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Residues of the Cork Industry as Carriers for the Production of Legume Inoculants

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Abstract. Growth and survival of two rhizobial strains, *Rhizobium leguminosarum* bv. *trifolii*, and *Mesorhizobium ciceri*, were used to evaluate residues from the cork industry as potential carriers for production of rhizobial inoculants. A peat-based carrier was used as a control. 41 days after inoculation, the number of viable bacteria was high, about 10^9 bacteria g^{-1} for all carriers. Rhizobial survival during storage (450 days) remained stable, between 10^8 and 10^9. The cork residues based carriers showed good handling properties and water holding capacities, they were non-toxic and had a nearly neutral pH. All these factors indicate that the use of these carriers is viable for the production of rhizobial inoculants.
Key words: carriers; cork industry; legume inoculants; *Rhizobium*

Resíduos da Indústria dos Aglomerados da Cortiça como Potenciais Substractos para a Produção de Inoculantes para Sementes de Leguminosas

Sumário. O crescimento e a sobrevivência de duas estirpes de *Rhizobium*, uma de *Rhizobium leguminosarum* bv. *trifolii*, e outra de *Mesorhizobium ciceri*, foram usados para avaliação de resíduos da indústria dos aglomerados da cortiça como potenciais substractos para a produção de inoculantes para sementes de leguminosas. A turfa foi usada como controlo. 41 dias após a inoculação, o n.º das bactérias viáveis era elevado e semelhante em todos os substractos, aproximadamente 10^9 bactérias g^{-1}. A sobrevivência durante os 450 dias de armazenamento permaneceu estável, entre 10^8 e 10^9. Os resíduos da cortiça mostraram possuir boas características de manuseio, boas capacidades de retenção de água, não apresentaram toxicidade, possuíam pH perto da neutralidade, indicando poderem ser utilizados como substractos alternativos à turfa na produção de inoculantes para leguminosas..
Palavras-chave: inoculantes; resíduos da indústria da cortiça ; *Rhizobium*

Résidus de l'Industrie du Liège comme Substrats pour la Production des Inoculums pour les Légumineuses

Résumé. La croissance et la survivance de deux souches de bactéries, *Rhizobium leguminosarum* bv. *trifolii* et *Mesorhizobium ciceri*, ont été employées pour évaluer l'utilisation des résidus de l'industrie de la fabrication des agglomérés du liège comme potentiels substrats pour la production d'inoculants pour les semences des légumineuses. La tourbe a été utilisée comme témoin. 41 jours après inoculation, le nombre de bactéries viables était élevé et semblable pour tous les substrats, environ 10^9 bactéries g^{-1}. La survivance pendant le stockage, 450 jours, est

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restée stable, entre 10^8 et 10^9. Les résidus de l'industrie du liège ont montré avoir de bonnes propriétés de manipulation et de rétention d'eau. De même ils étaient non toxiques, pouvant être employés comme substrats possibles pour la production des inoculums rhizobiens.
Mots clés: inoculums; liège; résidus de l'industrie; *Rhizobium*

Introduction

Legume seeds inoculation with nitrogen-fixing root nodule bacteria, generically known as rhizobia, is an old agricultural practice used since the end of 19th century. It is a "success story" of applied Soil Microbiology, probably the largest and oldest experience of voluntary microbial release and dissemination in the environment (CATROUX *et al*., 2001). The inoculation of legume seeds with rhizobia had contributed to increase N_2 fixation and yield in legume crops, in situations where the natural N_2 fixation was not optimal and exogenous nitrogen fertilizers and were not applied.

Legume inoculants provide a carrier for transporting the rhizobial bacteria from the laboratory to the soil. A carrier must support rhizobial growth and survival over an acceptable time period. Other characteristics of a solid carrier should also include: high water-holding capacity, high water retention, physical uniformity, non-toxic, non-polluting, nearly neutral pH or easily adjustable, manageable in the mixing and packing operations, suitable for all rhizobia, adequate supply and reasonable cost (SMITH, 1992; STEPHENS and RASK, 2000).

Finely ground peat has been a standard carrier and the most commonly used carrier for the inoculant industry. However, peat is not available everywhere and the need to preserve ecosystems makes the extraction of peat undesirable in some countries and other potential materials are needed (DAZA *et al*., 2000).

