Asian Journal of Plant Pathology

ISSN 1819-1541

Academic Journals Inc.

www.academicjournals.com

Asian Journal of Plant Pathology 9 (2): 33-45, 2015
ISSN 1819-1541 / DOI: 10.3923/ajppaj.2015.33.45

Construction of a Large Mutational Library from a Defoliating *Verticillium dahliae* Strain and its Evaluation

[1,2]Ahmed A. El-Sharawy, [1]Dongfang Hu, [1]Xiaoping Hu and [1]Jiarong Yang
[1]State Key Laboratory of Crop Stress Biology for Arid Areas, College of Plant Protection, Northwest A and F University, Yangling, 712100, China
[2]Department of Plant Production, Faculty of Environmental and Agricultural Sciences, Suez Canal University, Egypt

Corresponding Author: Jiarong Yang, State Key Laboratory of Crop Stress Biology for Arid Areas, College of Plant Protection, Northwest A and F University, Yangling, 712100, China

ABSTRACT

Agrobacterium tumefaciens Mediated Transformation (ATMT) was used to establish an insertional mutant library. Firstly, we have developed and optimized ATMT system using defoliation strain of *V. dahlia*, which is the causal agents of wilt on cotton. Furthermore, a library consisted of 4890 T-DNA insertion mutants were generated. Compared with wild-type strain, 34.7% of mutants significantly reduced their colony growth rates among the 196 mutants that were randomly selected, while 34.8% of mutants increased their colony growth rate, 16.8% of mutants lost their ability to produce microsclerotia, 30.0% of mutants decreased their sporulation ability and 27.1% of mutants reduced their pathogenicity. Especially, mutant VdAG30, VdAG153, VdAG151 and VdAG95 showed significant reduce in pathogenicity. In this study, the constructed ATMT mutant library was found to be effective and suitable for defoliating *V. dahliae* strain and could lay a foundation for cloning candidate pathogenicity-related genes and development-related genes in *V. dahliae* in the future.

Key words: *Verticillium dahliae*, ATMT, mutant, pathogenicity

INTRODUCTION

Cotton (*Gossypium* sp.) is an important economic crop and it is grown in more than 80 countries, especially upland cotton (*G. hirsutum* L.), which produces more than 95% of the annual cotton crop world wide (Chen *et al*., 2007) and provides the natural fiber for textile industry (Thibodeaux and Evans, 1986; Wakelyn *et al*., 2010). China is the world largest cotton producer and it is estimated that 32.0 million bales of cotton would be produced in 2013/14 (Johnson *et al*., 2014), however, more than 2 million ha of cotton crop was under the attack of *Verticillium* wilt that caused about 10-15% yield loss due to such a disease (Johnson *et al*., 2014). Cotton *Verticillium* wilt is one of the most important diseases and difficult to control (Fradin and Thomma, 2006). Schnathorst and Mathre (1966) first showed that different levels of pathogenicity of *V. dahliae* isolates from cotton existed in California and classified them as defoliating and non-defoliating pathotypes according to their ability to completely defoliate cotton leaves or to cause only wilt symptoms and no defoliation on cotton cultivars Tanguis (*G. barbadense*), Acala 4-42 and Delta Pine 15 (DPL) (*G. hirsutum*), respectively. Since then, the defoliating strains of *V. dahliae* of cotton were reported in Spain (Perez-Artes *et al*., 2000; Mercado-Blanco *et al*., 2003), Turkey (Dervis *et al*., 2010), Italy (Colella *et al*., 2008), Tunisia (Bellahcene *et al*., 2005) and Egypt

(El Said *et al*., 2012). In China, defoliating type of *V. dahliae* from cotton was first reported in 1983 in Jiangsu Province (Lu *et al*., 1983) and it had increased up to 83.8% by 2008 (Lin *et al*., 2012). A similar situation was reported in Xinjiang Autonomous Region, the largest cotton growing region in China, where the defoliating type proportion in Xinjiang was reported to be 39.4 and 33.3% in 2011 and 2012, respectively (Han *et al*., 2011, 2012).

Therefore, understanding the molecular mechanism between the defoliating type of *V. dahliae* and cotton interaction has the fundamental importance to design novel control strategies for *Verticillium* wilt. An effective way to identify pathogenicity or virulence genes is adopted by random mutagenesis, following to screen virulence-reduced mutants on a particular host plant (Jeon *et al*., 2007). Transferring DNA (T-DNA)-based random insertional mutagenesis provide a unique opportunity to generate genetic mutations in a manner that facilitates subsequent isolation of gene identification (Michielse *et al*., 2005; Jeon *et al*., 2007). Using genome the sequences of *V. dahliae* will be useful for easy identification of T-DNA insertion loci based on detailed genetic blueprints of these pathogens and the potential pathogenicity factors.

Agrobacterium tumefaciens Mediated Transformation (ATMT) has been confirmed to be a useful method to obtain insertional mutant libraries and to identify pathogenicity-related genes. Several publications (Fradin and Thomma, 2006; Gao *et al*., 2010; Klosterman *et al*., 2011; Maruthachalam *et al*., 2011; Tzima *et al*., 2011, 2012) had identified some *V. dahliae* pathogenicity and virulence genes. A mitogen-activated protein kinase gene, VMK1, was approved to have a role in pathogenicity (Rauyaree *et al*., 2005). One glutamic acid-rich protein 1 (VdGARP1), a hydrophilic protein, was achieved through T-DNA insertional mutagenesis library of *V. dahliae* strain V592 and identified to play a role in pathogenicity (Gao *et al*., 2010). Maruthachalam *et al*. (2011) identified several genes involved in pathogenicity on *V. dahliae* strain VdLs17 from lettuce through ATMT insertional mutagenesis: VdEg-1, VdHMGS, VdMFS1 and VdGPIM3. Tzima *et al*. (2012) presented that VGB disruption mutant of *V. dahliae* race 1 of tomato severely impaired virulence on tomato and eggplant. Alternative approaches are needed to address the genetic complexity of this fungus in order to identify the genes that cause the pathogenicity.

In this study, we aim to (1) Optimize conditions of ATMT for a large number of transformants carrying a single copy of T-DNA, (2) Establish a mutant library of a defoliating *V. dahliae* strain XJ2008 from cotton, (3) Evaluate the library and (4) Get pathogenicity related mutants.

