Title: Development of Micropropagation and Acclimation Protocols for the Commercialization of a New Bonsai Ornamaental Tree for the California Market.

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Introduction:

A novel phenotype from a *Pistacia chinensis* hybrid cross was discovered in 1991 and published in 2003. The trees are highly dwarfed, giving a 'Bonsai' appearance (Figure 1). The trees have much shortened internodes, miniature leaves, and a thickened bark. Fall coloration ranges from yellow to red. Tree and leaf form vary among the dwarf genotypes. These trees have potential as subjects for the bonsai tree market, since they are naturally dwarfed as well as displaying good fall color in relatively low chill environments. They are described in Parfitt (2003).

The phenotype is the result of a recessive mutation occurring in two specific parents, and is expressed only when these parents are crossed. The seedling progeny express the dwarf in a 1:4 dwarf:normal ratio. Because 3/4 of the progeny are normal, a nurseryman desiring to produce these dwarf trees will have to discard 3/4 of the progeny after selecting for the dwarfs. The dwarf trees have never flowered or produced fruit (some are more than 10 years old) and therefore cannot be used to produce seedlings with a higher percentage of dwarf progeny. The non-dwarf progeny should be 50% heterozygous for the gene, but do not mature reproductively for more than 10 years. The prospect of trying to produce the dwarf trees from seed is an unattractive proposition from an economic perspective. Further, the variability in phenotype among the progeny means that some forms may be less desirable and would be less saleable. The mutation has never been observed in a homozygous condition except for the progeny from the specific cross described in Parfitt (2003). For commercial development of this potentially valuable plant, a clonal propagation procedure is needed.

During the last 15 years, the Parfitt lab has developed improved protocols to propagate pistachio by micropropagation (Parfitt and Almehdi 1994) and direct rooting of cuttings (Almehdi et al. 2002). Preliminary studies exploring the possibility of producing somatic embryos from protoplasts (Chan et al. 2002) were also conducted.

The project funded by the Slosson grant was undertaken to develop a method for clonal propagation of the 'Bonsai' dwarf pistachio, with the eventual goal of being able to commercially propagate them. The project was initiated in the spring of 2006. Both tissue culture micropropagation and grafting techniques were evaluated for efficacy.

Materials and Methods:

Shoot Micropropagation: An initial population of 62 dwarf 'Bonsai' pistachio trees, maintained in 1 gallon pots was available for the initial micropropagation study. These materials were moved to the greenhouse and grown until good populations of shoots were present. The best plants were brought into the lab and grown under lights while cuttings were excised. Due to the slow growth rate and small size of the trees, only limited numbers of cuttings were available for use. Cuttings were taken until no actively growing dwarf shoots were present, at which time the plants were returned to the greenhouse for regeneration.

Fifteen mother miniature pistachio trees were used for the matrix experiment. Approximately 0.25-0.5 inch terminal shoots were taken from each plant. Because of the limitation on the number of terminal shoots from each plant, only six cuttings could be taken for each treatment. Two treatments were tested. Cuttings were surface sterilized with 1% household bleach solution for 10 minutes with shaking. Tthe cuttings were rinsed three times with sterile water for 30 minutes and soaked overnight in 4°C in fresh sterile water.

Cutting numbers from the source plants were limited and were slow growing. Therefore the initial focus of the research was to increase shoot numbers, with rooting studies to be conducted later. Treatments were modified from the PA2 medium (Almehdi et al., 2002) included varying amount of BA, Kinetin, and 2,4D, individually and in combination (Table 1). The pH of the medium was adjusted to 5.7 with Potassium Chloride and autoclaved at 121°C for 20 minutes. The cultured shoots were placed under 55 micromole/sec/meter light and room temperature (25°C). Treatments are shown in Table 2. After the dwarf mother plants produced enough shoots, the matrix was tested again with sand instead of gelrite. 10 mg of liquid medium plus 12 g of sand were added to each culture vessel using the combinations of growth regulators shown in Table 2.

Rooting of Microshoots: In vitro multiplied shoots were excised and then dipped in 0, 10, 20 and 100mg/L potassium salt of IBA solutions for 30 seconds. The shoots were transferred to a medium lacking growth regulators and containing one g/L Gelrite and 12 g sand in each culture tube.

