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EFFECT OF CYTOKININS ON SHOOT REGENERATION FROM ROOT EXPLANTS OF BIRDSFOOT TREFOIL *(LOTUS CORNICULATUS* L.)

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The effect of three different cytokinins KIN, 2iP and BA at concentration 0.2 and 0.5 mg l^{-1} on shoot regeneration from root explants of domestic birdsfoot trefoil *(Lotus corniculatus* L.) cv. Bokor has investigated. The significant differences in shoot regeneration and development were found among the cytokinins tested. The high frequency of shoot production was achived on the medium containing BA at 0.2 mg l^{-1}. About 1000 plants were obtained from one root explant on the same medium after 120 days of culture. The plantlets were regenerated directly from root segments or via callus, depending upon the type of cytokinin used. KIN and 2iP have provoked spontaneously root formation.

Key words: birdsfoot trefoil, cytokinins, root explant, shoot regeneration

Ključne reči: žuti zvezdan, citokinini, eksplantati korena, regeneracija pupoljaka

INTRODUCTION

Lotus corniculatus L. (birdsfoot trefoil) is a tetraploid (2n = 24) (W e r n s m a n et al., 1964) perennial forage legume. It has softer stems, lower cellulose content and more carbohydrates. This legume good grows on poor, acid and salt soils. Because of its good nutritive composition and the other biological characteristics, today, birds foot trefoil is wide-spread forage crop in the world.

This culture technology has been applied successfully to *L. corniculatus.* Fertile plants were regenerated from calli of different explants: internode segments (O r s h i n s k y et al., 1983; O r s h i n s k y & T o m e s, 1985; S w a n s o n & T o m e s, 1980), anthers (N i i z e k i & G r a n t, 1971), leaves (M a r i o t i et al., 1984) and nodes (T o m e s 1979; P u p i l l i et al., 1992). The plants also were obtained directly from roots (W e b b et al., 1986; R y b c z y n s k i & B a d z i a n, 1987) and leaf explants (W e b b et al., 1986).

In this study we investigate effect of three different cytokinins on shoot regeneration and development from root explants of birdsfoot trefoil cv. Bokor. This cultivar was produced by a polycross method in the Centre for Agricultural and Technological Research in Zaječar and it is well adapted to climate conditions of the Timočka Krajina region. The cultivar Bokor has good chemical composition (19.5% albumens, 19.4% cellulose, 3.2% oil and 103.3 mg/kg β-carotene), stem height (50-55 cm) and it regenerates very fast after mowing.

The aim of this investigation is to establish efficient regeneration system to be applied *in vitro* selection and genetic transformations methods in plant improvement programs.

MATERIALS AND METHODS

Plant material

The seeds of birdsfoot trefoil *(L. corniculatus* L.) cv. Bokor, were surface sterilized in 20% sodium hypochlorite solution (20 min) and washed three times with sterile destilated water. They were aseptically germinated on a 0.45% agar (SIGMA) MS (M u r a s h i g e & S k o o g, 1962) medium, at 25 ± 2°C under $16/8^h$ photoperiod. Non-meristematic root segments 5 mm long were excised from 6 days old seedlings.

Culture media and conditions

A basal medium of MS supplemented with 3% sucrose, 0.45% agar (SIGMA) and (in mg l^{-1} each): glycine 2, nicotinic acid 0.5, B_1 1 and B_6 1 was used.

Different cytokinins, KIN, 2iP and BA at concentration 0.2 and 0.5 mg per liter each were added in the media and pH was adjusted to 5.8 prior to autoclaving. Root explants were grown in 100 ml erlenmayer flasks, containing 40 ml of the media. The cultures were transfered every 20 days to the same, fresh medium. The regenerated plantlets (2-3 cm height) were rooted on hormon free medium.

All cultures were kept at 25 ± 2°C under white fluorescent light (47 μmol $m^{-2}s^{-1}$), in a 16^h day period.

RESULTS

The shoot regeneration from root explants of cv. Bokor was compared to media containing various cytokinins (KIN, 2iP and BA) at 0.2 and 0.5 mg per liter. The type of cytokinin affected shoot regeneration and differentiation. The plants were obtained directly or indirectly via callus.

