Journal of Experimental Biology and Agricultural Sciences

http://www.jebas.org

ISSN No. 2320 – 8694

Effect of culture medium composition on somatic embryos induction and maturation of pineapple [*Ananas comosus* (L.) var. (Smooth Cayenne)]

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Received – August 11, 2022; Revision – December 11, 2022; Accepted – February 27, 2023
Available Online – February 28, 2023

DOI: http://dx.doi.org/10.18006/2023.11(1).140.149

KEYWORDS

Smooth Cayenne

Pineapple

Somatic embryogenesis

Embryos induction

Embryos maturation

Côte d'Ivoire

ABSTRACT

The cultivation of pineapple contributes 1.6% of the gross Ivorian national product (GDP). However, this crop is facing a severe production crisis due to the aging of the orchards. Revising this sector requires the rejuvenation of orchards with healthy and improved planting material. This work was conducted to study the conditions for the efficient in vitro production of restorative pineapple planting material by somatic embryogenesis. The effects of seven culture media consisting of a different combination of nitrogen sources (casein hydrolyzate, glutamine, and glycine), cytokinins (kinetin or BAP), and auxins (2,4-D or picloram) were tested on somatic embryos induction and maturation in pineapple. Results of the study revealed that EIM_1 (EIM added with 3 $mg.L^{-1}$ picloram, 0.05 $mg.L^{-1}$BAP, 2 $mg.L^{-1}$ glycine, 1000 $mg.L^{-1}$glutamine, 100 $mg.L^{-1}$casein hydrolyzate) and EIM_5 (EIM added with 2 $mg.L^{-1}$glycine, 100 $mg.L^{-1}$casein hydrolyzate, 0.2 $mg.L^{-1}$kinetin) media induced the highest numbers of embryogenic cells, i.e., 154 and 149 cells respectively. Further, the EIM_5 medium was more embryogenic, with the most significant number of mature embryos (66 mature embryos), and allowed the observation of all embryonic maturation stages. Embryogenic cell induction in pineapple is thought to be controlled by a low NH_4^+/NO_3^- ratio in interactions with phytohormones. In the presence of 2,4-D, embryogenic cell maturation was improved by kinetin addition to the culture medium containing glycine and casein hydrolyzate.

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Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

Production and Hosting by Horizon Publisher India [HPI] (http://www.horizonpublisherindia.in/).

