*Original Research Article*

Effect of Culture Medium Composition and Phenolic Markers on In Vitro Conversion of Somatic Embryos into Plantlets in Pineapple (*Ananas comosus* L. var. Smooth Cayenne) Grown in Côte d’Ivoire

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ABSTRACT

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| Pineapple is a strategic crop in Côte d'Ivoire. However, its declining productivity requires the renewal of orchards using high-performance vitroplants. The aim of this study was to optimize the conversion of mature somatic embryos into plantlets, by evaluating the influence of culture medium composition and phenolic compounds. Six germination media (MIE1 to MIE6) composed of a combination of auxins (picloram or 2,4-D), cytokinins (BAP or kinetin) and amino acids (glycine, glutamine, casein hydrolysate) were tested for their effectiveness in inducing in vitro conversion. In addition, phenolic compounds present in embryos were identified and quantified by spectrophotometry and high-performance liquid chromatography (HPLC). The identified phenolic markers of germination were then added to recalcitrant media to assess their effect on conversion. Results showed that MIE2 (3 mg/L 2,4-D + 1000 mg/L glutamine) and MIE5 (3 mg/L 2,4-D + 2 mg/L glycine + 100 mg/L casein + 0.2 mg/L kinetin) media achieved the best germination rates (75.21% and 79.95%) and the highest mean numbers of germinated embryos (22.7 and 64, respectively). HPLC analysis revealed that resveratrol, trans-methoxycinnamic acid, astragalin, gallic acid, genistein, quercetin and naringenin were associated with in vitro germination. However, exogenous addition of these compounds did not improve germination and caused necrotic browning of the calli, suggesting that only endogenous metabolites promote germination. The MIE5 medium not only promoted the accumulation of these markers, but also stimulated the de novo biosynthesis of gallic acid, genistein, quercetin and naringenin. These results underline that the hormonal and mineral combination of the MIE5 medium is the most efficient for converting pineapple somatic embryos and offers new perspectives for the improvement of vitroplants production. |

*Keywords: Pineapple, somatic embryogenesis, in vitro germination, phenolic markers, in vitro culture, HPLC*

1. INTRODUCTION

Pineapple (Ananas comosus L.) is a Bromeliaceae cultivated in tropical regions for its fruit (Akbar et al., 2003; Yapo, 2013). In Côte d'Ivoire, pineapple cultivation contributes 0.6% to the national GDP, and fresh pineapple exports generate more than 30 billion FCFA per year (CNE, 2022). Smooth Cayenne variety, mainly grown in Côte d'Ivoire, has made the country the leading supplier to the European Union (97% of the market). However, since the late 1980s, pineapple production in Côte d'Ivoire has been steadily declining (Vagneron et al., 2009; Africa 24, 2022). This decline can be explained by the degeneration of plant material and the failure to respect good agricultural practices, resulting in the accumulation of chemical residues beyond the maximum residue limit and an increase in fruit acidity. Consequently, Ivorian pineapple has lost considerable value in the international market in favor of the MD2 variety from Costa Rica. Today, Côte d’Ivoire holds less than 2% of the European market share (Kouadio, 2018; FAO, 2023).

In this context, the renewal of the Ivorian orchard with high-performance varieties is essential to improve fruit quality and yield of smooth Cayenne. To this end, the work of Yapo (2013) and Kouadio et al. (2017) has shown that in vitro plant tissue culture by somatic embryogenesis is an interesting tool for pineapple varietal improvement. However, the acquisition of embryogenic competence by cells is strongly influenced by several factors including growth regulators, carbohydrates, amino acids and nitrate (Yapo et al., 2011a; Cacaï et al. 2021; Kessel-Domini et al. 2022). Thus, one of the main challenges during somatic embryogenesis lies in the culture medium's ability to support the conversion of mature somatic embryos into plantlets for large-scale production of high-quality planting materials (Kouadio et al., 2023). Indeed, the low conversion capacity of somatic embryos hinders the production of sufficient healthy vitroplants. However, the work of Kouakou (2009) in cotton highlighted the central role of phenolic compounds in the acquisition of embryogenic competence. He reported that embryogenic competence depends on both the type and concentration of phenolic compounds. Thus, embryogenic cells accumulate significantly higher levels of phenolic compounds than non-embryogenic cells (Meguellati et al., 2022). This suggests that the addition of specific phenolic compounds to the culture medium could potentially enhance the conversion of embryos into plantlets. It is therefore crucial to identify the optimal culture medium conditions that stimulate the biosynthesis of these phenolic compounds and to understand their role in the in vitro conversion process. The aim of this study was to optimize the conversion of mature somatic embryos of the smooth Cayenne pineapple variety into plantlets. Specifically, it aimed to examine the effect of culture medium composition on the production of phenolic compounds during the in vitro conversion of mature somatic embryos into plantlets and to assess the impact of their exogenous addition to recalcitrant media on embryo conversion.

