In Vitro Shoot-apex Grafting of Mulberry (*Morus alba* L.)

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Abstract. A protocol to achieve in vitro shoot apex grafting of *Morus alba* is described. Decoated seeds were incubated at 26C on Murashige and Skoog (MS) medium in 0.2% Gelrite for 10 days in darkness and 2 weeks with a 16-hour daily photoperiod. The shoots were excised from seedlings, and the shoot bases were soaked in 500 μM IBA for 30 min. The shoots were transferred to a vermiculite support medium moistened with half-strength MS salts and were incubated at 26C in darkness for 10 to 12 days. Seedling stems, now ≈ 8 mm in circumference, were used as rootstocks. Shoot apex scions 1 to 2 mm long were taken from adult plants of several major commercial cultivars of *M. alba*. The average successful frequency of in vitro shoot-apex grafts was 75% to 80%, indicating that thickening the circumference of rootstock stems was a critical factor. Chemical name used: indole-3-butyric acid (IBA).

In 1990, China planted 770,000 ha of mulberry as a food source for silkworm (*Bombix mori* L.) (Zhu, 1993). In addition, *Morus alba* plantings are a part of a sustainable strategy for water and soil conservation in the north loess plateau of China.

The genetic characteristics of superior *M. alba* cultivars are maintained by the vegetative propagation of selected cultivars. Rooting may be a problem with cuttings (Hossain et al., 1992). Grafting is preferred for propagation of new or superior *M. alba* cultivars, despite the higher cost and limited duration of the season.

In some plant species, propagation through tissue culture offers a ready method to obtain a large number of uniform plants. *Morus* spp. have been micropropagated from adventitious buds forming on cotyledon, leaf, hypocotyl, shoot tip, nodal segments, or axillary and winter buds of seedling or mature trees (Hossain et al., 1992; Ivanicka, 1987; Kim et al., 1985; Mhatre et al., 1985; Ponchia and Gardiman, 1992). However, especially with shoot-tip culture, difficulties have been encountered in rooting proliferated shoots and transplanting these plantlets to soil (Hossain et al., 1992; Kim et al., 1985; Ponchia and Gardiman, 1992). Shoot apex grafting, as perfected for citrus (Navarro et al., 1975), may be an effective propagation method to overcome such difficulties. Inserting the citrus shoot apex scion in a small slit 1 to 2 mm from the top of the rootstock gave 90% to 100% success frequency (Ma and Murashige, 1989), a success rate double that of the standard method with an

inverted-T incision. The process of shoot apex grafting has been reviewed (Jonard, 1986), especially in woody fruit species, including citrus (Navarro et al., 1975), apple (*Malus domestica* Borkh.) (Huang and Millikan, 1980), and peach [*Prunus persica* (L.) Batsch] (Deogratias et al., 1986). To our knowledge, in vitro shoot-apex grafting of *M. alba* has not been reported in the literature. Our objectives were 1) to determine the feasibility for in vitro shoot apex grafting of *M. alba*, 2) to investigate how the condition of the rootstock segments affects the survival rate of the shoot apex grafted plantlets, and 3) to develop a protocol for in vitro shoot apex grafting of economically important cultivars of *M. alba* in China.

Materials and Methods

Culture of rootstocks under aseptic conditions. *Morus alba* seeds were moistened and decoated (including pericarp) by hand with the aid of a surgical blade and then were surface-sterilized by immersing in 1% solution of sodium hypochlorite for 15 min, followed by three rinses with sterilized water. The 100-ml Erlenmeyer culture flasks were prepared containing 25 ml of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) in 0.2% Gelrite (a gellan gum, a gel-forming hydrocolloid; Kelco Division of Merck & Co., San Diego). Ten decoated and sterilized seeds were placed in each flask, and the flasks were held for 10 days in a culture chamber in darkness at 26C with $\approx 50\%$ of seeds germinating. Three methods then were used to obtain rootstock seedlings. Flasks were held in a culture chamber for 2 weeks at 26C with a 16-h daily photoperiod ($\approx 100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ from cool-white fluorescent lamps). Shoots were excised from the seedlings at cotyledon nodes. Ex-

cised shoot bases were soaked in 25 ml sterilized solution of 500 μM IBA for 30 min. Ten shoots were transferred to each 100-ml Erlenmeyer flask with 25 ml vermiculite support medium moistened with 5 ml half-strength MS salts. For the first method (i), the flasks were placed in an incubator at 26C in darkness for 10 to 12 days; for the second method (ii), the newly germinated seedlings were incubated in darkness at 26C for 3 weeks; and for the third method (iii), the decoated seeds were soaked in (2*RS*,3*RS*)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1*H*-1,2,4-triazol-1-yl)pentan-3-ol (paclobutrazol) (Chemical Factory of Jiangsu, China) solution at 8.5 μM for 15 min before planting on a vermiculite support medium moistened with MS medium, followed by incubation in darkness at 26C for 4 weeks with 16-h daily photoperiod in a culture chamber.

