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Physiological and cytological studies on the inhibition of *Striga* seed germination by the plant growth-promoting bacterium *Azospirillum brasilense*

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Abstract

Striga hermonthica (Del.) Benth. is an obligate parasitic weed of tropical cereals whose rhizosphere can also be colonised by bacteria of the genus *Azospirillum*. A previous study demonstrated that the two organisms (*Azospirillum* and striga) interacted during cerealroot colonisation. Two strains of *A. brasilense* isolated from an African sorghum rhizosphere prevented the germination of striga seeds although they were stimulated to germinate by the presence of sorghum roots. *Azospirillum* cells suspended in a synthetic germination stimulant (GR24) did not inhibit striga seed germination, but did block radicle elongation. Those radicles had an abnormal morphology, and contained no vacuolated cells in the root elongation zone. Lipophilic compounds extracted from the medium of bacteria in the log and stationary growth phases prevented the germination of striga seeds.

Introduction

Bacteria of the genus *Azospirillum* are free-living, nitrogen-fixing micro-organisms that are commonly found in soil, but mainly in association with the roots of plants (Döbereiner et al., 1976; Tyler et al., 1979). These rhizosphere bacteria have been studied because of their ability to promote growth of numerous plant species, including grasses. They encourage greater root development, resulting in improved crop yields (for review, see Okon and Labandera-Gonzalez, 1994; Bashan and Holguin, 1997). Thus, *Azospirillum* bacteria are classified as plant growth-promoting bacteria (PGPB) (Bashan and Holguin, 1998).

Grass rhizospheres in tropical regions (notably in Africa) can also be colonised by *Striga hermonthica* (Del.) Benth. an obligate parasite of tropical cereals. Several mechanisms regulate seed germination and attachment of this root hemi-parasite angiosperm to its host. Striga seeds must first be moistened (preconditioned), before they can respond to a chemical stimulant exuded by host roots and germinate. The radicles then grow towards the host roots and become fixed by radicle-tip transformation into a structure called the haustorium. This transformation is also triggered by a chemical stimulant exuded by the host. The seedlings must rapidly attach to their host roots or they will die, because their tiny seeds have very little food reserves (for review, see Berner et al., 1995; Olivier, 1996). This weed is becoming an uncontrollable pest for food-producing crops, and there is no real effective means of controlling striga in Africa (Sallé et al., 1995).

In a previous study, *Azospirillum* strains were isolated from an African soil under sorghum cultivation. This soil was sampled from a sorghum field where two sites were distinguishable: in the first one, sorghum was infested by striga, whereas in the other site, plants were not infested by the weed (Kabir et al., 1996).

Azospirillum has been mostly studied for its ability to enhance plant growth, but it may have a potential as a biocontrol agent as well. There are a few cases of antagonistic effects of *Azospirillum*

towards other micro-organisms including inhibition against *Agrobacterium* (Bakanchikova et al., 1993) and *Staphylococcus* (Holguin and Bashan, 1996). Moreover, some *Azospirillum* strains produce a bacteriocin (Tapia-Hernandez et al., 1990). Even though no reports have been made on interactions between *Azospirillum* and parasitic plants, the hypothesis was proposed that the protection of sorghum against striga was due to competition between *Azospirillum* strains and the weed for the same host plants.

A previous experiment showed that two of the strains isolated from the uninfested site of the field (*A. brasilense* L2 and L4) inhibited the root-induced germination of striga seeds. The L4 strain also acted as a PGPB on sorghum inoculated in microcosms (Bouillant et al., 1997). This strain may therefore both stimulate plant growth and protect it against striga in field. The mechanisms by which *Azospirillum* inhibits striga seed germination were therefore studied, in order to improve this inhibitory effect.

Materials and methods

Striga seeds treatment

Striga hermonthica seeds obtained from Sikasso, Mali were surface-sterilised in 70% ethanol for 2 min, then in 1% (w/v) filtered calcium hypochlorite with three drops of Tween 20 for 20 min. Seeds were rinsed with sterile water and placed on 6-mm diameter glass fibre discs that were themselves placed on moistened Whatman glass fibre papers in Petri dishes (about 50 seeds per disc). The closed Petri dishes were placed in the dark at 31 °C for at least 14 days for preconditioning.

