

## Induction of vitro-plants of two varieties of pineapple (*Ananas comosus*) L. Var. Cayenne smooth and MD2 grown in Côte d'Ivoire and assay of some biochemical compounds

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### Abstract

Pineapple (*Ananas comosus* (L.) MERR) is the most widely consumed fruit in the world after the sweet banana. It's a fruit plant that plays a key role in the Ivorian economy. However, its cultivation is facing numerous problems due to the ageing of orchards and the abusive use of phytosanitary products, leading to a drop in fruit quality and yield. The revival of this sector is all the more important as it occupies a strategic position in the Côte d'Ivoire economy. Biotechnology seems to be a highly effective way of dealing with aging orchards. In this study, the influence of variety on *in vitro* shoot induction in two pineapple varieties (Smooth Cayenne and MD2) was tested to determine the mass production of healthy and high-performing shoots and then the biochemical compound content of the two pineapple varieties during shoot induction was tested. The results show that MD2 has the highest shoot induction capacity (87.93 shoots) and the highest phenol, flavonoid and leaf pigment content. Phenolic compounds and flavonoids are thought to be markers of shoot induction in pineapple. The MD2 variety is best suited to shoot induction.

**Keywords:** *In Vitro*; Shoots; *Ananas Comosus*; Biochemical Compounds

### 1. Introduction

A monocotyledon belonging to the Bromeliaceae family, pineapple (*Ananas comosus* (L.) MERR) is a perennial herbaceous plant. It accounts for around 20 % of the world's tropical fruit production and is the second most cultivated exotic fruit after bananas [1]. Over the past 25 years, world pineapple production has doubled to around 25.4 million tons per year. According to the FAO, more than 80 countries produce pineapple, 65 % of which is destined for export [2]. The fresh pineapple market is dominated by Costa Rica, Brazil and the Philippines. In West Africa, Côte d'Ivoire, Ghana, Togo and Benin are the biggest producers. In 2017, Côte d'Ivoire was West Africa's leading pineapple exporter to the European Union (27300 t) [3]. In recent decades, Cote d'Ivoire has lost its strategic position as the world's leading pineapple exporter, due to the emergence of new varieties developed by Latin American countries [4]. Exports, which represented almost 200,000 tons in 2001, have fallen drastically to around 30,000 tons in 2019 [5]. The drop in yield may be due to several factors including high pest pressure, which has led to disease, the ageing of the Ivorian orchard, the lack of crop rotation and soil impoverishment [6]. Also, the excessive use of agricultural inputs and phytosanitary products has led to acidity and a high level of chemical residues in the fruit. All these factors have contributed to lowering the quality of pineapple from Côte d'Ivoire. This makes it less competitive on the international market [7]. Biotechnology through its various fields, including *in vitro* culture, could be a tool for this varietal improvement. In fact,

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*in vitro* vegetative propagation is of great importance, as it makes it possible to take a diseased plant and regenerate healthy, disease-free plants in mass, thereby acquiring defense capabilities. It can also be used to monitor the compounds present in the plant. Pineapple contains phenolic compounds such as flavonoids, which have antioxidant effects. These phenolic compounds help prevent many diseases, such as cancer, by fighting free radicals. The general objective of this study is therefore to produce *in vitro* healthy and vigorous plants of two pineapple varieties (smooth Cayenne and MD2) for the improvement of Ivorian production.

## 2. Materials and methods

### 2.1. Plant material

The plant material used in this study consisted of rejects of two pineapple varieties from the Bonoua farmers' plantations (Côte d'Ivoire). These are the Cayenne Smooth and Extra Sweet (commonly known as MD2) varieties, the most widely grown in Côte d'Ivoire and highly productive.



A Cayenne Smooth

B Extra Sweet (MD2)

**Figure 1** Rejects from two pineapple varieties

## 3. Methods

### 3.1. Culture medium

The basic medium used for this study was [8] medium supplemented with vitamin B5 [9] (MSB5) generally used for pineapple tissue cultures [10]; [11]. For this purpose, 30 g/l glucose was added to the medium as a carbon source. This medium was supplemented with 0.2 g/l glutamine and 0.01 mg/l Kin. The medium was solidified by adding 7.2 g/l Agar Agar. The pH of the medium was adjusted to 5.8 with 1N HCl and/or NaOH. The culture media were then brought to the boil, dispensed into jars at a rate of 30 ml/jar and autoclaved (Autester) for 30 min at 121°C under a pressure of 1 bar.

