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Evaluation of the bean qualities of cocoa clones after propagated from somatic embryogenesis culture

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Abstract

A series of field experiments were conducted to assess the bean qualities of elite cocoa clones (Malaysian Cocoa Board Clone 1 or MCBC1, Prang Besar Clone 230 or PBC230, Koko Klon Mardi 22 or KKM22, and Klon Koko Mardi 4 or KKM4) after regenerated through somatic embryogenesis culture and grafting. The bean of all clones was morphologically and physically evaluated before quantified for the physiochemical changes after the fermentation and drying processes. No abnormalities were found in the morphological characteristics of beans for all treatments. However, the KKM4 clone of immature zygotic embryo culture yielded distinctly different lightest individual seed fresh weight (4.13 g), shortest seed length (21.2 mm), and seed width (11.5 mm) compared with those from the staminode culture and grafting. From this study, some clones such as KKM4 exhibited variation after propagated from different types of explants during somatic embryogenesis culture. Nevertheless, the dried bean moisture content (7.31%) and cut test score (685.0), which fall within the standard range, validated that the bean quality is not affected after the somatic embryogenesis culture process.

Keywords: Cocoa, Bean, Fermentation, Drying, Somatic embryogenesis culture

1. Introduction

Theobroma cacao L. or commonly known as cocoa, is a tropical crop tree species which highly valued as a main raw material for the confectionary and cosmetic making industries. Cocoa, which belongs to the family of Sterculiaceae, is one of the major agricultural export products for several producing countries in Africa, Latin America, and Asia [1,2]. The raw cocoa seed is naturally bitter and have unpleasant taste, thus must be well fermented and dried to generate flavor precursors for a better quality of cocoa bean products [3]. Various biochemical reactions occurred during fermentation and these induced chemical precursors which developed the specific flavour, aroma, and colour of beans. During fermentation, several microorganisms such as yeast, lactic and acetic bacteria converted the organic acid and sugars from cocoa pulps into ethanol and organic acids, i.e., lactic and acetic acid [4]. These organic acids then penetrated the seed and induced high temperatures through an aerobic process that leads to the embryo death and tissue acidification. After the death, numerous compounds and enzymes react inside the seed to form flavour. The cocoa beans are usually dried to a moisture content of 6 to 8% after the fermentation process to prevent mould infestation during subsequent bean storage and improve the flavours formation further [5].

The genotype of clones and handling procedures during fermentation and drying have been reported as the main factors that influence the quality of cocoa bean final products [6]. Some studies [3,7] showed that cocoa clones with different seed physical characteristics induced different bean physiochemical changes during both fermentation and drying processes. In cocoa, physiochemical changes in temperature, pH, moisture content, total soluble solid (TSS), and cut test score (CTS) are monitored to achieve the maximum fermentation and

drying processes. Thus, for somatic embryogenesis cultured plants, which are usually observed with the occurrence of variation [8], it is imperative to carry out a rigorous evaluation of the quality standard of the cocoa bean before it is distributed for consumption. There is minimal research [9,10] conducted on the performance of cocoa plant following somatic embryogenesis culture and these are only focused on-field performance. To our knowledge, the present study is the first study that combines field performance and fermented bean quality of somatic embryogenesis-cultured cocoa clones. Hence, this study aims to quantify and compare the morphological, physical, and fermented bean qualities of staminode and immature zygotic embryo cultured clones compared with clones from the conventional method of grafting.

