academicJournals

Vol. 7(22), pp. 2712-2718, 28 May, 2013
DOI: 10.5897/AJMR2013.5793
ISSN 1996-0808 ©2013 Academic Journals
http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Optimization of factors affecting protoplast preparation and transformation of smoke-tree wilt fungus *Verticillium dahliae*

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Accepted 17 May, 2013

Verticillium dahliae **is a causal agent of vascular wilt smoke-tree** ***Cotinus coggygrial*****. The genetic transformation of** ***V. dahliae*** **has been carried out by** ***Agrobacterium*** **and polyethylene glycol-mediated transformation. Due to technical simplicity and efficiency, PEG-mediated transformation is the most commonly used method for genetic transformation in filamentous fungi. However, polyethylene glycol (PEG)-mediated transformation is still deficient in** ***V. dahliae*****. In this study, we studied the main factors affecting the release of protoplasts from mycelia of** ***V. dahliae*** **such as lytic enzymes, enzymolysis buffers and incubation time. The optimal combination for protoplast preparation from smoke-tree wilt fungus** ***V. dahliae*** **can release 2.8 × 10^6 protoplasts/ml from fresh mycelia of smoke-tree wilt fungus. The majority of spheroplast contained nuclei and well regenerated. After PEG mediated transformation, green fluorescent protein (GFP) expressing** ***V. dahliae*** **strains were obtained. Therefore, this study provides a protocol suitable for protoplast preparation and transformation of smoke-tree wilt fungus** ***V. dahliae*****.**

Key words: *Verticillium dahliae*, smoke-tree wilt, protoplast preparation, polyethylene glycol (PEG) transformation.

INTRODUCTION

Verticillium dahliae is a soil-borne vascular wilt fungus which infects over 200 plant species and causes annual crop losses of billions of dollars. Vascular wilts caused by fungal pathogens are widely spread and are very destructive plant diseases; wilts caused by *Verticillium* species are among the most devastating. *V. dahliae* enters and colonizes the plant xylem system, disrupting water transport, and causing the characteristic symptoms of wilting, and often vascular discoloration (Klosterman et al., 2009; Klosterman et al., 2011). There are currently no fungicides to control *Verticillium* wilts once plants are infected. Despite its economic importance and considerable phytopathological attention, in recent functional analysis of *V. dahliae*, few genes have been investigated, such as Vdh1 (Klimes and Dobinson, 2006; Klimes et al., 2008), Sge1 (Santhanam and Thomma, 2013), VGB (Tzima et al., 2012), so very little has been discovered of the molecular basis of *Verticillium* diseases or the molecular disease-

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Abbreviations: PEG, Polyethylene glycol; **ATMT,** *Agrobacterium* tumefaciens-mediated transformation; **GFP,** green fluorescent protein; **PDA,** potato dextrose agar **DAPI,** 4', 6-diamidino-2-phenylindole.

causing mechanisms (Fradin and Thomma, 2006). In Beijing Fragrant Hills Park, the smoke-tree, *Cotinus coggygria*, is one of the most important cultivated plant species for the landscape ecology, and is the main component of the red leaf scenery during autumn. It was initially infected by *V. dahliae* during the 1990s, and the disease soon spread widely through the region. The main symptoms are stunted stem growth, early leaf senescence, and severe mortality, with seriously detrimental effects on the red leaf scenery (Wang et al., 2008).

The genetic transformation of *V. dahliae* has been established and can be carried out utilizing different transformation methods including *Agrobacterium* and polyethylene glycol (PEG)-mediated transformation (Dobinson, 1995; Amey et al., 2002). The *Agrobacterium* tumefaciens -mediated transformation (ATMT) system of *V. dahliae* has been successfully established for several years (Mullins et al., 2001; Dobinson et al., 2004), while PEG-mediated protoplast transformation was established reported in *V. dahliae* in 1995 (Dobinson, 1995),. However, low transformation efficiency is a main limitation for its wide use. More recently, using PEG-mediated protoplast transformation method, a green fluorescent protein (GFP)-tagged strain of the smoke-tree wilt fungus was successfully obtained by means of this modified protoplast transformation system, which enabled the colonization process between *V. dahliae* and the plant to be easily observed (Wang et al., 2013). But obtaining high quantity and quality protoplasts favorable for genetic transformation is still a big challenge. The effectors such as lytic enzymes, enzymolysis buffers and incubation time affecting protoplast preparation from *V. dahliae* are little understood.

