January 2022

THE SECOND INTERNATIONAL CONFERENCE ON GENETIC RESOURCES AND BIOTECHNOLOGY: Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture

Committees: The Second International Conference on Genetic Resources and Biotechnology

Cite as: AIP Conference Proceedings **2462**, 010002 (2022); https://doi.org/10.1063/12.0008934
Published Online: 19 January 2022

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AIP Conf. Proc. 2462, 010002-1–010002-3; https://doi.org/10.1063/12.0008934
Published by AIP Publishing. 978-0-7354-4172-9/$30.00

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Cite as: AIP Conference Proceedings **2462**, 010001 (2022); https://doi.org/10.1063/12.0006897
Published Online: 19 January 2022

Preface: The Second International Conference on Genetic Resources and Biotechnology

The Second International Conference on Genetic Resources and Biotechnology, which is the continuation of the first event held in 2018, focuses on topics related to advances in biotechnology to create more opportunities for effective conservation and sustainable utilization of genetic resources for food and agriculture. This year conference's theme is Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture. The conference was organized by Indonesian Agency for Agricultural Research and Development (IAARD), Ministry of Agriculture, Indonesia, in collaboration with Indonesian Biotechnology Consortium and held on 24th-25th of May 2021virtually due to the pandemic of COVID-19.

The conference aims to share and exchange current scientific information and technological developments on biotechnology and their applications for conservation and sustainable use of genetic, to encourage and promote quality, efficiency, and modernization of management and utilization of genetic resources, and to facilitate national and international collaboration among participants. There are five scopes discussed in this conference. They are effective management of conservation and sustainable use of genetic resources for food and agriculture, application of genomics and molecular markers for genetic resource conservation and crop adaptation to climate change, application of innovative crop improvement techniques for conservation and sustainable use of plant genetic resources for food and agriculture, plant cell and tissue culture for conservation and effective utilization of genetic resources, and the use of microbial genetic resources as biological control agents of agricultural pests and diseases, and for soil bioremediation.

Five speakers from the United States of America, Japan, India and Indonesia were invited to discuss about their expertise and knowledge on relevant subjects in the plenary sessions. This conference was attended by more than 100 participants including 75 presenters and 44 listeners worldwide. They came from diverse governmental, private, or academic institutions and also scientific communities. The presented materials have undergone peer review processes and only qualified papers were selected. Furthermore, all papers were subjected to double blind peer-review and expected to meet the scientific criteria of significance and academic excellence to be published in a conference proceedings indexed in a well-known, reputable service.

We would like to express our sincere gratitude to our speakers, presenters and all participants for their contributions in this conference. We would also like to express our appreciation for the generosity of our sponsors that support this conference: PT CropLife, PT ITS Science Indonesia, PT Fajar Mas Murni and PT Prima Instrument Analitika. Lastly, special thanks to all committee members for their exceptional work and contributions in the conference and publication.

Chair of Organizing Committee

Dr. Toto Hadiarto

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AIP Conf. Proc. 2462, 010001-1–010001-1; https://doi.org/10.1063/12.0006897
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Location: Bogor, Indonesia
ISBN: 978-0-7354-4172-9
Editors:
I Made Tasma, Dwinita Winkan Utami, Ika Roostika, Yadi Suryadi, Chaerani, Eny Ida Riyanti, Puji Lestari, Toto Hadiarto, Reflinur, Joko Prasetyono, Fatimah, Surya Diantina, Tru Puji Priyanto, Kusumawaty Kusumanegara, Wening Enggarini, Rerenstradika Tizar Terryana and Dani Satyawan
Volume number: 2462
Published: Jan 19, 2022
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Location: Bogor, Indonesia
ISBN: 978-0-7354-4172-9
Editors:

I Made Tasma, Dwinita Winkan Utami, Ika Roostika, Yadi Suryadi, Chaerani, Eny Ida Riyanti, Puji Lestari, Toto Hadiarto, Reflinur, Joko Prasetyono, Fatimah, Surya Diantina, Tru Puji Priyanto, Kusumawaty Kusumanegara, Wening Enggarini, Rerenstradika Tizar Terryana and Dani Satyawan
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Identifying potential seedless citrus accessions through floral structure and pollen performance

Cite as: AIP Conference Proceedings **2462**, 020021 (2022); https://doi.org/10.1063/5.0076922
Published Online: 19 January 2022