To make the preconditioned seeds germinate, a stimulant must be added. Rather than using complex sorghum exudates, which gave irregular germination rates despite the adjustment made by Weerasuriya et al. (1993), the seeds were stimulated with GR24 (1 μg/ml). This chemical germination stimulant is a synthetic strigol analogue commonly used for *in vitro* studies (Zwanenburg et al., 1994).

Inhibition of germination by Azospirillum *exudates*

Azospirillum brasilense L2 and L4 were grown on TY (tryptone yeast extract) (Beringer, 1974) for 4 h and collected by centrifugation. The cells were suspended in 10 ml Nfb (nitrogen-free broth) (Nelson and Knowles, 1978) to obtain an OD_{540} of 0.2 (10^7 cfu/ml) and incubated at 28 °C with shaking. After 7, 13, 23, 31, 46 and 53 h, bacterial cfu were measured by plating on Nfb. Cells were removed by centrifugation and the supernatant extracted with an equal volume of ethyl acetate. The solvent was evaporated under vacuum and the residue suspended in ultra-pure water (1 volume of bacterial supernatant).

To test their effect on striga seed germination, supernatant extracts of increasing bacterial ages were put into the wells of a tissue culture plate, each well containing one disc of striga seeds. GR24 was diluted into supernatants extracts to stimulate weed germination (three repetitions were done for each kinetic point). The controls contained GR24 only, diluted into sterile Nfb medium extract, to ensure that the stimulation of seed germination was not blocked by this protocol of extraction. The plates then placed in the dark at 31 °C for 3 days before germinated seeds were counted under a microscope. The results were statistically analysed by a χ^2 test.

Cytological study of radicles

Azospirillum cells suspended in a GR24 solution (10^{10}/ml) were added to striga seeds. This did not affect the weed germination rate, but the radicles had an abnormal morphology. Abnormally germinated seeds were fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.2) for 1.5 h under vacuum and post-fixed in OsO_4 2% in veronal buffer ($NaCH_3COO$ 0.034 M, $NaC_8H_{11}N_2O_3$ 0.025 M, pH 7.2) in the same conditions. They were dehydrated in ethanol series and propylene oxide, and embedded in Epon resin. For light microscopy, sections (1 μm thick) were stained with 1% toluidine blue and 2.5% Na_2CO_3 for 5 min and washed with water.

Results and discussion

The mechanisms by which *A. brasilense* L2 and L4 inhibit striga seed germination were tested using this inhibition in a simplified system. The complex sorghum root exudates were replaced with a chemical germination stimulant: GR24 (Zwanenburg et al., 1994), which permitted the control of stimulant concentration in replicate experiments.

Azospirillum cells suspended in GR24 stimulation solution caused striga seed germination to abort. Seeds started to germinate, but the radicles that emerged did not lengthen (Figure 1c). This resulted in the formation

