New *Rhizobium leguminosarum* bv. *trifolii* isolates: collection, identification and screening of efficiency in symbiosis with clover

T. Šimon

Research Institute of Crop Production, Prague-Ruzyne, Czech Republic

ABSTRACT

Suitable clover stands (field and meadow) were chosen to isolate new *R. leguminosarum* bv. *trifolii* strains. Together 49 strains were isolated, labelled and maintained. Ten primers were used for identification and amplification products were detected by agarose electrophoresis. Amplification products of individual primers did not make possible to distinguish all isolates; a combination of at least two primers was necessary. No differences in genetic variability level between field and meadow isolates were found. Hydroponic perlite experiments were used for the screening of rhizobial isolates and the tetraploid red clover variety Amos was used as a host plant. Shoots were weighed and the root volume was determined at the onset of anthesis. Total nitrogenase activity (TNA) of the symbiotic system was measured and the root dry weight was determined. High differences among individual isolates were found in all the characteristics. Statistically significant were differences among individual isolates for TNA, growth characteristics did not differ significantly. Strong positive relationships between fresh forage, dry forage, root volume and root dry weight were determined (r = 0.69–0.98; P > 0.05). Nine isolates were selected as effective, based on the mean values of all isolates screened. Selected isolates achieved the above average values in most measured characteristics. Out of the nine isolates, two were isolated from root nodules of clover cultivated in field stands, seven strains were isolated from clover grown in natural meadows. Additional study of isolates in soil condition will follow.

Keywords: *R. leguminosarum* bv. *trifolii* isolates; collection; identification; screening in hydroponic experiments; selection

The legume N_2 fixation plays a key role in the maintenance of world crop production. Rhizobia specific for clover (*Trifolium* spp.) are commonly present in soils of Central Europe and symbioses of these two partners are able to fix hundreds kg of nitrogen per hectare annually. Hardarson et al. (1987) calculated for *Trifolium* spp. 673 kg N/ha/year as the maximum fixation. However, the individual rhizobia widely differ in efficiency in nitrogen fixation and this well documented fact is still used as one of the criteria in selection programmes of rhizobia and maintaining the culture collection of rhizobial strains (Date 2000). In addition, native rhizobia isolated from organically manured fields are able to fix more nitrogen, compare to rhizobia isolated from fields with no organic fertilization (Šimon 2003). At present, maintaining small, separate, and thoroughly characterized collections is preferred (Lupwayi et al. 2000). The cultures must be viable, properly identified and must have a known history. Culture collection records should include the date and the location of isolate collection, host type and soil type of collection site, date of preservation, and as complete a history as possible for the strain (Lupwayi et al. 2000). *Rhizobium* strains for such collection must be selected on the basis of their high competitiveness and efficiency in N_2 fixation upon nodule formation. There is a need to select strains for specific geographic, soil and environmental regions and, ideally, for individual varieties of a given legume species (Stephens and Rask 2000). Plant genotypes are steadily bred and it is necessary to find and screen new suitable *Rhizobium* strains for them. The aim of the study was the isolation, maintaining, identification and screening of new *Rhizobium*

Supported by the Ministry of Agriculture of the Czech Republic, Project No. QF 3051.

leguminosarum bv. *trifolii* strains for inoculation of developed tetraploid red clover cultivars.

MATERIAL AND METHODS

Isolation and maintaining. In spring 2003 suitable clover stands were chosen in selected localities of northern Moravia. Both production area of red clover planting (locality of Breeding station Hladké Životice) and natural meadows near river Odra (together 25 stands) were found to collect the appropriate red clover plants. No inoculants had been applied there for a long time. Several healthy plants with rootballs were taken from each stand. The roots of plants were thoroughly washed and ten nodules of typical appearance were isolated and sterilised. Each nodule was crushed and the content of the nodule was transferred onto a Petri dish with YEM agar (Vincent 1970). Petri dishes were kept in a thermostat at 28°C until typical colonies of rhizobia appeared. Colonies of rhizobia were examined microscopically, and purified by a series of successive dilutions. Isolates were inoculated to a slope of pea agar and stored. Together 49 *Rhizobium leguminosarum* bv. *trifolii* strains were isolated and maintained. Strains were labelled (locality, host, operator, date of isolation) and a working collection was established. Control of isolates is done every three months. Strains were numbered as follows 1/1, 1/2, 2/1, 2/2 etc. to 25/2. First number indicates the plant and second one indicates the nodule from which the strain was isolated.

