Title：

**Cyclic Secondary Somatic Embryogenesis And Plant Regeneration *In Vitro* Leaf Culture of *Rosa multiflora* Thunb. var. *carnea* Thory**

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Running title: Repetitive Secondary Somatic Embryogenesis And High Frequency Plant Regeneration of *Rosa multiflora*

**Abstact**

In this study, a plant optimal regeneration system, via repetitive secondary somatic embryogenesis, is developed and optimized in *Rosa multiflora* Thunb. var. *carnea* Thory. Primary somatic embryogenesis was initiated from leaf tissues of two accessions of *R. multiflora* var. *carnea* (namely RmcType2 and RmcType4). The highest induction frequency, 4.63% for RmcType2 and 5.01% RmcType4, was achieved after 2-3 months culture on the medium MS + 400 mg l-1 L-proline +10 mg l-1 AgNO3 + 30 g l-1 Glucose + 3.0 g l-1 Phytagel supplemented with 4.0 mg l-1 2, 4-D and 5.0 mg l-1 2, 4-D respectively. Cyclic secondary somatic embryogenesis was long maintained by periodical proliferation. Effective proliferation was obtained on the medium MS + 1.0 mg l-1 2, 4-D + 0.01 mg l-1 BA + 30 g l-1 Glucose + 3.0 g l-1 Phytagel in the light intensity of 50-100 lx and on the medium MS + 1.0 mg l-12, 4-D + 30 g l-1 Glucose + 3.0 g l-1 Phytagel in the darkness respectively. The highest secondary somatic embryo germination rate, 92% for RmcType2 and 83% RmcType4, was obtained on the same medium MS + 0.5 mg l-1 BA + 1.0 mg l-1 TDZ + 30 g l-1 Glucose + 3.0 g l-1 Phytagel. The whole plantlets of the accessions were recovered from germinated secondary somatic embryos on the medium MS + 0.5 mg l-1 BA + 0.01 mg l-1 NAA + 30 g l-1 Glucose + 3.0 g l-1 Phytagel. The optimized regeneration system here supplies a referenced model for other woody plants and made a foundation of the transformation for *R. mutiflora*.

**Key words:** Leaf, Primary somatic embryos induction, Cyclic secondary somatic embryogenesis, Plant regeneration

**Introduction**

*Rosa multiflora*, distributing around the world but many in Asia, with characteristics of fast growth, disease and heat resistance. Usually *R. multiflora* is used as grafted rootstocks, as well as breeding parent, for other *Rosa* species, such as *Rosa hybrida*, *Rosa rugosa*. While the varied genotypes of *R. multiflora* lead to the great diversity among different cultivars, particularly, limited adaptability restricts the geographical distribution of the optimal plants. It is an important prerequisite to improve the *R. multiflora* and to select an optional plant used as rootstock and breeding parent in related species. The traditional ways to propagating and breeding *R. multiflora* need long time and has great boundedness. Tissue culture and genetic engineering provides an opportunity to expand the optimal plants in short time and overcome the restrictions in traditional propagations.

Somatic embryogenesis had been verified to be an efficient method in regeneration and genetic engineering on more and more woody species (Prakash and Gurumurthi 2010, Vergne et al. 2010, Paul et al. 2011, Martín et al. 2012). Successful establishment of regeneration systems via somatic embryogenesis for a number of rose species have been documented (Li et al. 2002, Estabrooks et al. 2007, Vergne et al. 2010, Bao et al. 2012). Cyclic secondary somatic embryogenesis had not been reported in most of previous literatures related to regeneration of Rosa species. There also are limited reports describing to use those mature tissues as plant materials for vitro culture proceeding somatic embryogenesis (Kintzios et al. 1999, Bao et al. 2012). Repetitive secondary somatic embryogenesis derived from mature tissue of optional plants not only supply an effective method for regeneration, but also provide a foundational material for further transformation. There are many optimal plants of *R. multiflora* that haves great adaptabilities to particular regions, which can be used as rootstocks or breeding parents to perform other *Rosa* species breeding or cultivating. While, until now, no reports presented a cyclic secondary somatic embryogenesis regeneration protocol on plant regeneration of *R. multiflora* except for other regeneration pattern like protocorm-like bodies which induced from rhizoids that developed from leaf explants (Tian et al. 2008). Therefore, establishing a cyclic secondary somatic embryogenesis will facilitate to the future rose breeding and cultivation.

