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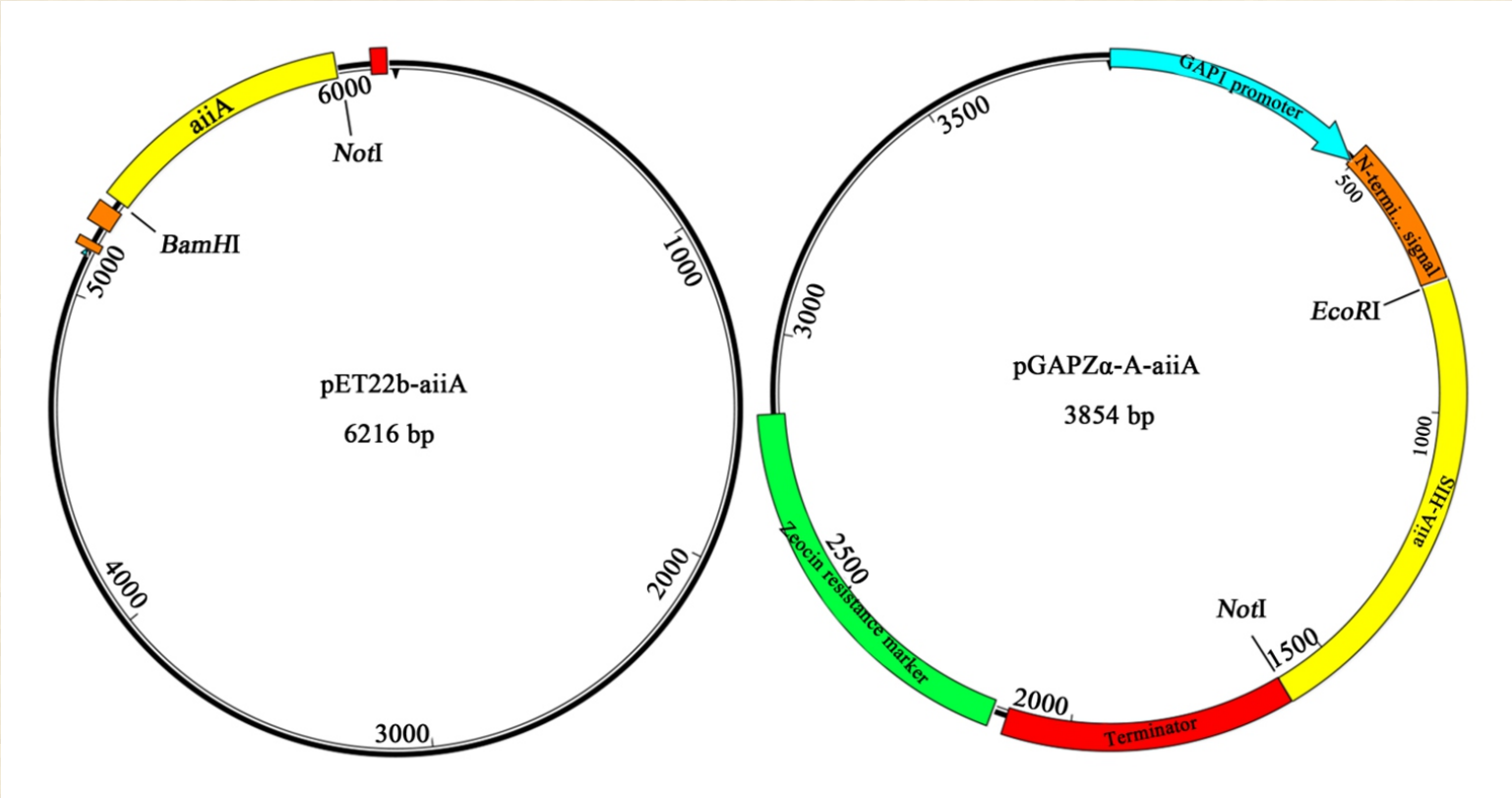

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Isolation and Characterization of Hydrocarbon Utilizing Yeast (HUY) Isolates from Palm Wine

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Abstract

Several studies have reported on the capabilities of different yeasts to utilize hydrocarbons hence the need to ascertain the petroleum hydrocarbon biodegradation potential of yeast isolated from palm wine. The study aimed at isolation, characterization and testing of yeasts from palm wine for biodegradation of petroleum hydrocarbons. Hydrocarbon utilizing yeasts (HUY) were isolated from palm wine, a rich sugar substrate from a non-oil impacted environment using Bacto Bushnell Haas broth. The medium composed of 0.2 g $MgSO_4$, 0.02 g $CaCl_2$, 1 g KH_2PO_4, 1 g K_2HPO_4, 0.05 g $FeCl_2$, 1 g/L NH_4NO_3, pH (7.2 ± 0.2) and mineral salt broth comprising of 4.4 g KH_2PO_4, 8 g $(NH_4)_2SO_4$, 2 g $Na_2HPO_4{\cdot}H_2O$, $MgSO_4{\cdot}7H_2O$, 0.1 g $FeSO_4$, 0.01 g/L $CaCl_2$, pH (4.5 ± 0.2) supplemented with palm wine (0.5%) and crude oil (1%). Yeasts isolated from an enriched medium and palm wine showed similarities. Yeast isolates were further screened for hydrocarbon degrading potentials using the Bacto Bushnell Haas broth containing 2, 6-dichlorophenol indophenols as the indicator agent. The isolates were identified using microscopic, macroscopic, fermentation and molecular characteristics as *Candida adriatica* (ZIM 2468) and *Candida taoyuanica* (MYA-4700).

Keywords

Hydrocarbon Utilizing Yeasts (HUY), Hydrocarbons, Palm Wine, *Candida* Species

1. Introduction

Diverse microbial population bacteria, yeasts or molds have been reported for degradation of hydrocarbons. The reported efficiency of biodegradation ranged from 6% to 82% for fungi, 0.13% to 50% for soil bacteria, and

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0.003% to 100% for marine bacteria [1].

Yeasts are eukaryotic microorganisms, classified in the kingdom fungi, with about 1500 species currently identified and described. They are estimated to be 1% of all fungal species [2] and measures up to 3 - 4 µm in diameter some can reach over 40 µm depending on the species [3].

Most young yeast colonies are moist, somewhat slimy in appearance and may also appear mealy. The colour of most colonies are whitish, cream-coloured or pink and change little with age, but others become dry and wrinkled. Yeasts are oxidative, fermentative or both. Oxidative yeast (film yeasts) may grow as a film or scum on the surface of a liquid medium where as fermentative yeasts grow throughout the liquid [4]. They are chemoorganotrophs, hence they use organic compounds as source of energy [5], they could be aerobes, facultative anaerobes but never strict or obligate anaerobes [6] [7].

Yeasts are widely dispersed in the environment. They grow best in neutral or slightly acidic pH environment. Some are normal flora on skin surfaces; others are parasitic or symbiotic and are mostly common in environments where there is a sugar-rich material. Some are associated with soil and insects [7] [8].

[9] reported that palm wine is a suspension of different types of microorganisms including bacteria, filamentous fungi, and yeasts. Yeasts occur in palm wine as indigenous micro flora and are mainly from the genus *Saccharomyces*, *Schizosaccharomyces*, *Pichia*, *Candida*, *Kleockera*, *Hansenula*, *Endomycopsis* and Saccharomycoides [10]-[12].

Several studies have reported on the capabilities of different yeasts to utilize hydrocarbons. The yeasts implicated in previous studies belong to the genera: *Candida*, *Clavispora*, *Pichia*, *Sporobolomyces*, *Sporidiobolus*, *Stephanoascus*, *Debaryomyces*, *Lodderromyces*, *Leucosporidium*, *Metschnikowia*, *Rhodotorula*, *Rhodosporidium*, *Trichosporon* and *Yarrowia* [7] [13] [14]. Irrespective of the reports from these studies, there is a dearth of information on the capability of yeast obtained from non oil polluted environment to degrade hydrocarbons which underscores the relevance of this study.

The study aimed at isolation and identification of yeast species in palm sap (palm wine), to ascertain their ability to degrade petroleum hydrocarbons. The objectives of this study were to:

1) Isolate and characterize yeasts from palm wine using morphological and molecular method.

2) Test the isolates for biodegradation of petroleum hydrocarbons.

2. Materials and Methods

2.1. Sample Collection

The crude oil used in this research was the Nigerian Bonny light crude oil obtained from Shell Petroleum Development Company (SPDC) Limited, Port Harcourt, Nigeria.

Yeasts used in this study were isolated from fresh palm wine gotten from raffia palm (*Raphiaraphia*) by palm wine tappers in Choba, Obia/Akpo Local Government Area, Rivers State. The samples were transported immediately to the laboratory in sterile containers packed in coolers with ice packs for analysis.

2.2. Experimental Design

2.2.1. Isolation of Test Organisms

One milliliter of palm wine was inoculated into 200 ml sterilized mineral salts broth [15] as described by [16]. Crude oil samples were filtered and autoclaved at 121˚C for 15 mins for sterility and when cooled added to the inoculums and swirled for proper mixing at a concentration of 1% (v/v). Incubation was done for fourteen days at room temperature (25˚C ± 2˚C) without shaking.

The spread plate technique was used to inoculate the viable culturable isolates. A glass spreading rod that has been sterilized in alcohol and flamed was used for this procedure. A 0.1 ml of the above enrichment medium was inoculated onto mineral salt agar plates in triplicates.

According to [17], sterile filter papers (Whatman No. 1) saturated with crude oil was placed on the inside of the cover plate of each Petri dish. The inoculated Petri dishes were kept in an inverted position and incubated at 25˚C ± 2˚C for five days. These filter papers supplied the hydrocarbons by the vapour phase transfer to the subcultured isolates.

The unadapted microorganisms were obtained by inoculating 0.1 ml of the fresh palm wine onto sterile mineral salt agar plates in triplicates using the spread plate method. Agar plates contained crude oil-soaked filter paper and incubated at 25˚C ± 2˚C for fourteen days in inverted position.

Viable isolates from the above media were purified using Sabouraud dextrose agar (balanced peptone water No.1 10 g, Dextrose 40 g, agar No.2 12 g, pH 5.6 ± 0.2). The adapted and unadapted pure isolates obtained were aseptically inoculated into the agar slants in the McCartney bottles. These were incubated at 25˚C ± 2˚C for 48 hours. These stock cultures were preserved in the refrigerator at 4˚C for future use. Yeast isolates obtained were prefixed with the letters Pw (Palm wine) e.g. Pw 01, Pw 02 for the first and second isolates respectively. Identification of yeast isolates were based on macroscopic and microscopic examination of the morphological and biochemical properties [18]. Wet mounts of a 48 hour culture were used for microscopy.

Sugar fermentation test was carried out to determine the ability of the isolates to metabolise some carbohydrates such as glucose, lactose, galactose, trehalose, inositol, maltose, dulcitol, sucrose and raffinose with the production of either acid, gas or both.

2.2.2. Screening for Biodegradation Potentials

The modified method of [19], was used for the screening test. A loopful of a 48 hour culture of the isolates were inoculated into a sterile 100 ml of Bacto Bushnell Haas broth containing 1 ml of sterile crude oil (1% v/v) and 1 ml of redox indicator (2% v/v of 2,6-dichlorophenol indophenols). A control was also set up containing no microorganisms. The set up was left for 15 days. Total hydrocarbon content (Oil and Grease) was determined according to API-RP45 method using a Spectrophotometer. The sample was extracted twice with 1:10 ratio of Xylene to sample. The combined extract after centrifuging was read in the spectrophotometer using Xylene as the reference material. The spectrophotometer had been previously calibrated with crude oil. Readings obtained from the spectrophotometer were traced out on the calibration graph and used to calculate the concentration of THC (Oil and Grease) in mg/l [20]. Total Hydrocarbon Content was absorbed at 420 nm using the spectrophotometer Corning 253.

Calculation:

$$\text{THC} = \text{absorbance} \times \text{gradient} \times \text{extraction volume} \times \text{volume of diluent.}$$

2.2.3. Molecular Identification of Test Isolates

Pure cultures of the potential strains maintained on Sabouraud dextrose agar slants in McCartney bottles were identified at the Biotechnology Centre, Federal University of Agriculture, Abeokuta, Ogun state (FUNAAB) and Microgen U.S.A. employing deoxyribonucleic acid (DNA) extraction, deoxyribonucleic acid (DNA) sequencing and sequence blasting on National Centre for Biotechnology Information (NCBI). The DNA extraction was carried out using the NORGEN BIOTEK CORP, Fungi/Yeast Genomic DNA Isolation Kit.

One milliliter of the washed microorganisms was transferred to a microcentrifuge tube and centrifuged using spectrafuge 24D, Labnet international, Inc. at 14,000 rpm gentle vortexing using the Stuart vortex mixer, SA8. Agarose gel electrophoresis was used to determine the quality and integrity of the DNA by size fractionation on 1.0% agarose gels. Agarose gels (biotechnology grade) were prepared by dissolving and boiling 1.0 g agarose in 100 ml 0.5× tris acetate ethylene diamine tetraacetic acid (TAE) buffer solution. The gels were allowed to cool down to about 45˚C and 10 µL of 5 mg/ml ethidium bromide was added, mixed together before pouring it into an electrophoresis chamber set with the combs inserted. After the gel has solidified, 3 µL of the DNA with 5 µL sterile distilled water and 2 µL of 6× loading dye was mixed together and loaded in the well created. Electrophoresis was done using the Consort EV231 electrophoresis machine at 80 V for 2 hours. The integrity of the DNA was visualized and photographed on UV light source by the UV documentation.

Polymerase chain reaction (PCR) was carried out using the MJ research thermal cycler (PTC-200). The amplicon was further purified prior to sequencing using 2 M sodium acetate wash technique.

The primer used for the reaction is 18S forward and reverse. The samples were loaded on the machine and the data in form A, C, T, and G were released.

The sequencing was carried out using the applied biosystem; ABI 3130X1 model. The isolates' genes were sequenced using the ITS 1 and ITS 2 primer [21]. The primer sequences are as shown below:

Primer Name Sequence (5′-3′).

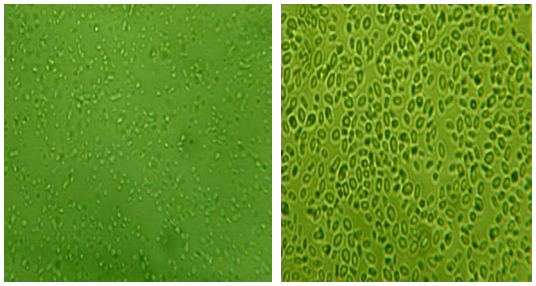
ITS 1 TCCGTAGGTGAACCTGCGG

ITS 2 GCTGCGTTCTTCATCGATGC

Sequence results obtained from above were compared with known sequences in the Genbank using the Basic Local Alignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI). Species were identified based on the percentage (%) similarity with the known species (sequences in the database).

3. Results and Discussion

In this study, thirteen yeast isolates were isolated from the enriched medium and fresh palm wine. Results from the enrichment medium (adapted microorganisms) and the palm wine (unadapted microorganisms) showed that microorganisms were of the same type based on colonial and cellular morphologies (**Table 1**). According to the colonial morphology on solid media, it was found that both isolates (Pw 1 and Pw 3) have cream colour but Pw 3 appears dull with age. They (adapted and unadapted microorganisms) also exhibited same reactions to biochemical test (**Table 2**). The yeast isolates were Gram positive, metabolized glucose with the production of acid and gas. Yeast isolates were identified tentatively as *Saccharomyces* species (Pw 1) and *Schizosaccharomyces* species (Pw 3) (**Plate 1**). The yeasts morphology under Celestron digital microscope was studied and found that *Schizosaccharomyces* species cells were small and *Saccharomyces* species cells were larger and spherical in shape.



(a) (b)

Plate 1. Celestron digital microscope micrographs of yeast cells at 25˚C ± 2˚C. (a) *Schizosaccharomyces* species; (b) *Saccharomyces* species.

Table 1. Cultural and morphological characteristics of yeast isolates.

Isolate	Cell shape	Form	Colour	Margin	Elevation	Density	Colony size	Probable isolate
Pw 1	Ellipsoidal budding cells	Circular large colonies	Cream colonies	Entire	Flat	Opaque	0.05 mm	*Saccharomyces* species
Pw 2	Ellipsoidal budding cells	Circular large colonies	Cream colonies	Entire	Flat	Opaque	0.05 mm	*Saccharomyces* species
Pw 3	Ovoid cylindrical fission cells	Circular small colonies	Cream white colonies, dull with age	Entire	Raised	Opaque	0.02 mm	*Schizosaccharomyces* species
Pw 4	Ellipsoidal budding cells	Circular large colonies	Cream colonies	Entire	Flat	Opaque	0.05 mm	*Saccharomyces* species
Pw 5	Ellipsoidal budding cells	Circular large colonies	Cream colonies	Entire	Flat	Opaque	0.05 mm	*Saccharomyces* species
Pw 6	Ovoid cylindrical fission cells	Circular small colonies	Cream white colonies, dull with age	Entire	Raised	Opaque	0.02 mm	*Schizosaccharomyces* species
Pw 7	Ellipsoidal budding cells	Circular large colonies	Cream colonies	Entire	Flat	Opaque	0.05 mm	*Saccharomyces* species
Pw 8	Ovoid cylindrical fission cells	Circular small colonies	Cream white colonies, dull with age	Entire	Raised	Opaque	0.02 mm	*Schizosaccharomyces* species
Pw 9	Ellipsoidal budding cells	Circular large colonies	Cream colonies	Entire	Flat	Opaque	0.05 mm	*Saccharomyces* species
Pw 10	Ellipsoidal budding cells	Circular large colonies	Cream colonies	Entire	Flat	Opaque	0.05 mm	*Saccharomyces* species
Pw 11	Ellipsoidal budding cells	Circular large colonies	Cream colonies	Entire	Flat	Opaque	0.05 mm	*Saccharomyces* species
Pw 12	Ovoid cylindrical fission cells	Circular small colonies	Cream white colonies, dull with age	Entire	Raised	Opaque	0.02 mm	*Schizosaccharomyces* species
Pw 13	Ellipsoidal budding cells	Circular large colonies	Cream colonies	Entire	Flat	Opaque	0.05 mm	*Saccharomyces* species

Table 2. Sugar fermentation test.

Isolate	Gram's reaction	Glucose	Galactose	Sucrose	Raffinose	Trehalose	Inositol	Dulcitol	Lactose	Maltose
Pw 1	+	A/G	A/G	A/	−	−	−	−	−	−
Pw 2	+	A/G	A/G	A/	−	−	−	−	−	−
Pw 3	+	A/G	−	−	−	−	−	−	−	−
Pw 4	+	A/G	A/G	A/	−	−	−	−	−	−
Pw 5	+	A/G	A/	A/	−	−	−	−	−	−
Pw 6	+	A/G	−	−	−	−	−	−	−	−
Pw 7	+	A/G	A/G	A/	−	−	−	−	−	−
Pw 8	+	A/G	−	−	−	−	−	−	−	−
Pw 9	+	A/G	A/	A/G	−	−	−	−	−	−
Pw 10	+	A/G	A/G	A/	−	−	−	−	−	−
Pw 11	+	A/G	A/G	A/G	−	−	−	−	−	−
Pw 12	+	A/G	−	−	−	−	−	−	−	−
Pw 13	+	A/G	A/G	A/	−	−	−	−	−	−

KEY: A/G: Acid and gas production; A/−: Acid and no gas production; +: Positive; −: Negative.

3.1. Screening for Biodegradation Potentials

All yeast isolates were subjected to screening for biodegradation potentials using the 2, 6-dichlorophenol indophenols as indicator agent. There was a gradual colour change in the setup from deep blue (the colour of the indicator agent) to purple to maroon and finally colourless. This colour change suggests that yeast isolates have biodegradative potentials. During the screening for biodegradation potentials, the total hydrocarbon content (THC) was reduced suggesting that yeast isolates were potential hydrocarbon degraders. Previous studies have shown that the bio-augmentation technology could significantly improve the efficiency of bioremediation (Pozdnyakova *et al*., 2008; Jacques *et al*., 2008). Irrespective of the findings, other researchers have reported on the ineffectiveness of exogenous microorganisms to repair the oil contaminated soil (Marianoand Kataoka, 2007; Ueno *et al.*, 2006; Liu and Wang, 2008). In this present study, the addition of exogenous yeasts isolated from palm wine biodegraded petroleum hydrocarbon thereby corroborating earlier reports in this regard. This research was based on the principle of allochthonous bio-augmentation since the yeasts were isolated from a different source other than petroleum hydrocarbon contaminated sites.