1 Introduction

Pineapple, originating from America, is eaten fresh or used for producing canned foods (Dick et al. 2015; Lucas 2020). It ranks third among tropical fruits, after banana and mango, with an average production of approximately 30 million tons (FAO 2020). In Côte d'Ivoire, fresh pineapple exports generate more than $223 million annually, and its cultivation contributes 1.6% to agricultural GDP (Nouza 2011; Anonymous 2022). The smooth cayenne is the main cultivated variety in Côte d'Ivoire because of its adaptation to climatic conditions (Leal and Coppens d'Eeckenbrugge 1996). Indeed, the smooth cayenne has been a pillar of the international pineapple trade. Côte d'Ivoire was the first pineapple supplier to the European Union because of the hegemony of the smooth cayenne (97% of the market). However, Ivorian production has steadily decreased since the end of the 1980s (Vagneron et al. 2009; Africa 24, 2022). For example, the production was 238,000 tons in 2000, dropping to less than 50,000 tons in 2021, revealing a drastic drop of more than 79% (Houessionon 2022). The degeneration of the plant material can explain this decrease. To overcome this difficulty, producers resort to higher than regular doses of phytosanitary products such as fibrophos (400 kg/ha), dolomite (500-750 kg/ha), kieserite (500 kg/ha), NPK (200 kg/ha), urea (1 g/plant), and potassium sulfate (2.5 g). Excess supply of these phytosanitary products causes an accumulation of chemical residues in the fruit beyond the maximum residue limit (for example: ethoprophos 0.01 mg/kg; fosetyl alumin 15 mg/kg; ethephon 2ppm) and makes the fruits more acidic. This is associated with the drop in exports from Côte d'Ivoire to the European market. Today, Côte d'Ivoire shares less than 2% in the European market (EUROSTAT 2014). In this context, the renewal of orchards with high-performance varieties is essential for improving smooth cayenne's fruit quality and yield. Thus, varietal selection programs based on interspecific hybridization have been initiated. However, they did not lead to the creation of varieties that could replace smooth cayenne because the conventional improvement of pineapple is difficult (Leal and Coppens d'Eeckenbrugge 1996; Akbar et al. 2003; Kouadio et al. 2017). However, *in vitro* culture of plant tissues by somatic embryogenesis appears to be an exciting tool for pineapple improvement. Indeed, somatic embryogenesis induces the formation of plants that conform to the mother plant, are rejuvenated, healthy, homogeneous, and have a high yield. In addition, these plants are free of contamination and pesticide residues, making them ideal materials for the renewal of orchards (Yapo et al. 2011). Many researchers have highlighted the mass multiplication of pineapples by somatic embryogenesis (Yapo 2013; Kouadio et al. 2017; Cacaï et al. 2021; Kessel-Domini et al. 2022). However, somatic embryogenesis is impacted by several culture parameters such as plant genotype, environmental conditions, cultural medium composition, and level of phytohormones (Rhimi et al. 2006; Kessel-Domini et al. 2022). Thus, the selection of culture media and phytohormones must meet the plant's nutrient requirements to allow its genetic potential to be fully expressed (Kouakou 2003; Usman et al. 2011). Therefore, growth regulators strongly influence somatic embryogenesis, and the most commonly used phytohormones include auxins and cytokinins (Kouadio et al. 2017; Cacaï et al. 2021). Also, many authors have suggested that a significant concentration of carbohydrates, amino acids, and nitrates are required for embryogenic skills acquisition during embryogenesis (Thiruvengadam et al. 2006; Yapo et al. 2011; Kouadio et al. 2017).

According to Daquinta et al. (1996), dicamba (auxin) induced embryogenic calli in smooth cayenne leaves and reported low embryogenic callus induction (approximately 55%). Sripaoraya et al. (2003) developed a methodology for somatic embryogenesis induction from the cultivar Phuket (Queens group) leaves. These authors reported an embryo induction rate of 58.3% using MS medium supplemented with sucrose (3%) and picloram (3.0 mg/L). However, the induction rate decreased with the increase in picloram concentration. Subsequently, different explants were used in smooth cayenne to develop more efficient protocols to induce somatic embryogenesis (Firoozabady and Moy 2004). After embryogenesis initiation, the maturation of embryos is obtained by culturing the embryogenic cells onto other media different from the induction media (Yapo 2013). However, the uses of the same medium for somatic embryo induction and maturation have been used by some researchers and not reported much difference. To simplify the somatic embryogenesis protocol and reduce the costs of pineapple production, this study sought a modified culture medium optimal for induction, followed by efficient somatic embryo maturation for healthy, high-performing plant material mass production. This study aimed to investigate the appropriate cultural medium and *in vitro* conditions for efficient and healthy pineapple planting material production by somatic embryogenesis.

2 Material and methods

2.1 Plant material

Pineapple (*Ananas comosus* var. Smooth cayenne, cv. CI 16) suckers used in this study were collected from the National Center of Agronomic Research (Anguédédou Station, Côte d'Ivoire). This study used the leaf base as the explant for the induction of somatic embryogenesis.

2.2 Methods

2.2.1 Disinfection of explants and shoot induction

The explants were disinfected using the method described by Yapo et al. (2011). Pineapple suckers were cleared of mature leaves and

root bases using a knife. Terminal buds were trimmed to approximately 2 cm. Under a laminar air flow hood, these buds were thoroughly washed with water. Then, they were disinfected for the 20s with alcohol (70%) and soaked in 3.6% (active chlorine) sodium hypochlorite for 30 min. With sterile distilled water, the buds were rinsed four times. The buds were removed entirely from the leaves using a blade mounted on a scalpel. These surface disinfected explants were transferred onto shoot initiation medium (MS basal medium with vitamin B_5, which was added with 0.2 $g.L^{-1}$ of glutamine, 0.01 $mg.L^{-1}$ of kinetin, and 30 $g.L^{-1}$ of sucrose). The pH of the prepared medium was adjusted to 5.8, and the medium was solidified by adding phytagel (2.5 $g.L^{-1}$).

