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Selection of a super-growing legume root culture that permits controlled switching between root cloning and direct embryogenesis

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Abstract Root cultures, displaying vigorous growth and high embryogenic capacity, were established in the legume forage species *Lotus corniculatus* (bird's-foot trefoil). Root cloning as well as plant regeneration was achieved on hormone-free medium, in agitated culture in the dark or under stationary conditions in the light, respectively. These qualities of vigorous growth and regeneration faded with time in hormone-free culture, with slow-growing roots turning brown in color. Addition of the synthetic cytokinin-like hormone benzylaminopurine to the culture medium, however, re-established the aging tissue's capacity for somatic embryogenesis and plant formation. During continuous initiation of new cultures, it was possible to obtain one root culture (selected from 11 960 seeds at a 65% germination rate) which did not show the typical decline of qualities after prolonged proliferation but distinguished itself by displaying even faster growth and more vigorous embryogenic plant production on hormone-free medium. There was no decline since its initiation 9 months earlier. This super-growing root culture produces plants that show no morphological differences as compared to wild-type regenerants or seedlings. Roots, dissected from plantlets derived from super-root embryogenesis, expressed all the super-root qualities again when cultured in vitro. This is the first report on somatic embryogenesis from sustained root cultures without exogenous hormone application. Such a hormone-free, continuous root culture should provide a superior experimental system for genetic or developmental studies that might be sensitive to exogenous hormones, such as somaclonal variation in transgenesis or, since introduced in a legume species, nodulation in vitro.

Key words Bird's-foot treefoil · *Lotus corniculatus* · Root culture · Somatic embryogenesis · Super-growth

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Introduction

Roots were the first plant organs to be cultured in vitro. In his classical paper, White (1934) described potentially unlimited growth of excised tomato root tips in a liquid medium. A few years later, he demonstrated the applicability of his technique to a number of different dicotyledonous species (White 1938). Since then, there have been numerous publications offering species-specific improvements and attempts at generalization. Surprisingly, successes comparable to White's tomato root cultures have remained rare (Said and Murashige 1979). Depending on the species examined, various modifications of medium or culture conditions have been shown to be inhibitory as well as stimulatory, or even essential as well as lethal, for root growth in vitro (Butcher and Street 1964). Most investigations that utilize cultured roots as an experimental system work with excised young roots that elongate in culture for a limited time, but not with continuous, true-root cultures. In true-root cultures, the root explants have grown in vitro, separated from the shoots, for a time long enough to establish shoot-independent growth. This essential difference between excised roots in culture and true-root cultures has not always been sufficiently taken into account in previous investigations. Hairy root cultures, so-called because of their hairy

appearance due to neoplastic branching caused by *Agrobacterium rhizogenes* infection (Signs and Hector 1990; Tepfer 1990) and often used to produce secondary metabolites in vitro (Hamill et al. 1987), are of limited use for developmental and genetic studies. In rye, Whitney (1996) was able to grow selected callus-derived roots on hormone-free medium for an extended time. However, the donor-callus was produced from radicles on 2,4-dichlorophenoxyacetic acid-containing medium. The culture showed the morphological characteristic of hairy growth, typical for root cultures transformed with *A. rhizogenes*. The establishment of true continuous root cultures remains problematic, and this may be the reason why this field has not been reviewed rigorously for quite some time (Butcher and Street 1964; see also Schiefelbein and Benfey 1991).

Symbiotic fixation of atmospheric nitrogen by leguminous plants is the principal process for maintaining nitrogen fertility in agriculture. Considerable knowledge about this process has been gathered in past years but much more insight needs to be gained before we will be able to successfully manipulate the efficiency of the process or even increase the species range. Plant tissue-culture techniques have provided an opportunity to study some aspects of this symbiotic relationship between higher plants and bacteria under in vitro conditions (Holsten et al. 1971). Early work in this field utilized cultures of excised legume roots (Raggio et al. 1957, 1959), but this system could not be sufficiently reproduced to become a basis for in vitro nodulation studies. The question of whether true continuous root cultures can nodulate at all remains unanswered.

