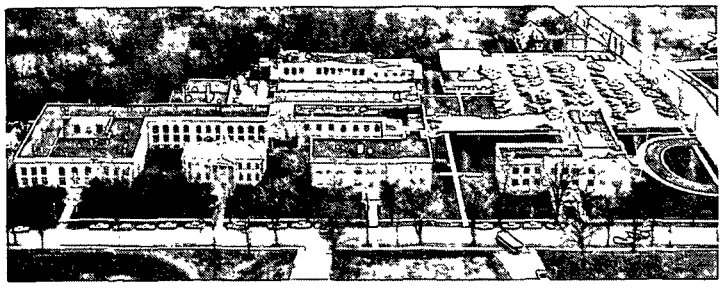

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MICROPROPAGATION OF FERRAGNES ALMOND (*PRUNUS AMYGDALUS* BATSCH)

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MICROPROPAGATION OF DIFFICULT-TO-PROPAGATE ALMOND (*PRUNUS AMYGDALUS* BATSCH) CULTIVAR

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SUMMARY

An *in vitro* culture scheme has been developed which allows a rapid clonal multiplication of shoots starting with excised shoot apices (0.4-0.7 mm) from flushing buds of a difficult-to-root almond cultivar, "Ferragnes." Up to 55% of these shoots could then be rooted *in vitro* for establishment in the soil.

After an initial 3 to 4 subcultures on media allowing slow growth, shoots were placed on Murashige and Skoog (MS) medium with 0.9% agar, 0.7 mg/L 6-benzylamino-purine (BAP), and 0.01 mg/L α-naphthalene acetic acid (NAA). Shoot multiplication rates of six could be obtained with 20 days subculture continued for at least 16 months. At any point these shoots could be elongated by placing them on an identical medium, lowering the BAP (0.2 mg/L), and omitting NAA. Each shoot was then reduced to 2 to 3 leafy microcuttings for rooting tests. Rooting was induced quickly in the dark in about 55% of the microcuttings by using the medium of Bourgin and Nitsch (BN) with only the macronutrients reduced to half, but containing 0.9% agar and 1 mg/L NAA or 1 mg/L γ-(indole-3)-butyric acid (IBA). Further proliferation of roots was successfully achieved in the liquid medium in the absence of any added auxin; sterile vermiculite served as the support. Some plants have been established in the soil.

INTRODUCTION

There is a great need to develop efficient methods for vegetative propagation of proven and established cultivars of fruit trees. The genus *Prunus* represents a

number of economically important fruit species demanding rapid clonal propagation methods if the world's demand for the various fruits and nuts of this genus are to be met.

Because woody species are generally difficult to root beyond the juvenile (seedling) stage by conventional methods, recourse to tissue culture methods offers an attractive alternative. Indeed, many species of Prunus and their interspecific hybrids have been cultured in vitro [1-3], and some progress has been reported in the propagation of species such as cherry, plum, and peach by aseptic culture [4,5].

Almond (Prunus amygdalus Batsch) is a species in which attempts to root cuttings even from seedlings have been unsuccessful. Little work or progress has been reported in the propagation of this species by tissue culture. One solitary report [6] indicates that problems remain in achieving a satisfactory shoot proliferation from the buds and subsequent rooting. In this paper, we describe an in vitro culture scheme which has enabled us to root up to 55% of the shoots proliferated in vitro from buds of a widely known cultivar of almond which does not otherwise root by cuttings.

MATERIALS AND METHODS

Explant Source

Terminal buds from shoots of a six-year-old tree of the almond cultivar "Ferragnes" growing in central Italy were collected in April, 1980. The buds had already flushed at this time. The buds were surface-sterilized in a 7% (w/v) solution of calcium hypochlorite containing 0.07% (w/v) Tween-80 as a wetting agent. Shoot tips (0.4 - 0.7 mm) were microdissected aseptically for initiating cultures.

Aseptic culture leading to shoot proliferation

The culture conditions and media compositions used in various steps are reported in Table 1. All studies were done with media solidified by agar, unless otherwise indicated (see Table 2). Sterile explants were placed singly in (12 x 100 mm) test tubes on initiation medium and subcultured twice on to fresh medium (Table 1). Shoots so developed were sectioned into uni- or binodal pieces to be transferred singly in test tubes (16 x 200 mm) containing 10 mL of establishment medium. Following two subcultures, the cultures were transferred onto shoot proliferation medium for rapid proliferation. This was accomplished by culturing 10 bi- or trinodal shoots in deep petri dishes (150 x 80 mm) with 100 mL of medium. At the end of each subculture, the rate of shoot multiplication was recorded. When a sufficient number of shoots had proliferated, some were left on the proliferation medium for multiplication, and the rest were placed on shoot elongation medium using the deep petri dishes with the same incubation conditions (see legend to Table 1).

