doi: 10.17221/124/2016-PPS

Effect of $MgSO_4$ Nutrition on *Theobroma cacao* L. Susceptibility to *Phytophthora megakarya* Infection

Emile MINYAKA[1,3], Collette Vanessa MADINA BANEN[1], Barbara KUSZNIEREWICZ[4], Oumar DOUNGOUS[2,3], Soungouna HAOUNI[1], Joseph HAWADAK[1], Nicolas NIEMENAK[3] and Denis Ndoumou OMOKOLO[3]*

[1]Biochemistry Laboratory, Faculty of Science, University of Douala, Douala, Cameroon; [2]Biotechnology Laboratory, Institute of Agricultural Research for Development, IRAD Ekona Regional Research Centre, Buea, Cameroon; [3]Laboratory of Plant Physiology and Biochemistry, Department of Biological Science, Higher Teachers Training College of Yaoundé, Yaoundé, Cameroon; [4]Department of Food Chemistry, Technology and Biotechnology, Faculty of Chemistry, Gdańsk University of Technology, Gdańsk, Poland

**Corresponding author: minyakae@yahoo.fr*

Abstract

Minyaka E., Madina Banen C.V., Kusznierewicz B., Doungous O., Haouni S., Joseph Hawadak J., Niemenak N., Omokolo D.N.: Effect of $MgSO_4$ nutrition on *Theobroma cacao* L. susceptibility to *Phytophthora megakarya* infection. Plant Protect. Sci.

A new strategy to reduce the severity of black pod disease (BPD) in *T. cacao* plants using $MgSO_4$ nutrition was investigated. The dynamics of the tolerance to BPD of 18 susceptible *T. cacao* plantlets coming from the cross (♀SNK64 × ♂UPA14) was monitored during weekly (8 weeks) supply of $MgSO_4$ into the soil. Prior to $MgSO_4$ application, disease scores of the 18 plantlets (in six sets of three plantlets per set) were varying between 3.5 (susceptible) and 5 (highly susceptible). After $MgSO_4$ application, a substantial decrease in disease scores was observed compared to the control. The percentage of disease tolerance gain of plantlets versus $MgSO_4$ supplied (0–2.96 g) presented a quasi-hyperbolic curve with asymptotic line corresponding to 60% (day 28) and 70% (day 56). Cysteine content was not significantly different between the six triplets before $MgSO_4$ nutrition. On days 28 and 56 of $MgSO_4$ supplementation, cysteine content presented a pattern similar to the tolerance gain of plantlet sets. The monitoring of glutathione content versus $MgSO_4$ supplementation (compared to day 0) showed sigmoid (day 28) and hyperbolic (day 56) curves which were associated with defined mathematical laws determined by MALAB software. Negative and highly significant correlations were observed between disease scores, cysteine and glutathione contents in leaves while positive and highly significant correlations were observed between cysteine and glutathione contents in leaves. These data might mean that $MgSO_4$ nutrition significantly improved the tolerance of *T. cacao*. The mechanism of tolerance improvement might be associated with the synthesis of sulphur-containing compounds (cysteine and glutathione) which might be directly or indirectly used by *T. cacao* against *P. megakarya*.

Keys words: cocoa; profitability; sulphur; defence; pathogen

Cocoa is one of the most important cash crops in Cameroon and other producing African countries (Tcharbuahbokengo 2005). Cocoa culture suffers severe yield losses due to the black pod disease of cocoa induced by *P. megakarya* attacks (Nyasse *et al.* 2007). Developing strategies for the management of this disease in Cameroon are the way out to improve cocoa production, productivity, and profitability.

doi: 10.17221/124/2016-PPS

Two main approaches are used to protect *T. cacao* against diseases, and these include: (a) the application of pesticides (chemicals) against the pathogen *P. megakarya*, but these chemicals have negative impacts on the human health, environment, and sustainable agriculture; (b) breeding which aims to develop genetically tolerant/resistant genotypes to black pod disease (Nyassé *et al*. 2007). However, in *T. cacao*, tolerance/resistance to black pod disease (BPD) is a multigenic character. Parents used in breeding programs for resistance to BPD are almost always heterozygotes for the character. Consequently, the progeny is highly heterogeneous (for this character) with most plants susceptible to BPD (Efombagn *et al*. 2011; Nyadanu *et al*. 2012).

In the early 1990s, in Scotland, Schnug *et al.* (1995a) reported a dramatic increase of fungal diseases simultaneously with a decrease in atmospheric sulphur (S). Dubuis *et al.* (2005) demonstrated a clear link between the sulphur nutritional status of oilseed rape and its resistance against *Leptosphaeria maculans*, *Botrytis cinerea*, and *Phytophthora brassicae*. Moreover, these authors investigated the antimicrobial activity of plant extracts and found a very significant reduction of antimicrobial activity in S-deficient plants that had reduced glucosinolate content. We hypothesise that sulphur may be involved directly or indirectly in resistance of *T. cacao* against *P. megakarya*.

In the absence of an effective method of the BPD control, other strategies that could be exploited alone or in combination with genetic improvement such as the use of nutritive "Molecule which could Reinforce the Natural Defence System" (MRNDS) are highly solicited. $MgSO_4$ is a non-pollutant salt which can be used as a source of sulphur. It is naturally present in soil as Mg^{2+}, SO_4^{2-}. $MgSO_4$ is a component of DKW (Driver & Kuniyuki 1984) salt medium mostly used (with good results) in *T. cacao* somatic embryogenesis and plant regeneration (Minyaka *et al.* 2008, 2010).

