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RESEARCH ARTICLE

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# Cytogenetic Analysis and Anther Culture Response of Indonesian Aromatic Local Rice (*Oryza sativa* L.) Crosses

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## ABSTRACT

Rice (*Oryza sativa* L.) is one of the world's major food crops. Increasing the quantity and quality of yields is carried out by hybridization of two superior parents. To accelerate the development of superior genotype homozygosity, this can be done by assembling double haploid lines through anther culture. This study aims to analyze the cytogenetic stability of the parents and hybrids of tropical aromatic rice japonica (Rojolele) × Inpari 32 and determine the best media for anther culture. The research was conducted from June to September 2025 at the Plant Tissue Culture Laboratory, Faculty of Agriculture, Universitas Jenderal Soedirman. This study used a completely randomized design with 6 replications. The treatments consisted of F1 results from the cross of aromatic japonica rice (Rojolele) x Inpari 32. There were 4 formulations of callus induction media with different types of carbon sources and auxins. Cytogenetic analysis shows a diploid number of chromosomes (2n = 24) with varying shapes and lengths of chromosome arms. The best formulation medium for callus induction was Medium 1 (N6 supplemented with 2mg/L NAA, 0.5mg/L kinetin, 60g/L sucrose, 5mg/L $AgNO_3$, and 1g/L activated charcoal) produced significantly the highest early callus induction percentage (13.88%), and anthers from the apical part of the panicle showed a highest response than from the basal part.

**Keywords:** Auxin; Carbon source; Chromosome; Japonica; Karyotype.

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## INTRODUCTION

Rice (*Oryza sativa* L.) is a primary food source for more than 3.5 billion people worldwide, who depend on it as a staple food (Mohidem et al., 2022). An estimated 756 million metric tons (MMT) of rice is needed annually to meet the food needs of approximately 8.6 billion people worldwide by 2030 (Hashim et al., 2024). Global demand for rice will increase significantly to reach around 515 million tons of milled rice in 2050 (Shi et al., 2023). Therefore, efforts to increase productivity through breeding superior varieties, the application of modern cultivation technologies, and sustainable environmental management are crucial to ensure food security and global socio-economic stability (Huang & Wang, 2024). In Indonesia, rice has extensive genetic diversity, including local rice groups from the tropical japonica subspecies (often called javanica) (Long et al., 2022). Aromatic rice varieties have long been known in Indonesia, with widespread distribution throughout the country. These varieties have high economic value due to their superior quality, for examples include Rojolele and Menthik Wangi from Central Java, Pandan Wangi from West Java, Anak Daro or Kuniang Daro from West Sumatra, and Kamba and Lamale from Central Sulawesi (Purwaningsih & Indrasari, 2019). This group of rice is generally known for its distinctive aroma derived from the volatile compound 2-acetyl-1-pyrroline (2AP) (Kongchum et al., 2022) , and 18 other volatile compounds found in raw rice and 22 volatile compounds found in cooked rice, these compounds can be potential biomarkers for aromatic rice plant breeding (Kasote et al., 2021). The key aroma compound 2-acetyl-1-pyrroline (2-AP) is biochemically formed from the proline and ornithine metabolic pathways (Renuka et al., 2022), and genetically, the aromatic trait is a simply recessive trait controlled by a non-functional *BADH2* gene (Addison et al., 2020). However, most tropical aromatic japonica varieties still face agronomic constraints, such as long plant

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life, susceptibility to lodging, and relatively low productivity (Mokhaer et al., 2025). One of the breeding methods that can be used to improve these characteristics, so that superior rice genotypes are obtained in terms of rice age, number of tillers, productivity and maintaining the quality of rice aroma, one of which is through crossing aromatic rice with superior inbred rice (Ndikuryayo et al., 2022). These trait improvements are not only aimed at increasing productivity and yields, but also have strategic implications for cultivation efficiency and the competitiveness of local varieties. Therefore, the Javanica aromatic rice breeding program aims not only to maintain the stability of its distinctive aroma but also to produce early-maturing, high-yielding varieties. This approach will produce varieties that are not only favored by consumers for their aromatic but also required by farmers for their superior agronomic performance. Through double haploid (DH) technology in breeding programs, the *BADH2* allele can be stabilized in a homozygous, so that aroma expression can be consistent across generations and accelerate the formation of aromatic lines.

Cytogenetic analysis is essential in the crossing process, as it helps ensure successful hybridization by evaluating chromosome stability and genetic compatibility (Harun et al., 2024). Through cytogenetic analysis, early identification of potential genetic imbalances caused by structural or numerical variations in chromosomes can be carried out, so that the risk of failure in hybrid formation can be minimized (Kumar, 2023). This cytogenetic information plays a role in supporting biotechnology-based breeding programs, including doubled haploid production (Jeberson et al., 2021). Cytogenetic analysis on progeny was carried out as a basis for reference of ploidy and chromosome number to produce double haploid.

