Role of Na\(^+\) manipulating genes in *Phragmites australis* adaptation to different habitats

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**Abstract**

*Phragmites australis* adapts to different habitats and tolerate drought and/or salt stress. In the present study, *P. australis* was collected from mesophytic, brackish water, sand dune, fresh water and salt marsh habitats. The leaf fresh weight was optimum in mesophytic habitats and it was negatively affected by stress found in other habitats. Water content followed the same trend for the fresh weight. The decrease in water content could be a tolerance strategy. Na\(^+\) content was higher in roots than in leaves for all habitats. In contrast, K\(^+\) content was higher in leaves than in roots. K\(^+\)/Na\(^+\) ratio was higher than unity in leaves for all habitats even within roots collected from salt marsh. Except for leaves collected from mesophytic habitats, K\(^+\)/Na\(^+\) ratio was less than one (1.1). NHX1 expression level was higher in roots than that in leaves suggesting that this plant may accumulate Na\(^+\) inside root vacuoles to restrict its movement to shoot. However, the transcript level of *SOS1* was not detected in any root collected from different habitats. Therefore, this reed may have other mechanisms for Na\(^+\) extrusion. However, *SOS1* transcript level was detected in leaves collected from different habitats suggesting that *SOS1* could play different roles in *P. australis* tolerance.

**Keywords**: brackish water; fresh water; mesophytes; reeds; salt marsh; sand dune; NHX1; SOS1

**Introduction**

Abiotic stress becomes major problems that threaten crop productivity. Up to 20% of irrigated lands are salt affected [1]. Many researches have been focused on studying the salt tolerance mechanism in glycophytes, which are naturally salt sensitive species. Therefore the attention should be shed towards the naturally adapted species to these harsh environments. Detailed understanding of the tolerance mechanisms that play roles in species acclimation, thrive and maintain growth in stress environments can lead to develop a proper breeding programs for crop tolerance [2].

Both salt and drought stresses decrease water potential and hence limit the available water to plants causing osmotic stress. In addition to osmotic stress, plants expose to salinity suffer from ionic stress due to salt accumulation [2-4].

Salt tolerance is a complicated mechanism and many genes regulate this process. Sodium compartmentalization and extrusion are two main mechanisms of salt tolerance mechanisms [3,5,6].
The vacuolar Na⁺/H⁺ antiporter (NHX1) has been reported to regulate the sequestration of sodium into vacuole to prevent the toxicity of cytosol and hence sodium will play as a cheap osmolytes [7,8]. Proton generated from the vacuolar H⁺-PPase and H⁺-ATPase achieved the movement of Na⁺ cross the tonoplast [9, 10]. Plasma membrane Na⁺/H⁺ antiporter (SOS1) controls the extrusion of sodium cross the plasma membrane back to the media [11]. Also this mechanism is energized by proton generated from plasma membrane H⁺-ATPase [12].

It is well known that dicotyledonous halophytes overcome the external, excess of salts by accumulating salts inside their vacuoles. Contrarily, monocotyledonous halophytes tend to exclude sodium to maintain high (more than unity) K⁺/Na⁺ ratio that seems to be crucial for salt tolerance [13, 14].

Phragmites australis (Cav.) Trin, ex Steud. (Poaceae) is an emergent grass and hydrophytic species whose typical habitats are fresh and brackish water of swamps, riversides, estuaries and coasts. Moreover, the plant can adapt to different habitats such as drought and salinity [15-17]. This study aims to investigate the contribution of sodium manipulating genes in the adaptation of P. australis to different habitats.

Materials and Methods

Plant materials

P. australis was collected from fresh and brackish water, sand dune, mesophytic and salt marsh habitats in the north eastern section of the Nile Delta. Plant samples were collected during July, 2013 at the midday to keep the unity of sampling. Samples were collected when P. australis reaches the maximum growth rate. To measure the growth parameters, samples were collected in plastic bags to prevent the loss of water. Others were collected in liquid nitrogen and then were kept at -80°C for the biochemical analyses. Soil and water samples were collected from the rhizosphere around the roots of the plants.

Soil and water analyses

Salinity was measured in terms of electric conductivity using YSI Model 33 S-C-T Meter. Soil extract (1:5) was prepared to measure Na⁺ and K⁺ by flamm photometer (PFP7, Jenway). Na⁺ and K⁺ concentration were determined from the standard curve in the range of 20-100 ppm for each ion. Three separate replicates were made.

Leaf fresh weight and water content

Fresh weight of leaves was measured and then leaves were dried on the oven at 60°C to calculate the water content. Water content was calculated as a percentage of the differences between the fresh and dry weights on fresh weight basis.

Leaf Na⁺ and K⁺ contents

Na⁺ and K⁺ have been extracted as described by [18]. About 100 mg of dried leaf or root tissue was firstly homogenized in 2 ml of boiled water. The mixture was kept in water bath at 100°C for 1 h. The residue was removed by centrifugation at 14000 rpm for 20 min. The diluted supernatant (1:10 with H₂O) was used to measure Na⁺ and K⁺ ions by flame photometry. The concentrations of Na⁺ and K⁺ ions were measured by using standard curves in the range of 20-100 ppm for Na⁺ and K⁺. Three replicates were used for each treatment.

Quantification of gene expression by semi-quantitative RT-PCR

Total RNA was extracted from about 50 mg of frozen leaves using TRI-reagent (Biovision, Egypt) according to the manufacture’s protocol. To prevent DNA contamination, the extracted RNA was treated with DNA-free kit (Thermo scientific, UK) for 30 min at 37°C. Then, poly A tail mRNA was isolated by reacting 10 µl of RNA with 2 µl of oligo dT₁₆ and 3 µl free RNase and DNase H₂O for 5 min at 70°C and then the reaction was terminated onto ice for at least 2 min. The reverse transcription was conducted by using MMLuV-reverse transcription kit according to the supplier’s recommendations (Thermo scientific, UK).

The primers for each gene were designed according to the sequence of P. australis found in the gene bank NCBI using primers 3 program. The primers used in amplifying NHXI, SOSI and 18S rRNA and the number of cycles are listed in table 1. PCR conditions were adjusted as follows initial denaturation at 94°C for 3 min, followed by 35-40 cycles according to each gene of denaturation at 94°C for 30 sec, annealing for 52°C for 30 sec and extension at 72°C for 50 sec. the PCRs were adjusted for the number of cycles.
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to ensure the intensity of bands within the linear phase of the amplification.

For each gene, three replicates were used from three different isolations of RNA; one of them is shown here. PCR products were resolved by electrophoresis in 1% agarose gels, stained with ethidium bromide and visualized by UV. The intensity of bands was estimated by using Image Studio “V 3.1” program. 18S rRNA was used as a reference in normalization.

Table 1. Primers used in quantification of genes by semi-quantitative RT-PCR. Number of cycles inside the linear phase is shown for each gene. N=No. of cycles.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHX1</td>
<td>GAAGGTTTGTGAATGATGC</td>
<td>CAATGTCCAATGCATCCATC</td>
<td>35</td>
</tr>
<tr>
<td>SOS1</td>
<td>TCCGTGAAAGCTGTTT</td>
<td>AAACAATTCCAAACGCAAGG</td>
<td>40</td>
</tr>
<tr>
<td>18S rRNA</td>
<td>CCACCCATAATCAAGAAAGAG</td>
<td>GCAAATTACCCAATCCTGAC</td>
<td>30</td>
</tr>
</tbody>
</table>

Statistical analysis

All statistical methods were performed according to [19], using SPSS, version 13.0. We used one-way ANOVA followed by LSD analyses.

Results

Salinity, Na⁺ and K⁺ of different habitats

The highest salinity was recorded for salt marsh habitats (33.66 mS cm⁻¹) followed by slightly saline water habitat as shown in table 2.

Leaf fresh weight and water content

Leaves collected from mesophytic habitats recorded the highest significant fresh weight followed by that collected from salt marsh habitats (Fig. 1A). Water content was in parallel relationship with the different habitats in terms of salinity. The highest water content was recorded in leaves collected from mesophytic habitat followed by fresh water, sand dune, brackish water and finally that of leaves collected from salt marsh habitats (Fig. 1B).

Leaf and root Na⁺ content

The highest accumulation of Na⁺ was recorded within leaves collected from salt marsh habitats (1046.719 ± 39 µmol g⁻¹ DWt) followed by brackish water (933 ± 115 µmol g⁻¹ DWt). The lowest accumulation was in leaves collected from mesophytic habitats (554 ± 49 µmol g⁻¹ DWt) (Fig. 2A). The same trend was found in roots (Fig. 2B), where the most significant accumulation was found within roots collected from salt marsh (3946 ± 189 µmol g⁻¹ DWt).

Generally, the accumulation in roots was significantly higher than that detected in leaves for all habitats (Fig 2 A and B).

Leaf and root K⁺ content

In contrast to Na⁺, the highest accumulation of K⁺ was found in leaves collected from mesophytic habitats where the low salt concentration (1782±20 µmol g⁻¹ DWt) (Fig. 3A).

Table 2. Soil chemical characteristics of P. australis growing in the different habitats of the NE part of the Nile Delta. Data is the mean of triplicates samples ± SE. Data labelled with different letter are significantly different at p ≤ 0.05 according to LSD test.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Fresh water</th>
<th>Mesophytic</th>
<th>Salt marsh</th>
<th>Sand dune</th>
<th>Brackish water</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salinity (mS cm⁻¹)</td>
<td>0.75 ± 0.1 c</td>
<td>0.50 ± 0.0 c</td>
<td>33.66 ± 2.3 a</td>
<td>0.37 ± 0.07 c</td>
<td>1.67 ± 0.1 b</td>
</tr>
<tr>
<td>K⁺ (µmol g⁻¹ Dwt)</td>
<td>3.29 ± 0.1 c</td>
<td>6.39 ± 0.56 d</td>
<td>215.16 ± 12.6 a</td>
<td>20.25 ± 1.26 c</td>
<td>94.92 ± 6.3 b</td>
</tr>
<tr>
<td>Na⁺ (µmol g⁻¹ Dwt)</td>
<td>49.70 ± 0.8 e</td>
<td>93.09 ± 1.57 d</td>
<td>7100.68 ± 0 e</td>
<td>157.79 ± 0 c</td>
<td>2998.06 ± 157.0 b</td>
</tr>
</tbody>
</table>
While the least significant accumulation was within leaves collected from salt marsh habitats (1264±9 μmol g⁻¹ DWt). The same trend was recorded in roots, while the highest significant accumulation was in roots collected from mesophytic habitats (1129 ± 29 μmol g⁻¹ DWt) and the lowest one was in roots from salt marsh habitats (877 ± 12 μmol g⁻¹ DWt) (Fig. 3B).

Generally, the accumulation of K⁺ in roots was significantly lower than that in leaves (Fig. 3 A and B).

**K⁺/Na⁺ ratio**

For all habitats, K⁺/Na⁺ ratio of leaves was higher than unity. The highest ratio was about 3.6 for mesophytic habitat and the lowest one was about 1.2 for salt marsh habitat (Fig. 4A). In contrast to leaves, K⁺/Na⁺ was less than one in roots except for roots collected from mesophytic habitats (1.1) (Fig. 4B).
Expression of NHX1

NHX1 transcript level was higher in roots than in leaves. In brackish water habitat, NHX1 mRNA was significantly higher in roots by about 40%. Also in sand dune habitat, the transcript level showed significant increase by about 75% in roots compared to that recorded in leaves. NHX1 mRNA was not detected in the roots of salt marsh, mesophytic or fresh water habitats. The highest significant transcript level detected in leaves was in the brackish water habitats (Fig. 5A and B).

Expression of SOS1

No detectable bands were recorded in any root for all habitats. The highest significant transcript level was found in brackish water habitat followed by sand dune and fresh water habitats (Fig. 6A and B).

Fig. 3 Leaf (A) and root K⁺ content (B) collected from different habitats. Data is the mean of triplicates samples ± SE. Bars labelled with different letters are significantly different at p≤0.05.

Fig. 4 Leaf (A) and root (B) K⁺/Na⁺ collected from different habitats. Data is the mean ± SE. Bars labelled with different letters are significantly different at p≤0.05.
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Fig. 5 Semi-quantitative RT-PCR of NHX1 in leaves collected from different habitats. A: Ethidium bromide-stained gels after different cycles showing the growth of the bands before reaching the plateau (saturated cycles) and B: quantification of expression in terms of band volumes. BW: brackish water, FW: fresh water, M: mesophytic, Sd: sand dune and SM: salt marsh.

Discussion

*P. australis* has an ability to adapt to different habitats including saline, dry and hydrophytic ones. Its acclimation to these different habitats is accompanied by changing in morphological, structural, and physiological and could be genetic features [20]. *P. australis* not only adapted to saline habitat but also it has an ability to maintain growth and production contrasting the glycophytes species especially monocotyledous ones that fail to survive under salt condition. The main reason has been attributed to the disability of monocot glycophytes to maintain higher K$^+$/Na$^+$ ratio (see introduction).

In the present study, the optimum growth could be found in mesophytic habitats whose salt concentration was low (Table 2). Leaf fresh weight was negatively affected by the concentration of external salt (Fig. 1A). Leaf fresh weight was higher in salt marsh than that in fresh water habitat, which could be attributed to the area and the thickness of leaf. Also,
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photosynthetic parameters may play a role in that growth variation (Unpublished data). Furthermore, the decrease in leaf fresh weight could be attributed to decrease in water content rather than dry weights.

Fig. 6 Semi-quantitative RT-PCR of S O S 1 in leaves collected from different habitats. A: Ethidium bromide-stained gels after different cycles showing the growth of the bands before reaching the plateau (saturated cycles) and B: quantification of expression in terms of band volumes. BW: brackish water, FW: fresh water, M: mesophytic, Sd: sand dune and SM: salt marsh

Water content reflected the adaptation of *P. australis* to different habitats. Where, water content significantly decreased by increasing the magnitude of outer stress condition (Fig. 1B). The decrease in water content during the exposure to harsh conditions seems to be a tolerance mechanism for many plants including halophytes [21,22].

The highest Na\textsuperscript{+} content was recorded in leaves collected from salt marshes and the lowest one was in leaves collected from mesophytic habitat. However, the highest Na\textsuperscript{+} content was found in roots collected from brackish water habitat. Generally, root Na\textsuperscript{+} content was significantly higher than that of leaves in all habitats (Fig. 2 A and B). Contrarily to Na\textsuperscript{+} content, the highest K\textsuperscript{+} content was detected in
leaves collected from mesophytic habitats and the lowest one was within leaves in salt marsh habitats. The same trend was recorded for roots. Generally, K⁺ content was significantly higher in leaves than that in roots for all habitats (Fig. 3).

Perusal data showed that P. australis could prevent the accumulation of Na⁺ inside its leaves and accumulated them inside the roots as a strategy for tolerance. K⁺/Na⁺ ratio (Fig. 4 A and B) could confirm this hypothesis, where P. australis could maintain this ratio more than unity inside its leaves in all habitats even in salt marsh. However, this ratio was less than one in its roots that could be attributed to the direct contact of roots to the salt in the soil.

NHX1 and SOS1 expressions were measured in leaves and roots collected from different habitats to evaluate their roles in maintaining K⁺/Na⁺ ratio higher than one especially for leaves. NHX1 expression was higher in roots compared to that in leaves. The highest significant expression was detected within roots and leaves collected from brackish water followed by those collected from salt marsh habitats (Fig. 5).

SOS1 transcript level was not recorded in any root collected from different habitats. Meanwhile, the highest expression value was detected in leaves collected from brackish water habitats (Fig. 6).

From these expression data, NHX1 may be more effective than SOS1 in plant roots and that could be a trial to accumulate Na⁺ inside root parts to restrict the transport of ions to leaves. P. australis use this strategy even with mesophytic habitats. However, the expression of SOS1 in leaves could be to remove the excess of Na⁺ by re-circulating them via the phloem or other pathways to prevent the toxicity of cytosol. SOS1 could play role in K⁺ homeostasis [23]. Additionally, the long cytoplasmic tail of SOS1 may have a vital role in oxidative stress response via the interaction between this tail and the regulator of oxidative stress response (RCD) [24].

Understanding the mechanism by which P. australis could maintain its productivity under these harsh environments especially under saline one could help in enhancing the crop tolerance especially monocots to survive under stress conditions. Further studies would be effective to investigate other genes that control the ion channels inside the roots and the leaves of this reed.

References

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ملخص العربية

دور الجينات المتحكمة بعنصر الصوديوم في تأقلم نبات البوص للبيئات المختلفة

ريهام ندا، ممدوح سراج، عبد الحميد خضر، نها النجار
قسم النبات - كلية العلوم - جامعة دمياط

النبات البوص قدرة على التكيف مع البيئات المختلفة وذلك يتيح للنبات الحفاظ على النمو والتنمو في بيئات مختلفة، بتحمل لاحظ في تحمل الصدأ في البيئات المختلفة وزيادة نسبة الملوحة. و في دراستنا الحالية تم تجميع نبات البوص من خمس بيئات مختلفة (البيئة المتوسطة، المياه متوسطة الملوحة، الكثبان الرملية، المياه العذبة، والأراضي الملحية) بدلًا من النباتات بشكل توليقي وسيلة بالإجهاد الموجود بالبيئات الأخرى. و وبين أن التغير في المحتوى المائي والمحتوى الملح في المحتوى المائي للورقة إستراتيجية للتكيف مع البيئات المختلفة. أظهرت الدراسة ارتفاع محتوى الصوديوم في الجذور عن الأوراق في كل البيئات بالعكس فإن محتوى البوتاسيوم كان أعلى في الأوراق عن الجذور. وكانت نسبة البوتاسيوم على الصوديوم أعلى من الوارد الصحيح في الأوراق في البيئات المختلفة من البيئات الشديدة الملوحة بينما نسبة البوتاسيوم على الصوديوم كانت أقل من الوارد الصحيح في كل الجذور من جميع البيئات ما بعد البيئة البيئية. إن الجين المسئول عن تجميع الصوديوم داخل الفجوة كان أعلى في الجذور من الأوراق و ذلك قد يكون نتيجة أن النبات يراكم الصوديوم داخل فجوة الجذور لاحظ من حركة الصوديوم إلى الساق. وعلى الرغم من ذلك فإن الجين المسئول عن تجميع الصوديوم خارج الجدار الخلوي لا يمكن تحديده في أي من الجذور المجمعة. و ذلك من الممكن وجود جينات غير المعترف عليها لطرد الصوديوم. و على الرغم من ذلك فإن هذا الجين كان معبرا في جميع الأوراق المجمعة، وربما يدل ذلك على أن هذا الجين قد يلعب أدوارا أخرى في قدرة نبات البوص على التكيف.
Optimization of exopolysaccharides production by *Bacillus subtilis*

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**Abstract**

The environmental and nutritional parameters were studied aiming to give a maximal exopolysaccharides (EPS) production by *Bacillus subtilis*. The basal medium (BM) was appeared to be the optimum medium among the seven tested media at 150 rpm. The highest production of EPS was obtained after 3 days incubation period, pH 7.0 and 25°C. Sucrose was the best carbon source stimulating the maximum production of EPS followed by mannitol. The maximum production was achieved at concentration 4.5% of sucrose. The highest production of EPS was recorded by using yeast extract with a concentration of 0.22%.

**Keywords:** optimization, exopolysaccharides, production, *Bacillus subtilis*

**Introduction**

Polysaccharides are a highly diverse group of polymers, of which the functional features are determined by their structural characteristics that may differ in molecular weight, saccharide linkage type, degree of branching and chemical composition [1]. This diversity led to broad applications in industry. Most of the polysaccharides used in the food industry as thickeners, stabilisers, texturizing and gelling agents are derived from plant (e. g. starch, pectin, gum arabic) or seaweeds (e. g. alginate, carrageenan). In the last decades several microbial exopolysaccharides (EPS) have been described as alternatives for plant polysaccharides. Microbial polysaccharides have rheological properties that match the technological demands and can be produced in large amounts and high purity. Microbial polysaccharides can be divided based on their location into (i) capsular polysaccharides (CPS) associated with the cell surface, and believed to belong to the virulence characteristics of pathogens (*Streptococcus pneumoniae*), and (ii) extracellular polysaccharides (EPS) secreted in the environment of the cell. It is suggested that CPS play a role in protection of microbial cells against desiccation, phagocytosis, phage attack, antibiotics and toxic compounds, and provide the cell with the capability to adhere to solid surfaces [2].

The ability of EPS production is widely spread among bacteria and less among yeasts and
fungi [3]. In the last years several EPS producing lactobacilli were described. Most of these lactobacilli were isolated from dairy products e. g. fermented milk, yoghurts and kefir grains [4]. EPS seem not to serve as nutrient reserve, since the most EPS producing bacteria are not able to degrade the EPS they produce [5]. Based on their composition EPS can be classified into homo- and heteropolysaccharides. Homopolysaccharides consist of one monosaccharide (mostly fructose or glucose), and are usually produced in large amount from sucrose by the action of glycansucrases [6].

