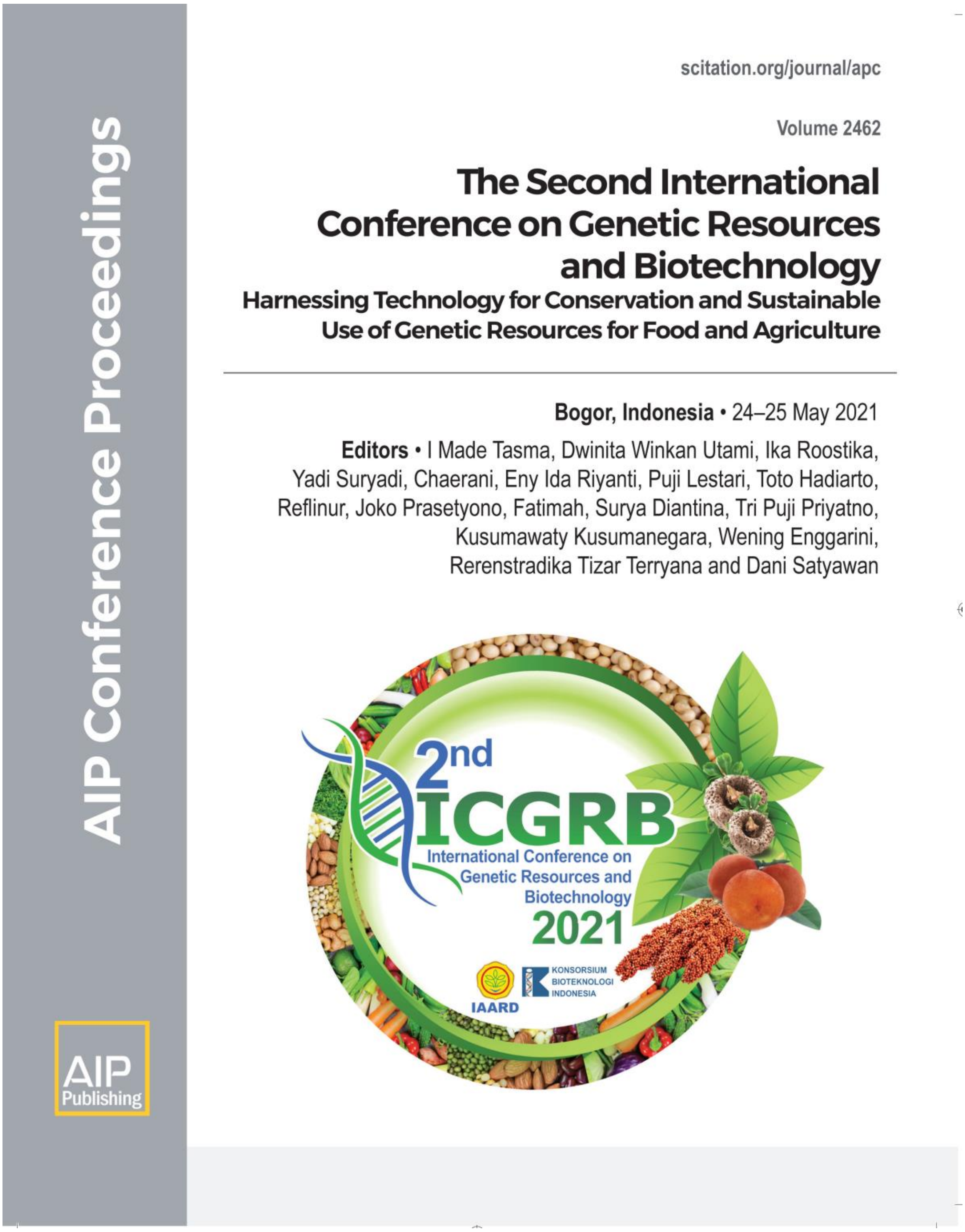


January 2022

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

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# 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 24$^{th}$-25$^{th}$ 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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# Table of Contents

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

PREV

Conference date: 24–25 May 2021
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- all

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## Harnessing plant genetic resources through biotechnology for food security in Indonesia

Mastur, Reflinur, Nurul Hidayatun, Sustiprijatno, Fatimah, Tri Puji Priyatno and Puji Lestari
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## DNA barcoding of *Vatica bantamensis*, a critically endangered tree endemic to Banten, Indonesia

Muhammad Rifqi Hariri, Iyan Robiansyah, Dipta Sumeru Rinandio, Dodo, Desi Siti Sundari, Cecep H. Sukmawan and Bayuntoro Ardi
AIP Conference Proceedings **2462**, 020002 (2022); https://doi.org/10.1063/5.0075529

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## Genetic parameters of agronomic traits in soybean (*Glycine max* [L.] Merrill) genotypes tolerant to drought

Made J. Mejaya, Suhartina, Purwantoro, Novita Nugrahaeni and Titik Sundari
AIP Conference Proceedings **2462**, 020003 (2022); https://doi.org/10.1063/5.0075159

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-
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## Yield stability performance of soybean (*Glycine max* [L.] Merrill) lines tolerant to drought

Suhartina, Purwantoro, Novita Nugrahaeni, Abdullah Taufiq and Made Jana Mejaya
AIP Conference Proceedings **2462**, 020004 (2022); https://doi.org/10.1063/5.0075158

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## Polymorphisms and associations of the *RACK1* genes with antibody response to Newcastle disease in KUB chickens

Ifa Manzila, Puji Lestari, Tike Sartika, Tri Puji Priyatno, Risa Indriani, Kristianto Nugroho and Rerenstradika Tizar Terryana
AIP Conference Proceedings **2462**, 020005 (2022); https://doi.org/10.1063/5.0075622

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## Rice grain quality evaluation of promising lines of rice under irrigation and for salinity tolerance

Dody D. Handoko, Nafisah, Aris Hairmansis, Trias Sitaresmi, Heni Safitri, Satoto, Ali Imamuddin, Cucu Gunarsih and Untung Susanto
AIP Conference Proceedings **2462**, 020006 (2022); https://doi.org/10.1063/5.0075956

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  - PDF
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## Existing diversity profile for kernel characteristics of maize germplasm in IAARD-ICABIOGRAD gene bank

Andari Risliawati, Sobir, Trikoesoemaningtyas, Willy B. Suwarno and Puji Lestari
AIP Conference Proceedings **2462**, 020007 (2022); https://doi.org/10.1063/5.0075178

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## Characterization of Japansche citroen rootstock somaclones and *in vitro* selection for aluminium tolerance

Deden Sukmadjaja, Mia Kosmiatin and Tiwi Wati
AIP Conference Proceedings **2462**, 020008 (2022); https://doi.org/10.1063/5.0077888

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  - PDF
  - E-READER
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## Resistance to brown planthoppers (*Nilaparvata lugens* Stål) in rice accessions originated from Sumatra Island, Indonesia

Dodin Koswanudin, Nurul Hidayatun and Muhamad Ace Suhendar
AIP Conference Proceedings **2462**, 020009 (2022); https://doi.org/10.1063/5.0075680

