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Ultrasonic Influence on Carob tree (*Ceratonia siliqua* L.) rooting under in-vitro conditions

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Abstract

The purpose of this study is to develop an appropriate protocol aiming to improve the rooting capacity of carob (*Ceratonia siliqua* L.) explants grown in vitro. Rooting is known to be difficult for this species, which therefore hinder the propagation of productive genotypes. Explants prepared from germinated seeds of *C. siliqua* L. were exposed to two sonication durations 15 and 30min at a frequency of 42 KHz. A combination of optimal sonication (15min) with hormonal (IBA) treatment was applied by immersing the basal part of the explants in three different concentrations (0.5, 1, and 2 mg/l). The results of sonication were significant on the parameters of aerial and root growth. The exposure time of 15 min is the best compared to the 30 min. Hormonal treatment (alone or in combination with sonication) has a positive effect on the length of the plant, and the number and length of the roots. The results show significant differences with control at 2 mg/l IBA treatment combined with 15min sonication, or 15min sonication followed by 2 mg/l IBA treatment (dark, 12 h, and 5°C). The results of these experiments show that sound wave stimulation can certainly promote plant growth. Furthermore, this protocol offers a new root induction pathway for cultivars characterized by low rooting capacity in nature.

Keywords: Sound wave Stimulation, IBA, *Ceratonia siliqua*, *In vitro* multiplication.

Introduction

Plants as living organisms get affected by external stimuli such as light, temperature, wind, and a variety of internal chemical signals. Sound, spread in form of waves, capable of moving through elastic media, is characterized by specific frequencies. Many researchers have used sound wave frequencies as external stimuli to study their effect on plants growth performances. Sound waves with specific frequencies and intensities believed to have positive effects on various plant biological mechanisms including seed germination, root elongation, plant height, callus growth, cell cycling signaling transduction systems, enzymatic and hormonal activities, and gene expression (Hassanien et al., 2014; Chowdhury et al., 2014). Although the mechanical stress induced by the sound waves does not give any obvious changes in the plant DNA, they have influenced the acceleration of the synthesis of RNA and soluble protein, which led to an increased level of transcription, so more proteins are produced for plant growth and survival (Wang et al., 2003).

Recent studies have revealed that audible sound stimulation has a great potential to improve plant growth and the quality of products. Plant acoustic frequency technology (PAFT) is the treatment of plants with a specific sound frequency. This treatment was found to increase the yield and quality, and strengthen disease-resistance in pepper, cucumber, and tomato (Tian et al., 2009). As to the physiological and biochemical effects, PAFT increased the content of growth hormones like indoleacetic acid (IAA) and polyamine compounds (Li, 2002). These growth regulators directly influence cell division and formation of vascular bundles, leaves and flower buds. They were also closely related to plant

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disease resistance (Qin, 2003). Moreover, the net photosynthetic rate, maximum fluorescence, and photochemical efficiency of photosystem II were also markedly increased by sound in strawberry leaves (Zhou et al., 2010; Meng et al., 2012).

Carob tree (*Ceratonia siliqua* L.) is a long-lived fruit species and forms an important part of the Mediterranean vegetation (Ciccarese et al., 1988; Batlle and Tous 1997). Carob tree is well known in the Mediterranean countries for its ornamental, nutritional, and medicinal values. Due to its particular biological and agro-ecological features, such as resistance to salinity, adaptation to poor soils and minimal cultural requirements, the carob tree forms part of the national list of priority as important forest resources for conservation in Morocco. This species is used naturally against soil erosion and land in most of Mediterranean areas (Janick and Paull, 2008).

Adventitious root formation in cuttings is an essential step for successful vegetative propagation of many woody plants. However, in several tree species, rooting is still a major problem. The potential of *Ceratonia siliqua* cuttings to form adventitious roots decreased with increasing plant age. Plant Acoustic Frequency Technology is believed to be the treatment with specific sound frequencies that boost crop yield and quality, as well strengthening disease-resistance.

The objective of this work is to test the effect of audible sound on the growth and the rooting of the carob tree as well as to establish a model protocol to implement this potential technology on the rooting recalcitrant plants.

