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Full Length Research Paper

Root-nodule bacteria isolated from native *Amphithalea ericifolia* and four indigenous *Aspalathus* species from the acidic soils of the South African fynbos are tolerant to very low pH

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Indigenous root-nodule bacteria isolated from the acid sands of the Cape using *Aspalathus linearis*, *Aspalathus hispida*, *Aspalathus carnosa*, *Aspalathus capensis* and *Amphithalea ericifolia* as trap hosts showed considerable tolerance to low pH. Isolates from *A. ericifolia* and *A. carnosa* could even grow in YMB medium at pH 3. Although, all strains grew well at pH 4, 5 and 6, the isolates from *A. carnosa* exhibited the highest growth rate at each of the three pH regimes. The isolates from *A. linearis* subsp. *linearis*, *A. capensis* and *A. carnosa* grew significantly better when re-cultured from pH 3 in pH 5 or same pH 3 medium as compared to first-time culture in pH 3. With isolates from *A. capensis* and *A. linearis*, growth of cells from pH 3 re-cultured in pH 5 or same pH 3 was not significantly different. Except for isolates from *A. carnosa*, which showed a marked increase and *A. capensis* with a major decrease, no differences were observed in bacterial growth when cells from pH 5 were re-cultured in pH 3. Providing 0.5% of root metabolites from *A. linearis* subsp. *linearis* to its microsymbiont at pH 3 significantly reduced cell growth from 0.8 to less than 0.1 OD units. At pH 5, however, bacterial growth was neither inhibited nor promoted by the addition of root extract.

Key words: Bacterial isolates, acid soils, optical density, Western Cape.

INTRODUCTION

Soil acidity is a major problem constraining increased yields of agricultural crops, especially symbiotic legumes. Low pH can affect the growth of the legume host, the microsymbiont or their interaction (Glenn and Dilworth, 1994) through the direct effects of high concentrations of H, Al and Mn ions, and/or low supply of Ca, P and Mo (Marschner, 1991). Transcription of *nod* genes in root-nodule bacteria is also altered by acidic rhizospheres (McKay and Djordjevic, 1993) via changes in the profile of root exudates released by legumes (Howieson et al., 1992). Decreased cell growth and impaired nodule formation can occur from the extrusion of Ca and K ions under low pH conditions (Aarons and Graham, 1991).

With some bacterial species, however, adaptation to low pH provides protective effects including improved resistance to a variety of environmental factors such as temperature and osmotic stress (Leyer and Johnson, 1993). This presumably occurs through changes in cell surface properties and enhanced intracellular pH homeostasis. Such an adaptation to low-pH stress generally stems from the ability of the strains to synthesize acid shock proteins in response to increasing internal acidification as a consequence of low external pH (Aarons and Graham, 1991; Foster, 1993; Del Papa et al., 2003; Kiss et al., 2004; Draghi et al., 2010).

In the Cape flats and Cederberg mountains of South Africa, the soils are extremely high in acidity, ranging from pH 2.9 to 5.0 (Muofhe and Dakora, 1998); yet they support growth of many native legumes as well as nodulation and N_2 fixation with their homologous bacterial symbionts in the soil (Muofhe and Dakora, 1999). *Aspalathus linearis* subsp. *linearis* (*A. linearis*) is one such indigenous legume with considerable nodulation specificity (Dakora, 1998). The ability of root-nodule bacteria, which infect *Aspalathus* and other species, to survive and persist under low pH conditions such as pH 2.9 to 5.0 implies their adaptation to

the naturally acidic soil environment. The aim of this study was to determine whether root-nodule bacteria isolated from selected indigenous legumes growing in the acid soils of the Western Cape are naturally tolerant to low pH stress.

MATERIALS AND METHODS

Isolation of N_2-fixing bacteria from root nodules

Nodules were collected from field plants of *A. linearis* subsp. *linearis*, *Aspalathus capensis*, *Aspalathus carnosa*, *Aspalathus hispida* and *Amphithalea ericifolia* to isolate the microsymbionts (Vincent, 1970). Nodules were washed free of soil, tissue-dried and immersed in 75% ethanol for 3 min followed by another 3 min exposure to 0.1% acidified $HgCl_2$ solution. After rinsing 10 times with sterile de-ionized water, each nodule was dissected and the pink bacteroid tissue was crushed, and a drop of the turbid suspension was used to streak onto yeast mannitol agar (YMA) plates and incubated at 28°C. Isolated single colonies were selected, re-streaked and authenticated to be the nodule-forming bacteria (Vincent, 1970; Dakora and Vincent, 1985) prior to their use as stock culture in various experiments in this study. These bacterial isolates were in general, slow-growing to medium-growing on YMA plates. However, because the 16S rDNA gene was not sequenced to show the genetic relatedness of these isolates to *Bradyrhizobium* in this study, they are simply referred to here as root-nodule bacteria.

