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Direct and callus-mediated regeneration of *Curcuma soloensis* Valeton (Zingiberaceae) and *ex vitro* performance of regenerated plants

Shijun Zhang[a], Nian Liu[a], Aiwu Sheng[a], Guohua Ma[b], Guojiang Wu[b,*]

[a] College of Horticulture and Landscape Architecture, Zhongkai University of Agriculture and Engineering, Guangzhou 510225, China
[b] South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, China

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ABSTRACT

Protocols are outlined for the regeneration of *Curcuma soloensis*, an attractive tropical ornamental plant, from young vegetative bud explants. We used both direct and callus-mediated regeneration techniques to produce material suitable for mass propagation and the development of transgenic plants. During direct plantlet propagation, the presence of thidiazuron (TDZ) in the growing medium induced more than three times as many shoots as 6-benzylaminopurine (BA), with a mean of 18.7 shoots per explant on MS medium containing 2.5 μM TDZ compared to 5.0 shoots with 40 μM BA. Subsequently, the shoots rooted readily on MS basal medium that was free of plant growth regulators. During indirect plantlet regeneration, TDZ combined with BA and 2,4-dichlorophenoxyacetic acid (2,4-D) had significant effects on embryogenic callus induction and multiplication. The frequency of callus formation was 91.1% for explants cultured on MS basal medium supplemented with 2.5 μM TDZ, 2.0 μM BA and 1.2 μM 2,4-D. On average 7.1 shoots were produced per callus mass cultured on MS medium supplemented with 2.5 μM TDZ, 9.0 μM BA and 1.2 μM naphthaleneacetic acid (NAA). Regenerated shoots were transferred to MS medium supplemented with 2.5 μM TDZ, to produce multiple shoots. *In vitro* cultured plantlets readily acclimatized to greenhouse conditions, showing 100% survival rates in a sphagnum, perlite and sand (1:1:1) medium. These plants were transplanted into pots or planted in the field. The *ex vitro* acclimated plants grew vigorously and produced showy inflorescences 5–6 months after planting. The high-frequency of shoot multiplication and rapid flowering of tissue-cultured plants indicate that *C. soloensis* has great potential in the floricultural market.

1. Introduction

The genus *Curcuma* (family Zingiberaceae) is widely distributed across tropical Asia and as far as Africa and Australia; it comprises more than 60 species of rhizomatous perennial herbs, including plants used for medicine, dyes and spices such as turmeric (Tyagi et al., 2007). In addition, due to the colourful bracts around the inflorescences, considered to be the plant's showiest feature, many species have also been developed as herbaceous ornamentals and sold as potted plants and cut flowers in Europe and N. America (Larsen and Larsen, 2006). *Curcuma alismatifolia*, the Siam tulip, is very popular (Bunya-atichart et al., 2004; Roh et al., 2006). *Curcuma soloensis*, native to the Solomon Islands and widely cultivated in tropical Southeast Asia, has slender lance-shaped foliage, and long cylindrical inflorescences (30–50 cm long) with green basal bracts and several pink apical bracts that form cup-like structures. The bracts subtend small yellow flowers. Owing to its outstanding ornamental value, *C. soloensis* could be grown as a bedding plant, potted plant or for cut flowers with a post-harvest life of over three weeks. In China, it is already considered a novel ornamental plant in the floricultural market (Yan, 2010).

