THE EFFECTIVENESS OF INBREEDING METHOD IN THREE TYPES OF GAMBIER (*UNCARIA GAMBIER* (HUNTER) ROXB.) OF WEST SUMATRA ORIGIN

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(Received 2nd Apr 2024; accepted 30th Aug 2024)

Abstract. Gambier is the main export commodity in West Sumatra with versatile benefits and has good market prospects because it is used as an industrial raw material. Population diversity caused by natural cross-pollination systems needs screening by increasing homozygosity through inbreeding. This study aims to obtain an effective inbreeding method from the three varieties of gambier from West Sumatera. This research was conducted at UPT Experimental Garden and Tissue Culture Laboratory the Faculty of Agriculture, Andalas University from July to December 2020. Determination of sample plants and cluster selection on sample plants was done by purposive sampling, while single flower selection was made by purposive random sampling. The observed variables were the number of single flowers in compound flowers, the percentage of fertilization, the number of fruits formed, the percentage of harvested fruit, the number of harvested fruits, the fertility and sterility of pollen, and the viability of pollen. Observation variable data is then analyzed statistically. The results showed that the most effective inbreeding method for all three varieties of gambier, such as udang, Cubadak, and Riau Mancik, is through artificial cross-pollination. This is evidenced by the percentage of fertilization produced through artificial cross-pollination of the three varieties of gambier by 87.33% compared to the fertilization rate through natural geitonogamy pollination of 0.46% and artificial geitonogamy pollination of 2.67%.
Keywords: *fertility, geitonogamy, pollination, sterility of pollen, viability of pollen*

Introduction

West Sumatra Province is the center of Indonesia’s largest gambier (*Uncaria gambier* (Hunter) Roxb.) plantation. This plantation commodity is one of the typical tropical plants with various benefits and has good market prospects because it is used as industrial raw material.

The area of gambier land in West Sumatra, Indonesia increased by 48.5% from 21,411.5 ha to 31,791.25 ha from 2012 to 2016. However, gambier production was only able to increase by around 19.8%, from 14,220 tons to 17,036.03 tons. Based on these data, it can be seen that the expansion of gambier land has not been able to increase gambier production significantly. The low production of gambier is because farmers generally still use mixed seeds resulting from free pollination (cross-pollination).

Judging from the process of pollination, gambier plants are cross-pollinated plants. This is because the gambier plant has hermaphrodite flowers with a higher stigma position than the anther, and the anther ripens earlier than the stigma (protandry) (Hayati, 2020). The pollination system through the pollen/ovule ratio approach (Nepal et al., 2023). The gambier plant showed a log value of the pollen/ovule ratio close to the log value of the pollen/ovule ratio in cross-pollinated plants (xenogamy).

Gambier cultivation using free-pollinated seeds (cross-pollination) will result in high segregation so that if planted, a mixed population will be obtained. The cross-pollinated plant populations result in random mating, which causes the population to be classified as heterozygous with wide phenotypic and genetic variability. The diversity of this population will affect the quality of the results, including yield capacity, yield quality, and the type of yield produced. The propagation of gambier plants can be done in two ways, namely generatively using seeds and vegetatively using cuttings, grafts and mounds. In general, gambier plants are propagated generatively by seeds.

The population diversity caused by the cross-pollination system that commonly occurs in gambier plants needs to be screened by increasing homozygosity through inbreeding. Inbreeding is a cross of one individual or a cross of two closely related individuals (Zhang et al., 2020). Inbreeding was carried out to obtain pure lines. If inbreeding is carried out continuously, it can lead to an increase in homozygosity from generation to generation (and the level of homozygosity is positively correlated with symptoms of inbreeding depression in both vegetative and generative characters of a plant; Maltecca et al., 2020). Inbreeding depression affects plant height, leaf area, leaf weight, yield, and generative components in *Capsicum annuum* L. (Ulas et al., 2020), *Lolium perenne* L. (Tokian et al., 2019).

