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Transformation of *Metarhizium anisopliae* by *Agrobacterium* Mediated Gene Transfer and Electroporation

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ABSTRACT. *Synthetic chemical pesticides have dominated the pesticide market for well over 50 years. Because of the hazardous nature of these pesticides, there is a trend towards the use of biologically based formulations. Metarhizium anisopliae provides an ideal platform for use as potent and eco-friendly mycopesticide. However, in order to overcome several weaknesses it possesses, M. anisopliae should be made amenable to genetic transformation be it to study the genes involved via insertional inactivation or to make the necessary molecular improvements through transgenes.*

The objective of the study was to establish proper transformation machinery for M. anisopliae. Both Agrobacterium tumefaciens mediated transformation (ATMT) and electroporation done in order to achieve this objective. ATMT was accomplished using strain LBA4404 harboring the binary T-DNA vector pLG12Hm. According to the histochemical GUS assay seven transformants per 2.68×10^6/mL conidia and 60% transformants with mycelial palettes were observed. Electroporation was done using an undeleted lac Z pUC 19 vector to a 3.2915×10^6 spores/mL spore suspension. X-Gal positive mycelia were confirmed by Southern hybridization with a Dig labeled linearized pUC 19 vector probe. According to the Southern blot, sample B1 gave three positive signals, one parallel to the pUC 19 vector and two other bands by homologous recombination with the M. anisopliae chromosome.

INTRODUCTION

Synthetic chemical pesticides have dominated the pesticide market for well over fifty years. However, with an increasing awareness about the hazardous nature of these pesticides, now there is a trend towards the dependence on biologically based formulations. *Metarhizium anisopliae*, the second most widely used and well studied entomopathogenic fungus (Roberts and Yendo, 1971) is regarded as one of the most promising species known in the development of practical insect biological controlling agents (BCA). As a successful

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biological controlling agent, it has a broad host range and it is cheap and easy to mass propagate.

The development of an efficient method for genetic transformation is a prerequisite for the application of molecular biology for the improvement of a biological control agent. Previous transformation methods for *M. anisopliae* have been developed using protoplast-Polyethylene Glycol (PEG) (Bernier *et al*., 1989) and biolistic methods (Inglis *et al.*, 2000). However, these procedures are time consuming and their transformation efficiencies are usually low. As many authors suggest, it is difficult to achieve efficient gene transfer using most of the popular transformation systems for entomogenous fungi (Reis *et al.,* 2004).

Recombinant *Agrobacterium* strains, in which their natural genetic make up has been changed with genes of interests (inserts) are considered as the most promising as well as efficient methods to transfer foreign genes into plant and microbial cells. Due to numerous research efforts over the past few decades *Agrobacterium tumefaciens* mediated transformation (ATMT) method has been well studied and optimized for several plant and microbial species.

The ATMT approach has been shown to be an important alternative to other fungal transformation methods as it facilitates high number of transformants, does not require special equipment, is easier to perform, and provides high percentage of homologous recombination (Michielse *et al.*, 2005; Brakhage and Langfelder, 2002). Electroporation is another transformation method that could be versatile in molecular improvements because it allows the transfer of an intact plasmid. In this study, we describe successful procedures for the genetic transformation of conidia and mycelial pellets of *M. anisopliae via* electroporation and ATMT.

MATERIALS AND METHODS

Bacterial and fungal strains

Fungal strain *M. anisopliae* (sample ARSEF 1727), was obtained from United States Department of Agriculture collection of fungal cultures (USDA-ARSEF). Sample ARSEF 1727 was originally collected from Paddy Breeding Station, Coimbatore, Tamil Nadu, India. The original culture was sub cultured on either glucose yeast extract basal salts (GYBS) agar medium or on potato dextrose broth (PDB).

A. tumefaciens strain LBA4404 harboring vector pLG121Hm maintained on yeast extract broth (YEB) was used for transformation of *M. anisopliae*.

Agrobacterium tumefaciens mediated transformation

Agrobacterium tumefaciens strain LBA 4404 was grown overnight at 28^{o}C with continuous shaking in 25 mL YEB.

