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German cacao of Cameroon – new facts on a traditional variety fallen into oblivion

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Summary

"German" cacao cultivated in Cameroon has emerged from a mixture of different gene pools with a large proportion of Trinitario and with a pronounced content of polyphenols. In order to characterize this old genotype, polyphenols and polyphenol oxidase were compared with hybrid selected genotypes. Epicatechin (25 mg/g - 52 mg/g fat free dm) and catechin (0.5 - 1.9 mg/g fat free dm) content of German cacao seeds were of similar range with hybrid investigated samples. German cacao is characterized by its high content of anthocyanins especially cyanidine-3-arabinoside which ranges from 8.84 mg/g to 17.51 mg/g fat free dm. Hybrid genotypes displayed 1 mg/g to 6.4 mg/g fat free dm of cyanidine-3-arabinoside. PPO activity was 10 to 20-fold higher in German cacao seeds compared to hybrid. Anthocyanin and PPO through the oxidation of phenols to quinone are involved in colour development and pests and diseases resistance. Pigment is one of the most important factors for the colour of cocoa powder. We discuss the high content of anthocyanin and PPO activity in German cacao in relation with the reddish colour of cocoa powder derived from Cameroonian cacao.

Key words: anthocyanin, polyphenol oxidase, polyphenols, cacao, Cameroon

Introduction

Cacao was first introduced to Africa in the beginning of the 18th century by the Portuguese. They brought cacao of the Amelonado type (Lower Amazon Forastero) from Bahia (Brazil) to the island of Principe around 1822 (Aikpokpodion, 2012). It arrived in Sao Tomé in 1850 (Bartley, 2005). In 1854 the Spanish brought cacao probably from Sao Tomé to the Island of Fernando Po (now Bioko, Equatorial Guinea) to the coast of Cameroon. Cacao from Fernando Po became the major source of planting material into the mainland of West Africa (Aikpokpodion, 2012).

The first species of recorded cacao reached Cameroon in 1876. It was probably introduced from Trinidad (Aikpokpodion, 2012; Efombagn et al., 2008). Thirteen plants imported by British missionaries from the Royal Botanic Gardens of England were planted on Cameroon Mountains in the south western region. Towards 1892, under the German colonial administration, Lower Amazon Forastero (Amelonado) from Sao Tomé and Fernando Po was introduced by Preuss, the curator of the Victoria (now Limbe) Botanical Garden (Efombagn et al., 2008; Aikpokpodion, 2012). Around 1895, Forastero plants (332 plants) from Trinidad were introduced (Aikpokpodion, 2012; Preuss, 1901).

In the year 1900 several varieties of the Forastero plants originating from South and Central America were introduced by Preuss (e.g. 'Puerto-Cabello', 'Venezuela', 'Maracaibo' and 'La Guira' from Venezuela, 'Guayaquil' from Ecuador, 'Soconusco' from Mexico, 'Nueva Grenada' from Colombia, 'Suriname', 'Criollo' and 'Forastero' (Aikpokpodion, 2012)). At the beginning of the twentieth century the cacao diversity in Cameroon had enlarged to one of the most diverse collections (Bartley, 2005).

The first cacao breeding process in Cameroon took place in the 1950s by selection of the dominating local Trinitario population. These were identified as selections from the Nkoemvone (SNK) accessions (research station, southern Cameroon, Efombagn et al., 2008). In the 1950s and 1960s the local cacao germplasm was broadened by introducing the Upper Amazon Forastero types (Efombagn et al., 2008). In the following years, the local Trinitario species was crossed with this Upper Amazon Forastero. In the 1970s and 1980s hybrids with a high yield potential and precocity were selected and established in Cameroon. In addition, some cacao genotypes were selected and cultivated by smallholders because of their low susceptibility to diseases (Efombagn et al., 2008). Therefore these cacao trees were only slowly replaced by new breeding materials. Because cacao was first introduced in Cameroon under the German colonial administration, this traditional cacao is known as "German" cacao. The German cacao presents a high level of admixture of diverse genes. Genes of Lower Amazon cacao (54%) were found to be predominant followed by Upper Amazon cacao (33%) and Criollo (7%) (Efombagn et al., 2008). Only a low content of Trinitario was found in the examined samples (Efombagn et al., 2008).

