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RESEARCH PAPER

The beneficial endophyte *Trichoderma hamatum* isolate DIS 219b promotes growth and delays the onset of the drought response in *Theobroma cacao*

Hanhong Bae[1,*], Richard C. Sicher[1], Moon S. Kim[1], Soo-Hyung Kim[2], Mary D. Strem[1], Rachel L. Melnick[3] and Bryan A. Bailey[1,†]

[1] USDA-ARS-Beltsville Agricultural Research Center, Beltsville, MD 20705, USA
[2] College of Forest Resources, UW Botanic Gardens, University of Washington, Box 354115, Seattle, WA 98195, USA
[3] Department of Plant Pathology, Pennsylvania State University, University Park, PA 16802, USA

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Abstract

Theobroma cacao **(cacao) is cultivated in tropical climates and is exposed to drought stress. The impact of the endophytic fungus** ***Trichoderma hamatum*** **isolate DIS 219b on cacao's response to drought was studied. Colonization by DIS 219b delayed drought-induced changes in stomatal conductance, net photosynthesis, and green fluorescence emissions. The altered expression of 19 expressed sequence tags (ESTs) (seven in leaves and 17 in roots with some overlap) by drought was detected using quantitative real-time reverse transcription PCR. Roots tended to respond earlier to drought than leaves, with the drought-induced changes in expression of seven ESTs being observed after 7 d of withholding water. Changes in gene expression in leaves were not observed until after 10 d of withholding water. DIS 219b colonization delayed the drought-altered expression of all seven ESTs responsive to drought in leaves by ⩾3 d, but had less influence on the expression pattern of the drought-responsive ESTs in roots. DIS 219b colonization had minimal direct influence on the expression of drought-responsive ESTs in 32-d-old seedlings. By contrast, DIS 219b colonization of 9-d-old seedlings altered expression of drought-responsive ESTs, sometimes in patterns opposite of that observed in response to drought. Drought induced an increase in the concentration of many amino acids in cacao leaves, while DIS 219b colonization caused a decrease in aspartic acid and glutamic acid concentrations and an increase in alanine and γ-aminobutyric acid concentrations. With or without exposure to drought conditions, colonization by DIS 219b promoted seedling growth, the most consistent effects being an increase in root fresh weight, root dry weight, and root water content. Colonized seedlings were slower to wilt in response to drought as measured by a decrease in the leaf angle drop. The primary direct effect of DIS 219b colonization was promotion of root growth, regardless of water status, and an increase in water content which it is proposed caused a delay in many aspects of the drought response of cacao.**

Key words: Drought stress, fungal endophyte, *Theobroma cacao*, *Trichoderma hamatum*.

Introduction

Theobroma cacao (cacao), the source of chocolate, is an understory tropical tree (Wood and Lass, 2001). Cacao is intolerant to drought (Mohd Razi *et al.*, 1992; Belsky and Siebert, 2003), and yields and production patterns are severely affected by periodic droughts and seasonal rainfall patterns. There is interest in the development of drought tolerance in cacao through plant breeding and crop management practices (Belsky and Siebert, 2003).

Cacao trees support a diverse microbial community that includes many endophytic fungi (Arnold *et al.*, 2003; Rubini

* Present address: School of Biotechnology, Yeungnam University, Geongsan 712-749, Korea.
† To whom correspondence should be addressed. E-mail: bryan.bailey@ars.usda.gov
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et al., 2005; Bailey *et al.*, 2008). Recent research has identified isolates of many *Trichoderma* species that are endophytic on cacao including above-ground tissues (Holmes *et al.*, 2004; Bailey *et al.*, 2008). In cacao, *Trichoderma* species are primarily being studied for their ability to control disease (Bastos, 1996; Evans *et al.*, 2003; Holmes *et al.*, 2006; Bailey *et al.*, 2008). *Trichoderma* species are commonly considered as saprophytic inhabitants of soil but some exist as opportunistic, avirulent plant symbionts (Wilson, 1997; Harman *et al.*, 2004). Beneficial activities attributed to *Trichoderma*/plant interactions include induced disease resistance, plant growth promotion, and tolerance of abiotic stresses including drought (Harman *et al.*, 2004).

Several classes of endophytic microorganisms, in addition to *Trichoderma* species, are known to alter the response of plants to abiotic stresses. Endophytes of cool-season grasses have been studied for their ability to induce tolerance to multiple biotic and abiotic stresses (Malinowski and Belesky, 2000). Some of the mechanisms used by the cool-season grass endophytes to alter the drought response include drought avoidance through morphological adaptations, drought tolerance through physiological and biochemical adaptations, and enhanced drought recovery (Malinowski and Belesky, 2000). Mycorrhiza also alter the response of plants to abiotic stresses (Augé, 2001). Of the many possible mechanisms employed by mycorrhiza to enhance drought tolerance, the most studied mechanism relates to enhanced growth and is commonly associated with increased phosphorous acquisition (Augé, 2001). Evidence supports several mechanisms as being important in *Trichoderma*-induced plant growth promotion including enhanced nutrient uptake and inhibition of deleterious root microflora (Harman *et al.*, 2004).

Recently, Bailey *et al.* (2006) characterized the interactions between four *Trichoderma* species and cacao at the molecular level. The observed changes in gene expression patterns in these *Trichoderma*/cacao interactions raised the possibility that *Trichoderma* species could induce tolerance to abiotic stresses, possibly including drought, in cacao. The objective was to characterize the effect of endophytic colonization of cacao by *Trichoderma hamatum* isolate DIS 219b on the responses of cacao to drought. Colonization of cacao seedlings resulted in a delay in many aspects of the drought response. It is proposed that this effect is mediated through enhanced root growth, resulting in an improved water status allowing cacao seedlings to tolerate drought stress.

Materials and methods

Trichoderma inoculation

Trichoderma hamatum isolate DIS 219b was isolated from a pod of *Theobroma gileri* in Guadual, Lita, Esmeraldas Province, Ecuador (Evans *et al.*, 2003). DIS 219b was grown on cornmeal dextrose agar plates at 23 °C for 5 d without light before use. Two agar plugs (0.5 cm in diameter) were added to a soilless mix (2:2:1, sand:perlite: promix), in double magenta boxes (77×77×194 mm; Magenta, Chicago, IL, USA). The magenta boxes contained 9 cm sterile soilless mix and had four holes (0.5 cm diameter) sealed with tape on the bottom. Sterile water (25 ml) was added to the soilless mix at the time of inoculation. The magenta boxes were maintained in growth chambers as described below for 14 d before being planted with cacao seed.

Plant materials

Seeds of *Theobroma cacao* variety comun (Lower Amazon Amelonado type) were obtained from the Almirante Cacau, Inc. farm (Itabuna, Bahia, Brazil). After removal of the seed coat, seeds were surface sterilized in 14% sodium hypochlorite for 3 min and then washed three times with sterile distilled water. Sterilized seeds were germinated on 1.5% water agar plates under fluorescent lights for 3 d at 22 °C. The germinated seeds were planted 3 cm deep into the sterile soilless mix in double magenta boxes with or without DIS 219b.

Phenotypic and molecular analyses of the cacao drought response

Magenta box-grown seedlings were produced as described above and grown in a controlled environment chamber (model M-2; EGC Corp., Chagrin Falls, OH, USA) with the following conditions: 12-h light/12-h dark photoperiod at 25 °C, 50% relative humidity, and 50 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR). The upper boxes and tape on the lower boxes were removed after 2 weeks. Seedlings were watered every other day for 32 d of growth in the magenta boxes. Before altering the watering cycles, the length of the hypocotyls and epicotyls for the non-colonized and colonized seedlings were measured. Watering was stopped for 13 d for the drought treatment, while control seedlings continued to be watered every other day. Six independent seedlings were harvested for each treatment combination (plus and minus DIS 219b and plus and minus drought) at 0, 7, 10, and 13 d after initiating the drought treatment. Roots and the largest leaves were harvested for quantitative real-time reverse transcription PCR (qPCR) analysis. A second leaf was harvested for fluorescence image analysis.

Direct effects of DIS 219b on gene expression in 9-d-old cacao seedlings

To assess further the direct effects of DIS 219b on gene expression in 9-d-old seedlings, expression patterns of transcripts were analysed using primers for newly identified drought-responsive expressed sequence tags (ESTs). The methods which have been described previously include pre-germination of sterile cacao seed on water agar for 3 d and colonization of the resulting seedlings for 6 d by four *Trichoderma* isolates including DIS 219b (Bailey *et al.*, 2006). The seedlings were frozen in liquid nitrogen and total RNA was isolated from the whole seedlings minus the cotyledons.

qPCR

Total RNA was isolated from cacao seedlings as described by Bailey *et al.* (2005). Procedures for qPCR and analysis were as described by Bae *et al.* (2008). To obtain the relative transcript levels, the threshold cycle (C_T) values for all genes of interest ($C_{T•GOI}$) were normalized to the C_T values of ACT ($C_{T•ACT}$) for each replication [$\Delta C_T=(C_{T•ACT})-(C_{T•GOI})$]. Relative transcript levels were calculated with respect to cacao *ACTIN* transcript levels (% of *ACTIN*). The fold changes in transcript were obtained from the following equation: fold change=[(*E*) ΔC_T] as described previously by Pfaffl (2001). Average values were calculated from six biological replications and the error bars indicate the standard error of the mean. The sequence information of the 20 primer sets used, including *Actin*, is given in Table 1.

Measurement of leaf gas exchange and leaf water potential

Net photosynthesis (P_n) and stomatal conductance (g_s) were measured using a portable photosynthesis system (LI-6400; LI-COR, Inc., Lincoln, NE, USA) with a leaf chamber fluorometer (LI6400-40). All measurements were made under the following conditions: 300 μmol m^{-2} s^{-1} (PAR), 25.9 °C (leaf temperature), 1.13 kPa (vapour pressure deficit), 400 μmol mol^{-1} (CO_2).

