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Research Article

Interaction Effects of Salinity and Ultrasound Pretreatment on the Phytochemical Compounds of Clover Sprouts

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Abstract

This study investigated the interaction effects of salinity and ultrasound pretreatment on the phytochemical composition and growth of clover sprouts. Clover seeds were pretreated with ultrasound at 20, 28, and 40 kHz for 30 min at 30 °C and soaked for 9 h in deionized water, 1000 and 2000 ppm NaCl solution, then sprouted in the dark for 3 days. Clover sprout length significantly ($p \leq 0.05$) decreased with increasing salinity concentration to 2000 ppm NaCl, and increased for the ultrasound pretreated samples, suggesting a reduction in the salinity damage to clover sprout growth. The phytochemical compounds in clover sprout were identified by GC/MS/MS analysis. Some phytochemicals detected in the clover sprouts (7,8,3,4-tetramethoxyflavone, 3,7,8,2'-tetramethoxyflavone and docasane, 4-methyl) were not identified in the seeds. The results showed an increase in the methionine in the clover sprout with increasing salinity concentration. In contrast, no significant difference was observed on the methionine with the ultrasound treated samples. The phenolic compound salicylic acid in clover sprouts increased with increasing ultrasound frequency levels. It inhibited the ethylene production and induced salinity tolerance of the clover seedling. A novel mechanism of protecting the sprout tissues from the damage effect of salinity by the ultrasound pretreatment was proposed.

Keywords: Clover Seed Sprout; Phytochemicals; Salicylic Acid; Salinity; Ultrasound

Introduction

The phytochemicals are biologically active compounds of plant origin that provide functional benefits, not the basic nutritional benefits [1]. Moreover, the effects of these biologically active components additively or synergistically may be responsible for the health benefits of diets [2]. Clover plant (*Medicago falcate* L.) belongs to the family Fabaceae (Leguminosae). For centuries, it has been used in feeding livestock, provided as green, hay, or pellet feeds. Nowadays, clover and alfalfa sprouts are widely consumed by humans as a garnish and leaf protein concentrates, and the dehydrated plant comprises many nutritional supplement products [3]. A research report shows that using seed sprouts in the human

diet can supply both the basic nutrients and phytochemical compounds with the health-promoting properties [4]. Several studies have reported the presence of phenolics and flavonoids [5-9], vitamins [10] and terpenoids [11,12] in legume seeds.

Germination is one of the numerous factors that affect phenolic compounds in legume plants. The germination process is an inexpensive and simple method of improving the nutritional value of legume seeds. An increase in phenolic compounds after sprouting legume seeds was reported [9,10]. It was also reported that germination modifies the quantitative and qualitative phenolic compounds of legumes and these changes influenced the functional properties of the legumes, such as the antioxidant activity [13]. Phenolic compounds have antioxidant properties that may make them to act as reducing agents (free radical terminators), hydrogen donors, singlet oxygen quenchers, and metal chelators [14-16].

Soaking seeds into lower NaCl concentration solutions has been a cheap and effective approach for improving clover seeds' germination under water stress. The soaking effect of NaCl solution could be associated with the increase in levels of endogenous gibberellic acid (G.A.) and indole-3-acetic acid (I.A.A.) through activating amylases. Consequently, the soaking of the clover seeds in NaCl solution could remarkably enhance the antioxidant metabolism during the seed germination [17]. It is well-established that auxin (I.A.A.) controls the biosynthesis of the gaseous plant growth regulator, during root development [18,19]. Habibi [20] found that using saline water for the sprouting of wheat grain resulted in lower phenolics and flavonoids using tap water. However, salt stress stimulates the activity of the antioxidant system [21,22].

Ultrasound technology is a novel, convenient route to enhance seed germination; therefore, it is a promising technology in the area of seed science [23,24]. Ultrasound treatment reduces the seed soaking time for chickpeas [25,26] and navy beans [27]. This improvement in the hydration process of seeds has been attributed to a greater reduction in internal resistance than the external resistance [28]. As well as possible changes in the seed microstructure caused by the acoustic cavitation (micro-channel formation) and/or the so called sponge effect "causing internal flow" generated by the ultrasound irradiations [29]. However, Miano [30] reported that the ultrasound technology-enhanced barley grain vigor during the first four days of germination improves the germination speed. Within the scope of our literature studies, there are only a few scientific researches on the phytochemical compounds of legume seed sprouts, especially clover. Besides, the study on the sprouting of ultrasonic pretreated legume seeds using saline water seems to be scarce. Therefore, the present work aimed to identify the phytochemicals of three-day-old etiolated clover sprout pretreated with ultrasound and germinated using saline solution vs. deionized water.