Studies using 12 microsatellite loci suggest that 25.5% of cacao from 400 farm accessions is still closely related to the traditional Amelonado variety (Efombagn et al., 2009; Parentage analysis). This is probably due to a relatively small number of hybrids (Efombagn et al., 2006). Another 46.3% were found to be direct descendants from 24 parental clones used in biclonal seed gardens, and 28.3% appeared to have come from uncontrolled pollination events in cacao farms. This could be the effect of a common practice of cacao growers, who use seeds collected in their own farm for new planting (Efombagn et al., 2009).

Preuss probably got the Trinitario varieties from Venezuela but also from West Indies. They had distinctive red podded fruits and an unusually red coloured powder (Preuss, 1901; Wood, 1991; Laird et al., 2007). These Trinitario varieties are still cultivated in the region around Mount Cameroon (Laird et al., 2007), whereas in South and East Cameroon these varieties were natural hybridized with Amelonado genotypes from Fernando Po (Laird et al., 2007).

Genetic diversity studies found a higher consistency with local Trinitario cacao in the East region of Cameroon and Upper Amazon cacao and its hybrids in the Centre-South regions (Efombagn et al., 2006). The genetic diversity of the farmers' planting material is not very high, and it is genetically close to parental genotypes available in genebanks (Efombagn et al., 2006).

The "German" cacao has small seeds and takes a long time to grow, but it is described as durable and more resistant to pests and diseases (Laird et al., 2007). Many farmers prefer the "German" cacao for new planting because of its constantly running harvest, its longevity and its low susceptibility to pod rot (*Phytophthora megakarya*, Paulin

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et al., 2003). New bred varieties of cacao do indeed have a much higher yield than the local “German” cacao, but farmers criticize their higher susceptibility to black pod disease and their shorter life span. For this reason, the replacement of traditional varieties by new varieties is slow.

In Cameroon, cacao has been increasingly grown since the 1960s. Today, about 400 000 hectares of cacao are cultivated in more than 200 000 farms (EFOMBAGN et al., 2006). Mostly, these are very small family farms. On average, cacao plantations are about 6.5 hectares in size, and averagely 1200 trees are planted per hectare (PAULIN et al., 2003). The annual production is about 200 000 tonnes (ICCO, 2012). The majority of plantations are more than 30 years old (about 50%, PAULIN et al., 2003). According to JAGORET et al. (2011), nearly 70% of cacao plantations in Cameroon are more than 40 years old, and 40% of these plantations in Southern Cameroon were planted before 1960 (PAULIN et al., 2003). They have very low yields (e.g. 150 to 300 kg dry cacao per hectare) (EFOMBAGN et al., 2006). In well managed plantations with high performance clones, significantly higher yields of up to 2500 kg per ha can be achieved.

The objective of the current paper is to describe the characteristics of the traditional “German” cacao of Cameroon. As already described, some traditional cacao varieties of Cameroon have distinctive red coloured seeds. For a more detailed analysis of these characteristics, the content of epicatechin and anthocyanins were analyzed and the activity of the polyphenoloxidase was determined. Phenolic compounds as well as oxidative enzymes contribute to the quality of raw cacao.

Material and methods

Material

Traditional “German” cacao varieties were collected from different cacao plantations from the region of Yaounde:

- Mbangasina about 100 km North of Yaounde, a dry region. Pods with a yellowish, light brown colour.
- Awae about 50 km East of Yaounde. Pods with a yellow colour.
- Ngat about 40 km South East of Yaounde. According to the owners description, the trees of this plantation were at least 50 years old. The fruits were much less affected by pests than the fruits of the other plantations. The fruits were classified in red and yellow according to their colour.

