Mycorrhiza

Spring stomatal response to vapor pressure deficit as a marker for desert truffle fruiting

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Abstract:	The cultivation of desert truffle Terfezia claveryi using Helianthemum almeriense as a host plant has recently become a solid alternative crop in the Mediterranean region due to its adaptation to arid and semiarid ecosystems, which are expected to increase during the following years because of climate change. However, management models are still being developed in order to improve and stabilize the production, which varies greatly from one year to another. According to gatherers and farmers, one of the key factors for desert truffle production is the plant phenology in spring which, in turn, depends on environmental conditions. In this manuscript we have characterized the physiological, morphological and molecular responses of the mycorrhizal plants in spring, coinciding with the fructification period of the plant and fungal species. Thanks to this characterization, a sigmoidal relationship between stomatal conductance and vapor pressure deficit (VPD) was found, that can be used as a marker of plant phenological switch. In order to confirm that this phenology status is related to desert truffle fructification, this marker has been successfully correlated to total truffle production. The results of this manuscript suppose a big step forward that will help to develop management models for the desert truffle crop.

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Dear Dr Colpaert,

Thank you very much for improving the reading and understanding of our manuscript "Spring stomatal response to vapor pressure deficit as a marker for desert truffle fruiting" for its publication in *Mycorrhiza* journal.

I look forward to hearing from you soon.

Best regards,

Asun Morte

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Spring stomatal response to vapor pressure deficit as a marker for desert truffle fruiting

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Abstract

The cultivation of desert truffle *Terfezia claveryi* using *Helianthemum almeriense* as a host plant has recently become a solid alternative crop in the Mediterranean region due to its adaptation to arid and semiarid ecosystems, which are expected to increase during the following years because of climate change. However, management models are still being developed in order to improve and stabilize the production, which varies greatly from one year to another. According to gatherers and farmers, one of the key factors for desert truffle production is the plant phenology in spring which, in turn, depends on environmental conditions. In this manuscript we have characterized the physiological, morphological and molecular responses of the mycorrhizal plants in spring, coinciding with the fructification period of the plant and fungal species. Thanks to this characterization, a sigmoidal relationship between stomatal conductance and vapor pressure deficit (VPD) was found, that can be used as a marker of plant phenological switch. In order to confirm that this phenology status is related to desert truffle fructification, this marker has been successfully correlated to total truffle production. The results of this manuscript suppose a big step forward that will help to develop management models for the desert truffle crop.

Keywords

Ectendomycorrhiza; Desert truffle; *Terfezia*; Spring phenology; Vapor pressure deficit (VPD); Stomatal conductance.

Introduction

Helianthemum almeriense Pau is a drought-deciduous Mediterranean perennial shrub belonging to *Cistaceae* family. It usually appears in open dry, stony, limestone, mica or marl places with gypsum or sandy soils, at altitudes between 0 to 500 m. Its habitat is the southeast semiarid regions of the Iberian Peninsula, although it can also be found in the north of Morocco (Morte and Honrubia 1997).

Despair the inherent difficulties of the cultivation of edible mycorrhizal fungi (Hall et al. 2003), the emergence of the cultivation of the desert truffle *Terfezia claveryi* Chatin (*Pezizaceae*, *Ascomycota*) during the last 20 years has conferred an important ecological, economical and commercial value to *H. almeriense*, since it is used as the host of this mycorrhizal edible fungus (Morte et al. 2008, 2017). Desert truffles are represented by several genera of edible hypogeus ascomycetes that are adapted to extreme conditions on arid and semiarid areas (Kovács and Trappe 2014). Among these, *Terfezia* genus and particularly the species *T. claveryi* is one of the most studied and appreciated members, mainly because of its nutritional value (Murcia et al. 2002), and the possibility of its cultivation (Kagan-Zur and Roth-Bejerano 2008; Morte et al. 2008). It was the first desert truffle to be cultivated (Honrubia et al. 2001; Morte et al. 2008) and, since 1999, several orchards have been established using *H. almeriense* as a host plant, to produce *T. claveryi* ascocarps.

Desert truffle fructification usually occurs after 2-3 years of plantation and happens yearly since then, reaching average productions of 350-400 kg ha^{-1} after 6-7 years, although with high interannual fluctuations in production (Morte et al. 2017). Site suitability, season and framework of the plantation, management practices (Morte et al. 2017), biotic factors (Navarro-Ródenas et al. 2016), as well as agroclimatic parameters (Morte et al. 2012; Andrino et al. 2019), are the major limiting factors to obtain high and stable productions. With regard to agroclimatic parameters, precipitations during autumn, soil water potential from fall to spring (including winter) and precipitations plus temperature and vapor pressure deficit (VPD) in spring, are the major candidates to explain the interannual variations in *T. claveryi* production in Mediterranean orchards (Morte et al. 2012; Andrino et al. 2019). The most common dates for desert truffle collection are from February to May, but the first fructifications can occur as early as in December and the last fructifications as late as in June. According to the experience of gatherers and farmers, the total production is greatly related with the starting and ending dates of the collecting period, mild temperatures together with some soil moisture are needed for fructifications. Moreover, desert truffles co-occur with some phenological changes of the plant: *i.e.* the start of the fructification coincides with the blooming, while the end is related to flower disappearance and leaf senescence. In addition, Andrino et al. (2019) have recently found that mainly soil water potential and VPD are the most important factors that determine the production of desert truffle during spring.

Phenology is defined as the study of periodic events in the life cycles of living beings, as influenced by the environment. The annual phenology of *H. almeriense* is typical of other Mediterranean summer-deciduous or semi-deciduous shrubs (Nilsen and Muller 1981; Haase et al. 2000; Gulías et al. 2009) and consists of a vegetative period that lasts from autumn (bud breaking) to spring, blooming events that start at the end of winter and finish in spring and leaf senescence at the end of spring. Maximum photosynthesis is found in winter. Mycorrhizal colonization in the field is mainly intracellular at above 40% of mycorrhization average, except for summer, when few signs of mycorrhiza are found (Gutiérrez et al. 2003;

Morte et al. 2010; Navarro-Ródenas et al. 2015). Spring phenology of plants is closely related to climate change (Badeck et al. 2004; Cleland et al. 2007), especially for Mediterranean shrubs (Bernal et al. 2010; León-Sánchez et al. 2018). In fact, climate change is already pushing spring to occur earlier in the year (Corell 2005), and the forecasted changes for the Mediterranean regions point to increases in temperature and VPD and to a lower availability of soil moisture (NOAA 2015). It is, thus, necessary to provide new insights in the spring phenology of crops such as desert truffles, which are a good alternative crop for arid and semiarid areas.