Inbreeding can be done through three methods, namely natural geitonogamy pollination, artificial geitonogamy pollination, and artificial cross-pollination. Geitonogamy pollination (neighbor pollination) is the transfer of pollen from one flower to the stigma of another flower, either in one cluster or between clusters but still on the same plant. At the same time, artificial cross-pollination is the attachment of pollen to the stigma of another flower of a different plant but still some types or related. Gambier plants can undergo pollination with flowers from different parts of the same plant. (geitonogamy). The autogamous pollination system may occur in addition to geitonogamous pollination in gambier plants because the position of the stigma and anther is parallel before the occurrence of anthesis. The pollen sticks to the stigma of the same flower. Reception of stigma occurs one day after anthesis, and pollen in the anther of the same flower is usually gone by the time of anthesis due to pollination vectors such as wind and insects. Autogamous pollination system has a smaller chance of pollination than geitonogamous pollination due to temporal mechanical barriers (Fattorini and Glover et al., 2020). Zainal et al. (2020) have analyzed the percentage of autogamy pollination on gambier plants using a pipette cover of 85.6% and oil-paper of 94.4%. Although the percentage of autogamous gambier pollination is high, it is not able to form fruit at all. Rizki et al. (2020) have analyzed the percentage of fertilization through natural geitonogamy pollination by covering the plant as a whole using a thin white cloth of 10.68% and artificial geitonogamy pollination by 27.5%, so it can be said that gambier plants are capable of geitonogamous pollination.

Gambier genetic screening experiment through several methods of deep cross-pollination (inbreeding) using three varieties of gambier widely cultivated in West Sumatran smallholder plantations, namely Shrimp, Cubadak, and Riau Mancik. These three varieties of gambier show differences in morphological characters, yield components, and productivity. The results of this genetic screening certainly need to be studied further, such as knowing the level of homozygosity of an individual by

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2206_57535766

observing the appearance of the two alleles through a molecular approach, seeding aspects, agronomic characters, etc.

Based on the interests that have been described, inbreeding is carried out to obtain pure genetics from the three varieties of gambier, which later, if carried out continuously, there will be no more segregation so that each locus has the same allele (homozygous), which is useful for creating pure strains. The pure strains that have been screened have been selected and can be used as parental crosses to create superior gambier cultivars. This study aimed to obtain an effective inbreeding method of the three varieties of gambier from West Sumatra.

Materials and methods

This research was carried out at the UPT Experimental Garden and Tissue Culture Laboratory, Faculty of Agriculture, Andalas University, Padang, from July to December 2020 using experimental methods, namely by determining sample plants, conducting natural geitonogamy pollination, conducting artificial geitonogamy pollination, cross-pollinating, observing sample plants, conduct sample testing in the laboratory, and collect data. The determination of the sample plants and the selection of clusters on the sample plants were carried out by purposive sampling. In contrast, purposive random sampling carried out the selection of single flowers. The gambier plants selected as samples are gambier plants that are eight years old, free from pests and diseases in the flowering phase. Then the data were analyzed statistically (mean, standard deviation).

Natural geitonogamy pollination requires six stems of gambier plants consisting of two varieties of Shrimp (UU), two varieties of Cubadak (CC), and two varieties of Riau Mancik (Rm). Artificial geitonogamy pollination requires three stems of gambier plants consisting of one Shrimp variety (UU), one Cubadak variety (CC), and one Riau Mancik variety (Rm). Artificial cross-pollination requires three stems of gambier plants as male parents and three stems of gambier plants as female parents. The number of plants needed is six gambier plants consisting of two varieties of Shrimp (UU) two varieties of Cubadak (CC). And two varieties of Riau Mancik (Rm). Then in each sample plant, several clusters were selected. The selected cluster is a cluster that is in the large flower bud phase (F2) which starts from the appearance of the flower crown from the fruit ovary until the flower crown opens, which is the beginning of the open flower phase (anthesis) (F3). A cluster is a flower stalk that forms fruit, on one stalk there are many fruits

In natural geitonogamy pollination, there must be a minimum of ten clusters in the sample plants, which will later serve as the parents of both male and female crosses. All flowers in the cluster were sampled. Artificial geitonogamy pollination and cross-pollination must have at least five clusters in the sample plants that will serve as female parents. Each cluster was selected as many as ten single flowers at random so that the planting material needed for artificial geitonogamy pollination and artificial cross-pollination were 150 single flowers each.

Pollen fertility testing using bromothymol blue staining method and pollen viability testing using germination media requires one cluster to be used as a male parent, each of which comes from plants that have previously carried out geitonogamy pollination both natural and artificial and carried out artificial cross-pollination. Then for each cluster, four single flowers were selected, which were in the anthesis (F3) phase to be used as

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2206_57535766

samples for pollen fertility testing using the bromothymol blue staining method, and four single flowers were used for pollen viability testing with Brewbaker and Kwack germination media (Brewbaker and Kwack, 1964). Fertility and pollen viability tests were carried out at the Tissue Culture Laboratory, Faculty of Agriculture, Andalas University.

Results and discussion

Amount of single interest in compound interest (cluster)

The gambier flowers have an unlimited compound flower structure (inflorescentia racemosa or inflorescentia centripetal) which appear opposite each other in the axils of the leaves (*Fig. 1*). Gambier flowers have compound flowers whose mother stalks can grow continuously with branches that branch again and have an acropetal arrangement. The younger they are, the closer they are to the tip of the mother stalk.

