ASEAN
Journal of Scientific and Technological Reports
Online ISSN:2773-8752

Research article

Effect of Chlorine Dioxide on Micropropagation of *Gymnocalycium mihanovichii* LB2178 Agua Dulce (Cactaceae)

Phakarat Rotduang[1], Supawadee Ramasoot[2*] and Tassanee Khawniam[3]

[1] Department of Creative Innovation in Science and Technology, Faculty of Science and Technology, Nakhon Si Thammarat Rajabhat University, Nakhon Si Thammarat, 80280, Thailand; phakarat_rot@nstru.ac.th
[2] Department of Biology, Faculty of Science and Technology, Nakhon Si Thammarat Rajabhat University, Nakhon Si Thammarat, 80280, Thailand; supawadee_ram@nstru.ac.th
[3] Department of Agricultural Innovation and Management Division, Faculty of Natural Resources, Prince of Songkla University, Songkhla, 90110, Thailand; tassanee.kh@psu.ac.th
* Correspondence: supawadee_ram@nstru.ac.th

Citation:
Rotduang, P.; Ramasoot, S.; Khawniam, T. Effect of Chlorine Dioxide on Micropropagation of *Gymnocalycium mihanovichii* LB2178 Agua Dulce (Cactaceae). *ASEAN J. Sci. Tech. Report.* **2024**, *27*(2), 103-111. https://doi.org/10.55164/ajstr.v27i2.252123.

Article history:
Received: December 19, 2023
Revised: February 27, 2024
Accepted: March 1, 2024
Available online: March 6, 2024

Abstract: *Gymnocalycium mihanovichii* LB2178 Agua Dulce is a succulent species popular in many areas. It has been cultivated for a long time. It is considered one of the easiest cacti to grow in Thailand. Therefore, the objective of this study was 1) to study the effect of Chlorine dioxide (ClO_2) on culture medium sterilization on micropropagation of *G. mihanovichii* LB2178 Agua Dulce. and 2) to study the effect of Chlorine dioxide on shoot and root induction of *G. mihanovichii* LB2178 Agua Dulce. The *G. mihanovichii* LB2178 Agua Dulce seeds were used as plant material. After 2 months of culture, the results showed that MS medium supplemented with 50 ppm ClO_2 gave the highest survival rate at 54.17%. After 3 months of culture, the results showed that shoot and root induction can be achieved in *G. mihanovichii* LB2178 Agua Dulce seeds were cultured on MS medium supplemented with 50 ppm ClO_2 gave the highest number of roots at 9.00 roots/explant, length of root 3.45 cm, length of shoot 0.54 cm and diameter of shoot 0.44 cm.

Keywords: *Gymnocalycium mihanovichii* LB2178 Agua Dulce; Chlorine dioxide; *in vitro*

1. Introduction

Cactus is a plant in the family Cactaceae (*Mila* sp.) and is a native plant that originates in the desert. It is a plant with a strange stem shape and is different from other plants in that the stems of almost all varieties are leafless. The trunk has a wax coating to reduce dehydration on the trunk surface. The stem has green chlorophyll, which performs photosynthesis instead of leaves [1]. A study report from The Global Succulent & Cactus Plants Market Report 2021 revealed that the cactus and succulent plant market will have an average overall growth of 16.8% per year for over 6 years until 2027. Many species of cactus grow only in certain areas. It tends to grow slowly, making it popular among people who love cacti and collectors especially interested in rare plant species [2].

In Thailand, Gymnocalycium cacti are popularly called "Gymno". There are many species, but *G. mihanovichii* LB2178 Agua Dulce is a popular species to

grow. It is the cactus species *G. mihanovichii* LB2178 Agua Dulce. The code LB2178 is the code that a Dutch botanist, an expert in this genus named Mr. C.A. Ludwig Bercht, found and collected samples from its origins in the Agua Dulce area, Alto Province, Paraguay, since 2000. It has a round stem. The number of lobes in LB2178 usually ranges from 9 up to 13 lobes. The ridges are thin. The next distinctive feature is the surface. You will see a green pattern alternating with dark green or black. It will look slightly raised. This pattern is called the "chevron" or bone pattern. The more frequent the chevrons, the more beautiful they look, and the price will be higher. Because it is easy to grow and beautiful, *G. mihanovichii* LB2178 Agua Dulce is a popular cactus. It has been cultivated for a long time. It is considered one of the cacti that are easy to grow in Thailand, and it is also a type that is popular worldwide. Furthermore, some types are popular and have a higher price than normal [3].

