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# Production of Multiple Shoots and Plant Regeneration from Leaf Segments of Fig Tree (*Ficus carica* L.)

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**High frequency of multiple shoots and plant regeneration has been obtained from the leaf segments of fig tree (*Ficus carica* L.). Budbreak from dormant buds is highly dependent upon cultivar, so we chose cv. Seungjung Dauphine because it shows an excellent degree of budbreak. Tissue-browning can be an important limiting factor during *in vitro* culture. This phenomenon could be substantially delayed or reduced by treating the tissues with 0.5 mM phloroglucinol, thus oxidizing the phenolic substances exuded from the segments. Wounded leaf explants cultured on MS medium supplemented with TDZ in combination with IBA produced more multiple shoots than did other combinations of auxin and cytokinin. For example, 2 mg $L^{-1}$ IBA along with either 0.5 or 1.0 mg $L^{-1}$ TDZ resulted in 8.1 or 10.8 multiple shoots per explant, respectively. We achieved a frequency of approximately 90% when tissues were first maintained under darkness in the culture medium for one week before being transferred to the light. Regenerated shoots rooted best in a full-strength MS basal medium. *In vitro* regenerated plantlets were then successfully transferred to greenhouse conditions. Here, we have demonstrated a regeneration protocol that is suitable for use in conservation as well as genetic transformation studies of figs and related species.**

*Keywords:* growth regulators, phloroglucinol, regeneration, tissue-browning, wounding

*Ficus carica* L., a member of the family Moraceae, was one of the earliest cultivated for its figs. Although it is an important tree in many rural, marginal areas, it is typically neglected and underutilized, especially in the Mediterranean region where it originated (Morton, 1987). Its fruits have high nutritional and pharmacological value (Moon et al., 1997; Vinson, 1999; Canal et al., 2000; Jeong et al., 2002; Pèrez et al., 2003; Konyaléoğlu et al., 2005), and plants also produce rubber (Kang et al., 2000).

The common fig has bisexual forms - the functional male caprifig and the unisexual female (edible figs) within the syconium. The caprifig is monoecious, and the edible figs contain only long-styled female flowers. Because functional male trees are hermaphroditic, the species is considered morphologically gynodioecious but functionally dioecious (Valdeyron and Lloyd, 1979). Coevolution of *F. carica* and the fig wasp (*Blastophaga psenes*, Agaonidae Chacidoidea) (Weiblen, 2002) has resulted in a complex breeding system involving the two tree morphs (caprifig and edible fig), three floral forms (long-styled female, short-styled female, and male flowers), and an insect pollinator (Beck and Lord, 1988). Thus, traditional breeding methods have required lengthy efforts to improve quality traits, such as the production of parthenocarpic varieties.

Because fig seeds are non-viable, trees must be propagated via cuttings or grafts. Though the propagation of *F. carica* by vegetative cuttings insures uniformity, relatively low multiplication rates are achieved because those materials can be obtained only from upright branches, which results in poor rooting (Kumar et al., 1998). Therefore, multiplication by tissue culture techniques could be a viable alternative, providing numerous, relatively uniform plants within a shorter time period. Over the last two decades, newly developed micropropagation protocols have been used for rapid and large-scale production of a number of fruit trees (Bajaj, 1986). Adventitious shoot development is a key factor when applying genetic engineering techniques to the breeding of woody plant species. *F. carica* is the most recalcitrant in its production of adventitious shoots, but *in vitro* propagation is possible when one uses either single shoot tips (Murithi et al., 1982; Demiralay et al., 1998; Gella et al., 1998; Hepaksoy and Aksoy, 2006) or apical buds (Kumar et al., 1998). Yakushiji et al. (2003) have devised a method for inducing organogenesis from leaf explants of *F. carica*, but their frequency of adventitious bud differentiation has been relatively low.

