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Ultrasonic Treatment Enhances Germination and Affects Antioxidant Gene Expression in Soybean (*Glycine max* L. Merr)

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Abstract: Ultrasound technology has been recently used to enhance and increase seed germination with no negative effect on seedling development. This study investigated the effects of ultrasound exposure for 10, 20, and 30 min on seed germination, seedling growth, and gene expression of three soybean varieties grown under glasshouse conditions. Ultrasonic treatments showed different effects on most of the studied traits compared with the untreated controls. Ultrasonic exposure increased germination percentage, root and shoot lengths, seedling dry matter, and vigor index of the three soybean varieties. Antioxidant gene expression was examined in the seedling tissues and indicated a significant stimulatory effect of ultrasonication on catalase and superoxide dismutase antioxidant gene expression. Scanning electron microscopy results showed multiple changes in soybean varieties. Seed coat rupturing appeared as pores and cracks on the waved seed coat and possibly increased seed germination. Soybean varieties revealed different abilities to germinate, grow, and develop, as well as different antioxidant gene expression in response to ultrasound treatments. In light of the results obtained, ultrasonication can be widely used to include other crops that face serious challenges in germination.

Keywords: soybean; ultrasonication; antioxidant gene expression

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1. Introduction

Seed germination is a vital stage in the life cycle of plant species. Several germination treatments are usually used to enhance seed germination and/or accelerate seedling growth, including chemical compounds, hormones, and hydration [1]. Most of these, however, are time consuming and relatively costly [2]. Physical treatment, using ultrasound waves that may generate heat, exerts mechanical and chemical effects on seeds within a short time; it is easier to implement and saves time compared with other seed priming methods [3]. Ultrasound is a new technique practiced in a wide range of biological applications, especially in seed priming. Various cellular and/or sub-cellular irreversible damages can occur throughout the different stages of seed development, presumably resulting in deteriorative alterations, including those in field weathering, harvesting, and seed storage [4]. These issues can cause losses exceeding 25% of the harvested crop and thus low productivity. The deterioration rate varies substantially not only from one species to another but also between varieties belonging to the same species [5]. The seeds of each plant species feature distinct morphological and physiological characteristics that impact the appropriate biological, physical, and chemical practices to enhance seed germination [6]. Seed priming may recover the biotic and abiotic stress tolerance of germinated seeds; plants

developed from primed seeds show an enhanced capacity to activate stress-responsive systems and establish the germinating state early [7]. Several seed priming techniques can generate sufficient abiotic stress levels to improve the stress tolerance of germinated seeds and subsequent stages of plant growth via "stress-memory" [8,9]. Ultrasonic stimulation can augment seed coat cavitation and contribute to a high rate of permeability, which in turn increases the germination rate and/or speed in deteriorated seeds [10]. Ultrasonic treatments have been reported to enhance germination in various crops, including black bean [11], corn [12], barley [13], alfalfa, broccoli [14], chickpea, wheat, watermelon, and pepper [15]. Goussous et al. [16] suggested that ultrasonic treatment assists in the penetration of moisture into plant cells and eventually increases the moisture content inside the seeds. Furthermore, the combination of chemicals, heat, and ultrasound treatment enhances seed germination by better killing of seed internal microbes [17]. Wang et al. [18] observed that sonication temperature exerts an effect on germination, whereas output power significantly affects seedling growth. However, the cultivation of soybean (*Glycine max* L.) as a valuable multi-purpose crop presents many challenges, as it is likely one of the most highly sensitive crops for long-term seed preservation. Dried seeds can be preserved for a relatively long time. However, compared with cereal crops, oil crops including soybean have seeds with high lipid contents, which may speed up deterioration. Therefore, extending the longevity and enhancing the germination potential of stored soybean seeds from different aspects have been increasingly investigated [19]. Locally, the cultivation and improvement of soybean yield faces many difficulties, with low germination rate as the most challenging. This problem is related to inappropriate maturity conditions at the end of the previous growing season [6,20]. Accordingly, the current study was proposed to investigate the stimulus effect of ultrasonication as an alternative technique of possible use to enhance soybean seed priming.

