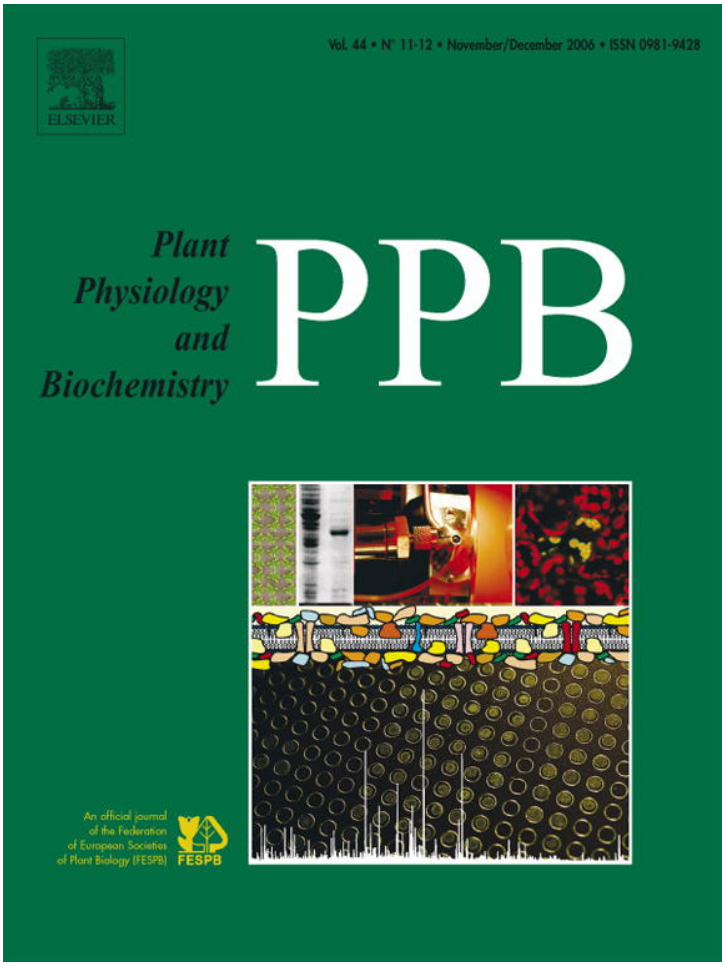

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Research article

Root phospholipids in *Azospirillum*-inoculated wheat seedlings exposed to water stress

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Abstract

Azospirillum–plant association is accompanied by biochemical changes in roots which, in turn, promote plant-growth and tolerance to water stress. To shed light on the possible factors underlying these effects, roots from *Azospirillum brasilense* Sp245-inoculated *Triticum aestivum* seedlings growing in darkness under osmotic stress were analyzed for phospholipid (PL) composition, fatty acid (FA) distribution profiles and degree of unsaturation of the major PL classes. *Azospirillum* inoculation diminished ion leakage and increased 2,3,5-tripheniltetrazolium reducing ability in roots of well irrigated and water-stressed wheat seedlings. Total root PL content remained unaltered in all treatments. Six PL classes were detected, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) comprising over 80% of the total. While water stress increased PC content and diminished that of PE, none of these changes were observed either under *Azospirillum* inoculation alone or when both treatments were combined. The major FAs found in both PC and PE were 16:0, 18:0, 18:1, 18:2, and 18:3. Higher PC and lower PE unsaturation than in well irrigated controls were observed in roots from *Azospirillum*-inoculated, water-stressed seedlings. *Azospirillum* inoculation could contribute to protect wheat seedlings from water stress through changes in the FA distribution profiles of PC and PE major root phospholipids.

