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Callus Induction and Plant Regeneration from Broccoli (*Brassica oleracea* var. italica) for Transformation

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We evaluated the efficiency of callus induction and plantlet regeneration from hypocotyl explants of broccoli (*Brassica oleracea* var. italica). The cultivars were 'Marathon', 'Greenbelt', and 'Shogun'. Transformation success was not affected by the presence of tobacco feeder-cell layers on the culture media. The frequency of shoot regeneration was greater from 10-d-old hypocotyls than from 14-d-old hypocotyls. Both 'Marathon' and 'Greenbelt' had higher potentials for tissue regeneration than did 'Shogun'. We found that for transformation selection, the optimum concentration was either 50 mg/L kanamycin or 100 mg/L geneticin.

Keywords: broccoli, callus induction, hypocotyls, plant regeneration, tobacco-feeder-cell layers, transformation

Broccoli (*Brassica oleracea* var. italica) is a universally important vegetable. However, because its heads easily turn yellow and become unmarketable within 1 to 3 d (Forney, 1995), genetic engineering is required for maintaining its quality. Therefore, researchers are looking for a suitable *Agrobacterium*-mediated transformation system in order to obtain a high ratio of transgenic plants.

Although tissue-culture techniques have been used extensively for vegetable crops, the efficiency for transferring genes into plants is restricted by the choice of culturing method. Shoot regeneration from explants of leaves, cotyledons, hypocotyls, and protoplasts could prove useful for gene transfer via *Agrobacterium* (Cervera et al., 1998; González et al., 1998; Luo and Jia, 1998; Collén and Jarl, 1999; Cogan et al., 2001). Because the rate of regeneration is generally reduced when explants are treated with *Agrobacterium*, a good system is required for obtaining higher transformation frequencies (Oka and Ohyama, 1981). Researchers (Cha et al., 2002; Thomas TD, 2002) require a means for comparing numerous variables in order to optimize the cell culture method. Manipulating either the explants and/or the bacterium to enhance virulence will increase efficiency (Henzi et al., 2000). However, the success of such manipulations in callus induction and regeneration depends on the age of the hypocotyl explants, the feasibility of using feeder-cell layers, or the selection of an appropriate medium. Plant regeneration from protoplasts has been obtained in *B. oleracea* (Jourdan and Earle, 1989; Jourdan et al., 1990; Hansen and Earle, 1994). Likewise, Metz et al. (1995) have reported a procedure that uses *Agrobacterium tumefaciens* for transforming *B. oleracea*. Therefore, the aim of the current study was to test that protocol from Metz et al. (1995), and to determine how callus induction and tissue regeneration influence transformation of broccoli.

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MATERIALS AND METHODS

Seed Germination and Testing for Transformation Success

We used three commercial cultivars of broccoli (*B. oleracea* var. italica)-'Greenblt', 'Marathon', and 'Shogun'. The seeds were surface-sterilized with 70% EtOH for 5 min, immersed for 20 min in 1.5% (w/v) sodium hypochlorite with 2 drops of Tween 20 (Sigma), then rinsed in sterile, purified water three times for 5 min each. They were then germinated in the dark at 24°C ± 2°C for 1 week on MS standard media (Murashige and Skoog, 1962). For testing, we took 5- to 7-mm-long hypocotyls from the developing plants. To determine the higher capacity for tissue regeneration, 10- and 14-d-old explants were used.

For our vector, we obtained *A. tumefaciens* strain GV3101 from PGEL (Plant Genetic Engineering Laboratory) at the University of Queensland, St. Lucia, Brisbane, Australia. Transgenic plants were confirmed

using the intron GUS expression assay (GUS staining method).

