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Original article

Seedlings growth promotion by *Azospirillum brasilense* under normal and drought conditions remains unaltered in Tebuconazole-treated wheat seeds

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ABSTRACT

The wide use of pesticides in modern agriculture may cause side effects on the non-target microflora. Data on the fungicide Tebuconazole effects on *Azospirillum*-wheat association are scarce. We analyzed the effects of Tebuconazole on: (a) *Azospirillum brasilense* Sp245 growth in pure culture, (b) *A. brasilense* Sp245 colonization of *Triticum aestivum* cv ProINTA Oasis roots, (c) *A. brasilense* Sp245-inoculated seedlings growth under normal and water stress conditions in the presence of 20% polyethylene glycol 8000. Seeds were separated in Tebuconazole-free and Tebuconazole-treated lots. Inoculated and non-inoculated seedlings were grown in hydroponics in the dark at 20 °C for 72 h. Root surface, coleoptile length, fresh and dry (DW) weights in both tissues and diazotrophic bacterial most probable number in roots were determined. Water contents and shoot-to-roots DW ratio were calculated. Neither *Azospirillum* growth nor root colonization was affected by Tebuconazole. Under normal growth conditions most of the growth parameters analyzed, revealed a clear positive effect of *A. brasilense* on wheat seedlings up to 72 h treatments. The characteristic *Azospirillum* enhancing effects observed on roots remained unaltered by Tebuconazole. The present study shows that Tebuconazole is compatible with *A. brasilense* Sp245-wheat inoculation.

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1. Introduction

The potential of various soil microorganisms to exert beneficial effects on various crops is now well established. They are often called plant growth promoting bacteria (PGPB). Amongst them, the species of *Azospirillum* have gained the reputation of being the most studied plant-associative bacterium [6]. When present in plants in proper amounts, they stimulate the

density and length of root hairs, the rate of appearance of lateral roots and root surface area [25]. These effects cause roots to take up more water and mineral nutrients resulting in faster plant growth. Under appropriate agronomic conditions, these processes would increase crop yield.

Plant water stress, often times caused by drought, can have major impacts on plant growth and development [27]. *Azospirillum* inoculation improves plant growth under water

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stress conditions [14]. It has been demonstrated that *A. brasilense* Sp245 improved water status in wheat seedlings under salt and osmotic stresses [1,4,12,13], and in other species [19].

On the other hand, the wide use of pesticides in modern agriculture may cause harmful side effects on the non-target soil microflora. Field and laboratory research on the effects of pesticides on soil microorganisms in the past decades indicated that most pesticides have only transient or minor influence on the soil microflora [18]. Studies on the influence of various pesticides on the growth of *Azospirillum* spp., showed that none of them had significant effects on the growth even at very high concentrations. By contrast, some fungicides were toxic to it [8]. Moreover, many pesticides, particularly herbicides, are toxic to cultivated plants [18] and also their performance is often affected by environmental factors such as water deficit situations [31,32].

Taking into account the increasing use of *Azospirillum* as PGPB and the fact that seeds and plants are frequently exposed to different pesticides, studying the combination of these factors on wheat seedlings growth assumed utmost importance. One of such pesticides is Tebuconazole. This fungicide is widely used to treat wheat seeds to prevent diseases caused by *Ustilago* spp., *Tilletia* spp. and *Fusarium* spp. [9]. However, Tebuconazole has been mentioned as the most significant residue when its metabolism was analyzed in wheat studies [17]. Therefore, the presence of Tebuconazole in the rhizosphere of wheat could be disturbing not only plant metabolism but also plant-microorganism relationships under normal or drought conditions.

The aims of the present work were to study Tebuconazole effects on: (1) *Azospirillum* growth under pure culture conditions; (2) root colonization by *Azospirillum* in wheat seedlings growing in hydroponics under normal or osmotic stress conditions; (3) growth parameters in *Azospirillum*-inoculated wheat seedlings treated as described in (2).

