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Flavones in Cocoa Defence against *Phytophthora megakarya*

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Research Article

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ABSTRACT

Cross-pollination of suitable genotypes and earlier selection of elite offspring using adequate plants-associated defence markers in plantlets leaves are decisive for development of T. cacao genotypes tolerant to black pods disease (BPD). T. cacao plantlets from manual cross-pollination of ♀SNK64 × ♂UPA143 were analyzed for their susceptibility to BPD using leaf disc test. Subsequently, leaves (healthy, wounded and wound+infected) of selected E9 (tolerant), E13 (moderate susceptible) and E32 (most susceptible) young hybrid genotypes from ♀SNK64 × ♂UPA143 were used for flavones analysis using a HPLC/LC/MS system. Leaf disc test showed 62.5% of the progeny with disease score rates lower than the mean value of both parents. About 79.17% and 20.83% of the progeny were lesser susceptible than the most susceptible parent (UPA143) and the tolerant parent (SNK64) respectively. Total flavones contents were abiotic and biotic stresses-dependant. Under infection, tolerant hybrid genotype (E9) displayed the highest flavones contents, which might control tolerance to BPD. Individual flavones analysis revealed differential patterns depending on flavones, treatment and hybrid genotype. Luteolin rutinoside isomer (tR=12.5), isoorientin (tR=12.0) and apigenin-pentosyl-hexoside (tR=10.9) appeared to be characteristic of tolerant hybrid genotype (E9) during infection. Reversely, high content of Apigenin-hexoside (tR=10.1) and apigenin-hexoside (tR=11.6) might be associated to the susceptible hybrid genotypes (E13) and(E32). Hence, ♀SNK64 × ♂UPA143 could be used to develop hybrid genotypes tolerant to BPD. Pools of above flavones, reported here for the first time in T. cacao defence, might be useful markers to develop T. cacao hybrid genotypes tolerant to BPD.

INTRODUCTION

Disease (black pods disease) and pest incidences are the most stumble stones of cocoa productivity and profitability in many African cocoa producing countries [1-4]. High-yielding varieties which are tolerant to diseases are missing; it has been challenging to develop such genotypes combining both features. For decades, farmers attempted to deal with the harmful effect of *P. megakarya* (causal agent of black pods disease) on *T. cacao* by the constant application of synthetic pesticides [5,6]. However, this approach is not cost effective; it also has detrimental impact on farmers' health and environment [7]. For this reason, development of sustainable practices which are environmental friendly for disease management in cocoa plantations are gaining ground. These practices usually include the development of *T. cacao* genotypes genetically tolerant to black pods disease [8,9]. Genetic improvement (breeding for pathogen resistance and productivity) of cocoa through generative strategy is mostly used [10,11]. This strategy is based on the aptitude of a given couple of parents (genotypes or clones) to generate progeny with desirable traits. Unfortunately, many couples of cocoa clones are not yet tested in this purpose, such as ♀SNK64 × ♂UPA143. SNK64 was reported to be less sensitive and to black pod disease and less productive; while UPA143 is known to be moderate sensitive to black pod disease and highly productive [12].

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The selection of progeny (from a given couple) with tolerant trait can be earlier monitored via the analysis of metabolites markers of tolerance in young plant leaves. Phytoalexins pools, namely flavonoids in general are reported to be markers of tolerance in plant defence against biotic stress [13]. However, there are more than 6000 flavonoids already identified with a wide range of biological functions: protection against ultraviolet radiation and phytopathogens, signalling during nodulation, male fertility, auxin transport, as well as the coloration of flowers [14,15].

Flavones, a sub-group of flavonoids, are a large group of metabolites with different biological functions in higher plants. These compounds are considered as endogenous antibiotic (antimicrobial) in plant protection against pathogens: nematodes, molluscs, fungi, oomycetes, and bacteria [16].

Research papers reported the implication of phenol compounds in *T. cacao* defense against *P. megakarya* in general, but don't mention flavones or types of flavones specifically in *T. cacao* defence mechanism against *P. megakarya* infection. Little is known about the implication of flavones in *T. cacao* tolerant against black pod disease caused by the oomycete *P. megakarya*.

The present research aims to evaluate the implication of flavones in *T. cacao* defence against *P. megakarya* for their potential use as earlier selection markers for *T. cacao* genotypes tolerant to black pods disease. Firstly, the study evaluated the susceptibility to black pods disease of a *T. cacao* progeny (from ♀SNK64 × ♂UPA143) using leaf disc test according to adapted method by Nyassé et al. [12]. Subsequently, quantitative and qualitative analysis of flavones were conducted in healthy (untreated), wounded and wounded+infected (with mycelia of *P. Megakarya*) leaves from young plants of three types of hybrid genotypes (from ♀SNK64 × ♂UPA143) different in their susceptibility to black pod disease (less, moderate and highly sensitive to black pod disease).

MATERIALS AND METHODS

T. Cacao Plant Material

T. cacao seeds from mature pods of ♀SNK64 × ♂UPA143 obtained by manual pollination at Barombi Kang (South-West Region, Cameroon) seeding farm were used to set up a nursery. Leaves of four months old plants of full sib progeny from ♀SNK64 × ♂UPA143 (of the above nursery) were used for leaf disc test, total and individual flavones families analysis.

P. megakarya Isolate Used to Test the Susceptibility of ♀Snk64 × ♂Upa143 Progeny

To test the susceptibility of full sib progeny from ♀SNK64 × ♂UPA143, middle aggressive isolate was freely offered to us by the Plant Pathology Laboratory from the IRAD (Institut de Recherche Agricole pour le Development) of Nkolbisson (Yaoundé, Cameroon). In our laboratory (Biochemistry Laboratory, Faculty of Sciences, University of Douala–Cameroon), this *P. megakarya* isolate has been preserved in the laboratory by frequently subcultures on 1.5% (w/v) pea-based agar medium. To maintain its virulence, the isolate was periodically inoculated onto cocoa pods.

Zoospore Production

Zoospores (or inoculums) were obtained according to adapted method. Zoospores were obtained from l0-days-old cultures. Cultures with sporangia were induced to liberate zoospores by adding sterile distilled water at 4°C. After 1 hour at room temperature, the zoospore concentration was adjusted to 3×10^5 zoospores/mL with a MALASSEZ hemati-meter.

Screening for Susceptibility of ♀SNK64 × ♂UPA143 Progeny to *P. Megakarya*

Leaf discs test used for screening was adjusted to method by Nyassé et al. [12]. The experimental design was made of three replicates and completely randomized 7 blocks of leaf discs (Ø=1.5 cm) per hybrid genotype. Hence, a total of 28 discs were used per hybrid. For each hybrid of the progeny, leaf discs were obtained from the slightly lignified young leaves (2.5-3 months old). Leaf discs were placed in trays and pre-incubated for 24 hours at 25 ± 1°C in darkness prior to inoculation. After the 24 hours, leaf discs were inoculated by depositing 10 µL ($3 \times l0^5$ zoospores/mL) of zoospores suspension on either side in the middle of each leaf disc and incubated in darkness (at 25 ± 1°C). The scoring (from 0: tolerant to 5: highly sensitive) of susceptibility (through the necrosis size) of each leaf discs (for each hybrid) was registered on 4th, 5th, 6th, 7th and 8th day after inoculation.

Inoculation of Whole Leaves for Flavones Pools Analysis

Whole leaves (2.5-3 months old) from E9, E13 and E32 hybrid genotypes different in their disease score (susceptibility) were arvested early in the morning. After harvesting, leaves were washed successively with tap and distilled sterilized water prior to re-incubation for 24 hours in darkness (25 ± 1°C) on trays. After the 24 hours (for each hybrid genotype) leaves were separated nto three groups: healthy (untreated) leaves, wounded leaves and wounded+infected leaves. Wounded+infected leaves were noculated with mycelium disc (Ø=0.5 cm) from 5-days-old cultures. Untreated and wounded leaves groups received a disc (Ø=0.5 m) of agar without mycelium. The three groups of leaves from the three hybrid genotypes were then incubated for 3 days in arkness at 25 ± 1°C. After the incubation, leaves were lyophilized and used for flavones analysis.

lavones Extraction and Analysis

The extraction and analysis of flavones were done on three groups of leaves (untreated, wounded and wounded+infected) arvested from three *T. cacao* hybrid genotypes (E9, E13 and E32) differed in their susceptibility (disease scores) to *P. megakarya*.

