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Peroxidase and polyphenol oxidase activities associated to somatic embryogenesis potential in an elite hybrid genotype of *Theobroma cacao* L.

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Cross-fertilization and somatic embryogenesis stand as potential useful tools for genetic improvement and vulgarization of agronomical interesting cocoa planting material for sustainable cocoa culture. Manual cross-fertilization (♀SNK13×♂UPA143) was conducted. ♀SNK13×♂UPA143 derived hybrid genotypes were tested for their susceptibility to black pods disease (BPD). The most tolerant hybrid genotype (KHACa12) was monitored for its precocity, yielding and somatic embryo potential. Peroxidase and polyphenol oxidase activities were evaluated in association to embryogenesis in KHACa12. The cross-fertilization ♀SNK13×♂UPA143 generated around 60% of offprint's tolerant or less susceptible to BPD. When transferred in experimental farm, KHACa12 produced flowers 18 months after planting. Mature pods from KHACa12 bearded 55±5 seeds/pod. Seventy-five pods were harvested from KHACa12 during the first season. These pods generated 6 kg of fermented-dried cocoa seeds. Somatic embryo responsive revealed that 71.041 ± 7.91% and 50.64 ± 9.48% staminodes and petals derived explants, respectively produced direct and indirect somatic embryos. Morphologically, the somatic embryos obtained were similar to zygotic embryos from the same hybrid genotype (KHACa12). Peroxidase and polyphenol oxidase activities profiles in zygotic and somatic embryos increase during the first stages of embryogenesis, then decrease when cotyledons developed. These results might indicate that, ♀SNK13×♂UPA143 could be used to develop cocoa (*Theobroma cacao* L.) hybrid genotypes tolerant or less susceptible to BPD. KHACa12 is an agronomic interesting hybrid genotype that could be produced in large scale and vulgarized using somatic embryogenesis process. The initiation and development of somatic embryo in KHACa12 can be monitored and modulated biochemically through peroxidase and polyphenol oxidase activities profiles.

Key words: Breeding, tissue culture, somatic, explants, hybrid.

INTRODUCTION

The chocolate and cocoa butter tree (*Theobroma cacao* L.) is a perennial tropical crop widely cultivated in Africa, Asia and America. Consumption of cocoa increases by 7% each year (ICCO, 2016). However, cocoa production is challenged with reliable planting material crisis. Mostly used planting material are low yielding and highly sensitive to disease (*Phytophthora megakary* specially). Improved planting material is not available or accessible to farmers. In many African cocoa producing countries, 25% of cocoa farms are made of improved planting

material. Additionally, most cocoa farms are old and need to be renewed with elite cocoa genotypes. Moreover, the ongoing cocoa culture practices that use chemical fertilizers and pesticides are costly and environmentally unfriendly. Therefore, developed and vulgarized elite cocoa genotypes are the ways out for sustainable cocoa production and profitability. Tremendous efforts have been put on cross-fertilization of targets *T*. *cacao* clones in order to provide farmers with elite cocoa genotypes. Breeding for resistance to black pods disease, precocity and yield are some of the characters expected in offprint (Efombagn et al., 2007). Since the parental clones are always heterozygote for these characters, the offprints from cross-fertilization of targets *T*. *cacao* clones are highly heterozygote. Additionally, the proportion of hybrid genotypes for the character such as tolerance *P. megakarya* did not exceed 1/3 (Tahi et al., 2000; Nyasse et al., 2003).

There is still much cross-fertilization to be tested. ♀SNK13×♂UPA143 has not been tested. Usually, pods or plantlets from interesting crossing are distributed to farmers. However, the allogamous character of *T*. *cacao* limits the vulgarization of elite hybrids genotypes. These explain why in cocoa producing country such as Cameroon, in spite of the distribution of these cultivars, cocoa plantations continue to be low yielding, with an average of 200 to 600 kg of dry cocoa per ha (Efombagn et al., 2007). An efficient and reliable vegetative multiplication of elite genotype was needed. Classic vegetative multiplication methods (grafting and the rooted cuttings) were studied. However, these vegetative propagation methods of cacao lead to plants highly sensitive to winds and dryness in African countries (Tahi et al., 2006). Somatic embryogenesis has been studied by many research teams (Omokolo et al., 1997; Li et al., 1998; Maximova et al., 2002; Traoré et al., 2003; Minyaka, et al., 2008, 2010; Minyaka, 2009). Cacao somatic embryogenesis allows rapid vegetative and large-scale multiplication of elite genotypes, genetic improvement, collection and conservation of cacao germoplasm, efficient diffusion of plant material as plantlets to farmers, etc. However, the limiting factor in using somatic embryogenesis to vulgarize an elite cocoa genotype is its somatic embryogenic responsive/ recalcitrance.

Peroxidase and polyphenol oxidase enzymes participate in the metabolism of auxins, coumaric acid and other phenolic compounds which are important in plant embryogenesis (Duchovskis et al., 2009). Changes in peroxidase and polyphenol oxidase acitivities in *T*. *cacao* embryogenesis (zygotic and somatic embryogenesis) have never been investigated. Such investigation enhances our understanding of the developmental processes underlying the formation of somatic and zygotic embryos in *T*. *cacao*. It could also aid in the development of stage-specific biochemical markers that might be used to optimize somatic embryogenesis protocols (Kormuťák et al., 2003).

In the present investigation, an agronomical interesting hybrid genotype from ♀SNK13×♂UPA143 was developed. Somatic embryogenesis potential, peroxidase and polyphenol oxidase activities during embryogenesis of this hybrid genotype (♀SNK13×♂UPA143) were monitored.

MATERIALS AND METHODS

The present study was conducted from September 2012 to October 2016.

Screening for susceptibility to *P. megakarya* of the progeny ♀SNK13×♂UPA143

Plant

T. cacao seeds from two pods of ♀SNK13×♂UPA143 (KHA) obtained by manual pollination, which were used to establish a nursery. Three months old plant leaves were used as plant biological material in leaf disc test (Nyasse et al., 1995) to evaluate the susceptibility of *T*. *cacao* plants to black pod disease.

