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Original Research Article

Use of Ethyl Methane Sulfonate to Produce Salt Resistant Mutant Strains of Bacterial Species Isolated from Sugarcane Callus Culture

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ABSTRACT

Three species of bacteria (Lactobacillus sp, Pseudomonas sp. and Staphylococcus sp.) were isolated from Sugarcane callus culture and treated with EMS to produce mutant strains which were salt resistant. The results showed the presence of some colonics inside the inhibition zone formed due to the treatment with EMS, This indicates that the bacterial colonics undergo mutation. Results of the effect of NaCl on bacteria showed a variation among the seven isolates in their tolerance to the different concentration of NaCl .there is no grow at 20% NaCl concentration. The synergistic effect of both EMS and NaCl resulted in a decrease in growth of both genera Lactobacillus sp. and Staphylococcus sp. While Pseudomonas sp. didn't affect. We research for this paper to get salinity resistant gene from microbial after subjected to mutation , the growth culture treated with sodium chloride only and other samples of growth treated with both NaCl + EMS mutagen with certain proportions and dilutions to get the gene-carrying bacteria resistant to salinity and diagnosis these mutant microbial species by ARMS-PCR assay, used specific primers designed around the mutation locus of salinity tolerance genes according to the principle of tetra-primer ARMS-PCR to test genomic DNA of three species of Bacteria. The result obtained, Lactobacillus sp. and Staphylococcus sp. suffered from mutation when subjected to both NaCl +EMS and not separately, while Pseudomonas sp. did not suffer from any mutation of any addition.

Keyword: EMS; NaCl, ARMS-PCR assay; Lactobacillus sp; Pseudomonas sp; Staphylococcus sp.; salinity tolerance gene

INTRODUCTION

Salinity is one of the most brutal environmental factors limiting the productivity of crop plants because most of these crops are sensitive to salinity caused by high concentration of salts in the soil, Sugarcane is one of these crops that's effected by salinity, attempts to improve yield

under stress condition by plant improvement have been largely un successful , primarily due to the mutagenic origin of the adaptive responses, Therefore, well-focused approach combining the molecular, physiological, biochemical and metabolic aspects of salt tolerance is essential to develop salt-tolerant crop varieties. Genetic engineering is one of the most important technology and play role of salt tolerance in plant by transgenic technology and has been proved to be an efficient approach to the development of salinity-tolerant plants, and this approach will become more powerful as more candidate genes associated with salinity tolerance are identified and widely utilized,[1,2and3], There are three ways in which to induce mutation, by either using :

- Biological agents such as transposes and T-DNA.
- 2- Physical agents such as fast neutron, UV, and X-ray irradiation.
- 3- Chemical agents such as N-methyl-N-nitrosourea (MNU); 1,2,3,4 diepoxy butane (DEB) or ethyl methane sulfonate (EMS).

Among these compounds, EMS has become one of the most effective, reliable powerful and frequently used chemical mutagens in plants. EMS mainly induces C-to –T substitution resulting in C \G to T \A transition and at a low frequency , T \A transversion through 7-ethyl guanine hydrolysis or A\T to G\C transitions through 3-ethyl adenine pairing errors. High degree of mutation saturation can be achieved with a mutagen like EMS that does not cause a lot of collateral DNA damage.[4].

The main aim of this study is to product salinity tolerance bacteria on the complex molecular mechanisms to get resistance salinity gene and identification of its genetic sequence by a process of sequencing which will able the Bacteria to resist high salinity that will help us to get resist high salinity transgenic sugarcane in further researches.

MATERIALS AND METHODS

Amplification Refractory Mutation System – PCR (ARMS – PCR)

A standard ARMS-PCR consists of **two complementary reactions** (two tubes) and utilizes **3 primers.** One primer is constant and complementary to the template in both reactions, the other primers differ at their 3' terminal residues and are specific to either the wild type DNA sequence or the mutated sequence at a given base - only one of these primers is used per tube. That is mean in ARMS-PCR, 2 pairs of primers in a single PCR tube, can simultaneously amplify both mutant and normal alleles as well as allow amplification of an internal DNA control.