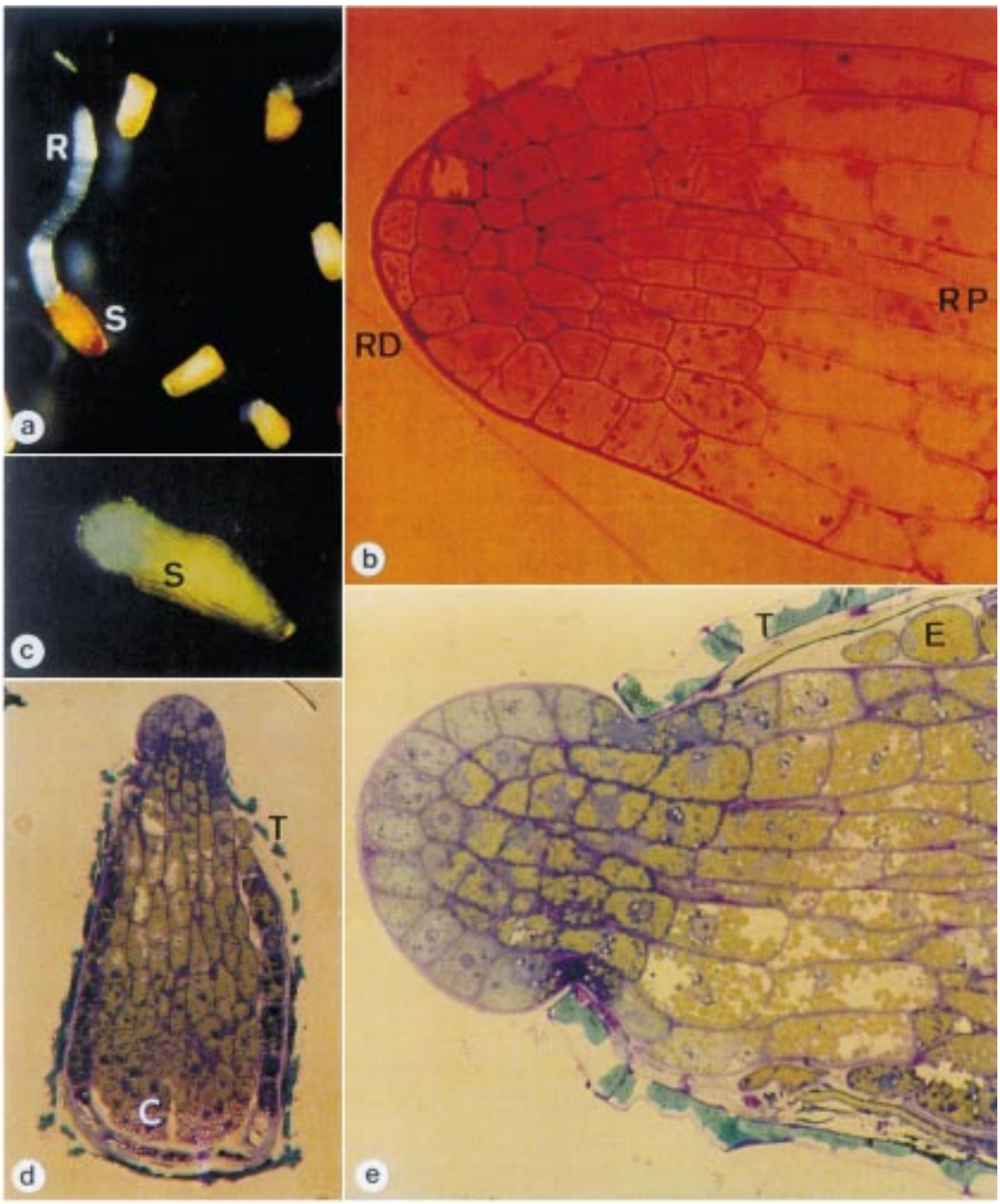

Figure 1. Morphology of *Striga hermonthica* seeds that have germinated in contact with GR24 alone (a and b), or with GR24 and *Azospirillum brasilense* L2 cells (c, d and e); a and c: germinated striga seeds (×40 and ×100 respectively); d: axial section of a germinated striga seed (×220); b: apical cells of a control radicle (×800); e: enlargement of d (×800). *Abbrevations:* C, cotyledons; E, endosperm; R, radicle; RD, radicle distal region; RP, radicle proximal region; S, seed; T, tegument.

of abnormal germ tubes that were shorter and thicker than controls (Figure 1a). The control germ tubes had a classical polar cellular structure (with a proximal region made of vacuolated cells and a distal one formed by meristematic cells) (Figure 1b), but the radicles that emerged in the presence of *Azospirillum* cell suspensions did not contain any vacuolated cells (Figures 1d and 1e). This lack of differentiated cells in the elongation zone could be the cause of the lack of germ tube growth.

Azospirillum cells made striga seed germination abort in the presence of synthetic stimulant (GR24), whereas these bacteria fully blocked it in a previous experiment using sorghum extracts (Bouillant et al., 1997). *Azospirillum* must therefore require specific environmental conditions to synthesise inhibitory molecules, which were not produced in this assay (may be because the medium used was nutrient deficient). No *Azospirillum* cells were detected at the surface or inside the striga radicles, perhaps because the medium did not allow them to produce adhesive substances (proteins and extracellular surface polysaccharides) needed for *Azospirillum* attachment (Bashan and Levanony, 1988; Michiels et al., 1991).

The inhibition of striga radicle growth by *Azospirillum* could be caused by the bacterium producing phytohormones that interfered with the hormonal balance of the weed. Several studies have shown that *Azospirillum* can act on the morphology of its hosts roots, in particular in the root-elongation zone (Levanony and Bashan, 1989) and can alter the cell arrangement in the outer layers of the cortex (Lin et al., 1983; Kapulnik et al., 1985). The effects of *Azospirillum* on root development are also directly related to the concentration of bacterial cells, which suggested that phytohormones or phytohormone-like compounds produced by the bacteria, especially IAA, are involved (Tien et al., 1979; Kolb and Martin, 1985; Fulchieri et al., 1993). A high over-optimal inoculum concentration (10^8 cfu/ml) causes a significant decrease in root growth, including the root-elongation zone (Lin et al., 1983).

