MK	2.4±0.2	5.1±0.2	34±0.4
2	FT8-7MK-01	4.2±0.2	6.3±0.3	76±0.2
3	FT8-7MK-02	4.5±0.3	6.8±0.2	84±0.4
4	FT8-7MK-03	4.3±0.3	6.5±0.4	80±0.3
5	FT8-7MK-04	4.7±0.2	7.0±0.3	88±0.2
6	FT8-7MK-05	5.1±0.4	7.7±0.2	95±0.2
7	FT8-7MK-06	3.9±0.4	6.0±0.4	72±0.4
8	FT8-7MK-07	3.4±0.2	5.8±0.3	65±0.3
9	FT8-7MK-08	4.1±0.2	6.2±0.4	75±0.3
10	FT8-7MK-09	4.8±0.3	7.0±0.2	89±0.2
11	FT8-7MK-10	4.3±0.4	6.5±0.4	81±0.3
12	FT8-7MK-11	4.0±0.2	6.1±0.3	74±0.4
13	FT8-7MK-12	4.9±0.3	7.1±0.2	90±0.2

CCA – Colloidal Chitin Agar; PDA – Potato Dextrose Agar

Enzyme activity

The protein production of self-fusant (FT8-7MK-05) and parent (T8-7MK) was measured in the presence of colloidal chitin as the carbon source. An increase in protein content was recorded in FT8-7MK-05 (56±0.56 μg/ml) compared to that in the parent strain (38±1.12 μg/ml). Enhanced chitinase activity in self-fusant FT8-7MK-05 was also confirmed. The enzyme activity of the 7.02±0.15 unit was measured in the case of self-fusant FT8-7MK-05 which had a 1.79 fold increase as compared to the parent strain. The values are the means of three replicates with standard error.

Isolation of transformants

For monitoring of FT8-7MK-05 fusant in the environment by GFP, it was cotransformed with the plasmid pDL2 and p3SR2 containing *gfp* and *amdS*, respectively. Stable transformants were initially selected using selective medium containing acetamide. Among 500 transformants, 100 were selected on the basis of their ability to grow on selective media. The selected stable transformants for the *amdS* were also found to have GFP activity. Among these transformants, the FT8-7MK-05-2 was selected for further study.

***In vitro* biological control**

To test whether the cotransformation of FT8-7MK-05 by *gfp* and *amdS* affects its antifungal activity, confrontation experiments were carried out. Inverted microscopy study, demonstrated that the biocontrol FT8-7MK-05-2 hyphae reached the *R. solani* hyphae and grew on its surface and then penetrated into the pathogen cell wall (Fig. 2). When *T. harzianum* wild type (as the control) or transformant and *R. solani* were grown in the same PDA Petri dish, they produced a lytic zone in the *R. solani* mycelia after 4 days. 5FT8-7MK was able to control the growth of *R. solani* and then sporulated in the PDA medium. The same behaviour was observed when the untransformed fusant strain was used as the control (Fig. 3). Therefore, transformation with the *gfp* gene did not affect the antagonistic ability of *T. harzianum* FT8-7MK-05.

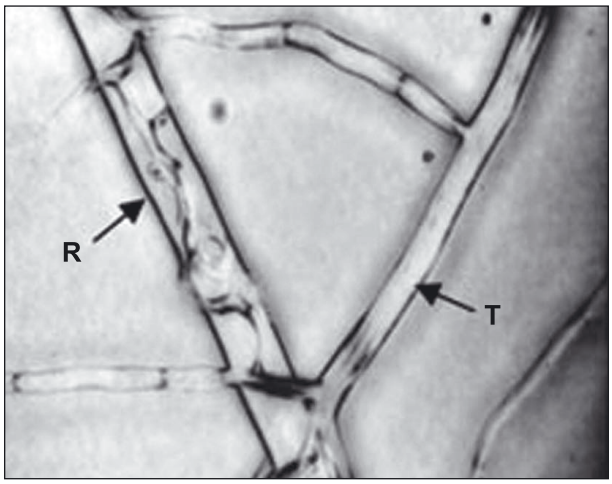

Fig. 2. Invert micrographs of biocontrol of *R. solani* by FT8-7MK-05, magnified ×1,000; T – *T. harzianum* FT8-7MK-05; R – *R. solani*