Baiq Dina Mariana, Anis Andrini and Sri Andayani

Identifying Potential Seedless Citrus Accessions through Floral Structure and Pollen Performance

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Abstract. Seedless character is one of important traits for fresh fruit citrus. Since there are many mechanisms to obtain seedless fruit, it is important to identify which mechanism is possibly used in it. The first to be observed is usually the pollen characteristic. Abnormality in pollen could lead to seedless fruit production. In this study, we observed several potential seedless citrus accessions in the germplasm collection to obtain the information on the floral structure and pollen characteristics supporting the production of seedless fruits. The study was conducted at Plant Breeding Laboratory of Indonesian Citrus and Subtropical Fruits Research Institute, Batu, East Java in 2019. The flower and pollen were observed on their structure, morphology, viability, and germination percentage. The result showed that at anthesis, the anther position could be located below, above and parallel with the stigma. Flowers with anthers located below the stigma have lower chance to be self-pollinated and in the absence of pollinators it increases the opportunity to produce seedless fruit. Pollen morphology observations indicated that most accessions had elliptical shape with either acute, circular or rectangular apices. Pollen viability ranged from 64–95% with pummelo accession Bageng showed the highest percentage, and lime accession Nipis Borneo was the lowest. In addition, Nipis Borneo also has the lowest germination percentage, around 17.69%, while the highest one was observed on lemon Montaji Agrihorti which was 57.52%. This study showed that Nipis Borneo has the highest potential as seedless citrus just based on at its pollen performance, while on the other hand, Montaji Agrihorti has the highest possibility of producing seedy fruit. For more comprehensive information, further study needs to be conducted especially on the interaction of pollen and pistil.

INTRODUCTION

The significance of citrus for agriculture in the world is shown by its worldwide distribution and its massive-scale production. Citrus is commercially grown in more than 80 countries around the world in tropical and subtropical climates. Citrus production worldwide (including oranges, grapefruit, tangerines, mandarins, lemons, and limes) has increased from 115 million tons in 2008 to 130 million tons in 2015 [1].

Seedlessness is one of the fruit properties that is appreciated in both fresh fruit and processed products [2] due to more convenient for consumption and less process for getting rid of the seeds. A plant is considered seedless when its fruits have no seed, contains aborted seeds or less number of seeds [3, 4].

Despite the economic importance of citrus, limited research has been done to characterize the reproductive biology of the genus and its related genera. It could partly contribute by the lack of an established borderline between species, subspecies, and interspecific hybrids, and also the paucity of knowledge of their self-incompatibility reaction and parthenocarpic behavior. A study by Distefano *et al*. [5] showed that parthenocarpy in mandarin citrus is related to an uncoupling of the onset of fruiting during the reproductive process. Other than that, there are many possible factors inducing seedless fruits, namely female or male sterility, defective ovules and embryo sac abortion, self-incompatibility, polyploidy, certain environmental condition, and application of gibberellins [6–8].

One of the ways to observe the seedlessness mechanism is to study the pollen characteristics. Characterizing the pollen performance, especially for some economically important genus like *Citrus*, in which the failure of the sexual

The Second International Conference on Genetic Resources and Biotechnology
AIP Conf. Proc. 2462, 020021-1–020021-7; https://doi.org/10.1063/5.0076922
Published by AIP Publishing. 978-0-7354-4172-9/$30.00

reproductive process resulting in parthenocarpic fruit development. Seedlessness is a prized character particularly for fresh consumption citrus [9] and has been one of the main requirements in the market [10].

Initial observations in the citrus collection of Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI) showed the potential of several accessions having seedless character. However, none of them has been characterized in terms of their reproductive biology related to seedless fruit character. Several studies emphasized the role of pollen in producing seedless fruit. Information about the ability of pollen grains to germinate when they reach the stigmas of flowers of their own species is valuable both for horticultural purposes and general botanical research. Viability tests provide means of assessing the potential of pollen to germinate on the stigma [11]. By having low viability of pollen, the chance of having seedless citrus fruit increases, as reported in pummelo [12], mandarin [13], and lemon [14]. This study was aimed to obtain information of floral structure and pollen characteristic (viability and fertility) of several citrus species showing the potential seedless character.

