

Full Length Research Paper

Somatic embryogenesis and plantlet regeneration of *Mangifera persiciforma* C.Y. Wu & T.L. Ming

Hong-Wen Li^{1,2}, Jian-jun Liu³, Ke-Ling Chen², Jian He², Jian-Hui Wang² and Xu-Feng Li^{1*}

¹Key Laboratory of Bio-resources and Eco-environment, Ministry of Education, College of Life Science, Sichuan University, Chengdu 610064, China.

²Horticultural Research Institute, Sichuan Academy of Agricultural Sciences, Chengdu 610066, China.

³Sichuan Academy of Agricultural Sciences, Chengdu 610066, China.

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A method for somatic embryogenesis and plant regeneration from immature nucellus (30–40 days old after pollination) of *Mangifera persiciforma* C.Y. Wu & T.L. Ming has been developed. Browning and necrosis of the nucellar explants were effectively controlled by pre-treating in an antioxidant solution and by pre-culturing in liquid medium in a constant temperature shaker. The explants were cultured for 2 weeks in the dark at $23 \pm 2^\circ\text{C}$ on induction medium and then moved to the light for 4 weeks at $25 \pm 2^\circ\text{C}$. Pro-embryonic masses have gradually been generated from the explants. The pro-embryogenic masses were subcultured on appropriate proliferation medium in the light for 5 weeks at $25 \pm 2^\circ\text{C}$, secondary embryogenesis and organogenesis was observed. Entire plantlets have been produced. Plantlets developed in 3-4 weeks, were acclimatized and transplanted to the greenhouse. Morphogenic competence was sustained for more than one year.

Key words: *Mangifera persiciforma*, nucellus, browning, pro-embryogenic masses, somatic embryogenesis, organogenesis.

INTRODUCTION

Mango (*Mangifera indica* L.) is one of the most widely distributed tropical fruit crops in the world. Breeding programs in several countries have resulted in the development of many mango cultivars. However good mango rootstocks are still lacking. Many features of the scion, such as environmental stress resistance, growth vigor and growth habit, precocity of fruit bearing, production level, fruit size and appearance are affected by the rootstock (Wertheim, 1998). *Mangifera persiciforma* C.Y. Wu and T.L. Ming is a wild *Mangifera* species endemic to China. It is poly-embryonic, exhibits moderate vigor and is generally resistant to environmental stresses such as drought, high temperatures and air pollution. It also exhibits resistance to common fungal disease of mango such as anthracnose, powdery mildew and gummosis. *Magnifera persiciforma* is grafted

compatible with commercial mango. An efficient and reliable regeneration system is a prerequisite for the application of gene transfer or other biotechnology techniques to the improvement of woody species (Litz and Gray, 1992; Litz and Lavi, 1997; Litz, 2004). Several biotechnological advances in mango have been described (Hare Krishna and Singh, 2007). Somatic embryogenesis and plant regeneration using nucellus tissues has been reported for several mango cultivars (Litz et al., 1982; Litz, 1984; Laxmi et al., 1999; Ara et al., 2000; Ara et al., 2000; Patena et al., 2002; Flórez-Ramos et al., 2007; Xiao et al., 2004; Wu et al., 2007). There are also a few reports describing production of mango rootstock from tissue culture (Yang and Ludders, 1993; Chaturvedi et al., 2004). In the experimental system described in this paper, we provide an effective and reliable protocol for somatic embryogenesis and plantlet regeneration for *M. persiciforma*. This system is benefit to genetic transformation, embryo rescue, micro-propagation and *in vitro* conservation studies of *Mangifera* species.

*Corresponding author. E-mail: xfli@scu.edu.cn. Tel: +86-28-85405389. Fax: +86-28-85410957.

MATERIALS AND METHODS

Plants material

Immature fruits (30–40 days after pollination) of *M. persiciforma* were collected from the orchards located in Pan Zhihua City, Sichuan province, China (Figure 1A).

Explant excision and pretreatment

Immature fruits were washed thoroughly with soap and then rinsed with tap water. They were then surface sterilized using 0.1% mercuric chloride containing sodium lauryl sulfate (0.1%) as surfactant for 15 min and rinsed with sterile double distilled water 3–4 times. Each rinse was 3 min long. The sterilized fruits were cut longitudinally into two halves and the intact ovules were removed.