2. Materials and methods

2.1 Experimental designs

The experiment was conducted based on the two factors randomized complete block design of four types of elite cocoa clones from the Trinitario group (Malaysian Cocoa Board Clone 1 or MCBC1, Prang Besar Clone 230 or PBC230, Koko Klon Mardi 22 or KKM22, and Klon Koko Mardi 4 or KKM4) × three types of propagation (immature zygotic embryo culture, staminode culture, and grafting). Following surface sterilization, the immature zygotic embryo and staminode explants were cultured onto Driver and Kuniyaki medium (DKW) which have been added with 1.0 mg/L 2, 4-Dichlorophenoxyacetic acid (2,4-D) and 25 µg/L Thidiazuron (TDZ) for callus induction. All cultures were incubated at room temperature (25-26 ˚C) and in a dark condition. After two weeks, the callus was transferred onto other culture medium with 2.3 g/L McCown's salts, 1.0 mg/L B5 vitamins, 2.0 mg/L 2,4-D, and 50 µg/L (6-Benzylaminopurine) 6-BA for embryogenic callus induction. All cultures were then incubated at room temperature (25-26 ˚C) and in light conditions of 1,850 lux provided by a cool white, fluorescent lamp. To develop the somatic embryo, embryogenic callus was transferred onto new fresh DKW medium with 30 g/L sucrose and 1.0 g/L activated charcoal. Only somatic embryos with the fully developed cotyledon were transferred onto the medium with half-strength Murashige and Skoog (MS) macro, DKW micro and vitamins, 0.01 mg/L (1-Naphthaleneacetic acid) NAA, and 0.02 mg/L (Gibberellic acid) GA_3 for the maturation and germination. After six weeks, the fully developed somatic embryos were once again transferred onto a medium with half-strength MS medium, DKW micro and vitamins, 10 g/L glucose, 5 g/L sucrose without plant growth regulators for plantlet regeneration. At the same time, the grafted cocoa clones were also established. The scions for the grafting were collected from the mother clones of MCBC1, PBC230, KKM22, and KKM4 that produce a high yield in the fields. A root stock of three to four weeks old cocoa clones from the Trinitario group (unknown) were then chosen. Plastic tape (5 to 7 mm) was used to tie and cover the graft union. Seven replications of cocoa clones derived from each propagation type were evaluated for this study.

2.1.1 Field establishment and site description

On 21st March 2016, 84 cocoa trees with height averaged ~0.6 m and diameter averaged ~0.5 mm were transplanted at 3×3 m spacing in the field station. Each cocoa tree was transplanted at 3×3 m spacing. The soil in the field station is classified as a Red Yellow Podzolic Soil (USDA) with a pH of 5.76, phosphorus of 20 me/g, calcium of 5.1 me/g, magnesium of 3.0 me/g, potassium of 0.43 me/g, and cation exchange capacity of 0.45 me/g. The soil A horizon consisted of clay loam texture, weak fine crumbly structure and friable to a firm consistency while the B horizon consisted of clay only texture and firm consistency coarse sub angular blocky structure. The cocoa trees were covered with a black plastic netting for the first six months to provide them with an adequate shade (60% light penetration) and minimize the transplanting shock. The weeds were controlled by hand when necessary. The cocoa trees were also watered manually twice a day with 500 mL tap water for the first year during dry periods. The Nitrogen: Phosphorus: Potassium (NPK) Blue compound fertilizer (12:12:17:2MgO) was applied four times a year at the rate of 960 g per tree.

2.1.2 Cocoa pod storage and breaking

Twenty healthy and fully ripe cocoa pods from each treatment were collected from the experimental station for the physical measurement and bean fermentation. After two days of storage, these pods were opened using a knife. Only the fresh and healthy beans were selected for the studies.

2.1.3 Cocoa bean fermentation and drying

The fermentation was conducted inside a wooden box measuring 35 × 35 cm^2 for 120 h according to the Malaysian standard protocol for cocoa bean fermentation [6]. These beans were fractioned into 3 kg per sample

for each treatment. The heap and top of the beans were covered with a fresh banana leaf to provide an adequate insulation inside the wooden box. The cocoa beans were also thoroughly mixed from top to bottom every 48 h to facilitate adequate aeration. The fermentation experimentations were conducted in triplicate. The drying process was modified [6] and conducted for four days, from 8 am to 6 pm. The cocoa beans were sun dried at one bean thickness on a drying platform and mixed manually every 4 h.

2.2 Measurements

2.2.1 Morphological characteristics of seed

The seed morphological characteristics observed were seed shape, cotyledon, and pulp colours. The cotyledon was examined under a stereo zoom microscope, and the colour was classified based on [11].

2.2.2 Physical characteristics of seed

A total of 25 seeds were randomly measured from each treatment. The seeds were cleaned by removing their pulp without fermentation for this physical data collection. The seed length and width were measured with a common ruler. Individual seed fresh weight was measured by using a digital analytical balance.