Identification. A molecular biology method was used for the identification of rhizobial isolates. Individual isolates were cultivated at the 15 ml YEM liquid medium (Vincent 1970) in 28°C overnight. Genomic DNA was extracted according to the standard protocol of QIAGEN (DNeasy® Tissue Handbook 07/2003). Amplification was done on the PCT-200 (MJ Research) amplifier in the following reaction mixture: 2 units of *Taq* DNA polymerase, PCR buffer (Promega), 2.0mM $MgCl_2$, 0.1mM dNTP, 1.5μM primer, 25 ng bacterial DNA. The amplification cycle involved the following sequences: 94°C/5 min, 40 repetitions of denaturation in 94°C/1 min, primer adherence in 35°C/1 min and primer elongation in 72°C/1 min. At the end, the mixture was incubated for 5 min at 72°C. Ten primers (OPI03, OPI04, OPI06, OPI07, OPI09, CS12, CS14, CS15, CS16 and CS31) were used for identification. Amplification products were detected by agarose electrophoresis and stained with ethidium bromide. GeneRuler™ 100 bp DNA Ladder Plus (Fermentas, Lithuania) was used as a marker.

Screening of rhizobial isolates efficiency in symbiosis with tetraploid red clover. Two hydroponic perlite experiments conducted in the greenhouse were used for the screening of rhizobial isolates in 2004. Tetraploid red clover variety Amos (bred in 1998 in Breeding Station Hladké Životice) was used as a host plant. A set of two plastic pots was used for experiments. The bottom pot contained a nutrient solution without nitrogen (Šimon 1991). The upper pot was filled with expanded perlite. Both pots were connected with glass fibres that conducted the flow of nutrients to the plant roots. Clover was seeded in the pots with perlite and after the emergence plants were inoculated of 1 ml (1×10^9 CFU) of individual rhizobia suspension. Clover seeds were sterilised with 0.2% $HgCl_2$ solution before sowing (Vincent 1970). The nutrient solution was regularly replaced. Every treatment had six replications. Additional light was provided by sodium-vapour lamps and the temperature was partly regulated (20–24°C day/12–16°C night). A 16 hour photoperiod was used. At the onset of anthesis plants were carefully removed from the pots, shoots were weighed (in a fresh and dry state) and roots were placed into the infusion bottles for determination of their volume. Total nitrogenase activity (TNA) (μmol C_2H_4/plant/hour) of the symbiotic system was measured by a routine indirect method of acetylene reduction to ethylene as an indication of N_2 fixation level (Hardy et al. 1973). Root dry weight was determined. Standard deviations (*SD*) were calculated and the obtained data were processed by analysis of variance followed by the Tukey test that evaluates the significance of differences among the variants. The correlations for the main characteristics were calculated.

RESULTS AND DISCUSSION

Each of *Rhizobium legumonisarum* bv. *trifolii* isolates was amplified with ten primers and RAPD (random amplified polymorphic DNA) product profiles with individual primers were recorded. As an example, RAPD product profiles with primer OPI03 for *Rhizobium leguminosarum* bv. *trifolii* isolates (12/2–25/2) are shown in Figure 1. Amplification products of individual primers did not make possible to distinguish all isolates. Combination of at least two primers was