Material and Methods

Materials

Dry seeds of clover (*Medicago falcate* L.) were obtained from a local seed market in Zhenjiang, Jiangsu province, China. The seeds were sorted out and cleaned to remove impurities. NaCl was obtained from Sigma-Aldrich Company, Shanghai, China.

Ultrasound Pretreatment

The ultrasound pretreatment was done using an ultrasound bath device, operating at three frequencies of 20, 28, and 60 kHz (Meibo Biotechnology Co. Ltd., Zhenjiang, China) (Figure 1). Three different soaking liquids, including deionized water, 1000 and 2000 ppm NaCl solution, were employed in the sonication process. Briefly, 2.0 g of the clover seeds were immersed in 150 ml of the soaking liquid in a 500 ml Erlenmeyer flask. The ultrasonic bath reactor was filled with 5 L of water, and the flask was put in the center of the bath; to guarantee the reach of ultrasound irradiations to the entire sample. The soaked seeds were sonicated at different ultrasonic frequencies (20, 28, and 40 kHz), a power density of 60 W/L, and a process temperature of 30 °C and time of 30 min. The ultrasound device was operated using a pulsed on-time of 10 s and off time of 3 s. The sonication experiment was performed without mechanical stirring. The temperature of the system was kept constanr at 30 °C with the air of a thermostat-controlled circulating water bath. Control samples were prepared by soaking the seeds in the liquid at 30 °C for 30 min; without applying ultrasound irradiations.

Seed sprouting

The sprouting of untreated and ultrasound pretreated clover seeds was done using the glass jar method described in the literature with modification [31,32]. In brief, the Clover seeds were immersed in a soaking liquid (deionized water, 1000 and 2000 ppm NaCl solutions) and placed in a 0.7 L capacity glass jar (household

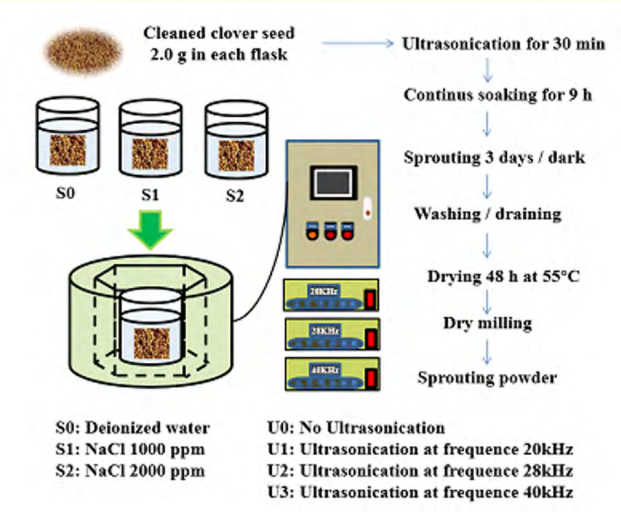

Figure 1: Flowchart of ultrasonication and sprouting clover seeds using saline water.

version), which was then covered with cheesecloth secured by a rubber band. The jar was stored in the dark for 9 hr to allow seeds to soak at room temperature. After soaking, water was discarded, and the seeds were rinsed with water in the jar (approximately 1.0 min.). The rinse water was discarded, the jar was inverted at a 45° angle and stored at room temperature in the dark for 12 h. The rinse-store procedure was repeated 8 times until 72 h cumulative time had been completed (harvest time).

At the end of the sprouting period, the seeds were removed, dried in a vacuum oven at 55°C for 48 h, milled to pass a 0.2 mm mesh screen, and then stored in an airtight container at 4 °C for the subsequent analyses.

Sprout length measurement

The length of the fresh clover seed sprouts of the untreated and ultrasound pretreated samples was measured immediately after their removal from the germination glass jar.

Phytochemical analysis

The phytochemical compounds in the clover seed sprout were measured using a GC/MS/MS (Agilent Technologies 7890A, Beijing, China) equipped with polar Agilent HP-5ms (5% phenyl methyl polysiloxane) capillary column (30 m × 0.25 mm inside diameter, and 0.25 µm coated film thickness) and a mass selective detector (M.S.D., Agilent 7000, Beijing, China). Identification of the compounds was based on a comparison of their mass spectra and retention times with those of the authentic reference compounds and by computer matching with NIST and WILEY library, and by comparison of the fragmentation pattern of the mass spectral data with those (*12*).

