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Full Length Research Paper

Establishment of a high frequency plant regeneration system of *Rauvolfia verticillata* via somatic embryogenesis

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Rauvolfia verticillata **is a traditional Chinese medicinal plant which contains a variety of terpenoid indole alkaloids such as ajmalicine, reserpine, rescinamine, serpentinine and yohmbine. The present investigation has successfully established the high frequency plant regeneration system of** ***Rauvolfia verticillata*** **via somatic embryogenesis. Leaves and stems were used as explants to induce calli on MS medium with 5 mg/L 2,4-D, 0.5 mg/L NAA, 0.2 mg/L 6-BA and half-strength MS medium supplemented with 0.8 mg/L IBA, respectively. Embryogenic cells emerged when sub-culturing calli induced from stems on MS medium supplemented with 2.0 mg/L 6-BA and 0.05 mg/L NAA, while calli initiated from leaves could not form embryogenic cells under the same condition. Embryogenic cells were separated from ordinary calli and propagated. After a sub-culturing period of 2 weeks on basic MS medium, shoots emerged on embryogenic cells and could root well on half-strength MS medium containing 0.8 mg/L IBA to become intact plants. Our present research laid basic foundation for improving yield of terpenoid indole alkaloid in** ***R. verticillata*** **by taking advantage of transgenic technology.**

Key words: *Rauvolfia verticillata,* regeneration, somatic embryogenesis, embryogenic cell.

INTRODUCTION

Rauvolfia verticillata, which has a long cultivation history in China as well as other countries of Asia, is a rare and important medicinal shrub belonging to Apocynaceae family. The species plays an important role in pharmacology for its capacity in providing numerous terpenoid indole alkaloids (TIAs). *Rauvolfia* alkaloids, such as ajmalicine and reserpine are therapeutically applied for hypertension and cardiac disorders due to their antihypertensive and antiarrythmic properties (Anitha and Kumari, 2006). Reserpine was firstly isolated in 1952 from the dried root of *Rauwolfia serpentina* (Klohs et al., 1953), the anti-hypertensive actions of which were contributed to its ability to deplete catecholamines from peripheral sympathetic nerve endings. Ajmalicine, also known as raubasine, is structurally related to yohimbine and other yohimban derivatives which act as α_1-adrenergic receptor antagonist. In China, *R. verticillata* is the main resource of reserpine and ajmalicine.

However, natural resources of *R. verticillata* are limited and accumulations of medicinal TIAs in roots are also very low. The increasing demand for reserpine and ajmalicine incurred detrimental destruction to wild *R. verticillata* resources. Even though the chemical

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Abbreviations: 2, 4-D, 2, 4-dichlorophenoxyacetic acid; **6-BA,** 6-benzylaminopurine; **NAA,** naphthaleneacetic acid; **IBA,** indole-3-acetic acid; **MS,** Murashige and Skoog; **GA3,** gibberllic acid.

synthesis of reserpine and ajmalicine is possible, it costs more than extracting it from natural resources. So it is urgent to find an efficient way to provide natural sources of pharmaceutical TIAs.

Plant tissue culture is a powerful tool for the production of medicinal secondary metabolites as well as for pursuing genetic transformation efforts. With the elucidation of enzymatic biosynthesis of TIAs, metabolic engineering biotechnology is deemed as a promising way to solve the problem. For example, a host of remarkable achievements of transgenic research in medicinal plants including *Catharanthus roseus* (Liu et al., 2007), *Atropa belladonna* (Rothe et al., 2003) and *Artemisia annua* L. (Aquil et al., 2009) have been obtained by over-expressing key enzymatic genes.

However, there has been no report about transgenic achievements in *Rauvolfia* plants. One of the possible reasons accounting for this phenomenon is the difficulty in establishing high regeneration system of woody plants, which is a great handicap in carrying out the transgenic research.

In the present study, we successfully established high frequency regeneration system of *R. verticillata* via somatic embryogenesis, which would not only greatly facilitate the transgenic researches on TIAs in the future, but also propose a feasible method for preservation of the rare medicinal plant.

MATERIALS AND METHODS

Plant material and establishment of *in vitro* culture system

Seeds of *R. verticillata* were collected from Chongqing academy of Chinese material medica. After removing pericarp, seeds were immersed in water for 2 h to pick out full seeds depending on the conditions of submergence. Subsequently, full seeds were washed thoroughly under running tap water for 5 h. As a control, half of seeds were removed tough seed coats. To break rest period, GA_3 was used to treat seeds for 16 h. For surface sterilization, the seeds were then immersed in 70% (v/v) ethanol for 1 min and washed three times with distilled water.