Hybrid varieties were collected in seed garden from SODECAO in Meigang. Fruits were transported to Hamburg and freeze dried (Tab. 1).

Methods

Before further analyses, freeze-dried seeds were defatted. First, the testa and the radicals were removed and 10 ml of n-hexane was added to approximately 2 g seed material and it was crushed in a ball mill by shaking for 10 minutes at a frequency of 25/s. The homogenate was rinsed out of the ball mill with about 75 ml of petroleum ether (bp 40-60 °C), then filtered and dried in a vacuum drying oven.

Polyphenols analysis

50 mg of defatted cocoa powder was stirred with 3 ml methanol for 30 sec with an ultraturrax (T25, Janke & Kunkel). After 15 min on ice with constant homogenisation, the samples were centrifuged for 10 min at 5000 rpm. The supernatant was decanted into 50 ml conical flasks. This extraction was repeated twice for only two minutes on ice and 20 seconds by an ultraturrax (T25, Janke & Kunkel).

The samples were concentrated by drying on a rotary evaporator at 40 °C and 100 mbar, and then resuspended in 1.5 ml methanol (gradient grade). After subsequent filtration through a 0.45 µm filter (Multoclear 25 mm PTFE, CSChromatographie Service), 20 µl of each sample were injected to the HPLC.

The polyphenols were measured by a PDA-Detector (Photo-Diode-Array-Detector, Waters 996) within a wavelength range from 225 nm to 540 nm. The polyphenols were quantified at a wave length of 280 nm and the anthocyanins at 540 nm. A LiChroCART 100 RP-18 end-capped column (particle size 5 µm, length 250 mm, inner diameter 4 mm) with a Lichrospher 100 RP-18 end-capped pre-column was used for separation. The column temperature was 26 °C and the eluents: A: 2% acetic acid; B: acetonitrile / deionized water / acetic acid (v / v / v 400: 90: 10) (Tab. 2). Catechin, epicatechin, cyanidin-3-galactoside and cyanidin-3-arabinoside were used as standard for quantification.

Tab. 2: RP-HPLC gradient used for the separation of polyphenols

Time (min)	Flow rate (mL min^{-1})	A (%)	B (%)
0	1.2	90	10
8	1.2	90	10
38	1.1	77	23
50	1	60	40
70	1	10	90
73	1	10	90
78	1.2	90	10
93	1.2	90	10

Polyphenoloxidase (PPO)

For each determination of the PPO activity, the cotyledons of one seed were used. The weights were determined and after adding 0.1 g polyvinylpolypyrrolidone and 10 ml iced cyanide Sörensen buffer pH 6.4 (Sörensen buffer: 738 ml KH_2PO_4-solution 67 mmol/l, 262 ml Na_2HPO_4-solution 67 mmol/l, pH 6.4. For cyanide Sörensen buffer, the buffer contained 5 mmol/l KCN and 1.7 mmol/l H_3PO_4), samples were homogenized by an ultraturrax (T25, Janke & Kunkel). Subsequently, the samples were centrifuged for 30 min at 14000 g (RC5C, Sorvall Instruments). The PPO activity was measured in the pellet and the supernatant. 50-100 mg of the pellet were suspended in 1 ml Sörensen buffer and further diluted, depending on the level of PPO activity. To measure the PPO activity in the supernatant, the cyanide-containing supernatant was purified from the cyanide with a PD10 column (Sephadex™ G-25M, Amersham Biosciences). For this purpose, the PD-10 column was first equilibrated with 25 ml Sörensen buffer. Subsequently, 2.5 ml of the sample solution were added to the column, which were eluted with 3.5 ml of Sörensen buffer. The cyanide was added in order to protect the enzymes to tanning reactions by quinones produced during extraction (STOLL, 2010).

Tab. 1: Different clones used for analysis comparison with the German cacao.

