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Influence of *Gigaspora margarita* and *Acaulospora tuberculata* on tolerance to *Phytophthora megakarya* in *Theobroma cacao* under plant nursery conditions

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ABSTRACT

The effect of *Gigaspora margarita* and *Acaulospora tuberculata* inoculation on the tolerance of hybrid genotypes of cocoa (*Theobroma cacao* L.) to stress caused by *Phytophthora megakarya,* was studied in a greenhouse and thereafter in the laboratory. An assessment of the susceptibility of parental and hybrid genotypes of the family F79SA (T79/501x SNK413) was performed after artificial infection with *P. megakarya* in the absence and presence of arbuscular mycorrhizal (AM) fungi by the leaf disc test. Variance analysis of necrotic surfaces showed a significant date effect and genotype effect (P>0.05) in the offspring. In addition, 68% of hybrid genotypes had a lower necrosis surface than the most active parent in the absence of the AM fungi. Under the control of AM fungi, 86% of hybrid genotypes were very tolerant and 14% were tolerant on day 7. The map of tolerance analysis under the control of AM fungi shows a strong dominance of the black color in all hybrid genotypes thus translating very small necrotic surfaces. These results indicate that the *G. margarita* and *A. tuberculata* significantly reduced susceptibility to *P. megakarya* in *T. cacao*. Although, the progeny came from fathers tolerant to *P. megakarya*, there tend to be many incidents with susceptibility to this fungus.

INTRODUCTION

The cocoa tree (*Theobroma cacao* L.) is a perennial plant of the family *Malvaceae*, native to Central and South America. Its seed is used as raw material in cosmetics, pharmaceuticals, food industries and therefore constitute an economic interest in producing countries (Janny et al., 2003; Jagoret, 2011). The marketing of cocoa beans enhances the purchasing power of farmers. World production of cocoa bean during the 2015-2016 season reached 4.154 million tons and Cameroon with a production of 232 000 tons is ranked as the 5th world producer. However, in Cameroon as elsewhere, this production although important, is still inferior to the food demand for this product. This insufficient production is due, on one hand, to population growth and on the other hand, to the aging of orchards, the low use of improved varieties and above all, the high parasite pressure in production areas (ICCO, 2007). In Cameroon, black pod disease caused by *Phytophthora megakarya* induces substantial yield losses of up to 90 to 100% depending on the region, genotype, and environmental conditions in the absence of phytosanitary treatments (Ndoumbé-Nkeng et al., 2004; Nyadanu et al., 2012). Despite efforts to

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develop cocoa for resistance to this phytopathogenic Oomycete, no fully resistant cocoa genotypes have been found to date (Efombagn et al., 2013). Chemical control although widely used, is expensive, polluting, presents a risk of toxicity to the peasant and therefore does not constitute a crop management process for sustainable development (Deberdt et al., 2007; Opoku et al., 2007; Nyadanu et al., 2012). In Cameroon, as in many producing countries, the improvement of cocoa farming will continue to progress through the use of orchards, which are potential centers of genetic resources. To this end, the selection of cocoa plants that are not very sensitive or tolerant to black pod disease remains a priority objective based on the selection of hybrid genotypes in producing countries. This selection aims at creating hybrid genotypes by crossing between two varieties in order to benefit from the heterosis effect in the first generation and to bring together the offsprings, which leads to interesting characteristics such as productivity and tolerance to diseases (Simo et al., 2014).

In most producing countries, cocoa cultivation faces many constraints such as the use of chemicals, lack of control of fungal diseases and lack of control over new cultivation techniques (Koulibaly et al., 2018). In the face of the constraints facing cocoa-based cropping systems in Cameroon, there is no satisfactory and universal solution, but a large number of possible solutions that must be adopted (Meynard et al., 2001).

For decades, improving crops such as cocoa has been through the irrational use of chemicals, a source of environmental contamination and risks to human health worldwide (Maroni et al., 2006). Today, the environmental challenges facing global agriculture call into question conventional production systems, hence the need for agricultural research to propose alternatives to reduce or even eliminate the use of chemicals. To face these issues related to respect for the environment and human health, the use of arbuscular mycorrhizal fungi (AMF) seems to be an alternative for the improvement of Cameroonian cocoa farming.

The AMF play an important role in reducing the incidence of plant diseases (Akthar and Siddiqui, 2008). Several species of AMF have been found to control plant pathogens (Küçükyumuk et al., 2014). For this purpose, Tchameni et al. (2012) showed the ability of *Gigaspora margarita* and *Funneliformis mosseae* to reduce the susceptibility of cocoa plants to *P. megakarya*. These fungi, found in the soils of most ecosystems, form symbiotic associations with the roots of many terrestrial plant species (Smith and Smith, 2012). In exchange for the carbon resources received from the host plant, these fungi reduce the effect of pathogenic infections (Smith and Read, 2008). Mycorrhizal symbiosis has been shown to increase tolerance to abiotic and biotic stress and improve the living conditions of the plant.

The present work aims to contribute to the improvement of the tolerance of a hybrid family of *cocoa* against *P. megakarya* through the evaluation of the susceptibility of the hybrid genotypes of the F79SA hybrid family of *T. cacao* after infection of the leaves with *P. megakarya* before and after inoculation of the *G. margarita* and *A. tuberculata*. The results obtained will allow to evaluate the effect of these AMF against the attack of the pathogen and to identify the tolerant hybrid genotypes in order to popularize them to the cocoa growers.

