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SONICATION (ULTRASOUND) AFFECTS *IN VITRO* GROWTH OF HYBRID *CYMBIDIUM*

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Abstract

Teixeira da Silva J.A., Dobránszki J., 2014: Sonication (ultrasound) affects *in vitro* growth of hybrid *Cymbidium* [Sonikacija (ultragarsas) skatina hibridinių *Cymbidium* augimą *in vitro*]. – Bot. Lith., 20(2): 121–130.

The use of ultrasound or sonication has been shown to stimulate growth and development of several plant species. No study exists on the impact of sound on *Cymbidium* growth *in vitro*. Using sonication at 60 Hz for several time periods, the response on new protocorm-like body (*neo*-PLB) formation on Teixeira *Cymbidium* (TC) medium was examined, as was the response on *in vitro* plant organogenesis. Sonication for 5 or 10 min stimulated *neo*-PLB formation significantly more than plant growth regulator (PGR)-free TC medium without sonication (negative control) and more than 1, 20 or 45 min sonication, but significantly less than control TC medium containing PGRs NAA and Kin (positive control) after 60 days in culture. Sonication, when applied to PLBs, did not influence most plantlet-related growth parameters. Flow cytometric analyses registered an increase in endoreduplication in sonicated PLB tissues. Sonication at 60 Hz has a PLB-promoting effect, but is not as effective as PGRs.

Keywords: orchid, PLB, sonication, sound waves, Teixeira *Cymbidium* (TC) medium.

INTRODUCTION

Orchid *in vitro* regeneration protocols for several orchid genera are well established (Hossain et al., 2013). Plant growth regulators, or PGRs, are the most common way to induce protocorm-like bodies (PLBs) in orchids (Teixeira da Silva, 2013a, 2013b; Teixeira da Silva et al., 2013). PLBs, which are considered to be somatic embryos (Teixeira da Silva & Tanaka, 2006), spontaneously form shoots and adventitious roots, when maintained on the same medium, although the time periods for this response differ from genus to genus and even between cultivars within a single species. The *in vitro* developmental response of hybrid *Cymbidium* (Orchidaceae) has been well studied (Teixeira da Silva & Tanaka, 2006; Teixeira da Silva, 2013b; Teixeira da Silva & Dobránszki, 2013) and PLBs can be used in applications ranging from synthetic seeds or synseed (Teixeira da Silva, 2012a) to cryopreservation (Sharma et al., 2013).

Sonication is a physical treatment in which sound energy is applied to a sample and the higher the frequency, the stronger the effect on biological processes (Mason, 2007; Rokhina et al., 2009). Acoustic sound is a mechanical wave with frequency ranges between 20 Hz and 20 kHz, while ultrasound is a longitudinal pressure wave with a frequency above 20 kHz. During ultra-sonication, an ultrasound of frequencies between 15 and 100 kHz is also applied in biotechnology, low-frequency ultrasound is used for enhancing target biological processes (Rokhina et al., 2009). Ultrasound causes thermal and mechanical stresses to living organisms and low intensity ultrasound has several biological effects mainly due to cavitation and acoustic microstreaming, including modification

