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DIRECT SHOOT FORMATION IN SPONTANEOUSLY OCCURRING ROOT PSEUDONODULES OF ALFALFA (*MEDICAGO SATIVA* L.)

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Summary

A simple and rapid procedure for direct organogenesis from root nodulelike structures of alfalfa (*Medicago sativa* L.) line SGg, spontaneously induced on growth regulator-free Gamborg (B_5) medium, was developed. Prolific adventitious shoot initiation was obtained using a combination of 1.0 mg/liter TIBA and 0.5 mg/liter 2iP. Transfer of shoots to a medium containing 0.5 mg/liter ABA and reduced concentration of TIBA (0.5 mg/liter) before rooting markedly stimulated shoot development. Regenerated shoots rooted easily and revealed the early appearance of nodulelike structures on basal medium (B_5) lacking growth regulators. Analysis of endogenous growth regulator levels of SGg roots maintained on growth regulators free media, showed that spontaneous shoot appearance was correlated with high cytokinin-to-auxin ratios.

Key words: alfalfa; *Rhizobium;* nodulation; organogenesis; endogenous growth regulators

Introduction

Alfalfa as a model species is widely used for fundamental studies. An effective and rapid procedure for the regeneration of plants from cell and tissue cultures is essential in genetic engineering. The typical pathway for alfalfa regeneration is direct (10,11,25) or indirect (3,4,7,18,28) somatic embryogenesis. As a rule, plant growth regulators are needed to trigger regeneration of alfalfa plants. Alfalfa is also known for its ability to nodulate as a result of its interaction with *Rhizobium*. Until recently, it was assumed that *Rhizobium* is indispensable for triggering nodulation (16,22). However, an occurrence of the so-called NAR phenomenon (26) (i.e., the capacity of aseptic alfalfa plants to nodulate in the absence of *Rhizobium* spp.) has been reported (15).

This study describes an alfalfa line, SGg, characterized by its ability to produce spontaneously nodulelike structures and reports data on existing correlation between endogenous growth regulator levels of tissue and their capacity for induction of these structures. Furthermore, we present a novel approach for alfalfa regeneration—direct differentiation of shoots from nodulelike structures.

Materials and Methods

Plant material. The experiments were carried out with two tetraploid alfalfa cultivars: Mongolian cv. Burgaltay, which has been found to be highly embryogenic (data unpublished) and cv. Rangelander.

The seeds were sterilized with 70% ethanol for 30 s and 0.2% $HgCl_2$ solution for 3–4 min and rinsed five times with sterile distilled water.

In vitro cultivation. Growth-regulator-free medium B_5 (12) was used for seed germination, induction of nodulelike structures on roots, adventitious shoots, rooting, and maintenance of plants. Attempts to reduce the period of regeneration and to improve the effectiveness of organogenesis from nodulelike structures were made by using various concentrations of kinetin (1.0, 2.0 mg/liter), triiodobenzoic acid (TIBA) (0.1, 0.5, 1.0 mg/liter), isopentenyladenine (2ip) (0.1, 0.5, 1.0, 2.0 mg/liter), N6-benzylaminopurine (6-BAP) (0.1, 0.5, 1.0 mg/liter), and abscisic acid (ABA) (0.5 mg/liter). Growth regulators were added after filter sterilization (0.22 μm Millipore Corp., Bedford, MA, USA) to agar medium (autoclaved 30 min. at 120° C). Root explants (3–4 cm long) with differentiated, nodulelike structures were excised from 50-day-old seedlings and plated on agar B_5 media in petri dishes. Five petri dishes (diameter 9 cm) with six to eight root explants were used for each tested media containing different combination of growth regulators. The experiments were performed in three replications. Significant differences between treatments were tested using a one-way analysis of variance (ANOVA).

All plant material were cultivated in growth chambers at 26° C, 70% humidity, a 16/8 light/dark photoperiod, and 2500–3000 lux light intensity. After developing a good root system and reaching the four thrifoliate leave stage on B_5 media, the regenerants were planted in autoclaved soil. The adaptation period took place in a greenhouse under "mist" conditions.