2. Materials and Methods

A glasshouse experiment was carried out at the Department of Field Crops, College of Agriculture, University of Anbar–Iraq in 2020. Seeds of the three soybean varieties that were of the same age and size were obtained from the Directorate of Agricultural Research/Baghdad/Iraq. Fifteen seeds from each of the tested varieties, namely Giza-111, Giza-22, and Senaia-2 (V1, V2, and V3, respectively), that showed relatively low germination percentage (*GP*) were subjected to osmohardening treatments through ultrasonication for 0, 10, 20, and 30 min using Crest Power Sonic P230 ultrasonic equipment (US Ultrasonics. LLC, Virginia Corporation, Willoughpy, OH, USA).

2.1. Scanning Electron Microscopy (SEM)

Two seeds from each of the twelve treatment groups were randomly chosen for SEM test. The selected seeds were completely dried at room temperature then fixed on an aluminum pellicle with double-sided carbon tape and covered with gold. The prepared seeds were observed under MIRA3 TSCAN SEM (TESCAN, Brno, Czech Republic) at 15 kV accelerating voltage.

2.2. Germination Percentage (GP%)

Ten out of fifteen treated soybean seeds from each treatment were planted directly after sonication in 30 cm diameter plastic pots filled with soil under glasshouse conditions (day/night temperature range of 25–30 °C/15–20 °C). Pots were irrigated as needed. Two weeks after seedling emergence, germination percentage (*GP*) was estimated according to the following equation described by [21]:

$$GP = \frac{Number\ of\ germinated\ seeds}{Number\ of\ total\ seeds} \times 100$$

2.3. Root Length

Ten seedlings were gently uprooted and washed repeatedly with running water to remove residual soil. The average root length was measured for each replicate, starting from the seed to the tip of the radical. The values were recorded and expressed in cm as root length at the end of a standard germination test [22].

2.4. Shoot Length

The average shoot length of the 10 seedlings was estimated from each replicate, starting from the seed to the tip of the leaf blade; the values were recorded and expressed in cm as shoot length at the end of a standard germination test [22].

2.5. Seedling Dry Matter

Seedling dry weight (g) was calculated by obtaining 10 seedlings from each treatment used for dry weight and keeping them in a hot-air oven drier at 60 ± 2 °C until a constant weight was achieved; then, their dried weight was determined.

2.6. Seedling Vigor Index (SVI)

SVI was calculated according to the formula suggested by [23]:

$$SVI = \frac{(Radicle\ length + Plumule\ length) \times Germination\ percentage}{100}$$

2.7. Real-Time Polymerase Chain Reaction (RT-PCR)

Fresh leaves were collected from soybean seedlings of the twelve treatment combinations at the first node stage and then dipped in Trizol reagent contained in a 1.5 mL micro tube. Total RNA was extracted from the collected samples following the Trizol procedure. One-step RT-PCR Kit (QIAGEN, Hilden, Germany) containing 1 mM dithiothreitol, 0.1 mM ethylene diamine tetra acetic acid, 0.5% Nonidet P-40, 0.5% Tween® 20, 50% glycerol, stabilizer (pH 9.0) Tris-Cl, KCl, $(NH4)_2SO_4$, 12.5 mM $MgCl_2$, and 10 mM each of dATP, dCTP, dGTP, and dTTP was used to amplify the targeted genes in accordance with the supplier's instructions.

Two pairs of specific primers were used to amplify and assess the expressions of the two selected *Glycine max* cDNA genes, superoxide dismutase (SOD) (forward: 5-TTCCGAATTCAAAGGTCCAG-3; reverse: 5-TAAGATCAGCCACCCTCAGC-3) and catalase (CAT) (forward: 5-CTGCTGGAAACTATCCTGAGTG-3; reverse: 5-ATTGACCTCTTC ATCCCTGTG-3) [24]. The following thermal profile was adopted in amplifying the SOD gene: 94 °C for 1 min; 33 cycles at 94 °C for 0.5 min, 55 °C for 1 min, and 72 °C for 1 min; a final extension at 72 °C for 7 min. The CAT gene was amplified according to the following thermal profile: 94 °C for 1 min; 29 cycles at 94 °C for 0.5 min, 54 °C for 1 min, and 72 °C for 1 min; a final extension at 72 °C for 7 min. Standard curve method was adopted for absolute quantification of SOD and CAT gene expression, where CT values were used in generating standard curves (ten-fold dilutions) for computing the absolute mRNA out of the total extracted RNA from each of the twelve treatment combinations included in the experiment.