The nucellus from each ovule was extracted and quickly transferred into one of three different antioxidant treatment solutions. Treatment 1: consisted of L-cysteine HCl at 0.1 mg/ml; treatment 2 was L-cysteine HCl (0.1 mg/ml) + L-ascorbic acid (0.1 mg/ml) + citric acid (0.1 mg/ml); and treatment 3 was sodium thiosulphate (200 mg/ml). The nucellar cultures were maintained on a shaker with a rotating speed set of 30 r min⁻¹ at 26°C for 60 min.

Induction experiments

After pre-treating with antioxidant solutions, the explants from treatment 2 were transferred into all the different liquid induction medium with the pH 5.8 (Table 1) and pre-cultured for one day on a constant temperature shaker set at 26°C and 30 r min⁻¹ in light. Each 350 ml jar contained 150 ml of liquid medium and about 20 explants. The explants were transferred onto the same induction medium in semi-solid (Table 1) supplemented with 6.5 g/l agar. The pH of all media was adjusted to 5.8 using 0.1 N KOH or 0.1 N HCl before autoclaving at 121°C for 15 min. Nucellar explants were cultured in petri dishes with the incision surface in contact with induction medium A, B, C, D1 and D2 (Table 1). During the 2-week induction period, the explants were kept in dark at 23 ± 2°C. During the first week, the explants were transferred to the fresh medium of the same composition every day. Then, the explants were maintained under 16/8 h photoperiod for 4 weeks at 25 ± 2°C. Cultures in the D1 medium treatment were transferred onto D2 medium. Cultures in all other treatments were transferred onto the same medium.

Regeneration experiments

After four weeks, proembryonic masses (PEMs) developed on the explants. The PEMs were separated and sub-cultured onto proliferation medium (Table 1) in jars and were maintained under 16/8 h photoperiod for 5 weeks at 25 ± 2°C. Each 350 ml culture jar contained 100 ml proliferation medium and six PEMs. Somatic embryos developed, germinated and formed entire plants with roots and green cotyledons and shoot tips. When the length of roots or shoots was 1 - 2 cm long, the plants were transplanted to the greenhouse.

Each treatment consisted of three petri dishes or three jars containing 6 explants each. Data were collected after the beginning of the induction and regeneration experiments. The entire experiment was repeated three times.

Statistical analysis

Somatic embryogenic response was evaluated as the number of

nucellus that produced PEMs or the number of somatic embryos generated. Adventitious bud numbers are presented as the means (± standard error) of buds regenerated per PEM. Regeneration rate is expressed as the average percentage (± standard error) of PEMs observed. Data were analyzed by analysis of variance.

RESULTS AND DISCUSSION

Pre-treatment and pre-culture

In vivo phenol, *in vitro* phenolic exudation, phenylalanine ammonialyase, peroxidase and polyphenol oxidase were affected by pre-treatment with antioxidants (Hare Krishna, 2008). Pre-treatment with antioxidant's solution was found to remarkably affect explant browning during post-culture stages (Hare Krishna et al., 2008). Pretreatment of explants using liquid shaker culture helps in overcoming the exudation of phenolics from the cut ends of mango explants (Raghuvanshi et al., 1995). Our results showed that pre-culture in liquid medium could also affect the browning propensity of explants and promote the development of PEMs. The constant temperature shaking accelerated *in vivo* polyphenol exudation and the action with antioxidants. The resistance to browning of combination with pre-treatment and pre-culture was proved to be superior to single treatment or single pre-culture. Pre-treatment 2 and pre-culture in medium D1 exhibited least activities of phenols and oxidative enzymes (Table 2).

Secondary embryogenesis and organogenesis

After 2 weeks of incubation in the dark, some development was observed in all induction media. Some of the explants started to turn brown. Frequent culturing to fresh medium was beneficial to control browning. When exposed to light conditions and sub-cultured, PEMs developed rapidly; some of them became brownish. Induction occurred earliest in treatment 2 on medium D1/D2. This treatment combination also had the least browning. The fastest browning was seen in explants of treatment 3 on all media (Table 2).

In medium D2 with treatment 2, the PEMs developed faster and were compact than when treatment 2 was combined with medium A, B or C. Whitish, spongy tissue developed within 2 weeks after nucellar excision. A few PEMs in this treatment turned brown. However, PEMs still developed to a good size (2.2 - 4.0 cm²) and quality (80 - 90% forming in the inner layers whitish). In comparison, PEMs from treatment A were only 0.8 - 2.3 cm² and about 73.8% turned brown. Those from treatment B were somewhat larger than those from treatment, attaining a diameter of 1.5 - 3.2 cm². Fewer (58.7%) were brown. PEMs from treatment C were 1.3 - 2.6 cm²; and 63.5% turned brown.

