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Construction of *Isaria fumosorosea* Blastospore-Transforming System by Agrobacterium-Mediated Transformation with Benomyl-Resistance Gene

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Abstract.- The green fluorescent protein (GFP) and benomyl resistance gene, the former biomarker for the study of host-pathogen interactions, has been expressed in a variety of prokaryotic and eukaryotic organisms. Here we report the expression of GFP in the entomopathognic fungus *Isaria fumosorosea* through Agrobacterium-mediated transformation (ATMT) using the pK-Ben-GFP vector that confers resistance to benomyl. All transformants expressed GFP whose signal was readily detected by fluorescence microscopy. Furthermore, fluorescent hyphae and conidia were easily distinguished on insect host, *Plutella xylostella*, and blastospores were also detected in the hemolymph of the diamondback moth larvae. Our results show that GFP-tagged strains of *I. fumosorosea* can be used to study the developmental fate of the fungus within its insect hosts and for analysis of the expression of tagged genes. At the same time, genetic transformation of *Isaria* with benomyl resistant gene indicates that these fungi can be used in combination with some fungicides to protect crop from insects as well as plant disease.

Key words: *Isaria fumosorosea, Plutella xylostella*, green fluorescent protein, benomyl resistant gene, Agrobacterium mediated transformation.

INTRODUCTION

Entomopathogenic fungi have received considerable attention owing to their potential in insect pest management especially as they are considered to be environment friendly alternative to chemical insecticides (Ortiz-Urquiza and Keyhani, 2013). These biological control agents may occur naturally and/or can be introduced into the agroecosystem as part of an integrated pest management (IPM) strategy. *Isaria fumosorosea*, a common soil-inhabiting filamentous fungus, causes diseases in a wide variety of insects (Huang *et al*., 2010a,b; Xu *et al*., 2011; Zhou *et al*., 2010; Mascarin *et al*., 2013), occasionally resulting in natural epizootics. These properties have led to the development and commercialization of *I. fumosorosea* as a mycoinsecticide (Butt *et al*., 2001). However, *I. fumosorosea* is very sensitive to several commonly used fungicides (Huang *et al*., 2008), which makes it difficult for *I. fumosorosea* to play a stable role in controlling crop pests when fungicides are being used for plant disease management. A number of studies on the compatibility of entomopathogenic fungi with chemical fungicides have been conducted by evaluating the effect of fungicides on mycelia growth, sporulation, conidial germination and efficacy of the entomopathogenic fungi (Jaros-Su J, *et al*., 1999; Shah *et al*., 2009; Martins *et al*., 2012).

The development of efficient transformation systems in entomopathogenic fungi can provide essential information on genes responsible for pathogenicity and virulence, in turn opening up the possibility of targeted molecular improvements for biological control of insect pests. Gene transfer systems have previously been reported for *I. fumosorosea* (formerly named *Peacilomyces fumosoroseus*) using a conventional method employing polyethylene glycol and particle bombardment (protoplast-PEG method) (Barreto *et al*., 1997; Cantone and Vandenberg, 1999; Inglis *et al*., 1999). *Agrobacterium tumefaciens* mediated transformation (ATMT) system was employed to increase efficacy of genetic manipulation for *I. fumosorosea* (Lima *et al*., 2006). ATMT was described by Bundock *et al*. (1995) for the yeast

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Table I.- List of primers and enzymes.

Primer name	Sequence (5' to 3')	Restriction enzyme site
PF	5'---CCCAAGCTTGGCTCTAGAGACGTTAACTGATATTGAAGGAGC---3'	*Hind* III / *Xba* I
PR	5'---ACAGGCGCGCATCGATGCTTGGGTAGAATAGGTAAGTCAGA---3'	*Asc* I
BF	5'---TTCTACCCAAGCATGCATAGGGGGCCTTCCACCCTTCCAAAAG---3'	
BR	5'---GTAACGTTAAGTGGATCAGCTTGGCCAGC---3'	
TF	5'--- GTAACTGCAGTTGATCCACTTAACGTTACTGAAATC---3'	*Pst* I
TR	5'---CCCAAGCTTCCGCTCGAGAACCCAGGGGCTGGTGACGGAATTT---3'	*Hind* III / *Xho* I
GF	5'---AAGCATCGATGCGCGCCTATGGTGAGCAAGGGCGAGGAGCTGT---3'	*Asc* I
GR	5'--- TAAGTGGATCAACTGCAGTTACTTGTACAGCTCGTCCATG ---3'	*Pst* I

Note: Underlined sequences are restriction site.

