J Korean Soc Appl Biol Chem (2013) 56, 377–382
DOI 10.1007/s13765-013-3088-7

ORIGINAL ARTICLE

Inhibition of *Botrytis cinerea* Spore Germination and Mycelia Growth by Frequency-specific Sound

Mi-Jeong Jeong · DongWon Bae · Hanhong Bae · Soo In Lee · Jin A Kim · Sung Chul Shin · Sung Han Park · Soo-Chul Park

Received: 16 April 2013 / Accepted: 20 May 2013 / Published Online: 31 August 2013

Abstract The effect of sound waves on mycelial growth of *Botrytis cinerea* was investigated to explore whether frequency-specific sound could be used as a practical alternative to chemical fungicides to control plant diseases. The fungus was exposed to wave frequencies ranging from 1 to 5 kHz, and then observed using light and scanning electron microscopy to assess changes in several physiological and morphological aspects. Of the frequencies tested, 5 kHz sound wave significantly inhibited mycelial growth and spore germination. Furthermore, morphological changes, including low mycelial density, swollen mycelial tips, and irregular mycelial surfaces, were observed. Most internal hyphae were empty, and the ends of hyphae were significantly thinner or swollen. These observations suggest that 5 kHz sound waves create stressful growth conditions for the fungus, which leads to the inhibition of mycelia growth and spore germination. It is possible that sound wave treatment could represent an environmentally-friendly alternative to chemical fungicides. These results broaden our knowledge regarding the effective management of noxious nectrotrophic fungal pathogens by a non-chemical approach.

Keywords biocontrol · *botrytis cinerea* · frequency-specific sound · inhibition · mycelia growth · spore germination

M. J. Jeong (✉) · S. I. Lee · J. A Kim · S. H. Park · S.C. Park (✉)
National Institute of Agricultural Biotechnology, Rural Development Administration (RDA), Suwon 441-707, Republic of Korea
E-mail: center1097@korea.kr

D.W. Bae · S. C. Shin
Gyeongsang National University, Jinju 660- 701, Republic of Korea

H. Bae
Yeungnam University, Gyeongsan 712-749, Republic of Korea

Introduction

When the mechanical energy of a sound wave passes through gas, liquid or solid, its energy is absorbed. Several reports suggest that sound waves of specific frequency and strength can significantly accelerate plant growth (Weinberger and Burton 1981; Takahashi et al., 1992; Creath and Schwartz, 2004). Jomdecha and Prateepasen (2011) showed that low-intensity ultrasonic sound enhances the growth of *Saccharomyces cerevisiae.* Takahashi et al. (1992) reported that treatment of germinating rice or cucumber seeds with a 50 Hz vibration for 72 h increase the hypocotyl elongation rate. Similar results have been reported for Arabidopsis (Johnson et al., 1998). Through biochemical analyses, Bochu et al. (2004) demonstrated that increased levels of indole acetic acid (IAA) and decreased levels of abscisic acid (ABA) correlated with sound wave stimuli. Following treatment of chrysanthemum with a specific sound wave frequency (1.4 kHz, 0.095 kdB, 10 days) levels of IAA and ABA were significantly higher and lower than untreated one, respectively. The researchers determined that greater relative levels of IAA/ABA favor development of the callus and differentiation of the mature callus (Bochu et al., 2004; Yiyao et al., 2002). They concluded that sound waves contribute to the regulation of endogenous hormone levels and the control of callus growth. Yiyao et al. (2002) reported that a certain range of sound waves enhanced the growth of chrysanthemum callus; however, treatment with sound waves of greater energy resulted in the opposite effect. This observation suggests that the effect of sound on plant growth depends on the intensity and frequency of the sound wave. The expression levels of several genes change in response to external stimuli. For example, touch (TCH) genes are induced by mechanostimulation (Braam et al., 1977; Braam, 1992; Sistrunk et al., 1994; Braam, 2005; Chehab et al., 2009). In Arabidopsis, expression of TCH genes can be up-regulated 10-to100-fold by mechanical stimuli, such as touch and wounding, followed by exposure to darkness, temperature extremes, and certain growth-promoting hormones. The existence of distinct gene sets that respond to different stimuli suggests that specific receptors and signal transduction pathways are utilized in response to alterations in environmental conditions. In previous research, we found that the transgenic rice plants harboring a *Aldolase* promoter:*GUS* reporter gene showed significant increase in *GUS* expression in mRNA levels at 125, 250 Hz or 1 kHz. In contrast, they showed significant decrease of *GUS* mRNA expression at 50 Hz, suggesting that the *Ald* promoter responds to sound with frequency-specific manner (Jeong et al., 2008). Hongbo et al. (2008) reported that when chrysanthemum stems were grown on MS medium with sound treatment, the content of DNA did not change, but the levels of RNA and soluble proteins increased to varying degrees. This result suggests that sound can stimulate the expression of several different genes. Sound wave exposure can change the deformability of the cell membrane, and the deformability was closely related to the frequency of the sound wave. In other words, as the frequency increased, the deformability of the cell membrane decreased (Bochu et al., 2001). Mechanotransduction has been reported in animal cells and tissues, and mechanical stresses stimulate various signal transduction mechanisms (Bhagyalakshmi et al., 1992; Mizoguchi et al., 1996; Johnson et al., 1998). Previous research to examine the effects of music and/or noise on plants has been controversial (Weinberger and Das, 1972; Weinberger and Measures, 1978; Galston and Slayman, 1979) and has been proved difficult to replicate, because the precise experimental conditions were not specified. Thus, despite the ecophysiological importance of sound wave acclimation, little is known about the molecular-physiological mechanisms underlying such a response.

