The effect of variations in media type and panicle length on the development of maize anther culture (*Zea mays* L. var. NK-212 and BISI-18)

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Abstract. The development of maize anther culture is strongly influenced by genetic factors and in vitro media conditions. This study aimed to determine the effects of varieties (NK-212 and BISI-18) and Yu-pei media forms (solid, liquid, and solid–liquid) on culture development based on color change, anther enlargement, and anther dehiscence. Panicles from each variety were grouped into three lengths (20, 25, and 30 cm), and the one with the highest percentage of viable microspores was selected. Selected panicles were given cold treatment at 7°C for 7 days, then cultured and incubated for eight weeks. Data were collected every two weeks. The results showed that varieties and media forms did not significantly affect anther development ($p > 0.05$). However, the solid–liquid medium tended to produce the highest responses in both varieties, with anther enlargement reaching 39.00% in NK-212 and 47.00% in BISI-18, and anther dehiscence reaching 30.67% in NK-212 and 36.33% in BISI-18. Anther color changed gradually from green to yellowish brown during incubation. Overall, anther culture development was more influenced by varietal genetic characteristics and incubation duration than by media form variation.

1 Introduction

Maize (*Zea mays* L.) is one of the major cereal crops that plays an important role as a food source due to its high carbohydrate content. In Indonesia, maize ranks second after rice as a food commodity with the largest production area. In addition to its use as food, maize also has a significant economic role as animal feed and for industrial needs [1]. Based on data from Statistics Indonesia (BPS) in 2023 [2], the national maize harvested area reached 2.48 million hectares, decreasing by 0.29 million hectares or 10.43% compared to 2022, which

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reached 2.76 million hectares. This decline indicates challenges in meeting the increasing demand for maize, thus requiring strategies to improve productivity.

One of the strategic steps to increase maize production is through plant breeding in order to obtain superior cultivars that are pest-resistant, tolerant to environmental stresses, and able to maintain the superior characteristics of existing varieties. Plant breeding can be carried out through two approaches, namely conventional and non-conventional methods. The conventional method is generally conducted through selection and hybridization processes. However, this approach requires a relatively long period of time, approximately 7–10 years, to obtain stable lines [3,4]. Therefore, modern breeding strategies are needed to accelerate the development of superior varieties.

One of the effective non-conventional approaches is double haploid technology, which enables the production of homozygous lines within a single generation [5,6]. The production of double haploid lines can be carried out through anther culture utilizing microspore cells [7]. The success of anther culture has been widely reported, yet the plant regeneration rate through anther culture remains low and is strongly influenced by the physiological conditions of microspores, including the developmental stage and panicle length [8-10]. The relationship between microspore stage and panicle length indicates that certain panicle sizes result in a higher proportion of uninucleate stages compared to those that are too short or too long [11]. Although panicle length can be used as a morphological indicator to determine the microspore stage, a microspore viability test is still required to ensure cell quality before culture is performed.

Endogenous factors also play a crucial role in determining the success of embryogenesis induction in anther culture. Therefore, this study utilized two hybrid maize varieties with different genetic backgrounds for evaluation. The varieties used were NK-212 from PT Syngenta Seed Indonesia and BISI-18 from PT BISI International Tbk. The NK-212 variety is known for its wide adaptability to various environmental conditions, tolerance to drought stress, potential for high yield (reaching approximately ±10.8 tons/ha of dry shelled grain), and ears that are fully filled with tightly closed husks, which help reduce the risk of ear rot. Meanwhile, the BISI-18 variety has high yield potential (up to 12 tons/ha of dry shelled grain), vigorous plant growth, large and uniform ear size with good tip-filling, and resistance to lodging as well as major diseases such as downy mildew and leaf rust. Through anther culture, haploid plants can be produced and then doubled into doubled haploid plants to obtain pure lines capable of maintaining and stabilizing the superior characteristics of these varieties.

