IN VITRO REGENERATION OF KUNYIT HITAM (CURCUMA CAESIA ROXB.)

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ABSTRACT

Curcuma caesia Roxb. is one of the species under genus Curcuma. It is called ‘Kunyit Hitam’ (Malay), Black Zedoaria (English), Nilkantha (Bengal) and Manupasupu (Telugu). It is a non-native plant in Malaysia. The present study was to establish an efficient surface sterilization technique and protocol for C. caesia. Leaves and rhizome buds of C. caesia were used as explants. The leaf explants part was lamina and midrib. They were surface sterilized using 70% ethanol and Clorox®. Axenic lamina and midrib were transferred into full solid MS medium supplemented with 1.0, 2.0, 3.0, or 4.0 mg/L of 2, 4-Dichlorophenoxyacetic acid (2, 4-D) in combination with 0.5 mg/L of 6-benzylaminopurine (BAP) for callus induction. The highest number of explants induced calli was in medium 1.0 mg/L of BAP with 0.5 mg/L of BAP. However, some of explants that produced calli started to become yellowish brown, dry and then died. Some of them were still alive but went slow growth of callus. As for rhizome buds, they were surface sterilized by using 70% (v/v) of ethanol for 1 minute, followed by 40% (v/v) of Clorox® (20 minutes) added with a few drops of Tween-20 and rinsed with sterile distilled water. Then, the explants were inoculated in ½ MS (Murashige and Skoog) basal medium for two weeks. The percentage of axenic explants obtained after surface sterilization was 28%. After that, the axenic explants were cultured in ½ MS supplemented with 1.0, 3.0 and 5.0 mg/ml of BAP either alone or with 0.5 mg/L of indole-3-butyric acid (IBA) for multiple shoot induction. The explants produced multiple shoots after 2 to 3 weeks of culture. Rooting and callus formation also had been recorded. Higher percentage of axenic explants also had been recorded when media was supplemented with 4 mg/L of plant preservative mixture (PPM) and 4 mg/L of tetracycline. The highest multiple shoot proliferations were recorded in medium supplemented with 1.0 mg/L BAP.

Keywords: Curcuma caesia, surface sterilization, callus induction, shoot multiplication, Murashige and Skoog (MS)

ABSTRAK

Curcuma caesia Roxb. adalah salah satu species berasal daripada genus Curcuma. C. caesia juga dikenali sebagai 'Kunyit Hitam' (Melayu), Black Zedoaria (Inggeris), Nilkantha (Bengal) and Manupasupu (Telugu). Ia bukan berasal dari Malaysia. Kajian ini dilaksanakan untuk menghasilkan satu sterilan teknik dan protocol untuk C. caesia. Daun dan pucuk rizom digunakan sebagai eksplan. Eksperimen dimulakan dengan mensterilkan permukan lamina dan tulang daun. Eksplan disteril dengan menggunakan 70% of ethanol dan Clorox®. Semua eksplan yang axenic dipindahkan ke media yang ditambahkan dengan 1.0, 2.0, 3.0, atau 4.0 mg/L 2, 4-Dichlorophenoxyacetic acid (2, 4-D) dikombinasikan dengan 0.5 mg/L of 6-benzylaminopurine (BAP) untuk induksi callus. Media yang mengandungi paling banyak eksplan yang menghasilkan kalus adalah media 1.0 mg/L dikombinasikan dengan 0.5 mg/L of BAP. Tetapi, sesetengah daripada eksplan ini mula menjadi perang kekuningan, kering dan kemudian mati. Bagi eksplan yang masih hidup, kebanyakannya mengalamai kadar induksi kalus yang sangat perlahan. Bagi pucuk rizom pula, ia disterilkan dengan 70% ethanol selama 1 minit, dikuiti dengan 40% (v/v) Clorox® (20 minit) ditambah dengan beberapa titis Tween-20 dan dibulas dengan air steril. Kemudian, eksplan itu akan dikutur dalam ½ MS (‘Murashige dan Skoog’) media selama dua minggu. Peratusan axenic eksplan yang didapati untuk process sterilan adalah 28%. Kemudian, axenic eksplan itu akan dikuturkan di ½ MS yang ditambahkan dengan 1.0, 3.0 dan 5.0 mg/L of BAP sahaja atau dicampurkan dengan 0.5 mg/L of indole-3-butyric acid (IBA) untuk penghasilan pucuk. Pucuk baru akan kelihatan dalam minggu ke-2 atau 3. Pengakaran dan penghasilan kalus juga direkodkan dalam ½ MS media dengan BAP sahaja atau dicampurkan dengan IBA. Peratusan yang lebih tinggi dapat dicapai dengan media yang ditambahkan dengan 4 mg/L PPM dan 4 mg/L tetracycline. Bilangan pucuk yang dihasilkan paling banyak adalah di media yang telah ditambahkan dengan 1.0 mg/L BAP.

