Malaysian Applied Biology
https://doi.org/10.55230/mabjournal.v54i1.2936

Research Article

An Effective Disinfection Protocol for The Development of Tissue Culture Mango Plant (*Mangifera indica* L.) cv. Harumanis

Zun Yip Lee[1], Amir Hamzah Ahmad Ghazali[1], Bee Lynn Chew[1], Sreeramanan Subramaniam[1,2]*

1. School of Biological Sciences, Universiti Sains Malaysia (USM), Georgetown, 11800 Penang, Malaysia
2. Centre for Chemical Biology (CCB), Universiti Sains Malaysia (USM), Bayan Lepas, 11900, Penang, Malaysia

*Corresponding author: sreeramanan@usm.my

ABSTRACT

Harumanis mango is one of the highest demand mango cultivars in Malaysia due to its exceptional sweetness and fabulous fragrance. However, the production of this mango cannot meet the market demand due to limited grafting activity and the limited number of seeds (only one harvest season per year). These make vegetative and non-vegetative propagation of Harumanis time and labour intensive. Micropropagation using tissue culture techniques is a reliable and effective alternative for mass *in vitro* propagation of Harumanis at a consistent and faster rate, producing clones of the mother plants. Nevertheless, deep-seated contaminants are the major problem faced in the micropropagation of this plant. The objective of the present study is to develop an effective disinfection protocol for *Mangifera indica* cv. Harumanis. A sterility rate of 87.5% was achieved by spraying the mother plants with 0.5 mL/L azoxystrobin two days before the experiment (pre-treatment) and surface sterilising nodal segments at the immature green leaf stage with 40% Clorox® for 20 min, 0.5 g/L benomyl for 1 hr and 300 mg/L cefotaxime for 5 min. The transverse thin layer (tTCL) technique was applied to the nodal segments and cultures were maintained at WPM with 0.5 g/L benomyl and 300 mg/L cefotaxime. Endophytic bacteria and fungi were observed in the axillary buds using scanning electron microscopy (SEM). Both histological and SEM analysis showed that the xylem vessels of the nodal segments at these two ends of the stem tended to have lower numbers of endophytic bacteria. This suggests that the nodal segments at the upper and middle ends of the stem are the best starting material for future experiments. The present results show the importance of pre-treatment and culture maintenance in reducing endophytic contamination. Furthermore, the results showed the potential of tTCL-treated Harumanis cultures in minimising the contamination rate.

Key words: Endophytes, histology, mango, scanning electron microscopy, surface sterilisation, tTCL

INTRODUCTION

Mangifera indica, commonly known as mango, is a globally prized tropical fruit with a millennia-old history originating in northeast India (Singh, 2016; Yadav & Singh, 2017). Almost all parts of the mango plant, including the leaves, roots and bark, are used in folk medicine for their potential role in combating ageing processes, diabetic complications and diseases related to oxidative stress (Ndoye *et al*., 2018). As one of the world's most widely cultivated fruits, the mango is of great economic importance and is one of the three most traded tropical fruits by export volume in 2021 (FAO, 2022). The mango can be marketed in various forms, including fresh fruit, peel, pickled fruit and processed products such as puree, pulp and concentrate. The pulp of the mango can also be used to enrich or flavour secondary products such as ice cream, yoghurt, beverages and soft drinks (Owino & Ambuko, 2021).

Harumanis, the most famous cultivar of *Mangifera indica* in Malaysia, is prized for its distinctive fragrance and the sweet, fibre-free flesh it produces. Harumanis characterised by its green exterior and orange-coloured flesh, is mainly grown in Perlis, Malaysia, as indicated by its registration as MA 128 in 1971 according to the Department of Agriculture of Malaysia (Jabatan Pertanian Malaysia, 2023). In Malaysia, the predominant mango varieties in the market are Chok Anan and Harumanis, with production for 2021 of 204,146 tonnes and 45,417 tonnes respectively (Unit Perangkaan Pertanian *et al*., 2022). Despite Chok Anan's shown higher production volume, this report revealed that the net profit from the sale of Harumanis was about 1.7 times higher than that of Chok Anan, suggesting that an increase in Harumanis production could lead to a significant increase in income for Malaysia.

Propagation of *M. indica* is a challenge, especially in seed propagation, where recalcitrant traits, only one harvest season per year and limited progeny per fruit are a major hurdle. The common practise is therefore labour-intensive vegetative propagation, mainly by grafting, which requires skilled workers. Besides, 6 months old of rootstocks germinated from the limited number of seeds produced by Harumanis per year with low germination rate further slows down the propagation of Harumanis (Buniamin *et al*., 2020). Micropropagation using tissue culture techniques is a more reliable and effective alternative for mass *in vitro* propagation of Harumanis at a consistent and faster rate, producing clones of the mother plants. To ensure the success of this process, effective surface sterilisation of the explants is essential to prevent microbial contamination and necrosis. This includes a thorough disinfection protocol using surface sterilisers, fungicides, antibiotics and biocides. In the previous reports, shoot tips

Article History
Accepted: 21 February 2025
First version online: 27 March 2025
Cite This Article:
Lee, Z.Y., Ghazali, A.H.A., Chew, B.L. & Subramaniam, S. 2025. An effective disinfection protocol for the development of tissue culture mango plant (*Mangifera indica* L.) cv. Harumanis. Malaysian Applied Biology, 54(1): 12-23. https://doi.org/10.55230/mabjournal.v54i1.2936

and/or nodal segments were used in the micropropagation of mango trees (cv. Terpentine, Gomera, Sabre, Alphonso, Totapuri, Banganapalli, Arka Anmol, Amrapalli, Zebda, Hindy Sinnara, Sukkary, 13-1, Irwin, Ataulfo, Gomera-4, and 'Keitt') but with little success (Tilahun *et al*., 2013; Ahmadpoor *et al*., 2022; Ho *et al*., 2022; Anjum *et al*., 2023; Verma *et al*., 2023). The three major problems limiting the success rate are the browning (necrosis) of explants, phenols excreted to the media, and deep-seated microbial contaminants.