Botrytis cinerea is one of the most important plant pathogens, which is able to infect at least 235 plant species, resulting in serious economic losses in vegetable, fruit, and ornamental flower productions (Jarvis et al., 1977; Prins et al., 2000). *B. cinerea* growth is difficult to control due to its complex etiology and various routes of aerial infection. Despite research efforts to develop effective growth-control strategies, regular application of fungicides is still one of the main methods used to control *B. cinerea* growth. However, there is an intense interest worldwide in reducing the use of chemical fungicides, because they contribute to environmental pollution and ecosystem disturbance. These concerns have promoted the exploration of alternatives to synthetic fungicides to control plant disease, including gray mold rot, to develop an environmentally-friendly approach for the control of plant pathogens.

The objective of the current study was to investigate the effect of sound on the growth of *B. cinerea* and to explore an alternative method for the control of plant pathogens. The effects of frequency-specific sound waves on the spore germination and mycelium growth of *B. cinerea* were evaluated. Sound waves significantly affected fungal mycelia growth and spore germination rate in a wave strength-dependent manner. The use of sound wave energy to control problematic nectrotrophic fungi represents a novel strategy for plant pathogen control in closed systems such as greenhouses.

Fig. 1 Noiseless chamber.

Materials and Methods

Organisms and sound treatment. *B. cinerea* (Korean Agricultural Culture Collection, NO. 40574) was cultured on Potato Dextrose Agar (PDA, Difco Laboratories, USA) in Petri plates and incubated at 25°C. The single-frequency signal was generated using Adobe Audition 3.0 software (Adobe System Company, USA). The speaker volume was 100 dB. To prevent effects from extraneous noise, experiments were conducted inside a custom-made, noiseless chamber (Korea Scientific Technique Industry Co., Korea) (Fig. 1). The sound level in the soundless chamber was approximately 40 dB, whereas that in a commercial growth chamber is generally about 80 dB.

