



Biological control of *Macrophomina phaseolina* on cowpea (*Vigna unguiculata*) under dry conditions by bacterial antagonists

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ABSTRACT

Macrophomina phaseolina, the causative agent of charcoal rot of cowpea and many other crops, is a devastating pathogen in many regions worldwide. Single control measures are ineffective or not feasible under farmers' conditions. In order to promote biological control as a component of an integrated management approach under arid ecological conditions, 20 bacterial antagonists were isolated from soil samples collected from the rhizosphere of healthy as well as *M. Phaseolina*-infected cowpea from fields located in the dry savannah zone of West Africa. In dual culture tests with four media, antagonistic activity was variable and depended on the medium used. Growth inhibition was generally good on tryptic soy agar on which two antagonists also inhibited microsclerotia production by *M. phaseolina*. Effective antagonists were identified as *Bacillus subtilis*, *B. sphaericus* and *Paenibacillus polymixa*. In greenhouse experiments, seed treatment with *B. subtilis* strain A11 reduced the incidence of *M. phaseolina* by 89.29% over the untreated control plants, and lower pathogen quantities in plants were confirmed by DAS-ELISA. *Bacillus subtilis* A11 was rhizosphere competent and maintained high population densities of up to 6 Log₁₀ CFU/g fresh weight on the roots and 5.73 Log₁₀ CFU/g fresh weight on the hypocotyls of cowpea plants over three weeks after inoculation. This antagonist is recommended for seed treatment in combination with other cultural practices for the management of *M. phaseolina* under arid conditions.

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Keywords: *Bacillus subtilis*, charcoal rot, rhizosphere colonization, seed treatment.

INTRODUCTION

Charcoal rot caused by *Macrophomina phaseolina* (Tassi) Goid. is an economically important disease of a broad range of crops (Srivastava et al., 2001), particularly in regions with warm and dry weather conditions during the growing season as they are prevalent in the dry savannah zone of Niger,

West Africa. Agronomically important hosts in Niger include cowpea (*Vigna unguiculata* (L.) Walp), sorghum (*Sorghum vulgare*), sesame (*Sesamum indica*), okra (*Hybiscus esculentus*) and groundnut (*Arachis hypogaea*) (Adam, 1986). Chemical control of the disease is difficult and economically not affordable for low income small scale farmers.

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Much of the effort to control *M. phaseolina* has focused on soil fumigation (Pearson et al., 1984), and applying irrigation water to reduce the disease promoting effects of drought (Kendig et al., 2000). But Niger, like many African countries, is characterized by farming at small scale level and incomes are extremely low making chemical control unsuitable. Additionally, water supply in this region is difficult. Although a few resistant cultivars have been reported, they often exhibited only partial levels of resistance (Demooy et al., 1989) and are not available to the farmers.

Other recommended control practices include solarization (Lodha et al., 1997), planting later-maturing cultivars (Pearson et al., 1984) and crop rotation (Singh et al., 1990). These practices are applicable and affordable in Niger, and could be implemented in combination with biological control to manage microsclerotial populations of *M. phaseolina* in the soil. For the biological control of *M. phaseolina*, antagonistic bacteria and fungi have been investigated (Pal et al., 2001; Singh et al., 2002; Baird et al., 2003). Combined application of two bacilli isolates reduced the *Macrophomina*-induced charcoal rot of maize by 54% (Pal et al., 2001). Among the fungal antagonists, *Trichoderma* spp. are generally the most frequently reported. Application of *T. harzianum* as seed treatment, suspension for soil drenching or wheat husk bran culture reduced infection of *Rhizoctonia bataticola* (former name of *M. phaseolina*) to 18%, 28% and 14%, respectively, as compared to 70% in the control variant (Parakhia et Vaishnav, 1986). However, most of these potent antagonists are either patented, not available, or would not withstand the dry climatic conditions prevailing in Niger. Therefore, this study was undertaken to isolate and evaluate ecologically adapted microbial organisms to be used as a component of an integrated management of charcoal rot in Niger.

