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ADVENTITIOUS SHOOT REGENERATION FROM *FAGUS SYLVATICA* LEAF EXPLANTS *IN VITRO*

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SUMMARY

Adventitious shoots were induced on transversally divided expanding leaves from *Fagus sylvatica* shoot cultures of juvenile origin. Adventitious shoot buds formed mainly on callus that developed on the petiole stump or on the cut across the midrib of distal leaf halves. However, sometimes they arose directly from leaf tissue. An anatomical study confirmed both the direct and indirect origin of the adventitious buds. The best results were obtained by culturing proximal leaf sections on woody plant medium supplemented with 2.9 μ*M* indole-3 acetic acid in combination with 8.9 μ*M* benzyladenine or 2.3 μ*M* thidiazuron (TDZ). Proximal explants were more responsive than distal explants in terms of both callus formation and bud regeneration, regardless of the induction medium or clone tested. Bud formation capacity was influenced by the genotype of the stock shoot culture and was enhanced by an initial 10 d darkness, but was inhibited by longer periods of darkness. Caulogenic competence was significantly affected by the duration of exposure to TDZ; in particular, adventitious shoot length was depressed by increasing the exposure period. Three weeks culture with TDZ was the most efficient treatment for shoot production and elongation. Further shoot development was promoted by subculturing the explants to the same medium used for the maintenance of the stock shoot cultures. Shoots so obtained were multiplied and rooted producing plantlets of adventitious origin.

Key words: adventitious buds; European beech; leaf culture; shoot regeneration; thidiazuron.

INTRODUCTION

The European beech is among the economically most important broadleaved trees of temperate forests in the Northern hemisphere. Beech is mainly propagated by seedlings, but beech trees do not produce fertile seeds until they are approximately 35–40 yr old, and good harvests occur only every 4–6 yr. Hybridization is very limited and the genetic basis for selection of elite types is thus restricted (Nadel et al., 1991). Genetic engineering of forest trees offers several possibilities to complement traditional tree breeding programs. The development of gene transfer techniques for *Fagus sylvatica* L. may give breeders an additional method for improving single characters. An efficient adventitious shoot regeneration system from leaves of European beech could potentially be used for the application of genetic transformation methods in improvement programs of this species.

Several recent studies have indicated the morphogenetic potential of explanted leaves of woody dicotyledons. Although most have referred to fruit trees (Chevreau et al., 1989; Jones, 1993), leaves of several hardwood trees, including elm (Boylard et al., 1991), aspen (Park and Son, 1988), birch (Leege and Tripepi, 1993), and black locust (Arrillaga and Merkle, 1993), can also be used as explants to obtain adventitious shoots.

In vitro propagation of members of the Fagaceae has proved to be difficult, especially for the genus *Fagus*, although recent progress in bud culture and plantlet regeneration of *Fagus sylvatica* via proliferation of axillary shoots is noteworthy (Chalupa, 1985; Vieitez et al., 1993; Meier and Reuther, 1994). In this genus, adventitious buds have to date only been induced on the intact hypocotyls of whole seedlings and on isolated cotyledon and hypocotyl segments of *F. sylvatica*, when cultured on media supplemented with benzyladenine (BA) and naphthaleneacetic acid (NAA) and 1 mo. of darkness incubation being applied (Vieitez et al., 1993).

In this study, we describe procedures for adventitious bud induction on leaf explants from several *in vitro Fagus sylvatica* clones derived from 2-mo.-old or 3-yr-old seedlings. The effect of stock plant genotype, the duration of an initial darkness period, and the exposure time to the thidiazuron treatment on the regeneration process are also evaluated. In view of the recalcitrant nature of this species, the achievement of the present study has been the development of a plantlet regeneration system from leaves that can be readily obtained from shoot cultures maintained *in vitro*. Histological examination characterized the origin and development of the adventitious buds.

MATERIALS AND METHODS

Plant material and culture conditions. In vitro shoot culture clones were initiated from axillary buds of 2-mo.-old (5B, FS-104, 138) or 3-yr-old (P3) beech seedlings (one clone per seedling) as previously described (Vieitez et al., 1993). These were maintained by sequential subculturing of shoot tips and basal shoot segments (10 mm long) every 6 wk. The medium consisted of woody plant medium (WPM) (Lloyd and McCown, 1980) to which 2.2 μ*M* BA, 9.1 μ*M* zeatin, 2.9 μ*M* indole-3-acetic acid (IAA), 30 g/l sucrose, and 7 g/l agar (Sigma No. A 1296, Sigma Chemical Co., St. Louis, MO) were added. The medium was brought to pH 5.7 before autoclaving at 120° C for 20 min and dispensed into 500-ml glass jars (80 ml/jar), with glass lids fixed with transparent plastic film. The stock cultures were incubated in a growth chamber with a 16 h photoperiod (provided by cool-white fluorescent lamps at a

TABLE 1

EFFECT OF CYTOKININ TREATMENT AND EXPLANT TYPE (DISTAL OR PROXIMAL) ON BUD REGENERATION FREQUENCY (%) AND THE NUMBER OF BUDS PER REGENERATING EXPLANT, FOR TWO *FAGUS SYLVATICA* CLONES CULTURED ON REGENERATION MEDIUM CONTAINING 2.9 μ*M* INDOLE-3-ACETIC ACID (IAA)[a]

