



RESEARCH PAPER

Biochemical changes during the development of witches' broom: the most important disease of cocoa in Brazil caused by *Crinipellis pernicios*

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Abstract

Witches' broom disease (WBD) is caused by the hemibiotrophic basidiomycete fungus *Crinipellis pernicios*, which is one of the most important diseases of cocoa in the western hemisphere. In this study, the contents of soluble sugars, amino acids, alkaloids, ethylene, phenolics, tannins, flavonoids, pigments, malondialdehyde (MDA), glycerol, and fatty acids were analysed in cocoa (*Theobroma cacao*) shoots during the infection and development of WBD. Alterations were observed in the content of soluble sugars (sucrose, glucose, and fructose), asparagine and alkaloids (caffeine and theobromine), ethylene, and tannins. Ethylene and tannins increased prior to symptom development and declined with the death of the infected tissues. Furthermore, MDA and glycerol concentrations were higher in infected tissue than in the controls, while fatty acid composition changed in the infected tissues. Chlorophylls *a* and *b* were lower throughout the development of the disease while carotenoids and xanthophylls dropped in the infected tissue by the time of symptom development. These results show co-ordinated biochemical alterations in the infected tissues, indicating major stress responses with the production of ethylene. Ethylene levels are hypothesized to play a key role in broom development. Some of the other biochemical alterations are directly associated with ethylene synthesis and may be important for the modification of its effect on the infected tissues.

Key words: Asparagine, basidiomycete, biotrophic, ethylene, oxidative stress, saprotrophic, tannins.

Introduction

Witches' broom disease, caused by the basidiomycete fungus *Crinipellis pernicios* (Stahel) Singer, is one of the most important diseases of cocoa (*Theobroma cacao* L.) in Latin America and the Caribbean islands, causing large-scale losses (de Arruda *et al.*, 2003; Griffith *et al.*, 2003). In Brazil, it is found throughout the Amazon basin and in southern Bahia, where it is one of the most destructive diseases of this crop (Santos Filho *et al.*, 1998).

C. pernicios basidiospores have the ability to infect any meristematic tissues of cocoa and are the only known infective propagules for this pathogen (Evans, 1980). The disease shows two distinct stages: a biotrophic and a necrotrophic/saprotrophic phase. In the biotrophic phase the fungus exists as an intercellular monokaryotic mycelium, that causes hypertrophy and hyperplasia of the tissues, loss of apical dominance, and proliferation of axillary shoots, which results in the formation of abnormal stems called a green broom. In the second stage, the fungus changes to the saprotrophic phase, with the spread of intracellular dikaryotic mycelium, which causes necrosis and death of infected tissues distal to the original infection site, thus forming the dry broom (Evans, 1980; Lawrence *et al.*, 1991). In the initial stage of broom development, the fungal density inside the plant tissue is very low and only shows

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a major increase after the death of the broom (Penman *et al.*, 2000). Basidiocarp production and spore formation can occur on any infected necrotic tissue, after alternating wet and dry periods (Almeida *et al.*, 1997; Rocha and Wheeler, 1985) thus completing the disease cycle.

The biotrophic phase mycelium depends on the host tissue for nutrients to sustain its growth and development. One marked characteristic of the parasitic lifestyle of plant pathogens is the unidirectional transfer of nutrients from the plant host to pathogen (Wei *et al.*, 2004). Specific carbohydrates are hypothesized to have a key role in the transition from the parasitic to saprophytic stage (Rocha and Wheeler, 1985; Orchard and Hardwick, 1988). Evans (1980) suggested that substances, for example, reducing sugars, present in significant quantities in living tissue, may delay or prevent dikaryotization of the saprophytic mycelium.

Only two previous reports have studied the biochemical interaction of *Theobroma cacao*–*Crinipellis pernicioso*. Aneja and Gianfagna (2001) analysed the induction of caffeine synthesis in young cocoa leaves and reported higher levels in leaves infected with *C. pernicioso* ($109 \pm 35 \mu\text{g g}^{-1}$), which were approximately 7–8 times greater than in healthy leaves ($14 \pm 1 \mu\text{g g}^{-1}$). Orchard and Hardwick (1988) studied photosynthesis, carbohydrate translocation, and metabolism in cocoa seedlings infected by *C. pernicioso*, and observed that the photosynthetic capacity of infected leaves is dependent on the severity of the infection and that the fungus did not cause a preferential movement of carbohydrates from healthy parts of the plant to the broom.

Other similar biological models are also found in the literature. Conceição *et al.* (1997) analysed the content of several substances in cupuaçu leaves (*Theobroma grandiflorum*) from healthy plants and from plants infected by *C. pernicioso*. In general, higher levels of soluble sugars (SS), starch, chlorophylls, phenolics, and tannins were observed in healthy leaves compared with infected leaves. Ndoumou *et al.* (1996) studied cocoa pods infection by *Phytophthora megakaraya*, the causal agent of black pod, and observed, in three clones with different susceptibilities to the disease, variations in the content of carbohydrates, amino acids, and phenolics. Using the same plant–pathogen system, Omokolo *et al.* (2002) analysed the amino acid and carbohydrate contents in nine clones of *T. cacao* with different degrees of susceptibility to the disease and observed a negative relationship between the lesion size and the amino acid and carbohydrate contents in the cortex.

In this study, cocoa seedlings were infected with *C. pernicioso* and the content of several biochemical compounds were followed during the development of the disease. In addition, diseased and healthy branches from adult plants were collected from the field and analysed for their composition of fatty acids, glycerol, and malondialdehyde (MDA). The aim of this study was to increase knowledge regarding the physiological changes that occur in the plant during the disease and try to define specific

changes that might be related to the transition of the fungus from the biotrophic to the saprotrophic phase. The possible implications and consequences of these biochemical changes on the plant/pathogen interaction are discussed

Materials and methods

Plant material, infection procedure and sample collection

Two hundred and eighty seedlings of cocoa, *Theobroma cacao* cv. Comum, grown in a greenhouse (Almirante Cacau Agrícola, Itajuípe, BA) for 40 d were used in this study. Shoots were cut to stimulate the growth of uniform meristematic buds to obtain homogenous plant material. After 20 d, 140 plants were inoculated by pipetting 30 μl of a basidiospore suspension of *C. pernicioso* (Frias *et al.*, 1995) (viable spores $1.7 \times 10^6 \text{ ml}^{-1}$, strain FA 42 isolated from infected *T. cacao* in the Almirante Cacau Agrícola (Rincones *et al.*, 2003). Infected buds and shoots from 20 plants (one from each plant) were collected at 3, 7, 14, 21, 35, 61, and 132 d after inoculation (DAI). As controls, similar groups of uninfected plant tissues (buds and shoots) were collected over the same periods. Except for ethylene analysis, the sampled materials were frozen in liquid nitrogen at the time of collection and freeze-dried for later processing.

For the evaluation of ethylene emission, individual buds and shoots from approximately 15 plants/treatment (in most case, the remaining bud from the same plants mentioned above) were collected, incubated for 48 h in a sealed flask and the ethylene concentration of this atmosphere was determined by gas chromatography (Hardy *et al.*, 1968). The results were expressed as nmol per fresh weight (FW)/dry weight (DW) ratio–nmol/(DW/FW) due to the high concentration of water present in the green broom (Orchard *et al.*, 1994).

In addition, samples were collected from adult plants growing in the field. For these samples, sections of diseased and healthy branches were collected. For each infected branch, GB for green broom and DB for dry broom; see Fig. 1C, two 8 cm sections were collected: one anterior to the infection node (apparently healthy), labelled '1', and one distal to the infection node representing the diseased tissue, labelled '2'. For example, GB1 means the anterior and apparently healthy section of a green broom branch and GB2 the diseased section. Corresponding sections were collected from healthy branches and were defined as green stems (GS) and dry stems (DS). This field material was also freeze-dried for further analysis.

Extractions and analyses

The plant material for subsequent extraction was prepared as follows: each sample represented a mixture of the tissue from 3–4 plants at the same stage and, for each analysis, averages were produced after three independent determinations, made using three different samples (replicates). Approximately 100 mg from each sample were ground and extracted three times in 2 ml 70% ethanol at 60 °C for 30 min. The 2 ml fractions were combined to form the soluble fraction (SF) used for the analysis of soluble sugars, amino acids, nitrate, secondary metabolites (theobromine, caffeine, phenolics, flavonoids, and tannins), and photosynthetic pigments (chlorophylls *a* and *b*, carotenoids, and xanthophylls) (Fry, 1988). The remaining material from the ethanolic extraction was ground and used to extract starch (twice in 5 ml DMSO 90% for 14 h at room temperature [RT]), pectic fraction (2.5 ml 1 M oxalacetic acid for approximately 16 h at RT), and hemicellulose (twice in 10 ml 6 M NaOH 0.1% NaBH₄, for 16 h at RT) (Fry, 1988). The soluble sugars (SS), starch, and hemicellulose were determined by the anthrone method (Yemm and Willis, 1954). The pectic fraction was quantified by a modified sulphamate/*m*-hydroxyphenyl assay (Filisetticozzi and Carpita, 1991).

