USE OF RHIZOSPHERIC MICROORGANISMS TO PROMOTE SUNFLOWER PLANT AGAINST HAZARDOUS EFFECT OF DROUGHT AND SALINITY

A thesis

Submitted for the degree of ph.D. in Microbiology

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ABSTRACT

The characterization and ability of halotolerant microbes to improve sunflower tolerance against salinity and drought were studied. A total of 56 bacteria, 10 actinomycetes and 12 fungi were isolated from rhizosphere of halophyte and xerophyte plants and screened for their ability to tolerate salt and water stress. Isolates with high salt tolerance were screened for their ability to produce salicylic acid, exopolysaccharide, proline and indoles and tested for their capacity to alleviate harmful effect of salt stress on sunflower seed germination *in vitro*. Halotolerant isolates namely 25Ag, 45Nr, A2, A6, F3 and F4 were found to be the best isolates and were identified as *Exiguobacterium acetylicum*, *Halomonas elongate, Streptomyces mutabilis, Sterptomyces tuirus, Eurotium cristatum* and *Aspergillus terreus* respectively. Pot experiment was conducted to investigate the ability of these isolates to promote sunflower growth under water and salt stress. Results showed that the selected isolates improved the growth parameters of sunflower in addition they maintaned the relative water content and relative membrane permeability of leaves and resulted in higher accumulation in K^+ and Ca^{++} levels in root and shoot. They also caused an increase in total carbohydrates, total protein percentages and proline content in shoot. A field experiment was conducted in salt affected soil and results showed that inoculation with selected isolates positively influenced the growth parameters of sunflower plants subjected to salt stress and enhanced sunflower seed yield and yield components. Maximum increments were obtained from plant inoculated with fungal strain *E. cristatum*. Treatment of salty soils with these halotolerant isolates can ameliorate the deleterious effects of salt stress on nutrition and growth parameters of sunflower plants under salinity conditions.

LIST OF ABBREVIATIONS

ACC:	**Aminocyclopropane carboxylase**
APX:	**Ascorbate peroxidase**
CAT:	**Catalase**
DAP:	**Diaminopimelic acid**
dS/m:	**DeciSienens per meter**
EC:	**Electrical conductivity**
EDTA:	**Ethylenediamine tetraacetic acid**
EXP:	**Exopolysaccharide**
GA:	**Gibberellic acid**
GPX:	**Glutathione peroxidase**
IAA:	**Indole acetic acid**
LSD:	**Least significant difference**
LXP:	**Lipopolysaccharide**
meq/l:	**Milliequivalent per liter**
mmhos:	**Millimhos per centimeter**
NA:	**Nutrient agar**
PCR:	**Polymerase chain reaction**
PDA:	**Potato dextrose agar**
PEG:	**Polyethylene glycol**
PGPR:	**Plant growth promoting rhizobacteria**
POX:	**Peroxides**
RMP:	**Relative membrane permeability**
ROS:	**Reactive oxygen speacies**
RWC:	**Relative water content**
SA:	**Salicylic acid**
SOD:	**Superoxide dismutase**

1. INTRODUCTION

In nature plants are often exposed to multiple stresses, and their response to a variety of stresses determines their capacity to survive. The development of plant growth promoting microorganisms (PGPM) that enhance stress tolerance in plants is a promising new strategy for sustainable agricultural productions.

Sunflower plant (*Helianthus annuus* L.) represents one of the important oil crops in Egypt (Mohamedin *et al*., 2004). It contains high percentage of poly-unsaturated fatty acids and low cholesterol level, the seed contains 25-48 % oil and 20-27 % protein. Sunflower is moderately sensitive to soil salinity, it can tolerate salinity up to E.C equals to 1.7 dS/m (Allen *et al*., 1998). It gained much attention in order to meet the increasing demand for vegetable oil, especially it could be cultivated in different types of soils and climate conditions in the newly reclaimed soils (Osman and Awed, 2010).

Plant-associated microorganisms can play an important role in conferring resistance to abiotic stresses. These organisms include rhizoplane, endophytic bacteria and symbiotic fungi. They operate through a variety of mechanisms like triggering osmotic response and induction of novel genes in plants (Grover *et al*., 2011).

