An Efficient Regeneration System for Four O'clocks (*Mirabilis jalapa*)

Xinjia Xu, Donald Hunter and Michael S. Reid
Department of Environmental Horticulture, University of California, One Shields Ave.
Davis, CA 95616
USA

Keywords: Floral senescence, ethylence insensitivity, meristem formation, thidiazuron, IAA

Abstract

As a model system for studying ethylene-independent floral senescence we have selected Four o'clocks, *Mirabilis jalapa*. As a first step to developing a transformation/regeneration (T/R) system, we have investigated ways to obtain rapid and efficient regeneration of *Mirabilis jalapa* plants. After testing a wide range of explants, media, and culture conditions, we determined that we could obtain high rates of meristem formation using cotyledons excised from mature embryos. The cotyledons and their subtending petioles were isolated from mature seeds and cultured in the dark for one week on Murashige and Skoog (MS) medium containing 1mg.L^{-1} IAA and 1mg.L^{-1} thiadiazuron (TDZ). The cotyledons were then transferred to regeneration media and cultured in the light. The regeneration medium contained MS salts and vitamins and 2 mg.L^{-1} TDZ. After four weeks, shoot meristems appeared on more than 80% of the petioles. Most of the meristems could develop to mature shoots, and then to intact plants.

INTRODUCTION

Most molecular studies on floral senescence have used ethylene-sensitive flowers, such as petunia and carnation. Senescence in these model systems is accelerated by application of ethylene, and can be delayed by treating the flowers with inhibitors of ethylene synthesis or action. Both species have been transformed and regenerated so as to modify expression of genes encoding key steps in the ethylene biosynthesis or transduction pathways, with predictable results. In contrast, studies on ethylene-insensitive senescence have used monocotyledonous models such as daylily, iris, and daffodil, species that are difficult to transform and regenerate, and where the process of determining the effect of up- or down-regulation of a gene may take several years. As a model system for studying ethylene independent floral senescence, we have selected *Mirabilis jalapa*, Four O'clocks, also known as Marvel of Peru, and Bella di Notti. The large nocturnal flowers of this member of the *Nyctaginaceae* open in the afternoon (hence some of the common names), are fully open at midnight, and senesce shortly after dawn.

Although senescence of *Mirabilis* flowers is associated with a small peak of ethylene production (Li et al., 1994) and can be accelerated by exogenous ethylene (Gookin et al., 2001), natural senescence is not affected by the ethylene action inhibitors STS or 1- methylcyclopropene (1-MCP) (Gookin et al., 2001). Therefore, we conclude that *M. jalapa* exhibits what we term a 'mixed' pattern of floral senescence, similar to that seen in daffodils, in which senescence can proceed via the ethylene-sensitive or the ethylene- insensitive senescence pathway.

M. jalapa can go from seed to seed in as little as three months. Being dicotyledonous, we anticipate that it will readily be transformed and regenerated, allowing relatively rapid examination of the function of genes associated with senescence of its ephemeral flowers. We report here an efficient regeneration system for *Mirabilis*, which is an essential prelude to developing a transformation/regeneration system to allow us to test the function of senescence-associated genes.

MATERIALS AND METHODS

Media

The media used in this protocol were as follows:

Basal medium:

MS salts and vitamins (Murashige and Skoog, 1962), 3% sucrose, 0.8% agar and is adjusted to pH 5.8.

Shoot induction medium (SI-medium):

Basal medium + 1 mg l^{-1} IAA + 1mg l^{-1} TDZ (thiadiazuron).

Shoot regeneration medium (SR-medium):

Basal medium + 2 mg l^{-1} TDZ.

Root induction medium (RI-medium):

1/2 MS salts + 1/2 MS vitamins, 1% sucrose, 0.8% agar and adjusted to pH 5.8.

Plant Material and Handling

Mature fruits were harvested from greenhouse grown plants of a cultivar with uniform pink flowers. The black coat was removed from the seeds which were then surface sterilized in 15% commercial bleach (5% hypochlorite) for 15 min and washed three times in sterile water. Cotyledons and their substending petioles were excised. Explants were washed once in sterile water to remove starch and placed, adaxial surface of the cotyledons up, in Petri dishes containing SI-medium.

Induction of Shoots and Roots

Explants were kept in the dark for at least 7 days for shoot induction. After shoot induction, they were transferred to SR-medium under day/night conditions (16 h light [40-50 μE $m^{-2}s^{-1}$], 8 h darkness, 25ºC temperature). When shoots reached 0.5 cm of height, they were detached from the explants and transferred to RI-medium.

