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Diterpenes of *Coffea* seeds show antifungal and anti-insect activities and are transferred from the endosperm to the seedling after germination

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ABSTRACT

Species of the genus *Coffea* accumulate diterpenes of the *ent*-kaurane family in the endosperm of their seeds, of which cafestol and kahweol are the most abundant. The diterpenes are mainly stored in esterified form with fatty acids, mostly palmitate. In contrast to the numerous studies on their effects on human health and therapeutic applications, nothing was previously known about their biological and ecological role *in planta*. The antifungal and anti-insect activities of cafestol and cafestol palmitate were thus investigated in this study. Cafestol significantly affected the mycelial growth of five of the six phytopathogenic fungi tested. It also greatly reduced the percentage of pupation of larvae and the pupae and adult masses of one of the two fruit flies tested. By contrast, cafestol palmitate had no significant effect against any of the fungi and insects studied. Using confocal imaging and oil body isolation and analysis, we showed that diterpenes are localized in endosperm oil bodies, suggesting that esterification with fatty acids enables the accumulation of large amounts of diterpenes in a non-toxic form. Diterpene measurements in all organs of seedlings recovered from whole seed germination or embryos isolated from the endosperm showed that diterpenes are transferred from the endosperm to the cotyledons during seedling growth and then distributed to all organs, including the hypocotyl and the root. Collectively, our findings show that coffee diterpenes are broad-spectrum defence compounds that protect not only the seed on the mother plant and in the soil, but also the seedling after germination.

1. Introduction

The genus *Coffea* (Rubiaceae) comprises at least 130 species (Davis and Rakotonasolo, 2021), mostly distributed in tropical Africa and in islands in the western Indian Ocean, and includes two widely cultivated species, *Coffea arabica* L. and *Coffea canephora* Pierre ex A. Froehner, which account for almost all coffee production worldwide. Coffee seeds are albuminous, comprising a tiny spatulate embryo encapsulated in a copious living cellular endosperm, which represents more than 98% of the mature seed dry mass in *C. arabica* for instance (Joët et al., 2009). As the main storage tissue, the endosperm accumulates nutrient reserves, mainly cell-wall polysaccharides, triacylglycerols (TAGs), globulins and sucrose. During embryo germination and seedling growth, nutrients are transferred via the apoplast from the endosperm to seedling cotyledons, which progressively invade and resorb the endosperm (Eira et al., 2006).

In addition to storage compounds, the coffee endosperm also accumulates significant amounts of peculiar secondary metabolites, including alkaloids (caffeine, trigonelline), phenolics (chlorogenic acids) and diterpenoids (Anthony et al., 1993; De Roos et al., 1997). These secondary metabolites may also be remobilized to nourish the seedling. For instance, the alkaloid trigonelline serves as a nicotinic acid reservoir for rapid NAD re-synthesis during coffee seed germination through trigonelline demethylase activity (Shimizu and Mazzafera, 2000). However, coffee seed secondary metabolites are thought to mainly play essential roles in seed and seedling defence against herbivores and pathogens (De-La-Cruz Chacón et al., 2013), thereby contributing to *Coffea* species reproductive fitness. The purine alkaloid caffeine acts as chemical protection to ward off a wide variety of

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pathogens and herbivores and may also inhibit the growth of neighbouring plants (Ashihara et al., 2008). During seed germination and seedling growth, caffeine is rapidly transferred from the endosperm to the cotyledons and part of this pool is thereafter excreted into the soil by the radicle where it has an allelopathic effect (Baumann and Gabriel, 1984). Esters formed between hydroxycinnamic and quinic acids, collectively known as chlorogenic acids, also have a wide range of biological activities, including antibacterial and antifungal effects (Clifford et al., 2017). Moreover, when transferred from the endosperm to the cotyledons during germination, they are rapidly remobilized for seedling lignin metabolism, thereby contributing to plant defence, since lignin makes tissues more resistant to microbial attack (Aerts and Baumann, 1994).

The functional role of the third main class of secondary metabolites that accumulate in coffee seeds, i.e. diterpenoids of the *ent*-kaurane family, the main ones being cafestol and kahweol, is far less documented. Diterpenoids form a large class of predominantly polycyclic 20-carbon isoprenoids that derive from geranylgeranyl pyrophosphate. The diterpenes cafestol and kahweol are pentacyclic alcohols with an *ent*-kaurene skeleton and belong to the large group of labdane-related diterpenoids, which contains more than 7000 known natural products (Mafu and Zerbe, 2018). A few labdane-related diterpenoids, including gibberellin phytohormones, occur broadly across the plant kingdom and play key roles in plant metabolism and development. However, most of these molecules are specialised secondary metabolites with limited taxonomic distribution, whose accumulation may be further limited to specific cell types, tissues, or organs (Gershenzon and Dudareva, 2007; Mafu and Zerbe, 2018). Many such diterpenoids have anti-microbial effects and provide a wall of defence against bacterial and fungal pathogens, such as diterpene phytoalexins in cereals or diterpene resin acids in conifer trees, while others deter herbivory or mediate allelopathic plant-plant interactions (Keeling and Bohlmann, 2006; Schmelz et al., 2014). More specifically, several studies have shown the involvement of kaurane-type diterpenes in plant defence. This is the case of kauralexins in maize (Schmelz et al., 2014), which are phytoalexins that inhibit the growth of the fungi *Rhizopus microsporus* and *Colletotrichum graminicola*, and also show antifeedant activity against the moth *Ostrinia nubilalis*.

While many studies have shown the pharmacological and anticancer properties of cafestol and kahweol (Ren et al., 2019), their biological role in coffee plants remains unknown. However, based on the literature cited above, the most likely hypothesis is that they contribute to seed defence against herbivores or pathogenic fungi. Assuming they confer defence, whether they are transferred from the endosperm to the cotyledons to protect the seedling is also not known. All *Coffea* species analysed so far accumulate cafestol and kahweol in their seeds, where they may represent up to 1.5% of the seed dry mass (De Roos et al., 1997). In mature coffee seeds, diterpenes are predominantly found in esterified form with different fatty acids, mostly palmitic and linoleic acids (Speer and Kölling-Speer, 2006), suggesting they may be stored in oil bodies together with TAGs. For example, free cafestol represent only 3% of total cafestol in *C. arabica* seeds (Speer and Kölling-Speer, 2006). In the present study, we tested the effect of free and esterified forms of cafestol on the growth of a panel of tropical pests including six phytopathogenic fungi (*Aspergillus niger, Colletotrichum gloeosporioides, Fusarium oxysporum, Fusarium proliferatum, Fusarium solani* and *Talaromyces stollii*) and two species of fruit flies (*Bactrocera dorsalis* and *Ceratitis capitata*). For both insects and fungi, species were chosen to include a pest that attacks coffee seeds and fruits, *A. niger* and *C. capitata* (Taniwaki et al., 2003; Wharton et al., 2000), and, since many specialists have evolved counterdefenses to plant secondary compounds (Després et al., 2007; Kettle et al., 2015), other generalist pests for which coffee is not a known host, but which however cause important losses to several major tropical crops (Clarke et al., 2005; Drenth and Guest, 2016). Bioactivity assays were conducted using diterpene concentrations considerably lower than levels in seeds of *Coffea* species. The bioactivity of kahweol was not tested because it is very unstable *in vitro*, especially in its free form (Speer and Kölling-Speer, 2006). The localization of diterpenes in endosperm cells was investigated using confocal imaging and chemical analysis of purified oil bodies. To determine the fate of diterpenes after germination, cafestol and kahweol were analysed in the endosperm, in the embryo and in different seedling tissues at various developmental stages in two *Coffea mauritiana* Lam. and *C. arabica* genotypes chosen for their high seed cafestol and kahweol levels, respectively.