	WAA	SNK 13	ICS 40	T 79/501	SNK 64	ICS 84	German cacao	Catongo
n =	11	3	3	3	3	3	10	3

The measurements were performed at 25 °C with air-saturated Sörensen-buffer (6.4) and 4-methylcatechol (56 mg 4-methylcatechol in 2 ml deionized water) as standard substrate. Autoxidation of methylcatechol was measured in a solution containing 2.9 ml Sörensen-buffer and 100 µl 4-methylcatechol solution. The PPO activity was evaluated within a solution containing 2.7 ml Sörensen-buffer, 200 µl enzyme suspension, 100 µl 4-methylcatechol solution. The enzyme reaction was started after two minutes of stirring the enzyme suspension in the buffer by adding the substrate solution. The oxygen consumption was recorded over a period of 6 min with a flat bed recorder. The PPO activity was determined from the increase of the curve 45 seconds after addition of substrate, by applying a tangent to the curve. The slope was calculated as a percentage of oxygen consumption per unit of time. Of these, the rate of the autoxidation was subtracted. According to Meyer (1975), the oxygen consumption of 1% per hour correlates with 6.7 nmol per hour.

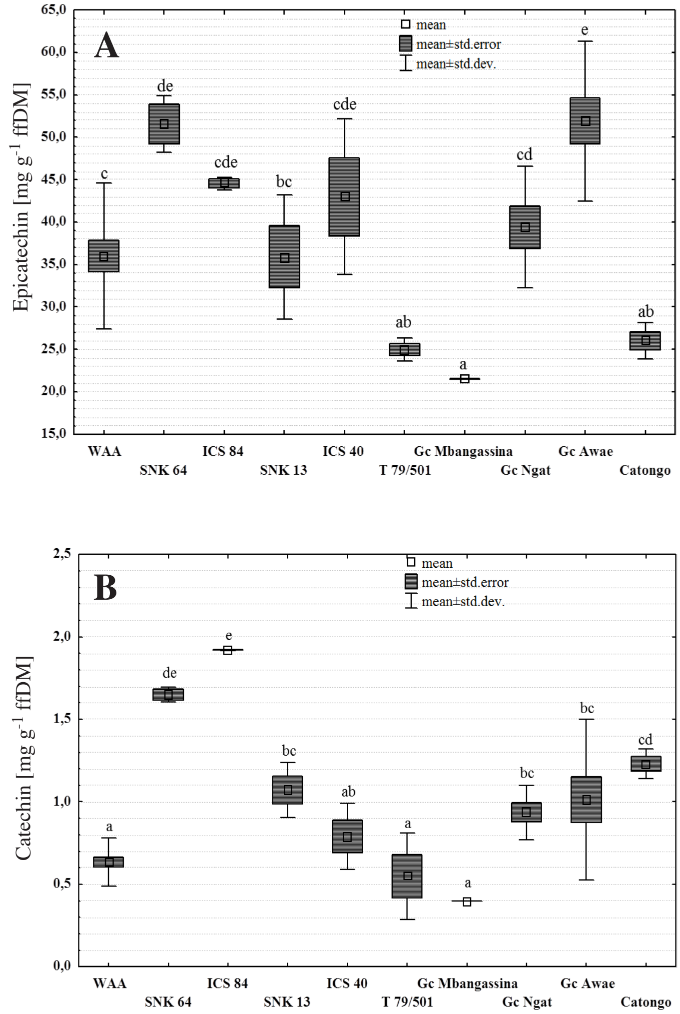

Fig. 2: Epicatechin (A) and catechin (B) content from traditional German cacao (Gc) and hybrid varieties. Milled defatted cacao seeds were extracted in methanol and polyphenol analysis was done by HPLC. Mean values (n = 6) with different letters differed significantly at $P < 0.05$, Post hoc Test: Fisher LSD, $P < 0.05$. Mean is the result of two analyses from three independents cacao pods.