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E9 (disease score=0) is the tolerant hybrid genotype, E13 (disease score=2.6) moderate susceptible hybrid genotype and E32 (disease score=4.8) the most susceptible hybrid genotype.

Chemicals

Luteolin 6-C-glucoside (isorientin) and apigenin-6-C-glucoside (isovitexin) were purchased from LGC Standards. Methanol and formic acid were obtained from Merck. All solvents were of HPLC grade, and water was of Milli-Q quality.

Extracts Preparation

Lyophilized *T. cacao* leaves were ground in grinder and powders (60 mg) were placed in centrifuge tubes. To each tube, 1 mL of methanol was added and the samples were sonicated for 30 min. The samples were centrifuged (3000 rpm, 15 min) and clear supernatants collected. The extraction procedure was repeated with a new portion of solvent (1 mL). The supernatants from two repeated extractions were combined to give about 2 mL of the final extract.

LC-DAD-ESI-MS Analysis

Phenolic compounds in the methanolic leaves extracts were characterized using an Agilent 1200 series HPLC system with DAD detector and ESI interface and mass spectrometer (MS) (Agilent 6130 Quadrupole LC/MS). Chromatographic separation was carried out using Phenomenex Kinetex XB-C18 100A column (150 × 4.6 mm, 5 µm particle size). The resolution of phytochemicals was carried out using a mobile phase composed of 0.1% formic acid in water (solvent A) and 0.1% formic acid in methanol (solvent B) at a flow rate of 0.8 mL/min; the injection volume of all samples was 10 µL. The elution gradient profile used was 30-50% B in 15 min, 50-100% B in 5 min followed by 10 min 100% B. The column was allowed to equilibrate between the injections for 5 min with initial concentration of mobile phase. Absorbance spectra were recorded between 190 and 700 nm every 2 s with a bandwidth of 4 nm, while the chromatograms were monitored at 270 and 335 nm. MS parameters were as follows: capillary voltage, 3000 V; fragmentor, 120 V; drying gas temperature, 350°C; gas flow (N_2), 12 L/min; nebulizer pressure, 35 psig. The instrument was operated both in positive and negative ion modes, scanning from m/z 100 to 1000. Individual phenolic compounds were identified by comparing their retention times with those for standards or on the basis of available literature data and UV and mass spectra. Since reference compounds were not available for most of the detected phenolics, luteolin glycosides were quantified as isoorientin and apigenin glycosides as isovitexin (at 335 nm). Total and individual flavones contents are expressed in mg per gram of dry weight of cocoa leaves (mg/g of dw).

Data Analysis

Data were represented as the mean ± standard deviation (SD) of at least three independent experiments. All of the statistical analyses were conducted using SPSS 20.0 software (SPSS, Inc., Chicago, IL, USA). The one-way analysis of variance (ANOVA) with Student-Newman-Keuls tests was used to compare differences between means when significant F values were observed at $p<0.05$.

RESULTS

Leaf Disc Test of the Progeny ♀SNK64 × ♂UPA143

The analysis of disease scores from 0 (tolerant) to 5 (highly susceptible) of the progeny ♀SNK64 × ♂UPA143 showed that the 7th day of leaf discs incubation best discriminates hybrid genotypes compared to other dates (4th, 5th, 6th and 8th day). At this date, 13 subgroups of hybrid genotypes were obtained using the Student Newman and Keuls test at 5%. Disease scores of the progeny were ranging between 0 (E9, J5 and C31) to 4.8 ± 0.07 (E32). Intermediate disease score was observed with E13 (2.6 ± 0.1). About 79.17% of the progeny were lesser sensitive than the most sensitive parent (UPA143). Approximately, 62.5% of the progeny were tolerant than the mean disease score (2.05) of both parents (♀SNK64 and ♂UPA143). Additionally, 20.83% of the progeny exhibited disease scores lower than the less susceptible parent (SNK64). Moreover, we observe 12.5% of progeny with disease scores lower than the most tolerant genotype (SCA6) reported **(Table 1)**.

Table 1. Means of necrosis rates (from 0: tolerant to 5: highly sensitive) comparison between hybrids, parental genotypes (SNK64 and UPA143) nd reference genotypes (SCA6 and SNK10).

Hybrid genotypes	Means of disease scores
(*) E9	0.0 ± 0.00^{a}
J5	0.0 ± 0.00^{a}
C31	0.0 ± 0.00^{a}
D11	0.2 ± 0.00^{ab}
E22	0.2 ± 0.00^{ab}
J17	0.4 ± 0.00^{abc}
E6	0.4 ± 0.00^{abc}
C37	0.8 ± 0.00^{bc}

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D18	1.2 ± 0.00^{c}
D1	1.6 ± 0.11^{cd}
J11	1.6 ± 0.00^{cd}
D4	2.0 ± 0.06^{cde}
C1	2.0 ± 0.00^{cde}
E39	2.0 ± 0.09^{cde}
(*) E13	2.6 ± 0.10^{def}
C29	2.8 ± 0.00^{defg}
E24	2.8 ± 0.00^{defg}
D15	3.4 ± 0.00^{efgh}
D32	3.2 ± 0.00^{efgh}
C19	4.0 ± 0.00^{fghi}
C32	4.0 ± 0.00^{fghi}
J25	4.2 ± 0.00^{ghi}
J23	4.4 ± 0.00^{hi}
(*) E32	4.8 ± 0.07^{i}
Parental genotypes	**Means of disease scores**
♀SNK64 (Female)	0.3 ± 0.00^{abc}
♂UPA143 (Male)	3.8 ± 0.00^{fghi}
Reference genotypes	**Means of disease scores**
SCA6 (most tolerant)	0.1 ± 0.02^{ab}
SNK10 (very susceptible)	3.3 ± 0.00^{efgh}

Disease scores were hanging from 0 (no necrosis) to 4.8 (true necrosis). Disease scores at 7th day of leaves discs incubation are expressed in term of Means ± SD (n=28). In disease scores column, values affected with the same letter are not significantly different according to the Student, Newman and Keuls test at 5%. (*) represents the hybrid genotypes with the lowest (0.0), middle (2.6) and highest (4.8) disease scores used for flavones studies.

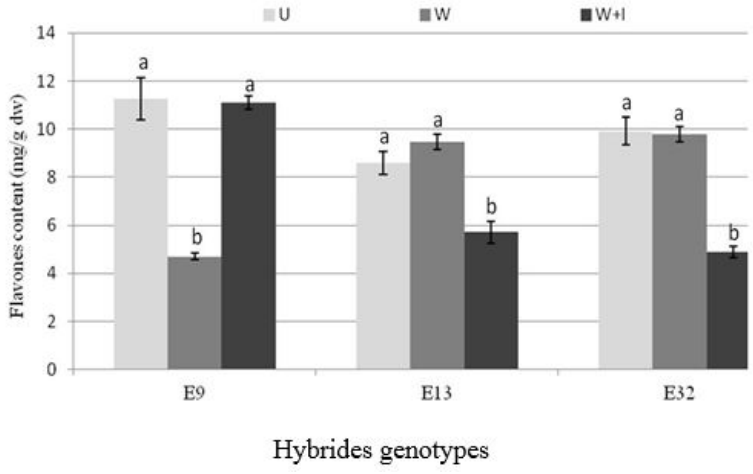
Flavones Analysis

Total flavones content

Total flavones content was estimated in untreated (U), wounded (W) and wounded+infected (W+I) leaves of three hybrid genotypes (E9, E13 and E32) of *T. cacao*.

In E9, a significant decrease in total flavones contents was observed when untreated leaves were wounded. The infection of wounded leaves leaded to a significant increase in total flavones contents in this hybrid genotype (E9). In E13, there was no significant difference in total flavones contents between untreated and wounded leaves. However, infected wounded leaves of E13 exhibited total flavones contents significantly lower than untreated and wounded leaves. E32 displayed total flavones contents pattern similar to E13.

In untreated leaves, the highest content of flavones is observed in E9. But, there was no significant difference in flavones content between E13 and E32 in untreated leaves. After wounding, total flavones contents significantly decrease in E9 (less susceptible genotype), while in E13 and E32 (most susceptible genotype) the content in flavones remained quasi constant. In wounded and infected leaves, total flavones contents significantly decrease from E9 to E13 and E32 **(Figure 1)**.



igure 1. Influence of treatment on flavones content in *T. cacao* leaves from E9, E13 and 32 hybrides: U=Untreated leaves; W=wounded leaves; /+I=wounded and infected leaves; dw=dry weight; Data are expressed in term of mean ± SD, (n=9). For a given hybrid genotype, total flavones ontents affected with the same letter are not significantly different according to the Student, Newman and Keuls test at 5%.

