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Novel polymorphisms in GPX5 gene and their association with reproductive and productive traits in Lumsniang (*Hampshire* x *Niang Megha*) pigs under subtropical hilly region of Meghalaya

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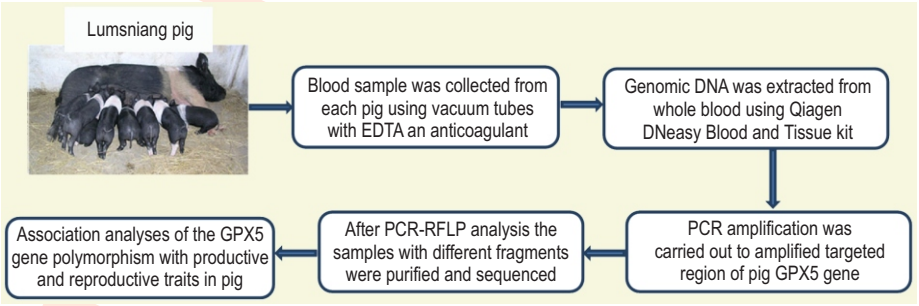
Abstract

Aim: To detect genetic variants of GPX5 gene and their associations with reproductive and productive traits in the Lumsniang (*Hampshire* x *Niang Megha*) pigs.

Methodology: In this study, a total of 87 Lumsniang pigs were selected for collecting blood samples (3-5 ml) from Nucleus Pig Breeding Farm of ICAR Research Complex for North Eastern Hilly region, Umiam, Meghalaya. Genomic DNA was extracted from the blood leukocytes using the Qiagen DNeasy Blood and tissue kits and diluted to working concentration (50 ng μl^{-1}) and stored at -20°C, which were used as templates for association of GPX5 variants with polymerase chain reaction. The data for reproductive and productive traits were recorded from all the animals taken in this study. The reproductive and productive traits were analyzed using a general linear model (GLM) procedure of SPSS Version 16.0.



Results: Polymorphisms at Intron 1 of the GPX5 gene was determined by PCR-RFLP with HinfI restriction enzyme and DNA sequencing analyses. Two alleles and three genotypes were identified by HinfI digestion of the GPX5 gene. In the SNP-HinfI (g.1896A>G) locus, significant associations were found between GPX5 genotypes and litter size at birth. The genotype AA demonstrated significantly ($P<0.05$) superior litter size at birth as compared to AG and GG genotypes at the SNP-HinfI locus in Lumsniang pig.

Interpretation: The study reported probable associations of GPX5 variants with reproduction and production traits in Lumsniang pig. The genotype AA at locus SNP-HinfI improved the litter size at birth in the studied population. The GPX5 gene may be considered as a DNA marker for marker-assisted selection after validation in a large pig population.

Key words: GPX5 gene, Lumsniang pig, Polymorphism, Productive traits, Reproductive traits

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Introduction

Livestock farming is an imperative subsector of agriculture and pig rearing is one of the important livestock modules of the tribal population of Meghalaya. Among all the livestock species, pig plays an important role in the livelihood of tribal people of Meghalaya, accounting 1.69% of the total livestock population (BAHS, 2019). It provides livelihood security to the rural population and plays a significant role in improving the socio-economic status of tribal population and vulnerable sections of the communities (Talukdar *et al.,* 2019). Among different kinds of meat, pork is the most desired meat and contributes more than 70% of the total meat consumption in this region (Kumaresan *et al.,* 2006b). There is a substantial gap between supply and demand of pork in the North-East (41.02 percent) region of India as compared to the remaining states of the country (27.40 percent) (Mahajan *et al.,* 2015). To bridge this gap, the institute developed two breed crosses of pigs (Lumsniang) by combining the inheritance of indigenous breeds, Niang Megha (NM), and exotic breeds, Hampshire, to increase pig productivity and production of high-quality pork in accordance with consumer preferences in the region (Kadirvel *et al.,* 2021).

Pig farming productivity and profitability are influenced by sow reproductive efficiency (measured in piglets weaned per sow per year) (Dall'Olio *et al.,* 2013). Improvements in sow reproductive performance are slow in porcine production due to low heritability of reproductive traits (Goddard and Hayes, 2009; Bidanel, 2011). Litter size is one of the important reproductive traits in pig production, as an increase in the number of pigs weaned per sow will increase economic returns for the swine farming sector (Sato *et al.,* 2016; Metodev *et al.,* 2019; An *et al.,* 2019). Genetic selection is employed in conjunction with traditional selection methods using suitable markers for improving reproductive traits in swine, therefore, it is necessary to identify and test suitable genetic markers. While using marker-assisted selection (MAS) programmes on different livestock populations, several researchers have discovered that the positive selection of pigs is associated with specific genes related to lactation, reproduction, carcass quality and growth traits (Fang *et al.,* 2014; Fischer *et al.,* 2015; Matika *et al.,* 2019; Zhuang *et al.,* 2020).

Recent development of sequencing and genotyping tools for pigs have permitted the investigation of genomic evidence of selection and the discovery of potential genes linked with desired traits (He *et al.,* 2021). For example, the bone morphogenetic protein receptor type 1B (BMPR-1B) gene in sheep may have foremost effect on their reproduction (Qi *et al.,* 2020). The equivalent gene in pigs is estrogen receptor gene (ESR) and follicle-stimulating hormone beta subunit (FSHB) and several other genes are the most promising candidate genes associated with reproductive traits (Bernard and Tran, 2013). Apart from the ESR and FSHR genes, sows litter size can be affected by mutations in cytochrome B, prolactin (PRL), swine leukocyte antigen 11 (SLA-11), and other genes (Korwin-Kossakowska *et al.,* 2009; Pradhan *et al.,* 2018; Zhang *et al.,* 2019). Quantitative traits, which are mostly affected by minor genes, are being used to assess porcine reproductive performance. In an attempt to discover quantitative trait locus (QTL) in animals, researchers employ two methods: linkage analysis to map genes and analyzing the effect of polymorphisms in candidate genes for the trait of interest (Polasik *et al.,* 2017). There are 29,865 QTL associated with 688 unique traits, according to Pig QTLdb (Hu *et al.,* 2019). Glutathione peroxidase 5 gene (GPX5) is located in a swine chromosome region 7 (SSC7), where several quantitative trait loci (QTL) have been identified for reproductive traits in pigs (Bertani *et al.,* 1999).

GPX5 is a noncanonical selenium-independent GPX gene, which encodes an epididymis-specific enzyme in mice, rats, pigs, monkeys as well as in humans (Terman *et al.,* 2016). GPX5 accounts for more than 95% of epididymal GPX mRNA and protein when combined with GPX3 (Brigelius-Flohé and Maiorino, 2013). GPX5 protects epididymis-transiting sperm cells against ROS-mediated loss of integrity by acting as a functional reactive oxygen species scavenger (Noblanc *et al.,* 2011). Therefore, GPX5 linkage studies demonstrated that this gene is strongly related to the major histocompatibility complex (MHC), which has been associated with reproductive traits in pigs (Buske *et al.,* 2005). The purpose of this study was to estimate relative genotype and allele frequencies of single nucleotide polymorphisms (SNPs) in intron 1 of the GPX5 gene in Lumsniang crossbred pigs (*Hampshire* x *Niang Megha*) as well as to determine possible association between individual genotypes and some reproductive and productive traits. This study contributes to the understanding of genetic variants of the GPX5 gene and molecular marker-assisted selection in pig breeding.

Materials and Methods

Geographical location and climatic description: The experimental farm is located in the subtropical eastern Himalayan hilly climate and temperature rises in the summer season (May to August), ranging between 28°C to 29.30°C, and experiences maximum rainfall from May to September with annual precipitation ranging from 2500 to 3000 mm whereas winter season (November to February) is very cold with temperature ranging from 12.3°C to 5.5°C. The experimental plan of the study was duly permitted by the Institution of Animal Ethics Committee (IAEC: RC/IAEC/2020/2) of the ICAR Research Complex for the North Eastern Hilly (NEH) Region, Umiam, Meghalaya, India.

Experimental animals and data collection: A total of 87 multiparity Lumsniang (*Hampshire* x *Niang Megha*) sows of three families with 267 litters were collected from nucleus pig breeding farm of ICAR Research Complex for North Eastern Hilly region, Umiam, Meghalaya. All animals were reared under uniform housing and feeding conditions. The sows were moved to a farrowing unit twenty to thirty days before the estimated farrowin date. The data for reproductive and productive traits were recorded for all the animals taken in this study. These traits included age at puberty, age at first conception, age at first

farrowing, farrowing intervals, litter size at birth, litter size at weaning, average birth weight, average weaning weight; and body weight were recorded at monthly intervals.