### 3.2. Disinfection of shoots and cultivation of meristems

In order to obtain contaminant-free explants for good micropropagation in pineapple, shoots of each variety were taken from village plots in Bonoua. Once in the laboratory, these rejects were washed in tap water with liquid soap for 10 min before being stripped of leaves and trimmed to reduce the pseudotrunc to 2/3 of its volume. Next, in a laminar flow hood, the trimmed rejects were soaked in 70 % (v/v) ethanol for 30 seconds, then rinsed three times with sterile distilled water for disinfection before being transferred to a 3.8 % (m/v) calcium hypochlorite bath with 0.1 % tween 20, for 10 minutes. After rinsing three times with sterile distilled water, the shoots were trimmed again using a sterile blade mounted on a scalpel, until the apical meristem measuring 1 cm x 0.5 cm x 0.5 cm was obtained. The explant thus obtained was cultured on shoot induction medium at a rate of one explant per 22 mm ø by 150 mm long test tube. These test tubes containing the explants were closed and sealed with cling-film, and placed in the culture room for four weeks.

### 3.3. Growing conditions

The growing room is regulated at a temperature of  $26 \pm 2$  °C and a relative humidity of 70 %. The room was lit with 200 lux white lamps for a 12h/24h photoperiod.

### 3.4. Evaluation of *vitro* plant production

After four weeks of cultivation, the number of shoots, leaves and roots was determined by hand-counting from each jar. Leaf shoot counts were carried out on two pineapple varieties, using 30 jars for each variety.

### 3.5. Study of biochemical compounds during *in vitro* plant production

#### 3.5.1. Extraction and assay of leaf pigments

Leaf pigments (chlorophylls (Chl) and carotenoids (Cart)) were extracted using the method described by [12], modified and adapted to our plant material. To this end, 100 mg of leaves cut into small fragments were ground in the presence of a pinch of Fontainebleau sand and calcium carbonate in 10 ml of 20 % acetone. The crushed material was centrifuged at 5000 rpm for 15 min at 4 °C. The supernatant obtained was the crude leaf pigment extract. Chlorophyll pigments and carotenoids were determined using the method described by [13]. To do this, 3 mL of the supernatant was taken and the optical density (OD) was measured with a spectrophotometer at 663 nm and 647 nm for chlorophyll and 470 nm for carotenoid against a control sample made with acetone. The various concentrations were calculated according to the following formulas

- $\text{Chl a (g/ml)} = [12,25 \times \text{DO}_{663} - 2,79 \times \text{DO}_{647}] \times V/1000 \text{ m}$
- $\text{Chl b (g/ml)} = [21,5 \times \text{DO}_{647} - 5,10 \times \text{DO}_{663}] \times V/1000 \text{ m}$
- $\text{Chl t (g/ml)} = [7,15 \times \text{DO}_{663} + 18,71 \times \text{DO}_{647}] \times V/1000 \text{ m}$
- $\text{Cart (g/ml)} = [1000 \times \text{DO}_{470} - 1,82 \times \text{chl a} - 85,02 \times \text{chl b}] / 189 \times V/1000 \text{ m}$
- V is the volume of crude extract (ml) and m is the mass of fresh leaves used (g).

### 3.6. Extraction and determination of phenolic compounds

Phenolic compounds were extracted using the method of [14]. A 500 mg sample of leaves from each pineapple variety was placed in 10 ml of pure methanol (96 %). The whole set was incubated in the dark for 18 hours (the time required to extract phenolic compounds at 4 °C). After centrifugation at 2000 rpm for 10 min, the supernatant constituted the crude phenolic extract (PE) to be analyzed. Phenolic compounds were determined using the method of [15]. 0.5 ml of 0.5 N Folin-Ciocalteu reagent was added to 0.9 ml distilled water. 0.1 ml of phenolic extract was added. After stirring at room temperature, 1.5 ml of 17 % (w/v) sodium carbonate and 6 ml of distilled water were added to the solution. The resulting mixtures were incubated for 35 minutes. The intensity of coloration proportional to polyphenol concentration was monitored using a spectrophotometer at 765 nm. The phenolic extract was replaced by distilled water. Total phenols are determined using a standard curve ( $y = 0.586x + 0.053$ ;  $R^2 = 0.999$  where y is absorbance and x is gallic acid concentration) performed with different concentrations of a gallic acid stock solution (200 µg/ml). Phenol levels were expressed in micrograms per gram of fresh matter (µg/g of MF) using the formula below:

