

Endogenous hormone concentrations correlate with fructan metabolism throughout the phenological cycle in *Chrysoleaena obovata*

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• **Background and Aims** *Chrysoleaena obovata*, an Asteraceae of the Brazilian Cerrado, presents seasonal growth, marked by senescence of aerial organs in winter and subsequent regrowth at the end of this season. The underground reserve organs, the rhizophores, accumulate inulin-type fructans, which are known to confer tolerance to drought and low temperature. Fructans and fructan-metabolizing enzymes show a characteristic spatial and temporal distribution in the rhizophores during the developmental cycle. Previous studies have shown correlations between abscisic acid (ABA) or indole acetic acid (IAA), fructans, dormancy and tolerance to drought and cold, but the signalling mechanism for the beginning of dormancy and sprouting in this species is still unknown.

• **Methods** Adult plants were sampled from the field across phenological phases including dormancy, sprouting and vegetative growth. Endogenous concentrations of ABA and IAA were determined by GC-MS-SIM (gas chromatography–mass spectrometry–selected ion monitoring), and measurements were made of fructan content and composition, and enzyme activities. The relative expression of corresponding genes during dormancy and sprouting were also determined.

• **Key Results** Plants showed a high fructan 1-exohydrolase (EC 3.2.1.153) activity and expression during sprouting in proximal segments of the rhizophores, indicating mobilization of fructan reserves, when ABA concentrations were relatively low and precipitation and temperature were at their minimum values. Concomitantly, higher IAA concentrations were consistent with the role of this regulator in promoting cell elongation and plant growth. With high rates of precipitation and high temperatures in summer, the fructan-synthesizing enzyme sucrose 1-fructosyltransferase (EC 2.4.1.99) showed higher activity and expression in distal segments of the rhizophores, which decreased over the course of the vegetative stage when ABA concentrations were higher, possibly signalling the entry into dormancy.

• **Conclusions** The results show that fructan metabolism correlates well with endogenous hormone concentrations and environmental changes, suggesting that the co-ordinated action of carbohydrate metabolism and hormone synthesis enables *C. obovata* to survive unfavourable field conditions. Endogenous hormone concentrations seem to be related to regulation of fructan metabolism and to the transition between phenophases, signalling for energy storage, reserve mobilization and accumulation of oligosaccharides as osmolytes.

Key words: ABA, IAA, fructan metabolism, *Chrysoleaena obovata*, *Vernonia herbacea*, Asteraceae, inulin, hormone signalling, carbohydrate reserves, rhizophore, Cerrado, seasonal dormancy.

INTRODUCTION

The Cerrado is a tropical savanna that covers about 23 % of the central region of Brazil, being surpassed in area only by the Amazon Rain Forest (Ratter *et al.*, 1997). This biome hosts a high biodiversity and is considered one of the world hotspots (Simon *et al.*, 2009). The Cerrado soil is deep, porous, permeable, and contains a mixture of sand and clay with overall low water retention, poor in nutrients and acid, due partly to high levels of Al³⁺ (Eiten, 1972). The climate is characterized by a marked dry winter (3–6 months) and a wet summer, presenting an average precipitation of 800–2000 mm and

average annual temperatures ranging between 18 and 28 °C (Dias, 1992).

Water restriction and the wide temperature range are some of the selective pressures acting on Cerrado species. In this biome, plants developed adaptive strategies to cope with seasonal water deficit and temperature stress, including enlarged underground organs, thick corky bark, hard sclerophyllous leaves, and leaf senescence in the dry season (Ratter *et al.*, 1997). Seasonal growth and reproductive patterns are also typical and related to the climatic seasonality of the Cerrado, as well as of other tropical savannas (Williams *et al.*, 1997; Batalha and

Mantovani, 2000). Metabolic adjustments, including production of compatible solutes and activation of the antioxidant system, complete the multiple plant stress response mechanisms in Cerrado species.

Fructans are soluble, osmotically active carbohydrates thought to act on membrane stabilization by binding to choline and phosphate groups, increasing tolerance to drought and low temperature (Pilon-Smits *et al.*, 1995; Carvalho *et al.*, 2007; Hinch *et al.*, 2007; Kawakami *et al.*, 2008; Valluru and Van den Ende, 2008; Garcia *et al.*, 2011). Fructans are widely distributed in herbaceous species of the Cerrado, showing changes in contents and composition associated with the phenological cycle, exposure to low temperature, water availability and other environmental factors prevailing in this biome (Carvalho and Dietrich, 1993; Isejima and Figueiredo-Ribeiro, 1993; Vieira *et al.*, 1995; Portes *et al.*, 2008; Garcia *et al.*, 2011; Oliveira *et al.*, 2013).

Fructans have been regarded as second to starch in importance as storage carbohydrates, being present in approx. 15 % of the contemporary angiosperms (Hendry and Wallace, 1993). Species containing fructans are distributed within a diverse range of families including Poaceae and Asteraceae. The diversity of distribution and the occurrence of fructans among highly evolved families indicate that genes for fructan metabolism in angiosperms may have arisen in response to one or a few selective pressures in the relatively recent past (Hendry, 1987).

Chrysolaena obovata (Less.) Dematt., previously named *Vernonia herbacea* (Vell.) Rusby, belongs to the Asteraceae family, is herbaceous, perennial and is native to the Cerrado. It accumulates inulin-type fructans in its underground reserve organs, the rhizophores. Inulin synthesis in *C. obovata* occurs predominantly in younger tissues of rhizophores (distal regions) by the action of the enzymes sucrose:sucrose 1-fructosyltransferase (1-SST; EC 2.4.1.99), that catalyses the formation of the trisaccharide 1-kestose, and fructan:fructan 1-fructosyltransferase (1-FFT; EC 2.4.1.100) that is involved in both the increase and decrease of fructan chain size. Fructan depolymerization occurs mainly in segments of the rhizophores near buds that originate new shoots (proximal regions), by the action of fructan 1-exohydrolase (1-FEH; EC 3.2.1.153) (Portes and Carvalho, 2006; Portes *et al.*, 2008; Asega *et al.*, 2011). The gene coding for 1-FEH in *C. obovata* was isolated and its activity in inulin hydrolysis was confirmed by heterologous expression in the *Pichia pastoris* system (Asega *et al.*, 2008).

Fructan metabolism seems to be tightly correlated with the phenological cycle of *C. obovata* in association with climatic seasonality. Sprouting of these plants in spring is followed by vegetative growth and flower development. Vegetative growth lasts until early autumn, when the aerial organs senesce and go through abscission. From this stage on, plants remain dormant until the end of winter, when buds sprout to develop new aerial organs. A decrease in fructan contents during sprouting indicates reserve mobilization to provide substrate for growth, followed by an increase in fructan contents during the vegetative growth phase (Carvalho and Dietrich, 1993).

Sugar metabolism is under hormonal control, and abscisic acid (ABA) is known to regulate key enzymes and transcript accumulation in different carbohydrate synthesis pathways (Trouverie *et al.*, 2003; Yang *et al.*, 2004). Different phytohormones are known to regulate fructan metabolism (Van den

Ende *et al.*, 2002). In the promoter region of the gene *1-FEH IIa* isolated from *Cichorium intybus*, Michiels *et al.* (2004) identified motifs for response to auxin, ABA, ethylene, gibberellins (GAs) and salicylic acid, revealing the complexity of the regulation of fructan metabolism genes.

In *Allium cepa*, Chope *et al.* (2006) detected the concomitant decrease in fructan and ABA contents in bulbs stored at low temperature, and reported the onset of sprouting at a critical minimum concentration of endogenous ABA, associated with carbohydrate mobilization. In *Hordeum vulgare*, another fructan-accumulating species, plant acclimation to cold occurred due to a concomitant increase in soluble sugars and ABA concentrations (Bravo *et al.*, 1998).