Anther culture is an effective technique for accelerating the achievement of homozygosity in breeding programs, which allows direct plant regeneration from microspores (Chen et al., 2025), which are then doubled to fully homozygous doubled haploid (DH) in a single generation (Lantos et al., 2022). It can significantly shorten the breeding cycle compared to conventional methods for producing homozygous lines in cereal breeding, which require typically 6-7 generations of inbreeding to achieve approximately 98% homozygosity (Lantos et al., 2023), it's process can takes at least 2–4 years, or possibly longer, that use the pedigree method by repeatedly selecting desirable plants and self-pollinating them for multiple generations until the offspring are genetically uniform and homozygous (Ghalagi et al., 2023). The characteristics of doubled haploid (DH) plants are stable and heritable because they are completely homozygous at every genetic locus, they also have identical alleles for each gene, so there is no genetic segregation in subsequent generations produced by self-pollination (Starosta et al., 2023). In addition, because all genetic loci are homozygous, both traits controlled by dominant and recessive genes can be fully expressed (Luo et al., 2021). This makes the selection process more efficient than conventional methods, especially for traits controlled by recessive genes (Ali & Wani, 2021), such as aromatic traits in rice plants.

The success of anther culture in rice is influenced by plant genotype factors. In general, Japonica type rice shows a higher response to callus formation and plant regeneration compared to Indica type rice. This difference is caused by genetic variations that affect the ability of microspore cells to undergo embryogenesis and develop into double-haploid plants (Sandaruvini & Ratnasekera, 2022). The aromatic rice, which often shows a low response (Nurhasanah et al., 2015). Therefore, an initial evaluation of anther culture response in tropical aromatic rice crosses is essential to determine the most suitable culture conditions (Nurmansyah et al., 2021).

The composition of the growth medium is a factor in the success of embryogenic callus induction that influenced by the composition of the growth medium, including the type of carbon source and auxin used. Carbon sources are components that play a role in providing energy for cell division and differentiation. The two most commonly used carbon sources in callus induction testing on rice genotypes are sucrose and maltose, as both are capable of supporting embryogenic callus formation with varying efficiencies depending on the genotype and culture medium conditions (Chen et al., 2025). Besides that, auxins specifically 2,4-D and NAA, are essential for callus induction in rice anther culture. Their type and concentration significantly influence the process, with optimal combinations varying by genotype, enhancing callus formation and subsequent plant regeneration (Rahman et al., 2021). This study aims to analyze the cytogenetic characteristics of progeny from crosses between tropical aromatic rice subspecies japonica and to evaluate the initial response of these genotypes to various carbon sources and auxin formulations in anther culture medium.

## MATERIALS & METHODS

### Plant Material and Experimental Design

This study was conducted on June to September 2025 in Plant Tissue Culture Laboratory, Faculty of Agriculture, Jenderal Soedirman University. The materials used in chromosome analysis activities are rice plant meristem roots, 45% glacial acetic acid solution, 1 N HCl solution, 2% acetocarmine solution, 70% alcohol, immersion oil, and distilled water. The materials used for anther culture are N6, NAA (Naphthalene Acetic Acid), 2,4-D (2,4-Dichlorophenoxyacetic Acid), Kinetin, $AgNO_3$ (Silver nitrate), sucrose, maltose, $HgCl_2$ (Mercury chloride), agar, activated charcoal, distilled water, PPM (Plant Preservative Mixture). The plant material used was anthers from the Rojolele x Inpari 32 rice progeny.

The tools used in chromosome analysis activities are dropper pipettes, hot plates, beakers, Erlenmeyer flasks, glass funnels, filter paper, plastic, aluminium foil, cover glasses, slides, scalpels, tweezers, 2µl tubes, Olympus CX 43 microscopes, refrigerators, stationery, markers, tissues, clear nail polish, and cameras. The tools used in anther culture include laminar air flow, magnetic stirrers, pH meters, autoclaves, glassware, and dissecting kits.

### Experimental Design

The anther culture experiment was arranged in a Completely Randomized Design (CRD) with medium formulation as factor with six replications. Statistical analyses were conducted using Analysis of Variance (ANOVA), and when significant differences were detected, means were compared using Duncan's Multiple Range Test (DMRT) at a 5% significance level. Chromosome analysis was performed on a representative root-tip sample to determine the somatic chromosome number, characterize karyotype morphology through arm ratio and relative length measurements, and construct a detailed ideogram illustrating chromosomal structure.

### Cytological and Chromosome Analysis

### Sample Preparation and Pretreatment

Rice meristem root sampling for chromosome analysis followed the method Aristya et al. (2024) conducted between 08.55-09.05 WIB. Meristem roots were taken with a length of ± 2cm. Fixation was carried out by cold acetic acid fixation methods. The macerated samples were added with 1 N HCl solution until submerged and incubated using a hot plate at a temperature of 60°C for 15 minutes. After the plant samples were incubated, they were washed with distilled water 3 times until all the solution was completely used up. Plant samples were stained with aceto-carmine solution and left for 2 hours. Preparation of chromosome analysis using the squash method so that the chromosomes to be observed were scattered and made easier to observe the chromosomes.

