Tropical Plant Pathology
https://doi.org/10.1007/s40858-022-00521-0

ORIGINAL ARTICLE

Flavan-3-ol and flavonol analysis in healthy and infected parents and progenies of cocoa leaves (*Theobroma cacao* L.) with *Phytophthora megakarya* Bras. and Grif

Jules Christian Djoko Kouam[1,2,3] · Jude Manga Ndjaga[2,3] · Simon Perrez Akoa[3,4] · Martine Louise Ondobo[3] · Pierre Effa Onomo[3,4] · Pierre François Djocgoue[2,3] · Nicolas Niemenak[3] · Sonia Collin[1]

Received: 8 April 2022 / Accepted: 3 July 2022

Abstract

Flavonoids are phenolic compounds involved in defense mechanisms against black pod disease (BPD). Crossing between Forastero and Trinitario-resistant cocoa genotypes usually produce resistant progenies with high contents of bioactive components. This study aims at analyzing flavan-3-ol and flavonol content in the defense against *P. megakarya* in *T. cacao* genotypes for their potential selection as markers of partial resistance to BPD. Assessment of necrosis development and biochemical markers of stress (total polyphenols (TPP), total flavonoids (TF), and condensed tannins (CT)) through spectrophotometric methods of 19 genotypes identified eight hybrids that contained higher amounts (P<0.05) of bioactive components than the better parent T79/467. The necrosis length revealed to be negatively correlated with metabolite concentrations ($P<0.05$). Flavan-3-ols and flavonols were analyzed by normal phase (NP) and reverse phase (RP) HPLC–DAD-ESI (-)-MS/MS. Among the best genotypes, flavan-3-ol monomers ((+)-catechin, (-)-epicatechin), derived procyanidins (especially B2 and four of its isomers, C1, and one pentamer), and flavonols (quercetin, quercetin-3-O-arabinoside, quercetin-3-O-glucoside, quercetin-3-O-rhamnoglucoside, and one isomer) were evidenced both in healthy and infected leaves. Analyzed metabolites increase following infection, but infection does not trigger the synthesis of new compounds. The order of accumulation of biomolecules is as follows: (-)-epicatechin (196%), procyanidin C1 (184%), pentamers (140%), procyanidin B2 (135%), quercetin (80%), quercetin derivatives (57–69%), and (+)-catechin (57%). Yet their accumulation after infection revealed to be genotype-dependent. This breeding approach is designed to select partial resistant cocoa genotypes against BPD evidenced useful biomarkers in hybrids with high flavonoid content.

Keywords Phytochemicals · *Phytophthora megakarya* · *Theobroma cacao* · Partial resistance

Introduction

Stagnation of cocoa production in Cameroon for several decades is partly due to a combination of parasitical pressure coupled to the lack of tolerant and productive genotypes with regard to environmental pressure and parasitical threat. Black pod disease (BPD), caused by *Phytophthora megakarya*, is one of the most prevalent diseases in the genus *Theobroma* with damages in Cameroon estimated at 80% yield losses per year (Nyasse et al. 2007). An environmentally, economically, and biologically reasonable control strategy often employed is hybridization of known genotypes associated to nursery and/or field observations, genetic screening, and molecular tests (Djocgoue et al. 2010; Ondobo et al. 2017; Simo et al. 2018). To increase pathogen resistance and yield, breeders have capitalized

✉ Pierre Effa Onomo
peffafr@yahoo.fr

1 Unit of Brewery and Food Sciences, LIBST Institute, Faculty of Bioscience Engineering, Université Catholique de Louvain, Croix du Sud 2, Box L7.05.07, B-1348 Louvain-la-Neuve, Belgium

2 Department of Plant Biology, Faculty of Science, University of Yaounde I, P.O Box 812, Yaounde, Cameroon

3 Laboratory of Plant Physiology, Department of Biological Sciences, Higher Teacher Training College, P. O. Box 47, Yaounde, Cameroon

4 Department of Biochemistry, Faculty of Science, University of Yaounde I, P.O Box 812, Yaounde, Cameroon

Published online: 01 August 2022

on heterosis that occurs in crosses between trees with different desirable traits and of different geographical origins or genetic groups. Cocoa germplasm in Cameroon is mainly made up of Forastero and Trinitario genotypes. Nevertheless, a recent geographical and genetic population differentiation of *T. cacao* germplasm reveals 10 genetic clusters, as opposed to the two groups (Criollo, Forastero) traditionally recognized within the species (Motamayor et al. 2008). Hybrid progenies with high hybrid vigor were obtained from cross-pollination between high Amazonian clones with other Forastero or Trinitario origins (Oracz et al. 2015; Minyaka et al. 2017).

Regarding establishment of host/parasite relationship, phytopathologist distinguish between two main strategies of plants to cope with pathogens: tolerance and resistance. Tolerance reduces the negative effects of infection and requires an evaluation of yield and quality, whereas resistance reduces the multiplication rate of the pathogen. More tolerant varieties better endure the effects of infection than do less tolerant ones at the same level of pathogen attack. The emphasis on equivalent level of pathogen attack is necessary to differentiate tolerance from partial resistance. Pathogen attack has been quantified most often as the pathogen load sustained by the host, which depends on the rate of within-host pathogen multiplication, i.e., on the resistance/susceptibility of the host, all else being equal (Pagan and Garcia-Arenal 2020).

Monitoring of partial resistance traits via the analysis of metabolites markers was done during nursing and on older trees (Manga et al. 2018). In addition to constitutive barriers, plants acquire tolerance and resistance to stress factors, due to their ability to activate defense mechanisms such as hypersensitivity responses, strengthening of the cell wall, oxidative species scavenging, and production of specialized metabolites such as flavonoids (Morrissey and Osbourn 1999; Tasiu 2019). Flavonoids are a subclass of polyphenols widely reported to play a key role in plants and specifically in cocoa disease resistance mechanism (Djocgoue et al. 2010; Minyaka et al. 2017). These secondary metabolites may be in the form of phenolic acids, flavonols, flavones, flavan-3-ol monomers (mainly (-)-epicatechin; up to 30% of total flavonoids in healthy leaves (Chaves and Gianfagna 2007)), and procyanidins (condensed tannins). Proanthocyanidins are more actively synthesized in newly emerging leaves than in mature leaves and seeds (Liu et al. 2013). Flavan-3-ol monomers, procyanidins, and flavonols have gained special attention because they have shown benefits to plants (defense against insects, pathogens, and microbes; absorption of free radicals and UV light) but also to human health (Chan et al. 2013; Ofori et al. 2015; Irondi et al. 2017). Flavonoids are consistently used as biochemical markers of resistance against pathogenic microorganisms (Nyadanu et al. 2012; Minyaka et al. 2017).

Evaluation of the role of plant metabolites in the production of hybrids gives a better insight in the knowledge of plant genetic diversity giving way to better choice of parents for the eventual selection of resistant genotypes. A close relationship has been established between biochemical traits and lesion size on one hand and components of resistance to *P. megakarya* on the other hand, thus confirming the importance of individual polyphenolic compounds in cocoa genotypes as a resistance mechanism against black pod disease (Effa et al. 2016; Ondobo et al. 2017). Therefore, it would be possible to select cocoa genotypes that possess high amounts of these compounds of interest. Several tests based on leaf inoculation (Manga et al. 2018) and detached pod inoculation (Djocgoue et al. 2010; Nana et al. 2011) have been used so far to investigate plant susceptibility to pathogens and the defense mechanisms involved. Nevertheless, expression of biochemical markers of cocoa resistance against fungal and other pathogenic micro-organisms among hybrid offspring remains controversial. Elwers et al. (2009) found no genetically determined differences in the amounts of total polyphenols and (-)-epicatechin among cocoa seed from different genetic groups and geographical origin, while other authors (Oracz et al. 2015; Febrianto and Zhu 2019) reported a wide range of flavan-3-ols, anthocyanins, and flavanols in cocoa beans to be genotype dependent. The later also provided evidence that the content of phenolic compounds differentiates not only individual varieties, but also cocoa tree species and cocoa tree-growing regions. A deeper insight on cocoa genetic diversity could pave way to better choices of parents for the creation of new hybrids.

