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ORIGINAL ARTICLES

Effects of Cytokinins on *In Vitro* Seed Germination and Early Seedling Morphogenesis in *Lotus corniculatus* L.

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ABSTRACT

We determined the effects of zeatin (ZEA), isopentenyl adenine (2iP), kinetin (KIN), benzyladenine (BA), and thidiazuron (TDZ) on seed germination, elongation of seedling shoots and roots, frequency of regeneration, and the number of regenerants per seedling in *Lotus corniculatus* L. Sterilized seeds were cultured *in vitro* on Murashige and Skoog (1962) medium containing 3% sucrose, 0.7% agar, and various cytokinins (0, 0.08, 0.22, 0.35, 0.80, 2.20, and 3.50 μM). After 30 days, seedlings were transferred to cytokinin-free medium for another 60 days. All cytokinins stimulated the rate and percentage of seed germination at least twofold in optimum concentrations; TDZ and ZEA were the most active, followed closely by BA, whereas KIN and 2iP stimulated germination in higher concentrations only. Elongation of shoots and roots was strongly inhibited at the lowest TDZ and BA concentrations, whereas ZEA, KIN, and 2iP exerted moderate, dose-dependent inhibition. The frequency of regenerant-producing seeds was highest on ZEA and BA, whereas the greatest number of regenerants per seedling was found on TDZ. It is concluded that the culture of seeds on cytokinin-containing media, followed by transfer to cytokinin-free medium, is a suitable procedure for rapid production of a large number of uniform regenerants. The presumed role of particular cytokinins is discussed.

Key words: Cytokinins; *In vitro* cultures; Seed germination; Shoot elongation; Root elongation; Shoot regeneration.

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INTRODUCTION

Lotus corniculatus L. (bird's foot trefoil) is a perennial leafy forage legume that in many areas competes in

cultivation with white clover and alfalfa due to its high nutritional value and tolerance to adverse environmental conditions. Numerous authors (for example, Rybczyński and Badzian 1987; Arcioni and others 1988; Webb and Watson 1991) have already noted that the species is readily amenable to tissue culture techniques. This was also confirmed for the Bokor and Zora domestic cultivars at the Center for Agricultural and Technological Research in Zaječar (Nikolić and others 1997). The Bokor cultivar was genetically transformed using as vectors *Agrobacterium rhizogenes* (Nikolić and others 2003/4) and *A. tumefaciens*, which carried the *bar* gene (Nikolić, unpublished). Cytokinins seem to be the only exogenous hormones required for regeneration in *L. corniculatus* (Badzian and Rybczyński 1994). In an attempt to simplify methods of obtaining regenerants *in vitro* and to shorten the period required for establishment of vegetative clones, we found that this could be achieved simply by putting intact seeds on cytokinin-containing media. Several authors have noted certain advantages of using seeds—that is, intact seedlings—as primary explants (Malik and Saxena 1992a, 1992b, 1992c; Victor and others 1999). If this method is followed, quiescent embryonic cells are directly exposed to a hormonal stimulus, dedifferentiation of parenchymatous cells and callus induction are bypassed, the seedlings' integrity is retained, and wounding is avoided.

While we were performing the experiments on regeneration, we noted that cytokinins had a positive effect on seed germination, and these data are also included in the present article. Because it is commonly known that various cytokinins differ in their activity, we used two isoprenoid (2iP and ZEA) and two aromatic (KIN and BA) cytokinins and thidiazuron (TDZ, *N*-phenyl-*N*′-(1,2,3,thidiazol-5-yl urea) to investigate their effects on seed germination, shoot and root elongation, early regeneration events, and subsequent regenerant multiplication.

Materials and Methods

All experiments were initiated in 2003 with field-grown seeds of *L. corniculatus*, cv. Bokor, a cultivar registered in Serbia upon its selection at the Center for Agricultural and Technological Research, Zaječar (Mijatović and others 1986). Seeds for the present experiments were produced by the polycross method between three mother plants maintained for seed production in the Zaječar Center's experimental field. Mature pods were harvested manually to avoid seed loss due to early pod shattering. Seeds were dried at room temperature and stored in tightly closed paper bags in the laboratory. For experiments, seeds were carefully selected for uniformity by discarding small seeds, pale seeds, and those with shrunken seed coats. Three seed batches were used for germination experiments: seeds collected in the years 1998, 2000, and 2002, with average germination capacities of 7.5%, 22.0%, and 34.0%, respectively. Because preliminary trials indicated that seedling length and regeneration capacity were not affected by seed age—once the seeds had germinated—the experiments concerning these properties were performed with seeds collected in the years 2000 and 2002.

