

Optimisation of shoot bud surface sterilisation technique for *Curcuma xanthorrhiza* Roxb.

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Abstract

Curcuma xanthorrhiza, also known as Java turmeric, is a medicinal plant that has been used for centuries. Because of the growing global market demand for this species, several biotechnology studies involving *in vitro* propagation and production have been conducted. Unfortunately, high costs are incurred as a result of the high contamination level of shoot bud explants. As a result, it is addressed in this study in order to solve the contamination problem. Surface sterilisation is extremely difficult in most plants, making sterilisation procedures critical for successful cultures. A total of twelve distinct sterilisation methods were assessed on the explants where 70% ethanol and 5% sodium hypochlorite were added with fungicide or mercuric chloride with different time explore for surface sterilisation with the goal of identifying the best method to establish an *in vitro* culture. The sterilised shoot bud explants were then cultured on Murashige & Skoog (MS) media with no additional vitamin or plant growth regulators under light below 25°C. Based on this study, a combination of 70% ethanol + 5% NaOCl + 0.1% HgCl₂ for 10 min was the best to reduce the contamination rate to 25% with a 65% survival rate of *C. xanthorrhiza* explant.

1. Introduction

Tissue culture is one method to propagate plants faster and efficiently and can be done by direct or indirect organogenesis (Oo *et al.*, 2018). A part of the plant is excised from the mother plant and brought to an aseptic environment to be grown in culture medium with desired nutrients. Plants grown using this method are usually called *in vitro* plants.

Curcuma xanthorrhiza Roxb. belongs to the Zingiberaceae family and is widely cultivated throughout Indonesia, Malaysia, Thailand, Vietnam, and the Philippines. However, due to it is dormant throughout the drought season and breaks dormancy during the rainy season. Temulawak dormancy, as well as its availability, has been an obstacle for off-season cultivation (Ratri *et al.*, 2018). Moreover, a large amount of rhizomes is needed due to their low vegetative propagation (Kusumastuti *et al.*, 2014). Traditionally, *C. xanthorrhiza* has been extensively used throughout its local distribution area as a component of herbal supplements and medicines or to treat and control a wide range of illnesses and disorders, including lack of

appetite, stomach disorders, liver problems, constipation, bloody diarrhoea, dysentery, arthritis, children's fevers, hypotriglyceridemia, haemorrhoids, vaginal discharge, rheumatism, and skin eruptions (Rahmat *et al.*, 2021). *In vitro* is known to be one of the methods to overcome these problems in temulawak propagation (Ratri *et al.*, 2018).

Despite having a lot of benefits in every aspect of study, tissue culture techniques usually face the same challenge, which is contamination that may be caused by microorganisms from bacteria, fungi and viruses (Oo *et al.*, 2018). This can inhibit the growth of *in vitro* plants, as well as causing the necrosis of plants. In this case, the *in vitro* plant will be discarded immediately, which can reduce production quantity and quality. In order to ensure that contamination in an *in vitro* plant does not happen, an efficient surface sterilisation protocol needs to be discovered and followed. This may not ensure a totally free contamination culture, but it can reduce the contamination rate in tissue culture, which is favourable to every study.

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Different species of plants require different surface sterilisation methods. This was done during surface sterilisation steps during the tissue culture procedure. The purpose of surface sterilisation is to reduce or eliminate microbial organisms that might attach to the planting materials during explant isolation (Irmawati *et al.*, 2022). To produce healthy *in vitro* plants, efficient sterilisation techniques are necessary to eliminate microorganism contamination (Hardjo and Krisnawan, 2020). Chemicals used as sterilising agents are mostly toxic to plant cells and may lower the viability of the explant (Sandhyarani *et al.*, 2018).

Some of the common surface sterilisation agents used are ethanol, sodium hypochlorite (NaOCl) or bleach, and mercuric chloride (HgCl₂). Some studies include the use of bactericides and fungicides during this step. According to Rout *et al.* (2020), bacteria and fungi are the main cause of microbial contaminants. Furthermore, different concentrations and time exposure are necessary for different types of explants, considering their plant species and plant parts. It is crucial to produce contamination-free *in vitro* plants.

