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# Effect of *Glomus intraradices* in improving the tolerance of a hybrid family of *Theobroma cacao* L. against *Phytophthora megakarya*

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**Key words:** *Theobroma cacao*, *Phytophthora megakarya*, *Glomus intraradices*, Necrosis, Tolerance, Phenolic compounds.

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## Abstract

The cocoa tree (*Theobroma cacao* L.) is a perennial plant of economic importance grown in several cocoa producing countries. *Phytophthora megakarya* is an oomycete that has a negative impact on cocoa production in Cameroon causing substantial yield losses (up to 100%). To remedy this, the use of genetic resources for the selection of highly tolerant genotypes is necessary for its production. The leaf necrosis of cacao in absence and in the presence of *Glomus intraradices* was measured, the accumulation of phenolic compounds was evaluated in the leaves of genotypes after *Phytophthora megakarya* infection in the absence and presence of *Glomus intraradices*. The analysis maps of the development of necrosis and the accumulation of phenolic compounds obtained on the basis of the necrotic area and the contents of phenolic compounds was used to appreciate the degree of tolerance of the hybrid genotypes againts *Phytophthora megakarya*. In addition, the development of necrosis on surfaces less than 0.5 $cm^2$ in the presence of *Glomus intraradices* was observed in 75% against 94% (without control of *G. intraradices*) of hybrid genotypes. *Glomus intraradices* have significantly improved the tolerance of these hybrid genotypes. Moreover, it has been found that some hybrid genotypes of the Ficup offspring exhibiting better tolerance with necrotic surfaces between 0 and 0.5 $cm^2$ in the presence *of G. intraradices*. Sensitive hybrid genotypes in the presence of *G. intraradices*, increased their degree of tolerance against the attack of *Phytophthora megakarya*. This *G. intraradices* can therefore be used by farmers in cocoa plantations to improve their yield.

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**Introduction**

Cacao is a perennial plant of economic importance grown for its beans in several cocoa producing countries. This plant with multiple virtues is mainly grown in developing countries. Cocoa seed have nutritional, cosmetic and pharmaceutical interests. They are grown for the manufacture of chocolate, biscuit and candy in the food industry; for the manufacture of milkshakes and cosmetics in the cosmetic industries and finally for the manufacture of drugs in the pharmaceutical industry. The cocoa tree is a cash crop with more than 40 million people dependent on the transaction of this plant in the world. Cameroon being ranked fourth world producer during the 2015-2016 campaign, produced more than 232,000 tonnes in 2015 for an orchard of 400,000 ha with nearly 400,000 Cameroonian families who derive most of their income from this plant. Income estimated at more than 173,000,000 USD (Effa *et al*., 2017). However, despite being profitable, this crop still faces multiple problems: parasitic hazards, aging orchards, unavailability and underutilization of improved plant material. In addition, some diseases can destroy the entire production when conditions are favorable for the disease. Brown rot is the major scourge of cocoa production over the past three decades with substantial losses of up to 100 % (Ndoumbe-Nkeng *et al*., 2004; Nyadanu *et al*., 2012). *Phytophthora megakarya* is the most aggressive causative agent of this disease present in Central and West Africa (Nyasee *et al*., 1995; Shahin *et al*., 2017a; Shahin *et al*., 2017b). In the long term, high hopes are placed in the selection and multiplication of tolerant varieties to brown rot. The main difficulty is that no complete resistance has been detected until now with this disease (Pokou *et al*., 2008; Effa *et al*., 2017); hence the interest of finding the host most tolerant to this brown rot. Pesticides have a bad image in the public because of their low yield and are increasingly in the hot seat for toxicity and pollution issues. There is also the problem of the effectiveness of plant protection products which, like antibiotics, is showing tolerance (Fagbohun and Aderiye, 2017). Faced with these limitations, the development of new active substances by the agrochemical industry seems more difficult. However, there are ways to break this deadlock that completely revise the paradigms governing the fight against cultural enemies. One of them is to give plants the means to defend themselves or to strengthen their own defense instead of directly fighting the aggressor (Nyadanu *et al*., 2012). In this category is the natural selection of tolerant plants that can be done by evaluating the effect of inoculation of Arbuscular Mycorrhizal Fungi (AMF), followed by the evaluation of phenol content. AMFs are fungi that live in plant roots through the symbiotic relationship. Numerous studies have shown that these AMFs have positive impacts on plant growth, water pollution control, biosynthesis of phenolic compounds and improved tolerance (Tchameni *et al*., 2012). Among them, *Glomus intraradices* is an endomycorrhizal fungus that stimulates the growth and development of different plant species. Phenolic compounds are involved in various ways in the defense of plants. As pre-infectious compounds whose contents may increase during infection, they participate in constitutive defense mechanisms. Damodaran *et al.* (2009) and Ondobo *et al.* (2014) reported that tolerant clones and cocoa tolerant hybrids contain high levels of phenolic compounds. The establishment of a host-parasite interaction can therefore lead to significant changes in phenolic metabolism involving the activation, regulation, but also the new expression of specific genes in the host, assuming that the relationship between *Glomus intraradices* and the plant will require the absorption of water and nutrients, protect it against various diseases (*P. megakarya*) and stimulate the polyphenoloxidase. This study has as general objective to study within a hybrid family, some effects of *Glomus intraradices* screwed *Phytophthora megakarya* by evaluating the impact of *P. megakarya* infection on leaf discs in the cocoa tree in the absence and presence of *Glomus intraradices*, by determining the phenols content in the hybrid genotype leaves studied under healthy and infected conditions, in the absence and in the presence of *Glomus intraradices*, and finally by identifying within the hybrid family, hybrid genotypes with improved tolerance screwed of this pathogen.

**Materials and methods**

*Plant material*

The plant material used consisted of two clones of *T.* cacao L.: a Trinitario from Trinidad and Tobago (ICS 40) and an Amazonian from Forastero of Ghana (UPA 134) to produce a hybrid family. The offspring was obtained by manual pollination in the genomic library in the field. The experimental material used consisted of 36 hybrid genotypes of Ficup cocoa descendants. The fungal material consisted of a local strain of *Phytophthora megakarya* and a local strain of *Glomus intraradices*.

*Experimental set-up*

Plastic pots, 30 cm high and 12 cm wide, were filled with humified earth obtained from a mixture of dead leaf powder, sawdust and undergrowth soil from the Faculty of Sciences of the University of Douala. Before sowing the beans, the pots were well watered very early in the morning. The beans were sown flat at a depth of 1 cm. The nursery was watered daily for 15 days to trigger germination of the beans. After this period, watering was continued once every two days. Regular manual weeding of the pots was done to eliminate weeds and prevent water and nutrient competition.

*Inoculation of Glomus intraradices (biofertilizers)*

*Glomus intraradices* strains were chosen according to their ability to increase nutrient uptake by their host plants to a greater degree and to increase tolerance to pathogenic infections (Ngonkeu, 2009; Tchameni *et al.*, 2012). The inoculum was composed of a mixture of spores, mycelium, root fragments and coarse sand. Fifty grams of the mixture (containing 50 spores of *Glomus intraradices* were inoculated in the pots 3 to 5 centimeters below the surface of the growing plants). A two-month period after inoculation was necessary for the biofertilizers to colonize the Plant roots.

*Root coloring and evaluation of mycorrhization*

The root staining technique used was that described by Philips and Hayman (1970) with Trypan Blue which allows chitin staining of the mushroom walls. The frequency and intensity of mycorrhization were evaluated according to the formulas used by Marx *et al.*, 1977 and Trouvelot *et al.*, 1986.

Infection of *P. megakarya* on the hybrid genotypes of cocoa before and after AMF Isolation and maintenance of the *Phytophthora megakarya* strain from a pod.

The methods used to evaluate the sensitivity of these genotypes were the same before and after *Glomus intraradices* control. This isolation method and the cultivation of *P. megakarya* were done according to the method of Nyassé *et al.* 1995 and taken over by Coulibaly *et al.*, 2013. From an infected pod, the whitish down corresponding to the spores was scraped off and placed on an agar-pea culture medium containing per liter of solution 65 g of pea, 15 g of agar previously sterilized by autoclaving for 30 minutes at 121°C. After sterilization, the medium was hot-poured into the Petri dishes under the horizontal laminar flow hood and previously disinfected. For subculture of *P. megakarya*, after cooling and solidification of the culture medium at room temperature, the whitish down were deposited in the center of the box containing the fresh culture medium at the rate of one disc per petri dish. On this medium, the thallus had developed in the absence of bacteria. After the thallus had formed, a mycelial fragment was taken from the growth front of the culture and transferred to petri dishes on pea agar medium. Incubation was carried out at 26 °C in the dark for 6-7 days. The virulence of the strain was preserved through weekly transplanting on pea agar medium.