We hypothesize that throughout the spring, there should be a generalized switch in the physiology of the mycorrhizal plant. This phenological switch must be explained by one or several environmental parameters that also change throughout the spring. If plant phenology is related somehow to desert truffle production, the environmental factors that cause this phenological switch should also be able to explain differences in desert truffle yield. The aim of the present work is to characterize the responses of the desert truffle plant *H. almeriense* to the environmental changes in spring. This work focuses on the search for morpho-physio-molecular parameters that could help us to easily track the changes in phenology that may be related to desert truffle yield. To achieve this goal, leaf anatomy, water relations, gas-exchange and expression of leaf rubisco and aquaporin (AQP) genes were analyzed. The increase of knowledge in these relationships could give us insight into the dynamics of desert truffle production.

Materials and methods

Experimental site and procedure

The experimental *H. almeriense* x *T. claveryi* site used for this study was located in Espinardo, Murcia (Spain) (38°01'20"N 1°10'00"W) at an altitude of 91 m. In May 2008, 40 mycorrhizal plants inoculated with *T. claveryi*, as explained in Morte et al. (2008), were transplanted at 1 x 1 m of distance one to another in a square pattern and were well-irrigated until February 2009 in order to ensure the proper establishment and initial development of the plants. Temperature (ºC), relative humidity (%) , VPD (kPa), radiation (W m^{-2}) and rainfall (mm) data were collected from an automatic weather station (Campbell Scientific Ltd, Shepshed, UK) sited on the experimental plot. Soil water potential (Ψ_{soil} expressed in kPa) was monitored using watermark sensors (Irrometer, Spain) located in four different spots of the orchard. Temperature and VPD surrounding the leaf were measured using a portable photosynthesis system (LI-6400, Li-Cor, Inc., Lincoln, NE, USA) simultaneously to the estimation of gas-exchange parameters.

Gas-exchange parameters, shoot water potential at midday (Ψ_{md}), leaf mass per area (LMA) and AQP and Rubisco gene expression were monitored during the spring of 2016 (April to June). Taking into account the large differences in meteorological conditions throughout the spring, and based on stomatal conductance (g_s) and Ψ_{md} regressions with VPD_{air} and Ψ_{soil}, respectively, threshold values of VPD_{air} and Ψ_{soil} were established, and spring season was divided between the first and second half of spring. For the experimental period, all data collected before May 8th were considered to belong to early spring, those collected after May 9th belong to late spring. No irrigation was applied to the experimental site during the period of study.

Gas-exchange parameters and shoot water potential at midday (Ψ_{md})

Leaf gas exchange parameters: net assimilation rate (A_N), g_s, transpiration rate (E) and intracellular carbon (C_i), were estimated during twelve weeks from April to June 2016, every two days, in six plants. They were measured in the morning from 9 am to 1 pm and then in the afternoon from 2 pm to 7 pm. Leaf gas exchange was estimated with a portable photosynthesis system (LI-6400xt, Li-Cor, Inc., Lincoln, NE, USA), on fully expanded leaves that were placed in a 2 cm^2 leaf cuvette. Due to the small leaves of this species, two or three of them were placed carefully in the cuvette and after the measurements, they were collected to measure their area using an Olympus SHZ stereomicroscope coupled to an Olympus DP73 camera. Area calculation was made using the image analysis software cellSens v1.14 (Olympus, Japan). Intrinsic water use efficiency ($_i$WUE) was calculated as the ratio between A_N and g_s. Measurements were taken at an air flow of 200 µmol s^{-1}, a controlled CO_2 concentration of 390 µmol s^{-1} achieved using the injection system and compressed CO_2 cylinders, at saturating light or PPDF (photosynthetic photon flux density) of 1500 µmol m^{-2} s^{-1} and at ambient air temperature and relative humidity. Throughout the same week, measurements were taken at different times to obtain data from all the different time periods and, therefore, to obtain a weekly diurnal pattern of gas-exchange parameters.

For Ψ_{md} measurements, five cm-long plant apices from the same six plants that were used to measure gas-exchange parameters were cut at noon and immediately placed in a pressure chamber (Soil Moisture Equipment Co; Santa Barbara, CA, U.S.A.), according to Scholander et al. (1965).

Leaf mass per area (LMA)

Once every two weeks, three leaves per plant from the second and third node from the apex, from six plants, were cut and their area without petiole was measured as explained in section 2.2. Afterwards, leaves were dried at 60 ºC for 72h and their dry weights were measured. LMA was calculated as the ratio of dry weight per area (g m^{-2}).

RNA extraction and quantitative real-time PCR

Once per week in the morning and once in the afternoon, after gas-exchange measurements, leaves from six plants were collected and immediately frozen in liquid nitrogen. 100 mg of frozen leaf material was homogenized in liquid nitrogen with the help of mortar and pestle. RNA was extracted with the CTAB method according to Chang et al. (1993). cDNA was synthetized by Reverse-Transcription Polymerase Chain Reaction (RT-PCR) from 0.5 µg of total RNA from each sample using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), following manufacturer´s instructions.

Expression of five *H. almeriense* AQPs (*HaPIP1.1*, *HaPIP1.2*, *HaPIP2.1*, *HaPIP2.2*, and *HaTIP1.1*) previously characterized (Navarro-Ródenas et al., 2013) and *H. almeriense* ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*HarbcL*) was studied by real-time PCR using a QuantStudioTM 5 Flex (Applied Biosystems, Foster City, California, USA). Primers described in Navarro-Ródenas et al. (2013) were used for the five *H. almeriense* AQPs. Primers used for quantification of *HarbcL* were designed for this study using *H. almeriense rbcL* gene sequence deposited in GenBank (FJ492025.1) by Guzmán and Vargas (2009). *HarbcL* primers (Table S1) were designed using http://www.idtdna.com

and tested to prove their specificity and efficiency. Each 15 µl of reaction medium contained 1.5 µl of 1:10 cDNA template, 0.11 µl of primer mix 5 µM each and 7.5 µl of SyBR Green Master Mix (Applied biosystems, Foster City, California, USA). The PCR program consisted of 10 min incubation at 95 °C, followed by 40 cycles of 15s at 95 °C and 1 min at 60 °C, where the fluorescence signal was measured.