The present research aims at analyzing flavan-3-ol and flavonol content in the defense against *P. megakarya* in *T. cacao* genotypes for their potential selection as markers of partial resistance to BPD. Our findings assess the level of resistance of some Trinitario and Forastero cocoa hybrids against *P. megakarya* and provide useful information on the variation in composition and amounts of some flavonoid compounds involved in partial resistance to BPD.

Material and methods

Plant material

Field experiments were conducted in the Cameroon Cocoa Development Corporation (SODECAO) station at Mengang (82 km from Yaounde; 3°53′ NL, 12°03′ EL in the center region of Cameroon). Mengang station falls under a bimodal forestry zone recording an annual rainfall of 1500–2000 mm and an average temperature of 25 °C. The soil is ferralitic, clay in nature, and has an acid pH range of 5.2 to 5.7 (Tsozué et al. 2015). Three parental clones of different susceptibilities were used in this study, namely

ICS40 (susceptible to BPD and highly productive) of the Trinitario group, SCA12 (moderately resistant to BPD and productive), and T79/467 (resistant to BPD and less productive) both belonging to Forastero group (Ondobo et al. 2014; Manga et al. 2016). Four hybrid progenies were produced from reciprocal crosses through hand pollination: **F10**: (♀) T79/467 × (♂) SCA12, **F80**: (♀) SCA12 × (♂) T79/467, **F12**: (♀) SCA12 × (♂) ICS40, and **F40**: (♀) ICS40 × (♂) SCA12. Three-month old, light green (semi-ripened), and healthy cocoa leaves were used during the experiments.

Inoculum preparation and leaf inoculation

P. megakarya-Nkoem isolate (moderately aggressive) came from the Central Laboratory of Phytopathology at IRAD Nkolbisson Centre (Institute for Agricultural development). It was cultured on V8 medium (20% V8 vegetable juice, 0.3% $CaCO_3$, 250 mg of penicillin, 250 mg of ampicillin, 20 mg of nystatin, 2% agar–agar, and distilled water in a final volume of 1000 mL) and incubated in the dark at 25 ± 2 °C. *P. megakarya* sporangia were induced to liberate zoospores by adding sterile distilled water at 4 °C at room temperature for 1 h. The zoospore concentration was then adjusted to 3×10^5 zoospores/mL with a MALASSEZ hemocytometer (Nyasse et al. 1995; Efombagn et al. 2011).

Inoculations were carried out during the wet season. For each cocoa genotype used in the trial, twenty sterile young whole leaves (five per each side of the canopy: N, S, E, W) (Djocgoue et al., 2010) per tree were inoculated by depositing 150 µL of zoospore suspension on the midrib scarified with sterilized scalpel. After infection, scars were covered with a disc of filter paper (6 mm in diameter) placed on top of each drop to obtain a uniform-inoculated zone (Nyasse et al. 1995). Necrotic lesions appeared 2 days after inoculation, and the size of these lesions was measured every 2 days for 6 days (Ondobo et al., 2017). Uninoculated leaves were divided in two groups: (1) healthy leaves and (2) scarified leaves inoculated with 150 µL of sterile water.

Chemicals

Acetone (100%), acetic acid (99.8%), acetonitrile (99.9%), formic acid (99.8%), sulfuric acid (95%), methanol (99.9%), dichloromethane (100%), and cyclohexane were obtained from VWR (Leuven, Belgium). (-)-Epicatechin-4β-8-(-)-epicatechin (B2, 90%), (-)-epicatechin-4β-8-(-)-epicatechin-4β-8-(-)-epicatechin (C1, 99%), and kaempferol (≥ 90%) were furnished by PhytoLab GmbH & Co KG (Germany). (+)-Catechin (98%), (-)-epicatechin (98%), quercetin (95%), and quercetin 3-O-rhamnoglucoside (rutin) (95%) were from Sigma-Aldrich (Bornem, Belgium). HPLC-grade water was obtained from Milli-Q purification system (Millipore, Bedford, MA, USA). Penicillin, ampicillin, and nystatin were purchased in local drug store.

Phytochemical composition of cocoa genotypes

Polyphenol extraction was done in duplicate. Fifty milligrams of ground freeze-dried cocoa leaves were mixed twice with 1 mL of extraction solution (70% acetone, 28% water, 2% acetic acid) at room temperature during 1 h in the dark. The pooled supernatant phases were subjected to a triplicate washing with cyclohexane to eliminate lipids, carotenoids, and chlorophyll (Wieslaw et al. 1988). The total polyphenol (TPP) content was determined using the Folin-Ciocalteu method using a 1:10 dilution ratio for extracts (Georgé et al. 2005). The total flavonoid (TF) content was determined using the method described by Zou et al. (2004) with aluminum trichloride ($AlCl_3$). Finally condensed tannins (CT) or proanthocyanidin content was determined using the vanillin method involving sulfuric acid (Hagerman 2002). Dilution of raw extracts (1:2) with anhydrous methanol was performed before tannin quantitation. A Shimadzu UV-240 spectrophotometer was used for all the above quantitations. Final concentrations were expressed as (±)-catechin equivalent (CE) in mg/g dry weight (DW). All analyses were performed in triplicate.

Extraction of flavonoids for HPLC–DAD-ESI(-)-MS/MS analysis was done in duplicate (De Taeye et al. 2014; Eyamo et al. 2016). Ten grams of ground freeze-dried material were extracted with 3 × 100 mL 70% acidified acetone with kaempferol as internal standard (500 mg/kg as calculated for leaves). Solvent-free extracts were flushed with nitrogen and stored at − 80 °C before freeze-drying for 3 days. A 5000 mg/L leaf extract dissolved in solvent A (dichloromethane, methanol, acetic acid, water; 82:14:2:2; v/v/v/v) was prepared and filtered through a 0.45-µm PET acrodisc syringe filter for NP-HPLC analysis. Similar preparation with anhydrous methanol was prepared for RP-HPLC analysis.

HPLC–DAD-ESI(-)-MS/MS analysis of flavonoids

An AS3000 autosampler injected 5 µL of leaf extract in duplicate into the column kept at 20 °C. A P4000 quaternary pump eluted compounds at a flow rate of 200 µL/min. The compounds were monitored with a UV6000LP diode array set at 280 nm for flavanols and 360 nm for flavonols. The mass detector was operated in negative ion mode with an LCQ ion trap mass spectrometer equipped with an ESI source (Thermo Fisher). Collision-induced dissociation spectra of flavan-3-ols were recorded at 38%, 26%, and 22% collision energy for single-charged $[M\text{-}H]^{-1}$ ions of monomers (m/z 289), dimers (m/z 577), and trimers (m/z 865), respectively. For tetramers (m/z 1153),

pentamers (m/z 1441), and hexamers (m/z 1729), a 20% collision energy was applied. Dissociation spectra of flavonols were obtained at 45% collision energy for quercetin (m/z 301), 38% for quercetin-3-O-glucoside (m/z 463), 36% for quercetin-3-O-arabinoside (m/z 433), and 32% for quercetin-3-O-rhamnoglucoside (m/z 609). The ESI inlet conditions were as follows: source voltage 4.5 kV, capillary voltage − 4 V, capillary temperature 250 °C, and sheath gas 39 psi. Data collection and manipulation were controlled with the Xcalibur software version 1.2 (Thermo Fisher Scientific, Austin, TX, USA).

The system was flushed with either methanol or dichloromethane every morning before separating any sample solution. (i) *RP-HPLC analysis*: (+)-catechin, (-)-epicatechin, proanthocyanidin B2 (and isomers), and quercetin-3-O-rhamnoglucoside (and one isomer) were determined on a C18 Prevail column (150 × 2.1 mm, 3 µm) (Grace, Deerfield, IL, USA).