This genus of cacti has been cultivated and bred for a long time, resulting in hybrids that are different from the original, such as the spotted gymnosperm or some with longer and larger thorns resistant to hot weather. In addition, the cactus species *G. mihanovichii* LB2178 Agua Dulce is also in demand in the market because it is a beautiful ornamental plant. It can be propagated by separating shoots, or if you want a large number, you must use seeds. This takes approximately 1-3 months, causing delayed propagation and easy diseases such as fungal diseases. This is usually found during the rainy season due to the humid air of cacti that have been cultivated and bred for a long time, resulting in hybrids that are different from the original, such as the spotted gymnosperm, or some with longer and larger thorns that are resistant to hot weather [4]. This results in the death of the cacti, and there is not enough for commercial production. Plant tissue culture techniques have been introduced to help increase the number of plants to a large quantity in a short period. It can also be applied in plant breeding to propagate plants commercially. Studies have shown that plant cell culture is a process of growing cells under sterile conditions, reducing the time needed to produce new plants and creating new characteristics in the plant cells we want to culture through induced mutations [5]. Tissue culture can quickly increase the number of plants in large quantities. Techniques for sterilizing culture media are an important step that affects the success of plant tissue culture. An autoclave is a standard method for sterilizing media preparation before plant parts are placed in the culture. This requires expensive equipment and electricity for sterilization. Chemical methods, such as adding silver nanoparticles, can synthesize options for making media sterile. This may involve using toxic chemicals in the synthetic process and be expensive [6]. And the addition of Chlorine dioxide (ClO_2), a synthetic chemical that can eliminate bacteria, fungi, and viruses. It is effective in a wide pH-alkaline range from 3.0 to 9.0, resulting in a high sterility percentage. Does not react with organic substances. Able to remove contaminants well, even with low concentrations. Moreover, it saves time. The preparation method is not difficult and provides better efficiency in developing new plants than sterilization with a steam autoclave [7]. There are reports of using ClO_2 for plant tissue culture, such as Krajood culture, by adding ClO_2 at a concentration of 25 mg/l in the culture medium to create sterile conditions for cultivating krajood in a bioreactor, immersion, and aeration system [8]. To test the chemical sterilization of culture medium using ClO_2 for *in vitro* gerbera cultivation. ClO_2 could replace autoclaving with the production of a sterilized culture medium without phytotoxic problems to Gerbera *in vitro* cultivation [9]. In addition, it has been reported that ClO_2 is used to sterilize the medium for potato tissue culture. To identify alternative methods of sterilizing culture conditions, the disinfection effects of ClO_2 at 88.0, 29.3, 17.6, 12.6, and 8.8 μM were evaluated in potato medium and vessels. The potato seedlings had similar morphological features as those grown on autoclaved medium, with some exceptions. [10]

Currently, there are studies on the tissue culture of cactus seeds of various species, resulting in large quantities of plants quickly. Healthy seedlings with healthy shoots and roots will increase their survival rate after being removed from sterile conditions. Using tissue culture technology increases the potential for development in breeding, resulting in a new species of cactus reducing the removal of plants from their natural state. Currently, there are no reports of using ClO_2 in cacti tissue culture in sterile conditions. Therefore, this research studied the effect of ClO_2 on the sterilization of culture media and the effect of ClO_2 on the induction of shoots and roots of the cactus strain *G. mihanovichii* LB2178 under sterile conditions.- As an alternative to solving the problem of microbial contamination in tissue culture, it helps increase work speed by reducing steps in preparing synthetic food instead of using an autoclave.

2. Materials and Methods

2.1 Plant materials

After pollination, a planted parent plant, *G. mihanovichii* LB2178 Agua Dulce seed pieces, was extracted from the pods and immersed in distilled water to select and discard floating seeds. The seeds were wrapped in nylon cloth and tied tightly with sewing thread. After that, they were soaked in 70% alcohol for 30 seconds and then dipped in a 20% sodium hypochlorite (Chlorox) solution for 20 minutes. I added 2 drops of Tween 20 and rinsed it with distilled water sterile. Three times in an aseptic environment with a laminar flow cabinet.