Several alternative solid materials have been evaluated as inoculant carriers, such as: soil, vermiculite, perlite, ground rock phosphate, polyacrylamide gel, alginate, decomposed sawdust, coal, compost made from bagasse, sawdust, plant compost, farmyard manure, volcanic pumice, etc. (STRIJDOM and DESCHODT, 1976; EINNARSSON *et al*., 1993; BROCKWELL and BOTTOMLEY, 1995; STEPHENS and RASK, 2000). In this study our objective was to evaluate the possibility of using, as carriers for rhizobial inoculants, two residues from the manufacture of the agglomerated cork industry, a natural and renewable material, the coating of the trunk and branches of the cork oak tree (*Quercus suber* L.), following the common procedures used for production of peat-based inoculants.

Materials and methods

Rhizobia, carrier materials and preparation

Bacterial strains used in this work were: a Portuguese strain, *Rhizobium leguminosarum* bv. *trifolii* named 123Ts2a, used for the production of inoculants for *Trifolium subterraneum* and a *Mesorhizobium ciceri* strain, CP36 (ICARDA designation), used for production of inoculants for *Cicer arietinum*. Cultures were maintained on yeast mannitol agar (YMA) (VINCENT, 1970).

Three carriers were used: peat, used

as reference, and two residues from the manufacture of the agglomerated cork industry: "mould", a residue from cork granules manufacture and "dust", a residue from agglomerated cork manufacture.

The peat collected in Portugal has been used for production of rhizobial inoculants since 1965. After dried in the open air, the peat was screened to remove debris and milled in a hammer mill to allow the peat to pass a 250 μm sieve. As the peat was quite acid (pH - 4.5), finely ground Ca CO_3 was added to raise the pH to 6.5-7.5.

Residues from cork stoppers industry and corks of inferior quality, triturated, sieved and cleaned, originate cork granules and a rejection product, the mould, similar to an organic soil. The cork granules bounded together under heat and pressure constitute the agglomerated cork, usually used in building construction (insulator). From this technological process a residue known as dust from manufacture of the agglomerated cork (insulation cork board) is obtained, being normally used in agriculture (preparation of substrates) and as source of energy (burning).

Cork residues (mould and dust), after sieved using a 250 μm sieve, were used. Mixtures, on a dry weight basis, of a sieved (250 μm) calcaric soil and cork residues, at the ratios 1.5:1 (soil:mould) and 3:1 (soil:dust), were made to obtain mixtures with physical characteristics similar to the peat, allowing to follow the procedures used for production of peat-based inoculants. After soil addition the pH raised to 7.59 and 7.83 for mould and dust, respectively. Analyses of used materials are given in Table 1.

The carrier (inoculant) should be moist but not soggy, friable in texture, to facilitate handling and packing. Distilled water was added to obtain critical moisture content, well bellow moisture-holding capacity. Final moisture content was 45, 36 and 34% (wet weight basis) for peat, mould and dust, respectively.

Carriers were packed (100 g) in heat-sealed polyethylene bags, perforated with a pointed small needle and autoclaved for 1 hour at 121°C. After sterilization, bags were inoculated aseptically with 10 ml of rhizobial suspensions containing $7x10^8$ and 10^9 cells ml^{-1} in the log phase of growth, for strains 123Ts2a and CP36, respectively, being vigorously shaken to distribute the bacteria and incubated at 27°C for 41 days. During incubation, the bags were shaken by hand twice each week. After incubation, bags were transferred to a refrigerator (4°C) and the survival under low temperature was evaluated. The inoculants were maintained in the refrigerator in larger plastic bags to prevent desiccation. One g of each inoculant was removed from the bags periodically for viable-bacteria counts, by plating 10-fold serial dilutions on Congo-red-YMA. Duplicate samples were made for each inoculant. Populations were transformed to a logarithmic basis before statistical analysis (ANOVA). Statistical significance was determined at $p<0.05$, according Fisher's LSD.

Table 1 - Properties of soil, neutralized peat and cork residues

	Soil	Peat	Mould	Dust
pH (H_2O)	8.23	7.64	5.79	6.03
Total N (%)	0.14	1.00	0.66	0.60
Total C (%)	0.99	37.49	49.28	64.70
Total P (mg kg^{-1})	1127.00	20.00	56.00	104.00
Total K (mg kg^{-1})	3533.00	331.00	1150.00	1638.00

Water retention of the carriers

To evaluate the water retention capacity of the carriers, 50 g of each carrier, prepared for packing and sterilization, were placed in 160 ml open glass flasks, and maintained in a forced-air oven at 30°C, during 145 hours.

The moisture contents of inoculants (bags) were also evaluated at the end of the experiment (450 days after inoculation).