$$TPT(\mu\text{g/g}) = 2X.Vf/m$$

TPT = Total Phenol Content; m: mass of leaves sampled; Vf: volume after centrifugation and X:  $Y/0.586$  (Y being OD).

### 3.7. Tannin extraction and dosage

The tannin extraction method is identical to that described by [14] for the extraction of phenolic compounds. Next, the tannin content of different Cayenne smooth and MD2 pineapple leaves was determined using the method of [16]. Thus, 1 ml of supernatant from the polyphenol extraction was taken, to which 5 ml of vanillin reagent (0.1 mg/ml vanillin in 70 % (v/v) sulfuric acid) was added. Tubes were left to stand for 20 min in the dark, and absorbance was read on a spectrophotometer (JASCO V530) at 500 nm against white. A calibration range was carried out using a 0.1 mg/ml tannic acid standard solution. The content was calculated using the following formula

$$TT (\text{mg}/100\text{g}) = [(DO \times 103) / 3,11 \times m]$$

TT = Tannin content; OD = Optical Density; m = sample mass

### 3.8. Flavonoid extraction and assay

Flavonoids were extracted in the same way as above (extraction of phenolic compounds). Flavonoids were determined using the method of [17]. To this end, 0.5 ml of supernatant from the polyphenol extraction was taken and added successively to 0.5 ml distilled water; 0.5 ml aluminum chloride (10 %, w/v); 0.5 ml sodium acetate (1M) and 2 ml distilled water. Tubes were left to stand for 30 min in the dark, and absorbance was read on a spectrophotometer (JASCO V530) at 415 nm against blank. A calibration range was carried out using a 0.1 mg/ml quercetin standard solution. Flavonoid content was calculated as follows:

$$TF (mg/100g) = [(DO \times 2 \times 103) / 18,12 \times m]$$

TF = Flavonoid content; OD = Optical Density; m = sample mass

### 3.9. Statistical analysis

Statistical analyses were performed using Statistica 7.1 software. Analysis of variance (ANOVA) with one classification criterion was performed on the means obtained. When a significant difference was observed, the Newman-Keuls test at the 5 % threshold was used to separate the means. In addition, a Principal Component Analysis (PCA) was performed to understand the influence of different biochemical compounds on shoot induction in the two pineapple varieties.

## 4. Results

### 4.1. Varietal influence on shoot, leaf and root induction in pineapple vitroplants

The results of the influence of variety on shoot, leaf and root induction are recorded in Table 1 and showed that shoot induction varied very significantly ( $p \leq 0.003$ ) according to pineapple variety. In fact, the MD2 variety produces more shoots (87.93) than the Cayenne variety (38.41).

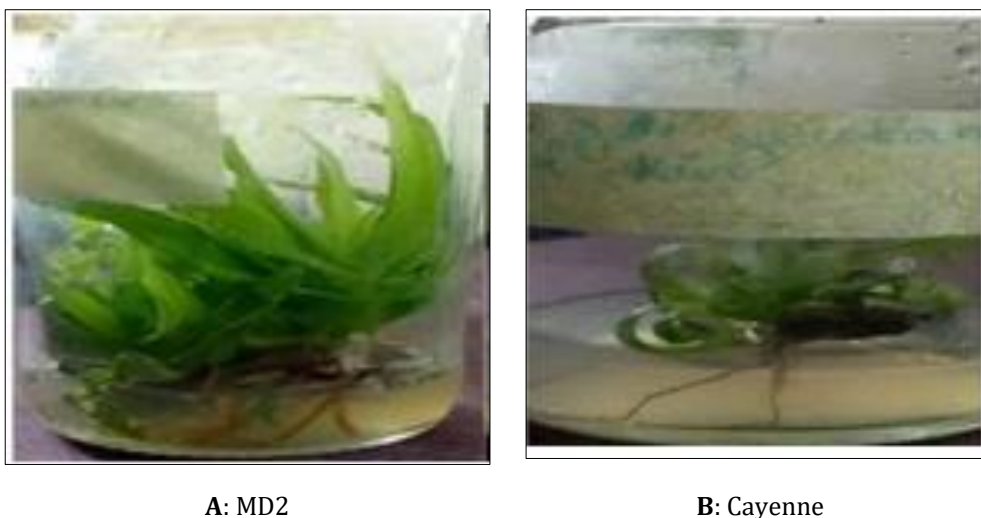
Analysis of the results also showed that the induction of leaves per shoot varied significantly ( $p \leq 0.002$ ) from one variety to another. The highest number of leaves per shoot was recorded for the MD2 variety (9.67 leaves) compared with 5.90 leaves for the Cayenne variety.

