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Inhibitory Effect of Kanamycin on *In Vitro* Culture of *Lycopersicon esculentum* Mill cv. *Mt11*

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ABSTRACT

Excised cotyledons of tomato (*Lycopersicon esculentum* Mill cv. *MT11*) were cultured on selective medium containing kanamycin at various concentrations (50, 100, 200, 300 mg/L). Significant toxic effects were observed when the cotyledon explants were grown on MS medium supplemented with 5 mg/L kinetin and 100 mg/L kanamycin. The regeneration of callus was decreased as the concentration of kanamycin increased from 200 to 300 mg/L. Explants grown on MS medium supplemented with 5 mg/L kinetin and 50 mg/L kanamycin showed the least toxic effects (mean survival rate 48.0% ± 0.19) compared to the rest of the concentrations tested. Even though 100 mg/L of kanamycin allows the non-transformed explants to grow on the medium, the shoot primordia would not develop further. The result suggests that 100 mg/L of kanamycin can be used effectively to differentiate between non-transformed and transformed *MT11* tomato explants with a death rate of more than 82% of non-transformed explants, after 4 weeks of incubation on selection medium. Therefore, 100 mg/L kanamycin is suitable for minimal inhibition concentration for *MT11* and true transformants can be selected at this concentration for the transformation system.

Keywords: Kanamycin, *Lycopersicon esculentum* cv. *MT11,* minimal inhibitory concentrations (MIC), tomato cotyledon explants

ABSTRAK

Kotiledon tomato (*Lycopersicon esculentum* Mill cv. *MT11*) yang dipotong telah dikultur ke atas medium memilih yang mengandungi pelbagai kepekatan kanamicin (50, 100, 200, 300 mg/L). Kesan toksik bererti telah dicerapi apabila eksplan kotiledon ditanam di atas medium MS yang mengandungi 5 mg/L kinetin dan 100 mg/L kanamicin. Regenerasi kalus susut apabila kepekatan kanamicin meningkat daripada 200 ke 300 mg/L. Eksplan yang ditanam di atas medium MS yang mengandungi 5 mg/L kinetin dan 50 mg/L kanamicin menunjukkan kesan toksik yang paling kurang (purata kadar kemandirian 48.0% ± 0.19) berbanding dengan kepekatan lain yang telah dikaji. Walaupun eksplan yang tidak transform dapat tumbuh di atas medium yang mengandungi 100 mg/L, primodia pucuk tidak dapat berkembang seterusnya. Keputusan ini menunjukkan bahawa 100 mg/L of kanamicin boleh digunakan dengan berkesan untuk membezakan diantara tomato *MT11* yang tidak transform dan yang transform, dengan kadar kematian eksplan yang tidak transform melebihi daripada 82%, berlaku selepas 4 minggu pengeraman di atas medium pemilihan. Dengan demikian, 100 mg/L kanamicin adalah kepekatan perencatan minima yang sesuai untuk tomato *MT11* dan pemilihan transforman boleh dibuat menggunakan kepekatan tersebut untuk sistem transformasi.

Kata kunci: Kanamicin, *Lycopersicon esculentum* cv. *MT11*, kepekatan perencatan minima (KPM), eksplan kotiledon tomato

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INTRODUCTION

The cultivated tomato (*Lycopersicon esculentum* Mill) belongs to the genus *Lycopersicon* of the Family Solanaceae. There are several tomato varieties that are cultivated both in the highlands and lowlands in Malaysia. The tomato varieties that are cultivated in the highlands of Malaysia are Cameron Highland varieties, *L24* and *K22*. Currently, more lowland tomato varieties are being cultivated such as *MT1* and *MT11, Serdang 2* and *King Kong* (F1). For lowland cultivation, *MT1* and *MT11* are recommended with a harvesting quantity of 15 to 25 t/ha (Agri-Food Business Development Center, 2004). Tomato variety *MT11* is more suitable for the processing industry and has better resistance towards diseases caused by bacteria compared to *MT1*. Therefore, it is important to improve the *MT11* and other lowland tomato varieties for better resistance and higher yield of tomato fruit. In addition, the tomato plant is also susceptible to several pests and viruses, which attack the flowers, leaves and immature fruits causing reduction in *MT11* tomato production. Thus, the improvement of the plant defense system *via* gene regulation is required to establish a suitable transformation system for *MT11* either *via* DNA bombardment or the *Agrobacterium*-mediated transformation technique.