This study aimed to address deep-seated contaminants in micropropagation, focusing on optimising the disinfection protocol through a comprehensive approach that includes treatment of mother plants (pre-treatment), explant surface sterilisation and culture post-treatment. Unlike previous studies, which focused on individual steps, this research integrates all these aspects. Histological and SEM analyses were performed to better understand the viability of the explants and the mechanisms of infection by deep-seated contaminants.

MATERIALS AND METHODS

Plant materials

The Harumanis mango trees were sourced from the Malaysia Agricultural Research and Development Institute (MARDI) in Perlis, Malaysia and planted in a Harumanis mango farm at Universiti Sains Malaysia. The nodal segments with a single axillary bud were cut from the stem of the field plants.

The shoot growth of the mango tree comprises 9 distinct stages (Ramírez *et al*., 2014). These stages describe the development from the dormant stem to the final mature, determinate stage. We refer to this timeline to observe the growth stages of the vegetative shoot of Harumanis. The nodal segments with a single axillary bud were cut from the stem of the 4- to 6-year-old field plants. The bud segments at immature green leaf (IGL) stage as shown in Figure 1 were selected.

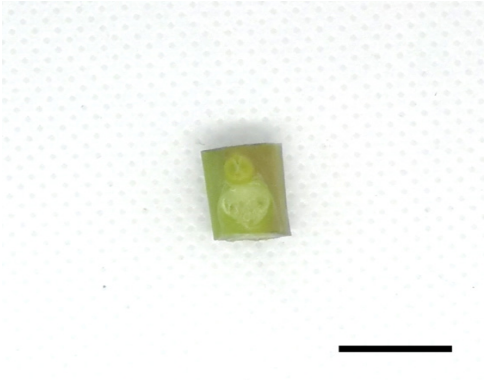

Fig. 1. Nodal segment excised from the stem of the field plants (Scale bar = 1 cm)

Various ways of reducing the degree of browning of the explants and excretion of phenols were tested with and without antioxidants (Thomas & Ravindra, 1997), as follows:

1. The explants were soaked in sdH_2O with ascorbic acid and citric acid for 1 hr after surface sterilisation.
2. Ascorbic acid and citric acid were added to the sterilised media by filter sterilisation.
3. PVP was added to the media and autoclaved together with them.
4. Activated charcoal was added to the media and autoclaved together with them.
5. The explants were soaked in sdH_2O and kept in the dark for about 13-18 hr.
6. The cultures were kept in the dark for the first 7 days.
7. The cultures were subcultured at intervals of 2-5 days, 1-4 weeks and 1 month.
8. The concentration of the MS medium and WPM were reduced to what from what?.

The tested concentration of the antioxidants was 0.5 mM for ascorbic acid, 0.5 mM for citric acid, 1 g/L for PVP, and 0.5, 1, and 2.5 g/L for activated charcoal.

Disinfection protocols of transverse Thin Cell Layer (tTCL) treated explants.

Effect of tTCL in combination with benomyl on explant sterility

The mother plants grew in the field were sprayed thoroughly with 5 g/L Fosetyl-Al (Aliette®) two weeks before the experiment. IGL stage stems were collected, cut into shorter segments and sterilised with a 5% Dettol solution and 2 drops/100 mL Tween® 20. Nodal segments (8-12 mm) with a single axillary bud were rinsed for 1 hr and then surface sterilised with 40% Clorox® and 2 drops/100 mL Tween® 20 for 20 min. They were then soaked in sterilised 0.5 g/L benomyl solution for 1, 2 and 3 hr and in a 200 ppm AgNP solution for 3 hr. The nodal segments were dried, subjected to the tTCL technique and divided into upper and lower halves, including the peduncle, which served as explants (Figure 2).

These explants were cultured in ½ WPM with 60 g/L sucrose, 2.75 g/L Gelrite® (Duchefa Biochemie, The Netherlands), 200 ppm AgNP and 0, 0.5, or 1 g/L benomyl. The pH of the culture medium was adjusted between 5.7 and 5.8 and the cultures were maintained under white LED light with a 16-hr photoperiod (20 µmoL/s) at 25 ± 2°C and 55% humidity (Khair *et al*., 2016). Subcultures were established on days 7 and 14 to reduce brown colouration and phenol excretion. The percentage of sterility, percentage of fungal contamination and percentage of bacterial contamination were recorded after 1 month of culture and calculated as follows:

$$\text{Sterility percentage} = \frac{\text{Total number of explant-Number of contaminated explant}}{\text{Total number of explant}} \times 100\%$$

$$\text{Fungal contamination percentage} = \frac{\text{Total number of fungal contaminated explant}}{\text{Total number of explant}} \times 100\%$$

$$\text{Bacterial contamination percentage} = \frac{\text{Total number of bacterial contaminated explant}}{\text{Total number of explant}} \times 100\%$$

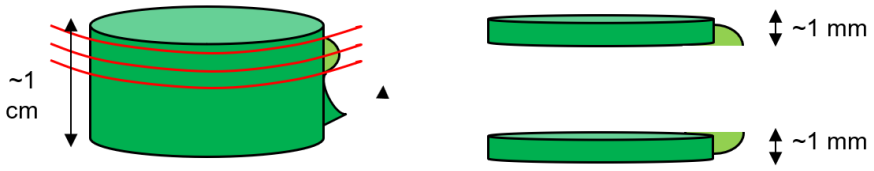

Fig. 2. Upper and lower half of the axillary bud along with the peduncle

The bacterial and fungal contamination were determined based on the appearance and morphology of the contaminants. When an explant was simultaneously infected by both bacteria and fungi, the type of contamination —whether bacterial or fungal — was determined by which microbe was the first to infect the explant.

The 15 combinations of treatments which consist of the 3 factors at 3 levels Box-Behnken design (BBD) was used to evaluate the effects of soaking time, benomyl concentration and benomyl medium on culture sterility. In addition to the design of the 15 combinations of treatments, visualisation, optimisation and ANOVA based on BBD analysis of the results were performed using the Minitab 18.1 programme, Minitab Inc, at a 95% confidence interval with a replicate containing 10 explants per treatment and cultured in individual test tubes.

Effect of tTCL in combination with cefotaxime on explant sterility

Stems at the IGL stage were processed by removing the lower 3 nodal segments. These stems were then cut into shorter segments, surface sterilised and nodal segments of about 1 cm with an axillary bud were obtained. After surface sterilisation, which included a 20-min treatment with 40% Clorox containing 2 drops/100 mL Tween® 20 and a 1-hr autoclaving with 0.5 g/L benomyl solution, the segments were subjected to tTCL treatment, resulting in halves for use as explants.

