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Short Communication

ECONOMIC MAXIMIZATION OF ALFALFA ANTIMICROBIAL EFFICACY USING STRESSFUL FACTORS

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ABSTRACT

Objective: The present study addresses the effect of water deficit stress on the antimicrobial capacity of alfalfa (*Medicago sativa*) plants.

Methods: Methanolic extracts of alfalfa plants grown in different soil types, varying in sand proportion, either alone or combined with various levels of water regimes were assessed for antibacterial and antifungal activities following cup plate method. The phytochemical profiles of plant extracts were also qualitatively screened using appropriate chemical reagents. Moreover, data were intensively processed *via* two different statistical designs.

Results: Increasing sand amount induced the inhibitory effect of plant extracts on *Escherichia coli*, *Klebsiella pneumonia*, *Proteus vulgaris*, *Salmonella typhi*, *Mucor circinelloides*, *Rhizopus azygosporus* and *R. microsporus* with less pronounced action on *Shigella flexneri*, *Staphylococcus epidermidis*, *Candida albicans* and *Emericella quadrillineata*; as well as a reversed influence on *Pseudomonas aerugenosa* and *Streptococcus pyrogenes*. Furthermore, withholding irrigation water enhanced the plant suppressive action on *E. coli, Salmonella typhi*, *Staphylococcus epidermidis*, *Candida albicans* and *R. microsporus* with less marked or reversed influence on the other tested microbes. However, *Pseudallescheria ellipsoidea*, two species of *Penicillium* and five of *Aspergillus* could resist the studied plant extracts. The results also revealed that the extracts of water-unsatisfied plants generally contained higher amounts of alkaloids, amino acids, flavonoids, glycosides, phytosterols, saponins, steroids, tannins, terpenoids and reducing sugars.

Conclusion: The employed biological evaluations point out to promising antimicrobial efficiency of alfalfa plants particularly when stressed.

Keywords: Alfalfa, Sand, Drought, Antibacterial, Antifungal, Phytochemicals

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For centuries, desertification and drought could affect many parts of the world with remarkable negative impact on land productivity. Almost every year, new sectors of the earth are hit by theses stresses bringing about paramount disturbance in agricultural yield [1]. Therefore, many studies have been registered to estimate the consequences of such constraints on plant performance, the response of plants to these devastating factors and also the techniques that could be employed to ameliorate their adverse effects. Nevertheless, it is to somewhat scarce to find studies on how to take advantage from these stressful conditions that threaten vast tracts of ecosystems.

For a known medicinal plant species, certain molecules with pharmacological activity may be over-synthesized when it is grown under a new set of environmental conditions, and this would elucidate the improvement of its beneficial effects under stress. In this connection, the levels of bioactive phytochemicals were documented to fluctuate with the environmental conditions [2]. Generally, comparative analyses manifested that the content of various secondary metabolites in plants suffering water shortage is higher than that in their synonyms growing under optimum circumstances [3].

The failure of some available antibiotics produced by pharma-cological industries to combat some pathogenic microbes, along with the baleful side effects of antibiotic overuse and misuse have forced researchers to investigate the antimicrobial activity of medicinal plants [4]. Alfalfa (*Medicago sativa* L., family Fabaceae) is a perennial herb with seeds that have long been used in traditional medicine for prevention and cure of various ailments [5]. However and up to our knowledge, few studies scrutinizing the antimicrobial capacity of alfalfa vegetative parts can be recorded [6].

Therefore, the present study aims at exploring stress utilization as an effectual simple, low-cost and low-risk strategy to promote alfalfa efficacy as a medicinal plant. In a trial to maximize its antimicrobial efficiency, alfalfa plants would be grown with little water supply employed to different levels either alone or combined with elevated sand proportion in the growing soil.

Seeds of alfalfa (*Medicago sativa* L., cultivar Nubaria 1) were obtained from Al Nubaria Agricultural Organization, El Biheera Governorate, Egypt. The seeds were sown in 3 groups of pots packed with clay and sand mixed to obtain 3 types of soil with 33, 67 and 100% sand proportion. Each group of pots was then subdivided into 3 sets; (1) control: plants were irrigated when required, (2) moderate drought: stress was imposed by withholding 33% of irrigation water and (3) severe drought: stress was imposed by withholding 67% of irrigation water; with drought starting from the day 45 for further 30 d.

