BIOLOGIA PLANTARUM 50 (4): 729-732, 2006

BRIEF COMMUNICATION

Direct shoot organogenesis and plant regeneration in *Fortunella crassifolia*

L. YANG*, C.-J. XU*, G.-B. HU** and K.-S. CHEN*[1]

*The State Agriculture Ministry Laboratory of Horticultural Plant Growth, Development and Biotechnology, Huajiachi Campus, Zhejiang University, Hangzhou 310029, P.R. China**
*Department of Fruit Science, South China Agricultural University, Guangzhou 510642, P.R. China***

Abstract

An efficient *in vitro* regeneration system in kumquats (*Fortunella crassifolia* Swingle) was established. Explant types and orientations, concentrations and combinations of plant growth regulators were evaluated for their influences on efficiency of plant regeneration. It was found that the optimum explant and its orientation was epicotyl planted vertically with upper part upward, and a shoot regeneration frequency of 1.48 shoots per explant was obtained on Murashige and Skoog (1962; MS) medium supplemented with 22.19 μM 6-benzyladenine (BA). A rooting percentage as high as 74 % was obtained on 1/2 MS supplemented with 0.54 μM 1-naphthaleneacetic acid (NAA), 9.29 μM kinetin (KN), and 0.5 g dm^{-3} activated charcoal.

Additional key words: auxins, cotyledon, cytokinins, epicotyl, explant, hypocotyl, kumquat, rooting.

Kumquats (*Fortunella* spp.), also called kinkan, are small evergreen shrubs of *Rutaceae*, which are closely related to plants of *Citrus* genus. Ripe fruits of kumquats are quite similar to cherry tomatoes in size and shape; the colour ranges from orange yellow to orange red. Kumquats fruits are rich in fiber, vitamins A and C, and contain traces of calcium and iron, offering many nutritional or clinical benefits. The most notable aspect of kumquat fruit, however, is its deliciously sweet rind, providing a special taste than other citrus fruits. As a result, kumquats are commercially cultivated in China, Japan, United States, *etc*. Furthermore, kumquats are popular ornamental plants because of their attractive shiny leaves, delicate white flowers and orange fruits.

Kumquats are routinely propagated by grafting, however, strictly technical and seasonal requirements largely handicapped its application. Therefore, development of a micropropagation system through tissue culture is necessary to provide an alternative way to produce uniform plantlets through the year. In addition, the availability of such a system is also necessary for genetic improvement and helpful for genetic resource conservation.

In vitro regeneration of citrus has been described in most common *Citrus* species such as *C. sinensis* Osbeck (Bordón *et al*. 2000, Chen *et al*. 2000, Costa *et al*. 2004), *C. aurantium* L. (Bordón *et al*. 2000), *C. reticulata* Blanco (Hassanein and Azooz 2003, He *et al*. 1997, Singh *et al*. 1994), *C. limonia* Osbeck (Costa *et al*. 2004), *C. aurantifolia* (Christm.) Swingle (Al-Bahrany 2002), *C. grandis* Osbeck (Huang *et al*. 2002), *C. paradisi* Macf. (Costa *et al*. 2004) as well as *Poncirus trifoliata* (L.) Rafin. (Beloualy 1991) and some plants of *Citrus* related genera (Hiregoudar *et al*. 2005, Ling and Iwamasa 1997). For kumquats, however, studies on tissue culture are quite limited. Jia *et al*. (1997) described a protocol for indirect shoot organogenesis and plantlet regeneration from hypocotyls *via* callus phase. Here we present an efficient plant regeneration system *in vitro* from epicotyl segments of kumquat by direct organogenesis.