Keywords: Azospirillum; Wheat; Roots; Water stress; Phospholipids; Fatty acids

1. Introduction

The lack of water is one of the main constraints to plant-growth and crop yield even in humid areas. Seedling survival to water stress in the days following germination is one of the major limitations to the establishment of species in many habitats [32]. Even though wheat is generally grown in water stress-prone parts of the world, soil water potential strongly affects seedling emergence [32]. In particular, wheat seedlings water-stressed in darkness with 20% polyethylene glycol (PEG) 6000, presented lower shoot height, fresh weight and total protein concentration than control ones [5]. However, part of the negative effects of water stress on shoot growth were attenuated in seedlings previously inoculated with *Azospirillum* [1], where a noticeable improvement in the plant water status was evident [17,18]. In general, *Azospirillum* genus is capable of stimulating growth in graminaceous as well as in nongraminaceous plants all over the world [7]. One of the first observations regarding plant-growth promotion exerted by *Azospirillum* inoculation was on roots [30]. This effect was attributed mainly to the bacterial ability to produce phytohormones [21,22]. In addition, recent results show that nitric oxide could be the inducing molecule participating in the *Azospirillum*-promoted lateral root formation in tomato seedlings [16]. In general and regardless of the mediator involved, one of the most evident effects that plant-growth promoting bacteria (PGPB) exert in plants is the change they produce in root metabolism which, in turn, stimulates growth [40]. How-

Abbreviations: FA, fatty acids; DW, dry weight; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEG, polyethylene glycol; PL, phospholipid; TTC, 2,3,5-triphenyltetrazolium chloride; fatty acids are indicated by the convention, number of carbon atoms: number of double bonds.

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ever, knowledge on the biochemical effects the bacteria could elicit on roots and how these responses could change the plant physiology is still scarce, being direct effects on plant cell membranes one of the hypotheses suggested [8].

Cellular and subcellular membranes are important sites of injury in plants exposed to water deficit [14], where submicroscopic changes ultimately lead to alterations in membrane permeability [32]. Hence, the rate of injury to cell membranes induced by drought may be easily estimated through the measurement of electrolyte leaked from the cells [12]. In addition, the activity of membrane-associated enzymes, such as reductases, is affected by drought-induced damage [15].

There is a complex relationship between membrane damage, susceptibility to stress, and lipid composition [24,39]. It is widely accepted that changes in the physical state of membrane lipids triggered by specific modifications in the microenvironment could have important consequences on the function of many membrane-associated proteins. Among others, these changes may be accomplished by varying the amount of total phospholipids (PL) and/or their class distribution, by changing the kind of fatty acids (FAs) [38] incorporated into them during synthesis or turnover, or by regulating the degree of unsaturation of the fatty acyl moieties [31]. In general, drought-sensitive plant species or cultivars undergo larger changes in lipid composition following drought stress than do drought-resistant ones [31]. In particular, total lipid unsaturation and phosphatidylcholine (PC) turnover in coleoptiles collected from wheat seedlings grown in darkness, were affected by osmotic stress [36]. Under these experimental conditions, no data concerning *Azospirillum*'s effects on root PL has been published so far.

Taking these considerations into account, the aim of the present work was to study the PL composition, FA distribution profiles and degree of unsaturation of major PL classes, in roots from *Azospirillum*-inoculated wheat seedlings growing in darkness under osmotic stress.

2. Results

Coleoptiles from wheat seedlings exposed to 20% PEG 8000 had shorter height and lower projected surface than well irrigated controls (Fig. 1A, B). Roots were also shorter than controls, but their projected area remained unaltered (Fig. 1C, D).

Inoculation of germinated seeds of *Triticum aestivum* cv. ProINTA Oasis with viable *A. brasilense* Sp245 cells resulted in a total *Azospirillum* colonization of ca. 8×10^6 and 2×10^7 cells g DW^{-1} root tissue in plants grown in water and in 20% PEG, respectively. In contrast, less than 100 bacterial cells g DW^{-1} were detected in control seedlings inoculated with previously autoclaved *Azospirillum* cells (data not shown).

Azospirillum inoculation promoted significant increases ($P < 0.05$) in coleoptile length, coleoptile projected area, and root length both in the absence and in the presence of 20% PEG 8000 (Fig. 1). However, the effect of *Azospirillum* on root projected area was evident only in seedlings not exposed to water stress (Fig. 1D).