Heteropolysaccharides are mostly composed of identical repeating units consisting of two or more monosaccharides e. g. galactose, glucose, rhamnose and fructose (Table 1). Several linkages can occur at the same time in one polysaccharide. Heteropolysaccharides are produced intracellularly as subunits and usually in small amounts up to 1.5 g l\(^{-1}\). Sugar nucleotides play an essential role in the synthesis of heteropolysaccharides due to their function in sugar interconversions as well as sugar activation [5].

### Table 1. Overview of some heteropolysaccharides producing bacteria

<table>
<thead>
<tr>
<th>EPS</th>
<th>Trivial name</th>
<th>Producing microorganism</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)-glucan</td>
<td>Dextran</td>
<td>Leuconostoc mesenteroides</td>
</tr>
<tr>
<td>Mutan</td>
<td></td>
<td>Streptococcus mutans</td>
</tr>
<tr>
<td>Pullulan</td>
<td></td>
<td>Aureobasidium pullulans</td>
</tr>
<tr>
<td>Fructan</td>
<td></td>
<td>Lactobacillus sanfranciscensis</td>
</tr>
<tr>
<td>(\beta)-glucan</td>
<td>Cellulose</td>
<td>Acetobacter xylinum</td>
</tr>
<tr>
<td>Curdlan</td>
<td></td>
<td>Alcaligenes sp., Rhizobium sp.</td>
</tr>
<tr>
<td>Fructan</td>
<td>Levan</td>
<td>Streptococcus salivarius</td>
</tr>
<tr>
<td>Inulin</td>
<td></td>
<td>Streptococcus mutans</td>
</tr>
<tr>
<td>Fructan and glucan</td>
<td>Polygalactan</td>
<td>Lactobacillus reuteri</td>
</tr>
<tr>
<td>Polygalactan</td>
<td></td>
<td>Lactococcus lactis sp. cremoris</td>
</tr>
</tbody>
</table>

Dextran synthesized by \(L.\) mesenteroides was one of the first biopolymers produced on industrial scale in 1948 [7] and was found several applications in medicine, separation technology and biotechnology [8]. Xanthan from \(Xanthomonas\) campestris and gellan (Gelrite) from \(Sphingomonas\) paucimobilis are also produced in large amounts and have found various food and non-food applications [3]. The interest of the food industry in developing “multifunctional additives”, that not only provide the desired improvement of the texture but also have additional nutritional properties, led to extensive search for polysaccharides with prebiotic attributes. Prebiotics are non-digestible food ingredients that affect the host beneficially by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improve host health [9]. Fructo-oligosaccharides (FOS), xylo-oligosaccharide and inulin are some prebiotics available for the human consumption [10]. FOS with prebiotic properties (e. g. kestose, nystose) are polymers of D-fructose joined by \(\beta(2\rightarrow1)\) linkages and terminated with a D-glucose molecule linked to fructose by an \(\alpha(1\rightarrow2)\) bond as in sucrose [11]. The degree of polymerization (DP) can vary from 2-35. FOS with DP of 3-5 are called neosugars [12] and are enzymatically synthesized from sucrose using fructosyltransferase obtained from \(Aspergillus\) niger [13]. McKellar and Modler showed that the maximum activity of \(\beta\)-fructosidase responsible for the hydrolysis of inulin type polysaccharides by bifidobacteria was observed with neosugars [14].

**Role of polysaccharide to the producing bacterium**

The production of exopolysaccharides is a significant energy cost to bacteria, and yet direct observation of bacterial cells in a wide variety of natural and industrial environments show, unequivocally, that all such cells are surrounded by structured exopolysaccharides and that many produce very large amounts of extracellular glycocalyx material.

Ecological studies of bacterial growth in natural systems have discovered certain pattern or strategies that used by bacteria in whatever environment they find themselves. Unlike test
tube bacteria, wild strains produce large amounts of exopolysaccharide and they use this external component to colonize specific surfaces, to mediate specific associations with other bacteria and with tissues, to produce the matrix of a protective biofilm, and to trap and concentrate nutrients from flowing fluids. In ecosystems with a finite supply of nutrients, these molecules are adsorbed and metabolized by bacteria within adherent biofilms until starvation overtakes any cells that penetrate past this nutrient rich zone. These starved cells reduce their cell size and their polysaccharide production and remain as minute, non adherent propagules until they encounter sufficient concentrations of nutrients to resume normal metabolic activity [15].

The aim of the present work was to optimize exopolysaccharide production by Bacillus subtilis.

Materials and methods

Isolation and identification of isolated bacterium

Isolated species of bacterium was identified using growth characteristics and various physiological and biochemical activities according to Bergey’s Manual of Systematic Bacteriology [16] and The Procaryotes [17].

Growth and maintenance of bacterial strains

Bacterial strains were maintained and routinely cultured on nutrient agar medium that has the following composition (g l⁻¹): beef extract, 3; peptone, 5; agar, 20; dist. water, 1L and the pH was adjusted to 7.0. The inoculated media were incubated at 30°C for 24 hours.

Screening of exopolysaccharides production by bacteria

Exopolysaccharide screening of the bacterial strains was carried out on the solid basal media containing either glucose or sucrose (20 g l⁻¹) as a carbon source. Basal medium (BM) contains the following gradients per 1 liter distilled: yeast extract 0.5; CaCl₂ 0.05; (NH₄)₂SO₄ 0.6; KH₂PO₄ 3.18; K₂HPO₄ 5.2; MgSO₄.7H₂O 0.3; FeSO₄.7H₂O 0.0006; ZnSO₄.7H₂O 0.0002; CuSO₄.5H₂O 0.0002; MnSO₄.xH₂O 0.0002; CoCl₂. 6H₂O 0.0002; agar 20. The pH was adjusted to 7.0.

The bacteria were grown on agar plates at 30 ± 1°C for 3 days and the plates were visually inspected for slime production.

Single spore cultures of the bacterial strains were grown on nutrient agar slopes at 30°C for 24 hours, and suspension of the investigated bacteria were used to inoculate flasks containing exopolysaccharide liquid basal medium (50 ml). The culture was incubated at 30 ± 1°C at 150 rev.min⁻¹ on rotary incubator shaker for 24 hour. Production flasks were then inoculated using 6% inoculum (24-h seed culture). The bacterial growth was measured using Turbidity meter.

The bacteria were routinely grown in conical flask (250 ml) containing liquid basal exopolysaccharide medium that has sucrose as carbon source (20 g l⁻¹). The pH of the liquid medium was initially adjusted to 7.0 before autoclaving. After inoculation, cultures were incubated at 30°C with shaking at 150 rev. min⁻¹ for 3 days.

Extraction of exopolysaccharides (EPS) from bacteria

After the incubation period, the cultures were harvested by centrifugation at 10000 rpm for 45 min. at 4°C using a microcentrifuge (Denver Instrument Microcentrifuge). Culture supernatants were used in precipitating exopolysaccharides. The EPS was precipitated from the supernatant by addition of four volume of 95% (v/v) aqueous ethyl alcohol. The mixture was agitated during addition of alcohol to prevent local high concentration of the precipitate, and the resulting solution was kept at 4 °C overnight before being centrifuged at 7000 rpm for 20 min. The precipitate was collected and dried at 80 °C to constant weight.

Factors affecting the exopolysaccharides production by Bacillus subtilis on submerged culture

Factors affecting exopolysaccharide (EPS) and biomass production by Bacillus subtilis were studied using shaking flask culture on rotary incubator shaker at 150 rev. min⁻¹ and 30 ± 1 °C in 250 ml flask containing 50 ml medium. A standard inoculum was used to inoculate a 50 ml medium (2 ml 50 ml⁻¹ media). Both dry weight of EPS and biomass were determined.

Studies on exopolysaccharides production using different types of media were carried out. Seven different types of media including BM medium (namely Yeast extract-malt extract
Optimization of exopolysaccharides production by Bacillus subtilis.

The effect of different seven types of media on production of EPS by Bacillus subtilis was tested. The basal medium stimulated the EPS production and appeared to be the optimum medium among the seven tested media (Fig. 1 a and b).

![Fig. 1 Effect of medium type on exopolysaccharide (EPS) production (a) and growth (b) of Bacillus subtilis. Data are represented by means of three replica ± SE.](image-url)
The effect of incubation period on the production of EPS and growth of *Bacillus subtilis* was shown in Fig. 2. The production of EPS increased during the growth phase of the culture, and the period for the maximum production was reached after 72 hours (the 3rd day). In the 4th day the production of EPS declined gradually, and then reached the minimum level at the end of incubation period (7th day). The maximum growth of *Bacillus subtilis* was reached at the 3rd day followed by stationary region through 4th and 5th days. There is no significant decrease in growth at decline phase which reached at 6 to 7 days.

The maximum growth of *Bacillus subtilis* was reached at the 3rd day followed by stationary region through 4th and 5th days. There is no significant decrease in growth at decline phase which reached at 6 to 7 days.

The effect of pH on the production of EPS was studied by adjusting the pH of growth medium from pH 5.0 to pH 10.0 (Fig. 3). EPS production increased gradually at pH 5.0 and 6.0 and the optimum pH for EPS production occurred at pH 7.0. After that, there is a decrease in EPS level at pH 8.0 to 10.0.

Maximum growth production by *Bacillus subtilis* was obtained at pH 7.0 and then decreased gradually at pH 8.0 to 10.0.

The effect of pH on the production of EPS was studied by adjusting the pH of growth medium from pH 5.0 to pH 10.0 (Fig. 3). EPS production increased gradually at pH 5.0 and 6.0 and the optimum pH for EPS production occurred at pH 7.0. After that, there is a decrease in EPS level at pH 8.0 to 10.0.

The maximum growth production by *Bacillus subtilis* was obtained at pH 7.0 and then decreased gradually at pH 8.0 to 10.0.

The effect of a range of carbon sources on the EPS production and growth were tested and the results are shown in Fig. 4 a and b. *Bacillus subtilis* was able to grow in medium supplied with nine different carbon sources which were maltose, lactose, sorbose, galactose, arabinose, xylose, sucrose, glucose and mannitol. It has been found that sucrose was the best carbon source stimulating the maximum production of EPS followed by mannitol. It is appeared that there is an increase of EPS production when using sucrose comparing with all carbon sources used. At the same time there is a slight difference in production of EPS between the other carbon sources.

Glucose, maltose and mannitol also supported a good biomass production, but the minimum growth was obtained by sorbose.

The effect of sucrose concentration on EPS production and growth of *Bacillus subtilis* was shown in Fig. 5. EPS production increased with increasing the sucrose concentration until the maximum production was achieved at concentration 4.5% followed by 5.0%. Above this concentration EPS production was decreased.

The biomass was increased with the increasing of the sucrose concentration with the optimum concentration reached at 5.5%.

The production of EPS was tested by using ten different nitrogen sources as shown in Fig. 6 a and b. The highest production of EPS was recorded by using yeast extract, KNO₃ and peptone as nitrogen sources, respectively. Also meat extract, ammonium sulphate, ammonium sulphate plus yeast extract (AS + YE) gave low production of EPS. However, the other nitrogen sources as tryptone, NH₄NO₃, ammonium phosphate and ammonium oxalate led to minimum EPS production.

The highest production of biomass was reached when using KNO₃ as nitrogen source followed by peptone and meat extract. Tryptone,
yeast extract and \( \text{NH}_4\text{NO}_3 \) also stimulate the growth of \textit{Bacillus subtilis}, respectively, however, the other nitrogen sources as ammonium sulphate plus yeast extract (AS + YE), ammonium phosphate, ammonium oxalate and ammonium sulphate slightly supported the bacterial growth.

The effect of different yeast extract concentration on the growth and production of EPS was examined and the results are shown in Fig. 7. The EPS production increased from 0.02% up to 0.32% after which there was a gradual decline down to 0.47%. Also, the growth increased from 0.02% up to the optimum at 0.22% then declined at the higher concentrations.

### Table 2. Morphological characteristics of isolate no. 1.

<table>
<thead>
<tr>
<th>Colony Shape</th>
<th>Consistency</th>
<th>Color</th>
<th>Transparency</th>
<th>Pigmentation</th>
<th>Margin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Irregular</td>
<td>slimy</td>
<td>creamy</td>
<td>opaque</td>
<td>no rhizoid</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Shape of bacteria</th>
<th>Shape</th>
<th>Gram response</th>
<th>Diameter of the cell</th>
<th>Endospore</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rod</td>
<td>Present</td>
<td>+</td>
<td>&lt; 1.0 um</td>
<td>Shape</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ovoid</td>
</tr>
</tbody>
</table>

**Fig. 4** Effect of different carbon sources on EPS production (a) and biomass production (b) by \textit{Bacillus subtilis}. Data are represented by means of three replica ± SE.

**Characterization and identification of the selected bacterial strain growth**

Agar colonies of strain no. 1 are dull, yellowish, irregular, flat, opaque, highly spreading on nutrient agar medium (Table 2).

According to the morphological and physiological studies, strain 1 indicated that it should be classified in the genus \textit{Bacillus}. Following the information provided in Bergey’s Manual of systematic Bacteriology and The Procraryotes, strain 1 was closely related to \textit{Bacillus subtilis} on the results stated above and could be classified as \textit{Bacillus subtilis}.

Isolate no. 1 had rod-shaped cells. The cell width is less than 1.0 \( \mu \text{m} \) and the length is about 1.0 \( \mu \text{m} \). The isolate is Gram positive, and endospore forming and the sporangium is not swollen. Spore is spherical in shape.
Optimization of exopolysaccharides production

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and central in position (Table 2).

Following the information provided in Bergey’s Manual of systematic Bacteriology and The Prokaryotes, strain 1 was closely related to *Bacillus subtilis* on the results stated above and could be classified as *Bacillus subtilis*.

**Discussion**

A variety of high-molecular-weight polysaccharides produced by bacteria (alginate, gellan, and xanthan) find applications as viscosifying, stabilizing, emulsifying, gelling, or water-binding agents in food and nonfood industries [2,3]. All of these polysaccharides are used as additives, however, and therefore they are considered less desirable in the food industry.

Different bacterial species produce exopolysaccharides (EPSs), located outside the cell wall, which results in mucoid, slimy colonies [18]. It is known that microbial production of EPS and sugar composition are both influenced by culture conditions. In addition, EPS production is stimulated by excess carbohydrate in the growing medium and by low temperatures [2].

Microbes release polysaccharides extracellularly as exopolysaccharides (EPSs) into the environment in the form of capsules or slime. Naturally occurring polysaccharides possess a unique combination of functional properties and environmentally friendly features. They are renewable in nature, nontoxic, and biodegradable [19]. Microbial polysaccharides are water soluble polymers and may be ionic or nonionic. Microbial EPSs, containing 90% or more polysaccharides [20], could be categorized into 2 broad classes: homopolysaccharides, which are compounds of single units of monosaccharide, and heteropolysaccharides, which are composed

**Fig. 5** Effect of sucrose concentration on production of EPS and biomass by *Bacillus subtilis*. Data are represented by means of three replica ± SE.

**Identification**

According to the morphological and physiological studies, strain 1 indicated that it should be classified in the genus *Bacillus*.

**Fig. 6** Effect of different nitrogen sources on production of EPS (a) and biomass (b) by *Bacillus subtilis*. Data are represented by means of three replica ± SE.
of 2 or more units of monosaccharide.

EPSs are highly important to any bacterium as a defense mechanism; to prevent desiccation [20]; for adhesions by forming biofilms [21]; and in industries as gelling agents, biosurfactants, emulsifiers, viscosifiers [22], biosorbents [23], and biologically active antimicrobials, anticancer agents, and antioxidants [24].

EPS is often produced at a lower temperature than is required for optimum growth [25]. It also requires higher carbon content in the medium and decreased nitrogen quantity [4] Factors that could influence the production of EPS are the composition of the medium, especially carbon and nitrogen sources, and parameters like pH, temperature, and incubation time. The commercial value of EPS would be determined by the ease of production, the quality produced, the composition of the polysaccharide, and the mode of harvest. A huge variety of biopolymers such as polysaccharides, polyesters, and polyamides are naturally produced by microbes. They range from a viscous solution to plastic, and their physical properties are dependent on the composition and molecular weight of the polymer. The genetic manipulation of the microorganism opens up an enormous potential for the biotechnological application with tailored properties suitable for tissue engineering and drug delivery.

Among seven types of media, the basal media supported the maximal production of EPS followed by XP media while YG media supported the minimal EPS production.

In our study the production of EPS increased during the growth phase of the culture, and the period for the production was reached after 72 hours (the 3rd day) of incubation period. The maximum growth of *Bacillus subtilis* was reached at the 3rd day followed by stationary region through 4th and 5th days.

### Table 3. Biochemical activities of isolate no. 1.

<table>
<thead>
<tr>
<th>Test</th>
<th>Test result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degradation of Casein</td>
<td>+</td>
</tr>
<tr>
<td>Starch</td>
<td>+</td>
</tr>
<tr>
<td>Gelatin</td>
<td>-</td>
</tr>
<tr>
<td>Urea</td>
<td>+</td>
</tr>
<tr>
<td>Enzyme activity</td>
<td></td>
</tr>
<tr>
<td>Proteolysis on egg yolk</td>
<td>+</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>+</td>
</tr>
<tr>
<td>Hydrogen sulphide production</td>
<td>-</td>
</tr>
<tr>
<td>Formation of indole</td>
<td>-</td>
</tr>
<tr>
<td>Dihydroxy acetone production</td>
<td>+</td>
</tr>
<tr>
<td>Degradation of tyrosine</td>
<td>-</td>
</tr>
<tr>
<td>Degradation of citrate</td>
<td>-</td>
</tr>
<tr>
<td>Degradation of propionate</td>
<td>-</td>
</tr>
<tr>
<td>Degradation of phenylalanine</td>
<td>-</td>
</tr>
<tr>
<td>Production of gas from glucose</td>
<td>-</td>
</tr>
<tr>
<td>Catalase test</td>
<td>+</td>
</tr>
<tr>
<td>Voges Proskauer test</td>
<td>+</td>
</tr>
<tr>
<td>Effect of NaCl conc.</td>
<td></td>
</tr>
<tr>
<td>0%</td>
<td>+</td>
</tr>
<tr>
<td>2%</td>
<td>+</td>
</tr>
<tr>
<td>5%</td>
<td>+</td>
</tr>
<tr>
<td>7%</td>
<td>+</td>
</tr>
<tr>
<td>10%</td>
<td>-</td>
</tr>
<tr>
<td>Production of acid from carbohydrate</td>
<td></td>
</tr>
<tr>
<td>D- glucose</td>
<td>+</td>
</tr>
<tr>
<td>L- arabinose</td>
<td>+</td>
</tr>
<tr>
<td>D- xylose</td>
<td>+</td>
</tr>
<tr>
<td>D- mannitol</td>
<td>+</td>
</tr>
<tr>
<td>Growth at different pH</td>
<td></td>
</tr>
<tr>
<td>pH 5.7</td>
<td>+</td>
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<tr>
<td>pH 6.8</td>
<td>+</td>
</tr>
<tr>
<td>Growth of different strains at different temperatures</td>
<td></td>
</tr>
<tr>
<td>15 °C</td>
<td>+</td>
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<tr>
<td>20 °C</td>
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<td>25 °C</td>
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<td>45 °C</td>
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<tr>
<td>50 °C</td>
<td>+</td>
</tr>
<tr>
<td>55 °C</td>
<td>-</td>
</tr>
</tbody>
</table>
According to Decho [26] and Manca et al. [27], the largest quantity of EPS was released during stationary growth phase in laboratory culture. The results obtained on Klebsiella aerogenes in liquid media, show that, the rate of polysaccharide production was greatest during the exponential phase of growth and gradually decreased thereafter [28]. Norval [29] reported that the rate of polysaccharide synthesis in washed cell suspension by log-phase and stationary-phase cells grown in several different media was very similar. The ability to synthesize extracellular polysaccharide was lost from older cultures (48 hr) in synthetic media and was very low in cells grown in media lacking a utilizable carbohydrate.

In the present study, the data showed that the maximum growth production by Bacillus subtilis was obtained at pH 7.0 and then significantly decreased. The maximum production of EPS also occurred at pH 7.0 after a slightly increase in EXP level at pH 5.0 and 6.0.

Results obtained from this study show that Bacillus subtilis was able to grow in medium supplied with nine different carbon sources which were maltose, lactose, sorbose, galactose, arabinose, xylose, sucrose, glucose and mannitol. It has been found that sucrose was the best carbon source which stimulates the maximum yield of EPS followed by mannitol. It is appeared that there is a significant increase of EPS production by sucrose comparing with all carbon sources used. At the same time there is no significant difference in production of EPS between the other carbon sources. Sucrose also supported the best growth of Bacillus subtilis which followed by glucose. Maltose and mannitol also support a good biomass production, but the minimum growth was obtained by sorbose.