Yang *et al.* (2004) reported that the ABA concentration increased in wheat stems under drought, correlated with increases in sucrose-phosphate synthase (EC 2.4.1.14) and 1-FEH, and a reduction in 1-SST. Plants treated with an inhibitor of ABA synthesis showed a reduction of sucrose synthesis and in the activities of 1-FEH and acid invertase (EC 3.2.1.26). An opposite response was detected when plants were treated with exogenous ABA. Further clear evidence of the regulation of fructan metabolism by ABA was presented by Ruuska *et al.* (2008). Using individual wheat culms fed with ABA, the authors showed increased *1-FEH* and *6-FEH* transcript accumulation and inhibition of *1-SST*, *6-SFT* and *1-FFT* gene expression. Transcriptome analysis of *Arabidopsis thaliana* and rice treated with exogenous ABA showed induction of 3.5 and 2.5 % of the genes, respectively (Seki *et al.*, 2002; Rabbani *et al.*, 2003). In fact, Seki *et al.* (2002) reported that carbohydrate metabolism is among the most affected by ABA treatment in *A. thaliana*.

Despite the importance of auxin in the regulation of plant development, there are only a few studies looking at the association of fructan metabolism and endogenous auxin concentrations. Barreto *et al.* (2010) reported an increase in fructan concentrations in plants of *Agave tequilana* in the presence of 1-naphthaleneacetic acid. However, Trevisan *et al.* (2014) suggested that auxin could act as a negative regulator of fructan-synthesizing enzymes in *in vitro* plants of *C. obovata*. More recently, Zhang *et al.* (2014) found a single nucleotide mutation in the promoter region of the wheat *1-FEH w3* gene, located at an auxin response element (ARE). The authors suggest that this mutation in ARE could affect *1-FEH* gene expression and enzyme activity, affecting wheat stem fructan remobilization to grain in different genotypes.

Major changes in phytohormone concentrations occur during the phenological cycle of several species. Zhao *et al.* (2013) pointed out that environmental factors can regulate plant hormone concentrations and determine the physiological state of plant development. McAdam and Brodrigg (2014), studying water use strategies of five phylogenetically distant species cohabiting in the monsoonal tropics of northern Australia, a region characterized by marked seasonal variation in precipitation, found different patterns of ABA sensing, synthesis and metabolism in leaves over the course of the year. According to the authors, the contrasting strategies found among the studied species represent an evolutionary pathway for adaptation in plant water use.

Although a correlation between phytohormones, fructan metabolism and tolerance to drought and cold has been shown in some studies, the implications of endogenous hormonal

changes in response to water and cold stress in nature and at different phenological phases are poorly understood. Considering that *C. obovata* is an excellent model for studying the regulation of fructan metabolism by environmental factors, this study aimed to evaluate endogenous concentrations of ABA and auxin during the transition to the dormant phase (dry season) and to the sprouting phase (wet season), in order to correlate hormone concentrations and fructan metabolism in the rhizophore, as the sink and source organ, in plants growing in the Cerrado.

MATERIALS AND METHODS

Plant material

Four adult plants of *Chrysolaena obovata* (Less.) Dematt. were collected in a preserved Cerrado area at the Reserva Biológica e Estação Experimental de Mogi Guaçu, SP, Brazil (22°18'S, 47°11'W) at each of the following phenological phases: one harvest at early dormancy (ED) (low water availability), three harvests during sprouting, from early (S1) through two more advanced stages (S2, S3), two harvests during the vegetative growth phase (V1, V2) (high water availability) and one harvest at dormancy (D) (low water availability), totalling seven sampling dates (Table 1). At each harvest, samples of the aerial organs (stems and leaves) at the same developmental stage were used for dry mass determination and hormone analyses. Rhizophores were washed immediately after harvest and sample fragments were cut from the distal and proximal regions, as described (see fig. 1 in Portes and Carvalho, 2006; Supplementary Data Fig. S1). Samples from the two regions were weighed and frozen in liquid nitrogen for biochemical and molecular analyses.

Environmental conditions

Meteorological data were provided by two companies located near the Biological Reserve where the study was carried out. Maximum and minimum temperatures were obtained from International Paper do Brasil LTDA. (22°22'S, 46°58'W) and rainfall data from two weather stations in the nearby area, belonging to AES Tietê S/A (22°22'S, 46°58'W) (Fig. 1A). The highest precipitation period occurred in summer, between December 2012 and January 2013, and the lowest occurred between the end of autumn and winter, from May to September 2012 and from April to July 2013, when the temperature range

TABLE 1. Harvesting dates and corresponding phenological phases of plants of *Chrysolaena obovata* at Reserva Biológica e Estação Experimental de Mogi Guaçu, SP, Brazil

Date	Phenological phase	Abbreviation
26 June 2012	Early dormancy	ED
14 August 2012	Sprouting	S1
11 September 2012	Sprouting	S2
5 October 2012	Sprouting	S3
16 January 2013	Vegetative	V1
17 April 2013	Vegetative	V2
1 July 2013	Dormancy	D

increased (Fig. 1A). The average temperature and the accumulated precipitation during the 7 d preceding each sampling date were calculated (Fig. 1B). Precipitation was close to zero between August and October (sprouting stage). The highest precipitation values were observed in January (vegetative stage). The highest temperature range was registered in October, and the lowest in January.

Soil samples were collected at the harvest area in triplicate at depths of 0–10, 20–30 and 40–50 cm, for estimation of soil water retention curves (Cruz *et al.*, 2005) at Instituto Agrônômico de Campinas (IAC), SP, Brazil. For soil water retention curves, see Supplementary Data Fig. S2.

Water status of soil and plants

Soil moisture was measured gravimetrically in soil samples obtained from the plant rhizosphere (Blake, 1965). Water content (WC) of aerial organs and rhizophore segments were determined in four samples at each phenological phase, using the equation $WC = (f. wt - d. wt) / f. wt \times 100$, where f. wt is the fresh weight and d. wt the dry weight of the sample. The d. wt of aerial organs was obtained after drying tissues at 60 °C to constant weight, and the d. wt of rhizophores was obtained after sample lyophilization.

The water potential of rhizophore segments was determined in four samples, in squeezed cell sap, using a vapour pressure osmometer (Model VAPRO 5520, Wescor, Logan, UT, USA), according to Oliveira *et al.* (2013). Osmolarity was measured in $mmol\ kg^{-1}$ and transformed into MPa, using the Van't Hoff equation, $MPa = mmol\ kg^{-1} \times 2.58 \times 10^{-3}$ (Santa-Cruz *et al.*, 2002).

Determination of endogenous concentrations of ABA and IAA by gas chromatography–mass spectrometry–selected ion monitoring (GC-MS-SIM)

The extraction was done as described in Ludwig-Muller *et al.* (2008) using frozen samples of aerial organs and rhizophores ground in a mortar, in liquid N₂. An isopropanol:acetic acid (95:5) solution was added to the tissue powder (4:1 v/w) together with 0.5 µg of each labelled ABA standard ([²H₆]ABA) and indole acetic acid (IAA) standard ([¹³C₆]IAA). The mixture was kept under shaking at 4 °C for 2 h and centrifuged at 13 000 g. The supernatant was collected and concentrated to 50 µL under an N₂ gas flow, followed by the addition of 0.2 mL of ultrapure water. Samples were then fractionated with 0.5 mL of ethyl acetate and the organic phase was transferred to another tube and dried under N₂ gas flow. The material was methylated with diazomethane, dried under N₂ gas flow and re-suspended in ethyl acetate. The analysis was performed on a Hewlett-Packard (Wilmington, DE, USA) gas chromatograph model 6890 coupled to a mass spectrometer model 5973 in selective ion monitoring mode (GC-MS-SIM). The chromatograph was equipped with an HP-1701 column (30 m, ID 0.25 mm, 0.50 µm thick internal film) using helium as the carrier gas at a flow rate of 4 mL min⁻¹ in the following program: 3 min at 150 °C, followed a ramp by 5 °C min⁻¹ to 210 °C and 15 °C min⁻¹ to 260 °C. Ions with a mass ratio/charge (*m/z*) of 130 and 189 (corresponding to endogenous