ARMS-PCR assay was performed for detection of point mutations directly by the presence or absence of amplification using allele-specific primers in mutant gene. For the diagnosis of specific point mutation a pair of allele-specific primers one of which has its 3’ terminal nucleotide complementary to the point mutation (Mt ARMS primer) and other to the normal DNA sequence (N ARMS primer) the method was carried out according to a method described by[5].

Bacteria Isolation

Forty callus culture tubes of sugarcane contaminated with bacteria from tissue culture laboratory in the marine science center were used in this study, the bacteria were streaked on Laurie – Bertani (LB) agar; Pseudomonas agar and Staphylococcus media 110 agar. Twenty-six isolates of bacteria were isolated from contaminated callus culture tube, both morphological and biochemical identification were done on the isolates,the identification showed that these 26 isolated belong to three genera*: Lactobacillus* sp*, Pseudomonas* sp *and Staphylococcus* sp. seven isolates were chosen to complete the study, two isolates belong to both genera *Pseudomonas* sp. *and*

Staphylococcus sp, and three isolates belong to genus *Lactobacillus sp.*

Effect of EMS on bacteria

The seven bacterial isolates were cultured in 250 ml conical flasks containing 100 ml of nutrient broth and incubated in shaker incubator (120 rpm) at 35 C° for 18 h then 0.1 ml of the overnight broth were taken by pipette and L- shaped on nutrient agar plates then incubated for 1 hour then four pores were done on the plate by using cork porer 5 mm diameter, these pores were filled with 50 µl from EMS with 5% concentrations the plates incubated over night at 35 C°.

The effect of salinity on bacteria

Five concentration of NaCl were prepared (2, 5, 10, 15, 20 % respectively) by adding a certain weight of NaCl to nutrient agar, then the NaCl nutrient agar plates were inoculated with the bacterial isolates and incubated for 18 h at 35 C°.

The synergy tic effect of both EMS and NaCl on bacteria

The seven bacterial isolates were grown in growth media containing either EMS, NaCl, or both of them by inoculating the bacterial isolates on:

- nutrient broth containing NaCl only
- nutrient broth containing EMS only
- 3- nutrient broth containing both EMS, NaCl
- The flasks were incubated at 35 c° for 18h

Sample Processing:

The genomic DNA(s) for all samples of all species extracted by using Promega wizard genomic DNA isolation kit in the biotechnology lab. – marine science center. Isolated DNA(s) further investigated for quality check by agarose gel electrophoresis as shown in figure (1). Then quantified by using Nano drop (260/280 ratio). The contamination of samples with RNA and protein was detected and samples were processed further for ARMS-PCR assay. For each species which consist of three samples , prepared three ARMS -PCR reactions (three tubes , wild type or normal , mutant of salt only and mutant of both salt&EMS) , one for identifying the presence of the normal allele (using the normal primer) , one for identifying the presence of mutant allele that cause by adding salt as mutagen only and the third for identifying the presence of mutant allele that cause by adding both salt & EMS mutagen (using same mutant primer) . (Note), Same mutant allele primer used for all species to get the gene-carrying bacteria resistant to salinity.The three separate ARMS -PCR reactions, were carried out in a single PCR run for each species first group: for *Pseudomonas sp* that subjected to the salt only then treated with both salt & EMS mutagen. Second group: *Lactobacillus sp.* and the third group: *Staphylococcus*. were also treated with same mutagens as shown in Table (1 - a).