Materials and methods

Plant material and explant preparation

Mature seeds were collected from a female Carob tree located in the region of Chefchaouan (Northern Morocco). The seeds were aseptically scarified and disinfected with a 95% sulfuric acid solution for 20 min and sterilized by immersion in 0.01% mercury chloride ($HgCl_2$) for 30 min. After disinfection, the seeds were rinsed three times for 10 min with sterile distilled water, and then each seed was placed in a sterile agar-water tube autoclaved at 121°C for 20 min. The cultures were maintained in the culture chamber at 25±2°C. After germination, hypocotyl sections were prepared from seedlings obtained by cutting the stem about 2 cm above the root collar. Those hypocotyls were used as explants for the next experiments.

Ultrasound treatment and culture media

The sonication was performed at a frequency of 42 kHz, a power of 70% (420 W) and at room temperature using an ultrasonic cleaning bath (Selecta, model 3000512).

All treated explants were transferred to pre-sterilized glass vials with a plastic cap containing 50 ml sterile 0.7% agar. The cultures were maintained in the culture chamber at 25±2°C under a photo-period of 16/8 h of cold white light of 1500 lux.

Effect of sonication on carob tree growth

To evaluate the effect of ultrasound on carob explants we tested the ability of this 42 kHz frequency technique to initiate an extended response for aerial growth and rooting. We exposed the explants to two sonication periods: 15 and 30 min. Explants without sonication treatment "control" were also considered.

The explants were put into pre-sterilized 150ml glass vials containing 50ml of sterile distilled water. Sonication was performed in an ultrasonic bath with a frequency of 42 KHz. Then, the explants were transferred into bottles containing 50 ml sterile 0.7% agar water, and were kept in the culture chamber. The reading was done after a month.

Hormonal effect combined with sonication

We tested the effect of IBA as a hormonal stimulator on improving root induction. IBA was used alone as a "control" and in combination with the application of ultrasound to test the effect of sonication on the improvement of integration of IBA into the cells.

The explants were put into pre-sterilized 150 ml glass vials containing 50ml of sterile distilled water. Three different concentrations of IBA (0.5, 1, and 2 mg/l) were added to the explants. To determine the appropriate hormone treatment time in conjunction with the ultrasound application, we changed the IBA addition time, either at the same time as the ultrasound application or immediately after sonication. Then the explants were kept in the dark at two different temperatures: 5 and 30°C for 12 h, in order to determine the best incubation temperature on the root induction. The 5ºC experiment was maintained in the refrigerator, and that of 30 °C maintained in the incubator.

After 12 h of incubation in the dark, all the treated and control explants were transferred into culture in flasks containing 50 ml of sterile 0.7% agar. The results are read after one month.

Estimation of the results

To estimate the effect of sonication on carob explants after one month of culture, we measured the total length of the aerial part, the length of the apical bud, the number of newly formed buds, the number of internodes, and the number of leaves. Regarding the root part, we made measurements on the number of roots, the maximum length of roots, and finally the percentage of rooting for each seedling.

The criteria used to estimate the effect of hormonal treatment with IBA alone or in combination with sonication are the total length of the plant, the number of roots, and the maximum length.

Statistical analysis

Each treatment comprised 30 explants in replicates of three. All data were subjected to one-way ANOVA followed by Duncan Post-hoc tests. Results were expressed as mean±standard error (SE). Significance level set to p=0.05 or 95% confidence level (IBM, SPSS ver. 20).

Results

The results (Table 1) show that sonication has a positive effect on the stimulation of major growth parameters of carob seedlings. Thus, the seedlings treated with sonication have a vigorous and qualitative appearance, compared to controls (0 min sonication).

Regarding the effect of duration exposure to sonication, we note that the duration of 15 min is significantly effective for all the measured parameters.

An average of 5.750±0.092 cm in plant length was obtained for an exposure time of 15 min. Then, this value is about 5.427±0.092cm for an exposure time of 30 min, and in controls, this average does not exceed 4,270±0,092 cm.

The number of roots was strongly reported with an average of 6.733±0.354 of the seedlings exposed to duration of 15 min of sonication. Then, a value of 4.133±0.354 was obtained in the plants exposed to 30 min ultrasound, and a value of 1.100±0.354 recorded in the control plants.

The average maximum root length was obtained in plants exposed to 15 min sonication with an average of 1.247± 0.144 cm and 1.947±0.065 cm in plants exposed to 30 min sonication. The control reached a low average of 0.330± 0.144 cm.

The length of the buds was significantly pronounced with an average of 2.250±0.065 cm for the plants exposed to 15 min sonication, 1.947±0.065 cm for

30min duration, and finally, 1.557± 0.065 cm for controls.