2. Materials and methods

All chemicals were of analytical grade. Polyethylene glycol (PEG) 8000 and Tebuconazole were purchased from SIGMA, St. Louis (USA) and from Supelco, Bellefonte, Pennsylvania (USA), respectively. Bacteria were grown in the following media: OAB medium [11] or OAB medium supplemented with 1 µg of Tebuconazole mL⁻¹ filtrated by 0.22 µm Millipore filter (this fungicide concentration was calculated on the basis of the recommended agricultural dose for seeds and the culture media volume used). Growth conditions were as described earlier [13]. Bacterial concentration in the growing media was calculated by determining both absorbance at 540 nm and Most Probable Number (MPN mL⁻¹) with nitrogen free Nfb-agar [12,28].

Wheat seeds (*Triticum aestivum* cv ProINTA Oasis) (Instituto Nacional de Tecnología Agropecuaria, EEA Balcarce, Argentina) were selected, disinfected in 1% NaClO for 3 min, and thoroughly rinsed in sterile water as described earlier [15]. Tebuconazole was applied when corresponding as a flowable powder, by stirring seeds with the chemical in a closed plastic bag up to the 2.5 mg kg⁻¹ seeds (this fungicide concentration was calculated on the basis of the

recommended agricultural dose and the inoculum volume used per seed). Germination was accomplished in a humid growth chamber for 72 h in the dark at 20 °C. *Azospirillum brasilense* Sp245 inoculation was performed afterwards as described earlier [12]. Briefly, uniform 2-cm coleoptile size seedlings were selected from each germinated pool and roots were submerged in a 10⁶ cells/mL inoculum for 3 h. All *A. brasilense* suspensions were made in phosphate buffer (66 mM, pH 6.8). Autoclaved bacteria were used as controls. After inoculation, seedlings growth proceeded in hydroponics in distilled water for an additional 72 h in the dark at 20 °C. When corresponding, immediately after inoculation mild water stress was imposed by replacing distilled water with a 20% polyethylene glycol (PEG) 8000 solution. This concentration provides an osmotic potential of -0.54 MPa [23]. The eight following treatments were assayed: non-treated control (C); Tebuconazole-treated (T); *Azospirillum*-inoculated (A); Tebuconazole-treated and *Azospirillum*-inoculated (TA); water-stressed (S); Tebuconazole-treated and water-stressed (TS); *Azospirillum*-inoculated and water-stressed (AS); and Tebuconazole-treated and *Azospirillum*-inoculated and water-stressed (TAS) seedlings. After 48 and 72 h growth, representative samples of seedlings (see experimental design below) were taken from each batch of treatments. Roots were washed with distilled water and 0.5 g used to determine MPN as described earlier [12]. Coleoptile length was measured with a ruler. Root surface was determined by titration and expressed as mL of 0.03 N NaOH [16]. Fresh (FW) and dry (DW) weights were determined in both tissues. Dry weights were determined by drying coleoptiles and roots in an oven at 65 °C until constant weight. Water contents (WC) were calculated as follows: $WC = (FW - DW) \times DW^{-1} \times 100$. Shoot DW/roots DW ratio was also calculated.

Experiments were a factorial combination of two inoculation levels (with and without bacteria) and two Tebuconazole concentrations (0 and the recommended dose) in complete randomized blocks, with four replicates and five seedlings in each treatment. Both normal and water stress conditions at 48 and 72 h treatments were analyzed as independent experiments. The results were analyzed with the PROC GLM procedure in the SAS statistical package and means compared by Duncan test ($P < 0.05$) [29]. Pearson correlation coefficient ($P < 0.05$) was calculated to establish the lineal correlation between RS and coleoptile growth parameters (coleoptile length and DW).

3. Results

Fig. 1 shows *A. brasilense* Sp245 growth curves in the absence or in the presence of 1 µg of Tebuconazole × mL⁻¹ OAB medium. Tebuconazole was completely harmless at the concentration tested, as lower OD values observed in Tebuconazole treatments after 14 h of growth did not correlate with a lower MPN.

Table 1 shows MPN of endophytic, N₂-fixing microorganisms present in roots of wheat seedlings exposed to the different treatments. Tebuconazole did not affect *A. brasilense* Sp245 root colonization when present in the hydroponic media.