The efficiency of the primer sets was evaluated by performing real-time PCR on several dilutions of cDNA. Real-time PCR threshold cycle (Ct) was determined in triplicate. 2-ΔΔCt method was used to evaluate the expression of each gene (Livak and Schmittgen 2001) normalizing gene expression to the geometric mean of *H. almeriense ATP synthase* (AF035907.1, GenBank) and *H. almeriense 18S* (Navarro-Ródenas et al. 2013) levels (Table S1). These genes have been confirmed as suitable reference genes under different conditions using the geNorm algorithm, included in qbase+ software, version 3.0 (Biogazelle, Zwijnaarde, Belgium (www.qbaseplus.com). Real-time qPCR experiments were carried out in six separate biological samples and non-template controls were performed in all reactions.

Relation between desert truffle yield and VPD

From spring 2001 to spring 2019, all truffles harvested from an experimental site located in Zarzadilla de Totana, Murcia, Spain (37°52'15.5"N 1°42'10.5"W) at an altitude of 870 m (more details in Andrino et al. 2019), were weighed and total production was expressed as fresh weight per hectare (kg ha^{-1}). These data were analyzed together with environmental data collected from the nearest meteorological station located in La Paca (Lorca, Spain IMIDA LO41, 37º51'18.7"N 1º49'6.6"W, http://siam.imida.es). In order to highlight long-term trends and to identify the day of the year in which the VPD stably reaches a certain threshold of VPD (DOY_{VPDt}), the simple moving sum of seven days for VPD was calculated. Linear regression analysis between production and DOY_{VPDt} and student's t test between years with below average production and years with above average production (average production of 355 kg ha^{-1} $year^{-1}$, according to Andrino et al. 2019) were performed.

Statistical analysis

Student's t test, analysis of variance ANOVA, Tuckey's post-hoc tests and Pearson´s correlation analysis were performed using R programming language with Rstudio software v1.1.456. Data plotting, regression analysis and model fitting were carried out using Sigmaplot v 10.0 (Systat Software, UK).

To visualize the dissimilarities between early and late spring, principal coordinate analysis (PCoA) calculated on an Euclidean dissimilarity matrix of plant physiological parameters (net assimilation rate, stomatal conductance, transpiration, intercellular CO_2 concentration, midday shoot water potential, intrinsic water use efficiency, and the expression of aquaporins and rbcL) was performed. To confirm the effect of the factors, a permutational multivariate analysis of variance (perMANOVA) and an analysis of similarities (ANOSIM) were conducted on the same Euclidean matrix, using the adonis function of the vegan package (Oksanen et al. 2007), and 999 permutations.

Results

Gas-exchange parameters, Ψ_{md} and LMA responses

VPD, radiation and precipitation profiles from spring 2016 were typical of Mediterranean regions, with a characteristic increase in average air VPD during spring. Precipitations were scarce during the study period, having minimum values in late spring and at the beginning of summer (Fig. S1). When analyzing Pearson's correlation table between environmental and plant physiological parameters, several correlations appeared, with the gas-exchange parameter g_s being most strongly correlated with environmental parameters during spring (Fig. 1). Correlations coefficients between g_s and VPD_{air} (-0.63, $p < 0.001$) and VPD_{leaf} (-0.88, $p < 0.001$), were higher than those with Ψ_{soil} (0.53, $p < 0.01$). Ψ_{md}, showed its main correlation with Ψ_{soil} (0.82, $p < 0.001$), while no correlations were found with VPD_{air} (-0.44, no significance) (Fig. 1).

A sigmoid regression following Eq. 1 proved to be the best fit for relations between g_s with VPD_{air} and Ψ_{md} with Ψ_{soil} (Figs. 2a, 2b). For both regressions, the points where g_s or Ψ_{md} starts a linear decrease, where it achieves its minimum and the inflection point can be estimated thanks to the sigmoid fit. When average VPD_{air} increased from 0.8 to 1.1 kPa, a sudden linear decrease of stomatal conductance occurred from 0.25 to 0.03 mol m^{-2} s^{-1}, and an inflection point of 0.93 kPa was calculated (Fig. 2a). A similar behavior was observed with Ψ_{md}, as at Ψ_{soil} between -50 and -80 kPa approximately, Ψ_{md} experimented a sudden linear decrease from -1,4 MPa to -2,4 MPa, with an inflection point at -65 kPa (Fig. 2b). The inflection points were used to establish threshold values of VPD and Ψ_{soil} and to separate the data into two groups: early and late spring.

$$f = \frac{yo + a}{\left(1 + exp\left(-\frac{x - xo}{b}\right)\right)}$$

Eq. 1. Sigmoid regression from g_s *vs* VPD_{air} and Ψ_{md} *vs* Ψ_{soil}.

In order to reveal which parameter explained best the overall differences in plant performance throughout spring, we performed PCoA and perMANOVA analyses grouping the data based on VPD threshold (VPDt) (Fig. 2c) or on Ψ_{soil} threshold (Fig. 2d), respectively. For both cases, the two groups of data were significantly different, revealing a significant generalized switch in plant performance between early and late spring. In the case of VPDt based perMANOVA (Fig. 2c), better significance levels, ANOSIM values and no overlapping dispersion ellipses were found compared to Ψ_{soil} threshold based perMANOVA (Fig. 2d) and, for the following analyses, we used this threshold (VPD = 0.93 kPa) to differentiate between early and late spring measurements. When comparing A_N, g_s, E, $_i$WUE, Ψ_{md} and LMA average values between early and late spring, all the parameters were different (Table 1) and the A_N and g_s dairy dynamics also changed (Fig. 3).

Rubisco and AQP gene expression profiles

The expression profile of *HarbcL* over time changed from early to late spring in a similar way to the rest of parameters studied. A decrease in the expression level of *HarbcL* from early to late spring was observed, finding its minimum during the first week of June, when it had a 6-fold down-regulation, compared to the start date of the study (Fig. 4a). *HarbcL* and stomatal conductance showed almost parallel patterns during this period, as it is shown in Fig. 4a and their relationship fitted an exponential regression following Eq. 2, with an r^2 of 0.88 (Fig. 4b).

$$f = yo + a * (1 - exp(-b * x))$$

Eq. 2. Exponential fit from *HarbcL* relationship with g_s.