Media and Culture Conditions

In all three cultivars, we tested the protocol of Metz et al. (1995), who had reported that transformation efficiency could be increased by pre-culturing the explants. For 3-d prior to co-cultivation, we used a pre-culture medium (pH 5.7) that contained 1/10 MS salts, 10 mL/L B5 vitamins, 100 mg/L I-inositol, 3% sucrose, 1 mL/L BA, and 8 g/L bacto-agar. After co-cultivation, the explants were planted on an MS standard medium with 100 μM acetosyringone. To identify the optimum antibiotic concentration for eliminating the *Agrobacterium*, we used a basal regeneration medium (pH 5.7) comprised of 4.3 g/L MS salts, 10 mL/L B5 vitamins (Gamborg et al., 1968), 100 mg/L I-inositol, 30 g/L sucrose, 2 mg/L BA, and 5 g/L Phyto-agar, all combined with two antibiotics, kanamycin and geneticin, at different concentrations (0, 25, 50, or 100 mg/L). We then used a regeneration medium with 0.5 g/L cefotaxin to select for the transformed cells. The media first were autoclaved at 121°C for 20 min. Afterward, all the cultures were kept in a growth room at 24°C ± 2°C, under a 16-h photoperiod provided by white fluorescent lamps. All the subsequent Petri dishes were sealed with Micropore surgical tape (3M Co.).

During the culture period, the percentages of callus or shoot induction were recorded. Regenerated shoots from the explants were excised and cultured in 1/4 MS medium. The rooted green shoots were then transferred to vermiculite-filled plastic pots, and covered with plastic bags to maintain high relative humidity before being placed in a growth chamber at 24°C under a 16-h photoperiod. Holes were gradually cut into the bags over 7 to 10 d before the bags were completely removed. All experiments were replicated three times.

Preparation of Tobacco Cell Suspensions

According to Metz et al. (1995), increased shoot regeneration and transformation frequencies can be obtained by using a tobacco nurse-cell layer during *Agrobacterium* co-cultivation. Therefore, we used three optimized protocols for transformation: 1) no tobacco cell layer on the regeneration medium; 2) sterile filter paper placed on a tobacco callus cell-feeder layer on the regeneration medium; and 3) tobacco callus cells placed directly on the regeneration medium. These feeder-cell layers, from NT-1 tobacco cell suspensions, were made by re-suspending 1 cm^3 of NT-1 tobacco cell callus in 50 mL of a liquid tissue-culture medium (4.3 g/L salts, 30 g/L sucrose, 10 mL/L B1-Inositol, 6 g/L KH_2PO_4, 0.5 mg/L BAP, and 400 μL/L 2,4D; pH 5.5 to 5.7). After being shaken at 120 rpm for 7 d before subculturing, 48 mL of the liquid medium was inoculated with 5 mL of the tobacco cells, at weekly intervals. In addition, 5 mL of re-suspended tobacco cells were maintained in an NT-1 tobacco-callus solid medium (as described above, but solidified with 0.8% Bacto-agar).

RESULTS AND DISCUSSION

The Effect of Pre-Culturing and Hypocotyl Age on Regeneration

The first radicals were seen emerging 3 d after the broccoli seeds were planted. At 7 d, the germination rates were 95% for 'Marathon', 80% for 'Greenbelt', and 72% for 'Shogun'. Ten days after planting, 47.8% of the 'Marathon' hypocotyls were >5 cm in length and 52.2% were <5 cm. Likewise, for 'Greenbelt', 20% of its hypocotyls were >5 cm long, while 21.7% of the 'Shogun' hypocotyls were >5 cm at day 10.

The overall potential for tissue regeneration was higher with 'Marathon' than for either 'Greenbelt' or 'Shogun' (Figs. 1 and 2). In our test of the 3-d preculture treatment on an MS standard medium before co-cultivation, we found no significant differences between the pre-treated and normal cultures (Fig. 1). This result was consistent with those of Christey et al. (1997) and Cogan et al. (2001), who, therefore, also chose not to pre-culture the broccoli to improve transformation success.

For all three cultivars, regeneration rates were always

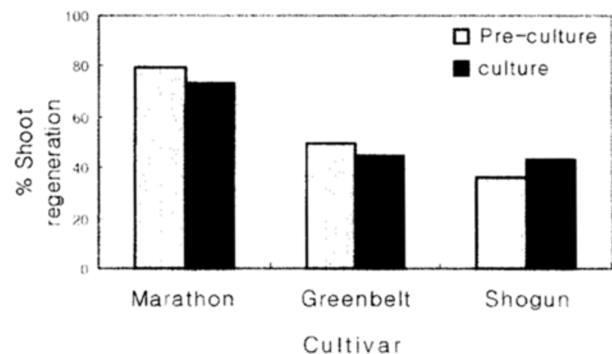


Figure 1. Shoot regeneration of three broccoli cultivars. No significant effects were observed in the pre-culture of 'Marathon' ($t = 1.224$, $p > 0.2$), 'Greenbelt' ($t = 0.423$, $p > 0.5$), and 'Shogun' ($t = -2.43$, $p > 0.01$).

