Factors that influence the yield and viability of protoplasts from *Carica papaya* L.

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The effects of four main factors (the concentration of cellulase R-10, macerozyme R-10 and D-mannitol, and duration of enzyme incubation) determining yield and viability of protoplasts isolation were investigated using Taguchi experimental design to optimize protoplasts isolation conditions from the leaves of *Carica papaya* L. seedlings. The results showed that the concentration of cellulase R-10 and incubation time were key factors affecting the protoplast yield; D-mannitol concentration played a significant role in protoplast viability. Moreover, the result of single factor tests revealed that pH of the enzyme solution significantly influenced both yield and viability of protoplasts. The highest yield of more than $1.5 \times 10^7$ protoplasts g$^{-1}$ fresh weight (FW) with more than 90% viability were consistently obtained by the optimum isolation conditions: enzyme combination of 1.2% cellulase R-10, 0.3% macerozyme R-10 and 0.44 M D-mannitol, pH 5.8 and incubation for 13 h at 26°C with a shaker speed of 60 rpm in darkness. This study will facilitate to obtain sufficient yields and viability of protoplasts for subsequent novel applications in papaya biotechnology such as the gene functional analysis and mechanism of PRSV infection in papaya protoplasts.

**Key words:** *Carica papaya* L., isolation conditions, protoplasts, Taguchi experimental design, viability, yields.

INTRODUCTION

With significant advances in genomics, proteomics and metabolomics, in addition to their role in the generation of novel plants by protoplast fusion (Davey et al., 2005), a new application of protoplasts has focused on gene functional analysis as a novel reverse-genetic approach by double-stranded (ds) RNA interference (RNAi) in protoplasts, which has been established in several plants such as *Arabidopsis thaliana*, *Coptis japonica*, rice, poplar and provides a rapid, high-throughput and cost-efficient initial screening of genes of interest for further studies in plants (Zhai et al., 2009; Dubouzet et al., 2005; Miki et al., 2004; Meyer et al., 2004). In addition, a transient expression system using *Nicotiana benthamiana* protoplasts was recently developed to determine the silencing efficiency by using a green fluorescent protein (GFP) marker gene fused to inverse repeat constructs targeting a gene of interest (Jiang et al., 2010). The efficient DNA transformation and the success of RNAi silencing of genes in protoplasts relies on high yield and stable viability of protoplasts. Hence, it is necessary to study the critical factors affecting the protoplast isolation to enable protoplasts to be used in various areas of biotechnology.

*Carica papaya* L. is a popular and important economical fruit tree of tropical and subtropical countries because of its high nutritional value, medicinal and industrial applications (Chandrika et al., 2003). Since the recent sequencing of the papaya genome (Ming et al., 2008), the functional identification of papaya genes has become increasingly important. For protoplast fusion

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**Abbreviations:** ds, Double-stranded; RNAi, RNA interference; GFP, green fluorescent protein; PRSV, Papaya ringspot virus; CP, coat protein; FW, fresh weight; PVP, polyvinylpyrrolidone; FDA, fluorescein diacetate.

#These authors contributed equally to this work.
studies, isolation of protoplasts from papaya leaves was reported more than 30 years ago (Litz et al., 1979), but the yield and viability of the isolated protoplasts was low. Moreover, the yield and viability of protoplasts obtained by repeating this procedure was insufficient for gene functional analysis for papaya. Furthermore, protoplasts may also provide a single cell model system to study the infection and replication of plant virus. Papaya ringspot virus (PRSV, genus Potyvirus, family Potyviridae) is considered to be the most destructive disease and occurs in most of the major papaya plantation areas of the world (Bateson et al., 1994; Gonsalves, 1998). Although transgenic papaya resistance to PRSV generally, are considered the best strategy for long-term virus control, different specificities of PRSV resistance were displayed in transgenic papaya lines expressing different PRSV coat protein (CP) gene toward PRSV isolates from different geographic origins and the application is limited in other areas of the world because of strain-specific resistance (Chiang et al., 2001; Bau et al., 2003). For understanding of molecular events associated with PRSV infection, PRSV-induced gene silencing and PRSV-host interactions to develop new strategies against PRSV, establishing an efficient protocol for high yield and viability of protoplasts from papaya leaf is essential to further study the infection and replication of PRSV.

