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WRIST ACTION SHAKER: A NOVEL METHOD FOR COLLECTING POLLEN GRAINS FROM THE INFLORESCENCE OF COCONUT (*COCOS NUCIFERA* L.) FOR ITS STABLE NUTRITIONAL COMPONENTS

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ABSTRACT: To predict the cocos anther dehiscence, a novel method was devised to compile the colossal amount of pollen grains. The matured inflorescence enveloped by the spathe was collected and the anther dehiscence were noticed day by day until the shedding of entire pollen. This study was experimented for a period of eight months between (February) 2018 to 2018 (September). After an experimental trails, sieve and a new tool wrist action shaker methods were used to collect the healthy pollen grains. Proximate analysis was determined by food analysis-FSSAI and association of analytical chemicals method. The complete process of anther dehiscence took 12 -18 days and after an experimental trials the high amount of pollen grains were obtained on the twelfth day. During the month of June amount of pollen i.e. 16.21 ± 0.05 g per 100 g of the fresh flower weight was recorded in wrist action shaker whereas 2.36 ± 0.018 g per 100 g of the flower weight found in sieve method. The average dry weight of pollen grains was (9.759 g) and 0.11 ± 0.03% of moisture content was found in wrist action shaker. In addition the quality of pollen was stable at – 4 ℃ (freeze drying). The p value ($p = 0.002 < 0.05$) is lesser than 0.05, which indicates that weight of the pollen grains is significant for wrist action shake. The total carbohydrate and protein were higher and zinc and iron were also abundant. The conclusive proof out of the two methods wrist action shaker was more efficient, reliable and cost-effective for collection and storage to use the pollen grains in the fields of pollination biology, nutraceuticals, modern herbal medicine and genetic resource conservation.

INTRODUCTION: The coconut palm *Cocos nucifera* L., (Fam: Arecaceae) popularly known as kalpa virshu, provides numerous benefits to our lives [1].

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Coconut inflorescence is monoecious, consisting both male and female flowers on the same inflorescence known as spadix, which developed from a woody sheathe or spathe.

During the flowering stage, the spathe splits lengthwise to expose the spadix [2]. Each spadix measures 1–1.5 m in length and contains 40–60 spikelets that bear the flowers. The male flowers are always more in number than the female flowers in the same spadix and this variation may be somewhere between a few hundreds and thousands

depending upon the number of ramification in the spadix [3]. The male flower has six yellow floral leaves arranged in two circles. The centre of each flower is an abortive pistal which forms the teeth at the apex, each bearing a nectar gland and six hammer-shaped stamens that yield large quantities of powdery yellow pollen [4].

Coconut pollen grains are monocolpate, spherical and smooth in nature when fresh, and they become ellipsoidal when dry [5]. When hydrated a pollen tends to gain its original shape. Each pollen measures about 65 µm to 69 µm in length and 0.028 to 0.30 mm in diameter [6]. In a male flower, the number of pollen grains present per inflorescence have been estimated about 88,000 to 1,21,000 [7, 8].

Early method of pollen collection and isolation was carried out by different technology using shed microspore method by applying a force using magnetic stirrer, but this has only possible to view the image. Another method was done by placing the spikelets in water so that the pollens will fall [9] were collected by using a camel hair brush, but this method had two disadvantages – very small amount of pollen was collected and also the content was reduced and the stability were loss during storage of the pollen [10].

Availability of large quantities of pollen production for controlled pollinations are possible by means of storing the pollen at freeze dry condition for germination and the viability also increased, by means of fluid bed drying method. This method of collection was efficient only for pollination and does not comprise any chemical constituents due to drying at 40 ℃ all chemical components are deteriorated [11].