The explants were cultured in WPM with 2.75 g/L Gelrite® and varying concentrations of cefotaxime, benomyl and sucrose. The pH was adjusted to 5.7 and 5.8, and the cultures were maintained under white LED light with a 16-hr photoperiod (20 µmoL/s) at 25 ± 2°C and 55% humidity. Subcultures were established on days 7 and 14 to minimise brown colouration and phenol excretion. The percentage of sterility, percentage of fungal contamination and percentage of bacterial contamination were recorded after 1 month of culture.

The 15 combinations of treatments which consist of the 3 factors at 3 levels Box-Behnken design (BBD) was used to evaluate the effects of the concentration of cefotaxime, benomyl and sucrose in the media on the sterility of the cultures. In addition to the design of the 15 combinations of treatments, the results were visualised using the Minitab 18.1 programme (Minitab Inc) with a 95% confidence interval, optimised and an ANOVA was performed based on the BBD analysis, culturing one replicate with 10 explants per treatment in individual test tubes.

Effect of tTCL in combination with benomyl and optimal concentration of cefotaxime on explant sterility

The mother plants grew in the field were sprayed thoroughly with 5 g/L Fosetyl-Al (Aliette®) two days before the experiment. Stems at IGL stage were collected, cut into shorter segments and sterilised with a 5% Dettol solution and 2 drops/100 mL Tween® 20. Nodal segments (8-12 mm) with a single axillary bud were rinsed for 1 hr and then surface sterilised for 20 min with 40% Clorox® and 2 drops/100 mL Tween® 20. They were then soaked in sterilised 0.5 g/L benomyl solution for 1 hr and 300 mg/L cefotaxime solution for 5 min. The nodal segments were dried, subjected to the tTCL technique and divided into upper and lower halves, including the peduncle, which served as explants.

These explants were cultured in WPM with 30 g/L sucrose, 2.75 g/L Gelrite®, 1 g/L activated charcoal, 300 mg/L cefotaxime and 0 – 1.50 g/L benomyl. The pH of the culture medium was adjusted between 5.7 and 5.8 and the cultures were maintained under white LED light with a 16-hr photoperiod (20 µmoL/s) at 25 ± 2°C and 55% humidity. The subculture was established on day 7 to reduce brown colouration and phenol excretion. The percentage of sterility was determined after 1 month of culture.

The data obtained were analysed with one-way ANOVA followed by Dunnett's test at a 95% confidence interval using the Minitab 18.1 programme, Minitab Inc. This experiment was performed with 3 replicates, each replicate containing 8 explants/treatment. 1 explant was cultured in 1 test tube with WPM.

Histology and scanning electron microscopy analyses

Histological analysis

The histological examinations were carried out on nodal segments of IGL stage stems of the field plants. The segments were fixed in formaldehyde (FAA), dehydrated with tertiary butyl alcohol (TBA) and ethanol according to a graded series and then treated with xylene and paraffin wax. The samples were successively treated with wax (wax I, II, III) and then cut into 0.8µm slices using a microtome. The slices were stained with safranin and fast green and observed under a light microscope (Spence, 2001).

Scanning electron microscopy analysis

A 1 cm long nodal segment from an IGL stage stem was fixed in McDowell-Trump fixative in a phosphate buffer solution at 4°C for 4 days. It was then fixed in osmium tetroxide, dehydrated in an ethanol series and immersed in hexamethyldisilazane (HDMS) before being air-dried in a desiccator. The segment was then attached to a SEM specimen stub with double-sided adhesive tape, labelled with 5-10 nm gold and observed with a scanning electron microscope (SEM - Leo Supra 55VP-Ultra High Resolution analytical FESEM) (Leamy, 1982).

RESULTS

Plant materials and culture conditions

The *in vitro* cultures showed progressive browning, which was accompanied by darkening of the medium due to phenol excretion. Various methods were tested to mitigate these problems. In these methods, the explants were soaked in antioxidant solutions, antioxidants were added to the sterilised media and the concentration of the culture medium was reduced. Reducing the media concentration and adding 1 g/L of activated charcoal effectively solved the problems, albeit with gradual browning over time. The other methods tested had little success, with cultures surviving less than two months. Cultures using WPM and lower medium concentrations showed less browning and phenol excretion.

Disinfection protocols of tTCL-treated explants.

Effect of tTCL in combination with benomyl on explant sterility

After 1 month of culture, the percentages of sterility, bacterial contamination and fungal contamination were documented in Table 1. With an adjusted R-squared of only 17.58% for the percentage of sterility, no statistical analysis was performed. Instead, the focus shifted to analysing fungal contamination. A Shapiro-Wilk test confirmed that the data for fungal contamination were normally distributed (W=0.955, p>0.1), which allowed the use of ANOVA.

As the interaction terms are not significant, the interaction terms are removed from the model to avoid false conclusions (Engqvist, 2005). According to the ANOVA analysis, the concentration of benomyl solution (B1), the concentration of benomyl in the media (C1) and their quadratic terms (B12, C12) significantly affect the percentage of fungal contamination.

In the regression model, an R-squared of 96.41% and a predicted R-squared of 78.42% were achieved. Given the high predicted R-squared, an optimisation plot (Figure 3) was generated. After analysing the optimisation plot, a composite desirability value of 1 was determined. The optimal conditions to achieve the lowest percentage of fungal contamination (≤0%) are as follows: 1 hr rinsing with benomyl solution (A1), 0.4343 g/L benomyl solution concentration (B1) and 0.697 g/L benomyl concentration in the media (C1). In Figure 3(B), the benomyl concentration in the media (C1) was maintained at 0.5 g/L, which predicts a percentage of fungal contamination of ≤0%.

It can be concluded that soaking the explants in 0.5 g/L benomyl solution for 1 hr and adding 0.5 g/L benomyl to the media is recommended for future experiments to achieve the lowest percentage of fungal contamination of the *in vitro* cultures.

