A NEW SAND POUCH-PLANT INFECTION TECHNIQUE FOR ENUMERATION OF RHIZOBIA IN SOIL

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ABSTRACT

A sand pouch-plant infection technique for counting most probable numbers of rhizobia in soil is described. Populations of *Rhizobium leguminosarum* bv. *trifoli* detected by the plant infection method performed in enclosed glass tubes or in sand pouches did not differ significantly. The described method was used to assess numbers of *R. leguminosarum* bv. *trifoli* (*R.l.t.*) and bv. *viciae* (*R.l.v.*) in 20 soils of Poland. Logarithms of the populations of *R.l.t.* in the tested soils ranged from not detectable level to 4.76 and those of *R.l.v.* from 2.23 to 5.84 in g^{-1} of soil dry mass. Numbers of *R.l.t.* were significantly correlated with soil clay, C org. and total N contents but not with the soil pH (in KCl), while numbers of *R.l.v.* showed significant correlation only with the soil pH.

KEY WORDS: assessment, nodulation, *Rhizobium leguminosarum*, soil, *Trifolium repens*, *Pisum arvense*.

INTRODUCTION

Although root-nodule bacteria (rhizobia) are culturable on different synthetic or semi-synthetic media, there is generally no selective medium available for making plate counts of these bacteria in contaminated material, particularly in soil. Attempts have been made to develop such selective media, for example to isolate *Sinorhizobium meliloti* (Bromfield et al. 1994) or *Bradyrhizobium japonicum* (Gault and Schwinghamer 1993) directly from soil. The selectivity of these media was, however, low and identification of rhizobial colonies required the use of quite sophisticated methods, e.g. a species-specific DNA probe and colony blot hybridization (Bromfield et al. 1994; Sadowsky and Graham 1998). For these reasons a serial soil dilution-plant infection method is most widely used to estimate most probable numbers (MPN) of native root nodule bacteria in soils (Brockwell 1963; Vincent 1970; Weaver and Frederick 1971; Amarger 1980; Toomsan et al. 1984). In this method, seedlings of a chosen leguminous plant are grown in enclosed glass tubes with N-free agar medium (or other materials, e.g. vermiculite) to support plant growth. Seedlings are then inoculated with serial dilutions of a soil sample and after 3-4 weeks of growth the presence or absence of nodules is scored. Formation of one or more nodules on the host roots indicates the presence of rhizobia in a given soil dilution and the total numbers of tubes with nodulated plants are than used to calculate the MPN of these bacteria in the tested soil (Brockwell 1963; Vincent 1970). The plant infection-glass tube method generally works well with small-seeded legumes such as clover or alfalfa, though there is one limitation concerning the harmful effect on seedling growth and nodulation of high quantities of soil microorganisms introduced to the tubes with the first dilutions of soil (e.g. 10^{-1}). Vincent (1970) suggested that such "skip" tubes should be scored as positive but this might pose serious problem in the proper assessment of root nodule bacteria in soils containing low populations of these bacteria.

More problems occur when the plant infection-soil dilution method is used to estimate soil populations of rhizobia nodulating large-seeded legumes, such as pea, lupine, bean or faba bean which are difficult to grow in enclosed tubes. To overcome these problems, several approaches have been proposed. The most common one deals with growing (in big tubes) of a smaller legume species that is nodulated readily by the same group of rhizobia, e.g. serradella (*Ornithopus sativa* L.) instead of lupine (Gault et al. 1986), but this substitution is not always possible. Thus, Toomsan et al. (1984) dwarfed chickpea seedlings by excising their cotyledons and grew such plants successfully in the test tubes. Large legumes can also be grown in Leonard jars with sterilised sand but preparation of these assemblies is difficult and time-consuming (Vincent 1970; Toomsan et al. 1984).

In this paper, a simpler sand pouch technique of growing leguminous plants for most probable counts of rhizobia in soil is presented.