Saccharomyces cerevisiae and subsequently for several filamentous fungi (De Groot *et al*., 1998). This bacterium has the ability to transfer a segment of DNA (so-called T-DNA) into plant cells so that the T-DNA integrates presumably at random into the host genome (Hooykaas and Beijersbergen, 1994). This ATMT approach can be a good alternative to protoplast-PEG method resulting in higher numbers of transformants (Amey *et al*., 2002; Fitzgerald *et al*., 2003; Meyer *et al*., 2003). Furthermore, the approach generates a higher percentage of transformants containing just a single-copy of the integrated T-DNA at random chromosomal sites in the fungal genome allowing for random insertional mutagenesis studies (Mulliuns *et al*., 2001; Rolland *et al*., 2003; Tsuji *et al*., 2003; Leclerque *et al*., 2004; Li *et al*., 2005).

In this research work, we developed a transformation system for *I. fumosorosea* in which the benomyl resistance gene marker (*ben*) was introducted into the fungus using *Agrobacterium* mediated transformation. The transformation system enabled us to use bio-molecular techniques to study genes responsible for pathogenicity and virulence in *I. fumosorosea* and to provide a basis for making targeted improvements in genetic makeup of fungus. At same time, genetic transformation of entomopathogenic fungus with the benomyl resistant gene has the potential to allow the use of *I. fumosorosea* in combination with corresponding fungicides to facilitate protection of crops from both insects and fungal plant diseases.

MATERIALS AND METHODS

Fungal and bacterial strains

Strain PF01-N4 of *I. fumosorosea*, deposited at the Engineering Research Center of Biological Control, South China Agricultural University, was originally isolated from a *Bemisia tabaci* nymph (Huang and Ren, 2004), and was maintained in 10% glycerol at -80ºC. *I. fumosorosea* was cultured on Potato Dextrose Agar (PDA) medium and incubated for 10 days at 26±2ºC, L:D=14:10. *Escherichia coli* DH5a was used to propagate and maintain plasmids following standard procedures. *Agrobacterium tumefaciens* LBA4404 was employed for *I. fumosorosea* transformation. The wild type *A. tumefaciens* was grown in Luria-Bertani (LB) medium with 25 µg/ml rifampicin. When *A. tumefaciens* carried the binary vectors, an additional 5 µg/mL of benomyl was added to the media.

Expression vector construction

The *trpC* promoter (*PtrpC*) and *trpC* terminator (*TtrpC*) sequences were amplified using *Aspergillus nidulans* genomic DNA as a template with the primers (PF/PR for *PtrpC* fragment amplification, TF/TR for *TtrpC* fragment). Primer sequence and the sites of restriction enzymes introduced into the primers are listed in Table I. The benomyl resistant gene fragment (*ben* gene), the green fluorescent protein (*GFP*) gene were amplified from pMD-Ben plasmid and pMD-GFP plasmid as a template with primers BF/BR and GF/GR, respectively (Table I). The T-*ben* fragment containing the *trpC* terminator and *ben* gene were

