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ANALYSIS OF MORPHOLOGICAL TRAITS OF BIRD'S FOOT TREFOIL PLANTS *CV*. BOKOR TRANSFORMED WITH *AGROBACTERIUM RHIZOGENES*

Radomirka NIKOLIĆ and Nevena MITIĆ

Agricultural Research Institute "Serbia", Center for Agricultural and Technological Research, 19000 Zaječar, Serbia and Montenegro

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An efficient method for genetic transformation and shoot regeneration was achieved in bird's foot trefoil cv. Bokor using *A. rhizogens*. The transformed shoots were regenerated on hairy root segments in high frequency. After rooting and acclimation, transformed T_0 plants were grown in experimental field. Analysis of morphological traits and chemical content in ten unintentionally chosen T_0 bird's foot trefoil plants (genotypes no. 2 and no. 5) was performed. They were compared to those of control non-transformed plants. The traits as a number of stems per plant, length of internodes in longest stem, number of flowers per plant and plan high were very significant differed than the same traits in control plants, while there were no significant differences in the leaf area. No signs of the *rol* genes genotype and "T" phenotype were present. The transformed plants had significantly higher content of cellulose, while the protein and nitrogen contents of are in the range of control plants.

Key words: *Lotus corniculatus*, morphological traits, transformed plants, hairy roots, *A. rhizogenes*, *rol* genes

Corresponding author: Dr Radomirka Nikolić, ARI "Srbija", ARI "Serbia", Center for Agricultural and Technological Research, Grljanski put b.b., 19000 Zaječar, Serbia and Montenegro
Tel: 019/420-222, Fax: 019/421-668; E-mail: czpiti@ptt.yu

INTRODUCTION

Bird's foot trefoil (*Lotus corniculatus* L.) is an important forage legume, which is in many areas replacing white clover and alfalfa, for its tolerance to adverse environmental conditions and high nutritive value. Large literature exists demonstrating that bird's foot trefoil is easily amenable to tissue culture techniques (early review by ARCIONI *et al*., 1988), and to *Agrobacterium* - mediated transformation (e.g. DAMIANI *et al*., 1993; WEBB *et al*., 1999). In order to further improvement of agronomically important traits in domestic bird's foot trefoil cultivar Bokor by biotechnological methods, we were researched genetic transformation with *Agrobacterium tumefaciens* and *A. rhizogenes*. The results obtained indicated that *A. rhizogenes* was most efficient for genetic transformation of cv. Bokor (NIKOLIĆ, 2000). Some authors reported that bird's foot trefoil plants transformed by *A. rhizogenes* could have some morphological alterations compared to seed-growing plants (GUERCHE *et al*., 1985; TEPFER and CASSE-DELBART, 1987; SCHMÜLLING *et al*., 1988). In this report we presented morphological traits of *A. rhizogenes* transformed bird's foot trefoil plants grown in field.

MATERIALS AND METHODS

Transformed plants were regenerated from *A. rhizogenes* transformed hairy roots induced on bird's foot trefoil cv. Bokor seedlings. The hairy roots were induced by inoculating an overnight bacterial suspension into 3-4 cm tall stems. To induce hairy roots, 3-4 cm tall seedlings, cultured *in vitro* on an agar medium, were punctuated by a needle, dipped into the overnight bacterial suspension, about 1 cm above the medium. The bacterial strain was *A. rhizogenes* A4M70GUS, comprising the *uidA* gene integrated in the TL region of pRiA4 plasmid (TEPFER and CASSE-DELBART, 1987). Five mm tips of hairy roots were cultured in hormone-free liquid MS (MURASHIGE and SKOOG, 1962) medium supplemented with 50 mg l^{-1} cefotaxime; when root segments were transferred to solid medium supplemented with 0.2 mg l^{-1} BAP, the putative transformed shoots regenerated and were cultured afterwards on hormone-free MS medium. *A. rhizogenes* cells were eliminated with 50 mg l^{-1} cefotaxime ("Jugoremedia", Zrenjanin). The cultures were maintained at 25 ± 2 ^{0}C, in a 16/8 h light regime, under 47 µmol m^{-2} s^{-1} fluorescent light. The rooted shoots were acclimated in a greenhouse and transferred subsequently to the experimental field. In primary transformants and in seedlings of T_1 generation the GUS expression was assayed with X-gluc according to JEFFERSON *et al*. (1987), and by PCR analysis. Morphological traits of T_0 plants grown in field were studied and compared with control plants from the some genotypes.

