

**STUDIES ON POLLEN VIABILITY, STIGMA RECEPTIVITY AND
POLLINATION BEHAVIOR OF *Annona muricata* L.**

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ABSTRACT

In order to achieve yield and quality improvement of *Annona muricata*, precise knowledge on its floral biology is essential. A study was conducted to evaluate pollen characteristics and pollination behavior of *A. muricata*. Pollen viability was studied by culturing pollen in liquid media on a cavity slide under different incubation temperatures, and identified the best medium and temperature used for longevity assessment. The ideal stage of stigma receptivity was determined by pollination during 3-6 days of anthesis where pollination success was assessed under four pollination methods; natural pollination, manual cross pollination, autogamy and geitonogamy. The best pollen germination rate was observed in the medium with 10 % sucrose, 0.01 % Boric acid and 0.01 % Calcium nitrate at a temperature of 32°C. The best time of pollen collection for cross pollination was 8.30 a.m., and the pollination was successful on the day five of flower anthesis which coincides with the best stigma receptive stage of flower. There was no significant effect of accessions on pollen viability longevity, stigma receptivity and pollination behavior. High yields and high number of fruits can be obtained from *Annona muricata* by manual cross pollination.

Key words: Manual cross pollination, Pollen germination and longevity

STUDIES ON POLLEN VIABILITY

INTRODUCTION

Annonaceae is the largest family within the order Magnoliales. It consists of 120 - 135 genera and 2,000 - 2,500 species. (Cronquist, 1981; Gottsberger, 1989, 1999; Kelly, 2000; Stevens, 2001; Lara, 2016). *Annona muricata* L. (Soursop) is originated in tropical America, and later reported in West Indies, regions from Southern Mexico to Peru, Argentina, regions from South eastern China to Australia and the lowlands of Eastern and Western Africa.

Although it is considered as an underutilized fruit crop in Sri Lanka, It has now become a valuable crop for fruit crop due its medicinal purposes. *A. muricata* is distributed throughout the country except certain parts of Nuwara Eliya district (Pushpakumara *et al.*, 2011). Information on floral biology and pollination behavior of some Annona species are available, however, information is meager in *Annona muricata* L.

It is generally considered that Annonaceae are outcrossers. (Norman and Clayton, 1986; Norman *et al.*, 1992; Andrade *et al.*, 1996; Momose *et al.*, 1998; Ratnayake *et al.*, 2006, 2007). Annonaceae species are protogineous (i.e. receptive stigma before anther dehiscence) and show synchronized dichogamy (i.e. most flowers within a single individual are on the female phase or on the male one). (Gottsberger, 1989; Kuchmeister *et al.*, 1998; Carvalho and Webber, 2000; Ratnayake *et al.*, 2007).

Development of reliable methods for determining the functional quality of pollen helps in monitoring pollen vigor during storage, genetic evaluations, pollen stigma interaction studies, crop improvement and breeding, and incompatibility and fertility studies (Sulusoglu *et al.*, 2014). In order to determine the amount of viable pollen, germination tests are necessary (Sulusoglu *et al.*, 2014). Although it is time consuming and laborious, the results of controlled pollination experiments are useful to assess breeding pattern of plants in the field (Ratnayake, 2005). In this context, the knowledge of the floral biology and the reproductive behavior of the species is fundamental importance, both to support the conducting of breeding programmes and to adopt precise management practices. Sri Lanka possesses a great diversity of germplasm of Annona species and particularly there is a potential to develop Soursop varieties for its yield, quality and medicinal values. However, there is no recommended variety for commercial cultivation available in the country. One of the main obstacles to improve crop productivity is lack of knowledge on the reproductive biology of *Annona muricata* L.

The present study aimed to evaluate the effect of pollination method on fruit set, the optimum period of stigmatic receptivity to achieve maximum viability and longevity of pollen grains and high yield, which are essential for initiating breeding programme of the species.

MATERIALS AND METHODS

Study site and materials.