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## Morphological identification of underutilized local fruits in Kutai Barat Regency to support their conservation and sustainable use

Fitri Handayani, Nurbani and Asep Pebriandi
AIP Conference Proceedings **2462**, 020010 (2022); https://doi.org/10.1063/5.0075594

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## Genetic resources of adlay (*Coix lacryma-jobi* L.) in East Kalimantan as source of functional food

Fitri Handayani, Muhammad Amin and Muhammad Taufiq Ratule
AIP Conference Proceedings **2462**, 020011 (2022); https://doi.org/10.1063/5.0075593

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## Screening of soybean genotypes resistance to rust disease (*Phakopsora pachyrhizi*)

Sumartini and Kurnia Paramita Sari
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## Identification of soybean promising lines resistant to pod-sucking bug, *Riptortus linearis* (Fabricius)

M. Muchlish Adie, Titik Sundari, Kurnia Paramita Sari and Ayda Krisnawati
AIP Conference Proceedings **2462**, 020013 (2022); https://doi.org/10.1063/5.0075343

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## Variation in pod shattering resistance among black soybean genotypes associated with agronomic traits

Ayda Krisnawati, Titik Sundari and M. Muchlish Adie
AIP Conference Proceedings **2462**, 020014 (2022); https://doi.org/10.1063/5.0075338

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## Preliminary characterization and identification of genetic integrity of velvet bean germplasm in IAARD-ICABIOGRAD gene bank

Nurwita Dewi, Andari Risliawati and Nurul Hidayatun
AIP Conference Proceedings **2462**, 020015 (2022); https://doi.org/10.1063/5.0076355

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## Plant parasitic nematodes infesting three minor legumes (velvet bean, lablab bean, and jack bean)

Chaerani, Try Zulchi P. Hariyadi and Nurwita Dewi
AIP Conference Proceedings **2462**, 020016 (2022); https://doi.org/10.1063/5.0075204

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## Proactive management approach of seed PGRFA conservation during the pandemic of coronavirus disease (COVID-19) in Indonesia

Nurul Hidayatun, Andari Risliawati, Nurwita Dewi, Lina Herlina and Dodin Koswanudin
AIP Conference Proceedings **2462**, 020017 (2022); https://doi.org/10.1063/5.0075531

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## Evaluation of mung bean accessions in saline soil based on quantitative morphological characters

Trustinah, Ratri Tri Hapsari, Rudi Iswanto and Rudy Soehendi
AIP Conference Proceedings **2462**, 020018 (2022); https://doi.org/10.1063/5.0075324

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## Screening and evaluation of 100 upland rice accessions for developing high-yielding upland rice varieties tolerant against acid soil

Lina Herlina and Yusi N. Andarini
AIP Conference Proceedings **2462**, 020019 (2022); https://doi.org/10.1063/5.0075550

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## Morphological characters of sugarcane mutant (*Saccharum officinarum* L.) from *in vitro* selection for drought stress

Rr. Sri Hartati, Sri Suhesti and Nurya Yuniyati
AIP Conference Proceedings **2462**, 020020 (2022); https://doi.org/10.1063/5.0075656

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## Identifying potential seedless citrus accessions through floral structure and pollen performance

Baiq Dina Mariana, Anis Andrini and Sri Andayani
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## Secondary characters based selection of Indonesian kenaf (*Hibiscus cannabinus* L.) germplasm for developing superior varieties

Taufiq Hidayat R. S., Marjani, Nurindah, Muhammad Rasyidur Ridho, Cynthia Lestari Hertianti and Widya Fatriasari
AIP Conference Proceedings **2462**, 020022 (2022); https://doi.org/10.1063/5.0075716

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## Genetic relationship of pigmented rice (*Oryza sativa* L.) collected from Eastern Indonesia based on morpho-agronomical traits and SSR markers

Yusi Nurmalita Andarini, Willy Bayuardi Suwarno, Hajrial Aswidinnoor and Hakim Kurniawan
AIP Conference Proceedings **2462**, 020023 (2022); https://doi.org/10.1063/5.0075706

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## Rejuvenation and morphological characterization of local rice from the province of Yogyakarta

Setyorini Widyayanti, Sutarno, Endang Wisnu Wiranti and Kristamtini
AIP Conference Proceedings **2462**, 020024 (2022); https://doi.org/10.1063/5.0075721

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## Characterization of plant architecture and yield trait of castor (*Ricinus communis* L.) germplasm suitable for mechanical harvesting

Tantri Dyah Ayu Anggraeni and Rully Dyah Purwati
AIP Conference Proceedings **2462**, 020025 (2022); https://doi.org/10.1063/5.0075155

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## Characterization and interrelationships of the number of vessel bundles with yield components in various genotypes of soybean (*Glycine max* [L.] Merrill)

Anna S. Karyawati and Dyah P. Fitrawantio
AIP Conference Proceedings **2462**, 020026 (2022); https://doi.org/10.1063/5.0075693

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-
  - PDF
  - E-READER
  - ADD TO FAVORITES
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## Tuber starch content of edible canna (*Canna indica* L.) from different geographical origins

Surya Diantina, Randy Sanjaya, Kristina Dwi Atmini, Ace Suhendar and Dodin Koswanudin
AIP Conference Proceedings **2462**, 020027 (2022); https://doi.org/10.1063/5.0075922

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## The diversity of morpho-agronomic characters and identification of early maturity cassava (*Manihot esculenta* Crantz.) germplasm

Tinuk Sri Wahyuni, Kartika Noerwijati and Made J. Mejaya
AIP Conference Proceedings **2462**, 020028 (2022); https://doi.org/10.1063/5.0075658

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- 
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  - E-READER
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## Radiosensitivity and phenotypic characterization of gamma ray-induced mutant population of four *Capsicum annuum* L. cultivars grown in screen house

Andri Fadillah Martin, Dyah Retno Wulandari, Tri Muji Ermayanti, Betalini Widhi Hapsari, Erwin Al Hafiizh and Laela Sari
AIP Conference Proceedings **2462**, 020029 (2022); https://doi.org/10.1063/5.0075173

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## Morphological performances of mutant butterfly pea (*Clitoria ternatea* L.)