Another critical problem associated with *in vitro* culture of explants – the darkening or browning of the culture medium that can prove toxic to plant growth (Taji and Williams, 1996) – is attributed to the phenolic substances that are exuded from the cut edges of explants and accumulate in that medium (Laukkanen et al., 1999). Such challenges must be circumvented if we are to be successful in regenerating and transforming fig trees. Thus, the objective of our

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Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxy acetic acid; IBA, indole-3-butyric acid; MES, 2-(N-morpholino) ethanesulfonic acid; MS, Murashige and Skoog; NAA, $\alpha$-naphthalene acetic acid; TDZ, thidiazuron (N-phenyl-N′-1,2,3-thidiazol-5-yl urea)

study was to optimize the conditions required for the induction of adventitious buds and the regeneration of multiple shoots from leaf tissues of *Ficus carica*.

## MATERIALS AND METHODS

### Collection and Plant Materials

Seven mature cultivars of *F. carica* - Seungjung Dauphine, Bongraesi, Banane, Brunswick, Violet Dauphine, Brown Turkey, and The King - were kindly provided by the Subtropical Fruit Crops Experiment Station (Haenam County, Korea) and the Agricultural Technology Center of Yeongam County (Korea). Each was amplified and maintained in the greenhouse of Kumho Life and Environmental Science Laboratory. From these mother plants, stems were cut into 5- to 10-cm pieces, each containing a single node with a dormant axillary bud. The explants (apical shoot buds and nodal explants) were surface-sterilized in 70% ethanol for 10 min, followed by immersion for 10 min in commercial bleach (Youhan Rox, Korea) containing 4% sodium hypochlorite (NaOCl). Later they were thoroughly rinsed with sterile distilled water, and were blotted on sterile dried filter paper to remove the excess moisture.

### Control of Explant-Browning

To prevent or reduce the browning of tissues in culture, we tested several methods: 1) placing the explants on layers of filter paper in the medium; 2) placing the explants on a medium containing 3 g $L^{-1}$ activated charcoal; 3) sealing the Petri dishes with porous tape, or 4) culturing the explants on a medium containing either 0.5 mM phloroglucinol (a degradation product of phloridizin that reduces tissue-browning); or 20 mM $AgNO_3$ (an inhibitor of ethylene action) or adding antioxidants such as 500 mg $L^{-1}$ citric acid and 2 g $L^{-1}$ ascorbic acid. The phloroglucinol, $AgNO_3$, citric acid, and ascorbic acid were all filter-sterilized and added to the media after autoclaving. Cultures were placed in a 25ºC growth room either in darkness or under light (40 μmol $m^{-2}$ $s^{-1}$, 18-h photoperiod).

### Budbreak and Shoot Maintenance

Buds from the seven cultivars were placed on half-strength MS (Murashige and Skoog, 1962) basal salts and vitamins supplemented with 1.0 mg $L^{-1}$ 6-benzylaminopurine (BAP), 0.5 mM phloroglucinol, 3 mM 2-(N-morpholino) ethanesulfonic acid (MES), 3% (w/v) sucrose, and 0.7% (w/v) Gellix® plant agar (Ventech Bio, Korea). Each 100 × 40 mm Petri dish (SPL Life Sciences, Korea) contained 50 mL of this medium plus four buds positioned horizontally. The leaves that developed were exposed to cool white fluorescent light (40 μmol $m^{-2}$ $s^{-1}$) under a 16-h photoperiod at 25 ± 1ºC for four to six weeks. Later, these explants were placed on a shoot induction medium consisting of MS basal salts and vitamins supplemented with 0.04 mg $L^{-1}$ IBA (indole-3-butyric acid), 1.0 mg $L^{-1}$ BAP, 3% (w/v) sucrose, and 0.7% (w/v) Gellix® plant agar (pH 5.8). All shoots were sub-cultured to fresh media at two- to four-week intervals.