2.2.2 Callus induction

In this study, calli were induced using Murashige and Skoog (1962) basal medium including vitamin B_5 (MSB_5 medium), supplemented with 3 $mg.L^{-1}$ picloram, 2 $mg.L^{-1}$ glycine, 1000 $mg.L^{-1}$ glutamine, 100 $mg.L^{-1}$ casein hydrolysate, 30 $g.L^{-1}$sucrose (Kouadio et al. 2017). The pH of the culture medium was adjusted to 5.5 and then solidified by adding 6 $g.L^{-1}$ of agar. The prepared medium was autoclaved under a pressure of 1 bar for 30 min at 121°C. Approximately 5 mm of leaf base from regenerated shoots was seeded onto the callus induction medium under a laminar flow hood. The jars containing the explants were incubated for four weeks. The incubation was carried out in a culture room at 25°C, with a 16 h photoperiod and a light flow of 2000 lux. The resulting friable calli were transferred to a somatic embryo induction medium.

2.2.3 Somatic embryos induction

Embryo induction medium (EIM) is composed of solid Murashige and Skoog and Gamborg B-5 (Gamborg et al. 1968) basal medium supplemented with vitamin B_5 (MSB_5) and the double concentration of KNO_3, the half concentration of NH_4NO_3 (MSB_5 - ½ [NH_4NO_3] + [KNO_3]), agar (6 $g.L^{-1}$), sucrose (30 $g.L^{-1}$), a combination of amino acids (glutamine, glycine, and casein hydrolysate), and hormones (BAP, kinetin, picloram, or 2,4-D). Seven embryo induction media selected from Kouadio et al. (2017) were tested in this study (Table 1). MIC_7 BAP medium (picloram-glycine-glutamine-casein-BAP) identified by Kouadio et al. (2017) as the best callogenesis medium served as a control in this study. Unlike the other test media, the MSB_5 basal medium was not modified.

Under a laminar flow hood, 1000 mg of friable calli were seeded into jars containing 10 mL of EIM medium with two calli explants per dish. Calli were subcultured on renewed media to maintain cell viability after four weeks of incubation.

After two subcultures, embryogenic structures (proembryos: clusters of rounded, regularly outlined, thick-walled cells with dense cytoplasm) were researched using a light microscope (Nikon TMS) (Vits et al. 1994; Nomura and Koumamine 1995). Then, the embryogenic structures observed were counted using a colony counter (COMECTA SA), and the embryogenic cell induction rate (ECIR) or embryogenic index (Cao Jing-Lin et al. 2008) was evaluated as follows:

$$\text{ECIR} = \frac{\text{NEC}}{\text{TNIC}} \text{ X } 100$$

Here NEC = number of embryogenic cells induced by medium; TNIC = Total number of induced embryogenic cells

2.2.4 Somatic embryos maturation

Approximately 500 mg of embryogenic calli were cultured on 10 mL of EIM medium with two explants per Petri dish for embryo maturation. The different cultures were incubated for three months in a dark room with monthly subcultures. At the end of each subculture, embryos were collected and observed under a laminar flow hood using a stereo microscope (Nikon TMS) at 100x or 400x magnification to look for evidence of embryo maturation according to the method of Thiruvengadam et al. (2006). Observed mature embryos were counted using a colony counter (COMECTA SA).

