OPTIMIZING MEDIA STERILIZATION VIA CHLORINE DIOXIDE AND AUTOCLAVING OF PAULOWNI MICROPROPAGATION

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ABSTRACT:
This study was aimed to investigat integrated system for *in vitro* growth of paulownia plants by assessing the efficacy of chlorine dioxide (ClO_2) as an alternative to autoclave in sterilizing culture medium. Therefore, this study was devised to compare autoclave sterilization at three different times (5, 10, and 15) minutes and three different concentrations of ClO_2 (0, 0.4, 0,8, 1) mg/L. The results showed that, compared with (0.4) mg/L concentration, concentrations of (0.8 and 1) mg/L are more effective at sterilizing the culture medium. ClO_2 sterilization improved individual single node growth more than autoclave sterilization. Since ClO_2 is non-toxic, it could be used as a safe alternative to autoclave when propagating paulownia *in vitro*. Culture medium sterilization in the autoclave takes only 5 minutes, compared with the standard 15 minutes. At initiation stage, growing single nodes in the Murashige and Skoog medium (MS) prepared with 0.5 mg/L Benzyl Adenine (BA) resulted in a 100% response rate, while doing the same in the Woody Plant Medium (WPM) resulted in a 20% response rate. The 1 BA + 0 a-Naphthalene Acetic Acid (NAA) mg/L treatment was effective during vegetative multiplication stage, the highest average number of shoots produced by a plant treated with the mentioned concentration was 6.40 shoot per explant. During the rooting stage, Indole Butyric Acid (IBA) at a concentration of 2 mg/L was more effective than NAA, the typical number of roots produced by with 27.40 root per shoot. After two months in their natural environment, the plants' acclimatization rate was at a perfect 100%.

Key word: tissue culture, sterilize medium, plant propagation, plant growth regulators.

تأثير تعقيم الوسط الغذائي بثنائي اوكسيد الكلور والموصدة في الأكثار الدقيق لنبات الباولونيا

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المستخلص:
الهدف من هذه الدراسة معرفة طريقة جديدة لتعقيم الاوساط الغذائية بأستعمال chlorine dioxide (CLO_2) مقارنةً بالموصدة، ومن ثم رسم نظام متكامل لأكثار نبات الباولونيا خارج الجسم الحي. لذلك صممت هذه الدراسة للمقارنة بين التعقيم بثلاث مستويات من CLO_2 (0، 04، 08 و 1 ملغم /لتر) والتعقيم بجهاز الموصدة (Autoclave) وبثلاثة مدد (5، 10، 15 دقيقة). . أشارت النتائج إلى أن التركيزين 0.8 و 1 ملغم /لتر أفضل في تعقيم الأوساط الغذائية مقارنة بالتركيز 0.4 ملغم /لتر. وأن التعقيم باستعمال CLO_2 أدى إلى نمو العقد المفردة بصورة افضل من نموها عند استعمال الموصدة. وبذلك يمكن استعمال CLO_2 في تعقيم الاوساط الغذائية بديلاً من الموصدة، من غير تأثيرات سامة عند أكثار الباولونيا خارج الجسم الحي. كما يمكن استعمال الموصدة في تعقيم الاوساط الغذائية لمدة 5 دقيقة فقط بدلاً من 15 دقيقة. كما بينت النتائج نجاح تقانة الاكثار الخضري الدقيق لنبات الباولونيا، وذلك بزراعة العقد المفردة في الوسط الغذائي Murashige and Skoog medium (MS) والمجهز o.5 ملغم /لتر Benzyl Adenine (BA) والذي اعطى نسبة استجابة بلغت 100% في حين اعطى الوسط الغذائي Woody Plant Medium (WPM)، والمجهز o.5 ملغم /لتر BA نسبة استجابة 20% لنمو العقد المفردة. أما في مرحلة التضاعف الخضري فقد تفوقت معاملة 1ملغم . لتر$^{-1}$ BA + 0 ملغم . لتر$^{-1}$ من NAA(a–Naphthalene Acetic Acid) , اذ اعطت اعلى معدل لعدد الافرع بلغ 6.40 فرع.جزء نباتي$^{-1}$. وبالنسبة لمرحلة التجذير فقد تفوق الاوكسين Indole Butyric Acid (IBA) عند التركيز 2 ملغم . لتر$^{-1}$ اذ اعطى اعلى معدل لعدد الجذور بلغ 27.40 جذر / فرع$^{-1}$، وبلغت نسبة نجاح عملية الاقلمة 100 % بعد شهرين من نقل النبيتات الى الظروف الطبيعية.