After 5 - 7 days in proliferation medium development slowed compared to that attained during the induction period.

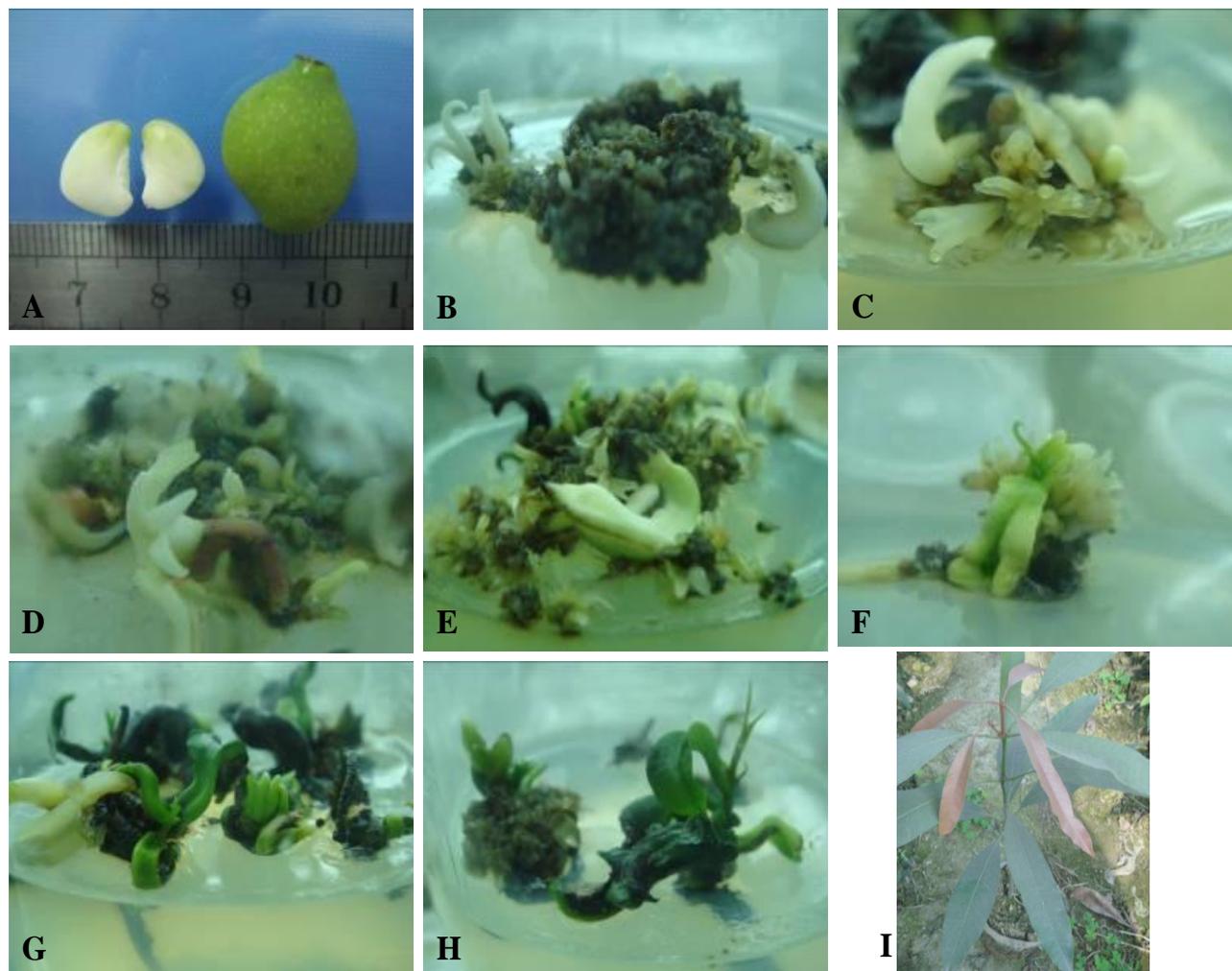


Figure 1. A-I, Somatic embryo induction and plantlet regeneration from nucellar explants of immature fruit of *Mangifera persiciforma*. A Immature fruit (30 - 40 days after pollination); B PEMs, 2 weeks after transfer to the proliferation medium and exposure to light; C PEMs, 3 weeks after transfer to proliferation medium and exposure to light; D, Germinating somatic embryos; E, Large scale somatic embryo germination; F, Adventitious bud development; G, Plantlet development; H, Regenerated plantlet with shoots and roots; I, Plantlet in greenhouse.