2. material and methods

**2.1 Plant material**

The plant material consisted of pineapple suckers (Ananas comosus var. Smooth Cayenne, cultivar CI 16) from the collection of the National Center for Agronomic Research (CNRA) of Côte d'Ivoire (Fig. 1).



**Fig. 1. Pineapple suckers (*Ananas comosus* var. Smooth Cayenne, cultivar CI 16)**

**2.2 Methods**

**2.2.1 Initiation of pineapple leafy shoots**

After disinfecting the explants using the method of Yapo et al. (2011a), the apical buds were carefully excised using a scalpel-mounted blade. These explants were then transferred to a shoot initiation medium. This medium consisted of MS basal medium (Murashige and Skoog, 1962) supplemented with vitamin B5, 0.2 g/L glutamine, 0.01 mg/L kinetin and 30 g/L sucrose.) The pH of the medium was adjusted to 5.8. The medium was solidified by adding 2.5 g/L phytagel.

**2.2.2 Callus induction**

Callus induction was performed according to the method described by Kouadio et al. (2017), using MSB5 basal medium supplemented with 3 mg/L picloram, 2 mg/L glycine, 1000 mg/L glutamine, 100 mg/L casein hydrolysate, and 30 g/L sucrose. The resulting friable calli were transferred to a somatic embryo induction medium.

**2.2.3 Somatic embryo induction and maturation**

Somatic embryo induction and maturation were carried out according to the method of Kouadio et al. (2023). After four weeks of culture, approximately 1000 mg of induced calli were seeded on embryo induction and maturation medium (MIE). This medium consists of the basic MSB5 medium modified by an ammonium-nitrate balance and supplemented with combinations of amino acids (glutamine, glycine and casein hydrolysate) and hormones (BAP or kinetin and picloram or 2,4-D) as shown in Table 1. The selection of culture media was based on the work of Kouadio et al. (2023). Thus, the MIE1 callogenesis medium (containing the picloram / BAP hormone combination) was used as the control in this study.

**Table 1. Somatic induction embryogenic medium (MIE) components**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Medium components | Culture media | | | | | |
| **MIE1** | **MIE2** | **MIE3** | **MIE4** | **MIE5** | **MIE6** |
| Picloram (mg/L) | 3 | - | - | - | - | - |
| 2.4-D (mg/L) | - | 3 | 3 | 3 | 3 | 3 |
| BAP (mg/L) | 0.05 | - | - | - | - | - |
| Kinetin (mg/L) | - | - | - | 0.2 | 0.2 | 0.2 |
| Glycine (mg/L) | 2 | - | 2 | - | 2 | 2 |
| Glutamine (mg/L) | 1000 | 1000 | - | 1000 | - | 1000 |
| Casein hydrolysate (mg/L) | 100 | - | 100 | - | 100 | 100 |

**2.2.4 Germination of mature somatic embryos**

*2.2.4.1 Effect of culture media on embryos germination*

Portions of calli containing mature somatic embryos were excised and subcultured onto the same maturation media to evaluate the germination of mature embryos. After four weeks of culture in darkness, the germinated embryos were transferred to a temperature-controlled room with a 16-hour photoperiod for two weeks to promote optimal photosynthetic activity. The germinated embryos were counted in jars using a Colony counter (COMECTA SA), and the embryo germination rate (EGR) was then calculated using the following formula:

**EGR (embryo germination rate) = (number of germinated embryos) / (number of germinated embryos) x 100**

*2.2.4.2 Identification of phenolic markers of embryos germination*

For this study, embryogenic calli from the best-performing germination media and those from the control medium, which did not allow any germination, were selected.

**Extraction and purification of phenolic compounds:** Total phenol extraction was performed on dry material using the method of Kouakou (2009). Three samples per culture medium were analyzed. A total of 500 mg of lyophilized calli were placed in 10 mL of 96% methanol for 10 h at 4°C. After centrifugation at 3000 rpm for 10 min, the supernatant was filtered through a Millipore membrane (0.45 µm) and constituted the crude phenolic extract.

**Quantification of Total Phenols by Spectrophotometry:** Total polyphenols were quantified according to the method of Swain and Hillis (1959), modified and adapted to the plant material. The presence of phenols in the reaction mixture was revealed by the addition of 0.5 mL Folin Ciocalteu reagent (0.5 N) and 1.5 mL sodium carbonate (Na2CO3) 17% (w/v) to 0.5 mL of extract. The final mixture was shaken and incubated for 45 minutes in the dark at room temperature. A blank control was prepared by replacing the extract with distilled water. The intensity of the blue coloration produced by this reaction was measured using a UV/VIS spectrophotometer (A & ELAB) at a wavelength of 765 nm. Total phenol content, expressed in milligrams of gallic acid equivalents per gram of dry matter (mg EAG/g DM), was determined using a calibration curve prepared from various concentrations of a stock solution of gallic acid (100 µg/mL) (y = 0.586x; R2 = 0.999, where y is the absorbance and x is the gallic acid concentration).