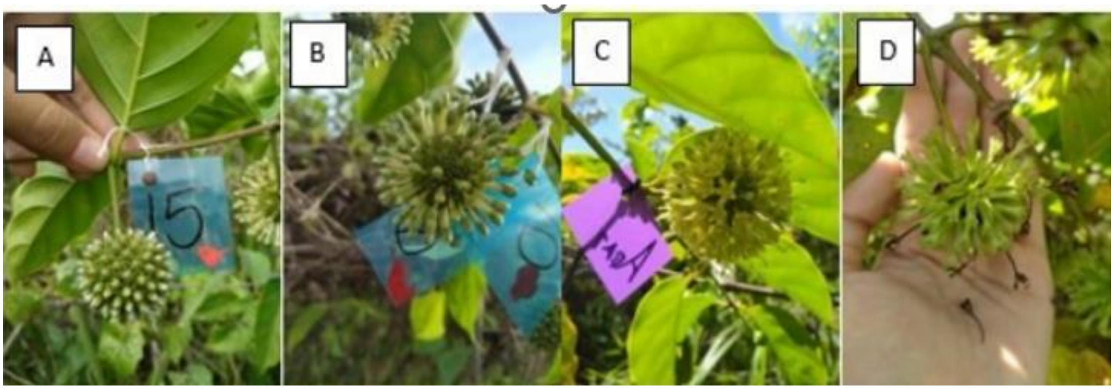

Figure 1. *The development phases of gambir flowers until producing seeds; (A) the phase of small buds, (B) the phase of large buds lasts for 1-3 days until the flower blooming, (C) the phase of the blooming flower lasts for 1-3 days until polling and fertilization being done, (D) the phase of fruit development characterized by the loss of petals*

Gambier flower is a complete flower that is characterized by the completeness of the basic flower organs such as the presence of sepals (calyx), petals (corolla), stamen (androecium), and carpel (pistil) or gynoecium in one individual flower. The shape of the gambier flower is like a hump (capitulum), which is a compound flower that resembles a cupped flower. Still, without supporting, leaves and the tip of the mother stalk usually swells so that the compound interest is composed of individual flower buds strung together according to a ball-like pattern (Hayati, 2020).

The number of single interest buds in compound interest (clusters) is calculated according to *Table 1*.

Based on *Table 1*, it can be seen that in several varieties of gambier, both Shrimp, Cubadak, and Riau Mancik, the number of single flowers in a compound flower (cluster) varies, namely GAUU1 = 51–102, GAUU2 = 65–117, GACC1 = 87–116, GACC2 = 79–93, GARm1 = 78–109, and GARm2 = 80–119. The number of single flowers in compound interest (cluster) is calculated when the gambier flower enters the large bud phase (F2) when the length of the flower crown reaches ± 2 cm as measured from the base of the flower.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2206_57535766

Table 1. *The number of single interest in the cluster*

Sample	Amount of single interest in cluster										
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**Average**
GAUU1	94	93	71	93	51	73	102	89	79	78	82.3
GAUU2	71	79	108	105	104	117	89	101	105	65	94.4
GACC1	109	99	87	116	104	104	102	100	101	96	101.8
GACC2	93	87	84	86	87	90	92	79	85	88	87.1
GARm1	109	103	89	78	84	90	92	95	91	84	91.5
GARm2	113	109	119	110	80	89	81	100	106	102	100.9

GAUU = Shrimp, GACC = Cubadak, GARm = Riau Mancik are sample plants that carry out natural geitonogamy pollination

Fertilization percentage

Inbreeding can be done through three methods, namely natural geitonogamy pollination, artificial geitonogamy pollination, and artificial cross-pollination. Inbreeding is said to be successful if the ovary develops to form a fruit. The fruit formed can be observed by looking at the development of the flower base to become large, long, and fresh green in color. The number of fruits formed through several cross-sectional methods was counted, and then the percentage of fertilization was analyzed, as shown in *Table 2*.