Biomonitor of *T. harzianum* FT8-7MK-05-2 in soil

The *gfp*-tagged hyphae were easily observed in the soil due to their fluorescence, when using florescent microscopy. The results indicated that the conidia and new germinated conidia were first observed after 2 days of incubation. Fluorescent mycelia, conidia, and conidiospores of FT8-7MK-05-2 were visible after 10 days of incubation. After 20 days of incubation, most structures of FT8-7MK-05-2 were fluorescent chlamydospores (Fig. 4). After 60 days of incubation, hyphal growth of FT8-7MK-05-2 significantly increased and the number of conidia decreased.

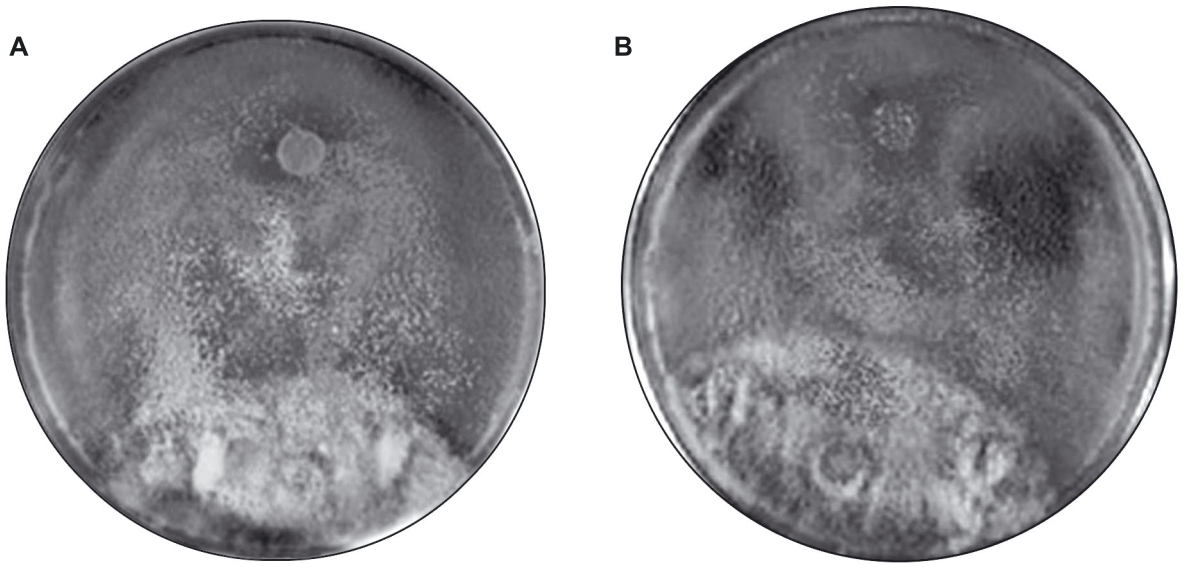

Fig. 3. *In vitro* inhibition of *R. solani* by *T. harzianum* FT8-7MK-05 (A) and the control (B)

