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Flood proofing of Ranjit, a popular variety of North-Eastern India through transfer of *Sub1* rice QTL by modified marker-assisted backcross breeding

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Abstract

Flooding is one of the major constraints of rice production in the rainfed lowland rice ecosystem. To cope with the frequent flooding in the climate change regime, tolerant cultivars are urgently needed to protect the paddy crop from flash floods. With the help of molecular markers, a major QTL for submergence tolerance "*Sub1*" was introgressed into a rainfed lowland mega variety Ranjit of N.E. India, by backcrossing followed by two generations of selfing. In this modified marker-assisted backcrossing (MABC), a gene based marker Sub1BC$_2$ and 50051 SNPs were used for foreground and background selection, respectively, in backcrosses between a *Sub1* donor, Swarna-*Sub1* and the recipient variety, Ranjit. The recombinant selection was skipped and background selection was deferred to BC$_2$F$_2$ generation. The size of the donor fragment was found to be 1.65Mb. The introgression of this small segment was possible due to large population and strict selection in segregating generations for the recurrent parent with the help of 62 DUS characters. Ranjit-*Sub1*, a selection in BC$_2$F$_2$ generation, showed background recovery of 96.54% and submergence tolerance similar to the tolerant donor parent. Yield, yield-components and grain physico-chemical properties showed successful recovery of these traits in Ranjit-*Sub1*, with yield potential ranging from 6.5 to 7.0 t/ha, not significantly different from the recurrent parent, Ranjit. Therefore, Ranjit-*Sub1* has been recommended for Zone III for submergence stress conditions in India. The study demonstrates a rapid and highly precise strategy adopted to introgress a major QTL by BC$_2$F$_2$ generation into a modern rice variety using a modified MABC.

Key words: Marker Assisted Backcross Breeding (MABB), *Sub1* QTL, Ranjit, rainfed lowland rice, submergence tolerance

Introduction

Submergence caused by flash-flooding is one of the important stress in agriculture of tropical Asia. In Assam state located in north eastern part of India, rice is cultivated in an area of 2.6 million ha, of which around one million ha is affected by flash flooding. Flash floods regularly affect rainfed lowland rice ecosystems, where floodwater stagnates for 2 weeks. Traditional varieties adapted to the submergence prone environments are low yielders. Therefore, improved varieties with submergence tolerance are required for this type of ecosystem to improve productivity of Eastern India including Assam. Because of the susceptibility of rice and the prevalence of this stress, submergence tolerance has been an important breeding objective for decades in rainfed lowland areas. However, many varieties have not been adopted by the farmers in submergence prone areas, due to lack of submergence tolerance and the quality. Though in recent years, a number of submergence tolerant high yielding varieties have been developed, these varieties have not been accepted by farmers due to the lack of desirable characteristics of the popular and widely grown varieties, such as plant stature and good grain quality (Septiningsih et al. 2009). The submergence tolerant varieties with *Sub1* are still susceptible to many diseases and other abiotic stresses and these stresses need to be overcome through further breeding efforts (Ismail et al. 2013). There are several rice varieties grown in Assam, of which Ranjit is the most popular, high yielding variety covering more than 50% area.

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Unfortunately, this mega-variety is susceptible to submergence, which causes major yield losses under submergence.

Mackill (2006) proposed that adoption of a completely new variety could take considerable time, whereas the chances of rapid adoption of popular varieties converted through marker assisted backcrossing (MABC) were relatively higher. MABC is a precise and effective method to introgress a single locus controlling a trait of interest while retaining the essential characteristics of the recurrent parent (Collard and Mackill. 2008). MABC has three main advantages over conventional backcrossing. Firstly, DNA markers can be used for simple and efficient selection of the target locus (foreground selection). Secondly, the size of the donor chromosome segment containing the target locus can be minimized (recombinant selection). Thirdly, the recovery of the recurrent parent genome (RPG) can be accelerated by selecting backcrossed lines with a higher proportion of RPG (background selection) as described by Ellur et al. (2016). This approach has been used with great success for 'enhancing' rice varieties for traits such as submergence tolerance through transfer of *Sub1* (Neeraja et al. 2007 and Septiningsih et al. 2009). However, despite these successes, there is still need to utilize flood-prone ecosystems for enhancing rice production needs attention. The MABC approach described here is a simplified version of MABC approach designed to achieve maximum benefit of submergence tolerance in a relatively short time with lesser laboratory work.