of cellular ultrastructures, the stability of enzymes, the growth properties of the cells, breaking extracellular polymers, releasing DNA from the nucleus, causing a decrease in the stability of cells, altering the permeability of cell membranes and thereby increasing the mass transport of cells and modifying charges on the cell surface (Rokhina et al., 2009). Ultrasound has been shown to stimulate development in several plant species depending on the frequency and exposure time applied. Cell growth rate of carrot (*Daucus carota* L.) suspension culture was enhanced by about 5% when exposed to ultrasound (28 kHz) for 2 sec, but the growth rate was reduced by 57% relative to the control, when ultrasound was applied for 40 sec (Wang et al., 1998). Applying ultrasonic stimulation to rice (*Oryza sativa* L.) 'Nipponbare' cell culture with the same frequency (28 kHz), cell growth was enhanced after exposure to 2 and 5 sec. However, by increasing the exposure time (30–120 sec), cell growth and proliferation was inhibited (Liu et al., 2003a). When a suspension culture of callus cells originated from the leaves of aloe (*Aloe arborescens* Mill.) was sonicated (28 kHz, for 2–60 sec), the greatest cell growth was detected after exposure to 5 sec (Liu et al., 2003b). In the same study, callus cells cultured on solid medium were treated with a frequency of 20 kHz for 2, 5 and 10 sec at 1–10W with continuous waves or with a cycle of 10% at 2 W (Liu et al., 2003b). The relative growth of callus (fresh weight basis after 15 days of culture) depended on ultrasound power, while at 1 and 2 W it increased with exposure period up to 80.7%, but above 5 W, sonication inhibited callus growth (relative growth decreased by 66.25%). H^+ transport in vacuolar membrane and ATP hydrolysis activity were enhanced. Chrysanthemum callus sonicated by sound wave with a frequency of 1.4 kHz and 0.095 kDb (kilodecibels) treated twice daily for two 0.5-h periods for 20 days accelerated callus growth (unquantified) and stimulated the differentiation of shoots (28% higher shoot forming index than the control) (Wang et al., 2004). The effects of power (28 kHz, 100–500 W, for 3 min) and exposure period (28 kHz, 300 W for 1–8 min) of ultrasound on PLB to shoot conversion were examined in *Dendrobium officinale* (Wei et al., 2012). Ultra-sonication improved the conversion rate with an optimum of 300 W for 5 min (80.6 shoots/g PLB compared to 6.5 shoots/g PLB of control 40 days after sonication). In both studies (Wang et al., 2004; Wei et al., 2012), the authors found that sonication changed the auxin (free IAA) level and thus the shoot number. Ultrasound, which is an abiotic stress, stimulated the production of reactive oxygen species (ROS) and thereby increased the antioxidative defense systems of *Porphyridium cruentum* by increasing the activities of superoxide dismutase (SOD) by 53.5%, catalase (CAT) by 24.3% and membrane-bound ATPases by 67.7–69.3%, when ultrasound with an amplitude of 21% and 5 sec pulse was applied for 60 sec at the early log phase of growth (Chen et al., 2008). However, no comparison was made with later phases of cell growth. Besides antioxidant enzymes, the carotenoid (quencher of singlet oxygen) and glutathione (antioxidant) contents of cells increased by 27% and 56%, respectively, compared to non-sonicated cells, suggesting the activation of both enzymatic and non-enzymatic defense systems to protect the integrity of membranes. Change in the membrane integrity was proved through a 48% increase in malondialdehyde (MDA) content and a 48.6% increase in electrolyte leakage. Similarly, ultrasound treatment increased SOD and CAT activity by about 57% and 36%, respectively, during the conversion of PLBs to shoots in *Dendrobium* (Wei et al., 2012).

Closely related to ultrasound is its application in *Agrobacterium*-mediated transformation, a new method for the transformation of plants introduced by Trick & Finer (1997) for soybean, cowpea, wheat, maize, white spruce and buckeye, and was termed sonication-assisted *Agrobacterium*-mediated transformation (SAAT). SAAT enabled an increase in the transformation efficiency and plant transformation via *Agrobacterium* also in plant species, which were previously recalcitrant. Sonication was an effective way to improve the genetic transformation of chrysanthemum (*Dendranthema* × *grandiflora* Ramat. Kitamura cv. 'Lineker' and 'Shuhou-no-Chikara') (Teixeira da Silva & Fukai, 2003), tobacco (*Nicotiana tabacum* L.) (Teixeira da Silva, 2005) and *Dendrobium* (Zheng et al., 2011) with 60 Hz for 20 min in the former two studies and with 40 kHz in the latter study. However, improved genetic transformation required a trade-off between cellular damage and transformation efficiency since excessive damage not only destroyed cells, but also allowed the

transformation agent, *Agrobacterium*, to grow excessively, even in the presence of high (normally inhibitive) concentrations of antibiotics.

A recent review expands on the topic of sonication in plant growth and genetic transformation using SAAT (Teixeira da Silva & Dobránszki, 2014).

Based on the premise that sound can stimulate plant cell growth (*sensu lato*), this experiment tested the impact of sonication on hybrid *Cymbidium* organogenesis. Since this is an unexplored topic in orchid research, basic research on this topic is required.

MATERIALS AND METHODS

All protocols (experimental design, chemicals, reagents, explant preparation and treatment analysis) follow Teixeira da Silva (2012b), almost *verbatim*, in parts.

Chemicals and reagents

All chemicals and reagents were of the highest analytical grade available and were purchased from either Sigma-Aldrich (St. Louis, USA), Wako Chemical Co. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), the cheapest choice at the highest tissue-culture grade, unless specified otherwise.

Plant material and culture conditions

PLBs of hybrid *Cymbidium* Twilight Moon 'Day Light' (Bio-U, Tokushima, Japan) originally developed from shoot-tip culture on Vacin & Went (VW) agar medium (Vacin & Went, 1949) without PGRs, were induced and subcultured (PLB induction and proliferation medium) every two months on TC medium (Teixeira da Silva, 2012b) supplemented with 0.1 $mg \cdot l^{-1}$ α-naphthaleneacetic acid (NAA) and 0.1 0.1 $mg \cdot l^{-1}$ kinetin (Kin), 2 $g \cdot l^{-1}$ tryptone and 20 $g \cdot l^{-1}$ sucrose, and solidified with 8 $g \cdot l^{-1}$ Bacto agar (Difco Labs., USA) after Teixeira da Silva et al., (2005) and Teixeira da Silva & Tanaka, (2006). Even though several basal media can support the induction and development of *Cymbidium* PLBs *in vitro* (Teixeira da Silva et al., 2005), Teixeira *Cymbidium* (TC) No. 1 medium (Teixeira da Silva, 2012b), optimized for this cultivar, was used in this study. All media were adjusted to pH 5.3 with 1 N NaOH or HCl prior to autoclaving at 100 kPa for 17 min. Cultures were kept on 40 ml medium in 100-ml Erlenmeyer flasks, double-capped with aluminium foil, at 25°C, under a 16-h photoperiod with a light intensity of 45 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by 40-W plant growth fluorescent lamps (Homo Lux, Matsushita Electric Industrial Co., Japan). Longitudinally dissected segments, i.e. PLBs cut into two equally sized pieces of 3–4 mm in diameter, 10/flask, were used as explants for PLB induction and proliferation. Culture conditions and media followed the recommendations previously established for medium formulation (Teixeira da Silva et al., 2005), biotic (Teixeira da Silva et al., 2006b) and abiotic factors (Teixeira da Silva et al., 2006a) for PLB induction, formation and proliferation.

Response of Cymbidium to sonication

The effect of six exposure periods of sonication (0 (control), 1, 5, 10, 20 and 45 min at 60 Hz) using an Iuchi® (Tokyo, Japan) bath sonicator was tested. Half-PLBs were placed in 2-ml Eppendorf tubes (AsOne, Osaka, Japan) containing 1.5 ml of sterilized (autoclaved) liquid TC medium (without PGRs) using the experimental design of Teixeira da Silva (2012b). Each Eppendorf tube contained 5 half-PLBs, which were totally submerged in the liquid TC medium. The logic behind removing PGRs was to assess whether sound treatment could effectively induce PLBs and thus test its growth-inducing ability. Eppendorf tubes were randomly placed in polystyrene floats with lids above the water surface and the body of the Eppendorf tubes clearly in a 25°C water bath. After each sonication period, Eppendorf tubes were inverted onto two layers of Whatman No. 1 filter paper on the clean bench, excess liquid TC medium was dabbed off and half-PLBs were plated onto PGR-free solidified (7 $g \cdot l^{-1}$ agar) TC medium to induce *neo*-PLBs. The number of *neo*-PLBs that formed was assessed and quantified at 60 days post-plating, 60 days being the optimal time for sampling (Teixeira da Silva & Dobránszki, 2013). *Neo*-PLB clusters were transferred to fresh solid TC medium and allowed to spontaneously form shoots for the next stage of the experiment. Explants were photographed using stereo light microscopy and/or a digital camera.

Using the protocol of Teixeira da Silva et al. (2007) for the acclimatization of *Cymbidium* to *ex vitro* conditions, shoots 4 cm long containing three

unsheathed leaves derived from all sonication treatments were placed in PGR-free Hyponex medium solidified with 7 $g \cdot l^{-1}$ agar. Plantlet growth was quantified after 90 days by the number of new leaves and roots, plant height (base in contact with medium to shoot tip), fresh and dry weight of shoots and roots. Chlorophyll content in the third leaf (counting downward from the apex) of the plantlets was measured as the SPAD value by a chlorophyll meter (SPAD-502, Minolta, Japan).

Flow cytometry

The protocol was used, almost *verbatim*, after Teixeira da Silva & Tanaka (2006). Nuclei were isolated from 0.5 cm^2 of sonicated or unsonicated PLBs as well as leaves of plantlets derived from sonicated or unsonicated PLBs by chopping in a few drops of nucleic acid extraction buffer (Partec Cystain UV Precise P, Germany), digesting on ice for 5 min. The nuclear suspension was then filtered through a 30 µm mesh size nylon filter (CellTrics®) and five times of Partec Buffer A (2 µg/ml 4,6-diamidino-2-phenylindole (DAPI), 2 mM $MgCl_2$, 10 mM Tris, 50 mM sodium citrate, 1% PVP K-30, 0.1% Triton X-100, pH 7.5; Mishiba & Mii, 2000) was added at room temperature for 5 min. Thereafter, nuclear fluorescence was measured using a Partec® Ploidy Analyser. Diploid barley (*Hordeum vulgare* L.) cv. 'Ryufu' served as the internal control. Three independent samples were used for measurements, and relative fluorescence intensity of the nuclei was analysed when the coefficient of variation between samples was < 3%. A minimum of 5000 nuclei were counted for each sample.