Pathogen

The pathogen material used in this investigation was *P. megakarya* strain ELEG-8 (characterized by RADP at CIRAD, Montpellier-France). This strain was graciously offered by the laboratory of plant pathology of Institut de Recherche Agronomique pour le Developpement (IRAD) at Nkolbisson (Yaoundé, Cameroon). In the laboratory (Biochemistry Laboratory, Faculty of Sciences, University of Douala, Cameroon), 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.

Zoospore production

Zoospores (or inoculums) were obtained according to Nyasse et al. (1995) adapted method. Zoospores were obtained from l0-day-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 a MALASSEZ hemati-meter.

Screening for susceptibility to P. megakarya of ♀SNK13×♂UPA143 (KHA) progeny

Leaf discs test was used for screening for susceptibility of hybrid genotypes from ♀SNK13×♂UPA143 progeny according to Nyasse

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et al. (1995) adapted method. The experimental design was made of three replicates and completely randomized 7 blocks of leaf discs (Ø = 1.5 cm) per hybrid. Hence, a total of 27 discs were used per hybrid. For each hybrid of the progeny, leaf discs were obtained from the slightly lignified young leaves (3 months old). Leaf discs were placed in trays and incubated for 24 h (at 25±1°C) in darkness prior to inoculation. After the 24 h, leaf discs were inoculated by depositing 10 µL (3×10^5 zoospores/mL) of zoospores 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 discs (for each hybrid) was registered on 4, 5, 6, 7 and 8th day after inoculation.

Field transfer of seeds-derived plantlets

All seeds-derived plantlets with disease score ranging from 0 to 0.2 were planted in an experimental plot (Faculty of Science, University of Douala, Cameroon). The transfer was made when plantlets were six months old. Growing parameters were recorded every three months. The date of first flowers was registered. For the hybrid genotype with the best precocity, the number of seeds per mature pods and weight of dried seed were registered. The hybrid with the best agronomic characters was submitted to embryogenesis investigation.

Somatic embryogenesis

Plant and explants

Plant material used in this investigation was made of immature flower buds of an elite hybrid genotype ♀SNK13×♂UPA143 from an experimental plot (Faculty of Science, University of Douala, Cameroon). Staminodes and petals isolated from immature flower buds were as explants.

Explants culture

This experiment used the protocol developed by Minyaka et al. (2008). Explants were cultured in induction medium and incubated for 14 days in darkness at 25±1°C. The induction medium was made of DKW basal salts as described by Driver and Kuniyuki (1984) and supplemented with 250 $mg.L^{-1}$ glutamine, 100 $mg.L^{-1}$ myoinositol, 1 $ml.L^{-1}$ DKW vitamin stock (100 $mg.ml^{-1}$ myo-inositol, 2 $mg.ml^{-1}$ thiamine-HCl, 1 $mg.ml^{-1}$ nicotinic acid, and 2 $mg.ml^{-1}$ glycine), 20 $g.l^{-1}$ glucose, 18 µM 2,4-dichlorophenoxyacetic acid (2,4-D), and 45.4 nM TDZ.

After 14 days in induction medium, explants were transferred in maintenance medium and incubated in the same condition as previously. The maintenance medium was similar to induction medium except that, glutamine and myoinositol were absent; 45.4 nM TDZ were replaced by 250 $\mu g.L^{-1}$ kinetin and the concentration of 2,4-D was 9 µM. The pH of induction and maintenance media was at 5.8 prior to addition of 0.2% (w/v) phytagel.

In vitro morphogenetic structures from maintenance medium were transferred into embryo development medium and incubated for 21 days (darkness, 25±1°C). After the 21 days, two additional subcultures were done in the same medium at 21 days' intervals and incubated in darkness (25±1°C). The embryo development medium was made of DKW basal salts, 0.7% (w/v) $MgSO_4$, 3% (w/v) sucrose and 1 $ml.L^{-1}$ DKW vitamin stock. The pH of embryo development medium was at 5.7 prior to addition of 0.2% (w/v) phytagel.

Collection of data

The aspect of explants was recorded in induction, maintenance and embryo development media. At the end of the culture cycle (day 91), percentages of staminodes and petal-derived structures bearing embryo were estimated. Additionally, the date of observation of first embryo was written down.

Zygotic embryogenesis

Pods at different maturation stages were harvested from an elite hybrid genotype ♀SNK13×♂UPA143 at an experimental plot (Faculty of Science, University of Douala, Cameroon). For each pod, three portions were considered viz., apical, median and distal. After fixations on gallows, pods were sectioned (2 mm teak) transversally from distal to apical end using a blade of lancet. At each transversal of a pod, morphological characteristic of a giving ovum (axial diameter), embryo (developmental stage) and endosperm (consistence of endosperm) were noted down.

The developmental stage of embryo was appreciated with arbitrary symbols, from E.0 to E.14: E.0 (no visible embryo), E.1 (globular stage), E.2 (torpedo stage), E.3 (hearth shape stage) and E.4 to E.14 (different cotyledonary stages). Endosperm and embryos at different maturation stage were collected separately and kept at -20°C and used for peroxidase and polyphenol oxidase extraction and analysis.

Peroxidase and polyphenol oxidase in endosperm, zygotic embryo and *in vitro* morphogenetic structures

Peroxidase and polyphenol oxidase extraction

Peroxidase and polyphenol oxidase extracts were obtained from different *in vitro* morphogenetic structures, endosperm and zygotic embryos (at different developmental stages). Peroxidase and polyphenol oxidase extracts preparation were obtained from the aforementioned biological material. Fresh biological material tissues (1 g) were homogenized 1 g in mortar with 3 ml of potassium phosphate buffer (50 mM, pH 6.0). The homogenate was then centrifuged (4°C, 30 min, 6000 g) and the supernatant (soluble peroxidase and polyphenol oxidase extract) was collected. The pellet was re-suspended in potassium-phosphate buffer and re-centrifuged under the same conditions, and the new supernatant was added to the first.