Agrobacterium-mediated transformation protocols have been developed in tomato interspecific hybrids for a wide variety of purposes, from expression analysis of agronomically important genes and their promoter elements such as those involved in flower development to the analysis of the genomic distribution and genetic behavior of introduced genes (Fillatti *et al*., 1987; Bird *et al*., 1988; Twell *et al*., 1991; Eyal *et al*., 1995). These protocols reported the success rates of different transformation processes that are dependent on factors such as the cultivar genotype, *Agrobacterium* strain (which specifies its growth capacity), antibiotic selection of transformants, and the utilization of acetosyringone, which is effective in enhancing the transformation process (Joao *et al*., 1993). In this study, the minimal inhibitory concentration (MIC) of antibiotic was determined in order to differentiate between the transformed and non-transformed *MT11* tomato explants. A number of studies had been conducted to determine the optimum minimal inhibitory concentration of kanamycin on transgenic tomato plants. The usual concentration to isolate the transformed from non-transformed tomato explants ranges from 50 mg/L (Thompson *et al*., 2000; Riggs *et al*., 2001; Li *et al*., 2003) to 100 mg/L of kanamycin (Krasnyanski *et al.,* 2001; Mathews *et al*., 2003; Li *et al*., 2003). Higher concentrations such as 150 to 200 mg/L of kanamycin added to selection medium can cause death, even for the transgenic cells. High kanamycin concentration might be used for natural selection, not for transformation purposes. Therefore, the objective of this study was to determine the minimal inhibitory concentration of kanamycin that inhibited the growth of tomato (*Lycopersicon esculentum* Mill cv. *MT11)* explants.

MATERIALS AND METHODS

Plant Materials

MT11 tomato seeds were obtained from the Malaysian Agricultural Research and Development Institution (MARDI). The seeds were surface-sterilized with 70% (v/v) ethanol for 5 minutes and a sterilant consisting of 20% (v/v) bleach (with 1.5% sodium hypochlorite activity) and one drop of Tween 20 (polyoxyethelene-sorbitan monoleast) for 15 minutes. The sterilant was then discarded and the seeds were rinsed with sterile distilled water thrice or until traces of the sterilant were completely removed. The seeds were then blot-dried on sterile filter paper and transferred onto a half MS hormone-free medium. The seeds were incubated at 26 + 2 °C under a 16 hour photoperiod, 2000 lux light regime for 2 weeks. Cotyledons from the seedlings were excised and then cultured onto selection medium.

Selection of *MT11* using Kanamycin

Excised cotyledons of *MT11* were cultured onto MS medium (3% sucrose, pH 5.7) supplemented with 5 mg/L kinetin (regeneration medium) and various concentrations of kanamycin (50, 100, 200 and 300 mg/L), and observations of explant viability were made weekly for four weeks. The cotyledon pieces were placed on the medium with the abaxial side up. A control experiment was carried out for *MT11* cotyledon using MS medium (3% sucrose, pH 5.7) supplemented with 5 mg/L kinetin but without the addition of kanamycin. The explants were incubated for 4 weeks at 26 ± 2 °C under a 16 hour photoperiod. A total of 50 excised cotyledons were cultured onto each medium and the experiment was repeated three times. Assessment of the effect of different levels of kanamycin on *MT11* explants was made to determine the suitable concentration for selection of *MT11* transformants.