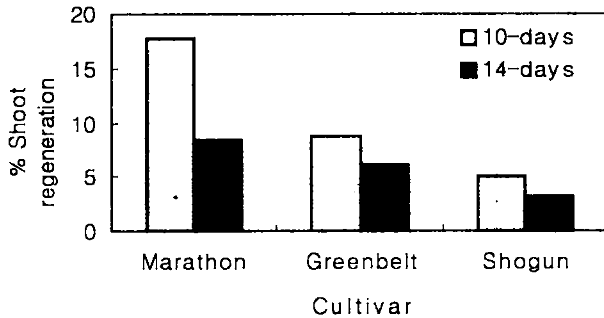
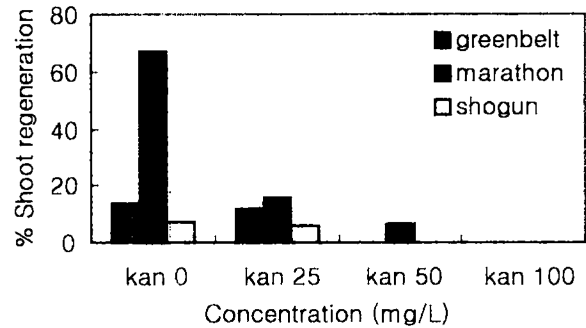


Figure 2. Shoot-regeneration efficiencies for 'Marathon', 'Greenbelt', and 'Shogun' after co-cultivation. Explants from 10- and 14-d-old seedlings were used for *Agrobacterium* transformation. The number of regenerated shoots was recorded after eight weeks.



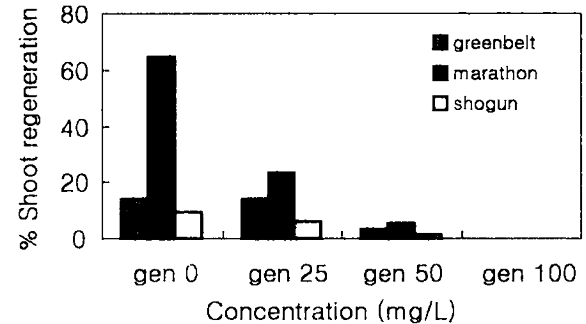


Figure 3. Shoot-regeneration efficiencies (%) of three broccoli cultivars under various antibiotic concentrations. Over a period of 4 weeks, hypocotyl explants from 10-d-old seedlings were cultured for 7 d in regeneration media containing various concentrations (0, 25, 50, or 100 mg/L) of kanamycin and geneticin. Afterward, the number of regenerated shoots was recorded. Each experiment was replicated three times. kan = kanamycin; gen = geneticin.

higher in 10- rather than 14-d-old tissues (Fig. 2), demonstrating that younger hypocotyls were more useful for efficient transformations. These differences were highly significant in the 'Marathon' cultivar ($t = -4.062$, $p < 0.005$). Hansen et al. (1999) also have reported that regeneration rates are higher from the early-stage protoplasts of *B. oleracea* because of lower environmental impacts, and because fewer genes are involved than during the advanced stages. Many other researchers (Izhar and Power, 1977; Coleman et al., 1990; Cheng and Veilleux, 1991) have also agreed that the age of the explants is a critical factor in determining the success of micropropagation.

