



• Original Article

Phytotoxicity of glyphosate in the germination of *Pisum sativum* and its effect on germinated seedlings

Subinoy Mondal^{1,2}, Mousumi Kumar¹, Smaranya Haque², Debajyoti Kundu²

¹Department of Environmental Science, Shyamsundar College, Syamsundar, India; ²Department of Environmental Science, University of Burdwan, Burdwan, India

The present study evaluated the effects of glyphosate on *Pisum sativum* germination as well as its effect on the physiology and biochemistry of germinated seedlings. Different physico-chemical biomarkers, viz., chlorophyll, root and shoot length, total protein and soluble sugar, along with sodium and potassium concentration, were investigated in germinated seedlings at different glyphosate concentrations. This study reports the influence of different concentrations of glyphosate on pea seeds and seedlings. Physico-chemical biomarkers were significantly changed by glyphosate exposure after 15 days. The germination of seedlings under control conditions (0 mg/L) was 100% after 3 days of treatment but at 3 and 4 mg/L glyphosate, germination was reduced to 55 and 40%, respectively. Physiological parameters like root and shoot length decreased monotonically with increasing glyphosate concentration, at 14 days of observation. Average root and shoot length (n=30 in three replicates) were reduced to 14.7 and 17.6%, respectively, at 4 mg/L glyphosate. Leaf chlorophyll content also decreased, with a similar trend to root and shoot length, but the protein content initially decreased and then increased with an increase in glyphosate concentration to 3 mg/L. The study suggests that glyphosate reduces the soluble sugar content significantly, by 21.6% (v/v). But internal sodium and potassium tissue concentrations were significantly altered by glyphosate exposure with increasing concentrations of glyphosate. Biochemical and physiological analysis also supports the inhibitory effect of glyphosate on seed germination and biochemical effects on seedlings.

Keywords Phytotoxicity, Glyphosate, Germination, *Pisum sativum*, Biochemistry

INTRODUCTION

Pea (*Pisum sativum* L.) is a well known, valuable, popular food source from the plant family Leguminosae (Fabaceae) throughout the world. Generally, green and dry pea are essential crop plants among others due to their high iron, starch and protein content [1]. Herbicides, applied to protect crops and promote agricultural productivity, are used in usual management practice in Indian agriculture and are of vital significance. Glyphosate (N-[phosphonomethyl]-glycine) is a non-selective [2] category IV (the least toxic) herbicide [3] used in agriculture, forestry and in site management for the removal of unwanted weeds. Accord-

ingly, use of glyphosate has been amplified in recent years [4] and its annual global use went above 907 000 tons in 2007 [5]. Glyphosate is a biologically active substance rapidly mineralized in soil [6].

Glyphosate is not bioaccumulated and its detection procedure is complicated. Glyphosate is absorbed and translocated to meristematic tissue which is the major site of herbicidal activity. In laboratory conditions the half-life of glyphosate is 30-40 days [7], while in field soils it can differ from 2197 days (average 30 days) [8].

Glyphosate inhibits aromatic amino acid biosynthesis via the shikimate pathway; however, it has some other secondary ef-

Correspondence: Subinoy Mondal
Department of Environmental Science,
Shyamsundar College, Syamsundar, West
Bengal 713424, India
E-mail: msubinoy@gmail.com

Received: March 17, 2017
Accepted: August 8, 2017
Published: August 16, 2017

This article is available from: <http://e-eh.t.org/>

fects on plant physiology [9]. Glyphosate-based herbicides have been revealed to have different types of effects on seed germination including deleterious [10], little and no effect [11], but the precise mechanism of its effect on the germination process is still unclear. It also causes impairment in protein synthesis and photosynthesis by lowering the rate of amino acid biosynthesis (aromatic and phenolic) [12]. Glyphosate toxicity is also linked with plant micronutrient conditions [13]. So, glyphosate toxicity is related to amino acid biosynthesis as well as photosynthesis; this may be the reason for different physiological and biochemical changes in seedlings and decreased seed germination.