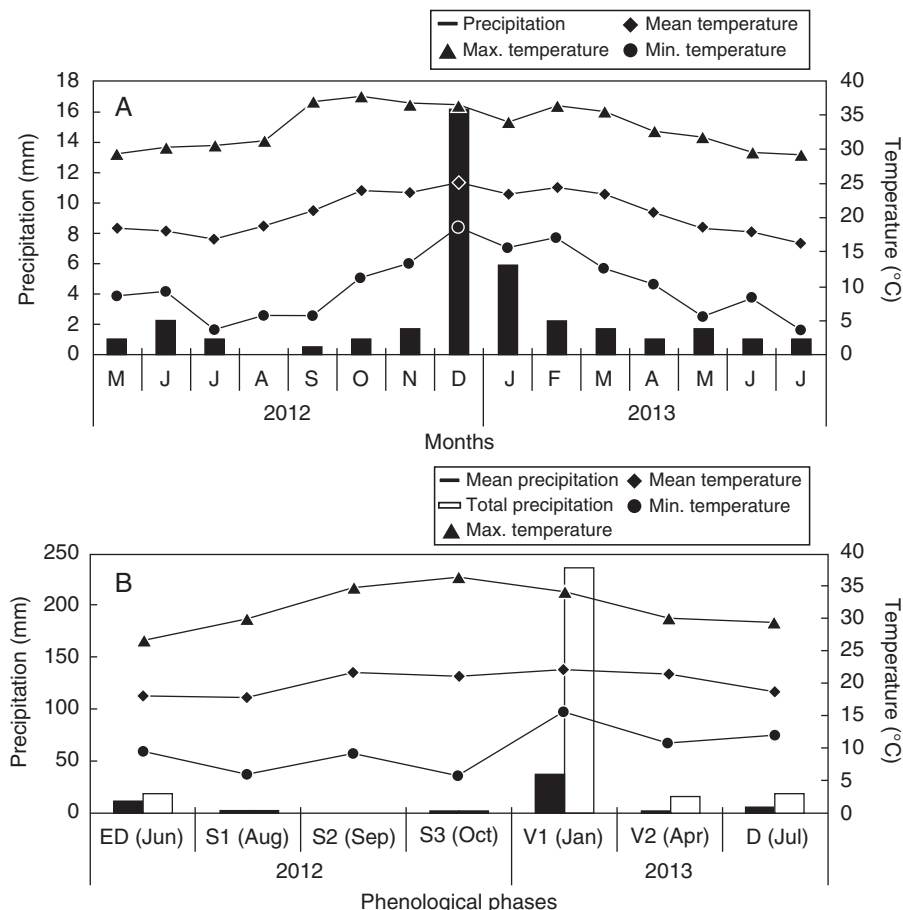


FIG. 1. (A) Monthly average precipitation, and monthly mean, maximum and minimum temperatures between May 2012 and July 2013 in the study area. (B) Average and accumulated precipitation, and mean, maximum and minimum temperatures 7 d before the harvest dates.

IAA) and 136 and 195 (corresponding to [$^{13}\text{C}_6$]IAA), and also ions with (m/z) 134, 162 and 190 (corresponding to endogenous ABA) and 138, 166 and 194 (corresponding to [$^2\text{H}_6$]ABA) were monitored. Concentrations were calculated based on extracted chromatograms at m/z 130 and 136 for IAA and 162 and 266 for ABA. The extractions were performed in triplicate.

Enzyme extraction and assays

Frozen rhizophore samples were pulverized in liquid N_2 and homogenized in 0.05 M McIlvaine buffer (pH 5.5); (1:3, w/v) containing 2 mM EDTA, 2 mM β -mercaptoethanol, 5 mM ascorbic acid and 10 % polyvinylpyrrolidone (PVPP; w/w) as in Asega and Carvalho, (2004). Proteins were precipitated with $(\text{NH}_4)_2\text{SO}_4$ at 80 % saturation, and re-suspended in the ratio of approx. 10 g fresh mass equivalent per mL in extraction buffer. The final extract was used for enzyme assays and protein determination (Bradford, 1976), using bovine serum albumin as standard. Enzymes were assayed by incubation of the protein extract with different substrates mixed 1:1 (v/v). Substrates were prepared in 0.05 M McIlvaine buffer (pH 4.5 for 1-FEH, and pH 5.5 for 1-SST and 1-FFT). The extracts were incubated at 30 °C at a final concentration of 0.2 M sucrose (Sigma-Aldrich) for 1-SST, 0.2 M 1-kestose for 1-FFT and 5 % inulin

from *C. obovata* for 1-FEH activity. Incubation time was 1 h for 1-SST and 1-FEH, and 2 h for 1-FFT. For determination of 1-SST, 1-FFT and 1-FEH activities, samples of the reaction mixtures were diluted and analysed using high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC/PAD) with a 2×250 mm CarboPac PA-1 column on a Dionex system (model ICS 3000, Dionex, Sunnyvale, CA, USA), as described in Oliveira *et al.* (2013). The activities of 1-FEH, 1-SST and 1-FFT were calculated by direct measurement of fructose, 1-kestose and nystose, respectively, using the external standard method. All procedures were done at 5 °C and extractions were performed in triplicate.

Soluble carbohydrate analyses

Soluble carbohydrates were extracted from freeze-dried rhizophores (Portes and Carvalho, 2006). The crude extract was submitted to ethanol precipitation and the fructo-oligosaccharide [hexoses, sucrose and fructans with degree of polymerization (DP) 3–27] and fructo-polysaccharide (mainly fructans with DP 10–60) fractions were separated by centrifugation. Free and combined fructose was measured in crude extracts and in the two fractions separately, using a ketose-specific modification of the anthrone reaction and fructose (Sigma-Aldrich) as

standard (Jermyn, 1956), and the fructo-oligo:fructo-polysaccharide ratio was calculated. Soluble carbohydrates in crude extracts were de-ionized through ion exchange columns (Carvalho and Dietrich, 1993), and analysed using HPAEC/PAD with the same conditions as described above for 1-FEH activity analysis. Extractions were performed in quadruplicate.

Total RNA extraction and gene expression analysis

Total RNA was isolated from the proximal and distal regions of rhizophores from plants in sprouting (S1 and S3), vegetative (V1) and dormant (D) phases, using an RNeasy Plant Mini Kit (Qiagen). For cDNA synthesis, duplicates of 1 µg of total RNA from each sample were treated with DNase I, RNase-free (Thermo Scientific) and then reverse transcribed using random hexamers and RevertAid H Minus Strand cDNA Synthesis (Thermo Scientific).

The quantitative real-time PCRs (qRT-PCRs) were performed in technical triplicates using the reagent EXPRESS SYBR GreenER qPCR SuperMix (Invitrogen) in a thermocycler Mastercycler ep realplex 2S (Eppendorf AG, Hamburg, Germany) with the following program: 2 min at 50 °C, 2 min at 95 °C followed by 40 cycles of 15 s at 94 °C, 1 min at 60 °C. For the melting curve, the temperature was increased gradually over 20 min, from 60 to 95 °C. The mRNA expression values were determined according to Pfaffl (2001), relative to the vegetative phase (control). Relative gene expression analysis was performed for *1-FEH* and *1-SST* genes, and expression levels were normalized to the reference genes *elongation factor (EF)* and *18S rRNA (18S)*, which were ranked as the most stable reference genes for gene expression analysis in *C. intybus* (Maroufi et al., 2010). For primer details, see Supplementary Data Table S1. The primer specificity was validated by melting curves showing amplification of a single product. PCR efficiencies varied from 93 to 99 %. Results were obtained from two biological replicates and a duplicate of the reverse transcription step, as suggested by Bustin et al. (2009).

Statistical analyses

The correlation coefficients were calculated between edaphoclimatic, biochemical and physiological parameters – temperature, precipitation, soil moisture, water content of plants, water potential of rhizophores, phytohormone and soluble carbohydrate contents, and enzyme activities – using Pearson's correlation. Statistical significance of results was tested using independent Student's *t*-tests. One-way analyses of variance (ANOVAs) were performed using the software SISVAR 4.2 for comparison of the means over the sampling period. When significant differences were found, a post-hoc Tukey's honestly significantly different (HSD) test was also performed at $P \leq 0.05$. Data of distal and proximal rhizophore segments were also compared at each sampling date with one-way ANOVA. Principal component analysis (PCA) was carried out using the following parameters: average precipitation and temperature, water content in aerial organs, water content and water potential of rhizophores, and biochemical and physiological parameters. PCA was performed from the correlation matrix with data transformed by logarithm $[(\log(x+1))]$.

The randomization test (999 permutations) was used to choose the PCA interpretation dimension ($P < 0.05$) using the program PC-ORD 6.0.

