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Intrinsic antibiotic resistance of different *Bradyrhizobium Japonicum* and *Rhizobium Galegae* strains

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Abstract

Thirty-six strains of Bradyrhizobium japonicum and three strains of Rhizobium galegae selected from a collection of the Soil Science Institute were examined to study their intrinsic resistance to different concentrations of several antibiotics, namely: ampicillin, tetracycline, chloramphenicol, gentamicin, streptomycin, erythromycin, cephalexin, trimethoprim, clindamycin and penicillin. The results of this investigation clearly indicate a diversity among the strains tested regarding their intrinsic resistance to different concentrations of antibiotics. Most B.japonicum strains tested on YMA had good growth on clindamycin, trimethoprim and chloramphenicol in concentrations of 150-175 µg·ml^{-1}. The lowest degree of intrinsic resistance of most B.japonicum strains tested was expressed to the other antibiotics, especially cephalexin and erythromycin in concentrations ranging from 2-25 µg·ml^{-1}.

Unlike B.japonicum strains, those of R.galegae had no growth at all or weak growth on most antibiotics applied in concentrations of 2-10µg·ml^{-1}, except clindamycin and trimethoprim, to which they showed a high degree of intrinsic resistance in concentrations of 150-175 µg·ml^{-1}.

Cluster analysis (dendogram) based on the intrinsic resistance to antibiotics showed that all tested rhizobia strains formed two main groups with 48% dissimilarity, each of them consisting of several subgroups. The first group formed four subgroups at 80% disagreement. The other main group includes 29 strains arranged into five dissimilarity-based subgroups. R.galegae strains showed 37% dissimilarity with B.japonicum strains within the first group, while they all together had 48% dissimilarity with the other group.

Keywords: Antibiotics, growth, resistance, strains of *B.japonicum* and *R.galegae*.

Introduction

Resistance of nodule-forming bacteria (rhizobia) refers to their intrinsic resistance to antibiotics in terms of normal growth. Hartman and Amarger, 1991, [1] and Tas et al., 1996 [2] emphasized the intrinsic resistance to antibiotics of different rhizobial strains belonging to the same species as a significant phenotypic characteristic. Applying antibiotics, typified *Rhizobium* isolates according to their nodulation efficacy in leguminous plants [3, 4]. Intrinsic resistance to antibiotics was also used as a crucial characteristic in characterizing *Rhizobium* spp. (*Cicer arietinum*) [5]. Based on plasmid profile and intrinsic resistance to ampicillin, novobiocin and kanamycin, natural isolates of *R.meliloti* were determined [6]. A broad variation regarding susceptibility of individual rhizobial strains to antibiotics was found in different rhizobial species, as well as significanly higher susceptibility of the fast-growing than slow-growing rhizobia to chloramphenicol, tetracycline, penicillin, viamicyn**,** vancomycin and streptomycin [7, 6].

Mode of action of antibiotics against bacteria depends on the chemical composition of any antibiotic and on bacterial cell morphology, primarily cell walls. For example, penicillin and ampicillin are known to prevent the formation of cell wall, especially in Gram positive bacteria [8], while chloramphenicol, kanamycin, neomycin, streptomycin, tetracycline and

erythromycin prevent cell protein or membrane synthesis [6]. Neomycin also prevents RNA synthesis, while nistatine disturbes the functioning of cell membrane.

Different rhizobial strains show different degrees of susceptibility to antibiotics, which is why this property is being used for their identification [9]. The resistance of rhizobial strains (mutants) to antibiotics is being widely used in investigation of their survival in soil and for monitoring their compatitiveness as a 'label' for nodulation of the host plant and effectivenes of nitrogen fixation [10, 11, 12, 13, 14].

The objective of this investigation was to characterize 36 strains of *B.japonicum* and three strains of *R.galegae* selected from a collection of the Soil Science Institute based on their intrinsic resistance to different concentrations of antibiotics, namely to: ampicillin, tetracycline, chloramphenicol, gentamicin, streptomycin, erythromycin, cephalexin, trimethoprim, clindamycin and penicillin. Data showing the resistance of rhizobial strains against different antibiotic concentrations can be used in further research of their survival in soil, and for monitoring their compatitiveness in nodulation of host plants and effectiveness of nitrogen fixation.