Elution was performed with the solvent system water-acetonitrile-formic acid 97:1:2 (v/v/v) (A) and acetonitrile-formic acid 98:2 (v/v) (B). Gradient elution was as follows: from 97 to 91% A in 5 min, from 91 to 85% in 25 min, from 85 to 64% in 35 min, from 64 to 10% in 10 min, and isocratic for 20 min. (ii) *NP-HPLC analysis*: quercetin and its monoglycosides, procyanidin C1, and flavan-3-ol pentamers were analyzed using an Alltima column (250 × 2.1 mm, 5 µm) with the solvent system dichloromethane-methanol-acetic acid–water (A) 82:14:2:2 (v/v/v/v) and (B) methanol-acetic acid–water 96:2:2 by volume. Gradient elution was as follows: from 100% A to 98% in 10 min, from 98 to 88% in 10 min, from 88 to 75% in 30 min, from 75 to 13% in 10 min, followed by isocratic elution with 100% A for 25 min. Quantitations of (-)-epicatechin, (+)-catechin, B2, C1, quercetin, and quercetin-3-O-rhamnoglucoside were done with reference calibration curves of pure commercial substances. The reference curves of the internal standard (kaempferol) was used for two quercetin heterosides (quercetin 3-O-arabinoside and quercetin 3-O-glucoside) and that of C1 for pentamers. Concentrations were expressed in mg/kg of individual flavonoids.

Statistical analysis

Data (necrotic length, TPP, TF, and CT) were subjected to analysis of variance (ANOVA) using IBM SPSS software 24th· version (SPSS, Inc., Chicago, IL, USA). Tukey's test was used to compare and separate the means ± standard deviation ($P < 0.05$). Principal component analysis (PCA) on necrosis length and bioactive components (TPP, TF, and CT) was realized with SPAD 5.5.

Results

Development of necrosis length on leaves and phytochemical characteristics of cocoa genotypes

The reaction of 19 cocoa genotypes (16 hybrids and three parents) to *P. megakarya* is presented in Table 1. Significant differences ($P < 0.05$) in lesion size were observed in all the genotypes 2, 4, and 6 days after inoculation (dai) (Fig. 1). 6 dai, smaller necrotic lesions were observed for partial resistant hybrids (F12.05, F12.07, F40.03, F40.05, F10.07, F10.08, F80.03, F80.08) with values ranging from 1.16 to 2 cm compared to the most partial resistance parent T79/467 (3.63 cm). Other hybrid genotypes with values ranging from 5.05 to 7.60 cm were classified as susceptible (F80.06, F10.05, F12.04, F12.02, F80.01, F10.09, F40.08, and F40.02) compared to the susceptible parent ICS40 (5.86 cm) (Table 1). The third parent SCA12 (4.70 cm) has an intermediate position.

The phytochemical traits in healthy and scarified leaves of cocoa genotypes did not vary significantly ($P < 0.05$), while these traits in healthy and inoculated leaves varied significantly ($P < 0.05$) among the partial resistant and susceptible genotypes (Table 1). In fact, partial resistant cocoa genotypes displayed higher accumulation of total polyphenol, total flavonoids, and condensed tannin content following infection of leaves. After inoculation with *P. megakarya*, these contents ranged from 8.17 to 21.75 for TPP, 2.07 to 6.50 for TF, and 1.08 to 5.22 for CT (mg CE/g DW) and were higher than in healthy leaves (Table 1). Constitutively, TPP, TF, and CT in the leaf of partial resistant genotypes were significantly higher than the susceptible ones.

A principal component analysis (PCA) based on the partial resistance level and phytochemical traits (PPT, FT, and CT) of the 19 cocoa genotypes was examined. The visualized dispersion represented about 94.01% (PC1 (87.17%) and PC2 (6.84%)) of variance of the studied system (Fig. 2). Three groups were differentiated and revealed a diagonal opposition between the development of the necrosis and phytochemical traits in the partial resistant (groups 2 and 3 with four genotypes each) and susceptible genotypes (group 1 made up of 11 genotypes) (Fig. 2). Owing to these observations, two hybrids with different phenolic profiles and sensitivity to infection with *P. megakarya* were selected for HPLC analysis of specific flavonoids. The F12.07 hybrid with low sensitivity to pathogen and higher polyphenolic contents was selected against the F40.02 hybrid with higher sensitivity and lower phenolic contents.

Table 1 Average necrosis length (6 dai) in the midrib and bioactive components of healthy, scarified, and infected cocoa leaf genotypes

Genotypes	Lesion size (cm)	Total polyphenols (mg CE/g DW)			Total flavonoids (mg CE/g DW)			Condensed tannins (mg CE/g DW)		
		Healthy	Scarified	Infected	Healthy	Scarified	Infected	Healthy	Scarified	Infected
Parents										
ICS40	5.86 ± 0.35 cd	7.93 ± 0.38 c*	8.10 ± 0.22 a*	10.03 ± 0.11 b**	2.70 ± 0.20 b*	2.82 ± 0.12 b*	3.70 ± 0.18 b**	1.10 ± 0.10 a*	1.52 ± 0.14 a*	2.20 ± 0.18 ab**
SCA12	4.70 ± 0.40 c	10.94 ± 0.17 e*	11.57 ± 0.11 c*	12.46 ± 0.39 c**	3.30 ± 0.10 bc*	3.55 ± 0.17 d*	5.60 ± 0.33 d**	0.90 ± 0.02 a*	1.03 ± 0.24 a*	2.30 ± 0.20 ab**
T79/467	3.63 ± 0.45 b	11.78 ± 0.49 f*	12.92 ± 0.35 cd*	17.19 ± 0.52 e**	2.40 ± 0.01 ab*	3.03 ± 0.21 cd*	6.50 ± 0.24 e**	1.20 ± 0.09 a*	1.58 ± 0.28 a*	2.80 ± 0.11 b**
F12.02	6.17 ± 0.36 d	6.96 ± 0.21 b*	7.51 ± 0.18 ab*	9.26 ± 0.35 ab**	2.30 ± 0.20 ab*	2.61 ± 0.11 b*	3.90 ± 0.19 b**	0.90 ± 0.07 a*	0.61 ± 0.06 a*	1.44 ± 0.09 a**
F12.04	5.93 ± 0.31 cd	7.87 ± 1.11 c*	8.35 ± 0.81 b*	11.52 ± 1.21 bc**	3.51 ± 0.43 bc*	3.22 ± 0.28 c*	3.50 ± 0.19 b*	1.01 ± 0.10 a*	1.13 ± 0.08 a*	1.60 ± 0.12 a*
F12.05	2.00 ± 0.38 a	12.03 ± 0.78 fg*	13.25 ± 0.48 cd*	18.66 ± 0.93 ef**	4.09 ± 0.57 c*	4.83 ± 0.26 d*	5.72 ± 0.78 d**	2.03 ± 0.07 b*	2.38 ± 0.33 b*	3.50 ± 0.14 c**
F12.07	1.16 ± 0.26 a	14.43 ± 0.21 h*	15.61 ± 0.19 de*	21.75 ± 0.49 g**	4.42 ± 0.11 c*	5.09 ± 0.32 de*	6.02 ± 0.24 e**	2.53 ± 0.12 b*	2.78 ± 0.12 c*	5.22 ± 0.42 e**
F40.02	7.60 ± 0.21 e	4.55 ± 1.12 a*	5.12 ± 0.39 a*	8.17 ± 0.63 a**	1.81 ± 0.52 a*	2.02 ± 0.01 b*	2.21 ± 0.67 a*	0.80 ± 0.01 a*	0.71 ± 0.07 a*	1.08 ± 0.07 a*
F40.03	1.91 ± 0.53 a	9.60 ± 0.25 d*	10.02 ± 0.27 bc*	14.49 ± 0.38 d**	4.60 ± 0.50 cd**	3.21 ± 0.19 c*	3.30 ± 0.15 b*	2.88 ± 0.21 b*	3.09 ± 0.15 b*	4.80 ± 0.20 d**
F40.05	1.90 ± 0.25 a	12.49 ± 0.17 fg*	14.54 ± 0.21 d*	18.28 ± 0.33 ef**	4.70 ± 0.37 cd*	4.83 ± 0.08 d*	5.10 ± 0.26 d*	2.13 ± 0.12 b*	2.99 ± 0.22 b*	5.90 ± 0.24 e**
F40.08	6.61 ± 0.26 d	6.22 ± 0.31 b*	6.91 ± 0.45 ab*	8.46 ± 1.02 a**	2.10 ± 0.19 ab*	2.07 ± 0.41 b*	2.94 ± 0.41 a*	0.60 ± 0.05 a*	1.01 ± 0.08 a*	1.71 ± 0.49 a**
F10.05	5.56 ± 0.54 de	7.53 ± 0.88 c*	8.12 ± 0.31 b*	10.06 ± 0.21 b**	1.52 ± 0.87 a*	1.55 ± 0.03 a*	2.07 ± 0.64 a**	0.80 ± 0.01 a*	1.21 ± 0.19 a**	1.60 ± 0.04 a**
F10.07	2.18 ± 0.43 ab	11.06 ± 0.11 e*	12.14 ± 0.12 c*	18.53 ± 0.16 ef**	2.30 ± 0.34 ab*	2.65 ± 0.33 b*	3.29 ± 0.13 b*	1.89 ± 0.50 ab*	2.26 ± 0.11 b*	3.03 ± 0.75 bc**
F10.08	3.55 ± 0.39 b	9.67 ± 0.14 d*	9.98 ± 0.09 bc*	11.55 ± 0.29 bc**	2.90 ± 0.27 b*	3.38 ± 0.07 c*	4.52 ± 0.36 c**	1.56 ± 0.82 ab*	1.87 ± 0.23 ab*	2.97 ± 0.51 b**
F10.09	6.19 ± 0.61 d	6.61 ± 0.27 b*	7.02 ± 0.14 ab*	8.65 ± 0.61 a**	1.80 ± 0.22 a*	1.89 ± 0.08 a*	2.05 ± 0.23 a*	0.70 ± 0.11 a*	0.79 ± 0.09 a*	1.20 ± 0.10 a**
F80.01	6.18 ± 0.30 d	7.74 ± 0.11 c*	7.97 ± 0.20 ab*	9.53 ± 0.19 ab**	1.74 ± 0.58 a*	1.81 ± 0.09 a*	2.09 ± 0.06 a*	0.90 ± 0.02 a*	1.01 ± 0.05 a*	1.60 ± 0.12 a**
F80.03	2.52 ± 0.16 ab	9.71 ± 0.53 d*	10.27 ± 0.71 bc*	13.25 ± 0.79 cd**	3.17 ± 0.30 bc*	3.25 ± 0.32 c*	4.50 ± 0.16 c**	1.97 ± 0.62 ab**	1.12 ± 0.14 a*	2.48 ± 0.17 ab**
F80.06	5.05 ± 0.44 cd	7.83 ± 0.94 c*	7.95 ± 0.71 ab*	8.68 ± 0.53 a*	1.88 ± 0.44 a*	1.94 ± 0.21 a*	2.23 ± 0.51 a**	0.70 ± 0.07 a*	0.88 ± 0.21 a*	1.30 ± 0.04 a**
F80.08	2.25 ± 0.47 ab	8.83 ± 0.29 d*	9.17 ± 0.18 bc*	11.77 ± 0.15 bc**	1.40 ± 0.10 a*	1.62 ± 0.12 b*	3.10 ± 0.16 b**	1.22 ± 0.21 a*	1.19 ± 0.22 a*	2.07 ± 0.06 ab**