Table 2. *Percentage of flowers forming fruit through natural geitonogamy pollination, artificial geitonogamy pollination, and artificial cross pollination*

Sample	1	2	3	4	5	6	7	8	9	10	(%) ± SD
1. Natural geitonogamy pollination											
GAUU1							1		.		0.10 ± 0.50
GAUU2										12	1.14 ± 3.61
GACC1											0.00 ± 0.00
GACC2					9						1.03 ± 3.27
GARm1											0.00 ± 0.00
GARm2			1		1				3		0.49 ± 0.93
Fertilization average (%)											0.46 ± 1.38
2. Artificial geitonogamy pollination											
GBUU	1										2.00 ± 0.44
GBCC											0.00 ± 0.00
GBRm	3										6.00 ± 1.34
Fertilization average (%)											2.67 ± 0.60
3. Artificial cross pollination											
UU x UU	10	10	8	6	10						88.00 ± 17.90
CC x CC	3	10	9	10	10						84.00 ± 30.49
Rm x Rm	10	9	10	6	10						90.00 ± 17.32
Fertilization average (%)											87.33 ± 21.90

GAUU = Shrimp, GACC = Cubadak, GARm = Riau Mancik are sample plants that carry out natural geitonogamy pollination; GBUU = Shrimp, GBCC = Cubadak, GBRm = Riau Mancik are sample plants that carry out artificial geitonogamy pollination; UU = Shrimp, CC = Cubadak, Rm = Riau Mancik are sample plants that perform artificial cross-pollination; SD = standard deviation

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2206_57535766

Based on the data obtained in *Table 2*, it is known that the inbreeding method through artificial cross-pollination is more effective than natural geitonogamy pollination and artificial geitonogamy pollination. This can be seen from the data on the percentage of fertilization produced through artificial cross-pollination of 87.33%, much higher than the percentage of fertilization in natural geitonogamy pollination of 0.46% and artificial geitonogamy pollination of 2.67%.

Pollination, artificial geitonogamy pollination, and artificial cross-pollination

In natural geitonogamy pollination, the percentage of fertilization produced from several varieties of gambier, both Shrimp, Cubadak, and Riau Mancik, was very low at 0.46%, with the highest percentage of fertilization found in sample plants GAUU2 of 1.14% and the lowest percentage of fertilization was found in on the sample plants GACC1 and GARm1 by 0%. The low percentage of fertilization produced is due to the absence of transfer of pollen to the stigma of other flowers because the pollen transfer process relies on pollination vectors such as wind and insects. The possibility of pollination occurs during anthesis or after the time of anthesis. According to Jamsari et al. (2007), the anthesis phase is characterized by the complete blooming of the flower bud where the petals open completely while the stigma begins to come out of the sheath petals.

The time of anthesis of gambier flowers varies both in one cluster and in different clusters. If the self-pollination process occurs during anthesis, the pollen attached to the stigma is in a viable state because the anther first ruptures during anthesis so that the chances of fruit formation will be greater. On the other hand, if pollination occurs after the time of anthesis, the stigma is not in a receptive state, and pollen has low viability because it has been heavily influenced by environmental factors such as changes in temperature and humidity which cause most of the pollen to die (sterile) (Abril et al., 2019), so it will not form fruit.

Gambier plants have a short anthesis time that lasts for 4–7 days, and the anthers rupture simultaneously as anthesis. The short duration of this anthesis causes the chances of fruit formation through natural geitonogamy pollination to be smaller (Pant et al., 2020).

After the anthesis period ends, the flower structures such as petals, anther, and stigma begin to wither and slowly turn brown and then fall off one by one. If it succeeds in forming fruit, the flower base will remain fresh green, large, and long, while the flower base that does not succeed in forming fruit will be brown, and the flower base will fall.

The natural geitonogamy pollination process in this study was carried out by covering the plant as a whole. The transfer of pollen from one flower to another flower stigma only relied on pollination vectors such as wind. Autogamous pollination usually occurs in addition to geitonogamous pollination. When the flower was opened before anthesis, it was found that the stigma and anther were parallel. Pollen was found to have attached to the stigma of the same flower when anthesis occurred, so this caused autogamous pollination on gambier plants (Zainal et al., 2020; Rizki et al., 2020). The pollination process will occur autogamy if there is no pollination vector such as wind.

Geitongamy pollination can form fruit because the stigma is pollinated by pollen from different flowers. The pollen allele is different from the allele produced in the pistil, causing compatible mating. However, suppose the pollen does not transfer to the stigma of another flower. In that case, autogamous pollination will occur, which causes

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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failure to form fruit due to the interaction of the two S genes protein between the pollen and the stigma, which causes inhibition of pollen germination or extension of the pollen tube so that it fails to form fruit. With self-incompatibility. This aligns with Zainal et al. (2020) research that none of the fruits were successfully formed due to autogamous pollination on gambier plants.

The self-incompatibility mechanism is controlled by genes located at a locus denoted by the S locus. Each S locus consists of two essential genes that control self-incompatibility. One gene will be active in the pistil, while the other gene will be active in the anther. The protein interaction of the two S genes causes inhibition of pollen germination or extension of the pollen tube. However, if a male determinant interacts with a female determinant that is not from the same flower, then fertilization will occur (Ahmad et al., 2022).