The involvement of sulphur in resistance against fungal diseases in certain plants coupled with its importance in *T. cacao* micropropagation emphasised the need to focus on a sulphate salt ($MgSO_4$) nutrition in *T. cacao* protection against the destructive fungal pathogen, *P. megakarya*. Moreover, the success of MRNDS would protect *T. cacao* against more than one disease.

This paper aims to study the effect of sulphate ($MgSO_4$) nutrition on the resistance of *T. cacao* plants against BPD caused by *P. megakarya*.

MATERIAL AND METHODS

Plant material. *T. cacao* seeds from pods of ♀SNK64 × ♂UPA14 obtained by manual pollination were used to establish a nursery. Leaves from three to four months old plantlets were used as plant biological material in a leaf disc test to evaluate the susceptibility of *T. cacao* plants to BPD according to the adapted method of Nyasse *et al.* (1995). The most sensitive hybrids (leaf disc test disease scores between 3.5 and 5) to BPD were used for subsequent experiments.

Pathogen material. The pathogen material used in this investigation was *P. megakarya* strain ELEG-8 (characterised by RADP at CIRAD, Montpellier-France). This strain was graciously offered to us by the laboratory of plant pathology of IRAD (Institute of Agricultural Research for Development) at Nkolbisson (Yaoundé, Cameroon). In our laboratory, the strain of *P. megakarya* was preserved by frequent subcultures on 1.5% (w/v) pea-based agar medium. To maintain its virulence, the strain was periodically inoculated onto cocoa pods.

Experimental design. Eighteen of the most sensitive (leaf disc test disease scores between 3.5 and 5) plantlets from ♀SNK64 × ♂UPA14 were randomly selected and placed (in the nursery; 14 h light/10 h darkness at ≈ 26 ± 1°C) in six sets of three plantlets per set.

Three plantlets of each set received the same and fixed quantity of $MgSO_4$ in 250 ml distilled water (every 7 days for 56 days) except the control (three plantlets) which received the equivalent quantity of distilled water. The quantity of $MgSO_4$ applied in a given set (of three plantlets) was defined based on the quantity of $MgSO_4$ (0.74 g/l) found in DKW salt

Table 1. Different sets (6) of plantlets and quantities of $MgSO_4$ supplied weekly for each plantlet of a given set

	Plantlet sets					
	S_I (control)	S_{II} (negative control)	S_{III} (positive control)	S_{IV} (test 1)	S_V (test 2)	S_{VI} (test 3)
$MgSO_4$ (g)	0	0.37	0.74	1.48	2.96	5.92
Distilled water (ml)	250	250	250	250	250	250

doi: 10.17221/124/2016-PPS

medium. $MgSO_4$ quantities for each set of plantlets are presented in Table 1. The $MgSO_4$ quantities were determined through the following mathematical law:

$$Q_n = Q_0 2^{n-1} \qquad Q_0 = 0.74 \text{ g}; 0 \le n \le 4$$

where: Q_0 – positive control, quantity in g of $MgSO_4$, i.e. the quantity of $MgSO_4$ in 1 l of DKW salt complex (Driver & Kuniyuki 1985)

Prior to $MgSO_4$ nutrition on day 0, the eighteen plants (in six sets of three plants) aged 3–4 months (with 6–7 leaves) were submitted to leaf disc tests in three independent experiments (or triplicate).

Zoospore production. Zoospores (or inoculums) were obtained according to the Nyasse *et al.* (1995) adapted method. Zoospores were obtained from 10-days-old cultures. Cultures with sporangia were induced to liberate zoospores by adding sterile distilled water at 4°C. After 1 h at room temperature, the zoospore concentration was adjusted to $3 \times l0^5$ zoospores/ml with Malassez hemocytometer (Assistant; Inter-Equipement, Bordeaux-Mérignac, France).

Screening for susceptibility of ♀SNK64 × ♂UPA143 progeny to P. megakarya. The screening for susceptibility of ♀SNK64 × ♂UPA143 progeny to *P. megakarya* was conducted on days 0, 28, and 56 of sulphate nutrition.

A leaf disc test was used for screening for susceptibility of the 18 hybrids from ♀SNK64 × ♂UPA143 progeny according to the Nyasse *et al.* (1995) adapted method. The experimental design consisted of three replicates and completely randomised 5 blocks of leaf discs (Ø = 1.5 cm) per hybrid. Hence, a total of 20 discs were used per hybrid. For each hybrid of the progeny, leaf discs were obtained from the slightly lignified young leaves (2–2.5 months old). Leaf discs were placed in trays and incubated for 24 h (at 25 ± 1°C) in darkness prior to inoculation. After 24 h, leaf discs were inoculated by depositing 10 µl (3×10^5 zoospores/ml) of zoospore suspension on either side in the middle of each leaf disc and incubated in darkness (at 25 ± 1°C). The necrosis rate (from 0 – tolerant to 5 – highly sensitive) of susceptibility (through the necrosis size) of each leaf disc (for each hybrid) was registered on day 4, 5, 6, 7, and 8 after inoculation.