The average of the neo-formed buds is 0.733±0.076. This value is the same for both sonication durations (15 and 30 min), while it is zero for the controls in which no neo-formed bud is noticed. The average number of internodes was significantly recorded at the same value of 3.067±0.108 for seedlings exposed to both sonication durations (15 and 30 min). Then, a value of 2.233±0.108 was obtained in the controls. The average number of leaves reaches a value of 6.600±0.206 for 15 min of sonication and 5.500±0.206 for 30 min sonication, while the controls reached a value of 4.300±0.206.

The percentage of explants response to rooting is significant for an exposure time of 15 min and 30 min with rates of 80% and 70% respectively compared to the control that recorded only 46.7%.

Table 1. Effect of duration exposure to sonication on indexed parameters of plant growth.

Variables	**Time of sonication (min)**	**Mean±SE***
Plant length (cm)	0	4.270±0.092c
	15	5.750±0.092a
	30	5.427±0.092b
Root number	0	1.100±0.354c
	15	6.733±0.354a
	30	4.133±0.354b
Secondary Root length (cm)	0	0.330±0.144b
	15	1.247±0.144a
	30	1.227±0.144a
Apical buds length(cm)	0	1.557±0.065c
	15	2.250±0.065a
	30	1.947±0.065b
Number of buds	0	0.000±0.076b
	15	0.733±0.076a
	30	0.733±0.076a
Number of internodes	0	2.233±0.108b
	15	3.067±0.108a
	30	3.067±0.108a
Number of leaves	0	4.300±0.206c
	15	6.600±0.206b
	30	5.500±0.206b
Rooting %	0	46.7±0.048b
	15	80.0±0.048a
	30	70.0±0.048a

*Means followed with the same letter within a treatment group are not significantly different at 5% level of probability using Duncan Multiple Range Test

Considering the hormonal effect of IBA auxin treatment at different concentrations, the observation of the results obtained in Table 2 shows a positive effect on the root activity. However, the response varies and progressively evolves according to the concentration tested. Indeed, the concentration of 2 mg/l gives the best result concerning the length of the plant and the number of roots, as well as their size. These averages, for a concentration of 2 mg/l, are respectively 5.200±0.090 cm; 4.700±0.217, and 0.937±0.044 cm. The averages obtained for the controls of these parameters are respectively 4.270± 0.090 cm, 1.100±0.217, and 0.330± 0.044 cm.

The combination of both treatments; sonication for 15 min and IBA (0.5, 1, and 2 mg/l); leads to a significant improvement in plant growth and rooting. Table 3 presents a comparative study of the effect of sonication combined with IBA in one hand and treatment with IBA alone on other hand. The average is significantly noted of 5.983±0.091 cm for the length of the plant using 1 mg/l of IBA in combination with 15 min of sonication, while the same treatment without sonication gives a value of 5.413±0.091 cm. For the root number, an average of 8.267±0.283 is obtained with 2 mg/l in combination with 15 min of sonication, while the same treatment without sonication gives a value of 4.700±0.283. The maximum root length is also influenced by the combination of 2 mg/l and 15min of sonication, with a value of 0.910±0.048 cm.

Table 4 presents the evaluation of two treatments; before and after seedlings immersion in IBA solution in parallel with the application of sonication. The treatment (before) corresponds to an application of IBA before exposure to 15 min sonication, while the treatment (after) corresponds to an application of IBA immediately after 15 min sonication. The results for the three measured parameters namely; the length of the plant, the number and the length of the roots, are significantly better for treatment (be-fore). The averages are res-

Table 2. Hormone effect of IBA on plant rooting.

Variables	IBA concentration	Means±SE*
total length of the plant (cm)	0	4.270±0.090b
	0.5	5.397±0.090a
	1	5.413±0.090a
	2	5.200±0.090a
Number of secondary roots	0	1.100±0.217c
	0.5	3.200±0.217b
	1	3.467±0.217b
	2	4.700±0.217a
Length of secondary roots	0	0.330±0.044d
	0.5	0.460±0.044c
	1	0.787±0.044b
	2	0.937±0.044a

*Means followed with the same letter within a treatment group are not significantly different at 5% level of probability using Duncan Multiple Range Test.

Table 3. Combined effect of sonication (15 min) and hormonal treatment with IBA.