Owing to its simple technique and equipment requirements, PEG-mediated transformation is the most widely used technique in filamentous fungi (Liu and Friesen, 2012). The three major steps in the basic procedure for PEG-mediated transformation are protoplast preparation, transforming DNA uptake, and regeneration on selective media. The general method of preparation of transformable protoplasts is to use cell wall degrading enzymes to digest the cell walls of mycelia or germinating spores. The lytic enzymes frequently used are helicase, cellulase, 1,3-glucanase, chitinase and driselase. Different lytic enzymes may vary in the effectiveness of their cell wall degradation, and it is necessary to test each batch and each enzyme combination. An osmotic buffer including sorbitol or high salt is used for stabilizing the resulting protoplasts (Fincham, 1989). Previously, Novozym 234 was the most frequently used lytic enzymes for preparation of fungal protoplasts (Ferrer et al., 1985; Roncal et al., 1991; Vazquez and Figueroa, 1996; Petrini and Petrini, 2001; Robinson and Deacon, 2001; Amey et al., 2002). In *Verticillium funicola*, Novozyme 234 was used to prepare the protoplasts (Amey et al., 2002). Unfortunately, the manufacturing of Novozym 234 was discontinued in the late 1990s. Recently, several alternative lytic enzymes are are available and used. For example, in the taxol-producing fungus *Ozonium sp.,* the study on the effects of some factors on protoplast isolation and regeneration were investigated and provided the foundation to develop genetic engineering (Zhou et al., 2008). In *Rhizoctonia solani*, the lytic enzymes used were driselase, lysing enzymes, and cellulase (Liu et al., 2010).

The efficient PEG-mediated protoplast transformation in the smoke-tree wilt fungus *V. dahliae* was required for functional characterization of pathogenicity genes. Consequently, based on the previous protocol developed in our lab, the overall objective of the present study was to investigate key aspects that could help to obtain high quantity and quality protoplasts favorable for stable PEG-mediated transformation for *V. dahliae*. Adapted from protocols of preparing protoplasts of *Aspergilus* (He et al., 2007) and *Fusarium* (Curragh et al., 1993), we identified the effectors including lytic enzymes, enzymolysis buffers and incubation time affecting protoplast production. The optimal combination was 0.7 M of NaCl with 10 mg/ml of lysing enzyme and 20 mg/ml of driselase incubated at 30°C and 70 rpm for 6 h, could release 2.8×10^6 / ml of protoplasts. After 4', 6-diamidino-2-phenylindole (DAPI) staining, we found nearly 91.67% of the protoplasts had nuclei. Finally, GFP was transformed and expressed in smoke-tree wilt fungus *V. dahliae* using this improved protocol. Thus, this protocol is suitable for protoplast preparation from the smoke-tree wilt fungus, *V. dahliae*.

MATERIALS AND METHODS

Fungal isolates and age of mycelia

In this study, *V. dahliae* strain XS11 was used, a single-spore cultural isolate from smoke-tree wilt plants, growing in Fragrant Hills Park, Beijing. Conidia were washed from cultures grown on potato dextrose agar (PDA) for 5 or 6 days. Mycelia were obtained from fresh conidia cultured in YEPD broth containing yeast extract, peptone, and dextrose for 24 h.

Preparation of protoplasts

A culture disk was placed in the middle of a PDA plate and grown for 5–6 days, then 4 ml of YEPD broth was used to wash off high concentrations of spores (about 10^6/ml); these were transferred into 100 ml of YEPD in 200 ml Erlenmeyer flasks and cultured at 25°C and 130 rpm for 24 h. To gather fresh fungal mycelia, two 50 ml Falcon tubes were used to horizontally centrifuge at 4,000 rpm for 5 min. The mycelial mass was filtered onto a piece of Miracloth (Calbiochem, USA) using two pieces of gauze placed on top of a 200 ml beaker. Mycelia were washed using the relevant buffer for enzymolysis until very few spores were observed under the microscope. A 40 ml beaker was used to hold lytic enzyme and the enzyme was dissolved in 20 ml of relevant buffer for enzymolysis. Using mixed enzymes, in particular, 10 mg/ml of lysing enzyme (L 1412, Sigma) and 20 mg/ml of driselase (D9515, Sigma), the enzymes were first dissolved in 30 ml of buffer, and then stirred for 20~30 min before filtered through a 0.45 µm membrane (Millex GP, Millipore) into a 100 ml flask. Usually about 20 ml was gained for further use. After the mycelia were placed on Miracloth into the buffer containing lytic enzyme and then, enzymolysis began. In this experiment, we focused on five different buffers in seven different

Table 1. The highest number of protoplasts released in different conditions (×10^4 protoplasts/ml).