MATERIALS AND METHODS

Sand pouch test for growing red clover and pea

Sets of 4 plastic pouches 15 cm high and 2.5 cm wide were prepared at the laboratory by welding folded polypropylene foil. The pouches were filled to within 2 cm of the top with about 30 g of washed dry sand, placed in baskets and auto-

Fig. 1. Sand pouch tests with red clover for MPN of *R. leguminosarum* bv. *trifoli* in different soils. In the lowest row soil nr 9 containing no detectable *R.l.t.* and in the uppermost row soil nr 15 with high population of these bacteria. In pots from left to right – seedlings inoculated with soil dilution from 10^{-1} to 10^{-6}. In the second and the third rows from the top, soils nr 1 and 6, respectively.

claved for 30 min. at 100 kPa (1 atm). Before autoclaving the pouches were perforated at the bottom with a needle to facilitate air escape when the sand was moistened from the top. After cooling, the sand-filled pouches were placed in plastic pots to hold the pouches up-right and protect plant roots from the light. These pots were made of a PVC hydraulic pipe (5 cm in diam.) cut into pieces 13 cm long, which were then glued to a plastic plate (Fig. 1). We constructed sets of 6 pots 2 cm apart on one PVC plate (50 cm × 8 cm × 0.5 cm). Each pot contained a set of 4 sand pouches. The sand in pouches was moistened to ≈ 60% of WHC with about 5 ml of N-free autoclaved Thornton nutrient solution (Vincent 1970). The nutrient medium can be delivered from the top or alternatively from the bottom. The second option is more convenient and can be done by pouring about 5 ml per one pouch of the medium to the bottom of the pot and allowing the medium to be sucked-up by the sand. Seeds of red clover (*Trifolium repens* L., cv. Hruszowska), which had been surface disinfected by soaking for 10 min. in 5% H_2O_2 followed by several rinses with sterile water, were placed on sterile 1% water agar in Petri dishes to germinate at 25°C for 48 hours. Healthy, uniform seedlings were selected and planted, two per pouch, into slits made with a sterile spatula in the surface layer of the moist sand in the pouches. Seedlings were then inoculated with 1 ml of 10-fold soil dilutions. In these studies we used six dilution steps (10^{-1}-10^{-6}) and 4 replicated pouches for each soil dilution (Fig. 1). After inoculation, all sets of pouches were transferred to a plant growth chamber (Hereaus HPS 1500-2000). Two days later, when seedlings became taller, about 0.5 cm layer of sterile sand was poured into each pouch. The growth chamber was running at 16 h/8 h day/night regime and temperature 22°/15°C, respectively. After 4 weeks of growth, pouches were cut lengthwise with a scalpel, roots of seedlings gently washed in tap water and inspected for the presence of nodules in each dilution and the total number of positive cases counted.

The same technique was also used in the plant infection test with field pea (*Pisum arvense* L, cv. Grapis), as the host to count population of *R. leguminosarum* bv. *viciae* in soil. The difference concerned only the size of the assemblies, which were scaled-up. To grow pea, 20 cm × 5 cm pouches containing about 180 g of dry sand were used. The pouches were planted not with pea seedlings but with two pea seeds, which had been surface desinfected as described above. The seeds were slightly pressed into the moist sand to a depth of about 1 cm and then inoculated with 1 ml of a soil dilution. After inoculation the seeds were covered with 1 cm layer of sterile sand. Plants were inspected for nodulation after 4 weeks of growth in the growth chamber.

Comparison of the conventional plant infection-glass tubes test and the sand pouch test for most probable numbers of R. leguminosarum bv. trifoli

Soil samples were collected in May of 1998 from a 2-year old stand of red clover grown at the Experimental Station in Puławy. The soil at this location has the following basic characteristics: 31% silt, 18% clay, 1% org. C, pH (H_2O) 6.7. Several cores of the soil were taken from the surface (0-20 cm) layer, bulked, thoroughly mixed and passed through a 2 mm sieve. Next day sub-samples, each weighing 10 g (equivalent to soil dry mass) were suspended in 90 ml of sterile water in bottles, shaken for 10 min on a rotary shaker, serially diluted and used to inoculate seedlings of red clover, cv. Hruszowska. Three parallel sub-samples were analysed by the plant infection method using the glass tubes test with agar as described in Vincent (1970) and sand pouch test (SPT) described above. In both cases six dilution steps with four replicated tubes or pouches inoculated with 1 ml of each 10-fold soil dilution (10^{-1}-10^{-6}) were used. Most probable numbers (MPN) of *Rhizobium leguminosarum* bv. *trifoli* in the tested soil were calculated from Fisher & Yates tables (Vincent 1970).