2.2.3 Total soluble solids (TSS)

The TSS of fresh cocoa bean pulp were measured before the fermentation process. 2 g of cocoa ground nibs from each treatment were homogenized in 2 mL boiled distilled water. The mixtures were homogenate for two minutes and filtered with Whatman No. 4 filter paper. The filtrate was then collected and prepared in triplicate. The TSS was measured by placing 100 µL of filtrate onto the refractometer with a slight modification method from [12]. The average TSS value was reported in the Brix unit.

2.2.4 Temperature

The temperatures for all treatments were measured by inserting a digital thermometer at the centre of the fermented bean masses. The measurement of temperature was measured in triplicate after 24, 48, 72, 96, and 120 h.

2.2.5 pH

The pH was also determined in triplicate after 24, 48, 72, 96, and 120 h of fermentation. 10 g of the cocoa ground nib from each treatment were homogenized in 90 mL boiled distilled water for two minutes before filtered with Whatman No. 4 filter paper. The filtrates were divided into three aliquots and left to cool at room temperature (25-26 ˚C) for 10 min. 25 mL of aliquot was pipetted into a beaker for pH evaluation using a digital pH meter, which has been calibrated with a buffer solution.

2.2.6 Moisture content

Moisture content (%) of the dried cocoa beans was evaluated after four days of sun drying. The beans moisture content (Equation 1) was performed in triplicate and measured by using a digital analytical balance [13].

$$\text{Moisture content (\%)} = (W1 - W2)\,x\frac{100}{W1\text{-}W0} \quad (1)$$

Where W0 is the weight of the empty dish with lid, W1 is the initial weight of the beans before drying, and W2 is the weight of the beans after four days of sun dried.

2.2.7 Cut test score (CTS)

A total of 30 dried bean samples were collected randomly from each treatment. The dried beans were cut lengthwise into half to expose their maximum surface. These cut surfaces were arranged in a board for classification into four colour categories [14] such as fully brown, purple brown, fully purple, and slatey [11]. To validate the classification, these beans were also inspected under a stereomicroscope. The CTS value was calculated as in Equation 2 [13,15].

$$\text{CTS} = (10 \times \%\text{Fully brown}) + (5 \times \%\text{Purple brown}) + (0 \times \%\text{Fully purple and slatey}) \quad (2)$$

2.3 Data analysis

The statistical analyses were conducted using [16]. The means for each variable parameter were reported, and associated variations were established by the Tukey HSD test at $p<0.05$. A Two-Way Analysis of Variance was performed to test the differences and interaction among cocoa clones and the type of propagation.

3. Results and discussion

3.1 Morphological characteristics of seed

The cocoa seeds from somatic embryogenesis cultured clones showed normal morphological characteristics similar with cocoa seeds from the conventional propagation of grafting. Other studies by [9,10] also revealed the comparable finding for the somatic embryogenesis cultured cocoa clones from the Trinitario group. The number of seed rows in each fruit indicated the total number of loculi within the ovary of the cocoa clones. For example, four rows of seeds mean four loculi inside the ovary of PBC230 clone, whereas five rows of seeds in MCBC1, KKM22, and KKM4 clones showed the presence of five loculi inside their ovaries (Figure 1). The colour of seed pulp was yellow (5 Y 8/4) and the colour of seed cotyledon was purplish-red (2.5 R (5/6 to 5/10))). The seed from these cultured clones varied in shapes including ellipsoid and oblong. These multiple forms of seed shapes, however, did not reflected abnormality as they were also observed in the grafted clones. Thus, it is not recommended to classify cocoa clones based on their seed shapes due to the inconsistent expression of the Trinitario group of clones which formed from the hybridization of several cocoa varieties such as Forastero and Criollo [17,18]. Detailed observation revealed the presence of translucid tissue inside the seed cotyledon of all clones. In cocoa, this translucid tissue reacts with flavour precursors such as sugar and polyphenol during the subsequent cocoa bean fermentation process [19].