Kata Kunci: Curcuma caesia, sterilan, induksi kalus, penghasilan pucuk, 'Murashige dan Skoog' (MS)
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<th>Description</th>
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<td>MS</td>
<td>Murashige and Skoog</td>
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<tr>
<td>BAP</td>
<td>6-benzylaminopurine</td>
</tr>
<tr>
<td>IBA</td>
<td>Indole-3-butyric acid</td>
</tr>
<tr>
<td>2, 4-D</td>
<td>2, 4-dichlorophenoxyacetic acid</td>
</tr>
<tr>
<td>PPM™</td>
<td>Plant Preservative Mixture</td>
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<tr>
<td>TET</td>
<td>Tetracycline</td>
</tr>
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<td>2iP</td>
<td>6-(y, y-Dimethylallylamino) purine</td>
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<tr>
<td>IAA</td>
<td>Indole-3-acetic acid</td>
</tr>
<tr>
<td>Dicamba</td>
<td>3, 6- dichloro-o-anisic acid</td>
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<td>Picloram</td>
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1.0 INTRODUCTION

Curcuma is one of the largest genera in Zingiberaceae which has around 80 species (Larsen, 2005). This genus is widely distributed in the tropics of Asia, Africa and Australia (Sasikumar, 2005). According to Velayudhan, Muralidharan, Amalraj, Gautam, Mandal, and Dinesh (1999), there were around 40 species of Curcuma originated from India. Around 50 species of Curcuma found in Thailand but only 26 species has been identified (Sirirugsa, 1999). The origin of Curcuma caesia Roxb. species is from India (Velayudhan et al., 1999) and native to Bengal (Envis Centre on Medicinal Plants, 2011). It is a rhizomatous herb plant (Envis Centre on Medicinal Plants, 2011).

It is used in condiments, medicine and perfumes (Kumar, 1991). Their dried leaves can also be used as fuel source. It is also used to treat blood diseases, animals and insects bites, congestions and scabies (Velayudhan et al. 1999). For the Northern tribe in India, it is used as a talisman to keep spirits away (Raju, n.d.)

There are some species of Curcuma that were cultured successfully using in vitro culture methods. They are Curcuma aromatica (Nayak, 2000), and Curcuma longa (synonyms C. domestica) (Prathanturarug, Soonthornchareonnong, Chuakul, Phaidee, & Saralamp, 2004), Curcuma amada (Prakash, Elangimathavan, Seshadri, Kathiruavam, & Ignacimuthu, 2004) and Curcuma zedoria Rose. (Christine, 2007). Besides that, report on in vitro propagation of C. caesia is still limited.

Prathanturarug et al. (2004) had reported that micropropagation has many advantages over conventional propagation methods. It can help to maintain the uniformity and consistency in producing true-to-type plants in a short time (Selvakumar, Balakrishnan, & Lakshami, 2007).
Generally, *C. caesia* will be difficult to improve through plant breeding techniques because they rarely flower (Velayudhan et al., 1999). The rate of propagating *C. caesia* through rhizome parts is very slow. So, *in vitro* plant tissue culture can be used as an alternative way to accelerate the plant multiplication rate. Besides that, there is a report concluded that *C. caesia* has the possibility to become important in the economic aspects because of its phenolic compound that higher than *C. amada* (Katalinic, Milos, Kulisic, & Jukie, 2006). So, this technique offers an opportunity to produce mass number of superior clones in limited time and space (Shukla, Shukla, Vijaya, & Mishra, 2006).