Table 1. Effects of combinations between the duration of soak in benomyl solution, the concentration of benomyl solution, and the concentration of benomyl in the media on the percentages of sterility, bacterial contamination, and fungal contamination of *in vitro* cultures (n=1)

Treatment	Duration of soak in benomyl solution (A_1, hr)	Concentration of benomyl solution (B_1, g/L)	Concentration of benomyl in media (C_1, g/L)	Sterility percentage (%)	Fungal contamination percentage (%)	Bacterial contamination percentage (%)
1	1	0.5	1.0	0	0	100
2	2	0.0	1.0	0	10	90
3	1	0.5	0.0	0	50	50
4	3	1.0	0.5	0	20	80
5	2	0.5	0.5	20	0	80
6	3	0.5	0.0	10	70	20
7	2	1.0	0.0	0	100	0
8	3	0.0	0.5	20	10	70
9	2	0.0	0.0	0	80	20
10	1	1.0	0.5	30	0	70
11	2	1.0	1.0	10	40	50
12	1	0.0	0.5	30	20	50
13	2	0.5	0.5	20	0	80
14	3	0.5	1.0	0	10	90
15	2	0.5	0.5	0	0	100

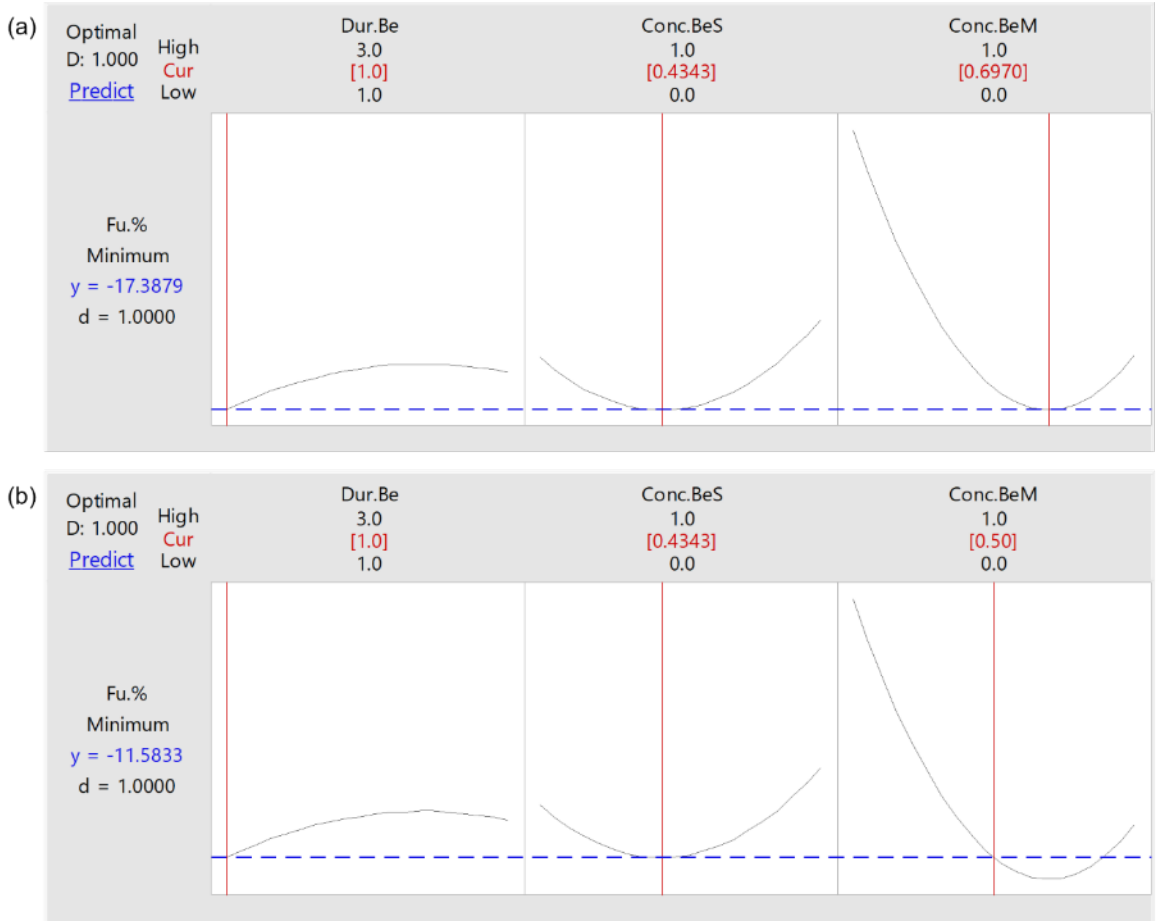

Fig. 3. Optimization plot for the percentage of fungal contamination. (A) Optimization plot with A_1 held at 1 hr. (B) Optimization plot with A_1 held at 1 hr and C_1 held at 0.5 g/L. Optimisation plot for BBD showing optimal values. The red line shows the current factor settings and the blue line shows the response to the current setting.

Effect of tTCL in combination with cefotaxime on explant sterility

After 1 month of culture, the percentages of sterility, bacterial contamination and fungal contamination of the *in vitro* cultures were reported in Table 2. As the adjusted R-squared for the percentage of sterility was only 52.27%, the statistical analysis was not included in the results. Instead, the percentage of bacterial contamination was analysed. A Shapiro-Wilk test was performed for the percentage of bacterial contamination and showed that the data were normally distributed (W=0.967, p>0.1).

Based on this result, a parametric test, namely ANOVA, was performed. As the interaction terms are not significant, the interaction terms are removed from the model to avoid false conclusions (Engqvist, 2005). The statistical analysis in Table 3 shows that the concentration of cefotaxime in the media (A2) and its quadratic term (A22) have a significant effect on the percentage of bacterial contamination.

For this regression model, the R-squared is 98.01% and the predicted R-squared is 92.9%. Since the predicted R-squared is high, an optimisation plot (Figure 4) was constructed. After analysing the optimisation plot, the composite desirability value is 1. The optimal condition for the lowest percentage of bacterial contamination = 0% is as follows: Concentration of cefotaxime in the medium (A2) = 224.66 mg/L, concentration of benomyl in the medium (B2) = 0 g/L, concentration of sucrose in the medium (C2) = 20 g/L. The surface plot and the contour plot show that A2 has a positive effect on the percentage of bacterial contamination. It can be concluded that the addition of 250 mg/L of cefotaxime to the media is recommended for future experiments to achieve the lowest percentage of bacterial contamination of the *in vitro* cultures.