Several abiotic factors, such as enzyme mixtures, osmotic potential, incubation time and digestion pH, affect the isolation of plant protoplasts. Each plant species requires specific optimal conditions for protoplasts isolation, and there are many reports on the optimal isolation conditions for different species (Tohjima et al., 1996; Sinha et al., 2003; Rizkalla et al., 2007; Pongchawee et al., 2006). However, there are no detailed reports on the factors affecting protoplasts isolation from papaya. Conventional optimization procedures involve altering of one parameter at a time, keeping all other parameters constant, which enables one to assess the impact of those particular parameters on the process performance. These procedures are time consuming, cumbersome, require more experimental data sets and cannot provide information about the mutual interactions of the parameters. Since Taguchi experimental design allows a maximum number of main effects to be estimated in an unbiased (orthogonal) fashion with a minimum number of experimental runs, it has found successful application in different areas of biotechnology for the optimization of various processes and in product development (Taguchi, 1987; Rao et al., 2008). In this paper, different factors determining yield and viability of protoplasts isolation from the leaves of papaya were first studied systematically in an orthogonal design and in single factor tests, and a stable and efficient method for mass high-quality protoplasts isolation from papaya mesophyll was established. We showed that the optimum protocol allows production of more than 1.5 x 10^5 protoplasts g^-1 fresh weight (FW) with viability of more than 90% by an optimum isolation conditions. This study aims to obtain sufficient yields and viability of protoplasts for subsequent novel applications in papaya biotechnology such as the gene functional analysis and mechanism of PRSV infection in papaya protoplasts.

MATERIALS AND METHODS

Plant material

Plant materials used were seedlings of C. papaya L. cultivar SZH NO.48, obtained from Hainan Agriculture and Science Academy Fruit Plant Research Institute (Haikou, China), which were cultured at 20 to 25°C with 60% humidity in the greenhouse. The 3rd to 4th upper fully expanded leaves from 2 to 3 months old plantlets were cut into small pieces (0.5 to 1 mm) with a sharp scalpel for protoplast isolation after they were surface-sterilized with 50% ethanol for 30 s, and with 1% sodium hypochlorite for 10 min, and then washed thoroughly with sterile distilled water.

Protoplasts isolation

The enzyme combination solution [0.9 to 1.5% w/v cellulase R-10 (Yakult Pharmaceutical Ind. Co. Ltd), 0.3 to 0.5% w/v macerozyme R-10 (Yakult Pharmaceutical Ind. Co. Ltd), 0.3 to 0.5% D-mannitol, 20 mM KCl and 20 mM 2-(N-morpholino) ethanesulphonic acid (MES)] was prepared before the leaves of papaya were digested, and then heated at 55°C for 10 min to inactivate proteases and enhance enzyme solubility. It was cooled to room temperature before adding 10 mM CaCl_2 and 0.5% polyvinylpyrrolidone (PVP) (w/v), then the enzyme solution was sterilized by 0.22 µm filter. After placing the leaves (0.500 g) in a Petri dish (Ø = 6 cm) containing 10 ml enzyme solution, vacuum was applied for 30 min (-0.07 ~ -0.08 MP). The Petri dish was sealed with parafilm, and the mixture of leaves and enzyme solution were incubated at 26°C for 9 to 13 h on a table concentrator (60 rpm) in darkness.

Protoplasts purification

After incubation, 10 ml filter-sterilized W5 buffer (154 mM NaCl, 125 mM CaCl_2, 5 mM KCl, 2 mM MES and 5 mM D-glucose) were added to the Petri dish. Undigested tissues and coarse protoplasts were separated through 50-µm nylon net. The protoplast suspension was centrifuged at 800 rpm for 5 min in 50-ml tubes, and then the debris and the enzyme solution were removed completely using a Pasteur pipette. Protoplasts were washed twice with 10 ml of W5 (800 rpm for 5 min). The purified protoplasts were resuspended in 10 ml of W5.

