4. Results and Discussion
4.1. Isolation and Identification of Halophilic Fungi

4.1.1. Soil Sampling and Analysis

Table 1 summarizes the physical and chemical characteristics of the soil of Little Rann of Kutch (Fig. 1). High EC value (indicating high salinity), neutral pH, and low N, C and P contents were the salient features of the soil.

In any soil sampling and analytical study due consideration should be given to the methodology adopted. Sampling methodology, sample area and design, instruments used, soil depth, soil volume collected and soil moisture content at the time of sampling are a few factors significantly influencing the desired soil parameters.
Table 1. Physicochemical properties of the soil of Little Rann of Kutch, India

<table>
<thead>
<tr>
<th>Property</th>
<th>Measured value</th>
<th>Methodology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand (g/Kg)</td>
<td>210</td>
<td>Hydrometer method</td>
<td>Tan (1996)</td>
</tr>
<tr>
<td>Silt (g/Kg)</td>
<td>650</td>
<td>“</td>
<td>“</td>
</tr>
<tr>
<td>Clay (g/Kg)</td>
<td>86</td>
<td>“</td>
<td>“</td>
</tr>
<tr>
<td>pH</td>
<td>6.63±0.3</td>
<td>Glass electrode</td>
<td>Smith and Doran (1996)</td>
</tr>
<tr>
<td>Electrical conductivity</td>
<td>8.61±0.28</td>
<td>EC Probe</td>
<td>Jackson (1973)</td>
</tr>
<tr>
<td>(EC&lt;sub&gt;1:1&lt;/sub&gt;) (dSm&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic C content (%)</td>
<td>0.23</td>
<td>Walkley-Black method</td>
<td>Walkley (1947); Jackson (1973)</td>
</tr>
<tr>
<td>Total N content (%)</td>
<td>0.1</td>
<td>Modified Kjeldahl method</td>
<td>Bremner (1960); Jackson (1973)</td>
</tr>
<tr>
<td>Total P content (%)</td>
<td>0.2</td>
<td>Chlorostannous–reduced molybdophosphoric blue color method, in sulfuric acid system</td>
<td>Jackson (1973)</td>
</tr>
<tr>
<td>Elemental analyses (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.39</td>
<td>Flame emission spectrophotometer</td>
<td>Jackson (1973)</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0.4</td>
<td>“</td>
<td>“</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0.35</td>
<td>“</td>
<td>“</td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.41</td>
<td>“</td>
<td>“</td>
</tr>
</tbody>
</table>
4.1.2. Isolation of Fungi

86 soil fungi were isolated. Based on colony characteristics and digital microscopy, 35 distinct fungi were identified. These belonged to genus *Fusarium* (11), *Penicillium* (10), *Aspergillus* (8), *Curvularia* (2), *Rhizopus* (2) and *Dreschlera* (2) (Fig. 2). The isolates were designated as KFI1 – KFI35 in ascending order.

Identification studies revealed that the majority of isolates belonged to genera *Fusarium*, *Penicillium* and *Aspergillus*. Microbial diversity of the region was found to be low, in accordance with the studies conducted in other hypersaline habitats around the world (Borut 1960; Ranzoni 1968; Salama et al. 1971; Khodair et al. 1991; Gunde-Cimmerman et al. 2000; Grishkan et al. 2003; Evans et al. 2013).

![Species richness of the study area represented as percentage occurrence of each phylum.](image-url)
4.1.3. Determination of Halophily of Fungal Isolates

Calculation of tolerance index value revealed that out of 35 isolates, two fungi – KFI 4 and KFI 6, exhibited halophily. 19 fungi were halotolerant while 14 isolates were halosusceptible. The presence of only two halophiles in the soil of this hypersaline region supports important observations made in earlier reports; prevalence of fungi in saline habitats that are halotolerant rather than halophilic (Hujslova et al. 2010; Nayak et al. 2012); halotolerant and halophilic traits are intrinsic characters of a particular species, rather than an adaptation in response to salt stress (Frisvad 2005); hypersaline environments do not harbor a characteristic fungal community of specialized taxa (Evans et al. 2013). It can hence be said that the fungi inhabiting hypersaline soils of Little Rann of Kutch have limited diversity, are mostly non-halophilic and exhibit cosmopolitan nature.