The capability of several yeast species to use n-alkanes and other aliphatic hydrocarbons as a sole source of carbon and energy is reportedly mediated by the existence of multiple microsomal Cytochrome P450 forms. These cytochrome P450 enzymes had been isolated from yeast species such as *Candida maltosa*, *Candida tropicalis*, and *Candida apicola* (Scheuer *et al.*, 1998). Sood and Lal (2009) reported that the ability of the strain to grow in MSM with hydrocarbons as the sole carbon source is indicative of its ability to utilize the hydrocarbons.

3.2. Molecular Identification of Yeast Isolates

Yeast isolates' DNA were isolated with the DNA extraction kit, amplified using the polymerase chain reaction and purified. Using the ITS 1 and ITS 2 primers, the genes were sequenced and BLAST carried out to compare these with those in GenBank as described by Mrinalini and Jayanthi (2011). The yeast isolates that were identified tentatively as *Schizosaccharomyces* species and *Saccharomyces* species when molecularly identified based on the 18S-rRNA were *Candida adriatica* ZIM 2468 and *Candida taoyuanica* MYA-4700 as shown in **Table 3**.

The study showed that isolates from the enrichment medium (adapted microorganisms) and the palm wine (unadapted microorganisms) were of the same type based on colonial and cellular morphologies (**Table 1**). Yeast isolates were identified tentatively and with the Celestron digital microscope as *Saccharomyces* species and *Schizosaccharomyces* species.

Table 3. Molecular identification by 18S-rRNA.

Isolate	Tentative identification	Ascension number	Species	Identification (%)
Pw 1	*Saccharomyces* species	HE799682.1	*Candida taoyuanica* MYA 4700	100
Pw 2	*Saccharomyces* species	HE799682.1	*Candida taoyuanica* MYA 4700	100
Pw 3	*Schizosaccharomyces* species	JQ812705.1	*Candida adriatica* ZIM 2468	98
Pw 4	*Saccharomyces* species	HE799682.1	*Candida taoyuanica* MYA 4700	100
Pw 5	*Saccharomyces* species	HE799682.1	*Candida taoyuanica* MYA 4700	100
Pw 6	*Schizosaccharomyces* species	JQ812705.1	*Candida adriatica* ZIM 2468	98
Pw 7	*Saccharomyces* species	HE799682.1	*Candida taoyuanica* MYA 4700	100
Pw 8	*Schizosaccharomyces* species	JQ812705.1	*Candida adriatica* ZIM 2468	98
Pw 9	*Saccharomyces* species	HE799682.1	*Candida taoyuanica* MYA 4700	100
Pw 10	*Saccharomyces* species	HE799682.1	*Candida taoyuanica* MYA 4700	100
Pw 11	*Saccharomyces* species	HE799682.1	*Candida taoyuanica* MYA 4700	100
Pw 12	*Schizosaccharomyces* species	JQ812705.1	*Candida adriatica* ZIM 2468	98
Pw 13	*Saccharomyces* species	HE799682.1	*Candida taoyuanica* MYA 4700	100

Molecular identification was carried out by sequencing the isolates' genes using the 18S-rRNA for fungi [21]. It will interest us to know that when the sequences were marched with the GenBank database using the Basic Local Allignment Search Tool (BLAST) of the National Centre for Biotechnology Information (NCBI), *Schizosaccharomyces* species showed 98% similarity with *Candida adriatica* ZIM 2468 and *Saccharomyces* species 100% with *Candida taoyuanica* MYA-4700. In the works of Tuntiwongwanich and Leenanon (2009), *Candida* species were also isolated from palm wine obtained from different palms.

Ability of the isolates to produce colour change in the medium during the screening for biodegradative potential is due to the reduction of the indicator agent; 2,6-dichlorophenol indophenols by the oxidized products of hydrocarbon degradation. The colour change supports the fact that the isolates are potential hydrocarbon utilizers. [19], in a related work also, observed a change in colour from deep blue to colourless. [22], said that another criterion to determine isolates' biodegradative potentials is the rupture of oily surface of the culture medium which we also, observed during the study. The reduction in the residual total hydrocarbon content (THC) during the screening suggests that isolates are hydrocarbon utilizers.

It is interesting to note that yeast isolates normally present in palm wine; a non-oil impacted environment, has the capability to utilize crude oil/petroleum hydrocarbons as sole source of carbon and energy. [17], in their work isolated yeasts capable of utilising kerosine and diesel as sole source of carbon from palm wine.

According to [11], microbial communities exposed to hydrocarbons adapt to this exposure through selective enrichment and genetic changes resulting in an increase in hydrocarbon-degradation. This pre-exposure of microorganisms make them better suited to degrade the pollutant through higher growth and reproduction and more efficient metabolism thus maximizing the rate of hydrocarbon removal from the soil.

4. Conclusions

Thirteen yeast isolates were collected from the enrichment medium and palm wine in this study. Two different strains were identified as *Candida adriatica* ZIM 2468 and *Candida taoyuanica* MYA 4700 based on the use of 18S rRNA for fungi. Under the Celestron digital microscope, *Candida adriatica* cells were small, oval creamy but turn gold with age while *Candida taoyuanica* cells were larger and ovoid. Both strains were hydrocarbon utilizers as observed in the biodegradation screening which were indicated by the change in colour from deep blue to colourless and rupturing of the oily surface of the culture medium.

The exogenous yeast isolated from palm wine that is not polluted with petroleum hydrocarbons was screened for biodegradation potentials; the total hydrocarbon content (THC) was reduced suggesting that yeast isolates were potential hydrocarbon degraders.

Authors' Contributions

This work was carried out in collaboration between all authors. Authors T. L. Ataikiru and P. O. Okerentugba designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Author P. O. Okerentugba is the supervisor under whose guide the entire execution of the work and corrections were done to this present standard. Author T. Ichor effected the corrections of the study, managed the literature searches, was responsible for sending the article and effected all the corrections that arose thereof from the publishers. The authors declare no competing interest.

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Association of XbaI *GLUT*1 Polymorphism with Susceptibility to Type 2 Diabetes Mellitus and Diabetic Nephropathy

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Abstract

Objectives: Diabetic nephropathy (DN) is one of the chronic microangiopathic complications of type 2 diabetes (T2DM) and has become the most frequent cause of end-stage renal disease. The XbaI polymorphism in the glucose transporter (GLUT1) has been suggested in the development of DN. We examined the association between XbaI polymorphism of GLUT1 and susceptibility to T2DM and development of DN. Methods: The study included 227 T2DM patients divided into 107 without DN (DM − DN) and 120 with DN (DM + DN), in addition to 100 apparently healthy controls. Genotyping was done by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). Results: The GLUT1 XbaI T allele was associated with increased susceptibility to T2DM, when comparing the healthy controls to the whole diabetic group, odds ratio (OR) = 1.899, 95% confidence interval (CI) (1.149 - 3.136), p = 0.011. This association was also significant between healthy controls and DM − DN OR = 1.997 (1.079 - 3.699), p = 0.026 as well as between healthy controls and DM + DN OR = 1.818 (1.016 - 3.253), p = 0.042. However there was no significant association of XbaI polymorphism with DN when comparing DM − DN to DM + DN OR = 0.910 (0.474 - 1.747), p = 0.777. Conclusion: XbaI T allele is associated with increased susceptibility to T2DM, but not to development of DN. Further studies are needed to replicate such findings.

Keywords

Type 2 Diabetes Mellitus, Diabetic Nephropathy, Glucose Transporter 1, XbaI, Polymorphism

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1. Introduction

Diabetic nephropathy (DN) is a major long term chronic microangiopathic complications of diabetes mellitus, leading to end-stage renal disease [1]. It is defined by occurrence of albuminuria and/or proteinuria in a diabetic patient with no evidence of nondiabetic renal conditions [2]. It is characterized also by elevated arterial blood pressure, decline in glomerular filtration rate (GFR), and high risk of cardiovascular morbidity and mortality [3].

Glucose transporters (GLUTs) encompass a family of facilitative transporters classified into three classes [4]. GLUT1 is a member of class 1 and it is highly expressed in the glomeruli, mesangial, endothelial cells and podocytes [5]. *GLUT*1 gene (*SLC*2*A*1) (rs841853) is located on chromosome 1p34.2, and it contains 10 exons and 9 introns [6] [7]. It has a central role in the pathogenesis of DN because over expression of GLUT-1 in glomerular mesangial cells is expected to augment the basal glucose uptake [8], and activate cellular pathways involved in cellular growth and in accumulation of the extracellular matrix [9]. The expression and activity of mesangial GLUT1 in diabetic patients comprise great individual variability attributed mostly to genetic causes. From this perspective it becomes clear why only a certain group of diabetics are predisposed to the development of DN, and could also clarify the reason for the poor correlation between glycemic control and progression of nephropathy in a subset of diabetics [10]. Thus investigating the genetic susceptibility to the development of DN may shed light on the pathogenesis of renal involvement in diabetes mellitus. Several single nucleotide polymorphisms (SNP) of *GLUT*1 gene have been examined in relation to DN, of which is XbaI G > T, located within intron 2, which represents transversion of guanine (G) to thymine (T) [9].

The conflicting data from worldwide studies done concerning XbaI G > T *GLUT*1 polymorphism in relation to DN [1] [9], and the fact that there were no studies in Egyptian population concerning this issue, made it noteworthy to study this polymorphism.

2. Subjects and Methods

2.1. Selection of Study Participants

We performed a case-control study of 227 diabetic patients attending the Teaching Hospital of Medical Research Institute, Alexandria University. All patients enrolled in this study were diagnosed as type 2 diabetes mellitus (DM2) according to the criteria based on the American Diabetes Association criteria (ADA) [11]. One hundred healthy subjects were also recruited from the same population. The study was explained to all participants and written informed consents were required. The experimental design was approved by the local ethical committee, and all participants gave their informed consent. All diabetics were receiving oral hypoglycemic agents.

Based on the guidelines of the ADA, albumin was measured in a spot urine sample collected as the first urine in the morning to identify nephropathy. Accordingly, diabetics were classified into non diabetic nephropathy patients (DM − DN) who were the normoalbuminuric with normal urinary albumin excretion (<30 mg/gm urine creatinine), while diabetic nephropathy patients (DM + DN) were those with persistently increased urinary albumin excretion (≥30 mg/gm urine creatinine) based on the consensus of at least two consecutive overnight samples collected over a 3 - 6 month period [11].

General exclusion criteria included: DM2 with less than 10 years duration, type 1 diabetes, secondary diabetes, smoking, pregnancy, heart failure, previously diagnosed nondiabetic kidney disease. During urine sample collection; acute fever, diabetic ketoacidosis, significant bacteriuria or hematuria, or patients who performed excessive exercise within 24 hours were excluded and repeated after condition resolution. Ultrasound examination was done to exclude other non-diabetic organic kidney disease.

2.2. Examination, Sampling and Biochemical Analysis

All subjects had a standardized physical examination and provided detailed history regarding diagnosis and complications of DM as well as the type of anti-diabetic treatment received. Anthropometric measurements (weight and height) and calculation of body mass index were done to all participants.

Following an overnight 12 hour fast, eight milliliters of whole venous blood were withdrawn from each participant; whole EDTA blood was used for genomic DNA extraction and serum was used for routine clinical chemistry (concentrations of urea, creatinine, uric acid, triglycerides, cholesterol and its high density fraction). In addition, urinary albumin and creatinine were measured from morning urine sample. Glycated hemoglobin

was determined using immunoturbidimetric assays. Analyses were conducted on the Olympus AU400 clinical chemistry analyzer (Beckman Coulter Inc., Brea CA, USA). Calculations of low density lipoprotein fraction using the Friedwald formula, estimated glomerular filtration rate (GFR) using the Cockroft and Gault formula, and urinary albumin to creatinine (UAC) ratio were done.

2.3. Genotyping of XbaI Polymorphism of the GLUT1 Gene

Genomic DNA was extracted using a commercially available kit (Qiagen). The concentration and purity of extracted DNA were determined by NanoDropTM 1000 spectrophotometer (Thermo Fisher Scientific) at 260 and 280 nm. The polymerase chain reactions were carried out in a Veriti thermal cycler (Applied Biosystems) according to the method described by Grabellus *et al.* (2010) [12]. The PCR reaction was carried out in a total volume of 25 µL, containing 10 µL of genomic DNA, 12.5 ml of DreamTaq™ Green PCR Master Mix, 20 pmol/l of each primer, 2.1 µL of nuclease free water. The primer sequences were; forward 5'-TGC AAC CCA TGA GCT AAC AA-3' and reverse 5'-GAA CCC AGC ACT CTG TAG CC-3' [12]. PCR program was initial denaturation 94˚C for 3 min followed by annealing and extension for 30 cycles of 45 s at 94˚C and 45 s at 56˚C and 72˚C for 45 s, with a final extension step of 7 min at 72˚C. The PCR products were digested with XbaI restriction enzyme (Roche Molecular Dagnostics, Germany) and incubated in 37˚C for 1 hour. In the mutant form, guanine (G) has been transverse to thymine (T) and abolished the recognition site. Hence, T allele which did not contain the XbaI restriction enzyme site remained undigested as 305 bp fragments, whereas G allele yields 232- and 73-bp fragments [12]. The resultant PCR products were visualized by electrophoresis on 3% agarose gel stained with ethidium bromide for visualization under UV light (**Figure 1**). In 5% of the samples genotyping was performed in duplicate and was fully concordant.

2.4. Statistical Analysis

Continuous results that satisfied a normal distribution were expressed as mean ± standard deviation. The Student's t-test, chi-square test, and Fisher exact test were used to assess the general characteristics between groups. The Hardy-Weinberg equilibrium was performed using chi square test by comparing the observed to the expected genotype frequencies. The association between variant alleles and their susceptibility to disease was assessed, and odds ratios (ORs) with 95% confidence intervals and chi-square tests were calculated. All statistical tests were two-tailed and p-values less than 0.05 were considered statistically significant. Statistical Program for Social Sciences (SPSS) version 20 was used for analysis of data.

3. Results

This case-control study included 100 healthy adults (56% males) with age 47 (34 - 66) years and 227 unrelated type 2 diabetics, stratified according to occurrence of diabetic nephropathy into; 107 diabetics without diabetic nephropathy (DM − DN) and 120 diabetics with diabetic nephropathy (DM + DN).

Although duration of diabetes was higher in (DM + DN) when compared to (DM − DN) but this did not reach the level of statistical significance ($p = 0.348$). HbA1c was significantly higher in (DM + DN) when compared to (DM − DN). As expected, the studied renal parameters were higher in (DM + DN) when compared to (DM − DN). The lipid profile showed no significant difference among groups, except for higher triglyceride level in (DM + DN) when compared to (DM − DN) (**Table 1**).

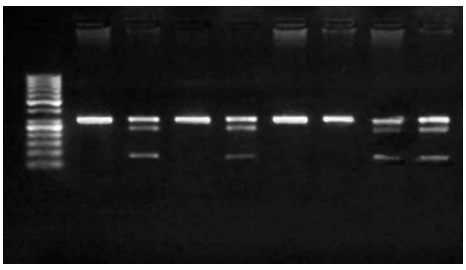

Figure 1. Agarose gel electrophoresis showing patterns of XbaI polymorphism of the GLUT1 gene. Lane 1: 50 bp ladder; lanes 2, 4, 6, 7 denote TT genotype; lanes 3, 5, 8, 9 denote GT genotype.

Table 1. Demographic and biochemical parameters of the studied sample.

Variable	Whole diabetics	DM − DN	DM + DN	*p* value
Number (%)	227 (100)	107 (47.1)	120 (52.9)	-
Gender (% men)	110 (48.2)	49 (45.8)	61 (50.8)	0.448
Age (years)	51.0 (32.0 - 66.0)	52.0 (38.0 - 65.0)	51.0 (32.0 - 66.0)	0.850
Duration of diabetes (years)	12.0 (10.0 - 22.0)	11.0 (10.0 - 20.0)	14.0 (11.0 - 22.0)	0.348
BMI (Kg/m^2)	31.2 (21.5 - 39.5)	32.0 (21.5 - 39.5)	31.0 (23.4 - 39.1)	0.951
HbA_{1C} (%)	8.65 ± 1.83	8.19 ± 1.53	9.07 ± 0.97	<0.001*
UAC (mg/gm)	46.0 (4.6 - 1749.0)	14.5 (4.6 - 29.0)	172.7 (43.3 - 1749.2)	<0.001*
Serum creatinine (mg/dl)	1.0 (0.6 - 5.7)	0.9 (0.7 - 1.3)	1.1 (0.6 - 5.7)	<0.001*
eGFR (ml/min)	85.7 (14.6 - 155.0)	91.5 (62.5 - 155.0)	72.0 (14.6 - 132.7)	<0.001*
Uric acid (mg/dl)	4.7 (3.2 - 10.2)	4.5 (3.2 - 6.7)	5.6 (3.7 - 10.2)	<0.001*
Total cholesterol (mg/dl)	219.0 (130.0 - 286.0)	219.0 (168.0 - 269.0)	221.5 (130.0 - 286.0)	0.661
Triglycerides (mg/dl)	168.0 (68.0 - 272.0)	158.0 (78.0 - 266.0)	171.0 (68.0 - 272.0)	0.029*
HDL-C (mg/dl)	43.0 (25.0 - 78.0)	45.0 (26.0 - 78.0)	43.0 (25.0 - 67.0)	0.275
LDL-C (mg/dl)	143.7 ± 29.2	143.3 ± 27.1	144.1 ± 31.1	0.830

DM − DN: diabetic patients without diabetic retinopathy, DM + DN: diabetic patients with diabetic retinopathy, BMI: body mass index, HbA1c: glycated hemoglobin, UAC: urinary albumin creatinine ratio, eGFR: estimated glomerular filtration rate, HDL-C, LDL-C: high and low density lipoprotein cholesterol.

All genotype groups obeyed the Hardy-Weinberg equilibrium; $p = 0.07$ in healthy control and $p = 0.145$ in whole diabetics group. We did not find any homozygous (GG) genotype in the studied population neither in healthy controls, nor in diabetics. TT genotype was significantly more frequent in whole diabetics (82.38%), DM − DN (83.2%) and DM + DN (81.7%) than in the healthy controls (69%). The difference in the genotype frequencies was also reflected in the allelic frequencies. XbaI polymorphism T allele was associated with increased susceptibility to diabetes, when comparing healthy controls to that of the whole diabetic group, odds ratio (OR) = 1.899, 95% confidence interval (CI) (1.149 - 3.136), $p = 0.011$. This significant difference was also present between healthy controls and DM – DN, OR = 1.997 (1.079 - 3.699), $p = 0.026$ as well as between healthy controls and DM + DN OR = 1.818 (1.016 - 3.253), $p = 0.042$. However, there was no association of XbaI polymorphism between DM − DN and DM + DN, OR = 0.910 (0.474 - 1.747), $p = 0.777$ (**Table 2**). We examined the studied parameters according to genotypes of XbaI polymorphism, but no significant difference was noted (**Table 3**).

4. Discussion

Diabetic nephropathy (DN) occurs in 20% - 40% of diabetics and is the single leading cause of end stage renal disease. It imposes a high social and economic burden. Persistent albuminuria in the range of 30 - 299 mg/ 24 h has been shown to be an early stage of DN in type 1 diabetes and a marker for development of nephropathy in type 2 diabetes. In addition, DN is a well-established marker of increased cardiovascular risk [11]. Apparently, hyperglycemia can no longer be the sole etiological factor in development of DN. This is supported by familial clustering of renal complications and ethnic variations. Therefore, genetic causes are considered a major contributor of occurrence and progression of DN [10]. The *GLUT*1 is a plausible candidate gene in diabetes research,

Table 2. Genotype and allele frequency of XbaI polymorphism of *GLUT*1 in the studied population.

Genotype/allele	Healthy (n = 100)	Whole diabetics (n = 227)	DM − DN (n = 107)	DM + DN (n = 120)	*p* value	OR, 95% CI
GG	0	0 (0)	0 (0)	0 (0)		
GT	31	40 (17.62)	18 (16.8)	22 (18.3)	0.007*	2.100 (1.219 - 3.610)
TT	69	187 (82.38)	89 (83.2)	98 (81.7)	0.017#	2.221 (1.148 - 4.300)
					0.029~	2.000 (1.069 - 3.747)
					0.766¶	0.901 (0.454 - 1.789)
Allele						
G	31 (15.5)	40 (8.8)	18 (8.4)	22 (9.2)		
T	169 (84.5)	414 (91.2)	196 (91.6)	218 (90.8)	0.011*	1.899 (1.149 - 3.136)
					0.026#	1.997 (1.079 - 3.699)
					0.042~	1.818 (1.016 - 3.253)
					0.777¶	0.910 (0.474 - 1.747)

DM − DN: diabetic patients without diabetic retinopathy, DM + DN: diabetic patients with diabetic retinopathy. OR: odds ratio, CI: confidence interval. *Comparison between healthy control and whole diabetics; #Comparison between healthy control and DM − DN; ~Comparison between healthy control and DM + DN; ¶Comparison between DM − DN and DM + DN.