### Adventitious Shoot Regeneration from Leaf Segments

Each leaf harvested from an apical bud of the *in vitro* grown plants was transversely cut three to four times across the midrib, but without fully separating the segments. Leaf segments (about 1 $cm^2$) were placed adaxial side up on MS salts and vitamins supplemented with various combinations of auxins [2,4-dichlorophenoxy acetic acid (2,4-D; 0.1, 1.0, or 3.0 mg $L^{-1}$), α-naphthalene acetic acid (NAA; 0.1, 1.0, or 3.0 mg $L^{-1}$), or IBA (0.5 or 1.0 mg $L^{-1}$)] plus cytokinins [BAP; 0.1 or 1.0 mg $L^{-1}$) or thidiazuron (TDZ; 0.5, 1.0, 2.0, or 5.0 mg $L^{-1}$)]. In addition, these media contained 0.5 mM phloroglucinol, 3 mM MES, 3% (w/v) sucrose, and 0.8% Gellix® plant agar (pH adjusted to 5.8 before autoclaving). The ca. 1 $cm^2$ leaf segments were wounded by puncturing their surfaces with fine forceps about 10 times. All cultures were kept at 28 ± 1ºC in the dark for one week, and then incubated under an 18-h photoperiod. After four to six weeks, we calculated the percentage of explants that had produced single or multiple shoots.

### Rooting and Acclimatization of Regenerated Shoots

*In vitro*-regenerated shoots were cultured individually in culture vessels (4.0 × 10 cm) containing full- or half-strength MS media supplemented with 3% sucrose and 0.8% Gellix® plant agar. Plantlets that developed roots were transplanted to a soil mixture comprising vermiculite and perlite (1:1) and placed in a growth room under plastic cover to maintain high humidity. After two to three weeks, these plants were transferred to the greenhouse.

## RESULTS

### Reducing Phenolic Compounds from Explants

One major obstacle associated with the *in vitro* establishment of many woody plant species is the deleterious effects of oxidizing phenols. Our experiments with *F. carica* revealed that compounds exuded from the wounded (cut) edges of the leaf explants were responsible for browning in the culture medium. Therefore, we tested various treatments (Table 1) to reduce this problem. The first, which

**Table 1.** Relative browning of disc sections (bud and leaf segments) from *F. carica* cv. Seungjung Dauphine exposed to different treatments.

| Treatment | Bud | Leaf segment |
|---|---|---|
| Control | +++ | +++ |
| Filter paper on medium | +++ | +++ |
| Petri dish sealed with porous tape | +++ | +++ |
| Addition of activated charcoal to medium | +++ | +++ |
| Addition of phloroglucinol to medium | + | + |
| Addition of $AgNO_3$ to medium | +++ | +++ |
| Addition of citric acid to medium | +++ | +++ |
| Addition of ascorbic acid to medium | +++ | +++ |
| Addition of phloroglucinol and Petri dish sealed with porous tape | + | + |

+, low discoloration; ++, medium; +++, high (darkened).

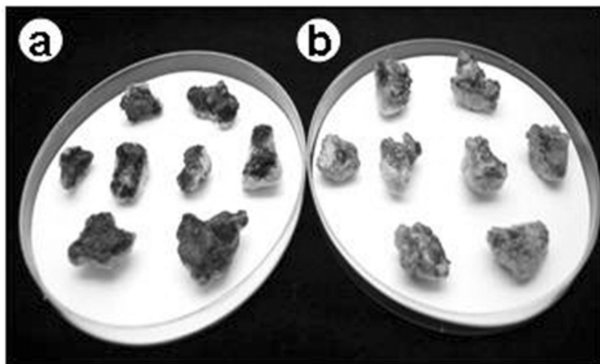


**Figure 1.** Effects on tissue-browning when explants are cultured on medium without (**a**) or with (**b**) phloroglucinol.

