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Metabolism of phenolic compounds during infection of cotton (*gossypium hirsutum* l. cv. R405-2000) *in vitro* plants and callus by *Fusarium oxysporum f. sp. vasinfectum*, a causal agent of *Fusarium* wilt

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Introduction

the cotton plant is a multipurpose textile and protein-oil crop (charvet 2020). cotton is côte d'ivoire's 3rd largest export product after cocoa and coffee (schwartz, 2012 ; ndayishimiye, 2013). it is the 5th largest agricultural export in terms of value after cocoa, cashew nuts, coffee and palm oil (icac 2019). it is therefore a very important source of foreign currency for the national economy. cotton is the main raw material for the textile industry and the oil and protein-rich seeds are used in human and animal nutrition (charvet 2020). however, the cotton plant is threatened by various pests and diseases that reduce production, fibre and seed quality (vaissayre, 1994). of all the cryptogamic disease attacks, fusariosis caused by *fusarium oxysporum* f. sp *vasinfectum* (fov) seems to be the most dreaded. however, studies have shown that when faced with pathogen aggression, plants produce secondary metabolites, notably phenolic compounds, to defend themselves (bruneton, 2015). thus, the cotton plant produces a large number of phenolic compounds that are a determining factor in disease resistance (konan et al, 2014). wiese and devay (1970) showed that the level of phenolic compounds increases in cotton during the cotton-verticillium interaction. vessere (1980) noted that the degradation or oxidation product of phenols has a bactericidal action, while konan (2015) and n'cho (2019) reported their antifungal activity. in order to understand the role of phenolic compounds in the cotton-fov interaction, we followed the evolution of fusarium head blight symptoms and analysed the metabolism of phenolic compounds after infestation of vitroplants and callus by the fungus. this allowed us to assess the susceptibility of the plant material in relation to phenolic metabolism, and hence to envisage studies to promote the resistance of cotton to this dreaded fungus.

ABSTRACT

This work was carried out to study the response of phenolic metabolism in vitro plants and callus to infections caused by fov. this allowed us to assess the susceptibility of the plant material in relation to phenolic metabolism, and hence to envisage studies to promote cotton resistance to this dreaded fungus.*In vitro* plants and callus from *in vitro* culture of cotton seeds of the R405-2000 variety of cotton (*gossypium hirsutum* l), were infected with 0;10;20;30;40;50µl of fov inoculum. thus, the study of the metabolism of phenolic compounds in the in vitro plants and callus of cotton (*gossypium hirsutum* l.) infected with fov, showed a regular decrease in the level of soluble phenols, while the level of bound phenols was stable compared to healthy in vitro plants and callus. the activity of soluble pal and tal also decreased in the infected material, while the activities of soluble peroxidases (soluble and insoluble), free ppo and aia oxidase were high. At the end of this study we note that the doses of inoculum used produced the same symptoms, although the 40 and 50 µl increased the damage. after 7 days of incubation, the plants showed the characteristic signs of fusarium wilt. all these changes could be considered as defence reactions of the plant to the infection. therefore, as a result of this study we can consider:

- aia oxidase and tal as potential markers of *fusarium oxysporum. f sp vasinfectum* infection of cotton *in vitro*.

- ppo as a potential marker of callus infection in cotton

key words: cotton, *gossypium hirsutum* l.; phenolic metabolism; fusarium; cal; vitroplant

Material and methods

Plant material and fungal material

the plant material consisted of cotton seeds of the variety gossypium hirsutum l.cv. r 405-2000 supplied by the compagnie ivoirienne du développement du textile (cidt). R405-2000 is a cultivar originating from côte d'ivoire (local cultivar) generally produced in the regions of niellé, m'bingué, korhogo. the strain of fusarium oxysporum f. sp vasinfectum was provided by the phytopathology laboratory of esa (ecole supérieur d'agronomie)

Methods

In vitro seed germination

seeds were first disintegrated (fibre removed) with concentrated sulphuric acid, then sterilised in a laminar flow hood by a rapid soak (1 min.) in 70% ethanol, followed by a 20 min. immersion in sodium hypochlorite (3.6% active chlorine). after three successive rinses with sterile distilled water for 3 to 5 min. the seeds were soaked in sterile distilled water. one to two seeds were placed in each test tube (20 mm diameter), containing approximately 35 ml of sterile distilled water. the test tubes were then placed in the dark for 48 h to promote radicle development. seeds with the radicle pointing after imbibition were stripped of their seed coats and placed in test tubes each containing 10 ml of solid ½ ms medium (macro and micro element content halved) to which 3% sucrose was added. the test tubes, sealed with plastic caps, were placed in the dark for 4 days to stimulate in vitro seed germination. they were transferred to light for 3 days. after 7 days of incubation, seedlings of about 15 cm in length with two cotyledons, a hypocotyl and roots were obtained.