Methionine Measurement

The methionine content was determined by hydrolyzing a sample in 6 M HCl under a vacuum at 110 °C for 24 h. The hydrolysate was dried in a vacuum oven set at 60 °C and then dissolved in a citrate buffer (pH 2.2). The methionine content was measured using an automatic amino acid analyzer (Sykam S - 433 D, Germany) [33].

Statistical analysis

Data were means of three replicating samples. The data were analyzed by a two-way analysis of variance (ANOVA), and the means were compared using L.S.D. test ($p \leq 0.05$). All analyses were conducted using S.A.S. software [34].

Results and Discussion

Effect of salinity and ultrasound pretreatment on contents of phytochemical compounds of clover seed sprouts

The results of phytochemical analysis illustrated that 31 phytochemical compounds were identified in the untreated and pretreated clover seed sprouts (Table 1). As can be seen, 28 phytochemical compounds were identified in the dry clover seeds, which were decreased to 26 compounds after sprouting the seeds in deionized water, without ultrasonic pretreatment (S0U0). The seeds soaked in the deionized water after ultrasonic pretreatment (28 kHz (S0U2) and 40 kHz (S0U3)) showed a total of 30 phytochemical compounds, which was higher than the number of compounds identified in S0U0 (Table 1). All the seeds (pretreated and unpretreated) soaked in the 1000 ppm NaCl saline solution (S1U0, S1U1, S1U2, and S1U3) recorded a total of 30 phytochemical compounds. On the other hand, the seeds soaked in the 2000 ppm NaCl solution, without the ultrasonic pretreatment (S2U0) showed 29 compounds, which decreased to 28 compounds in the seed sprouts pretreated with the ultrasonic frequency of 20 kHz (S2U1), followed by an increase to 29 compounds and 30 compounds in the seed sprout samples pretreated with the ultrasonic frequencies of 28 and 40 kHz, respectively (i.e., S2U2 and S2U3).

No.	R.T. Min	Name	Relative peak area (%)												
			S0U0	S0U1	S0U2	S0U3	S1U0	S1U1	S1U2	S1U3	S2U0	S2U1	S2U2	S2U3	Dry seeds
1	5.311	3',4'-Dimethoxy-2' -hydroxychalcone	4.26	3.41	3.59	3.48	4.07	4.16	2.62	4.75	4.24	2.73	2.78	2.04	5.15
2	12.888	Phytol	2.79	2.1	1.76	1.72	1.38	0.8	0.5	1.25	1.78	1.81	1.47	1.26	1.03
3	13.059	Citronellyl tiglate	1.95	0.99	0.82	1.09	0.74	1.28	0.55	0.8	0.89	0.81	0.54	1.45	0.69
4	13.185	Levomenthol	2.29	1.69	1.03	1.29	0.83	0.76	0.56	0.85	1.43	1.32	0.92	1.04	1.40
5	13.405	5,7,3',4',5' -Pentahydroxyflavone	1.39	0.69	1.02	1.3	1.4	0.94	1.41	0.71	0.86	1.36	1.51	2.03	1.00
6	13.723	Afromosin 7-O-glucoside	6.11	6.93	6.42	7.18	6.41	5.61	7.6	4.53	6.26	6.95	8.57	8.78	2.99
7	13.833	6,4'-Dimethoxy-7 -hydroxyisoflavone	2.41	1.54	1.23	1.04	1.54	1.55	1.4	1.37	2.2	1.59	1.63	1.21	3.72
8	14.505	Thunbergol	3.68	1.65	0.76	2.05	1.46	2.87	1.65	2.59	0.95	1.74	2.36	1.61	1.87
9	14.868	Isolongifolol	15.65	20.12	16.12	18.23	15.04	11.62	25	12.47	17.37	26.29	33.24	30.74	5.57
10	14.896	Dihomo-γ-linolenic acid	8.22	8.84	8.8	7.32	8.94	8.73	6.41	6.85	5.58	10.74	8.79	11.53	11.33
11	15.255	2',4'-Dimethoxy-3 -hydroxy-6-methylflavone	0.58	0.57	0.63	0.68	0.85	0.61	0.87	1.01	2.82	0.51	1.08	0.61	0.91
12	15.744	Quercetin-4'-methylether	4.03	1.96	5.21	7.52	9.36	9.57	5.4	8.62	0.93	1.25	1.24	2.72	9.43
13	16.428	Luteolin 5,7,3',4' -tetramethylether	1.48	1.7	1.8	2.51	1.16	2.13	0.89	2.1	1.48	1.52	1.43	1.39	0.75
14	16.816	2',3'-Dimethoxyflavone	1.64	0.94	1.14	1.56	0.72	1.36	0.55	0.71	5.24	0.5	1.1	0.49	0.87
15	17.259	3,4,5-Trimethoxycinnamic acid	1.02	1.32	1.3	1.1	1.49	1.97	1.67	1.63	0.57	9.74	0.67	1.13	1.47
16	17.834	Salicylic acid β-D-O-glucuronide	1.15	1.39	1.66	2.11	1.24	2.05	1.82	1.92	0.68	1.16	1.12	1.15	1.58
17	18.599	7,8,3',4' -Tetramethoxyflavone	0.82	1.08	1.18	1.29	1.56	1.13	0.91	0.96	1.96	1.17	0.74	0.99	-
18	18.893	3-Hydroxy-7,8,2',3' -tetramethoxyflavone	0.41	0.54	0.61	1.34	1.4	1.15	0.65	1.03	8.94	0.96	0.8	0.51	0.63
19	19.162	Hexa-hydro-farnesol	0.45	0.62	0.76	0.66	1.07	1.17	1.08	1.64	1.04	0.87	0.53	0.49	1.15
20	19.561	Vitamin E	-	2.4	2.94	3.5	2.69	2.37	1.81	4.8	-	-	1.42	2.31	4.67
21	19.871	3,7,8,2'-Tetramethoxyflavone	-	1.47	1.06	0.66	1.15	1.25	1.31	1.7	-	-	-	1.23	-
22	20.066	Quercetin 3,5,7,3',4' -pentamethyl ether	-	-	2.16	1.05	1.35	1.36	1.35	1.18	0.5	0.71	1.73	0.51	2.56
23	20.62	Geranyl isovalerate	1.22	0.84	0.89	0.88	1.31	0.82	0.95	1.11	0.69	1.26	1.21	0.95	0.62
24	21.11	Propyl gallate	0.97	0.83	0.34	1.67	1.47	1.85	1.15	2.01	12.52	1.09	0.76	1.41	1.96