In vitro environmental conditions, particularly the physical form of the culture medium, also play an important role in determining the success of embryogenesis induction. The anther culture process begins with the induction phase, which is the stage when microspores are diverted from the gametophytic developmental pathway to the sporophytic pathway to form callus or embryos. During this critical phase, appropriate medium support becomes a key factor to promote microspore cell reprogramming. Each type of medium has its own advantages and limitations. In solid media, nutrient absorption tends to be less optimal because explants can only absorb nutrients from the area in direct contact with the surface of the medium. Nevertheless, solid media have the advantage of lower contamination risk, and activated charcoal is often added to absorb inhibitory compounds and provide a dark color that helps reduce light intensity. Conversely, liquid media allow explants to absorb nutrients more effectively because their contact with the medium is wider compared to solid media. However, the use of liquid media has weaknesses, including a higher risk of contamination and the potential for hyperhydricity. To overcome the limitations of both forms of media, a solid–liquid medium (double layer) was developed, combining a solid layer at the bottom and a liquid layer on top, with the aim of integrating the advantages of both solid and liquid media while minimizing their drawbacks.

Although many studies have reported the success of anther culture in maize, most still focus on the effects of media composition and the addition of natural substances to improve culture responses. Studies on the effects of media shape variation, such as solid, liquid, and double-layer (solid–liquid) media, are still limited. Therefore, this study was conducted to examine the effects of variety and media shape variation on the development of anther cultures in maize. Through this research, it is expected that scientific information will be obtained regarding the developmental response of anthers in the NK-212 and BISI-18 varieties, which may improve the efficiency and success of haploid and doubled haploid plant production, as well as serve as a reference for future studies.

2 Research Method

2.1 Plants materials

Hybrid maize seeds used in the anther culture were obtained from various varieties produced by several seed companies. The NK-212 variety is produced by PT. Syngenta Seed Indonesia, and BISI-18 is produced by PT. BISI International, Tbk. Seeds from each variety were planted in polybags until the panicles appeared at the compost house of Universitas Airlangga, Surabaya.

2.2 Determination of panicle length

The panicles used in this study consisted of three sizes, namely 20 cm, 25 cm, and 30 cm, with the condition still in bud form. One panicle will be selected as the explant based on the highest viability percentage.

2.3 Fresh observation

After the panicle was harvested, fresh observations were conducted regarding the morphology of the panicle, spikelets, anthers, and microspore cytology. For cytological observation, three spikelets from the panicle were taken, then the anthers were placed into microtubes that had been filled with medium B. Using a sterile toothpick, the anthers were pressed against the microtube wall until the microspore cells were released. Microspore viability was then observed using an inverted microscope.

2.4 Isolation and culture of anthers

Isolation of anther culture was carried out aseptically inside a laminar air flow (LAF) cabinet. First, the panicle sheath was opened, then several spikelets were placed into a bottle that had been added with Clorox and two drops of Tween 80. The bottle was gently shaken for 7 minutes, then rinsed with sterile distilled water 3–4 times. The rinsed spikelets were placed on a petri dish lined with filter paper. The anthers were then removed and placed into culture bottles containing Yu-Pie (YP) medium with variations in media form, namely solid, liquid, and semi-solid (double layer). Each bottle was filled with 12 anthers, then incubated at 25°C with stages of 2 weeks (Dark 1), 4 weeks (Dark 2), 6 weeks (Light 1), and 8 weeks (Light 2).

2.5 Qualitative analysis

The response of the anthers after being cultured was observed at each incubation stage based on changes in color, the number of anthers that enlarged, and the number of anthers undergoing dehiscence. Each observed change was documented using a micro camera.

2.6 Quantitative Analysis

Quantitative analysis was conducted to determine the highest percentage of microspore viability as well as the percentage of enlarged anthers and anthers undergoing dehiscence after 8 weeks of culture. The obtained data were analyzed using two-way analysis of variance (ANOVA) to examine the effects of variety and media form variation on the development of anther culture. Significance testing was performed at $\alpha = 0.05$. When significant differences were found, mean separation was carried out using a Post Hoc Test.

3 Result and Discussions

3.1 Microspore Viability

Microspore viability testing was carried out as an initial step to ensure the quality of anther explants before the induction process of anther culture. In this study, the microspore viability of two maize varieties, namely NK-212 and BISI-18, was observed at three panicle lengths (20, 25, and 30 cm) through fresh observation on day 0 after harvest. Microspores were categorized as viable if they had a round, intact, and turgid shape with dense cytoplasm. Conversely, non-viable microspores were characterized by plasmolysis, excessive vacuolization, pollen tube formation, and starch accumulation. Observations were carried out using an inverted microscope, then the number of viable and non-viable microspores was counted to obtain the percentage value. The results of microspore cytology observations are presented in Figure 3.3.