The study was conducted over the period of 2015-2017 at Fruit Research and Development Institute, Horana. Twelve uniform, mature, eight-year old healthy grafted plants belong to three *Annona muricata* accessions each containing four trees, grown under similar cultural practices was selected for the study. Pollen viability and longevity was investigated by stereo microscope under magnification of 10× in the laboratory conditions.

Pollination behavior

Four pollination treatments were employed to assess the pollination success of *Annona muricata*. The treatments were natural or open pollination, cross pollination, autogamy (pollination from the same flower) and geitonogamy (pollination from other flowers in the same tree).

Natural or open pollination-Perfect flowers from each accession were tagged before anthesis and allowed to remain in the trees for on fruit setting. Total number of 120 flowers, 40 flowers from each accession, was subjected for observations from fruit setting to fruit maturation in three seasons.

Cross pollination-Hand cross pollination was done for 120 flowers in one season including 40 flowers from each accession, and continued for three seasons during 2016 and 2017. The flowers with closed inner petals were covered with oil paper bags 3 or 4 days before anthesis to prevent geitonogamy. Then, the fully opened flowers which have yellow color outer petals turned outward with glistening sticky stigmas were manually pollinated at the 5th day of flower anthesis at 9.00 a.m. Two different *Annona muricata* accessions were used as male and female parents.

Pollen shedding of *Annona muricata* occurs around 6.00 p.m. and small amount of pollen remained in the floral chamber on the next day morning. Pollen was collected

STUDIES ON POLLEN VIABILITY

from the matured flowers when the color of stamens changed from yellow to yellowish ash. The selected flowers were bagged on the previous day around 3 p.m. and pollens were collected on the next day around 8.30 a.m. The collected pollens were applied on the stigma of mother plant at 9.00 a.m. on the same day by using a small paint brush. Then the flowers were covered again with the oil paper bags. The bags were removed after one week, and the pollinated flowers were observed from fruit formation to fruit maturity.

Hand self-pollination-Flowers with closed inner petals were bagged 3-4 days before anthesis to avoid cross pollination. Then the fully matured flowers were pollinated with the pollen of another flower in the same plant (geitonogamy). These pollinated flowers were bagged again until one week's time, and allowed to remain on the trees for fruit set.

Bag self-pollination-The flowers with closed inner petals were bagged and allow self-pollination to occur. The bags were removed after one week of pollen shedding and the dormant structures were observed from fruit formation to maturity.

Pollen viability and longevity

Budded plants of three *Annona muricata* accessions in the same age were selected to collect flowers for pollen viability and longevity testing studies under in vitro condition. Three plants from each accession and three flowers from each plant were selected at a time of pollen harvest.

The matured flowers were bagged around 3.00 p.m. at the day of anther dehiscence and pollens were collected on the following day around 8.30 a.m. to be used for pollen viability and longevity studies.

The collected pollens were kept on dry blotting paper in a petri dish. The fresh pollens were inoculated on cavity slides by using a small paint brush. Then two drops of liquid medium were added onto the cavity slide using a dropper before incubation. Pollen viability was analyzed by assessing the ability of pollen grains to germinate in *vitro* in liquid medium on cavity slides.

The liquid media were composed of three sucrose concentrations (5, 10 and 15%) boric acid (0.01 %) and Calcium nitrate (0.01 %). The distilled water was used as the

control medium. The pollen cultures were incubated in to ten temperatures ranged from 27°C to 36°C in a cooling incubator in order to detect the optimum temperature to test pollen viability. Germinated pollen grains were observed in a stereo microscope under 10 × magnifications after three hours of incubation. Pollen was considered as germinate when the length of the pollen tube exceeded the diameter of its pollen grain. Pollen viability was estimated by calculating the ratio of the number of germinated pollen grains to the total number of pollen grains.

Pollen were collected from the open field at set times (8.30 a.m., 11.30 a.m. and 2.30 p.m.) on the same day and inoculated on cavity slides just after collection to assess the pollen longevity. Viability of pollen grains at set times were counted by following the same procedure used for assessing pollen viability.