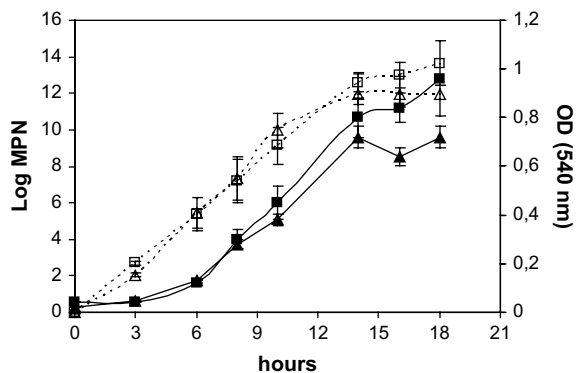


Fig. 1 – *Azospirillum brasilense* Sp245 growth curves in the absence (control) or in the presence of Tebuconazole ($1 \mu\text{g mL}^{-1}$). Bacterial concentration was determined both by measuring optical density (OD) at 540 nm and by Most Probable Number (MPN). Dashed lines, log MPN; solid lines, OD. Δ , with Tebuconazole; \square , without Tebuconazole. Results are shown as mean \pm SD ($P < 0.05$).

Fig. 2 shows Tebuconazole effects on coleoptile length in control and *Azospirillum*-inoculated wheat seedlings growing either under normal or water stress conditions after inoculation. While coleoptile length was promoted by *Azospirillum* in well irrigated seedlings 48 h after inoculation, this parameter remained unaltered under the presence of Tebuconazole. However, 72 h after inoculation coleoptile length was significantly higher ($P < 0.05$) than controls both in T- and

Table 1 – *Azospirillum brasilense* Sp245 root colonization of *Triticum aestivum* cv ProINTA Oasis seedlings exposed to different treatments

Treatment	Time elapsed after inoculation (h)	<i>Azospirillum</i> root colonization MPN (g of roots) $^{-1}$
Control	48	<100
	72	<100
Tebuconazole	48	<100
	72	<100
<i>Azospirillum</i>	48	$4 \times 10^7 \pm 1 \times 10^6$
	72	$7 \times 10^7 \pm 5 \times 10^6$
<i>Azospirillum</i> + Tebuconazole	48	$3 \times 10^7 \pm 4 \times 10^6$
	72	$6 \times 10^7 \pm 4 \times 10^6$
Control + stress	48	<100
	72	<100
Tebuconazole + stress	48	<100
	72	<100
<i>Azospirillum</i> + stress	48	$6 \times 10^6 \pm 4 \times 10^5$
	72	$8 \times 10^6 \pm 2 \times 10^5$
<i>Azospirillum</i> + Tebuconazole + stress	48	$7 \times 10^6 \pm 4 \times 10^5$
	72	$7 \times 10^6 \pm 6 \times 10^5$

Results are expressed as Most Probable Numbers (MPN). Results are shown as means \pm SD ($P < 0.05$), obtained from three replicates of 10 seedling roots each.

TA-treated seedlings (Fig. 2A). The growth promotion effect of the fungicide alone was also evident under stress conditions after 72 h treatment (Fig. 2B).

Fig. 3 shows Tebuconazole effects on root surface in control and *Azospirillum*-inoculated wheat seedlings growing either under normal or water stress conditions after inoculation. *A. brasilense* Sp245 inoculation enhanced root surface after 48 h of growth in well irrigated wheat seedlings (Fig. 3A) and had no effect under water stress (Fig. 3B). However, after 72 h the bacteria had a significant effect on this parameter both in well irrigated and stressed seedlings, either in the absence or in the presence of Tebuconazole (Fig. 3A, B).

Table 2 shows the correlation coefficients ($P < 0.05$) between coleoptile length or dry weight and root surface in control and *Azospirillum*-inoculated T-treated and non-treated seedlings growing under normal conditions after inoculation. A high positive correlation was detected between coleoptile length and root surface after 48 h of growth in *Azospirillum*-inoculated plants. This correlation showed a negative value in control non-inoculated plants. Tebuconazole-treated plants showed no correlation. Dry weight correlations were also small. After 72 h of growth, again coleoptile length and root surface showed a high correlation coefficient in *Azospirillum*-inoculated plants. Tebuconazole-treated plants also showed a high value. The combination of factors diminished this correlation. Dry weight correlated with the root surface only in T-treated plants.

