

Study of Bacteria and Fungi Isolate From Contaminated Banana Tissue Culture

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ABSTRACT: Contamination in Plant tissue culture poses a serious threat to plant tissue culture industry, more the complex media greater the chances of contamination, so to control this contamination viz. bacterial and fungal a potent drug methyl isothiazolinone (MIT) is used in this present study. MIT have been shown heat tolerant capacity and bacteriostatic and bacteriocidal effect on fungi and microbial contamination respectively. MIT don't have phytotoxic effect and give normal plant growth with controlling the contamination effectively. Normal concentration of MIT controlling the contamination was found to be 1.5%.

KEYWORDS: drug methyl isothiazolinone, bacteriocidal, bacteriostatic, phytotoxic effect

I. INTRODUCTION

Plant tissue culture techniques evolved due to the necessity of higher food demand along with unequal distribution of wealth which has subsequently increased stress on the food producers [1,2]. This demand and pressure of fulfilling the demand have given rise to new technologies that provides abundant yield of the crops with better quality.

Bacterial and fungal contaminations are one of the serious challenges that demands continued attention for successful crop propagation [3]. These contaminants if not controlled properly cause great loss to the agriculture industries as well as sectors dependent on them. This includes loss of expensive culture, time and production of infected plants used for plantation that collectively hamper growth and yield [4]. A wide range of antibiotic drugs have been used for controlling microbial and fungal contamination in plant tissue culture and in several cases multidrug combinations demonstrated superior results [5].

Heat sensitivity of classical antibiotics prevents their inclusion in the media prior to sterilization process which is one of the primary hurdles for its extensive use in commercial set up. This has given rise to adoption of different methods that employ synthetic anti microbial and anti fungal agents in plant tissue culture. In this study, we attempted to evaluate the response of a set of commonly found and persistent microbial as well as fungal contaminants encountered in a banana tissue culture laboratory environment towards MIT (methyl isothiazolinone) and explore the feasibility of its use as a heat tolerant component of the tissue culture media formulation.

II. MATERIALS AND METHOD

Isolation of microbial contaminants from infected Tissue culture bottles

Banana tissue culture bottles infected with frequently encountered bacterial and fungal contaminants were used as the starting material for this study.



Fig 1 Contaminated tissue culture bottles

Suspensions with 1 g of the contaminated sample from tissue culture bottles and 9 mL of sterile distilled water were prepared; each suspension was diluted from 10⁻¹ until 10⁻⁸. From these dilutions, 0.1 mL were inoculated in plates with Luria Agar and Muller Hilton Agar for bacteria and Potato dextrose agar for fungi and incubated at 37°C for 24 hours for bacteria and 28°C for 72 h for fungi. Colonies with different characteristics were selected and preserved at 4°C. Growth curve of each bacterial isolates was determined for 24 h at regular interval of 2h.

Testing of different antibiotics sensitivity to microbes by Disc diffusion method

Prepare MH agar according to the manufacturer's instructions, with supplementation for fastidious organisms. 5ml sterile LB broth inoculated with bacterial colony, the culture suspension O.D adjusted to McFarlands standard (0.5). Spread 1ml of adjusted culture suspension on sterile Muller Hinton's Plate using sterile glass spreader precaution should be taken that the culture should be spread evenly. Antibiotic strips used for ascertaining sensitivity were procured from Hi media. Place the antibiotic disc on plates Using sterile forceps. strips used for ascertaining sensitivity was procured from Hi media. Incubate the plates for 24 hours at 37°C.

Results recorded using ruler.

Determining the MIC and MBC using drug methyl isothiazolinone on ELISA reader machine

5ml sterile LB broth inoculated with bacterial colony incubated for 24 hrs at 37°C, 40ml sterile LB was prepared in 250ml flask each and autoclaved at 15 psi for 15 min. Desired concentration of drug added in Flask (Conc. 0.05%, 0.1%, 0.5%, 1% and 1.5%) In each flask 200uL of culture is added And incubated for 24 hrs at 37°C. 200uL Sample loaded in each well, Results (MIC) recorded using 96-well micro- titre plate technique.

The concentration at which complete inhibition of growth was observed from that flask 100uL of culture broth was spread on sterile LB Agar plate and results were recorded after 24 hours.