Following Kosem *et al.* [7], 20 g of the powdered air-dry vegetative plant tissues was extracted with 200 ml of 50% methanol (El Gomhuria Company) for a week at 37 °C. The antimicrobial activity of the plant methanolic extracts, as well as the negative control (methanol) and positive ones (streptomycin as antibacterial and nystatin as antifungal agents), was performed following cup plate method as adopted by Nair and Chanda [8]. The assayed microbial strains were provided by the Laboratory of Bacteriology and that of Mycology, Botany Department, Faculty of Science, Mansoura University, Egypt. According to Harborne [9] and Kokate [10], methanolic extracts were analyzed to identify phytoconstituents of the plant extracts qualitatively.

Using CoHort/CoStat software, descriptive analysis was performed to determine standard deviation. In addition and in an ANOVA (Analysis Of Variance)-type experiment, two different designs were applied. The first involved all treatments as a single factor with the subjects arranged in "One Way Completely Randomized" type, while the second involved two separate factors (sand proportion as a subplot and watering level as the main plot) with the subjects arranged in "Split Plot" way. *Via* both designs, small letters were denoted according to the values of least significant difference (LSD) and mean standard error (MSE) at $P<0.05$ where different letters refer to significant variation.

As shown in table 1, the inhibitory effect of alfalfa extracts on all the tested bacteria generally increased with raising sand amount in the

cultivation soil except for *Pseudomonas aerugenosa* and *Streptococcus pyrogenes*. In addition, water withholding induced the retardant impact of the plant extracts on all the checked bacteria except for *Shigella flexneri* and *Streptococcus pyrogenes*.

Table 1: Effect of different water regimes on the antibacterial activity of alfalfa plants grown in different soil types. Data listed represent the mean values±standard deviation where different letters refer to significant variation with least significant difference (LSD) and mean standard error (MSE) at P<0.05