Growth regulator analyses. Roots of 35-, 50-, 57-, and 65-d-old seedlings from two alfalfa lines, SGg and SGw, of var. Burgaltay and cv. Rangelander-A70 were collected and studied for endogenous cytokinin and indole-3-acetic acid (IAA) content. Root material (0.5–1.0 g/FW) was extracted (100% methanol 10 ml/g FW) and centrifugated. The supernatant was divided in two parts: for cytokinin analysis (with addition of about 16 Bq ^{3}H-zeatin, Amersham, as an internal standard) and for IAA analysis (addition of about 240 Bq ^{3}H-IAA, Amersham) The quantitative determination was performed by ELISA, based on polyclonal antibodies raised against indole-3-acetic acid (IAA), zeatin riboside (ZR), and isopentenyl odenosine 2iPA, respectively (14). Validation of the methods and establishment of the purification procedures required prior to ELISA were carried out by HPLC on-line fluorometric detection for IAA-analysis (21) and by LC-MS and by RIA, based on chicken polyclonal antibodies (results not shown).

Cytological analysis. Young roots from newly rooted cuttings were pretreated in colchicine solution (2.5%, 2h, 26° C), fixed in a 3:1 (vol/vol) mixture of ethanol and acetic acid, and stained with 4% acetocarmine.

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Squash preparations were made in 45% acetic acid and chromosome number was counted from the root-tip cells in methaphase.

Results

Organogenesis on growth-regulator-free media. No differences in phenotypic behavior of both Burgaltay-SG and Rangelander-A70 seedlings were observed during 5-wk cultivation on basal medium lacking growth regulators. Considerable changes of the var. Burgaltay root system occurred between the 6th and 7th wk after germination, with the earliest changes seen at Day 40. Initially, 35.5% of the roots became rather thick and green, while the rest stayed white, similar to the root systems of all A70 seedlings. Based on visual assessment of SG seedling roots, two alfalfa lines (one with green and one with white roots) were selected for further detailed study and were subsequently designated SGg and SGw.

After an additional week of culture, about 20% of the SGg roots developed spontaneously root-derived structures that morphologically resembled root nodules (Fig. 1 *A*). Two types of nodulelike structures were observed: green, multilobed structures spread most frequently on the tap-root (Fig. 1 *B*) and white, single ones formed mainly on root branches (Fig. 1 *C*). The number of root nodulelike structures per seedling varied over a wide range (from 52 to 137). Nine weeks after germination, however, only about 1.6–3% of root nodulelike structures developed adventitious shoots. Within 10–15 d, the emerging shoots were grown further into plantlets directly on intact seedling roots (Fig. 1 *D*). When the regenerant shoots were excised and transferred to fresh B_5 medium, fast rooting and a more rapid appearance of nodulelike structures occurred. The capacity to induce root-derived structures has been maintained during clonal propagation of the regenerants for more than 2 years. By contrast, none of the SGw and Rangelander seedlings grown on basal medium were capable of inducing any nodulelike structures on the roots.

Growth regulator analysis. Marked differences in the endogenous cytokinin levels between the tested alfalfa genotypes were found (Fig. 2). While there were no significant changes in cytokinin content on the Rangelander-A70 roots, a drastic increase was observed in the var. Burgaltay. The two selected lines SGg and SGw, possessing different abilities to form nodulelike structures, also showed different cytokinin contents (Fig. 2). The 3.5 times increase of cytokinins for SGw roots and the 16-fold increase for SGg were found after 65 d of cultivation. The shifts in IAA levels were similar for all of the tested alfalfa lines (SGg, SGw, and A70). IAA concentration decreased with the age of roots.

Organogenesis on growth-regulator-containing media. The observed phenomenon in the SGg alfalfa line prompted us to investigate whether the existing nodule-derived shoots can be used as an applicable regeneration system. Thus, SGg root explants with induced nodulelike structures were cultivated on media supplemented with different growth regulator combinations. Among the tested growth regulators, the best effect on shoot induction was found to be the combination of 2iP and TIBA. Shoot induction was achieved in all tested concentrations of these growth regulators at a variable frequency (Table 1), except root induction that occurred on medium III. At 0.5–1.0 mg/liter concentrations (media II, Y, and YII), TIBA and 2iP induced a higher frequency of shoot formation than the rest of the treatments. On these media, shoots were induced directly (without callusing) from nodulelike structures. Furthermore, sporadic prolific shoots continuously developed on the same media and gave rise to plantlets within 18–25 d of culturing. Growth regulator combinations of TIBA with kinetin and BAP were ineffective in inducing formation of adventitious shoots. These