Extraction of Genomic DNA: Blood samples (3-5 ml) were collected from the anterior vena cavaunder sterile conditions using lithium coated vacutainer tubes containing heparin as an anticoagulant. Genomic DNA was extracted from the blood leukocytes using the Qiagen DNeasy Blood and tissue kits and diluted to working concentration (50 ng μl^{-1}), stored at -20°C.

PCR amplification and restriction digestion: Amplification reaction was carried out in a 25 µl volume containing 50 ng μl^{-1} genomic DNA, 1.0 µm of each forward and reverse primers, PCR Master Mix (2X) (Thermo Fisher) of 12.5 µl and 8.5 µl water. Primers used in this study were F-TTCATGTAGAACTTATTTCTG and R-TGACTTACCCATTCTTCAG (Buske et al.2006)and targeted a span of 501 bp of swineGPX5. The PCR program included in following stages: initial denaturation at 95°C for 3 min, followed by 37 cycles of denaturation at 95°C for 30 sec, annealing at 52°C for 45 sec, extension at 72°C for 1 min, and a final extension at 72°C for 5 min in an Eppendorf Thermal cycler. Amplified products were electrophoresed on 1.8% agarose gel at constant voltage and 1X TAE for 1 hr. The gels were stained with ethidium bromide and visualized by GeNei™ Imaging System. The restriction digestion was carried out in 20 µl of reaction mixture of each sample containing 10 µl of PCR product, 9.0 µl of 10 X buffer, and 1.0 µl of Hinf1 enzyme (Promega). The reaction mixture was incubated overnight at 37 °C, and the resulting products were separated by 3.5% agarose gel and visualized on a U.V. transilluminator.

Statistical analyses: The population parameters were estimated by PopGene version 1.32 (Yeh *et al.,* 1999). Association between SNP locus with reproduction and production traits were investigated using a general linear model technique of SPSS Version 16.0.

Results and Discussion

An amplified PCR product of 501 bp size was observed in the studied breed on amplification of Intron 1 of the GPX5 gene (Fig. 1A).The PCR product of similar size has also been reported by Buske *et al.* (2006) in commercial pig population (Large White x Landrace) and Terman *et al.* (2016) found in wild boars by amplification of this intron of the GPX5. When these fragments were digested with a restrictionenzyme, Hinf1, revealed three band patterns (Fig.1B) in the studied breed, which indicated that the GPX5 region under study was polymorphic. The fragments detected in the present study showed that the presence of one restriction site on one allele resulted in the appearance of two bands of 234, 94bp for allele 2B (A), 298, 94 for allele 1B (G) and the fragments of 64, 53, 33 and 23 bp length were not visible on agarose gels due to its small size.

The genotypic frequencies of AA, AG and GG were 0.157, 0.478 and 0.365 and allelic frequencies of A and G were 0.396 and 0.604, respectively, in the studied breed. The frequency of 1B2B (AG) genotype obtained in the present study is in consonance with the findings of Terman *et al.* (2016), who reported 1B2B genotype was the most frequent genotype with a frequency of 0.50 and the 1B1B genotype was least frequent (0.12) in wild boars. Likewise, Buske *et al.* (2006) also found that

