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ULTRASOUND AND SALINITY EFFECTS ON GROWTH CHEMICAL COMPOSITION AND PROTEIN QUALITY OF CLOVER SPROUTS

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Abstract

Recent researches show that using seed sprouts in the diet as a healthy food not only being a good source of basic nutrients but also contain chemical compounds with health-promoting properties. This study aimed to evaluate the effect of China clover seeds soaking in NaCl (0, 1000 and 2000 ppm) solution and ultrasound irradiation levels (0, 20, 28 and 40 kHz) and their interactions on clover seed sprouting; sprout character, chemical composition and protein quality of clover sprouts. The obtained result revers that China clover seed sprouting increased the protein content in sprout samples. Using NaCl solution in combination with ultrasound seed priming also resulted in better sprout characters, mineral content, total essential amino acids (TEAA), protein efficient ratio (PER), essential amino acid index (EAAI), biological value (BV) and nutritional index (NI) compared with deionized water control. Essential amino acid methionine (Precursor of ethylene biosynthesis) increased in clover sprout with increasing NaCl concentration and inhibit clover sprout growth. Salicylic acid a phytochemical phenolic compound increased with increasing pretreatment ultrasound frequency levels which inhibited ethylene production. Therefore, Ultrasound with NaCl seed priming could be a simple approach for enhancing tolerance to salinity stress in China clover. Moreover, clover sprouting influenced the protein content and quality of sprouts as a functional food.

Key words : Clover sprout; Protein quality; Amino acid profile; mineral content

Introduction

Clover crop was first cultivated thousands of years ago in China and Egypt before pyramid building (Abdallah, 2008; Lewis-Jones *et al.*, 1982). However, clover and alfalfa sprouts are a sensorially attractive food product. It's more important to define accurately the amount and quality of protein required to meet human nutritional needs. Clover or alfalfa sprouts are considered as high quality for the health, due to its rich nutritional profile (>50 % protein) (Abdallah, 2008; El-Gebaly *et al.*, 2018). Clover seed germination and production of sprouts is an old habit that was adapted days of sprouting to be the optimum period for clover growing in the dark to produce etiolated sprouts. Recent research shows that using seed sprouts in the diet as a healthy food not only being a good source of basic nutrients but also contain chemical compounds with health-promoting properties (Kurtzweil, 1999; Tahany *et al.*, 2018). Salinity affects seed germination (Kandil *et al.*, 2012; Sairam *et al.*, 2002) and seedling characters plants (Tezara *et al.*, 2003), by slow or less recruitment of reserve foods (Kayani *et al.*, 1990) and injuring hypocotyls (Assadian and Miyamoto, 1990). Salinity can affect germination and seedling growth either by creating an osmotic pressure that prevents water uptake or by toxic effects of sodium and chloride ions seed germination (Akbarimoghaddam *et al.*, 2011; Goussous *et al.*, 2010). Seed soaking with lower NaCl concentration is a cheap and effective approach for improving the germination of clover seeds under water stress. This NaCl effect could be associated with increases in endogenous gibberellic acid (GA) and indole-3-acetic acid (IAA) levels through activating amylases and finally soaking with NaCl could remarkably enhance antioxidant metabolism during clover seed germination (Cao *et al.*, 2018). It is well-established that auxin (IAA) control the gaseous plant growth regulator ethylene

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biosynthesis, during root development (Benková and Hejátko 2009; Muday *et al.,* 2012)**.** Moreover, it is well-established in some spices that ethylene inhibits root elongation during early root development (Markakis *et al.,* 2013; Ruzicka *et al.,* 2007; Swarup *et al.,* 2007).

Ultrasound (US) novel technology show enhancement in seed germination and is a promising technology in the area of seed science (Goussous *et al.*, 2010; Rifna, E.J. *et al.,* 2019)**.** Ultrasound treatment was shown to reduce the seed soaking time for chickpeas (Ranjbari *et al.*, 2013; Yildirim *et al.*, 2010) and navy beans (Ghafoor *et al.,* 2014)**.** This improvement of hydration process of seeds has been attributed to a greater reduction of internal resistance than external resistance (Cunningham *et al.,* 2008) as well as possible changes in microstructure by cavitation (micro-channel formation) and /or the so-called “sponge effect causing internal flow” (Patero and Augusto, 2015). However, (Miano *et al.*, 2015) reported that ultrasound’s technology enhanced barley grain vigor during the first four days of germination and improves the germination speed. Ultrasound treatment to stimulate germination has been investigated in many seed types including carrots, radish, maize, barley, rice, wheat, chickpea and sunflower (Aladjadjiyan, 2002; Carbonell *et al.*, 2000; Flórez *et al.*, 2007; Goussous *et al.*, 2010; Hebling and Silva, 1995; Miyoshi and Mii, 1988; Shimomura, 1998; Shors *et al.*, 1999; Yaldagard *et al.*, 2008a, 2008b). These investigations indicated that the effects of US on seed germination depend on frequency and exposure time and appear to vary widely between the different species and cultivars. This research aimed to determine whether NaCl and US irradiation and their interaction are usable as a seed priming method for China clover crop. Also, aimed for sprout production to achieve fast sprout establishment of China clover sprouts and to determine the chemical composition and protein quality of clover sprout. The feasibility of this research was evaluated under favourable room sprouting temperatures. Additionally, NaCl and US irradiation take place in water bath. The research also ran paralleled treatment in which seeds were soaked in water only. This was important to eliminate the effect of water in the NaCl, US and their interaction treatments as control treatment.