Variables	Times of sonication(min)	concentration of IBA(mg/l)	*Mean±SE
Total length of the plant (cm)	0	0.5	5.397±0.091ab
		1	5.413±0.091a
		2	5.200±0.091b
	15	0.5	5.813±0.091ab
		1	5.983±0.091a
		2	5.750±0.091b
Number of secondary root	0	0.5	3.200±0.283b
		1	3.467±0.283b
		2	4.700±0.283a
	15	0.5	5.433±0.283b
		1	6.233±0.283b
		2	8.267±0.283a
Length of secondary roots	0	0.5	0.530±0.048b
		1	0.787±0.048a
		2	0.937±0.048a
	15	0.5	0.827±0.048b
		1	1.143±0.048a
		2	0.910±0.048a

*Means followed with the same letter within a treatment group are not significantly different at 5% level of probability using Duncan Multiple Range Test pectively 5.550± 0.115 cm; 7.367±0.237, and 0.900± 0.053 cm with respect to the (after) treatment in which their values are respectively 5.133±0.115 cm; 5.767± 0.237, and 0.660±0.053 cm.

Table 5 shows the effect of incubation temperature (12 h in the dark) of carob explants without hormone treatment and without sonication on their growth and rooting. The best averages are recorded with 5°C for measured parameters; the length of the seedlings, the number and the length of the roots which are respectively: 5.292±0.090 cm, 4.867±0.188, 0.688± 0.053 cm, while under 30 °C, the averages recorded for these parameters are respectively: 4.700±0.090 cm, 2.200± 0.188, and 0.267±0.053 cm.

Table 4. Effect of IBA treatment before or after sonication.

Variables	Treatments	Means±SE*
Length of the plants	Before	5.550±0.115a
	After	5.133±0.115b
Number of roots	Before	7.367±0.237a
	After	5.767±0.237b
Root maximum length	Before	0.900±0.053a
	After	0.660±0.053b

*Means followed with the same letter within a treatment group are not significantly different at 5% level of probability using Duncan Multiple Range Test.

Table5. Effect of incubation temperature 12h in the dark.

Variables	Temperature	Means±SE*
Seedlings length	5	5.292±0.090a
	30	4.700±0.090b
Number of roots	5	4.867±0.188a
	30	2.200±0.188b
Length of roots	5	0.688±0.053a
	30	0.267±0.053b

*Means followed with the same letter within a treatment group are not significantly different at 5% level of probability using Duncan Multiple Range Test.

Table 6. Effect of incubation temperature in combination with 15 min sonication and IBA (2 mg/l)treatments

Variables	Incubation Temperature (°C)	Means±SE*
Seedlings length	5	5.750±0.127a
	30	4.967±0.127b
Number of roots	5	8.267±0.266a
	30	3.567±0.266b
Maximum length of roots	5	0.910±0.075a
	30	0.500±0.075b

*Means followed with the same letter within a treatment group are not significantly different at 5% level of probability using Duncan Multiple Range Test.

Table 6 shows the effect of incubation temperature (5 and 30°C, in the dark for 12 h) on the growth and rooting of the explants treated with 15 min sonication combined with immersion in 2 mg/l IBA. After one month of culture, significant results for the three measured parameters were recorded for seedlings incubated under 5°C compared to those incubated under 30°C. The averages for 5°C are respectively; 5.750±0.127 cm, 8.267±0.266, and 0.910±0.075 cm. While those related to 30°C are respectively; 4.967±0.127 cm, 3.567±0.266, and 0.500±0.075 cm.

Discussion

Compared with controls, this study shows that stimulation of carob seedlings with sound waves at a frequency of 42 KHz has a positive effect on seedlings stem and root growth. The application of ultrasound has improved plant growth capacity and root induction, leading to the same results as research on rice, cucumber, lettuce, and wheat (Hou, 2009; Ran, 2015). Yi and colleagues (2003) reported that sound stimulation increased root metabolism and thus chrysanthemum growth. In contrast, plant growth is thought to be the sum of cell proliferation and subsequent cell elongation, essential for the production of new organs that have a significant impact on overall plant growth. In recent decades, various biological effects attributable to ultrasound treatment have been described for different plant species (Ananthakrishnan et al., 2007).

Regarding the effects of sound frequency on the plants cell structure, some researchers have concluded that appropriate frequency stimulation of acoustic processing has played an important role in the thermodynamic behavior of cell walls. Indeed, after ultrasonic treatment the membrane structure of plant cells adopt some characteristics such as increased fluidity and number of free lipids (Shen, 1999; Wang, 2003), which is not only facilitated the transfer of material, energy, and information, but also facilitated cell division (Li, 2001).