obtained by using the mixture of *TtrpC* fragment and *ben* gene fragment as a template with the primer BF/TR. The T-*ben*-P fragment containing *trpC* terminator and *ben* gene, *trpC* promoter were obtained by using a mixture of the T-*ben* fragment and the *PtrpC* fragment as a template with primer PF/TR. The P-GFP-T fragment containing *trpC* terminator, GFP gene and *trpC* promoter were obtained by using the same method with different primer sets. All fragments clones were amplified in a 50 µl reaction system (2 µl mixture template described above, 0.2 µM each primer, 0.2 mM each dNTP, 2.5mM $MgCl_2$, 1×*Taq* polymerase buffer, and 2.5 U *Taq* Polymerase) by denaturation at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 4 min at 72°C, and terminating with a final extension at 72°C for 10 min. The amplified fragments were sequenced at Invitrogen (Life Technologies, China) to verify the sequence. Sequence identity of cloned fragments to the *trpC* promoter, *trpC* terminator, *ben* gene and *GFP* gene were compared by online BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST).

The initial plasmid pCAMBIA-1300 (GenBank: AF234296) and the T-*ben*-P fragment were digested with the enzymes *Xho* I, respectively, and then Probest DNA Polymerase was employed for the flat end of enzyme incision before digested with *Hind* III (TaKaRa, Dalian, China). The resultant fragment between CaMV35S promoter and hygromycin was discarded and the rest were ligated to the T-*ben*-P fragment digested with enzymes *Hind* III to construct a new plasmid pK-Ben. The pK-Ben plasmid was transformed into *E. coli DH5a* for propagation, followed by isolation with Plasmid Miniprep Kit (Omega, Doraville, GA, USA). The P-GFP-T fragment was digested with *Xba* I / *Hind* III enzymes, and then was cloned into pK-Ben plasmid cut with *Xba* I / *Hind* III, resulting in an inverted insertion of P-GFP-T fragment between the trpC promoter and *Hind* III site in the pK-Ben, yielding plasmid pK-Ben-GFP (Fig. 1, construction of pK-Ben-GFP vector). The presence of the Ben and GFP gene in expression plasmid was examined by PCR and *Asc* I / *Pst* I enzymes.

Agrobacterium-mediated transformation (ATMT)

I. fumosorosea was transformed by using the method described by Lima *et al*. (2006), with some modifications. The *A. tumefaciens* strains LBA4404 harboring the pK-Ben-GFP binary vector were grown at 28°C for 24h in liquid LB medium, supplemented with 50 µg/mL of kanamycin and 50 µg/mL rifampicin. The culture was diluted to an optical density at 660 nm (OD_{660}) of 0.15 in 20 ml of induction medium (IM) (Reis *et al*., 2004), in the presence of 200 µmol/L acetosyringone (AS) and grown under the same conditions until an OD_{660} of 0.6 was reached. *I. fumosorosea* conidia were obtained by harvesting sporulated cultures grown on agar plates composed of minimal medium (Pontecorvo *et al*., 1953). Co-cultivation between *A. tumefaciens* and *I. fumosorosea* was performed by addition of 100 µl of bacterial culture in 100 µl of conidial suspension (1×10^6 conidia/ml). This mix was plated on to nitro-cellulose filters (0.45 µm pore; MFS-Japan, Tokyo, Japan) on co-cultivation medium (same as IM, expect containing 5 mmol/l glucose). After 2 days co-cultivation at 28°C for 48 h, the membranes were transferred to M-100 (Stevens, 1974) agar that contained benomyl (5 µg/mL) as the selection agent for fungal transformants, kanamycin (50 µg/mL) to inhibit growth of *A. tumefaciens* cells and 50 µg/mL rifampicin. All putaive transformants were transferred to fresh M-100 agar containing 5 µg/mL of benomyl for a second round of selection. Control experiments were carried out in the absence (IM-AS) of AS. The stability of 200 randomly selected puative transformants was assessed through successive sub-culturing on M-100 medium for five rounds of sporulation, after which transformants were transferred to selective medium (M-100 medium + 5 µg/mL of benomyl). Plates were incubated at 28°C for 15 days.