A matured spathe of coconut palm (*Cocos nucifera*) which shoots 12–14 inflorescences per year [12]. The coconut palm in general, is allogamous (out-breeding), because even though the male and female flowers are located close to each other in each inflorescence, the pollen is shed before the female flowers are ready for it. Pollens were shed during the bursting of anthers when the spikelets gets split automatically. The major phytochemicals present in *C. nucifera* are lignin, tannin, furfural, pentose and cellulose many researches are on-going to produce these compounds that can be used in various biological and pharmacotherapeutic applications [13-15]. Coconut pollen grains contain enormous amount of carbohydrate, proteins, lipids and secondary metabolites such has phenols, flavonoids, anthocyanins, phospholipids, minerals and vitamins and generally it is very difficult to separate these components from pollen extract [16]. Recently, increasing evidence of coconut pollen possess potential therapeutic benefits including antioxidant, antibacterial, anti-inflammatory, chemotherapeutic activity, and they are also good source of food for foraging bees. Collecting large amount of pollen grain is a challenging task though. To overcome the defects observed in the earlier methods of pollen collection, this research focuses to design a new method for collecting a healthy amount of pollen grains directly from fresh spikelets.

MATERIALS AND METHODS:

Study Site: The research work was conducted at the Eco-restoration located at Pondicherry – near Ousteri Lake, about 10 km west of Pondicherry city. The matured spathe of coconut flower- *Cocos nucifera* was collected during the period of February 2018 to September 2018 – timing is between 8.00 am to 10.00 am.

Protection of the Inflorescence: The split spathe was kept in a clean, dry butter paper. When the matured inflorescence starts dehiscence by longitudinally splitting between spikelet, the whole process hardly took about 3 to 8 days. During these days, the pollen quantity was less due to the premature inflorescence [16].

The collected flowers were batched for a period of 8 months – the collection was done from 1st to 19th day after opening of spathe, *i.e.* the period between the opening of first female flower, which generally coincides with the bursting of the spathe and abscission of the male flowers (termed as male phase). The spadices are ivory in colour and turn brownish orange at mature stages. The mature male flowers break open the bracts to release the yellow colour pollen grains from the anthers.

Collection of Spikelets: The split spikelets were separated and collected at regular intervals in order to avoid the deviation of environmental parameters,

which in turn affect the pollen release. During the period of anthers' dehiscence, the pollen grains get released in the form of a fine dust of yellow-coloured powder. It takes 3 to 12 days to release the entire pollen from the anthers' bract [17].

To separate the pollen grains, we devised two experimental methods that optimize the standard protocol for convenient and continuous collection of a healthy amount of pollen grains.

Isolation of Pollen Grains:

Method-1:

Sieve Method: Exactly 100 g of dried coconut flowers was taken and then transferred into a pharmaceutical sieve mesh size no. 40. The flowers were sieved for 5 min using a butter paper to collect the pollen grains separately. The yellow-coloured powder obtained after this was then weighed and the percentage of the yield was calculated. To avoid moisture, the pollen grains were immediately transferred in to an Eppendorf tube and stored at 4 ℃ to prevent contamination and germination, to improve shelf life and to prevent the viability of the pollens [9].

Method-2:

Wrist Action Shake Method: Exactly 100 g of the dried flowers was taken in a sterilized round bottom flask and the flask was connected to a wrist action shaker that was run for 15 min at 250 oscillations in order to separate the powdery pollens from its stamen. On continuous shaking, pollen grain got released from the woody flowers and this mixture was then transferred into a 50-ml conical tube and centrifuge at 3000 rpm for 15 min. The supernatant was discarded and the pollen grains settled at the bottom were collected and then finally sieved. The sieved pollen grains are kept in a desiccator for 1 h. to remove the moisture and then stored at freezing -4 ºC in air tight container.

Determination of Proximate Composition: The proximate composition (*i.e.* fibre, moisture, protein, carbohydrates, lipid, ash and dry matter) of the coconut pollen grains was determined according to the method of AOAC [17].

Total Proteins: The total protein content in the coconut pollen grains sample was measured using Kjeldhal method (Kelplus- Supra LX). Take 0.4 g of the sample add 5 ml of Conc. Sulphuric acid and subjected to digestion and further distilled after adding 40% Sodium hydroxide. It was collected in a flask containing 4% of boric acid and titrated with 0.1N Hydrochloric acid. The percentage of nitrogen quantified was converted into nitrogen by multiplying with 6.25 as conversion factor 9 [18].

