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Laboratory prescreening of *Bradyrhizobium japonicum* for low pH, Al and Mn tolerance can be used to predict their survival in acid soils

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ABSTRACT

We examined whether strains of *Bradyrhizobium japonicum* selected for growth on acid media *in vitro* would also survive and grow better in acid soils. Four agar screening media for acid-tolerant rhizobia, which differed in the number of acid soil stresses imposed (pH, low calcium (Ca) and phosphorus (P), high aluminum (Al) and manganese (Mn)), were assessed for their effects on the survival of 14 Indonesian strains and two commercial strains of *B. japonicum*. Survival of *B. japonicum* in the agar media was compared with that in two acid soils. A repeat stab inoculation method which provided a declining range of inoculum cell number to 10^3 cells per stab was used to assess the daily growth of the strains on the screening media at 5 pH levels (3.8, 4.2, 4.5, 5.0, and 6.8). The growth and survival of the 16 strains were then measured at days 1, 8, 18, and 28 after inoculation in two acid soils (pH 4.24 and 4.35) sterilized using γ-irradiation at 5.0 Mrad. Selectivity of the agar media improved as more acid stress factors were incorporated in the media. Those strains of *Bradyrhizobium* identified as acid, Al and Mn-tolerant in acidic agar media, also had better survival in the low pH soils. There was no relationship between acid or alkali production on agar media and acid tolerance on agar or in soil. There was no apparent relationship between symbiotic performance and acid tolerance, and one acid-tolerant strain was as effective as the commercial inoculant strain CB1809. The most acid-tolerant strain was also the most ineffective.

1. Introduction

The survival and competitiveness of an introduced strain of *Bradyrhizobium japonicum* in soil may be adversely affected by poor growth at low pH and competition for nodulation by naturalized (acid-tolerant) bradyrhizobia of unknown effectiveness. This results in the formation of a small number of nodules by the inoculant strain and consequently a small population left in the soil after plant death. It is important therefore to be able to select inoculant strains tolerant of acid soil stress factors that are both effective in N_2-fixation and competitive.

Soil acidity and associated infertility and mineral toxicities are major constraints to agricultural production in several parts of the world (Rao et al., 1993). Acid soils are distributed over extensive areas of the humid tropics and subtropics (Van Wambeke, 1976), where they represent an important but fragile resource covering 850 million ha in tropical America, 450 million ha in tropical Africa, and 210 million ha in tropical Asia (Rao et al., 1993). In Indonesia itself, the area of good, fertile lands is decreasing, so that agricultural production is forced onto infertile acid soils. These are mainly Ultisols and Oxisols and comprise 57.4 million ha or 28.6% of Indonesia's land area (Utomo and Sunyoto, 1995). These acid soils are of vital importance in the expansion of croplands, particularly for transmigration settlement (Berek et al., 1995).

Acid soil infertility can be caused by toxic levels of hydrogen (H^+), aluminum (Al^{3+}) and manganese (Mn^{2+}), and deficiencies of calcium, magnesium, phosphorus and molybdenum (Foy et al., 1978; Foy, 1984). These infertility factors have been shown to affect symbiotic nitrogen fixation through their effects on any stage of the legume – *Rhizobium* symbiosis (Alva et al., 1987, 1988).

We examine the hypothesis that effective bradyrhizobia, previously selected for effectiveness in N_2-fixation with soybean (*Glycine max* (L.) Merr.), and which are tolerant of soil acidity factors on acid media which simulate soil conditions *in vitro*, will also be tolerant of similar conditions in soil. Furthermore, we examine whether the relative growth of the strains *in vitro* matches that in acid soil, thus validating the laboratory screening as an appropriate method for selecting bradyrhizobia for inoculation trials in acidic soils.

Abbreviations: ATR, acid tolerance response; YMA, yeast-mannitol agar.

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Liquid media have been employed for pre-selection of rhizobia tolerant of acid soil stresses such as high aluminum (Al), low phosphorus (P), and low pH (Date and Halliday, 1979; Keyser and Munns, 1979). Tolerance is measured by an increase in turbidity indicating growth of rhizobia in the presence of the selected factors. Changes in medium pH from the metabolism of rhizobia have led to inaccurate classification of acid-tolerant rhizobia. Keyser and Munns (1979) attempted to avoid changes in the medium pH by using low inoculum potentials. Richardson and Simpson (1989) used a medium based on Keyser and Munns (1979) containing different concentrations of Al and P at various pH's, and showed that 5 μM P prevented shifts in medium pH with growth of subclover (*Trifolium subterraneum* L.) rhizobia, *Rhizobium leguminosarum* bv. trifolii. Date and Halliday (1979) screened *Stylosanthes* rhizobia and showed that substitution of arabinose or galactose for mannitol prevented the formation of alkali in broth culture. These sugars were adopted by other researchers (Ayanaba et al., 1983; Wood and Cooper, 1985; Wood and Shepherd, 1987) to prevent errors that arise where the strain growth increases media pH.

Ayanaba et al. (1983) developed an agar plate medium based on Keyser and Munns (1979) basal constituents together with a pH indicator. They characterized isolates of *Bradyrhizobium* and *Rhizobium* sp. into groups of strains tolerant and sensitive to Al and low pH. Gemell et al. (1993) lowered the calcium (Ca) concentration (from 300 μM to 50 μM) and increased the manganese (Mn) concentration (from 1.0 μM to 1000 μM) of the Ayanaba et al. (1983) medium. They found that this low Ca media was more selective than Keyser and Munns (1979) and Ayanaba et al. (1983) media in identifying the tolerance of *R. leguminosarum* bv. trifolii to soil acidity related stress.