Effect of tTCL in combination with benomyl and optimal concentration of cefotaxime on explant sterility

After one month of cultures, the percentage of sterility of the cultures was recorded in Table 4. A Shapiro-Wilk test was performed and showed that the data were normally distributed (W=0.979, p>0.1). Based on this result, the ANOVA parametric test was used to analyse the result. Although all mean values of the tested benomyl concentrations showed no significant difference from each other, the results analysed by Dunnett's test showed that 0.5 g/L benomyl added to the media had a significant effect on the percentage of sterility.

The cultures treated with 0.5 g/L benomyl showed the highest percentage of sterility at 87.5±12.50%. As the benomyl concentration increases, the degree of browning of the explants and the phenolics excreted into the media increases. This phenomenon was recorded in an experiment on the effect of benomyl concentration in the media on the amount of phenolics excreted from the cultures into the media.

Table 2. Effects of combinations between the concentration of cefotaxime in media, the concentration of benomyl in media, and the concentration of sucrose in the media on the percentages of sterility, bacterial contamination, and fungal contamination of *in vitro* cultures

Treatment	The concentration of cefotaxime in media (A_2, mg/L)	The concentration of benomyl in media (B_2, g/L)	Concentration of sucrose in media (C_2, g/L)	Sterility percentage (%)	Fungal contamination percentage (%)	Bacterial contamination percentage (%)
1	0	0.8	40	0	20	80
2	250	0.4	40	60	40	0
3	500	0.4	20	40	60	0
4	250	0.4	40	40	50	10
5	250	0.8	60	60	40	0
6	250	0.4	40	30	70	0
7	0	0.4	60	0	30	70
8	250	0.8	20	50	50	0
9	250	0.0	20	10	90	0
10	250	0.0	60	0	100	0
11	0	0.0	40	0	40	60
12	500	0.4	60	20	80	0
13	500	0.0	40	0	100	0
14	0	0.4	20	0	40	60
15	500	0.8	40	20	80	0

Table 3. ANOVA analysis of the effect of duration of soak in benomyl solution, concentration of benomyl solution, and concentration of benomyl in media on the percentage of bacterial contamination of the *in vitro* cultures

Source	Sum of squares	Degree of freedom	Mean square	*F*-value	*p*-value
Model	13106.7	6	2184.44	65.53	0.000*
A_2	9112.5	1	9112.50	273.38	0.000*
B_2	50.0	1	50.00	1.50	0.256
C_2	12.5	1	12.50	0.38	0.557
A_2^2	3800.6	1	3800.64	114.02	0.000*
B_2^2	0.6	1	0.64	0.02	0.893
C_2^2	31.4	1	31.41	0.94	0.360

R^2 = 0.9801, Adj. R^2 = 0.9651, and Pred. R^2 = 0.9290

The denoted asterisk (*) indicates significance at $p<0.05$.

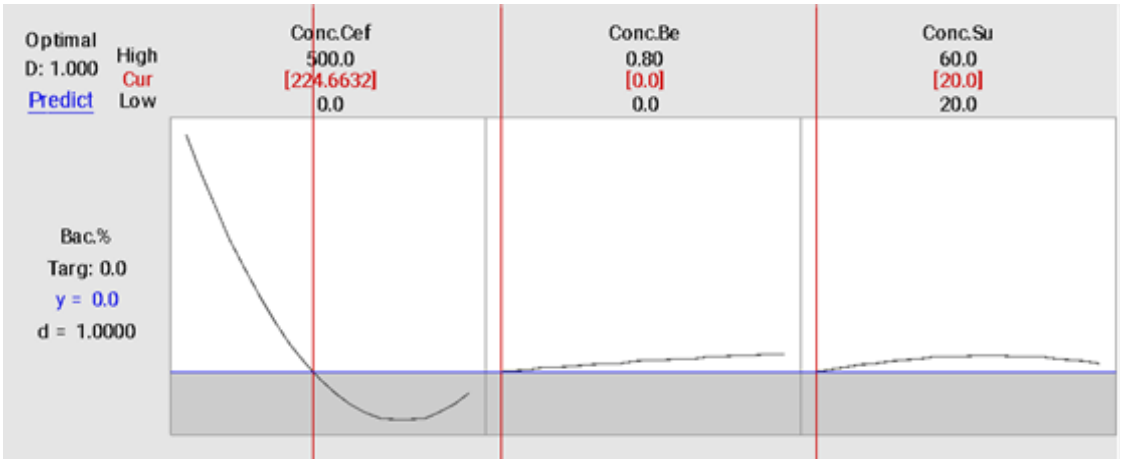

Fig. 4. Optimization plot of the percentage of bacterial contamination. Optimization plot for BBD with the optimal values. The red line shows the current factor settings and the blue line shows the response to the current settings.

Table 4. Effect of various concentrations of benomyl in WPM on the percentage of sterility

The concentration of benomyl in WPM (g/L)	Sterility percentage mean ± SE in %
0	41.70^b ± 11.00
0.50	87.50^a ± 12.50
0.75	79.17^b ± 4.17
1.00	75.00^b ± 12.50
1.25	79.20^b ± 11.00
1.50	79.20^b ± 11.00

Mean values ($\bar{x}$ ± s.e.) followed by different letters were significantly different (Dunnett's test, $p<0.05$).

Histology and scanning electron microscopy analyses

Histological analysis

It was observed that the apical meristem part and the leaf primordia were heavily stained. This indicates that the axillary buds are capable of forming shoots. Besides, the size of axillary buds and their number of bud scales are decreased from top to bottom.

Figure 5 shows the xylem vessels infected with bacteria. The biomass of bacteria in the xylem vessels was also observed in Figure 5(c) and (d). It was found that more bacteria-infected xylem vessels and biomass of bacteria were found in the xylem of the lower 3 axillary buds. The same methodology was performed to observe the endophytes residing in the xylem of hazelnut shoots and birch (Hand *et al*., 2016; Covelo *et al*., 2019).