In the SF, soluble sugars were also analysed by anion-exchange HPLC with pulsed amperometric detection (Johnson *et al.*, 1993). The

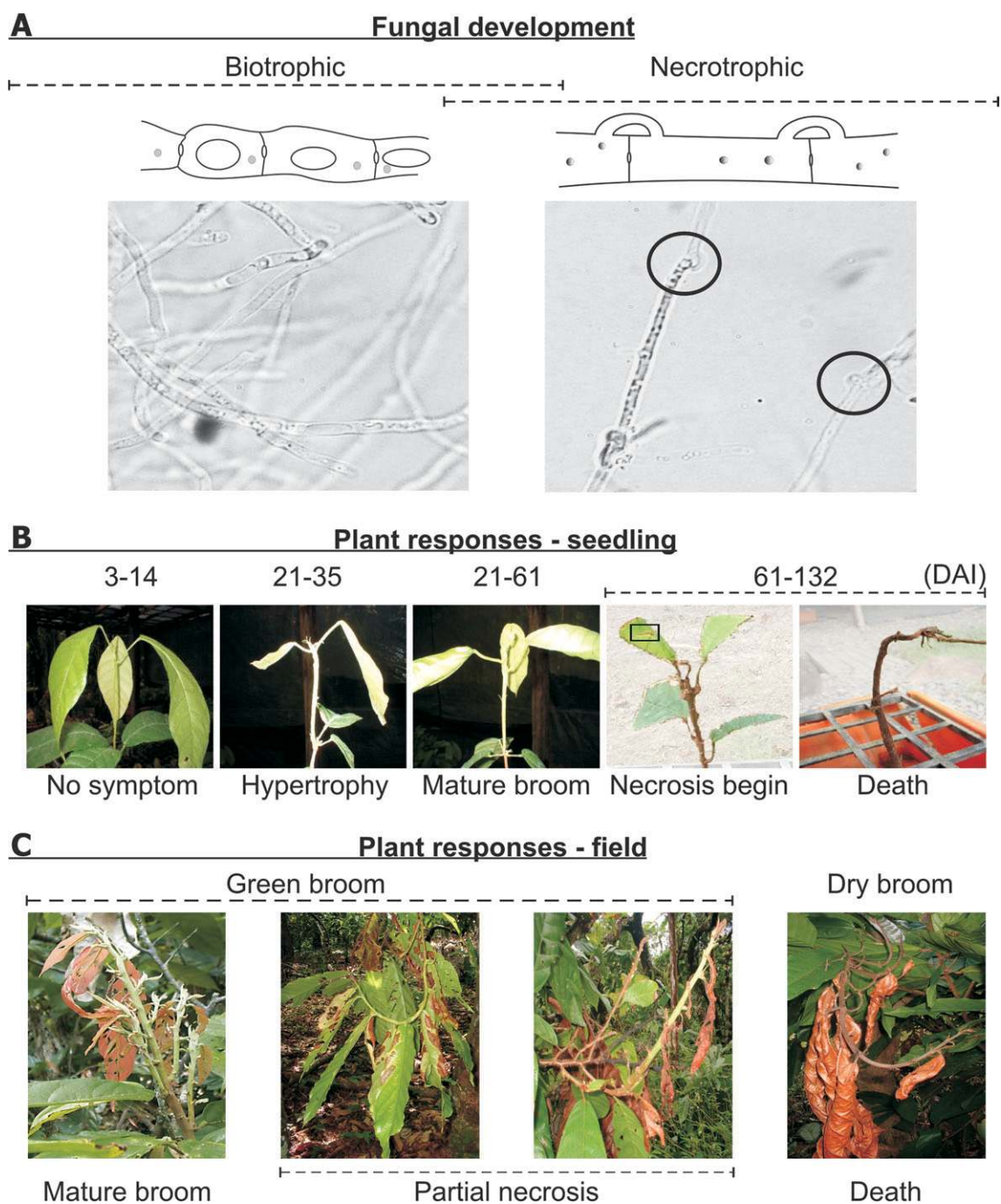


Fig. 1. (A) Biotrophic and necrotic phase of *C. perniciosa*. (B) WBD time line and symptoms showed by infected cocoa plants inoculated with *C. perniciosa* at different time periods (days after inoculation, DAI). (C) Classical symptoms of green and dry broom in the field.

amounts of chlorophyll *a* and *b*, and the sum of carotenoids and xanthophylls were obtained using spectrophotometric analysis (Lichtenthaler and Wellburn, 1983). Amino acids were quantified by the ninhydrin method (Cocking and Yemm, 1954). The individual distribution of amino acids was determined by reversed phase liquid chromatography, using the OPA-borate method (Jarret *et al.*, 1986). The proline content was determined by reacting with ninhydrin in an acid medium (Ringel *et al.*, 2003). Nitrate content was quantified using salicylic acid (Cataldo *et al.*, 1975). Phenolics were determined with the folin ciocalteau reagent (Swain and Hillis, 1959), the flavonoids

using aluminium chloride (Grayer, 1989), and the tannin content was determined with ferric ammonium sulphate (Porter, 1989).

The alkaloids, theobromine and caffeine, were extracted from approximately 15 mg of freeze-dried tissue of each sample (21, 35, and 61 DAI and the corresponding uninfected controls) in 80% methanol at 50 °C for 30 min followed by separation and determination by HPLC (Mazzafera *et al.*, 1991).

Samples from field material were prepared by mixing four sections of the same type of tissue (for example, four sections of GB1 from different brooms), and 4 g of each sample were used for fatty acids

extraction with hexane (8 h in a Soxhlet apparatus). Fatty acid composition was analysed by capillary GC (Agilent 6850) with a DB-23 column (AOCs Official Method Ce2-66), after the formation of methyl esters (Metcalf *et al.*, 1966). Determination of lipid peroxidation was estimated from the MDA levels and assayed by spectrophotometry (Heath and Packer, 1968). Glycerol concentrations were determined by HPLC with a NH_2 column using methodology adapted from Gerber *et al.* (1988). Extracts obtained after grinding in ice-cold 0.1% (v/v) phosphoric acid and boiling in water for 10 min were purified through a 4 ml Dowex column (1/1 w/w dowex 50 and dowex 1). Results were analysed statistically by analysis of variance and differences between means by Tukey test (5%, 1%, and 0.1%) using VarPC software.

Results

Symptoms

In order to obtain the different phases of the WBD, seedlings were inoculated with *C. perniciosa* spores as described above and visually inspected for 132 d (Fig. 1B). Strong similarity was observed in the plant phenotypes after inoculation. In the first collections (3–14 DAI), no symptoms were observed on any plant. However, these stages were very important due to the establishment of the fungus in the plant tissues (biotrophic phase, Fig. 1A). Green broom symptoms begin to form after 21 DAI, with 50% of the plants showing stem swelling and morphological alterations of the apex, with deformed, curved, light green coloured leaves. After 35 DAI, 100% of the plants showed the symptoms described above. At around 50 d, necrotic spots could be seen at the border of the upper leaves, which are some of the first external signs of broom senescence. The base of the brooms started drying after 61 DAI and after 132 DAI 100% of the brooms were dead and dried.

Ethylene emission

Ethylene levels are known to increase in stress-induced plant tissues. In order to determine whether ethylene varies during WBD, ethylene emission was quantified in control and inoculated plants (Fig. 2). Ethylene emissions were similar until 14 DAI, when the levels in the inoculated material increased significantly to around 260 nmol/(DW/FW) at 21 DAI and maintained these levels to 61 DAI ($P \leq 0.05$). Between 61 and 132 DAI the ethylene levels dropped significantly in the inoculated material ($P < 0.001$) presumably due to tissue death.

Determination of carbohydrate during the disease

One frequently observed resistance phenomenon in plants is the so-called 'high sugar resistance' phenotype which is characterized by an enhanced defence against pathogens mediated through elevated levels of soluble carbohydrates and corresponding alterations in primary metabolism (Horsfall and Dimond, 1957). To investigate possible changes in host carbohydrates (uptake, utilization, and synthesis) during the disease process, soluble and cell wall

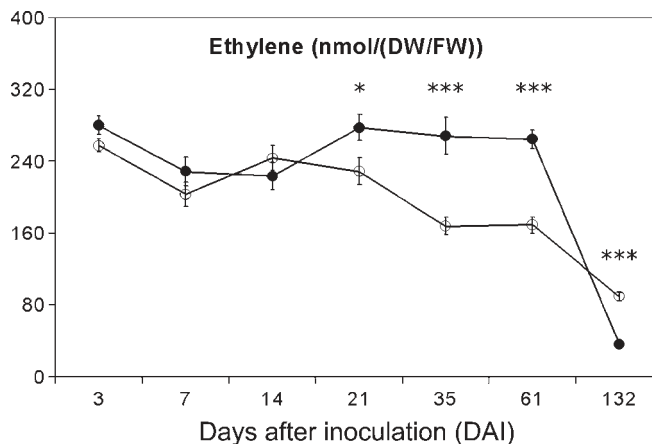


Fig. 2. Ethylene emission (nmol/(DW/FW)) in cocoa shoots from control tissue (open circles) and tissue inoculated with *C. perniciosa* (closed circles) at different time periods (days after inoculation, DAI). Mean of three repetitions \pm standard error (* $P < 0.05$ and *** $P < 0.001$).

carbohydrates levels were measured in the infected and control seedlings.