Values in the same column with different letters were significantly different ($p < 0.05$) ($n = 6$). 6 dai = 6 days after infection

Values in the same line with different asterisk for the same bioactive component were significantly different ($p < 0.05$) ($n = 6$)

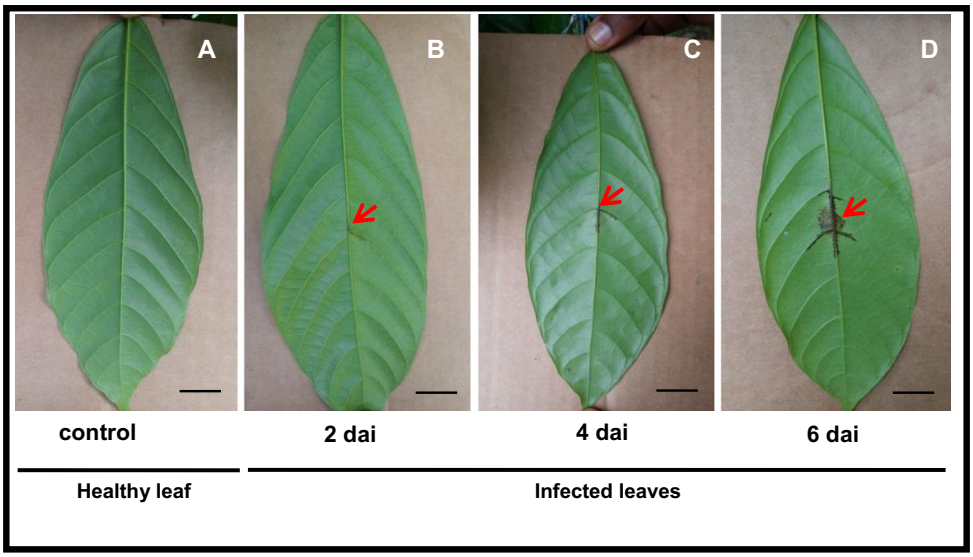


Fig. 1 Behavior of necrosis on cocoa leaves midrib (SCA12) following artificial inoculation in field. **A** Healthy leaf. **B** Infected leaf 2 days after inoculation. **C** Infected leaf 4 days after inoculation. **D** Infected leaf 6 days after inoculation. **dai,** day after infection. Black dash = 1 cm. The red arrow indicates the presence of *P. megakarya* on the leaves across the necrosis

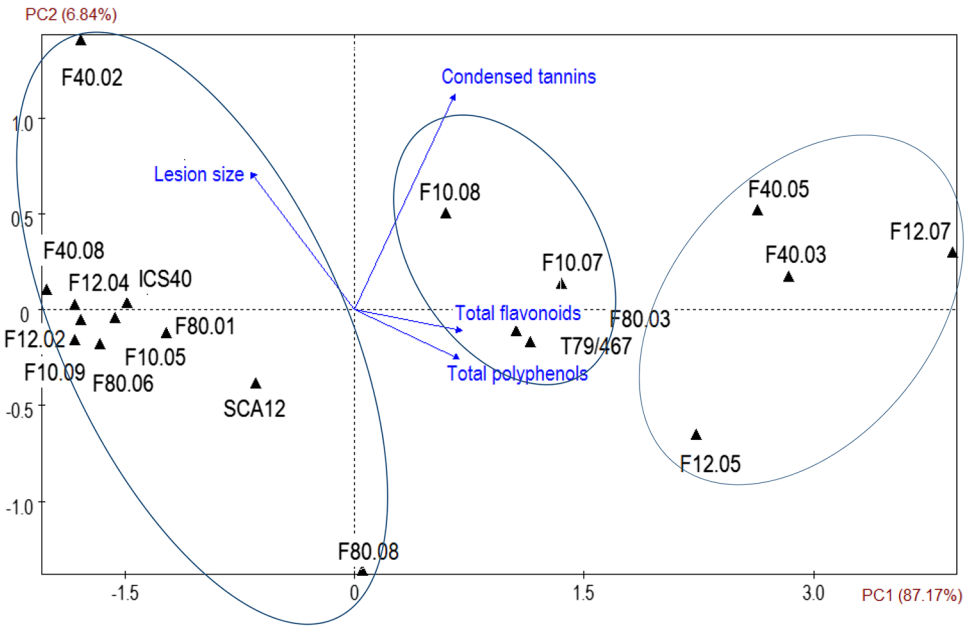


Fig. 2 A two-dimensional biplot of the principal component analysis based on necrosis length and bioactive components (total polyphenols, total flavonoids, and condensed tannins) on 19 cocoa genotypes. PC1, principal component 1; PC2, principal component 2

HPLC compound identification

Figure 3 summarizes the spectral data obtained by HPLC–MS/MS–ESI(-) for the identification of flavan-3-ols and flavonols. The spectrometric study of cocoa leaves through MS^2 fragmentation confirms a similar qualitative profile for all cocoa leaves, both healthy and infected. Nine compounds were clearly identified (by matching retention time and spectra of commercial standards) in different leaf extracts: two flavan-3-ol monomers (at m/z 289: (+)-catechin (peak 6) and (-)-epicatechin (peak 7)), three procyanidins ((at m/z 577: dimer B2 (peak 8), m/z 865: trimer C1 (peak 4), and m/z 1441: pentamers (peak 5)), and four flavonols (at m/z 301: quercetin (peak 1), m/z 433: quercetin-3-O-arabinoside (peak 2), m/z 463: quercetin-3-O-glucoside (peak 3), m/z 609: quercetin-3-O-rhamnoglucoside (peak 13)) (Fig. 3 and Table 2). In addition, four unknowns suspected to be dimers (m/z 577) were detected within healthy and infected leaves of all cocoa genotypes (peak 9 (Rt = 36.05 min), peak 10 (Rt = 40.22 min), peak 11 (Rt = 42.40 min), and peak 12 (Rt = 47.39 min)). One rutine (quercetin-3-O-rhamnoglucoside) isomer was also detected at m/z 609 (peak 14: Rt = 48.09 min) (Fig. 3 and Table 2).