Cultural studies

Two expedite experiments were performed to search possible problems of toxicity of the carriers. For these studies, only the Portuguese rhizobial strain 123Ts2a was used, due to his importance for production of inoculants for *Trifolium subterraneum.*

The first experiment was based on the growth or survival of rhizobia in water extracts from peat, soil and based cork carriers (mould and dust). Extracts were obtained by shaking 20 g of dry material in 200 ml of distilled water during 24 hours, followed by a pre-filtration of the supernatant. The extracts were sterilised by a second filtration through 0.2 μm pore size membranes, capable of retaining the microorganisms. From each material two extracts were prepared: one, the original extract, using the distilled water, and another one by adding to the water suspension, the nutrients of the yeast mannitol broth (VINCENT, 1970). Tubes containing 6 ml of extract were inoculated with 0.1 ml of a rhizobial suspension containing 7.3 x 10^5 cells ml^{-1} and were shaken for 144 hours at 28°C. Viable rhizobial cells counts were determined by a serial dilution and plating on Congo-red-YMA. Duplicate samples were made from each extract.

The second experiment was based on the possible direct effects of the peat and the cork residues (mould and dust), sterilized by moist heat, on the rhizobia bacteria growth, when incorporated into Congo-red-YMA plates containing 10^4-10^5 rhizobial cells ml^{-1}.

Results and discussion

Growth and survival of rhizobial strains in peat and in cork residues based carriers

Viable rhizobia counts, in the peat and in the mould and dust based carriers, kept at 27°C, were determined 8, 20, 41, 160, 300 and 450 days after inoculation. The results obtained for strains 123Ts2a and CP36 are presented in Figures 1 and 2, respectively. During the incubation time, the strains showed significantly different growth rates with all carriers. Strain 123Ts2a showed to be more sensitive (Figure 1) than strain CP36 (Figure 2), when the cork residues were used, decreasing the size of the population after inoculation. However, 41 days after inoculation, these negative effects disappeared and the number of bacteria reached about 10^9 g^{-1} of carrier. Survival of both strains, in the 3 carriers, was maintained at high numbers during storage, the rhizobial numbers ranging between $5x10^8$ (dust+123Ts2a) and $4x10^9$ (mould+123Ts2a). These numbers fulfilled the criteria for acceptable quality of legume inoculants. Current minimum standards for rhizobial cells g^{-1} of inoculant vary from $5x10^7$ in Thailand (BOONKERD, 1991) to 10^9 in France (CATROUX, 1991) and Australia (THOMPSON, 1991). For each strain, survival was similar with the three

carriers, except for the strain 123Ts2a at the 160 days, the number of bacteria being significantly different ($p<0.05$) for all carriers. In general, strain CP36 had a better growth rate and maintained higher values during storage than strain 123Ts2a, showing a superior adaptation for growth and survival in different carriers.

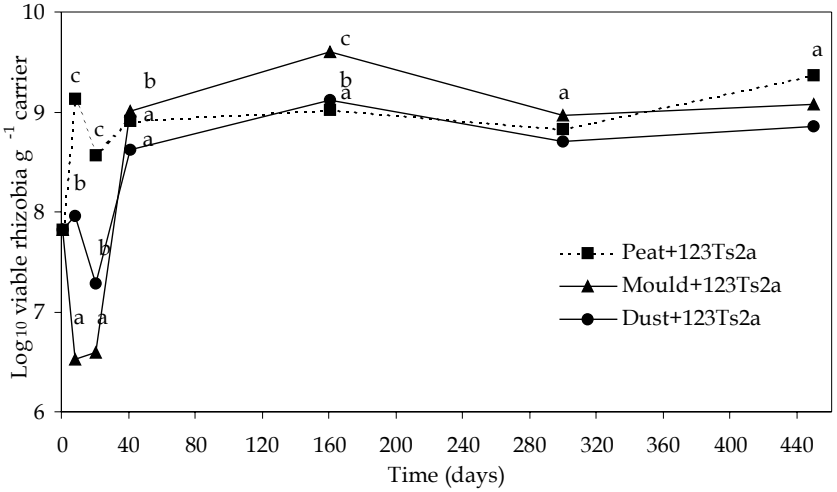

Figure 1 - Growth and survival of rhizobial strain 123Ts2a in peat and in cork residues based carriers. Values followed by different letters, within the same sampling day are significantly different