MATERIALS AND METHODS

Ecological conditions

The study was conducted in the nursery of the University of Douala (4°01 N, 9°44 E, altitude: 13 m above sea level, with total annual rainfall of 3597 mm and average temperature of 27°C). The soil properties are sandy soil.

Plant material

Two parental clones SNK413 and T79/501 from the Research Institute for Agricultural Development (IRAD) at Barombi-Bikang (Kumba) and 24 hybrid genotypes from cross pollination of parents (T79/501 × SNK413) were used. Clone SNK413 a local Trinitario is characterized by a tolerance against *P. megakarya* and clone T79/501 a high Amazonian, is susceptible to *P. megakarya.*

Fungal material

The strain of *P. megakarya* is a local isolate, isolated at the Plant Biotechnology Laboratory of IRAD Ekona from the naturally infected cocoa pod in a peasant cocoa farm in Ekona (South-West, Cameroon). The strain of AMF from the Research Institute for Agricultural Development (IRAD) at Nkolbisson (Yaoundé) is a mixture of *G. margarita* and *A. tuberculata*. They were chosen according to their ability to increase nutrient uptake by their host plants and to increase tolerance to pathogenic infections in other plants (Ngonkeu, 2009; Tchameni et al., 2012). The inoculum was composed of a mixture of spores, mycelium, root fragments and coarse sand. The prepared inoculum was stored under laboratory conditions at 15 to 20°C after drying. Fifty gram of the mixture was inoculated at 5 cm below the surface of the growth medium. A two-month period after inoculation was required for the AMF for root colonization and spore germination.

Establishment of the nursery

The three-liter plastic pots perforated in small diameters

(Θ = 0.5 cm) at their bases were filled with humified soil obtained from a mixture of surface soil from a plot of fallow fields of the Faculty of Science of the University of Douala and fine sawdust 3: 1 (v / v). This soil was air dried, then sieved to remove stones and plant debris. The seeds taken outside the tips of the cocoa pod were washed in tap water added to the fine sand to remove the mucilage. On the eve of sowing, the pots were watered and the next day the cocoa seeds were sown at a rate of one bean per pot. Each seed was placed in the middle of each pot at 1 cm with the large end of the seed down to maintain the right pivot. After sowing, the pots were watered daily at 6 am for the first 15 days after sowing, then every 2 days so as to maintain the soil moisture.

Root staining and evaluation of mycorrhization

The roots were stained according to the method described by Phillips and Hayman (1970) with Trypan blue to allow the staining of chitin on the walls of the fungus. The frequency and intensity of mycorrhization were determined according to the methods described by Marx et al. (1977) and Trouvelot et al. (1986).

Isolation and culture of the *P. megakarya* strain

Isolation and culture of *P. megakarya* was done according to the method of Nyassé et al. (1995) taken over by Coulibaly et al. (2013). The *P. megakarya* strain was isolated from a pod that was naturally affected by brown rot, whose necrosis was evolving. This pod was used to infect a healthy pod previously washed with distilled water by placing them side by side in a plastic container soaked with distilled water and incubated at 26°C in the dark for two weeks. This new infected pod was washed with tap water, and then disinfected in a series with 95% ethanol for 30 s in 10% sodium hypochlorite for 2 min, then in 70% ethanol for 2 min; so as to eliminate the microorganisms present on the epidermis of the cortex. The pod was then rinsed three times with sterile distilled water to remove traces of disinfectant. The sampling area was chosen and the superficial tissues were stripped with a sterile scalpel. Five cubic-shaped fragments of about 7 mm in size were removed from the subcortical tissues with a sterile punch at the growth front of the necrosis. Each fragment was placed on agar medium containing pea in petri dishes of diameter 90 mm. Incubation was done in the dark in a hot air oven at 26°C for 5 days. After colony formation, the isolates obtained were purified by successive subcultures on pea-based agar medium.

Preparation of inoculum and zoospore counting

The method used for the preparation of the inoculum and the counting of the zoospores was that of Nyassé et al. (1995). Fourteen days before the planned date of inoculation, the strain to be inoculated was transplanted into pea-based agar medium in 90 mm diameter petri dishes and incubated in the dark in an oven at 26°C for 7 days. The dishes were alternately placed for 7 days in the dark and in the light of an incandescent lamp (60 W) (photoperiod 12 h/12 h) at 26°C in order to obtain the formation of sporocysts which are at the same time origin of zoospore production. On the day of inoculation, 4 to 5 ml of sterile distilled water was added to each dish. These Petri dishes were placed in a refrigerator at 4°C for 30 min to allow the cold shock to promote the release of zoospores contained in the sporocysts. The dishes were then placed at 26°C for 2 h so that the zoospores could be released into the water and for each dish the solution containing the zoospores was recovered in a beaker. The zoospores were immobilized with two drops of methylene blue. The zoospore suspension thus obtained in the beaker was counted using a Malassez hematimetric cell at the concentration of 101 × 105 zoospores/ml. The count was done on objective 40 optical microscope. The inoculation with the calibrated suspension was done as quickly as possible to prevent the zoospores from losing their ability to swim in the water.