Cysteine and glutathione extraction. Simultaneously with the leaf disc test screening, cysteine was extracted from leaves of the 18 hybrids on days 0, 28, and 56 of $MgSO_4$ nutrition. Leaves 2.5 months old were slightly ground in a mortar in the presence of 5 ml acetone (to remove chlorophyll) and dried for 5 min at room temperature on Whatman N°1 filter paper.

For cysteine extraction, 0.5 g of chlorophyll-free leaves was ground in the presence of 2.5 ml of ethanol 80° and centrifuged for 30 min at 6000 *g*. The supernatant was collected for cysteine quantification.

Glutathione was extracted by grinding 0.5 g of chlorophyll-free leaves in 2.5 ml of Tris-HCl buffer (50 mM, pH 7.4) followed by centrifugation (30 min, 6000 *g* at 4°C). The supernatant was used to quantify glutathione in leaves on days 0, 28, and 56 of $MgSO_4$ nutrition.

Cysteine and glutathione quantification. Cysteine content was determined according to the Gaitonde (1967) method. Cysteine extract (0.15 ml) was mixed with 0.35 ml of acidic ninhydrin reagent [1.3% (w/v) ninhydrin in 1 : 4 concentrated HCl : CH_3COOH]. The mixture was heated at 100°C for 10 min, then it was cooled in ice bath to allow PING colour development. The optical density was read at 560 nm against the control in which 0.15 ml of cysteine extract was replaced by an equal volume of ethanol 80°.

Glutathione was quantified using DTNB/EDTA according to the Elman (1959) method. 50 µl of phosphate buffer (100 mM pH 6.8) containing 8 mM DTNB and 19 mM EDTA were mixed with 100 µl glutathione crude extract and 1 ml of Tris-HCl buffer (0.5 M pH 7.4) and incubated for 25 min at room temperature. The optical density was read at 412 nm against the control in which the crude extract was replaced by 100 µl of Tris-HCl buffer (50 mM, pH 7.4).

Data analysis. Collected data were firstly subjected to descriptive statistics and analysis of variance (ANOVA). Then, the means were separated using the Student-Newman-Keuls test (at 5% significance level). Spearman's correlation analysis between variables ($MgSO_4$ mass, disease scores, cysteine and glutathione contents on days 0, 28, and 56) was conducted to evaluate the dependence between these variables. These statistical analyses were performed by SPSS v17.0 software. Data on glutathione content in leaves versus quantities of $MgSO_4$ supplied into the soil were converted in a mathematical model (mathematical equation) using MATLAB software.

RESULTS

The effect of weekly exogenous $MgSO_4$ nutrition on *T. cacao* tolerance to *P. megakarya* was monitored simultaneously with cysteine and glutathione

doi: 10.17221/124/2016-PPS

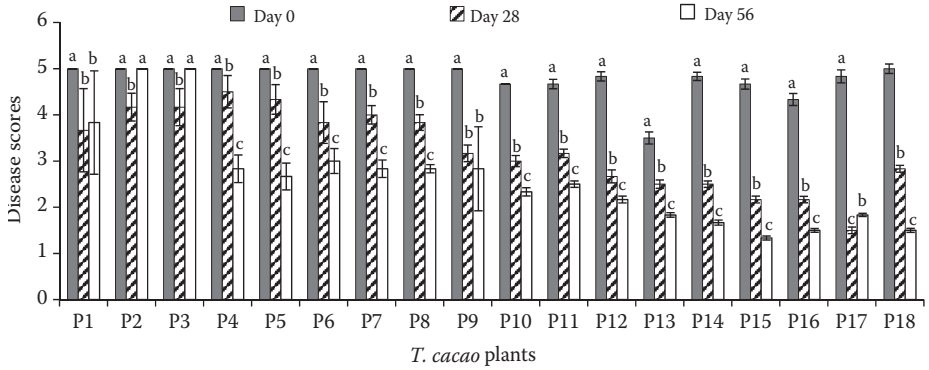

Figure 1. Disease scores of *T. cacao* plants (genotypes) on day 0, day 28, and day 56 of $MgSO_4$ nutrition

Plantlets are ranged in sets of three plantlets receiving the same quantity of $MgSO_4$ weekly; sets of plantlets received increasing quantities of $MgSO_4$; values are expressed as means and standard deviations; values that are significantly different for a given plantlet (in same set of three plantlers) at the 5% level of significance are indicated with different letters

contents in young leaves of *T. cacao* on days 0, 28, and 56 of $MgSO_4$ supply.

Dynamics of the tolerance (to P. megakarya) of T. cacao plants during exogenous sulphate supply to the soil. On day 0 (before sulphate supply as $MgSO_4$), 55.6% of plants presented the highest disease score (5) while 38.86% showed a disease score of 4 and 5.56% indicated a disease score of 3.5. When sulphate was supplied four times (day 28), there was a substantial decrease in plant susceptibility to *P. megagarya* in the five sets of plants used as the test ones while there was no change in plants used as the control. The decrease in plant susceptibility was amplified when additional four sulphate doses were applied to soil (day 56) (Figure 1).

The decrease in disease score versus $MgSO_4$ was followed on days 0, 28, and 56. It appears that on day 0 there was no significant difference between disease scores (mean value obtained from disease scores of three plantlets of each set) of the six sets of plantlets. Four-week (day 28) or eight-week (day 56) supply of $MgSO_4$ induced a significant decrease in disease score while $MgSO_4$ supply increased (Figure 2).