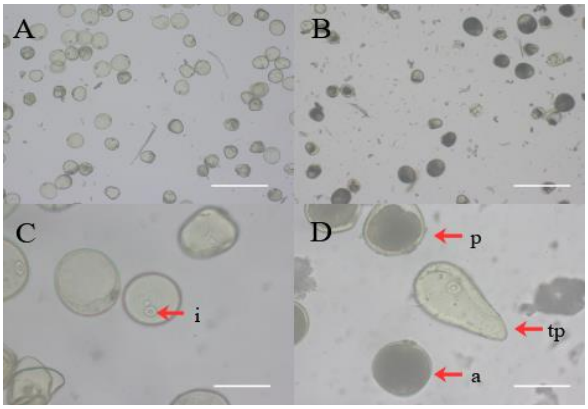

Fig. 1. Development of microspores before and after cold shock treatment. (A) Microspores before cold shock; (B) Microspores after cold shock; (C) Late uninucleate-stage microspores with (i: nucleus) positioned at the periphery; (D) Non-viable microspores: (p) plasmolysis, (tp) pollen tube, (a) starch-accumulating microspores. Bar A = 100 µm, B and C = 40 µm.

Before the cold shock treatment (Figure 1.A), the microspores appeared round, intact, and turgid with dense cytoplasm. This condition indicates that the microspores were still in the normal gametophytic developmental pathway. After the cold shock treatment (Figure 1.B),

the responses of the microspores became more diverse as a result of low-temperature stress induction. Some microspores maintained their turgid morphology due to having higher stress tolerance, while others experienced plasmolysis and structural damage due to disrupted membrane stability. In addition, microspores that formed pollen tubes also appeared, indicating that although cold shock treatment was applied, not all microspores were able to undergo a shift from the gametophytic pathway to the sporophytic pathway. The cold shock treatment is an important stage in anther culture because it functions to suppress normal gametophytic development and direct the microspores toward the sporophytic pathway. Low temperature causes a slowdown in cellular metabolism and stabilizes the membrane and cytoplasm, allowing the microspores to remain in a phase that is responsive to embryogenesis induction. In addition, the cold shock treatment acts as a physiological signal that triggers the reprogramming of cell development, as reported in various cereal species [12].

Microspores at the late uninucleate stage with the nucleus pushed to the periphery (Figure 1.C) appeared as the cells most responsive to induction, while non-viable microspores showed signs of plasmolysis, pollen tube formation, and starch accumulation (Figure 1.D). The accumulation of starch indicates that the microspore has returned to the pollen maturation pathway or is undergoing cellular degeneration. Overall, the differences between the conditions before and after cold shock illustrate that the effectiveness of microspore reprogramming is strongly influenced by the developmental stage of the cells and the ability of each microspore to tolerate low-temperature stress. This information serves as a reference in the process of calculating the viability percentage, the results of which are presented in Table 3.1.

Table 1. Percentage of microspore viability in maize plants (*Zea mays* L. var. NK-212 and BISI-18)

Varietas	Viability(%)		
	Panicle length		
	20 cm	25 cm	30 cm
NK212	76,85 ± 6,57 [a]	29,76 ± 13,15 [a]	13,99 ± 6,46 [a]
BISI-18	19,68 ± 2,74 [a]	24,23 ± 8,25 [a]	9,10 ± 3,76 [a]

Note : The results of the two-way ANOVA showed a significant effect; however, the Tukey HSD post hoc test at α = 5% did not identify any significant differences among treatment combinations, so all means are marked with the same letter notation.

3.2 Development of corn anther culture (*Zea mays* L. var. NK-212 and BISI-18)

3.2.1 Anther Color Changes During Incubation Period

Changes in anther color during the incubation period on the three types of media (solid, liquid, solid-liquid) are presented in Table 4.2. Based on Table 4.2, in the dark phase 1 (2 weeks), the anthers from both varieties generally still exhibited a green color. The green color indicates that chlorophyll remained relatively stable because it had not yet undergone intense degradation. According toTouraev *et al.* (1997), dark conditions at the early stage of culture are indeed required to suppress photosynthetic activity so that microspores can enter the sporophytic pathway [13]. In liquid and solid–liquid media, some anthers began to show a greenish-yellow color, indicating the initial stages of chlorophyll degradation due to in vitro culture stress, in accordance with the report of Shariatpanahi et al. (2006) stating that dark conditions accelerate the transformation of plastids from chloroplasts to amyloplasts [14].