When the circumference of the stem reached ≈ 5 to 10 mm, 2-cm segments with two to three nodes were used as rootstocks. The segments were covered by a damp cotton cloth and kept in an upright position in petri dishes (10 cm in diameter) to maintain their physiological polarity. The sections were cut before the grafting was performed. In a separate experiment, rootstock sections of 1.0, 1.5, and 2.0 cm were prepared to examine the effect of rootstock length on the survival of shoot apex grafted plantlets.

Preparation of scions under aseptic conditions. Thirty adult plants of the commercial cultivars 707, Heyiebai, Tongxiangqing, and Xinyizhilai were used as a source of the shoot apex scions. Shoot apices were taken from the new spring growth of outdoor-grown plants. Cultivar 707 was used in developing the initial protocol to obtain suitable shoot apices as scions for shoot-apex grafting in vitro. Shoot apices 1.0 cm in length were cut, surface-

Table 1. Influence of methods used on resultant rootstock seedling height, circumference *M. alba* stems, and percent survival of shoot apices as scions 4 weeks after grafting to 2-cm-long rootstock segments.

Method ^a	Ht seedling (cm)	Stem circumference (mm)	Grafting survival rate (%)
i	3.6 a ^y	8.0 a	92 a
ii	4.7 a	4.5 b	69 b
iii	4.5 a	2.7 c	0 c

^aTen decoated and sterilized seeds were placed in each flask. The flasks were held for 10 days in darkness at 26C. Rootstocks then were obtained by excising shoots at cotyledon nodes after holding flasks for 2 weeks at 26C with a 16-h daily photoperiod. Excised shoot bases were soaked in 500 μM IBA for 30 min. Ten shoots were transferred to each flask with vermiculite support medium moistened with half-strength MS salts. Three methods were used: i) flasks were placed at 26C in darkness for 10 to 12 days, ii) newly germinated seedlings were incubated in darkness at 26C for 3 weeks, iii) decoated seeds were soaked in paclobutrazol solution at 8.5 μM for 15 min before planting on a vermiculite support medium moistened with MS medium followed by incubation in darkness at 26C for 4 weeks with 16-h daily photoperiod.

^yMean separation within each column by Fisher's protected least significant difference test at $P \leq 0.05$.

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sterilized in 1% sodium hypochlorite for 15 min, and then rinsed three times with autoclaved water. Shoot apex scions 1 to 2 mm long were excised under aseptic conditions with the aid of a microscope with a fluorescent illuminator below. Once the protocol was established with cultivar 707, the other major commercial cultivars noted also were used to obtain suitable shoot apices as scions.

Micrografting procedures. Under aseptic conditions, a 1.5- to 2.5-mm incision was made on the top of the upright 2-cm-long rootstock using a razor blade sliver attached to a surgical handle. The 1- to 2-mm-long shoot apex scion was inserted into the incision. Its top was higher than or even with the cut surface of the rootstock. Bases of the grafted rootstocks were inserted into culture medium in the culture tubes (2.5 × 15 cm) with 20 ml MS salts in 0.2% Gelrite. The culture tubes were stopped with cotton balls. In a separate experiment, the effectiveness of rootstocks selected from higher or lower portions on the seedlings was investigated (i.e., success of shoot tips grafted to the top of the lower half of seedlings as rootstocks vs. success of those grafted to the top of upper half of seedlings as rootstocks).

Maintenance of the grafted plantlets in vitro. The culture tubes with the shoot apices–rootstock grafts were incubated in a culture chamber at 26°C with a 16-h daily photoperiod ($\approx 100 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ from cool-white fluorescent lamps) and were observed periodically to

evaluate the survival of scions and rooting of rootstocks. The percentages of survival and rooting of these grafted plants were recorded 2 weeks after grafting, while the number of roots per grafted shoot and elongation of the scion were determined 4 weeks after grafting. Adventitious shoots arising from the rootstocks were removed aseptically with a pair of surgical scissors.

Hardening and transplanting the grafted plants to soil. About 4 to 6 weeks after in vitro shoot apex grafting, grafted plants ≈ 1.5 to 2.0 cm tall with at least three expanded leaves grown from the scion-derived shoot apices were moved to natural lighting for hardening for 7 to 10 days. The Gelrite medium was washed off with tap water, and the grafted plants were transplanted to a nursery bed with a 25-cm-deep mixture of 1 fine sand : 1 yellow cotton soil : 1 compost (by volume). The nursery bed was covered with arched transparent plastic sheeting to minimize moisture loss and was shaded with nylon netting. The plastic sheets were opened 10 days later and removed after a total of 2 to 3 weeks. The plants were allowed to grow under natural light. The percent survival, shoot height, and number of branches per plant were recorded 2 months after transplanting to the nursery fields.