Counting of rhizobia in different soils

SPT was used to enumerate the most probable numbers of *R. leguminosarum* bv. *trifoli* and bv. *viciae* in 20 soils from 9 locations in Poland. The locations were as follows: Laskowice (soils: 1-3; loamy sand), Żelislawki (4, 5; light loam), Błonie (6-8; loamy sand), Wierzbno (9, 10; light loamy sand), Baborówko (11,12; loamy sand), Borusowa (13-15; heavy loam), Wielichowo (16-18; light loamy sand), Kępa (19; loam) and Osiny (20; loamy sand). Basic physico-chemical characteristics of these soils and crop sequences during 1995-1998 are given in table 2. Samples of these soils were collected in October of 1998, sieved and stored moist at 4°C in a refrigerator until analysed, but not longer than 1 month. Soil analyses of: organic C (dichromate digestion), total N (Kjeldahl), pH (1*N* KCl) and clay content (sedimentation) were done by standard methods at the Central Laboratory of the Institute of Soil Science and Plant Cultivation in Puławy.

RESULTS AND DISCUSSION

Table 1 presents the most probable numbers (MPN) of clover rhizobia, *Rhizobium leguminosarum bv. trifoli* (*R.l.t.*) in the soil under red clover as detected by the plant infection tests performed in glass tubes and in sand pouches. Mean (of 3 replicated tests) log numbers of *R.l.t.* assessed by the glass tube test (4.07) and by the sand pouch test (3.91) did not differ significantly (at P = 0.05), indicating that both tests detected similar counts of root nodule bacteria in soil. However, marked differences between these two tests were observed with respect to nodulation pattern and seeding growth. To illustrate these differences, the results of one test performed in glass tubes and one using sand pouches (Table 1) are compared in Table 2. In the case of the glass tube test the total score of positive cases (+) was taken as 14, even though clover seedlings in 3 of 4 tubes inoculated with the first soil dilution (10^{-1}) had no nodules on their roots due to harmful effects of contaminating soil microorganisms on nodulation. These kinds of nodulation problems are often met when using agar medium to support plant growth in the glass tube-plant infection tests for counting rhizobia in soil or other contaminated materials (Robinson 1968; Toomsan et al. 1984; Vincent 1970). Contrary to that, in the sand pouch test, all seedlings inoculated with the first soil dilution nodulated well. This is particularly important when the population of rhizobia in the examined soil is low, e.g. soil nr 6 (Fig. 1 – second row from the bottom). Moreover, the growth of nodulated clover seedlings in the SPT was also more vigorous than in the GTT (Table 2). In the sand pouch technique seedlings are not fully protected against airborne contamination or cross-contamination by water splash. Unexpected nodulation, e.g. in the non-inoculated control treatments, of seedlings grown in sand pouches was, however, very rare. Such an unexpected nodulation occasionally occurs also when leguminous plants are grown in enclosed tubes and it is a result of incomplete seed disinfection rather than contamination. In the described sand pouch technique, airborne contamination is reduced to a minimum by introducing a layer of sterile sand on top of the pouches after their inoculation and by leaving the top 2 cm of the pouches unfilled. Moreover, plants were watered, as needed, only by pouring appropriate volume of water, usually about 5 ml, into the bottom of the plastic pots containing sand pouches. This volume of liquid is quickly absorbed by the sand in the pouches. By doing this, cross-contamination of seedlings during watering is almost completely eliminated.

TABLE 1. Log of MPN of *Rhizobium leguminosarum* bv. *trifolii* in 1 g of soil under red clover as detected by the plant infection test performed in glass tubes and in sand pouches.