Materials and Methods

Materials, ultrasound pretreatment and seed sprouting

Cleaned with no impurities seed of clover (*Medicaga falcate* L.) were obtained from a local seed market in Zhenjiang, Jiangsu province, China. 2 g of seed were immersed in 150 ml of three different soaking liquid including distilled water, 1000 and 2000 ppm NaCl solution in a 500 ml Erlenmeyer flask for employed in sonication experiments. The ultrasonic bath reactors were filled with 5 L of water and the flask with seeds was put in the center of the bath to guarantee irradiation reach to the entire sample (Fig. 1). The sonication used different ultrasonic frequencies (20, 28 and 40kHz), power intensity of 0.2 W/cm^2, power density of 60 W/L, reaction temperature of 30°C and time of 30 min. Control samples were prepared by soaking the seeds in the liquid at 30°C min without applying ultrasound irradiation. Untreated and ultrasound pretreated clover seeds were left in liquid for 9 h soaking after Kr sonication, then 50 soaked seeds were used for sprouting in Petri dishes for measuring sprout characters. The rest of 2 g seeds were left to sprout using the glass jar method described by (Abdallah, 2008)**.** Soaking (9 h) and sprouting period (3 days) were done in the dark at room temperature (~25°C). After the end of the sprouting period, the fresh sprout characters were measured, and the left sprout were dried in a vacuum oven at 55°Cfor 48 h milled to pass a 0.2 mm mesh screen and then stored at 4°C for the subsequent analysis.

Sprouting characteristics

1. Sprouting percentage (SP) was taken after 3 days from seed soaking and expressed as percentages according to the following equation:

$$\mathrm{SP}(\%)=\frac{\text{Number of sprouted seeds}}{\text{Total number of seed tested}}\times 100$$

2. Sprouting index (SI), was calculated as ratio of sprouting percentage of each treatment to sprouting percentage of control and calculated by the following equation

$$\mathrm{SI}(\%)=\frac{\text{SP of treatment}}{\text{SP of control}}\times 100$$

3. Relative injury rate of salt or ultrasound was calculated as difference between SP of control and SP in NaCl concentration or ultrasound level of each treatment to SP of control and calculated by the following equation:

Relative injury rate = [SP of control – SP of treatment] / SP of control

4. Hypocotyl length, the length of ten sprouts from the base of radical to the tip of the cotyledons were recorded and expressed in centimeters (cm) as hypocotyl length.

5. Radical length: measured for the ten sprouts from hypocotyl base to the tip of the radical and expressed in centimeters (cm) as radical length

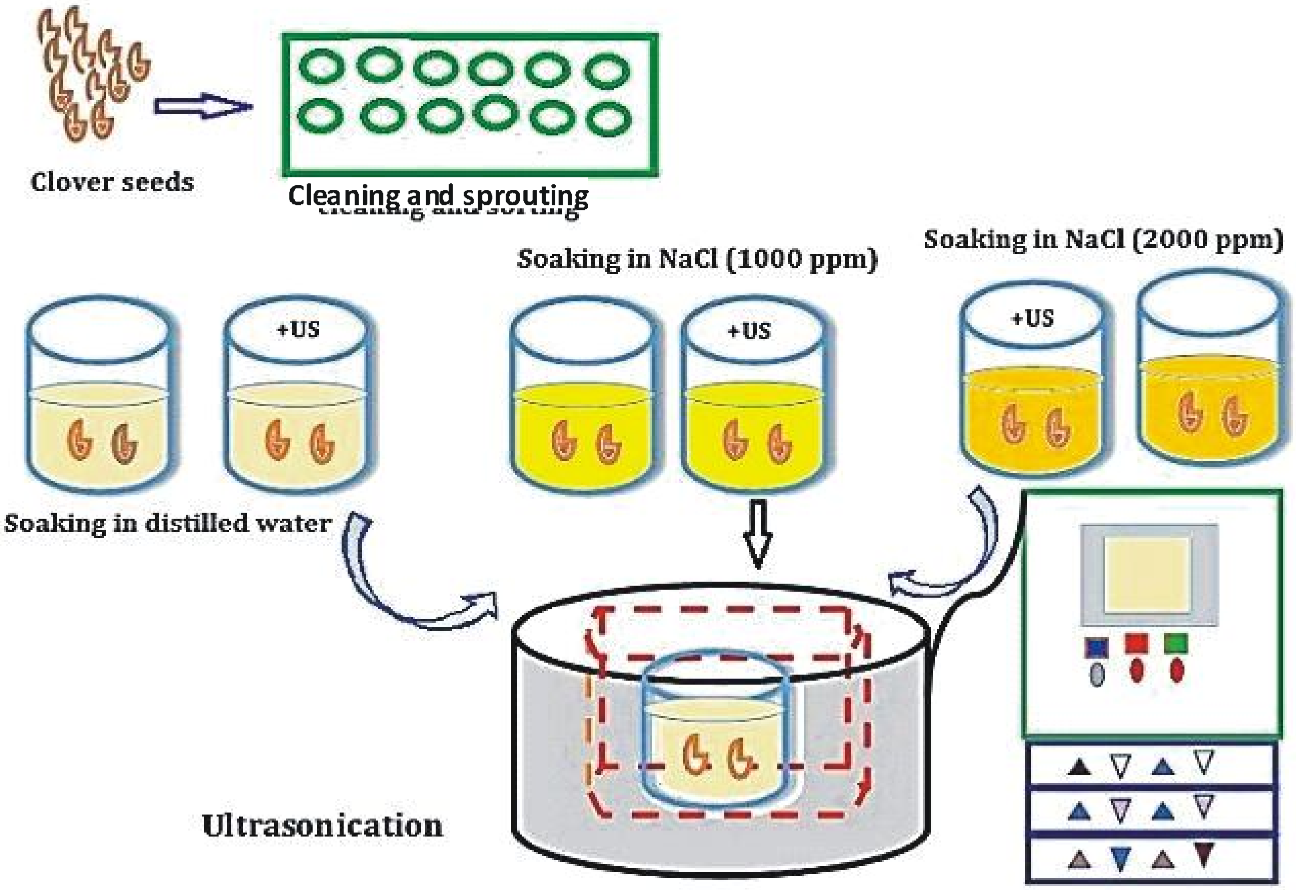

Fig. 1: Flowchart of ultra-sonication and sprouting clover seeds using saline water.