2.2 Effect of Chlorine dioxide (ClO_2) on G. mihanovichii LB2178 Agua Dulce micropropagation through culture medium sterilization

The seeds of *G. mihanovichii* LB 2178 Agua Dulce were cultured on autoclaved Murashige and Skoog (1962) [11] (MS) free medium at 1.05 kg/cm^2, 121^{o}C for 15 min and MS medium supplemented with 0, 10, 25, 50, 75 and 100 ppm ClO_2. All culture mediums were supplemented with 30 g/l of sucrose and 8 g/l of agar. The pH was adjusted to 5.7. The culture was kept in 3000 lux light-intensity for 14 hours per day at 25±2 °C and subcultured every 4 weeks on the same medium component for 3 months. The percentage of contamination of the culture medium, percentage of contamination over a four week, and percentage of survival rate were recorded. The germination was evaluated daily for 20 days after the first seed germinated, considering the emission of radicle as evidence of germination. The variables used in the analysis were germination (%G), mean germination time (MGT), and germination speed index (GSI) [12]. The experiment design was completely randomized design (CRD), and the mean differences were compared using Duncan's multiple range test (DMRT).

mean germination time (MGT)

$$MGT = (\sum n_i\ t_i)/\sum n_i) \quad (1)$$

Where n_i = number of seeds that germinated in a day "i," and t_i = day "i" in evaluated time

germination speed index (GSI)

$$GSI = \sum(n_i/t_i) \quad (2)$$

where n_i = number of seeds that germinated in day "i" t_i = day "i" in evaluated time

2.3 Effect of Chlorine dioxide on proliferation and induction of shoot and root of G. mihanovichii LB2178 Agua Dulce

The shoots of *G. mihanovichii* LB 2178 Agua Dulce derived from previous experiments were tested. They were cultured on autoclaved MS-free medium at 1.05 kg/cm^2, 121 °C for 15 min and MS medium supplemented with 0, 10, 25, 50, 75 and 100 ppm ClO_2. All culture media were supplemented with 30 g/l of sucrose and 8 g/l of agar. The pH was adjusted to 5.7. The culture was placed in 3,000 lux light-intensity for 14 hours per day at 25±2 °C and subcultured every 4 weeks on the same medium component for 3 months. The number of roots, root length, shoot length, and shoot diameter were recorded. The experiment design was ~~planned using a~~ completely randomized design (CRD), and the mean differences were compared using Duncan's multiple range test (DMRT).

2.4 Statistical analysis

A completely randomized design with 3 replicates (each with 10 explants) was performed for experimental design and statistical analysis. Data was analyzed using ANOVA.

3. Results and Discussion

3.1. Effect of Chlorine dioxide on G. mihanovichii LB2178 Agua Dulce micropropagation through culture medium sterilization

The seeds were cultured on autoclaved MS-free medium and MS medium supplemented with various concentrations of ClO_2. The result found that MS medium supplemented with 0 and 10 ppm ClO_2 gave the highest percentage of contamination, 100 %, followed by MS medium supplemented with 25, 50, and 75 ppm ClO_2, 70.17%, 55.83%, and 45.83%, respectively. Autoclaved MS medium and MS medium supplemented with 100 ppm ClO_2 gave the same percentage of contamination, 0.00 %, significant difference at $p \leq 0.05$. For the percentage of survival rate, the result found that seeds of *G. mihanovichii* LB 2178 Agua Dulce were cultured on MS medium supplemented with 50 ppm ClO_2 gave the best survival rate at 54.17 %, followed by MS medium supplemented with 75 ppm ClO_2 and autoclaved MS medium, 32.50% and 21.26% respectively, significant difference at $p \leq 0.05$. (Table 1 and Figure 1)

This study found that high concentrations of ClO_2 were effective in killing a germ both in seeds of *G. mihanovichii* LB 2178 Agua Dulce and culture media. Still, low concentrations of ClO_2 were not able to control contamination. According to reports, using an Oreactor system and 100 parts per million ClO_2 effectively eliminated fungal contamination in Phulae pineapple cultivation. However, high levels of ClO_2 cause the growth of Phulae pineapple to stop in the early stages and improve growth. After culturing for 2 months, ClO_2 has been reported to be used in the culture of other plants [13], such as using ClO2 to sterilize the medium for potato tissue culture. To substitute techniques for disinfecting culture conditions, the disinfection effects of ClO_2 at 88.0, 29.3, 17.6, 12.6, and 8.8 μM were evaluated in potato medium and vessels. During a 30-minute fumigation process, the ≥12.6 μM gaseous ClO_2 efficiently disinfected the vessel, and its aqueous solution similarly effectively disinfected the potato medium. In the presence of 12.6 μM ClO_2 in the medium, with a few exceptions, the potato seedlings' morphological characteristics were comparable to those of those grown on autoclaved medium [14]. Comparing media sterilized by autoclave to media sterilized with ClO_2 for Gerbera tissue culture is consistent with this finding. It was found that Gerbera grew better in the killed media sterilized with ClO_2. It also grows better than those grown on media sterilized media using an autoclave. ClO_2 is effective in killing bacteria, fungi, and viruses. It is stable in solutions with a pH of 3.0-9.0. Moreover, sterilizing media with ClO_2 is not easy. Fortunately, there is no need to use the autoclave method, which necessitates costly instruments and electrical power [15]. Additionally, it states that ClO_2 is a gas that dissolves in water and does not react with it. Effective in killing microorganisms even when used in small amounts. and does not create chloramine, which is toxic to animals. This research found that there was no statistically significant difference in the fresh weight of seedlings grown on autoclaved media and media supplemented with ClO_2. It demonstrates that, in sterile conditions, Black Miracle Nepenthes seedlings are not toxically affected by low concentrations of ClO_2. Additionally, it was discovered that there was no difference in the growth outcomes of seedlings grown on autoclaved media and media supplemented with 5 ppm ClO_2. Although the mode of action of Chlorine in microorganisms is not fully elucidated, the ClO_2 acts by destroying cell membranes and oxidizing intracellular components of microorganisms [16]. Tested ClO_2 and liquid Chlorine in water and demonstrated that ClO_2 killed several types of bacteria more effectively than liquid Chlorine and is superior to Chlorine against spore-forming bacteria; thus, they concluded that ClO_2 was a safe and effective alternative disinfectant for the treatment of water. ClO_2 acts by transferring electrons; it penetrates the cell wall easily, dehydrating and destroying the cell membrane and finally oxidizing the intracellular components of both Gram-positive and Gram-negative microorganisms [17]. The polar nature of ClO_2 contributes to its sanitizing and sporicidal action due to its higher solubility in complex organic molecules such as the components of most viruses and bacteria [18].