MATERIALS AND METHODS

Fungal and bacterial strains: A defoliating strain XJ2008 of *V. dahlia* was isolated from a *Verticillium* wilt of cotton plant sampled in 2008 in Xinjiang Autonomous Region, China. Potato Dextrose Agar (PDA) medium was used to grow XJ2008 and its transformants. *Agrobacterium tumefaciens* strains AGL-1 and EHA105 were used in this study. The pCT-Hyg vector carrying hygromycin B phosphotransferase gene (Hyg) under control of *Aspergillus nidulans* trpC promoter was used throughout this study (Liu *et al*., 2013).

Sensitivity of *V. dahliae* and *A. tumefaciens* to hygromycin B: The wild type strain XJ2008 of *V. dahliae* was inoculated onto PDA plates containing hygromycin B 25, 50, 75, 100 and 150 g mL^{-1} and without containing hygromycin B as the control and then plates were cultivated at 25°C for 7 days. The sensitivity of *V. dahliae* to hygromycin B was evaluated by mycelium growth. *A. tumefaciens* strain AGL-1 and EHA105 were grown on YEP (Yeast Extract and Peptone) plates with different concentrations of cefotaxime sodium as 100, 200 and 300 g mL^{-1}. The experiment was repeated three times and each replicate had three plates.

***Agrobacterium tumefaciens* mediated transformation:** The ATMT referred to the method described by Maruthachalam *et al*. (2008) was followed with some modifications. Two different types of *A. tumefaciens* strains i.e., AGL-1 and EHA105 harboring the pCT-Hyg were streaked onto YEP medium plate (10 g L^{-1} bacto peptone, 10 g L^{-1} yeast extract, 0.5 g L^{-1} NaCl and 1.5 g L^{-1} agar pH 7.0) containing 50 g mL^{-1} kanamycin (Kan) and 50 g mL^{-1} rifampicin (Wang *et al*., 2013) and cultivated at 28°C for 2 days. The single colony was transferred into a 10 mL test tube with 5 mL of Minimal Media (MM) (Adachi *et al*., 2009) amended with 5 g mL^{-1} thiamine and 5 g mL^{-1} of kan and cultivated at 28°C with 220 rpm for 48 h in an orbital shaker. The culture was collected through centrifugation at 12000 rpm for 1 min, then the supernatant was discarded and the pellet was re-suspended in 5 mL IM medium (Bundock *et al*., 1995) and it re-suspended in fresh IM medium containing acetosyringone (AS) (200 mol L^{-1}) at OD_{600} = 0.15, after cultivated for 6-14 h at 28°C with 220 rpm to an OD_{600} of 0.2, 0.4, 0.6, 0.8, 1.0 and 1.2. The conidial suspension of *V. dahliae* contained 1×10^6 conidia per milliliter and cells of *A. tumefaciens* were mixed (1:1, v/v) in a 1.5 mL eppendorf tube and kept at room temperature for 5 min. Then 200 μL of this mixture was spread on the co-cultivation plates and cultured following five different co-cultivation times as 12, 24, 36, 48 and 60 h on a cellophane in three different temperature treatments as 23, 25°C and 28°C. The cellophane was transferred onto a new PDA plates augmented with 200 g mL^{-1} of cefotaxime and 50 g mL^{-1} of hygromycin B using sterile forceps and cultivated for 5-7 days. Typically, the colonies that could grow on the new PDA plates containing 50 g mL^{-1} hygromycin B were assumed as putative mutants. And the positive transformants were then stored in 25% (v/v) sterilized glycerol at -80°C until use.

Optimization of ATMT conditions for *V. dahliae*: We optimized the conditions of ATMT using two different types of *A. tumefaciens* strains AGL-1 and EHA105, the association parameters were systematically investigated and optimized, including the concentration of *Agrobacterium* and AS, co-cultivation duration and co-cultivation temperature.

DNA manipulation: Genomic DNA from powdered mycelium of individual transformants and wild-type strain XJ2008 were extracted based on a procedure established by Moller *et al.* (1992). For Southern blot analysis, genomic DNA was digested with *EcoRI*, which cuts once in the T-DNA. Electrophoresis and blotting followed standard protocols (Sambrook and Russell, 2001). PCR was conducted with specific primer pair *Hyg*-F (5'-CCT GAA CTC ACC GCG ACG TC-3') and *Hyg*-R (5'-CTA TCC TTT GCC CTC GGA CGA GTG-3') using a volume of 25 L. The PCR program was as follows: 5 min at 95°C; 35 cycles of 1 min at 95°C, 1 min at 60°C and 1 min at 72°C and 10 min at 72°C for the final extension. The PCR production was separated by electrophoresis on a 1% agarose gel, stained with Ethidium Bromide (EtBr) and photographed.

Mitotic stability of transformants: To evaluate the stability of inserted T-DNA in transformants, hygromycin B resistant colonies on selection plates were randomly selected and cultured on PDA without hygromycin B at 25°C for 7 days. New mycelia from the colony edge were punched and inoculated onto a fresh PDA plate without hygromycin B following five successive generations. Subsequently, the mycelium from each transformant was inoculated for growth onto the fresh PDA amended with 50 g mL^{-1} hygromycin B to determine its mitotic stability (Dos Reis *et al*., 2004; Michielse *et al*., 2005; Maruthachalam *et al*., 2011).

Assays for biological characteristics of transformants: The wild-type strain XJ2008 and transformants were inoculated onto PDA plates and cultivated in dark at 25°C for 7 days. The growth rates of transformants were calculated based on colony size measured once a week over a 2 week period. The sporulation ability of the wild-type strain XJ2008 and transformants were preceded by adding 200 μL of the conidial suspension of 1×10^6 spore mL^{-1} to 50 mL of liquid Czapek Dox medium, incubating the cultures at 25°C, 150 rpm for 7 days. A hemocytometer was employed to count the amount of the sporulation ability. Furthermore, the ability of microsclerotia formation was estimated through spreading 150 μL of the conidial suspension of 10^6 spore mL^{-1} on the Basal Modified Medium (BMM) covered with a sterilized permeable cellophane disc (HC273 film, Beijing Dongguo Co., China) as describe by Hu *et al.* (2013). The experiment was repeated three times and each replicate had three plates.