Sucrose concentration at 4.5% stimulated the maximal EPS production followed by 5.0%. Above this concentration EXP production was slightly decreased while the optimum growth occurred at concentration 5.5%.

On the other hand, Gandhi et al. [30] pointed out that ratios of carbon and nitrogen sources play the most important role in cellular growth and exobiopolymer production. At 100 g l⁻¹ sucrose, it was best source for EPS production from B. licheniformis 221a, at 13.57 g EPS l⁻¹ of medium [31]. Sucrose, a disaccharide, upon hydrolysis produces glucose and fructose. Higher yield is obtained, since sucrose apparently acts as a precursor of EPS synthesis. As the concentration of the sugars increased above 2%, the cell growth and the yield were found to decline. This is mostly due to the elevation of osmotic pressure in the cellular system, thereby causing plasmolysis, leading to cell death [32]. Various studies have been carried out to learn the effects of different carbon substrates on EPS production. A concentration of 2% maltose in the production medium was able to produce 3.5 g EPS 1⁻¹ from Cordyceps jiangxiensis [33]. A maximum of 44.49 mg l⁻¹ of EPS was produced from Lactobacillus fermentum when the medium was supplemented with 2% glucose and 0.5% whey protein concentrate [34]. Sugars like fructose, lactose, glucose, and sucrose were used for EPS production in Streptococcus thermophilus ST1 from skim milk, yielding 64.52 mg l⁻¹, 66.39 mg l⁻¹, 69.35 mg l⁻¹, and 73.28 mg l⁻¹, respectively [35].

Various nitrogen sources were observed for their effects on EPS yield from our isolate. Organic nitrogen sources were inferred to yield a higher amount of EPS than inorganic nitrogen substrates. Yeast extract was found to produce the maximum yield of EPS by KNO₃ and peptone as nitrogen sources, respectively. Meat extract, ammonium sulphate, ammonium sulphate plus yeast extract (AS + YE) supported less production of EPS. However, the other nitrogen sources as tryptone, NH₄NO₃, ammonium phosphate and ammonium oxalate supported minimum EPS production, respectively.

The highest production of biomass was reached by using KNO₃ as nitrogen source followed by peptone and meat extract. Tryptone, yeast extract and NH₄NO₃ also stimulate the growth of Bacillus subtilis, respectively, but the other nitrogen sources as ammonium sulphate plus yeast extract (AS + YE), ammonium phosphate, ammonium oxalate and ammonium sulphate slightly supported the bacterial growth.

Both EPS production and growth of Bacillus subtilis was recorded at from concentration 0.02% and 0.07% to concentration 0.12% and 0.17%. Then a slightly increase in EPS production occurred until the maximum amount of EPS reached at concentration 0.32%, after this concentration EPS production was decreased. The optimal growth of Bacillus subtilis was occurred at concentration 0.22% of yeast extract.

It was suggested that certain essential amino acids cannot be synthesized from inorganic nitrogen components [36], because of which bacterial cells might neither fully grow nor undergo metabolism, and hence the deterioration
of EPS yield. It was also reported that the primary role of heterotrophic bacteria is classically considered to be decomposition and mineralization of dissolved particulate organic nitrogen. This might be an obvious cause of higher production of EPS by \textit{B. subtilis}. As the concentration of the nitrogen sources was increased, the growth rate was found to ascend, but the mitigation of EPS production was observed. Reports suggest that nitrogen limitation and higher amounts of carbon in the medium could yield a maximum amount of EPS. A study showed that EPS production from \textit{Rhizobium meliloti} was higher when the nitrogen source was in minimal quantity. Similarly, pullulan was generated by \textit{Aureobasidium pullulans} when it was grown in a medium with lesser amounts of nitrogen source.

References


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الملخص العربي

تحديد الظروف المثلى لإنتاج السكريات العديدة الخارجية بواسطة باسيليس سيتيليس

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3 مركز الجهاز الهضمي والكبد - جامعة المنصورة

تم دراسة الظروف البيئية لتحديد الظروف المثلى لإنتاج مادة السكريات العديدة الخارجية بواسطة باسيليس سيتيليس وذئو في مزارع مغمورة عند درجة حرارة 30 درجة مئوية وتم الحصول على النتائج الآتية: دعت البيئة الأساسية لإنتاج مادة السكريات العديدة الخارجية وكانت هي الأفضل من ضمن سبعة بيئة مستخدمة. كما وجد ان الانتاج الأعلى حدث في اليوم الثالث (الثناة وسبعون ساعة) من فترة التحضير. كما وجد ان الهدف يوغيني 7 درجة حرارة 25 درجة مئوية دعا اعلى إنتاج لمادة السكريات العديدة الخارجية. كما انتجت هذه السلاسة من البكتيريا على كمية من هذه المادة عند استخدام السكرز كمصدر كربوني وتذ ذكر عند تركيز 4.5%، كما وجد أن اعلى ان إنتاج لمادة السكريات العديدة الخارجية تم تسجيله عند استخدام مستخلص الخمور كمصدر كربوني عند تركيز 22%.
Relationship between efficiency of antioxidants in wheat and tolerance to NaCl

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Abstract

Application of NaCl at 75, 150 and 225 mM to 9-d-old seedlings of two wheat cultivars (Misr1 and Sakha93) for 15 days significantly decreased fresh and dry weights of only Sakha93. Growth parameters were most likely similar in both cultivars grown under normal conditions. Lipid peroxides and H$_2$O$_2$ were greatly accumulated in both cultivars particularly in Sakha93; significant increases were only detected in Misr1 treated with 225 mM. On the contrary, 150 and 225 mM NaCl led to great diminution in reducing power of Sakha93; however, there was no significant change in Misr1. In contrast to reducing power the phenolic contents were highly elevated in Sakha93 by NaCl at all concentrations but the effect of NaCl in Misr1 seemed to be nonsignificant. Also, all concentrations of NaCl decreased both protein content GSH in Sakha93 while the decrease in Misr1 was restricted to 225 mM. On the other hand, the activities of catalase (CAT), peroxidase (POD), glutathione-S-transferase (GST) and glutathione reductase (GR) were increased in Misr1 by 75 and 150 mM NaCl but inhibited in Sakha93 by all concentrations. The results of growth parameters suggest that Misr1 is a more tolerant cultivar to NaCl than Sakha93. The concomitant accumulation of MDA and H$_2$O$_2$ in Sakha93 and in the meantime the decrease in GSH reveal that ROS scavenging is less efficient in the more susceptible than the tolerant cultivar. Consequently, the cultivar Misr1 seemed to tolerate NaCl may be due to the more efficient induction of potential antioxidants to cope with salinity conditions.

Keywords: enzymatic antioxidants; non-enzymatic antioxidants; salinity; tolerance; wheat

Introduction

Salt stress is one of the major abiotic stresses that affect biochemical and physiological processes in plants, causing growth inhibition and yield loss [1]. NaCl is the most important constituent of a saline environment. An increase in salinity stress induces both a reduction in the percentage of seeds germinating and a delay in the initiation of the germination process, but also can cause complete inhibition of the germination process at salinities beyond the tolerance limits of the species. The negative effects of salinity have been attributed to increase in Na$^+$ and Cl$^-$ ions in different plants. The outcome of these effects may cause membrane damage, nutrient...
imbalance, altered levels of growth regulators, enzymatic inhibition and metabolic dysfunction which ultimately lead to plant death [2]. Plants respond and adapt to stress by complex molecular responses. Salinity generates reactive oxygen species (ROS) in plants. ROS typically result from the excitation of O2 to form \( \cdot \text{O}_2 \) or from the transfer of one, two or three electrons to O2 to form, respectively, a superoxide radical (O2\(^-\)), hydrogen peroxide (H2O2) or a hydroxyl radical (HO\(^-\)) [3-5]. ROS are highly reactive and may cause cellular damage through oxidation of lipids, proteins and nucleic acids [6-8]. Production of ROS is increased under saline conditions [9] and ROS-mediated membrane damage has been demonstrated to be a major cause of the cellular toxicity by salinity in different crop plants [8,10,11]. It was expected that up-regulation of antioxidant system protect plants against NaCl-induced oxidative damage. Salinity treatments caused significant increase in H2O2 and lipid peroxidation in wheat seedlings, which were higher in salt-sensitive cultivar than salt tolerant cultivar [12]. Increased lipid peroxidation and levels of H2O2 was observed with increased salinity in *B. napus* and *T. aestivum* [13]. These detrimental effects of the overproduction of ROS could limit the plant tolerance to stresses [14]. However, plants have developed a battery of complex defense system to detoxificate and eliminate the generated ROS [3-5,15]. Such system includes nonenzymatic antioxidants as glutathione and enzymatic antioxidants as glutathione reductase (GR), glutathione-S-transferase (GST), catalase (CAT) and peroxidase (POD) [3,16-18]. Therefore, this work aims at studying the potentialities of antioxidants in two cultivars of wheat varied in tolerance to salinity in order to elucidate the relationship of antioxidant efficiency with plant tolerance to salinity.

**Materials and methods**

**Plant materials and growth conditions**

The grains of two wheat cultivars (*Triticum aestivum* Misr1 and Sakha93) differentially tolerate NaCl were surface sterilized by immersing in 3% sodium hypochlorite solution for 10 min and thoroughly washed. The grains were soaked for 8 h, germinated in perlite in 7 cm-diameter pots and watered with tap water. On the 7\(^{th}\) day, seedlings were thinned to only one per pot. The pots were placed in 40 \( \times \) 60 cm trays containing 100% long Ashton nutrient solution for 2 days and then divided into 4 sets for NaCl treatments (0, 75, 150 and 225 mM). Each set was represented by 10 replications. The seedlings were distributed randomly into trays and kept for the following 15 days under controlled conditions (25/10 °C day/night regime, 60% RH, 10 h photoperiod and 300 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) photosynthetic photon flux density). The nutrient solution was consistently made up to the appropriate volume daily and renewed every 4 days. At harvest, the plants were carefully removed from the perlite, washed thoroughly with the relevant nutrient solution, dipped into deionized water and plotted dry. Shoot system was separated and used for determination of fresh and dry weights. Other samples were frozen in liquid N\(_2\) for the subsequent analyses.

**Determination of lipid peroxides and H\(_2\)O\(_2\) contents**

Lipid peroxides were extracted in 150 mM KCl. After centrifugation at 7000 xg for 15 min, 1 ml of the supernatant was incubated at 37 °C for 2 h with 1 ml of 0.6 M trichloroacetic acid (TCA). One ml of supernatant was taken with 1 ml of thiobarbituric acid and placed in a boiling water bath for 10 min, cooled and diluted with 1 ml distilled water. The absorbance was read at 535 nm [19]. H2O2 was extracted in 200 mM perchloric acid and centrifuged at 5000 xg for 10 min. The assay mixture contained 0.4 ml 12.5 mM 3-dimethylaminobenzoic acid in 375 mM phosphate buffer pH 6.5, 0.08 ml 1.3 mM 3-methyl-2-benzothiazolinone hydrazone and 0.02 ml (0.25 units) horseradish peroxidase. The increase in absorbance at 590 nm was monitored for 3 min [20].

**Determination of protein and glutathione (GSH) contents**

About 200 mg of frozen leaves from 5 seedlings were homogenized in 80 mM Tris-HCl, pH 7.4. After centrifugation at 14000 xg for 5 min, the extracted protein was precipitated overnight at 4°C by adding 10% chilled trichloroacetic acid in acetone (w/v). Protein pellets were separated by centrifugation at 12000 xg for 15 min and reconstituted in the buffer. Protein was determined using Commassie Brilliant Blue G-250 at 595 nm [21]. GSH was extracted in TCA (5%, w/v) containing 5 mM EDTA and
centrifuged at 12,000 xg for 15 min [22]. GSH was assayed in 100 mM phosphate buffer, pH 6.8 containing 5 mM EDTA and 1 mM 1-chloro-2,4- dinitrobenzene (CDNB). The reaction was started by adding 1.0 U equine Glutathione-S-transferase (GST) and incubated at 35 °C for 30 minutes. The absorbance was recorded at 340 nm.

**Determination of reducing power and phenolic contents**

The Fe³⁺ reducing power of the extract was determined according to the method of Oyaizu [23]. The extract (2 ml) was mixed with 0.2 M phosphate buffer, pH 6.6 (2 ml) and 1% potassium ferricyanide (2 ml) and incubated at 50°C for 20 min. The the reaction was stopped by 10% trichloroacetic acid (2 ml) and then centrifuged at 10000 xg for 10 min. The upper layer of supernatant (2 ml) was mixed with distilled water (2 ml) and 0.1% FeCl₃ solution (0.5 ml) and the absorbance was measured at 700 nm. Phenolic extraction was performed in methanol at 37°C. The extract was collected and concentrated by evaporation. Total phenolic contents were assessed by using the Folin-Ciocalteu phenol reagent. To 1 ml of the extract, 0.5 ml of 10% Folin-Ciocalteu reagent and 0.5% sodium carbonate were added, thoroughly mixed and allowed to stand for 30 min and absorption at 750 nm was measured. The total phenolic contents were expressed as gallic acid equivalents.

**Measurement of antioxidant enzyme activities**

The enzyme were extracted in 50 mM sodium phosphate buffer (pH 6.9) containing 2 mM EDTA and 5 mM β mercaptoethanol and centrifuged at 12,000 xg for 10 min at 4 °C. Glutathione reductase (GR) was assayed in reaction mixture contained 100 mM phosphate, pH 7.5, 0.5 mM EDTA, 0.75 mM 5,5-dithiobis-(2-nitrobenzoic acid), 0.1 mM NADPH and 1 mM oxidized glutathione (GSSG) [24]. The reaction mixture was incubated at 35 °C meanwhile absorbance at 412 nm is measured up to 5 min. Glutathione-S-transferase GST was extracted in 100 Mm Tris-HCL (pH 7.5) containing 2 mM EDTA, 14 mM β-mercaptoethanol and 7.5% (w/v) polyvinylpolyprollidine then centrifuged at 15,000 xg for 15 min and ammonium sulfate was added to 80% saturation [25]. GST was assayed in 100 mM phosphate buffer (pH 6.5) containing 5 mM GSH and 1 mM CDNB. After incubation for 1 h at 35 °C, 3 ml of 0.33 N HCl were added to stop the reaction and absorbance was measured at 340 nm. GST activity was assayed using the extinction coefficient E = 9.6 mM⁻¹ cm⁻¹. Catalase (CAT) activity was measured by following the consumption of H₂O₂ at 240 nm in 50 mM sodium phosphate buffer (pH 7.0) containing 5 mM H₂O₂ [26]. The absorbance was read at zero time and after 1 min. Peroxidase (POD) activity was measured in 50 mM sodium phosphate buffer (pH 6.9) containing 3.2 mM guaiacol and 0.4 mM H₂O₂ [27]. The absorbance was measured at 470 nm.

**Statistical analysis**

All values are means (± SD) of at least six determinations from two independent experiments. The full data were first subjected to analysis of variance (ANOVA) followed thereafter by least significant differences (LSD) at 5% level.

**Results**

In Fig. 1, treatment with NaCl resulted in significant decrease in fresh weight of Misr1 cultivar only by 225 mM (about 32%). In Sakha93, all concentrations induced significant decreases; NaCl at 75, 150 and 225 mM caused a reduction in fresh weight by about 42, 71 and 85%, respectively. Also, dry weight was greatly affected by salinity; however, 75 mM NaCl seemed not significant in Misr1 while 150 and 225 mM led to significant decrease by about 21 and 28%, respectively. On the other hand, all concentrations of NaCl (75, 150 and 225 mM) induced significant decreases in Sakha93 by about 53, 76 and 79%, respectively.

Treatment with NaCl at all concentrations resulted in great accumulations of lipid peroxides in both cultivars (Fig. 2). The accumulation of lipid peroxides reached in Misr1 about 55, 91 and 180% of control values following treatment with 75, 150 and 225 mM NaCl, respectively and in Sakha93 about 109, 227 and 235%, respectively. Meanwhile, H₂O₂ content was highly increased in both cultivars, following NaCl treatment, although only 75 mM resulted in non significant effect in Misr1 but 150 and 225 mM induced increases of about 33 and 59%. Whilst all concentrations were of significant effect in...
Sakha93; 75, 150 and 225 mM led to an accumulation of about 97, 103 and 106%, respectively.

![Graph](image1)

**Fig 1.** Changes in shoot fresh and dry weight of nine-d-old seedlings of two wheat cultivars (Misr1 and Sakha93) after 15 days from treatment with 75, 150 and 225 mM NaCl. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars represent LSD at 5% level.

![Graph](image2)

**Fig 2.** Changes in lipid peroxides as MDA and \( \text{H}_2\text{O}_2 \) of nine-d-old seedlings of two wheat cultivars (Misr1 and Sakha93) after 15 days from treatment with 75, 150 and 225 mM NaCl. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars represent LSD at 5% level.

The results depicted in Fig. 3 represent that reducing power was higher in Sakha93 than in Misr1 by about 11%. However, treatment with NaCl resulted in significant decrease in reducing power in seedlings of both cultivars; the decrease was more pronounced in Sakha93 than in Misr1. Nonetheless, only 225 mM NaCl was of significant effect in Misr1 inducing an inhibition of about 33%. On the contrary, all NaCl concentration resulted in significant inhibition in Sakha93, 75, 150 and 225 mM caused a decrease in reducing power by about 31, 50 and 78%, respectively of the control values. In addition, phenolics content exhibited higher values in Misr1 than in Sakha93 by about 14% (Fig. 3). In this account, phenolics content was further increased by NaCl treatment, the magnitude of increase was greater in Sakha93 than in Misr1.
Only 225 mM NaCl seemed to significantly increase phenolics content in Misr1 by about 47%. On the contrary, all NaCl concentrations induced significant increases in Sakha93, the content of phenolics increased following treatment with NaCl at 75, 150 and 225 mM by about 69, 74 and 102%, respectively of the control values.

![Graph](image_url)

**Fig 3.** Changes in reducing power and phenolic content of nine-d-old seedlings of two wheat cultivars (Misr1 and Sakha93) after 15 days from treatment with 75, 150 and 225 mM NaCl. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars represent LSD at 5% level.

In Fig. 4, Protein content was only 5% higher value in Misr1 cultivar than in Sakha93 under control conditions. Treatment with 75 mM NaCl seemed with no significant effect upon Protein content of Misr1 seedlings while the other concentrations induced significant reductions as compared to the control values. On the contrary, all concentrations of NaCl resulted in significant reductions Protein content of Sakha93 as compared to the control values. The magnitude of reduction due NaCl treatment was higher in Sakha93 than in Misr1 seedlings and augmented with increasing concentrations. The decrease in Protein content in Misr1 reached about 5, 16 and 28% of control values following treatment with 75, 150 and 225 mM NaCl, respectively whereas these decreases were greater in Sakha93 and reached about 49, 69 and 72%, respectively. The cultivar Misr1 contained higher GSH content than Sakha93 by about 6% under control conditions (Fig. 4). Nonetheless, decreases were detected in these contents following NaCl treatment particularly in Sakha93, the magnitude of decrease augmented with increasing NaCl concentrations. However, GSH content seemed non significantly changed in Misr1. The magnitude of decrease of GSH content decreased in Sakha93 by about 44, 63 and 77%, respectively of the control values following treatment with NaCl at 75, 150 and 225 mM, respectively.

In Fig. 5, there was a marked difference in GR activity among both cultivars normally-grown under control conditions; the activity was higher by 9% in Misr1 than in Sakha93. The treatment with NaCl at all concentrations resulted in non significant effect in GR activity in Misr1 seedlings; however, all concentrations significantly inhibited the enzyme activity in Sakha93. NaCl at 75, 150 and 225 mM caused a significant inhibition in GR activity of Sakha93 seedlings by about 57, 64 and 67%, of the control value respectively. The activity of GST was higher in Misr1 than in Sakha93 by only about 7% (Fig. 5).

Nonetheless, NaCl at all concentrations seemed with no significant effect upon GST activity in only Misr1 cultivar; however, the activity was significantly inhibited in Sakha93. The activity of GST decreased following treatment with NaCl at 75, 150 and 225 mM by about 44, 63 and 77%, respectively of the control values in Sakha93. The activity of CAT was most likely similar in magnitude in both cultivars. Treatment with NaCl seemed with no significant effect upon GST activity in only Misr1 cultivar, however, the enzyme activity was significantly inhibited in Sakha93 by all concentrations. The
inhibition in CAT activity reached about 64, 66 and 67% of control values in Sakha93 following treatment with 75, 150 and 225 mM NaCl, respectively. There was a higher activity of POD in Misr1 than in Sakha93 by only about 8% (Fig. 5). Nonetheless, the POD activity in Misr1 cultivar seemed to be non-significantly affected by NaCl at all concentrations but exhibited significant inhibition in Sakha93. The activity of POD in Sakha93 was inhibited following treatment with NaCl at 75, 150 and 225 mM by about 58, 62 and 64%, respectively of the control values.