At the dark phase 2 (4 weeks), the pattern of color change became more evident. In the NK-212 variety, anthers on the solid medium turned yellow, while those on the liquid medium still appeared green because exposure to liquid nutrients can delay chlorophyll degradation, whereas anthers on the semi-solid medium had turned yellow-brown. The BISI-

18 variety showed a tendency toward yellow-brown coloration across all medium types, indicating that this variety undergoes tissue aging (senescence) and browning more rapidly than NK-212. Browning in anther culture is strongly influenced by genotype and the ability of tissues to withstand culture stress. With the absence of light during the dark phase, the process of chlorophyll degradation progresses more rapidly, making the color transition from green to yellow-brown increasingly apparent.

Table 2. Color changes of anthers in maize (*Zea mays* L. var. NK-212 and BISI-18) anther culture during the incubation period on solid, liquid, and solid-liquid media.

Incubation Period (weeks)	Media type	Morphology	
		Color	
		NK-212	BISI-18
2	Solid	Green	Green
	Liquid	Green	Yellowish green
	Solid-liquid	Yellowish green	Yellowish green
4	Solid	Yellow	Brownish yellow
	Liquid	Green	Brownish yellow
	Solid-liquid	Yellow	Brownish yellow
6	Solid	Yellow	Brownish yellow
	Liquid	Yellow	Yellow
	Solid-liquid	Yellow	Brownish yellow
8	Solid	Yellow	Brownish yellow
	Liquid	Yellow	Brownish yellow
	Solid-liquid	Yellow	Yellow

Entering the light phase 1 (6 weeks), the anthers of both varieties increasingly lost their green color. Light at this stage accelerates chlorophyll degradation and tissue senescence. The NK-212 variety generally showed a yellow color, while the BISI-18 variety showed a yellow-brownish color on solid and semi-solid media. In liquid media, the anthers tended to appear more yellow because the liquid phase can suppress the accumulation of phenolic compounds, causing browning to progress slightly more slowly. This phenomenon is consistent with the findings of Orłowska et al. (2020) on barley anther culture, which demonstrated that the use of liquid media can improve culture response and suppress browning [15].

In the light phase 2 (week 8), the anthers in almost all treatments exhibited yellow to yellowish-brown coloration. The brownish color at this stage reflects increased phenolic oxidation and advanced tissue senescence. This pattern of color change is consistent with the browning phenomenon observed in anther cultures of various plant species, as reported by Ferrie & Caswell (2011) in *Brassica napus* [12]. Interestingly, the anthers of BISI-18 in solid–liquid media maintained a more yellow coloration compared to other treatments, indicating that the solid–liquid medium combination may provide a more stable in vitro environment, thereby minimizing browning.

3.2.2 Enlarged Anthers

The second parameter analyzed in anther culture development was anther enlargement. The results of the statistical analysis regarding the percentage of anther enlargement are presented in Table 4.3. Based on Table 3, the statistical analysis using the two-way ANOVA test during the 2-week incubation period showed that the type of medium had no significant effect on the percentage of enlarged anthers ($p > 0.05$), therefore no further test was required. Nevertheless, descriptively, there appeared to be a tendency for differences among media. At

this stage, the highest percentage of enlarged anthers in both NK-212 and BISI-18 varieties was observed in the solid–liquid medium. This pattern is likely related to the more uniform availability of nutrients and oxygen in liquid or solid–liquid media, allowing the microspores within the anthers to enlarge more easily as an initial response toward the formation of embryogenic structures. Several studies on anther culture in cereal crops have shown that liquid media can create a more efficient culture environment, particularly through enhanced nutrient diffusion and reduced mechanical stress on the anthers. These conditions not only facilitate the uptake of nutrients by anther tissues but also support the physiological activity of the microspores, which is generally reflected through anther enlargement during the early stages of culture. A similar phenomenon has been reported in wheat and maize anther cultures, where liquid media induced a more active microspore response and supported the early development of embryo-like structures [12,13]. The lowest percentage of enlarged anthers in the NK-212 variety was found in the liquid medium, while in the BISI-18 variety, the lowest value was observed in the solid medium. These differences may be caused by the varying abilities of each variety to utilize nutrients or tolerate the physical conditions of the medium during the early incubation phase. This initial response is also often influenced by the physiological condition of the anther, the microspore maturity level, and genotypic factors that determine sensitivity to cultural environmental changes.