In the present paper, in order to expand and preserve the optimal two strong heat resistance *R. multiflora* plants, an embryogenesis based plant regeneration system, including primary somatic embryogenesis initiation, cyclic secondary somatic embryogenesis maintenance by periodical proliferation and embryos germination, were indicated. The effects of plant growth regulators and other culture conditions on the primary somatic embryos induction and secondary somatic embryos proliferation were also indicated in detail. The developed regeneration model here in *R. multiflora* suggests a referenced pattern for other woody plants and is significant to other *Rosa* species propagation and breeding.

**Materials and methods**

**Plant materials and culture conditions**

Unexpanded leaves, used for primary somatic embryos induction, were collected from the in-vitro culture sterile shoots through stem micropropagation. The originated shoots explants were harvested from 2 optional plants of rose (*R. multiflora* var. carnea) growing in the field of Huazhong Agricultural University, Wuhan, China and cultured on MS medium supplemented with 0.5 mg l-1 6-Benzyladenine (BA), 0.01mg l-1 α-Naphthalene acetic acid (NAA), 30 g l-1 Sucrose and 7.5 g l-1 Agar (Dingguo Changsheng Biotechnology, Beijing, China). The sterile shoots were recultured every 4 weeks on the same medium. All culture conditions are the same to that reported by Ning et al. (2007). Unless mentioned otherwise, the basal culture medium used throughout the experiments consisted of MS basal salts and vitamins (Murashige and Skoog 1962) supplemented with 30g l-1 glucose, and solidified with 3g l-1PhytagelTM(Sigma-Aldrich, China).

**Primary somatic embryos induction**

In this step, there sets of factors were investigated to find the optimal culture condition. These are as following: 1) MS medium supplemented with 30g l-1 glucose and 3g l-1 Phytagel composed the basal medium, then the leaflets, from RmcType2 and RmcType4, were plated with their adaxial side down onto the basal medium supplemented with the combinations of 2, 4-Dichlorophenoxyacetic acid (2, 4-D) (1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 mg l-1) and (0, 400mg l-1) L-proline. The cultures were incubated in darkness for 2 months, and subcultured onto the same fresh medium every 4-5 weeks. 2) The induction basal medium supplemented with 3.0 mg l-1 2, 4-D and 400mg l-1 L-proline was used to investigate the effect of AgNO3 on primary embryos induction with varied concentration(0, 5, 10, 15 and 20 mg l-1). 3)The basal medium containing 10 mg l-1AgNO3, varied combinations of 2, 4-D (3.0, 4.0, 5.0, 6.0 and 7.0 mg l-1) and L-proline (0, 400mg l-1) were used again to optimize the primary somatic embryos induction.

**Proliferation and maintenance of somatic embryos**

To optimize secondary somatic embryos proliferation for the two genotypes, MS medium supplemented with 1.0 mg l-12, 4-D, and 3g l-1 Phytagel, combining with varied glucose(30, 45 or 60 g l-1). Additionally, the medium containing 30 g l-1 glucose and 3 g l-1 Phytagel were tested deployed to investigate the effect of plant growth regulators (PGRs) on embryos proliferation by supplementing with complete combinations of 2, 4-D (0, 0.5 or 1.0 mg l-1) and BA (0, 0.005 or 0.01 mg l-1).