الكلمات المفتاحية: الزراعة النسيجية، تعقيم الوسط الغذائي, إكثار النباتات، منظمات النمو النباتية.

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INTRODUCTION

Culture medium sterilization is a crucial part of plant tissue culture technology, which consists of a series of interrelated steps and processes. Sterilization is thus one of the most fundamental necessities for creating sterile cultures. Eliminating the fungi, bacteria, and their metabolites that rapidly proliferate on the surface of the culture media and ultimately destroy the cultivated explant requires a high degree of precision. The culture media is typically autoclaved at a temperature of 121 degrees Celsius, 1.04 kg / cm^2 pressure for 15-20 minutes. There are a number of drawbacks to this method includes the reality that it consumes an extra power, needs prolonged sterilization, calls for specialized containers and tubes that can endure high temperatures, and could be damage organic and inorganic components of culture media (21; 34). The Paulownia tree (*Paulownia tomentosa*) is a member of the Lamiaceae family and is an evergreen member of the genus *Paulownia*. It's one of the quickest growing and most common trees, and it has anywhere from 6 to 17 different species. Even though Japan, Korea, the United States of America, and Vietnam all have very different climates from China, the paulownia tree spread rapidly from China to these other regions. Amazingly, this tree is hardy in many different climates and can even be found in the Middle East and North Africa (40). With a flowering time of 4-8 weeks and a range of colors from white to purple and an aromatic scent, this tree is ideally suited for use as an ornamental in urban areas, green spaces, and forests (8; 18). Paulownia tree, according to scientific research, has properties that allow it to withstand climatic changes (1; 19). Its leaves have a high protein content—up to 18 percent—and are used as animal feed. It also has a low water requirement, requiring only once-weekly irrigation during the summer and never being watered at all during the winter, Antioxidants, antispasmodics, and anti-inflammatory medicinal compounds are present in its explants (leaves, bark, wood, and fruits; 2; 24; 39). Traditional seed-based paulownia plant propagation cannot be relied upon for clonal propagation due to the paulownia seed's susceptibility to fungal, bacterial, and viral diseases, as well as its weak germination and slow growth (27; 33). Vegetative propagation and other forms of plant multiplication have been given a significant boost by advances in plant tissue culture technology (28, 29, 30). because unlike conventional methods, it could be rapidly generate a large quantity of genetically uniform plant stock. Tissue culture has many purposes, one of the most common being vegetative propagation (37). Following various methods of differentiation and morphological formation, such as the formation of adventitious buds, axillary buds stimulating, and induction of somatic embryos, are the royal method for propagating plants vegetatively, as described by (4 , 13). Finding chemicals with high effectiveness against fungi, bacteria, and viruses, and at a lower cost than the autoclave device, has been shown in scientific studies as an alternative method for sterilizing agricultural medium. Chlorine dioxide, peracetic acid, and dithyl pyrocarbonate are examples of such substances (12; 15; 38). Chemical sterilization with chlorine dioxide (CLO_2) to sterilize the nutritional medium for Persian Violet plant propagation at concentrations (15, 10.5 mg) gave no contamination rates, and CLO_2 had no toxic effect on the growth of the mentioned plant (36). The objectives of this study : developing a chlorine dioxide–based sterilization process for culture media as an alternative to autoclave, which would save time.