*Production of zoospores*

Ten days before the planned date of inoculation, the strain to be inoculated was transplanted on small agar medium agar plates in Petri dishes (4 to 5 in diameter: 10 cm). The plates were alternately placed for 7 days in the dark and in the light (photoperiod 12h / 12h) at 25 °C in order to obtain the formation of the sporocysts which were at the origin of the production of the zoospores. On the day of artificial infection, 4 to 5 ml of sterile distilled water was added

to each dish. The dishes were then placed in a refrigerator at 4 °C for 30 min. The dishes were then placed at 26 °C for 2 hours so that the zoospores could be released into the water. Then, for each box, the solution containing the zoospores was recovered in a beaker. The zoospores were immobilized with a drop of methylene blue. The zoospore suspension thus obtained in a test tube was counted using a Malassez cell at the concentration of 101 × $10^5$ zoospores / ml. Counting was done on an optical microscope at objective 10 (Fig. 1). 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 infection, incubation and measurement of necrosis diameters*

*Artificial infection of cocoa leaves and incubation in laboratory*

Leaves of approximately two months of age (50 to 60 days) were collected very early in the morning on each hybrid genotype (Nyassé *et al.*, 1995), discs of 1.5 cm in diameter were cut from these leaves and placed at the same time in plastic dishes on toilet paper moistened with water. Covered dishes were incubated overnight at approximately 25 °C. Artificial infections were performed the next morning with 6 µl of *P. megakarya* suspension calibrated at 101 × $10^5$ zoospores / ml previously prepared as previously described and deposited with a micropipette with 3 repetitions.

*Evaluation of the development of necrosis*

Measurement of the size of the necrotic lesion was made at 3, 4, 5, 6 and 7 days after artificial infection. The diameters of the necrotic spots were measured and the area calculated using the formula of Blaha and Lotodé, 1976.

*Artificial infection on whole leaves in a greenhouse*

Leaves approximately two months old attached to the plants were used. Some leaves were scarified (with fine sand) and infected with *P. megakarya* (6 µl of 101×$10^5$ zoospore / ml calibrated suspension) and others served as controls (healthy leaves). The infected area was covered with absorbent cotton and plaster. The plants were watered daily for 7 days to create the moisture necessary for the development of *P. megakarya*. 7 days after artificial infection, these leaves were harvested and stored for 24 hours at 4 °C to facilitate grinding and used for biochemical analyzes.

*Phenolic compounds*

*Extraction of phenolic compounds*

The extraction of the polyphenols was done according to the method of Taquet, 1985 and Simo *et al.*, 2014. Twenty mg of fresh leaf cut to 2 mm of the necrotic part was ground with 2 g of sterile fine sand in 5ml of Methanol 80° then centrifuged at 6000 g for 20 minutes. The supernatant S1 is collected and the pellet put back into 3 ml of methanol 80° and centrifuged at 6000 g for 20 minutes. The supernatant S2 was collected and the pellet 2 removed. The supernatants S1 and S2 constituted the extract for the quantitative analysis of the phenolic compounds.

*Determination of the content of phenolic compounds*

The content of phenolic compounds were determined according to the method of Marigo (1973) which uses the Folin and Ciocalteu reagent (mixture of phosphomolybdic acids). This method was based on the fact that this reagent would be reduced in the presence of phenolic compounds to a blue molybdenum complex. This complex had an absorption maximum at 725 nm.

The reaction mixture was homogenized and incubated in an oven at 40 °C for 20 minutes. After cooling to room temperature, the absorbance of the blue complex formed was read using a spectrophotometer (BK-UV-1600PC) at 725 nm, against a blank in which the extract was replaced by methanol 80°.

The content of phenolic compounds was determined by reference to the calibration curve established with Gallic Acid. The amounts of phenolic compounds were expressed in $mg.g^{-1}$ of fresh weight of leaves.

*Statistical analyzes*

Analysis of variance (ANOVA) of clones and hybrid genotypes were performed by the Duncan test at P > 0.05 with SPSS software version 16.0 for Windows comparing the susceptibility level of hybrid genotypes and the content of phenolic compounds. The data was expressed as means ± SD.

The different groups of hybrid genotypes according to the degree of their tolerance were obtained through the hierarchical classification carried out with the SPAD software version 4.1 for Windows. The analysis map of the tolerance of hybrid genotypes was carried out with the software Mev version 4.9.0 for Windows.

**Results and discussion**

*Foliar disk infection test*

The development of foliar disk necrosis ($cm^2$) was assessed in parental genotypes as well as in hybrid genotypes of Ficup progeny without control of *Glomus intraradices*. Variance analysis indicated that there was a significant difference (P > 0.05) in day and genotype in the development of necrosis. On day 3 after infection, necrosis was observed in parental genotypes and in 94% of hybrid genotypes.

**Table 1.** Development of necrosis ($cm^2$) on leaves of parental genotypes and hybrids of Ficup offspring without control of *Glomus intraradices*.

| Genotypes | Day 3 | day 4 | day 5 | day 6 | day 7 |
|---|---|---|---|---|---|
| ICS40 | 0.01±0$^{a}$ | 0.031±0$^{a}$ | 0.22±0.05$^{ab}$ | 0.51±0.13$^{abc}$ | 1.07±0.10$^{ef}$ |
| UPA134 | 0.02±0.01$^{3a}$ | 0.25±0.05$^{abc}$ | 0.69±0.09$^{bcd}$ | 1.77±0$^{h}$ | 1.77±0$^{h}$ |
| Ficup1 | 0.89±0.09$^{c}$ | 1.01±0.10$^{fghi}$ | 1.20±0.11$^{ef}$ | 1.40±0.12$^{gh}$ | 1.54±0$^{gh}$ |
| Ficup2 | 0.78±0$^{c}$ | 1.14±0.19$^{ghij}$ | 1.47±0.12$^{fg}$ | 1.77±0$^{h}$ | 1.77±0$^{h}$ |
| Ficup3 | 0.15±0.04$^{a}$ | 0.32±0.06$^{abc}$ | 0.46±0.07$^{abcd}$ | 0.46±0.07$^{abc}$ | 0.46±0.07$^{abc}$ |
| Ficup4 | 0.13±0.06$^{a}$ | 0.35±0.1$^{abc}$ | 0.43±0.13$^{abc}$ | 0.50±0$^{abc}$ | 0.55±0.08$^{abc}$ |
| Ficup5 | 0.02±0.03$^{a}$ | 0.03±0$^{a}$ | 0.07±0.11$^{a}$ | 0.16±0.11$^{a}$ | 0.33±0.16$^{ab}$ |
| Ficup6 | 1.19±0.11$^{d}$ | 1.40±0.12$^{ij}$ | 1.77±0$^{g}$ | 1.77±0$^{h}$ | 1.77±0$^{h}$ |
| Ficup7 | 0.17±0.09$^{a}$ | 0.29±0.09$^{abc}$ | 0.43±0.13$^{abc}$ | 0.46±0.07$^{abc}$ | 0.50±0a$^{bc}$ |
| Ficup8 | 0.12±0$^{a}$ | 0.35±0.06$^{abc}$ | 0.64±0$^{abcd}$ | 1.20±0.11$^{fg}$ | 1.20±0.11$^{f}$ |
| Ficup9 | 0.08±0.06$^{a}$ | 0.18±0.09$^{ab}$ | 0.26±0.13$^{ab}$ | 0.35±0.06$^{abc}$ | 0.46±0.07$^{abc}$ |
| Ficup10 | 0.08±0.06$^{a}$ | 0.17±0.04$^{ab}$ | 0.20±0.08$^{ab}$ | 0.35±0.06$^{abc}$ | 0.38±0$^{abc}$ |
| Ficup11 | 0.68±0.08$^{c}$ | 1.01±0.10$^{fghi}$ | 1.20±0.11$^{ef}$ | 1.40±0.12$^{gh}$ | 1.77±0$^{h}$ |
| Ficup12 | 0.78±0$^{c}$ | 1.27±0.3$^{hij}$ | 1.54±0.22$^{fg}$ | 1.61±0.13$^{h}$ | 1.69±0.13$^{h}$ |
| Ficup13 | 0.63±0b$^{c}$ | 0.78±0$^{defg}$ | 0.89±0.09$^{cde}$ | 1.01±0.10$^{ef}$ | 1.40±0.24$^{g}$ |
| Ficup14 | 0.03±0.04$^{a}$ | 0.07±0.06$^{a}$ | 0.10±0.09$^{a}$ | 0.14±0.08$^{a}$ | 0.25±0.05$^{a}$ |
| Ficup15 | 0.02±0.01$^{a}$ | 0.07±0.01$^{a}$ | 0.13±0.02$^{ab}$ | 0.21±0.02$^{ab}$ | 0.47±0.14$^{abc}$ |
| Ficup16 | 0.11±0.07$^{a}$ | 0.11±0.07$^{a}$ | 0.16±0.08$^{ab}$ | 0.26±0.13$^{ab}$ | 0.46±0.07$^{abc}$ |
| Ficup17 | 0.03±0$^{a}$ | 0.06±0$^{a}$ | 0.13±0.06$^{ab}$ | 0.18±0.11$^{a}$ | 0.39±0.11$^{abc}$ |
| Ficup18 | 0.07±0$^{a}$ | 0.13±0.06$^{a}$ | 0.18±0.09$^{ab}$ | 0.35±0.06$^{abc}$ | 0.64±0$^{bcd}$ |
| Ficup19 | 0.01±0$^{a}$ | 0.07±0$^{a}$ | 0.15±0.04$^{ab}$ | 0.23±0.05$^{ab}$ | 0.26±0.11$^{a}$ |
| Ficup20 | 0±0$^{a}$ | 0.11±0.03$^{a}$ | 0.25±0.05$^{ab}$ | 0.25±0.05$^{ab}$ | 0.32±0.06$^{ab}$ |
| Ficup21 | 0.05±0.04$^{a}$ | 0.15±0.04$^{ab}$ | 0.35±0.06$^{ab}$ | 0.47±0.18$^{abc}$ | 0.55±0.14$^{abc}$ |
| Ficup22 | 0.09±0.03$^{a}$ | 0.30±0.15$^{abc}$ | 0.49±0.25$^{abcd}$ | 0.54±0.31$^{abc}$ | 0.86±0.32$^{de}$ |
| Ficup23 | 0.225±0.05$^{a}$ | 0.47±0.18$^{abcd}$ | 0.60±0.20$^{abcd}$ | 0.73±0.09$^{cde}$ | 0.90±0.20$^{de}$ |
| Ficup24 | 0.005±0$^{a}$ | 0.24±0.13$^{ab}$ | 0.33±0.16$^{ab}$ | 0.33±0.16$^{abc}$ | 0.36±0.13$^{abc}$ |