Glass tube test	Sand pouch test
4.23, 4.23, 3.76 Mean: 4.07[a]	4.23, 4.00, 3.49 Mean: 3.91a

[a] Means are not significantly different according to Tuckey's test (P = 0.05)

One of the reasons for developing the sand pouch technique was that we could not grow large-seeded legumes, e.g. pea or bean, in glass tubes, even in large ones. In a preliminary study it was found that these plants grow and nodulate well in sand pouches. Therefore, the sand pouch-plant infection test was used to estimate populations of *R. leguminosarum* bv. *trifoli* and bv. *viciae* in 20 samples of soils collected from 9 locations throughout Poland (Table 3). These soils differed with respect to their chemical and physical, as well as agronomical (crop sequences) characteristics. Log numbers of *R.l.t* in the tested soils ranged from no detectable level of these bacteria in soil nr 9 to 4.76 in soil nr 19 and those of *R.l.v.* from 2.23 in soils nr 18 and 20 to 5.84 in soils nr 2, 8 and 11. Therefore, populations of *R.l.v* in the examined soils were generally higher but less differentiated than those of *R.l.t.* The differences in the MPN counts of *R.l.t.* and *R.l.v.* in the tested soils could be attributed to their chemical and physical properties rather than to the crop sequences. As it can be seen from Table 3 red clover (the host of *R.l.t*) was not grown during last 4 years on any of the soils. Pea (the host of *R.l.v.*) was

TABLE 2. Pattern of nodule formation and fresh shoot mass of clover seedlings in the glass tube test (GTT)[a] and the sand pouch test (SPT)[a].

Soil dilution	GTT		SPT	
	Nodulation (+) in 4 tubes	Mean fresh mass of shoots (mg)	Nodulation (+) in 4 pouches	Mean fresh mass of shoots (mg)
10^{-1}	+ – – –	292	+ + + +	881
10^{-2}	+ + + +	370	+ + + +	803
10^{-3}	+ + + –	320	+ + + +	651
10^{-4}	+ + – –	210	+ – – –	109
10^{-5}	+ – – –	185	– – – –	61
10^{-6}	– – – –	91	– – – –	57
Control (Uninoculated)	– – – –	92	– – – –	59
Control (+ *R.l.t*)	+ + + +	350	+ + + +	735

[a] Tests shown in Table 2, GTT = 3.76; SPT = 3.49.

TABLE 3. Physico-chemical characteristics and log of MPN of *Rhizobium leguminosarum* bv. *trifolii* (*R.l.t.*) and *R. leguminosarum* bv. *viciae* (*R.l.v.*) in 1 g of different soils as assessed by the plant infection-sand pouch technique.

Soils	C org. (%)	Total N (%)	Clay (%)	pH in KCl	Crop sequences in 1995-1998	Numbers of rhizobia	
						R.l.t.	R.l.v.
1	0.65	0.052	5	4.7	c-ww-o-ww[a]	2.49	3.76
2	0.72	0.064	5	6.7	ww-c-sw-wr	2.76	5.84
3	0.80	0.065	2	5.3	r-r-c-ww	3.00	2.76
4	0.58	0.054	6	4.7	sb-sb-ww-sb	<0.78	3.76
5	0.84	0.083	11	6.2	p-ww-ww-**p**	3.23	4.77
6	0.67	0.066	6	6.5	p-ww-s-sb	1.76	4.77
7	1.04	0.092	6	6.3	sw-ww-sb-s	3.00	4.23
8	0.97	0.091	6	6.2	s-sb-sb-ww	3.23	5.84
9	0.55	0.042	1	6.2	c-t-r-sb	0	3.23
10	0.76	0.072	4	5.6	r-wb-c-sw	2.00	5.25
11	0.63	0.058	6	6.1	sw-sr-sb-**l**	4.23	5.84
12	0.62	0.055	4	6.2	sb-sr-ww-c	1.23	4.23
13	0.99	0.105	13	6.3	c-sw-s-sb	3.00	3.23
14	1.55	0.140	18	6.5	sb-wr-ww-sb	3.23	3.23
15	1.66	0.120	19	6.3	ww-s-sw-ww	4.23	3.76
16	0.61	0.054	5	6.6	ww-wr-sw-s	3.23	5.25
17	1.10	0.095	4	6.3	wr-sw-s-ww	2.76	4.23
18	0.63	0.045	3	5.6	s-ww-sb-wr	1.23	2.23
19	1.11	0.109	16	5.7	ww-c-sb-**fb**	4.76	3.76
20	0.93	0.085	7	5.4	sb-wr-ww-sb	2.76	2.23
				95%	fiducial limits	± 0.58	