Try Zulchi, Ali Husni, Dwinita Wikan Utami, Reflinur, Mia Kosmiatin, Tarkus Suganda and Agung Karuniawan
AIP Conference Proceedings **2462**, 020030 (2022); https://doi.org/10.1063/5.0075592

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## Screening of beta carotene and its correlation with tuber flesh color in sweet potato

Kristina Dwi Atmini, Surya Diantina, Muhamad Sabda and Dodin Koswanudin
AIP Conference Proceedings **2462**, 020031 (2022); https://doi.org/10.1063/5.0075618

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## Evaluation of morpho-agronomical characters and grain quality of red rice lines

Heni Safitri and Puji Lestari
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## Growth variation and relationship of clove progenies of high-yielding mother trees collected from various regions in Indonesia

Mariana Susilowati, Sri Wahyuni, Adi Setiadi and Nurliani Bermawie
AIP Conference Proceedings **2462**, 020033 (2022); https://doi.org/10.1063/5.0075824

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-
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## Screening on bast fiber plants resistant to spiral stem borer, *Agrilus acutus* (Coleoptera: Buprestidae)

Sujak, Nurindah, Dwi Adi Sunarto, Marjani and Nurul Hidayah
AIP Conference Proceedings **2462**, 020034 (2022); https://doi.org/10.1063/5.0075691

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## Characteristic of indigenous *Leuconostoc mesenteroides* EN 17-11 protease and its stability during storage at cold and freezing temperatures

Tatik Khusniati, Ika, Harry Noviardi and Sulistiani
AIP Conference Proceedings **2462**, 020035 (2022); https://doi.org/10.1063/5.0076004

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  - EXPORT CITATION

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## Performance of introduced lines based on morphological markers for diversity enrichment of Indonesian chili pepper (*Capsicum annuum* L.) varieties

Rinda Kirana, Catur Hermanto, Reflinur and Derek W. Barchenger
AIP Conference Proceedings **2462**, 020036 (2022); https://doi.org/10.1063/5.0075186

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-
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## The growth of garlic internal sprout on different storage condition

Chotimatul Azmi, Imas Rita Saadah, Nazly Aswani and Asih Kartasih Karjadi
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## Genetic diversity analysis of *Castanopsis argentea* using random amplified polymorphic DNA markers

Muhammad Imam Surya, Lily Ismaini, Decky Indrawan Junaedi, Aisyah Handayani, Taufikurrahman Nasution, Muhammad Efendi, Andes Hamuraby Rozak, Zaenal Mutaqien, Musyarofah Zuhri, Imawan Wahyu Hidayat, Fitri Kurniawati, Vandra Kurniawan, Dwinda Mariska Putri and Risha Amilia Pratiwi
AIP Conference Proceedings **2462**, 020038 (2022); https://doi.org/10.1063/5.0077390

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## Current status of tidal swamp rice varieties and its improvement for Fe toxicity tolerance and biofortification

Muhamad Sabran, Dwinita Wikan Utami and Susilawati
AIP Conference Proceedings **2462**, 030001 (2022); https://doi.org/10.1063/5.0075202

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## Agroforensic, a new emerging study using molecular marker technique

Edy Listanto, Ahmad Warsun, Ahmad Dadang, Eny Ida Riyanti, Saptowo Jumali Pardal, Sustiprijatno and Mastur
AIP Conference Proceedings **2462**, 030002 (2022); https://doi.org/10.1063/5.0075164

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## Molecular diversity comparison in local rice accessions originated from Kalimantan and other islands of Indonesia

Puji Lestari, Rerenstradika Tizar Terryana, Kristianto Nugroho, Andari Risliawati, Nurul Hidayatun, Priatna Sasmita, Yudhi Sastro, I. Gusti Komang Dana Arsana and Ikhwani
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Joko Prasetiyono, Tio Fadel Rafsanjani, Tri Aminingsih, Tasliah and Sugiono Moeljopawiro
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  - SHARE
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## The genome sequence of Ciherang, an Indonesian rice mega variety, revealed the footprints of modern rice breeding

Ida Rosdianti, Dani Satyawan, Muhamad Yunus and Dwinita Wikan Utami
AIP Conference Proceedings **2462**, 030005 (2022); https://doi.org/10.1063/5.0075676

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- 
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
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## Field adaptation and molecular characterization of Code-*qTSN4* and Code-*qDTH8* rice lines at two different locations

Tasliah, Kurniawan Rudi Trijatmiko and Joko Prasetiyono
AIP Conference Proceedings **2462**, 030006 (2022); https://doi.org/10.1063/5.0075661

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- 
  - PDF
  - E-READER
  - ADD TO FAVORITES
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## Hybrid purity assessment in $F_1$ hybrids segregating for phytophthora root rot resistance genes of chili pepper (*Capsicum annuum* L.)

Fatimah, Reflinur, Joko Prasetiyono, Wartono, Kristianto Nugroho, Rinda Kirana, Dani Satyawan, Rerenstradika Tizar Terryana, Aqwin Polosoro, Puji Lestari and I. Made Tasma
AIP Conference Proceedings **2462**, 030007 (2022); https://doi.org/10.1063/5.0075160

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-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

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## Characterization of genomic variation on three Indonesian oil palm genotypes analyzed using next-generation sequencing HiSeq

I. Made Tasma, Habib Rijzaani, Dani Satyawan, Ida Rosdianti, Edy Supriyanto and Razak Purba
AIP Conference Proceedings **2462**, 030008 (2022); https://doi.org/10.1063/5.0075392

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-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

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## Cytological and molecular identifications of seedless tangerine derived from endosperm culture

Chaireni Martasari, Mia Kosmiatin, Ali Husni, Kurniawan Budiarto and Innez Candri Gilang Purnama
AIP Conference Proceedings **2462**, 030009 (2022); https://doi.org/10.1063/5.0076395

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- 
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

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## Improvement of sex determination of salak plant using sequence characterized amplified regions

Reflinur, Ma’sumah, Namira Nur Arfa, Budi Setiadi Daryono and Azis Natawijaya
AIP Conference Proceedings **2462**, 030010 (2022); https://doi.org/10.1063/5.0075698

- SHOW ABSTRACT
- 
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

# Table of Contents

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

_PREV

Conference date: 24–25 May 2021
Location: Bogor, Indonesia
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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
DISPLAY :

- 20
- 50
- 100
- all

## APPLICATION OF INNOVATIVE CROP IMPROVEMENT TECHNIQUES FOR CONSERVATION AND SUSTAINABLE USE OF PLANT GENETIC RESOURCES FOR FOOD AND AGRICULTURE

FreeJanuary 2022

### Design and *in vitro* test of sgRNA for the CRISPR/Cas9 plasmid construct of the *SQS* gene of *Artemisia annua* L.

Sri Koerniati
AIP Conference Proceedings **2462**, 040001 (2022); https://doi.org/10.1063/5.0075695

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

### The efficacy of genetically modified (GM) corn Bt11 against *Ostrinia furnacalis* (Guenee) and *Helicoverpa armigera* (Hubner)

Bahagiawati and Diani Damayanti
AIP Conference Proceedings **2462**, 040002 (2022); https://doi.org/10.1063/5.0075312

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Construction and introduction of OsAER1::LeAlaAT cassette to improve the nitrogen use efficiency in rice cv. Mekongga

Atmitri Sisharmini, Aniversari Apriana, Intan Kamila, Aqwin Polosoro, Wening Enggarini, Tri Joko Santoso, Toto Hadiarto, Bahagiawati A. Husin and Kurniawan Rudi Trijatmiko
AIP Conference Proceedings **2462**, 040003 (2022); https://doi.org/10.1063/5.0075458

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Environmental safety assessment of genetically engineered potato resistant to late blight caused by *Phytophthora infestans*