RESULTS

Water status of soil and plants

In accordance with the highest accumulated precipitation shown in Fig. 1B, soil moisture was highest in the vegetative phase (V1) (Fig. 2A). At early dormancy (ED), the highest water potential was observed (-0.52 MPa in proximal and -0.6 MPa in distal segments), while in sprouting (S1 and S3) and vegetative (V2) phases, the lowest values were detected (between -0.73 and -0.92 MPa in both segments), without marked differences between distal and proximal segments (Fig. 2B). Water content in the aerial organs remained constant during sprouting and vegetative phases, differing significantly only at ED. In S1 and dormancy (D), the plants presented no aerial organs (Fig. 2C). In rhizophores, the water content presented small variations throughout the analysed period and remained higher in the proximal segment, in comparison with the distal ones, with significant differences at ED, S1 and D (Fig. 2D).

Endogenous concentrations of ABA and IAA

The ABA content in the aerial organs tended to increase, from 508 ng g⁻¹ d. wt in S2 to 3868 ng g⁻¹ d. wt in V2, with the lowest content (22 ng g⁻¹ d. wt) at ED (Fig. 3A). Stages S1 and D were not sampled due to lack of aerial organs. In rhizophores, no variation in ABA contents between proximal and distal segments occurred, except at D, when the distal segment presented a value significantly higher than the proximal one. The highest ABA concentrations were found in the proximal segment at V2 (3031 ng g⁻¹ d. wt), and the lowest, during sprouting (S1 and S2), with values near zero (Fig. 3B).

An opposite profile was observed for IAA contents in aerial organs; the value detected at V2 (110 ng g⁻¹ d. wt), was twice as low as in S2 (208 ng g⁻¹ d. wt) (Fig. 3C), although no statistical differences were detected. IAA in rhizophores showed no significant differences between the two segments, and the lowest value was detected in S2 (20 ng g⁻¹ d. wt in the distal segment; Fig. 3D).

Soluble carbohydrates

Fructo-polysaccharide contents did not vary significantly among the proximal segments during the analysed period. Among the distal segments, a difference was observed only between S3 and D. In the latter, the content in the distal segments was significantly higher than in the proximal segment (Fig. 4A). Conversely, fructo-oligosaccharide contents did not differ between the rhizophores segments; however, values differed among phenological phases, with the lowest values sampled during sprouting (Fig. 4B).

The sharp reduction of fructo-oligosaccharides during sprouting is evidenced by the fructo-oligo:fructo-polysaccharides

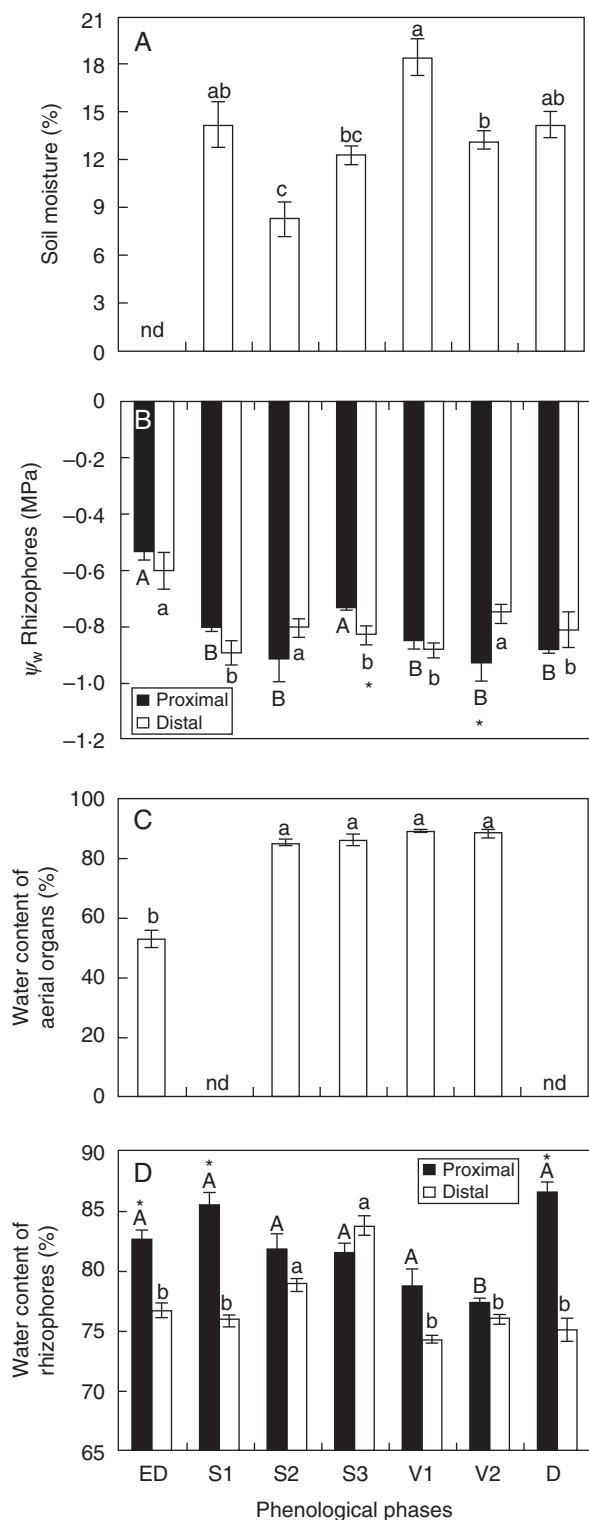


FIG. 2. (A) Soil moisture content, (B) water potential (Ψ_w) in proximal and distal rhizophore segments (as indicated in the key), (C) water content of aerial organs and (D) water content of proximal and distal rhizophore segments of plants of *Chrysolaena obovata* at different phenological phases. Values are the means \pm s.e. ($n=4$). Upper case letters indicate a comparison of proximal segments and lower case letters indicate a comparison of distal segments during the experimental period ($P < 0.05$). An asterisk (*) denotes a comparison of the segments (proximal and distal) within each harvest date ($P < 0.05$). nd, no data.

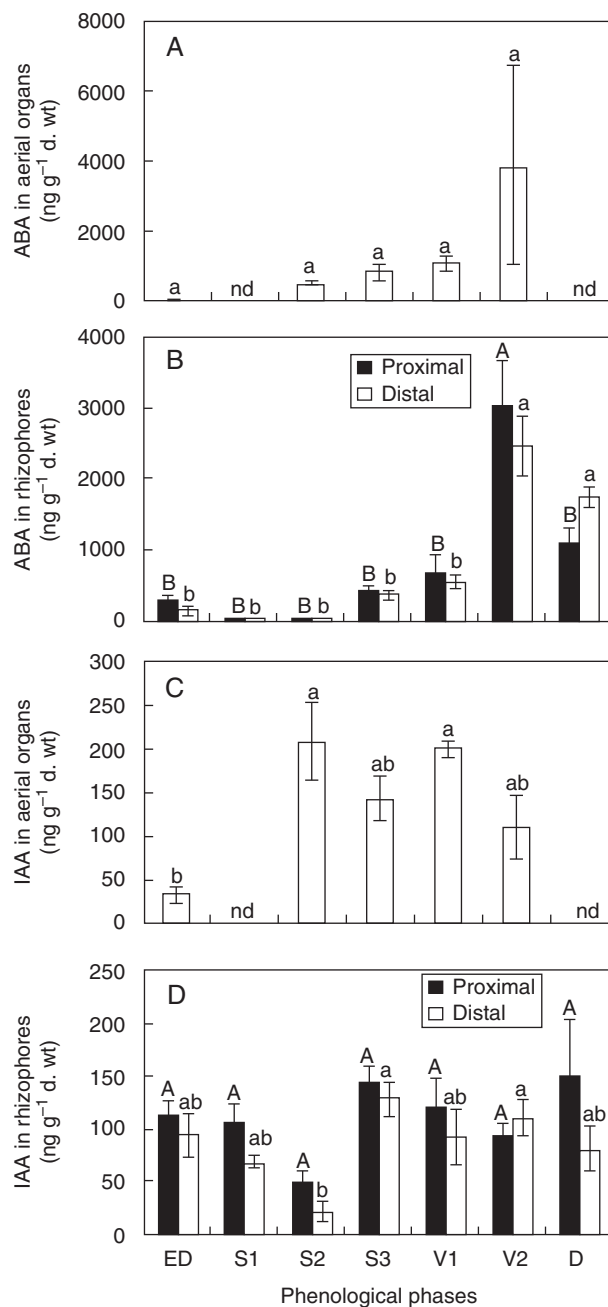


FIG. 3. (A) ABA content in the aerial organs and (B) in the proximal and distal rhizophore segments (as indicated in the key), and (C) IAA content in the aerial organs and (D) in the proximal and distal rhizophore segments of plants of *Chrysolaena obovata* at different phenological phases. Values are the means \pm s.e. ($n=3$). Upper case letters indicate a comparison of proximal segments and lower case letters indicate a comparison of distal segments during the experimental period ($P < 0.05$). An asterisk (*) denotes a comparison of the segments (proximal and distal) within each harvest date ($P < 0.05$). nd, no data.

ratio, and the increase in this ratio in proximal segments at D was due to a reduction of fructo-polysaccharides (Fig. 4C).