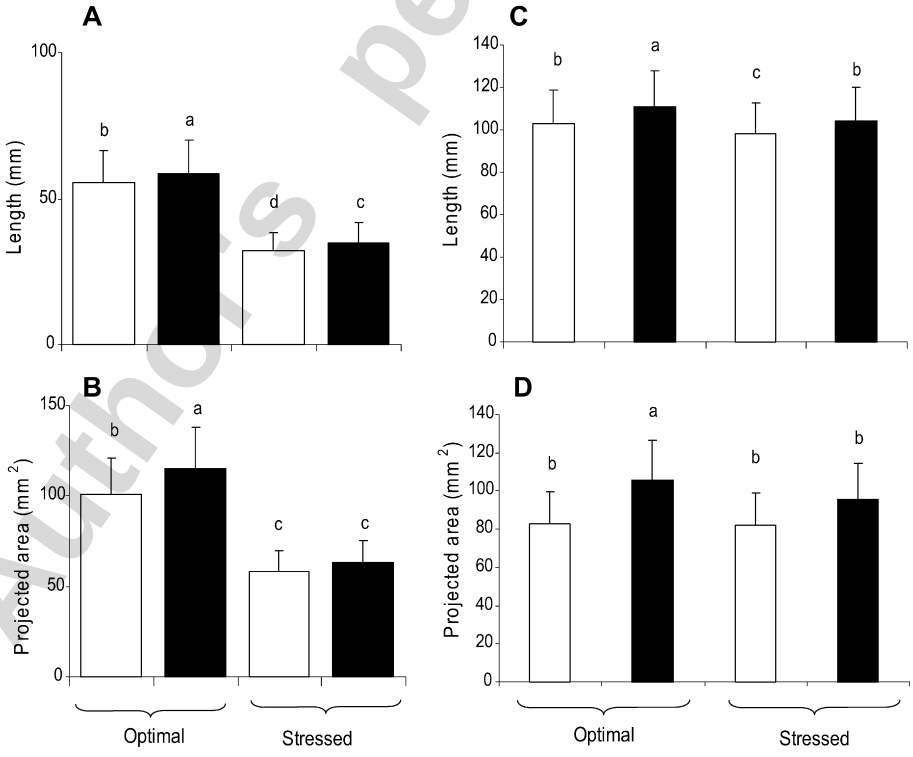

Fig. 1. Length **(A)** and projected area **(B)** in coleoptiles, length **(C)** and projected area **(D)** in roots of *Azospirillum*-inoculated wheat seedlings grown under normal and water stress conditions. Seedlings were grown for 96 h in the dark and inoculated either with previously autoclaved *A. brasilense* Sp245 cells (□), or with live bacteria (■). Each of these groups was then grown for another 48 h, either in sterile distilled water (optimal) or in 20% polyethyleneglycol 8000 (stressed). Results are shown as mean ± S.D. ($P < 0.05$), obtained from five replicates of 30 seedlings each. Columns denoted by different letters differ significantly at $P < 0.05$.

While water stress alone did not produce any significant changes in root electrolyte leakage or in 2,3,5-triphenyltetrazolium chloride (TTC) reducing ability, *Azospirillum* inoculation diminished ion leakage in both well irrigated and water-stressed wheat seedlings (Fig. 2). Moreover, *Azospirillum* inoculation increased the TTC-reducing ability in both roots from well irrigated and water-stressed wheat seedlings (Fig. 2).

Under the present growth conditions, total PL content of roots was not statistically ($P < 0.05$) affected either by water stress, inoculation, or by the combination of both treatments (Table 1). Six PL classes were detected in the TLC analyses of total PL: PC, phosphatidylethanolamine (PE), phosphatydilinositol, phosphatidylserine, phosphatidylglycerol, and diphosphatidylglycerol (Table 1). Phosphatidic acid (PA) remained undetected in the treatments tried. Both PC and PE accounted for well over 80% of the total PL content found in all treatments (Table 1). Water stress increased PC content and tended to diminish that of PE in roots of *T. aestivum* cv. Pro INTA Oasis seedlings growing in darkness (Fig. 3). However, none of these changes were observed either under *Azospirillum* inoculation alone or when both inoculation and water stress treatments were combined (Fig. 3).

Palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) were the major FAs found in both PC and PE (Table 2).

In the PCs, the ratio of total saturated vs. total unsaturated FAs in well irrigated seedlings was 60:40, which changed to 50:50 in all treatments (Table 2). In water-stressed conditions, the unsaturation rise was mainly due to a decrease in 18:0 vs. an overall increase in the unsaturated ones. In the *Azospirillum* inoculation treatment alone, the unsaturation rise was

Table 1
PL composition (nmol g^{-1} DW) in roots from *Azospirillum*-inoculated wheat seedlings grown under normal and water stress conditions

	Treatment			
	Optimal irrigation		Water stress	
	Dead	Live	Dead	Live
PC	8.42 ± 0.42	8.83 ± 0.41	11.50 ± 0.55	9.82 ± 0.41
PE	7.81 ± 0.34	8.98 ± 0.44	6.30 ± 0.32	9.98 ± 0.39
PS	0.72 ± 0.14	0.69 ± 0.15	0.59 ± 0.18	0.62 ± 0.12
PI	0.72 ± 0.12	0.67 ± 0.14	0.59 ± 0.16	0.62 ± 0.14
PG	0.72 ± 0.17	0.68 ± 0.20	0.64 ± 0.14	0.80 ± 0.12
DPG	0.72 ± 0.19	0.64 ± 0.14	0.64 ± 0.13	0.64 ± 0.12

Seedlings were grown for 96 h in the dark and inoculated either with previously autoclaved (dead) *A. brasilense* Sp245 cells, or with live bacteria. Each of these groups was then grown for another 48 h, either in sterile distilled water (optimal) or in 20% polyethyleneglycol 8000 (stressed). PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol. Results are shown as mean ± S.D. ($P < 0.05$), obtained from five replicates of 50 seedlings each.

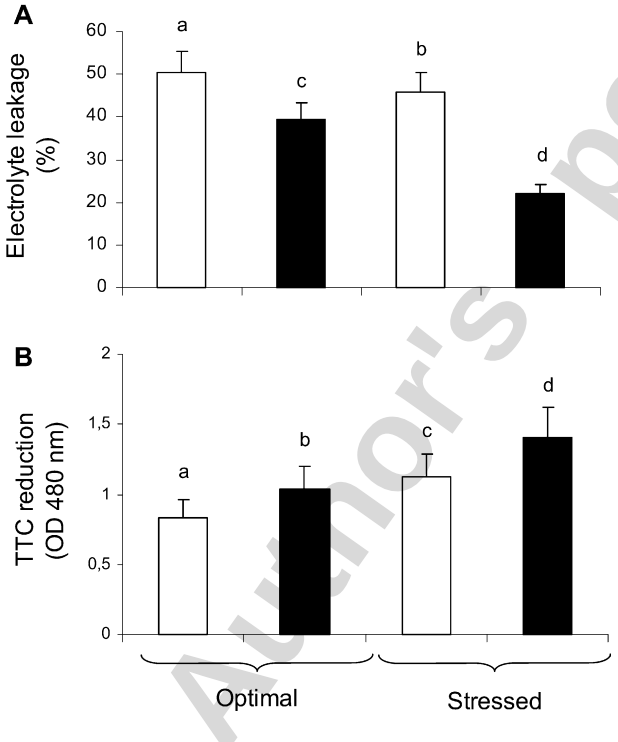

Fig. 2. Electrolyte leakage **(A)** and TTC reduction ability **(B)** in roots of *Azospirillum*-inoculated wheat seedlings grown under normal and water stress conditions. Seedlings were grown for 96 h in the dark and inoculated either with previously autoclaved *A. brasilense* Sp245 cells (□), or with live bacteria (■). Each of these groups was then grown for another 48 h, either in sterile distilled water (optimal) or in 20% polyethyleneglycol 8000 (stressed). Results are shown as mean ± S.D. ($P < 0.05$), obtained from three replicates of 15 seedlings each. Columns denoted by different letters differ significantly at $P < 0.05$.