The development of stress tolerant crop varieties through genetic engineering and plant breeding is essential but a long drawn process, whereas microbial inoculation to alleviate stresses in plants could be cost effective, environmental friendly option which could be available in a shorter time frame. Taking the current leads available, concerted future research is needed in this area, particularly on field evaluation and application of potential organisms (Grover *et al*., 2011).

Aim of the work

This work aimed to evaluate the role of halotolerant microorganisms in ameliorating the hazardous effects of salt and drought stress on sunflower crop.

2- REVIEW OF LITERATURE

Soil salinity is one of the major problems for agriculture in semi arid regions. In Egypt, plants are subjected to extreme climatic factors such as high temperatures and drought. Under these conditions, dissolved salts may accumulate in soils because of the insufficient leaching of ions. Accumulation of salt in upper soil layers may also occur due to unsuitable irrigation management. Soil or water salinity adversely affects growth, development and yields of crop plants (Eisa *et al*., 2012). Soil salinity causes plant stress in two ways, by making water uptake by the roots more difficult and by causing plant toxicity via accumulation of high salt concentrations in the plant (Munns and Tester, 2008).

Moreover high salinity constitutes an environmental stress for rhizospheric bacteria. It inhibits various physiological processes, ranging from energy and nutrient uptake to inhibition of DNA replication and macromolecule biosynthesis (Bartels and Sunkar, 2005). In addition, alteration of proteins involved in the initial attachment steps (adsorption and anchoring) of bacteria to plant roots in symbiotic interaction as well as inhibition of bacterial nodulation and nitrogen fixation activity. Similarly, alteration of exopolysaccharide (EPS) and lipopolysaccharide (LPS) composition of the bacterial cell surface and impairment of molecular signal exchange between bacteria and their plant host due to the alteration of membrane glucan contents was reported. Inhibition of bacterial mobility and chemotaxis toward plant roots were also reported (Jofre´ *et al.*, 1998).

To overcome salinity effects, scientists are using transgenic approaches to obtain genetically modified plants (Ashraf and Akram, 2009; Mittler and Blumwald, 2010). These approaches are time consuming and costly due to the impressive charges required to validate the consumption or cultivation of genetically modified plants. Several factors limit the success of producing salt-tolerant cultivars through genetic engineering, in most cases only a single gene has been transformed, although salt stress resistance is polygenic.

Plants in their natural environment are colonized both by endocellular and intracellular microorganisms (Gray and Smith, 2005). Rhizosphere microorganisms, particularly beneficial bacteria and fungi, can improve plant performance under stress environments and, consequently, enhance yield both directly and indirectly (Dimkpa *et al.*, 2009).

2.1 Halophiles

Halophiles are a group of microorganisms that live in saline environments and in many cases require salinity to survive. According to Parthiban *et al.*, (2010), halophiles can be classified according to their salt requirement and growth pattern to slight halophiles which grow at 2-5% NaCl, moderate halophiles optimally grow at 5-20% NaCl and extreme halophiles which grow at 20-30% NaCl.

Halophiles include a great diversity of organisms, like moderately halophilic aerobic bacteria, cyanobacteria, sulphur-oxidizing bacteria, heterotrophic bacteria, anaerobic bacteria, archaea, protozoa, fungi, algae and multicellular eukaryotes. Halophilic microbes have the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts (Nieto and Vargas, 2002). Moreover, halophiles have been used as a source of compatible solutes or hydrolytic enzymes (Mellado and Ventosa, 2003).

Moderately halophilic microbes have the potential for exciting and promising applications. Not only do many of them produce compounds of industrial interest (enzymes, polymers, and osmoprotectans), but also they possess useful physiological properties which can facilitate their exploitation for biotechnology. Most of them can grow at high salt concentrations (Vreeland, 1992). In addition, they are easy to grow, and their nutritional requirements are simple thus the majority can use a large range of compounds as their sole carbon and energy source (Ventosa *et al*., 1998).

2.2. Adaptation of microorganisms to saline environments

Halotolerance is the adaptation of living organisms to conditions of high salinity. Microorganisms that live at high salt concentrations are exposed to media of low water activity, and must have mechanisms to avoid water loss by osmosis. There are two basic strategies within the microbial world which enable microorganisms to cope with the osmotic stress inherent to the presence of high salt concentrations 'salt-in' strategy and 'compatible solute' strategy (Ventosa, *et al.*, 1998).