Table 1 MS media composition of somatic embryos induction medium (EIM)

Components /Medium	Culture media						
	MIC_7BAP (control)	EIM_1	EIM_2	EIM_3	EIM_4	EIM_5	EIM_6
Glycine ($mg.L^{-1}$)	2	2		2		2	2
Glutamine ($mg.L^{-1}$)	1000	1000	1000		1000		1000
Caseinhydrolyzate ($mg.L^{-1}$)	100	100		100		100	100
Picloram ($mg.L^{-1}$)	3	3					
2.4-D ($mg.L^{-1}$)			3	3	3	3	3
Kinetin ($mg.L^{-1}$)					0.2	0.2	0.2
BAP ($mg.L^{-1}$)	0.05	0.05					

The rate of mature embryos (RME) was calculated using the following equation:

$$\text{RME} = \frac{\text{NMEC}}{\text{NEP}} \text{ X } 100$$

Here NMEC =number of mature embryogenic cells; NEP = number of embryos present on the callus

2.2.5 Culture condition

Apart from embryo maturation, all cultures were incubated under a 16 h photoperiod, with illumination (2000 lux) provided by long light tubes in a culture room at 25°C.

2.2.6 Statistical analysis

The embryogenesis culture media effect was assessed based on the embryos' average number, induction rate, and maturation rate. Before the study, percentage rates were submitted to an angular transformation (arcsin√x). The analyses of data were performed using Statistica 7.1 software. One and two-criterion variance analyses were carried out on the mean values of parameters. Newman-Keuls test (5%) was used to classify the means when a significant difference was revealed between the two means.

3 Results

3.1 Influence of the culture medium composition on somatic embryos induction

Evaluation of the influence of different medium compositions on somatic embryo induction revealed that all culture media induced embryogenic cells (Figure 1). The composition of the selected media influenced the embryogenic cell induction rate (ECIR) and the number of induced embryogenic cells (NEC). The results also revealed that EIM_1 and EIM_5 induced the highest number of NEC (154 and 149 cells, respectively). These were followed by EIM_3 (50 cells) and EIM_2 (48 cells) media with statistically identical numbers of embryogenic cells. Furthermore, EIM_4 (10 cells) and EIM_6 (3 cells) media showed the lowest NEC (Table 2). These

