Effect of Gamma Rays Irradiation and \textit{In Vitro} Selection on \textit{Citrus nobilis} (L.) ‘Siam Banyuwangi’ to \textit{Huanglongbing} (HLB) Disease

Dumaris Priskila Purba$^1$, Ali Husni$^2$, Alina Akhidaya$^2$, Mia Kosmiatin$^2$ and Agus Purwito$^3$

$^1$ Master Program of Plant Breeding and Biotechnology, Faculty of Agriculture, IPB University, Bogor, West Java, Indonesia
$^2$ Indonesia Center of Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRD), Bogor, West Java, Indonesia
$^3$ Faculty of Agriculture, IPB University, Bogor, West Java, Indonesia

\section*{INTRODUCTION}

\textit{Siam} orange (\textit{Citrus nobilis} L.) provides great economic value and social importance, despite its susceptibility to \textit{Huanglongbing} (HLB) disease caused by \textit{Ca. Liberabacter} sp., classified as specific bacterium phloem vessels marked by the formation of callouses covering plasmodesmata. This research aims to improve the tolerance of \textit{Siam} orange from Banyuwangi (SB) to HLB disease. The experiment was undertaken by performing a randomized design (CRD) with one factor (irradiation dose). This study consists of two interrelated experiments, which include: induction of embryo mutations with gamma rays irradiation, and \textit{in vitro} selection of putative mutant. The embryo of SB was irradiated by gamma rays with doses of 0, 45, 50, and 55 Gray. Each treatment was repeatedly undertaken for five times with 20 embryos. After 24 weeks, \textit{in vitro} selection of putative mutant shoots was screened by HLB pathogen suspension. The observation indicated that treatment of gamma rays irradiation in various doses influenced embryo germination. In general, gamma ray irradiation gave significant effects on embryo germination and plant morphological characters. \textit{In vitro} selection results for putative mutant on doses of 45, 50, and 55 indicated tolerance to HLB pathogen after selection.
symptoms include blotching or mottling on leaves developed from the tip of the plant (yellow shoots) or chlorosis on the leaves, leading to yellowish plant, slowly drying and dying (da Graça et al., 2015; Mira, Yu, & Matia, 2019). Symptoms of HLB have been associated with phloem collapse and blockage of translocation streams (Achor et al., 2010).

Strategies for HLB control and appropriate management have been conducted by using healthy planting material, eradicating the infected plants and applying the chemical spraying for vectors (Machado, Cristofani-Yaly, & Bastianel, 2011), despite less evident to prove its effectiveness. Thus, a more sustainable approach for HLB management would be to cultivate Citrus cultivars that are unaffected, or resistant to infection (Gottwald, 2010). Efforts to improve the resistancy of the varieties are by establishing high genetic diversity and selection. Increase genetic diversity is performed through hybridization, mutation, protoplast fusion, and genetic engineering (Wulandari, Purwito, Susanto, Husni, & Ermayanti, 2018). Hybridization however is not deemed effective since all sweet orange and mandarin varieties are susceptible to HLB (Machado, Cristofani-Yaly, & Bastianel, 2011). In traditional breeding and sexual crossing, various biological barriers, such as seed polyembryony, sexual incompatibility, high heterozygosity and sterility, conducted resulted in longer period of harvesting and high plant breeding cost (Latado, Tulmann Neto, & Figueira, 2012).

High genetic diversity becomes the key to success in achieving the expected targets in plant breeding (Kosmiatin & Husni, 2018). Improvement of plant varieties through mutations with gamma rays is considerably more effective in increasing the diversity of Citrus (Arisah & Mariana, 2017). Gamma irradiation could produce high mutations to create new variants which are different from the wild orange type (Rattanpal, Singh, & Gupta, 2019).

Gamma rays are the most widely used mutagenic radiation in plant mutation breeding; however, detailed characteristics of mutated DNA sequences have been inadequately clarified (Li, Shimizu, Nishio, Tsutsumi, & Kato, 2019). Evaluation for the physiological effects on the growth rate of propagules sensitivity test for mutagen was previously performed at different doses to obtain a lethal dose (Latado, Tulmann Neto, & Figueira, 2012). The lethal doses (LD50) were applied to determine the gamma doses that killed half (50%) of the plantlets (Agisimanto, Mohd Noor, Ibrahim, & Mohamad, 2016). An example of the successful mutation breeding results includes the seedless mandarin orange (Goldenberg, Yaniv, Porat, & Carmi, 2014), considered as a resistant mutant of sweet orange to Citrus cancer under artificial inoculation (Mba et al., 2009), which could increase the production and fruit quality (Bermejo, Pardo, & Zaragoza, 2015).