6. Hypocotyl / radical ratio: was calculated as ratio of hypocotyl length to radical length by the following equation: Hypocotyl / radical ratio = Hypocotyl length / radical length

7. Sprout length measured by additive hypocotyl length + radical Length and expressed in centimeters (cm)

8. Sprout length reduction per cent (SLR%), was calculated using the following equation:

$$SLR\% = \left(\frac{Sprout\ length\ of\ control - sprout\ length\ of\ treatment}{sprouts\ length\ of\ control} \right)$$

9. Sprout fresh weight. The weight of ten sprouts was measured and expressed milligram (mg) as ten sprouts fresh weight.

10. Sprout dry weight: The weight of ten sprouts were recorded and expressed in milligram (mg) as ten sprout dry weights after vacuum oven drying at 55°C for 48 h.

11. Sprouts yield ratio. The sprout yield ratio was calculated as the ratio of sprout fresh weight to seed fresh weight by the following equation:

$$\text{Sprout yield ratio} = \frac{10\ \text{sprout fresh weight (mg)}}{10\ \text{seeds fresh weight (mg)}}$$

Minerals determination

From the dried sample, phosphorus (P %), Potassium (K %) and Calcium (Ca %),

Magnesium (Mg ppm), Manganese (Mn ppm) and Cupper (Cu ppm) were analyzed by atomic absorption spectrophotometry 3300 Perken Elmer according to the methods described in the (AOAC, 2005).

Determination of crude protein

Total nitrogen was determined by the usual Kjeldhal method according to (AOAC, 2005). The crude protein was calculated by multiplying the total organic nitrogen by 6.25 using the following equation:

$$\text{Protein \%} = \%\ N \times 6.25$$

Amino acid measurement

The amino acids content were determined by hydrolyzing a sample each in 6 MHCl 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 amino acids content were measured by using an automatic amino acid analyzer (Sykam S - 433

Sprout nutritional quality

Sprout samples nutritional quality was determined using amino acid profiles and calculated essential amino acid index (EAAI) according to (Labuda *et al.,* 1982) method cited by Tahany *et al.,* 2018) according to following equation:

$$EAAI = \sqrt[8]{\frac{Lys \times Ileu \times Val \times Thr \times Leu \times Phe \times His \times Meth)a}{Lys \times Ileu \times Val \times Thr \times Leu \times Phe \times His \times Meth)b}} \times 100$$

Where (Lys, Thero, Val, Thr, Leu, Phe, His and Meth) in test sprout sample and (content of the same amino acids in casein as standard protein)

Nutritional index percentage of the sprout samples was determined using the following equation:

$$Nutrition\,index\,(NI) = \left(\frac{EAAI \times protein}{100}\right)$$

Biological value (BV) was calculated according to (Oser, 1959) method cited by

(Tahany *et al.,* 2018) using the following equation:

$$BV = [1.09 \times EAAI - 11.7]$$

Protein efficiency ratio (PER) estimated according to (Alsmeyer *et al.,* 1974) equation cited by (Tahany *et al.,* 2018) as follows:

$$PER = [\,-0.468 + 0.454\,(LEU) - 0.105\,(TYR)]$$

Other protein quality parameters included total amino acid (TAA), total essential amino acids (TEAA), total nonessential amino acid (TNEAA), and the ratio of TEAA/ TAA) also estimated.

Salicylic acid phytochemical compound measurement

Salicylic acid compound in the clover seed sprout measured using GC/MS/MS as reported by Santana *et al.,* 2013.

Statistical analysis

The data were analyzed by the two-way analysis of variance (ANOVA) in a complete randomized design with three replicates and the means were compared using LSD test (P<0.05). All analyses were conducted using SAS software (SAS, 2013)**.**

Results and Discussion

Effect of ultrasound and salinity on clover sprout characteristics

The Chinese clover seed sprouting percentage (SP) increased with increasing NaCl concentration, but it was more pronounced in seeds with ultrasound pre-treatment table 1, which is consistent with earlier findings of (Goussous *et al.*, 2010; Rifna, E. J. *et al.*, 2019). Lower sprouting may be due to limited water uptake by Chinese clover seeds as cold winter crop, similar to water uptake was also reported by (Dodd and Donovan, 1999)**.** The pre-treatment with ultrasound improved SP as reported before by (Goussous *et al.*, 2010; Rifna, E. J. *et al.*, 2019).

Interaction of ultrasound and NaCl concentration on SP was found to be significant. It is quite clear that Chinese clover seeds primed with ultrasound proved to be effective in inducing salt tolerance at the sprouting stage in winter clover crop. The highest sprouting index was obtained from ultrasound treatment with increasing levels. The lowest sprouting index was obtained from the control treatment (100%) and increased with increasing salinity especially with ultrasound combination. The relative salt and ultrasound injury rate were increased as the NaCl concentration increased. Data was more pronounced with ultrasound combined with salinity. The relative salt and ultrasound injury rate recorded no damage in all treated treatment. The higher injury was recorded in control treatment using deionized water while all NaCl concentration alone or combination with ultrasound recorded no salinity or ultrasound injury (Table 1).