The main objective of the present study was to convert the mega rice variety, Ranjit into a submergence tolerant variety by incorporating *Sub*1, a major QTL on chromosome 9 (Hospital. 2003).

Materials and methods

Plant material and crossing scheme

Swarna-*Sub1*, a submergence tolerant version of popular mega variety of Eastern India, Swarna (MTU 7029) was used as donor of *Sub1* gene (Neeraja et al. 2007). Swarna-*Sub1* is not so popular in Assam due to its dwarf plant stature. The thirty days old seedling height of this variety is only around 25cm which is not suitable for transplanting in the flood prone, lowland rainfed ecosystem of Assam. Therefore, the widely grown mega variety of Eastern India, Ranjit, which is susceptible to submergence, is used as the recipient for transferring *Sub1*. Ranjit is a semi-tall variety derived from cross, Pankaj/Mahsuri. Moreover, the 30 days old seedling height of Ranjit is around 35cm suitable for transplanting in the rainfed, lowland flood prone ecosystem. Swarna-*Sub1* was used as the *Sub1* donor due to its similarity and shared genealogy with the variety, Ranjit (Rani et al. 2014).

Marker Assisted Selection (MAS) scheme followed in transferring *Sub1* to Ranjit is depicted in Fig 1. Ranjit was crossed with Swarna-*Sub1* to obtain F_1 seeds. These F_1s were backcrossed to Ranjit and BC_1F_1 seeds were thus obtained. A total of 1090 BC_1F_1 plants were screened using gene based marker, Sub1BC_2 (Septiningsih et al. 2009) and the selected heterozygous plants were further backcrossed to Ranjit to obtain BC_2F_1 generation. A total of 2100 BC_2F_1 plants were screened with Sub1BC_2 maker. The plants selected for crossing had more than 80% morphological similarity to Ranjit and the introgressed *Sub1* QTL. Selection was based on plant height, days to 50% flowering, grain characters along with 80% similarity of other DUS characters in BC_1F_1 and BC_2F_1 generations. Out of these, 2100 BC_2F_1 plants, 971 plants were found to be carrying *Sub1* gene in heterozygous condition. Based on morphological characteristics, the plant population was reduced to 26 plants. These 26 desirable plants were named A to Z, selfing was carried out and a population size of 384 BC_2F_2 of each line was further raised. Foreground selection in these 384 plants of 26 populations, totalling 9,984 BC_2F_2 plants were raised and allowed to mature, and prior screening of the population was done morphologically. 493 Ranjit type plants were thus selected and molecular marker screening of these plants (foreground selection) was carried out. Of these, 70 lines have shown the presence of *Sub1* gene in homozygous condition. These selected lines were further screened phenotypically and 25 plants which showed close resemblance to Ranjit were selected. Background selection was carried out only in 20 BC_2F_2 plants that were selected on the basis of DUS parameters. Finally one plant, E-6 was selected based on its morphological similarity to Ranjit. To know the size of the introgressed segment in BC_2F_2 population, an analysis was carried out with the help of flanking markers RM 316 (1.8cM) and RM8300 (8.4cM).

Molecular marker analysis and foreground selection

DNA was extracted from young leaves of 2-week-old plants using a modified protocol as described by Zheng et al. (1995). PCR was performed in 20µl reactions

containing 25ng of DNA sample, 2µl of 10X PCR buffer (200mM Tris-HCl pH 8.3, 500mM KCl, 15mM $MgCl_2$), 0.50 µl of 1mM dNTP, 0.50 µl each of forward and reverse primers and 1 µl of Taq DNA Polymerase (4U/µl) using an Eppendorf dual 96-well thermal cycler. After initial denaturation for 5 min at 94°C, each cycle comprised of 1min denaturation at 94°C, 1min annealing at 55°C, 1 min extension at 72°C and a final extension at 72°C for 2 min at the end of 35 cycles. The PCR products were visualized on 3.5% agarose gel.

For the foreground selection gene based marker Sub1BC_2 located between *Sub1B* and *C* gene at a position of 6.2-6.3Mb or 4.4-6.8cM on chromosome 9 was used due to its clear co-dominant nature and easily scorable bands with a difference of 29bp which can be conveniently separated in an agarose gel (Septiningsih et al. 2009).