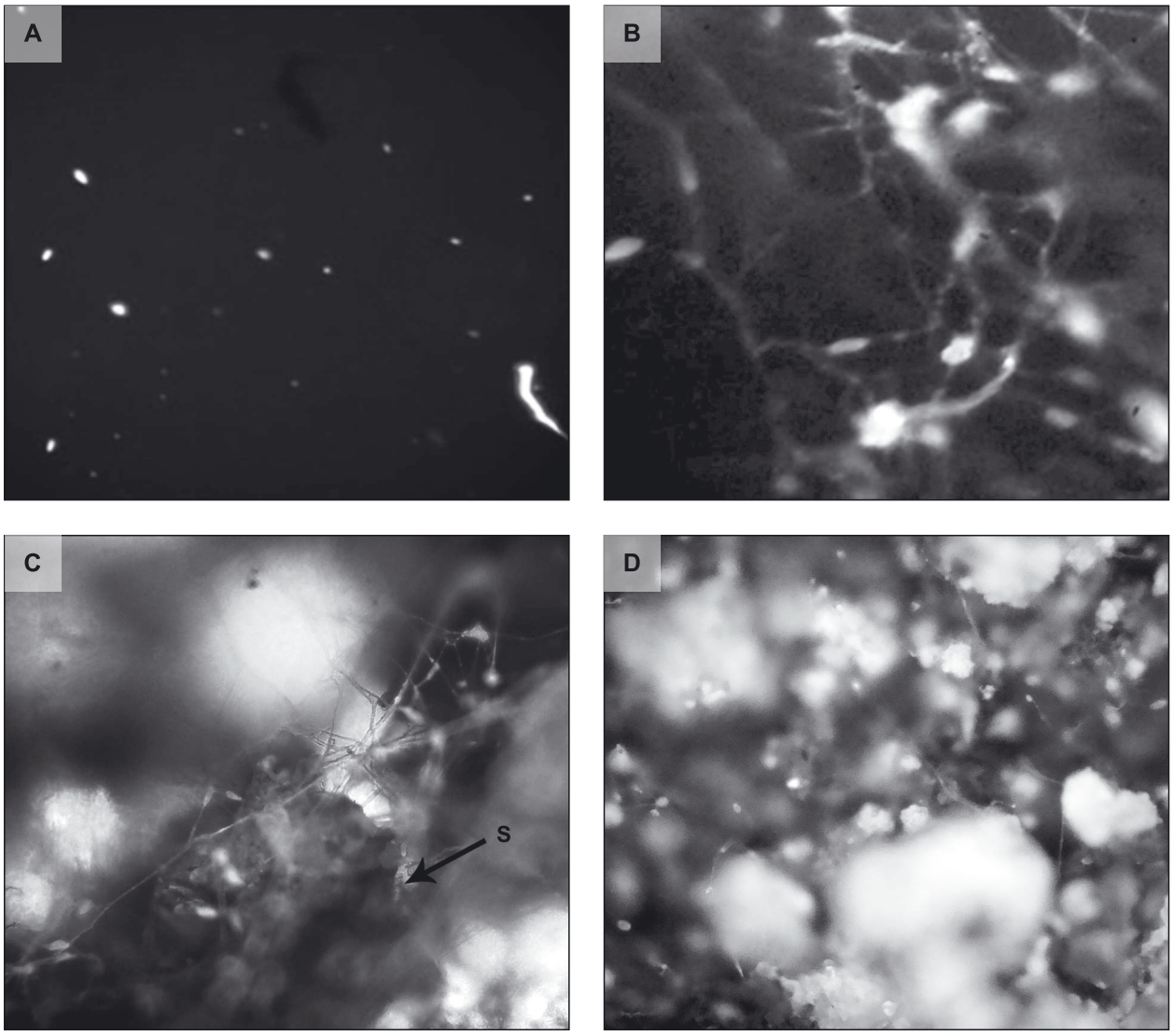

Fig. 4. Fluorescent conidia after 2 days of inoculation (A); fluorescent mycelia were observed in soil after 4 days (B); colonised soil (S) particles with FT8-7MK-05-2 in unsterilised soil after 10 days (C); fluorescent conidia after 20 days (D)