Table 3. Demographic data and biochemical parameters according to genotypes of XbaI polymorphism in type 2 diabetics.

Item	TT	GT	*p* value
Number (%)	187 (82.4%)	40 (17.6%)	-
Gender (% men)	88 (47.1%)	22 (55.0%)	0.362
Age (years)	51 (38 - 66)	52 (32 - 66)	0.550
Duration of diabetes (years)	12.0 (10.0 - 22.0)	14.0 (10.0 - 22.0)	0.390
BMI	31.0 (21.50 - 39.10)	32.0 (22.0 - 39.50)	0.716
Fasting plasma glucose (mg/dl)	169 (78 - 346)	167 (79 - 327)	0.330
HbA_{1C} (%)	8.63 ± 1.81	8.77 ± 1.93	0.652
UAC ratio (mg/gm)	46.0 (4.6 - 1749.2)	49.2 (5.3 - 954.5)	0.622
Serum creatinine (mg/dl)	1.0 (0.6 - 5.7)	1.0 (0.6 - 5.7)	0.723
eGFR (ml/min)	85.7 (14.6 - 155.0)	89.5 (14.6 - 136.5)	0.716
Uric acid (mg/dl)	4.7 (3.2 - 10.2)	4.7 (4.1 - 8.9)	0.358
Total cholesterol (mg/dl)	219 (134 - 286)	224 (130 - 265)	0.466
Triglycerides (mg/dl)	168 (78 - 272)	188 (68 - 266)	0.350
HDL-C (mg/dl)	43 (25 - 67)	43 (26 - 78)	0.955
LDL-C (mg/dl)	143.7 ± 28.9	143.9 ± 31.2	0.977

BMI: body mass index, HbA1c: glycated hemoglobin, UAC: urinary albumin creatinine ratio, eGFR: estimated glomerular filtration rate, HDL-C, LDL-C: high and low density lipoprotein cholesterol.

because GLUT1 is the main facilitative glucose transporter. In this case control study, we found that the T allele of XbaI *GLUT*1 gene polymorphism was associated with increased risk of susceptibility to T2DM, but not to development of DN.

The genotypes reported in our study obeyed the Hardy-Weinberg equilibrium. The G and T allele frequencies in the healthy control group were 15.5% and 84.5% respectively. We did not find GG genotype in any of the

studied subgroups. This was quite different from other studies [9] [10] [13] [14]. Variation in XbaI polymorphism genotype frequencies in healthy population is noted among different ethnicities for example the frequency of T allele in British Caucosid [15] was more than twice that reported in Chinese (51% in former and 21% in latter) [10], taken into consideration that both studies did not deviate from Hardy-Weinberg equilibrium.

In this case-control study, the *GLUT*1 XbaI T allele was associated with increased susceptibility to T2DM, when comparing the healthy controls to the whole diabetic group, odds ratio (OR) = 1.899, 95% confidence interval (CI) (1.149 - 3.136), $p = 0.011$. This association was also significant between healthy controls and DM − DN OR = 1.997 (1.079 - 3.699), $p = 0.026$ as well as between healthy controls and DM + DN OR = 1.818 (1.016 - 3.253), $p = 0.042$. The XbaI polymorphism has been previously studied in association to risk of DM, but results were inconsistent. Significant association was reported in Japanese [16] [17] and Italians [18], but other studies could not confirm such association [19] [20]. The XbaI polymorphism has an intronic nature and it can hardly cause changes in the protein sequence. Thus, it can be assumed that the XbaI polymorphism is in linkage disequilibrium with another locus which does have significant functional implications at the protein level [9].

In the current study, *GLUT*1 XbaI polymorphism was not associated with DN. Over the past years, conflicting results were reported from different studies concerning the association of GLUT1 Xba polymorphism and DN in type 2 diabetic patients [9] [10] [13] [14] as well as in type 1 diabetic patients [15] [21] [22]. The non significant association of the XbaI polymorphism of *GLUT*1 gene with DN found in the present study was in agreement with studies done in T2DM in Caucasian Mediterranean population [23] and in Tunisians [14]. Also in type 1 DM no significant association of the XbaI polymorphism with DN was reported in Danish population [21]. On the other hand, a study by Liu *et al*. indicated that type 2 diabetic patients with XbaI (T) allele of the *GLUT*1 gene may be prone to DN in Chinese subjects [10]. A finding that was supported by later studies in different populations including; a study on Mediterranean Caucasian population which showed an association with T2DM and possibly a severe form of it that leads to the development of DN. It further showed a statistically significant association between the XbaI (T) carriage and the presence of arterial hypertension in type 2 DM patients. However when they considered hypertension as a confounding factor, the association between *GLUT*1 XbaI G > T polymorphism and DN was no longer significant [9]. Also, another study on European Americans found that those having homozygous XbaI T allele were associated with DN, and suggested that enhancer 2 (Enh2) SNP, and not XbaI, is the causative polymorphism associated with diabetic albuminuria [24]. There is great heterogeneity in genetic studies; ethnicity of the studied population, demographics including different age and gender distribution. And above all, non uniformity in selection criteria of cases, for example definition of DN can range from microalbuminuria in spot urine sample, proteinuria in 24 hr urine and/or impaired renal function, thus making comparability of results quite difficult [1].

It is worth mentioning that a meta-analysis including several studies upon different populations concluded that XbaI polymorphhism in *GLUT*1 gene may represent a genetic susceptibility to DN. However, it did not support the association between XbaI and the severity of DN [25]. Nevertheless, (Grzeszczak *et al*., 2001) study in Caucasians from Poland was not in agreement with all the aforementioned studies and it even suggested that the XbaI (T) allele protects against the development of DN, given that the frequency of the XbaI (GG) genotype increased with each stage of DN. They found that patients with microalbuminuria had a higher frequency of XbaI (GG) genotype than those with normoalbuminuria and in the group with proteinuria/chronic renal failure the frequency of XbaI, (GG) genotype was the highest [13].

5. Conclusion

In conclusion, the association between *GLUT*1 XbaI polymorphism and DN is still debatable up to date due to the contradictory reports from different populations. In the same context, the present study was only able to demonstrate an association between this polymorphism and susceptibility to T2DM but not DN. Further studies on larger sample size are needed to replicate such findings.

Disclosure

Authors have not conflict of interest to disclose.

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Constitutive and Secretory Expression of the AiiA in *Pichia pastoris* Inhibits *Amorphophallus konjac* Soft Rot Disease

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Abstract

Amorphophallus konjac is an important economic crop widely cultivated in Southeast Asia and Africa. However, _A. konjac_ is seriously infected by soft rot pathogen. The endocellular acyl homoserine lactonase (AiiA) which is generated by _Bacillus_ species has inhibitory effect on soft rot pathogen through disrupting the signal molecules (N-acylhomoserine lactones, AHL) of their Quorum Sensing system. The aim of our study is to obtain recombinant yeast which produces AiiA protein. The recombinant yeast _Pichia pastoris_ GS115 was constructed to constitutive expression of the _AiiA_ gene. The results of reverse transcript PCR analysis showed that the _AiiA_ gene was expressed successfully in the yeast. Proteins extracted from YPDS showed the highest inhibition efficacy to _E. carotovora_ compared with the other two mediums (YPD and LB) under tested conditions.

Keywords

***Amorphophallus konjac*, Acyl Homoserine Lactonase, *Pichia pastoris*, Fusion-Expression**

1. Introduction

The genus *Amorphophallus* has been used as food, medicine, fodder and wine production [1]. *Amorphophallus konjac* which is one of the most widely cultivated species has been grown in China for more than 2000 years [2] [3]. *A. konjac* is a perennial plant with a huge commercial value to produce Glucomannan, which is a polysaccharide consisting of glucose and mannose residues at a molar ratio of 2:3 with β-1, 4 linkages [4]. Several clinical trials show that Glucomannan is responsible for lowering systolic blood pressure, total cholesterol and gly-

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cemia [5] [6]. Unfortunately, bacterial soft rot disease has a large impact on the yield of *A. konjac*. The soft rot *Erwiniae* which is the main soft rot disease bacterial on *A. konjac*, usually exits in soli, ground water and plant surface. Once inside the plant they reside in the vascular tissue and intercellular spaces of suberized or thin-walled parenchymatous tissues and will develop when environmental conditions become suitable, including free water, oxygen availability and temperature [7] [9]. During the *Erwiniae*-plant interaction, multiple cell wall degrading enzymes (exoenzymes) are secreted by *Erwiniae*, including pectinases, cellulases and proteases, which break down plant cell walls and release nutrients for bacterial growth. With successful release of nutrients during infection, other non-pectolytic bacteria will co-grow on the plant and, indeed, other pectolytic and non-pectolytic bacteria are often isolated from diseased plant tissues [8]. The facultative anaerobic pathogen *Erwiniae* causes maceration and rotting of parenchymatous tissues of all organs, eventually resulting in plant death [10]. For a long time, this disease has been a huge problem to *A. konjac* production.

Quorum signaling (QS) is an intercellular communication mechanism and widely employed by bacteria to coordinate behaviors such as bioluminescence, antibiotic synthesis, biofilm formation, adhesion, swarming, competence, sporulation, virulence, and others [11]. Using chemical signals organisms can detect their local population and change their gene expression so as to competitively optimize their behavior in their local environment. In the case of *Erwiniae*, N-acylhomoserine lactones (AHLs) are chemical signal molecules that responsible for regulating the production of plant cell wall degrading exoenzymes [9] and the antibiotic carbapen-3-em carboxylic acid which function in competing with other bacteria [12]-[14]. Degradation of AHLs has been proven as the efficient control of bacterial infections in transgenic plants [15].

The first AHLs degradation enzyme was identified from a soil bacterial isolate *Bacillus* species belonging to Gram positive bacteria, encoded by *aiiA* gene [16]. The enzyme has been subjected to study and is of interest as its resultant effect to disrupt bacteria's ability to communicate. The *Bacillus* aiiA enzyme is a metalloprotease containing two zinc ions in close proximity. AiiA degrades AHLs in a tail length independent manner but the tail is required for activity [17].

As one of the anti-quorum signaling strategies, degradation of AHL-signaling molecules using aiiA enzyme could have potential applications in attenuating plant disease. In this paper, a safely and constitutive expressional recombinant yeast was reported that efficiently produces aiiA protein using for attenuating plant disease. The recombinant yeast *Pichia pastoris* GS115 was constructed to constitutive expression of the *aiiA* gene. The *aiiA* gene expression was confirmed by reverse transcript PCR analysis in the yeast. AiiA enzyme products were extracted from yeast fermentation broth and effectually inhibited the biassay of *Erwinia carotovora*.

2. Materials and Methods

2.1. Bacteria, Medium, and Culture Conditions

Escherichia coli DH5*α* and BL21 were grown in Luria-Bertani broth (LB) (10 g/L of tryptone, 5 g/L of yeast extract, 10 g/L of NaCl, pH 7.0) at 37˚C for propagation of plasmids and protein expression, respectively. The strain of *P. carotovora* subsp. *carotovora* (P.c.c) was isolated from an infected corm of konjac from Hubei province in China (Registry number: FJ463871). *Bacillus thuringiensis* strain 4Q7 was cultured in LB medium at 28˚C. Host strain *P. pastoris* GS115 was purchased from Invitrogen (USA). YPD (1% yeast extract, 2% peptone, 2% glucose) and YPDS (YPD, 1 M sorbitol) medium were prepared as described in the manual of the *Pichia* Expression Kit (Invitrogen, Carlsbad, CA, USA).

2.2. Extraction of Total DNA, RNA, PCR and Sequencing

Based on the sequence of *aiiA* gene from the NCBI (NC_018877.1), gene specific primers were designed for *aiiA* gene cloning (P1: 5'GTCGGATCCATGACAGTAA AGAAGCTTTA3', P2: 5'GTCGCGGCCGCCTATATATACTCAGGGAACA3'). DNA was isolated from *B. thuringiensis* or *P. pastoris* by the modified CTAB method [18]-[20]. Total RNA from *P. pastoris* was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. To eliminate genomic DNA contamination, purified RNA was treated with RNase-free DNase I (Takara, Dalian, China) before final ethanol precipitation. Next, 3 μg of total RNA was reversely transcribed into cDNA by reverse-transcription with Superscript II (Invitrogen) and an oligo-dT20 primer. The final cDNA was stored at −20˚C before use.

Polymerase chain reactions (PCRs) were carried out in a PTC-100 Thermal Cycler (MJ Research, Massachu-

setts, USA) with gene-specific PCR primers following the procedure: initial denaturation at 94˚C for 3 min, 35 amplification cycles of 94˚C for 20 s, 54˚C for 30 s, 72˚C for 1 min and final polymerization step of 72˚C for 7 min. Each PCR mixture (25 μl) contained 0.1 μM primers of P1 and P2, 20 mM dNTP (Dingguo Ltd., China) (10 mMol of each), 40 mM $MgCl_2$, Taq polymerase buffer, 10 ng template-DNA, 1U Taq polymerase (Promega Co., China) and 15 μl mineral oil were added to each of the reaction solutions. The final PCR product was resolved in 1% agarose gel and purified using the AxyPrep gel purification kit (Axygen, Union City, CA, USA). PCR products sequencing was performed by Sangon biotech (Shanghai, China). The protein O-glycosylation site was identified by NetOGlyc 4.0 Server (http://www.cbs.dtu.dk/services/NetOGlyc/) [21].

2.3. Plasmid Construction

For *aiiA* gene expression in *E. coli*, the full length of *aiiA* gene without stop codon was amplified using gene-specific PCR primers as described above. After restricted by *BamH*I and *Not*I, the 762 bp fragment was cloned into the expressional vector pET22b between *BamH*I and *Not*I restriction enzyme sites to form pET22b-aiiA (**Figure S1**). Similarly, *aiiA* gene without stop codon was cloned into the expressional vector pGAPZ*α*-A between *EcoR*I and *Not*I restriction enzyme sites for expression in *P. pastoris* to form pGAPZ*α*-A-aiiA. The primers using for vector construction were list in **Table 1**.

2.4. Expression, Purification and Detection of Recombinant AiiA in *E. coli*

E. coli BL21 cells (10 μL) containing a phagemid pET22b-aiiA was inoculated into 20 mL of LB liquid medium plus 100 μg/mL ampicillin and incubated overnight at 37˚C on a shaker (200 rpm). The next day, 2 mL of the cultured cells was transferred to 100 mL of LB medium with the antibiotics and grown to an OD 600 value of 0.5. After addition of IPTG to a final concentration of 0.4 mM, the culture was incubated at 16˚C for 12 h with shaking. The cells were collected by centrifugation (10 min, 3000 g, 4˚C). The resulting pellets were resuspended in 1 mL of buffer containing 30 mM Tris/HCl and 1 mM EDTA and stored for 15 min on ice, followed by ultrasonication. After centrifuging (10,000 g, 15 min, 4˚C), the supernatant was collected and dialyzed by Ni-NTA-agarose (Qiagen, Chatsworth, CA) and the pellet was resuspended in 8 M-urea.

Proteins were separated by 12% (w/v) SDS-PAGE and transferred to nitrocellulose membranes. Immunoblot was developed using a 1:5000 diluted monoclonal anti-His antibody and AP-conjugated goat anti-mouse IgG antibody. The colorimetric reaction was carried out by using the BCIP/NBT Color Development Kit (Boster). The immunoblot membranes were scanned with Epson perfection V500 Photo.

2.5. Pichia Pastoris Transformation

The recombinant plasmid pGAPZ*α*-A-aiiA was linearized by digestion with *Bgl*II and then transformed into *P. pastoris* GS115 by electroporation at 1.5 kV with a 2 mm cuvette. Then, 800 μL ice-cold sorbitol was immediately added to the cuvette, and the mixture was spread on YPDS plates containing 100 μg/mL Zeocin. Then the plates were incubated at 30˚C for 3 days until colonies form. The selected transformants were inoculated into new YPDS plates containing 100 μg/mL Zeocin. Ten of Zeocin-resistant *P. pastoris* transformants were chosen for the presence of insert detection.

Table 1. Sequences of primers used for this study.

Primer	Sequence (5'-3')
aiiAF	CTGCGGATCCGACAGTAAAGAAGCTTTATTTCATCC
aiiAR	GTCGCGGCCGCTATATATTCTGGGAACACT
aiiA-His-F	AGTAGCGTATGGATATCGGAATTAAT
aiiA-His-R	CAAAAAACCCCTCAAGACCCG
pGAP-F	GTCCCTATTTCAATCAATTGAA
AOX1	GCAAATGGCATTCTGACATCC

2.6. Fermentation of Recombinant AiiA in Shake-Flasks

Two positive transformants were selected and inoculated into 300 mL YPD, YPDS or LB medium. Flasks were cultured at 16˚C and 30˚C, for one to three days at 200 rpm. The supernatant were collected by centrifugation at 5000 g, 4˚C for 10 min. Crystalline ammonium sulfate is slowly added to the supernatant layer to a final concentration of 450 g/L (70% saturation), and the mixture is stirred for 2 h at 4˚C. Proteins were collected by centrifugation (13000 g, 15 min, 4˚C) and resolved by PBS.

2.7. *In Vitro* Bioassay of Purified Proteins on P.c.c

The CPA media was used to assay the growth of P.c.c instead of *A. konjac* leave or shoot basal discs. The CPA media contained 1% hydroxypropyl methylcellulose, 1% pectin, 5.6 g/L NH_4NO_3, 0.5 g/L of KH_2PO_4, 0.25 g/L of $MgSO_4 \cdot 7H_2O$ and 1% Agarose (Low melting point). The antibacteria activity of aiiA protein was studied by agar diffusion test using CPA media. Overnight cultures of P.c.c were diluted 1:10,000 into fresh, prewarmed CPA media (35˚C) and 20 mL mixture was added into a 9 cm petri dish. Wells were made in each plate using a 5 mm sterile puncher and 50 µL aqueous solutions of protein or control buffer were placed in respective wells. The plates were incubated at 28˚C for 3 d and then photographed.

3. Results

3.1. Gene Cloning and Sequence Analysis

The full length without stop codon (750 bp) was amplified from *B. thuringiensis* strain 4Q7 using the primers aiiAF/aiiAR or aiiA-His-F/aiiA-His-R to clone into pET22b expression vector (expressed in *E.coli*) or pGAPZ*α*-A expression vector (expressed in *P. pastoris*). The recombination *aiiA* gene encoded a 269 (in *E.coli*) or 369 (in *P. pastoris*) amino acid polypeptide with N-terminal secretion peptides and C-terminal His-tag. After splicing, the fusion protein was secreted with a calculated molecular mass of 29.2 kDa and a pI of 4.87. No potential O-glycosylation site was identified by NetOGlyc 4.0 Server.

3.2. Expression and Purification of Recombinant AiiA in *E. coli*

To investigate whether the fusion aiiA protein would inhibit *E. carotovora in vitro*, we expressed the fusion aiiA protein by *E. coli* expression system. After addition of IPTG (0.4 mM) for 6 h, the fusion aiiA protein was significantly detected by SDS-PAGE compared with the no IPTG addition sample (**Figure 1(a)**). The aiiA fusion

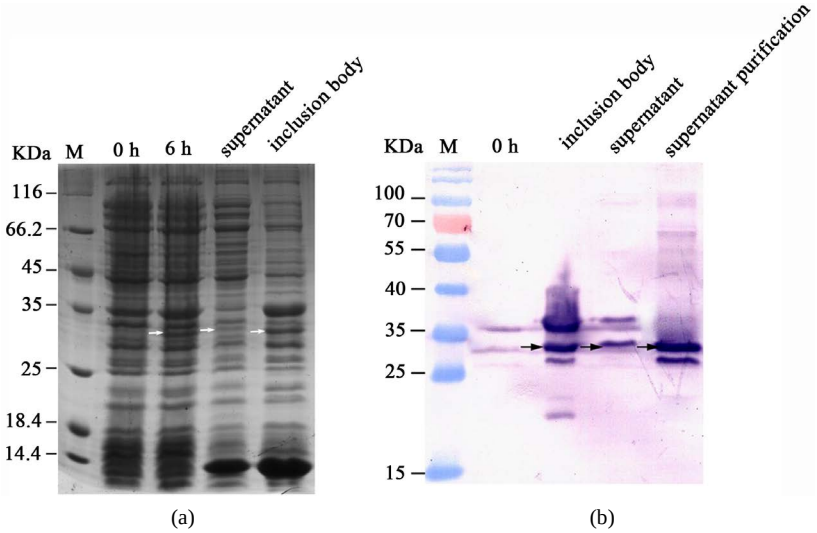

Figure 1. SDS-PAGE and Western blot analysis of proteins extracted from transgenic *E. coli*. (a) SDS-PAGE analysis of total proteins extracted from *E. coli* cultured for 0 h, 6 h and 12 h (supernatant and inclusion body) after adding IPTG; (b) Western blot analysis of proteins in supernatant and inclusion body extracted from *E. coli* cultured for 12 h after adding IPTG. M, protein molecular mass standards; White arrows labeled the aiiA protein band.

protein existed both in supernatant and precipitate (**Figure 1(a)**). This indicated the toxicity of aiiA fusion protein to *E. coli* cell. When adding IPTG to 1 mM or changing the expression condition to 28˚C, most of aiiA fusion proteins formed inclusion bodies (data not shown). This phenomenon supplementary verified our hypothesis. To increase the production of aiiA fusion protein, the concentration of 0.4 mM IPTG was chosen to induce protein expression. After culturing for 12 h at 16˚C, *E. coli* cells were collected for protein extraction and purification and the purification soluble protein concentration was 200 ng/μL (**Figure 1(b)**).