Protoplasts yield and viability

The suspended protoplasts (500 µl) were mixed with 12 µl fluorescein diacetate (FDA) stock solutions (5 mg/ml aceton), then 20 µl of suspended protoplasts were placed on a haemocytometer slide. After 5 min, protoplasts were counted on the haemocytometer under white light, results were expressed as protoplast yield g^-1 FW, and all yield assessments were repeated at least five times. The viability of protoplasts was examined by FDA staining assay under B-2A illumination using an epifluorescence microscopy (NIKON ECLIPSE 80i). Viability expressed as percentage is determined as the number of protoplasts that fluoresced yellow-green under UV light out of the total number of isolated protoplasts observed in the
Table 1. Taguchi experimental design L₉ (3⁴) and test results for the mesophyll protoplasts isolation in papaya.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Factor</th>
<th>Viable yield (× 10⁶ g⁻¹ FW)</th>
<th>Yield (× 10⁶ g⁻¹ FW)</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
</tr>
<tr>
<td>Exp. 1</td>
<td>1</td>
<td>(0.9%) 1 (0.3%) 1 (0.36 M)</td>
<td>1 (9 h)</td>
<td>3.067±0.152</td>
</tr>
<tr>
<td>Exp. 2</td>
<td>1</td>
<td>2 (0.4%) 2 (0.44 M) 2 (11 h)</td>
<td>4.467±0.033</td>
<td>6.067±0.035</td>
</tr>
<tr>
<td>Exp. 3</td>
<td>1</td>
<td>3 (0.5%) 3 (0.52 M) 3 (13 h)</td>
<td>4.800±0.116</td>
<td>7.167±0.088</td>
</tr>
<tr>
<td>Exp. 4</td>
<td>2</td>
<td>1 (1.2%) 2 3 1</td>
<td>14.03±0.117</td>
<td>15.60±0.100</td>
</tr>
<tr>
<td>Exp. 5</td>
<td>2</td>
<td>2 2 3</td>
<td>5.667±0.145</td>
<td>6.067±0.167</td>
</tr>
<tr>
<td>Exp. 6</td>
<td>2</td>
<td>3 1 2</td>
<td>8.633±0.088</td>
<td>12.07±0.067</td>
</tr>
<tr>
<td>Exp. 7</td>
<td>3</td>
<td>1 (1.5%) 3 2 1</td>
<td>11.33±0.133</td>
<td>11.67±0.140</td>
</tr>
<tr>
<td>Exp. 8</td>
<td>3</td>
<td>2 1 3</td>
<td>8.800±0.115</td>
<td>12.93±0.167</td>
</tr>
<tr>
<td>Exp. 9</td>
<td>3</td>
<td>3 2 1</td>
<td>5.467±0.181</td>
<td>7.100±0.163</td>
</tr>
</tbody>
</table>

Duncan’s multiple range test of viable yield

| k1  | 4.1111ᵇ | 9.4778ᵃ | 6.833３ᵇ | 5.133３ᶜ |
| k2  | 9.4444ᵃ | 6.3111ᵇ | 8.3889ᵃ | 8.1444ᵇ |
| k3  | 8.933３ᵃ | 6.7000ᵇ | 7.2667ᵇ | 9.2111ᵃ |
| Range | 5.333３ | 3.1667 | 1.5556 | 4.0778 |

Data are means ± standard error of triplicate. Means in the same column not sharing the common superscript letter are significantly different and determined by LSD (p < 0.05); A: cellulase R-10, B: macerozyme R-10, C: D-mannitol, D: incubation time; pH 5.8, temperature 26°C, shaken at 60 rpm. k: the average value of the yield of viable protoplasts (× 10⁶ g⁻¹ FW).

Experimental treatments of protoplasts

In experiment 1, the factors (the hydrolytic enzymes concentration, D-mannitol concentration and incubation time) were studied using an orthogonal design L₉ (3⁴) to determine efficient conditions for protoplasts isolation and their relations (Table 1). The concentrations of cellulase R-10 [0.9, 1.2 and 1.5% (w/v)], the macerozyme R-10 [0.3, 0.4 and 0.5% (w/v)] and D-mannitol [0.36, 0.44 and 0.52 (M)] were tested, respectively, the pH of the enzyme solution was 5.8 and digestion was performed on a table concentrator (26°C, 60 rpm) for 9, 11 and 13 h in darkness. Following the experiment 1 study (experiment 2), pH of the enzyme solution (5.0, 5.4, 5.8 and 6.2) were tested for their effects on the isolation of protoplast in a single factor test to obtain the best combination of factors. Each experiment had three replicates.

Data analysis

All data were analyzed by one-way analysis of variance (ANOVA) and means were compared using Duncan’s multiple range tests. All statistical analyses were carried out using the Statistical Analysis System (SAS) 9.0 software.

RESULTS

Effects of factors (enzymes concentration, D-mannitol concentration and incubation time) on mesophyll protoplasts isolation in papaya

Nine different treatment combinations were used in protoplast isolation, the yield and viability of protoplasts isolation from the leaves of papaya was investigated using ANOVA and intuitive analysis (Tables 1 and 2). Four factors had significant effects on the yield of viable protoplasts (P < 0.01; Table 2). Since a higher F-value indicated a greater effect on yield and viability of protoplasts, the cellulase R-10 and incubation time were the key factors affecting the yield and viability of protoplasts isolation and the effect of D-mannitol concentration on the yield and viability of protoplasts isolation was least.