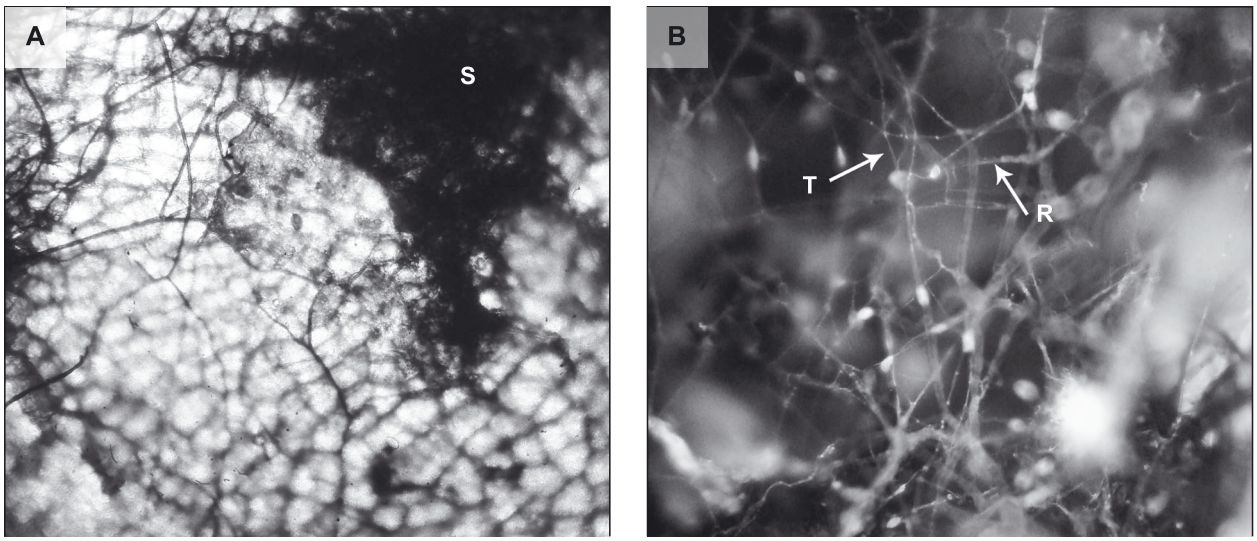

Fig. 5. The compacted black masses of sclerotia (S) on potato tubers (A); the *R. solani* (R) mycelium from sclerotia were surrounded by fluorescent FT8-7MK-05-2 (T) (B). The images were obtained with a 20 objective

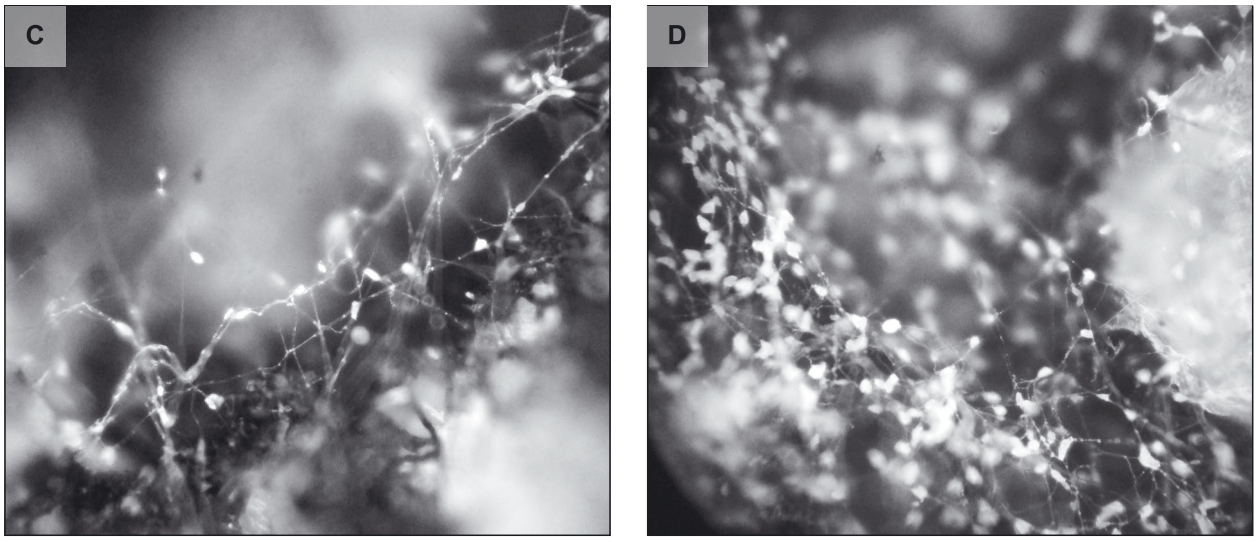

Fig. 5. *Continuation.* The hyphal growth of 5-2FT8-7MK continued after 12 days (C); completely colonised sclerotia and lots of bright spores during the 20 day time period (D). The images were obtained with a 20 objective