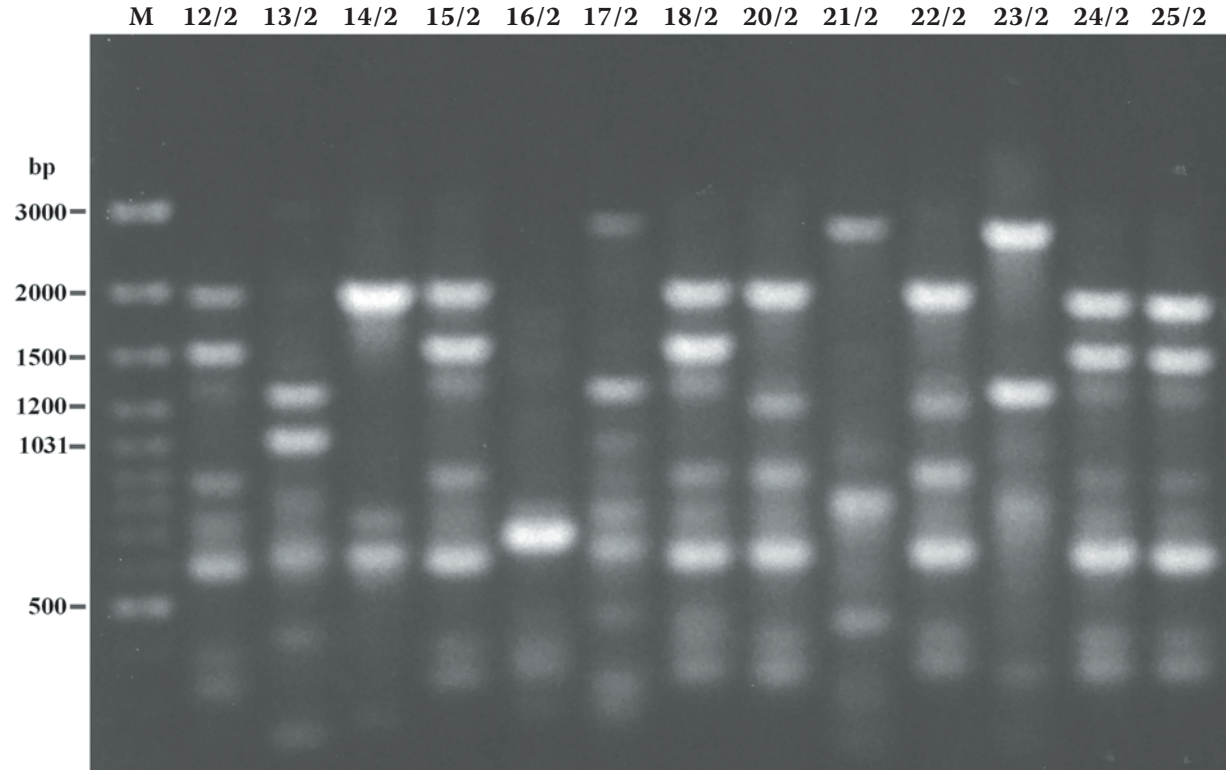

Figure 1. RAPD product profiles with primer OPI03, lines 12/2 to 25/2 are *Rhizobium leguminosarum* bv. *trifolii* isolates; line M is standard of molecular weight GeneRuler™ 100 bp DNA Ladder Plus (Fermentas, Lithuania)

necessary for identification. Hebb et al. (1998) used PCR-RAPD profiles for identification of inoculant strains and non-inoculant strains isolated from clover root nodules. Out of 766 isolates examined by PCR amplification, 260 could not be identified on the basis of matching amplification profile as an inoculant strain of *Rhizobium leguminosarum* bv. *trifolii.* Among these unknown isolates, several predominant amplification profile types were evident. Similarly, several main amplification profile types were identified in the collection of our isolates. No differences in genetic variability level between the field and meadow isolates were found.

Results of isolates screening for growth and symbiotic characteristics are summarized in Tables 1 and 2. High differences among individual isolates were found in all characteristics determined and the wide ranges of the lowest and the highest mean values of measurements were recorded (fresh forage = 7.32–66.65 g/plant; dry forage = 0.77–13.56 g per plant; root volume = 17.63–77.38 ml; root dry weight 0.36–5.52 g/plant; TNA = 1.24–8.56 μmol C_2H_2/plant/hour). Significant differences between individual isolates were found for TNA (Tables 1 and 2), growth characteristics did not differ significantly due to the high variability inside the isolate replications. Significant positive relationships among fresh forage, dry forage, root volume and root dry weight were determined (r = 0.69–0.98; $P > 0.05$). Sirois and Peterson (1981) who screened *Rhizobium meliloti* isolates for their nitrogenase activity with alfalfa found a significant positive relationship between the nitrogenase activity of those isolates which reduced more than 60 nmol C_2H_2/plant/hour and the dry weight of the shoots of the nodulated plants in 2 weeks of growth. No significant relationship between nitrogenase activity and yield was identified in the set of our isolates after four months of planting. Mean values of all evaluated characteristics of all screened isolates were calculated as the basic parameter for isolates ranking and selection of the best of them (Table 3). Based on the mean values, three progressive ranges of values for each characteristic were defined that an individual isolate must reach to be selected as effective (Table 3). Together nine isolates were selected as effective. These isolates achieved high values in most of the measured characteristics; the main emphasis was put on nitrogenase activity as a parameter characterizing the ability of isolates to fix atmospheric nitrogen. Percentage comparison with mean values of all screened isolates was done for the selected isolates (Table 4) and percentage values of selected isolates were counted up to create the final rank of isolates: 1. 1/2; 2. 14/2; 3. 20/2;

