

The Second International Conference on Genetic Resources and Biotechnology

**Harnessing Technology for Conservation and Sustainable
Use of Genetic Resources for Food and Agriculture**

Bogor, Indonesia • 24–25 May 2021

Editors • I Made Tasma, Dwinita Winkan Utami, Ika Roostika,
Yadi Suryadi, Chaerani, Eny Ida Riyanti, Puji Lestari, Toto Hadiarto,
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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 24th-25th of May 2021 virtually 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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Deden Sukmadjaja^{1, a)}, Mia Kosmiatin¹ and Tiwi Wati²

¹Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, IAARD, Jln. Tentara Pelajar No. 3A, Bogor 16111, West Java, Indonesia

²Udayana University, Jln. Raya Kampus Unud, Jimbaran 80361, Bali, Indonesia

^{a)}Corresponding author: deden_sukmadjaja@yahoo.com

Abstract. Japansche citroen (JC) is a type of citrus which is widely used as rootstock in citrus cultivation. In order to expand citrus cropping on marginal acid land, a JC citrus clones that are tolerant of acid soil conditions are needed. Therefore, it is necessary to assemble the citrus genotype by increasing genetic diversity to obtain tolerant traits for aluminum and low pH. In this study, mutation induction experiments with gamma ray irradiation were carried out, followed by *in vitro* selection methods. The aims of this research was to increase genetic variation of JC citrus through induced mutation by gamma ray irradiation and to obtain candidates of JC citrus somaclones that are tolerant to Al at low pH. The research was conducted at the Tissue Culture Laboratory, Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Bogor. The plant material used was JC citrus plantlet from immature embryos treated with gamma ray irradiation at doses of 0, 4.5, 5, and 5.5 Gy. Evaluation was conducted to morphological characters, followed by *in vitro* selection for tolerance to Al at low pH by using selecting agent of aluminium trichloride hexahydrate. The results showed that morphological variations found in JC citrus somaclones after being irradiated with gamma rays, including the formation of the winged leaves (*unifoliolatus*) that are not found in the control JC. There were 32 selected somaclones to be tolerant to Al at low pH.

INTRODUCTION

Orange is one type of fruit that is popular and widely consumed by the people of Indonesia. The consumption of citrus fruits in Indonesia has increased from year to year. Consumption of citrus fruit in the years 1995–2014 has increased by 11.65% per year [1]. Meanwhile, the national citrus productivity tends to decline. In 2011 the production of oranges amounted to 1,721,880 tons to 1,498,394 tons in 2012, in 2014 the production of oranges was 1,785,256 tons to 1,744,330 tons in 2015 [1]. The reduced productivity of oranges is caused by pests and diseases, especially citrus vein phloem degeneration (CVPD), and a reduction in fertile land due to the shift in function outside the agricultural sector [2].

The change in the function of the planting area on fertile soils out of the agricultural sector causes the agricultural area to be pushed over. Therefore, the use of marginal land is one way that can be used as agricultural area. Indonesia has marginal acid land that can be used for the development of annual crops including citrus plants of 50.94 million ha spread across Sumatra, Kalimantan, Papua, Maluku, Java, and Sulawesi [3]. The problem of using marginal acid land for agricultural land is that it requires high costs and labor, because it requires high liming and fertilization to improve the quality of the soil [4].

With the widespread marginal acid dry land and the transition of fertile agricultural areas, the use of citrus rootstock plant types or cultivars that are tolerant of acid soils is the most effective and efficient way to overcome the development of citrus farming in acid soils, so that production costs can be minimized possible [4]. In addition, these plants must also have high genetic compatibility with scion, be disease resistant and have a good root system [5]. Japansche citroen (JC) is a type of citrus that is widely used as rootstock by citrus breeders in Indonesia. The rootstock of JC is most preferred because of its wide adaptability, drought tolerance, and high salinity. However, JC

is sensitive to several diseases, such as CVPD, *Citrus tristeza virus* (CTV), *Citrus exocortis viroid* (CEV), and root and stem rot disease [6]. It is easily available in various regions in Indonesia, such as East Java, Central Java, South Sulawesi, North Sumatra, and Bali [7]. In addition, JC in Indonesia is widely used as a rootstock because it has several advantages, one of which is the compatibility between rootstock and scion, so that it has a positive effect on plant survival and productivity [8]. Although JC can provide several good properties, JC oranges are not tolerant to aluminum (Al) stress and low pH, and are not resistant to root rot disease of *Phytophthora* sp. [9].