4.1.4. Identification of Halophilic Fungal Isolates

4.1.4.1. Morphological identification

Growth characteristics on different media, conidial pigmentation, mycelia and rear side colony colour were determined 8 d post inoculation. KFI4 culture featured thick mycelium with velvety texture and greyish green conidia suggesting that the fungus belonged to genus Aspergillus (Fig. 3 A-B). The stipes were smooth and 300-400 µm long, vesicles were pyriform-shaped, biseriate and measured 10-12 µm in diameter. The conidia were globose and finely roughened. The observations indicated that the isolate could be Aspergillus versicolor (Fig. 3 C-D). The KFI6 culture was characterized by thick yellowish green mycelium with profuse conidiation (Fig. 4 A-B). The stipes were 600-700 µm long with spiny texture and pale green colour. The vesicles were elongate, uniseriate and 30-40 µm in diameter. The conidia were globose and finely roughened. The features suggested the isolate’s identity to be Aspergillus flavus (Fig. 4 C-D).
Fig. 5 Morphology of the halophilic isolate KFI4 cultured on CYA plates. Figures (A) and (B) represent the front side and reverse side colony morphology, respectively. Scanning electron micrographs depicting vesicle (C) and conidia (D).

Fig. 6 Morphology of the halophilic isolate KFI6 cultured on CYA plates. Figures (A) and (B) represent the front side and reverse side colony morphology, respectively. Scanning electron micrographs depicting vesicle (C) and conidia (D).
4.1.4.2. Molecular identification

ITS 1- 5.8S- ITS 2 region of the rDNA was sequenced for molecular identification. Based on sequence homology and phylogenetic tree analysis, KFI 4 and KFI 6 were identified as *Aspergillus versicolor* and *Aspergillus flavus*, respectively. The sequences have been submitted in NCBI GenBank as *Aspergillus versicolor* KR87 (Accession no. KT164811) and *Aspergillus flavus* KDP3 (Accession no. KT164810).
4.2. Plant – Microbe Interaction Studies

4.2.1. Assessment of Salt Tolerance Potential of Groundnut

At 50mM NaCl, 50% seed germination was observed ($LD_{50} = 50$ mM) whereas at 100 mM NaCl, only 10% seeds germinated. No germination was observed at and above 150 mM (Fig. 7). 50 mM NaCl concentration was taken as standard salinity for all further experiments.

![Assessment of germination potential of groundnut seeds at different salinities. 100% seed germination was recorded in non-saline medium (dH$_2$O). $LD_{50}$ was observed at 50 mM NaCl. No germination was observed at 100 and 150 mM NaCl.](image)

The results indicate that groundnut var. TG 37A is a salt-sensitive variety showing susceptibility to even very low salinities, with only 70% and 50% seeds germinating at 25 mM and 50 mM NaCl, respectively. Groundnut is categorized as a salt-sensitive crop. It has been reported earlier that salinity hinders seed germination and seedling growth and dry biomass production (Janila et al. 1999). Salinity has also shown to cause Ca, K and Fe deficiencies in groundnut, resulting in yield losses (Singh et al. 2004; Hunshal et al. 1991). It can be inferred from the results that the TG 37A variety of groundnut is salt sensitive, capable of tolerating very low concentrations of salt.
4.2.2. Screening of Halophilic Fungal Isolates for Plant Pathogenicity

The *in vitro* interaction studies revealed that *A. versicolor* KR87 could be a potential plant growth promoter. *A. flavus* KDP3 did not show any growth promotion or inhibition. *A. versicolor* KR87 was selected for further interaction studies.

*A. flavus* is widely recognized as a pathogen of groundnut, causing aflatoxin contamination in seeds. However, aflatoxin production capacity varies with *A. flavus* strains. The non-pathogenic effect of *A. flavus* KDP3 on the growth of groundnut suggested that this strain did not produce aflatoxin. The same was confirmed by a plate assay in which an aflatoxin producing strain of *A. flavus* culture, when observed under UV light, glows around the colony edges. No such feature was observed in *A. flavus* KDP3, indicating that the fungus lacked aflatoxin-producing capacity.