Kill Curves for Geneticin and Kanamycin in Broccoli Explants

Because we observed inadequate kanamycin tissue selection at the recommended concentration of 25 mg/L, we performed a kill-curve experiment using geneticin and kanamycin at 0, 25, 50, and 100 mg/L. In this experiment, hypocotyl explants from 10-d-old seedlings were held in a regeneration medium before being placed in the designated geneticin and kanamycin concentrations for 4 weeks. As before, 'Marathon' explants showed considerably better shoot regeneration rates than did 'Greenbelt' and 'Shogun'. Differences were highly significant both for kanamycin concentration ('Greenbelt', $p < 0.00005$; 'Marathon', $p < 0.0000005$; and 'Shogun', $p < 0.000005$) and for geneticin concentration ('Greenbelt', $p < 0.00005$; 'Marathon', $p < 0.00005$; and 'Shogun', $p < 0.001$). A kanamycin concentration of 50 mg/L was sufficient to inhibit shoot formation in the 'Greenbelt' and 'Shogun' explants, but not in those from 'Marathon'. In addition, each concentrations of 100 mg/L for the geneticin and kanamycin were required to fully inhibit shoot formation in all cultivars (Fig. 3). However, no significant differences were found at the 95% confidence level between kanamycin and geneticin at all concentrations (0 mg/L, $p = 0.989$; 25 mg/L, $p = 0.28$; 50 mg/L, $p = 0.388$). Interestingly, calli were formed even in the presence of kanamycin and geneticin concentrations of 100 mg /L for all cultivars, which indicates that, although shoot formation had been fully inhibited, most explants were still viable (Fig. 4).

Although Metz et al. (1995) had used a kanamycin concentration of 25 mg/L for transformation selection, our results suggest that comparable levels of shoot regeneration are possible with 50 mg/L for 'Greenbelt' and 'Shogun'. Likewise, a concentration of 100 mg/L kanamycin was found to be suitable for 'Marathon'. Based on our results, we also propose that 100 mg/L of geneticin is a desirable selection concentration for all three broccoli cultivars.

As stated above, shoot regeneration was observed at kanamycin or geneticin concentrations of 50 mg/L, with

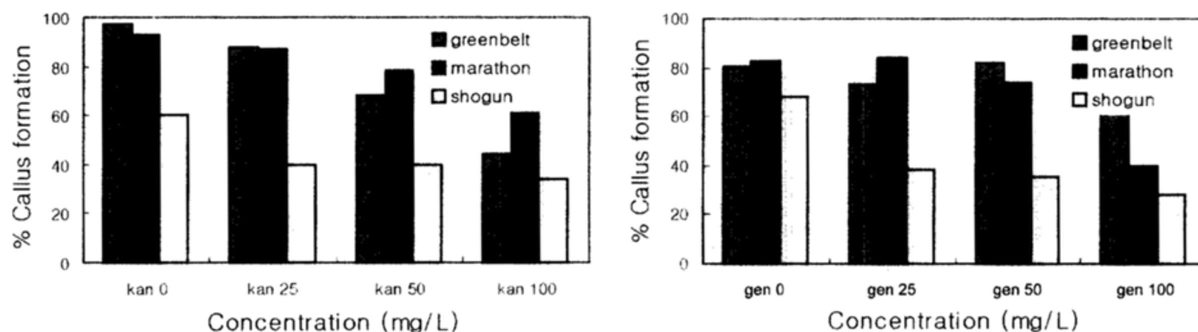


Figure 4. Callus-formation efficiencies (%) of three broccoli cultivars treated with various concentrations (0, 25, 50, or 100 mg/L) of kanamycin and geneticin. Hypocotyl explants from 10-d-old seedlings were held for 4 weeks in the regeneration media. Afterward, the number of calli formed per explant was recorded. The varying kanamycin concentrations caused highly significant differences in calli formation for 'Greenbelt' ($p = 2.7 \times 10^{-8}$), 'Marathon' ($p = 0.000187$), and 'Shogun' ($p = 0.000919$), as determined via ANOVA. Likewise, the concentration of geneticin also resulted in highly significant differences in Greenbelt ($p = 0.021$), 'Marathon' ($p = 1.33 \times 10^{-6}$), and 'Shogun' ($p = 0.000421$).