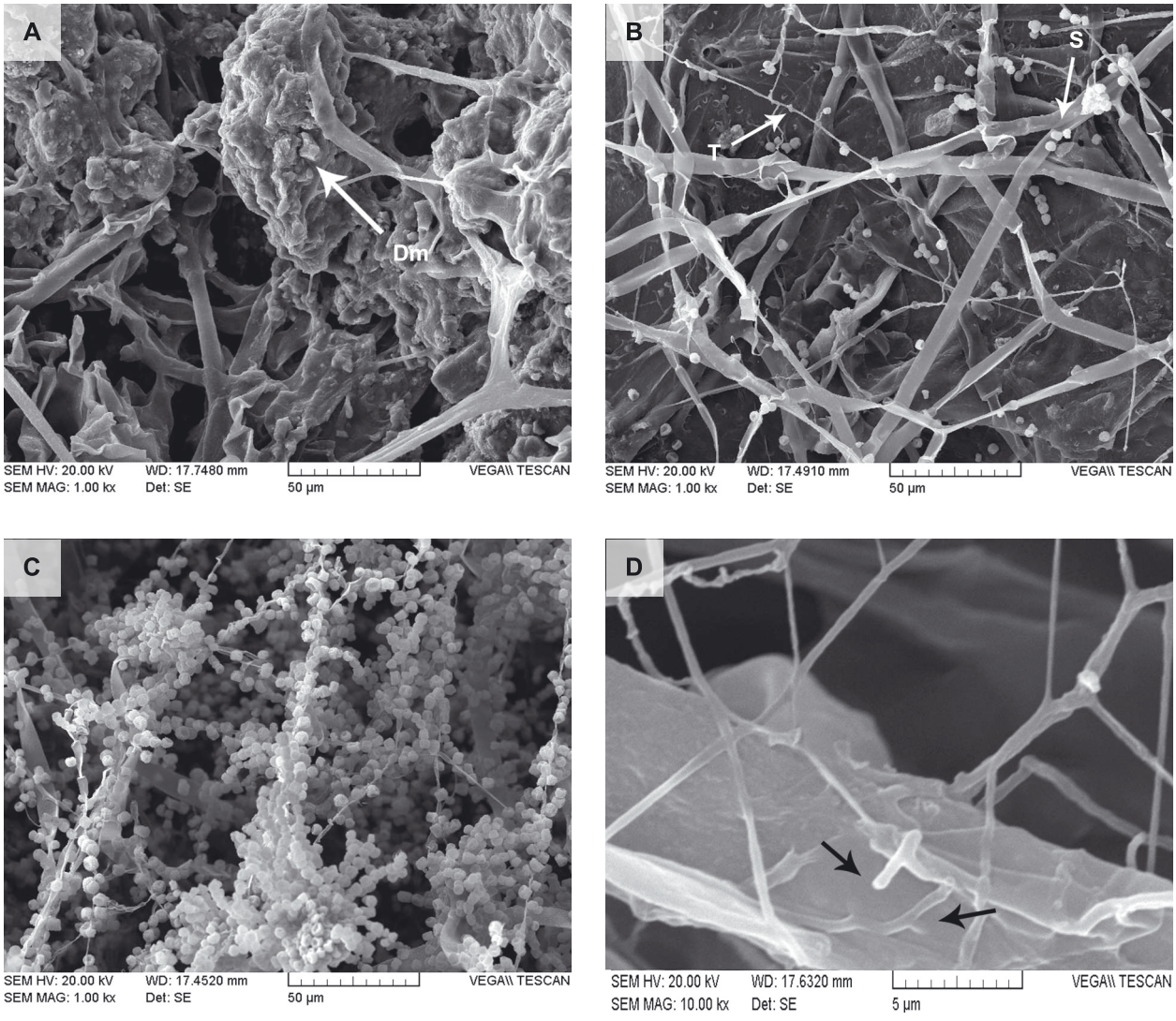

Fig. 6. *In vivo* examination of sclerotia colonisation by FT8-7MK-05-2 using SEM microscopy: A – Dense mass (Dm) of sclerotia (×1,000); B – colonisation of FT8-7MK-05-2 (T) on sclerotia (S) (×1,000); C – the T8-7MK sporulated rapidly in 10 days (×1,000); D – the FT8--7MK-05-2 penetration in pathogen cell wall. Appressorium indicated by black arrows (×10,000)