RESULTS AND DISCUSSION

The entire procedure starting from shoot inoculation to completed acclimation took 70-85 days. Initially, 51 seeds (= genotypes) were inoculated in three experiments, and 25 (51%) genotypes produced adventitious roots at the inoculation site after 3 weeks. Regeneration was induced by transferring root segments

(0.5-1.0 cm) to solid medium with 0.2 mg l^{-1} BAP. From the initial 25 genotypes that produced hairy roots, a total of 177 shoots were obtained. On the hormone-free medium these shoots elongated, branched by tillering and spontaneously rooted, thus producing whole plants. When potted, 136 (77%) plants survived the treatment. Thirty five plants, belonging to the genotypes 2 and 5, were planted in the

Fig. 1. A transformed T_0 flowering plant of the genotype no. 2, after 83 days of acclimation

experimental field, where they flowered (Fig. 1) and set T_1 seeds. Their morphological traits were compared with controls (Table 1). Untransformed control plants from the same genotypes, derived from *in vitro* cultures, were planted in the same field, but in the required distance to prevent cross pollination. None of the transformants displayed the phenotype typical for the presence of *rol* genes (SCHMÜLLING *et al*., 1988). Although hairy root regenerants have often been reported to exhibit a characteristic transformed (T) phenotype with short internodes, reduced apical dominance, and wrinkled leaves (GUERCHE *et al*., 1985; TEPFER and CASSE-DELBART, 1987), transformed bird's foot trefoil plants of cv. Bokor did not show morphological differences which characterize "T" phenotype, with respect to longer stems, less shoots and a lower number of flowers per plant compared to control plants (Table 1). They did not differ significantly in the leaf length and width, growth habit, and date of flowering. In the other cases where plants have been transformed by *A. rhizogenes* the manifestation of normal appearance was also documented (PETIT *et al*., 1986; SPANO *et al*., 1987; TABAEIZADEH, 1993). The transformed plants obtained were fertile and the average number of seeds per plant was 84, 65 and 56 in the genotypes 2, 3, and 5, respectively. According to results obtained after chemical analysis of transformed and control plants, the

transformed plants had significantly increased content of cellulose while there were not found the significant differences in protein and nitrogen content (Table 2).

Table 1. Some morphological traits of the transformed L. corniculatus plants, grown in the experimental field

Trait	Genotype	Transformants	Controls
Average height	2	35.2**	28.8
[cm]	5	35.0*	31.4
No. of stems	2	32.0**	40.0
[plant^{-1}]	5	29.6**	34.8
Length of the longest stem	2	61.6**	31.8
[cm]	5	55.8**	41.6
No. of internodes	2	12.6*	11.2
[longest stem-1]	5	12.6ns	12.2
Length of the internodes of the longest stem	2	4.84**	2.90
[cm]	5	4.38**	3.40
Length of upper leaves	2	1.20ns	1.28
[cm]	5	1.20ns	1.22
Length of lower leaves	2	1.46ns	1.38
[cm]	5	1.42ns	1.38
Width of upper leaves	2	0.50ns	0.52
[cm]	5	0.50ns	0.50
Width of lower leaves	2	0.76ns	0.78
[cm]	5	0.78ns	0.76
Flowering date	2	20ns	22
(No. of days after 01.06.)	5	20ns	21
No. of flowers	2	272.0**	340.2
[plant^{-1}]	5	264.0**	308.4
Flowers	2	Yellow	Yellow
Color	5	Yellow	Yellow
Leaves	2	Light green	Green
Color	5	Light green	Green
Plant growth	2	Erect	Erect
Habit	5	Erect	Erect
Stem	2	Round	Round
shape	5	Round	Round

Differences significant at * - P≤0.05; ** - P≤0.01; ns - non-significant

Table 2. Chemical amount of plants transformed and control plants

Character	Genotype	Transformants	Controls
Proteins [%]	2	23.3ns	20.2
	5	22.3ns	20.7
Celulose [%]	2	17.7**	15.3
	5	17.6*	15.8
Nitrogen [%]	2	3.7ns	3.2
	5	3.6ns	3.4

Differences significant at * - P≤0.05; ** - P≤0.01; ns - non-significant

From the results presented here it can be concluded that the transformation of bird's foot trefoil cv. Bokor with A. *rhizogenes* results in plants with good agronomic qualities. Most important is the fact that no morphological alterations, due to the presence of *rol* genes and "T" phenotype, were observed. Since the cultivar Bokor was selected for its high green mass production, high crude protein yield and good tolerance to local climatic conditions of eastern Serbia, the prospects of its genetic modification using *A. rhizogenes* as a vector for desirable genes seem recommendable.