In general, there are two types of self-incompatibility, namely gametophytic self-incompatibility (GSI) and sporophytic self-incompatibility (SSI). Gametophytic self-incompatibility is determined by alleles present in pollen gametes. Meanwhile, sporophytic self-incompatibility is determined by alleles found in female (maternal) plants (Zhang et al., 2024).

Gametophytic self-incompatibility (GSI) is the most common form of self-incompatibility found in closed seed plants (angiosperms), which include: Solanaceae, Scrophulariaceae, Rosaceae, Fabaceae, Onagraceae, Campanulaceae, Papaveraceae, Liliaceae, Graminae, and Poaceae. Vijayakumar (2018) has reported sporophytic self-incompatibility (SSI) found in many Brassicaceae families. In sporophytic self-incompatibility (SSI), pollen will not grow to form a pollen tube if it pollinates a pistil with the same allele as the allele produced by the pollen.

Gambier plants are thought to be classified as gametophytic self-incompatibility (GSI), where pollen can still germinate on the pistil/stigma, which is the beginning of the fertilization process with the consequence of fruit formation. This is because pollination that occurs by geitonogamy means pollination from different pollens and stigmas but on the same plant. It is suspected that one of the alleles produced by the pollen when attached to the stigma is different from the allele produced by the pistil/stigma so that the pollen can germinate on the stigma and successfully form fruit. In contrast, the gambier plant, which does not succeed in developing fruit, is caused by the absence of pollination vectors such as wind. So that the gambier plant performs autogamous pollination from pollen and pistils from the same flower so that it fails to form fruit. This is presumably because the allele produced by pollen has no difference from the allele produced by pistil, so the pollen cannot germinate and fails to form fruit.

In artificial geitonogamy pollination, the percentage of fertilization produced in several varieties of gambier, both Shrimp, Cubadak, and Riau Mancik, was very low at 2.67%, with the highest percentage of fertilization in the sample plants of GBRm at 6% and the lowest percentage of fertilization in plants GBCC sample is 0%. The low percentage of fertilization is due to self-incompatibility, climate, pollen count with high viability.

Self-incompatibility is the inability of hermaphrodite plants to pollinate/breed by themselves or relatives to produce a zygote. This mechanism in higher plants prevents inbreeding and forces cross-pollination due to self-incompatibility’s genetic control mechanism. In addition, self-incompatibility plays an essential role in the evolutionary process of higher plants by preventing self-pollination and the resulting inbreeding

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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depression (Zhang et al., 2024) and as a control of self-pollination in the presence of failure of fertilization at pre-zygotic or post-zygotic levels. Generally, self-incompatibility is caused by: 1) failure of the pollen tube to germinate on the stigma, 2) failure of the pollen tube to penetrate the stigma and grow down the stylus tube, 3) failure of the pollen tube to penetrate the ovule layer, 4) male gametes that have already entered into the embryo sac failed to unite with the egg (Nasrallah, 2019; Ahmad et al., 2022). The incompatibility event was shown when the pollen grains fell on the stigma. The pollen could not germinate even though the pollen was in good condition, fertile, and not damaged.

In pollination and fertilization, it is necessary to be compatible (compatible) between male and female gametes. This fertilization will be successful if the pollen can germinate above the stigma. The pollen tube can grow and penetrate the ovary until finally, the pollen can fertilize the egg. Self-incompatibility occurs in bisexual angiosperms (covered seed plants), especially hermaphrodites (male and female sex organs in the same flower) (Broz and Bedinger, 2021) The failure of the fruit formed was thought to be due to the incompatibility of the gambier plant.

Information about the mating system is useful for finding the effective methods needed for breeding a type of plant. Breeding results are not optimal if a species classified as self-incompatible is propagated through artificial pollination without considering the allele possessed by the male parent (pollen donor) at the S locus, which is the locus responsible for the flowering/fertilization process. The fertilization process cannot occur because it is inhibited if the alleles in the male and female parents are the same (Claessen et al., 2019; Zainal et al., 2023). The self-incompatibility mechanism is controlled by genes located at a locus denoted by the S locus. Each S locus consists of two essential genes that control self-incompatibility. One gene will be active in the pistil, while the other gene will be active in the anther. According to Vijayakumar (2018), physically, the two genes are close to each other; even genetically, they are linked to one another and inherited as a unit. The protein interaction of the two S genes causes inhibition of pollen germination or pollen tube elongation. Fertilization will occur if the male determinant interacts with a female determinant, not from the same flower. Artificial geitonogamy pollination can form fruit because the stigma is pollinated by pollen from different flowers, so the allele S locus in pollen is not the same as the S locus produced by the pistil/stigma resulting in mating harmony.