Table (1- a): Three ARMS- PCR reactions for each species which subjected first, to both NaCl & EMS and second to NaCl mutagen only

Species	Mutagen	Three ARMS –PCRreactions (three tubes)
Pseudomonas sp.	NaCl	1. wild type allele primer (N) 2. mutant allele primers (M)(NaCl only)
	NaCl & EMS	3. mutant allele primers (M)(NaCl & EMS)
Lacto bacillus sp.	NaCl	1. wild type allele primer (N)
	NaCl & EMS	2. mutant allele primers (M))(NaCl only) 3. mutant allele primers (M)(NaCl& EMS)
Staphylococcus sp.	NaCl	1. wild type allele primer (N)
	NaCl & EMS	2. mutant allele primers (M))(NaCl only) 3. mutant allele primers (M)(NaCl& EMS)

For ARMS-PCR reactions two specific primers designed, one with mutant allele primers and one with wild-type allele primers and the third one for Internal Control Primer . as shown in Table (1- b).

ARMS-PCR master mix was prepared by using Solis Bio-Dyne kit in 96-well plates. The reaction master mix consisted of a 12.5µl master mix,1µl from each of primers (forward and reverse primers),1µl of the sample then complete the size of reaction to 25µl, add 9.5µl add water. Then, all the PCR tubes transferred into Existing vortex centrifuge at 3000rpm for 3 minutes. Then placed in PCR Thermo cycler. With specific conditions of each species and primer as shown in Table (2 - a).

Table (1- b): Oligonucleotide primers used in the study

Pseudomonas sp.	Wild type allele primers	Forward	GGGAGGAAGGGCAGTAAGTT
		Reverse	ACCACCCTCTACCGTACTCT
	Mutant allele primers	Forward	TGGATGCCATCAATCAACGC
		Reverse	GCGATAATATTCAGCGGCGT
Lactobacillus sp.	Wild type allele primers	Forward	GAGGCAGCAGTAGGGAATCT
		Reverse	GATAACGCTTGCCACCTACG
	Mutant allele primers	Forward	TGGATGCCATCAATCAACGC
		Reverse	GCGATAATATTCAGCGGCGT
Staphylococcus sp.	Wild type allele primers	Forward	AGGTGGGGATGACGTCAAAT
		Reverse	TCACCGTAGCATGCTGATCT
	Mutant allele primers	Forward	TGGATGCCATCAATCAACGC
		Reverse	GCGATAATATTCAGCGGCGT

ARMS-PCR Thermocycler Conditions:

Table (2–a): ARMS-PCR program (1): To detect the mutations for *Pseudomonas* sp. caused by NaCl, NaCl & EMS

PCR step	Temp.	Time	Repeat
Initial Denaturation	94 c	3 min	1
Denaturation	94 c	30 sec	35
Annealing	59 c	30 sec	
Extension	72 c	30 sec	
Final extension	72c	10 min	1
Hold	4 c	Forever	_

ARMS-PCR program (2): To detect the mutations for *Bacillus sp.* caused by NaCl, NaCl & EMS

PCR step	Temp.	Time	Repeat
Initial Denaturation	94 c	3 min	1
Denaturation	94 c	1min	35
Annealing	58 c	1min	
Extension	72 c	1min	
Final extension	72c	10 min	1
Hold	4 c	Forever	_

ARMS-PCR program (3): To detect the mutations for *Staphylococcus sp*. caused by NaCl, NaCl & EMS.

PCR step	Temp.	Time	Repeat
Initial denaturation	94 c	3 min	1
Denaturation	94 c	30 sec	35
Annealing	59 c	30 sec	
Extension	72 c	30 sec	
Final extension	72c	10 min	1
Hold	4 c	Forever	_

RESULTS AND DISCUSSION

Effect of EMS on bacteria

From the result, in (table 3) we can see that the exposure of the bacterial isolates results in presence of some colonies inside the inhibition zone around the pones in which the EMS was injected this indicates that these colonies undergo mutation.

The effect of salinity on bacteria

As shown in (table 3)there is a variation among the seven isolates in their tolerance to the different concentration of NaCl, but no one can grow in 20% NaCl concentration these findings are in agreement with [6]who stated that tolerance to salinity is to some extent due to the physiological mechanisms and morphological adaptation .