Analysis of root induction results for both varieties revealed a significant effect ( $p \leq 0.03$ ) on root formation. Thus, the MD2 variety induced the highest number of roots (10.25 roots). On the other hand, root formation was very low (2.64 roots) in the Cayenne variety (figure 2).

**Table 1** Effect of variety on shoot induction in pineapple

	Nbr shoots	Nbr leaves	Nbr root
Cayenne	38.41 ± 0.19 b	5.90 ± 0.39 b	2.64 ± 0.20 b
MD2	87.93 ± 0.23 a	9.67 ± 0.59 a	10.25 ± 0.57 a

In the same column, means followed by the same letter are statistically identical (5% Newman Keuls test); Nbr : Number



**Figure 2** *In vitro* root induction in two pineapple varieties

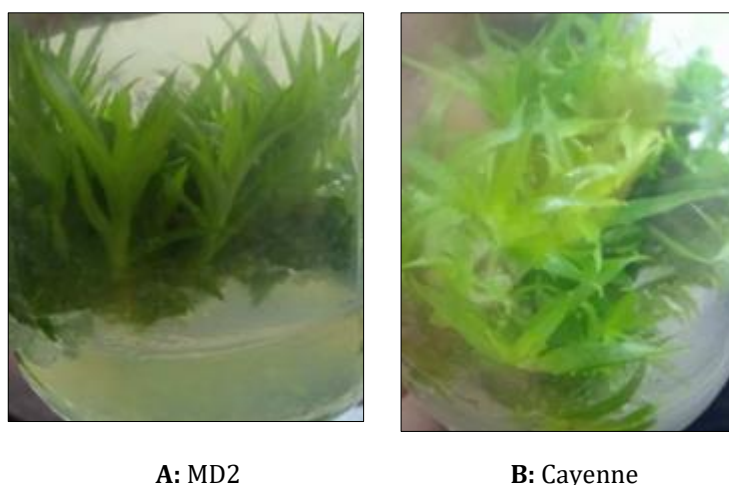
#### 4.2. Evaluation of leaf pigment content during shoot induction in two pineapple varieties

The results for leaf pigment content are recorded in Table 2 and showed that the MD2 variety expressed high levels of chlorophyll a (0.087 g/ml), b (0.069 g/ml) and t (0.156 g/ml) compared with the Cayenne variety (chlorophyll a (0.05 g/ml), b (0.051 g/ml) and t (0.101 g/ml)). The same applies to carotenoid content, which was higher in the MD2 variety (192.089 g/ml) than in the Cayenne variety (99.8 g/ml). Leaf color varies from one variety to another (figure 3).

**Table 2** Leaf pigment content of two pineapple varieties

Leaf pigment content (g/ml)				
Varieties	Chl (a)	Chl (b)	Chl (t)	Cart
Cayenne	0.050 ± 0.002b	0.051±0.002b	0.101 ±0.05b	99.8 ±0.03b
MD2	0.087 ±0.022 a	0.069±0.0023a	0.156 ±0.06a	192.089 ±0.03a

In the same column, means followed by the same letter are statistically identical (5% Newman Keuls test). Chl: Chlorophyll; Cart: Carotenoid



**Figure 3** Color of pineapple leaves of cultivated varieties

#### 4.3. Evaluation of biochemical compound content in two pineapple varieties

Analysis of this table shows that in pineapple, the MD2 variety produces more phenolic compounds (327.11 µg/g/MF) than the Cayenne variety (215.32 µg/g/MF). Flavonoid content results for both varieties showed that variety had no significant effect on flavonoid content ( $p \leq 0.35$ ). Flavonoid content is statistically identical in both varieties (168.42 and

165.31 mg/100 g Cayenne and MD2 respectively). The results also revealed a higher tannin content in the smooth Cayenne variety (94.12 mg/100g) than in the MD2 variety (63.31 mg/100g).