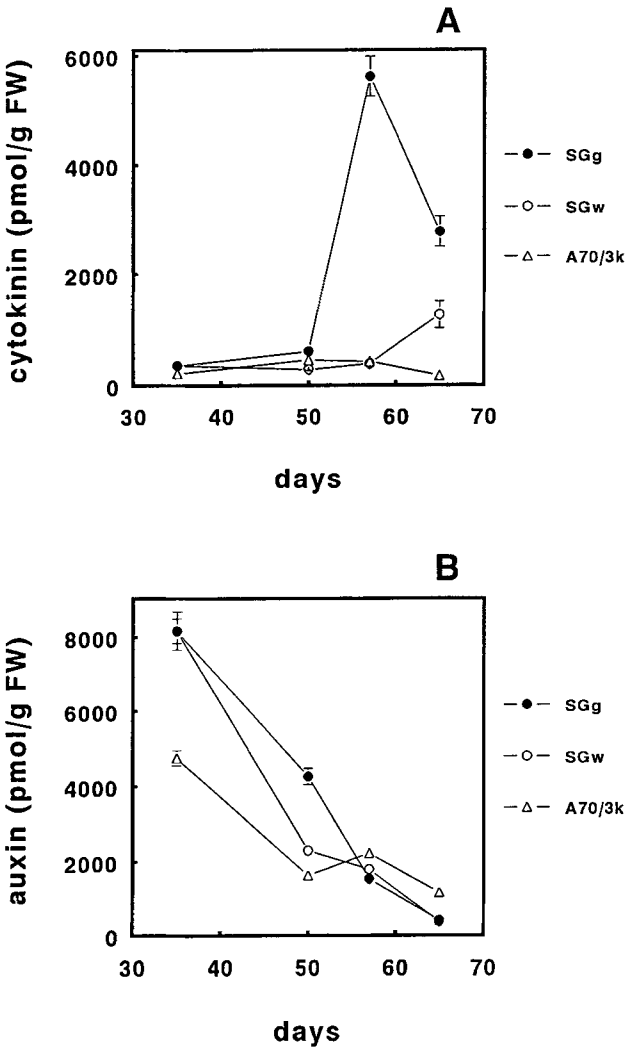

Fig. 1. Endogenous levels of cytokinin (*A*) and IAA (*B*) in roots of SGg, SGw, and A70 alfalfa seedlings 65 d after cultivation on hormone-free media.

growth regulators tested in the range of 0.1–2.0 mg/liter and were found to be particularly unsuitable because they resulted in callus formation and cessation of further differentiation of the root structures. Root structures lacking any growth regulator treatment (control) did not exhibit any further stages of differentiation. However, most of the shoots induced on the media II, Y, and YII containing TIBA and 2iP did not easily grow to plantlets. Therefore, a two-step treatment, including varying combinations of these three media was attempted. Efficient regeneration of shoots and plantlets was observed when the nodulelike structures, produced on B_5 basal medium along with the root explants, were first cultured on media II, Y, and YII for shoot induction. The emerging shoots were excised and transferred to a second medium, Y and YII, where they developed into plantlets. Shoot regeneration occurred in all media combinations used. However, significantly higher regeneration efficiency was obtained from the following media combinations: C, D, and K (Table 2), where the average percentages of shoot regeneration were 65.2, 66.7, and 71.4, respectively, and the average numbers of responding regenerants per shoot were 4.0, 6.3, and 4.7, the latter being rather low. The best overall shoot recover was obtained