Pathogenicity test: Four-week-old cotton seedlings of cultivar Jimian11 (susceptible to *Verticillium* wilt) were used to evaluate the pathogenicity of *V. dahliae* strain XJ2008 and its mutants by rootdip-inoculation method with conidia suspension of 1×10^6 spore mL^{-1} as described by Hu *et al.* (2013). The wild-type strain XJ2008 and sterile distilled water served as positive and negative controls, respectively. Plants were then incubated in the green house under the control of temperature at 25°C. The symptoms of disease scoring were as follows: for, the severity of the inoculated plants was recorded at 3 weeks post-inoculation (wpi) following five categories: 0 = healthy plants, no symptoms on leaves, 1 = One or two cotyledonous leaves showing symptoms; 2 = A single true leaf showing symptoms, 3 = More than two leaves showing symptoms and 4 = dead plant. The Disease Index (DI) was calculated using the following formulate:

$$DI = \frac{\sum N_i \times i}{4\sum N_i}$$

where, N_i is number of plants with a disease score of i

Data analysis: All data was analyzed using SPSS software (Version 16.0, SPSS Inc.). A one-way analysis of variance (ANOVA) and Student-Newman-Keuls (S-N-K) tests were employed to evaluate the statistical significance ($p<0.05$) of the results.

RESULTS

Optimized condition for ATMT: Experimental results showed that the growth of *V. dahliae* strain XJ2008 hyphae was completely inhibited at 50 g mL^{-1} hygromycin B with 200 g mL^{-1} cefotaxime sodium. Also, we found out that the transformation efficiency increased with rising concentrations of AS, which had obvious effects on transformation efficiency. The presence of AS (200 mol L^{-1}) was the optimal concentration for *V. dahliae* transformation and yielded 89.75±5.40 transformants that identified per 10^6 conidia. However, fewer transformants were observed when the concentration of AS was increased (Fig. 1a). *Agrobacterium tumefaciens* concentration was increased from OD_{600} = 0.6-0.8 and obtained 69.25±1.65 and 80.00±3.13, respectively (Fig. 1b). In this study, optimal transformation frequency was attained when the mixture was co-cultivated for 36 h and obtained 82.00±1.47 transformants per 10^6 spore, while extended the incubation time could reduce transformation efficiency (Fig. 1c). The results indicated that the optimal temperature for achieving the highest transient transformation was 25°C with 82.75±3.94 (Fig. 1d). Strain AGL-1 yielded much more transformants than EHA105.

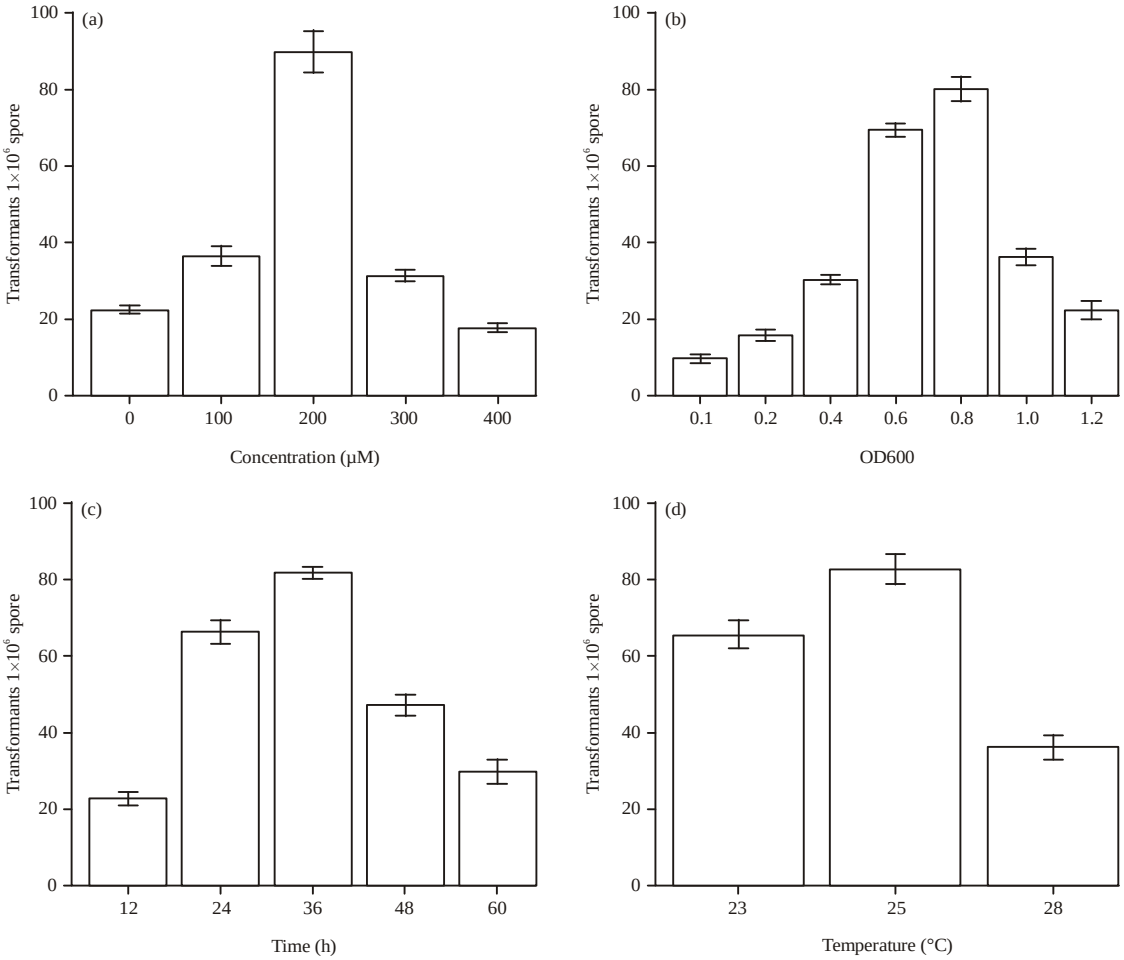

Fig. 1(a-d): Optimization of ATMT for wild-type strain XJ2008 of *Verticillium dahliae*, (a) Efficiency of acetosyringone (AS) on transformation was tested by adding to the IM plates with AS concentrations of 0, 100, 200, 300 and 400 µM, (b) *Agrobacterium tumefaciens* concentration. Two hundred microlitre of *A. tumefaciens* cell suspension (OD_{600} = 0.4, 0.6, 0.8, 1.0 and 1.2) was mixed with 200 of fresh spore suspension (10^6 spore mL^{-1}) and then incubated on an IM agar plate containing 200 mol L^{-1} of AS at 25°C for 36 h. (c) Co-Culture time. Two hundred microlitre of *A. tumefaciens* cell suspension (OD_{600} = 0.8) was mixed with 200 of fresh spore suspension (10^6 spore mL^{-1}) and then incubated on an IM agar plate containing 200 mol L^{-1} of AS at 25°C for 12, 24, 36 and 48 h (d) Co-Culture temperature. Two hundred microlitre of *A. tumefaciens* cell suspension (OD_{600} = 0.8) was mixed with 200 of fresh spore suspension (10^6 spore mL^{-1}) and then incubated on an IM agar plate containing 200 mol L^{-1} of AS at 23, 25 and 28°C for 36 h. Each value indicates the mean and standard error