Fig. 4 shows Tebuconazole effects on coleoptile water content in control and *Azospirillum*-inoculated wheat seedlings growing either under normal or water stress conditions. Under normal irrigation, while no differences on WC among treatments were observed after 48 h, after 72 h Tebuconazole had a negative effect on this parameter while the bacteria enhanced it. However, the combination TA did not significantly alter WC in coleoptile after 72 h treatment (Fig. 4A). Under water stress, both A and TA treatments increased coleoptile's WC after 48 h. These effects disappeared after 72 h, with WC in TA treatment even lower than controls (Fig. 4B).

Fig. 5 shows Tebuconazole effects on root WC in control and *Azospirillum*-inoculated wheat seedlings growing either under normal or water stress conditions after inoculation. In well irrigated seedlings and after 48 h treatment, Tebuconazole presence lowered roots' WC significantly but this effect was reverted 72 h after treatment (Fig. 5A). Moreover, WC was significantly enhanced in roots of *Azospirillum*-inoculated wheat seedlings 72 h after inoculation (Fig. 5A). In contrast, in roots exposed to water stress during 48 h the inoculation lowered WC and TA raised it, but neither treatment had effect after 72 h inoculation (Fig. 5B).

Fig. 6 shows Tebuconazole effects on shoot DW to root DW ratio (S/R ratio) in control and *Azospirillum*-inoculated wheat seedlings growing either under normal or water stress conditions after inoculation. In contrast to *Azospirillum* stimulation, Tebuconazole diminished ($P < 0.05$) S/R ratio in well irrigated seedlings 48 h after inoculation (Fig. 6A). While this effect remained in T-treated seedlings for an additional 24 h, it was not significant for those treated with the combination TA (Fig. 6A). Under water stress condition, only T treatment had a significant effect in stimulating this ratio after 72 h (Fig. 6B).

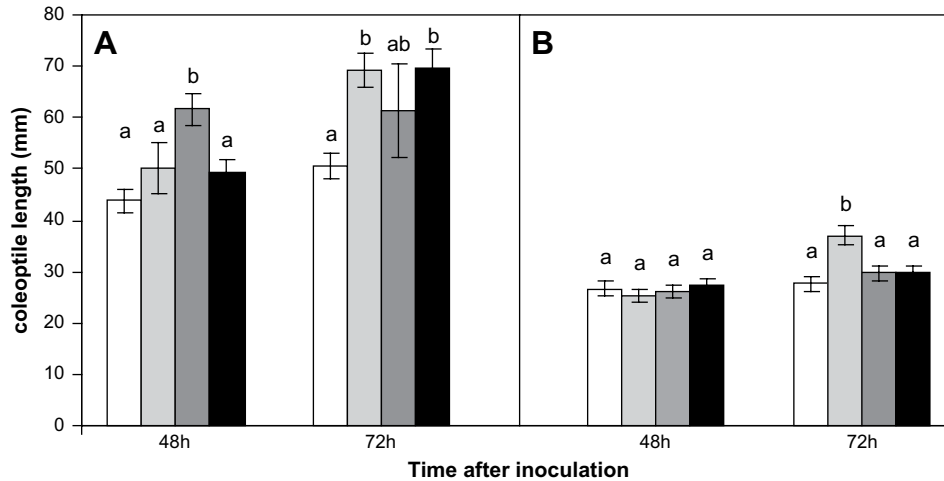


Fig. 2 – Coleoptile growth in *Triticum aestivum* cv. ProINTA Oasis seedlings exposed to Tebuconazole treatment (2.5 mg kg/ seeds) and *Azospirillum brasilense* Sp245 inoculation. A, growth in distilled water; B, growth in 20% PEG 8000 (–0.54 MPa). □, Control inoculated with autoclaved bacteria; ■, Tebuconazole-treated seedlings; ▒, *Azospirillum*-inoculated seedlings; ■, Tebuconazole/*Azospirillum*-treated seedlings. Results are shown as mean \pm SD ($P < 0.05$), obtained from four replicates of five seedlings each. Columns denoted by different letters differ significantly at $P < 0.05$.

