

In vitro growth response to bacterial wilt pathogen of banana (var. lakatan, *Musa acuminata* Colla) plantlets regenerated from ethyl methanesulfonate-treated shoot explants

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ABSTRACT

Bacterial wilt caused by *Ralstonia solanacearum* leads to death of infected suckers and reduces the yield of commercially important banana varieties like Lakatan. Among the many varieties of banana, no germplasm with bacterial wilt resistance has been identified yet (Tripathi et al 2004).

Mutation induction in plants to develop disease resistance genes using physical or chemical mutagens has been used as alternative to harmful pesticides. To induce mutation for the possible development of resistance to bacterial wilt, shoot tips of Stage 2 in vitro-grown Lakatan plantlets were exposed to 0.1% and 0.2% ethyl methanesulfonate (EMS) for 12 and 24h. Treated and untreated explants were cultured in vitro to regenerate plantlets.

Shoots emerged two days after in vitro inoculation of explants treated with 0.1% EMS for 12h. Significantly longer shoots also developed from the cultures compared to the untreated explants. The other explants exposed to other treatments had shoot emergence one to three days later. Falcate, curled, irregularly-shaped, and yellowish leaves and pseudostems also developed in EMS-treated cultures.

Untreated plantlets exhibited at least one bacterial wilt symptom such as leaf spots, necrosis at pseudostem base, and death six days from the introduction of *Ralstonia solanacearum* in vitro. Plantlets from explants exposed to 0.1% EMS for 12h did not exhibit disease symptoms even after ten days of exposure to the pathogen and had 100% survival. Seventy one percent of plantlets from explants exposed to 0.1% EMS for 24h and 55% from explants treated with 0.2% EMS for 24h also survived without infection. The surviving plantlets need to be studied further for their ex vitro responses to the pathogen and determine possible genetic changes due to the chemical mutagen treatment.

Keywords: in vitro growth of banana, Ethyl methanesulfonate, Bacterial wilt screening, *Ralstonia solanacearum*

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INTRODUCTION

Banana (*Musa* sp.) is the largest agricultural commodity of the Philippines (PSA 2016). The three major banana cultivars in the country are the Cavendish, Saba, and Lakatan. Lakatan (*Musa acuminata* Colla) is the fifth most important banana crop in world trade. This variety can lower blood pressure because of its high potassium and low salt contents (Dadang 2014). However, Lakatan production in the Philippines is severely affected by many disease-causing bacteria such as the bacterial wilt organism *Ralstonia solanacearum*. Since no banana germplasm has the gene for resistance to the disease (Tripathi et al 2004), an alternative solution to the use of pesticides is to develop resistance genes by inducing mutation through the use of chemical mutagens like Ethyl methanesulfonate (EMS). Chemically-induced mutant genes have been developed for resistance to the *Fusarium oxysporum* f. sp. *Cubense* in Brazil banana, and *Pseudomonas syringae* in tomato. EMS is a very effective mutagen for creating somaclonal variations in banana (Omar et al 1989). Hence, it was used in this study for the possible induction of genetic changes that may lead to the development of resistance to bacterial wilt in tissue-cultured shoots of Lakatan.

MATERIALS AND METHODS

Preparation of Explants and Treatment with EMS

The study was done in factorial set up in Complete Randomized Design with three replicates, five samples per treatment per replicate. Stage 2 Lakatan shoots (Figure 1) were obtained from the Experiment Station Laboratory of the Department of Agriculture in Balinsasayao, Abuyog, Leyte. Shoots were soaked in either sterile 0.1% EMS, 0.2% EMS, or in distilled water (control) for 12 or 24h as treatments (Table 1).



Figure 1. Stage 2 in vitro grown shoots of Lakatan used as source of explants

Table 1. Treatments for the induction of mutagenesis in Lakatan shoot tip explants of Lakatan

Treatment Code	EMS concentration	Duration of exposure (h)
T01	Distilled water (No EMS)	12
T02		24
T11	0.1%	12
T12		24
T21	0.2%	12
T22		24

Medium Preparation and Culture of Treated Explants

Murashige and Skoog (1962) medium with supplements suitable for banana culture was prepared following standard procedures. Fifteen milliliters of the medium was dispensed to each culture vessel. The medium was autoclaved at 121°C and 15psi for 20mins and cooled for one day before use.

After exposure to treatments, tightly-packed layers of leaf sheaths were removed from the shoots to expose the shoot tip. The actively dividing region was excised aseptically and inoculated individually into each culture vessel. Cultures were incubated at 26±1°C under 8h per day lighted condition until plantlets had developed three to four leaves. The explants were observed weekly to monitor growth.