Hence, the objectives of this study were:

a) To establish an efficient surface sterilization technique for *C. caesia*.

b) To develop an efficient protocol for *in vitro* regeneration of *C. caesia*.
2.0 LITERATURE REVIEW

2.1 Taxonomic classification and nomenclature

According to Velayudhan et al. (1999), *Curcuma caesia* is also called Kala haldi (Bengal), Kali haldi (Hindi), Black zedoaria (English), Kunyit Hitam (Malay), Nar kacchur (Marathi), and Manupasupu (Telugu).

The taxonomic classification and nomenclature of *C. caesia* are shown in Table 2.1.1 below.

Table 2.1.1 The taxonomy of *Curcuma caesia* Roxb.

<table>
<thead>
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<td>Phylum</td>
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<tr>
<td>Class</td>
<td>Liliopsida</td>
</tr>
<tr>
<td>Order</td>
<td>Zingiberales</td>
</tr>
<tr>
<td>Family</td>
<td>Zingiberaceae</td>
</tr>
<tr>
<td>Genus</td>
<td>Curcuma</td>
</tr>
<tr>
<td>Species</td>
<td><em>Curcuma caesia</em> Roxb.</td>
</tr>
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(Catalogue of Life, 2012)
2.2 Distribution and habitat

In the Zingiberaceae family, one of the largest genera is Curcuma which consists of 80 species (Larsen, 2005). *C. caesia* Roxb. is originated from India and can be found mostly in West Bengal, Himalaya region, North-Eastern, Central India and Nepal as indigenous plants. *C. caesia* is considered as non-native plant to Malaysia as it spread through cultivation by using rhizome part. The habitat of this *Curcuma* species is in the plains and hills (Velayudhan et al., 1999).

According to Velayudhan et al. (1999), *Curcuma lanceolata*, *C. sylvestris* Ridl. and *C. xanthorrhiza* are originated from Malaysia. Llamas (2002) reported that *Alpinia zerumbet*, *Burbidgea schizocheila*, *Curcuma roesceoeana*, *Etlingera elatior*, *Globba atrosanguinea* and *Zingiber spectabile* are species that native to Malaysia. As for *Curcuma ecomata* Craib. and *Curcuma rhabdota* P.Sirirugsa & M. Newman, they are native to Thailand (Velayudhan et al., 1999). They also showed that mostly *Curcuma* sp. in this world is originated from India. For examples, *Curcuma zedoaria*, *Curcuma haritha* and *Curcuma ferrugenia*. The famous *Curcuma longa* is from New Guinea (Velayudhan et al., 1999).

The Figure 2.1.1 showed the distribution of Curcuma sp. through South Asia, East Asia and Pacific.

![Figure 2.1.1 Distribution of Curcuma sp](image-url)
2.3 Botanical description

The characteristics of this black zedoria are large green leaves with erect rhizomatous herb, low wavy of leaves margins with rough upper side, and deep violet-red patch color in the middle of the midrib (Mangla, Shuaib, Jain, & Kashyap, 2010) as showed in Figure 2.1.2. The flower petal is deep pink or red in color and the bracts it is green with a ferruginous tinge (Scrib Inc., 2011) (Figure 2.1.5). The identification of this species can be done through the morphological characteristics of the rhizome which is bluish black in color and the shape is oblong and fusiform (Figure 2.1.3 and 2.1.4). These rhizomes have bitter and hot in smell with pungent smell (Karmakar, Saha, Sarkar, Bhattacharya, & Haldar, 2011).

Figure 2.1.2 Whole plant

Figure 2.1.3 Inner part

Figure 2.1.4 Rhizome buds

Figure 2.1.5 Flower
2.4 Chemical constituents

One of the chemical compounds in *Curcuma* species is curcumin. It is orange colored and soluble in methanol, ethanol, acetone, dichloroethylene and glacial acid; sparingly soluble in water and diethyl ether (Velayudhan et al., 1999). This curcumin is anti-bacterial, anti-HIV, anti-oxidant, anti-tumor and anti-carcinogenic (Chatoopadhyay, Biswas, Bandyopadhyay, & Banerjee, 2004). Different species of *Curcuma* consist of different amounts of curcumin (Velayudhan et al., 1999).