Efficiency of methyl isothiazolinone at high temperature was determined by addition of MIT before autoclaving.

Comparison between methyl isothiazolinone with traditionally used Tetracycline at high temperature

Same procedure used in MIC only difference is that the addition of drug (Tetracycline) before autoclaving and after autoclaving. Results obtained compared with the result of methyl isothiazolinone.

Isolation of fungus

Contaminated bottles were examined for fungal growth and that growth was spot inoculated on sterile Potato Dextrose agar plate. Incubated at 28°C for 72 hours. Again spot inoculated on new plates.

Effect of drug on Fungus

From the thick spore suspension 100uL is spread on the PDA plates containing the different concentration of methyl isothiazolinone. Plates incubated at 28°C for 72 hours. Results were recorded.

Evaluating the efficiency of methyl isothiazolinone in Murashige Skoog medium

Methyl isothiazolinone added in MS media in different at different concentrations (Conc. 0.05%, 0.1%, 0.5%, 1% and 1.5%) before autoclaving then Stored at room temperature near sunlight, Tubes examined after 1 week and results recorded on the basis of the visible growth.

MIT Effect On Banana Plants

MIT was added in tissue culture media at a concentration of 1.5%. Suckers were then subsequently inoculated into the initiation media.

III. RESULT

Determination of Growth curve

After examining the contaminated bottles and respective plates incubated and the colonies with unique features are isolated and named as M1, M2, M3, M4 and fungal contaminant as F1, F2, F3, F4. And growth curve of bacterial isolates were determined.

Formula for calculating generation time: at absorbance 0.6 the time recorded as T_1 and when the absorbance is 0.2 this time recorded as T_2 .

Then $T_2 - T_1 = \text{Generation time}$

The generation time suggested that all the microbe isolated are fastidious growing which will cause depletion of nutrient present in the media.

After checking the generation time it was important to check the effect of different drugs on the isolated microbes. For this purpose different antibiotics sensitivity to microbes by Disc diffusion method was carried out and the results are noted. (Table 2 and fig 2)

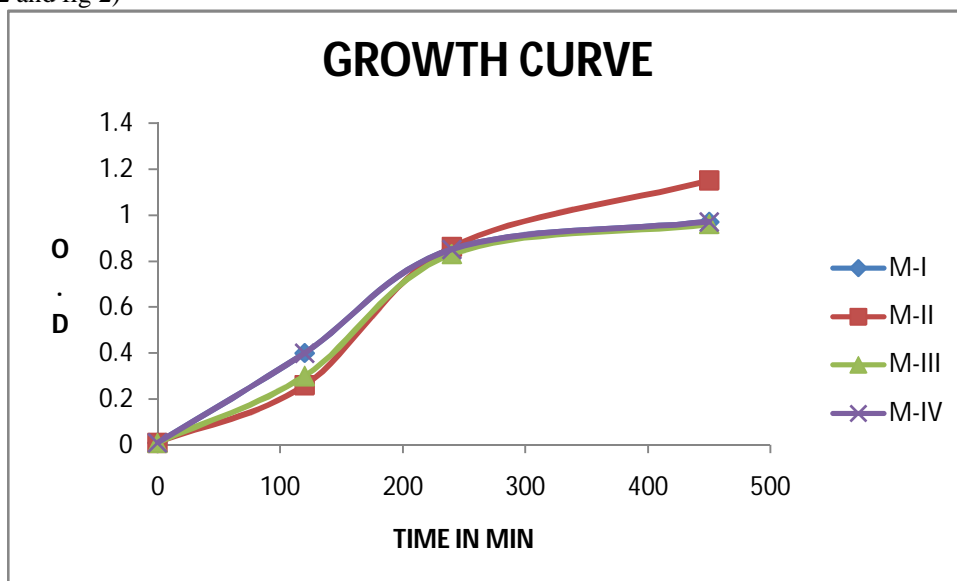


Fig2 Growth curve of microbial contamination

NAME OF MICROBE	GENERATION TIME	GRAM CHARACTER
M1	24 min	Gram negative rods in chain
M2	39 min	Gram positive cocci in pairs
M3	24min	Gram positive rods
M4	30min	Gram positive cocci in cluster

Table1 Generation Time From Growth Curve

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 5, Issue 3, March 2016

Determining the MIC Of Individual Microbe Using methyl isothiazolinone: (Drug addition after autoclaving)

Drug used:

Methylisothiazolinone solution was procured from Sigma Aldrich, USA. A 0.5% solution was prepared using sterile deionized distilled water as stock solution for all experimental purposes.