The media used in this study were based on our preliminary experiments and the desirable media on other citrus plants as reported in Chen *et al*. (2000), Ghorbel *et al*. (2000) and Wong *et al*. (2001). All media were based on Murashige and Skoog (MS) or 1/2 MS medium supplemented with 3 % (m/v) sucrose and 0.7 % (m/v) *Bacto* agar at a pH of 5.8. Seeds were sown on 1/2 MS (seed germination medium, SGM), adventitious shoots

Received 19 October 2004, *accepted* 18 October 2005.
Abbreviations: BA - 6-benzyladenine; IAA - indole acetic acid; IBA - indole butyric acid; KN - kinetin; MS - Murashige and Skoog; NAA - 1-naphthaleneacetic acid; RM - rooting medium; SGM - seed germination medium; SIM - shoot induction medium.
Acknowledgements: The study was supported by the National Natural Science Foundation of China (30100125), the Natural Science Foundation of Zhejiang Province (301291), and the National 973 Project of China (G2000046806).
[1] Author for correspondence, fax: (+86)57186971931, e-mail: akun@zju.edu.cn

were induced on MS media supplemented with various plant growth regulators (shoot induction medium 1 - 10, SIM_{1-10}, Table 1), and roots were induced on 1/2 MS supplemented with 2.46 μM indole butyric acid (IBA) and 0.5 g dm^{-3} activated charcoal (rooting medium 1, RM_1), MS supplemented with 1.07 μM 1-naphthaleneacetic acid (NAA), 1.14 μM indole acetic acid (IAA), and 0.5 g dm^{-3} activated charcoal (RM_2) or 1/2 MS supplemented with 0.54 μM NAA, 9.29 μM kinetin (KN), and 0.5 g dm^{-3} activated charcoal (RM_3). Seed germination, shoot regeneration, and rooting were conducted at temperature 26 ± 1 ºC, 16-h photoperiod with an irradiance of approximately 45 μmol m^{-2} s^{-1} and a relative humidity of about 60 % unless otherwise stated.

Seeds were collected from ripe kumquat (*Fortunella crassifolia* Swingle cv. Jindan) fruit and surface sterilized after seed coat removal with 70 % ethanol for 1 min, followed by 0.2 % mercuric chloride for 8 min, and finally 5 rinses with sterile distilled water. The sterilized seeds were sown on SGM and cultured at 26 ± 1 ºC in dark for two weeks, then at a condition described above for 2 - 3 additional weeks before explants were prepared from the seedlings.

The most suitable explant type and the best orientation were evaluated firstly. Epicotyls, cotyledons, and hypocotyls were excised from 4 to 5-week-old *in vitro* raised seedlings. Cotyledons were then bisected into halves, and epicotyls and hypocotyls were cut into 1.0 cm segments. The explants were cultured on MS supplemented with 8.88 μM 6-benzyladenine (BA) and 2.69 μM NAA (SIM_7). For epicotyls or hypocotyls, three orientations were applied: horizontal, vertical with upper part upward and vertical with lower part upward.

To determine the best shoot induction medium, epicotyl explants were inserted into SIM_{1-10} (Table 1) with upper part upward. Shoot regeneration percentage, defined as percentage of explants with adventitious shoot, and shoot regeneration frequency, defined as average number of adventitious shoots formed per explant, were recorded four weeks later. To promote the initiation and development of root systems, adventitious shoots of 1.0 cm in length were excised from epicotyl explants of previous cultures and transferred to RM_{1-3}. The percentage of explants rooted and average length of roots produced were recorded 4 weeks afterwards.

Table 1. Effects of plant growth regulators on shoot regeneration from epicotyl explants. Values within a column followed by different letters are significantly different at $P \leq 0.01$.