To overcome this, it is necessary to assemble citrus through increasing genetic variation followed by *in vitro* selection methods in order to obtain traits that are tolerant of Al and low pH. The combination of physical (irradiation) and chemical treatment (Al simulation with low pH) on embryogenic cell populations of JC citrus in *in vitro* media is one way to obtain superior JC citrus plants, which can be used as rootstocks that are tolerant of Al toxicity and low pH in acid soils [4]. In addition, mutation induction with physical mutagens such as gamma ray irradiation in tissues or a group of cells (callus) is an alternative to obtain the desired variant. This variant can be seen from the change in morphology of the irradiated plants [10]. This is evident from the research conducted by Pramono [11] that the morphological form of the first leaves of the *iles-iles* plant at a dose of 10 Gy is the malformation of the leaves, as well as the emergence of variegata leaves (leaves that have a different color from the original, which are generally located adjacent to one part of the plant), the abnormality does not appear in the second or third leaves. In addition, plants irradiated with 10 Gy had a longer vegetative period and resulted in a longer plant life than the control. Therefore, in this study, mutation induction with gamma rays and *in vitro* selection was carried out using Al with different doses in the direction to analyze morphological characters and to obtain JC rootstock that tolerant to Al at low pH.

Several studies to increase cell tolerance to Al have been conducted. *In vitro* selection to obtain Al tolerant plants has been carried out on potato plants [12]. Sutjahjo [13] also carried out an *in vitro* selection for tolerance to Al who carried out research on four genotypes of maize. In addition, Mariska *et al.* [14] conducted research on soybean plants and found that *in vitro* selection could be used to increase soybean plant tolerance to Al stress and low pH, where the resulting traits could be passed on to the next generation. This research aims to obtain the variability of JC citrus through induction of gamma ray mutations as well as to obtain candidates for JC citrus plants that are tolerant of Al toxicity at low pH.

MATERIALS AND METHODS

The research was conducted from December 2018 to May 2019. Gamma ray irradiation was carried out at the Central Laboratory for Isotope and Irradiation Application (PAIR), BATAN, Jakarta. The research was conducted at the Tissue Culture Laboratory, Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development (ICABIOGRAD), Bogor.

The research was carried out in two stages: (1) increasing the genetic variation by induced mutation by gamma ray irradiation, and (2) *in vitro* selection to Al stress at low pH. The plant material used in the first stage of the study was an embryo measuring ± 3 mm from 1-month-old young JC citrus. The JC embryos were irradiated with gamma rays at doses of 0, 4.5, 5, and 5.5 Gy. A total of 10 embryos were planted on Murashige & Skoog basal media with Morel & Weitmore vitamins (VMW media) in 200 ml culture jar, incubated in a culture room with ± 800 lux fluorescent light, for 16 h/day at 21–25°C. The experimental design used was a completely randomized design (CRD) with one factor, namely the dose of gamma ray irradiation. Observations were conducted on culture growth with the parameters of the number of germinated embryos, leaves, roots, lateral shoots, root length, and leaf morphology.

The sprouts or plantlets grown from each treatment in the first stage of the experiment were transferred to selection media containing $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ at low pH. The selection media used were modified MS basic media (MSKd) with the selection component of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ at concentrations of 0, 250, 500, and 750 ppm with low acidity at pH 4 and VMW media with pH 4 and 5.8 as controls. The research design in the *in vitro* selection experiment for Al tolerance at low pH with the selection component of $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ was a two-factor CRD. Each treatment was repeated seven times. Selection was carried out for 8 weeks. After the selection, the culture was transferred to VMW recovery media without ZPT. During selection and recovery the cultures were stored in the culture room in light conditions (1,000 lux) for 16 h/day at 22–24°C [4]. Observations were conducted on shoot height, number of shoots, chlorotic leaves, number of leaves, number of nodes, number of roots, and root length. Data were analyzed using analysis of variance (ANOVA) analysis of variance with SPSS v22.0 for Windows at the real level $\alpha < 5\%$.