Rooting schemes

After 18-20 days of culture on elongation medium, vigorous shoots were cut into two to three leafy microcuttings (12 to 20 mm long) and used for rooting tests. Two schemes of rooting using dark conditions were employed. In the first scheme, vigorous shoots from the elongation medium were placed on agar-solidified media containing various auxins for root initiation. After 14 days, all the shoots were transferred to sterile vermiculite containing only the liquid medium without any growth regulator. In the second scheme, agar-solidified media were used throughout, but a switch in auxins from NAA to IAA was employed at four days to allow root elongation.

Transfer to soil

Rooted plantlets were transferred from the aseptic culture to 100-mL clay pots in different soils to find a mixture suitable for adequate development. The

establishment of rooted plantlets was attempted under controlled environmental conditions with a relative humidity of about 90% and a photoperiod of 16 hours per day at 23°C.

RESULTS

With the exception of rooting, our studies were carried out at 23°C using a 16-hour photoperiod. At 20 days, most of the excised shoot tips developed small single shoots in the initiation medium containing 0.5 mg/L of BAP and 0.01 mg/L of IBA as growth regulators. Higher BAP concentrations (1-3 mg/L) resulted in stunted shoots with callus at the base. After 2 subcultures, the shoots were transferred to the establishment medium, which is essentially full-strength Murashige and Skoog (MS) medium, but with the macronutrients reduced to half strength. After two consecutive subcultures on this medium, the shoots still grew slowly but, significantly, without callus formation. However, little proliferation of axillary buds occurred and leaves often turned yellow. Rapid shoot proliferation was achieved when the shoots were transferred to MS medium with the macronutrients restored to full strength (proliferation medium, Table 1). The basal buds sprouted on shoots with short internodes, with multiplication rates mounting to 6 in 20 days (Fig. 1 and 2). Partial vitrification of shoots was observed when 0.7% agar was used. Substantial improvement was obtained with 0.9% agar; normal shoots grew with a mixture of 0.7% agar and 0.5% pectin in the medium. Even the vitrified shoots could be restored to normalcy by storing them for 15 days at 4°C followed by a routine subculture.

Our attempts to further supplement the proliferation medium with additional nitrogen sources did not result in any significant improvements (data not shown). These additives included 600 mg/L NH_4NO_3 or casamino acids (Difco, 0.2% w/v) or amino acid supplements identical to the casamino acids.

Elongation of the laterally proliferated shoots was obtained satisfactorily on MS medium lacking auxin but containing reduced BAP as the cytokinin (elongation medium, Table 1). From each shoot, it was possible to obtain two or three leafy microcuttings with 3-4 nodes by the end of a single 20-day culture. Parallel treatments were run when elongation medium was supplemented with a range of GA_3 concentrations (0.1 to 1 mg/L). None of these improved the rate of shoot elongation over the controls (data not shown). The rooting percentages in Table 2 represent averages of 4 successive trials. Several conclusions can be drawn from this table. In Scheme 1, NAA is preferred to IBA, the latter usually resulting in much variability. The naturally occurring auxin (IAA) produced practically no roots. Supplementing NAA with zeatin riboside at 0.1 mg/L was not desirable and resulted in abundant callus formation at the base of shoots (data not shown). GA_3 (1 mg/L) completely inhibited rooting. Addition of riboflavin and quercitin did not significantly alter rooting percentage. When concentrations of quercitin greater than 0.3 mg/L were used, roots and basal parts of the shoots turned brown. Scheme 2 was just as effective as Scheme 1; however, the roots produced were brown and hence not as vigorous as those produced in Scheme 1. The number of roots per cutting averaged from 2 to several in both schemes. We have noted definite improvements in the rooting percentage with prolonged subculturing. For example, our most recent rooting trial estimates rooting of more than 70% of the microcuttings (Fig. 3).

The composition of soil mixture found satisfactory for survival of plantlets in the greenhouse is given in Table 1. Figure 4 shows that plantlets with a well-developed root system can be obtained under these conditions.