Assessing natural acid tolerance in indigenous root-nodule bateria

Bacterial tolerance of low pH was tested by growing each of the five isolates in yeast mannitol broth (Vincent, 1970). Different pH levels were obtained by adjusting the media with NaOH or HCl while keeping P content the same at each pH. One millilitre of bacterial culture prepared from single-colony isolates of each bacterium was added to sterile 200 ml yeast mannitol broth maintained at pH 3, 4, 5 or 6. In one instance, media with pH 7 and 8 were included in test the range of pH tolerance of the isolate from *A. linearis*. The bacterial cultures were maintained on a shaker and cell growth was monitored up to 35 or 74 h by reading optical densities at A_{600} on a spectrophotometer. The pH was measured at each sampling time. Four replicate cultures were used for each strain.

Determining the adaptive response of indigenous root-nodule bacteria to low pH

To assess the adaptive response of these indigenous root-nodule bacteria to low pH, the bacteria were cultured in yeast mannitol broth at pH 3 (1 ml cell suspension in 200 ml broth) and maintained with shaking for 14 days to test cell survival at this extremely low pH. The cells were then re-cultured in either pH 3 or 5, and growth was compared with first-time culture at pH 3. Similarly, bacteria grown at pH 5 for 14 days were re-cultured in media with same pH 5 or 3, and growth was measured at A_{600} for comparison with that of first-time culture at pH 5. In all instances, four replicate cultures were used for each strain and pH was measured at the beginning and end of the experiment.

Testing the effects of *A. linearis* root metabolites on growth of its microsymbiont at low pH

The effects of root metabolites on growth of *A.* bacterial symbionts at pH 3 and 5 were tested using 0.5% (1 ml root extract to 200 ml broth medium) concentration of sterile root extract from *A. linearis*. The root extract was obtained by grinding 1 g fresh weight of root tissue in 10 ml of 80% HPLC grade methanol, centrifuging and autoclaving the supernatant. After adding 1 ml bacterial cells to pH 3 or 5 media containing 0.5% metabolites, growth rates of each culture were measured at A_{600} over a 35-h period from lag phase to stationary phase. The medium pH was monitored each time. Four replicate cultures were used for each strain.

Statistical analysis

Data on rates of bacterial cell growth were analysed statistically using one-way ANOVA with STATISTICA software Program version 7.1.

RESULTS AND DISCUSSION

Response of bacterial isolates to growth in different pH levels

Time-course measurements of cell growth at pH 3, 4, 5, 6, 7 and 8 over a 74-h period showed that a slow-growing, N_2-fixing isolate from *A. linearis* could survive pH 3 and 4 conditions, and improved its growth as the medium acidity increased from pH 5, 6, 7 to 8 from 36 to 74 h (Figure 1). In contrast, the slow-growing bacterial isolates from *A. ericifolia*, *A. carnosa* and *A. hispida* showed significantly better growth at pH 3 (Figure 2a). Although, all isolates grew at pH 4, *A. carnosa* and *A. ericifolia* were again more tolerant to this pH level, followed by *A. capensis*, especially after 25 h (Figure 2b). Although, all isolates grew well at pH 5 and 6 (Figure 2c and d), those from *A. linearis*, *A. hispida*, *A. carnosa*, *A. capensis* and *A. ericifolia* could also survive in laboratory media at pH 3 and 4 (Figures 1 and 2), levels low enough to constitute acid stress. This suggests that these slow-growing, N_2-fixing strains isolated from acidic soils of the Western Cape can tolerate very low pH conditions. Some strains were however more adapted to low-pH stress than others. For example, the isolate from *A. carnosa* significantly outgrew the other isolates at all pH levels tested, except at pH 3, where *A. ericifolia* isolate showed the best growth (Figure 4). These data support the view that native populations of root-nodule bacteria in acidic soils are naturally tolerant to the low pH conditions prevailing in their niche (Lindstrom and Myllyniemi, 1987).