| | | | | | |
|---|---|---|---|---|---|
| Ficup25 | 0±0$^{a}$ | 0.02±0.01$^{a}$ | 0.09±0.03$^{a}$ | 0.20±0$^{ab}$ | 0.32±0.06$^{ab}$ |
| Ficup26 | 0.13±0$^{a}$ | 0.39±0.11$^{abc}$ | 0.59±0.08$^{abcd}$ | 0.64±0.14$^{bcd}$ | 0.64±0.14$^{bcd}$ |
| Ficup27 | 0.63±0.04$^{bc}$ | 1.20±0.11$^{hij}$ | 1.539±0$^{fg}$ | 1.54±0$^{h}$ | 1.766±0$^{h}$ |
| Ficup28 | 0.15±0.04$^{a}$ | 0.35±0.06$^{abc}$ | 0.502±0$^{abcd}$ | 0.50±0$^{abc}$ | 0.64±0.14$^{bcd}$ |
| Ficup29 | 0.008±0$^{a}$ | 0.04±0.02$^{a}$ | 0.13±0.06$^{ab}$ | 0.20±0$^{ab}$ | 0.39±0.11$^{abc}$ |
| Ficup30 | 0.09±0$^{a}$ | 0.26±0.12$^{abc}$ | 0.32±0.22$^{ab}$ | 0.49±0.25$^{abc}$ | 0.69±0.16$^{cd}$ |
| Ficup31 | 0.32±0.22$^{a}$ | 0.69±0.16$^{cdef}$ | 0.95±0.17$^{de}$ | 0.95±0.17$^{def}$ | 1.01±0.20$^{ef}$ |
| Ficup32 | 1.36±0.51$^{d}$ | 1.49±0.47$^{j}$ | 1.55±0.37$^{fg}$ | 1.55±0.37$^{h}$ | 1.77±0$^{h}$ |
| Ficup33 | 0.86±0.32$^{c}$ | 1.12±0.55$^{ghij}$ | 1.34±0.73$^{efg}$ | 1.62±0.25$^{h}$ | 1.69±0.13$^{h}$ |
| Ficup34 | 0.38±0$^{ab}$ | 0.89±0.09$^{efgh}$ | 1.26±0.11$^{ef}$ | 1.54±0$^{h}$ | 1.77±0$^{h}$ |
| Ficup35 | 0.18±0.09$^{a}$ | 0.60±0.20$^{bcde}$ | 0.92±0.36$^{cde}$ | 0.92±0.36$^{def}$ | 1.01±0.20$^{ef}$ |
| Ficup36 | 0.07±0$^{a}$ | 0.16±0.11$^{ab}$ | 0.33±0.16$^{ab}$ | 0.39±0.11$^{abc}$ | 0.42±0.07$^{abc}$ |

CS 40 = Imperial College Selection, UPA 134 = Upper Amazon, Ficup = ICS Family 40 x UPA 134. Values with the same letter on the same column are not significantly different at P>0.05.

On this day, a significant difference was been observed in some hybrid genotypes. Both parental genotypes and hybrid genotypes had small necrotic surfaces except for the Ficup 6 and Ficup 32 hybrid genotypes which showed the highest necrotic surfaces. From day 4 after infection, necrosis was observed in all hybrid genotypes followed by progressive evolution until day 7.

The appearance of necrosis in the center of infected leaf disks in the laboratory and on the leaves attached to the nursery was due to the presence of *P. megakarya* zoospores.

These results are similar to those obtained by Djocgoue *et al.*, 2007 and Ondobo *et al.*, 2014 who showed that the evolution of black pod disease was due to the presence of this pathogen on the plant.

A significant difference (P > 0.05) was observed between the two parents and their genotypes hybrids over time. On day 7, the parental genotype ICS 40 showed the lowest necrotic area compared to the parent UPA 134.

**Table 2.** Development of necrosis (cm²) on the leaves of hybrids of Ficup progeny under control of *Glomus intraradices*.

| Genotypes | day 3 | day 4 | day 5 | day 6 | day 7 |
|---|---|---|---|---|---|
| Ficup1 | 0±0$^{a}$ | 0.005±0$^{ab}$ | 0.02±0$^{ab}$ | 0.05±0$^{abc}$ | 0.18±0.01$^{abcd}$ |
| Ficup2 | 0.07±0.06$^{a}$ | 0.10±0.08$^{bcde}$ | 0.15±0.04$^{bcdefg}$ | 0.29±0.09$^{efghi}$ | 0.51±0.12$^{defgh}$ |
| Ficup3 | 0.02±0.01$^{a}$ | 0.04±0$^{abcd}$ | 0.09±0.03$^{abcde}$ | 0.18±0.09$^{abcdefg}$ | 0.47±0.14$^{cdefgh}$ |
| Ficup4 | 0±0$^{a}$ | 0.01±0$^{ab}$ | 0.03±0$^{ab}$ | 0.13±0.06$^{abcde}$ | 0.39±0.11$^{bcdefgh}$ |
| Ficup5 | 0.008±0$^{a}$ | 0.04±0.02$^{abcde}$ | 0.07±0$^{abcd}$ | 0.13±0$^{abcde}$ | 0.35±0.06$^{abcdefg}$ |
| Ficup6 | 0.005±0$^{a}$ | 0.005±0$^{ab}$ | 0.005±0$^{a}$ | 0.07±0.06$^{abc}$ | 0.22±0.16$^{abcde}$ |
| Ficup7 | 0.03±0$^{a}$ | 0.07±0$^{abcde}$ | 0.07±0$^{abcd}$ | 0.15±0.04$^{abcdef}$ | 0.42±0.07$^{cdefgh}$ |
| Ficup8 | 0.008±0$^{a}$ | 0.03±0$^{abc}$ | 0.04±0.02$^{abc}$ | 0.13±0.06$^{abcde}$ | 0.32±0.11$^{abcdefg}$ |
| Ficup9 | 0.071±0$^{ab}$ | 0.071±0$^{abcde}$ | 0.071±0$^{abcd}$ | 0.13±0.06$^{abcde}$ | 0.29±0.09$^{abcdef}$ |
| Ficup10 | 0.13±0$^{bc}$ | 0.20±0$^{f}$ | 0.20±0$^{defg}$ | 0.32±0.06$^{fghi}$ | 0.64±0.14$^{ghi}$ |
| Ficup11 | 0.02±0.01$^{a}$ | 0.07±0$^{abcde}$ | 0.07±0$^{abcd}$ | 0.20±0.08$^{bcdefg}$ | 0.42±0.07$^{cdefgh}$ |
| Ficup12 | 0.03±0$^{a}$ | 0.04±0.03$^{abcd}$ | 0.15±0.04$^{bcdefg}$ | 0.22±0.05$^{cdefgh}$ | 0.42±0.07$^{cdefgh}$ |