25	21.305	Vitexin	0.54	0.73	1.05	0.63	0.83	2.81	1.14	1.19	1.06	1.16	1.08	1.12	0.43
26	21.773	Nerolidol	1.55	0.71	0.99	0.65	1.06	0.95	0.66	0.65	2.64	2.6	1.3	0.78	1.38
27	21.859	Gentisic acid	31.75	31.88	31.02	23.6	24.33	24.42	26.19	26.06	3.63	13.42	18.07	17.66	32.65
28	22.034	3-(3,4-Dimethoxyphenyl)-4-methylcoumarin	0.91	0.66	1.41	1.33	2.35	1.89	1.68	2.03	0.88	0.64	0.82	0.54	1.22
29	22.278	Nobiletin	2.73	0.41	0.62	1.15	1.01	1.42	0.75	1.45	0.5	4.32	0.76	0.73	1.75
30	22.792	Astilbin	-	2.01	1.67	1.39	1.79	1.39	1.45	2.04	11.15	-	2.32	1.58	1.23
31	22.902	Docosane, 4-methyl	-	-	-	-	-	-	-	-	1.21	1.79	-	-	-

Table 1: Interaction effects of using saline water (S) and ultrasonic pretreatment (U) on the content of phytochemical compounds in clover sprout.

S0: 0 NaCl; S1: 1000 ppm NaCl; S2: 2000 ppm NaCl; U0: Untreated; U1: 20 kHZ; U2: 28 kHZ; U3: 40 kHZ.

The phytochemical compounds detected in the studied clover seed sprouts included phenolics, flavonoids, terpenoids, vitamins, aromatic compounds, fatty acids, and other organic compounds. However, vitamin E, quercetin, and astilbin identified in the dry clover seeds were completely lost after sprouting the seeds in the deionized water (S0U0) and saline solution (S0U1, S2U0, and S2U1). It was worth noticing that these phytochemical compounds were partially retained in the seed sprouts, pretreated at 28 and 40 kHz, and germinated in the saline solution or the deionized water (Table 1). Moreover, the compound 7,8,3,4-tetramethoxyflavone, which was not detected in the dry seeds, was found in all the studied seed sprouts prepared using the deionized water or the saline solution, with or without the ultrasonic pretreatment. Likewise, 3,7,8,2'-tetramethoxyflavone was not detected in the dry seeds, but was noticed in all the deionized water clover sprouts pretreated with ultrasound. Similarly, this organic compound was also observed in all the low concentration saline solution sprouts (S1U0, S1UI, S1U2, and S1U3) and the high concentration saline solution sprout pretreated at 40 kHz (S2U3). However, the relative content of 3,7,8,2'-tetramethoxyflavone in the deionized water clover sprouts showed a decreasing tendency in the order of S0UI, S0U2, and S0U3 and that in the lower concentration saline solution sprouts showed an increasing tendency in the order of S1U0, S1UI, S1U2, and S1U3.