Treatment	Diameter of Clear Zone (mm)							
	Escherichia coli	***Klebsiella pneumoniae***	***Proteus vulgaris***	***Pseudomonas aerugenosa***	***Salmonella typhi***	***Shigella flexneri***	***Staphylococcus epidermidis***	***Streptococcus pyrogenes***
33% sand+100% watering	0 ^{f}±0	14 ^{g}±0	21 ^{d}±1	19 ^{cd}±1	0 ^{h}±0	16 ^{e}±0	14 ^{f}±0	12 ^{d}±0
67% sand+100% watering	14 ^{e}±0	15 ^{fg}±1	25 ^{c}±1	0 ^{f}±0	0 ^{h}±0	24 ^{b}±0	16 ^{e}±0	12 ^{d}±0
100% sand+100% watering	15 ^{e}±1	16 ^{ef}±0	18 ^{e}±0	17 ^{de}±1	0 ^{h}±0	18 ^{d}±0	20 ^{b}±0	14 ^{c}±0
33% sand+67% watering	19 ^{d}±1	18 ^{cd}±0	29 ^{a}±1	21 ^{c}±1	14 ^{fg}±0	17 ^{de}±1	18 ^{cd}±0	18 ^{b}±0
67% sand+67% watering	21 ^{c}±1	19 ^{bc}±1	26 ^{bc}±0	26 ^{b}±0	15 ^{ef}±1	17 ^{de}±1	16 ^{e}±0	0 ^{e}±0
100% sand+67% watering	14 ^{e}±0	17 ^{de}±1	21 ^{d}±1	31 ^{a}±1	18 ^{c}±0	21 ^{c}±1	17 ^{de}±1	0 ^{e}±0
33% sand+33% watering	24 ^{b}±0	16 ^{ef}±0	14 ^{f}±0	32 ^{a}±0	16 ^{de}±0	18 ^{d}±0	19 ^{bc}±1	0 ^{e}±0
67% sand+33% watering	14 ^{e}±0	17 ^{de}±1	22 ^{d}±0	0^{f}±0	17 ^{cd}±1	0 ^{f}±0	18 ^{cd}±0	0 ^{e}±0
100% sand+33% watering	18 ^{d}±0	20 ^{b}±0	26 ^{bc}±0	0 ^{f}±0	28 ^{b}±0	22 ^{c}±0	18 ^{cd}±0	0 ^{e}±0
Negative Control	14 ^{e}±0	14 ^{g}±0	13 ^{f}±1	15 ^{e}±1	13 ^{g}±1	16 ^{e}±0	10 ^{g}±0	12 ^{d}±0
Positive Control	28 ^{a}±0	22 ^{a}±0	28 ^{ab}±0	24 ^{b}±0	30 ^{a}±0	33 ^{a}±1	26 ^{a}±0	20 ^{a}±0
LSD at P<0.05	1.63	1.88	2.09	2.09	1.63	1.88	1.33	4.8 x 10^{-7}
MSE at P<0.05	0.74	0.85	0.95	0.95	0.74	0.85	0.60	2.2 x 10^{-7}
Significance Degree	***	***	***	***	***	***	***	***
Factor: Sand Proportion								
33% sand	14.33 ^{b}	16.00 ^{b}	21.33 ^{b}	24.00 ^{a}	10.00 ^{b}	17.00 ^{b}	17.00 ^{b}	10.00 ^{a}
67% sand	16.33 ^{a}	17.00 ^{ab}	24.33 ^{a}	8.67 ^{c}	10.67 ^{b}	13.67 ^{c}	16.67 ^{b}	04.00 ^{c}
100% sand	15.67 ^{a}	17.67 ^{a}	21.67 ^{b}	16.00 ^{b}	15.33 ^{a}	20.33 ^{a}	18.33 ^{a}	04.67 ^{b}
LSD at P<0.05	1.33	1.15	1.33	1.33	0.94	3.00	0.94	1.77 x 10^{-7}
Significance Degree	*	*	*	***	***	***	*	***
Factor: Watering Level								
100% watering	09.67^{b}	15.00 ^{a}	21.33 ^{b}	12.00 ^{b}	00.00 ^{c}	19.33 ^{a}	16.67 ^{b}	12.67 ^{a}
67% watering	18.00 ^{a}	18.00 ^{a}	25.33 ^{a}	26.00 ^{a}	15.67 ^{b}	18.33 ^{a}	17.00 ^{b}	06.00 ^{b}
33% watering	18.67 ^{a}	17.67 ^{a}	20.67 ^{b}	10.67 ^{b}	20.33 ^{a}	13.33 ^{b}	18.33 ^{a}	00.00 ^{c}
LSD at P<0.05	1.17	3.10	2.34	2.34	1.17	3.51	1.17	4.05 x 10^{-}[15]
Significance Degree	**	ns	*	**	***	*	*	***

Results in table 2 indicated that the extracts of plants grown under different conditions had no inhibitory effect on the growth of the five species of *Aspergillus*, the two species of *Penicillium* and *Pseudallescheria ellipsoidea*. Meanwhile, stress imposed by increasing sand proportion and/or drought level generally enhanced the ill impact of the plant extracts on the growth of *Candida albicans*, *Emericella quadrilineata*, *Mucor circinelloides*, *Rhizopus azygosporus* and *R. microsporus*.

The antimicrobial activity of the considered alfalfa plants as revealed from the present investigation coincides with many reports that proved that the extracts of many plant species could significantly inhibit the growth of different microbes including bacteria and/or fungi [11]. In the current study, the assayed enhancement of alfalfa antimicrobial activity under stressful conditions can be ascribed to the stimulatory effect of stress on the biosynthesis of various phytochemicals that could be implicated as a tolerance strategy as previously assumed by Ramakrishna and Ravishankar [12].

Herein, the results in table 3 cleared that the extracts of alfalfa plants grown under different water regimes in various soil types contained alkaloids, amino acids, flavonoids, glycosides, phytosterols, saponins, steroids, tannins, terpenoids and reducing sugars; with higher concentrations indicated in most cases by withholding irrigation water. On the other hand, only anthraquinones were not detected in the extracts of all plants.

For alkaloids, they were intensively reported to possess dignified antimicrobial due to their potency for DNA intercalation and/or topoisomerase inhibition [13]. The functional antimicrobial activity of flavonoids may be because of their capability of complexing with soluble extracellular proteins and with the microbial cell walls [14]. The cellular toxicity of steroids to microbial cells was similarly well documented with retarding microbial cell growth, respiration and some essential enzymatic activities [15]. The potent antimicrobial action of saponins has also been reported and attributed to their membranolytic properties [16].

