AFTERCARE PROCEDURES REQUIRED FOR FIELD SURVIVAL OF TISSUE CULTURE PROPAGATED *ACACIA KOA*

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Abstract. A system of treatments was developed which permitted frequent rooting of *Acacia koa* shoots grown aseptically from shoot tip callus, successful transplanting of the plantlets from aseptic to normal growing conditions, and eventual field establishment of the tissue-culture propagated plants. Rooting was promoted by removing the shoots from a source of benzyladenine for a month or more before providing them a rooting stimulant. To induce roots to become functional, rooted plantlets were grown in a hydroponic medium (Hoagland's solution) for a month or more after their removal from an agar-gelled medium. To date, 82 plants of one clone have been established in the field.

Acacia koa Gray is a large forest tree of Hawaii that has become increasingly valuable as a source of cabinet wood, but like other valued timber species it is becoming short in supply. There is a need to increase the population of straight, well-formed trees of the type best suited for veneer and lumber production. Toward this end, a tree improvement program has been undertaken with the species that includes clonal propagation of superior trees among its aims. Normal techniques of vegetative propagation have proven difficult with this species, but one of the techniques with which we have had some success has been aseptic callus culture of shoot tip tissue. We have grown and field-planted 82 plants produced from the tissue of one shoot tip. The tissue culture technique by which plantlets were first obtained was described earlier (10). This paper reports the results of research that have enabled us to readily root the shoots produced, remove the tissue-culture propagated plantlets from their flasks, cause the root system to become functional, and eventually successfully field-plant the young trees.

REVIEW OF LITERATURE

Although a large amount of literature has been devoted to studies of propagation of forest trees by tissue culture, few of these studies have been aimed at establishing the tissue-culture propagules in the field (3,8). Populus is the only genus for which considerable success in field establishment has been reported (6). Many researchers (1,2,9,11) specifically mention serious rooting difficulties with tissue-culture propagated shoots. In our work with one clone of *Acacia koa*, we have achieved 60 percent success in rooting and 80 percent survival

of the plantlets produced after removal from aseptic culture. This success required considerable experimentation.

MATERIALS AND METHODS

Shoots were grown from callus derived from a seedling shoot apex by methods already described (10) but summarized below. The basal nutrient medium of Murashige and Skoog (7) gelled with 0.8 g/l agar and containing various hormone additions was used for all aseptic growth and rooting experiments. Rooted plantlets were potted in both aseptic and non-sterile Hoagland's solution (4) and in a potting mix of peatmoss, vermiculite, and perlite.

Continuous light at photon flux densities of 50 to 120 u mol $m^{-2}sec^{-1}$ of photosynthetically active radiation was used in the experiments.[3] A constant temperature of 25°C was maintained. The growth of regulators used in the experiments were coconut water, benzyladenine, naphthaleneacetic acid, and indolebutyric acid. The coconut water was derived from green nuts and kept frozen until used. Plantlets growing in one liter containers of potting mix were fertilized once a month with 25 ml of a 20:20:20 liquid fertilizer. Plants were grown in a nursery and then planted in the forest at three locations similar in elevation to that of the tree that provided the seed from which they were all grown.

RESULTS

Shoot formation and growth. Shoot meristems formed in a subculture of callus derived 1 year earlier from a seedling shoot tip. The solid medium on which they formed contained 10 mg/l of benzyladenine. The callus, with shoot meristems, was then transferred to a medium with 1 mg/l benzyladenine for 1 month and then to one containing 10 percent coconut water for another month. Then it was transferred back to a 1 mg/l benzyladenine medium for another month and again followed by transfer to the 10 percent coconut water medium. These sequential transfers caused the meristems to proliferate and begin shoot elongation. Following these transfers, the shoots were maintained and multiplied on a 1 mg/l benzyladenine medium by dividing and subculturing the callus and shoots at approximate monthly intervals.

Initiation of roots. Rooting experiments were undertaken as soon as shoots had produced two or more leaves. We placed the shoots, some with callus and others without, onto media containing 0.2, 0.3, 1.0 mg/l indolebutyric acid and 0.5 and 1.0 mg/l naphthaleneacetic acid. No roots formed in any of these exper-

[3] Light terminology and measurements follow those of Incoll et al. (5).

iments, so after about 3 months, several of the shoots were transferred to a nutrient medium without growth regulators in an attempt to "dilute" the amount of auxin they had taken up.