Mutagenesis is typically performed by in vitro or in vivo mutagenesis. In vitro mutagenesis is a combination of in vitro culture and mutation induction which provides the opportunity to increase genetic variability (Ling, Chia, Hussein, & Harun, 2008). The application of in vitro mutagenesis has been considered to solve the problems in conventional by providing an expected outcome if the putative mutant is directed by in vitro selection through a screening agent simulated with real conditions in the field (Kosmiatin & Husni, 2018). Previously, in vitro selection was successfully undertaken by Kosmiatin, Martasari, Yunimar, Akhdiya, & Husni (2020) with an artificial HLB infection technique by using HLB suspensions derived from Citrus plants with positive HLB, depicting high HLB specific symptoms (registered patent POD2019093190021909319). Such artificial infection technique enables a selection simulation to test the resistance of HLB in several populations of in vitro orange shoots from mutagenesis and breeding results. In vitro testing methods are proved to save cost and time for testing, in which the testing result could be confirmed before testing on the fields, since only clones that are resistant in in vitro testing are eligible for further testing. Thus, this study aims to increase the genetic diversity of Citrus nobilis (L.) from Banyuwangi (Siarn Banyuwangi) resistant to HLB disease by the application of gamma rays irradiation and in vitro selection.

MATERIALS AND METHODS

The research was conducted from February 2019 to April 2020 at the tissue culture laboratory of the Indonesia Center of Agricultural Biotechnology and Genetic Resources Research and Development (ICABIIOGRAD), and KP Cikeumeuh Greenhouse (ICABIIOGRAD). Gamma rays irradiation treatment was applied at the Isotope and Radiation Application Center of National Nuclear Energy Agency (PAIR-BATAN), Jakarta. The source of irradiation was derived from the Cobalt 60 gamma rays through the C220 gamma cell irradiator.
The plant material applied in this study was the juvenile fruit derived from Siam orange Banyuwangi (SB) and Lime (from Indonesian Citrus and Subtropical Fruits Research Institute [ICSFRI]), having a 2.5–3.0 cm in diameter. In the in vitro selection stage, Ca. Liberabacter asiaticus (CLas) suspension was employed as a selection component. Other materials included media formulations such as: growth regulator (ZPT), GA3 (Giberelic Acid) of 1 ppm, Phytagel, Alcohol of 70% and 96%, distilled water, filter paper sterile, tissue, glucose and cefotaxime of 400 ppm.

**Preparation of Plant Sources and Gamma Rays Irradiation**

Juvenile embryos were isolated in a laminar airflow cabinet. The selected Citrus fruits were sterilized by soaking the fruit in 96% alcohol and flamed on Bunsen lamp for 4-5 times. Further, the sterilized fruits were peeled to remove the seeds. Meanwhile, the embryos were isolated and cultured on germination medium of MS modified with VMW. Embryos treated with gamma irradiation doses of 0, 45, 50, and 55 (Gy). The irradiated embryos were then planted on the prepared media, enriched by GA3 at 1 mg/l. Additionally, planting medium plays a pivotal role for the embryos’ growth (Agisimanto, Normah, & Ibrahim, 2019). Mutant shoots were sub-cultured every two months. Reservations for the percentage of germinated embryos (PGE) were weekly prepared until 12 weeks, by the following formula:

\[
\text{PGE} (\%) = \frac{\text{NGS}}{\text{TNS}} \times 100
\]

Where: NGS = number of germinated seeds; TNS = total number of cultured seeds

**In Vitro Selection of HLB Disease**

Putative mutant shoots were selected and normally grown for 24 weeks, followed by the selection process by using suspension (CLas) (Kosmiatin, Martasari, Yunimar, Akhdiya, & Husni, 2020). Lime shoots were utilized as a negative control. The average shoots used had 3 or more segments planted on selection media of MS+VMW with CLas suspension with ratio of 1:1. Inoculation was undertaken in 8 days. In addition, limes shoots indicated a characteristic symptom, which was the death of plant tissue marked with brown-white color on the leaves. The shoots were flushed with cefotaxime (400 ppm), and were cultured on medium recovery to observe the appearance of browning symptoms. Observation was weekly conducted by analyzing color changes on leaves and stems for two months. The shoots with symptoms of chlorosis were further calculated to navigate the disease incidence by the following formula:

\[
\text{DI} = \frac{X}{N} \times 100\%
\]

Where: X = number of the plant affected by HLB; N = number of plants observed.