Callus culture

hypocotyl segments of about 0.5 cm in length were taken from cotton seedlings and used for callus initiation. five explants were placed in petri dishes (90 mm diameter) each containing 30 ml of callogenesis medium or one per test tube containing 10 ml of callogenesis medium. this medium is represented by ms of solid base to which 3% glucose and hormones (0.1 mg/l of 2.4-d and 0.5 mg/l of kinetin) are added. petri dishes were sealed with parafilm. these explants were incubated for 8 weeks under a 14 h photoperiod.

Transplanting and purification of fov

the fungus was transplanted according to the method of vakili (1968) on pda (potato dextrose agar) medium: autoclaved at 121 °c for 30 min. under a pressure of 1 bar. agar explants of about 0.5 cm in diameter are taken from the test tubes containing the fungus on slant agar medium. they are then transferred under a hood to petri dishes containing pda medium. the inoculated petri dishes are incubated for 7 days in a culture room at a temperature of 25 ± 2 °c under a 12 h photoperiod. these conditions are those recommended by messiaen and cassini (1968) to obtain the expression of the desired characteristics.

Demonstration of pathogenicity

the pathogenicity of *fusarium oxysporum f. sp. vasinfectum* was demonstrated by inoculating 20 7-day-old cotton seedlings. these seedlings were obtained by sowing sterile cotton seeds

(under in vitro germination conditions) in pots containing soil autoclaved at 121°c for 30 min under a pressure of 1 bar. inoculation was done using agar explants carrying the pure fungus. these explants are mixed with soil containing the seedling at the two cotyledonary leaf stage

Inoculation of vitroplants and callus

Preparation of the culture filtrates

the inoculum was prepared from 5 mm diameter explants taken with a punch from fungal colonies growing on pda medium. the explants were placed in liquid media previously autoclaved at 121 °c for 30 min. under a pressure of 1 bar. the foil-covered erlenmeyer flasks are placed on a rotary shaker at 200 rpm in the culture room at 28 ± 2 °c. after 7 days of incubation, the solutions contained in the erlenmeyer flasks are frozen and then thawed to cause the release of the toxic metabolites contained in the spores and mycelia. the whole is filtered with sterile filter paper. the filtrate obtained then constitutes the inoculum.

Inoculation

inoculations of seedlings from in vitro germination were made by injecting 10, 20, 30, 40 and 50 µl of culture filtrate per plant into the culture substrate near the roots. the 2-month-old callus was inoculated using the same volumes of inoculum as above.

Assay

the assay was carried out on healthy and *fusarium oxysporum f. sp. vasinfectum*-infected in vitro plants and callus. for the extraction of the different compounds, the plant material was ground in a laboratory mortar. we used a selecta centrifuge and a milton roy spectrophotometer (spectronic 601) for the different manipulations. five enzymes of phenolic metabolism were analysed; two biosynthetic enzymes and three degradation enzymes of phenolic compounds. all extraction and purification steps are conducted at low temperature (4 °c) using ice packs.

Extraction and determination of phenolic compounds

Extraction

0.5 g of plant material (seedling and callus) was ground in 5 ml of 80% methanol to which 0.5 ml of 0.5% sodium metabisulphite was added. after centrifugation at 5000 rpm for 5 min, the supernatant obtained is the crude extract of the soluble fraction. the residue obtained is then ground with 80% methanol and 100% acetone. the grind is taken up with 1 ml of 4n sodium

hydroxide (NaOH) followed by a one-hour decantation (alkaline hydrolysis) to isolate the wall-bound phenolic compounds. after centrifugation under the same conditions as before, the supernatant was removed and the residue was taken up in 2 ml of pure ethyl acetate. the combined supernatant and pellet constituted the crude extract on which the determination of total phenols was performed