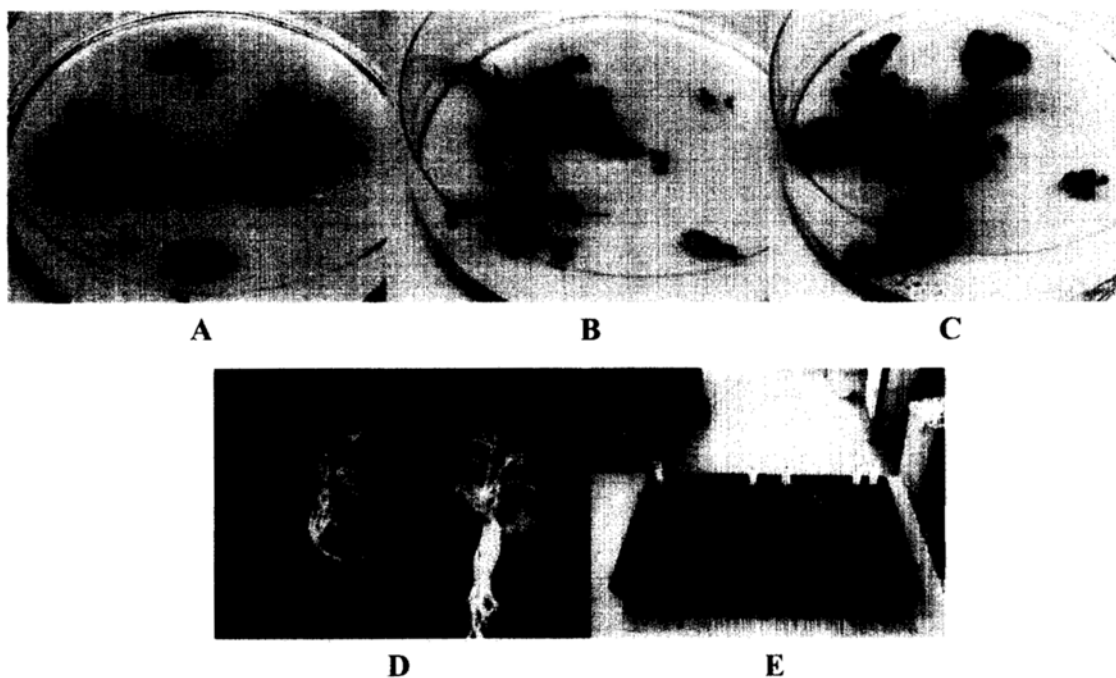


Figure 5. A. Plantlet regeneration from calli of hypocotyls from 'Greenbelt'. The arrow indicates callus formation. **B, C.** Organo-shoots (at arrow) for 'Marathon' and 'Shogun', respectively. **D.** Rooted regenerants of 'Greenbelt' (left) and 'Marathon' (right). **E.** Well-developed plantlet in vermiculite, transferred to the greenhouse at 8 to 10 weeks after co-cultivation. The 'Shogun' regenerants were not further evaluated because they had been contaminated.

explants from all cultivars still being viable at 100 mg/L of either, as indicated by their frequency of callus formation. Therefore, we suggest that selection of transformed cells could be carried out using 100 mg/L of kanamycin or geneticin. Although we have not yet been able to confirm transformation in broccoli, we do believe we have obtained an optimum protocol for rapidly inducing callus formation and regeneration in that species (Fig. 5).

Effect of Tobacco Feeder-Cell Layer

Christey and Sinclair (1992) and Metz et al. (1995) reported that the use of a tobacco feeder layer during

co-cultivation gave the best transformation results for *A. tumefaciens*-mediated transformation in broccoli. The presence of a tobacco cell layer during co-cultivation also increased, by 1.3-fold, the percentage of explants exhibiting transformation (Henzi et al., 2000). Nevertheless, we were unable to obtain transformants when using either a tobacco cell-suspension layer or a tobacco cell layer on the media. Therefore, we believe that an optimized method for *Agrobacterium*-mediated transformation of broccoli remains to be developed and applied. Cogan et al. (2001) have demonstrated that transformation efficiency is restricted by the choice of the target genotype, which suggests that plant genetic factors determine the response to *Agrobacterium*-mediated transformation. Therefore, a first step would be to use a more efficient *Agrobacterium* gene for transfers to the plant cell. Furthermore, the use of compounds that are known to enhance virulence in *Agrobacterium*, such as mannopine, arginine, and acetosyringone (Henzi and Christey, 1999) should be investigated.

