Antimicrobial activity in a drought-tolerant lentil variety under some stress conditions

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Abstract. Broomrapes are the most difficult plant parasites to control among all biotic stresses affecting plants in the Mediterranean, Europe and Asia. Lentil is sensitive to broomrape (*Orobanche crenata*), which causes significant yield losses in this species around the Mediterranean region. Our study was aimed at the evaluation of antibacterial properties of *Lens culinaris* cv. 'Sultan-1' under drought stress and broomrape infection stress conditions. The antimicrobial activity of the ethanol extract of 'Sultan-1' was assayed against the test for microorganisms by methods of diffusion in agar and dilution in broth. The ethanol extracts from different studied treatments showed antimicrobial activities, with diameters of the inhibition zone ranging from 7 mm to 15 mm and from 2.5 to 20 μg mL^{-1}, respectively. The highest antimicrobial activity against *A. baumannii* ATCC 19606 was demonstrated by the extract of 'Sultan-1' at combined stress (drought stress + broomrape infection) on the 1st day. Furthermore, the combined stress treatment promoted antibacterial effect against some Gram-negative bacteria on the 1st day.

Key words: Antimicrobial activity, broomrape, drought stress, lentil, 'Sultan-1'

Introduction

Abiotic and biotic stress factors limit the yields (Haklı 2008). Drought, salinity, low or high temperature, and contamination are the leading abiotic factors that decrease yields in agricultural production (Demirbaş & Acar 2008). Drought claims the greatest share of all stress factors (Kalefetoğlu & Ekmekçi 2005) and is one of the most important factors limiting plant production (Boyer 1982). One of the strongest limiting biotic factors in lentil production is broomrape (*Phelipanche* spp., *Orobanche* spp.) (Rubiales & al. 2003). Broomrapes are the most difficult plant parasites to control among all biotic stresses affecting plants in the Mediterranean, Europe, and Asia. Lentil – *Lens culinaris* Medik., is sensitive to broomrape – *Orobanche crenata* Forskk., which causes significant yield losses in this species around the Mediterranean region (Jurado-Exposito & al. 1997).

Plants contain antimicrobial properties important in drug design against diseases (Das & al. 2010; Bhattacharjee & Islam 2015). Antibiotic resistance has become global concern (Westh & al. 2004) for clinical efficacy of many existing antibiotics is threatened by the emergence of multidrug-resistant pathogens. Therefore, there is an urgent need in developing new antimicrobial compounds that are more active against the newly-emerging infectious diseases (Rojas & al. 1992). On the other hand, antimicrobial activity has been reported to increase due to oxidative stress caused by environmental stress (Sharma & al. 2018; Schmidt & al. 2019). Moreover, relation between plant-based antimicrobial activity and pathogen-

associated proteins (PRs), such as phytoalexins, is manifested under environmental stress (Li & al. 2011; Acar & Hacioğlu Doğru 2019; Schmidt & al. 2019).

In this context, medicinal plants, which produce and accumulate bioactive substances such as flavonoids, phenolic acid and anthocyanin, are another important natural resource for the production of new bioactive compounds (Karakaş & Türker 2013). This study was conducted to evaluate the antimicrobial activities of ethanolic extracts of *L. culinaris* 'Sultan-1' exposed to two stress factors for seven days.

Material and methods

Plant material: Drought-tolerant variety *Lens culinaris* 'Sultan-1' was used for this study and obtained from Geçitkuşağı Central Research Institute (Eskişehir/Turkey). *Orobanche crenata* seeds were obtained from the Adana Plant Protection Research Institute (Adana/Turkey). The seeds of 'Sultan-1' were sterilized by washing with sodium hypochlorite solution (20 %, for 5 min) and with sterile distilled water for 7.5 min. Sterile seeds were germinated in sterile petri dishes and transferred to pots containing perlite. All seedlings were watered with Hoagland nutrient solution (Steward 1983). Fourteen-day old seedlings were grown in water culture (25 ± 2 °C, 16/8 photoperiod).

Orobanche crenata seeds were washed with 70 % ethyl alcohol for 2 min, then sterilized by sodium hypochlorite (5 %, 10 min) and washed with water. Sterilized seeds were incubated for 1 week in a plant-growth cabinet at 22 °C. *Orobanche crenata* seeds were induced with 1ppm GR24 and the roots of 21-day-old lentil seedlings were infected. Drought stress was created by applying polyethylene glycol 6000 (10 % PEG 6000, –1.5 MPa) (Gökçay 2012). Antimicrobial properties were determined in leaf samples taken on the first and the seventh day. Lentil seedlings (21st day) were divided into four groups for the 1st and 7th day samples: (1) control (1st day); (2) broomrape infected (1st day); (3) drought stressed (1st day); (4) drought stressed + broomrape infected (1st day); (5) control (7th day); (6) broomrape infected (7th day); (7) drought stressed (7th day); (8) drought stressed + broomrape infected (7th day).