Discussion

The general response of plants to salinity is reduction in growth [28]. In spite of having most likely similar values of growth parameters under normal conditions in the present study, the growth of the wheat cultivar Sakha93 was more inhibited than Misr1. These findings could conclude that Sakha93 might be considered as a more sensitive cultivar to NaCl than Misr1. This varied sensitivity to NaCl could be related to deteriorations in various processes. Parida and Das [29] indicated that salt stress induces various biochemical and physiological responses in plants and affects almost all plant metabolic processes. Salinity causes alterations in the integrity of cell membranes [30] and inhibition of different enzymatic activities [31]. Moreover, one of the most effects of salinity stress is the accumulation of ROS that would react with lipids, proteins and pigments causing lipid peroxidation and membrane damage [3,5,32]. ROS initiate a variety of autooxidative chain reactions on membrane unsaturated fatty acids, producing lipid hydroperoxides and thereby cascade of reactions ultimately leading to destruction of organelles and macromolecules [16].

In the present results, the accumulation of lipid peroxides (as MDA) in both wheat cultivars could point to an occurrence of an oxidative stress status induced due to salinity treatment. However, MDA was more accumulated by NaCl in Sakha93 than in Misr1 concluding that the oxidative stress is related to the sensitivity to salinity. Therefore, a relationship could be established between the deleterious effects of NaCl and the sensitivity of plants to salinity. Yasar et al. [33] reported that NaCl treatments led to a gradual increase in the levels of MDA in green bean and its accumulation was higher in the sensitive cultivar than tolerant one. Also NaCl caused greater accumulation of H$_2$O$_2$ in the more sensitive cultivar than in the less sensitive one. In confirmation, the reducing power was greatly affected by NaCl more in Sakha93 than in Misr1. Moreover, salinity highly induced induction in phenolic compounds Sakha93 concluding that this cultivar is more sensitive to NaCl relative to Misr1. Similar effects were also
detected regarding protein content revealing that salinity has a negative effect on the structural or functional protein of Sakha93.

To cope with oxidative stress, plants develop an antioxidant system which plays an important role in the defense mechanism against stress. The correlation between the resistance to environmental stresses and the efficiency of the antioxidant system has been established [33]. Plants are endowed with an array of non-enzymatic antioxidants such as GSH and enzymatic antioxidants such as GR, GST, CAT and POD for removal of ROS [3,5,16]. The present results indicate greater effects of NaCl on GSH of Sakha93 than Misr1 pointing out that antioxidants are less efficient in the less tolerant cultivar than in the more tolerant one.

<table>
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<td>GR activity (kat)</td>
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Fig 5. Changes in activities of glutathione reductase (GR), glutathione-S-transferase (GST), catalase (CAT) and peroxidase (POD) of nine-d-old seedlings of two wheat cultivars (Misr1 and Sakha93) after 15 days from treatment with 75, 150 and 225 mM NaCl. Data are means (±SD) of at least six replications from two independent experiments. Vertical bars represent LSD at 5% level.

GSH is the most abundant low molecular weight non-enzymatic antioxidants in plant cells participating in ROS scavenging through the AsA-GSH cycle [4]. Nonetheless, during ROS scavenging, GSH is oxidized to GSSG while GR maintains glutathione in its reduced form. GST activates the protection of plants from some xenobiotics and ROS [3]. Therefore, the decrease in GSH content and in activities of GST and GR in Sakha93 reveals that the scavenging of ROS is less efficient in the less tolerant cultivar suggesting that there is a severe state of stress imposed by NaCl occurs particularly in Sakha93, the more sensitive cultivar.

In conclusion, both cultivars, Misr1 and Sakha93, responded differentially to NaCl in spite of having similar values of growth parameters. The results concluded that Sakha93 is more sensitive to NaCl treatment than Misr1. Greater was the induction of lipid peroxides and H$_2$O$_2$ by NaCl in Sakha93 than in Misr1. Similar increases were also detected regarding phenolic compounds. On the contrary, proteins as well as antioxidants either enzymatically or non-
enzymatically were inhibited by salinity more in Sakha93 than in Misr1. So, Sakha93 could be concluded to suffer from NaCl treatment more than Misr1. These findings support that Sakha93 is more sensitive to NaCl than the other cultivar, Misr1 which can tolerate and combat salinity tolerance. The ability of the plant to combat environmental stress is determined by its efficiency to sense the stress and activate its defense machinery. These results confirm that there is a relationship between the potential antioxidants and NaCl stress tolerance in wheat.

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الملخص العربي

العلاقة بين كفاءة المواد المضادة للأكسدة في القمح وتحمله لكلوريد الصوديوم

مذود نعمة الله، جابر مختار أبو جاد الله، ايناس بدران
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تم معاملة بادرات صنفين من القمح ( مصر 1 وسخا 93 ) بتكرات 75 و150 و225 مللي مولار من كلوريد الصوديوم لمدة 15 يوما. شهد أن كل الكرات ادت إلى انخفاض كبيرو في الأوران الرطبة والجافة فقط في صنف سخا 93، بينما بد صنف مصر 1 لا يتأثر كثيرا. كانت لأقاس في البادرات النامية في ظل الظروف الطبيعية مماثلة لكل الصنفين. وتركبت البروتينات الدهنية وفوق أكسيد الهيدروجين في كل الصنفين وكان الكرات أكثر وضوحا في صنف المعالمة بالكرات 225 و250 مللي مولار إلى حد من القدرة الاختزالية في صنف سخا 93. وكانت المحتويات الفينولية مرتفعة للغاية بينما كان ان التغير طفيفا في صنف مصر 1. أفاد أن كل الصنفين تحت الظروف الطبيعية لا يختلفان في القدرة الاختزالية أو المحتويات الفينولية. والانخفاض البروتيني والجلوتاثيوني في صنف سخا 93 في حين اقل الامتصاص في صنف مصر 1 على المعالمة بتكرات 225 مللي مولار. لم يكن هناك فرق كبير سواء في محتوى البروتيني أو الجلوتاثيوني بين بادرات كل الصنفين النامية في ظل الظروف الطبيعية. وزارت أنشطة مضادات الأكسدة الإزدامة في صنف مصر 1 بفعل كلوريد الصوديوم عند تركتين 75 و150 مللي مولار ولكنها ثابت في صنف سخا 93. وتشير النتائج إلى أن كلوريد الصوديوم من صنف سخا 93. وتؤكد كفاءة أقل في التخلص من مسببات الإجهاد التأكسدي في الصنف الأقل تحملًا للملوحة بينما تحمل صنف مصر 1 كلوريد الصوديوم قد يكون بسبب كفاءة أكثر لمضادات الأكسدة للتلغلب على الإجهاد التأكسدي الناتج عن الملوحة.
Isolation and characterization of some multi-antibiotic resistant bacterial pathogens associated with nosocomial infections

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Abstract

Nosocomial infections are frequent complications of hospitalization, caused by opportunistic pathogens that gain access to hosts undergoing invasive procedures. This study was carried out to investigate the presence of antibiotic resistance bacteria from hospital environment of Damietta, Egypt. Two hundred and six clinical bacterial isolates were collected from different samples (sink, floor, bed, bed cover, toilet floor, bed pan, ward wall and hospital staff apparel (protective gowns, hand groves and face-shield) within reception hall, maternity ward, convalescing wards, surgical theaters, intensive care, dental unit, pharmacy and laboratory) from (Kafr Saad General Hospital and El-Azhar University Teaching Hospital). Four bacterial species were identified as Bacillus subtilis, Bacillus cereus, Klebsiella pneumoniae and Enterobacter aerogenes using standard morphological, biochemical tests and sequencing of 16S rRNA gene. Eleven antibiotics (Trimethoprim/sulphamethoxazole (25 µg); Rifampicin (5 µg); Piperacillin/tazobactam (110 µg); Ofloxacin (5 µg); Nitrofurantoin (300 µg); Imipenem (10 µg); Gentamicin (120 µg); Ceftriaxone (30 µg); Cefotaxime (30 µg); Amikacin (30 µg) and Amoxicillin/clavulanic acid (30 µg) were tested against the bacterial isolates using disc diffusion method to determine the multi-drug resistance bacteria. Imipanem was found to be as the most effective drug against Klebsiella pneumoniae while ofloxacin, amikacin, piperacillin/tazobactam, gentamicin and rifampcin had low effect against Klebsiella pneumoniae and no effect of amoxicillin/clavulanic acid, cefotaxime, ceftriaxone, nitrufurantion and trimethoprim/sulphamethoxazole was recorded against Bacillus subtilis.

Keywords: multi-antibiotic resistant, nosocomial infections, Bacillus subtilis, Bacillus cereus, Klebsiella pneumoniae and Enterobacter aerogenes

Introduction

Nosocomial or hospital-acquired infections are usually defined as infections that are identified at least 48–72 hours following admission to health institutions [1]. Nosocomial infections are also important public health problems in developing countries as well as in developed countries [2]. The most frequent types of nosocomial infections are urinary tract infections (UTIs), surgical-wound infection, pneumonia, and bloodstream infections (BSIs) [3]. Nosocomial infections are
caused by bacteria, viruses and fungi contracted by the contaminated equipment of the hospital. Many of the organisms associated with hospital-acquired infections exhibit two particular features: firstly, they are pathogens of well-established medical importance and secondly, they can withstand the severity or harshness of the hospital environment. The common bacterial pathogens present in the BSIs and UTIs are *Staphylococcus aureus*, Coagulase Negative *Staphylococci* (CoNS), *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Escherichia coli*, *Enterobacter* sp., *Enterococcus* sp., and *Acinetobacter* sp. [4]. As the result of extensive uses of antimicrobial agents, nosocomial pathogens have shifted away from easily treatable bacteria towards more resistant bacteria. This change is important problem for nosocomial infection control and prevention [5]. Nosocomial infections comprise one of the leading causes of preventable injuries and deaths in hospitals, affecting 5% to 10% of hospitalized patients and contributing to increased morbidity, mortality, length of stay and cost [6].

*Enterobacter* species are among the most common causes of gram-negative health care-associated infections, causing 8% of nosocomial bacteremia cases, and are the second most common gram-negative pathogens causing pneumonia in patients admitted to intensive care units (ICUs) [7]. In addition, in recent years, they are an increasing cause of community-acquired infections as well [7]. Resistance to a variety of broad-spectrum antimicrobials among *Enterobacter* strains, including β-lactams, is frequently encountered. Moreover, the emergence of resistance to extended-spectrum cephalosporins occurs often during therapy [8].

Nosocomial *Klebsiella* infections are caused mainly by *Klebsiella pneumoniae*, the medically most important species of the genus. To a much lesser degree, *Klebsiella oxytoca* has been isolated from human clinical specimens. It is estimated that *Klebsiella* sp. causes 8% of all nosocomial bacterial infections in the United States and in Europe. No great geographical variations in frequency have been noted. *Klebsiella* accounts for 3 to 7% of all nosocomial bacterial infections, placing them among the eight most important infectious pathogens in hospitals [9].

*Bacillus* species have been reported to cause bacteremia, endocarditis, pneumonia, meningitis, and other invasive infections, particularly in immunocompromised patients [10]. However, due to the wide distribution of *Bacillus* spores in nature (in soil, dust, water, and other animal sources) and in the hospital environment, this organism is usually considered a saprophyte or contaminant when detected in clinical specimens of different sources [10]. Dissemination of *Bacillus* species among hospitalized patients has previously been reported [11]. Most of these events were later considered nosocomial pseudoepidemics and were frequently secondary to the contamination of equipment and environments such as a fiber-optic bronchoscope, an air filtration system, a ventilator, a water bath, and a radiometric blood culture analyzer in microbiology laboratories [12].

*Bacillus subtilis* is an aerobic, spore-forming bacterium with soil as its natural habitat. It belongs to the family *Bacillaceae* but unlike its relatives *Bacillus cereus* and *Bacillus anthracis* it is not pathogenic. The bacterium has served as a model organism for the Gram positive order for the past 50 years and its biochemistry, genetics and physiology has been extensively mapped. The genome of *B. subtilis* strain 168 was completely sequenced in 1997 [13].

*Bacillus cereus* is a ubiquitous, gram-positive, endospore-forming rod. Occasionally, it can cause wound infection, gastrointestinal infection, pneumonia, meningitis, septicemia or food poisoning [14]. Intraocular infections caused by *Bacillus cereus* are very rare, and most cases are related to trauma [15]. In several case series, vision outcomes were uniformly poor, with 75%–91% of patients experiencing a loss of light perception, eyeball evisceration, or enucleation [10]. Serious infections caused by bacteria that have become resistant to commonly used antibiotics have become a major global healthcare problem in the 21st century. The greatest threat to the use of antibiotics is the emergence and spread of resistance in pathogenic bacteria that consequently cannot be treated by previously successful regimens [16].

Extended-spectrum β-lactamases in gram-negative pathogens have been implicated as enzymes responsible for resistance to β-lactam antibiotics such as ceftazidime and aztreonam [17]. Initially these enzymes were identified in isolates in Western Europe, where major outbreaks of ceftazidime resistant members of the family Enterobacteriaceae have been described, particularly involving *Klebsiella pneumoniae* [18]. *Enterobacter* species, in particular, *Enterobacter cloacae* and *Enterobacter aerogenes* are able to compromise
antibacterial treatment by over expressing the chromosomal AmpC beta lactamase [19]. Emergence and spread of Class A extended-spectrum beta-lactamases (ESBLs) among these species are further complications [20].

The extended spectrum β-lactamases have been identified most often among strains of \textit{Klebsiella pneumoniae} responsible for outbreaks of nosocomial infections particularly in intensive care units but also in oncology units and chronic care facilities [21]. This investigation was designed to isolate and characterize some multi-drug resistant nosocomial bacteria from two hospitals environment at Damietta.

**Materials and methods**

**Sites of samples collection**

Sixty swab samples were obtained from various items in the wards and staff apparels of two selected hospitals in Damietta Governorates; these include Kaf Saad General Hospital and El-Azhar University Teaching Hospital (30 samples from each hospital). The items from which the samples were collected include sink, floor, bed, bed cover, toilet floor, bed pan, ward wall and hospital staff apparel (protective gowns, hand groves and face-shield) within reception hall, maternity ward, convalescing wards, surgical theaters, intensive care, dental unit, pharmacy and laboratory of each hospital.

Sterile swab tubes each containing prepared Cled broth were labeled appropriately and were taken to the hospital. Swabbing of the surface of each item was made using sterile cotton wool soaked with Cled broth. Many portions as possible of each item were swabbed and more than one swab stick was used for each subject. The swabs were inoculated into each labeled tube and covered. All the samples were immediately conveyed to the laboratory for processing [22].

**Antibiogram (antibiotic sensitivity test)**

Antibiotic susceptibility of the bacteria isolates was assayed according to the disc diffusion method [23]. Few colonies of each bacterial isolates were picked with a wire loop from the original culture plate and introduced in to test tubes containing 4 ml of nutrient broth medium. The tubes were incubated for 3 to 4 hours at 37°C. Petri dishes 9 cm were used with cled agar, plates were dried for about 30 min before incubation. Each bacterial broth suspension was streaked evenly in three planes into the surface of the medium with a sterilize cotton swab. After the inoculum was dried, standard commercial paper discs containing known amounts of the selected antibiotics (Trimethoprim/sulphamethoxazole (25 µg); Rifampicin (5 µg); Piperacillin/tazobactam (110 µg); Ofloxacin (5 µg); Nitrofurantoin (300 µg); Imipenem (10 µg); Gentamicin (120 µg); Ceftriaxone (30 µg); Cefotaxime (30 µg); Amikacin (30 µg) and Amoxicillin/clavulanic acid (30 µg) were gently passed down with flamed forceps to ensure contact and the plates were kept in refrigerator at 4°C for 1-2 hour, then the plates were incubated at 37°C for 24 hour. After the incubation, inhibition zone around each disc was measured for each disc and used to classify the organisms as sensitive or resistant to an antibiotic according to the interpretive standard of the clinical and laboratory standards institute [24]. The bacterial isolates were designated Sensitive (S), Intermediate (I) or Resistant (R).

**Morphological and biochemical characteristics**

Colonial characters, Microscopic examination of bacterial isolates (Gram and Endospore stain), Motility test, Biochemical test: Catalase test [25]. Coagulase tests [26]; Urease Test [27]; Triple sugar iron (TSI) test [28]; Oxidase test [29]; Methyl red test (MR); Voges Proskauer test; Citrate utilization test [30] and Reduction of nitrates. The bacterial isolates were identified by the following bacterial key [30,31].

**Extraction of DNA and PCR amplification**

The bacterial genomic DNA extraction from bacterial cells was carried out using DNA purification kit (QIAGEN) according to manufacturer's instructions.

The universal Primers F (5'-AGAGTTTGATCCTGCTCAG-3’) and R (5'-AAGCAGGTTGATCCTCCAGG-3’), corresponding to the polymorphic region of bacterial 16S rRNA [32] were used to amplify the 16S rRNA gene. Briefly, 1 µl of the forward and reverse primers was added to 2.5 µl Taq polymerase buffer 10x (Promega, Madison, USA) containing a final concentration of 1 mM MgCl₂, 0.2 mM dNTPs and 0.2 µl Taq polymerase (5U/ µl) in a final reaction volume of 25 µl. PCR reaction conditions were initial denaturation at 95°C for 5 min, 34 cycles at 95°C for 1 min, 60°C for 1 min.
and 72°C for 1 min. Final extension at 72°C for 10 min was done. The results were visualized on 1.5 % agarose gel stained by ethidium bromide and photographed using gel documentation system.

The amplified PCR products were sequenced using forward primer. Sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and model 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Alignment and phylogenetic analysis

Blast was performed to obtain the DNA sequence match with the best similarities with other related 16S rRNA genes on database. Pair wise and multiple DNA sequence alignment were carried out using CLUSTALW multiple sequence alignment programme version 1.82 [33]. Bootstrap neighbour joining tree was generated using MEGA version 4 [34]. The Streptomyces coelicolor was used as an outgroup strain.

Results

Sixty clinical site swabs were collected from various items of Kafr Saad General Hospital and El-Azhar University Teaching Hospital and investigated. From the 60 sites swabs processed, 37 (61.2 %) swabs yielded at least one bacterial isolate and the remaining 23 (38.8 %) swabs did not yield any bacterial growth. Direct gram stain recovered 206 organisms from the swabs in the form of bacilli, cocci or a mixture of the two. The bacterial isolates were cultured on specific media and observed as the most frequent and were given serial code numbers.

Based on the morphological characterization (Table 1) and some specific biochemical reactions (Table 2) of the most resistant, intermediate and sensitive bacteria, the isolates were identified as Bacillus subtilis, Bacillus cereus, Enterobacter aerogenes and Klebsiella pneumoniae.

The results of this study indicated that most of the isolated bacterial strains (Table 3) are susceptible to most the tested antibiotics. From the results it was observed that Bacillus subtilis (the most resistant isolate) was resistance to Amoxicillin/clavulanic acid (30 µg), Cefotaxime (30 µg); Ceftriaxone (30 µg); Nitrofurantoin (300 µg); Rifampicin (30 µg) and Trimethoprim/Sulphamethoxazole (1.25 µg) while, Enterobacter aerogenes and Klebsiella pneumoniae (intermediate isolates) showed resistance toward Amoxicillin/ clavulanic acid (30 µg), Cefotaxime (30 µg) and Ceftriaxone (30 µg), Nitrofurantoin (300 µg), respectively. On the other hand, Bacillus cereus (the most sensitive isolate) was appeared to be sensitive to all tested antibiotics.

Table 1. Morphological characteristics of the most resistant, intermediate and sensitive clinical bacterial isolate.