**Separation and quantification of phenolic compounds by high-performance liquid chromatography (HPLC):** HPLC was performed according to the method of Rodriguez-Delgado et al. (2001), modified by Kouakou et al. (2009). Separation of phenolic compounds was carried out on an Agilent LC series system, using the method of Rodriguez-Delgado et al. (2001). A reverse-phase C18 column (Eclipse XDB-C18, 150 x 4.6 mm, 5 μm, Agilent) was used with a solvent system consisting of: (A) filtered water/trifluoroacetic acid at 0.1% (99.99/0.1) and (B) acetonitrile/trifluoroacetic acid at 0.1% (99.99/0.1). The applied elution gradient was: 10% B (0–30 min), 30% B (30–40 min), 50% B (40–45 min), 100% B (45–48 min), and 10% B (48–60 min).

Separation and quantification of phenolic compounds were carried out in HPLC, controlled by a microcomputer (Workstation system). Approximately 10 µL of the previously obtained hydro-methanolic extract was injected at a flow rate of 0.8 mL/min. Each analysis was repeated three times. Chromatograms were detected at 284 nm. The identification of phenolic compounds (peaks) was based on retention times and NMR (nuclear magnetic resonance) spectra compared to a reference library of known pineapple phenolics (Py et al., 1984; Macheix et al., 1990; Gorinstein et al., 1999; Chao et al., 2007; Yapo et al., 2011b).

*2.2.4.3 Evaluation of the effect of exogenous addition of phenolic germination markers on the conversion of somatic embryos from recalcitrant media*

After autoclaving the germination media at 121°C for 30 min under 1 bar pressure, the phenolic compounds previously identified in the calli as embryo germination markers were added to the recalcitrant media using a sterile 0.22 μm Millipore filter. Phenols were added at concentrations similar to those identified in the embryogenic calli. The concentrations used were close to those levels found in the embryogenic calli. Then, calluses containing mature embryos were placed on the media. After four weeks of culture at 25°C, under the same conditions as previously described, the calli were observed to detect germinated embryos.

**2.2.5 Statistical analyses**

The effect of the different culture media on somatic embryo germination was evaluated through the mean number and germination rate. The rates, expressed as percentages, were subjected to an angular arcsin (√x) transformation before analysis. Statistical analyses were performed using Statistica 7.1 software. Analyses of variance were performed on the mean values of the measured parameters. When a significant difference was revealed between two means, the Newman-Keuls test at the 5% threshold was used to rank the means.

3. results

**3.1 Effect of culture media composition on somatic embryos germination**

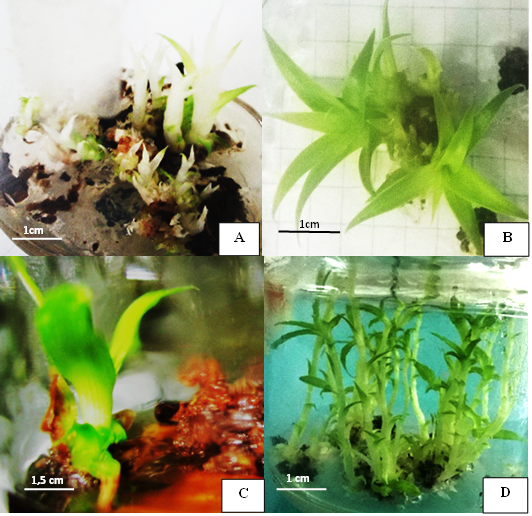
Culture media composition had differential effects on the rate and number of germinated embryos (Table 2). The MIE5 medium (MIE + 3 mg/L 2,4-D + 2 mg/L glycine + 100 mg/L casein hydrolysate + 0.2 mg/L KIN) and MIE2 medium (MIE + 3 mg/L 2,4-D + 1000 mg/L glutamine) recorded the highest embryo germination rates (EGR), which were statistically similar (79.95 and 75.21% respectively). Regarding the number of germinated embryos (NGE), the MIE5 medium yielded the highest number (64 embryos), followed by MIE2 (22.67 embryos). MIE4 medium produced the lowest NGE (2.67 embryos) (Fig. 2). However, analysis of Table 2 shows that not all culture media favored embryo germination. For example, mature embryos observed on MIE1 (MIE + 3 mg/L picloram + 2 mg/L glycine + 1000 mg/L glutamine + 100 mg/L casein hydrolysate + 0.05 mg/L BAP) and MIE6 (MIE + 3 mg/L 2,4-D + 2 mg/L glycine + 1000 mg/L glutamine + 100 mg/L casein hydrolysate + 0.2 mg/L KIN) degenerated. These media therefore did not allow embryos germination. Consequently, the best germination media (MIE5 and MIE2) and the control medium (MIE1) were used for quantitative and qualitative analyses of the phenolic compounds in the calli.