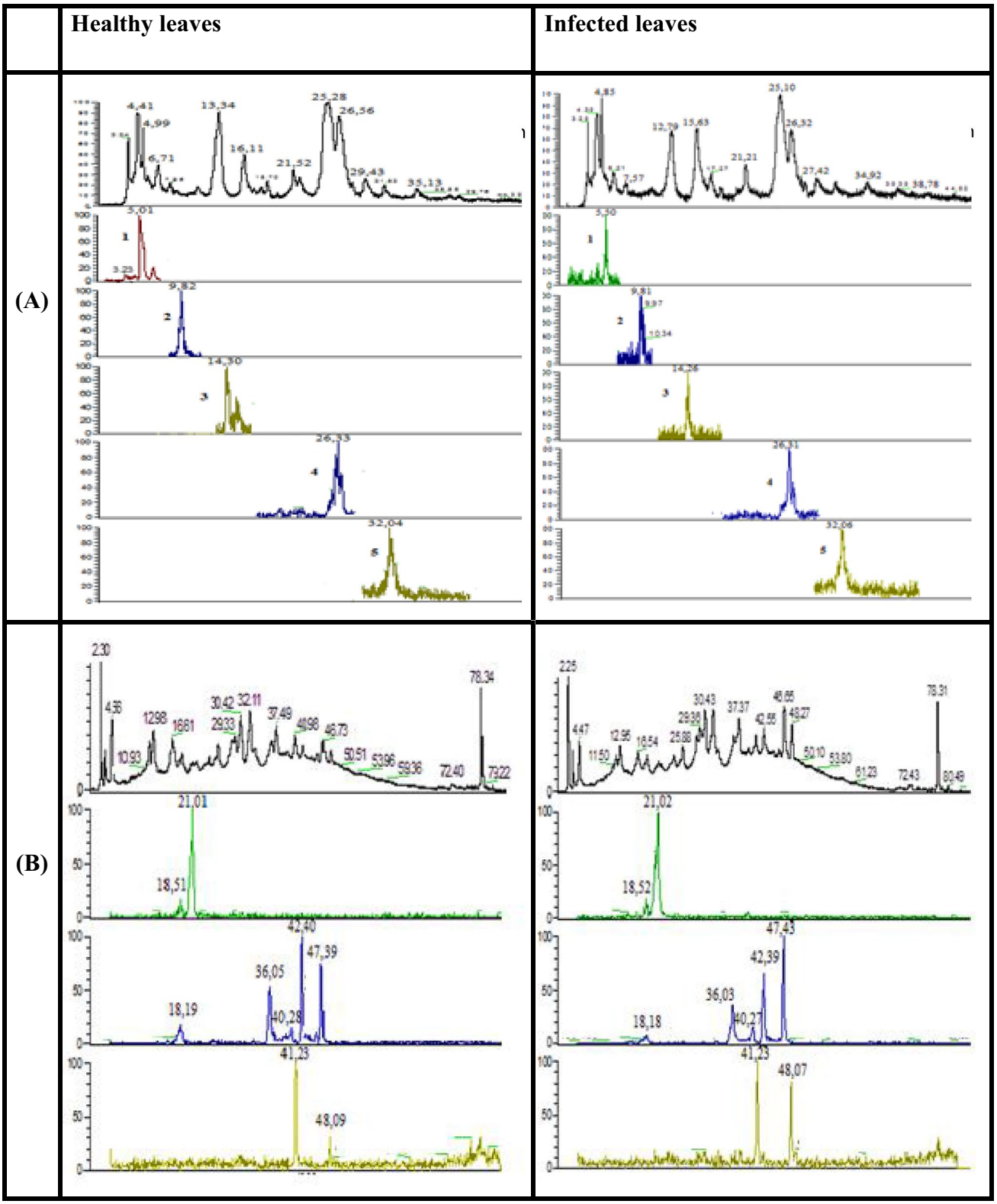
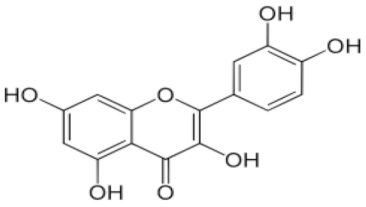
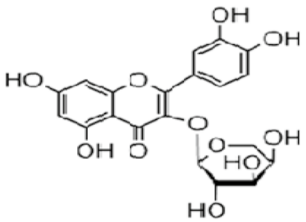
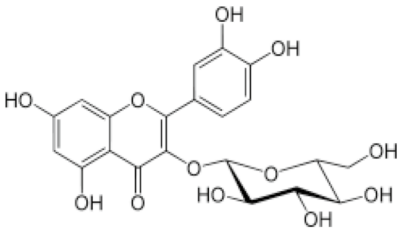
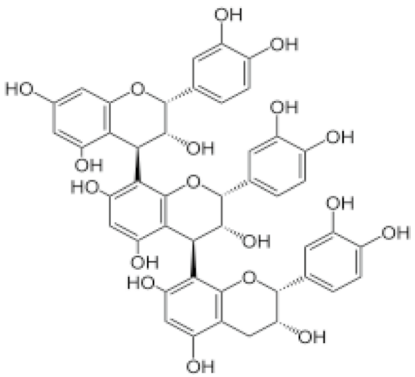


Fig. 3 Chromatograms of cocoa flavonoids from healthy and infected leaves of the F12.07 partial resistant hybrid using NP-HPLC–DAD-ESI(-)-MS/MS (A) and RP-HPLC–DAD-ESI(-)-MS/MS (B). The Arabic numeral reference of phytochemical compounds: 1 = quercetin, 2 = quercetin-3-O-arabinoside, 3 = quercetin-3-O-glucoside, 4 = procyanidin C1, 5 = pentamers, 6 = (+)-catechin, 7 = (-)-epicatechin, 8 = procyanidin B2, 9–12 = B2 isomers, 13 = quercetin 3-O-rhamnoglucoside, 14 = quercetin-3-O-rhamnoglucoside isomer

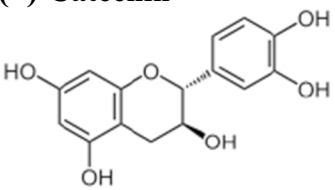
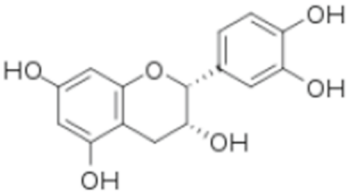
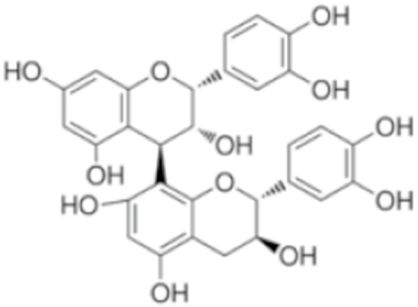
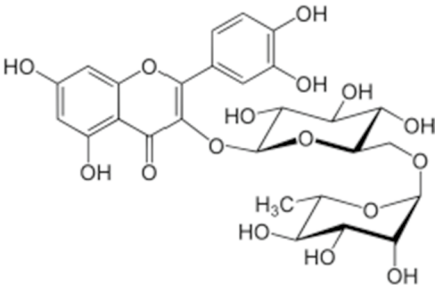
Table 2 Flavan-3-ols and flavonols detected in healthy and infected leaves of *T. cacao*. Peak number refers to the F12.07 HPLC chromatograms showed in Fig. 3