| | | | | | |
|---|---|---|---|---|---|
| Ficup13 | 0.02±0.01$^{a}$ | 0.05±0.04$^{abcde}$ | 0.05±0.04$^{abc}$ | 0.32±0.06$^{fghi}$ | 0.55±0.08$^{efgh}$ |
| Ficup14 | 0.03±0$^{a}$ | 0.09±0.03$^{abcde}$ | 0.09±0.03$^{abcde}$ | 0.13±0$^{abcde}$ | 0.32±0.06$^{abcdefg}$ |
| Ficup15 | 0±0$^{a}$ | 0±0$^{a}$ | 0±0$^{a}$ | 0.01±0$^{a}$ | 0.07±0$^{a}$ |
| Ficup16 | 0.008±0$^{a}$ | 0.03±0$^{abc}$ | 0.03±0$^{ab}$ | 0.07±0$^{abc}$ | 0.29±0.09$^{abcdef}$ |
| Ficup17 | 0±0$^{a}$ | 0.01±0$^{ab}$ | 0.02±0$^{ab}$ | 0.05±0.04$^{abc}$ | 0.14±0.12$^{abc}$ |
| Ficup18 | 0±0$^{a}$ | 0.02±0.01$^{abc}$ | 0.08±0.05$^{abcd}$ | 0.35±0.06$^{ghi}$ | 0.59±0.08$^{ghi}$ |
| Ficup19 | 0.02±0.01$^{a}$ | 0.06±0.02$^{abcde}$ | 0.13±0$^{abcdef}$ | 0.42±0.07$^{ij}$ | 0.69±0.08$^{hi}$ |
| Ficup20 | 0.02±0.01$^{a}$ | 0.07±0$^{abcde}$ | 0.17±0.04$^{cdefg}$ | 0.25±0.05$^{defghi}$ | 0.46±0.07$^{cdefgh}$ |
| Ficup21 | 0.04±0.03$^{a}$ | 0.09±0.03$^{abcde}$ | 0.17±0.04$^{cdefg}$ | 0.29±0.09$^{efghi}$ | 0.51±0.12$^{defgh}$ |
| Ficup22 | 0±0$^{a}$ | 0.003±0$^{a}$ | 0.01±0$^{a}$ | 0.04±0.03$^{ab}$ | 0.15±0.07$^{abc}$ |
| Ficup23 | 0.07±0$^{ab}$ | 0.07±0$^{abcde}$ | 0.11±0.03$^{abcdef}$ | 0.22±0.05$^{cdefgh}$ | 0.59±0.08$^{fghi}$ |
| Ficup24 | 0.07±0$^{ab}$ | 0.13±0$^{de}$ | 0.20±0$^{defg}$ | 0.22±0.05$^{cdefgh}$ | 0.42±0.07$^{cdefgh}$ |
| Ficup25 | 0.008±0$^{a}$ | 0.03±0$^{abc}$ | 0.07±0$^{abcd}$ | 0.13±0$^{abcde}$ | 0.38±0$^{bcdefgh}$ |
| Ficup26 | 0.04±0.03$^{a}$ | 0.05±0.03$^{abcde}$ | 0.09±0.05$^{abcde}$ | 0.18±0.11$^{abcdefg}$ | 0.43±0.18$^{cdefgh}$ |
| Ficup27 | 0.07±0$^{ab}$ | 0.11±0.03$^{cde}$ | 0.17±0.04$^{cdefg}$ | 0.25±0.05$^{defghi}$ | 0.51±0.12$^{defgh}$ |
| Ficup28 | 0.06±0.05$^{a}$ | 0.13±0.06$^{e}$ | 0.20±0.08$^{efg}$ | 0.29±0.09$^{efghi}$ | 0.51±0.12$^{defgh}$ |
| Ficup29 | 0.003±0$^{a}$ | 0.02±0.01$^{abc}$ | 0.05±0.03$^{abc}$ | 0.09±0.05$^{abcd}$ | 0.32±0.11$^{abcdefg}$ |
| Ficup30 | 0±0$^{a}$ | 0±0$^{a}$ | 0±0$^{a}$ | 0.01±0$^{a}$ | 0.08±0.05$^{a}$ |
| Ficup31 | 0.07±0$^{ab}$ | 0.13±0$^{de}$ | 0.23±0.05$^{fg}$ | 0.38±0$^{hi}$ | 0.69±0.08$^{hi}$ |
| Ficup32 | 0.13±0.06$^{c}$ | 0.20±0.08$^{f}$ | 0.43±0.18$^{h}$ | 0.51±0.12$^{j}$ | 0.85±0.25$^{i}$ |
| Ficup33 | 0.04±0.02$^{a}$ | 0.06±0.02$^{abcde}$ | 0.13±0$^{abcdef}$ | 0.29±0.09$^{efghi}$ | 0.51±0.12$^{defgh}$ |
| Ficup34 | 0.13±0.06$^{c}$ | 0.20±0.08$^{f}$ | 0.25±0.05$^{g}$ | 0.42±0.07$^{ij}$ | 0.64±0.14$^{ghi}$ |
| Ficup35 | 0±0$^{a}$ | 0±0$^{a}$ | 0.013±0$^{a}$ | 0.04±0$^{ab}$ | 0.20±0.08$^{abcd}$ |
| Ficup36 | 0±0$^{a}$ | 0±0$^{a}$ | 0±0$^{a}$ | 0.01±0$^{a}$ | 0.09±0.01$^{ab}$ |

CS 40 = Imperial College Selection, UPA 134 = Upper Amazon, Ficup = ICS Family 40 x UPA 134. Values with the same letter on the same column are not significantly different at P>0.05.

It is therefore, considered the best parent. In addition, 69 % of the hybrid genotypes showed a necrotic surface area lower than that of the best parent (Ficup 36, Ficup 3, Ficup 18, Ficup 19, Ficup 5, Ficup 14, Ficup 15, Ficup 16, Ficup 23, Ficup 24, Ficup 25, Ficup 29, Ficup 10, Ficup 17, Ficup 20 and Ficup 9) The hybrid genotypes Ficup 6 and Ficup 32 showed large necrotic surfaces (Table 1). These results corroborate those obtained by Djocgoue *et al.*, 2010; Manga *et al.*, 2016 and Effa *et al.*, 2017 on detached pods. This confirms the presence of an additive trait in tolerance to cocoa brown rot (Pokou *et al.*, 2008). Some authors found similar results showing that tolerance to brown pod rot was under genetic control and could be improved; explaining that the presence of susceptible and resistant genotypes in the same hybrid family confirms an excellent combination of parental genes (Cilas *et al.*, 2004; Manga *et al.*, 2016; Effa *et al.*, 2017; Minyaka *et al.*, 2017; Ondobo *et al.*, 2017).

The development of leaf disc necrosis was also evaluated in hybrid genotypes controlled by *Glomus intraradices*. Analysis of variance (ANOVA) indicated that there was a significant difference (P > 0.05) depending on the day and hybrid genotype in the development of necrosis. On day 3 after infection, necrosis was observed in parental genotypes and in 75 % hybrid genotypes. There was no significant difference (P > 0.05) between hybrid genotypes with low necrotic surface area except the Ficup 32, Ficup 34, and Ficup10 hybrid genotypes, which showed the highest necrotic area at this date with respective values of 0.13 ± 0.06, 0.13 ± 0.06 and 0.13 ± 0. From

the 4th day after infection, necrosis was observed in 88 % of the hybrid genotypes tested with very weak necrotic surfaces and in 91 % of the hybrid genotypes on day 5 with an evolution increasing until day 7. 64 % of hybrid genotypes showed necrotic surfaces less than 0.5 cm² which means they are very tolerant. It was found that only the hybrid genotypes Ficup 2, Ficup 10, Ficup 13, Ficup 18, Ficup 19, Ficup 21, Ficup 23, Ficup 27, Ficup 28, Ficup 31, Ficup32, Ficup 33 and Ficup 34 showed necrotic surfaces greater than 0.5 cm² and more, none of the hybrid genotypes reached a necrosis area of 1 cm² (Table 2). Under control of *Glomus intraradices*, artificial infection was performed in a greenhouse.

In this treatment condition, the values of leaf sensitivity of hybrid genotypes in the presence of *G. intraradices* were less than 1.

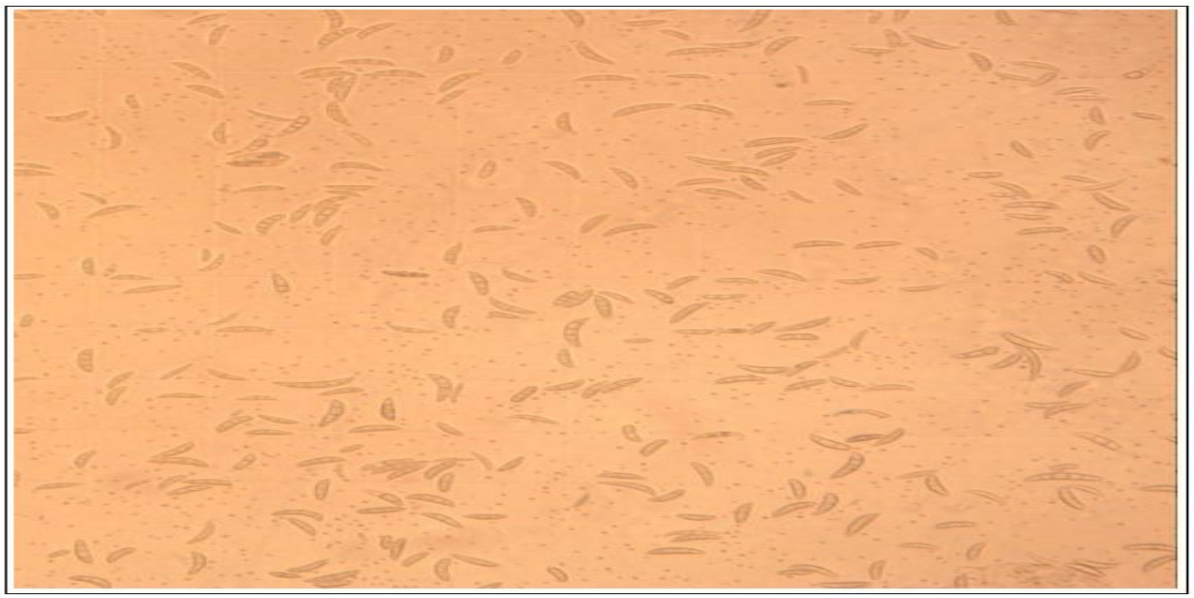

**Fig. 1.** Zoospores of *P. megakarya* observed under an optical microscope at objective 10.

The low average susceptibility values of *P. megakarya* leaves compared to the control (without *G. intraradices*) reflects the inhibitory action of *G. intraradices*, which significantly reduced the frequency of necrotic lesions due to *P. megakarya*. Similar results were obtained by Bowers *et al.* (2001) on Trichoderma which showed in a study that the application of *Trichoderma* sp on leaf discs allowed the reduction of foliar sensitivity to *P. megakarya*. This reducing action resulted from the germination of *G. intraradices* spores on the leaf area, which probably inhibited or interfered with the germination of *P. megakarya* zoospores. This germination ability of *G. intraradices* spores would stimulate the defense mechanisms, thus enhancing resistance to parasite penetration and spread. These results corroborate the work of Harman *et al.* (2004) in beans, cotton, chili and corn. A significant difference in date effects and hybrid genotypes of cocoa for *P. megakarya* tolerance was also observed.