METHODS

Experimental Design

Healthy uniform pea seeds were chosen as the test plant, and were soaked in distilled water (DW) overnight prior to germination. Before germination the seeds were sterilized using 0.1% mercury chloride (Sisco Research Laboratories, Mumbai, India) for 30 seconds, washed in DW several times to remove excess chemical and then dried to eliminate fungal attack. Twenty selected seeds were placed on filter paper inside a sterilized 15 cm Petri plate for seed germination and seedling growth. In each Petri plate 20 mL of glyphosate (Roundup, Marysville, OH, USA) at different concentrations (0.0, 1.0, 2.0, 3.0 and 4.0 mg/L doses), or DW as the control treatment, was added for 72 hours. Germination and seedling growth were recorded up to 14 days at intervals of 24 hours. The expected environmental concentration 2.6 mg/L [14], was considered as a realistic value of exposure level, comparable to those mentioned by other authors such as 1.87 mg/L [15] and 3.73 mg/L [16]; higher values have also been estimated (e.g., 10.13 mg/L) [17].

Tissue Sampling

After the experiment (14 days) desirable tissues from glyphosate-treated and control plants were collected. Then tissues were washed in saline solution (0.75%), homogenized in 0.2 M pH 7.4 phosphate buffer (2 mL) and centrifuged (8000 rpm) at 4°C for 25 minutes. The supernatants were kept in Teflon tubes and stored at -80°C to perform the biochemical analysis of carbohydrate and total protein. Leaves were separated and put in acetone (80%, Sisco Research Laboratories) for the analysis of chlorophyll, i.e., chlorophyll a (Chlo-a), chlorophyll b (Chlo-b) and total chlorophyll (Chlo-t) [18].

Biochemical Analysis

Total Protein

For the estimation of total protein content, 10 mg of plant ma-

terial was weighed out and crushed first with 10 mL of DW. This was transferred into a centrifuge tube and centrifuged for 10 minutes at 1000 rpm. One milliliter from the supernatant liquid was pipetted out into a test tube, then 5 mL of alkaline copper sulfate (Sisco Research Laboratories) reagent was added to each of the test tubes before incubation for 15 minutes at room temperature. Then 0.5 mL of Folin (Merck Life Science, Mumbai, India) reagent was added and the mixture allowed to stand for 30 minutes. Then the absorbance was measured at 660 nm [19].

Soluble Sugar

About 1.0 g of plant material was weighed and crushed with a pestle in a mortar with 10 mL of DW. This was transferred into a centrifuge tube and centrifuged for 10 minutes at 10 000 rpm. The supernatant was collected, then 1 mL of this solution was pipette out into a test tube, 4 mL freshly prepared 0.2% anthrone (Sisco Research Laboratories) was added to it and the mixture heated in a boiling water bath for 10 minutes at 100°C. This was allowed to cool to room temperature. The absorbance of the developed green to dark green color was measured at 630 nm using UV-vis double beam spectrophotometer (UV-260; Shimadzu, Tokyo, Japan) [20].

Sodium and Potassium

For the estimation of sodium (Na^+) and potassium (K^+), 0.5 g of dried and ground tissues was digested in Kjeldahl flasks with the addition of an acid mixture of nitric acid (15 mL) and 60% perchloric acid (0.5 mL, Merck Life Science) along with sulfuric acid (0.5 mL, Merck Life Science) until evaporated to dryness. After that, the residue was cooled at room temperature following dilution with DW (up to 15 mL), filtered with a Whatman No. 42 filter paper and the final volume made up to 50 mL using DW. This process was used for estimation of Na^+ and K^+ by flame photometer (Systronics, Mumbai, India) [21].

Statistical Analysis

Observations were statistically proved using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Probit analysis was performed for percentiles, survival probabilities and cumulative probabilities for the distribution of a stress. Two-way analysis of variance was used to compare mean values of biochemical parameters at different concentrations and differences were considered as statistically significant at p -value < 0.05 . All biochemical values were represented as mean \pm standard deviation (SD) ($n = 10$).

Table 1. Correlation of different parameters of glyphosate-treated pea seedlings for 14 days