Artificial leaf infection and assessment of necrosis

Two-month-old leaves (60 days) of non-lignified twigs as described by Nyassé et al. (1995) were selected very early in the morning on each hybrid genotype. Discs, 15 mm in diameter were cut from these sheets (three per sheet) and placed upside down in plastic dishes on toilet paper soaked in distilled water. Artificial infections of the leaves was done the morning of the following day with 6 µl of suspension of *P. megakarya* calibrated at 101×10^5 zoospores/ml previously prepared as described above and deposited with a repeating micropipette. Measurement of the size of the necrotic lesion was done on the 3^{rd}, 4^{th}, 5^{th}, 6^{th} and 7^{th} days after infection. These artificial infections were done before and after inoculation of the AMF. The diameters of the necrotic spots were measured and the area was calculated using the formula of Blaha and Lotodé (1976).

Statistical analysis

The results are represented in the form of mean ± standard deviation of the three repetitions. The one-way analysis of variance (ANOVA) by the Duncan test was performed to compare the differences between the means at the significance level P>0.05 using SPSS software version 16.0 for Windows. The hierarchical classification of the different groups of hybrid genotypes

Table 1. Development of necrosis (cm^2) on leaf discs of parental genotypes and F79SA hybrid without control of *G. margarita* and *A. tuberculata*.

Genotypes	Day 3	Day 4	Day 5	Day 6	Day 7
SNK413	0.005±0.004^{a}	0.057±0.023^{ab}	0.154±0.072^{ab}	0.546±0.077^{bcd}	1.07±0.104^{c}
T79/501	0.008±0^{a}	0.108±0.032^{abc}	0.463±0.068^{de}	1.332±0.205^{f}	1.766±0.104^{d}
F79SA1	0.050±0.036^{a}	0.196±0^{c}	0.225±0.050^{abc}	0.225±0.050^{b}	0.225±0.050^{ab}
F79SA2	0.785±0^{ab}	1.272±0.298^{f}	1.544±0.220^{f}	1.615±0.131^{e}	1.690±0.131^{d}
F79SA3	0.029±0.036^{a}	0.044±0.023^{ab}	0.044±0.023^{a}	0.086±0.095^{a}	0.196±0^{a}
F79SA4	0.024±0.041^{a}	0.029±0.036^{a}	0.029±0.036^{a}	0.322±0.109^{ab}	0.322±0.109^{ab}
F79SA5	0.003±0.005^{a}	0.029±0.036^{a}	0.029±0.036^{a}	0.063±0.055^{a}	0.089±0.032^{a}
F79SA6	0.024±0.041^{a}	0.065±0.113^{ab}	0.128±0.222^{a}	0.212±0.367^{ab}	0.212±0.367^{ab}
F79SA7	1.766±0^{c}	1.766±0^{g}	1.766±0^{f}	1.766±0^{e}	1.766±0^{d}
F79SA9	0.633±0.430^{ab}	1.196±0.114^{f}	1.539±0^{f}	1.539±0^{e}	1.766±0^{d}
F79SA10	0.000±0^{a}	0.008±0^{a}	0.029±0.036^{a}	0.254±0.050^{ab}	0.254±0.050^{ab}
F79SA11	0.000±0^{a}	0.008±0^{a}	0.008±0^{a}	0.173±0.040^{a}	0.225±0.050^{ab}
F79SA12	0.071±0^{a}	0.385±0^{e}	0.424±0.068^{d}	0.756±0.331^{d}	1.112±0.566^{c}
F79SA13	0.010±0.018^{a}	0.016±0.013^{a}	0.037±0.032^{a}	0.317±0.059^{ab}	0.424±0.068^{ab}
F79SA14	0.042±0.073^{a}	0.133±0.109^{abc}	0.204±0.177^{ab}	0.390±0.110^{abc}	0.429±0.126^{ab}
F79SA15	1.408±0.326^{b}	1.615±0.131^{f}	1.615±0.131^{f}	1.615±0.131^{e}	1.766±0^{d}
F79SA16	0.131±0.063^{a}	0.463±0.068^{e}	0.591±0.077^{e}	0.636±0^{cd}	0.636±0^{b}
F79SA17	0.000±0.000^{a}	0.173±0.040^{bc}	0.351±0.059^{bcd}	0.351±0.059^{abc}	0.502±0^{ab}
F79SA18	0.037±0.032^{a}	0.283±0^{d}	0.385±0^{cd}	0.385±0^{abc}	0.385±0^{ab}
F79SA20	0.000±0^{a}	0.023±0.013^{a}	0.071±0^{a}	0.225±0.050^{ab}	0.351±0.059^{ab}
F79SA21	0.000±0^{a}	0.050±0.036^{ab}	0.160±0.110^{ab}	0.254±0.050^{ab}	0.283±0^{ab}
F79SA22	0.000±0^{a}	0.099±0.086^{abc}	0.160±0.110^{ab}	0.254±0.050^{ab}	0.254±0.050^{ab}
F79SA23	1.539±0^{c}	1.766^{g}	1.766±0^{f}	1.766±0^{e}	1.766±0^{d}
F79SA24	0.016±0.013^{a}	1.539±0^{f}	1.766±0^{f}	1.766±0^{e}	1.766±0^{d}

Values with the same letter in a column are not significantly different at P>0.05 from Duncan's test.

according to their ability to tolerate the pathogen was carried out with SPAD software version 4.1 for Windows. The hybrid genotype tolerance analysis map was performed with the Mev software version 4.9.0 for Windows.