### Karyotype Analysis

The chromosome numbers of each genotype were determined by direct microscopic observation at the prometaphase stage using Image Raster v3. Chromosome analysis was performed through karyotyping, which included measuring the short (p) and long (q) arms, determining total chromosome length, calculating the centromeric index, identifying chromosome morphology, and constructing the ideogram. These analyses were conducted manually with Image Raster v3, Photoscape v3.6, CorelDRAW X6, and Microsoft Excel 2007. Chromosome length measurements were recorded in Excel for subsequent calculation of total length, centromeric index, and arm ratio (long arm/short arm). Chromosomal size, shape, and configuration were analyzed descriptively. The karyotype was organized in descending order of absolute chromosome length using CorelDRAW X6, and the resulting karyotype served as the basis for constructing the ideogram in Microsoft Excel 2007. Chromosomal classification followed the criteria established by Levan et al. (1964) The total chromosome length (c) for each pair was obtained by summing the mean lengths of the short arm (s) and long arm (l), expressed as c = s + l. Chromosomes were categorized based on their centromeric index (CI) values as follows: telocentric (CI = 0), acrocentric (CI between 0 and 12.5), sub-telocentric (CI between 12.5 and 25.0), submetacentric (CI between 25.0 and 37.5), and metacentric (CI between 37.5 and 50.0).

### In Vitro Culture Methods

### Explant Preparation and Sterilization

Anthers were taken from panicles at the booting stage in the morning (07.00–09.00 WIB), then given pre-treatment in the form of storage at a temperature of 4-8°C for 8–10 days in dark conditions (Safitri et al., 2010). Pretreatments such as cold shock (low-temperature incubation of donor anthers) improve microspore reprogramming to the sporophytic pathway.

Sterilization was carried out by immersing the panicles in a 0.1% $HgCl_2$ solution for 5 minutes, followed by five rinses with sterile distilled water under aseptic conditions. To improve safety and reproducibility, we have also included an alternative sterilization method using 0.26% NaOCl for 5 minutes as a non-toxic option. The proper disposal of $HgCl_2$ waste collection in labeled containers. Sterile workflow quality was ensured by periodic monitoring of culture contamination and browning rates.

### Culture Media and Condition

Culture media are formulated with various carbon source substantially influences androgenesis (Ali et al., 2021). Addition of silver nitrate ($AgNO_3$) to induction media acting as an ethylene action inhibitor to enhance embryogenic response and reduce tissue senescence in anther culture (Ravi et al., 2019). Besides that, auxin types and concentration (most often 2,4-D or NAA) critically influence callus induction on anther culture (Arisandi et al., 2020). Anthers were cultured on the following 4 medium formulations:

Medium 1 : N6 + 2.0mg/L NAA + 0.5mg/L Kinetin + 60g/L sucrose+ 5.0mg/L $AgNO_3$ + 1g/L activated charcoal + 7g/L agar; pH 5.8.
Medium 2 : N6 + 0.5mg/L 2,4-D + 0.5mg/L Kinetin + 60g/L sucrose+ 5.0mg/L $AgNO_3$ + 1g/L activated charcoal + 7g/L agar; pH 5.8.
Medium 3 : N6 + 2.0mg/L NAA + 0.5mg/L Kinetin + 30g/L maltose+ 5.0mg/L $AgNO_3$ + 1g/L activated charcoal + 7g/L agar; pH 5.8.
Medium 4 : N6 + 0.5mg/L 2,4-D + 0.5mg/L Kinetin + 30g/L maltose+ 5.0mg/L $AgNO_3$ + 1g/L activated charcoal + 7g/L agar; pH 5.8.

The cultured anthers were incubated in dark conditions at a temperature of 25 ± 2°C for 12 weeks to induce callus formation. Relative humidity in the culture room at 50–70%.

Callus observations were conducted at 3-day intervals for a total of 6 weeks. For each treatment, the number of explants producing callus was recorded and the induction frequency was expressed as a percentage of explants forming callus = (number of explants with callus / total explants per bottle) × 100.

Contamination rate was calculated as the percentage of culture units exhibiting microbial contamination during the observation period: contamination percentage (%) = (number of contaminated units / total units) × 100.

Browning of explants or calli was quantified by the percentage of browned calli per treatment (number of explant / total explant observed × 100)

## RESULTS AND DISCUSSION

### Cytogenetic Analysis

Mitotic phases were successfully observed in the Rojolele, Inpari 32, and Rojolele × Inpari 32, indicating normal cell division activity at the root tips (Fig. 1).

According to Wang & Yu, (2016), mitotic activity in rice roots was reported to occur optimally between 09:00 and 12:00. However, in this study, the results showed that the genotypes had an optimum time for prometaphase division, namely at 08:55 and 09:15. This time was the optimum time to obtain clear and easily counted prometaphase chromosome distribution. This difference is thought to be influenced by environmental conditions and plant physiological factors, each variety and growing condition can have a different mitotic rhythm pattern, so optimization was necessary before karyotype analysis.

The results of the chromosome number analysis show that the Rojolele, Inpari 32, and and Rojolele × Inpari 32 have a chromosome number of 2n = 24 (Fig. 2). This is in accordance with research Jha, (2021), which indicates that the rice genotype has 2n = 24 chromosomes, whereas wild types such as *Oryza minuta, Oryza latifolia, Oryza eichingeri*, and *Oryza alata* have a chromosome number of 2n = 4x = 48. In addition, research from Cica & Isnawati (2023) also states that several rice varieties that are widely cultivated by farmers in Indonesia have a chromosome number of 2n = 24.