The experiments were designed according to the Randomized Complete Block Design (RCBD) with three replicates. Randomly selected three visual areas of the slide including about 100 pollen grains were counted in each replicate. Since pollen germinability was similar in both years, data were combined and the means were used in statistical analysis.

Stigmatic receptivity duration

The flowers which started blooming but have closed inner petals were bagged in the 2nd day of bud splitting. In order to find out the best stage for manual pollination and to get a high percentage of fruit set of *Annona muricata*, hand cross pollination was performed on the 3rd, 4th, 5th and 6th day at 9.00 a.m. by depositing fresh pollen collected from another accession over the different stages of stigmas.

Thirty flowers from each tree, totaling to 360 flowers at pistillate stage were cross pollinated from three *Annona* accessions in two seasons. The percentage of fruit set was calculated as the percentage of harvested fruits over the number of pollinated flowers.

Statistical Analysis

Logistic regression analysis was used to find the best fitting model to describe the relationship between the dichotomous characteristic of interest (count of the pollens that germinated and fruit set percentage) and the independent variables (*Annona muricata*

STUDIES ON POLLEN VIABILITY

accessions, pollen germination media, incubation temperature, time of pollen collection and the day of Anthesis) The statistical analysis of the data was performed using SAS® University Edition.

RESULTS AND DISCUSSION

Pollen viability

The results revealed that accession, medium and temperature had no effect on pollen germination (Table 1). Since none of the interaction terms of accession was significant, it appears that there is no effect of accession on pollen germination. A significant interaction effect was observed in medium × temperature on pollen germination of *A.muricata*.

Table 1. Effect of independent variables for pollen germination of *Annona muricata*

Effect	DF	Wald Chi-Square	Pr > ChiSquare
Medium	3	5.3496	0.1479
Accession	2	1.5684	0.4565
Medium x Accession	6	3.8267	0.7001
Temperature	9	9.2946	0.4105
Medium x Temperature	27	64.5694	<.0001
Accession x Temperature	18	9.1202	0.9569
Medium x Access x Temper.	54	32.0200	0.9925

Table 2 presents the results two medium and temperature effect which again revealed that the interaction effect between medium and temperature is significant.

Table 2. Effect of medium and temperature on pollen germination of *Annona muricata*

Effect	Joint Tests results		
	DF	Wald Chi-Square	Pr > Chi Square
Medium	3	12.3138	0.0064
Temperature	9	25.3827	0.0026
Medium x Temperature	27	182.5430	<.0001

As there is a significant interaction between the medium and temperature, it is not possible to explain the effect of each variable on pollen viability independently. Therefore, as shown in Table 3, separate models were fitted for each of the four media with the aim of explaining the effect of temperature at a fixed level of pollen germination medium.

Table 3. Odds Ratio Estimates for pollen germination in different temperatures and media.

Temperature Effect relative to 27 °C	Odds Ratio Estimate (95% Confidence Interval)			
	Medium 1	Medium 2	Medium 3	Control
28 °C	1.9 (1.2 to 2.9)	1.8 (1.1 to 3.1)	2.3 (1.1 to 4.7)	2.0 (0.8 to 5.1)
29 °C	2.2 (1.5 to 3.4)	5.2 (3.2 to 8.2)	1.8 (0.8 to 3.7)	3.2 (1.3 to 7.7)
30 °C	2.5 (1.6 to 3.7)	8.5 (5.4 to 13.4)	1.0 (0.4 to 2.3)	3.7 (1.5 to 8.7)
31 °C	3.9 (2.6 to 5.7)	10.5 (6.7 to 16.5)	0.8 (0.3 to 1.9)	1.9 (0.8 to 5.0)
32 °C	4.6 (3.1 to 6.7)	13 (8.3 to 20.3)	0.5 (0.2 to 1.4)	4.0 (1.7 to 9.4)
33 °C	5.4 (3.6 to 7.9)	9.5 (6.1 to 15.0)	0.4 (0.16 to 1.2)	3.4 (1.4 to 8.0)
34 °C	2.8 (1.8 to 4.2)	5.0 (3.1 to 7.9)	0.2 (0.07 to 0.9)	1.8 (0.7 to 4.6)
35 °C	2.7 (1.8 to 4.03)	2.4 (1.5 to 4.0)	0.15 (0.03 to 0.7)	1.8 (0.7 to 4.7)
36 °C	1.5 (1.0 to 2.3)	1.4 (0.8 to 2.5)	0.2 (0.04 to 0.8)	1.1 (0.4 to 3.1)