MATERIALS AND METHODS

Bacterial and fungal strains

The isolates of *M. phaseolina* N1, N3, were obtained from plants' samples, N4 from soils, and N7 from seeds collected in Niger, whereas isolates 93-23, 94-188 and 96-42 were provided by Dr Florini from the International Institute of Tropical Agriculture (IITA) -Ibadan. The bacterial antagonists (A1, A4, A6, A8, A9, A10, A11) were obtained from field soil samples collected from the rhizosphere - up to a soil depth of 10 cm of healthy cowpea, groundnut and sorghum plants, while A2, A3, A5, B6, B10, C7, C11, C12, C18, B3, C17 and C20 were isolated from diseased cowpea, groundnut and sorghum plants collected during surveys in the major agricultural production zones from Niamey to Zinder in the Republic of Niger. Bacterial antagonists were maintained on tryptic soy agar (TSA; 20 g Agar, 20 g tryptic soy broth in 1,000 ml H₂O, pH 7.0) at 4 °C, and *M. phaseolina* as microsclerotia on toothpicks at room temperature.

Agrobacterium tumefaciens, *Bacillus megaterium*, *Escherichia coli*, *Erwinia amylovora* and *Pseudomonas phaseolicola* were obtained from the collection of Wolf, Plant Pathology and Crop Protection Division, University of Göttingen.

Optimum temperature for the growth of *M. phaseolina*

Eight isolates of *M. phaseolina* (N1, N3, N4, N7, 93-23, 94-188 and 96-42) from different origins were grown on potato dextrose agar (PDA) in Petri dishes (Ø 85 mm, 4 replications per isolate) at different temperatures (13 °C, 19 °C, 20 °C, 23 °C, 25 °C, 27 °C, 30 °C, 31 °C, 35 °C, 39 °C, 40 °C and 42 °C), and radial growth of the colonies was measured after 48h.

Isolation of antagonists

Isolations were performed using the agar layer method (Herr, 1959). Ten grams of soil were suspended in 50 ml sterile tap water

in a beaker and stirred for 10 min. One ml of the suspension was sixfold diluted by 1:10 in 9 ml sterile water. One ml from the dilutions from 10^{-3} to 10^{-6} , in 5 replicates, was uniformly mixed in a Petri dish with 10 ml TSA, soil extract agar (SEA; 20 g agar in 500 ml field soil extract and 500 ml H₂O, pH 7.0) or King's medium B (KB; 20 g proteose peptone, 25 g KH₂PO₄, 15 ml glycerol, 6 g MgSO₄·7H₂O, 15 g Agar in 1000 H₂O, pH 7.0) stabilised at 42-45°C (seeding layer). After solidifying, 5 ml water agar (20 g Agar in 1000 ml H₂O) cooled to 42 - 45 °C was spread as a second layer over the seeding layer, in order to prevent overgrowth of the seeding layer by fast growing microorganisms. The plates were incubated at 32 °C for 48 h to allow growth of antagonists prior to pouring a third layer (test layer) composed of propagules of *M. phaseolina*. For preparation of the test layer, mycelium/microsclerotia of *M. phaseolina* from 48h old cultures grown in 100 ml potato dextrose broth (PDB; 24 g potato dextrose broth in 1,000 ml H₂O, pH 5.5) were blended in 200 ml sterile water after filtrating off the culture medium. Two hundred microliters of mycelial/sclerotial suspension of *M. phaseolina* were added on top of the seeding layer and evenly mixed with 7 ml PDA stabilised at 42 – 45 °C. The plates were incubated at 32°C for another 3-4 days. Zones of inhibition appeared around colonies of antagonistic microorganisms contained in the seeding layer. These colonies were harvested, and used in the following trials.

Antagonist tests *in vitro*

Bacterial antagonists were streaked in the middle of Petri dishes containing 15 ml PDA, TSA, SEA or KB. After 48h incubation at 32°C, mycelial discs of *M. phaseolina* (Ø 3mm) cut from an actively growing 4 days-old culture were laid 3 cm apart from the streak. The plates were incubated at 32 °C for one week, or until the colonies of *M. phaseolina* in the control plates had covered the plate. Inhibition zones of growth of *M. phaseolina*

due to antagonistic activity were measured. Similarly, the effect of the antagonists on five bacterial species was tested. Inhibition zones of 10 mm or more were considered as important, and at 30 mm, the inhibition of *M. phaseolina* was total.

Identification of antagonists

Identification of selected antagonists was carried out by microscopic observation of 24 h old cultures under a phase-contrast microscope, by heat-treating 3 week-old bacterial cultures at 80 °C for 10 min, and by performing amino peptidase (Merck), KOH tests and using API 50 CH strips (bioMérieux) according to the manufacturers' instructions.

