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Carrier formulations and their effect on rhizobial growth

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Abstract

Effects of 15 carrier formulations on the growth of 4 strains of Bradyrhizobium japonicum, 3 strains of Shinorhizobium meliloti, 8 strains of Rhizobium leguminosarum (3 of which bv. phaseoli, 3 bv. viciae and 2 bv. trifolii), 2 strains of Rhizobium loti and 2 strains of Rhizobium galegae, all part of a collection of the Soil Science Institute, have been investigated and compared to control. The formulations examined were found to have different effects on the growth of strains belonging to different, as well as identical species of rhizobia. The peat+maize meal carrier was found to have the highest stimulating effect on the growth of B. japonicum, R. leguminosarum bv. phaseoli and bv. viciae, R. loti and R. galegae strains applied, compared to control, while negative effect was recorded for the peat+soybean meal, where no rhizobia were found present in the 10^{-7} inoculant dilution of some strains of rhizobia. The most remarkable stimulating effect on the growth of S. meliloti strains, compared to control, was found for the carrier peat+lucerne meal, and the most negative effect for the peat+Agrostemin+Minazel and the peat+Agrostemin+charcoal carriers. In contrast to R. leguminosarum bv. phaseoli and bv. viciae strains, the highest stimulating effect on the growth of R. leguminosarum bv. trifoli strains, compared to control, was found for the carrier peat+Minazel+charcoal, while the peat+soybean meal, the peat+maize meal, and the peat+Agrostemin had negative effect on their growth.

Keywords: carrier formulation, growth, rhizobia strains, moist inoculant form.

Introduction

Biological fixation of atmospheric nitrogen is very important as one of the main sources of replenishment of deficient N in the soil. Nitrogen deficiency in the soil is one of the main limiting factors in plant production as N is one of the most important biogenic elements in plant nutrition, being a crucial component of proteins and other vital compounds.

Symbiosis between nodule-forming bacteria (rhizobia) of the genera *Rhizobium, Sinorhizobium* and *Bradyrhizobium* and legumes has a special place in the process of biological nitrogen fixation. Rhizobia in symbiosis with the host plant are able to fix atmospheric N and thus make the plant less dependent on soil N and mineral nitrogen fertilization. This property of rhizobia is widely exploited in practice for producing microbiological N fertilizers, which are microbiological N-inoculants with rhizobial species as the active agents specific to different leguminous plants.

The basic problem in acquiring a quality microbiological N-inoculant is the high titer of effective rhizobia in terms of nodulation and nitrogen fixation of the host plant. It is therefore essential to find a nutrient medium, pH, temperature and aeration degree optimal for their growth in fermenting units. It is also important to choose the most appropriate carrier for moist or granular forms of inoculant in order to acquire or preserve the high rhizobial titer needed for inoculation of legumes. Regardless of the ultimate form of inoculant, the technological procedure for acquiring it includes the following stages: maintenance of rhizobial cultures, choosing a most appropriate form of inoculant (moist, liquid, granular), acquiring a quality inoculum broth, choosing a most appropriate carrier and preparing it, inoculant maintenance and quality control of an inoculant as a finished product.

First artificial inoculants were liquid bacterial cultures, whose application dates back in 1932 when they were either used to treat leguminous seeds or incorporated directly in soil, and their use expanded rapidly in the United States, especially in soybean crops due to easy mechanical application to that crop [1]. It was found out soon; however, that peat inoculant was superior to the culture broth, as the culture broth has no protection effect for the rhizobia as does the peat during seed inoculation [2, 3, 4, 1, 5, 6, 7]. Use $CaSO_4$ granules impregnated with rhizobial cultures as inoculants, but the product failed to secure widespread application [8]. Freeze-dried rhizobia cultures or a mixture of talc and freeze-dried culture were used as commercial inoculants in Australia for several years [9, 1, 10]. The main disadvantage of freeze-dried inoculants is their incapacity to protect post-inoculation survival of rhizobia on seeds [11, 12]. A most remarkable breakthrough in inoculation production was achieved by using peat as the carrier enriched with other nutrients. Those nutrient supplements included: lucerne meal, straw, yeast, sucrose [13, 14]; soybean meal and charcoal [15]; Nile mud [16]; vermiculite, sawdust, perlite, rice husk [17]; maize cob, bentonite [18, 19]. In some countries, however, peat is not suitable as a carrier and is therefore replaced by charcoal, sugar cane meal, filtered mud, vermiculite, polyacrylamides, mineral salts, vegetable oils or plant residues [20, 21].

The objective of this investigation was to identify the most suitable type of carrier for the moist form of inoculant to enable optimal growth (high titer) and long survival of effective rhizobial strains verified for the production of quality microbiological nitrogen inoculant intended for specific legumes.