Mycelial palettes (collected from PD broth) and conidia (2.68×10^{6}/ mL) were added separately to hundred micro liters of *A. tumefaciens* (10^{8} CFU/mL) and transferred to an induction medium supplemented with 150 mM acetocyringone, 5 mM glucose and 5 mM

arabinose. Induced conidia and mycelia were co-cultivated at 29^{o}C for 48 hrs. Co-cultivated conidia and mycelia were then plated on water agar supplemented with 500 µg/mL cefotaxime and 200 µg/mL GUS (5-Bromo-4-chloro-3-indolyl-B-D-Glucuronic acid Cyclohexylammonium salt).

Electroporation mediated transformation

Electroporation mediated transformation was carried out as described by Chakraborty and Kapoor (1990). *M. anisopliae* spore suspension of 3.2915×10^{6}/mL was used for the electroporation. Four washing steps were carried out with ice-cold de-ionized water (followed by a 3000 g centrifugation for 2 min each time). One hundred micro liter (3.2915×10^{6} spores/mL) samples were mixed with 1 µL (0.25 µg) of lacZ pUC19 vector and subjected to electroporation using the Bio-Rad Gene Pulser apparatus at 1.8 kV, 200 Ω resistance and 25 µF capacitance.

After electroporation, conidia (50 µL) were plated on Sabouraud's dextrose agar (SDA) supplemented with 4 µL (200 mg/mL) IPTG (Isopropyl-ß-D-thiogalactopyranoside) and 80 µL (10 mg/mL) XGal (5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside).

Isolation of DNA and Southern hybridization

Lac Z positive *M. anisopliae* colonies were cultured on SDA (broth) with continuous shaking for 2 weeks. Then mycelial pellets were collected by filtration through cheesecloth, frozen in liquid nitrogen, and dried mycelia were ground with a mortar and pestle and resuspended in 1 mL of 100 mM/L LiCl, 50 mM EDTA, 10 mM Tris pH 8.0 and 4% Sodium Dodecyl Sulfate (SDS).

After heating the suspension to 55^{o}C in a water bath, the mixture was extracted first with 1 M Tris (pH 8.0) saturated phenol, followed by phenol:chloroform (1:1, v:v) and then RNA was precipitated from the aqueous phase by the addition of an equal volume of ice cold 6 M/L LiCl and the mixture was incubated at -20^{o}C for two to three hours. After centrifugation at 12000 g for 20 min at 4^{o}C, the supernatant was transferred to two volumes of ethanol and cooled to -20^{o}C for 15 min to precipitate the DNA. After centrifugation as described above, the DNA pellet was washed with 70% ethanol, dried and resuspended in TE Buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). Twenty microliters of DNA from each sample and 15 µL of pUC19 vector, digested in *HindIII* (37^{o}C, 1 hr) were subjected to gel electrophoresis with 0.8% agarose denaturing gel and blotted to nylon membrane by vacuum transfer technique.

One microliter of freshly denatured DNA (heated in a boiling water bath for 5 min and chilled quickly on ice) was used for Dig labeling. The DNA was cross linked on membrane by baking at 80^{o}C for 2 hrs. The membrane was pre-hybridized in a sealed plastic box using 20 mL of Dig easy hyb buffer.

The nylon membrane was hybridized to probe with the Dig labeled linearized pUC19 probe. The blotting and hybridizations were carried out according to the instruction manual (Roche Applied Science).

Assessment of growth rate of *M. anisopliae*

Diameters of transformed and non-transformed mycelial cultures were measured using metric ruler up to 12 days after germination.

RESULTS AND DISCUSSION

Agrobacterium mediated Transformation

GUS analysis

Blue color colonies were observed in successfully transformed *M. anisopliae* cultures using ATMT. Blue color could easily be observed when growing in water agar plates. When the medium is supplemented with sugars/nutrients the GUS expression was delayed. Seven mycelial culture plates from transformed spores were GUS positive (7/16 = 44%), but more than 60% culture plates from transformed mycelia showed the blue color development (10/16 = 62.5%). This could be due to the filamentous structure of hyphae which promotes better binding of *Agrobacterium* (Brian *et al*., 2002).

Electroporation Mediated Transformation

Screening with X-gal

Blue color colonies were observed in successfully transformed *M.anisopliae* cultures.