RESULTS AND DISCUSSIONS

Increased Genetic Diversity with Gamma Ray Irradiation and Germination of JC Globular Embryos

Table 1 shows the number of nucellar embryos that germinated after being treated with mutation induction at several doses of gamma ray irradiation. In general, radiation administration increased the number of normal germinating nucellar embryos, however, an increase in radiation dose did not show a correlation with an increase in the number of normal sprouts.

TABLE 1. Germination of JC embryos after induced mutation at several levels of irradiation, 30 days age.

Irradiation (Gy)	Number of embryo cultured	Normal		Abnormal	
		Number	(%)	Number	(%)
0	34	10	29.41	24	70.59
4.5	96	49	51.04	47	48.96
5.0	199	98	49.25	101	50.75
5.5	114	74	64.91	40	35.09

Irradiation treatment caused decreasing the number of organs formed of gamma-irradiated cultures. This can be seen from all the parameters of the irradiated plant organs that have lower values than those without irradiation (Table 2). Fig. 1 shows the cultures of irradiated JC embryos. The number of organs was not significantly different, except the formation of lateral shoots. The increase in variation could be detected in leaf morphology. It indicated that the shape, tip, and edge of the leaves were not different, but the base of the leaves was different, which are called winged leaves and wingless leaves (Fig. 2). The number of leaf bases without wings was higher than that of winged leaves. The base of the leaves without wings in each treatment is more than 50%. The highest number of winged base leaves was at a dose of 5.5 Gy with as much as 35.63%. The control also found a winged base, but it had the lowest percentage compared to irradiated explants (Fig. 3).

TABLE 2. Number of organs from gamma-irradiated cultures, 8 weeks after planting.

Irradiation (Gy)	Number of							
	Leaf	SD	Root	SD	Lateral shoot	SD	Node	SD
0	6.98	3.63	1.58	1.21	1.02	0.74	4.88	2.35
4.5	4.94	2.10	0.99	0.74	0.91	0.61	3.46	1.77
5.0	4.25	1.68	0.95	0.84	0.89	0.70	3.09	1.29
5.5	4.45	1.71	1.05	0.85	0.55	0.55	3.21	1.77

SD = standard deviation.

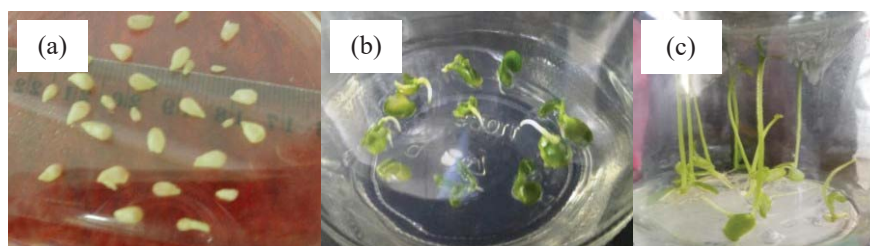


FIGURE 1. Germination of orange JC embryos after induced mutation by gamma ray irradiation. (a) Explant of nucellar embryos. (b) Germination of nucellar embryos. (c) Plantlets from nucellar embryos used for testing of tolerance to aluminum.

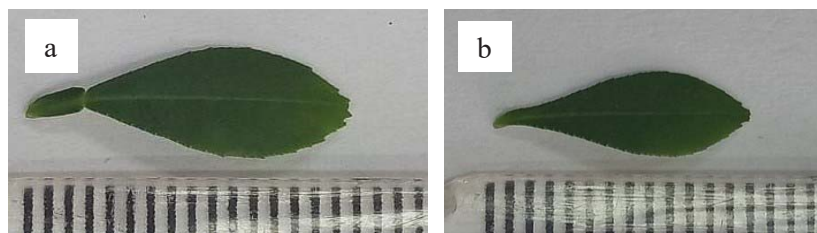


FIGURE 2. Morphological variation of JC citrus leaves. (a) Petiole winged. (b) Without petiole wing.