The effect of both EMS and NaCl on bacteria

The bacterial isolates that classified as belonged to the genera *Lactobacillus and Staphylococcus* respectively, didn't affect by neither NaCl alone nor EMS alone, the same speech is true for *Pseudomonas*, while the bacterial isolates exposure to the mixture of EMS and NaCl, *Lactobacillus and Staphylococcus* isolates were affected. There a decrease in growth of these isolates, while *Pseudomonas* didn't affect (table 3).

Table 3: Effect of Nacl and EMS on bacteria

Isolation No.	5% EMS	% NaCl con.					5% EMS+% NaCl con				
		2%	5%	10%	15%	20%	2%	5%	10%	15%	20%
Lactobacillus 14	G g	W g	G g	W g	Very week	Ng	W g	W g	W g	W g	W g
Lactobacillus 15	G g	W g	G g	Ng	Ng	Ng	W g	W g	W g	W g	W g
Lactobacillus 19	G g	W g	G g	Ng	Ng	Ng	W g	W g	W g	W g	W g
Staphylococcus 15	G g	W g	G g	W g	Very week	Ng	W g	W g	W g	W g	W g
Staphylococcus 17	G g	W g	G g	W g	Very week	Ng	W g	W g	W g	W g	W g
Pseudomonas 19	G g	W g	G g	Ng	Ng	Ng	G g	G g	G g	G g	G g
Pseudomonas 24	G g	W g	G g	Ng	Ng	Ng	G g	G g	G g	G g	G g

Ng –No growth, Gg- Good growth, Wg –week growth

DNA Isolation:

DNA extracted for all species were resolved on 1% agarose gels are shown in Figure (1).

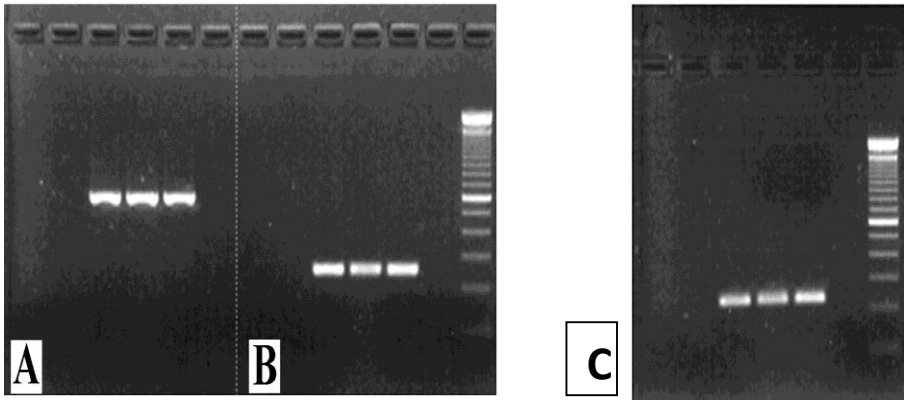

Fig. 1: Genomic DNA bands extracted from
A – *Pseudomonas* sp. of the three samples on 1 % agarose gel
B – *Staphylococcus* sp. of the three samples on 1 % agarose gel
C - *Lactobacillus* sp. of the three samples on 1 % agarose gel

ARMS-PCR technique was used for molecular screening three species of Bacteria which subjected to two types of chemicals mutagens (EMS & NaCl) and NaCl only to get salinity tolerance gene using a specific set of primers for mutation and a set of internal control primers. The internal control product of 876 bp molecular weight was showed in ARMS-PCR reactions for *Pseudomonas sp*, 845 bp for *Staphylococcus sp.* and 814bp for *Lactobacillus sp.* as shown in figure (2). For normal cases, the ARMS-PCR products were found within the normal primer reactions while in positively diagnosed mutation, ARMS-PCR products were found only within mutant primer reactions in homozygous cases or within both normal and mutant reactions in heterozygous cases.