RESULTS

Foliar disc inoculation test before and after inoculation of *G. margarita* and *A. tuberculata*

Assessment of necrosis in the absence of G. margarita and A. tuberculata

The development of leaf disc necrosis was evaluated in parental genotypes and their offspring in the absence of *G. margarita* and *A. tuberculata*. At day 3 after artificial infection of the leaves, necrosis was observed in all parental genotypes and in 73% of hybrid genotypes existing at that date. At this date, no significant difference was observed between the two parents and most of the hybrid genotypes with small necrotic surfaces (cm^2) except for the individuals F79SA15, F79SA23, F79SA7 which had the highest necrotic surfaces. From day 4 after artificial infection, necrosis was observed in all individuals tested, which increased until day 7. A significant difference was observed between parental genotypes and hybrid genotypes from day 4 to day 7. On day 7, the parental genotype SNK413 showed the lowest necrotic surface area compared to parent T79/501 and therefore considered to be the best parent. On day 7, 68% of hybrid genotypes showed a lower necrotic surface compared to the most active parent. The hybrid genotypes F79SA2, F79SA7, F79SA9, F79SA15, F79SA23 and F79SA24 had the highest necrotic surfaces and therefore very sensitive. In contrast, the hybrid genotypes F79SA5, F79SA3, F79SA6, F79SA11, F79SA1, F79SA10, F79SA22, F79SA21, F79SA4, F79SA20, F79SA18, F79SA13 and F79SA14 had the lowest necrotic surfaces and therefore very tolerant (Table 1).

Table 2. Development of necrosis (cm^2) on leaf discs of F79SA hybrids under the control of *G. margarita* and *A. tuberculata*.

Genotypes	Day 3	Day 4	Day 5	Day 6	Day 7
F79SA1	0±0^{a}	0±0^{a}	0.008±0^{a}	0.131±0.063^{a}	0.173±0.040^{a}
F79SA2	0±0^{a}	0±0^{a}	0±0^{a}	0.008±0^{a}	0.031±0^{a}
F79SA3	0±0^{a}	0.021±0.017^{a}	0.05±0.037^{a}	0.094±0.054^{a}	0.154±0.072^{a}
F79SA4	0±0^{a}	0.024±0.004^{a}	0.052±0.007^{a}	0.121±0.014^{a}	0.178±0.090^{a}
F79SA5	0±0^{a}	0.023±0.013^{a}	0.023±0.013^{a}	0.058±0.023^{a}	0.058±0.023^{a}
F79SA6	0±0^{a}	0.010±0.002^{a}	0.024±0.004^{a}	0.045±0.007^{a}	0.055±0.006^{a}
F79SA7	0±0^{a}	0±0^{a}	0.044±0.023^{a}	0.154±0.072^{a}	0.406±0.220^{a}
F79SA9	0±0^{a}	0.013±0.002^{a}	0.05±0.036^{a}	0.094±0.054^{a}	0.154±0.072^{a}
F79SA10	0±0^{a}	0±0^{a}	0.023±0.013^{a}	0.071±0^{a}	0.154±0.072^{a}
F79SA11	0±0^{a}	0±0^{a}	0.024±0.004^{a}	0.047±0.007^{a}	0.086±0.009^{a}
F79SA12	0.005±0.004^{a}	0.016±0.013^{a}	0.044±0.023^{a}	0.076±0.048^{a}	0.131±0.063^{a}
F79SA13	0±0^{a}	0.023±0.013^{a}	0.113±0.072^{a}	0.150±0.040^{a}	0.254±0.050^{ab}
F79SA14	0±0^{a}	0.044±0.023^{ab}	0.010±0.009^{a}	0.178±0.091^{a}	0.259±0.109^{ab}
F79SA15	0.010±0.002^{a}	0.024±0.004^{a}	0.089±0.032^{a}	0.113±0.072^{a}	0.149±0.040^{a}
F79SA16	0±0^{a}	0±0^{a}	0.010±0.009^{a}	0.361±0.154^{b}	0.790±0.157^{d}
F79SA17	0.013±0002^{a}	0.071±0^{b}	0.259±0.109^{b}	0.641±0.141^{c}	0.641±0.142^{cd}
F79SA18	0.008±0^{a}	0.023±0.013^{a}	0.044±0.023^{a}	0.076±0.047^{a}	0.108±0.032^{a}
F79SA20	0±0^{a}	0±0^{a}	0±0^{a}	0.005±0.004^{a}	0.013±0.002^{a}
F79SA21	0±0^{a}	0.0027±0.005^{a}	0.003±0^{a}	0.016±0.013^{a}	0.037±0.032^{a}
F79SA22	0±0^{a}	0±0^{a}	0.005±0.004^{a}	0.040±0.032^{a}	0.113±0.072^{a}
F79SA23	0.013±0.002^{a}	0.021±0.017^{a}	0.121±0.014^{a}	0.455±0.300^{b}	0.596±0.163^{c}
F79SA24	0±0^{a}	0±0^{a}	0.003±0^{a}	0.016±0.013^{a}	0.029±0.036^{a}

Values with the same letter in a column are not significantly different at P>0.05 from the Duncan test.

Necrosis development in AMF inoculated plants

The development of leaf disc necrosis was also evaluated in hybrid genotypes controlled by *G. margarita* and *A. tuberculata*. Variance analysis revealed low variability in necrosis between days and between genotypes. On the third day after artificial leaf infection, necrosis was observed in only 23% of hybrid genotypes with very low necrotic surfaces. On this date, no significant difference was observed between hybrid genotypes. Similarly, no significant difference was observed in most of the hybrid genotypes between day 4 and 6 with the exception of the hybrid genotypes F79SA14 and F79SA17; F79SA17, F79SA16, F79SA17 and F79SA23 which had relatively high necrotic surfaces respectively at these different dates. On day 4, necrosis was observed in 59% of genotypes generally with very weak necrotic surfaces and in 100% of hybrid genotypes on day 6. On day 7, 86% of hybrid genotypes had necrotic surfaces less than 0.5 cm^2 and are therefore considered very tolerant. The hybrid genotypes F79SA23, F79SA17 and F79SA16 have necrotic surfaces between 0.5 and 1 cm^2 and are therefore considered tolerant (Table 2).