2.8. Statistical Analysis

Treatments in the experiment were laid out in a factorial arrangement with completely randomized design of four replicates. The recorded data were subjected to the analysis of variance (ANOVA) using SAS software SAS (r) version 9.1 (SAS Institute Inc., Raleigh, NC, USA, 2004). Treatment means were separated and compared using the least significant difference test (LSD) at $p \leq 0.05$.

3. Results

Ultrasound durations significantly affected most of the studied traits of soybean seedlings compared with the controls (Figure 1). Thus, the positive effect of seed priming via ultrasonic durations can be easily detected. Significant variation was also observed in the performance of experienced varieties, as they revealed relatively different responses to ultrasound durations.

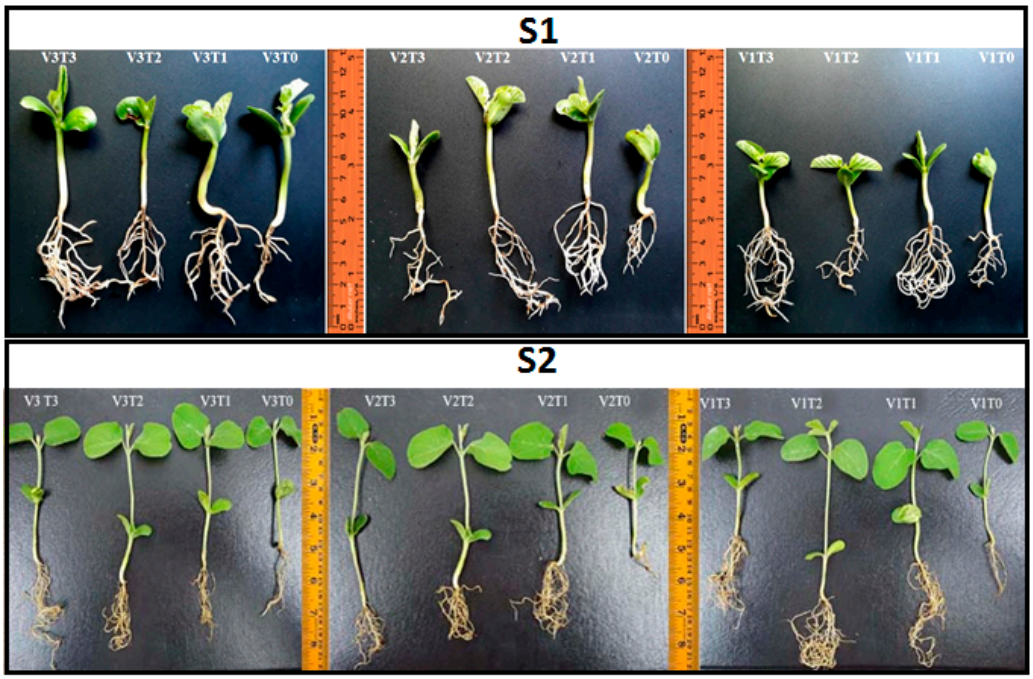

Figure 1. Priming seed ultrasonication in three durations (T0: control, T1: 10 min, T2: 20 min, and T3: 30). Priming seed ultrasonication in three durations (T0: control, T1: 10 min, T2: 20 min, and T3: 30 min) on three soybean variety seedlings (V1: Giza-111, V2: Giza-22, and V3: Senaia-2). S1: Two-leaf stage (ruler length, 13 cm). S2: First node stage (ruler length, 20 cm).

3.1. Seedling Growth Parameters

Ultrasonication enhanced the germination parameters of the three tested soybean varieties and at different exposure periods (Figure 2). As illustrated in Figure 2A, the (*GP*%) of the 30 min ultrasonication treatment group differed significantly compared with the controls (0 min) in the three varieties. The highest *GP* was obtained at the 30 min exposure period, resulting in 80%, 75.5%, and 60% for V2, V1, and V3, respectively. The two other ultrasound durations (10 and 20 min) showed partial significant response against controls, as a significant difference was observed in V1 and V3 by 10 min of ultrasound waves, and that in V2 and V3 was obtained after the 20 min ultrasound exposure. The lowest *GP* values were for untreated controls of the three varieties, giving 24.4, 55.5, and 51.1% for V1, V2, and V3, respectively.