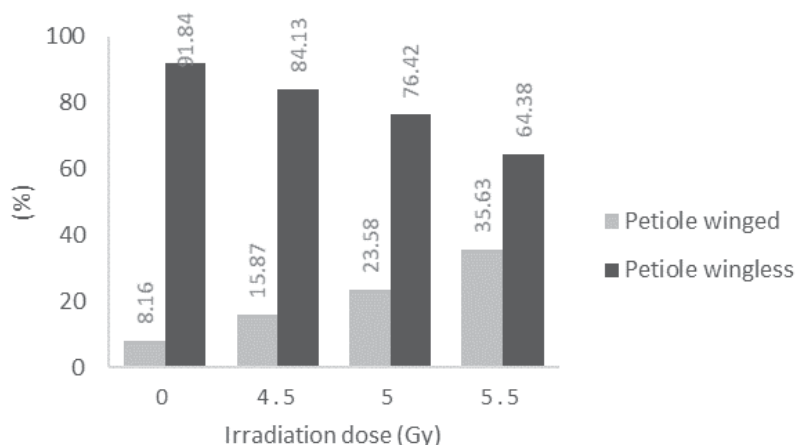


FIGURE 3. Percentage of winged and wingless JC petiole leaves after gamma ray irradiation.

***In Vitro* Selection for Tolerance to Aluminum (Al) Stress**

In general, the growth of all organs on the VMW control media (both at pH 5.8 and 4) was better than that in the selection media. The growth of cultures planted on MS media with the addition of vitamin VMW was better than those planted on MSKd media. This is because the VMW media contains Ca-pantothenate and biotin, which play a role in the formation of cell membranes and the process of mitosis, and increase the multiplication of shoots and roots in *in vitro* culture. Meanwhile, biotin functions as an enzyme cofactor, where the use of biotin can stimulate callus growth, optimize embryogenesis, and increase the number and size of embryos.

Irradiation treatment tend to suppress culture growth in most of the growing organs. Meanwhile, an increase in the concentration of the Al selection component reduced the growth in some plant organs, but on the other hand it did not show an effect on other organs. High Al concentrations caused damage plant cells. Al is toxic to plants in the form of the trivalent Al^{3+} ion, which is dominant in acid conditions [15]. The low increase in leaf number is also influenced by genotype and environment. Leaf position in plants (number of plastochrons), which is mainly controlled by genotype, also has a significant effect on leaf growth rate [16]. Some plant organs have determinate growth patterns, while other organs are indeterminate. The determinate growth pattern is characterized by the growth of the organ until it reaches its maximum size, then the growth stops, the organ grows old (senescence) and finally falls out. One of the plant organs that has a determinate growth pattern is leaves [17].

The dose of 5 Gy was able to provide root addition in all media treatments, both VMW and MSK, with the addition of Al. It was assumed that the application of gamma ray irradiation can increase genetic variation, including to increase their tolerance to Al and low pH. Induction treatment at a dose of 5 Gy was able to increase root growth in the treatment of selection media with 750 ppm Al, which at other doses including control did not cause additional roots. The selection media containing of 750 ppm Al did not affect on the number of root at 0, 4.5, and 5.5 Gy. This is probably because Al is able to stop the phytochemical properties and biological functions of plants.

TABLE 3. Number of organs originating from explants without irradiation on various selection media, 8 weeks after planting.

No.	Media selection	Number of				Root length	Shoot height
		Leaf	Node	Root	Shoot		
1	VMW pH 5.8	2.43	2.00	0.29	0.29	3.33	0.62
2	VMW pH 4.0	1.57	2.29	0.71	0.00	3.43	0.77
3	MSKd Al 0	1.57	1.14	0.29	0.43	0.82	0.59
4	MSKd Al 250	2.29	1.43	0.14	0.43	0.14	0.65
5	MSKd Al 500	2.29	1.57	0.00	0.43	0.23	0.53
6	MSKd Al 750	1.43	1.71	0.00	0.14	0.10	0.39

VMW = MS basal media with Morel & Weitmore vitamins, MSKd = modified MS basal media.