Alberta Dinar Ambarwati, Eny Ida Riyanti, Edy Listanto, Tri Joko Santoso, Toto Hadiarto and Kusmana
AIP Conference Proceedings **2462**, 040004 (2022); https://doi.org/10.1063/5.0075612

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES

- SHARE
- EXPORT CITATION

FreeJanuary 2022

## Backcrossing of soybean lines containing aluminium tolerance gene into superior soybean variety, Biosoy

Saptowo J. Pardal, Amalia Prihaningsih, Suharsono, Ratna Utari and Riri Sundasari
AIP Conference Proceedings **2462**, 040005 (2022); https://doi.org/10.1063/5.0075187

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Phenotypic and genetic stability evaluation of the targeted *GA20ox-2* gene mutation in CRISPR/Cas9 mutant rice derived from Mentong cultivar

Aniversari Apriana, Tri Joko Santoso, Atmitri Sisharmini, Reflinur, A. Dinar Ambarwati, Toto Hadiarto, Sustiprijatno and Nuryati
AIP Conference Proceedings **2462**, 040006 (2022); https://doi.org/10.1063/5.0075603

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Transformation of *csp* gene into tobacco plant mediated by *Agrobacterium tumefaciens*

Sustiprijatno, Seagames Waluyo and Suharsono
AIP Conference Proceedings **2462**, 040007 (2022); https://doi.org/10.1063/5.0075571

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

## PLANT CELL AND TISSUE CULTURE FOR CONSERVATION AND EFFECTIVE UTILIZATION OF GENETIC RESOURCES

FreeJanuary 2022

### The application of gamma ray irradiation to increase triterpenoid compounds in embryogenic calli of *Centella asiatica* L. Urban

Ika Roostika, Suci Rahayu and Nurliani Bermawie
AIP Conference Proceedings **2462**, 050001 (2022); https://doi.org/10.1063/5.0076402

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

### The effect of $FeSO_4$ concentration on the callus growth of two chili (*Capsicum annuum* L.) varieties

Rossa Yunita, Endang Gati Lestari, Iswari S. Dewi, Mastur and Bambang Sapta Purwoko
AIP Conference Proceedings **2462**, 050002 (2022); https://doi.org/10.1063/5.0075223

- SHOW ABSTRACT
-
  - PDF

- E-READER
- ADD TO FAVORITES
- SHARE
- EXPORT CITATION

FreeJanuary 2022

## Evaluation of ratooning ability in several sweet sorghum (*Sorghum bicolor* [L.] Moench) mutant lines

Endang Gati Lestari, Iswari Saraswati Dewi, Rossa Yunita and Amin Nur
AIP Conference Proceedings **2462**, 050003 (2022); https://doi.org/10.1063/5.0075542

- SHOW ABSTRACT
- 
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Response of gamma ray irradiation derived-cultures of three sugarcane varieties to drought stress induced by polyethylene glycol

Ragapadmi Purnamaningsih and Suci Rahayu
AIP Conference Proceedings **2462**, 050004 (2022); https://doi.org/10.1063/5.0075185

- SHOW ABSTRACT
- 
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Sucrose and putrescine increased callus induction in tomato anther culture

Iswari Saraswati Dewi, Imam Nur Kholis, Bambang Sapta Purwoko and Ratna Ningsih
AIP Conference Proceedings **2462**, 050005 (2022); https://doi.org/10.1063/5.0075666

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Field evaluation of elephant grass mutant lines (*Pennisetum purpureum* Schumach.) in highlands

Ali Husni, Muhammad Rifay, Mia Kosmiatin and Vyta W. Hanifah
AIP Conference Proceedings **2462**, 050006 (2022); https://doi.org/10.1063/5.0076418

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Increasing drought tolerance of sugarcane through gamma ray irradiation and *in vitro* selection

Sri Suhesti, Syafaruddin, I. Ketut Ardana, Endang Hadipoentyanti and Rr. Sri Hartati
AIP Conference Proceedings **2462**, 050007 (2022); https://doi.org/10.1063/5.0076155

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Cells density affects cell production of *Citrus limonia* in flask and air-lift bioreactor cultures and limonin farming

Dita Agisimanto, Farida Yulianti and Hidayatul Arisah
AIP Conference Proceedings **2462**, 050008 (2022); https://doi.org/10.1063/5.0075651

- SHOW ABSTRACT
- 
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

# THE USE OF MICROBIAL GENETIC RESOURCES AS BIOLOGICAL CONTROL AGENTS OF AGRICULTURAL PESTS AND DISEASES, AND FOR SOIL BIOREMEDIATION

FreeJanuary 2022

## *In Silico* functional prediction of CAS2, a protein specifically expressed in appressorium and required for pathogenicity of *Colletotrichum gloeosporioides*

Tri Puji Priyatno, Farah Diba Abu Bakar, Rohaiza Ahmad Redzuan, Abdul Munir Abdul Murad and Ifa Manzila
AIP Conference Proceedings **2462**, 060001 (2022); https://doi.org/10.1063/5.0075625

- SHOW ABSTRACT
- 
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Biofertilizer increases nutrient use efficiency (NUE) of nitrogen, phosphorus, and potassium at leaves level of *Artemisia annua* L.

Wiguna Rahman, Arthur A. Lelono, Erwin Al Hafiizh and Tri Muji Ermayanti
AIP Conference Proceedings **2462**, 060002 (2022); https://doi.org/10.1063/5.0075503

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Effect of nitrogen fixation and phosphate solubilizing bacteria on growth and yield of lowland rice in different soil type

Ikhwani, Higa Afza, Siti Yuriyah and Waluyo
AIP Conference Proceedings **2462**, 060003 (2022); https://doi.org/10.1063/5.0077914

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Morphological, physiological, and molecular identification and characterization of yeast isolated from Indonesian fruits and woods

Rerenstradika Tizar Terryana, Nazhirotul Ilmiyah, Inda Setyawati, Titin Haryati, Karden Mulya, Eny Ida Riyanti, Yudi Sastro and Puji Lestari
AIP Conference Proceedings **2462**, 060004 (2022); https://doi.org/10.1063/5.0075170

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## The effect of coating application using chitosan enzymatic depolymerization on anthracnose disease suppression in mango (*Mangifera indica* L.) cv. 'Arumanis'

Yadi Suryadi, Dwi Ningsih Susilowati, I. Made Samudra, Alina Akhdiya and Karsinah
AIP Conference Proceedings **2462**, 060005 (2022); https://doi.org/10.1063/5.0075183

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Understanding yeast tolerance as cell factory for bioethanol production from lignocellulosic biomass

Eny Ida Riyanti and Edy Listanto
AIP Conference Proceedings **2462**, 060006 (2022); https://doi.org/10.1063/5.0075157

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Isolation and pathogenicity test of fusarium basal rot and purple blotch fungal pathogens from shallot and *Allium* spp

Chaerani, Ragapadmi Purnamaningsih and Suci Rahayu
AIP Conference Proceedings **2462**, 060007 (2022); https://doi.org/10.1063/5.0075209