The salt-in strategy is employed by true halophiles, including halophilic archaea and extremely halophilic bacteria. These microorganisms are adapted to high salt concentrations and cannot survive when the salinity of the medium is lowered. They generally do not synthesize organic solutes to maintain the osmotic equilibrium. This adaptation involves the selective influx of K^+ ions into the cytoplasm. All enzymes and structural cell components must be adapted to high salt concentrations for proper cell function (Shivanand and Mugeraya, 2011).

Compatible solute strategy is employed by the majority of moderately halophilic and halotolerant bacteria, some yeasts, algae and fungi. In this strategy cells maintain low concentrations of salt in their cytoplasm by balancing osmotic potential through the synthesis or uptake of organic compatible solutes. Hence these microorganisms are able to adapt to a wide range of salt concentrations. The compatible solutes include polyols such as glycerol, sugars and their derivatives, amino acids and their derivatives, and quaternary amines such as glycine betaine and ectoines (Ventosa *et al*., 1998).

Few groups of halophiles use salt (mainly KCl) to provide osmotic balance with the outside medium. Potassium is the main intracellular cation in all cases, and chloride is used as the dominant intracellular anion (Oren, 2002).

The accumulation of compatible organic solutes may provide osmotic pressure and the concentrations of such osmotic solutes are regulated according to the salt concentration in which the cells live (Galinski and Louis, 1999), and they can be rapidly adjusted as required when the outside salinity is changed.

The presence of high intracellular salt concentrations requires special adaptation of the proteins and other macromolecules of the cells. However, proteins of halophilic microorganisms contain an excess ratio of acidic to basic amino acids and are resistant to high salt concentration. Proteins in extreme halophiles have structure–function stability only in the presence of salt and their enzymes require salts for activity. Surface negative charges prevent denaturation and precipitation of proteins at high salt concentrations (Shivanand and Mugeraya, 2011).

Moderate halophiles accumulate high cytoplasmatic concentrations of low-molecular-weight organic compounds to cope with the osmotic stress and to maintain positive turgid pressure. The ability to produce and accumulate high concentrations of these compounds makes moderate halophiles useful for the biotechnological production of these osmolytes. Some compatible solutes, especially glycine, betaine and ectoines, have gained considerable attention in recent years, because they may be used as stress protectants against high salinity, thermal denaturation, desiccation, freezing. (Louis *et al*., 1994 and Galinski, 1995).

2.3 Amelioration of crop resistance to salinity and drought by halotolerant microbes.

Dehydratation, salinity, low as well as high-temperature and other abiotic stresses lead to metabolic toxicity, membrane disorganization, generation of reactive oxygen species, inhibition of photosynthesis, reduced nutrient acquisition and altered hormone levels (Wang *et al*., 2003).

Accumulation of osmoprotectants, production of superoxide radical scavenging mechanisms, exclusion or compartmentation of ions by efficient transporter and production of specific enzymes involved in the regulation of plant hormones are some of the mechanisms that plants have evolved for adaptation to abiotic stresses (Des Marais & Juenger, 2010; Santner *et al*., 2009 and Shao *et al*., 2009).

An alternative strategy to improve crop salt tolerance is by introducing salt-tolerant microbes that enhance crop growth. Soil microbes have enhanced the growth of many different crops grown in a wide range of root-zone salinities, they do this through several mechanisms.

Inoculation with the rhizosphere bacterium *Azospirillum brasilense* NH, originally isolated from salt affected soil in northern Algeria, greatly enhanced growth of durum wheat ((*Triticum durum* var. waha) under saline soil conditions. At salt stress conditions (160 and 200 mM NaCl) *A. brasilense* NH restored almost completely vegetative growth and seed production (Nabti *et al.,* 2010). Similarly, Bacilio *et al.,* (2004) reported that under high NaCl concentration, inoculation of wheat seedling with modified *Azospirillum lipoferum* reduced the deleterious effects of NaCl. A similar result was obtained by Barassi *et al.,* (2006) who found that *Azospirillum*-inoculated lettuce seeds had better germination and vegetative growth than non-inoculated controls after being exposed to NaCl.

Timmusk and Wagner (1999) were among the first to show that inoculation of *Paenibacillus polymyxa* confers drought tolerance in *Arabidopsis thaliana* through the induction of drought