Grafting Experiments: 47 of the 62 original source plants were available for grafting at the inception of the grafting experiments. The rootstocks used in the grafting experiment were non-dwarf plants obtained from the same cross from which the dwarf pistachios were derived (*P. integrima* x *P. chinensis*). An additional 700 dwarf plants and 2200 standard size plants were generated by repeating the original cross used to produce the dwarfs (Parfitt, 2003). Seed was collected from the cross, and seedlings grown out in the greenhouse. Dwarf plants and a sub-population of standard size seedlings from the same cross were selected for later grafting experiments.

Results and Discussion:

Shoot Micropropagation: Initial results were not positive, apparently because these selections do not do grow well in gelrite media. Replacement of gelrite with sand resulted in much better survival of the explants (Figure 2). Some were maintained for more than six months. However, examination of the microshoots suggested that additional micronutrient supplementation or a better method of inducing nutrient absorbtion by the shoots is needed.

Rooting of Microshoots: While the emphasis of the project was to multiply shoots in-vitro, some cuttings from the trial were also used in a rooting trial. Rooting has proven to be difficult, because a) few explants have been available for use in rooting matrices and b) the roots are very fine and the root systems that develop are weak and difficult to transplant. This was a major reason for exploring the use of grafted plants, which have the benefit of a rootstock with a robust root system. Cuttings from two of the fifteen initial source plants have been rooted. Both plants produced one rooted cuttings (Figure 3). The cuttings took from three months to approximately five months to root. Previous results showed cuttings rooted from three to 14 months. The specific source plants used for propagation appeared to influence the ability to successfully culture shoots and subsequently root them.

Somatic embryogenesis: One of the more interesting observations to date was the apparent formation of somatic embryos on the leaves and stems of selected clones (Figures 4 and 5). These resulted from planting the cuttings in the original rooting treatment #1 and dipping the cuttings in 2 mg/L BA. This treatment yields embryo-like structures. These structures were replanted in the original medium supplemented with 2 mg/L BA. This resulted in the structures reverting into callus.

Grafting: A grafting experiment was conducted to overcome the limitations of the in vitro experiments (the limited number of cuttings, and therefore shoots, obtained from each source plant to be cultured and the presence of only two source dwarf plants that gave rootable in vitro cuttings). Cuttings from the dwarf plants were grafted onto standard size rootstocks from the same cross to avoid potential problems of graft incompatibility. The only type of grafting that succeeded was the approach graft when both the scion and the rootstock were actively growing. Several grafted dwarf pistachio were obtained from a few of the dwarf source plants (Figure 6).

Conclusions:

The micropropagation approach to obtain clonal plants was not very effective. This was due to the slow growth rate and small diameter of the explants that could be obtained from the source dwarf trees. The micropropagated shoots did not grow very fast and did not form axillary shoots. Therefore, micropropagation was not a practical method for rapid multiplication of clonal plants.

The grafting experiments were not completed at the end of the project. Grafting appeared to be a more useful approach to producing the dwarf trees than micropropagation, because the explants were being grafted to vigorous rootstocks and avoided the limitations of a weak root

system. However, the small diameter of the explants and the slow growth rate of the scion explants made the production of successful grafts difficult. Modifications to the technique are needed to make this a successful solution to the problem. Discussions were initiated with a commercial tissue culture nursery. The owners felt that grafted trees would be more useful in a commercial environment than micropropagated trees for the previously mentioned reasons.

Literature Cited:

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Table 1. Media composition used with growth regulator combinations from Table 2.

1 pk/L MS medium plus the following:	mg/L
Casein enzymatic hydrolysate	500
Ascorbic acid	50
H_3BO_3	0.960
$Zn(NO_3)_2$	1.3
KNO_3	100
Glycine	20
Nicotinic acid	5
Pyridoxine-HCl	5
Thiamine-HCl	1
Myo-inositol	100
Kinetin	2
2,4-D	2
Glutamine	250
Sucrose	30

Table 2. Growth regulator treatment combinations tested for shoot initiation:

Treatment #	BA	Kinetin	2,4D
1	0	0	0
2	0	2	0
3	0	0	2
4	0	2	2
5	1	0	0
6	1	2	0
7	1	0	2
8	1	2	2
9	5	0	0
10	5	2	0
11	5	0	2
12	5	2	2
13	10	0	0
14	10	2	0
15	10	0	2
16	10	2	2

Figure 1. Examples of ‘Bonsai’ dwarf pistachios showing fall coloration.