Molecular analysis

Fungal genomic DNA was extracted from mycelia of non-transformed and transformed strains grown in shake cultures for 3 days at 28°C in complete medium (Pontecorvo *et al*., 1953; Reader and Broda, 1985) as described by Reis *et al*. (2004). PCR analysis of putative transformants was performed using the primer sets PF/GR that amplify about 1.1 kb fragment containing *trpC* promotor and *GFP* gene with an initial denaturing cycle of 4 min

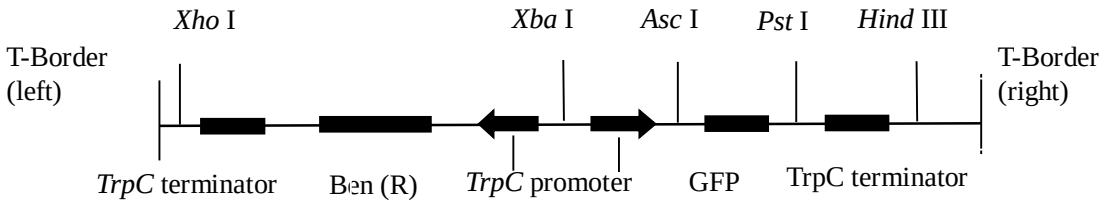

Fig. 1. Construction of pK-Ben-GFP vector.

at 95, followed by 35 cycles of 95°C for 30s (denaturation), 1 min at 55°C, 72°C for 2 min (annealing) and 72°C for 1min (elongation).

For Southern blots, the fragment used as a probe with total 1.1 kb fragment, containing the sequence of *trpC* promoter initiation codon (ATG) to the sequence of *GFP* fragment stop codon (TAA), was amplified using the primers PF/GR using plasmid pK-Ben-GFP as the template. Southern blotting was performed using genomic DNA (10 µg) digested with enzymes *Pst* I, *Xba* I, and *Pst* I / *Xba* I (Takara Co.), respectively. The digested DNA was resolved on 0.8% agarose gel and was transferred onto a Biodyne B nylon membrane (Gelman Laboratory, Shelton, WA, USA). Probe preparation, membrane hybridization, and visualization were performed using DIG High Prime DNA Labeling and Detection Starter Kit II with chemiluminescence detection method (Roche, Penzberg, Germany). The hybridization and stringent washing procedures were performed overnight at 42°C and then at 68°C for 2×15 min, respectively.

Bioassay

Five concentrations of the non-transformed and transformed strains of *I. fumosoroseus* (1×10^3, 1×10^4, 1×10^5, 1×10^6, 1×10^7 conidia/ml) were prepared using the method as described by Huang *et al*. (2010). Leaves with the second instar larvae of *P. xylostella* were dipped into the five concentrations of *I. fumosoroseus* for 15 seconds, and then removed to air dry before being transferred to clean glass petri dishes (20 cm diameter). A piece of filter paper (20 cm in diameter) was placed at the bottom of the dish along with a few drops of water for daily moisture. Leaf disks were replaced every two days except during the pupal stage. Control dishes were treated with 0.02% Tween-80. The insects were placed in air-condense room at 25±1°C, 75% - 90% R.H, and a photoperiod of 14:10 (L:D). Each treatment was replicated three times, and each replicate contained 30 insects. The mortalities of *P. xylostella* were recorded at 24 h intervals until adult emergence. The experiment was repeated three times. A χ^2 test was used to determine the homogeneity of the variance of the repeats ($P<0.05$). The mortality data obtained were subjected to Probit analysis (SAS Institute, 2000).

RESULTS

We employed *Agrobacterium*-mediated transformation with a vector (Fig. 1, construction of pK-Ben-GFP vector) that confers resistance to the selection marker, benomyl. This strategy was pursued because *I. fumosorosea* is very sensitive to benomyl, which is a commonly used fungicide. A number of transformants expressed the benomyl resistance as well as the green fluorescent protein as detected under long-wave UV light, indicating successful transformation and expression via the constructed vector (pK-Ben-GFP). Thirty putative transformants were randomly selected from a large number of transformants screened on 5 µg/ml benomyl and sub-cultured for 5 generation on selective medium (M-100 + 5 µg/mL of benomyl). The rate of false positive transformants was 6.67 % (2/30, Fig. 2 PCR test with primers PF/GR).