Total Carbohydrates: The difference between100 and sum of crude protein, fat, moisture, ash and fibre is called total carbohydrate. This is referred to as total carbohydrate by difference and is calculated by the following formula [19].

100 - (Weight in grams [protein + fat + water + ash + alcohol] in 100 g of food)

Ash Content: Ash content was determined by using a muffle furnace by taking 2 g of coconut pollen grain in a crucible and kept inside a muffle furnace (Technico) heated to 550 ℃ for 4 h. The resulting ash was measured in an electronic weighing balance. The ash content of coconut pollen grain greatly depends on the variety and climatic condition [20].

Total Lipids: Total lipid determined by extraction using organic solvents. Extraction of Crude fat by treating with diethyl ether and petroleum ether. Evaporate and calculate the residual weight [21].

$$\text{Fat \% (w/w)} = \text{Weight of Extracted Fat} \times 100$$

Weight of Dample:

Moisture Content: One gram of pollen grains was accurately weighed in a porcelain evaporating dish and was heated at 60 ℃ in an oven for 1 h. The percentage of moisture content of the pollen grain was calculated for the sample using the below formula [19].

$$\text{Moisture content \%} = M_2\text{-}M_1 / M_2$$

Where M_1 is weight of the sample after drying and M_2 is weight of the sample before drying

Determination of Mineral Components: The mineral components were determined by using atomic absorption spectroscopy [22].

Statistical Analysis: The statistical analysis was performed using SPSS tool and the result obtained as mean ± standard deviation. Comparisons were carried out by Student's t-test and Levene's Test for equality of variances; tests were with $p < 0.05$ level of significance.

Storage: Under atmospheric condition, pollen grains tend to loss their potency for germination as well as denaturation of protein happens due to the temperature of above 30 ℃ and humidity [23]. Some researchers proved pollen grains are normally stored at room temperature [24]. Some researchers reported that the pollen grains are stored according to freeze drying method in a closed natural atmosphere (sealed capsules) corresponding to relative humidity and rotary pump vacuum at 6 × 10 mm of Hg.

Storage could be possible at different temperatures like 4 ℃ (refrigerator), –20 ℃ and –80 ℃ (freezer). On the basis of different periods of pollen collection, the moisture content and the percentage of yield and storage temperature will be recorded. The process of drying has been referred to as auto freezing [25, 26],

Pollen grains belonging to a wide variety of species are best stored at temperatures 4 °C and –20 °C for short-term. Dry pollens kept at between 4 °C and –20 °C remain viable for a few days to a year, which may be adequate for use in breeding programs [27, 28].

RESULT:

Mean weight of Pollen Obtained from the Inflorescence of *Cocos nucifera* at Different Period of Anther Dehiscence: The effective time period for pollen grains to get released from the anther of an inflorescence was 1 to 18 days – this may depend on the climatic conditions and the species. To demonstrate the developmental difference of pollen shed day by day were observed and revealed the mean weight of the pollen grains obtained by wrist action handshake method at different time periods (*i.e.* number of days), after the release of pollen grains in **Table 1**.

TABLE 1: MEAN WEIGHT OF POLLEN OBTAINED FROM THE INFLORESCENCE OF *COCOS NUCIFERA* AT DIFFERENT PERIOD OF ANTHER DEHISCENCE

Month	Total weight of the flowers	No of days after the release of pollen grains in grams			Total weight of the pollen(g) Mean ± SD
		1-4 days	5-8 days	9-12 days	
May	100 g	1.5±0.1	3.3±0.1	7.8±0.1	12.6±0.1
June	100g	2.3±0.51	6.68±0.05	11.8±0.05	16.21±0.05
July	100 g	1.7±0.051	4.3±0.1	9.2±0.3	15.9±0.1