Bird's-foot trefoil (*Lotus corniculatus* L.), a widely cultivated forage legume, has been used as a model plant for transformation studies in legumes (Stogaard et al. 1986; Petit et al. 1987). In tissue culture, *L. corniculatus* regenerates well from callus through caulogenesis (Niizeki and Grant 1971; Tomes 1979; Swanson and Tomes 1980) and embryogenesis (Mariotti et al. 1984; Akashi et al. 1998). Regenerable calli have been induced from a number of different tissues and also from hypocotyl- and cotyledon-derived protoplasts (Ahuja et al. 1983; Vessabutr and Grant 1995). Rybczynski and Badzian (1987) demonstrated that root-segment explants, isolated from young seedlings of *L. corniculatus*, produce shoots at the proximal end of the explant on hormone-free medium. The number of shoots could be increased through cytokinin (Badzian and Rybczynski 1994).

In the present communication, we report the establishment of a root culture of *L. corniculatus* in hormone-free medium and plant regeneration from these sustained cultures through somatic embryogenesis. Furthermore, we have isolated a super-growing root culture which is characterized by accelerated growth and long-term regeneration capacity. The super-growth characteristics did not disappear with an interposed plant regeneration step. This research was initiated with the eventual goal of in vitro-nodulation in true-root cultures.

Materials and methods

Plant material

Seeds of bird's-foot trefoil (*L. corniculatus* L.) cv Viking were obtained in 1996 from the Snow Brand Seed Company, Japan. Seeds were surface sterilized in 70% (v/v) ethanol for 1 min followed by sterilization in 2% sodium hypochloride for 20 min, then rinsed five times with sterile distilled water and germinated on hormone-free BGMM medium (Bergersen 1961), containing 0.8% (w/v) agar, pH 6.8, at 27°C and 16 h of light (germination rate 65%).

Root culture

A summary of explant selection and culture is given in Fig. 1. Root explants, with or without a hypocotyl, were collected from 5–9 days old seedlings. One explant was isolated per seedling; it was approximately 2-cm long when dissected immediately below the hypocotyl, or 2.5-cm long when taken with the hypocotyl section attached. Roots with damaged root tips or brown pigmentation were rejected, resulting in only 1–2% of the seeds sown yielding root explants. Five root explants, all belonging to one of the two types (with or without a hypocotyl), were placed into a 125-ml Erlenmeyer flask containing 20 ml of either liquid hormone-free Raggio root (Rr) medium (Raggio et al. 1957) at pH 6.8 or liquid hormone-free Murashige and Skoog (MS) medium (Murashige and Skoog 1962) at either pH 5.8 (regular) or 6.8 (modified to match the pH of the Rr medium). Both media contained 30 g/l of sucrose. The cultures were kept at 25°C in the dark on a rotary shaker at 80 rpm.

After 3 weeks in primary culture, the roots, now approximately 20 cm long and with multiple branches, were divided into four sub-explants (Fig. 1). Explant I is a 1-cm segment containing the root tip but no branches. The remaining root was cut into two pieces, yielding explant II and a long segment containing the original cut (with or without a hypocotyl) and the longest branches. From this segment one long and thick branch with an intact white tip was chosen as explant III. The remaining piece is explant IV, which was transferred to conditions for plant regeneration (stationary, light). Five pieces of each of explant types 1 to 3, respectively, were cultured per flask in fresh medium for root cloning and subcultured thereafter at intervals of 2 weeks with aliquants tested for regeneration at the end of each subculture period. Root growth was assessed as the fresh weight increase per unit of time.

Root cultures were also re-initiated from selected plants regenerated from cultured roots. In these cases, the strongest root was cut approximately 2 cm above the tip and cultured as described above.