Ultrasound treatments significantly affected root length of the three soybean varieties (Figure 2B). At 30 min ultrasound seed exposure of V2, and at 20 min seed exposure of V1 and V2, the longest significant seedling roots were obtained, with root lengths more than 11 cm compared to less than 9 cm in other treatments, which showed long roots but were not significantly different from those of the untreated controls.

Ultrasound waves showed variable responses in terms of seedling shoot length in the three soybean varieties (Figure 2C). Soybean seedlings resulting from seeds exposed to 10 and 20 min of ultrasound waves showed the highest shoots in the three varieties. Shoot length in the 10 and 20 min ultrasound seed treatments ranged from 10.7 cm to 11.3 cm and from 11.5 cm to 13.2, respectively. Shoot lengths of the three varieties subjected to 30 min

ultrasound ranged from 10.2 cm to 11.3 cm. The lowest shoot height (9.6 to 11.3 cm cm) was in the untreated control seedlings.

Seedling dry weight of the three soybean varieties was affected by ultrasound duration as the seed priming technique (Figure 2D). Soybean seedlings obtained from seeds treated for 20 min with ultrasound waves showed the highest dry weight, ranging from 1.59 g to 1.66 g in the three varieties. Other treatments (10 and 30 min ultrasound exposure) resulted in seedling dry weights ranging from 1.49 g to 1.59 g and from 1.48 g to 1.60 g, respectively. Soybean seedlings of untreated seed sonication gave the lowest seedling dry weight, ranging between 1.1 g to 1.4 g.