Material and Methods

Mineralized peat of pH 6.5-7.0 and organic matter content >50% was used in this investigation as the main type of carrier for producing the moist form of inoculat for particular legumes. Before use, peat was dried to 7-8% moisture, ground and sieved to 0.02-0.04 mm parcile size. Prepared this way, it was mixed with other materials at weight ratios shown in tables (**1** and **2**). The following carrier formulations were examined: peat+perlite (control); peat+lucerne meal; peat+soyben meal; peat+maize meal; peat+Agrostemin; peat+talc; peat+Minazel; peat+charcoal; peat+maize cob; peat+ashes; peat+lucerne meal+Agrostemin; peat+lucerne meal+Minazel; peat+lucerne meal+charcoal; peat+Agrostemin+Minazel; peat+Agrostemin+charcoal; peat+Minazel+charcoal. The peat supplemented with these other materials was packed into polyethylene bags 40-50 μm thick that allow gas exchange and minimal moisture loss, and then sterilized with 50 kGy gamma radiation. The investigation included 4 strains of *Bradyrhizobium japonicum* (525, 526, 532 and 542); 3 strains of *Sinorhizobium meliloti* (224, 234 and 236); 8 strains of *Rhizobium leguminosarum*, of which 3 were bv. *phaseoli* (110, 131 and 134), 3 bv. *viciae* (316, 318 and 321) and 2 bv. *trifolii* (461 and 464); 2 strains of *Rhizobium loti* (612 and 615) and 2 strains of *Rhizobium galegae* (801 and 802), all part of the Soil Science Institute collection. Inoculum broths of the rhizobial strains tested were obtained in fermenting units on sterile YMB by adding their respective cultures from Erlenmayer flasks and allowing them to incubate further at 26ºC, aerated in 5 l sterile air per l of medium per h. Over the 48 h phase of exponential growth of fast-growing rhizobia, and 72 h phase of slow-growing bacteria, the inoculation of polyethylene bags containing sterile carriers at a ratio of 100 ml of inoculant per 200 g of carrier was done aseptically so as to provide 55% moisture of the ready-to-use inoculum. Inoculum density at leaving the fermenting unit varied from 1.12 to1.22$\cdot 10^9$ ml^{-1} rhizobia depending on strain and species. Rhizobial titer in the moist form of inoculant obtained was determined after the begs were incubated for 2–3 weeks at 26º C according to the method of decimal solution from 10^{-1} to 10^{-7} on Congo red YMA medium with 12 replicates.

Results and Discussion

The main carrier component used in this study was mineralized peat, the same one used in a microbiological N-inoculant for legumes, i.e. a product of the Soil Science Institute named ″Azotofiksin″ – and for the following reasons: it is widespread in our country, non-toxic to rhizobia, inexpensive, has a high organic matter content (>50%), high moisture absorption capacity (300 $m^2 \cdot g^{-1}$), it is suitable for sterilization by autoclaving and γ-radiation, has a good adhesion capacity for seed inoculation and an appropriate pH of 6.5-7.0.

Growth of unicellular microorganisms, including rhizobia, can be measured using two different parameters: cell mass or cell number, which may not coincide. The mass of individual cells may vary in different stages of growth and increases continuously over time, while increase in cell numbers occurs at intervals and results from their successive partition at particular time intervals. Generation time of the fast-growing rhizobia is 2-4 h, and of slow-growing 6-8 h. In this study, growth of the rhizobial strains applied to peat as the main carrier supplemented with different other materials was determined according to increase in their cell numbers (titre). The results of this research shown in tables (**1** and **2**) clearly indicate different effects of the carrier formulations applied on the growth of rhizobial strains under investigation.

Regarding rhizobial numbers, the carrier peat+maize meal was found to have the highest stimulating effect on the growth of *B. japonicum* strains tested as the titer increased against control from 1.77 times in strain 542 to 3.55 times in strain 526, varying from 976.25-2626.25$\cdot 10^7$ rhizobia per gram of inoculant. Good growth of these strains was found also on the carriers: peat+charcoal, peat+Agrostemin+charcoal, peat+ashes and peat+talc. Negative effect on their growth, compared to control, was found for the carrier peat+soybean meal, whose titer ranged from 6.87$\cdot 10^7 \cdot g^{-1}$ in strain 532 to 27.50$\cdot 10^7$ rhizobia per gram of inoculant in strain 526. Weak growth of these strains, compared to control, was found also for the carrier peat+lucerne meal, peat+lucerne meal+Minazel and peat+lucerne meal+charcoal. The carriers applied showed different effects on the growth of *B.japonicum* strains tested. For example, the carrier peat+lucerne meal+Agrostemin had a stimulating effect on the growth of strain 525, but not other *B.japonicum* strains, while peat+talc stimulated the growth of strains 525, 532 and especially 542, but not strain 526.