Plant regeneration from cultured roots

Experiments on shoot regeneration from cultured roots were carried out: (1) with explant type IV 3 weeks after culture initiation, and (2) at the end of every subculture period with explant types I to III, up to 3 months after culture initiation. Five pieces of explant IV or approximately 1 g of subcultured roots, consisting of randomly selected pieces from material contained in one Erlenmeyer flask and chopped into segments of 1–4 cm in length, were placed in a 9-cm Petri dish with 20 ml of liquid hormone-free MS medium. Petri dishes were kept stationary at 16 h light and 25°C. To rejuvenate non-regenerating roots, root segments were placed on MS medium supplemented with 8 g/l of agar and six different concentrations (0.01–1 mg/l) of 6-benzylaminopurine (BAP).

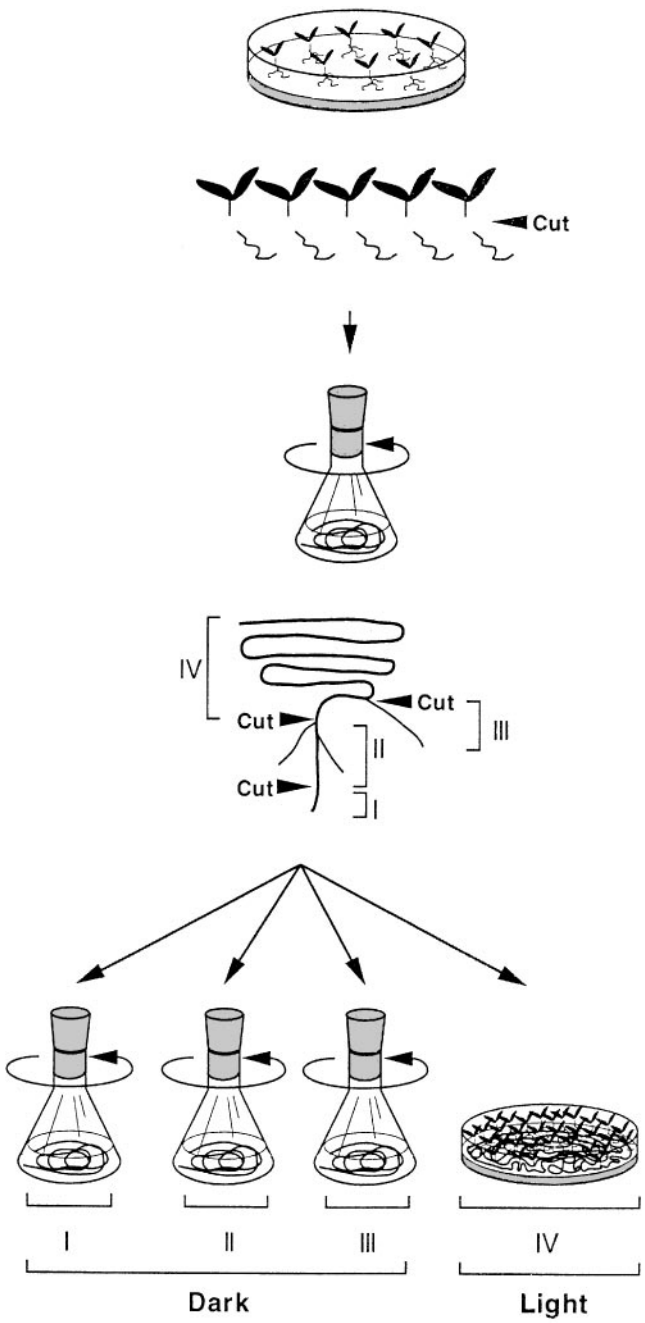

Fig. 1 Initiation of root cultures from seedlings of *L. corniculatus* (bird's-foot trefoil). Five root explants derived from five different seedlings were cultured per flask in hormone-free MS medium. After 3 weeks in primary culture, the roots (approximately 20-cm long) were cut into four defined explant types (*I to IV*). *Types I to III* were subcultured in the dark on a shaker for root cloning, and *type IV* was transferred to stationary subculture under light for plant regeneration

Regenerated shoots of approximately 1.5 cm height were cut off the cultured roots and transferred to half-strength hormone-free MS medium (Magenta culture box) supplemented with 0.8% (w/v) agar for rooting and further development. Recovery of potted plants was close to 100% (Akashi et al. 1998).