On day 7, 64 % of hybrid genotypes showed necrotic surfaces less than 0.5 cm² and none of these hybrid genotypes had a necrotic surface equal to 1 cm² suggesting a strong tolerance towards the pathogen. These results show that the mycorrhizal fungus (*Glomus intraradices*) had significantly improved the tolerance of these hybrid genotypes to *P. megakarya*. These results are in agreement with those obtained by Tchameni *et al.* (2012) and Nana *et al.* (2016) who showed that leaf sensitivity of young *T. cacao* plants decreased with inoculation of biofertilizers of the genus *Glomus intraradices* and *Gigaspora margarita*. Tchameni *et al.* (2017) in their work found that disease symptoms on *T. cacao* leaves were significantly reduced ($P > 0.05$) in seedlings treated with *Trichoderma* and *Asperellum* isolates. This increase in tolerance can be explained by the fact that

these AMFs are able of producing elicitors that can stimulate the increase to hydrogen peroxides and peroxidases which contribute to the hypersensitivity reaction and to the reinforcement of the wall.

*Classification of hybrid genotypes in the absence of Glomus intraradices*

The necrotic surfaces of the days mentioned above made it possible to hierarchically classify the parental genotypes and the hybrid genotypes of the Ficup offspring. This hierarchical classification was obtained based on the leaf necrosis surface from day 3 to day 7 in the absence of *Glomus intraradices*.

Parental genotypes and hybrid genotypes were 95 % homogeneous. This hierarchical classification made it possible to distinguish 13 groups of individuals. Groups 2, 9 and 10 consisting of 3 hybrid genotypes each were characterized by very large necrotic surfaces and therefore considered very sensitive.

Group 8 consisted of 3 hybrid genotypes were characterized by medium necrotic surfaces and therefore considered sensitive.

Groups 3, 7 and 11 consisted of 3 hybrid genotypes each characterized by small necrotic surfaces and therefore considered tolerant. Group 6 consisting of 2 hybrid genotypes and groups 1, 4, 5, 12 and 13 each consisted of 3 hybrid genotypes were characterized by very small necrotic surfaces and therefore considered very tolerant (Fig. 2).

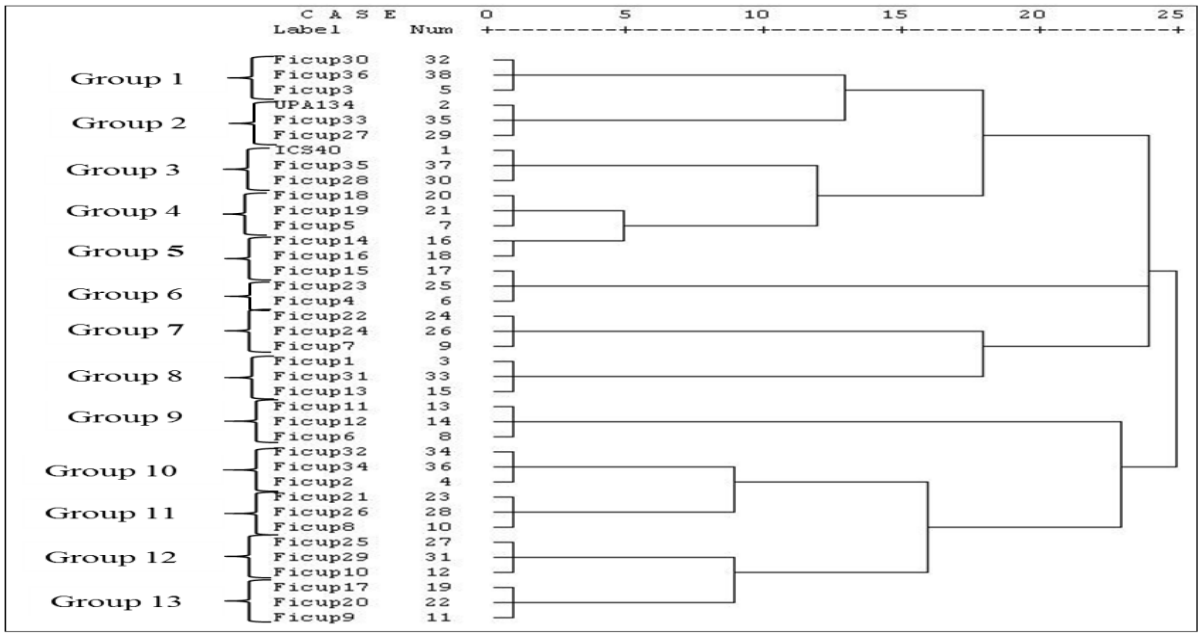


**Fig. 2.** Hierarchical classification of Ficup progeny hybrids obtained on the basis of leaf necrosis surface from day 3 to day 6 without control of *Glomus intraradices*.

These results are similar to those obtained by Simo *et al.*, 2014 on cocoa pod in the same plant.

*Frequency and intensity of mycorrhization*

The evaluation of the frequency of mycorrhization showed a rate greater than 70 % in all the hybrid genotypes studied. This rate shows a symbiotic association between *Glomus intraradices* and *Theobroma cacao*. However, no significant difference ($P > 0.05$) was noted between the hybrid genotypes (Fig. 3A). Evaluation of mycorrhizal intensity showed less than 50 % mycorrhization in all hybrid genotypes tested.

There was also no significant difference ($P > 0.05$) in all hybrid genotypes (Fig.3B). These results are similar to those obtained by Nzeweundji *et al.*, 2015 on the diversity of arbuscular mycorrhizal fungi associated with *prunus africana* where the frequency of mycorrhization ranged between 80 and 91 %.

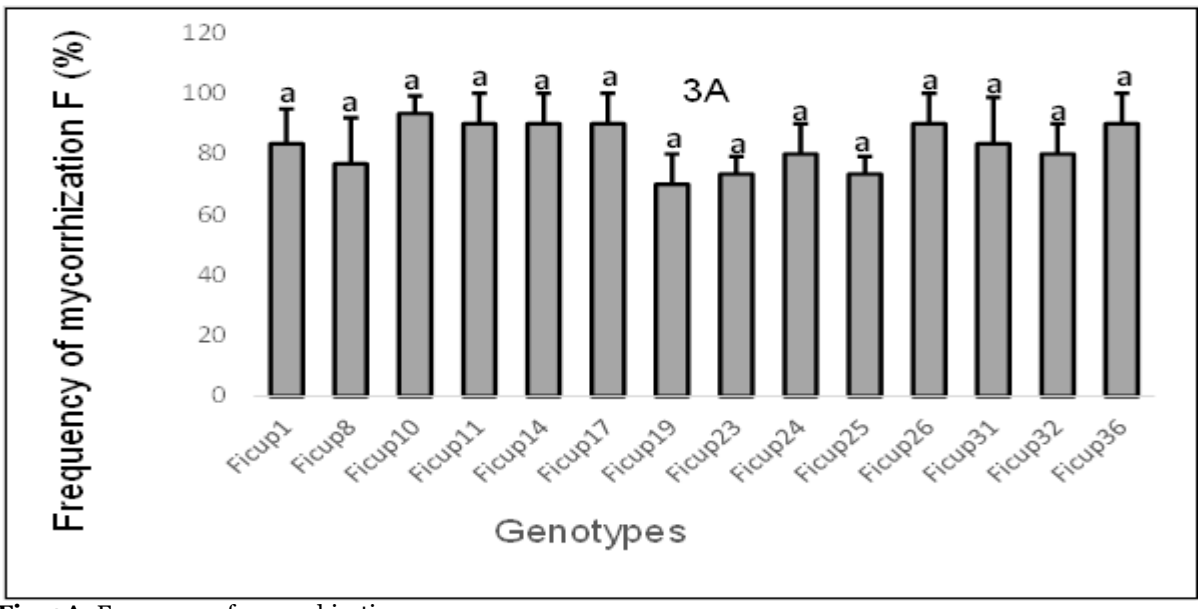


**Fig. 3A.** Frequency of mycorrhization

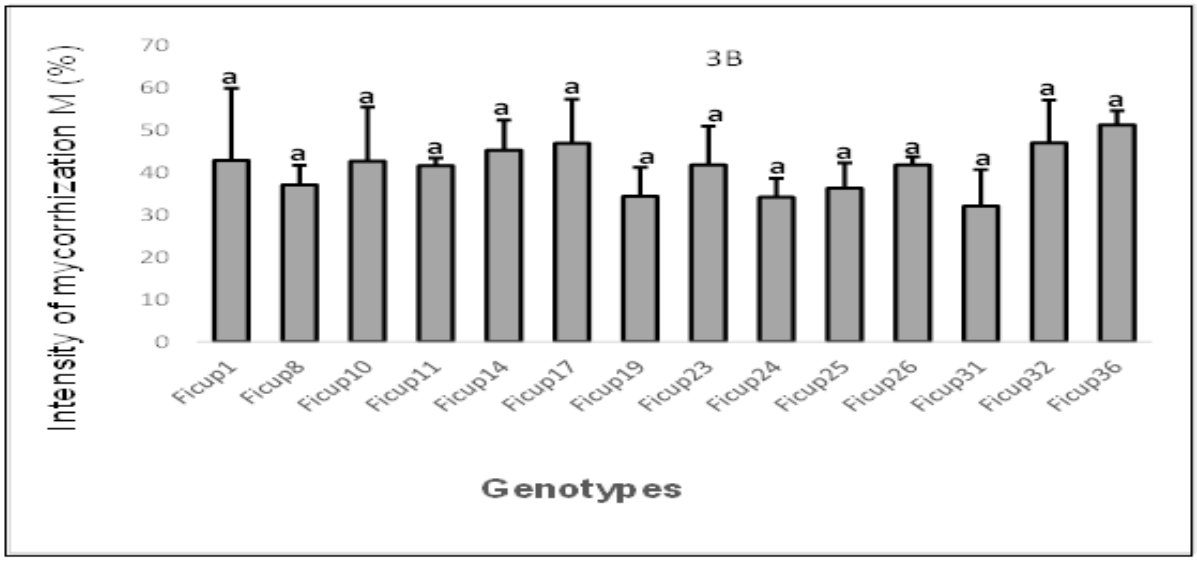


**Fig. 3B.** Intensity of mycorrhization.

On the other hand, mycorrhization intensity results showed a low frequency of mycorrhization that is less than 50 % in all hybrid genotypes studied. These results are in agreement with those obtained by Ajeesh *et al.*, 2015 who showed that the fertility level of the substrate, soil fertility, would inhibit mycorrhizal infection.