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-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Morphological characters and efficacy of thirteen entomopathogenic fungi of *Aschersonia aleyrodis* Webber isolates on whitefly (*Bemisia tabaci* Gennadius)

Yusmani Prayogo, Marida Santi Yudha Ika Bayu, Sri Wahyuni Indiati and Made Jana Mejaya
AIP Conference Proceedings **2462**, 060008 (2022); https://doi.org/10.1063/5.0076067

- SHOW ABSTRACT
-
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Physicochemical characteristics of yoghurt from various beans and cereals

Heny Herawati, Diana Nur Afifah, Eni Kusumaningtyas, Sri Usmiati, Agus S. Soemantri, Miskiyah, Elmi Kamsiati and Muchamad Bachtiar
AIP Conference Proceedings **2462**, 060009 (2022); https://doi.org/10.1063/5.0075712

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- 
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## The potential use of zeolite and exopolysaccharide bacteria for reduction of degradation and carbon emission on oil palm plantation in tropical peatland

Laksmita P. Santi, Haryo T. Prakoso and Donny N. Kalbuadi
AIP Conference Proceedings **2462**, 060010 (2022); https://doi.org/10.1063/5.0075506

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- 
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

FreeJanuary 2022

## Application of phosphate solubilizing microbes to promote the effectiveness of rock phosphate on cacao seedling growth in acid soil

Kurnia Dewi Sasmita, Iswandi Anas, Syaiful Anwar, Sudirman Yahya and Gunawan Djajakirana
AIP Conference Proceedings **2462**, 060011 (2022); https://doi.org/10.1063/5.0075843

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- 
  - PDF
  - E-READER
  - ADD TO FAVORITES
  - SHARE
  - EXPORT CITATION

# Cytological and molecular identifications of seedless tangerine derived from endosperm culture

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Chaireni Martasari, Mia Kosmiatin, Ali Husni, et al.

# Cytological and Molecular Identifications of Seedless Tangerine Derived from Endosperm Culture

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**Abstract.** Endosperm cultures have been established on tangerine citrus cv. Siam Madu. To verify the ploidy levels of the regenerant derived-plants, both cytological and molecular assessments were applied. The research was conducted from March until June 2017 at the Laboratory of Plant Breeding and Genetic Resources of the Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI). A total of 25 tangerine citrus accessions served as the evaluated materials after they were grafted into JC rootstock for 16 weeks. The results showed that the stomatal density, length, and width were varied among the tested accessions. The lowest stomatal density was observed in SM42. While, SM11 showed the highest stomatal density with smallest value in stomatal size trait. The highest ratio of stomatal length and width was detected in SM56. Based on the cytological observation, 13 accessions were observed to have triploid, 11 accessions were diploids, and one accession had haploid chromosome structures. Molecular characterization using SSR primers (TTTA41, TAA15, and CAT01) resulted that most of tested accessions had the same number of amplified alleles, except for SM18, SM47, SM49, SM51, and SM54 which showed polymorphic bands compared to the controls.

## INTRODUCTION

Among the marketable citrus fruits, tangerine (*Citrus nobilis* Lour.) is the most preferred citrus by Indonesian consumers. The planting area covers 60–70% of total commercial citrus plantation [1, 2]. Tangerine citrus cv. Siam Madu, or also known as Siam Medan, is one of the common local citrus varieties that has higher market share commpared to other tangerines due to it has several competitive characteristics, such as sweet taste with medium fruit size and longer storage durability (8–10 days after harvest) [3]. On the other hand, the citrus variety is also categorized as seedy citrus, and the amount of seed can reach 15–21 seeds per fruit [4], which is considered as an undesirable trait [5, 6].

Seedlessness is an important economic trait relating to fruit quality. There are many desirable characteristics of seedless fruit, including its high quality and taste, which are greatly valued by both consumers and the processing industry [7]. The presence of a large number of seeds in citrus fruits greatly hinders consumer acceptability, even if it has good organoleptic properties. Therefore, the development of seedless fruit cultivars has become a major goal for fruit breeders [8] and one of most promising ways to promote seedlessness in plants is through induction of polyploids [9]. Among polyploids, triploids are of utmost importance as they have been reported to produce seedless fruits and vegetables, including citrus [10].

Plant breeders have tried to investigate suitable methods applicable in developing plants that produce seedless or nearly seedless fruit through sexual recombination, such as by cross combination of diploids and tetraploids (2n × 4n), as well as tetraploids and diploids (4n × 2n), through embryo rescue technique, spontaneous triploids,

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irradiation, endosperm culture, and other biotechnology tools [11–13]. The endosperm is a unique tissue and developed from the fusion product of three haploid nuclei; two polar nuclei and one pollen nucleus, in most angiosperms, is triploid [14]. With the development of tissue culture techniques, it is now possible to get embryogenesis from cultured endosperm. This technique enables the production of triploid plants [15, 16].

The successful development of complete plant or plantlet from endosperm culture should be verified by ploidy level identification. This identification acted as preliminary selection for the targeted triploid plantlets. The process can be carried out through direct observation on the number of somatic chromosomes and molecular assessment [17–20], and indirect assessment on the characteristic of stomatal anatomy [21, 22]. The selected plants were further maintained in the field and induced for flowering to verify seedless traits.

Genetic improvement program of tangerine cv. Siam Madu have been initiated since 2015 and developed plantlets derived from endosperm culture have been established. The desired improvement is expected on the absent or decrease on number of seeds, while the other original characters, like sweet taste and easiness of peeling, were remained unchanged. Cytological and molecular assessments in the study were carried out to verify the ploidy levels of the putative triploid plantlets and genetic variation among plantlets derived from endosperm cultures.

## MATERIALS AND METHODS

The research was conducted from March until June 2017 at the Laboratory of Plant Breeding and Genetic Resources of the Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI). The plant materials used were the population of tangerine cv. Siam Madu derived from endosperm culture, namely SM7, SM9, SM11, SM12, SM17, SM22, SM23, SM36, SM37, SM38, SM39, SM41, SM42, SM45, SM47, SM49, SM51, SM55, SM56, SM57, SM58, and SM59. The scion of these plants was grafted into Japansche citroen (JC) rootstocks and optimally maintained for 16 weeks. The field collection diploid plant, the source of intial endosperm expants, served as the control.

### The Observation of Stomatal Anatomy

The observation of stomatal anatomy was carried out on the stomata located on the abaxial part of fully expanded leaves [23]. The leaf was traced on a piece of graph paper and the leaf surface area was estimated. The leaf section (between parallel veins) was painted using clear fingernail polish and allowed the fingernails polish to completely dry. A piece of clear tape was placed over the dried nail polish and firmly pressed the tape onto the leaf. The tape was then peeled from the leaf and placed the tape sticky side down onto a microscope slide. The tape which contained an impression of leaf cells was examined under the light microscope with the 400× magnification.