**Plant conversion from secondary somatic embryos**

Cotyledonary somatic embryos derived from the secondary embryos of two optional plants were cultured on MS or 1/2MS basal medium containing 30g l-1glucose and 0.5 mg l-16-Benzyladenine (BA), was supplemented with 0.1mg l-1Gibberellic acid (GA3), 1.0 mg l-1Thidiazuron (TDZ) or 0.05 mg l-1 α-Naphthalene acetic acid (NAA) separately to find the optimal conditions for embryo germination. As the shoots or bipolar plantlets were observed on somatic embryos, they were transferred to the MS medium supplemented with 0.5 mg l-1BA, 0.01 mg l-1NAA, 30 g l-1glucose and 3g l-1Phytagel to grow the generated plantlets.

**Statistical analysis**

For all above experiments, each treatment contained 3 replicates (each replicate 12-22 explants). All data were analyzed using ANOVA and means were compared using the least significant difference (LSD). Data analysis was performed using the SAS software. The proliferation coefficient of somatic embryos was calculated as reported by Bao et al. (Bao et al. 2012). Percentage data were transformed by arcsine before analysis.

**Results**

**Primary somatic embryogenesis initiation**

Two optional plants (Fig. 1 A, B) of *R. multiflora* had been used to generate sterile shoots via *in vitro* stem culture. Unexpanded leaflets were deployed to promote primary embryos initiation. After 2 weeks of culture on embryo induction medium, adventitious roots were usually observed on petioles. Few of leaf explants formed somatic embryos through continuously cultured for 2-3 months (Fig. 1 C). Low concentration 2, 4-D (1.0, 2.0 mg l-1) causes the petioles to swell and gradually become brown, whereas high (3.0, 4.0, 5.0, 6.0, 7.0 mg l-1) concentrations promote the more callus formation and becoming brown faster, with adventitious roots formation (Fig. 1 C).

For investigating the effect of 2, 4-D and L-proline, most of explants from RmcType2 and RmcType4 had not formed somatic embryos on the induction medium (Table 1). With L-proline, primary somatic embryos were observed on medium supplemented with various concentration 2, 4-D. There were also no differences on the frequency of somatic embryo formation (Table 1) among different concentrations of 2, 4-D in each genotypes. The maximum frequency of primary embryos formation was 4.63% in RmcType2 with the induction medium containing 4.0mg l-1 2.4-D and 400 mg l-1 L-proline, and 5.01% in RmcType4 on medium containing 5.0mg l-12.4-D and 400 mg l-1L-proline.

The only AgNO3 level that affected primary embryo formation was the highest level of 20 mg l-1(Table 2).

**Proliferation and maintenance of somatic embryos**

Although the rate of primary somatic embryogenesis initiation was low (4-5%), secondary somatic embryos could be efficiently induced from primary embryos (Fig. 1 D-G). The optimal levels of glucose for somatic proliferation was 30g l-1 glucose for RmcType4 (Fig. 1 D-F) and 45 g l-1 glucose for RmcType2 (Fig. 1 G) (Table 3). All the stages of those secondary somatic embryos formation and development were observed, like globular (Fig. 1 D) and cotyledonary somatic embryos (Fig. 1 E-F) without browning and abnormal morphology.

The proliferation efficiencies on medium containing 1.0 mg l-1 2, 4-D was higher than that of 0.5mg l-1in both genotypes. Those proliferated embryos appeared healthily and become golden-yellow color on medium supplemented with1.0 mg l-1 2, 4-D and 0.01 mg l-1 BA in RmcType2, while on the medium with 0.5 mg l-1 2, 4-D and 0.005 mg l-1 BA in RmcType4. Generally, the added BA promoted secondary somatic embryos proliferation (Table 4), but usually the proliferated somatic embryos turned green and germinated as the concentration of BA increased(Fig. 1 G). In addition, it was observed that cyclic secondary somatic embryos developed vigorously and proliferated well in the dark, and that light caused a negative effect on embryos proliferation (data not show).