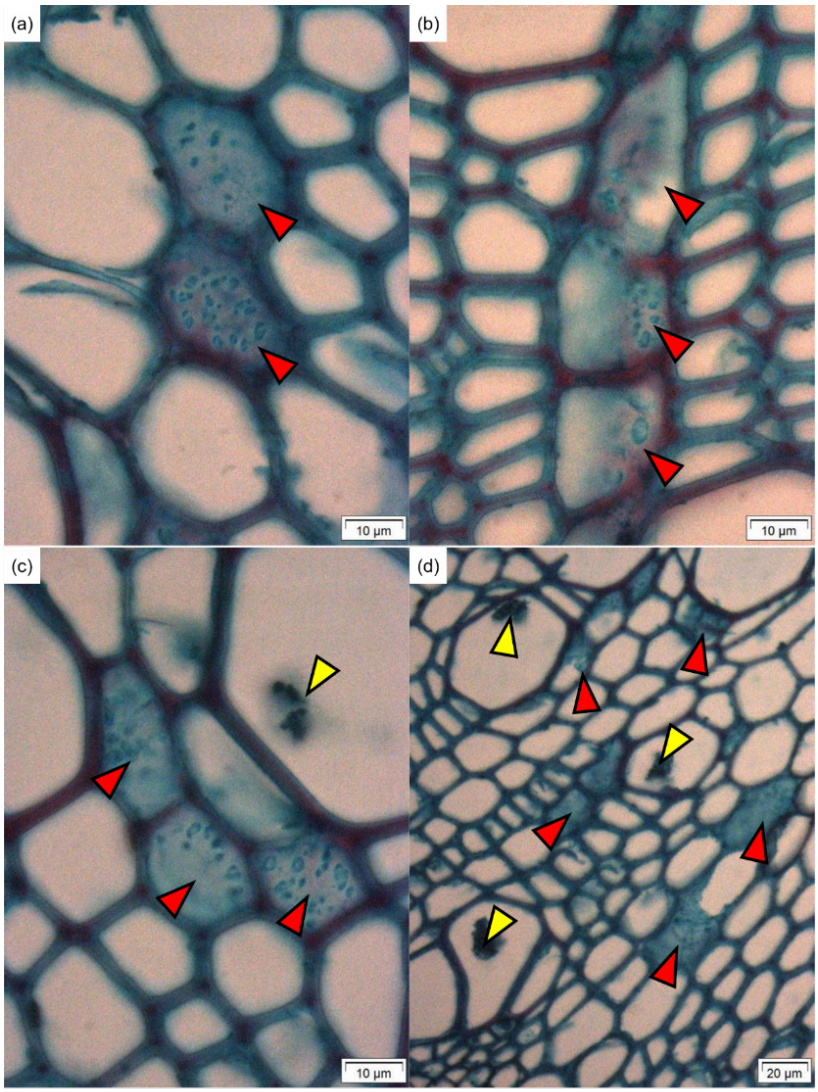

Fig. 5. Histology of xylem tissues of the axillary buds obtained from a field plant. (a), (b), (c) 7th axillary bud (d) 9th axillary bud. (Red arrow: xylem cells infected by bacteria; Yellow arrow: biomass of bacteria; Scale bars: (a), (b), (c) = 10 µm, (d) = 20 µm)

Scanning electron microscopy analysis

The images from the SEM show the morphological characteristics of the xylem cells in the stem and leaf primordia cells in the axillary bud obtained from the field plant. Figure 6(a) shows many xylem vessels infected with bacteria. The bacteria have formed a biofilm that blocks the xylem vessels.

Figure 6(b) shows fungal hyphae in the gap between the leaf primordia of the axillary bud. In addition, bacterial biofilms were observed on the leaf primordia.