On the contrary, the relative content of 7,8,3,4-tetramethoxyflavone detected in the deionized water clover sprout increased. While in the saline solution, the content decreased with the increase in the frequency level. Interestingly, a new organic compound docasane, 4-methyl, was detected for the first time in the clover seed sprouts, prepared in the 2000 ppm NaCl saline solution, without or with 20 kHz ultrasonic pretreatment (S2U0 and S2U1, respectively).

Dried sprouts are used instead of fresh ones to increase the nutritional value of food products and avoid bacterial contamination [35]. The beneficial effect of clover sprouts may arise from the combined action of several bioactive phytochemical compounds, such as phenolics and flavonoids, terpenes, vitamins and other phytochemicals (Table 2). It has been reported that some phytochemical compounds found in seed sprouts have many beneficial effects on human health, such as reducing the risk of cardiovascular diseases and type 2 diabetes [2,9,36]. Furthermore, the relative content of vitexin, gentisic acid, and astilbin found in the untreated dry clover seeds increased after sprouting the seeds in the deionized water and saline solutions, without and with the ultrasonic pretreatment. These compounds' relative content increased gradually with the increase in the frequency level of the ultrasonic pretreatment. Some phytochemicals detected in the clover sprouts have potent biological and pharmaceutical activities. Therefore, they can be incorporated into the industrial functional food channel to produce safe, healthy foods with good nutritional values. For instance, the flavonoid compound vitexin has a major antioxidant activity [37], and the phenolic compound gentisic acid has antioxidant and radio-protective properties, which are exerted by its phenoxyl group [38]. The astilbin (flavonoid compound) shows an insecticidal ac-

tivity, antibacterial activity [39], and activity on burn wound healing [40]. It is also used in traditional Chinese medicine [41]. Luteolin 5,7,3,4-tetramethylether, another important member of the flavonoid compound, was also detected in the clover seed sprout. The relative content of this compound in all the studied clover sprouts was higher than that in the dry seed. Besides, the content of the compound in the seed sprouts increased with the increasing frequency level of the ultrasonic pretreatment.

Effect of salinity and ultrasound pretreatment on phenolics, flavonoids, and terpenoids

The changes in phenolics, flavonoids, and terpenoids fractions in the clover sprouts are illustrated in table 3 and figure 2 A, B, and C. Germination of the clover seeds in the saline solution for three days result in a decrease in the phenolic compounds compared with those soaked in the deionized water; the decrease was more pronounced ($P \leq 0.05$) with using the higher salinity concentration (NaCl 2000 ppm) than the lower one (Figure 2A1). Using ultrasound pretreatment did not significantly influence ($P > 0.05$) the phenols in the 3 days old clover sprouts (Figure 2A2). The results also indicated that the percentage of flavonoids in the clover sprout increased significantly ($P \leq 0.05$) with increasing NaCl concentration and decreased in the ultrasound pretreated samples, irrespective of the frequency level used (Figure 2B1 and 2B2). Nevertheless, the observed lower percentage of phenols versus the higher percentage of flavonoids in the saline clover sprouts, especially in 2000 ppm NaCl (Figure 2A1 and B1), could be a result of the transformation of some phenolics, such as gentisic acid into flavonoids, such as astilbin or tetramethoxyflavone during the sprouting process in the high concentration of the saline solution of 2000 ppm NaCl (Table 1) and also complex conversion and degradation of phenolics of free forms by enzymes [42].

In regard to the terpenoids, the clover seeds sprouted into 2000 ppm NaCl solution showed a significantly higher ($P \leq 0.05$) content than the seeds germinated in 1000 ppm NaCl solution and deionized water (Figure 2C1). The sprouts of the clover seeds pretreated with 28 kHz ultrasound irradiations revealed a significantly higher ($P \leq 0.05$) content of terpenoids compared with the untreated seed sprouts (Figure 2C2). The higher content of terpenoids in the clover sprouts sprouted using the saline solution medium could be ascribed to the protection against loss of terpenoids provided by the increased water absorption by the sprout, resulting from the