The percentage of disease tolerance (compared to day 0) as a function of $MgSO_4$ supply to soil was represented by a quasi-hyperbolic curve for day 28 and day 56 with the respective highest value of 54.11% and 65.88% for days 28 and 56 of $MgSO_4$ supply. The asymptotic line seems to correspond to the values 60 and 70% for days 28 and 56, respectively (Figure 3).

Dynamics of cysteine content in leaves during exogenous $MgSO_4$ supply. Cysteine content was analysed on days 0 (before $MgSO_4$), 28 (after 4 times $MgSO_4$), and 56 (after 8 times $MgSO_4$ supply) in the six triplets of plants with gradually increasing contents of $MgSO_4$. On day 0, cysteine content of individual plants was variable from one plant to another. The addition of $MgSO_4$ in test triplets of

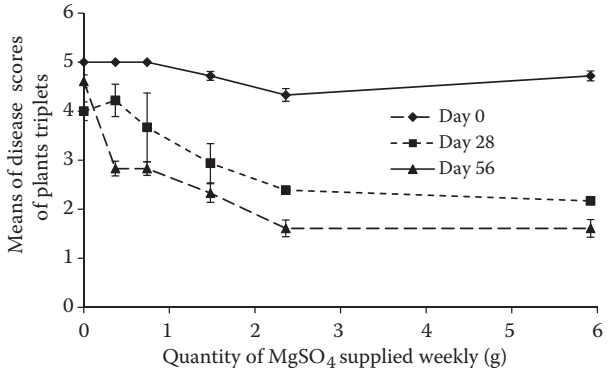

Figure 2. Profile of the means of disease scores versus $MgSO_4$ supplied into the soil weekly

Disease scores are means ± SD (n = 28 × 3)

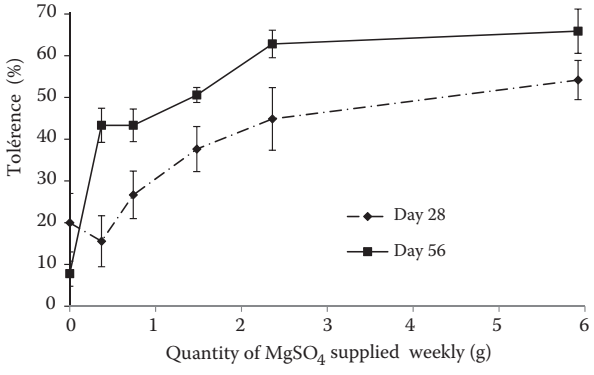

Figure 3. Plant tolerance changes (%) subsequent to $MgSO_4$ supplied into the soil

Disease scores are means ± SD (n = 28 × 3)

doi: 10.17221/124/2016-PPS

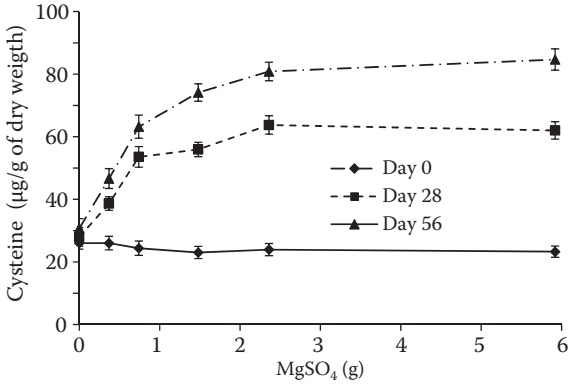

Figure 4. Cysteine content versus $MgSO_4$ supplied into the soil

Cysteine contents are means ± SD (n = 3 × 3)

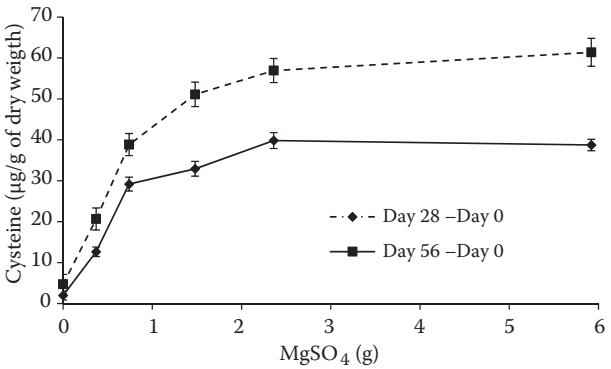

Figure 5. Increase in cysteine content (day 28–day 0 and day 56–day 0) versus sulphur (from $MgSO_4$) content

Cysteine contents are means ± SD (n = 3 × 3)

plants for four times (day 28) showed a significant increase of the sulphurous amino acid in each plant except the three plants used as control. Four more additions (day 56) of $MgSO_4$ increased the cysteine content in leaves (Figure 4).

Curves of the means of cysteine contents of each triplet of plants receiving the same quantity of $MgSO_4$ as a function of increasing $MgSO_4$ supply showed no significant difference between triplets on day 0. Reversely, the fourfold supply of $MgSO_4$ (day 28) resulted in an increase in cysteine contents as the $MgSO_4$ supply to soil increased from one triplet of plants to another. The same shape was observed when four additional doses of $MgSO_4$ were supplied (day 56). However, the shapes of both curves (day 28 and day 56) were not linearly proportional to the mathematical law of $MgSO_4$ supply. In fact, day 28 and day 56 curves appeared hyperbolic while the $MgSO_4$ supply mathematical law is an exponential function (Figure 5).