[a]c – corn; **fb** – faba bean; **l** – lucerne; o – oats; **p** – pea; r – rye; s – sugar beat; sb – spring barley; sr – spring rape; sw – spring wheat; t – triticale; wb – winter barley; wr – winter rape; ww – winter wheat.

TABLE 4. Correlation coefficients between numbers of *R. leguminosarum* bv. *trifoli* (*R.l.t.*) and bv. *viciae* (*R.l.v.*) and some physico-chemical properties of soils shown in Table 3.

Log MPN of rhizobia	Clay (%)	C org. (%)	Total N (%)	pH (KCl)
R.l.t.	0.660**	0.581**	0.616**	0.065
R.l.v.	-0.118	-0.185	-0.108	0.440*

* Significant at $p = 0.05$; ** significant at $p = 0.01$

grown only on soils no 5 and 6, but populations of *R.l.v.* in these soils were lower than in some other soils without pea in the crop rotation during last 4 years (e.g. soils nr 2, 8, 10, 11 and 16). Nutman and Hearne (1979) detected relatively high populations of clover and field bean rhizobia (log 4.06-4.34) in soil under long-term cereal monocultures but alfalfa rhizobia almost disappeared in the absence of the host (alfalfa). Similar results were reported by Martyniuk at al. (1999). Table 4 shows correlation coefficients between the estimated numbers of rhizobia in the studied soils and some physico-chemical characteristics of these soils. Numbers of *R.l.t* were significantly correlated with clay, C org. and total N content but not with soil pH (in KCl). In contrast, numbers of *R.l.v.* showed significant correlation only with the pH of the soils. Amarger (1980) assessed populations of rhizobia in different soils and found that soil pH markedly influences the persistence of these bacteria in soil, particularly those of *S. meliloti* and *Bradyrhizobium* sp. (*Lupinus*). Results presented here indicate that other soil properties also modify populations of rhizobia and that different species of these bacteria respond differently to soil conditions. Further studies are investigating populations of several species of root-nodule bacteria in numerous soils of Poland and we found out that the plant infection-sand pouch technique is suitable for this purpose.

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NOWA TECHNIKA OSZACOWANIA LICZEBNOŚCI RHIZOBIÓW W GLEBIE

STRESZCZENIE

Opisano nowy biotest do oszacowania liczebności rhizobiów w glebie. W bioteście tym siewki roślin motylkowatych rosnące w woreczkach z folii polipropylenowej zaszczepiane są różnymi rozcieńczeniami badanej gleby. Badania porównawcze wykazały, że liczebności *Rhizobium leguminosarum* bv. *trifolii* w glebie, oznaczone za pomocą biotestu w woreczkach z piaskiem oraz biotestu w probówkach szklanych, nie różniły się istotnie. Opisany biotest wykorzystano od oceny populacji *R. leguminosarum* bv. *trifoli* (*R.l.t.*) i bv. *viciae* (*R.l.v.*) w 20 glebach Polski. Liczebności *R.l.t.* (wyrażone w logarytmach) w badanych glebach wahały się od 0 do 4,76 a *R.l.v.* od 2,23 do 5,84 w g^{-1} suchej masy gleby. Liczebności *R.l.t.* były istotnie skorelowane z zawartościami C org. i N całkowitego w glebach, a także z odczynem (pH w KCl) tych gleb. W przypadku bakterii brodawkowych *R.l.v.* istotną korelację stwierdzono tylko pomiędzy ich liczebnościami i pH gleb.

SŁOWA KLUCZOWE: *Rhizobium leguminosarum*, liczebność, koniczyna czerwona, groch, brodawkowanie, gleba.