The ability of these symbiotic isolates to grow in a wide range of acidic conditions has also been observed in some pathogenic bacteria such as *Salmonella typhimurium* (Foster et al., 1994). This adaptation to low pH is apparently triggered in most bacteria by an acid protection system controlled by pH-regulated genes, which induce increased resistance to acid stress (Foster et al., 1994; Glenn and Dilworth, 1994; Tiwari et al., 1996a, b). In *Sinorhizobium meliloti*, *actR* and *actS* genes were found to be responsible for sensing and response to low pH, while *actA* gene directly controlled acid tolerance, as its deletion resulted in acid-sensitivity in an otherwise acid-tolerant strain (Tiwari et al., 1996a). Whether these same genes regulate acid tolerance in the indigenous strains from the Cape, remains to be determined.

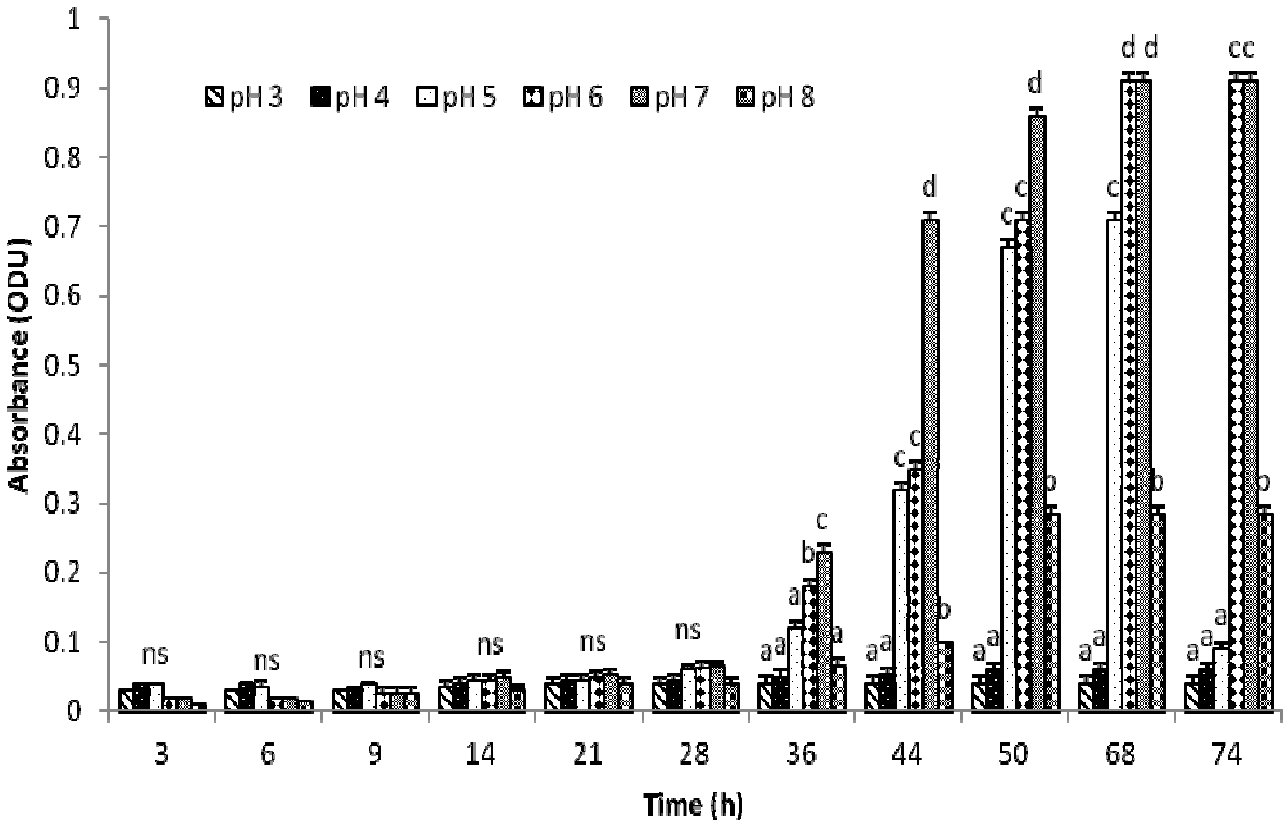

Figure 1. Responses of a slow-growing, N_2-fixing isolate from *A. linearis* subsp. *linearis* to growth in different pH levels. Values with dissimilar letters within each grouped bar chart are significantly different at $p < 0.05$ using one-way ANOVA. ns = Not significant.