Table 1. Growth and symbiotic characteristics of *Rhizobium leguminosarum* bv. *trifolii* isolates screened in the first experiment (mean values ± *SD*)

Isolate	Fresh forage (g/plant)	Dry forage (g/plant)	Root volume (ml)	Root dry weight (g/plant)	TNA (μmol/plant/h)
1/1	31.01 ± 17.08NS[1]	3.54 ± 1.82NS	36.08 ± 8.34NS	1.99 ± 0.74NS	2.56 ± 1.09 abcd[2]
2/1	33.96 ± 25.81	3.91 ± 3.13	34.56 ± 16.91	1.31 ± 0.79	2.71 ± 1.58 abcde
3/1	44.25 ± 16.98	5.12 ± 1.83	46.83 ± 12.52	2.33 ± 0.62	2.91 ± 1.85 abcdef
4/1	38.46 ± 27.47	4.61 ± 3.46	40.30 ± 19.10	1.84 ± 1.03	3.05 ± 1.71 abcdef
5/1	37.91 ± 24.56	4.59 ± 2.89	46.80 ± 21.71	2.11 ± 1.36	1.82 ± 1.23 abc
6/1	31.57 ± 13.93	3.76 ± 1.48	42.86 ± 15.07	2.17 ± 0.46	2.08 ± 1.12 abc
7/1	22.94 ± 14.74	2.55 ± 1.62	44.92 ± 14.55	1.66 ± 0.78	1.65 ± 0.72 ab
8/1	17.43 ± 7.19	2.09 ± 0.99	31.50 ± 7.98	1.12 ± 0.59	2.08 ± 0.84 abcd
9/1	29.85 ± 13.55	3.33 ± 1.46	32.14 ± 8.47	1.43 ± 0.53	6.35 ± 2.35 abcdef
10/1	26.84 ± 12.24	2.92 ± 1.22	34.90 ± 11.82	1.46 ± 0.57	5.60 ± 3.81 abcdef
11/1	40.71 ± 23.77	4.53 ± 2.41	44.36 ± 19.93	1.92 ± 0.82	5.07 ± 2.30 abcdef
12/1	23.90 ± 17.56	2.54 ± 1.85	28.18 ± 13.91	1.14 ± 0.74	5.37 ± 3.56 abcdef
13/1	41.61 ± 28.15	4.91 ± 3.20	53.44 ± 15.43	2.21 ± 0.95	4.72 ± 2.32 abcdef
14/1	43.19 ± 12.21	4.98 ± 1.37	55.18 ± 20.68	2.46 ± 0.69	8.02 ± 3.21 f
15/1	38.53 ± 22.98	4.30 ± 2.20	34.17 ± 12.45	1.46 ± 0.62	3.49 ± 2.39 abcdef
16/1	38/31 ± 17.40	4.73 ± 2.31	50.45 ± 9.69	2.00 ± 0.37	7.31 ± 3.08 cdef
17/1	42.18 ± 29.03	5.39 ± 1.87	44.46 ± 26.05	2.43 ± 1.60	2.92 ± 1.23 abcdef
18/1	37.35 ± 20.49	4.31 ± 2.16	38.16 ± 8.77	2.07 ± 0.39	4.18 ± 2.35 abcdef
19/1	35.91 ± 15.08	4.18 ± 1.70	42.38 ± 11.73	2.19 ± 0.59	4.54 ± 2.60 abcdef
20/1	39.09 ± 26.79	4.37 ± 3.04	45.65 ± 21.82	2.33 ± 1.08	3.40 ± 0.59 abcdef
21/1	55.67 ± 15.91	6.48 ± 2.24	55.95 ± 16.65	2.70 ± 0.31	4.78 ± 2.45 abcdef
22/1	60.85 ± 25.27	7.15 ± 3.30	63.43 ± 16.99	2.74 ± 0.63	2.80 ± 1.48 abcdef
23/1	54.80 ± 34.05	6.34 ± 3.85	35.58 ± 15.23	2.70 ± 1.31	5.66 ± 2.25 abcdef
24/1	7.32 ± 5.68	0.77 ± 0.56	17.63 ± 6.60	0.36 ± 0.23	1.24 ± 0.60 a
25/1	40.02 ± 31.16	4.68 ± 3.60	48.00 ± 19.32	2.22 ± 1.48	2.72 ± 2.73 abcdef

