Short Communication

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Effectiveness of Sonication on Diminishing Bacterial Overgrowth in *Agrobacterium*-mediated Transformation

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Abstract

This study was carried out to trace the biological effects of exposing *Agrobacterium tumefaciens* and sugarcane cell clumps of suspension culture to ultrasonic waves. A diluted suspension of *Agrobacterium tumefaciens,* strains EHA101 and LBA4404, was treated with 44 kHz sonication which remarkably reduced the frequency of colony formation in the inoculated medium of both strains. The cell clumps of the suspension culture were co-cultured with *Agrobacterium,* and then sonicated. Following 3 days repeating co-culture in solid medium, the cell clumps were transferred to the MS-1e medium. Without sonication treatment, 31% of cell clumps revealed bacterial overgrowth, whereas no overgrowth was found in the sonication treatment after 14 days of culture. However, no significant differences in the rates of growth and shoot regeneration between the sonication-treated cell clumps and the untreated cell clumps.

Key words: *Agrobacterium tumefaciens,* bacterial overgrowth, sonication, sugarcane, transformation.

Abbreviations

SAAT, sonication-assisted *Agrobacterium*-mediated transformation.

Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) was established by Trick and Finer (1997) to enhance the transformation efficiency of plant species recalcitrant to *Agrobacterium*-mediated transformation. SAAT is a method of subjecting the target tissue to ultrasound while it is immersed in an *Agrobacterium* suspension (Santarem *et al.*, 1998). This procedure mechanically disrupts and wounds cells via a sonic wave effect, and is thought to allow more *Agrobacterium* penetration into the explant tissue (Meurer *et al.*, 1998). Hence, there are some reports evaluating the effects of sonication on the structural integrity of the explants. However, to our knowledge, there is no report concerning the effects of sonication on the *Agrobacterium* cells themselves and on bacterial overgrowth, using particularly sugarcane culture which is recalcitrant to *Agrobacterium*-mediated transformation (Arifin *et al.*, 2002).

The calli of sugarcane cv. NiF 4 (*Saccharum.* spp. hybrids) were desegregated with forceps and proliferated in N6-2 medium (**Table 1**). Strains EHA101 and LBA4404 of *Agrobacterium tumefaciens*, which carry a binary vector pMLH7133-GUS, were used. The binary vector pMLH7133-GUS was a derivative of pMLH7133-mwti1b (Mochizuki *et al.*, 1999), in which the mwti1b gene was replaced with the β-glucronidase (GUS) gene instead of the mwti1b gene. The pMLH7133-GUS contains the neomycin phosphotransferase II gene and the hygromycin phosphotransferase gene as selectable markers, and an enhanced CaMV 35S promoter (Mitsuhara *et al.*, 1996) fused with the GUS ORF as a reporter gene. EHA101 and LBA4404 were streaked on LB medium supplemented with 50 mg l^{-1} hygromycin and 50 mg l^{-1} kanamycin for 48 h. The grown bacteria were collected and suspended in liquid medium.

The suspension of *Agrobacterium* (EHA101) cells in the N6-2 medium was diluted with sterile water to obtain 0.5, 0.05, or 0.005 OD_{600}. The bacterial suspension (10 ml) was sonicated in a 50-ml centrifuge tube (polypropylene) in a bath sonicator (Ultrason Velvo Clear VS-150H, 44 kHz) filled with 1500 ml water for mediation of the ultrasonic

Table 1 Composition of culture media.

Culture step	Inorganic salts	Carbon source	Additives and pH
Callus induction(MS-1)	MS [1]	30 g l^{-1} sucrose	2 mg l^{-1} 2,4-D, 500 mg l^{-1} casamino acids, 5% coconut water, 0.9% agar, pH 5.8
Cell suspension culture (N6-2)	Modified N6 [2]	30 g l^{-1} sucrose	2 mg l^{-1} 2,4-D, 500 mg l^{-1} casamino acids, 5% coconut water, pH 5.8
Coculture with *Agrobacterium* (liquid N6-2c and solid MS-1c)	N6-2 MS-1	30 g l^{-1} sucrose	50 mg l^{-1} acetosyringone, pH 5.2
Bacterial elimination(MS-1e)	MS-1	30 g l^{-1} sucrose	200 mg l^{-1} carbenicillin, pH 5.8
Shoot regeneration(MS-R9)	MS	15 g l^{-1} sucrose 15 g l^{-1} sorbitol	0.5 mg l^{-1} IAA, 1 mg l^{-1} BA, 500 mg l^{-1} casamino acid, pH 5.8

All media supplemented with 50 mg l^{-1} nicotinic acid, 100 mg l^{-1} thiamine HCl, 50 mg l^{-1} pyridoxine HCl, 10 g l^{-1} myo-inositol, 200 mg l^{-1} glycine.

[1] Murashige and Skoog (1962).

[2] Matsuoka and Sugimoto (1997).