The range of the data obtained is given within parenthesis. Pollen viability at different temperatures and media (medium 1 = 5 % sucrose, 0.01 % boric acid, 0.01 % calcium nitrate, medium 2 = 10 % sucrose, 0.01 % boric acid, 0.01 % calcium nitrate, medium 3 = 15 % sucrose, 0.01 % boric acid, 0.01 % calcium nitrate, control = Distilled water). Odds ratios for each temperature were calculated relative to 27°C.

As indicated in the Table 3, the highest odds ratio records for 33°C in medium 1, indicating that the germinability of pollens increases 5.4 times in 33°C compared to 27°C. In medium two, the germinability of pollen was 13 times higher in 32°C than in 27°C. Both Medium 3 and distilled water (control) showed lower odds ratios compared to Medium 1 and Medium 2. In overall, medium 2 (10 % sucrose) resulted the best germination rate at a 32°C (Plate1), followed by medium 1. Mendes *et al.*, (2012) obtained an average pollen germination rate of 52.5 % in Brazilian seedless sugar apple (*Annona squamosa* L) in 10% sucrose medium.

Increasing sucrose content in the medium from 10 % to 15 %, pollen germinability has been decreased in considerable proportion. It was also observed during the study that the cell plasma started to get shriveled in pollen cultured in 15 % sucrose medium,

STUDIES ON POLLEN VIABILITY

and after three hours of incubation the shrunken pollen could be seen. Normal *Annona muricata* pollens are tetrads (Plate 2). When germinating, it was rarely noted that, four pollen tubes developed from one tetrad. In most cases, at least one pollen tube can be seen from one tetrad. Azeez *et al.*, (2014) observed that compound pollen grains in the form of tetrad in *Annona muricata*, *Annona senegalensis*, *Annona squamosa*, *Annona reticulata*, *Monodora tenifolia* and *Xylopia aethiopica* and Tetragonal tetrads (in which the center of the grains define a tetrahedron) in *Annona muricata* and *Annona squamosa*.



Plate 1. Germinated *Annona muricata* pollens in 10x magnification under Stereo microscope in 10 % Sucrose, 0.01 % boric acid, 0.01 % Calcium nitrate medium in 32 °C after three hours of incubation.



Plate2. Tetragonal tetrad structure of normal *Annona muricata* pollens in 10x magnification under Stereo microscope.

Pollen longevity

There was no significant effect of accession or accession \times time interaction on pollen longevity (Table 4). However, the time of pollen collection was having a significant impact on the number of pollens that germinated.

Table 4. Effects of Accession, time and Accession x Time interaction for pollen longevity

Effect	DF	Joint Tests	
		Wald Chi-Square	Pr > Chi Square
Accession	2	3.699	0.1573
Time	2	17.668	0.0001
Accession X Time	4	2.369	0.6681

Therefore, while excluding the accession variable and the interaction term, which were previously found to be non-significant, the time effect on pollen longevity is presented in Table 5.

Table 5. Effects of time of pollen collection for pollen longevity.

Type 3 Analysis of Effects			
Effect	DF	Wald Chi-Square	Pr > Chi Square
Time	2	67.5953	<.0001

According to the results shown in Table 6, the pollen collected at 8.30 a.m. are viable about 12 times more than the pollen collected at 14.30 p.m. The collection at 11.30 a.m. had about seven times higher germination rate than that at 14.30 p.m. When the time of pollen collection and longevity of pollen are compared, the most number of germinations were seen with the earliest collection at 08.30 a.m. The second best germination rate was observed in 11.30 a.m. collection followed by the 14.30 p.m. collection which had the lowest longevity.