In our study, a frequency of 42 KHz significantly favored the response of different growth parameters of carob seedlings measured after one month of culture. In addition, indole acetic acid (AIA) is an essential plant hormone that helps the growth and development of plants. It is suggested that the acoustic frequency of 42 Khz significantly stimulates the production of endogenous hormones in plants, such as AIA and AG. Studies on several vegetables, including cucumber, tomato, embroidered melon, cowpea, and eggplant confirm these findings compared to control (Zhu et al., 2011; Jiang et al., 2011; Meng et al., 2012).

In addition, the application of IBA significantly increased the length of the plant, the number of roots per seedling, and the length of the roots. This is in agreement with the study conducted by De Klerk et al. (1999) and Hartmann et al. (1997). Where the application of IBA has improved the rooting percentage and the number of roots compared to the untreated control. IBA has been reported to significantly increase adventitious root formation in many species. Our studies also showed a positive effect of IBA treatment with 2mg/L in combination with 15 min ultrasound on root induction. These results agree with that of Qi et al (2010). The authors reported that sound waves favor strawberry growth. They showed the influence of sound wave stimulation on the surface / dimensions of strawberry leaves, photosynthetic characteristics, and other physiological responses. They concluded that the frequency of audible sounds could stimulate the opening of leaf stomata and thus facilitate the absorption of light energy by the plant and help it to develop better.

In our case, it is suggested that the rigid pectocellulosic cell wall is a barrier to the transfer of molecules within the attributable cells. However, the cavitations caused by ultrasound treatment cause thousands of micro-wells on the seedlings surface. These micro-wells allow growth hormones to penetrate deeper into the cells, increasing by such their metabolic activity. Ultrasonic cell administration has been demonstrated *in vitro* by uptake of extracellular fluid, drugs and DNA into cells (Koch et al., 2000; Wu et al., 1993) and plant tissues (Zhang et al., 1991). In addition, the sonication method has already demonstrated the efficacy of Agrobacterium transformation in host cells in several plant species (Khanna et al., 2004; Liu et al., 2005; Gurel et al., 2009; Chandrasekaran et al., 2015).

Considering the conditions and particularly the temperatures to which our seedlings are subjected, the latter can have an influence on the morphogenetics of the tissues cultivated *in vitro*. In our working conditions, the temperature of the culture chamber was constantly kept at 25°C. According to Gaspar (1988), a high temperature (±30°C) can favor the initiation of primordial roots, whereas a temperature (±25ºC) favors their development. In our case, we tested the effect of temperature during the incubation of seedlings in 12h immersion in the IBA in the dark. The results show that the temperature of 30°C affects negatively the vigor of the plant itself and on the quality of IBA absorption. The reason why this temperature did not support a significant root growth, as indicated in our study. In contrast, seedlings incubated at 5°C with

2 mg/l IBA favored plant growth. This allows us to suggest that the incubation at 5°C ensures the stability of the active molecule of IBA and consequently allow its better absorption.

Finally, the results of this study confirm the studies by Hassanien et al (2014). He found that ultrasound could be used as a rooting stimulator since it can activate root-stimulating enzymes; possess antifungal properties, and antiseptics, and therefore promotea strong and a healthy root growth. The absence of any kind of contamination during our *in vitro* cultures, also suggests that sonication helps plants prevent bacterial or fungal problems, allowing them to stay healthy and robust.

Conclusion

This study was conducted to evaluate the effects of ultrasound on the growth and rooting of carob seedlings. Ultrasound treatment for 15 min improved the aerial and root growth parameters of the plants compared to control. The efficacy of IBA has been demonstrated on rooting. Combined sonication treatment at 15 min with immersion in 2 mg/l solution of IBA increased the hormonal absorption reflected by a maximal response to rooting. Ultrasound at 42 KHz as a form of stress has positive effects on growth and root induction of Carob seedlings. Ultrasonic treatment with certain sound frequencies may help a plant absorb more water and nutrients into its culture medium and may influence the growth of certain plant organs.

However, further research is needed to optimize processes by modifying variables such as the power, frequency, time, and temperature of the ultrasound treatment. Based on the results obtained, ultrasound seems to be a promising technique to improve the quality of carob plants and to optimize the amount of roots.

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