4.2.3. *Ex vitro* Plant – Microbe Interaction

Figure 8 represents the agronomical characteristics of groundnut cultivated at different salinities. The salt-stressed inoculated plants had 50% longer shoot, 20% longer root and 30% higher number of leaves as compared to their non-inoculated counterparts. The root/ shoot length and leaf count in salt treated - inoculated plants were almost equal to that of those grown in non-saline soil.

Proline content in plant tissues decreased significantly with enhanced NaCl levels. Plants grown at 50 mM NaCl showed lower proline content as compared to the control, whereas the metabolite’s content was highest in plants co-cultivated with *A. versicolor*. Proline accumulation helps to stabilise proteins at high ionic strength or at low water activity. Salt tolerant plants have enhanced levels of proline at high salinities. Inability of groundnut to accumulate proline at high salt levels might be one of the reasons for the plant’s salt sensitivity. Decrease in proline levels might possibly be due to greater proline utilization than synthesis caused by NaCl stress (Ayala-Astorga and Alcaraz-Melendez 2010). Increase in proline contents in plant tissues in presence of fungal inoculum indicates that the fungus might have some role in
enhancing the plant’s proline content and its resultant ability to survive at high salinity.

Protein levels were slightly lower in plants grown at 50 mM as compared to the control. Potassium is required for protein synthesis. Salt stress leads to removal of $K^+$ ions from plant roots, thereby perturbing plant growth and development (Chen et al. 2007). Prolonged salt stress affects protein synthesis and eventually leads to decline in plant growth (Caplan et al. 1990). Plants co-cultivated with A. versicolor showed significantly higher soluble protein levels corresponding to the plant’s enhanced ability of surviving at 50 mM NaCl.

The total soluble carbohydrate contents in the plants increased with salinity. This finding is in accordance with previous studies in which plants, under saline stress, accumulated starch and soluble carbohydrates (Rathert 1984). The chief reason for this accumulation is the compromised carbohydrate utilization under abiotic stress (Munns and Termaat 1986).

The CAT and SOD enzyme activities decreased at 50 mM NaCl as compared to the control. However, in presence of the fungus, enhanced enzymatic activity was recorded. Abiotic stresses such as salt stress lead to enhanced generation of reactive oxygen species (ROS) such as $O_2$, $H_2O_2$ and OH. These radicals are highly reactive and negatively affect cellular metabolism via oxidative damage to membranes, proteins and nucleic acids. The complex antioxidant system of plants inhibits the damaging effects of ROS. Enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), peroxidases, etc. form the chief component of this system. The degree of tolerance of a plant against abiotic stresses depends on its ability of scavenging these free radicals (Aghaei et al. 2009). Increased activity of ROS and CAT in groundnut treated with A. versicolor KR87 might explain the plant’s survival at 50 mM NaCl. Micrographs of the plant roots treated with A. versicolor KR87 revealed the existence of an intricate hyphal network, indicating the endophytic nature of the organism. The absence of the same was reported in the non-inoculated plant roots. The finding suggests that one of the ways A. versicolor KR87 executes its plant growth promoting activity is by colonizing the roots of the treated plants.
Fig. 8 (A) Root and shoot morphology of the control and treated plants. (B) Micrograph of a root section colonised by *A. versicolor*. Arrows indicate the intricate hyphal network in the root tissue. Graphical representation of the agronomical traits (C) and biochemical parameters (D) of groundnut cultivated in different experimental setups. Data expressed as mean values of three replicates ± SD.
4.2.4. Study of Plant Growth Promoting Properties of *A. versicolor* KR87