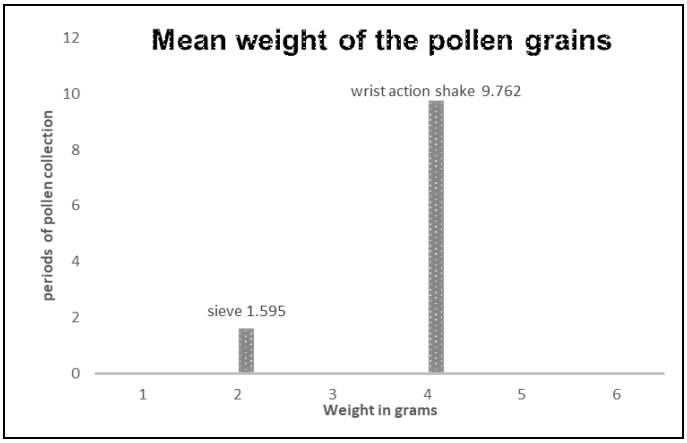

FIG. 1: MEAN WEIGHT OF THE POLLEN GRAINS

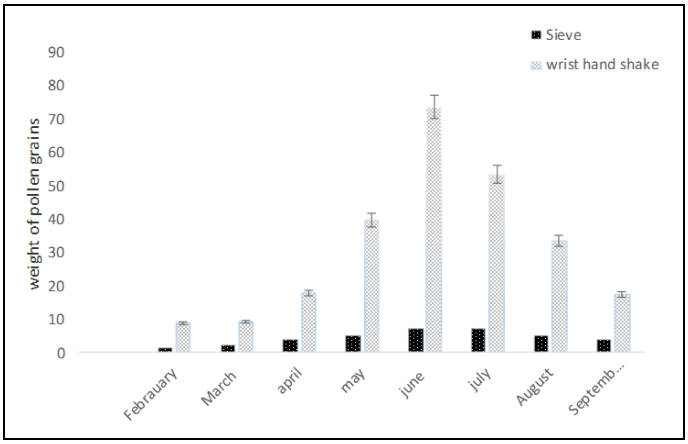

FIG. 2: PERCENTAGE OF POLLEN GRAINS OBTAINED BY TWO DIFFERENT METHODS

Pollen collection was carried out over a period of 8 months (from February 2018 to September 2018) by two different methods as shown in **Fig. 2**. Results indicate that the maximum amount of pollen grains obtained using sieve method was found to be 2.36 ± 0.018g/100 g of flower and in wrist action shake method it is about 17.89 ± 0.97 g/100 g of flower. The moisture content also differed between these two methods: a low moisture content of 0.11 ± 0.03% was observed in wrist action shake method and in sieve method the moisture content was found to be 0.29 ± 0.07% represented in **Table 2**. The maximum amount of pollen grains obtained by wrist action method was during the months of June and July and they had less moisture content as shown in **Fig 3**.

Proximate Analysis: The proximate analysis of coconut pollen grains with various parameters – total carbohydrate: 15.903 ± 0.105 mg/g, total

protein: 18.56 ± 0.115 mg/g, lipid: 11.833 ± 0.075 mg/g, moisture content: 0.970 ± 0.008% and ash: 0.896 ± 0.020%. Summarized in **Table 4** and shown in **Fig. 3** are the estimated values of the mineral components of the dried powder of coconut pollen grains – magnesium: 3.54 ± 0.034 ppm, calcium: 3.066 ± 0.049 ppm, manganese: 0.273 ± 0.020 ppm, copper: 38.58 ± 0.549 ppm, iron: 53.43 ± 0.445 ppm, zinc: 131.7 ± 0.359ppm, potassium: 4.733 ± 0.208 ppm and sodium: 4.233 ± 0.283ppm were summarized in **Table 3**.