The sugar distribution patterns (SS, starch, pectin, and hemicellulose) were similar for control and inoculated samples, throughout the process (Fig. 3A, B, C, D, respectively). SS contents in inoculated tissues were only significantly higher at 14 DAI (Fig. 3A). The starch levels were higher in the inoculated samples with significant differences at 14 and 21 DAI (Fig. 3B). No differences were observed for hemicellulose and pectin (Fig. 3C, D). The SS fraction was analysed for sucrose, glucose, and fructose (Fig. 3E, F, G, respectively). Differences in sugar contents between infected and control plants are concentrated at 21 and 35 DAI. Sucrose amounts were much higher than other sugars in both materials at any collected periods with significantly higher amounts in the control at 21 DAI, and in the inoculated material at 35 DAI (Fig. 3E). Significant differences in glucose levels were observed from 21–61 DAI (Fig. 3F). Interestingly, the observed changes in the glucose and sucrose contents had similar variation patterns while fructose was different at 7 DAI and 35 DAI (Fig. 3G).

Pigment contents

A colour change in the leaves accompanied the infection. The leaves were a lighter-green colour when compared with healthy plants (Fig. 1B, C). To confirm whether this effect is due to photosynthetic pigment alterations, the chlorophylls *a* (Fig. 4A) and *b* (Fig. 4B), carotenoids and xanthophylls (Fig. 4C) contents were evaluated. The concentrations of all pigments were reduced in infected tissues. The chlorophyll *a* and *b* levels were lower in the inoculated material at all collected points, with significant differences at 35 DAI and 61 DAI for chlorophyll *a*, and from 7–61 DAI for chlorophyll *b*. The carotenoids and xanthophylls showed similar levels in both control and inoculated materials until 14 DAI, when levels in the inoculated

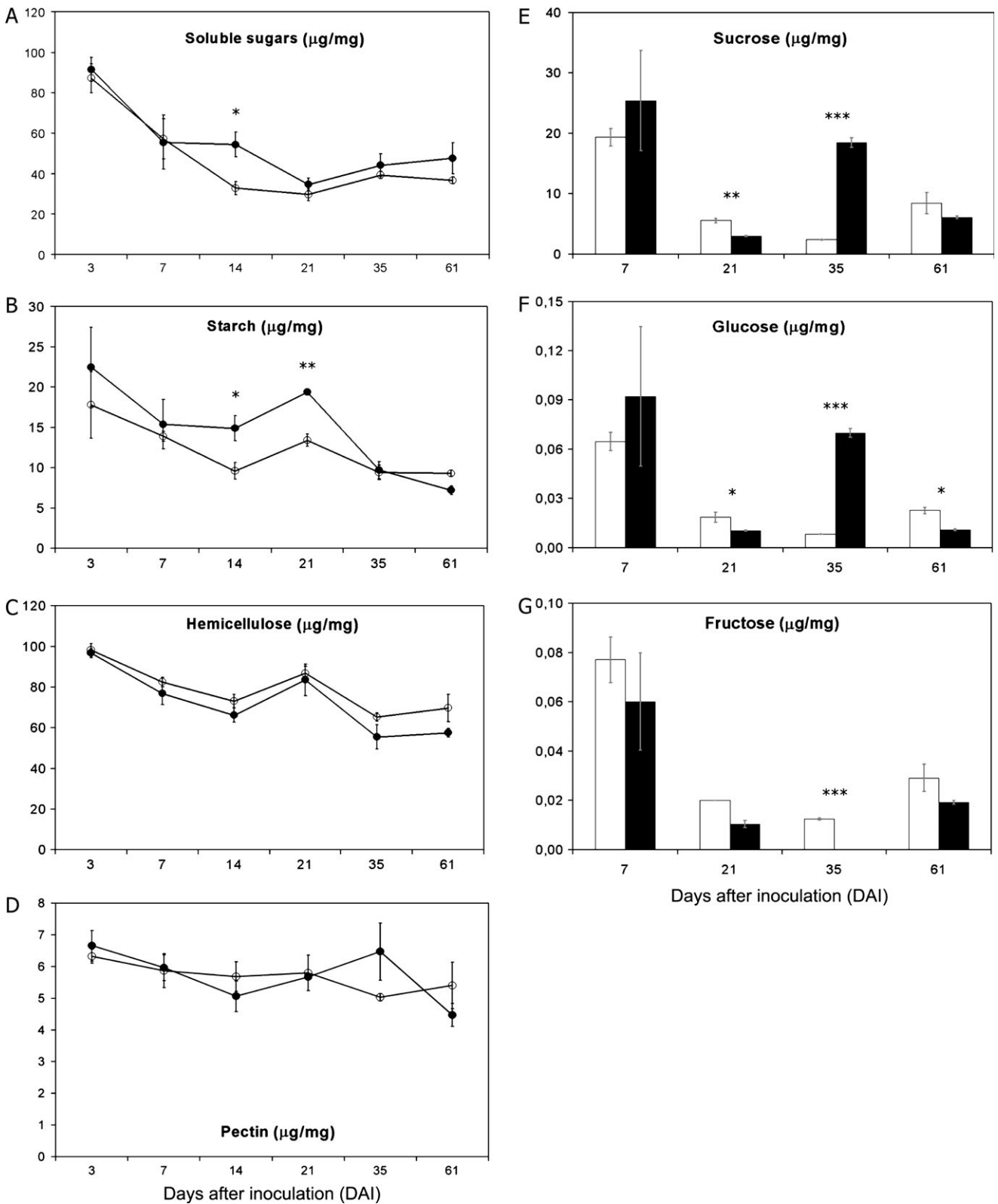


Fig. 3. Levels of total soluble sugars (A), starch (B), hemicellulose (C), pectin (D), sucrose (E), glucose (F), and fructose (G) ($\mu\text{g mg}^{-1}$ dry tissue) in cocoa shoots from control tissue (open circles) and tissue inoculated with *C. perniciosa* (closed circles) at different time periods (days after inoculation, DAI). Means of three repetitions \pm standard error (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

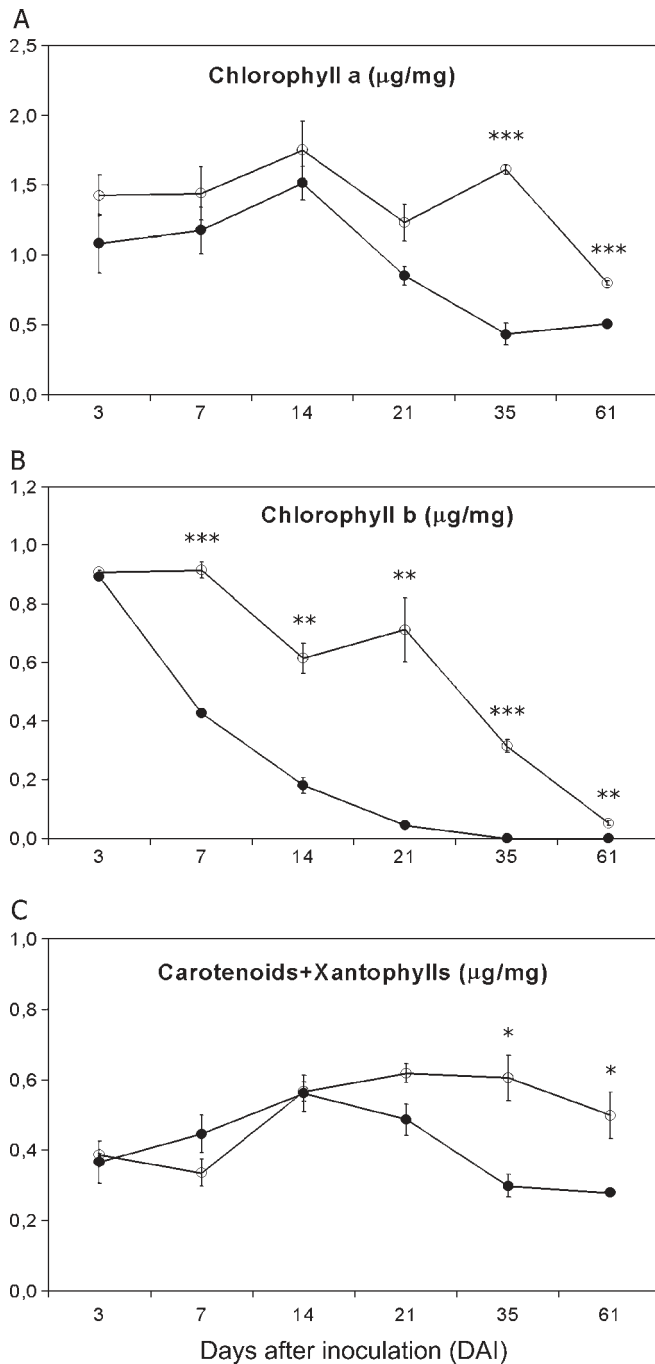


Fig. 4. Levels of chlorophylls *a* (A), *b* (B) and carotenoids+xanthophylls (C) ($\mu\text{g mg}^{-1}$ dry tissue) in cocoa shoots from control tissue (open circles) and tissue inoculated with *C. perniciosa* (closed circles) at different time periods (days after inoculation, DAI) Mean of three repetitions \pm standard error (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

material began to drop and showed significant differences at 35 and 61 DAI ($P < 0.05$).