Mutant library construction of defoliating strain XJ2008 and its evaluation: We got 4890 mutants from wild-type strain XJ2008 of *V. dahliae* using ATMT method. The transformants were identified using Hyg B resistance and Hyg-specific PCR analysis. A single expected band of about 1 kb was amplified from all of them using primers Hyg-F and Hyg-R with pCT-Hyg as positive control and the DNA from wild-type strain XJ2008 was used as the negative control (Fig. 2) and Southern blot analysis was performed from eleven PCR positive transformants with

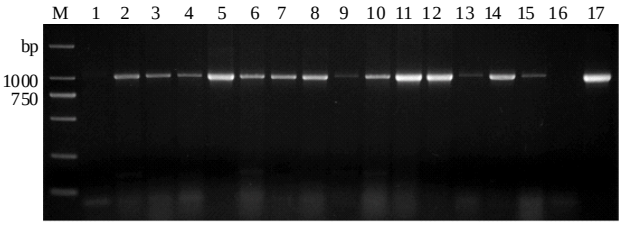

Fig. 2: Transformant identification of *Verticillium dahliae* strain XJ2008 using *hph* gene specific primers Hyg-F and Hyg-R. Lane M: DNA ladder (D2000, Beijing, Tiangen Biotech Co.,China), Lanes 1-15: Genomic DNA isolated from putative transformantsVdAG2, VdAG6, VdAG7, VdAG9, VdAG11, VdAG16, VdAG18, VdAG20, VdAG23, VdAG25, VdAG26, VdAG27, VdAG28, VdAG30, VdAG33 and VdAG36, respectively, Lane 16: Genomic DNA of wild-type XJ2008, Lane 17: Vector pCT-Hyg as positive control

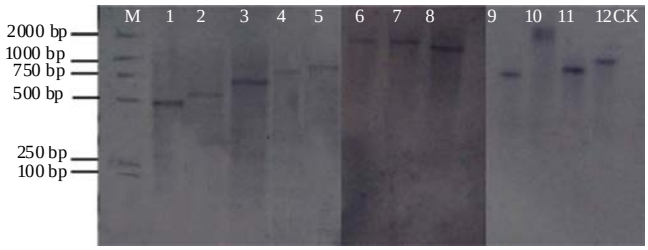

Fig. 3: Southern blot analysis of T-DNA integration from eleven PCR positive transformants and a wild-type strain was digested with *EcoRI*. M DNA molecular weight markers with bases indicated on the left. Lanes 1-11: Transformants of strain XJ2008, Lane 12: Linearized pCT-Hyg as positive control and CK genomic DNA of wild-type XJ2008

wild-type strain DNA as negative control and linearized pCT-Hyg as positive control. The hybridization yielded only a single-copy insertion and the insertion sites are different for these 11 transformants. Moreover, the presence of different sized bands in the Southern blot revealed random integration of the T-DNA of the majority of the transformants. No hybridizing band was detected in the lane loaded with DNA from the untransformed wild-type strain (Fig. 3).

Mitotic stability of transformants is important feature of an effective mutagenesis system. After five subcultures in the absence of hygromycin B, *V. dahliae* transformants could still grow on PDA containing 50 μg mL^{-1} hygromycin B, suggesting that the *hph* gene was stably maintained in all the transformants.

The growth rate for wild-type strain was 6.75±0.18 mm per day. The 34.7% of mutants was significantly reduced the growth rate ($p<0.05$) andVdAG151had the lowest growth rate only it had 4.8±0.12 mm per day. Also 34.8% of mutants increased their growth rate, 16.8% lost their ability to produce microsclerotia when culturing on BMM for 30 days and 30% decreased their sporulation ability (Table 1). The 22.9% of mutants showed abnormal morphological characteristics including the deformation and fan-shaped colonies. There were 12.7% of mutants grew significantly colony edges that had more mycelia compared with the wild-type strain, aerial mycelia grew vigorously and the colony surface was swollen. The rest mutants had similar productivity to that of wild type strain XJ2008 that was folded (Table 1).

Table 1: Comparison of some important biological characteristics and pathogenicity between wild-type strain XJ2008 and its mutants

Mutant	Disease index 21dpi	Growth rate (mm d^{-1})	Sporulation (10^6 mL^{-1})	Microsclerotiashape (plate)
XJ2008	56.67	6.75±0.18	2.10±0.05	Present
VdAG9	21.67	6.12±0.49	2.10±0.02	Absent
VdAG20	28.33	5.36±0.58	1.16±0.14	Absent
VdAG26	20.00	8.32±0.24	2.45±0.02	Absent
VdAG45	16.67	5.46±0.09	1.68±0.04	Present
VdAG61	18.33	8.16±0.13	1.73±0.07	Present
VdAG30	8.33	7.61±0.63	0.85±0.10	Absent
VdAG31	16.60	7.61±0.63	1.61±0.03	Absent
VdAG95	11.67	5.66±0.16	1.48±0.08	Present
VdAG94	25.00	5.84±0.03	2.08±0.06	Absent
VdAG150	23.33	5.86±0.02	2.10±0.07	Present
VdAG153	10.00	7.72±0.45	1.33±0.07	Present
VdAG161	15.00	5.76±0.16	1.65±0.10	Present
VdAG36	31.67	6.45±0.08	1.68±0.09	Absent
VdAG73	23.33	8.91±0.42	1.28±0.08	Present
VdAG27	13.33	8.03±0.23	1.63±0.08	Absent
VdEH266	80.00	7.65±0.11	3.05±0.07	Present
VdEH227	75.00	5.73±0.09	2.83±0.41	Present
VdEH229	73.33	6.02±0.41	2.68±0.04	Present
VdAG165	45.00	6.03±0.28	2.00±0.07	Present
VdAG151	6.67	4.88±0.12	0.90±0.07	Present
VdAG18	30.00	5.47±0.37	2.00±0.07	Absent
VdAG57	38.33	6.15±0.02	2.06±0.07	Absent
VdEH252	65.67	5.89±0.04	2.65±0.32	Present
VdAG107	26.67	5.58±0.34	1.16±0.10	Absent
VdAG33	46.67	6.08±0.04	2.38±0.04	Absent

dpi: Days post inoculation. The values in the table above were the average of three replicates for wild-type strain XJ2008 and its transformants, respectively and the value following by "±" was standard error. Values were significantly different ($p<0.05$) in S.N.K test of one-way ANOVA.