MATERIALS AND METHODS

The research was conducted at Plant Breeding Laboratory of ICSFRI, Batu, East Java in 2019. Initial observations were made to determine accessions that showed the potential of having seedless fruit (observed in 10–20 fruits). Accessions were selected based on the number of seed, less than five seeds per fruit. Plant materials used were flowers derived from 6 citrus accessions with potential seedless character and 1 accession served as a reference for seedy accession. All accessions were taken from Punten Experimental Station of ICSFRI. The list of accession used were given in Table 1.

TABLE 1. Citrus accessions observed in this study.

Accession	Latin name	Average number of seed per fruit
Jemari Taji	*Citrus sinensis*	<5
Montaji Agrihorti	*C. limon*	<5
Nipis Borneo	*C. aurantifolia*	<5
Pamelo Bageng	*C. maxima*	<5
Tongheng	*C. japonica*	<5
Topazindo Agrihorti	*C. reticulata* × *C. sinensis*	<5
Batu 55*	*C. reticulata*	>10

*Reference for seedy accession.

Flower anatomy (pollen and pistil position) was observed using Nikon ShuttlePix, while pollen characteristics were observed using hanging drop technique under microscope Olympus BX51. Overall, there were two activities done as follows.

Flower and Pollen Morphology Observation

For flower anatomy, the focus was on the position of pollen and pistil (stigma) at anthesis. To determine the anthesis phase, balloon stage flowers and the ones that had slight opened petals were collected in the morning. Ten flowers were observed and then documented under microscope. For pollen morphology, the size (length and width) at equatorial view were measured from 100 pollens. In addition, the shape was also observed to see whether there is any abnormality that could hamper pollen development.

Identification of Pollen Viability and *In Vitro* Germination

The viability of the pollen was identified in the following step: ten flowers harvested from plants and then placed in room temperature. When stamen broke, the pollen grains were collected in a vial and given 2–3 drops of 1% acetocarmine solution and let immersed for 3 h. The pollens were spread on slide glass and observed under microscope with 100–200× magnification. Light red to red pollens were counted as viable while crooked, half stained pollens were counted as nonviable [15]. Percentage of viable pollen was calculated per 100 pollens and repeated three times.

For *in vitro* germination, pollens were collected and prepared similar to the one in pollen viability observation. After that, they were dispersed in a 2 ml tube containing liquid medium which consisted of 10% sucrose, 100 ppm H_3BO_3, 300 ppm $Ca(NO_3)2.4H_2O$, 100 ppm KNO_3, and 200 ppm $MgSO_4.7H_2O$, the final solution was measured to pH 5.5 [16]. The pollens were incubated for 24 h in room temperature and then stored in refrigerator. Pollens were considered germinated when the length of pollen tube reached its diameter [17]. The percentage of germinated pollen was counted in the same manner as pollen viability. Both experiments were arranged in a completely randomized design (CRD) and data obtained was analyzed using GenStat software.

RESULTS AND DISCUSSIONS

Flower and Pollen Morphology

Citrus accessions observed showed different position of anthers relatively to stigma at anthesis. There are four positions of anthers observed, which were lower, middle, parallel, and higher than stigma (Fig. 1). Lower anthers were observed on Jemari Taji and Nipis Borneo. Meanwhile, anthers reaching the middle of stigma were observed on Montaji Agrihorti and Pamelo Bageng. On the other hand, Topazindo Agrihorti showed parallel position of stigma and anthers of which the anthers can reach the upper part of the stigma while Tongheng had the highest position of anthers, towering the stigma below them. To be able to access the ovule, pollen must reach the upper part of the stigma. If the pollen is compatible, the pollen will germinate and form pollen tube [18]. After germination on the surface, pollen tube will penetrate its style toward the ovule. Four of these accessions, Jemari Taji, Montaji Agrihorti, Nipis Borneo, and Pamelo Bageng, have anthers position that could not reach the upper part of the stigma. Therefore, in the absence of pollinator, wind or human intervention, it is difficult for pollination to take place. Plants with this flower structure have higher chance to have seedless fruit.