Isolation of Bacterial Wilt Causal Organism from Infected Banana Tissues

Ralstonia solanacearum was isolated from the glosses of banana fruits exhibiting symptoms of the disease known as “bugtok” in Leyte, Philippines, and cultured at the Plant Disease Diagnostic Laboratory of the Visayas State University. Terazolium Chloride Agar (TCA) medium was used to verify the virulence of the isolated bacteria. Pink bacterial colonies with cream peripheral growth were observed which are characteristic of very virulent bacterial wilt pathogen (Figure 2a & 2b).



Figure 2a. Colonies of *Ralstonia solanacearum* in TCA medium

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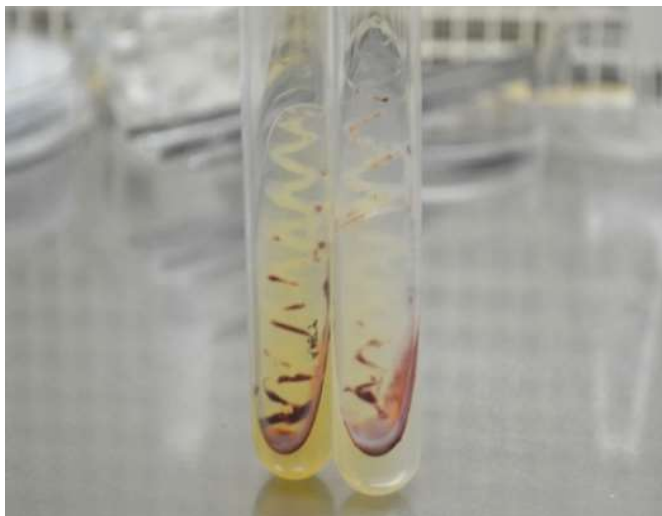


Figure 2b. Isolated *Ralstonia solanacearum* used in the preparation of bacterial suspension inoculated into the Lakatan cultures

In vitro* Screening for Response to *Ralstonia solanacearum

The bacterial suspension was prepared by adding sterile distilled water to the culture, scraping the bacterial colonies and shaking the solution well. One milliliter of the bacterial suspension was introduced into each culture with the untreated and EMS-treated regenerated plantlets inside a laminar flow hood to avoid contamination. The cultures were observed for symptoms of the bacterial infection for ten days after the introduction of the pathogen.

RESULTS AND DISCUSSION

Effects of EMS Treatments on the Growth of the Explants

Ethyl methanesulfonate (EMS) treatments enhanced the development of shoots from the explants, suggesting that 0.1% and 0.2% concentrations did not cause damage to the explants. Shoots emerged from the explants treated with 0.1% EMS for 12h, two days after inoculation (Figure 3). Explants treated with 0.1% EMS for 24h and those treated with 0.2% EMS for 12h developed shoots on the third day. Those exposed to 0.2% EMS for 24h and the untreated explants had shoot emergence four to five days from inoculation. A similar study by Dhakshanamoorthy et al (2010) also showed that the longer the duration of EMS treatment, the longer the time for the explants to grow. Jabeen and Mirza (2004) explained that the growth of shoots is influenced by many genes (polygenic) and higher mutagen concentration might affect at least one of these genes.

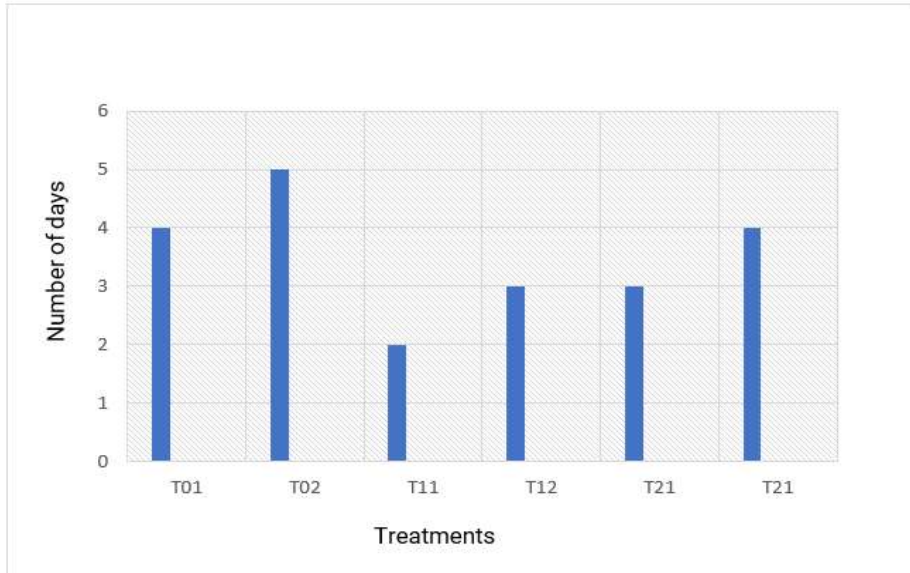


Figure 3. Number of days to shoot emergence from Lakatan explants

Secondary shoots were produced in all explants except in the control which was soaked for 12h in water. Some control explants did not even develop the primary shoot (Figure 4). Although the average number of shoots was higher in explants exposed to 0.1% EMS for 24h, differences in shoot production among treatments were not significant.