In the horticultural production of *C. soloensis*, the underground rhizomes are the traditional planting materials, and are referred to as seed rhizomes (Hagiladi et al., 1997; Paz et al., 2003). However, the slow propagation rate and high occurrence of rhizome rot disease during storage and cultivation considerably limit its production. To make better use of this promising ornamental, efficient propagation and regeneration systems involving tissue culture, which would facilitate horticultural production and genetic improvement, are required.Tissue culture techniques have been reported for several species of the genus *Curcuma*, including *Curcuma longa* (Shirgurkar et al., 2001; Salvi et al., 2000, 2001, 2002; Prathanturarug et al., 2003, 2005; Tyagi et al., 2007), *Curcuma aromatica* (Nayak, 2000), *Curcuma zedoaria* (Loc et al., 2005) and *Curcuma amada* (Das et al., 2010). Micropropagation of *C. soloensis* has been achieved via direct shoot regeneration induced by

Abbreviations: BA, 6-benzylaminopurine; NAA, naphthaleneacetic acid; 2,4-D, 2,4 dichlorophenoxyacetic acid; TDZ, thidiazuron.

* Corresponding author. Tel.: +86 20 37252703.
E-mail address: wugj@scbg.ac.cn (G. Wu).

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cytokinins (Tyagi et al., 2004), but the efficiency of plant regeneration (2.8–5.0 shoots per culture) is relatively low.

Herein, however, we present ways to achieve both direct shoot formation and indirect plantlet regeneration through culturing *C. soloensis* calli. The main aims of the described investigations were to increase the multiplication and rapid production of pathogen-free plant material for growers, independently of the growing season. A further aim was to provide a platform for genetically improving *C. soloensis* via particle bombardment or *Agrobacterium*-mediated transformation, thereby facilitating the production of transgenic plants with desirable horticultural traits.

2. Materials and methods

2.1. In vitro culture

2.1.1. Plant materials and culture conditions

Sprouted rhizomes of *C. soloensis* were obtained from the South China Botanical Garden for use as initial explants. Excised shoots (1–2 cm long) were surface-sterilized with 70% (v/v) ethanol for 30 s, rinsed in sterile distilled water, soaked in 0.1% (w/v) mercuric chloride solution for 10 min, and finally washed 7 or 8 times with sterilized distilled water. The shoots were then placed on initial culture medium, consisting of MS (Murashige and Skoog, 1962) basal medium with 30 g L^{-1} sucrose, 0.55% (w/v) agar (Huankai Microbial Sci. & Tech, Co., Ltd., China) and various permutations of plant growth regulators (see below), pH adjusted to 5.8 before autoclaving at 121 °C and 104 kPa for 20 min. Three explants were inoculated on 30 ml of medium per glass vessel (5 cm diameter × 10 cm deep), with 15 replicate vessels per treatment (combination of plant growth regulators). Cultures were maintained at 27 ± 1 °C under a 16-h photoperiod, with 50 μmol m^{-2} s^{-1} light intensity provided by cool, white fluorescent lamps. For callus induction, all explants were kept in the dark.

2.1.2. Shoot initiation and multiplication

Surface-sterilised shoots were cultured on MS medium supplemented with BA at either 4.0 or 8.0 μM in combination with NAA (0.5, 1 μM) for shoot initiation. Shoots resumed growth, produced leaves and were measured after 4 weeks of culture to assess the effect of varying concentrations of BA and NAA on their length.

Subsequently, single *in vitro* shoots produced on the medium containing 4.0 μM BA and 0.5 μM NAA were subcultured on MS medium supplemented with varying concentrations and combinations of BA (8, 20, 40 μM), TDZ (0.5, 1.5, 2.5, 5 μM) and NAA (0.5, 2.5 μM) for further shoot multiplication. The multiple shoots obtained after 30 days were separated into single shoots and subcultured at 30-day intervals on the same medium. From the second subculture onwards, the multiplication rate and shoot length did not significantly change, so the propagation rates and shoot lengths were recorded 60 days later. The regenerated shoots were subsequently transferred to MS medium devoid of growth regulators for further elongation and induction of roots.

2.1.3. Callus induction

Shoot base sections (0.5 cm long) from 30-day-old, fully-grown plantlets raised *in vitro* on MS medium devoid of growth regulators were cut and inoculated on MS medium supplemented with different concentrations and combinations of BA (2.0, 4.0 μM), 2,4-D (1.2, 2.4 μM) and TDZ (1.5, 2.5, 5.0 μM) for callus induction. Calli were subcultured at 30 day intervals. The number of explants forming callus material was recorded as the induction frequency, and the mass of callus was also noted after 30 days of culturing.