Some of these shoots, together with basal callus, were placed on a medium containing 0.2 mg/l indolebutyric acid after having been on the regulator-free medium for about 1 month. Two of these rooted. This suggested to us that after having been grown on a medium containing benzyladenine, the shoots had to be grown for a time without growth regulators to "clear" or dilute the benzyladenine. Furthermore, they would only root from that portion of the stem surrounded by callus tissue, so callus surrounding the stem base was essential.

Actually, several empirical experiments were needed to demonstrate that these were indeed the answers to success in rooting koa shoots. These experiments demonstrated that a period of at least 3 weeks on regulator-free medium was required in order to overcome the inhibitory effect of the 1 mg/l benzyladenine treatment. A concentration of 0.3 mg/l indolebutyric acid produced better root growth than did a concentration of 0.2 mg/l, or than 0.5 mg/l naphthaleneacetic acid. Shoots without basal callus invariably died. Using a period of 1 month in regulator-free medium and 0.3 mg/l indolebutyric acid in the rooting medium, we achieved approximately 60 percent success in rooting.

Initiation of autotrophic growth. The first six rooted plantlets removed from aseptic media and transplanted to a rooting medium rapidly desiccated and died. Microscopic examination showed a good connection between the stem and root vascular systems, but the plants appeared to die of moisture stress.

A second group of six plants was potted and placed under intermittent mist. These survived until they were removed from the mist. They then all died of apparent moisture stress.

A series of experiments were then made to seek a conditioning method that would result in survival of the plantlets after removal from the aseptic rooting medium. Several of these tests required continued aseptic growth to improve root systems, while others involved immediate transfer from aseptic to non-sterile media.

The aseptic treatments consisted of growing the plantlets in erlenmeyer flasks containing distilled water; agar-gelled water; agar-gelled basal medium without sucrose and growth regulators; without growth regulators; the three media darkened with activated charcoal or by mixing with peatmoss and vermiculite; full-strength Hoagland's solution in agar-gelled form; and moist peatmoss-vermiculite without a nutrient solution added.

The non-sterile methods were: placing the roots in Hoagland's solution with the tops open to the air or enclosed in polyethylene bags; transplanting to water, perlite, or peatmoss-vermiculite; and keeping the plantlets under intermittent mist or in polyethylene bags. The Hoagland's solution and water experiments included tests with light excluded from the roots.

Of these experiments, only two had promising results, the aseptic medium without growth regulators and the non-sterile Hoagland's solution treatments. Roots grew more extensively in the basal medium without growth regulators, but did not grow well in the minus sucrose, or darkened basal medium. The other treatments that produced good root growth were the non-sterile Hoagland's solution trials. The roots grown in flasks of solution from which light was excluded were larger than those open to light. Plantlets in the Hoagland's solution quickly desiccated and died unless kept covered with polyethylene bags.

All the plantlets that survived the various treatments and retained their vigor were transplanted to peatmoss-vermiculite-perlite and kept under polyethylene except, of course, those that were already in this medium under intermittent mist. These were simply removed from the mist. Soon after they were transplanted, all the plantlets died except those from the non-sterile Hoagland's solution treatments. All of these survived and gradually began to grow autotrophically.

In examining roots grown in agar-gelled media and those grown in Hoagland's solution, we found that the roots from the liquid medium had root hairs while those from agar did not (Figure 1). Also, roots grown in agar frequently lacked a fully developed vascular system while those from Hoagland's solution always had such a system.