Phenolics content

Phenolics are substances that are involved in plant–pathogen interactions. Therefore, the contents of total soluble phenolics, flavonoids, and tannins were determined in the soluble fraction.

The total soluble phenolic content decreased with seedling growth and except for 14 DAI, at which a peak was observed in the infected tissues, they were similar in both treatments (Fig. 5A). Flavonoids in both treatments were similar up to 14 DAI, but were significantly lower in infected tissues at 21 DAI and 61 DAI (Fig. 5B). Condensed tannins were significantly higher in the inoculated material, at 3, 14, 21, and 35 DAI (Fig. 5C), but were lower than the controls at 61 DAI, when necrosis begins.

Alkaloid determination

Alkaloids have been described as components of plant defence responses and the two main alkaloids found in cocoa are caffeine and theobromine. These compounds were determined in material from only three collections because of a lack of plant material (Fig. 6A, B). For both alkaloids, the infected tissues showed a very high content at 21 DAI, approximately 10 times higher than in healthy plants. Although the alkaloid levels were always higher in infected treatment, a marked decrease was observed in both treatments from 21–35 DAI.

Amino acid and nitrate contents

Amino acids are important nutrients that are transferred from the plant to the fungus and, like nitrates, can function as nitrogen sources for the fungus. Analysis of the total amino acid content showed higher levels in the inoculated material at all collected periods, except at 7 DAI, with significant differences at 21, 35, and 61 DAI (Fig. 7A). When amino acids were individually analysed, the proline contents were very low in both materials and did not show any significant difference throughout the experiment (data not shown) and amino acids that showed very low amounts are not presented. Remarkably, a clear change was observed in the patterns of amino acids in the infected tissues from 21 DAI. Among the most abundant amino acids observed, aspartic acid (ASP), glutamic acid (GLU), and asparagines (ASN), significantly higher levels of ASN and lower levels of GLU were observed in the inoculated samples (Fig. 7C, D). For glutamine (GLN), serine (SER), alanine (ALN), GABA, and glycine+threonine (GLY+THR), the levels were clearly reduced in infected plants (Fig. 7E, F). The nitrate contents in both treatments present the same pattern until 14 DAI. At 21 DAI the infected tissue was significantly lower than the control, but showed a linear increase in nitrate accumulation thereafter, with no corresponding tendency in the control (Fig. 7B).

Fatty acids, glycerol and lipid peroxidation in field material

Plant fatty acid contents and composition can vary during plant–pathogen interactions by reacting with reactive oxygen species (ROS) or can be utilized for growth by the

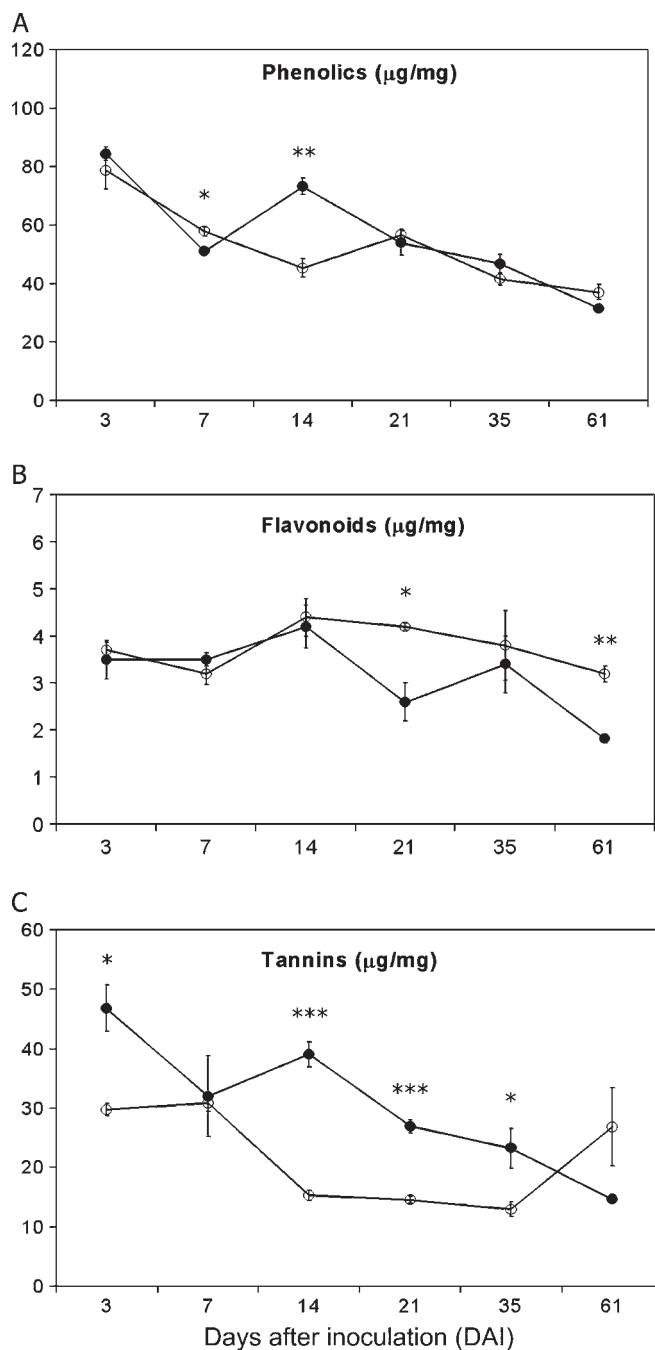


Fig. 5. Levels of phenolics (A), flavonoids (B), and tannins (C) ($\mu\text{g mg}^{-1}$ dry tissue) in cocoa shoots from control tissue (open circles) and tissue inoculated with *C. perniciosa* (closed circles) at different time periods (days after inoculation, DAI). Mean of three repetitions \pm standard error (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

pathogen. The fatty acid composition showed that palmitic (C16:0), stearic (C18:0), oleic (C18:1:cis-9), and linoleic (C18:2) acids were the most abundant in the analysed materials (Fig. 8A). The main differences were observed for palmitic and oleic acids (cis-9) in the comparison between the green broom (GB2) and the healthy stem (GS), suggesting modifications in the lipid metabolism during

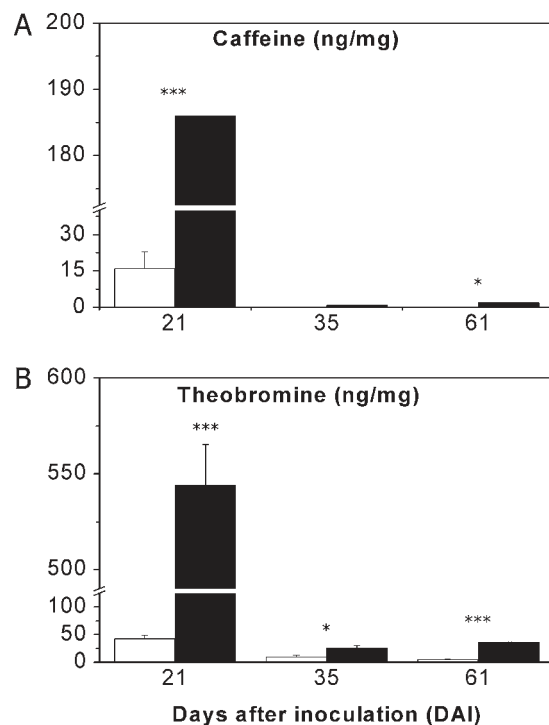


Fig. 6. Levels of caffeine (A) and theobromine (B) (ng mg^{-1} dry tissue) in cocoa shoots from control tissue (open circles) and tissue inoculated with *C. perniciosa* (closed circles) at different time periods (days after inoculation, DAI). Mean of three repetitions \pm standard error (* $P < 0.05$ and *** $P < 0.001$).

the disease. These alterations seem to be transitory, since the fatty acid pattern of the dry broom (DB2) is similar to that from a dried healthy stem (DS) (Fig. 4A). The contents of glycerol were significantly different in field materials (Fig. 8B). The highest content was observed in GB2, followed by GB1, DB1, and GS, with DS and DB2 having significantly lower levels. Lipid peroxidation was determined as the amount of malondialdehyde (MDA). Curiously, MDA contents followed the same pattern as observed for glycerol (Fig. 8C). Higher levels were detected in GB2 samples and lower levels in GB1 and DB1, and in the healthy materials (GS and DS).