According to Sarangthem, and Haokip (2010), the chemical compounds in *C. caesia* are curcuminoids, flavonoids, phenolics, amino acids, protein, volatile oil, and also alkaloid. Velayudhan et al. (1999) reported that the mother rhizome of *C. caesia* contained 0.031% curcumin while the sessile tuber had only 0.018%. The curcuminoids available in this plant is important as anti-flammatory, antimicrobial, anticoagulant; wound healing, and hypoglycemia (Maheshwari, Singh, Gaddipati, & Smimal, 2006). As for the flavonoids and phenolics component, they are crucial as antioxidant and anti-flammatory (Miller, 1996); analgesic, locomotor depressant, anticonvulsant and muscle relaxants effect (Karmakar et al., 2011).

Krishnaraj, Manibhushanrao, and Mathivanan (2010) reported that *C. caesia* has higher phenolic content than the *Curcuma amada*. According to Katalinic et al. (2006), this phenolic content is responsible for the antioxidant activity and the amount of the phenolic can be related to the antioxidant properties. They also concluded that *C. caesia* had radical scavenging activities higher than *C. amada"*. Therefore, *C. caesia* have the potential to become economically important plant due to its antioxidant properties in the future (Katalinic et al., 2006).
Curcuma Longa L. is famous as medicinal plant that has been widely used to treat various diseases. According to Chatoopadhyay et al. (2004), turmeric contains protein, fat, mineral, carbohydrate, α-phellandrene, sabinene, cineol, borneol, zingiberebe and sesquiterpines. Tumeric also comprises of curcumin which curcumin I, curcumin II and curcumin III. This curcumin can act as antibacterial, antimoebic, anti-HIV, antioxidant, antitumor and anticarcinogenic activities (Chatoopadhyay et al., 2004).

2.5 Economic Importance

C. caesia had been reported to be widely used as medicinal herbs. The tribes in Gohpur use fresh rhizome of this plant mixed with Musa balbisiana fruit bark ash to treat gout, sprains and bruises (Saikia, 2006). It is also consumed in order to treat diarrhea and cough (Kala, 2005); piles, leprosy, bronchitis, cancer, epilepsy, fever, wounds, impotency, fertility, menstrual disorder, toothache, and vomiting (Raju, n.d.); relief rheumatic pain (Sarangthem, & Haokip, 2010); as diuretic, stimulant, carminative, asthma, and allergic eruption (Hussain, & Hore, 2006).

According to Usia et al. (2006), the rhizomes part of the Curcuma aeruginosa is used to treat rheumatism, obesity, scabies and as anthelmintic agent. As for C. xanthorrhiza had anti-flammatory, anti-coagulation of blood cells, anti-tumor, inhibit fibrosis in lung tissue and activating T and B cell-mediated immune functions (Chu, 2011).

There is limited report on the roles in C. caesia in religious, cultural and social aspects. In the historic time, it is used to represent the ceremonial color and a magical symbol (Farrel, 1990). Turmeric is essential as an offering to deity in the temples in India. Its powder is also applied to the devotees’ foreheads as symbolic to be auspicious and holy to Hindus (Velayudhan et al., 1999).
The other economic uses of turmeric are as spices and condiment for the people in South East Asia and Indochina. It is also used as dye with some alkalines to color the silk, cotton, and coloring material in pharmacy, confectionery and food industries (Velayudhan et al., 1999). According to Chaveerah et al (2008), *Curcuma* sp. is also important as tropical ornamental plants and perfumes industry.

### 2.6 Micropropagation

#### 2.6.1 *In vitro* propagation history

According to Kyte, and Kleyn (1996), the word micropropagation can be called as *in vitro* culture and plant tissue culture. It is an aseptic culture of cells, tissue, organs and components under *in vitro* conditions. The basis of plant tissue culture was originally proposed by Gottlieb Haberlandt in the experiment on culture of single cells. He was the Father of plant tissue culture (Trevor, 2007).