involved layering filter paper on the medium, did absorb those secreted phenolic substances, but was not effective in preventing the browning of tissue. Moreover, the addition of activated charcoal to the medium did not reduce browning, a result similar to that reported by Fráguas et al. (2004). For better ventilation, we also used porous tape as a sealing material, but this also was not successful, and the browning continued. Supplements of the ethylene inhibitor $AgNO_3$ as well as two antioxidants, citric acid and ascorbic acid, also failed to decrease the amount of phenolic compounds produced from both buds and leaf segments. However, when the explants were placed on a medium containing phloroglucinol, tissue-browning was diminished (Fig. 1); phloroglucinol treatment plus sealing with porous tape had a synergetic effect in delaying browning. We also found that sub-culturing the explants to fresh media at least every two to three weeks helped in either reducing the exudation of phenolic substances or delaying the browning. Therefore, the remaining experiments included phloroglucinol as a media component at every stage.

## Comparison of Budbreak among Cultivars

We compared the degree of budbreak from dormant, cultured dormant buds (Table 2). 'Seungjung Dauphine' achieved excellent budbreak within four weeks of the culture initiation (Fig. 2), along with less incidence of tissue-browning. 'Bongraesi' and 'Brown Turkey' also showed good frequency, with their buds breaking within five weeks of the culture period. However, the remaining cultivars - 'Banane', 'Brunswick', 'Violet Dauphine' and 'The King' - had poor budbreak after the first five weeks, and their dissected buds exuded phenolic compounds that caused

**Table 2.** Comparison of *in vitro* budbreak from the cultured buds of seven *F. carica* cultivars.

| Cultivar | Budbreak | Time frame |
|---|---|---|
| Seungjung Dauphine | +++ | 3 to 4 weeks |
| Bongraesi | ++ | > 5 weeks |
| Banane | + | > 5 weeks |
| Brunswick | + | > 5 weeks |
| Violet Dauphine | + | > 5 weeks |
| Brown Turkey | ++(+) | > 5 weeks |
| The King | + | > 5 weeks |

+, poor; ++, good; +++, excellent.

severe tissue-browning and eventual death. Thus, we conclude that budbreak is highly cultivar-dependent. In our further examinations, we used 'Seungjung Dauphine' as our source material.

## Effects of Plant Growth Regulators on Morphogenesis of Leaf Segments

Leaf segments from 'Seungjung Dauphine' were cultured on MS media supplemented with different concentrations and combinations of auxin and cytokinin. When 2,4-D (in particular, 1.0 mg $L^{-1}$) was combined with 1.0 mg $L^{-1}$ BAP, we observed only an enlargement of the leaf segments, up to two-fold over their original size. In contrast, callus was induced when 2,4-D (especially at 1.0 mg $L^{-1}$) was combined with 0.1 mg $L^{-1}$ BAP.

For all combinations of NAA and BAP, callus was induced only on the edges of the cultured leaf segments and tissue-browning was apparent, but no further morphogenesis occurred (data not shown). In contrast, a relatively high frequency of callus induction was attained with 2.0 mg $L^{-1}$ 2,4-D plus low levels of TDZ (i.e., 0.1 or 0.5 mg $L^{-1}$). Less regeneration was found from leaf segments cultured on a medium with 0.5 mg $L^{-1}$ 2,4-D and 0.1 mg $L^{-1}$ TDZ (Fig. 3). In fact, except for that combination, no shoot morphogenesis occurred from explants cultured on any other test combinations of auxins and cytokinins (data not shown).

We also evaluated the regeneration capability of leaf segments in response to IBA/TDZ (Table 3). The rate of callus induction was excellent when explants were cultured in 0.5 mg $L^{-1}$ IBA plus either 0.5 mg $L^{-1}$ (78.6%) or 1.0 mg $L^{-1}$ TDZ (82.1%). Their respective frequencies of shoot regeneration were 78.6 and 67.9%, and each explant produced an average of three to four plantlets. In contrast, shoot regeneration was 28.9% when we used a combination of 1.0 mg $L^{-1}$

**Figure 2.** Budbreak and shoot development of *F. carica* cv. Seungjung Dauphine. (**a**) Cultured buds, (**b**) shoot development from bud, and (**c**) plant materials for present study.