RESULTS AND DISCUSSION

An initial survey of the regeneration capacity of different Four o'clock tissues revealed that only cotyledons from mature seeds are capable of regeneration. Results from an initial experiment with different hormone combinations showed that high frequency of regeneration was only obtained with the combination of TDZ and IAA (results not shown). After one week of culture in darkness, the attached petioles had obviously expanded and the cotyledons were yellow or yellow-green. Adventitious shoot meristems became visible within two weeks of transferring explants to SR-medium and light. During this period the blades of the cotyledons expanded and turned green. All of the meristems arose in the petioles and most of them developed into shoots by the end of the fourth week (Fig. 1). Once the shoots had reached 0.5 cm in height, they were excised from the petioles and transferred to RI-medium. Shoots continued to grow on the RI-medium and roots were usually induced within 2-3 weeks. Roots could be induced in almost all the regenerated shoots and the plantlets could then be transferred to pots in the greenhouse.

Culture in darkness is required for shoot regeneration. When cotyledons were cultured in the light directly the petioles elongated instead of expanding. Development of even those meristems that did form was arrested so that they failed to develop into shoots.

In initial experiments, we tested different cytokinin-auxin combinations. Each combination included one cytokinin chosen among TDZ, kinetin, and BAP and one auxin chosen among IAA, NAA and 2,4-D. For each combination, we tested different concentrations of the hormones varying from 0-2 mg l^{-1}. This survey used hypocotyls and epicotyls from sterile seedlings as explants instead of cotyledons from mature seeds. The effectiveness of the hormone combinations was evaluated by their ability to induce callus from the epicotyl. The combination of TDZ and IAA proved to be optimal. TDZ has been shown to promote in vitro regeneration in many dicotyledonous species (for review, see Lu, 1993; Murthy et al. 1998) and in monocotyledons such as wheat and barley (Shan et al., 2000). In our study it was the most effective of the cytokinins in promoting shoot

regeneration.

From studies of regeneration of other species, the importance of explant source has been well established (Luo et al., 1999; Saito and Suzuki, 1999; Rani and Grover, 1999; Hemphill et al., 1998). In this study, we found that even though callus could be generated from hypocotyls and epicotyls, they never formed adventitious shoots. Consulting the methods used to regenerate monocotyledonous plants we decided to use embryos as explant source. So far cotyledons are still the only Four o'clock tissue from which we have successfully regenerated shoots.

To conclude, regenerated plants of *M. jalapa* can be obtained from the cultivar Pink Uniform. Cotyledons isolated from mature seeds should be placed on SI-medium in the darkness for at least one week. Then the explants should be transferred to SR-medium and cultured in the light for four weeks. Shoots regenerated from the petioles were easy to root on RI-medium. Plantlets are transferred to soil when roots are well developed. The efficiency of regeneration is about 80%, which is high enough for transformation. However, the regeneration is not via callus, which could make *A. tumefaciens* infection and subsequent selection difficult. Attempts to find a regeneration protocol via callus are now under way.

ACKNOWLEDGEMENTS

This study was partially supported by a grant to Michael Reid from the American Floral Endowment. We also appreciated the advice from Dr. Michele Zaccai.

Literature Cited

Gookin, T. 2001. Evaluation of *Mirabilis jalapa* as a model system for studying floral senescence. HortScience 36: 599.

Hemphill, J.K., Maier, C.G.A. and Chapman, K.D. 1998. Rapid in vitro plant regeneration of cotton *Gossypium hirsutum* L. Plant Cell Rep. 17: 273-278.

Li, X.Z., Hou, J.Z., Shao, L.M., Yin, W.Y. 1994. Physiological, biochemical and cytological changes in senescing corolla of four o'clock flower. Acta Botanica Sinica 36: 116-122.

Lu, C.Y. 1993. The use of thidiazuron in tissue culture. InVitro Cell. Dev. Biol. – Plant 29: 92-96.

Luo, J.P., Jia, J.F., Gu, Y.H. and Jiu, J. 1999. High frequency somatic embryogenesis and plant regeneration in callus cultures of *Astragalus adsurgens* Pall. Plant Sci. 143: 93-99.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plantarum 15: 473-497.

Murthy, B.N.S., Murch, S.J., Saxena, P.K. 1998. Thidiazuron: a potent regulator of in vitro plant morphogenesis. In Vitro Cell. Dev. Biol – Plant 34: 267-275.

Rani, G. and Grover, I.S. 1999. In vitro callus induction and regeneration studies in *Withania somnifera*. Plant Cell Tiss. Org. Cult. 57: 23-27.

Satio, A. and Suzuki, M. 1999. Efficient shoot regeneration from calli of apple rootstock (*Malus* × *prunifolia* var, ringo Asami Mo84-A) and cultivar (*Malus* × *domestica* cv. Fuji). J. Plant Physiol. 155: 620-624.

Shan, X., Li, D. and Qu, R. 2000. Thidiazuron promotes in vitro regeneration of wheat and barley. In Vitro Cell. Dev. Biol – Plant 36: 207-210.

Figures

Fig. 1. Adventitious shoots on petiole of Four o'clock cotyledon explant, four weeks after being transferred to SRI medium in the light.