2. Materials and methods

2.1. Plant materials

Fresh mature seeds of *Coffea arabica* (var. Marsellesa) were provided by the 'Nicafrance' Experimental Station in La Cumplida (Matagalpa, Nicaragua) and those of *Coffea mauritiana* were taken from the field collection of the Coffea Biological Resource Centre (Saint-Pierre, La Réunion Island, France). For seed germination, batches of 15 seeds were placed on 375 mL of vermiculite fully imbibed with 150 mL of sterile water in closed glass jars. The jars were incubated in the dark at 27 °C for 14 days, the photoperiod was then changed to 12 h of light and 12 h of dark. Both batches of seeds were of high quality with more than 98% normal seedling development. A batch of 50 embryos of *C. arabica* were also extracted from seeds after disinfection and two days of imbibition in sterile water, and cultivated *in vitro* as described in Dussert et al. (1997). For seedlings obtained from whole seed germination, tissue sampling was performed at five developmental stages for *C. mauritiana* and six stages for *C. arabica* (Fig. 4). Stages were defined according to phenotypic criteria: ST1: ungerminated imbibed seed; ST2: germinated seed lying on vermiculite with curved hypocotyl (apical hook) and radicle together measuring ca. 10 mm; ST3: seed above the vermiculite due to hypocotyl lengthening (mean value of 12.9 mm) and apical hook half unfolded; ST4a: apical hook fully unfolded and straight hypocotyl measuring 22.7 mm; ST4b: further elongation of the hypocotyl up to 35.8 mm; ST5: unfolding of the cotyledonary leaves out of the residual endosperm. For *in vitro* plantlets derived from embryo extraction, tissues were sampled at three developmental stages (Fig. 4). ST3 and ST4 were assigned based on phenotypic criteria (apical hook unfolding and hypocotyl length) and developmental periods similar to those defined in the whole seed germination process. By contrast, since the emergence of cotyledonary leaves from residual endosperm could not be a criterion in isolated embryos, ST5 was defined by an extended culture period (79 days). At each developmental stage, three samples were collected and each sample was made of tissues sampled from 15 seeds for seedlings recovered from whole seed germination and five embryos for *in vitro* seedlings. Samples were frozen directly in liquid nitrogen after sampling, freeze-dried, weighed and ground for chemical analysis (Supplementary Table 1).

2.2. Diterpene analysis by HPLC-DAD

All reagents used for extraction were of analytical grade (Merck, Darmstadt, Germany). Acetonitrile was of HPLC grade and was purchased from Honeywell (Seelze, Germany). Cafestol and kahweol standards were purchased from Santa Cruz Biotechnology (Heidelberg, Germany). Total lipids were extracted and quantified from 6 to 600 mg samples of freeze-dried powder, using a modified Folch method as described in Dussert et al. (2008). To quantify total diterpenes, i.e. including diterpenes from diterpene esters, ester hydrolysis was achieved through total lipid saponification. The unsaponifiable fraction was obtained as described in Dussert et al. (2008), dried under nitrogen and dissolved in 4 mL of acetonitrile by sonication at 30 °C for 10 min. For HPLC analysis, 600 μL of each sample was mixed with 400 μL of water and then filtered through a 0.2 μm filter membrane (UptiDisc). Unsaponifiable lipid extracts were injected directly into the Agilent Infinity II HPLC system (Santa Clara, CA, USA). The column was a reverse phase

Uptisphere UP5ODB-250/046 column (250 × 4.6 mm internal diameter 5 μm) (Interchim, Montluçon, France). Isocratic elution using acetonitrile/water (60:40, v/v) was performed for 25 min at a flow rate of 1 mL/min and an oven temperature of 30 °C. Cafestol and kahweol were detected at 230 nm and 290 nm, respectively, and identified by comparing their retention time with that of standards. Each diterpene was quantified using a calibration curve generated with seven concentrations ranging from 0.025 to 200 mg/L. Tissue diterpene content is expressed as the mass of diterpenes (μg) per seed or seedling (Supplementary Table 1).

2.3. Isolation of oil bodies and diterpene analysis

Oil bodies were extracted and purified from 10 g of *C. arabica* seeds using the method described by Tzen et al. (1997) with minor modifications. Seeds were first ground to a fine powder in an analytical grinder (IKA A10, Staufen, Germany). The powder was then homogenised in 50 mL of extraction medium (10 mM phosphate buffer, pH 7.5, 0.5 M NaCl, 0.6M sucrose and 2% (w/v) PVPP), using an Ultra Turrax at a speed of 8000 rpm for 40 s. After filtration through three layers of Miracloth (Calbiochem, La Jolla), 15 mL aliquots of the homogenate were placed at the bottom of a 50 mL centrifuge tubes on ice, gently overlaid by 15 mL of flotation medium (10 mM phosphate buffer, pH 7.5, 0.5 M NaCl and 0.4M sucrose), and centrifuged at 10,000×*g* for 20 min at 4 °C in a swinging bucket rotor. The oil body fraction on the top was collected and resuspended in 15 mL of urea medium (10 mM phosphate buffer, pH 7.5, 0.25 M sucrose, 9 M urea), homogenised and then overlaid by 15 mL of flotation medium (10 mM phosphate buffer, pH 7.5) and centrifuged at 10,000×*g* for 20 min at 4 °C. The oil body fraction at the top was collected and resuspended in 2 mL of phosphate buffer (10 mM phosphate buffer, pH 7.5) and mixed with 1 mL of hexane for 1 min. After removal of the upper hexane layer, the oil bodies were resuspended in 25 mL of phosphate buffer, centrifuged at 10,000×*g* for 20 min at 4 °C, and collected. To check oil body integrity prior to chemical analyses, purified oil bodies were mixed with a Nile red solution (1:1000 v/v) for 10 min and then observed using an Axio imager Z2 microscope (Zeiss) at 63x, first with transmitted light using a DIC contrast method to visualise the oil bodies, and then with the DSred filter (EX BP 545/25, BS FT 570 and EM BP 605/70) to visualise neutral lipids (Supplementary Fig. 1). Total lipids were extracted from purified oil bodies using 8 mL of methylene chloride/methanol (2/1) containing 5 mg of triheptadecanoin (Merck) as internal standard. Each lipid extract was then divided into two equal parts to quantify total diterpenes and TAGs, respectively. Total diterpenes were measured as described above. For TAG measurement, fatty acid methyl esters (FAMEs) were prepared according to the ISO-5509 standard and analysed using gas chromatography (Dussert et al., 2008). Analyses were performed using an Agilent 7820A gas chromatography system with flame ionization detection. A Famewax capillary column (Restek, France), 30 m × 0.25 mm × 0.25 m, was used. Analyses were carried out from 185 to 225 °C at 4 °C/min and then at 225 °C for 10 min. The carrier gas was helium at 40 cm/s. Both injector and detector were at 230 °C. TAGs were then quantified by comparing the sum of areas of all FAME peaks to that of heptadecanoic acid.

2.4. Imaging of diterpene localization

Seeds were first hydrated for 48 h, then cut transversally into two pieces and dipped in 10 mL of fixation medium (EDC 4% w/v and PFA 4% v/v in PBS 1X) for 12 h. Sections (100 μm) of fresh endosperm were cut using an HM650V vibrating blade microtome (Microm, Waldorf, Germany). Spectral imaging of endosperm sections and pure cafestol palmitate and kahweol palmitate standards was performed using a multiphoton Zeiss 880 NLO microscope (Zeiss, Iena, Germany), equipped with a tuneable laser Chameleon Ultra II Ti-Sapphire (Coherent, Santa Clara, California) and a 40x/1.1W objective. Optimal excitation was obtained at $\lambda = 720$ nm and band-pass emission in the 365–700 nm range using an array of 32 channel photomultiplier tube detector (Zeiss), each with a 8.9 nm bandwidth. Spectral analysis was carried out as described by Talamond et al. (2015), using the advanced linear unmixing function (ZEN2 Zeiss software) (Supplementary Fig. 2). Observations were made on 5 seeds and 3 sections per seed. To visualise the oil bodies, endosperm sections were dipped in a Nile red solution (1:1000 v/v) for 10 min in the dark and then washed with PBS 1X. Confocal imaging of endosperm sections was then performed using a confocal laser Zeiss 880 microscope. Nile red was excited using a 561 nm laser.