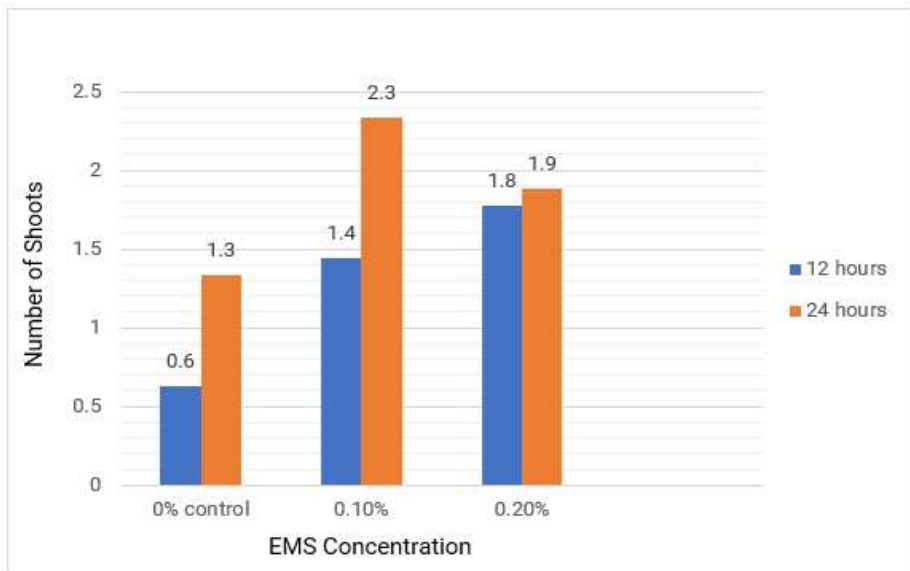


Figure 4. Average number of shoots produced per explant after EMS treatment

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EMS treatment significantly improved the growth of shoots. Shoots of EMS-treated explants generally grew faster than the control and shoots from explants treated with 0.1% EMS for 12h were significantly longer than the untreated ones (Figure 5). However, at the same concentration of EMS, longer exposure to treatment resulted in shorter shoots. Nevertheless, the negative effect of longer exposure to higher EMS concentration on shoot growth was not significant.

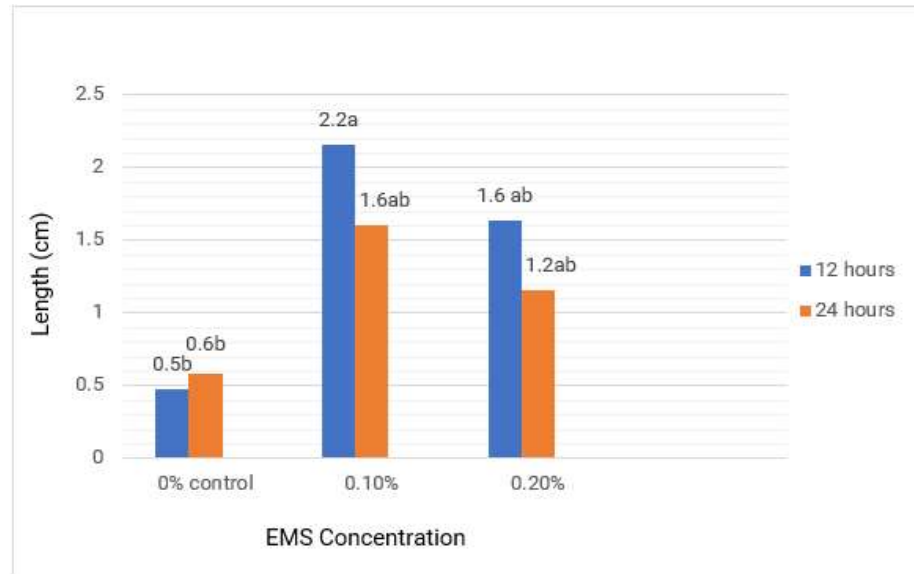


Figure 5. Average length of shoots produced by untreated and EMS-treated explants

The production of the shortest shoots in the control was not in line with most reports on the effects of EMS treatment on plants. A study on mutation induction in sugarcane showed that the untreated plants had longer shoots than the treated ones (Khan 2009). According to Konzak et al (1965), high concentrations of mutagenic agents can cause biological damage like seedling injury, lethality, and sterility, which increases with the increase in dosage and at a faster rate than the mutations. The significant difference in length of shoots between the explants treated with 0.1% EMS for 12h and the control might be due to enhanced production of hormones responsible for growth in the former.