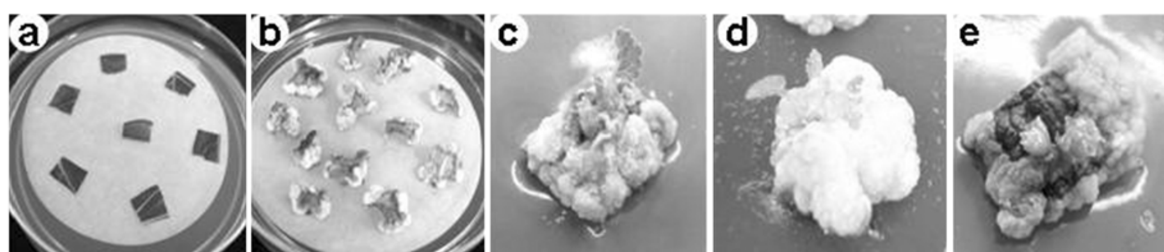

**Figure 3.** Plant regeneration from *F. carica* leaf segment. (**a**) Cultured fig leaf segments, (**b**) callus induction, and (**c**-**e**) shoot regeneration: (**c**, 0.5 mg $L^{-1}$ 2,4-D/1.0 mg $L^{-1}$ TDZ; **d**, 0.5 mg $L^{-1}$ IBA/0.5 mg $L^{-1}$ TDZ; **e**, 0.5 mg $L^{-1}$ IBA/1.0 mg $L^{-1}$ TDZ).

**Table 3.** Effects of IBA and TDZ on callus induction and plant regeneration from leaf segments of *F. carica* cv. Seungjung Dauphine.

| PGR (mg $L^{-1}$) | | Callus induction (%) | Shoot development (%) | No. of shoots per explant[d] | Callus growth[e] |
|---|---|---|---|---|---|
| Auxin | Cytokinin | | | | |
| IBA 0.5 | TDZ 0.5 | 22[a]/28[b] (78.6) | 22[c]/28[b] (78.6) | 3.9 | +++ |
| IBA 0.5 | TDZ 1.0 | 23/28 (82.1) | 19/28 (67.9) | 3.1 | +++ |
| IBA 0.5 | TDZ 2.0 | 16/28 (57.1) | 0 | 0 | +++ |
| IBA 0.5 | TDZ 5.0 | 12/28 (42.9) | 0 | 0 | ++ |
| IBA 1.0 | TDZ 0.5 | 12/28 (42.9) | 8/28 (28.9) | 1.0 | ++ |
| IBA 1.0 | TDZ 1.0 | 14/28 (50.0) | 0 | 0 | ++ |
| IBA 1.0 | TDZ 2.0 | 15/28 (53.6) | 0 | 0 | ++ |
| IBA 1.0 | TDZ 5.0 | 12/28 (42.9) | 0 | 0 | ++ |

[a]Number of leaf segments inducing calli.
[b]Number of leaf segments cultured.
[c]Number of leaf segments producing shoots.
[d]Number of shoots per responding explant.
[e]+, poor; ++, good; +++, excellent.

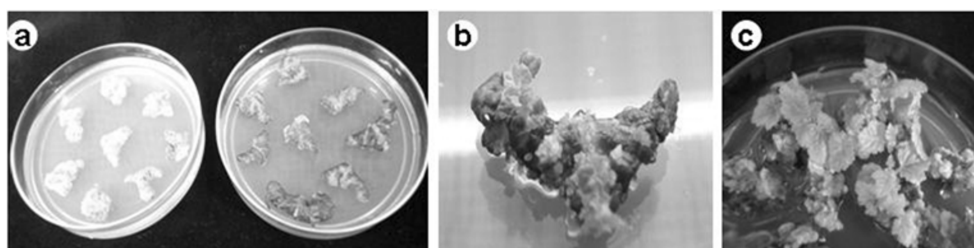

**Figure 4.** Effect of illumination and wounding on multiple shoot production from leaf segments. (**a**) Left, continuous darkness; right, one week of darkness followed by 18-h light treatment; (**b**, **c**) multiple shoots developing from leaf segment cut with forceps.