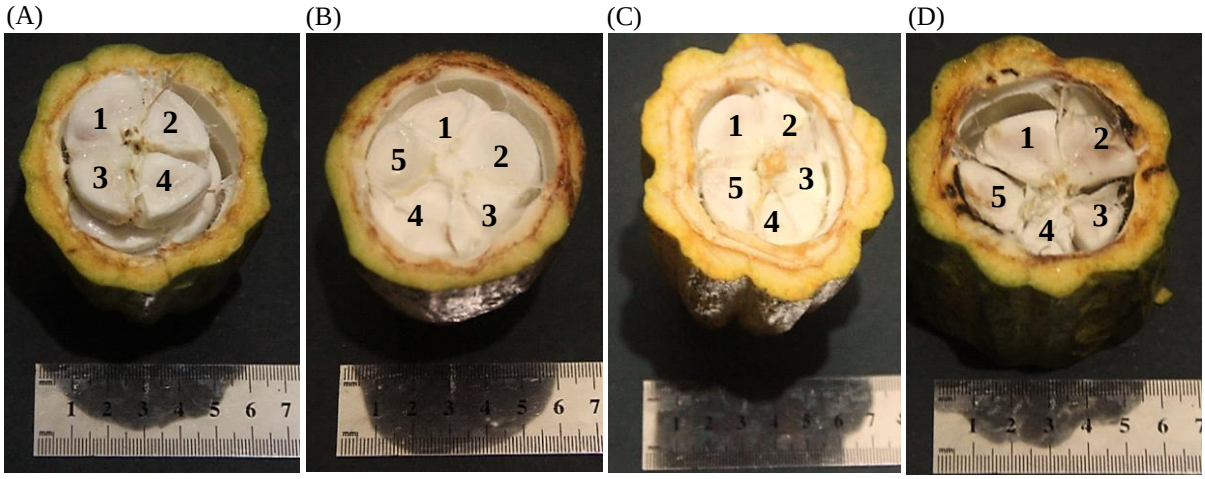

Figure 1 The seeds arrangements for cocoa clones regenerated from staminode culture. The seeds were arranged in four rows for PBC230 (A) and five rows for MCBC1 (B), KKM4 (C), and KKM22 (D) clones.

3.2 Physical characteristics of seed

Propagation type and cocoa clone interaction was significant in the KKM4 clone of immature zygotic embryo culture for seed length, seed width, and seed fresh weight (Table 1). This clone observed with the lowest measurements of seed length, seed width, and fresh seed weight compared with other treatments. Comparable studies by [9,10] also found variability in the somatic embryogenesis cultured trees for those parameters when compared with grafted and seed donor trees. The physical characteristics of seed is a crucial in cocoa plant [20] as they regulate the physiochemical changes inside beans during the fermentation process. The study by [20] found that some cocoa beans fail to reach the maximum temperature and pH for effective fermentation due to the seed morphometry characteristics. For instance, the smaller seeds which formed compact fermentation masses inside the fermentation boxes will minimize the oxygenation reaction of the aerobic phase during the fermentation process.

Table 1 The seed morphometric measurements for all cocoa clones.

Cocoa Clones	Propagation type	Seed length (mm)	Seed width (mm)	Individual seed fresh weight (g)
MCBC1	ST	21.8	12.0	4.30
	IZ	22.1	12.2	4.33
	G	21.7	12.0	4.26
	Mean	21.9	12.1	4.30
PBC230	ST	21.7	12.5	4.28
	IZ	22.1	12.3	4.30
	G	22.4	12.0	4.26
	Mean	22.1	12.3	4.28
KKM22	ST	22.1	12.3	4.33
	IZ	22.1	12.5	4.34
	G	22.4	12.4	4.31
	Mean	22.2	12.4	4.33
KKM4	ST	22.3	12.4	4.32
	IZ	21.2	11.5	4.13
	G	22.4	12.3	4.32
	Mean	22.0	12.1	4.26
	P-value for propagation	0.164	0.354	0.473
	P-value for clones	0.416	0.087	0.139
	P-value for Propagation*Clones	<0.05	<0.05	<0.05

ST, staminode culture trees; IZ, Immature zygotic embryo culture trees; G, Grafted trees; Tukey HSD, Tukey honestly significance difference.

3.3 Temperature

The pattern of increasing and decreasing temperature indicated the typical fermentation characteristic of cocoa beans from the Trinitario group (Table 2). These patterns of temperature, which rose from ~25 ˚C to ~48 ˚C for 24 to 96 h, then declined to ~45 ˚C at the end was also reported before in other studies [12,21]. In other study by [22], such condition was due to cocoa pulp, which encloses seed was wiped out by the successive action of microorganisms such as yeast, lactic, and acetic acid bacteria. These microorganisms transformed the organic acids and sugars inside cocoa pulp into ethanol, lactic acid, and acetic acid via the aerobic phase. Subsequently, the transformations then induce an exothermic reaction [4]. The high temperature produced from the reaction which observed at 96 h of fermentation causes the death of the embryo, cotyledon cracking, and tissue acidification [20]. During the 120th, whereby the activities of microorganisms and endogenous enzymes are completely degraded, the temperature was slightly decreased than before. At this point, the fermentation process was terminated.