The shoots were formed directly from root explants which cultured on medium containing KIN (0.2 and 0.5 mg l^{-1}) 15 days after culture initiation (Fig. 1). The number of produced shoots increased during the culture on the same media. No calli formation was observed on media consisted KIN. The average number of shoots per explant was lower and similar on both media (0.2 and 0.5 mg l^{-1} KIN) (7.1 and 6.2 shoots after 60 days of culture, Tab. 1 and Tab. 2). Obtained plants had very elongated internodes (Fig. 2). The average height of plants was 7.3 to 7.6 cm and did not depend upon KIN concentration. The most of plants formed well developed roots on these media.

Tab. 1. – Effects of three different cytokinins at concentration 0.2 mg l^{-1} on shoot regeneration from root explants of cv. Bokor

Cytokinin	No. explants	Callus formation (%)	Shoot regeneration (%)	Average No. of shoots per explant*	Average plants height (cm)
KIN	25	0	8 (32)	7.1	7.3
2iP	17	13 (76)	3 (23)	6.4	6.7
BA	22	20 (91)	17 (85)	62.0	3.6

*The data were recorded after two months of culture.

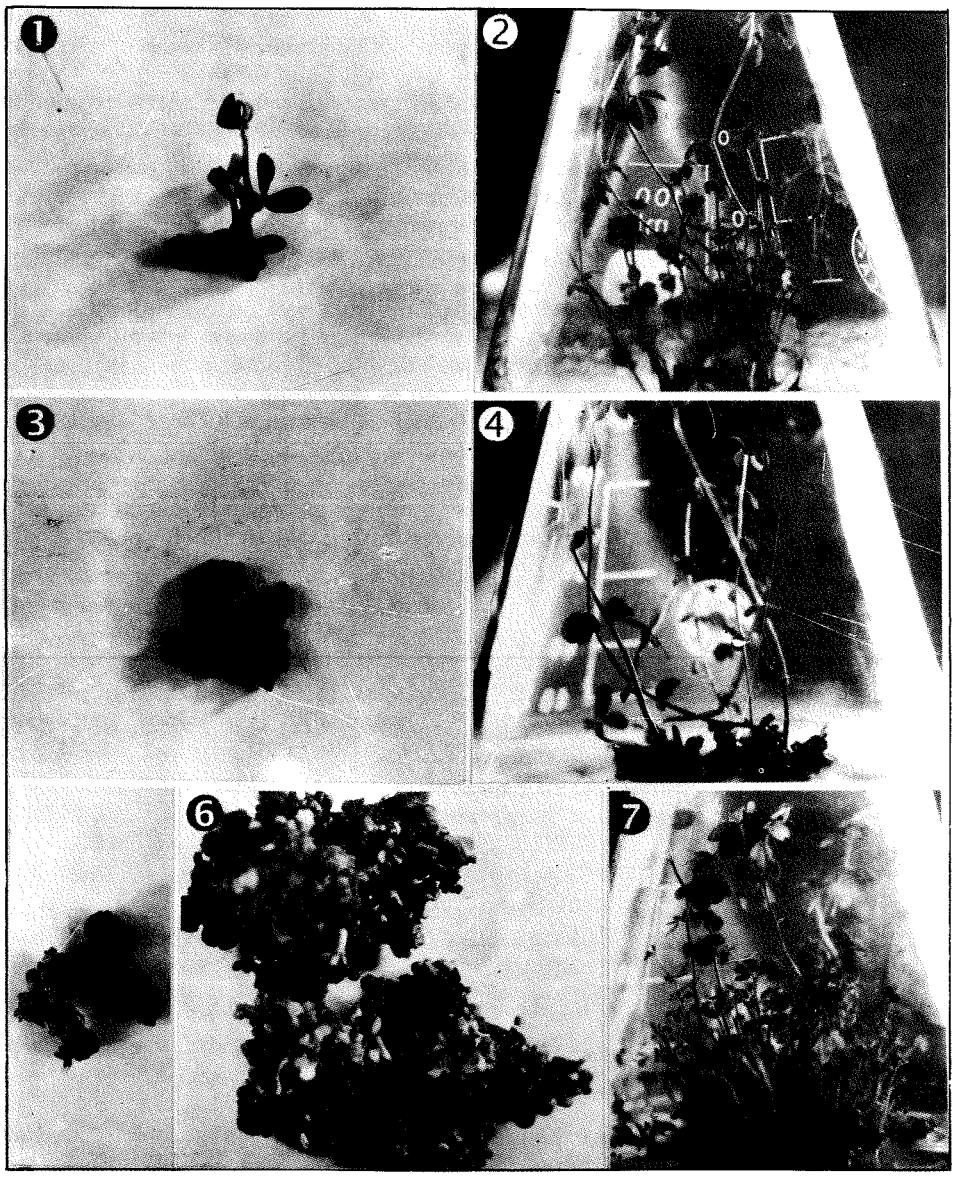
Tab. 2. – Effects of three different cytokinins at concentration 0.5 mg l^{-1} on shoot regeneration from root explants of cv. Bokor