Determination

the determination of total phenols is done by the method of singh et al (2002), modified and adapted to our plant material. the reaction mixture is mainly composed of phosphotungstic acid and phosphomolybdic acid which will be reduced in alkaline medium, in parallel with the oxidation of phenols. the presence of the phenols is revealed by the addition of 0.5 ml of folin-ciocalteu 1 n reagent, 1.5 ml of 17 % sodium carbonate and 0.5 ml of crude extract. the intensity of the blue coloration produced by this reaction, which is proportional to the concentration of phenolic compounds in the extract, is monitored with a spectrophotometer at a wavelength of 765 nm. during the assay, a control is carried out where the phenolic extract is replaced by distilled water. the level of total phenols is determined using a standard curve with different concentrations of a stock solution of paracoumaric acid (200 µg/ml) and is expressed as mg/g mf. extraction and determination of enzymes

Extraction of enzymes

the buffer required for the extraction of the enzyme substances depended on the enzymes studied and was therefore specified later for each of them. the enzymes were extracted cold at 4 °c by grinding fresh plant material (seedling and callus) in extraction buffer. during grinding 1.2 ml of an extraction medium solution consisting of 0.5 ml polyethylene glycol 6000 (peg 6000), 0.25% sodium thiosulphate, 15% glycerol, 1 mm edta and 15 mm mercaptoethanol was added. after centrifugation, at 5000 rpm for 20 min, the resulting supernatant represented the crude extract of cytoplasm-soluble enzymes. the residue was depleted by 3 times as before. then 1 ml of 1% triton x 100 and 1 ml of the extraction medium were added. after centrifugation (under the same conditions), the supernatant was removed, the residue was taken up in the same buffer for a new extraction conducted in the presence of polyvinylpyrrolidone (pvp), followed by one hour of incubation. after centrifugation, the resulting supernatant represented the crude wall-bound enzyme extract.

Purification of the crude extracts

dowex 2 was dissolved in the crude extracts and incubated for 30 min. under stirring. centrifugation at 5000 rpm for 5 min was performed to remove the dowex 2 (which bound the inhibitor ions). the supernatant obtained was the purified enzyme fraction ready for analysis.

Determination of the enzymes

Ammonia-lyases

these are pal and tal. the reaction catalysed by these two enzymes is irreversible and the spectrophotometric evolution of the rate of production of cinnamic and p-coumaric acids, respectively, reflects the enzymatic activity.

the determination of these two enzymes was carried out using the method described by regnier, (1994) modified and adapted to our plant material. the basic buffer used was 0.2 m sodium borate at ph 8.8. the reaction mixture contained: 0.1 ml of enzyme extract, 1 ml of 0.1 m phenylalanine for pal or 0.1 m tyrosine for tal and 1.9 ml of 0.2 m sodium borate buffer at ph 8.8. after 10 min incubation at room temperature, the activity of pal and tal, which is proportional to the amount of cinnamic acid and p-coumaric acid respectively, is monitored by spectrophotometer at a wavelength of 290 nm. in the assay, a control test was performed for each extract in which phenylalanine or tyrosine is replaced by 0.2 m sodium borate buffer at ph 8.8. the activity of pal and tal is expressed in millimoles of cinnamic acid or p-coumaric acid formed per minute per gram of fresh material, respectively, assuming that the molar extinction coefficient of cinnamic acid is equal to 19600 cm-1 mol-1 and that of p-coumaric acid is equal to 17600 cm-1 mol-1.

Peroxidases

peroxidase activity was determined according to the technique described by santimone (1973). the base buffer used was 0.1 m sodium phosphate at ph 7.5. the reaction mixture was composed of 0.2 ml of enzyme extract and 2.8 ml of substrate consisting of a 10-2 m guaiacol and 10-2 m hydrogen peroxide solution (v/v). after shaking, the mixture was incubated for 10 min. in the dark to prevent partial destruction (by light) of the red-brown oxidation product formed from guaiacol in the presence of hydrogen peroxide. a delay of one minute was allowed between tubes when adding the enzyme extract to the substrate. the oxidation of guaiacol was monitored with a spectrophotometer at a wavelength of 470 nm, always respecting the one minute delay between tubes. a control was made where the substrate was

replaced by 0.1 m sodium phosphate buffer at ph 7.5. peroxidase activity is expressed in millimoles of product formed per minute per gram of fresh material. the molar extinction coefficient of the product formed at a wavelength of 470 nm is equal to 26.6 cm-1 mol-1 (santimone, 1973).