Materials and Methods

Effects of ten antibiotics, namely: ampicillin, tetracycline, chloramphenicol, gentamicin, streptomycin, erythromycin, cephalexin, trimethoprim, clindamycin and penicillin on the growth of 36 strains of *B.japonicum* and three strains of *R.galegae* on YMA were examined. The following antibiotic forms and concentrations were tested: 250 mg capsules of ampicillin in sulphate form dissolved in sterile distilled water in concentrations of 5, 10, 20, 25, 50, 100 and 150 $\mu g \cdot ml^{-1}$; 500 mg capsules of tetracycline in chloride form dissolved in sterile distilled water in concentrations of 10, 25, 50, 100, 125, 150 and 175 $\mu g \cdot ml^{-1}$; 250 mg capsules of chloramphenicol dissolved in ethyl alcohol in concentrations of 10, 20, 50, 100, 125, 150 and 175 $\mu g \cdot ml^{-1}$; 80000 $iu \cdot ml^{-1}$ ampoule of gentamicin in sulphate form in concentrations of 2, 5, 25, 50, 75, 100 and 125 $\mu g \cdot ml^{-1}$; 100 $mg \cdot ml^{-1}$ injection ampoule of streptomycin in sulphate form in concentrations of 5, 10, 25, 50, 75, 100, 150 and 200 $\mu g \cdot ml^{-1}$; 250 mg capsules of erythromycin dissolved in ethyl alcohol in concentrations of 5, 10, 25, 50 and 100$\mu g \cdot ml^{-1}$; 500 mg capsules of cephalexin dissolved in a mixture of ethyl alcohol and sterile distilled water in concentrations of 2, 5, 25, 50 and 100$\mu g \cdot ml^{-1}$; 400 mg tablets of trimethoprim in sulphate form dissolved in sterile distilled water in concentrations of 10, 25, 50, 100, 125, 150 and 175 $\mu g \cdot ml^{-1}$; 250 mg capsules of clindamycin dissolved in ethyl alcohol in concentrations of 10, 25, 50, 100, 125, 150 and 175 $\mu g \cdot ml^{-1}$; 800000 $iu \cdot ml^{-1}$ injection of penicillin dissolved in sterile distilled water in concentrations of 10, 25, 50, 100, 125 and 150 $\mu g \cdot ml^{-1}$.

Sterilization of antibiotic solutions was done using 0.2 µm membrane filters. The solutions were kept in a freezer at -20ºC. Petri dishes containing YMA and appropriate antibiotic concentrations were prepared 24 h before use. The rhizobial strains were examined by adding their respective cultures, established on YMB medium with approximately 10^{-9} cells per ml, onto YMA media supplemented with appropriate antibiotic concentrations using multiple inoculator with 48 injectors, according to „Handbook for Rhizobia“ [15] and with four replicates. After incubation in air bath at 26ºC for 4-7 days, visual assessment of growth was carried out using three marks: + (good growth), ± (weak growth) and – (no growth), as compared to control.

Cluster analysis was done according to a UPGA method, using Euclidean distance to express dissimilarity between groups of the strains tested. Based on dissimilarity found

bewteen the strains, a dendogram was constructed determining the degree of similarity or dissimilarity between the strains investigated.

Results and Discussion

Slow-growing (*B.japonicum*) and fast-growing (*R.galegae*) rhizobia are rod-like cells 0.5-09. μm and 1.2-3.0 μm in length, respectively, asporogenous, Gram negative and motile with a single subpolar flagellum. They are mostly chemo-organotrophs growing well in the presence of O_2 and using relatively simple carbohydrates and amino compounds for growth. Fast-growing rhizobia acidify YMB media, while slow-growing rhizobia alkalize them.

Growth of unicellular microorganisms, including rhizobia, can be measured using two different parameters: cell mass and cells numbers, which need not coincide. Mass of individual cells may vary at different stages of growth and increases continuously over time, while cell number increase is a process with interruptions, resulting from the process of successive cell partitions at certain time intervals. Generation time for fast-growing rhizobia is 2-4 h, and 6-8 h for slow-growing. In this investigation, the growth of rhizobial strains applied to different antibiotic concentrations was visually determined based on cell mass increase. The results of our research shown in tables (**1** and **2**) clearly indicate a diversity among the strains investigated, especially *B. japonicum*, in terms of their intrinsic resistance or susceptibility to the antibiotic concentrations applied.

Most of the *B.japonicum* strains investigated had good growth on YMA supplemented with clindamycin, trimethroprim and chloramphenicol in concentrations 150-175 $\mu g \cdot ml^{-1}$. Especially high was the intrinsic resistance found in strains 520, 524, 527, 528 and 538 on clindamycin; 528 and 530 on trimethoprim; and 528 and 532 on chloramphenicol in 175 $\mu g \cdot ml^{-1}$ concentration. In contrast to those strains were strains 534, 535, 536 and 539, which had weak or no growth at all on 10-25 $\mu g \cdot ml^{-1}$concentrations of these antibiotics. The lowest degree of intrinsic resistance of most *B.japonicum* strains tested was to the other antibiotics applied, especially cephalexin and erythromycin in concentrations 2-25 $\mu g \cdot ml^{-1}$. Strains 519, 520, 527, 528, 529 and 537 were exceptions as they had good growth on 100-150 $\mu g \cdot ml^{-1}$ ampicillina; 519, 524, 527, 530, 540 and 542 on 125-175 $\mu g \cdot ml^{-1}$ tetracycline; 509, 517, 522, 524, 542 and 554 on 75-150 $\mu g \cdot ml^{-1}$ gentamicin; 519, 520, 524, 526, 527, 530 and 542 on 75-100 $\mu g \cdot ml^{-1}$ and 532 on 200 $\mu g \cdot ml^{-1}$ streptomycin; 509, 522, 538 and 542 on 25-50 $\mu g \cdot ml^{-1}$ erythromycin; 520, 526, 527 and 537 on 25-50 $\mu g \cdot ml^{-1}$ and 528 on 100 $\mu g \cdot ml^{-1}$ cephalexin; and 519, 527, 528 and 529 on 125-150 $\mu g \cdot ml^{-1}$ penicillin.