Table 3. Average percentage of enlarged anthers in maize anther culture (*Zea mays* L. var. NK-212 and BISI-18) during incubation on solid, liquid, and solid-liquid media.

Media Type	Varieties	Enlarged Anther (%)			
		Incubation Period (weeks)			
		2 (Dark 1)	4 (Dark 2)	6 (Light 1)	8 (Light 2)
Solid	NK212	18,67 ± 4,619 [a]	25,00 ± 0,000 [a]	27,00 ± 5,196 [b]	21,67 ± 9,815 [a]
	BISI-18	18,67 ± 4,619 [a]	26,67 ± 4,619 [a]	22,00 ± 5,196 [b]	24,67 ± 8,505 [a]
Liquid	NK212	30,00 ± 5,196 [a]	19,00 ± 5,196 [a]	30,33 ± 13,204 [ab]	21,67 ± 9,815 [a]
	BISI-18	33,33 ± 8,505 [a]	38,67 ± 23,671 [a]	30,00 ± 5,196 [ab]	41,67 ± 8,505 [a]
Solid-Liquid	NK212	13,33 ± 4,619 [a]	30,33 ± 13,204 [a]	39,00 ± 5,196 [a]	21,67 ± 9,815 [a]
	BISI-18	44,33 ± 20,599[a]	27,33 ± 33,486 [a]	47,00 ± 19,053 [a]	41,33 ± 21,733 [a]

The statistical analysis using the Kruskal–Wallis test at 4 weeks of incubation showed that the variation in media forms did not have a significant effect on the percentage of enlarged anthers (Asymp. Sig > 0.05), so further post-hoc tests were not required. Although the differences were not statistically significant, the response patterns among treatments still showed variation between varieties and media types. In the NK-212 variety, the highest percentage of enlarged anthers was observed in the semi-solid medium, while the lowest value was found in the liquid medium. Meanwhile, in the BISI-18 variety, the highest response occurred in the liquid medium, and the lowest percentage was found in the solid medium. These differences in response patterns between varieties indicate a strong genotype-dependent characteristic in the physiological adaptation of microspores to the culture environment, even though the effect of media has not yet appeared significantly. Several studies have reported that anther enlargement responses are often not directly aligned with media effectiveness, especially during the mid-culture phase. Touraev et al. (1997) and Ferrie & Caswell (2011) demonstrated that microspore responses to the medium are highly influenced by the developmental stage of the cells, whereby the medium tends to exhibit more pronounced effects once the microspores enter the reprogramming phase toward

embryogenesis, rather than during the initial enlargement stage [12,13]. This may explain why no significant differences were observed among media at 4 weeks of incubation.

Statistical analysis using the Kruskal–Wallis test at 6 weeks of incubation showed that the form of the medium had a significant effect on the percentage of enlarged anthers (Asymp. Sig < 0.05). Therefore, a post-hoc test using Mann–Whitney was conducted to determine the differences among the medium treatments. Based on the observations, the highest percentage of enlarged anthers in both varieties was found in the semi-solid medium. Conversely, the lowest percentage in both varieties was obtained in the solid medium.

The significant effect observed at the 6-week incubation stage indicates that the culture medium began to exert a more noticeable influence on the dynamics of anther enlargement at this phase. The semi-solid medium appeared to provide more favorable conditions, likely due to the combined advantages of both medium phases. The liquid portion of the semi-solid medium allows for more effective diffusion of nutrients and hormones, while the solid component provides structural support so that the anthers do not experience excessive mechanical stress. This semi-liquid condition has been reported in tissue culture studies as an environment capable of enhancing physiological activity and accelerating early responses of organs or microspores [12,13]. In contrast, the solid medium tended to produce lower enlargement responses. This may be due to limited nutrient uptake, as water availability and mobility of nutrients are lower compared to liquid or semi-solid media. Studies on anther culture in cereals also indicate that solid media may induce higher osmotic stress, which can inhibit anther enlargement during the early stages of microspore differentiation. Overall, the results at week 6 demonstrate that the mid-phase of culture development is the point at which the medium begins to have a significant effect, and the semi-solid medium provides the most optimal conditions to support anther enlargement in both varieties.