Climatic conditions such as rain can be an inhibiting factor in the process of artificial geitonogamy pollination (Rizki et al., 2020) This condition was found during the pollination process, where rain caused the pollen to be difficult to stick to the stigma because the stigma was wet, causing the mucus on the stigma to decrease.

The availability of pollen and the amount of pollen with high viability conditions need to be a concern. The availability of pollen is strongly influenced by wind speed because pollen is small, so the wind easily carries it. Pollen from Shrimp and Cubadak varieties have diameters of 86 mμ and 91 mμ, respectively, but can still be seen with the naked eye and pollen size on corn plants of 80 mμ. The amount of pollen with high viability also needs to be considered when pollinating artificial geitonogamy because a large amount of pollen with high viability can increase the chances of success in forming fruit. Pollen viability must always be considered because it plays a vital role in pollination. Although the amount of pollen available in large quantities has low pollen viability, fertilization will not occur because pollen cannot germinate and penetrate the stigma (Williams and Reese, 2019).

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
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In artificial cross-pollination, the percentage of fertilization produced in several gambier varieties, Shrimp, Cubadak, and Riau Mancik, was very high at 87.33%, with the highest percentage of fertilization, namely in sample plants Rm x Rm of 90% and the highest percentage of fertilization. Low, namely in the sample plants CC x CC by 84%. This is in line with the research of Zainal et al. (2020), which states that the percentage of fertilization through the artificial cross-pollination system in UU x UU, CC x CC, and Rm x Rm in tall gambier plants is 86%, 64%, respectively and 100%.

The determination of cross pairs with incompatibility or compatibility characteristics is based on the percentage of fruit formed. Compatible crosses when producing fruit > 20%, partially incompatible crosses when producing 10-20% fruit, and incompatible crosses when producing fruit < 10%. Fruits formed through artificial cross-pollination are compatible because they can produce fruit > 20%.

Inbreeding that succeeded in forming fruit showed that the crossed flowers had compatibility, namely the compatibility between pollen and stigma, so that they were able to form fruit. This fertilization will be successful if the pollen can germinate above the stigma. The pollen tube can grow and penetrate the ovary until finally, the pollen can fertilize the egg. In addition, compatibility is closely related to the genes contained in male and female parents. If the S gene of the male parent interacts with the female S gene that is not from the same flower, it will lead to compatible marriages. Still, suppose there is an interaction of the protein of the two S genes in the male and female parents. In that case, it will cause inhibition of pollen germination and extension of the pollen tube so that it does not cause marital harmony (incompatible).

Percentage of harvested fruit

A change in skin color characterizes physiologically ripe fruit on gambier plants to brown and dry. Gambier fruit that is ripe will usually break or dehiscent after a while when the fruit has reached a certain water content. The splitting of the fruit is thought to be one of the mechanisms in the effort to spread the seeds of the gambier plant as a strategy for the preservation of the generation of the species concerned. Given the structure of gambier seeds which have wings and are very light, the spread of this species will be significantly helped by the movement of the wind.

The number of successfully harvested fruit was counted, and then the percentage of fruit harvested was analyzed. The percentage of fruit harvested through natural geitonogamy pollination, artificial geitonogamy pollination, and artificial cross-pollination is presented in *Table 3*.

Based on the data obtained in *Table 3,* it can be seen that the percentage of fruit harvested through artificial cross-pollination was 60.67% higher than through natural geitonogamy pollination of 3.22% and artificial geitonogamy pollination of 0.00%. Suppose it is seen from the percentage of fertilization through several inbreeding methods such as natural geitonogamy pollination, artificial geitonogamy pollination, and previous artificial cross-pollination. In that case, it can be said that the fruit formed is unable to survive or undergoes miscarriage (abortion) before the fruit is physiologically ripe. The occurrence of fruit miscarriage can occur during fruit development, starting from young fruit to old fruit. The occurrence of fruit miscarriage can be caused by two factors, namely internal factors, and external factors.

Genetic barriers cause internal factors that affect harvested fruit. The genetic barriers significantly affect fertilization (Bandeira e Sousa et al., 2021). The loss of young fruit

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2206_57535766

can be caused by incompatibility after fertilization because the endosperm fails to develop so that it cannot support the growth and development of the embryo. That miscarriages of ovules are caused by failure of embryo formation that occurs due to genetic barriers in the form of gametophytic incompatibility, namely inhibition of development of the pollen tube to the embryo sac so that the embryo becomes abnormal due to natural geitonogamy pollination and artificial geitonogamy pollination in some cases. Gambier varieties include Shrimp, Cubadak, and Riau Mancik. This incompatibility event occurs randomly and is part of the ecosystem of plants that already have natural mechanisms to anticipate them.