Treatment (μ*M*)	Regeneration %		Number of buds[b]	
	Distal	Proximal	Distal	Proximal
Clone 5B				
BA[c] 4.4	6.3 ± 3.0	43.8 ± 9.9	1.0 ± 0.4	1.5 ± 0.2
BA 8.9	9.0 ± 4.6	36.0 ± 6.2	1.6 ± 0.7	2.1 ± 0.2
BA 17.8	4.0 ± 1.4	28.0 ± 3.5	1.3 ± 0.6	1.7 ± 0.2
TDZ 0.2	13.2 ± 4.5	35.7 ± 7.6	3.6 ± 0.7	3.4 ± 0.5
TDZ 2.3	24.3 ± 5.4	49.4 ± 8.9	2.7 ± 0.5	3.4 ± 0.5
TDZ 4.5	13.6 ± 2.6	54.5 ± 6.9	2.2 ± 0.2	2.5 ± 0.3
TDZ 9.1	8.8 ± 3.0	47.0 ± 10.3	1.0 ± 0.4	2.5 ± 0.1
Clone FS-104				
BA 4.4	1.7 ± 1.4	17.0 ± 1.3	0.3 ± 0.3	2.6 ± 0.4
BA 8.9	10.0 ± 4.1	27.2 ± 8.5	1.3 ± 0.6	1.7 ± 0.3
BA 17.8	0	2.2 ± 0.9	0	0.7 ± 0.3
TDZ 2.3	4.4 ± 2.4	35.6 ± 6.6	3.1 ± 1.4	5.6 ± 0.7

Analysis of Variance	Clone 5B				Clone FS-104			
Source of variation	Regeneration %		Number of buds		Regeneration %		Number of buds	
	F-test	LSD 5%	F-test	LSD 5%	F-test	LSD 5%	F-test	LSD 5%
Treatment (A)	2.262[ns]	—	6.899***	1.34	6.684**	15.04	9.822**	2.30
Explant (B)	69.434***	6.27	4.795*	0.54	23.431**	8.68	7.124[ns]	—
A × B	0.541[ns]	—	0.621[ns]	—	1.369[ns]	—	0.965[ns]	—

[a] Means ± standard errors in four experiments, each with 30 replicates per treatment/explant/clone combination.

[b] In thidiazuron (TDZ) treatments of clone 5B, the number of bud values actually represent the number of bud clusters. F-test: ns, not significant; *P = 0.05; **P = 0.005; ***P = 0.0001

[c] BA = benzyladenine.

photon flux density of 30 μmol·m^{-2} s^{-1}) and 25° C day/20° C night temperatures. These standard culture conditions were also applied to the cultures described below unless otherwise stated.

General method for adventitious bud regeneration. Explants were obtained from leaves removed from 6-wk-old shoot cultures. The two uppermost unfurled expanding leaves (6–11 mm long) were excised from the donor shoot apex leaving 2–3 mm of petiole attached. Leaves were cut transversally across the mid-vein into distal and proximal (basal) halves that were placed abaxial face down in 90 × 15-mm petri dishes containing 25 ml of medium. Five leaves so divided were cultured in each dish; petri dishes were sealed with Parafilm®.

Basal bud regeneration medium consisted of WPM containing 30 g/l sucrose and 7 g/l agar. In a preliminary growth regulator experiment, leaf explants were placed on regeneration medium supplemented with BA (2.2 or 4.4 μ*M*), alone or in combination with an auxin [1.0 μ*M* indole-3-butyric acid (IBA), 1.1 μ*M* NAA or 2.9 μ*M* IAA]. In subsequent experiments, IAA (2.9 μ*M*) was used together with various concentrations of BA (4.4, 8.9, or 17.8 μ*M*) or thidiazuron (TDZ; 0.2, 2.3, 4.5, or 9.1 μ*M*). For each treatment, at least 30 leaves (six petri dishes) were used for each genotype tested, and the experiments were repeated four times.

In most cases, explants were incubated for 40 d at 25° C in the dark, and then for another 20 d under the standard lighting conditions described above. Bud-forming capacity was then determined in terms of the percentage of leaf halves forming shoot buds and the number of shoot buds formed per responsive explant. Data were analyzed by two-way (Table 1) or one-way (Fig. 2 and Tables 2,3, and 4) analysis of variance (ANOVA) followed by the least significant difference (LSD) test at the P = 0.05 level to compare means. Percentage data were subjected to arcsine transformation before analysis, but are presented here untransformed. In some experiments, callus production was also evaluated by recording the percentage of leaf sections forming callus (Table 2).

Duration of initial darkness. In order to determine the effect of incubation in darkness on adventitious bud formation leaf sections of clone 5B were cultured on regeneration medium with 8.9 μ*M* BA and 2.9 μ*M* IAA, in the dark for 0, 10, 20, 30, 40, 50, or 60 d and then under the standard 16 h photoperiod for the remainder of the total 60-d experimental period. Each dark treatment was applied to 40 leaf halves (20 leaves, 4 dishes) in each of three repeated experiments.