Data Collection and Analysis

Observations and data collection were performed after one week in culture. The numbers of explants showing formation shoots primordia were recorded. The shoot primordia were isolated from the pre-existed explants and transferred onto fresh medium and incubated at 26 ± 1 °C for further elongations. Shoot elongation was determined by observing the increment in the height of individual shoots formed. The percentage of mean value and standard deviation survival rate of *MT11* explants were analyzed using ANOVA (Analysis of variance) and DMRT (Duncan Multiple Range Test). The frequencies of shoot primordia formation and shoot elongation were determined for the experiment using the following formula:

$$\text{Shoot primordia formation (\%)} = \frac{\text{number of shoot primordia developed}}{\text{Number of explants}} \times 100\%$$

$$\text{Shoot elongation formation (\%)} = \frac{\text{number of shoot elongated}}{\text{Number of shoot primordia}} \times 100\%$$

RESULTS AND DISCUSSION

Toxicity of Kanamycin on Tomato *MT11* Explants and Formation of Shoots Primordial

Variation among the cotyledon explants were observed after the first week in culture. Those cultured on kanamycin-free regeneration medium (control) and on regeneration medium containing 50 mg/L kanamycin produced calli at the wounded sides (Figure 1). On the contrary, the explants cultured onto regeneration medium containing 100, 200 and 300 mg/L of kanamycin did not show any response after one week. On the second and third week, the control cultures showed formation of shoot primordia (Figure 1c). As shown in Table 1; 48, 25, 12 and 15% of the explants cultured onto regeneration medium containing 50, 100, 200 and 300 mg/L kanamycin, respectively, also produced shoot primordia. By the end of the third week, the explants that turned brown (Figure 1f) or bleached in cultures were discarded from the plates and the surviving ones were transferred onto fresh medium for another week before further observations were carried out.

Toxicity of Kanamycin on Tomato *MT11* Explants and Shoots Elongation

After four weeks in culture, the control cultures (95%) and those cultured in the presence of 50 mg/L kanamycin (88%) elongated and were transferred onto rooting medium. Fifty-two percent of shoot primordia from 100 mg/L kanamycin-treated cultures showed stunted shoot formation. The shoot primordia from 200 and 300 mg/L kanamycin treated cultures experienced 95 to 97% of necrosis, browning and bleaching, and eventually death. The cultures from 50 mg/L kanamycin showed a highly significant difference ($P < 0.01$) for survival of *MT11* explants with a mean value of 48.0% ± 0.19, 50 mg/L kanamycin gave the lowest percentage of cell death for *MT11* due to low inhibition from the antibiotic used. Cultures placed on medium containing 100 mg/L of kanamycin showed a significant difference ($P< 0.01$) with a mean value of 27.5% ± 0.22 for the survival rate of *MT11* explants. The cultures placed on medium containing 200 and 300 mg/L kanamycin were not significantly different in terms of their survival of the explants, where the mean values obtained were 12.0% ± 0.00 and 12.0% ± 0.47, respectively (Table 1). These high concentrations of kanamycin inhibit the growth and development of non-transformed cells.