Discussion

A typical analysis of plants infected by fungi has revealed changes in the enzyme activities of both the plant and the fungus and in metabolite concentrations within the infected host tissues. However, due to the intricate nature of fungal/host interactions, it is an exceedingly difficult task to delineate the individual change caused by the host or pathogen. Witches' broom disease, caused by the infection of cocoa tissues by *C. perniciosa*, is followed by very distinctive symptoms in the plant, which parallel the different phases of the fungus (Evans, 1980; Lawrence *et al.*, 1991). It is assumed that in the green broom the *C. perniciosa* cells are monokaryotic and live in the

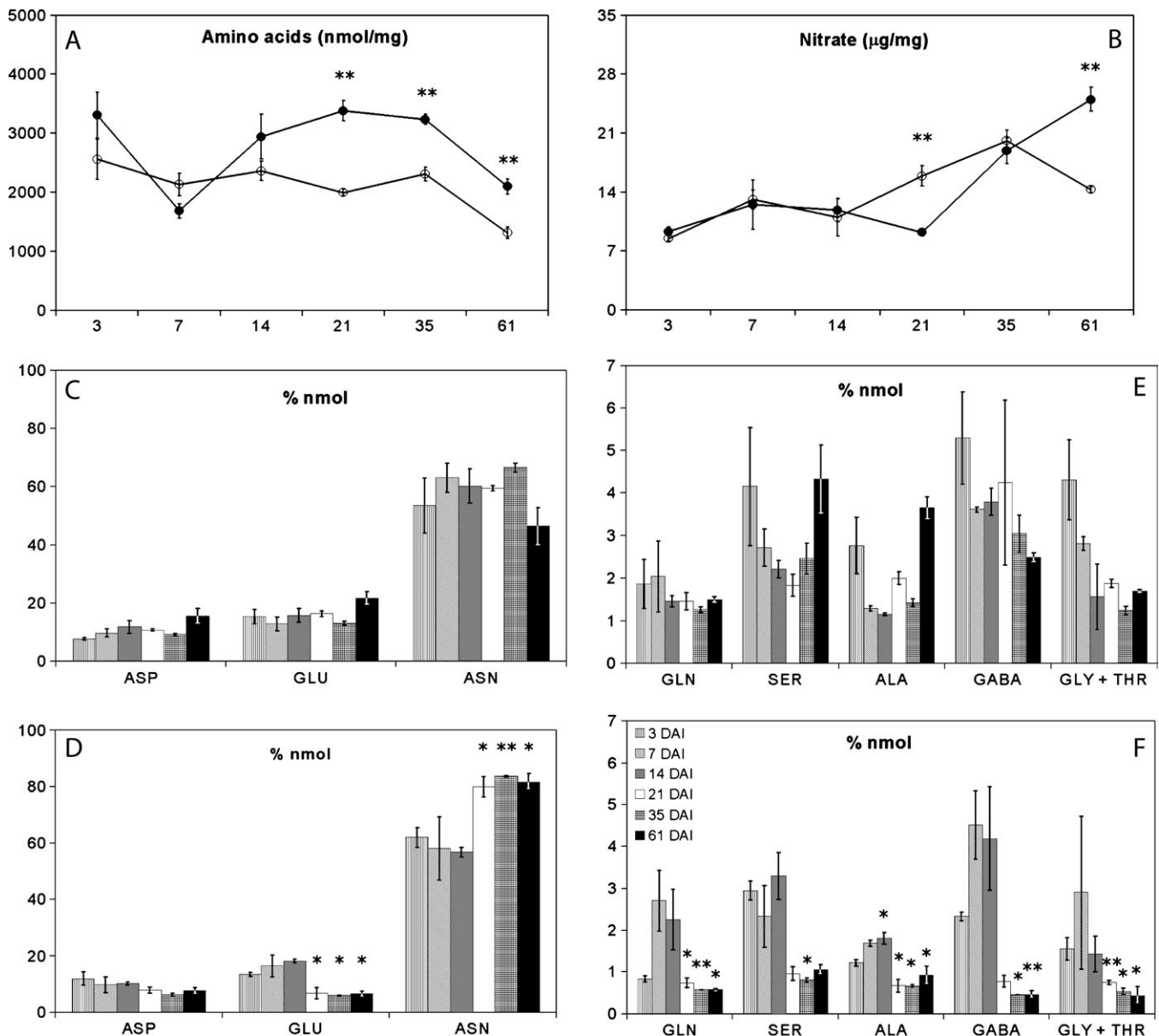


Fig. 7. Levels of total amino acids (A) (nmol mg^{-1} dry tissue) and nitrate (B) ($\mu\text{g mg}^{-1}$ dry tissue), in cocoa shoots from control tissue (open circles) and tissue inoculated with *C. perniciosia* (closed circles) at different time periods (days after inoculation, DAI). Mean of three repetitions \pm standard error. Specific amino acid levels for ASP, GLU, and ASN (% nmol) in control cocoa tissue (C) and in cocoa tissue inoculated with *C. perniciosia* (D). Levels of GLN, SER, ALA, GABA, GLY+THR (% nmol) in control cocoa tissue (E) and in cocoa tissue inoculated with *C. perniciosia* (F) at different time periods (days after inoculation, DAI) (* $P < 0.05$ and ** $P < 0.01$).

apoplast of the intercellular space (Purdy and Schmidt, 1996). In the necrotrophic/saprotrophic phase, triggered by unknown factors, the fungus changes to the dikaryotic state and through, yet again, unknown processes initiate necrosis and death of the infected tissue (Calle *et al.*, 1982). In this study, an attempt was made systematically to analyse changes in a number of key plant biochemical parameters for *T. cacao* infected with the hemibiotrophic fungus *C. perniciosia* during the various stages of the disease process and to correlate those changes with disease symptoms and possibly with changes in the biology of the fungus.

Initially the progression of the disease was accompanied in seedlings under controlled conditions, confirming the general cycle reported in the literature (Purdy and Schmidt, 1996). After inoculation and up to 14 DAI there were no symptoms on any of the plants, suggesting a period of fungal establishment. Green broom symptoms were initially observed as stem swelling and morphological alterations of the shoots, such as loss of apical dominance and deformed, light-green-coloured leaves that were curled. These symptoms were evident in 50% of the plants at 21 DAI and in 100% at 35 DAI. Drying of the brooms

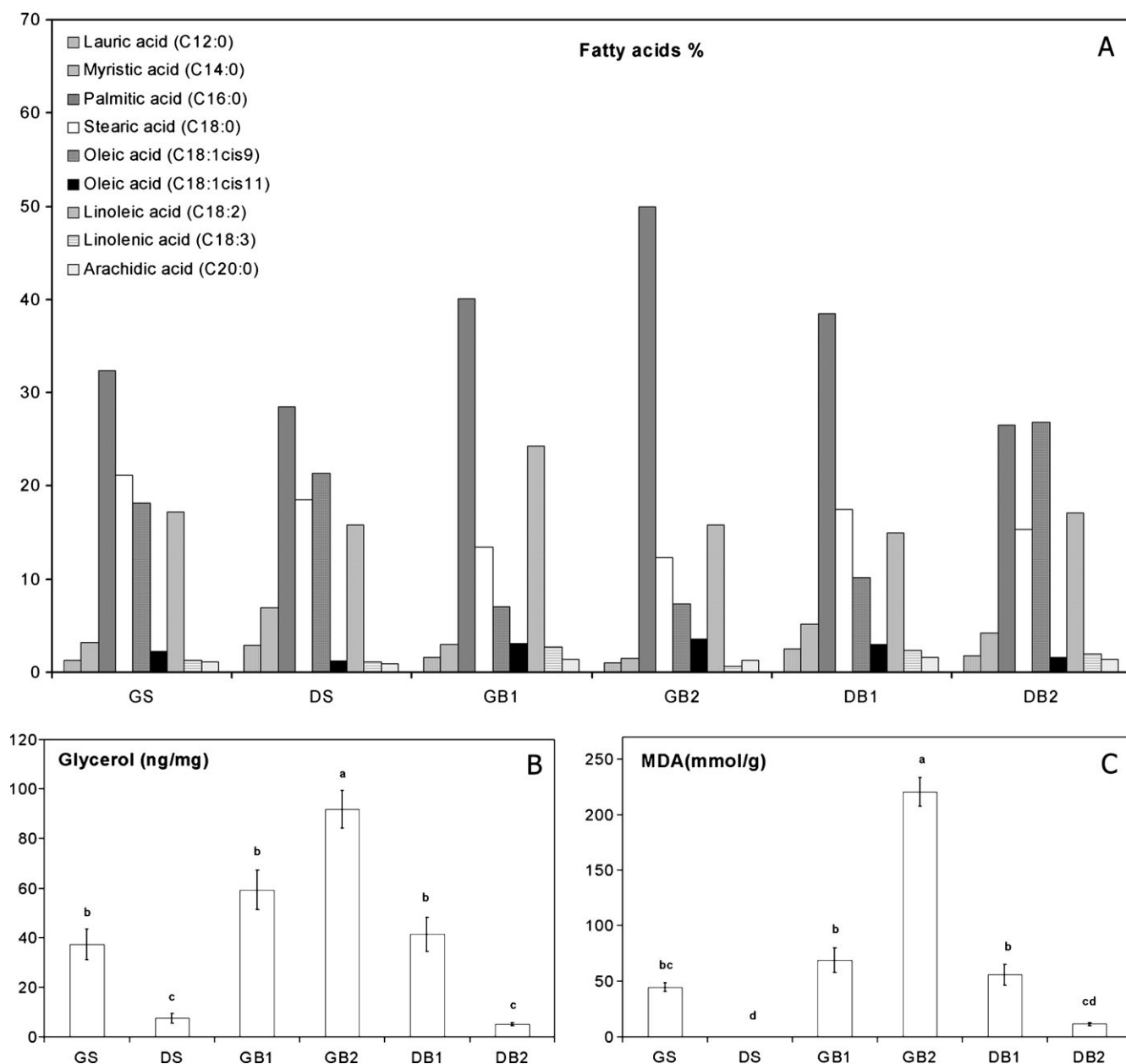


Fig. 8. Fatty acid composition (%) (A)*, glycerol (ng mg^{-1} dry tissue) (B), and MDA levels (mmol g^{-1} dry tissue) (C) of the cocoa field materials: green stem (GS), dry stem (DS), anterior section to the infection node of green broom (GB1), green broom (GB2), anterior section to the infection node of dry broom (DB1), dry broom (DB2). Different letters indicated statistically significant differences. *Data from two independent experiments with consistent results.

began at around 61 DAI and continued to the last sampling at 132 DAI.

