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Effects of salt stress factors on antimicrobial activity of two *Triticum aestivum* L. varieties

Original Article

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Abstract:
Salinity is one of the most common environmental stress factors that adversely affect plant growth and crop production in cultivated areas worldwide. Herbal or 'alternative' medicine is gaining popularity and scientific research about wheat grass as a "functional food" is becoming more available and popular. Wheat grass, *Triticum aestivum* L. has a long history and is widely used as a health food supplement. It is found to be used as a treatment for minor ailments, and also as a preventive dietary supplement and therapeutic drug. Current study was aimed at evaluate antimicrobial properties of the two varieties of *T. aestivum* L. [cv. Tosunbey (drought tolerant) and cv. Sultan 95 (drought sensitive)], grown in three different conditions [(1) control; not treated with salt or acetyl salicylic acid; (2) treatment with sea water; (3) sea water and pre-treatment of seeds with acetyl salicylic acid]. The antimicrobial activity of the ethanol extracts of the two varieties of *T. aestivum* were assayed against *Escherichia coli* NRRL B-3704, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 13315, *Acinetobacter baumanii* ATCC 19606, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *S. haemolyticus* ATCC 43252 and *Candida albicans* ATCC 10231 test microorganisms by agar disc diffusion method and broth microdilution method**.** The results showed that the ethanol extracts from the different treatments showed antimicrobial activities, with the diameters of the inhibition zone ranging from 8 to 13 mm and 2.5 to 20 µg/mL, respectively**.** The highest antimicrobial activity was demonstrated against *P. aeruginosa* ATCC 27853 by the extract of *T. aestivum* cv. Sultan 95, which grown in sea water and whose seeds were pre-treated with acetyl salicylic acid.

Key words:
antibacterial activity, T. aestivum cv. Tosunbey, T. aestivum cv. Sultan 95

Apstract:
Efekat sonog stresa na antibakterijsku aktivnost dva varijeteta *Triticum aestivum* L.

Salinitet je jedan od najčešćih sredinskih stresnih faktora koji nepovoljno utiču na rast biljaka i produkciju useva u kultivisanim područjima širom sveta. Biljna ili "alternativna" medicina dobija na popularnosti i naučna istraživanja na temu pšenice kao "finkcionalne hrane" postaju sve dostupnija i popularnija. Pšenica, *Triticum aestivum* L., ima dugu istoriju i veoma je zastupljena kao dodatak ishrani. Utvrđeno je da se koristi kao tretman za blaža oboljenja i kao preventivni dodatak ishrani i terapeutski agens. Ovo istraživanje imalo je za cilj da utvrdi antimikrobni potencijal dva varijeteta *T. aestivum* L. [cv. Tosunbey (tolerantna na sušu) i cv. Sultan 95 (osetljiva na sušu)], uzgajanih u tri različita uslova [(1) kontrola; netretirana semena; (2) tretman morskom vodom; (3) tretman morskom vodom i pretretman semena acetil salicilnom kiselinom]. Antimikrobna aktivnost etanolnih ekstrakata dva varijeteta *T. aestivum* testirana je u odnosu na *Escherichia coli* NRRL B-3704, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 13315, *Acinetobacter baumanii* ATCC 19606, *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *S. haemolyticus* ATCC 43252 i *Candida albicans* ATCC 10231, korišćenjem metoda disk difuzije na agaru i mikrodilucije. Rezultati su pokazali da etanolni ekstrakti iz različitih uslova gajenja poseduju antimikrobnu aktivnost, sa dijametrima inhibicionih zona od 8 to 13 mm i minimalnim inhibitornim aktivnostima od 2.5 do 20 µg/mL. Najveća antimikrobna aktivnost, utvrđena u odnosu na *P. aeruginosa* ATCC 27853, pokazana je od strane ekstrakta *T. aestivum* cv. Sultan 95, dobijenog od biljaka gajenih u morskoj vodi i čija su semena pretretirana acetil salicilnom kiselinom.