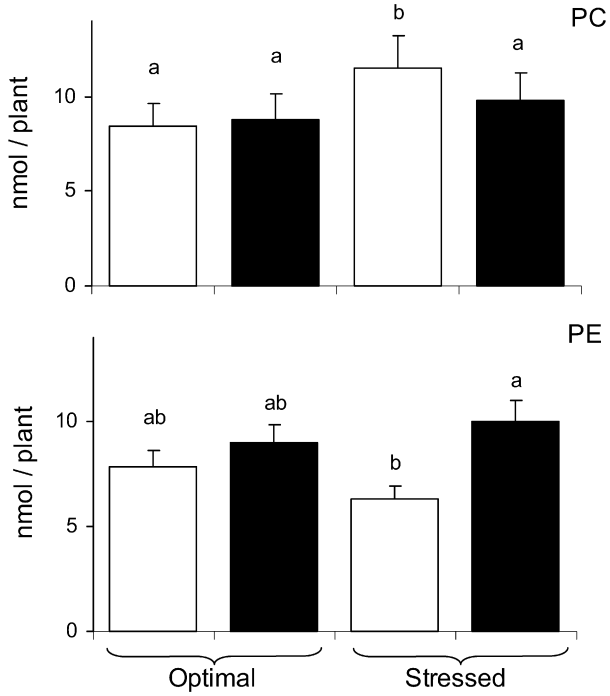

Fig. 3. **PC** and **PE** contents in roots from *Azospirillum*-inoculated wheat seedlings grown under normal and water stress conditions. Seedlings were grown for 96 h in the dark and inoculated either with previously autoclaved *A. brasilense* Sp245 cells (□), or with live bacteria (■). Each of these groups was then grown for another 48 h, either in sterile distilled water (optimal) or in 20% polyethyleneglycol 8000 (stressed). Results are shown as mean ± S.D. ($P < 0.05$), obtained from five replicates of 50 seedlings each. Columns denoted by different letters differ significantly at $P < 0.05$.

Table 2
FA composition (percentages) in PC and PE extracted from roots from *Azospirillum*-inoculated wheat seedlings grown under normal and water stress conditions

	PC				PE			
	Treatment				Treatment			
	Optimal irrigation		Water stress		Optimal irrigation		Water stress	
	Dead	Live	Dead	Live	Dead	Live	Dead	Live
16:0	38.0 ± 3.0	36.8 ± 4.5	33.3 ± 4.5	30.8 ± 3.6	24.5 ± 2.6	31.0 ± 4.9	40.3 ± 5.2	33.3 ± 4.0
18:0	21.5 ± 1.0	12.5 ± 1.9	13.0 ± 1.7	18.2 ± 2.1	18.3 ± 4.4	19.8 ± 3.2	15.3 ± 1.8	16.3 ± 3.0
18:1	17.5 ± 5.8	14.7 ± 0.7	23.3 ± 2.3	18.5 ± 1.6	22.5 ± 1.0	19.3 ± 3.0	13.3 ± 1.3	13.0 ± 1.6
18:2	12.8 ± 4.3	25.3 ± 2.6	16.5 ± 1.3	21.8 ± 2.4	19.5 ± 3.8	21.0 ± 2.5	21.0 ± 1.8	23.8 ± 1.2
18:3	10.8 ± 6.3	11.3 ± 1.9	14.3 ± 1.5	11.3 ± 1.3	15.5 ± 1.6	9.0 ± 2.6	10.0 ± 0.9	13.8 ± 0.3
DBI	1.4 ± 0.4 (a)	2.1 ± 0.5 (b)	2.2 ± 0.3 (b)	2.0 ± 0.3 (b)	2.6 ± 0.6 (c)	1.9 ± 0.6 (b)	1.6 ± 0.6 (a)	2.1 ± 0.3 (b)