Karyotype analysis (Fig. 3) shows differences in chromosome composition between the Inpari 32 and Rojolele varieties. Inpari 32 has a karyotype formula of 8 submetacentric (SM) + 16 metacentric (M), while Rojolele consists entirely of metacentric chromosomes (24 M). This difference reflects genetic variation between the two varieties, where the presence of submetacentric chromosomes in Inpari 32, according to (Correia-da-Silva et al., 2014) has greater diversity in chromosome structure and the possibility of expressing superior traits. This is in line with the agronomic performance of Inpari 32, which is known to have high productivity and better resistance compared to local varieties. In contrast, the homogeneous karyotype of Rojolele with all metacentric chromosomes indicates relatively high genetic stability. In addition, the results of crosses between the two varieties (Rojolele × Inpari 32) showed a karyotype formula of 24 M, due to a more stable cell division regulatory mechanism factor in metacentric chromosomes (Steckenborn & Marques, 2025). This is in accordance with previous research that metacentric chromosomes have higher stability during mitotic and meiosis, due to a more balanced distribution of spindle thread pulling forces around the centromere (Cuacos et al., 2015). The results of this study indicate the homogeneity of metacentric chromosomes in the results of the Rojolele × Inpari 32 cross which indicates genetic stability and minimizes the risk of chromosome aberration during cell division.