### Chromosome Observation

The observation of chromosome was carried out according to method as described by Kosmiatin *et al.* [23]. The terminal and auxiliary bud flushes were collected in the morning (7.00–8.30 AM local time) and placed in sterile distilled water. The collected materials were then immersed under 0.002 M 0.8-Hidroxyquinoline for 3–4 h under 5°C. The materials were transferred into 45% acetic acid for 10–15 min under 60°C. After being immersed in acetic acid, the specimens were put into mixture of hydrochloric acid:acetic acid (3:1, v/v) for 15–20 min under 20°C and followed in 2% aceto-orcein for 5–10 min. The specimens were then put under glass slides and given 1–2 drops of 2% aceto-orcein. The materials were squashed, covered with glass clips and slightly heated on Bunsen burner. The preparates were then observed under light microscope (Olympus BX51) the magnification of 1,000×. The targeted images were then captured using digital evolution LC color camera.

### Molecular Observation

Genomic DNA was extracted from 0.5 g young leaves tangerine accessions using CTAB method described by Jannati *et al.* [24]. The quality of the DNA samples was checked using 0.8% agarose gel electrophoresis in 0.5× TBE buffer at 100 volts for 30 min. The electrophoresis result was visualized under UV light using BioDoc analyzer.

Amplification reactions were performed in a 20 µl of FastStart PCR Master (Roche) reaction mixture with 5 µM of each primer (forward and reverse). The primers used with following sequences were TAA41

(F: AGGTCTACATTGGCATTGTC, R: ACATGCAGTGCTATAATGAATG), TAA15 (F: GAAAGGGTTACT-TGACCAGGC, R: CTTCCCAGCTGCACAAGC), and CAT01 (F: GCTTTCGATCCCTCCACATA, R: GATCCCTACAATCCTTGGTCC) [25]. After denaturation at 94°C for 4 min, the reaction mixture was subjected to amplification for 30 cycles consisting of 50 seconds at 94°C, annealing at 51°C for 1 min, and a final extension at 72°C for 10 min. The amplification products were separated by 3% agarose gel electrophoresis in 1× TBE buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA [pH 8.0]). The gel was stained with ethidium bromide (0.8 g/ml) and visualized under UV light.

## RESULTS AND DISCUSSIONS

The observation of the stomata characteristics revealed that the stomatal density, length, and width among the tested accessions were varied greatly. The lowest stomatal density was observed in SM42 accession, and the highest ones was in SM11 (Table 1). Accession SM11 also had smaller stomata respected to smaller stomatal length and width of stomata compared to the rest of tested plants. The highest length and width ratio of stomata was found in SM56.

**TABLE 1.** Stomatal density, length, width, and ratio between stomatal length and width of the tested tangerine cv. Siam Madu accessions derived from endosperm culture.

| Accessions | Stomatal characteristics | | | |
|---|---|---|---|---|
| | **Density (unit/mm)** | **Length (µm)** | **Width (µm)** | **Ratio of stomatal length and width** |
| SM7 | 4.86 | 218.04 | 168.6 | 1.29 |
| SM9 | 8.15 | 164.65 | 136.39 | 1.21 |
| SM11 | **13.81** | **118.92** | 111.85 | **1.06** |
| SM12 | 9.04 | 181.08 | 158.40 | 1.14 |
| SM17 | 10.93 | 160 | 120.41 | 1.33 |
| SM22 | 5.79 | **230.21** | 178.52 | 1.28 |
| SM23 | 8.53 | 185.68 | 145.56 | 1.22 |
| SM36 | 6,19 | 166.7 | 136.56 | 1.21 |
| SM37 | 8.54 | 179.8 | 148.46 | 1.14 |
| SM38 | 9.58 | 143.87 | 126.19 | 1.37 |
| SM39 | 7.17 | 149.95 | **109.06** | 1.29 |
| SM41 | 8.1 | 171.33 | 133.04 | 1.29 |
| SM42 | **4.28** | 214.11 | 165.47 | 1.27 |
| SM45 | 13.14 | 139.59 | **198.88** | 1.23 |
| SM47 | 6.79 | 152.07 | 123.54 | 1.28 |
| SM49 | 11.76 | 143.85 | 112.1 | 1.19 |
| SM51 | 5.65 | 154.3 | 129.97 | 1.29 |
| SM55 | 9 | 139.86 | 108.33 | 1.18 |
| SM56 | 9.04 | 157.08 | 133.44 | **1.38** |
| SM57 | 6.58 | 162.05 | 117.43 | 1.28 |
| SM58 | 9.98 | 132.51 | 103.43 | 1.24 |
| SM59 | 12.06 | 143.38 | 115.98 | 1.24 |

The ploidy level of the tested tangerine cv. Siam Madu accessions derived from endosperm culture also varied which had haploid, diploid, and triploid chromosome structures with somatic diploid accounted for 18 chromosomes (Table 2 and Fig. 1). The incidence was putatively resulted from the nucellar embryo contamination during endosperm cultures [23].

**TABLE 2.** Number of chromosomes of the tested tangerine cv. Siam Madu accessions derived from endosperm culture.

| Accession | Chromosome number | Accession | Chromosome number |
|---|---|---|---|
| SM 7 | 18 | SM 39 | 27 |
| SM 8 | 27 | SM 41 | 27 |
| SM 9 | 18 | SM 43 | 18 |
| SM 10 | 27 | SM 45 | 18 |
| SM 11 | 18 | SM 47 | 27 |
| SM 12 | 27 | SM 49 | 18 |
| SM 17 | 27 | SM 51 | 27 |
| SM 18 | 27 | SM 54 | 18 |
| SM 23 | 9 | SM 56 | 27 |
| SM 35 | 18 | SM 57 | 27 |
| SM 36 | 27 | SM 58 | 18 |
| SM 37 | 18 | SM 59 | 18 |
| SM 38 | 27 | Control | 18 |

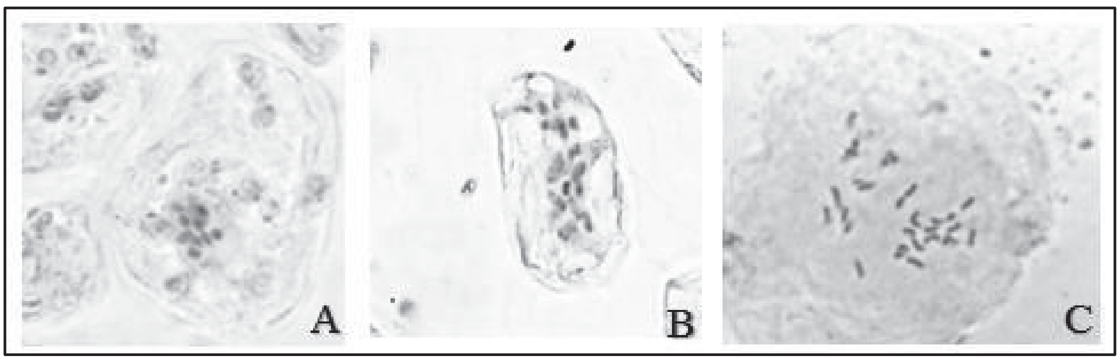


**FIGURE 1.** Variation on ploidy levels: (a) haploid (2n = 9), (b) diploid (2n = 18), and (c) triploid (2n = 27) of tangerine (*Citrus nobilis* Lour.) cv. Siam Madu derived from endosperm culture (1,000× magnification).