Table 1. Primer sequences used for qPCR analysis in cacao

Putative gene function (EST designation)	Accession no.	Sequence (5′ to 3′)[a]	Expected size (bp)	Max identities/E-value (accession no.)
Rubisco small subunit (*TcrbcS*)	CF973934	F: GAACCTCAGGATACCTGGGTACTAT R: GTCCCTACAAGGGAACAAGTCTAAT	241	79/3E-90 (Y07779)
ATP-binding cassette transporter (*TcABC-T*)	CF974185	F: ACAGCTTCAATTACCTGACTCCAT R: TAGTCCACTAGTAGGCTCATCAAGG	217	59/2E-57 (AY734542)
Histidine kinase (*TcHK*)	CF972897	F: GCACTAATCACATCAGCTCCATACT R: TACTTCTGGTGTTTGTAACTTGACC	243	83/3E-85 (AJ298990)
Receptor-like protein kinase (*TcRPK*)	CF973313	F: TCCAGAGAATGCATTTACAACTACC R: CTGATACCTGATCTCTGATTCCAGT	232	70/5E-49 (U77888)
MAP kinase 3 (*TcMAPK3*)	CF974368	F: TGAATAGAAAGCCTCTATTTCCTGG R: GAGATCAATAGCCAATGGATGAAC	204	86/1E-88 (AF153061)
MAP kinase kinase 4 (*TcMKK4*)	CF974565	F: CAAGCTCCATATATCTCCAAGGTAA R: CTTGCCTTACTTACATGCCCATA	239	74/1E-73 (AY691333)
Serine/threonine kinases/receptor kinase (*TcSTK*)	CF973647	F: ACTTCAATATCTCCAGCAAGCATAG R: CCAAACTCTGACAATAGCCTAGAAA	230	67/4E-67 (NM104491)
Senescence-associated protein (*TcSEN1*)	CF973886	F: ATACCCTCCAGCAATGTCTGTAAC R: AGGACTCCTGAAGAGTTCAGTGC	237	71/1E-57 (DQ116561)
Cellulose synthase 3 (*TcCESA3*)	CA798419	F: CCCACCAACTACTCTCCTCATTAT R: CAGAATCGACCATACGACAACAAT	208	96/4E-74 (AY221087)
Protein phosphatase 2C (*TcPP2C*)	CF972808	F: ACAGCTTCTTGATTGCTTAGGTG R: TACAGGTTTCCTAGATCTATCGGTG	215	90/2E-90 (AJ277744)
Allene oxide cyclase (*TcAOC*)	CF973875	F: CTTTAGACTGAAAAAGCTGGCATAG R: CAGGTTAAGCTGCAGCAAATTGT	247	78/4E-41 (AB095986)
Lipoxygenase (*TcLOX*)	CF973956	F: ACATACTCAGTCACCCATTGTTTG R: TTGGCCAAGTATTATCTAGAGGTTG	218	69/4E-86 (X96405)
Trehalose-6-phosophate phosphatase (*TcTPP*)	CA796499	F: CAATTTATATTGGAGACGACAGGAC R: GCTCCTAATTTCTTCAAGCTTCTTC	204	76/2E-69 (AY570725)
Pathogenesis-related protein 5 (*TcPR5*)	AY766059	F: ATTAGATGCACGGCAGATATCATAG R: CAGAACACAACCCTGTAGTTAGTCC	242	79/9E-121 (AF364864)
Neutral invertase (*TcNI*)	CF974742	F: GACAGGCATAAGTCTGATCCTTAAT R: CTATAAGGACAAAGCAAAGCAAGG	228	84/2E-81 (Y16262)
Sorbitol transporter (*TcSOT*)	CF974712	F: AGTAATTCTTTCCAGACGCCTTC R: TCTTGGCTATTGGTGTTATAGCG	229	81/1E-62 (AB125648)
Nitrate reductase (*TcNR*)	CF974302	F: ATGACTTGATAAATTGGCGTGATAC R: TTTGGGTCACATTGAATATACGTG	120	96/7E-29 (AY138811)
Zinc finger binding protein (*TcZFP*, *TcP13*)	DW246138	F: CGATAAACATCGTTGTGGATCTGTA R: ATAACGATACTAAAGTTCGCTTCGC	230	88/2E-39 (AJ632084)
Tonoplast intrinsic protein (*TcTIP*, *TcP31*)	DW246146	F: GAAAGGTAACATTGGGATCATTGC R: ATGAAGAAGATCTCATAGACAACGG	216	81/5E-70 (DQ202710)
Actin (*TcACT*)	CF973918	F: CAGACTTTGAGTTCACTTGACACAG R: AGTGTCTGGATTGGAGGATCTATCT	200	85/1E-30 (AY305729)

[a] F, Forward; R, reverse.

Multispectral fluorescence imaging system

Multispectral fluorescence images from the third largest cacao leaves were obtained as described by Kim *et al.* (2003). The excitation light source was as follows: four 12 W, long-wave UV-A fluorescent lamps (Model XX-15A; Spectronics Corp., New York, NY, USA), 0.33 mW cm^{-2} intensity with an emission max at 360 nm in the target area. The radiation from the UV lamps was filtered with Schott UG-1 glass to eliminate wavelengths >400 nm. A back-illuminated and thermoelectrically cooled CCD camera (PixelVision, OR, USA) was used to capture the fluorescence images. A Nikon f 1.4/35 mm lens was coupled to a common aperture multispectral adapter (MSAI-04; Optical Insights, AZ, USA) to collect the fluorescence emissions. Blue and green filters were used in this study: 450 nm with a 25 nm full width at half maximum (FWHM) and 530 nm with a 25 nm FWHM, respectively.

Metabolite measurement

Seedlings in magenta boxes (six seedlings per treatment) were grown in a controlled environment chamber as described above. Seedlings were watered every other day for 32 d of growth in the magenta boxes after which the watering cycle was altered. The watering cycle was then changed to 3 d or 13 d and maintained for 13 d. The mature first flush leaves were harvested separately for each seedling and frozen in liquid nitrogen, lyophilized, and dry weights obtained prior to carbohydrate and amino acid analysis.

For amino acid analysis, leaf samples were ground to a fine powder in a mortar and pestle using liquid nitrogen. Fifty milligrams of each lyophilized sample were extracted with 2 ml of 70% aqueous methanol in a ground-glass tissue homogenizer. The homogenates were incubated in a H_2O bath at 45 °C for 15 min, centrifuged for 15 min at 5800 *g*, and the supernatants were evaporated to dryness under a stream of N_2 at 37 °C. Samples were resuspended in 0.5 ml of 20 mM HCl and filtered using a 0.22 μm Ultrafree-MC membrane filter unit (Millipore Corp., Bedford, MA, USA). Soluble amino acids were determined by HPLC using the AccQTag pre-column derivatization method (Waters Corp., Milford, MA, USA). Separations were performed using a Waters 600E Multisolvent Delivery System equipped with a 3.9×150 mm AccQTag C_{18} column. The elution gradient, based on recommendations in the AccQFluor kit, used a pH of 5.0 and a column temperature of 37 °C. Detection was with a Gilson 121 fluorometer using excitation and emission wavelengths of 250 nm and 395 nm, respectively. The detector output was monitored using Empower2 software from Waters and standard curves were prepared with known mixtures of amino acids.

For carbohydrate analysis, leaf tissue (50 mg of lyophilized tissue) was extracted twice with 1 ml of 80% ethanol in ground-glass tissue homogenizers at 4 °C. The homogenates were transferred to 15 ml conical tubes and centrifuged at 4000 *g* for 15 min. The pellets were washed with 1 ml of 80% ethanol and centrifuged as above. The supernatant fractions were combined and the extracts were evaporated to dryness under N_2 at 37 °C. The dried samples were reconstituted in 1 ml of deionized H_2O and transferred to a sealed cryovial for storage at –20 °C. Pellets were gelatinized in 2 ml boiling H_2O for 30 min and leaf starch was hydrolysed by the action of α-amylase and amyloglucosidase. Soluble sugars and glucose from starch were quantified spectrophotometrically in coupled enzyme assays (Sicher, 2001).

Biomass and metabolite analyses of the cacao drought response

Seedlings in magenta boxes were grown in a controlled environment chamber (model M-2, EGC Corp., Chagrin Falls, OH, USA) with the following conditions: 12 h light/12 h dark photoperiod at 25 °C and 90 μmol m^{-2} s^{-1} PAR. The upper boxes and tape on the bottoms were removed after 2 weeks.

In the initial experiment, carried out at 80% humidity, seedlings were watered every other day for 32 d of growth. The watering cycle was then changed to 1, 3, 7, or 10 d and maintained for 21 d. Leaf production by cacao seedlings proceeds in cycles (flushes) of two to four new leaves at a time. The first flush leaves were harvested separately for each seedling and frozen in liquid nitrogen. The stem and second flush leaves were harvested and weighed. The roots of each seedling were collected, washed, dried on paper towels, and weighed. After measuring fresh weights, the tissues were oven dried (50 °C for 7 d) and dry weights were determined.

In the second experiment, carried out at 40% humidity, seedlings were watered every other day for 32 d of growth, after which the watering cycle was altered. The watering cycle was then changed to 3 d or 5 d and maintained for 15 d. At the end of each 5 d cycle leaf angles were measured. Leaf angle was determined as the drop of the leaf blade tip from horizontal using a protractor. The maximum drop in leaf angle was set at 90°. The stem, first flush leaves, and second flush leaves were harvested separately for each seedling and weighed. The roots of each seedling were collected, washed, dried on paper towels, and weighed. The tissues were then oven dried (50 °C for 7 d) and dry weights were determined.