Southern hybridization was used to analyze the genomic DNA of 9 transformants (selected randomly from the 28 putative positive transformants) digested with *Bam* HI, an enyme which cuts once within the benomyl resistance gene but does not cut the *GFP* gene fragment. Southern blot analysis confirmed that the *GFP* gene integrated into *I. fumosorosea* genome, when the 1.1-kb fragment amplified by PCR with the primers PF / GR containing the *trpC* promotor and *GFP*

gene was used as a probe. At least 50% of the transformants analyzed appeared to have a single integrated copy of the gene as a single band of differing size was detected. A variety of hybridization patterns were observed from the transformants examined (Fig. 3). The banding pattern seen for transformants #20 suggested a tandem integration of pK-Ben-GFP at a homologous site, giving rise to linearized plasmid with flanking fragment containing genomic DNA. Additionally, some integrations may have occurred at heterologous sites or integration events resulted in rearrangements of the plasmid.

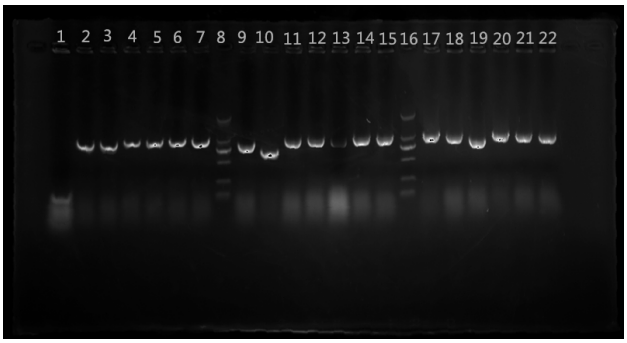

Fig. 2. DNA (7 µg/lane) was separated by electrophoresis on a 0.8% agarose gel. Lane 1, wild type of *I. fumosorosea*; Lane 8 and 16, marker; Lane 2-7, 9-15 and 17-22, transformants.

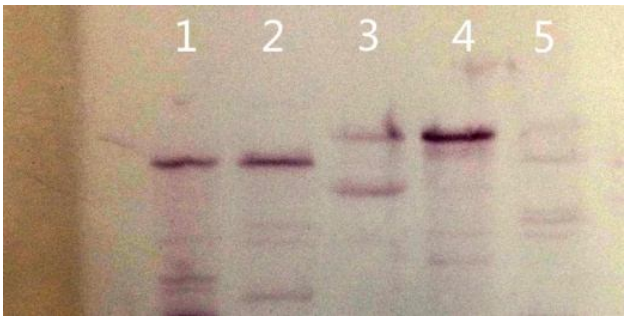

Fig. 3. Southern blot analysis of strain *Isaria fumosorosea* transformed with pK-Ben-GFP. DNA (7 µg/lane) was separated by electrophoresis on a 0.8% agarose gel, transferred to Nytran membranes, and hybridized a ^{32}P-labeled 1.1-kb insert from pK-Ben-GFP. Lane 1-4, DNA from transformant No.14, 17, 20, 22 digested with *Bam* HI. Lane 5, wild type of strain *I. fumosorosea* linearized with *Bam* HI.