Dynamics of glutathione content in leaves during exogenous $MgSO_4$ supply. Glutathione content in leaves was evaluated on day 0 of $MgSO_4$ supply in 18 (in 6 triplets of) *T. cacao* plants. It appeared that the content of glutathione in young leaves of the 6 triplets of plants was not significantly different between triplets. Four times $MgSO_4$ supply (day 28) led to a significant increase in glutathione content (compared to the control where $MgSO_4$ was not applied to the soil). On the day 56 of $MgSO_4$ nutrition, glutathione contents in leaves were more consistent compared to days 0 and 28. However, the graphs of glutathione contents in leaves as functions of increasing $MgSO_4$ supply had almost the same pattern on days 28 and 56 (Figure 6).

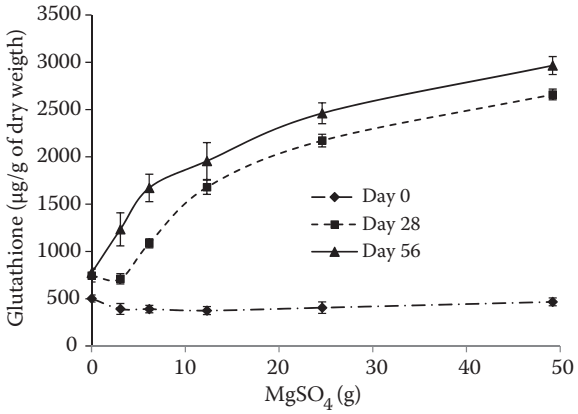

Figure 6. Glutathione content versus $MgSO_4$ supplied into the soil

Cysteine contents are means ± SD (n = 3 × 3)

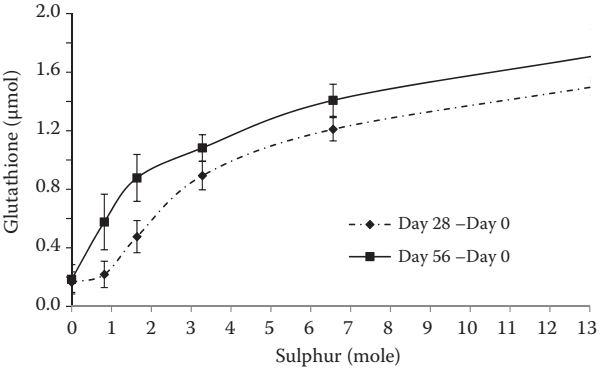

Figure 7. Increase in gluthatione content (day 28–day 0 and day 56–day 0) versus sulphur (from $MgSO_4$) content

Cysteine contents are means ± SD (n = 3 × 3)

doi: 10.17221/124/2016-PPS

Dynamic differences (day 28–day 0 and day 56–day 0) in glutathione content and mathematical laws associated. Curves for the differences in glutathione contents between day 28 and day 0 on the one hand and day 56 and day 0 on the other hand showed quasi sigmoid and hyperbolic shapes (Figure 7). When data of these curves were submitted to MATLAB software analysis the following mathematical laws were obtained:

$$F_{day28-day0}(x) = \frac{1}{2623}x^3 - \frac{1}{54}x^2 + \frac{1}{4}x + \frac{1}{10} \quad \text{for day28–day0 (1)}$$

$$F_{day56-day0} = (x) = \frac{1}{429.73}x^3 - \frac{1}{18.01}x^2 + \frac{1}{2.26}x + \frac{1}{4.47} \quad \text{for day56–day0 (2)}$$

where: $F_{day28-day0}(x)$ – glutathione content in µg/g of leaves; x – quantity of $MgSO_4$ in the soil

Both mathematical laws are similar and indicate that at the limit threshold of $MgSO_4$, glutathione contents become constant. Additionally, glutathione content in leaves is not linearly proportional to $MgSO_4$ supply.

Correlation between cysteine, glutathione contents, $MgSO_4$ supply, and plant tolerance to *P. megakarya*. Spearman's correlation test showed negative and highly significant correlations between $MgSO_4$ supplied to soil, cysteine contents, glutathione contents, and disease scores. While a positive and highly significant correlation was observed between the quantity of supplied $MgSO_4$ and cysteine, glutathione contents on days 28 and 56 of $MgSO_4$ supply. A positive and highly significant correlation exists between cysteine and glutathione on days 28 and 56 (Table 2).