Ključne reči:
antibakterijska aktivnost, T. aestivum cv. Tosunbey, T. aestivum cv. Sultan 95

Introduction

Antibiotic resistance has become a global concern (Westh et al., 2004) and clinical efficiency of many existing antibiotics is being threatened by the emergence of multi-drug resistant pathogens as reported by Bandow et al. (2003). So, there is an urgent need to develop new antimicrobial compounds which are more active against newer and re-emerging infectious diseases (Rojas et al., 1992). Medicinal plants have bioactive compounds which are used for curing of various human diseases and play an important role in healing. Biosynthesis and accumulation of substances such as flavonoids, phenolic acid and anthocyanin enhances the biological activity (antimicrobial, antioxidant, etc.) of plant material (Karakaş and Türker, 2013).

Wheat grass, *Triticum aestivum* L. has a long history and is widely used as a health food supplement. It is found to be utilized as a treatment for minor ailments and serious life-threatening diseases, and as a preventive dietary supplement and therapeutic drug.

Salinity is one of the most common environmental stress factors that affect plant growth and crop production in cultivated areas worldwide. Primary saline conditions appear naturally in environment, while anthropogenic activities are responsible for secondary salinity. Increased urbanization and deforestation are two important human-derived activities for salinity. A number of reports on soil salinization and their influences on crop productivity, land degradation and ecological disturbances are reported worldwide. Excess amount of salts affected physical, chemical, as well as the biological properties of soils. Plant health in saline soils considerably declines owing to poor nutrition, osmotic stress and reduced microbial diversity (Paul and Lade, 2014; Vimal et al., 2018).

The response of plants to physiological stress conditions (e.g. salinity) activates genes encoding pathogen-related proteins (PRs) (Sehgal and Mohamad, 2018). Therefore, PRs are commonly synthesized in plants not only under biotic stress conditions, but also under abiotic stress conditions (such as osmotic shock, chemical injury, and aging). In fact, there are inducible defense proteins classified into 17 PRs families in plants (Van Loon et al., 2006; Sehgal and Mohamad, 2018). The relationship between antifungal (Schmidt at al., 2019) and antimicrobial resistance (Sharma et al., 2018) has been shown in proteins induced by oxidative stress caused by environmental stresses. In addition, phytoalexins synthesized in response to stresses such as antimicrobial attack and physical injury in plants (Ejike et al., 2013) have been shown to increase antimicrobial activity in the rice plant in relation to the physiology of chloroplast and mitochondria (Li et al., 2011). Accordingly, it is believed that increases in antimicrobial activity are a consequence of oxidative stress caused by environmental stresses.

In this study, the effects of seawater induced salt stress (24-26% NaCl, 24 h) and salicylic acid (SA) priming combinations were investigated on antimicrobial activity in 21-day-old seedlings of the two wheat varieties [cv. Tosunbey (drought tolerant) and cv. Sultan 95 (drought sensitive)].

Material and methods

The plants material:

The seeds were sterilized by washing with 5% sodium hypochlorite solution for 5 min (1 time) and with sterile distilled water for 2.5 min (3 times). Seawater is taken from the coast of Kepez (Çanakkale), 30 m from the coast, and filtered in the laboratory to remove the particles. Salt concentration of clear seawater is 24-26% (Türkoğlu, 2006) and it was mixed with distilled water to obtain 50% concentration (SW 50%), while full concentration (SW 100%) was also used in experiments. Seedlings were planted in perlite:peat mixture (1:3) containing pots. Plants were watered with Hoagland nutrient solution (100%; Steward, 1983) for 21 days. Seedlings of *T. aestivum* L. cv. Tosunbey (drought tolerant) and cv. Sultan 95 (drought sensitive) were divided into three groups: (1) control; not treated with salt or acetyl salicylic acid; (2) treatment with 100 ml sea water (50% and 100 %); (3) sea water and pre-treatment of seeds with 1000 mg acetyl salicylic acid (during 2 hours in 2 x 500 mg aspirin tablets solution). Leaf sampling was collected after 24 hours of salt treatment of 21d old plants.

Test microorganisms

Gram-negative bacteria (*Escherichia coli* NRRL B-3704, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 13315, *Acinetobacter baumanii* ATCC 19606), Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Staphylococcus haemolyticus* ATCC 43252) and yeast (*Candida albicans* ATCC 10231) were used for determining the antimicrobial activities of the two *T. aestivum* varieties.