Increasing salinity levels from 0 to 2000 ppm NaCl decreased sprout radical length with no significant effect on sprout hypocotyl length. Highlight salinity levels (2000 ppm) produced the shortest sprout radical length. Ultrasound levels increased both hypocotyl and radical length. Seed primed with salinity in combination with ultrasound had higher sprout hypocotyl and radical length. The tallest hypocotyl and radical length were recorded with deionized water, but with ultrasound the tallest hypocotyl was recorded with 40 kHz treatment and tallest radical with 20 kHz. Moreover, the combination of three salinity concentrations (0.0, 1000 and 2000 ppm NaCl) with 20 kHz ultrasound recorded the tallest radical length and with 40 kHz recorded the tallest hypocotyl length. The same results showed with hypocotyl length were recorded in sprout length (Hypocotyl + radical length) also (Table 1). The radical length and hypocotyl length are the most important parameters for salt stress because radicals are in direct contact with absorb water from growing media and supply it to the hypocotyl and the rest of the plants, for this reason, radical and hypocotyl length and ratio provides an important clue for sprout response to salt stress. Data in table 1 showed increased in hypocotyl/radical ratio increased with increasing NaCl

Table 1: Effect of sprouting using saline water (S); ultrasound pretreatment (U) and their interaction on clover sprouting and sprout characters.

Character Treatments	Sprouting %	Sprouting index (%)	Relative injury	Sprout hypocotyl length (cm)	Sprout radical length (cm)	Hypocotyl radical ratio (cm)	Hypocotyl	Sprout length reduction (%)	Sprout fresh weight (mg)	10 sprout dry weight (mg)	Sprout yield ratio
	Effect of salinity										
S0 (NaCl 0.0 ppm)	75.5B	124.6B	-0.18A	1.94A	2.52A	0.78B	4.46A	-26B	211a	22.7A	5.38A
S1 (NaCl 1000 ppm)	2.3AB	136.3AB	-0.25AB	1.92A	2.33A	0.83AB	4.25A	-19B	214A	23.4A	5.48A
S2 (NaCl 2000 ppm)	84.3A	139.7A	-0.27B	1.81A	1.94B	0.95A	3.75A	-6A	219A	24.2A	5.60A
	Effect of ultrasound										
U0 (0.0 KHz)	70.9C	117.7B	-0.12A	1.42C	1.90B	0.76C	3.32B	7A	206B	25.1A	5.27B
U1 (20 KHz)	88.0A	149.6A	-0.30B	1.88B	2.48A	0.77BC	4.36A	-22B	213AB	24.3A	5.45AB
U2 (28 KHz)	80.0A	132.9AB	-0.24B	2.06AB	2.29A	0.91AB	4.35A	-22B	217AB	23.8a	5.56AB
U3 (40 KHz)	84.0AB	138.4A	-0.27B	2.21A	2.39A	0.97A	4.60A	-29B	222A	20.4A	5.67A
	Effect of salinity × ultrasound interaction										
S0U0	60.7e	100c	0.0a	1.49de	2.07bcde	0.73b	3.56def	0a	1970b	20.7a	5.04b
S0U1	83.3abcd	137.7ab	-0.27bc	2.01abc	2.79a	0.72b	4.81a	-35ef	219ab	22.0a	5.34ab
S0U2	84.0abcd	139.1ab	-0.28bc	2.03abc	2.53ab	0.81b	4.56abc	-28def	215ab	23.3a	5.49ab
S0U3	74.0de	121.7bc	-0.18b	2.23a	2.69a	0.84b	4.92a	-39f	222ab	24.7a	5.67ab
S1U0	75.3cd	125.4bc	-0.18b	1.42e	1.92de	0.74b	3.37ef	6ab	228ab	19.3a	5.32ab
S1U1	86.7abcd	143.1ab	-0.29bc	1.80cd	2.48abc	0.72b	4.28abc	-20cdef	214ab	24.0a	5.47ab
S1U2	78.0bcd	129.5ab	-0.22bc	2.22e	2.36abcd	0.95ab	4.58ab	-28def	217ab	25.0a	5.55ab
S1U3	89.3ab	14.2ab	-0.32bc	2.23ab	2.53ab	0.89b	44.77a	-34ef	218ab	25.3a	5.58ab
S2U0	76.7bcd	127.7b	-0.20b	1.35e	1.67c	0.81b	3.01f	15a	213ab	21.3a	5.46ab
S2U1	94.0a	155.9a	-0.35c	1.81bcd	2.17bcde	0.85b	3.98bcde	-12bcd	217ab	25.3a	5.56ab
S2U2	78.0bcd	128.9ab	-0.22bc	1.92abc	1.98cde	0.97ab	3.90cde	-10bcd	220ab	24.7a	5.63ab
S2U3	88.7abc	146.4ab	-0.31bc	2.17a	1.93de	1.17a	4.10bcd	-16ab	225a	25.3a	5.76a

concentration and ultrasound levels and their combinations.