Finally, the seeds were treated with 0.1% $HgCl_2$ solution for 15 min with constant shaking and washed five times with sterile double distilled water. After getting rid of excess water on surface with sterile filter paper, seeds were placed on basic MS medium (Murashige and Skoog, 1962) with 3% sucrose and 0.7% agar. The pH was adjusted to 5.85 with 1N NaOH before autoclaving. Seeds were kept at 25±1.0°C under 16 h photoperiod and light extensity was set as 55 μmol· m^{-2}·s^{-1}.

Induction and propagation of calli from different explants

Sterile leaves and stems were respectively used as explants to induce calli. For the induction of calli from leaves, 0.5 to 1cm leaf segments were excised from sterile plantlets and placed on MS medium supplemented with 10 mg/L 2, 4-D, 0.5 mg/L NAA and 0.2 mg/L 6-BA in darkness at 25±1.0ºC. To obtain calli from stems, 5 to 6 cm stems were placed on half-strength MS medium supplemented with 0.8 mg/L IBA under the same cultural condition. Both kinds of calli were excised from explants and maintained on MS medium supplemented with 5 mg/L NAA and 0.5 mg/L 6-BA for propagation and sub-cultured every 4 weeks in darkness at 25±1.0°C.

Establishment of embryogenic cell suspension culture

After propagating for 2 to 3 weeks, both kinds of calli were transferred to MS medium supplemented with 2.0 mg/L 6-BA and 0.05 mg/L NAA. About 3 weeks later, embryogenic cells emerged on the surface of stem calli. Subsequently, embryogenic cells were disintegrated with a 0.5 mm sieve and transferred to 150 ml flasks containing 30 ml of MS liquid medium supplemented with 3.0 mg/L 2,4-D for propagation. The cultures were maintained in shake incubator at the speed of 120 rpm in darkness at 25°C. Cell suspension cultures were sub-cultured every 2 weeks. As a control, calli induced from leaves were undergone the same treatment.

Plant regeneration from embryogenic cells

Embryogenic cell aggregates with proper size were collected and placed on MS basal medium to regenerate intact plants. Cultures were maintained at 25±1.0°C for 16 h light condition under 55 μmol· m^{-2}·s^{-1} light extensity. As a control, calli induced from leaves were also placed on the same medium under the same culture condition. Shoots regenerated from embryogenic cells were excised and transferred to half-strength MS medium containing 0.8 mg/L IBA.

RESULTS AND DISCUSSION

The establishment of *in vitro* culture system

To investigate the effects of tough testa on the germination rate of *R. verticillata*, half of seeds were removed seed coat. The contrast was obvious: seeds removed seed coats germinated about 2 weeks after sterilized and germination rate reached up to 80%. While, as a control, seeds with tough seed coats did not germinate even after 2 months.

The present study indicates that seed coat is a key factor which affects the germination rate of *R. verticillata.* After removing tough seed coat, the germination rate and ratio improved significantly. It maybe due to that seed coat handicapped the absorption of oxygen, water and other nutritional factors and the same situation was observed in other plants such as *Mimosa Invisa* (Chauhan and Johnson, 2008) and Arabidopsis (Debeaujon et al., 2000).

The induction of embryogenic cells

In the present work, two kinds of explants: leaf and stem were respectively used as initial materials for inducing calli. Calli of leaves formed on the surface of wounds two weeks after cultivated on MS medium added with 10 mg/L 2, 4-D, 0.5 mg/L NAA and 0.2 mg/L 6-BA. Calli of stems formed after 20 days cultured on half strength MS medium added with 0.8 mg/L IBA. Calli induced from different explants showed distinguished appearance