Duration of TDZ treatment. With a view to obtaining more elongated shoots, an experiment was performed to study the effect of the duration of exposure to TDZ on bud induction and elongation. According to the results of the previous experiments, leaf halves of clone 5B were incubated on regeneration medium with 2.3 μ*M* TDZ and 2.9 μ*M* IAA for 1, 2, 3, 4, 5, 6, or 8 weeks. After these times, the explants were transferred to multiplication medium to give a total experimental time of 8 wk. In all cases, leaf dishes were transferred to standard lighting following 10 d initial darkness. At the end of the 8 wk, the percentage and productivity of explants forming buds were determined, as were the proportions of responsive explants forming at least one shoot less than 2-mm long, 2–5-mm long, or longer than 5 mm (classes 1, 2, and 3). Each TDZ treatment was applied to 40 leaf halves (20 leaves, 4 dishes) in each of three repeated experiments.

Histology. After 4 or 8 wk culture, leaf sections that had been cultured in different induction treatments were fixed in FAA (18:1:1 vol/vol/vol, 50% ethanol/glacial acetic acid/formalin), dehydrated by passage through an ethanol/butyl alcohol series, and embedded in paraffin wax. Sections 10-μm thick were cut and stained with Safranin-Fast green.

RESULTS

Effects of growth regulators, explant type, and genotype on bud regeneration. Leaf sections cultured in the absence of growth regu-

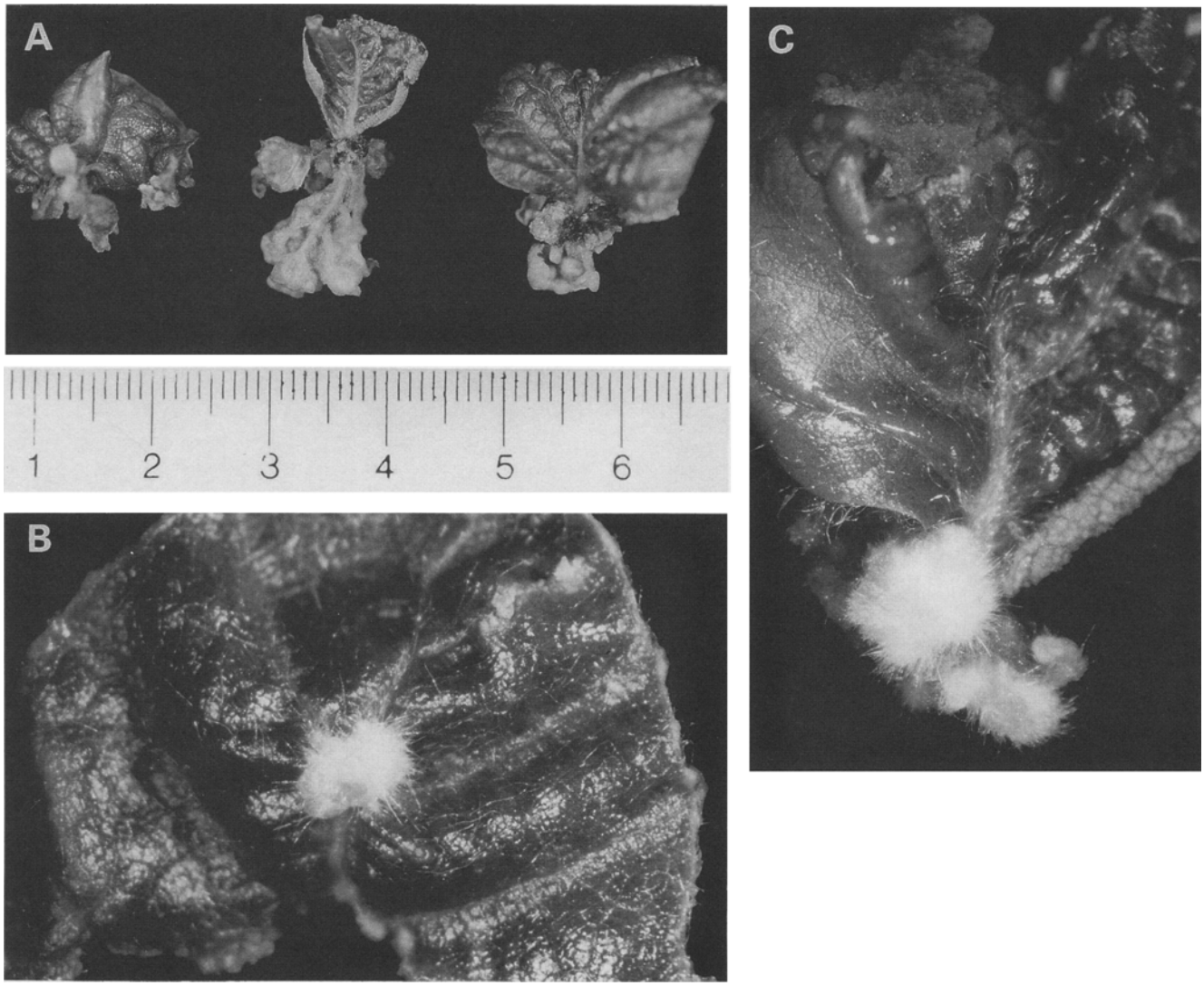

FIG. 1. Adventitious shoot bud differentiation on half-leaves of *Fagus sylvatica* cultured *in vitro*. *A*, Bud regeneration on proximal explants cultured on medium containing 8.9 μ*M* benzyladenine (BA) and 2.9 μ*M* indole-3-acetic acid (IAA) (clone FS-104, scale in cm). *B*, Buds formed directly on the midrib of a distal explant cultured on medium with 4.5 μ*M* thidiazuron (TDZ) and 2.9 μ*M* IAA (clone 5B), ×8. *C*, Bud clusters on petiole stub callus on a proximal explant cultured on medium with 4.5 μ*M* TDZ and 2.9 μ*M* IAA (clone 5B), ×8.