2.5. Diterpene activity against fungi and insects

To test antifungal activity, stock solutions of 300 mM cafestol and 100 mM cafestol palmitate were prepared in dimethyl sulfoxide (DMSO). For the anti-insect assays, stock solutions of 300 mM cafestol and 100 mM cafestol palmitate in ethanol were prepared. The concentrations of stock solutions corresponded to solubility maxima of the two compounds in both DMSO and ethanol. Cafestol and cafestol palmitate were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

To test antifungal activity, diterpene solutions in DMSO were incorporated in culture media at 60 °C before distribution (2 mL per well) and solidification in 12-well plates. Five final diterpene concentrations were tested in either potato dextrose agar (PDA) or Sabouraud (SAB) medium (10, 100, 500, 1000 and 3000 μM), but the DMSO concentration in the media was always 1% v/v. Preliminary tests using different fungal strains showed that 1% DMSO in the culture medium had no significant effect on fungal growth. The highest concentration tested for cafestol and cafestol palmitate (3000 μM and 1000 μM, respectively) was imposed by their solubility threshold in DMSO and the percentage of DMSO in the culture medium. Based on the molecular mass of cafestol or kahweol, the percentage of diterpenes in dry seeds of various coffee species (De Roos et al., 1997) and the water content of mature coffee seeds (ca. 50% of the seed fresh mass; Joët et al., 2009), it can be estimated that the seed diterpene concentration ranges between ca. 5000 and 20,000 μM in the different coffee species analysed so far. Therefore, the highest concentrations tested in the present bioactivity assays were well below levels found in coffee seeds. Another preliminary experiment, performed at a cafestol concentration of 500 μM with the two culture media and three fungi, showed that the variability between two independent inoculations was very low (Supplementary Fig. 3). Experiments were thus subsequently done in triplicate in the same plate. The effect of each concentration of diterpene was tested against six pathogenic fungi: *Aspergillus niger* (BP094), *Colletotrichum gloeosporioides* (MG1), *Fusarium oxysporum* (BP369), *Fusarium proliferatum, Fusarium solani* (BP395) and *Talaromyces stollii* (BP642). Each strain was pre-cultured on PDA at 25 °C in the dark. For the antifungal assays, a 2 mm agar plug of a pre-culture plate was inoculated on the medium in the centre of the well (350 mm^2). Fungi were then grown at 25 °C in the dark for seven days. Fungal growth was estimated by daily measurement of the mycelial area on photographs analysed using the ImageJ software.

The anti-insect activity was tested against second instar larvae raised in laboratory cultures (*Pôle de Protection des Plantes 3P*, CIRAD, La Réunion) of *Bactrocera dorsalis* and *Ceratitis capita*. The effect of the diterpene concentration in the larvae diet was tested in triplicate (three independent layings at different dates) using batches of 60 larvae bred in open 5 cm Petri dishes containing 10 g (8.925 mL) of the diet described in Duyck and Quilici (2002). Three final cafestol concentrations in the diet (100, 1000 and 3000 μM) and two final concentrations of cafestol palmitate (100 and 1000 μM) were tested. Cafestol palmitate low polarity and solubility in ethanol made it impossible to reach 3000 μM in the diet. Diterpene solutions in ethanol were directly incorporated into the diet prior to distribution in Petri dishes. Whatever the diterpene concentration, the ethanol concentration in the diet was always 1% v/v. Preliminary trials showed that neither species was affected by the presence of 1% ethanol in the larvae diet. Each Petri dish was placed in a

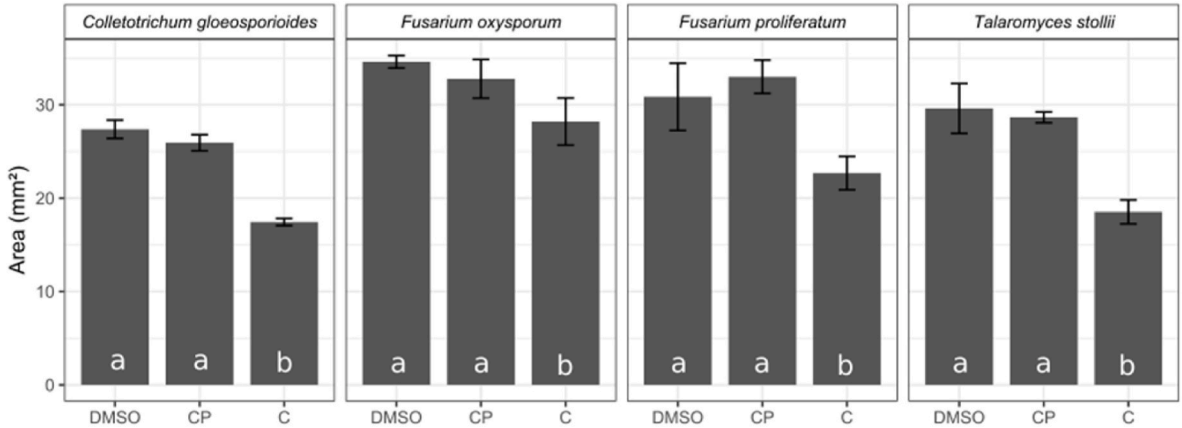

Fig. 1. Effect of 1000 μM of cafestol palmitate (CP) or cafestol (C) in the culture medium on mycelial growth after 72 h of culture of the four pathogenic fungi *Colletotrichum gloeosporioides, Fusarium oxysporum, Fusarium proliferatum and Talaromyces stollii*. Fungal growth was measured by the mycelium area (mm^2). Data are shown as the mean ± s.d. Bars containing the same letter were not significantly different at p = 0.05 according to the Waller-Duncan post-hoc test.

plastic screened box containing sand to allow pupation and incubated at 25 °C. At maturity, larvae crawl out of petri dishes containing the diet and move to the sand to pupate. The number of pupae and their weight was recorded daily after careful sifting of the laying sand. Pupae were immediately replaced in the sand after weighing. After complete hatching, adults were counted and weighed after drying for 72 h in an oven at 52 °C. Anti-insect activity was assessed using the following variables: (i) the final pupation percentage, (ii) the time to reach 50% of pupation (T50) as determined by fitting a logistic model to daily pupae numbers (Supplementary Fig. 4), (iii) the hatching percentage, (iv) the pupae fresh mass and (v) the adult dry mass.

2.6. Statistical analyses

One-way analyses of variance (ANOVA) were performed using R software. Means were compared using the Waller-Duncan post-hoc test (Package agricolae version 1.3–3) at a level of significance of 0.05. Logistic regression analyses were performed using Statistica (Tibco software, Palo Alto, California, USA).

3. Results

3.1. Antifungal activity of cafestol

The effect of cafestol and cafestol palmitate on the growth of four generalist fungal plant pathogens (*C. gloeosporioides*, *F. oxysporum*, *F. proliferatum* and *T. stollii*) was first assessed at high final concentrations in the PDA culture medium, i.e. 1 mM (316 and 556 μg/mL for cafestol and cafestol palmitate, respectively). Cafestol significantly affected the growth of all the fungi tested, as the mycelial growth of *C. gloeosporioides* and *T. stollii* was reduced by ca. 40% after 72 h of incubation, and that of *F. oxysporum* and *F. proliferatum* by ca. 25% (Fig. 1; Supplementary Table 2). By contrast, regardless of the fungal strain, cafestol palmitate had no inhibitory effect on mycelial growth. Since this assay was performed at the maximum concentration of cafestol palmitate that can be solubilised in the culture medium using 1% DMSO, this compound was not tested further.