3.3. Effect of Purified aiiA Fusion Proteins on *E. carotovora*

Agar diffusion test was used to detect the inhibition of aiiA fusion proteins on *E. carotovora*. The *E. carotovora* cells were mixed with CPA media medium and perforated with sterile puncher. The well adding 200 ng/μL aiiA fusion protein remarkably inhibited *E. carotovora* growth. Howerer, the well adding PBS (control to aiiA fusion protein), the purified protein from *E. coli* and the purified protein from control bacteria (bacteria transformed with empty plasmid vector instead of expressing aiiA fusion protein) were fully covered with *E. carotovora* colonies (**Figure 2**). Our results indicated the aiiA fusion protein had the ability to inhibit *E. carotovora* growth and was valuable for further reproduction.

3.4. Expression and Fermentation of Recombinant AiiA in *P. pastoris*

With the expression vector pGAPZ*α*, the aiiA protein was transformed into *P. pastoris* GS115. The presence of insert expression cassette was detected using PCR and the 1100 bp fragment was amplified by primers pGAP-F/AOX1 (**Table 1**; **Figure 3(a)**). To verify the aiiA fusion gene was expressed in transcriptional level, RNA was extracted from two positive *P. pastoris* transformants and was reverse transcript into cDNA. The 770 bp aiiA transcriptional fragment was amplified using primers aiiAF/aiiAR (**Figure 3(b)**).

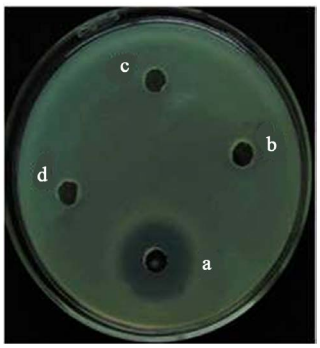

Figure 2. Bioassay of purified proteins from transgenic *E. coli* on *E. carotovora.* The activity of *aiiA* protein was studied by agar diffusion test using CPA media. 50 μL samples were placed in respective wells. The plates were incubated at 28˚C for 3 d and photographed. a: *aiiA* fusion protein; b: PBS; c: the purified protein from *E. coli*; d: the purified protein from control bacteria (bacteria transformed with empty plasmid vector instead of expressing aiiA fusion protein).

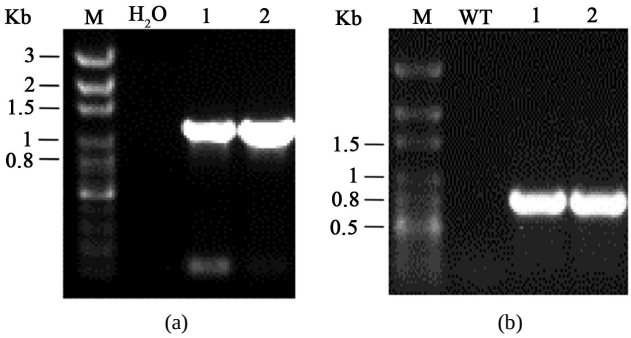

Figure 3. PCR and RT-PCR analysis of transgenic *P. pastoris.* Amplicons of aiiA gene from transgenic *P. pastoris* genome (a) and transcript (b). M; DNA molecular marker, 1; transgenic *P. pastoris*-1, 2; transgenic *P. pastoris*-2; H_2O and wild-type cDNA were used as negative control, respectively.

Three fermentation mediums including LB, YPD and YPDS were selected to research the efficacy of aiiA fusion protein production at the temperature of 16˚C and 30˚C. Proteins were extracted 3 d post inoculated from the supernatant of fermentation broth. The antibateria of extracting solution was measured by agar diffusion as describe above. With the same condition, proteins extracted from YPDS showed the highest inhibition efficacy to *E. carotovora* compared with the other two mediums (YPD and LB) (**Figure 4**). Protein expression at 30˚C was higher than at 16˚C (date not shown). Finally the YPDS was chosen to express the aiiA fusion protein at 30˚C for 3 d.

4. Discussion

The soft rot is a serious damage to *A. konjac* production which is mainly caused by *E. carotovora*. Current protective measures rely on chemical control, producing bactericide-resistant pathogens and other undesirable environmental consequences [22]. Alternative strategies must be found to protect *A. konjac* crops from *Erwiniae* pathogens. The use of biochemical tools is gaining great momentum in crop protection and these may be a supplement or an alternative to chemical pesticides control. The increased understanding of the quorum sensing has made possible using the enzyme aiiA as a diagnostic tool [23] [24]. AiiA have been expressed in several pathogens or transgenic plants [10] [16] [25]-[27]. However, these transgenic strains were not suitable for aiiA production due to the attenuated growth caused by aiiA or the bacterial security. To improve the production of aiiA, aiiA was constitutively and secretory expressed in an atoxic fungi *P. pastoris.*

E. carotovora secretes exoenzymes including pectinases, cellulases and proteases, that contribute to the pathogenesis of plant. AHLs regulate the production of plant cell wall degrading exoenzymes in *E. carotovora* [12]. *In vitro* bioassay of *E. carotovora* growth, the cell number was comparatively rare at 200 ng/μL aiiA proteins (extracted from *E.coli*). That indicated AHLs were degraded by aiiA fusion proteins and the transcription of the exoenzyme structural genes were not coordinated to high levels by AHLs in *E. carotovora.*

The promoter of the gene (GAP) encoding the GAPDH protein has recently been characterized and shown to express recombinant proteins to high levels in *P. pastoris*, depending on the carbon source used [28]. The level of expression seen with the GAP promoter can be slightly higher than that obtained with the AOX1 promoter (an inducible promoter usually used in *P. pastoris* expression) [29]. Moreover, the GAP promoter is a constitutive promoter and it is convenient for aiiA fusion protein production. After cloning the aiiA-His gene to GAP promoter expression cassette, the expression cassette was transformed into *P. pastoris* and inserted into the genome sequence. The transcription of aiiA-His was detected by RT-PCR. Three days post inoculation, high level transcription of the aiiA-His gene was detected in transgenic *P. pastoris*.

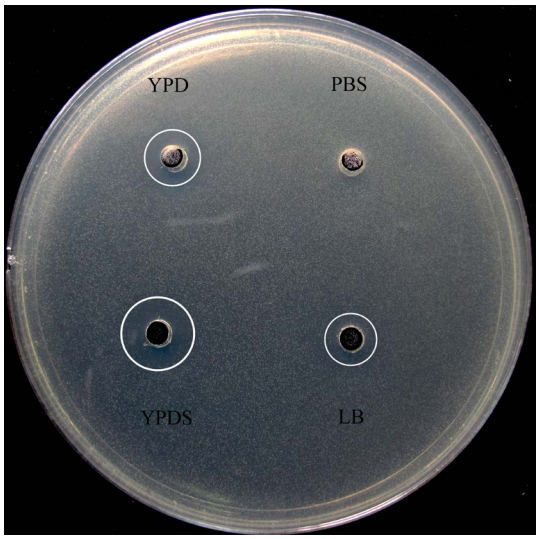

Figure 4. Bioassay of purified proteins on *E. carotovora* from different transgenic *P. pastoris* fermentation broth. AiiA proteins were extracted from *P. pastoris* fermentation broth (LB, YPD and YPDS) 3 d post inoculation. 50 μL aqueous solutions of protein were placed in respective wells. The plates were incubated at 28˚C for 3 d and photographed. PBS was used as negative control.

To improve the production of aiiA-His, three fermentation mediums (LB, YPD and YPDS) were selected to research the efficacy of aiiA fusion protein production under 16˚C and 30˚C. According to the result of agar diffusion test, highest antibacteria activity was observed in YPDS-grown cells, although aiiA-His was constitutively expression in all three fermentation mediums at 30˚C. That was consist with the Northern blot analysis of total RNA isolated from *P. pastoris* cells grown on glucose-carbon sources [28].

5. Conclusion

In conclusion, we constitutively expressed of the AiiA-His gene in *P. pastoris* and achieved high-yield fermentation of AiiA-His protein. The AiiA enzyme products extracted from yeast fermentation broth effectually inhibit the growth of *E. carotovora*. Our results indicated that direct application of AHL-lactonase to control *E. carotovora* infection might be an effective alternative of chemical control to avoid the emergence of bactericide-resistant strains.

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Supplement

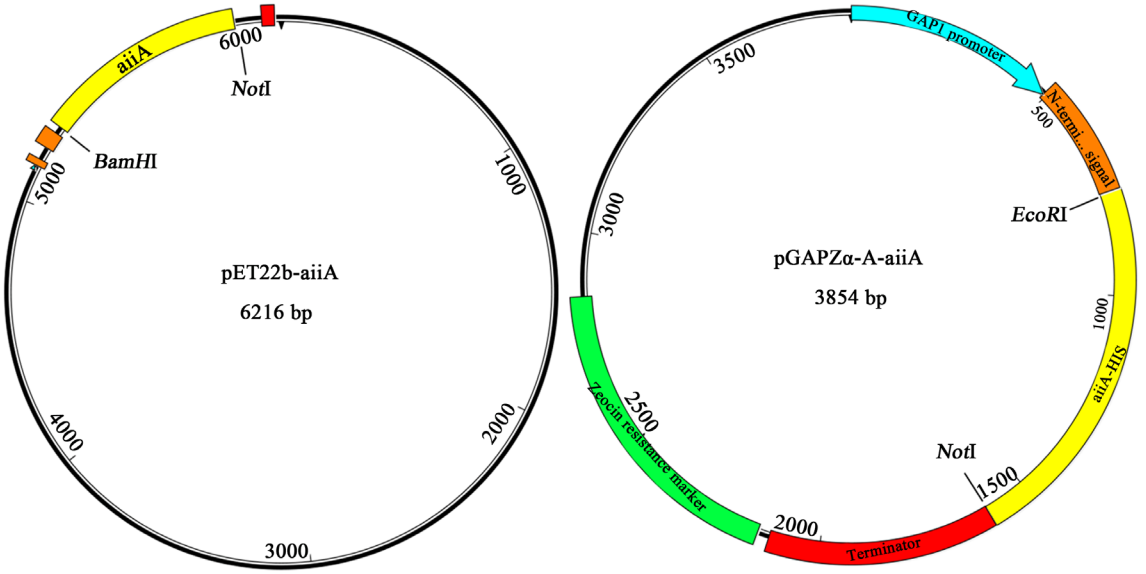

Figure S1. Plasmids used in this study.

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Quantitative Gene Expression of Peroxidase, Polyphenoloxidase and Catalase as Molecular Markers for Resistance against *Ralstonia solanacearum*

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Abstract

Brown rot or bacterial wilt of potato caused by *Ralstonia solanacearum*, is an economically important disease. Potato, cv. Nicola, was found to be relatively highly resistant to the infection with *R. solanacearum* and showed 15.12% wilt disease index, meantime, cv. Kara showed intermediate resistance with 37.40% disease index while, cv. Spunta was susceptible and showed 80.33% disease index. The role of defense-related enzymes in imparting resistance in potato against *R. solanacearum* was investigated by quantifying enzymes activity and gene expression of three defense-related enzymes, peroxidase, polyphenol oxidase and catalase. Peroxidase showed maximum activity 0.488 $min^{-1} \cdot g^{-1}$ early at 12 h after pathogen inoculation in the cv. Nicola, whereas in susceptible cultivar Spunta showed lower activity of maximum 0.226 $min^{-1} \cdot g^{-1}$ later at 48 h after inoculation. While, the moderately resistant cultivar Kara showed intermediate activity for the peak and its time. Meanwhile, polyphenol oxidase showed similar trends to that of peroxidase. On the contrary, catalase showed the highest activity values in the susceptible, cv. Spunta, while, in relatively highly resistant (cv. Nicola) and the moderately resistant (cv. Kara) showed lower values of activity and up to 96 h after inoculation. Meanwhile, gene expression of related enzymes the RT-PCR was used. At zero time, the relatively highly resistant potato cultivar, Nicola, showed the highest values of gene expression for both Peroxidase (POD) and Poly Phenol Oxidase (PPO). While, the susceptible potato cultivar, Spunta showed the lowest values. On the contrary, Catalase (CAT) gene expression was the highest in the susceptible, cv. Spunta, and was the lowest in the relatively highly resistant, cv. Nicola, while, was of intermediate values in the intermediate resistance, cv. Kara. Results show that peroxidase and polyphenol oxidase activities can be used as biochemical markers to reveal the resistance and susceptibility nature of potato cultivars against bacterial wilt disease of potato caused by *R. solanacaerum*.

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Keywords

RT-PCR, Defense Related Enzymes, Gene Expression, Potato Brown Rot, *Ralstonia solanacearum*

1. Introduction

Potato (*Solanum tuberosum* L.) is one of the most consumed crops in the world with global production of approximately 367,753,014 tons, produced from approximately 19,454,997 hectares [1] around the world. Meantime, it is considered one of the most important vegetable crops in Egypt. Potato production is approximately 4,800,000 tons, production from approximately 178,000 hectares, which making Egypt Africa's No. 1 potato producer [1]. However, brown rot or bacterial wilt of potato caused by *Ralstonia solanacearum* constitutes a threat to potato cultivation in Egypt and in all tropical agriculture [2]. *R. solanacearum* has a large host range of more than 200 species in 50 families [3] and affects a wide range of economically important crops such as tomato, potato, eggplant, chili and non solanaceous crops such as banana and groundnut [4]. Bacterial wilt causes 15% to 55% crop losses around the world. Disease resistance in plants is associated with activation of a wide array of defense responses that slow down or halt infection at certain stages of the host-pathogen interaction. These defense mechanisms include preexisting physical and chemical barriers that interfere with pathogen establishment. In response to the infection, the host induces a cascade of pathogen inducible enzymes, which are implemented in defense against phytopathogens. Early and elevated levels of expressions of various defense enzymes are an important feature including peroxidase (POD), polyphenol oxidase (PPO) and catalase (CAT) during host pathogen interactions. Polyphenol oxidase (PPO) is one of the main SAR related enzymes in plant, and their activities are related to plant resistance [5] [6]. Increases in POD activity are often associated with a progressive incorporation of phenolic compounds within the cell wall during incompatible plant-microbe/elicitor interactions. CAT is an antioxidative enzyme involved in oxidative burst generated transiently in plant-pathogen interactions. CAT is involved in regulation of H_2O_2 levels in plant tissues [7]. Real Time PCR (RT-PCR) has become an extensively applied technique in molecular plant pathology and it has been extensively used for quantification of different enzymes gene expression activities [8]. RT-PCR proved to be a successful tool for the evaluation of possible resistance in wheat [9].

Therefore aims of the present study were to:

1) Investigate the reaction of different potato cultivars to infection with *R. solanacearum*.

2) Investigate the potential of peroxidase (POD) and catalase (CAT) enzymes activity in relation to potato resistance to *R. solanacearum*.

3) Quantifying the gene expression levels of peroxidase, polyphenol oxidase and catalase enzymes using Real Time PCR.

2. Materials and Methods

2.1. Plant and Fungal Materials

Certified tubers of cvs.Spunta, Nicola and Kara potato were purchased from the International Potato Center (CIP) Kafr El-Zayat, Egypt. These three potato varieties were previously proved to have different degree of susceptibility against *R. solanacearum.*

A highly virulent isolate of *R. solanacearum* was obtained from *R. solanacearum* collection bank, Plant Pathology Dept., Faculty of Agriculture, Damanhour University.

Potato tubers of the three cultivars Spunta, Kara and Nicola were surface sterilized with 1% sodium hypochlorite for five minutes, washed with sterile water, and planted in plastic pots 30 cm diameter filled with sterile peat moss (one tuber per pot). When plant reached 15 - 20 cm tall (25 days after plantation), stems were inoculated by injecting by a sterilized needle 0.25 ml, bacterial suspension (10^9 cfu/ml) into the stems at 5 cm above the soil level according to (Prior and Steva, 1990) [10]. Ten replicates were used in the experiment and plants injected with sterile distilled water were served as control. Inoculated plants were placed in a greenhouse at 25˚C ± 2˚C.

2.2. Disease Assessments

After 5 weeks of inoculation, wilt severity was assessed according to [11] as follows: 0:extremely resistant, no wilting; 1: highly resistant, 1% - 25% of total leaves wilted; 2: moderately resistant, 26% - 50% of total leaves wilted; 3: susceptible, 51% - 75% of total leaves wilted and 4: highly susceptible, 76% - 100% of total leaves wilted or plant died. Disease severity as disease index was calculated according to the formula:

$$\text{Disease index}(\%) = \left[\Sigma\left(n_i \times v_i\right)/\left(V \times N\right)\right] \times 100$$

where n_i = number of plants with respective disease rating; v_i = disease rating; V = the highest disease rating; and N = the number of plants observed.

2.3. Assessment of Potato Growth Parameter

Five weeks after inoculation plants were uprooted and examined for the following growth parameters:

- Shoot fresh weight (g).
- Root fresh weight (g).
- Total tubers weight/plant (g).

Then, percentage of reduction according to the control was calculated in each potato cultivar.

2.4. Determination of Photosynthetic Pigments

Fresh leaves samples of inoculated and non-inoculated potato cultivars were collected after 5 weeks from inoculation for estimation of photosynthetic pigments.

Chlorophyll A, B and β-carotene were determined according to [12] as follows: half gram fresh leaves were ground in a pestle and mortar and extracted by 15 ml of 80% acetone (1:100 w/v) and 0.5 g calcium carbonate. The mixture was filtered through a glass funnel and the residue was washed with a small volume of acetone and completed to 25 ml. The optical density (O.D) of a constant volume of filtrate was measured at a wave length of 662 nm, 644 nm and 440 nm for chlorophyll A, chlorophyll B and carotene, respectively. The experiment was repeated thrice.

The following equations were used:

Chl.A = 9.784 E.662 − 0.99 E.644 = mg/L

Chl.B = 21.426 E.644 − 4.65 E.662 = mg/L

Carotene = 4.695 E.440 − 0.268 (Chl.A − Chl.B) = mg/L

where, E. = Optical density at the wavelength indicated.

2.5. Determination of the Defense Related Enzyme Activity in Potato Cultivars against *R. solanacearum*

2.5.1. Estimation of Peroxidase Activity

The peroxidase (POD) activity was assayed as described by [13]. The peroxidase activity was measured at 0, 3, 6, 12, 24, 48, 72 and 96 hours (h) after inoculation. Extraction was carried out by homogenizing 1 g of the fresh leaves of inoculated potato samples in 2.6 mL of 0.1 M sodium phosphate buffer (pH 6.5) using pre chilled pestle and mortar (4˚C). The homogenate was centrifuged at 10,000 rpm for 15 mins at 4˚C. The supernatant was served as enzyme source for the reaction mixture which consisted of 1.5 mL of 0.05 M pyrogallol, 0.5 mL of enzyme extract, and 0.5 mL of 1% H_2O_2. The reaction mixture was incubated at 28˚C ± 2˚C. At the start of enzyme reaction, the absorbance of the mixture was set to zero at 420 nm in the spectrophotometer and the change in the absorbance was recorded at 20 s intervals for 3 mins. Boiled enzyme preparation was served as a control. Peroxidase activity was expressed as change in the absorbance of the reaction mixture $\text{min}^{-1} \cdot \text{g}^{-1}$ protein of fresh tissue. All the experiments were repeated thrice.