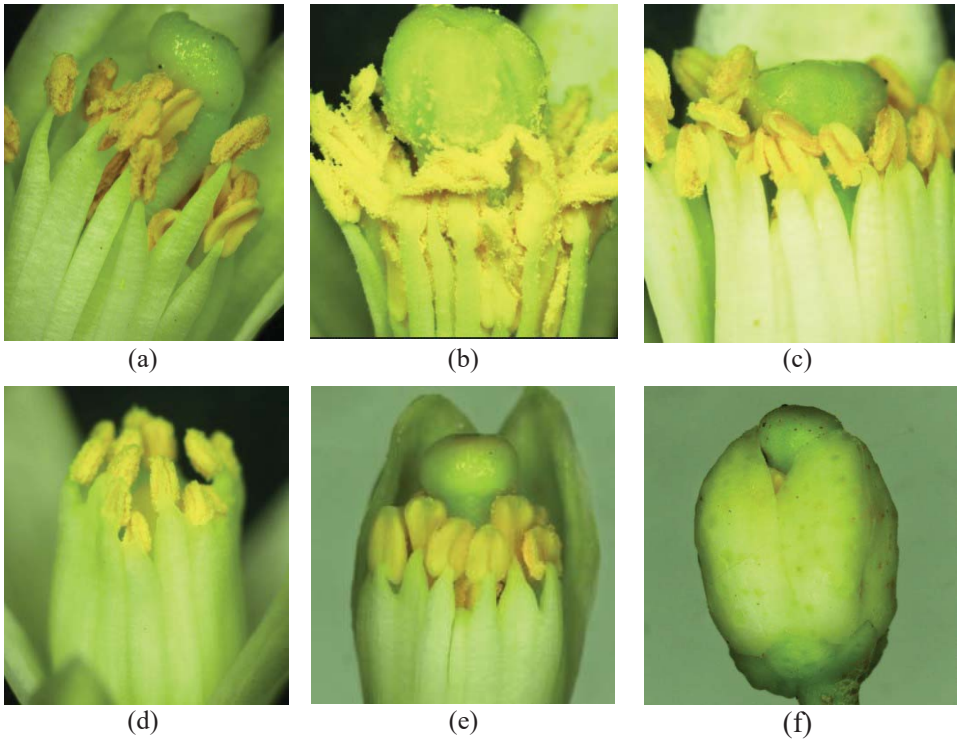
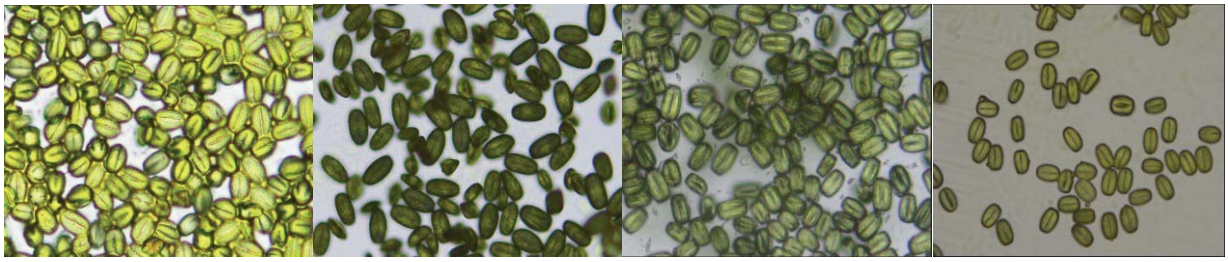


FIGURE 1. The position of anthers compared to stigma at anthesis observed on potential seedless citrus accessions: lower than stigma (a), reaching the base (b), parallel with the stigma (c), and higher than stigma (d); flower structure of seedy reference accession (e, f).

The reference seedy accession (Batu 55) had lower anthers similar to Jemari Taji and Borneo. However, this accession showed different character of which the stigma already emerged, even though the flower has not opened yet (Fig. 1f). The character was not found in any other accessions. This typical development favored for cross pollination since the stigma is already accessible for pollination before flower opens. If there are overlapping bloom and pollinators, pollination will occur [19]. In the absence of pollinators, there should be similar result among the accessions. Nevertheless, they have different number of seeds observed, so there should be other reasons which needed further investigations.

To be able to access the ovule, pollen must reach the upper part of the stigma. If the pollen is compatible, the pollen will germinate and form pollen tube [18]. After germination on the surface, pollen tube will penetrate the style toward the ovule. Four of these accessions, namely Jemari Taji, Montaji Agrihorti, Nipis Borneo, and Pamelo Bageng, have anthers that could not reach the upper part of the stigma. Therefore, in the absence of pollinator, wind or human intervention, it is difficult for pollination to take place. Plants with this flower structure has higher chance to have seedless fruit. This particular structure also observed on the mutant of mandarin SoE along with less fertile pollen which then resulted in seedless fruits [20].