The expression pattern of four plasma intrinsic proteins (PIPs) and one tonoplastic intrinsic protein (TIP) was also studied during spring 2016. Almost no correlation was found between the expression pattern of all the AQPs analyzed and the rest of the parameters studied. When AQP expression data from the early and late spring were compared (Student's t test), no significant differences were observed, but interesting results came out from Pearson's correlations test. No correlations were observed in early spring, but all four *HaPIPs* were significantly correlated with environmental (VPD_{leaf}) and physiological (Ψ_{md}) parameters during late spring (Fig. S2). The increase in VPD in late spring resulted in *HaPIP1.1* and *HaPIP2.1* upregulation (Figs. 5a, 5c) and *HaPIP1.2* and *HaPIP2.2* downregulation (Figs. 5b, 5d). *HaTIP1.1* showed no correlation with any parameter neither in early nor in late spring (Fig. S2). *HaPIP1.1* and *HaPIP2.1* on the one hand, and *HaPIP1.2* and *HaPIP2.2* on the other, seemed to respond to the increase in VPD observed in spring as different clusters, and when their expressions were studied against each other, they showed linear regressions with r^2 higher than 0.62 in all the cases (Fig. S3).

Desert truffle production correlation to VPDt

A VPDt of 0.93 kPa was used as a marker for the general switch that occurs between early and late spring (Fig. 2a). A correlation analysis was performed between desert truffle production and the day of the year (DOY) at which VPDt was reached (DOY_{VPDt}). A significant positive correlation was found between DOY_{VPDt} and truffle production (Fig. 6) and the years with below average production showed significant earlier DOY_{VPDt} (April 30th) than the years with above average production (May 19th).

Discussion

Desert truffle *T. claveryi* cultivation was started in 1999 as a result of several years of research (Morte et al. 2008, 2017). Since then, its cultivation has grown exponentially and nowadays, *T. claveryi* cultivation is becoming an economical resource at regional, national and international level (Morte et al. 2017; Oliach et al. 2019). All these years of *H. almeriense* x *T. claveryi* association studies have led to a general knowledge about its phenology (Navarro-Ródenas et al. 2015; Morte et al. 2010, 2017) but detailed information about morpho-physio-molecular responses of the mycorrhizal plants during the spring (a key period of time for *H. almeriense* phenology and desert truffle fructification) was lacking.

Mediterranean springs represent the start of the dry season: temperature increases, relative humidity decreases and, consequently VPD increases. Furthermore, rainfalls are scarce during this season, resulting in low soil water availability. The results of the correlation analysis (Fig. 1), as well as regressions (Figs. 2a, 2b), statistically demonstrate that most of changes in physiological parameters can be explained by VPD and Ψ_{soil}, and therefore, these environmental parameters and their complex interactions, determine the responses of the plants to water-stress, as it happens in most Mediterranean plants in the dry season (Flexas et al. 2014). More importantly, we proved that in this species a generalized phenological switch occurs during spring and that the VPD is the main factor determining it, since the switch in plant parameters is better explained by VPD ($p = 0.001$) than by Ψ_{soil} ($p = 0.02$) (Figs. 2c, 2d).

With regard to LMA, the majority of deciduous and evergreen shrubs adapted to arid and semiarid conditions are considered as high-LMA species and, therefore, possess a conservative strategy (Poorter et al. 2009). The LMA increase in *H. almeriense* during spring (Table 1) could be considered as a response to drought and can play a role in the adaptation of this species to arid ecosystems, since it has been shown that decreases in water availability normally result in increases in LMA (Poorter et al. 2009). In several plant species, water-stress is also known to induce a decrease in Rubisco activity (Boyer 1976; Lawlor 1995; Flexas et al. 2006a; Abdallah et al. 2018); the mechanism of these decreases can vary from biochemical regulation to a direct regulation of Rubisco genes or indirectly, via regulation of Rubisco inhibitors (Flexas et al. 2006a; Parry et al. 2008). The transcriptional repression of *HarbcL* during the study period was highly correlated to stomatal closure (Fig. 4), reinforcing the idea of stomatal closure as the triggering event for the down-regulation of Rubisco through a decrease in the concentration of CO_2 in the chloroplast (Flexas et al. 2006a, 2006b), especially in Mediterranean species (Galmés et al. 2011). Normally, AQPs expression response to water-stress is difficult to analyze, since induction can be a way of increasing hydraulic conductance under stress conditions but repression could also be a way to minimize water loss (Alexandersson et al. 2005). The absence of correlations of AQP expression levels with VPD and Ψ_{md} in early spring and the subsequent later appearance of these correlations when environmental conditions become more stressful (Fig. 5) implies a fine-tune regulation (Lovisolo et al. 2007) of *H. almeriense* leaf AQPs, confirming in field conditions what was previously described in nursery conditions by Navarro-Ródenas et al. (2013). Only in late spring, *HaPIP1.1* and *HaPIP2*.1 increased its expression with VPD increase, while *HaPIP1.2* and *HaPIP2.1* were repressed when VPD increased. This means that AQPs are differentially regulated only in environmental restrictive conditions. Furthermore, it has been proven that *HaPIP1.1* increments CO_2 permeability (Navarro-Ródenas et al. 2013), so its induction in response to the increase in VPD increase could also be a way of facilitating the path of CO_2 through the mesophyll, therefore maximizing photosynthesis with reduced stomatal conductance. The co-expression of *HaPIP1.1* with *HaPIP2.1* and *HaPIP1.2* with *HaPIP2.2*, respectively (Fig. S3), could be an indicative of *PIP1-PIP2* heteromerization process that has been proposed to be necessary to their correct function (Yaneff et al. 2014; Bienert et al. 2018). It is also important to take into account that only five AQPs were analyzed within a family that presumably counts more than thirty members, so in order to fully understand the AQP response of the plant during spring, more AQPs should be identified and studied.