Table 3. *Percentage of fruit harvested through natural geitonogamy pollination, artificial geitonogamy pollination, and artificial cross pollination*

Number of harvested fruits											Harvested fruits
Sample	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**(%) + SD**
1. Natural geitonogamy pollination											
GAUU1											0.00 ± 0.00
GAUU2										4	3.33 ± 1.27
GACC1											0.00 ± 0.00
GACC2					9						10.0 ± 2.84
GARm1											0.00 ± 0.00
GARm2			1		1				1		6.00 ± 0.48
Average of harvested fruits (%)											3.22 ± 0.76
2. Artificial geitonogamy pollination											
GBUU											0.00 ± 0.00
GBCC											0.00 ± 0.00
GBRm											0.00 ± 0.00
Average of harvested fruits (%)											0.00 ± 0.00
3. Artificial cross pollination											
UU x UU	10	10	8	3	4						70.00 ± 33.16
CC x CC	1	3	6	4	5						38.00 ± 19.23
Rm x Rm	9	7	10	4	7						74.00 ± 23.02
Average of harvested fruits (%)											60.67 ± 25.14

GAUU = Shrimp, GACC = Cubadak, GARm = Riau Mancikare sample plants that carry out natural geitonogamy pollination; GBUU = Shrimp, GBCC = Cubadak, GBRm = Riau Mancikare sample plants that carry out artificial geitonogamy pollination; UU = Shrimp, CC = Cubadak, Rm = Riau Mancikare sample plants that perform artificial cross-pollination. SD = standard deviation

External factors such as environmental conditions can cause miscarriage (abortion). That environmental conditions significantly affect the success of fruit production. Climatic conditions such as low temperature, high rainfall, and wind speed can physiologically affect fruit development to maturity. During the research, it was often rained with high intensity accompanied by strong winds, which caused the fruit that was formed previously through natural geitonogamy pollination, artificial geitonogamy pollination, and artificial cross-pollination to fall out. In addition, the activity of other organisms can also cause fruit damage during fruit development to

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DOI: http://dx.doi.org/10.15666/aeer/2206_57535766

physiological maturity. During this research, it is often found that insects such as caterpillars and ants attack young and old fruit, causing damage to the fruit and causing the fruit to fall out.

Pollen fertility and pollen viability in vitro

Pollen fertility is a critical stage in the life cycle of a plant). Pollen fertility is vital for the efficiency of the sexual reproduction of a plant because it greatly affects the success of forming fruit in crosses. A lot or a small amount of pollen produced by a plant does not indicate that the pollen produced can fertilize the ovules properly. Pollen will fertilize the ovules if the pollen can live. Hence, the purpose of observing pollen fertility is to determine the ability of plants to produce live pollen. The number of fertile and sterile pollen was calculated on the field of view of the glass object. Then the percentage of fertility and pollen sterility was analyzed according to *Table 4*.

Table 4. *Fertility percentage and pollen sterility*

Gambier varieties	Sample	Fertility (%)	Sterility (%)
Shrimp	GAUU1	27.49	72.51
	GAUU2	48.40	51.60
	GBUU1	54.34	45.66
	UU x UU	75.00	25.00
	Rata-Rata	51.30	48.70
Cubadak	GACC1	67.34	32.66
	GACC2	73.75	26.25
	GBCC1	45.44	54.56
	CC x CC	68.00	32.00
	Rata-Rata	63.63	36.37
Riau Mancik	GARm1	19.96	80.04
	GARm2	22.67	77.33
	GBRm1	9.53	90.47
	Rm x Rm	21.46	78.54
	Rata-Rata	18.40	81.60

GAUU = Shrimp, GACC = Cubadak, GARm = Riau Mancikare sample plants that carry out natural geitonogamy pollination; GBUU = Shrimp, GBCC = Cubadak, GBRm = Riau Mancikare sample plants that carry out artificial geitonogamy pollination; UU = Shrimp, CC = Cubadak, Rm = Riau Mancikare sample plants that perform artificial cross-pollination

Based on the data obtained in *Table 4,* it is known that the highest percentage of pollen fertility was found in the flower sample of the Cubadak variety, which was 63.63%. In contrast, the lowest percentage of pollen fertility was found in the flower sample of the Riau Mancik variety, which was 18.40.