Seeds selected for experiments were washed with liquid detergent, surface sterilized with 20% NaOCl (v/v) for 60 min, and rinsed five times with sterile distilled water. They were then soaked in sterile water at room temperature for 30 min prior to culturing. Single seeds were put into 100 × 10 mm test tubes containing 5 ml of plain 0.7% agar (v/w) (Torlak, Belgrade, Serbia) or on MS (Murashige and Skoog 1962) mineral medium containing 3% sucrose (v/w) in addition. Both media were supplemented with the following cytokinins: KIN (kinetin), BA (benzylademine), 2iP (isopentenyl adenine), ZEA (zeatin), and TDZ, in concentrations of 0, 0.08, 0.22, 0.35, 0.8, 2.2, and 3.5 μM. Thidiazuron and ZEA were sterilized using 0.2 μm Millipore filters and added after autoclaving. The pH of the media was adjusted to 5.8 prior to autoclaving. Germination was determined as protrusion of the radicle, after 6, 12, and 18 days. Germinated seeds were retained in the same test tubes for the first 30 days in culture. The effect of cytokinins on seedling elongation was measured during that period (after 20 days of culture), using a flexible ruler from outside the test tubes. Between 25 and 30 days, swellings and protuberances appeared on roots and shoots, which was taken as a sign of regeneration processes and served to determine the regeneration frequency. On the 30th day after sowing, seedlings with regenerant initials were transferred to Erlenmayer flasks containing the so-called MS regeneration medium lacking any growth regulators. Consequently, the experiments lasted 90 days: the seeds were maintained on cytokinin-containing media for 30 days, and then transferred to cytokinin-free media for two subsequent subcultures of 30 days each. The cultures were maintained at a temperature of 25° ± 2°C under conditions of a 16-h photoperiod (irradiance of 47 μmol m^{-2} s^{-1}).

The experiments were repeated three times, each repetition comprising 25 seeds (tubes). All data were subjected to analysis of variance (ANOVA).