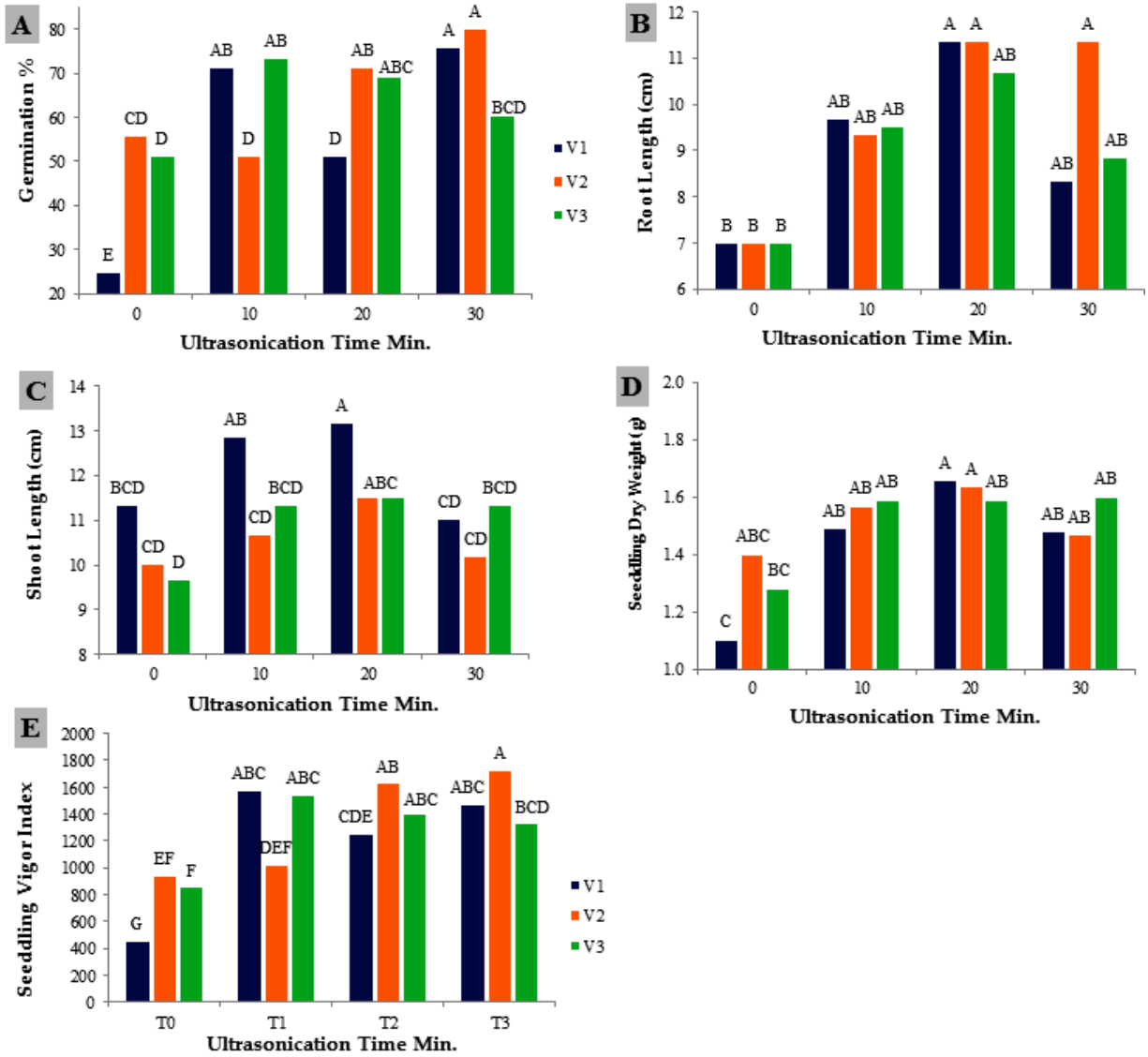

Figure 2. Ultrasonication enhancement in various durations (T0: control, T1: 10 min, T2: 20 min, and T3: 30 min) and germination parameters of the three soybean varieties (V1: Giza-111, V2: Giza-22, and V3: Senaia-2). (**A**) *Germination percentage GP* (%); (**B**) root length (cm); (**C**) shoot length (cm); (**D**) seedling dry matter (g); (**E**) *Seedling vigor index SVI*. Different letters express significant differences.

The seedling vigor index (*SVI*) reflects the significant difference in seed priming by ultrasound waves at all seed exposure durations (Figure 2E). All varieties showed positive responses with ultrasound waves at the three durations (except 10 min exposure of V2) of treatments compared with the controls. In general, the soybean seedlings treated with 10 and 20 min of ultrasound waves showed superior response in terms of root and shoot lengths compared with those treated for 30 min. Meanwhile, most of the soybean

treatments at the three ultrasound durations showed significant differences in terms of *SVI* traits compared with untreated controls. This result is due to the superiority of the *GP* under 30 min ultrasound wave duration (Figure 2A).

3.2. Antioxidant Gene Expression

The quantification of expression of the targeted genes indicated the significant stimulating effect of ultrasonication on the expression of SOD and CAT antioxidant genes (Table 1, Figure 3). In general, the three tested soybean varieties showed modest expressions for both CAT and SOD genes in the control treatment with no sonication (T0). However, the highest level of expression was recorded for the SOD-encoded gene in the V2 variety (Giza-22). Ultrasonic treatment of 20 min (T2) was the most effective duration in the expression of SOD with a mean of 67.62 copies, followed by T1 duration (10 min) that resulted in 40.78 copies of the SOD gene in the three treated varieties of soybean. However, the control treatment (T0) produced the minimum number of copies of the SOD gene (13.67 copies). The mean expression of the CAT gene across the tested varieties was varied in response to the ultrasonic durations. The T2 (20 min) duration revealed the maximum copies of the CAT gene (58.69 copies); meanwhile, T3 (30 min) and T0 ultrasonic durations expressed the minimum copies of the investigated gene (26.16 and 17.49 copies, respectively). The genotype of the treated soybean varieties had a major role in outlining the SOD and CAT response. The second variety V2 (Giza-22) was the most responsive genotype to the ultrasonic provocation, showing the highest number of SOD copies (55.73 copies), followed by V1 (Giza 111) that scored 52.13 copies of the SOD gene, whereas V3 (Senaia-2) had a modest response, revealing the lowest number of copies (28.32 copies) of the same gene.

Table 1. Effect of four ultrasonic durations (T0: control, T1: 10 min, T2: 20 min, and T3: 30 min) on the expression of CAT and SOD antioxidant genes in the three soybean varieties (V1: Giza-111, V2: Giza-22, and V3: Senaia-2).