lators produced no callus or adventitious buds. Preliminary experiments revealed that shoot buds were developed by only 10% of leaf explants cultured on regeneration medium containing only 2.2 or 4.4 μ*M* BA. In the absence of other growth regulators, BA failed to induce callus and instead promoted the growth of the leaf lamina. Auxin addition (IBA, NAA, or IAA) to the BA-containing media promoted callus development, although the bud regeneration frequency was not enhanced by IBA or NAA. The combination of 4.4 μ*M* BA and 2.9 μ*M* IAA yielded the highest incidence of callus production (90%) and adventitious bud induction (20%). Consequently, 2.9 μ*M* IAA was included in the regeneration medium in all subsequent experiments.

Explants grown on media supplemented with BA or TDZ plus IAA responded by forming callus on the cut surfaces, especially the petiole stub. When adventitious buds formed they generally developed within 5–7 wk on the petiole stub callus of proximal explants (Fig 1 *A*) or on the callus formed close to the wound of the mid-vein in distal explants. In addition, some buds appeared to form directly on the adaxial leaf blade in association with the midrib or major vasculature (Fig. 1 *B*).

Table 1 compares, for clones 5B and FS-104, the bud formation capacities of the two types of explant in media containing different concentrations of BA and TDZ. In both clones, callus formation occurred on media with TDZ at a higher frequency than on media with BA (data not shown). Also, browning of the explants was more extensive in BA media than in TDZ media. Adventitious buds induced in BA media had more expanded leaves and were less pubescent than those originated in TDZ (Fig. 1 *A*). In clone 5B, the number of buds per explant in TDZ media was difficult to quantify because buds arose in compact clusters and were stunted and densely pu-

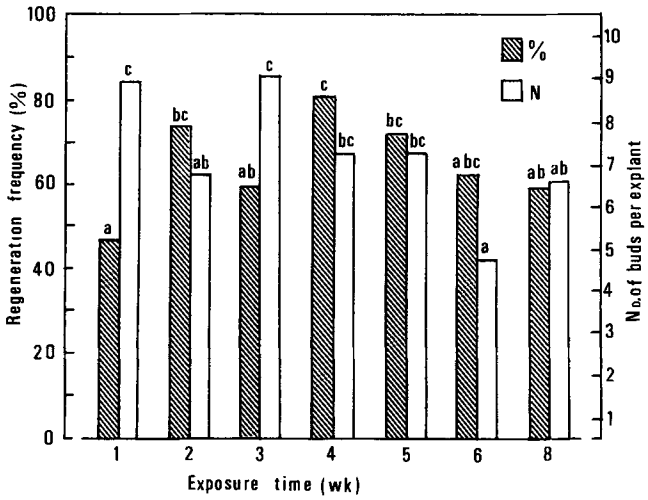

FIG. 2. Effect of the duration of exposure to 2.3 μ*M* thidiazuron (TDZ) on regeneration frequency (%) and the number of buds per regenerating explant (N), for proximal leaf sections of *Fagus sylvatica* (clone 5B). For each variable, bars with the same letter are not significantly different at the $P = 0.05$ level according to the least significant difference (LSD) test.

bescent (Fig. 1 *C*). With regard to TDZ treatments of clone 5B, the "number of buds" values shown in Table 1 actually represent the number of bud clusters.

Analysis of variance showed that in clone 5B explant type had a more significant effect on the two variables evaluated than did the growth regulator treatment. Regardless of the induction medium, the percentage of explants producing shoots and their productivity were significantly greater for proximal explants than for distal ones. The growth regulator treatment significantly affected the number of shoots but not the percentage of explants producing shoots. In clone FS-104, on the other hand, the bud induction treatment was more significant than explant type for both the dependent variables; explant type only significantly affected the regeneration frequency. Neither for the dependent variables nor for the clone was there any significant statistical interaction between induction treatment and explant type. In general, the best results in terms of both regeneration frequency and explant productivity were obtained with proximal explants cultured in media supplemented with 2.9 μ*M* IAA and either 8.9 μ*M* BA or 2.3 μ*M* TDZ.

The ability of TDZ (2.3 μ*M*) to induce adventitious buds on leaf explants depended on genotype (Table 2). Clone 5B was the most productive and clone P3 the least, although its regeneration rate was not significantly different from that of clone 138. Callus production was significantly less in P3 than in the other clones. Whatever the genotype, caulogenic reponse and callus formation were much greater in proximal than in distal explants. Callus was always formed on at least twice as many proximal explants as distal explants. Callus formation was not correlated with bud-forming capacity.