Preparation of extracts for antimicrobial activity

Air-dried samples of two wheat varieties were grounded into a fine powder in a grinding mill. Pulverized plant samples (1 g) were extracted with 10 mL of 80% ethanol, (1:10 w/v) using an orbital shaker for 8 h at room temperature. The extract was separated from the solids by filtration with Whatman No. 1 filter paper. The remaining solids

Table 1. Antimicrobial activity results of the two varieties of *T. aestivum* extracts

Test microorganisms	Plant extracts																	
	*Disc Diffusion[a] (mm)									MIC (µg/mL)								
	cv. Tosunbey				cv. Sultan 95					cv. Tosunbey				cv. Sultan 95				
	T1	T2	T3	T4	S1	S2	S3	S4	Control P10/ NY100	T1	T2	T3	T4	S1	S2	S3	S4	Control ST/ NY100
E. coli NRRL B-3704	8.0 ± 0.21	11.0 ± 0.2	8.0 ± 0.03	8.0 ± 0.03	12.0 ± 0. 01	11.0 ± 0.03	7.0 ± 0. 01	7.0 ± 0. 01	16.0	20.0	2.5	20.0	20.0	2.5	2.5	20.0	20.0	4.0
P. aeruginosa ATCC 27853	10.0 ± 0.11	9.0 ± 0.02	7.0 ± 0.2	10.0 ± 0.01	11.0 ± 0.12	7.0 ± 0.01	10.0 ± 0.02	13.0 ± 0.13	8.0	5.0	5.0	20.0	5.0	2.5	20.0	10.0	2.5	1.0
P. vulgaris ATCC 13315	13.0 ± 0.01	10.0 ± 0. 01	8.0 ± 0.1	11.0 ± 0.14	12.0 ± 0.12	9.0 ± 0.03	9.0 ± 0.02	8.0 ± 0.01	13.0	2.5	5.0	20.0	5.0	2.5	10.0	10.0	20.0	4.0
A. baumanii ATCC 19606	10.0 ± 0. 01	9.0 ± 0.12	8.0 ± 0.01	10.0 ± 0.12	10.0 ± 0.01	7.0 ± 0.03	10.0 ± 0.04	10.0 ± 0.12	12.0	10.0	20.0	20.0	10.0	10.0	20.0	20.0	20.0	2.0
B. subtilis ATCC 6633	7.0 ± 0.14	8.0 ± 0.2	7.0 ± 0.12	9.0 ± 0.03	11.0 ± 0.12	9.0 ± 0.03	8.0 ± 0.12	10.0 ± 0.12	14.0	20.0	20.0	20.0	5.0	2.5	10.0	20.0	5.0	4.0
S. aureus ATCC 6538P	11.0 ± 0.02	9.0 ± 0.12	8.0 ± 0.04	10.0 ± 0.1	10.0 ± 0.01	8.0 ± 0.01	12.0 ± 0.01	7.0 ± 0. 01	15.0	2.5	5.0	20.0	5.0	5.0	20.0	2.5	20.0	4.0
S. haemolyticus ATCC 43252	9.0 ± 0.03	10.0 ± 0.1	8.0 ± 0.12	10.0 ± 0.1	11.0 ± 0.1	10.0 ± 0.2	10.0 ± 0. 01	8.0 ± 0. 01	14.0	5.0	5.0	20.0	5.0	5.0	5.0	5.0	20.0	5.0
Candida albicans ATCC 10231	7.0 ± 0.1	8 . 0 ± 0.1	10 ± 0.1	11.0 ± 0. 01	8.0 ± 0.1	7.0 ± 0.12	7.0 ± 0.03	7.0 ± 0.12	16.0	20.0	20.0	10.0	5.0	20.0	20.0	20.0	20.0	2.5

T*: T. aestivum* cv. Tosunbey

S: *T. aestivum* cv. Sultan 95

T1: control; T2: treatment with acetyl salicylic acid; T3: treatment with salinity stress; T4: salinity stress and pre-treatment of seeds with acetyl salicylic acid

S1: control; S2: treatment with acetyl salicylic acid; S3: treatment with salinity stress; S4: salinity stress and pre-treatment of seeds with acetyl salicylic acid
Inhibition zone (mm); a includes diameter of disk (6 mm); P10 = Penicillin (10 ug/disc); ST: Streptomycin; NY100 Nystatin 100 ug/disc

were extracted twice with the same solvent and the extracts were combined. Extracts were stored in a refrigerator (4 °C) until analyzed (Sultana et al., 2007).