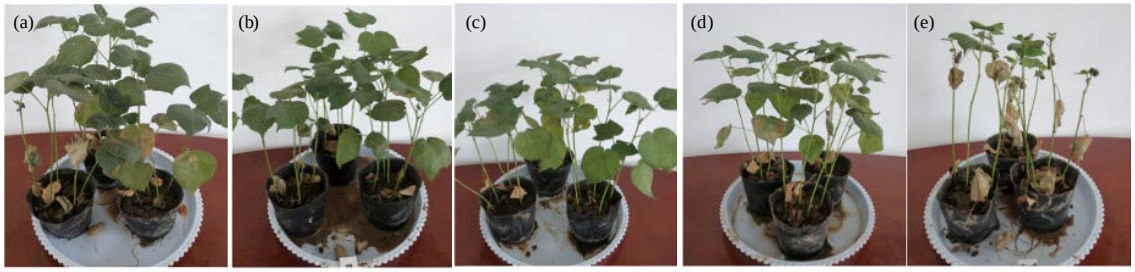

Fig. 4(a-e): Pathogenicity test of mutants using Jimian 11. Fourteen-day-old cotton seedlings were inoculated with conidia of wild-type or random T-DNA insertion mutants of *Verticillium dahliae*. The disease was scored at 21 days after inoculation based on a five categories: (a) Healthy plants, no symptoms on leaves, (b) One or two cotyledonous leaves showing symptoms, (c) A single true leaf showing symptoms, (d) More than two leaves showing symptoms and (e) Plant dead. The pathogenicity of mutant VdAG30, VdAG153, VdAG151 and VdAG95 was reduced relative to WT

The pathogenicity test results showed that about 27.1% of mutants reduced their pathogenicity without development of symptoms, including VdAG30, VdAG151, VdAG153 and VdAG95 (Table 1 and Fig. 4). The other 57.0% of mutants caused similar symptoms to the wild-type and 15.9% of mutants were caused higher pathogenicity (Table 1).

DISCUSSION

Random mutagenesis through ATMT followed by inoculation of the generated mutants on host plants has been widely used to identify pathogenicity or virulence genes in several plant pathogens

(Mullins *et al*., 2001; Jeon *et al*., 2007; Huser *et al*., 2009; Michielse *et al*., 2009; Munch *et al*., 2011; Giesbert *et al*., 2012). In present study, we optimized the conditions of ATMT that affect the transformation efficiency of inserted T-DNA in defoliating Strain of *V. dahliae*. Thereafter, a library consisted of 4890 mutants was constructed.

To carry out the transformation, we checked the optimal concentration of hygromycin B for wild type XJ2008. It was found that 50 μg mL^{-1} hygromycin B was completely inhibited the growth of wild type *V. dahliae*. This result suggests that this concentration can be used in selecting transformants and hygromycin B that is a suitable marker for *V. dahliae*.

The efficiency of transformation was 8-100 transformants per 10^6 spore, which was not very high compared to those of other fungi (De Groot *et al*., 1998; Hirabayashi *et al*., 1989; Mullins *et al*., 2001). The efficiency of fungal ATMT is affected by several factors, including: a) concentration of AS, b) *A. tumefaciens* strain and its growth conditions and (c) Numbers of fungal and bacterial cells were mixed for co-cultivation (Bundock *et al*., 1995; De Groot *et al*., 1998; Islam *et al*., 2012; Mullins *et al*., 2001; Rho *et al*., 2001). Several factors affecting the efficiency of transformation were tested and optimized in this study, such as influence of *Agrobacterium* strains, AS concentration; co-cultivation temperature; recipient *Agrobacterium* concentration and co-cultivation time were determined. This study facilitates strain improvement of defoliating Strain XJ2008 by molecular approaches and is a good reference for genetic study of other fungi.

AS was found to be necessary for *A. tumefaciens* mediated transformation, adjustments of AS concentration during the induction and co-cultivation stages may help to improve the transformation efficiency, which is also validated by our results in which an optimum amount of AS (200 mol L^{-1}) is added. In contrast to previous reports AS in IM was dispensable for the growth of bacterial cells prior to co-cultivation (Michielse *et al*., 2005). AS is typically added to fungal transformations at a concentration of 200 mol L^{-1} (De Groot *et al*., 1998; Covert *et al*., 2001; Dos Reis *et al*., 2004). However, a high concentration of AS hindered the transformation, probably due to the toxic effects of high AS concentration posed on the recipient conidia, for example, inhibiting the germination of the spore.

The ratios of bacterial and fungal mixtures during co-cultivation may have a considerable effect on the transformation efficiency (Maruthachalam *et al*., 2008). In our study, Fig. 1b, the highest transformation frequency was observed with an *A. tumefaciens* density between OD_{600} = 0.6 and 0.8. However, the number of transformants decreased, when the concentration of *A. tumefaciens* reached to OD_{600} = 1.0 and 1.2. The increased concentration of *A. tumefaciens* may result in its overgrowth and lead to limit fungal growth, If the huge growth of *A. tumefaciens* also rises difficulty at the selection stage (Michielse *et al*., 2004; Yamada *et al*., 2009).

Up-till now, optimal temperatures were usually defined among 23, 25 and 28°C. Although, lower and higher values had been tested as well (Michielse *et al*., 2005). Our results demonstrated that higher transformation efficiency could be achieved at 25°C, the different efficiencies among these temperatures suggested that a closely low temperature during co-culture is beneficial for *A. tumefaciens* to transfer its T-DNA to the host (Combier *et al*., 2003; Gardiner *et al*., 2004; Michielse *et al*., 2004). Even though at 28°C hygromycin-resistant transformants also appeared (Fullner and Nester, 1996).

Generally, more transformants appeared by a longer co-cultivation period in most fungi (Yang and Lee, 2008; Zhang *et al*., 2008; Ando *et al*., 2009). In our study, we found that the transformation efficiency was improved by increasing the co-cultivation time up to 36 h. With the

36 h of co-cultivation the transformants were generated around 84 transformants per 1×10^6 spore mL^{-1} and increased time might lead to *Agrobacterium* background growth. The current study suggests that co-cultivations for 36 h appeared as the optimum time to obtain an adequate number of single copy of transformants.