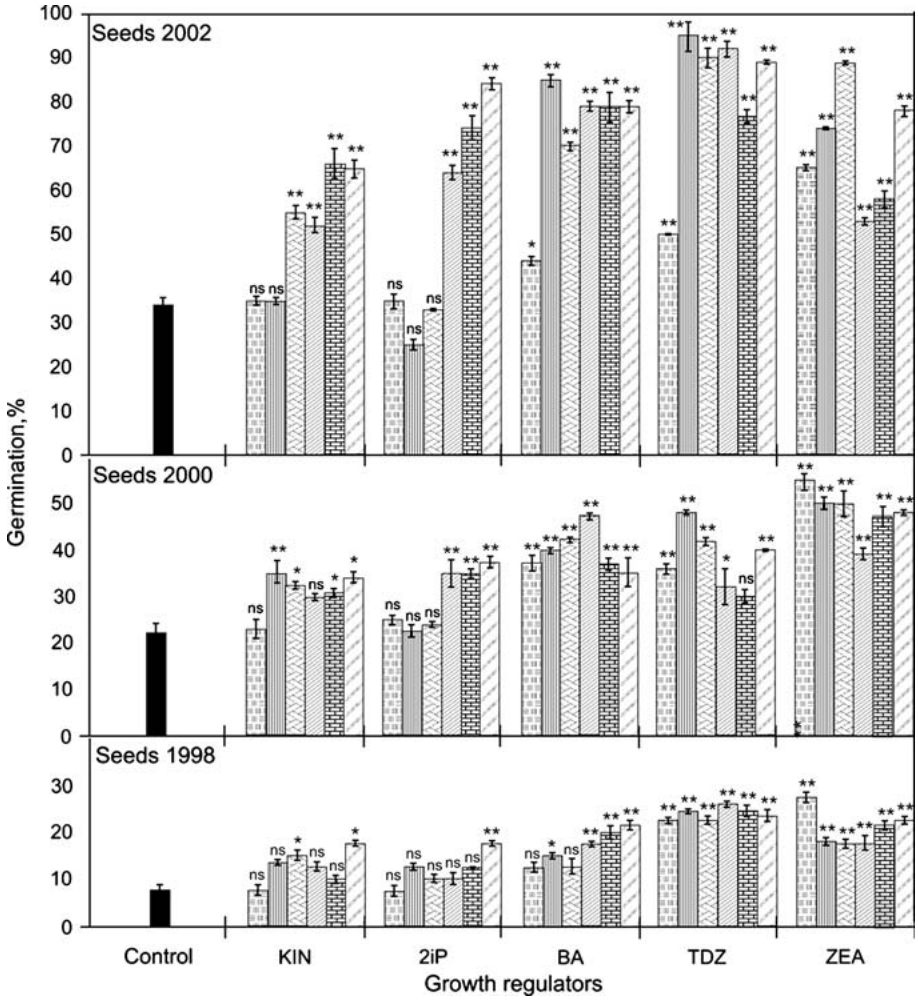

Figure 1. Percent germination of *L. corniculatus*, seeds collected in years 1998, 2000, and 2002. Germinated seeds counted after 18 days of culture on media without or with five cytokinins at increasing concentrations: 0 (■), 0.08 (▩), 0.22 (▥), 0.35 (▨) 0.80 (▧), 2.20 (▦), and 3.50 (□) μM. Values are means ± SE of three replicates, containing 25 seeds each. Asterisks indicate significant differences from control at $p \leq 0.05$ (*) and $p \leq 0.01$ (**).

Germination and regeneration data were transformed (arcsin) before statistical analysis. The LSD test was used to estimate significant differences among mean values of the treatments.

Results

Effect of Cytokinin Treatment on Seed Germination

Cytokinins were applied to stimulate subsequent regeneration in seedlings. However, an unexpected effect was noted with respect to seed germination. All cytokinins used were able to accelerate the germination rate and increase the final germination percentage. In controls, out of the total number of germinated seeds, about 12% germinated in the first 6 days and the rest germinated evenly over the next 12 days. However, in the presence of optimum cytokinin concentrations, between 50% and 80% seeds germinated during the first 6 days and reached almost the maximum by the 12th day. After 18 days, the percentage of seed germination in all three seed batches was stimulated. Zeatin and TDZ displayed the highest activity, followed by BA, whereas KIN and 2iP stimulated germination only in higher concentrations (Figure 1). The response to cytokinins was commensurate with seed viability in the three seed batches, which were harvested in subsequent years. In experiments in which cytokinins were added to the plain agar medium, essentially the same results were obtained. These results are not presented here, but they have been taken as an indication that mineral salts and sucrose did not play an essential role in the response of *Lotus* seeds to cytokinins.

Effect of Cytokinins on Seedling Elongation

Seeds cultured in test tubes on control medium lacking cytokinins displayed normal development, and in about 20 days some seedlings reached a length of up to 6.5 cm. On media containing different cytokinins, elongation of seedlings was