Conditions used	0.1 g lysing	0.1 g lysing +0.2 g driselase
0.7 M NaCl	128 ± 4.0[a]	280 ± 2.6[b]
1.2M KCl	123 ± 1.6[a]	140 ± 11.1[b]
0.7 M KCl+10 mM $CaCl_2$	116 ± 7.5[a]	N.D.

Data standard deviation represents the means of five replicates. Values followed by the same letter are not significantly different ($P < 0.05$) according to Fisher's least significant difference test. N.D., Not determined.

treatments. In single enzyme treatment, only 10 mg/ml of lysing enzyme was used with 0.7 M of NaCl, 1.2 M of KCl and 0.7 M KCl + 10 mM $CaCl_2$, respectively, and incubated at 30°C and 70 rpm for 4.5 h. We began protoplast counting from 1.5 h and this was done using a blood counting plate every 30 min. In mixed enzyme treatment, 10 mg/ml of lysing enzyme and 20 mg/ml of driselase were used separately with 0.7 M NaCl and 1.2 M KCl. The first two were incubated at 30°C and 70 rpm for 8 h; at 34°C and 70 rpm for 0.5 h followed by 30°C for 3 h. Counting began from 2 h and was conducted every hour. Three replicates for each treatment were used and three times of the experiments were conducted.

Staining of nuclei from protoplasts

Nuclei of protoplasts were stained with DAPI stain consisting of 10 ppm DAPI, 0.1 M NaH_2PO_4, and 0.1 M Na_2HPO_4, pH 7 (Liu et al., 2010). On a glass slide, 10 µl of protoplast solution with isopycnic DAPI solution was mixed and the product heated at 55°C for 6 min. Then the slide was covered with a cover slip and examined using a Leica fluorescence microscope (DM 2500).

Regeneration of protoplasts and GFP expression

Protoplasts were gently suspended in STC buffer (20% sucrose, 10 mM Tris-HCl, pH 8.0, and 50 mM $CaCl_2$). Following the protocol of PEG-mediated transformation protocol in our lab (Wang et al., 2013), 250 µL protoplast suspension was well mixed with 25 µL eGFP cassette including the HPH marker in a polystyrene Falcon tube and incubated at room temperature for 20 min, followed by the addition of 1.5 mL 60 % PEG solution (60 % PEG4000 in STC). Finally, 10 mL of TB_3 liquid (3 g yeast extract, 3g casamino acids, and 20% sucrose in 1L H_2O) was added in the tube and incubated at room temperature for 2 min, then followed by another 10 mL of TB_3 liquid and overnight incubation (approx. 16 h) at room temperature. After regeneration for 16 h, GFP expression was observed under a Leica fluorescence microscope (DM2500). Using hygromycin B, the transformants were screened, and GFP-expressing transformants were obtained.

RESULTS

Effect of lytic enzyme treatments on protoplast release

Transformable protoplasts are prepared through digestion of the cell walls of fresh mycelia using cell wall degrading enzymes. Among seven different treatments tested, the results showed that 20 ml of 0.7 M NaCl with mixed enzymes (0.1 g lysing enzyme and 0.2 g driselase) was capable of releasing the greatest number of protoplasts from mycelia of *V. dahliae* strain XS 11 after incubation at 30°C for 6 h; a significantly higher number of protoplasts (2.8×10^6 /ml) than in the other treatments (Table 1). When 0.2 g of lysing enzyme in 20 ml of 0.7 M NaCl was only used, only 0.2 g of lysing enzyme in 20 ml of 0.7 M NaCl, the number of protoplasts declined to 1.28×10^6 /ml. In 1.2 M KCl; when driselase was added, the number increased only a little, from 1.23×10^6 to 1.4×10^6 /ml. The other three treatments were capable of releasing about 1.2×10^6 /ml (Table 1). It is important to note that Solution I with mixed lytic enzymes resulted in the appropriate protoplast concentration in the least incubation time (3 h), and moreover, these protoplasts appeared larger than those from other treatments.