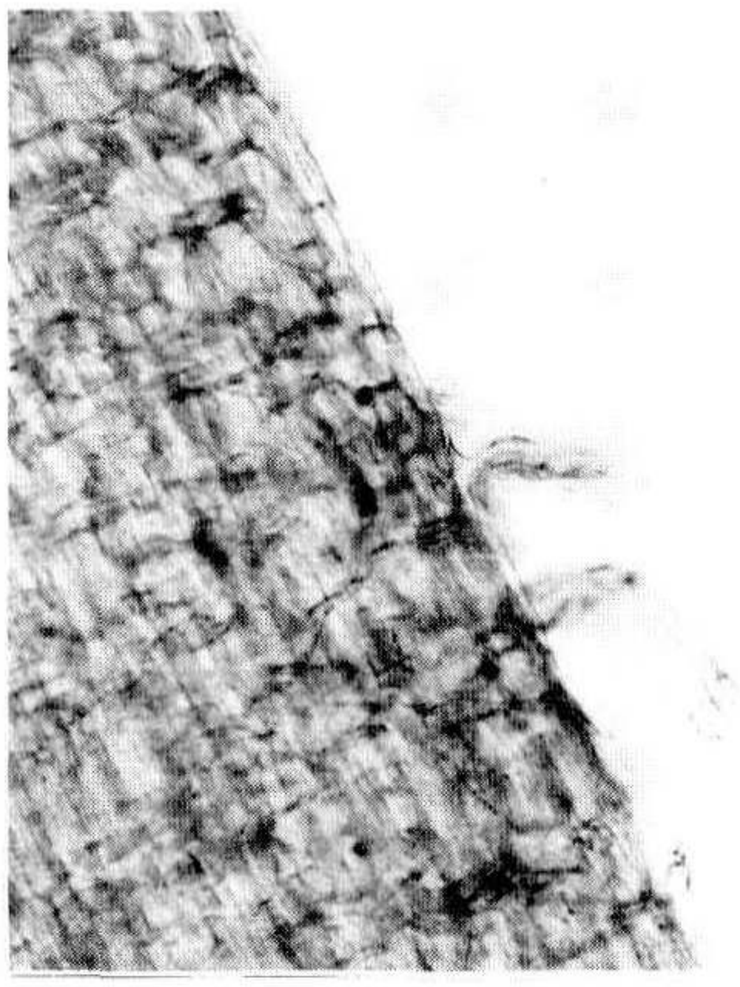

Figure 1. Root hairs on a root after growth in Hoagland's solution.

Once the method of growing plantlets in Hoagland's solution was worked out, a system was perfected in which the roots were suspended in 50 ml flasks of solution, covered from light with aluminum foil, and the entire plantlets surrounded by polyethylene bags for 1 month. The well-rooted plantlets were then transplanted to potting mix. Survival of the plantlets moved from agar to Hoagland's solution was 83 percent. Those that failed generally had short (2-3 cm) rather than normal size (5-8 cm) root systems.

Hardening for field planting. The plantlets were potted in a mixture of peatmoss, perlite, and vermiculite and fertilized once a month with 25 ml of a 20:20:20 liquid fertilizer per one liter container of potting mix. It was found necessary to cover them with polyethylene for 4 to 6 weeks after transplanting to reduce transpiration.

Empirical trials of light requirements indicated that rapid growth required photon flux densities more than 100 u mol m^{-2} sec^{-1}. Plants supplied only 60-80 u mol m^{-2} sec^{-1} grew very slowly. Best growth was achieved with continuous light at 120 u mol m^{-2} sec^{-1} supplied by Gro-lux (Westinghouse) lamps.

Buds became enlarged, indicating rapid root growth after 2 to 3 months in the laboratory. At this stage they were moved to a greenhouse for further growth in stronger light (70% of ambient sunlight) and higher temperature. Survival of the plantlets moved from Hoagland's solution to potting mix was 77 percent.

The elevated temperature of the greenhouse (30-40°C) frequently induced the early formation of phyllodes, the mature leaf form of the species (Figure 2).

Figure 2. Phyllodes formed on tissue-culture propagated plants when grown in a very warm greenhouse.

The first field planting was made with 16 plants taken di-

rectly from the greenhouse. Nine of these 16 died within a week of planting. Soon after this, a group of 35 plants was shipped to the island where they would be planted and spilled out of their pots in shipment. Sixteen of the 35 were in satisfactory condition and were immediately planted; the other 19 were placed on an outdoor nursery bench to recover from the damage in shipping. Six of the 16 plants planted directly after shipment died soon after planting. All 19 of the plants that had been stored outdoors survived planting when they were planted 3 months later.

Since then, we have found that the plants require a period of growth of at least 2 months in full sunlight before field planting. Otherwise, their leaves are bleached and fall off. This occurs with both true leaves and phyllodes.

Although none of these plants has been inoculated with rhizobial bacteria, 16 of the 82 that have been planted were observed to be nodulated at the time of planting (Figure 3). The nodulation occurred during growth in the greenhouse or outdoor nursery.