Effect of darkness period. Darkness was not necessary for bud induction, and shoot regeneration ability generally increased with the length of light periods (Table 3). For proximal explants, regeneration frequency was maximum with a 10-d darkness treatment and the number of adventitious shoots per responsive explant fell significantly after 20 d darkness. The response achieved with 10 d darkness was halved when cultures were kept in darkness for 50 d or more.

Effect of duration of exposure to thidiazuron. Caulogenic competence was affected in a minor but statistically significant way by the duration of culture in the presence of TDZ (Fig. 2). Among proximal explants, regeneration frequency was highest after 4 wk exposure (significantly higher than after 1 wk or 8 wk) and the number of shoot buds per responsive explant was greatest after 1 or 3 wk (significantly greater than after 2, 6, and 8 wk). Distal explants exhibited the same trends (data not shown). The development of adventitious shoots (more than 2-mm long) decreased with increasing time of exposure to TDZ (Table 4). Transfer of proximal explants to multiplication medium after 1–3 wk on TDZ favored shoot elongation, increasing the proportion of class 3 (> 5 mm) explants. Exposure times longer than 3 wk significantly reduced the number of class 3 explants, the percentage of class 2 explants was significantly depressed after 6 weeks, but none of the exposure periods significantly affected the proportion of class 1 explants. The proportion of explants developing shoots more than 2-mm long (class 2 + class 3 explants) after 6 wk exposure to TDZ was less than half the figure recorded after 1-wk exposure. For distal explants, the maximum number of class 3 explants (7%) was obtained after 3 wk exposure to TDZ, but there were

TABLE 2

EFFECT OF GENOTYPE ON CALLUS PRODUCTION (PERCENTAGE), BUD REGENERATION FREQUENCY (%), AND NUMBER OF BUDS PER REGENERATING EXPLANT, FOR DISTAL (D) AND PROXIMAL (P) LEAF SECTIONS OF *FAGUS SYLVATICA* CULTURED ON REGENERATION MEDIUM CONTAINING 2.3 μ*M* THIDIAZURON (TDZ) AND 2.9 μ*M* INDOLE-3-ACETIC ACID (IAA)[a]

	Callus Percentage[b]		Regeneration %[b]		Number of buds[b,c]	
Clone	D	P	D	P	D	P
5B	37 ± 8.9b	94 ± 3.1b	33 ± 7.0c	63 ± 10.7c	2.8 ± 0.6a	3.6 ± 0.9b
FS-104	35 ± 5.6b	87 ± 3.5b	5 ± 1.9b	33 ± 5.6b	3.7 ± 1.2b	6.1 ± 0.7c
138	28 ± 2.2b	90 ± 2.6b	0a	11 ± 1.2a	—	1.5 ± 0.2a
P3	1 ± 0.5a	41 ± 4.9a	0a	7 ± 2.1a	—	2.1 ± 0.3ab
	****[d]	****	*	****	**	***

[a] Means ± standard errors in four experiments, each with 30 replicates per treatment.

[b] Within each column, values followed by the same letter are not significantly different at the $P = 0.05$ level according to the least significant difference (LSD) test.

[c] In clone 5B, the number of bud values actually represent the number of bud clusters.

[d] F test: *$P = 0.05$; **$P = 0.01$; ***$P = 0.0005$; ****$P = 0.0001$

TABLE 3

EFFECT OF THE DURATION OF INITIAL DARKNESS ON REGENERATION FREQUENCY (%) AND NUMBER OF BUDS PER REGENERATING EXPLANT, FOR DISTAL AND PROXIMAL LEAF SECTIONS OF *FAGUS SYLVATICA* (CLONE 5B) CULTURED ON REGENERATION MEDIUM CONTAINING 8.9 μ*M* BENZYLADENINE (BA) AND 2.9 μ*M* INDOLE-3-ACETIC ACID (IAA)[a]

Darkness period (days)	Regeneration %[b]		Number of buds[b]	
	Distal	Proximal	Distal	Proximal
0	22.0 ± 1.8c	48.3 ± 8.3ab	2.3 ± 0.1a	2.6 ± 0.3b
10	18.5 ± 6.8bc	60.0 ± 2.4b	2.3 ± 0.2a	2.8 ± 0.1b
20	6.7 ± 2.7ab	53.3 ± 1.4ab	1.3 ± 0.6a	2.0 ± 0.2a
30	7.5 ± 1.4abc	43.3 ± 3.6ab	1.5 ± 0.3a	1.9 ± 0.1a
40	8.3 ± 3.6abc	53.3 ± 8.3ab	1.3 ± 0.6a	1.7 ± 0.2a
50	3.3 ± 1.4a	35.0 ± 7.1a	1.0 ± 0.5a	1.4 ± 0.1a
60	5.0 ± 2.4ab	33.3 ± 6.8a	1.0 ± 0.5a	1.6 ± 0.2a
	*[c]	*	ns	**

[a] Means ± standard errors in three experiments, each with 20 replicates per treatment.

[b] Within each column, values followed by the same letter are not significantly different at the $P = 0.05$ level according to the least significant difference (LSD) test.