In contrast to *B.japonicum* strains tested, *R.galegae* strains had no growth on ampicillin, tetracycline and cephalexin in concentrations of 2-10 $\mu g \cdot ml^{-1}$. Weak growth of these strains was found on streptomycin and erythromycin in concentration of 5 $\mu g \cdot ml^{-1}$. A high degree of intrinsic resistance of these strains was recorded on clindamycin and trimethoprim in concentrations 150-175 $\mu g \cdot ml^{-1}$.

The results obtained in this research agree with those reported by other authors [16, 10, 17] who found a wide diversity of reactions by *B.japonicum* and *R.leguminosarum* bv *viciae* strains regarding resistance to antibiotics.

Based on their intrinsic resistance to antibiotics, the strains investigated formed 2 main groups with 48% disagreement, each containing several subgroups (**Fig 1**. – dendogram). Four subgroups were formed within the first main group at 80% similarity. The first subgroup (a) of the first group includes *R. galegae* strains 801, 802 and 804. Strains 801 and 802 are identical, while strain 804 is 4% dissimilar. Strains 537 and 521 form two separate subgroups (b and d).

Strains 536 and 535 (4% disagreement) together with 539 and 534 (5% disagreement) and strain 500x form a common fourth subgroup (c) at 86% similarity. This subgroup is characterized by weak growth or no growth at all under low concentrations of clindamycin, trimethoprim and chloramphenicol. The other main group consists of 29 strains distributed into 5 subgroups with different similarity percentages. The first subgroup (a) of the second group includes strain 528, which disagrees more than any other strain in the group (32% disagreement) and is the only one resistant to 100µg·ml^{-1} cephalexin. The second subgroup (b) of the second group includes strains 554 and 517, which are resistant to high concentrations of gentamicin, while the third subgroup (c) consist of strains 527 and 520 especially resistant to high concentrations of clindamycin. The fourth subgroup (d) consists of strains 519 and 531 (20% disagreement between all). Dissimilarity between the second, third and fourth subgroups is 20-25%. The fifth subgroup (e) includes 22 strains with 10-16% mutual disagreement**.**

R. galegae strains characteristically showed 37% dissimilarity with the other *B. japonicum* strains of the first group, while they all together had 48% disagreement with the other group.

Shishido and **Pepper**, 1990, [6] showed in their research that *R. meliloti* isolates with identical plasmid profiles had similar resistance to antibiotics in 94.5% of all cases. **Raza** et al., 2001, [18] grouped isolates collected from *Lupinus albus* nodules into four groups according to their resistance to antibiotics. **Jošić,** 2004, [19] showed in her research a heterogenous susceptibility of *R.leguminosarum* bv. *tifolii* isolates within each particular soil type and that isolates from brown soils on limestone and eutric cambisols had the highest dissimilarity regarding resistance to antibiotics, while disagreement between isolates from chernozem, hydromorphous black soil, sandy soils and pseudeogley was considerably smaller. **Zilli** et al., 2004, [20] investigated the diversity of rhizobial populations in eight regions of Brazil under soybean and rice crop rotation, using *Vigna unguinculata* as the host plant. Based on instrinsic resistance to antibiotics, a collection of isolates from *V. unguiculata* nodules was grouped into five profiles.

Conclusion

The results obtained in this study clearly show a diversity among the rhizobial strains tested, *B.japonicum* strains in particular, regarding their intrinsic resistance to different antibiotic concentrations:

Most *B.japonicum* strains tested showed good growth on clindamycin, trimethropirm and chloraphenicol in concentrations of 150-175 µg·ml^{-1}. A particularly high degree of intrinsic resistance was found in strains 520, 524, 528 and 538 on clindamycin; 528 and 530 on trimethroprim; 528 and 532 on chloramphenicol in concentration 175 µg·ml^{-1}, and 532 on 200 µg·ml^{-1} streptomycin.

The lowest degree of intrinsic resistance of *B.japonicum* strains was found to the other antibiotics applied, especially cephalexin and erythromycin in concentrations 2-25 µg·ml^{-1}.

Unlike *B.japonicum* strains, the examined strains of *R.galegae* had weak or no growth at all on most antibiotics applied in concentrations of 2-10 µg·ml^{-1}, except on clindamycin and trimethoprim, to which they showed a high degree of intrinsic resistance in concentrations 150-175 µg·ml^{-1}.

Cluster analysis (dendogram) based on intrinsic resistance to antibiotics showed that all rhizobial strains examined formed two major groups with 48% disagreement, each of which consisted of several subgroups. Four subgroups were identified within the first group at

80% similarity. The other main group includes 29 strains distributed into five subgroups based on their disagreement percentage. *R. galegae* strains had 37% dissimilarity with the other *B. japonicum* strains in the first group, while they together had 48% disagreement with the other group.

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