From all the gas-exchange parameters studied, we focused on the relationship of stomatal conductance with VPD in order to identify environmental and physiological markers that revealed the

generalized phenological switch that *H. almeriense* plants experienced. Stomatal conductance showed a strong correlation with VPD (Fig. 1), even stronger than with Ψ_{soil}, suggesting a major importance of VPD in stomatal closure regulation of *H. almeriense*, as reported for several species (Ohsumi et al. 2008; Aliniaeifard and Van Meeteren 2013). Moreover, the response of g_s to average VPD_{air} fitted a sigmoidal curve (Fig. 2a), which is similar to that found in different gravepine cultivars (*Vitis vinifera*) (Prieto et al. 2010), consisting of a maximum g_s at mild VPDs and a threshold value determining a linear decrease in g_s, until reaching a minimum value. Using the inflection point calculated in the sigmoidal adjustment from Fig. 2a**,** we determined an average VPD threshold of 0.93 kPa. In order to prove that desert truffle production is related to the generalized phenological switch, we compared desert truffle production from a 20-year-old orchard with the day of the year at which VPD reached the VPD threshold (DOY_{VPDt}). These regression analyses statistically demonstrate that this value (DOY_{VPDt}) explains part of the interannual differences in the production of desert truffle previously discussed by Andrino et al. (2019), and agree with the idea hinted by desert truffle gatherers and farmers who confirm that the years with early summers (early DOY_{VPDt}) are below average years in terms of total truffle yield.

In conclusion, mycorrhizal *H. almeriense* desert truffle plants present a generalized physiological, morphological and molecular response to the environmental transition from spring to summer, showing a clear phenological switch. By using the VPD-g_s relationship as a marker of the phenological state of the plant, the interannual fluctuations in desert plant production can be partially explained. This marker can be further used as a tool for plantation design (selecting potential places with historically late DOY_{VPDt}), plantation diagnosis and management through VPD control (*via* shadowing and/or water mist devices), and will allow future basic and applied research to fully understand the relationship between plant phenology and desert truffle production.

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Figure captions

Fig. 1 Pearson's correlation table between plant and environmental parameters during the experimental period. Different colors represent positive (blue) or negative correlations (red) and color intensity represents Pearson's correlation coefficient. Asterisks indicate statistical correlations. * = $p \leq 0.05$; ** = $p \leq 0.01$ and *** = $p \leq 0.001$.

Fig. 2 Threshold values of VPD and soil water potential (Ψ_{soil}), their relationship with stomatal conductance (g_s) and midday shoot potential (Ψ_{md}), respectively, and ordination of the data according to principal coordinate analysis. Each point represents the average of 1-week measurements. (**a**) Sigmoid regression between g_s and VPD_{air}. (**b**) Sigmoid regression between Ψ_{md} and Ψ_{soil}. (**c**) and (**d**) Principal coordinate analysis (PCoA) of *H. almeriense* morpho-physio-molecular variables separating the points into early (blue circles) and late (red triangles) spring according to VPD threshold (**c**) or Ψ_{soil} threshold (**d**). Ellipses were drawn according to the standard deviation and perMANOVA and ANOSIM tests were performed using the same Euclidean dissimilarity matrix used for PCoA.

Fig. 3 *H. almeriense* net assimilation and stomatal conductance diurnal patterns in early and late spring. Each point represents the average for a certain time lapse in early (circles) and late (triangles) spring measurements and bars represent standard error.

Fig. 4 Rubisco expression relationship with vapor pressure deficit (VPD) and stomatal conductance (g_s). (**a**) g_s values and *HarbcL* expression levels expressed as log2 fold-change during the course of spring 2016, where 1, 2, 3, 4 are respectively the first, second, third and fourth weeks in a month. (**b**) Exponential relationship between g_s and *HarbcL* expression levels, every point represents the average of 1-week measurements.

Fig. 5 Relationship and linear regression between VPD and *HaPIP1.1* (**a**), *HaPIP1.2* (**b**), *HaPIP2.1* (**c**) and *HaPIP 2.2* (**d**) expressions in early and late spring 2016. Each point represents a single measurement (three biological and technical replicates) in early (circles) or late (triangles) spring.

Fig. 6 Desert truffle production from 2001 to 2019 compared to the day of the year when a vapor pressure deficit (VPD) threshold of 0.93 kPa is reached. Each point represents the total production in a single year.

Fig. S1 Vapor pressure deficit (VPD), soil water potential (Ψ_{soil}) and precipitations (Prec) evolution during the period of study.

Fig. S2 Correlation between AQP expression, vapor pressure deficit surrounding the leaf (VPD_{leaf}) and midday shoot water potential (Ψ_{soil}) in early and late spring. Asterisks indicate statistical correlations. * = $p \leq 0.05$; ** = $p \leq 0.01$ and *** = $p \leq 0.001$.

Fig. S3 Gene expression of the studied leaf HaPIPs and their reciprocal correlation in spring. (**a**) *HaPIP1.1 vs HaPIP 2.1.* (**b**) *HaPIP1.2 vs HaPIP2.2.* (**c**) *HaPIP1.1 vs HaPIP2.2.* (**d**) *HaPIP1.2 vs HaPIP2.1.*

Table 1

Table 1 Plant parameters in early and late spring. Mean of net assimilation (A_N), stomatal conductance (g_s), transpiration (E), intrinsic water use efficiency ($_i$WUE), midday shoot water potential (Ψ_{md}), and leaf mass per area (LMA) ± standard error are represented. Different letters represent significant differences according to Student's t-test ($p < 0.05$).

	A_N ($\mu mol\ m^{-2}\ s^{-1}$)	g_s ($mol\ m^{-2}\ s^{-1}$)	E ($mmol\ m^{-2}\ s^{-1}$)	$_i$WUE ($\mu mol\ mol^{-1}$)	Ψ_{md} (MPa)	LMA ($g\ m^{-2}$)
Early Spring	6.99 ± 0.64a	0.133 ± 0.020a	3.04 ± 0.3a	70.64 ± 7.7a	-1.65 ± 0.07a	170.11 ± 7a
Late Spring	3.14 ± 0.54b	0.023 ± 0.003b	1.21 ± 0.2b	134.26 ± 9.5b	-2.37 ± 0.08b	244.48 ± 10b

Figure 1

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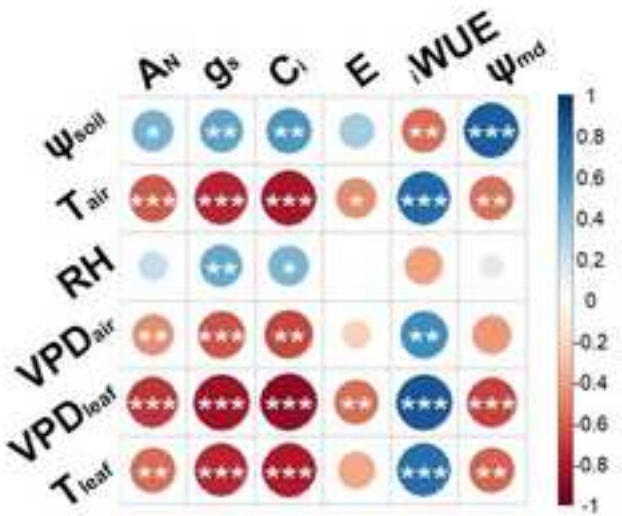