Table 1. The percentage of contamination and survival rate of *G. mihanovichii* LB2178 Agua Dulce was cultured on an autoclaved culture medium and sterilized with different concentrations of Chlorine dioxide

Chlorine dioxide (ppm)	Contamination (%)		Contamination (%)				Survival rate (%)
	With explants	Without explants	1 week	2 weeks	3 weeks	4 weeks	
sterilized	0.00^{d}	0.00	0.00^{b}	40.00^{b}	0.00^{d}	0.00^{b}	21.26^{b}
0	100.00^{a}	0.00	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	0.00^{c}
10	100.00^{a}	0.00	100.00^{a}	100.00^{a}	100.00^{a}	100.00^{a}	0.00^{c}
25	70.17^{b}	0.00	0.00^{b}	14.17^{c}	0.00^{d}	0.00^{b}	0.00^{c}
50	55.83^{c}	0.00	0.00^{b}	0.00^{d}	25.00^{b}	0.00^{b}	54.17^{a}
75	45.83^{c}	0.00	0.00^{b}	0.00^{d}	15.83^{bc}	0.00^{b}	32.50^{b}
100	0.00^{d}	0.00	0.00^{b}	0.00^{d}	11.67^{c}	0.00^{b}	0.00^{c}
F-test	*	-	*	*	*	*	*
C.V.(%)	0.45	-	0.63	0.57	0.59	0.63	1.53

* = significant difference at $p \leq 0.05$

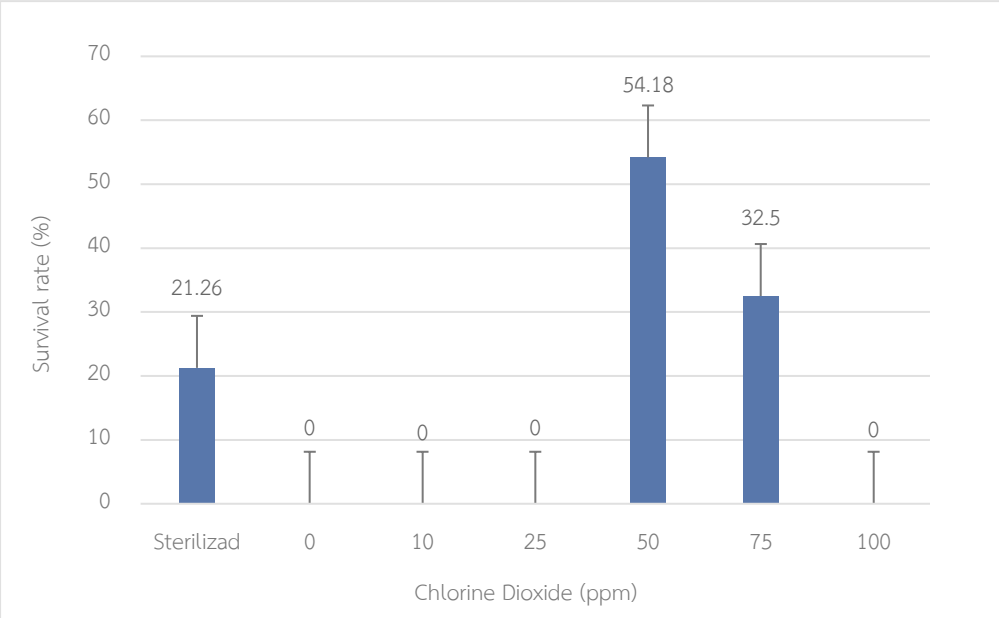

Figure 1. Survival rate formation of *G. mihanovichii* LB2178 Agua Dulce was cultured autoclaved or sterilized with different concentrations of Chlorine dioxide

