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Improved callogenesis and somatic embryogenesis using amino acids and plant growth regulators combination in pineapple [*Ananas comosus* (L.) merr. (Bromeliaceae)]

[1] Kouadio Oi Kouadio Samuel, [2] Yapo Edwige Sopie Salomé, [3] Kouassi Kan Modeste, [4] Silue Oumar, [5] Koffi Edmond, [*6] Kouakou Tanoh Hilaire

[1, 6] Université Nangui Abrogoua, UFR des Sciences de la Nature, Laboratoire de Biologie et Amélioration des Productions Végétales, Abidjan, Cote d'Ivoire

[2] Université Jean Lorougnon Guédé, UFR Agroforesterie, BP 150 Daloa, Côte d'Ivoire

[3, 4, 5] Centre National de Recherche Agronomique (CNRA), Station de Recherche de Bimbresso, 01 BP 1536 Abidjan 01, Côte d'Ivoire

Abstract
The pineapple (Smooth Cayenne) in Côte d'Ivoire is subject to many production problems whose causes are mainly of agronomic order. The revitalization of this sector requires that the smooth Cayenne be cleaned and improved. Biotechnology through its various methods has been used as a working tool. Among them, callogenesis and somatic embryogenesis were adopted in the present study. The calli induction and proliferation as well as somatic embryos formation were evaluated on the basal medium of Murashige and Skoog (MS) supplemented with Gamborg vitamins (vitamins B_5), 30 g/l of sucrose and solidified with 6 g/l of agar. This medium was supplemented with the polyamines glycine, glutamine and casein hydrolyzate, alone or in combination with different concentrations of growth regulators such as picloram (PIC), acid 2,4-dichlorophenoxyacetic (2,4-D), kinetin (KIN) and benzylaminopurine (BAP). The results revealed that the medium MIC_7BAP (3.0 mg/l picloram, 2.0 mg/l glycine, 1,000 mg/l glutamine and 0.05 mg/l BAP) was the best for callus induction while the medium MIE_1 (MIE, 3.0 mg/l picloram, 2.0 mg/l glycine, 1,000 mg/l glutamine and 100 mg/l casein hydrolyzate in combination with 0.05 mg/l BAP) media was identified as the most embryogenic cells induction medium (77 cells). Callogenesis and embryogenesis in smooth Cayenne were favored by the combination of amino acids in presence of growth regulators.

Keywords: pineapple, callus, somatic embryos, amino acids, plant growth regulator

1. Introduction

Pineapple is a tropical plant, native to South America. It is mainly known for its edible fruit, which is in fact a fructification. It is popular for its strong flavor and its high nutrients content. Pineapple is very important for the economy of many developing countries. Thus, it is of considerable economic and social importance because it supports a substantial part of the farmers and is also an important source of foreign exchange. Côte d'Ivoire is the 3rd African pineapple producer after Nigeria and Kenya. It produces on average 188 000 tons of pineapples per year, of which 150 000 tons are exported to Europe [1]. In Côte d'Ivoire, pineapple accounts for 1.2 % of exports and generates nearly 34 billion FCFA revenue per year [1]. It contributes 1.6 % to agricultural gross domestic product and 0.6 % to national gross domestic product [2, 3]. In Côte d'Ivoire, the Smooth Cayenne variety is the most cultivated because of its adaptation to the pedoclimatic conditions of the country and especially its potential for yield and appreciation on the international market. The country has long been the largest supplier of pineapple in the European Union (97 % of the market) but this dominant position is now occupied by Costa Rica. The quasi-hegemony of the Smooth Cayenne culture in Côte d'Ivoire has led, after several decades of intensive cultivation and without fallow, a degeneration of the plant material with enormous phytosanitary risks. This situation led to a significant 70 % reduction in pineapple production in Côte d'Ivoire, which varied from 213 620 to 73 000 tons between 1999 and 2008 [4]. The use of phytosanitary products, mainly ethephon, to counter the problems of declining production, has also led to the accumulation of chemical residues in pineapple [5]. Thus, Côte d'Ivoire pineapple have a high residue limit (MRL) and a high acidity in the fruit. This situation has led to the depreciation of pineapple from Côte d'Ivoire on the international market in favor of the Mary Dillard 2 (MD2), variety from Costa Rica. In addition, the importation of suckers by farmers is also expensive and cannot meet the market requirements [6]. Addressing the problems faced by the cultivation of Smooth Cayenne, interspecific hybridization has been recommended for varietal improvement. But it has had little success because pineapple is a monocotyledonous, self-incompatible, highly heterozygous plant; which makes it difficult to conventional breeding [7]. Given its economic importance, the decline in production and the depreciation of the pineapple of Côte d'Ivoire, breeding program is essential for the crop improvement [3]. Biotechnology covering many

fields like tissue culture concepts and genetic engineering to the applications seems to be an ideal tool for crop improvement. Among *in vitro* culture techniques, callogenesis is an important step because callus is the ideal starting material for others *in vitro* cultures such cell suspensions, somatic embryogenesis and organogenesis [8]. In pineapple, several culture media have been developed to initiate callus. Among them, MS medium [9] including vitamins B_5 [10] and supplemented with picloram or 2.4-D allow callus formation at a high frequency [11, 12]. Compounds other than auxins also play an important role in the production of pineapple calli. These include glycine and glutamine [13, 14, 15, 16], and cytokinins such as BAP or kinetin in combination or not with auxins [17, 12]. These results showed that responses to callogenesis in pineapple differs according to the type of amino acid and the hormonal regime [18]. Therefore, the objective of this study is to develop an efficient protocol of call genesis for the induction of somatic embryogenesis in pineapple. The induction of somatic embryos will contribute to the regeneration of healthy pineapple plants free of pesticide residues in view of the renewal of the orchard and hence the revival of the pineapple crop in Côte d'Ivoire.

2. Materials and methods

2.1 Plant material, preparation and disinfection of explants

Pineapple (*Ananas comosus* L. var. Smooth Cayenne, cv. CI 16) suckers were used as plant material in this study. They were harvested from the plantations of CNRA's production station in Anguédédou (Dabou Road, Côte d'Ivoire, West Africa). The Smooth Cayenne was chosen for this study because it is the main cultivated variety on a large scale in Côte d'Ivoire. Suckers from 12-month-old pineapple were transported in coolers to the laboratory to be washed extensively under tap water. The adult leaves were excised by a knife and the root portion of the suckers were removed (Figure 1A-C). The apical buds were reduced to small size (2 cm) and washed with tap water and then with distilled water. Under a hood with laminar flow, the buds were soaked in 70 % alcohol for 20 s and then in sodium hypochlorite (3.6 %) for 30 min. They were then rinsed four times in sterile distilled water. After rinsing, the bud was stripped of the remaining 2 or 3 rows of leaves using a blade mounted on scalpel, and the explants thus obtained were cultured on the shoot initiation medium (Figure 1D).

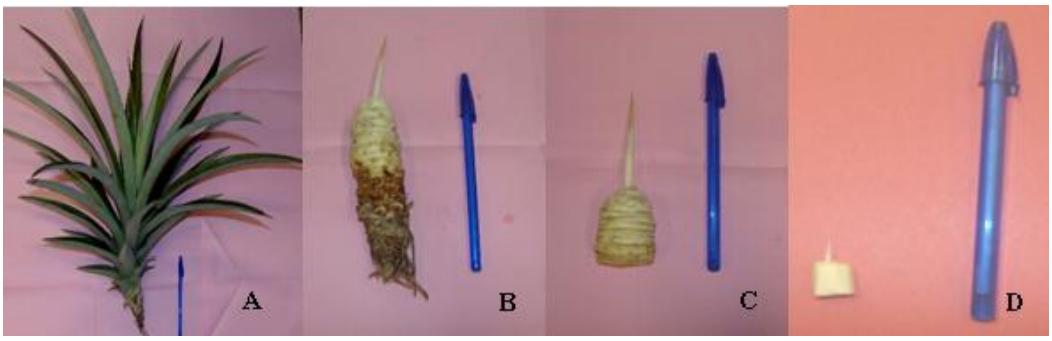

Fig 1: Explants obtained from pineapple suckers
A: suckers collected from field; **B-C:** terminal bud; **D:** terminal bud reduced to 2 cm in length;

2.2 Initiation of shoots culture

The sterilized explants were placed in Pyrex test tubes (21 x 150 mm) containing 10 ml of initiation medium for shoot induction (MIP). This medium is composed of MS [9] basic medium supplemented with 30 g/l sucrose, 0.01 mg/l kinetin (KIN), 0.2 g/l glutamine, 2.0 g/l activated charcoal and solidified with 2.5 g/l phytagel. After one month of culture in a controlled culture room, young shoots were obtained (Figure 2). Shoot leaves were cut into segments of 5 mm in length and used as explants for callus induction. All the explants were transferred for callus induction on various growth regulators and amino acids concentrations.