2.1.4. Shoot regeneration from organogenic callus

Callus clumps measuring 4–5 mm in diameter, produced on MS medium containing 2.0 μM BA, 1.2 μM 2,4-D and 2.5 μM TDZ, were transferred to growth media with different concentrations and combinations of BA (8.0, 9.0, 10.0 μM), NAA (1.2, 2.4 μM) and TDZ (1.5, 2.5 μM) to evaluate shoot regeneration. The number of calli-forming shoots was recorded as the induction frequency after 30 days. The number of shoots produced per explant and shoot length were noted. Regenerated shoots were then transferred to MS medium containing TDZ (2.5 μM) for shoot multiplication. New subcultures were started at intervals of 30 days and the regenerated shoots were subsequently transferred to MS medium devoid of growth regulators for further elongation and induction of roots.

2.2. Ex vitro performance of the regenerated plants

Plantlets cultured on growth regulator-free MS basal medium were acclimatized to *ex vitro* conditions a month later, when they had developed 2–4 roots 5–7 cm long, in two stages as described below.

2.2.1. One-month greenhouse acclimatization

Complete plants were removed from the culture vessels after rooting for one month on MS basal medium, washed in water and transplanted into 50-cell plug trays containing three types of culture medium—sphagnum:perlite (1:1, v/v), sphagnum:sand (1:1, v/v) and sphagnum:perlite:sand (1:1:1, v/v)—to assess their effectiveness for promoting plantlet survival and development. One plantlet was planted in each cell (5 cm diameter × 6 cm deep), there were six replicate trays per treatment, and the trays were placed in a greenhouse maintained at 27 ± 2 °C with 12 h photoperiods (average light intensity 90 μmol m^{-2} s^{-1} during light phases). Plantlets were watered at 3-day intervals and fertilized weekly with one fourth-strength MS salt solution. One month later, the survival rate, number of roots, root length, number of leaves and height of the plants growing in each medium were recorded.

2.2.2. Subsequent growth and blooming

Because the average minimum temperature in the latter half of March is over 15 °C at Guanngzhou, it was possible to grow the plants outdoors after mid-March. Therefore, we evaluated their outdoor growth and flowering parameters by transferring sets of 45 plants, after acclimatization in the greenhouse for a month, to the open field, pots in a greenhouse and pots outdoors on 16 March 2009. The potted plants were placed in sets of three per 30-cm pot containing sphagnum:perlite:sand (1:1:1, v/v) while those cultured in the open field were grown in sandy loam soil with 30 cm × 30 cm spacing. The temperature in the greenhouse was 27 ± 2 °C, while mean monthly temperatures outdoors and in the open field recorded from March to November 2009 were 17.9, 21.9, 25.1, 22.5, 29.2, 29.5, 28.9, 25.6 and 17.5 °C, respectively. All treatments included natural photoperiods (11–13.5 h light) with a light intensity (at noon) of 500–1200 μmol m^{-2} s^{-1}. Plants were watered at 2-day intervals and fertilized once a week with 150 ppm compound fertilizer (15% N, 15% P_2O_5, 15% K_2O; Stanley Fertilizer Stock Co., Ltd., China).

To evaluate the growth and flowering performance of the plants under the three treatments, days to anthesis were recorded, and the following parameters were measured at the end of November 2009, when plants outdoors entered dormancy: flower-stem length and plant height (from the surface of the growing medium), rhizome formation rate and rhizome circumference. To evaluate whether the presence of a rhizome was a prerequisite for bloom production, the rhizome formation rate was recorded by dividing the number of blooming plants that had developed rhizomes by the total number of blooming plants (defining blooming as occurring when the inflo-