DISCUSSION

An increasing number of reports indicates that tissue culture methods are demonstrating considerable promise in situations in which conventional vegetative propagation is impractical. In woody species, cuttings from mature trees are often

impossible or very difficult to root. Fortunately, all trees continue to produce new meristems every year, and it should be possible to exploit the inherent totipotency of these meristems by applying the principles of rapid plant propagation which have proven so successful with many of the herbaceous plant species.

Several recent studies have reported positive results with shoot tip or bud cultures of mature woody plants which are difficult to root by cutting. Significant examples include apple [7], teak [8], eucalyptus [9], and Prunus [10]. Tabachnik and Kester cultured dormant shoot buds from almond and almond-peach hybrids [6]. They used the same basal medium and attempted to go through various propagation stages by manipulation of the exogenously supplied growth regulators. In the present study, the same medium was used, but only as the initiation medium. Since this medium is deficient, particularly in K^+ and a reduced nitrogen source, we found it inadequate for proliferation of shoots. When the shoots were not vigorous, they would not root easily.

We are not quite sure of the necessity of steps which involved consecutive use of initiation and establishment media leading to the production of single shoots from each shoot tip, but we observed that use of MS medium directly for excised shoot tips resulted in much callus. It is possible that this "slow growth phase" adapted the explant to in vitro culture resulting later in a concerted favorable response in proliferation of shoots and their subsequent rooting. The results presented indicate a need for optimizing levels of growth regulators and selecting a proper basal medium for obtaining an active multiplication rate. The almond cultivar used requires a relatively low level of cytokinin (0.7 ppm BAP) and a trace level of NAA in the proliferation medium. Perhaps the almond shoots synthesize their own cytokinin, as has been demonstrated recently in dormant rootlet shoots [11].

The culture medium used successfully here for rapid growth of almond shoots was also successful in producing callus from leaves of the same cultivar with NAA

(5 mg/L) and zeatin riboside (0.1 mg/L) as growth regulators (data not shown). However, the same medium was not able to support growth of cell suspensions unless supplemented with an amino acid mixture or casein hydrolysate [12].

The culture conditions established in this report enable one to obtain numerous clonal shoots by repeated subcultures. It is important to note that addition of pectin along with agar was advantageous in this study. Under these conditions, vigorous shoots could be generated repeatedly without vitrification problems. One must be cautious, however, since addition of pectin to the medium results in a drastic drop in pH on autoclaving. This problem can be alleviated by adjusting the pH of an autoclaved portion of the pectin and then freeze- or oven-drying the sample for use in media preparations. Whether or not addition of pectin to the medium contributed nutritionally to the growth of shoots remains to be determined.

Our results with respect to rooting schemes are also significant. Selection of the BN medium was not arbitrary. The macronutrients of this medium compare very well with one-half strength MS, but the concentration of certain microelements (B, Zn) exceeds even the full-strength MS medium levels while others are maintained at the same level as in full-strength MS medium. BN medium is also enriched in a number of vitamin supplements. Preliminary studies of callus initiation on almond leaves produced roots from petioles only in this medium (data not shown). We believe the results are quite satisfactory with NAA used as auxin. Unlike results obtained with Prunus persica [5], addition of quercitin and riboflavin did not seem to promote rooting in the present study. Similarly, addition of GA_3 or ABA, successfully used by others in root induction in some species of Prunus [1], completely inhibited rooting in the "Ferragnes" almond cultivar. Our observation that rooting efficiencies increased with subculturing is consistent with recent work on apple [7].

We did not conduct extensive investigations on establishing plantlets in the soil, but we have given the composition of a soil mixture which enabled us to establish some plantlets with extensive root systems (Fig. 3). With the existing facility, further work in this direction could not be carried out. The progress reported here indicates that the goal of rapid clonal propagation of the difficult-to-root "Ferragnes" cultivar of almond is well within reach.

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TABLE 1

COMPOSITION OF MEDIA AND CULTURE CONDITIONS USED FOR VARIOUS STAGES OF MICROPROPAGATION OF ALMOND CULTIVAR.

The pH of all media was adjusted to 5.8 prior to autoclaving. Under "Additional features" only the best treatments are given. The details of other treatments which were studied can be found in the text. All experiments except the rooting phase (which took place in complete darkness) were conducted in a growth chamber at 23°C with a 16-hour photoperiod and with a light intensity of 4500 lux (Gro-lux and white).