The initial germination of *G. mihanovichii* LB 2178 Agua Dulce was observed 20 days following the experiment. The different concentrations of ClO_2 interfered in germination for each variable under study, and there was interaction between mediums in the evaluated variables. It was found that *G. mihanovichii* LB 2178 Agua Dulce seeds germination on MS medium supplemented with 50 ppm ClO_2 gave the highest germination at 61.67 %. Regarding *G. mihanovichii* LB 2178 Agua Dulce's germination time, the result found that *G. mihanovichii* LB 2178 Agua Dulce seeds germination on MS medium supplemented with 50 ppm ClO_2 gave the highest germination time at 15.50 days, followed by MS medium supplemented with 75 ppm ClO_2 (10.23 days) and autoclaved MS free medium (7.77 days), respectively. While MS medium supplemented with 0, 10, 25, and 100 ppm, ClO_2 gave germination time at 0.00 day, significant difference at $p \leq 0.05$. Germination speed index studies, the result found that seeds of *G. mihanovichii* LB 2178 Agua Dulce were cultured on MS medium supplemented with 50 ppm ClO_2 gave the highest germination speed index at 0.11, followed by MS medium supplemented with 75 ppm ClO_2 (0.06) and autoclaved MS free medium (0.04). While MS medium

supplemented with 0, 10, 25, and 100 ppm, ClO_2 gave a germination speed index of 0.00, a significant difference at p≤0.05. (Table 2). MS medium supplemented with 50 ppm ClO_2 gave the highest germination than other mediums because it is a concentration that does not damage seeds and helps reduce their dormancy period. As a result, seeds grown on MS medium with 50 ppm ClO_2 germinate at the fastest pace possible. Furthermore, ClO_2 effectively destroys viruses, fungi, and bacteria in the culture medium. This helps reduce the percentage of contamination in the culture medium. Corresponding with the report, the nutrient media recommended for *in vitro* germination of *Micranthocereus flaviflorus* is 1/2MS since it is the most economical option and has nutrients to allow healthy initial development of seedlings. Similar results were found for other plants. [19] Similar results were found for other plants, such as the effect of culture medium on seed germination and seedling performance, which was studied with a significantly higher number of seeds germinated per day on 1⁄4 and 1⁄2 MS media strengths than on full and 1⁄4 strengths of MS media. The highest GRI (7.74 seeds/day) was obtained for the gelrite culture, which differed significantly from the GRI for all strengths of MS media except the 1⁄4 strength. However, these seed cultures proved unsustainable for seedling establishment, which was best supported on a full-strength MS medium. *In vitro* seed propagation of *Hibiscus coddii* subsp. *barnardii* could be beneficial for faster commercial production of seedlings. [20]

Table 2. Effect of Chlorine dioxide on germination (%G), mean germination time (MGT), and germination speed index (GSI) of *G. mihanovichii* LB2178 Agua Dulce seeds were cultured on autoclaved culture medium and sterilized with different concentrations of Chlorine dioxide

Chlorine dioxide (ppm)	Germination (%)	Mean Germination Time (days)	Germination speed index
sterilized	31.67^{b}	7.77^{b}	0.04^{b}
0	0.00^{c}	0.00^{c}	0.00^{c}
10	0.00^{c}	0.00^{c}	0.00^{c}
25	0.00^{c}	0.00^{c}	0.00^{c}
50	61.67^{a}	15.50^{a}	0.11^{a}
75	39.17^{b}	10.23^{b}	0.06^{b}
100	0.00^{c}	0.00^{c}	0.00^{c}
F-test	*	*	*
C.V.(%)	1.18	1.42	1.54

* = significant difference at $p \leq 0.05$

3.2. Effect of Chlorine dioxide on proliferation and induction of shoot and root of G. mihanovichii LB2178 Agua Dulce

The shoots of *G. mihanovichii* LB 2178 Agua Dulce were cultured on autoclaved MS-free medium and MS medium supplemented with 0, 10, 25, 50, 75, and 100 ppm ClO_2. The result found that shoots of *G. mihanovichii* LB 2178 Agua Dulce were cultured on MS medium supplemented with 50 ppm ClO_2 and gave the highest number of roots at 9.00 roots/explant, root length at 3.45 cm, shoot length at 0.54 cm and shoot diameter at 0.44 cm., significantly different (p≤0.05) (Table 3). Shoots generated from *G. mihanovichii* LB 2178 Agua Dulce seeds germinated in a medium supplemented with 50 ppm ClO_2 were round and dark green. The characteristics of the roots are fibrous roots, long and slender, and light brown (Figure 2).