Frequency and intensity of mycorrhization

The frequency and intensity of mycorrhization were evaluated two months after inoculation of *G. margarita* and *A. tuberculata*. The results show a frequency of mycorrhization greater than 86% in all the hybrid genotypes studied. These observations show a good mycorrhizal symbiosis between *G. margarita* and *A. tuberculata* and *T. cacao* (Figure 1). However, no significant difference (P>0.05) was observed in the hybrid genotypes studied. On the other hand, the results of mycorhization intensity show a rate of less than 50% in all the hybrid genotypes studied (Figure 2). No significant difference (P>0.05) was also noted in all the hybrid genotypes studied.

Hierarchical classification of AMF uninoculated hybrid cocoa genotypes

Hierarchical classification at 95% level of homogeneity of the parental genotypes and the F79SA progeny obtained on the basis of the necrosis surface of the leaf discs from day 3 to day 7 without control of *G. margarita* and *A.*

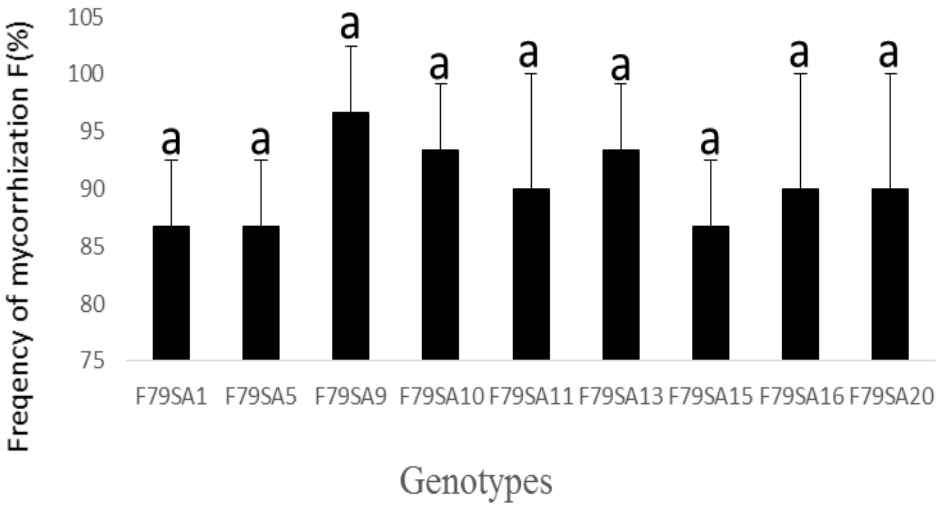

Figure 1. Frequency of mycorrhization of cocoa roots by *G. margarita* and *A. tuberculata*.

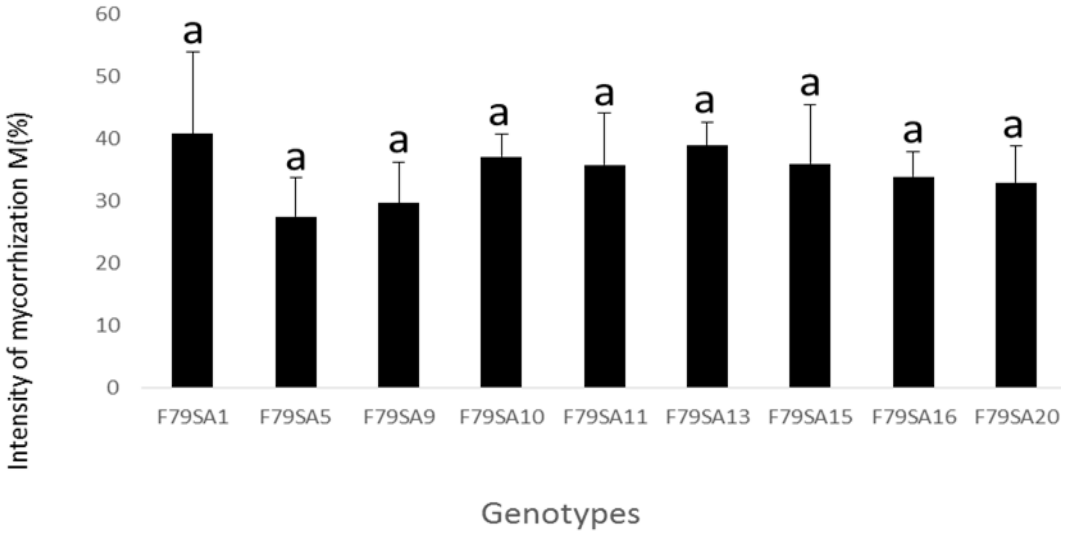

Figure 2. Intensity of mycorrhization of cocoa roots by *G. margarita* and *A. tuberculata*.

tuberculata made it possible to distinguish 7 groups (Figure 3). Groups 1 and 3 consisting of the parental genotype T79/501 and hybrid genotypes characterized by large necrotic surfaces. Group 6 consisting of parental genotype SNK413 and hybrid genotypes characterized by medium necrotic surfaces and groups 2, 4, 5 and 7 consisting of hybrid genotypes, characterized by small necrotic surfaces.