Phloem vessels on resistant and susceptible shoots were observed with Scanning Electron Microscope (SEM). Observation data were analyzed in terms of the variance at error level (α) = 5% and continued with Least Significant Difference (LSD) test by employing Statistical Tools for Agriculture Research (STAR) IRRI V2.0.1 program. The LD50 value was analyzed with Curve Expert 1.3 along with qualitative data which were descriptively analyzed.

**RESULTS AND DISCUSSION**

**Mutant Shoots Germination**

Gamma rays irradiation influenced the germination juvenile Citrus embryos (SB) after 12 weeks. A proportionate reduction in germination was observed along with increased doses of gamma rays. The highest percentage of germination (96%) was found at untreated embryo. The total germinated embryos were the commutative number of normal and abnormal germination. At 45 and 50 Gy, the number of total germinated embryos were lower with negligible differences with untreated embryo. The percentage of normal germinated embryos of these treatments, however, was significantly lower. Both percentage of total germination and normal germination of 55 Gy was significantly decrease from the other treatments. These condition indicated that irradiation gamma rays affected plant growth and development by inducing genetical, cytological, biochemical, physiological, and morphogenetic changes in cells and tissues in accordance with the levels of irradiation (Ikram, Dawar, Abbas, & Zaki, 2010).

The normal germination of embryo under irradiation treatments differ significantly from those of 0 Gy (control). The results are classified into three groups (Table 1). The first group consists of control with 93% germinated; the second and third groups comprise of embryo treated with 45-50 Gy and 55 Gy. Only 21% of the embryo could be normally
germinated under 55 Gy gamma rays irradiation. The increasing dose of gamma rays however decreased the ability of the embryo to germinate normally, alike sprouts without plumule or radicle, even increasing the percentage of mortality. The highest result of abnormal germination was obtained on dose of 50 Gy, indicating that changes in plant chromosomes due to gamma rays are unpredictable due to random physical mutations. These was because gamma rays radiation is ionizing radiation contributing to free radicals and subsequently causing damage or modification of chromosomes in plants. Gamma rays are absorbable by DNA, leading to spontaneous damage to DNA molecules (Asadi, 2013). The reduction of seed germination due to mutagenic treatments might be due to e damage in cell constituents at molecular level or at altered enzyme activity (Goyal & Khan, 2010). High dose would however kill the plants since mutagens have a direct and lethal effect on plant tissue (Bodele, 2013).

The decreasing percentage of juvenile embryo in Citrus germination is often associated with the level of radio sensitivity of the irradiated material. Radio sensitivity has been applied to determine the level of sensitivity of irradiated materials to irradiation dose. Fig. 1 indicates the results of Curve Expert analysis with several regression models. The best equation model based on the percentage of germination is the Quadratic Fit curve with $S = 10.95440505$ and $r = 0.96881969$ and the equation of $Y = 9.6 + 3.6x - 8.4x^2$. The $r$-value is near to be 1, thus the correlation between the doses of Gamma irradiation to the percentage of embryo germination is significantly strong. Radio sensitivity is measured based on the lethal dose of 50 (LD50) value, causing the loss of 50% of the irradiated population (Asadi, 2013). LD50 becomes the parameters to establish an adequate irradiation dose and to induce mutation in plant breeding programs (Álvarez-Holguín et al., 2019). Generally, LD50 has been obtained in the dose range of 49.9, which is in line with prior report (Wulansari, 2013) that obtaining LD50 53.25 Gy on callus from C. nobilis protoplast culture.

