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Survival of Rhizobia in Two Soils as Influenced by Storage Conditions

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Abstract

Two soils were kept moist at 4°C, –20°C or air-dried at 20–22°C and after one week, one month, two months and six months of storage at these conditions changes in soil populations of *Rhizobium leguminosarum* bv. *trifolii* (*Rlt*) and *Rhizobium leguminosarum* bv. v*iciae* (*Rlv*) were examined. In one air-dried soil (from Grabów) markedly lower numbers of both *Rlt* and *Rlv*., as compared to the refrigerated or frozen samples, were found already after 1 week of storage. In the case of the second soil (from Osiny) air-drying significantly reduced numbers of the rhizobia after 2 and 6 months of storage. The soil from Osiny contained higher amounts of C org, total N and clay than the Grabów soil. Both soils stored moist in a refrigerator (4°C) or frozen (–20°C) retained similar populations of the examined rhizobia throughout the entire storage period, indicating that soil freezing is not detrimental for the examined rhizobia.

K e y w o r d s: rhizobia, storage conditions, , survival in soil

Root-nodule bacteria, commonly known as rhizobia, represent an important group of soil bacteria that are able to fix atmospheric nitrogen in the symbiosis with roots of leguminous plants. Between symbiotic phases rhizobia survive in soil as saprophytes and their populations depend on many physical and chemical properties of the soil environment and on the frequency of planting of legumes in a given area or field (Nutman and Hearne, 1979; Amarger, 1980; Sadowsky and Graham, 1998; Martyniuk *et al.*, 1999). Although rhizobia are culturable on various synthetic or semi-synthetic media there is generally no selective medium available for making plate counts of these bacteria in soils and in other contaminated materials. For these reasons a serial soil dilution-plant infection method is most widely used to estimate most probable numbers (MPN) of native or introduced rhizobia in soils (Weaver and Frederick, 1972; Nutman and Hearne, 1979; 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 4–6 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). As indicated above this method is rather a long-lasting one and for this reason soil samples need to be stored for a longer period of time, *e.g.* in case of a test failure. Storage of soil samples is also necessary when a complex biological characterisation of soil is planned. While relatively abundant data are available on the effects of storage conditions on such soil parameters as: respiration, microbial biomass C, or enzymes activities (Ross *et al*., 1980; Shan-Min *et al*., 1987; Zelles *et al*., 1991) little is known about the survival of various species of microorganisms in soils stored at various conditions. In this work changes in populations of *Rhizobium leguminosarum* bv. *trifolii* (*Rlt*) and *Rhizobium leguminosarum* bv. *viciae* (*Rlv*) in two soils stored moist at 4°C, –20°C or air-dried at 20–22°C were examined after one week, one month, two months and six months of storage.

In August of 2006 after winter wheat harvest two field soils (Albic Luvisols) were sampled for the purpose of this study. The first soil (loamy sand) having

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the following basic characteristics: pH(H_2O) = 7.0; 0.7% C org.; 0.08% total N; 7% clay, originated from an experimental field located in Grabów Experimental Station (51°20' N; 21°39' E) of the Institute of Soil Science and Plant Cultivation (ISSPC). The second soil (loamy sand: pH(H_2O) = 6.7; 1.05% C org.; 1.2% total N; 9% clay) was sampled from a field in Osiny Experimental Station (51°27' N; 22º2' E) of ISSPC. On Grabów soil the following crops are grown in rotation: peas-winter wheat-potato-spring barley and Osiny soil is planted to: red clover grass mixture-winter wheat-potato-spring barley. Bulked samples of both soils consisted of twenty soil cores (3 cm × 25 cm) collected throughout the fields. The same day in the laboratory the bulked samples were thoroughly mixed, put through a sieve with 2 mm openings and than divided into three 0.5 kg sub-samples. The sub-samples designated to be air-dried were spread on sterile pieces of aluminium foil on a laboratory bench at room temperature. After one week of drying the soils were put into zipped bags and stored at room temperature (20–22°C) in the dark. Other 0.5 kg sub-samples of the soils (field moist) were stored in a refrigerator at 4°C. The sub-samples designated to be frozen were further divided into five samples, each weighing 100 g, and stored under freezing at –20°C. The frozen samples were stored in the zipped plastic bags and the refrigerated samples were kept in similar bags, which were slightly folded to facilitate gas exchange. Next day the refrigerated samples were used to perform the first assessment of rhizobial counts in the soils (day 0). Further samplings and analyses were done after one week, one month, two months and six months of storage. At each sampling time the frozen soils were removed from a freezer 24 hours before examination and allowed to thaw under room temperature.