Greenhouse experiments

Methods for soil inoculation with M. phaseolina

Inoculation of plants with *M. phaseolina* isolate N3 was carried out by soil drenching. Four day-old microsclerotial / mycelial mat of *M. phaseolina* grown on potato dextrose broth (PDB) was blended in tap water. The suspension was adjusted to an optical density of 0.6 at 660 nm. Two hundred ml of the inoculum were mixed with 1,900 g of soil, which were equally spread on top of 5 pots initially filled each with 2,000 g of the compost / sand mixture. The control pots received the same treatment, except that the 1,900 g soil was mixed with water only.

Plant growth

Pots (11 x 11 x 12 cm) were filled with a mixture of compost (steamed at 95 °C for 2 h) and sand (1:1 w/w) prior to being sown with 5 seeds per pot. Five pots were used per treatment, and each experiment was repeated at least twice. Cowpea plants genotype IT93K-734 (IITA - Ibadan, Nigeria) were grown in the climate chamber (11 h light at 30,000 Lux), at relative humidity of 50-60% and temperatures between 30 and 35°C for 13 h during the day, and 22 and 25 °C in the night, simulating the growth conditions in Niger. Watering was carried out twice per week.

Seed treatment with bacterial antagonists

Bacillus spp strains A11, B6, C17 and C20 were selected for *ad planta* tests.

Twenty-four hour old colonies of bacterial antagonists of each strain were inoculated to 500 ml flasks containing 100 ml tryptic soy broth (TSB; 20 g tryptic soy broth in 1,000 ml H₂O, pH 7.0) and incubated for 24 h at 30 °C on a rotating shaker (100 rpm). The bacterial cells were harvested by centrifugation at 10,000 x g for 10 min and resuspended in 0.1 M MgSO₄. The bacterial suspensions (1.7 x 10⁹ cfu/ml) were applied by 10 min soaking to cowpea seeds, which were air-dried before sowing in soil inoculated with *M. phaseolina*. Control seeds were soaked in 0.1 M MgSO₄ and sown in soil inoculated with *M. phaseolina* (positive controls K+), or in non-inoculated soil (negative control K-). After six weeks, percentage disease incidence (DI) was calculated by dividing the number of diseased plants by the total number of plants per pot. Five plants were then randomly selected per treatment, removed and analysed by DAS-ELISA. This experiment was performed twice.

Studies on the rhizosphere competence of *Bacillus subtilis* A11

For studies on the establishment and colonisation of the rhizosphere of the host plant by *Bacillus subtilis*, isolate A11 was used to produce spontaneous antibiotic double mutants, resistant against 100 ppm rifampicin and 500 ppm streptomycin sulphate. These mutants were tested again on PDA for their ability to inhibit the growth of *M. phaseolina* *in vitro* prior being finally selected for *in planta* tests. Seeds were treated with washed bacterial cells as mentioned above before sowing. The roots, hypocotyl and epicotyl of 5 plants were weighted and sampled at weekly intervals for three weeks to study the population dynamics of the antagonist.

Bacterial populations were quantified by dilution plating the macerate of the respective plant organ on TSA supplemented with 100 ppm rifampicin + 500 ppm streptomycin sulphate. Rifampicin-streptomycin-resistant

colonies were counted after 24 h of incubation at 32 °C.

Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)

DAS-ELISA was performed as described by Clark and Adams (1977) using antibody 848/3 (Afouda et al., 2009) to analyse 6 weeks-old plants with or without symptoms grown from antagonist-treated seeds on soil inoculated with *M. phaseolina*.

Statistical analysis

Disease incidence data was analysed by SPSS, and means were separated according to Student–Newman–Keuls after square root transformations. For ELISA and *in vitro* antagonist tests, standard errors were determined.

RESULTS

Temperature optimum for growth of *M. phaseolina*

Optimal growth of eight isolates of *M. phaseolina* from different origins on PDA was observed at temperatures between 30 and 35 °C (Figure 1). This temperature was chosen for the selection of antagonists.

Test of antagonists

Using TSA, SEA or KB in the seeding layer, 47 antagonists, of which 45 bacteria and 2 fungi, were isolated from soil samples collected from fields infected with *M. phaseolina* as well as non-infected ones. Colonies of bacterial antagonists appeared in the seeding layer, surrounded by zones of inhibition in the test layer. Some of these colonies were used for further tests.