Protein quantification

Protein contents in soluble peroxidase and polyphenol oxidase extracts were determined according to the method of Bradford (1976) and bovine serum albumin was used as standard.

Polyphenol oxidase assay

Polyphenol oxidase (PPO) activity was determined by measuring the increase in absorbance at 330 nm according to the method of Van Kammen and Broumer (1964). The reaction mixture incubated at 25°C contains: 2.7 ml of 1/15 M phosphate buffer pH 6.1 and 0.3 ml of 10 mM catechol. The reaction was initiated by adding 40 µl of enzymatic extract. The enzyme activity was determined according to the change in optical density at 330 nm after 30 s. This activity was expressed on a fresh weight basis, compared to the protein content.

Table 1. Average disease score of the progeny ♀SNK13×♂UPA143.

Progeny	♀SNK13×♂UPA143
Average disease score	3.68±1.47

Peroxidase assay

Peroxidase activity was determined according to Thorpe and Gaspar (1978) method by monitoring the formation of guaïacol at 420 nm. Five milliliters of reaction mixture (1V of 0.2% H_2O_2; 2V of 1% guaiacol; 5V of 1/15 M phosphate buffer, pH 6) was added to 10 µl of the extract. One unit of enzyme activity corresponded to 0.1 is degraded per min, at 420 nm. Peroxidase activity was expressed on a fresh weight basis (unit g^{-1} FW).

Data analysis

Collected data were submitted to descriptive statistics which generated means and standard deviation. Means were compared using the Student Newman and Keuls test. Correlations between variables were evaluated with Pearson correlation test. All statistical analysis was done using SPSS 17.0 software.

RESULTS

Screening for susceptibility to *P. megakarya* of the progeny ♀SNK13×♂UPA143

The screening for susceptibility to *P*. *megakarya* of the progeny ♀SNK13×♂UPA143 revealed a mean disease score of 3.68±1.47 (Table 1). The classification of hierarchical clusters of disease scores of the progeny displayed three mains groups of hybrid genotypes susceptibility: (1) A group of tolerant or less susceptible hybrid genotypes with disease scores [0; 2]. This group is subdivided into two disease scores subgroups ([0.12; 0.62] and [1.12; 2]); (2) A group of middle susceptible hybrid genotypes exhibiting disease scores [2.0; 3.6]. In this group, two subgroups were observed ([2.25; 2.62] and [3; 3.57]); (3) A group of highly susceptible hybrid genotypes displaying disease scores [3.75; 5].

In this progeny, the most tolerant hybrid genotypes were: KHACa12 (0.12), KHACb8 (0.37), KHACb26 (0.62), KHACb3 (1.12), KHACb9 (1.25), KHACb24 (1.25), KHACb22 (1.62), KHACb1 (1.71) and KHACa35 (1.71) (Figure 1 and Table 2). The hybrid genotype KHACa12 (0.12) appeared therefore to be the most hybrid genotype of the progeny ♀SNK13×♂UPA143.

Field transfer of seed-derived plantlet from ♀SNK13×♂UPA143

The seeds-derived plantlets from ♀SNK13×♂UPA143 were transferred in an experimental plot when plantlets were 4 months old. The monitoring of the plantlets on the field revealed that, the hybrid genotype (KHACa12 with disease score 0.12) submitted in the present investigation produced first flowers 18 months after planting. Mature pods from this hybrid were entirely harvested. A total of 75 pods were harvest during the first cocoa season. The number of cocoa seeds per pod was 55±5. After fermentation, the cumulate weight of dried cocoa seeds from the 75 pods was 6.0 kg. During this cocoa production season, none of the pods were attacked by black pod disease due to *P*. *megakarya* (Table 3).

Somatic embryogenesis potential

Morphogenesis and somatic embryo responsive of floral explants from hybrid genotype KHACa12

Staminodes and petals explants when cultured in induction medium, developed calli between 10th and 14th day of culture incubation. Immature explants appeared to be the most callogenic. The development of calli was amplified in maintenance medium. The differentiation of somatic embryo was observed when explants from maintenance medium were transferred in embryo development medium. Two types of somatic embryogenesis were observed: direct somatic embryogenesis and indirect somatic embryogenesis. Both types emerged from browning explants. Somatic cotyledonary embryos were morphological similar to zygotic cotyledonary embryos (Figure 2).

The staminods-derived explants appeared to be more embryogenic (71.041±7.91%) than petals-derived explants (50.64±9.48). The number of embryo per explants was higher in staminods-derived explants (4 to 10) compared to petals-derived explants (Table 4).

Zygotic embryogenesis

The developmental stage of embryo was appreciated with arbitrary symbols (from E.0 to E.14). The consistence of endosperm and sizes of ovum were also inspected. The sizes of the embryos ranged between 0.2 (for globular stage) to 1.6 cm in cotyledonary stages. The ovum sizes were embryo development stage-independent. Endosperm consistence was liquid in globular stage of embryo and viscous, colloidal or cellularized as embryo develops cotyledons (Figure 3).

