Applications of Shock Waves in Agriculture Research

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ABSTRACT

Controlled shock waves can be generated in any medium of interest in the laboratory using various methods. The instantaneous increase in pressure and temperature, in the medium of propagation behind a shock wave, can be used for many novel industrial applications. In this paper we describe two new applications of shock waves developed for the agriculture research. An underwater shock wave generator has been used designed, calibrated and successfully used to generate under water shock waves. About 100 J of energy is deposited between two stainless steel electrodes immersed in water for about 350 ns for generating the micro-shock waves in water. Utilizing the instantaneous jumps in pressure and temperature behind these spherical micro-shock waves in water, desired DNA has been successfully injected inside (*Agrobacterium tumefaciens*) cells at a measured overpressure level of 50 bar. Reporter gene assay were also recorded in tomato plants to confirm the successful transformation of the naked plasmid DNA. These micro-shock waves are also used as a means to induce Abiotic stress in germinated finger millet seeds. Substantial reductions in root and shoot lengths were observed in these seedlings after exposure to shock waves confirming the induction of Abiotic stress. These new applications of shock waves will be useful in the field of agriculture research.

1. INTRODUCTION

The phenomenon of *shock waves* is commonly associated with aerospace engineering/astronautics and in particular with supersonic flight. Shockwaves appear in nature whenever the different elements in a fluid approach one another with a velocity higher than the local speed of sound. Any sudden release of energy (within few μs) will invariably result in the formation of shock waves since they are one of the efficient mechanisms of energy dissipation observed in nature. The dissipation of mechanical, nuclear, chemical, and electrical energy in a limited space will usually result in the formation of a shock wave. Because of the dissipative nature of shock waves they invariably need a medium both for generation as well as for propagation. Physically the occurrence of shock wave is always characterized in a fluid flow by instantaneous changes in pressure, velocity and temperature. In recent times utilizing the instantaneous changes brought about by shock waves various industrial and biological applications have been developed or being proposed. Owing to the success in creating shock waves in laboratory, has resulted in the development of innovative applications [1-3] in biological and medical fields. For example, shock wave assisted lithotripsy is one of the most useful and proven treatments for kidney stones and gall bladder diseases and is now widely used by the doctors in many countries. In this study we successfully demonstrate, two novel applications of underwater micro-shock waves in the emerging area of plant bio-technology. While one of them involves introduction of a desired naked plasmid DNA into bacteria without killing the cell, the other pertains to using underwater micro-shock waves for inducing *Abiotic stress* in germinated agricultural crop seeds.

Bacterial transformation is one of the key steps in the recombinant DNA technology. In this study we have attempted to transform a genus of bacteria that has great importance in Plant Biotechnology, *Agrobacterium tumefaciens*. Agrobacterium is used as a vehicle for successful transfer of foreign DNA into recipient plant cell [4-5]. The stable introduction of foreign gene into Agrobacterium is a prerequisite for Agrobacterium mediated gene transfer technology. In the present paper we are reporting the shock wave assisted uptake of foreign DNA in Agrobacterium. The transformed Agrobacterium

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showed stable integration of the gene of interest as evidenced by transient gene expression assay in tomato leaves.

Plant growth and development is regulated by various different Abiotic factors such as water, light intensity, pH and salt concentration in soil solution, temperature etc. Plants growing in natural environment are continuously exposed to the changes in these environmental factors (stresses) at different growth stages. The plants that are acclimated to the changing environmental conditions synthesize specific stress-responsive proteins upon inductions. Synthesis of proteins associated with stress tolerance are commonly seen when plants are exposed to a sub-lethal stress, known as induction. These proteins are involved in altering stress related biochemical processes necessary for adaptation [6]. In this context we have examined the influence of shock waves in modifying growth of crop plant seedling, with an ultimate aim of using shock waves for induction of stress in crop seeds. Germinated crop plant seeds showed inhibition of growth upon exposure to shock waves suggesting that the waves can act as Abiotic stress. In this paper we describe the underwater shock wave generator in IISc that has been used for carrying out the experiments first followed by the details of the Agrobacterium cell transfer experiments. This is followed by the description of the Abiotic stress induction experiments.