Except for the strains from *A. capensis* and *A. linearis*, the other isolates grew significantly better when transferred from pH 3 to 5 as compared to first-time growth in pH 3, or when re-cultured in same pH 3 (Figure 3a). Also, all isolates previously cultured in pH 3 grew significantly better when re-cultured in same pH 3 when compared with first-time growth in pH 3, with the exception of bacteria from *A. ericifolia* and *A. hispida* (Figure 3a). In contrast, all isolates except that from *A. carnosa* exhibited a significantly decreased cell growth when re-cultured from pH 5 in pH 3 (Figure 3b). There was also a significant decrease in growth of cells during first-time culture of *A. ericifolia*, *A. carnosa* and *A. hispida* isolates in pH 5 as compared to re-culture from pH 5 in pH 5 (Figure 3b). The better growth of bacteria when transferred from pH 3 to 5 relative to re-culturing in same pH 3 (Figure 3a) could be attributed to the induction of new proteins at pH 5. On the other hand, re-culturing cells from pH 5 in pH 3 significantly reduced growth as a consequence of pH shock, especially when these were compared with pH 5 cells re-cultured in same pH 5 (Figure 3b). Although, these slow-growing bacterial symbionts may have survived the acid stress in soils at pH 3 or 4, cell growth was apparently limited, and became greatly enhanced when root exudates elevated rhizosphere pH from 3 or 4 to pH 6.8 (Muofhe and Dakora, 2000).

The significant growth exhibited by pH 3-tolerant isolates from *A. ericifolia* and *A. carnosa* on re-culturing in pH 5 (Figure 3a) suggests the versatility of these strains to survive different pH levels. Furthermore, the ability of the isolates from *A. capensis* and *A. linearis* to maintain at the same level of cell growth at both pH 3 and 5 following transfer from a previous pH 3 culture, does not only suggest strain differences in acid tolerance, but also differences in the nature and profile of proteins used to control acid tolerance. The significantly decreased growth when pH 5 cells were re-cultured in pH 3 medium (Figure 3b) could suggest the requirement for new proteins to be synthesized for cell growth to occur at the lower pH level.

In this study, viable cell count was not done as evidence of acid tolerance (Thorton, 1984; O'Hara et al., 1988; Clarke et al., 1993), however, the significantly rapid growth obtained with the transfer of bacteria from pH 3 or 5 to same or new pH level could imply that the optical densities measured were most likely those of live, viable cells, and not exo-polysacharrides. Although, nutrient limitation including low carbon supply in culture medium, can cause rapid decline in cell viability of rhizobia (Clarke et al., 1993), in this study, a rich YMB medium was used (Vincent, 1970), thus eliminating such a possibility of a decrease in cell viability. So, the reduced bacterial growth observed at lower pH levels, and the changes in OD units obtained with re-culturing isolates from pH 5 to 3 can only be attributed to