Test for microorganisms: Gram-negative bacteria (*Escherichia coli* NRRLB 3704, *Pseudomonas aeruginosa* ATCC 27853, *Proteus vulgaris* ATCC 13315, *Acinetobacter baumanii* ATCC 19606), Gram-positive bacteria (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus haemolyticus* ATCC 43252) and yeast (*Candida albicans* ATCC 10231) were used for determining the antimicrobial activities of 'Sultan-1'.

Preparation of extracts for antimicrobial activity: Air-dried leaf samples of 'Sultan-1' were ground into 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 were extracted twice with the same solvent and extracts combined. Extracts were stored in a refrigerator (4 °C) until analyzed (Sultana & al. 2007).

Screening for antimicrobial activities: Antimicrobial activities of 'Sultan-1' were screened with Disc Diffusion (CLSI 2006) and Minimum Inhibitor Concentration (MIC) (CLSI 2006; Teanpasian & al. 2017). Our study was aimed at evaluation of the antibacterial properties of 'Sultan-1' under drought stress and broomrape infection conditions, between the 1st and 7st day, with controls. Antimicrobial activity of the ethanol extract of 'Sultan-1' variety was assayed against the test for microorganisms, by methods of diffusion in agar and dilution. Empty sterilized antibiotic discs with a diameter of 6 mm (Schleicher and Schull No. 2668, Dassel, Germany) were each impregnated with 50 µL of 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 studied yeast culture was incubated in Malt Extract Broth (Difco Laboratories, MI, USA) at 25 ± 0.1 °C for 48 h. An inoculum containing 106 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 kept at 4 °C for 2 h, plaques injected with the yeast culture were incubated at 25 ± 0.1 °C, and bacteria were incubated at 35 ± 0.1 °C for 24 h (CLSI 2006). At the end of the period, the inhibition zones formed in the medium were evaluated in millimeters. Studies were repeated 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 carried out according to the instruction of the Clinical and Laboratory Standards Institute (CLSI 2006). The lowest concentration of extracts inhibiting visible growth of each test microorganism was taken as MIC. The medium, 0.1 % (w/v) Streptomycin (ST), Nystatin (NYS100) and 10 % DMSO were used as non-treated, positive and negative controls, respectively (Teanpasian & al. 2017).

Results

Broomrape infection and drought stress treatments showed different antimicrobial activities in the extract of 21-day-old lentil seedlings. According to our results, diameters of the inhibition zone (IZ) and minimal inhibitory concentration (MIC) ranged from 7–15 mm and 2.5–20.0 μg/mL, respectively.

The highest antimicrobial activity of the extract of 'Sultan-1' recorded against S. *aureus* ATCC 6538P was demonstrated by the extract of 'Sultan-1' grown under drought stress and broomrape infection stress conditions (S1 in Table 1). Lentil extracts treated with both broomrape (S2) and drought stress + broomrape (S4) showed a higher zone of inhibition in all Gram-negative bacteria, as compared to the controls (P10), especially in *A. baumanii* ATCC 19606 and *P. aeruginosa* ATCC 27853. This is an indication that the Gram-negative test bacteria are more effective than the Gram-positive test bacteria. Furthermore, an increase in antimicrobial activity was observed in infected with *O. crenata* Forsk. 'Sultan-1' plants (S2, 1st day, in Table 1). This has been recorded especially in *A. baumanii* ATCC 19606 test microorganisms, with diameters of the IZ ranging from 14.0 mm. However, antimicrobial activity decreased with drought appication and again increased under drought stress and pre-treatment of seeds with broomrape (S2 and S4 in Table 1, 1st day).

Table 1. Disc diffusion, MIC ratios of *Lens culinaris* 'Sultan-1' extracts.

Test microorganisms	Plant extracts																	
	*Disc diffusiona									MIC								
	cv. 'Sultan-1'								Control P10/ NY100	cv. 'Sultan-1'								Control ST/ NY100
	S1	S2	S3	S4	S5	S6	S7	S8		S1	S2	S3	S4	S5	S6	S7	S8	
***E.coli* NRRL B-3704**	7.0	10.0	9.0	10.0	7.0	–	–	–	16.0	20.0	5.0	20.0	5.0	20.0	20.0	20.0	20.0	4.0
***P. aeruginosa* ATCC 27853**	9.0	9.0	11.0	12.0	9.0	7.0	10.0	10.0	8.0	5.0	1.0	2.5	2.5	5.0	20.0	10.0	5.0	1.0
***P. vulgaris* ATCC 13315**	11.0	10.0	9.0	11.0	8.0	7.0	7.0	7.0	13.0	2.5	5.0	20.0	5.0	20.0	20.0	20.0	20.0	4.0
***A. baumanii* ATCC 19606**	9.0	14.0	8.0	13.0	–	–	9.0	10.0	12.0	10.0	2.5	20.0	2.5	20.0	20.0	10.0	10.0	2.0
***B. subtilis* ATCC 6633**	7.0	7.0	8.0	7.0	8.0	7.0	8.0	9.0	14.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	20.0	4.0
***S. aureus* ATCC 6538P**	15.0	10.0	9.0	11.0	7.0	–	10.0	9.0	15.0	2.5	5.0	20.0	5.0	20.0	20.0	10.0	20.0	4.0
***S. haemolyticus* ATCC 43252**	12.0	10.0	9.0	9.0	9.0	8.0	9.0	10.0	14.0	5.0	5.0	20.0	20.0	20.0	20.0	20.0	20.0	5.0
***Candida albicans* ATCC 10231**	–	8.0	9.0	11.0	7.0	8.0	8.0	9.0	16.0	20.0	20.0	10.0	5.0	20.0	20.0	20.0	20.0	2.5