No.	Groups	Name
1	A	3',4'-Dimethoxy-2'-hydroxychalcone
2	A	Salicylic acid β-D-O-glucuronide
3	A	Propyl gallate
4	A	Gentisic acid
5	B	5,7,3',4',5'-Pentahydroxyflavone
6	B	Afromosin 7-O-glucoside
7	B	6,4'-Dimethoxy-7-hydroxyisoflavone
8	B	2',4'-Dimethoxy-3-hydroxy-6-methylflavone
9	B	Quercetin-4'-methylether
10	B	Luteolin 5,7,3',4'-tetramethylether
11	B	2',3'-Dimethoxyflavone
12	B	7,8,3',4'-Tetramethoxyflavone
13	B	3-Hydroxy-7,8,2',3'-tetramethoxyflavone
14	B	3,7,8,2'-Tetramethoxyflavone
15	B	Quercetin 3,5,7,3',4'-pentamethyl ether
16	B	Vitexin
17	B	3-(3,4-Dimethoxyphenyl)-4-methylcoumarin
18	B	Nobiletin
19	B	Astilbin
20	C	Phytol
21	C	Citronellyl tiglate
22	C	Levomenthol
23	C	Thunbergol
24	C	Isolongifolol
25	C	Hexa-hydro-farnesol
26	C	Geranyl isovalerate
27	C	Nerolidol
28	D	Dihomo-γ-linolenic acid
29	D	3,4,5-Trimethoxycinnamic acid
30	D	Vitamin E
31	D	Docosane, 4-methyl

Table 2: Classifications of the phytochemical compounds in the clover sprout.

A: Phenols and their derivatives; B: Flavonoids and their derivatives; C: Tarpenoids and their derivatives;

D: Other phytochemical compounds.

Note: The compounds were classified based on data found on Dr. Dukes phytochemicals, NIST and WILEY library.

Character Treatments	Phenols (peak area %)	Flavanoids (peak area %)	Total terpenoids (peak area %)	Salicylic acid (peak area%)	Methionine (%)	Sprout length (cm)
	Effect of salinity					
S0 (NaCl 0.0 ppm)	35.78A	25.53B	27.0B	1.58B	0.023C	4.47A
S1 (NaCl 1000 ppm)	34.12A	31.26A	23.87B	1.76A	0.046B	4.25A
S2 (NaCl 2000 ppm)	21.12B	29.17A	35.85A	1.03C	0.066A	3.75B
	Effect of ultrasound pretreatment					
U0 (0.0 KHz)	30.10A	35.70A	26.42B	1.02B	0.045A	3.31B
U1 (20 KHz)	29.46A	26.01B	28.56AB	1.53A	0.041A	4.36A
U2 (28 KHz)	32.49A	26.01B	31.88A	1.53A	0.042A	4.35A
U3 (40 KHz)	29.29A	28.57B	28.75AB	1.73A	0.052A	4.60A
	Effect of salinity x ultrasound interaction					
S0U0	38.13a	23.05e	29.58bcd	1.15d	0.019d	3.56f
S0U1	37.51ab	21.23e	28.72cd	1.39cd	0.023cd	4.81a
S0U2	36.61ab	27.21cde	23.13de	1.66bc	0.023cd	4.57ab
S0U3	30.86b	30.63bcd	26.57cde	2.11a	0.025cd	4.92a
S1U0	31.11b	32.88bc	22.89de	1.24d	0.049bcd	3.37ef
S1U1	32.48ab	34.17b	20.27e	2.05a	0.038bcd	4.28abc
S1U2	38.13a	27.36cde	30.95bc	1.82ab	0.044bcd	4.58ab
S1U3	34.74ab	30.63bcd	21.36e	1.92ab	0.052abc	4.77a
S2U0	21.07c	44.78a	26.79cde	0.68e	0.067ab	3.01f
S2U1	18.40c	22.64e	36.70ab	1.16d	.061ab	3.98bcde
S2U2	22.73c	24.81de	41.57a	1.12d	0.058ab	3.90cde
S2U3	22.26c	24.44de	38.32a	1.15d	0.079a	4.10a

Table 3: Interaction effects of using saline water and ultrasound pretreatment on contents of total phenolics, flavonoids, terpenoids, salicylic acid, methionine and length of clover sprout.

Within a column of each treatments, means with different capital letters or small letters are significantly different ($P \leq 0.05$). S0: 0 NaCl; S1: 1000 ppm NaCl; S2: 2000 ppm NaCl; U0: 0 kHz; U1: 20 kHz; U2: 28 kHz; U3: 40 kHz.

reduction in the water potential due to receiving large amounts of salt by the root cells during the sprouting process in the salty solution [43]. In contrast to that, the deionized water used for the sprouting of clover seeds might enhance the synthetic enzyme responsible for transforming some terpenoids into phenolic compounds; therefore, a decrease in terpenoids. Also, the increase in the content of terpenoids observed in the clover sprout with using ultrasonic pretreatment and higher saline sprouting solution (2000 ppm NaCl) could be arising from the increase in porosity for loss of water, which protect against increased membrane permeability rather than membrane disruption with water uptake during hydration [25-27]. However, more research is necessary to prove the effect of ultrasound pretreatment on increasing terpenoids using higher saline concentration for sprouting solution. Other phytochemical compounds, including dihomo-**γ**-linolenic acid, 3, 4, 5-trimethoxycinaamic acid, and vitamin E were also identified in the clover seed sprouts (Table 1).