Moreover, sprout length (hypocotyl + radical length) decreased with increasing NaCl concentration while increased with priming with ultrasound especially with higher levels (40 kHz). On the other hand, sprout length reduction % increased with increasing NaCl concentration up to 2000 ppm while decreased with using ultrasound at all levels (20, 28 and 48 kHz). The higher ultrasound levels recorded increment in sprout length with no sprout length reduction percentage (average -29%) as compared with no ultrasound treatment (average 7%). The ten sprouts dry weight was not statistically affected by increasing NaCl concentration or ultrasound levels and their interaction (Table 1). However higher ultrasound level (40 kHz) increased average ten sprout fresh weight and data was more pronounced with higher NaCl concentration (2000 ppm) interaction (S2U2) as compared with control (S0U0) as shown in Table 1. The clover sprout fresh yield ratio increased from 5.04 in control treatment to the higher ratio 5.76 in higher NaCl concentration (2000 ppm) combined with higher ultrasound levels (40 kHz) in S2U3 treatment. The increment in the sprout ratio regarded to the higher fresh weight of sprouts since the sprout had greater water content than the original on the fresh weight basis. The data were more pronounced with longer sprout hypocotyl and radical length. However, this yield ratio (about 1:5) was obtained before by (Abdallah, 2008; Tahany *et al.*, 2018)**.** In this study, clover seeds soaking with NaCl (1000 and 2000 ppm) concentration significantly increase sprouting seed percentage (75.3 and 76.7%) compared with control soaked in distilled water (67.7%). The results suggest that seed soaking with 1000 and 2000 ppm of NaCl could be a simple approach for entrancing tolerance to water stress in clover as reported before by (Cao *et al.*, 2018)**.** They also found that NaCl induced seed sprouting associated with the increases in endogenous gibberellic

Table 2: Effect of sprouting using saline water (S); ultrasound pretreatment (U) and their interaction on minerals content of clover sprout.

Character Treatments	N %	P %	K %	Ca %	Mg ppm	Zn ppm	Fe ppm	Mn ppm	Cu ppm
	Effect of salinity								
S0 (NaCl 0.0 ppm)	7.94A	0.70A	0.78A	0.32A	0.26A	61.39A	54.29B	19.74A	16.59A
S1 (NaCl 1000 ppm)	7.77A	0.74A	0.73A	0.12B	0.24A	56.60A	69.16A	18.25A	6.64B
S2 (NaCl 2000 ppm)	7.80A	0.71A	0.72A	0.11B	0.24A	57.65A	55.42B	17.94A	5.46B
	Effect of ultrasound								
U0 (0.0 KHz)	8.04A	0.71A	0.75A	0.17B	0.24AB	61.83A	57.66A	19.70A	15.01A
U1 (20 KHz)	7.75A	0.73A	0.74A	0.16B	0.25AB	56.12A	61.80A	18.63A	6.80C
U2 (28 KHz)	8.04A	0.71A	0.78A	0.31A	0.27A	58.51A	59.11A	18.10A	10.23B
U3 (40 KHz)	7.50A	0.72A	0.70A	0.10C	0.23B	57.72A	59.93A	18.13A	6.21C
	Effect of salinity × ultrasound interaction								
S0U0	8.08a	0.67a	0.85a	0.28b	0.27ab	67.87a	61.0b	21.93a	32.47a
S0U1	8.05a	0.71a	0.74abc	0.21bc	0.25ab	60.01ab	53.09bc	19.97abc	8.31c
S0U2	8.03a	0.67a	0.75abc	0.66a	0.29a	60.81ab	58.46b	18.57abc	18.83b
S0U3	7.61a	0.76a	0.78abc	0.13def	0.24ab	56.86ab	44.61c	18.49abc	6.76cd
S1U0	8.06a	0.74a	0.72abc	0.12def	0.22b	60.98ab	53.10bc	19.68abc	7.11cd
S1U1	6.89a	0.73a	0.66bc	0.09f	0.24ab	40.26b	81.76a	15.87bc	6.26cd
S1U2	8.14a	0.77a	0.83ab	0.17cde	0.27ab	55.67ab	61.32b	17.19bc	6.30cd
S1U3	8.01a	0.70a	0.71abc	0.09f	0.23b	60.50ab	80.46a	20.24ab	6.87cd
S2U0	8.01a	0.71a	0.67bc	0.10ef	0.24ab	56.65ab	53.87b	17.49bc	5.44d
S2U1	8.32a	0.75a	0.82ab	0.18cd	0.27ab	59.09ab	50.55bc	20.06ab	5.82cd
S2U2	7.97a	0.68a	0.76abc	0.09f	0.24ab	59.06ab	57.54bc	18.55abc	5.57d
S2U3	6.89a	0.71a	0.62c	0.07f	0.22b	55.79ab	54.73bc	15.67c	4.99d

acid (GA) and indole-3-acetic acid (IAA) levels through activating amylases leading to improved amylolysis under salinity stress. Moreover, IAA is mainly related to root growth as spray (Guilfoyle *et al.,* 2015; Vanstraelen and Benkovã, 2012). Soaked clover seeds pre-treated with ultrasound (20, 28 and 40 kHz) effectively alleviated salt-caused inhibition on some sprout characteristics under salt stress. These study results suggest that ultrasound pre-treatment significantly improve salt tolerance of China clover seeds and this effect is dependent on ultrasound levels.

Effect of ultrasound and salinity on clover sprout mineral content

Data in table 2 indicated that sprouting process did not induce any significant changes in N%, P%, K%, Zn ppm and Mn ppm by ultrasound pre- treatment and NaCl concentration effects. However, the levels on Ca% and Cu ppm in clover sprout were decreased with increasing NaCl concentration and with increasing ultrasound levels from 20 kHz to 40 kHz. Moreover, the interaction between higher NaCl concentration (2000 ppm) and higher ultrasound levels (40 kHz) recorded the lowest Ca % and Cu ppm levels in clover sprouts. However, the lower contents of Ca and Cu in clover sprouts treated with ultrasound or NaCl and their interaction might be due to leaching out the minerals into the soaking water as a result of salt injury and ultrasound making halls and fishers.