Statistical design. The experiments were randomized complete block in design. Except where noted, each treatment had 20 grafted plantlets with three replications. All experiments were repeated at least once, with similar

results. All sets of data were subjected to analysis of variance. In cases where the variance ratio was significant at $P < 0.05$, Fisher's protected least significant differences tests were used to check the level of significance.

Results and Discussion

Grafting method. The frequency of successful in vitro shoot apex grafts of *M. alba* was highest in rootstock seedlings obtained from method (i). Nearly all of the 2-cm-long segments from these rootstock seedlings yielded successful shoot apex grafts (Table 1). The rootstock seedlings obtained from method (ii) yielded $\approx 20\%$ fewer, and method (iii) resulted in no successful shoot apex grafts. The stem circumference of the resultant seedlings from method (i) reached 8 mm, which is $\approx 80\%$ and 190% more than that of the seedlings obtained from methods (ii) and (iii), respectively. The rootstock seedlings obtained from method (iii) supported no successful shoot apex grafting; they remained green, with more than two expanded leaves, but had the smallest stem circumference (Table 1). Method (i) was used successfully on all four major commercial cultivars of *M. alba* we tested. The overall average frequency of successful shoot apex grafts reached 75% to 80%.

Germinated seedlings of *M. alba*, whether grown in the light or etiolated, as is the case with the seedling obtained by using methods (ii) or (iii) (Table 1), could not be used directly

Fig. 1. A schematic procedure for producing shoot-apex-grafted plants of *Morus alba*.

as rootstocks for shoot apex grafting in vitro. This failure may be due to the small seed size of *M. alba* (≈ 1.47 to 1.49 g/1000 seeds) and to the limited size of the resultant seedlings. Our results indicated that thickening of the rootstock stem with method (i) is a critical step for successful, in vitro, shoot-apex grafting of *M. alba*. The photoperiod, the pretreatment of rootstock segments in IBA for 30 min, or the length of rootstock (≈ 2 cm) may have contributed to the success of the shoot apex grafts and their better growth.

Location of rootstock seedlings. Shoot tips grafted to the top of the lower half of seedlings as rootstocks gave $\approx 50\%$ more growth by 4 weeks than those grafted to the top of the upper half of seedlings as rootstocks of *M. alba* cv. 707 (20.9 vs. 13.5 mm). Using either of these segments as rootstocks resulted in similar survival rates of grafted plantlets, rooting percentage, and number of roots per shoot. However, the rootstocks used in grafted plants from the lower-half segments of the seedlings appeared more woody than those from the upper-half segments.

Length of rootstock segments. The longer the rootstock segments, the greater the elongation of the shoot-tip scions of *M. alba* cv. 707. When the length of rootstock segments was 1.5 or 2.0 cm, rather than 1.0 cm, the elongation of the scions increased by $\approx 75\%$ or 185% , respectively (detailed data not shown). Graft survival rate, rooting percentage, and number of roots per shoot were not significantly different with increasing length of rootstocks segments, although the means of these three variables were highest with 2-cm segments as rootstocks. This result indicates that a 2-cm-long rootstock segment is the preferred length for this type of grafting.

Growth of grafted plants in the field. The grafted plants of all four commercial cultivars of *M. alba* were transplanted to a field nursery

from Apr. to June 1992. About 85% of the plants survived. The plants grew well in the field, with average shoot length per plant reaching 209 cm, with 5.2 branches per plant, in Fall 1992.

Attempts at shoot-tip culture in vitro of many commercial varieties of *Morus* spp. encountered difficulty in rooting the proliferated shoots (Kim et al., 1985; Ponchia and Gardiman, 1992). Transplanting the plantlets to soil was even more difficult. Shoot differentiation could not be easily induced in certain cultivars. These obstacles were overcome with the techniques of in vitro shoot-apex grafting. Our study yielded $\geq 90\%$ successful, in vitro, shoot-apex grafts for *M. alba* compared to the average of 30% to 50% successful shoot-apex grafts in other woody species (Navarro, 1988; Navarro et al., 1975).

The restored rooting competence and increased growth rates of shoots represent a partial restoration of juvenile traits. Therefore, freely rooting clones of *M. alba* could be developed with the shoot-apex grafting procedures in vitro. We have obtained the fourth generation of grafted *M. alba* plants. Whether the rooting competence continues in these scion-derived shoots remains to be determined.

Since the palatability and nutritive qualities of *M. alba* leaves for silkworms vary tremendously among trees, our shoot-apex grafting method will be useful for rapid clonal multiplication of difficult-to-root and superior cultivars. To our knowledge, this study is the first to use shoot-apex grafting to develop a practical propagation procedure for major commercial cultivars of *M. alba*. A brief schematic procedure for producing plantlets via shoot-apex grafting of *M. alba* is outlined (Fig. 1). Our suggested protocol will assist the programs of virus elimination, clonal breeding, and reproduction of superior clones for *M. alba*.

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