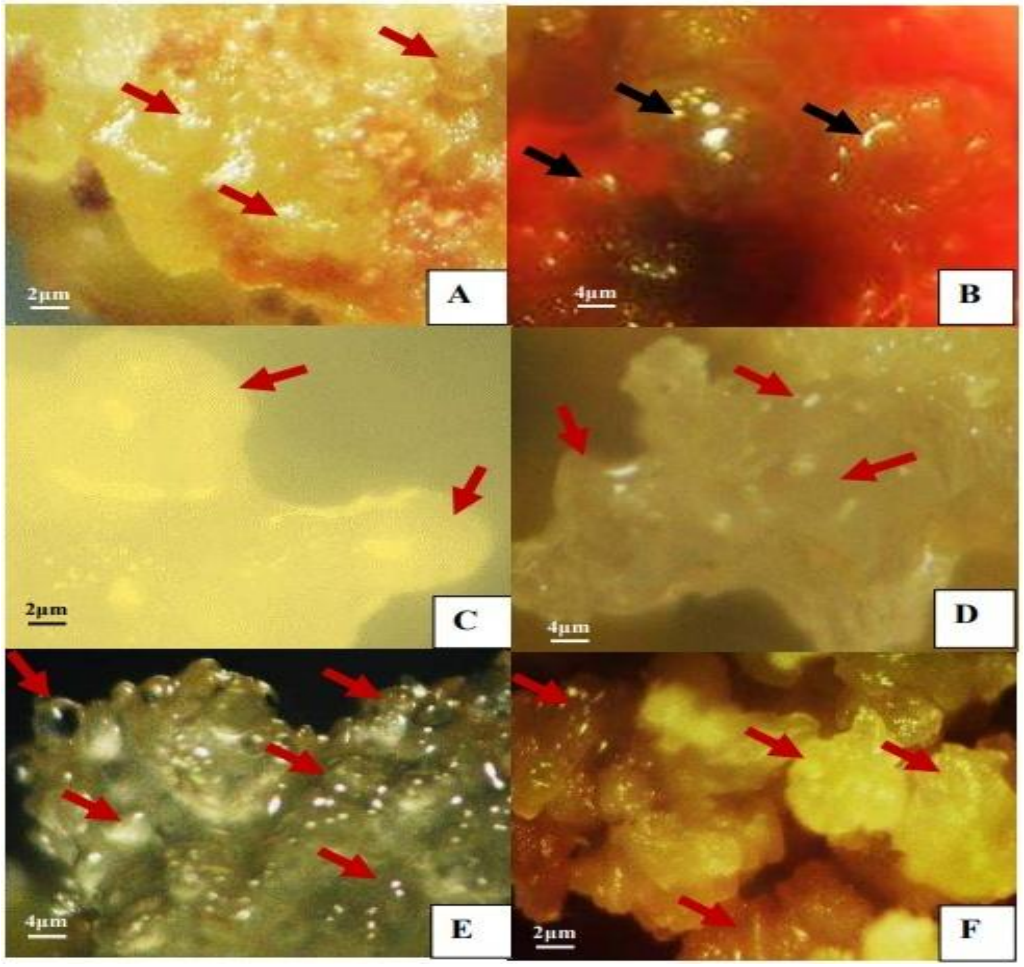

Figure 1 Embryogenic cell observed on pineapple calli from the selected cultures media; A: EIM_1; B: EIM_2; C: EIM_3; D: EIM_4; E: EIM_5; F: EIM_6; Arrows indicate the embryogenic cells.

results showed that the EIM1 medium induced a higher NEC (154 cells) and was reported lowest in EIM_6 media (3 cells). These two media were different, and the hormonal combination significantly influenced NEC.

In addition, analysis of EIM_5 media composition revealed that the addition of the kinetin (cytokinin) to the medium containing glycine and casein significantly improved embryogenic cell induction (149 in EIM_5 media) as compared to the EIM_3 (50). However, the EIM_2 and EIM_4 media composition analysis revealed that adding kinetin to the EIM2 medium containing glutamine inhibited the formation of embryogenic cells, showing only 48 and 10 cells, respectively. Similar trends were reported in the embryogenic cell induction rate (ECIR) (Table 2).

3.2 Influence of culture medium composition on the maturation of somatic embryos

Figure 2 revealed a significant evolution of the embryogenic structures on all selected culture media. Further, the culture

Table 2 Influence of culture media composition on the embryogenic cells induction

Culture media	Somatic embryogenesis parameters	
	Induction rate of embryogenic cells (%)	Number of embryogenic cells (NEC)
MIC_7BAP (control)	0 ± 0^{e}	0^{e}
EIM_1	37.20 ± 2.37^{a}	154^{a}
EIM_2	11.59 ± 2.07^{b}	48^{b}
EIM_3	12.08 ± 1.01^{b}	50^{b}
EIM_4	2.42 ± 1.53^{c}	10^{c}
EIM_5	35.99 ± 3.61^{a}	149^{a}
EIM_6	0.72 ± 0.46^{d}	3^{d}

In a column, numbers with the same letter are not significantly different (Newman-Keuls test at the 5% threshold).