Southern Hybridization

The X-gal selected blue color single spore isolates of *M. anisopliae* were subjected to whole genome DNA extractions and digested using *Hind III* enzyme. According to the results of the Southern blot (Plate 1) lanes P, B1 and B2 gave positive signals with the Dig labeled linearized pUC19 vector probe. Sample P (pUC19) gave one positive signal. Sample B1 gave three positive signals of which one was parallel to the pUC 19 vector (band B), and another band was observed in the upper part of the membrane (band A), which can be a homologous recombination with *M. anisopliae* chromosome (single cross over). The third band (band C) found in the lower part of the membrane, could be the result of an integration of smaller fragment of vector DNA into the host chromosome (double cross over).

In recombinant *M. anisopliae* culture sample B3, unlike in the other 2 samples (B1and B2), there was no positive signal. Since the initial transformants were selected on *lac Z* basis the blue color development could be due to transient transformation with an unstable degenerative plasmid.

Assessment of growth rate of *M. anisopliae*

As shown in Figure 1, there was a low growth rate in the first 14 days in transformed cultures than in the normal untransformed cultures. However, growth rates appeared to be similar after 2 weeks. This may be due to the stress created by the transformation process.

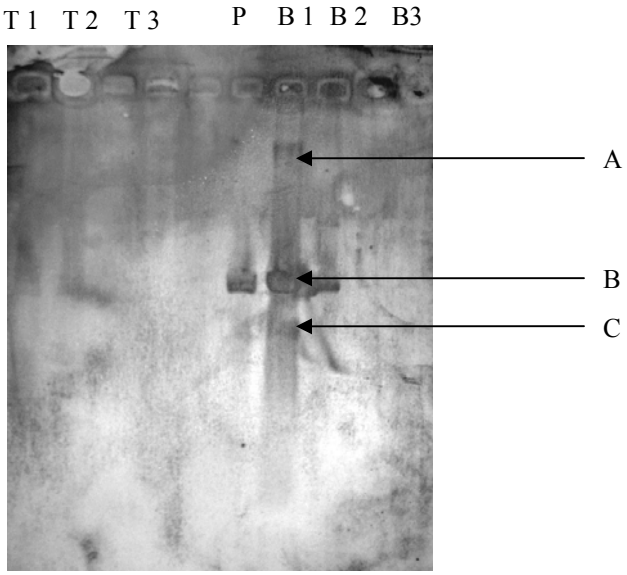

Plate 1. Southern blot of electroporated samples and normal *M. anisopliae* cultures.

Note: Wild-type *M. anisopliae* culture samples (lanes T1, T2 and T3) as negative controls. pBluescript vector sample as a positive control (lane P), Transformant in which homologous recombination has occurred (lane B1), Transformant with no homologous recombination (lane B2) and Transformant with transient transformation of plasmid (lane B3).

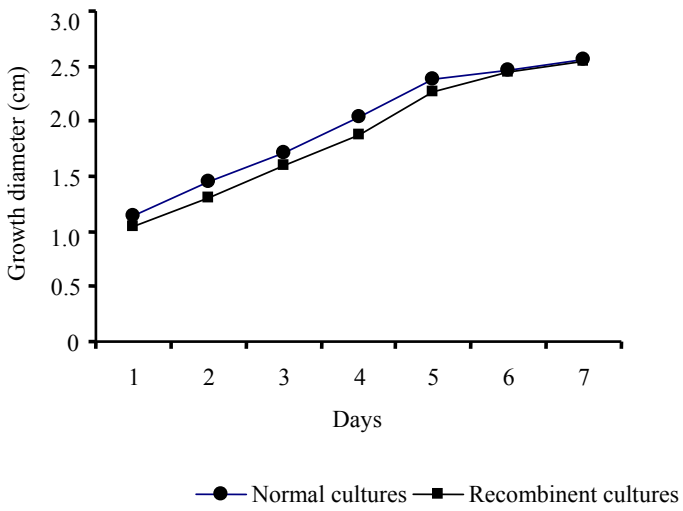

Figure 1. Growth rates of *M. anisopliae* cultures.

CONCLUSIONS

The success of the *Agrobacterium* mediated transformation and electroporation of *M. anisopliae* is a confirmation of efficiency of these methods in the transformation of filamentous fungi. Employment of the vector pLG121Hm produced up to 7 transformants per 2.68×10^6/mL conidia and 60% transformants with mycelial palettes.

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