Physiological response in beans of three cacao (*Theobroma cacao* L.) cultivars to micro-environmental growing conditions in cacao agroforestry systems and monocultures under conventional and organic management

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Abstract

Cocoa beans are produced all across the humid tropics under different environmental conditions provided by the region but also by the type of production system and the season. Among other ecosystem services, agroforestry systems and organic farming differ from conventional monocultures in their soil quality, i.e. water holding capacity and enhanced nutrient cycling that can affect crop nutrient uptake and their molecular characteristics. Additionally, agroforestry systems provide a buffered microclimate, implying that environmental stressful conditions and strong variation in the course of the year can be reduced.

We analyzed cocoa beans from three cacao cultivars, TSH-565, ICS-1xIMC-67 and the local cultivar IIa-22, growing in different production systems comprising monoculture and agroforestry system, both under conventional and agroforestry systems, and a successional agroforestry system in Alto Beni (Bolivia). Beans were harvested at the beginning and at the end of the dry season to determine the physiological response to climate during fruit maturation. We measured the total phenolic content from milled cotyledons according to the Folin-Ciocalteu`s assay and polyamines from defatted cocoa powder via HPLC as indicators for abiotic stresses.

Conventional farming in monocultures was increasing tree growth and production compared to organic monocultures. The reduced water availability when pods were ripening during the dry season increased the total phenolic content and reduced the concentration of spermine, a polyamine. Effects of environmental growing conditions were not strong, but can explain variations in cocoa bean quality apart from post-harvest processing. Especially when analyzing cocoa beans from different origins the climatic conditions, soil water availability during harvesting season and the shade conditions of the production systems should be taken into consideration.

Introduction

Cocoa beans are produced under tropical conditions around the world. Annual precipitation in producing countries ranges from 1300 mm to 2800 mm that might by uniformly distributed over the year or concentrated in one or two rainy seasons (Carr and Lockwood, 2011). Additionally, cocoa beans are harvested in many countries, like in Bolivia, almost all year round (Schneider et al., 2017) even though the cacao trees are exposed to seasonally varying environmental conditions (Niether et al., submitted). Not only the country of production and the season influence the climatic growing conditions of the cacao tree, but also the production system. Cacao trees produce under full-sun conditions as well as in agroforestry systems (Rice and Greenberg, 2000). Shade trees provide a microclimate for the understory cacao where climatic extremes are buffered (Niether et al., submitted). Organic and conventional farming practices can be applied on monocultures and agroforestry systems, what is the more common strategy. Higher yields are usually obtained under conventional management, but organic agriculture enhances ecosystem services, that improve system sustainability (Altieri, 1999).

The cacao tree is sensitive to drought (Zuidema et al., 2005) and a prolonged desiccation period results in a reduction of yield (Schwendenmann et al., 2010). Beside the drought effect on total bean production, the water availability during maturation may also have an effect on the beans itself in terms of a physiological plant response to abiotic stress like in other seed crops (Alqudah et al., 2011). Plants react to abiotic stress with a cascade of biochemical reactions that enhance the stress tolerance. Polyamines are secondary metabolites with antioxidant properties that are involved in development and stress response (Ruf and Schroth, 2010). Bae et al. (2008) found increased polyamine-levels, i.e. putrescine, spermidine and

spermine, in cacao leaves and flowers in response to drought together with enhanced stress tolerance, while spermine and spermidine were identified in beans (do Carmo Brito et al., 2017). An evaluation of stress response in beans in relation to the growing conditions is to our knowledge not yet done. Also phenolic substances are reported to play a role in plants' stress response due to their antioxidant properties (Ramakrishna and Ravishankar, 2011). Phenolic compounds are accumulated in the cocoa bean during the phase of fruit development (maturation phase) together with other substances and proteins that are involved in biotic and abiotic stress response (Wang et al., 2016) and may serve as an indicator for environmental conditions such as drought and light that are influenced by the production system and the harvesting season during the seed maturation phase.

We harvested beans from mature pods at the beginning and at the end of the dry season and expected an increase in water stress related total phenolic content and polyamine concentration. Further, the pods were produced in five different production system comprising monocultures and agroforestry systems, both under conventional and organic management, and a successional agroforestry system. We expected that agroforestry systems provide a buffer for the cacao tree and therefore less changes in the concentration of the stress indicators. Finally, the beans from two international cultivar ICS-1xIMC-67 and TSH-565 and from one local cultivar IIa-22 from the region Alto Beni, Bolivia, were analyzed with the aim to show success of the local breeding programs to local climate and production systems.