Tolerance analysis map of AMF uninoculated plants

The tolerance analysis map based on the necrosis surface of artificially infected cocoa leaf discs of parental genotypes and F79SA offspring from day 3 to day 7 without control of *G. margarita* and *A. tuberculata* showed an increasing trend in necrosis from day 3 to day 7. This evolution is characterized by the gradual decrease of the black color responsible for the tolerance in favor of the progressive appearance of the red color responsible for the sensitivity. The analysis of this map also showed the progressive decrease of the black color in favor of the red color as one evolves from the very tolerant genotype to the very sensitive genotype. This observation makes it possible to have an idea about the degree of tolerance of each hybrid genotype. This analysis map also allow us to

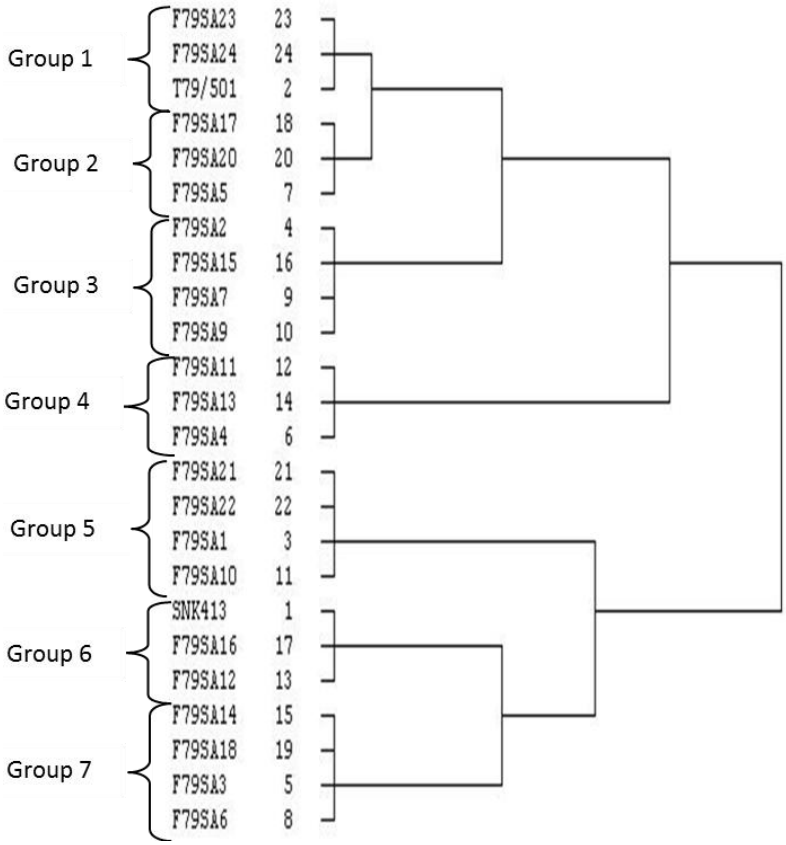

Figure 3. Hierarchical classification of parental genotypes and hybrid genotypes of F79SA offspring obtained on the basis of leaf necrosis surface from day 3 to day 7 in the absence of *G. margarita* and *A. tuberculata*.

be able classify hybrid genotypes from the most sensitive to the most tolerant (Figure 4).

Hierarchical classification of AMF inoculated hybrid genotypes

The hierarchical classification of the hybrid genotypes of the F79SA progeny obtained on the basis of the necrosis surface of leaf discs from day 3 to day 7 under the control of *G. margarita* and *A. tuberculata* allowed to distinguish 9 groups. The group 9 consisted of hybrid genotypes, was characterized by small tolerant necrotic surfaces and the groups 1 to 8 consisted of hybrid genotypes characterized by very small necrotic surfaces (Figure 5).

Tolerance analysis map of AMF inoculated cocoa plants

The tolerance analysis map based on the necrosis surface of artificially infected cocoa leaf discs of the hybrid genotypes of F79SA offspring from day 3 to day 7 in the presence of *G. margarita* and *A. tuberculata* showed very small necrosis in all hybrid genotypes from day 3 to day 5. These small areas of necrosis are characterized by a black color, a sign of tolerance in all these hybrid genotypes at these different dates. From the 6th day, there was a small gradual decrease of the black color and a small gradual reappearance of the red color, and sign of a small decrease of the tolerance level in the hybrid genotypes F79SA17, F79SA23 and F79SA16 (Figure 6).