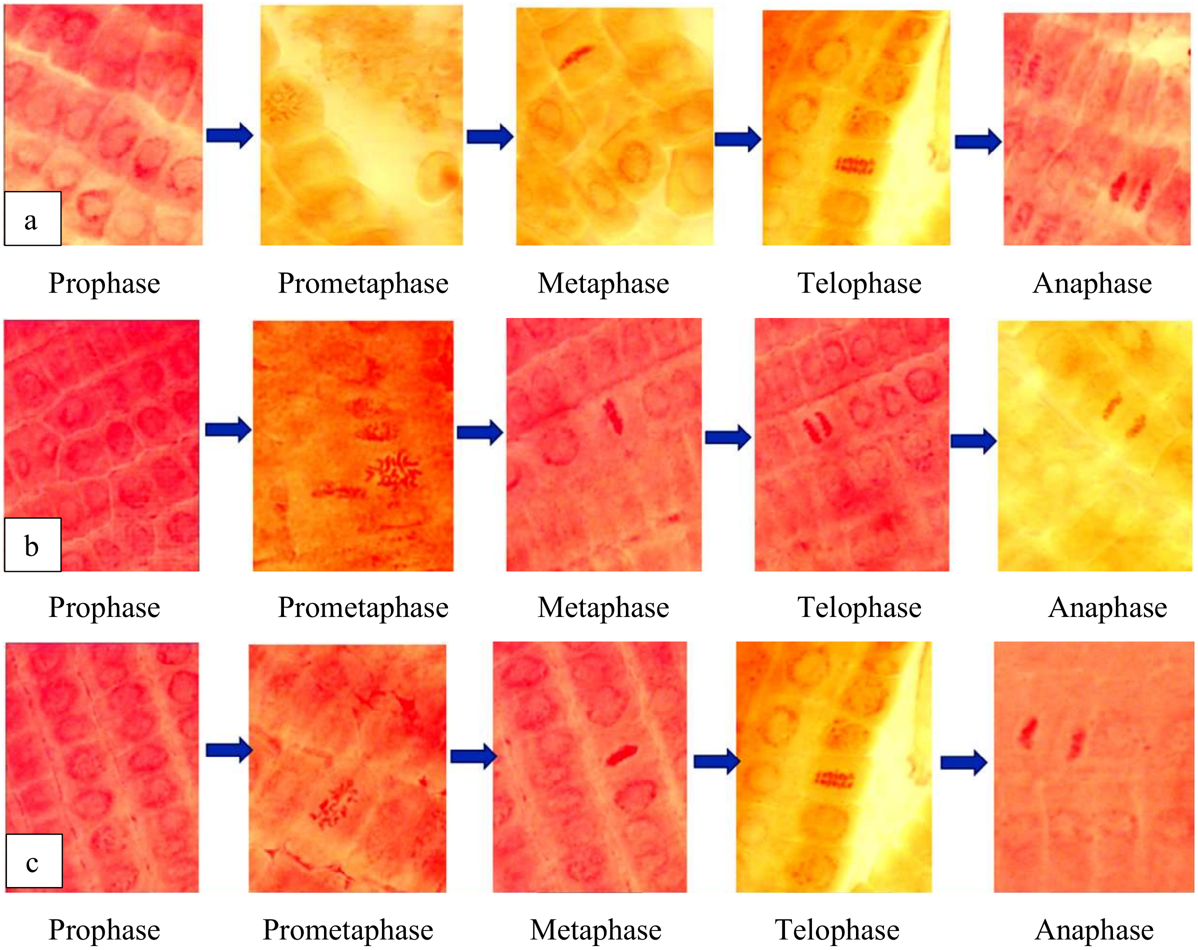

**Fig. 1:** Mitotic phase of a) Rojolele, b) Inpari 32, c) Rojolele × Inpari 32.

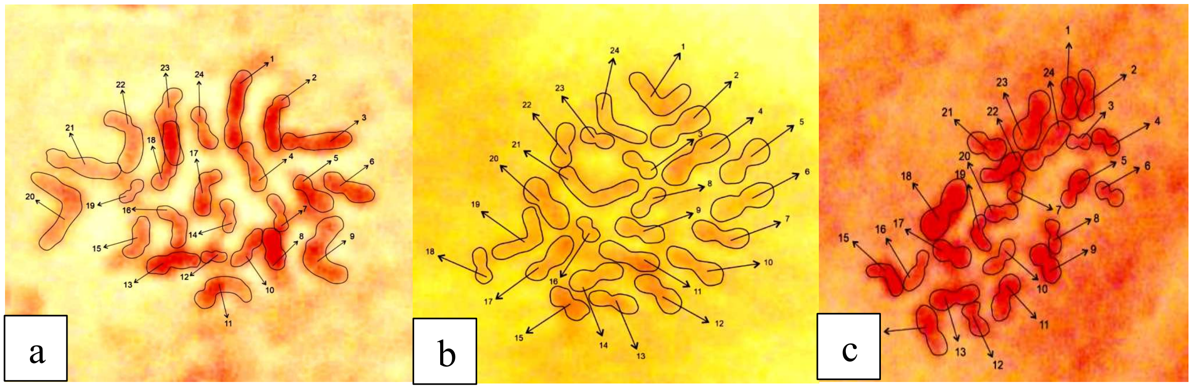

**Fig. 2:** Number of chromosome of a) Rojolele (2n = 24), b) Inpari 32 (2n = 24) and c) Rojolele × Inpari 32 (2n= 24).

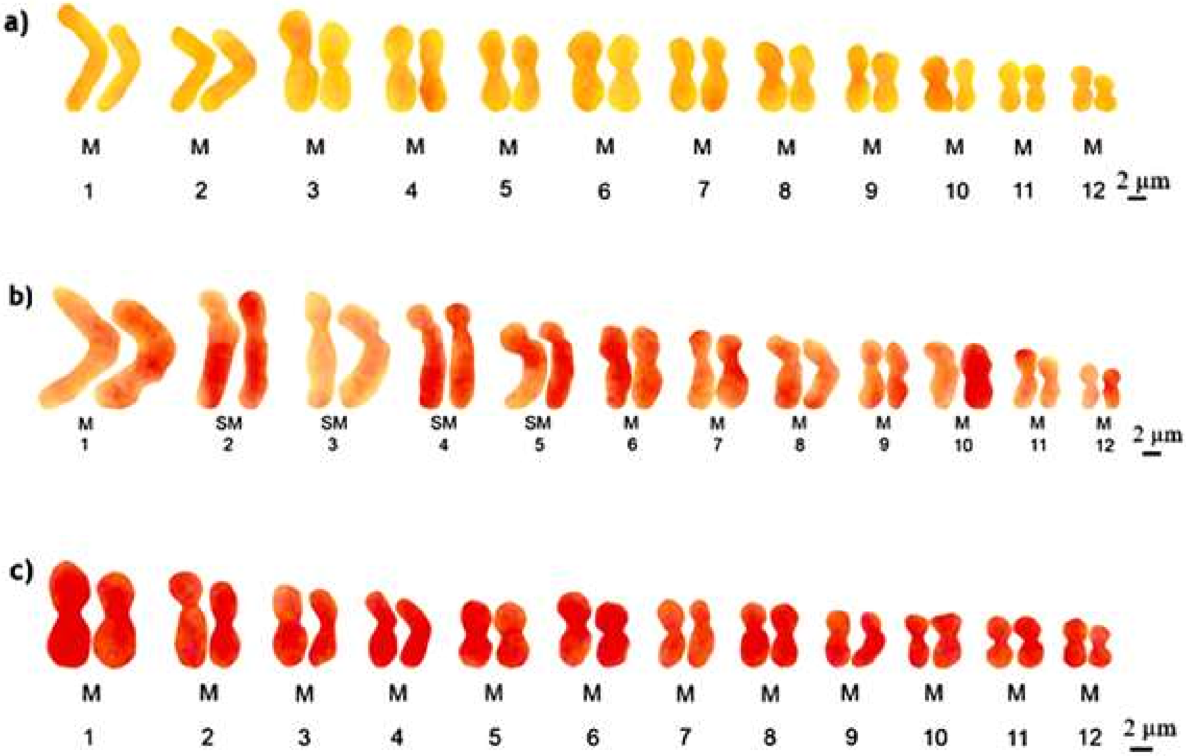

**Fig. 3:** Karyotype Formula of a) Rojolele = 24 Metacentric ; b) Inpari 32 = 16 Metacentric + 8 Submetacentric; c) Rojolele × Inpari 32 = 24 Metacentric.