Effect on amino acid content

The results of the amino acid determination are shown in tables 3, 4. Most of the amino acid showed increment with increasing NaCl concentration up to 1000 ppm and then decrement with higher NaCl concentration 2000 ppm except essential amino acids (THR, MET, HIS and Lys) and non-essential amino acid ASP ALA, CYS and ARG, which increased up to 2000 ppm NaCl. Aspartic acid (non-essential amino acid) found to be the most abundant in clover sprouts counted 56% at 1000 ppm NaCl more than control followed by SER 49.6%, GLU 37%, ALA 38% and Proline 35.6% (Table 4). On the other hand, all essential amino acid counted more than 40% at 1000 ppm NaCl more than control, and the most abundant was methionine (MET) which counted 146% followed by LEU 53.7% and THR 50%. The changes in amino acid content can be attributed to release of amino groups from decrement amino acids to oxaloacetate in the shift from storage protein to functional protein during sprouting as reported before by (Dagnia *et al.,* 1992). However, the

Table 3: Effect of sprouting using saline water (S); ultrasound pretreatment (U) and their interaction on essential amino acid (EAA) content (g/100 g protein) of clover sprout.

EAA Treatments	THR	VAL	MET	ILEU	LEU	PHE	HIS	LYS
	Effect of salinity							
S0 (NaCl 0.0 ppm)	2.39B	2.33B	0.15C	2.50B	4.59B	3.24B	3.31B	3.06B
S1 (NaCl 1000 ppm)	3.94A	2.87A	0.31B	3.08A	5.80A	4.13A	4.10A	3.83A
S2 (NaCl 2000 ppm)	3.08A	2.89A	0.46A	2.97A	5.62A	3.95A	4.33A	4.06A
	Effect of ultrasound							
U0 (0.0 KHz)	2.83A	2.71A	0.30A	2.81A	5.13A	3.63A	3.76A	3.53A
U1 (20 KHz)	2.77A	2.65A	2.28A	2.89A	5.48A	3.96A	3.88A	3.55A
U2 (28 KHz)	2.88A	2.69A	0.28A	2.84A	5.45A	3.65A	3.91A	3.67A
U3 (40 KHz)	2.72A	2.73A	0.38A	2.84A	5.29A	3.85A	4.09A	3.84A
	Effect of salinity × ultrasound interaction							
S0U0	2.22d	2.19d	0.13d	2.29d	3.97e	2.99d	3.08d	2.85d
S0U1	2.50cd	2.31cd	0.15cd	2.55cd	4.76cde	3.24bcd	3.31cd	2.94cd
S0U2	2.58bcd	2.41bcd	0.15cd	2.59bcd	5.03bcde	3.23cd	3.36cd	3.25bcd
S0U3	2.26d	2.43bcd	0.17cd	2.56cd	4.61de	3.50bcd	3.48bcd	3.19bcd
S1U0	3.33a	3.12a	0.32bcd	3.30a	6.10ab	4.23ab	4.30abc	4.01ab
S1U1	2.58bcd	2.92abc	0.30bcd	3.28ab	6.35a	4.75a	4.12abcd	3.75abc
S1U2	2.90abcd	2.67abcd	0.29bcd	2.85abcd	5.36abcd	3.68bcd	3.89abcd	3.73abcd
S1U3	2.95abcd	2.75abcd	0.34bc	2.87abcd	5.38abcd	3.83abcd	4.08abcd	3.83abc
S2U0	2.93abcd	2.82abcd	0.45ab	2.85abcd	5.31abcd	3.66bcd	3.90abcd	3.73abcd
S2U1	3.24ab	2.73abcd	0.39b	2.84abcd	5.33abcd	3.87abcd	4.22abc	3.97ab
S2U2	3.17abc	2.98abc	0.39b	3.08abc	5.95ab	4.05abc	4.49ab	4.02ab
S2U3	2.96abcd	3.02ab	0.61a	3.10abc	5.88abc	4.22ab	4.71a	4.52a

increased in free amino acid content in favorable as the protein quality of food depends not only on its amino acid composition but also on the availability of those amino acids. However, the higher main non-essential amino acids detected in NaCl treatments were ASP, GLU, ARG, ALA and PRO. These results indicated that China clover at sprout growth stage showed salt-tolerance, and the salt-induced changes in the free amino acid content play an important role in the response to salt stress of sprout. (Alhadi *et al.,* 2012) reported that sufficient levels of ARG, GLU and MET enhance seed germination and the germination was assumed to be regulated more or less by these amino acids.

Thus the effect of salinity in changes amino acid composition in the clover seed germination and sprout stages can be noticed to change in response with clover germination demands. Concerning the effect of pre-treatment ultrasound effect on amino acid content, data in table 3, 4 showed no significant effect of ultrasound levels on all amino acids content. However, the combination between ultrasound especially at higher levels 40 kHz with higher NaCl concentration increased amino acid content especially ASP, GLU, ARG and PRO (non-essential amino acid) and MET essential amino acid. The control treatment without salinity or ultrasound (S0U0) using distilled water for sprouting process recorded the majority of amino acids content decrease and the most dramatic increases occur using NaCl at 1000 ppm and 200 ppm with increasing ultrasound levels. The high level of amino acids present in sprouted clover seeds might be involved as precursors / stimulates in several different branches of metabolic pathway for synthesis of other amino acids in clover seed germination and sprout development. In particular Arginine and Glutamate as reported before by (Alhadi *et al.,* 2012)**.**

Effect on protein content and nutritional quality

Table 5 contains crude protein percentage and other nutrient content of clover sprout. The protein percentage showed no significant effect with NaCl concentration and ultrasound levels or their interactions. However, the protein percentage ranged between 43.05% in S1U1 treatment to 52.02% in S2U1 treatment. The amino acid data indicated that total amino acids (TAA), total essential amino acids (TEAA) and total nonessential amino acids (TNEAA) increased significantly with increasing NaCl concentration with no difference between NaCl 1000 and 2000 ppm. However, no different effect of salinity on TEAA/TAA ratio. Moreover, no statistical difference

Table 4: Effect of sprouting using saline water (S); ultrasound pretreatment (U) and their interaction on non-essential amino acids (NEAA) content (g/100 g protein) of clover sprout.