The culture filtrate of *A. versicolor* KR87 was analysed for its indole-3-acetic acid content. Figure 9 depicts the IAA concentrations in the culture filtrate at different time intervals. As evident from the graph, the IAA levels in the filtrate increased gradually with duration, peaking at 12.16 µg mL\(^{-1}\) on 6\(^{th}\) day post inoculation, after which there was a gradual decline in the IAA levels. The detection of IAAs in the culture filtrate of *A. versicolor* suggests that the secretion of this phytohormones may be one of the factors causing growth promotion of groundnut plants under salinity stress. Further, it also indicates the presence of an IAA synthesis pathway. Previous reports also confirm that fungal endophytes produce phytohormones. Plants harbouring endophytes show a higher stress tolerance capacity than those lacking such interaction. The role of phytohormone – secreting endophytes in plant growth promotion under salt stress has been reported earlier in soybean and rice (Khan et al. 2011; Redman et al. 2011).

Phosphorus is the second important key element after nitrogen as a mineral nutrient in terms of quantitative plant requirement. Phosphorus is required during early phases of plant growth and development of reproductive parts. It plays significant role in enhancing root strength, thereby imparting vitality and disease resistance capacity to plant. Poor availability or deficiency of phosphorus impedes plant size and growth.

The fungus was tested for the ability to solubilize inorganic phosphates. The maximum PO\(_4\) concentration was detected at 50.21 mg 100mL\(^{-1}\) on 12\(^{th}\) day of incubation (Fig. 10). This indicates that the fungus is an excellent phosphate solubilizer.
Fig. 9 IAA levels detected in the culture filtrate of *A. versicolor* at different incubation periods.

Fig. 10 Solubilised P levels detected in the culture filtrate at different incubation periods.
P is abundant in soils in both organic and inorganic forms. However, its availability is restricted as it occurs mostly in insoluble forms. The average soil P content is about 0.05% (w/w) but only 0.1% of the total P is available to plant because of poor solubility and its fixation in soil (Illmer and Schinner 1995). To satisfy crop nutritional requirements, P is usually added to soil as chemical P fertilizer. However synthesis of chemical P fertilizer is highly energy intensive processes and has long term impacts on the environment in terms of eutrophication, soil fertility depletion and carbon footprint. In this regards phosphate-solubilizing microorganisms (PSM) have been seen as best eco-friendly means for P nutrition of crop. *A. versicolor* KR87 possesses high phosphate solubilisation potential. Mobilization of soil P leading to its enhanced uptake by groundnut may explain its growth promoting activity under salt stress.

The aforementioned results suggest that the susceptibility of *A. hypogaea* TG 37A to high salinities can be attributed to the organism’s lack of an inbuilt metabolic machinery for countering salt stress. The proline, protein and carbohydrate contents and the SOD and CAT activities were lower in groundnut plants cultivated at 50 mM NaCl as compared to those grown in absence of salt. In other words, an increase in salinity caused decrease in the concentration of stress metabolites, thereby inhibiting plant growth as evident from its lower root and shoot length and number of leaves. Groundnut co-cultivated with *A. versicolor* KR87 at 50mM NaCl, however, showed healthy growth features, with agronomical traits equivalent to those growing in normal soil. Biochemical analyses revealed greater proline, protein and carbohydrate contents as well as enhanced SOD and CAT activities. The results suggest that *A. versicolor* KR87 promotes plant growth by enhancing the stress metabolites’ levels in plant tissues, up-regulating the activities of critical enzymes of the plant’s antioxidant system, secretion of phytohormones and mobilization of P.
4.3. Defining the Chemical Architecture of Halophilic Fungi – the Metabolomics Approach

4.3.1. Analysis of Fungal Extracts by Liquid Chromatography - Mass Spectrometry (LC-MS)

Crude extracts of *A. versicolor* KR87 cultivated on CDB yielded 23 different metabolites belonging to 13 different compound classes. Majority of compounds belonged to alkaloids (18%), amines (18%), terpenes (8%), peptides (8%) and sterols (8%) (Fig. 11). Presence of a polyamine, N-carbamoyl putrescine, was detected in the extract, the identity of which was confirmed by the compound’s mass spectrum.