Dual culture tests

Twenty bacterial antagonists growing well on KB, TSA, SEA and PDA were selected and tested in dual culture against *M. phaseolina* on these media. Growth inhibition was best on TSA with ≥ 10 mm inhibition zone for all tested antagonists except strain C18 and B3 (Table 1). On TSA, five antagonists (A8, A9, C7, C11, B6) inhibited

growth of *M. phaseolina* totally and eight antagonists produced an inhibition zone of 15-20 mm and maintained it over a period of 2 months. On PDA and on KB, antagonists A2, and A2 and A3, respectively, also showed a good inhibition. Some antagonists (A2, A3 and A9) were efficient on several of the media used. Strains A5 and A6 differed from the other antagonists by their ability to inhibit microsclerotial production by *M. phaseolina* on PDA.

Identification of antagonists

Based on their cultural characteristics and physiological properties, the selected antagonists were identified as *B. Subtilis* (A1, A2, A3, A4, A6, A8, A9, A10, A11, B6, B10, C7, C11, C12, C18), *B. sphaericus* (B3, C20) and *Paenibacillus polymixa* (A5, C17) by API 50 CH strips kit, with a probability ranging from 99 to 99.7% (data not shown).

Effect of seed treatment with *Bacillus* spp. on *M. phaseolina* in the greenhouse

Antagonists A11, B6 and C20 reduced respectively disease incidence by 89.29%,

85.71% and 78.57% over the control K+, and DAS-ELISA analysis of whole plants (with or without symptoms) showed lower values for plants grown from seeds treated with these antagonists than for untreated plants (Table 2).

When tested on TSA, SEA and KB, *Bacillus subtilis* A11 showed no antibacterial effect against *Agrobacterium tumefaciens*, *Bacillus megaterium*, *Escherichia coli*, *Erwinia amylovora* and *Pseudomonas syringae* pv. *phaseolicola* (data not shown).

Rhizosphere competence of *B. subtilis* A11

Population densities of antibiotics-resistant mutants of *B. subtilis* A11 were high (over 6 Log₁₀ CFU/g fresh weight) for three weeks in the roots of cowpea plants inoculated with this antagonist. Population densities of *B. Subtilis* was also high on the hypocotyls after 3 weeks (≥ 4.46 Log₁₀ CFU/g fresh weight), but slightly less than in the roots (Table 3). On the epicotyls, low population densities (4 Log₁₀ CFU/g fresh weight) of the bacteria were detectable only at the end of the 1st week (data not shown).

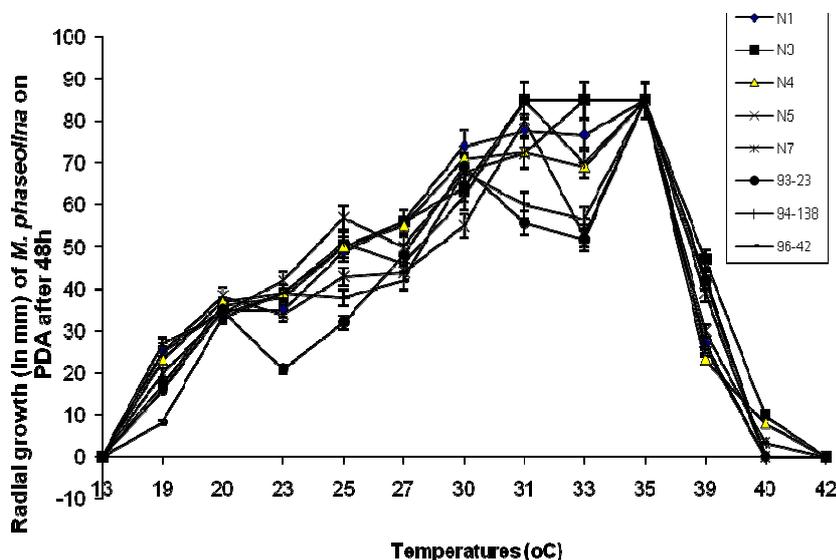


Figure 1: Growth of eight isolates of *M. phaseolina* at 10 temperatures (13 °C, 19 °C, 20 °C, 23 °C, 25 °C, 27 °C, 30 °C, 31 °C, 35 °C, 39 °C, 40 °C and 42 °C) on potato dextrose agar after 48 hours incubation.

Table 1: Inhibition zones [mm] produced at 35 °C *in vitro* by bacterial antagonists on *M. phaseolina* growth on four agar media, PDA, KB, TSA and SEA.