Results

Initiation of root culture and spontaneous caulogenesis

Based on preliminary studies with *Lotus* (unpublished) and experience with *Glycine* (Hoffmann et al. submitted), we tested root explants with and without a hypocotyl in two different media, Rr (Raggio et al. 1957) and MS (Murashige and Skoog 1962). Although care was taken to ensure phenotypic explant homogeneity, some explants turned brown in culture within 1–2 weeks and stopped growing. The necrotization process seemed to be catalytic, i.e. often either none or all of the five roots contained in a single flask were affected. Browning was far more frequent in Rr medium as compared to MS. Overall, MS was also the better medium in supporting vigorous growth and differentiation. Differences between MS at pH 5.8 and 6.8 could not be found. We randomly chose pH 6.8 for further experimentation. Our pre-screening data, summarized in Table 1, clearly suggested that MS is the preferable medium and that inclusion of the hypocotyl is not necessary. Quantification of these data is laborious and would not provide further information critical to our research project.

Within 2–3 weeks of the initial culture, all growing roots (Fig. 2 a), derived from explants with or without a hypocotyl, developed a short longitudinal split at the cut end with a microscopic callus between the halves and one to two pale shoots emerging from that callus (Fig. 2 b).

Root cloning

After 3 weeks in culture, roots, approximately 20 cm in length, were separated into the four explant types described in Materials and methods (Fig. 1) and subcultured per type to fresh medium for root cloning (explant types I to III) or regeneration (explant type IV). Root cloning was initially successful with all three

Table 1 Pre-screening of different culture conditions for the initiation of root cultures in *L. corniculatus* (bird's-foot trefoil)

Culture condition (medium[a] and explant type[b])	Overall root growth[c]	Degree of branching[c]	Formation of somatic embryos[c]
MS/with hypocotyl	+ + +	+ +	+ +
MS/without hypocotyl	+ + +	+ + +	+ + +
Rr/with hypocotyl	+ +	−	+
Rr/without hypocotyl	+ +	−	+

[a] Media used are MS (Murashige and Skoog 1962) and Rr (Raggio et al. 1957)
[b] Explant types are seedling-derived roots with or without hypocotyl
[c] Pre-screening rating is − for none, + for poor, + + for moderate and + + + for good