2.5.2. Estimation of Polyphenol Oxidase Activity

One gram of the leaf sample was homogenized in 2 mL of 0.1 M sodium phosphate buffer (pH 6.5) in a pre-chilled pestle and mortar. The homogenate was centrifuged at 10,000 rpm for 15 min at 4˚C and the supernatant served as enzyme source. Polyphenol oxidase activity was determined according to the procedure given by [14]. The reaction mixture consisted of 1.5 mL of 0.1 M sodium phosphate buffer (pH 6.5) and 200 µL of the enzyme

extract. To start the reaction, 200 µL of 0.01 M catechol was added. The reaction mixture was incubated at room temperature and the absorbance was set to zero at 495 nm. The changes in absorbance were recorded at 30 s interval for 2 min and the activity was expressed as change in absorbance $min^{-1} \cdot g^{-1}$ of fresh tissue. The polyphenol oxidase activity was measured at 0, 3, 6, 12, 24, 48, 72 and 96 hours after inoculation. All the experiments were repeated thrice.

2.5.3. Estimation of Catalase (CAT) Activity

Catalase (CAT) activity was measured according the methods given by [15] with a slight modification. Extraction was carried out by homogenizing 1 g of the fresh leaves of inoculated potato samples in 2.6 mL of 0.1 M sodium phosphate buffer (pH 6.5) using pre chilled pestle and mortar (4˚C). The assay mixture contained 2.6 mL of 50 mM potassium phosphate buffer (pH 7.0), 0.4 mL of 15 mM H_2O_2 and 0.04 mL of the enzyme extract. The extract was centrifuged at 4˚C for 20 min at 12,500 rpm. The supernatant was used for enzyme assay. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. The enzyme activity was expressed in mixture $min^{-1} \cdot g^{-1}$ protein. The catalase activity was measured at 0, 3, 6, 12, 24, 48, 72 and 96 hours after inoculation. All the experiments were repeated thrice.

2.6. Quantification of the Enzyme Genes Expression Associated with Resistance to *R. solanacearum*

2.6.1. RNA Isolation and Preparation for RT-PCR

Total RNA was extracted from plant tissue using Green Start™ RNA Isolation kit II (guanidiumthiocynate) according to the manfacture procedures.

Reverse transcription (RT) or first strand reaction was performed for converting the mRNA to complementary DNA (cDNA) in the presence of dNTPs (deoxynucleotide triphosphates) reverse transcriptase. The components are combined with a DNA primer in a reverse transcriptase buffer for an hour at 42˚C. The exponential amplification via reverse transcription polymerase chain reaction provides a highly sensitive technique, where a very low copy number of RNA molecules can be detected.

Reverse transcription reaction was performed using oligo (dT) primer (5'-TTTTTTTTTTTTTTT-3'). Each 25 µl reaction mixture contained 2.5 µl (5×) buffer with $MgCl_2$, 2.5 µl (2.5 mM) dNTPs, 1 µl (10 pmol) primer, 2.5 µl RNA (2 mg/ml) and 0.5 unit reverse transcriptase enzyme. PCR amplification was performed in a thermal cycler programmed at 42˚C for 1 hr, 72˚C for 10 min (enzyme killing) and the product was stored at 4˚C until use.

2.6.2. Estimation of Quantitative Enzyme Gene Expression Using RT-qPCR

According to [16] with some modifications. Samples were analyzed using the Fermentasekit: Each reaction contained 12.5 µl of 2× Quanti tech SYBR® Green RT Mix, 1 µl of 25 pm/µl forward primer, 1 µl of 25 pm/µl reverse primer,1 µl of the cDNA (50 ng), 9.25 µl of RNase free water for a total of 25 µl. Samples were spun before loading in the Rotor's wells.

The real time PCR program was as follows: initial denaturation at 95˚C for 10 min; 40 cycles of at ˚C for 15 sec.; annealing at 60˚C for 30 sec and extension at 72˚C for 30 sec. Data acquisition performed during the extension step. This reaction was performed using Rotor-Gene-6000-system (Qiagen, USA) with a set of primers for peroxidase, polyphenol oxidase and catalase genes (**Table 1**).

Table 1. Sequence of primers used in the real-time PCR.

Primers	Primer sequence 5'→3'	Annealing (˚C)
Catalase (F)	AGGAGGCGGATCTAGCCTTA	60
Catalase (R)	TGTCAAGAAAGGGGTGTCGT	
Peroxidase (F)	GCTTTGTCAGGGGTTGTGAT	60
Peroxidase (R)	TGCATCTCTAGCAACCAACG	
Polyphenol oxidase (F)	CATGCTCTTGATGAGGCGTA	60
Polyphenol oxidase (R)	CCATCTATGGAACGGGAAGA	

(F) Forward primer; (R) Reverse primer.

2.6.3. Data Analysis

Comparative quantification analysis was done using Rotor-Gene-6000 Series Software according to [17].

$$\left(\text{Experimental}/\text{control}\right)=\frac{\text{Fold change in target gene expression }\left(\text{expt}/\text{control}\right)}{\text{Fold change in reference gene expression }\left(\text{expt}/\text{control}\right)}$$

$$\text{Fold change in target gene expression}=\frac{\text{copy number experimental}}{\left(\text{expt}/\text{control}\right)\text{copy number control}}$$

$$\text{Fold change in reference gene expression}=\frac{\text{copy number reference}}{\left(\text{expt}/\text{control}\right)\text{copy number control}}$$

The equation shows a mathematical model of relative expression ratio in real-time PCR. The ratio of a target gene is expressed in a sample versus a control in comparison to a reference gene.

The sample and control dataset of real-time PCR data were analyzed with appropriate Bioinformatics and Statistical program for the estimation of the relative expression of genes using real-time PCR and the result normalized to ITS housekeeping gene (Reference gene). The data were statistically evaluated, interpreted and analyzed using Rotor-Gene-6000 version 1.7.

2.7. Statistical Analysis

The obtained data were statistically analyzed using the American SAS/STAT Software, version 6 and means were compared by the least significant difference test (LSD) [18].

3. Results

3.1. Susceptibility of Different Potato Cultivars to *R. solanacearum*

Data in (**Table 2**) shows that potato, cv. Nicola was the most resistant potato cultivar to *R. solanacearum* and shows disease index as low as 15.12%. Meantime, cv. Kara, showed moderate resistance as exhibited 37.40% wilt disease index. On the other hand, cv. Spunta was highly susceptible and showed 80.33% disease index to the infection with *R. solanacearum* the incitant of the potato bacterial wilt disease (**Table 2**).

3.2. Potato Growth Parameters

Data in (**Table 3**), illustrated in (**Figure 1**) showed that there was no significant variations among the tested non inoculated (healthy) potato cultivars for the assessed vegetative traits, *i.e.* shoot fresh weight/plant, root fresh weight/plant and total tuber fresh weight/plant. However, after the inoculation with *R. solanacearum*, the tested potato cultivars showed significant variations for the tested vegetative traits. Potato, cv. Nicola, showed the most resistance expressed in terms of the lowest reduction in shoot fresh weight/plant (11.74%), root fresh weight/plant (13.71%) and total tuber fresh weight/plant (12.59%). This comparing to high percentage of reductions in the most susceptible potato, cv. Spunta, being 63.98%, 71.25% and 62.68% for the same traits, respectively. Meanwhile, cv. Kara showed intermediate values for the previous traits being 30.54%, 43.29% and 40.54%, respectively.

Table 2. Wilt disease index (severity) developed on potato plants of different cultivars artificially inoculated with *R. solanacearum* under greenhouse condition, five weeks after inoculation.

Potato cultivars	Wilt disease index (%)	
	Inoculated	Uninoculated
Nicola	15.12	0.0
Kara	37.40	0.0
Spunta	80.33	0.0

Data are means of ten replicates.

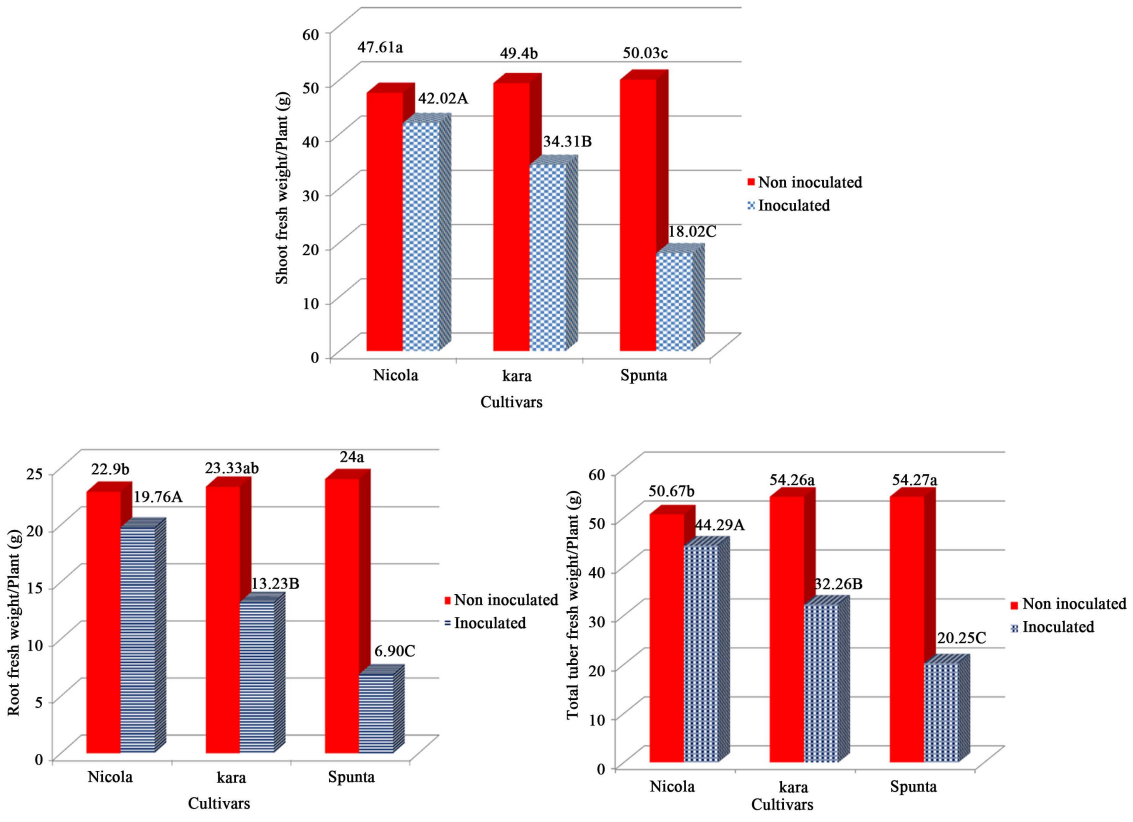


Figure 1. Shoot fresh weight/plant, root fresh weight/plant and total tuber fresh weight/plant of three potato cultivars (Nicola, Spunta and Kara) inoculated with *R. solanacearum*, under greenhouse conditions, five weeks after inoculation.

Table 3. Values of vegetative traits of three potato cultivars artificially inoculated with *R. solanacearum* under greenhouse conditions five weeks after inoculation.

Traits Cv.	Shoot fresh weight/plant (g)			Root fresh weight/plant (g)			Total tuber fresh weight/plant (g)		
	Non inoculated	Inoculated	Reduction (%)	Non inoculated	Inoculated	Reduction (%)	Non inoculated	Inoculated	Reduction (%)
Nicola	47.61^{a}	42.02^{a}	11.74^{C}	22.9^{a}	19.76^{a}	13.71^{C}	50.67^{a}	44.29^{a}	12.59^{C}
Kara	49.4^{a}	34.31^{b}	30.54^{B}	23.33^{a}	13.23^{b}	43.29^{B}	54.26^{a}	32.26^{b}	40.54^{B}
Spunta	50.03^{a}	18.02^{c}	63.98^{A}	24^{a}	6.90^{c}	71.25^{A}	54.27^{a}	20.25^{c}	62.68^{A}
Mean	49.02^{A}	31.45^{B}		23.41^{A}	13.3^{B}		53.1^{A}	32.3^{B}	

Data are means of four replicates; growth parameters were assessed five weeks after potato inoculation with *R. solanacearum*; values followed by different letters for each parameter are significantly different at $p = 0.05$.

3.3. Potato Chlorophyll Content

Data in (Table 4), illustrated in (Figure 2) shows that, there was no significant variations between healthy non-inoculated plants of cv. Nicola (relatively highly resistant) and cv. Spunta (susceptible) for the measured chlorophyll contents, chlorophyll A, chlorophyll B and the carotene. However, after the inoculation with *R. solanacearum*, considerable variations were revealed where the inoculationwith *R. solanacearum* resulted in significant decrease in all pigment contents, chlorophyll A, chlorophyll B and the carotene. Meantime, cv. Spunta exhibited the lowest pigment contents being 0.05, 0.11 and 0.1 for chlorophyll A, chlorophyll B and the carotene, respectively. This comparing to 0.09, 0.16 and 0.28 for the same pigments in cv. Nicola (relatively high resistance). While, for most pigments there was no significant differences between cv. Spunta (susceptible) and cv. Kara (intermediate resistance).

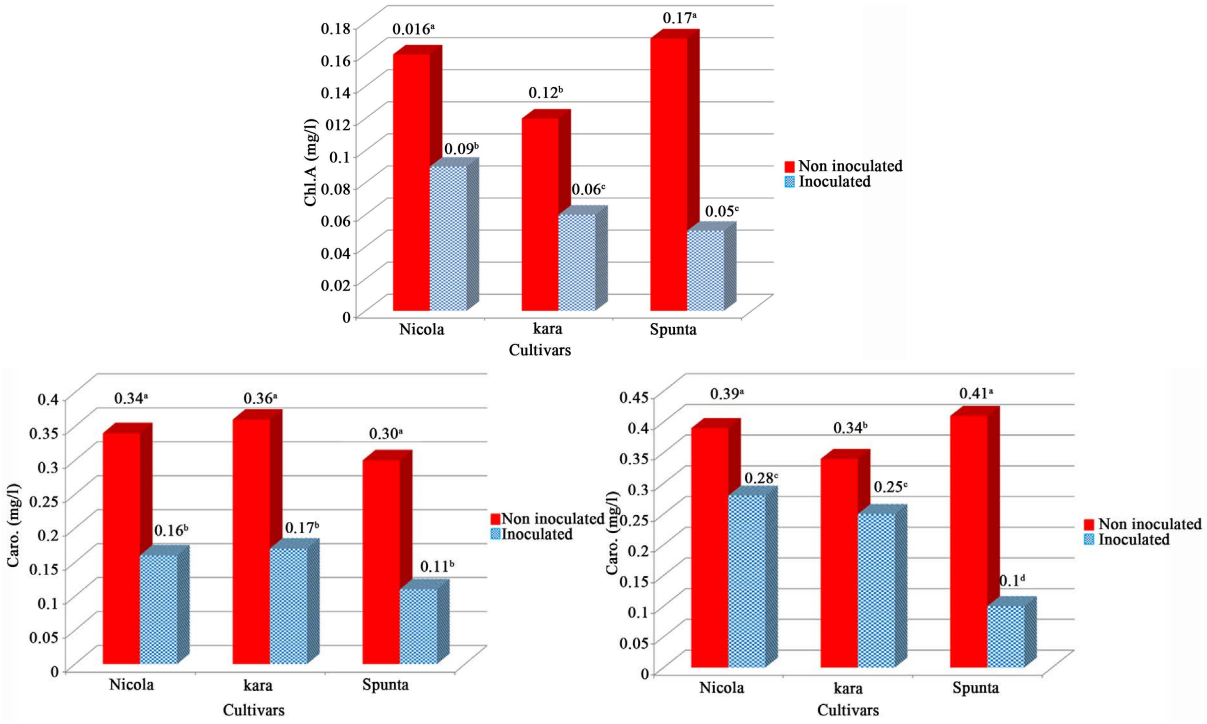


Figure 2. Effect of artificial inoculation with *R. solanacearum* on chlorophyll A, chlorophyll B and carotene contents in three potato cultivars tested, five weeks after inoculation under greenhouse conditions.

Table 4. Effect of artificial inoculation with *R. solanacearum* on chlorophyll A, chlorophyll B and carotene contents in the three tested potato cultivars, five weeks after inoculation under greenhouse conditions.

Traits Cv.	Chlorophyll A (mg/L)		Chlorophyll B (mg/L)		Carotene (mg/L)		Mean
	Non inoculated	Inoculated	Non inoculated	Inoculated	Non inoculated	Inoculated	Reduction (%)
Nicola	0.16^{a}	0.09^{b}	0.43^{a}	0.16^{b}	0.39^{a}	0.28^{c}	0.25^{A}
Kara	0.12^{b}	0.06^{c}	0.36^{a}	0.17^{b}	0.34^{b}	0.25^{c}	0.21^{B}
Spunta	0.17^{a}	0.05^{c}	0.30^{a}	0.11^{b}	0.41^{a}	0.10^{d}	0.19^{B}
Mean	0.15^{A}	0.06^{B}	0.36^{A}	0.14^{B}	0.38^{A}	0.21^{B}	

Data are means of three replicates. Photosynthetic pigments were examined five weeks after potato inoculation with *R. solanacearum*. Values followed by different letters for each parameter are significantly different at $p = 0.05$.

3.4. Potato enzyme Activity Associated with Resistance to *R. solanacearum*

Peroxidase (POD), polyphenol oxidase (PPO) and catalase (CAT) activities were assessed spectrophotometrically in the potato tuber tissues after inoculation with *R. solanacearum*. Data illustrated in (**Figure 3**) showed that, in the relatively highly resistant cultivar (Nicola) a drastic increase in peroxidase (POD) activity was noticed after inoculation and reached its peak (0.488 $min^{-1} \cdot g^{-1}$) soon at 12 h, and kept at the higher level up to 72 h after inoculation. On the contrary, in the susceptible, cv. Spunta, the POD activity peak was as low as 0.226 $min^{-1} \cdot g^{-1}$ and was recognized later at 48 h, after inoculation (**Figure 3**). Potato, cv. Kara, however, of the intermediate resistance to *R. solanacearum*, showed intermediate POD activity for the peak value and its time.

Concerning polyphenol oxidase (PPO) activity, in relatively highly resistant, cv. Nicola, the peak activity of PPO was the highest (0.445 $min^{-1} \cdot g^{-1}$) at 24 h, and kept at high level up to 72 h after inoculation. However, in susceptible, cv. Spunta, the maximum activity was 0.364 $min^{-1} g^{-1}$ and was recognized as later at 48 h after inoculation. Meanwhile, cv. Kara, of the intermediate resistance showed intermediate values (**Figure 3**).

On the contrary, catalase (CAT) activity, in the susceptible, cv. Spunta, was the highest and remained at the high levels up to 96 h after inoculation, while, in relatively highly resistant one, cv. Nicola, and moderately resistant one, cv. Kara, showed lower values of activity.

3.5. Quantitative Enzyme Gene Expression Associated with Potato Resistance to *R. solanacearum*

The relative expression of peroxidase (POD), polyphenol oxidase (PPO) and catalase (CAT) genes were quantified by real-time reverse transcription RT_qPCR analysis at 0, 3, 6, 12, 24, 48, 72 and 96 hours after inoculation of the three tested potato cultivars, Nicola, Kara and Spunta with *R. solanacearum*. Data illustrated in (**Figure 4**) shows that, at zero time, the relatively highly resistant potato cv. Nicola showed the highest values of gene expression for both POD and PPO. However, the susceptible potato cultivar, Spunta, showed the lowest values, while, the intermediate resistance potato cultivar, Kara, exhibited intermediate values.

Meanwhile, POD and PPO exhibited their peaks sooner at 12 h and 24 h, respectively, after inoculation of the relatively highly resistant potato cultivar, Nicola, while for the susceptible, cv. Spunta, the POD and PPO peaks were recognized later at 48 h and with considerable low level.

On the contrary, for CAT, gene expression was the highest in cv. Spunta (susceptible) and was the lowest in, cv. Nicola, (relatively highly resistant) while, it was of intermediate values in cv. Kara (intermediate resistance) (**Figure 4**).