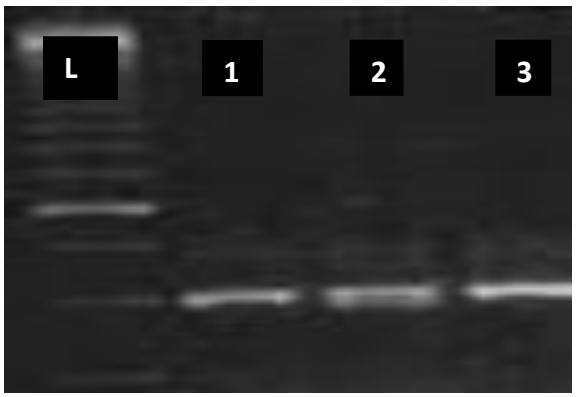

Fig. 2: Internal control product for the three species

Lane L: 1000-bp DNA ladder
Lane 1: *Lactobacillus sp.*(814 bp).
Lane 2: *Staphylococcus sp*.(845 bp)
Lane 3 : *Pseudomonas sp.* (876 bp).

As shown in figure (3).All species did not get the positive result for mutation as a result of subjected the species for NaCl only. All contains an amplified products for all species in the normal (N) but lack in the mutant (M) primers, hence, there is no mutation caused in any of species which subjected to salt only.

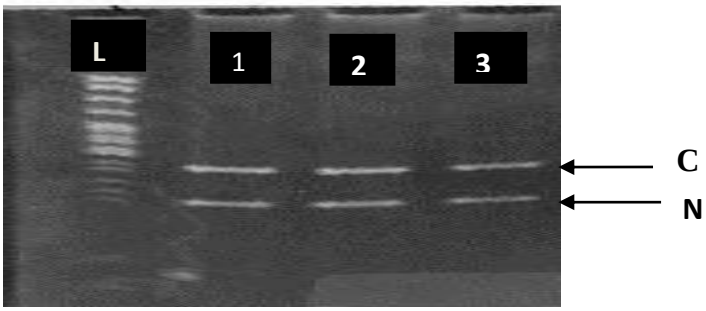

Fig. 3: ARMS-PCR products of the three species that adjusted to NaCl only

L: ladder DNA; N: Wild type allele or normal; C: Internal control product.

Lane: *Staphylococcus* sp., contains an amplified product in the normal (N) but lacks it in the mutant (M) primer, hence, there is no mutation caused by salt only.

Lane 2: Pseudomonas sp., also contains an amplified product in the normal (N) but lacks it in the mutant (M) primer

Lane 3: *Lactobacillus* sp., contains an amplified product in the normal (N) but lacks it in the mutant (M) primer. That is mean all species did not suffer from mutation result in subjected to NaCl.

But, as shown in figure (4), there was different result, ARMS-PCR products of all species that adjusted to both EMS & NaCl as following :L: ladder DNA ; N: (wild type allele) or normal;M : mutant allele; C: Internal Control product, these results may be occurred by adding EMS to bacteria species caused permanent change of the nucleotide sequence of the genome . Bertram et al [7] refer that Mutations result from damage to DNA which is not repaired or to RNA genomes. Mutations may or may not produce discernible changes in the observable characteristics (phenotype) of an organism, This mutation in the structure of genes is known Point mutations, often caused by chemicals or malfunction of DNA replication, exchange a single nucleotide for another. A point mutation can be reversed by another point mutation, in which the nucleotide is changed back to its original state (true reversion) or by second-site reversion (a complementary mutation elsewhere that results in regained gene functionality) [8].