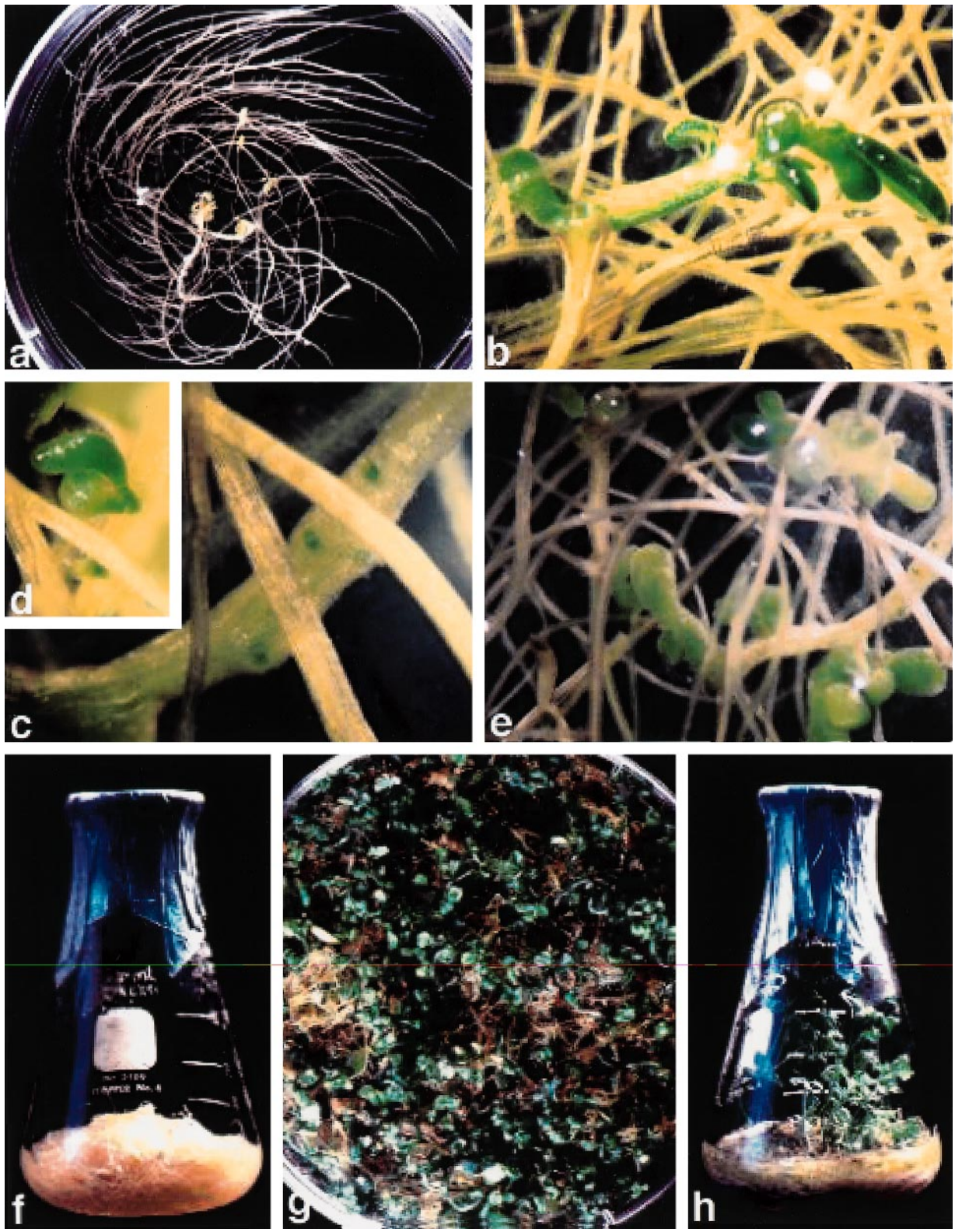

Fig. 2a–h Root cloning, somatic embryogenesis and plant regeneration in normal and super-growing root cultures of *L. corniculatus* (bird's-foot trefoil). **a** Roots after 3 weeks of initial culture (transferred to a 9-cm Petri plate for photography); yellowish plant regeneration spots formed at the wound sites of the five root explants are visible. **b** Plant regeneration from wound callus formed between the split ends of a cultured root. **c** Green spots on cultured roots indicating the onset of somatic embryogenesis. **d** Embryo formed by the uppermost green spot in **c**. **e** In the vicinity of the green spots, as magnified in **c**, are numerous additional older regeneration sites. **f** A 125-ml Erlenmeyer flask containing a super-growing root culture displaying the typical root density at the end of a subculture period. **g** Plant regeneration in a stationary culture of super-roots (9-cm Petri plate). **h** Yellowish shoots, formed at wound sites of super-growing roots, turned green and grew vigorously when placed on a shelf under light. **a** through **e** show normal root cultures but are also fully representative of super-roots at this stage. Magnification: bar in **c** = 1 mm

explant types, but most vigorous in the case of the root-tip explants (type I), which formed a dense root cushion extending above the medium at the end of the culture period. Cultures from all explant types developed root hairs. Occasional spontaneous plant formation from wound callus continued in subcultures. However, root growth as well as plant regeneration ceased gradually with time and stopped after 4–6 subcultures (3–4 months).

Embryogenesis from cultured roots

Cultured roots, when transferred to regeneration conditions (stationary, hormone-free liquid culture in light), usually carried a few wound-callus-derived yellowish shoots. These shoots turned green and continued to grow vigorously under regeneration conditions. Beginning after 1 week under light, the roots developed distinct green spots (Fig. 2 c), and these spots formed somatic embryos (Table 1) without prior wounding or the formation of visible callus (Fig. 2 d). Furthermore, wounded areas continued to form small calli from which somatic embryos developed. These embryos germinated at high rates while still attached to the roots (Fig. 2 e), probably only inhibited by competition for space, and were easily rooted and transferred to soil with close to 100% success (Akashi et al. 1998). The highest plant regeneration rate was found when cultures were young, being the best in the original explant IV. From a single Petri plate hundreds of plantlets can be harvested within 4 weeks when a decline in embryogenic activity becomes visible, but we never attempted to isolate such numbers or to even maintain the embryo yield by transfer to fresh medium.