Another dehiscence of *A.muricata* takes place around 6.00 p.m. At the beginning of another dehiscence outer and inner petals and anthers started to shed gradually. Released anthers with yellow color sticky pollens remained inside the floral chamber for short time period. In the next day morning, a small amount of released pollens remained with 2 or 3 petals on the tree. Bagging of flowers is necessary on the day of anther dehiscence around 3 - 4 p.m. to collect the pollen for cross pollination in the next day morning. If the

STUDIES ON POLLEN VIABILITY

flower was removed from the tree previous day around 3 – 4 p.m. to collect pollen in the next day morning, a low germination percentage of pollen was observed compared to the pollen collected on the same day in the morning for cross pollination (data not present). However, Thanthirige (2001) reported that the flowers should collect previous day at 3.00 p.m. to obtain the pollen for cross pollination.

Table 6. Contrast Estimation and Testing Results for pollen germination with time of pollen collected.

Contrast	Odds ratio Estimate	Standard Error	Confidence Limits		Wald Chi-Square	Pr > ChiSq
Time 08.30 vs 14.30	11.9	3.5777	6.6	21.4	67.1875	<.0001
Time 11.30 vs 14.30	7.4	2.2756	4.1	13.5	42.7146	<.0001
Time 08.30 vs 11.30	1.6	0.2754	1.1	2.2	7.4002	0.0065

Stigma receptivity

Table 7 presents the effects of pollination day and the accession on stigma receptivity where no significant effect was shown by the accession. But, the day of anthesis was significant in predicting pollination success (p <0.0001).

Table 7. Effects of day of pollination and accession on stigma receptivity of *Annona muricata*.

Type 3 Analysis of Effects			
Effect	DF	Wald Chi-Square	Pr > ChiSq
Accession	2	0.3812	0.8265
Day	3	262.4617	<.0001

As shown in Table 8, when the cross pollination is considered, flowers at 5th day of anthesis were 71 times more receptive than those at 3rd day of anthesis whereas flowers at 6th day of anthesis was 50 times more receptive than the flowers at 3rd day of anthesis. With the highest odds ratio of 71, crosspollination of the flowers at 5th day of Anthesis at 9 a.m. resulted the best stigma receptivity. However, compared to the flowers at 3rd and 4th days of anthesis, ones at 6th day of anthesis at 9.00 a.m. also showed good stigma receptivity.

Table 8. Contrast odds ratio estimates for pollination success in different stigma receptivity stages.

Contrast	Odds ratio Estimate	Standard Error	Confidence Limits		Wald Chi-Square	Pr > Chi-Sq
Day 4 vs 3	7.1	2.758	3.313	15.2	25.4	<.0001
Day 5 vs 3	70.8	26.465	34.059	147.3	130.04	<.0001
Day 6 vs 3	50.3	18.758	24.231	104.5	110.5	<.0001
Day 5 vs 4	10.0	1.867	6.918	14.4	151.3	<.0001
Day 5 vs 6	1.4	0.213	1.046	1.9	5.2	0.0239
Day 6 vs 4	7.1	1.315	4.929	10.2	111.5	<.0001

During this study it was observed that anthesis of *Annona muricata* flower take 6 days to reach its full blooming stage and anther dehiscence take place on 6th day around 6 p.m. Flowers at first and second day of anthesis are in bud splitting stage and only outer layer of petals separated and yellowish green color remains in petals. The stigma and stamens are white in color in first three days of flower anthesis. In the third and fourth day of flower anthesis, buds are partially opened and outer petals separate widely, but still inner petals remain closed. On the 5th and 6th day, inner petals also detached and make small hole and outer petals became yellow in color and curved out ward.

The stigmas of 3rd and 4th day flowers were white in color, no exudates and not sticky. Stigmas of the 5th day flowers had more exudates, sticky and glistening than other flowers. The flowers at the 5th day and 6th day of anthesis could be identified separately by the color of stamens. The stamens in the 6th day flowers were grayish yellow while stamens in the 5th day flowers were dark yellow in color.