NEAA Treatments	ASP	SER	GLU	GLY	ALA	CYS	TYR	ARG	PRO
	Effect of salinity								
S0 (NaCl 0.0 ppm)	11.28B	4.02B	9.90B	2.89B	3.31B	1.16B	2.29C	6.79B	3.31B
S1 (NaCl 1000 ppm)	14.66A	4.76A	12.0A	3.55A	4.10A	1.43A	2.98A	8.04A	4.02A
S2 (NaCl 2000 ppm)	16.34A	4.35AB	10.89AB	3.53A	4.25A	1.45A	2.60B	8.37A	3.89A
	Effect of ultrasound								
U0 (0.0 KHz)	13.43A	4.42A	11.16A	3.27A	3.79A	1.19B	2.52A	7.68A	3.79A
U1 (20 KHz)	14.09A	4.31A	10.80A	3.27A	3.80A	1.49A	2.78A	7.77A	3.73A
U2 (28 KHz)	13.98A	4.44A	10.55A	3.26A	3.90A	1.39AB	2.60A	7.42A	3.60A
U3 (40 KHz)	14.87A	4.33A	11.21A	3.49A	4.06A	1.31AB	2.60A	8.06A	3.84A
	Effect of salinity × ultrasound interaction								
S0U0	10.16d	3.73d	9.66d	2.69c	3.14c	1.01ef	1.95d	6.58e	3.23b
S0U1	11.09cd	3.81cd	9.64d	2.89de	3.21c	1.31bcdef	2.38cd	6.84de	3.29b
S0U2	11.81bcd	4.60abcd	10.52cd	2.99cde	3.40bc	1.36abcde	2.33cd	6.78e	3.36b
S0U3	12.06abcd	3.94bcd	9.76d	2.98cde	3.49abc	0.95f	2.49bcd	6.96de	3.37b
S1U0	15.89abc	5.58a	13.23a	3.76ab	4.34ab	1.43abcd	3.09ab	8.53abc	4.38a
S1U1	14.08abcd	4.94abc	12.86abc	3.68abc	4.06abc	1.74a	3.44a	8.72ab	4.20a
S1U2	13.56abcd	4.94abcd	11.17abcd	3.37bcde	3.93abc	1.26cdef	2.83abc	7.26cde	3.76ab
S1U3	15.09abcd	4.02bcd	10.75bcd	3.40abcde	4.08abc	1.29cdef	2.57bc	7.65bcde	3.73ab
S2U0	14.23abcd	3.16bcd	10.59cd	3.34bcde	3.90abc	1.14def	2.52bcd	7.93bcde	3.77ab
S2U1	17.10ab	4.18bcd	9.89d	3.24bcde	4.14abc	1.44abcd	2.51bcd	7.75bcde	3.69ab
S2U2	16.56ab	4.23bcd	9.95d	3.42abcd	4.36ab	1.54abc	2.64bc	8.23abcd	3.67ab
S2U3	17.44a	5.01ab	13.12ab	4.10a	4.60a	1.69ab	2.73bc	9.56a	4.44a

between control and ultrasound levels (20, 28 and 40 kHz) was obtained on TAA, TEAA, TNEAA and TEAA/TAA ratio. On the other hand, the interaction between lower ultrasound level (20 kHz) with lower NaCl concentration (1000 ppm) and between higher ultrasound levels (28 and 40 kHz) with higher NaCl concentration (2000 ppm) recorded the higher percentage of TAA, TEAA and TNEAA. The percentage ratio of TEAA/TAA in China clover sprout samples ranged between 32.71 to 34.76%. These values were well above the 26 % considered adequate for ideal protein food for children and 11% for adults (FAO/WHO/UNU, 1985)**.** Regarding the results of other nutritional quality table 5, the most widely used method for measurement of protein quality is the protein efficiency ratio (PER) test which is the weight gained by the rats (biological assays) divided by the weight of protein consumed. Nowadays, (Alsmeyer *et al.*, 1974) equation cited by (Tahany *et al.*, 2018) for estimated PER is less expense and time required for the assay test. The values of PER table 5 of clover sprout samples were higher with lower NaCl concentration (1000 ppm) and lower ultrasound value (20 kHz) and decreased with increasing NaCl concentration (2000 ppm) and higher ultrasound levels (28 and 40 kHz). The interaction of lower NaCl concentration (1000 ppm) with or without low level of ultrasound (20 kHz) (S1U1 and S1U0 respectively) recorded higher PER than 2.0 followed by S1U2, S1U3, S2U0 and S2U1 which recorded PER than 1.8 and higher than control (S0U0) which recorded 1.2. Moreover, increasing NaCl concentration combined with higher ultrasound levels (28 and 40 kHz) (S2U2 and S2U3) recorded PER less than 1.0. However, PER of China clover sprout with salinity and lower ultrasound level were lower than 2.5 in reference (Oyarekua and Eleyinmi, 2004) but favorable comparable to 1.21 in cowpea and 1.62 in popcorn casein (Ijarotimi and Keshinro., 2013; Oyarekua and Eleyinmi, 2004) and also comparable to 0.95 in Egyptian clover sprout (Tahany *et al.*, 2018)**.**

Also, protein quality can be measured using biological values (BV) and essential amino acid index (EAAI). Increasing NaCl concentration recorded higher EAAI and BV compared with deionized water. Ultrasound levels showed no significant effect on EAAI and BV. However, higher ultrasound levels (20, 28 and 40 kHz) coupled with higher NaCl concentration (2000 ppm) had the higher EAAI (61.1%, 64% and 69%). Similarly, the BV of clover sprouts were 58.1% and 63.5% with ultrasound higher levels (28 and 40 kHz) in combination with higher NaCl

Table 5: Effect of sprouting using saline water (S); ultrasound pretreatment (U) and their interaction on nutrient content of clover sprout.