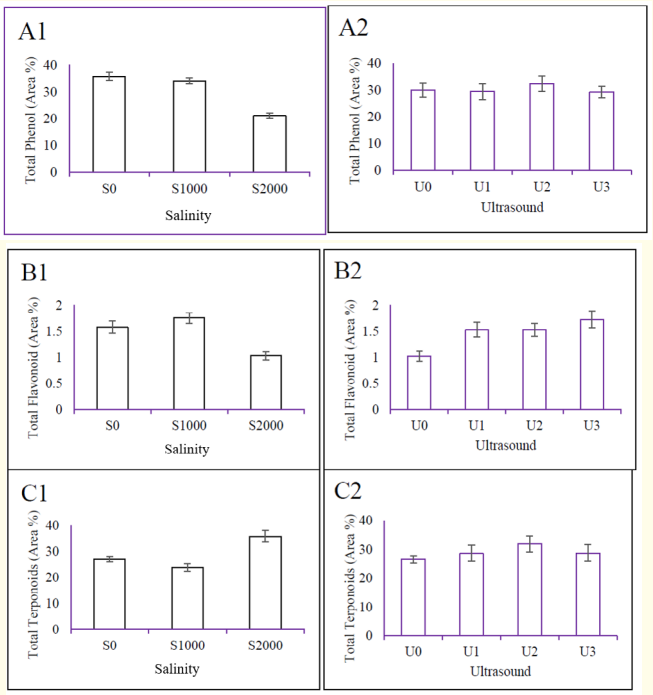

Figure 2: Effect of salinity (S) concentration and ultrasound pretreatment (U) levels on the content of total phenolics (A1 and A2), total flavonoids (B1 and B2) and the total terpenoids (C1 and C2) of 3 days old clover sprout.

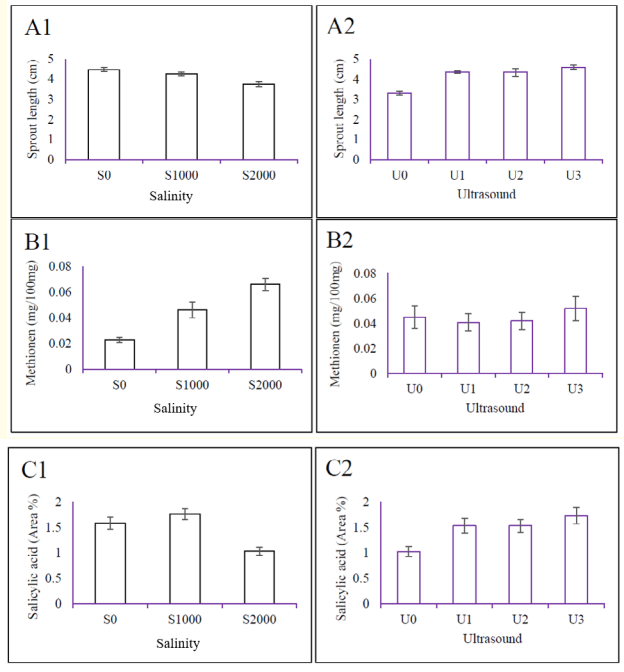

Figure 3: Effect of salinity (S) concentration and ultrasound pretreatment (U) levels on the on clover sprout length, methionine and salicylic acid contents after three days etiolated growth.

Clover sprout length, salicylic acid, and methionine contents

The clover sprout length was significantly decreased by increasing the NaCl salinity concentration to 2000 ppm in the sprouting medium (Table 3 and Figure 3 A1). Similar studies showed that NaCl treatment decreased seedling length with increasing salt concentration [20]. The sprout length (Table 3 and Figure 3A2) was observed to significantly increase ($P \leq 0.05$) for the ultrasound pretreated samples. However, there exist no significant differences between the various levels of ultrasound frequencies (20, 28, and 40 kHz). These results indicated that ultrasound pretreatment reduced the damaging action of salinity on the growth of clover sprouts. Salinity stress is one of the major factors limiting the growth and increasing damage to tissues of plant species by enhancing ethylene production.