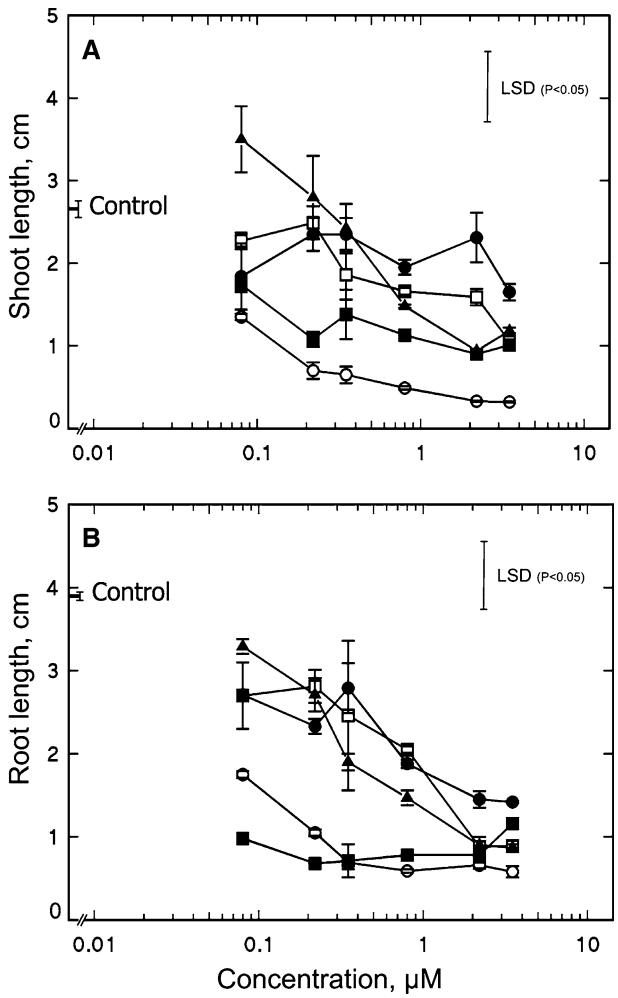

Figure 2. Effects of KIN (□), 2iP (●), ZEA (▲), BA (■), and TDZ (○) on *L. corniculatus* seedling shoot **(A)** and root **(B)** length (± SE) after 20 days of culture. Horizontal lines on the ordinate indicate lengths of controls (± SE). Vertical bars indicate LSD values.

suppressed, at the expense of both roots and shoots. With respect to inhibition of axial organs, the cytokinins used fall into two groups: TDZ and BA had the strongest effect and even in the lowest concentrations (0.08 and 0.22 μM) retarded elongation to about one third of control lengths. In contrast, ZEA, KIN, and 2iP slightly inhibited elongation at lower concentrations (0.08, 0.22, and 0.35 μM), whereas at higher concentrations (0.8, 2.2, and 3.5 μM) the inhibition was stronger, but still below that exerted by BA and TDZ (Figure 2). Not all parts of shoots were equally affected. The hypocotyls in control plants did not elongate much, and they were not particularly inhibited by cytokinins. The first internodes (together with the second ones if they occurred), comprised the bulk of shoot length and were the targets of cytokinin inhibitory action. For example, in a representative experiment the hypocotyl in control seedlings was about 9 mm long, and the total length of the first and second internodes was 28 mm. At 0.8 μM of TDZ, the hypocotyl was 7.5 mm, which equaled the entire plant's length, because the internodes were not visible at all. At 0.8 μM of BA, the hypocotyl and the first and second internodes were 7.5 and 5 mm long, respectively. At 0.8 μM of 2iP, KIN, and ZEA, the lengths of hypocotyls and internodes were between those in the presence of BA and the control values. The point of Figure 2 is to show the strong inhibitory effect of BA and TDZ, in contrast to KIN, 2iP, and ZEA. Due to high variability, the LSDs between the three latter cytokinin effects were not significant for roots, and at the significance limits for shoots.

Effect of Cytokinins on Organogenesis and Plantlet Regeneration

The first signs of organogenesis in seedlings appeared about 20 days after seed exposure to cytokinins. Although the control seedlings did not show any morphological abnormalities, the shortened shoots and roots on cytokinin-containing media displayed swellings and many protuberances, which gave rise to green outgrowths of various shapes (Figure 3A). Some of them developed as bunches at the cotyledonary node, sometimes surrounded by a callus tissue; they might have originated at the nodes by multiplication of axillary buds (Figure 3B). Numerous green structures were observed along the roots (Figure 3C) and hypocotyls, and on the cotyledon surface. They undoubtedly represented a direct regeneration process. However, without histological examination, it was not possible to distinguish whether they were buds or somatic embryos. They will therefore be called simply ''regenerants'' hereinafter.