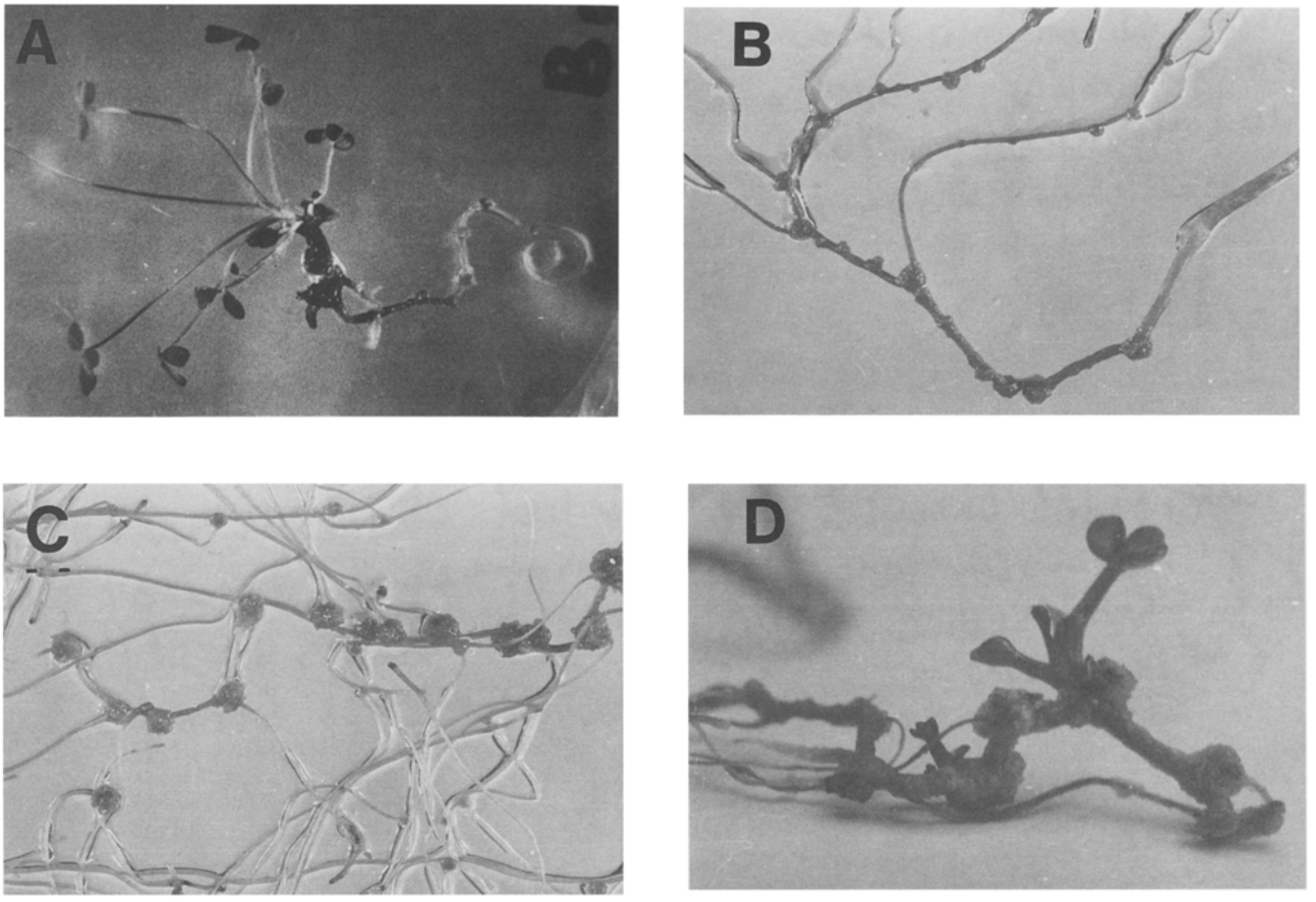

FIG. 2. Direct morphogenesis in spontaneously induced pseudonodules on alfalfa roots. *A*, alfalfa seedlings with "nodules" induced on hormone-free media. Two types of nodulelike structures: *B*, green—located predominantly on the main root and *C*, white-spreaded on the branches. The green "nodules" developed adventitious shoots 9 wk after germination, and they further grew into plantlet within 2 wk (*D*).

when shoots were induced on B_5, containing 0.5 mg/liter 2iP and 1.0 mg/liter TIBA and were subcultured on the same concentration but with reduced TIBA (0.5 mg/liter) to promote further development of adventitious shoots (Table 2, Var. D). Incorporation of ABA (0.5 mg/liter) in the second step enhanced the percentage of shoots recovered. In addition, there was a marked reduction of the shoot regeneration period (within 27–35 d) (Table 2). After transferring to medium without growth regulators, the regenerants showed a good ability for rooting and retained the capacity of initial seedlings to produce nodulelike structures (data not shown).

No morphological abnormality and changes in the chromosome number of the obtained regenerants were found.

A comparative evaluation of regeneration efficiency between seedlings and newly obtained plantlet root explants was carried out. No differences in numbers of induced nodulelike structures but significant enhancement in numbers of regenerated shoots in the newly obtained plants was observed. Furthermore, a faster differentiation of shoots (within 18–35 d) induced on root explant of the regenerants compared to that of the initial seedlings was observed (Table 3).

The capacity of intact SGg seedling roots to induce nodulelike structures on hormone-free media was compared with that on regeneration media (with 2ip and TIBA). The presence of growth regulators in the medium inhibited the formation of the nodulelike structures, as well as subsequent root growth. In regard to the morphogenic behavior of the other two alfalfa lines, both SGw and Rangelander-A70 cultured on growth-regulator-supplemented media demonstrated lack of any symptoms for nodulelike structure formation.