Statistical analyses

Experiments were organized according to a randomized complete block design (RCBD) with three blocks of 10 replicates per treatment (each sonication period). All experiments were repeated in triplicate (n = 30, total sample size per treatment). The data were subjected to the analysis of variance (ANOVA) with mean separation by Duncan's multiple range test (DMRT) using SAS® version 6.12 (SAS Institute, Cary, NC, USA). All percentage data were arc-sine transformed prior to analysis. Significant differences between means were assumed at $p \leq 0.05$.

RESULTS AND DISCUSSION

Sonication for 5 or 10 min stimulated PLB formation significantly more than PGR-free TC medium without sonication (negative control) and more than 1, 20 or 45 min sonication, but significantly less than control TC medium containing PGRs NAA and Kin (positive control) after 60 days in culture (Table 1). Consequently, the number of *neo*-PLBs was significantly higher from half-PLBs sonicated for 5/10 min compared to the negative control (Fig. 1; Table 1). The number of new leaves on plantlets derived from sound-derived *neo*-PLBs was the same as the control treatment after 90 days in culture (Table 2) independently of the exposure period of the sound wave treatment. However, some negative impact of sound wave on plant height was evident, particularly 10/20 min (Table 2). Shoot and root FW and DW of plantlets was significantly lower than the same parameters of positive control plantlets, but significantly higher than the same parameters of negative control plantlets (Tables 2, 3). The SPAD value of plantlets derived from any sonication treatment or from controls were not significantly different (Table 2). The root : shoot ratio on a FW and DW basis indicated that sonication for 5 and 10 min resulted in plantlets with practically the same root : shoot ratio as in the positive control. Relative to plantlets developed from unsonicated PLBs and grown on TC medium containing PGRs, the root : shoot ratio of plantlets developed from unsonicated PLBs and grown on PGR-free TC medium (negative control) was 42% and 22% lower (FW and DW, respectively) (Table 3). Considering all the measured parameters, the negative control (TC medium without sonication and without PGRs) tended to perform significantly worse than all sonication treatments and the positive control (TC medium without sonication, but with PGRs NAA + Kin) (Tables 1–3). This suggests that sonication has a growth-promoting effect for *neo*-PLB formation after 60 days of culture and subsequently, after 90 days of culture, enhanced the conversion of PLBs to plantlet, but was not as effective as PGRs. This is confirmed by the fact that sonication in the presence of PGRs did not further enhance the growth and development of *neo*-PLBs (data not shown). Sonication increased the level of endoreduplication in PLB cells, but plantlets derived from sonicated PLBs showed normal levels of nucle-

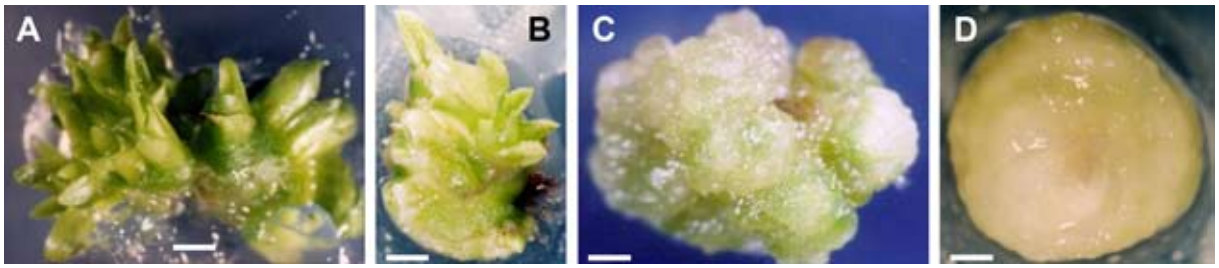

Fig. 1. Growth and development of hybrid *Cymbidium* Twilight Moon 'Day Light' *neo*-PLBs on solid basal TC (Teixeira da Silva, 2012b) medium in response to sonication 60 days after treatment application. (A) Multiple shoots developed from *neo*-PLBs following 5 min sonication. (B) Spurious shoot development from a few *neo*-PLBs that formed after 10 min sonication. (C) Clear inhibition of shoot formation (as a result of no *neo*-PLB formation) following 20 min sonication, inhibition being even more accentuated after 45 min sonication (D). In all cases, sonication was at 60 Hz. Bars = 1.0 mm (A, B), 0.1 mm (C), 0.2 mm (D)