The percentage of potentially regenerating seedlings, as scored after 30 days on cytokinin-containing media, was rather variable in different groups. Generally, at the three lower concentrations of all cytokinins (0.08, 0.22, and 0.35 μM), regeneration was scarce, except on BA. Benzyladenine induced regenerants in 11.7% of seedlings at 0.08 μM, 13.3% at 0.22 μM, and 25.6% at 0.35 μM. At higher concentrations (0.8, 2.2, and 3.5 μM), ZEA was the most effective cytokinin, inducing regeneration in 59.5%, 48.3%, and 46.4% of the seedlings, respectively. The regeneration potential of BA was 45.7% at 0.8 μM, 40.5% at 2.2. μM, and 32.1%

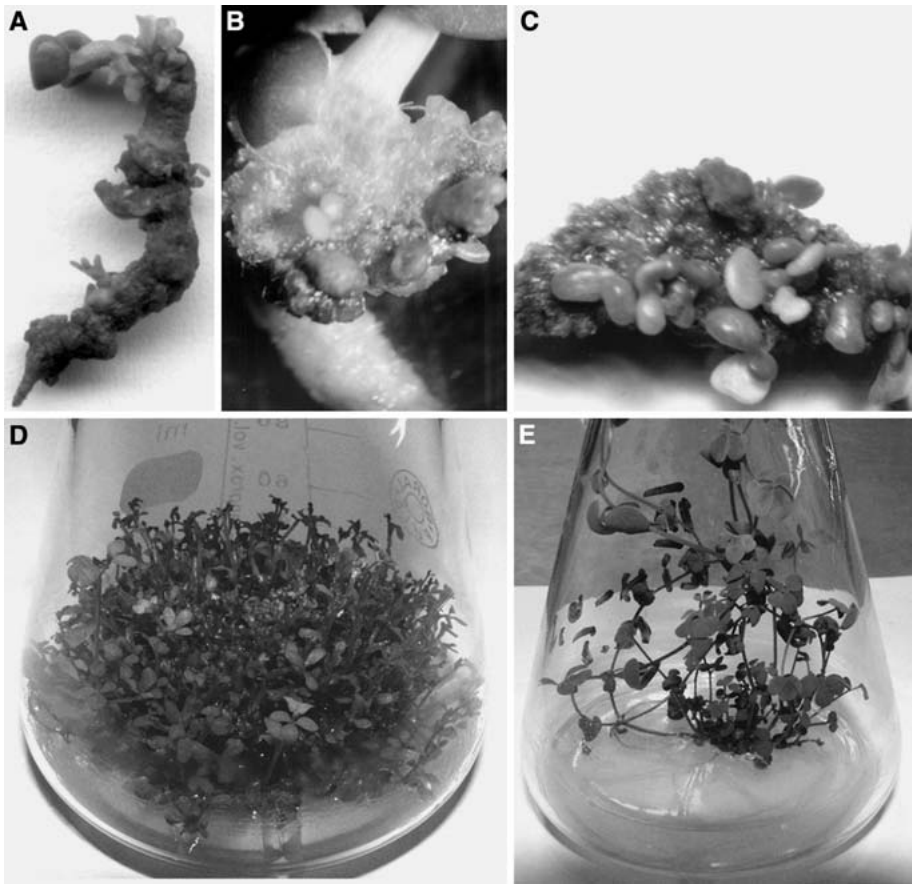

Figure 3. Regeneration from germinating seeds of *L. corniculatus*. Seeds were cultured for 30 days on cytokinin-containing media and then transferred to cytokinin-free media for two subcultures of 30 days each. **(A)** Seedling with regenerants' initials, grown for 30 days on 2.2 μM of BA. **(B)** Cotyledonary node region with callus tissue and regenerants, arising probably from axillary buds; grown for 30 days on 0.8 μM of BA. **(C)** Root segment with regenerants; grown for 30 days on 2.2 μM of BA. **(D)** Regenerants from a single seed, grown for 30 days on 0.8 μM of TDZ. **(E)** Regenerants from a single seed, grown for 30 days on 0.8 μM KIN. Photographs A, B, and C taken on the 32nd day from the beginning of experiments (2 days after transfer to cytokinin-free medium); photographs D and E taken on the 80th day from the beginning of experiments (50 days after transfer to cytokinin-free medium).

at 3.5 μM. Thidiazuron induced regeneration in 28.6% of seedlings (at 0.8 μM), 35.0% (at 2.2 μM), and 21.5% (at 3.5 μM). The optimum concentration was 0.8 μM for ZEA and BA, and 2.2 μM for TDZ and 2iP. The response to 2iP and KIN remained low, and at optimum concentrations (2.2 and 3.5 μM, respectively) it did not surpass 20.5% (2iP) and 15.9% (KIN) of regenerable seedlings.