Duncan’s multiple range test of the yield of viable protoplasts (k value in Table 1) showed that the A₂ (1.2% cellulase R-10) (k₂ = 9.4444) was better than A₁ (0.9% cellulase R-10) (k₁ = 4.1111), but not significantly different from A₃ (1.5% cellulase R-10) (k₃ = 8.9333). Theoretically, we could choose A₂ or A₃; however, to reduce costs, A₂ was better than A₃. Since Duncan’s multiple range test of the yield of viable protoplasts also showed that B₁ (0.3% macerozyme R-10) (k₁’ = 9.4778), C₂ (0.44M D-mannitol) (k₂* = 8.3889), D₃ (13 h) (k₃* = 9.2111) were the better treatment, respectively, the optimal conditions for protoplasts isolation were A₂B₁C₂D₃ (1.2% cellulase R-10, 0.3% macerozyme R-10, 0.44 M D-mannitol and incubation for 13 h) (Exp.4, Table 1) and the yield of protoplasts in our experiments was 15.60 ± 0.100 × 10⁶ g⁻¹ FW. Fresh protoplasts and viable protoplasts from papaya mesophyll, isolated in the A₂B₁C₂D₃ treatment in Experiment 4, are shown in Figure 1A and B.

ANOVA showed that incubation time was the key factor affecting the yield of protoplasts (F = 575.48) (Table 2,

same microscopic field under normal light.
Table 2. Yield of viable protoplasts, yield and viability (ANOVA).

<table>
<thead>
<tr>
<th>Source</th>
<th>Viable yield ANOVA</th>
<th>Yield ANOVA</th>
<th>Viability ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>F-value</td>
<td>Pr &gt; F</td>
<td>F-value</td>
</tr>
<tr>
<td>A</td>
<td>262.27</td>
<td>&lt;.0001</td>
<td>445.76</td>
</tr>
<tr>
<td>B</td>
<td>90.33</td>
<td>&lt;.0001</td>
<td>103.87</td>
</tr>
<tr>
<td>C</td>
<td>19.52</td>
<td>&lt;.0001</td>
<td>41.71</td>
</tr>
<tr>
<td>D</td>
<td>135.44</td>
<td>&lt;.0001</td>
<td>575.48</td>
</tr>
</tbody>
</table>

A: Cellulase R-10, B: macerozyme R-10, C: D-mannitol, D: incubation time; pH 5.8, temperature 26°C, shaken at 60 rpm.

Figure 1. Fresh protoplasts (A) and viable protoplasts (B) from mesophyll of papaya. The isolation conditions used were 1.2% cellulase R-10, 0.3% macerozyme R-10, 0.55 M D-mannitol, incubation for 13 h and digestion pH of 5.8. Viable protoplasts were determined by FDA staining method, the green/yellow fluorescing protoplasts were viable (UV-B, 40×).

The protoplasts from mesophyll of papaya were isolated respectively at pH 5.0, 5.4, 5.8 and 6.2 of enzyme solution. The result showed that pH influenced the yield and viability of protoplasts and the highest yield (17.45 ± 0.77 × 10^6 g⁻¹ FW) with 91.10 ± 1.95% viability of protoplasts were isolated at pH 5.8. The yield of viable protoplasts obtained from pH5.8 was not significantly different from that of pH 5.4 (14.50 ± 0.26 × 10^6 g⁻¹ FW), but significantly different from that of pH 5.0 (12.90 ± 0.77 × 10^6 g⁻¹ FW) and 6.2 (12.85 ± 0.85 × 10^6 g⁻¹ FW). The yield (16.20 ± 0.63 × 10^6 g⁻¹ FW) of viable protoplasts and protoplast viability (79.47 ± 2.30%) were the least at pH 5.0. Therefore, pH 5.8 was optimum for protoplast isolation from mesophyll of papaya (Table 3).

**DISCUSSION**

In this study, Taguchi method was applied to test the relative importance of four main factors (the concentration of cellulase R-10, macerozyme R-10 and D-mannitol, duration of enzyme incubation) determining yield and viability of protoplast isolation to optimize protoplast isolation conditions from the leaves of *C. papaya* L. seedling. The results showed that enzyme concentrations (especially cellulase R-10) and incubation time were the dominant factors affecting the yield of viable protoplasts of protoplasts from papaya, whereas protoplast yield was mainly affected by incubation time. Within any one incubation time, the yield and the yield of viable protoplasts can be improved by increasing the cellulase R-10 concentration from 0.9 to 1.2% (Table 1), but a higher enzyme concentration (1.5%) resulted in lower yield and viability of protoplasts (Table 1), possibly due to enzyme toxicity, since protoplasts are fragile and sensitive to...
Table 3. Effects of pH of the enzyme solution mesophyll protoplast isolation in papaya.