Morphology of Shoots from EMS-Treated and Untreated Explants

Different colors and shapes of leaves were observed among shoots from different treatments. Untreated shoots had no color change and elliptic and lanceolate leaves were the most common (Table 2, Figures 6 & 7). Shoots of explants exposed to 0.1% EMS for 12h had yellowish pseudostem with leaf shape similar to the control, while those from explants treated with the same concentration for 24h developed curled and falcate leaves, in addition to yellowish pseudostem. Shoots from cultures exposed to 0.2% EMS for 12h were distinctly yellow including the leaf margins but the shape was similar to those developed in

explants treated with lower EMS concentration. Exposure to 0.2% EMS for 24h caused the development of falcate and irregularly-shaped leaves in entirely yellowish shoots. Color changes are common in mutant plants.

Table 2. Morphology of shoots from EMS-treated and untreated explants of Lakatan

Treatment	Color	Leaf Shape
T ₀₁ : no EMS, 12h	Green	Elliptic
T ₀₂ : no EMS, 24h	Green	Lanceolate
T ₁₁ : 0.1% EMS, 12h	Yellowish	Elliptic
T ₁₂ : 0.1% EMS, 24h	Yellowish pseudostem	Curled Elliptic, Falcate
T ₂₁ : 0.2% EMS, 12h	Yellow including the leaf margins	Elliptic, Lanceolate
T ₂₂ : 0.2% EMS, 24h	Yellow entirely	Lanceolate, Elliptic, Falcate, Irregular