Gradually, the surfaces of the PEMs turned brown or black and the cells on the surface died. However, the inner layers remained whitish or light green. Secondary embryogenesis was apparent within 2 weeks of transfer to proliferation medium. 4 weeks later, the brown surface had enlarged and continued to produce new embryos as long as the inner layers remained whitish or light green (Figure 1B). The different somatic embryo stages (globular, heart-shaped, torpedo and cotyledon) were observed (Figure 1B, C and D). Both normal and abnormal somatic embryogenesis occurred, but only the normal somatic embryos germinated. Newly emerged shoots were generally off-white, but turned green.

The greatest regeneration was observed in the D2 treatment, although the B2 treatment was comparable. The least amount of regeneration occurred in the C2 treatment (Figure 2). Shoots of the regenerated plantlets

were shoots 5 - 8 mm in height and the roots were 2 - 4 mm in length (Figures 1F and G). Five weeks later, the shoots had grown to 15 - 25 mm in height and the roots 12 - 15 mm in length (Figure 1H). PEMs from treatment D2 continued to produce plantlets for more than one year. However, morphogenic capability gradually decreased after 9 months in proliferation medium. Conversely, morphogenic masses developing from treatment B2 maintained regeneration ability for 7 - 9 months. Treatment C2 allowed PEMs to maintain regeneration ability for 5 - 7 months. Regeneration ability of PEMs from treatment A2 was limited to 3 months.

During somatic embryos germination, plantlets with roots and green shoots have been obtained (Figure 1H). However, the roots are mainly taproots only, largely lacking root hairs. A few somatic embryos only produced shoots without any roots. Shoot tip necrosis and shoot

Table 1. Induction and regeneration media used in the different experiments.

| Constituent | Induction media | | | | | Proliferation medium |
|-------------------------|-----------------|------|------|------|------|----------------------|
| | A | B | C | D1 | D2 | |
| Macro-elements | MS/2 | B5 | LP | DKW | DKW | DKW |
| Microelements | MS | B5 | LP | DKW | MS | MS |
| FeNa ₂ EDTA | MS | B5 | LP | DKW | DKW | DKW |
| Nicotinic acid (mg/ l) | 0.5 | 1.0 | 0.5 | 1.0 | 1.0 | 1.0 |
| Glycine (mg/ l) | 2.0 | - | 2.0 | 2.0 | 2.0 | 2.0 |
| Pyridoxine HCl (mg/ l) | 0.5 | 1.0 | 0.5 | - | 0.5 | 0.5 |
| Thiamine HCl (mg/ l) | 0.1 | 1.0 | 1.0 | 2.0 | 2.0 | 2.0 |
| Ascorbic acid (mg/ l) | - | - | 10 | - | 10 | 10 |
| Citric acid (mg/ l) | - | - | 10 | - | 10 | 10 |
| Myo-inositol (mg/ l) | 100 | 100 | 100 | 100 | 100 | 100 |
| BA (mg/ l) | - | 3.0 | 3.0 | - | 3.0 | 0.8 |
| TDZ (mg/ l) | 2.0 | - | - | - | - | - |
| IBA (mg/ l) | - | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| GA ₃ (mg/ l) | - | - | - | - | - | 0.1 |
| 2,4-D (mg/ l) | - | - | - | 1.0 | - | - |
| Sucrose (mg/ l) | 60 | 60 | 60 | 60 | 60 | 30 |

MS: Murashige and Skoog (1962); B5: Gamborg et al., (1968); LP: Quoirin and Lepoivre, (1977); DKW: Driver and Kuniyuki, (1984).

Table 2. Effect of browning with different pre-treatment and pre-culture of nucellar explants.