Figure 3. Nodules formed on the root systems of some of the tissue-culture propagated trees.

Trees of the oldest plantings — now 16 months old — are growing normally, although not as rapidly as nearby natural seedlings (Figure 4). They exhibit a normal form for the species, but so far lack the uniformity that should occur in a single clone.

DISCUSSION

The procedure we have developed to produce plantable trees from shoot tip callus consisted of 14 steps (Table 1). About 16 months are required to carry the process from meristem in

Figure 4. Tissue-culture propagated tree (arrow) has grown normally, but more slowly than nearby natural seedlings about 6 months after planting.

callus to planted tree in the forest, but the process is a continuous pipeline once started.

Table 1. Sequential treatments required to grow *Acacia koa* plants from callus cultures.

Step	Condition	Culture time	Treatment
1	Seedling shoot tip callus	1-2 months	10 mg/l benzyladenine
2	Shoot meristems in callus	1	1 mg/l benzyladenine
3	Shoot meristem in callus	1	10% coconut water
4	Shoot meristems elongating	1	1 mg/l benzyladenine
5	Shoot meristems proliferating	1	10% coconut water
6	Shoot meristems elongating	1	1 mg/l benzyladenine
7	Shoots proliferating, callus growing	1	1 mg/l benzyladenine
8	Leafy shoots + callus	1	No growth regulators
9	Leafy shoots + callus	2-3	0.3 mg/l Indolebutyric acid
10	Rooted plantlets	1	Hoagland's solution
11	Functional roots	2-3	Potting mix
12	Rapid growth	1-2	Greenhouse
13	Plantable seedling	2	Outdoor nursery
14	Plant in field		

The key parts of the procedure are step 8, during which benzyladenine is depleted and step 10 during which nonfunctional roots become functional. We attribute the enhanced

rooting after a period of growth on a regulator-free medium to the absence of the auxin-inhibition of the cytokinin benzyladenine. How the benzyladenine is altered during the period of growth on the regulator-free medium is not known. It may simply be diluted by greater dispersion due to continued cell division.

The formation of root hairs and root vascular systems in the liquid medium which made the roots functional may be a physical rather than chemical effect resulting from the absence of agar. The evidence to support this conclusion is that none of our agar-gelled Hoagland's solution cultures formed functional roots. The gelled media may have mechanically inhibited root hair development. The greater gas exchange that was possible in the non-sterile treatment may also have improved root development.

The growth of the plants in an outdoor nursery, proved essential (Step 13). *Acacia koa* is a species that grows best in the field if exposed to full sunlight immediately on emergence from the soil. The leaves formed in reduced light bleach in bright light so a period of hardening in bright sunlight greatly increased the survival of the propagules.

It is interesting that the genetic programming required for phyllode formation as a result of high temperature, and for nodulation in response to rhizobial bacteria was retained through the callus culture process. The temperature control of phyllode formation in the species has been observed repeatedly in seedlings grown in the same greenhouse.

We believe that our techniques for improving rooting by "diluting" the cytokinin and improving root functioning by a period of hydroponic growth may be useful to other tissue culturists experiencing these common rooting problems.

Although we have only produced plants from one clone so far, the system developed shows promise. After more than 3 years, shoots continue to proliferate without signs of diminishing. If most of the shoots and callus had not been sacrificed for other experiments, many more than the 82 trees now planted would have been produced.

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PROPAGATION OF TROPICAL FOLIAGE PLANTS

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Most foliage house plants come from the tropics. These plants require a temperature around 60 to 70° F to grow; they will stop growing at 50° F and will be badly damaged at 45° F. Other species come from the temperate zone and can tolerate temperatures to almost freezing and, if hardened off, can survive being frozen.

Our customers learn about the different foliage plants through a magazine or a book or advertisement where they see a perfect plant — no broken leaves, no leaf spots, and the plant has perfect shape. So, of course, that is what they want to buy.

There is our challenge, or half of it, to produce a perfect plant. The other half of our challenge is to grow those perfect plants and make a profit for the company even with the price of pots going up all the time and wages for our employees going up. This all means that of the 100 plants you start you had better sell 100 or you will not make it. There are several things that help. As you think about the kinds of house plants that are popular today you will notice two things: