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ORIGINAL PAPER

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Elicitor-like effects of low-energy ultrasound on plant (*Panax ginseng*) cells: induction of plant defense responses and secondary metabolite production

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Abstract In this work we examined the elicitor-like effects of low-energy ultrasound (US) on plant cells with respect to the induction of plant defense responses and secondary metabolite production. *Panax ginseng* cells in suspension culture were exposed to US (power ≤0.1 W/cm^3 at 38.5 kHz fixed frequency) for short periods of time (30 s–6 min). Two early events in plant defense metabolism and signal transduction pathways, the increased cross-membrane ion fluxes (Ca^{2+} influx and K^+ efflux/H^+ influx) and the production of active oxygen species (AOS), were detected in sonicated cultures within 2 min after US exposure. These responses could be induced with small doses of US energy, 0.6–0.8 J/cm^3, and enhanced by increasing US energy within a non-inhibitory range. US treatment stimulated the synthesis of useful secondary metabolites, saponins of ginseng cells, without causing any net loss of the biomass yield of ginseng cell cultures. These results suggest that US can act as a potent abiotic elicitor to induce the defense responses of plant cells and to stimulate secondary metabolite production in plant cell cultures.

Introduction

Plant cell culture is a promising bioprocess for mass production of plant-derived natural products, particularly the secondary metabolites of naturally rare and slowly growing plant species. The process has drawn considerable research efforts in the last few decades, but has so far found only few commercial applications. The lack of commercial significance is in many cases due to the low yields of most secondary metabolites in plant cells. Therefore, various strategies have been exercised to induce and stimulate secondary metabolite biosynthesis of plant cells.

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The treatment of plant cells with biotic and abiotic elicitors has been one of the most effective means to improve the yields of secondary metabolites in plant cell cultures (Dörnenburg and Knoor 1995; Roberts and Shuler 1997). The strategy is based on the fact that the accumulation of most secondary metabolites in plants is part of the defense response to pathogen infection and environmental stimuli. The agents that induce plant defense responses are generally referred to as elicitors. Elicitor-induced plant defense responses involve a cascade of physiological events in the challenged cells, such as changes in membrane potential and ion fluxes, the production of active oxygen species (AOS), the activation of defense-related genes, and the synthesis of antimicrobial secondary metabolites (phytoalexins) (Low and Merida 1996; Ebel and Mithöfer 1998). The increase in membrane ion fluxes, e.g., Ca^{2+} influx and K^+ efflux/H^+ influx exchange, and the production of AOS such as hydrogen peroxide (H_2O_2), which is referred to as the oxidative burst, are two early and important events in plant defense.

The elicitors used in previous studies to stimulate secondary metabolite production in plant cell cultures were mostly chemical agents, such as carbohydrate fractions of fungal and plant cell walls, methyl jasmonate, chitosan and heavy metal salts (DiCosmo and Misawa 1985; Dörnenburg and Knoor 1995). There have been few reports on the use of physical or mechanical stimuli to enhance the production. It is well-established in plant sciences that physical and mechanical stresses such as wounding and rigorous stirring induce plant defense responses in plant tissues and cells (Low and Merida 1996). However, mechanical stress also has been found deleterious to the growth and viability of many plant cells, causing negative effects on secondary metabolite production in plant cell cultures (Namdev and Dunlop 1995; Zhong 2001).

Ultrasound (US) is a special type of physical stimulus that has a range of biological effects (reviewed in Williams 1983; Nyborg and Ziskin 1985; Miller et al. 1996). Although high-intensity US is generally destructive to biological materials, mild US may stimulate biological activities, such as enzymatic and microbial bio-

conversions (Sakakibara et al. 1996; Barton et al. 1996; Schläfer et al. 2000), and cellular biosynthesis (Joersbo and Brunstedt 1992). As the sonic power is propagated through the biological medium via wave motion rather than mechanical agitation, US can produce mechanical effects on biological materials in the medium non-intrusively. We have recently found that low-intensity US can dramatically increase the yields of secondary metabolites in plant cell cultures, e.g., ginsenoside saponins of *Panax ginseng* cells (Lin et al. 2001) and shikonins of *Lithospermum erythrorhizon* cells (Lin and Wu 2002). We proposed that US acts as an abiotic elicitor of plant defense responses to stimulate the secondary metabolite biosynthesis of plant cells.

The main objective of this work was to examine the elicitor-like effects of US by measuring the characteristic events of plant defense responses after exposure to US, such as changes in membrane ion fluxes and the production of H_2O_2. Experiments were carried out in suspension cultures of *P. ginseng* cells exposed to low-intensity US. *P. ginseng* is a precious and well-known oriental herb, especially the root, which is widely used in traditional Chinese medicine and the formulation of health foods. Saponins are secondary metabolites of ginseng cells and the principal active ingredients of ginseng. The mechanisms of US induction of plant defense responses and US stimulation of secondary metabolite (saponin) biosynthesis in plant cell cultures are also discussed.

Materials and methods

Plant cell cultures

Suspension cultures of *Panax ginseng* cells used in this work were routinely maintained on MS medium (Murashige-Skoog 1962) in Erlenmeyer flasks. The cultures were shaken constantly on an orbital shaker at 110–120 rpm and are generally referred to as shake-flask cultures. The ginseng cell line used in the present study had been maintained in shake-flask culture for more than 6 years when this work started. Further details of the culture conditions can be found elsewhere (Wu and Ho 1999).

Exposure of ginseng cells to US

An ultrasonic cleaning bath (model 1875, Crest Ultrasonics, Trenton, N.J.) with a fixed frequency of 38.5 kHz and variable power levels (1–9) was used to insonate the ginseng cells. All cells tested in the US exposure experiments were taken from the shake-flask culture described above during the mid-exponential growth phase (day 5–10). The cells were transferred to 125-ml Erlenmeyer flasks (glass) containing 25 ml MS medium. For exposure, the flasks were dipped in the ultrasonic bath to a depth at which the liquid in the flasks was about 1.0 cm below the liquid in the bath. For uniform exposure, the flasks were clamped to an orbital shaker and shaken at 80 rpm. The ultrasonic bath temperature was maintained at 25±0.5 °C during the exposure. All treatments were duplicated or triplicated; each data point reported is the mean of two or three replicate measurements.

Table 1 shows the actual US power (power density in mW/cm^3, measured calorimetrically) transmitted into the culture flasks corresponding to the power levels employed in this work. The total US energy (E) transmitted to the culture, which equals the product of US power P and exposure period t ($E=P\times t$), is a useful parameter for quantifying the US dosage at various US power levels and exposure periods. Details of the US device and power measurement have been given elsewhere (Lin et al. 2001).

Table 1 Power parameters of the US exposure system. *Power (density)* Ultrasound power transmitted to the culture (per volume), *energy (density)* product of power (density) and exposure period

Power level	Power (mW/cm^3)	Energy (J/cm^3)			
		1 min	2 min	4 min	6 min
1	3.4	0.20	0.41	0.82	1.22
2	13.7	0.82	1.64	3.28	4.93
4	61.4	3.68	7.37	14.74	22.10
6	113.7	6.82	13.64	27.29	40.93

Measurement of cell density, viability and saponin content

The cells were separated from the liquid by filtration through Whatman filter paper (no. 1–6) under vacuum. The cell mass in the filter was rinsed twice with deionized water and then weighed as the fresh weight (fw) of the cells. The dry weight (dw) of the cells was obtained by drying the fresh cell mass in an oven at 50 °C until constant weight. Cell viability was determined by the exclusion of Evans blue staining. The Evans blue (Sigma, St. Louis, Mo.) was dissolved at 2.5% (w/v) in a 0.6 M sorbital solution, which was mixed with an equal volume of the sample cell suspension for about 5 min. Viable and dead cells in the sample were then enumerated with a hemocytometer under a microscope.

The total saponin content of ginseng cells was extracted from powdered dry ginseng cells with water-saturated *n*-butanol, isolated by thin-layer chromatography (TLC), and then quantified with the vanillin-perchloric acid method, as described in detail elsewhere (Lin et al. 2001). Saponin released into the medium was no more than 5% of the total saponin content (even after sonication) and not determined in this work.

Measurement of membrane ion-fluxes

The cross-membrane fluxes of Ca^{2+}, K^+ and H^+ were determined by measuring the changes of ion concentrations and pH (for H^+) in the extracellular medium, based on methods used previously (Atkinson et al. 1990; Bach et al. 1993). The US-induced ion fluxes were tested at various US power levels (2, 4, 6) and exposure periods (0.5, 1, 2 min) (total US energy 0.82–13.6 J/cm^3). In some of the ion-flux tests, La^{3+}, a plasma membrane Ca^{2+}-channel blocker, was added at 0.5 mM to the culture or assay medium. La^{3+} was supplied as a 100×-concentrated solution of $La(NO)_3$, obtained by dissolving La_2O_3 in HNO_3 and adjusting the pH to 4.0 with NaOH.

The pH change was directly measured in the culture medium with a cell density of 60 g fw/l using an Orion SA520 pH meter (Orion Research, Boston, Mass.). The meter has a resolution of 0.01 pH, and US treatment had no influence on its function. To analyze Ca^{2+} and K^+ fluxes, the fresh mass of ginseng cells from a shake-flask culture was rinsed thoroughly with, and then resuspended in an assay buffer (0.175 M mannitol, 0.5 mM K_2SO_4, 0.5 mM Ca_2Cl, 5 mM MES; pH adjusted to 6.0 with Tris buffer). The final cell density in the assay buffer was 40 g fw/l for K^+ and 100 g fw/l for Ca^{2+} assay. Cells in assay buffer were incubated in shake-flask culture for about 1 h before US exposure. During and after US exposure, samples (1–1.5 ml each) were withdrawn from the flasks at selected intervals. To determine the concentrations of ions in the sample (cell suspension), the medium was separated from the cells immediately after withdrawal of the sample from the culture flasks by pipeting the sample through a cotton-plugged

pipet tip. The concentrations of Ca^{2+} and K^+ were measured by atomic absorption (AA) spectroscopy with an Analyst 100 (PerkinElmer, Shelton, Conn.) using KCl as a standard for K^+ and $CaCO_3$ (dissolved in HCl) for Ca^{2+}. For K^+ measurement, 1 ml of 5 g CsCl/ml was added to 5 ml of sample medium prior to AA spectroscopy.

Bach et al. (1993) suggested that cell-wall binding of Ca^{2+} may interfere with measurements of elicitor-induced membrane Ca^{2+} influx in cell suspensions. However, our observation and that by Tavernier et al. (1995) showed that cell-wall adsorption of Ca^{2+} had no significant influence on the results of membrane Ca^{2+}-flux measurements in our experiments.

Measurement of H_2O_2 production

Hydrogen peroxide produced by the cells and released into the culture medium was detected by oxidative quenching of fluorescent pyranine (trisodium salt, Aldrich, Gillingham, Dorset, UK) as described by Yahraus et al. (1995). A cell suspension from the shake-flask culture was mixed with fresh medium at 1:3 volume ratio, and 25 ml of fresh cell suspension (containing 1.5 g fw cells) was added to a 125-ml Erlenmeyer flask (glass). To each flask, 50 µl of 1 mg/l pyranine solution was added just prior to US exposure. The cells in the flasks were then exposed to US at selected power levels and exposure periods. The fluorescence intensity of the culture medium taken from the flasks was determined with a luminescence spectrometer (model LS50B, PerkinElmer) at 405 nm excitation and 512 nm emission.

Results

Effects of US on cell growth, saponin production, and cell viability

The time course of the change in dry cell wt in the culture (Fig. 1) shows that initially (1 day) cell growth was slightly depressed after US exposure, but then gradually recovered, reaching nearly the same cell density (in about 3 days) as the control culture. On the other hand, the saponin content of cells increased significantly after US treatment, being about 45% higher on day 9 and 80% higher on day 15 than in the control culture. These effects of US on cell growth and saponin accumulation of ginseng cell cultures are similar to those reported in our previous work (Lin et al. 2001).

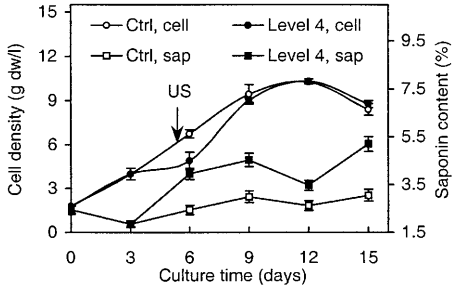

Fig. 1 Effects of ultrasound (US) on cell growth and saponin production of ginseng cell culture. US exposure on day 5 at level 4 US power for 2 min. *Ctrl* Control, *sap* saponin. *Error bars* Standard errors of duplicate treatments

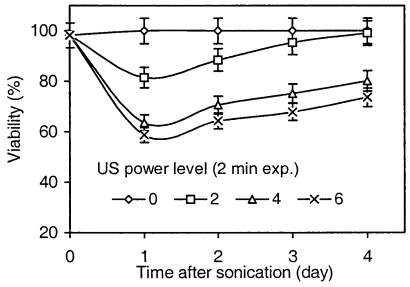

Fig. 2 Effect of US on cell viability of ginseng cell culture. *Error bars* 5% maximum relative standard errors of duplicate treatments

Sonication of the cells at US powers up to level 6 for 30 s or at level 1 for up to 5 min had no significant effect on cell viability (data not shown). At higher US power levels (2–6) and longer exposure periods (2–6 min) (Fig. 2), cell viability decreased significantly with increasing US power 1 day after exposure. In the following 2–3 days after exposure, however, the cell viability of sonicated cultures recovered to reach significantly higher levels. This viability trend with respect to US dosage and culture time is consistent with the increase in cell growth (Fig. 1).

Membrane ion fluxes induced by US

Changes in extracellular Ca^{2+}, K^+ and pH vs US power level and exposure period are shown in Fig. 3A–C. In general, US treatment of cells resulted in rapid changes of the extracellular ion concentrations and pH, reaching a plateau within 5–10 min after exposure; the amount of change was dependent on the US dosage (power level and exposure period). The extracellular Ca^{2+} concentration (Fig. 3A) at various US doses except for very large ones (e.g., level 6, 1 min) showed a sharp rise immediately (~30 s) after exposure and then dropped rapidly below the initial level and that of the control. The lower extracellular Ca^{2+} concentration of sonicated cultures than of control cultures represents a net influx of Ca^{2+} through the cell membrane induced by US. With very large US doses, e.g., 1 min at level 6, however, the extracellular Ca^{2+} concentration remained higher than that of control for most of the 30-min post-exposure period. The increase in extracellular Ca^{2+} induced by US was due mostly to US-stimulated release of intracellular contents, since the medium became cloudy after US exposure. In the presence of La^{3+}, a membrane Ca^{2+}channel blocker, the extracellular Ca^{2+} concentration did not decline from the high level gained after sonication, which was apparently due to the blockage of the membrane Ca^{2+}channel and Ca^{2+}influx by La^{3+}.

The extracellular K^+ concentration (Fig. 3B) and pH (Fig. 3C) increased with increasing US dosage. It may be argued that the increase in K^+ concentration and pH of the extracellular medium could be attributed to the release of intracellular contents that is enhanced by son-

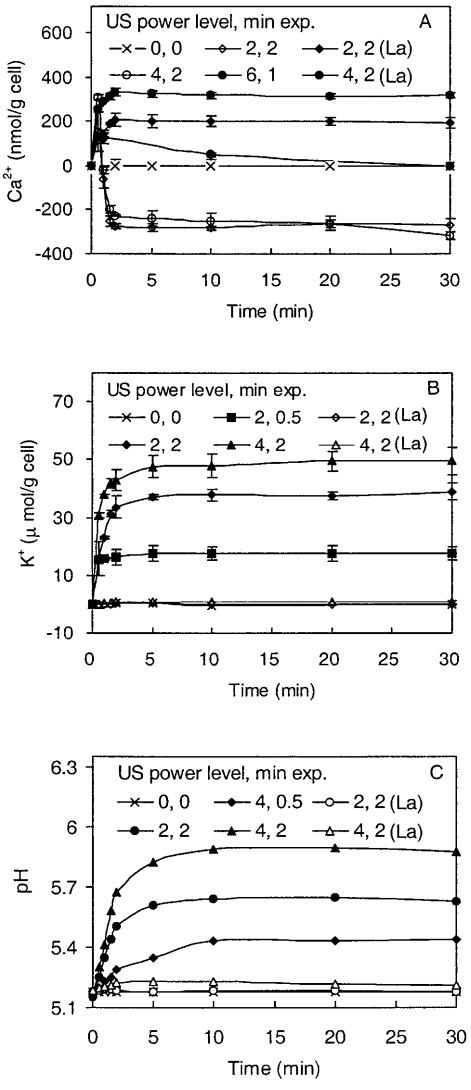

Fig. 3 US-induced changes of **A** extracellular Ca^{2+}, **B** K^+, **C** pH: effects of US dosage and La^{3+}. Time includes the period of US exposure. *Error bars* Standard errors of triplicate treatments. Maximum standard error of pH measurements was about the size of the symbols (triplicate flasks.) *First number* Power level, *second number* exposure period (min), *(La)* treated with 0.5 mM La^{3+}

ication. However, the US-induced rises of extracellular K^+ and pH were completely (at lower power levels) or partially (at higher power levels) inhibited by La^{3+}. This means that the ion-fluxes detected in the sonicated cells at relatively low US doses were mainly a result of the active responses of the cells rather than the US-induced release of intracellular contents. On the other hand, it indicates that the induction of cell membrane K^+ efflux/H^+ influx by US is dependent on and associated with increased membrane Ca^{2+} influx, as is the case with ion fluxes induced by biotic elicitors (Atkinson et al. 1990).

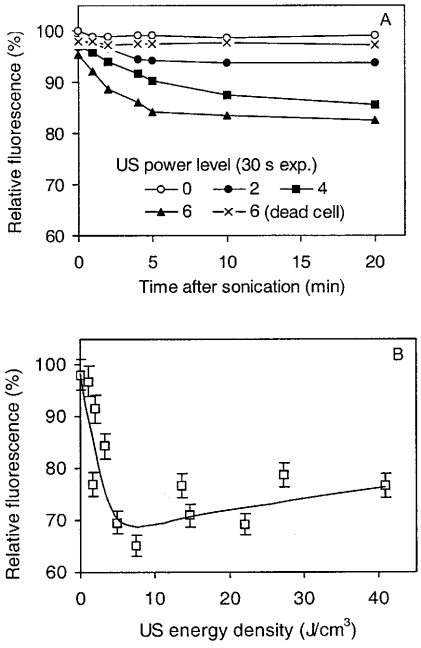

Fig. 4A, B US-induced H_2O_2 production (oxidative quenching of fluorescence) in ginseng cell culture. **A** Time course at various US power levels, **B** correlation to US dosage (total energy). *Error bars* Maximum standard error, 4% of the mean of triplicate treatments

Oxidative burst induced by US

The fluorescence intensity of the ginseng cell suspension supplemented with fluorescing pyranine declined rapidly after a 30-s exposure to various levels of US power (Fig. 4A), indicating the production and release of AOS, such as H_2O_2, by the cells. At sufficiently high US powers, level 4–6, oxidative quenching of fluorescence in the sonicated cultures was detectable immediately after exposure, while its onset delayed by about 3–4 min at a lower US power, level 2. The amount of oxidation was enhanced with increasing US power, from level 2 to level 6, within the 20-min observation period. The duration of the stimulated oxidative reaction, i.e., the period with significant fluorescence quenching, was only about 5–10 min at various US power levels. Since the extinction was only marginal or negligible when cell-free medium or medium containing dead cells was sonicated even at the highest power level and longest exposure period tested (data not shown for cell-free medium), the oxidative reaction in the sonicated ginseng cell cultures may be attributed mainly to the oxidative burst of intact cells stimulated by US. The cell growth and viability data (Figs. 1 and 2) also support this view.