Statistical analysis using a two-way ANOVA test for the 8-week incubation period showed that variations in media form did not significantly affect the percentage of anther enlargement ($p > 0.05$), so further testing was not necessary. Based on the data in Table 3, the percentage of anther enlargement in the NK-212 variety was relatively uniform across all media forms. For the BISI-18 variety, the highest value was obtained in liquid media, while the lowest value was found in solid media. The absence of a significant effect of media at the 8-week incubation stage indicates that the anther enlargement process at this stage has entered its final stage, where the anther's ability to increase in size decreases. The uniformity of response in the NK-212 variety indicates that this variety tends to be stable but less responsive to media modifications in the final phase of culture. In contrast, the BISI-18 variety still showed little variation across media, with liquid media providing the highest values. This response may be related to the ability of liquid media to provide better nutrient and water mobility, which can help maintain anther enlargement even during the extended incubation phase. However, this difference was not statistically strong enough to be concluded as a treatment effect. This 8-week incubation pattern indicates that the effect of the media on anther enlargement decreases with increasing incubation time, and the effectiveness of the media is more dominant in the middle phase (e.g., week 6) than in the early or late phases.

3.2.3 Anther Dehiscence

The third parameter analyzed in the development of anther culture was anther dehiscence. The results of the statistical analysis regarding the percentage of anther dehiscence are presented in Table 4.4. Based on Table 4, the results of the two-way ANOVA test after 2 weeks of incubation indicate that the media form had no significant effect on the percentage of anther enlargement ($p > 0.05$), so further testing was not necessary. However, descriptively, differences in response between media were still visible, with the highest

percentage of anther enlargement in both varieties recorded on the solid-liquid medium. This condition is thought to be related to a more even distribution of nutrients and oxygen, thus supporting the physiological activity of microspores and facilitating anther enlargement as an early response to embryogenesis. A similar finding has been reported in cereal anther cultures, where liquid media increases nutrient diffusion and reduces mechanical stress, resulting in a more active microspore response and supporting the formation of early embryoid structures [12,13]. The lowest percentages at this stage were found on the liquid medium for NK-212 and on the solid medium for BISI-18, indicating variation in the ability of genotypes to utilize nutrients or tolerate the physical conditions of the media.

Table 4. Average percentage of dehisced anthers in maize anther culture (*Zea mays* L. var. NK-212 and BISI-18) during incubation on solid, liquid, and solid-liquid media.

Media Type	Varieties	Dehisced Anther (%)			
		Incubation Period (weeks)			
		2 (Dark 1)	2 (Dark 1)	2 (Dark 1)	2 (Dark 1)
Solid	NK212	18,67 ± 4,619 [a]	21,67 ± 9,815 [a]	19,00 ± 5,196 [a]	30,33 ± 13,204 [a]
	BISI-18	13,33 ± 4,619 [a]	25,00 ± 0,000 [a]	33,33 ± 8,505 [a]	16,33 ± 8,505 [a]
Liquid	NK212	18,67 ± 4,619 [a]	19,00 ± 5,196 [a]	22,00 ± 5,196 [a]	41,67 ± 8,505 [a]
	BISI-18	27,33 ± 19,630 [a]	25,00 ± 17,000 [a]	35,67 ± 13,204 [a]	13,33 ± 4,619 [a]
Solid-Liquid	NK212	33,00 ± 17,000 [a]	30,00 ± 5,196 [a]	30,67 ± 9,815 [a]	52,33 ± 17,214 [a]
	BISI-18	19,89 ± 12,067 [a]	29,56 ± 16,094 [a]	36,33 ± 9,815 [a]	25,00 ± 0,000 [a]

Statistical analysis using the Kruskal–Wallis test at 4 weeks of incubation showed that variations in the form of culture media did not have a significant effect on the percentage of anther dehiscence (Asymp. Sig > 0.05), therefore no further post-hoc test was required. At this stage, the highest percentage of anther dehiscence was found in the solid–liquid medium for both varieties. The combination of solid–liquid media provides a more balanced physical and nutritional environment, thereby supporting the hydration process and internal pressure within the anther to facilitate the rupture of the anther wall. Conversely, the lowest percentage of anther dehiscence at 4 weeks of incubation for the NK-212 variety was found in the liquid medium, while in BISI-18 the lowest value was obtained in the solid medium. The low response in liquid media for NK-212 may be associated with the potential occurrence of overhydration, which may inhibit the formation of stable internal pressure required to trigger dehiscence [12]. Meanwhile, in the BISI-18 variety, solid media tend to restrict water and nutrient diffusion, thereby reducing the likelihood of dehiscence [13]. The differences in responses between the varieties in each medium indicate physiological variations that influence anther sensitivity to culture conditions. Overall, although not statistically different, the solid–liquid medium continued to provide the most consistent and relatively high anther dehiscence response in both varieties at this incubation stage.