Monitoring of *T. harzianum* FT8-7MK-05-2 on potato tubers having black scurf

To test whether the FT8-7MK-05-2 transformed by *gfp* was able to colonise and also be identifiable on black scurf, the potato tubers covered by black scurf were planted into agricultural soil containing FT8-7MK-05-2. The compacted black masses of sclerotia on diseased tubers were visible (Fig. 5A). Examination by fluorescent microscopy of mycelial growth of FT8-7MK-05-2 was also achieved after 4 days. Colonisation of biocontrol on *R. solani* black scurf was observed on the potato tuber surface (Fig. 5B). The mycelial fluorescent intensity demonstrated that hyphal growth of FT8-7MK-05-2 continued for approximately 12 days (Fig. 5C). After 20 days of incubation, the *gfp*-tagged *Trichoderma* completely colonised on *R. solani* sclerotia and mostly produced conidia which totally covered the black scurf (Fig. 5D).

In vivo examination of *T. harzianum* FT8-7MK-05-2 colonisation by SEM microscopy

A scanning electron microscope showed periderm of potato tubers having black scurf. A dense mass of *R. solani* sclerotia on the surface of the tuber was observed which was tightly attached to the periderm via their mycelia (Fig. 6A). Colonised periderm tissues and sclerotia with FT8-7MK-05-2 were examined by SEM in order to study the detail of interactions. Colonisation of biocontrol on the potato tubers surface and sclerotia is shown in figure 6B. Also, a switching to sporulation and then more profuse sporulation after 10 days of inoculation were observed (Fig. 6C). The diameter of hyphae of FT8-7MK-05-2 were 1–2 μm as compared to the sclerotial hyphae which were 5–7 μm. Such diameters meant they could easily be distinguished from each other (Fig. 6D). The biocontrol frequently grew parallel to the pathogen mycelia and formed new hyphal branches that further extended toward the *R. solani.* The branches coiled around the sclerotial hyphae and then eventually penetrated into the cell wall (Fig. 6D).

R. solani AG3 sclerotia germination

To test the viability of sclerotia after *T. harzianum* FT8-7MK-05-2 colonisation, the sclerotia were collected and rinsed in sterile-distilled water and dry-blotted on sterile paper towels. Germination and growth rate were evaluated on PDA. The mycelial growth of sclerotia was not observed after 8 days of incubation, whereas the germination of untreated sclerotia (as the control) started at a shorter period of time (after 2 days) (Fig. 7). The radial growth of the treated scelrotia on PDA indicated a lower growth rate (23 mm) than that of untreated sclerotia as the control (94 mm) after 10 days of incubation.

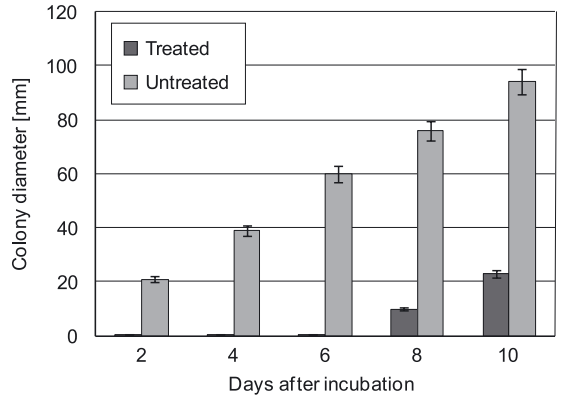

Fig. 7. The sclerotia germination in samples treated with FT8-7MK-05-2 and untreated samples. Results are the average of three replicates