Furthermore, it can be observed in table 3 and Figure 3B1 that the methionine content of the clover sprouts increased with increasing salinity concentration. Accordingly, the higher level of methionine in the clover sprouts might be indirectly responsible for limiting their growth because methionine can be converted to ethylene as a precursor of the intermediate aminocyclopropane-1-carboxylic acid. Previous studies proved the fundamental role of ethylene in inhibiting seedling elongation during early root development [44-46].

It can be seen that the results showed a significant increase ($P \leq 0.05$) in the content of salicylic acid in the clover sprouts when applying a higher ultrasonic frequency level (40 kHz). Also, a decrease ($P \leq 0.05$) in the content of salicylic acid when using higher salinity concentration in the sprouting solution (2000 ppm NaCl) was observed (Table 3 and Figure C1 and C2). As stated previously,

the ultrasound pretreatment reduced the destructive action of salinity on clover seedling growth. Likewise, the salicylic acid treatment was also found to reduce the damaging action of salinity on wheat seedling growth. Moreover, garden cress seedling length was significantly decreased by increasing salinity, and seed priming by salicylic acid improved the germination [47]. Accordingly, the above results suggested that the action of ethylene primarily regulates sprout elongation. Therefore, ultrasonic pretreatment may indirectly enhance the inhibition of ethylene production by promoting the biosynthesis of ethylene inhibitors, such as salicylic acid. In addition to its role in the inhibition of ethylene synthesis, salicylic acid inhibits the synthesis of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (A.C.C.) as well. Moreover, the inhibitory action of salicylic acid most closely resembled that of dinitrophenol, a known inhibitor of ethylene forming enzyme [48]. Therefore, salicylic acid might be involved in the regulation of winter plant responses to the stress as reported by many researchers [48-52]. These data suggested that the ultrasound pretreatment can protect the clover sprout under saline conditions by increasing the production of salicylic acid in the sprout tissues.

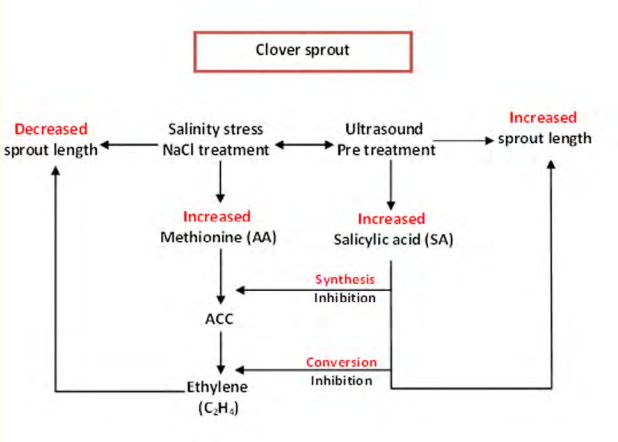

Figure 4: Hypothesis for the effect of ultrasound pretreatment on protected clover sprout under saline condition by increasing salicylic acid (SA) production. (AA= amino acid, ACC = 1-amino cycle-propane 1-carboxylic acid).

Finally, the mechanical effect generated by the ultrasound irradiations caused numerous small holes in the seed coatings and fissures on the pericarp that resulted in a notable rise in the moisture of seedlings and an increase in the oxygen availability; consequently, seed germination is enhanced [24]. Salicylic acid involved in the regulation of growth and development of plant was reported to reduce salinity damage by suppressing the excess ethylene formation [53]. This research's novelty is the increase in the production of salicylic acid in the clover sprouts by the aid of ultrasonic pretreatment, which inhibits the expected ethylene wound due to salinity or the harmful effects of the ultrasound irradiations. Since the mode of action of salicylic acid on ethylene production is well known, and that of the ultrasound pretreatment is unfortunately unknown; therefore, a proposed mode of action of ultrasound pretreatment on the ethylene production and sprout growth is explained in figure 4.

Conclusion

The synergetic combination of sprouting clover seed with or without ultrasound pretreatment using saline solution vs. deionized water was evaluated. The use of saline water for sprouting decreased the clover sprout length with increasing salinity concentration. The ultrasound pretreatment interacts with salinity and led to an increase in the sprout length. Also, ultrasound pretreatment reduced the destructive action of salinity on the growth of the clover sprouts. The other important conclusion is that this is the first report on the interaction effects of ultrasound pretreatment and salinity on clover sprout phytochemical composition. A novel hypothesis was used to explain the mode of action of ultrasound pretreatment on protecting sprout tissues from the damaging effect of salinity through ethylene biosynthesis. Nevertheless, further studies are required to prove the effect of ultrasound pretreatment on some phytochemical compounds under higher saline concentration for sprouting solution.

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