4. Discussion

The necessity to improve crop disease management through the integration of cultural, agronomic and plant resistance strategies has been stressed. Amongst these, recent developments have invigorated PGPB research [30]. Moreover, in view of the high input of agrochemicals in contemporary crop production and the likelihood that *Azospirillum* inoculation could be regularly used in the near future in regular crop production, studies on the interaction of *Azospirillum* inoculants with common pesticides are essential. In this regards, only a few tests have been conducted so far mostly under laboratory conditions [7]. Although herbicides are the obvious class of pesticides affecting plant growth, crops are regularly

treated with the less toxic fungicides. However, these compounds should be specific for their targets, and have no effects on other soil microorganisms. In addition, if fungicides somehow alter plant metabolism and growth, its application should be the less damaging response to the worse situation resulting from an unchecked pest attack [20]. Besides its effects on preventing fungal attacks, fungicidal treated seedlings build natural defences that provide additional protection from disease and insect pests. However, the possibility that in the long run these pesticides could be altering microbial ecosystems in the rhizosphere or their interaction with plants should not be discarded. In this work, Tebuconazole a common fungicide used as a seed treatment for the control of smuts, bunt, seed rots and seedling blights on wheat [9] was chosen as a model.

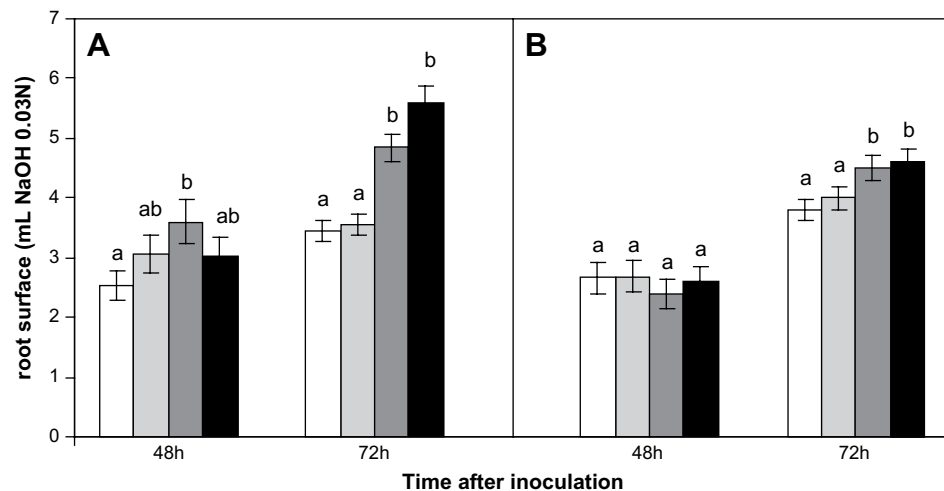


Fig. 3 – Root surface in *Triticum aestivum* cv. ProINTA Oasis seedlings exposed to Tebuconazole treatment (2.5 mg kg/ seeds) and *Azospirillum brasilense* Sp245 inoculation. A, growth in distilled water; B, growth in 20% PEG 8000 (–0.54 MPa). Details as in Fig. 2.

Table 2 – Correlation coefficients ($P < 0.05$) between root surface and coleoptile length or dry weight in *Triticum aestivum* cv ProINTA Oasis seedlings exposed to different treatments

Coleoptile	Root surface							
	48 h after inoculation				72 h after inoculation			
	C	T	A	AT	C	T	A	AT
Length	-0.7638	0.2664	0.8200	0.1831	-0.3610	0.6920	0.7134	0.4383
Dry weight	-0.6830	0.4149	0.3249	0.0419	0.3000	0.6869	0.3653	0.2684

C, control; T, Tebuconazole; A, *Azospirillum*.