| Pre-treatment + Pre-culture | 2 Weeks | 2 Weeks | 3 Weeks | 4 Weeks | p |
|-----------------------------|-------------|-------------|-------------|-------------|-------|
| | (in dark) | (in light) | (in light) | (in light) | |
| Browning % | | | | | |
| Treatment 1 + medium A | 19.4±1.5 b | 55.6±3.6 c | 61.1±1.8 c | 63.9±1.6 b | ≤0.05 |
| Treatment 2 + medium A | 17.7±1.1 bc | 38.9±2.5 ef | 47.2±1.4 e | 52.8±2.0 d | ≤0.05 |
| Treatment 3 + medium A | 30.6±2.4 a | 88.9±1.4 a | 94.7±1.0 ab | 94.7±2.3 a | ≤0.05 |
| Treatment 1 + medium B | 16.7±1.4 bc | 47.2±1.7 d | 52.8±2.0 d | 58.3±1.3 c | ≤0.05 |
| Treatment 2 + medium B | 13.9±1.3 c | 33.3±1.8 f | 41.7±2.2 e | 44.4±1.4 e | ≤0.05 |
| Treatment 3 + medium B | 27.8±1.8 a | 83.3±1.8 ab | 88.9±1.9 b | 91.7±1.1 a | ≤0.05 |
| Treatment 1 + medium C | 16.7±1.7 bc | 50.0±2.4 cd | 58.3±2.0 c | 61.1±2.0 bc | ≤0.05 |
| Treatment 2 + medium C | 13.9±1.4 c | 38.9±2.1 ef | 44.4±2.0 e | 50.0±1.2 d | ≤0.05 |
| Treatment 3 + medium C | 30.6±2.6 a | 86.1±2.1 ab | 91.7±1.6 a | 94.7±1.1 a | ≤0.05 |
| Treatment 1 + medium D1/D2 | 13.9±1.0 c | 44.4±2.4 de | 52.8±2.1 d | 58.3±0.9 c | ≤0.05 |
| Treatment 2 + medium D1/D2 | 8.3±1.0 d | 19.4±1.5 g | 25.0±1.0 f | 27.8±1.0 f | ≤0.05 |
| Treatment 3 + medium D1/D2 | 27.8±1.5 a | 80.6±3.8 b | 91.7±1.6 ab | 91.7±1.0 a | ≤0.05 |

The values are expressed in mean browning percent ± standard error.

etiolating were also observed.

Regenerated plantlet transplanting to soil

The regenerated plantlets were very sensitive to environmental stress and disease. The survival rate was usually very low and was about 20 - 30%. Humidity and temperature control were crucial to plantlet survival.

Before the plantlets were transplanted into greenhouse, their roots were dipped in a solution of indole butyric acid (IBA) (500 mg/l) for 30 – 60 s. On the first day or second day after transplanting (Figure 11), the plantlets should be sprayed with fungicides. The first week, the relative humidity and temperature must be maintained at 90 - 95% and 26 ± 2°C, respectively. After this, aeration can be used to decrease the relative humidity and the temperature may be increased to 28±2°C. After weeks,

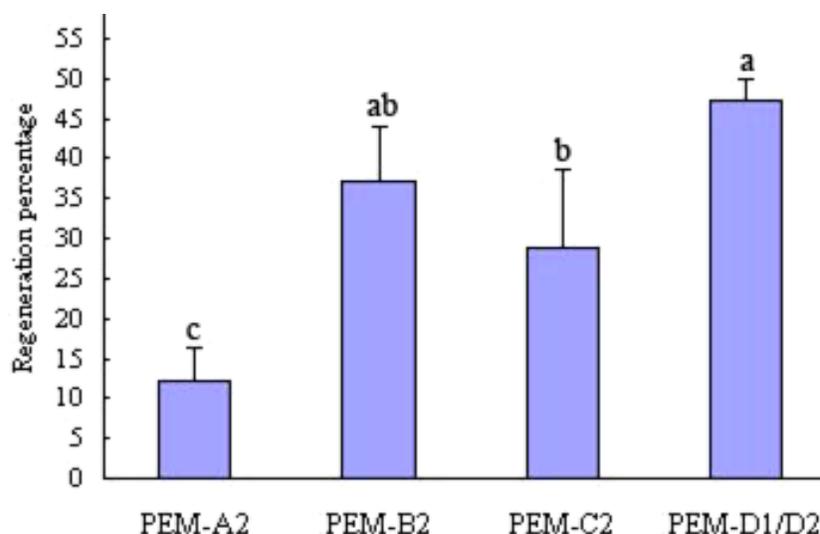


Figure 2. Average percentage of PEMs that developed into differentiated plantlets data were collected 4 weeks after the PEMs were transferred to light. Means with the same letter are not significantly different from each other at $p=0.05$.

the plantlet developed some new roots and the survival rate could be increased to about 75%.

The results indicate that the combination of pre-treating in antioxidant solution and pre-culturing in liquid medium significantly controlled explants browning. Use of modified DKW medium was beneficial in improving embryogenic response and enhancing regeneration rate from nucellar explants. An effective greenhouse management strategy was used to increase the survival rate of regenerated plantlets.

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