DISCUSSION

Artificial infection test on leaf disc of AMF inoculated and uninouclated plants

The development of necrosis in the absence of *G. margarita* and *A. tuberculata* showed a significant effect based on the day and genotype. These results are in agreement with those obtained by Effa et al. (2017) on

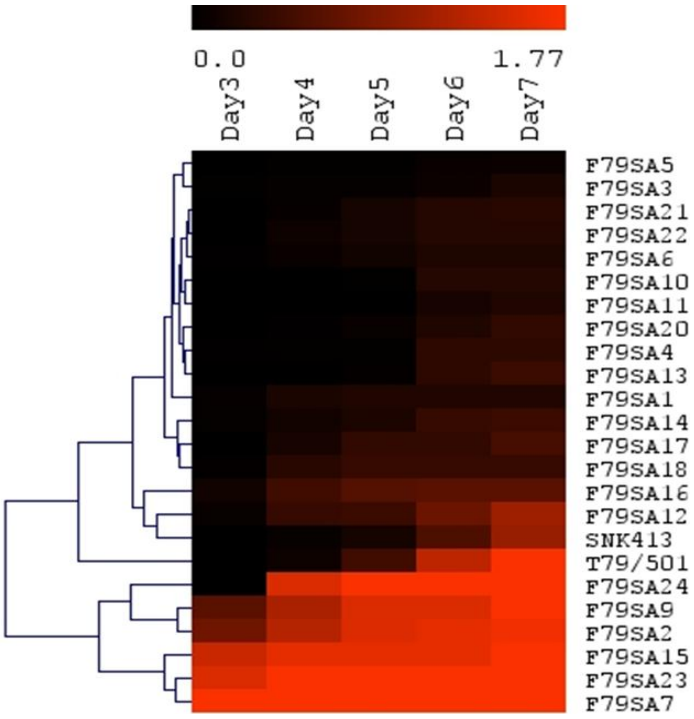

Figure 4. Safety analysis map based on cocoa leaf necrosis surface of parental genotypes and F79SA offspring from day 3 to day 7 in the absence of *G. margarita* and *A. tuberculata*.

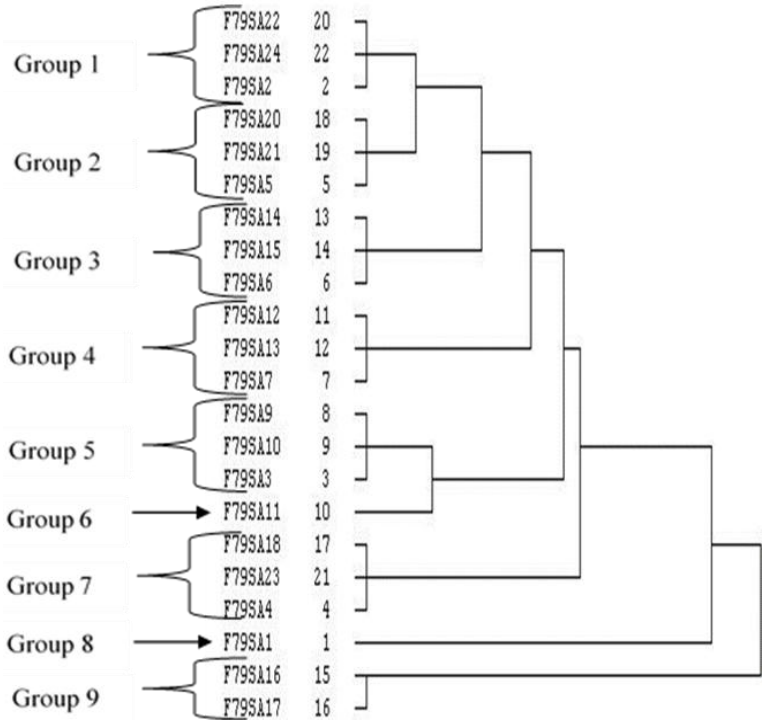

Figure 5. Hierarchical classification of hybrids of F79SA offspring obtained based on necrosis surface of leaf discs from day 3 to day 7 in the presence of *G. margarita* and *A. tuberculata*.

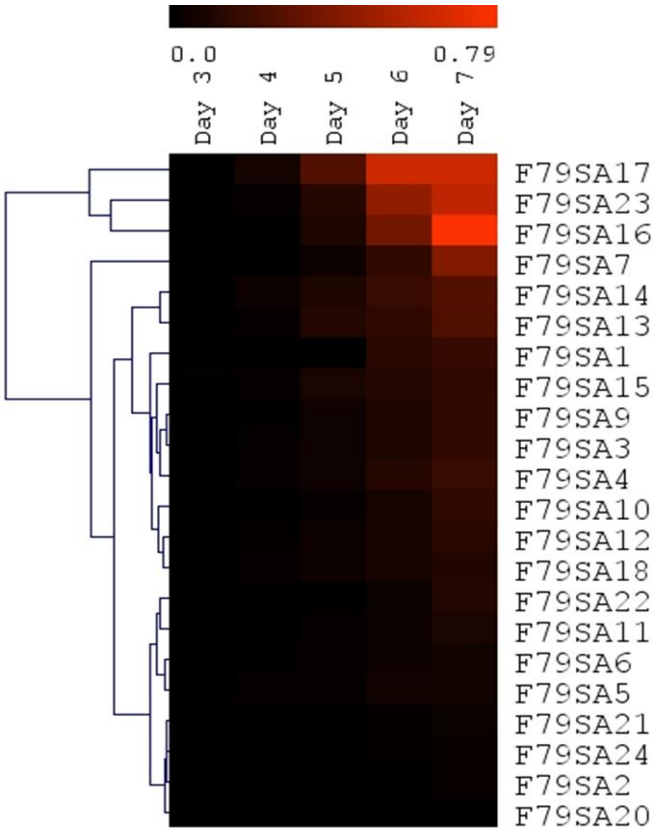

Figure 6. Tolerance analysis map based on surface area of cocoa leaf necrosis of genotypes of F79SA offspring from day 3 to day 7 in the presence of *G. margarita* and *A. tuberculata*.

detached pods and Ondobo et al. (2017) on leaves attached to seedlings of the same plant.