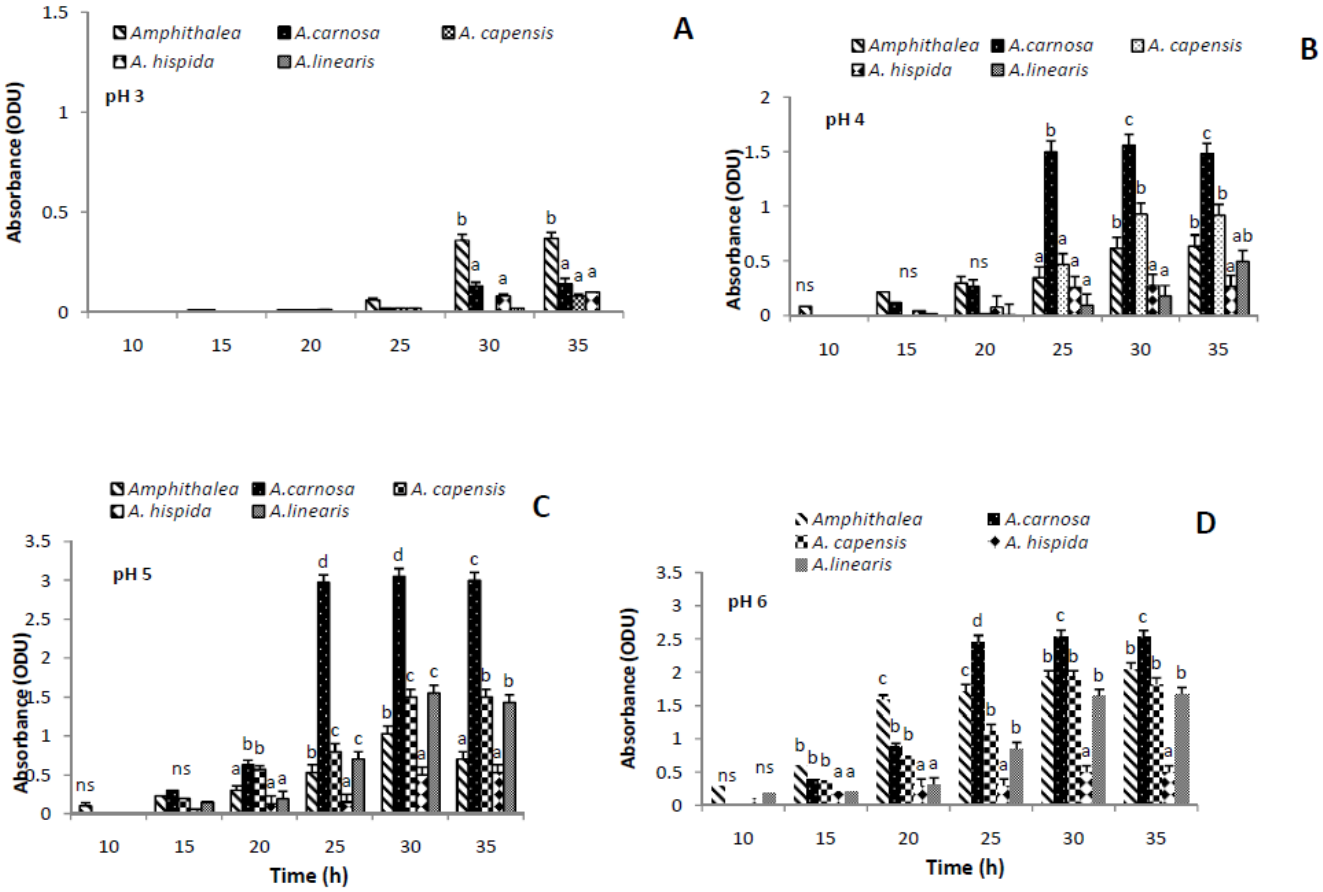

Figure 2. pH effects on growth of N_2-fixing bacterial isolates from root nodules of five indigenous legumes. Values with dissimilar letters within each grouped bar chart are significantly different at $p < 0.05$ using one-way ANOVA.

acid shock and its possible effects on protein synthesis.

Effect of *Aspalathus* root metabolites on growth of its bacterial symbiont

Growing the microsymbiont of *A. linearis* at pH 3 with 0.5% of the legume's root metabolites significantly reduced cell growth from 0.8 to less than 0.1 OD units (Figure 4a). However, at pH 5, these root extracts neither promoted nor inhibited bacterial growth (Figure 4b). This growth response to root compounds could be a mechanism for controlling carbon cost of nodule formation under conditions of proton stress. If not, *A. linearis* must have some mechanism for modifying its rhizosphere pH (usually pH 2.9 to 5.0) in order to overcome this growth inhibition of its microsymbiont in the highly acidic soils of the Cederberg. It has been reported elsewhere that plants of *A. linearis* can elevate their rhizosphere pH from 4.0 to 6.8 in order to promote symbiotic establishment in the acidic soils of the Cederberg (Muofhe and Dakora, 2000). That way, the bacterial symbionts within the legume's rhizosphere environment would probably not experience the actual pH 3 or 4 found in non-rhizosphere bulk soils.