The mean weight of the pollen grain are analysed statistically by means of Independent Sample t-test and Levene's test for Equality of Variances were observed that mean weight of pollen grain for the Seive method is 1.5975 and Wrist action shake method is 9.7262. The p value ($p = 0.002<0.05$) is lesser than 0.05, which indicates that the weight of the pollen grains is not same for sieve method and wrist action shake method in **Fig. 2**

Accordingly by the two methods, the collected pollens were stored in Eppendorf tube and wrapped with a cellophane sheath and kept at –4 ºC and it is maintained viable for up to 3 months of storage. During this storage period, the protein and other nutrient values has no changes observed and remains same when compared those with their initial values.

TABLE 2: COLLECTION OF POLLEN GRAINS OBTAINED BY TWO DIFFERENT METHODS

Months	Method -1 Sieve		Method-2 Wrist action Shake	
	(weight in gms)	Moisture content	(weight in gms)	Moisture content
February	0.95±0.018	0.26±0.04	2.92±0.060	0.19±0.15
March	0.94±0.018	0.29±0.07	3.26±0.06	0.11±0.03
April	1.83±0.018	0.08±0.04	5.386±0.052	0.06±0.01
May	1.74±0.017	0.08±0.05	13.56±0.039	0.07±0.00
June	2.36±0.018	0.14±0.04	16.21±0.02	0.02±0.01
July	2.33±0.018	0.27±0.02	15.76±0.04	0.06±0.02
August	1.93±0.018	0.54±0.00	12.86±0.01	0.04±0.00
September	1.76±0.018	0.67±0.01	7.86±0.05	0.06±0.01

Values are the Mean ± Standard deviation (n=3)

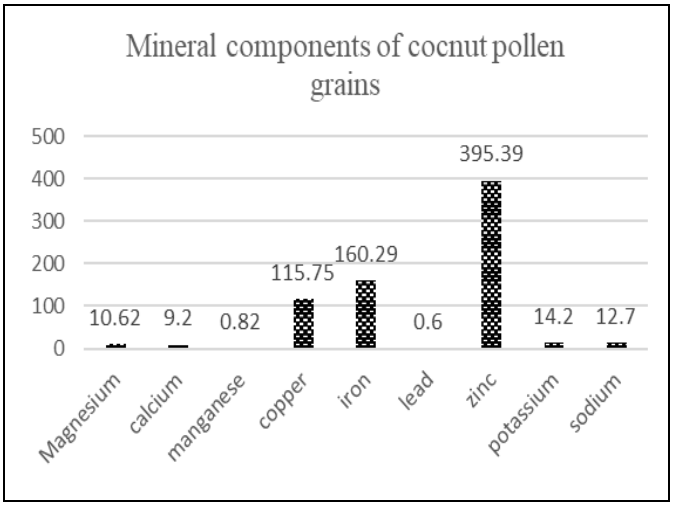

FIG. 3: MINERAL COMPONENTS OF COCONUT POLLEN GRAINS

TABLE 3: PROXIMATE COMPOSITION OF COCONUT POLLEN GRAINS

Component	Concentration%
Total carbohydrates	15.903±0.105
Total protein	18.56±0.115
lipid	11.833±0.075
Moisture content	0.970±0.008
Ash	0.896±0.020

TABLE 4: MINERAL COMPOSITION OF COCONUT POLLEN GRAINS

Minerals	Concentration ppm
Magnesium	3.54±0.034
calcium	3.066±0.049
manganese	0.273±0.020
copper	38.58±0.549
iron	53.43±0.445
lead	0.01±0.005
zinc	131.7±0.359
Potassium	4.733±0.208
Sodium	4.233±0.283

Values are the Mean ± Standard deviation (n=3)