DRUG	M-I	M-II	M-III	M-IV
VA30	1.8	0.8	1.3	1.2
CFM5	3	0.5	0	0
TE30	1.2	1.5	1.4	1.5
S10	1.6	0.5	1.7	1.3
RIF5	1.1	2.4	1.5	0
LE5	3.6	1.7	1	2.6
CPD10	1	0.6	1.6	1.4
CTR30	3.2	0.6	1.7	1
CD2	1.6	2	2	2.5
AMC30	1.4	0	1.2	0.6
AK30	2.4	0.6	1.7	1
C30	0	1.4	2	0

Table 2 Zone of Inhibition shown by antibiotics

DRUG CONCENTRATION IN μ l	M-I	M-II	M-III	M-IV
CONTROL	1.014	1.272	1.8745	0.1606
40 μ L	0.5026	0.111	1.331	0.0824
200 μ L	0.1526	0	1.2344	0
400 μ L	0.0146	0	1.1394	0
600 μ L	0	0	1.0428	0

Table3 MIC of different microbes

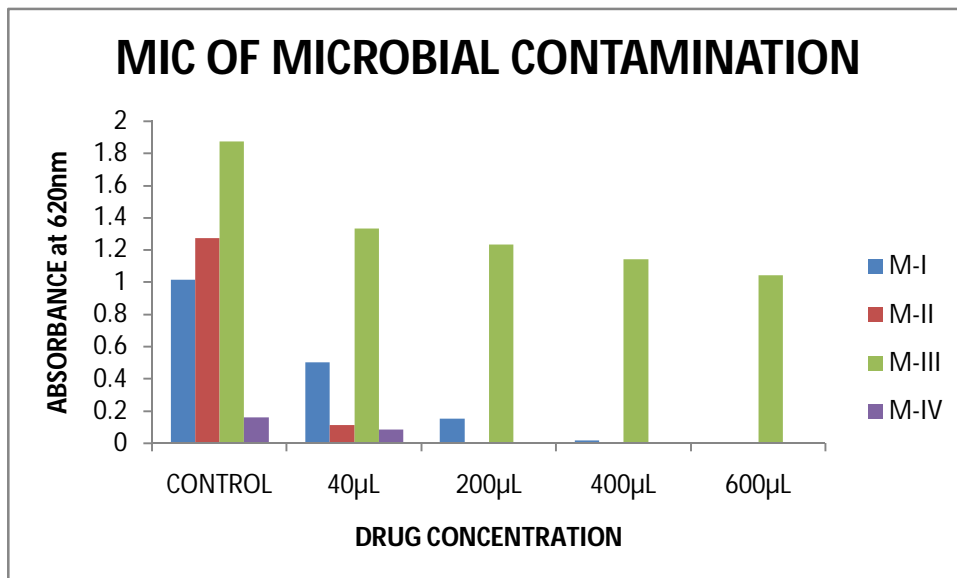


Fig 3 Determination of Minimum Inhibitory concentration of microbial contaminant against different concentration of MIT

MIT is considered as the heat tolerant drug to check its efficiency drug is added before autoclaving in concentration previously used. This test was carried out on the M4 contaminant.

	CONTROL	BLANK	0.10%	0.50%	1%	1.50%
BEFORE AUTOCLAVING	0.8785	0.049	0.09225	0.071	0.04825	0
AFTER AUTOCLAVING	0.76	0.0809	0.084	0	0	0

TABLE05: Different concentration of MIT before and after autoclaving on M4 contaminant