TABLE 4. The number of organs derived from explants irradiated by 4.5 Gy on various selection media, 8 weeks after planting.

No.	Media selection	Number of				Root length	Shoot height
		Leaf	Node	Root	Shoot		
1	VMW pH5.8	3.00	2.43	0.14	0.00	3.34	0.70
2	VMW pH4.0	3.57	2.57	0.57	0.14	8.66	0.81
3	MSKd Al 0	5.43	3.57	0.86	0.29	0.74	0.47
4	MSKd Al 250	1.57	1.29	0.00	0.00	0.14	0.10
5	MSKd Al 500	2.00	1.71	0.00	0.14	0.20	0.41
6	MSKd Al 750	2.71	2.43	0.00	0.00	0.33	0.25

VMW = MS basal media with Morel & Weitmore vitamins, MSKd = modified MS basal media.

TABLE 5. The number of plant organs derived from explants irradiated by 5 Gy on various selection media, 8 weeks after planting.

No.	Media selection	Number of				Root length	Shoot height
		Leaf	Node	Root	Shoot		
1	VMW pH5.8	3.71	3.71	0.43	0.14	8.29	0.69
2	VMW pH4.0	3.43	3.14	0.71	0.29	4.26	0.81
3	MSKd Al 0	2.29	1.86	0.71	0.00	3.47	0.55
4	MSKd Al 250	1.43	1.29	0.29	0.00	0.28	0.43
5	MSKd Al 500	1.71	1.71	0.29	0.00	0.30	0.31
6	MSKd Al 750	2.00	1.14	0.14	0.00	0.35	0.30

VMW = MS basal media with Morel & Weitmore vitamins, MSKd = modified MS basal media.

TABLE 6. Number of plant organs derived from explants irradiated by 5.5 Gy on various selection media, 8 weeks after planting.

No.	Media selection	Number of				Root length	Shoot height
		Leaf	Node	Root	Shoot		
1	VMW pH5.8	2.00	1.86	0.00	0.00	0.96	0.63
2	VMW pH4.0	3.14	2.57	0.29	0.14	4.05	0.47
3	MSKd Al 0	1.43	1.00	0.57	0.00	0.34	0.27
4	MSKd Al 250	1.57	1.00	0.00	0.29	0.15	0.33
5	MSKd Al 500	1.29	1.29	0.14	0.00	0.39	0.19
6	MSKd Al 750	0.86	1.14	0.00	0.00	0.15	0.19

VMW = MS basal media with Morel & Weitmore vitamins, MSKd = modified MS basal media.

Selection media containing Al affects the formation and growth of roots. The roots in the selection medium generally experience a color change from whitish green to brownish green to brown and morphology. Roots that are formed in media that do not contain Al will form root hairs and have a root cap that is useful for accelerating nutrient absorption. Meanwhile, those grown on media containing Al looked compact at the root tips and no root caps were visible. Roots that have tolerance to Al will form new roots or root branching (Fig. 4). Root damage in plants begins with interference with the root cap, which has a signal and is a detector of gravity force and mechanical tolerance, so that in turn it reduces the secretion of root cap cell mucilage where the cell is a source of endogenous growth regulators. According to Jones and Kochian [18], roots treated with Al will inhibit the extension of the cells and cause the root tips to swell and when given Al for longer it causes inhibition of elongation and division of root cells. Yamamoto *et al.* [19] found that Al toxicity in addition to causing plant nutrient deficiency also changes the structure and function of the plasma membrane and inhibits cell division at the root tips.