Statistical analysis

Data for biomass, carbohydrates, amino acids, stomatal conductance, and net photosynthesis were statistically analysed for significance using the general linear model PROC GLM analysis, followed by a Tukey test using the SAS program (SAS Institute Inc., Raleigh, NC, USA). A 95% confidence level was used for all analyses, so that $P \leqslant 0.05$ was considered to be significant.

Results

Physiological and fluorescence image data

Prior to the shift in watering cycles at 32 d, cacao seedlings treated with DIS 219b had taller hypocotyls (Fig. 1A). After withholding water for 13 d, the leaves of cacao seedlings not

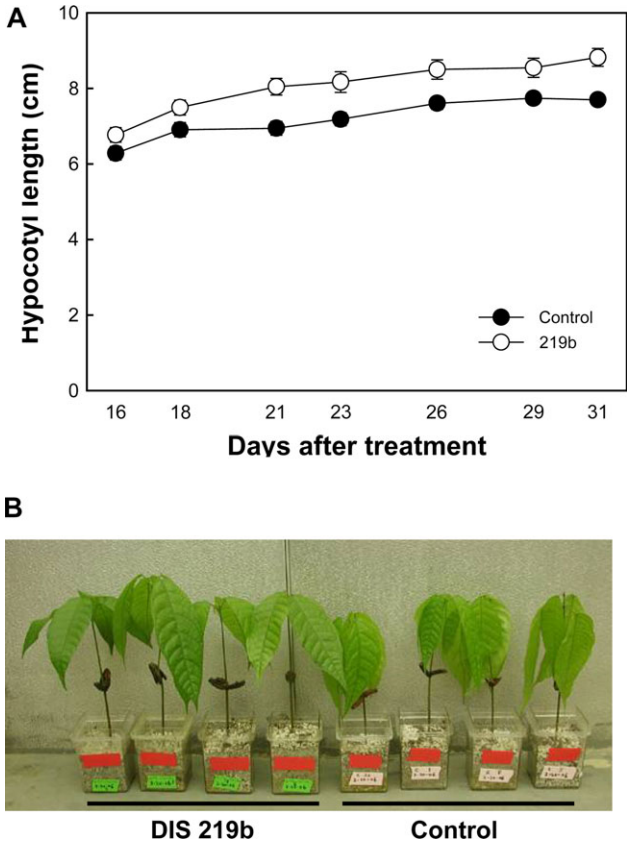

Fig. 1. The effect of drought and DIS 219b colonization on cacao seedling growth. Seedlings were grown in magenta boxes in a controlled environment chamber (model M-2; EGC Corp., Chagrin Falls, OH, USA) with the following conditions: 12 h light/12 h dark photoperiod at 25 °C, 50% relative humidity, and 50 μmol m^{-2} s^{-1} PAR. The seedlings were either colonized with *Trichoderma hamatum* isolate DIS 219b or not colonized (Control). After emergence, seedlings were watered every other day for 32 d of growth in the magenta boxes. Before altering the watering cycles, the length of the hypocotyls for the non-colonized and colonized seedlings were measured. After 32 d of growth, watering was stopped for 13 d (drought treatment), while control seedlings continued to be watered every other day. (A) Hypocotyl length after 32 d of growth without drought treatment. Bars show means ±standard error. (B) The photograph of the seedlings was taken after water was withheld for 13 d.

colonized with DIS 219b were noticeably more droopy than those of cacao seedlings colonized by DIS 219b (Fig. 1B). Net photosynthesis and stomatal conductance were significantly reduced as the time without water increased from 7 d to 13 d (Table 2). Treatment with DIS 219b suppressed the reduction in net photosynthesis and stomatal conductance (Table 2). The interaction between drought and DIS 219b treatment was not significant. Values for stomatal conductance were reduced due to drought by 48% 7 d post-watering (PW) (Table 2) and to 96% 13 d PW. Net photosynthesis was reduced due to drought by 29% 7 d PW and to 96% 13 d PW (Table 2). Non-colonized seedlings averaged an 85% reduction in stomatal conductance and a 76% reduction in net photosynthesis across the 7, 10, and

Table 2. The effect of drought and DIS 219b colonization on stomatal conductance (g_s) and net photosynthesis (P_n)

The readings for the non-colonized seedlings at 7, 10, and 13 d PW were divided by the readings for 3 d watered non-colonized seedlings at those same time points, and the readings for the DIS 219b-colonized seedlings at 7, 10, and 13 d PW were divided by the readings for 3 d watered DIS 219b-colonized seedlings at those same time points within each replication. These values were subtracted from 1 and multiplied by 100 to determine percentage reduction. Statistical analysis was carried out as described in the text. The interaction between drought and *Trichoderma* was not significant so means are presented for drought effect over both *Trichoderma* treatments (colonized or non-colonized) and for the *Trichoderma* effect over all drought treatment time points (7, 10, and 13 d).

Treatment	% reduction	
	g_s	P_n
Drought effect		
7 d	48 B[a]	29 C[a]
10 d	82 AB	63 B
13 d	96 A	96 A
Trichoderma effect		
Non-colonized	85 A	76 A
DIS 219b-colonized	64 B	46 B

[a] Means within columns for the individual treatments of drought or *Trichoderma* followed by the same letter are not significantly different at the $P \leq 0.05$ level.

13 d watering cycles compared with the 3 d watering cycle. The DIS 219b-colonized seedlings averaged a 64% reduction in stomatal conductance and 46% reduction in net photosynthesis across the same watering cycles.

Increased leaf fluorescence was observed in the green (F530) spectral range starting 10 d PW in non-colonized seedlings (Fig. 2). The fluorescence emissions of adaxial leaf surfaces of non-colonized seedlings increased 26% and 22% over that of colonized seedlings 10 d and 13 d PW, respectively. Pair-wise comparisons of the relative intensities for the green fluorescence band at 530 nm were significantly different at α=0.05 at 10 d and 13 d PW.

Drought-induced changes in transcripts levels

Changes of transcript levels were monitored during drought treatment in leaves and roots of cacao seedlings (Figs 3–6). Nineteen ESTs (Table 1) were identified as responsive to drought in leaves and/or roots of cacao. The transcript level of *TcrbcS* declined at 10 d PW by 74% in non-colonized seedlings compared with a decline of only 39% in colonized seedlings (Fig. 3).

TcSOT, *TcPR5*, and *TcNI* were induced by drought starting at 10 d PW in leaves (Fig. 4A). *TcTPP* was also induced by drought but not until 13 d PW in leaves (Fig. 4A). *TcTPP* and *TcSOT* were induced by drought starting at 7 d PW in roots (Fig. 4B), while *TcPR5* was induced by drought starting at 10 d PW (Fig. 4B). *TcCES3* was repressed by drought in roots (Fig. 4B). The change in gene expression in response to drought was delayed in colonized

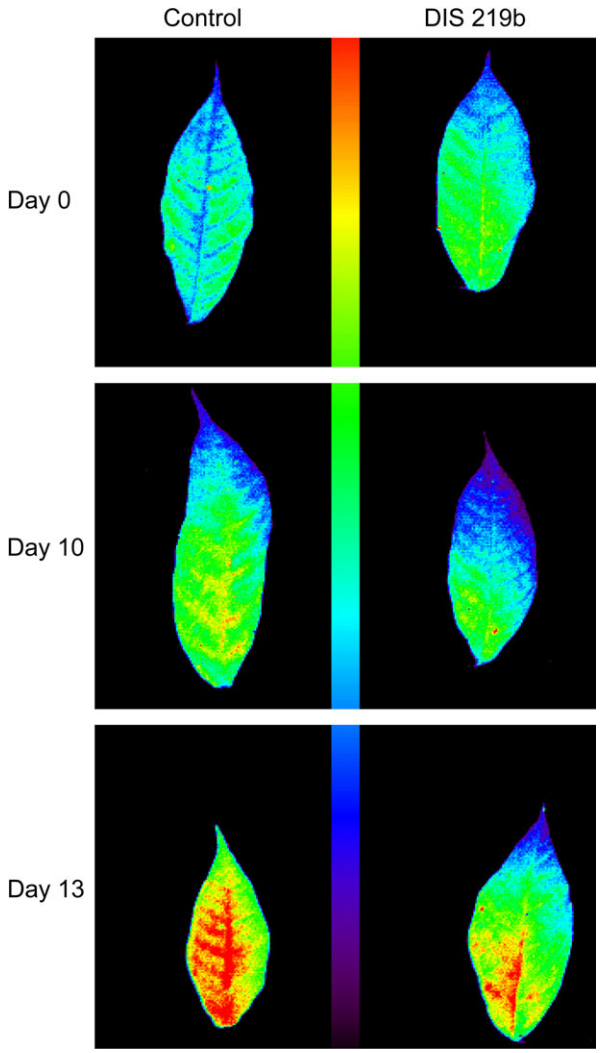

Fig. 2. Representative fluorescence responses of cacao leaves during drought treatment (0, 10, and 13 d without watering) of non-colonized (Control) and colonized (DIS 219b) cacao seedlings. Seedlings were watered every other day for 32 d of growth. After 32 d of growth, watering was withheld for 13 d. Fluorescence emissions of adaxial surfaces of cacao leaves at 530 nm (F530) were measured using six biological replications. Relative fluorescence intensity is given in the vertical colour scale in the middle.