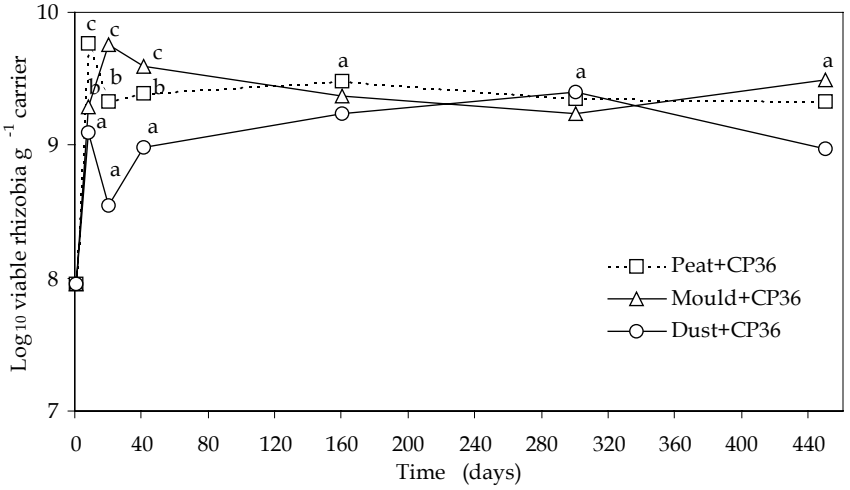

Figure 2 - Growth and survival of rhizobial strain CP36 in peat and in cork residues based carriers. Values followed by different letters, within the same sampling day are significantly different

Growth and survival of rhizobia in peat, in soil and in cork based carriers water extracts and toxicity of solid carriers

Numbers of rhizobia (strain 123Ts2a) evaluated 48 and 144 hours after inoculation, in water extracts and in the nutrient enriched water extracts, were quite different. In water extracts (Figure 3), 144 hours after inoculation, the rhizobial populations, were still higher than 10^4 bacteria ml^{-1} for peat extract and higher than 10^2 bacteria ml^{-1} for soil extract. In the cork-based extracts, mould and dust, the population decreased very quickly, and 144 hours after inoculation, viable cells were not detected. These results showed that the soil and the peat have an amount of nutritional water-soluble nutrients required by rhizobia superior than cork extracts. These results can be due to the fact that the nutrients in cork based carriers are in chemical forms not bioavailable. In nutrient enriched extracts (Figure 4), a general increase in rhizobial populations was observed during the experiment (144 hours) and a satisfactory viable rhizobial population, not significantly different, was reached (10^9-10^{10} bacteria ml^{-1}), supplying the yeast mannitol broth adequate nutrients for bacterial growth.

When the sterilized solid materials that constitute the carriers (peat, mould and dust) were incorporated into the Congo-red-YMA plates, containing rhizobial cells, their growth was not affected, indicating that the water soluble products of the carriers did not had negative effects on rhizobial growth.

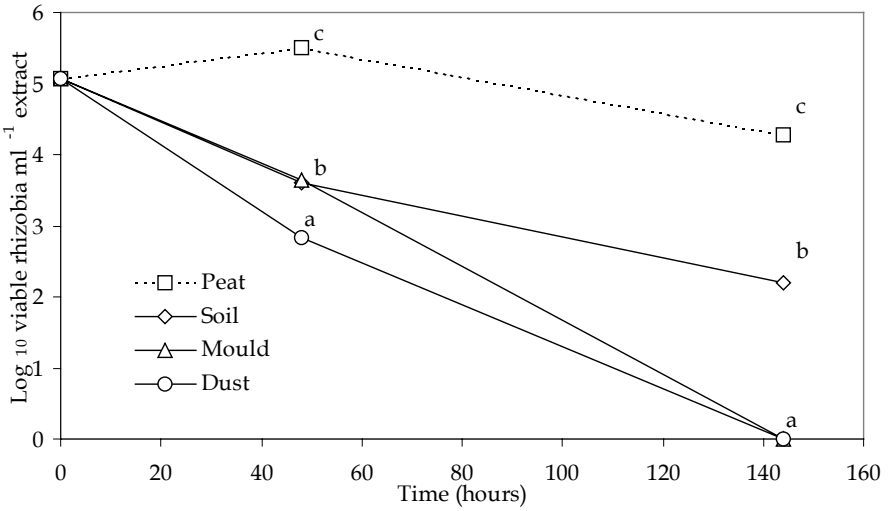

Figure 3 - Growth and survival of rhizobial strain 123Ts2a in peat, soil and cork residues water extracts. Values followed by different letters, within the same sampling time are significantly different