**Table 2. Effect of culture media composition on somatic embryo germination**

|  |  |  |
| --- | --- | --- |
| **Culture media** | **Embryos germination parameters** | |
| **NGE (nb)** | **EGR (%)** |
| MIE1 | 0,00 ± 0,00**c** | 16,74 ± 0,00**c** |
| MIE2 | 22,67 ± 2,18**b** | 75,21± 1,95**a** |
| MIE3 | 18,00 ± 0,58**b** | 64,05 ± 1,91**b** |
| MIE4 | 2,67 ± 1,20**c** | 57,66 ± 8,29**b** |
| MIE5 | 64,00 ± 3,60**a** | 79,95 ± 0,27**a** |
| MIE6 | 0,00 ± 0,00**c** | 16,74 ± 0,00**c** |

*In the same column, means followed by the same letter are not significantly different (Newman-Keuls test at the 5% threshold).*

*nb (number); MIE (MSB5 – ½ [NH4NO3] + [KNO3]); MIE1 (MIE + 3 mg/L picloram + 2 mg/L glycine + 1000 mg/L glutamine + 100 mg/L casein hydrolysate + 0.05 mg/L BAP); MIE2 (MIE + 3 mg/L 2,4-D + 1000 mg/L glutamine); MIE3 (MIE + 3 mg/L 2,4-D + 2 mg/L glycine + 100 mg/L casein hydrolysate); MIE4 (MIE + 3 mg/L 2,4-D + 1000 mg/L glutamine + 0.2 mg/L KIN); MIE5 (MIE + 3 mg/L 2,4-D + 2 mg/L glycine + 100 mg/L casein hydrolysate + 0.2 mg/L KIN); MIE6 (MIE + 3 mg/L 2,4-D + 2 mg/L glycine + 1000 mg/L glutamine + 100 mg/L casein hydrolysate + 0.2 mg/L KIN). NGE (Number of germinated embryos); EGR (Embryo germination rates)*

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**Fig. 2. Vitroplants from the germination of pineapple somatic embryos on different culture media**

*A: MIE2 (MIE + 3 mg/L 2,4-D + 1000 mg/L glutamine); B: MIE3 (MIE + 3 mg/L 2,4-D + 2 mg/L glycine + 100 mg/L casein hydrolysate); C: MIE4 (MIE + 3 mg/L 2,4-D + 1000 mg/L glutamine + 0.2 mg/L KIN); D: MIE5 (MIE + 3 mg/L 2,4-D + 2 mg/L glycine + 100 mg/L casein hydrolysate + 0.2 mg/L KIN)*

**3.2 Quantitative analysis of phenolic compounds in calli**

The culture medium significantly influenced the total phenol content in the calli. Evaluation of total phenolic content showed that pineapple calli cultured on MIE5 medium had the highest total phenolic content (16.01 mg/g fresh calli), followed by those on MIE2 medium (14.07 mg/g fresh calli). In contrast, the results showed that calli from MIE1 medium yielded the lowest total phenol content (9.70 mg/g fresh calli) (Table 3).

**Table 3. Total phenolic contents of calli**

|  |  |
| --- | --- |
| **Culture Media** | **Total phenolic contents (mg/g of fresh calli)** |
| MIE1 | 09,70 ± 0,17b |
| MIE2 | 14,07 ± 1,74ab |
| MIE5 | 16,01 ± 1,54a |

*Means followed by the same letter are not significantly different (Newman-Keuls test at the 5% threshold), MIE (MSB5 – ½ [NH4NO3] + [KNO3]); MIE1 (MIE + 3 mg/L picloram + 2 mg/L glycine + 1000 mg/L glutamine + 100 mg/L casein hydrolysate + 0.05 mg/L BAP); MIE2 (MIE + 3 mg/L 2,4-D + 1000 mg/L glutamine); MIE5 (MIE + 3 mg/L 2,4-D +2 mg/L glycine + 100 mg/L casein hydrolysate + 0.2 mg/L KIN)*

**3.3 Qualitative analysis and identification of phenolic compounds in calli**

High-performance liquid chromatography (HPLC) analysis enabled the identification of phenolic compounds present in pineapple calli cultured on different media (MIE1, MIE2, and MIE5). A total of 20 phenolic standards were chromatographed beforehand under the same conditions as the samples. This process allowed the determination of the various retention times of the phenolic standards (Table 4).