IBA and 0.1 mg $L^{-1}$ TDZ, and only one plantlet per explant was produced. Thus, the best shoot development from leaf segments was obtained with 0.5 mg $L^{-1}$ IBA and 0.5 mg $L^{-1}$ TDZ (Fig. 3d).

In separate observations, we noted positional and illumination effects for leaf segments on the culture medium containing 0.5 mg $L^{-1}$ IBA and 0.5 mg $L^{-1}$ TDZ. For example, when the axial surface was placed downward, no plantlets were regenerated, but when the adaxial side touched the media, the shoot regeneration frequency exceeded 80%. Moreover, when the segments were cultured under continuous darkness, they became bleached and showed no further differentiation (Fig. 4a). Finally, more plants were regenerated when tissues were cultured first in the dark for one week, then moved to an 18-h photoperiod, compared with the performance of explants exposed to either continuous dark or light conditions.

## Production of Multiple Shoots from Leaf Segments

To induce multiple shoots, we wounded the surfaces of our leaf explants with forceps, then placed them on media supplemented with various combinations of IBA and TDZ (Table 4). At low levels of IBA (0.5 or 1.0 mg $L^{-1}$), only callus was induced, without further development or differentiation. When 2.0 mg $L^{-1}$ IBA was combined with different TDZ concentrations, only a few calli were induced, but the frequency of shoot regeneration was considerably high, ranging from 28.6 to 92.9%. Furthermore, shoot regeneration frequencies were 89.3 or 92.9% for explants exposed to 2.0 mg $L^{-1}$ IBA plus either 0.5 or 1.0 mg $L^{-1}$ TDZ, respectively, and the number of multiple shoots averaged 8.1 or 10.8, respectively, per explant (Fig. 4b, c). Callus induction rates ranged from 39.3 to 42.3% when 5 mg $L^{-1}$ IBA was applied with TDZ, although no shoot organogenesis occurred

**Table 4.** Effects of IBA and TDZ on callus induction and the production of multiple shoots from wounded leaf segments of *F. carica* cv. Seungjung Dauphine.

| PGR (mg L$^{-1}$) | | Callus induction (%) | Shoot development (%) | No. of shoots per explant[d] | Callus growth[e] |
|---|---|---|---|---|---|
| Auxin | Cytokinin | | | | |
| IBA 0.5 | TDZ 0.5 | 24[a]/28[b] (85.7) | 0 | 0 | +++ |
| IBA 0.5 | TDZ 1.0 | 25/28 (89.3) | 0 | 0 | +++ |
| IBA 0.5 | TDZ 2.0 | 22/28 (78.8) | 0 | 0 | +++ |
| IBA 1.0 | TDZ 0.5 | 19/28 (67.9) | 0 | 0 | +++ |
| IBA 1.0 | TDZ 1.0 | 19/28 (67.9) | 0 | 0 | +++ |
| IBA 1.0 | TDZ 2.0 | 19/28 (67.9) | 0 | 0 | +++ |
| IBA 2.0 | TDZ 0.5 | 5/28 (17.9) | 25[c]/28[b] (89.3) | 8.1 | + |
| IBA 2.0 | TDZ 1.0 | 2/28 (7.1) | 26/28 (92.9) | 10.8 | + |
| IBA 2.0 | TDZ 2.0 | 2/28 (7.1) | 8/28 (28.6) | 1.0 | + |
| IBA 5.0 | TDZ 0.5 | 11/28 (39.3) | 0 | 0 | ++ |
| IBA 5.0 | TDZ 1.0 | 12/28 (42.3) | 0 | 0 | ++ |
| IBA 5.0 | TDZ 2.0 | 12/28 (42.3) | 0 | 0 | ++ |

[a]Number of leaf segments inducing calli.
[b]Number of leaf segments cultured.
[c]Number of leaf segments producing shoots.
[d]Number of shoots per responding explant.
[e]+, poor; ++, good; +++, excellent.