In situations where the nodule tissue pH is higher than external soil pH, microsymbionts released from senescing nodules into the acidic soil environment are likely to incur low cell viability as a consequence of pH shock. Also, inoculant strains prepared at neutral pH for field application in low pH soils could suffer rapid loss of cell viability due to low pH stress (Clarke et al., 1993). More importantly, the versatility in response exhibited by some strains to different pH regimes is a potentially useful trait for agriculture and land reclamation through their use as inoculants for soils with differing pH levels. In conclusion, N_2-fixing bacteria isolated from the acidic soils of the Cape fynbos were found to be tolerant to very low pH levels in laboratory media, indicating their adaptation to the environment of their origin.

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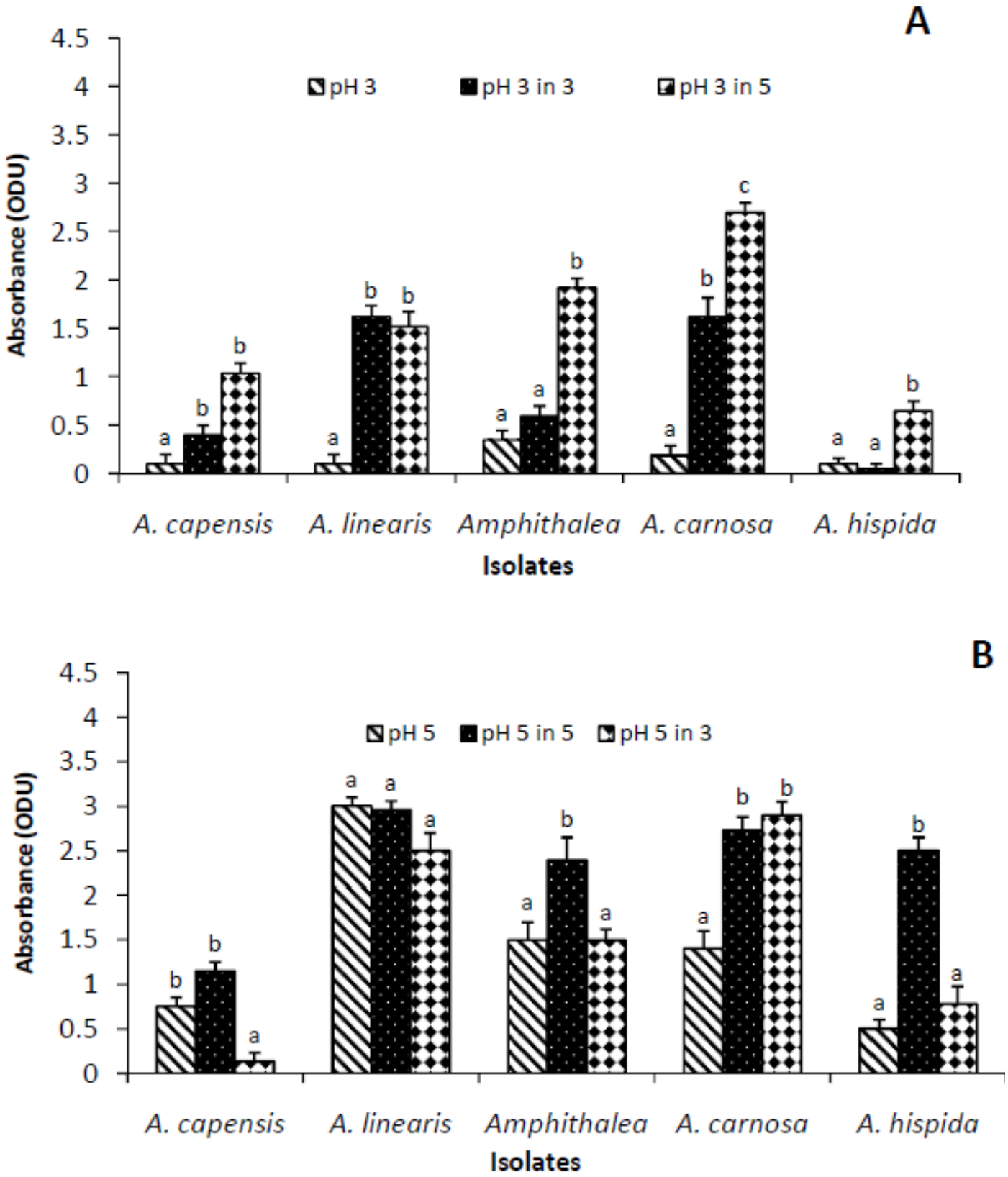

Figure 3. Response of slow-growing bacterial isolates to changes in pH: A) First-time culture in pH 3 when compared with pH 3 cells regrown in pH 3 and pH 3 cells regrown in pH 5; B) first-time culture in pH 5 when compared with pH 5 cells regrown in pH 5 and pH 5 cells regrown in pH 3. Values with dissimilar letters for each species are significantly different at $p < 0.05$ using one-way ANOVA.