Table 2. Spearman's correlations between cysteine, glutathione contents, $MgSO_4$ supply, and plant tolerance to *P. megakarya*

		Mass $MgSO_4$	ds day 0	ds day 28	ds day 56	cys day 0	cys day2 8	cys day 56	gsh day 0	gsh day 28	gsh day 56
Mass $MgSO_4$	Correlation coeff	1.000									
	Sig. (2-tailed)	0.000									
ds day 0	Correlation coeff	–0.695**	1.000								
	Sig. (2-tailed)	0.001	0.000								
ds day 28	Correlation coeff	–0.874**	0.763**	1.000							
	Sig. (2-tailed)	0.000	0.000	0.000							
ds day 56	Correlation coeff	–0.937**	0.707**	0.818**	1.000						
	Sig. (2-tailed)	0.000	0.001	0.000	0.000						
cys day 0	Correlation coeff	–0.097	–0.156	0.110	0.160	1.000					
	Sig. (2-tailed)	0.701	0.538	0.665	0.527	0.000					
cys day 28	Correlation Coeff	0.834**	–0.612**	–0.663**	–0.817**	–0.005	1.000				
	Sig. (2-tailed)	0.000	0.007	0.003	0.000	0.984	0.000				
cys day 56	Correlation coeff	0.960**	–0.668**	-0.851**	–0.907**	–0.120	0.885**	1.000			
	Sig. (2-tailed)	0.000	0.002	0.000	0.000	0.636	0.000	0.000			
gsh day 0	Correlation Coeff	–0.016	0.025	–0.103	–0.072	0.029	–0.065	0.010	1.000		
	Sig. (2-tailed)	0.951	0.921	0.683	0.777	0.909	0.798	0.968	0.000		
gsh day 28	Correlation coeff	0.956**	–0.726**	–0.891**	–0.904**	–0.154	0.800**	0.918**	0.181	1.000	
	Sig. (2-tailed)	0.000	0.001	0.000	0.000	0.542	0.000	0.000	0.473	0.000	
gsh day 56	Correlation Coeff	0.981**	–0.643**	–0.871**	–0.934**	–0.103	0.826**	0.958**	0.102	0.950**	1.000
	Sig. (2-tailed)	0.000	0.004	0.000	0.000	0.683	0.000	0.000	0.687	0.000	0.000

Mass $MgSO_4$ – mass of $MgSO_4$ supplied; dsday0: disease score on day 0 of $MgSO_4$ supplementation; ds day 28 – disease score on day 28 of $MgSO_4$ supplementation; dsday56: disease score on day 56 of $MgSO_4$ supplementation; cys day 0 – cysteine content on day 0 of $MgSO_4$ supplementation; cys day 28 – cysteine content on day 28 of $MgSO_4$ supplementation; cys day 56 – cysteine content on day 56 of $MgSO_4$ supplementation; gsh day 0 – glutathione content on day 0 of $MgSO_4$ supplementation; gsh day 28 – glutathione content on day 28 of $MgSO_4$ supplementation; gsh day 56 – glutathione content on day 56 of $MgSO_4$ supplementation; **correlation is significant at the 0.01 level (2-tailed); *correlation is significant at the 0.05 level (2-tailed)

doi: 10.17221/124/2016-PPS

DISCUSSION

Sulphur, an essential element in plants, is provided mainly from the root absorption of sulphate (Kataoka *et al*. 2004). After absorption, sulphate is distributed into different plant organs, tissues, cells and organelles where it is subjected to a reduction-assimilation process that leads to variable sulphur-containing compounds (primary and secondary metabolites) with variable biological functions in plants (Saito 2004).

In the present study we monitor the effect of exogenous sulphate (as $MgSO_4$) nutrition on *T. cacao* against an oomycete, *P. megakarya*, the most destructive pathogen of cocoa production in cocoa-producing countries of central and western Africa. Simultaneously with exogenous $MgSO_4$ nutrition, disease scores, cysteine and glutathione contents were monitored in young leaves of *T. cacao* plants from the same progeny (♀SNK64 × ♂UPA143). In plants, contents of cysteine and glutathione are considered as markers of primary sulphate assimilation and stress response (Kruse *et al*. 2007).

The 18 plants (in six sets) from ♀SNK64 × ♂UPA143 progeny tested for their susceptibility to black pod disease prior to sulphate nutrition showed heterogeneity in disease scores between plantlets. This heterogeneity is due to the fact that tolerance or susceptibility of *T. cacao* to BPD is a polygenic character and *T. cacao* clones used as parents to generate a progeny are always heterozygotes for this character (Nyassé *et al*. 2003; Pokou *et al*. 2008).

When plantlets were subjected to sulphate nutrition, their disease scores significantly decreased with increasing quantities of $MgSO_4$. This might indicate that, when sulphate is supplied, *T. cacao* plantlets gain tolerance or resistance to black pod disease. However, the gain of tolerance/resistance (evaluated in percentage) was not linear to the quantity of $MgSO_4$ supplied in the soil. Instead, the curve (of tolerance gain versus $MgSO_4$ supplied) appeared to be hyperbolic. However, the gain of tolerance/resistance of plantlets seems to be associated with the quantity of $MgSO_4$ supplied into the soil. This set of results might reveal the role of $MgSO_4$ in the tolerance/resistance of *T. cacao* against BPD (due to *P. megakarya*). The beneficial effect of $MgSO_4$ in the protection of *T. cacao* against BPD is surely provided by SO_4^{2-} or sulphur. In fact, Williams *et al.* (2002) reported an accumulation of sulphate in the vascular tissues and leaves of resistant cultivars of tomato. To explain this observation, Cooper and Williams (2004) brought up a hypothesis of sulphite (SO_3^{2-}) oxidation in plant cells, leading to elementary sulphur which has been reported as an induced antifungal substance in plant defence (Cooper & Williams 2004). Besides the elementary sulphur and sulphate, these authors also hypothesised the implication of organic molecules containing sulphur such as cysteine and glutathione in plant defence (Cooper & Williams 2004). Hence, this positive impact of $MgSO_4$ on *T. cacao* protection against BPD might also result from the implication of sulphur in the defence platform operations in plants when exposed to biotic stress (Rausch & Wachter 2005; Ströher & Dietz 2006).