<table>
<thead>
<tr>
<th>Microscopy and cultural characters</th>
<th>Bacillus subtilis</th>
<th>Bacillus cereus</th>
<th>Enterobacter aerogenes</th>
<th>Klebsiella pneumoniae</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gram Stain</strong></td>
<td>+ ve</td>
<td>+ ve</td>
<td>- ve</td>
<td>-ve</td>
</tr>
<tr>
<td><strong>Shape</strong></td>
<td>Rods</td>
<td>Rods</td>
<td>Rods</td>
<td>Rods</td>
</tr>
<tr>
<td><strong>Spore formation</strong></td>
<td>+ ve</td>
<td>+ ve</td>
<td>- ve</td>
<td>- ve</td>
</tr>
<tr>
<td><strong>Pigmentation on cled agar media</strong></td>
<td>Forming a green pigment</td>
<td>Forming a white pigment</td>
<td>Forming a yellow pigment</td>
<td>Forming a green pigment</td>
</tr>
<tr>
<td><strong>Growth on cled agar media</strong></td>
<td>Grow on cled medium, circular; opaque; regular; glistening and convex</td>
<td>Grow on nutrient medium, circular; opaque; regular</td>
<td>Grow on Cled medium, circular; opaque; regular; glistening and convex</td>
<td>Grow on nutrient medium, circular; opaque; regular; glistening and convex</td>
</tr>
<tr>
<td><strong>(isolation media)</strong></td>
<td>No formation of pink colony (-ve)</td>
<td>No formation of pink colony (-ve)</td>
<td>No formation of pink colony (+ve)</td>
<td>No formation of pink colony (+ve)</td>
</tr>
<tr>
<td><strong>Haemolysis on blood agar</strong></td>
<td>- ve</td>
<td>- ve</td>
<td>α-haemolysis (green) - ve</td>
<td>- ve</td>
</tr>
<tr>
<td><strong>Triple Sugar Iron (TSI) Agar</strong></td>
<td>Orange pink, no black color</td>
<td>Pink, no black color</td>
<td>Yellow, no black color</td>
<td>Yellow, no black color</td>
</tr>
</tbody>
</table>

* + ve, positive - ve, negative
Table 2. Biochemical reactions of the most resistant, intermediate and sensitive clinical bacterial isolate.

<table>
<thead>
<tr>
<th>Biochemical tests</th>
<th>Bacillus subtilis</th>
<th>Bacillus cereus</th>
<th>Enterobacter aerogenes</th>
<th>Klebsiella pneumoniae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidase test</td>
<td>-ve</td>
<td>-ve</td>
<td>- ve</td>
<td>- ve</td>
</tr>
<tr>
<td>Lactose Fermentation</td>
<td>-ve</td>
<td>- ve</td>
<td>+ ve</td>
<td>+ ve</td>
</tr>
<tr>
<td>Indole test</td>
<td>-ve</td>
<td>+ve</td>
<td>- ve</td>
<td>- ve</td>
</tr>
<tr>
<td>Urease test</td>
<td>-ve</td>
<td>+ve</td>
<td>- ve</td>
<td>+ ve</td>
</tr>
<tr>
<td>H2S production</td>
<td>-ve</td>
<td>-ve</td>
<td>- ve</td>
<td>- ve</td>
</tr>
<tr>
<td>Catalase test</td>
<td>+ve</td>
<td>+ve</td>
<td>- ve</td>
<td>+ ve</td>
</tr>
<tr>
<td>Voges-Proskauer</td>
<td>+ve</td>
<td>+ve</td>
<td>+ ve</td>
<td>+ ve</td>
</tr>
<tr>
<td>Methyl Red</td>
<td>-ve</td>
<td>+ve</td>
<td>- ve</td>
<td>- ve</td>
</tr>
<tr>
<td>Citrate utilization</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ ve</td>
</tr>
<tr>
<td>Casein Hydrolysis</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>- ve</td>
</tr>
<tr>
<td>Starch Hydrolysis</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ ve</td>
</tr>
<tr>
<td>Gelatin Liquefaction</td>
<td>+ve</td>
<td>-ve</td>
<td>- ve</td>
<td>- ve</td>
</tr>
<tr>
<td>Nitrile reduction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nitrite</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ ve</td>
</tr>
<tr>
<td>Ammonia</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ ve</td>
</tr>
<tr>
<td>Tyrosine Hydrolysis</td>
<td>-ve with</td>
<td>+ve</td>
<td>- ve</td>
<td>+ ve with pigmentation</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>-ve</td>
<td>-ve</td>
<td>- ve</td>
<td>- ve</td>
</tr>
<tr>
<td>determination</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Utilization of sugars</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salicin</td>
<td>-ve</td>
<td>-ve</td>
<td>+ ve acid</td>
<td>+ ve</td>
</tr>
<tr>
<td>Glycerol</td>
<td>-ve</td>
<td>+ve acid</td>
<td>- ve acid</td>
<td>+ ve</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>+ve</td>
<td>-ve</td>
<td>+ ve acid</td>
<td>+ ve</td>
</tr>
<tr>
<td>D-Mannitol</td>
<td>+ve</td>
<td>+ve acid</td>
<td>+ ve acid</td>
<td>+ ve</td>
</tr>
<tr>
<td>L-Arabinose</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ ve</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>+ve</td>
<td>+ve</td>
<td>+ ve acid</td>
<td>+ ve</td>
</tr>
<tr>
<td>Maltose</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ ve</td>
</tr>
<tr>
<td>Rhamnose</td>
<td>-ve</td>
<td>- ve</td>
<td>+ ve</td>
<td>+ ve</td>
</tr>
<tr>
<td>Galactose</td>
<td>-ve</td>
<td>- ve</td>
<td>- ve</td>
<td>- ve</td>
</tr>
<tr>
<td>Mannose</td>
<td>-ve</td>
<td>- ve</td>
<td>+ve</td>
<td>+ ve</td>
</tr>
<tr>
<td>Fructose</td>
<td>+ve</td>
<td>+ve acid</td>
<td>+ ve acid</td>
<td>+ ve</td>
</tr>
<tr>
<td>Trehalose</td>
<td>-ve</td>
<td>+ ve</td>
<td>+ ve acid</td>
<td>+ ve</td>
</tr>
<tr>
<td>Myo-Inositol</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ ve</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+ve</td>
<td>+ve acid</td>
<td>+ ve acid</td>
<td>+ ve</td>
</tr>
<tr>
<td>Sorbose</td>
<td>-ve</td>
<td>-ve</td>
<td>- ve</td>
<td>- ve</td>
</tr>
<tr>
<td>Acid from Glucose</td>
<td>+ve</td>
<td>-ve</td>
<td>- ve</td>
<td>+ ve</td>
</tr>
</tbody>
</table>

* + ve, positive - ve, negative

PCR amplicon with specific forward 16S rRNA primers of the four bacterial isolates were subjected to DNA sequence analysis. Data showed that 663 bp was obtained for *Bacillus subtilis*, 660 bp for *Bacillus cereus*, 597 bp for *Enterobacter aerogenes* and 764 bp for *Klebsiella pneumoniae* isolates.

BLAST homology search for the resulted sequences of the four isolates revealed that, *Bacillus subtilis* isolate possessed 99% identity with other strains of *Bacillus subtilis, Bacillus subtilis subsp. subtilis, Bacillus subtilis subsp. spizizenii, Bacillus subtilis subsp. inaquosorum, Bacillus sp, Bacillus tequilensis, Bacillus*.
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amylo liquefaciens and Bacillus methylotrophicus. The Bacillus cereus isolate is identical with strains of other Bacillus cereus, Bacillus sp., Bacillus thuringiensis and Bacillus anthracis by 100% identity. Also, Klebsiella pneumoniae isolate possessed 100% identity with other strains of Klebsiella pneumoniae, Klebsiella pneumoniae subsp. pneumoniae, Klebsiella pneumoniae subsp. rhinoscleromatis, Klebsiella sp., Klebsiella varicola and Klebsiella rhinoscleromatis. The Enterobacter isolate showed 99% identity with other members of Enterobacteriales species such as Enterobacter hormaechei, Enterobacter ludwigii and Enterobacter cloacae, Enterobacter cancerogenus. The similarity distance between the four isolates and other related strains was represented as a phylogenetic tree (Fig. 1).

Table 3. Antibiotic susceptibility profile (sensitivity test) of clinical bacterial isolates against different antibiotics drugs.

<table>
<thead>
<tr>
<th>No. of Bacterial isolates</th>
<th>Antibiotic drugs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>Amikacin (30 µg) AK*</td>
<td>17 S</td>
</tr>
<tr>
<td>Amoxicillin/clavulanic acid (30 µg) AMC</td>
<td>0 R</td>
</tr>
<tr>
<td>Cefotaxime (30 µg) CTX</td>
<td>0 R</td>
</tr>
<tr>
<td>Ceftriaxone (30 µg) CRO</td>
<td>0 R</td>
</tr>
<tr>
<td>Centamicin (10 µg) CN</td>
<td>15 S</td>
</tr>
<tr>
<td>Imipenem (10 µg) IPM</td>
<td>16 S</td>
</tr>
<tr>
<td>Nitrofurantoin (300 µg) F</td>
<td>0 R</td>
</tr>
<tr>
<td>Ofloxacin (5 µg) OFX</td>
<td>23 S</td>
</tr>
<tr>
<td>Piperacillin/Tazobactam (110 µg) TPZ</td>
<td>22 S</td>
</tr>
<tr>
<td>Rifampicin (30 µg) RD</td>
<td>2 R</td>
</tr>
<tr>
<td>Trimethoprim/ Sulphamethoxazole (1.25 µg) SXT</td>
<td>0 R</td>
</tr>
</tbody>
</table>

* Abbreviation of the antibiotics; Diameters of inhibition zone in millimeter; R: Resistant; S: Susceptible and I: Intermediate

Discussion

Nosocomial infections occur worldwide and affect both developed and developing countries [35]. Many of these infections are associated with microorganisms that are resistant to antibiotics and can easily spread by hospital personnel [36]. Guidelines for antibiotic therapy can be helpful for clinicians to select more appropriate antibiotics for effective treatment and prevent the development of drug resistance [22]. This study shows the distribution of antibiotic resistance of bacterial species associated with nosocomial infections at a hospital in Damietta, Egypt, and this showed that they have become multi-resistant to these therapeutic agents, thus rendering these drugs ineffective as treatments of choice for infections caused by these pathogens.

Antibiotic resistance is a problem that continues to challenge the healthcare sector. Furthermore, in developing countries drugs are available to the public and thus people may practice self-administration of antibiotics and further increase the prevalence of drug resistant strains [37].

From the antibiotic susceptibility profile of the bacterial isolates, four strains labeled and identified as Bacillus subtilis, Bacillus cereus, Enterobacter aerogenes and Klebsiella pneumoniae were selected as most resistant, most sensitive and two intermediates, respectively.

The result of this study is consistent with Jalalpoor and Ebadi [38] who reported Bacillus species was the most frequent bacteria isolated in hospital environment followed by Enterobacteriace. Also this result correlates with the previous study on bacterial epidemiology in hospitals, where Bacillus species and Staphylococcus species were the major bacteria that were isolated from the hospital environment [39].
Fig. 1. Phylogenetic tree analysis based on the 16S rDNA sequence alignment for *Klebsiella pneumoniae*, *Enterobacter aerogenes*, *Bacillus subtilis* and *Bacillus cereus* with some other related members which possess the best similarity.

Nowadays, about 70% of bacteria causing infections in hospitals are resistant to at least one of the drugs most commonly used for treatment. Imipenem has proved to have a broad spectrum and high activity against all the selected Gram positive and Gram negative bacterial isolates.
These results are in accordance with data reported in previous studies of Tohamy et al. [41]. Moreover, the entire organisms showed higher resistance to Amoxicillin/clavulanate except Bacillus cereus. This finding is similar to the work of Muhammad et al. [22] who recorded higher resistance of Amoxicillin/clavulanate among both gram negative and Staphylococcus aureus. Enterobacteriaceae isolates resistant to multiple antibiotics have also been reported from several parts in the world [42].

The 16S rRNA sequence analysis confirmed the classical identification of the four isolated strains as they possessed a very high identity reach to 99-100%.

In spite of Bacillus subtilis isolate showed 99% identity with Bacillus tequilensis, Bacillus amyloliquefaciens and Bacillus methylotrophicus and other Bacillus subtilis strains, the classical biochemical tests were more related to Bacillus subtilis. The Bacillus cereus isolate is identical with strains of Bacillus thuringiensis, Bacillus anthracis and other strains of Bacillus subtilis by 100 % identity, but the classical biochemical tests forced us to identify it as Bacillus cereus. Also, Klebsiella pneumoniae isolate which possessed 100 % identity with other strains of Klebsiella pneumonia, Klebsiella varicola and Klebsiella rhinoscleromatis, the classical biochemical identification suggested to name it as Klebsiella pneumoniae. The species of Enterobacter is isolated named aerogenes according to the classical biochemical tests, although it showed 99 % identity with Enterobacter hormaechei, Enterobacter ludwigii Enterobacter cloacae, Enterobacter cancerogenus and other Enterobacter aerogenes strains.

The antibiotic susceptibility results of the four bacterial isolates showed that, these organisms have been well exposed to the tested antimicrobials and they have developed mechanisms to evade or avoid these antibiotics which full agreement with Celik et al. [2]. Because of the prevalent of multiple antibiotic resistant bacteria search for new antibiotics effective against multi-drug resistant pathogenic bacteria is presently an important area of antibiotic research.

References

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Resistant pathogens and nosocomial infections

الملخص العربي

عزل وتعريف بعض الأجناس البكتيرية المقاومة للمضادات الحيوية والمرتبطة بعدوى المستشفيات

تم عزل مائتي وست عزلة بكتيرية من أماكن مختلفة من مستشفى كفر سعد العام بدمياط ومستشفى الأزهر التعليمي بدمياط الجديدة. وتم دراسة تأثير أحد عشر مضاد حيوياً (ميثوبريم/سلفاميثوكزازول (25 ميكروجرام)، الريفامبين (5 ميكروجرام)، البيبريسيلين/تازوبكتام (110 ميكروجرام)، أوفلوكساسين (5 ميكروجرام)، التيترافورانتونين (300 ميكروجرام)، الإيثاميسين (10 ميكروجرام)، الجنتاميسين (120 ميكروجرام)، سيفاتواكسيم (30 ميكروجرام)، أميكاسينون (30 ميكروجرام) وحمض الكلوفالانك/أموكسيسيلين (30 ميكروجرام). وتم تعريف بعض الأنواع على أنها باسيلس ساتلس، باسيلس سيرز، كيلبسيلا بنيومنيا، وأنتيروباكتر اروجينز. ووجد أن أكثر الأنواع حساسية وغير مقاومة للمضادات الحيوية باسيلس سيرز بينما باسيلس ساتلس أدت مقاومة لستة أنواع من المجموعة المستخدمة. أظهرت أنتيروباكتر اروجينز مقاومة حمض الكلوفالانك/أموكسيسيلين وسيفاتواكسيم، بينما أبدت كيلبسيلا بنيومنيا مقاومة سيفترياكسون والتيترافورانتونين.
Performance and chemical composition of three Echinochloa grasses over short term experiment

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Abstract

Three weedy grasses, Echinochloa crus-galli (L.) P. Beauv. Echinochloa colona (L.) Link. and Echinochloa stagnina (Retz.) P. Beauv. were grown for four months in the greenhouse of Botany Department, Faculty of Science at New Damietta to examine the effect of age on plant biomass, ash, proteins, total carbohydrates, lipids and fiber content. Plants were collected from the field and replanted in greenhouse, with the same age and size. Biomass and ash content increased by increasing the age in E. colona and E. stagnina but increased in E. crus-galli till the fourth harvest then decreased. Protein, crude lipid and crude fiber content were decreased gradually by the increase of the age, in the three species throughout the progressive harvests. By the increase of the age total carbohydrates increased in E. crus-galli and E. colona but remained largely unchanged in E. stagnina. The crude fiber was a relatively high in E. crus-galli and E. colona but this was not good for forage use of the grass. The amount of PEPC and Rubisco protein was higher in E. stagnina than in E. crus-galli and E. colona and this lead to increase in plant biomass of E. stagnina than E. crus-galli and E. colona. It is recommended using E. stagnina as fodder additives due to its high content of protein and biomass and low fiber content.

The obtained results will be useful for the optimum managing strategy of these three grasses as invasive weeds in field crop as well as use them as a source of natural forage in particular in summer.

Keywords: Ash, Biomass, Carbohydrates, Echinochloa, Fibers, Forage, Lipids PEPC, Rubisco

Introduction

Echinochloa is a cosmopolitan genus belonging to Subfamily Panicoideae of the family Poaceae comprised of 20 to 25 species [1]. Although considered a weed in many agricultural systems, it is also valued by wildlife managers as a food source for waterfowl [2]. The genus Echinochloa includes serious weeds in agriculture being the third and fourth most important weeds in the world [3,4]. Echinochloa crus-galli (L.) Beauv. is a C4 weed that grows in paddy fields and distributed worldwide. It has adapted to temperate regions and anaerobic conditions such
as rice fields and wetlands, but it is also used for reclamation of saline soil especially in Egypt [5]. The broad ecological adaptation, such as the ability to mimic rice, quick germination, rapid growth and abundant seed production, make it a successful weed [6]. E. crus-galli includes many sub-species and varieties. Echinochloa colona, is a vigorous C4 annual species, is one of the world’s most serious grass weeds in rice [7,4]. More than 60 countries have reported it as a weed problem in 35 crops, including rice (Zea mays L.), sorghum (Sorghum bicolor L.), cotton (Gossypium hirsutum L.), and peanuts (Arachis hypogea L.) [7]. E. colona is an example of crop mimicry because it closely resembles rice at the seedling stage and is sometimes unintentionally transplanted into fields together with the crop. By the time the weed can be easily recognized by farmers and removed, crop yield losses may already be unavoidable [7]. E. colona had been reported to reduce yield of direct-seeded rice by as much as 76% [8]. Echinochloa stagnina, is a C4 plant, a perennial grass, it presents as weeds in deep-water paddies and river channels [9,10].

Nutritive value of forage refers to its chemical composition, digestibility and the nature of digested products [11,12]. The amount of forage consumed by the animal is very important, as it affects total nutrient intake and therefore the animal’s response. The efficiency of ruminant production systems based on forages as the main source of protein and energy is strongly dependent on forage maturity, which is considered a primary factor decreasing its nutritional quality [13]. These modifications are well evident in forages at different stages of development (e.g. vegetative vs. reproductive or mature). Age and the prevailing environmental conditions may also affect the growth and nutritive value of the used grass. Variation in growth patterns, dry matter accumulation and tiller production [14].

The highest known productivity in natural vegetation is for a C4 perennial grass which achieves about of 100 t (dry matter) ha\(^{-1}\) year\(^{-1}\) [15,16]. Some of the world’s most productive crops and pasture, such as maize (Zea mays), sugarcane (Saccharum officinarum), sorghum (Sorghum bicolor), Bermuda grass (Cynodon dactylon), and Rhodes grass (Chloris gayana) are C4 plants. In addition, the most troublesome weeds like nutgrass, crabgrass and barnyard, are also C4 species. Although C4 plants represent only a small portion of the world’s plant species, accounting for only 3% of the vascular plants, they contribute about 20% to the global primary productivity because of the highly productive C4 grass-lands [17].

The objective of the present study was to examine the effect of age on plant biomass, ash, proteins, total carbohydrates, lipids and fiber content of the three weedy grasses in the coastal area of Damietta.

Materials and methods

Studied species

*Echinochloa crus-galli* is an erect to decumbent, often branched at base, tufted, stout annual, graminoid; up to 3 ft. (100 cm) tall or more, fibrous roots, culms often branching at base, and branching culms ascending from decumbent branches; sometimes rooting at lower culms nodes, culms stout, up to 60 in. (50-150 cm) high, glabrous, nodes slightly swollen [18-22].

*Echinochloa colona* is a tufted, erect to spreading and semi-prostrate, annual graminoid; up to 16 in. (60 cm) tall or more, fibrous roots, culms freely branching at base and lower nodes, culms 8-16 in. (20-60 cm) or more high, glabrous or nodes pubescent [18-23].

*Echinochloa stagnina* is an annual or perennial, coarse, often succulent grass which floats in deep water or creeps with rhizomes and stolons, to 2 m long, readily and sprouts at nodes, stems up to about 1 m long under non-flooded conditions and elongating up to 2 m in flooded areas, under flooded conditions, the stem trails on the surface of the water but the leaves and inflorescence are held in an upright position above the water [18,24,25].

Experimental design

The study was conducted using a randomized complete block design with 3 replicates, 9 blocks represent all treatments, the area of each block approximately 0.5 m\(^2\). The abovementioned grasses were collected from the natural habitat and replanted in green house in the third week of May 2011, the age of the plant was one month before beginning the experiment in both species *E. crus-galli* and *E. colona* but in *E. stagnina* similar cuts of 3 internodes are planted. Nitrogen fertilizer was applied a sure a in the third week of experiment. The first harvest began after one month for two species *E. crus-galli* and *E. colona*
Performance and chemical composition of *Echinochloa* Scientific Journal for Damietta Faculty of Science 3 (1) 2014, 43-51

and after two months for *E. stagnina*. The other harvest was taken monthly till the end of experiment.

**Chemical Analysis**

*E. crus-galli*, *E. colona* and *E. stagnina* shoots were harvested and oven-dried at 65°C for 72 hr. Biomass was calculated as dry weight per plant. Dried shoots were used for chemical analysis.