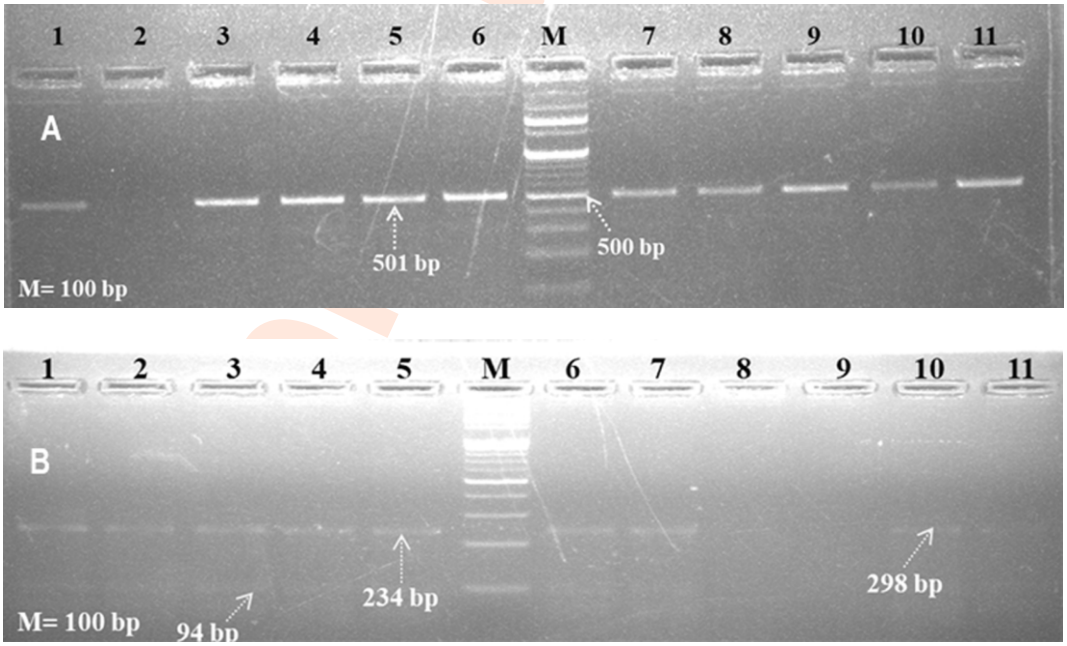

Fig. 1: GPX5 gene in Lumsniang pig: (A) PCR amplification product and (B) Electrophoresis pattern of polymorphisms.

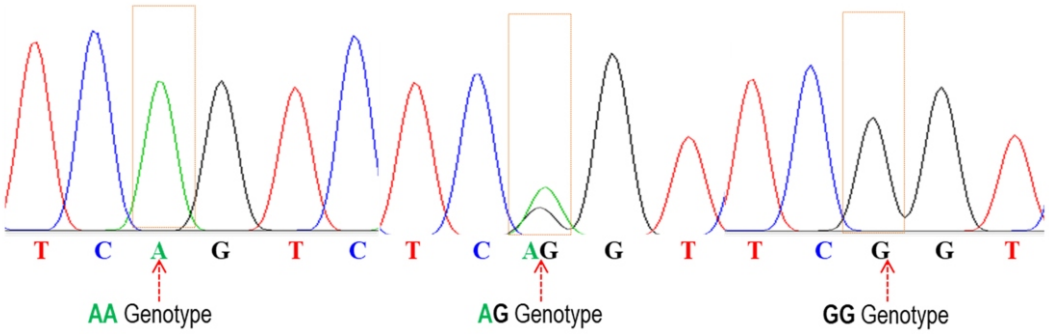

Fig. 2: Chromatogram showing polymorphism at SNP-Hinf1 locus (g.1896A>G) of GPX5 in Lumsniang Pig. (SNPs, single nucleotide polymorphisms; GPX5, Glutathione peroxidase).

Table 1: Least squares mean (LSMEANS) and standard errors (SE) for reproductive traits of different genotypes of GPX5 gene in Lumsniang pig.

SNP	Genotypes	AP (Days)	AFC (Days)	AFF (Days)	FI (Days)	LB (No.)	LW (No.)	ABW (Kg)	AWW (Kg)
SNP- Hinf1	AA (20)	288.83±1.06	331.18±2.52	451.96±1.21	209.18±1.02	10.67±0.16[a]	8.92±0.46	1.33±0.02	10.89±0.15
(g.18	AG (29)	287.41±1.56	328.37±1.40	450.41±1.86	210.32±1.91	8.45±0.35[b]	8.04±0.17	1.17±0.41	9.76±0.45
96 A>G)	GG (38)	288.59±1.04	328.49±1.19	450.84±1.90	208.88±2.10	8.12±0.24[b]	7.56±0.24	0.95±0.13	9.02±0.26

AP=Age at puberty; AFC=Age at first conception; AFF=Age at first farrowing; FI=Farrowing intervals; LB=Litter size at birth; LW= Litter size at weaning; ABW=Average Birth weight; AWW= Average weaning weight. Figures in parenthesis are number of animals; Figures with dissimilar superscript differ significantly.