Results and discussion

According to the Barry Callebaut's communication officer at the ISM (International supplier trade fair for the sweets and snacks industry) launch in Cologne (Germany) 2012, the milk chocolates created with the red cacao from Cameroon are of outstanding quality. The reddish colour of the Cameron cacao is world-wide known and the derived cacao powders are perfectly suited for use in bakery application. However, the scientific and biological backgrounds underlying this specific property are not well uncovered. In incubation experiments (Kadow et al., 2013), German cacao showed distinctly stronger red coloured medias by a leakage of anthocyanins from the cotyledons than other examined genotypes (data not shown). Based on this observation, further analyses with traditional cacao seeds were carried out. Fresh seeds of German cacao contain a very high content of polyphenols, especially anthocyanins.

Fig. 2A shows the epicatechin content of different cacao genotypes. The epicatechin content varies between 25 mg/g ffdm in seeds of T 79/501 and 52 mg/g ffdm in seeds of SNK 64. The epicatechin content of German cacao lies in a similar range. There is no significant difference to the epicatechin content of the other analyzed genotypes. The catechin content of the German cacao seeds was also found to be in a similar range as the content of the other investigated samples. The samples examined contained between 0.5 mg/g ffdm and 1.9 mg/g ffdm catechin (Fig. 2B). The polyphenol content of German cacao seeds of different regions in Cameroon was compared

Fig. 1: Root aspect of traditional German cacao, Gc (A) and hybrid ICS40 clone (B) 6 days after germination in distilled water. The high accumulation of anthocyanin in the root is observed in Gc.