The results of chromosome analysis (Table 1) showed variations in chromosome length and karyotype formula between the Rojolele, Inpari 32, and their crossed genotypes. The Rojolele genotype has the longest chromosome of 17.40µm and the shortest 4.74µm, with a total chromosome length of 255.09µm and an average length of 10.62µm. Inpari 32 shows different characters with the longest chromosome reaching 26.19µm and the shortest 6.30µm. The total chromosome length reaches 328.48µm with an average length of 13.68µm, greater than Rojolele. The cross between Rojolele and Inpari 32 resulted in the longest chromosome measuring 10.13µm and the shortest chromosome measuring 4.50µm, with a total chromosome length of 164.17µm and an average length of 6.84µm. Research (Jha, 2021) also indicates that cultivated rice exhibits longer chromosome arms than wild-type rice. The Inpari 32 variety exhibits the longest (26.19µm) and shortest (6.3µm) chromosome lengths, higher than Rojolele, which has the longest chromosome measuring 17.40µm and the shortest 4.74µm. This indicates that Inpari 32 has a relatively larger chromosome size range, which may reflect differences in the level of chromosome condensation and the influence of genetic factors on its chromosome structure.

The cross between Rojolele × Inpari 32 actually showed a basal longest chromosome length (10.13µm) compared to its two parents, although the shortest chromosome length (4.5µm) was relatively close to that of Rojolele. The karyotype analysis of the Rojolele × Inpari 32 cross showed that the average chromosome size was shorter compared to both parents. This result cannot be directly interpreted as an indication of a decrease in superior traits, but rather reflects the process of genetic recombination and chromosome restructuring that commonly occurs in crosses. The consistent stability of chromosome number in all genotypes indicates that both Rojolele and Inpari 32 varieties, as well as Rojolele × Inpari 32, are in normal cytogenetic condition. According to (Jha, 2021), variation in chromosome length in rice cultivars is more influenced by genetic factors, domestication, and chromosome reorganization than by a direct relationship to agronomic traits. The shorter chromosome length in crossbreds does not eliminate the potential for superior traits, as these traits are controlled by a combination of alleles at various genetic loci, not just by total chromosome length (Li et al., 2020). Generational selection plays a crucial role in integrating and stabilizing the superior traits of both parents. Therefore, even though the chromosome length in crossbreds is relatively shorter, the potential for genotypes with improved resistance or higher productivity remains (Hou et al., 2025).

## Chromosome Idiogram

Idiograms was a visual representation of chromosomes that illustrating the relative lengths of chromosome arms and the positions of centromeres. This graphical map enables to accurately distinguish individual rice chromosomes and their corresponding arms, facilitating comparative karyotype analysis and the detection of structural variations among genotypes (Ohmido et al., 2010). The ideogram provides a general overview of the chromosomal characteristics of Rojolele, Inpari 32, and the Rojolele × Inpari 32 cross (Fig. 4). Both cultivars have a diploid chromosome formula (2n) with a chromosome number of 24. This represents 12 pairs of chromosomes in each genotype. The chromosome number is indicated by the similarity between the three genotypes studied, indicating their equivalence at the species level. The difference between the three genotypes lies in their chromosome length. This difference is seen phenotypically in various agronomic aspects based on the growth characteristics of the three genotypes (Zhao et al., 2022).

## Callus Induction

The callus growth stage (Fig. 5) showed that a) the microspores are still on the gametophytic developmental pathway, namely towards the formation of functional pollen grains. Microspores are usually in the early to middle uninucleate phase. In Fig. 5b and 5c, the microspore developmental pathway begins to shift from

**Table 1:** Length of the longest chromosome (µm), length of the shortest chromosome (µm), total length of chromosomes (µm), average length of chromosomes (µm), and diploid karyotype formula of rice genotypes

| Genotype | Length of longest chromosome (µm) | Length of shortest chromosome (µm) | Total Chromosome Length (µm) | | | Average Chromosome Length (µm) | | | Diploid karyotype formula |
|---|---|---|---|---|---|---|---|---|---|
| | | | p | q | p+q | p | q | p+q | |
| Rojolele | 17.40 | 4.74 | 116.38 | 138.71 | 255.09 | 4.85 | 5.78 | 10.62 | 2n = 24 M |
| Inpari 32 | 26.19 | 6.3 | 129.17 | 199.31 | 328.48 | 5.38 | 8.30 | 13.68 | 2n = 16 M + 8 SM |
| Rojolele x Inpari 32 | 10.13 | 4.5 | 74.97 | 89.20 | 164.17 | 3.12 | 3.71 | 6.84 | 2n = 24 M |

Note = p: length of short arm (µm), q: length of long arm (µm), p+q: length of short + long chromosome arms (µm); M: Metacentric; SM: Submetacentric.