Our results (Fig. 1) showed that cell growth was not hampered by Tebuconazole during exponential growth up to 14 h, when bacteria reached the metabolic optimum conditions to interact with seedling roots and colonize them. We presumed that the lower OD values observed in Tebuconazole treatments in pure cultures after 14 h of growth could be related to some kind of cell aggregation without affecting cell viability, because these diminished values did not correlate with a lower MPN. Neither *Azospirillum lipoferum* nor *A. brasilense* growth was affected when growing in pure culture in the presence of three insecticides and 10 herbicides [2]. Cycoń et al. [15] demonstrated that nitrogen-fixing bacterial numbers augmented in accordance to increased levels of Tebuconazole in soil. However, amongst 20 different pesticides, fungicides were the most toxic compounds to *A. brasilense* Cd in *in vitro* growth [18]. Omar and Abd-Alla [26] observed a progressive decrease in the growth of *A. brasilense* Sp245 and *A. lipoferum* 215 with increasing concentrations of a mixture of two thiocarbamates and copper oxychloride fungicides, a benzoic acid herbicide or a pyrethroid insecticide. Our results showed that the active compound Tebuconazole is compatible with *Azospirillum* growth in pure culture. Nevertheless, the inhibitory effect on bacterial growth in many commercial fungicide formulations may be due to the presence of excipient compounds. Each one of them might be particularly assayed to assess its compatibility with *Azospirillum*.

We next studied if the presence of Tebuconazole could alter the bacterial colonization of roots in wheat seedlings either in normal or water stress conditions. Tebuconazole did not affect *A. brasilense* Sp245 root colonization when present in the hydroponic media (Table 1) in either normal or water stress conditions. In this regard, contradictory results were reported. Bozhkov et al. [10] reported that various pesticides caused a considerable reduction of the inoculum exposed to roots and even lead to its total destruction. On the other side, Madhuri and Rangaswamy [22] found that after 24 h of incubation in fungicide treated soils, *Azospirillum* populations reached a maximum stimulation at different fungicide doses.

It is worth to point out, that this experiment provide only direct evidence of the Tebuconazole effect on wheat seedlings in respect to the plant capacity to support bacterial root colonization and not about the direct effect of Tebuconazole on the microorganism growth or PGPR activity, because the inoculation methodology separated the active principle and bacteria spatially.

However, the possibility that Tebuconazole could alter several growth parameters in wheat seedlings cannot be discarded. Forty eight hours after inoculation and under normal conditions, only *Azospirillum* growth promotion effects were detected as inoculated seedlings coleoptiles were the longest ones (Fig. 2A). Inoculated Tebuconazole-treated seedlings showed no difference with T-treated non-inoculated controls. On the contrary,

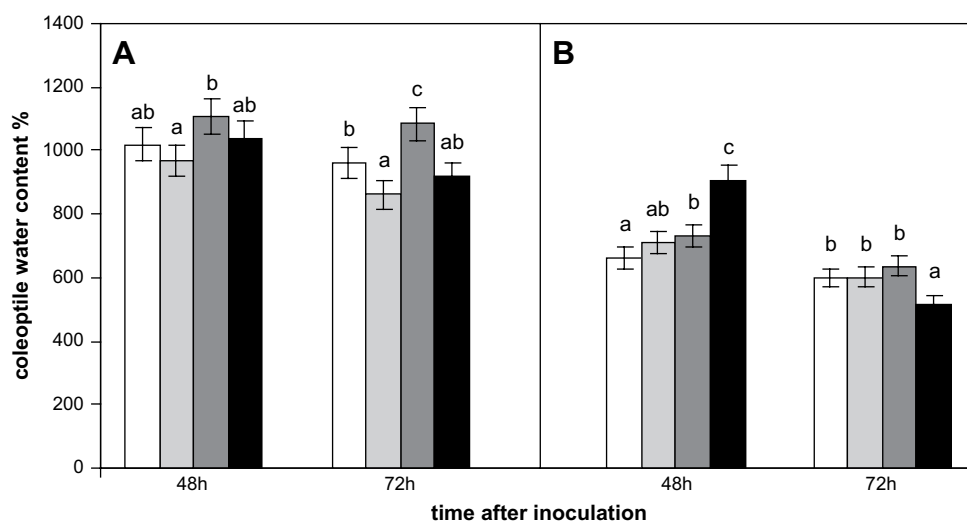


Fig. 4 – Coleoptile Water Content (WC) in *Triticum aestivum* cv. ProINTA Oasis seedlings exposed to Tebuconazole treatment (2.5 mg kg/seed) and *Azospirillum brasilense* Sp245 inoculation. WC was calculated as $WC = (FW - DW) \times DW^{-1} \times 100$. A, growth in distilled water; B, growth in 20% PEG 8000 (-0.54 MPa). Details as in Fig. 2.