**Table 1.** Germination of juvenile Citrus embryos (SB) after irradiated by gamma rays on 12 weeks

| Dose | Percentage of germination | Percentage of normal germination | Percentage of abnormal germination |
|------|---------------------------|---------------------------------|-----------------------------------|
| 0 Gy | 96.0±5.48$a$              | 93.9±6.50$a$                    | 6.0±6.52$c$                       |
| 45 Gy| 84.0±7.42$a$              | 65.0±16.96$b^*$                 | 22.0±12.04$^{ab}$                 |
| 50 Gy| 76.0±19.49$a$             | 41.0±27.70$b^*$                 | 35.0±9.35$^{a*}$                  |
| 55 Gy| 37±34.57$b^*$             | 21.0±19.49$c^*$                 | 16±15.57$^{bc}$                   |

Remarks: *Mean values within a column followed by the same letters are not significantly different at p>0.05 according to Least Significant Difference (LSD)

**Fig. 1.** Quadratic fit analysis in germination of juvenile Citrus embryos (SB) after irradiated by gamma rays on 12 weeks
Fig. 2. *In vitro* selection of putative mutant shoot in SB: a. The shoots were selected with medium of VMW + Isolate CLas suspension; b. The shoot mutant was symptomatic; c. Normal vascular vessels with a magnification of 220 x 100μm; d. Infected vascular vessel, indicated by callose covering vascular (220 x 100μm magnification); e. Vascular vessels of resistant plant, without callose and bacteria; f. Vascular vessels of the resistant plant with bacteria, but no callose formation; g. Three mutant shoots were resistant before grafted; h. The mutant shoots were resistant after grafted in 2 months
The LD50 is expected to produce high diversity by maintaining the ability of plants for a normal growth. Variation in leaf morphology was visible, marked by the shape of leaves and leaf edges (Tahir, Riniarti, Ersan, & Kusuma, 2019; Yani, Khumaida, Ardie, & Syukur, 2018). In some studies on mutation breeding, variation would generally occur in LD50 to produce genetic changes disparaging from the wild Citrus plant type. Similarly, Zanzibar & Sudrajat (2016) reported that doses between LD20 and LD50 are considered as optimal doses to increase the varieties for plant mutation breeding programs. Prior study reported that only 50% of plantlets presented normal morphology in Citrus leaf (Wulansari, 2013).

**In Vitro Selection of Putative Mutant Shoots**

The expected variation tolerance to HLB disease was observed by in vitro selection testing. The shoots obtained from embryo germination indicated the normal growth within 24 weeks. Lime as a negative control would be selected as a parameter in the selection process. After the selection process, the observation of lime on morphological symptom, was carried out. Prior studies reported that Lime was susceptible to HLB disease in Oman (Al Fahdi et al., 2018; Lopez-Buenfil et al., 2017).

The media formulation with the suspension indicated a characteristic HLB symptom response both in lime as control and in SB (Fig. 2a). In lime shoots, HLB symptoms during in vitro selection or inoculation were apparently observed shoot morphology and cytology, and the time of appearance of symptoms was diminutive (± 8 days). Susceptible shoots on selection media would indicate leaf chlorosis resulting in dying plant (Fig. 2b). Shoots which were tolerant to HLB disease were fresh and green. Previously, most studies on cultivar assessment were based on HLB symptomatology (Ramadugu et al., 2016). Foliar asymmetrical chlorosis and blotchy mottle appearance became the most recognized characteristic of HLB symptomatology (Deng et al., 2019).

Table 2 indicates the effect of in vitro selection of HLB into SB. Incidence of HLB disease on 24 shoots of 0 Gy (wild type) indicates that symptomatic response on plants was up to 100%, confirming that the resistant gene of SB shoots does not exist. Currently, the updated reports regarding citrus resistance to the HLB is limited, unless the impact of HLB resulted in 100% damage in commercial citrus orchard (Deng et al., 2019). There are 4 shoots indicating asymptomatic on putative mutant of SB. No symptoms of HLB disease indicate that SB genetic has changed to be tolerant, despite less than 10%. Then mutant shoots were observed with Scanning Electron Micrographs (SEM) to evaluate the cytology level in phloem tissue (Koh et al., 2012). Pathogen infection indicates plants response to induce defense formation in cell walls and through metabolite secondary compounds (Bendix & Lewis, 2018). Plant defense also depends on the performance of plant metabolism (Killiny & Hijaz, 2016).