Ethanol is a very common sterilisation agent that is used as a pre-treatment and is responsible for phototoxicity and dehydration of plant tissue (Rosle *et al.*, 2021). NaOCl is a sterilisation agent that has been proven to significantly reduce contamination rates for *in vitro* growth. Tween 20, which acts as a surfactant and wetting agent, plays an important role, including eliminating the infestation and disrupting the surface tension of the explants to increase the permeability of sterilising agents (Rosle *et al.*, 2021). In some cases where the pathogen is hard to eliminate, the use of HgCl₂ is needed despite its toxicity characteristics (Mng'omba *et al.*, 2012).

Apart from using effective surface sterilisation agents, time exposure of the explant to surface

sterilisation is also crucial. The underlying cause is that sterilisation agents are mostly toxic to plant cell tissue, and a longer exposure time could cause cell necrosis. However, if the explant was exposed to sterilisation agents for a short amount of time, the presence of endophytic pathogens in the plant cells could later cause contamination during the incubation period. Thus, choosing the best concentration of agents and time exposure for surface sterilisation agents is vital.

In this study, the combination of surface sterilisation agents and time exposure is used to determine the best protocol in reducing the *in vitro* contamination rate of *C. xanthorrhiza* species.

2. Materials and methods

Rhizome bud of *Curcuma xanthorrhiza* Roxb was used as an explant in this study. Murashige & Skoog basal medium was used as culture medium and was supplemented with 30 g/L of sucrose, agar, pH at 5.7 to 5.9 and then autoclaved at 121°C for 20 min. Different surface sterilisation agents such as 70% ethanol, 5% sodium hypochlorite (NaOCl) and mercury chloride (HgCl₂), and time exposure (5, 10 and 15, 60, and 120 min) were used for treatment such as shown in Table 1. Rhizome bud was brought to the UiTM Jasin tissue culture laboratory and ran under tap water for 1 hr to remove dirt and debris from its surface. The explant was then brought to a laminar flow hood to undergo the surface sterilisation stage. The explant was air-dried on sterilised filter paper. Using sterile scalpel and forceps, a final size of 0.3 to 0.5 cm was used and transferred to basal medium and brought into an incubation room with 24 h light and 25±2°C. Percentage of contamination, survival rate, as well as explant colour were observed; the data were collected 7 days after inoculation.

Table 1. Different surface sterilisation agents and time exposure used in reducing contamination rate, n = 20.

Treatment code	Sterilisation methods	Time (min)
S1	70% ethanol + 5% NaOCl	5, 5
S2	70% ethanol + 5% NaOCl	10, 10
S3	70% ethanol + 5% NaOCl	15, 15
S4	1 g/L Benomyl (fungicide), 70% ethanol, 5% NaOCl + Tween 20	60, 5, 5
S5	1 g/L Benomyl (fungicide), 70% ethanol, 5% NaOCl + Tween 20	60, 10, 10
S6	1 g/L Benomyl (fungicide), 70% ethanol, 5% NaOCl + Tween 20	60, 15, 15
S7	1 g/L Benomyl (fungicide), 70% ethanol, 5% NaOCl + Tween 20	120, 5, 5
S8	1 g/L Benomyl (fungicide), 70% ethanol, 5% NaOCl + Tween 20	120, 10, 10
S9	1 g/L Benomyl (fungicide), 70% ethanol, 5% NaOCl + Tween 20	120, 15, 15
S10	70% ethanol + 5% NaOCl + 0.1%HgCl ₂	5, 5, 5
S11	70% ethanol + 5% NaOCl + 0.1%HgCl ₂	10, 10, 10
S12	70% ethanol + 5% NaOCl + 0.1%HgCl ₂	15, 15, 15

3. Results and discussion

The explant was assessed visually after 7 days of inoculation by observing the appearance of bacteria and/or fungus. Based on Table 2, S12 has the lowest contamination rate of 10% compared to S1, S2, S4 and S5 with a 100% contamination rate. The appearance of microbial organisms was recorded to appear two to three days after the inoculation date such as shown in Figure 1. Table 2. Percentage of survival, necrosis and contamination rate of explant in response to different surface sterilisation agents and time exposure, n = 20.