To further investigate the antifungal activity of cafestol, dose-response growth inhibition assays were conducted against six phytopathogenic fungi (*A. niger* and *F. solani* in addition to the four previous

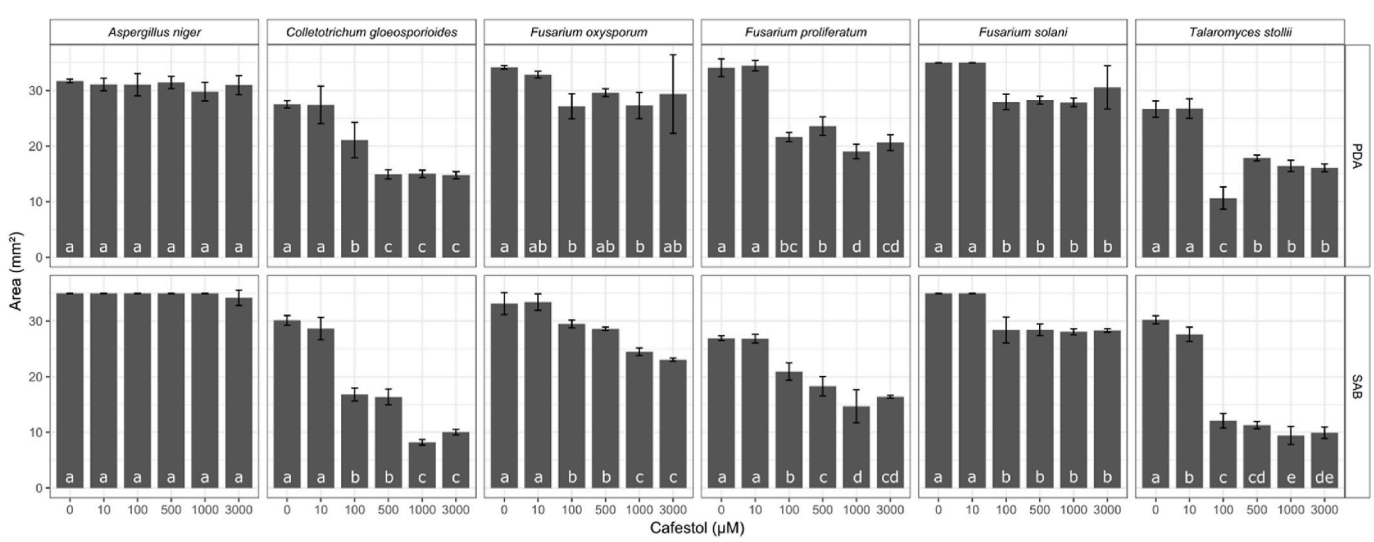

Fig. 2. Effect of the concentration of cafestol in the culture medium on mycelial growth after 72 h of culture of the six pathogenic fungi *Aspergillus niger*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum, Fusarium proliferatum*, *Fusarium solani* and *Talaromyces stollii*. Fungal growth was measured in triplicate by the mycelial area (mm^2). Fungi were cultivated on two media (Potato-Dextrose-Agar: PDA; Sabouraud: SAB). Data are shown as the mean ± s.d. Bars containing the same letter were not significantly different at p = 0.05 according to the Waller-Duncan post-hoc test.

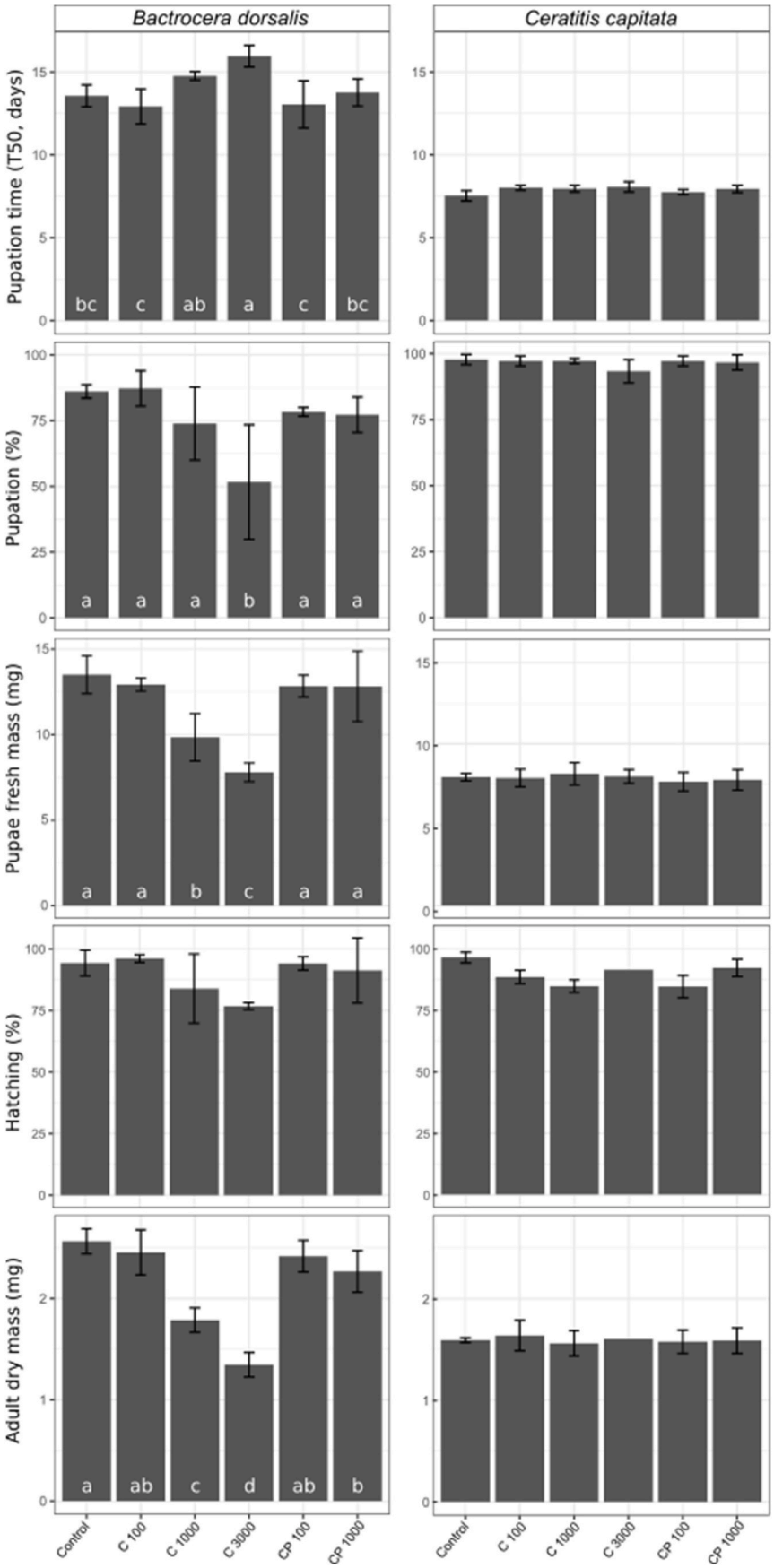

Fig. 3. Effect of the concentration of cafestol (C) and cafestol palmitate (CP) in the larvae diet on five developmental variables of *Bactrocera dorsalis* and *Ceratitis capitata*: time to reach 50% of pupation (T50, days), the final pupation rate (%), the pupae fresh mass (mg), the hatching rate (%), and the adult dry mass (mg). C 100 = cafestol 100 μM; C 1000 = cafestol 1000 μM; C 3000 = cafestol 3000 μM; CP 100 = cafestol palmitate 100 μM; CP 1000 = cafestol palmitate 1000 μM. Data are shown as the mean ± s.d. Means were compared using the Waller-Duncan post-hoc test when ANOVA showed a significant effect of the concentration of diterpene. Bars containing the same letter were not significantly different at p-value = 0.05.