Media	BA [μM]	IBA [μM]	NAA [μM]	KN [μM]	Shoot regeneration [%]	Shoot number [explant^{-1}]
SIM_1	4.44	0	0.54	0	54.4 ± 6.8	0.69 ± 0.19b
SIM_2	2.22	0.49	0	0	51.2 ± 2.7	0.65 ± 0.01b
SIM_3	4.44	0.49	0	0	58.3 ± 8.5	0.81 ± 0.08ab
SIM_4	6.66	0.49	0	0	62.1 ± 2.2	0.73 ± 0.01b
SIM_5	8.88	0.49	0	0	56.5 ± 7.0	0.84 ± 0.06ab
SIM_6	4.44	2.46	0	0	55.4 ± 0.3	0.81 ± 0.13ab
SIM_7	8.88	2.46	0	0	58.3 ± 1.8	0.72 ± 0.08b
SIM_8	33.29	0	5.37	0	51.7 ± 13.9	0.98 ± 0.15ab
SIM_9	22.19	0	0	0	45.9 ± 10.1	1.48 ± 0.21a
SIM_{10}	8.88	0	5.37	4.65	50.0 ± 3.2	0.94 ± 0.11ab

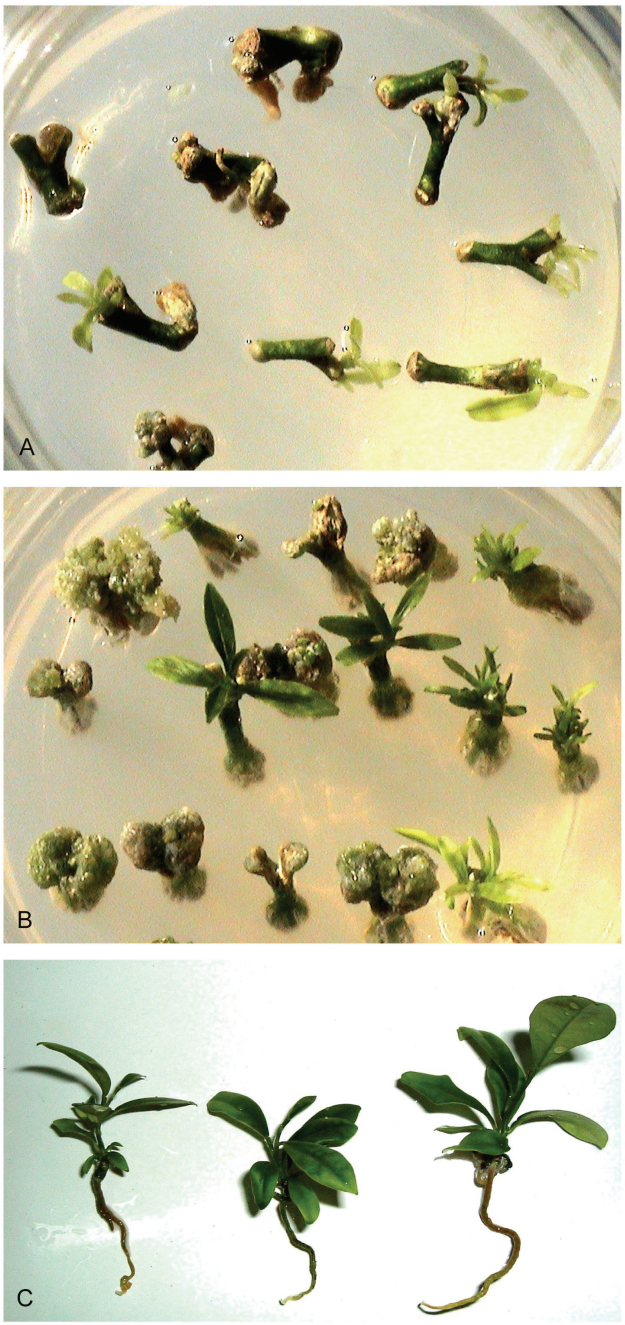

Fig. 1. Shoot regeneration and root formation of *Fortunella crassifolia*: *A* - Shoot regeneration from epicotyl explants horizontally cultured on SIM_7; *B* - Shoot regeneration from epicotyl explants vertically cultured on SIM_7 with upper part upward; *C* - Plantlets resulting from rooting of adventitious shoots on RM_1 (*left*), RM_2 (*middle*) and RM_3 (*right*).