(a) (b) (c) (d)

FIGURE 2. Morphology of seedless citrus accessions showing elliptical shape at equatorial view, with round apices (a), acute apices (b), and rectangular apices (c); pollen morphology of seedy reference accession with elliptical shape and rectangular apices (d).

Fig. 2 showed the pollen morphology of the accessions observed. Majority of the accessions have elliptical shape at equatorial view with slightly round apices (Jemari Taji, Montaji Agrihorti, Tongheng, and Topazindo Agrihorti), acute apices (Nipis Borneo), and rectangular apices (Pamelo Bageng and Batu 55). The result is similar to the finding of Inyama *et al*. [21] where they compared the pollen morphology of several citrus species, including five species that were also tested in this study (excluding *C. japonica*). This result revealed that pollen shape is related to the diversity of pollen morphology in citrus family. The character does not seem to have direct influence on seedless trait since seedless accession and seedy accession could have similar shape and also size. However, pollens which showed irregular size and form in the colony may have influences on the pollen performance, such as observed in Nipis Borneo and Topazindo Agrihorti. Both accessions have similar polymorphic pollen (Fig. 2b). The small ones showed irregular or crook shape. This finding was confirmed on the observation of pollen viability showing some pollens of these accessions had more irregular shape pollen than other accessions. Perhaps to some degree, their pollens undergo abnormal development resulted in crooked and defective pollens which affected their performance.

TABLE 2. Pollen measurement of citrus accessions at equatorial view.

Accession	Equatorial outline (µm)	
	Length	Width
Jemari Taji	32.34±3.01	19.21±0.52
Montaji Agrihorti	39.74±0.86	22.63±0.63
Nipis Borneo	33.51±0.44	18.36±0.29
Pamelo Bageng	28.66±0.62	17.24±0.38
Tongheng	24.95±0.68	18.40±1.32
Topazindo Agrihorti	28.66±1.23	21.00±1.14
Batu 55*	27.15±0.22	20.23±0.31

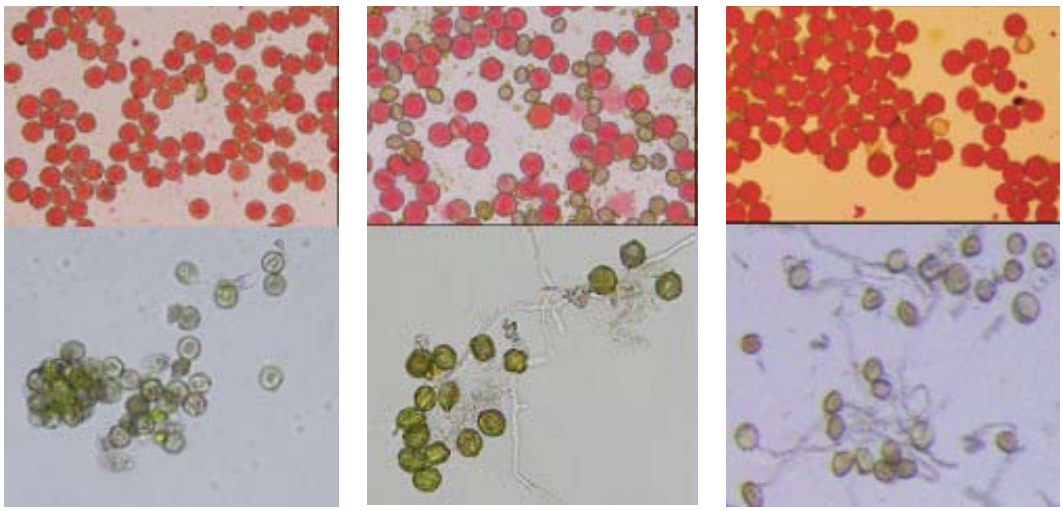
*Reference for seedy accession.

The result of observation on the pollen size also showed there is no clear relation between pollen size and seedless character (Table 2). Overall, Montaji Agrihorti has the biggest size of pollen among accessions observed. On the other hand, Tongheng has the smallest pollen. From this study, it is not clear whether size of pollen has influence in the viability and germination rate. Although Montaji Agrihorti also had the highest germination percentage, the second biggest one, Nipis Borneo, however, had the lowest percentage for both viability and germination percentage (presented in Table 3).