High concentrations of kanamycin bleached (due to lost of chlorophyll) almost 100% of the explants which resulted in cell death of the non-transformed cotyledon explants (Figure 2). The survived shoot primordia (Figure 2d) from explants treated with 100 mg/L kanamycin showed elongation of shoots. Treatments with 200 and 300 mg/L of kanamycin were not suitable even though both selection media gave the highest cell death. These high concentrations of kanamycin added into regeneration medium might inhibit or kill transformed cells. Adding 50 mg/L of kanamycin to the regeneration medium of tomato cv. *MT11* resulted in too many 'escapes' (growth of non-transformed plants which escape the antibiotic selection system) (unpublished data). In several studies, 50 mg/L of kanamycin was used for the selection medium for tomatoes (Thompson *et al.*, 2000; Riggs *et al.*, 2001) and *Arabidopsis thaliana* (Li *et al.*, 2003). However, in this study 50 mg/L kanamycin was not suitable for screening transformants. A higher concentration of antibiotic such as 100 mg/L of kanamycin was more suitable for selection of transformed cells of tomato cv. *MT11*. Similar observations have been reported in yet other tomato varieties (Krasnyanski *et al.*, 2001; Mathews *et al.*, 2003). However, for other tomato cultivars, such as *Daniela 144, Brillante 179, Annan 3017, Galina 3019* and *Bernadine 5656*, higher concentrations of kanamycin (150 to 200 mg/L) have been used to avoid regeneration of the 'escapes' (Velcheva *et al.,* 2004); whereas in another tomato variety such as *Zhongshu No. 5*, 80 mg/L of kanamycin was optimal for selection of transgenic tomato (Ying *et al.,* 2008). Thus, this suggests that the selection of transgenic tomato plant is dependent on the genotypes of tomato cultivars. Higher concentrations of kanamycin (200-300 mg/L) have also been used as selection systems for other plants such as tea plants (*Camellia sinensis*) (Matsumoto and Fukui, 1998) and rubber trees (*Hevea brasiliensis* Muell Arg.) (Jayashree *et al.,* 2003). Yet, in some transformation procedures, no selection was made until the cells developed into plantlets, and selection for transformation was carried out using molecular analysis (Wang *et al.*, 2001). The results showed no calli formation due to stringent selection pressure exerted by 100 mg/L kanamycin.

For tomato cv. *MT11*, the leaf explants showed severe toxicity effects such as browning and bleaching, and eventually died within three weeks in selection media, which contained concentrations higher than 100 mg/L kanamycin. The results above were similar to the studies done by Li *et al.* (2003) and Mathews *et al.* (2003) on tomato (inbred line, L4783) and *Arabidopsis thaliana* transgenic plants. The 100 mg/L kanamycin selection media killed 82.5% of the non-transformed; which is a fair amount of deaths for non-transformed explants and therefore, is suitable to be incorporated in selection medium after inoculation with *Agrobacterium*.