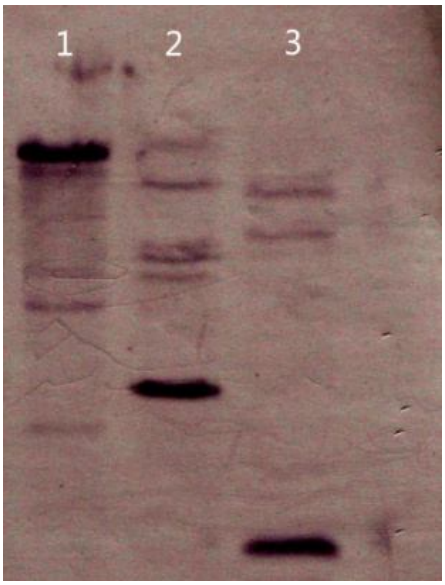

Fig. 4. Southern blot analysis of strain *Isaria fumosorosea* transformed with pK-Ben-GFP. DNA (7ug/lane) from transformant No.14 was separated by electrophoresis on a 0.8% agarose gel, transferred to Nytran membranes, and hybridized a ^{32}P-labeled 1.1-kb insert from pK-Ben-GFP. Lane 1 and 2, DNA digested with *Pst* I and *Xba* I, respectively. Lane 3, DNA linearized with enzymes *Pst* I / *Xba* I with 1.1 kb fragment.

In other analyses, genomic DNA of transformant #14 was digested with *Xba* I, *Pst* I and probed with the *Xba* I - *Pst* I insert from pK-Ben-GFP (total 1.1 kb fragment containing the *trpC* promoter and the *GFP* cDNA) (Fig. 4). These two enzymes do not cut the benomyl resistance gene or the *GFP* gene fragment. These results suggest that tranformant #14 has a single insert in its genome of the linearized plasmid.

Confirmed *GFP* transformants of *I. fumosorosea* displayed the characteristic emerald green fluorescence when grown in a varity of media. Fluorescence was apparent in hyphae of transformant #14 grown on PDA plates, as well as in the blastspores isolated from hemolymph of infected insect larvae, and in the resulting conidia isolated from cuticle of infected insect larvae. *GFP* signals were observed at the individual cell as well as colony levels (Fig. 5). No fluorescence was apparent in hyphae, conidia, or any other cell type examined of the wild type *I. fumosorosea* parent strain (Fig. 5).