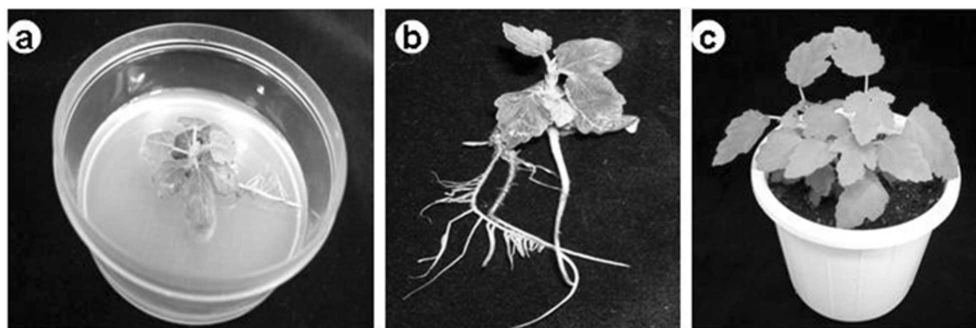


**Figure 5.** Production of *F. carica* from leaf segments. (**a**) *in vitro*-regenerated plant, (**b**) plant with well-developed roots, and (**c**) plant growing in greenhouse.

from these calli or the explants.

All regenerated plantlets were transferred to rooting media containing full- or half-strength MS, where the majority of those new shoots eventually produced roots. The full-strength basal MS proved more effective, with >90% of those shoots showing root proliferation and development (Fig. 5a, b). The successfully rooted plantlets were transferred to a soil mixture for acclimatization and later moved to the greenhouse (Fig. 5c). None of them showed any detectable variations in their growth habits or morphological characteristics.

## DISCUSSION

*F. carica* is known to be recalcitrant to *in vitro* morphogenesis. Historically, plantlets have been produced from shoot tips (Murithi et al., 1982; Demiralay et al., 1998; Gella et al., 1998; Hepaksoy and Aksoy, 2006), apical buds (Kumar et al., 1998), or leaf tissues (Yakushiji et al., 2003). However, common problem has been the browning of that explant material. Therefore, we investigated this phenomenon, using both buds and leaf segments. Polyphenol oxidases contribute to browning through the oxidation of phenolic acids (Webb, 1966; Vaughn and Duke, 1984; Dowd and Norton, 1995). Regardless of the culture period, the degree of tissue-browning is generally higher with buds than with leaf tissue. In the present study, the addition of phloroglucinol to the culture medium as well as sealing the Petri dishes with porous tape resulted in reduced or delayed browning. Phloroglucinol, a degradation product of phloridizin, regulates this behavior. Also called phloroglucin, this 1,3,5-trihydroxybenzene is the genin of many glycosides, found particularly in the bark of apple and other trees. Although free phloroglucinol is not abundant in plants, its derivatives, e.g., flavonoid compounds, are distributed widely (Jones, 1976). Phenolics, such as phloroglucinol and resorcinol, can inhibit the activity of some polyphenol oxidases (PPO). The effectiveness of certain phenolic substances in enhancing shoot formation is well established in plant tissue culture protocols. Likewise, other factors that affect auxin levels within those tissues are important in controlling their growth

and morphogenesis. Therefore, we showed here that the enhancement of multiplication may have been due to the interaction between phloroglucinol and sucrose, as well as the protection of auxin by phloroglucinol. Di- and tri-hydroxyphenols and their polymers possibly protect the auxin from peroxidase-catalyzed oxidation by keeping the cells at a lower redox potential (Lee et al., 1982).