Reducing sugars showed a decreasing tendency from ED to S2, followed by an increase at S3, and a further decrease until D (Fig. 4D). These results were also observed in the chromatographic profiles, with an increase of the hexoses glucose and fructose during sprouting, especially at S3 (Fig. 5). In the

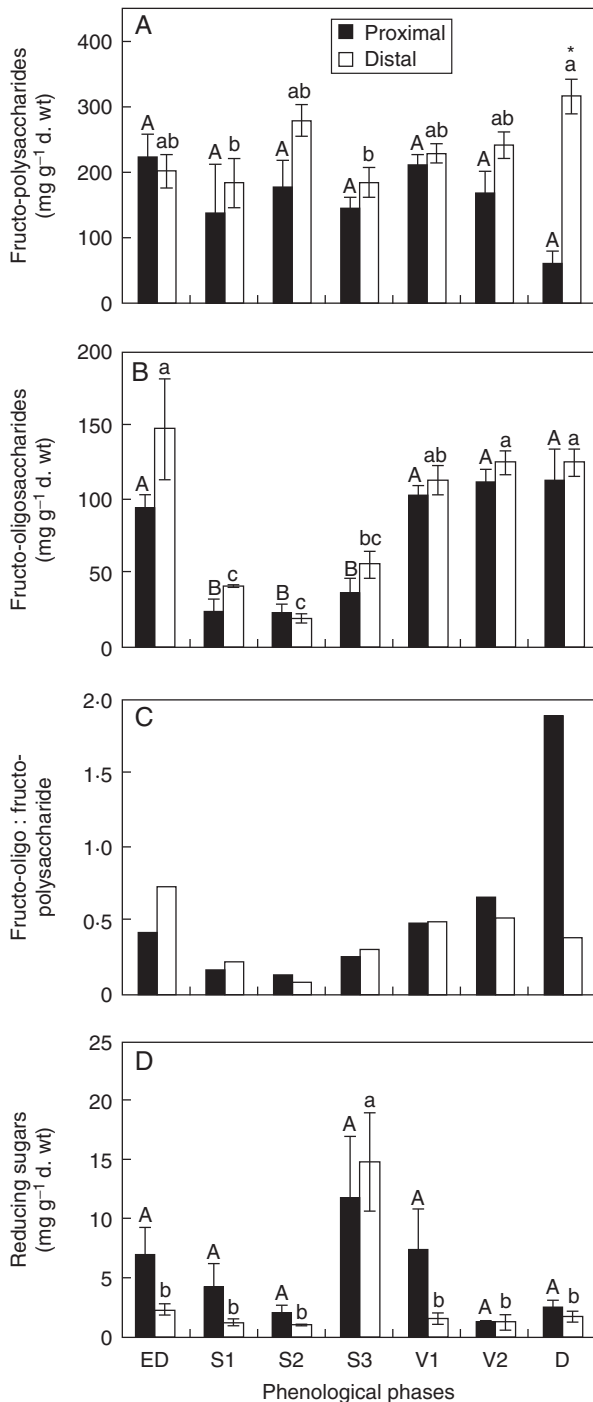


FIG. 4. (A) Fructo-polysaccharides, (B) fructo-oligosaccharides, (C) fructo-oligo:fructo-polysaccharides ratio and reducing sugars (D) in the proximal and distal rhizophore segments (see key) of plants of *Chrysolaena obovata* at different phenological phases. Upper case letters indicate a comparison of proximal segments and lower case letters indicate a comparison of distal segments during the experimental period ($P < 0.05$). An asterisk (*) denotes a comparison of the segments (proximal and distal) within each harvest date ($P < 0.05$).

proximal segment, following V1 and until D, a gradual increase in fructans with medium DP (5–15) was observed. In the distal segment, an increase in the 1-kestose peak was detected only at V1.

Enzyme activities and gene expression of fructan metabolism

The activity of 1-FEH was highest in the proximal segments of rhizophores throughout the experimental period, with significant differences between the two segments in S1, V1 and V2. Moreover, the overall activities were higher during sprouting (Fig. 6A). The *1-FEH* gene showed higher expression during sprouting, being five times more greatly expressed in the proximal segment at S3 (Fig. 7A). The *1-FEH* transcript abundance showed a moderate increase in distal segments, twice as high as at the vegetative stage (V1), used as the reference (Fig. 7A).

In contrast, 1-SST showed the lowest activities during sprouting and was most active in the distal segments at ED and V1 (Fig. 6B). The *1-SST* gene showed the highest expression in V1, with a marked inhibition at S1, S3 and D (Fig. 7B).

The enzyme 1-FFT remained active through all the phenological phases analysed, with a marked decrease at S3 and higher activity at ED, S1 and S2 (Fig. 6C). The activity was significantly higher in the distal segment only at S1.

Principal component analysis

Principal component analysis summarized 55.2 % of the total data variability on the two first axes (Fig. 8), which were considered significant at the randomization test ($P < 0.005$). All the samplings at sprouting, except for the distal segments at S2, and at D were positioned on the positive side of axis 1, which indicated a correlation with rhizophore water content ($r > 0.5$) and 1-FEH activity (1-FEH) ($r > 0.7$). Conversely, on the negative side of axis 1, distal segments at S2, distal segments at ED and all the samplings of the vegetative phase were correlated with average temperature (AT) ($r > -0.6$), average precipitation (AP) ($r > -0.58$), water content (H₂O AO), ABA ([ABA] AO) and IAA ([IAA] AO) concentrations in aerial organs and 1-SST activity (1-SST) ($r > -0.7$). Concerning axis 2, the proximal segments at the vegetative phases, and all samplings of S2 and S3 were correlated with ABA and IAA concentrations in aerial organs ($r > 0.58$) and average temperature ($r > 0.6$), and also water content in aerial organs ($r > 0.5$), while ED and D were correlated with fructo-oligosaccharide contents ($r > -0.6$), and average precipitation ($r > -0.6$). Consequently, the PCA axes showed the relationship of environmental and endogenous variations and the phenological phases of *C. obovata*.

DISCUSSION

This work describes for the first time changes in fructan metabolism and in endogenous concentrations of ABA and IAA associated with climatic conditions and water status during the developmental cycle of a Cerrado species growing naturally in the field. We hypothesized that endogenous concentrations of hormones have a regulatory role in determining the transition between different phenological phases and in the induction of changes in fructan metabolism under field conditions.