Striga seeds with short swollen germ tubes were observed by Logan and Stewart (1991) when the seeds were germinated in high hormonal concentrations. Most of classical phytohormones being hydrophobic, the production of lipophilic molecules during bacterial growth in Nfb medium was monitored, and their ability to block striga seed germination tested.

In contrast to results obtained with *Azospirillum* cells, extracts of *A. brasilense* L4 supernatants reduced

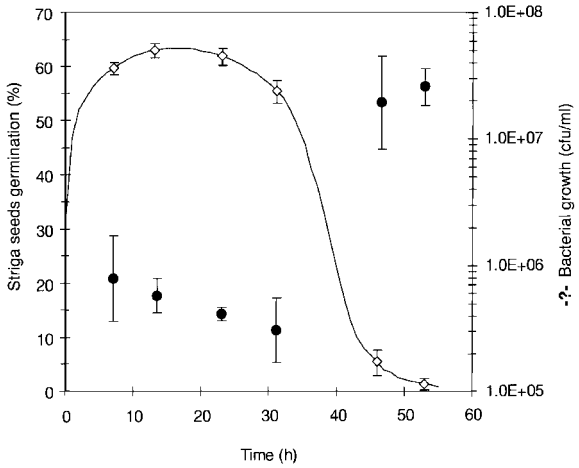

Figure 2. Germination percentage of *Striga hermonthica* seeds in contact with GR24 and lipophilic extracts of *Azospirillum brasilense* L4 supernatants, according to bacterial growth stage. •: germination percentage; ◇: cfu/ml. Points represent the means ± SE of three replicates. Germination percentage of control striga seeds (GR24 alone) was 55.8 ± 3.2%.

the striga seed germination rate: less than 20% of striga seeds germinated in presence of bacterial extracts, instead of 55.8 ± 3.2% with GR24 alone (Figure 2). These results are reminiscent of Dirar's study (1995), which showed that *Xanthomonas* inhibited striga seed germination via molecules secreted into the culture medium. In our study, the bacterial molecules were extracted with a rather lipophilic solvent, ethyl acetate, which suggests that the inhibition of striga seed germination involves the production of small lipophilic compounds; and not proteins, peptides, or sugars, which are not soluble in this solvent. The inhibition was statistically uniform at least until 31 h, as the χ^2 test showed no significant difference with time (not shown). The last two points in the Figure 2 show that bacteria can no longer inhibit striga seed germination when cell viability falls. This suggests the inhibitor(s) does not accumulate in the medium, or may be unstable or volatile.

This is the first report that striga seed germination has been inhibited by a product of a bacterium that is beneficial for sorghum growth. It opens up new prospects for combating the parasite in Africa.

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References

Bakanchikova TI, Lobanok EV, Pavlova Ivanova LK, Red'kina TV, Nagapetyan ZA and Majsuryan AN (1993) Inhibition of tumor formation process in dicotyledonous plants by *Azospirillum brasilense* strains. Mikrobiologiya (Russian Federation) 62: 515–523 (In Russian)

Bashan Y and Holguin G (1997) *Azospirillum*–plant relationships: environmental and physiological advances (1990–1996). Can J Microbiol 43: 103–121

Bashan Y and Holguin G (1998) Proposal for the division of plant growth-promoting rhizobacteria into two classifications: biocontrol-PGPB (plant growth-promoting bacteria) and PGPB. Soil Biol Biochem 30: 1225–1228

Bashan Y and Levanony H (1988) Active attachment of *Azospirillum brasilense* Cd to quartz sand and to a light-textured soil by protein bridging. J Gen Microbiol 134: 2269–2279

Beringer JF (1974) R factor transfer in *Rhizobium leguminasorum*. J Gen Microbiol 84: 188–198

Berner DK, Kling JG and Singh BB (1995) Striga research and control. A perspective from Africa. Plant Disease 79: 652–660

Bouillant ML, Miché L, Ouedraogo O, Alexandre G, Jacoud C, Sallé G and Bally R (1997) Inhibition of *Striga* seed germination associated with sorghum growth promotion by soil bacteria. C R Acad Sci Paris 320: 159–162