The LC-MS chromatogram of *A. flavus* exhibited 22 different metabolites belonging to 13 different compound classes. The metabolites chiefly belonged to phenols (24%), alkaloids (21%), flavonoids (19%), amines (19%) and terpenes (9%) (Fig. 12). The absence of any type of aflatoxin in the chemoprofile corroborated the earlier observation that *A. flavus* KDP3 is a non-aflatoxin producer.

On comparing the metabolite profile of *A. versicolor* against *A. flavus*, the former was found to be qualitatively richer in carbohydrates, polyamines and unsaturated fatty acids. An organism’s metabolome is greatly dependent on culture conditions such as nutrient source, light, temperature, pH etc. (Calvo et al. 2002; Yin and Keller 2011). Further detailed studies need to be carried out to formulate a medium that is ideal for secondary metabolite production. However, the findings of the current study suggest that CDB is the medium of choice for studying the fungi’s metabolome. Earlier works also report CDB as an excellent media for metabolite production (Jennessen et al. 2005).
Fig. 11 (A) ESI+ time-of-flight (TOF) MS total ion chromatogram of *A. versicolor* KR87. (B) Pie-chart depicting the chemoprofile of *A. versicolor*. Metabolite diversity is represented as % abundance of metabolites of each compound class.
Fig. 12 (A) ESI+ time-of-flight (TOF) MS total ion chromatogram of *A. flavus* KDP3. (B) Pie-chart depicting the chemoprobe of *A. flavus*. Metabolite diversity is represented as % abundance of metabolites of each compound class.
4.3.2. Biochemical characterization

Total phenol, flavonoid and alkaloid contents in *A. flavus* were found to be 130.7±0.13 µg mg⁻¹ Gallic Acid equivalent (GAE), 63±0.20 µg mg⁻¹ Quercetin equivalent (QE) and 81.02±0.29 µg mg⁻¹ Pilocarpine nitrate equivalent (PNE), respectively.

The phenol, flavonoid and alkaloid contents in *A. versicolor* were estimated at 268.77±0.18 µg mg⁻¹ GAE, 73.537±0.20 µg mg⁻¹ QE and 71.23±0.41 µg mg⁻¹ PNE, respectively.

Abiotic stresses such as salt and drought stress lead to generation of high amount of free radicals, which are responsible for hindering cell growth and metabolism. Phenols, flavonoids and alkaloids are known to exert antioxidant activities. High concentrations of these compounds may be produced by the organisms to counter the abiotic stresses.
4.4. Bioassays

4.4.1. Antibacterial Assay

*A. flavus* KDP3

Figure 23 gives a graphical representation of antibacterial activity of *A. flavus*, measured as zone of inhibition, against the test organisms *K. pneumonia*, *B. cereus*, *E. coli* and *S. aureus*. Clear zones were observed against *E. coli* (10.4±0.58 mm) at 10 µg mL\(^{-1}\), whereas no activity was observed at 10 µg mL\(^{-1}\) against *K. pneumonia*, *B. cereus* and *S. aureus*. Significant inhibition was observed against *K. pneumonia* (12±0.58 mm), *B. cereus* (11.7±0.58 mm) and *S. aureus* (10.4±0.58 mm) at a minimum concentration of 20 µg mL\(^{-1}\) and above. In general, the antibacterial efficacy of fungal extract increased with increasing concentration.

Calculation of the MIC of FE revealed that *E. coli* and *S. aureus* were the most susceptible test pathogens, with an MIC value of 32 µg mL\(^{-1}\) each, followed by *B. cereus* (64 µg mL\(^{-1}\)) which was slightly more resistant to the extract. *K. pneumoniae* was the most resistant pathogen with an MIC value greater than 128 µg mL\(^{-1}\). Since *K. pneumoniae* is Gram negative in nature, the high MIC value could be attributed towards its complex periplasmic structure, i.e. the presence of lipopolysaccharides. On the contrary, MIC against *E. coli*, another Gram negative bacterium, was observed to be unexpectedly low at 32 µg mL\(^{-1}\). Seemingly, this particular strain of *E. coli* possibly has certain channels in its outer membrane which facilitate entry of extract or its any compound(s).
Fig. 13 (A) Antibacterial activity of *A. flavus* KDP3 measured as zone of inhibition. (B) MIC values calculated by micro-broth dilution method.
**A. versicolor KR87**