Pollen Viability and *In Vitro* Germination

Fig. 3 showed the variability of pollen viability and *in vitro* germination of the accessions. Both pollen viability and *in vitro* germination provide means to see the potential of pollen germinating on stigma [11]. At least there were three results observed when compare the viability to the *in vitro* germination. First, there were seedless accessions with high pollen viability but low germination percentage, such as Bageng and Tongheng. Second, low pollen viability and also low germination as observed in Nipis Borneo. Third, high pollen viability and also high germination percentage as observed in Montaji Agrihorti and also the reference Batu 55. Nipis Borneo had the most pollen that were not stained. This probably the irregular, crooked and smaller pollen that were seen previously in morphology observation. In addition, the result of this observation on Batu 55 is just as expected. With both high number of pollen viability and germination, consequently, the pollen is most fertile and it is easy to grow the pollen tube which later could fertilize the eggs resulted in seed production.

The result of observation on pollen viability and *in vitro* germination was given in Table 3. The result indicated that both viability and germination percentage varied among accessions. Nipis Borneo showed low percentage for both viability and germination percentage, 64.40 and 17.69, respectively. On the other hand, the accessions with high percentage of viability do not necessarily have high germination percentage, as seen in Pamelo Bageng. Compare with viability percentage, the ratio of germinated pollen was lowest in Jemari Taji (22%) and highest in Montaji Agrihorti (66%). This finding of Montaji Agrihorti (*C. limon*) is in line with the result of a study by Khan and Perveen [22] in which they compared *in vitro* pollen germination of five species of citrus, including *C. limon*, *C. sinensis* and *C. reticulata*. In the study, they found that *C. limon* had highest pollen germination among the species observed, reaching 67% for fresh pollen germination and 70% after four weeks storage.



(a) (b) (c)

FIGURE 3. Pollen viability and *in vitro* germination of seedless citrus accessions (a, b) and the reference seedy accession (c).

TABLE 3. Pollen viability and germination percentage of citrus accessions.

Accession	Viability (%)	Germination (%)
Jemari Taji	84.87 c	18.54 ab
Montaji Agrihorti	87.35 cd	57.52 d
Nipis Borneo	64.40 a	17.69 a
Bageng	95.11 e	24.25 b
Tongheng	92.20 de	34.36 c
Topazindo Agrihorti	74.89 b	34.78 c
Batu 55*	97.86 e	55.60 d

Means within a column followed by the same letters were not significantly different by DMRT at α 0.05. *Reference for seedy accession.

There are several factors that could affect pollen performance, as in viability, germination, and tube growth [8, 23]. These include genotype, temperature, flower type, media, and also duration of incubation. The different of tube growth could be seen in Fig. 3. Montaji Agrihorti and Tongheng had similar pollen tube growth. The tubes were long and dense with pollen cytoplasm and nucleus. On the other hand, pollen tube of Nipis Borneo was lean and devoid of any substance, albeit its long growth. In addition, only few pollens of this accession were seen germinated. Pollen tube development was also seemed to be arrested in Pamelo Bageng. It was similar to Nipis Borneo which only several colonies of pollen could be seen germinating.

The media used for this experiment was based on study by Aprilia *et al*. [24]. The media was tested in a preliminary study using several accessions. However, it seemed that the media should be adjusted according to the requirement of each genotype (accession) since the ones that have high viability logically should have high germination in a suitable media. Others studies also mentioned the important of temperature for the pollen to have optimal germination [25, 26] and also storage [27]. More experiment is needed for observation on pollen and pistil interaction such as using artificial pollination [28] and fluorescence to provide more information on the seedless mechanism of the accessions [29]. Pollen and pistil interaction is very important to be observed since there are many factors contribute to the seedlessness that related to this interaction [30].

CONCLUSION

Citrus accessions have different floral structures especially at anthesis which could affect the pollination. There were three positions of anthers in relation to stigma observed, namely above, below, and parallel with the stigma. In the absence of pollinators, accession with lower anthers will have higher chance to produce seedless fruit. Citrus accessions tested had elliptical shape at equatorial view with acute, circular, and rectangular apices. The morphology seems to be related more to the character of the species rather than to seedless character. Nipis Borneo had the lowest viability and germination rate, 64.40 and 17.69, respectively, making it to be the most potential seedless accession among the accessions observed.

ACKNOWLEDGMENTS

This research was funded by Indonesian Agency for Agricultural Research and Development. We thanked Farida Yulianti and Susi Purwiyanti for their help in data analysis.

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