**Table 4. Retention times of phenolic standards detected at 284 nm**

|  |  |  |
| --- | --- | --- |
| **N°** | **Phenolic compounds** | **Retention time (min)** |
| 1 | Arbutin | 4,471 |
| 2 | Gallic acid | 7,888 |
| 3 | Protocatechic acid | 8,911 |
| 4 | 3-Hydroxybenzoic acid | 10,078 |
| 5 | 4-Hydroxybenzoic acid | 10,545 |
| 6 | Ellagic acid | 10,762 |
| 7 | Catechin | 11,544 |
| 8 | Syringic acid | 11,570 |
| 9 | Gentisic acid | 11,922 |
| 10 | Chlorogenic acid | 12,111 |
| 11 | Isovanillic acid | 13,364 |
| 12 | Epicatechin | 13,954 |
| 13 | Vanillin | 16,112 |
| 14 | Vanillic acid | 15,046 |
| 15 | Quercitrin | 16,112 |
| 16 | Ferulic acid | 18,274 |
| 17 | Rutin | 18,670 |
| 18 | Salicylic acid | 21,014 |
| 19 | Kaempferol | 29,470 |
| 20 | Quercetin | 35,925 |

Analysis of the chromatograms in Fig. 3 revealed that the phenolic profiles of pineapple calli cultured on MIE1, MIE2, and MIE5 media showed both similarities and differences. Indeed, chromatogram analysis revealed that five compounds are common to all three tested culture media (MIE1; MIE2 and MIE5). Thus, compounds (2); (3); (4); (5) and (6) were synthesized in all calli from the three media. However, HPLC results showed that compounds (7); (8) and (9) were biosynthesized exclusively in calli from MIE5 and MIE2 media. A comparison of the phenolic profiles of calli grown on MIE2 medium (containing 2,4-D without cytokinin) and those grown on MIE5 medium (containing 2,4-D supplemented with 0.2 mg/mL kinetin) revealed a de novo synthesis of compounds (1), (10), (11), and (12) in the calli cultured on MIE5. Additionally, an overlay of the chromatographic profiles (Fig. 3) highlighted differences in the peak amplitudes of phenolic compounds common to both media. Calli cultured on MIE5 exhibited significantly higher peak amplitudes for compounds (2), (4), (5), (6), (7), and (8) compared to those from MIE2 (without kinetin).

By comparing with standard compounds whose retention times and NMR profiles are cataloged in the reference library, all detected phenolic compounds were identified based on their retention times and NMR spectra. Thus, the compounds were identified as (1) Gallic acid (7.923 min); (2) Protocatechic acid (8.887 min); (3) 3-hydroxybenzoic acid (10.080 min); (4) Epicatechin (13.951 min); (5) Vanillin (16.111 min); (6) Myricetin (18.890 min); (7) Resveratrol (23,060 min); (8) Trans-methoxycinnamic acid (26,466 min); (9) Astragalin or Kaempferol 3-O-glucoside (29,471 min); (10) Genistein (33,750 min); (11) Quercetin (35,922 min); (12) Naringenin (43,870 min).



**Fig. 3. Chromatographic profile of polyphenols detected during pineapple calli germination at 284 nm**

*(1) Gallic acid (7.923 min); (2) Protocatechic acid (8.887 min); (3) 3-hydroxybenzoic acid (10.080 min); (4) Epicatechin (13.951 min); (5) Vanillin (16.111 min); (6) Myricetin (18,890 min); (7) Resveratrol (23,060 min); (8) Trans-methoxycinnamic acid (26,466 min); (9) Astragalin or Kaempferol 3-O-glucoside (29,471 min); (10) Genistein (33,750 min); (11) Quercetin (35,922 min); (12) Naringenin (43,870 min).*

*MIE (MSB5 - ½ [NH4NO3] + [KNO3]); MIE1 (MIE + 3 mg/L picloram + 2 mg/L glycine + 1000 mg/L glutamine + 100 mg/L casein hydrolysate + 0.05 mg/L BAP); MIE2 (MIE + 3 mg/L 2,4-D + 1000 mg/L glutamine); MIE5 (MIE + 3 mg/L 2,4-D + 2 mg/L glycine + 100 mg/L casein hydrolysate + 0.2 mg/L KIN)*

**Comparison of average contents of phenolic compounds identified in calli:** Table 5 presents the average contents of phenolic compounds identified in pineapple calli cultured on MIE1, MIE2, and MIE5 media. Compounds (2) protocatechic acid; (3) 3-hydroxybenzoic acid; (4) epicatechin; (5) vanillin and (6) myricetin are common to all three media. However, comparison of their concentrations showed that the levels of certain compounds doubled from MIE1 to MIE5. For example, the concentrations of protocatechic acid (4.67 μg/g); epicatechin (14.10 μg/g); vanillin (10.75 μg/g) and myricetin (4.11 μg/g) observed in calli from MIE1 medium increased to 14.60 μg/g, 20.14 μg/g, 23.17 μg/g and 13.40 μg/g respectively in calli from MIE5 medium.

Regarding MIE2 and MIE5, both containing 2,4-D as the auxin source, it was noted that resveratrol, trans-methoxycinnamic acid, and astragalin, specific to these two media, were present at nearly equal concentrations. However, the addition of kinetin to MIE5 medium not only increased the concentrations of protocatechic acid (from 5.84 to 14.60 μg/g), epicatechin (16.77 to 20.14 μg/g), vanillin (19.58 to 23.17 μg/g), and myricetin (4.70 to 13.40 μg/g), but also induced the de novo synthesis of new compounds such as gallic acid (6.13 μg/g), genistein (13.09 μg/g), quercetin (14.22 μg/g), and naringenin (3.96 μg/g). Thus, the specific combination of hormones and amino acids in the MIE5 medium promoted both the biosynthesis and accumulation of certain phenolic compounds.