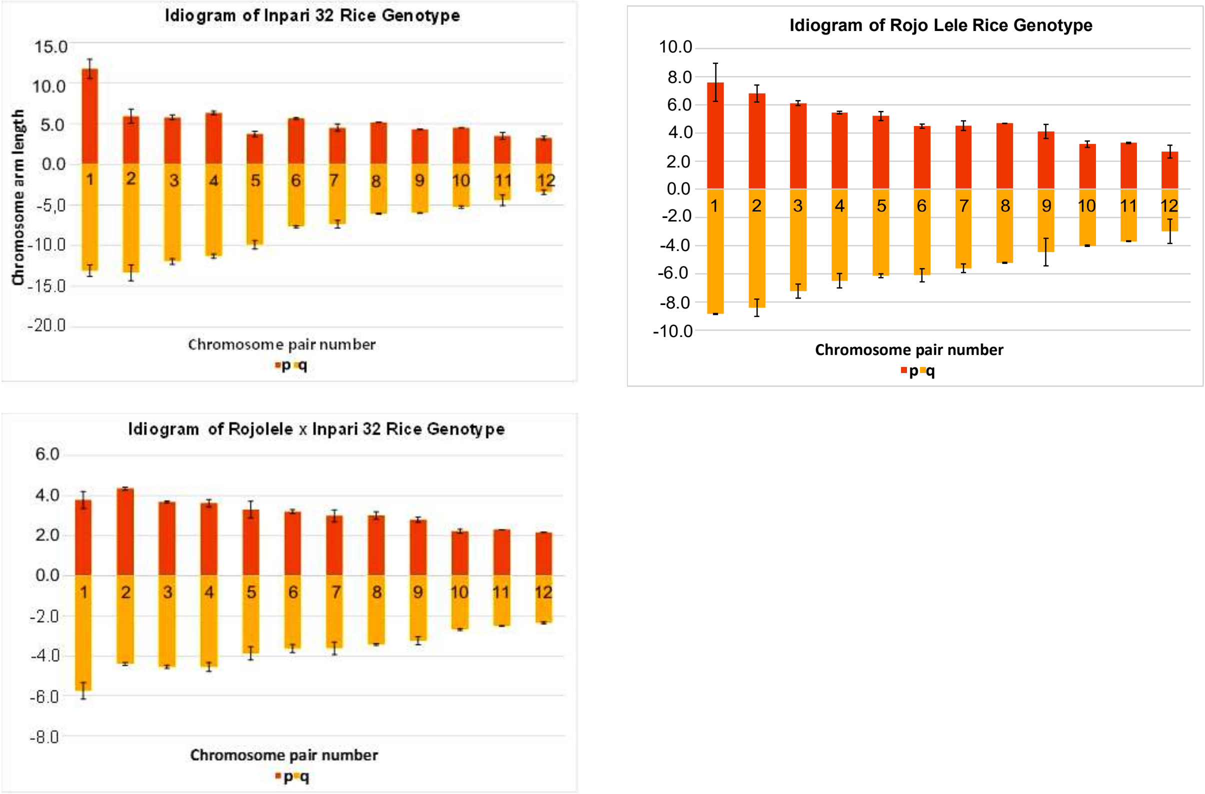

**Fig. 4:** Combined chromosome ideogram of the a) Rojolele; b) Inpari 32; and c) Rojolele × Inpari 32. Short arms (p) are colored orange, long arms (q) are colored yellow.

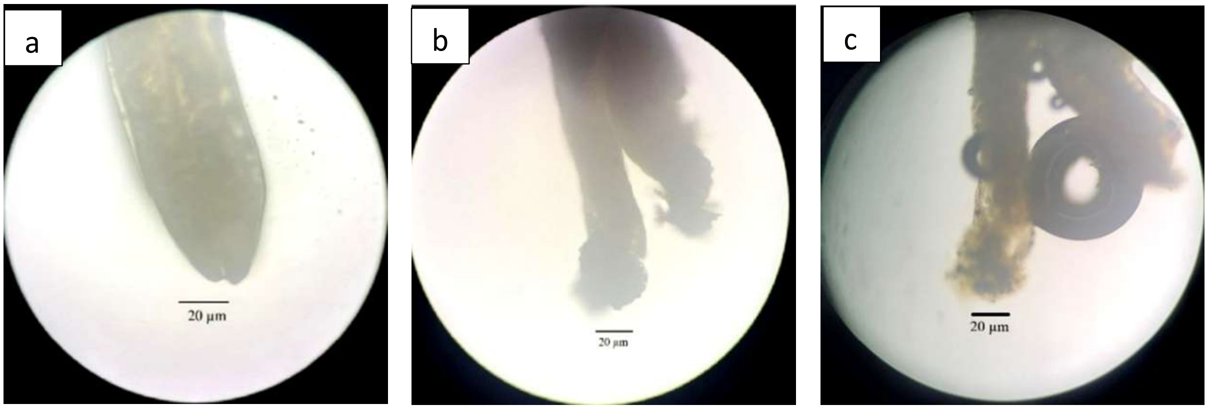

**Fig. 5:** Callus development based on external morphology and tissue integrity (12 Weeks After Culture), a) no callus development; b) small and fragile callus (<40µm); c) medium callus (>40µm), pale, compact.