DISCUSSION

The alfalfa line SGg was selected from the commercially available var. Burgaltay. Two specific features of this line are: (a) capacity to develop structures on their roots that closely resemble normal nodules and (b) ability for regeneration from these nodulelike structures on growth-regulator-free media. The direct organogenesis from root nodulelike structures in alfalfa is described for the first time in this study.

Clearly, formation of nodules on the roots of legumes is a complex process, involving both the host's and the bacterial genome. However, development of root-derived structures from uninoculated alfalfa plants can take place. Several reports have previously found that under aseptic conditions, certain alfalfa plants are able to develop the so called "empty nodules" lacking infection threads and bacteria (13,26). Expression of the early nodulin gene (Enod 2) was found in cells of these nodules (26).

In the present study, we reported that spontaneous induction of

TABLE 1

MORPHOGENIC RESPONSE OF ROOT NODULELIKE STRUCTURES ON B5 MEDIA UPPLEMENTED WITH DIFFERENT GROWTH REGULATORS[a]

Media No.	Growth regulators				Response		
	Kinetin	TIBA (mg/l)	2iP	BAP	Morphology	Induction (%)[b]	Regeneration (%)[c]
I	–	0.1	1.0	–	S	20.3 ± 0.88	–
II	–	0.5	1.0	–	S	55.0 ± 1.52	5.3 ± 0.43
III	–	1.0	1.0	–	R	–	–
IY	–	1.0	0.1	–	S	10.6 ± 0.38	–
Y	–	1.0	0.5	–	S	95.8 ± 0.78	5.9 ± 0.17
YI	–	1.0	2.0	–	S	6.70 ± 0.36	–
YII	–	0.5	0.5	–	S	97.2 ± 1.16	2.2 ± 0.44
YIII	–	0.1	–	0.1	C	–	–
IX	–	0.1	–	0.5	C	–	–
X	–	0.1	–	1.0	C	–	–
XI	1.0	0.1	–	–	C	–	–
XII	2.0	0.1	–	–	C	–	–
Control	–	–	–	–	–[d]		

[a] Data scored from 50–60 root nodulelike structures per treatment after 4 wk of culture.
[b] Mean ± SE; percentages of root nodulelike structures showing shoot induction.
[c] Mean ± SE; percentages of shoots developing.
[d] Inhibition of root structures development. S = shoot induction, C = callusing, R = rooting.

nodulelike structures in the absence of *Rhizobium* occurs in selected clones of alfalfa. We have documented the morphogenic capacity of root nodulelike structures and their use as an alternate approach for alfalfa regeneration.

The influence of the genetic background on differentiation under in vitro conditions has been reported earlier (6,19). In this paper, significant differences in morphogenic behavior among three alfalfa lines (SGg, SGw, and A70) were established. While SGg displayed a natural tendency for direct organogenesis, both SGw and A70 seedling roots were recalcitrant to direct shoot formation. The fact that the "nodulation" pattern of the naturally occurring SGg line is reproducible during in vitro cultivation suggests that genetic determinants may be involved in the observed phenomenon.

The nodulelike structures were able to undergo the process of shoot organogenesis on media lacking growth regulators. This is unlike the situation in the alfalfa tissue where shoot induction is promoted by exogenously added growth regulators. One plausible explanation is that spontaneous organogenesis is due to the presence of the correct endogenous growth regulator levels in the roots of the SGg line. This assumption was supported by growth regulator analysis.

A dramatic increase of the endogenous cytokinins responding to a sharp decrease of the IAA levels in roots of SGg, maintained on growth-regulator-free medium, was detected at the time of visible shoot formation. The shift in growth regulator status suggests that increased endogenous cytokinin-to-auxin ratios can induce cells to undergo morphogenesis. This conclusion is in agreement with the results of tobacco and cumber plants transformed with the isopentenyl transferase (*ipt*) gene, where the elevated cytokinin-to-auxin ratios were associated with a shooty phenotype (1,24). Since, in general, a corresponding increase in IAA-levels was not observed, we speculate that cytokinin-producing cells may provide a signal for inducing auxin-independent growth. In favor of this hypothesis. Binns et al. (5) have shown that tobacco cells carrying the Ti *ipt* gene also exhibit auxin-autonomous growth. On the other hand, endogenous growth regulators have been shown to play an important but as yet undefined role in nodule induction. Hirsch et al. (13) have shown that outgrowths could be elicited on alfalfa roots by