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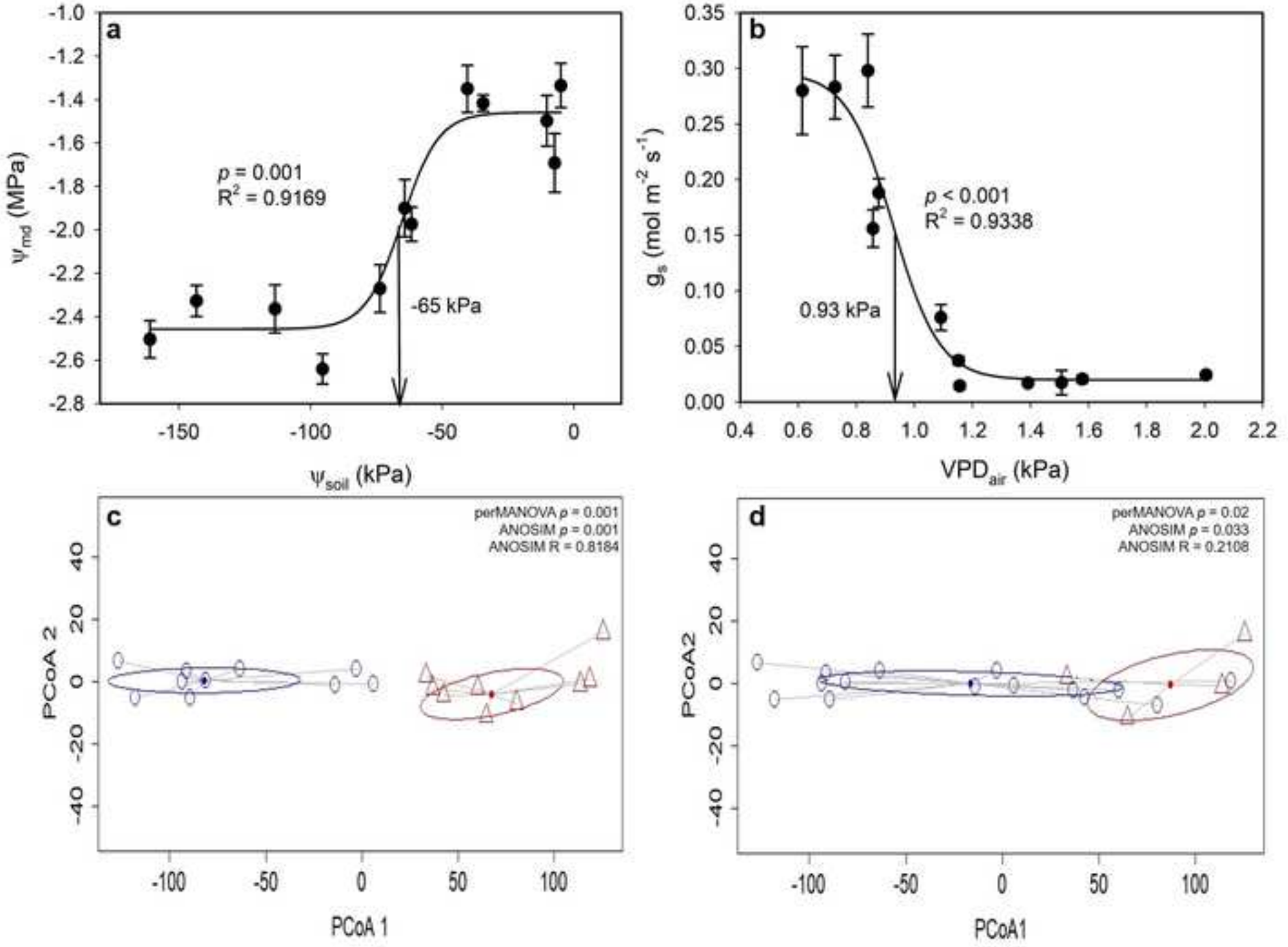

Figure 3

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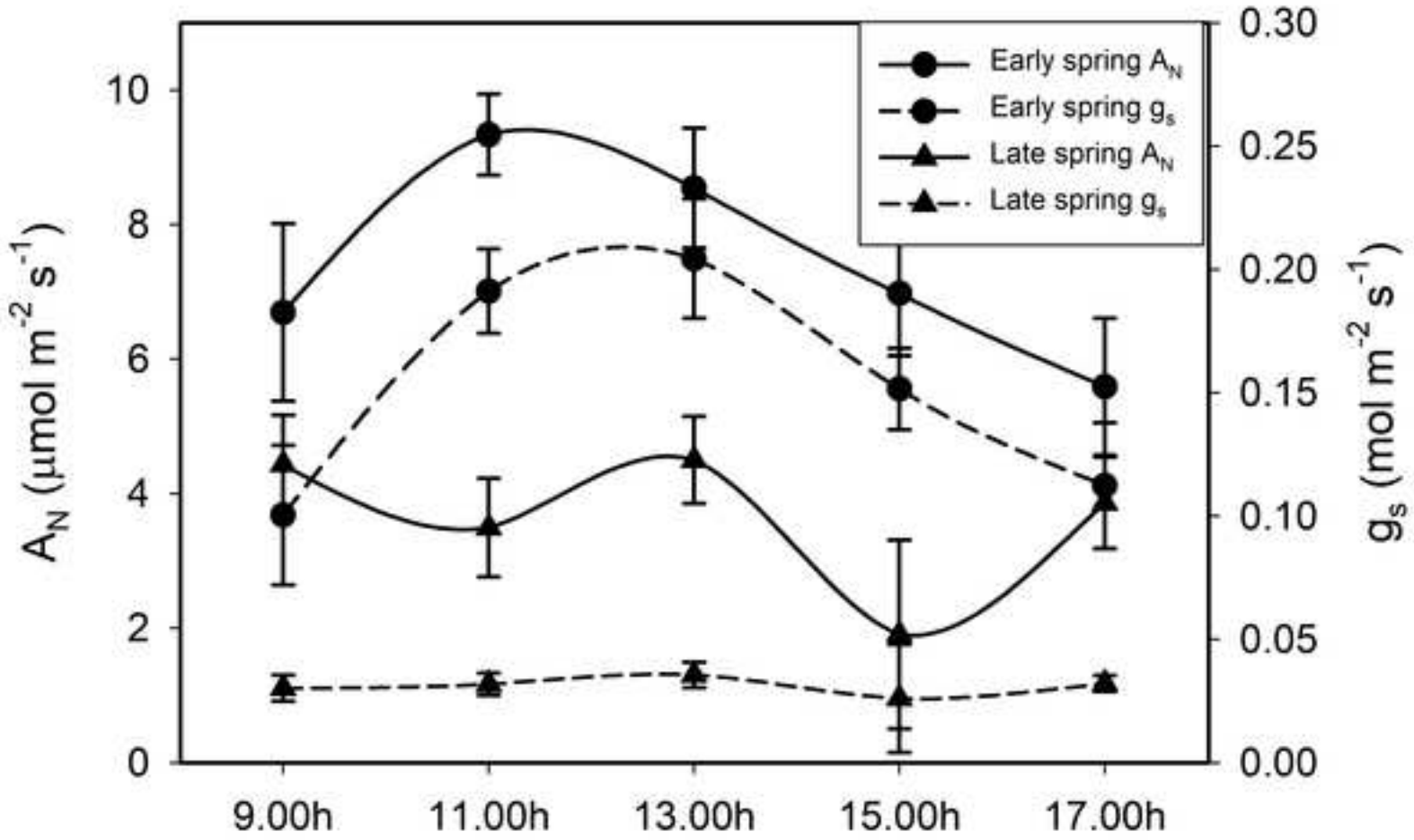