**Group classification of phenolic compounds identified in calli:** Table 6 shows the average contents of the various phenolic groups identified in pineapple calli depending on the culture medium used. Analysis of Table 6 showed that approximately three phenolic groups were identified in the calli cultured on MIE1, MIE2, and MIE5. The concentrations of phenolic acids and flavonoids in calli from MIE1 doubled and then quadrupled in calli from MIE2 and MIE5, respectively. In contrast, the content of stilbenoids did not vary significantly between MIE2 and MIE5. Additionally, flavonoids had the highest concentrations, followed by phenolic acids in calli cultured on media containing 2,4-D as the auxin. Therefore, flavonoids were the predominant phenolic group in pineapple calli, followed closely by phenolic acids. Stilbenoids were synthesized in very small amounts or not at all in some media (MIE1).

Regarding the phenolic pool, the MIE5 medium yielded the highest accumulation (131.89 μg/g), followed by MIE2 (70.93 μg/g) and MIE1 (37.45 μg/g).

**Table 5. Content of polyphenols (μg/g) identified in pineapple calli at 284 nm**

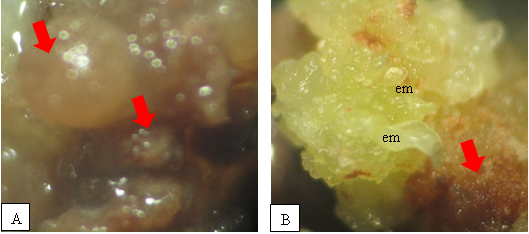
|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  |  | **Phenolic compound content (μg/g)** | | |
| **Phenolic groups** | **Phenolic compounds** | **MIE1** | **MIE2** | **MIE5** |
| Phenolic acids  (hydroxybenzoic acid) | (1) Gallic acid | nd | nd | 6,13 ± 0,30d |
| Phenolic acids  (hydroxybenzoic acid) | (2) Protocatechic acid | 4,67 ± 0,20de | 5,84 ± 0,10d | 14,60 ± 0,40b |
| Phenolic acids  (hydroxybenzoic acid) | (3) 3-hydroxybenzoic acid | 3,82 ± 0,50e | 7,33 ± 0,40d | 5,19 ± 0,20d |
| Phenolic acids  (hydroxybenzoic acid) | (5) Vanillin | 10,75 ± 0,60c | 19,58 ± 0,30ab | 23,17 ± 0,50a |
| Phenolic acids  (hydroxycynnamic acid) | (8) Trans-methoxycinnamic acid | nd | 2,68 ± 0,30ef | 3,36 ± 0,10e |
| Stilbenoids (stilbene) | (7) Resveratrol | nd | 3,83 ± 0,20e | 4,38± 0,30de |
| Flavonoids (flavanol) | (4) Epicatechin | 14,10 ± 0,30b | 16,77 ± 0,50b | 20,14 ± 0,80ab |
| Flavonoids (flavonol) | (6) Myricetin | 4,11 ± 0,10de | 4,70 ± 0,10de | 13,40 ± 0,60bc |
| Flavonoids (flavonol) | (9) Astragalin | nd | 10,20 ± 0,40c | 10,25 ± 0,70c |
| Flavonoids (isoflavone) | (10) Genistein | nd | nd | 13,09 ± 0,40bc |
| Flavonoids (flavonol) | (11) Quercetin | nd | nd | 14,22 ± 0,30b |
| Flavonoids (flavanone) | (12) Naringenin | nd | nd | 3,96 ± 0,10e |
| nd, not detected or absent; S, standard error; within the same column and within the same row, means followed by the same letter are not significantly different (5% Newman-Keuls test); values represent the mean of three replicates. MIE1 (MIE + 3 mg/L picloram + 2 mg/L glycine + 1000 mg/L glutamine + 100 mg/L casein hydrolysate + 0.05 mg/L BAP); MIE2 (MIE + 3 mg/L 2,4-D + 1000 mg/L glutamine); MIE5 (MIE + 3 mg/L 2,4-D + 2 mg/L glycine + 100 mg/L casein hydrolysate + 0.2 mg/L KIN) | | | | |