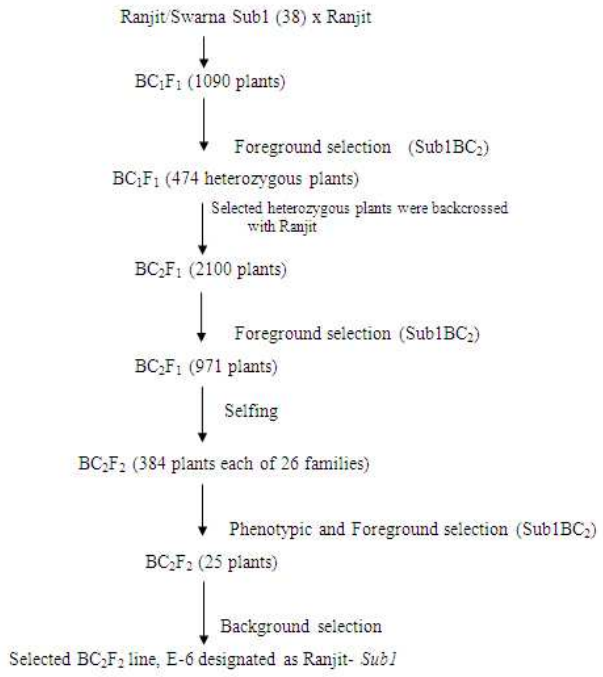


Fig. 1. Scheme for the development of the submergence tolerant Ranjit-Sub1

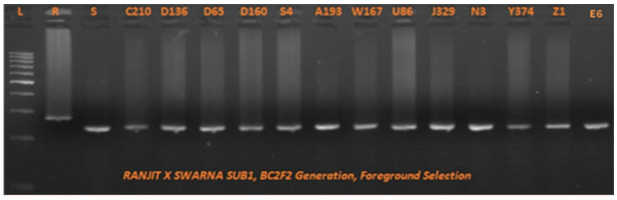
Determination of genetic background and screening for submergence tolerance

High quality DNA (260/280 and 260/230 values in the range of 1.8-2.0) was used on Affymetrix chip. The steps involving DNA amplification, fragmentation, chip hybridization, and ligation was based on the Affymetrix SNP 6.0 protocol. Approximately 0.75-1µl genomic DNA was labelled overnight at 25°C using 3 volumes of the Bio Prime DNA labelling reactions. The labelled DNA was ethanol precipitated, re-suspended in 40µl of H_2O and then added to the Affymetrix SNP 6.0 hybridization cocktail. Staining and scanning was performed using Gene Titan integrated platform (http://www.affymetrix.com). An in-house designed Affymetrix rice SNP genotyping chip 'OsSNPnks' (Singh et al. 2015) was used for background selection of 20 BC_2F_2 lines for each donor-recipient combinations. The assay includes 50,051 high quality non-redundant SNPs mostly representing single-copy (SC) genes from whole rice genome with an average distance of 7.77 Kb between adjacent SNPs. Moreover 62 morphological characters of Ranjit, Swarna-*Sub 1* and 25 BC_2F_2 lines were also recorded for background analysis.

Submergence screening was performed using standard protocols (Xu et al. 2000). Seeds of the selected plants of BC_2F_2 along with parents and resistance check FR13A, susceptible check IR42 were germinated in rows in submergence tank of 20m x 10m. The depth of the tank was 2.5m. Ten-day-old seedlings were submerged for 14 days. The survival of plants was scored 10 days after de submergence (calculated as a percentage). The percent survivals of the lines were calculated after de-submergence.

Results

The hybridity of F_1s were confirmed using foreground marker Sub1BC_2 and only 38 true F_1s were used for backcrossing. Plants heterozygous at *Sub1* locus were selected in each backcross generation. Recombinant selection was skipped in this modified MABC. For this, a large population was grown in both the generation to get a desirable recombinant. However, in most of the selected BC_2F_2 plants *Sub1* locus was fixed for donor parent allele (Fig. 2). To know the segment of donor