Table 1. The growth and developmental response of hybrid *Cymbidium* Twilight Moon 'Day Light' half-PLBs to sonication after 60 days (after Teixeira da Silva & Dobránszki, 2013) in culture

Treatment	Percentage of half-PLBs forming *neo*-PLBs (%)*	Number of *neo*-PLBs formed per half-PLB	Fresh weight (mg) of half-PLB + *neo*-PLBs[3]
TC + PGRs + no SON[1]	100 a	8.3 a	526 a
TC – PGRs + no SON[2]	47 c	1.8 d	129 c
TC – PGRs + 1 min SON	44 c	2.3 d	144 c
TC – PGRs + 5 min SON	61 b	6.1 b	361 b
TC – PGRs + 10 min SON	67 b	4.8 c	328 b
TC – PGRs + 20 min SON	19 d	2.2 d	177 c
TC – PGRs + 45 min SON	8 e	0.6 e	93 d

SON – sonication
* All percentage data were arc-sine transformed prior to analysis
Mean values followed by the same letter in the same column are not significantly different based on DMRT ($p = 0.05$). $n = 90$ ($10 \times 3 \times 3$). PGR – plant growth regulator; PLB – protocorm-like body; TC – Teixeira *Cymbidium* medium No. 1 (Teixeira da Silva, 2012b)
[1] control with no sonication, but including TC PGRs: 0.1 0.1 mg·l^{-1} NAA + 0.1 0.1 mg·l^{-1} Kin
[2] control with no sonication and excluding PGRs
[3] In fact, the average fresh weight of initial half-PLB explants is 54 mg ($n = 10$)

Table 2. Shoot (derived from *neo*-PLBs) growth and development of hybrid *Cymbidium* Twilight Moon 'Day Light' in response to sonication 90 days in culture after transfer to PGR-free Hyponex medium solidified with 7 g·l^{-1} agar

Treatment	Number of new leaves[3]	Plant height (mm)[4]	Shoot fresh weight (mg)[5]	Shoot dry weight (mg)[6]	SPAD value[7]
TC + PGRs + no SON[1]	3.6 a	11.4 a	968 a	128 a	44.3 a
TC – PGRs + no SON[2]	1.9 b	7.1 c	635 d	63 c	39.8 a
TC – PGRs + 1 min SON	3.1 a	10.8 a	884 b	94 b	41.3 a
TC – PGRs + 5 min SON	3.1 a	9.9 ab	796 c	91 b	43.5 a
TC – PGRs + 10 min SON	3.2 a	9.6 b	781 c	88 b	41.1 a
TC – PGRs + 20 min SON	3.1 a	9.5 b	769 c	86 b	40.3 a
TC – PGRs + 45 min SON	3.4 a	9.8 ab	801 c	88 b	41.4 a

SON – sonication
Mean values followed by the same letter in the same column are not significantly different based on DMRT ($p = 0.05$)
$n = 90$ ($10 \times 3 \times 3$). PGR – plant growth regulator; PLB – protocorm-like body; TC – Teixeira *Cymbidium* medium No. 1 (Teixeira da Silva, 2012b)
[1] control with no sonication, but including TC PGRs: 0.1 0.1 mg·l^{-1} NAA + 0.1 0.1 mg·l^{-1} Kin
[2] control with no sonication and excluding PGRs
[3] at the beginning of each treatment, rootless shoots contained three full-grown leaves
[4] measured from the level of medium to the tallest leaf tip
[5] shoots were dabbed on dry tissue paper to remove *in vitro* flask moisture before weighing
[6] shoots were cut at the base from roots, wrapped in two layers of newspaper, labelled and dried in a hot-air convection oven at 65°C for one week
[7] SPAD value is a measurement of the chlorophyll content

Table 3. Root (derived from *neo*-PLBs) growth and development of hybrid *Cymbidium* Twilight Moon 'Day Light' in response to sonication 90 days in culture after transfer to PGR-free Hyponex medium solidified with 7 g·l^{-1} agar

Treatment	Number of roots[3]	Root fresh weight (mg)[4]	Root dry weight (mg)[5]	Root : shoot ratio FW/DW basis[6]
TC + PGRs + no SON[1]	3.6 a	361 a	74 a	0.373/0.578
TC – PGRs + no SON[2]	1.3 c	118 c	29 d	0.186/0.460
TC – PGRs + 1 min SON	2.8 b	273 b	62 b	0.301/0.660
TC – PGRs + 5 min SON	2.6 b	260 b	54 b	0.327/0.593
TC – PGRs + 10 min SON	2.4 b	247 b	51 b	0.316/0.580
TC – PGRs + 20 min SON	2.4 b	236 b	44 c	0.301/0.512
TC – PGRs + 45 min SON	2.7 b	254 b	59 b	0.317/0.670

