

## Somatic Embryogenesis and Plant Regeneration in Tissue Cultures of *Artemisia annua* L.

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**ABSTRACT** Mature seeds of *Artemisia annua* L. were placed onto Murashige and Skoog's (MS) medium supplemented with 4.52  $\mu$ M 2,4-dichlorophenoxyacetic acid (2,4-D). After 6 weeks of culture, off-white, compact calluses were formed on the plumule of seedlings at a frequency of 5.9%. Calluses were subcultured on the same medium. After an additional 2 weeks of subculture, calluses produced a few somatic embryos at a frequency of 28.8%. Upon transfer to MS basal medium, calluses producing a few somatic embryos gave rise to numerous somatic embryos, which subsequently developed into plantlets. Plantlets were successfully transplanted to potting soil and grown to maturity in a greenhouse.

### Introduction

*Artemisia annua* L. is an annual plant belonging to the Compositae. This plant is noted for the source of artemisinin, an antimalarial drug efficacious against multidrug-resistant strains of Plasmodium (the malarial parasite) (Luo and Shen 1987). Because of the complex structure of artemisinin, chemical synthesis of this compound is not cost-effective for a large-scale production. Production of artemisinin has been attempted using cell, hairy root, or shoot-tip cultures, but none of them seem to be practical (Ferreira and Janick 1996). Until now, the primary method of commercial production is to extract this compound directly from the plant. To obtain elite plants with high artemisinin content, germplasm collection could be a useful source of genetic material for plant selection and breeding based on artemisinin content.

However, genetic improvement of *A. annua* is difficult because the flowers are minuscule (approximately 1 mm in diameter) and systems for controlled pollination have not been devised (Ferreira and Janick 1995).

Somaclonal variation can provide the basis of an alternative to germplasm collection and breeding for genetic improvement of *A. annua*. Variation among plants regenerated from cultured cells or tissue have been demonstrated to be a useful source for genetic improvement (Larkin and Scowcroft 1981). Genetic improvement based on somaclonal variation requires a reliable plant regeneration system for a species of interest. In *A. annua*, plant regeneration via organogenesis was reported by (Fulzele et al. 1991) and their modified protocol was used for its genetic transformation (Vergauwe et al. 1998). However, plant regeneration via somatic embryogenesis of this species has not been reported. In this study, we established somatic embryogenesis and plant regeneration in seed cultures of *A. annua*. Embryogenic calluses obtained in

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this study were able to be maintained by subculture for a prolonged period retaining competence to produce numerous somatic embryos, which then developed into plantlets. Therefore, these calluses will be useful to produce a number of variants from which elite plants with high artemisinin content can be selected.

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## Materials and Methods

### Plant Material

Seeds of *Artemisia annua* L. were surface-sterilized with 70% (v/v) ethanol for 1 min and 0.4% (v/v) sodium hypochlorite for 10 min with occasional agitation, and then rinsed three times with sterile distilled water.

### Culture medium and conditions

The basal culture medium consisted of Murashige and Skoog (MS) (1962) inorganic salts, 100 mg l<sup>-1</sup> myo-inositol, 0.4 mg l<sup>-1</sup> thiamine·HCl, 3% (w/v) sucrose, and 0.4% (w/v) Gelrite. The pH of all media was adjusted to 5.8 before autoclaving. Twenty-five ml of medium was dispensed into plastic each Petri dish (87 x 15 mm). Unless mentioned otherwise, all cultures were incubated at 25°C in the dark.

### Somatic embryogenesis and plant regeneration

To induce callus, surface-sterilized seeds were placed onto MS medium supplemented with 0, 0.45, 2.26, 4.52, 9.05, or 22.62 µM 2,4-dichlorophenoxyacetic acid (2,4-D). Each treatment consisted of 100 seeds per dish with 6 replicates. After 6 weeks of culture, the number of seeds producing calluses was determined. Calluses formed on the plumule of germinated seedlings were detached with a scalpel and forceps and subcultured on medium with 4.52 µM 2,4-D. After an additional 2 weeks of subculture, calluses that formed a few somatic embryos on the surfaces were identified as embryogenic calluses. To produce a large number of somatic embryos and subsequent plantlets, embryogenic calluses were transferred to MS basal medium and cultured in the light (approximately 3 Wm<sup>-2</sup> from