Treatments		Number of Copies		Gene Expression vs. Control (%)	
		SOD	CAT	SOD	CAT
V1	T1	11.73	18.36	−19.2	43.8
	T2	93.94	80.50	547.1	530.6
	T3	50.73	23.36	249.4	83.0
V2	T1	60.91	64.70	407.4	119.5
	T2	92.02	71.89	666.6	143.9
	T3	14.27	28.36	18.9	−3.8
V3	T1	49.70	36.38	243.3	71.9
	T2	16.89	23.67	16.6	56.8
	T3	18.36	26.77	26.8	61.8
T0	V1	14.52	12.76	-	-
	V2	12.00	29.48	-	-
	V3	14.48	10.23	-	-
Mean	SOD	T0: 13.67	T1: 40.78	T2: 67.62	T3: 27.79
	CAT	T0: 17.49	T1: 39.81	T2: 58.69	T3: 26.16
	SOD	V1: 52.13		V2: 55.73	V3: 28.32
	CAT	V1: 40.74		V2: 54.98	V3: 28.94

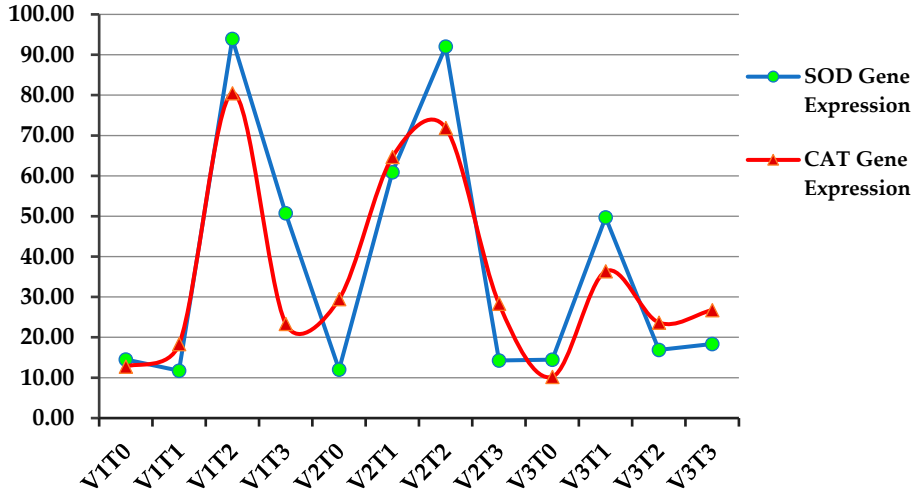

Figure 3. Expressions of CAT and SOD antioxidant genes in three soybean varieties (V1: Giza-111, V2: Giza-22, and V3: Senaia-2) subjected to four durations of ultrasound (T0: control, T1: 10 min, T2: 20 min, and T3: 30 min).

The general performance of the CAT gene was slightly different compared to the previously described gene, although the genotypic effect still drove the general response of the CAT gene to the experienced ultrasonic durations. The studied varieties showed that the number of CAT copies ranged between 54.98 copies in V2 and 28.94 copies in V3. The longest duration of ultrasonic waves (T3: 30 min) showed a similar effect, resulting in low gene expression of both investigated genes, except in the V1 variety (Giza-111), which achieved a higher level of CAT gene expression (T3) compared with the two other varieties treated with the same ultrasonic duration. The SOD gene was more responsive, reaching a higher level of expression in response to the increased duration of sonication, especially in the seedlings of V2 (Giza-22) when the maximum value was achieved compared with the two other varieties. On the other hand, CAT gene expression was less sensitive to the practiced durations of ultrasound; however, its performance remained close to that of the SOD gene, as a close number of copies of the two targeted genes was detected. The estimated expression of the two genes against control (%) indicated that almost all treatments had a positive induction effect in the expression of both investigated genes. However, the highest induction resulted from subjecting the V2 variety (Giza-22) to 20 min (T2) of ultrasound, which positively regulated the expression of the SOD gene up to 666.6% compared to the control. On the other hand, the T1 duration (10 min) negatively regulated the expression of the SOD gene in the V1 variety (Giza-111) with −19.2%. V2 (Giza-22) showed a similar negative response to T3 duration of ultrasound (30 min) with −3.8%.