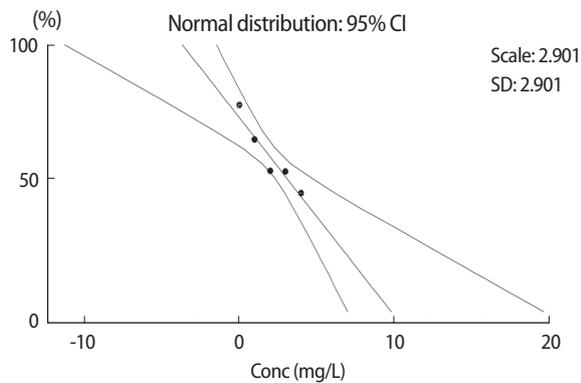
	Conc	RL	ST	Chlo-a	Chlo-b	Chlo-t	TP	SS	Na ⁺	K ⁺
Conc	1.000									
RL	-0.771	1.000								
ST	-0.927	0.922	1.000							
Chlo-a	-0.717	0.997	0.891	1.000						
Chlo-b	-0.733	0.998	0.901	1.000	1.000					
Chlo-t	-0.725	0.998	0.895	1.000	1.000	1.000				
TP	-0.860	0.959	0.970	0.941	0.948	0.944	1.000			
SS	-0.946	0.845	0.973	0.804	0.818	0.810	0.952	1.000		
Na ⁺	-0.967	0.779	0.881	0.732	0.744	0.738	0.806	0.859	1.000	
K ⁺	-0.934	0.946	0.981	0.918	0.926	0.922	0.964	0.943	0.928	1.000

Conc, concentration; RL, root length; ST, shoot length; Chlo-a, chlorophyll a; Chlo-b, chlorophyll b; Chlo-t, total chlorophyll; TP, total protein; SS, soluble sugar; Na⁺, sodium; K⁺, potassium.

Table 2. Descriptive statistical analysis 14 days after glyphosate application

	RL	ST	Chlo-a	Chlo-b	Chlo-t	TP	SS	Na ⁺	K ⁺
Mean	3.455	4.672	0.019	0.010	0.031	1.103	0.572	2.932	2.438
SD	3.606	3.523	0.040	0.020	0.062	1.117	0.328	1.657	1.223
Minimum	1.450	1.800	0.001	0.001	0.001	0.185	0.224	0.930	1.290
Maximum	9.875	10.233	0.092	0.046	0.142	2.972	1.039	5.080	4.433

RL, root length; ST, shoot length; Chlo-a, chlorophyll a; Chlo-b, chlorophyll b; Chlo-t, total chlorophyll; TP, total protein; SS, soluble sugar; Na⁺, sodium; K⁺, potassium; SD, standard deviation.

**Figure 1.** Germination probability plot. Conc, concentration; CI, confidence interval; SD, standard deviation.

RESULTS

Seed Germination

Seed germination was negatively correlated with increasing concentration of glyphosate (Table 1). In this study, 0 and 1 parts per million (ppm) glyphosate concentrations showed the highest percentage of germination, 90 and 75%, respectively, while the germination rate decreased to 55% at 2 and 3 ppm and showed a similar pattern. Treatments at 4 ppm decreased germination to 40% (Figures 1 and 2).

Root Length and Shoot Length

Average root and shoot length showed a regular decrease with increasing glyphosate concentration, reduced to 14.7 and 17.6%,

respectively, at 4 ppm. The magnitude of reduction at all concentrations was proportionate to the concentration (Figure 3A).

Protein and Soluble Sugar

There was a decreasing tendency in total protein content but it showed a marked (16.05 times) reduction at 4 ppm compared to the control. There was an increase (184.61%) in total protein content at 3 ppm compared to at 2 ppm (Figure 3B). Similarly, soluble sugar content also showed a decreasing trend in the control and treatment at 1, 2 and 4 ppm (Figure 3B) while the content at 3 ppm (0.44 mg/g) and 2 ppm (0.39 mg/g) was similar.

Chlorophyll

The chlorophyll content was also reduced significantly ($p < 0.05$) at all concentrations compared to the control (Table 2) and showed a regular trend (Figure 3C). Both Chlo-a and Chlo-b as well as Chlo-t remarkably decreased at 1 ppm, by nearly 90%. This may be due to the breakdown of chlorophyll during stress or inhibition of chlorophyll biosynthesis, a primary symptom of fluoride-induced chlorosis.