After 60 days on cytokinin-free media, the average number of regenerants per seedling showed that cytokinins again fall into two groups: KIN and 2iP belong to the group with low activity, whereas TDZ, BA, and ZEA constitute the active group (Figure 4). Individual variation between seedlings was high, because in certain plantlets initial swellings and protuberances did not continue development and dried up without producing shoots. The absolute record-holder was a single seed that at 0.8 μM TDZ produced 178 regenerated buds (Figure 3D), diverging so much from the rest of the seeds, that it was excluded from statistical evaluation. By way of comparison, only 11 shoots developed on 0.8 μM KIN (Figure 3E). The great differences in regeneration frequency between plants grown at close cytokinin concentrations (Figure 4) are probably genotype dependent. Similar genotypic variability has been noted before in *Lotus* by Badzian and Rybczyński (1994), as well as in a previous work of ours (Nikolić and others 1997).

Discussion

Cytokinins are plant hormones implicated in the regulation of various processes of growth and development. Among their multiple activities, the

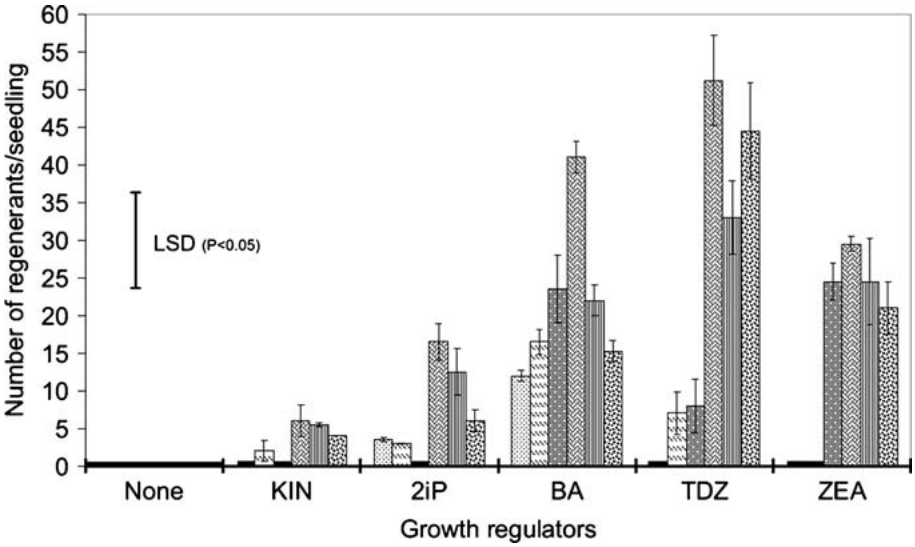

Figure 4. Mean number (±SE) of regenerants per seedling of *L. corniculatus*, after 30 days on cytokinin-containing media followed by 60 days on cytokinin-free media. Cytokinin concentrations 0.08 (▨), 0.22 (▨), 0.35 (▨), 0.80 (▨), 2.20 (▨), and 3.50 (▨) μM. Vertical bar represents LSD value.

effects of cytokinins on seed germination stand apart from their role in shoot morphogenesis. Modern analytical methods have shown their very active metabolism in all phases of germination, from imbibition to radicle emergence and the start of seedling establishment (Stirk and others 2005; Chiwocha and others 2005). On the other hand, exogenous cytokinins have various effects on seed germination in different species. Their promotive effects are mostly related to the alleviation of stress factors. Kinetin was one of the growth regulators that alleviated both innate and salinity-induced seed dormancy in many halophytes (Khan and Ungar 1997; Khan and others 2004); however, kinetin did not affect germination in some halophytic grasses (Gulzar and Khan 2002). Heavy metal (Pb and Zn) stress induced increases in endogenous ZEA and zeatin riboside content in chick pea seeds (Atici and others 2005), whereas kinetin alleviated the harmful effects of Cu and Zn in *Lupinus termis* (Gadallah and El-Enany 1999). Kinetin was also reported to prolong the viability of recalcitrant seeds, which was interpreted as possible protection of cell membranes against oxidative stress (Chaitanya and Naithani 1998). Although the occurrence of cell membrane lesions in dehydrated *L. corniculatus* seeds has been reported (McKersie and Stinson 1980), the possible protective effect of cytokinins in seeds of that species remains to be investigated. In seeds of several large-seeded grain legumes, cytokinins did not affect seed germination (Malik and Saxena 1992a).