A sand pouch-plant infection method was used to assess most probable numbers (MPN) of the rhizobia in the studied soils (Martyniuk *et al*., 2000). Shortly, in this method seedlings of the test leguminous plants

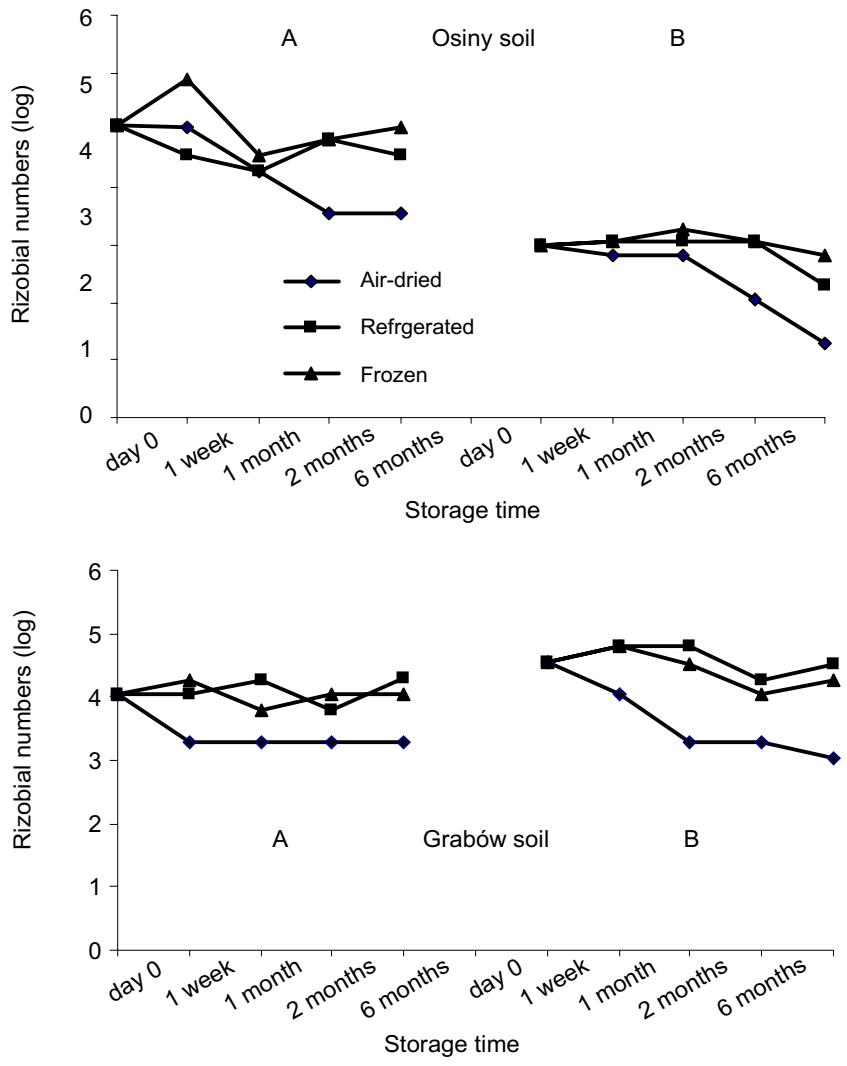

Fig.1. Changes in the numbers (log) of *Rhizobium leguminosarum* bv. *trifolii* (A) and *R. leguminosarum* bv. *viciae* (B) in two soils as influenced by storage conditions (95% confidence limit = 0.58)