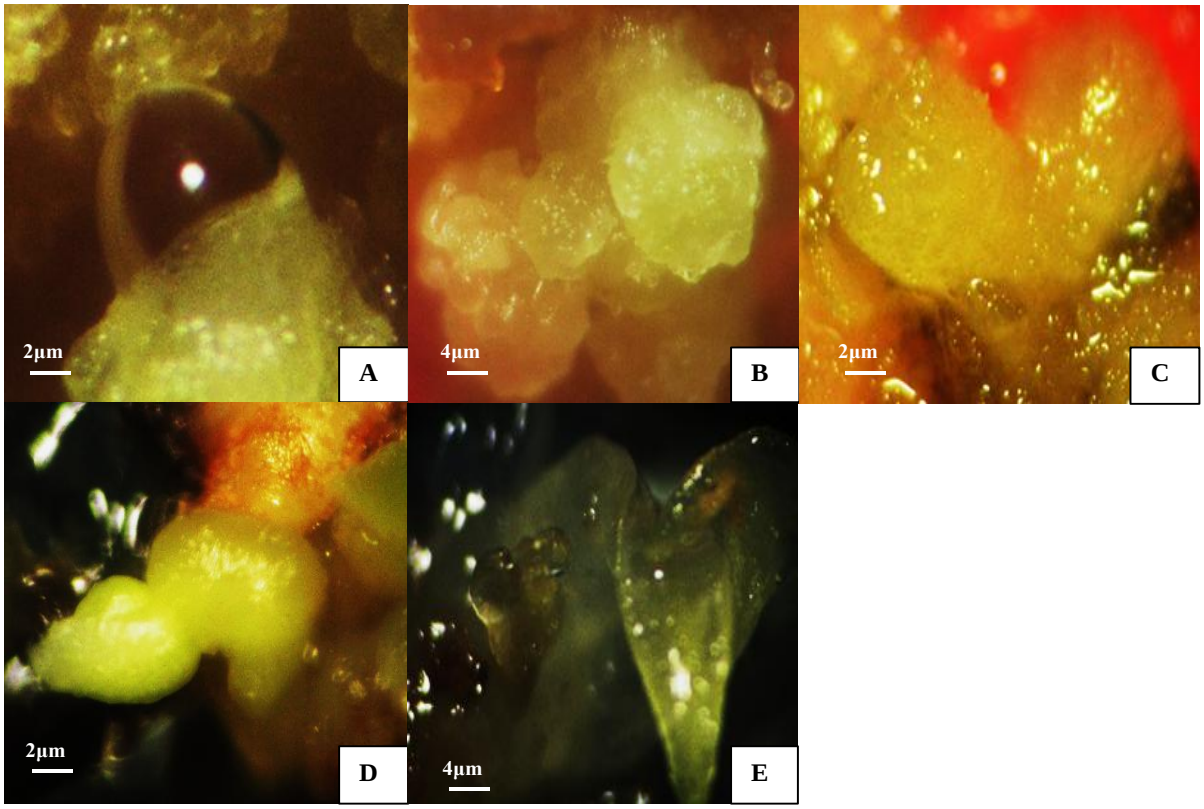

Figure 2 Different stages of evolution of pineapple somatic embryos observed on EIM_5 maturation medium; EIM_5 (EIM added with 3 $mg.L^{-1}$ of 2,4-D,0.2 $mg.L^{-1}$ of KIN,2 $mg.L^{-1}$ of glycine, 100 $mg.L^{-1}$ of casein hydrolyzate); A: globular stage embryo; B: cordiform stage embryo; C: heart stage embryo; D: torpedo stage embryo; E: cotyledonary stage embryo

Table 3 Influence of culture media composition on the maturation of the somatic embryo in pineapple

Culture media	Parameters	
	Mature embryo rate (%)	Number of mature embryogenesis cells/callus (num)
MIC_7BAP (Control)	0 ± 0^{c}	0^{d}
EIM_1	30.40 ± 1.27^{b}	19.67 ± 0.88^{b}
EIM_2	76.74 ± 1.81^{a}	23.00 ± 2.52^{b}
EIM_3	72.76 ± 3.38^{a}	22.33 ± 0.88^{b}
EIM_4	54.40 ± 2.00^{ab}	3.33 ± 0.88^{c}
EIM_5	76.33 ± 2.59^{a}	66.00 ± 4.91^{a}
EIM_6	35.58 ± 1.84^{b}	2.02 ± 0.65^{c}

In a column, numbers with the same letter are not significantly different (Newman-Keuls test at the 5% threshold)

medium composition significantly influenced the mature embryo rate (RME) (Table 3). EIM_2 and EIM_5 media induced a significantly higher rate of mature embryos (76.74 and 76.33%, respectively). These were followed by the EIM_1 (30.40%) and EIM_6 (35.58%) media. Analysis of variance also showed that medium composition strongly influenced the number of mature embryogenic cells (NMEC). The highest number of mature embryos (66 embryos) was recorded in the EIM_5 medium, followed by EIM_2 (23 embryos), EIM_3 (22.33 embryos), and EIM_1 (19.67 embryos), which had a statistically identical NMEC. Among the tested media, EIM_4 (3.33 embryos) and EIM_6 (1.33 embryos) media were found to be least effective in the induction of mature embryos. The EIM_5 medium, which gives a good evolution of embryos towards maturation with 66 mature embryos, was retained as the maturation medium. Globular, cordiform, heart, torpedo, and cotyledonary embryo stages were observed in this medium (Figure 2).