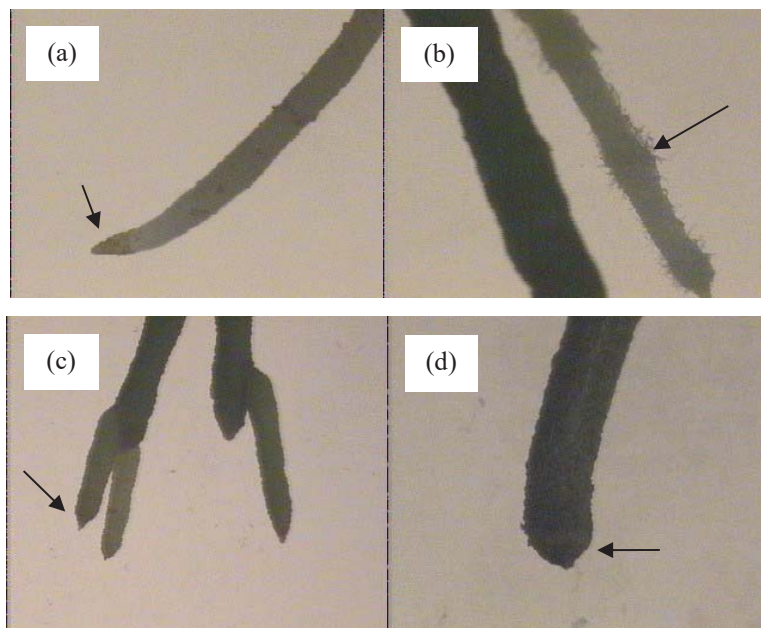


FIGURE 4. Results of root observation under a 10×10 magnification microscope. (a) Root cap. (b) Root hairs. (c) Root branches. (d) Compact root tips.

The increase in the number and height of shoots was only slightly in all treatments, this indicates that the presence of Al is thought to have caused damage to the root cap, thereby disrupting growth and culture development. According to MacDiarmid and Gardner [20], plants will reduce their root system and show various symptoms of nutrient deficiency due to Al poisoning.

JC citrus cultures that were susceptible to Al will show symptoms of wilting, leaves experiencing chlorosis, shoots turning brown, and stunted plant growth (Fig. 5). Enggarini and Marwani [21] stated that the symptoms of Al stress include stunted root growth, chlorosis, nutritional deficiency, and stunted plants.

Deficiency symptoms in explants, such as brown or yellow leaves, cause the development and growth of plants to be disturbed. Santoso and Nursandi [22] stated that browning is a character that appears brown or black, which often prevents growth and development of cultured plants. Chlorosis in plants begins with the appearance of yellow and brown on the leaves until finally all parts of the explants appear to be wilted.

Based on the results of observations of root growth, there were 32 culture numbers that grew on 500 and 750 ppm Al selection media. This result is considered an Al tolerant candidate because it has increased root length and not damage of root tips. Reported by Samuel *et al.* [23] who determined that there are two criteria for plants that are tolerant of Al stress, one of which is that the roots are able to grow continuously, and the root tips are not damaged. Somaclones considered Al tolerant are then numbered for further research on these plant mutants.

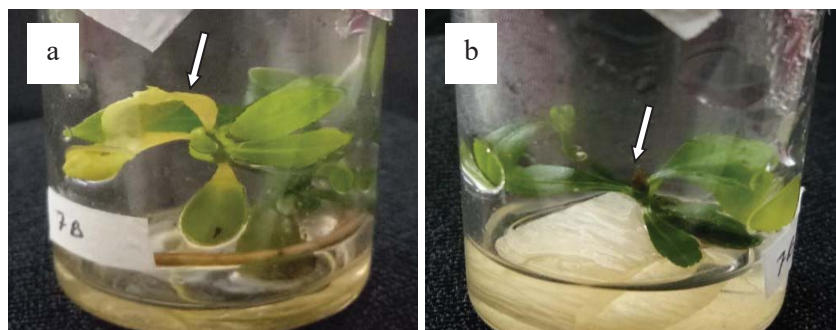


FIGURE 5. Symptoms of deficiency in JC citrus culture on selection media containing Al. (a) Leaves that turn yellow on MSKd 750 ppm Al media. (b) Shoots tip turn brown on MSKd 250 ppm Al media.

CONCLUSION

Increasing genetic variation of JC *in vitro* cultures occurred after gamma ray irradiation of nucellar embryos. *In vitro* selection by using $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ selecting agent combined with low acidity (pH 4) of media culture could screened Al tolerant somaclones. There were 32 somaclones of JC citrus that were tolerant to Al stress.

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