**Table 3** Biochemical compound content of the two varieties

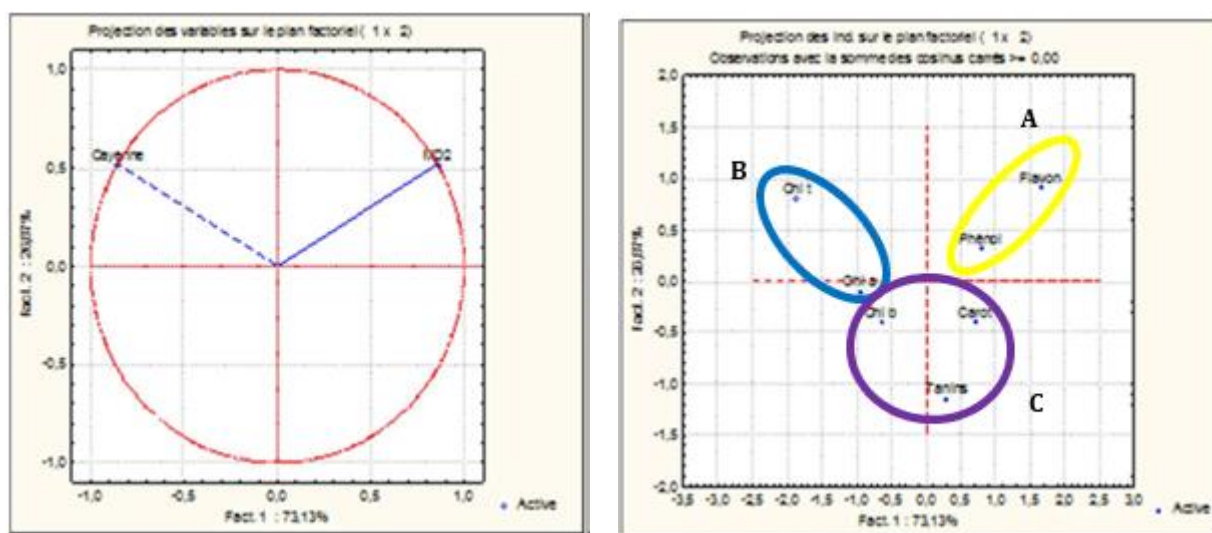
Varieties	TPT ( $\mu\text{g/g/MF}$ )	TF (mg/100g)	TT (mg/100g)
Cayenne	215.32 $\pm$ 0.16b	168.42 $\pm$ 0.12a	94.12 $\pm$ 0.04a
MD2	327.11 $\pm$ 0.19a	165.31 $\pm$ 0.11a	63.31 $\pm$ 0.06b

In the same column, means followed by the same letter are statistically identical (5% Newman Keuls test).; TPT: Total phenol content, TF: Flavonoid content, TT: Tannin content, MF: fresh material

#### 4.4. Principal component analysis (PCA)

To better understand the influence of different biochemical compounds on shoot induction, a principal component analysis (PCA) was carried out.

PCA showed that axes 1 and 2 expressed 73.13 and 26.87 % of variability respectively. The distribution of the various compounds on the two axes has enabled us to identify three very distinct groups (Figure 4). Group A (phenols and flavonoids) correlates with the MD2 variety, group B (chlorophyll pigments, total chlorophyll and chlorophyll b) correlates with the Cayenne variety and group C (chlorophyll a, carotenoids and tannins) does not correlate with any factor.



**Figure 4** Distribution of biochemical compound content variables according to variety in the 1-2 plane of a principal component analysis

## 5. Discussion

The study of morphological parameters revealed that the MD2 variety induced the highest number of shoots, leaves and roots. This could be explained by the fact that the MD2 variety is better at assimilating the compounds present in the culture medium.