Peroxidase and polyphenol oxidase activities in endosperm, zygotic embryo and in vitro morphogenetic structures

Peroxidase activities were monitored in endosperm and zygotic embryos at different developmental stages. In the endosperm, peroxidase activities remained low and quasi constant during zygotic embryo development. Reversely,

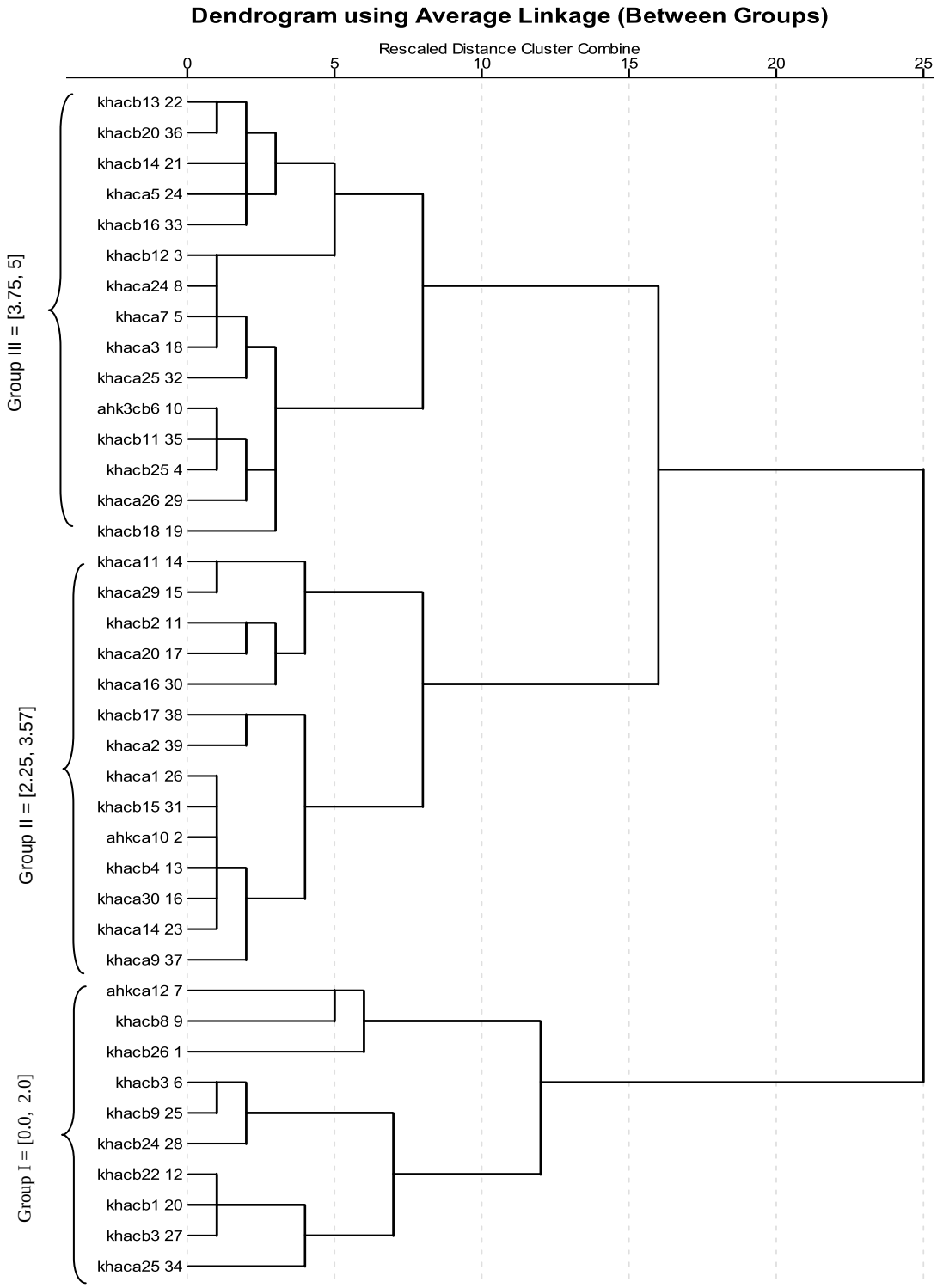

Figure 1. Dendrogram of disease score of hybrid family KHA. $khaca_i$=family KHA, pod a, seed i; $khacb_j$=family KHA, pod b, seed j.

in zygotic embryo, peroxidase activities increased from stage E0 to stage E2. There was no significant difference in the peroxidase activities between stages E5 and E14 (Figure 4).

Polyphenol oxidase (PPO) in endosperm exhibited a profile not quite different from peroxidase activities. PPO appeared to be low almost low in endosperm (compared zygotic embryo) during zygotic embryo maturation. In

Table 2. Disease scores and percentages of hybrid genotypes per group of susceptibility of progeny ♀SNK13×♂UPA143.

Group	Hybrid genotypes susceptibility categories	Disease scores interval	Percentage
I	Less susceptible	[0.0, 2.0]	25.64
II	Middle susceptible	[2.0, 3.6]	35.90
III	Highly susceptible	[3.75, 5]	38.46

Table 3. Agronomic characteristics of the selected hybrid genotype.

Age of hybrid genotype at first flowers buds	Number of seeds/mature pod	Weight of dried seeds from 75 pods (kg)	Number of pods during first cocoa season	Number of pods attacked by BPD
18 months	55±5	6.0	75	0

zygotic embryos, PPO activities increase from 0+ to E2, then decrease from E2 to E4. No significant fluctuation in PPO activities was observed between stages E4 and E14 (Figure 5).

At day 28 of cultures incubation, peroxidase and polyphenol oxidase activities appeared to be higher in staminods than petals. At day 56 of cultures incubation, embryogenic calli exhibited higher peroxidase and polyphenol oxidase activities than none embryogenic calli.

During somatic embryo maturation, it appeared that, peroxidase activities were significantly higher in globular stage of somatic embryo than cotyledonary stage. The same profile was observed with polyphenol oxidase. The activities of this enzyme were significantly higher in globular stage than cotyledonary stage of somatic embryo development (Figures 6 and 7).

DISCUSSION

Disease score evaluation of the offprint from ♀SNK13×♂UPA143 was conducted for the selection of hybrid genotypes tolerant to *P. megakarya*. Variability in disease scores between hybrid genotypes was observed. The heterogeneity in disease scores in the offprint might result from the heterozygote statute of the parents (♀SNK13 and ♂UPA143) for "resistance to black pod disease" character which has been reported to be additive and probably polygenic (Nyasse et al., 2007).