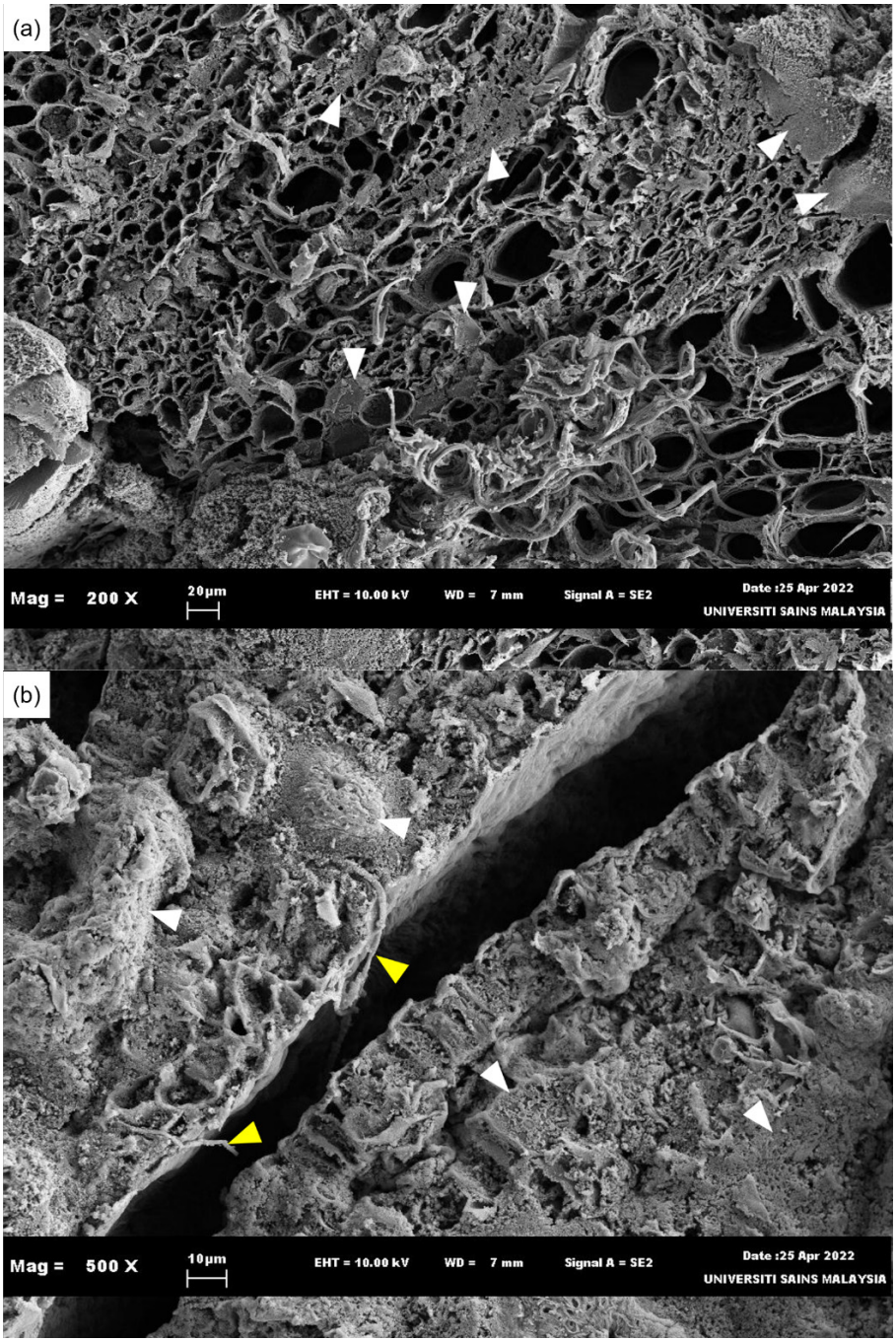

Fig. 6. SEM image of the nodal segment obtained from the field plant. (a)xylem vessels (b)axillary bud. (White arrow: bacterial biofilm; Yellow arrow: fungal hyphae. Scale bars: (a) = 20 µm, (b) = 10 µm)

DISCUSSION

Plant materials and culture conditions

The tTCL-treated nodal segments used as explants were taken from shoots at the immature green leaf stage, as younger stems are fragile and susceptible to bleaching during surface sterilisation. In addition, axillary buds are not yet fully developed at younger stages. Mature nodal segments at the green leaf stage were also avoided as their stem surface is harder and has a higher wood fibre content, which makes dissection more difficult.

Alternate spraying of mother plants with a combination of 0.5 g/L benomyl and 0.5 mL/L azoxystrobin fungicide is recommended to mitigate endophytic fungal infections in mango trees and to prevent potential resistance development in pathogens such as *Colletotrichum gloeosporioides* (Kumar *et al*., 2007). The optimum percentage of sterility *in vitro* cultures was observed when explants were harvested two days after fungicide application, although there is uncertainty, especially during the rainy season. Studies using shoot tips or nodal segments in mango micropropagation have had limited success due to problems such as browning of explants, phenolics exuded into the media, and deep-seated microbial contamination (Yang & Lüdders, 1993; Thomas and Ravindra, 1997; Chandra *et al*., 2004; Samaan *et al*., 2007; Krishna *et al*., 2008; Tetsumura *et al*., 2016; Conde *et al*., 2023). This study showed that the addition of 1 g/L activated charcoal and the use of WPM as a culture medium

effectively solved these problems. Rinsing with antioxidants did not significantly reduce brown colouration and phenol excretion, which is consistent with the results of Thomas and Ravindra (1997). Selection of the correct type and concentration of medium was crucial; cultures on ½ WPM and WPM showed fewer problems than those on ½ MS and MS media (results not shown). This result is consistent with the findings of Tetsumura *et al*. (2016), who reported that the survival rate of nodal explants of *M. indica* cv. Irwin was higher when cultured on ½ WPM and WPM compared to ½ MS medium and MS medium. However, this result is in contrast to the findings of Shamsul (2021), who reported that the shoot tip of *M. indica* cv. Harumanis shoot tip cultured in MS media with 1.5 mg/L ascorbic acid and 2.0 g/L activated charcoal had a lower degree of browning (84%) than the shoot tip cultured in WPM with 1.5 mg/L ascorbic acid and 1.5 mg/L citric acid, which had a degree of browning of 80%.

Disinfection protocols of tTCL-treated explants

With the tTCL technique, the size of the explants was reduced from 5 to 9 mm (nodal segments) to 1 mm in length. The smaller the explants are, the more cells within the explants are "morphogenetically responsive" due to their close contact with the growth medium (Nhut *et al*., 2003). In addition to the plant cells, the endophytic bacterial and fungal cells also come into contact with the antibiotic- and fungicide-containing medium. This increases the effectiveness of the antibiotic and fungicide in combating microbial contamination and reduces the contamination rate. This statement agrees with that of Karjadi *et al*. (2022), who reported that the 0.5 mm meristem cultures had lower contamination than the shoot tip cultures of Solanum tuberosum L.

Effect of tTCL in combination with benomyl on explant sterility

When investigating the effects of benomyl on the percentage of fungal contamination, both fungal and bacterial contamination rates were monitored. It was observed that bacteria appeared first when fungal growth was inhibited and vice versa. Increased concentrations of benomyl in the media correlated with increased browning of the explants and increased phenol excretion. Therefore, the concentration of benomyl in the media was maintained at 0.5 g/L in Figure 3(B). The ANOVA analysis also shows that the addition of benomyl to the media had a greater effect on the percentage of fungal contamination than the benomyl solution used in the surface sterilisation. However, the predicted R-squared is only 78.42%, suggesting that the interaction between bacteria and fungi within the culture could influence the rate of fungal emergence.

Effect of tTCL in combination with cefotaxime on explant sterility

When investigating the influence of cefotaxime on the percentage of bacterial contamination, it was found that the addition of cefotaxime to the media significantly reduced contamination. Cultures supplemented with 250 and 500 mg/L cefotaxime showed no significant differences in browning and phenolic excretion. This finding is noteworthy as it allows for higher cefotaxime concentrations of up to 500 mg/L during periods of increased bacterial contamination, especially during the rainy season. Da Silva and Fukai (2001) emphasised the low phytotoxicity of cefotaxime to chrysanthemums compared to carbenicillin and vancomycin. Although cefotaxime is effective against *Agrobacterium* overgrowth on apple tree leaves, it inhibits shoot induction at 500 mg/L, while 100–200 mg/L promotes shoot regeneration (Verma *et al*., 2023). The effect of concentration on shoot growth from the axillary bud of *M. indica* cv. Harumanis remains unknown, as the growth of leaf primordia of tTCL-treated cultures always stops when they reach 1-2 mm in length.