Sodium and Potassium

Glyphosate reduced the Na⁺ content and significant ($p < 0.05$) differences were detectable with an increase in glyphosate concentration. In contrast, Na⁺ content at 2 ppm (3.47 mg/g) and 3 ppm (3.55 mg/g) was more or less the same (Table 2). Related observations were also noticed for K⁺ in treated and control plants. The

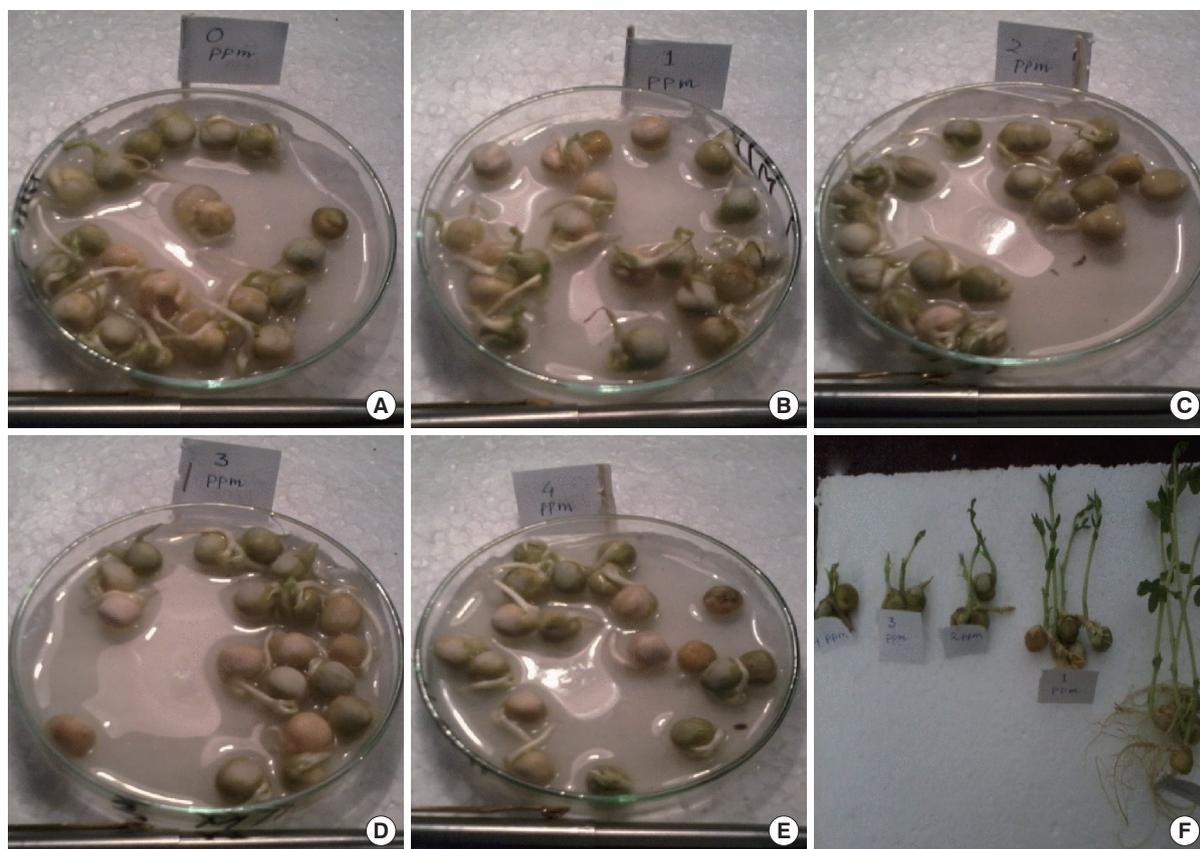


Figure 2. Comparative germination images of pea seeds in (A) control, (B) 1 ppm, (C) 2 ppm, (D) 3 ppm, (E) 4 ppm, and (F) glycosate concentrations in Petri dishes. The comparative images show seedling root and shoot length at 14 days at various glycosate concentrations. ppm, parts per million.