MATERIALS AND METHODS

Initiation stage: This study was implemented on a paulownia tree, eight years old at the plant tissue culture lab / plant protection office/Ministry of Agriculture/Abu Ghraib from February2023 till October 2023. Two different procedures were applied to sterilize the medium:

A- Different concentrations of Chlorine dioxide independently 0, 0.4, 0.8, and 1 mg/L (12).

B- Autoclaves for 5, 10, and 15 minutes.

Medium were routinely checked up for contamination and after ten days of incubation, contamination percentage was calculated (for both procedures) by applying the following equation:

Contamination % = (contaminated test tubes / total test tubes) * 100.

For culture initiation, 5-centimeter-long of young and healthy stem cuttings were excised, washed for 30 minutes with tab water, disinfected in a laminar flow air for 10 minutes with NaOCl (commercial minor FAS solution, 6% concentration, 7% concentration), and then rinsed three times with distilled water (5). Explants that have been sterilized are grown vertically on culture media. Typically, 1 cm-long pieces of the nodal stem are removed lengthwise, and the area containing the bud is cultivated with the cut surface in contact with the media. Different basal culture media were tested in order to identify the optimal medium for *in vitro* establishment of paulownia, including MS and WPM (17; 26). Both basal media were fortified with 0.5 mg/L BA.

Vegetative multiplication stage

An experiment was applied to examine the effects of various concentrations of BA 0, 0.5, 1, 2, and 4 mg/L separately or interaction with NAA 0.0 and 0.2 mg/L(14; 31). The average number of shoots and their length were recorded after 6 weeks.

Rooting stage

The third experiment examined the effectiveness of two auxins, IBA and NAA, at various concentrations for root induction. The elongated shoots (4.0 cm in length) were cultivated on half strength MS medium that had been enriched with 3% sucrose, 0.7% agar, and IBA 0.0, 0.2, 0.5, 1.0, 2.0 mg /L or NAA 0.0, 0.2, 0.5, 1.0, 2.0 mg /L (23; 32). After 6 weeks, Data were calculated on:

A-Mean number of roots per plant

B- Root length (cm)

The medium used in all of the mentioned experiments had their pH adjusted to 5.7 before being autoclaved at 121°C for 5 minutes. The culture was incubated in a controlled environment (16/8 hours of photoperiod, 21°C, and 50% relative humidity). Weekly notes were documented and statistically evaluated.

Acclimatization stage

Both 1:1:1 and 1:1:0 mixtures of sand, peat moss, and high-purity sand were evaluated and incubated in the growth chamber for eight weeks within plastic boxes at 27 + 2°C and under illumination intensity of 3000 lux for sixteen hours of light and eight hours of darkness. After 8 weeks in the lab, they were released into the field and their survival rates were determined (3, 4).

Statistical analysis

Each treatment had 10 replicas in each experiment, with 1 explant in each test tube. The experiments were designed using Completely Randomized Design (25). Significant differences between means were recorded using least significant differences (L.S.D) at 0.05 level.

RESULTS AND DISCUSSION

Initiation Stage: Sterilization of culture medium: Table 1 shows that the percentage of pollution decreased with increasing concentrations of chlorine dioxide, from 0% for the control treatment (without ClO_2) and autoclave sterilization to 10%, 0%, and 0%, respectively at concentration 0.4 – 0.8 -1 mg/L respectively (Figure 1). The acidity (pH) of the medium, the readiness of the elements, and the hardness of the nutrient medium were all measured before and after the chemical sterilization of the medium with ClO_2. There was no toxic effect on Paulownia plant tissue cultures at any of the concentrations tested. One possible explanation is that the chemical composition of culture medium is less likely to shift after ClO_2 chemical sterilization than during wet thermal sterilization. It also has potent antimicrobial, antiviral, and antifungal properties. It is stable in solutions between a pH of 9.0 to 3.0, with the maximum efficiency in sterilizing at neutral pH (10; 35), it does not form harmful chloramines when added to culture medium.