The carrier peat+lucern meal+charcoal had the highest stimulating effect of all tested carriers on the growth of *S.meliloti* strains, whose titer increased, compared to control, from 2.38 times in strain 236 to 6.11 times in strain 224, varying from 275.20-1375,00 $\cdot 10^7$ rhizobia per gram of inoculant. Good growth of strains 224 and 234 was found on most carriers applied, in contrast to strain 236, whose growth was significantly weaker on most carriers, compared to control, except peat+maize meal. Negative effect on the growth of *S.meliloti* strains, compared to control, was recorded for the carriers peat+Agrostemin+Minazel, and peat+Agrostemin+charcoal.

Effects of the carriers applied on the growth of *R.leguminosarum* bv. *phaseoli* strains tested varied. The carrier peat+maize meal had the highest stimulating effect on their growth with titer increasing against control from 1.32 times in strain 110 to 9.2 times in strain 134, and varying from 330.62-805.00$\cdot 10^7$ rhizobia per gram of inoculant. Stimulating effect on the growth of these strains, especially strain 110, was found also for the carriers peat+lucerne meal+charcoal and peat+ashes. Negative effect on the growth of these strains, compared to control, was found for the carrier peat+soybean meal, where no presence of rhizobial strain 131 was found at inoculant dilution 10^{-7}.

The carriers applied had different effects on the growth of different strains tested. The carrier peat+lucerne meal+charcoal had a more stimulating effect on the growth of strain 110 ($575.00 \cdot 10^7 \cdot g^{-1}$); peat+maize cobs on the growth of strain 131 ($345.00 \cdot 10^7 \cdot g^{-1}$) and peat+lucerne meal+Minazel on the growth of strain 134 ($230.00 \cdot 10^7 \cdot g^{-1}$). We may assume that the difference in growth between these different strains resulted from an interaction between carrier and rhizobial strain caused by each carrier's chemical composition and the biochemical and genetic characteristics of strains within identical rhizobial species.

The growth of *R.leguminosarum* bv. *viciae* strains tested on the carriers applied differed as well. The carrier peat+maize meal was found to have the highest stimulating effect on their growth with titer increasing against control from 1.28 times in strain 321 to 3.90 times in strain 316, and varying from 225.25-459.00 10^7 rhizobia per gram of inoculant. Good growth of these strains was recorded also for the carrier peat+charcoal. Strain 321 had better growth on most carriers applied than on the control carrier, in contrast to strains 316 and 318, whose growth was considerably weaker. Negative effect on the growth of these strains, compared to control, was recorded for the carriers: peat+soybean meal, peat+Agrostemin, peat+Minazel, peat+ashes, peat+Agrostemin+Minazel, peat+Agrostemin+charcoal and peat+lucerne meal+charcoal. No strain 321 rhizobia were found on the carrier peat+soybean meal at inoculant dilution 10^{-7}.

In contrast to *R. leguminosarum* bv. *phaseoli* and bv. *viciae* strains, the highest stimulating effect on *R. leguminosarum* bv. *trifolii* strains tested was found for the carrier peat+Minazel+charcoal with titer increasing against control from 2.11 times in strain 464 to 10.62 times in strain 461, and varying $142.50\text{-}810.00 \cdot 10^7$ rhizobia per gram of inoculant. Good growth of these strains, especially strain 461, was recorded also on the following carriers: peat+lucerne meal+Minazel, peat+lucerne meal, peat+charcoal. Strain 461 had weaker growth on most carriers applied than on control. Negative effect on the growth of these strains was found for the carriers: peat+soybean meal, peat+maize meal, peat+Minazel, peat+lucerne meal+Agrostemin. No presence of rhizobial strain 464 was found in inoculant dilution 10^{-7} on the carrier peat+soybean meal.

The carrier peat+maize meal had the highest stimulating effect on the growth of *R. loti* strains tested with titre increasing against control from 3.38 times in strain 615 to 4.23 times in strain 612, which is $1003.75\text{-}1141.75 \cdot 10^7$ rhizobia per gram of inoculant. Most of the carriers applied had stimulating effect on the growth of both strains applied, compared to control. Rhizobial strain 615 was not found on the carrier peat+soybean meal in inoculant dilution 10^{-7}.

The carriers examined were found to have the least stimulating effect on the growth of *R. galegae* strains, compared to all other rhizobial species tested. The following carriers were found to have stimulating effects on *R. galegae* strains: peat+lucerne meal+Minazel, peat+maize cob, peat+maize meal, peat+lucerne meal+charcoal and peat+Agrostemin + Minazel. No rhizobia were found present on the carrier peat+soybean meal in inoculant dilution 10^{-7}. Effects of the carriers applied on the growth of these strains differed. Strain 802, in contrast to strain 801, had weaker growth on most carriers applied, compared to control.