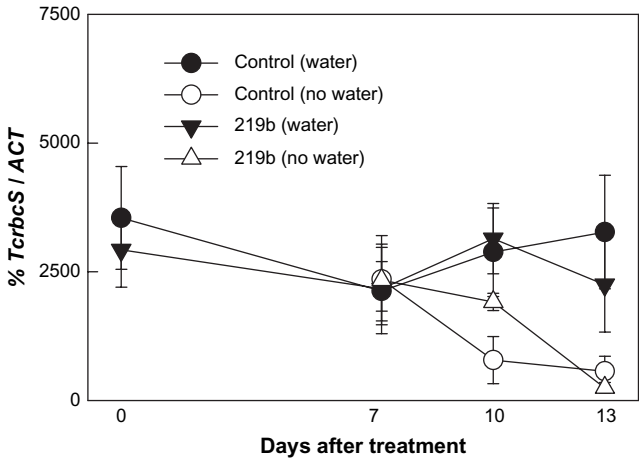

Fig. 3. Expression pattern for EST *TcrbcS* (putative Rubisco small subunit). Seedlings were watered every other day for 32 d of growth before drought treatment. The largest leaves were harvested 0, 7, 10, and 13 d after the last watering. Treatments: Control (water), non-colonized seedlings watered every other day; Control (no water), non-colonized seedlings with water withheld 13 d; 219b (water), colonized seedlings watered every 2 d; 219b (no water), colonized seedlings with water withheld 13 d. Relative mRNA levels were calculated for qPCR results with respect to *ACTIN* transcripts (% of *ACTIN*). Bars show means ±standard error (n=6).

seedlings for *TcTPP*, *TcSOT*, *TcPR5*, and *TcNI* in leaves (Fig. 4A) and for *TcPR5* and *TcCESA3* in roots (Fig. 4B).

In roots, drought-induced *TcLOX* and *TcSEN1* (Fig. 5). *TcAOC*, *TcABC-T*, *TcTIP*, and *TcNR* were repressed by drought treatment 10 d PW (Fig. 5). DIS 219b colonization did not alter the drought-induced changes in *TcAOC*, *TcLOX*, *TcABC-T*, *TcNR*, or *TcSEN1* (Fig. 5).

TcMAPK3 and *TcZFP* were induced by drought in leaves of non-colonized seedlings (Fig. 6). The induction of *TcMAPK3* and *TcZFP* by drought in leaves was delayed in DIS 219b-colonized seedlings. In roots, drought induced expression of *TcHK*, *TcMAPK3*, *TcPP2C*, and *TcZFP*, and repressed expression of *TcRPK*, *TcMKK4*, and *TcSTK* (Fig. 6). Colonization by DIS 219b delayed the drought induction of *TcMAPK3* and *TcZFP* in roots.

Direct effects of DIS 219b gene expression in 9-d-old cacao seedlings

RNA isolated from 9-d-old seedlings (DIS 219b-colonized and non-colonized) grown on agar plates during a previously published study (Bailey *et al.*, 2006) was used to study further the early influence of DIS 219b colonization on expression of drought-responsive ESTs identified in research first presented here. *TcHK* and *TcMAPK3*, inducible by drought, were also induced by DIS 219b colonization (Fig. 7). Also significant was the observed induction of *TcSTK* and *TcMKK4*, and *TcNR*, ESTs that were repressed by drought (Fig. 7). *TcNR* was the most highly induced DIS 219b-responsive EST (1200-fold induced).

Changes in carbohydrates and soluble amino acids

Mean foliar starch and sucrose concentrations did not change significantly in response to water cycle or DIS 219b colonization. Glucose accumulated in response to the 13 d water cycle (Table 3). Total amino acid content increased in response to the 13 d water cycle, but not to DIS 219b colonization (Table 3). The concentrations of His, Arg, Pro, γ-aminobutyric acid (GABA), Val, and Leu increased significantly in response to the 13 d water cycle (Table 3). For DIS 219b-colonized seedlings, the concentrations of Ala and GABA increased, while the concentrations of Asp and Glu were reduced compared with non-colonized seedlings (Table 3). The concentration of Asp increased significantly in response to the 13 d water cycle only in

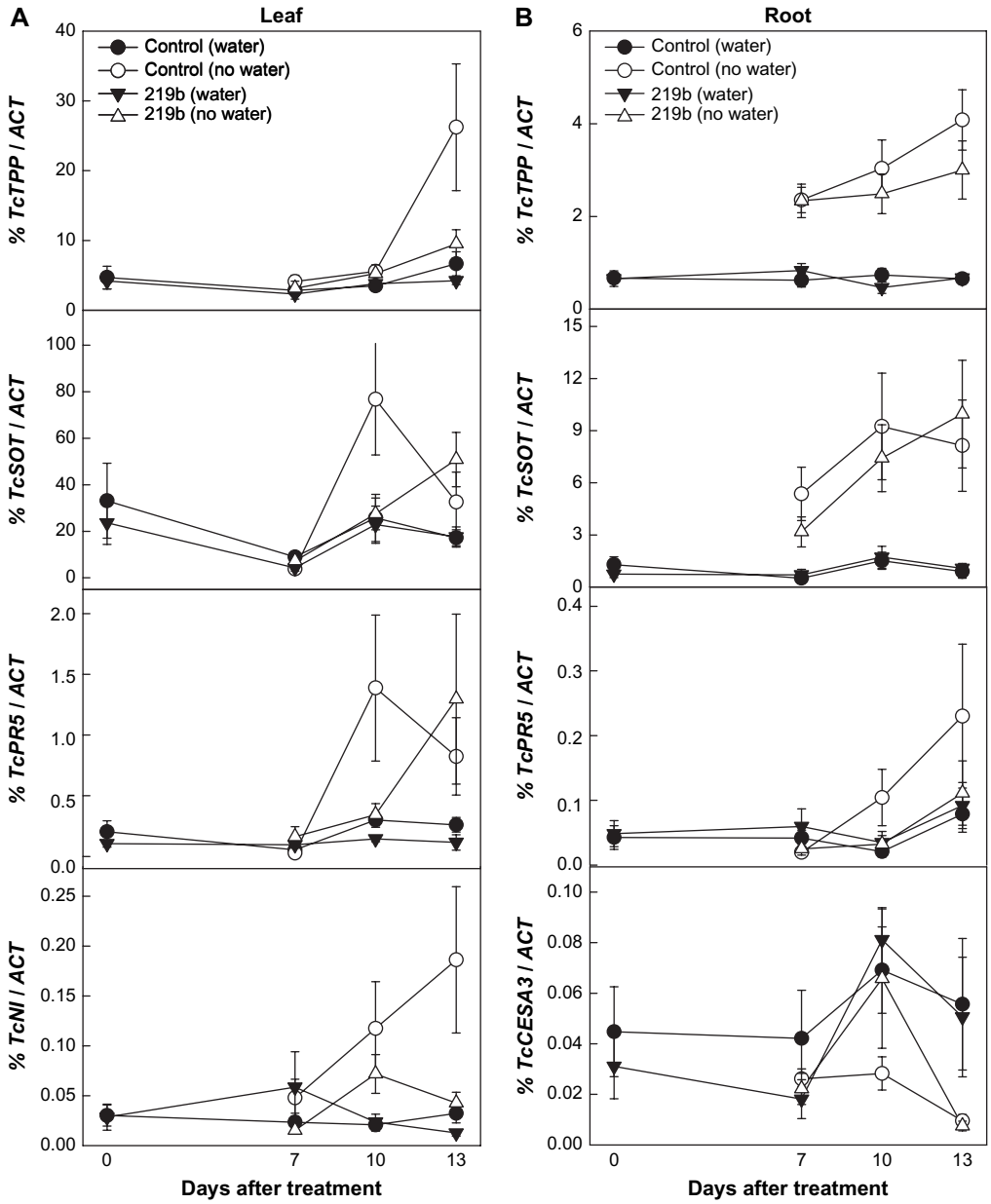

Fig. 4. Expression patterns for ESTs putatively involved in osmoprotectant production in (A) leaves including *TcTPP*, *TcSOT*, *TcPR5*, and *TcNI* and (B) roots including *TcTPP*, *TcSOT*, *TcPR5*, and *TcCESA3*. Seedlings were watered every other day for 32 d of growth before drought treatment. The roots and largest leaf were harvested 0, 7, 10, and 13 d after the last watering. Treatments: Control (water), non-colonized seedlings watered every other day; Control (no water), non-colonized seedlings with water withheld 13 d; 219b (water), colonized seedlings watered every 2 d; 219b (no water), colonized seedlings with water withheld 13 d. Relative mRNA levels were calculated for qPCR results with respect to *ACTIN* transcripts (% of *ACTIN*). Bars show means ±standard error (n=6).

non-colonized seedlings, while Lys concentrations increased in response to the 13 d water cycle only in DIS 219b-colonized seedlings (data not shown).