*Analysis map of the necrosis in the absence and presence of Glomus intraradices*

The tolerance analysis map based on the necrosis surface of the cocoa leaf disks of the parental genotypes and those of the Ficup progeny from day 3 to day 7 without control of *Glomus intraradices* made it possible to establish a classification of hybrid genotypes in order of increasing tolerance (Fig. 6) with respectively the hybrid genotypes mentioned above. This map showed a progressive decrease of the black color, sign of the tolerance and a progressive appearance of the red color, sign of the sensitivity in certain hybrid genotypes of the day 3 to the day 7.

Thus, high tolerance was observed in hybrid genotypes of group 1, 4, 5, 6, 12 and greater sensitivity in hybrid genotypes of group 2, 9 and 13 at these different dates (Fig. 4).

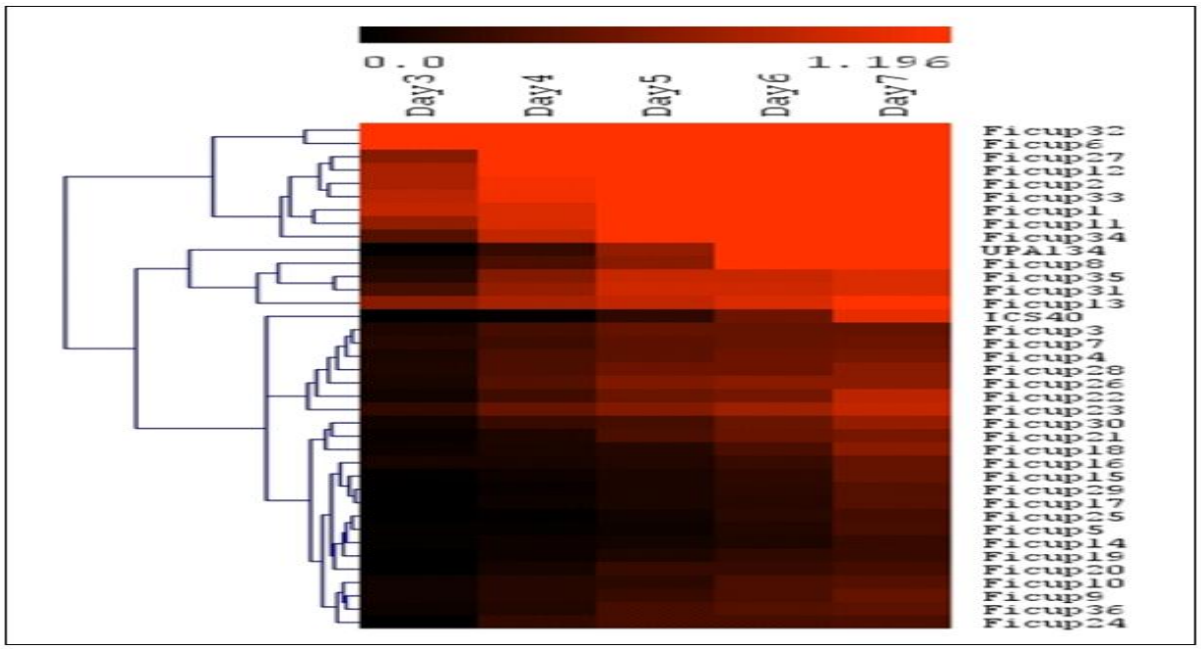


**Fig. 4.** Tolerance analysis map based on cocoa leaf necrosis surface of parental genotypes and Ficup offspring from day 3 to day7 without control of *Glomus intraradices*.

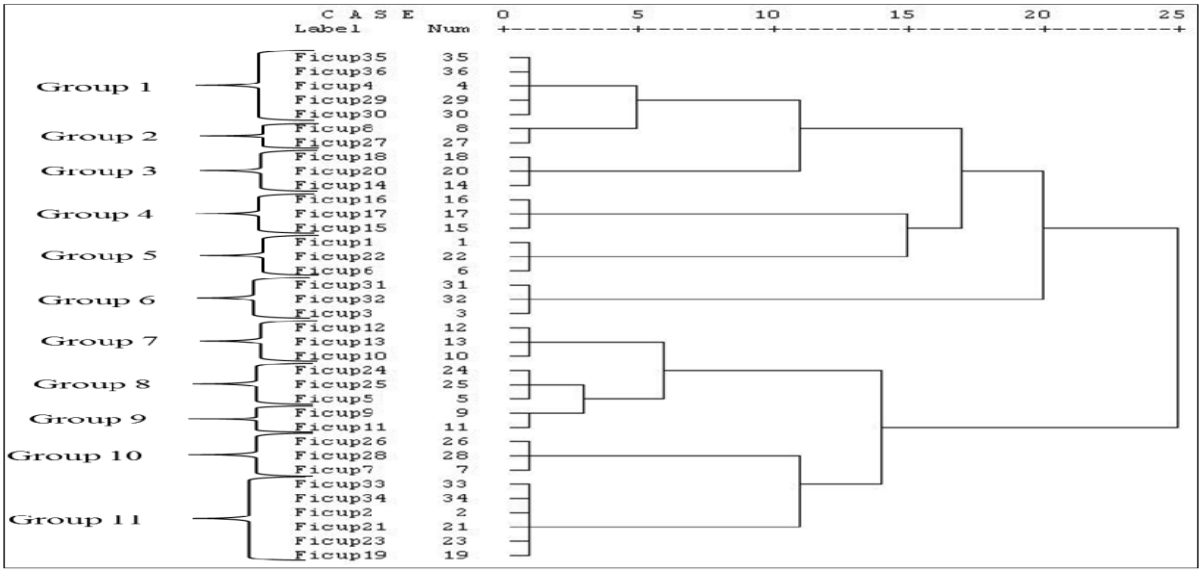


**Fig. 5.** Hierarchical classification of Ficup progeny hybrids obtained on the basis of leaf necrosis surface from day 3 to day 7 under control of *Glomus intraradices*.

In addition, Fig. 7 shows the hierarchical classification of hybrid genotypes of Ficup offspring also obtained based on the necrosis surface of leaf discs from day 3 to day 7 under control of *Glomus intraradices*.

This classification made it possible to distinguish 11 groups. Group 6 consisting of 3 hybrid genotypes are characterized by medium necrotic surfaces and therefore considered sensitive. Groups 2, 3, 7 and 11 consisting of 2, 3, 3 and 6 hybrid genotypes each as well as groups 8, 9 and 10 also consisting of 3, 2 and 3 hybrid genotypes each are characterized by small necrotic surfaces and therefore considered tolerant. Groups 1, 4 and 5 consisting of 5, 3 and 3 hybrid genotypes each are characterized by very small necrotic surfaces and therefore considered very tolerant (Fig. 5). Moreover, the Tolerance Analysis map, also based on the necrosis surface of cocoa leaf disks of parental genotypes and hybrid genotypes of

Ficup progeny on the days mentioned above under the control of *Glomus intraradices*, allowed to establish a classification of hybrid genotypes in the order of increasing tolerance (Fig. 8) with respectively the hybrid genotypes mentioned above. This map shows a progressive decrease of the black color, sign of the tolerance and a progressive appearance of the red color, sign of the sensitivity in certain hybrid genotypes of the day 3 to the day 7. For this, a great tolerance was observed in group 1, 4 and 5 genotypes on day 3 that progresses to day 5 and a mean tolerance in group 6 genotypes on day 6 and 7 (Fig. 6). Furthermore, under control of *Glomus intraradices*, the black coloration was more accentuated, a sign of the improvement of tolerance.