Materials and methods

Study area and experimental design

The study site Sara Ana is located in Alto Beni at the foothill of the Bolivian Andes with 25.2 °C mean annual temperature and 83.0% mean annual relative humidity.78% of 1439 mm annual precipitation fall in the rainy season from October to April (Niether *et al.*, submitted). The dry winter coincides with the main cacao harvesting period. The long-term trial comprised five cacao production systems within a fully randomized complete block design with four repitions: full-sun monocultures (MONO) and agroforestry systems (AF) both under certified organic (ORG) and conventional (CONV) farming and a highly diverse cocoa successional agroforestry system (SAFS) under organic farming. Each plot size was 48 by 48 m with cacao tree spacing of 4 by 4 m. Stem density and leaf area index increased from MONO to AF to SAFS (Niether et al., submitted). Further plot characteristics and management are shown in detail in (Schneider et al., 2017). Twelve cultivars were planted with a regular pattern in every plot. For this study, three different cultivars were selected: one local clone (IIa-22), one foreign clone (TSH-565) and a hybrid cultivar (ICS-1 x IMC-67), all cultivated from Trinitario in different selection series.

Cacao stem diameter, yield and cacao beans sampling

Cacao stems were measured in 2014 at 30 cm above the ground. Only data from trees that were already in production were used in the calculation, as some trees were replanted after 2008 and did not yet produce fruits.

Cocoa beans were harvested from April to November 2014 every 15 days. Number of ripe pods per tree was counted and beans were taken off the pod. The bean sampling was repeated two times during the harvesting period: the first harvest ('wet') took place in April 2014, the second ('dry') in September 2014. Since cacao pod production takes five to six months until maturity, flowering for the harvest A was in November and pods developed during the rainy season (1707 mm of rainfall in 6 months), while the fruits for the harvest B were pollinated in April and matured during the dry season (539 mm of rainfall in 6 months) (Niether et al., 2017).

Sample preparation and measurements

Raw cocoa beans were manually deshelled. The cotyledons were lyophilized and milled using a rotor mill (ZM100, Retsch, Germany) with a 2 mm sieve.

The total phenolic content of milled cotyledons was measured photometrically according to the Folin-Ciocalteu's assay (Singleton and Rossi, 1965) at 735.8 nm. A calibration curve was established with gallic acid and the results are expressed as gallic acid equivalents (mg GAE g^{-1} milled cotyledons, dry matter).

Ten grams of milled cotyledons were defatted by acid exploration according to the Weibull-Stoldt Method (200 ml 12.5% hydrogen chloride for one hour) followed by extraction with petroleum ether for 5 h with a Soxleth-apparatus (Matissek and Steiner, 2006). The extraction and derivatization of the polyamines with 1-dimethylamino-naphthalene 5-sulfonic acid chloride using 0.1 g of defatted cocoa powder followed the method described by Smit et al. (2014).

Poylamines were analyzed via High Performance Liquid Chromatography (HPLC, LC-2000 Series, Jasco, Germany) as described by Smit et al. (2014) with 15 µl injection volume, fluorescence was detected at 254

nm and emission wavelength was set at 510 nm. Determination and quantification limits were defined according to Kromidas (2011). The results are expressed as $\mu g\ g^{-1}$ defatted cocoa powder (dry matter).

Statistical analyses

A student's t-test was used to determine the differences in total phenolic content and the polyamines between the two harvests. Afterwards, we applied linear mixed-effect models to assess the response of cocoa yield and stem diameter and of stress indicators separately for the two harvests to the fixed factors cultivar and production system. Block entered the model as a random factor. Orthogonal contrasts were fixed a priori to compare the different levels of the production systems: monocultures were compared with agroforestry systems (MONO vs. AF), agroforestry systems were compared with SAFS (AF vs. SAFS) and within monocultures and agroforestry systems, conventional and organic management were compared (MONO CONV vs. MONO ORG and AF CONV vs. AF ORG). Orthogonal contrasts were also fixed for the cultivars where we compared the local to the foreign cultivars (ICS-1 x IMC-67 vs. IIa-22 and IIa-22 vs. TSH-565).
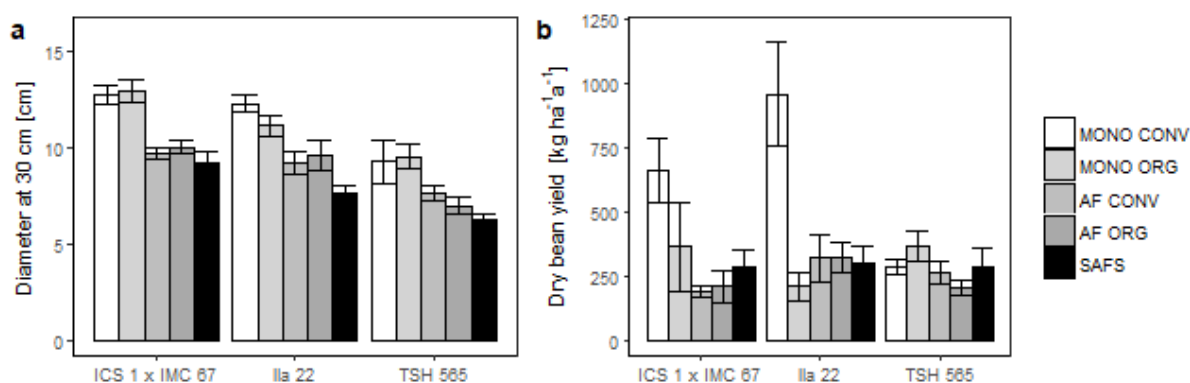
Results and discussion