SON = sonication
Mean values followed by the same letter in the same column are not significantly different based on DMRT ($p = 0.05$)
$n = 90$ (10 × 3 × 3). PGR – plant growth regulator; PLB – protocorm-like body; TC – Teixeira *Cymbidium* medium No. 1 (Teixeira da Silva, 2012b)
[1] control with no sonication, but including TC PGRs: 0.1 mg·l^{-1} NAA + 0.1 0.1 mg·l^{-1} Kin
[2] control with no sonication and excluding PGRs
[3] unlike shoots, no roots existed on shoots when first plated, thus number of roots = new roots
[4] roots were dabbed on dry tissue paper to remove *in vitro* flask moisture before weighing
[5] roots were cut at the base from shoots, wrapped in two layers of newspaper, labelled and dried in a hot-air convection oven at 65°C for one week
[6] FW – fresh weight; DW – dry weight

ar DNA (Table 4), suggesting that damage induced by sonication is only temporary, and is not permanently transmitted to older plant tissues as the plant matures. Damage caused by explant preparation, excessive wounding or excessively small explant size is known to reduce *neo*-PLB induction, and even to induce endopolyploidy, but in all cases, plants that are derived from such *neo*-PLBs always revert back to the original ploidy following regeneration (Teixeira da Silva & Tanaka, 2006).

Table 4. Flow cytometric analyses of hybrid *Cymbidium* Twilight Moon 'Day Light' PLBs (60 days old) exposed to sonication (60 Hz, 20 min) or not (control) as well as leaf tissue of plantlets (90 days old) derived from those treatments

Plant material analysed	2C : 4C : 8C : 16C*
Unsonicated PLBs	84 : 12 : 4 : 0
Sonicated PLBs (20 min)	83 : 9 : 6 : 2
Leaves of plantlets from unsonicated PLBs	92 : 8 : 0 : 0
Leaves of plantlets from sonicated PLBs (20 min)	91 : 9 : 0 : 0

*Three samples were measured, and relative fluorescence intensity of the nuclei was analysed, when the coefficient of variation was < 3%. A minimum of 5000 nuclei were counted for each sample

The growth-promotive effects in this study correspond to the growth- and development-promoting effects of sonication observed in other plant species when sound wave (1.4 kHz, 0.095 kdb, 0.5-h-long treatment twice daily for 20 days) was applied to chrysanthemum callus (Wang et al., 2004), or short exposure (2–5 sec) of low frequency ultrasound was applied to a suspension culture of carrot (Wang et al., 1998), rice (Liu et al., 2003a) or aloe (Liu et al., 2003b) at frequency of 28 kHz and for an aloe callus culture at a frequency of 28 kHz (Liu et al., 2003b). Similarly, exposure of *Dendrobium officinale* PLBs (Wei et al., 2012) to a low frequency (28 kHz, 300 W) of ultrasound for a short period (5 min) improved the conversion of PLBs to shoots.

Sound waves, and therefore sonication, are a form of abiotic stress for plants (Wang et al., 2006). Sonication with sound wave or ultrasound of low frequency (< 100 kHz) may affect the growth and development of plants, but the mechanism of how sound wave influences plant growth is not clear yet.

Wang et al. (2003) exposed *Chrysanthemum* stem segments to sound wave irradiation of 1000 Hz and 100 dB intensity for 3–15 days, daily for 60 min. They found no difference in the DNA content of stimulated stem segments compared to the non-stimulated stems. However, a significant increase in the RNA and protein content of stimulated stems was observed and the highest RNA/protein content was detected when the stimulation was for 9 days, indicating altered gene expression under sound wave stimulation. Shao et al. (2008) found that gene expression changed after sound stress by sonicating

chrysanthemum stem segments with a sound wave of 1000 Hz and 100 dB for 9 days, for 60 min daily. The total RNA content increased in stem segments exposed to sound stress and three differential bands were identified (SA3, SG7-1 and CA2). In stem segments treated by sound wave SA3 was expressed differentially, SG7-1 preferably, but the expression of CA2 was limited.