Dried shoots were ground to pass a 1 mm sieve and stored in airtight containers prior to chemical analysis. Total ash was determined by igniting at 550°C for 2 h. Protein content of dry powdered plant material was determined according to the method of Bradford [26]; while, carbohydrates were determined according to the method of Schortemeyer et al. [27]. Crude lipids and crude fibers were estimated according to the method of Nesamvuni et al. [28].

**Measurement of PEPC and Rubisco**

The protein content of fresh plant material was determined according to the method of Bradford [26].

**SDS-PAGE of protein for PEPC and Rubisco quantification**

Proteins were resolved as described by Laemmlli [29] using the BioRad Mini Protean 3 (BioRad laboratories, Hercules, CA, USA). The resolving gel contained 11% acrylamide and the stacking gel contained 5% acrylamide. Proteins from 3 mg leaf fresh weight were loaded onto each lane. The proteins were resolved at 100 V for 90 min. The gels were stained with brilliant blue R-250 (BioRad) and then destained with 20% methanol, scanned and used for PEPC and Rubisco quantification.

**Statistical analysis**

One-way ANOVA-LSD was performed by entering the data into two computer programs SPSS 18.0 and CoStat 6.311.

**Results**

Dry weight of *E. colona* and *E. stagnina* increased but in *E. crus-galli* it increased until the fourth harvest then decreased in the last one, the highest dry weight was found in the fourth harvest of *E. stagnina* (115.16 g plant⁻¹) and the lowest one was found in the first harvest of *E. crus-galli* (6.34 g plant⁻¹) (Fig. 1).

![Fig. 1. Changes in dry biomass over monthly harvests of *E. crus-galli*, *E. colona* and *E. stagnina*. h1 (55 days), h2 (80 days), h3 (105 days), h4 (130 days), h5 (156 days), (ND) not detected. Data is mean±SE. Bars labeled with different letters are significantly different at p<0.05.](image1)

Ash content was increased by increasing the age of both *E. colona* and *E. stagnina* but in *E. crus-galli* it increased in the third harvest then decreased in the fourth and the fifth harvest, the highest ash content was found in the fourth harvest of *E. stagnina* (19.67%), the lowest value was observed in the first harvest of *E. stagnina* (11%) (Fig. 2).

![Fig. 2. Changes in ash content over monthly harvests of *E. crus-galli*, *E. colona* and *E. stagnina*. h1 (55 days), h2 (80 days), h3 (105 days), h4 (130 days), h5 (156 days), (ND) not detected. Data is mean±SE. Bars labeled with different letters are significantly different at p<0.05.](image2)
Monthly harvests indicated different changes in the chemical composition of *E. crus-galli*, *E. colona* and *E. stagnina*. Protein content decreased by increasing the age in the three plants gradually and significantly along the progressive harvests, except that in *E. colona*, it similar in the third harvest to the first one, the highest protein content was observed in the first harvest of *E. crus-galli* (266.43 mg g$^{-1}$) and the lowest one was found in the fourth harvest of *E. stagnina* (83.23 mg g$^{-1}$) (Fig. 3).

By increasing the age, total carbohydrates of *E. crus-galli* and *E. colona* increased but remained largely unchanged in *E. stagnina*. The highest total carbohydrate content (Fig. 4) was found in the third harvest of *E. stagnina* (2.2 mg g$^{-1}$) and the lowest one was observed in the first harvest of *E. crus-galli* (0.76 mg g$^{-1}$). The crude lipids content decreased by increasing the age of *E. colona*, but in *E. crus-galli* and *E. stagnina* where it decreased by increasing the age then increased in the last harvest (Fig. 5). The highest crude lipid content was found in the fourth harvest of *E. stagnina* (3.23%) and the lowest one was observed in the fourth harvest of *E. crus-galli* (0.78 %).

Crude fibers decreased by increasing the age of plants in *E. crus-galli*, but not in *E. colona* and *E. stagnina* where it decreased in the second harvest then increased in the last harvest than the first. Highest crude fibers was found in the third harvest (Fig. 6) of *E. colona* (81.3%) and the lowest one was found in the second harvest of *E. stagnina* (36.9%). The amount of PEPC protein in *E. stagnina* was higher than that in *E. crus-galli* and *E. colona* (3-fold and 95.4%, respectively). Rubisco protein content in *E. stagnina* was slightly higher than those of *E. crus-galli* and *E. colona*. The ratio of Rubisco/PEPC in *E. crus-galli* was higher than in *E. colona* and *E. stagnina* where increased by 38% and 2.58-fold, respectively (Fig. 7 A&B).
Fig. 6. Changes in fiber content over monthly harvests of *E. crus-galli*, *E. colona* and *E. stagnina*. h1 (55 days), h2 (80 days), h3 (105 days), h4 (130 days), h5 (156 days), (ND) not detected. Data is mean±SE. Bars labeled with different letters are significantly different at *p*<0.05.

**Discussion**

The biomass (dry weight) increased by increasing the age. In *E. crus-galli* it increased from 6.3 g plant⁻¹ in 55-d old to 81 g plant⁻¹ in 130-d old. In *E. colona* it increased from 6.5 g plant⁻¹ in 55-d old to 22 g plant⁻¹ in 105-d old. In *E. stagnina* it increased from 75 g plant⁻¹ in 55-d old to 115 g plant⁻¹ 130-d old. The present results of dry weight in line with wheat grass (*Thinopyrum paniticum*) 94-780 g plant⁻¹ [30].

Ash content was increased by increasing the age of *E. crus-galli*, *E. colona* and *E. stagnina*. It increased in *E. crus-galli*, and *E. stagnina* from 12.9 and 11% in 55-d old, respectively to 15.6 and 19.6 % in 130-d old, respectively. And in *E. colona* it increased from 13% in 55-d old to 18.3 % in 105-d old. Ash content of three species of *Echinochloa* were similar to that of *Artriplax ummularia* 18.1 % [31], lower than that of *Artriplax halimus* 26.9 % [32], but higher than those reported for *Bromus erectus, Horedum marinum* and *Dactylis glomerata* [33].

Total carbohydrates content increased by increasing the age of *E. crus-galli*, *E. colona* and *E. stagnina*. It increased in *E. crus-galli* and *E. stagnina* from 0.7 and 1.9 mg g⁻¹ in 55-d old to about 2, 2.2 mg g⁻¹ in 130-d old. And in *E. colona* it increased from 0.8 mg g⁻¹ in 55-d old to 1.26 mg g⁻¹ in 105-d old. Results of our study are in agree with Donaghy and Fulkerson [34] who reported that carbohydrates and its composition in grass had been correlated with its growth.

Fig. 7. (A): SDS-page shows the protein bands of PEPC and Rubisco in (1) *E. crus-galli*, (2) *E. colona* and (3) *E. stagnina*. (B) Level of PEPC, Rubisco and Rubisco/PEPC in *E. crus-galli*, *E. colona* and *E. stagnina*. Data is mean±SE.
Also, the obtained results are in agreement with those of cocksfoot (*Dactylis glomerata*) where total carbohydrate increased from 101 mg g\(^{-1}\) in September to 214 mg g\(^{-1}\) in November [35]. Carbohydrate content of *Echinochloa* sp. was lowest than that of other grasses crested wheatgrass (*Agropyron cristatum*) 39 g g\(^{-1}\) and wide leaf orchard grass (*Dactylis glomerata*) 23 g g\(^{-1}\) [36]. The nonstructural carbohydrates and structural polysaccharides or dietary fiber which comprises the principal components of cell walls [37] are considered to be the primary energy source for ruminant animals.

However, the fiber content of ryegrass can vary from being deficient in a vegetative state (between May and August) [38], to excess [39], after seed set or where moisture is deficient in summer. Non-structural carbohydrates are important in that they are a readily fermentable source of energy in the rumen and may also be important in synchronizing with rumen ammonia from the high protein intakes and common in cows grazing pasture [40].

Protein content was high in young plants in *E. crus-galli* and *E. stagnina* which began with 266 mg g\(^{-1}\), 207 mg g\(^{-1}\) in 55-d old, respectively then decreased to 113 mg g\(^{-1}\), 83 mg g\(^{-1}\) in 130-d old, respectively. It increased by the increase of the age in *E. colona* where began with 219 mg g\(^{-1}\) in 55-d old and reached to 262 mg g\(^{-1}\) in 105-d old. Tuna et al. [41] reported that the protein content of some grasses was between 385 and 780 mg g\(^{-1}\), these values are not in agreement with the present study where protein content of *Echinochloa* sp. is between 83 and 266 mg g\(^{-1}\). The results of *E. colona* are in line with Minson [42] who showed that the crude protein content was lowest in mid-summer (170 g kg\(^{-1}\)) and highest in autumn (230 g kg\(^{-1}\)), due to the increased proportion of leaf in the forage. Results of *E. crus-galli* and *E. stagnina* agree with Lima et al. [43] which reported that hay components decreased linearly with increased growth age of *Echinochloa* sp. (rice grass). Also, the results agree with those in soybean *HLW-18* where it began with 228 mg g\(^{-1}\) in July and decreased to 177 mg g\(^{-1}\) in October [44]. Protein content of *Echinochloa* sp was similar to those of *Medicago sativa* and *Trifolium pretense* (206, 242 g kg\(^{-1}\)), respectively [45], but higher than that of *Panicum maximum* 177 g kg\(^{-1}\) [46] and *Atriplex nummularia* (92-131 mg g\(^{-1}\)) [31] and lowest than soybean (310-340 g kg\(^{-1}\)) [44].

Lipid content decreased by the increase of the age in *E. crus-galli* and *E. colona* but the contrast happen in *E. stagnina*. In *E. crus-galli* began with 3.15% in 55-d old and decreased to 1.6% in 156-d old. In *E. colona* it began with 3.16% in 55-d old and decreased to 1.3% in 105-d old. In *E. stagnina* began with 1.8% in 55-d old and reached to 3.2% in 130-d old. Lipid content of *Echinochloa* sp highest than other in *Medicago sativa, Trifolium pretense* 1.3, 1.8%, respectively [38] and *Atriplex halimus* 1.8 % [32].

Fiber content was decreased by increasing the age of *E. crus-galli* it began with 61% in 5-d old and decreased to 40% in 156-d old. But increased in both *E. colona* and *E. stagnina* from 68.6, 47% in 55-d old, respectively to 81, 80% in 130-d old, respectively. Fiber content of *Echinochloa* sp was higher than other in *A. halimus* 12.8% [32], *Atriplex nummularia* 2.15 % [31] and soybean (HLW-18) 58.0 % [44] and *Panicum maximum* 71.3 % [46].

Protein and carbohydrates content changes in relation to season [47,48], stage of growth [49,50], time of day [51], soil fertility or fertilizer application rate (particularly nitrogen (N)) [52] and probably soil moisture status.

Photosynthetic efficiency depends on the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39). When the atmospheric CO\(_2\) decreased and O\(_2\) increased, CO\(_2\) concentration mechanisms evolved to reduce photorespiration (oxygenase activity of Rubisco) [53].

The primary CO\(_2\) fixation to PEP is catalyzed by phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) in mesophyll cells. C4 acids are transported to bundle sheath cells, where they provide CO\(_2\) to Rubisco [53,54]. Plants with C4 photosynthesis have advantages in extreme growth conditions such as high temperature, low water availability, high irradiation or saline soils [53].

The amount of PEPC and Rubisco protein was higher in *E. stagnina* than in *E. crus-galli* and *E. colona* and this lead to increase in plant biomass of *E. stagnina* than *E. crus-galli* and *E. colona*. The present results are in line with Piedade et al. [15] and Long [16] who reported that C4 plants exhibit higher photosynthetic and growth rates due to gains in the water, carbon and nitrogen efficiency uses, The highest known productivity in natural vegetation is for a C4 perennial grass in the central Amazon, which achieves a net production of 100 t (dry matter) ha\(^{-1}\) year\(^{-1}\).
Conclusion

The present study indicated that *E. crus-galli*, *E. colona* and *E. stagnina* had a relatively high proteins in young plants, but because the low carbohydrate content were low in these plants, therefore, it can be considered as a source of protein as well as additives to the fodder. Crude fibers were relatively high in *E. crus-galli* and *E. colona* and this was not good for forage use. It is recommended to use *E. stagnina* as a summer fodder in marshlands where these grasses are dominant. It is a cheap source of natural forage. Furthermore, the obtained results will be useful for managing these invasive weedy grasses in field crops.

References


[49] J.F. Ayres, K.S. Nandra, A.D. Turner, The...


Evaluation of some plant extracts for controlling mycoflora causing spoilage of stored cereals and legumes

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Abstract

This study aimed to isolate and identify seed-borne fungi associated with some cereals and legumes and the possibility of their control by some plant extracts. The studied cereals and legumes were Sorghum bicolor, Triticum aestivum, Oryza sativa, Lens esculentus, Vigna sinensis, Arachis hypogea and Vicia faba. Thirteen fungal species were isolated from these cereals and legumes. Five fresh plants called Allium sativum, Aloe vera, Mentha basilicum, Musa acuminate and Eucalyptus rostrata and eleven dried plants called Datura stramonium, Zingiber officinale, Azadirachta indica, Jatropha curcas, Euphorbia peplis, Ocimum basilicum, Carum carvi, Rosmarinus officinalis, Nigella sativa, Cuminum cyminum and Citrullus colocynthis as medicinal plants were screened for their antifungal activities. Aqueous extracts of all mentioned plants were tested against Aspergillus niger, A. flavus and Penicillium chrysogenum in vitro because they represented the highest percentage of occurrence on seeds and grains. Allium sativum and Zingiber officinale exhibited maximum antifungal activity, whereas Datura stramonium and Citrullus colocynthis showed moderate activity. In addition, these plant extracts were tested against the germination of seeds and grains by using blotter plate method. Treated seeds and grains by plant extracts showed an increase of their germination (%) and reduction of seed-borne fungal infection.

Keywords: Antifungal activity, Cereals, Legumes, Medicinal plants, Seed-borne mycoflora

Introduction

Legumes (Fabaceae) are one of the most important plant families all over the world. It is used for human feeding, animals, poultry and other consumption. Legumes are the second after grasses (cereals) in providing food crops for world agriculture [1]. In comparison to cereal grains, the seeds of legumes are rich in high quality protein, providing man with a highly nutritious food resource [2].

Cereal grains have been the principal component of human diet for thousands of years and have played a major role in shaping human civilization. Around the world, rice, wheat, and maize, and to a lesser extent, are important...
staples critical to daily survival of billions of people. More than 50% of world daily caloric intake is derived directly from cereal grain consumption [3].

Seed-borne fungi have been found to affect the growth and productivity of crop plants including legumes and cereals [4, 5]. Presence or absence of seed-borne fungi on seed surface is one of the important aspects that determine the quality of seeds and grains. Attempts have been made to reduce seed-borne fungi by chemical treatment of the seeds and some successes have been reported. Seed dressings are used to eliminate most surface infestation of seeds but have relatively little effect on internally borne organisms [6]. However, the application of these fungicides may not always be desirable, owing to their toxic effects on non-target organisms [7].

An urgent need for alternatives to fungicides for the control of seed-borne fungi is important. In recent years, much attention has been given to nonchemical systems for seed treatment to protect them against seed-borne pathogens. Plant extracts have played a significant role in the inhibition of seed-borne pathogens and in the improvement of seed quality and field emergence of plant seeds. Many authors reported the effective and safe use of plant extracts for controlling seed-borne fungi [8-11]. However, in this study, the effects of water extracts of some plants on seed-borne fungi of some legumes and cereals; and on seed germination were investigated.

Materials and methods

Collection of legumes and cereals

Some stored cereals and legumes of economically important crops were collected from some markets in Damietta Governorate, Egypt such as sorghum (Sorghum bicolor), wheat (Triticum aestivum), rice (Oryza sativa), lentil (Lens esculentus), cowpea (Vigna sinensis), peanut (Arachis hypogea) and faba bean (Vicia faba).

Isolation and identification of Fungi

Fungal isolation from collected samples was carried out by using direct plating method. First, grains and seeds were surface sterilized using 2.5% sodium hypochlorite for 1 min and rinsed with distilled water and dried between sterile Whatman No. 1 filter papers. Then, five grains or seeds spaced out in the Petri dishes containing PDA medium (200 g potato + 20 g glucose + 20 g agar in liter distilled water). An antibacterial agent chloramphenicol (50 ppm) was used to inhibit the growth of bacteria. The Petri dishes were incubated at 27±2 °C for 5-7 days and examined daily for fungal growth [12]. The observed fungal colonies were sub-cultured to get pure culture. All isolates were identified according to their microscopic and macroscopic characteristics according to some authors [13-19].

Preparation of plant extracts

For dried samples, the plant materials were air-dried under the shade at 25-29°C until they became dry and crispy. Dried parts of the plants (Table 1) were ground using a blender and sieved to remove coarse particles. Ten grams from each fine powdered plant material was placed in a 250 ml beaker and extracted with 25 ml of distilled water for 48 hr. Mixture was vigorously stirred for 5 min. three times per day to prevent decay of water extracts. For fresh samples, water extract of fresh samples were prepared as follow: ten grams of each plant material were cut into small pieces using a sharp knife. The cutting plant material was mixed with 25 ml of distilled water by using electrical blender. The homogenate was placed in a closed container and was left for 48 hr. [20].

Assay of antifungal activity

The antifungal activity assay was carried out by the standard method according to Gurgel et al. [21]. Potato dextrose agar media were sterilized by autoclaving at 121°C for 15 min and cooled to 45°C. Then, about 20 ml of PDA medium was poured into Petri dishes and allowed to solidify. Plant extracts were tested against Penicillium chrysogenum, Aspergillus niger and A. flavus because they exhibited the highest percentage of occurrence on seeds and grains. The fungus was spread over the media by using sterile cotton swab. Five-mm diameter of sterile filter paper (Whatman No.3) discs previously soaked in aqueous plant extract (400 and 500 mg 1⁻¹) were placed on the surface of the plates. The plates were incubated at 28°C for 48-72 hr. The experiment was performed in triplicates to minimize the error ratio. At the end of incubation period, the inhibition zones around each disc
were measured to an accuracy of 0.1 mm and the effect was calculated as a mean of triplicate tests to evaluate the antifungal activity.

**Effect of plant extracts on incidence of fungi and seed germination**

Three replicates of 25 seeds per Petri-dish for each of the treated seeds including the controls were plated using the Blotter method as recommended by Mathur and Kongsdal [22]. These gave a total of 75 seeds for each treatment. They were then incubated for 7–10 days and then examined for seed-borne fungi. Records on incidence of seed-borne fungi and germination of treated seeds were then taken.

**Statistical analysis**

Analysis of variance (ANOVA) was performed on all transformed data (i.e. using the arcs in percentage transformation) collected in respect of parameters studied on effects of plant extracts and separation of treatment means was done using the LSD at 5% level of significance.

**Results**

**Fungi isolated from grains and seeds**

Results obtained in Table 1 showed that thirteen fungal species were isolated from different seeds and grains. *Aspergillus niger*, *A. flavus* and *P. chrysogenum* showed the highest percentage of occurrence.

**Antifungal activity of plant extracts against fungal species**

Warm water extracts of five fresh plant samples namely Garlic, Aloe, Peppermint, Banana and Camphor were tested against some fungal isolates (*Aspergillus niger*, *A. flavus* and *P. chrysogenum*), only Garlic bulb had the highest antifungal activity. It is worth to mention that the antifungal activity of Garlic extract varied from one fungal species to another. The diameter of inhibition zone for *Aspergillus niger*, *A. flavus* and *P. chrysogenum* were 22.83, 12.50 and 10.83 mm at 400 mg ml\(^{-1}\), while at 500 mg ml\(^{-1}\) they were 24.33, 18.0 and 13.17 mm, respectively (Fig. 1).

**Table 1.** List of the isolated fungal species from grains and seeds.

<table>
<thead>
<tr>
<th>Isolated fungal species</th>
<th>Sorghum bicolor</th>
<th>Triticum aestivum</th>
<th>Oryza sativa</th>
<th>Lens esculentus</th>
<th>Vigna sinensis</th>
<th>Arachis hypogaea</th>
<th>Vicia faba</th>
<th>% of occurrence</th>
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<tr>
<td>Acremonium gramineum</td>
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<td>5.4</td>
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<td>-</td>
<td>5.4</td>
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<tr>
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<td>Epicoccum nigrum</td>
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<td>2.7</td>
<td>2.7</td>
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<td>-</td>
<td>-</td>
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<td>2.7</td>
</tr>
<tr>
<td>Penicillium chrysogenum</td>
<td>5.4</td>
<td>8.1</td>
<td>2.7</td>
<td>8.1</td>
<td>2.7</td>
<td>5.4</td>
<td>8.1</td>
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<tr>
<td>Rhizopus oryzae</td>
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<td>2.7</td>
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<td>2.7</td>
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<td>5.4</td>
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<td>Rhizopus stolonifer</td>
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<tr>
<td>Trichothecium roseum</td>
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</table>

- = absence

The antifungal activities of eleven dried plant water extracts (Datura, Ginger, Bitter apple, Jatropha, Basil, Neem, Caraway, Rosemary, Euphorbia, Black seeds and Cumin) were tested against the fungal species. Datura leaves extract exhibited the highest antifungal activity against all tested fungi except *Aspergillus niger*. The highest sensitive species to water extract of Datura leaves were *Aspergillus flavus* and *Penicillium chrysogenum* with inhibition zone of 12.50 and 12.33 mm at 400 mg ml\(^{-1}\), and 14.0 and 20.83 at 500 mg ml\(^{-1}\), respectively (Fig. 2).
Water extract of dried powdered Ginger rhizome exhibited antifungal activity against all tested fungi with inhibition zone of 8.83, 8.167 and 8.0 mm at 400 mg ml⁻¹, and 9.83, 11.67 and 16.83 at 500 mg ml⁻¹, respectively (Fig. 3). Water extract of dried bitter apple fruit showed antifungal activity against *Penicillium chrysogenum* only with inhibition zone of 22.83 mm at 500 mg ml⁻¹ (Fig. 4).