Peak	Rt (min)	$[M-H]^{-1}$ (m/z)	MS/MS ions m/z (%)	Name and structure
(1)	5.01	301	179 (100) 151 (44) 273 (18)	**Quercetin** 
(2)	9.82	433	300 (100) 301 (54) 433 (10)	**Quercetin-3-O-arabinoside** 
(3)	14.30	463	301 (100) 300(45) 463(4)	**Quercetin-3-O-glucoside** 
(4)	26.33	865	739 (100) 713 (64) 577 (58)	**Procyanidin C1** 
(5)	32.04	1441	1422 (100) 1315 (76) 865 (60)	**Pentamers**

HPLC quantitation of specific flavonoids

Following identification of nine flavonoids, quantitation was done using reference curves of authentic standards. The average distribution of flavan-3-ol monomers, procyanidin oligomers, and flavonols in cocoa leaves is presented in Fig. 4. The average (-)-epicatechin level in healthy leaves was 18.5 mg/kg, representing about 53% of the total flavan-3-ol content. The other flavan-3-ols were (with decreasing lower values): B2 (~ 9.3 mg/kg), C1 (~ 5.0 mg/kg), (+)-catechin (~ 2.3 mg/kg), and pentamers (~ 1 mg/kg). There were more quercetin-3-O-rhamnoglucoside (~ 7.4 mg/kg) than quercetin-3-O-arabinoside (~ 1.3 mg/kg) and quercetin-3-O-glucoside (0.7 mg/kg) in all cocoa

Table 2 (continued)

Peak	Rt (min)	$[M-H]^{-1}$ (m/z)	MS/MS ions m/z (%)	Name and structure
(6)	18.51	289	245 (100) 205 (30) 179 (14)	**(+)-Catechin** 
(7)	21.01			**(-)-Epicatechin** 
(8)	18.19	577	425 (100) 451 (35) 559 (20)	**Procyanidin B2** 
(9)	36.05		269(100), 577(20)	**B2 isomers** (unknowns)
(10)	40.28		457(100), 353(60)	
(11)	42.40		413(100), 293(30)	
(12)	47.39		457(100), 353(60)	
(13)	41.23	609	301 (100) 300 (56)	**Quercetin-3-O-rhamnoglucoside** 
(14)	48.09		300 (100) 299 (60) 301 (25)	**Quercetin-3-O-rhamnoglucoside isomer** (unknowns)

Rt retention time; %, relative abundance of the ions obtained after fragmentation

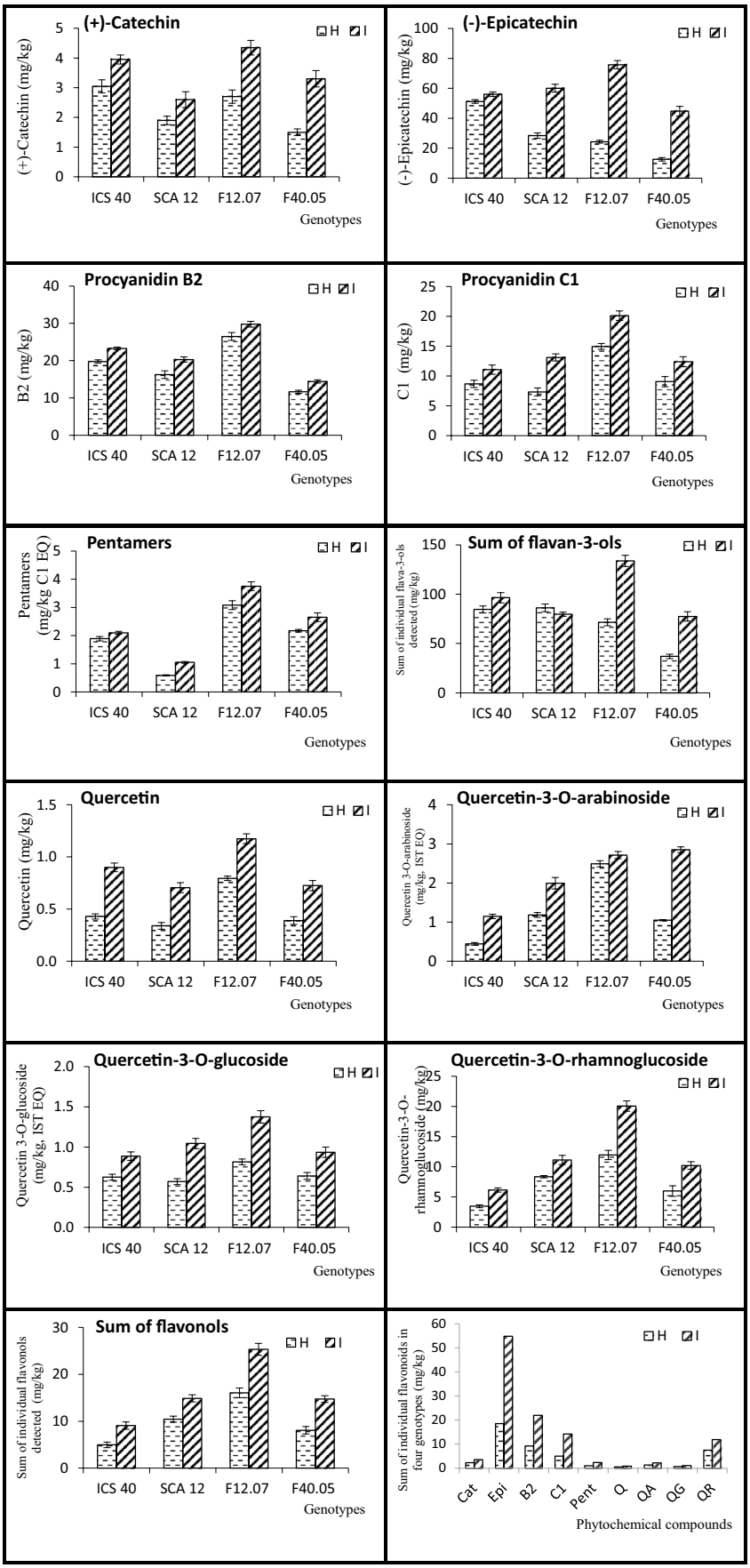


Fig. 4 Phytochemical contents in cocoa leaves from ICS40, SCA12, F12.07, and F40.05 genotypes with bar with horizontal line and letter H that indicates healthy leaves and bar with diagonal line and letter I that indicates infected leaves

leaves. Free quercetin (~0.5 mg/kg) was found to be less present in our samples.

For all the flavonoids here investigated, the mean increase in infected leaves (calculated for all cocoa genotypes) was above 50%. This enrichment reached 196% for (-)-epicatechin, 184% for procyanidin C1, 135–140% for procyanidin B2 and pentamers, 80% for quercetin, and 57–69% for (+)-catechin, quercetin-3-O-arabinoside, quercetin-3-O-rhamnoglucoside, and quercetin-3-O-glucoside (Fig. 4). This increment was generally higher in the F12.07 hybrid than the other genotypes.