[1]NS = non-significant differences; [2]means within the column followed by the same letter do not differ significantly as determined by Tukey multiple range test ($P < 0.05$)

4. 9/2; 5. 21/2; 6. 22/2; 7. 14/1; 8. 21/1; 9. 23/1. The isolate 1/2 achieved the balanced high values for all evaluated characteristics (Table 4), other isolates (14/2, 20/2, 22/2) exceeded mean values of all isolates by 30% in dry matter of forage and total nitrogenase activity, as well. In conclusion, the results of selected isolates were compared with the results of two effective collection strains D711 and D716 (*Rhizobium* collection, RICP) tested in 2003 in the same way (Table 5). The comparison showed that the selected isolates achieved similar values of TNA and six of them increased the dry biomass of host plant above the level of collection strains.

Out of the nine selected isolates, two (1/2, 9/2) were isolated from root nodules of clover cultivated in production area of plant breeding station, seven strains were isolated from clover growing in natural meadows. The possibility to choose the natural meadow clover stands where no long-term nitrogen fertilization, inoculation and other treatments were done as an appropriate source of effective rhizobia for clover inoculation resulted from that proportion. However, effective rhizobia

Table 2. Growth and symbiotic characteristics of *Rhizobium leguminosarum* bv. *trifolii* isolates screened in the second experiment (mean values ± *SD*)

Isolate	Fresh forage (g/plant)	Dry forage (g/plant)	Root volume (ml)	Root dry weight (g/plant)	TNA (μmol/plant/h)
1/2	65.35 ± 43.17NS	10.29 ± 6.67NS	61.90 ± 22.90NS	4.00 ± 1.68NS	5.11 ± 3.98 abcd
2/2	74.90 ± 46.89	10.77 ± 6.75	75.26 ± 44.19	4.80 ± 1.25	3.98 ± 2.16 abcd
3/2	74.89 ± 47.36	11.20 ± 7.99	68.18 ± 27.18	2.66 ± 1.75	1.84 ± 1.44 ab
4/2	85.39 ± 55.32	11.60 ± 6.76	57.97 ± 17.61	2.52 ± 1.56	1.98 ± 1.53 abcd
5/2	52.24 ± 44.28	8.38 ± 7.57	53.03 ± 32.51	4.51 ± 1.71	1.43 ± 0.29 a
6/2	85.83 ± 34.25	13.56 ± 6.80	77.38 ± 10.19	4.02 ± 1.32	2.18 ± 1.62 abcd
7/2	70.73 ± 45.86	10.08 ± 6.85	63.12 ± 27.34	4.11 ± 1.41	1.87 ± 1.63 abc
8/2	19.23 ± 15.76	2.28 ± 1.23	28.00 ± 12.26	2.71 ± 0.93	2.64 ± 1.72 abcd
9/2	66.65 ± 40.04	8.66 ± 7.72	65.60 ± 33.14	3.05 ± 1.12	4.51 ± 0.47 abcd
10/2	54.32 ± 12.64	8.22 ± 2.32	58.12 ± 17.67	3.19 ± 1.23	3.68 ± 1.19 abcd
11/2	74.87 ± 39.75	11.40 ± 5.85	71.25 ± 9.71	5.52 ± 1.64	3.42 ± 2.16 abcd
12/2	47.30 ± 42.08	6.31 ± 5.83	51.72 ± 27.15	2.73 ± 1.19	3.47 ± 1.53 abcd
13/2	55.82 ± 33.75	8.17 ± 4.59	57.75 ± 17.43	3.04 ± 1.03	2.16 ± 1.49 abcd
14/2	56.45 ± 38.80	8.38 ± 6.12	65.22 ± 17.69	4.15 ± 1.81	5.47 ± 0.43 abcd
15/2	37.93 ± 15.38	5.23 ± 2.23	49.38 ± 20.59	2.03 ± 0.94	3.06 ± 1.86 abcd
16/2	11.95 ± 5.46	1.66 ± 0.78	22.75 ± 6.29	0.93 ± 0.40	4.00 ± 2.05 abcd
17/2	35.44 ± 17.88	6.22 ± 3.68	40.43 ± 13.02	2.76 ± 2.15	3.31 ± 2.33 abcd
18/2	48.10 ± 45.12	6.81 ± 6.38	46.03 ± 26.62	2.82 ± 1.95	5.34 ± 2.06 abcd
20/2	52.83 ± 33.16	8.43 ± 5.52	53.44 ± 19.92	3.25 ± 1.46	7.72 ± 5.61 bcd
21/2	47.34 ± 32.93	6.84 ± 4.84	52.27 ± 27.00	3.42 ± 1.41	6.23 ± 2.55 abcd
22/2	54.69 ± 23.29	8.01 ± 3.75	47.67 ± 23.36	2.81 ± 1.58	5.66 ± 3.18 abcd
23/2	42.54 ± 25.95	6.44 ± 4.04	71.60 ± 63.80	2.66 ± 0.94	4.09 ± 2.49 abcd
24/2	27.31 ± 21.11	3.66 ± 2.78	39.35 ± 24.52	2.18 ± 1.78	6.52 ± 3.65 abcd
25/2	31.71 ± 29.83	4.65 ± 3.90	43.57 ± 27.87	1.89 ± 0.74	8.56 ± 3.82 bcd