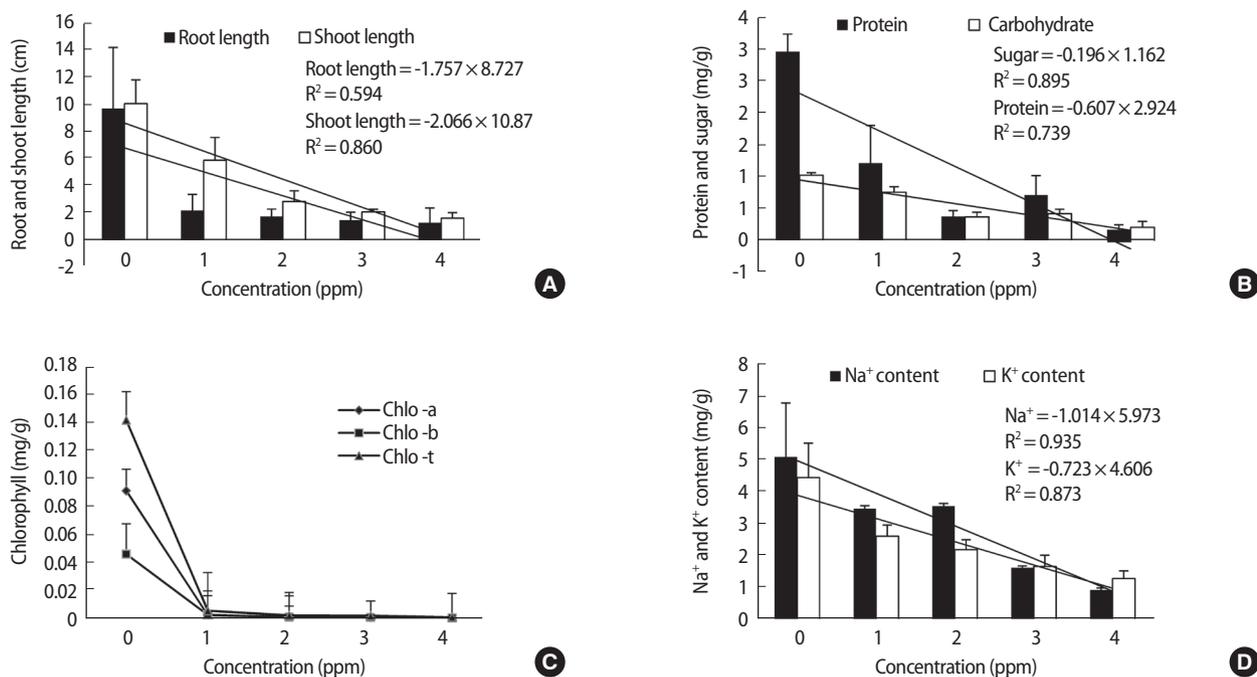


Figure 3. (A) Root and shoot length, (B) protein and soluble sugar concentration, (C) chlorophyll a (Chlo-a), chlorophyll b (Chlo-b), and total chlorophyll (Chlo-t), and (D) sodium (Na⁺) and potassium (K⁺) content of seedlings after 14 days of glycosate treatment. Regression equations and R² values are shown inside each box. ppm, parts per million.

reduction of K^+ content was significant ($p < 0.05$) (Table 2) and less likely to obtain a linear pattern than Na^+ (Figure 3D).

DISCUSSION

The metabolic reactions concerning synthesis, molecule degradation during development and germination processes in seeds are challenging to determine, as cell integrity as well as metabolism are associated with a variety of enzymes functions, which are particular to every species [22]. In a study on seed germination, crops planted immediately after glyphosate application was not injured and germination was not affected. It has been reported that glyphosate does not inhibit the germination of turf grasses [23], but at subtoxic concentrations, glyphosate can be a growth stimulant, as reported by Baig et al. [24]. The results reveal that reticence in pea germination is correlated with the concentration of glyphosate. The entry of water-soluble allelochemicals into the seeds may be the reason for inhibition of germination and growth [25]. On the other hand, the impact of glyphosate on indole-3-acetic acid, the main endogenous auxin in the plant, reduces the rate of germination and growth [26].

Application of glyphosate on a rare endemic shrub, *Pimelea spicata*, 2 common native plants and 2 environmental weed species under glass house conditions showed reduced growth and productivity of plants [27]. Seedling emergence and growth in field pea [24], *Zea mays*, *Glycine max*, and *Sorghum halepense* [28] determined the effects of glyphosate application pre-harvest and at various stages of maturity, revealing that shoot meristematic cell moisture above 40% reduced seedling germination and fresh weight of shoots.

Physiological parameters other than root length are much weaker indices of herbicide activity in different plant species measured after 6 days of germination. Inhibition of shoot growth and a lack of seed germination in watergrass, transgenic and non-transgenic soybean using glyphosate were observed by Kohata et al. [29]. The effect of glyphosate is more pronounced in roots and seedlings than matured plants. Glyphosate significantly reduces the activity of acid invertase, hydrolyzing sucrose to hexose sugars for energy production, which ultimately affects plant growth and maintenance.