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Comparison between Treatments of Individual Flavones Profile for Each T. Cacao Hybrid Genotype Tested

A total of 13 flavones were detected in untreated, wounded and wounded+infected leaves of the three *T. cacao* hybrid genotypes (E9, E13 and E32) tested. For each hybrid genotype, the content of the individual flavones was monitored in untreated (U) wounded (W) and wounded+infected (W+I) leaves.

Hybrid Genotype E9

In untreated leaves, the most abundant flavones were apigenin-pentosyl-hexoside (t_R=7.8), apigenin-pentosyl-hexoside (t_R=8.1), apigenin-pentosyl-hexoside (t_R=8.7), apigenin-pentosyl-hexoside (t_R=10.9), isovitexin (t_R=14.4) and apigenin rutinoside isomer (t_R=14.8). The wound was associated to significant reduction of the content of the individual flavones. Flavones such as apigenin-pentosyl-dehexoside (t_R=10.7) present in healthy leaves was absent in wounded leaves. When wounded leaves were infected, there was a significant increase of the individual flavones. Isoorientin (t_R=12) absent in untreated and wounded leaves appeared when wounded leaves were infected. Luteolin rutinoside isomer (t_R=12.5) absent in wounded leaves reappeared with the infection. Infection seems to induce the synthesis of additional flavones in this hybrid genotype **(Figure 2)**.

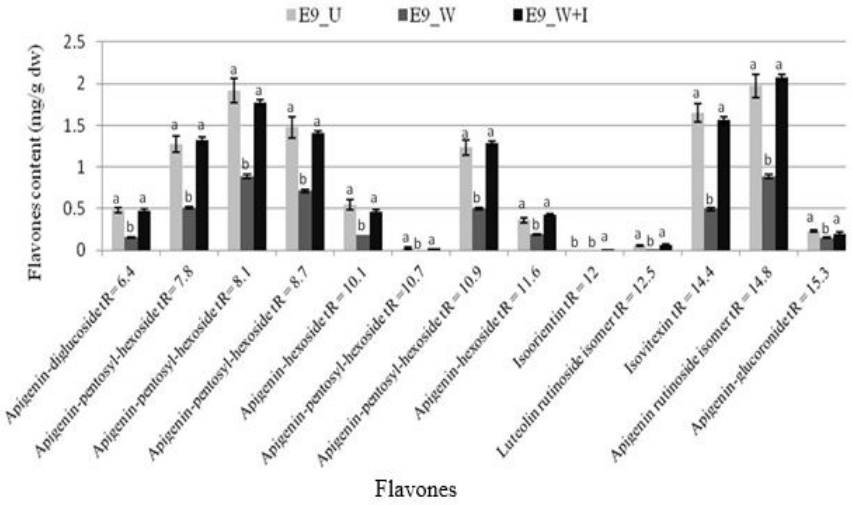
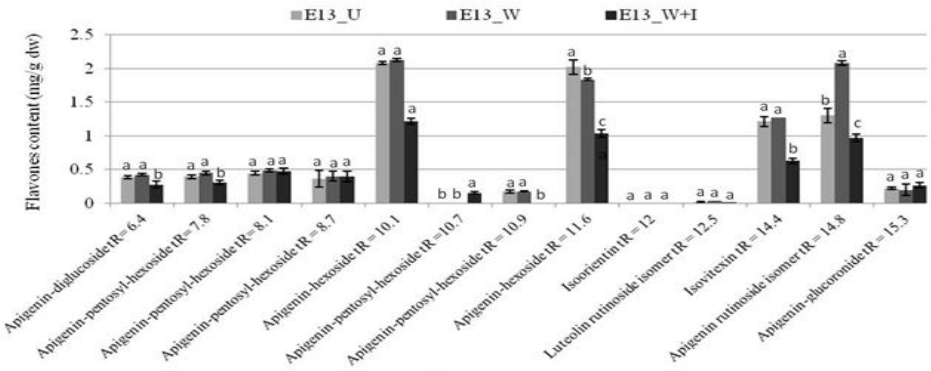


Figure 2. Comparaison of flavones profile in Untreated (U), Wounded (W) and wounded+infected (W+I) leaves from E9 hybrid genotype of *T. cacao*: dw=dry weight; Data are expressed in term of Mean ± SD (n=9). For a giving flavone, treatments affected with the same letter are not significantly different according to the Student, Newman and Keuls test at 5%.

Hybrid Genotype E13

Apigenin-hexoside (t_R=10.1), apigenin-hexoside (t_R=11.6), isovitexin (t_R=14.4) and apigenin rutinoside isomer (t_R=14.8) were the most abundant flavones in untreated leaves of genotype E13. The wound did not significantly influence the content of the individual flavones except for apigenin rutinoside ismer (t_R=14.8) which content significantly increases with wound. When leaves were wounded and infected, there was significant decrease in apigenin-diglucoside (t_R=6.4), apigenin-pentosyl-hexoside (t_R=7.8), apigenin-hexoside (t_R=10.1), apigenin-pentosyl-hexoside (t_R=10.9), apigenin-hexoside (t_R=11.6), luteolin rutinoside isomer (t_R=12.5), isovitexin (t_R=14.4) and apigenin rutinoside isomer (t_R=14.8). Moreover, apigenin-pentosyl-hexoside (t_R=10.9) and luteolin rutinoside isomer (t_R=12.5) disappeared with the infection. Apigenin-pentosyl-hexoside (t_R=10.7), which was absent in untreated and wounded leaves, was induced with infection; while isoorientin (t_R=12.0) remained absent in E13 **(Figure 3)**.



igure 3. Comparaison of flavones profile in Untreated (U), Wounded (W) and wounded+infected (W+I) leaves from E13 hybrid genotype of *T. acao*: dw=dry weight; Data are expressed in term of Mean ± SD, (n=9). For a giving flavone, treatments affected with the same letter are not gnificantly different according to the Student, Newman and Keuls test at 5%.

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Hybrid Genotype E32

Apigenin-hexoside (t_R=10.1), apigenin-hexoside (t_R=11.6) and isovitexin (t_R=14.4) were the most abundant flavones in E32 genotype. As with E13, wound did not significantly change the content of the individual flavones. But when wounded leaves were infected, the individual content of apigenin-diglucoside (t_R=6.4), apigenin-pentosyl-hexoside (t_R=7.8), apigenin-pentosyl-hexoside ($t_{R=8.1}$), apigenin-hexoside (t_R=10.1), apigenin-pentosyl-hexoside (t_R=10.9), apigenin-hexoside (t_R=11.6), isoorientin (t_R=12.0), isovitexin (t_R=14.4), apigenin rutinoside isomer (t_R=14.8) and apigenin-glucoronide (t_R=15.3) significantly decrease. As with E13, apigenin-pentosyl-hexoside (t_R=10.7) which was absent in untreated and wounded leaves was induced with infection; while isoorientin (t_R=12.0) remained absent **(Figure 4)**.