Effect of sound treatment on mycelia growth of *B. cinerea*. To determine whether sound waves effectively inhibited mycelial growth, *B. cinerea* was treated with various sound waves. *B. cinerea* inoculum discs (5 mm in diameter) were cut using a cork-borer from the periphery of an actively growing 3-day-old colony growing on a Potato Dextrose Agar (PDA) plate, and placed individually at the center of a Petri plate. To minimize culture variability, inoculum discs were removed from the periphery of the colonies at a point equidistant from the center. Mycelia were subjected to treatment with a single-sound frequency. To determine the treatment time that effectively inhibited mycelial growth inhibition, agar disks (5 mm in diameter) of *B. cinerea* were placed in the center of Petri plates and treated with 5 kHz for various times. After 6 days, the diameter of the mycelia was measured to assess mycelial growth.

Effect of sound treatment on germination of *B. cinerea* spores. Spore germination assays were carried out on 1/2 PDA plates. Spores were harvested from 10–14-day-old cultures by agitating small pieces of agar containing mycelia and conidia in a glass tube containing 2 mL of sterile water and 0.01% (wt/vol) Tween 80. The suspension was filtered through cheesecloth, and the spore concentration was calibrated with a hemocytometer and adjusted to 1×10^5 spores per ml. The 1/2 PDA plates were inoculated with the spore suspension and treated with 5 kHz sound waves for 1 day. After sound treatment, plates were incubated at 25°C for 7 h in a growth chamber.

Light microscopy. To examine the morphological changes in *B. cinerea* after sound treatment, *B. cinerea* mycelia were transferred to 1/2 PDA plates and treated with 5 kHz sound waves for 2 days. Mycelia were transferred from the margins of actively growing colonies and examined under 400× magnification using a light microscope (Olympus, Japan).

Scanning Electron Microscopy (SEM). A 5-mm diameter disc from a 3-day-old *B. cinerea* culture grown on PDA was inoculated on a 1/2 PDA plate and treated with 5 kHz sound waves for 2 days. Segments of new growth measuring 5–10 mm were cut at the periphery of an actively growing colony, fixed with modified Karnovsky's fixative at 4°C overnight, and post-fixed with 1% Osmic acid. Subsequently, specimens were dehydrated using increasing ethanol concentrations of 50, 70, 90, 95 and 100%, each for 30 min, and then air dried. Specimens were gold-coated by evaporating ionized gold onto the surface with a sputter coater. Specimens were examined using a scanning electron microscope S-2460N (Hitachi, Japan) at an accelerating voltage of 20 kV.

Statistical analysis. Analysis of variance for experimental datasets was performed using JMP software version 5.0 (SAS Institute Inc., USA). Significant effects of treatment were determined by the magnitude of the F value (P=0.05). When a significant F test was obtained, separation of means was accomplished by Fisher's protected LSD at P=0.05.

Results and Discussion

Inhibition of *B. cinerea* mycelial growth by different sound frequencies. The mycelia growth of *B. cinerea* was affected by treatment with certain frequencies of sound waves. Radial mycelial growth was calculated from mean values of colony diameter in treated and untreated control Petri plates. Sound frequency and the degree of mycelial growth inhibition were positively correlated; as the wave frequency increased, mycelial growth inhibition increased (Fig. 2). Following treatment with 5 kHz, mycelia growth was completely inhibited when compared to that of the control. Treatments at 1, 2, 3, and 4 kHz also inhibited *B. cinerea* mycelia growth, but to a lesser extent than that of the 5 kHz treatment. Furthermore, the degree of inhibition was similar among these treatments despite the increasing frequency. After establishing 5 kHz as a benchmark for growth inhibitory treatment, experiments were performed to investigate the time-dependence profile for inhibition over a period of 1–7 days. Mycelial growth was inhibited after 3 days (Fig. 3).

Inhibition of *B. cinerea* spore germination by sound treatment. Assays were performed to determine the effect of sound waves on spore germination. Sound waves caused a noticeable reduction in *B. cinerea* spore germination when compared to that of the untreated control (Fig. 4). Because spores of plant pathogenic fungi are a main source of secondary infection in plant disease, inhibition of spore formation and germination are closely related to disease control. In that sense, it is significant that sound waves can inhibit the germination of spores. Further studies to examine the mechanism of inhibition by sound waves are warranted. The use of sound waves as an alternative treatment to synthetic chemicals is compelling in view of the potentially hazardous effects on the environment when chemical fungicides are made.