Fig. 1 Cacao tree performance: (a) stem diameter and (b) dry bean yield of three cultivars and five production systems from left to right: monoculture conventional (MONO CONV), monoculture organic (MONO ORG), agroforestry conventional (AF CONV), agroforestry organic (AF ORG) and successional agroforestry system (SAFS).

Table 1 Results from linear mixed models with the fixed factors treatment and cultivar showing the t-values from orthogonal contrasts. Asterisks indicate differences according to levels of significance (* <0.05; **<0.01; ***<0.001).

response variable	harvest	MONO CONV vs. MONO ORG	AF CONV vs. AF ORG	MONO vs. AF	AF vs. SAFS	ICS-1 x IMC-67 vs. IIa-22	IIa-22 vs. TSH-656
stem diameter		0.5	-0.1	8.9***	6.0***	7.1***	8.6***
yield		3.3**	0.4	3.0**	0.4	-0.7	0.8
total phenolic content	wet	0.6	1.3	-0.9	0.3	-2.7*	-0.2
	dry	0.2	0.1	0.4	-0.6	-2.5*	1.3
putrescine	wet	1.0	-1.3	0.9	2.5*	-0.1	-0.0
	dry	0.2	0.3	0.6	2.8**	0.3	0.3
spermidine	wet	0.6	-1.3	0.7	2.1*	0.3	0.9
	dry	-0.6	1.0	0.6	3.6**	0.9	0.8
spermine	wet	-1.1	-1.5	-0.1	0.9	0.3	1.2
	dry	-0.8	0.7	1.4	2.1*	2.7*	1.3

Table 2 Results from student's t-test comparing harvest 'wet' and 'dry'. Asterisks indicate differences according to levels of significance (* <0.05; **<0.01; ***<0.001).

	harvest		
stress indicator	wet	dry	t-value
total phenolic content [mg g^{-1}]	5.3	7.4	-4.8***
putrescine [$\mu g\ g^{-1}$]	1.25	1.24	0.1
spermidine [$\mu g\ g^{-1}$]	4.97	4.20	1.7

spermine [µg g^{-1}]	2.71	1.94	3.2**

Cacao stem diameter decreased with increasing stem density of the system from MONO to AF to SAFS (Fig. 1, Table 1). The cacao yield was highest in MONO CONV. Organic and conventional management had no effect on the yield and the stem diameter in agroforestry systems (Schneider et al., 2017). The hybrid cultivar had the highest stem diameter across all production systems, followed by the local clone IIa-22 and the foreign clone TSH-565. Hybrid cultivars in general have a higher stem with ramification at approximately 1 m compared to clones were the branches develop close to the ground (Schneider et al., 2017). That may be an explanation for the higher stem diameter at already 30 cm. Instead, both TSH-565 and IIa-22 have short stems with ramification close to the ground level and the local cultivar has a higher stem diameter across all production system that the foreign cultivar implying better use of available resources for biomass production but not for pod production.