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REFERENCES

ARCIONI S., D. MARIOTTI, F. DAMIANI, and M. PEZZOTTI (1988): Birds foot trefoil (*Lotus corniculatus* L.), crown vetch (*Coronilla varia* L.) and sainfoin (*Onobrychis vicifolia* Scop.). *In*: BAJAJ Y.P.S. (ed.): Biotechnology in Agriculture and Forestry Vol 6, Crops II. Pp. 548-572. Springer, Berlin, Heidelberg, New York.

DAMIANI F., E. NENZ, F. PAOLOCCI, and S. ARCIONI (1993): Introduction of hygromycin resistance in *Lotus* spp. trough *Agrobacterium rhizogenes* transformation. Transgenic Res., *2*, 330-335.

GUERCHE G., P. DHAESE, M. VAN MONTAGU, and J. SCHELL (1985): DNA flux across genetic barriers the crown gall phenomenon. *In*: HOHN B. F.S. DENIS (eds) Genetic flux in plants. Pp. 11-47. Springer, Wien.

JEFFERSON R.A., T.A. KAVANAGH, and M.W. BEVAN (1987): GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J., *6*, 3901-3907.

MURASHIGE T. and F. SKOOG (1962): A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant., *15*, 473-497.

NIKOLIĆ R. (2000): Genetic transformation of bird's foot trefoil (Lotus corniculatus L.) using *Agrobacterium rhizogenes* and *Agrobacterium tumefaciens*. PhD thesis, Biological Faculty, University of Belgrade. (in Serbian).

PETIT A., A. BERKALOFF, and J. TEMPÉ (1986): Multiple transformation of plant cells by *Agrobacterium* may be responsible for the complex organization of T-DNA in crown gall and hairy root. Mol. Gen. Genet., *202*, 388-393.

SCHMÜLLING T., J. SCHELL, and A. SPENA (1988): Single genes from *Agrobacterium rhizogenes* influence plant development. EMBO J., *7*, 2621-2629.

SPANO L., D. MARIOTTI, M. PEZZOTTI, and F. DAMIANI (1987): Hairy root transformation in alfalfa (*Medicago sativa* L.). Theor. Appl. Genet., *73*, 523-530.

TABAEIZADEH Z. (1993): Transformation of bird's foot trefoil (*Lotus corniculatus*). *In*: BAJAJ Y.P.S. (ed.): Biotechnology in Agriculture and Forestry Vol 23, Plant Protoplasts and Genetic Engineering IV. Pp. 249-258. Springer-verlag, Berlin, Heidelberg.

TEPFER M. and F. CASSE-DELBART (1987): *Agrobacterium rhizogenes* as a vector for transforming higher plants. Microbiological Sci., 4, 24-28, 1987.

WEBB K.J., O. HUMPHREYS, L. SKØT, M. GIBBS, and J. GATEHOUSE (1999): Inheritance and expression of transgenes in T2 and T3 generation of *Lotus corniculatus* transformed using *Agrobacterium tumefaciens*. Euphytica, *108*, 169-179.

ANALIZA MORFOLOŠKIH KARAKTERISTIKA BILJAKA ŽUTOG ZVEZDANA CV. BOKOR TRANSFORMISANIH SA *AGROBACTERIUM RHIZOGENES*

Radomirka NIKOLIĆ i Nevena MITIĆ

Institut "Srbija", Centar za poljoprivredna i tehnološka istraživanja, 19000 Zaječar, Srbija i Crna Gora

Izvod

Uspostavljen je efikasan metod za transformaciju žutog zvezdana cv. Bokor pomoću *A. rhizogenes*. Transformisani pupoljci su regenerisani na segmentima adventivnih korenova u visokoj frekvenci. Posle ožiljavanja i aklimatizacije transformisane T_0 biljke su gajene na eksperimentalnom polju. Izvršena je analiza morfoloških karakteristika deset slučajno odabranih T_0 biljaka žutog zvezdana (genotipovi br. 2 i br. 5). One su upoređivane sa istim osobinama kontrolnih, netransformisanih biljaka. Osobine kao što su broj stabala po biljci, dužina internodija najdužeg stable i broj cvetova po biljci su bile signifikantno različite od istih osobina kontrolnih biljaka, dok nisu utvrđene značajne razlike u površini listova. Nije bilo znakova prisustva genotipa sa *rol* genima. Transformisane biljke su imale povećan sadržaj celuloze, dok je sadržaj proteina i azota bio na nivou kontrolnih biljaka.

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