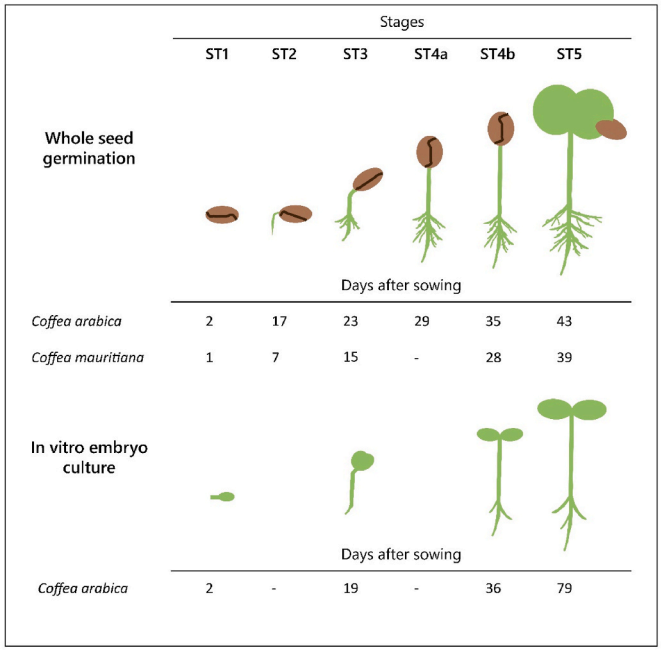

Fig. 4. Schematic representation of the developmental stages (ST1-5) sampled in seedlings obtained from whole seed germination and plantlets recovered from embryos extracted from the endosperm and germinated *in vitro*. Since stages were assigned according to phenotypic criteria, the number of days after sowing varied with the species and with the initial material (whole seed or isolated embryo): ST1: ungerminated imbibed seed or isolated embryo; ST2: germinated seed lying on vermiculite with a curved hypocotyl (apical hook) and radicle together measuring ca. 10 mm; ST3: seed above the vermiculite due to lengthening of the hypocotyl (mean value of 12.9 mm) and apical hook half unfolded; ST4a: apical hook fully unfolded and straight hypocotyl measuring 22.7 mm; ST4b: further elongation of the hypocotyl up to 35.8 mm; ST5: unfolding of the cotyledonary leaves out of the residual endosperm.

fungal species), using two different culture media, PDA and SAB, and six concentrations of cafestol ranging from 0 to 3000 μM (Fig. 2 and Supplementary Fig. 5). *A. niger* was chosen because it infests coffee seeds (Taniwaki et al., 2003). The five other strains allow to test the activity of cafestol against a broad spectrum of generalist tropical pathogenic fungi. Cafestol did not affect *A. niger* growth in the range of concentrations tested (Fig. 2). By contrast, after 72 h of culture, mycelial growth was significantly reduced at cafestol concentrations as low as 100 μM in the five other fungal strains, whatever the culture medium used (Fig. 2; Supplementary Table 3). A significant effect was even observed at 10 μM in *T. stollii* cultivated on SAB. In *C. gloeosporioides*, *F. oxysporum*, *F. proliferatum* and *T. stollii* cultivated on SAB, the reduction in mycelial growth increased significantly with increasing concentrations of cafestol. This was also observed with *C. gloeosporioides* grown on the PDA culture medium, while for the four other fungal species, the extent of growth reduction on PDA was similar at all concentrations higher than or equal to 100 μM. The highest inhibitory effect was observed at 1000 and 3000 μM cafestol in *C. gloeosporioides* and *T. stollii*, with about 70% reduction in mycelial growth after 72 h of culture on SAB medium (Fig. 2; Supplementary Table 3). For these two fungal strains, at 3000 μM cafestol, mycelial growth was still reduced by ca. 35% at the end of the 7-day culture period (Supplementary Fig. 5).

3.2. Anti-insect activity of cafestol

To test the anti-insect activity of cafestol and cafestol palmitate, two fruit flies originating from contrasted geographical areas were chosen. The natural distribution area of *C. capitata*, which is a pest of cultivated coffee species, is similar to that of African species of the genus *Coffea*. By contrast, *Coffea* species could not be a natural host of *B. dorsalis* since it is endemic to Southeast Asia. No-choice bioassays were conducted using various cafestol concentrations in the diet fed to the fruit fly larvae (Fig. 3). Neither of the two forms (free cafestol and cafestol ester) had a significant effect against *C. capitata* during larvae development, pupation and hatching. By contrast, cafestol had a marked effect against *B. dorsalis* at different development stages significantly affecting four of the five variables measured, i.e. pupation time, the final pupation percentage, as well as pupae and adult masses. At 3000 μM, cafestol delayed the time to achieve 50% pupation (T50) by 2.4 days while the final pupation percentage, and the pupae and adult masses were all reduced by more than 40% (Supplementary Table 4). Although no significant effect was found at a concentration of 100 μM, pupal and adult masses were already reduced by 27% and 30%, respectively, at a concentration of 1000 μM cafestol (Supplementary Table 4). At the highest concentration tested (1000 μM), cafestol palmitate had no effect on pupation time, pupation and hatching percentages, or on pupal fresh mass, but had a small yet significant effect (12% reduction) on *B. dorsalis* adult dry mass.

3.3. Fate of endosperm diterpenes during seed germination and seedling development

The period from seed imbibition (stage 1) and germination (i.e. radicle protrusion, stage 2) to the fall of the residual endosperm due to the unfolding of cotyledonary leaves (stage 6) required ca. 40 days in both *C. mauritiana* and *C. arabica* (Figs. 4 and 5). During this period, almost 80% of the endosperm was digested in both species as estimated by the decrease in its dry mass (Fig. 5A–B). Diterpenes were monitored in different organs of germinating and developing *C. arabica* and *C. mauritiana* seedlings, and total cafestol and kahweol contents (including free and esterified forms) were compared both in the endosperm and in the seedling (Fig. 5C–F). The endosperm of mature seeds of the *C. arabica* genotype used (variety Marsellesa) contained mostly kahweol (Fig. 5C and E). During development of the *C. arabica* seedlings, the amount of kahweol in the endosperm decreased from 1000 to 300 μg (Fig. 5C). Over the same period, the amount of kahweol in the seedling increased by more than 500 μg, suggesting that kahweol was not catabolised during endosperm digestion, but mostly transferred to the