Table 2: Least squares mean (LSMEANS) and standard errors (SE) for productive trait of different genotypes of GPX5 gene in Lumsniang pig

SNP	Genotypes	BW (30 days)	BW (60 days)	BW (90 days)	BW (120 days)	BW (150 days)	BW (180 days)	BW (210 days)	BW (240 days)
SNP- Hinf1	AA (20)	6.40±0.08	10.33±0.27	15.32±0.15	22.10±0.31	30.01±0.24	43.43±0.31	51.22±0.16	64.38±0.84
(g.18	AG (29)	5.77±0.12	9.62±0.08	14.94±0.18	22.49±0.24	29.67±0.16	44.50±0.67	50.54±0.36	63.85±0.52
96A>G)	GG (38)	5.48±0.02	10.11±0.13	14.31±0.01	21.17±0.53	28.34±0.63	44.83±0.24	52.31±0.72	61.85±0.02

BW: Body weight in kg. Figures in parenthesis are number of animals.

the 1B1B genotype had the lowest frequency (0.06) in crossbred (Large White x Landrace) sows x Leicome boars. However, Mackowski *et al.* (2004) found a higher frequency of 2B2B genotype (0.51) and a similar frequency of 1B2B genotype (0.46) in boar semen. This discrepancy might be the result of breed differences or sampling variations in the populations under investigation. For assessing the Hardy Weinberg equilibrium (HWE) of the studied population, genotype and allele frequencies were determined, and the Chi-square (χ^2) test was used.

The Chi-square value obtained was 8.331, which was higher than the critical value. It was not determined to be in Hardy-Weinberg equilibrium because the population under investigation had been under selection for production and reproduction traits for years. Thus, genetic variations could be probably affected by artificial selection, because selection may significantly change the genotypic and allelic distribution of GPX5 gene. Terman *et al.* (2016) also found that the investigated pig populations were not in Hardy-Weinberg equilibrium, which is compatible with our findings. In this study, a total of one SNP locus in GPX5 *i.e.,* SNP-HinfI (g.1896A>G) detected in the Intron 1, and no mutation caused a change of amino acid (Fig. 2). There is evidence to suggest that Intron plays an important role in regulating post-transcriptional regulation, mRNA splicing, and gene expression although Intron does not code protein (Dwyer *et al.,* 2021), hence, the SNP located in the intron could be significant for the function of protein into full play. The values of the population genetics parameters in Lumsniang pig population for GPX5 gene were as follow: Effective number of alleles (ne) was 1.917, Shannon's Information Index (I) 0.671, Expected heterozygosity (Nei) 0.478 and Polymorphism Information Content (PIC) 0.364.

The PIC value (0.364) indicated that the GPX5 gene in the investigated population had medium (0.25<PIC<0.5) genetic diversity. Association analyses of the GPX5 gene polymorphism with production and reproduction traits are presented in Table 1 and 2. The results showed that least square mean (LSM) values for the genotype AA (10.67±0.16) demonstrated significantly

(P<0.05) superior litter size at birth compared to AG (8.45±0.35) and GG (8.12±0.24) genotypes, while the LSM values for other reproduction traits *viz.,* age at puberty, age at first conception, age at first farrowing, farrowing intervals, litter size at weaning, average birth weight and average weaning weight in different genotypes varied, but these differences were not statistically significant (P>0.05) in the studied population (Table 1).

Association of the porcine g.1896A>G polymorphism with production traits is shown in Table 2. There was no significant association of porcine g.1896A>G polymorphism with any production traits in the Lumsniang pigs. However, the sows with the AA genotype had higher body weight values than the sows with the AG and GG genotypes, but these differences were found to be statistically non-significant (P>0.05). Thus, the porcine g.1896A allele seems to be a beneficial allele for litter size traits in pigs. These findings are consistent with those of Zhang *et al.* (2010), who reported that GPX5 mutations were not substantially linked with production traits in a population of F1 hybrid pigs. Similarly, Buske *et al.* (2006) did not find association between GPX5 polymorphism and performance traits in commercial F2-sows. Anastasiadou *et al.* (2017) reported that the GPX5 variants had no significant effects on any of the reproductive traits in Greece pigs. However, in another study, this GPX5 gene exhibited 1B1B genotype which was favourable for all reproductive traits in large white x landrace crossbred sows (Polasik *et al.*, 2017).