Seedlings were grown for 96 h in the dark and inoculated either with previously autoclaved (dead) *A. brasilense* Sp245 cells, or with live bacteria. Each of these groups was then grown for another 48 h, either in sterile distilled water (optimal) or in 20% polyethyleneglycol 8000 (stressed). DBI, double bond index. Results are shown as mean ± S.D. ($P < 0.05$), obtained from five replicates of 50 seedlings each. Columns denoted by different letters differ significantly at $P < 0.05$.

caused mainly by a 18:0 decrease accompanied by a 18:2 increase. When inoculated seedlings were exposed to water stress, the unsaturation rise was caused mainly by a decrease in 16:0 and a rise in 18:2. In short, *Azospirillum* inoculation may have some effects in rising 18:2 in PCs from wheat seedling roots growing both under normal and stressed conditions. These effects on the FA distribution profile of PC was reflected in the double bond indexes, which rose with every treatment tried (Table 2).

In the PEs, the ratio of total saturated vs. total unsaturated FAs in well irrigated seedlings was 40:60, which changed to 50:50 in all treatments (Table 2). In water-stressed conditions, the unsaturation fall was mainly due to a 16:0 increase accompanied by a decrease in both 18:1 and 18:3 (Table 2). In the *Azospirillum* inoculation treatment alone, the unsaturation fall was caused mainly by a 16:0 increase accompanied mainly by a 18:3 fall (Table 2). When inoculated seedlings were exposed to water stress, 16:0 rise was accompanied by a 18:1 fall (Table 2). These effects on the FA distribution profile of PE was reflected in the double bond indexes, which fell with every treatment tried (Table 2). However, the effect water stress alone had on this parameter was partially reverted in *Azospirillum*-inoculated seedlings (Table 2).

3. Discussion

Water stress affected several coleoptile growth parameters in seedlings from different wheat cultivars [1,5,18]. Water-stressed seedlings of *T. aestivum* cv. Pro INTA Oasis also had both lower coleoptile height and projected surface than those of well irrigated ones. However, while these changes took place in the aerial part, root length and projected area remained unaltered. Preserving root size while diminishing that of the aerial part could be part of a mechanism that could help seedlings of this cultivar to cope with a mild water stress as the one imposed by 20% PEG 8000.

On the other hand, *Azospirillum*–plant interactions have been extensively studied since the seventies [40]. The bacteria stimulate plant-growth even in the presence of several stressors, such as drought [17], salt [25,2], heavy metals [10], humic acid [3], and also, mitigates high pH effects in microalgae [19]. Appropriate root colonization is known to result in growth promotion of wheat and maize seedlings [1,13,18], which are more evident under water stress [1,18]. With this degree of colonization, a larger root system was evident in *Azospirillum*-inoculated wheat seedlings growing either under well irrigated or water stress conditions. However, the growth promotion effect on root projected area was only evident under water stress conditions. Even though the bacteria can stimulate the rate of root elongation, its mode of action is still under discussion [8]. It has been assumed that as a consequence of deeper plant rooting, inoculated plants may enhance the uptake of minerals and water, which in turn could benefit crops growing in water-deficient soils [30]. In this regard and in order to successfully cope with other changing environmental conditions, the plant must rely mainly on the permeability barrier constituted by their plasma membranes [11]. *Azospirillum* inoculation affects proton efflux activities in intact wheat roots and cowpea [6] reduces the membrane potential in soybean seedlings [6], and changes the PL content in cowpea plant membranes [6,20]. Thus, root membranes could represent the first point of contact for *Azospirillum* in order to initiate its growth promotion effects in plants [8].

The permeability of roots, as measured by ion leakage, has been directly correlated to membrane integrity [4]. A lower ion leakage in plants exposed to drought, has been considered indicative of a relative tolerance to water stress [11]. In our situation, electrolyte leakage did not increase either in water-stressed or inoculated seedlings, indicating uninjured root membranes. Moreover, the degree of stress imposed by 20% PEG 8000 has been considered moderate [18]. A stronger stress could cause collateral damaging effects that could mask the eventual mechanisms of tolerance.