The efficiency of two strains of *A. tumefaciens* namely, AGL-1 and EHA105 were tested for genetic transformation in this study. *Agrobacterium* strain AGL-1 presented the highest transformation rate at 54%, followed by EHA105 with 45%. Using the EHA105 strain, Cruz-Mendivil *et al*. (2011) have reported efficiencies of transformation up to 19%, whereas Sun *et al.* (2006) have reported transformation rates ranging from 4-36% with EHA105 and it is derived from super virulent strain A 281 (Hood *et al*., 1993). Studies which compare the relative transformation efficiencies of *A. tumefaciens* strains have demonstrated that strain AGL-1 is superior to C58, GV3101 and EHA105 in its ability to deliver T-DNA into Switch grass seedlings (Chen *et al*., 2010). Figure 2 showed that the PCR results revealed 1 kb of fragments were present in all five transformants, but not in the wild type strain. These results indicate that hygromycin B resistant colonies were obtained by ATMT system.

Mitotic stability of transformants is important feature of an effective mutagenesis system. After five subcultures in the absence of hygromycin B, *V. dahliae* transformants grew on PDA containing 50 μg mL^{-1} hygromycin B. The results suggested that the *hph* resistances were stably maintained in all the transformants.

Based on the above described results, we obtained 4890 mutants from wild-type strain XJ2008, Two hundred microlitre of fresh spore suspension (10^6 spore/mL) was mixed with an equal volume of *A. tumefaciens* cell suspension (OD_{600} = 0.8) and was spread onto cellophane placed on IM plates amended with 200 mol L^{-1} of AS. After Co-Cultivation at 25°C for 36 h, the cellophane was transferred onto a new PDA plates augmented with 200 g mL^{-1} of cefotaxime and 50 g mL^{-1} of hygromycin B and cultivated for 5-7 days to select transformants. Improved transformation efficiency was compared with that previously reported (Maruthachalam *et al*., 2011).

Screening of 196 mutants was distinct in providing virtually complete coverage of the value of the ATMT methodologies in investigating the molecular mechanisms that involved of defoliating *V. dahliae* and identification of genes directly or indirectly by characterizing the biological properties as well as pathogenicity of transformants.

Moreover, we report a high frequency of isolating pathogenicity/virulence-defective mutants compared with other fungi (Jeon *et al*., 2007; Talhinhas *et al*., 2008), because these mutants that were selected for pathogenicity assay showed interesting phenotypes on PDA medium, such as slow and fluffy growth, different colony color or deformation and fan-shaped colonies, decreased or increased the sporulation ability and lost the ability to produce microsclerotia. Among the identified genes through ATMT insertional mutagenesis, two had an insertion in genes that had previously been characterized and known to be involved in pathogenicity in other fungi and bacteria (De Lorenzo *et al*., 1997; Goswami and Kistler, 2004; Maruthachalam *et al*., 2011). In the determination of virulence and sporulation, the sporulation was reduced; however, it could be effective to spread the *V. dahliae* and would not have any difference in its virulence. This assay is preferred because it is rapidly compared with the conventional greenhouse assay in the soil medium for16 weeks (Klosterman and Hayes, 2006).

In addition, studying the impact on pathogenicity of *V. dahliae* is greatly important. We have developed two basic tools in order to study fungal interaction i.e., i) that is an efficient tool of ATMT

to increase transformation and (ii) a transformation protocol to establish a mutant library in a virulent defoliating Strain XJ2008. These tools will provide a favorable reference for gene in the defoliating strain. Importantly, the *V. dahliae* defoliating strain is potentially useful for development-related genes in *V. dahliae* in the future.

ACKNOWLEDGMENTS

This research was supported by NSFC (31371888) and by 111 Project from Education Ministry of China (B07049).

REFERENCES

Adachi, H., S. Tani, S. Kanamasa, J.I. Sumitani and T. Kawaguchi, 2009. Development of a homologous transformation system for *Aspergillus aculeatus* based on the sC gene encoding ATP-sulfurylase. Biosci. Biotechnol. Biochem., 73: 1197-1199.

Ando, A., Y. Sumida, H. Negoro, D.A. Suroto, J. Ogawa, E. Sakuradani and S. Shimizu, 2009. Establishment of *Agrobacterium tumefaciens*-mediated transformation of an oleaginous fungus, *Mortierella alpina* 1S-4 and its application for eicosapentaenoic acid producer breeding. Applied Environ. Microbiol., 75: 5529-5535.

Bellahcene, M., K. Assigbetse, Z. Fortas, J.P. Geiger, M. Nicole and D. Fernandez, 2005. Genetic diversity of *Verticillium dahliae* isolates from olive trees in Algeria. Phytopathol. Mediterranea, 44: 266-274.

Bundock, P., A. den Dulk-Ras, A. Beijersbergen and P.J.J. Hooykaas, 1995. Trans-kingdom T-DNA transfer from *Agrobacterium tumefaciens* to Saccharomyces cerevisiae. EMBO J., 14: 3206-3214.

Chen, Z.J., B.E. Scheffler, E. Dennis, B.A. Triplett and T. Zhang *et al*., 2007. Toward sequencing cotton (*Gossypium*) genomes. Plant Physiol., 145: 1303-1310.

Chen, X., R. Equi, H. Baxter, K. Berk, J. Han, S. Agarwal and J. Zale, 2010. Research A high-throughput transient gene expression system for switchgrass (*Panicum virgatum* L.) seedlings. Biotechnol. Biofuels, Vol. 7.

Colella, C., C. Miacola, M. Amenduni, M. D'Amico, G. Bubici and M. Cirulli, 2008. Sources of *Verticillium* wilt resistance in wild olive germplasm from the Mediterranean region. Plant Pathol., 57: 533-539.

Combier, J.P., D. Melayah, C. Raffier, G. Gay and R. Marmeisse, 2003. *Agrobacterium tumefaciens*-mediated transformation as a tool for insertional mutagenesis in the symbiotic ectomycorrhizal fungus *Hebeloma cylindrosporum*. FEMS Microbiol. Lett., 220: 141-148.

Covert, S.F., P. Kapoor, M.H. Lee, A. Briley and C.J. Nairn, 2001. *Agrobacterium tumefaciens*-mediated transformation of *Fusarium circinatum*. Mycol. Res., 105: 259-264.