The first true plant tissue culture was obtained from cambial tissue of *Acer pseudoplatanus* by Gautheret. Other explants of *Ulmus campestre, Robinia pseudoacacia,* and *Salix capraea* also obtained through culture in agar-solidified medium of Knop’s solution, glucose and cycteine hydrochloride. Then, the accessibility of indole acetic acid and addition of B vitamin had more or less simultaneous demonstrations with carrot root tissues and tumor tissue of *Nicotiana glauca* with *Nicotiana langsdorffii* hybrid which did not need auxin (Trevor, 2007).

According to Bhojwani, and Radzan (1996), *in vitro* techniques had been widely used to culture different types of plants during 1990s. The plants were cereals and grasses, legumes, vegetable crops and potato. The role of cytokinin and auxin was later discovered. Between 1970 and 1980, the totipotency of protoplasts and protoplasts fusion was used in crop improvement by genetic manipulation of somatic cells.
Nadagauda and companions was the first to perform plant tissue culture on *Curcuma* sp. It was *in vitro* propagation used stem tips of *C. domestica* and the shoot formation was successfully achieved but not on the callus induction (Chougule, 2008). According Chougule (2008), Nayak is the first person who successfully produced *in vitro* multiplication and microrhizome in *C. aromatica*. This induction of microrhizomes in *in vitro* produced disease-free plantlets of turmeric at low cost and increased the commercial production of disease-free turmeric (Chougule, 2008).

### 2.6.2 Contamination Problems

Contamination is a constant problem in *in vitro* propagation even though there is some improvement in the *in vitro* propagation techniques (Enjalric, Carron, & Lardet, 1988). This is because microorganisms such as fungi, bacteria, molds and other organisms are always present in our atmosphere (Hartmann, Kester, & Davies, 1990).

Kyte, and Kylen (1996) stated that bacteria and fungi also refer as biological contaminants that can be found on or within plants or in laboratory. Contamination can be caused by endogenous and exogenous bacteria, fungi and other organisms. The present of exogenous microorganisms usually cause by human or laboratory environment or from the explants itself. As for endogenous contamination, it is because of the plants itself which has internal bacteria and fungi. But usually, the problem of contamination arises because of the newly initiated culture derived from the intact plant itself from endogenous viral, bacterial or fungal contaminants rather than poor aseptic technique (Collin, & Edwards, 1992).

Ley (2006) reported that some microorganisms that lived inside the tissue of the explants are *Bacillus subtilis*, *Erwinia* sp and *Pseudomonas* sp. These microorganisms will not grow immediately as they are probably controlled by the medium which is high in acid and cytokinin (Ley, 2006). The outer part of the ginger plants that is easily infected is the
rhizome part which is located inside the soils that contain fungi, bacteria and other organisms (Hosoki, & Sagawa, 1977).

Plant preservative mixture (PPM™) which is a heat-stable, broad spectrum biocide is commonly used to control fungi and also decrease the microbial contamination cause by the airborne, waterborne and also human contact (Ley, 2006). Bacterial infections can be treated in antibiotics such as tetracycline, penicillin, rifampicin, and ampicillin at recommended doses such as 10, 15 and 50 mg/ml respectively (Ley, 2006). Fungicide such as Benomyl at 0.1% is also usually used to reduce the contamination.

Naz, Ilyas, Javad, and Ali (2009) reported that it is very difficult in obtaining contamination-free culture by using underground rhizomes of turmeric as explants. But, they had obtained better result by using 70% of ethanol during surface sterilization and 5.0 mg/L PPM™ (plant preservative mixture) in which more than 70 % explants remain contamination free.

2.6.3 Media selection

According to Jha, and Ghosh (2005), the nutritional requirement for particular plants must be known by trial and error because it is difficult to suggest a same and suitable formulation for all the plants. The types of culture media available are Gamborg’s B5 medium, Linsmaier and Skoog (LS) medium and Schenk and Hilderbrandt (SH) medium (Dixon, & Gonzales, 1994).