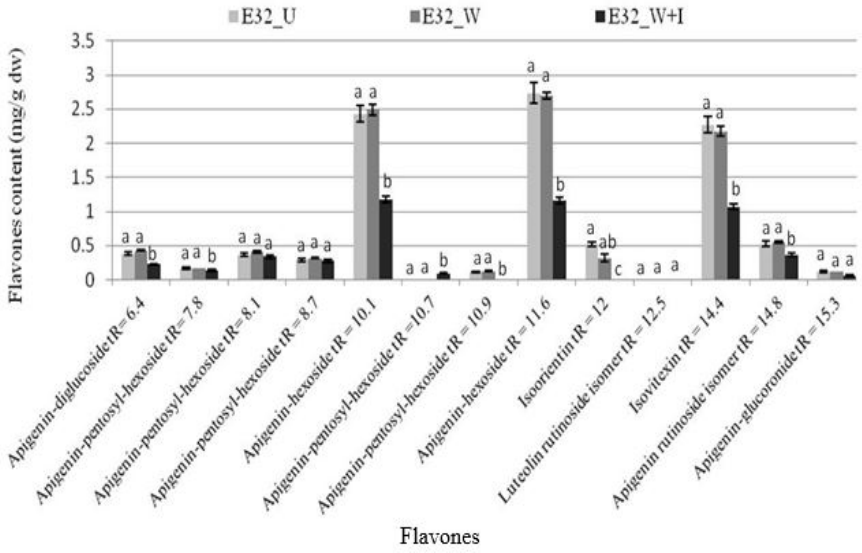


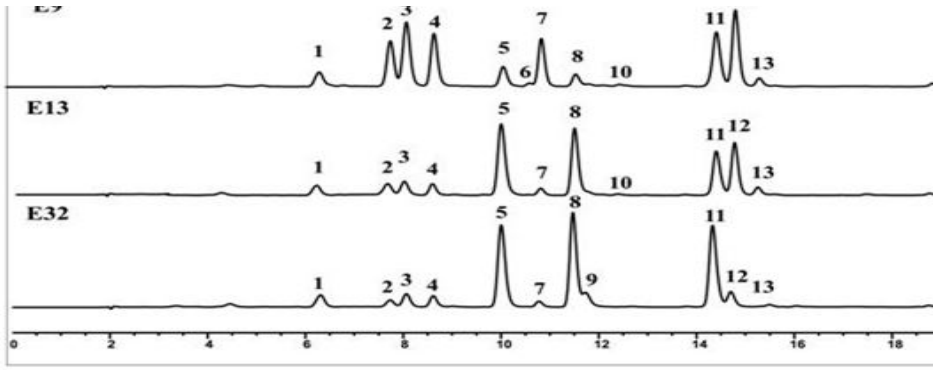
Figure 4. Comparaison of flavones profile in Untreated (U), Wounded (W) and wounded+infected (W+I) leaves from E32 hybrid genotype of *T. cacao*: dw=dry weight; Data are expressed in term of mean ± SD, (n=9). For a giving flavone, treatments affected with the same letter are not significantly different according to the Student, Newman and Keuls test at 5%.

Flavones profiles (quantity and quality) in *T. cacao* hybrid genotypes leaves appeared to be treatment, abiotic or biotic stress-dependant.

Comparison of Individual Flavones Profile between the Three Hybrid Genotypes for Each Treatment

Untreated leaves

In untreated leaves of the three genotypes (E9, E13 and E3) tested, 13 flavones discriminated by their individual retention time (t_R) were detected: apigenin-diglucoside (t_R=6.4), apigenin-pentosyl-hexoside (t_R=7.8), apigenin-pentosyl-hexoside (t_R=8.1), apigenin-pentosyl-hexoside (t_R=8.7), apigenin-hexoside (t_R=10.1), apigenin-pentosyl-hexoside (t_R=10.7), apigenin-pentosyl-hexoside (t_R=10.9), apigenin-hexoside (t_R=11.6), isoorientin (t_R=12.0), luteolin rutinoside isomer (t_R=12.5), isovitexin (t_R=14.4), apigenin rutinoside isomer (t_R=14.8), apigenin-glucoronide (t_R=15.3). The distribution of the 13 families of flavones was variable (number and concentration) among the three genotypes **(Figure 5)**.



igure 5. The chromatographic profiles of major flavones detected in healthy (untreated) leaves of three genotypes (E9, E13 and E3) of *T. acao*, recorded at 335 nm during HPLC-DAD-MS analyses. The number of a peak corresponds to compounds: 1- apigenin-diglucoside (t_R=6.4), -apigenin-pentosyl-hexoside (t_R=7.8), 3-apigenin-pentosyl-hexoside (t_R=8.1), 4-apigenin-pentosyl-hexoside (t_R=8.7), 5-apigenin-hexoside $_R$=10.1), 6-apigenin-pentosyl-hexoside (t_R=10.7), 7-apigenin-pentosyl-hexoside (t_R=10.9), 8-apigenin-hexoside (t_R=11.6), 9-isoorientin (t_R=12.0), 0-luteolin rutinoside isomer (t_R=12.5), 11-isovitexin (t_R=14.4), 12-apigenin-rutinoside isomer (t_R=14.8), 13-apigenin-glucoronide (t_R=15.3).

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The comparison of the contents of flavones in the three *T. cacao* hybrid genotypes showed that, apigenin-di glucoside (t_R=6.4) was present in the three genotypes: E9 (0.485 ± 0.033 mg/g dw), E13 (0.386 ± 0.025 mg/g dw) and E32 (0.380 ± 0.024 mg/g dw). The content of this secondary metabolite significantly decreases from E9 (tolerant genotype) to E13 and E32 (susceptible genotypes). It appears that, the content of this group of flavones decreases while the susceptibility of genotypes increases. Apigenin-glucoronide (t_R=15.3) showed a pattern similar to apigenin-diglucoside (t_R=6.4) between E9, E13 and E32. Luteolin rutinoside isomer (t_R=12.5) detected in E9 (0.061 ± 0.004 mg/g dw) and E13 (0.018 ± 0.003 mg/g dw) was absent in E32 (the most susceptible genotype tested). Apigenin-pentosyl-hexoside (t_R=10.7) observed in E9 (0.027 ± 0.008 mg/g dw) was absent E13 and E32. Reversely to apigenin-pentosyl-hexoside (t_R=10.7) pattern, isoorientin appears to be specific to the most susceptible genotype, E32 **(Figure 6)**.

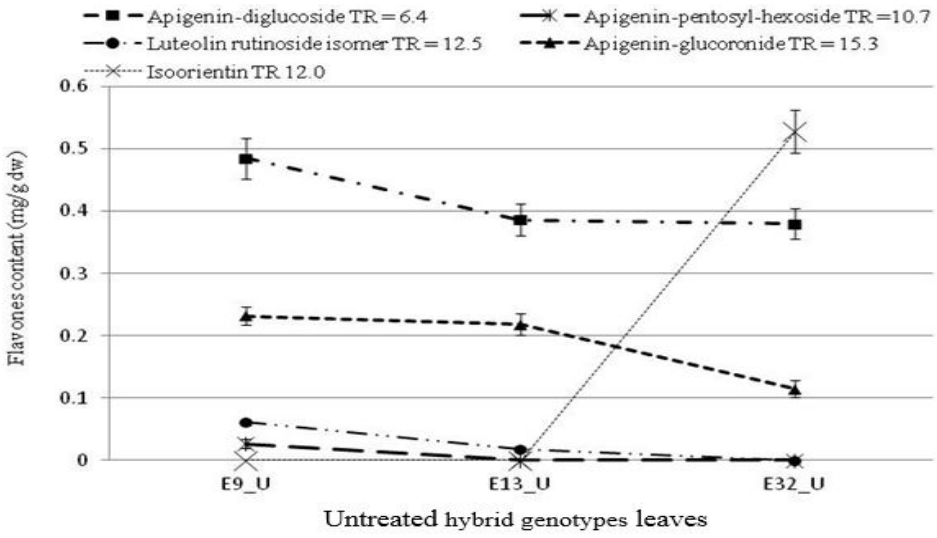
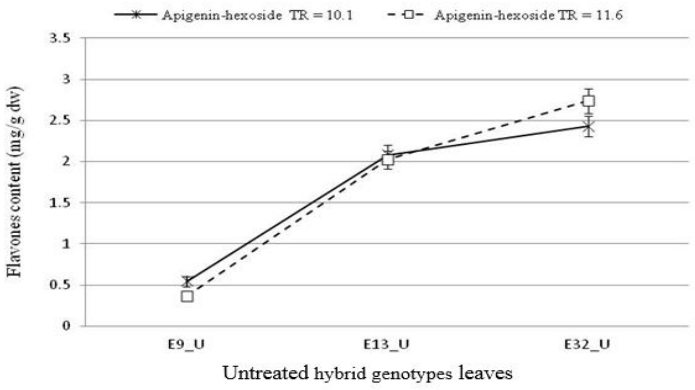


Figure 6. Flavone content in untreated leaves versus *T. cacao* genotypes susceptibility to *P. megakarya*. U=untreated; dw=dry weight; Data are expressed in term of mean ± SD, (n=9).