Based on these data, a disease development scheme or time line can be proposed. DAI 1–14 represents spore germination, initial infection, and the establishment period. DAI 14–21/35 represents the period where the fungus induces or mediates metabolic changes that result in the development of the classic symptoms of WBD (green broom). Sometime between DAI 35 and DAI 61 the biotrophic phase of the fungus changes to the saprotrophic phase resulting in the death of the tissues,

which apparently begins in the leaves, distant from the point of infection (Fig. 1B, C).

It is important to mention that, characteristically, green broom tissues are typically longer and wider than healthy tissues, which has the appearance of a collar at the initial site of infection (Orchard *et al.*, 1994). Anatomical analyses of the broom tissue by these authors revealed that all cell types and the overall internal organization of the tissues were unchanged with an increase in the average cell size. However, the vascular tissues were less defined with less

defined xylem vessels and a reduction in the ratio of phloem fibres and sieve tubes to phloem parenchyma cells. Furthermore, they also found lower DW/FW ratios for the infected tissues. Most importantly, fungal mycelia were only rarely found in the infected tissues throughout most of the development period and only became apparent in the final stages of the broom (Orchard *et al.*, 1994). It could be hypothesized that the progression of the broom symptoms in the entire branch reflects a concerted physiological response of the plant to fungal infection, which eventually leads to the elimination of the affected organ.

Initially, the infected branches show several signs of general biochemical defence mechanisms, such as increased levels of alkaloids and phenolics. In the infected material at 21 DAI, theobromine and caffeine levels were much higher than in the control, which may suggest a critical stage in the plant defence system. Aneja and Gianfagna (2001) found significantly higher amounts of caffeine in cocoa stems infected by *C. pernicioso* ($109 \pm 35 \mu\text{g g}^{-1}$), approximately 7–8 times greater than in healthy stems ($14 \pm 1 \mu\text{g g}^{-1}$). In other species, like in tea (*Camellia sinensis*), there is a correlation between the levels of caffeine and resistance to disease in the different cultivars (Kumar *et al.*, 1995). For witches' broom, however, there is no clear correlation between cocoa resistance to *C. pernicioso* and caffeine levels (data not shown). Indeed, although the caffeine and theobromine levels have been sharply increased upon infection, indicating an induction of plant defence mechanisms, these levels seem not to be sufficient to contain the fungal progression. This is consistent with the fact that these compounds *in vitro* only poorly inhibit mycelium growth (Aneja and Gianfagna, 2001).

Phenolics, predominantly the tannins showed an increased concentration during infection. These compounds have been documented to have antimicrobial properties (Ndoumou *et al.*, 1996). Furthermore, procyanidins (condensed tannins) can inhibit basidiospore germination and can cause altered germ-tube morphology of the biotrophic phase of *C. pernicioso*, however, the saprophytic phase is not affected by these compounds (Brownlee *et al.*, 1990). The significant increase in tannin concentrations observed at 3 DAI could, therefore, be a response to the basidiospore inoculation. The levels increased again after 14 DAI, remaining significantly higher than the controls until sometime after 35 DAI, which appears to correlate with the symptom development (levels rose just prior to symptoms) and with the death of the green broom (tannins levels dropped prior to necrosis). Since there is a change from the biotrophic to the saprotrophic phase of the fungus during the progression of the disease, this high concentration of tannins could possibly be a constraint for the growth of the monokaryotic fungal cells; i.e. it could serve as a selection factor favouring the proliferation of the necrotrophic mycelium. Unlike tannin levels, phenolics and flavonoids could not be correlated with symptom development. Phen-

olics were only higher at 14 DAI in infected tissue, but were near control levels for the other samplings, while flavonoids paralleled the controls up to DAI 14, but were lower for the remainder of the samplings.

Another key symptom during green broom development is the lighter green colour. This colour change of the green broom could indicate alterations in plastid metabolism. During the disease process, a decrease in chlorophyll *b* levels was observed soon after infection, which was followed by lower decreases in chlorophyll *a* and the carotenoids/xanthophylls levels. This decrease probably leads to a reduction in photosynthesis, previously reported for *T. cacao* infected with *C. pernicioso* (Orchard and Hardwick, 1988). In this work, a number of possible biochemical connections for this phenomenon was found.

It has been demonstrated that chlorosis can be a consequence of lipid peroxidation with an increase in MDA formation and a subsequent reduction of the antioxidant defences in chloroplasts, which leads to decreased chlorophyll concentrations and photosynthetic activity (Munne-Bosch and Alegre, 2002). Consistent with this idea, there is a 4–5-fold higher level of MDA in green brooms than in non-infected tissues, which could indicate pigment oxidation.

Furthermore, the reduced photosynthesis could be a negative feedback response to the augmented levels of soluble sugars in the infected tissue. In plants, sugars can work directly as gene regulation signals, attenuating the expression of several plastid-localized nuclear genes required for normal chloroplast development (Mullet *et al.*, 1990), and their presence could reduce the need for photosynthesis and, therefore, the need of pigment synthesis (Lee and Daie, 1997; Ludewig *et al.*, 1998). The high levels of sucrose and glucose in the infected tissues have been observed previously in cocoa (Orchard and Hardwick, 1988) and in other biotrophic pathosystems (Chou *et al.*, 2000). This study corroborates those previous findings and found that infected plants at 14 DAI had a significant increase in soluble sugar concentrations when compared with the non-infected plants. It is important to note that, at this point in the disease process, no symptoms were observed and this peak of soluble sugars could represent a response of the plant to infection. Significantly higher starch levels in the infected tissues at 14 DAI and 21 DAI could also suggest that some of the soluble sugars are being mobilized into starch, which could be an overflow mechanism to store the carbohydrate in excess in the infected region. However, after 21 DAI the accumulated starch seems to be utilized, a possibility that would explain the increase of glucose at 35 DAI, which was not accompanied by an increase in fructose in infected tissues. In spite of the absence of fructose, there is also a clear accumulation of sucrose in the infected tissue at this point. Since this augmentation is not accompanied by an increase in photosynthesis, this effect might be caused by translocation from other tissues. Effectively, the green broom shows an intense formation of young developing

leaves and shoots (Fig. 1C), which are known to be major carbohydrate sinks. On the other hand, formation of these new tissues could also be stimulated by the high sucrose, an effect previously demonstrated for other plants (Munier-Jolain and Salon, 2003). Moreover, the decreases in the chlorophyll concentrations during senescence has been demonstrated to be followed by increases in the concentration of soluble sugars and starch (Jongebloed *et al.*, 2004), which are somewhat similar to characteristics found in this study. This phenomenon, which is triggered by the *C. pernicioso* infection, may parallel events involved in natural plant senescence.

Free amino acids are important indicators of the plant conditions, arising as a consequence of protein degradation in tissues under programmed cell death (PCD) or senescence (Hurst and Clark, 1993). However, before being loaded into the vascular system for remobilization, they are extensively converted to amides (Buchanan *et al.*, 2001). This seems to be the situation in the green broom. From 21 DAI on, there is a clear decrease in the concentration of most amino acids and a corresponding increase in the amide asparagine, therefore suggesting again that the green broom tissues is under senescence/PCD.

Another characteristic of senescence/PCD in plants is the generation of ROS (Blokhina *et al.*, 2003), which would increase the levels of MDA, mentioned above, and alter the composition of fatty acids (Fox *et al.*, 1994). Fatty acid analysis revealed that the relationship between palmitic and oleic acid (*cis*-9) is much higher in GB2 (5:1) than in normal tissue (GS) (1.5:1) or even dry broom (DB2) (1:1). Alterations in fatty acid composition may be associated with mechanisms of the necrotic phase of *C. pernicioso* since fatty acids have been shown to regulate sporulation and filamentous growth in *Aspergillus* and *Ustilago* (Calvo *et al.*, 1999; Klose *et al.*, 2004).