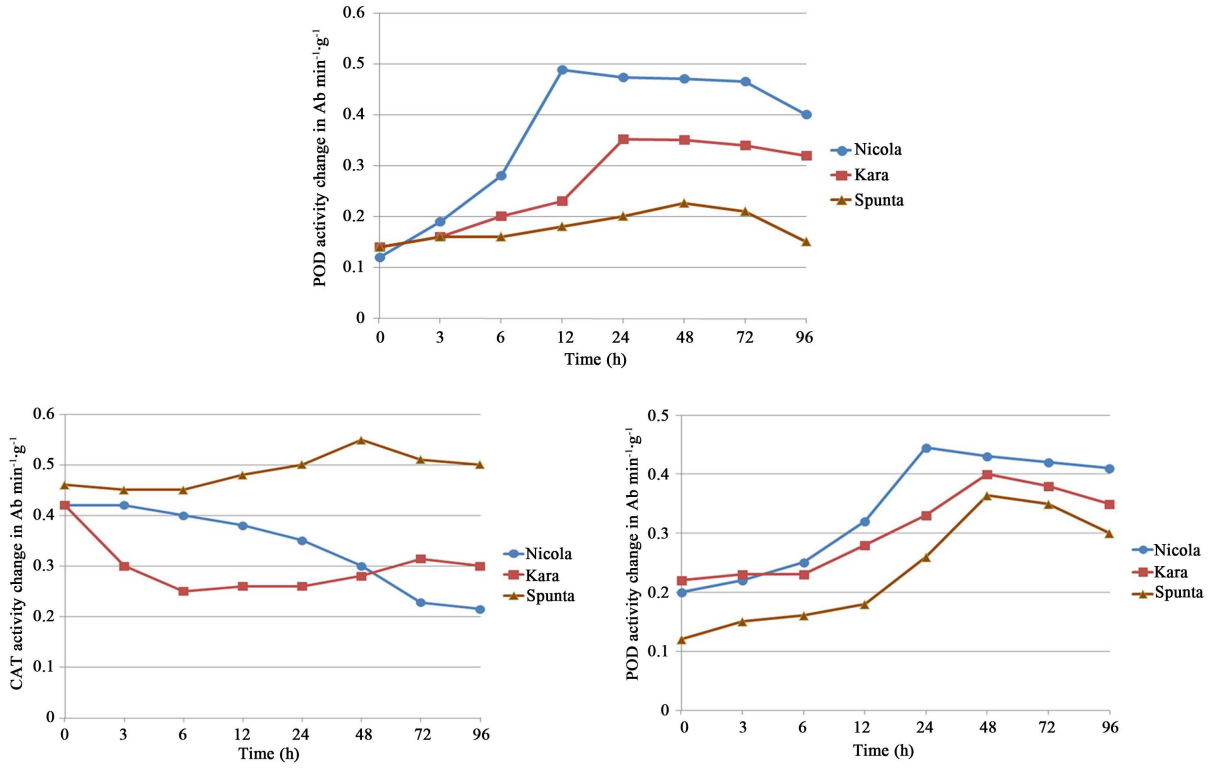
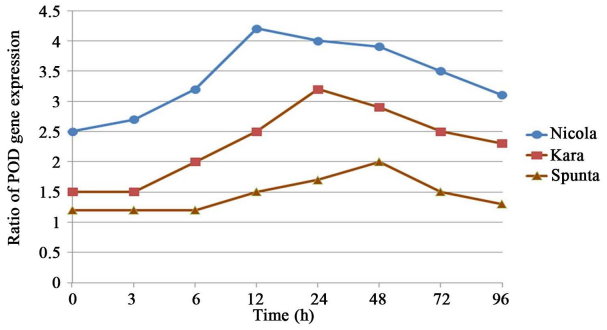


Figure 3. Peroxidase (POD), polyphenol oxidase (PPO) and catalase (CAT) activity in potato cultivars, after inoculation with *R. solanacearum.*



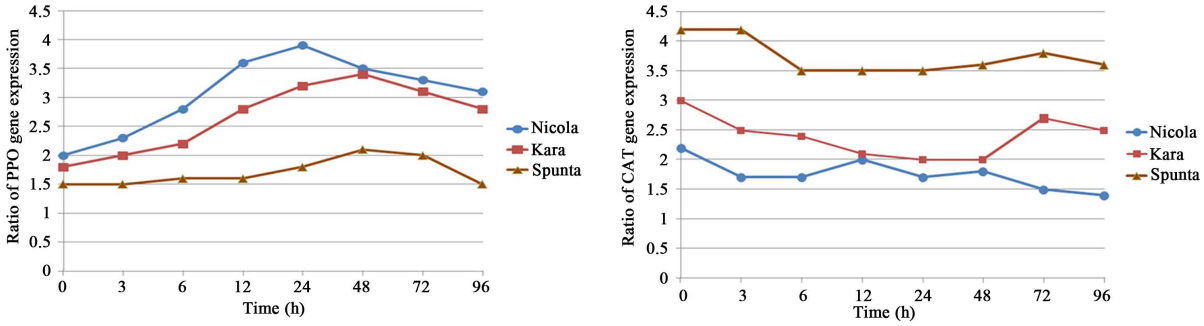


Figure 4. Peroxidase (POD), polyphenol oxidase (PPO) and catalase (CAT) gene expression in potato cultivars, after inoculation with *R. solanacearum.*

4. Discussion

In the present study potato, cv. Nicola was the relatively highly resistant potato cultivar to *R. solanacearum* and showed disease index as low as 15.12%. Meantime, cv. Kara was shown to be moderately resistant as exhibited 37.40% wilt disease index; on the other hand, cv. Spunta, was susceptible and showed 80.33% disease index to the bacterial wilt disease. The study indicated that there was no significant variations among the tested uninoculated (healthy) potato cultivars for the assessed vegetative traits, shoot fresh weight/plant, root fresh weight/plant and total tuber fresh weight/plant. However, after the inoculation with *R. solanacearum*, the tested potato cultivars showed significant variations for the assessed vegetative traits. Potato plants, cv. Nicola, showed the relatively highly tolerance expressed in terms of the lowest reduction in shoot fresh weight/plant (11.74%), root fresh weight/plant (13.71%) and total tuber fresh weight/plant (12.59%). This in comparison with high percentage of reductions in the susceptible potato, cv. Spunta, being 63.98%, 71.25% and 62.68% for the same traits, respectively. Meanwhile, cv. Kara, showed intermediate values for the previous traits being 30.54%, 43.29% and 40.54%, respectively.

There was no significant difference in the uninoculated healthy potato plants between, cv. Nicola, (relatively highly resistant) and, cv. Spunta, (susceptible) for the measured pigments contents, chlorophyll A, chlorophyll B and the carotene. However, after the inoculation with *R. solanacearum*, more variations were revealed where, cv. Spunta, exhibited the lowest pigment contents. While, cv. Nicola (relatively highly resistant) exhibited higher pigment contents. Meanwhile, for most pigments there was no significant difference between cv. Spunta (susceptible) and cv. Kara (intermediate resistance).

Inoculation of potato plants of different cultivars with *R. solanacearum* showed low levels of chlorophyll pigments in the leaves which indicated a process of senescence. During senescence, leaf cells undergo drastic metabolic degeneration of cellular structures, starting with chlorophyll catabolism as well as protein and RNA degradation, with loss of photosynthetic activity and chlorophyll content, which were greater in susceptible potato cvs. compared to the tolerant potato cvs. [19]. It was obvious that rate of chlorophyll loss due to infection with *R. solanacearum* was negatively correlated with tolerance of potato cvs. to *R. solanacearum*. Increased plant defense-related enzyme activities were observed in potato cultivars tolerant to the infection with bacterial pathogen compared to the susceptible ones [20]-[23]. The oxidative enzymes such as POD and PPO, can catalyze the formation of lignin and other oxidative phenols, and contribute in formation of defense barriers by changing the cell structure defense system get actuated against pathogens [24]. The resistance induced by systemic acquired resistance (SAR) is generally effective against a broad range of pathogens and this type of resistance is associated with an increase in the activity of POD [25], PAL [26] and PPO in the plant [26].

In present study, the relatively highly resistant cultivar (Nicola) showed an increase in POD activity in which it was noticed initially and reached its peak 0.488 $min^{-1} \cdot g^{-1}$ at 12 h, and kept at the higher level up to 72 h after inoculation. On the contrary, in the susceptible, cv. Spunta, the POD activity peak was as low as 0.226 $min^{-1} \cdot g^{-1}$ and was recognized later at 48 h, after inoculation. Potato, cv. Kara, however, of the intermediate resistance to *R. solanacearum*, showed intermediate POD activity for the peak value and its time. In tomato, POD is one of the enzymes believed to catalyse the last step in the lignification pathways. The reinforcement of the plant cell wall by phenolics and lignin increases plant resistance to wall-degrading enzymes produced by pathogens, and acts as a mechanical barrier to toxin ingress and to physical penetration toward the protoplast [28].

POD activity in plants can increase in response to a variety of stresses including biotic stress, indicating that POD activities involved in defense against attack by pathogens [29]. In the present study, we reported the quick response of POD when potato inoculated with *R. solanacearum*, indicating a possible role of the enzyme during pathogen infection and host-resistance. The fact that POD activity was higher in resistant and moderately resistant cultivars than in susceptible cultivar indicates that POD might have played a specific role in triggering the development of host resistance. Our findings are similar with the results of the earlier studies of Chittoor *et al.* [30] on rice in case of *Xanthomonas oryzae* pv. *oryzae* and Vanitha & Umesha [31] on tomato in case of *R. solanacearum* path-systems. In the relatively highly resistant cultivars, the activity of POD was higher when compared to the un-inoculated plants and also with the susceptible and highly susceptible cultivars.

Under stress conditions, the enhanced POD activity in the intercellular spaces, stimulating cell wall stiffening, probably reduces cell growth which might represent a mechanical adaption to adverse conditions [32]. Meanwhile, an enhanced POD activity was demonstrated during various pathogenesis systems [29].

Polyphenol oxidase, a copper containing enzyme, oxidizes phenolics to highly toxic quinines and is involved in the terminal oxidation of diseased plant tissue and is attributed for its role in disease resistance [33].

Concerning PPO activity, in resistant cultivar (Nicola) the peak of activity of PPO was the highest (0.445 $min^{-1} \cdot g^{-1}$) early at 24 h, after inoculation with *R. solanacearum*. However, in susceptible cultivar (Spunta), maximum activity was lower (0.364 $min^{-1} \cdot g^{-1}$) and was recognized as later at 48 h after inoculation. Meanwhile, cv. Kara showed intermediate values. PPO is a copper-containing enzyme known to be involved in resistance against *R. solanacearum* in resistant tomato cultivars [34]. Besides, overexpression of PPO in transgenic tomato plants enhanced their resistance to *Pseudomonas syringae*, another bacterial pathogen of tomato [35]. Also, there are reports that PPO activities increased in resistant tomato cultivars more than those in susceptible and highly susceptible cultivars after inoculation with *Xanthomonas axonopodis* pv. *vesicatoria* [36]. Increased PPO activity contributed to disease resistance due to its property to oxidize phenolic compounds to more toxic quinines which badly affect pathogenic micro-organisms [37].

On the contrary, CAT activity, in the highly susceptible cv. Spunta, was always higher than its level in both, the resistant cultivar (Nicola) and moderately resistant one (Kara). CAT is the key H_2O_2 detoxifying enzyme in plant which keeps the balance of the active oxygen species (AOS), such as H_2O_2 level during plant defense. H_2O_2 is associated with hypersensitive response (HR) during systemic acquired resistance. Higher concentrations of H_2O_2 in resistant than in susceptible tomato cultivars have been reported in tomato-*Ralstonia* interactions [7]. Another study reported that the restriction of *R. solanacearum* growth could be due to the antimicrobial activity of H_2O_2, which is strongly increased around bacterial cells. Consequently, the high CAT susceptible cultivars restrict H_2O_2 action against *R. solanacaerum*.

To investigate the association of gene expression of potato cultivars and resistance to *R. solanacaerum*. The real-time qPCR technique was used to evaluate changes in the transcription levels of three genes peroxidase, polyphenol oxidase and catalase. At zero time, the relatively highly resistant potato cultivar, Nicola, showed the highest values of gene expression for both POD and PPO. However, the susceptible potato cultivars, Spunta, showed the lowest values, while, the intermediate resistance potato cultivars, Kara, exhibited intermediate values.

On the contrary, the CAT, gene expression was the highest in the susceptible potato, cv. Spunta, and was the lowest in the relatively highly resistant potato, cv. Nicola, while, was of intermediate values in cv. Kara (intermediate resistance).

Meanwhile, gene expression reached its peak (4.2) for POD in cv. Nicola after 12 hours while, it was at 48 hours for Spunta and at low peak of 2.0. Intermediate values were recognized for cv. Kara. Similar trend was revealed for PPO gene expression.

However, an opposite trend was revealed for CAT where cv. Nicola (relatively highly resistant) showed the lowest values while, cv. Spunta (susceptible) showed the highest values. Also, cv. Kara showed intermediate values. These results were in harmony with findings of Navodit & Prabir [38]. Peroxidase gene expression in tomato was shown to be induced differentially in resistant and susceptible lines by elicitors of the fungal pathogen *Verticillium albo-atrum* [39].

Also, catalase expression and activity change during plant-pathogen interactions and a decrease in plant catalase activity occurs in resistant plants in response to attempted infection by viruses [40]. It is thought that this allows H_2O_2 to accumulate, resulting in antimicrobial activity through strengthening of the plant cell wall, activation of defense genes, hypersensitive cell death and a subsequent halt to pathogen infection [41]. In contrast,

in susceptible hosts increases in host catalase activity have been observed by Havelda & Maule, [42] Kuzniak & Sklodowska, [43] and Pompe-Novaka *et al*., [44], exogenously applied catalase can result in decreased hypersensitive cell death [45] and increased penetration by pathogens of normally resistant hosts (Borden & Higgins, [46] and Abel *et al*., [47].

5. Conclusions

Finally, Peroxidase (POD), Polyphenol oxidase (PPO) and Catalase (CAT) gene expressions and enzymes activities can be used as molecular and biochemical markers to reveal the resistance or susceptibility nature of potato cultivars against bacterial wilt disease of potato caused by *R. solanacaerum*.

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Genetic Variation among Fragmented Populations of *Atriplex halimus* L. Using Start Codon Targeted (SCoT) and ITS1-5.8S-ITS2 Region Markers

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Abstract

Two forms of *A. halimus* shrubs: erect habit (*A. halimus*) and bushy habit shrub (*A. schweinfurthii*) are used naturally isolated by a considerable distance from each other and occupy the same area. To explore the effect of natural isolation on the genetic basis of the two forms, Start Codon Targeted (SCoT) and the phylogenetic relationships of *A. halimus* by sequencing ITS1-5.8S-ITS2 regions of the ribosomal DNA are used. Significant isolation-by-distance relationship was found (r = 0.62, *P* = 0.001). Soil factors did not influence molecular variations. The natural isolation of *A. halimus* habitats restricts gene flow among the populations and the observed high within-population genetic diversity (74.19%) in this species is best explained by its outcrossing behaviour, long-lived individuals and overlapping generations. The UPGMA analysis of the SCoT results showed that all the studied populations were divided into two discrete genetic groups with significant separation of the two forms in Burg El-Arab area (Populations 1 and 2) and insignificant separation between two forms in El-Hammam area (population 5 and 6). The sequencing of the ITS1-5.8S-ITS2 rDNA regions also showed the insignificant separation of the two *A. halimus* forms. We conclude that gene flow depending on habitat fragmentation was the main factor affecting the population genetic differentiation. We suggest that the two forms do not merit specific rank in presence of interference between the two forms and absence of a breeding barrier fail to separate the different populations when they become sympatric.

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Keywords

Atriplex halimus*, SCoT, ITS1-5.8S-ITS2 rDNA Regions, *Atriplex schweinfurthii

1. Introduction

Atriplex halimus L. (Chenopodiaceae), a C4 perennial shrub, highly outbreeding, is found in semi-arid and arid environments. This species, particularly well adapted to arid and salt-affected areas, is valued as livestock forage in low rainfall Mediterranean areas [1]-[4] and is considered as a promising forage plant for large-scale plantings [5]. *A. halimus* is able to accumulate heavy metals in plant tissue without displaying symptoms of toxicity during growth [6] [7]. In Egypt only one species viz., *halimus* with one variety viz; *schweinfurthii* was mentioned by Täckholm [8], while Boulos [9] mentioned only one species *A. halimus*. Recently the taxonomic revision of *Atriplex halimus* L. in Egypt revealed the presence of two subspecies namely: subsp. *halimus* L. and subsp. *schweinfurthii* Boiss [10]. This treatment was based on the pollen diversity. Both subspecies of *Atriplex halimus* L., are extremely heterogenous in terms of their morphology, ecology and productivity [11]. Amer & Abdo [10] noted a high degree of morphological variations in 52 populations of *A. halimus* (subsp. *halimus* and subsp. *shweinfurthii*), which was supported by the earlier works cited by Le Houérou [1]. Moreover, the two subspecies were confirmed by Walker *et al.* [12], who divided *A. halimus* into two groups diploid (2×), named *A. halimus* subsp. *halimus* and tetraploid (4×), named *A. halimus* L. subsp. *Schweinfurthii* Bioss. Hcini *et al.* [13] mentioned that *A. halimus* is dominant in the semi-arid and sub-humid areas, while *A. schweinfurthii* is more common in arid areas. However, the existence of intermediate morphotypes complicates the designation of plants as one or the other subspecies [14] [15]. Determination of DNA content showed that certain populations with morphologies intermediate between those considered typical of subsp. *halimus* and *schweinfurthii* were tetraploid [14].

In recent years, changing environmental conditions and the resulting threats to the survival of existing populations have resulted in increased interest to study how genetic variation is maintained in natural populations [16]. Among the environmental factors, the habitat variability and gene flow by seed or pollen dispersal can affect genetic diversity [17] [18]. Habitat variation often generates ecological barriers against gene flow and thus enhances genetic differentiation between local populations [16] [19]. In the past, *A. halimus* occupied a large area of distribution in the Mediterranean area of Egypt. Currently, its habitat has been severely fragmented; thus its genetic resources is required to be catalogued.

The first study of genetic variability of *A. halimus* was made by Haddioui & Baaziz [4] analysing the isoenzyme polymorphisms of nine populations from several locations in Morocco. Ortíz-Dorda *et al.* [20] extended the study to include ten countries in the Mediterranean basin using RAPD and ITS markers marker. Bouda *et al.* [21] applied RAPD analysis and AMOVA technique to determine the pattern and extent of genetic variations within and between natural populations of *A. halimus* from Morocco. Morphological, physiological and isozyme-based studies showed high genetic diversity in Moroccan populations of *A. halimus* [4] [22] [23].

Recently, new marker techniques have been developed depending on gene-targeted markers. A novel marker system called Start Codon Targeted (SCoT) Polymorphism [24] was developed based on the short conserved region flanking the ATG start codon in plant genes. SCoT markers are generally reproducible, and are similar to RAPD and ISSR because the same single primer is used as the forward and reverse primer [24] [25]. It is suggested that primer length and annealing temperature are not the factors determining reproducibility. These dominant markers could be used for genetic analysis, quantitative trait loci (QTL) mapping and segregation analysis [24]. These markers have been successfully used to study diversity in peanut, grape, potato and *Dendrobium nobile* [26]-[29].

The present study used two forms of *A. halimus* shrubs: erect habit (*A. halimus*) and bushy habit shrub (*A. schweinfurthii*). The two morphotypes are used naturally isolated by a considerable distance from each other and occupy the same area to explore the effect of natural isolation on the genetic basis of the two forms using Start Codon Targeted (SCoT) and the phylogenetic relationships of *A. halimus*by sequencing ITS1-5.8S-ITS2 regions of the ribosomal DNA. Besides, the previous results obtained from both isozymes and RAPDs markers collected from the Mediterranean basin populations were compared with the results of the present markers.

2. Material and Methods

2.1. Plant Populations

A total of 18 accessions of *A. halimus* were collected from six populations growing naturally in the Western Mediterranean Desert, Egypt (**Table 1**). Samples were collected from six populations. Three populations in the Mediterranean coastal land: Two distinctive forms (erect and bushy habit) from Burg Al-Arab (40 Km from Alexandria) and one population from El-Ghabaniat (45 Km from Alexandria). The other three populations were collected from El-Hammam (60 Km from Alexandria) (**Figure 1**). Soil physical and chemical characteristics were analysed by Richards [30] and Klimer & Alexander [31]. Soil characteristics supporting the six study populations are shown in **Table 2**.