The infection of HLB is indicated by the accumulation of starch in phloem tissues of Citrus plant, known as callose, a polymer of β-1,3-glucan units playing important role in intercellular water transport, cell growth and differentiation (Granato, Galdeano, Da Roz D’Alessandre, Breton, & Machado, 2019). Callose becomes an important mechanism of defense against invasive tissue pathogens as a general mechanism of cell wall in strengthening or blocking the plasmodesmata cells (Koh et al., 2012). Callose formation in sieve tubes of Citrus plants infected by Las could lead to a restriction of phloem transport (Etxeberria & Narciso, 2012).

Vascular vessels infected with HLB pathogen depict callose that covers the vascular tissue (Fig. 2d). There are 2 types of the vascular vessel in resistant plant, which are: without bacteria and callose on vessels and with bacteria without callose on vessels. Genetic changes caused by irradiation might change different responses in each

| Dose  | Number of plants affected by HLB | Number of Plant Observed | Percentage of Symptomatic |
|-------|---------------------------------|--------------------------|---------------------------|
| 0 Gy  | 24                              | 24                       | 100%                      |
| 45 Gy | 22                              | 24                       | 91.7%                     |
| 50 Gy | 23                              | 24                       | 95.8%                     |
| 55 Gy | 23                              | 24                       | 95.8%                     |
shoot. Changes due to gamma rays irradiation are unpredictable because they are random, requiring further testing (Lisdyanyanti, Anwar, & Darmawati, 2019). Induction of mutation was able to produce mutants with a level of diversity in various selected characters (Setiawan, Khumaida, & Dinarti, 2015). However, this method requires further confirmation. The mutant shoot which was resistant to HLB disease was grafted for further evaluation in the field (Fig. 2h).

CONCLUSION

Gamma rays irradiation provides a significant effect on embryo germination. The lethal dose 50 (LD 50) obtained in this study is 49.9 Gray. Gamma rays irradiation at in vitro combined with in vivo selection using CLas suspensions has resulted in 4 putative mutants that are considerably resistant to HLB diseases.

ACKNOWLEDGEMENT

The author would like to thank KP4S 2018 (No.31.5/PL.040/H.1/02/2018) and Indonesia Center of Agricultural of Biotecnology and Genetic Resources Research and Development for being willing to fund this work in a scheme of DIPA APBN 2019 with registration number of 1798.201.054.A.

REFERENCES

Achor, D. S., Etxeberria, E., Wang, N., Folimonova, S. Y., Chung, K. R., & Albrigo, L. G. (2010). Sequence of anatomical symptom observations in citrus affected with Huanglongbing disease. Plant Pathology Journal, 9(2), 56–64. https://doi.org/10.3923/ppj.2010.56.64

Agisimanto, D., Normah, Mohd. N., & Ibrahim, R. (2019). Rapid somatic embryogenesis of Citrus reticulata Blanco cv. Madu in an air-lift bioreactor culture. AGRIVITA Journal of Agricultural Science, 41(2), 284–294. https://doi.org/10.17503/agrivita.v41i2.2237

Agisimanto, D., Normah, Mohd. N., Ibrahim, R., & Mohamad, A. (2016). Gamma irradiation effect on embryogenic callus growth of Citrus reticulata cv. limau madu. Sains Malaysiana, 45(3), 329–337. Retrieved from https://www.ukm.my/jsm/pdf_files/SM-PDF-45-3-2016/02 Dita Agisimanto.pdf

Al Fahdi, A., Al-Mamari, A., Shahid, M. S., Maharachchikumbura, S. S. N., Carvalho, C. M., Elliot, S. L., & Al-Sadi, A. M. (2018). Characterization of Huanglongbing disease associated with acid lime (Citrus aurantifolia Swingle) in Oman. Journal of Plant Pathology, 100(3), 419–427. https://doi.org/10.1007/s42161-018-0088-9

Álvarez-Holguín, Alan Morales-Nieto, C. R., Avendaño-Arrazate, C. H., Corrales-Lerma, R., Villarreal-Guerrero, F., Santelion-Estrada, E., & Gómez-Simuta, Y. (2019). Mean lethal dose (LD50) and growth reduction (GR50) due to gamma radiation in Wilman lovegrass (Eragrostis superba). Revista Mexicana de Ciencias Pecuarias, 10(1), 227–238. https://doi.org/10.22319/rmcp.v10n1.4327