We evaluated four agar media used to screen rhizobia for acid soil tolerance. The various media were used to evaluate the effect of acidity (low pH) alone, or in combination with stress related to low concentrations of Ca and P, and elevated concentrations of Al and Mn, on the growth of 16 strains of *B. japonicum*. The validity of screening in agar media was assessed by comparison with the survival of the *B. japonicum* strains in sterile acid soils. A trial to assess the effectiveness in N_2-fixation of the strains at neutral pH was conducted to determine if there was any correlation between tolerance to acid stress factors and effectiveness. The effectiveness of the bradyrhizobia strains in fixing N_2 was assessed by comparison with the commercial strain CB1809 when used to inoculate soybean grown in sand culture at neutral pH.

The objective was to establish a reliable and easy to use method for selecting potential *B. japonicum* inoculant strains for soybean to be grown in acid soils that would survive in acid soil.

2. Materials and methods

2.1. Screening of B. japonicum in acidic agar media

The growth of 14 bradyrhizobia strains isolated from nodules of soybean plants grown at various sites in Indonesia and two commercial strains (CB1809, CB2940) was compared on the following media: (i) Keyser and Munns (1979); (ii) Date and Halliday (1979) which reduced the P concentration from 1000 μM to 5 μM; (iii) Ayanaba et al. (1983) which added 50 μM Al as $KAl(SO_4)_2$; and (iv) Gemell et al. (1993) which reduced the Ca concentration from 300 μM to 50 μM and increased Mn concentration from 1.0 μM to 1000 μM (Table 1). Each medium was adjusted to five pH values before autoclaving which was one day before use (pH 3.8, 4.2, 4.5, 5.0, and 6.8). For media of Ayanaba et al. (1983) and Gemell et al. (1993) the Al as $KAl(SO_4)_2$ was filter sterilized (0.22 μm) before addition to the agar medium held at 50 °C. The pH was then re-adjusted with 1 M HCl and/or 1 M NaOH before pouring. Bromocresol green was used as a pH indicator in the media at pH 3.8–5.0, while bromothymol blue was used at pH 6.8. Plates of medium were dried overnight at 28 °C after pouring and before use.

Table 1
Composition of four media used for screening strains of *B. japonicum* for tolerance to acid soil stress factors.

Component	Type of medium[a]			
	K + M	D + H	Aya	Gem
$MgSO_4 \cdot 7H_2O$	300 μM	←[b]	←	←
$CaCl_2$	300 μM	←	←	50 μM
Fe EDTA	100 μM	←	←	←
KCl	10 μM	←	←	←
$MnCl_2 \cdot 4H_2O$	1 μM	←	←	1000 μM
$ZnSO_4 \cdot 7H_2O$	0.4 μM	←	←	←
$CuCl_2 \cdot 2H_2O$	0.1 μM	←	←	←
$Na_2MoO_4 \cdot 2H_2O$	0.02 μM	←	←	←
$Co(NO_3)_2 \cdot 6H_2O$	0.002 μM	←	←	←
KH_2PO_4	500 μM	5 μM	←	←
K_2HPO_4	500 μM	0	←	←
Arabinose	0	5 g	←	←
Galactose	0	5 g	←	←
Mannitol	10 g	0	←	←
$KAl(SO_4)_2$	0	←	50 μM	←
Na glutamate	1.1 g	1.8 g	←	←
Biotin	0	←	←	0.1 mg
Thiamin	0	←	←	1.0 mg
Bromocresol green	0	0.005%	←	←
Distilled water	1 L	←	←	←
Agar	15 g	←	←	←
pH	3.8–6.8	←	←	←

[a] K + M = Keyser and Munns (1979), D + H = Date and Halliday (1979), Aya = Ayanaba et al. (1983), Gem = Gemell et al. (1993).
[b] Concentration does not differ from that shown in the immediate left column.

All strains were sub-cultured onto yeast-mannitol agar (YMA) plates at pH 6.8 (Vincent, 1970) and incubated for 7 days at 28 °C before using this culture for stab inoculation. Stab inoculation enables study of the growth of strains of bradyrhizobia from a range of inoculum cell populations in one plate (Gemell et al., 1993). A sterile wooden orange stick was run across the growth on the plate then stabbed onto the test YMA plate at 15 points to about half the depth of the agar with 1.5 cm between stabs. There were 3 replicate plates per strain. Growth of bradyrhizobia was observed daily and rated using a numerical scale of 0 for "no growth", 1 for "sparse", 2 for "good", and 3 for "very good growth". Any changes in pH were noted.

The inoculum potential of rhizobia applied in the stabs was determined for two representative strains with different colony types, FCB152 (raised pinpoint cream/white colony) and FCB166 (flat watery gummy colony). To establish the size of the inoculum at the different stab positions, the number of rhizobia cells in stab numbers 1, 3, and 15 were counted by excising the agar surrounding these stabs. This portion of agar was homogenized in sterile water, serially diluted and plated on a sterile YMA plate at neutral pH. Both spread plate and drop count plate methods were used to count the number of rhizobia cells at each of these stab positions (Table 2).

Table 2
The inoculum potential (number of viable rhizobia $\times 10^3$ at selected stab inoculation positions) of FCB152 (raised pinpoint cream/white colony) and FCB166 (flat watery gummy colony) inoculated into YMA medium (mean of 4 replications).

Strain	Population at inoculation stab position		
	1st	3rd	15th
FCB152	106	25.4	2.25
FCB166	153	37.5	1.04