The monitoring of cysteine contents (in the six sets of plantlets of our experimental design) indicated that before sulphate nutrition, the content in cysteine was quasi identical in the six sets of plantlets which did not present a significant difference in disease scores (between sets on day 0). The supplementation of sulphate into the soil resulted in a gradual increase in cysteine contents in plants leaves. This result firstly indicates that sulphate supplied into the soil was absorbed and assimilated in cysteine. However, the sulphate was supplied following an exponential mathematical law (equation) while cysteine content in plantlet (in sets of three plantlets) leaves showed a hyperbolic curve which might be associated with a logarithmic mathematical law (equation). These observations should mean that, in *T. cacao* plants, sulphate absorption and assimilation processes to produce cysteine are regulated. This could also mean that cysteine synthesised under availability of sulphate is partially used for the biosynthesis of other sulphur-containing molecules in *T. cacao* plants.

The comparison of cysteine contents and disease score patterns in the six sets of plantlets showed that both variables presented opposite patterns. The increase in cysteine content in *T. cacao* plantlet leaves was associated with a decrease in disease scores of *T. cacao* plantlets. This might highlight the protective incidence of cysteine against biotic stress due to *P. megakarya*. *T. cacao* plants might therefore use a cysteine pool to reinforce their tolerance to or control of *P. megakarya*. This finding, reported here in *T. cacao* for the first time, means that the concentration of cysteine in *T. cacao* tissues might directly be related to the tolerance/resistance of genotypes (hybrids) to black pod disease. In tomato plants, Vidhyasekaran (2000) reported a closed link between the amino acid

doi: 10.17221/124/2016-PPS

pool in plant tissues and the susceptibility of plants to pathogens. Resistant (to powdery mildew) tomato plant tissues were rich in sulphur-containing amino acids. Zook and Hammerschmidt (1997) attested that in *Arabidopsis thaliana* cysteine induces the synthesis of some phytoalexins such as camalexin. In defined pathosystems and controlled nutritional conditions Kruse *et al*. (2007) reported an activation of plant sulphur metabolism in several incompatible and compatible interactions. Contents of cysteine and glutathione as markers of primary sulphate assimilation and stress response showed increases in *Arabidopsis thaliana* upon infection, coinciding with the synthesis of sulphur-containing defence compounds (Mou *et al*. 2003).

Glutathione content in leaves of *T. cacao* plantlets prior to sulphate nutrition showed a pattern similar to that of cysteine content before sulphur supply in soil. During the sulphate nutrition, glutathione content (day 28–day 0 or day 56–day 0) versus the quantity of supplied sulphate presented hyperbolic (day 28–day 0) or quasi-sigmoid (day 56–day 0) curves which might testify the biological regulation of glutathione pool in *T. cacao* plant tissues. As observed with cysteine, glutathione content and disease score presented antagonist patterns. Showing that, the pool of glutathione in *T. cacao* plant tissues leads to an improvement of the tolerance of cocoa plantlets to *P. megakarya*. Therefore, it could be assumed that sulphate supplied into soil led to an increase in the glutathione pool in *T. cacao* plant tissues; and the high pool of glutathione in tissues participates in the tolerance/resistance of cocoa genotypes (hybrids) to *P. megakarya*. This finding with *T. cacao* reminds the redox-active properties of glutathione (GSH/GSSH) which fulfils protective functions when plants are exposed to biotic or abiotic stress (Rausch *et al*. 2007). Moreover, Bloem *et al.* (2004, 2007) reported that the infection of *Brassica napus* L. with *Pyrenopeziza brassicae* increased cysteine and glutathione contents, as well as the activity of L-cysteine desulfhydrase. It could therefore be assumed that the tolerance of *T. cacao* hybrids to *P. megakarya* depends on the availability and mobilisation ability of sulphate, cysteine, and glutathione which are used for the synthesis of sulphurous antibiotic molecules (metabolites, peptides, ...) against the biotic stress due to *P. megakarya*. Therefore, as with *B. napus*, *T. cacao* might mobilise sulphur, cysteine, and glutathione to react to *P. megakarya* infection. This hypothesis is backed up through the negative and significant correlations observed between disease scores and cysteine and glutathione contents.

CONCLUSION

Sulphate supplied (as $MgSO_4$) into soil improved the tolerance of *T. cacao* genotypes to *P. megakarya*. The improvement of the tolerance is associated with the increase of cysteine and glutathione pool in *T. cacao* plants. Therefore, disease (BPD) incidence due to *P. megakarya* on *T. cacao* could be minimised (up to 70%) through the sulphate nutrition which reinforces the defence system of this economically important plant. For the *T. cacao* defence against *P. megakarya*, sulphur appears to be used directly or indirectly through cysteine, glutathione or others sulphur-containing defence compounds such as antimicrobial small cysteine-rich peptides called defensins and thionins.