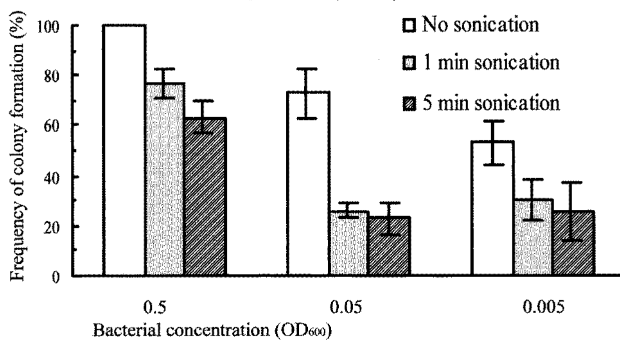

Fig. 1 Effect of sonication on colony formation of *Agrobacterium* (EHA101) in pricked media. The number of colonies per plate was determined visually after 5 days of incubation. The values are the means ± S.D. of 3 replicates.

waves. Sonication was conducted for 0, 1, or 5 min at ambient temperature. Sterile platinum needles were immersed for one minute in the treated suspension and wiped with sterile filter paper. The *Agrobacterium* was inoculated into a solid MS-1 medium (**Table 1**) in petri dishes by pricking the treated needle, and was then incubated at 26℃ for 5 days. The experiment was replicated 3 times, with 10 spots of needle-prick inoculation per petri dish in each replication. The frequency with which colonies developed was recorded.

In another experiment, a bacterial suspension of EHA101 was adjusted to $1x10^{-6}$ OD_{600} and sonicated for 0, 1, 2, 5, 10 min using the same method as described above, and 50 ml treated bacterial suspension was plated on MS-1 medium and cultured to determine the number of developing single colonies.

The *Agrobacterium* strain EHA 101 was re-suspended in N6-2c medium (**Table 1**) and diluted to 0.5 OD_{600}. It was then co-cultured with cell clumps from the suspension culture and shaken at 125 rpm. The medium was discarded after a single day of co-culture, and the cell clumps were washed with sterile water 3 times prior to sonication. The cell clumps were then re-suspended in N6-2 medium (30 ml) and sonicated in a 50 ml centrifuge tube in a bath sonicator. After the sonication treatment, the cell clumps were cultured on MS-1c medium (**Table 1**) for 3 days and then transferred to the MS-1e medium (**Table 1**). The number of *Agrobacterium* overgrown cell clumps was measured after 2 weeks of culture.

The cell clumps of the suspension culture in the N6-2 medium were sonicated for 0, 1, or 5 min with the same method as described above, but without *Agrobacterium*. These cell clumps (15 clumps with 5 replications) were weighed and cultured on MS-1 medium in a petri dish. After 2 weeks, the cell clumps were weighed and transferred to MS-R9 medium (**Table 1**) for shoot formation. The frequency of shoot formation was measured after 3 weeks of culture.

The biological effects of exposing *Agrobacterium* to ultrasonic waves were evaluated. All inoculated *Agrobacterium* from the non-sonicated suspension of 0.5 OD_{600} in the pricked medium grew well and developed into colonies 5 days after inoculation (**Fig. 1**). The frequency of developed colonies fell to 77% on average with 1 min sonication. In this population, the maximum reduction of the developed colonies was obtained by 5 min sonication. In the lower population (0.05 OD_{600}), 73% of the inoculated samples developed into colonies for the control, while the colony formation rate of 1 min sonication decreased to 26%. No significant difference was detected between 1 and 5 min sonication. The water temperature used for the wave mediation

after 5 min of sonication was less than 30℃, below the critical temperature for *Agrobacterium*. Thus, the death of the bacterial cells was attributable to the disruption from the ultrasound waves and not to lethal temperature.

By sonication at 44 kHz, the number of single colonies of *Agrobacterium* strain EHA101 decreased drastically from 170 (non-sonication) to 14 within 5 min (**Fig. 2**). The number of single colonies of strain LBA4404 also decreased from 246 (non-sonication) to 66 per plate on average with 5 min sonication (data not shown). Cid *et al.* (1998) found that the percentage of cell lyses in yeast is proportional to the period of sonication treatment. Joyce *et al.* (2001) reported that sonication is effective in killing bacteria (*Bacillus* species) in suspension, and that the destruction rate increased with prolongation of the sonication time in the low- kilohertz range (20 and 38 kHz). As shown in this experiment, we also obtained similar results in *Agrobacterium*.

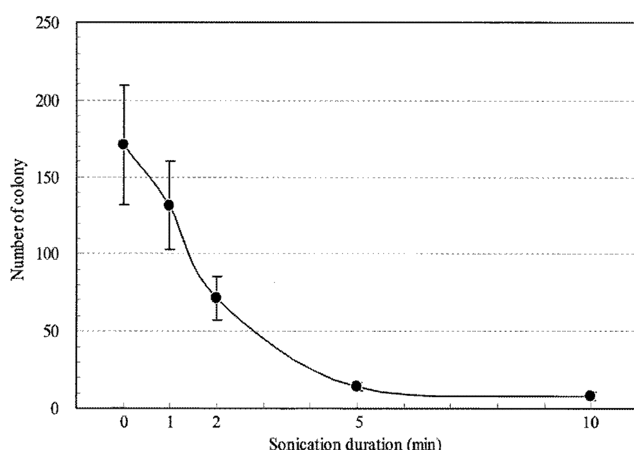

Fig. 2 Effect of sonication duration on the colony formation of *Agrobacterium* (EHA101).
The number of single colonies per plate was determined visually after 6 days of incubation. The values are the means ± S.E. of 5 replicates.