Light microscopy analysis. Mycelial morphology was analyzed by light microscopy after treatment for 2 days with 5 kHz sound waves. The mycelial growth of *B. cinerea* treated with sound waves was significantly inhibited, and several morphological changes were observed (Fig. 5). The mycelia septa were thickened, and the mycelia interior was almost empty, with contents vacuolated (Fig. 5B and 5C). Aggregates of mycelium-forming sclerotia were observed more frequently in sound-treated fungi than in the non-treated control (Fig. 5D). In addition, the hyphae were significantly thinner, and the hyphal tips were expanded and swollen at the ends. These various morphological changes indicate that fungal growth was severely stressed by sound treatment. This observation is compelling, as similar morphological

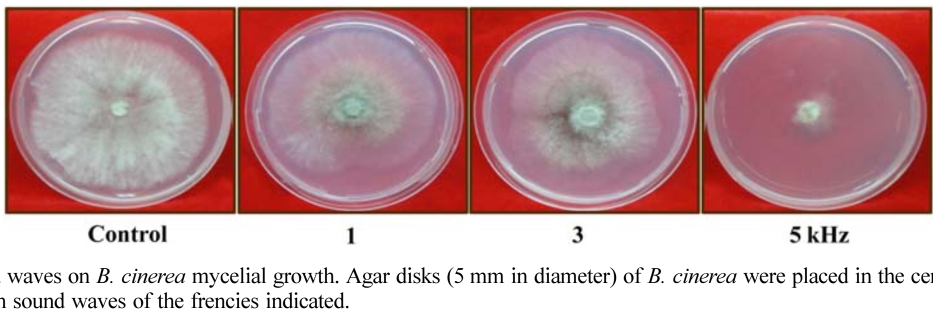

Fig. 2 Effect of sound waves on *B. cinerea* mycelial growth. Agar disks (5 mm in diameter) of *B. cinerea* were placed in the center of Petri plates and treated for 5 days with sound waves of the frencies indicated.

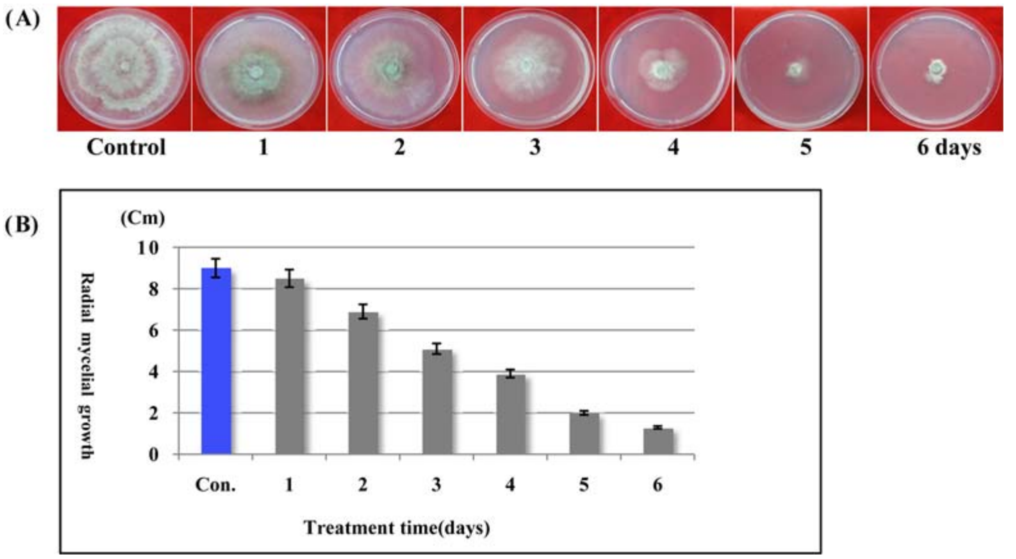

Fig. 3 Inhibitory effects of sound waves on *B. cinerea* mycelia growth. Agar disks (5 mm in diameter) of *B. cinerea* were placed in the center of Petri plates and treated with or without (control) sound waves for times indicated. After 6days, the diameter of mycelia was measured. (A) Mycelia growth on 1/2 PDA with or without sound treatment. (B) Mycelial growth profiles of *B. cinerea* following various duration of at different sound treatment.