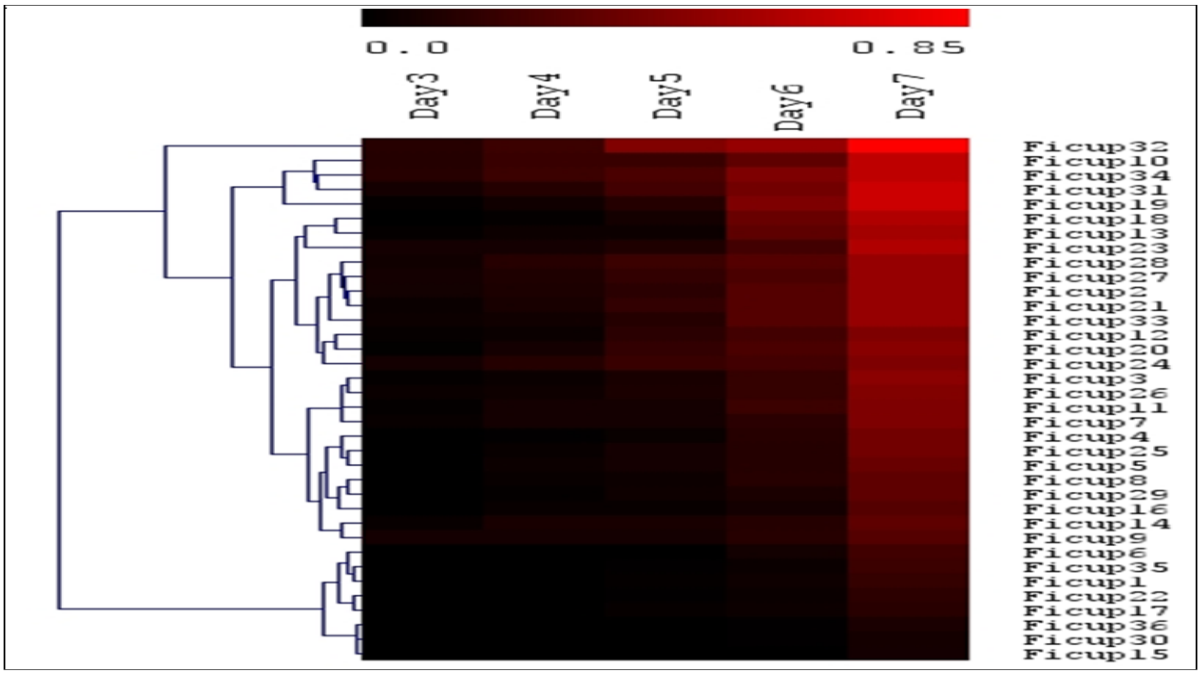


**Fig. 6.** Tolerance analysis map based on cocoa leaf necrosis surface of parental genotypes and hybrid genotypes of Ficup progeny from day 3 to day 7 under control of *Glomus intraradices*.

*Determination of the content of phenolic compounds*

The content of phenolic compounds was determined in the parents ICS 40, UPA 134 and the hybrid genotypes Ficup 11, Ficup, 14 Ficup 17, Ficup 19, Ficup 23, Ficup 24, Ficup 25, Ficup 26, Ficup 31, Ficup 32 and Ficup 36.

The biochemical analyses consisted of the measurement of the polyphenol contents of the semi-augured leaves (in triplicate) on 13 groups representing the 13 plants (genotypes) of *T.* cacao in healthy and infected conditions without control of *Glomus intraradices* obtained in vitro. (Fig. 7A).

In a healthy condition, although the phenol content was higher in the parent ICS 40 (223 ± 0.570 mg.g$^{-1}$ of FW) compared to the parent UPA 134 (212 ± 0 mg.g$^{-1}$ of FW), there was no significant difference ($P > 0.05$). A significant difference in the content of phenolic compounds was observed within the different hybrid genotypes and between the two treatments.

In these hybrid genotypes in healthy condition, the phenol content was higher in the genotype Ficup 36 (306 ± 43.20 mg.g$^{-1}$ of FW), Ficup 26 (303 ± 15.58 mg.g$^{-1}$ of FW) followed by hybrid genotypes Ficup 24 (273.33 ± 10.06 mg.g$^{-1}$ of FW), Ficup 25 (231.67 ± 24.82 mg.g$^{-1}$ of FW) and Ficup 23 (225 ± 10.39 mg.g$^{-1}$ of FW).

In infected condition, there was an increase in the content of phenolic compounds in parental genotypes and in hybrid genotypes.

These increases were greater in the parental genotype ICS 40 (9.30 %) and in the hybrid genotypes Ficup 24 (62.46 %), Ficup 19 (57.28 %), Ficup 14 (48.04 %) and Ficup (24.83 %) (Fig. 7B). These results are similar to the work of Iwaro *et al.*, 2001, who evaluated the relationship between phenolic compounds and *P. megakarya* resistance in cocoa hybrids found that highly productive and tolerant genotypes were high in phenolic compounds while the less tolerant and productive had a low content.

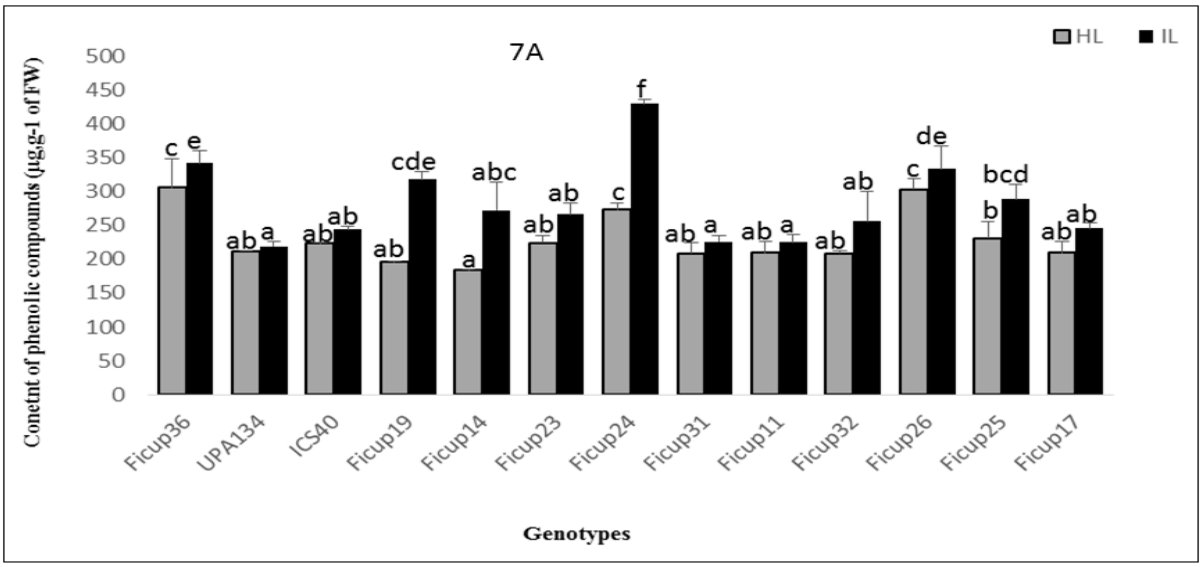


**Fig. 7A.** Contents of phenolic compounds in the leaves of UPA134 and ICS40 clones and hybrid genotypes of the Ficup family under different conditions of non-control *Glomus intraradices* treatment.

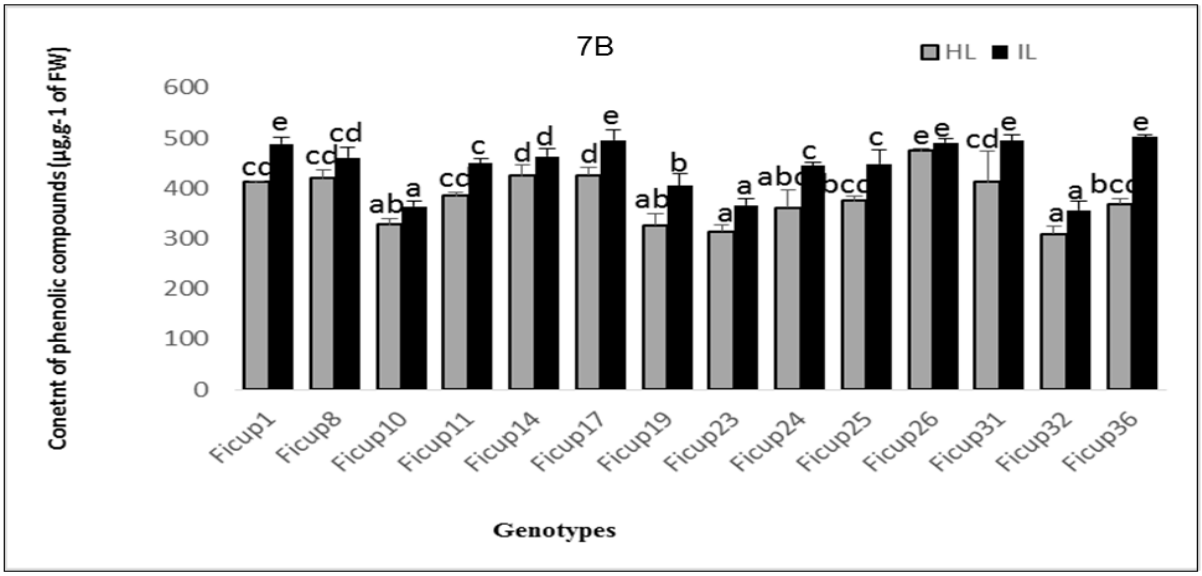


**Fig. 7B.** Phenolic compound content in leaves of hybrid genotypes of the Ficup family under different treatment conditions and under control of *Glomus intraradices*.

Under the control of *Glomus intraradices*, the content of phenolic compounds in the leaves of the hybrid genotypes was also evaluated at different treatment conditions. This content was evaluated in the semi-augured leaves (in triplicate) in 14 groups representing the 14 plants (genotypes) of *T. cacao* under healthy and infected conditions under control of *Glomus intraradices*.

A significant difference in the content of phenolic compounds was also observed within the different hybrid genotypes and between different treatments.

In these hybrid genotypes in healthy condition, the phenol content was lower in the genotype Ficup 32 (309.82 ± 14.18 mg.g$^{-1}$ of FW), Ficup 23 (314.10 ± 12.45 mg.g$^{-1}$ of FW), Ficup 19 (326.14 ± 23.01 mg.g$^{-1}$ of FW) and Ficup 10 (328.47 ± 12.14 mg.g$^{-1}$ of FW).