2. MATERIALA AND METHODS

2.1 Generation of shock waves

An underwater electric discharge device was designed, fabricated and successfully used for creating spherical micro-shock waves in the laboratory (Figure1). Spherical micro-shock waves (peak overpressures up to 100 MP) were generated in water by instantaneously depositing electrical energy (100 J) between a pair two stainless steel electrodes (1 mm apart) for about 0.35 μs. The electrical energy locally vaporizes the water between the electrodes and creates a bubble. This spherical bubble grows with time and eventually breaks up generating a spherical shock wave. This wave propagates at supersonic velocities only for about few mm before attenuating into a Mach wave. Considering the size of the wave we refer to it as *Micro shock wave*. The high voltage applied between the electrodes can be varied to generate shock wave of requisite strength. A high precision mechanical traverse system (Figure 2) was used to hold the test tubes containing Agrobacterium cells with naked plasmid DNA above the electrodes. The distance between the bottom of the tube and electrodes was maintained at 1.5 to 3 mm and the corresponding shock overpressure measured using PVDF Needle hydrophones (Ms Muller, Germany). The typical signature of the underwater micro-shock wave measured in the vicinity of the electrodes is shown in Fig. 3. The average pressure (~50 bar) felt by the Agrobacterium cells with naked plasmid DNA inside the test tube is plotted in Fig. 4. Because the pressure pulse lasts only for few microseconds the bacterial cell remains viable even after exposure to shock waves.

2.2. Bacterial transformation

2.2.1 Preparation of competent cells of Agrobacterium tumefaciens by $CaCl_2$ method

The overnight grown fresh culture of Agrobacterium strain GV 2260 and GV3101 was inoculated into 100mL of LB media and grown to OD600 of 0.4-0.6 in a specific antibiotic medium (LB containing

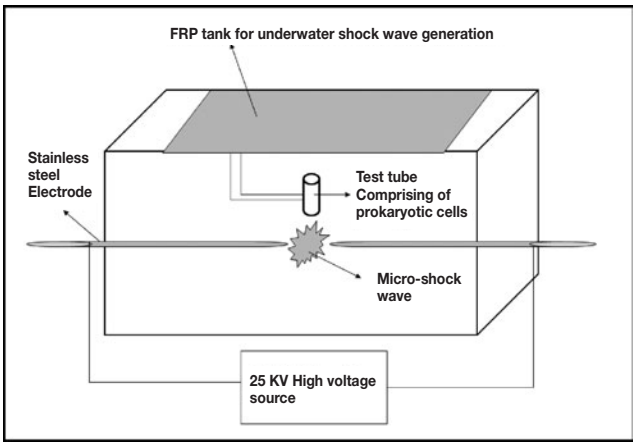

Figure 1. Schematic diagram of the underwater shock wave generator in IISc

Carbencillin, Streptomycin and Rifampicin for strain GV 2260 & Gentamycin for strain GV 3101) at 28˚C. The culture was cooled in ice and subjected to centrifugation at 4000rpm for 5min at 4˚C to collect healthy cells. The collected cells were suspended in 10mL of chilled 20mM $CaCl_2$ and centrifuged at 4000 rpm to collect the cells.

After washing the cells were suspended in 2mL of 10mM CaCl2 and stored in aliquots at -70˚C after freezing in liquid nitrogen.

2.2.2. Transformation

To transform Agrobacterium traditional freeze-thaw method was followed. Known volume (100ul) of cells were mixed with naked plasmid DNA (1000ng), frozen in liquid nitrogen and incubated at 37˚C for 5 min. After incubation, 1 ml LB was added and incubated at 28˚C for 2-4 h at constant shaking. About 100 μl of transformed cells were plated onto LB medium containing appropriate antibiotics (Kanamycin 50 g/mL). The isolated colonies were counted to express the transformation efficiency as CFU/μg of plasmid DNA.

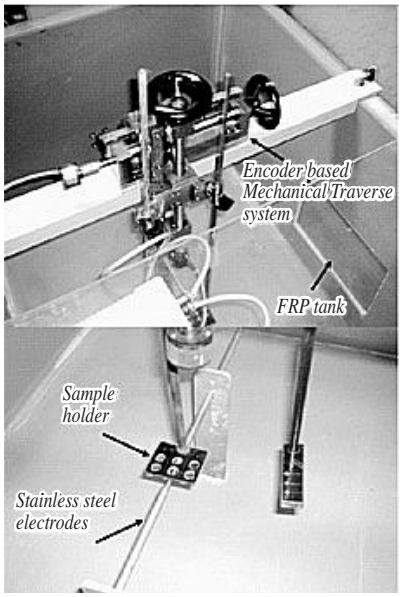

Figure 2. A photograph of the encoder based mechanical traverse system and the sample holder in the underwater shock wave generator

2.2.3. Shock Wave Assisted Transformation

For shockwave assisted method of transformation, naked plasmid (pBI121 containing nptII and gus reporter genes, Clontech, USA) DNA (100ng) was mixed with 100 μl of competent cells before exposing to shock waves in an underwater shockwave generator. After exposing to different levels of shockwaves, the cells were grown in antibiotic free medium for 2-3hours before transferring to antibiotic selection. The incubation temperatures were 28˚C. The transformation efficiency was calculated as CFU/ g of plasmid DNA.

2.2.4. Transient Gene Assay

The efficiency of the transformed Agrobacterium by shockwave method in inducing infection and expression of gene of interest (gus gene) was confirmed by transient gene expression assay. Fresh over night grown transformed Agrobacterium cells were diluted to O.D.600 1.0-1.5 and incubated in infiltration media (glucose 1%, sucrose2%, MES buffer 10mM, *Acetosyringone* 200 M, $MgCl_2$ 10mM) for 2-3 hours. Then the cells in the infiltration media were infiltrated into mature tomato leaves using needless syringe. These infiltrated leaves were subjected for gus gene assay five days post infiltration [7]. The leaf bits were incubated in X-gluc solution (sodium phosphate buffer 50mM, X-gluc 1mM, triton X-100 0.1%, potassium ferricyanide 4mM, chloramphenicol 1%) overnight at 37^0C. Leaf pigments were removed by incubating the tissue in ethanol overnight and observed for the blue coloration.

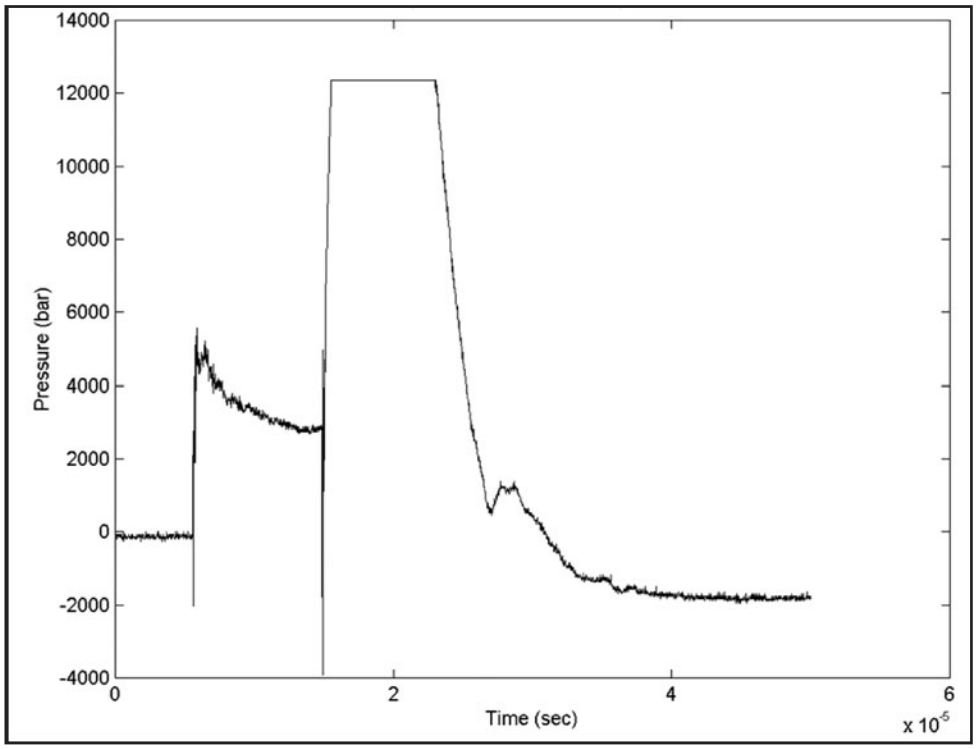

Figure 3. Typical pressure history of the overpressure behind the shock wave: measured at a distance of 1.5 mm from the electrodes: The power source was operated at 17 kV