Dirar HA (1995) Bacteria that scavenge germination stimulants? Haustorium 30: 1

Döbereiner J, Marriel IE and Nery M (1976) Ecological distribution of *Spirillum lipoferum*. Beijerinck. Can J Microbiol 22: 1464–1473

Fulchieri M, Lucangeli C and Bottini R (1993) Inoculation with *Azospirillum lipoferum* affects growth and gibberellin status of corn seedling roots. Plant Cell Physiol 34: 1305–1309

Holguin G and Bashan Y (1996) Nitrogen-fixing by *Azospirillum brasilense* Cd is promoted when co-cultured with a mangrove rhizosphere bacterium (*Staphylococcus* sp.). Soil Biol Biochem 28: 1651–1660

Kabir M, Faure D, Heulin T, Achouawk W and Bally R (1996) *Azospirillum* populations in soils infested by a parasitic weed (*Striga*) under sorghum cultivation in Mali, west Africa. Eur J Soil Biol 32: 157–163

Kapulnik Y, Okon Y and Henis Y (1985) Changes in root morphology of wheat caused by *Azospirillum* inoculation. Can J Microbiol 31: 881–887

Kolb W and Martin P (1985) Response of plant roots to inoculation with *Azospirillum brasilense* and to application of indole acetic acid. In: Klingmüller W (ed) *Azospirillum* III: Genetics, Physiology, Ecology (pp 215–221) Springer-Verlag, Berlin, Heidelberg

Levanony H and Bashan Y (1989) Enhancement of cell division in wheat root tips and growth of root elongation zone induced by *Azospirillum brasilense* Cd. Can J Bot 67: 2213–2216

Lin W, Okon Y and Hardy RWF (1983) Enhanced mineral uptake by *Zea mays* and *Sorghum bicolor* roots inoculated with *Azospirillum brasilense*. Appl Environ Microbiol 45: 1775–1779

Logan DC and Stewart GR (1991) Role of ethylene in the germination of the hemiparasite *Striga hermonthica*. Plant Physiol 97: 1435–1438

Michiels KW, Croes CL and Vanderleyden J (1991) Two different modes of attachment of *Azospirillum brasilense* Sp7 to wheat roots. J Gen microbiol 137: 2241–2246

Nelson LM and Knowles R (1978) Effect of oxygen and nitrate on nitrogen fixation and denitrification by *Azospirillum brasilense* grown in continuous culture. Can J Microbiol 24: 1395–1403

Okon Y and Labandera-Gonzalez CA (1994) Agronomic applications of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. Soil Biol Biochem 26: 1591–1601

Olivier A (1996) La relation entre le *Striga hermonthica* et ses hôtes: une synthèse. Can J Bot 74: 1119–1137

Sallé G, Raynal-Roques A and Tuquet C (1995) Un fléau en Afrique, les *Striga*. La vie des sciences 12: 27–46

Shah S, Karkhanis V and Desai A (1992) Isolation and characterization of siderophore, with antimicrobial activity, from *Azospirillum lipoferum* M. Curr Microbiol 25: 347–351

Tapia-Hernandez A, Mascarua-Esparza MA and Caballero-Mellado J (1990) Production of bacteriocins and siderophore-like activity by *Azospirillum brasilense*. Microbios 64: 73–83

Tien TM, Gaskins MH and Hubbell DH (1979) Plant growth substances produced by *Azospirillum brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). Appl Environ Microbiol 37: 1016–1024

Tyler ME, Milam JR, Smith RL, Schank SC and Zuberer DA (1979) Isolation of *Azospirillum* from diverse geographic regions. Can J Microbiol 25: 693–697

Weerasuriya Y, Siame BA, Hess D, Ejeta G and Butler LG (1993) Influence of conditions and genotype on the amount of *Striga* germination stimulants exuded by roots of several hosts crops. J Agric Food Chem 41: 1492–1496

Zwanenburg B, Mangnus EM and Thuring JWJF (1994) Strigol analogues: design, synthesis and biological activity. In: Pieterse AH, Verkley JAC, Ter Borg SJ (eds) Proceedings of the Third International Workshop on Orobanche and Related Striga Research, Amsterdam (pp 187–197) Royal Tropical Institute Publisher, The Netherlands