Phloroglucinol can also enhance the growth rates of apple shoots (Jones, 1976) and potato shoot tips (Sarkar and Naik, 2000), and can act as an auxin synergist during the auxin-sensitive phase of root initiation. Using *in vitro* cultures of fig, Yakushiji et al. (2003) and Hepaksoy and Aksoy (2006) have shown that the addition of phloroglucinol is effective in inducing the formation of adventitious buds. However, those studies do not mention the influence of phloroglucinol on reducing or delaying tissue-browning. Here, we demonstrated that this phenolic compound had a beneficial effect, ultimately resulting in an increased survival rate and morphogenesis.

Antioxidants are not always effective in solving this problem, because, for example, ascorbic acid is rapidly destroyed in the plant tissue culture media (Elmore et al., 1990). However, Mante and Tepper (1983) have used such antioxidant compounds to delay browning. We also found that the addition of an ethylene inhibitor, $AgNO_3$, to the medium was not effective. Pua and Chi (1993) have reported that leaf discs derived from mustard plants are highly regenerative on a shoot regeneration medium supplemented with $AgNO_3$, but its presence inhibits this regeneration when discs are derived from plants grown with that compound.

Plant regeneration is associated with the dissected edges of leaf explants and their vascular tissue. Sarwar and Skirvin (1997) have proposed that these cut surfaces provide a means for nutrients and plant growth regulators to be taken up and utilized efficiently. Here, we showed that the highest frequency occurred when the leaf adaxial surface was in contact with the medium, similar to observations made from apple (Fasolo et al., 1989). This response was due to increased oxygen exchange, because the stomata are located axially (Blancke and Belcher, 1989), and the adaxial palisade parenchyma are better able to transport nutrients and growth regulators from the medium into the explants (Welander, 1988). Higher regeneration rates also were achieved by using young leaf explants that were still developing, less differentiated, and possedded more metabolically active cells.

In the present study shoots formed from leaf segments cultured in a combination of 0.5 mg $L^{-1}$ IBA plus either 0.5 or 1.0 mg $L^{-1}$ TDZ. This effect of IBA on multiple-shoot induction has also been reported in *Cymbopogon nardus* (Chan et al., 2005). Moreover, TDZ is effective for the regeneration of adventitious shoots from woody plants (Huetteman and Preece, 1993) and in promoting the growth of *Centella asiatica* (Kim et al., 2004). It is more efficient than BAP for inducing organogenesis in fig (Yakushiji et al., 2003), mulberry (Thomas, 2003), apple (de Bondt et al., 1996; Sarwar and Skirvin, 1997), and black and wild cherries (Hammatt and Grant, 1998). Here, we found that, for a medium containing 0.5 mg $L^{-1}$ IBA with 0.5 or 1.0 mg $L^{-1}$ TDZ (Table 4), the cut edges preferentially formed more calli than shoots compared with explants that were not wounded (Table 3). In contrast, when a high concentration of IBA (2.0 mg $L^{-1}$) was combined with 0.5 or 1.0 mg $L^{-1}$ TDZ, multiple shoots developed from the cut edges (Table 4), suggesting that both growth regulators and wounding play key roles. Yakushiji et al. (2003) also have successfully produced fig plants from leaf tissue, but at lower frequencies, i.e., 22.5% for shoot formation and with an average of 1.0 to 2.5 shoots per responding explant. Here, we achieved about 90% shoot production and 10.8 shoots per responding explant. Therefore, although leaf-wounding may disrupt the existing meristems within the explants, it may also cause increased meristematic activity that leads to multiple shoot formation.

In summary, we have now identified several important factors for improving multiple shoot production and plant regeneration from fig leaf segments. Phloroglucinol added to the medium delays the exudation of phenolic substances. Likewise, tissue-wounding and efficient combinations of IBA and TDZ enhance the frequency and numbers of multiple shoots per responding explant. This protocol could be very useful for genetic engineering studies, particularly those involving transformation.

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