Antagonist code	Inhibition zone on			
	PDA	KB	TSA	SEA
A1	6 (±1)	8,17 (±0,76)	15,67 (±1,15)	10,17 (±0,58)
A2	18,67 (±1,15)	10 (±0)	18,5 (±0,5)	8 (±0,87)
A3	9,67 (±1,15)	10,33 (±0,58)	15,83 (±0,29)	8,83 (±0,29)
A4	4 (±1)	9,33 (±0,58)	11,67 (±0,58)	8,83 (±0,29)
A5	8,17 (±1,04)	3,83 (±0,29)	10,83 (±1,29)	15,17 (±0,76)
A6	9,17 (±0,76)	5 (±0)	10,17 (±0,76)	14,83 (±0,29)
A8	9,17 (±0,57)	7,67 (±0,29)	30 (±0)	8 (±0)
A9	9,77 (±1,17)	30 (±0)	30 (±0)	10 (±0,87)
A10	8,9 (±1,01)	8 (±0)	18 (±1)	10,17 (±1,04)
A11	5,4 (±0,96)	6,33 (±0,76)	15,17 (±0,76)	10 (±1,32)
B3	0	0	9,83 (±0,58)	17,5 (±0,5)
B6	5,4 (±0,66)	0	30	10,17 (±0,76)
B10	5,33 (±0,58)	0	14,83 (±0,29)	10 (±0)
C7	3,5 (±0,5)	5,67 (±0,29)	30 (±0)	9,83 (±0,29)
C11	2,83 (±0,29)	8,67 (±0,29)	30 (±0)	6,17 (±0,76)
C12	0	2,33 (±0,29)	18,5 (±0,5)	2 (±0)
C17	9 (±0,7)	4 (±0)	11,83 (±0,29)	7,67 (±0,29)
C18	0	0	5,83 (±0,29)	8,33 (±0,58)
C20	4,97 (±0,25)	0	20,33 (±1,15)	10,17 (±0,58)
C27	10,17 (±0,58)	8,5 (±0,87)	18 (±1)	3 (±0)

Values are average of 3 replications repeated twice, and data in parentheses indicate standard error (SE).

Table 2: Population densities (Log₁₀ CFU/g) of antibiotics-resistant mutants *Bacillus subtilis* A11 in the roots and epicotyls of cowpea plants at 1 to 3 weeks after treatment.

Plant part	Mean colonization in Log ₁₀ CFU/g fresh weigh		
	Week 1	Week 2	Week 3
Root	6,04 (±0.039)	6,68 (±0.036)	6,16 (±0.043)
Hypocotyl	5,95 (±0.048)	4,46 (±0.151)	5,73 (±0.046)

Values are average of 3 replications repeated thrice, and data in parentheses indicate standard error (SE).

Table 3: Effect of seed treatment with isolates A11, B6, C17 and C20 of *Bacillus subtilis* on *M. phaseolina* in the greenhouse.

Treatment	Disease incidence (in %)	ELISA value (E ₄₀₅)
<i>B. subtilis</i> A11	6 ^c	0.24 (±0.05)
K+	56 ^a	0.79 (±0.21)
K-	0 ^c	0.11 (±0.03)
B6	8 ^c	0,38 (±0.08)
C17	24 ^b	0,5(±0.04)
C20	12 ^c	0,4(±0.03)

Values within one column followed by the same letter are not significantly different at p= 0.05 according to SNK test E₄₀₅= DAS-ELISA value at 405 nm. Values are average of 3 replications repeated thrice, and data in parentheses indicate standard error.

DISCUSSION

In the present study promising antagonists were isolated from the rhizosphere of healthy as well as diseased cowpea, groundnut and sorghum plants in Niger. Most of the isolated bacterial antagonists irrespective of the site of their isolation inhibited the growth of *M. phaseolina*. However, the antagonist *B. subtilis* A11, most effective *ad planta* was isolated from the rhizosphere of plants in a cowpea field not infected by *M. phaseolina*. Similarly, antagonist isolates from diseased and healthy gram plants, or from potato fields heavily infected with *M. phaseolina* were reported to be effective against *M. phaseolina* (Broadabent et al., 1977; Parakhia and Vaishnav, 1986). As demonstrated in various studies (Pusey and Wilson, 1984; Tilcher, 1995; Montesinos et al., 1996), strains of pseudomonads, bacilli and actinomycetes, isolated from the soil were successfully used to control pathogens causing damages in aerial parts of the plants. Differences in inhibition zone diameter observed between different isolates of *Bacillus* on the same medium suggest that antibiotic production is strain dependent, as also reported by (Becker and Cook (1988) and Landa et al. (2003). Thus, the inhibition zone produced *in vitro* indicates antibiotic production and is regarded as an important criterion for the selection of potent antagonists *ad planta*. The isolation of numerous antibiotic-producers from naturally suppressive soils indicates that antibiotics are involved in the naturally occurring biological control of pathogens (Raaijmakers, 1998). Various authors (Utkhede and Rahe, 1980; Loeffler et al., 1986; Pal et al., 2001) reported the production of antibiotics with antifungal effect by *B. subtilis* strains. Besides bacterial strains, inhibition of *M. phaseolina* in agar culture by fungal species was also reported (Parakhia and Vaishnav, 1986; Pal et al., 2001). In the greenhouse experiments, the *Bacillus* antagonists A11, B6, C20, which were to a different extent efficient *in vitro*, revealed effective *ad planta*. Strain A11 inhibited totally the growth of *M. phaseolina*