These results also indicated that on day 7, the parental genotype SNK413 had the smallest necrotic surface comparatively to the parental genotype T79/501 and therefore was considered to be the most active parent. In addition, 68% of hybrid genotypes had a lower necrosis surface than the best parent on this date. Similar results have been found by several authors (Nyasse et al., 2002; Efombagn et al., 2011; Ondobo et al., 2014, 2017; Effa et al., 2017; Minyaka et al., 2017), which showed that tolerance to black pod disease was under genetic control and could be improved genetically. Otherwise, the existence within the same family of sensitive and tolerant hybrid genotypes confirms the best aptitude for the combination of parental genes. These results are similar to those obtained in cocoa (Cilas et al., 2004; Ondobo et al., 2013, 2017). In addition, the presence of hybrid genotypes with necrotic surfaces lower than that of the best parent shows that tolerance to black pod disease is an additive trait of the combination of genes (Pokou et al., 2008; Efombagn et al., 2011). This tolerance to black pod disease of hybrid genotypes relative to the best parent is a direct consequence of the heterosis effect that occurs in the F79SA hybrid family.

The development of necrosis in the presence of AMF also showed low variability in necrosis between days and between hybrid genotypes. On day 7, 86% of hybrid genotypes showed necrotic surfaces less than 0.5 cm^2 and are therefore considered very tolerant. The hybrid genotypes F79SA23, F79SA17 and F79SA16 showed necrotic surfaces between 0.5 and 1 cm^2 and are therefore considered tolerant. These results reveal that the AMF significantly improved the tolerance of these hybrid genotypes to *P. megakarya*. These results corroborate with those of Tchameni et al. (2011, 2012) and Nana et al. (2016) who also showed that leaf sensitivity of young *T. cacao* seedlings decreased with inoculation of AMF. These results are also similar to those reported by Tchameni et al. (2017), who found that disease symptoms on *T. cacao* leaves were significantly reduced in seedlings treated with *Trichoderma asperellum* isolates. This ability of hybrid genotypes colonized by *G. margarita* and *A. tuberculata* to resist to the pathogen attack may be explained by the fact that mycorrhizae modifies several aspects of the host plant

and the environment that allow for better resistance to environmental stress. AMFs improve the tolerance level of the plant because they would allow the plant to have better access to nutrients and substrate water, which promotes its growth and allows it to better withstand periods of environmental stress such as the biotic stress caused by the attack pathogens (Helgason and Fitter, 2005; Plenchette, 2005; Dalpe, 2006; Fortin et al., 2008).

Frequency and intensity of mycorrhization

Mycorrhizal frequency results showed high values between 86 and 96% in hybrid genotypes studied. These results are similar to those obtained by Benjelloun et al. (2014) which have a frequency of 98% in corn under stress conditions. These results reflect a symbiotic association between AMF and cocoa. In addition, the results of the mycorrization intensity of *G. margarita* and *A. tuberculata* in cocoa, show values of less than 50% in all hybrid genotypes tested. These results are contrary to those obtained by Benjelloun et al. (2014) who showed that mycorhization intensity is greater than 50% in corn under stress conditions.

Hierarchical classification of AMF inoculated cocoa genotypes

The hierarchical classification of parental genotypes and F79SA offspring obtained from the necrosis surface of leaf discs from day 3 to day 7 without AMF inoculation allowed us to distinguish 7 groups. The highly tolerant or highly sensitive hybrid genotypes constituting the different groups confirm the genetic variability between the different hybrid genotypes. Similar results were obtained by Effa et al. (2017) on the same plant. Moreover, this difference in reaction of hybrid genotypes during artificial infection with *P. megakarya* is confirmed by the tolerance analysis map based on the necrosis surface of the cocoa leaf discs of the parental genotypes and the F79SA offspring from day 3 to day 7 without control of *G. margarita* and *A. tuberculata* which shows a progressive decrease in the color black in favor of the red color as one evolves from the very tolerant hybrid genotype to the very sensitive hybrid genotype.

The hierarchical classification of F79SA progeny genotypes obtained on the basis of the necrosis surface of leaf discs from day 3 to day 7 inoculated with *G. margarita* and *A. tuberculata* allowed us to distinguish 9 groups. Group 9 consisting of tolerant genotypes and the other eight groups consisting of highly tolerant genotypes further confirms the positive effect of *G. margarita* and *A. tuberculata* on improving the tolerance of cocoa during the interaction with the pathogen *P. megakarya*. In addition, the tolerance analysis map based on the necrosis surface of the cocoa leaf disks of the hybrid genotypes of the F79SA progeny from day 3 to day 7 inoculated with *G. margarita* and *A. tuberculata* showed a strong dominance of black coloration in all the hybrid genotypes thus showing very small necrotic surfaces. These results confirm once again that the AMF *G. margarita* and *A. tuberculata* improve the tolerance of cocoa during its interaction with the pathogen *P. megakarya*. These results are similar to those obtained by Mpika et al. (2009) who showed that the application of *Trichoderma* isolates significantly reduced the leaf sensitivity of six cocoa clones after infection with *Phytophthora palmivora*.

Conclusion

T. cacao responds to mycorrhization, it could be a non-obligate mycotic plant. These results indicate that the biofertilizers *G. margarita* and *A. tuberculata* significantly reduced susceptibility to *P. megakarya* in *T. cacao*. Although the progeny come from fathers tolerant to *P. megakarya*, tend to be many incidents with susceptibility to this fungus.

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