[c] F test: ns, not significant; $*P = 0.05$; $**P = 0.01$.

no significant differences with respect to other exposure times (data not shown).

Histology. Meristemoids and bud primordia became recognizable as polar structures after 3–4 wk culture (Fig. 3 *A*). After 6–8 wk, these structures had grown into adventitious buds exhibiting both an apical meristem and vascular strands connected with vascular tissue located in the callus or in the original explant (leaf veins).

Histological examination of responsive leaves confirmed that adventitious buds were induced both directly and indirectly (via callus). Most frequently, buds arose from a callus produced at the cut end of the petiole (Fig. 3 *B*). The callus was formed by parenchymatic cells and numerous tracheids. The callus surface exhibited areas of meristematic cells that evolved into bud primordia. When adventitious buds originated directly, they differentiated in association with main-vein tissues, from a small protuberance of parenchymatic tissue produced by the subepidermis and bundle sheath cells located in the adaxial part of the main-vein. Successive divisions of parenchyma cells at the surface of the protuberance led to the formation of meristemoids, which differentiated into adventitious buds (Fig. 3 *C*).

There were differences between adventitious buds induced by BA and those induced by TDZ. Buds originated in BA medium were well-defined, with well-developed leaf primordia and vascular tissue (Fig. 3 *D*). On the contrary, buds induced by TDZ were less individualized in general, and in most cases it seemed as though several buds were fused together, giving rise to very compact bud clusters or fasciate buds, especially with the highest (9.1 μ*M*) TDZ concentration.

Micropropagation of adventitious buds. After 8 wk culture in regeneration medium containing BA, explants with adventitious buds were transferred to the same medium used for maintenance of the stock cultures. Shoot elongation was promoted by transferring the explants to fresh medium every 2 wk. After 3 mo., when the adventitious shoots were about 2-cm long, they were excised from the original leaf explant, sectioned, and subcultured. Different shoot culture lines identified by their adventitious shoot of origin were established following the outgrowth of axillary shoots. The rooting capacity of two shoot lines derived from adventitious buds of clone 5B was evaluated and compared with that of the stock shoot cultures from which the original leaf sections had been isolated. Fifty-four explants (3 repetitions with 18 shoots each) from each lot were rooted by dipping their basal ends in 4.9 m*M* IBA solution for 2 min and subsequent transfer to medium with half-strength WPM macronutrients and no auxin, in which they spent the first 8 d in darkness (Fig. 3 *E*). The rooting frequency of the two 5B shoot lines (80 and 88%) did not differ significantly from that of the stock cultures of origin (83%).

Shoot lines of adventitious origin of 5B and FS-104 clones have been maintained by sequential subculturing for over 2 yr.

Attempts to elongate and micropropagate adventitious buds originated after 8 wk culture in TDZ medium were unsuccessful. After several successive transfers to fresh multiplication medium the shoots became necrotic and died.

Discussion

The results achieved in this work represent a reproducible plant regeneration system through organogenesis from leaf explants of *F. sylvatica* cultures. The efficiency of shoot regeneration depended on leaf explant type (proximal or distal half leaf), on the cytokinin added to the basal medium, and on genotype.

Caulogenic response was polar, proximal explants responding better than distal half leaves. Economou and Maloupa (1995) reported no significant difference in shoot formation ability between proximal and distal leaf explants of *Elaeagnus angustifolia*, but such differences have been found in *Prunus* species (Escalettes and Dosba, 1993) and apple (Kouider et al., 1984). The fact that the caulogenic response of proximal beech explants is invariably associated with the petiole or main leaf veins suggests the involvement of the vascular tissues, by supplying shoot induction factors (Nair et al., 1984; Brand and Lineberger, 1988) or by mediating quantitative changes in nutrients and growth factors (Brown and Thorpe, 1986). Polar caulo-

TABLE 4

EFFECT OF THE DURATION OF EXPOSURE TO 2.3 μ*M* THIDIAZURON (TDZ) ON THE PERCENTAGE (%) OF RESPONSIVE PROXIMAL EXPLANTS FORMING SHOOTS LESS THAN 2-MM LONG (CLASS 1), 2–5-MM LONG (CLASS 2), AND LONGER THAN 5 MM (CLASS 3), IN *FAGUS SYLVATICA* (CLONE 5B)[a]

Exposure time (weeks)	Class 1 (%)[b]	Class 2 (%)[b]	Class 3 (%)[b]
1	60.5 ± 2.2a	29.5 ± 1.7b	10.0 ± 3.1e
2	65.4 ± 5.5a	28.5 ± 4.7b	5.9 ± 1.4cde
3	59.7 ± 2.5a	32.1 ± 2.0b	7.9 ± 0.4de
4	65.4 ± 3.5a	31.4 ± 3.3b	3.4 ± 0.4bcd
5	70.1 ± 3.8a	28.4 ± 3.2b	1.5 ± 0.8ab
6	84.4 ± 3.9a	15.5 ± 4.0a	0 ± 0a
8	88.9 ± 1.8a	8.1 ± 0.8a	3.0 ± 1.1bc
	ns[c]	*	**

[a] Means ± standard errors in three experiments, each with 20 replicates per treatment.