Discussion

Developing *T. cacao* genotype partial resistance to BPD can be done through the reciprocal crosses of target parental genotypes and selection of progenies with desirable traits using reliable test and markers of resistance. This study investigates the susceptibility to BPD of three genitors (ICS40, SCA12, and T79/467) and hybrids of their four progenies (F12, F40, F10, and F80) produced from reciprocal crosses through hand pollination. The physical perception of infection was examined in field through the size of necrotic lesions on leaves, due to the presence of *P. megakarya* oomycete. Hybrids with lower necrotic length relative to the best parent T79/467 were considered partial resistant, while hybrids with higher necrotic length than the susceptible parent ICS40 were considered susceptible to BPD. This is in accordance with the findings obtained by Ondobo et al. (2014) in nursery and by Manga et al. (2016) in nursery and field on leaves attached to the plant (healthy, scarified, scarified, and inoculated). Similar observations were made on detached cocoa leaves (healthy, scarified, scarified, and inoculated) by Boudjeko et al. (2007) and on pepper (*Capsicum annuum* L.) leaves (scarified and inoculated) by Koç and Üstün (2012).

Among the 19 cocoa genotypes (three clones and their sixteen progenies) studied, eight hybrids (F12.07, F12.05, F40.03, F40.05, F10.07, F10.08, F80.03, and F80.08) exhibited lower lesion size and higher phenolic content (TPP, TF, CT) as compared to their genitors. Yet it is worth noting that among the three parental clones, T76/467 was resistant to BPD, while ICS40 and SCA12 were susceptible or moderately resistant. Authors who studied the heritability of resistance level to *P. megakarya* in two hybrid populations of *T. cacao* suggested the absence of maternal effect in the developmental of necrotic lesions (Djocgoue et al. 2007; 2010). The eight partial resistant hybrids exhibited hybrid vigor (heterobeltiosis) with reference to the better parent as described by Djocgoue et al. (2010), Nyadanu et al. (2012), and Manga et al. (2018). Hybrid vigor manifestation would be due to the presence of the additive and dominant gene effect in transmission of character.

Oomycete growth is arrested when exposed to effective concentrations of antifungal compounds (Morrissey and Osbourn 1999; Manga et al. 2018). Various defense mechanisms have been enumerated in the literature: hypersusceptible response (Camagna and Takemoto 2018), the formation of papilla which serves as barrier to pathogen propagation, strengthening of the cell wall (Beraldo-Hoischen et al. 2021), oxidative species scavenging, and production of secondary metabolites (Tasiu 2019). Polyphenols are known as bioactive compounds that help to slow down disease progression in plants. The accumulation of TPP, TF, and CT revealed here to be higher in the partial resistant genotypes than the susceptible ones ($P < 0.05$), in agreement with previous reports (Nyadanu et al. 2012; Ofori et al. 2015; Minyaka et al. 2017). Monomeric flavan-3-ols (catechin, epicatechin), procyanidins (B2, C1 and pentamers), and flavonols (quercetin, quercetin-3-O-arabinoside, quercetin-3-O-glucoside, quercetin-3-O-rhamnoglucoside) here identified both in healthy and infected leaves of all our cocoa genotypes could participate to the partial resistance of *T. cacao* against invasion of *P. megakarya*. For all bioactive compounds studied, partial resistant cocoa genotypes displayed higher accumulation of compounds in healthy and infected leaves. Similar results were previously observed in resistant hybrids of cocoa with two flavones (luteolin and apigenin derivatives) and some hydroxycinnamic acid derivatives (Boudjeko et al. 2007; Djocgoue et al. 2007). On the other hand, our findings contrast those of Minyaka et al. (2017) who showed that a high content of apigenin-hexosides was associated with susceptible hybrid genotypes. Inversely, luteolin, a rutinoside isomer, isoorientin, and apigenin-pentosyl-hexoside were only found in infected resistant hybrid genotypes. Besides their oxidation into quinines (Pourcel et al. 2007), the metal-binding properties of procyanidins through O-diphenol group complexation was suggested to be an important mechanism of antimicrobial activity (Dixon et al. 2005). Some authors also suspect the implication of flavan-3-ols ((-)-epicatechin and B2/C1 derivatives) in the resistance of cocoa pod cortex against *P. megakarya* (Nana et al. 2011).

(-)-Epicatechin level revealed to be the most impacted by leaf infection, suggesting that this compound could be a key determinant in cocoa defense versus *P. megakarya*. Monomers in uninfected cocoa leaves represent about 30% of total flavan-3-ols (Chaves and Gianfagna 2007). Cocoa young leaves have higher concentrations of soluble procyanidins than seeds, which were previously considered being the major source of polyphenols in chocolate (Liu et al. 2013). Cocoa leaf, flower, or fruit procyanidins are composed almost entirely of epicatechin units (only 0.5 to 2% catechin units) (Liu et al. 2013). These authors isolated

three genes (*TcANR*, *TcANS*, *TcLAR*) encoding flavan-3-ol synthesis enzymes and hypothesized that free epicatechin could be issued from non-stereospecific depolymerization of procyanidins. Investigations on expression of genes involved in flavonoid biosynthetic pathways of different genotypes tissues and treatments should be carried out to better understand the intra- and inter-specific variations of these metabolites in cocoa.

As expected, crossing between Trinitario (ICS40) and Forastero (SCA12) gives hybrids that generally show much better partial resistance to disease compared to hybrids derived from crossings involving two Trinitario or Forastero (Djocgoue et al. 2007, 2010; Effa et al. 2016; Ondobo et al. 2017). Of course, plant biochemical contents vary according to geographical origin, level of maturity, climatic conditions, or cultivar. Researchers showed that cocoa beans of the Forastero group from Brazil exhibited the highest content of individual flavan-3-ols and anthocyanins, while the samples of the Trinitario type from Papua New Guinea had the lowest polyphenolic levels (Oracz et al. 2015). Also, genetic origin of the male parent influences agronomic traits and phytochemical quality of cocoa hybrids (Akoa et al. 2021).

Flavan-3-ol and flavonol levels in cocoa leaves increase following infection with *P. megakarya*, but infection did not trigger the synthesis of new compounds. The accumulation of flavan-3-ols and flavonols in *T. cocoa* hybrids was genotype dependent. The reciprocal crossing of ♀SCA12 and ♂ICS40 generates hybrid genotypes more partial resistant to BPD. A breeding approach using crosses between Forastero and Trinitario partial resistant cocoa genotypes could help to produce partial resistant progenies with high contents of bioactive components.

Acknowledgements The authors express their gratitude to the Cameroon Cocoa Development Corporation (SODECAO) for the field used. This research was supported by the Agence Universitaire de la Francophonie (AUF) via a grant to Jules Christian Djoko Kouam (Grant n° S0020ADM10507L).

Author contribution All authors contributed to the study conception and design. Jules Christian Djoko Kouam produced the samples, did the lab experiment, and wrote original graft. Jude Manga Ndjaga produced the samples and analyzed the data (software). Simon Perrez Akoa produced the samples. Sonia Collin, Pierre Effa Onomo, and Pierre François Djocgoue supervised this work, research activity planning, and execution. Martine Louise Ondobo and Nicolas Niemenak provided the study materials.

Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Conflict of interest The authors declare no competing interests.

References

Akoa SP, Effa PO, Manga JN, Ondobo ML, Djocgoue PF (2021) Impact of pollen genetic origin on compatibility, agronomic traits, and physicochemical quality of cocoa (*Theobroma cacao* L.) beans. Sci Hortic 287:110278

Beraldo-Hoischen P, Hoefle C, López-Sesé AI (2021) Fungal development and callose deposition in compatible and incompatible interactions in melon infected with powdery mildew. Pathogens 10(7):873

Boudjeko T, Djocgoue PF, Nankeu DJ, Mbouobda HD, Omokolo ND, El Hadrami I (2007) Luteolin derivatives and heritability of resistance to *Phytophthora megakarya* in *Theobroma cacao* Australasian Plant Pathology 36:56–61

Camagna M, Takemoto D (2018) Hypersusceptible Response in Plants. eLS 1–7

Chan CH, Ngoh GC, Yusoff R (2013) Power and energy requirement for microwave-assisted extraction of antidiabetic compounds from cocoa leaves. The 6th AUN/SEED-Net regional conference on Chemical Engineering, Century Park Hotel, Manila, Philippines NPR2

Chaves FC, Gianfagna TJ (2007) Cacao leaf procyanidins increase locally and systemically in response to infection by *Moniliophthora perniciosa* basidiospores. Physiol Mol Plant Pathol 70:174–179