Biomass production

Fresh weights, dry weights, and water weights of roots, stems, and second flush leaves were determined in a study using non-colonized and DIS 219b-colonized seedlings exposed to 1, 3, 7, or 10 d watering cycles for 21 d (Table 4). Humidity in the chamber was maintained at 80%. Increasing the watering cycle from 3 d to 10 d resulted in a reduction in fresh and dry weights of stem and second leaf flush and these changes were not altered by DIS 219b colonization (data not shown). Nor did DIS 219b colonization have a direct effect on the weight of these tissues. Limiting watering from a 7 d to a 10 d cycle caused a reduction in root fresh weight and root water weight. The watering cycle had a mixed effect on root dry weight with the 1 d watering cycle having the smallest root systems. The

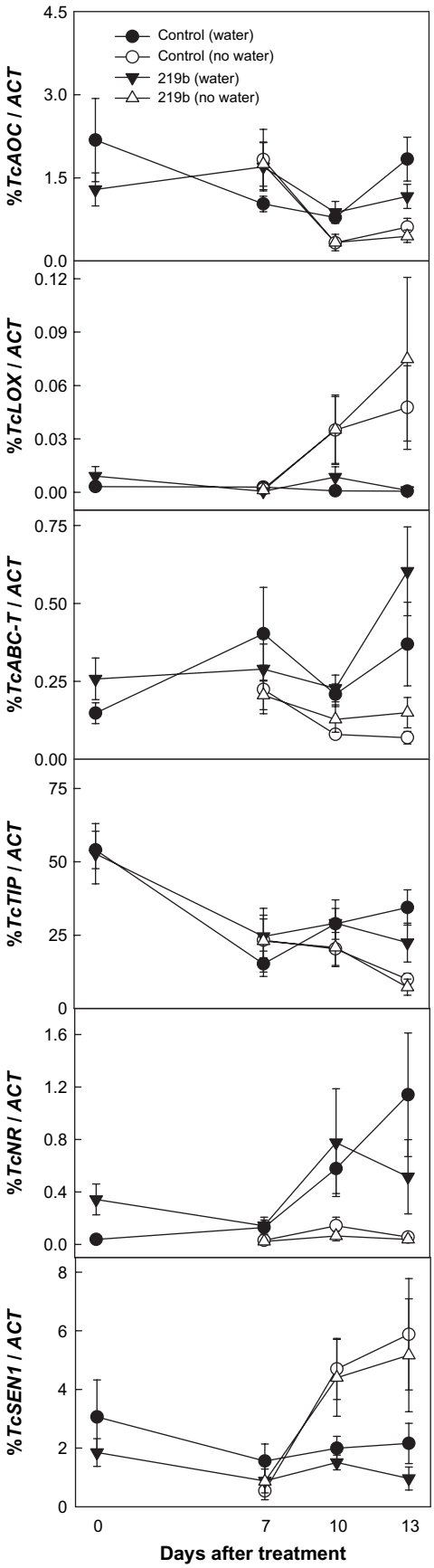

Fig. 5. Expression patterns in roots for ESTs putatively involved in hormone production (*TcAOC*, *TcLOX*), membrane function (*TcABC-T*, *TcTIP*), and other physiological processes (*TcNR*, *TcSEN1*). Treatments: Control (water), non-colonized seedlings watered every other day; Control (no water), non-colonized seedlings with water withheld 13 d; 219b (water), colonized seedlings watered every 2 d; 219b (no water), colonized seedlings with water withheld 13 d. Relative mRNA levels were calculated for qPCR results with respect to *ACTIN* transcripts (% of *ACTIN*). Bars show means ±standard error (n=6).

DIS 219b treatment, on the other hand, was consistently associated with an increase in root fresh weight, root dry weight, and root water weight, regardless of the watering cycle.

In a second biomass study, fresh weights, dry weights, and water weights of roots, tops (leaves and stems), and total seedlings were determined using non-colonized and DIS 219b-colonized seedlings on 3 d and 5 d watering cycles (Table 5). In addition, the ratio of root dry weight to top dry weight was determined. Leaf angle was measured at the end of each 5 d watering cycle. In this study the humidity was maintained at 40%. Only data where DIS 219b effect or its interaction with drought was significant are presented. During the experiment, some seedlings died under the 5 d watering cycle treatment and the weights of their tissues were not included in the statistical analysis. Limiting watering from a 3 d to a 5 d cycle caused a reduction in root fresh weight and root dry weight, total fresh weight, root water weight, and total water weight, along with an increase in the root dry weight/top dry weight ratio. DIS 219b colonization resulted in an increase in the root fresh weight, root dry weight, total fresh weight, root water weight, and total water weight, along with an increase in the root dry weight/top dry weight ratio. The water cycle×DIS 219b treatment interaction was significant for top fresh weight, top dry weight, and total dry weight. For all three measurements, DIS 219b colonization was associated with an increase in weight under the 3 d watering cycle but not for the 5 d watering cycle. Top fresh weight, top dry weight, and total dry weight decreased in response to the 5 d watering cycle regardless of DIS 219b treatment.

By separating each seedling into 0.05 g weight class increments based on root dry weight, it is apparent that, for the 3 d watering cycle, DIS 219b seedlings dominated the two heaviest weight classes (0.5 g dry weight and larger) (Fig. 8). For the 5 d watering cycle, DIS 219b-colonized seedlings dominated the 0.55 g and larger weight class but seedlings in the 0.5–0.54 g weight class were lost to smaller weight classes and some seedlings died. The non-colonized seedlings dominated the dead class with 2.5 seedlings per block dying compared with one seedling per block for the DIS 219b-colonized seedlings.

Leaf angle was measured at the end of each 5 d watering cycle (Table 6). After cycle 1, drought caused a significant increase in the leaf angle drop and the DIS 219b effect was not significant, although it tended towards reducing the degree of drop. After the second and third watering cycle, drought again caused an increase in the leaf angle drop, and

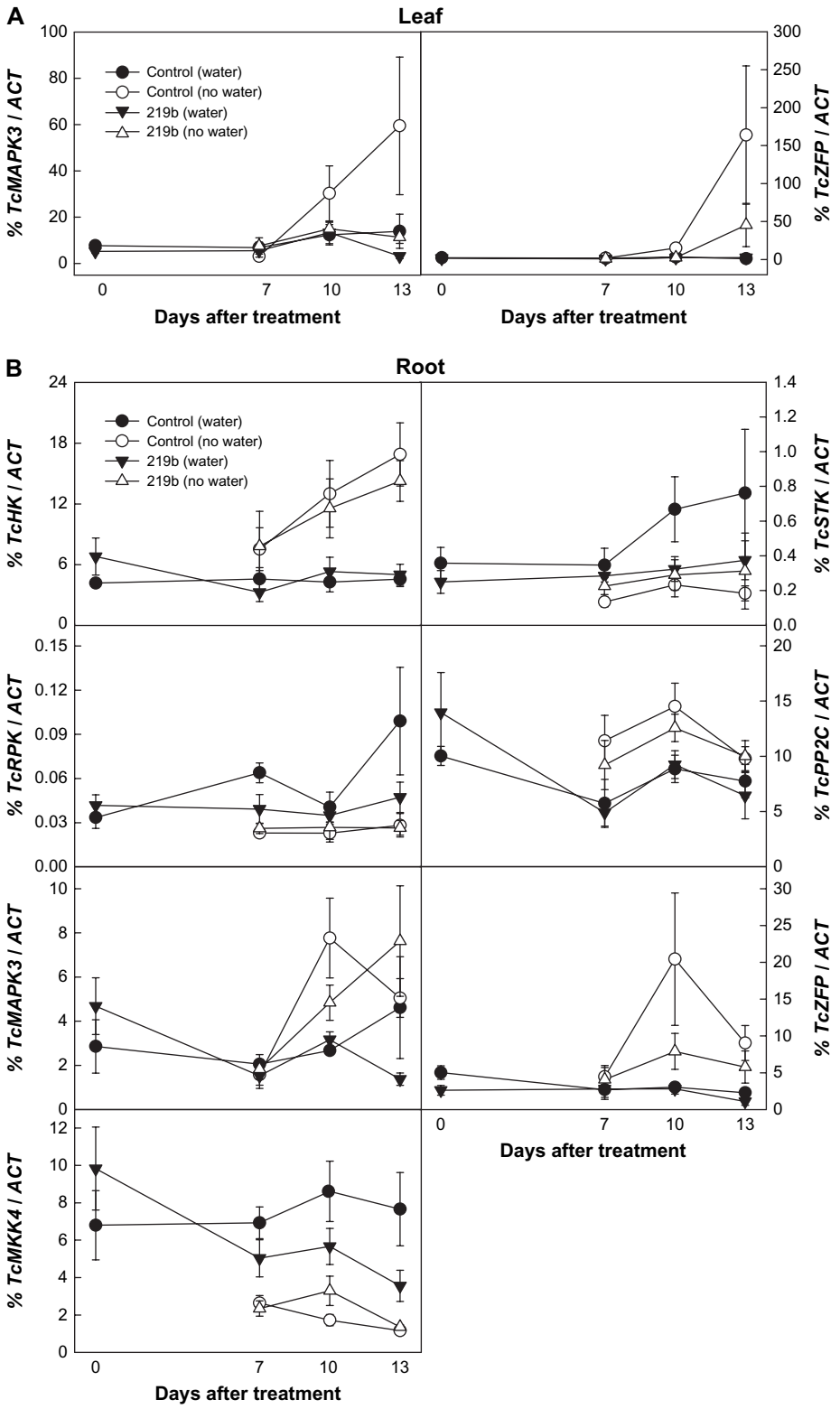

Fig. 6. Expression patterns for ESTs putatively involved in signal transduction, transcription and post-transcriptional regulation. ESTs responding to drought included (A) T*cMAPK3* and *TcZFP* in leaves, and (B) *TcHK*, *TcRPK*, T*cMAPK3*, *TcMKK4*, *TcSTK*, *TcPP2C*, and *TcZFP* in roots. Treatments: Control (water), non-colonized seedlings watered every other day; Control (no water), non-colonized seedlings with water withheld 13 d; 219b (water), colonized seedlings watered every 2 d; 219b (no water), colonized seedlings with water withheld 13 d. Relative mRNA levels were calculated for qPCR results with respect to *ACTIN* transcripts (% of *ACTIN*). Bars show means ±standard error (n=6).