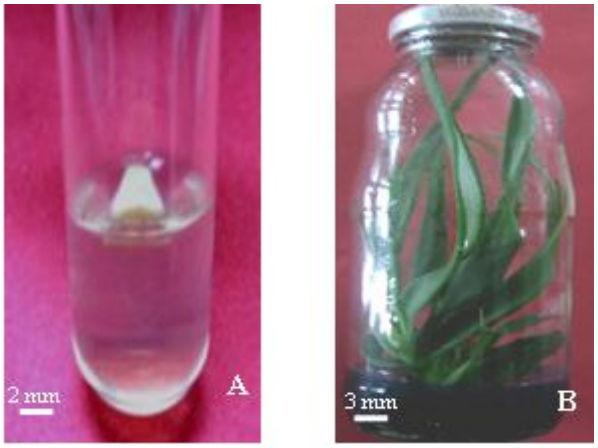

Fig 2: Induction of in vitro pineapple shoot
A: terminal bud on initiation medium in a Pyrex test tube; **B:** four-week-old pineapple shoot

2.3 Callus induction

Callus was induced from sections of leaf base (5 x 7 mm) of *in vitro* pineapple shoot. Five explants were placed in jars containing 10 ml of MS [9] basic medium with vitamins B_5 (MSB_5). This medium was supplied with 30 g/l sucrose, 2.4-dichlorophenoxyacetic acid (2.4-D) or picloram (4-amino-3,5,6-trichloropicolinic acid), 6-benzylaminopurine (BAP) or kinetin (KIN), and combined or not with glutamine, glycine and casein hydrolyzate (amino acids) to obtain callus induction medium. Three types of callus culture medium were realized according growth regulators and amino acids combination. All the media were solidified with 6.0 g/l agar-agar and 0.75 g/l $MgCl_2$.

2.3.1 Effect of growth regulators and amino acids

2.3.1.1 Effect of amino acids in the presence of picloram and or without BAP

Callus induction media (MIC) were composed of medium MSB_5 containing 3 mg/l picloram (medium M1) supplemented with the three amino acids and combined or not with 0.05 mg/l BAP. These culture media are designated as follows in table 1.

Table 1: Effect of amino acids on callus induction in the presence of picloram and or without BAP

Components/Medium	Culture media													
	MIC_1	**MIC_2**	**MIC_3**	**MIC_4**	**MIC_5**	**MIC_6**	**MIC_7**	**MIC_1B**	**MIC_2B**	**MIC_3B**	**MIC_4B**	**MIC_5B**	**MIC_6B**	**MIC_7B**
Picloram (mg/l)	3	3	3	3	3	3	3	3	3	3	3	3	3	3
BAP (mg/l)	-	-	-	-	-	-	-	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Glycine (mg/l)	-	2	-	-	2	2	2	-	2	-	-	2	2	2
Glutamine (g/l)	-	-	1	-	1	-	1	-	-	1	-	1	-	1
Casein hydrolyzate (mg/l)	-	-	-	100	-	100	100	-	-	-	100	-	100	100

2.3.1.2 Effect of amino acids in presence of 2,4-D and or without kinetin

Callus induction media (MIC') consists of MSB_5 medium to which are added 3.0 mg/l 2.4-D (M2 medium), the amino acids and combined or not with 0.2 mg/l kinetin and then designated as follows in table 2.

Table 2: Effect of amino acids on callus induction in the presence of 2, 4-D and or without kinetin

Components/Medium	Culture media													
	MIC'_1	**MIC'_2**	**MIC'_3**	**MIC'_4**	**MIC'_5**	**MIC'_6**	**MIC'_7**	**MIC'_1K**	**MIC'_2K**	**MIC'_3K**	**MIC'_4K**	**MIC'_5K**	**MIC'_6K**	**MIC'_7K**
2.4-D (mg/l)	3	3	3	3	3	3	3	3	3	3	3	3	3	3
Kin (mg/l)	-	-	-	-	-	-	-	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Glycine (mg/l)	-	2	-	-	2	2	2	-	2	-	-	2	2	2
Glutamine (g/l)	-	-	1	-	1	-	1	-	-	1	-	1	-	1
Casein hydrolyzate (mg/l)	-	-	-	100	-	100	100	-	-	-	100	-	100	100

2.3.1.3 Effect of amino acids in presence of picloram and kinetin

Callus induction medium (MIC^+) was composed of MSB_5 medium containing 3.0 mg/l picloram (M1 medium), 0.2 mg/l kinetin and to which amino acids were supplemented then arranged as follows in table 3.

Table 3: Effect of amino acids on callus induction in presence of picloram and kinetin

Components /Medium	Culture media						
	MIC^+_1K	**MIC^+_2K**	**MIC^+_3K**	**MIC^+_4K**	**MIC^+_5K**	**MIC^+_6K**	**MIC^+_7K**
Picloram (mg/l)	3	3	3	3	3	3	3
Kinetin (mg/l)	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Glycine (mg/l)	-	2	-	-	2	2	2
Glutamine (g/l)	-	-	1	-	1	-	1
Casein hydrolyzate (mg/l)	-	-	-	100	-	100	100

2.4 Establishment of embryogenic culture

Primary calli obtained from best cultures media according to the PCA analysis, were chopped with a scalpel according to Yapo *et al.* [12] and transferred on embryos induction media. Approximately, 1,000 mg of healthy, friable and necrosis-free calli were placed in jars containing 10 ml of embryos induction medium (MIE) during 4 weeks. The calli were then transferred twice successively onto a new MIE each month. MIE medium was MS basic medium containing vitamin B_5 (MSB_5) with double KNO_3 and half NH_4NO_3 (MSB_5 - ½ [NH_4NO_3] + [KNO_3]) and supplemented with sucrose 30 g/l sucrose, 6.0 g/l agar-agar, cytokinin (BAP or kinetin), and combined or not with glutamine, glycine and casein hydrolyzate which are amino acids. Six somatic induction embryogenic medium were realized and was ranged as follows in table 4.

Table 4: Somatic induction embryogenic medium (MIE) components

Components /Medium	Culture media					
	MIE_1	MIE_2	MIE_3	MIE_4	MIE_5	MIE_6
Picloram (mg/l)	3	-	-	-	-	-
BAP (mg/l)	0.05	-	-	-	-	-
2.4-D (mg/l)	-	3	3	3	3	3
Kinetin (mg/l)	-	-	-	0.2	0.2	0.2
Glycine (mg/l)	2	-	2	-	2	2
Glutamine (g/l)	1	1	-	1	-	1
Casein hydrolyzate (mg/l)	100	-	100	-	100	100

After three months on MIE medium with monthly subculture, sample of calli were harvested and observed under an optical microscope (Nikon TMS ®) to investigate callus embryogenic structures which clusters of rounded cells with regular contours, thick-walled and dense cytoplasm [19, 20]. These embryogenic structures were then counted using a Colony counter (COMECTA SA).

2.5 Culture conditions

The pH of shoot initiation medium was adjusted to 5.8 while that of callus induction medium and somatic embryos induction medium were adjusted to 5.5. All culture media were autoclaved during 30 min at 121 °C under one bar of pressure. Cultures were performed in a controlled culture room with a 16 h/8 h photoperiod and a light flow of 2000 lux provided by white fluorescent tubes, at 25 ± 2 °C. The effect of amino acids in combination of plant growth regulators was evaluated through the initiation rate (IR), water content (WC), fresh weight (FW), dry weight (DW), texture and the color of the callus after each experiment.

2.6 Statistical analysis

For all experiments undertaken, 30 explants per treatment were used and the experimental design was randomized. Statistical analyzes were carried out by using the Linear Model ANOVA/MANOVA of STATISTICA version 7.1 software. The treatment were discriminated by multiple mean comparison of after variance analysis followed by Newman–Keuls test [21]. A probability of $p < 0.05$ was considered as significant. To normalize the data, all percentage values were subjected to arcsin $x^{1/2}$ transformation before statistical analysis. Principal component analysis (PCA) was performed to identify the contribution of parameters in pineapple callus induction.