Changes observed at ED suggest an increase in sink strength of the rhizophore at this stage, evidenced by the increase in 1-SST and 1-FFT activities and in fructo-oligosaccharides, mainly in distal segments. Since the reserve tissues accumulate high fructan contents, the increase in IAA in these tissues might be an important factor for cell expansion, necessary for the

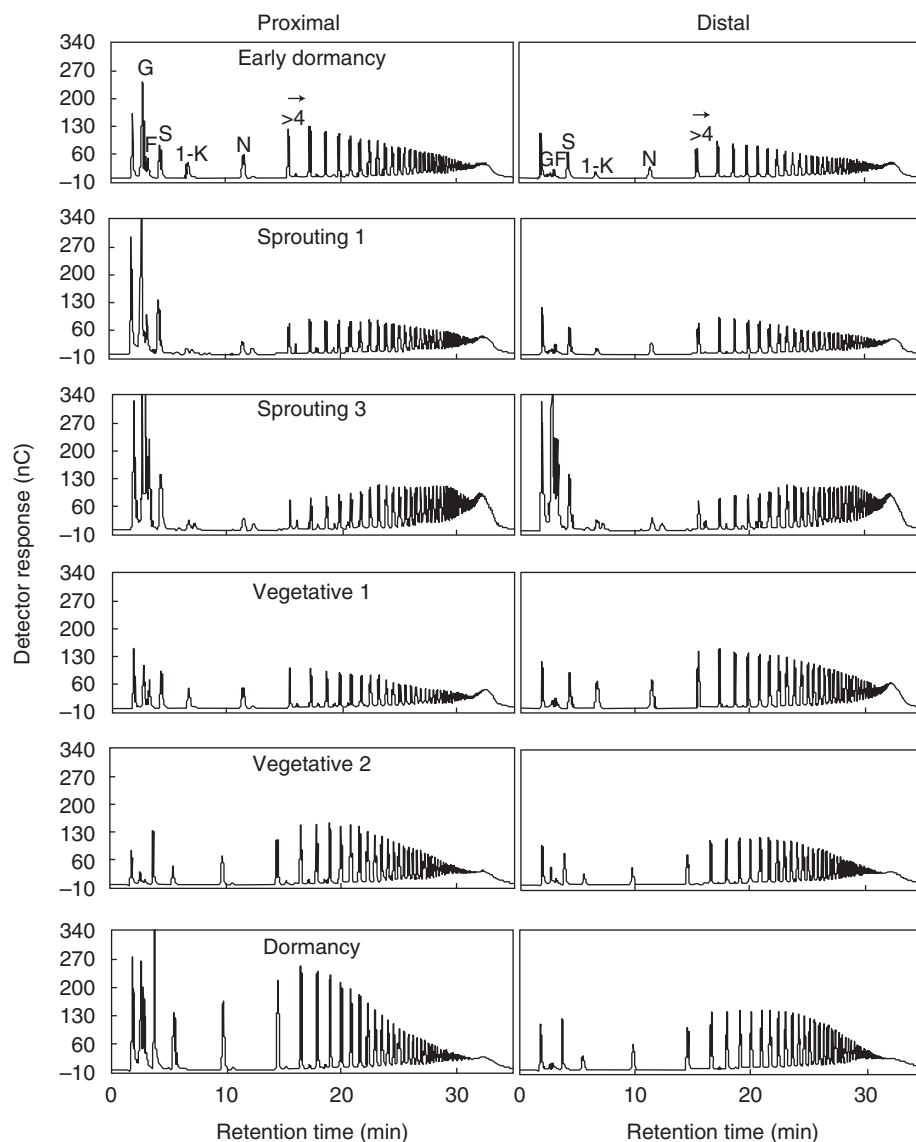


Fig. 5. HPAEC/PAD profiles of total fructans of proximal (left) and distal (right) rhizophore segments of plants of *Chrysoleaena obovata* at different phenological phases. G, glucose; F, fructose; S, sucrose; 1-K, 1-kestose; N, nystose; >4, fructans with DP higher than 4.

accumulation of reserve compounds. At this stage, photoassimilates from the senescing aerial organs are translocated to the underground reserve organ and used as substrate for fructan synthesis in a similar way to that reported for plants of the same species with fully established aerial organs (Portes and Carvalho, 2006) or under high atmospheric CO₂ concentration (Oliveira *et al.*, 2010) whose assimilated carbon, in excess for the aerial organs, is translocated downwards.

Following senescence and abscission of aerial organs at the dormant stage, sprouting of new shoots occurred at the end of winter, coinciding with the increase in temperature and a slight and gradual increase in precipitation (S1). At this stage, ABA concentrations were low, allowing sprouting of new shoots, similar to the report of Chope *et al.* (2006) for sprouting bulbs of *A. cepa*. The higher 1-FFT activity may have promoted a reduction of fructan chain sizes, thus facilitating hydrolysis by 1-FEH to supply the energy required for sprouting at S1 by the

mobilization of fructo-oligosaccharides ($r = -0.79$, $P < 0.05$). The increase in the proportion of hexoses and sucrose, associated with the increase in 1-FEH activity and expression, confirm the proximal region as the main site of fructan hydrolysis during sprouting as previously reported for this species (Asega and Carvalho, 2004; Portes and Carvalho, 2006; Asega *et al.*, 2011).

As sprouting and shoot growth progressed (S2), the expansion of photosynthetically active tissues was accompanied by a high IAA content. The low reducing sugar content observed in the rhizophores in the initial phases of sprouting (S1, S2) indicates a fast translocation towards the growing aerial organs. When the aerial organs are fully established (S3), the need for carbon supply decreases and accumulation of reducing sugars is detected, suggesting the transition of rhizophores from source to sink organs. This sink function is in accordance with the high IAA content in the rhizophores, probably acting in cell

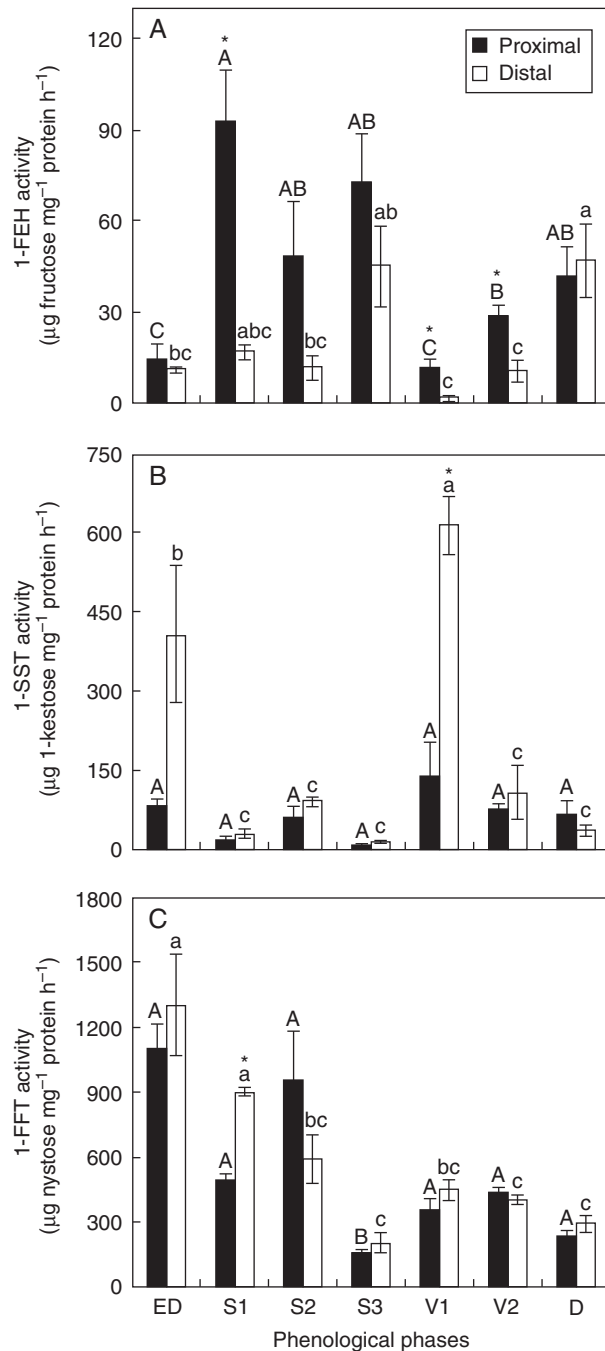


FIG. 6. 1-FEH (A), 1-SST (B) and 1-FFT (C) activities in the proximal and distal rhizophore segments (see key) of plants of *Chrysolaena obovata* at different phenological phases. Values are the means \pm s.e. ($n = 3$). Upper case letters indicate a comparison of proximal segments and lower case letters indicate a comparison of distal segments during the experimental period ($P < 0.05$). An asterisk (*) denotes a comparison of the segments (proximal and distal) within each harvest date ($P < 0.05$).