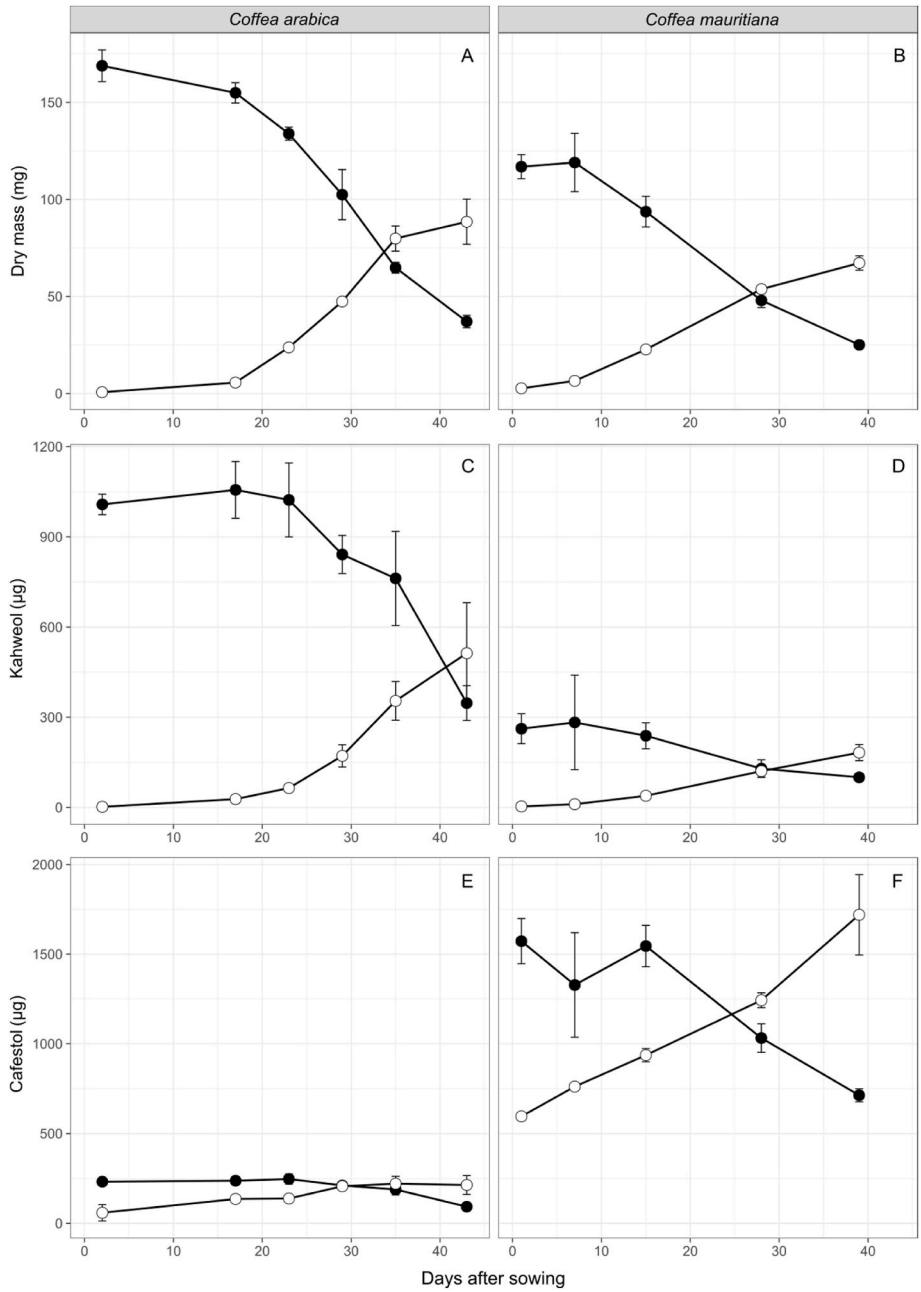

Fig. 5. Changes in dry mass (A and B) and in kahweol (C and D) and cafestol (E and F) amounts in the endosperm (black circle) and the seedling (white circle) during germination and seedling development in *C. arabica* (A, C, and E) and *C. mauritiana* (B, D and F). Data are shown as means ± s.d.

seedling. Although the original concentration of cafestol in the endosperm of mature *C. arabica* seeds was considerably less than that of kahweol, similar mirroring patterns were observed in the digesting endosperm and developing seedling (Fig. 5E). Indeed, cafestol decreased by ca. 150 µg in the endosperm and increased by the same amount in the seedling. In contrast to *C. arabica*, the main diterpene in the endosperm of *C. mauritiana* seeds was cafestol (Fig. 5D and F). However, although the cafestol to kahweol ratio was inverted, patterns similar to those found in *C. arabica* were observed in *C. mauritiana*: at each developmental stage, the increase in each diterpene in the seedling quantitatively corresponded to its decline in the endosperm. The underlying processes therefore seem to be common to both *Coffea* species and to not depend on the major diterpene accumulated during seed development.

To verify that diterpene accumulation in the seedling was not the result of *de novo* synthesis, diterpenes were measured in plantlets of *C. arabica* recovered from embryos extracted from the endosperm (isolated embryo, IE) and germinated *in vitro* (Figs. 4 and 6). *C. arabica* was preferred to *C. mauritiana* because isolated embryos of the latter already contained large amounts of diterpenes (ca. 600 µg, Fig. 5F). In contrast to the endosperm, the initial amount of cafestol in the embryo was higher (ca. 60 µg) than that of kahweol (ca. 2 µg, Fig. 6). No cafestol was detected in cotyledons of IE derived seedlings at any developmental stage (Fig. 6A). Small amounts of cafestol were present in the hypocotyl and roots, but they did not increase during IE derived seedling growth and their sum was equivalent to the amount originally measured in the embryo. The same pattern was observed for kahweol (Fig. 6B), which was not detectable in cotyledons and was present in only trace amounts in the hypocotyl and root. These barely detectable amounts of kahweol in IE derived seedling contrast with the very high kahweol levels measured in seedlings recovered from whole seed (WS) germination

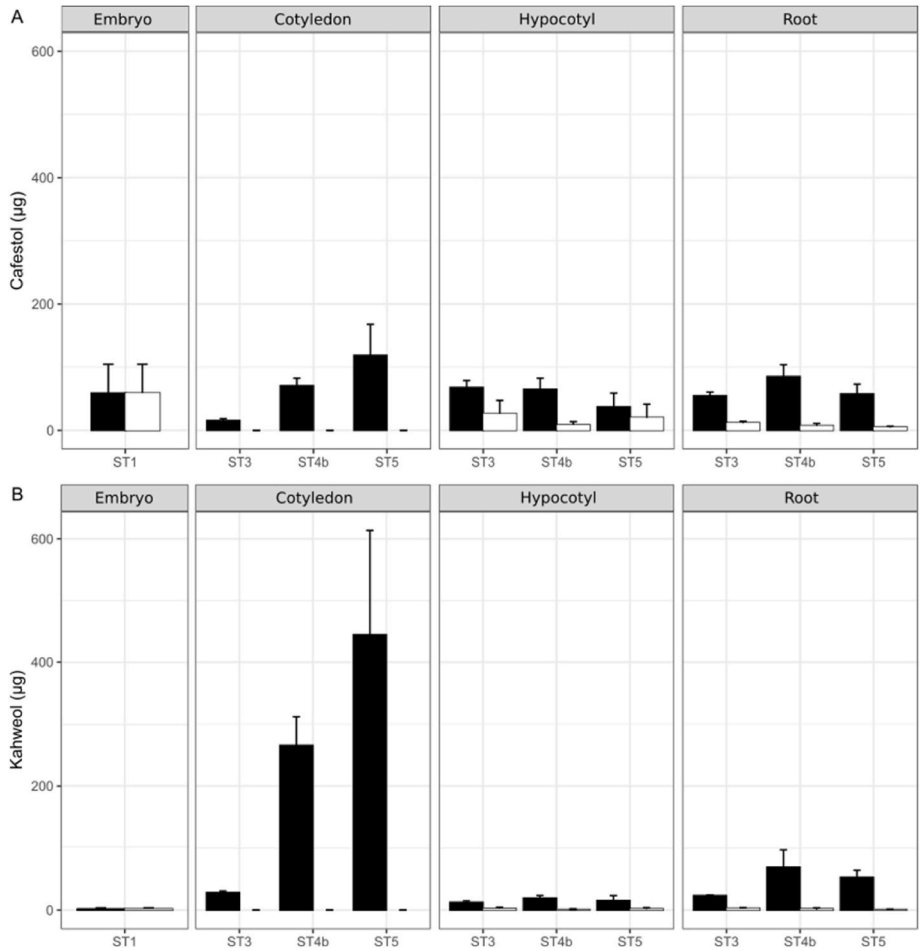

Fig. 6. Cafestol and kahweol amounts (µg) in imbibed embryos and in the cotyledons, hypocotyl and root of seedlings obtained from whole seed germination (black bars) and in plantlets recovered from embryos extracted from the endosperm and germinated *in vitro* (white bars) at three developmental stages (ST3, ST4b and ST5). Data are shown as the means ± s.d.