<table>
<thead>
<tr>
<th>pH</th>
<th>Viable yield (× 10⁶ g⁻¹ FW)</th>
<th>Yield (× 10⁸ g⁻¹ FW)</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>12.90±0.77⁵⁶</td>
<td>16.20±0.63⁵⁶</td>
<td>79.47±2.30⁵⁶</td>
</tr>
<tr>
<td>5.4</td>
<td>14.50±0.26⁵⁶</td>
<td>16.55±0.60⁵⁶</td>
<td>87.84±2.33⁵⁶</td>
</tr>
<tr>
<td>5.8</td>
<td>15.30±0.63⁵⁶</td>
<td>17.45±0.77⁵⁶</td>
<td>91.10±1.95⁵⁶</td>
</tr>
<tr>
<td>6.2</td>
<td>12.85±0.85⁵⁶</td>
<td>14.10±0.80⁵⁶</td>
<td>88.37±3.08⁵⁶</td>
</tr>
</tbody>
</table>

Data are means ± standard error of triplicate. Means in the same column not sharing the common superscript letter are significantly different and determined by LSD (p < 0.05); 1.2% cellulase R-10, 0.3% macerozyme R-10, 0.55 M D-mannitol, temperature 26°C, shaken at 60 rpm for 13 h.

digesting enzymes. When cellulase R-10 concentration was with any value, prolonged incubation time led to increased yield. However, the prolonged incubation of leaves in enzyme solution could lead to protoplast breakage and dysfunction. Such damage could be decreased by modifying the enzyme solution treatments (lower concentration of enzymes) or reducing duration (Navratilova et al., 2000). Hence, suitable enzyme concentration especially cellulose and incubation time for protoplast isolation for different plant species and different organs or tissues of the same species is very important. In our investigations, we confirmed that the high yield and viability of protoplast from papaya mesophyll can be isolated by the most appropriate enzyme concentration of 1.2% cellulase R-10, 0.3% macerozyme R-10 and incubation time of 13 h.

The appropriate concentration of osmotic-pressure-regulating agent can prevent protoplasts missing cell walls from bursting or shrinking. In general, osmotic potential is adjusted by adding D-mannitol, sorbitol, glucose or sucrose to the enzyme mixture (Navratilova, 2004), and D-mannitol is commonly effective. Osmotic values of the environment into which protoplasts are released are critically important. Isolation protoplasts from different plant species, tissues and organs used the different concentration of D-mannitol (Kamnoon et al., 2001; Duquenne et al., 2007). Generally, the concentration of D-mannitol is 0.23 to 0.90 M; however, protoplasts are more stable in a slightly hypotonic environment when compared to isotonic environment, as a higher osmotic potential prevents the protoplasts bursting, but can inhibit their division (Bhojwani et al., 1996). In the present study, we found that 0.52 M D-mannitol gave the highest viability (an average of 85.84%) but was not significantly different from 0.44 M D-mannitol. Since 0.44 M D-mannitol gave the highest yield of protoplasts, this concentration of D-mannitol was a suitable osmotic pressure for protoplasts isolation of papaya.

In addition, pH of the enzyme solution affecting the protoplasts isolation from papaya was studied in a single factor test. A low pH can strengthen enzyme activity, enhance membrane-lipid peroxidation level, damage cellular structure and finally lead to cell death; thus, the yield and viability of protoplasts is dropped under a low pH stress. On the contrary, it can inactivate or partially deactivate enzymes to reduce the yield and viability of protoplasts under a high pH stress. In the present study, it was concluded that pH 5.8 was suitable for isolation of papaya protoplasts, and it gave the highest yield.

In this work, the leaves of 2 to 3 months papaya seedlings were chosen as the best donor material for protoplast isolation of papaya because the protoplasts isolated were spheroidal and intact, and had many chloroplasts (Figure 1B). It is difficult to isolate protoplasts from older leaves because more lignin accumulated in cell walls of old cells, whereas protoplasts isolated from too young leaves were fragile. In addition, the nutritional status of plants may be very important for protoplast production. The yield of protoplasts (Table 3) was generally higher than that of Experiment 1 treatment (15.60 ± 0.10 × 10⁶ g⁻¹ FW, as shown in Table 1). The reason may be that the same donor seedlings were supplied nitrogen during experimental periods, thus, they had sufficient nutrition to grow vigorously, their leaves were verdant and had high water contents, and the cells were metabolically active, which may have enhanced their tolerance to external environmental stresses. The plantlet leaves in this condition were optimum for proto-plast isolation.

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