Figure 24 gives a graphical representation of the antibacterial activity of the fungal extract against the test organisms at different extract concentrations. At 10 µg mL\(^{-1}\) concentration, clear zones were observed only against *S. aureus* (11±1 mm). Significant inhibition zones in *K. pneumoniae* (12.33±1.52 mm), *E. coli* (11±1 mm) and *B. cereus* (12±1 mm) were observed at a minimum concentration of 20 µg mL\(^{-1}\) and above. In general, the antibacterial efficacy of the extract increased with increasing concentration.

With an MIC of 32 µg mL\(^{-1}\), *S. aureus* was found to be the most susceptible of the pathogens tested against *A. versicolor*, followed by *E. coli* and *B. cereus* (64 µg mL\(^{-1}\) each). *K. pneumoniae* was again found to be the most resistant pathogen with an MIC value greater than 128 µg mL\(^{-1}\).
Fig. 14 (A) Antibacterial activity of *A. versicolor* KR87 measured as zone of inhibition. (B) MIC values calculated by microbroth dilution method.
4.4.2. Cytotoxic Activity

Cytotoxicity of *A. versicolor* and *A. flavus* extract was studied *in vitro* against MCF-7 cancer cell line at different concentrations (Fig. 25). Based on results obtained from MTT assay after 24 h of incubation, it was established that extracts possessed significant cytotoxic activity against MCF-7 with IC$_{50}$ = 10 µg mL$^{-1}$ for *A. flavus* and IC$_{50}$ = 5µg mL$^{-1}$ for *A. versicolor*. With an increase in extract concentration, the percentage of cytotoxicity increased.

![Graph of A. flavus](image)

**A. flavus**

![Graph of A. versicolor](image)

**A. versicolor**

Fig. 15 Cytotoxic activity of *A. flavus* and *A. versicolor*.

The IC$_{50}$ values of *A. flavus* and *A. versicolor* were measured at 7.5 and 5 µg mL$^{-1}$, respectively.
The bioassays revealed that *A. flavus* and *A. versicolor* possess potent antibacterial and cytotoxic activities. The extracts displayed bactericidal activity against both Gram positive (*S. aureus* and *B. cereus*) and Gram negative bacteria (*E. coli* and *K. pneumoniae*), indicative of their broad spectrum antibiotic nature. Quite expectedly, Gram negative bacteria were more resistant against the extracts as compared to Gram positive strains, as evident from their respective MIC values. This phenomenon can be attributed to the differences in cell architecture between the two strains. Gram negative strains possess an outer lipopolysaccharide membrane, which inhibits the entry of molecules into the cell, thereby making the bacteria more resistant to bioactive compounds. The fungi also exhibited remarkable cytotoxic activity against MCF-7 cancer cell line.

The antibacterial and cytotoxic activities may be ascribed to the high content of phenols, flavonoids and alkaloids, which have been reported to be involved in inhibition of nucleic acid biosynthesis and other metabolic processes. Among the reported mechanisms of action of phenolic compounds are inhibition of hydrolytic enzymes, cellular membrane disruption, ATPase activity inhibition and release of intracellular ATP and other elements, inactivation of microbial adhesins, cell envelope transport proteins and non-specific interactions with carbohydrates. Alkaloids have been used for centuries in medicine and continue to be an active component of modern day drugs. Alkaloids exhibit antibiotic activity, antimalarial activity (quinine), antiarrhythmic effects (quinidine, sparteine) and anticancer activities (vincristine, vinblastine). Physostigmine (parasympathomimetic) and codeine (antitussive) are few examples of alkaloids serving as models for chemical synthesis of analogues. Significant amount of alkaloids might be one of the plausible mechanisms for the antibacterial and cytotoxicity nature of the fungal extract.

The findings advocate further isolation and identification of the active principles of the *A. flavus* KDP3 and *A. versicolor* KR87 extracts (Phongpaichit et al. 2007; Hung et al. 2014; Verekar et al. 2014).