In the range of concentrations used in our experiments, cytokinins can be divided into two groups, according to their physiological activities: 2iP and KIN exhibited rather weak effects, whereas ZEA, BA, and TDZ constituted a very active group. In promoting seed germination, ZEA, TDZ, and BA occupy the first place, with inconsistent differences between them (Figure 1). In inducing the onset of regeneration in individual seedlings, ZEA was the most effective, followed by BA, with TDZ in third place. In shoot multiplication, on the other hand, TDZ had the strongest effect, followed by BA and ZEA. A notable exception in the position of ZEA was found in its effect on shoot and root elongation (Figure 2), where it joined 2iP and KIN in the group of less active cytokinins.

The activity of various cytokinins in morphogenetic phenomena reflects their concentration in the tissue, which depends on the capacity for their synthesis and the activity of various inactivation mechanisms. The most important role in cytokinin inactivation is assigned to the enzyme cytokinin oxidase/dehydrogenase (CKX) (Galuszka and others 2001; Bilyeu and others 2003), formerly described as cytokinin oxidase (Hare and Van Staden 1994; Galuszka and others 2000). The enzyme catalyzes cleavage of the unsaturated N^6-side chain of most isoprenoid cytokinins. A good correlation exists between the overexpression of CKX in transgenic plants, content of endogenous cytokinins, and several cytokinin-regulated processes (Werner and others 2001). Thidiazuron, not being a cytokinin *per se*, is supposed to inhibit the action of CKX, thereby protecting cytokinins from destruction. This satisfactorily explains the high activity of TDZ in all cytokinin-regulated processes. All exogenous cytokinins transiently increased activity of the enzyme, but not all became inactivated to the same extent. The preferred substrate of CKX is 2iP and its riboside (Motyka and others 1996), both of which are

rapidly converted to inactive products; in contrast, the enzyme seems to have lower affinity for ZEA in most tissues. This may perhaps explain the low activity of 2iP and the high activity of ZEA in many developmental processes. Alternatively, the higher biological effects of ZEA may be due to the simultaneous presence of zeatin reductase (ZRED), which has been found in *Phaseolus* embryos (Martin and others 1989) and was recently confirmed in pea leaves (Gaudinová and others 2005). This enzyme converts ZEA to dihydrozeatin (DHZ), which is not attacked by CKX. As a consequence, the biological activity of ZEA may be conserved in the form of DHZ for a longer time. Because nothing is known about the presence of either enzyme in *Lotus* seeds, this hypothesis needs to be investigated.

The fact that in our experiments ZEA acted as a very active cytokinin in seed germination and regeneration, but as a weak cytokinin in elongation of roots and shoots, is hard to explain. It has frequently been reported that cytokinin action is different in different types of cells, for instance in root and shoot apical meristems (Werner and others 2001). Differential uptake of exogenous cytokinins can be considered one of the causes of this phenomenon (Auer and others 1999). Seed germination is most likely initiated in quiescent embryonic cells, and these cells perhaps become determined at the same time for subsequent regeneration. However, the stimulus for root and shoot elongation is probably perceived in the apical meristematic cells. They may differ from embryonic cells in endogenous cytokinin content and in uptake of exogenous hormones. Spichal and others (2004) have recently demonstrated that similar cytokinin receptors differ in recognizing cytokinins as ligands, as well as in transmitting their signals to biological processes.

The activities of aromatic cytokinins in *Lotus* organogenesis cannot be understood in terms of CKX activity. They are resistant to degradation by CKX (Galuszka and others 2000) and are degraded independently of each other (Strnad 1997). In lower plants, other enzymatic systems occur that specifically degrade kinetin (Gerhäuser and Bopp 1990). Strong evidence exists for the occurrence of such enzymes in higher plants as well (Strnad 1997).

In conclusion, the results presented here show that it is possible to improve the production of *L. corniculatus* plants *in vitro*. Moreover, *L. corniculatus* seeds and seedlings seem to be rewarding objects for further fundamental studies concerning the relationship between cytokinins and the systems that regulate their activities. Dry seeds containing quiescent embryonic cells represent novel objects on which to study the development of these functions.

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