Another interesting finding is the higher amounts of glycerol in green broom tissues which could, with the fatty acids, be the result of triglyceride degradation. Several other reports corroborate these observations of higher glycerol levels in infected plant tissues. Wei *et al.* (2004) reported that glycerol was a significant transfer metabolite from plant to pathogen in mallow leaves infected with *C. gloeosporioides*. Glycerol also has an important role for sustaining rhythmic growth and conidiation of this fungus *in vitro*, as well as maintaining the biotrophic phase (Wei *et al.*, 2004). Furthermore, it has been suggested that glycerol is an essential component of fungal pathogenesis, which is involved in the adhesion and penetration phases of infection (Yoder and Turgeon, 1996). Thus, accumulation of this compound may be advantageous for the fungus. Therefore, glycerol formation could be an important part of the disease mechanism, since this carbon source is practically absent in the dry broom.

The external appearance of the green broom has been repeatedly associated with the idea of hormonal imbalances

triggered by the infection. Indeed, increase in zeatin riboside has been reported upon infection (Orchard *et al.*, 1994) and this could alter the ratio of cytokinin/auxin, responsible for lateral budding formation and shoot production. In addition, it has been demonstrated *in vitro* that caffeine can mimic the physiological effect of cytokinins (Vitoria and Mazzafera, 1998). Thus, since this compound shows a peak during infection, it can be speculated that caffeine could contribute to the hormonal imbalance in the infected plant. However, exogenous cytokinin applied to normal stems was not able to mimic the green broom development (Abohamed *et al.*, 1981).

Although less evident, a very important characteristic of the green broom tissue is the hypertrophy (Orchard *et al.*, 1994), an effect that has been associated with ethylene in other plants. Hypertrophic growth has been shown in ethylene-induced aerenchyma cell formation in roots under hypoxic conditions (Gunawardena *et al.*, 2001). In addition, hypertrophic growth and increased water content has been shown in tissue culture, with plants derived from callus under high ethylene combined with cytokinins and hypoxic conditions, in a process called hyperhydricity, (Kamaluddin and Zwiazek, 2002; Saher *et al.*, 2004). Ethylene also seems to play a role in the degradation of chlorophyll (Trebitch *et al.*, 1993) and epinasty of leaf petioles and stems, symptoms that are characteristic of mature green broom tissues. Epinasty is a distinct bending of growing petioles and stems that requires turgor pressure (Woodrow and Grodzinski, 1989).

Indeed, ethylene levels in the *C. pernicioso* infected tissues showed an increase at 21 DAI that continued at a nearly constant level until the death of the broom. Ethylene production normally increases in tissues that suffer from some form of external stresses, like wounding, pathogens invasion, insect attack, and flooding (Chang and Meyerowitz, 1995; Leite and Pascholati, 1995). Stress-induced ethylene, in turn, leads to defence responses, such as accelerated senescence, abscission of infected organs, or the induction of specific defence proteins (Chang and Meyerowitz, 1995).

Taken together, the observed biochemical alterations associated with the infection suggest that, in the first stage, the plant uses unspecific mechanisms to try to eliminate the fungus, such as an increase in alkaloids, phenolics, and tannins. However, these mechanisms seem not to be sufficient to avoid the disease and a number of biochemical changes, such as increased levels of ethylene, asparagine, sugars, and MDA; alteration in the fatty acids profile and reduction of individual amino acids and pigments, suggests that a cascade of events has been triggered to cause the death of the infected organ. If this is true, the elevation of ethylene, which has been connected with several of these processes (Jongebloed *et al.*, 2004; Maruyama *et al.*, 1997; Munne-Bosch and Alegre, 2002), could be a key factor in triggering this concerted response.

The major question remaining is how the fungus causes or modulates these apparent responses in the plant. Database analysis of the genomic and EST sequences of *C. pernicioso*, a Brazilian Genome Project Initiative co-ordinated by our group (www.lge.ibi.unicamp.br/vassoura), showed a high number of potential pathogenicity genes (data not shown). Therefore, to verify these findings and advance an understanding of this complex pathogen–host interaction, additional biochemical studies of green broom development, as well as the detailed analysis of gene expression of both pathogenicity factors and host response factors, will be required. Along these lines, gene expression is currently being analysed in two cDNA libraries generated from resistant and susceptible *T. cacao* clones after inoculations with *C. pernicioso* and two cDNA libraries of *C. pernicioso* that have been induced with cocoa extract in an attempt to understand the gene expression during this disease. The sequencing of the plant libraries are now nearing completion, however, preliminary results (data not shown) corroborates the biochemical observations described in this paper.

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References

- Abohamed S, Collin HA, Hardwick K.** 1981. Biochemical and physiological aspects of leaf development in cocoa (*Theobroma cacao*). 6. Hormonal interaction between mature leaves and the shoot apex. *New Phytologist* **89**, 191–200.
- Almeida OC, Chiacchio FPB, Rocha HM.** 1997. Sobrevivência de *Crinipellis pernicioso* (Stahel) Singer em vassouras secas de cacauzeiros (*Theobroma cacao* L.) do estado da Bahia. *Agrotropica* **9**, 23–28.
- Aneja M, Gianfagna T.** 2001. Induction and accumulation of caffeine in young, actively growing leaves of cocoa (*Theobroma cacao* L.) by wounding or infection with *Crinipellis pernicioso*. *Physiological and Molecular Plant Pathology* **59**, 13–16.
- Blokhina O, Virolainen E, Fagerstedt KV.** 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Annals of Botany* **91**, (Special issue) 179–194.
- Brownlee HE, Mceuen AR, Hedger J, Scott IM.** 1990. Antifungal effects of cocoa tannin on the witches broom pathogen *Crinipellis pernicioso*. *Physiological and Molecular Plant Pathology* **36**, 39–48.
- Buchanan BB, Gruissem W, Jones RL.** 2001. *Biochemistry and molecular biology of plants*. Rockville, Maryland: American Society of Plant Physiologists.
- Calle H, Cook AA, Fernando SY.** 1982. Histology of witches broom caused in cacao by *Crinipellis pernicioso*. *Phytopathology* **72**, 1479–1481.
- Calvo AM, Hinze LL, Gardner HW, Keller NP.** 1999. Sporogenic effect of polyunsaturated fatty acids on development of *Aspergillus* spp. *Applied and Environmental Microbiology* **65**, 3668–3673.
- Cataldo DA, Haroon M, Schrader LE, Youngs VL.** 1975. Rapid colorimetric determination of nitrate in plant-tissue by nitration of salicylic-acid. *Communications in Soil Science and Plant Analysis* **6**, 71–80.
- Chang C, Meyerowitz EM.** 1995. The ethylene hormone response in *Arabidopsis*: a eukaryotic 2-component signaling system. *Proceedings of the National Academy of Sciences, USA* **92**, 4129–4133.
- Chou HM, Bundock N, Rolfe SA, Scholes JD.** 2000. Infection of *Arabidopsis thaliana* leaves with *Albugo candida* (white blister rust) causes a reprogramming of host metabolism. *Molecular Plant Pathology* **1**, 99–113.
- Cocking EC, Yemm EW.** 1954. Estimation of amino acids by ninhydrin. *Biochemical Journal* **58**, R12–R12.
- Conceição HEO, Mazzafera P, Rocha Neto OG, Stein RLB.** 1997. Biochemical composition of *Theobroma grandiflorum* leaves infected by *Crinipellis pernicioso*. *Revista Brasileira de Fisiologia Vegetal* **9**, 135–138.
- de Arruda MCC, Miller RNG, Ferreira MASV, Felipe MSS.** 2003. Comparison of *Crinipellis pernicioso* isolates from Brazil by ERIC repetitive element sequence-based PCR genomic fingerprinting. *Plant Pathology* **52**, 236–244.
- Evans HC.** 1980. Pleomorphism in *Crinipellis pernicioso*, causal agent of witches broom disease of cocoa. *Transactions of the British Mycological Society* **74**, 515–523.
- Filissettozzi TMCC, Carpita NC.** 1991. Measurement of uronic acids without interference from neutral sugars. *Analytical Biochemistry* **197**, 157–162.
- Fox TC, Kennedy RA, Rumpho ME.** 1994. Energetics of plant growth under anoxia: metabolic adaptations of *Oryza sativa* and *Echinochloa phyllopogon*. *Annals of Botany* **74**, 445–455.
- Frias GA, Purdy LH, Schmidt RA.** 1995. An inoculation method for evaluating resistance of cacao to *Crinipellis pernicioso*. *Plant Disease* **79**, 787–791.
- Fry SC.** 1988. *The growing plant cell wall: chemical and metabolic analysis*. London, UK: Longman Scientific and Technical.
- Gerber DW, Byerrum RU, Gee RW, Tolbert NE.** 1988. Glycerol concentrations in crop plants. *Plant Science* **56**, 31–38.
- Grayer RJ.** 1989. Flavonoids. In: Dey PM, Harborne JB, eds. *Methods in plant biochemistry*, Vol. 1. London, UK: Academic Press, 275–302.
- Griffith GW, Nicholson J, Nenninger A, Birch RN, Hedger JN.** 2003. Witches' brooms and frosty pods: two major pathogens of cacao. *New Zealand Journal of Botany* **41**, 423–435.
- Gunawardena AH, Pearce DM, Jackson MB, Hawes CR, Evans DE.** 2001. Characterisation of programmed cell death during aerenchyma formation induced by ethylene or hypoxia in roots of maize (*Zea mays* L.). *Planta* **212**, 205–214.
- Hardy RWF, Holsten RD, Jackson EK, Burns RC.** 1968. Acetylene–ethylene assay for N₂ fixation: laboratory and field evaluation. *Plant Physiology* **43**, 1185–1207.
- Heath RL, Packer L.** 1968. Photoperoxidation in isolated chloroplasts. I. Kinetics and stoichiometry of fatty acid peroxidation. *Archives of Biochemistry and Biophysics* **125**, 189–198.
- Horsfall JG, Dimond AE.** 1957. Interactions of tissue sugar, growth substances, and disease susceptibility. *Zeitschrift für Pflanzenkrankheiten, Pflanzenpathologie und Pflanzenschutz* **27**, 415–421.