1: *L. culinaris* cv. 'Sultan-1': control (1st day);

S2: pre- treatment of seeds with broomrape (1st day)

S3: drought stress (1st day);

S4: drought stress + pre-treatment of seeds with broomrape (1st day)

S5: control (7stday)

S6: pre-treatment of seeds with broomrape (7stday)

S7: drought stress (7stday)

S8: drought stress and pre-treatment of seeds with broomrape (7stday).

Inhibition zone (mm); a – includes diameter of disk (6 mm); P10 = Penicillin (10 ug/disc); ST: Streptomycin; NY100 Nystatin 100 ug/disc.

As a result, biotic stress caused by *O. crenata* infection increased the antimicrobial activity of 'Sultan-1'. In particular, one-day stress applications on *Candida albicans* ATCC 10231 have promoted increased antimicrobial activity. Furthermore, the first day of stress application increased antimicrobial activity, whereas the last day of stress application was not determined. Actually, combined stress treatment promoted the antibacterial effect against some Gram-negative bacteria on the 1st day of sampling (Table 1).

Discussion

Phytochemicals are secondary metabolites of plants and have important pharmaceutical properties. Thus, they could be used as a new antimicrobial source of food production and clinical settings (Sakarikou & al. 2020). It is well known that abiotic stress leads to a series of morphological, physiological, biochemical, and molecular changes, which affect adversely plant growth, productivity and antagonistic activity (Hayat & al. 2013; Ripa & al. 2019).

Our results have shown that the extract of 'Sultan-1' triggers out higher antimicrobial activity against the Gram-negative test bacteria than against the Gram-positive bacteria under broomrape and combined stress conditions. Similarly, it was noted that biostimulants are triggering out an increasing amount of total phenolic compounds in green bean (*Phaseolus vulgaris* L.) seed extracts, which resulted in antimicrobial activity against *Bacillus cereus* (Petropouos & al. 2019). Furthermore, the Gram-negative bacteria have a unique outer membrane. This outer membrane excludes certain drugs and antibiotics from penetrating the cell. This partially accounts for the fact why the Gram-negative bacteria are generally more resistant to antibiotics than the Gram-positive bacteria (Dülger & Dülger 2018). In fact, some plant-derived secondary metabolites, such as chalcone, cause oxidative stress and show antimicrobial activity by degrading the membrane of *Staphylococcus aureus*, a Gram-positive bacterium (Meier & al. 2019).

Lentils have bioactive compounds (Ganesan & Xu 2017) and antimicrobial activity. However, the antimicrobial activity of lentils grown under normal conditions was found to be low, as compared to conventional antibiotics (Nair & al. 2013). It was known that lentil plants have secondary metabolites such as lectins (Nair & al. 2013) and defensins (Drikvand & al. 2019), which induce antimicrobial activity. On the other hand, it was discovered that only broomrape leaf extracts have antimicrobial activity against the Gram-positive strains (Genovese & al. 2019), *Salmonella enteritidis* (Abbes & al. 2014) and six different pathogens (Gatto & al. 2015). Contrary to this, our results indicated the highest antibacterial activity against *A. baumanii* ATCC 19606 under drought-stressed and drought-stressed+broomrape infection applications among all groups (S2, 1st day, and S4 ,1st day, in Table 1). Moreover, all broomrape infections increased the MIC level to 20 µg/mL. Contrary to our research, Hsouna & al. (2019) found similar IZ (14-22 mm) and different MIC (62-250 µg/mL) data in *Lobularia maritima* under biostimulant application and stress conditions. This shows that the IZ and MIC results may vary depending on the plant and application.

This is the first report on antimicrobial activity in the lentil variety 'Sultan-1' under drought stress and broomrape infection conditions in literature. Accordingly, biotic and abiotic stress conditions may alter antimicrobial activity in lentils. In this context, our findings provide evidence about the antimicrobial activity of the drought-tolerant variety 'Sultan-1' and suggest that new antimicrobial investigations into the relationships between lentils and other stress factors are necessary for the future.

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