3. Results

3.1 Callus induction

3.1.1 Effect of amino acids in presence of picloram

The callus initiation was significantly affected ($p < 0.$) by the amino acids in the presence of picloram. All the studied media are therefore favorable to the initiation of the pineapple calli. The MIC_6 medium (containing glycine and casein) with mean dry weight calli of 28.84 mg, is the most callogenic because of its high solids content (Table 5). However, some amino acids used alone (MIC_2 medium) or in combination with other amino acids (MIC_7 medium), act negatively on the dry weight of the callus compared to the MIC_1 medium (control) which does not contain any amino acids. The calli obtained on these media are compact and aren't ideal for somatic embryos induction.

Table 5: Effect of amino acids alone or in combination in presence of picloram on pineapple callus induction after four weeks on MIC media

	Culture media						
Parameters	**MIC_1**	**MIC_2**	**MIC_3**	**MIC_4**	**MIC_5**	**MIC_6**	**MIC_7**
Initiation rate (%±s)	66.67 ± 9.62a	61.11 ± 14.72a	77.78 ± 15.55a	72.22 ± 14.67a	61.11 ± 11.11a	77.78 ± 14.47a	55.55 ± 15.51a
Fresh weight (mg±s)	44.08 ± 0.32a	18.00 ± 0.37c	15.66 ± 0.33c	15.66 ± 0.11c	5.25 ± 0.13d	33.14 ± 0.31b	31.66 ± 0.22b
Dry weight (mg±s)	22.33 ± 0.37b	2.75 ± 0.19d	15.23 ± 0.14c	12.01 ± 0.17c	4.58 ± 0.11d	28.84 ± 0.42a	3.28 ± 0.27d
Water content (%±s)	49.12 ± 4.08b	84,76 ± 0.22a	2.75 ± 0.42d	4.67 ± 0.19cd	12.49 ± 0.46cd	13.40 ± 0.43c	89.66 ± 0.11a
Texture	Compact	Compact	Compact	Compact	Compact	Compact	Compact
Color	whitish	whitish	Yellow	Yellow	whitish	Brownish	Yellow

MIC (Callus induction medium containing amino acids in combination with 3.0 mg/l picloram) ; M1 (MSB_5 + 3.0 mg/l picloram) ; MIC_1 (M1) ; MIC_2 (M1 + 2.0 mg/l glycine); MIC_3 (M1 + 1,000 mg/l glutamine); MIC_4 (M1 + 100 mg/l casein hydrolyzate); MIC_5 (M1+ 2.0 mg/l glycine + 1,000 mg/l glutamine); MIC_6 (M1+ 2.0 mg/l glycine + 100 mg/l casein hydrolyzate); MIC_7 (M1+ 2.0 mg/l glycine + 1,000 mg/l glutamine + 100 mg/l casein hydrolyzate). ±S: Standard deviation; Average values were calculated from three repetitions per condition of medium; in the line values followed of a same letter are not statistically different (test of Newman-Keuls at 5 %).

3.1.2 Effect of amino acids in presence of picloram and BAP

Table 6 shows that the combination BAP-amino acids significantly influence the fresh weight and dry weight of calli ($p < 0.001$). MIC_7BAP produces the highest mean dry weight (56.62 mg). All the calli obtained on the various media have a very high water content ranging from 24.59 to 91.76 %. However, they are all friable. Calli from the MIC_1BAP, MIC_2BAP, MIC_3 BAP media are yellow-green in color. The MIC_4BAP and MIC_6BAP media have greenish calli. And, those formed on the MIC_5BAP and MIC_7BAP media are yellowish. The data from Tables 5 and 6 show that all the calli obtained on the media without BAP are compact whereas those, which come from the media provided with BAP, are friable. From this analysis, the contribution of BAP improves the callus texture. Moreover, the comparison of the dry weight values of the call of the two groups of media reveals that the media with make it possible to obtain call whose quantity of dry matter is higher than the media without BAP. The addition of BAP therefore increases the dry matter of the calluses. However, it should be noted that the addition of BAP to the amino acid-free MIC_1 medium decreases the dry weight of the calli (6.29 mg) compared with that of the MIC_1 medium without BAP (22.33 mg). These calli are friable and then are ideal for the induction of somatic embryos.

Table 6: Effect of picloram in combination with BAP and amino acids on pineapple callus induction after four weeks on MICBAP media

Culture media							
Parameters	**MIC_1BAP**	**MIC_2BAP**	**MIC_3BAP**	**MIC_4BAP**	**MIC_5BAP**	**MIC_6BAP**	**MIC_7BAP**
Initiation rate (%±s)	77.78 ± 5.55a	77.78 ± 9.22a	83.33 ± 9.62a	83.33 ± 9.62a	77.78 ± 5.55a	66.67 ± 9.62a	88.87 ± 5.52a
Fresh weight (mg±s)	76.58 ± 0.93a	80.46 ± 0.37a	29,83 ± 0.25c	81,73 ± 0.23a	42,98 ± 0.24b	29,89 ± 0.29c	76,98 ± 0.39a
Dry weight (mg±s)	6.29 ± 0.17bc	9.61 ± 0.13c	14.22 ± 0.32c	30.0 ± 0.24b	32.39 ± 0.27b	7.97 ± 0.19b	56,62 ± 0.43a
Water content (%±s)	91.76 ± 0.32a	88.46 ± 1.16a	54.72 ± 7.91c	63.11 ± 1.39c	24.59 ± 2.20d	74.20 ± 2.62b	27.03 ± 3.22d
Texture	Friable	Friable	Friable	Friable	Friable	Friable	Friable
Color	Yellow-green	Yellow-green	Yellow-green	Brown	Yellow	greenish	Yellow

MIC Callus induction medium () was composed to picloram (3.0 mg/l) in combination with BAP (0.05 mg/l) and three types of amino acids (MICBAP). M1 (MSB_5 + 3.0 mg/l picloram) ; MIC_1BAP (M1 + 0.05 mg/l BAP); MIC_2BAP (M1 + 2.0 mg/l glycine); MIC_3BAP (M1 + 1,000 mg/l glutamine); MIC_4BAP (M1 + 100 mg/l casein hydrolyzate); MIC_5BAP (M1+ 2.0 mg/l glycine + 1,000 mg/l glutamine); MIC_6BAP (M1+ 2.0 mg/l glycine + 100 mg/l casein hydrolyzate); MIC_7BAP (M1+ 2.0 mg/l glycine + 1,000 mg/l glutamine + 100 mg/l casein hydrolyzate). ±S: Standard deviation; Average values were calculated from three repetitions per condition of medium; in the line values followed of a same letter are not statistically different (test of Newman-Keuls at 5 %).

3.1.3 Effect of amino acids in presence of 3 mg/l 2,4-D

Amino acids influenced very significantly ($p < 0.001$) fresh weight and dry weight of callus in presence of 2.4-D alone. MIC'_6 medium has the highest dry weight (53.83 mg). The MIC'_5 medium induced the lowest dry weight (1.38 mg) (Table 7). Calli obtained on these different media are compact and brown. The culture media tested in this study are favorable to the initiation of callus in pineapple. MIC'_6 seems to be the most callogenesis medium because of its possess the high dry matter of callus. However, addition of some amino acids reduced the callus dry weight compared to the control medium MIC'_1 and most of calli have a compact structure. These calli would not suitable for the somatic embryos induction. Only friable calli were used for the induction of somatic embryos.