(Fig. 5C). In particular, at the organ level, the cotyledon kahweol content dramatically increased in the growing WS seedling, while no kahweol was found in the cotyledons of seedlings recovered from IE. Collectively, these results therefore suggest the absence of *de novo* diterpene synthesis in the developing seedling, although it cannot be completely ruled out that the accumulation of diterpenes in the seedling results from neosynthesis triggered by a signal from the degrading endosperm. Moreover, since substantial amounts of cafestol and kahweol were measured in all organs of the WS seedlings, our observations suggest that cafestol and kahweol were transported from the cotyledons to the roots through the hypocotyl after their transfer from the endosperm to the cotyledons.

3.4. Subcellular localization of diterpene esters in the endosperm

Endosperm sections were first stained with Nile red to reveal the localization of oil bodies. Oil bodies, whose size ranged from ca. 0.2–1 µm (Supplementary Fig. 1), formed a continuous layer at the periphery of each endosperm cell at confocal imaging resolution (Supplementary Fig. 6). To determine the subcellular localization of diterpene esters, a linear unmixing function was applied on spectral images of endosperm sections using the cafestol palmitate spectrum, which was almost identical to that of kahweol palmitate (Supplementary Fig. 2). The spectral signature of cafestol palmitate was also detected at the periphery of endosperm cells, suggesting that fatty acid esters of diterpenes could be stored in the TAG matrix of oil bodies (Supplementary Fig. 6). This hypothesis was investigated by chemical analysis of the diterpene content in purified oil bodies (Supplementary Fig. 1). No significant difference was observed in the level of diterpenes between oil bodies isolated from the endosperm and the whole endosperm tissue as expressed in mg per g of TAG (65.1 and 69.4 mg/g TAG, respectively; Fig. 7), confirming that diterpene esters are located in endosperm oil bodies.

4. Discussion

A plethora of plant diterpenoids have been shown to have antimicrobial, antifungal, antifeedant or insecticidal activities (Keeling and Bohlmann, 2006; Schmelz et al., 2014). Based on this abundant literature, we hypothesised that *Coffea* specific seed diterpenes may be constitutive defences against pests. To our knowledge, the present work is the first to question the ecological role of coffee seed diterpenes, in contrast to the growing number of studies on their effect on human health, since they are present in the coffee brew, or their potential therapeutic applications (Ren et al., 2019). Cafestol significantly delayed the mycelial growth of five of the six fungi tested, and showed antifeedant and insecticidal activities against the larvae and pupae of the fruit fly *B. dorsalis.* These effects were observed at concentrations well below levels found in seeds of all coffee species studied so far (De Roos et al., 1997). As it is able to affect the growth, development or

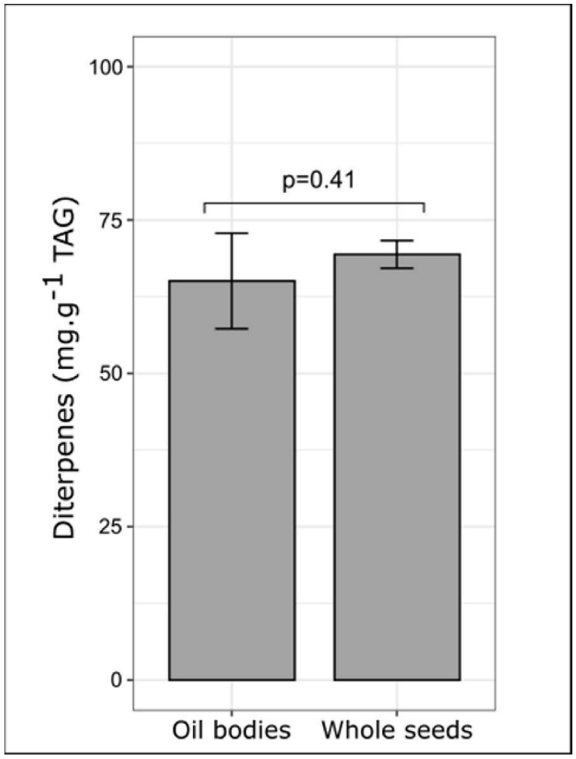

Fig. 7. Diterpene content in purified oil bodies and whole endosperm.

survival of both fungi and insects, cafestol may be thus considered a 'generalised defence compound', as conceptualised by Krischik et al. (1991) based on the inhibitory effect of nicotine against both herbivores and microbial pathogens. Since then, several secondary metabolites have been shown to have the ability to protect plants against several types of pests, including caffeine (Ashihara et al., 2008) and the iridoid glycosides produced by *Plantago lanceolata* (Biere et al., 2004). A central question that arises from this result is whether cafestol acts against insects and fungi via the same or different modes. Little is known about the mechanisms involved in diterpene toxicity to insects and pathogenic fungi (Gershenzon and Dudareva, 2007).

Although cafestol hampered the growth of five of the six fungi tested and compromised the development of *B. dorsalis* larvae and pupae, it had no effect on the mycelium of *A. niger* or on the juvenile life stages of the fruit fly *C. capitata*. This fungus and this herbivore, which were chosen because they are pests of coffee, may have thus evolved counterdefense mechanisms. Phytopathogenic fungi usually overcome plant chemical defences through enzyme-mediated detoxification reactions. For example, many *Fusarium* spp. fungi are able to degrade phytoalexins of the benzoxazolinone class produced by cereals thanks to a specific γ-lactamase enzyme (Kettle et al., 2015). Which mechanism protected *A. niger* mycelial growth from cafestol remains unknown. However, the ability of *A. niger* to transform a very large diversity of terpenoids, including kaurane diterpenes, may be involved in its resistance to cafestol (Parshikov and Sutherland, 2014). Multiple strategies and mechanisms enable insects to resist plant allelochemicals, including sequestration, excretion and detoxification via enzymes that cleave or modify defence compounds, mostly in the saliva or the gut (Després et al., 2007). The fruit fly *C. capitata* originates from the Afrotropical region, which is also the natural distribution area of African species of the genus *Coffea* (De Meyer et al., 2008). Coffee berries in plantations of cultivated coffee species in Africa can be infested by *C. capitata* (Wharton et al., 2000), but it has also been found in the fruits of wild coffee species, such as *Coffea racemosa* (Grové et al., 2017). Although at lower rates than in the endosperm, the coffee fruit pericarp also contains significant amounts of cafestol (Rafael et al., 2010). Therefore, our observations that *C. capitata* larvae were not affected by any concentration of cafestol may reflect a long-term counteradaptation of this herbivore to the specific diterpenes of *Coffea* in their common natural distribution area, through one or several of the mechanisms identified in other plant-predator interactions (Després et al., 2007).

Our bioactivity assays showed that cafestol palmitate had no effect on the growth and development of any of the fungi and insects tested. Since the fatty acid esters of diterpenes are the main form in the endosperm (ca. 97% of total diterpenes, Speer and Kölling-Speer, 2006), this major finding raises two key questions: i) what are the benefits of acylating cafestol and kahweol when they show no bioactivity, and ii) how diterpene esters are hydrolyzed to free diterpenes and protect the plant against pests. Constitutive plant defence compounds are often stored in a non-toxic form. Indeed, broad-spectrum constitutive plant allelochemicals, which appear to include cafestol, are often also toxic to the plant cells that produce and store them (Heiling et al., 2021). Specific decorations of defence compounds, such as glycolysation, hydroxylation, acetylation or acylation, are frequently used by plants to facilitate their storage, in particular their solubility in one of the cell compartments, and to solve the problem of autotoxicity. Fatty acid esters of bioactive terpenoids are less frequently encountered than glycosylated forms. However, fatty acid diterpene esters are found in seeds of numerous Euphorbiaceae, of which Croton and Jatropha phorbol esters have been abundantly documented, in particular their insecticidal properties (Devappa et al., 2012). Like coffee, *Jatropha curcas* produces albuminous seeds that store large amounts of oil and diterpene esters are mostly accumulated in the endosperm (Devappa et al., 2012). In a plant tissue that stores massive apolar TAG reserves in specialised structures called oil bodies, esterification with fatty acids may be the most efficient strategy to accumulate large quantities of diterpenes in a non-phytotoxic form. In support of this hypothesis, we showed through oil body purification and confocal imaging, that cafestol and kahweol are stored in the endosperm oil bodies. This calls for investigation of how TAG and diterpene biosynthesis are connected during coffee seed development. In this respect, it is worth noting that the major fatty acids in coffee seed TAGs and diterpene esters are the same (Joët et al., 2009; Speer and Kölling-Speer, 2006), suggesting their biosynthetic pathways use the same pool of acyl-CoA in the endoplasmic reticulum.