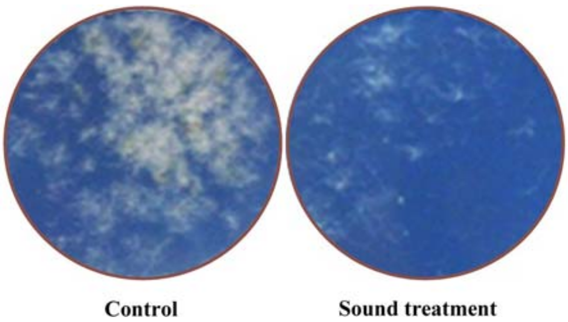

Fig. 4 Inhibition of *B. cinerea* spore germination by sound treatment. Known concentrations of *B. cinerea* spore suspension (2×10^5 spores/mL) were treated with 5 kHz sound waves for 24 hrs.

changes are observed upon treatment with chemical fungicides used to suppress mycelial growth.

Morphological analysis by SEM. The results of SEM showed normal morphology in untreated controls, with linear-shaped hyphae and smooth surfaces (Fig. 6A-C), and spores were observed in some places (Fig. 6A). However, in sound-treated specimens, hyphal growth and conidiogenesis were impaired (Fig. 6A). The distance between septa was decreased, and the mycelial surface was bumpy and cracked in comparison to that of the untreated control (Fig. 6B,C). These observations suggest that sound waves can act as an abiotic stress and thereby trigger abnormal mycelial growth and development. Further study is needed to determine the molecular mechanism by which sound waves inhibit mycelial growth. In this study, we investigated the effect of sound waves on the mycelial growth and spore germination of *B. cinerea*. When treated with 1 kHz sound waves, mycelia of *B. cinerea* showed no difference in growth when compared to that of non-treated *B. cinerea*. However, mycelia growth was significantly inhibited when treated with sound waves of 5 kHz. Changes in mycelial growth were closely related to changes in sound wave frequency, and hyphal growth was inhibited as the frequency of sound increased. Several studies report that sound waves stimulate the growth and development of plants (Bochu et al., 2001; Yiyao et al., 2002; Bochu et al., 2004; Braam, 2005,). Yiyao et al. (2002) showed that sound waves have dual effects on plant growth.

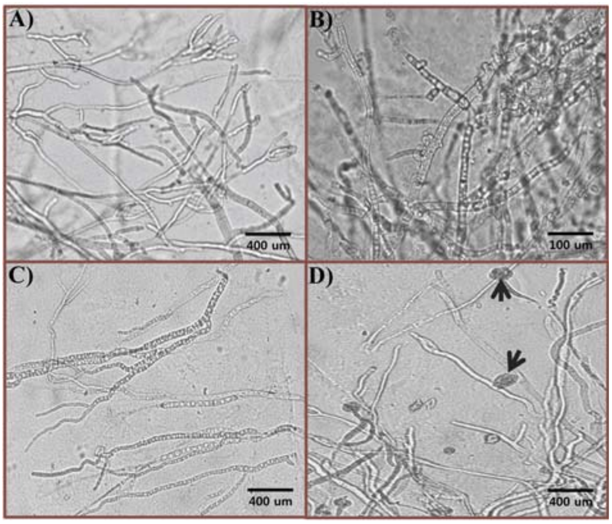

Fig. 5 Light microscopy visualization of *B. cinerea* mycelia treated with or without 5 kHz sound waves. Mycelia treated without sound (control) (A) and with sound (B-D). The arrow points to mycelium aggregates in the form sclerotia.