In vitro-regenerated and rooted plantlets were transplanted to pots containing potting mix (soil:sand: compost, 1:1:1). The plantlets were then acclimatized in the culture room by covering with a plastic film for first 3 d and for 8 h every day of next 4 d. The hardened plants were transferred to a greenhouse for further growth.

Each treatment consisted of 50 explants or adventitious shoots, and all data are representative of triplicate experiments.

F. crassifolia seeds germinated at approximately 10 d after sowing on SGM. Preliminary studies suggested that 4 to 5-week-old seedlings are suitable for explant preparation.

After 4-d culture of explants on SIM_7, the epicotyl and hypocotyl explants partially degreened and swelled at the cut ends, while no palpable change was observed on cotyledons. Four weeks later, shoot regeneration percentage was found to be significantly affected by explant type and orientation. Epicotyl explants have strongest regeneration potency, followed by hypocotyls, and cotyledons were found to be poorest, without any shoot regenerated. A significantly higher shoot regeneration percentage was observed for epicotyl or hypocotyl explants cultured with upper part up than those with horizontal orientation, and no shoot was regenerated when lower part up was applied, suggesting keeping normal plant growth polarity is necessary for high frequency shoot regeneration. Similar effects of explant type and orientation on shoot regeneration were also observed in 4 *Citrus* species and Troyer citrange (Bordón *et al*. 2000). The highest regeneration percentage (68 %) was obtained on epicotyls with upper part up, followed by epicotyls with horizontal orientation (30 %), then by hypocotyls with upper part up (8 %) and hypocotyls with horizontal orientation (0.67 %). It was also found that adventitious shoots regenerated directly from the epicotyl explants bypassing an intermediate callus phase though some calli were induced meanwhile (Fig. 1*A*,*B*), such non-organogenic calli were also observed in *C. reshni*, *C. sinensis* and *C. aurantium* (Bordón *et al*. 2000). *F. crassifolia* is quite similar to *Citrus* in shoot regeneration pathway as reported by Bordón *et al*. (2000). According to the report, indirect shoot regeneration through callus formation and differentiation was observed in Troyer citrange but not in *C. aurantium*, *C. macrophylla*, *C. reshni*, and *C. sinensis*.

Shoot regeneration percentage was not significantly different among SIM tested, while the shoot regeneration frequency varied with types and concentrations of auxins applied (Table 1). The highest regeneration frequency, 1.48 shoots per explant, was obtained on SIM_9 which was supplemented with 22.19 μM BA but without any auxins. It is found that the presence of IBA or NAA, even at a concentration as low as 0.49 μM and 0.54 μM, respectively, decreased the shoot regeneration frequency significantly (Table 1). An obvious difference in regeneration ability between kumquat and orange was observed when comparing the results here with what we obtained in previous experiments on *C. sinensis* (Chen *et al*. 2000). The regeneration percentage and frequency were 96 % and 5.00 shoots per explant, respectively, for orange epicotyl explants (Chen *et al*. 2000) while 51.7 % and 0.98 shoot per explant for kumquat on the same medium (SIM_8), suggesting a more difficult shoot regeneration in kumquat.

Among three media tested for rooting, RM_3 was the best, RM_1 intermediate, and RM_2 the worst. Highest percentage (74 %) of shoot rooted on RM_3, while a bit less (71.33 %) and much less (16.67 %) shoots rooted on RM_1 and RM_2, respectively. Moreover, strongest roots with an average length of 1.8 cm were produced for shoots rooted on RM_3, whereas the roots were only 1.5 cm in average length on RM_1 and RM_2 (Fig. 1*C*). Additionally, most vigorous growth of shoots was observed for those rooted on RM_3 as well (Fig. 1*C*). Plantlets with well-established shoot and root system were transplanted to pots filled with potting mix, and hardened for a week under acclimation conditions, with a final survival rate of near to 100 %.