The dynamics of protoplast release from *V. dahliae* were also studied in different enzyme combination and enzymolysis buffers. As for lysing enzyme single, three different buffers such as 0.7 M NaCl, 1.2 M KCl, and 0.7 M KCl + 10 mM $CaCl_2$ were tested. From Figure 1, the results showed that the highest number of protoplasts released in 0.7 M NaCl was 1.28×10^6 /ml protoplasts at 3.5 incubation hour; in 1.2 M KCl, it was 1.23×10^6/ml protoplasts, and in 0.7 M KCl + 10 mM $CaCl_2$ was 1.16×10^6/ml protoplasts (Figure 1). The number of protoplasts released from the three buffers was significantly different according to Fisher's least significant difference test ($P<0.05$). Next, the lytic enzyme combination of lysing and driselase were tested in 0.7 M NaCl and 1.2 M KCl, respectively. After incubation at 30°C and 70 rpm for 5 h, the number of protoplasts released from mycelia of *V. dahliae* using 1.2 M KCl increased to 1.4×10^6 /ml. At 6 h, the number of protoplasts in 0.7 M NaCl was 2.8×10^6 /ml and twice than the number released in 1.2 M KCl (Figure 2). Therefore, the combination of 0.1 g lysing and 0.2 g driselase in 0.7 M NaCl was selected for use in this study.

Effect of duration of enzyme treatment on protoplast release

During the preliminary test, the results suggested that that incubation of mycelia of *V. dahliae* strain XS11 with enzymes at 30°C was the most effective in releasing protoplasts from mycelia. Using lytic enzyme singly or in combination, protoplast release rose during early incubation and decreased with increasing time of incubation. For example, the number of protoplast released in NaCl

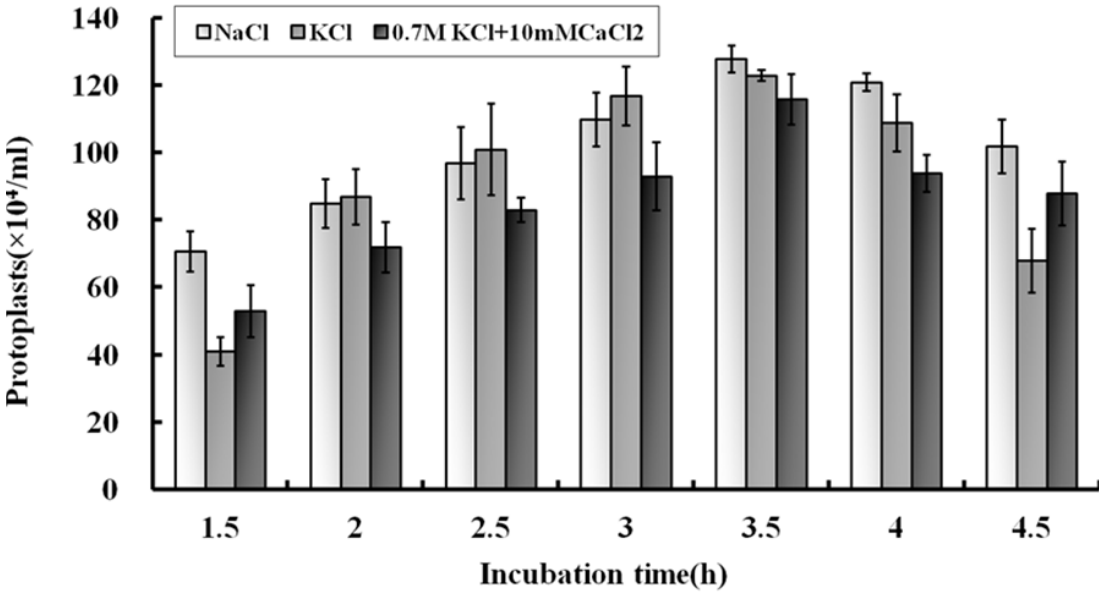

Figure 1. Protoplasts released from *V. dahliae* mycelia in three different buffers with lysing enzyme single. Smoke-tree wilt fungus *V. dahliae* strain XS11 was incubated with 0.2 g lysing enzyme single. Treatments consisted of incubation at 30°C and 70 rpm for 1.5, 2, 2.5, 3, 3.5, 4, and 4.5 h with the buffer 0.7 M NaCl, 1.2 M KCl, and 0.7 M KCl + 10 mM $CaCl_2$, respectively. Bars represent standard deviation of the means of three replicates.