Photoautotrophic micropropagation, which excludes the carbohydrate source (sucrose) in the media (Fujiwara *et al*., 1988; Paynter, 2022; Quynh Thi *et al*., 2020), restricts the growth of bacteria and fungi. Although a minimum sucrose concentration of 20 g/L was maintained in this study, as opposed to complete exclusion, the lack of a significant effect on the percentage of bacterial and fungal contamination suggests that even this minimum sucrose concentration may not be a significant factor in the percentage of bacterial and fungal contamination.

Effect of tTCL in combination with benomyl and optimal concentration of cefotaxime on explant sterility

After the optimal concentration of cefotaxime was determined to be 250 mg/L, 300 mg/L was used in subsequent experiments to ensure control over bacterial contamination, especially when harvesting explants on rainy days. As cefotaxime significantly reduced bacterial contamination, the focus shifted to determining the optimal concentration of benomyl in the media. The experiment on the effect of benomyl on the sterility of the explants indicated that the addition of 0.5 g/L benomyl was effective in reducing fungal contamination, but in this experiment, cefotaxime was omitted, resulting in sterility below 30%. In this section, the combined effects of the optimal cefotaxime concentration and different benomyl concentrations were investigated. As 0.5 g/L benomyl was predicted to be optimal, concentrations below this threshold were not tested. The highest sterility (87.5%) occurred with WPM supplemented with 0.5 g/L benomyl. However, treatments with a higher benomyl concentration, which resulted in sterility of more than 70%, increased browning of the explants and phenol excretion. For the subsequent experiments, WPM with 0.5 g/L benomyl and 300 mg/L cefotaxime was used. Nevertheless, sterility (40-70%) fluctuated in the subsequent shoot regeneration experiments, likely which was probably influenced by the weather and the timing of fungicide spraying of the mother plants. This is consistent with the findings of Singh *et al*. (2011) on increased bacterial contamination of bananas during the rainy season.

Histology and scanning electron microscopy analyses

Histological analysis

Before the introduction of the tTCL technique, nodal segments with a single axillary bud were used as explants, but after three years of studies, no bud breakage occurred. Histological analysis confirmed the viability of axillary buds of *M. indica* cv. Harumanis showed highly stained cells in the basal part and leaf primordia, indicating meristem cells.

Mango trees, like apples and walnuts, exhibit an acrotonic branching pattern (Brunel *et al*., 2002; Solar *et al*., 2011; Boudon *et al*., 2020). This type of tree tends to transport sap to the buds near the apical part of the stem, resulting in larger axillary buds

at the top that become smaller towards the bottom. Axillary buds near the tip show a higher percentage of bud burst, which is consistent with the acrotic growth pattern. The same phenomenon was also observed in apple and walnut trees. Morphological analysis of apple tree buds showed that the size of axillary buds was larger at the top and middle of the stem than at the bottom (Brunel *et al*., 2002). It was also found that the percentage of bud burst was highest in axillary buds at the top of the stem (100%), followed by buds in the centre (55%) in the same report. In addition, the author mentioned that no bud burst was observed at the axillary buds at the bottom of the stem.

Moreover, the viability of the basal nodal segments of hazelnut shoots decreased significantly compared to the nodal segments around the apical part of the stem (Hand *et al*., 2016). It has also been reported that the xylem parenchyma cells in the apical part of the stem of walnut trees have higher xylem sap concentration and sucrose uptake than the basal part of the stem during the season when bud break begins (Decourteix *et al*., 2008). The author mentioned that bud break was only observed on axillary buds in the apical part and not on axillary buds in the basal part of the stem.

Scanning electron microscopy analysis

In Figure 7(a), a strong bacterial infestation was observed in the protoxylem vessels of the xylem of immature green leaf shoots, which is consistent with the results in tomato and sugarcane (Olivares *et al*., 1997; Chalupowicz *et al*., 2012; Peritore-Galve *et al*., 2021). The bacteria preferentially infect the protoxylem vessels (the first part of the xylem) and not the metaxylem vessels (the xylem part formed after the protoxylem). Most of the protoxylem vessels were blocked by the bacterial biofilm. This bacterial blockage was evident in the protoxylem vessels infected with *Herbaspirillum rubrisubalbicans*. It can be assumed that nodal segments from the upper and middle part of the stem at the immature green leaf stage have a lower bacterial biomass in the xylem vessels than those from the lower part. For a comprehensive understanding of the bacterial infection mechanisms, a longitudinal analysis is recommended. In addition, SEM analysis of axillary buds revealed a fungus originating exclusively from the axillary bud. Despite thorough cleaning, the fungal infestation persisted, which is consistent with the results of Pasqualini *et al*. (2019) in bamboo. The study suggests that endophytic fungi impair the effectiveness of surface sterilisation in controlling microbial growth *in vitro* (Pasqualini *et al*., 2019). However, the SEM analysis was not performed on the surface of the axillary bud. Otherwise, it would provide a better picture of the mechanism by which the fungus infects the axillary bud.

CONCLUSION

It was found that pretreating the *M. indica* cv. Harumanis mother plants with 0.5 g/L azoxystrobin, sterilising the surface of the nodal segment at the immature green leaf stage with 40% Clorox®, 0.5 g/L benomyl solution and 300 mg/L cefotaxime solution, applying tTCL to the nodal segments and keeping the cultures in WPM with 0.5 g/L benomyl and 300 mg/L cefotaxime significantly improved the percentage of sterility. Although the success rate of this protocol was strongly influenced by weather conditions, sterility of the remaining plants was ensured even after 7 months of cultivation. This study proves that pre-treatment and subsequent crop care is as important as surface sterilisation. Although the growth of leaf primordia was observed from the tTCL-treated explants, growth stopped at 1 - 2 mm and the cultures experienced necrosis after 2 months. These phenomena raised questions about the viability of tTCL-treated axillary buds. The results of histological and SEM analysis showed that the nodal segments at the upper and middle ends of the stem were more mature and sterile at the immature green leaf stage. These results are as they will determine the appropriate source of explants for future studies.

ACKNOWLEDGEMENT

The authors wish to acknowledge Universiti Sains Malaysia for providing the Research University Team Grant Scheme [Grant No: 1001/PBIOLOGI/8580101].

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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