Table 1. Effect of chlorine dioxide (ClO_2) concentrations on the percentage of contamination of culture media used to propagate paulownia plants *in vitro*

Concentrations m g/L (ClO2)	Pollution of culture media (%)
(Sterilized via autoclave)	0.00
0.4	10.00
0.8	0.00
1	0.00

Table 2 shows that after autoclaving for 5, 10, and 15 minutes, there is no detectable contamination in the culture medium

Table 2. Effect of autoclave sterilization on the percentage of contamination of culture media used to propagate Paulownia plants *in vitro*

Extended thermal sterilization (minute)	pollution of culture media (%)
5	0.00
10	0.00
15	0.00

Figure 1. The effect of chemical sterilization using ClO_2 on the percentage of contamination of the medium and the growth of explants of the paulownia plant

Vegetative multiplication stage:

Effect of cytokinin BA and auxin IAA

Table 3 shows that at a concentration of 1 mg /L BA performs much better than the control. There was no significant difference in the average number of shoots produced between doses of 0.1 and 2.0 mg /L Explant^{-1}. The concentration was higher than 0.2 mg/L, as shown in the same table. The average number of shoots produced by plants treated with 1 of NAA was 3.60, substantially greater than the results obtained with any of the other concentrations. Explant. The impact of BA concentrations with 1 mg/L of NAA was detected. 1 BA + 0 mg/L of NAA performed exceptionally well, resulting in the greatest average number of shoots, at 6.40 (Figure 2). In contrast to the other treatments, Explant^{-1} produced 5.60 shoots when given 2 BA + 0.2 mg/L of NAA Explant. This might be because cytokinins, and BA in particular, encourage cell division and differentiation in plants, as well as nutrient uptake by treated explants (11). Cytokinins promote cell growth, division, and morphological differentiation, and they also inhibit the catabolism of protein and chlorophyll while activating enzymes involved in photosynthesis. Within the plant cell, cytokinins have a crucial role in the synthesis of RNA, proteins, and enzymes (6, 7).

Table 3.Effect of BA and NAA concentrations and their interaction on the rate of the number of multiplying shoots of Paulownia after 6 weeks of culture on MS medium sterilized with ClO_2

BA concentrations	NAA concentrations			average BA
	0	0.2	0.5	
0	0.00	0.00	0.00	0.00
0.5	2.40	3.80	5.40	3.87
1	6.40	5.20	3.40	5.00
2	2.60	5.60	2.60	3.60
4	2.40	3.40	1.80	2.53
LSD 0.05		0.96		0.56
NAA average	2.76	3.60	2.64	
LSD 0.05		0.43		

Figure 2. Multiplication of paulownia shoots grown on prepared 1 mg /L **BA MS medium**

According to Table 4, the average shoot length (cm) increased when BA concentrations were more than 4 mg/L. The average length of the shoots at 3.00 cm BA was not substantially different from the other concentrations. Regarding the effect of NAA concentrations, the same data reveals that 0.5 mg/L has the

greatest impact, The average length of the longest shoots increased to 2.31 cm in the presence of NAA, although this trend was consistent across all concentrations. The 1 and 0.5 mg/L application of BA and NAA in the culture media resulted in the fastest growth of shoots due to their combined impact. The average length of BA grown shoots was 3.20 cm, which was not substantially longer than that of 0.5 mg/L NAA+ 2 mg/L BA. The average length of the shoots dropped as the BA content increased, as seen in the same table. The greater rivalry amongst shoots might be to blame for the shorter average shoot length that has been seen since the number of shoots has grown.