**Fig. 1** Antifungal activity of fresh *Allium sativum* bulb water extract at 400 mg ml⁻¹ (a) and 500 mg ml⁻¹ (b) against fungal species.

**Fig. 2** Antifungal activity of dried *Datura stramonium* leaves water extracts at 400 mg ml⁻¹ (a) and at 500 mg ml⁻¹ (b) against fungal species.

Effect of plant extracts on seeds and grains germination and fungal growth.

Results obtained in Table 2 showed the effect of different plant extracts on seeds and grains germination and fungal growth. In this experiment, four different plant extracts were used to controlling seed-borne fungi on seeds and grains which were Bitter apple, Ginger, Datura and Garlic. Sorghum grains treated with these extracts showed high seed germination with 93.3, 96.0, 97.3 and 94.7 %, respectively, while the untreated (control) grains showed 90.7 %. The fungal growth appeared only on grains treated with Bitter apple extract with 72.0 %, while the control was 96.0 %. Wheat grains treated with these extracts showed high grain germination with 88.0, 94.7, 92.0 and 93.3 %, respectively, while the control was 65.3 %. The fungal growth appeared only on the grains treated with Bitter apple extract with 34.7 %, while the control was 90.7 %. Rice grains treated with these extracts
showed no germination. The fungal growth appeared only on grains treated with Bitter apple extract with 33.3 %, while the control seeds were 53.3 %.

**Fig. 3** Antifungal activity of dried *Zingiber officinale* powder extracts at 400 mg ml\(^{-1}\) (a) and at 500mg ml\(^{-1}\) (b) against fungal species.

Lentil seeds treated with these extracts showed high seed germination with 80.0, 98.7, 96.0 and 98.7 %, respectively, while the control seeds were 73.3 %. The fungal growth appeared only on the seeds treated with bitter apple and Datura extracts with 26.7 and 53.0 %, respectively, while the control was 88.0 %. Cowpea seeds treated with these extracts showed high seed germination with 77.3, 93.3, 90.7 and 97.3 %, respectively, while the control was 60.0 %. The fungal growth appeared only on the seeds treated with Bitter apple and Datura extracts with 17.3 and 8.0 %, respectively, while the control was 61.3 %. Faba been seeds treated with these extracts showed high seed germination with 78.7, 93.3, 90.7 and 98.7, respectively, while the control seeds was 77.3 %. The fungal growth appeared only on the seeds treated with Bitter apple extract with 48.1 %, while the control was 93.3 %.

**Discussion**

Seeds and grains play a vital role in the production of healthy crops. Healthy seeds and grains is the foundation of healthy plant; a necessary condition for good yields [23]. Many pathogenic fungi are seed transmitted, often reduce the germination ability or kill the infected plants or substantially reduce the productivity. Therefore, control of seed-borne fungi is extremely important and the damaging effects can be relieved through integrated approaches [24].

**Fig. 4** Antifungal activity of water extracts of dried *Citrullus colocynthis* fruit at 500 mg ml\(^{-1}\) against fungal species.

Deteriorations by fungi are due to unhygienic conditions of storage and this in turn is associated with initial high moisture content of the stored products or absorption of moisture during storage due to defects in the storage system [25]. In the present study, the most
common fungi isolated from seeds and grains were Aspergillus niger, A. flavus and Penicillium chrysogenum. These fungal species are very important for different reasons; they cause deteriorations in stored commodities [26-29], bring about reduction in the quality and quantity of agricultural products in storage and transit and also create health hazards in animals and human beings by producing toxic metabolites in the form of mycotoxins in the stored commodities [30,31]. Penicillium chrysogenum is a common fungus that can inhabit a wide variety of habitats [32].

From all samples extracted from fresh plants, only Garlic bulb exhibited the highest antifungal activity against isolated fungi. Lawson [33] reported that the most abundant sulfur compound in Garlic is allicin (S-allylcysteine sulfoxide), which is present at 10 mg g⁻¹ fresh Garlic or 30 mg g⁻¹ dry weight. Various chemical constituents in garlic products, including non-sulfur compounds such as saponins, may contribute to their antimicrobial activity [39]. The present results indicated that the treated seeds and grains of garlic attributed to the presence of antifungal compounds. Water extract of garlic rhizome exhibited significant antifungal activity against all tested fungi except Aspergillus niger. Datura plants contain tropane alkaloid such as hyoscyamine, scopolamine and atropine but the seeds, leaves and the flowers contain the highest level of alkaloids. Besides its hallucinogen activity, Datura reported to have antimicrobial activity [35, 36]. Hussain et al. [37] reported that the leaf extract of Datura stramonium reduced the development of rust pustules on the leaves of wheat. The inhibitory effect of the plant extracts might be attributed to the presence of antifungal compounds. Water extract of ginger rhizome exhibited significant antifungal activity against all tested fungi. Ficker et al. [38] reported that gingerols and gingerdial are the main antifungal principles presented in ginger rhizomes. Water extract of bitter apple fruit showed antifungal activity against Penicillium chrysogenum only. The antifungal effect of bitter apple attributed to the presence of the active compounds colocolithin and colocolithin alkaloids which may be disrupt cytoplasmic membrane of the microorganisms through their action on lipids and protein [39]. The present results indicated that the treated seeds and grains with plant extracts gave very good effects on their germination and also reducing seed-borne infections.

Table 2. Screening of plant species for antifungal activities

<table>
<thead>
<tr>
<th>Scientific name</th>
<th>Common name</th>
<th>Family name</th>
<th>Part used</th>
<th>Antifungal activity (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allium sativum L.</td>
<td>Garlic</td>
<td>Liliaceae</td>
<td>Bulb (Fresh)</td>
<td>10.83-24.33</td>
</tr>
<tr>
<td>Aloe vera L.</td>
<td>Aloe</td>
<td>Liliaceae</td>
<td>Leaves (Fresh)</td>
<td>-</td>
</tr>
<tr>
<td>Azadirachta indica A. Juss</td>
<td>Neem</td>
<td>Meliaceae</td>
<td>Seeds (Dry)</td>
<td>-</td>
</tr>
<tr>
<td>Carum carvi L.</td>
<td>Caraway</td>
<td>Apiaceae</td>
<td>Seeds (Dry)</td>
<td>-</td>
</tr>
<tr>
<td>Eucalyptus rostrata L.</td>
<td>Camphor</td>
<td>Myrtaceae</td>
<td>Leaves (Fresh)</td>
<td>-</td>
</tr>
<tr>
<td>Citrullus colocynthis L.</td>
<td>Bitter apple</td>
<td>Cucurbitaceae</td>
<td>Fruits (Dry)</td>
<td>22.83</td>
</tr>
<tr>
<td>Cuminum cyminum L.</td>
<td>Cumin</td>
<td>Apiaceae</td>
<td>Seeds (Dry)</td>
<td>-</td>
</tr>
<tr>
<td>Datura stramonium L.</td>
<td>Datura</td>
<td>Solanaceae</td>
<td>Leaves (Dry)</td>
<td>12.33-20.83</td>
</tr>
<tr>
<td>Euphorbia peplus L.</td>
<td>Euphorbia</td>
<td>Euphorbiaceae</td>
<td>Leaves (Dry)</td>
<td>-</td>
</tr>
<tr>
<td>Jatropha curcas L.</td>
<td>Jatropha</td>
<td>Euphorbiaceae</td>
<td>Leaves and seeds (Dry)</td>
<td>-</td>
</tr>
<tr>
<td>Mentha piperita L.</td>
<td>Peppermint</td>
<td>Lamiaceae</td>
<td>Leaves (Fresh)</td>
<td>-</td>
</tr>
<tr>
<td>Musa acuminate L.</td>
<td>Banana</td>
<td>Musaceae</td>
<td>Leaves (Fresh)</td>
<td>-</td>
</tr>
<tr>
<td>Nigella sativa L.</td>
<td>Black seeds</td>
<td>Ranunculaceae</td>
<td>Seeds (Dry)</td>
<td>-</td>
</tr>
<tr>
<td>Ocimum basilicum L.</td>
<td>Basil</td>
<td>Lamiaceae</td>
<td>Leaves and seeds (Dry)</td>
<td>-</td>
</tr>
<tr>
<td>Rosmarinus officinalis L.</td>
<td>Rosmary</td>
<td>Lamiaceae</td>
<td>Leaves (Dry)</td>
<td>-</td>
</tr>
<tr>
<td>Zingiber officinal Rosc.</td>
<td>Ginger</td>
<td>Zingiberaceae</td>
<td>Rhizome (Dry)</td>
<td>8.0-16.83</td>
</tr>
</tbody>
</table>

- = no antifungal activity

These results are in agreement with the findings of [40] who found that garlic extract was superior in terms of reducing seed-borne infections by Alternaria spp., Bipolaris sorokiniana, Curvularia lunata and Fusarium spp. infecting wheat grains. Mondall et al. [41]
reported that seed treated with the Garlic extract, reduced seed-borne prevalence and increased germination percentage of wheat seeds. In addition, aqueous extracts of Garlic bulb and ginger rhizomes were significantly exhibited antifungal activity against all tested fungi. These aqueous extracts were strongly inhibited seed infection.

The treated seeds and grains with the extracts of Datura showed very low infection by fungi rather than bitter apple. The effect of extracts depends on their concentrations. These results are in agreement with the findings of Reddy et al. [42]. They reported that the plant extracts completely inhibited A. flavus. Some important seed-borne pathogens like Fusarium oxysporum, Aspergillus niger, A. flavus, Penicillium spp. and P. vexans can be managed by using some plant extracts [43]. Exploitation of naturally available chemicals from plant protection would be a prominent role in development of future commercial pesticides for crop protection strategies, with special reference to manage plant diseases [44].

Conclusion

The study confirms that the natural plant extracts may be safer for human health than chemical fungicides to combat the fungi that cause deterioration of stored grains and seeds. The using of these extracts can also reduce wastage during storage process, as well as increasing seed germination during agriculture. Datura, bitter apple, garlic and ginger extracts are recommended as antifungal agents for preserving grains and seeds.

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الملخص العربي

تقييم بعض المستخلصات النباتية لمقاومة الفلورا الفطرية المسببة لفساد الحبوب والبقوليات المخزنة

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تهدف الدراسة إلى عزل وتعريف بعض الفطريات المسببة لفساد بعض الحبوب والبقوليات واستخدام بعض المستخلصات النباتية لتشتيط نمو هذه الفطريات ومعرفة تأثير هذه المستخلصات على نمو الحبوب والبذور وقدرتها على اختزال النمو الفطري. وقد تم دراسة حبوب وبذور نباتات النبتة العبيجة والقمح والأرز والعدس واللوبية والقول البلدي والقول السوداني. وقد تم عزل ثلاثة عشر فطرا من على هذه الحبوب والبذور. وكانت أكثرها حدوثا في اسبريليس نيجر واسبريليس فلافس وبينيسيلوم كريسوجيم. وقد تم اختبار مستخلصات خمسة نباتات طازجة وهي فصوص اللوئ والصبار والنعناع الفلفلى والجوز والكافور وأحد عشر نباتا جافا وهي الزنجبيل والثوم واللاتروفا واللوبية والريحان والكرونا والبقلة والكمون والحة السوداء والكركم والغاب طرية وثمرة الحنطل. مرت مستخلصات النباتية المائية لكل من فصوص اللوئ وأوراق الداتورا وريزومات الزنجبيل وثمرة الحنطل وأربع من هذه المستخلصات النباتية المائية كلاً من فصوص اللوئ وريزومات الزنجبيل كان لهما قدرة عالية في تثبيط نمو الفطريات الأكثر حدوثا. أما مستخلص أوراق الداتورا كان له قدرة عالية في تثبيط الفطريات الثلاثة بينما ثبت المستخلص المائي لأوراق الداتورا فقط. أما مستخلص فضاء وبينيسيلوم كريسوجيم كان له قدرة عالية في تثبيط الفطريات الثلاثة بينما ثبت المستخلص المائي لأوراق الداتورا فقط. وعلى مدار العشرة أيام، وجد أن هذه المستخلصات تنشط أنشطة هذه الفطريات والبذور كما أنها تختزل نمو الفطريات عليها عند مقارنتها بالحبوب غير المعالجة.
Reservoir quality of Hawaz formation, J oil field, concession NC186, NW Murzuq basin, SW Libya

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Abstract

The present paper deals with evaluation of petro-physical parameters of Hawaz Formation at J oil field, Concession NC186 in Murzuq basin through the analysis of well-log data available for ten exploratory wells, distributed in J oil field. A comprehensive formation evaluation has been applied through numbers of cross-plots and using their output parameters as input data for interactive petro-physics software (IP) in order to evaluate the lithological constituents and fluid saturations. The litho-saturation cross-plot indicated that the Hawaz Formation consists of sandstones with few shale. These sandstones are generally fine to very fine grains but occasionally coarse to very coarse grained. Generally coursing upward sequence is indicated. The litho-saturation cross-plots also illustrated that horizon H4 and H5 are the main reservoirs in Hawaz formation

Keywords: Hawaz Formation, J field, well logging, petro-physical parameters

Introduction

Murzuq Basin is located on the North African Platform (Fig. 1), covers an area of some 350,000 km², extending southwards into Niger [1]. The present-day borders of the basin are defined by erosion resulting from multiphase tectonic uplifts. It is not a sedimentary basin in the normally accepted sense, and could more accurately be described as an erosional remnant of a much larger Palaeozoic and Mesozoic sedimentary basin which originally extended over much of North Africa [2]. The flanks comprise the Tihemboka high to the W, the Tibesti high to the SE, and the Gargaf and Atshan highs to the N and NW. These uplifts were generated by various tectonic events ranging from middle Palaeozoic to Tertiary times, but the main periods of uplift took place during Middle Cretaceous to Early Tertiary Alpine movements.

The present-day Murzuq Basin contains a maximum sedimentary fill of about 4000m. Davidson et al. [2] pointed out that, despite successive erosive episodes during several phases of uplift and erosion throughout the history of the basin, the maximum sedimentary thickness probably never exceeded 5000 m at any single point in time.
This Basin has different concessions containing some oil fields. Each field has some wells drilled for the evaluation of subsurface geology and hydrocarbon potentialities of the Cambro-Ordovician Hawaz Formation intervals drilled by Repsol Oil Operations [4]. J field is one of the oil fields in concession NC186 that was encountered by ten exploratory and development wells, distributed in the field (J-NC186) on the northwestern flank of the Murzuq basin, southwest part of Libya (Fig. 1 and 2). The investigated J oil field lies between the latitudes 26° 45' and 26° 50' N and longitudes 12° 32' and 12° 36' E as shown below in (Fig. 2 and 3).

Generally, the petroleum system is represented by structural Hawaz paleo-high created during the post Hawaz erosional event. The main regional seal is the Silurian Tanezzuft shale Formation, and the basal Tanezzuft hot shale member acts as the main source rock in the
Reservoir quality of Hawaz formation, J oil field

This formation is considered the main target.

![location map of J oil field](image)

**Fig. 3** location map of J oil field, concession 186, Murzuq basin

This paper is devoted to study the hydrocarbon potentialities of Hawaz Formation in J oil field through analysis of the available well log data. A comprehensive analytical formation evaluation has been applied using interactive petrophysics (IP) software. The well log data comprise resistivity, sonic, neutron, density, nuclear magnetic resonance, spontaneous potential, caliper, gamma ray and natural gamma ray spectrometry logs.

**General Geologic and Structural Settings**

Murzuq basin is located on SW Libya and forms one of several intracratonic sag basins. The structural fabric imparted to the North African continental lithosphere during the late Proterozoic, Pan-African event has played an important role in controlling the subsequent structural and stratigraphic evolution of the basin. Early Palaeozoic tectonics created a series of NNW trending arches and sub-basins across North Africa, which filled with clastic continental and shallow marine deposits and transgressive open marine facies. Early Palaeozoic tectonism effectively controlled the distribution of late Ordovician reservoirs and distribution of Silurian “Hot Shale” which onlap early-fomed fault blocks [3,4].

The structure of the Murzuq basin is quite simple. The sub-horizontal or gently dipping strata is faulted and the faults are most frequently parallel to the axis. Tectonic movements affected the basin to a greater or lesser degree from middle Palaeozoic (Caledonian) to Post-Oligocene (Alpine) times [6]. Caledonian, Hercynian and Alpine tectonic events affected this basin evolution, specially Caledonian and Hercynian orogenies [6]. The Caledonian orogeny started in the Upper Silurian and persisted through the lower part of the Lower Devonian.. This has been provided in several localities in the south of the Ghabades Basin, in the Murzuq basin and also in the Kufra basin (Fig. 4).

The stratigraphic column of Murzuq basin ranges from the Pre-Cambrian to the Quaternary (Fig. 5). The maximum thickness in the basin center doesn’t exceed 4000 m. Hawaz formation rests conformably over the Ash Shabiyat formation. Both formations are cut by an erosive surface recognizable in outcrop and subsurface [7,8]. This surface is related to a glacial period lasting from Caradocian to Upper Ashgillian times. The deposition of the overlying Melaz Shuqran formation was controlled by a prominent palaeo topography including deeply incised valleys.

Hawaz formation has been described by Pierobon [9] as "typically consisting of cross-bedded, quartizitic sandstone with kaolinitic and thin shaley intercalations. Tigillites-bioturbated levels and ripple marks are conspicuous. The formation thickness ranges from 50 m (at Dor Al Qussah) to 300 m (at Al Qarqaf) in outcrops, and 30 m to 230 m in the subsurface. Palynological studies of this formation strongly indicate a Middle Ordovician (Llanvirnian-Llandeilian) age for the whole of the Hawaz formation, this is based on palynological data from Bras petro type well C1-NC58 [9].

**Well logging data analysis technique**

The processing of the well logging data in this study has been carried out utilizing constructing two cross-plots (Pickett and Hingle) for deriving formation water resistivity \( R_w \), cementation factor \( m \) and matrix parameters \( \rho_{ma} \Delta t_{ma} \). These parameters were used as input parameters for the interactive petrophysics software to evaluate Hawaz reservoir. The pay flag were computed using Vshale cut-off 40%, Porosity cut off 10% and Sw cut-off 50%. These cut-off percent's were determined from the
inspection of the logs and cross plots of the porosity versus Vshale. The output results are presented in the form of litho-saturation cross plots and maps.

Fig. 4 Location Map showing the general tectonic framework of Libya (After Fello, 2001)

Applications

The pickettcross-plot [10] is one of the simplest and most effective cross-plot methods in use. This technique is based on the observation that true resistivity (Rt) is a function of porosity (\( \phi \)), water saturation (\( S_w \)), and cementation factor (\( m \)). On the plot, a zone with constant water saturation will have data points along a single straight line trend. The slope of the straight line representing SW=100% represent \( -m \) and its intersection with 100% \( \phi \) is \( aR_w \). Figures 6 and 7 represent the Pickett plot for Hawaz formation in wells J11-NC186 and J13-NC186. It was found from the cross-plots that the average value of \( R_w \) equals to 0.31 \( \Omega \) m\(^2\) m\(^{-1}\), which is correletable with that obtained by core sample data executed by Repsol oil operation. It was also found that \( m \) is equal 2.

On the other hand, matrix parameters (\( \rho_{ma}, \Delta_{ma} \)) have been obtained from Hingle cross-plot. In this study, the matrix values have been determined by the intersection of SW=100% line and the horizontal axis. Figures 8 and 9 represent Hingle cross plot for Hawaz formation for J13-NC186 and J1-NC186 wells respectively. The results indicated that \( \rho_{ma} \) value is 2.66 g cc\(^{-1}\) and \( \Delta_{ma} \) is 55 \( \mu \)sec/ft. Diameter porosity cross plot between \( \rho_b \) versus \( \phi_N \) is used for evaluating matrix lithology and porosity. The lithological facies for Hawaz formation in J4-NC186 well consists mainly of sandstone matrix (Fig. 10). This sandstone is characterized by coarsening upward facies from horizon H8 at the bottom level to horizon H1 at the top (Fig. 11).