Arisah, H., & Mariana, D. (2017). Keragaman buah jeruk keprok SoE mutan generasi M1V2 hasil induksi mutasi sinar gamma. Buletin Plasma Nutfah, 23(2), 69–80. https://doi.org/10.21082/blpn.v23n2.2017.p69-80

Asadi. (2013). Pemuliaan mutasi untuk perbaikan terhadap umur dan produktivitas pada kedelai. Jurnal AgroBiogen, 9(3), 135–142. https://doi.org/10.21082/jbio.v9n3.2013.p135-142

Bendix, C., & Lewis, J. D. (2018). The enemy within: phloem-limited pathogens. Molecular Plant Pathology, 19(1), 238–254. https://doi.org/10.1111/mpp.12526

Bermejo, A., Pardo, J., & Zaragoza, S. (2015). Influence of gamma irradiation on seedless citrus production: Pollen germination and fruit quality. Acta Horticulturae, (1065), 229–237. https://doi.org/10.17660/ActaHortic.2015.1065.25

Bodele, S. K. (2013). Effect of gamma radiation on morphological and growth parameters of Andrographis paniculata (Burm.F) Wall. Ex. Nees. Indian Journal of Applied Research, 3(6), 55–57. https://doi.org/10.15373/2249555x/june2013/19

Bové, J. M. (2006). Huanglongbing: A destructive, newly-emerging, century-old disease of citrus. Journal of Plant Pathology, 88(1), 7–37. Retrieved from http://www.sipav.org/main/jpp/index.php/jpp/article/view/828

Coletta-Filho, H. D., Carlos, E. F., Alves, K. C. S., Pereira, M. A. R., Boscariol-Camargo, R. L., de Souza, A. A., & Machado, M. A. (2010). In planta multiplication and graft transmission of ‘Candidatus Liberibacter asiaticus’ revealed by Real-Time PCR. European Journal of Plant Pathology, 126(1), 53–60. https://doi.org/10.1007/s10658-009-9523-2
da Graça, J. V., Kunta, M., Sétamou, M., Rascoe, J., Li, W., Nakhtla, M. K., ... Bartels, D. W. (2015). Huanglongbing in Texas: Report on the first detections in commercial citrus. Journal of Citrus Pathology, 2(1), 1–6. Retrieved from https://escholarship.org/uc/item/99p100ts

Deng, H., Achor, D., Exteberria, E., Yu, Q., Du, D., Stanton, D., ... Gmitter Jr., F. G. (2019). Phloem deposition is a mechanism for Huanglongbing-tolerance of "Bears" lemon and "LB8-9" sugar Belle® mandarin. Frontiers in Plant Science, 10, 277. https://doi.org/10.3389/fpls.2019.00277

Exteberria, E., & Narciso, C. (2012). Phloem anatomy of citrus trees: Healthy vs. greening-affected. Proceedings of the Florida State Horticultural Society, 125, 1–4. Retrieved from https://crec.ifas.ufl.edu/media/crecfsufledu/faculty/exteberria/2012-FSHS.pdf

Goldenberg, L., Yaniv, Y., Porat, R., & Carmi, N. (2014). Effect of Gamma-Irradiation Mutagenesis for induction of seedlessness, on the quality of mandarin fruit. Food and Nutrition Sciences, 5(10), 943-952. https://doi.org/10.4236/fns.2014510105

Gottwald, T. R. (2010). Current epidemiological understanding of citrus Huanglongbing. Annual Review of Phytopathology, 48(1), 119–139. https://doi.org/10.1146/annurev-phyto-073009-114418

Goyal, S., & Khan, S. (2010). Induced mutagenesis in urdbean (Vigna mungo L. Hepper): A review. International Journal of Botany, 6(3), 194–206. https://doi.org/10.3923/ijb.2010.194.206

Granato, L. M., Galdeano, D. M., Da Roz D’Alessandre, N., Breton, M. C., & Machado, M. A. (2019). Callose synathese family genes plays an important role in the Citrus defense response to Candidatus Liberibacter asiaticus. European Journal of Plant Pathology, 155(1), 25–38. https://doi.org/10.1007/s10658-019-01747-6