Cytokinin	No. explants	Callus formation (%)	Shoot regeneration (%)	Average No. of shoots per explant*	Average plants height (cm)
KIN	30	0	14 (47)	6.2	7.6
2iP	26	8 (31)	5 (63)	5.5	7.6
BA	36	26 (72)	20 (77)	41.0	2.8

*The data were recorded after two months of culture.

In contrast with the root explants on KIN media, on media supplemented with 2iP and BA, they produced shoots indirectly, via callus stage. Calli initially were observed on both ends of root parts (Figs 3 and 5).

However, further regeneration process was different on 2iP and BA media. Calli, produced on 2iP media, had lower organogenic abilities and they have grown very slow in comparison to the calli on BA media. The average number of shoots from callus per explant was 6.4 on 0.2 mg l^{-1} or 5.5 on 0.5 mg l^{-1} 2iP. The regenerated plantlets had long internodes (6.7 to 7.6 cm stem height) (Tab. 1 and Tab. 2) (Fig. 4), and roots formation were observed to, as well as the plantlets on KIN media.



Figs. 1. – Birdsfoot trefoil shoot regenerated directly from root explant on KIN (0.2 mg l^{-1}) 15 days after culture initiation; 2. – Elongated plantlets 60 days old on KIN (0.2 mg l^{-1}); 3. – Organogenic callus formed from root segment on 2iP (0.2 mg l^{-1}) medium after 15 days of culture; 4. – Plantlets with long internodes on 2iP (0.2 mg l^{-1}); 5. – Calli formed on root segment on BA (0.2 mg l^{-1}); 6. – Organogenic callus 20 days old on medium with BA; 7. – Multiple plantlets on BA (0.2 mg l^{-1}), 60 days after culture initiation

BA was the most effective among the cytokinins tested. On media with BA well developed organogenic, green calli were produced. They formed buds after 20 days of culture (Fig. 6). The shoots number were increased rapidly with the time of culture. A lot of buds were regenerated from calli obtained on medium with 0.2 mg l^{-1} BA (62.0, Tab. 1). BA at this concentration was more effective on shoot regeneration frequency than BA 0.5 mg l^{-1} (41.0, Tab. 2), and on an average 250 regenerants were produced from one root explant after 90 days of culture. The regenerated plantlets had short internodes and lower height than the plantlets obtained on KIN and 2iP media (Fig. 7). Rooting was not found and elongated buds were rooted on MS medium lacking plant growth regulators. No abnormal plantlets were observed on all used media. Only cultures which growed on medium with 0.5 mg l^{-1} BA produced red coloured pigment in the medium.

DISCUSSION

Niizeki & Grant (1971) first obtained the plants of birdsfoot trefoil in *in vitro* conditions, using antheres as a explants. The cultivar Bokor was regenerated erlier in *in vitro* culture (Nikolić, 1995) from apical buds of the seedlings and the whole plants. The plants were obtained from calli on medium which was initially formulated for alfalfa (Saunders & Bingham, 1972).

In the present study, the parts of roots had very good organogenic ability. These explants regenerated shoots directly, or indirectly via callus, depend upon the type of cytokinin used. Only KIN has favourized direct organogenesis, in contrast with the 2iP and BA.