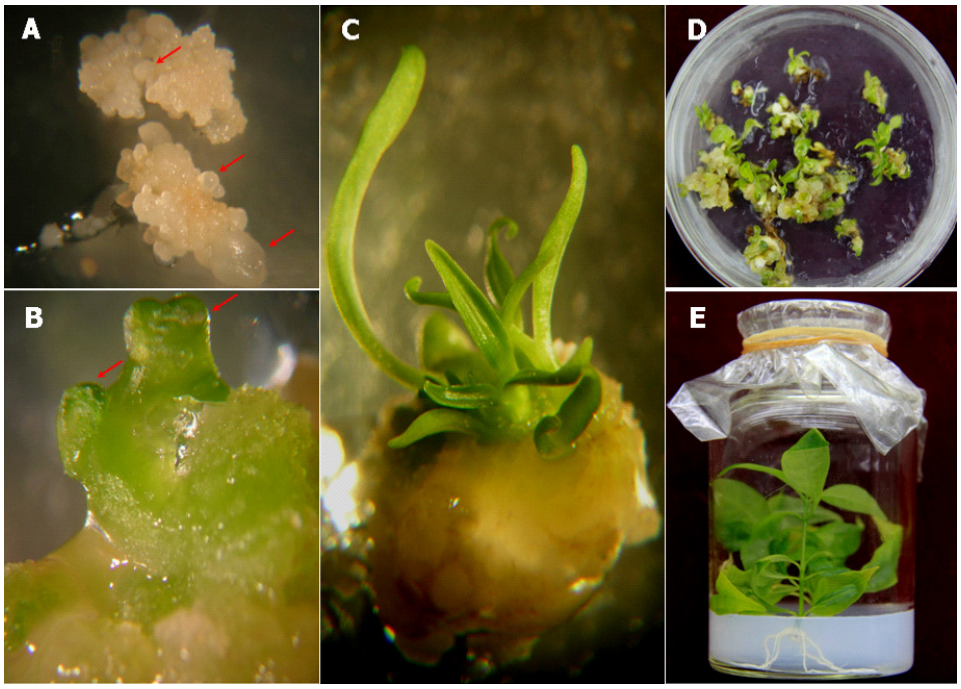

Figure 1. Plant regeneration of *R. verticillata*. A: Initiation of embryogenic cells (marked with an arrow) from calli obtained from stem; B: Bud (marked with an arrow) formation from embryogenic cells; C: Shoot formation from buds; D: High-frequency shoot formation; E: Well-rooted plantlets of *R. verticillata*.

patterns. Calli of stems were much more compacted than calli of leaves and the color of the former were much darker compared to that of the latter.Somatic embryogenic cells formed 3 weeks later when culturing calli induced from stems on MS medium supplemented with 2.0 mg/L 6-BA and 0.05 mg/L NAA, exhibiting pale-yellow globular structures. By contrast, when cultivating on the same medium, calli of leaves could propagate well but could not be able to form embryogenic cells.

A great variety of explants can be used for induction of somatic embryos including zygotic embryo (Oh et al., 2010; Kim et al., 2004), shoot apices (Liu et al., 2001), petiole (Zhang et al., 2005), leaf and stem (Wang et al., 2006). Our present work established an optimum efficient regeneration system of *R. verticillata* using stem as explant.

The regeneration of shoots from embryogenic cells

When transferred to MS basal medium, somatic embryos regenerated into buds after cultivating 2 weeks. Shoots grew well and could root on MS medium without any rooting auxins. To accelerate the propagation, shoots were cut from (Figure 1) embryogenic cells and transferred to half-strength MS medium containing 0.8 mg/L IBA for inducing calli again. Rooted regenerated plantlets could easily survive when transferred to potting soil. As a control, calli induced from leaves grew vigorously on MS medium added with 2.0 mg/L 6-BA and 0.05 mg/L NAA. However, when transferred to MS basal medium without phytohormone, calli could not grow well and became brown. The results proved the fact that somatic embryos can develop into the bipolarity structure in MS medium without phytohormone while the ordinary calli can not. Over the past two decades, somatic embryogenesis has been the major plant regeneration system (Fraley, 2009). It is widely accepted that somatic embryogenesis have many advantages over organogenesis such as good genetic stability and high propagation efficiency. Apart from that, somatic embryos of some plants can accumulate medicinal ingredients (Fulzele and Satdive, 2003). With the elucidation of enzymatic steps, metabolic engineering is deemed as a promising technology to improve alkaloid contents in natural resources. In order to obtain transgenic plants, besides choosing the most optimum transgenic method including Agrobacterium-mediated transformation (Tzfira and Citovsky, 2006), gene gun bombardment (Kao et al., 2008) and electroporation (Sorokin et al., 2000), the success greatly depends on the efficient regeneration system of plants. So far, significant progress on somatic embryogenesis and plant transformation has been achieved in a wide range of species such as walnut (Dandekar et al., 1998), soybean (Kita et al., 2007), California poppy (Park and Facchini, 2000), peanut

(Deng et al., 2001), tea (Mondal et al., 2001) and Sweet potato (Otani et al., 2007). However, there is no remarkable report on transgenic progress in Rauvolfia plants. Organogenesis regeneration systems of some Rauvolfia plants have already been reported in previous studies (Sudha and Seeni, 1996). In addition leaf protoplast (Trémouillaux-Guiller and Chénieux, 1991) and root segment (Sudha and Seeni, 2006) have been successfully used for inducing somatic embryos in Rauvolfia plants.

In this study, we established a high frequency plant regeneration system of *R. verticillata* via somatic embryogenesis using stems as initial materials for the first time. The high regeneration system we have established can be served as an alternative propagation method of *R. verticillata* to protect this endangered plant and facilitate genetic modification of *R. verticillata* plants, especially developing transgenic plants with higher levels of TIAs via engineering the TIAs biosynthetic pathway in transgenic plants.

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