Polyphenoloxidases (ppo)

the determination of ppo activity was done according to the method of cano et al (1980), modified and adapted to our plant material. the base buffer used was 0.1 m phosphate citrate at ph 6.5. for wall-bound ppos, before the addition of dowex 2, two volumes (2xvml) of 100% acetone (to precipitate enzymes contained in the supernatant) are added to volume (v) of supernatant. a final centrifugation under the same conditions as above yields a supernatant which represents the crude extract of wall-bound ppo. the reaction mixture, incubated for 10 min. at room temperature (25 °c) was composed of 0.1 ml of enzyme extract and 1 ml of 130 mm pyrocatechol. the oxidation of pyrocatechol is monitored by spectrophotometer at a wavelength of 500 nm against a control assay in which pyrocatechol was replaced by 0.1 m phosphate citrate buffer at ph 6.5. ppo activity was expressed as the change in optical density (Δdo) per minute per gram of fresh material (ngalani and crouzet, 1986).

AIA oxidase

the determination of aia oxidase activity was carried out according to the method of hoyle (1972) modified by el bellaj and el hadrami (1998). the base buffer used was 0.1 m sodium phosphate at ph 7.5. the reaction mixture was composed of 0.2 ml of enzyme extract to which were added: 0.3 ml 1 mm mncl2, 0.5 ml 1mm p-coumaric acid and 0.5 ml 0.5 mm IAA.

the reaction mixture was incubated for 20 min. 1.5 ml of salkowsky's solution (9 ml 0.5 m fecl3 ; 300 ml h2o and 180 ml h2so4) was added and incubated under stirring for 30 min. the intensity of the pink coloration characteristic of the presence of iaa is estimated at the wavelength of 535 nm by spectrophotometer against a control test in which the salkowsky reagent was replaced by 0.1 m sodium phosphate buffer at ph 7.5. the amount of oxidised iaa was estimated using a standard curve which was performed with different concentrations of an iaa stock solution (125 µ/ml) in the presence of the salkowsky reagents, 1 mm mncl2, 1mm p-coumaric acid and different concentrations of 0.1 m sodium phosphate buffer at ph 7.5.

Statistical analysis

the values corresponding to the different biochemical parameters studied represented the average of three separate experiments (each experiment consists of three trials). for the analysis of the results, two types of statistical tests were used:

analysis of variance with one classification criterion (anova 1) was performed to compare the different biochemical parameters. the method of smallest significant differences (lsd) is used to classify the parameters when there is a significant difference between them.

the principal component analysis (pca), which, based on the variables characterising a group of objects, made it possible to represent, in a reduced space, a maximum of information on the possible relationships between the variables. the various statistical analyses were carried out using statistica 7.0 software.

Results and discussion

Effect of fov inoculum on the vitroplants

the experiment conducted in the presence of fov inoculum revealed that all volumes (10µl, 20µl, 30µl, 40µl and 50µl) used induced the same symptoms. however, the aggressiveness was different according to the dose of inoculum. after 7 days of cultivation, seedlings inoculated with doses of 10 to 30 µl showed symptoms of fusarium head blight but remained alive. seedlings inoculated with 40 and 50 µl of fov inoculum showed wilted leaves, brown and wilted stems, softened and necrotic roots. the 40 and 50 µl fov inoculum doses were therefore lethal to the seedlings (figure 1).

Figure 1: Appearance of control and FOV-inoculated cotton plants

A: 50 µl FOV inoculum after 7 days incubation; B: 20 µl FOV inoculum after 7 days incubation; e: trial; t: control

Total phenols, Pal, Tal, peroxidase, polyphenoloxidase and AIAoxidase content in healthy and FOV-inoculated glass plants after 7 days of incubation (Table 1)