3.3. Scanning Electronic Microscope (SEM)

The SEM examination of the control (Figure 4A,B) and treated seeds (Figure 4C–F) of three soybean varieties showed multiple, clear cracks on the surface of the treated seed coat. The controls (untreated seeds) of the investigated varieties contained almost no cracks. These cracks probably resulted from ultrasonication, particularly in the Giza-111 variety (V2).

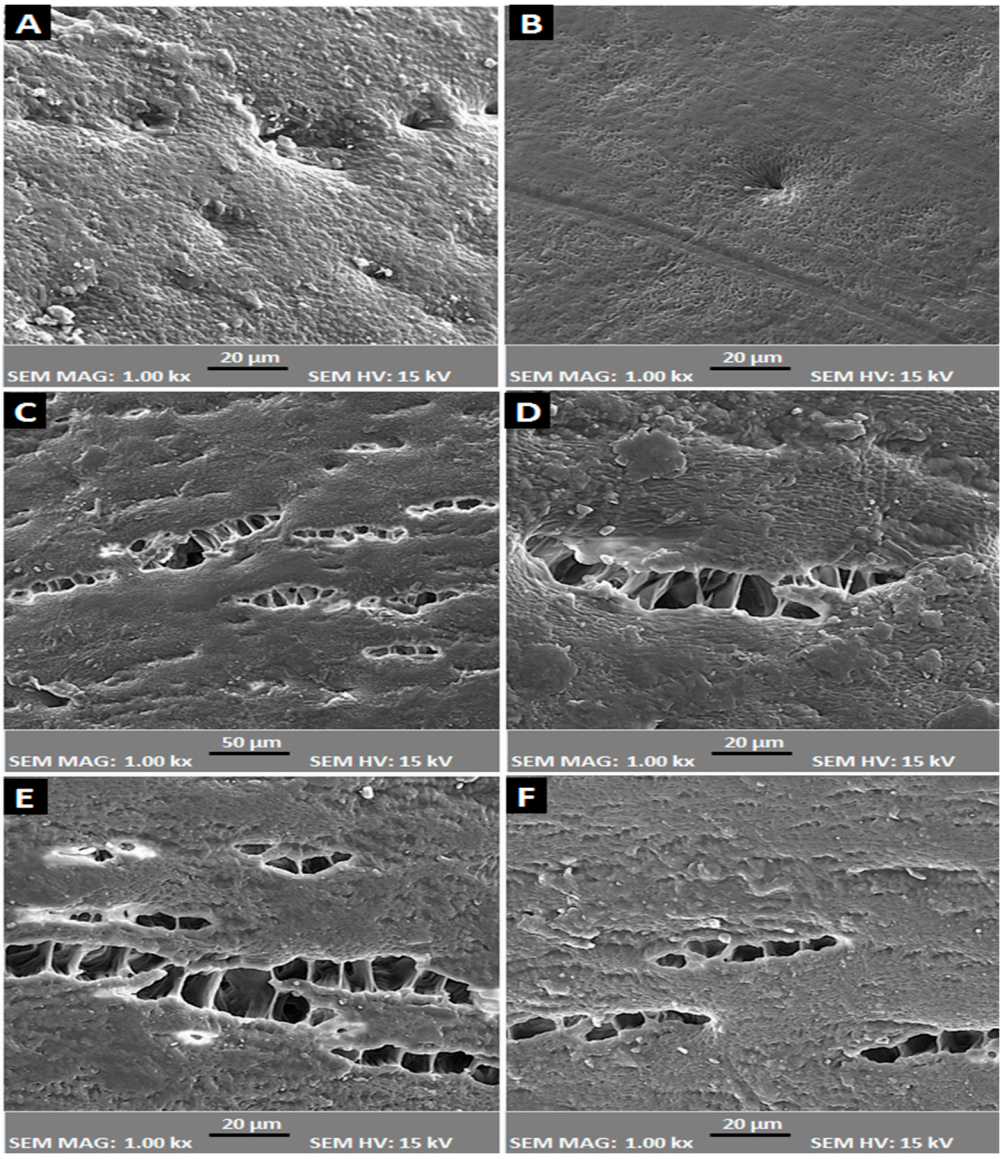

Figure 4. SEM (HV: 15.0 kv) of seeds of three soybean varieties (V1: Giza-111, V2: Giza-22, and V3: Senaia-2) subjected to three ultrasound durations (10, 20, and 30 min) and the control. (**A**,**B**) Control (V1T0 and V3T0), (**C**,**D**) V2T2, and (**E**,**F**) V3T2. Scale bars: 20 µm for (**A**,**B**,**D**–**F**) and 50 µm for (**C**).