Treatment code	Explant response (%)		
	Survived (free contamination)	Necrosis (free contamination)	Contaminated explants
S1	0	0	100
S2	0	0	100
S3	5	15	80
S4	0	0	100
S5	0	0	100
S6	0	10	90
S7	0	5	95
S8	10	0	90
S9	5	15	80
S10	15	0	85
S11	65	10	25
S12	40	50	10

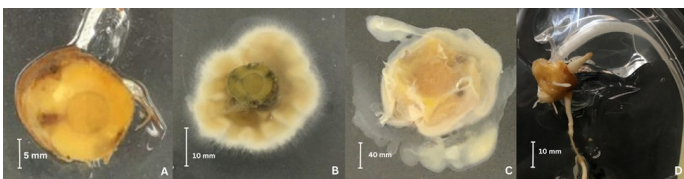


Figure 1. (A) Explant on 0-DAI, (B) and (C) Contaminated explant with the appearance of microbial organisms, (D) Healthy explant with the appearance of root 7-DAI.

The combination of 70% ethanol with 5% NaOCl + Tween 20 was observed to have no effect in reducing the contamination rate. Although S3 treatment used higher time exposure to the explant and was recorded to have 5% survival rate compared to S1 and S2, the contamination rate of the S3 treatment is still high (80%). This is because the explant was only able to survive up until 4 to 5 days after inoculation before the explant was infected with microbial organisms. It may be because the treatment was only able to eliminate epiphytic fungi infestation, while endophytic fungi were able to survive and appear later during the incubation period. In a study conducted by Nongalleima *et al.* (2013) on *Zingiber zerumbet* (L.), the use of 70% ethanol (10 min) and HgCl₂ (15 min) was able to reduce the contamination rate to 18.71%.

The use of fungicides as sterilising agents in this study has not been successful in reducing the contamination rate either. In comparison, Hardjo and

Krisnawan (2020) conducted a study on *Zingiber officinale* Roxb. using a combination of 100 mg/L Benomyl for 60 min, before using Cefotaxime for 60 min and NaOCl for five min was able to produce only a 10% contamination rate. This is contra to this study, where the application of fungicide does not reduce the contamination rate in *C. xanthorrhiza*.

On the other hand, S11 treatment was able to lower the contamination rate of the explant to 25%. A healthy explant can be seen to produce roots 7 days after inoculation date (Figure 1). The use of HgCl₂ as a sterilising agent successfully reduces the contamination rate, as well as increasing the survival rate. Mercuric chloride, despite being poisonous and toxic, is an effective steriliser and widely used to reduce contamination in tissue culture as well as stimulating seed germination (Nongalleima *et al.*, 2013). Previous study on *Zingiber zerumbet* (L.) by Nongalleima *et al.* (2013) noted that the use of HgCl₂ (15 min) reduced the contamination rate to 18.71% with the highest percentage of survival rate (83.6%). In a study conducted by Yusuf *et al.* (2007), the use of 0.1% HgCl₂ for 5 min recorded a low contamination rate in *Curcuma* species *in vitro* growth.

Even though S12 shows the lowest contamination rate of 10%, the survival rate of the explant is reduced to 40% compared to S11, with 65%. Longer time exposure of the explant to sterilising agents caused toxicity to the explant as the sterilising agents seeped into the plant and caused damage to the host tissue (Mng'omba *et al.*, 2012). Toxicity to the explant is caused by the increase in time exposure or high concentration of explant to HgCl₂ (Hashim *et al.*, 2021). This is proven by a study conducted on *C. mangga* by Sundram *et al.* (2012), where a concentration of 0.3% HgCl₂ showed the highest survival rate of 77% while increasing the concentration of HgCl₂ to 0.5% led to necrosis of 83% of the explants. Furthermore, the explant colour turns brown, stopping the growth and eventually dying and becoming necrotic (Figure 1).

4. Conclusion

In conclusion, the concentration of sterilising agents and time exposure are crucial in eliminating epiphytic and endophytic fungi in order to produce a healthy and viable plant. Based on this study, the combination of 70% ethanol + 5% NaOCl + 0.1% HgCl₂ for 10 min was able to reduce the contamination rate and had a higher survival rate compared to other sterilising agents. However, further study on the use of antibiotics to eliminate bacterial contamination on the explant should be done.

Conflict of interest

The authors declare no conflict of interest.

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