The study found that the concentration of ClO_2 affects the induction of shoots and roots of *G. mihanovichii* LB 2178 Agua Dulce seedlings. Because ClO_2 prevents cell expansion, high concentrations cause the plant to grow slowly, reducing growth in an autoclaved medium. This is because heat exposure during autoclaving might lead to the decomposition of some nutrients. As reported, Gerberas grown on medium supplemented with ClO_2 grew as well or better than gerberas grown on autoclaved medium. This may be because sterilization causes some nutrients to be lost from the use of high heat [21]. While reported on a study that contrasted the amounts of ClO_2 (1-25 mg/l) to the *in vitro* culture of fragrant rice parts to increase the number of shoots, it was found that bacterial and fungal contamination happened after the culture was kept

for three days. While the number of shoots could be increased up to 12 when the sterile parts were grown in a medium with 25 mg/l ClO_2, when compared to those grown in a medium sterilized by autoclave, it was found that the plates could be increased. The number of shoots can be doubled. This is because steam sterilization with an autoclave is a sterilization method for tissue culture medium that changes the chemical composition of the medium, such as some nutrients being lost from using heat, to make it sterile [22]. Additionally, sterilizing media with ClO_2 can prolong the sterilizing effect. However, it is also a strong oxidizing agent that can cause stress to potato seedlings. Thus, the effect of ClO_2 on the growth of potato seedlings requires further systematic evaluation. In this study, the morphological indices, including plant height, root length, branches, and biomass, decreased with increasing ClO_2 concentrations. Although some differences were observed among the three cultivars when cultured on Chlorine dioxide -sterilized media, seedlings cultured on 12.6 μM Chlorine dioxide -sterilized media were morphologically like those cultured on autoclaved media. In addition, microtubers induced on 17.6 μM ClO_2 sterilized media maintained an aseptic niche for two months, like autoclaved media [23]. The similar morphological features and rates of microtuber induction between autoclaved and ClO_2 sterilized media align with previous studies, including [24]. Recently, [25] reported that the elimination of epidermal wax from explants increased the *in vitro* growth of lily, suggesting that the status of explants is a probable factor that affects plant tissue culture. When disinfecting plant explants for tissue culture, a balance should be maintained between antimicrobial effects and plant cell viability [26]

Table 3. Effect of Chlorine dioxide on shoot and root induction of *G. mihanovichii* LB2178 Agua Dulce were cultured on an autoclaved culture medium and sterilized with different concentrations of Chlorine dioxide

Chlorine dioxide (ppm)	Number of roots (roots/explant)	Length of root (cm)	Length of shoot (cm)	Diameter of shoot (cm)
sterilized	2.53^{c}	0.53^{c}	0.23^{c}	0.18^{c}
0	0.00^{d}	0.00^{d}	0.00^{d}	0.00^{d}
10	0.00^{d}	0.00^{d}	0.00^{d}	0.00^{d}
25	0.00^{d}	0.00^{d}	0.00^{d}	0.00^{d}
50	9.00^{a}	3.45^{a}	0.54^{a}	0.44^{a}
75	4.07^{b}	1.38^{b}	0.34^{b}	0.29^{b}
100	0.00^{d}	0.00^{d}	0.00^{d}	0.00^{d}
F-test	*	*	*	*
C.V. (%)	0.71	1.02	0.79	0.69

* = significant difference at $p \leq 0.05$

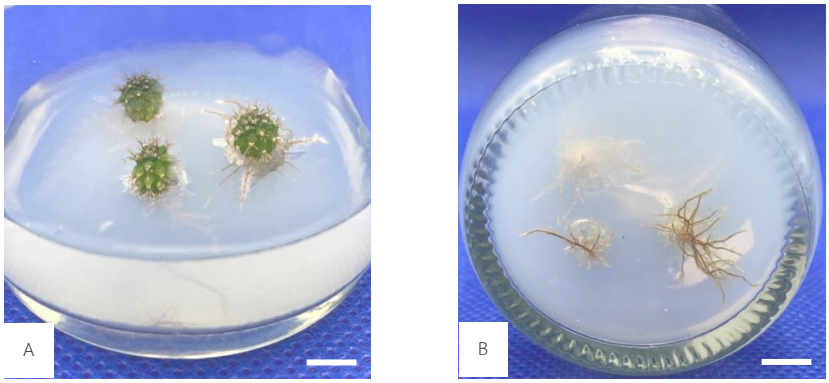

Figure 2. Characteristics of shoot and root formation of *G. mihanovichii* LB2178 Agua Dulce were cultured on MS medium supplemented with 50 ppm of Chlorine dioxide after culturing for 3 months (bar = 1 cm) (A) shoot formation (B) root formation