The proportion of less and middle susceptible hybrid genotypes was above 60% of the offprint. Usually, the proportion of cocoa tolerant offprint did not exceed 1/3 of the progeny (Thai et al., 2000). The high proportion of tolerant hybrid genotypes from ♀SNK13×♂UPA143 might be due to SNK13 which was reported among tolerant cocoa clones (Nyasse et al., 1995). This set of results might indicate that the present tested cross-fertilization (♀SNK13×♂UPA143) could be used to develop less or middle susceptible (to black pod disease) hybrid genotypes. Hybrid genotypes from cross-fertilization ♀SNK13×♂UPA143 could be used by farmers in other to reduce the use of pesticides and preserve the environment.

The less susceptible hybrid genotype, KHACa12, was monitored in the field. This hybrid genotype was able to produce flowers at the age of 18 months. This finding seems to be atypical. Most *T. cacao* genotypes produce first flowers between 28 and 36 months (Ndoumbe-Nkeng et al., 2001). KHACa12 hybrid genotype seems to be an outstanding hybrid genotype for its precocity. The number of seeds per pod ranged between 50 and 60. Additionally, 75 pods were harvested from KHACa12; the first year generated 6.0 kg of fermented dried 75 pods.

Somatic embryogenesis is reported as a reliable vegetative multiplication process for the vulgarization of allogamous plant such as *T. cacao* (Minyaka et al., 2008, 2010). However, the limiting factor in the use of this process in *T. cacao* is the recalcitrance and low responsive of many elites *T. cacao* genotypes to somatic embryogenesis (Tan et al., 2003). Somatic embryogenesis potential of KHACa12 hybrid genotype investigation was conducted in this study. KHACa12 hybrid genotype differentiated somatic embryos. Direct and indirect somatic embryos were observed from staminodes and petals-derived explants. Direct somatic embryogenesis is uncommon in *T. cacao* genotypes. The occurrence of direct somatic embryo in KHACa12 hybrid genotype implies the inhibition of callogenesis and ability of explants-cells to be directly competent and determined to change their differentiation pathways in other to become embryogenic (Minyaka, 2009). This might be due to stress-related compounds which are required for somatic cells to become embryogenic cells (Verdeil et al., 2007; Minyaka et al., 2008). Staminodes and petals explants were able to differentiate somatic embryos at different rates. Staminodes were more embryogenic than petals. This observation underlines the explants-type-dependence of somatic embryogenesis in *T. cacao* (Li et al., 1998; Minyaka, 2009). Somatic embryo responsive of both staminodes and petals explants was significantly