L= 100bp ladder, R= Ranjit, S= Swarna-*Sub1*

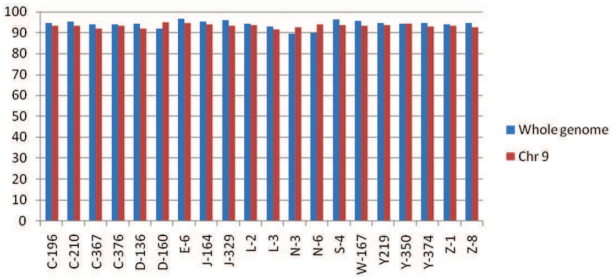
Fig. 2. Foreground selection for *Sub1* locus in BC_2F_2 population using Sub1BC_2 marker

chromosome introgression a pair of markers RM316 (1.8cM) and RM 8300 (8.4cM) flanking to *Sub1* locus were used in BC_2F_2 generation. The size of the donor

fragment was found to be 1.65Mb. Further, background selection and DUS characterisation of the BC_2F_2 plants homozygous for *Sub1* were carried out.

Screening of 26 lines was carried out and survival percentage of the *Sub1* introgressed lines were recorded which was in more than 80% cases as donor variety Swarna-*Sub1*. One of the line Y-355, line number 15 could not survive and it was found that *Sub1* QTL was absent in that genotype (Fig. 3).

In the present study the background selection was done using a high density SNP chip in 20 advanced BC_2F_2 lines derived from a cross of Ranjit/Swarna-



X axis - BC_2F_2 plants, Y axis- % RPG recovery and selected line E-6 showed 96.54% recovery of RPG

Fig. 4. Percent RPG recovery in the carrier chromosome 9 and in whole genome in *Sub1* introgressed lines in Ranjit background using 50K SNP chip

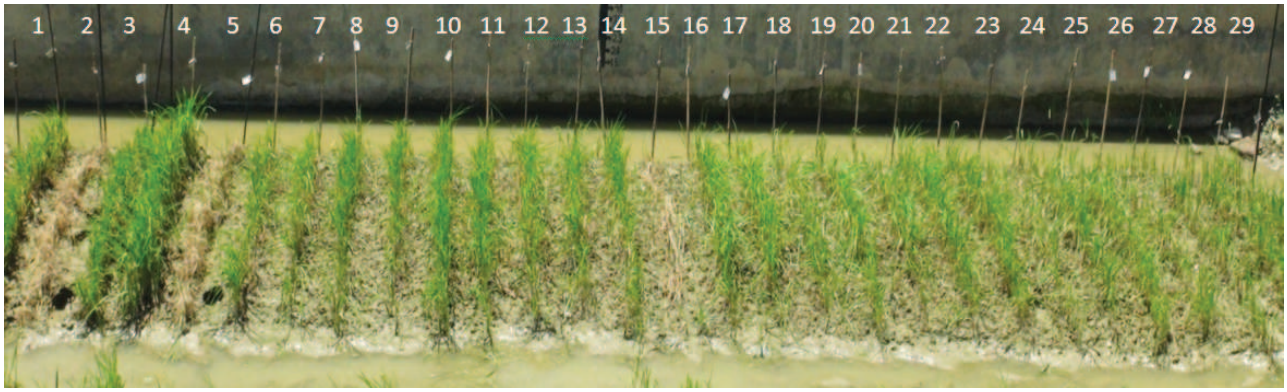


Fig. 3. Phenotypic screening of BC_2F_2 plants. 1=IR64- *Sub1*, 2=Swarna, 3=Swarna- *Sub1*, 4= FR13A, 5=Ranjit, 6-29 = Representative BC_2F_2 plants

Sub1. These lines were identified based on 62 agro-morphological DUS characteristics, grain yield and foreground trait phenotyping. The 20 BC_2F_2 lines showed 89.52-96.54% overall recipient parent genome recovery and 91.74-94.89% similarity for chromosome 9, which shows a very high recipient genome recovery (Fig. 4). Among the 20 BC_2F_2 lines the RPG recovery of 96.54% was observed in a plant (E-6) which was designated as Ranjit- *Sub1* (Fig. 5).