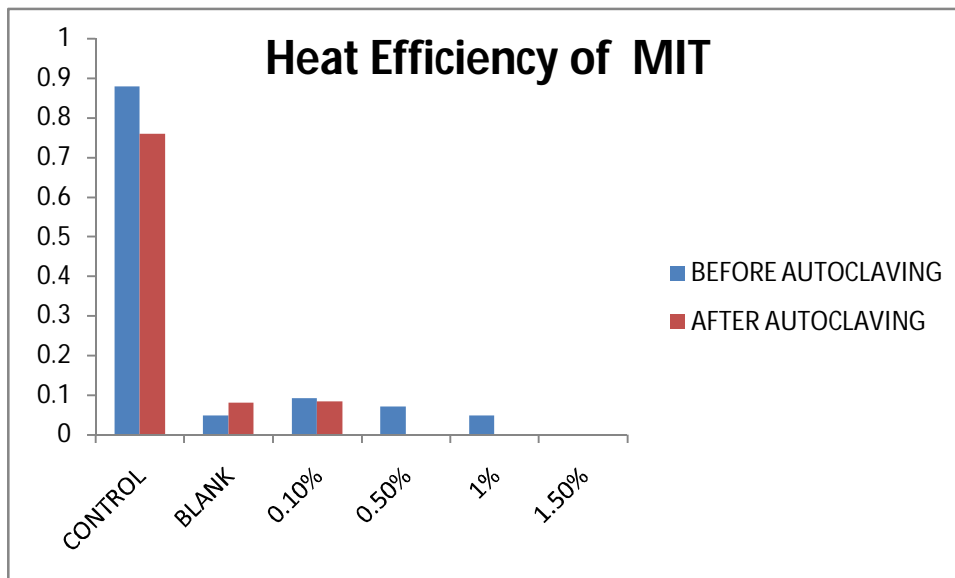


Fig 5 Determination of Heat Efficiency of MIT

Four very distinct and fast growing fungus was selected. Sabouraud dextrose agar dextrose agar plates were prepared of different concentration same concentration used for microbial contaminant

Efficiency of methyl isothiazolinone in Murashige Skoog medium

Effect of MIT was tested on Murashige and medium with the same concentration used above in MIC testing (BEFORE AUTOCLAVING) and it was observed that at the least concentration of MIT i.e 20 μ L (0.05%) the contamination was completely inhibited..

MIT Effect On Banana Plants

After completing the test on the contaminant and the medium used in plant propagation it was necessary to check its effect on the banana plants. The plants were shown to grow satisfactorily as compared to control (no MIT) indicating that 1.5% did not have any cytotoxic effect on banana plantlets when added in tissue culture media.

IV. DISCUSSION

Contamination causes economical loss:

Results indicate that microbial and fungal contamination in plant tissue culture is a serious problem in commercial farms. The loss in plants is 30% or more due to these contaminations. Use of antibiotics and anti fungal agents can control a major part of these contaminants but its use is tedious, time consuming and expensive.

Growth assay suggest that The data suggest that the contaminating microbes are fast growing and hence with capability of fast depleting the media of its vital constituents. It further indicate that if not bactericidal, at least a well performing bacteriostatic agent is required to halt the growth when contaminated with, thus enabling the plants to complete their growth cycle.

Antibiotic testing have shown that The antibiotics sensitivity generated mixed responses. While several of the antibiotics were found to be with potential to control the growth of the contaminants, some of the microbes were found to be resistant to them. This indicate that cheap and economical antibiotics no longer can control plant tissue culture microbes and in some cases expensive antibiotics are required.

MIT inhibited microbes at varying concentration:

Results indicate that all the four isolates were inhibited at varying concentration of MIT. This was estimated using an ELISA reader in order to allow multiple assays to be performed that can impart statistical robustness to the experiment. However, this experiment was performed by adding MIT post sterilization.

International Journal of Innovative Research in Science, Engineering and Technology

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Vol. 5, Issue 3, March 2016

Fungus showed mixed response to the drug:

The control of fungi was studied on a solid plate since its growth cannot be monitored using spectroscopy. Two important observations were noted.

1. The control of growth of the fungi was partial as observed on solid plate and further,
2. A laboratory contaminant of unknown origin prevented proper recording of data. This called for proper maintenance of cleanliness and sterility of the work place for such kind of studies.

Antibiotic gets damaged when subjected to high heat and pressure

Our results indicated that antibiotics get degraded when subjected to sterilization process. Tetracycline was used for this demonstration. Results indicated that media containing antibiotic (tetracycline) when subjected to high heat and pressure allowed bacterial culture to grow comfortably. This is because the antibiotic got degraded. On the other hand, when this antibiotic was added after sterilization, it successfully prevented growth of the contaminating microbes.