Stage	Reference for Media	Additional features (mg/L)
Initiation	The same as used by Tabachnik and Kester [6]	0.5 BAP, 0.01 IBA, 20,000 sucrose and 7,000 Bactoagar (Difco)
Establishment	MS medium [13]	The same as above but with macronutrients replaced by half-strength MS medium
Proliferation	MS medium [13]	Full strength MS with 0.7 BAP, 0.01 NAA, 9,000 Bactoagar or 7,000 agar + 5,000 pectin and 30,000 sucrose
Shoot elongation	MS medium [13]	The same as above with no auxin, BAP reduced to 0.2, and 9,000 agar
Rooting	Bourgin and Nitsch [14]	Only the macronutrients reduced to half strength, other components kept at the same levels, 1 NAA or 1 IBA
Root elongation[a]	Bourgin and Nitsch [14]	The same as above but with no growth regulator. Sterile vermiculite (1.3 g in 10 mL medium) replaced agar

[a]Following this step, plantlets were transferred to the greenhouse in clay pots containing 17% sand, 21% sphagnum peat moss, 8% perlite, 25% common soil, 25% black peat soil, 4% vermiculite percolated with Knop's macronutrients and Heller's micronutrients [15] plus 0.08% NH_4NO_3.

TABLE 2

ADVENTITIOUS ROOT FORMATION ON LEAFY MICROCUTTINGS OF AN ALMOND CULTIVAR

Leafy microcuttings (3 or 4 nodes, 12-20 mm in length) obtained from shoot-elongation medium were used in rooting schemes 1 and 2. Four rooting trials were conducted in the dark with 25 microcuttings each, and the number of rooted shoots was recorded at the end of 32 days. See text for details on rooting Schemes 1 and 2.

Supplement	Rooting (%) ± SD (average of 4 trials)
Rooting Scheme 1	
NAA 1 mg/L	54.0 ± 5.9
IBA 1 mg/L	35.6 ± 13.1
IAA 1 mg/L	0.0
NAA 1 mg/L + GA_3[a] 1 mg/L	0.0
NAA 1 mg/L + riboflavin[a] 0.1 mg/L + quercitin[a] 0.3 mg/L	56.6 ± 9.0
Rooting Scheme 2	
IAA 0.7 mg/L	56.6 ± 7.2
IAA 1 mg/L + riboflavin[a] 0.2 mg/L + quercitin[a] 0.3 mg/L	52.6 + 6.7
IAA 1 mg/L + ABA[a] 0.8 mg/L + GA_3[a] 0.1 mg/L	0.0
IAA 1 mg/L + ABA[a] 0.8 mg/L	0.0

[a]Added filter-sterilized.

ABBREVIATIONS

ABA, abscisic acid; BAP, 6-benzylaminopurine; BN, Bourgin and Nitsch; GA, gibberellic acid; IAA, indole-3-acetic acid; IBA, γ-(indole-3)-butyric acid; MS, Murashige and Skoog; NAA, α-naphthalene acetic acid.

Figure 1. Almond cultivar in shoot proliferation medium at 20 days.

Figure 2. Single crown of shoots arising from a bi- or trinodal explant at 20 days of culture.

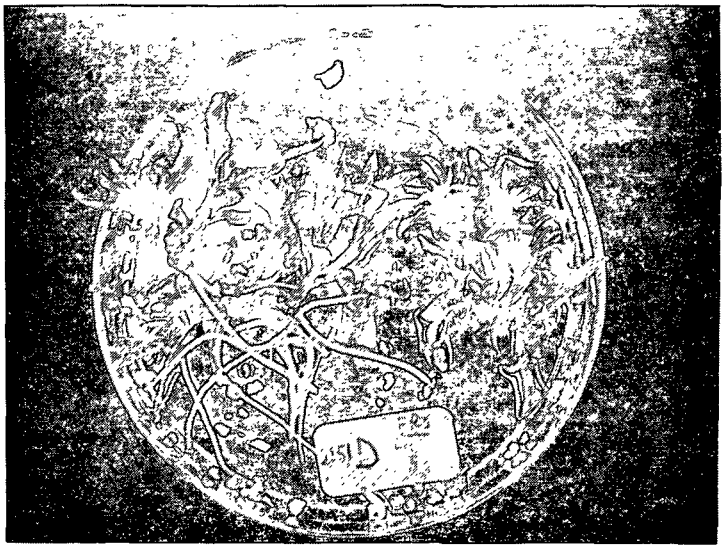

Figure 3. Adventitious root formation on almond shoots in a recent trial employing NAA as in rooting Scheme 1 (see results).

Figure 4. An example of an almond plantlet established in soil 3 weeks after transplanting.