can be also found in soils where the clover has been cultivated for a long time.

We are aware that the screening in hydroponic condition for *Rhizobium* isolates efficiency is only the first step to check the real isolates quality. Vincent (1965) emphasized that the rhizobial strains for inoculation must have the capacity to form highly effective nodules on the hosts under a wide range of field conditions. Simultaneously, the competitiveness of new isolates with native

Table 3. Mean values of determined characteristics for all screened isolates and three progressive ranges of values for each characteristic serving as criterions for isolate selection

	Fresh forage (g/plant)	Dry forage (g/plant)	Root volume (ml)	Root dry weight (g/plant)	TNA (μmol/plant/h)
Means of all isolates screened (n = 49)	44.81	6.02	48.44	2.52	4.07
Range	45–50	6–7	50–60	2.5–3	4–5
	50–60	7–8	60–70	3–4	5–6
	> 60	> 8	> 70	> 4	> 6

Table 4. Selected isolates signed by percentage comparison with mean values of all screened isolates (mean = 100%)

Selected isolates	Fresh forage (g/plant)	Dry forage (g/plant)	Root volume (ml)	Root dry weight (g/plant)	TNA (μmol/plant/h)
14/1	43.19 (96%)	4.98 (82%)	55.18 (114%)	2.46 (98%)	8.02 (197%)
21/1	55.67 (124%)	6.48 (106%)	55.95 (116%)	2.70 (107%)	4.78 (117%)
23/1	54.80 (122%)	6.34 (104%)	35.58 (73%)	2.70 (107%)	5.66 (139%)
1/2	65.35 (146%)	10.29 (169%)	61.90 (128%)	4.00 (159%)	5.11 (126%)
9/2	66.65 (149%)	8.66 (142%)	65.60 (135%)	3.05 (121%)	4.51 (111%)
14/2	56.45 (126%)	8.38 (138%)	65.22 (135%)	4.15 (165%)	5.47 (134%)
20/2	52.83 (118%)	8.43 (138%)	53.44 (110%)	3.25 (129 %)	7.72 (190%)
21/2	47.34 (106%)	6.84 (112%)	52.27 (108%)	3.42 (136 %)	6.23 (153%)
22/2	54.69 (122%)	8.01 (132%)	47.67 (98%)	2.81 (112 %)	5.66 (139%)

Table 5. Growth and symbiotic characteristics of effective *R. leguminosarum* bv. *trifolii* strains (*Rhizobium* collection, RICP)

Strain	Fresh forage (g/plant)	Dry forage (g/plant)	Root volume (ml)	Root dry weight (g/plant)	TNA (μmol/plant/h)
D 711	22.57	4.34	56.60	2.92	7.00
D 716	36.95	6.59	79.97	4.01	4.99

soil rhizobia should be determined (Date 2000). An additional study of isolates in soil condition will therefore follow.

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Received on February 16, 2005

Corresponding autor:

Ing. Tomáš Šimon, CSc., Výzkumný ústav rostlinné výroby, Drnovská 507, 161 06 Praha 6-Ruzyně, Česká republika
phone: + 420 233 022 253, fax: + 420 233 310 636, e-mail: simont@vurv.cz