Reversely to the previous mentioned flavones which presented a general decrease pattern of their contents from the less susceptible (E9) to the most susceptible (E32), apigenin-hexoside (t_R=10.1) and apigenin-hexoside (t_R=11.6) showed a significant increase content pattern from E9 to E32. Additionally, their individual content value in a given genotype was not significantly different. This seems to indicate a positive correlation between both flavones contents a given *T. cacao* hybrid genotype **(Figure 7)**.



igure 7. Flavone content in untreated leaves versus *T. cacao* genotypes susceptibility to *P. megakarya*: U=untreated; dw=dry weight; Data are xpressed in term of mean ± SD, (n=9).

Apigenin-pentosyl-hexoside (t_R=7.8), apigenin-pentosyl-hexoside (t_R=8.1), apigenin-pentosyl-hexoside (t_R=8.7), apigenin-entosyl-hexoside (t_R=10.7), apigenin-pentosyl-hexoside (t_R=10.9) and apigenin rutinoside isomer (t_R=14.8) have presented a ecrease pattern from E9 to E32. The content in apigenin-pentosyl-hexoside (t_R=7.8) was significantly low in E13 (0.391 ± 0.030 ıg/g dw) and E32 (0.166 ± 0.012 mg/g dw) compared to E9 (1.275 ± 0.100 mg/g dw). Apigenin-pentosyl-hexoside (t_R=8.1), pigenin-pentosyl-hexoside (t_R=8.7) and apigenin-pentosyl-hexoside (t_R=10.9) presented a pattern similar to apigenin-pentosyl-exoside (t_R=7.8). That is, the flavones content is significantly high in E9 and significantly low in E13 and E32. Additionally, the ontent of these previously mentioned flavones appeared to be significantly high in tolerant genotype (E9) and low in susceptible

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genotypes (E13 and E32); E32 being the most susceptible hybrid genotype (E9 less susceptible) to black pod disease. Apigenin rutinoside isomer (t_R=14.8) showed a quasi linear pattern (y=-0.7245x+2.7173 with R^2=0.9984) from E9 to E32. Isovitexin (t_R=14.4) is significantly high in E32 (2.268 ± 0.123 mg/g dw) compared to E9 (1.654 ± 0.111 mg/g dw) and E13 (1.213 ± 0.072 mg/g dw) **(Figure 5 and Figure 8)**.

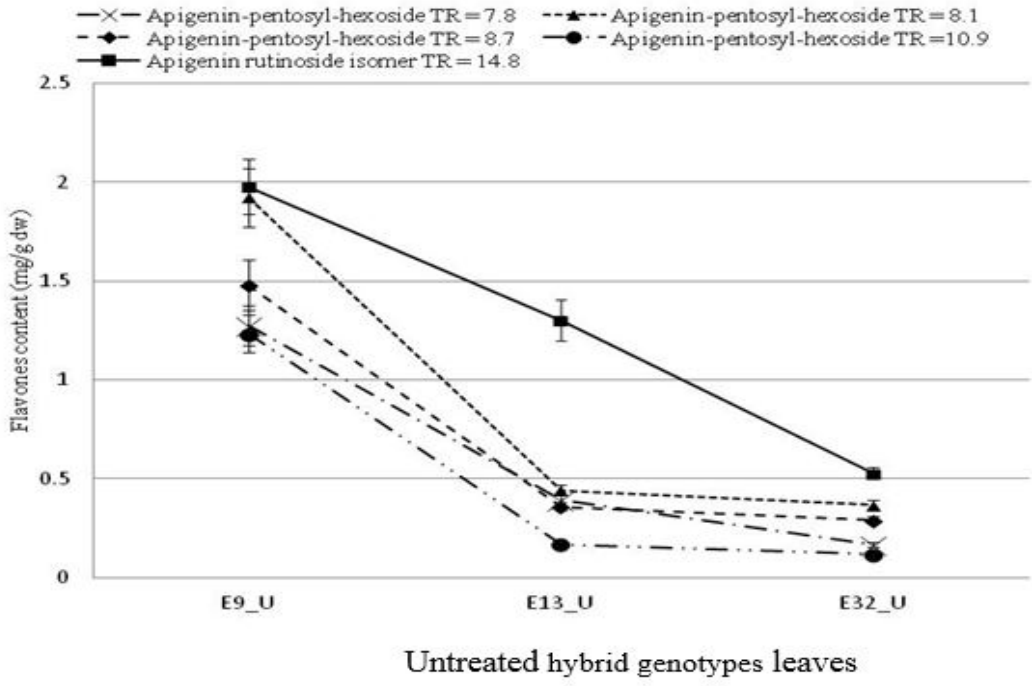
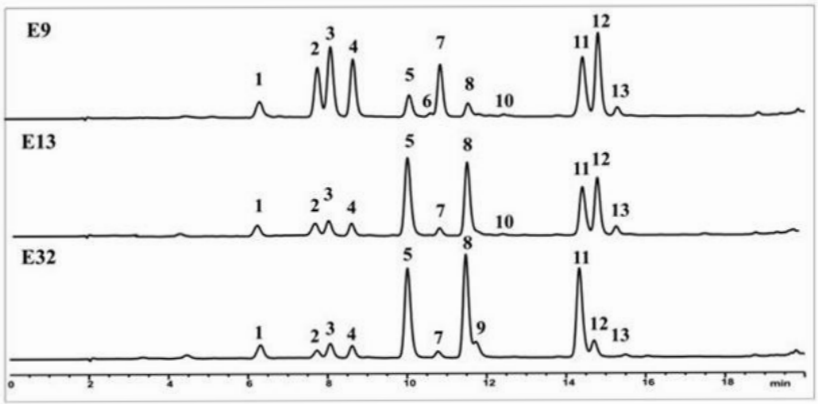


Figure 8. Flavone content in untreated leaves versus *T. cacao* genotypes susceptibility to *P. megakarya*: U=untreated (healty); dw=dry weight; Data are expressed in term of mean ± SD, (n=9).

Wounded leaves

In the wounded leaves, none of the genotypes has the 13 flavones. Among the 13 flavones detected, isoorientin (t_R=12.0) is the only absent flavones in E9 and E13 (but present in E32). Apigenin-pentosyl-hexoside (t_R=10.7) present in E9 is absent in E13 and E32. Luteolin rutinoside isomer present in E9 and E13 is absent in E32 **(Figure 9)**.



igure 9. The chromatographic profiles of major flavones detected in wounded leaves of different hybrids (E9, E13 and E32) of T. cacao, ecorded at 335 nm during HPLC-DAD-MS analyses. The number of a peak corresponds to compounds: 1- apigenin-diglucoside (t_R=6.4), -apigenin-pentosyl-hexoside (t_R=7.8), 3-apigenin-pentosyl-hexoside (t_R=8.1), 4-apigenin-pentosyl-hexoside (t_R=8.7), 5-apigenin-hexoside $_R$=10.1), 6-apigenin-pentosyl-hexoside (t_R=10.7), 7-apigenin-pentosyl-hexoside (t_R=10.9), 8-apigenin-hexoside (t_R=11.6), 9-isoorientin (t_R=12.0), 0-luteolin rutinoside isomer (t_R=12.5), 11-isovitexin (t_R=14.4), 12-apigenin-rutinoside isomer (t_R=14.8), 13-apigenin-glucoronide (t_R=15.3).

The content of the individual flavones (as function of hybrid genotypes susceptibility to BPD) showed variable patterns. pigenin-diglucoside (t_R=6.4) presented an opposite shape compared to untreated leaves. Apigenin-pentosyl-hexoside (t_R=10.7) ppeared to be absent in the wounded leaves of the three genotypes (this flavone was only present in E9 in untreated leaves). soorientin (t_R=12.0) has a similar shape as observed in untreated leaves. Luteolin rutinoside isomer (t_R=12.5) and apigenin-lucoronide t_R=15.3) have the analogous shapes (with a peak of content at E13) in wounded leaves **(Figure 10)**. While in untreated eaves, they presented a decrease shape from E9 to E32.