**Plantlets regeneration from cotyledonary somatic embryos**

The highest germination frequencies of somatic embryos germination, 92% in RmcType2 and 88% in RmcType4, were obtained by culture on MS medium supplemented on 0.5 mg l-1 BA , 1.0 mg l-1 TDZ and 30 g l-1 glucose. When cotyledonary secondary somatic embryos were transferred to the germination medium, the cotyledons or the whole embryo turned greening. Usually, it was observed that the germinated embryos showed green cotyledons with many rather long roots (Fig. 1 H). On the 0.5 mg l-1 BA medium, the addition of other hormones (GA3, TDZ or NAA) changed germination frequencies substantially (Table 5). There was an interaction in response among MS or 1/2MS medium and various hormones treatments (Table 5). After the embryos were transferred into MS medium supplemented with 0.5 mg l-1 BA, 0.01mg l-1NAA and 30 g l-1 Glucose and cultured for 2-3 months, about 95 % of the embryos regenerated plantlets (Fig. 1 I).

**Discussion**

In the present study, an alternative efficient plant generation system in which the plant is regenerated by proliferated secondary somatic embryos via cyclical secondary smatic embryogenesis is reported in two cultivars of *Rosa multiflora* var. *carnea*. The report not only supply a way to propagate *R. multiflora* plants but also establish a regeneration method for transformation.

Currently， no literature describes cyclic somatic embryogenesis in *R.multiflora*, although somatic embryogenesis or cyclic secondary somatic embryogenesis had been to be induced in other Rose species (Vergne et al. 2010, Bao et al. 2012). 2, 4-D is routinely used hormone in promoting somatic embryogenesis and usually primary somatic embryos could be induced in most species by combining with 2, 4-D (Jiménez 2001, Thorpe 2007, Bao et al. 2012). In the present study, 2, 4-D used alone and ranging in a certain concentration, had only promoted the initiation of somatic embryogenesis in RmcType4 plant in *R.multiflora*. L-proline can stimulate somatic embryogenesis and improve the quality of embryos in rose, thus the combination of L-proline and 2, 4-D could promote the initiation of somatic embryos (Marchant et al. 1996, Das 2010). Our experiments in *R. multiflora* also confirmed the deduced results and found that L-proline played a key role in primary somatic embryos induction with reducing the brown of leaflets and enhancing primary somatic embryogenesis.

AgNO3 has been reported to promote somatic embryogenesis from embryogenic callus in some woody plants (Pullman et al. 2003, Parimalan et al. 2011) and it is also reported to enhance direct somatic embryogenesis from immature zygotic embryos and primary embryos’ formation in durum wheat (Fernandez et al. 1999). In our study, no effects on the primary embryo induction were observed between the AgNO3 free and AgNO3 containing medium. In contrast, high concentration of AgNO3 promoted explants to brown and leaded to no somatic embryo formation.

Cyclic secondary somatic embryogenesis is a best way to creat and preserve somatic embryos, especially for these plants in that few of primary embryos were induced from explants. Adjustment of osmotic pressure is more important to the long maintenance of somatic embryos. Glucose not only provides the requirements of carbon sources but also influences the osmotic pressure in the culture medium. High concentration of carbohydrate engenders a high osmotic stress, which had been to promote somatic embryos formation (Agarwal et al. 2004). In our study, the optimal concentration of glucose used on RmcType2 was 45 mg l-1, and RmcType4 was 30 mg l-1. Higher concentration of glucose promoted the germination and produced abnormal embryos. When lower than the optimal concentration, the proliferation velocity of secondary somatic embryos increased with the concentration of glucose increased.

Cramer and Bridgen (1997) reported that BA concentration between1.0 - 2.0 mg l-1improved the proliferation of embryos in *Mussaenda* cultivars. Bao et al. (2012) also indicated that the combination of 2, 4-D and BA promoted the secondary somatic embryogenesis in *R. hybrida*. The same phenomenon was seen our study. Higher concentrations of 2, 4-D (3.0 - 6.0mg l-1) stimulated the induction of primary somatic embryogenesis, and lower concentration (0 - 1.0 mg l-1) or free of 2, 4-D facilitated to the secondary somatic embryogenesis. When the medium were paired with particular concentration of BA, the proliferation frequency of secondary somatic embryos improved. In addition, darkness had a positive effect on somatic embryo proliferation, without decreasing their quality. A similar phenomenon was reported on proliferation of somatic embryos in *Eucalyptus globulus* (Pinto et al. 2008).