TABLE 2

EFFECT OF TWO-STEP TREATMENTS ON SHOOT REGENERATION FROM ROOT NODULELIKE STRUCTURES

Combination of Media (1p − 2p)[a]	No. Shoots per Nodulelike Structure $\bar{x}$ ± SE	Regeneration (%)[b]	No. Regener. per Shoot $\bar{x}$ ± SE	Regeneration Period (days)
A (II-Y)	1.26 ± 0.23	26.3	1.30 ± 0.33	60
B (II-YII)	1.73 ± 0.30	22.2	0.33 ± 0.05	57
C (Y-Y)	4.73 ± 0.29	65.2	4.06 ± 0.57	49
D (Y-YII)	9.96 ± 0.72	66.7	6.33 ± 0.33	40
E (YII-Y)	1.60 ± 0.20	33.3	0.73 ± 0.37	58
K (YII-YII)	5.80 ± 0.30	71.4	4.66 ± 0.66	33
D[c] (Y-YII)	9.23 ± 0.43	100	11.3 ± 0.88	27
K[c] (YII-YII)	4.16 ± 0.44	76.5	4.00 ± 0.57	28
C[c] (Y-Y)	3.00 ± 0.57	76.9	4.33 ± 0.33	35

[a] Two-step media treatments including: 1st passage for induction of shoots from nodulelike structures and 2nd for shoot regeneration.
[b] Average percentages of shoots developing.
[c] Added ABA (0.5 mg/liter) to the second passage.

TABLE 3

EFFECT OF THE EXPLANT ORIGIN ON MORPHOGENIC RESPONSE FROM ROOT NODULELIKE STRUCTURES[a]

Root Explants From:	No. Shoot per Explant $\bar{x}$ ± SE	No. Regenerants per Explant $\bar{x}$ ± SE	Regeneration Period (days)
Seedlings	5.8 ± 0.58	3.0 ± 1.09	(45–65)
Regenerants	10.8 ± 2.88	11.6 ± 1.20	(18–35)

[a] Culture medium: B_5 containing 2iP (0.5 mg/l) and TIBA (1.0–0.5 mg/liter).

auxin transport inhibitors. The latter are reported to mimic factors normally triggering nodule development (25). Surprisingly, a cloned cytokinin (zeatin) biosynthesis gene has been found to be able to partially complement a nodABC-mutant of *R. meliloti*, resulting in "empty" alfalfa nodules in which Enod2 is expressed (17). Moreover, the Enod2 gene of the stem-nodulated legume *Sesbania rostrata* has been shown to be induced specifically and rapidly (within 2 h) by cytokinins (9). Thus, it was assumed that plant nodulin gene (Enod2) can be specifically regulated by growth regulators in the absence of other environmental control signals. We could also speculate that high cytokinin-to-auxin ratios in SGg roots may be serving as a signal for cell division and differentiation towards induction of nodulelike structures. Moreover, the developmental pathway responsible for the generation of bacteria-free nodules has been found to be the same as that for nitrogen-fixing nodules (27).

Our idea to develop a rather novel approach for alfalfa regeneration using a high morphogenic capacity of root structures was successfully achieved. Media containing the growth regulator combination of 2iP and TIBA were found to be most efficient for shoot regeneration. One possible explanation for the beneficial influence of these growth regulators on shoot formation could be that the auxin transport inhibitory effect of TIBA leads to the lowered endogenous IAA-levels in tissue. The presence of ABA in the growth media seems also to be a crucial factor favoring further stimulation of shoot development. Inclusion of ABA in this study was consistent with the earlier reports, where exogenously applied ABA has been shown to stimulate regulation of embryo development in caraway, *Brassica napus*, and alfalfa (2,8,10), and shoot morphogenesis in potato (23).

Another important aspect of this study was the possibility of obtaining newly formed regenerants with significantly enhanced morphogenic capacity, as compared to that of initial seedlings. This result has confirmed the importance of "recurrent" selection towards improving the morphogenic ability (18,20).

In conclusion, in this study we have investigated the possible relationship between "nodulation" capacity and morphogenic competence of alfalfa root explants and endogenous levels of growth regulators. The rapidity and simplicity of the direct regeneration procedure of alfalfa could be an advantage for *Agrobacterium*-mediated gene transfer. The nodulelike structure formation pattern of an isolated clone could provide an additional experimental system that may be of particular importance in studies on the interaction between leguminous plants and *Rhizobium*. Finally, the elucidation of alfalfa nodulation specificity will increase our knowledge on the molecular mechanism of this process and possibilities for establishing basic regulatory parameter.

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