According to the results, stigma receptivity of *A. muricata* flower gradually increased from day 3 to day 6 of anthesis. The day before anther dehiscence (flower at 5th day of anthesis) around 9 a.m. is the best for crosspollination. However, Thantirige (2001) reported that suitable stage of the flowers for pollination as partially open flowers and the best time was between 6 a.m. to 11 a.m. Kishore *et al.*, (2012) showed that in *Annona squamosa* L, the highest fruit set was recorded when pollination happened between 9 a.m. and 12 noon on the day of anthesis where the peak stigma receptivity takes place.

STUDIES ON POLLEN VIABILITY

Pollination behavior

As shown in Table 9, accession was not found to be a significant factor on pollination success. The method of pollination had the significant effect ($p < 0.0001$).

Table 9. Effect of accession and method of pollination on fruit set of *Annona muricata*.

Type 3 Analysis of Effects			
Effect	DF	Wald Chi-Square	Pr > Chi Square
Accession	2	0.3241	0.8504
Method	3	246.4661	<.0001

Describing the effect of pollination method on pollination success, Table 10 presents the effect of each method is compared to reference level (one of the remaining methods). The initial reference level used in the table was autogamy, as it had the lowest success rate.

Table 10. Contrast odds ratio estimates for pollination methods of *Annona muricata*.

Contrast	Odds ratio Estimate	Standard Error	Confidence Limits		Wald Chi-Square	Pr > ChiSq
Cross pollination vs Autogamy	288.1	206.6	70.7	1174.7	62.3695	<.0001
Natural pollination vs Autogamy	8.9	6.660	2.03	38.6	8.4407	0.0037
Geitonogamy vs Autogamy	63.2	45.468	15.5	258.8	33.2724	<.0001
Cross pollination vs Geitonogamy	4.5	0.7368	3.3	6.3	87.8726	<.0001
Cross pollination vs Natural pollination	32.5	8.809	19.1	55.3	164.8306	<.0001
Geitonogamy vs Natural pollination	7.1	1.968	4.1	12.2	50.6916	<.0001

As shown in Table 10, the success of cross pollination was 288 times higher than that of self-pollination (Autogamy). Geitonogamy and natural pollination had 63 and 10 times higher success rates, respectively, compared to autogamy. When natural pollination was compared with hand cross pollination, cross pollination showed

a 32.49 times more fruit set. According to the results cross pollination is prominent in *Annona muricata* L. Geitonogamy also shows significant success in fruit set. Success of manual cross pollination is very high compared to the fruit set of natural pollination. This may be due to the absence of pollinators in natural condition. Observations were done during day time from 8.00 a.m. to 5.00 p.m. to record pollinators and there were no any insects found on flowers other than ants. Ants may not have an influence on the process of pollination. Thanthirige (2001) showed that artificial pollination increased the fruit set in Soursop by 67 – 75% compared to 25% in natural pollination. It was found that a major impediment to fruit production of Atemoya was low fruit set due to inadequate pollinator visits (Jenkins, 2015). Further, Kahn *et al.*, (1994) stated that neither the self nor cross pollination take place effectively in *Annona* species. Referring to *Annona cherimola*, Schroeder (1941) indicated that 59 % hand pollinated flowers set fruit while only 2 % set fruit under natural pollination.

CONCLUSIONS

The best pollen germination medium for *Annona muricata* was 10 % sucrose, 0.01 % Boric acid and 0.01 % Calcium Nitrate at a temperature of 32°C. The most suitable time for pollen collection was at 8.30 a.m. But collection of pollen from the open field from bagged flowers was possible up to 11.30 a.m. The best stigma receptive stage of the flower for pollination was 5th day of anthesis where fully opened flower has dark yellow stamens and glistening stigmas. The *Annona muricata* is a highly cross pollinated crop. There was no significant effect from the accession in pollen viability and longevity, stigma receptivity and pollination behavior of *Annona muricata*. High yields and high number of fruits can be successfully obtained by application of manual cross pollination.

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STUDIES ON POLLEN VIABILITY

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TROPICAL AGRICULTURIST, VOL.166 (3), 2018

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