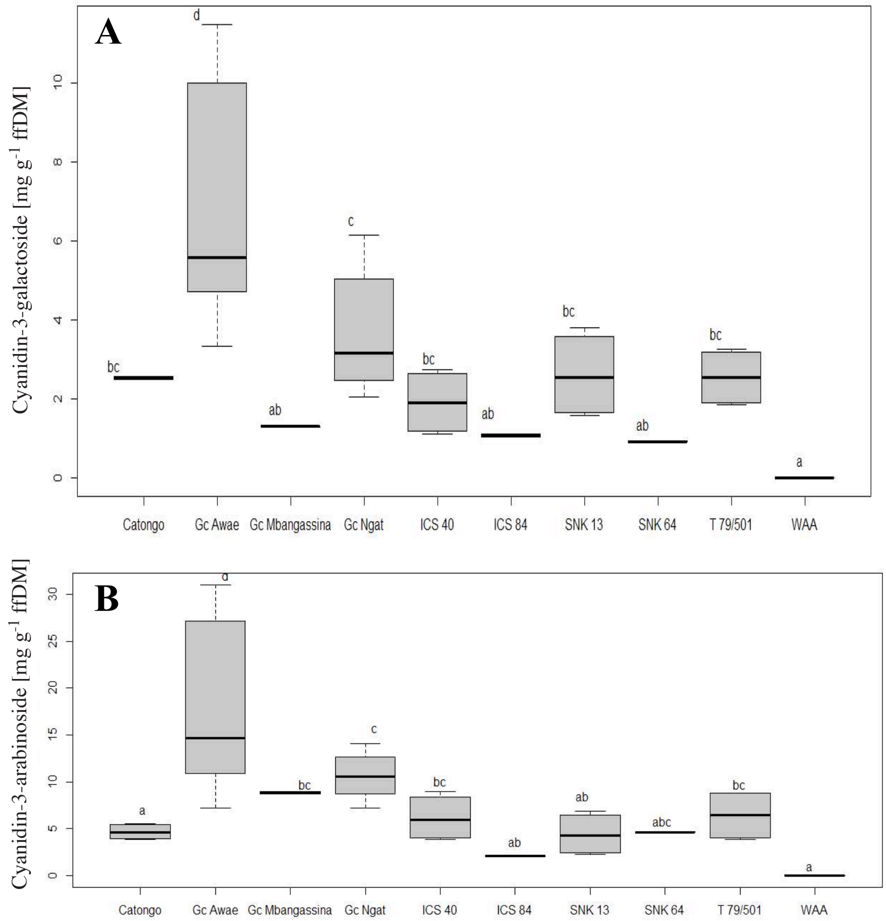

Fig. 3: Cyanidin-3-galactoside (A) and cyanidin-3-arabinoside (B) content from traditional German cacao (Gc) and hybrid varieties. Milled defatted cacao seeds were extracted in methanol and polyphenol analysis was done by HPLC. Mean values (n = 6) with different letters differed significantly at $P < 0.05$, Post hoc Test: Fisher LSD, $P < 0.05$. Mean is the result of two analyses from three independents cacao pods.

(data not shown). The highest content of all polyphenols were found in German cacao of Awae. The lowest contents were found in German cacao of the dry area of Mbangassina. The polyphenols quality of raw cacao beans is rarely diverse. However, their quantity rely on many factors such as the area of trees cultivation, bean maturity, macro- and micro-environment, the harvest period and store time after harvest (NIEMENAK et al., 2006; ORACZ et al., 2015).

The content of cyanidin-3-galactoside and cyanidin-3-arabinoside in German cacao seeds were significantly higher than in the seeds of the other samples examined (Fig. 3). This high anthocyanin content in cotyledon tissue corresponds to a high coloration of the root during germination (Fig. 1). Furthermore, it is a typical trait in German cacao and represents a potential phenotypic descriptor to morphologically identify the German cacao group.

Different developmental processes are regulated by flavonoids among which lateral root initiation well highlighted in *Arabidopsis* and tomato using transgenes and mutants. In Tomato mutants with reduced anthocyanin synthesis displayed reduction in lateral roots at the onsets of root initiation (NEGI et al., 2008; 2010).

In addition to the high polyphenol content, fresh German cacao seeds contain an approximately 12-fold higher PPO activity (Fig. 4) than seeds of other varieties examined. Fresh German cacao seeds contain in the supernatant an activity between 0.01 and 0.14 µkat/g fresh weight (fw) and in the pellet an activity between 5.2 and 6.7 µkat/g fw. Whereas in controlled samples only an activity between 0.003 and 0.026 µkat/g fw in the supernatant and between 0.1 and 0.7 µkat/g fw in the pellet were found. In both cases, most of the PPOs were membrane bound. PPO catalyses the oxidation of *ortho* (*o*)-phenols to *o*-quinone at the expense of molecular O_2. The *o*-quinone is a highly reactive electrophilic molecule and links with functional groups of proteins such as sulfhydryl, amine, amide, indole and amidazole substituents, forming protein-bound phenol (BITTNER, 2006). This catalytic action of PPO has an enormous impact on food quality and results in alteration of colour (browning), flavour, texture and nutritional value (VAMOS-VIGYAZO, 1981). However, in cacao, this browning has beneficial effects in improving chocolate flavour precursor's development in fermented and dried beans. Indeed, during the cacao cut-test chart the browning of the beans is one of the criteria to recognize good fermented cacao.

Further, the German cacao is well known by farmers to be black-pod resistant. The contribution of PPO and polyphenols to this feature may be significant. In fact, PAULIN et al. (2003) and OMOKOLO et al. (1996) showed that cacao genotypes resistant against pod rot are rich in polyphenols. The most frequently suggested role for PPO and polyphenols in plants has been in defence against herbivores and pathogens. The *o*-quinone-protein complex is known to reduce the nutritional value of the tissue and thereby reduce predation and can also participate in the formation of structural barriers against invading pathogens. Recently, ARAJI et al. (2014) showed that silencing PPO in Walnut (*Juglans regia*) caused major alterations in the metabolism of phenolic compounds and in the expression of phenylpropanoid pathway genes, suggesting a PPO role in secondary metabolism. The main advantage of German cacao in comparison to other cacao varieties is its very high resistance against pathogens, which is