Cruz-Mendivil, A., J. Rivera-Lopez, L.J. German-Baez, M. Lopez-Meyer and S. Hernandez-Verdugo *et al*., 2011. A simple and efficient protocol for plant regeneration and genetic transformation of tomato cv. Micro-Tom from leaf explants. HortScience, 46: 1655-1660.

De Groot, M.J., P. Bundock, P.J. Hooykaas and A.G. Beijersbergen, 1998. *Agrobacterium tumefaciens*-mediated transformation of lamentous fungi. Nat. Biotechnol., 16: 839-842.

De Lorenzo, G., R. Castoria, D. Bellincampi and F. Cervone, 1997. Fungal Invasion Enzymes and their Inhibition. In: The Mycota V. Part B, Plant Relationships, Carroll, G.C. and P. Tudzynski (Eds.). Springer, New York, pp: 61-83.

Dervis, S., J. Mercado-Blanco, L. Erten, A. Valverde-Corredor and E. Perez-Artes, 2010. *Verticillium* wilt of olive in Turkey: A survey on disease importance, pathogen diversity and susceptibility of relevant olive cultivars. Eur. J. Plant Pathol., 127: 287-301.

Dos Reis, M.C., M.H.P. Fungaro, R.T.D. Duarte, L. Furlaneto and M.C. Furlaneto, 2004. *Agrobacterium tumefaciens*-mediated genetic transformation of the entomopathogenic fungus *Beauveria bassiana*. J. Microbiol. Methods, 58: 197-202.

El Said, S.H., A.A. Hegazi and A.M.A. Allatif, 2012. Resistance of some olive cultivars to *Verticillium* Wilt. J. Applied Sci. Res., 8: 2758-2765.

Fradin, E.F. and B.P.H.J. Thomma, 2006. Physiology and molecular aspects of *Verticillium* wilt diseases caused by *V. dahliae* and *V. albo-atrum*. Mol. Plant Pathol., 7: 71-86.

Fullner, K.J. and E.W. Nester, 1996. Temperature affects the T-DNA transfer machinery of *Agrobacterium tumefaciens*. J. Bacteriol., 178: 1498-1504.

Gao, F., B.J. Zhou, G.Y. Li, P.S. Jia and H. Li *et al*., 2010. A glutamic acid-rich protein identified in *Verticillium dahliae* from an insertional mutagenesis affects microsclerotial formation and pathogenicity. PLoS One, Vol. 5. 10.1371/journal.pone.0015319

Gardiner, D.M., A.J. Cozijnsen, L.M. Wilson, M.S.C. Pedras and B.J. Howlett, 2004. The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus *Leptosphaeria maculans*. Mol. Microbiol., 53: 1307-1318.

Giesbert, S., J. Schumacher, V. Kupas, J. Espino and N. Segmuller *et al*., 2012. Identification of pathogenesis-associated genes by T-DNA-mediated insertional mutagenesis in Botrytis cinerea: A type 2A phosphoprotein phosphatase and an SPT3 transcription factor have significant impact on virulence. Mol. Plant-Microbe Interactions, 25: 481-495.

Goswami, R.S. and H.C. Kistler, 2004. Heading for disaster: *Fusarium graminearum* on cereal crops. Mol. Plant Pathol., 5: 515-525.

Han, H., P. Liu, F. Gao, Y. Ren, C. Li and G. Li, 2011. The pathogenicity differentiation and variability of *Verticillium dahliae* from cotton in northern Xinjiang. Acta Phytophylacica Sin., 38: 121-126.

Han, H.W., Y.Z. Ren, P.Y. Liu, L. Zhang, C. Li and G.Y. Li, 2012. Pathogenicity differentiation and variability of *Verticillium dahliae* Kleb. from cotton in southern Xinjiang. Cotton Sci., 24: 147-152.

Hirabayashi, K., S. Iwata, M. Ito, S. Shigeta, T. Narui, T. Mori and S. Shibata, 1989. Inhibitory effect of a lichen polysaccharide sulfate, GE-3-S, on the replication of human immunodeficiency virus (HIV) *in vitro*. Chem. Pharm. Bull., 37: 2410-2412.

Hood, E.E., S.B. Gelvin, L.S. Melchers and A. Hoekema, 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. Transgenic Res., 2: 208-218.

Hu, X., Y. Bai, T. Chen, D. Hu, J. Yang and X. Xu, 2013. An optimized method for *in vitro* production of *Verticillium dahliae* microsclerotia. Eur. J. Plant Pathol., 136: 225-229.

Huser, A., H. Takahara, W. Schmalenbach and R. O'Connell, 2009. Discovery of pathogenicity genes in the crucifer anthracnose fungus *Colletotrichum higginsianum*, using random insertional mutagenesis. Mol. Plant-Microbe Interactions, 22: 143-156.

Islam, M.N., S. Nizam and P.K. Verma, 2012. A highly efficient *Agrobacterium* mediated transformation system for chickpea wilt pathogen *Fusarium oxysporum* f. sp. ciceri using DsRed-Express to follow root colonisation. Microbiol. Res., 167: 332-338.

Jeon, J., S. Park, M. Chi, J. Choi and J. Park *et al*., 2007. Genome-wide functional analysis of pathogenicity genes in rice blast fungus. Nat. Genet., 39: 561-565.

Johnson, J., J. Kiawu, S. MacDonald, L. Meyer and C. Skelly, 2014. The world and United States cotton outlook. United States Department of Agriculture, February 21, 2014.

Klosterman, S.J. and R.J. Hayes, 2006. A soilless *Verticillium* wilt assay using an early flowering lettuce line. Plant Dis., 93: 691-698.

Klosterman, S.J., K.V. Subbarao, S. Kang, P. Veronese and S.E. Gold *et al*., 2011. Comparative genomics yields insights into niche adaptation of plant vascular wilt Pathogens. PLoS Pathog., Vol. 7. 10.1371/journal.ppat.1002137

Lin, L., R.Y. Zhang, X. Zhang, S. Deng, J.Y. Xu and Y.J. Zhou, 2012. Cultural characteristics and pathogenicity differentiation among strains of *Verticillium dahliae* from Cotton in Jiangsu province. Cotton Sci., 24: 199-206.

Liu, S.Y., J.Y. Chen, J.L. Wang, L. Li, H.L. Xiao, S.M. Adam and X.F. Dai, 2013. Molecular characterization and functional analysis of a specific secreted protein from highly virulent defoliating *Verticillium dahliae*. Gene, 529: 307-316.

Lu, J.Y., C. She, L. Ju and Z. Fang, 1983. Studies on differentiation of the *Verticillium dahliae* Kleb in Jiangsu province. J. Nanjing Agric. College, 1: 36-43.