Among the tested tangerine cv. Siam Madu accessions, 13 accessions were observed triploid (2n = 3x = 27), i.e. of SM8, SM10, SM12, SM17, SM18, SM36, SM38, SM39, SM41, SM47, SM51, SM56, and SM57 (Fig. 2). Eleven accessions (SM7, SM9, SM11, SM35, SM37, SM43, SM45, SM49, SM54, SM58, and SM59) were diploid (2n = 2x = 18) and one accession (SM 23) was counted as a haploid (2n = x = 9) plant.

Molecular assessment on tangerine cv. Siam Madu accessions using SSR primers of TTTA41, TAA15, and CAT01 was presented in Figs. 3, 4, and 5. According to Jannati *et al.* [24], these primers were able to identify 12 alleles with DNA fragment size ranged in 100–300 bp. The average number of alleles that appeared was 2 at the size of 100–200 bp, but in the accessions of SM 18, SM 47, SM 49, SM51, and SM 54 only one allele appeared at one locus (Fig. 3).

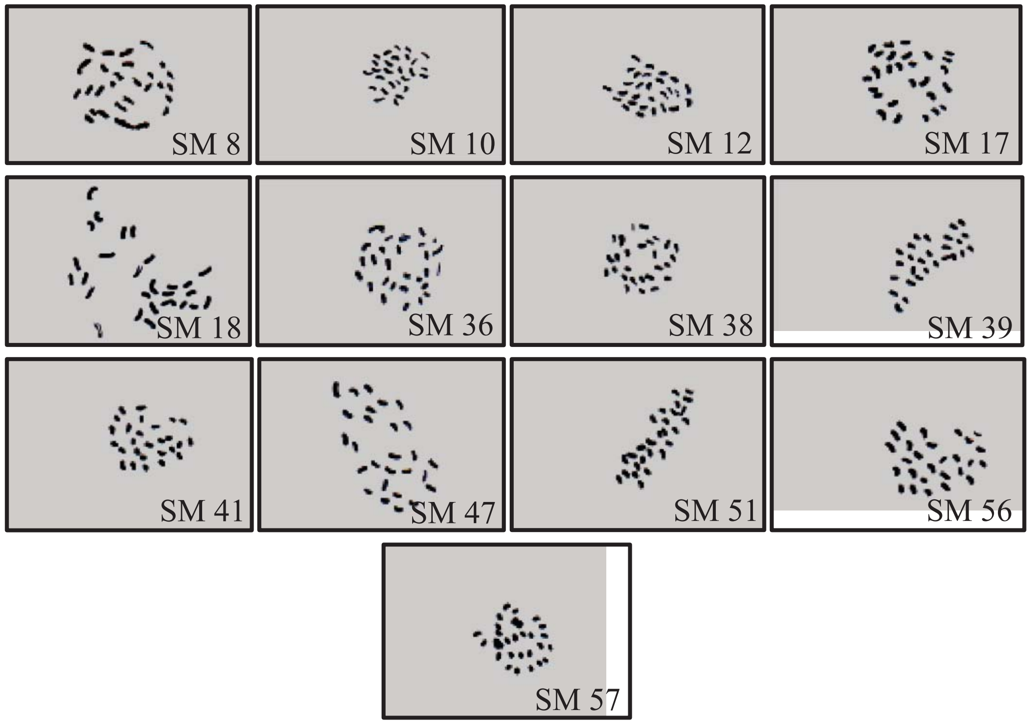


**FIGURE 2.** Chromosomal variability of tangerine cv. Siam Madu derived endosperm culture from triploid (2n = 3x = 27) accessions (1,000× magnifications).

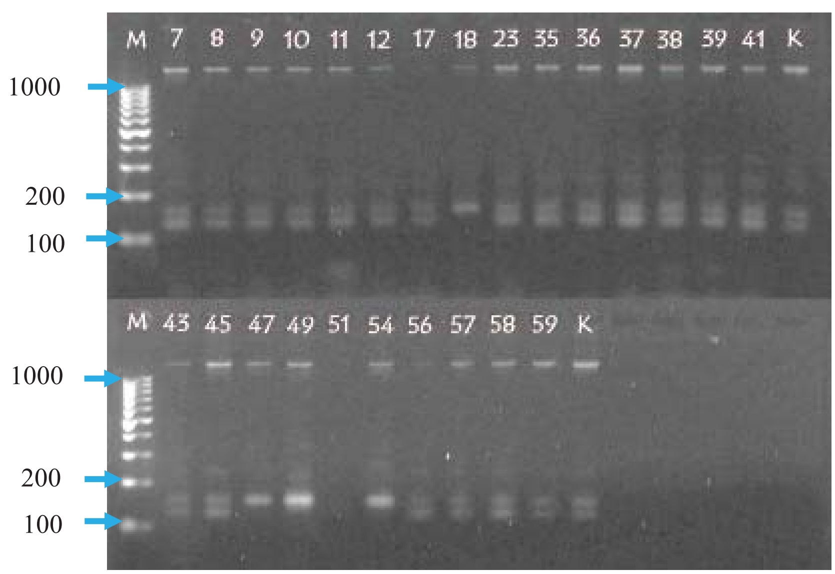


**FIGURE 3.** DNA amplification of tested tangerine cv. Siam Madu accessions using primer TAA41. M = 100 bp DNA ladder, 7–59 = tangerine accessions, K = control.

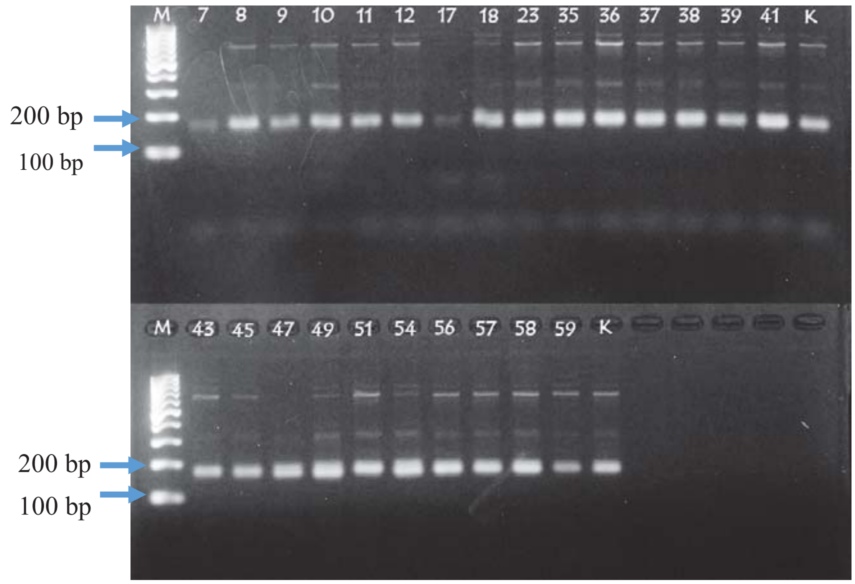


**FIGURE 4.** DNA amplification of tested tangerine cv. Siam Madu accessions using primer TAA15. M = 100 bp DNA ladder, 7–59 = tangerine accessions, K = control.