- Hurst PL, Clark CJ. 1993. Post-harvest changes in ammonium, amino-acids, and enzymes of amino-acid-metabolism in *Asparagus* spear tips. *Journal of the Science of Food and Agriculture* **63**, 465–471.
- Jarret HW, Coosky KD, Ellis B. 1986. The separation of o-phthalaldehyde derivatives of amino acids by reversed-phase chromatography on octylsilica column. *Analytical Biochemistry* **153**, 189–198.
- Johnson DC, Dobberpuhl D, Roberts R, Vandenberg P. 1993. Pulsed amperometric detection of carbohydrates, amines, and sulfur species in ion chromatography: the current state of research. *Journal of Chromatography* **640**, 79–96.
- Jongebloed U, Szederkenyi J, Hartig K, Schobert C, Komor E. 2004. Sequence of morphological and physiological events during natural ageing and senescence of a castor bean leaf: sieve tube occlusion and carbohydrate back-up precede chlorophyll degradation. *Physiologia Plantarum* **120**, 338–346.
- Kamaluddin M, Zwiazek JJ. 2002. Ethylene enhances water transport in hypoxic aspen. *Plant Physiology* **128**, 962–969.
- Klose J, de Sa MM, Kronstad JW. 2004. Lipid-induced filamentous growth in *Ustilago maydis*. *Molecular Microbiology* **52**, 823–835.
- Kumar NS, Hewavitharanage P, Adikaram NKB. 1995. Attack on tea by *Xyleborus fornicatus*: inhibition of the symbiote, *Monacroporium ambrosium*, by caffeine. *Phytochemistry* **40**, 1113–1116.
- Lawrence JS, Campêlo AMFL, Figueiredo JM. 1991. Enfermidades do cacauero. II. Doenças fúngicas que ocorrem nas folhas, ramos e tronco. *Agrotropica* **3**, 1–14.
- Lee JS, Daie J. 1997. End-product repression of genes involving carbon metabolism in photosynthetically active leaves of sugarbeet. *Plant and Cell Physiology* **38**, 887–894.
- Leite B, Pascholati SF. 1995. Hospedeiro: alterações fisiológicas induzidas por fitopatógenos. In Bergamin Filho A, Kimati H, Amorim L, eds. *Manual de fitopatologia: princípios e conceitos*, Vol. 1. São Paulo: Editora Agronômica Ceres, 393–416.
- Lichtenthaler HK, Wellburn AR. 1983. Determination of total carotenoids and chlorophylls *a* and *b* of leaf extracts in different solvents. *Biochemical Society Transactions* **11**, 591–592.
- Ludewig F, Sonnewald U, Kauder F, Heineke D, Geiger M, Stitt M, Muller-Rober BT, Gillissen B, Kuhn C, Frommer WB. 1998. The role of transient starch in acclimation to elevated atmospheric CO₂. *FEBS Letters* **429**, 147–151.
- Maruyama A, Yashima M, Adachi Y, Nanba H, Hasegawa R, Esashi Y. 1997. Possible participation of β-cyanoalanine synthase in increasing the amino acid pool of cocklebur seeds in response to ethylene during the pre-germination period. *Australian Journal of Plant Physiology* **24**, 751–757.
- Mazzafera P, Crozier A, Magalhaes AC. 1991. Caffeine metabolism in *Coffea arabica* and other species of coffee. *Phytochemistry* **30**, 3913–3916.
- Metcalf LD, Schmitz AA, Pelka JR. 1966. Rapid preparation of fatty acid esters from lipids for gas chromatographic analysis. *Analytical Chemistry* **38**, 514–515.
- Mullet JE, Klein PG, Klein RR. 1990. Chlorophyll regulates accumulation of the plastid-encoded chlorophyll apoproteins CP43 and D1 by increasing apoprotein stability. *Proceedings of the National Academy of Sciences, USA* **87**, 4038–4042.
- Munier-Jolain N, Salon C. 2003. Can sucrose content in the phloem sap reaching field pea seeds (*Pisum sativum* L.) be an accurate indicator of seed growth potential? *Journal of Experimental Botany* **54**, 2457–2465.
- Munne-Bosch S, Alegre L. 2002. Plant aging increases oxidative stress in chloroplasts. *Planta* **214**, 608–615.
- Ndoumou DO, Ndzomo GT, Djocgoue PF. 1996. Changes in carbohydrate, amino acid and phenol contents in cocoa pods from three clones after infection with *Phytophthora megakarya* Bra and Grif. *Annals of Botany* **77**, 153–158.
- Omokolo ND, Nankeu DJ, Niemenak N, Djocgoue PF. 2002. Analysis of amino acids and carbohydrates in the cortex of nine clones of *Theobroma cacao* L. in relation to their susceptibility to *Phytophthora megakarya* Bra. and Grif. *Crop Protection* **21**, 395–402.
- Orchard J, Collin HA, Hardwick K, Isaac S. 1994. Changes in morphology and measurement of cytokinin levels during the development of witches brooms on cocoa. *Plant Pathology* **43**, 65–72.
- Orchard JE, Hardwick K. 1988. Photosynthesis, carbohydrate translocation and metabolism of host and fungal tissues in cacao seedlings infected with *Crinipellis perniciosa*. In *10th International Cocoa Research Conference*.
- Penman D, Britton G, Hardwick K, Collin HA, Isaac S. 2000. Chitin as a measure of biomass of *Crinipellis perniciosa*, causal agent of witches' broom disease of *Theobroma cacao*. *Mycological Research* **104**, 671–675.
- Porter LJ. 1989. Tannins. In: Dey PM, Harborne JB, eds. *Methods in plant biochemistry*, Vol. 1. London, UK: Academic Press, 389–419.
- Purdy LH, Schmidt RA. 1996. Status of cacao witches' broom: biology, epidemiology, and management. *Annual Review of Phytopathology* **34**, 573–594.
- Rincones J, Meinhardt LW, Vidal BC, Pereira GAG. 2003. Electrophoretic karyotype analysis of *Crinipellis perniciosa*, the causal agent of witches' broom disease of *Theobroma cacao*. *Mycological Research* **107**, 453–458.
- Ringel C, Siebert S, Wienhaus O. 2003. Photometric determination of proline in quartz microplates: remarks on specificity. *Analytical Biochemistry* **313**, 167–169.
- Rocha HM, Wheeler BEJ. 1985. Factors influencing the production of basidiocarps and the deposition and germination of basidiospores of *Crinipellis perniciosa*, the causal agent of witches' broom disease on cocoa (*Theobroma cacao* L.). *Plant Pathology* **34**, 319–328.
- Saher S, Piqueras A, Hellin E, Olmos E. 2004. Hyperhydricity in micropropagated carnation shoots: the role of oxidative stress. *Physiologia Plantarum* **120**, 152–161.
- Santos Filho LP, Freire ES, Carzola IM. 1998. Estimativas de perda de produção de cacau causadas por vassoura-de-bruxa (*Crinipellis perniciosa* (Stahel) Singer) na Bahia. *Agrotropica* **10**, 127–130.
- Swain T, Hillis WE. 1959. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *Journal of the Science of Food and Agriculture* **10**, 63–68.
- Trebitsh T, Goldschmidt EE, Riov J. 1993. Ethylene induces *de novo* synthesis of chlorophyllase, a chlorophyll degrading enzyme, in Citrus fruit peel. *Proceedings of the National Academy of Sciences, USA* **90**, 9441–9445.
- Vitoria AP, Mazzafera P. 1998. Cytokinin-like effects of caffeine in bioassays. *Biologia Plantarum* **40**, 329–335.
- Wei YD, Shen WY, Dauk M, Wang F, Selvaraj G, Zou JT. 2004. Targeted gene disruption of glycerol-3-phosphate dehydrogenase in *Colletotrichum gloeosporioides* reveals evidence that glycerol is a significant transferred nutrient from host plant to fungal pathogen. *Journal of Biological Chemistry* **279**, 429–435.
- Woodrow L, Grodzinski B. 1989. An evaluation of the effects of ethylene on carbon assimilation in *Lycopersicon esculentum* Mill. *Journal of Experimental Botany* **40**, 361–368.
- Yemm EW, Willis AJ. 1954. The estimation of carbohydrates in plant extracts by anthrone. *Biochemical Journal* **57**, 508–514.
- Yoder OC, Turgeon BG. 1996. Molecular-genetic evaluation of fungal molecules for roles in pathogenesis to plants. *Journal of Genetics* **75**, 425–440.