expansion necessary for reserve accumulation, as shown for tubers of *Helianthus tuberosus* (Schubert and Feuerle, 1997). The increase in fructan synthesis was also detected in callus of *Viguiera discolor* (Itaya et al., 2005) and in plants of *A. tequilana* (Barreto et al., 2010) supplied with auxin *in vitro*, giving

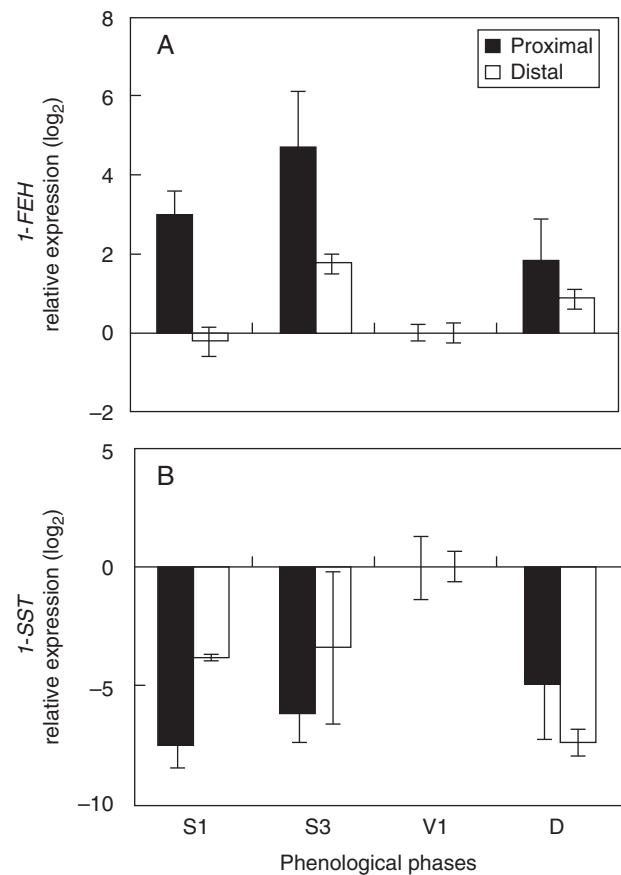


FIG. 7. Relative expression levels (log₂) of 1-FEH (A) and 1-SST (B) as assessed by qRT-PCR analysis in the proximal and distal rhizophore segments (see key) of plants of *Chrysolaena obovata* at different phenological phases (compared with the vegetative phase). Values are means (\pm s.e.) of two biological replicates with three technical replicates.

support to the hypothesis of a relationship between this growth regulator and fructan metabolism.

Specific primers for relative gene expression analyses were designed on the sequence of 1-FEH from *C. obovata* for which the activity of inulin hydrolysis was confirmed (Asega et al., 2008). In the present study, higher transcript accumulation detected in rhizophores during natural sprouting in the field is consistent with the increase in 1-FEH activity, suggesting a transcriptional regulation of 1-FEH, as previously observed for induced sprouting conditions (Asega et al., 2011).

The increase in sucrose and reducing sugars at the end of sprouting induced the reduction of 1-FEH activity at the beginning of the vegetative phase (V1), by feedback regulation. This may have triggered fructan biosynthesis (Pollock and Cairns, 1991), through the downward translocation of photoassimilates from the established aerial organs with the subsequent increase of sink capacity of the rhizophores and 1-SST activity and expression, correlated with precipitation ($AP \times 1-SST = 0.87$, $P < 0.05$). Accordingly, when supplied to plants of *C. obovata* cultivated *in vitro*, sucrose, glucose and fructose inhibited 1-FEH and promoted 1-SST gene expression (F. Trevisan et al., Instituto de Botânica, São Paulo, Brazil, pers. comm.)

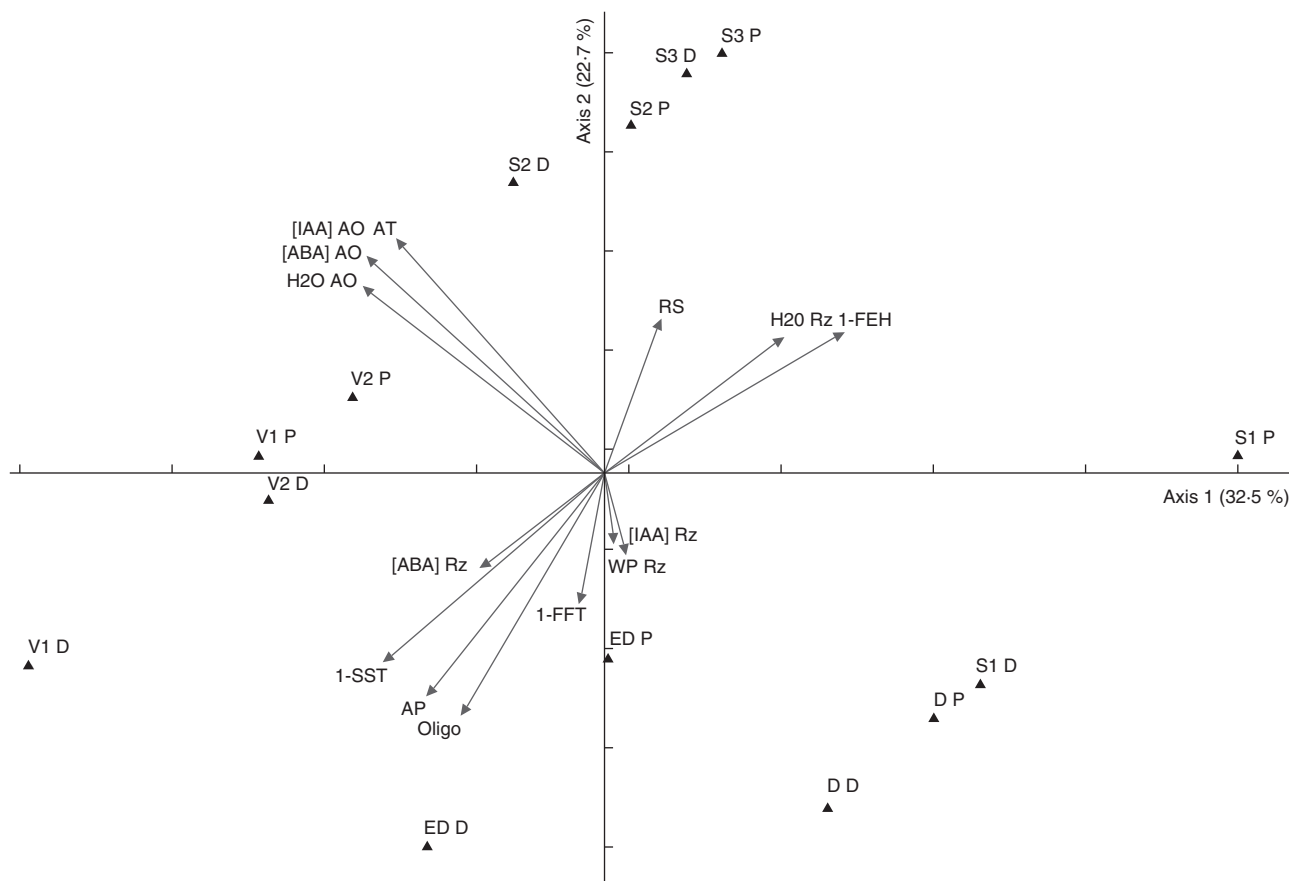


Fig. 8. PCA bi-plot of *Chrysoleaena obovata* at different phenological phases and environmental and endogenous parameters. Water content in aerial organs (H2O AO) and rhizophore (H2O Rz), water potential in rhizophore (WP Rz), temperature (AT) and precipitation (AP) average, ABA contents in aerial organs ([ABA] AO) and rhizophore ([ABA] Rz), IAA contents in aerial organs ([IAA] AO) and rhizophore ([IAA] Rz), fructo-oligosaccharide (Oligo) and reducing sugar (RS) contents, and 1-FEH, 1-SST and 1-FFT activities. Abbreviations for phenological phases are followed by P for proximal, and D for distal rhizophore segments.