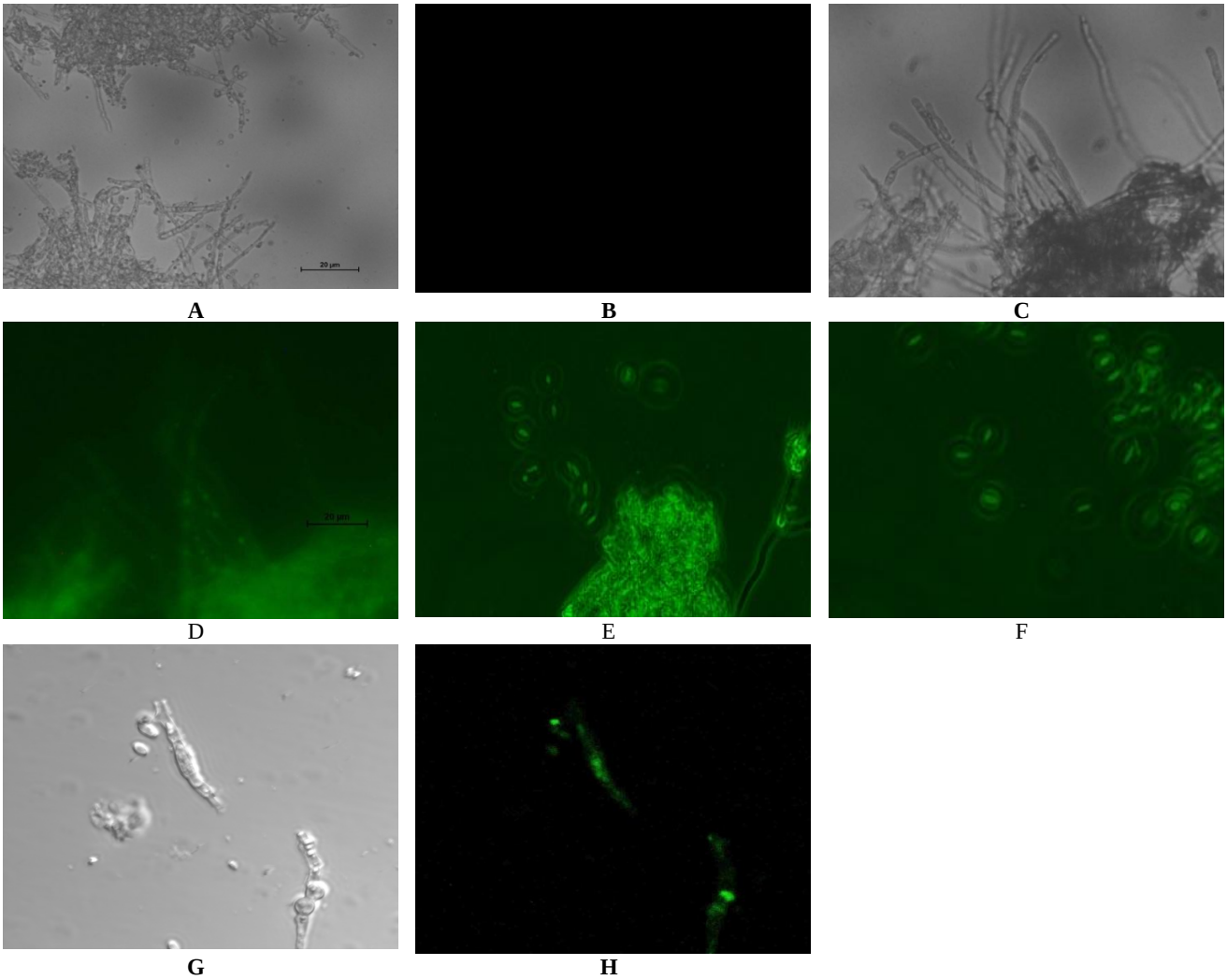

Fig. 5. Expression of the green fluorescent protein in transformant No.14 of *Isaria fumosorosea*. The spores and hyphae of wild type of *Isaria fumosorosea* was observed in optical microscope (A) and fluorescence microscope (B). The hyphae of transformant grown on PDA plates were observed in optical microscope (C) and fluorescence microscope (D). The spores of transformant in the hemolymph of infected *P. xylostella* larvae (E) and conidia of transformant isolated from cuticle f infected *P. xylostella* larvae (F) were observed in fluorescence microscope. The hyphae and spore of transformant grown on PDA plates were observed in optical microscope (G) and fluorescence microscope (H).

The virulence of *GFP*-expressed transformants was determined in bioassays using *P. xylostella* larvae. All the transformants were pathogenic and produced symptoms indistinguishable from those of the wild type. There were no significant differences in total mortality for the 2nd instar larvae (range from 80% to 94%) and time to death (range 3.5-4.8 days) for *I. fumosorosea* using a concentration of 1×10^6 conidia/ml. The mortality for the 2nd instar larvae treated by wild type and transformant #14 of *I. fumosorosea*, were 46%, 57%, 78%, 88%, 95% and 48%, 58%, 76%, 86%, 95%, respectively, for 1×10^3, 1×10^4, 1×10^5, 1×10^6 and 1×10^7 conidia/ml after 6 days of treatment, the mortality of the control were 8% and 7%. Based on the above initial mortality data, the concentration-mortality response regression analysis for *I. fumosorosea* was calculated by assaying five

Table II.- Regression analysis of probit mortality and log-concentration data of bioassay with *I. fumosorosea* against the 2nd instar *P. xylostella* larvae.

Fungal strain	Days	Slope	χ^2	LC_{50} value (95% fiducial limit)	LC_{95} value
Wild type	6	0.47	0.396	3.92×10^3 ($1.61\sim9.54\times10^3$)	1.26×10^7
Transformant #14	6	0.45	0.394	3.86×10^3($1.53\sim9.73\times10^3$)	1.81×10^7

concentrations (1×10^3, 1×10^4, 1×10^5, 1×10^6 and 1×10^7 conidia/ml) against newly molted the 2nd instar *P. xylostella* larvae, which were not significantly different from each other (χ^2, $\chi^2_{0.05}$). The LC_{50} values were 3.92×10^3 and 3.86×10^3 conidia/ml for *I. fumosorosea* against *P. xylostella* larvae (Table II). These data show no significant difference in virulence between the *GFP* expressing strains and its wild type parent.