A reduction in chlorophyll may decrease photosynthesis and thereby substantially decrease all metabolites, viz., total sugars, proteins and soluble amino acids. Its reduction at all concentrations is probably due to 1) degradation of chlorophyll pigments [30] and 2) a reduction of chlorophyll synthesis [31]. Relatively, photosynthesis along with the carbon export light period is inhibited by glyphosate although it has no noticeable effect on photosynthesis and the C3 cycle. But glyphosate translocation

and inhibition in carbon translocation overlap, validating that glyphosate represses the process of carbon export and eventually disrupts C3 cycle metabolism in bright light, a primary factor in the inhibition of photosynthesis and fast cessation of carbon and glyphosate translocation [32].

Changes in protein profiles and the sugar content of pea plants under different types of stress, both biotic and abiotic, have been characterized [33]. These stresses are correlated with anti-oxidant enzymes, along with superoxide dismutase and nucleoside diphosphate kinase. Plants absorb glyphosate from the soil matrix. It impedes the shikimate pathway, leading to a reduction in the biosynthesis of amino acids (aromatic) and resulting in a disturbance of protein synthesis and metabolic interruption in the phenylpropanoid pathway. The alteration in shikimic acid of cotton (*Gossypium hirsutum* L.) at different concentrations of glyphosate was obtained by Pline et al [34].

Glyphosate toxicity restrains 5-enolpyruvylshikimic acid-3-phosphate synthase (EPSP) enzyme activity. EPSP is essential for tyrosine, phenylalanine and tryptophan production, aromatic amino acids associated with protein synthesis and primary and secondary metabolism [35]. Glyphosate and phosphoenolpyruvate (PEP) may perform as competitive inhibitors, which necessitates precursors to amino acid production. Injury to meristematic tissue is attributed to inhibition of branched-chain amino acids in the region. It also affects amino acids from pre-existing proteins, which results in a deficiency in the protein store, present in mature tissues [36]. Inhibition of EPSP and PEP activity due to glyphosate toxicity may be responsible for reducing total protein content. The sucrose content is negatively correlated with the activity of soluble acid invertase due to intrusion in photochemical sugar biosynthesis.

Terrestrial plants evolved from sea bryophytes to flowering plants from a K^+ -rich system in the Cambrian era when the concentration of K^+ was lower than in the sea (oligotrophic system). This insufficiency developed the hydraulic cell movement of K^+ [37]. So, analysis is required of a feasible disorder in Na^+/K^+ actions, storage and uptake in cells or tissues in glyphosate toxicity, which may limit translocation of Na^+/K^+ to the shoots, root and cells.

Dealing with cations is a key problem for plants. K^+ plays a crucial role in plants, such as in activation of enzymes and coenzymes, protein formation, sugar transport and photosynthesis. Deficiency of K^+ in plants does not show any immediately visible symptoms; initially, it only inhibits the growth rate and later causes chlorosis and necrosis [38]. From the cradle of evolution in the sea, K^+ was used by organisms as a major cation to maintain electroneutrality and osmotic equilibrium in cells. Uptake of K^+ ions is desirable to build up osmotic potential and to ab-

sorb water and sustain turgor; however, Na⁺ acts as a toxic element [39]. K⁺ present in the cell regulates protein activity which is dependent on K⁺-protein interactions and thus helps in various biochemical processes. This interaction depends on the topology of their electrical charge-density, a unique electrochemical property of K⁺ ions, which are completely different from any other cationic process in their electron shell configuration and arrangement of the surrounding hydration shell.

In conclusion, negative side-effects on plant growth and nutrient status may be caused by glyphosate application at the recommended dosage. The physiological responses showed significantly reduced seed germination, shoot and root length. The chlorophyll content was reduced significantly at all concentrations, which may affect the sugar content. The correlation study revealed that there is a negative effect on Na⁺/K⁺ content like others. Finally, the maximum inhibitory effect was found in total protein content which may extensively diminish growth and production in addition to the food value of pea seeds.

CONFLICT OF INTEREST

The authors have no conflicts of interest associated with the material presented in this paper.

ORCID

Subinoy Mondal <http://orcid.org/0000-0001-6212-2423>
 Mousumi Kumar <http://orcid.org/0000-0001-9082-7762>
 Smaranya Haque <http://orcid.org/0000-0001-5634-8434>
 Debajyoti Kundu <http://orcid.org/0000-0002-3981-3055>

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