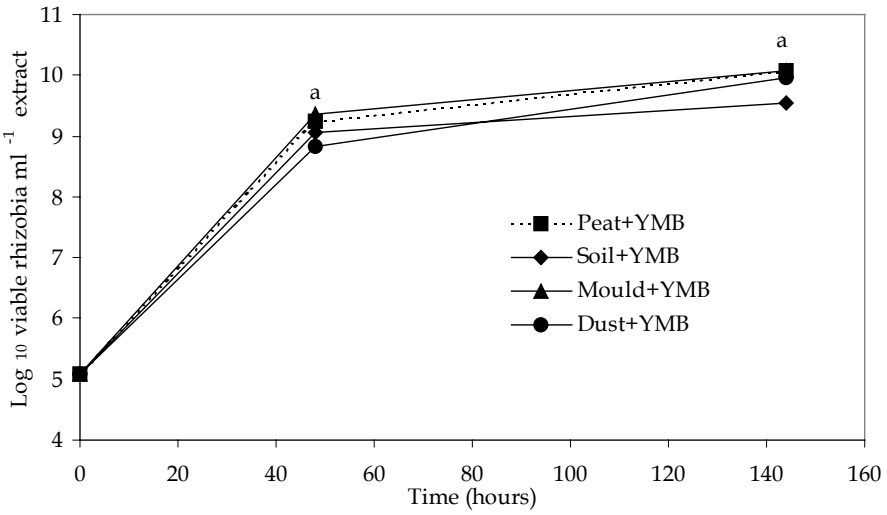

Figure 4 - Growth and survival of rhizobial strain 123Ts2a in peat, soil and cork residues nutrient enriched water extracts. Values followed by different letters, within the same sampling time are significantly different

Water retention of the carriers

The carriers, prepared with different moisture percentages to obtain critical moisture contents, 45, 36 and 34% (wet weight basis) for peat, mould and dust, respectively, showed to be different in their capacity for water retention. During the experiment (145 hours), the peat based carrier lost about 81% of the initial water content, whereas the mould and the dust based carriers lost about 91 and 96% respectively (Figure 5). The peat based carrier showed a slight water retention capacity than the cork-based carriers. These small differences in water retention could be due to the different physical properties of the original materials, and also to the different amounts of soil used for based cork extracts formulations.

In spite of this difference in water retention, which is an important characteristic of the inoculants for growth and survival of rhizobial bacteria (STEPHENS and RASK, 2000), the moisture content of the bags kept in the refrigerator, 450 days after inoculation, was 42, 39 and 35% (wet weight basis) for peat, mould and dust based carriers, respectively. SMITH, 1987; GRIFFITH and ROUGHLEY, 1992, reported, as adequate, values for moisture content ranging between 30 and 50%, according to different carriers and strains, showing that our results can be considered adequate for survival of *Rhizobium* bacteria.

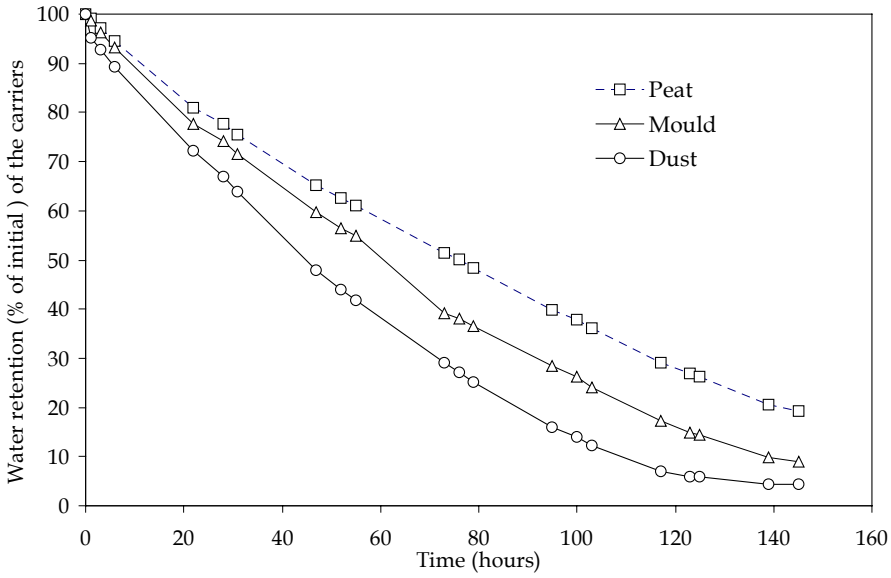

Figure 5 - Water retention (% of initial) of peat mould and dust based carriers, at 30ºC for 145 hours

Conclusions

The use of residues from cork industry as carriers for production of legume inoculants is possible. Our results showed that they fulfilled the main characteristics to be considered as good carriers: supported growth and survival (about 10^9 bacteria g^{-1} of carrier) of different rhizobial strains over a long time period, had a reasonable water-holding capacity and water retention, were non-toxic, nearly neutral pH and were suitable for the use of the common procedures for production of peat based inoculants. This new utilization of the residues from cork industry can be an alternative to the peat, increasing the economic value of an abundant and renewable Portuguese material, without environmental problems.

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