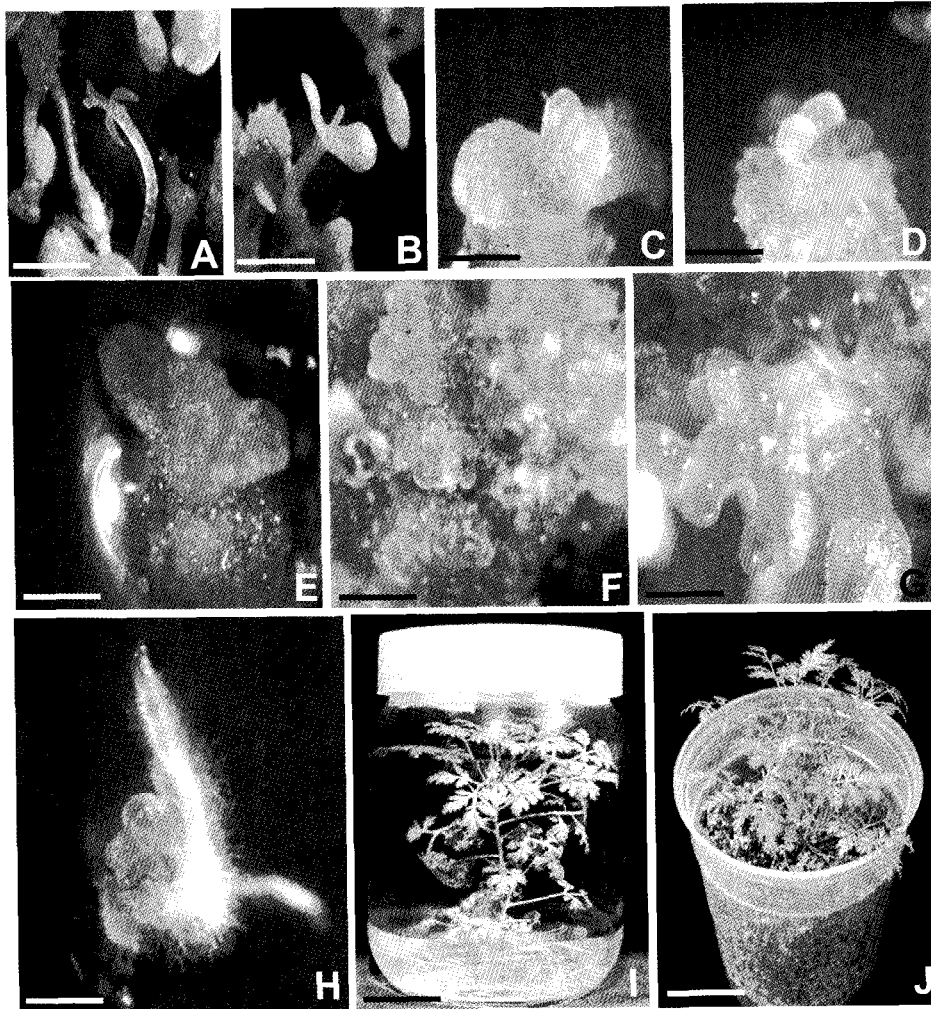
cool-white fluorescent lamps with 16-h photoperiods). Plantlets developed from somatic embryos on MS basal medium were subjected to acclimation, transplanted to potting soil, and maintained in a greenhouse.