[b] Within each column, values followed by the same letter are not significantly different at the $P = 0.05$ level according to the least significant difference (LSD) test.

[c] F test: ns, not significant; $*P = 0.01$; $**P = 0.001$

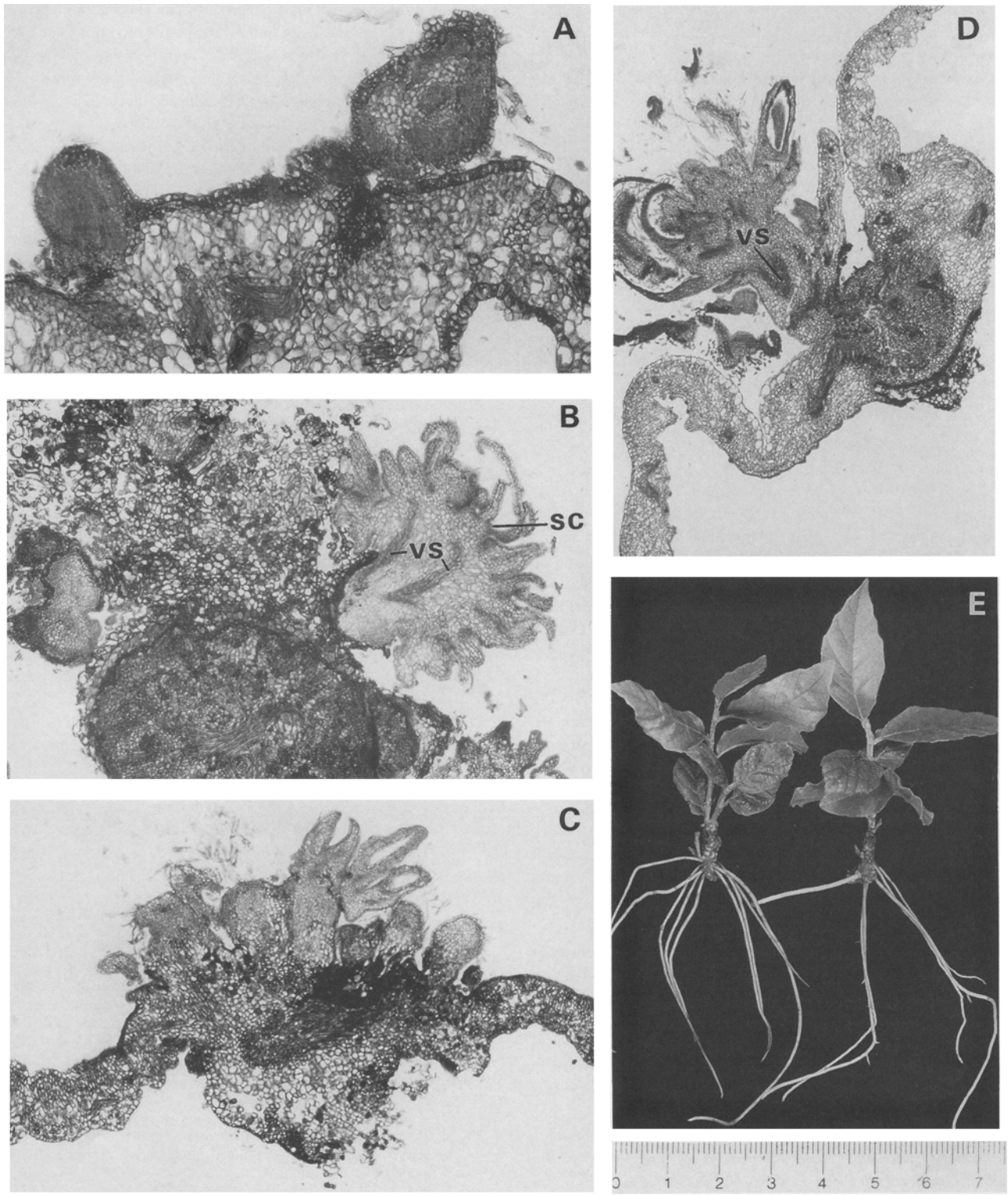

FIG. 3. Micrographs of the shoot-forming regions of leaf explants of *Fagus sylvatica*. *A*, Two bud primordia emerging from the leaf surface after 3 wk culture in medium containing 4.5 μ*M* thidiazuron (TDZ) and 2.9 μ*M* indole-3-acetic acid (IAA) (clone 5B), ×83. *B*, Petiole-stub callus showing bud clusters formed after 8 wk culture in the presence of 2.3 μ*M* TDZ and 2.9 μ*M* IAA (clone 5B), ×43. *C*, Multiple buds originated directly in association with the mid-vein tissues at the adaxial surface of an explant cultured on medium with 2.3 μ*M* TDZ and 2.9 μ*M* IAA (clone 5B), ×43. *D*, Bud formation as induced after 8 wk culture on medium supplemented with 8.9 μ*M* BA and 2.9 μ*M* IAA (clone FS-104), ×33. *E*, plantlets produced from shoots of adventitious origin (clone 5B) 1 mo. after root induction treatment (scale in cm). Key: *sc* = shoot cluster; *vs* = vascular strands.

genic response is of course suggestive of a relationship with polar transport of endogenous auxin (Paterson, 1883; Goh et al., 1994). In the case of European beech, it suggests that optimal auxin/cytokinin balance, necessary to induce regeneration, may exist in the petiole stump of proximal explants. However, polar response may involve both hormone transport and differences in tissue maturity between the distal and proximal leaf tissues (Welander, 1988). When adventitious shoots formed on a distal explant in our study, the proximal counterpart also differentiated adventitious shoots, indicating the importance of the physiological state of each particular leaf.

