Archives of Agriculture and Environmental Science 8(1): 55-61 (2023)
https://doi.org/10.26832/24566632.2023.080109

e-ISSN: 2456-6632

This content is available online at AESA

Archives of Agriculture and Environmental Science

Journal homepage: journals.aesacademy.org/index.php/aaes

ORIGINAL RESEARCH ARTICLE

Blast resistance (*Pi54*) introgression in temperate rice (*Oryza sativa* L.) K343 using marker assisted backcrossing

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ARTICLE HISTORY

Received: 21 January 2023
Revised received: 18 March 2023
Accepted: 21 March 2023

Keywords

Magnaporthe oryzae
Marker-Assisted Backcrossing
Pi54
Rice blast
RPG

ABSTRACT

In hill rice ecologies, *Magnaporthe oryzae* causes rice blasts and a significant biotic constraint. The study was aimed to develop rice blast-resistant lines/varieties through marker-assisted selection (MAS) by using the *Pi54* gene, which provides resistance against the most prominent blast fungus isolate (PLP-1). The blast resistance gene *Pi54* from the *indica* rice genotype DHMAS has been inserted into the genetic makeup of the temperate rice variety K343 in the current study. Three SSR markers (TRS26, TRS33, and RM206) are closely linked to the *Pi54* locus used in this study. Marker RM206, located 0.7cM from the *Pi54* gene, distinguished between donor and recipient alleles and co-segregated with the target gene. Thus, RM206 is used for *Pi54* gene foreground selection. Sixty-one plants have the homozygous allele for the *Pi54* in BC_1F_2, accounting for roughly 50% of the homozygous population. Polymorphic genome-wide SSR assessment of the BC_1F_2 genetic stock (K343*2/DHMAS) revealed recurrent parent genome (RPG) recovery above 75% in 4 plants. Therefore, *M. oryzae* race PLP-1, the dominant race used in this study, showed high resistance to the resistant response after inoculation.

Citation of this article: Hangloo, S., Mahdi, G. M. A., Sharma, M., Salgotra, R. K., Sharma, D., & Bhat, R. (2023). Blast resistance (*Pi54*) introgression in temperate rice (*Oryza sativa* L.) K343 using marker assisted backcrossing. *Archives of Agriculture and Environmental Science*, *8*(1), 55-61, https://dx.doi.org/10.26832/24566632.2023.080109

INTRODUCTION

Several biotic factors, including fungi, bacteria, and viruses, restrict rice production. *Magnaporthe oryzae*'s rice blast is a fatal disease that causes significant yield losses in rice (Helliwell *et al.*, 2013; Khush and Jena, 2009; Skamnioti and Gurr, 2009). Blast is the most severe biotic constrain, especially in the hilly and temperate regions of India, including Jammu and Kashmir, where rice is grown in an environment that is irrigated and cold at night during the Kharif season, favoring blast upsurge and ultimately causing blast outbreaks. In the hilly regions of the Jammu region, K343 (Chenab) (6.0-7.0 tons per hectare) is the most widely cultivated *indica* rice cultivar. However, reports have suggested that K343 is susceptible to responses to blast fungus and are only moderately resistant. Therefore, inducing resistance through genetic transformation is a straightforward pathway. Marker-assisted selection (MAS) has been extensively used to create rice blast-resistant cultivars because it has been proven to be the most effective and environmentally friendly method of controlling this disease (Dai *et al.*, 2018; Kanchiswamy *et al.*, 2016; Temnykh *et al.*, 2001). Rice blast resistance has been attributed to more than 100 R genes and 350 QTLs, of which 32 genes have been cloned and characterized (Devanna *et al.*, 2022; Liu *et al.*, 2010; Rani and Adilakshmi, 2011; Vasudevan *et al.*, 2015; Xiao *et al.*, 2020a, 2020b; Zhou *et al.*, 2020). Numerous blast-resistant genes, including *Pi1*, *Pita*, *Pi2*, *Pi9*, *Piz*, and *Pi54*, have been reported in the North Western Himalayan region. The *Pi54* gene, among these, offers broad-spectrum resistance to blast fungus and is very effective against the local blast pathogen. This gene was cloned and is located on rice's chromosome 11L (Kumari *et al.*, 2013; Sharma *et al.*, 2005; Thulasinathan *et al.*, 2020; Vasudevan *et al.*, 2015).

Multiple markers related to the *Pi54* gene have been identified (TRS26, TRS33, and RM206 for gene-derived *Pi54* MAS) for potential donors and are genetically related. Thus, segregating populations of crosses between resistant (here DHMAS) and susceptible genotypes (here K343) can successfully track this gene and induce resistance. By introducing the *Pi54* gene into the genetic makeup of K343-derived genetic stocks, the current study sought to develop rice blast resistance in subsequent strains and develop resistant genetic stocks for cultivar development. The diversity analysis (Sharma *et al.*, 2021) is a predefined pathway to work on this type of work, where microsatellite marker screening (Hangloo *et al.*, 2022) to assess the polymorphism builds the foundation for the following sequential works.