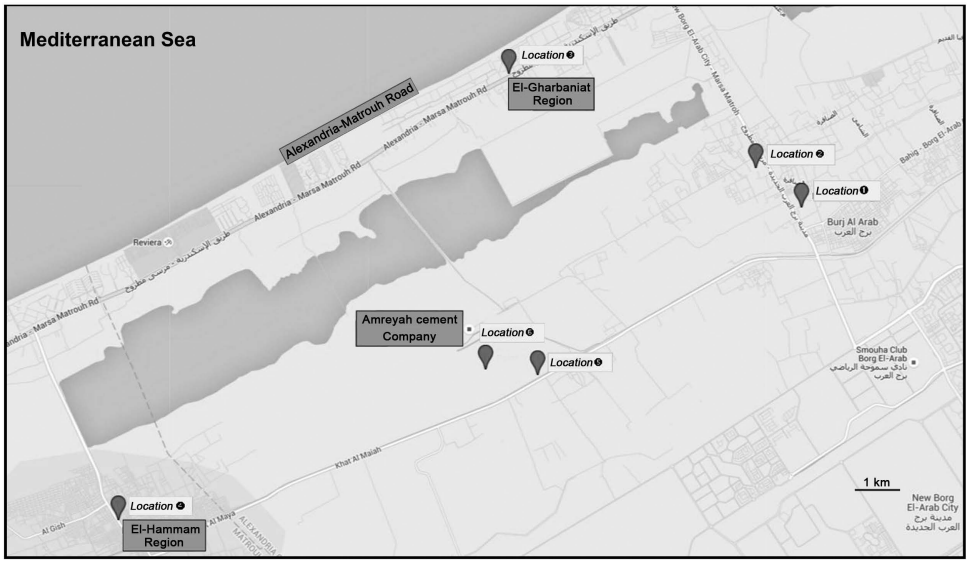

Figure 1. A map showing different locations from which the studied accessions of *A. halimus* were collected.

Table 1. Collection localities, habitats, dates and sample size of the studied populations of *A. halimus*.

Location	Population number	Site description	Growth form	Sample size	Sample code	Sampling site longitude and latitude
Burg El-Arab	1	Inland typical salt marches (Ideal habitat)	Bushy habit	4	1 - 4	30˚905' and 29˚536'
	2	Edges of run-road-transported sand to this area	Erect habit	3	5 - 7	30˚909' and 29˚533'
El-Gharbaniat	3	North slope, costal rocky ridge	Erect habit	3	8 - 10	30˚929' and 29˚474'
	4	Road run, Sandy plain	Erect habit	2	11 - 12	30˚847' and 29˚386'
El-Hammam	5	Polluted area (Cement factory)	Bushy habit	3	13 - 15	30˚862' and 29˚478'
	6	Polluted area (Cement factory)	Erect habit	3	16 - 18	30˚874' and 29˚475'

Table 2. The soil characteristics supporting the six studied populations of *A. halimus.*

Soil factor	Locations			
	Burg El-Arab Pop.1 & 2	El-Gharbaniat Pop.3	El-Hammam Pop.4	El-Hammam Pop.5 & 6
pH	8.6	8	8.3	8.5
Conductivity mmhos/cm	125	55.8	9.1	71.3
Sand%	13	60	48	31
Silt%	37	18	23	51
Clay%	50	22	29	18
$CaCO_3$ (mg/L)	45.6	55.8	37	16.5

2.2. DNA Extraction and SCoT-PCR Amplification

DNA was extracted from 2 g of young leaf tissue using DNA Plant Minipreps Kit (Bio Basic INC, Canada.), following the manufacturer instructions. All the PCR reactions were carried out in 25 µL volumes containing 50 ng of template DNA, 12.5 µl of PCR master mix buffer (2×) (Thermo, USA), and 20 pmol for each primer (**Table 3**). The reaction programs were set at 95˚C for 3 min, followed by 35 cycles of 30s at 95˚C, 30s at annealing 30˚C and 1 min at 72˚C, with a final extension at 72˚C for 10 min in a thermal cycler MyGene MG96+ (LongGene , USA). After completion of the amplification, 2.5 µL of 10× blue dye was added to the samples, and the amplified DNA was analyzed on 1.5% agarose gel in 1× TAE buffer at 65 - 70 V for 3 - 4 h. The amplified products were stained with ethidium bromide and photographed under UV illumination. Scoring for the presence or absence of DNA fragments was aided by the use of a 1 kb DNA ladder.

2.3. ITS1-5.8S-ITS2 rDNA Gene

For the phylogenetic the internal transcribed spacer ITS1 and ITS2 regions and the 5.8*S ribosomal DNA* (rDNA) regions were amplified by using universal primers ITS1 (5'-TCCGTA GGTGAACCTTGCGG-3') and ITS-4 (5'-TCCTCC GCTTATTGATATGC-3') [32]. Amplifications were performed in 25 µL volumes containing 50 ng of template DNA, 12.5 µl of PCR master mix buffer (2×) (Thermo, USA), and 20 pmol for each primer. PCR cycles were as follows: initial denaturation for 5 min at 95˚C followed by 34 cycles of 1 min at 95˚C, annealing at 58˚C for 1 min and extension at 72˚C for 1 min, and a final elongation step of 10 min at 72˚C. PCR products were then separated electrophoretically on agarose gel using 2% (w/v) agarose in 0.5× TBE buffer. The gel was stained with ethidium bromide. Then the PCR products were purified by EZ-10 Spin Column DNA Gel Extraction Kit (Bio Basic, Canada), following the manufacturer's instruction. Cycle sequencing was performed using T7SequencingTM kit (Pharmacia, Biotech, USA) and model 310 automated sequencer (Applied Biosystems, Foster City, CA, USA). This analysis was performed using a single plant from the six studied populations (**Table 1**). The DNA sequences were determined by Macrogene Company (Korea). The DNA sequences are deposited in GenBank http://www.ncbi.nlm.nih.gov under the accession numbers: KU555430 to KU555435.

2.4. Statistical Analysis

SCoT bands were binary scored; presence (1) or absence (0) characters to assemble the matrix of the SCoT phenotypes. Then, the indices of genetic diversity were calculated using POPGENE 3.2 Software [33] on the basis of gene frequencies. Hierarchical analysis of molecular variance (AMOVA) within and among populations was done using allele frequencies with ARLEQUIN V. 3.11 [34]. The Mantel test was applied using XLSTATARTVIS Software to test the significance of the association between the genetic distance and geographic distance matrices. The Pearson correlation between the genetic diversity index within population and ecological factors was analyzed using the SPSS 17.0 Software.

Table 3. Data of SCoT primers used in the present study and the extent of polymorphism**.**

Primer name	Primer sequence (5'-3')	Total no. of bands	No. of polymorphic bands	No. of monomorphic bands	% of Polymorphism (P)
S4	CAACAATGGCTACCACCT	18	17	1	94.44
S6	CAACAATGGCTACCACGC	21	20	1	95.23
S7	CAACAATGGCTACCACGG	19	18	1	94.73
S9	CAACAATGGCTACCACGT	13	13	0	100
S10	CAACAATGGCTACCAGCC	19	19	0	100
S12	ACGACATGGCGACCAACG	15	13	2	86.66
S17	ACCATGGCTACCACCGAG	16	16	0	100
S32	CCATGGCTACCACCGCAC	19	19	0	100
S34	ACCATGGCTACCACCGCA	14	14	0	100
S36	CATGGCTACCACCCGCCC	23	23	0	100
	Total	177	172	5	
	Average	17.7	17.2	0.5	97.10

Genetic similarity was calculated on the basis of genetic distance coefficient using the NTSYS-pc program [35]. The similarity matrix was subjected to cluster analysis by Unweighted Pair Group Method with Arithmetic averages (UPGMA) [36].

Pairwise and multiple DNA sequence alignment were carried out using CLUSTAL W version 1.81 (http://seqtool.sdsc.edu/CGI/BW.cgi; Thompson *et al*. [37]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 [38]. Neighbour-Joining (NJ) algorithm was employed to construct phylogenetic relationships. Using NJ, the evolutionary distances were computed using the Maximum Composite Likelihood model and reliability of the branches was assessed by bootstrapping the data with 1000 replicates. Phylogenetic comparisons included *A. canescens* (AM420672), *A. glauca* (AY873928) and *A. prostrate* (HM005857) as out-groups.

3. Results

3.1. SCoT-PCR

For analysis of variability of *A. halimus*, 10 primers were used for studying the SCoT banding patterns across the entire samples. A total of 177 amplification products were scored of which 172 were polymorphic, exhibiting 97.10% polymorphism. The amplification products using 10 primers ranged from 86.66% to 100% in producing polymorphic bands (**Table 3**). The primers S9, S10, S17, S32, S34 and S36 exhibited the highest level of polymorphism with the percentage of polymorphic bands to be 100% for all these primers (**Table 3** and **Figure 2**).

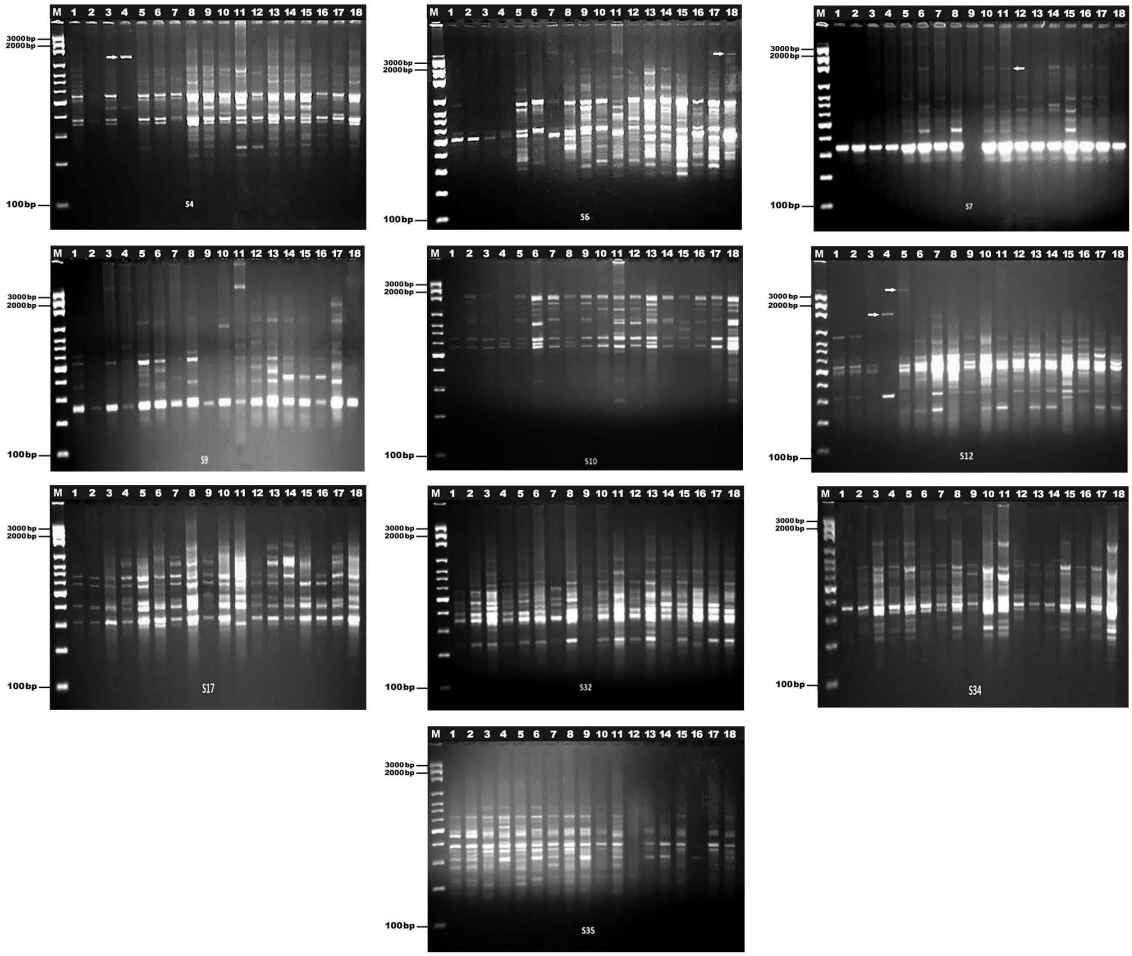

Figure 2. SCoT profiles generated from genomic DNA of *A. halimus* populations with primers; M: DNA marker, lanes 1-4: Burg El-Arab (1), lanes 4-7: Burg El-Arab(2), lanes 8-10: El-Gharbaniat, lanes 11-12: El-Hammam (1), lanes 13-15: El-Hammam (2) and lanes: 16-18 El-Hammam (3), arrows = monomorphic bands.

3.2. Genetic Diversity in Plant Populations

Ten primers (**Table 3**) generated a total of 177 SCoT bands (loci), 17.7 bands per primer on average. The number of amplification products per primer varied from 13 to 23, and these primers produced fragments ranging from 200 to 3000 bp in size (**Figure 2**). The observed number of alleles (N_a) and effective number of alleles (N_e) ranged between 1.355 - 1.553 and 1.284 - 1.412, respectively. Similarly, Nei's gene diversity (h) and Shannon's Information index (I) ranged between 0.226 - 0.327 with overall diversity of 0.28 and 0.13 - 0.24 with an average value of 0.43, respectively. The percentage of polymorphic loci (*PPL*) was estimated in the range of 35.51% to 55.37%. The gene flow value and the diversity among populations were found to be 0.362 and 0.881, respectively (**Table 4**). It was found that the genetic variation in population 6 growing in El-Hammam area reached the highest value (I = 0.327) and population 2 growing in road-run of Burg El-Arab attained the lowest value (I = 0.226).

3.3. Genetic Structure within and among Populations

The estimate of genetic structure of populations is significantly different from zero ($P < 0.0001$). Analysis of AMOVA showed that genetic variation (74.19%) was observed within the populations, whereas the variance among populations was 25.81% (**Table 5**), which was in accordance with the *Gst* (36.2%). The estimate of gene

Table 4. Genetic diversity and differentiation parameters for six natural populations of *A. halimus* in Northwest Egypt.

Populations	Sample size	Polymorphic loci (N_p)	Percentage population level (*PPL* %)	Observed number of alleles (N_a)	Number of effective alleles (N_e)	Shannon's Index of diversity (I)	Nei's gene diversity (h)	$h_S \pm$ SD	$h_t \pm$ SD	G_{ST}	*Nm*
Pop1	4	98	55.37	1.553	1.368	0.323	0.218				
Pop2	3	63	35.59	1.355	1.284	0.226	0.158				
Pop3	3	82	46.33	1.463	1.371	0.294	0.205				
Pop4	2	73	41.24	1.412	1.412	0.285	0.206				
Pop5	3	67	37.85	1.378	1.325	0.240	0.168				
Pop6	3	91	51.41	1.514	1.411	0.327	0.228				
Population level	3	79	44.63	1.445	1.361	0.282	0.197				
Species level	18	169	95.48	1.954	1.594	0.512	0.355	0.342 ± 0.022	0.218 ± 0.108	0.362	0.881

N_p = number of polymorphic loci; *PPL*% = percentage of polymorphic loci; Na = observed number of alleles; N_e = effective number of alleles; I = Shannon's information index; h = Nei's gene diversity; h_S = Gene diversity within population; h_t = total gene diversity; SD = standard deviation; G_{ST} = diversity among populations; *Nm* = gene flow 0.25 $(1 - G_{ST})/G_{ST}$.

Table 5. Hierarchical analysis of molecular variance (AMOVA) within and among natural populations of *A. halimus*in Northwest Egypt. The *P* values are the probabilities of having a greater variance component than the observed values by chance alone and are based on 1023 random permutations of the data matrix.

Source of variation	df	Sum of squares	Variance components	Percentage variation	*P*
Among populations	5	253.028	8.647 Va	25.81	***
Within populations	12	298.250	24.854 Vb	74.19	***
Total	17	551.278			
Fixation Index (*Fst*)		0.25813			***
Among regions	2	149.736	7.081	20.09	**
Among populations Within regions	3	103.292	3.310	9.39	**
Within populations	12	298.250	24.854	70.52	**

***$P < 0.0001$, **$P < 0.005$.

flow N_m based on *Gst* was 0.880 (**Table 4**). This result is equivalent to Fixation Indices (*Fst*) 0.258 $P < 0.0001$ calculated with the Arlequin program, which implies a low degree of differentiation among populations.

To identify the source of the highest genetic variation, AMOVA analysis was also performed among groups; Group (1) includes the erect form and Group (2) includes the Chameophyte form populations. Genetic variation among the two groups was found to be low, that reaching 1.9% of the total variation. The amount of genetic variation among regions and among populations was 20.09% and 9.39%, respectively, with the reminder (70.52%) occurring within populations, suggesting that there is no significant genetic differentiation of populations.

The Mantel test showed a significant correlation between genetic distance and geographic distance (r = 0.673, $P = 0.025$).

3.4. Cluster Analysis

The neighbour-joining dendrogram based on the genetic distance between populations revealed a similar pattern: the genetic distances among the populations showed a spatial pattern that corresponded to their geographic locations (**Figure 3**). Moreover, all six populations were clustered into two geographical groups. Clear geographical pattern of genetic diversity was identified between Burg El-Arab populations (1 and 2) and the rest of the studied populations.

Based on UPGMA clustering algorithm generated from the obtained SCoT dataset, the populations were grouped into two distinct groups (**Figure 3**). The four individuals (*A. halimus*) of population one of Burg El-Arab were included in one group and the other five populations were included in another group. Population 1 of Burg El-Arab is the most differentiated from the rest of the studied populations, even from population 2 which is the most geographically closely located population (4 Km apart). Calculated the cophenetic correlation coefficient (0.72) shows a relatively good fit of the data are obtained with the dendrogram. In the meantime, all populations of *A. halimus* could be discriminated from each other, except that of population 4, one individual (18) of population 5 from El-Hammam area and one individual (9) of population 3 from El-Gharbaniat area. The hierarchical AMOVA analysis indicating that the among-regions genetic variation was weak (25.81%) which agrees with the dendrogram which shows that most individuals from a given population tend to cluster together and are, therefore, more genetically similar than individuals from different populations.

3.5. Correlation between Genetic Diversity and Soil Factors

The correlation analysis indicated that the genetic diversity indices of different populations showed insignificant ($P > 0.05$) correlations between the genetic diversity indexes and the soil factors. This indicates that the soil factors had no effect on population's structure and there were no local adaptation of the studied populations.

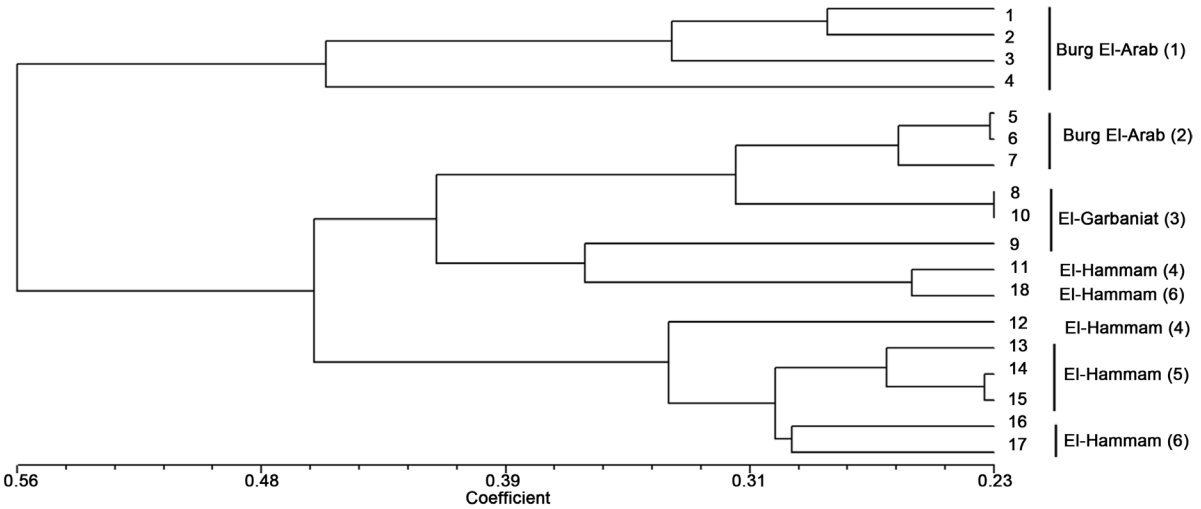

Figure 3. Dendrogram based on genetic distances and Jaccard coefficient computed from SCoT data using algorithm of Unweighted Pair Group Method with Arithmetic Averages (UPGMA) in the six *A. halimus* studied populations.