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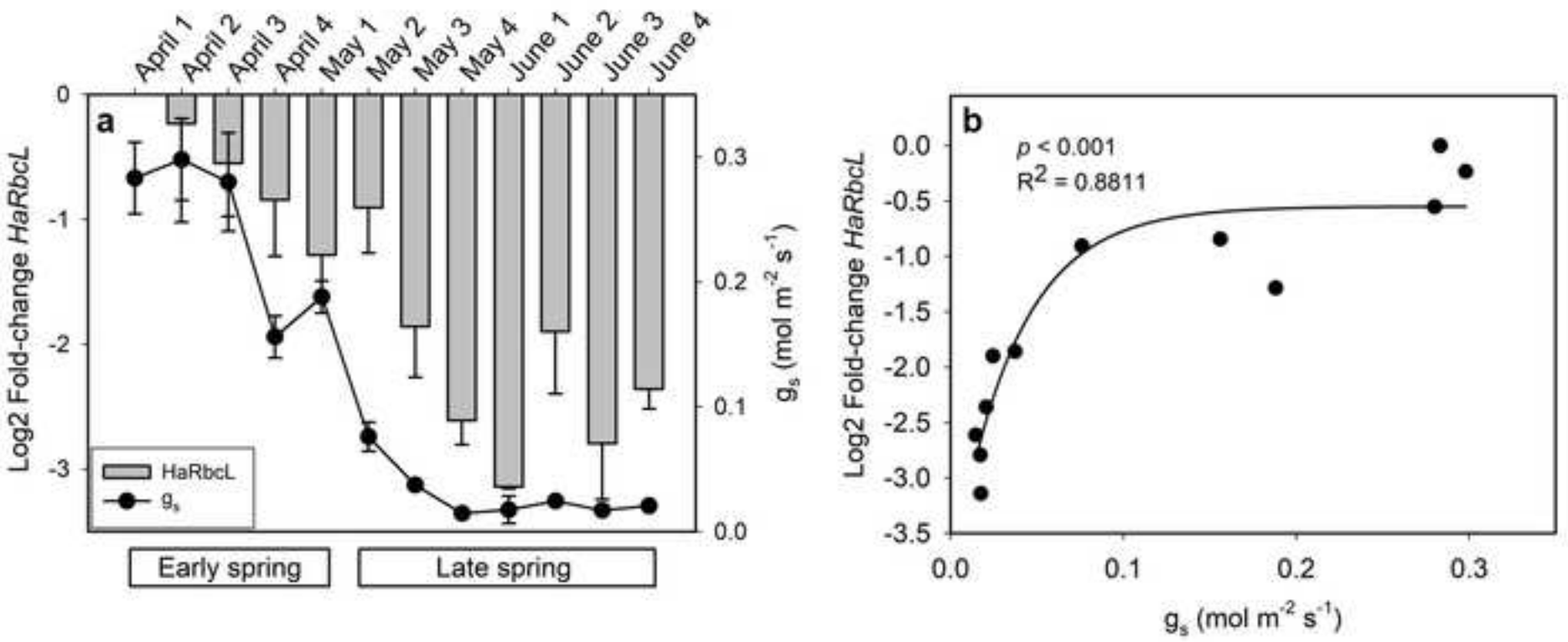