Thidiazuron, a nonpurine phenylurea, has been shown to have high cytokinin activity by influencing endogenous cytokinin biosynthesis or metabolism (Mok et al., 1987). A range of TDZ concentrations from 0.1 to 20 μM has been used to stimulate shoot organogenesis from leaves of woody species (Huetteman and Preece, 1993). This compound is very effective for induction of adventitious shoots in hardwood genera including *Fraxinus* (Bates et al., 1992; Tabrett and Hammatt, 1992), *Ulmus* (Boylard et al., 1991; George and Tripepi, 1994), and *Populus* (Chalupa, 1989), a list to which this study allows the addition of *Fagus*. For European beech, as for other woody species (Hammatt, 1994; Pawlicki and Welander, 1994), TDZ was more effective than BA in inducing shoot buds on leaf explants. As of yet, there are very few studies on the caulogenic action of TDZ on Fagaceae explants. Adventitious shoots were differentiated in the callus tissue developed at the basal part of oak shoots cultured on TDZ-containing medium (Chalupa, 1988). On the other hand, in an earlier study (Vieitez et al., 1993), BA + NAA supplemented medium induced adventitious shoot bud regeneration on only 20% of *F. sylvatica* hypocotyl and cotyledon sections, which are furthermore a less abundant starting material than leaf explants.

The concentration of TDZ and the length of exposure time are critical for optimizing caulogenesis. In this study, bud clusters produced by continuous exposure to TDZ were usually stunted and compact, and therefore more difficult to elongate than buds induced by BA media. Although only buds visible at the tissue surface were counted, histological examination showed that numerous other bud primordia had been initiated in response to TDZ but had not developed. Similar findings have been reported for *Limonium perigrinum* (Seelye et al., 1994), and the compactness of TDZ-induced shoots has been noted in the cases of apple (Fasolo et al., 1989), white ash (Bates et al., 1992), and American elm (George and Tripepi, 1994). Cytokinins commonly stimulate shoot proliferation and inhibit their elongation. Therefore, the inhibition of shoot elongation by TDZ may be consistent with its high cytokinin activity (Huetteman and Preece, 1993). For European beech, the problem can be overcome by reducing exposure time to 1–3 wk, in accordance with the two-stage culture procedure proposed by Huetteman and Preece (1993) (culture in TDZ medium followed by culture in a secondary medium often lacking TDZ or with other plant growth regulators).

The genotype of the explant is one of the most influential factors determining organogenic response (Brown and Thorpe, 1986). We found that leaves of all four genotypes tested were able to differentiate adventitious buds with greater or lesser efficiency. However, the differences in bud-forming capacity cannot only be ascribed to the genotype, as stock plant material of different age was used in this study. In general, the more juvenile the material, the more easily organ formation will occur *in vitro* (Thorpe and Patel, 1984). The low responsiveness of P3 leaves could also be an age effect, as this clone was derived from a 3-yr-old plant, while the other three clones had a 2-mo.-old seedling origin. Variation in shoot-forming capacity among genotypes has been found in explants of various species, including white ash (Bates et al., 1992), apple (Jones, 1993), eucalyptus (Lainé and David, 1994), and American elm (George and Tripepi, 1994). This variation may be due to different clones having different sensitivities to growth regulators (Lainé and David, 1994). Analysis of segregation ratios in crosses between cultivars of different shoot-regeneration capacity indicates that two or three genes control *in vitro* shoot formation in *Medicago sativa* and *Petunia hibrida*, respectively (Halperin, 1986).

For European beech, an initial darkness period had no positive influence on bud induction. Although adventitious buds did form on leaf explants subjected to continuous darkness, the best caulogenic response was achieved under continuous photoperiodic conditions or with only 10 d of initial darkness. This result contrasts with reports of a 1–4-wk initial dark period being necessary for optimum shoot regeneration from leaf tissues of apple (Fasolo et al., 1989; Famiani et al., 1994) or eucalyptus (Lainé and David, 1994), and of continuous darkness promoting successful shoot induction on black locust leaves (Arrillaga and Merkle, 1993) and olive petioles (Mencucciani and Rugini, 1993).

Histological evidence confirmed both the indirect and direct pattern of development, in the regenerated buds. Further studies on the physiological or anatomical factors controlling whether shoots develop directly or indirectly may contribute to the understanding of the fundamental principles underlying regeneration capacity in woody plants.

In conclusion, the results reported here demonstrate that the formation of adventitious shoots on leaf explants from *F. sylvatica* cultures and the regeneration of plantlets therefrom are possible. Definite trends could be established with regard to the induction requirements of different clones. Future research will attempt to increase the efficiency of this regeneration system by improving the elongation of shoots originated after different exposures to TDZ.

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