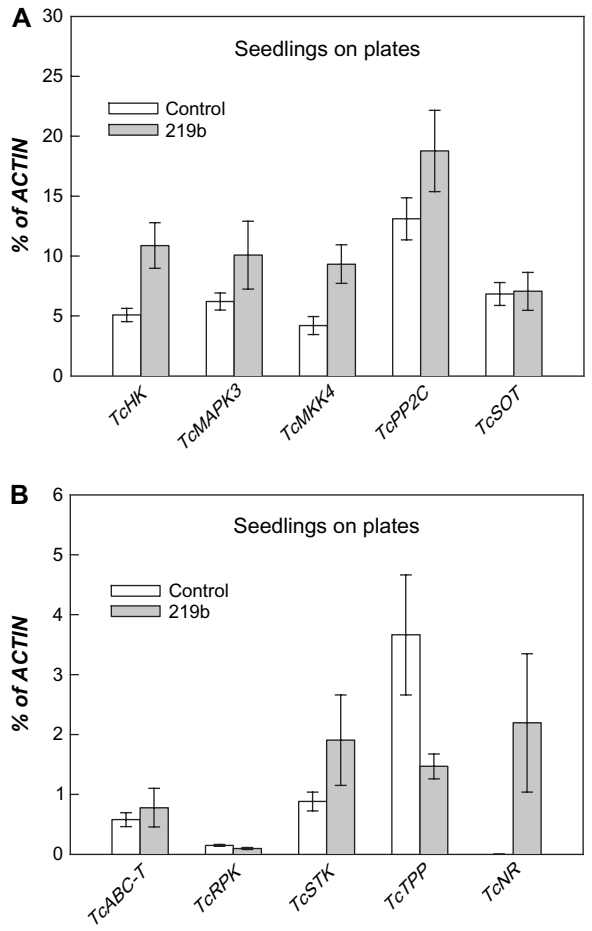

Fig. 7. Direct influence of DIS 219b colonization on gene expression in 9-d-old cacao seedlings germinated on 1.5% water agar plates. Colonized (219b) or non-colonized (Control) seedlings were grown for 9 d as described in the text. The whole seedling minus the cotyledons was harvested for qPCR analysis. A subset of drought-responsive ESTs was analysed. The relative mRNA levels of the ESTs were calculated with respect to *ACTIN* transcripts (% of *ACTIN*). Bars show means ±standard error (n=4).

at both time points the increase was reduced in DIS 219b-colonized seedling compared with non-colonized seedlings.

Discussion

Induced effects on cacao physiology and the drought response

During drought, plants can show the following symptoms: cessation of shoot growth, decreased stomatal conductance, reduced net CO_2 assimilation, impaired photosynthesis, accumulation of solutes, cessation of root growth, and leaf senescence and plant decline (reviewed by Passioura, 1996). In addition, the blue-green fluorescence of plants often increases in response to drought (Lichtenthaler and Miehé, 1997; Buschmann *et al.*, 2000; Hideg *et al.*, 2002). Previously, aspects of the drought response of cacao related to expression of genes involved in polyamine biosynthesis were

Table 3. The effect of water cycle and *Trichoderma* colonization on carbohydrate and amino acid concentrations in cacao leaves

Treatments were: colonized (219b) or non-colonized seedlings (Cont) watered every three days; colonized (219b) or non-colonized seedlings (Cont) with watering withheld 13 days. Statistical analysis was carried out as described in the text. Means are presented for water cycle effect over both *Trichoderma* treatments and for the *Trichoderma* effect over both water cycle treatments. The interaction between water cycle and *Trichoderma* was significant for ASP and LYS only and these are discussed in the text.

Metabolite	Water cycle effect			*Trichoderma* effect		
	3 day	13 day		Cont	219b	
	–μmol g^{-1}	DW–	SIG[a]	–μmol g^{-1}	DW–	SIG
Starch	403	386		413	375	
Glucose	113	126	*	114	120	
Sucrose	104	101		99	106	
	–pmol g^{-1}	FW–	SIG[a]	–pmol g^{-1}	FW–	SIG
ASP	1430	1789	*	1799	1421	*
SER	763	983		850	884	
GLU	3268	2670		3470	2536	*
GLY	422	660		474	593	
HIS	354	821	*	494	653	
ARG	879	1095	*	1033	936	
ALA	413	498		375	525	*
PRO	193	461	*	368	278	
GABA	443	818	*	406	848	**
VAL	338	463	*	394	401	
LYS	440	532		448	518	
LEU	61	90	*	68	81	
PHE	253	215		268	205	
Total AA	9257	11126	*	10447	9880	

[a] *,** indicates a significant difference at $P \leq 0.05$ and 0.01 level, respectively, between means of individual carbohydrates and amino acids for the individual treatments of water cycle or *Trichoderma*.

described (Bae *et al.*, 2008). Here it is shown that the drought-induced changes in net photosynthesis and stomatal conductance were delayed in DIS 219b-colonized seedlings (Table 2). The change in fluorescence emissions of cacao leaves was greater for non-colonized seedlings than colonized seedlings at 10 d and 13 d PW (Fig. 2).

The impact of endophytes on the plant drought response has been intensely studied in cool-season grasses (reviewed by Malinowski and Belesky, 2000). Endophyte-infected tall fescue showed improved tolerance to drought and faster recovery after a drought period. The cool-season grass endophytes often cause their plant host to respond to drought earlier, resulting in accelerated stomatal closure and reduced water loss (Malinowski and Belesky, 2000). However, a delayed drop in stomatal conductance and net photosynthesis due to drought was observed in colonized seedlings. Plants colonized by vesicular-arbuscular microrrhizae (VAM) under drought conditions can maintain stomatal conductance and leaf water potential (Davies *et al.*, 1996; Augé, 2001), which resembles the responses of DIS 219b-colonized cacao seedlings to drought. The primary impact of VAM on plant–water relationships has been attributed to changes in plant growth and development and is commonly associated with enhanced phosphorus acquisition (Augé, 2001).

Table 4. Changes in biomass and water content in cacao seedlings due to varying watering cycle (1, 3, 7, and 10 d) and *Trichoderma hamatum* isolate DIS 219b treatment (colonized or non-colonized)

Treatments were DIS 219b-colonized (219b) or non-colonized seedlings (Control) watered every 1, 3, 7, or 10 d. The seedlings were grown at 25 °C and an average of 80% humidity. The watering cycle was initiated after 32 d and continued for 21 d. Statistical analysis was carried out as described in the text. The interaction between water cycle and *Trichoderma* was not significant, so means are presented for water cycle effect over both *Trichoderma* treatments and for the *Trichoderma* effect over all water cycle treatments.

Measurement	*P*-value	Treatment	Weight (g)	Means separation[a]
Water cycle effect				
Root fresh wt	0.0006	1 d	1.91	A
		3 d	2.07	A
		7 d	1.95	A
		10 d	1.61	B
Root dry wt	0.0007	1 d	0.35	B
		3 d	0.40	AB
		7 d	0.44	A
		10 d	0.38	AB
Root water wt	<0.0001	1 d	1.56	A
		3 d	1.67	A
		7 d	1.51	A
		10 d	1.22	B
Trichoderma effect				
Root fresh wt	<0.0001	Control	1.74	B
		219b	2.06	A
Root dry wt	<0.0001	Control	0.36	B
		219b	0.43	A
Root water wt	<0.0001	Control	1.38	B
		219b	1.63	A

[a] Treatment means for measurements within the factors water cycle and *Trichoderma* followed by the same letter are not significantly different at the $P \leq 0.05$ level.

Table 5. Changes in biomass and water content in cacao seedlings due to varying watering cycle (3 or 5 days) and *Trichoderma hamatum* isolate DIS 219b treatment (colonized or non-colonized)

Treatments were DIS 219b colonized (219b) or non-colonized seedlings (Cont) watered every 3 or 5 days. The seedlings were grown at 25°C and 40% to 45% humidity. The watering cycles were initiated after 32 days and continued for 15 days. Statistical analysis was carried out as described in the text. Means for the interaction between water cycle and *Trichoderma* are presented only where significant. Otherwise means are presented for water cycle effect over both *Trichoderma* treatments and for the *Trichoderma* effect over both water cycle treatments.

Measurement	*P*-value	Treatment	Weight (g)	Means separation[a]
Water cycle effect				
Root Fresh	0.0005	3 day	2.471	A
Weight		5 day	2.186	B
Root Dry	0.0035	3 day	0.516	A
Weight		5 day	0.464	B
Total	0.0012	3 day	6.131	A
Fresh Weight		5 day	5.217	B
Root Water	0.0003	3 day	1.954	A
Weight		5 day	1.722	B
Total Water	0.0001	3 day	4.557	A
Weight		5 day	3.864	B
Root Dry Wt/	0.0382	3 day	0.493	B
Top Dry Wt		5 day	0.526	A
Trichoderma effect				
Root Fresh	0.0001	Cont	2.192	B
Weight		219b	2.492	A
Root Dry	0.0006	Cont	0.464	B
Weight		219b	0.521	A
Total Fresh	0.0012	Cont	5.507	B
Weight		219b	5.951	A
Root Water	<0.0001	Cont	1.729	B
Weight		219b	1.970	A
Total Water	0.0012	Cont	4.086	B
Weight		219b	4.420	A
Root Dry Wt/	0.0459	Cont	0.49	B
Top Dry Wt		219b	0.523	A
Water cycle by *Trichoderma* Interactions				
Top Fresh	0.0436	Cont/3 day	3.481	B
Weight		219b/3 day	3.844	A
		Cont/5 day	3.054	C
		219b/5 day	3.011	C
Top Dry	0.0090	Cont/3 day	0.991	B
Weight		219b/3 day	1.126	A
		Cont/5 day	0.904	C
		219b/5 day	0.876	C
Total Dry	0.0094	Cont/3 day	1.463	B
Weight		219b/3 day	1.687	A
		Cont/5 day	1.354	C
		219b/5 day	1.350	C

[a] Treatment means for measurements within the factors water cycle and *Trichoderma* and the water cycle by *Trichoderma* interaction followed by the same letter are not significantly different at the $P \leq 0.05$ level.