Statistical analysis using the two-way ANOVA test at 6 weeks of incubation showed that variations in the form of culture media did not have a significant effect on the percentage of anther dehiscence ($p > 0.05$), therefore no further post-hoc test was required. At this stage, the highest percentage of anther dehiscence in both varieties was found in the solid–liquid medium. The solid–liquid medium is assumed to provide more stable physical conditions compared to fully liquid or fully solid media, because the combination of a solid phase as support and a liquid phase as a nutrient source can more optimally support hydration and expansion of anther tissues. This condition potentially accelerates internal pressure on the anther wall, thereby increasing the likelihood of dehiscence [13]. Conversely, the lowest percentage of anther dehiscence for both varieties was found in the solid medium. Solid media tend to have more limited nutrient and water diffusion, causing the anther hydration process to proceed more slowly. This limitation in hydration may reduce the formation of

turgor and internal pressure required to trigger anther wall rupture. This indicates that although the differences among media were not statistically significant, biologically the solid–liquid medium provided more favorable conditions for the anther dehiscence process in both varieties compared to solid and liquid media. These findings are consistent with the general trend that the use of a double-layer system can overcome diffusion limitations and create a more effective culture environment in cereal microspore culture [12].

The results of statistical analysis using a two-way ANOVA test during the 8-week incubation period showed that variation in medium form did not have a significant effect on the percentage of anthers that dehisced ($p > 0.05$), therefore further post-hoc testing was not required. At 8 weeks of incubation, the highest percentage of anther dehiscence for the NK-212 variety was found in the solid–liquid medium, whereas for the BISI-18 variety the highest percentage was observed in the liquid medium. The increase in anther dehiscence percentage in this second light phase indicates that anthers that had previously enlarged reached the optimal stage of dehiscence as a result of the prolonged incubation period and increased metabolic activity triggered by illumination. In the solid–liquid medium, the combination of physical stability from the solid phase and nutrient supply from the liquid phase likely provided the most favorable conditions for NK-212, while BISI-18 appeared to be more responsive to the liquid medium, which allows for more uniform diffusion of nutrients and water. This difference in pattern once again emphasizes the genotype-dependent nature of adaptation to culture environments. Conversely, the lowest percentage of anther dehiscence at this stage was found in the liquid medium for NK-212 and in the solid medium for BISI-18. The low value in the liquid medium for NK-212 may indicate that excessive water saturation at the advanced incubation stage caused anther tissue to experience osmotic stress or degradation, thereby inhibiting the dehiscence process [12]. Meanwhile, the lowest value in the solid medium for BISI-18 was likely due to limited hydration and nutrient diffusion, which were no longer adequate in the late developmental phase when anthers require higher metabolic supply to complete maturation and eventually dehisce [13].

Overall, the variation in media form did not have a significant effect on the percentage of anthers undergoing dehiscence across all incubation phases ($p > 0,05$), although the data revealed distinct physiological response patterns between the two varieties. The NK-212 variety consistently exhibited the highest percentage of anther dehiscence on the solid–liquid medium, while the lowest values were found on the solid medium. Conversely, BISI-18 showed more variable responses, with the highest percentages occurring on the liquid medium at 2 weeks and the solid–liquid medium at 6 weeks, and the lowest values on the solid medium. These patterns confirm a genotype-dependent response to media conditions. Although not statistically significant, this biological tendency remains important because anther dehiscence represents the initial stage that enables microspore release and subsequent entry into the embryogenic pathway.

4 Conclusion

Varieties and media variations did not significantly affect the development of maize (*Zea mays* L. var. NK-212 and BISI-18) anther culture in all observed parameters ($p > 0.05$). However, both varieties displayed different physiological response patterns, with solid-liquid media tending to produce the highest percentage of anther enlargement and anther dehiscence in both varieties. Anther morphological development occurred gradually during incubation, characterized by color changes, enlargement, and anther rupture. These findings indicate that incubation duration and genetic varietal differences play a more dominant role than media variations in influencing anther culture response**s.**

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