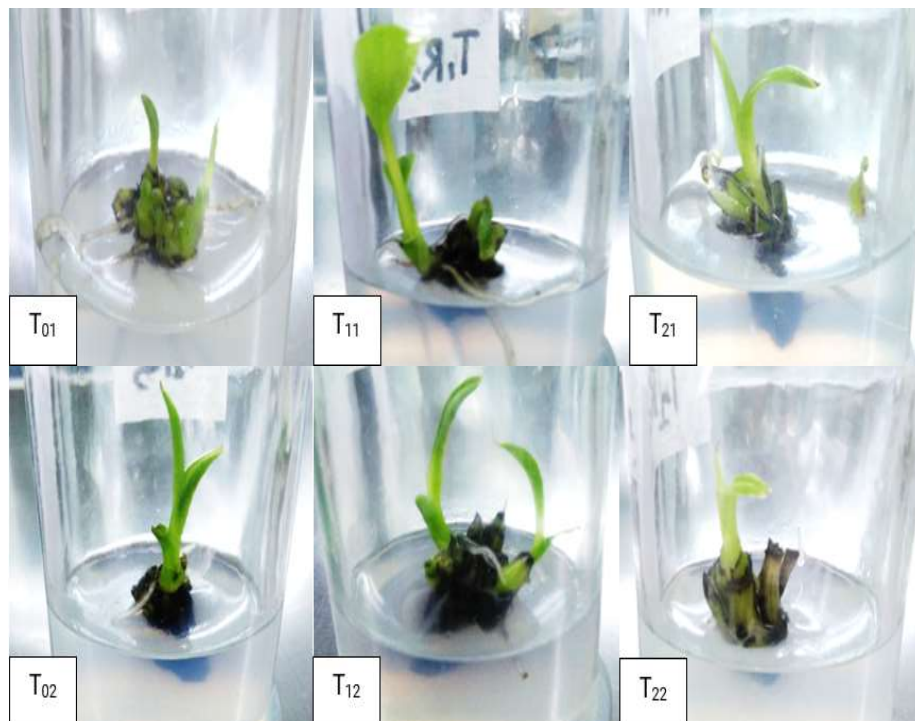


Figure 6. Colors of Lakatan shoots as affected by EMS treatment

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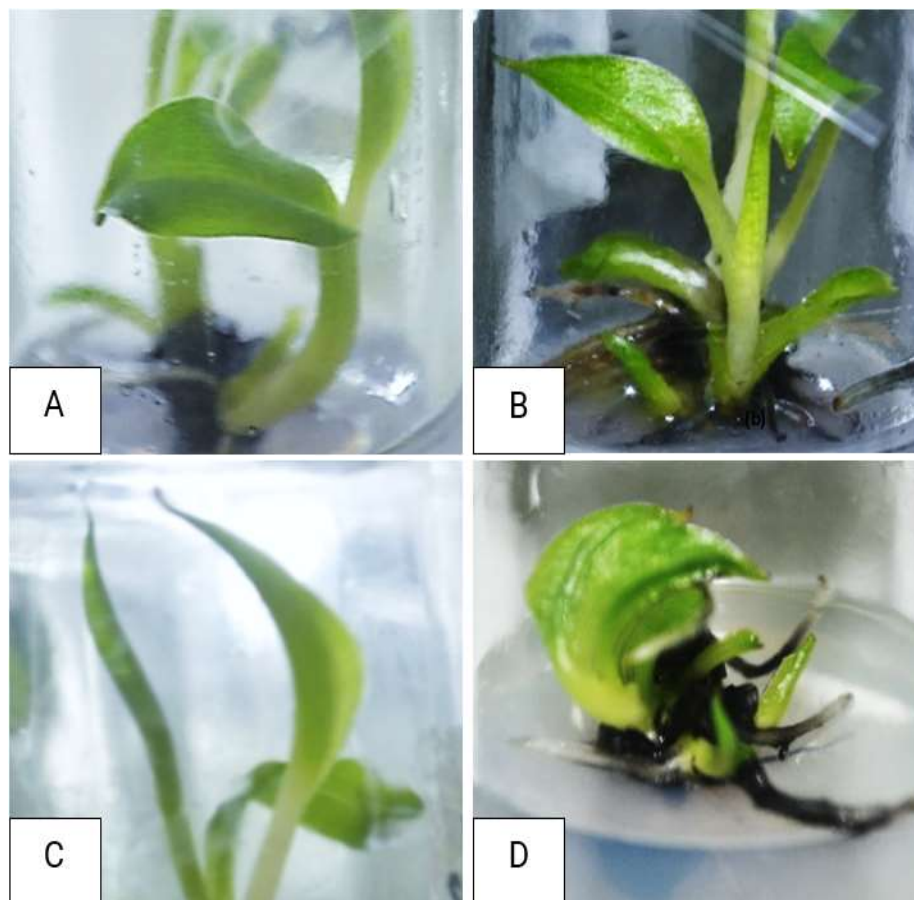


Figure 7. Leaf shapes of Lakatan shoots as affected by EMS treatment: (a) elliptic, (b) lanceolate, (c) falcate, and (d) curled/irregular

Response of Regenerated Shoots from Untreated and EMS-treated Explants to *Ralstonia solanacearum*

Bacterial colonies formed at the roots of cultures one day after inoculation. However, no bacterial wilt symptoms were observed on the shoots (Figure 8). As reported by Alvarez et al (2006), the bacteria can enter through physical wounds or natural openings, and attach at two precise root sites: the root elongation zones and axils of emerging or developed lateral roots. The root cortex of these zones may have the intercellular spaces invaded and filled with bacteria.

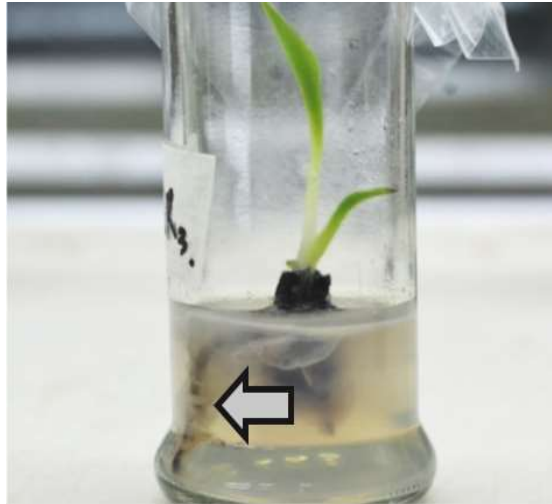


Figure 8. Formation of colonies of *Ralstonia solanacearum* in roots of an in vitro-grown Lakatan plantlet

Different bacterial wilt symptoms such as black and brown leaf spots, softening of tissues of the plants and death of plants were observed (Figure 9). Leaf spot was the most common. The control plants developed severe leaf spots while some EMS-treated plantlets had leaf spots which were not as severe as those in the control plantlets. Symptoms were first observed in the control plants six days after introduction of the pathogen (Table 3). Ten days after inoculation of the pathogen, disease symptoms also developed in some EMS-treated plants but the plantlets from explants treated with 0.1% EMS for 12h (T11) had no observable symptoms.