4 Discussion

4.1 Influence of the culture medium composition on somatic embryos induction

The results of this study revealed that the combination of EIM_5 media significantly affects somatic embryogenesis and development. In this manner, it can be established that MS media supplemented with glycine, 2,4-D, and kinetin is suitable for somatic embryogenesis. The results of this study agree with the findings of Cacaï et al. (2021), who suggested that somatic embryo induction in pineapple was favored by picloram combined with BAP in contrast to the kinetin and 2,4-D combination. Similarly, Zouzou et al. (2008) and Kouakou et al. (2009) also reported that the 2,4-D and kinetin combination was significantly effective for embryo induction in cotton. Kone (2010) also reported that the combination of 2,4-D and TDZ favor the embryogenesis in *Vigna subterranean*. These results suggest cytokinin-associated auxins' had a varietal effect in the induction of embryogenic structures (Dóra et al. 2020). Moreover, Yapo (2013) also reported that phytohormones also influence somatic embryo induction in pineapples. This supposes that pineapple calli produce endogenous picloram and BAP, which increases their stressful action to trigger cell differentiation into embryos. These results also suggest a robust synergistic action of picloram and BAP for embryogenic cell induction compared to the interaction of 2,4-D and kinetin. Furthermore, this difference in expression between EIM_1 and EIM_5 correlated with the types of amino acids (source of nitrogen). Similarly, Yapo et al. (2011) reported that somatic embryogenesis in pineapple is improved by adding the amino acids in the culture medium. These amino acids immediately provide a readily available nitrogen source to cells which initiates the stimulation of embryogenesis. These researchers suggested that nitrogen is selectively absorbed by cells in the form of nitrates to induce embryonic development. Thus, high nitrate levels significantly influenced the induction of embryogenic cells. A low NH_4^+/NO_3^- ratio induced the high number of embryogenic cells observed on the EIM_1 medium. Thus, nitrate appears to be essential for somatic embryo induction (Yapo 2013). The low embryogenic cell number observed in the EIM_3 medium compared to the EIM_5 medium showed that the kinetin addition to the culture medium stimulated the embryogenic cell induction. Arnold et al. (2002) have reported that phytohormones induce callus differentiation in polarized cells and initiate somatic embryogenesis. However, a decrease in embryogenic cells after adding 2-4D to the EIM_2 medium shows that somatic embryogenesis in pineapple depends on the combination of amino acids in the culture medium. Callus sensitivity to phytohormones is impacted by the presence of nitrogen in the culture medium (Fuentes-Cerda et al. 2006; Cangahuala-Inocente et al. 2007; Kouadio et al. 2017). The present study reported that the embryogenic cell induction in pineapple is controlled by a low NH_4^+/NO_3^- ratio and phytohormone interactions. EIM_1 and EIM_5 media are the most embryogenic in pineapple.

4.2 Influence of culture medium composition on somatic embryo maturation

This study reported that the composition of the culture medium influenced the induced embryo's maturation. Thus, all selected media allowed the initiation of embryogenic structures and had a similar effect on embryo maturation. These results corroborate with the authors, who have reported that somatic embryo maturation requires a proper combination of cytokinin and auxin (Fotso et al. 2008; Cacaï et al. 2021). According to Yapo (2013), weak auxin and cytokinin are essential to induce embryo maturation once embryogenesis is initiated. Indeed, the auxin and cytokinin combination stimulates cell division in the embryos, leading to their differentiation by allowing them to go through different embryonic stages (Fotso et al. 2008). In the current study, different stages (globular, cordate, heart, torpedo, and cotyledonary stages) of embryo evolution were observed, demonstrating that the embryos obtained are mature. Similar results were reported by Yapo (2013) for pineapple and Kouakou (2009) for cotton. These results suggest the influence of environment, nitrogen source, and phytohormones on maturation, and this could also be explained by the fact that embryos would accumulate starch reserves during maturation. The starch synthesized and accumulated in cells is an essential energy source for mitotic activities (Profumo et al. 1986).