4. Conclusions

The results showed that *G. mihanovichii* LB2178 Agua Dulce can be propagated using *in vitro* techniques. After 2 months of culture, the results showed that MS medium supplemented with 50 ppm ClO_2 gave the highest survival rate at 54.17%. The initial germination of *G. mihanovichii* LB 2178 Agua Dulce was observed 20 days following the experiment. It was found that *G. mihanovichii* LB 2178 Agua Dulce seeds germination on MS medium supplemented with 50 ppm ClO_2 gave the highest germination at 61.67 %, the highest germination time at 15.50 days, and the highest germination speed index at 0.11. After 3 months of culture, the results showed that shoot and root induction can be achieved in *G. mihanovichii* LB2178 Agua Dulce seeds were cultured on MS medium supplemented with 50 ppm ClO_2 gave the highest number of roots at 9.00 roots/explant, length of root 3.45 cm, length of shoot 0.54 cm and diameter of shoot 0.44 cm. Sterilization by ClO_2 may help increase efficiency and an alternative to solving the problem of microbial contamination in tissue culture. It also helps increase work speed by reducing steps in preparing synthetic food instead of using an autoclave.

5. Acknowledgements

We thank Miss Wariya Nuannut for supervising and controlling the tissue culture room at the science center. And thank the science center in the Faculty of Science and Technology, Nakhon Si Thammarat Rajabhat University.

Author Contributions: A short paragraph specifying their individual contributions must be provided for research articles with several authors. The following statements should be used "Conceptualization, P.R. and S.R.; methodology, P.R.; software, S.R.; validation, P.R., S.R. and T.K.; formal analysis, P.R.; investigation, P.R.; resources, P.R. and T.K..; data curation, P.R..; writing–original draft preparation, P.R.; writing–review and editing, S.R.; visualization, T.K.; supervision, S.R..; project administration, P.R. and S.R All authors have read and agreed to the published version of the manuscript." Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

Funding: This research received no external funding

Conflicts of Interest: The authors declare no conflict of interest.

References

[1] Supanantananon, P. *Cactus*. Bangkok: Amarin printing and publishing. **2019**, 3-6.

[2] Verified Market Research. *Global Succulent Plant Market Size By Types, By Application, By Geographic Scope And Forecas*t. Available online: https://www.verifiedmarketresearch.com/product/succulent-plant-market/. (access on 7 October 2023).

[3] Supanantananon, P*). Cactus*. Bangkok: Amarin printing and publishing. **2019**, 7-10.

[4] Kanchanakul, S. *Roiphan Phrueksa Cactus*. Bangkok: Amarin printing and publishing, 2014; 105-108.

[5] Ramasoot, S. *Textbook on plant tissue culture*. Nakhon Si Thammarat: Nakhon Si Thammarat Rajabhat University. **2016**, 106-108.

[6] Balashanmugam, P.; Balakumaran, M.D.; Murugan, R.; Dhanapal, K.; Kalaichelvan, P.T. Phytogenic synthesis of silver nanoparticles, optimization and evaluation of *in vitro* antifungal activity against human and plant pathogens. *Microbiological Research*. **2016**, *192*, 52-64. http://dx.doi.org/10.1016/j.micres.2016.06.004

[7] Cardoso, J. C.; da Silva, J. A. T. Micropropagation of Gerbera using chlorine dioxide (ClO_2) to sterilize the culture medium. *In Vitro Cellular & Developmental Biology-Plant*. **2012**, *48*(3), 362-368.

[8] Te-chato, S.; Yuso, A.; Domyoas, P. Proliferation of *Lepironia articulate* from Culturing Shoots by Air Bubble Bioreactor. *Princess of Naradhiwas University Journal*. **2017**, *9*(2), 83-88.