Screening of antimicrobial activity

Disc diffusion assay

Empty sterilized antibiotic discs having a diameter of 6 mm (Schleicher and Schull No. 2668, Dassel, Germany) were each impregnated with 50 µL of the extract (10 mg/disc). All the bacteria mentioned above were incubated at 35 ± 0.1 °C for 24 h by inoculation into Nutrient Broth (Difco Laboratories, MI, USA) and the yeast culture studied was incubated in Malt Extract Broth (Difco Laboratories, MI, USA) at 25 ± 0.1 °C for 48 h. An inoculum containing 10^6 bacterial cells or 10^8 yeast cells/mL was spread on Mueller Hinton Agar (MHA) (Oxoid Ltd., Hampshire, UK) plates (1 mL inoculum/plate). The discs injected with extracts were placed on the inoculated agar by pressing slightly. Petri dishes were placed at 4 °C for 2 h, afterwards the plate inoculated with the yeast culture was incubated at 25 ± 0.1 °C and bacteria were incubated at 35 ± 0.1 °C for 24 h (Collins et al., 1989). At the end of the period, inhibition zones formed on the medium were measured in millimeters. Studies were performed in triplicate. Treatments with penicillin (P10), and nystatin (NYS30) served as positive controls and treatments with ethanol, without bacterial or fungal materials served as negative controls.

Minimum inhibitory concentration assay

Minimum Inhibitory Concentration (MIC) was investigated as recommended by instructions of the Clinical and Laboratory Standards Institute (CLSI, 2006). The lowest concentration of extracts inhibiting visible growth of each test microorganisms was taken as the MIC. The medium, 0.1% (w/v) streptomycin (ST), nystatin (NYS100) and 10% DMSO were used as the non-treated, positive and negative controls, respectively (Teanpaisan et al., 2017).

Statistical analysis

The results of antimicrobial assay were mean ± SD of the three parallel assays.

Results and discussion

A total of eight plant extracts were tested against eight test microorganisms. Ethanol was selected as extraction solvent, because it is one of the best solvents used for the extraction of antimicrobial substances (Jonathan and Fasidi, 2003).

The results showed that the ethanol extracts from the different treatments studied have antimicrobial activities, with the diameters of the inhibition zone ranging from 8 to 13 mm and 2.5 to 20 µg/mL, respectively (**Tab. 1**). The highest antimicrobial activity was demonstrated against *P. aeruginosa* ATCC 27853 by the extract of *T. aestivum* cv. Sultan 95 which grown in sea water and whose seeds were pre-treated with acetyl salicylic acid (S4). However, the disc diffusion method is a qualitative technique and is mainly used for selecting extracts with antimicrobial activity, mostly when diameter zones of inhibition are ≥ 10 mm (Sánchez et al., 2016). So, *S. haemolyticus* ATCC 43252 was the second most susceptible strain against the tested plants extracts. The same was found when observing the results of micro-dilution method.

Although the disc diffusion method is one of the inexpensive and less laborious antimicrobial testing methods currently available, this procedure suffers with the demerit of not always producing reproducible results for plant extracts (Eloff, 1998; Vambe et al., 2018). This is primarily because some constituents do not diffuse readily in polarized agarose gels (Vambe et al., 2018).

When the same extracts were screened using microdilution method, five plant extracts, namely T1, T2, S1, S2, S3 demonstrated higher antibacterial activity than the control antibiotic. This discordance observed in the results obtained from these two bioassays could be attributed to several factors (Vambe et al., 2018). It is possible that antibacterial activity was masked in the disk diffusion assay by low diffusion potential.

Wheat is a grain containing protein, starch (Vida et al., 2014), fiber, phytochemicals and antioxidant substances which have a very important place in human nutrition (Andersson et al., 2013). While meeting the daily nutrition requirements with protein and starch, it facilitates digestion with fibers and thus reduces the risk of developing colon cancer (Huang et al., 2015). This plant has been also shown to have anti-inflammatory, antioxidant, anti-carcinogenic, immuno-modulatory, laxative, astringent, diuretic, antibacterial and anti-aging properties and is a part of formulations as a highly effective antimicrobial agent (Saha et al., 2018).