IP output results

The IP output results were presented in the form of lithosaturation cross plots for Hawaz Formation in the study area. This formation was subdivided into eight subzones H1, H2, H3, H4"H4a-H4b-H4c", H5, H6"H6a-H6b-H6c", H7 and H8. These subzones have its own petrophysical parameters (Vsh, Sw, Sh and \( \phi \)).
Fig. 5 Stratigraphic column of the Palaeozoic, Mesozoic and Cenozoic successions in NC186, NW Marzuq basin, SW Libya, (modified from Davidson et al. [2])

Fig. 6 Pickett plot for Hawaz Formation for J11-NC186 well, J oil field
Fig. 7 Pickett plot for Hawaz Formation for J13-NC186 well, J oil field.

Fig. 8 Hingle cross-plot for Hawaz Formation at J13-NC186
Reservoir quality of Hawaz formation, J oil field

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Fig. 9 Hingle cross-plot for Hawaz Formation at J1-NC186

Fig. 10 porosity $\rho_b$ vs $\theta_n$ cross-plot for Hawaz Formation for J4-NC186 well, J oil Field
Fig. 11 Sw vs Φ showing upward coarsening sequence, for Hawaz Formation at J1-NC186, J oil Field

Fig. 12 IP results for Hawaz Formation, J13-NC186 well. J Oil Field
The calculated shale volume Vsh for Hawaz formation in J13-NC186 well (Fig. 12) ranges from 5.7% to 19.6% with average 10.9%. The effective porosity ranges from 10.1% to 15% with average 13%. Water saturation ranges from 31% to 75.7% with average 56 %. The top of Hawaz formation is at 4281 ft with a gross thickness of 369 ft and net pay thickness is 98 ft. It is well known that H4 and H5 are potentially the most productive subzones.

The calculated shale volume of J14-NC186 well (Fig. 13) ranges from 5.2% to 19.2% with average 11.8%, the effective porosity ranges from 11.7% to 19.3% with average 15.3%, the water saturation ranges from 12.9% to 41% with average 26%.

The top of Hawaz formation is at 4408 ft with a gross thickness of 465 ft and net pay thickness is 388 ft. From H3 to H5 are potentially the most productive subzones. The litho-saturation cross-plot of well J1-NC186 (Fig. 14) displays that the calculated shale volume ranges from 0.2% to 4.5% with average 2.6%, the effective porosity ranges from 11% to 24.7% with average 14%, the water saturation ranges from 9% to 75% with average 53%. The top of Hawaz formation is at...
4354 ft with a gross thickness of 495 ft. and net pay thickness 294 ft. It is indicated also that subzones H3, H4 and, H5 are potentially the most productive zones (Fig. 14).

**Areal distribution of Hawaz reservoir**

The deduced petrophysical parameters are represented by a number of isoparametric maps showing the lateral distribution of Hawaz Formation. The gross reservoir thickness contour map (Fig. 15) shows that the maximum recorded reservoir thickness 558ft at J5-NC186 well, while it decreases gradually NW and west ward recording the minimum value of 304ft at the J6-NC186 well.
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Fig. 15 Gross thickness contour map for Hawaz reservoir in J-NC186 Concession

Fig. 16 Net Pay thickness contour map for Hawaz reservoir in J-NC186 Concession

Fig. 17 Average effective porosity contour map for Hawaz reservoir in J-NC186 Concession

Fig. 18 Average water saturation contour map for Hawaz Reservoir in J-NC186 Concession
Reservoir quality of Hawaz formation, J oil field

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Fig. 9 Average oil saturation contour map for Hawaz Reservoir in J-NC186 Concession

Conclusions

This paper has been carried out to study and evaluate the petrophysical characteristics of Hawaz formation in J oil field of concessions NC186. The conclusions which can be extracted are as follows:

- The analytical formation evaluation reveals that the hawaz reservoir is mainly clean sandston. This sandstone represents coarsening upward facies.
- H4 and H5 subzones are potentially the most productive in Hawaz formation as indicated from the litho-saturation cross-plots.

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الملخص العربي

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بحث الحالى يعنى بتعيين الخصائص البيروفيزيائية لمنشئي حواز بحقل J بالامتياز NC186 داخل حوض مرزق ليبيا، وذلك من خلال تحليل تسجيلات الآبار لعدد 10 آبار استكشافية منزورة بحقل J. تم تطبيق تقييم شامل على المتكون المراد دراسته وذلك من خلال رسم بعض العلاقات cross plots ثم استخدام نتائج هذه العلاقات في الحصول على بارامترات لإدخالها في برنامج Interactive Petrophysics software (IP) وذلك لتحسين المتكونات الصخرية للمكمن حواز وتحديد قيم تشبع للمكمن حوازLC186. النتائج التي أظهرت أن السحبة الصخرية لحالة المكمن تكون أساسا من حجر رملي مع قليل من الطين. والحجر الرملي يتكون من حبيبات دقيقة إلى دقيقة جدا ولكن أحيانا يكون خشن إلى خشن جدا. وهو يتدرجو الاتجاه من حبيبات الصخريات. أن الوحدات الصخرية لمنشئي حواز والمتمثلة في H5, H4 هم الفائدة من النظرة المكانية.
Petrophysical characteristics of Hawaz formation, H oil field, concession NC186, NW Murzuq basin, SW Libya

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Abstract

Petrophysical characteristics of the Hawaz Formation in H oil field, Concession NC186 of Murzuq basin have been evaluated through the analysis of well-logging data recorded for ten exploratory and development wells. These records have been analyzed through utilizing some cross-plots and use their outputs for controlling the interactive petrophysics software in order to identify the lithological constituents and fluid saturation parameters. The litho-saturation results indicated that Hawaz Formation is mainly oil-bearing Horizons H4 and H5 are the main productive zones. The evaluation reveals also that the lithological facies consists mainly of sandstone. This facies is characterized by coarsing upward.

Keywords: Hawaz Formation, H field of NC186, petrophysical parameters, well logging

Introduction

Murzuq Basin is one of several intracratonic basins located on the North Africa platform that have a predominantly marine Palaeozoic clastic infill. It is located on the southwestern part of Libya and has sub-circular shape and clearly visible on satellite images. The basin covers an area of some 350,000 km², extending southwards into Niger [1]. This basin has different concessions that have some oil fields. Each field contain some wells drilled for the evaluation of subsurface geology and hydrocarbon potentialities of the Cambro-Ordovician Hawaz Formation intervals drilled by Repsol Oil Operations. H field is one of the oil fields in concession NC186 that was encountered by several exploratory and development wells, distributed in the field (H-NC186) on the northwestern flank of the Murzuq basin, southwestern part of Libya (Fig. 1 and 2). Ten of these exploratory wells have been selected for this study. These investigated wells lie between the latitudes 26º 43' and 26º46' N and longitudes 12º 32' and 12º 36' E (Fig. 3). It measures about 36 km².

Oil was discovered in H field by H1-NC186 well drilled between April 21st and May 22nd, 2004. The well was tested in the interval 4365-4608 producing 950 STBOPD of 35° API gravity
by natural flow. Subsequently, three appraisal wells (H2, H3 and H4) were drilled in order to collect the required information to evaluate the commerciality and development feasibility of the field. Generally, the petroleum system is created during the post Hawaz erosional event. The main regional seal is the Silurian Tanezzuft shale Formation, and the basal Tanezzuft Hot Shale member acts as the main source rock in the area of study.

Fig. 1 Location Map of the Sedimentary Basins of Libya (After Fello [2])

Fig. 2 Location map of H oil field, concession 186

The present paper is devoted to study the petrophysical parameters and hydrocarbon potentialities of Hawaz Formation in H oil field. A comprehensive analytical formation evaluation program has been applied on the available well log data to identify the lithological constituents, petrophysical and fluid saturation parameters using interactive petrophysics (IP) software. The hydrocarbon potentialities have been evaluated through integration of the well logging and core data in order to deduce the petrophysical parameters. The log data comprise resistivity, sonic, neutron, density, spontaneous potential, caliper, gamma ray and natural gamma ray
spectrometry logs, where the geological data are represented by composite logs.

Murzuq Basin is roughly triangular in shape, narrowing towards the south from Libya into Niger. The sedimentary fill is predominately marine and continental Palaeozoic, with some Mesozoic and Cenozoic sediments overlying Precambrian crystalline basement. In the central part of the basin, the total sedimentary thickness exceeds 3500 m [1]. It is separated from the Illizi Basin, Algeria, to the west by the north-south ridge of the Ghat/Tikiumit Arch [3]. It is located between three tectonic elements: the Qarqaf uplift in the north, the Tibesti/Haruj uplift in the east and the Precambrian Hogger on the west which extends into Algeria and Niger.

The whole sedimentary succession is well exposed along much of the edge of the basin, as well as on the southern flank of the Qarqaf Arch. The full sedimentary succession is present only in few outcrop areas due to regional erosion connected with the Caledonian and Hercynian orogenies, and other lesser unconformities affecting on all formations. In the core of the Qarqaf arch the crystalline basement outcrops in relatively small areas [4]. The structure of the Murzuq Basin is quite simple. The sub-horizontal or gently dipping strata is faulted. These faults are most frequently parallel to the axis. Tectonic movements have affected the basin to a greater or lesser degree from middle Palaeozoic (Caledonian) to Post-Oligocene (Alpine) times [5].

The Tectonics, Caledonian, Hercynian and Alpine tectonic events have affected Murzuq Basin evolution, specially Caledonian and Hercynian orogenies [5]. The Caledonian orogeny started in the Upper Silurian and persisted through to the lower part of the Lower Devonian, Which is for some 25 million years. This has been provided in several localities in the south of the Ghadames Basin, Murzuq Basin, and also in the Kufra Basin (Fig. 4).
The area of study has been affected by the structural and tectonic movements of Murzuq basin mentioned above and created paleo-high during the post Hawaz erosional events. Figure 5 is a selective example of stratigraphic section illustrating part of paleo-high, which is clearly represented in the corresponding 2-D seismic line shown in (Fig. 6). This paleo-high is restricted in the area between wells H1 and H3.

The stratigraphic succession of the basin in the south western part of Libya was studied by many authors [5,7,8]. They concluded that, the stratigraphic column of Murzuq basin ranges from the Pre-Cambrian to the Quaternary (Fig. 7). The maximum thickness in the basin center is about 3500 m.

Hawaz formation is one of stratigraphic sequences and it is the main reservoir in Murzuq basin in the absence of Mamuniyat formation. It has been described by Pierobon [7] as "typically consisting of cross-bedded, quartizitic sandstone with kaolinitic and thin shaley intercalations. Tigillites-bioturbated levels and ripple marks are conspicuous. Hawaz Formation is conformably overlain by Melez Shuqran Formation. The formation thickness ranges from 50 m (at Dor Al
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Qussah) to 300 m (at Al Qarqa) in outcrops, and 30 m to 230 m in the subsurface. Palynological studies of the Hawaz Formation strongly indicate a Middle Ordovician (Llanvirnian-Llandeilian) age for the whole of the Hawaz Formation, this is based on palynological data from Braspetro type well C1-NC58 [7].

Data analysis and processing

Several specific analysis steps are employed in the well logging data in the study area for interpretation process:

1) Filtering the raw log response data to remove and correct anomalous data points.
2) Correcting neutron, sonic, density and resistivity logs for mud filtrate invasion.
3) Normalizing logs from all selected wells to determine the appropriate ranges of porosity, clay content, water resistivity, etc.

These data are integrated to verify the following petrophysical characterization and hydrocarbon potentialities of Hawaz sandstone within the H oil field. Reservoir characterization study is based on the following components: cross-plot, log evaluation, lithology and fluid
analysis, forward modeling and rock property relationships.

The processing of the well logging data in this study has been carried out utilizing analytical cross-plots (Pickett and Hingle) for deriving formation water resistivity ($R_w$), cementation factor ($m$) and matrix parameters ($\rho_{ma}$, $\Delta\rho_{ma}$), and then use these parameters for the interactive petrophysics software (IP). The shale volume ($V_{sh}$) was calculated using the gamma ray log data. The porosity ($\phi$) was calculated using neutron and density logs with shale correction when needed.

The sonic log ($\Delta T$) is used for determining matrix porosity values. The water saturation ($SW$) was then derived using connate water resistivity ($R_{wa}$) derived from, $R_{wa}$ technique. The cut off petrophysical parameters used for discriminating between pay and non-pay were: $V_{shale} \leq 40\%$, Porosity $\geq 10\%$ and $SW \leq 50\%$.

The output results obtained from the application of IP program, are presented as litho-saturation cross plots and iso-parametric maps. This is to evaluate the hydrocarbon potentialities of the studied reservoir. This lithosaturation crossplot (Fig. 8), comprise eight tracks from left to right as follows: gamma ray, caliper, and spontaneous potential (track 1) depth and tops of horizons (tracks 2 and 3). The fourth track includes resistivity data. The porosity logs ($\rho_b$, $\Phi N$ and $\Delta T$) are displayed in track no. 5. The output results are illustrated in tracks 6 and 7. Total porosity ($\Phi T$), effective porosity ($\Phi_{eff}$), flushed zone bulk volume water (BVW_{sx0}), and bulk volume water (BVW) values are presented in track 7. Volume fraction of the deduced lithological constituents ($\Phi$, $V_{cl}$ and $V_{san}$) are displayed in track 8.

**Fig. 8** Litho-saturation cross-plot for well H2-NC186
Analytical formation evaluation

The Pickett cross-plot [10] is one of the simplest and most effective techniques in use. It is based on the observation that true resistivity (Rt) is a function of porosity (ϕ), water saturation (SW), and cementation factor (m). It can be used for determining formation water resistivity (RW), water saturation (SW), cementation factor (m), and saturation exponent (n) through plotting effective porosity (ϕeff) versus true resistivity (RT) on two-by-three cycle log-log paper. On the plot, zones of 100% SW will land on a single straight line. Pickett cross-plot for wells H2-NC186 and H12NC-186 are shown in Figures 9 and 10.

![Pickett Plot for H2-NC186](image)

**Fig. 9** Pickett plot for Hawaz Formation, H2-NC186 well

![Pickett Plot for H12-NC186](image)

**Fig. 10** Pickett plot for Hawaz Formation, H12-NC186 well

The intersection of Ro line with the horizontal axis at 100% porosity represents a*Rw. The average value of Rw equals to 0.32 Ωm² m⁻¹ as “a” equals 0.62 for sandstone. This value is corrollable with that deduced by core analysis executed by Repsol oil operation. It was found that value -m was 1.85 (slope of 100% SW line). "n" has been taken as 1.7 as obtained from core data (Repsol oil operation). On the other hand, matrix density (ρma) has been obtained from Hingle cross-plot, which is the oldest of the resistivity versus porosity cross-plot methods. In this study, the matrix was found to be equals 2.66
g cc\(^{-1}\) (Fig. 11 and 12) which represent the intersection of Ro line with the x-axis.

The value of 2.66 g cc\(^{-1}\) for matrix reflects the sandstone nature for this reservoir which confirmed on the \(\rho_b/\phi_n\) cross-plot for well H7-NC186 (Fig. 13). Points migrated slightly over the sandstone line reflect the low density nature for the oil encountered in this reservoir which confirmed by low gravity oil represented by 35\(^{\circ}\)API [6]. The grain size can be obtained from semilog representation between porosity on logarithmic scale and water saturation (Sw) on linear one [11]. The Hawaz reservoir is characterized by coarsening upward sequence (Fig. 14). The upper part for this formation contains coarse sand with high porosity where the main reservoir is located.

The IP litho-saturation results of Hawaz formation

Hawaz Formation is divided into eight horizons (H1 to H8.). Each horizon represented by its own petrophysical parameters (Vsh, \(\phi\) and Sw). The following is a full interpretation for the lithosaturation crossplot for H2-NC186, H12-NC186 and H4-NC186 wells (Fig. 8, 15 and 16).

The calculated shale volume of H2-NC186 well (Fig. 8) ranges from 1% to 12.7% with average 7.4%. The effective porosity ranges from
10.3% to 15.6% with average 12.5%. Water saturation ranges from 23.7% to 99.6% with average 82%. The top of Hawaz formation is located at 4595 ft with a gross thickness of 635 ft. and net pay thickness is 114 ft. The results show that horizons H3 and H4 are potentially the most productive zones, where caliper denoted reduction of hole size corresponding to the presence of mud cake and hence good permeability. Also high resistivity and effective porosity together with low Sw values confirmed productive reservoir. Also these zones have low Bulk Volume Water (BVW) indicative of high oil saturation. The high separation between flushed zone Bulk Volume water (BVWsxo) and that of BVW reflect high mobility of the encountered oil. The lithosaturation results for H12-NC186 (Fig. 15) reflect the clean nature of the reservoir where Vsh ranges from 0.1% to 5.5%. The effective porosity $\Phi_{\text{eff}}$ ranges from 6.5% to 18.3% with average 12.9%, $\text{SW}$ ranges from 3% to 89% with average 54.5%. The top of Hawaz formation at this well is located at 4641 ft with a gross thickness of 407 ft. The net pay thickness is 217 ft. Horizons H2, H3, H4 and H5 are potentially representing the main reservoir zones as shown in the lithosaturation cross-plot (Fig. 15). Zone H5 which is the cleanest zone has high content of movable oil and very low BVW. The Oil Water Contact (O.W.C) may be located at 4862 ft where the resistivity decreased and Sw increased and also BVW increased.

Fig. 13 $p_b$ and vs $\Phi_n$ cross-plot for Hawaz Formation at H7-NC186

Fig. 14 Semilog representation between $\text{Sw}$ (linear) and $\Phi$ (logarithmic) for Hawaz Formation at H2-NC186 showing grading from coarse grain sand (CG) at the upper levels to very fine grain (VFG) at lower levels representing coarsening upward sequence
It is important here to notice that both BVW and BVWsxo coincide at high values confirming the wet nature at this depth. The most striking feature noticed here is the presence of residual oil in the base of H6c and top of zone H7 beneath the water sand seen from base H5 until the base of H6c. This residual oil encountered in zones of high gamma ray and high shale content with high resistivity which may reflect presence of source rock.

The lithosaturation cross-plot for well H4-NC186 (Fig.16) indicated the extremely low shale volume for Hawaz Formationn in this well. Vsh ranges from 0.1% to 1.2% with average 0.4%. The average $\Phi_{eff}$ is 13.6%. The average oil is 58.1%. The top of Hawaz formation is at 4584ft with a gross thickness of 642ft and net pay thickness 133.5 ft. It is clear from figure 16 that base H3, H4,H5 and H6 contain the main oil reservoir. The movable oil is small. Again H7 zone may be represent source rock as the shale is the main constituent with very high resistivity and extremely high gamma ray (out of scale). The top and bottom sand of zone H8 contain a percent of movable hydrocarbon.
The iso parametric maps of Hawaz reservoir.

A number of isoparametric maps showing the aerial distribution of the reservoir petrophysical parameters (net pay, $\Phi_{\text{eff}}$, And $S_w$) were constructed and presented in Figures 17, 18 and 19. The net pay thickness map (Fig. 17) indicates a general increase at the northern part of the area with maximum thickness of 471 ft at well H1-NC186, while it decreases gradually from the center to the east. The effective porosity contour map of this reservoir (Fig. 18) shows a general increasing towards NW and SW of the area recording a maximum value of 16.4% at well H7-NC186. The water saturation contour map (Fig. 19) of the reservoir illustrates a considerable distribution pattern with a general increase towards NE ward, recording a maximum value of 75% at well H2-NC186. This value decreases gradually in the center of the study area recording a minimum value of 34% at well H8-NC186.
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Conclusions

This research paper is focused on studying the petrophysical parameters and hydrocarbon potentialities of Hawaz Formation in H oil field, concession NC186. The analytical and graphical formation evaluation reveals that the reservoir consists mainly of clean sandstone. This sandstone is characterized by coarsening upward sequence from horizon H8 at the bottom level until horizon H1 at the top. The results indicated that the Hawaz reservoir is mainly oil-bearing.

The lithosaturation crossplots resulted through IP program indicated that horizons H4 and H5 contain the main oil reservoir. Also horizon H7 may be considered as possible source rock where the resistivity is exceptionally high for the shale encountered and also the exceptionally very high radioactivity levels. The isoparametric maps indicated that the northern part of the area of study contained the main productive wells.

References

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Fig. 17 Net pay thickness map for H oil field, Concession NC186, NW Murzuq basin, SW Libya

Fig. 18 Average effective porosity contour map H- NC186 for H oil field, Concession NC186, NW Murzuq basin, SW Libya.

Fig. 19 Average water saturation contour map for H oil field, Concession NC186, NW Murzuq basin, SW Libya.

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