Contrary to the present findings, Dall'Olio *et al.* (2013) reported that SNPs in BMPR1B, FUT1, GPX5, RBP4 and TGFBR1 genes showed significant association (P<0.003) with NBA (number of piglets born alive at first parity), EBVs (estimated breeding values for the number of piglets born alive at first parity) in Italian Large White purebred sows. In addition, Gondim *et al.* (2019) analyzed significant associations between MC4R, FABP3, and DGAT1 genes polymorphisms and the reproductive traits in pigs (Large White x Landrace Danish). Barranco *et al.* (2016) also observed the presence of GPX5 in seminal plasma and identified that boars with high GPX5 levels had higher farrowing rates and litter sizes than boars with low GPX5 levels. Moreover, Terman *et al.* (2016) studied the relationship between GPX5 genotypes and selenium concentrations in the liver and kidneys of wild boars and their findings revealed the highest selenium concentrations in the liver and kidneys of wild boars with the 2B2B genotype as compared to 1B1B and 1B2B genotypes of animals.

Recently, Rahman *et al.* (2021) studied polymorphism of ESR gene in doom pigs via gene sequencing and PCR-RFLP techniques, and its correlation with litter traits. They stated that the AB genotype had higher LSM for litter size at birth, litter size at weaning, litter weight at birth and litter weight at weaning compared to AA genotype, however, these differences were found to be statistically non-significant (P>0.05).Likewise, Michos *et al.* (2021) reported in significant relationship of GPX5 with litter sizes, however, significant (P>0.04) and positively correlated with farrowing rates (6.7%) in pigs. The results obtained in this study indicate that the GPX5 gene might play an important role in paternal (sperm quality) rather than maternal fertility in sows (Kmiec *et al.,* 2007; Dall'Olio *et al.,* 2012; Rui-lan *et al.,* 2015). Inconsistent results about the effect of SNP-Hinf1 locus could be attributed to difference in sample size, population structures, environmental factors and statistical models used, or epistatic interactions affecting litter size with population specific genetic backgrounds. Limited literature is available on association of the GPX5 gene with litter size and productive traits in pigs. However, numerous studies have demonstrated that polymorphism of GPX5 gene are associated with the litter size traits in sows. Therefore, it was explored whether the accumulated favorable alleles of the porcine GPX5 genotypes are associated with litter size traits in pigs. Interestingly, the significant effect of the favorable genotype (AA) of porcine GPX5 gene on litter size traits was exhibited.In addition, the accumulated favorable allele (A) was identified as a beneficial genotype with the highest values for reproductive and productive attributes.

This evidence indicates that there are strong additive effects of the accumulated favorable alleles of porcine GPX5 gene on the litter size traits and the increasing numbers of favorable genotypes seem to enhance litter size traits in these sows. Therefore, the favourable genotype gained can be exploited in marker-assisted selection to select individuals with superior litter size traits. These findings emphasize the importance of the porcine GPX5 gene in the reproductive traits of pigs. Therefore, the porcine GPX5 gene may be used as a potential candidate gene for the genetic improvement of litter size traits in the pig breeding industry. Further studies are required to confirm the association of this SNP with litter size traits in larger population and various commercial pig breeds.

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Add-on Information

Authors' contribution: R. Kumar: Conceptualization, Experimental designing, data compilation and manuscript preparation; **G. Kadirvel:** Revision, editing and fund acquisition; **G. Khargharia:** Blood sample collection; **S. Deori:** Revision and editing; **S. Doley:** Supervision and editing; **M. Das:** Methodology, revise and editing; **N. Uttam Singh:** Data analysis; **M. Singh:** Revision and editing; **K.K. Baruah:** Investigation and overall supervision of the experiment; **V. K. Mishra:** Overall support.

Research content: The research content of the manuscript is original and has not been published elsewhere.

Ethical approval: The animal study was approved by the Institution of Animal Ethics Committee (IAEC, Registration No.

RC/IAEC/2020/2).

Conflict of interest: No potential conflict of interest was declared by the author (s).

Data from other sources: Not applicable.

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology*.

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