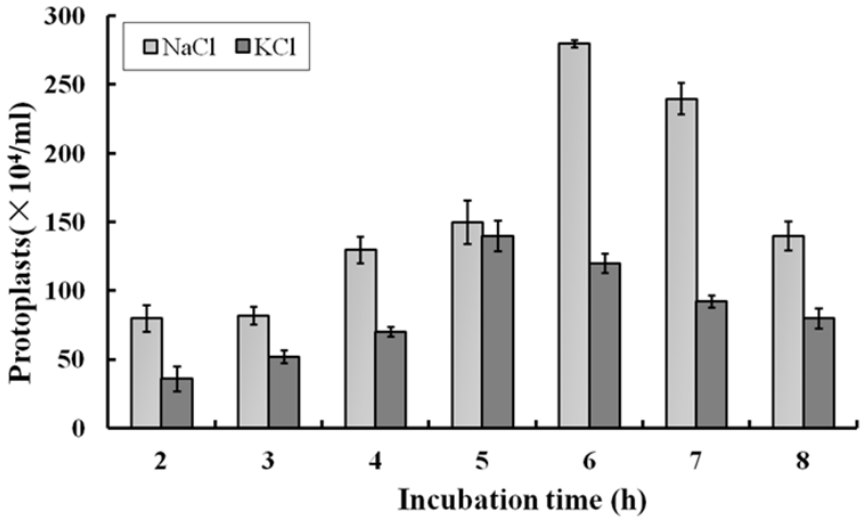

Figure 2. Protoplasts released from *V. dahliae* mycelia treated with lysing and driselase in the buffer 0.7 M NaCl and 1.2 M KCl. Smoke-tree wilt fungus *V. dahliae* strain XS11 was incubated with 0.1 g lysing and 0.2 g driselase enzyme combination. Treatments consisted of incubation at 30°C and 70 rpm for 2, 3, 4, 5, 6, 7, and 8 h with the buffer 0.7 M NaCl and 1.2 M KCl, respectively. Bars represent standard deviation of the means of three replicates.

with both lysing and driselase increased with increasing time of incubation and rose to the highest number at 6 h, but decreased thereafter (Figure 2). At the optimum incubation time of 6 h, about 2.8×10^6 /ml sprotoplasts were released.

Nuclear number of released protoplasts

The size of protoplasts of *V. dahliae* was around or less than 1 μm and many protoplasts were inclined to cluster together (Figure 3). The protoplast size of *V. dahliae* was

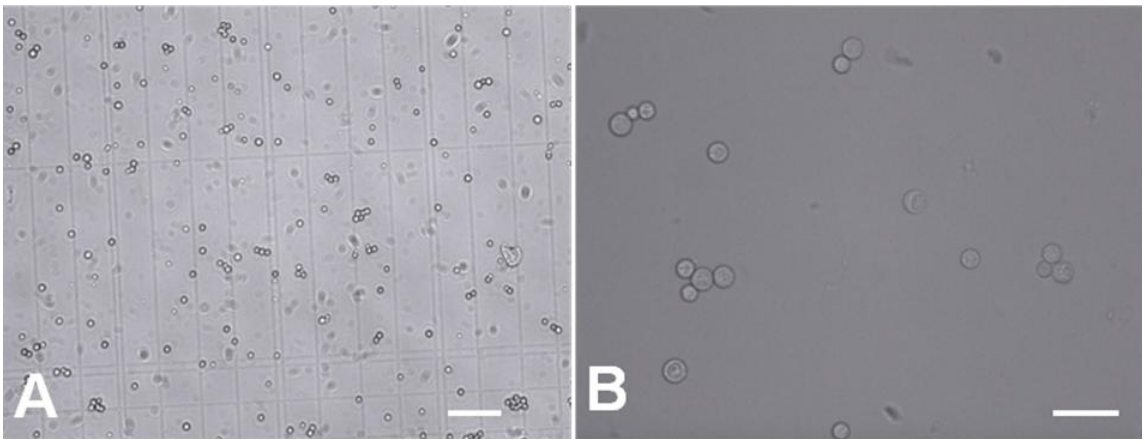

Figure 3. The protoplasts released from *V. dahliae*. These two photographs were taken under the treatment, 20 ml 0.7 M NaCl with 0.1 g lysing enzyme and 0.2 g driselase. The bar in A = 5 µm; scale bar in B = 2.45 µm.