The level of free phenols was higher than the level of wall-bound phenols in the glass plants. Thus, this study revealed that phenols are 13 times higher in the cytoplasm than in the walls (Table 1). It seems as if wall-bound phenols do not participate in the plant's defence reactions. The low level of these compounds in the walls may be one of the reasons. Indeed, TAN et al. (1992) and N'cho (2019) have shown that a low accumulation of phenols in the walls does not participate in the reinforcement and rigidification of the walls, which then become. Also, the observed decrease in the level of free phenols following infection is not in agreement with the results of many authors who have rather noted an increase in these compounds in infected materials (brzozowska et al., 1978, el bellaj and el hadrami,1998., n'cho et al., 2018). similarly, ziouti et al. (1996) and konan (2015) observed a high accumulation of free phenols, especially caffeoylshikimic acids, an essential component of the soluble phenolic composition of the root of resistant date palm (Phœnix dactylifera) plants against *Fusarium oxysporum f. sp. albedinis*. Furthermore, these results are in agreement with those of brzozowska and honower (1978) who observed a decrease in phenols in the cotton variety HAR 444-2 resulting from crosses between Gossypium hirsutum, Gossypium arboreum and Gossypium raïmondii during parasitic infections. It can therefore be assumed that cytoplasmic phenols do not have antifungal actions. This suggests that vitroplants are susceptible to infection by FOV, and that doses of 40 and 50 µl of filtrate of this fungus (inoculum) cause significant damage. However, it should be noted that the phenol contents measured at a given stage are the result of biosynthesis and transformation, in particular by oxidative degradation (kouakou, 2003). Indeed, the analysis of biosynthesis activities revealed an intense activity of free PAL and free TAL in healthy vitroplants, whereas the activity of bound PAL and bound TAL does not vary. Also, free PAL activity steadily decreases during infection while TAL activity becomes zero (Table 1). This decrease in the activity of these enzymes was reported in olive fruits (*Olea europaea L.*) by morrelo et al. (2005). In the same study, it was shown that the decrease in PAL activity resulted in a decrease in total phenols. This may therefore partly explain the decrease in free phenols observed in our infected material. Also, the significant decrease in free TAL suggests that in case of infection the synthesis of phenols takes place through the PAL pathway. Thus, the cinnamic acid pathway would be the most solicited for phenol biosynthesis in case of FOV infection. Indeed, our results showed that in the absence of infection, PAL contributes 63% to the biosynthesis of phenols against 27% for TAL, whereas with infection the contribution of PAL rises to almost 100%. This confirms the work of

richter (1993) who reported that PAL functions as a key enzyme in the branching of phenolic synthesis pathways, especially when the plant is under stress.

In addition, the analysis of the activities of the phenol degradation enzymes shows an increase in the activity of free peroxidases and free PPOs with the infection compared to the control, while bound peroxidases and bound PPOs do not undergo major modifications. These results are in agreement with those of many authors such as el bellaj and el hadrami (1998) who noted an increase in the activity of free peroxidases in date palm (phœnix dactylifera) against fusarium oxysporum f.sp. albedinis. ziouti et al (1996) had already suggested that the increase in peroxidase activities coincided with the decrease in free phenol content of the date palm root during infection. This is in line with our results.

Indeed, these results reveal that the contribution of peroxidases in the degradation of free phenols at baseline, which is 16% in healthy seedlings, increases to 50% during FOV infection. We can therefore think that during fungal stresses the phenolic compounds would be oxidised (hence a decrease in their content) into quinones which, according to nicholson and hammerschidt (1992), are remarkably toxic to parasites. similarly, constabel et al (1995) showed that the activity of free ppos increased during plant infection, which would indicate a significant oxidation of phenolic compounds. indeed, our results show that free ppos contribute to 84% of the degradation of free phenols in healthy plants, and to about 50% of the degradation of phenols in plants infected by fov. the oxidation of phenols by ppos would lead to the formation of diquinones whose beneficial effect in plant defence has been demonstrated by several authors (el bellaj and el hadrami, 1998; gouzi, 2014). this intense activity of ppos in healthy vitroplants suggests a strong presence of monophenols in this material. our study also revealed significant aia oxidase activity in healthy in vitro plants with, however, a more marked activity of free aia oxidase in fov-infected in vitro plants (4 times higher than the control). According to mérigouT (2006), IAA oxidase, an enzyme involved in the degradation of IAA, plays an important role in plant metabolism by catalysing the incorporation of phenolic compounds into the cell walls. In the same vein, el bellaj and his collaborators in 1998 specified that the oxidation of IAA generates H2O2 which is used by peroxidases in the attachment of phenolic compounds to cell walls. However, this intense IAA oxidase activity does not catalyse the incorporation of phenols into the cell wall, which would indicate that the incorporation of phenols is inhibited by endogenous factors. This could explain the sensitivity of our material to FOV.