Discussion

The present study clearly demonstrated the rapid and precise enhancement of the mega variety, Ranjit into Ranjit-*Sub1* with submergence tolerance by only with two backcrossing and two selfing generation. The specific aim of this modified MABC is to reduce the selection activities with molecular markers by skipping the recombinant selection. Using MABC, Neeraja et al. (2007) developed submergence tolerant version of the mega variety, Swarna with three backcrosses and two selfing generation with a recurrent parent genome

Fig. 5. A BC_2F_2 line (E-6) designated as Ranjit- *Sub1*at maturity stage

(RPG) recovery of 96.2% and an introgressed fragment of 2.3-3.4Mb. Similarly, the submergence tolerance version of BR11-*Sub1* was developed with the help of MABC with two backcrossing and two selfing generation, in which the RPG recovery was 99.8% and introgressed fragment of 800Kb (Iftekharuddaula

et al. 2011). Cuc et al. (2012) reported introgression size of only 0.3 Mb between ART5 and SC3 into the Vietnam elite rice variety- AS996 and recovery of about after three generations of backcrossing in BC_3F_1 generation with the help of MABC.

In this modified MABC, Ranjit-*Sub1* was developed with an RPG recovery of 96.54% which was achieved with the help of two backcrossing and two selfing generation only as in the case of BR11-*Sub1*. Further, the introgression from the donor parent was also only 1.65 Mb. Skipping of recombinant selection and analyzing the background selection at the final stage that too with only twenty selected lines helped in reducing laboratory expenditure to a considerable extent. However, there is considerable difference between the conventional backcrossing where introgressed fragments can easily be 50 cM or more (Salina et al. 2003). A chromosome fragment of 2 Mb could contain 200 genes. Young and Tanksley (1989) first proposed the idea of reducing the size of donor fragments containing target loci. They suggested that, using 1 cM flanking markers on each side of a target locus, the size of the introgressed segment could theoretically be 2 cM in two generations, in comparison with traditional backcross breeding, where it is expected to require 100 BC generations to obtain such a small segment. Previous research studies have reported the introgression of QTLs associated with submergence tolerance (Siangliw et al. 2003; Xu et al. 2004 and Toojinda et al. 2005), in which the extent of the size of the donor chromosomal segment was not estimated. The first report is the conversion of Swarna-*Sub1* resulted in an introgression size of 2.3–3.4 Mb in the BC_3F_2 and 6.5 Mb in the BC_2F_2 using RM316 (1.8cM) flanking marker at proximal end and RM219 (11.7cM) at distal end (Neeraja et al. 2007). The recent successes of MABC in improving complex traits as exemplified in the drought and salt tolerance cases could be contributed to the use of well-characterized major QTLs which tend to be less sensitive to environment and genetic background (Biao et al. 2014; Singh et al. 2016).

In the present study, it has been observed that by using a donor parent with a similar genealogy, the recipient parent along with strict selection in the BC_1F_1 and BC_2F_1 generation for the recurrent parent based on the DUS characters will also lead to the introgression of small segment of 1.65 Mb in BC_2F_2 generation. Also there was a great improvement of RPG recovery through phenotypic selection in segregating generations with the help of DUS

Table 1. The performance of Ranjit-*Sub1* during *Kharif* 2015 and 2016

	Year		Overall		Zone III		Zone IV		Zone VII	
			Yield (kg/ha)	% RP	Yield (kg/ha)	% RP	Yield (kg/ha)	% RP	Yield (kg/ha)	% RP
Ranjit-*Sub1*	2015	Sub	.....	.....	1643	82	.....	.....	.....	.....
		Water Stag	4792	3	4070	on par	4854	5	7559	7
	2016	Sub	.....	.....	3226	5	.....	.....	.....	.....
		Water Stag	4708	19	4481	36	5235	17	4635	-3
Ranjit	2015	Sub	.....		901		.....		.....	
		Water Stag	4655		4067		4632		7052	
	2016	Sub	.....		3071		.....		.....	
		Water Stag	3964		3305		4464		4784	

RP- Recurrent Parent, Sub- Submergence, Water Stag- Water Stagnation (ICAR-IIRR, 2017 progress report, varietal improvement)
Zone III = Orissa, Bihar, Jharkhand, West Bengal, Uttar Pradesh : Zone IV = Assam, Manipur, Tripura : Zone VII = Andaman & Nicobar, Andhra Pradesh, Telangana, Tamil Nadu, Kerala, Karnataka, Puducherry

Table 2. Rice grain quality characteristics of Ranjit- *Sub1* during *Kharif* 2016