Character Treatments	Protein %	TAA	TEAA	TNEAA	TEAA/TAA	EAAI	NI	BV	PER
	Effect of salinity								
S0 (NaCl 0.0 ppm)	49.64A	64.85B	21.57B	43.28B	33.23A	45.33B	22.48B	37.71B	1.49B
S1 (NaCl 1000 ppm)	48.58A	80.51A	27.05A	53.47A	33.62A	59.73A	29.0A	53.41A	2.01A
S2 (NaCl 2000 ppm)	48.73A	80.99A	27.34A	53.65A	33.80A	63.73A	30.83A	57.51A	1.34B
	Effect of ultrasound								
U0 (0.0 KHz)	50.30A	74.07A	24.70A	49.37A	33.32A	54.86A	27.58A	48.10A	1.73AB
U1 (20 KHz)	48.46A	75.57A	25.46A	50.11A	33.68A	55.79A	26.95A	49.11A	1.88A
U2 (28 KHz)	50.27A	74.71A	25.38A	49.34A	33.92A	55.80A	28.04A	49.12A	1.49BC
U3 (40 KHz)	46.89A	77.45A	25.74A	51.71A	33.29A	58.28A	27.17A	51.82A	1.37C
	Effect of salinity × ultrasound interaction								
S0U0	50.48a	60.28d	19.72d	40.56d	32.71cd	41.02d	20.71d	33.01d	1.23de
S0U1	50.33a	64.60cd	21.76cd	42.84cd	33.67abcd	45.69cd	23.0bcd	38.10cd	1.57cd
S0U2	50.16a	68.10bcd	22.61bcd	45.48bcd	33.17bcd	47.33bcd	23.74bcd	39.89bcd	1.69bcd
S0U3	47.58a	66.43bcd	22.20bcd	44.24bcd	33.38bcd	47.26bcd	22.49cd	39.82bcd	1.50cd
S1U0	50.36a	86.75a	28.72a	58.03a	33.12bcd	63.69a	32.08a	57.73a	2.14ab
S1U1	43.05a	83.37ab	28.06ab	55.31ab	33.68abcd	60.60ab	26.09abcd	54.35ab	2.26a
S1U2	50.85a	75.17abcd	25.39abcd	49.78abcd	33.81abc	56.05abc	28.50abc	49.39abc	1.81abc
S1U3	50.05a	76.77abcd	26.03abcd	50.74abc	33.87abc	58.59abc	29.32abc	52.16abc	1.84abc
S2U0	50.05a	75.17abcd	25.66abcd	49.51abcd	34.13ab	59.88abc	29.97ab	53.57abc	1.81abc
S2U1	52.02a	78.74abc	26.57abc	52.17abcd	33.69abcd	61.09ab	31.78a	54.89ab	1.81abc
S2U2	49.79a	80.88abc	28.13ab	52.75abc	34.76a	64.02a	31.87a	58.09a	0.98a
S2U3	43.05a	89.16a	29.01a	60.15a	32.62d	68.98a	29.70ab	63.49a	0.77e

concentration (2000 ppm) (Table 5). (Oser, 1959) reported that protein-based food is inadequate when it EAAI is below 70%. This research shows that consumption of China clover sprouts alone is inadequate without complement with other protein-based foods as cheese or egg for the consumer nutritional needs. Regarding nutritional index (NI) data in table 5 showed that etiolated clover sprouts in saline water increased nutritional index compared with deionized water control. Moreover, ultrasound (20 and 28 kHz) in combination with NaCl (2000 ppm) increased NI more than 31.7%.

Effect on salicylic acid production

The results showed increase in the content of salicylic acid in the clover sprout when applying a higher ultrasound frequency level (40 KHZ) (2.11,1.92 and 1.15 area %) compared to 0.0 ultrasound freqency (1.15, 1.24 and 0.68 area%) under saline NaCl concentration (0.0, 1000 and 2000 ppm respectively).

Shakirova *et al.*, (2003) Reported that garden cress seed priming by salicylic acid improved germination but seedling length significantly decreased by increasing salinity. Therefore, our data suggested that pretreatment with ultrasound can protect the clover sprout under salinity by increasing production of salicylic acid in sprout tissues. The novelty of our data is the production of salicylic acid in sprouts by ultrasound, which inhibit the expected wound ethylene as results of salinity. Therefore unknown mode of action of ultrasound can explained by inhibition ACC 1-amino cycle-propane1-carboxylic acid synthesis from methionine and inhibition conversion ACC to ethylene by increasing salicylic acid production under saline condition, which increased ethylene production.

Conclusion

China clover seed sprouting increased the crude protein content in sprout. Sprouting using saline water in combination with ultrasound seed priming resulted also in better sprout characters, mineral content, TEAA, PER, EAAI, BV and NI. Therefore, clover sprouting influenced the protein content and quality of sprouts as a functional food.

Ultrasound and NaCl seed priming could be a simple approach for enhancing tolerance to salinity stress in China clover.

Conflict of interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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