Table 1: Total phenols; Pal; Tal; Peroxidase; Polyphenoloxidase and AIAoxidase content of healthy callus and callus inoculated with 0;10;20;30;40 and 50 µl of FOV inoculum after 7 days incubation

	PHENOLS		PAL		TAL		PEROXYDASE		PPO		AIAoxydase	
Inoculum(µl)	FREE	Linked	FREE	Linked	FREE	Linked	FREE	Linked	FREE	Linked	FREE	Linked
0	45±0.1^{a}	3.4±1.03^{a}	160±0.001^{a}	90±0.50^{a}	95±0.56^{a}	750±1.5^{a}	0 .25±0.012	0.032±0.0012	1.3±0.33	1.1±0.66	1.1±0.66	0.9±1.13^{a}
10	35±0.023^{b}	3.4±1.03^{a}	140±0.012^{b}	90±0.48^{a}	0±0.02^{a}	750±1.4^{a}	2.5±1.32	0.032±0.001	1.8±0.85	1.1±0.65	1.1±0.65	1.8±0.014^{b}
20	35±0.02^{b}	3.4±1.03^{a}	140±0.022^{b}	90±0.50^{a}	0±0.01^{a}	750±1.5^{a}	2.5±1.23	0.032±0.0015	1.8±0.80	1.1±0.66	1.1±0.66	1.8±0.013^{b}
30	35±0.012^{b}	3.4±1.03^{a}	140±0 .013^{b}	90±0.65^{a}	0±0.001^{a}	750±1.6^{a}	2.5±1.30	0.032±0.0012	1.8±0.86	1.1±0.64	1.1±0.64	1.8±0.033^{b}
40	25±1.002^{c}	3.4±1.03^{a}	110±0.010^{c}	90±0.50^{a}	0±0.001^{a}	750±1.5^{a}	2.5±1.31	0.032±0.0011	1.8±0.79	1.1±0.67	1.1±0.67	1.8±0.023^{b}
50	25±1.02^{c}	3.4±1.03^{a}	110±0.021^{c}	90±0.45^{a}	0±0.012	750±1.47^{a}	2.5±1.29	0.032±0.001	1.8±0.80	1.1±0.66	1.1±0.66	1.8±0.010^{b}

PAL, phenylalanine ammonialyase; TAL, Tyrosine ammonialyase; PPO, Polypnenol oxidase; AIA, Indol acetic acid; FOV, Fusarium oxyporum vasynfectum ± S, standard error; within the same column and within the same row, means followed by the same letter are not significantly different. (5% SDPP test); values represent the average of three replicates

Effect of FOV inoculum on cotton callus

After 7 days of incubation, the callus turned brown regardless of the dose of FOV inoculated. However, with 40 and 50 µl of inoculum, the callus decreased in volume and the cells were destroyed. Both doses of FOV inoculum were therefore lethal to the callus (Figure 2).

Figure 2: Appearance of control and inoculated cotton callus at FOV

Total phenol content PAL, TAI , peroxidase Polyphenoloxidase and AIAoxidase in healthy callus and inoculated with FOV after 7 days of incubation (Table 2).

In calluses, our results show a decrease in free phenols with FOV infection. Bound phenols, while statistically identical regardless of callus health status, remained very low compared to free phenols. The ratio of free phenols to bound phenols therefore shows an abundance of phenols in the cytoplasm compared to phenols bound to the walls (table 2). These results are not in agreement with those of cvikova et al. (1996) who showed that in alfalfa callus is characterised by a greater accumulation of phenolic compounds in the walls. similarly, lozovaya et al (1996) revealed that regenerating calluses of strawberry contain more parietal phenolic acids than non-regenerating calluses. the deficit of phenols in the walls according to several authors, seems to explain the vulnerability of our callus to inoculations of applied fov. indeed, phenols would favour the lignification of the wall, which would thus become more resistant to fungal attacks (kouakou, 2003; kouadio et al. 2004: konan 2014). Also, the decrease in phenols with infection suggests that these compounds, although strongly represented in the cytoplasm, are not antifungal.