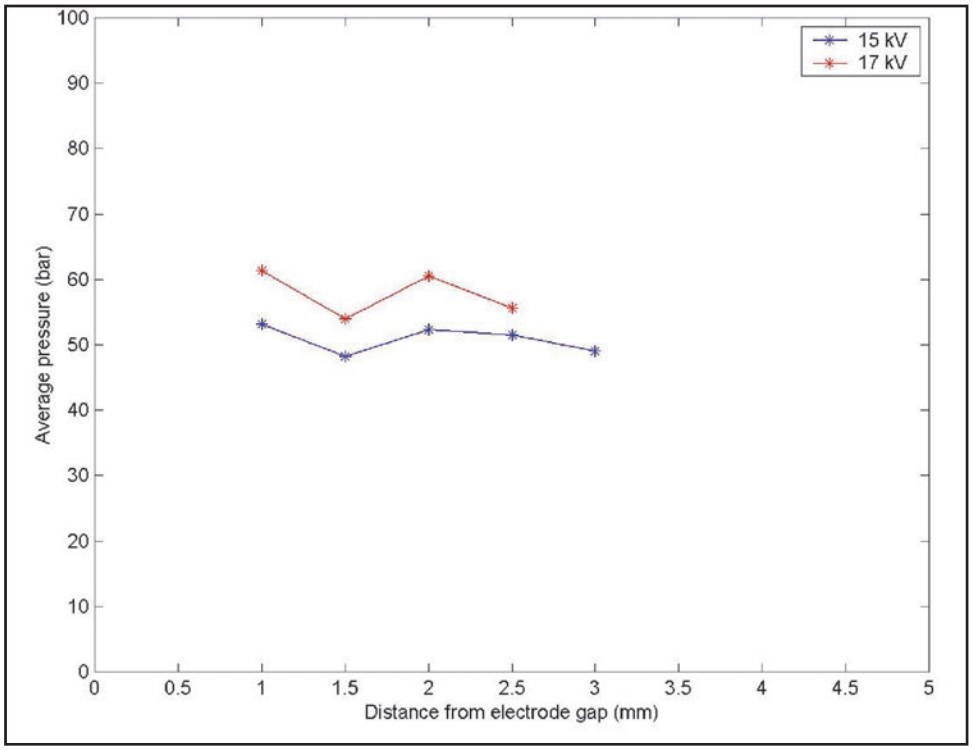

Figure 4. The average overpressure measured inside the tube containing the agro-bacterial cell suspension: The tip of the test tube was placed at a distance of 1.5 mm from the electrodes: The power source was operated at both 15 kV and 17 kV

3. DISCUSSIONS ON BACTERIALTRANSFORMATION

We have successfully demonstrated that the spherical micro-shock waves generated by underwater shockwave generator induced the prokaryotic cells to uptake naked DNA [3]. On the basis of this initial observation, we examined whether this phenomenon can be effectively used to develop transformation protocol to transform naked plasmid DNA into Agrobacterium. For this purpose we selected Agrobacterium tumefaciens strain and performed transformation, using 100ng of naked plasmid DNA (pBI121 containing nptII and gus reporter genes, Clontech, USA) by employing shock waves. The efficiency of transformation was compared with the other methods of bacterial transformation, which was found to be efficient under normal laboratory condition. The results indicated that there was uptake of foreign DNA by the Agrobacterium upon exposure to shockwaves (Figure 5). The shockwave pressure levels beyond 50 bar did not yield any transformed colonies. We confirmed the transformation event by isolating the plasmid from the transformed cells and subjecting the plasmid for restriction digestion and polymerase chain reaction to amplify the gene of interest.

The transient gene expression assay was carried out using the transformed cells by Agro-infiltration method in tomato leaves. The histochemical assay for gus gene product yielded blue color in the leaves infiltrated with transformed *Agrobacterium* cells (Figure 6). Transient expression (blue coloration) in the veins of leaf tissues was clearly observed due to -glucuronidase activity. The leaves infiltrated with untransformed cells did not show any blue color. It appears that the exposure of shock waves temporally enhances the cell membrane permeability, thereby enabling the transformation of Agrobacterium cells. There is a need to examine the actual mechanism of shock wave assisted cell transformation.