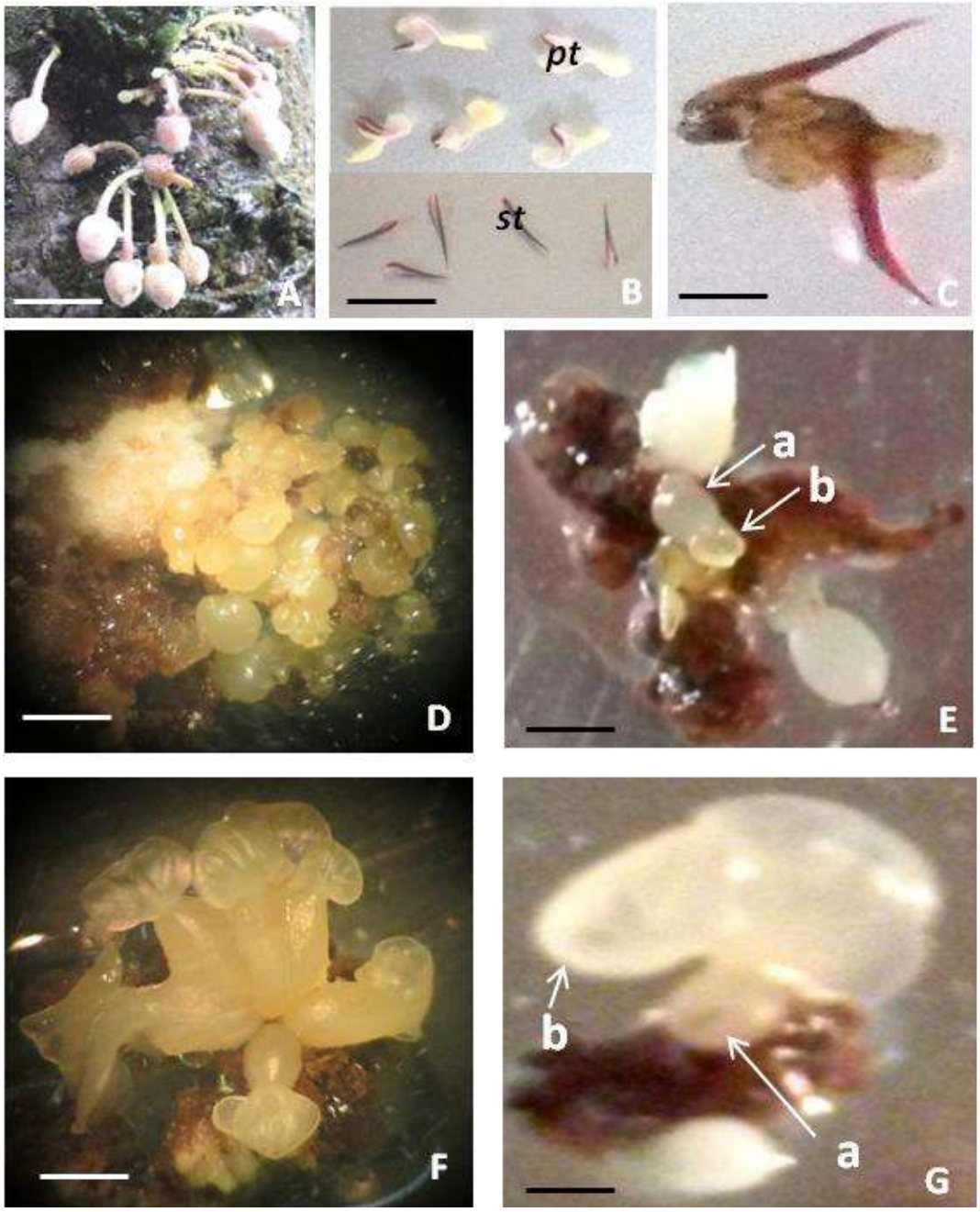

Figure 2. Different morphogenetic structures obtained from staminods and petals explants from hybrid genotype KHACa12. A. Flowers buds; B. staminods (*st*) and petals (*pt*) explants; C. staminods explants developing calli at day 10 of culture; D. callus bearing globular somatic embryo; E. callus bearing indirect somatic embryos at different maturation stages (a = radicul, b = cotyledons); F. callus bearing cotyledonary somatic embryo; G. petal-derived explant with direct cotyledonary somatic embryo (a: radicule, b: cotyledons). Bar = 0.25 cm.

Table 4. Embryo responsive of staminods and petals-derived explants.

Explant type.0	Number of embryos/explant	Embryogenic explants (%)
Staminods	4 - 10	71.041 ± 7.91
Petals	2 - 7	50.64 ± 9.48

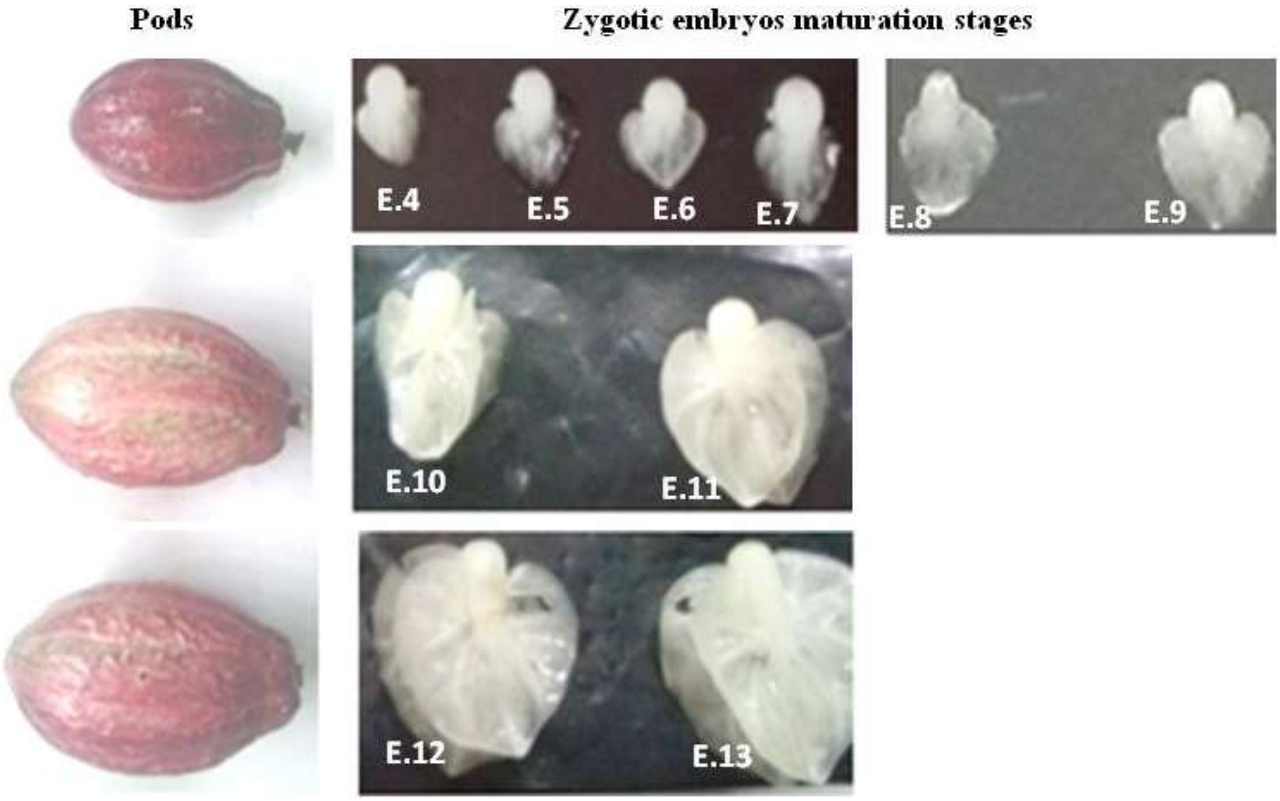

Figure 3. Different cotyledonary stages of zygotic embryo from hybrid genotype KHACa12 of *T. cacao*.