MS (1962) medium is most widely used by researcher to culture Curcuma species. For examples, plant regeneration of C. aromatica (Mohanty, Panda, Subudhi, & Nayak, 2008); efficient regeneration of C. amada Roxb. (Prakash, Elangomathavan, Seshadri, Kathiravan, & Ignacimuthu, 2004); in vitro conservation of C. longa L. (Tyagi, Agrawal &
Mahalakshmi, 2007) and micropropagation of *C. Zedoria Roscoe* by Loc, Duc, Kwon, & Yang, 2005.

For other species such as callus induction from leaf explants of *Cornukaempferia larsenii* also using MS (1962) medium as culture medium (Saensouk, Theerakulpisut, Kilwijan, & Bunnag, 2007). MS (1962) medium also had been used by Azma, Suffian, and Norzulaani (2011) to culture *Boesenbergia rotunda* L. For micropropagation of *Boesenbergia pulchella* (Ridley) Merrell, Gamborg B5 media was used (Hamirah, Sani, Boyce, & Sim, 2007).

### 2.6.4 Surface sterilization

The part of the plants that is most difficult to produce contamination-free cultures is the stem tissue of woody plants. This followed by the seed, roots, and rhizome that usually located in the soil and in contact with soil micro flora that is very difficult to be sterilized in compared to leaf part (Stafford, & Warren, 1993). All the explants will be cultured as fast as possible to prevent turgor decrease and also reducing the explosion to contamination.

The surface sterilization disinfectants that usually used are sodium hypochlorite (Clorox), ethanol, tween-20, hydrogen peroxide, and mercuric chloride. For some tissues such as waxy leaf, Tween-20 can be used as a surfactant and for tubers and fruits part, it is encouraged to use ethanol (Stafford, & Warren, 1993).

To obtain axenic explants, some of the researchers had used ethanol, sodium hypochlorite (Clorox) and mercuric chloride in different concentrations. For instance, Prathanturarug et al. (2005) reported that in surface sterilization stage of rhizomes for *C. longa*, they used 70% ethanol for 1 min, 1.5% of sodium hypochlorite with 10 μL of Tween 80 and then 75% of
sodium hypochlorite for 10 min for second time. Mature rhizome buds from C. zedoria were treated with 20% (v/v) sodium hypochlorite for 5 min, then again with 20 % hypochlorite with a few drops of Tween 20 for 15 minutes. Finally, the rhizomes were sterilized again with 70% (v/v) ethanol (Banisalam, Sani, Philip, Imdadul, & Khorasani, 2011).

According to Hamirah, Sani, Boyce, and Sim (2010), Zingiber montanum Koening syn. rhizomes part were surface sterilized by using 75% (w/v) ethanol for one minute, then agitated with 20, 30 or 40 % (w/v) Clorox (5.25% w/v sodium hypochlorite) added with 0.1ml/L Tween 20 and four drops of 25% hyphochloric acid (HCl) for 20 minutes with constant agitation. For Boesenbergia pulchella (Ridley) Merrell rhizome part, 75% (w/v) ethanol for one minute, then agitated with 30 % (w/v) Clorox (5.25% w/v sodium hypochlorite) added with 0.1ml/L Tween 20 with constant agitation were applied (Hamirah, Sani, Boyce, & Sim, 2010).

As for C. amada rhizome part, Prakash et al. (2004) used Teepol for 5 min, 70% of ethanol for 1 min and mercuric chloride for 5 min. Shukla et al. (2006) obtained axenic rhizome shoot buds by using 0.2 % of mercuric chloride for 15 min. Loc, Duc, Kwon, and Yang (2005) had used 70 % of ethanol for 1 min and 0.2 % mercuric chloride for 20 min for C.zedoria. For rhizomes of C. zedoria and Z. zerumbet, they were surface sterilized by mercuric chloride solution for 5 minutes and sterilized in 20% of Clorox with a few drops of Tween 20 for 10 minutes (Christine, & Chan, 2007). Islam, Kloppstech, and Jacobsen (2004) stated that axenic rhizome explants can be obtained through using of 70% ethanol for 30-40 seconds and then immersed in 0.1 % of mercuric chloride with two to three drops of Tween-20.