**Table 6. Average phenolic group contents (μg/g) in pineapple calli at 284 nm**

|  |  |  |  |
| --- | --- | --- | --- |
|  | **Phenolic compound content (μg/g)** | | |
| **Phenolic groups** | **MIE1** | **MIE2** | **MIE5** |
| Phenolic acids | 19,24 ± 0,11**f** | 35,43 ± 0,30**e** | 52,45 ± 0,23**d** |
| Flavonoids | 18,21 ± 0,50**f** | 31,67 ± 0,20**ef** | 75,06 ± 0,03**b** |
| Stilbenoids | nd | 3,83 ± 0,08**g** | 4,38 ± 0,11**g** |
| Phenolic pool | 37,45 ± 0,12**e** | 70,93 ± 0,18**c** | 131,89 ± 0,09**a** |
| nd, not detected or absent; S, standard error; within the same column and on the same row, means followed by the same letter are not significantly different (5% Newman-Keuls test); values ​​represent the average of three repetitions. MIE1 (MIE + 3 mg/L picloram + 2 mg/L glycine + 1000 mg/L glutamine + 100 mg/L casein hydrolysate + 0.05 mg/L BAP); MIE2 (MIE + 3 mg/L 2,4-D + 1000 mg/L glutamine); MIE5 (MIE + 3 mg/L 2,4-D + 2 mg/L glycine + 100 mg/L casein hydrolysate + 0.2 mg/L KIN) | | | |

**3.4 Evaluation of the Addition of Exogenous Phenolic Compounds on the Germination of Mature Embryos in Recalcitrant Media**

The results obtained show that the addition of exogenous phenolic compounds (markers of in vitro germination of somatic embryos) neither enabled the germination of mature embryos on the MIE1 medium, nor increased the germination rate of embryos on MIE2 medium. However, the calli showed browning followed by necrosis (Fig. 4).



**2μm**

**4μm**

**Fig. 4. Pineapple embryogenic calli obtained after addition of phenolic germination markers**

A: MIE1 (MIE + 3 mg/L picloram + 2 mg/L glycine + 1000 mg/L glutamine + 100 mg/L casein hydrolysate + 0.05 mg/L BAP); B: MIE2 (MIE + 3 mg/L 2,4-D + 1000 mg/L glutamine); em (germinating embryos); red arrows indicate browned callus.

4. discussion

The low germination rate of mature embryos is a major constraint to somatic embryogenesis in several species (Das et al., 1997; Cuenca et al., 1999; Odutaya et al., 2005). Consequently, various studies have been conducted to optimize the germination of mature embryos. In pineapple, Yapo (2013) showed that modified MS medium fortified with cytokinin (BAP) and glutamine improves embryos germination. This may explain the observed germination on most media (MIE2, MIE3, MIE4, and MIE5). In contrast, our results revealed that the MIE1 medium (MIE + 3 mg/L picloram + 2 mg/L glycine + 1000 mg/L glutamine + 100 mg/L casein hydrolysate + 0.05 mg/L BAP), although containing BAP, induced degeneration of the mature embryos. This result suggests a differential influence of BAP on mature embryos germination depending on the cultivar. This inhibition of germination may also be related to the combination of amino acids in the MIE1 medium.

Similarly, this study showed that MIE6 medium (MIE + 3 mg/L 2,4-D + 2 mg/L glycine + 1000 mg/L glutamine + 100 mg/L casein hydrolysate + 0.2 mg/L KIN) did not allow embryos germination. Comparison of the composition of MIE1 and MIE6 media suggests that the simultaneous presence of all three amino acids (glycine, glutamine and casein hydrolysate) inhibits embryo germination on these media. This suggests a competitive interaction between certain amino acids.

Furthermore, MIE5 medium (MIE + 3 mg/L 2,4-D + 2 mg/L glycine + 100 mg/L casein hydrolysate + 0.2 mg/L KIN) enabled the germination of a large number of mature embryos (64 embryos), followed by MIE2 medium (MIE + 3 mg/L 2,4-D + 1000 mg/L glutamine) with 22.67 embryos. These results demonstrate the influence of medium composition on embryo germination in pineapple. Similar results were reported by Matthews et al (1993) in cassava; Fki et al (2001) in date palm; and Pateña et al (2001) in mango. Indeed, these authors mentioned that the osmotic and/or hormonal shock induced by the medium composition leads to embryo cell desiccation, followed by an increase in intracellular osmotic pressure. This affects membrane permeability and alters endogenous abscisic acid content, thereby stimulating germination.