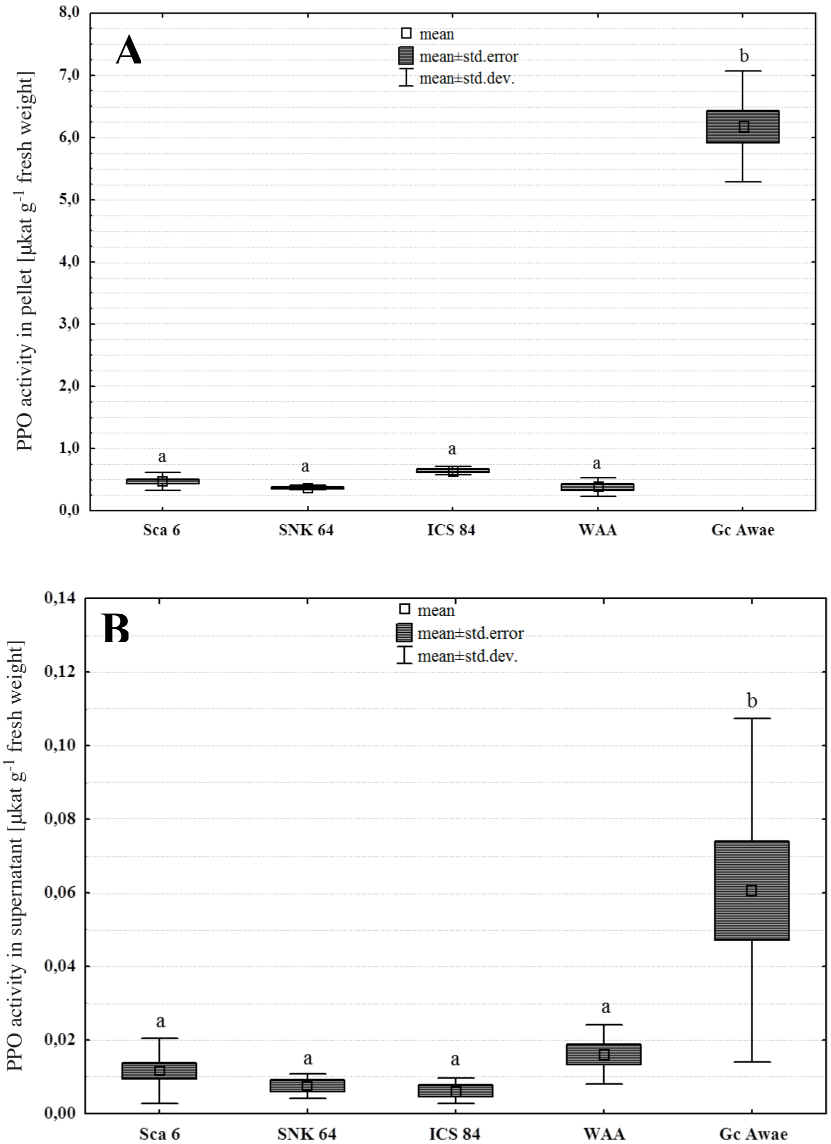

Fig. 4: Polyphenol oxidase activity in pellet (A, bound PPO) and supernatant (B, solubilized PPO) from extracted fresh seed of traditional German cacao (Gc) and hybrid varieties. Fresh seeds were extracted in Sörensen buffer and PPO estimated by using a polarographic method based on the consumption of oxygen (MEYER et al., 1975). Mean values ($n = 6$) with different letters differed significantly at $P < 0.05$, Post hoc Test: Fisher LSD, $P < 0.05$. Mean is the result of two analyses from three independents cacao pods.

mainly related to high PPO activity. This study highlights the positive impact of high anthocyanin content on reddish colour development necessary for producing premium cacao powder for chocolate related food.

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