There are few reports on the change of endogenous hormonal balance of treated plant tissue or cells in response to (ultra)sound, and thereby promoting growth and development. In chrysanthemum, sonication with 1.4 kHz sound wave promoted callus growth and shoot differentiation from callus, and a high rate of endogenous indole-3-acetic acid (IAA)/abscisic acid (ABA) was detected (125% higher than in the control) due to a 20.2% higher level of free IAA and 49.2% lower level of ABA compared to control callus (Wang et al., 2004). Ultrasound (28 kHz, 300 W for 5 min) of *Dendrobium officinale* PLBs (Wei et al., 2012), which enhanced the PLB-to-shoot conversion, increased the activity of IAA oxidase and decreased the activity of cytokinin (CK) oxidase resulting in about a 40% higher CK : IAA ratio due to a lower IAA content and higher total CK content in the treated cells compared to the control cells. This increased ratio of total CK to IAA might be responsible for the enhanced conversion of PLBs to shoots. During conversion of PLBs to shoots in *Dendrobium houshanense*, Wang et al. (2009) proved that the conversion rate was enhanced by an increased ratio of total CK to IAA, which was due to a decrease in the endogenous level of free IAA. Although the studies on changes in endogenous hormone levels after sonication are very limited and available only in the case of few plant species, it seems that sonication applied at a low frequency increases endogenous IAA level, while the increase of frequency into the ultrasound range (above 28 kHz) decreases the level of free IAA.

Sonication with low frequency ultrasound may alter the permeability of membranes inside and outside the cell (Rokhina et al., 2009), may change the activity and the conformation of membrane-bound enzymes (Wang et al., 2002; Liu et al., 2003b) and thereby may enhance the transport processes of cells.

Another typical effect of ultrasound, which may affect plant metabolism, is acoustic cavitation, i.e. bubble formation and collapse of cavities in liquids irradiated by ultrasound, by which high amount of energy may be released locally (Rokhina et al., 2009) and, therefore, chemical and physical changes may be induced in plant cells. One of the results of acoustic cavitation is that local turbulences and liquid micro-circulations may be formed (microstreaming) both in a cell suspension and within a cell (intracellular microstreaming), causing enhanced mass transfer and metabolic activity (Nyborg, 1982; Liu et al., 2003b; Rokhina et al., 2009). If the ultrasound stress is long-lasting or has a high intensity, plant growth is inhibited due to destruction of the metabolic activity caused by high intracellular microstreaming (Liu et al., 2003b). Free radicals formed as a result of acoustic cavitation can accelerate some chemical reactions by influencing reaction kinetics (Rokhina et al., 2009). If ROS production was stimulated, both the activity of antioxidant enzymes (SOD, CAT) and the level of protecting/preventive substances (carotenoids, gluthatione) increased, thus protecting cell membranes against sound stress (Chen et al., 2008; Wei et al., 2012).

The effect of sonication depends always on the frequency, intensity and duration of sound irradiation (Rokhina et al., 2009). On the other hand, the sensibility of irradiated plant material might affect its effect. There are different levels on which sound wave may affect the growing and developmental functions of plants. It can modify the plant growth and development by modifying the permeability of cell membranes and the conformation of membrane bound enzymes, by altering the activity and/or conformity of different enzymes involved in the hormonal regulation and antioxidant defense mechanism or by alterations in the transcription (Fig. 2).

In the present experiment, *Cymbidium* was sonicated by a sound wave with a low frequency (60 Hz), which promoted the formation of new PLBs, hypothetically through a process involving the CK-IAA ratio, hence promoting the development of new PLBs, as was achieved in the positive control when *neo*-PLB formation occurred by the exogenous application of PGRs (0.1 0.1 $mg \cdot l^{-1}$ NAA and 0.1 0.1 $mg \cdot l^{-1}$ Kin). Further studies would be needed in the future to learn whether this effect of low frequency (60 Hz) sound wave might affect the gene transcription of stress-inducible genes, whether it may alter the activity of enzymes involved in the endogenous hormonal