Moreover, when both treatments were applied together, electrolyte leakage was reduced; suggesting more stable cell membranes than those of seedlings subjected to water stress or inoculation treatments alone. Another cell viability assay applied to measure stress tolerance is the TTC-reducing ability, where the dye is reduced mainly by electrons coming from the mitochondrial electron transport chain [15]. In our case, *Azospirillum*-inoculated wheat seedling roots had higher values of TTC-reducing ability than the corresponding controls inoculated with autoclaved bacteria. In short, the results obtained with both electrolyte leakage and TTC-reducing tests

are compatible with a tendency of the cultivar to adapt to water stress conditions and an *Azospirillum* effect in enhancing the overall cell membrane performance.

A correct membrane performance under variable environmental conditions implies a dynamic hydrophobic frame where changes in lipid composition should be expected. Several studies [24] on plant lipid composition in response to water stress revealed a decrease in total PL content [29], an augmented PC: PE ratio, and no changes in total FA unsaturation [33]. In cowpea calli inoculated with *A. brasilense* Cd, a significative increase in total PL was observed, which could be indicating an important role of membranes in the biochemical response of plants to *Azospirillum* interaction [6]. In our experimental system, the total PL content of roots was not modified either by inoculation or by water stress treatments alone. However, a non-significant ($P < 0.05$) total PL tendency to increase could be appreciated when both inoculation and water stress were combined. This could be related to a higher projected root surface under the same conditions and also, to an increase in total TTC reduction ability.

Although six PL classes were detected, PC and PE were in the range 85–88% of total PL in all treatments.

T. aestivum cv. Pro INTA Oasis seedlings exposed to water stress showed a significant higher PC content and a lower PE content in roots than well irrigated controls, resulting in a higher PC:PE ratio. The decrease in PE was almost totally compensated by an increase in PC. This could be compatible with a methylation of PE leading to PC biosynthesis. Such speculation would be supported by a low amount of PA. The negligible amount of PA found in the lipid extracts is an important indication that roots homogenization with boiling isopropyl alcohol before extraction was effective in inactivating lipid-degrading enzymes [26]. Such finding also supports the speculation expressed above.

Changes in PC:PE ratio have been observed in many organisms and there is evidence suggesting that an enhanced PC content in plants could be part of a general adaptive mechanism to stress [27]. In addition, the root FA distribution profiles of *T. aestivum* cv. Pro INTA Oasis seedlings exposed to water stress revealed an increase in the degree of PC unsaturation and a decrease in that of PE. Thus, an increase in the fluidity of membranes could have occurred in these cells. Based on this assumption, one possible function of this increase could be to limit the loss of water thus preserving cell turgidity [27]. In short, both a higher PC:PE ratio and unsaturation in Pro INTA Oasis root seedlings exposed to less available water could be indicating that the cultivar has a certain degree of tolerance to water stress.

Regarding bacterial effects alone, although inoculated wheat roots showed no significant changes in the distribution of the major PL classes, PE became more saturated and PC more unsaturated than controls, the latter PL with a particularly high content of 18:2. There is evidence that FA desaturases bound to the endoplasmic reticulum could be part of an existing link between the correct level of membrane unsaturation and plant cell elongation [28]. *A. brasilense* could have an effect on the activity of desaturases of wheat root membranes. Changes in the FA distribution profiles of PC and PE may represent a novel control mechanism exerted by the bacteria on the plant and would be worthy of further studies.

When both *Azospirillum* inoculation and water stress treatments were combined, the distribution of the major PL classes remained unaltered while FA distribution profiles changed in both PC and PE. Among the FAs affected by inoculation followed by stress, the 18:2 content of PC increased the most. In PE, 16:0 was increased in all treatments. It has been reported that *Azospirillum* could mitigate negative water stress and saline stress effects on wheat [18]. The present work tends to shed light on the possible relationship between the amelioration effects of water stress on wheat, and the lipid composition of roots. In comparison, even though the halophyte *Salicornia bigelovii* oilseed is already adapted to survive in a harsh, saline environment, *Azospirillum* inoculation increased total lipid content and increased the percentage of 16:0 while decreasing that of 18:2, in seeds harvested from plants grown under seawater irrigation [9]. Many studies have suggested the involvement of auxins produced by *Azospirillum* on the root morphology [28]. These hormones are involved in the expression of ER-bound Δ^{12} desaturase, a key enzyme in the synthesis of 18:2. As a matter of speculation, this FA could be contributing to regulate the activity of key membrane-associated enzymes such as ATPases via modification of their lipid microenvironment [32]. Plasma membrane H^+-ATPases pump protons from the cytoplasm to the apoplastic space, where extensive acidification is believed to contribute to cell wall loosening, a prerequisite for cell growth [34]. In this regard, *Azospirillum* inoculation improved coleoptile growth in wheat seedlings growing in darkness under osmotic stress, an effect closely correlated with an improved water status and cell wall elasticity [18].