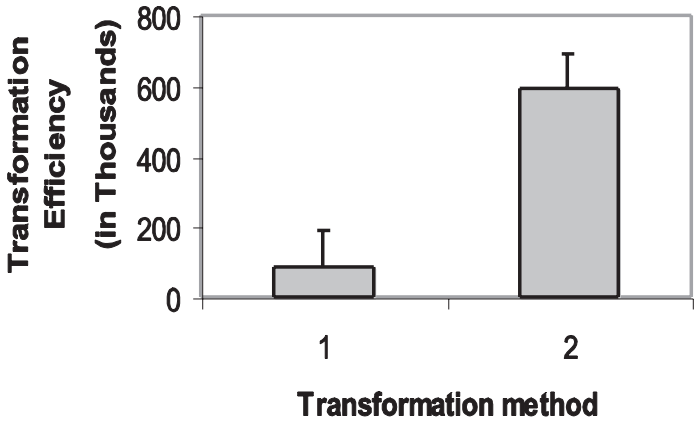

Figure 5. Transformation efficiency expressed as CFU/µg of plasmid DNA of Agrobacterium cells. 1 – Freeze thaw method and 2 – Shock wave assisted transformation method.

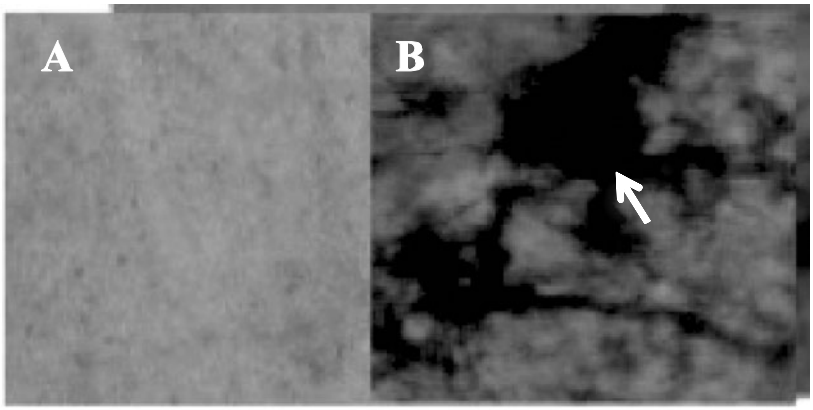

Figure 6. Transient expression of gus gene in tomato leaves. The gene expression was assessed five days post infiltration. A-Untransformed control and B- gus transformed. Blue color as indicated by the arrows depicts gus gene products.

4. ABIOTIC STRESS INDUCTION IN FINGER MILLET SEEDLING USING SHOCK WAVES

Finger millet (*Elucine coracana*) seeds were soaked in sterile water and allowed for germination at 37˚C in seed germinators for 48 hours. These germinated seeds were exposed briefly to shock waves. An underwater electric discharge device was used to create spherical micro-shock waves (over pressures 90 Bar). Shock waves with different levels of pressures were generated and germinated seeds were directly exposed at a distance 1.5mm from the electric discharge device. After exposure to shock waves the seeds were allowed to grow under controlled condition at 37˚C for 48 to 74 hours in seed germinator and the growth responses of the germinated seeds were examined by measuring root and shoot lengths. The focus of this study is to examine whether shock waves can be used as a means to load stress in the germinated finger millet seeds. It is presumed that shock waves may also act as abiotic stress factors such drought, high temperature, salinity etc., and hence might influence growth of plants. From this context, underwater shock generator device was used to create spherical micro-shock waves (over pressures 100 Bar). Shock waves with different levels of pressures were generated and germinated seeds were exposed to the waves. When the seedlings were assessed for recovery growth at 72 hours post exposure, there was significant reduction in root and shoot growth (Figures 7 -10).

The un-shocked control germinated seeds maintained normal growth as expected (Figure 7& 8). Several abiotic factors such as salinity, water deficit, high temperature etc., are known to induce similar types of responses in crop plants [8-10]. The modification in growth upon exposure to stressful factor is mainly due to the adjustments in different physiological processes after stress perception. The findings suggest that shock waves can also alter certain plant physiological process, which has resulted in reduction in growth. The precise role of shock waves on specific plant physiological processes needs to be examined. It is anticipated at this point of time that finger millet seeds after exposure to shock waves may perform better when exposed to adverse environment during the growth cycle. What precise effects the shock induced stress will have on the growth pattern of finger millet seeds needs further investigations.