Rejuvenation of cultured roots through BAP

The ability of cultured roots to undergo embryogenesis without exogenous hormone application declined gradually within 3 months and concurrent with the decline in growth of these root cultures. To rejuvenate the aging roots, they were randomly cut into short segments and placed on solid medium containing different concentrations of BAP. At this time, the root segments are brown in color and are releasing brown pigment. Table 2 shows the rejuvenating effect of BAP on those partially necrotic root segments, expressed as regained embryogenic activity. Under the influence of BAP, explants produced callus at both cut ends as well as from multiple wounded sites of the necrotic tissue. These calli regenerated embryos. Concentrations of 0.01 and 0.05 mg/l of BAP were ineffective; 0.5 mg/l produced the maximum amount of embryogenic callus. Embryogenic calli were smaller on 0.1 mg/l. However, these smaller calli were always first in showing embryogenic activity. Embryo maturation and rooting in hormone-free medium was not different in regenerants induced on BAP-containing medium as compared to those born hormone-free.

Selection of super-growing roots

One Erlenmeyer flask, containing original root explants 190–195 (selected from 11 960 seeds at a 65% germination rate), was found to be unusually densely packed with root tissue (Fig. 2 f), displaying long lateral roots and a normal density of root hairs. Eleven months after this super-growing root was found, the vigorous growth of this culture on hormone-free medium still continued. Subculture was performed by cutting the densely interwoven disk-shaped root cluster into four slices of equal size and transferring each quarter slice either for root cloning to another Erlenmeyer flask or to a Petri plate for regeneration. Root growth stabilized to quadrupling from a 5-g (fresh weight) quarter slice to a 20-g cluster in 2 weeks. A precise comparison of this growth rate with that of the wild-type cultures was not possible because explant type and culture density differed strongly and normal cultures declined steadily in quality. In this situation, the super-root should be compared to the best growth

Table 2 Re-activation of embryogenic potential in root explants of *L. corniculatus* (bird's-foot trefoil) that have been randomly selected from a formerly highly embryogenic root culture which had lost its embryogenic potential through gradual cessation. Root explants are brown and necrotic when cultured on medium with different concentrations of benzylaminopurine (BAP)

BAP concentration[a] (mg/l)	Number of explants inoculated	Number of embryogenic calli	Embryogenic calli per root explant	Embryos/shoots per callus	Embryos/shoots per root
0	119	0	0	0	0
0.1	123	161	1.4 ± 0.2	6.5 ± 0.4	9.7 ± 1.8
0.25	134	196	1.5 ± 0.1	7.9 ± 0.8	11.3 ± 1.1
0.5	124	209	1.6 ± 0.2	10.0 ± 1.1	16.3 ± 2.3
1.0	115	169	1.7 ± 0.2	10.0 ± 0.5	14.8 ± 1.9

[a] 0.01 and 0.05 mg/l were ineffective

found in the wild-type. If root tip-derived (explant type I) roots were pooled and started at 5 g per Erlenmeyer flask for the first subculture, the best cluster formed in 2 weeks was 12 g and consistently declined in subsequent subcultures.

When cultured in the dark on a shaker, super-roots, like the wild-type, produced a wound callus at the cut ends and regenerated pale shoots (compare Fig. 2 a) which turned green and grew vigorously when placed under stationary light conditions (Fig. 2 h). Once transferred, the roots produced embryos without an intervening callus. Within 4 weeks and without further transfer to fresh medium the Petri plate was covered with a lawn of green plantlets growing from the root cluster (Fig. 2 g). The embryogenic capacity of the super-roots did not decline in hormone-free culture during the 9 months following its discovery. Shoots harvested from super-root cultures could easily be converted to potted plants using the same protocol as employed for the wild-type. Flowering plants regenerated from super-roots looked morphologically normal as compared to wild-type regenerants or seedlings.