Drought-induced changes in cacao gene expression

Nineteen drought-responsive ESTs were newly identified in cacao. These ESTs were chosen based on their relatedness to orthologues in other plants with characterized involvement in various biological processes, including drought (Table 1). The majority of the drought-responsive ESTs (16 out of 19) were identified in previous studies characterizing the responses of cacao to other biotic and abiotic stresses (Verica *et al.*, 2004; Bailey *et al.*, 2006).

ESTs putatively involved in the production of osmoprotectants and/or regulatory metabolites were responsive to drought (Fig. 4). In a previous study, it was found that three ESTs (*TcODC*, *TcADC*, and *TcSAMDC*) putatively involved in polyamine biosynthesis are also responsive to drought in cacao (Bae *et al.*, 2008). Osmolytes are sugars, sugar alcohols, amino acids, and amines. Osmolytes accumulate under drought stress in order to maintain cell turgor and to stabilize cell proteins and structures (reviewed by Valliyodan and Nguyen, 2006; Seki *et al.*, 2007). *TcTPP* putatively encodes the enzyme trehalose-6-phosphatase, the enzyme that catalyses the final step in trehalose biosynthesis. Trehalose confers drought tolerance to microorganisms, various higher plants, and to transformants engineered to

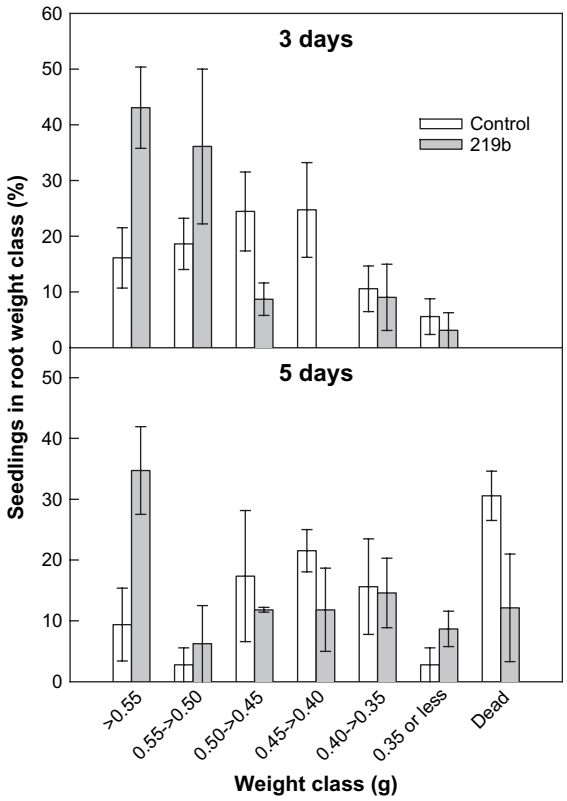

Fig. 8. The influence of DIS 219b colonization and watering cycle on the dry weight of cacao roots. For each treatment combination in the second biomass study, seedlings were grouped into 0.05 g weight class increments based on their root dry weights. The percentage of seedlings that died during the experiment is also included for each treatment combination. Bars show means±standard error.

Table 6. Changes in leaf angle in cacao seedlings due to varying watering cycle (3 d or 5 d) and *Trichoderma hamatum* isolate DIS 219b treatment (colonized or non-colonized)

Leaf angle was determined as the drop of the leaf blade tip from horizontal using a protractor. Treatments were DIS 219b-colonized (219b) or non-colonized seedlings (Control) watered every 3 d or 5 d. The seedlings were grown at 25 °C and 40–45% humidity. The watering cycles were initiated after 32 d and continued for 15 d. Leaf angle was measured three times at the end of each 5 d water cycle. Statistical analysis was carried out as described in the text. Means for the interaction between water cycle and *Trichoderma* are presented.

Measurement	*P* value	Treatment	Angle (°)	Means separation[a]
Leaf angle cycle 1	0.2227	Control/3 d	36.61	A
		219b/3 d	34.06	A
		Control/5 d	62.79	B
		219b/5 d	52.79	B
Leaf angle cycle 2	0.0158	Control/3 d	30.00	A
		219b/3 d	29.29	A
		Control/5 d	51.55	B
		219b/5 d	36.94	A
Leaf angle cycle 3	0.0097	Control/3 d	30.69	A
		219b/3 d	29.57	A
		Control/5 d	59.13	C
		219b/5 d	41.17	B

[a] Treatment means for measurements followed by the same letter are not significantly different at the $P \leq 0.05$ level.

overexpress enzymes in the trehalose pathway (Garg *et al.*, 2002). In cacao, *TcTPP* induction was an early response to drought in roots (7 d) but a late response in leaves (13 d). *TcSOT*, a putative sorbitol transporter, was induced 7 d PW in roots and 10 d PW in leaves. Sorbitol is the major phloem-translocated carbohydrate in some plant species and, similar to trehalose, is thought to be important in stress tolerance (Watari *et al.*, 2004). *TcPR5* was induced by drought 10 d PW in leaves and roots and encodes an osmotin-like protein. Osmotins and osmotin-like protein, members of the PR-5 protein family, are commonly associated with tolerance to osmotic stress and in plant defence (D'Angeli and Altamura, 2007). *TcNI* was induced by drought in leaves only. *TcNI* has close similarity to genes encoding alkaline/neutral invertases. Alkaline/neutral invertases participate in the hydrolysis of sucrose, providing a source of carbon for the biosynthesis of other osmoprotective substances and/or as a source of energy (Sturm, 1999). Lastly, *TcCESA3*, putatively encoding a cellulose synthase, was repressed in the roots by drought. Foyer *et al.* (1998) suggested that drought stress can increase sugar accumulation through inhibition of cellulose synthesis.

While *TcLOX* and *TcAOC* were chosen for their potential involvement in jasmonic acid biosynthesis, they do not show coordinated regulation in response to drought. *TcLOX* is induced by drought only in roots starting at 10 d PW (Fig. 5), putatively encoding a 13-lipoxygenase involved in jasmonic acid biosynthesis through a pathway that includes allene oxide cyclase (AOC). *TcAOC* putatively encodes an AOC, a plastid-associated protein (Hause *et al.*, 2003). *TcAOC* was repressed by drought only in roots starting 10 d PW (Fig. 5). In *Arabidopsis*, four AOCs were localized to the chloroplast (Stenzel *et al.*, 2003). Hause *et al.* (2003) verified that AOC in tomato is also associated with companion cells and plastids within sieve elements of vascular bundles, allowing for its possible function in cacao roots responding to drought.

The movement of molecules across membranes is critical for maintaining normal cell function. Aquaporins or major intrinsic proteins are responsible for the transcellular movement of water across the cell membranes (Steudle, 1994). In this study, *TcTIP* (P31), putatively encoding a tonoplast intrinsic protein, was repressed in the roots by drought at 7 d PW (Fig. 5). In an earlier study involving 9-d-old seedlings, colonization by DIS 219b also strongly repressed *TcTIP* (P31) expression (Bailey *et al.*, 2006). The repression of aquaporin gene expression may result in reduced membrane water permeability and encourage water conservation during periods of drought (Luu and Maurel, 2005; Secchi *et al.*, 2007). Expression of *TcABC-T* was also repressed in roots by drought beginning at 7 d PW. ABC transporters function in the movement of molecules across membranes in an ATP-dependent process (Jasinski *et al.*,

2003). The changes observed in the expression of these ESTs (*TcTIP*, *TcABC-T*, and *TcSOT*) allude to the significant impact of drought on the movement of molecules across membranes in cacao.

Orthologues of *TcSEN1* are found in many plant species and are induced by darkness and during senescence (Shimada *et al.*, 1998). Their function is unknown, but a recent study by Yang *et al.* (2003) suggested that the tobacco orthologue, *Ntdin*, plays a role in N- and S-metabolism functioning in molybdenum cofactor biosynthesis. Nitrate reductase (NR) is the first enzyme of primary N assimilation in plants (Ferrario-Mery *et al.*, 1998). As was observed in cacao roots for *TcNR* (Fig. 5), NR enzyme activity and transcript abundance are known to be sensitive to repression by drought (Ferrario-Mery *et al.*, 1998).

During drought stress, increases in plant cell osmolarity trigger signal transduction, resulting in activation of components of the drought response (reviewed by Beck *et al.*, 2007). In cacao roots, starting at 7 d PW, *TcRPK* (putative receptor-like protein kinase), *TcMKK4* [putative mitogen-activated protein (MAPK) possibly involved in the plant defence response], and *TcSTK* (putative serine/threonine protein kinase) were down-regulated in response to drought and *TcHK* (putative sensor type histidine kinase, possible osmoticum/cytokinin receptor), and *TcPP2C* (putative protein phosphatase 2C) were up-regulated in response to drought (Fig. 6). Histidine kinases are membrane-associated proteins that bind ligands serving as sensory preceptors, transmitting signals that participate in multiple gene regulatory cascades. HK can activate MAPK pathways (Zhu, 2002). *TcMAPK3*, a putative mitogen-activated protein kinase, was induced by drought but not until 10 d PW in both leaves and roots. *TcMAPK3* has close similarity to a family of MAP kinases that are inducible by various effectors including wounding, drought, cold, and salt, in addition to several plant hormones (Morris, 2001). Of particular interest is WIPK which is a transducer of the wounding signal leading to the synthesis of jasmonic acid (Morris, 2001) and TIPK of *Cucumis sativus* which is required for *Trichoderma*-conferred plant resistance (Shoresh *et al.*, 2006). *TcPP2C* was induced in cacao roots 7 d PW. PP2Cs are ubiquitous protein phosphatases found in all eukaryotes and several PP2Cs are involved in ABA responses (Tahtiharju and Palva, 2001; Saez *et al.*, 2004). Kinase-associated protein phosphatases serve as negative regulators for receptor-like kinases (Williams *et al.*, 1997; Li *et al.*, 1999). For example, in alfalfa (*Medicago sativa*), MAPKs were regulated by protein phosphatase 2C (MP2C) by dephosphorylation (Meskiene *et al.*, 2003). *TcZFP* (P13), a putative C2H2 zinc finger protein, was induced by drought, but not until 10 d PW in roots and 13 d PW in leaves. *TcZFP* (P13) was originally isolated by differential display and was shown to be induced by *Trichoderma* colonization in 9-d-old cacao seedlings (Bailey *et al.*, 2006). C2H2 zinc finger proteins may function as key transcriptional repressors involved in the responses of plants to stresses (Ciftci-Yilmaza and Mittler, 2008).