Analysis of phenolic metabolism through the biosynthetic enzymes showed a decrease in free PAL activity while bound PAL activity did not change with infection. Both free and bound TAL activity drop dramatically to zero during infection. This decrease confirms in part the decrease in the level of free phenols recorded. Indeed, the synthesis of these compounds, which was dependent on both PAL and TAL activity in healthy calluses, is now dependent only on PAL in infected calluses at FOV. This suggests that PAL is the preferred pathway for the activation of phenol synthesis during infections. Similarly, the results show the high activity of PAL compared to TAL in phenol biosynthesis during FOV infection (65.5% for PAL versus 32.5% for TAL in healthy calluses and 100% for PAL in FOV infected calluses).

Furthermore, analysis of phenol degrading enzyme activities in callus revealed significant peroxidase (free, bound) and PPO (free, bound) activity in FOV-infected callus compared to healthy callus. These results are in agreement with those of kouakou (2003) who reported an accumulation of these enzymes in calluses in subculture. Furthermore, the intense activity of these enzymes during infection indicates the availability of monophenols and diphenols in the callus due mainly to the biosynthetic activity of PAL. However, the results show a predominance of PPO activities in healthy and infected calluses. Indeed, PPOs are 12 times more active than peroxidases in healthy calluses, and 2 times more active in infected calluses.

This predominance of PPOs according to schantz (1966), mateille (1993) and gouzi (2014) could exert an inhibition on IAA oxidase through the quinone forms of oxidized polyphenols. This is in line with our results. Indeed, the results show a significant decrease in AIA oxidase activity in infected callus compared to healthy callus.

This could explain the low level of phenols in the walls of our calluses. According to EL bellaj and el hadrami (1998), AIA oxidase, by oxidation of IAA generates H202 which will be used by peroxidases in the coupling of phenolic compounds to cell walls.

Table 2: Total phenols; Pal; Tal; Peroxidase; Polyphenoloxidase and AIAoxidase content of healthy callus and callus inoculated with 0;10;20;30;40 and 50 µl of FOV inoculum after 7 days incubation

	PHENOLS		PAL		TAL		PEROXYDASE		PPO		AIAoxydase	
Inoculum(µl)	FREE	Linked	FREE	Linked	FREE	Linked	FREE	Linked	FREE	Linked	FREE	Linked
0	30±0.1^{a}	2.2±1.13^{a}	250±0.001^{a}	90±0.50^{a}	95±0.56^{a}	500±1.5^{a}	0.25±0.32^{a}	0.2±0.10^{a}	2.9±0.02^{a}	1.1±0.002	1.1±0.66	1±1.13^{a}
10	19±0.023^{b}	2.2±0.014^{b}	130±0.012^{b}	90±0.48^{a}	0±0.02^{a}	0±1.4^{a}	2±0.12^{b}	3±0.09^{b}	3.2±0.52	2±0.32	1.1±0.65	0.25±0.014^{b}
20	19±0.02^{b}	2.2±0.013^{b}	130±0.022^{b}	90±0.50^{a}	0±0.01^{a}	0±1.5^{a}	2±0.14^{b}	3±0.095^{b}	3.2±0.44	2±0.31	1.1±0.66	0.25±0.013^{b}
30	19±0.012^{b}	2.2±0.033^{b}	130±0.013^{b}	90±0.65^{a}	0±0.001^{a}	0±1.6^{a}	2±0.12^{b}	3±0.089^{b}	3.2±0.5	2±0.32	1.1±0.64	0.25±0.033^{b}
40	13±1.002^{c}	2.2±0.023^{b}	110±0.010^{c}	90±0.50^{a}	0±0.001^{a}	0±1.5^{a}	3±0.074^{b}	3±0.074^{b}	3.2±0.48	2±0.33	1.1±0.67	0.25±0.023^{b}
50	13±1.02^{c}	2.2±0.010^{b}	110±0.021^{c}	90±0.45^{a}	0±0.012	0±1.47^{a}	2±0.17^{b}	3±0.073^{b}	3.2±0.45	2±0.3	1.1±0.66	0.25±0.010^{b}

PAL, phenylalanine ammonialyase; TAL, Tyrosine ammonialyase; PPO, Polypnenol oxidase; AIA, Indol acetic acid; FOV, Fusarium oxyporum vasynfectum ± S, standard error; within the same column and within the same row, means followed by the same letter are not significantly different. (5% SDPP test); values represent the average of three replicates

In addition, statistical analyses show that IAA oxidase discriminates the infection of cotton seedlings and evolves in the same direction as the peroxidases (figure 3). Also the high activity of free and bound PPO would be characteristic of the infection of callus by FOV and evolves in the opposite way to IAA oxidase.