Table 2 The fermentation temperatures for all cocoa clones.

Cocoa clones	Propagation type	Temperatures (˚C)					
		0 h	24 h	48 h	72 h	96 h	120 h
MCBC1	ST	25.4	28.1	34.2	38.1	47.7	45.0
	IZ	25.4	28.2	34.6	37.8	47.8	45.0
	G	25.5	28.2	34.5	37.9	48.1	45.3
	Mean	25.4	28.2	34.4	37.9	47.9	45.1
PBC230	ST	25.7	28.6	34.3	38.5	47.7	45.0
	IZ	25.8	28.5	34.4	39.0	48.2	45.5
	G	25.5	28.5	34.9	38.4	48.2	45.0
	Mean	25.7	28.5	34.5	38.6	48.0	45.2
KKM22	ST	25.4	28.5	34.6	38.4	47.2	44.7
	IZ	25.7	28.8	34.6	38.3	47.8	45.2
	G	25.3	28.5	34.4	38.6	47.8	44.7
	Mean	25.5	28.6	34.5	38.4	47.6	44.9
KKM4	ST	25.6	28.9	34.8	38.7	48.3	45.1
	IZ	24.6	27.5	32.3	37.4	47.4	44.1
	G	25.4	29.1	34.6	38.5	48.1	45.1
	Mean	25.2	28.5	33.9	38.2	47.9	44.8
	Propagation type	0.788	0.104	0.098	0.313	0.489	0.964
	Cocoa clones	0.137	0.312	0.216	0.189	0.419	0.894
	Propagation*Clones	0.109	<0.05	<0.05	0.09	0.545	0.681

ST, staminode culture trees; IZ, Immature zygotic embryo culture trees; G, Grafted trees; Tukey HSD, Tukey honestly significance difference.

3.4 pH

The fermented cocoa nib pH was increasingly acidic from the beginning to 96 h of the fermentation process (Table 3). The Similar normal pH trends have also been reported by the previous studies [12,22]. The pH was increased due to the structural breakdown of both lactic and acetic acid inside cocoa pulp by microorganisms [12]. In contrast, [23] discovered significantly higher acidic beans, whereas [24] reported the less acidic beans of the West African cocoa plant. These differences may be due to the genotypic effect, duration, and methods applied during the fermentation process [3]. The failure to obtain the maximum pH during the end fermentation process in some study [1] resulted in the production of defective beans with mould formations during the subsequent drying process. In case of this study, the KKM4 clone from immature zygotic embryo culture produced less acidic bean within the first 48 h of fermentation when compared with other treatments. This lower bean acidity could be explained by a reduction in acid fermentation by lactic and acetic acid bacteria due to the poor aeration of the compact masses formed by the smaller beans of this KKM4 clone. Nevertheless, a rigid turning at 48 h of fermentation successfully increased the aeration for the maximum fermentation process for the clone.

Table 3 The pH of all cocoa bean pulps during the fermentation process.

Cocoa clones	Propagation type	pH					
		0 h	24 h	48 h	72 h	96 h	120 h
MCBC1	ST	6.23	5.92	5.43	4.84	4.75	4.79
	IZ	6.28	5.95	5.47	4.84	4.72	4.79
	G	6.26	5.90	5.50	4.85	4.72	4.78
	Mean	6.26	5.92	5.47	4.84	4.73	4.79
PBC230	ST	6.29	5.98	5.52	4.83	4.75	4.80
	IZ	6.26	5.89	5.48	4.82	4.74	4.81
	G	6.25	5.90	5.47	4.80	4.74	4.89
	Mean	6.27	5.92	5.49	4.82	4.74	4.83
KKM22	ST	6.27	5.95	5.45	4.85	4.74	4.79
	IZ	6.31	5.94	5.44	4.84	4.75	4.79
	G	6.28	5.88	5.40	4.82	4.70	4.80
	Mean	6.29	5.92	5.43	4.84	4.73	4.79
KKM4	ST	6.28	5.92	5.42	4.86	4.71	4.78
	IZ	6.29	6.09	5.62	4.91	4.78	4.82
	G	6.31	5.94	5.46	4.85	4.72	4.79
	Mean	6.28	5.96	5.50	4.87	4.74	4.80
	Propagation type	0.928	0.348	0.643	0.747	0.601	0.848
	Cocoa clones	0.742	0.493	0.121	0.581	0.845	0.943
	Propagation*Clones	0.621	<0.05	<0.05	0.288	0.518	0.669