The study described an efficient *in vitro* plantlet regeneration protocol for *F. crassifolia* which can be also used for development of a genetic transformation system for this plant.

References

Al-Bahrany, A.A.: Effect of phytohormones on *in vitro* shoot multiplication and rooting of lime *Citrus aurantifolia* (Christm.) Swing. - Sci. Hort. **95**: 285-295, 2002.

Beloualy, N.: Plant-regeneration from callus-culture of 3 citrus rootstocks. - Plant Cell Tissue Organ Cult. **24**: 29-34, 1991.

Bordón, Y., Guardiola, J.L., García-Luis, A.: Genotype affects the morphorgenic response *in vitro* of epicotyl segments of *Citrus* rootstocks. - Ann. Bot. **86**: 159-166, 2000.

Chen, D.-M., Xu, C.-J., Zhang, S.-L.: [Studies on a direct shoot regeneration system of *Citrus sinensis* L. Osbeck with a high frequency *in vitro*]. - J. Zhejiang Univ. **26**: 493-499, 2000. [In Chin.]

Costa, M.G.C., Alves, V.S., Lani, E.R.G., Mosquima, P.R., Carvalho, C.R., Otoni, W.C.: Morphogenic gradients of adventitious bud and shoot regeneration in epicotyl explants of *Citrus*. - Sci. Hort. **100**: 63-74, 2004.

Ghorbel, R., Domínguez, A., Navarro, L., Peña, L.: High efficiency genetic transformation of sour orange (*Citrus aurantium*) and production of transgenic trees containing the coat protein gene of citrus tristeza virus. - Tree Physiol. **20**: 1183-1189, 2000.

Hassanein, A.M., Azooz, M.M.: Propagation of *Citrus reticulata via in vitro* seed germination and shoot cuttings. - Biol. Plant. **47**: 173-177, 2003.

He, H., Pan, R.-C., He, Y.-W., Han, M.-L., Li, G.-G.: [A study on the tissue culture and plant regeneration of *Citrus reticulata* cv. Tankan]. - J. South China norm. Univ. (nat. Sci. Ed.) **1997** (4): 63-66, 1997. [In Chin.]

Hiregoudar, L.V., Ashok Kumar, H.G., Murthy, H.N.: *In vitro* culture of *Feronia limonia* (L.) Swingle from hypocotyl and internodal explants. - Biol. Plant. **49**: 41-45, 2005.

Huang, T., Peng, S.-L., Dong, G.-F., Zhang, L.-Y., Li, G.-G.: Plant regeneration from leaf-derived callus in *Citrus grandis* (pummelo): effects of auxins in callus induction medium. - Plant Cell Tissue Organ Cult. **69**: 141-146, 2002.

Jia, Y.-J., Chen, F., Lin, H.-H., Cao, Y.-L., Luo, Y.-Y.: [*In vitro* culture of different explant from *Fortunella margarita* (Lour.) Swing]. - J. Sichuan Univ. (nat. Sci. Ed.) **34**: 344-348, 1997. [In Chin.]

Ling, J.T., Iwamasa, M.: Plant regeneration from embryogenic calli of six *Citrus* related genera. - Plant Cell Tissue Organ Cult. **49**: 145-148, 1997.

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

Singh, S., Ray, B.K., Bhattacharyya, S., Deka, P.C.: *In vitro* propagation of *Citrus reticulata* Blanco and *Citrus limon* Burm.f. - HortScience **29**: 214-216, 1994.

Wong, W.-S., Li, G.-G., Ning, W., Xu, Z.-F., Hsiao, W.L.W., Zhang, L.-Y., Li, N.: Repression of chilling-induced ACC accumulation in transgenic citrus by over-production of antisense 1-aminocyclopropane-1-carboxylate synthase RNA. - Plant Sci. **161**: 969-977, 2001.