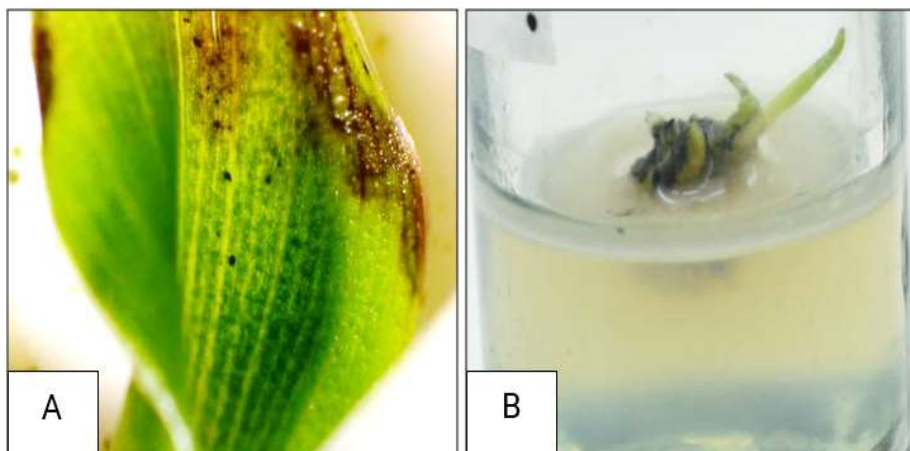


Figure 9. Bacterial wilt symptoms in in vitro-grown plantlets: (a) leaf spots in untreated plantlets, (b) softening of tissues resulting in slumping of the plantlets and (c) dead plantlets

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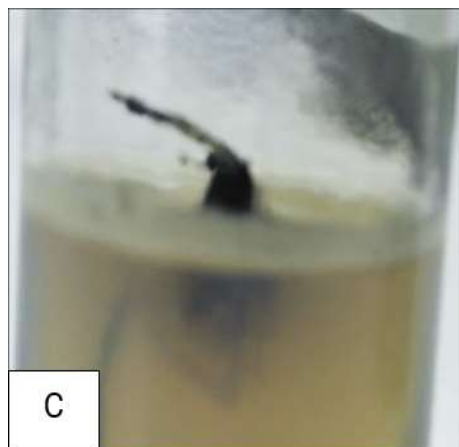


Figure 9. Continuation Bacterial wilt symptoms in in vitro-grown plantlets: (a) leaf spots in untreated plantlets, (b) softening of tissues resulting in slumping of the plantlets and (c) dead plantlets

Table 3. Duration from pathogen introduction to the first appearance of bacterial wilt symptoms

Treatment	Duration (days)
T ₀₁	6
T ₀₂	6
T ₁₁	no symptoms
T ₁₂	10
T ₂₁	10
T ₂₂	10

Dissected plantlets from the control explants (T₀₁ & T₀₂) also showed necrosis at the base of the pseudostems (Figure 10). All T₁₁ plantlets had clean pseudostem bases. Although some T₁₂ plantlets had leaf spots, their pseudostems were clean. Plantlets from explants treated with 0.2% EMS for 12h (T₂₁) had clean pseudostem bases but leaf spots were observed in lesser degree than those found in the control plantlets. Among explants exposed to 0.2% EMS for 24h (T₂₂), pseudostem rot was observed in some plantlets with leaf spots. These observations were similar to the study of Bhagwat and Duncan (1988) and of Saraswathi et al (2014) on mutation induction in banana for the development of genes against *Fusarium oxysporum* and Black Sigatoka, respectively.