US-induced H_2O_2 production by ginseng cells was also measured over a wide range of US power levels (2, 4, 6) and exposure periods (2, 4, 6 min) and correlated with the US dosage. The fluorescence intensity at 30 min post-US exposure at each US power and exposure period was plotted against the total US energy (Fig. 4B). Note that, at all US doses, H_2O_2 production (or decreasing flu-

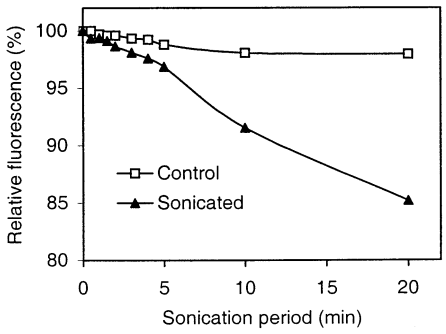

Fig. 5 H_2O_2 production in ginseng cell culture exposed to low-power US to determine the energy threshold for activation of the oxidative burst (see Table 1 for US energy at a given time). The maximum standard error in the data is 4% of the mean, based on triplicate treatments

orescence intensity) mainly occurred within 2–5 min after exposure, and the fluorescence intensity was little changed in the following 1–2 h, so that the intensity at 30 min was approximately at the lowest level. The level of H_2O_2 production increased with US energy at relative low levels, but declined at a much higher US energy range. The maximum level of H_2O_2 production (or the lowest fluorescence intensity) was found near 7.4 J/cm^3 (level 4, 2 min), although the level of H_2O_2 showed appreciable fluctuation with the change of US energy. The decline in H_2O_2 production from the peak level with increasing US dosage may be due to the negative effect of high US energy on the physiological activity of the cells.

The increase in H_2O_2 production with increasing US dosage within the low-energy range suggested that there may exist a threshold US dose for activating the oxidative response of plant cells. To detect this minimum US dose for ginseng cells, the cells were exposed to a very low US power, level 1 (3.4 mW/cm^3 power), continuously for 20 min, during which the fluorescence intensity of the culture was monitored frequently. The fluorescence intensity of each sample was measured 15 min after the sample was removed from the exposure system (which allowed sufficient time for the oxidative burst to evolve). US-stimulated H_2O_2 production (Fig. 5) was detectable at a 2–3 min exposure period, corresponding to an US energy of 0.41–0.62 J/cm^3, which may be considered as the threshold US dose to initiate H_2O_2 production in ginseng cells. At this low US power, the stimulating effect of US on H_2O_2 production increased steadily with exposure time or US energy received by the cells.

Discussion

Our experimental results show that sonication of plant cells with low-energy US induces the characteristic events of plant defense responses to pathogen infection and elicitor treatment, i.e. a transient increase in the Ca^{2+} influx and the K^+ efflux/H^+ influx exchange by the cell membrane and the transient production of AOS. This provides strong evidence that US may act as an abiotic elicitor of plant defense responses to stimulate the secondary metabolite biosynthesis of plant cells. Saponins, the steroid and triterpene glycosides synthesized in plants mainly through the mevalonic pathway, may also contribute to plant defense against pathogens and herbivores because these molecules often exhibit potent antifungal activity (Osbourn et al. 1998). However, saponins are frequently present in relatively high levels in intact plants and cells in culture, unlike phytoalexins, which are mainly synthesized in response to pathogen attack. Despite the wide distribution of saponins in plants, their role in plant defense has only been partially confirmed in a few species such as oats (*Avena*) and tomato (*Lysipersicon*) (Osbourn et al. 1998). Although there is still no experimental evidence for the function of saponins as disease-resistance agents in ginseng plants, it has been shown that saponin synthesis by ginseng cells in suspension culture is dramatically increased after elicitation with yeast extract and methyl jasmonate (Lu et al. 2001).