References

Bloem E., Riemenschneider A., Volker J., Papenbrock J., Schmidt A., Salac I., Haneklaus S., Schnug E. (2004): Sulphur supply and infection with *Pyrenopeziza brassicae* influence L-cysteine desulphydrase activity in *Brassica napus* L. Journal of Experimental Botany, 55: 2305–2312.

Bloem E., Haneklaus S., Salac I., Wickenhäuser P., Schnug E. (2007): Facts and fiction about sulphur metabolism in relation to plant-pathogen interactions. Plant Biology, 9: 596–607.

Cooper R.M., Williams J.S. (2004): Elemental sulphur as an induced antifungal substance in plant defence. Journal of Experimental Botany, 55: 1947–1953.

Driver J.A., Kuniyuki A.H. (1984): *In vitro* propagation of paradox walnut rootstock. Horticulture Science, 19: 507–509.

Dubuis P.H., Marazzi C., Staedler E., Mauch F. (2005): Sulphur deficiency causes a reduction in antimicrobial potential and leads to increased disease susceptibility of oilseed rape. Journal of Phytopathology, 153: 27–36.

Ellman G. (1959): Tissue sulfhydryl groups. Archive of Biochemistry and Biophysics, 82: 70–77.

Gaitonde M. (1967): A spectrophometric method for the direct determination of cysteine in the presence of other naturally occurring amino acids. Biochemistry Journal, 104: 627–633.

Kataoka T., Hayashi N., Yamaya T., Takahashi H. (2004): Root to-shoot transport of sulphate in *Arabidopsis*. Evidence for the role of SULTR3;5 as a component of low-

doi: 10.17221/124/2016-PPS

affinity sulphate transport system in the root vasculature. Plant Physiology, 136: 4198–4204

Minyaka E., Niemenak N., Issali E.A., Sangaré A., Omokolo N.D. (2010): Sulphur deplection altered somatic embryogenesis in *Theobroma cacao* L. Biochemical difference related to sulphur metabolism between embryogeneic and non embryogenic calli. African Journal of Biotechnology, 9: 5665–5675.

Minyaka E., Niemenak N., Fotso, Sangare A., Omokolo N.D. (2008): Effect of $MgSO_4$ and K_2SO_4 on somatic embryo differentiation in *Theobroma cacao* L. Plant Cell Tissue Organ Culture, 94: 149–160.

Mou Z., Fan W., Dong X. (2003): Inducers of plant systemic acquired resistance regulate NPR1 function through redox changes. Cell, 113: 935–944.

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

Nyassé S., Efombagn M.I.B., Kébé B.I., Tahi G.M., Despréaux D., Cilas C. (2007): Integrated management of *Phytophthora* diseases on cocoa (*Theobrama cacao* L.): impact of plant breeding on pod rot incidence. Crop Protection, 26: 40–45.

Nyassé S., Efombagn M.I.B., Bouambi E., Ndoumbe-Nkeng, M., Eskes A.B. (2003): Early selection for resistance to *Phytophthora megakarya* in local and introduced cocoa varieties in Cameroon. Tropical Science, 43: 96–102.

Pokou N.D., N'Goran J.A.K., Kebe I., Eskes A., Tahi M., Sangare A. (2008): Levels of resistance to *Phytophthora* pod rot in cocoa accessions selected on farm in Cote d'Ivoire. Crop Protection, 27: 302–309.

Rausch T., Wachter A. (2005): Sulphur metabolism: a versatile platform for launching defence operations. Trends in Plant Science, 10: 503–509.

Rausch T., Gromes R., Liedschulte V., Müller I., Bogs J., Galovic V., Wachter A. (2007): Novel insight into the regulation of GSH biosynthesis in higher plants. Plant Biology, 9: 565–572.

Saito K. (2004): Sulphur assimilatory metabolism. The long and smelling road. Plant Physiology. 136: 2443–2450.

Schnug E., Haneklaus S., Booth E., Walker K.C. (1995): Sulphur supply and stress resistance in oilseed rape. In: Rapeseed Today and Tomorrow. Proceedings 9th International Rapeseed Congress, July 4–7, 1995, Cambridge, UK: 229–231.

Ströher E., Dietz K.-J. (2006): Concepts and approaches towards understanding the cellular redox proteome. Plant Biology, 8: 407–418.

Tcharbuahbokengo N. (2005): Cocoa production in Cameroon. In: Brooks K.N., Ffolliott P.F. (eds): Moving Agroforestry into the Mainstream. 9th North American Agroforestry Conference Proceedings, June 12–15, 2005, St. Paul, USA.

Vidhyasekaran P. (2000): Physiology of Disease Resistance in Plants. Vol. II. Boca Raton, CRC Press, Inc.

Williams, J.S., Cooper, R.M. (2004): The oldest fungicide and newest phytoalexin – a reappraisal of the fungitoxicity of elemental sulphur. Plant Pathology, 53: 263–279.

Williams J.S., Hall S.A., Hawkesford M.J., Beale M.H., Cooper R.M. (2002): Elemental sulphur and thiol accumulation in tomato and defense against a fungal vascular pathogen. Plant Physiology, 128: 150–159.

Zook M., Hammerschmidt R. (1997): Origin of the thiazole ring of camalexin, a phytoalexin from *Arabidopsis thaliana*. Plant Physiology, 113: 463–446.

Received: 2016–08–19
Accepted after corrections: 2017–08–17
Published online: 2017–12–06