Discussion

For successful fusion and transformation, a high-quality and a large quantity of protoplasts is required. In this study, different parameters were studied for protoplast production. Maximum release of protoplast (5×10^8/ml) occurred in optimum conditions with a 24 h culture age in an inexpensive medium (Rice bran water), and using, 6.6 mg/ml Novozym L1412, with incubation at 30°C for 2 h. Different culture ages have been reported for release of protoplasts, 15–18 h in *Trichoderma viride* (Tomova *et al.* 1993), 24 h in *T. longibrachiatum* (Mrinalini and Lalithakumari 1998), 20 h in *T. reesei* (Kolar *et al.* 1985). Novozym L1412 used in this study, is a complex of different enzymes such as: chitinase, cellulase, protease, and *β*-glucanase, but Novozym 234 used in other reported studies lacks *β*-glucanase (Jung *et al.* 2000). The optimum concentration of enzyme used for the maximum release of protoplast was 6.6 mg/ml. A higher enzyme concentration decreased the rate of protoplast production. This effect may be due to a toxic level and a bursting of the released protoplasts. The same finding has also been reported by Naseema *et al*. (2008). The use of Novozym L1412 was the most advantageous and yields were higher than that reported elsewhere for *Trichoderma* species (Savitha *et al*. 2010; Balasubramanian *et al.* 2012; You *et al.* 2012). However, by using this lytic system, the period required for protoplast isolation (2 h) showed a result lower or similar to that reported for the same and other related species (ranging from 2 to 4 h) (Balasubramanian *et al.* 2003; Balasubramanian *et al.* 2012). Protoplast fusion of biocontrol agents is a novel approach for the improvement of biocontrol efficiency (Wang *et al.* 2009). We used chitin medium to select intra fusants, with growth rate as the selection marker. A similar method was used by Prabhavathy *et al*. (2006). This growth pattern on chitin medium indicated the enhanced production of chitinase and this could be directly related to the antagonistic activity improvement in *Trichoderma*. The increase in the expression of chitinase at the transcriptomic level based on quantitative real-time RT-PCR in this research, is in agreement with the other research finding (Vizcaino *et al.* 2006; Vizcaino *et al.* 2007; Lorito *et al.* 2010). Enhanced chitinase production of about 1.8 fold in FT8-7MK-05 fusant was also confirmed by quantitative assay. The protoplasts of *T. harzianum* FT8-7MK-05 fusant with suitable growth rate, enzyme production, and biocontrol activity was selected for transformation with *gfp* gene for monitoring.

Regeneration of untransformed protoplasts was inhibited at *amds* selective medium. Therefore, growth on this medium was used as the selection criterion for *amds*

transformants. The *gfp*-tagg transformant fungi can provide valuable tools for the monitoring and detection of interactions between pathogen-biocontrol agents on plant tissues and in soil. It provided a nondestructive sampling for visualisation of hyphae *in situ*. The wild-type and the selected genetically marked cotransformant, showed biocontrol activity. These results indicated that cotransformation and expression of transgens did not reduce the biocontrol ability. The stabilised cotransformants were visible by fluorescence microscopy from germinated conidia to mature mycelium. The hyphal growth increased rapidly up to 10 days in soil but subsequently decreased. After 20 days, the morphology switched from filamentous growth to spore structure. In the soil, abundant production of spores is critical for effective distribution of *T. harzianum* FT8-7MK-05-2. These conidia may play an important role as survival structures of introduced *T. harzianum* FT8-7MK-05-2 in natural ecosystems. Survival structures such as spores, have been reported in fungal which is pathogenic for plants and mammals (Lin and Heitman 2005).

Previous reported experiments were necessarily conducted using autoclaved soil, because it was impossible to differentiate hyphae from different fungi in raw soil and on potato tuber surface (Knudsen *et al*. 1991). In the present study, by using the expression of *gfp*, we were able to introduce *T. harzianum* FT8-7MK-05-2 into agricultural soil and monitor the presence of the biocontrol agent even after 60 days of incubation. Also, the *in situ* interaction and colonisation of this *gfp*-tagged biocontrol agent on tuber surface sclerotia were easily biomonitored. The results in this study also demonstrated that the antagonist reduced the viability of tuber-borne sclerotia of *R. solani*. This finding is in accordance with the previous studies, where *Trichoderma* spp. was found to reduce the viability of *R. solani* sclerotia (Brewer and Larkin 2005).

In conclusion, the observations in the present study clearly indicated that production of fusant of *Trichoderma* spp. and its transformation with *gfp* provides a potentially useful tool for monitoring hyphal growth patterns and the population of biocontrol agent isolates introduced into environmental systems.

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