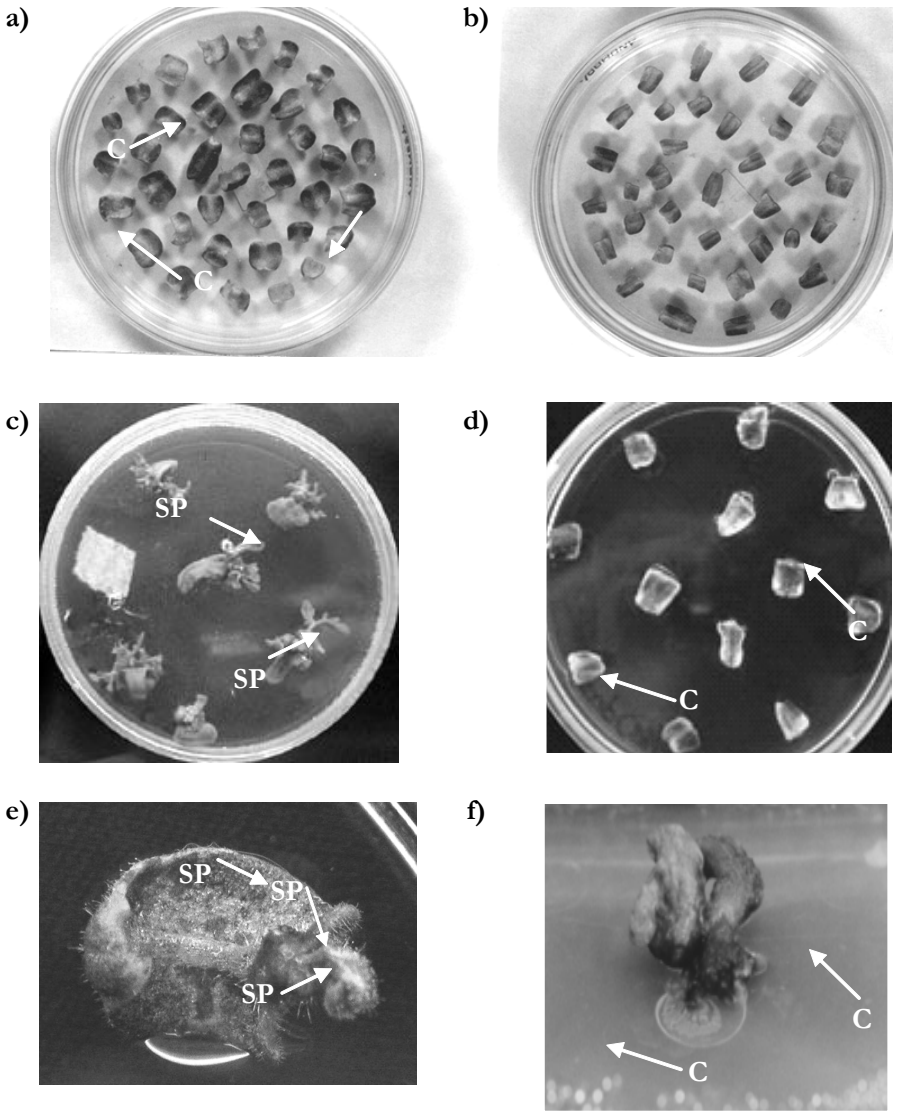

Fig. 1. *MT11* tomato cotyledons on selection and non-selection plates: a) MS medium supplemented with 5 mg/L kinetin after 1 week (control culture), and b) MS with 100 mg/L kanamycin and 5 mg/L kinetin after 1 week; c) shoots primordial regeneration from cultures on MS medium supplemented with 5 mg/L kinetin after 3 weeks (control cultures), and d) no callus or shoot primordial regenerated from cultures of MS medium supplemented with 100 mg/L kanamycin and 5 mg/L kinetin after 3 weeks, e) shoot primordial (SP) close-up, and f) shoot primordial turned brown in cultures on selection medium containing 100 mg/L kanamycin. The arrows indicate the presence of calli (C) and shoot primordia (SP).