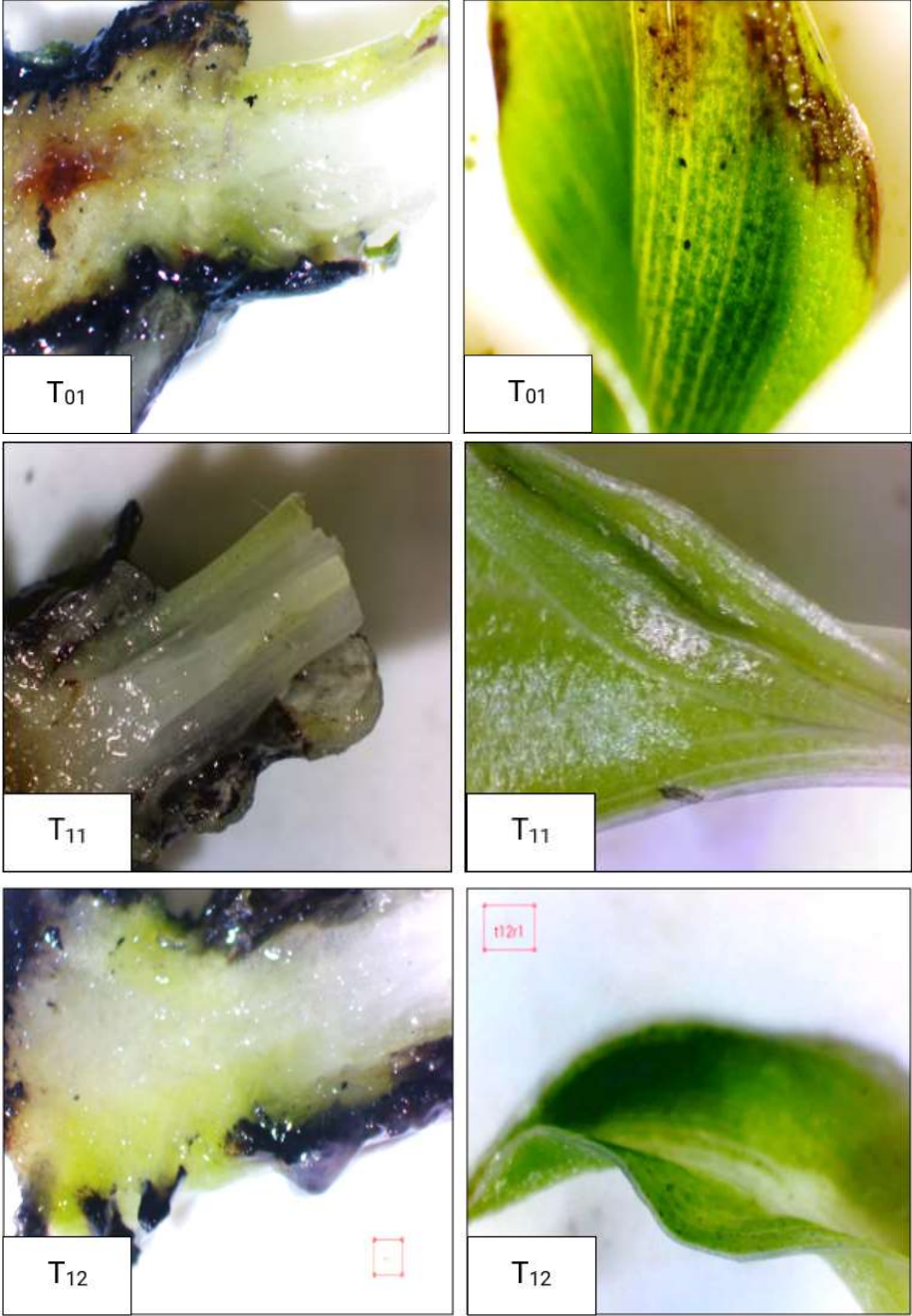


Figure 10. Dissected pseudostems and leaves of plantlets from untreated and treated explants

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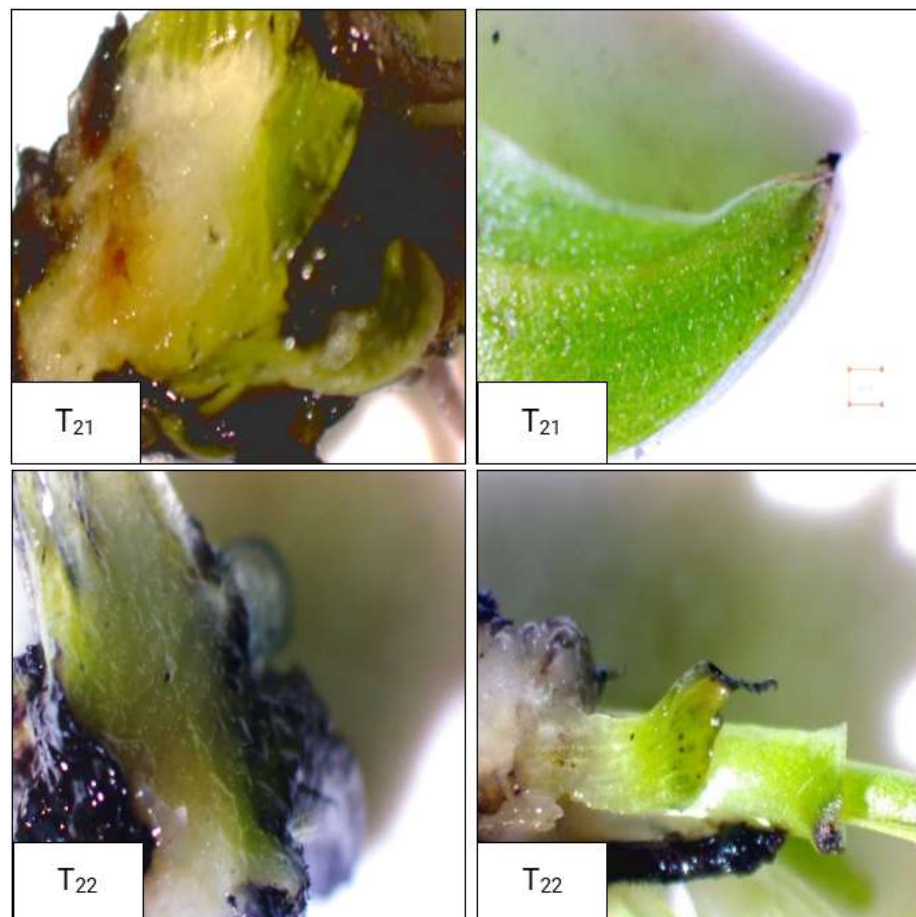