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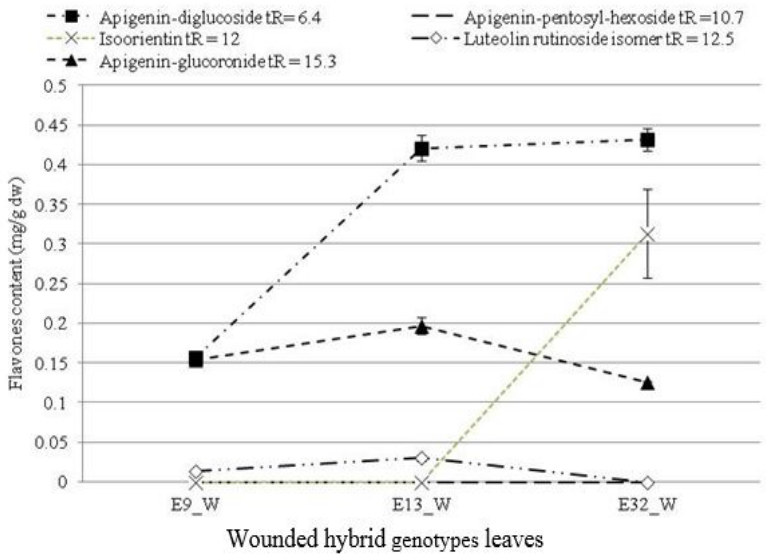


Figure 10. Flavone content in wounded leaves versus *T. cacao* genotypes susceptibility to *P. megakarya*. W=untreated; dw=dry weight; Data are expressed in term of mean ± SD (n=9).

As with untreated leaves, apigenin-hexoside (t_R=10.1) and apigenin-hexoside (t_R=11.6) presented positive correlated and increase shape from E9 to E32 in wounded leaves **(Figure 11)**.

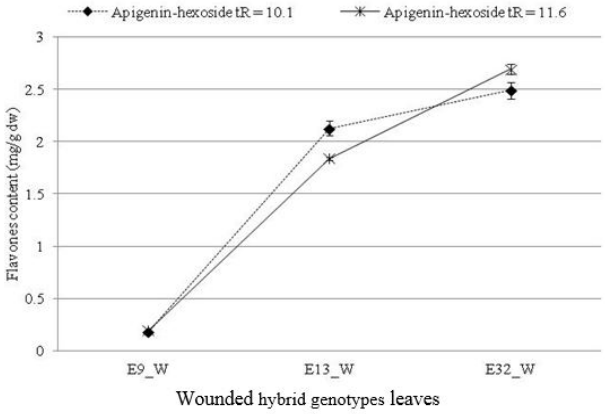
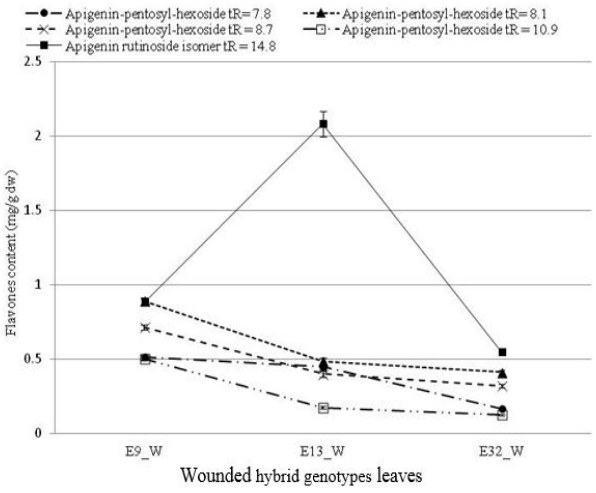


Figure 11. Flavone content in untreated leaves versus *T. cacao* genotypes susceptibility to *P. megakarya*. W=wounded; dw=dry weight; Data are expressed in term of mean ± SD (n=9).

In wounded leaves, apigenin-pentosyl-hexoside (t_R=7.8), apigenin-pentosyl-hexoside (t_R=8.1), apigenin-pentosyl-hexoside (t_R=8.7), apigenin-pentosyl-hexoside (t_R=10.7), apigenin-and pentosyl-hexoside (t_R=10.9) conserved a similar decrease shapes (from tolerant to susceptible genotypes) as observed in untreated leaves **(Figure 12)**.



igure 12. Flavone content versus *T. cacao* genotypes susceptibility to *P. megakarya* in untreated leaves: W=wounded; dw=dry weight; Data are xpressed in term of mean ± SD (n=9).

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Wounded and infected leaves

In wounded and infected (W+I) leaves, apigenin-pentosyl-hexoside (t_R=7.8), apigenin-pentosyl-hexoside (t_R=8.1), apigenin-pentosyl-hexoside (t_R=8.7), apigenin-pentosyl-hexoside (t_R=10.9), isovitexin (t_R=14.4) and apigenin rutinoside isomer (t_R=14.8) are most high in the tolerant genotype E9 compared the susceptible genotypes E13 and E32. While, apigenin-hexoside (t_R=10.1) and apigenin-hexoside (t_R=11.6) were significantly higher in E13 and E32 than E9. Apigenin-pentosyl-hexoside (t_R=10.7) present in E9 was absent in E13 and E32. Additionally, apigenin-glucoronide (t_R=15.3) absent in the most susceptible genotype (E32) is present in E13 and E9 **(Figure 13)**.

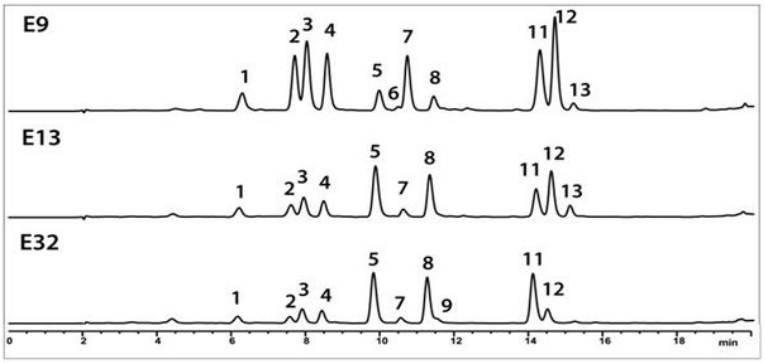
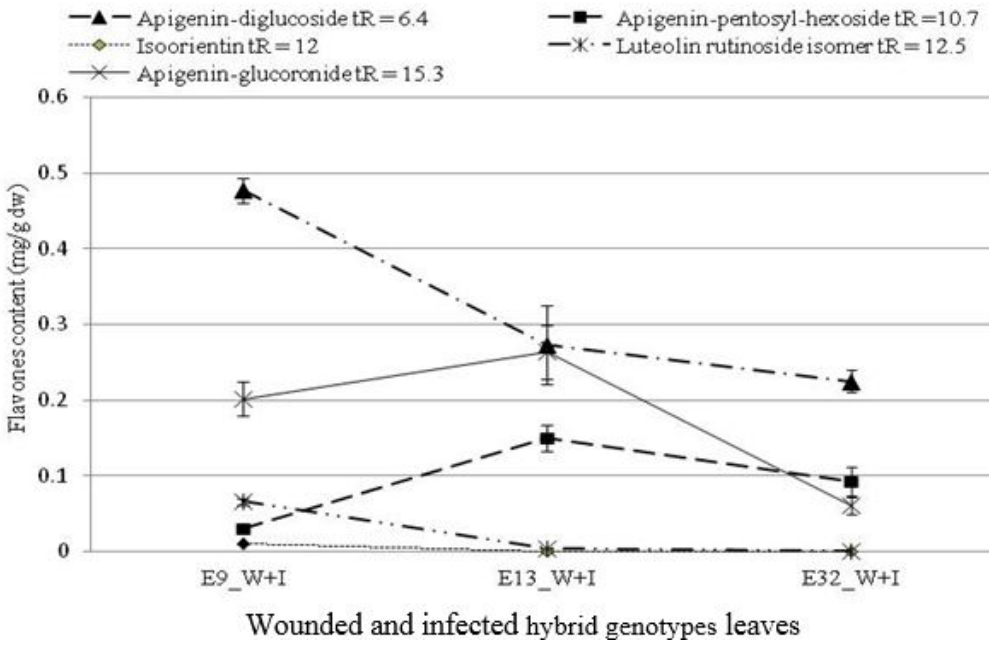


Figure 13. The chromatographic profiles of major flavones detected in wounded and infected leaves of different genotypes of T. cacao, recorded at 335 nm during HPLC analyses. 1-apigenin-diglucoside (t_R=6.4), 2-apigenin-pentosyl-hexoside (t_R=7.8), 3-apigenin-pentosyl-hexoside (t_R=8.1), 4-apigenin-pentosyl-hexoside (t_R=8.7), 5-apigenin-hexoside (t_R=10.1), 6-apigenin-pentosyl-hexoside (t_R=10.7), 7-apigenin-pentosyl-hexoside (t_R=10.9), 8-apigenin-hexoside (t_R=11.6), 9-isoorientin (t_R=12.0), 10-luteolin rutinoside isomer (t_R=12.5), 11-isovitexin (t_R=14.4), 12-apigenin rutinoside isomer (t_R=14.8), 13-apigenin-glucoronide (t_R=15.3).