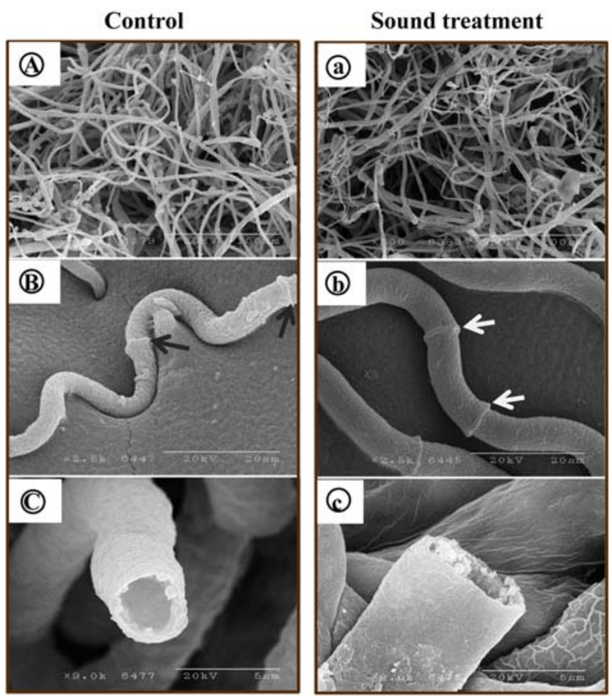

Fig. 6 Electromicrograph of *B. cinerea* mycelia treated for 2 days with or without 5 kHz sound waves. The arrows indicate the interval between the two septa.

Sound waves of specific, suitable intensity, and frequency can stimulate plant growth, whereas sound waves exceeding these parameters can inhibit plant growth. In this report, the wave frequencies tested ranged from 1 to 5 kHz, and several physiological and morphological aspects were observed by light microscopy and SEM (Figs. 5 and 6). Of the tested frequencies, the 5 kHz sound wave significantly inhibited mycelial growth, spore germination, and caused morphological changes including low mycelial density, and irregular mycelial surfaces of *B. cinerea.* Most hyphae were empty, thinner along the length, and significantly swollen at the tips. These results demonstrate that sound waves render mycelia growth under stress, and thereby fungal growth and spore germination is presumably inhibited. The molecular mechanism of *B. cinerea* sound perception and transduction is not yet understood. Previously, we analyzed the sound response gene in Arabidopsis, and found that a large number of genes are induced or repressed by sound treatment. More studies are needed to elucidate the mechanism by which sound waves inhibit mycelial growth and spore germination. Necessity of reduction in chemical use for plant disease control is increasing more and more in terms of environment and safety. Because spore is one of the major factors of secondary infection of plant disease, suppression of the spore formation and inhibition of the spore germination are very effective for controlling plant disease. In this experiment, we showed that spore germination and mycelia growth of plant pathogenic fungi can be inhibited by sound treatment, indicating that the treatment of sound wave can be utilized as a useful nonchemical means for plant disease control.

Acknowledgment We thank the support of grant from NAAS Agenda Program (Grant No. PJ 9070542013 and PJ9070492013) in RDA.

References

Braam J, Sistrunk ML, Sistrunk, DH Xu, Purugganan MM, Antosiewicz DM et al. (1977) Plant response to environmental stress: Regulation and function of the *Arabidopsis* TCH genes. *Planta* **203**, S53–41.

Braam J (1992) Regulated expression of the calmodulin-related TCH genes in cultures *Arabidopsis* cells: Induction by calcium and heat shock. *PNAS* **80**, 3212–6.

Braam J (2005) In touch: Plant responses to mechanical stimuli. *New Physiol* **165**, 373–89.

Bhagyalakshmi A, Berthiaume F, Reich KM, and Frangog JA (1992) Fluid shear stress stimulates membrane phospholipid metabolism in cultures human endothelial cells. *J Vasc Res* **29(6)**, 443–9.