A wide range of antimicrobial actions has also been assigned to tannins due to their ability to make microbial adhesins inoperative, deactivate enzymes and to form complexes with polysaccharides [17]. Terpenoids are also active against infectious microorganisms mainly through disrupting microbial membranes [18]. Amino acids similarly comprise an important constituent in the design of antimicrobials. In this regard, some amino acids could exhibit significant antibacterial activity [19]. Reducing sugars have also been reported to have antimicrobial properties [20]. Moreover, several glycosides could exert antibacterial effects particularly on *E. coli* [21].

Table 2: Effect of different water regimes on the antifungal activity of alfalfa plants grown in different soil types. Data listed represent the mean values±standard deviation where different letters refer to significant variation with least significant difference (LSD) and mean standard error (MSE) at P<0.05

Treatment	Diameter of clear zone (mm)						
	Aspergillus carneus	*Aspergillus flavus*	*Aspergillus fumigatus*	*Aspergillus sydowii*	*Aspergillus terreus*	*Candida albicans*	*Emericella quadrilineata*
33% sand+100% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	32 ^{e}±0	0 ^{c}±0
67% sand+100% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{g}±0	0 ^{c}±0
100% sand+100% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{g}±0	0 ^{c}±0
33% sand+67% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	33 ^{d}±1.41	0 ^{c}±0
67% sand+67% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	34 ^{c}±0	0 ^{c}±0
100% sand+67% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{g}±0	0 ^{c}±0
33% sand+33% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{g}±0	0 ^{c}±0
67% sand+33% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	34 ^{c}±0	0 ^{c}±0
100% sand+33% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	36 ^{b}±0	30 ^{a}±0
Negative Control	14 ^{b}±0	16 ^{b}±0	14 ^{b}±0	20 ^{b}±0	18 ^{b}±0	24 ^{f}±0	17 ^{b}±1.41
Positive Control	25 ^{a}±1.41	28 ^{a}±2.83	20 ^{a}±0	26 ^{a}±0	27 ^{a}±1.41	40 ^{a}±0	32 ^{a}±2.83
LSD at P<0.05	0.94	1.88	$6.7x10^{-7}$	$5.6x10^{-7}$	0.94	0.94	2.10
MSE at P<0.05	0.43	0.85	$3.0x10^{-7}$	$2.6x10^{-7}$	0.43	0.43	0.95
Significance Degree	***	***	***	***	***	***	***
Factor: Sand Proportion							
33% sand	0	0	0	0	0	21.67 ^{b}	0 ^{b}
67% sand	0	0	0	0	0	22.67 ^{a}	0 ^{b}
100% sand	0	0	0	0	0	12.00 ^{c}	10 ^{a}
LSD at P<0.05	-	-	-	-	-	0.67	2.63 x 10^{-}[14]
Significance Degree	ns	ns	ns	ns	ns	***	***
Factor: Watering Level							
100% watering	0	0	0	0	0	10.67 ^{b}	0 ^{b}
67% watering	0	0	0	0	0	22.33 ^{a}	0 ^{b}
33% watering	0	0	0	0	0	23.33 ^{a}	10 ^{a}
LSD at P<0.05	-	-	-	-	-	1.17	2.63 x 10^{-}[14]
Significance Degree	ns	ns	ns	ns	ns	***	***

Continued: Effect of different water regimes on the antifungal activity of alfalfa plants grown in different soil types. Data listed represent the mean values±standard deviation where different letters refer to significant variation with least significant difference (LSD) and mean standard error (MSE) at P<0.05