Table 7: Effect of amino acids in presence of 2,4-D on pineapple callus induction after four weeks on MIC' media

Culture media							
Parameters	**MIC'_1**	**MIC'_2**	**MIC'_3**	**MIC'_4**	**MIC'_5**	**MIC'_6**	**MIC'_7**
Initiation rate (%±s)	40.37 ± 6.12a	41.37 ± 9.12a	57.61 ± 10.62a	45.22 ± 11.64a	41.13 ± 7.23a	57.35 ± 10.47a	39.65 ± 12.03a
Fresh weight (mg±s)	18.75 ± 0.32c	12.75 ± 0.27d	53.67 ± 0.13a	12.75 ± 0.91d	2.04 ± 0.18e	55.37 ± 0.003a	31.42 ± 0.003b
Dry weight (mg±s)	11.25 ± 0.25bc	2.19 ± 0.16de	49.53 ± 0.94a	10.66 ± 0.85c	1.38 ± 0.11e	53.83 ± 0.39a	3.15 ± 0.14f
Water content (%±s)	39.85 ± 1.28c	82.76± 0.37b	7.71 ± 0.73f	16.05 ± 4.67e	31.78 ± 1.41d	2.78 ± 1.35e	89.96 ± 0.14a
Texture	Compact	Compact	Friable	Compact	Compact	friable	Compact
Color	Brown	Brown	Brown	Brown	Whitish	Brown	Brown

MIC' (Callus induction medium containing amino acids in combination with 3.0 mg/l 2,4-D); M2 (MSB_5 + 3.0 mg/l 2,4-D) ; MIC'_1 (M2); MIC'_2 (M2 + 2.0 mg/l glycine); MIC'_3 (M2 + 1,000 mg/l glutamine); MIC'_4 (M2 + 100 mg/l casein hydrolyzate); MIC'_5 (M2 + 2.0 mg/l glycine + 1,000 mg/l glutamine); MIC'_6 (M2 + 2.0 mg/l glycine + 100 mg/l casein hydrolyzate); MIC'_7 (M2 +2.0 mg/l glycine + 1,000 mg/l glutamine + 100 mg/l casein hydrolyzate). ±S: Standard deviation; Average values were calculated from three repetitions per condition of medium; in the line values followed of a same letter are not statistically different (test of Newman-Keuls at 5 %).

3.1.4 Effect of amino acids in presence of 2,4-D and kinetin

The results of Table 8 show a very significant difference between the different media for the dry weight of callus ($p < 0.001$). The MIC'_6KIN medium allowed the highest dry weight calli (54.91 mg). The MIC'_1Kin and MIC'_2KIN media induced the lowest dry weight calli, 5.47 and 8.82 mg, respectively. The calli produced on these culture media are mostly compact. Calli from the MIC'_1KIN media; MIC'_2KIN; MIC'_5KIN and MIC'_7KIN are yellowish, whereas those from the MIC'_3KIN and MIC'_6KIN media are yellowish green. A slight browning of the calli on the MIC'_4KIN medium is observed. The calli induced on these media have a very high water content ranging from 23.94 to 92.41%. The calli obtained on the media without kinetin are almost all compact, contrary to those induced on media provided with kinetin, which are mostly friable. We therefore deduce that kinetin's intake improved the callus texture. Thus, data from both tables (7 and 8) show that kinetin's intake improved the amount of dry matter in some media, but decreased it in other media such as the control (MIC'_1 = 11.25 mg and MIC'_1KIN = 5.47 mg) and the MIC'_6 (49.53 mg) and MIC'_6KIN (47.83 mg). The induction of callus in the presence of kinetin gave predominantly friable calli interesting for the induction of somatic embryos.

Table 8: Effect of 2,4-D in combination with KIN and amino acids on pineapple callus induction after four weeks on MIC'KIN media

Culture media							
Parameters	**MIC'$_1$Kin**	**MIC'$_2$ Kin**	**MIC'$_3$ Kin**	**MIC'$_4$ Kin**	**MIC'$_5$ Kin**	**MIC'$_6$ Kin**	**MIC'$_7$ Kin**
Initiation rate (%±s)	57.93 ± 3.95a	57.26 ± 5.62a	61.18 ± 4.23a	62.56 ± 7.23a	56.25 ± 3.24a	62.93 ± 7.32a	59.74 ± 4.12a
Fresh weight (mg±s)	72.00 ± 0.38b	78.16 ± 0.23a	70.33 ± 0.46b	76.91 ± 0.21a	43.58 ± 0.77d	58.11 ± 0.29c	76.83 ± 0.87a
Dry weight (mg±s)	5.47 ± 0.17f	8.82 ± 0.91e	47.83 ± 0.11d	27.79 ± 0.12c	33.08 ± 0.44b	54.91 ± 0.61a	52.11 ± 0.19a
Water content (%±s)	92.41 ± 0.06a	88.71 ± 0.06b	31.99 ± 1.08d	63.91 ± 1.08c	23.94 ± 1.08e	5.51 ± 0.42f	32.10 ± 0.73d
Texture	Compact	Friable	Friable	Friable	Friable	Friable	Friable
Color	Yellow	Yellow	Yellow-green	Brown	Yellow	Yellow-green	Yellow

MIC (Callus induction medium) was composed to 2.4-D (3 mg/l) in combination with KIN (0.2 mg/l) and three types of amino acids (MIC'KIN) ; M2 (MSB_5 + 3.0 mg/l 2,4-D) ; MIC'$_1$KIN (M2 + 0.2 mg/l kinetin); MIC'$_2$KIN (M2 + 2.0 mg/l glycine); MIC'$_3$KIN (M2 + 1,000 mg/l glutamine); MIC'$_4$KIN (M2 + 100 mg/l casein hydrolyzate); MIC'$_5$KIN (M2 + 2.0 mg/l glycine + 1,000 mg/l glutamine); MIC'$_6$KIN (M2 + 2.0 mg/l glycine + 100 mg/l casein hydrolyzate); MIC'$_7$KIN (M2 + 2.0 mg/l glycine + 1,000 mg/l glutamine + 100 mg/l casein hydrolyzate). ±S: Standard deviation; Average values were calculated from three repetitions per condition of medium; in the line values followed of a same letter are not statistically different (test of Newman-Keuls at 5 %).

3.1.5 Effect of amino acids in presence of picloram and kinetin

Analysis of the variances shows that the KIN-amino acid combination significantly influenced all the parameters studied ($p < 0.001$). However, not all culture media allowed the induction of calluses. The induced calluses are small. Except for MIC$^+_6$KIN and MIC$^+_7$KIN media that did not induce callus. The fresh weights of other calli induced on other media were very low (0.003 to 0.007 mg). The dry weight of the calli obtained varied from 0.0008 to 0.003 mg. The MIC$^+_3$KIN medium yielded the highest dry weight (0.0033 mg). All calli obtained on these media are compact and whitish in color (Table 9). The calli obtained on these media are compact and aren't ideal for somatic embryos induction.

Table 9: Effect of picloram in combination with KIN and amino acids on pineapple callus induction after four weeks on MIC$^+$KIN media

Culture media							
Parameters	**MIC$^+_1$KIN**	**MIC$^+_2$KIN**	**MIC$^+_3$KIN**	**MIC$^+_4$KIN**	**MIC$^+_5$KIN**	**MIC$^+_6$KIN**	**MIC$^+_7$KIN**
Initiation rate (%±s)	7.67 ± 0.14c	3.87 ± 0.05d	23.33 ± 0.51a	13.00 ± 0.42b	4.17 ± 0.07d	0 ± 0e	0 ± 0e
Fresh weigh (mg±s)	0.003 ± 0.001c	0.003 ± 0.002c	0.007 ± 0.001a	0.003 ± 0.001c	0.005 ± 0.001b	0 ± 0d	0 ± 0d
Dry weight (mg±s)	0.0008 ± 0.001c	0.0011 ± 0.001b	0.0033 ± 0.002a	0.0013 ± 0.001b	0.0007 ± 0.001c	0 ± 0d	0 ± 0d
Water content (%±s)	72.47 ± 1.35b	59.34 ± 3.01c	53.89 ± 2.84d	61.11 ± 2.24c	86.12 ± 0.63a	0 ± 0c	0 ± 0a
Texture	Compact	Compact	Compact	Compact	Compact	Compact	Compact
Color	Whitish	Whitish	Whitish	Whitish	Whitish	Whitish	Whitish

MIC$^+$ (Callus induction medium) was composed to picloram (3.0 mg/l) in combination with KIN (0.2 mg/l) and three types of amino acids (MIC$^+$KIN). M1 (MSB_5 + 3.0 mg/l picloram) ; MIC$^+_1$KIN (M1 + 0.2 mg/l kinetin) ; MIC$^+_2$KIN (M1 + 2.0 mg/l glycine + 0.2 mg/l kinetin) ; MIC$^+_3$KIN (M1 + 1,000 mg/l glutamine + 0.2 mg/l kinetin); MIC$^+_4$KIN (M1 + 100 mg/l casein hydrolyzate + 0.2 mg/l kinetin) ; MIC$^+_5$KIN (M1 + 2.0 mg/l glycine + 1,000 mg/l glutamine + 0.2 mg/l kinetin) ; MIC$^+_6$KIN (M1 + 2.0 mg/l glycine + 100 mg/l casein hydrolyzate + 0.2 mg/l kinetin) ; MIC$^+_7$KIN (M1 + 2.0 mg/l glycine + 1000 g/l glutamine + 100 mg/l casein hydrolyzate + 0.2 mg/l kinetin). ±S: Standard deviation; Average values were calculated from three repetitions per condition of medium; in the line values followed of a same letter are not statistically different (test of Newman-Keuls at 5 %).