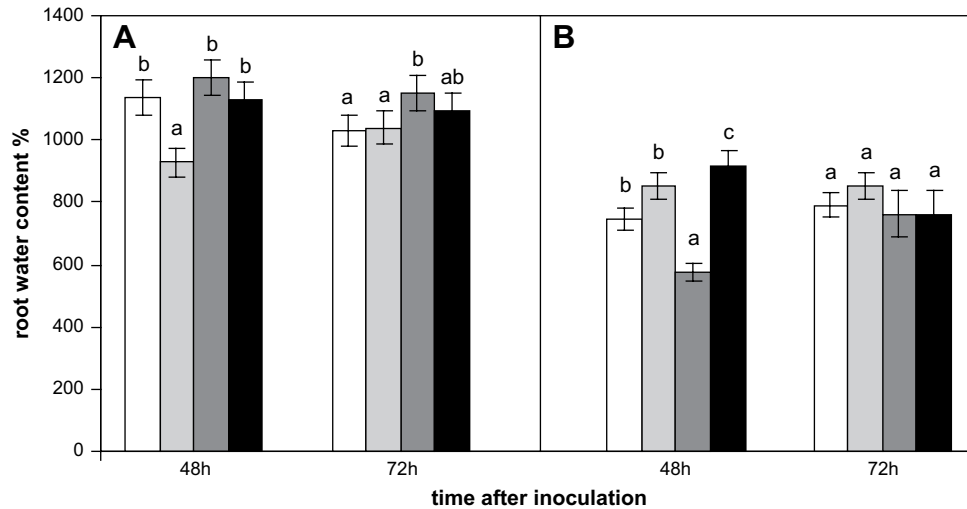


Fig. 5 – Root Water Content (WC) in *Triticum aestivum* cv. ProINTA Oasis seedlings exposed to Tebuconazole treatment (2.5 mg kg/seeds) and *Azospirillum brasiliense* Sp245 inoculation. WC was calculated as $WC = (FW - DW) \times DW^{-1} \times 100$. A, growth in distilled water; B, growth in 20% PEG 8000 (-0.54 MPa). Details as in Fig. 2.

a Tebuconazole growth promotion was recorded 24 h after application, since T-treated inoculated and non-inoculated seedlings showed larger coleoptiles. In this regard, contradictory results were found in different species treated with triazole fungicides. Some workers reported growth-retarding effects on shoots and roots [3] and Jardine and Bowden [21] reported that Tebuconazole shortened wheat coleoptiles and recommended to avoid deep planting of T-treated seeds. Other authors found growth stimulation [3]. A different situation was seen in stress conditions. Only Tebuconazole growth promotion was detected 72 h after inoculation. This effect was not detected in inoculated seedlings. Asare-Boamah et al. [3] reported a significant degree of protection of Triadimefon (another triazol) against drought by maintaining a high shoot water potential after water restriction had been imposed.

Nevertheless, no Tebuconazole induced changes were detected in coleoptile water content (Fig. 4). On the other side, *Azospirillum* growth promotion was evident by the observation of significant increases in root surface in either normal or water stress conditions (Fig. 3A and B). Tebuconazole itself did not show any particular effect on roots. We even found a positive correlation between coleoptile length and root surface after 48 and 72 h of growth in *Azospirillum*-inoculated plants, suggesting that the enhanced coleoptile's growth could be related to an augmented root surface induced by the bacteria on the seedlings, as was reported previously as characteristic positive effect [10,25]. Tebuconazole growth promotion effects were detected on coleoptile length while *Azospirillum* growth promotion effects were mainly detected on roots. Tebuconazole as well as *Azospirillum* treatments