In infection condition, there was also an increase in the content of phenolic compounds in the hybrid genotypes. These increases were greater in hybrid genotypes Ficup 1 (18.35 %), Ficup 11 (16.79 %), Ficup 19 (24 %), Ficup 24 (22.82 %) Ficup 25 (19.45 %), Ficup 31 (19.88 %) and Ficup 36 (36.03 %) in infected condition (Fig.7B).

However, it was found that the AMF *Glomus intraradices* resulted in a greater increase in phenolic content in all hybrid genotypes at different treatment conditions compared to the one obtained in absence of *Glomus intraradices*.

These increases are due to the activation of phenylalanine ammonia lyases (PAL) by the elicitors produced by *P. megakarya*.

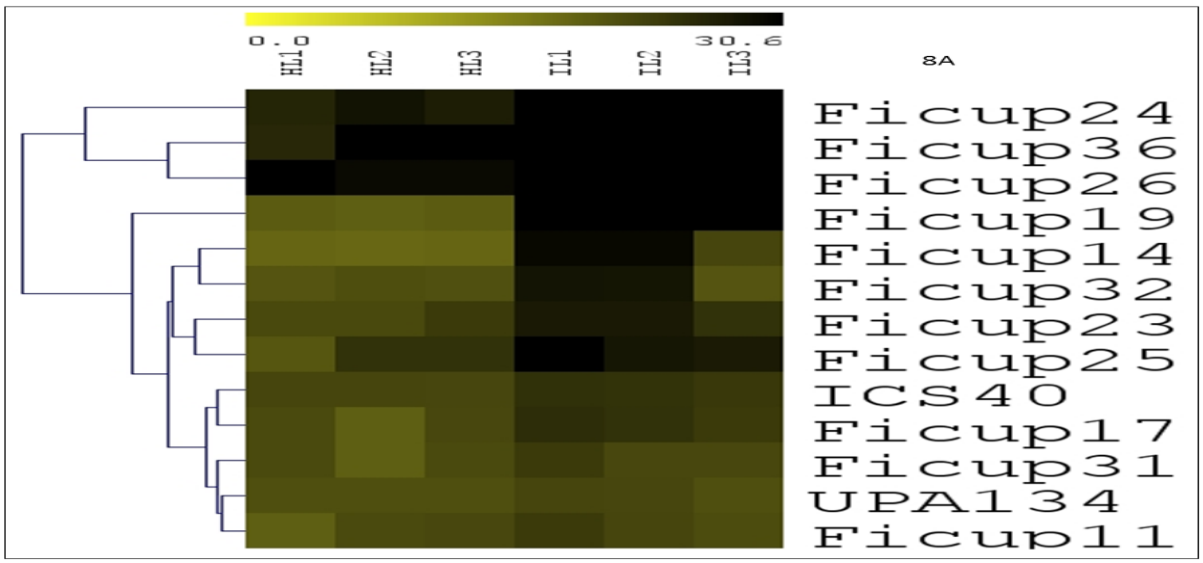


**Fig. 8A.** Analysis map of the content of phenolic compounds of healthy and infected cocoa leaves of parental genotypes and Ficup progeny without control of *Glomus intraradices*.

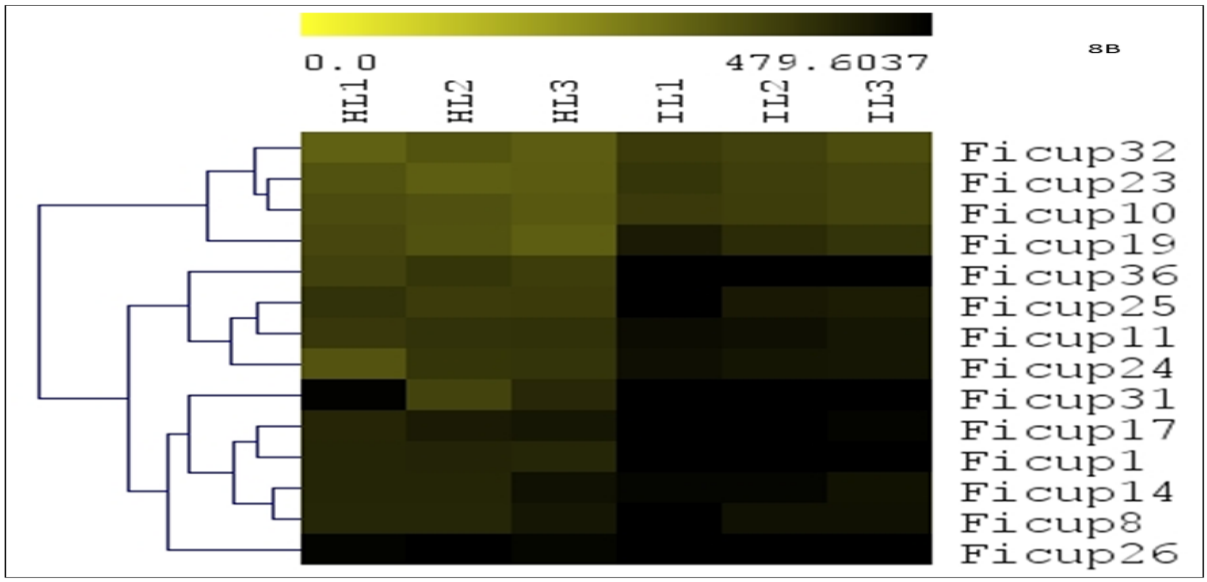


**Fig. 8B.** Analysis map of the content of phenolic compounds of healthy and infected cocoa leaves of Ficup progeny under control of *Glomus intraradices*.

These results are in agreement to those obtained by Constabel *et al.*, 2000 and Housti *et al.*, 2002 who showed in their studies that the increase of the content of phenolic compounds in *P. megakarya* infection condition was greater in the tolerant parent and in all hybrid genotypes except sensitive genotypes that accumulated more low levels compared to tolerant ones.

Furthermore, the results obtained after 60 days of inoculation with the AMF (*Glomus intraradices*), show that this AMF had undoubtedly favored the improvement of the tolerance of hybrid genotypes and the synthesis of phenolic compounds in *T. cacao*. These results are consistent with the work of Nana *et al.* (2002); Al-Askar *et al.* (2010) and Lu *et al.* (2015) who reported that inoculation of plants by AMF favored phenol biosynthesis in cowpea (*Vigna unguiculata*), common bean (*Phaseolus vulgaris L.*) and yam (*Dioscorea* spp.) respectively. AMF *G. intraradices* allowed susceptible hybrid genotypes to be more tolerant to the *P. megakarya* agent.

These results confirm the idea that the biosynthesis of phenolic compounds in cocoa plants (*T. cacao*) can be stimulated by several factors, in particular the AMF as indicated previously (Tchameni *et al.*, 2011). The increase in phenol content in cocoa leaves infected by *P. megakarya* may be explained by the fact that root colonization and root inoculation by *G. intraradices* may favor the production of elicitors which may stimulate activity of phenylalanine ammonia lyases (PAL), which are the enzymes most involved in phenolic metabolism.

*Analysis map of phenolic compounds*

The analysis map of the content of phenolic compounds of healthy and infected cocoa leaves of parental genotypes and hybrid genotypes of Ficup offspring was performed in the absence of *Glomus intraradices* collected on days 7, 10 and 14 after infection.

This map showed no sense of color variation in healthy condition over days in the hybrid genotypes tested. On the other hand, there was a gradual decrease in black color, a sign of a decrease in tolerance in favor of the appearance of the yellow color, a sign of the sensitivity over the days after infection in certain hybrid genotypes with the exception of hybrid genotypes Ficup 24, Ficup 36, Ficup 26, Ficup 19 where no noticeable decrease in black color, a sign of tolerance, is observed (Fig. 8A). The analysis map of the content of phenolic compound of healthy and infected cocoa leaves of parental genotypes and those of Ficup offspring was also performed in the presence of *Glomus intraradices*.

It was noted that the Ficup 32, Ficup 23, Ficup 10, Ficup 19, Ficup 24 and Ficup 11 genotypes had a more significant decrease in healthy and infected conditions on days 7, 10 and 14, which changed considerably over time and a higher content in hybrid genotypes Ficup 36, Ficup 17, Ficup 26, Ficup 1 and Ficup 31 in infected condition over time. This increase of phenolic content in these hybrid genotypes was significantly different ($p < 0.05$) in different conditions (Fig. 8B).

**Conclusion**

This work was carried out in order to evaluate the impact of the infection of *P. megakarya* on the leaves of the cacao tree in the absence and in the presence of *Glomus intraradices* by evaluating the development of necrosis and the content of phenolic compounds in hybrid genotypes studied under healthy and infected conditions, and finally to identify hybrid genotypes within the hybrid family with better tolerance towards this pathogen. The results of the tests carried out made it possible to note that the hybrid genotypes of the Ficup offspring presenting a better tolerance with respect to *P megakarya* are Ficup 3, Ficup 5, Ficup10, Ficup14, Ficup 15, Ficup 16, Ficup 17, Ficup 19, Ficup 20, Ficup 24, Ficup 25, Ficup 29, and Ficup 36 with very low necrotic surfaces of between 0 and 0.5 $cm^2$ and very high phenol contents in the presence of *G. intraradices*. These can be multiplied by in vitro culture in order to improve the yield of crops and offered to farmers for useful purposes. The results

also showed that the sensitive hybrid genotypes in the presence of *G. intraradices*, increased their degree of tolerance against the attack of *Phytophthora megakarya*. This *G. intraradices* can therefore be used by farmers in cocoa plantations to improve their yield.

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