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## Results and Discussion

Because of its small size, removal of the seed coat from *A. annua* seeds was tedious. Alternatively, intact seeds were cultured. After 1 week of culture, seedlings were protruded out of their seed coats. Germinated seedlings on medium with 2,4-D appeared abnormal to some extent: the radicle formed gray, wet, friable calluses that were not competent to produce any organized structure, and the hypocotyl and cotyledon were much smaller than those germinated on medium without 2,4-D after 6 weeks of culture (Figure 1A, B, C). Some of the seedlings formed off-white, compact calluses on the plumule on medium with greater than 4.52 µM 2,4-D (Figure 2; Figure 1C, D, E). The frequency of callus formation was reduced with an increasing concentration of 2,4-D (Figure 2). Off-white, compact calluses were excised from the plumule using a scalpel and forceps and subcultured on medium with 4.52 µM 2,4-D in the light, where the calluses became greenish (Figure 1F). After an additional 2 weeks of subculture, 28.8% of the calluses produced a few somatic embryos (Figure 1G), indicating that the off-white, compact calluses were embryogenic. However, the calluses that remained on the plumule did not become competent to produce somatic embryos even after an additional two weeks of culture (date not shown). Upon transfer to MS basal medium, off-white, compact calluses that produced a few somatic embryos gave rise to numerous somatic embryos (Figure 1H), which subsequently developed into plantlets (Figure 1I). Acclimated plantlets were successfully transplanted to potting soil (Figure 1J) and grown to maturity.

In a preliminary experiment, cotyledon and hypocotyl explants were cultured under the same conditions as seeds were placed on culture medium described in this study. However, these explants did not produce any organized structures. Therefore, it is suggested that embryogenic competence is expressed under the given culture conditions during an early



**Figure 1.** Somatic embryogenesis and plant regeneration in seed cultures of *A. annua* L. The plumules of germinated seedlings on medium supplemented with 0.45  $\mu\text{M}$  (A, bar = 1 cm), 2.26  $\mu\text{M}$  (B, bar = 0.5 cm), 4.52  $\mu\text{M}$  (C, bar = 0.1 cm), 4.52  $\mu\text{M}$  2,4-D (D, bar = 0.1 cm), or 22.62  $\mu\text{M}$  2,4-D (E, bar = 0.1 cm) after 6 weeks of culture; plumule-derived embryogenic calluses (F, bar = 0.5 mm); numerous somatic embryos developed from embryogenic calluses (G, bar = 0.1 cm); germination of somatic embryo (H, bar = 0.5 cm); plantlet regenerated via somatic embryogenesis (I, bar = 5 cm); and plant in potting soil (J, bar = 15 cm).

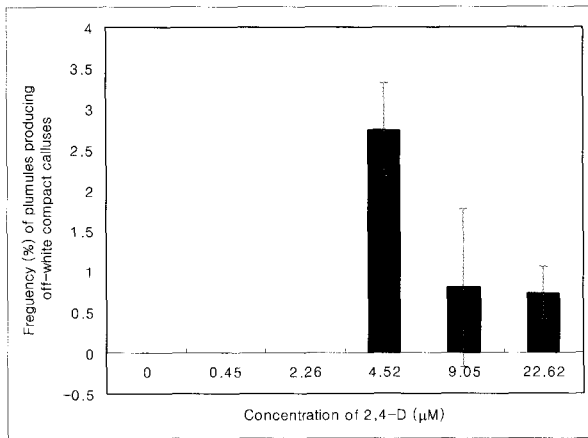
stage of seed germination.

Production of artemisinin by chemical synthesis or tissue culture is not economically feasible. Artemisinin is currently produced on a large scale by extraction directly from cultivated plants. Because a system for controlling pollination of *A. annua* is not available, breeding to produce elite genotypes with high artemisinin content cannot be carried out. In this study, alternatively, we produced embryogenic calluses with a high competence for plant regeneration that may provide a means for somaclonal variation. Somaclonal variation, variation among plants regenerated from cultured cells or tissue, has

been demonstrated to be a useful source for genetic improvement in many species including sugarcane (Evans 1989), potato (Secor and Shepard 1981), and rice (Adkins et al. 1990). A system for somatic embryogenesis and plant regeneration described in this study will be useful to produce a great number of variants from which elite plants with high artemisinin content can be selected.

#### Acknowledgements

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**Figure 2.** Frequency of plumules producing off-white compact calluses when placed on medium supplemented with various concentrations of 2,4-D. Data were collected after 6 weeks. Each treatment consisted of 100 seeds per dish with 6 replicates. Vertical bars indicate SD.

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