Indeed, in *in vitro* culture, explant response depends not only on the explant itself, but also on medium component and culture conditions [18]. The medium composition thus plays an important role, as it can either promote or inhibit the success of *in vitro* cultures. Hormones such as cytokinins, once in the medium, promote cell division and bud formation [19]. The MD2 variety is therefore the most suitable for inducing shoots on the medium used. In terms of physiological parameters, this study showed that total chlorophyll and carotenoid levels were higher in the MD2 variety. The MD2 variety presents a greener color, seems to be self-sufficient, ready to be acclimatized and put in nursery, this MD2 variety has a good photosynthetic activity. The culture medium used is therefore more favorable to shoot induction in this variety. According to [20], chlorophyll pigment biosynthesis pathways are closely linked to the uptake of salts present in the culture medium. In fact, chlorophyll is an essential element in plants, as it ensures photosynthesis. So, even if



photosynthetic activity is very low in *in vitro* culture, this chlorophyll content indicates the ability of vitroplants to photosynthesize. In the Cayenne variety, on the other hand, a pale green color was observed. This variety also has the elements needed for photosynthesis, but not in sufficient quantities to be self-sufficient.

Phenolic compound levels were higher in the MD2 variety. This result could be explained by the fact that the culture medium is stressful for the explant. In this way, this variety would develop early physiological capacities, such as the synthesis of phenolic compounds, enabling it to adapt to its new environment. It therefore becomes very self-sufficient, unlike the Cayenne variety, despite the fact that both are the same age. According to [21], these compounds regulate plant cell growth and are heavily involved in plant development. The work of [22] has also shown the involvement of phenolic compounds in organogenesis *in vitro*, while indicating that increasing their content in the plant causes early flowering. Thus, the high phenolic compound content would indicate that MD2 responds favorably to environmental conditions. When tested for flavonoid content, both varieties recorded statistically identical levels. Flavonoids have certain properties, such as reduced membrane permeability, as well as anti-inflammatory, anti-allergic and antioxidant activities. Thus, once on the culture medium, the young shoots would have secreted sufficient flavonoids to survive on the new medium. Our results are in line with those of [23], who showed that a high flavonoid content activates the plant's anti-allergic properties.

As for tannin levels, the presence of tannin is due to the fact that the MD2 variety is richer in phenolic acid (gallic acid). The Cayenne variety had a high tannin content (94.12 mg/100g) and would therefore be less equipped with phenolic acid. Our results are in agreement with those of [23] in work carried out on *Carlina acaulis*. According to [24], high tannin levels can have a negative influence on certain secondary metabolic processes. This could explain the low rate of inductive induction observed in this variety.

Principal Component Analysis was used to discriminate the biochemical compounds studied. The MD2 variety contains more phenolic compounds and is therefore more resistant to stress in the environment studied. MD2 would therefore be the best variety, as it possesses the elements essential for growth.

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## 6. Conclusion

The aim of this study was to evaluate the ability of two pineapple varieties (Cayenne smooth and MD2) to induce shoots and to determine the quantity of certain biochemical compounds. The results showed that the MD2 variety proliferated better on the medium used, with good vegetative growth (shoots, leaves and roots). Also, photosynthetic activity through chlorophylls a, b and t was highest in this variety. Finally, the synthesis of secondary metabolites such as phenols and flavonoids was greater in the MD2 variety. The MD2 variety responds more favorably to vegetative propagation. This variety is therefore better suited to *in vitro* shoot production in pineapple.

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## Compliance with ethical standards

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### *Disclosure of conflict of interest*

The purpose of this declaration is to confirm that all authors have read and agreed to the submitted manuscript. We affirm that the article represents the original work of the authors. We certify that the article has not been previously published and that there are no plans to publish it elsewhere. On behalf of all co-authors, the corresponding author takes full responsibility for the submission. All authors confirm that the list of authors is correct in both content and order, and that no changes may be made. Authors acknowledge that the decisions of the Editor-in-Chief regarding acceptance or rejection, as well as in the event of violation of the ethical principles of publication in the World Journal of Advanced Research and Reviews, are final.

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