Figure 5

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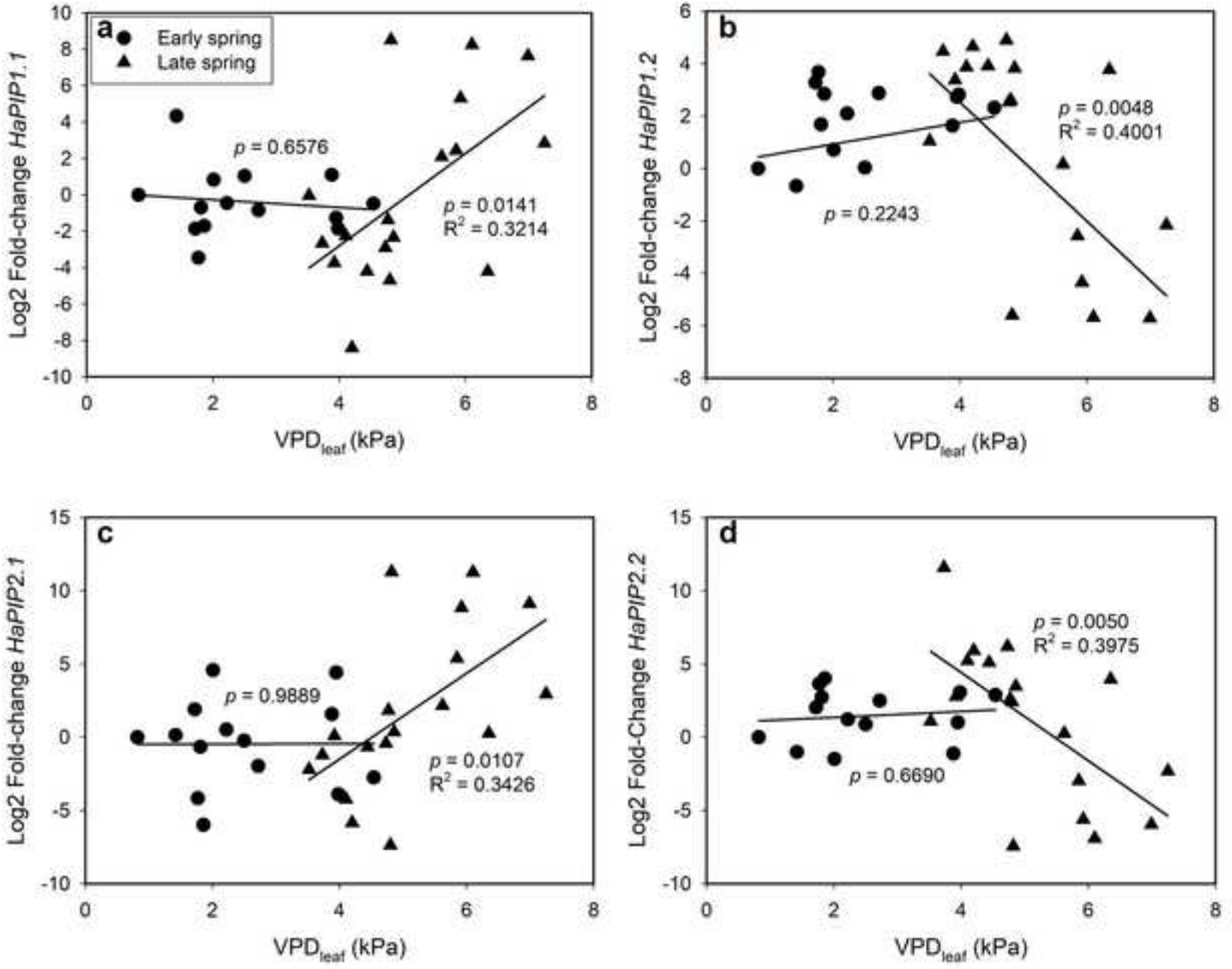

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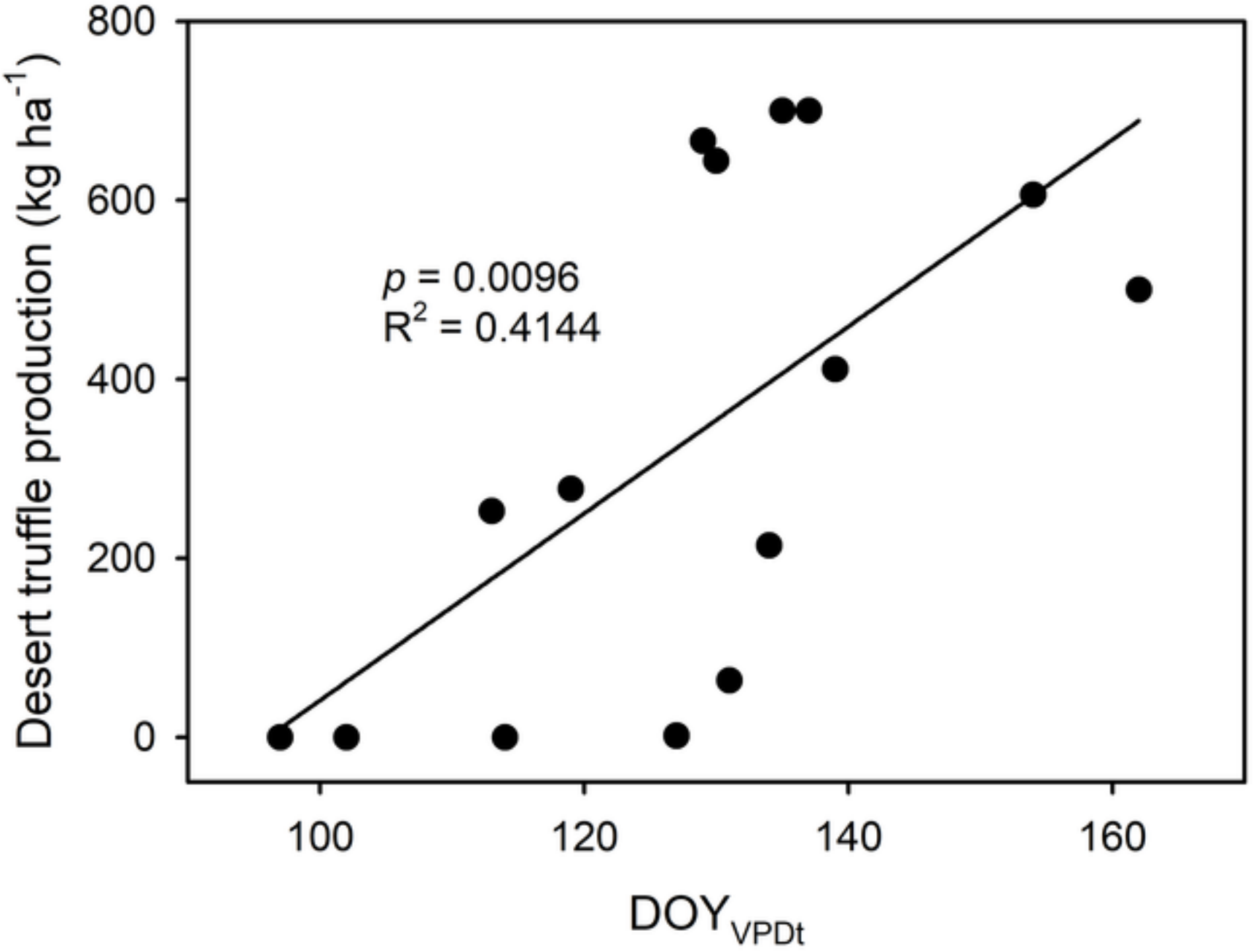

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