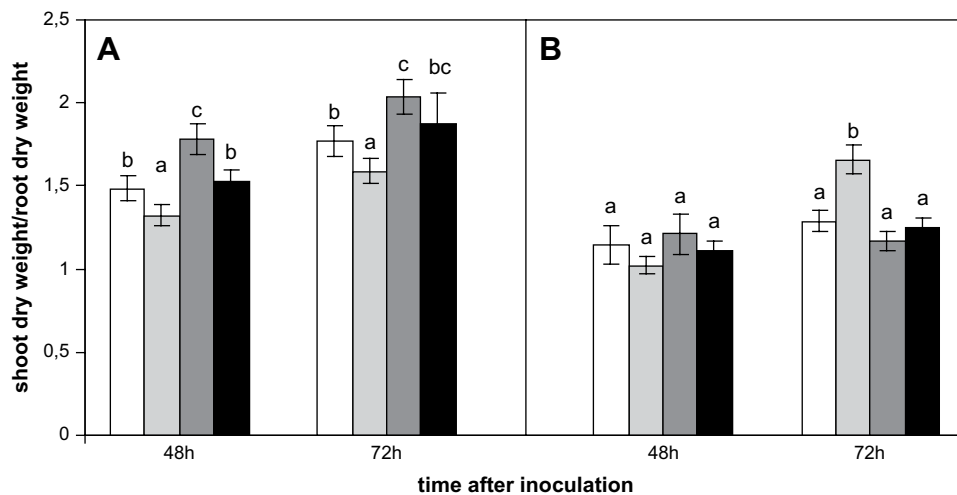


Fig. 6 – Coleoptile dry weight/root dry weight ratio in *Triticum aestivum* cv. ProINTA Oasis seedlings exposed to Tebuconazole treatment (2.5 mg kg/seeds) and *Azospirillum brasiliense* Sp245 inoculation. A, growth in distilled water; B, growth in 20% PEG 8000 (-0.54 MPa). Details as in Fig. 2.

alone improved seedling growth, but no additive effects were found when both were applied together.

Given the close relationship existing between shoot and root development in wheat [24] it is very important to reveal how chemicals applied to seeds could disturb this balance. Shoot-to-root ratio (S/R) in plants is affected by the balance between shoot-specific (carbon supply) and root-specific (mineral and water uptake) activities [5]. Factors participating in the partitioning of dry matter between the shoots and the roots are highly significant to optimal health and performance of plants. *Azospirillum* augmented S/R ratio 48 and 72 h after inoculation under normal conditions (Fig. 6), when coleoptile length increased in correlation with the root surface (Table 2). This effect was not detected under stress conditions. On the other hand, Tebuconazole negatively affected S/R ratio under normal growth conditions but this effect was reverted by *Azospirillum* (Fig. 6A). Under stress conditions, the only effect observed was an enhancement in S/R ratio exerted by Tebuconazole 72 h after application (Fig. 6B). This last situation is in agreement with the longer T-treated coleoptiles observed in Fig. 2B with no changes in T-treated roots (Fig. 3B). Another evidence of this is the absence of correlation between coleoptile length and root surface (Table 2). Bashan and Dubrovsky [5] reported that bacterial induced changes in the S/R ratio could contribute to different partitioning of carbon compounds within the plant (by an as yet unknown mechanism) in addition to its experimentally proven ability to enhance water and mineral uptake by the plant. Our results agreed with these observations under normal conditions and water stress might have delayed the changes beyond the 72 h assay period.

As concluding remarks, coleoptile length (Fig. 2A), root surface (Fig. 3A), coleoptile and root WC (Fig. 4A and Fig. 5A), and reserve partition to shoots (Fig. 6A) indicated a clear growth promotion effect of *A. brasilense* on wheat seedlings growing in hydroponics. Moreover, TA combination promoted coleoptile length (Fig. 2A) and root surface (Fig. 3A), indicating that seedlings growth promotion by *Azospirillum* under normal conditions remained unaltered in Tebuconazole-treated wheat seeds. The fact that AS and TAS treated seedlings also showed an increased root surface may be indicating that the beneficial *Azospirillum*-wheat association is not hampered by the presence of Tebuconazole, even when other stressing factors are present. When applied in combination with other fungicides in field trials, Tebuconazole did not affect winter and spring wheat grain yields of selected endophytic-rhizobacteria-inoculated plants [11].

In short, the present study shows that in the conditions described Tebuconazole is compatible with *A. brasilense* Sp245-wheat inoculation. Further studies must be taken to determine if this assertion can be generalized to the agricultural practice of seed inoculation.

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