The frequency of cell clumps revealing bacterial overgrowth was decreased by 5 min sonication. Without sonication treatment, 31% of the cell clumps revealed bacterial overgrowth, whereas none was found with 5 min sonication treatment after 14 days of culture on the MS-1e medium (**Fig. 3**). This suggested that 5 min sonication effectively controlled bacterial overgrowth in the cell clumps during the co-culture and selection process. In our experience with sugarcane transformation, necrotic cell clump and the inhibition of transformed cell clump growth sometimes occurred in the selection process, due to the overgrowth of *Agrobacterium*. To increase the transformation efficiency, the overgrowth of *Agrobacterium* after co-culture must be prevented. Increasing antibiotic concentration in the culture medium is not the best way to suppress bacterial overgrowth, since it may cause problems with the plant tissue growth and transformation rate, as reported by Ogawa and Mii (2002). In this experiment, we found that sonication treatment could decrease the concentration of carbenicillin in selection media from 500 to 200 mg l^{-1} without bacterial overgrowth during selection (data not shown). This suggested that 5 min sonication could reduce the chemical costs in sugarcane transformation.

The effect of sonication on the growth of target cells was determined by using sonication in *Agrobacterium*-mediated transformation protocol. Our results clarified that sonication up to 5 min did not affect the growth of sugarcane cell clumps of suspension culture (**Table 2**). Trick and Finer (1998) reported that sonication for 10 s and 60 s had a detrimental effect on soybean suspension culture tissue. Meurer *et al.* (1988) found that few explants (soybean cotyledonary nodes) survived for 10 days

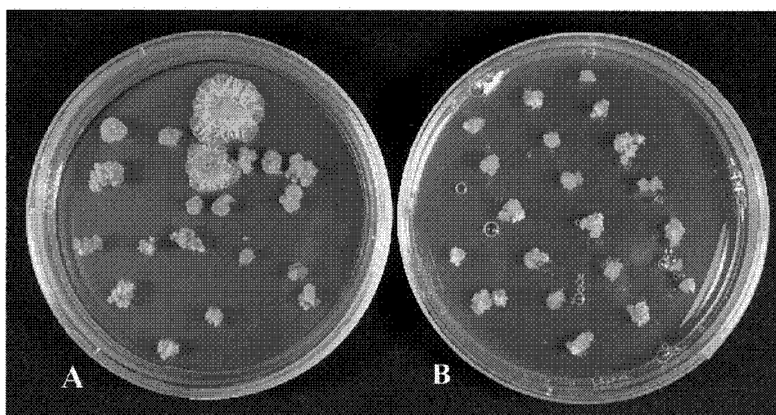

Fig. 3 Effect of sonication on the growth of *Agrobacterium* (EHA101).
Cell clumps with an overgrowth of *Agrobacterium* (A: no sonication) and cell clumps without an overgrowth of *Agrobacterium* (B: 5 min sonication) after 14 days of culture on a MS-1e medium.

Table 2 Effect of sonication on the growth and shoot formation rate of cell clumps of suspension culture.

Sonication Duration (min)	Fresh weight of cell clumps (g) per plate		Frequency of shoot formation (%)
	1st day	14th day	
0	0.19 ± 0.023	1.52 ± 0.161	63 ± 13.8
1	0.21 ± 0.028	1.82 ± 0.244	79 ± 11.0
5	0.18 ± 0.049	1.61 ± 0.267	69 ± 23.4

The fresh weight of the samples was measured on the 1st and 14th days after sonication. The frequency is expressed as a percentage of the cell clumps regenerating shoots. The values are the means ± S.D. of 5 replicates, each with 15 cell clumps of suspension culture.

when sonicated for 600 s. These results may be due to the different protocols used for sonication (type of target tissue, type of sonicator bath used, and type of vessel used for the tissue during sonication). In sugarcane, the shoot formation rate of the cell clumps was 63% for non-sonication, followed by 69% for 5 min sonication, and 79% for 1 min sonication (**Table 2**). These data indicated that there were no significantly different regeneration rates among the treatments.

In this report we studied the response of sugarcane cell clumps of suspension culture and of *Agrobacterium* in a sonication-assisted *Agrobacterium*-mediated transformation. Concerning *Agrobacterium*, it has been clarified that sonication can tremendously reduce the development of bacterial colonies and suppress bacterial overgrowth after co-culture. Sonication did not inhibit growth or shoot formation ability in sugarcane cell clumps of suspension culture. It can be concluded that 1-5 min sonication is applicable to the *Agrobacterium*-mediated transformation of sugarcane, and effectively reduces the detrimental effects resulting from bacterial overgrowth in the selection process. The method described here would be widely applicable to the *Agrobacterium*-mediated transformation of plants.

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