Conrath U, Pieterse CM, Mauch-Mani B (2002) Priming in plant-pathogen interactions. Trends Plant Sci 7(5):210–216

De Taeye C, Cibaka ML, Jerkovic V, Collin S (2014) Degradation of (−)-epicatechin and procyanidin B2 in aqueous and lipidic model systems. First evidence of "chemical" flavan-3-ol oligomers in processed cocoa. J Agric Food Chem 62:9002–9016

Dixon RA, Xie DY, Sharma SB (2005) Proanthocyanidins - a final frontier in flavonoid research? New Phytologist 165:9–28

Djocgoue PF, Boudjeko T, Mbouobda HD, Nankeu DJ, Hadrami E, Omokolo DN (2007) Heritability of phenols in the resistance of *Theobroma cacao* against *Phytophthora megakarya*, the causal agent of black pod disease. J Phytopathol 155:519–525

Djocgoue PF, Simo C, Mbouobda HD, Boudjeko T, Nankeu DJ, Omokolo ND (2010) Assessment and heritability of productivity and partial resistance level to *Phytophthora megakarya* in two hybrid populations of *Theobroma cacao*. J Plant Pathol 92(3):607–617

Effa PO, Akoa SP, Ondobo ML, Djocgoue PF (2016) Assessment of partial resistance level to *Phytophthora megakarya* in four hybrid populations of *Theobroma cacao* L. International Journal of Biosciences 3:53–64

Efombagn MIB, Bieysse D, Nyassé S, Eskes AB (2011) Selection for resistance to Phytophthora pod rot of cocoa (*Theobroma cacao* L.) in Cameroon: repeatability and reliability of screening tests and field observations. Crop Prot 30(2):105–110

Elwers S, Zambrano A, Rohsius C, Lieberei R (2009) Differences between the content of phenolic compounds in Criollo, Forastero and Trinitario cocoa seed (*Theobroma cacao* L.). Eur Food Res Technol 229:937–948

Febrianto NA, Zhu F (2019) Intravariety diversity of bioactive compounds in Trinitario cocoa beans with different degrees of fermentation. J Agric Food Chem 67:3150–3158

Eyamo EVJ, De Taeye C, Niemenak N, Youmbi E, Collin S (2016) Influence of acetic and lactic acids on cocoa flavan-3-ol degradation through fermentation-like incubations. LWT- Food Sci Technol 68:514–522

Georgé S, Brat P, Alter P, Amiot MJ (2005) Rapid determination of polyphenols and vitamin C in plant-derived products. J Agric Food Chem 53:1370–1373

Hagerman AE (2002) Acid butanol assay for proanthocyanidins. Tannin Handbook, Philadelphia, USA. Retrieved from https://www.academia.edu/25605508/The_Tannin_Handbook

Irondi EA, Olanrewaju S, Oboh G, Olasupo F, Boligon AA (2017) Inhibitory potential of cocoa leaves polyphenolics-rich extract on xanthine oxidase and angiotensin 1-converting enzyme. Journal Biochemistry Active Products Nature. https://doi.org/10.1080/22311866.2017.1293564

Koç E, Üstün AS (2012) Influence of Phytophthora capsici L. inoculation on disease severity, necrosis length, peroxidase and catalase activity, and phenolic content of resistant and susceptible pepper (*Capsicum annuum L.*) plants. Turkish J Biology 36(3):357–371

Liu Y, Shi Z, Maximova S, Payne MJ, Guiltinan MJ (2013) Proanthocyanidin synthesis in *Theobroma cacao*: genes encoding anthocyanidin synthase, anthocyanidin reductase, and leucoanthocyanidin reductase. BMC Plant Biol 13:202

Manga NJ, Effa OP, Ondobo ML, Djoko KJC Djocgoue PF (2016) Heritability of the partial resistance to *Phytophthora megakarya* Bras. and Grif. of *Theobroma cacao* L. in terms of their necrosis length, phenolic contents and activity of oxidative enzymes. International Journal Biosciences 8: 249-261

Manga NJ, Ondobo ML, Effa PO, Djoko KJC, Djocgoue PF (2018) Heterosis, heterobeltiosis, narrow-sense and broad-sense in two populations of *Theobroma cacao* L. Afr J Biotechnol 7(14):495–504

Minyaka E, Simo C, Kusznierewicz B, Madina BCV, MouenPiau JC, Niemenak N, Omokolo ND (2017) Flavones in cocoa defence against *Phytophthora megakarya.* Journal of Botanical Sciences 6(3):50–63

Morrissey JP, Osbourn AE (1999) Fungal resistance to plant antibiotics as a mechanism of pathogenesis. Microbiol Mol Biol Rev 63(3):708–724

Motamayor JC, Lachenaud P, Da Silva Mota JW, Loor R, Kuhn DN (2008) Geographic and genetic population differentiation of the Amazonian chocolate Tree (*Theobroma cacao* L.). PLoS One 3(10):33–11

Nana WL, Tchameni NS, Fokom R, Etoa FX, Debost M (2011) Flavonoïd compounds synthesis by cocoa Fruits (*Theobroma cacao* L.) in response to Phytophthora megakarya infection. Res J Agric Biol Sci 7(3):335–342

Nyadanu D, Akromah R, Adamako B, Kwoseh C, Lowor ST, Dzahini-Obiatey H, Akrofi AY, Owusu AF, Yaw OA, Assuah MK (2012) Biochemical mechanisms of resistance to black pod disease in cocoa (*Theobroma cacao* L.). Am J Biochem Mol Biol 3:20–37

Nyasse S, Cilas C, Herail C, Blaha G (1995) Leaf inoculation as an early screening test for cocoa (*Theobroma cacao* L.) resistance to Phytophthora black pod disease. Crop Prot 14(8):657–663

Nyasse S, Efombagn MIB, Kebe BI, Tahi M, Despreaux D, Cilas C (2007) Integrated management of *Phytophthora* diseases on cocoa (*Theobroma cacao* L.): impact of plant breeding on pod rot incidence. Crop Prot 26:40–45

Ofori A, Padi FK, Ameyaw GA, Dadzie AM, Lowor S (2015) Genetic variation among cocoa (*Theobroma cacao* L.) families for resistance to cocoa swollen shoot virus disease in relation to total phenolic content. Plant Breed 134:477–484

Ondobo ML, Effa PO, Djocgoue PF, Manga NJ, Boudjeko T, Omokolo ND (2014) Phenolic content and heritability of resistance in four hybrid populations of *Theobroma cacao* L. after leaves inoculation with *Phytophthora megakarya* Bras. and Grif. Int J Biol Chem Sci 8(1):17–30

Ondobo ML, Effa PO, Manga NJ, Djoko KJC, Djocgoue PF, Omokolo ND (2017) Breeding of parental and partial resistance hybrids of *Theobroma cacao* L. to *Phytophthora megakarya* Bras. and Griff. Int J Plant Physiol Biochem 9(2):9–21

Oracz J, Nesbeny E, Zyzelewicz D (2015) Changes in the flavan-3-ols, anthocyanins, and flavanols composition of cocoa beans of different *Theobroma cacao* L. groups affected by roasting conditions. Eur Food Res Technol 241:663–681

Pagan I, Garcia-Arenal F (2020) Tolerance of plants to pathogens: a unifying view. Annu Rev Phytopathol 58:9.1–9.20

Pourcel L, Routaboul JM, Cheynier V, Lepiniec L, Dedbeaujon I (2007) Flavonoid oxidation in plants: from biochemical properties to physiological functions. Trends Plant Sci 12:29–36

Tasiu I (2019) Stress and defense responses in plant secondary metabolites production. Biol Res 52:39

Tsozué D, Nghonda JP, Mekem DL (2015) Impact of land management system on crop yields and soil fertility in Cameroon. Solid Earth 6:1087–1101

Wieslaw O, Chang YL, Antoni WJ, Keith RP (1988) Identification of some phenolic compounds in apples. J Agric Food Chem 36(3):430–432

Zou Y, Lu Y, Wei D (2004) Antioxidant activity of a flavonoid-rich extract of *Hypericum perforatum* L. *in vitro* J Agric Food Chem 52:5032–5039