Opposing activities and expression profiles of 1-SST and 1-FEH ($r = -0.9$, $P < 0.05$) were detected, providing evidence that plants growing naturally in the Cerrado present a similar behaviour in this respect to that observed in cultivated plants (Asega and Carvalho, 2004; Portes and Carvalho, 2006; Oliveira *et al.*, 2010), supporting the hypothesis of a temporal control of fructan-metabolizing enzymes in the vacuole, under the regulation of sucrose, which activates 1-SST (Wagner and Wienkem, 1986) and inhibits 1-FEH (Marx *et al.*, 1997).

The marked decrease in 1-SST activity towards V2, also detected by Portes and Carvalho (2006) throughout the vegetative stage, is accompanied by an increase in sucrose content and higher concentrations of ABA. Gusta *et al.* (2005) suggested a relationship between increasing concentrations of ABA and sugar concentration to confer cold tolerance, and refer to studies in which high concentrations of glucose and/or sucrose are associated with high concentrations of ABA. In the present study, V2 was accompanied by low temperature and precipitation, with a decrease in water potential (Ψ_w) of the rhizophore, and the signal to maintain the water status of the plants under low water availability may have been provided by ABA.

Environmental and physiological changes observed at this stage signalled the beginning of dormancy (D), marked by an increase in 1-FEH activity, and a higher fructo-oligo:fructo-polysaccharide

ratio and water content in rhizophores, all of these converging to osmoregulation, drought and cold tolerance, and energy supply.

Temperature and precipitation values measured throughout the year matched the expected indexes for the Cerrado biome, especially for the studied area, with low precipitation during winter, reflected in low soil moisture and a high temperature range (Vuono *et al.*, 1986; Coutinho, 2002). However, the variation of water availability during the year was not sufficient to promote changes in plant water status, in contrast to what was observed in field experiments with *Dactylis glomerata* L. and *Lolium perenne* L. (Volaire *et al.*, 1998) and in potted plants of *C. obovata* (Garcia *et al.*, 2011) and *V. discolor* (Oliveira *et al.*, 2013) under water withholding. It is important to point out that rhizophores of potted plants dry out faster than in natural field conditions, due to the limited volume of soil surrounding it. Our results suggest that in the Cerrado, plants of *C. obovata* present strategies to maintain the water status during the dry season, similar to what was detected in plants of *Gomphrena marginata* Seub. in field conditions (Silva *et al.*, 2013).

Recent works suggest that auxin is also involved in tolerance to drought and cold stress; however, the precise role of this phytohormone in abiotic stress responses remains largely unknown. As recently shown by Shi *et al.* (2014), transgenic lines of

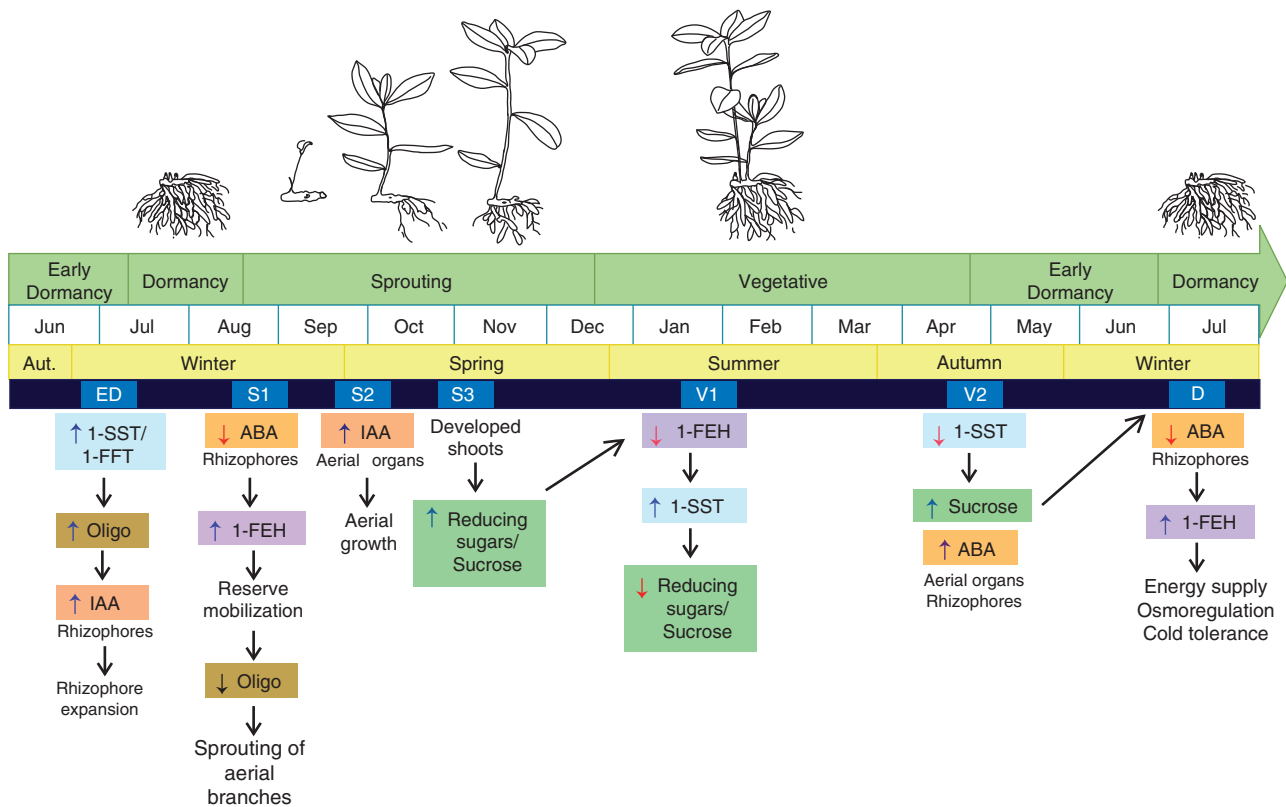


Fig. 9. Diagram representing the main phenological phases of *Chrysolaena obovata* and the concurrent physiological and biochemical changes observed.

A. thaliana with higher endogenous auxin concentrations show enhanced resistance to drought, by positively modulating some ABA-responsive genes and the accumulation of different metabolites with an osmotic function such as amino acids, organic acids, sucrose and other carbohydrates. The results reinforce the cross-talk between these two phytohormones in response to abiotic stresses, previously reported by Du *et al.* (2012). These authors showed that overexpressing a gene involved in the maintenance of IAA homeostasis in rice resulted in reduced ABA concentrations and increased resistance to cold stress. The maintenance of IAA homeostasis in aerial organs and rhizophores throughout the phenological cycle and the decline of ABA concentrations in the transition from vegetative to dormant phases, the coldest and driest season in the Cerrado, suggest that both hormones may be essential for *C. obovata* to cope with the climatic conditions during winter.

The PCA confirms what was pointed out previously, indicating the influence of 1-FEH in sprouting, especially in S1, and 1-SST in vegetative periods, as well as their opposing activities. The relationship found between temperatures, ABA, IAA and water contents in aerial organs with phases in which plants presented developing or well-established aerial organs (including ED) was opposite to that in which plants did not have aerial organs and temperatures were lower, such as dormancy and the beginning of sprouting. The association between ABA in the rhizophore and early dormancy, in spite of the high 1-SST activity, indicated the entry of plants in dormancy.

The diagram presented in Fig. 9 summarizes the phenological phases of *C. obovata*, its mechanisms of adaptation and the

strategies to endure abiotic stresses prevailing in the Cerrado. *Chrysolaena obovata* is able to up- and downregulate fructan metabolism in response to environmental changes, enabling plants to undergo unfavourable periods. Endogenous hormone concentrations seem to be related to regulation of fructan metabolism and to the transition between phenophases, signalling for energy storage, reserve mobilization and accumulation of oligosaccharides as osmolytes. In addition, variation in hormonal contents could directly contribute to tolerance to abiotic stresses, modulating pathways other than just those related to carbohydrates, not accessed in this study. Temperature and photoperiod could be other signals for the transition between phenophases; however, other environmental factors, such as radiation levels and relative air humidity, must also be considered in future studies.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxford-journals.org and consist of the following. Figure S1: image of *C. obovata*, highlighting the proximal and distal regions of rhizophores. Figure S2: soil water retention curves. Table S1: list of primers used for qRT-PCR.

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