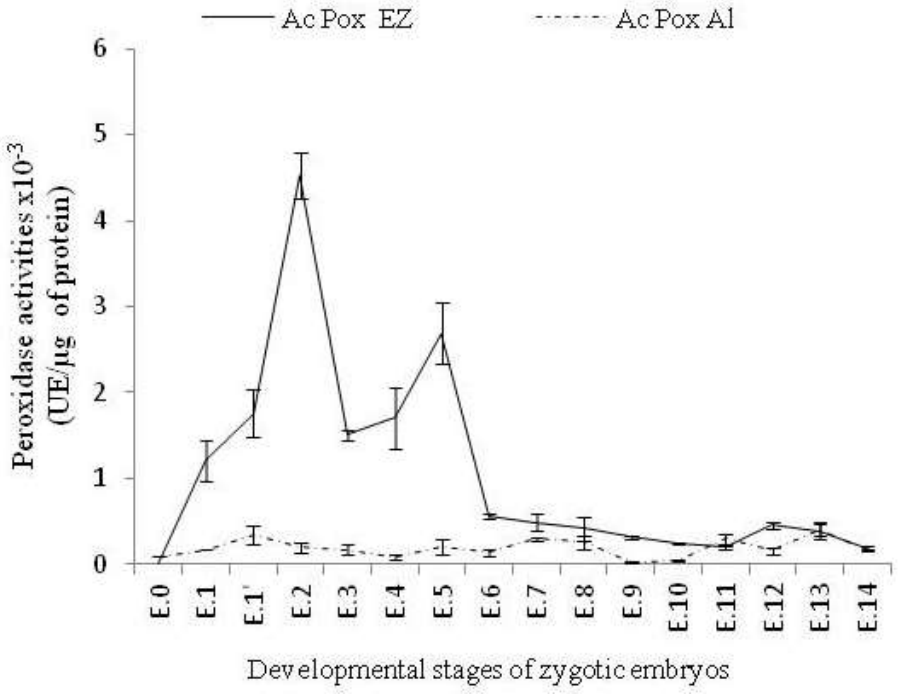

Figure 4. Peroxidase activities in zygotic embryos at different developmental stages and associated endosperm (albumen). Values are given in terms of mean ± SD (n = 9). Ac.Pox EZ, Peroxidase activities in zygotic embryo; Ac.Pox Al, peroxidase activities in endosperm.

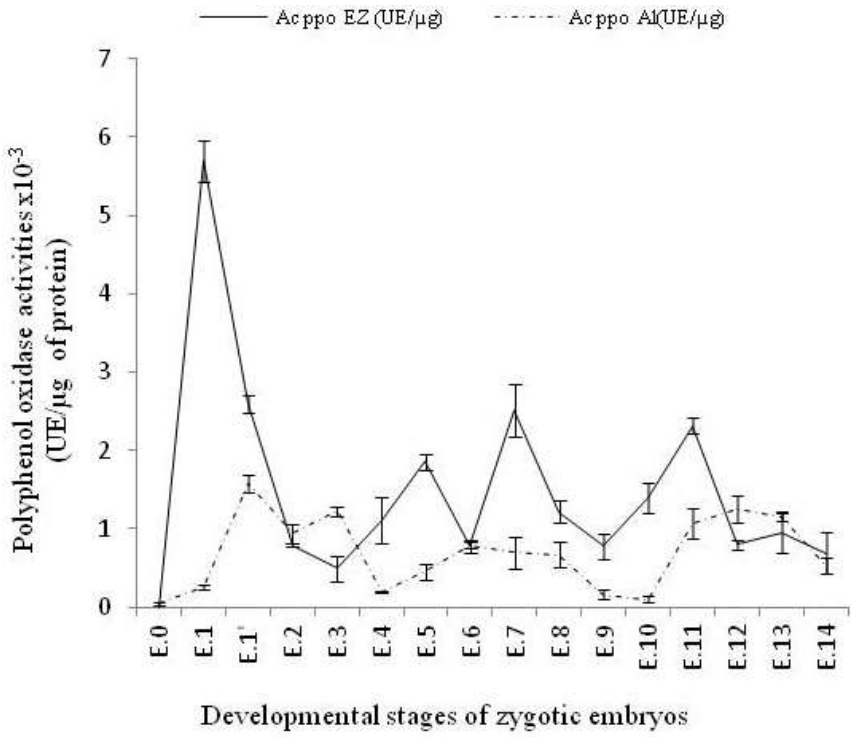

Figure 5. Polyphenol oxidase activities in zygotic embryos at different developmental stages and associated endosperm (albumen). Values are given in term of Mean ± SD (n = 9). Ac.ppo EZ (UE/µg), polyphenol oxidases activities in zygotic embryo; Ac.ppo Al (UE/µg), polyphenol oxidases activities in endosperm.

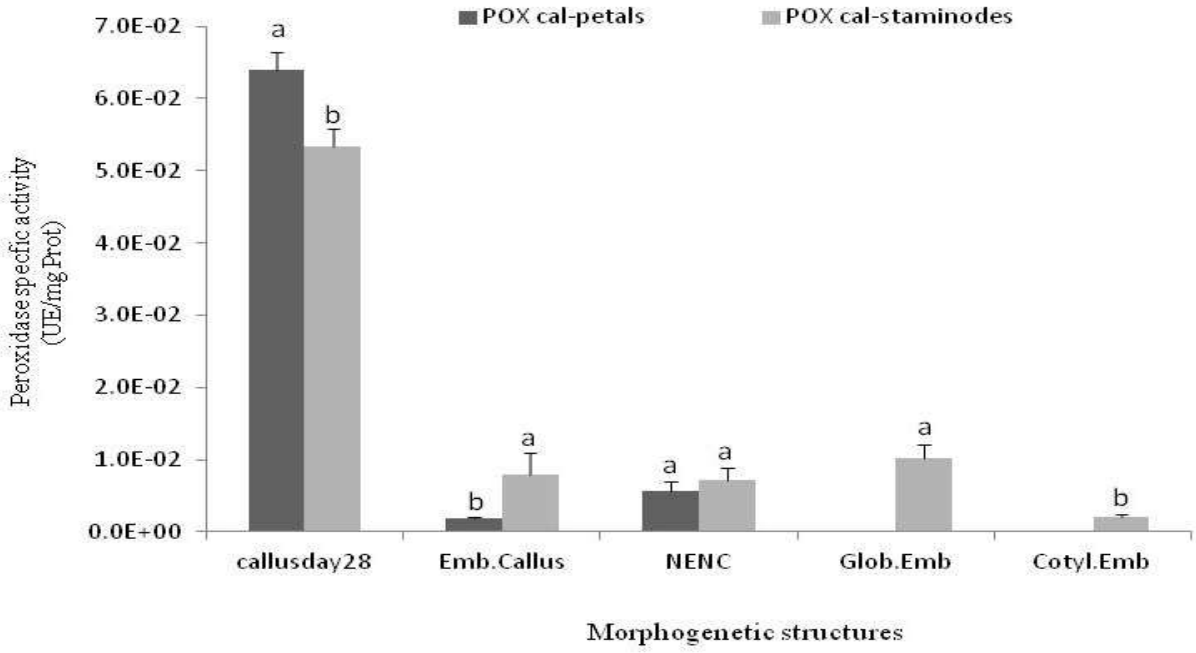

Figure 6. Peroxidase specific activities in different morphogenetic structures. Values are given in term of Mean ± SD (n = 9). Means were compared with Student Newman and Keuls test (5%). Callus day28, Callus at day 28 of cultures incubation; Emb.callus, embryogenic callus; NENC, non embryogenic necrotic callus; Glob.Emb, globular embryo; Cotyl.Emb, embryo with cotyledon. In callus day 28, Emb.callus and NENC significant difference between staminodes and petals are indicated in different letters. Significant difference between Glob.Emb and Cotyl.Emb are indicated by different letters.