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LITERATURE CITED

Cervera M, Pina JA, Juárez J, Navarro L, Peña L (1998) *Agrobacterium*-mediated transformation of citrange: Factors affecting transformation and regeneration. Plant Cell Rep 18: 271-278

Cha HC, Park HJ, Min BM (2002) Plant regeneration and morphology during in-vitro oganogenesis from *Heloniopsis orientalis* (Thunb) C. Tanaka. J Plant Biol 45: 56-61

Cheng J, Veilleux RE (1991) Genetic analysis of protoplast culturability in *Solanum phureja*. Plant Sci 75: 257-265

Christey MC, Sinclair BK (1992) Regeneration of transgenic kale (*Brassica oleracea* var. *acephala*), rape (*B. napus*) and turnip (*B. campestris* var. *rapifera*) plants via *Agrobacterium rhizogenes* mediated transformation. Plant Sci 87: 161-169

Christey MC, Sinclair BK, Braun RH, Wyke L (1997) Regeneration of transgenic vegetable brassicas (*Brassica oleracea* and *B. campestris*) via Ri-mediated transformation. Plant Cell Rep 16: 587-593

Cogan N, Harvey E, Robinson H, Lynn J, Pink D, Newbury HJ, Puddephat I (2001) The effects of anther culture and plant genetic background on *Agrobacterium rhizogenes*-mediated transformation of commercial cultivars and derived doubled-haploid *Brassica oleracea*. Plant Cell Rep 20: 755-762

Coleman M, Waugh R, Powell W (1990) Genetic analysis of *in vitro* cell and tissue culture response in potato. Plant Cell Tiss Org Cult 23: 181-186

Collén AM, Jarl CI (1999) Comparison of different methods for plant regeneration and transformation of the legume *Galega orientalis* Lam (goat's rue). Plant Cell Rep 19: 13-19

Forney CF (1995) Hot-water dips extended the shelf life of fresh broccoli. HortScience 30: 1054-1057

Gamborg OL, Miller RA, Ojima K (1968) Nutrient requirements for suspension culture of soybean root cells. Exp Cell Res 50: 151-158

González AE, Schöpke C, Taylor NJ, Beachy RN, Fauquet CM (1998) Regeneration of transgenic cassava plants (*Manihot esculenta* Crantz) through *Agrobacterium*-mediated transformation of embryogenic suspension cultures. Plant Cell Rep 17: 827-831

Hansen LN, Earle ED (1994) Regeneration of plants from protoplasts of rapid cycling *Brassica oleracea*. Plant Cell Rep 13: 335-339

Hansen LN, Ortiz R, Andersen SB (1999) Genetic analysis of protoplast regeneration ability in *Brassica oleraceae*. Plant Cell Tiss Org Cult 58: 127-132

Henzi MX, Christey MC (1999) *Agrobacterium rhizogenes*-mediated transformation of broccoli (*Brassica oleracea* L. var. *italica*) with an antisense l-aminocyclopropane-1-carboxylic acid oxidase gene. Plant Sci Shannon 143: 55-62

Henzi MX, Christey MC, McNeil DL (2000) Factors that influence *Agrobacterium rhizogenes*-mediated transformation of broccoli (*Brassica oleracea* L. var. *italica*). Plant Cell Rep 19: 994-999

Izhar S, Power JB (1977) Genetic studies with petunia leaf protoplasts. I. Genetic variation to specific growth hormones and possible genetic control on stages of protoplast development in culture. Plant Sci Let 8: 375-383

Jourdan PS, Earle ED (1989) Genotypic variability in the frequency of plant regeneration from leaf protoplasts of four *Brassica* spp. and of *Raphanus sativus*. J Amer Soc Hort Sci 114: 343-349

Jourdan PS, Earle ED, Mutschler MA (1990) Improved protoplast culture and stability of cytoplasmic traits in plants regenerated from leaf protoplasts of cauliflower (*Brassica oleracea* spp. *botrytis*). Plant Cell Tiss Org Cult 21: 227-236

Luo J-P, Jia JF (1998) Callus induction and plant regeneration from hypocotyl explants of the forage legume *Astragalus adsurgens*. Plant Cell Rep 17: 567-570

Metz TD, Dixit R, Earle ED (1995) *Agrobacterium tumefaciens*-mediated transformation of broccoli (*Brassica oleracea* var. *italica*) and cabbage (*B. oleracea* var. *capita*). Plant Cell Rep 15: 287-292

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays and tobacco tissue culture. Physiol Plant 15: 437-497

Oka S, Ohyama K (1981) *In vitro* initiation of adventitious buds and its modification by high concentration of benzyladenine in leaf tissues of mulberry (*Morus alba* L.). Can J Bot 59: 68-74

Thomas TD (2002) Advances in mulberry in tissue culture. J Plant Biol 45: 7-21