Treatment	Diameter of Clear Zone (mm)					
	Mucor circinelloides	*Penicillium citrinum*	*Penicillium purpurogenum*	*Pseudallescheria ellipsoidea*	*Rhizopus azygosporus*	*Rhizopus microsporus*
33% sand+100% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{b}±0	0 ^{c}±0	14 ^{e}±0
67% sand+100% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{b}±0	15 ^{b}±1.41	14 ^{e}±0
100% sand+100% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{b}±0	16 ^{b}±0	14 ^{e}±0
33% sand+67% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{b}±0	0 ^{c}±0	16 ^{d}±0
67% sand+67% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{b}±0	0 ^{c}±0	16 ^{d}±0
100% sand+67% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0 ^{b}±0	0 ^{c}±0	16 ^{d}±0
33% sand+33% watering	0 ^{c}±0	0 ^{c}±0	0 ^{c}±0	0^{b}±0	16 ^{b}±0	17 ^{c}±1.41
67% sand+33% watering	14^{b}±0	0 ^{c}±0	0 ^{c}±0	0 ^{b}±0	16 ^{b}±0	18 ^{b}±0
100% sand+33% watering	14^{b}±0	0 ^{c}±0	0 ^{c}±0	0 ^{b}±0	16 ^{b}±0	18 ^{b}±0
Negative Control	15 ^{b}±1.41	19 ^{b}±1.41	14 ^{b}±0	0 ^{b}±0	15 ^{b}±1.41	14 ^{e}±0
Positive Control	23 ^{a}±1.41	23 ^{a}±1.41	21 ^{a}±1.41	11 ^{a}±0	26 ^{a}±0	32 ^{a}±0
LSD at P<0.05	1.33	1.33	0.94	0.47	1.33	0.94
MSE at P<0.05	0.60	0.60	0.43	0.21	0.60	0.43
Significance Degree	***	***	***	***	***	***
Factor: Sand Proportion						
33% sand	0 ^{b}	0	0	0	5.33 ^{b}	15.67 ^{a}
67% sand	4.67 ^{a}	0	0	0	10.33 ^{a}	16.00 ^{a}
100% sand	4.67 ^{a}	0	0	0	10.67 ^{a}	16.00 ^{a}
LSD at P<0.05	5.52 x 10^{-}[15]	-	-	-	0.67	0.67
Significance Degree	***	ns	ns	ns	***	ns
Factor: Watering Level						
100% watering	0 ^{b}	0	0	0	10.33 ^{b}	14.00 ^{c}
67% watering	0 ^{b}	0	0	0	0 ^{c}	16.00 ^{b}
33% watering	9.33 ^{a}	0	0	0	16.00 ^{a}	17.67 ^{a}
LSD at P<0.05	6.76 x 10^{-}[15]	-	-	-	1.17	1.17
Significance Degree	***	ns	ns	ns	***	*

Table 3: Effect of different water regimes on phytochemical constituents in the methanolic extracts of alfalfa plants grown in different soil types

Treatment	Phytochemical Constituents										
	Alkaloids	Amino acids	Anthra-quinones	Flavonoids	Glycosides	Phytosterols	Saponins	Steroids	Tannins	Terpenoids	Reducing sugars
33% sand+100% watering	++	+	-	+	+	+	+	+	++	+	+
67% sand+100% watering	+	++	-	+	+	+	+	+	++	+	+
100% sand+100% watering	+	++	-	+	+	+	++	+	+	+	+
33% sand+67% watering	+++	++	-	+	+	+	++	++	+++	++	++
67% sand+67% watering	++	++	-	++	++	+	++	++	+++	++	++
100% sand+67% watering	++	++	-	+	++	+	++	++	+++	++	++
33% sand+33% watering	+++	++	-	+++	+++	+	++	++	+++	++	++
67% sand+33% watering	++	++	-	+++	++	+	+++	++	+++	++	+++
100% sand+33% watering	+	+++	-	+++	++	+	+++	++	+++	++	+++

(+: Low concentration,++: Medium concentration,+++: High concentration,-: Not detected)

The ineffectiveness of alfalfa extracts to inhibit the growth of some fungal genera may be a result of the protective effect of the microbial coats. Another probability of such poor efficacy may hide in the used concentration of the plant extract that may be insufficient to cause the microbial inhibition. The extraction technique and the extraction solvent are also among the critical factors controlling the antimicrobial activity of the studied plants. Therefore, it could not be ascertained that alfalfa plants had, in general, no inhibitory effect on the studied fungal strains that showed negative inhibition result unless further investigations are carried out in more variable manner.

As an illation, extracts of alfalfa plants proved to have great antimicrobial potential against some microorganisms, and so these plants can be promising sources of antimicrobial agents particularly when stressed. Stressed alfalfa plants can be further exploited for isolation and characterization of novel phytochemicals in the treatment of infectious diseases.

CONFLICT OF INTERESTS

All authors have none to declare.

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