Table 4. Effect of BA and NAA concentrations and their interaction on the rate of multiplying shoot lengths (cm) of Paulownia after 6 weeks of culture on MS medium sterilized with ClO_2

BA concentrations	NAA concentrations			average BA
	0	0.2	0.5	
0	0.00	0.00	0.00	0.00
0.5	2.93	2.72	2.12	2.59
1	2.00	2.90	3.20	2.70
2	3.00	2.54	3.15	2.90
4	3.42	2.51	3.06	3.00
LSD 0.05		0.28		0.16
NAA average	2.27	2.14	2.31	
LSD 0.05		0.13		

Figure 3. Rooting of paulownia shoots grown on MS medium with half the prepared salt strength of 2 mg/L IBA

Rooting stage

Effect of auxin IBA concentrations when using MS medium with half strength salts:

Table 5 shows the results of growing Paulownia shoots in MS media supplemented with IBA at a dose of 2 mg /L The typical number of roots produced by, was 27.40 (Figure 3). The number of shoot was dramatically different from all the others, with the exception of the 1 mg/L concentration. An average root length increase of 2 mg/L was seen after IBA was applied When compared to the other length rates, the longest one stands out at 6.80 cm. The explanation for this is that lowering the concentrations of the salts in the MS nutritional medium caused an increase in the quantity of roots and the lengths of those roots by affecting the ratio of carbohydrates to nitrogen (C/N). The impact of the salts was cut in half, while the effect of the sucrose, which was supplied at a constant 30 g, was amplified. Since sucrose is an energy source essential to the rooting process, its effect is amplified when salt concentrations are lowered (16, 22). This encourages the growth and development of roots. Not only does IBA effectively promote root development on emerging shoots, but it also Because it is resistant to degradation by auxin-catabolizing enzymes, a concentration of 2 mg /L of this compound has been shown to improve root growth rate (9).

Table 5. Effect of IBA concentrations on the average number of roots and their lengths of Paulownia shoots grown on MS medium with half the strength of its salts sterilized with ClO_2 after 6 weeks of planting

IBA concentrations (mg /L)	Number of roots	Root lengths (cm)
0	1.20	0.92
0.2	11.00	3.50
0.5	18.20	3.95
1.0	25.60	3.66
2.0	27.40	6.80
LSD	0.700	0.700

Effect of auxin NAA concentrations when using MS medium with half strength salts

Table 6 shows that 1 mg/L of NAA in half-strength MS culture medium yields the best results. The average number of roots produced by was 19.00 Roots.Shoot-1. One of the most dissimilar concentrations. The same table also shows that different concentrations of NAA had different effects on root lengths, with the longest roots being observed in the half-strength MS medium to which 1 mg/L of NAA

was added. The maximum length of the roots at 0 mg/L was 1.54 cm, which was much shorter than at any of the other doses. (no NAA).

Table 6. Effect of NAA concentrations on the average number of roots and their lengths of Paulownia shoots grown on MS medium with half the strength of its salts sterilized with ClO_2 after 6 weeks of culture

NAA concentrations (mg /L)	Number of roots	Root lengths (cm)
0	2.40	1.54
0.2	9.20	5.26
0.5	11.80	3.86
1.0	19.00	6.54
2.0	13.40	3.00
LSD	3.066	0.929

Stage of acclimatization and transportation of the soil: Table 7 shows that the percentage of acclimatized plants that survived was positively influenced by the type of composed used in their growth. The results showed the superiority of two composed consisting of sand, peat moss, and high-purity sand in ratios of 1:1:1 and 1: 1:0, with a 100% survival rate for the acclimatized plants in both cases (Figure 4). These two composed are preferred because they retain the necessary moisture for plant growth and because of the nutrients they contain, making them ideal for root development, plant expansion, and ultimately plant survival (20). Paulownia trees recycle agricultural soil substrates, which improves soil fertility and removes heavy metals, lowering water, soil, and air pollution. Besides surviving climate swings to offer a favorable setting for other plants, the tree maintains environmental balance, therefore its proliferation is crucial to sustainable agriculture.

Table 7. The effect of the type of growing medium on the percentage (%) of survival of Paulownia plants resulting from the rooting stage

Type of growing medium	Survival percentage (%)
river soil 1: Peat moss1: High purity sand1	100.00
river soil 0: Peat moss 1: High purity sand1	90.00

Figure 4. The stage of acclimatization of Paulownia plants and transfer to the soil

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