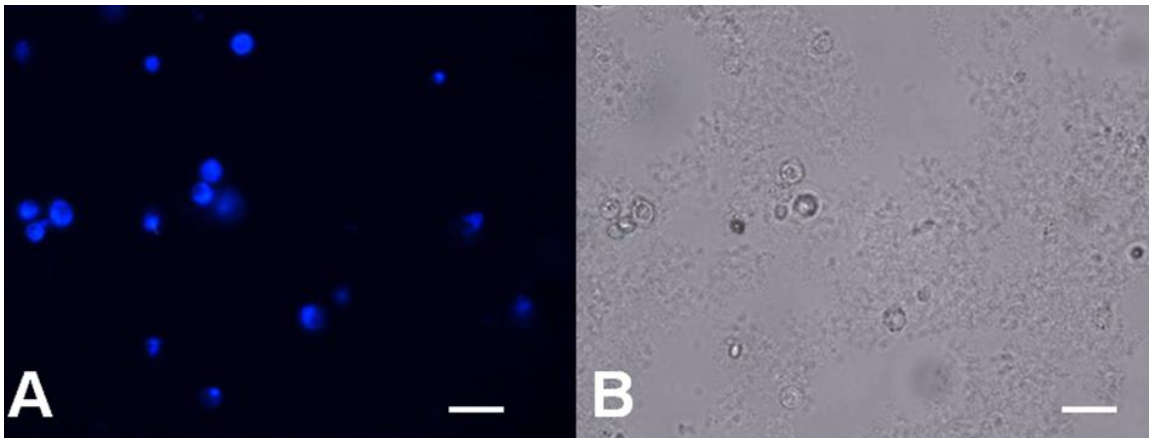

Figure 4. The staining of DAPI nuclei of released protoplasts. The released protoplast was stained with DAPI. Bright field and fluorescent microscopy of corresponding protoplasts stained with DAPI. A under fluorescence, and B under bright field. Almost all protoplasts have visibly stained nuclei. The high numbers of protoplasts with nuclei may indicate the high quality of released protoplasts. Scale bars in A and B = 2 µm.

much smaller than that of other filamentous fungi, the protoplast size of *R. solani* was 5.5 to 8.5 µm (Liu et al., 2010). To determine the relevant relationship between the number of nuclei and the quality of the protoplasts, DAPI was used to stain protoplasts after enzymolysis. It was clear that most released protoplasts had nuclei. Observing through the microscope at a magnification of ×40, 22 out of 24 protoplasts in the field of view had nuclei (Figure 4). Thus 91.67% of the protoplasts had nuclei. This showed that the quality of the protoplasts was good.

Regeneration of protoplasts and GFP expression

Protoplasts were regenerated at 20°C and 70 rpm for 14–16 h in TB_3. Almost half the protoplasts were regenerated and the hyphae could be seen very clearly (data not shown). We used the method described by Wang et al. (2013) with some modifications. After obtaining protoplast suspension, following the PEG-mediated transformation steps, we transformed gGFP and the hygromycin B resistance (hph) gene into protoplasts of *V. dahliae*. After about 16 h of regeneration, expression of GFP was observed under fluorescence microscope (Figure 5B). Under hygromycin B resistance, gGFP-tagged transformants where gGFP-tagged transformants were obtained, and the fluorescence observation showed that GFP expression was stable and strong (Figure 5). The results showed that the protocol is suitable for protoplast preparation and transformation of smoke-tree wilt fungus *V. dahliae*.

DISCUSSION

Our aim was to establish a stable PEG-mediated protoplast transformation system. For this purpose, we used