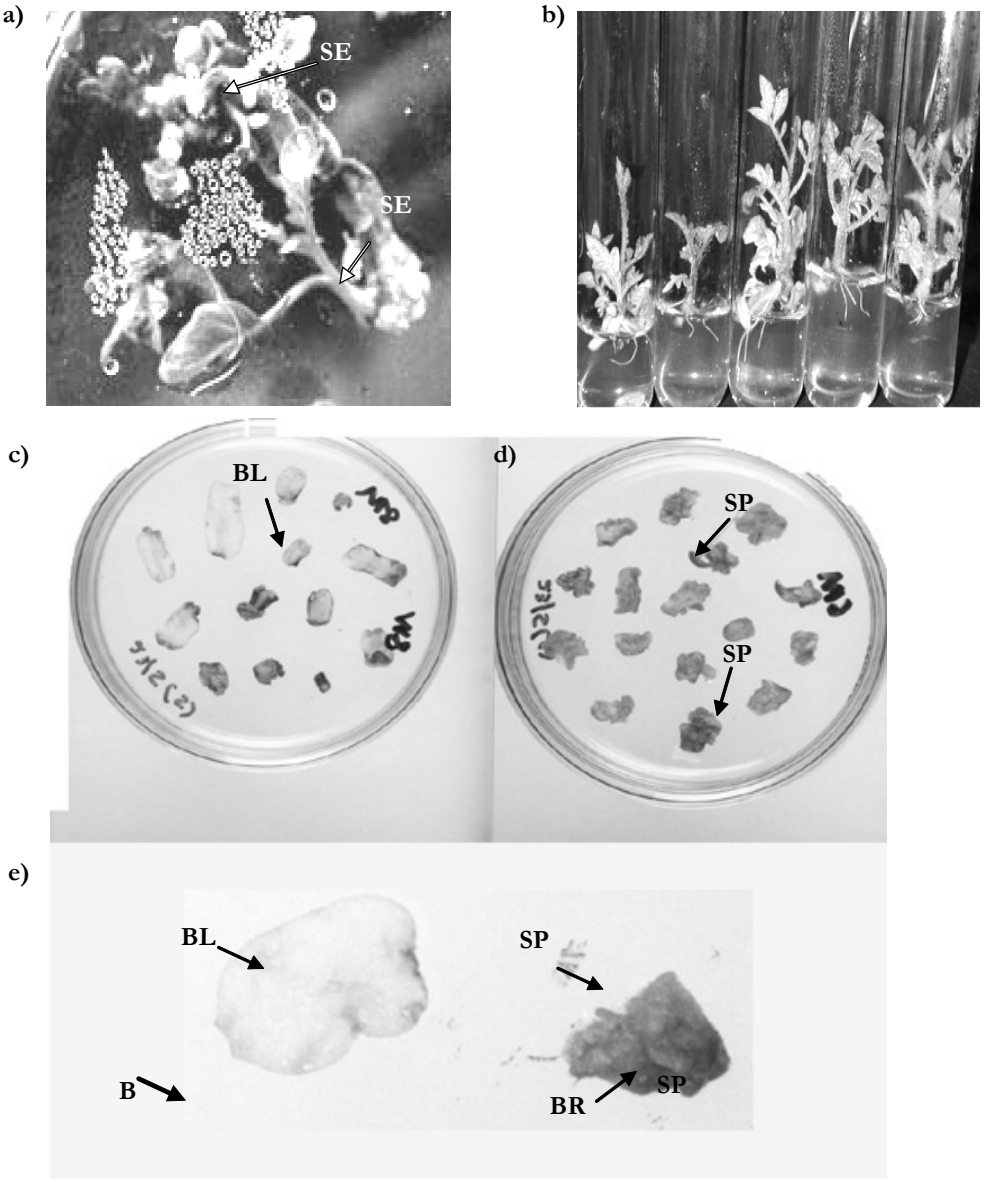

Fig. 2. The regeneration of *MT11* from cotyledons after the 4 weeks: a) Shoot elongation development from explants in MS medium + 5 mg/L kinetin, b) root development from the shoots and plantlets were obtained, c) and d) shoot primordial regeneration observed formed in MS medium supplemented with 100 mg/L kanamycin + 5 mg/L kinetin. Almost 95% of the callus did not grow into shoot primordial or elongation stage, and e) the cotyledon in selection medium experienced bleaching and browning effects along with the production of shoot primordial. The arrows indicate the presence of shoot elongation (SE), bleaching (BL), shoot primordia (SP), browning (BR) and rooting (R).

Table 1. Survival rate (%) of cotyledon explant of *MT11* tomato cultivar on regeneration medium supplemented with different concentrations of kanamycin.

[Kanamycin]**	*Means of % survival [1] ± SD
50 mg/L	48.0 ± 0.19[a]
100 mg/L	27.5 ± 0.22[b]
200 mg/L	12.0 ± 0.00[c]
300 mg/L	12.0 ± 0.47[c]

*Means with the same letters are not significantly different.
**Highly significant ($P < 0.01$) according to DMRT.
[1] c.v. = 7.5

These results showed that callus could not be obtained from a stringent selection pressure exerted by the presence of more than 100 mg/L kanamycin in the selection medium.
The explants also showed severe toxicity effects such as browning and death within 3 weeks on selective media which contained concentrations higher than 100 mg/L of kanamycin. The death and slow organogenic development is also probably due to the active release of toxic, phenolic compounds by the majority dead, non-transformed callus surrounding the explants as shown in 200 mg/L and 300 mg/L of kanamycin (Figure 2). Using 50 mg/L of kanamycin in the selection medium was not suitable due to the presence of too many escapes, and it would be difficult to screen for transformed cells after transformation. In conclusion, a minimal inhibitory concentration of 100 mg/L kanamycin was the best to be used in the transformation system for the selection of tomato cv. *MT11* transformants.

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