PGRs had been documented to have a significant influence on the germination of secondary somatic embryos. BA promotes the development of shoots and roots of somatic embryos and increases the frequency of bipolar germination in some other Rosa species (Sarasan et al. 2001, Kim et al. 2009, Chen et al. 2010). GA3 is particularly necessary to interrupt somatic embryo dormancy and promote the development of somatic embryos (Chen et al. 2010, Punja et al. 2004). TDZ promotes the maturation of somatic embryos (Khan et al. 2006, Shi et al. 2010, Bao et al. 2012). The combination of NAA and BA successfully lead to rooting of somatic embryos in *Bixa orellana* (Parimalan et al. 2011). In our study, the best result was obtained on those medium containing BA and TDZ together. Generally, though varied PGRs could lead the secondary somatic embryos to germinate effectively, it is found that the germination rate of embryos cultured on medium supplemented with BA and other PGRs were higher than that on those medium with BA alone. BA and other PGRs maybe promote the germination of embryos together. In addition, better results on germination were obtained on MS medium as far as the types of basal medium were concerned.

In conclusion, a primary and cyclic secondary somatic embryogenesis procedure was established starting with *in vitro* leaf culture from two genotypes of *R. multiflora*. The efficient secondary somatic embryogenesis regeneration model not only suggests a referenced ways of using somatic embeyos for other woody plants to expand and preserve germplasm, but also is significant to propagation and breeding of other *Rosa* species.

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**Table 1. Effect of 2, 4-D, L-proline and AgNO3 on induction of somatic embryogenesis in RmcType2 and RmcType4.**

|  |  |  |  |
| --- | --- | --- | --- |
| 2,4-D(mg l-1) | L-proline(mg l-1) | Frequency of somatic embryo induction（%） | |
|
| RmcType2 | RmcType4 |
| 3.0 | 0 | 0 | 0 |
| 4.0 | 0 | 0 | 0 |
| 5.0 | 0 | 0 | 1.59±2.75a |
| 6.0 | 0 | 0 | 0 |
| 3.0 | 400 | 4.36±0.19a | 0 |
| 4.0 | 400 | 4.63±0.33a | 3.04±2.64a |
| 5.0 | 400 | 1.52±2.72a | 5.01±0.25a |
| 6.0 | 400 | 0 | 4.36±0.19a |

Data were recorded after leaflets cultured on MS medium supplemented for 1 month with10 mg l-1 AgNO3 and varied combinations of 2, 4-D and L-proline, and then subcultured onto new fresh medium contain 1.0 mg l-1 2, 4-D in the dark for 2 months.

Values shown are means ± standard errors, values significantly different within columns at the 5% level of significance are indicated with different small letter.

**Table 2. Effect of AgNO3 on primary embryos induction of RmcType2 optional plant.**

|  |  |
| --- | --- |
| AgNO3 Concentration (mg l-1) | Frequency of somatic embryo induction（%） |
|
| 0 | 1.85±3.21a |
| 5 | 2.97±2.57a |
| 10 | 4.63±0.33a |
| 15 | 3.12±2.72a |
| 20 | 0 |

All data were recorded after leaflets cultured on basal culture medium with AgNO3, 3.0 mg l-1 2, 4-D and 400 mg l-1 L-proline in the dark for 1 moth, then subcultured onto new fresh medium supplemented with 1.0 mg l-1 2, 4-D in the dark for 2-3 months.

Values shown are means ± standard errors, values significantly different within columns at the 5% level of significance are indicated with different small letter.