DISCUSSION

In these studies, Southern blot analysis confirmed that both the benomyl resistance marker and the *GFP* gene integrated into *I. fumosorosea* genome, with at least 50% of the transformants analyzed appearing to have a single integrated copy of the gene as seen by single bands of differing size in our analyses. In previously reported transformation methods such as that described for *Peacilomyces fumosoroseus*, multiple integration sites of tandem arrays of the vector (Barreto *et al*., 1997) or homologous gene replacement (Inglis *et al*., 1999) were commonly seen recombination events in the transformants potentially limiting the application of the described methods. In our results, by applying ATMT to generate random insertional mutants, single-copy T-DNA integration in *P. fumosoroseus* was rather high, and the frequency of transformants with single-site integration could be optimized by adjusting the *A. tumefaciens* transforamation system with respect to culturing the *Agrobacterium* cells in the absence of AS prior to co-cultivation as reported for some species (Michielse *et al*., 2005). The ability of the fungus to express the benomyl resistant gene and *GFP* through sporulation suggests that the pK-Ben-GFP vector can be stably integrated in the genomes of the transformants. Two integrated copies of the *GFP* gene were observed in tranformant #20 during these studies. The variation in *GFP* among transformants could be explained by differences in sequence context due to integration of the plasmid at different positions in the chromosome (Spelling *et al*., 1996; Van den Wymelengerge *et al*., 1997). Perhaps sequences located near the integrated plasmid enhanced the activity as observed with the β-glucuronidase gene in *Fusarium oxysporum* (Couteaudier *et al*., 1993).

Strain improvement through selection or genetic manipulation requires knowledge of key factors, including transmission, infectivity, and persistence of the propagule (Ortiz-Urquiza *et al.*,, 2015). Our results show that expression of the benomyl resistant gene and the *GFP* gene in *Isaria* transformants did not affect pathogenicity of *I. fumosorosea*, as the strains tested showed symptoms indistinguishable from those caused by the wild type, including time to death and extent of sporulation. This is critical if the method is to be applied for molecular and genetic studies of the system (Ying *et al*., 2013; Fan *et al*., 2011). Therefore, the pk-Ben-GFP vector and the resulting transformants can be developed for a number of applications, including examination of the pathogenic interactions and analysis of the expression of specific genes. The application of *GFP* as a marker in the microbial biological control population can be extremely useful in collecting information on the process by which fungi invade their hosts. Because *GFP* cDNA under the control of *trpC* promoter is expressed in *I. fumosorosea*, the green fluorescence protein can be easily detected by fluorescence microscopy in hyphae, conidia or blastospores on artificial culture medium and insect hosts (Lima *et al*., 2006). In our studies, fluorescence was apparent in hyphae and conidia of transformants grown on PDA plates and spores isolated from the hemolymph of infected insect larvae (Fig. 5). Fungal structures were examined in real time and there was no need for intrusive or disruptive preparation procedures.

The efficacy of entomopathogenic fungi in the field is often influenced by several factors, such as the use of fungicides (Todorva *et al*., 1998). Our results showed that transformant containing the benomyl resistant gene can survive at high benomyl concentration (5 μg/mL), and expression of benomyl resistant gene in the transformants did not affect pathogenicity of *I. fumosorosea*. Resistance to benomyl is a selection marker that has also been used to transform *Metarhizium anisopliae* (Bogo *et al*., 1996).

In conclusion, our results showed that GFP-tagged strains of *I. fumosorosea* can be used to study the developmental fate of the fungus within its insect hosts and analysis of the expression of specific genes. At same time, genetic transformation of entomopathogenic fungus with benomyl resistant gene will allow for *I. fumosorosea* to be used in combination with some fungicides to protect crop from insects as well as plant disease.

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