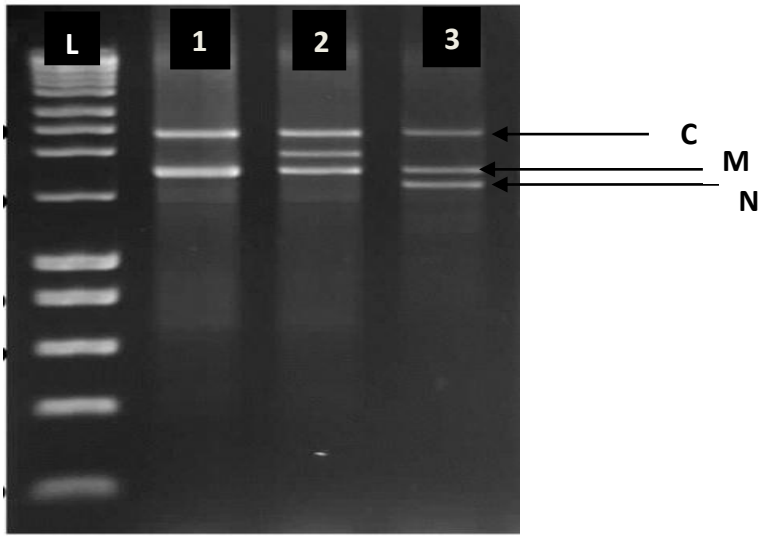

Fig. 4: ARMS-PCR products of the three species that adjusted to both EMS & NaCl

Lane 1*: Pseudomonas* sp*.,* contains an amplified product in the normal (N) but lacks it in the mutant (M) primer, hence implying a normal species .
Lane 2: *Staphylococcus* sp., contains an amplified product in both normal (N) and mutant (M) primers, assigning the individual is heterozygous genotype.
Lane 3: *Lactobacillus* sp., also contains an amplified product in both normal (N) and mutant (M) Primers, assigning the individual are heterozygous genotype.

CONCLUSIONS

The present study reveals genetic changes that detect in bacteria after additions of mutants so *Pseudomonas sp*., did not suffer from any mutation neither salt nor salt and EMS mutant. The DNA sequence of *Pseudomonas sp*. didn't suffer from any change in arrangement of its nucleosides or altered of them, Whereas *Lactobacillus sp*. and *Staphylococcus sp*. suffered from mutation that causes by both salt and EMS mutants together but salt only did not cause mutation, as a result of change happened in DNA sequence of *Bacillus sp*. and *Staphylococcus sp.*, from this two species of Bacteria we can get the salinity resistance gene.

CONFLICT OF INTEREST STATEMENT

Authors declare that they have no conflict of interest

REFERENCES

1. Bhaskar G, Bingru H. Mechanism of Salinity Tolerance in Plants: Physiological, Biochemical, and Molecular Characterization. Int J Gen 2014; 1-18.
2. Bohnert HJ, Jensen RG. Metabolic engineering for increased salt tolerance the next step. Australian J Plant Physiol 1996; 23: 661-667.
3. Flowers TJ, Yeo AR. Breeding for salinity resistance in crop plants. Australian J Plant Physiol 1995; 22: 875-884.
4. Serrat X, Esteban R, Guibourt N. EMS mutagenesis in mature seed-derived rice calli as a new method for rapidly obtaining TILLING mutant population. Plant Methods 2014; 10 (5): 1-13.
5. Kwok S, Kellogg DE, McKinney N, Spasic D, Goda L, Levenson C, Sninsky J J. Effects of primer-template mismatches on the polymerase chain reaction: human

immunodeficiency virus type I model studies. Nucl Acids Res 1990; 18: 999-1005.
6. Agwu O, Oluwagunke T. Halo tolerance of heterotrophic bacteria isolated from tropical coastal waters.Int J Sci Basic Applied Res 2014; 16 (2): 224-231.
7. Bertram J. The molecular biology of cancer. Mol Aspects Med 2000; 21(6): 167-223.
8. Freese and Ernst. The difference between spontaneous and base analogue induced mutation of phageT4D. Proc Natl Acad Sci 1959; 45(4):622-633.

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