Rybczynski & Badzian (1987) first reported of *L. corniculatus* plant regeneration from non-meristematic root segments. The organogenesis was direct, without callus formation, on hormone free medium. So, no cytokinins needed for shoot regeneration from roots, but the number of buds per one explant was small.

By using low concentration of cytokinins in the media, we found higher shoot regeneration per explant and the shoot number was increased with the time of culture. In this case BA at concentration 0.2 mg per liter, was the most satisfactory on buds multiplication than KIN and 2iP. The other investigaters used also low BA concentration for shoots regeneration through calli from internodes (Orshinsky et al., 1983) and from the apical shoot or node (Tomes, 1979; Pupilli et al., 1992) reported that BA was most effective cytokinin than KIN, and the number of shoots produced per single node of three *Lotus* spp. increased during the culture on BA medium. Our results also confirmed theirs observation that the shoots never rooted on media containing BA.

All these results indicate that the percentage of cultures which produced shoots were not influenced by the presence of cytokinin in the culture medium. However, the addition of a cytokinin, such as a BA, is necessary to increase the number of shoots produced. Using BA (0.2 mg l^{-1}), it would be possible to produce about 1000 plants from a single root explant within 4 months of culture.

Therefore, *in vitro* propagation of *L. corniculatus* by root culture can be efficient regeneration plant system to apply the *in vitro* selection and genetic transformation methods (Webb, 1986; Armstead & Webb, 1987; Petit, 1987) in birdsfoot trefoil breeding programs. By using these methods, it is possible to obtain cell lines and plants with improved characteristics (resistant to disease, herbicides, salt tolerant etc.).

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Rezime

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EFEKAT CITOKININA NA REGENERACIJU PUPOLJAKA IZ EKSPLANTATA KORENA ŽUTOG ZVEZDANA (*LOTUS CORNICULATUS* L.)

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Žuti zvezdan (*Lotus corniculatus* L.) Bokor je domaća sorta selekcionisana u Centru za poljoprivredna i tehnološka istraživanja u Zaječaru. U ovom radu ispitivan je efekat tri vrste citokinina (KIN, 2iP i BA) primenjenih u koncentraciji 0.2 i 0.5 mg l^{-1} na regeneraciju i razvoj pupoljaka iz korenskih eksplantata. Cilj ovog rada je uspostavljanje efikasnog regenerativnog sistema u kulturi *in vitro* radi primene metoda *in vitro* selekcije i genetičke transformacije u programima dobijanja biljaka žutog

zvezdana sa poboljšanim karakteristikama. Zapažene su razlike u regeneraciji i razvoju pupoljaka iz odsečaka korenova u zavisnosti od primenjenog citokinina. Biljke su dobijene direktnom organogenezom ili indirektno, preko kalusa. Samo na podlozi sa KIN biljke su dobijene direktno iz eksplantata, dok su 2iP i BA inicirali obrazovanje kalusa. Biljke razvijene na podlogama sa KIN i 2iP imale su izdužene internodije i spontano su se oživljavale. Visoka frekvenca produkcije pupoljaka postignuta je na medijumu sa 0.2 mg l^{-1} BA i oko 1000 biljaka je regenerisano iz jednog eksplantata posle 120 dana gajenja u kulturi. Oživljavanje nije zapaženo. Rybczynski & Badzian (1987) su prvi dobili direktnu regeneraciju pupoljaka iz ne-meristematskih segmenata korenova žutog zvezdana što ukazuje da dodatak citokinina nije neophodan za ovaj proces. Dodavanje niske koncentracije citokinina u medijum omogućilo je rapidno povećanje broja dobijenih pupoljaka tokom gajenja *in vitro*. Koristeći BA u koncentraciji 0.2 mg l^{-1} moguće je dobiti oko 1000 biljaka žutog zvezdana po jednom eksplantatu posle 4 meseci gajenja u kulturi. Na osnovu dobijenih rezultata može se zaključiti da se veliki broj biljaka žutog zvezdana sorte Bokor može brzo regenerisati iz eksplantata korenova tako da se ova metoda može primeniti u daljem radu na oplemenjivanju i selekciji žutog zvezdana.