3.1.6 Principal component analysis

The results showed that fifteen culture media make it possible to obtain friable calli. Thus, to better perceive the effect of different media on callogenesis, a principal component analysis (PCA) was carried out. Three parameters, such as fresh weight (FW), dry weight (DW) and water content (WC) were better characteristic of callus induction and quality, were retained. PCA has shown that axis 1 and 2 express a variability of 64.09 and 35.25 %, respectively. By considering selected parameters and distribution of the fifteen culture media as a function of the axes, four groups of media were obtained. Thus, group G1 (MIC'$_3$, MIC'$_6$, MIC'$_3$KIN, MIC'$_6$KIN, MIC'$_7$KIN and MIC$_7$BAP) is related to dry weight, G2 (MIC$_4$BAP and MIC'$_4$KIN) to fresh weight, G3 (MIC$_1$BAP, MIC$_2$BAP and MIC'$_2$KIN) to water content and G4 (MIC$_3$BAP; MIC$_6$BAP; MIC$_5$BAP and MIC'$_5$KIN) to no parameters (Figure 3). However, the media in G1 that give the most friable callus, therefore of good quality and likely to be used for somatic embryogenesis, are related to the dry weight of calli. This result clearly showed that MIC'$_3$KIN (3.0 mg/l 2,4-D + 1,000 mg/l glutamine + 2.0 mg/l kinetin); MIC$_7$BAP (3.0 mg/l picloram + 2.0 mg/l glycine + 1,000 mg/l glutamine + 100 mg/l casein hydrolyzate + 0.05 mg/l BAP) and MIC'$_7$KIN (3.0 mg/l 2,4-D + 2.0 mg/l glycine + 1,000 mg/l glutamine + 100 mg/l casein hydrolyzate + 2.0 mg/l kinetin) media are the most favorable to induce calli in pineapple. All these high quality calli from the G1 media are likely to be used for somatic embryogenesis. Thus, the DW parameter best characterize callogenesis in pineapple. Therefore, MIC$_7$BAP medium which has a significantly higher calli dry weight seems to be more conducive to the induction of pineapple

calli. Picloram (auxin) and BAP (cytokinin) are the best plant growth regulators to induce callus from pineapple, in combination with glutamine, glycine and casein hydrolyzate. The presence of these amino acids is essential to induce high quality of callus in pineapple. Thus, the culture media (G1) which produced the dry weight calli were retained for somatic embryogenesis.

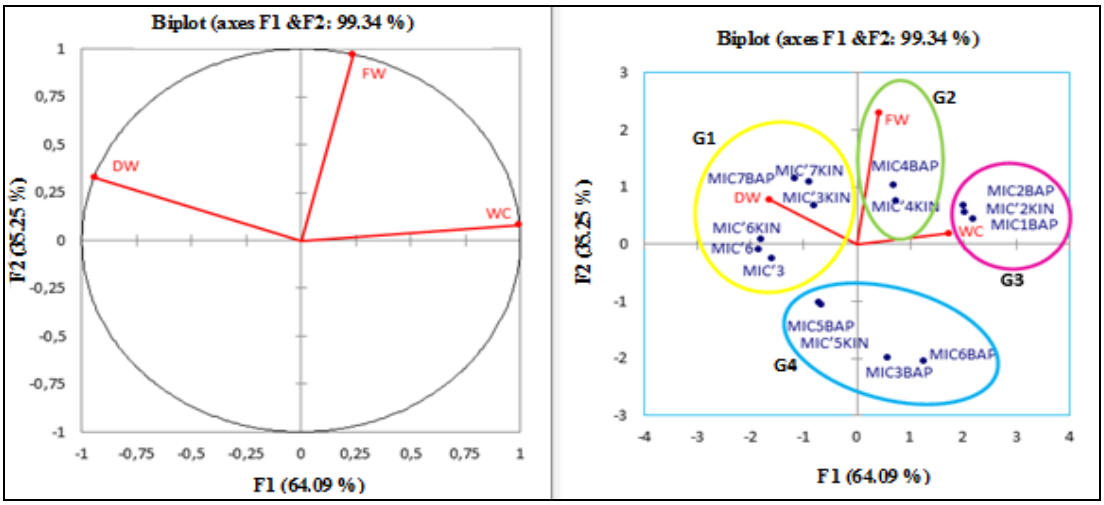

Fig 3: Distribution of callogenesis parameters and media culture in the plane formed by the factorial axes 1 and 2 of the correlation circle

MIC (Callus induction medium) ; DW (Dry weight); FW (Fresh weight); WC (Water content); G (Group); MIC'$_3$ (3.0 mg/l 2,4-D + 1,000 g/l glutamine) ; MIC'$_6$ (3.0 mg/l 2,4-D + 2.0 mg/l glycine + 100 mg/l casein hydrolyzate) ; MIC'$_3$KIN (3.0 mg/l 2,4-D + 1,000 g/l glutamine + 0.2 mg/l kinetin); MIC'$_6$KIN (3.0 mg/l 2,4-D + 2.0 mg/l glycine + 100 mg/l casein hydrolyzate + 0.2 mg/l kinetin) ; MIC'$_7$KIN (3.0 mg/l 2,4-D + 2.0 mg/l glycine + 1,000 g/l glutamine + 100 mg/l casein hydrolyzate + 0.2 mg/l kinetin) ; MIC$_7$BAP (3.0 mg/l picloram + 2.0 mg/l glycine + 1,000 g/l glutamine + 100 mg/l casein hydrolyzate + 0.05 mg/l BAP) ; MIC$_4$BAP (3.0 mg/l picloram + 100 mg/l casein hydrolyzate + 0.05 mg/l BAP); MIC'$_4$KIN (3.0 mg/l 2,4-D + 100 mg/l casein + 0.2 mg/l kinetin) ; MIC$_1$BAP (3.0 mg/l picloram + 0.05 mg/l BAP) ; MIC$_2$BAP (3.0 mg/l picloram + 2.0 mg/l glycine + 0.05 mg/l BAP) ; MIC'$_2$Kin (3.0 mg/l 2,4-D + 2.0 mg/l glycine + 0.2 mg/l kinetin); MIC$_3$BAP (3.0 mg/l picloram + 1,000 g/l glutamine + 0.05 mg/l BAP) ; MIC$_6$BAP (3.0 mg/l picloram + 2.0 mg/l glycine + 100 mg/l casein hydrolyzate + 0.05 mg/l BAP) ; MIC$_5$BAP (3.0 mg/l picloram + 2.0 mg/l glycine + 1,000 g/l glutamine + 0.05 mg/l BAP); MIC'$_5$Kin (3.0 mg/l 2,4-D + 2.0 mg/l glycine + 1,000 g/l glutamine + 0.2 mg/l kinetin)

3.2 Somatic embryogenesis

Effect of culture media on somatic embryos induction

Statistical analysis (Table 10) revealed that all culture media allowed the induction of embryogenic cells (Figure 4). However, the composition of these media did not influence (p = 0.26) the induction rate of embryogenic cells (ECIR) but rather that of embryogenic cells number (ECN) induced (p < 0.0001). MIE$_1$ and MIE$_5$ media induced the largest ECNs respectively 77 and 70.33 cells. MIE$_3$ (5 cells) and MIE$_6$ (1.33 cells) gave the lowest NCEs. Analysis of these results showed that MIE$_1$ (MIE + 3.0 mg/l picloram + 2.0 mg/l glycine + 1,000 mg/l glutamine + 100 mg/l casein hydrolyzate + 0.05 mg/l BAP) induced a greater NCE (77 cells) compared to that (1.33 cells) of MIE$_6$ medium (MIE + 3.0 mg/l 2,4-D + 2.0 mg/l glycine + 1,000 mg/l glutamine + 100 mg/l casein hydrolyzate + 0.2 mg/l kinetin). Since these two media differ in their hormonal regimen, the hormonal combination used significantly influenced ECN. Thus, the picloram-BAP combination induced the largest number of embryogenic cells (77 cells) compared to the results obtained with the 2,4D-kinetin combination (1.33 cells). The analysis of variances and the composition of MIE$_3$ (MIE + 3.0 mg/l 2,4-D + 2.0 mg/l glycine + 100 mg/l casein hydrolyzate) and MIE$_5$ (MIE + 3.0 mg/l 2,4-D + 2.0 mg/l glycine + 1,000 mg/l glutamine + 100 mg/l casein hydrolyzate + 0.2 mg/l kinetin) media revealed that the addition of cytokinin (kinetin) significantly stimulated the ability of calli to induce embryogenic cells on the MIE$_5$ medium. However, analysis of the results obtained with MIE$_2$ (MIE + 3.0 mg/l 2,4-D + 1,000 mg/l glutamine) and MIE$_4$ (MIE + 3.0 mg/l 2,4-D + 1,000 mg/l glutamine + 0.2 mg/l kinetin), respectively 23.67 and 5 cells, showed that the addition of kinetin to the MIE$_2$ medium inhibited the formation of embryogenic cells.