The total phenolic content was higher in beans from harvest at the end of the rainy season than in beans matured during the rainy season (Table 2), when water was sufficiently available (Niether et al., 2017). The seasonal influence on the beans was also supposed by Albertini et al. (2015), Camu et al. (2008), and Wang et al. (2016). Phenolic compounds are antioxidants that reduce the increased level of reactive oxygen species during drought, as shown for drought tolerant shrubs (Varela et al., 2016). Despite cacao is a drought susceptible species (Läderach et al., 2013), an increased level of the total phenolic compounds in the dry season may protect the bean, the plants' reproduction unit, during ripening from cell damage by reactive oxygen species.
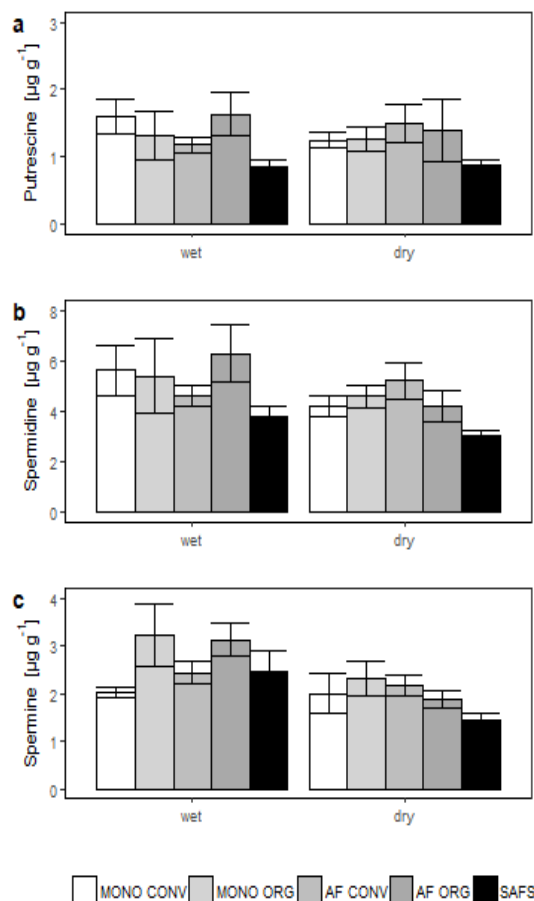

Fig. 2 Polyamine concentration in cocoa beans. (a) putrescine, (b) spermidine, (c) spermidine concentration during the wet and dry season in the cacao production systems: monoculture conventional (MONO CONV), monoculture organic (MONO ORG), agroforestry conventional (AF CONV), agroforestry organic (AF ORG) and successional agroforestry system (SAFS).
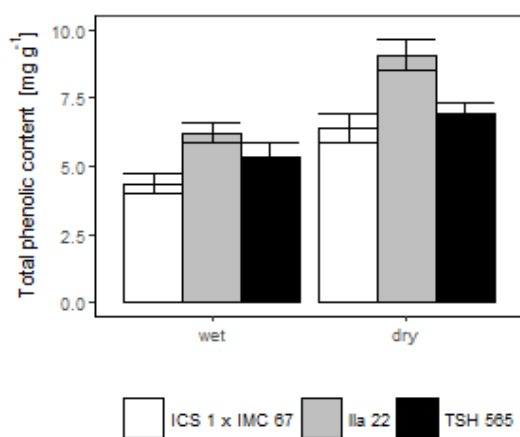
Fig. 3 Total phenolic content in cocoa beans during the wet and the dry season harvest of three cultivars.

While polyamine levels in flowers and leaves increase after a few days of drought (Bae et al., 2008), an opposite trend was observed for concentration of the polyamine spermine that was lower in the beans from the dry season harvest, while spermidine and putrescine did not change (Fig. 2, Table 2). This observation might be explained by the different plant organs that react to drought with distinct physiological responses to protect the beans or to enhance drought tolerance and maintain photosynthesis like in leaves. Another explanation can be the long-term stress response after a slowly decreasing precipitation and soil moisture over the dry season (as described in Niether et al., 2017). Furthermore, the control of the polyamine pathway is not finally described, and a back-conversion between the polyamines may be possible that avoid an accumulation (Alcázar et al., 2014). Polyamines were less abundant in beans from successional agroforestry systems at both harvests (Fig. 2, Table 1). These systems have a much higher diversity and stem density than the other agroforestry systems and the monocultures (Schneider et al., 2017). That might imply a higher competition between the individual trees for soil resources and light as well as allelopathic interactions between the species that are not identified yet. In contrast, the total phenolic content was not affected by the production systems, but by the cultivars: the local cultivar IIa-22 had the highest phenolic content in both harvests, followed by the foreign cultivar TSH-565 and finally the hybrid ICS-1 x IMC-67 (Fig. 3). The phenolic content of all cultivars was still much lower than in beans from other studies (e.g., do Carmo Brito et al., 2017) that might be explained by a strong decrease during the long fermentation time (Camu et al., 2008).

Conclusions

We found an effect of environmental growing conditions, i.e. production system and season, on the stress response of the cacao beans, as well as variation between cultivars. The effects were not strong in comparison to other post-harvest processes, and in still in the range of other studies, but they can explain variation in the chemical composition of beans from various origins, that can be countries with varying climate, and therefore should be taken into consideration when discussing the chemical composition.

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