Super-root cultures from super-root regenerants

Roots formed in vitro by shoots derived through super-root embryogenesis on hormone-free medium were removed and re-cultured in hormone-free medium. The resulting root culture showed super-root qualities again. This experiment was repeated ten times with identical results.

Discussion

The regular root culture that can easily be established from *L. corniculatus* in hormone-free medium grows vigorously in liquid culture and regenerates dramatically under hormone-free conditions if transferred to light and stationary placement. No differences were found between cultures derived from explants with or without the hypocotyl section attached. In soybean, inclusion of the hypocotyl was found to be essential under similar experimental conditions (Hoffmann et al. submitted). Also, bird's-foot root cultures produced abundant root hairs, while soybean, like many other species (unpublished), grows naked roots under in vitro conditions. The bird's-foot culture maintains sufficient quality for a considerable time to render itself as a useful experimental system, i.e. the root is detached from the shoot portion for a period long enough to be viewed as a true root culture. The interval between the second and third subculture, 2 months after culture initiation with the culture still vigorously growing and regenerating, seems to be the best period for experimentation. However, with time root growth ceases and regeneration stops. The culture is clearly not immortal under the hormone-free culture conditions employed. Partial rejuvenation is possible through exogenous cytokinin application. Plants regenerated by rejuvenated roots do not differ visibly from those regenerated hormone-free. They do not show inhibition of rooting as reported by Badzian and Rybczynski (1994) in root-segment explants of *L. corniculatus* treated with BAP. Rejuvenation with cytokinin was also obtained in long-term root cultures of *Citrus aurantifolia* (Bhat et al. 1992).

A decline in quality, defined in terms of root growth-rate and regeneration capacity, did not occur in super-growing roots. These cultures remained unchanged. Morphologically, no differences can be observed between super-roots and controls, apart from an increased length of the main root and its branches. Characteristics typical for transformed roots, such as a changed branching pattern, are not apparent. Regenerated plants derived from super-roots look morphologically normal. Root cultures re-established from these super-roots do not differ morphologically from controls. The question of whether the super-roots are habituated or stably mutated remains open. The fact that roots, formed on regenerated plants derived from super-roots, showed super-growth and regeneration in all cases when cultured in vitro seems to point towards a mutation. However, a genetic analysis needs to be conducted to answer this question. A clone of super-root regenerants is currently blooming and will be crossed with other ecotypes if attempts to self them prove unsuccessful. The original super-root culture was found in a flask containing five root explants (Fig. 1) derived from five different seedlings. We have no experimental evidence that only one of the seedlings produced the super-growing root culture but assume that this is the case. This assumption is in compliance with the mutation hypothesis.

Hormone-free legume root cultures, as reported here, should be especially suited to study nodulation in vitro. Hormones play an important, although not fully understood, role in nodulation (Hirsch and Fang 1994), and hormone-containing culture media can be expected to interfere with the process or even cause pseudo-nodules (Sarul et al. 1995; Kawaguchi et al. 1996). The mass regeneration possible from these roots could play an important part in evaluating the role of the shoot in nodulation (Francisco and Harper 1995). For the same two reasons, the system should be favorable for studying root development in vitro.

The mechanism of embryo formation observed in both the normal as well as the super-growing root cultures is quite distinct. The yellowish-white root tissue develops small green spots, initially without any visible swelling. Then, green protuberances emerge which soon display an indentation at the tip, leading to cotyledon formation. No prior wounding occurred, and the formation of interlinked callus could be excluded, without the need for a histological investigation,

through microscopical observation. On the same root, indirect somatic embryogenesis can be observed at wound sites, such as the original cut. The absence of exogenous hormones in our root cultures and the presence of direct embryogenesis could mean a reduction in somaclonal variation. This would make these cultures a favorable transformation target. The high rate of direct embryogenesis of differentiated cells from intact tissues suggests that the biolistic approach might be the gene-delivery method of choice for root cultures of *L. corninculatus*.

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