3.6. ITS1-5.8S-ITS2 rDNA Gene

The ITS1-5.8S-ITS2 rDNA gene was successfully amplified from the six individuals of the six populations (**Figure 4**). DNA sequencing results revealed an approximately 1200 bp fragment of the ITS1-5.8S-ITS2 rDNA. The edited nucleotide sequences of the six individuals ITS1-5.8S-ITS2 gene were subjected to sequence analysis using CLUSTAL W (1.81) multiple sequence alignment (**Appendix**). Comparative nucleotide sequence alignment revealed that the ITS1-5.8S-ITS2 rDNA gene was homologous between the studied individuals. The phylogenetic analysis based on ITS1-5.8S-ITS2 regions sequences presented in **Figure 5**. All population grouped together except population 4 from El-Hammam, which clearly separated in a node supported by a bootstrap value of 98. Using *A. canescens*, *A. glauca* and *A. prostrata* as outgroups in the phylogenetic analysis, *A. canescens*, *A. glauca* are the species closest to *A. halimus* from this group, while *A. prostrata* is the most distant.

4. Discussion

The study of genetic diversity of *Atriplex halimus* from diverse environment of the Mediterranean Basin showed a very high intra-populational diversity [4] [20] [21]. These authors found that two genetics groups of *A. halimus* can be distinguished and the genetic diversity of their collection was explained mainly by the within population component. The present work studied the two *A. halimus* forms (erect and bushy habit) occupying the same area and naturally isolated by a considerable distance from each other in Egypt with two types of genetic markers; SCoT and ITS.

Results of SCoT analysis showed differences in the genetic diversity among populations of *A. halimus* from different locations. The total gene diversity (hT) and the genetic parameters (*PPL*%, *I*, *h*, N_a, N_e) at population level were lower in Population 2 from Burg El-Arab and population 5 from El-Hammam than in the other

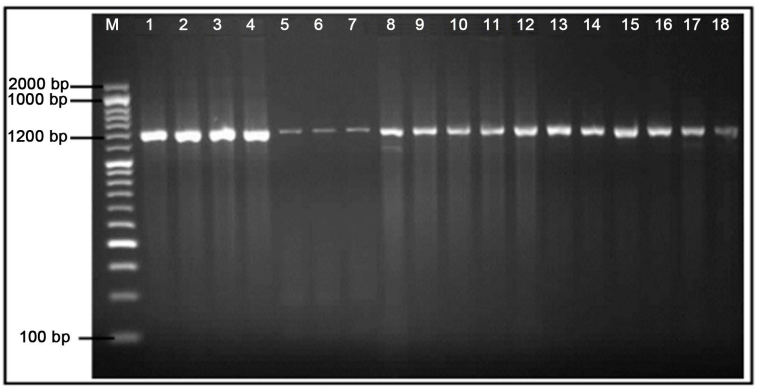

Figure 4. Amplified product of ribosomal DNA gene (approx. 1200 bp) of six *A. halimus* populations; M: DNA marker, lanes 1-4: Burg El-Arab (1), lanes 4-7: Burg El-Arab (2), lanes 8-10: El-Gharbaniat, lanes 11-12: El-Hammam (1), lanes 13-15: El-Hammam (2) and lanes 16-18: El-Hammam (3).

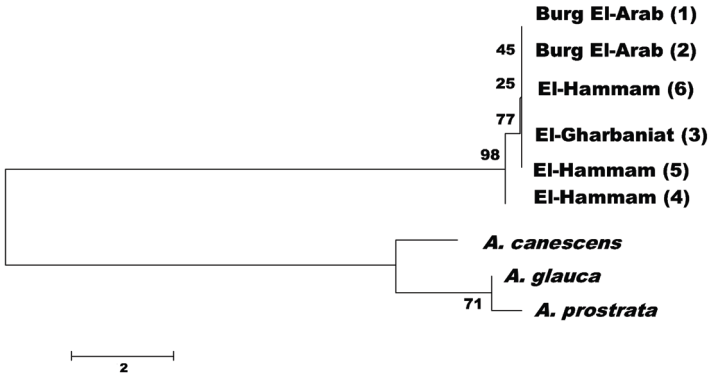

Figure 5. Phylogenetic tree calculated by Neighbor-Joining (NJ) method with 1000 bootstrap replications based on the nucleotide sequences of ribosomal DNA gene in the six *A. halimus* populations.

populations. In the present study, the mean value of Nei's gene diversity index (*h*) was 0.197, near to the minimum h value (0.174 - 0.328) of nine out-crossing plants summarized by Schoen and Brown [39]. The estimate of gene flow Nm based on *Gst* was 0.888, which indicates that gene flow among populations was low (**Table 5**). This result was equivalent to fixation indices (*Fst* = 0.258, *P* = 0.001) calculated with Arlequin program, which implies nearly moderate degree of differentiation among population according to Wright [40] (1965) (*Fst* > 0.15 and <0.25). This result agrees with Bouda *et al*. [41] who suggested that *A. halimus* populations from Morocco are very largely differentiated based on RAPD data (*Fst* = 0.334, *P* = 0.000). The estimate of *Gst* (0.362) at the species is consistent with the characteristics of outbreeding species, according to studies based on dominant markers (RAPD), which lead to the conclusion that population differentiation is lower in outbreeding than in inbreeding species (*Gst* < 0.23 and >0.5, respectively; Hamrick & Godt [42]; Nybom & Bartish [43]. The present study established that *A. halimus* in Egypt had a considerable amount of genetic variations at SCoT loci which are not correlated with soil factors. These results suggested that SCoT diversity is non-adaptive by natural selection and does not influence by soil factors and their interactions.

The analysis by AMOVA implied that 1/4 of genetic variation occurred among population and most of the variation (61.9%) settled within the populations. Our results are compatible with the pattern of species that are primarily outcrossing and long lived-wind pollinated shrub, which retain most of their genetic variability within populations [43]-[45]. The level of population differentiation 25.81% obtained in this study was lower than that found among 51 *A. halimus* populations (29.18%) from ten countries in the Mediterranean basin and 12 populations (33.43%) from Morocco, analysed by RAPDs [20]-[41]. However, Ortíz-Dorda *et al*. [20] studied six populations from Egypt (Matrouh area) and they concluded that these populations had the lowest value of genetic diversity among the populations studied and they all were clustered in one group.

Outcrossing species usually have a high within-population diversity and low population differentiation, whereas selfing species often have low within population diversity and high differentiation among populations [44] [46]-[49]. The genetic structure of plant populations is also influenced by the long-term evolutionary and ecological history of the species, which would include shifts in distribution, habitat fragmentation and population isolation [50]. Wu *et al*. [51] proposed that Tacca originated from the southern marginal area of the Palaearctic continent when Pangaea expanded to the Pacific Ocean for the first time. Later, this genus became differentiated in a succession of nearby environments.

The results (**Table 2**) indicate that 20.09% of the total variation is between regions (*P* < 0.001). The variation between regions is near to the variation among populations, which coincide with the isolation by distance (Mantel test r = 0.673, *P* = 0.025). Populations within regions accounts for 9% of the total variation and the variation within populations represent 74.19% of the total variations. This indicates that, there is a significant structuring and separation of populations.

The low estimates of gene flow (N_m = 0.881) among wind-pollinated *A. halimus* populations, correspond well with the geographic isolation of the populations, in which genetic differentiation among populations appears to be highly correlated with geographic distance between populations (r = 0.673, *P* = 0.025), although the detected geographical effect might be associated with differences between the type of isolation between populations (resort facilities, sand transport etc.). For example, Population 1 (bushy habit) and population 2 (erect habit) from Burg El-Arab are geographically close, but separated with a high genetic distance. The two locations are isolated by buildings of resorts, contrary to population 5 (bushy habit) and population 6 (erect habit) which are geographically and genetically close. The N_m would be low, with increased spatial isolation of small populations caused by habitat fragmentation [52]. Furthermore Limited gene flow due to habitat isolation could lead to a stronger genetic differentiation among populations compared with plants found in less-isolated habitats [49]. Our results from the cluster analysis based on the coefficients of genetic distances showed evident differentiation among the six *A. halimus* populations, where the genetic distance between two forms from Burg El-Arab is higher than the isolation by distance and a significant geographic sub-structure was confirmed by Mantel test. Genetic divergence was particularly low among populations of *A. halimus*, which may be explained by restricted gene flow. Similarly, significant correlation between genetic and geographic distances has been found in outcrossing species *Prunus mahaleb* [53] and long-lived perennial species *Quercus petrae* [54]. In contrast, no correspondence between geographic and genetic distances has been found in 12 populations from different locations in Moracco, long-lived, perennial species *Haloxylon ammodendron* [55] thus, isolation by distance has played an important role in establishing the genetic structure of this species. The significant correlation between the genetic and the geographic distances assumed that migration or gene flow rather than genetic drift or natural

selection was the main factors affecting the population genetic differentiation [56].

Another factor may play an important role in the evolution of *A. halimus*; morphogenesis of reproductive structures is remarkably plastic in *A. halimus*, since flowers of both architectural patterns might be both male, female or hermaphroditic and their distribution along the reproductive axes as well as their relative occurrence were dependent on environmental conditions. *A. halimus* is mainly considered a monoecious species which is occasionally dioecious. However, some authors have found individuals that present unisexual and hermaphrodite flowers so this species could be polygamous or, more precisely, trimonoecious [57]. Amer & Abdo [10] established that there was a relation between polyploidy and pollen morphological variations in *Atriplex halimus* L., in case of erect habit (*A. halimus*) the pollen sterility increases with the increase of salinity and aridity. *A. schweinfurthii* showed more adaptive character to arid and saline soil, and the pollen fertility increased under these harsh environmental conditions.

The present study used two forms of *A. halimus* shrubs; erect habit (*A. halimus*) and bushy habit shrub (*A. schweinfurthii*). The two morphotypes are used naturally isolated by a considerable distance from each other and occupying the same area to explore the effect of natural isolation on the genetic basis of the two forms using Start Codon Targeted (SCoT) and the phylogenetic relationships of *A. halimus* by sequencing ITS1-5.8S-ITS2 regions of the ribosomal DNA. Besides, we compared the previous results obtained with isozymes markers and RAPDs from Mediterranean basin to the present marker. According to previous reports, *A. halimus* includes two quite different groups in terms of habitat and morphology; subspecies *halimus* and subspecies *schweinfurthii*. However, these subspecies described by Le Houérou are not accepted taxonomical units. Ortíz-Dorda [20] distinguished two genetic groups are rather coincident with the characters described by Le Houérou [1]-[11]. The connection between the two genetic groups described by Ortíz-Dorda [20] and the two subspecies described by Le Houérou [1] is uncertain particularly in our study which revealed the uncertain genetic separation between the two types of morphologies of *A. halimus* using Start Codon Targeted (SCoT) marker and the sequencing of ITS1-5.8S-ITS2 regions. According to Ortíz-Dorda, [20] all studied individuals from Egypt belong to subspecies *schweinfurthii* (Bushy habit) contrasting to our collection in which the majority characterized by erect habit (subspecies *halimus*). Moreover, it was found that *A. halimus* is dominant in the semi-arid, sub-humid areas (population 3) and also in arid areas of *A. schweinfurthii* habitat.

5. Conclusion

Habitat fragmentation through land use change can limit connectivity between populations and gene flow between *A. halimus* populations. There is interference between the two forms of *A. halimus* viz. *A. halimus* and *A. schweinfurthii*. We conclude that the two forms do not merit specific rank in presence of intermediate morphotypes between the two forms and absence of a breeding barrier.

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Appendix

The sequences of the fragment ribosomal DNA amplified by PCR of the six studied populations.

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El-Hammam     (6)     CCGGGGAATCGCTTCGCCTTGGCGGGGCGTCCTTCCCGGCACAATAACGAACCCCGGCGC
Burg El-Arab  (2)     CCGGGGAATCGCTTCGCCTTGGCGGGGCGTCCTTCCCGGCACAATAACGAACCCCGGCGC
El-Gharbaniat (3)     CCGGGGAATCGCTTCGCCTTGGCGGGGCGTCCTTCCCGGCACAATAACGAACCCCGGCGC
El-Hammam     (4)     CCGGGGAATCGCTTCGCCTTGGCGGGGCGTCCTTCCCGGCACAATAACGAACCCCGGCGC
El-Hammam     (5)     CCGGGGAATCGCTTCCCCTTGGCGGGGCGTCCTTCCCGGCATAATAACCAACCCCGGCGC
Burg El-Arab  (1)     CCGGGGAATCGCTTCGCCTTGGCGGGGCGTCCTTCCCGGCACAATAACGAACCCCGGCGC
                      ************** ************************ ****** **********

El-Hammam     (6)     GGTCTGCGCCAAGGAACATGAATACAAGCGTGCCCTTCTCCGACCGGTTCGCCGGTCGTG
Burg El-Arab  (2)     GGTCTGCGCCAAGGAACATGAATACAAGCGTGCCCTTCTCCGACCGGTTCGCCGGTCGTG
El-Gharbaniat (3)     GGTCTGCGCCAAGGAACATGAATACAAGCGTGCCCTTCTCCGACCGGTTCGCCGGTCGTG
El-Hammam     (4)     GGTCTGCGCCAAGGAACATGAATACAAGCGTGCCCTTCTCCGACCGGTTCGCCGGTCGTG
El-Hammam     (5)     GGTCTGCGCCAAGGAACATGAATACAAGCGTGCCCTTCTCCGACTGGTTCGCCGGTCGTG
Burg El-Arab  (1)     GGTCTGCGCCAAGGAACATGAATACAAGCGTGCCCTTCTCCGACCGGTTCGCCGGTCGTG
                      **************************************** ***************

El-Hammam     (6)     GACGTGGCACCAAGTCGTATATAACATTAAACGACTCTCGGCAACGGATATCTCGGCTCT
Burg El-Arab  (2)     GACGTGGCACCAAGTCGTATATAACATTAAACGACTCTCGGCAACGGATATCTCGGCTCT
El-Gharbaniat (3)     GACGTGGCACCAAGTCGTATATAACATTAAACGACTCTCGGCAACGGATATCTCGGCTCT
El-Hammam     (4)     GACGTGGCACCAAGTCGTATATAACATTAAACGACTCTCGGCAACGGATATCTCGGCTCT
El-Hammam     (5)     GACGTGGCACCAAGTCGTATATAACATTAAACGACTCTCGGCAACGGATATCTCGGCTCT
Burg El-Arab  (1)     GACGTGGCACCAAGTCGTATATAACATTAAACGACTCTCGGCAACGGATATCTCGGCTCT
                      ************************************************************

El-Hammam     (6)     CGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
Burg El-Arab  (2)     CGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
El-Gharbaniat (3)     CGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
El-Hammam     (4)     CGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
El-Hammam     (5)     CGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCACAATCCCGTGAA
Burg El-Arab  (1)     CGCATCGATGAAGAACGTAGCGAAATGCGATACTTGGTGTGAATTGCAGAATCCCGTGAA
                      ********************************************** ***********

El-Hammam     (6)     CCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCTTTAGGTTGAGGGCACGCCTGCCT
Burg El-Arab  (2)     CCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCTTTAGGTTGAGGGCACGCCTGCCT
El-Gharbaniat (3)     CCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCTTTAGGTTGAGGGCACGCCTGCCT
El-Hammam     (4)     CCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCTTTAGGTTGAGGGCACGCCTGCCT
El-Hammam     (5)     CCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCTTTAGGTTGAGGGCACGCCTGCCT
Burg El-Arab  (1)     CCATCGAGTCTTTGAACGCAAGTTGCGCCCGAAGCCTTTAGGTTGAGGGCACGCCTGCCT
                      ************************************************************

El-Hammam     (6)     GGGCGTCACGCATCGCGTCTCCCCCCACCACCCCGTGTGGATGGGGAGGAGGATGATGGC
Burg El-Arab  (2)     GGGCGTCACGCATCGCGTCTCCCCCCACCACCCCGTGTGGATGGGGAGGAGGATGATGGC
El-Gharbaniat (3)     GGGCGTCACGCATCGCGTCTCCCCCCACCACCCCGTGTGGATGGGGAGGAGGATGATGGC
El-Hammam     (4)     GGGCGTCACGCATCGCGTCTCCCCCCACCACCCCGTGTGGATGGGGAGGAGGATGATGGC
El-Hammam     (5)     GGGCGTCACGCATCGCGTCTCCCCCCACCACCCCGTGTGGATGGGGAGGAGGATGATGGC
Burg El-Arab  (1)     GGGCGTCACGCATCGCGTCTCCCCCCACCACCCCGTGTGGATGGGGAGGAGGATGATGGC
                      ************************************************************

El-Hammam     (6)     CTCCCATGCCTCACCGGGCGTGGATGGCCTAAATATGGAGCCCCCGGTTACGAAGTGCCG
Burg El-Arab  (2)     CTCCCATGCCTCACCGGGCGTGGATGGCCTAAATATGGAGCCCCCGGTTACGAAGTGCCG
El-Gharbaniat (3)     CTCCCATGCCTCACCGGGCGTGGATGGCCTAAATATGGAGCCCCCGGTTACGAAGTGCCG
El-Hammam     (4)     CTCCCATGCCTCACCGGGCGTGGATGGCCTAAATATGGAGCCCCCGGTTACGAAGTGCCG
El-Hammam     (5)     CTCCCATGCCTCACCGGGCGTGGATGGCCTAAATATGGAGCCCCCGGTTACNAANTGCCG
Burg El-Arab  (1)     CTCCCATGCCTCACCGGGCGTGGATGGCCTAAATATGGAGCCCCCGGTTACGAAGTGCCG
                      ************************************************** ** *****

El-Hammam     (6)     CGGCAATTGGTGGAATACAAGGCCACGCCTAGGATGAAACGGTAGTCGCGCACATCGTGG
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Burg El-Arab  (2)     CGGCAATTGGTGGAATACAAGGCCACGCCTAGGATGAAACGGTAGTCGCGCACATCGTGG
El-Gharbaniat (3)     CGGCAATTGGTGGAATACAAGGCCACGCCTAGGATGAAACGGTAGTCGCGCACATCGTGG
El-Hammam     (4)     CGGCAATTGGTGGAATACAAGGCCACGCCTANGATGAAACGGTAGTCGCGCACATCGTGG
El-Hammam     (5)     CGGNNNTTGGTGGAATACAAGGNCACCCCTANGATGAAACGGTANTCGCGCACATCNTGG
Burg El-Arab  (1)     CGGCAATTGGTGGAATACAAGGCCACGCCTAGGATGAAACGGTAGTCGCGCACATCGTGG
                      ***   *************** *** **** ************ *********** ***

El-Hammam     (6)     CTCTTGAGGACTCGCAGGACCCTTACTTGTTTGCCCCTAGGGGCGGCAAAACCGTTGCGA
Burg El-Arab  (2)     CTCTTGAGGACTCGCAGGACCCTTACTTGTTTGCCCCTAGGGGCGGCAAAACCGTTGCGA
El-Gharbaniat (3)     CTCTTGAGGACTCGCAGGACCCTTACTTGTTTGCCCCTAGGGGCGGCAAAACCGTTGCGA
El-Hammam     (4)     CTCTTGAGGACTCGCAGGACCCTTACTTGTTTGCCCCTAGGGGCGGCAAAACCGTTGCGA
El-Hammam     (5)     CTCTTGANGACTNNNNNGACCCTTACTTGTTTGCCCCTANGGGCGGCAAAACCGTTGCGA
Burg El-Arab  (1)     CTCTTGAGGACTCGCAGGACCCTTACTTGTTTGCCCCTAGGGGCGGCAAAACCGTTGCGA
                      ******* ****     ********************** ********************

El-Hammam     (6)     CCCC-AGGTCAGGCGGGGCTACCCGCTGAGTTTAAGCATAT
Burg El-Arab  (2)     CCCC-AGGTCAGGCGGGGCTACCCGCTGANTTTAAGCATAT
El-Gharbaniat (3)     CCCC-AGGTCAGGCGGGGCTACCCGCTGAGTTTAAGCATAT
El-Hammam     (4)     CCCC-AGGTCAGGCGGGGCTACCCGCTGAGTTTAANCATAT
El-Hammam     (5)     CCCCCAGGTCAGGCGGNGCTACCCGCTGAGTTTAAGCATAT
Burg El-Arab  (1)     CCCC-AGGTCAGGCGGGGCTACCCGCTGAGTTTAAGCATAT
                      **** *********** ************ ***** *****
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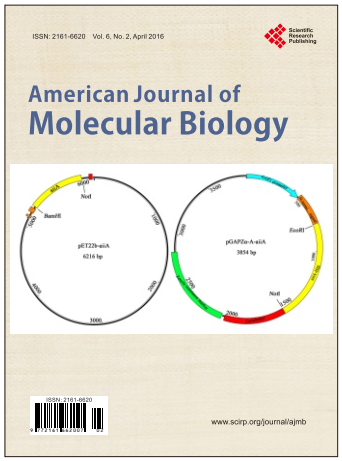

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