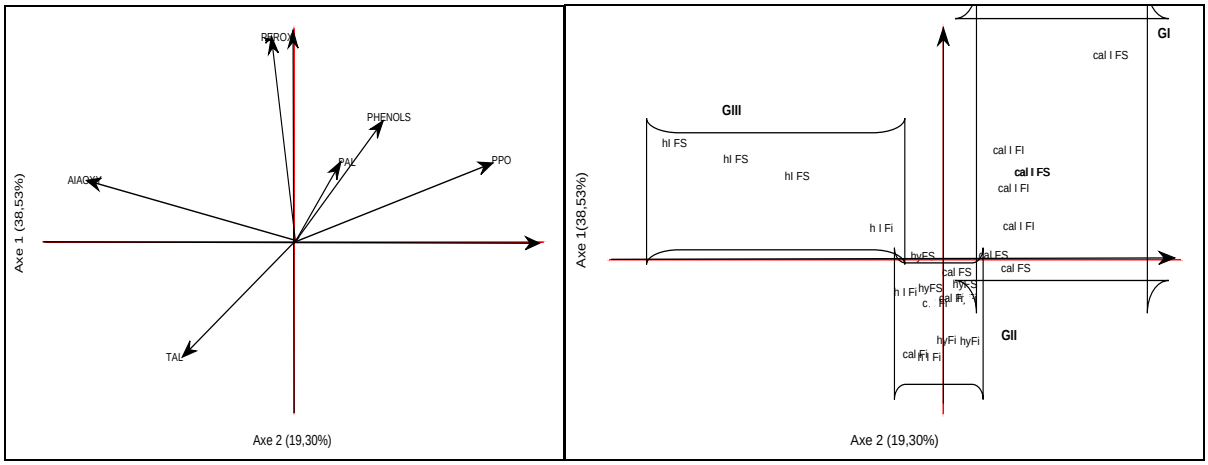

Figure 3: Distribution of cotton organs and variables during infection of in vitro plants and callus by FOV, in the 1-2 plane . A: Distribution of infected and non-infected cotton organs in the 1-2 plane, B: Distribution of variables in the 1-2 plane. HI: Infected seedling; Hy: Healthy seedling; Cal I: Infected Cal; Cal: Healthy Cal; FS: Soluble fraction; FI: Insoluble fraction

It is therefore as if FOV enhances the degradation reactions of phenols and the biosynthetic reactions of these compounds do not undergo major changes with this fungus. All these modifications of the phenolic metabolism in relation with the infection can be considered as defence reactions against the parasite. However, these reactions seem to be too late to avoid the appearance of wilting symptoms, which seem to be probably related to toxins produced by the parasite. This is in line with our results as the inoculation of callus and seedlings in tubes was done with culture filtrates of the fungus.

Conclusion

The objective of our work is to study the response of the metabolism of phenolic compounds of vitroplants and callus to infections caused by FOV. At the end of this study, we note that all the doses of inoculum used produce the same symptoms, but the 40 and 50 µl doses accentuate the damage. After 7 days of incubation, the plants showed the characteristic signs of Fusarium wilt. Analysis of the phenolic metabolism of the glass plants and callus in relation to the FOV infection revealed a decrease in the content of free phenols and a stable

level of wall-bound phenols. This situation is the result of an imbalance between the more or less passive activities of biosynthetic enzymes (PAL and TAL) and the transformative activities, in particular the very active oxidative degradation of enzymes such as PPO, peroxidases and AIA oxidase. All these modifications could be considered as defence reactions of the plant to the infection. However, they are either late or insufficient to prevent the appearance of these symptoms. Therefore, as a result of this study we can consider : AIA oxidase, TAL as potential markers of Fusarium oxysporum f. sp. vasinfectum infection of cotton plants R405-2000. PPO as a potential marker of Fusarium oxysporum f. sp. vasinfectum infection of callus of cotton plants R405-2000.

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