**Table 3. Effect of different glucose concentrations on somatic embryo proliferation in RmcType2 and RmcType4.**

|  |  |  |  |
| --- | --- | --- | --- |
| Genotype | Concentration of Glucose (g l-1) | Proliferation coefficient of somatic embryos | Growth state of somatic embryos |
|  | 30 | 2.54±0.25a | ++ |
| *RmcType2* | 45 | 3.23±0.60a | +++ |
|  | 60 | 1.86±0.39a | + |
|  | 30 | 3.47±0.51a | +++ |
| *RmcType4* | 45 | 2.42±0.27ab | ++ |
|  | 60 | 1.99±0.28b | + |

Data were recorded after secondary embryos cultured on MS medium supplemented with 1.0 mg l-1 2, 4-D, and 3.0 g l-1 Phytagel and different Glucose for 1 month.

Values shown are means ± standard errors, values significantly different within columns at the 5% level of significance are indicated with different small letter.

(+ indicates that somatic embryos grow normally; ++ indicates somatic embryos grow well; +++ indicates somatic embryos grow vigorously.).

**Table 4. Effect of different combinations of 2, 4-D and BA on somatic embryo proliferation in RmcType2 and RmcType4.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Genotype | 2,4-D(mg l-1) | BA(mg l-1) | Proliferation coefficient of somatic embryos | Growth state of somatic embryos |
|  | 0.5 | 0 | 2.47±0.31b | ++ |
|  | 0.5 | 0.005 | 3.19±0.30ab | +++ |
| *RmcType2* | 0.5 | 0.01 | 2.61±0.06b | ++ |
|  | 1.0 | 0 | 2.83±0.47b | ++ |
|  | 1.0 | 0.005 | 3.93±0.11a | +++ |
|  | 1.0 | 0.01 | 4.15±0.37a | +++ |
|  | 0.5 | 0 | 1.48±0.36b | + |
|  | 0.5 | 0.005 | 3.55±0.70a | +++ |
| *RmcType4* | 0.5 | 0.1 | 2.83±0.35ab | ++ |
|  | 1.0 | 0 | 2.36±0.50ab | ++ |
|  | 1.0 | 0.005 | 1.42±0.05b | + |
|  | 1.0 | 0.1 | 3.32±0.38a | ++ |

Data were recorded after secondary embryos cultured on MS medium supplemented with 30 g l-1 glucose and varied combinations of 2, 4-D and BA for 1 month.

Values shown are means ± standard errors, values significantly different within columns at the 5% level of significance are indicated with different small letter.

(+ indicates that somatic embryos grow normally; ++ indicates somatic embryos grow well; +++ indicates somatic embryos grow vigorously).

**Table 5. Effect of the mediums and plant grown regulator on the secondary somatic embryos germination of RmcType2 and RmcType4.**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Medium | GA3  (mg l-1) | TDZ  (mg l-1) | NAA  (mg l-1) | Germination frequency of somatic embryos (%) | |
| RmcType2 | RmcType4 |
| MS | － | － | － | 44.4±9.6c | 87.6±13.8a |
| 1/2MS | － | － | － | 36.1 ±4.8c | 46.7±5.8c |
| MS | 0.1 | － | － | 77.8±6.9ab | 46.7±6.7c |
| 1/2MS | 0.1 | － | － | 88.9±9.6a | 85.4±13.8a |
| MS | － | 1.0 | － | 91.7±8.3a | 88.3±4.4a |
| 1/2MS | － | 1.0 | － | 39.9±14.3c | 83.9±8.1a |
| MS | － | － | 0.05 | 79.2±8.2ab | 77.5±12.3ab |
| 1/2MS | － | － | 0.05 | 68.5±7.4b | 64.9±8.8b |

Data were recorded after secondary embryos cultured on MS or 1/2MS medium supplemented with 0.5mg l-1 BA, 30 g l-1 glucose and 3g l-1 Phytagel and different PGRs additionally for 2 months.

Values shown are means ± standard errors, values significantly different within columns at the 5% level of significance are indicated with different small letter.