Table 10: Effect of culture media on somatic embryos induction

	Culture media					
Parameters	**MIE$_1$**	**MIE$_2$**	**MIE$_3$**	**MIE$_4$**	**MIE$_5$**	**MIE$_6$**
ECIR (% ± s)	63.64 ± 9.40a	54.42 ± 9.51a	35.58 ± 4.65a	35.58 ± 9.81a	54.82 ± 9.41a	54.42 ± 9.41a
ECN (number ± s)	77.00 ± 2.52a	23.67 ± 2.73b	24.67 ± 0.88b	5.00 ± 1.15c	70.33 ± 4.91a	1.33 ± 0.33c

ECIR (embryogenic cells induction rate) ; ECN (embryogenic cells number) ; MIE (MSB_5 – ½ [NH_4NO_3] + [KNO_3]) ; MIE_1 (MIE + 2.0 mg/l glycine + 1,000 mg/l glutamine + 100 mg/l casein hydrolyzate + 0.05 mg/l BAP) ; MIE_2 (MIE + 3.0 mg/l 2,4-D + 1,000 mg/l glutamine) ; MIE_3 (MIE + 3.0 mg/l 2,4-D + 2.0 mg/l glycine + 100 mg/l casein hydrolyzate) ; MIE_4 (MIE + 1,000 mg/l glutamine + 0.2 mg/l kinetin) ; MIE_5 (MIE + 2.0 mg/l glycine + 100 mg/l casein hydrolyzate + 0.2 mg/l kinetin) ; MIE_6 (MIE + 2.0 mg/l glycine + 1,000 mg/l glutamine +100 mg/l casein hydrolyzate + 0.2 mg/l kinetin). ±S: Standard deviation; Average values were calculated from three repetitions per condition of medium; in the line values followed of a same letter are not statistically different (test of Newman-Keuls at 5 %).

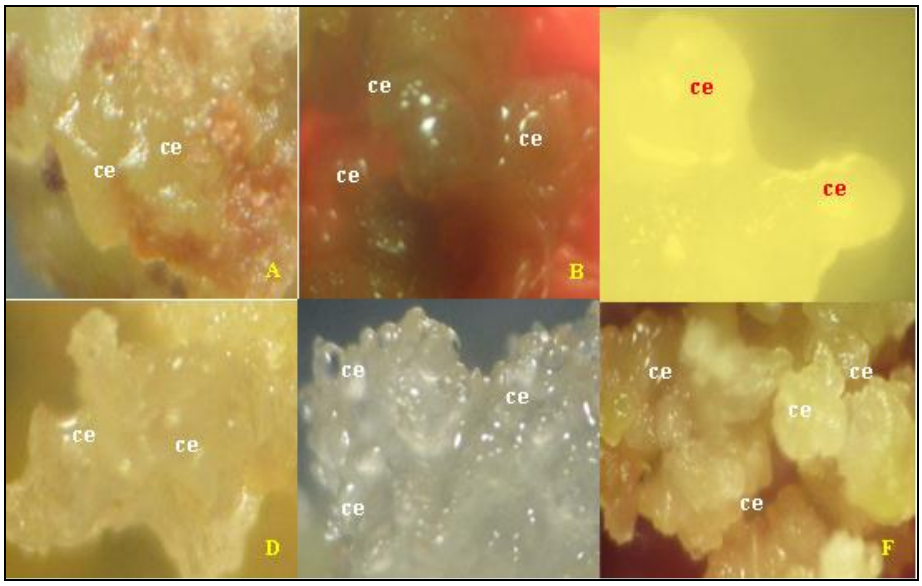

Fig 4: Somatic embryogenic cells observed on calli from somatic embryogenesis induction medium (MIE) Gx400 Embryogenic cells (ce). A : MIE_2 ; B : MIE_1 ; C : MIE_4 ; D : MIE_3 ; E : MIE_6 ; F : MIE_5

4. Discussion

All the media tested in this work, allowed callus formation. Excepted the glycine-casein supplemented medium and the control (medium without any amino acid), the addition of amino acids (alone or in combination) to the culture medium resulted in a decrease in the fresh and dry weights of callus. Amino acids inhibit the formation of callus. This inhibition is due to competition between auxin and amino acids to occupy binding sites [22, 23]. Zenk *et al.* [11] reported similar competition between glycine and glutamine. Indeed, glutamine is an important source of nitrogen which has already shown its positive effects on the production of organic matter in Bromeliaceae [24, 25]. However, its combination with glycine reduces the use of NH_4^+ by inactivating localized glutamate synthase in chloroplasts and cytoplasm. According to Morot-Gaudry *et al.* [26], the importance of glutamine is due to the fact that it is the precursor of all other amino acids. It would provide nitrogen for the biosynthesis of amino acids. This author noted that L-glutamate and L-glutamine are the first amino acids synthesized after integration of the ammonium ion to the carbon skeleton. By transamination, their nitrogen is then transferred to other carbon substrates (α-keto acid) to form new amino acids (aspartate, α -alanine, glycine...) which are precursors of all other amino acids. The synthesis of L-glutamate and L-glutamine is catalyzed by glutamate synthase (GOGAT) and glutamine synthetase (GS) in the presence of NADPH and ATP. Inactivation of these enzymes by glycine prevents the use of NH_4^+ for the synthesis of amino acids. However, the addition of arginine or ammonium ions (NH_4^+) would compensate this inactivation. Thus, glycine would inhibit the action of glutamine. This would explain the low dry weight of callus obtained on the glycine-glutamine-casein and glycine-glutamine supplemented-media. The high dry weight of callus on glycine-casein medium could be explained by a synergistic action between these molecules.

For media with BAP, the control medium (without amino acid) produced callus with a lower dry matter. On the other hand, addition of amino acids (alone or in combination) to these culture media (casein-BAP, glycine-glutamine-BAP and glycine-glutamine-casein-BAP) improved the dry weight of the calli. Thus, the association BAP-amino acids would allow a gain of dry matter of callus. Indeed, according to Gendy *et al.* [27], in some species, the addition of small amounts of cytokinin to the medium containing auxin may increase the percentage of callus formation. This increase in the amount of dry matter is mainly due to the role of cytokinin which actively stimulating cell division [28]. Various media tested in this work exhibited friable calluses with color varying from the yellowish to the greenish. These results have already been reported in *Abies alba* Mill [29]. These authors also observed browning of callus after addition of casein. BAP would cause greening of callus and improve their texture by transforming it from compact to friable. According to Seddiki and Chesnoy [30], BAP causes stimulation of chlorophyllogenesis and differentiation of plastids. It would therefore involve in photosynthesis. In contrary to the callus color observed in the present work with the cytokinin BAP, Chatibi *et al.* [31] reported that the addition of this plant growth regulator

induced the formation of whitish callus in pistachio. Therefore, the type of explant and the hormonal combination might influence the callus color. The BAP-amino acid interaction which improved the dry weight could be explained by the fact that the BAP acted in synergy with the amino acids for the production of dry matter. Indeed, according to Zenk *et al.* [11], this synergy allowed the release of nitrogen in amino acids and which would be available to the cells metabolism. The results reported in this work are in agreement with those of Chatibi *et al.* [31] and Merkle [32]. These authors observed a high level of callus on media containing amino acids and BAP respectively in *Pistacia vera* and *Liquidambar styraciflum*. It should be noted that the absence of a significant difference in the initiation rate of callus on different media is due to the fact that organic supplements such as amino acids (Glycine, Glutamine) and casein hydrolyzate influence formation and growth of callus in some herbaceous plants [33] including pineapple.