ST, staminode culture trees; IZ, Immature zygotic embryo culture trees; G, Grafted trees; Tukey HSD, Tukey honestly significance difference.

3.5 Moisture content

After the fermentation process, all cocoa beans were sun dried to achieve a moisture content averaged at 7.51% (Table 4). This moisture content was consistent with the standard dried bean moisture content value reported by [6] (7 to 8%) for Malaysian cocoa clones bean after four days of sun drying. According to Hii [6], cocoa beans were sun dried to this moisture content for the purpose of safe storage. The result of this study was also in agreement with those reported by other researchers [22,24]. In contrast, [25,26] reported variation in fermented bean moisture content due to cocoa variety and fermentation handling procedures. The over dried bean with moisture content less than 7% usually resulted in its hardness, which then inhibited the outward migration of acetic acid hence leading to the excessive bean acidity. These beans, which have a slatey appearance, is of low quality and deleterious to the chocolate flavour.

Table 4 All cocoa beans moisture content (%) after four days of sun dried.

Propagation type	Moisture content (%)				
	MCBC1	PBC230	KKM22	KKM4	Mean
ST	7.64	7.38	7.39	7.60	7.50
IZ	7.76	7.38	7.50	7.31	7.49
G	7.56	7.43	7.58	7.53	7.53
Mean	7.65	7.40	7.49	7.48	
	Propagation	Clone	Propagation*Clone		
P-value	0.608	0.419	0.257		

ST, staminode culture trees; IZ, Immature zygotic embryo culture trees; G, Grafted trees; Tukey HSD, Tukey honestly significance difference.

3.6 Cut test score

One of the indicators used to evaluate the final price of cocoa beans was their CTS value (Table 5). For the dried bean CTS value, no significant difference was found among treatments. The CTS value averaged 689.9 in terms of propagation type and 690.6 in terms of cocoa clones. These findings were consistent with those reported by [6,26] for Malaysian cocoa clones. The bean with a CTS value of between ~681 to ~696 was considered high quality and fully fermented. In Mexico, [22] also found a comparable finding for cocoa beans after four days of sun drying. Contradictorily, other separate research by [6] reported some cocoa bean with significantly lowest bean CTS value (<590). The researchers observed that inadequate fermentation and drying duration due to inconsistent Malaysian weather have contributed to such difference. Thus, it is suggested to conduct a bean drying process through a controlled artificial dryer such as a microwave oven.

Table 5 The CTS value of dried bean for all clones after four days of sun drying.

Propagation type	Cut test score				
	MCBC1	PBC230	KKM22	KKM4	Mean
ST	694.3	680.6	696.7	695.4	691.8
IZ	692.2	692.8	688.3	685.0	689.6
G	695.1	687.8	690.0	690.7	690.9
Mean	693.9	687.1	691.7	690.4	
	Propagation	Clone	Propagation*Clone		
P-value	0.478	0.744	0.096		

ST, staminode culture trees; IZ, Immature zygotic embryo culture trees; G, Grafted trees; Tukey HSD, Tukey honestly significance difference.

4. Conclusion

Some elite cocoa clones such as KKM4 exhibited variations in bean qualities after propagated from somatic embryogenesis culture by using immature zygotic embryo explant. This showed the effect of cocoa genotype (i.e., genetic constituent where some clones are prone to mutation) and type of explant in inducing mutation during somatic embryogenesis culture. Nevertheless, the bean CTS value as the final determinant of cocoa quality, which falls within the acceptable standard range, validated no adverse effects occurred after somatic embryogenesis culture. It is recommended to ensure that the fermented bean of the KKM4 clone from immature zygotic embryo culture has sufficient aeration through rapid turning during the fermentation process.

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