Numerous reports have shown different effects of carriers on the growth not only of strains of different rhizobial species but different strains of the same species as well [4, 12, 15, 22, 23, 24, 18, 25, 26, 27]. It may be assumed that the differences in growth found for the strains tested in this study are the result of different interactions between carriers and rhizobial strains, which are caused by the chemical composition of the carrier, and biochemical and genetic characteristics of strains belonging not only to different rhizobial species but to identical species as well.

It is important to stress that the quality of inoculant depends on rhizobial numbers and their effectiveness. One hundred rhizobia per seed is a sufficient number for a satisfactory inoculum intended for soils with good physico-chemical properties containing no legume-specific natural rhizobial population [22]. When soil contains a large number of ineffective rhizobia and has unfavourable conditions for their development, then the requirement per seed is 10^6. The amount of inoculant for different legumes varies depending on seed size and their number per kg. For example, a required inoculant amount for clover, lucerne and soybean is 5 $g \cdot kg^{-1}$ seed, while a corresponding number of rhizobia per seed for clover is $2.5\text{-}3.0 \cdot 10^3$; for lucerne $1.0\text{-}2.0 \cdot 10^4$ and for soybean $1.0\text{-}2.0 \cdot 10^6$ [21].

Conclusion

The carrier formulations examined showed different effects on the growth of strains belonging to different as well as identical rhizobial species.

The carrier peat+maize meal had the highest stimulating effect on the growth of *B. japonicum* strains examined, compared to control, with titre varying from $976.25 \cdot 10^7 \cdot g^{-1}$ in strain 542 to $2626.25 \cdot 10^7 \cdot g^{-1}$ rhizobia per gram of inoculant in strain 526. Negative effect on their growth, compared to control, was recorded for the carrier peat+soybean, whose titer ranged from $6.87 \cdot 10^7 \cdot g^{-1}$ in strain 532 to $27.50 \cdot 10^7$ rhizobia per gram of inoculant in strain 526.

In contrast to *B. japonicum* strains, the highest stimulating effect on the growth of *S. meliloti* strains tested, compared to control, was found for the carrier peat+lucerne meal+charcoal, whose titer varied from $275.20 \cdot 10^7 \cdot g^{-1}$ in strain 236 to $1375.00 \cdot 10^7$ rhizobia per gram of inoculant in strain 224. Negative effect on their growth, compared to control, was found for the carriers peat+Agrostemin + Minazel and peat+Agrostemin+charcoal.

Regarding *R. leguminosarum* bv. *phaseoli* and bv. *viciae* strains tested, the highest stimulating effect on their growth, compared to control, was found for the carrier peat+maize meal. The titre of *R. leguminosarum* bv. *phaseoli* strains varied from $330.62 \cdot 10^7 \cdot g^{-1}$ in strain 110 to $805.00 \cdot 10^7$ rhizobia per gram of inoculant in strain 134, while the titre of *R. leguminosarum* bv. *viciae* strains was from $255.25 \cdot 10^7 \cdot g^{-1}$ in strain 321 to $459.00 \cdot 10^7$ rhizobia per gram of inoculant in strain 316. Negative effect on the growth of *R. leguminosarum* bv. *phaseoli* and bv. *viciae* strains tested, compared to control, was found for the carrier peat+soybean meal, where no presence of strains 131 and 321 was found in inoculant dilution 10^{-7}.

In contrast to *R. leguminosarum* bv. *phaseoli* and bv. *viciae* strains, the highest stimulating effect on the growth of *R. leguminosarum* bv. *tifolii* strains, compared to control, was found for the carrier peat+Minazel+charcoal, whose titre varied from $142.50 \cdot 10^7 \cdot g^{-1}$ in strain 464 to $810.00 \cdot 10^7$ rhizobia per gram of inoculant in strain 461. Negative effect on the growth of these strains, compared to control, was found for the carrier peat+soybean, where no presence of rhizobial strain 464 was found in inoculant dilution 10^{-7}.

The carrier peat+maize meal had the highest stimulating effect on the growth of *R. loti* strains, compared to control, with titer varying from $1003.75 \cdot 10^7 \cdot g^{-1}$ rhizobia in strain 612 to $1141.75 \cdot 10^7$ rhizobia per gram of inoculatn in strain 615. On the carrier peat+soybean meal, no rhizobial strain 615 was found in inoculant dilution 10^{-7}.

Of all examined rhizobial species, the carriers tested were found to have the least stimulating effect on *R.galegae*, compared to control. Stimulating effect on their growth, compared to control, was found for the carriers: peat+lucerne meal+Minazel, peat+maize cob, peat +maize meal, peat+lucerne meal+charcoal and peat+Agrostemin+Minazel. No rhizobia were found on the carrier peat+soybean meal in inoculant dilution 10^{-7}.

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