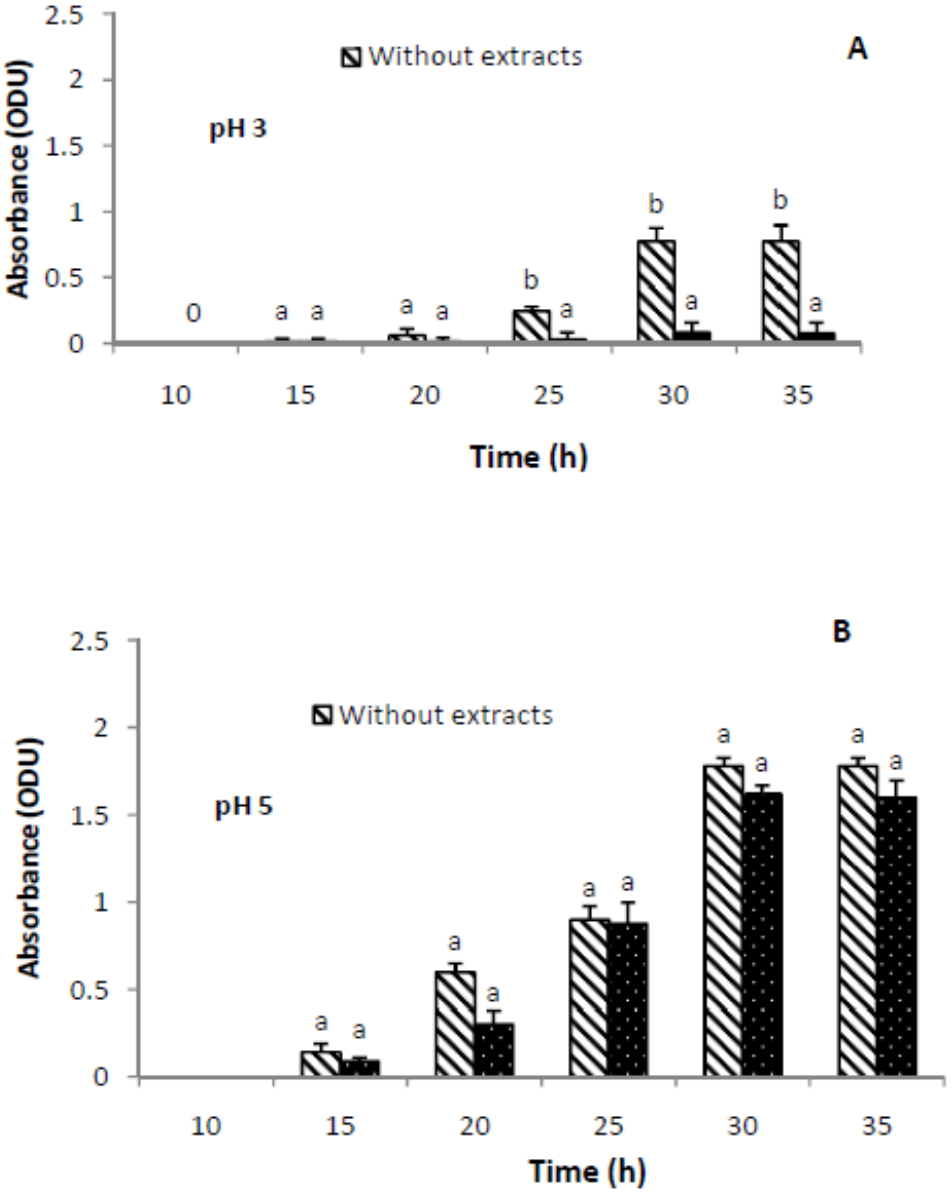

Figure 4. Root metabolite effect on growth in A) pH 3, and B) pH 5 of a slow-growing N_2-fixing strain isolated from *A. linearis* subsp. *linearis*. Values with different letters at each time point are significantly different at $p < 0.05$ using one-way ANOVA.

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