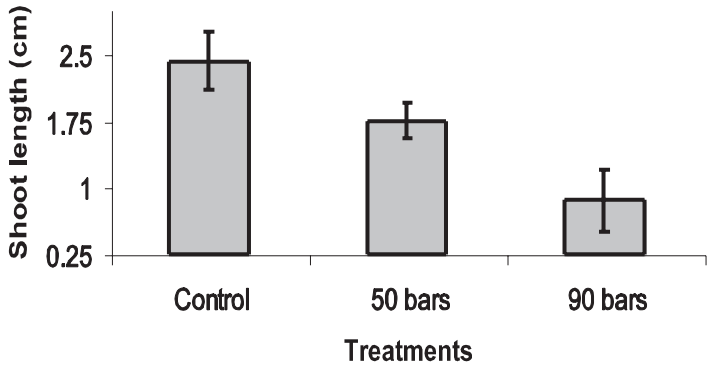

Figure 7. Inhibition of shoot growth of germinated seeds of finger millet (Elusine corocana L) when exposed to shock waves.

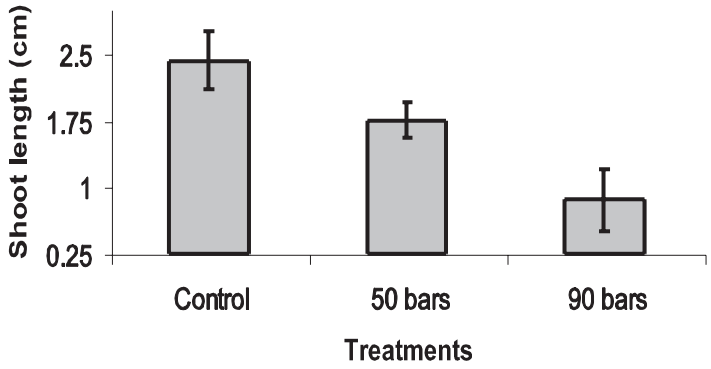

Figure 8. Inhibition of root growth of germinated seeds of finger millet (Elusine corocana L) when exposed to shock waves

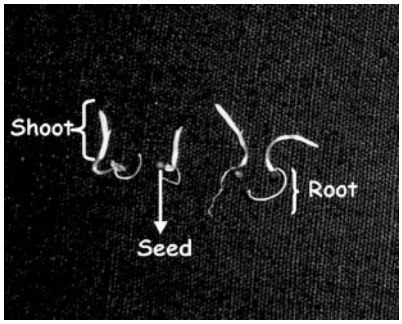

Figure 9. The growth of germinated seeds of finger millet (Elusine corocana) exposed to shock waves

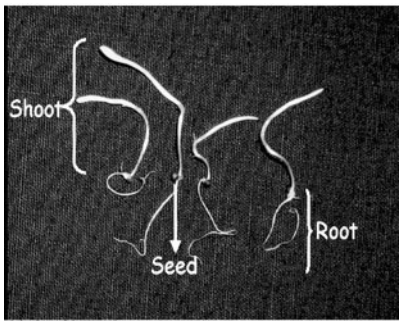

Figure 10. The growth of germinated seeds of finger millet (Elusine corocana) not exposed to shock waves

5. CONCLUSIONS

Shock waves have been largely confined to the realms of high speed flows in the classical aerospace engineering discipline for a long time. In recent times the unique characteristics of shock waves to enhance the fundamental physical structure in any medium of propagation has opened up new vistas and this has led to the development many innovative industrial applications using shock waves. In this paper we have described two such applications of shock waves that will have significant impact in the agricultural research have been described. Using the shock waves generated in water following the deposition of electrical energy, the Agrobacterium cells have been transformed. These micro-shock waves have also been successfully used to induce Abiotic stress in germinated finger millet seeds. In the coming years we will find many more useful applications of shock waves, which seems to be breaking the barriers of high speed flows and moving into many fascinating interdisciplinary research domains.

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