Maruthachalam, K., N.V. Nair, H.S. Rho, Y.H. Lee, 2008. Agrobacterium tumefaciens-mediated transformation in two *Colletotrichum* species; C. falcatum and C. acutatum. J. Microbiol. Biotechnol., 18: 234-241.

Maruthachalam, K., S.J. Klosterman, S. Kang, R.J. Hayes and K.V. Subbarao, 2011. Identification of pathogenicity-related genes in the vascular wilt fungus *Verticillium dahliae* by *Agrobacterium tumefaciens*-mediated T-DNA insertional mutagenesis. Mol. Biotechnol., 49: 209-221.

Mercado-Blanco, J., D. Rodriguez-Jurado, S. Parrilla-Araujo and R.M. Jimenez-Diaz, 2003. Simultaneous detection of the defoliating and nondefoliating *Verticillium dahliae* pathotypes in infected olive plants by duplex, nested polymerase chain reaction. Plant Dis., 87: 1487-1494.

Michielse, C.B., A.F.J. Ram, P.J.J. Hooykaas and C.A.M.J.J. Van den Hondel, 2004. Role of bacterial virulence proteins in *Agrobacterium*-mediated transformation of *Aspergillus awamori*. Fungal Genet. Biol., 41: 571-578.

Michielse, C.B., P.J. Hooykaas, C.A. van den Hondel and A.F. Ram, 2005. *Agrobacterium*-mediated transformation as a tool for functional genomics in fungi. Curr. Genet., 48: 1-17.

Michielse, C.B., R. Van Wijk, L. Reijnen, B.J. Cornelissen and M. Rep, 2009. Insight into the molecular requirements for pathogenicity of *Fusarium oxysporum* f. sp. lycopersici through large-scale insertional mutagenesis. Genome Biol., Vol. 10.

Moller, E.M., G. Bahnweg, H. Sandermann and H.H. Geiger, 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies and infected plant tissues. Nucleic Acids Res., 20: 6115-6116.

Mullins, E.D., X. Chen, P. Romaine, R. Raina, D.M. Geiser and S. Kang, 2001. *Agrobacterium*-mediated transformation of *Fusarium oxysporum*: An efficient tool for insertional mutagenesis and gene transfer. Phytopathology, 91: 173-180.

Munch, S., N. Ludwig, D.S. Floss, J.A. Sugui and A.M. Koszucka *et al*., 2011. Identification of virulence genes in the corn pathogen *Colletotrichum graminicola* by *Agrobacterium tumefaciens*-mediated transformation. Mol. Plant Pathol., 12: 43-55.

Perez-Artes, E., M.D. Garcia-Pedrajas, J. Bejarano-Alcazar and R.M. Jimenez-Diaz, 2000. Differentiation of cotton-defoliating and nondefoliating pathotypes of *Verticillium dahliae* by RAPD and specific PCR analyses. Eur. J. Plant Pathol., 106: 507-517.

Rauyaree, P., M.D. Ospina-Giraldo, S. Kang, R.G. Bhat, K.V. Subbarao, S.J. Grant and K.F. Dobinson, 2005. Mutations in *VMK1*, a mitogen-activated protein kinase gene, affect microsclerotia formation and pathogenicity in *Verticillium dahliae*. Curr. Genet., 48: 109-116.

Rho, H.S., S. Kang and Y.H. Lee, 2001. *Agrobacterium tumefaciens*-mediated transformation of the plant pathogenic fungus, *Magnaporthe grisea*. Mol. Cells, 12: 407-411.

Sambrook, J. and D.W. Russell, 2001. Molecular Cloning: A Laboratory Manual. 3rd Edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY., USA., ISBN-13: 9780879695774, Pages: 2344.

Schnathorst, W.C. and D.E. Mathre, 1966. Host range and differentiation of a severe form of *Verticillium albo-atrum* in cotton. Phytopathology, 56: 1155-1161.

Sun, H.J., S. Uchii, S. Watanabe and H. Ezura, 2006. A highly efficient transformation protocol for Micro-Tom, a model cultivar for tomato functional genomics. Plant Cell Physiol. 47: 426-431.

Talhinhas, P., S. Muthumeenakshi, J. Neves-Martins, H. Oliveira and S. Sreenivasaprasad, 2008. *Agrobacterium*-mediated transformation and insertional mutagenesis in *Colletotrichum acutatum* for investigating varied pathogenicity lifestyles. Mol. Biotechnol., 39: 57-67.

Thibodeaux, D.P. and J.P. Evans, 1986. Cotton fiber maturity by image analysis. Textile Res. J., 56: 130-139.

Tzima, A.K., E.J. Paplomatas, P. Rauyaree, M.D. Ospina-Giraldo and S. Kang, 2011. VdSNF1, the sucrose nonfermenting protein kinase gene of *Verticillium dahliae*, is required for virulence and expression of genes involved in cell-wall degradation. Mol. Plant-Microbe Interactions, 24: 129-142.

Tzima, A.K., E.J. Paplomatas, D.I. Tsitsigiannis and S. Kang, 2012. The G protein β subunit controls virulence and multiple growth-and development-related traits in *Verticillium dahliae*. Fungal Genet. Biol., 49: 271-283.

Wakelyn, P.J., N.R. Bertoniere, A.D. French, D.P. Thibodeaux and B.A. Triplett *et al*., 2010. Cotton Fiber Chemistry and Technology. CRC Press, New York.

Wang, C., X. Guan, H. Wang, G. Li, X. Dong, G. Wang and B. Li, 2013. *Agrobacterium tumefaciens*-mediated transformation of *Valsa mali*: An efficient tool for random insertion mutagenesis. Sci. World J., Vol. 2013. 10.1155/2013/968432

Yamada, M., K. Yawata, Y. Orino, S. Ueda and Y. Isogai *et al*., 2009. *Agrobacterium tumefaciens*-mediated transformation of antifungal lipopeptide producing fungus Coleophoma empetri F-11899. Curr. Genet., 55: 623-630.

Yang, Y.J. and I. Lee, 2008. *Agrobactrium tumefaciens*-mediated transformation of *Monascus ruber*. J. Microbiol. Biotechnol., 18: 754-758.

Zhang, P., B. Xu, Y. Wang, Y. Li and Z. Qian *et al*., 2008. *Agrobacterium tumefaciens*-mediated transformation as a tool for insertional mutagenesis in the fungus *Penicillium marneffei*. Mycol. Res., 112: 943-949.