MIT can be added like any media component prior to sterilization

This observation is very important because it indicates that MIT can be added while preparing the media and autoclaved. This will not damage or reduce its efficacy in controlling the growth of contaminating microbes. This is one of the unique superiority of MIT compared to traditional antibiotics.

V.CONCLUSION

The overall data indicate that MIT can be used prior to sterilization in controlling contaminating microbes in plant tissue culture. It does not have any cytotoxic effect on plants. It can replace an antibiotic that is traditionally added after sterilization. The process is economical and fast. It also has potential to save time and other resources. It can replace an antibiotic that is traditionally added after sterilization. The process is economical and fast. It also has potential to save time and other resources.

REFERENCES

- [1] Reed, B. M., Mentzer, J., Tanprasert, P., & Yu, X. Internal bacterial contamination of micropropagated hazelnut: identification and antibiotic treatment. *Plant cell, tissue and organ culture*, 52(1), P67-70 1998.
- [2] Bunn, E., & Tan, B. . Microbial contaminants in plant tissue culture propagation. *Microorganisms in plant conservation and biodiversity*, 307-335 2004.
- [3] Felise, H. B., Nguyen, H. V., Pfuetzner, R. A., Barry, K. C., Jackson, S. R., Blanc, M. P., ... & Miller, S. I. An inhibitor of gram-negative bacterial virulence protein secretion. *Cell host & microbe*, 4(4), 325-336 2008.
- [4] Sattigeri, V. J., Soni, A., Singhal, S., Khan, S., Pandya, M., Bhateja, P., ...& Mehta, A. . Synthesis and antimicrobial activity of novel thiazolidinones. *Arkivoc*, 2, 46-59 2005
- [5] Misra, P., Gupta, N., Toppo, D. D., Pandey, V., Mishra, M. K., &Tuli, R.. Establishment of long-term proliferating shoot cultures of elite *Jatropha curcas* L. by controlling endophytic bacterial contamination. *Plant Cell, Tissue and Organ Culture (PCTOC)*, 100(2), 189-197 2010
- [6] Kumar, D., Bux, F. B., Parmar, V., & Singh, A Thiazolidinone: synthesis and biological studies. *Der PharmaChemica*, 4, 538-543 2012
- [7] Jain, A. K., Vaidya, A., Ravichandran, V., Kashaw, S. K., &Agrawal, R. K. (2012). Recent developments and biological activities of thiazolidinone derivatives: A review. *Bioorganic & medicinal chemistry*, 20(11), 3378-3395 2012.
- [8] García-González, R., Quiroz, K., Carrasco, B., &Caligari, P. (2010). Plant tissue culture: Current status, opportunities and challenges. *Ciencia e Investigación Agraria*, 37(3), 5-30 2010.
- [9] Nagajyoti, P. C., Lee, K. D., &Sreeranth, T. V. M. Heavy metals, occurrence and toxicity for plants: a review. *Environmental Chemistry Letters*, 8(3), 199-216 2010
- [10] Reed, B. M., &Tanprasert, P. Detection and control of bacterial contaminants of plant tissue cultures. A review of recent literature. *Plant tissue culture and Biotechnology*, 1(3), 137-142 1995
- [11] Van den Houwe, I., Guns, J., &Swennen, R. Bacterial contamination in *Musa* shoot tip cultures. In *I International Symposium on Banana in the Subtropics 490* (pp. 485-492) 1997.
- [12] Isac, V., &Cristea, C. REZULTS OF MICROBIAL CONTAMINATION IN TISSUE CULTURE LABORATORY OF RIFG PITESTI. *Scientific Papers of the Research Institute for Fruit Growing Pitesti, Romania*, 27 2011
- [13] Swamy, R. D., Rao, N. K., &Chacko, E. K. Tissue-culture propagation of banana. *Scientia Horticulturae*, 18(3), 247-252 1983.
- [14] Niedz, R. P. Using isothiazolone biocides to control microbial and fungal contaminants in plant tissue cultures. *HortTechnology*, 8(4), 598-601 1998