Moreover, the work of Koné (2003) and Kouakou (2009) showed that cotton embryos that evolve to the cotyledonary stage (the most developed stage of maturation) have a dense cytoplasm, i.e., filled with starch reserves. According to Virgo-Brown (1987), the interaction of kinetin (0.5 $mg.L^{-1}$) and 2,4-D (2 $mg.L^{-1}$) in the N6 basal medium promoted embryo maturation in sorghum. Jayanthi et al. (2001) also showed that the association of 2,4-D and BAP or kinetin influenced embryogenesis. This combination also supports the maturation of embryos in most of the used culture media in this study.

However, this study revealed the non-maturation of some embryogenic cells during the induction phase. In addition, combinations of amino acids with cytokinins significantly affect the maturation of some embryogenic cells. In the current study, the association of glutamine with 2-4D inhibits the maturation of embryogenic cells, as observed for EIM_2 and EIM_4 media. These results suggest a toxic or competitive effect of 2-4D on glutamine. Furthermore, the abnormal cytoplasmic accumulation of starch reserves could explain the low rate of embryo maturation observed in EIM1 and EIM6. In addition, some cells without starch reserves accumulate phenolic compounds that inhibit maturation (Virgo-Brown 1987; Robert et al. 1989). The composition of these media inhibited hydrolases in particular and, consequently, low energy availability to embryogenic cells. Therefore, the embryos obtained remained blocked at the globular stage.

In contrast, adding kinetin to the EIM_3 and EIM_5 medium supplemented with glycine and casein hydrolyzate stimulated the maturation of the embryogenic cells. These results corroborate previous studies that reported that the cytokinin (BAP or kinetin) addition onto the culture medium supplemented with 2,4-D stimulates the maturation of the somatic embryos (Fotso et al. 2008; Jayanthi et al. 2001). These results could be explained by the synergistic effects of cytokinins and auxins on embryo maturation. Moreover, working on cotton plant embryos, Kouakou (2009) reported that cells have differential sensitivity to compounds in the culture medium that condition mitotic activity. He also noted that adding kinetin and 2,4-D onto the culture medium promoted the induction of embryogenic structures and embryo maturation in cotton. These results indicate the action of cytokinins and auxins on somatic embryo maturation depends on plant species.

Conclusions

The present study aimed to establish the appropriate conditions for efficient somatic embryogenesis and mass production of healthy and efficient pineapple plant material to renew the Ivorian orchard. Results of the study revealed the effects of seven culture media consisting of various amino acids (glutamine, glycine, and casein hydrolysate), cytokinins (kinetin and BAP), and auxins (2,4-D and picloram) on the induction and maturation of smooth cayenne (*Ananas comosus* L.) somatic embryos. Results of the study show that the embryogenic cell induction in pineapple was under the control of a low NH^+_4/NO^-_3 ratio and phytohormone interactions. Among the tested media combinations, EIM_5 medium (EIM added with 2 $mg.L^{-1}$ of glycine, 100 $mg.L^{-1}$ of casein hydrolyzate, and 0.2 $mg.L^{-1}$of kinetin) was the most embryogenic, and maturation of embryogenic cells media. These results suggested that adding kinetin to the culture medium containing casein hydrolysate and glycine in the presence of 2,4-D significantly affects the somatic embryo's initiation and maturation.

Author contribution

KOKS and KTH designed the experiment. KOKS, SO, and KD conducted the experiments and performed data analyses. KOKS and YSES prepared all of the figures, and all authors contributed to data interpretation. KOKS wrote the first draft of the manuscript, and KOKS and KTH edited the draft. All authors reviewed the manuscript.

Acknowledgments

The authors would like to acknowledge Nangui Abrogoua University and the National Center of Agronomy Research of Côte d'Ivoire for technical support.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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