In conclusion, *Azospirillum* inoculation could contribute to protect wheat seedlings from water stress through changes in the FA distribution profiles of the major PC and PE root PLs.

4. Methods

4.1. Wheat inoculation and colonization assessment

T. aestivum cv. Pro INTA Oasis seedlings were inoculated with *A. brasilense* Sp245 cells as previously described [18]. The same procedure was followed for controls, except that the inoculum was autoclaved prior to application. The degree of colonization was assessed as previously described [18].

4.2. Water stress treatments and plant material collection

Seedlings that had been treated with autoclaved or with viable bacteria were exposed for 48 h to sterile distilled water or to 20% PEG 8000 (SIGMA, St. Louis, MO). Roots were collected and washed twice with water before use. Length and projected area of coleoptiles and roots were determined by

applying GSRoot 5.00 software [23] to their digitalized images. The images were obtained in a flat bed scanner.

4.3. Root membrane integrity

Overall root membrane integrity was estimated by determining electrolyte leakage [37] and TTC-reducing ability [15] of roots.

4.4. Total lipid extraction, separation of lipid classes and FA analysis of major PLs

Roots were previously homogenized with boiling isopropanol [26] and then extracted with chloroform/methanol (2:1, v/v) containing 0.005% butyl-hydroxytoluol as an antioxidant. Total lipid extracts were washed according to Kates [26]. Total lipid amount and total PL–quantified on the basis of their phosphorus content–per root seedlings were determined as previously described [35]. The total lipid extract was sown on a silica gel (100–200 mesh, SIGMA) chromatography column and then fractionated into neutral lipids, glycolipids, and PLs by eluting with chloroform, acetone and methanol sequential, respectively [26]. The total PL fraction was separated into classes on precoated TLC plates (Merck) developed with chloroform/methanol/acetic acid/water (50:37.5:3.5:2, v/v/v/v) [26]. Each lipid class was identified by co-chromatography of authentic standards (Merck) under UV after spraying with 0.2% (w/v) 2′-7′-dichlorofluoresceine in ethanol [26]. The major PL constituents of the total polar lipids were separated and quantified on the basis of their phosphorus content as described by Rouser et al. [35]. They also were quantitatively assayed for their FA content, transferring them from the plates into test tubes. The FA-methylesters, obtained by transmethylation with 12% boron-trichloride in methanol (Supelco Inc., State College, Penna) were analyzed by GC using a Shimadzu GC-14B gas chromatograph equipped with a flame ionization detector and a Chromatopac C-R6A integrator. The operating conditions were as follows: nitrogen carrier gas flow: 30 ml min^{-1}, injector and detector temperatures: 250 °C and 280 °C, respectively; initial and final column temperatures: 130 and 230 °C, respectively; temperature program rate 4 °C min^{-1}. One microliter samples were injected into SP-2330 polar 30-m column (Supelco). Solvent blanks were checked periodically for impurities. FA were identified by co-chromatography with reference standards (Lipid Standard 99%, Supelco).

4.5. Statistical analysis of data

The experiments were a factorial combination of two inoculation levels and two water statuses in complete randomized blocks, with five replicates. The results were analyzed through PROC GLM procedure using SAS statistical package. The number of seedlings taken as replicates was as follows: 30 for both coleoptile and root length and projected areas; 15 for electrolyte leakage and TTC-reducing ability; and 50 for PC and PE content determinations.

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