**Table 2:** Type of medium, number of brownish anthers, number of contaminated anthers, number of callus, percentage of callus to anthers, percentage of callus-producing plants on anthers resulting from the Rojolele × Inpari 32

| Type of medium | Percentage of browning anthers | | Number of emerged callus | | Percentage of emerged callus (%) | |
|---|---|---|---|---|---|---|
| | Apical Part | Basal Part | Apical Part | Basal Part | Apical Part | Basal Part |
| Medium 1 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.83 ± 0.41 a | 0.00 ± 0.00 | 13.88 ± 6.80 a | 0.00 ± 0.00 |
| Medium 2 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 b | 0.00 ± 0.00 | 0.00 ± 0.00 b | 0.00 ± 0.00 |
| Medium 3 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 b | 0.00 ± 0.00 | 0.00 ± 0.00 b | 0.00 ± 0.00 |
| Medium 4 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.17 ± 0.41 b | 0.00 ± 0.00 | 2.77 ± 6.80 b | 0.00 ± 0.00 |

Note: Different letters within the same column indicate significant differences according to Duncan's Multiple Range Test (DMRT) at the 5% significance level.

gametophytic to sporophytic. At this stage, the microspores no longer develop into gametes, but instead undergo dedifferentiation and begin to divide mitotically to form an undifferentiated cell mass (callus).

Callus development first eight weeks, no callus formation was observed on the anthers. The lack of callus growth at this stage is likely due to the microspores not having reached a responsive developmental stage, such as the mid-to-late uninucleate stage, which is crucial for the transition from the gametophyte to the sporophyte pathway (Mayakaduwa & Silva, 2021). Small, fragile calli (<40µm) begin to appear around the tenth week after culture initiation, indicating the early stages of sporophyte development. This callus initiation is driven by the synergistic effect of auxin and cytokinin in the induction medium, which stimulates cell proliferation (Gao et al., 2024). By the eleventh week, medium-sized, pale, and compact calli (>40µm) were formed, indicating active cell division. This stage marked the transition toward embryogenic potential.

Panicle position was divided into apical and basal sections based on one-half divisions of the total panicle length. Apical anthers were collected from the apical one-half of the panicle, while basal anthers were taken from the basal one-half of the panicle. The position of anthers within the panicle is closely related to the developmental stage of microspores, which strongly influences their response in anther culture (Chen et al., 2025). The research reveals that anthers from the apical part of the panicle contain the maturity stage that microspores predicted at the mid to late uninucleate stage, that the most responsive stage for callus induction and haploid plant formation (Mayakaduwa & Silva, 2023). Although spikelet maturation in rice proceeds acropetally, the apical spikelet is the first to mature (Fushimi et al., 2021). The basal anthers of the panicle are generally at an earlier microspore stage than the apical anthers. Microspores at this stage are still in the early uninucleate phase, making them physiologically incompetent for embryogenesis, as they cannot transition from the gametophytic to the sporophytic pathway, resulting in no callus formation. This developmental unpreparedness causes the basal anthers to fail to respond to callus induction, despite favorable environmental conditions and growth media.

The results of anther culture induction research on the Rojolele x Inpari 32 cross (Table 2) showed that Medium 1 (M1) produced the highest percentage of early-emerged calli, at 13.88% in the apical anthers. Medium 4 (M4) only produced a 2.77% response, while Medium 2 (M2) and Medium 3 (M3) showed no callus formation in either the apical or basal anthers. These findings indicate that the combination of NAA (2mg/L) with a high sucrose concentration (60g/L) in M1 is quite effective in promoting microspore dedifferentiation, thus shifting from the gametophytic to the sporophytic pathway and forming callus. This medium composition is consistent with that used in the study (Safitri et al., 2010).

Differences in response between medium also indicate a significant role for carbon source composition (Arisandi et al., 2020). In previous studies, the standard carbon source for the rice anther culture process was sucrose, with a concentration of 3–6% Chen et al., 2025). High concentrations of sucrose act as both an energy provider and a source of osmotic stress, triggering microspores to alter their developmental pathways. Furthermore, the use of maltose in M3 and M4, did not produce significant initial callus, indicating that this is also influenced by genotype and interaction with auxin type. The addition of an ethylene action inhibitor such as silver nitrate ($AgNO_3$) commonly improves embryogenic responses and reduces tissue browning and senescence in anther cultures across species, including rice and cereals; optimized $AgNO_3$ concentrations and interactions with dark pretreatments or cold pretreatments are important to maximize benefits while avoiding phytotoxicity (Hassan & Islam, 2021).

### Conclusion

This study demonstrated that Rojolele, Inpari 32, and their hybrid possessed a diploid chromosome number of 2n = 24 but differed in karyotype composition. Rojolele exhibited a homogeneous karyotype (24M), Inpari 32 showed variation (16M + 8SM), and the hybrid (Rojolele × Inpari 32) displayed a return to homogeneity (24M). Among the tested media, Medium 1 (N6 with 2mg/L NAA, 0.5mg/L kinetin, 60g/L sucrose, 5mg/L $AgNO_3$, and 1g/L activated charcoal) produced the highest percentage of early callus formation (13.88%), while Medium 4 induced 2.77%, and no response occurred in Medium 2 and 3. Anthers from the apical panicle portion were more responsive than those from the basal portion, and the high sucrose concentration in Medium 1 effectively promoted microspore transition to the sporophytic pathway.

## DECLARATIONS

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**Data Availability:** The datasets generated and analyzed during the current study are available from the corresponding author upon reasonable request.

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