DISCUSSION: The study suggest that step by step spikelet's splits starting from bursting of spadix to anther dehiscence, which probably could facilitate pollen bank. The result demonstrate that the evolutionary steps to increase the anther dehiscence by interfering the pollination as an important selective force for anther dehiscence. Each stage was monitored and recorded from day-1 morning to evening, for inspection on several changes from bursting of spadix to shedding the pollen. The removal of coconut pollen grains are characterized

by several phenomenon of day by day variations of anther dehiscence were noticed on 3 to 18 days to shed their entire pollen. Overall our data revealed that step by step and day to day studies may promote the release of pollen grains increased gradually and shed their entire pollen at fourteenth day. We thus provide a direct experimental evidence for anther dehiscence. Collecting healthy amount of pollen grains is of critical task for pollen bank since coconut palm is monoecious and final step of anther dehiscence may leads to self-pollination by means of stomium and elongated pollen filaments. To prevent pollination the split spikelets were kept at sterile and controlled atmospheric room temperature for drying. Meanwhile for successful anther dehiscence a series of developmental events that leads to breakage of stomium and release of pollen grains must be synchronized with improved technique by an external force using a spontaneous shaking (wrist action shaker) and centrifugal force to separate the pollen grains. About 100 grams of dried flowers contains 17.5 to 19.8 gms/dry weight of pollen grams and it depends on the variety, weather, season and climatic condition.

Based on our observations and findings of the two methods Sieve and wrist action shaker, we suggest wrist action shake method was very effective in order to get enormous amount of pollen grains without any contamination and stability also maintained during storage (–4˚C) [27]. This is because pollen grains are surrounded by a pollen kit, so it cannot be released automatically by beating. Hence it needs some extra force to shed the pollen grains by shaking continuously for 15 minutes by means of wrist action shaker machine in which the pollen grains are released from the anthers and centrifuge at 3000 rpm for 15 min in order to separate and the entire pollen grains, despite being finer, less dense and damp, it is very difficult to collect the pollen grains conventionally; so we used the sieve technique by using a sieve which was the final step to remove the anthers and stored at air tight containers. Enormous amount of copper, potassium, magnesium and zinc are found in Coconut pollen grains. But the collection of pollen grains and its processing are of critical importance for establishing the pollen bank. However the moisture content of pollen grains plays a critical role to maintain its quality as well as the viability. If it is not stored properly contamination may occur and if it is dried at above 40 ºC the thermo labile compounds will be denatured, since it rich in protein the choice of drying is only freeze to restore the phyto-compounds. Abundant of zinc and iron consumption of coconut pollen grains could aid in various biochemical processes in our body and also act as certain enzyme catalysis [29-31]. Enormous amount of copper, potassium and magnesium of coconut pollen grains has improve osmoregulation in our body and regulate homeostatic balance. Hence it supports the proper functioning of body cells, nerves, bones and muscles [32].

CONCLUSION: Our result concludes that the manual method for collecting the matured spathe and complete detachment of flowers from spikelets and better understanding the development of anther dehiscence and pollen release mechanism helps to optimize the collection and harvesting the pollen to restore the pollen grains for future research. Moreover coconut inflorescence contains immense quantity of pollen and enriched with fortified nutrients especially proteins, minearls as zinc and steroids and flavonoids as phytocompounds are abundant. After a period of an extensive experimental trials we observed that the result findings conclude a successful methods for establishing the release of pollen grains without any contaminants depends on time and climatic conditions. Thus this research work would results in successful collection of pollen grains brought out by two novel methods: sieve method and wrist action action shake method, both of which enable us to collect viable, matured coconut pollen grains but the quantity and the stability are varied. Despite being finer, less dense and damp, it is very difficult to collect the pollen grains, and that too they quickly get rehydrated upon release from anthers. For this reason Wrist action is the wright choice for collection, desiccation and efficient to obtain quality and quantity of pollen grains. Meanwhile this study also found that even though coconut inflorescence flowers throughout the year, the maximum amount of pollen grains were obtained only during the months of May, June and July, which had the most favourable climatic conditions and during this period the moisture content was also found to be less.

Our investigation of proximate analysis revealed that the coconut pollen grains are enriched with protein, lipids, fats and lipid. Mineral components like zinc are present abundantly. These findings conclude that future this would be the choice for immune booster and best neutraceuticals for women health as it contains steroid and flavonoids.

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CONFLICTS OF INTEREST: Nil

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