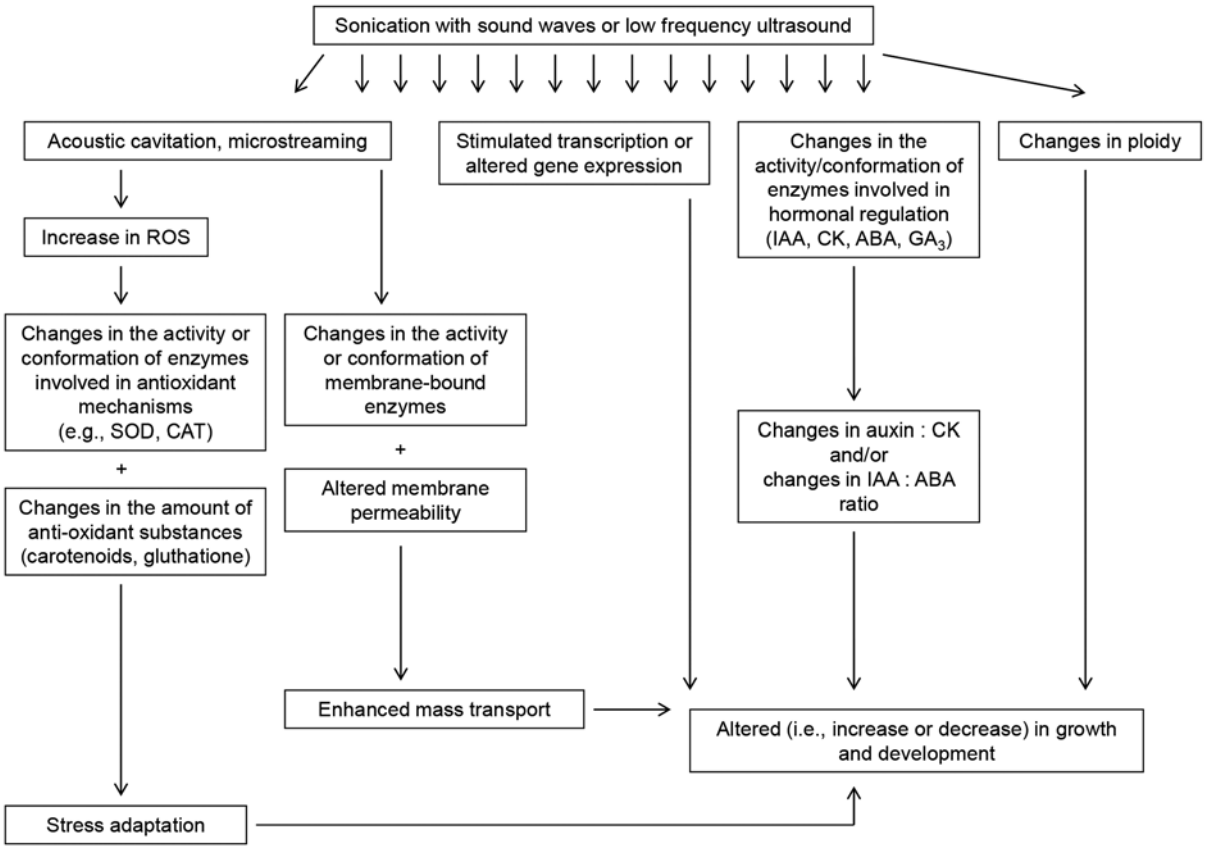

Fig. 2. Broad mechanisms by which sonication can affect a plant cell or organ. Mechanisms are based on different plants exposed to different levels of sonication and frequencies of acoustic sound and ultrasound. ABA = abscisic acid; CAT = catalase; CK = cytokinin; GA_3 = gibberellic acid; IAA = indole-3-acetic acid; ROS = reactive oxygen species; SOD = superoxide dismutase

balance or whether it may perhaps cause any change in the permeability of cell membranes hereby affecting transport-related processes of plant cells.

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SONIKACIJA (ULTRAGARSAS) SKATINA HIBRIDINIŲ *CYMBIDIUM* AUGIMĄ *IN VITRO*

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Santrauka

Straipsnyje parodyta, kad ultragarsas arba sonikacija skatina keleto augalų rūšių augimą ir vystymąsi. Nėra atlikta tyrimų analizuojant garso poveikį *Cymbidium* augimui *in vitro*. Naudojant 60 Hz sonikaciją per kelis laiko tarpus, buvo nustatytas naujų protokormo kūnelių (*neo*-PLB) formavimasis Teixeira *Cymbidium* (TC) terpėje, kaip ir *in vitro* augalų organogenezės atveju. Sonikacija, trunkanti 5 ar 10 min skatino *neo*-PLB susidarymą žymiai stipriau, nei TC terpė be augalų augimo reguliatorių (AAR) ir nepaveikus sonikacija (neigiama kontrolė), ar veikiant ultragarsu 1, 20 ar 45 min, bet žymiai silpniau, nei kontrolinė TC terpė, turinti AARs NAR ir Kin (teigiama kontrolė) po 60 dienų auginimo kultūroje. Ultragarsas, kai buvo taikomas PLBs, neturėjo įtakos daugeliui augalų plantlet-related augimo parametrų. Tėkmės citometrijos analizė užfiksavo endoreduplikacijos padidėjimą ultragarsu paveiktuose PLB audiniuose. Sonikacija esant 60 Hz turi PLB vystymąsi skatinantį poveikį, bet nėra tokia pat veiksminga kaip augalų augimo reguliatoriai (AAR).