(red clover and pea) were grown under semi-aseptic conditions in plastic pouches filled with sterile sand moistened with N-free nutrient solution to support plant growth. The seedlings were inoculated with 1 ml of 10-fold soil dilutions in water and grown in a plant growth chamber (Hereus HPS). In these tests we used six soil dilution steps (from 10^{-1} to 10^{-6}) and four replicated pouches (seedlings) for each dilution. The first soil dilution (10^{-1}) was prepared by weighting 10 g samples of the soils (at each sampling time) into 500 ml flasks containing 90 ml of sterile water followed by agitation for 15 min on a rotary shaker. The growth chamber was set at a 16 h/8 h light-dark regime and at 22°C day/15°C night temperature. After 4–6 weeks of growth the roots of the seedlings were gently washed in tap water and inspected for the presence of nodules in each dilution and the total number of positive cases counted. Based on these scores most probable numbers (MPN) of the rhizobia and 95% confidence limit were calculated (Vincent, 1970). The numbers of the rhizobia were $\log_{10}$ transformed for making graphs.

At the time of sampling (day 0) the soil originating from the field in Osiny Experimental Farm contained significantly higher populations of *Rlt* – rhizobia nodulating red clover, than *Rlv* – rhizobia nodulating vetch, peas and faba-been (Fig. 1). The opposite was true for the second soil used in this study, which was collected from a field in Grabów Experimental Farm. These soils differed mainly with respect to the cropping history. On Osiny soil for more than ten years a red clover-grass mixture has been grown in rotation with other non-leguminous crops, while on Grabów soil pea is included in the crop rotation. Cultivation of the host-plant is one of the most important factor influencing population densities of rhizobia in soils (Nutman and Hearne, 1979; Amarger, 1980; Sadowsky and Graham, 1998; Martyniuk *et al.*, 2005).

Of the storage factors studied in this work, air-drying had generally negative effect on the survival of the rhizobia in the soils during their storage (Fig. 1). In the air-dried samples of Grabów soil markedly lower numbers of both *Rlt* and *Rlv*, as compared to the refrigerated or frozen samples, were found already after 1 week of storage. In the case of Osiny soil storage conditions generally did not have significant influence on the numbers of the rhizobia surviving in this soil up to one month of storage, but after two and six months of storage numbers of the root-nodule bacteria detected in the air-dried Osiny soil were generally significantly lower than those in the frozen or refrigerated samples of this soil (Fig. 1). These differences could be related to slightly different physico-chemical characteristics of the soil used in this study. Osiny soil was richer in clay and organic matter contents as compared to Grabów soil. It has been shown that these soil parameters can protect soil microorganisms, including rhizobia, against soil desiccation (Marshall, 1964; Bushby and Marshall, 1977). Higher contents of clay and organic matter (C org.) in Osiny soil could result in better survival of the tested rhizobia in the air-dried samples of this soil during the first month of storage (Fig. 1).

The soils stored moist in a refrigerator (4°C) or frozen (–20°C) retained similar populations of the examined rhizobia throughout the entire storage period (Fig. 1). Although only two biovars of rhizobia were examined in this study, the obtained results indicate that soil freezing is not detrimental for these bacteria, and that when a longer storage of soil samples for microbial analyzes is needed the samples should be kept moist at 4°C or frozen at –20°C. Zelles *et al.* (1991) kept moist soils at 4, –18 and –140°C or air-dried at 21°C and examined changes in various indicators of soil microbial activity (ATP content, heat output, FDA hydrolysis) after 1, 2 and 20 months of storage at these conditions. They concluded that while air-drying caused a significant decrease in the soil microbial activity, the changes in the microbial indicators for soils stored at 4°C or at freezing were in most cases insignificant. These findings are therefore in accord with the results obtained in our study.

In conclusion, results of this study indicate that soils stored moist in a refrigerator (4°C) or frozen (–20°C) can retain similar populations of rhizobia (*Rlt* and *Rlv*) for at least 6 months of storage, while storage of air-dried soil samples at room temperature can markedly reduce surviving populations of the rhizobia.

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