The release of the bioactive molecule from its storage form has been extensively studied in the case of glycosylated defence compounds. A specific glucosidase is usually synthesised by the plant and stored separately from the pool of latent defence compounds, either in another compartment of the same cell, e.g. in the apoplast, while glycosylated compounds are stored in the vacuole (Gruhnert et al., 1994), or in different cells of the same tissue, such as in the well-known 'mustard oil bomb' (Kissen et al., 2009). In both cases, the hydrolysing enzyme and the conjugate come into contact upon cell or tissue disruption during fungi or insect attack, resulting in the release of toxic compounds. Alternatively, hydrolysis may be catalysed by glucosidases produced by the bioagressor (Bouarab et al., 2002). Regarding fatty acid esters of cafestol and kahweol, the release of active forms depends on lipase activity. Both pathogenic fungi (Feng et al., 2009) and herbivores (Weidlich et al., 2015) secret broad-spectrum lipases that could act on coffee diterpene esters. Alternatively, most oily seeds accumulate lipases in a subcellular compartment other than oil bodies to prepare for lipid mobilisation during germination (Zienkiewicz et al., 2014). This spatial separation resembles that described above for glucosidases and glycosylated defence compounds. The subcellular localization of lipases in coffee seeds is not known. However, transcripts encoding lipases are highly accumulated during coffee seed maturation (Cheng et al., 2018), lipase activity has been detected in mature coffee seeds prior to imbibition and germination (Patui et al., 2014) and, very interestingly, is responsible for the hydrolysis of diterpene esters during storage of coffee

seeds in suboptimal conditions (Speer and Kölling-Speer, 2006). Therefore, the endogenous enzymatic machinery necessary to release free cafestol and kahweol upon cell disruption due to fungus or insect damage is present in the coffee seed and could contribute to its defence.

Two general strategies have been adopted by plants to protect their juvenile seedlings, which are highly vulnerable to pathogenic fungi and herbivores (De-La-Cruz Chacón et al., 2013): i) secondary metabolites are synthesised and stored in the reserve organ during seed development and transferred to different tissues of the seedling after germination, or, ii) defence compounds are not accumulated in the developing seed but synthesised *de novo* by the seedling itself very soon after germination. Our diterpene measurements in the various tissues of the seed and the seedling, from germination to the opening of cotyledonary leaves, suggest that diterpenes stored in the endosperm are transferred to all the seedling organs. Along with caffeine, trigonelline and chlorogenic acids (Aerts and Baumann, 1994; Baumann and Gabriel, 1984), this is a remarkable example of how *Coffea* species maximize the resource investment dedicated to the synthesis of defence compounds, by conferring protection to the developing seed *in planta*, then to the mature seed on the soil awaiting favourable climatic conditions to germinate, and finally to the resulting seedling. As the metabolic cost of terpenoid accumulation is very high (Gershenzon, 1994), this reallocation strategy appears to be particularly effective in the case of cafestol and kahweol. Following their transfer from the endosperm, seedling tissue distribution differed between cafestol and kahweol. At the final developmental stage of *C. arabica* seedlings, kahweol amounts were very high in the cotyledons, moderate in the root and low in the hypocotyl, whereas similar amounts of cafestol were present in all three tissues. Like kahweol, caffeine and trigonelline concentrations also vary between organs in the young coffee seedling (Zheng and Ashihara, 2004).

By which mechanisms, and in which chemical forms, cafestol and kahweol are transferred from the endosperm to the embedded cotyledons, and then transported from the cotyledons to the roots through the hypocotyl, remain to be elucidated. In *Hevea brasiliensis*, which produces albuminous seeds, the cyanogenic linamarin stored in the endosperm is first glycosylated after germination, excreted in the apoplast and reincorporated in the cotyledons in its conjugated form, then either hydrolyzed in the cotyledons to form linamarin again or transported through the epicotyl to the leaves (Selmar, 1993). As fatty acid esters of diterpenes are insoluble in water, their hydrolysis appears to be necessary for apoplastic diffusion and uptake by the cotyledons. Specific membrane transporters of the ATP Binding Cassette (ABC) family have been identified for diterpene transport in various plant models (Pierman et al., 2017). Because gibberellins share the diterpenoid kaurane skeleton with cafestol and kahweol, recent progress in the identification of the gibberellin plasma membrane transporters SWEET13/14 and NPF3 and the long-distance transport mechanisms of gibberellins may also be of interest (Binenbaum et al., 2018). Transcriptome and proteome analysis of the cotyledons growing in the endosperm after germination should greatly facilitate the identification of the actors involved in coffee diterpene remobilisation.

5. Conclusions

In the majority of plant species, the seed coat and the endocarp are the first line of the seed defence against herbivores and pathogens (Radchuk and Borisjuk, 2014). In coffee seeds, both tissues are especially tenuous and confer very little protection. Instead, species of the genus *Coffea* accumulate a spectacular array of chemical defences within the endosperm, including alkaloids, phenolics and terpenoids, which may collectively account for more than 10% of the seed dry matter. During their evolutionary history, *Coffea* species colonised almost all forest ecosystems of the Afrotropic ecozone, including savannas, lowland evergreen forests, and mountain forests (Anthony et al., 2010). The biosynthesis of constitutive generalised defence compounds was maintained through migration and speciation, suggesting they confer major adaptive advantages. One of these advantages is certainly the capacity to transfer defence compounds from the endosperm to the seedling after germination. Indeed, coffee seeds are sensitive to desiccation (Eira et al., 2006) and, like in most desiccation-sensitive seed species (Vazquez-Yanes and Orozco-Segovia, 1993), the seeds germinate rapidly after dispersal and the seedling bank is thus more abundant than the soil seed bank. However, great interspecific variability was observed in the seed content of each class of defence compounds (Anthony et al., 1993; De Roos et al., 1997). This may reflect the varying biotic pressures encountered by *Coffea* species in the highly diverse habitats and geographical areas they occupy (Biere et al., 2004). Regarding diterpenes, not only their total content varies between *Coffea* species, but also their skeleton and decorations. For instance, a major diterpenoid compound of still unknown structure was found in two species of East Africa, *Coffea pseudozanguebariae* and *Coffea salvatrix* (De Roos et al., 1997), which also accumulate a glycosylated diterpene, termed mozambioside (Anthony et al., 1993). Major efforts are thus still needed to obtain a comprehensive view of the terpenoids of *Coffea* species and their biological activities.

Author contribution

Conceptualization: TJ, SD; Methodology: GA, VV, JCM, LC, TJ, SD; Formal analysis: GA, TJ, SD; Investigation: GA, VV, JCM, JP, GC, TJ, SD; Resources: JCM, JP, GC, LC; Writing - Original Draft: GA; Writing - Review & Editing: JCM, LC, IFB, TJ, SD; Supervision: TJ, SD; Funding acquisition: IFB, TJ, SD.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2022.12.013.

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