Concerning the inhibition observed on media without kinetin in presence of 2,4-D, Yemet *et al.* [22] showed that in the presence of picloram, amino acids inhibit callus formation due to competition between amino acids. This competition is very accentuated in the presence of 2,4-D. Indeed, 2,4-D is much more ready to occupy the binding sites. The results obtained in this work confirm those of Fotso *et al.* [34] in *Tylophora indica*, 2,4-D at very high concentrations induced tissue necrosis [35] and thus, browning of all most of callus observed on those culture media.

The increasing of dry matter observed in several culture media containing kinetin in presence of 2,4-D, could be explained by the fact that the addition of small quantities of cytokinin to the auxin-supplemented medium can improve the percentage of callus formation [34]. This result suggests a synergistic and/or complementary effect of auxins and cytokinins on callogenesis. This complementary effect on callogenesis has already been demonstrated in other woody species such as *Quercus suber*, *Mangifera indica* and *Citrus spp* [35, 36, 37]. However, 2,4-D concentrations reduces kinetin potentiality at higher levels as reported by Houndonougbo [38]. This mechanism of inhibition was briefly mentioned by Nielsen *et al.* [39]. These authors suggested that a receptor and two models of binding or binding sites allow the action of cytokinin in the cell. The receptor-active hormone complex is the only site used by endogenous hormones. High concentrations of exogenous hormones compete with endogenous hormones for occupancy of active sites or change the conformation (structure) of the receptor site and thus reduce the total number of functional complexes. This would differently influence nutrients uptake from culture medium. The analysis of the variances revealed that the culture media using picloram as auxin produced callus of best qualities that those using 2,4-D. This result confirms the statement made by Evans *et al.* [40], who asserts that a high concentration of auxin and a low concentration of cytokinins would promote abundant callus proliferation. Indeed, callogenesis carried out on culture media containing 3 mg/l of picloram and 0.05 mg/l of BAP is predisposed to a better callogenesis than that carried out on media with 3 mg/l of 2.4-D and 0.2 mg/l of kinetin. However, media with 2,4-D have induced low dry weight calli but have a very high friability index which, according to Yapo *et al.* [12], could be very interesting for somatic embryogenesis. Indeed, according to Trolinder and Goodin [41], embryo production from tissue culture begins with their ability to differentiate into friable calli. This characteristic would therefore be an indicator of a good aptitude for somatic embryogenesis. The very low performance of amino acids in the presence of picloram and kinetin observed during callogenesis was reported by Yapo *et al.* [12]. This hormonal combination would induce a low expression of the explant.

According to somatic embryogenesis, only friable calli induced somatic embryos. These results were observed by Firoozabady and Moy [15]. They claim that there are five types of embryogenic tissue, including friable cell clusters. According to them, somatic embryos are initiated in the presence of high concentrations of auxins such as picloram, dicamba and 2,4-D. This explains the relatively high induction rate of embryogenic cells (ECIR) observed on all culture media containing either picloram or 2,4-D in this study. However, these results indicate that the MIE_1 medium containing the hormonal combination picloram/BAP induced the greatest NCE compared to that of the MIE_5 medium containing the combination 2,4-D/kinetin. This indicates that picloram and BAP have a strong synergy action for the induction of embryogenic cells relative to that of the 2.4-D/kinetin interaction. Indeed, according to Yapo *et al.* [12], picloram has more significant effect on somatic embryo induction than 2.4-D. Also, Staden *et al.* [42] noted that this difference in action would be due to the cytokinin/auxin ratio (kinetin/2,4-D) which would not be qualitative and ideal for optimal callus expression. This difference in expression between MIE_1 (MIE + 2.0 mg/l glycine + 1,000 mg/l glutamine + 100 mg/l casein hydrolyzate + 0.05 mg/l BAP) and MIE_5 (MIE + 2.0 mg/l glycine + 100 mg/l casein hydrolyzate + 0.2 mg/l kinetin) would also be correlated with the difference in amino acids (source of nitrogen) that constitute them. Thom *et al.* [43] and Yapo *et al.* [12] have shown that the addition of amino acids to the culture medium immediately provides the cells with an available nitrogen source that would be rapidly used by these cells to stimulate embryogenesis. According to these authors, nitrogen is used selectively as nitrate for embryos induction. Thus, the high levels of nitrate would have a significant effect on embryogenic cells induction. The high number of embryogenic cells observed on the MIE_1 medium would therefore be due to a low NH^+_4/NO^-_3 ratio. Nitrogen in nitrate form therefore appears to be indispensable for somatic embryos induction [12]. The low number of embryogenic cells observed in MIE_3 relative to the MIE_5 medium, shows that the addition of kinetin to the culture medium stimulated embryogenic cell induction. Indeed, according to Arnold *et al.* [44], phytohormones induce the differentiation of calli in polarized cells that lead to somatic embryogenesis. However, the decrease in the number of embryogenic cells observed after addition of kinetin to the MIE_2 medium (MIE + 3.0 mg/l 2,4-D + 1,000 mg/l glutamine) shows that the action of kinetin during the embryogenic cells induction would depend on the amino acid combination of the culture medium. The sensitivity of the callus to a phytohormone is affected by the presence of nitrogen in the culture medium [45, 46]. At the end of this work, it is remembered that the induction of

embryogenic cells in pineapple would be under an interaction control of phytohormones and a low NH^+_4/NO^-_3 ratio. MIE_1 (MIE + 2.0 mg/l glycine + 1,000 mg/l glutamine + 100 mg/l casein hydrolyzate + 0.05 mg/l BAP) and MIE_5 (MIE + 2.0 mg/l glycine + 100 mg/l casein hydrolyzate + 0.2 mg/l kinetin) are the most embryogenic.

5. Conclusion

The main objective of this work was to determine an optimum medium inducing callogenesis and subsequent somatic embryogenesis, for varietal improvement of pineapple through the production of healthy pineapple releases free pesticide residues. A protocol was therefore developed to determine the combinations of 6-benzylaminopurine and kinetin and amino acids (glycine, glutamine, casein hydrolyzate) necessary to induce friable callus suitable to initiate somatic embryos in pineapples. At the end of this study on callogenesis and somatic embryogenesis in pineapple (*Ananas comosus*), MIC_7BAP (glycine-glutamine-casein-BAP) medium has a higher callogenic potential than other culture media. It provides friable and high dry weight calli. MIE_1 (MIE + 2.0 mg/l glycine + 1,000 mg/l glutamine + 100 mg/l casein hydrolyzate + 0.05 mg/l BAP) and MIE_5 (MIE + 2.0 mg/l glycine + 100 mg/l casein hydrolyzate + 0.2 mg/l kinetin) are the most embryogenic. It results from this comparison that the association of amino acids is beneficial for the initiation of pineapple calli. Callogenesis and somatic embryogenesis in pineapple (*Ananas comosus*) vary according to the organic compound and are strongly influenced by cytokinins such as 6-benzylaminopurine and kinetin.