[9] Cardoso, J. C. & Imthurn, A. C. P. Easy and efficient chemical sterilization of the culture medium for *in vitro* growth of Gerbera using chlorine dioxide (ClO_2). *Ornamental Horticulture*. **2018**, *24*(3), 218–224. http://dx.doi.org/10.14295/oh.v24i3.1222

[10] Duan, Y.; Zhang, H.; Sun, M.; Zhao, F.; Xue, T.; Xue, J. Use of chlorine dioxide to sterilize medium for tissue culture of potato. *Scientific Reports*. **2019**, *9*, 1-9. https://doi.org/10.1038/s41598-019-46795-4

[11] Murashige, T.; Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant*. **1962**, *15*, 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

[12] Brasil. *Rules for seed analysis. Ministry of Agriculture, Livestock and Food Supply*. Secretariat of Agricultural and Livestock Defense. Brasília: MAPA/ACS. **2009**, 148-224.

[13] Srichuay, W.; Te-chato, S. Effect of chlorine dioxide (ClO_2) on sterilization in micropropagation of pineapple cv. Phulae by bioreactor system. *KHON KAEN AGRICULTURE Journal*. **2014**, *42*(3), 75-80.

[14] Duan, Y.; Zhang, H.; Sun, M.; Zhao, F.; Xue, T.; Xue, J. Use of chlorine dioxide to sterilize medium for tissue culture of potato. *Scientific Reports*. **2019**, *9*, 1-9. https://doi.org/10.1038/s41598-019-46795-4

[15] Cardoso, J. C.; da Silva, J. A. T. Micropropagation of Gerbera using chlorine dioxide (ClO_2) to sterilize the culture medium. *In Vitro Cellular & Developmental Biology-Plant*. **2012**, *48*(3), 362-368.

[16] Lenntech. *Disinfectants: Chlorine dioxide*. Available online: http://www.lenntech.com/processes/disinfection/chemical/disinfectants-chlorine-dioxide.htm. (access on 10 October 2023).

[17] Huang, J.; Wang, L.; Ren, N.; Ma, F.; Ma, J. Disinfection effect of chlorine dioxide on bacteria in water. *Water Res.* **1997**, *31*, 607–613. https://doi.org/10.1016/S0043-1354(96)00275-8

[18] Srebernich, S.M. Using chlorine dioxide and peracetic acid as substitutes for sodium hypochloride in the sanitization of minimally processed green seasoning. *Ciência e Tecnologia de Alimentos*. **2007**, *27*(4), 744-750.

[19] Civatti, L.M.; Marchi, M.N.G.; Bellintani, M.C. Micropropagation of two species of *Micranthocereus* (Cactaceae) with ornamental potential native to Bahia, Brazil. *African Journal of Biotechnology*. **2017**, *16*(14), 749-762. https://doi.org/10.5897/AJB2016.15901

[20] Plessis, H.J.d.; Nikolova, R.V.; Kleynhans, R.; Egan, B.A. *In vitro* seed germination and seedling performance of *Hibiscus coddii* subsp. *Barnardii*. *Ornamental Horticulture.* **2020**, *26*(4), 598-606. https://doi.org/10.1590/2447-536X.v26i4.2191

[21] Cardoso, J. C.; da Silva, J. A. T. Micropropagation of Gerbera using chlorine dioxide (ClO_2) to sterilize the culture medium. *In Vitro Cellular & Developmental Biology-Plant*. **2012**, *48*(3), 362-368.

[22] Yusoh, A.; Te-chato, S. Propagation of hom Kra-Dang-Nga rice through tissue culture technic and its conservation *in vitro*. *Songklanakarin Journal of Plant Science.* **2015**, *2*(3), 12-16.

[23] Duan, Y.; Zhang, H.; Sun, M.; Zhao, F.; Xue, T.; Xue, J. Use of chlorine dioxide to sterilize medium for tissue culture of potato. *Scientific Reports*. **2019**, *9*, 1-9. https://doi.org/10.1038/s41598-019-46795-4

[24] Pais, A. K. *et al*. Sodium hypochlorite sterilization of culture medium in micropropagation of *Gerbera hybrida* cv. *Essandre*. *African Journal of Biotechnology*. **2016**, *15*, 1995–1998. https://doi.org/10.5897/AJB2016.15405

[25] Askari, N.; De Klerk, G.J. Elimination of epidermal wax from explants increases growth in tissue culture of lily. *Scientia Horticulturae-Amsterdam*. **2020**, *274*, 109637. https://doi.org/10.1016/j.scienta.2020.109637

[26] Duan, Y.; Zhao, F.; Li, H.; Zhou, Y.; Zhu, X.; Li, F.; Chen, W.; Xue, J. Evaluation of aqueous chlorine dioxide for disinfecting plant explants. *In Vitro Cellular and Developmental Biology*. **2016**, *52*, 38–44. https://doi.org/10.1007/s11627-015-9736-3