The analysis of individuals flavones contents as function of hybrid genotypes susceptibility to BPD indicates that apigenin-diglucoside (t_R=6.4) has a decrease shape (from E9 to E32) instead of an increase shape observed in wounded leaves. Apigenin-hexoside (t_R=10.7), which was absent in wounded leaves, reappears in wounded+infected leaves. E13 genotype presented the highest content in apigenin-hexoside (t_R=10.7) followed by E32 and E9. Apigenin-glucuronide (t_R=15.3) has presented a similar shape as -hexoside ($t_{R=10.7}$) in wounded+infected leaves. Isoorientin (t_R=12.0) and luteolin rutinoside isomer (t_R=12.5) appeared to be associated to less susceptible hybrid genotype E9 in wounded+infected leaves **(Figure 14)**.



igure 14. Flavones contents in wounded+infected leaves versus *T. cacao* hybrides susceptibility to *P. megakarya*: W+I=wounded and infected eaves; dw=dry weight; Data are expressed in term of mean ± SD (n=9).

In wounded+infected (W+I) leaves, apigenin-hexoside (t_R=10.1) and apigenin-hexoside (t_R=11.6) have conserved similar orrelated and increasing pattern (from E9 to E32) as observed with untreated and wounded leaves. The high content of both avones in *T. cacao* leaves (untreated, wounded or wounded+infected) seems to be synonym of *T. cacao* hybrid genotypes usceptibility to *P. megakarya* **(Figure 15)**.

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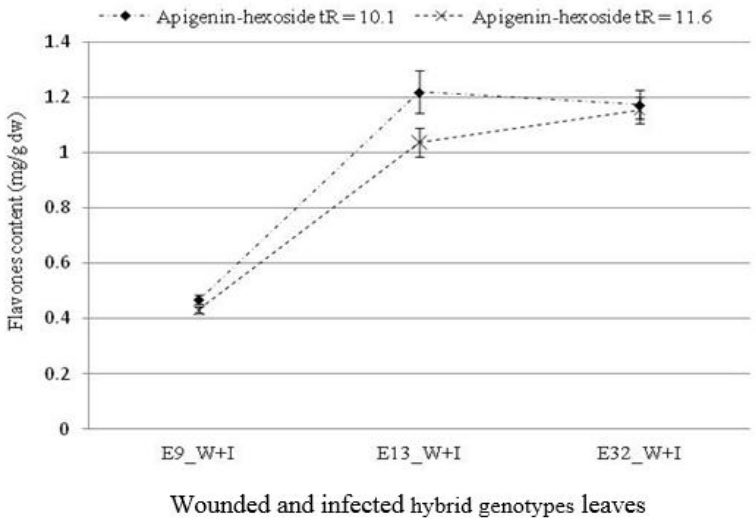

Figure 15. Flavones contents in wounded+infected leaves versus *T. cacao* hybrides susceptibility to *P. megakarya*: W+I=wounded and infected leaves; dw=dry weight; Data are expressed in term of mean ± SD (n=9).

In wounded+infected leaves, Apigenin-pentosyl-hexoside (t_R=7.8), Apigenin-pentosyl-hexoside (t_R=8.1), Apigenin-pentosyl-hexoside (t_R=8.7) and Apigenin-pentosyl-hexoside (t_R=10.9), have presented a decrease pattern (from E9 to E32) as observed with untreated and wounded leaves. The decrease in these flavones contents could be associated to decrease of tolerance to *P. megakarya* of *T. cacao* hybrid genotypes **(Figure 16)**.

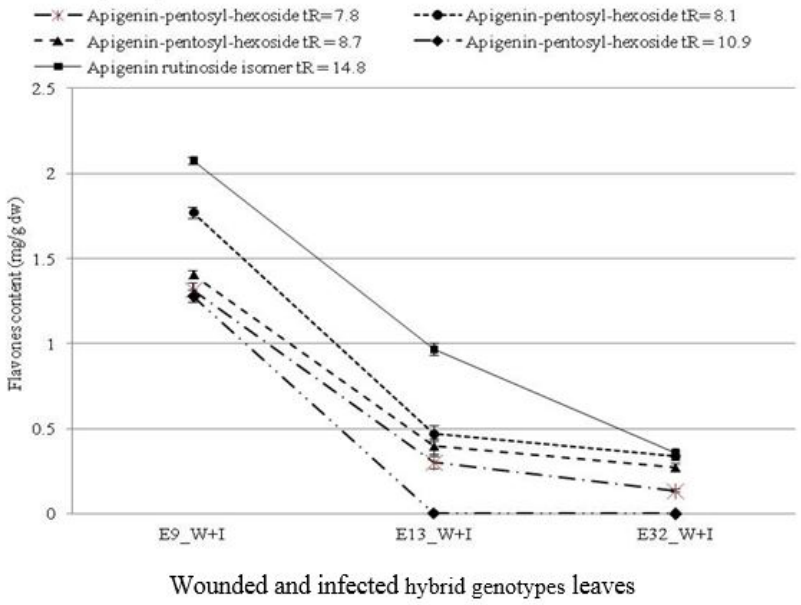

Figure 16. Flavones contents in wounded+infected leaves versus *T. cacao* hybrides susceptibility to *P. megakarya*: W+I=wounded and infected leaves; dw=dry weight; Data are expressed in term of mean ± SD (n=9).

DISCUSSION

Developing *T. cacao* genotypes resistant/tolerant to black pods disease (BPD) is the way out for sustainable cocoa culture. his can be done through the cross-fertilization of target parental genotypes and earlier selection of progenies with desirable traits sing reliable test and markers of tolerance. Unfortunately, there many untested cross-fertilization (such as ♀SNK64 × ♂UPA143). eveloped in Cameroon, SNK64 was reported has one of the most tolerant clone to BPD but less productive. Reversely, UPA143 productive but sensitive to BPD [17]. We studied the susceptibility to BPD of a *T. cacao* progeny (from ♀SNK64 × ♂UPA143) sing leaves disc test of the adapted method of [12]. Susceptibility of hybrid genotypes was monitored through disease scores. ence, ♀SNK64 × ♂UPA143 progeny exhibited disease scores between 0 and 4.8 while parental clones' ♀SNK64 and ♂UPA143 isplayed disease scores of 0.3 and 3.8 respectively. The presence of hybrid genotypes with disease scores lower 3.8 might ndicate that tolerant to BPD in *T. cacao* is an additive trait [2,3]. Furthermore, data analysis of leaf disc test presented 13 disease cores phenotypic subgroups of hybrid genotypes (from ♀SNK64 × ♂UPA143). This heterogeneity in disease scores might confirm he fact that tolerant to BPD disease in *T. cacao* is a multigenic character and cross-fertilized genotypes used are heterozygote

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for this character [18]. Nevertheless, high percentage of hybrid genotypes from ♀SNK64 × ♂UPA143 was less sensitive to BPD compared to the most susceptible parent (UPA143). Additionally, most hybrid genotypes presented a disease score lower than the mean disease score of both parents (♀SNK64 and ♂UPA143). An important proportion of hybrid genotypes exhibited disease scores lower than the best parent (SNK64) or the reference clone (SCA6). These results indicate that, cross-fertilization ♀SNK64 × ♂UPA143 could be used to develop *T. cacao* hybrid genotypes that are tolerant or less susceptible to BPD.

In presence of biotic or abiotic stress plants mobilize defence mechanism to cancel or minimize the incidence of stress. Plant defence mechanism includes variety of bio molecules. The effective molecules in plant defence are termed defence makers. Flavones are a subgroup of flavonoid metabolites with various biological functions in plants [19].

Relatively, nothing is known about flavones or flavones families in *T. cacao* defence against *P. megakarya*. Plant flavones are secondary metabolites that encompass several classes structurally diverse of natural products biogenetically arising from the shikimate-phenylpropanoids-flavonoids pathways. Plants need flavones for resistance to pathogens and for many other biological functions. Therefore, flavones represent adaptive characters. Plants synthesize a greater array of flavones because they cannot rely on physical mobility to escape their predators (pathogens) and have therefore evolved a chemical defence against such predators [20].