4. Discussion

4.1. Seedling Growth Parameters

Soybean seeds were exposed to ultrasound waves to comprehend its function in seed priming and seedling development. Recently, ultrasound waves have been successfully demonstrated to improve germination percentage (*GP*) and seed emergence by the activation of various enzymes activity in many crops, particularly legume seeds and small-seeded grasses [16]. Root length was increased under ultrasonication duration. This shows that ultrasound waves stimulate root elongation. In this regard, Liu et al. [3] reported that ultrasound treatment enhanced root and shoot length in aged seeds of tall fescue (stored for five years) in Russian wild rye. Ultrasound enhancement was also evident in the seedling shoot length for all durations used, which improved optimal growth and preparation, resulting in a strong plant. However, the increase in seedling shoot length was different from the control depending on the intensity of the ultrasound duration. Machikowa et al. [25] confirmed that all treatment durations resulted in higher shoot lengths of sunflower seedlings. The increase in soybean seedling dry matter seemed expected as a result of the increase in the

root and shoot length, which was enhanced by the activation of ultrasound. The current results are in line with what Shekari et al. [26] stated, that sesame seedling dry matter was significantly affected by the priming sonication technique. Furthermore, stimulation of seed germination by ultrasound had an expected response presented by seed vigor index (*SVI*), which was reflected positively in the graduation of optimal plant growth. In this regard, Nazari and Eteghadipour [27] mentioned that rapid germination and seedling emergence are critical factors for a successful establishment of plants and crops, and mild sonication can stimulate seedling development.

4.2. Antioxidant Gene Expression

The enhanced expression of SOD and CAT genes can effectively modulate the excessive cellular production of ROS in the aged soybean seeds, hence maximizing seed vigor. In this regard, the elevated temperature and porous effect of sonication may have acted as an abiotic stress that in turn provoked the SOD and/or CAT expression under coherent levels [28]. This can be described as an adaptive mechanism followed by the plant in an attempt to control the electron leakage that typically occurs under stress conditions [28–30]. The ultrasonication effect could occur directly and/or indirectly via creating micro-multi cracks that mediated seed coat disruption after seed inhibition. Furthermore, some recent studies reported that the significant effect of ultrasound may result from the exceeding temperature. In general, a long ultrasound duration is not always positively related to antioxidant gene expression, hence seedling growth parameters. The stimulative effect of ultrasound is not always involved in a positive relationship with the expression of SOD and CAT genes, where in general, the positive effect was limited to the first and second exposure durations. However, the level of expression of both targeted genes was negatively related to the increased duration of ultrasound in most investigated varieties, which may explain the deteriorated mean of some seedling traits in the maximal duration. The T2 treatment (20 min) seems to be the most inductive duration across the studied genotypes of soybean.

5. Conclusions

Ultrasound waves enhanced soybean seed germination by inducing cracks or pores of the seed coat, which led to higher water consumption and improved seed hydration. In addition, it was found that the rate of enzyme-catalyzed hydrolysis reactions within the soybean seeds, which enhanced enzyme activity and hydrolysis, accelerated germination and seedling growth as a result of the ultrasonic treatment.

Author Contributions: Contributions were as follows: Conceptualization, A.O.A. and A.A.A.; methodology, A.O.A. and A.A.A.; software, B.S.A.; validation, A.O.A. and A.F.A.; formal analysis, A.O.A. and A.A.A.; investigation, O.M.D.; resources, A.N.; data curation, A.F.A.; writing—original draft preparation, A.O.A., B.S.A. and A.A.A.; writing—review and editing, J.R.Q.; visualization, A.N.; supervision, J.R.Q.; project administration, O.M.D. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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