Bochu W, Hucheng Z, Yiyao L, Yi J, and Sakanishi A (2001) The effects of alternative stress on the cell membrane deformability of chrysanthemum callus cells. *Colloids and surfaces B: Biointerfaces* **20**, 321–5.

Bochu W, Jiping S, Biao L, and Chuanren D (2004) Soundwave stimulation triggers the content change of the endogenous hormone of the *Chrysanthenum* mature callus. *Colloids and surfaces B: Biointerfaces* **37**, 107–12.

Chehab EW, Eich E, and Braam J (2009) Thigmomorphogenesis: A complex plant response to mechano-stimulation. *J Exp Bot* **60(1)**, 43–56.

Creath K and Schwartz GE (2004) Measuring effects of music, noise, and healing energy using a seed germination bioassay. *J Altern Complement Med* **10(10)**, 113–22.

Galston AW and Slayman CL (1979) The not-so-secret life of plants. *Am Aci* **67**, 337–44.

Hongbo S, Biao L, Bochu W, Kun T, and Yilong L (2008) A study on differentially expressed gene screening of *Chrysanthemum* plants under sound stress. *Colloids and surfaces B: Biointerfaces* **331**, 329–33.

Jarvis WR (1977) *Botryotinia* and *Botrytis* Species-Taxonomy, *Physiology and Pathogenicity- a Guide to Literature*. Canada Department of Agriculture, Ca.

Jeong MJ, Shim CK, Lee JO, Kwon HB, Kim YH, Lee SK et al. (2008) Plant gene responses to frequency-specific sound signal. *Mol Breeding* **21**, 217–26.

Johnson KA, Sistrunk ML, Polisensky DH, and Braam J (1998) *Arabidopsis thaliana* responses to mechanical stimulation do not required ETR1 or EIN2. *Plant Physiol* **116**, 643–9.

Jomdecha C and Prateepasen A (2011) Effects of pulse ultrasonic irradiation on the lag phase of *Saccharomyces cerevisiae* growth. *Letters in Applied* **52(1)**, 62–9.

Mizoguchi T, Irie K, Hirayama T, Hayashida N, Yamaguchi-Shinozaki K, Matsumoto K et al. (1996) A gene encoding a mitogen-activated protein kinase is induced simultaneously with genes for a mitogen-activated protein kinase and an S6 ribosomal protein kinase by touch, cold, and water stress in Arabidopsis thaliana. *PNAS* **93**, 765–9.

Prins TW, Tudzynski P, von Tiedemann A, Tudzynski B, Have A, Hansen ME et al. (2000) Infection strategies of *Botrytis cinerea* and related necrotrophic pathogens. In: *Fungal Pathology*, J. Kronstad (ed.), pp. 3–34, Kluwer Academic Publishers, The Netherlands.

Sistrunk ML, Antosiewica DM, Purugganan MM, and Braam J (1994) The *Arabidopsis* TCH3 encodes a novel Ca^{2+} binding protein and shows environmentally induced and tissue-specific regulation. *Plant Cell* **6**, 1553–65.

Takahashi H, Suge H, and Kato T (1992) Growth promotion by vibration at 50 Hz in rice and cucumber seedlings. *Plant Cell Physiol* **32**, 729–32.

Weinberger P and Das G (1972) The effect of an audible and low ultrasound frequency on the growth of synchronized cultures of *Scenedesmus obtusiculus*. *Can J Bot* **50**, 361–6.

Weinberger P and Measures M (1978) Effects of the intensity of audible sound on the growth and development of Rideau winter wheat. *Can J Bot* **57**, 1036–9.

Weinberger P and Burton C (1981) The effect of sonication on the growth of some tree seeds. *Can J Forest Res* **11**, 840–4.

Yiyao L, Bochu W, Xuefeng L, Chuanren D, and Akio S (2002) Effects of sound field on the growth of *Chrysanthemum* callus. *Colloids and surfaces B: Biointerfaces* **24**, 321–6.