In this investigation, we analysed flavones pools in healthy (untreated), wounded and wounded+infected leaves from young plantlets (of the above progeny) in other to appreciate their potential use as markers of earlier selection of *T. cacao* genotypes resistant/tolerant to BPD.

From data of disease scores, E9 (less susceptible), E13 (moderate susceptible) and E32 (highest (susceptible) hybrid genotypes were used for flavones analysis in leaves (untreated, wounded and wounded+infected).

In untreated leaves from the three hybrid genotypes, it appears that, the total flavones contents were high in the tolerant genotype (E9) compared to the sensitive hybrid genotype E32. In wounded leaves, flavones were more abundant in the sensitive hybrid than the middle sensitive (E13) and less abundant in the tolerant one, E9. Reversely, in wooded and infected conditions, flavones were more abundant in E9 then gradually decrease from E13 to E32. This set of results might indicate that, flavones profiles pools in healthy, abiotic and biotic stressed *T. cacao* leaves are unmatchable. Therefore, flavones might be mobilized in *T. cacao* depending to a given status (healthy, wounded or infected). In fact, flavones are subgroups of flavonoids, which belong to phytoalexins, low molecular weight antimicrobial compounds mobilized by plants in response to abiotic or biotic stress [21].

During the infection, tolerant hybrid genotype (E9) displayed the highest flavones contents, which might be the backbone of its tolerance/resistance to BPD. Hence, decrease in total flavones contents in sensitive hybrids during infection might be associated to susceptibility to BPD of a giving genotype. This finding might confirm the implication flavones in plants defence against pathogens (oomycetes) such as *P. megakarya* [16,22].

Subsequently, to total flavones contents, we investigated profiles of individual flavones (in different leaves statutes) to understand if all flavones in *T. cacao* leaves are associated to resistance/tolerance of hybrid genotypes to BPD (which was an open question).

In *T. cacao* hybrids tested, most flavones displayed profiles depending to leaves status and hybrid genotypes. In highest and moderate sensitive hybrid genotypes (E32 and E13 respectively), the infection was associated to a decrease of flavones: apigenin-diglucoside (t_R=6.4), apigenin-pentosyl-hexoside (t_R=7.8), apigenin-hexoside (t_R=11.6), isovitexin (t_R=14.4), apigenin rutinoside isomer (t_R=14.8). Moreover, there was disappearance of isoorientin (t_R=12.0) but, light induction of apigenin-pentosyl-hexoside (t_R=10.7) in E13 due to infection. While, in tolerant hybrid genotype E9, infection leaded to significant increase in apigenin-diglucoside (t_R=6.4), apigenin-pentosyl-hexoside (t_R=7.8), apigenin-pentosyl-hexoside (t_R=8.1), apigenin-pentosyl-hexoside (t_R=8.7), apigenin-pentosyl-hexoside (t_R=10.9), isovitexin (t_R=14.4) and apigenin rutinoside isomer (t_R=14.8) and apigenin-glucoronide (t_R=15.3). This finding reveals differential flavones pools between tolerant and susceptible hybrid genotypes to BPD. This may highlight the fact that, during the infection, the production and adequate pools (quantitative and qualitative) of these flavones confer tolerance to BPD of less sensitive hybrid genotype. In fact, it was reported that the anti-pathogenic effect of flavonoids (including flavones) depends on their structure [23].

The analysis of individual flavones family profile versus sensitivity of the three hybrids (E9, E13 and E32), showed a decrease attern in the contents of apigenin-diglucoside (t_R=6.4), apigenin-pentosyl-hexoside (t_R=7.8), apigenin-pentosyl-hexoside (t_R=8.1), pigenin-pentosyl-hexoside (t_R=8.7), apigenin-pentosyl-hexoside (t_R=10.9), isoorientin (t_R=12.0), luteolin rutinoside isomer (t_R=12.5), isovitexin (t_R=14.4) and apigenin rutinoside isomer (t_R=14.8) from E9, E13 to E32. In plant such as *Bellis perennis* L., *dinandra nitida* and *Portulaca oleracea* L. Nayaka et al. [23] and V et al. [24] have reported antimicrobial properties of apigenin. In ıe interation *Colletotrichum sublineolum*/sorghum, Nicholson and Hammerschmidt [21] reported an accumulation of luteolin in ıe sorghum resistant cultivar than the susceptible one.

These authors demonstrated that, luteolin as well as apigenin were able to inhibit spore germination of *C. Sublineolum*. seems that, these flavones may use resistance mechanisms which refer to traits that inhibit or limit attack of *P. megakarya* gainst cocoa. Or, the above flavones may also use tolerance strategies which reduce or offset consequences on *T. cacao* fitness

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by adjusting its physiology to buffer the effects of diseases [18]. In one hand, these resistance strategies, may include physical and/or chemical barriers, mechanisms that rapidly clear infection and processes that limit the spread of damage within *T. cacao* (such as localized cell death). In other hand, tolerance often involves some degree of compensation for disease damage. Therefore, *T. cacao* might tolerate infection due BPD by increasing the chlorophyll concentration in leaves, delaying the senescence of infected tissues, and increasing the nutrient uptake [24].

This might indicate that, the above mention flavones could be considered as markers of tolerance to BPD in *T. cacao*. The stimulation of these flavones group can be due to the activation of jasmonic acid pathway during the development of different reactions of cocoa against *P. megakarya* [25]. In fact, in sorghum anthracnose resistance, Nicholson and Hammerschmidt [21] identified luteolin and apigenin as flavonoid phytoalexins. Additionally, in infection condition, luteolin rutinoside isomer (t_R=12.5), isoorientin (t_R=12.0) and apigenin-pentosyl-hexoside (t_R=10.9) appeared to be present in tolerant hybrid genotype (E9) but, completely absent in moderate (E13) and high (E32) susceptible hybrid genotypes. Protective effect of isoorientin against pathogen in *Linum usitatissimum* was reported [26]. Antipathogenic properties of luteolin rutinoside isomer (t_R=12.5), isoorientin (t_R=12.0) and apigenin-pentosyl-hexoside (t_R=10.9) may result, in part, from antioxidative properties of flavonoids. They may quench ROS (reactive oxygen species) generated both by the pathogens and the plant as a result of the infection [26]. They may also be directly involved in the inhibition of the pathogen's enzymes, especially those digesting the plant cell wall, by chelating metals required for their activity. These small secondary metabolites may also inhibit spore development and mycelium hyphae elongation [26]. Makoi and Ndakidemi [27] showed that, flavonoids inhibit root pathogens, especially fungal ones. In general, isoflavones, flavanes and flavanones were acknowledged as efficient anti-microbial agents [27].

Antipathogenic properties of luteolin rutinoside isomer (t_R=12.5), isoorientin (t_R=12.0) and apigenin-pentosyl-hexoside (t_R=10.9) could be characteristic of tolerant *T. cacao* hybrid genotype during infection. Reversely high content in apigenin-hexoside (t_R=10.1) and apigenin-hexoside (t_R=11.6) appeared to be characteristic of susceptibility to BPD. Their contents increase from E9, E13 and E32 in untreated, wounded and wounded+infected leaves from young *T. cacao* hybrid genotypes plantlets.

CONCLUSION

The cross-fertilization of ♀SNK64 and ♂UPA143 generates an important proportion of hybrid genotypes tolerant or less susceptible to BPD. During BPD infection, *T. cacao* hybrid genotypes mobilized flavones depending on the susceptibility of the hybrid genotype. During the infection, luteolin rutinoside isomer (t_R=12.5), isoorientin (t_R=12.0) and apigenin-pentosyl-hexoside (t_R=10.9) appeared to be characteristic of tolerant hybrid genotype (E9). High pools in apigenin-hexoside (t_R=10.1) and apigenin-hexoside (t_R=11.6) seem to be characteristic of susceptibility hybrid genotypes. Hence, ♀SNK64 × ♂UPA143 can be used to produce tolerant hybrid genotypes. Luteolin rutinoside isomer (t_R=12.5), isoorientin (t_R=12.0) and apigenin-pentosyl-hexoside (t_R=10.9) stand as useful makers of tolerance in cocoa exposed to *P. megakarya*. These flavones might also be useful in markers assisted selection in *T. cacao*.

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