	HULL	MILL	HRR	KL	KB	L/B	Grain type	Grain Chalk	VER	WU	KLAC	ER	ASV	AC	GC	Aroma
Ranjit- *Sub1*	74	66	53.5	5.0	1.88	2.65	MS	VOC	4.6	135	9.5	1.99	5	20.73	22	NS
Ranjit	72.9	62.2	50	5.1	1.93	2.64	MS	VOC	4.4	165	8.6	1.82	7	21.35	22	NS

(ICAR-IIRR, 2017 progress report)
HULL- Hulling %, MILL- Milling %, HRR- Head Rice Recovery %, KL- Kernel Length, KB- Kernel Breadth, L/B- Length and breadth ratio, Grain Chalk- Grain Chalkiness, VOC- Very occasionally present, A- Absent, KLAC- Kernal Length After Cooking, WU-Water Uptake, VER- Volume Expension ratio, ER- Elongation Ratio, ASV- Alkali Spreading Value, AC- Amylose Content %, GC-Gel Consistency, LB- Long Bold, SB-Short Bold, LS- Long Slender, MS- Medium Slender, SS-Short Slender

characters. To know the recovery of the twenty selected individual, background selection was carried out using 50K SNP chip. In case of MABC, for background selection higher precision in estimation of RPG recovery can be achieved by using SNP markers (Khanna et al. 2015). The best plant had 96.54% of the recipient genome by BC_2F_2 generation (Fig. 3). However, background selection was confined to the individuals which had the target gene as well as which minimized the size of the donor segment containing the QTL in the BC_2F_2 generation. Neeraja et al. (2007) used 56 SSR markers as initial background markers for the development of Swarna-*Sub1*. Since recombination events accumulate over time, the number of donor chromosome segments spread throughout the genome increases as their length decreases. Hence, more markers are required to detect them at more advanced backcross generations (e.g. BC_3) (Collard et al. 2008 and Hospital et al. 1997). Neeraja et al. (2007) used 32 additional background markers in the finally selected BC_2F_2 and BC_3F_2 version of Swarna-*Sub1* for ensuring the proper recovery of RPG. In the present study, we prefer phenotypic selection in the early back cross generation using 62 morphological traits to identify plants showing maximum similarity with Ranjit, which helped in reducing the population size and finally SNP marker approach for back ground analysis was performed only in selected BC_2F_2 plants. Screening for yield, yield-component parameters, grain physico-chemical properties and diseases or insect pest were also carried out during *Kharif* 2015 and *Kharif* 2016 in All India Coordinated Rice Improvement Program (AICRIP) network (Varietal Improvement citation from AICRIP). The successful recovery of the phenotypic traits of Ranjit with submergence tolerance was observed in Ranjit-*Sub1*. In Zone III, Ranjit-*Sub1* showed 82 and 5% yield advantage in 2015 and 2016, respectively under submergence conditions. While under water stagnation conditions, it showed 'on par' and 36% yield superiority over Ranjit in 2015 and 2016, respectively (Table 1). It is similar to Ranjit in all quality characteristics (Table 2) and reaction to disease or pest. Therefore, Ranjit-*Sub1* is recommended for Zone III including the states *viz*., Odisha, Bihar, West Bengal, Jharkhand and Uttar Pradesh in India.

The major drawbacks of the earlier improved mega varieties has been their dwarf stature, and some of them are of medium duration. In most of the Eastern India, including Assam, rice is cultivated in rainfed condition with long duration varieties. Sometimes at the time of transplanting water level remains around 20-30cm. As the seedling height of the converted mega varieties are less than 30cm it was not possible for the farmers to transplant in the waterlogged condition. This was the major limitation on adoption of Swarna-*Sub1* and other earlier improved mega varieties with *Sub1* in this region. This problem could be overcome by the *Sub1* version of Ranjit, as the plant is medium-tall in its stature and 30 days old seedling is around 40 cm tall. Hence, Ranjit-*Sub1* is expected to have better acceptance by the farmers of this region and will help in increasing the production as well as productivity of the flash flood affected areas of Eastern India.

Authors' contribution

Conceptualization of research (SKC, TA, NKS); Designing of the experiments (SKC, TA, MKM); Contribution of experimental materials (SKC, TA, NKS); Execution of field/lab experiments and data collection (RKV, MK, BB); Analysis of data and interpretation (RKV, MK, BB); Preparation of manuscript (SKC, BB, RKV, MK, NKS).

Declaration

The authors declare no conflict of interest.

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