Ikram, N., Dawar, S., Abbas, Z., & Zaki, M. J. (2010). Effect of (60cobalt) gamma rays on growth and root rot diseases in mungbean (Vigna radiata L.). Pakistan Journal of Botany, 42(3), 2165–2170. Retrieved from https://inis.iaea.org/search/searchxlrecord.aspx?recordsFor=SingleRecord&RN=42015203

Killiny, N., & Hijaz, F. (2016). Amino acids implicated in plant defense are higher in Candidatus Liberibacter asiaticus-tolerant citrus varieties. Plant Signaling & Behavior, 11(4), e1171449. https://doi.org/10.1080/15592324.2016.1171449

Koh, E.-J., Zhou, L., Williams, D. S., Park, J., Ding, N., Duan, Y.-P., & Kang, B.-H. (2012). Callose deposition in the phloem plasmodesmata and inhibition of phloem transport in citrus leaves infected with “Candidatus Liberibacter asiaticus.” Protoplasma, 249(3), 687–697. https://doi.org/10.1007/s00709-011-0312-3

Kosmiatin, M., Martasari, C., Yunimar, Akhdija, A., & Husni, A. (2020). In vitro selection to increase Huanglongbing tolerance of citrus-derived from in vitro breeding. IOP Conference Series: Earth and Environmental Science, 457, 012080. Retrieved from https://iopscience.iop.org/article/10.1088/1755-1315/457/1/012080

Kosmiatin, M., & Husni, A. (2018). Perakitan varietas jeruk tanpa biji melalui pemuliaan konvensional dan nonkonvensional. Jurnal Penelitian Dan Pengembangan Pertanian, 37(2), 91–100. https://doi.org/10.21082/jp3.v37n2.2018.p91-100

Latado, R. R., Tulmann Neto, A., & Figueira, A. (2012). In vivo and in vitro mutation breeding of citrus. Bioremediation, Biodiversity and Bioavailability, 6(Special Issue 1), 40–45. Retrieved from http://www.globalsciencebooks.info/Online/GSBOnline/images/2012/BBB_6(SI1)/BBB_6(SI1)-40-45c.pdf

Li, F., Shimizu, A., Nishio, T., Tsutsuami, N., & Katai, H. (2019). Comparison and characterization of mutations induced by gamma-ray and carbon-ion irradiation in rice (Oryza sativa L.) using whole-genome resequencing. G3: Genes, Genomes, Genetics, 9(11), 3743–3751. https://doi.org/10.1534/g3.119.400555

Ling, A. P. K., Chia, J. Y., Hussein, S., & Harun, A. R. (2008). Physiological responses of Citrus sinensis to gamma irradiation. World Applied Sciences Journal, 5(1), 12–19. Retrieved from http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.571.8020&rep=rep1&type=pdf

Lisdyanyanti, N. D., Anwar, S., & Darmawati, A. (2019). Pengaruh iradiasi sinar gamma terhadap induksi kalus dan seleksi tingkat toleransi padi (Oryza sativa L.) teradap cecekam salinitas secara In-vitro. Berkaa Bioteknologi, 2(2), 67–75. Retrieved from https://ejournal2.undip.ac.id/index.php/bb/article/view/6716

Lopez-Buenfil, J. A., Ramirez-Pool, J. A., Ruiz-Medrano, R., Del Carmen Montes-Horcasitas, M., Chavarin-Palacio, C., Moya-Hinojosa, J., ... Xoconostle-Cazaures, B. (2017). Dynamics of Huanglongbing-associated bacterium Candidatus Liberibacter asiaticus in Citrus...
Machado, M. A., Cristofani-Yaly, M., & Bastianel, M. (2011). Breeding, genetic and genomic of citrus for disease resistance. *Revista Brasileira de Fruticultura*, 33(special 1), 158–172. https://doi.org/10.1590/S0100-29452011000500019

Mba, C., Afza, R., Jankowicz-Cieslak, J., Bado, S., Matijevic, M., Huynh, O., & Till, B. J. (2009). Enhancing genetic diversity through induced mutagenesis in vegetatively propagated plants. In Q. Y. Shu (Ed.), *Induced Plant Mutations in the Genomics Era* (pp. 262–265). Rome, IT: Food and Agriculture Organization of the United Nations. Retrieved from http://www.fao.org/3/i0956e/I0956e.pdf