Figure 10 continued

Percentages of Bacterial Wilt Infected and Clean Plantlets in Untreated and Treated Cultures

All plantlets from untreated and 0.2% EMS treated explants exposed for 12h had bacterial wilt infection (Figure 11). Plantlets from explants treated with 0.1% EMS for 24h had 29% infection, while those regenerated from explants treated with 0.2% EMS for 24h had 44% infection. All plantlets from explants exposed to 0.1% EMS for 12h survived with no infection, suggesting that the plantlets might have improved ability to resist bacterial wilt infection. Uninfected surviving plantlets were also obtained from EMS-treated T₁₂ (71%) and T₂₂ (56%) explants.

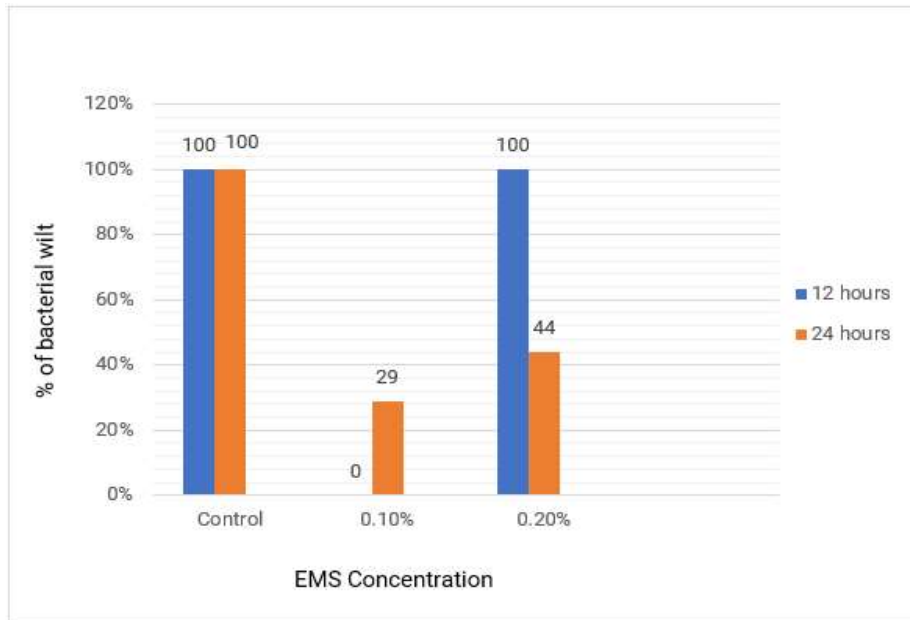


Figure 11. Percentage of bacterial wilt infected (susceptible) plantlets in untreated and EMS-treated cultures of Lakatan

EMS has the ability to enter the cells of living organisms and interact with the DNA, leading to changes in the genetic material, commonly with guanine (G) at the O position, forming O-6 ethylguanine, which can pair with thymine (T) instead of cytosine (C), resulting in base pair errors (Greene et al 2007). This genetic change is known as “missense mutation” since different amino acids may be assembled during translation following base pair errors. The resulting protein could perform a different function giving the organism a changed trait. This possibility should be verified in the surviving plantlets.

CONCLUSION AND RECOMMENDATION

This study showed that shoot tip explants from Stage 2 in vitro-grown Lakatan plantlets can tolerate exposure to 0.1% and 0.2% EMS concentrations for 12 to 24h. These EMS levels and durations of exposure did not inhibit growth of shoots from the treated explants. Treatment with 0.1% EMS for 12h apparently induced changes in the explants, which was manifested in this study as the absence of bacterial wilt symptoms up to ten days from the introduction of the pathogen during the early in vitro growth of 100% of the developing plantlets.

Mutation is a change in the genetic material. To confirm the presence of genetic changes in the surviving clean plantlets, ex vitro and molecular studies must be done.

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