A critical point is how US irradiation activates the defense responses of plant cells in suspension cultures. Although the specific mechanisms remain to be elucidated, they are probably related to the general mechanisms of US-induced bioeffects and elicitor-induced plant cell responses. The bioeffects of US on cells in liquid media are mostly attributed to the mechanical stress arising from US-induced fluid motion and hydrodynamic events, particularly acoustic cavitation and cavitation-induced microstreaming (Williams 1983; Nyborg and Ziskin 1985; Miller et al. 1996). As shown in our previous work (Lin et al. 2001), these events also occur in the exposure system used in our experiments.

On the other hand, plants and plant cells have acquired a defense system which allows them to stage an array of defense responses when challenged by pathogens as well as physical and chemical stresses. Although the elicitors or the triggers of the defense responses are generally thought to be compounds that are released during pathogen infection of plant tissues (DiCosmo and Misawa 1985; Nishi 1994), it has been recently proposed that mechanical signals may be more universal triggers of plant defense responses since alteration of the cell membrane occurs in plant cells infected by most pathogens (Yahraus et al. 1995). Previous studies have shown that physical and mechanical stresses such as hydrostatic pressure (Dörnenburg and Knoor 1997), mechanical agitation (Yahraus et al. 1995), and wounding (Watanabe and Sakai 1998) induce the oxidative burst and other elicitor responses of plant tissues and cells. Since mechanical stress is the most likely and principal cause of US-induced biological effects on suspended cells, mechanical signals may trigger the defense responses and secondary metabolite production of plant cells induced by US. Plant cells in suspension culture are constantly exposed to abiotic stress such as agitation and changes in the osmotic pressure of the medium which may induce a certain level of defense responses by the cells. With the introduction of US, a more intense abiotic stress to the culture, the defense response of cells was augmented.

The ion fluxes and oxidative burst induced by US is in some ways similar to the effects of other biotic and abiotic stresses with respect to the time course and dose dependence. However, one distinct characteristic of the US-induced events is perhaps their rapidity, which could be detected within seconds after a 30- s to 2-min exposure to US at suitable power levels. In comparison, the post-elicitation lag of the oxidative burst was 1–3 min in soybean cell cultures induced by osmotic shock (Yahraus et al. 1995), and 2–3 min by oligogalacturonides of plant origin (endogenous elicitors) (Legendre et al. 1993; Levine et al. 1994). Much longer lag periods have been reported for the oxidative burst induced by some biochemical elicitors of microbial origin, e.g., 4–8 min in soybean cell cultures treated by a glycoprotein fraction of fungus *Verticillium dahliae* (Apostol et al. 1989), and nearly 10 min by a glucan elicitor isolated from a fungal pathogen (Levine et al. 1994). The increase in membrane ion fluxes of plant cells induced by some biotic elicitors (Atkinson et al. 1990; Nürnberger et al. 1994) also appeared later than the US-induced changes observed in our work. The rapidity of the US-activated increase of ion fluxes and oxidative burst in cultured plant cells may be attributed in part to the rapid propagation of US waves through the culture medium and the simultaneous induction of hydrodynamic events such as cavitation and microstreaming in close vicinity of the cells. It may also be attributed to the sensitivity of plant cells to mechanical stress and perturbation, and to their ability to deploy immediate responses to mechanical stimuli.

Therefore, the present work has demonstrated the elicitor-like effects of low-energy US on plant cells, the induction of the early events of plant defense responses to biotic and abiotic elicitors as well as the stimulation of secondary metabolite production. The response was very sensitive and rapid, could be activated by a small dose of US energy, and was detectable within seconds after the exposure. These findings suggest that US is a potent elicitor of plant cell defense responses. Mechanical stress arising from US-induced fluid motion in the culture medium appeared to be the main inducer of the defense events, although further investigation is needed to identify the specific mechanisms of the US-induced effects in plant cell cultures. It is of fundamental interest to examine the effects of US on the expression of defense-related genes in the cells of different plant species. Finally, the present work indicates that low-energy US can effectively enhance secondary metabolite yield of plant cell cultures using a simple exposure system. Therefore, the potential of US-stimulated plant cell cultures for large-scale application deserves further exploration.

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