Figure 2. Dwarf pistachio microcuttings propagated on augmented sand substrate.

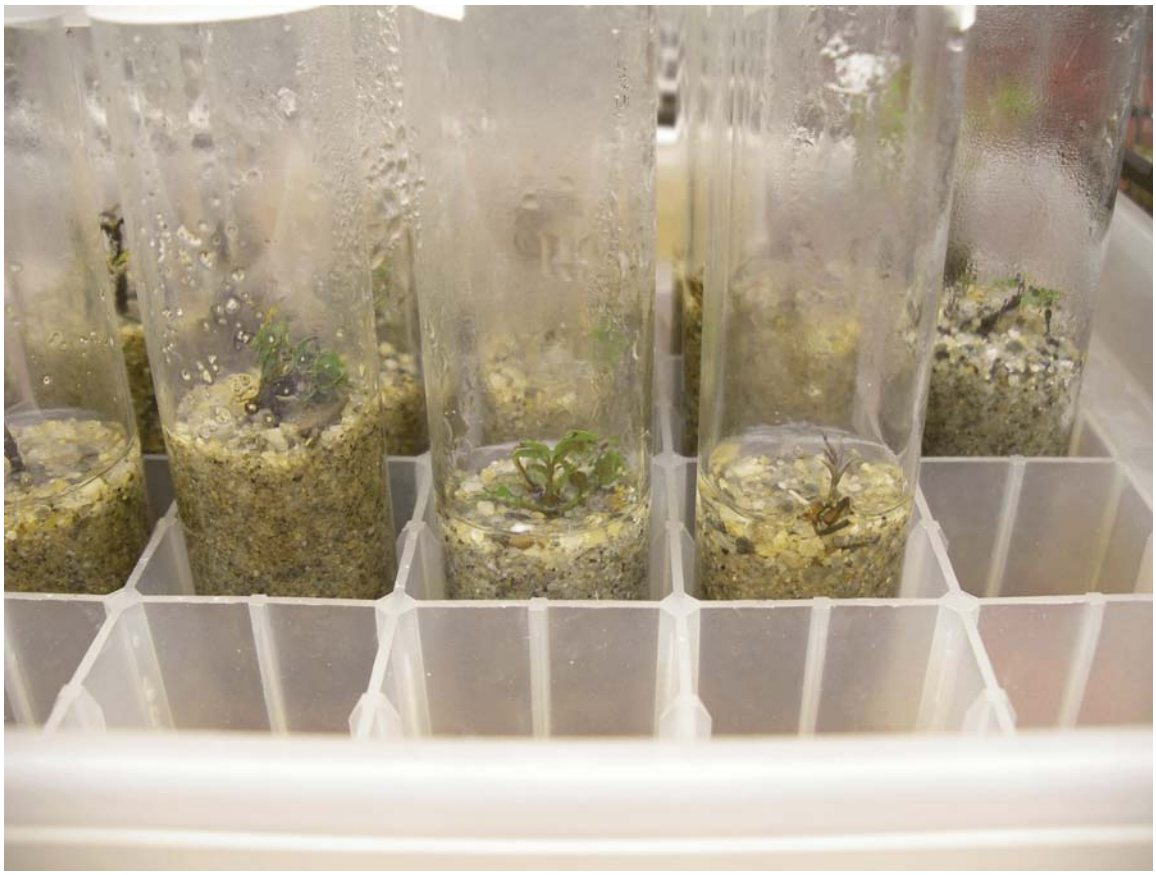

Figure 3. Rooted dwarf pistachio cutting in-vitro.

Figure 4. Possible somatic embryo formation on dwarf pistachio explant (leaf).

Figure 5. Possible somatic embryo formation on dwarf pistachio explant (shoot).

Figure 6. Grafted ‘Bonsai’ dwarf scion on standard sized rootstocks.