DIS 219b colonization delays drought-induced changes in gene expression

The drought-induced changes in gene expression patterns were delayed in leaves of colonized seedlings, whereas many of the drought-induced changes in gene expression observed in roots were not. For example, in leaves, the drought-altered expression of *TcrbcS* (Fig. 3), *TcTPP* (Fig. 4), and *TcMAPK3* (Fig. 6) was delayed in colonized seedlings. Examples where the delay did not occur in roots include *TcAOC*, *TcLOX*, *TcSEN1*, *TcNR*, *TcTPP*, *TcTIP*, *TcABC-T*, *TcHK*, *TcRPK*, and *TcMKK4* (Figs 4–6). DIS 219b caused a significant lag in the initiation of the drought response in the leaf but less so in the root.

Although consistent changes in cacao gene expression in watered seedlings over time in response to colonization were not observed, differences in gene expression due to colonization at specific time points were observed. These differences, for example for *TcNR* (Fig. 5), *TcABC-T* (Fig. 5), and *TcHK* and *TcZFP* (Fig. 6) at time zero, tended to be no more than 2-fold.

Changes in carbohydrate and amino acids in cacao during drought stress

Glucose content of cacao leaves increased during drought stress despite the reduction in carbon fixation due to both stomatal closure and down-regulation of the Calvin cycle. Foliar levels of starch and sucrose were not changed by drought or DIS 219b colonization. During drought stress, soluble carbohydrates (glucose, fructose, sucrose, stachyose, mannitol, and pinitol) can accumulate in leaves in a response that varies among plant species (reviewed by Valliyodan and Nguyen, 2006). Soluble carbohydrates serve as protectants, nutrients, and metabolite signalling molecules that modulate the transcription of genes involved in sugar sensing mechanisms (Ho *et al.*, 2001; Price *et al.*, 2004; Li *et al.*, 2006).

Concentrations of seven soluble amino acids were altered by withholding water for 13 d (Table 2). Concentrations of four soluble amino acids were altered by DIS 219b colonization. Pro concentrations increased in response to drought stress. A positive relationship between Pro accumulation and drought tolerance has been reported in many plant species (Bandurska, 2000; Nayyar and Walia, 2003; Chandra *et al.*, 2004; Simon-Sarkadi *et al.*, 2006). The influence of VAM and the grass endophytes on the drought-induced accumulation of Pro and other amino acids varies depending on the endophyte/plant interaction, the tissue sampled, the nutritional status of the plants, and many other factors (Malinowski and Belesky, 2000; Augé, 2001). Glu and Arg can function as Pro precursors. The trend was for a decrease in Glu concentration in non-watered seedlings, while Arg concentrations increased significantly in non-watered non-colonized seedlings. This result might indicate that Pro is synthesized mainly using Glu in cacao seedlings instead of Arg during drought treatment. In a previous study, the induction of polyamines in cacao was

characterized during drought treatment (Bae *et al.*, 2008). Glu might also be used for the synthesis of polyamine in cacao during drought stress. The accumulation of Arg in response to water deprivation was also observed in wheat (Galiba *et al.*, 1989) and *Brassica napus* (Good and Zaplachinski, 1994).

GABA concentrations in cacao leaves increased in response to drought and DIS 219b colonization (Table 3). Glu concentration decreased in DIS 219b-colonized seedlings. Glu and GLN usually are the principal amino acids present in foliar tissue. Ala concentrations also increased in response to DIS 219b colonization. The Ala response may be a by-product of the GABA shunt, as a result of the activity of GABA transaminase using pyruvate as the amino group acceptor (Shelp *et al.*, 1999). GABA is produced in response to various abiotic stresses including water stress (Bouché *et al.*, 2003; Oliver and Solomon, 2004; Fait *et al.*, 2005; Mazzucotelli *et al.*, 2006).

Initial DIS 219b colonization alters expression of drought-responsive genes

Under non-drought conditions, colonization by DIS 219b altered expression of several drought-responsive ESTs in 9-d-old seedlings (Fig. 7). It was in these same 9-d-old seedlings that induction by *Trichoderma* colonization of *TcODC*, a putative ornithine decarboxylase that is drought-responsive (Bae *et al.*, 2008), and *TcZFP* (P13), a putative C2H2 zinc finger protein, was noted earlier (Bailey *et al.*, 2006). It is of particular interest and a subject for further research that a dramatic increase in expression of *TcNR* (putative nitrate reductase) was observed in 9-d-old colonized seedlings (Fig. 7). This is in contrast to the effect of drought which typically causes a decrease in expression of the nitrate reductase genes (Ferrario-Mery *et al.*, 1998), a response also observed in cacao roots after drought exposure (Fig. 5). The endophytic fungus *Piriformospora indica* promoted growth of *Arabidopsis* and tobacco seedlings and stimulated nitrogen accumulation and the expression of a gene encoding NR in roots (Sherameti *et al.*, 2005). It was noted by Sherameti *et al.* (2005) that recruitment of nitrogen in endophytic interactions differs from mycorrhizal interactions in which the fungus preferentially recruits ammonium rather than nitrate, and the plant NR enzyme is down-regulated. By comparing the patterns of altered gene expression in 9-d-old seedlings colonized by DIS 219b with seedling responding to drought (32–45 d old) it is clear that, although the two treatments sometimes alter expression of the same ESTs, the direction of that influence is sometimes in opposite directions.

Plant growth promotion by Trichoderma hamatum isolate DIS 219b

In the initial biomass study, colonization of cacao seedlings by DIS 219b enhanced growth, resulting in an increase in root fresh and dry weight, and root water weight independent of the water cycle (Table 4). In a second biomass study, DIS 219b again enhanced growth independent of the water cycle, resulting in an increase in root fresh weight, root dry weight, total fresh weight, root water weight, total water weight, and the root dry weight/root fresh weight ratio (Table 5). In the second biomass study a delay in the drooping of cacao leaves in response to drought (Table 6) was associated with the DIS 219b colonization. In the second biomass study humidity was maintained at 40%, resulting in much drier conditions and a shorter time period until wilting than observed in the earlier studies. The ability of *Trichoderma* isolates to enhance plant growth has been characterized in other cropping systems, although the mechanisms involved have not been fully explained (Harman *et al.*, 2004). Plant growth promotion is often observed in response to *Trichoderma* colonization (Harman *et al.*, 2004; Lynch, 2004; Adams *et al.*, 2007). Enhanced nutrient availability through solubilization and chelation of minerals and increased nutrient uptake efficiency, among others, are proposed mechanisms involved in *Trichoderma*-induced plant growth promotion (Altomare *et al.*, 1999; Yedidia *et al.*, 2001; Harman *et al.*, 2004). The impact of endophyte-induced enhanced plant growth on drought tolerance has been extensively studied for VAM/plant associations and endophytes of cool-season grasses (Malinowski and Belesky, 2000; Augé, 2001). The abilities of larger plant tissues to store water as observed here are commonly cited mechanisms for enhancing drought tolerance in plants (Malinowski and Belesky, 2000; Augé, 2001).

Conclusions

Although net photosynthesis and stomatal conductance in cacao leaves were significantly altered in response to drought 7 d PW, leaf gene expression tended to remain unaltered until 10 d PW. By contrast, many of the ESTs studied that responded to drought in roots did so starting at 7 d PW, demonstrating the early perception of drought and rapid alteration of gene expression in roots. Much of the drought-altered expression of ESTs in roots was not influenced by DIS 219b colonization, whereas the ESTs responding to drought in leaves demonstrated a delayed drought response when the seedlings were colonized by DIS 219b. Most of the physiological measurements evaluated (leaf responses) showed a similar delay in response to drought when seedlings are colonized by DIS 219b. This leads to the suggestion that the delay in the cacao drought response observed in colonized seedlings is due to changes in the physiology of the seedling and not due to changes in the seedling's interaction with the soil at the time of drought exposure. The simplest explanation is that DIS 219b colonization enhanced root growth, resulting in improved water acquisition and an increase in water content. The roots of colonized seedlings perceive the dry soils and respond while the leaves take advantage of the increased water availability through the roots, resulting in a delayed drought response. Unfortunately, when plant growth promotion occurs, it can be very difficult to separate out the

influence of factors unrelated to plant size and nutritional status. The changes in plant growth may themselves be mediated through direct effects of DIS 219b colonization on cacao gene expression observed during the early stages of seedling colonization. The concentrations of several amino acids, notably GABA, were also directly responsive to DIS 219b colonization, leaving open the possibility that colonization may pre-adapt cacao to drought as a component of the altered signal transduction pathways observed in direct response to colonization. The colonization of cacao seedlings by *T. hamatum* isolate DIS 219b enhanced seedling growth, altered gene expression, and delayed the onset of the cacao drought response in leaves at the molecular, physiological, and phenotypic levels, a response that could prove valuable in the production of this important tropical crop.

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