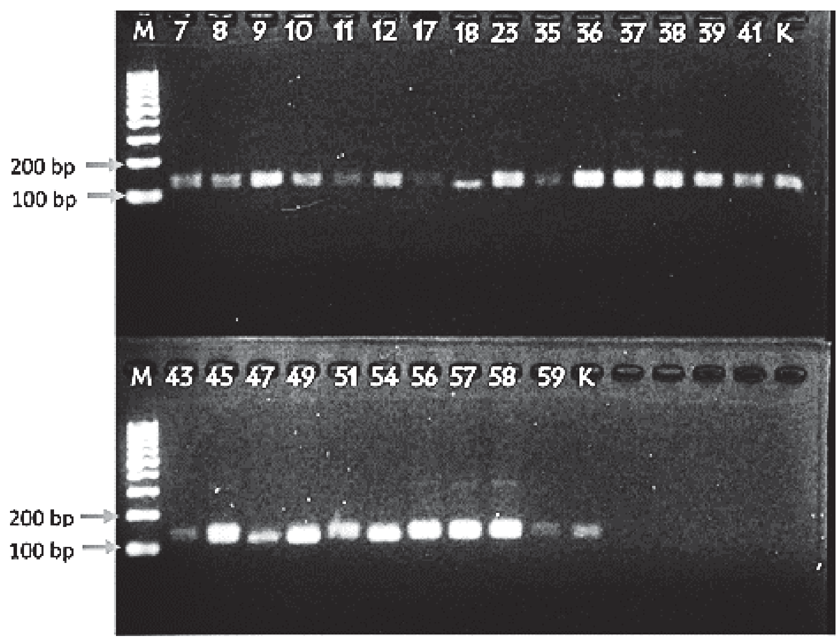


**FIGURE 5.** DNA amplification of tested tangerine cv. Siam Madu accessions using primer CAT01. M = 100 bp DNA ladder, 7–59 = tangerine accessions, K = control.

The desired ideotype from the triploid plant derived from endosperm culture is seedless fruit with the other original fruit characters remained the same with diploid plant (control). Variation on the number of alleles based on primer TAA41 indicated that aside from the internal variation among the triploids, the accessions also had close relationships (similarity) with the original diploid plant (Table 3).

**TABLE 3.** Number of alleles amplified by primers on triploid tangerine cv. Siam Madu derived from endosperm culture.

| Accession | Number of chromosome | Number of amplified alleles | | | Remarks |
|---|---|---|---|---|---|
| | | TAA15 | TAA41 | CAT01 | |
| SM 8 | 27 | 2 | 2 | 2 | √ |
| SM 10 | 27 | 2 | 2 | 2 | √ |
| SM 12 | 27 | 2 | 2 | 2 | √ |
| SM 17 | 27 | 2 | 2 | 2 | √ |
| SM 18 | 27 | 2 | 1 | 2 | - |
| SM 36 | 27 | 2 | 2 | 2 | √ |
| SM 38 | 27 | 2 | 2 | 2 | √ |
| SM 39 | 27 | 2 | 2 | 2 | √ |
| SM 41 | 27 | 2 | 2 | 2 | √ |
| SM 47 | 27 | 2 | 1 | 2 | - |
| SM 51 | 27 | 2 | - | 2 | - |
| SM 56 | 27 | 2 | 2 | 2 | √ |
| SM 57 | 27 | 2 | 2 | 2 | √ |
| Control | 18 | 2 | 2 | 2 | √ |

√ = identical with diploid, - = non-identical with diploid.

Mother cell of endosperm is originated from the fusion process of one pollen nucleus and two polar nuclei. In some citrus species, the development of endosperm cells is also accompanied by the nucellar tissues formation [22], which is originally diploid and haploid antipodal cell [25]. The endosperm culture resulted variation ploidy levels, usually haploid, diploid, and triploid, since naturally the endosperm tissues might contain cells with different ploidy levels [23]. Similar findings were also reported on the endosperm culture of *Azadirachta indica*. Endosperm culture of these plant produced 66% triploid (2n = 3x = 36) and 34% diploid (2n = 2x = 24) regenerants [26]. On parsley, viable regenerants derived from endosperm culture produced mostly diploid, while on *Diospyros kaki,* endosperm culture gave 60% dodecaploid (2n = 12x) and 40% hexaploid plants [27].

The nucleus of tangerine endosperm resulted from double fertilization process is nuclear endosperm. During mitotic division, the cytokinesis process is not directly accompanied by cell wall formation. The cellularization proceeded gradually, thus the development of endosperm cells were not uniform. The variation on the developmental stage of endosperm cells served as the nourishing cells for the embryo [28]. Similar findings were also reported by Kosmiatin *et al.* [25], that high variability of embryogenic callus formation was observed on the endosperm culture of tangerine cv. Siam Madu. The different cell growths reflected various developmental fates of endosperm cells, thus give different responses to the provided media formulation. The different growth response might also be resulted from nucellar proembryo and antipodal cells that incidentally included on the endosperm isolation and cultures [29].

Genetic variabilities reflected from DNA amplification of the tangerine cv. Siam Madu derived from endosperm culture indicated the putative occurrence of somaclonal variation during in vitro culture. During the culture, actually the use of variation-inducing substances had been reduced to avoid the presence of variation. These included the application of growth regulator was only in callus induction stage. On further development stages, regeneration process was carried out by regular deflasking of the propagules onto free-hormone media. The initial explants were also derived from the same protocols, the triploid control plants. The existing variation was then probably resulted from other uncontrollable factors like the indigenous features of diploid parental where the triploids produced.

In line with these putative causes, Hoshino *et al.* [30] stated that endosperm genome was structured from maternal and paternal genomes with the proportion of 2:1. This allelic variation would affect gene expression of the genotypes derived from endosperm cultures. Tangerines are commonly heterozygous plants [31] and consequently, the progenies from sexual reproduction would also be varied even they were produced from self-pollination. The variation due to heterozygous characteristics of the initial diploid plants might induce variation on the derived-triploid endosperm plants and expressed on the grafted/top working plants of the scion-triploid plants.

## CONCLUSION

The stomatal density, length, width, and the rasio between stomatal length and width were varied among the tested accessions. Among the tested tangerine cv. Siam Madu accessions, 13 accessions were observed to have triploid, 11 accessions were diploid, and one accession was haploid plant based on the cytological analysis. Molecular characterization using SSR primer TTTA41 resulted in five accessions (SM18, SM47, SM49, SM51, and SM54) which showed polymorphic bands compared to the control indicating genetic variation of the said accessions from the control.

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