In the literature there are many investigations about wheat grass antimicrobial activity. Pallavi et al. (2011), tested wheat grass extracts against the Gram-positive bacteria *S. aureus*, *B. subtilis* and Gram-negative *E. coli*, using Amoxicillin as standard. Certain extracts exhibited considerable activity against *B. subtilis* and moderate activity against *S. aureus* and *E. coli*. Ashok (2011) also reported antibacterial activity against *E. coli*, *P. aeruginosa* and *S. aureus*. Antifungal activity was also reported against *C. albicans.* Sundaresan et al. (2015), tested antimicrobial properties of 7th, 14th,

and 21st day wheat grass extracts obtained with five different solvents (water, ethanol, methanol, ethyl acetate and hexane). All these extracts showed antibacterial activity against seven food borne pathogens. Amongst them, hexane extracts from the seven-day-old wheat grass showed maximum antibacterial activity, especially against *Yersinia enterocolitica* and *Listeria monocytogenes*. Das et al. (2012) found that acetone (80%) extract of wheatgrass was effective against five foodborne microorganisms, including the fungus *Aspergillus niger*, a common contaminant of food.

There appears to be a lack of published scientific data on the salt stress effect to antimicrobial properties of wheat grass, thereby highlighting the need for continued research.

Plants are frequently subjected to a variety of harsh environmental stresses such as scarcity of water, extreme temperatures, high soil salinity, herbivore attack, and pathogen infection diminishing their productivity (Sewelam et al., 2016). Different salt concentrations induce physiological and metabolic changes in plants affecting their seed germination, growth, development, yield and also decreases the rate of respiration and photosynthesis in plants. In addition to the growth and yield, the composition of bioactive compounds present in the aromatic and medicinal plants is affected by salinity (Gil et al., 2002). The increased levels of plant secondary metabolites such as phenols, flavonoids, tannins, alkaloids and others under the influence of increased salt concentrations as a part of defense mechanism have been reported (Kate, 2008). Reactive oxygen species (ROS) are continuously produced under normal environmental conditions in plant cells. Bioactive compounds also trigger metabolic pathways in plant cells and result in a higher ROS accumulation. The common response observed in salt-stressed plants is the generation of ROS, highly reactive and responsible in damaging cell structures, nucleic acids, lipids and proteins. Plants possess medicinal value with anti-inflammatory and antimicrobial activities; acquired resistance to stress induced by ROS is due to the presence of several bio-active compounds (Foyer et al., 1994).

In the scientific literature, there are no data on the effect of salt stress on wheat grass antimicrobial activity. Kotagiri et al. (2017) investigated the secondary metabolites and antimicrobial potential of leaf, stem and root ethanol and chloroform extracts of five different *Coleus* species; *C. aromaticus*, *C. amboinicus*, *C. barbatus*, *C. forskohlii* and *C. zeylanicus* subjected to salinity stress. The up-regulation in the content of plant bioactive compounds, along with the antimicrobial activities of ethanol and chloroform extracts under the influence of salinity stress have been observed during the study in *Coleus*. The leaf, stem and root extracts of all the five *Coleus* species showed good antimicrobial activity against the tested pathogenic strains. The leaf extracts of *Coleus* showed higher inhibitory activity compared to the stem and root extracts. Ethanol extracts showed higher antimicrobial activity ranging from 1.5-100.0 mg/ml when compared with the chloroform extracts ranging from 0.97-250 mg/ml respectively. The leaf, stem and root extracts of *Coleus* showed effective antimicrobial activity against the pathogenic strains even under saline conditions is due to the up regulation of secondary metabolites which provides a scope of developing novel drugs to treat infectious diseases. Our results have a correlation with the results of Kotagiri et al (2017) about increasing antimicrobial activity under saline stress and pre-treatment of seeds with acetyl salicylic acid conditions, which is probably related to the up-regulation of secondary metabolites which provides a scope for developing novel drugs to treat *P. aeruginosa* ATCC 27853.

Conclusion

The screening of the plant extracts for antimicrobial activity has shown that higher plants represent a potential source of new anti-infective agents. Herbal medicines are a valuable and rapidly available resource for primary health care and complementary health care systems. The results of the present study provide evidence that *T. aestivum* plant varieties grown in different condition represent an important asset to the health care in communities. Some infectious diseases could be potentially managed using *T. aestivum* plant varieties, based on mono- or combination therapies. More pharmacological investigations including phytochemical analysis are necessary to find out which compounds are responsible for observed effects.

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