Mendonça, L., Badel, J., & Zambolim, L. (2017). Bacterial citrus diseases: Major threats and recent progress. *Journal of Bacteriology & Mycology*, 5(4), 340–350. https://doi.org/10.15406/jbmoa.2017.05.00143

Mira, A., Yu, S., & Matia, M. (2019). Evaluation of Huanglongbing tolerance in citrus breeding populations. *Journal of Productivity and Development*, 24(2), 371–390. Retrieved from https://www.semanticscholar.org/paper/EVALUATION-OF-HUANGLONGBING-TOLERANCE-IN-RASA-Mira-Yu/cd17d0d373e09f-c4e0d806ed34de26754c7eb8

Nurhadi. (2015). Penyakit Huanglongbing tanaman jeruk (Candidatus Liberibacter asiaticus): Ancaman dan strategi pengendalian. *Pengembangan Inovasi Pertanian*, 8(1), 21–32. Retrieved from http://balitjestro.litbang.pertanian.go.id/penyakit-huanglongbing-tanaman-jeruk-candidatus-liberibacter-asiaticus-ancaman-dan-strategi-pengendalian/

Ramadugu, C., Keremane, M. L., Halbert, S. E., Duan, Y. P., Roose, M. L., Stover, E., & Lee, R. F. (2016). Long-term field evaluation reveals Huanglongbing resistance in citrus relatives. *Plant Disease*, 100(9), 1858–1869. https://doi.org/10.1094/PDIS-03-16-0271-RE

Rattanpal, H. S., Singh, G., & Gupta, M. (2019). Studies on mutation breeding in mandarin variety Kinnow. *Current Science*, 116(3), 483 – 487. https://doi.org/10.18520/cs/v116/i3/483-487

Rustiani, U. S., Endah, A. S., Nurjanah, Prasetiawan, A., & Nurmaida. (2015). Deteksi bakteri penyebab CVPD pada jeruk menggunakan DNA asal tulang daun. *Jurnal Fitopatologi Indonesia*, 11(3), 79. https://doi.org/10.14692/jfi.11.3.79

Setiawan, R. B., Khumaida, N., & Dinarti, D. (2015). Induksi mutasi kulai embriogenik gandum (*Triticum aestivum*) melalui iradiasi sinar gamma untuk toleransi suhu tinggi. *Jurnal Agronomi Indonesia*, 43(1), 36–44. https://doi.org/10.24831/jai.v43i1.9589

Tahir, M., Riniarti, D., Ersan, & Kusuma, J. (2019). Genetic and leaf characteristic diversity on 10 mutant progenies of patchouli (*Pogostemon cablin*) provide insights to selection strategies. *AGRIVITA, Journal of Agricultural Science*, 41(1), 139–148. https://doi.org/10.17503/agrivita.v41i1.1908

Wulandari, D. R., Purwito, A., Susanto, S., Husni, A., & Ermayanti, T. M. (2018). Protoplast fusion between Indonesian *Citrus maxima* (Burm.) Merr. and *Citrus reticulata* L.: A preliminary report. *AGRIVITA, Journal of Agricultural Science*, 40(2), 233–241. https://doi.org/10.17503/agrivita.v40i0.950

Wulansari, A. (2013). Induksi keragaman genetik melalui iradiasi sinar gamma pada kulai embriogenik hasil kultur protoplas jeruk siam. Bogor Agricultural University. Retrieved from https://repository.ipb.ac.id/handle/123456789/63744

Yani, R. H., Khumaida, N., Ardie, S. W., & Syukur, M. (2018). Analysis of variance, heritability, correlation and selection character of M1 V3 generation cassava (*Manihot esculenta* Crantz) mutants. *AGRIVITA, Journal of Agricultural Science*, 40(1), 74–79. https://doi.org/10.17503/agrivita.v40i1.844

Zanzibar, M., & Sudrajat, D. J. (2016). Effect of gamma irradiation on seed germination, storage, and seedling growth of *Magnolia champaca* L. *Indonesian Journal of Forestry Research*, 3(2), 95–106. https://doi.org/10.20886/ijfr.2016.3.2.95-106