The low pollen fertility obtained is due to the pollen’s susceptibility to decline and death (sterile). Pollen that has regressed is characterized by a decrease in the viability of the pollen. The death of most pollen can cause a decrease in pollen fertility due to evaporation caused by changes in daily temperature and humidity in an open environment. The temperature and humidity are the main factors that affect pollen behavior and fertility (Pacini and Dolferus, 2019).

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The influence of environmental temperature causes pollen fertility to decrease due to changes in macromolecules into simple molecules rapidly. Carbohydrates such as starch will become sucrose, so the sucrose content will increase when the starch content decreases. Pollen still fertile consists of 5% sucrose, but when the ambient temperature increases, the sucrose content will also increase to 12% (Luo et al., 2020). The hydrolysis of sucrose will form fructose and glucose. Fructose plays a role in respiration, while glucose formed will provide energy. A high respiration process due to an increase in temperature causes the loss of food reserves in the pollen, causing the pollen to die (sterile).

The staining method is used to test pollen fertility by approaching the nutrient content in the pollen; if it is sufficient, it is considered fertile. The nutrient content is detected using a staining reaction with a specific solution. Staining with bromothymol blue is one of the most widely used dyes to predict pollen fertility. The component tested was callose content in the cell wall and pollen tube. Callose is a carbohydrate that separates microspore mother cells from other cells and covers pollen after meiosis (Li et al., 2023). Pollen will be colored blue if it contains callose. Kalose content indicates fertile pollen. Pollen will be blue in all nucleus parts except for the cell wall. If the cell nucleus of the pollen is blue, the pollen is fertile, while the light or partially light-colored pollen means the pollen is sterile.

Abnormal development of the generative organs can cause sterility, such as defective stamens or pistils, damaged pollen or pollen, or aborted egg cells. There are three types of sterile pollen: imperfect pollen, round but colorless pollen, and spherical pollen with only one vegetative nucleus. In contrast, fertile pollen is perfectly round and has a nucleus full of material. Cytoplasmic can be stained with aniline blue or bromothymol blue.

The success of pollination and fertilization is influenced by pollen fertility and pollen quality, such as viability or pollen ability to germinate. Pollen viability is the state of pollen that is ripe and ready to pollinate the stigma. Viable pollen will grow and form a pollen tube and show its ability to transport sperm to the ovule. Furthermore, if perfect fertilization occurs, the ovaries will form fruit. Low viability causes pollen not to germinate, and pollen tubes are not formed so that the fertilization process will not occur.

Pollen viability test was carried out using Brewbaker and Kwack (1964) germination medium consisting of 300 mg/l calcium nitrate ($Ca(NO_3)_2.4H_2O$), 100 mg/l boric acid (H_3BO_3), 200 mg/l magnesium sulfate ($MgSO_4.7H_2O$), 100 mg/l potassium nitrate (KNO_3) and 10% sucrose, but in this study, none of the pollen was successfully germinated (non-viable). Pollen is said to grow if the elongation of the pollen tube is equal to or more than the diameter of the pollen (Calabrese and Agathokleous, 2021)

In this study, pollen viability testing obtained lower results than pollen fertility using the bromothymol blue staining method. Infertile pollen can sometimes also be stained by the staining method. The absence of pollen germination in the in vitro germination medium was caused by the incompatibility of the pollen with the composition of the medium. Pollen germination of a plant varies with each species, depending on the substance (medium) required for its growth, genotype, environmental conditions, pollen maturity, composition, and pH of the media (Brandoli et al., 2024).

Pollen staining methods are sometimes unreliable for most species. Pollen germination in vitro is more practical to determine the fertility and viability of pollen. The ability to germinate pollen plays a vital role in fruit formation and the interaction between flowers and flower pollinators. Pollen germination and pollen tube growth are essential for fertilization and seed formation.

http://www.aloki.hu ● ISSN 1589 1623 (Print) ● ISSN 1785 0037 (Online)
DOI: http://dx.doi.org/10.15666/aeer/2206_57535766

Conclusions

The most effective inbreeding method for the three varieties of gambier, such as Shrimp, Cubadak, and Riau Mancik, is artificial cross-pollination. This is evidenced by the percentage of fertilization produced through artificial cross-pollination on the three varieties of gambier by 87.33% compared to the percentage of fertilization through natural geitonogamy pollination of 0.46% and artificial geitonogamy pollination of 2.67%.

Acknowledgements. We thank the Directorate General of Higher Education, Ministry of Research, Technology, and Higher Education of the Republic of Indonesia and LPPM Andalas University for Reputable publication research Batch II. 2023 with contract No.37/UN.16.19/PT.01.03/ food-RPB/2023, date 03 August 2023. SK Rektor No. 2133/UN16R/KPT/PT.01.00 /2023, date 1 August 2023.

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