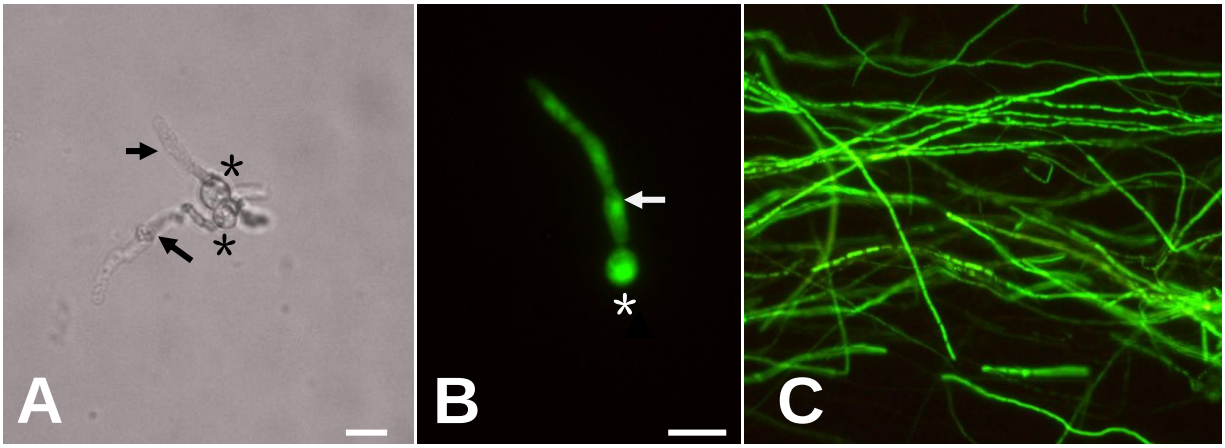

Figure 5. The regeneration of protoplasts and GFP expression in *V. dahliae*.

lysing enzyme and driselase with five different salt solutions to investigate the best conditions for maximum protoplast release. We used a GFP-tagged gene in PEG-transformation to demonstrate the quality and usability of the protoplasts. The above results indicate that the combination of lysing enzyme and driselase is more effective than only using lysing enzyme for the production of protoplasts from *V. dahliae*. Among seven treatments we found that 0.7 M NaCl with 10 mg/ml of lysing enzyme and 20 mg/ml of driselase incubated at 30°C and 70 rpm for 6 h, was the most efficient method for releasing maximum protoplasts. The numbers of protoplasts obtained using the other six ways were not significantly different from each other. Because large numbers of protoplasts were obtained by our best method, PEG-mediated transformation could proceed smoothly, and a GFP-tagged strain was successfully selected.

Main factors affecting protoplast release in fungi include lytic enzymes, osmotic buffers and incubation temperature. Liu (2010) obtained optimal protoplast numbers from the mycelia of *R. solani* using Solution I with 10 mg/ml of lysing enzyme and 20 mg/ml of driselase, incubated at 37°C for 15 min followed by 34°C for 105 min. We did several trials changing the incubation temperature and time to 34°C for 30 min followed by 30°C for 120 min, in order to find the optimal conditions. Liu and Friesen (2012) used 0.7 M of KCl + 10 mM of $CaCl_2$ as protoplasting buffer with three different lytic enzymes to gain high concentrations of protoplasts. Turgen (2010) used 0.7 M of NaCl with glucanex and driselase to gain protoplasts from *Cochliobolus heterostrophus*. Lysing enzyme and driselase purchased from Sigma have been commonly used because of their high efficiency and easy acquisition. Lytic enzymes singly or in combinations could be used for effective lysis of cell walls. For example, high quantity of protoplasts was produced by lysing enzyme singly in *Colletotrichum gloeosporioides* (Li et al., 2013). In this study, lysing enzyme and driselase in combination were required to prepare protoplasts in *V. dahliae*. In addition, the high percentage of protoplasts with nuclei is another factors influencing protoplasts transformation of fungi. In our study, protoplasts with nuclei were in the majority, and these were of very good quality. Through successful PEG-mediated transformation, the regeneration of released protoplasts was satisfactorily accomplished.

Protoplasts transformation of *V. dahliae* was studied as early as in 1995 by Dobinson (1995). It is not easy to obtain protoplasts from *V. dahliae*, or to gain high concentrations of these. Released protoplasts are usually small and easily damaged by the wrong osmotic buffer. The protoplasts produced by lysing enzyme and driselase have advantages in PEG-mediated transformation. The applications of the results of the present study provide a protocol suitable for protoplast preparation and transformation of *V. dahliae*. The establishment of PEG-mediated transformation in smoke-tree wilt fungus *V. dahliae* has laid a solid foundation for future work such as gene deletion and further functional genomic research, so that the molecular mechanisms of pathogenicity in *Verticillium* species can be further clarified.

ACKNOWLEDGMENTS

The research was supported by the Fundamental Research Funds for the Central Universities (NO. TD2011-06) and NSFC Project (31000302) to W.Y.

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