in vitro on TSA and was also the most effective antagonist to control the fungus in pot experiments. It is known that good correlation may exist between the efficiency of antagonists *in vitro* and their effect *ad planta* (Xu and Gross, 1986; Montesinos et al., 1996; Kempf, 1988) as there may also be lack of correlation between *in vitro* and *ad planta* results (Kempe and Sequeira, 1983; Green et al., 1995). The performance of antagonists can be influenced by various factors such as the humidity and structure of the soil and other uncontrolled, adverse environmental conditions (Broadabent et al., 1977). The high efficiency of *B. subtilis* A11 in the control of *M. phaseolina*, with an overall low disease incidence of 6% and low inoculum level detectable in the plants by ELISA ($E_{405} = 0.24$) may be associated not only to antibiotic production, but also probably to plant-growth promotion by the antagonist. Indeed, *B. subtilis* isolates are implicated in the production of organic acids, gibberellin and auxin, in the solubilization of phosphate or inhibition of deleterious root-colonising microorganisms or their toxins (Broadabent and Baker, 1977; Pal et al., 2001) and have been successfully used for the management of many plant pathogens (Pal et al., 2001; El-Hassan and Gowen, 2006; Schisler et al., 2002; Landa et al., 2004). Mechanisms of pathogen control by *Bacillus* spp. may be by competition in root colonisation and production of antifungal compounds (Pal et al., 2001) by promotion of plant growth, and/or induction of systemic resistance (Kloepper et al., 2004). Various communications also report the successful use of microbial antagonists to control *M. phaseolina ad planta* (Pal et al., 2001; Singh et al., 2002), which supports our results.

A good rhizosphere competence and colonization of the hypocotyls was observed for *B. subtilis* A11 three weeks after treatment under the dry and hot growth conditions, making this antagonist suitable for use in the field under the ecological conditions of arid ecozones. Also Sasse (1997) and Milus and Rothrock (1993) detected high population

densities of *B. subtilis* on the roots, and to a smaller extent on the stem of rapeseed 30 days after planting, and on the roots of wheat. Several studies have demonstrated that rhizobacteria must establish and maintain a threshold population density in the rhizosphere to prevent or limit pathogen infection (Raaijmakers et al., 1998). But, reports of rhizosphere colonisation by bacteria of the genus *Bacillus* are few (Pal et al., 2001), as compared to *Pseudomonas* spp., which were extensively investigated for their ability to colonise the rhizosphere (Pal et al., 2001; Singh et al., 2002; Landa et al., 2003). The results of the present study are, however, conflicting with those of Scher et al. (1984), who reported a general lack of root colonisation by Gram-positive bacteria. Numerous studies (Lemanceau et al., 1995; Smith and Goodman, 1999; Mazzola and Gu, 2002) have clearly shown that various factors, among them also the plant genotype, can influence the composition and activity of microorganisms in the rhizosphere, and that the effect of antagonists could therefore vary under field conditions.

Thus, the combination of the ability of *B. subtilis* to adapt to extreme environmental conditions such as high temperatures and drought, which favour the occurrence of *M. phaseolina*, with its capability to colonise the plant organs first attacked by the fungus, make this bacterium most suitable as a means to contribute significantly to the control of charcoal rot. In the specific case of Niger, coating seeds with *Bacillus subtilis* A11 in combination with cultural practices is feasible and is suggested to considerably ease the problem of *M. phaseolina*. Therefore, biological control should be one element in an integrated approach for the control of charcoal rot, further comprising the use of clean planting material, crop rotation, timing of the growth period, use of resistant varieties and solarisation.

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