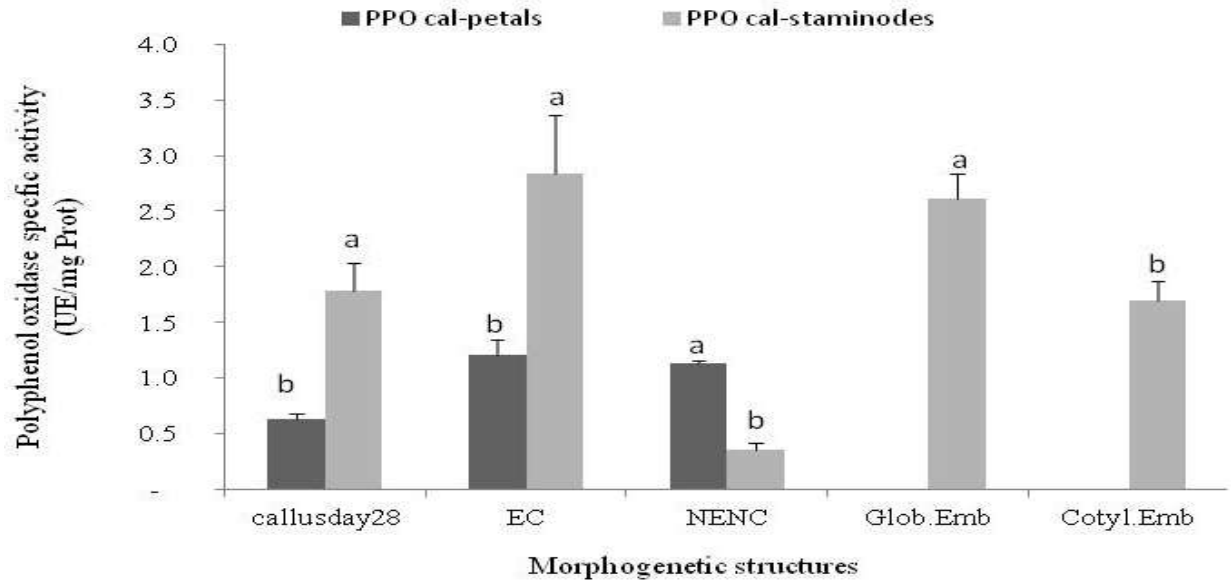

Figure 7. Polyphenol oxidase specific activities in different morphogenetic structures. Values are given in term of Mean ± SD (n = 9). Means were compared with Student Newman and Keuls test (5 %). Callusday28, Callus at day 28 of cultures incubation; Emb.callus, embryogenic callus; NENC, non embryogenic necrotic callus; Glob.Emb, globular embryo; Cotyl.Emb, embryo with cotyledon. In callus day 28, Emb.callus and NENC significant difference between staminodes and petals indicated different letters. Significant difference between Glob.Emb and Cotyl.Emb are indicated by different letters.

high in KHACa12 hybrid genotype compared to results reported by Tan and Furtek (2003) and Minyaka et al. (2008). In fact, these authors reported somatic embryogenesis responsive hanging between 10 and 40%. Hence, KHACa12 hybrid genotype appeared to be vulgarized through somatic embryogenesis.

Biochemical characterization of some woody plants zygotic and somatic embryogenesis is useful in the development of stage-specific biochemical markers that might be used to optimize somatic embryogenesis protocols (Kormuťák et al., 2003). Peroxidases and polyphenol oxidase activities were monitored during somatic and zygotitic embryos development in KHACa12 hybrid genotype. During zygotic embryogenesis in *T. cacao*, peroxidases and polyphenol oxidase activities increased during the earlier stages of zygotic embryogenesis, then decrease while cotyledons expanded. In somatic embryos, a decrease pattern of peroxidases and polyphenol oxidase activities was also observed from globular to cotyledonary embryos. Generally, somatic embryos (direct and indirect) emerged from brown staminodes and petals-derived explants. In *Fraxinus mandshurica*, Chun-Ping et al. (2015) reported the implication of peroxidases during somatic embryos differentiation in browning explants. The profile of peroxidase and polyphenol oxidase reported here in *T. cacao* for the first time matches with the observation reported by Amal and Hemmat (2015). These authors indicated that, in date palm, peroxidase activity was the highest level at embryogenic callus and then decreased gradually during the subsequent developmental stages. This set of results might reveal the participation of peroxidases and polyphenol oxidase in the initiation of zygotic and somatic embryogenesis in *T. cacao*. Hence, the stimulation of both enzymes in explants during *T. cacao* tissue culture could improve the somatic embryos responsive of cocoa genotypes.

Conclusion

The set of results presented in this investigation, might indicate that, ♀SNK13×♂UPA143 could be used to develop *T. cacao* hybrid genotypes tolerant or less susceptible to BPD. KHACa12 is an agronomic interesting hybrid genotype that could be produced in large scale and vulgarized by using somatic embryogenesis process. The initiation and development of somatic embryos from KHACa12 can be monitored and modulated biochemically through peroxidase and polyphenol oxidase activities profiles.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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