To better assess the effect of the culture medium on embryogenesis, biochemical studies were conducted. Several authors have reported the significant influence of phenolic compounds on somatic embryogenesis (Cvikrova et al., 1996; Kouadio et al., 1999; Kouakou, 2009). According to Dembinska-Migas et al. (1998), phenolic compounds exert specific effects on cell growth. In pineapple, our study demonstrated the influence of phenolic metabolism on the germination of mature embryos. HPLC analysis of the samples revealed the presence of a large number of phenolic compounds. However, the quality and quantity of these compounds varied depending on the culture medium. During embryogenesis, media MIE1, MIE2, and MIE5 all synthesized protocatechuic acid, 3-hydroxybenzoic acid, epicatechin, vanillin, and myricetin. The simultaneous presence of these phenolic compounds in the three media, which induced mature embryos, appears to be related to embryogenesis but may not favor embryo germination in pineapple. However, the study also showed that MIE2 and MIE5, which are rich in 2,4-D, supported embryo germination. The presence of resveratrol, trans-methoxycinnamic acid, and astragalin (kaempferol 3-O-glucoside), synthesized exclusively in calli containing mature embryos from these media, suggests a close relationship between phenolics and embryo germination. Indeed, Kouakou (2009) also reported the beneficial effect of resveratrol and kaempferol on cotton somatic embryogenesis. Furthermore, the results showed that MIE5 not only induced the highest germination rate but also promoted de novo synthesis of phenolic compounds (gallic acid, genistein, quercetin, and naringenin) following the addition of kinetin. This result suggests that the presence of gallic acid, genistein, quercetin, and naringenin in pineapple calli may be responsible for the high germination rate of mature embryos observed on MIE5. Similar results were observed in cotton. Kouakou (2009) showed that quercetin and gallic acid may serve as phenolic markers of embryogenesis. However, he also reported that genistein is among the phenolic compounds potentially responsible for inhibiting the induction of embryogenic structures. The effect of phenolic compounds thus appears to be species-dependent. This study also showed that, in addition to the de novo synthesis of specific phenolic compounds, the addition of kinetin to the MIE5 medium induced a high accumulation of phenolic compounds common to both MIE2 and MIE5 (resveratrol, trans-methoxycinnamic acid, and astragalin). This suggests a key role of the accumulation of these phenolic compounds in embryo germination in pineapple. Similar results have been reported by several authors in other plants (El Bellaj and El Hadrami, 1998; Luczkiewicz and Glod, 2003).

Furthermore, phenolic group classification of HPLC-identified compounds reveals that the flavonoid and phenolic acid pools doubled, then quadrupled on MIE2 and MIE5 media respectively. This suggests a very important role of flavonoids and phenolic acids in the acquisition of the capacity for mature embryo conversion in pineapple. Similar results have been reported by other authors. According to them, a significant accumulation of phenolic acids, flavonoids, and stilbenes in the cells promotes the formation of embryogenic structures in certain plant species (El Bellaj and El Hadrami, 1998; Kouakou, 2009). In contrast, the statistically identical content of stilbenoids (resveratrol) in both MIE2 and MIE5 media, as well as their absence in MIE1, suggests an influence of this phenolic group on germination but seems to have no correlation with the germination rate of mature embryos. Similar findings were reported by Kouakou (2009), who first demonstrated the beneficial effect of resveratrol on embryogenesis in cotton.

This study showed that the combination of certain hormones with amino acids enabled the biosynthesis and accumulation of specific phenolic compounds in MIE5 medium. This suggests that the germination capacity of embryos could be due to the biosynthesis and subsequent accumulation of certain phenolic compounds in pineapple calli. Resveratrol, trans-methoxycinnamic acid, astragalin, gallic acid, genistein, quercetin, and naringenin could be considered as phenolic markers of mature embryos germination in pineapple.

Regarding the addition of exogenous phenolic compounds to media that are not favorable (recalcitrant) to embryo germination, the present study showed that this did not stimulate the germination of mature embryos from these media. On the contrary, browning followed by necrosis of the calli were observed in the tested media. The phenolic compounds added to the culture medium could not be assimilated by the cells. They were probably oxidized by oxidoreductases secreted by the calli, leading to the formation of quinones, which rapidly condensed into brown polymers. These toxic polymers are responsible for the observed cell necrosis (Shetty et al., 1990; Baaziz et al., 1994; Kouakou, 2009) and inhibit germination. Moreover, Kouakou (2003) reported that phenolic compounds act as germination inhibitors. Kouakou (2009) also demonstrated that embryogenic cells possess specific phenolic compounds that are not synthesized by non-embryogenic cells. Thus, the germination or conversion of somatic embryos in pineapple appears to depend on the types of phenolic compounds present, but their direct addition to the medium does not seem to promote germination. In fact, according to the work of Kouakou (2009) in cotton, only a change in the endogenous phenolic metabolism of the cells could effectively stimulate embryogenesis in cultivars that are recalcitrant to somatic embryogenesis.

5. Conclusion

In pineapple, the combination of hormones and amino acids enabled the biosynthesis and accumulation of several phenolic compounds in the MIE5 medium, which produced the highest germination rate. The ability of embryos to germinate therefore appears to be linked to the biosynthesis followed by the accumulation of phenolic compounds such as resveratrol, trans-methoxycinnamic acid, and astragalin (kaempferol 3-O-glucoside), as well as gallic acid, genistein, quercetin, and naringenin in the pineapple calli. These phenolic compounds could be considered as phenolic markers of mature embryos germination or conversion in pineapple. However, these phenolic markers are exclusively of endogenous origin. Any exogenous supply of phenolic germination markers leads to browning followed by cell necrosis in pineapple.

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