6. References

1. Anonymous. Présentation de la Côte d'Ivoire. http://www.bnetd.ci. 04 february 2009.
2. Organisation Centrale des producteurs exportateurs d'Ananas et de Bananes. Rapport. annuel, document OCAB, Abidjan, 2000, 32.
3. Adomon S. Une filière aux abois, *in* Quot. l'Int., Group. Olym Ed., Côte d'Ivoire, Abidjan. 2007, 12.
4. Tanoh R. Ananas-La misère des planteurs, *in* Quot. Nord-Sud, Group. Olym Ed., Côte d'Ivoire, Abidjan, 2008, 12.
5. Anonymous. Nouvelles exigences de qualité de l'Union européenne pour l'ananas ivoirien. http://www.panapress.com. 04 february 2009.
6. Danso K, Aye K, Amiteye S, Amoatey H. Effect of 6-Benzylaminopurine and 3-Naphthalene Acetic Acid on *in vitro* production of MD2 Pineapple planting materials. World Appl. Sci. J. 2008; 3(4): 614-619.
7. Collins J. The pineapple *in* Leo. Hill Ltd, London, UK. 1968, 295.
8. Zrÿd J. Cultures de cellules, tissus et organes végétaux. Fondements théoriques et utilisations pratiques. Press. Polytechn. Romand. 1992, 308.
9. Murshige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissues culture. Physiol. Plant. 1962; 15: 473-497.
10. Gamborg O, Miller R, Ojima K. Nutriment requirements of suspension cultures of soybean root cells. Exp. Cell. Research 1968; 50: 151-158.
11. Zenk M, El-Shagi H, Schulte U. Anthraquinone production by cell suspension cultures of *Morinda citrifolia*. Planta. Med. Suppl. 1975; 1-79.
12. Yapo E, Tanoh K, Mongomaké K, Kouadio J, Kouamé P, Merillon J. *Et al.* La régénération de l'ananas Ananas *comosus* L. par embryogenèse somatique végétale. J. Bioch Plant Biotechnol. 2011a; 20 (2): 196-204.
13. Daquinta M, Cisneros A, Rodriguez Y, Escalona M, Perez M, Luna I. *et al.* Somatic embryogenesis in pineapple *Ananas comosus*. Acta Horticult. 1997; 425:251-257.
14. Sripaoraya S, Marchant R, Brian P, Davey M. Plant regeneration by somatic embryogenesis and organogenesis in commercial pineapple *Ananas comosus*. *In vitro* Cell. Dev. Biol. 2003; 39(5): 450-454.
15. Firoozabady E, Moy Y. Regeneration of pineapple plants via somatic embryogenesis and organogenesis. *In Vitro* Cell Dev Biol Plant, 2004; 40:67-74.
16. Yapo E, Kouakou K, Bognonkpe J, Kouame P, Kouakou T. Comparison of pineapple fruit characteristics of plants propagated in three different ways: by suckers, micro propagation and somatic embryogenesis. J. of Nutrit. and Food Sci. 2011b; 1(4): 1-8.
17. Wang Y, Wang X, MA Z, Zhang G, Han G. Somatic embryogenesis and plant regeneration from two recalcitrant genotypes of *Gossypium hirsutum* L. Agric. Sci. Chin. 2006; 5(5): 323-329.
18. Ika R, Ika M. *In vitro* culture of Pineapple organogenesis and somatic embryogenesis: Its utilization and prospect. Bulet. Agrobio. 2003; 6 (1): 34-40.
19. Vits L, Van Camp G, Coucke P, Wilson G, Schrander-Stumpel C. Schwarz C *et al.* MASA syndrome is due to mutations in the L1 CAM gene. Nature Genetics, 1994; 7: 408-413.
20. Nomura K, Komamime A. Physiological and biochemical aspect of somatic embryogenesis. Current Plant Science and Biotechnology in Agriculture, 1995; 20: 249-265.
21. Dagnelie P. Théorie et méthodes statistiques, application agronomique. Gembloux, Belgique: Press. agron. de Gemb. 1980; 2: 452.
22. Yemet A, Tarassenko L, Blume Y. Effcient callus formation and plant regeneration of goosegrass *Eleusine indica* L. Gaertn.] Plant Cell Rep. 2002; 21: 503-510.
23. Jheng F, Liauh Y, Chung J, Huang P. Enhancement of growth and regeneration efficiency from embryogenic callus culture of *Oncidium* Gower Ramsey by adjusting carbohydrate sources, Plant Sci. 2006; 170: 1133-1140.
24. Mercier H, Kerbay G. The importance of tissue culture technique for conservation of endangered Brazilian bromeliads from Atlantic rain forest canopy. Selb. 1998; 16: 147-149.
25. Hamasaki R, Purgatto E, Mercier H. Glutamine enhances competence for organagenesis in pineapple leaves cultivated *in vitro*. Braz. J. Plant. Physiol. 2005; 17(4): 383-389.
26. Morot-Gaudry J, Hirel B, Phillipson B, Murchie E, Suzuki A, Kunz C *et al.* Manipulating the pathway of ammonia assimilation in transgenic non-legumes and legumes. Zeitsch. für Pflanz. und Bod. 1998; 160 : 283-290.
27. Gendy C, Sene M, Le B, Vidal J, Van K. Somatic

embryogenesis and plant regeneration in *sorghum bicolor* L. Moench. Plant Cell Report. 1996; 15: 900-904.
28. Haberlandt G. Zur Physiologie der Zellteilung. Sitzber. K. Preuss. Akad. Wiss. 1913, 318.
29. Térèzia S, Jàn S. Somatic embryogenesis and plantlet regeneration from cotyledon explants isolated from emblings and seedling of hybrid firs. J. Plant Physiol. 2001; 158: 747-755.
30. Seddiki A, Chesnoy L. Multiplication végétative et embryogénèse somatique: étude de variants obtenus chez le riz Oryza sativa. L. Thèse de doctorat : Sci. biol. et fond. app. Psy. : Paris 7 France: 1994, 183.
31. Chatibi A, Kchouk M, Zemni H, Ghorbel A. Organogenèse *in vitro* du pistachier *(Pistacia vera* L.) CV. Mateur à partir de feuilles d'embryons zygotiques. Ci Heam Opt. Med. pp. 2007; 131-138.
32. Merkle S, Kimberly A, Battle P, Bailey R. Somatic embryogenesis and plantlet regeneration from immature and mature tissues of sweetgum *Liquidamber styraciflua*. Plant Sci. 1998; 132: 169-178.
33. Torres K, Mateo F, Melendez-Hevia E. Tissue culture of strawberry *Fragaria*. In *Tiss. Cult. Techn. for horticult. crops*, New York: Avi, 1989; 96-101.
34. Fotso S, Donfagsiteli T, Omokolo N. Comparaison des premières étapes de l'embryogenèse somatique chez *Baillonella toxisperma* et *Vitellaria paradoxa* Sapotacées. Biotechnol. Agron. Soc. Environ. 2008 ; 12 (2): 131-138.
35. El kbiach ML, Lamarti A, Abdali A, Badoc A. Culture *in vitro* des bourgeons axillaires de chêne-liège *Quercus suber* L. Bull. Soc. Pharm. Bordeaux. 2002 ; 141 : 89-104.
36. Lilian FP, Luzminda R, Carlos-Refuerzo, Ramon CB. Somatic embryogenesis and plantlet regeneration in mango *Mangifera indica* L. *In vitro* Cell. Biol.-Plant. 2002; 38: 173-177.
37. Label K, Handaji N, Brhadda N, Arsalane N, Gmira N, Essalhi E, *et al.* Optimisation de l'induction de callogenèse à partir des embryons excisés de mandariniers *Citrus spp*. J. Appl. Biosci. 2015 ; 89:8300-8310.
38. Houndonougbo A. Influence des différentes concentrations d'acide indole-3-acétique, d'acide naphtalène-acétique, d'acide 2,4-Dichlorophenoxyacétique et de kinétine sur la callogenèse et l'organogenèse *in vitro* de fragment d'entre nœud de deux variétés de patate douce *Ipomaea batatas* L. Agron. 1989; 9: 653-660.
39. Nielsen J, Hansen J, Brandt K. Synergism of thidiazuron and benzyladenine in axullary shoot formation depends on sequence of application in *Miscanthus x ogiformits* 'Gigantenus'. Plant cell. Tiss. Org. Cult. 1995; 41 : 165-170.
40. Evans D, Sharp W, Flick C. Growth and behavior of cell cultures: embryogenesis and organogenesis. In T. A. Thorpe Ed. Plant tissue culture: methods and applications in agriculture. Acad. press, New York. 1981; 45-114.
41. Trolinder N, Goodin J. Somatic embryogenesis and plant regeneration in cotton *Gossypium hirsutum* L Requirements for embryo development and plant regeneration. Plant Cell Tiss. Org. Cult. 1988; 12: 43-53.
42. Staden JV, Zazimalova E, George EF. Plant growth regulator II: cytokinins, their analogues and antagonists. *In*: Georges EF, Michael AH & De Klerk G-J Eds *Plant propagation by tissue culture*. United Kingdom: Springer, 2007; 205-226.
43. Thom M, Maretzki A, Komer E, Sakai WS. Nutrient uptake and accumulation by sugar cane cell culture in relation to the growth cycle. Plant Cell, Tissue and Organ Culture. 1981; 1: 3-14.
44. Arnold SV, Sabala I, Bozhkov P, Dyachok J, Filonova L. Developmental pathways of somatic embryogenesis. Plant Cell Tissue Org, 2002; 69: 233-249.
45. Fuentes-Cerda CF, Monforte-Gonzalez M, Mendez-Zeel M, Rojas-Herrera R, Loyola-Vargas VM. Modification of the embryogenic response of *Coffea arabica* by the nitrogen source. Biotechnology Letter. 2006; 23: 1341-1343.
46. Cangahuala-Inocente GC, Dal Vesco LL, Steinmacher D, Torres AC, Guerra MP. Improvement in somatic embryogenesis protocol in Feijoa *Acca sellowiana* Berg Burret: Induction, conversion and synthetic seeds. Scientia Horticulturae. 2007; 111(3): 228-234.