MATERIALS AND METHODS

The plant material consisted of two *indica* rice genotypes, i.e., K343, used as susceptible recipient/recurrent parent, and DHMAS (pyramided line with blast gene combination Pi*54*+*Pi1*+*Pita*) as donor parent for *Pi54* gene. Rice variety K 343 was developed and released by SKUAST-Kashmir for hill and temperate ecologies of J&K in 1996 and is a predominant rice cultivar in the hill zone of Jammu and Kashmir. The genomic DNA extraction of the parents, F_1s, and backcross genetic stocks (BC_1F_1, BC_1F_2) was done by the cetyl tri-methyl ammonium bromide (CTAB) method (Doyle and Doyle, 1990) with few modifications (Clarke, 2009). The quantity and quality of the isolated DNA were examined using spectrometric methods (Myspeq/Nanodrop) and in agarose gel electrophoresis. Polymerase chain reaction (PCR) reaction mixture contained 50 ng/µl of template DNA, 10x PCR buffer with 15Mm $MgCl_2$, ten pmol of each primer, 2.5 mM/µldNTPs and 5 U of Taq DNA polymerase (Sigma Aldrich, USA). Using a Universal Gradient Thermal Cycler (Eppendorf AG, Hamburg, Germany), the PCR was carried out with an initial denaturation of 5 min at 94°C, followed by a loop of 35 cycles (denaturation at 94°C for the 30s, annealing at 55-58°C for the 30s, and extension at 72°C for the 30s), and a final extension of 7 min at 72°C. The amplified product was separated using 3-3.5% MetaphorTM Agarose gel electrophoresis, visualized with an ultraviolet transilluminator, and photographed with a gel documentation unit (MiniLumi by DNR Bio-Imaging System, Israel). Three SSR markers closely linked to *Pi54*, i.e., TRS26 and TRS33 (flanking markers for *Pi54* gene with a distance of 0.7 and 0.5 cM, respectively) and RM206 (0.7 cM), were selected based on earlier studies (Dai *et al.*, 2018; Fjellstrom *et al.*, 2006; Kumari *et al.*, 2013; Rani and Adilakshmi, 2011; Sharma *et al.*, 2005; Thulasinathan *et al.*, 2020; Vasudevan *et al.*, 2015; Vijay Kumar *et al.*, 2018). All markers selected were individually screened for resolution on 3 percent agarose gel. We have used 450 SSR markers (Hangloo *et al.*, 2022) to spot polymorphic markers between parental lines. The markers that could noticeably discriminate among parents' alleles regarding the particular locus on 3% agarose gel were considered polymorphic. The hybridity of F_1 plants was confirmed using polymorphic SSR markers. The presence of both the parental alleles in plants confirmed the hybrid nature of F_1 plants, which backcrossed with a recurrent parent to produce BC_1F_1 seeds. The foreground selection was made for the *Pi54* gene on BC_1F_1 plants using closely linked markers, and only the gene-positive plants were allowed to selfing to produce the BC_1F_2 population (K 343*2/DHMAS). The SSR markers that showed polymorphism between donor and recipient parents were used to assess the recovery of the donor parent genome in both BC_1F_1 and BC_1F_2 stocks found positive for the target gene (*Pi54*).

Table 1. Agronomical and pathological status of genetic stocks with maximum RPG recovery.

A: K 343*2/DHMAS						
Gene-positive plants *Pi54*	**DHMAS**	**K 343**	**PP 41**	**PP40**	**PP11**	**PP32**
RPG (%)			80.30	78.60	76.60	75.20
Disease score	0	3	0	0	1	1
Plant height (cm)	132.53	124.09	107.40	108.90	114.10	100.40
Days to 50 per cent flowering	96.00	86.00	100.00	108.00	100.00	97.00
Days to maturity	128 .00	117.00	134 .00	139.00	133.00	135.00
Duration of grain filling	31.00	30.00	32.00	31.00	32.00	36.00
Panicle length (cm)	26.18	27.12	22.90	23.40	24.20	23.10
Effective tillers	9.00	8.00	10.00	9.00	7.00	6.00
Grain length (mm)	6.65	5.95	4.33	4.17	4.50	4.39
Grain breadth (mm)	2.09	2.51	2.46	2.47	2.57	2.45
Yield per plant (g)	33.38	30.75	44.30	30.6	32.4	33.50
1000 grain weight (g)	24.20	27.27	23.94	24.3	22.49	23.53

The genotyping of all plants of the BC_1F_2 population was done to analyze the recovery of the recurrent parent genome. SSR bands were scored manually as 'A' for their similarity with the recipient parent, 'B' for their similarity with the donor parent, and 'H' if both the donor and recipient alleles were present. The graphical representation of molecular marker genotyping data and overall recovery of recurrent parent genomes was done by the software GGT 2.0 (van Berloo, 2008). The Pathotyping of the BC_1F_2 plants for blast symptoms was performed with the most predominant type PLP-1 isolate of *M. oryzae* present in the North-Western Himalayan region (Devanna *et al.*, 2022; Sharma *et al.*, 2012; Wu *et al.*, 2015). The entire BC_1F_2 population and parents were inoculated with PLP-1 by spray (Bonman, 1986) in the polyhouse. Inoculating seedlings with *Magnaporthe oryzae* conidial suspension (1×105 spores/ml) was done at the three to the four-leaf stage (Sharma *et al.*, 2005). The inoculated plants in darkness (relative humidity (> 90%) for 24 h) were then placed in a polyhouse under a 16 h light/8 h dark regime with 80 percent relative humidity. Temperatures during the day and night were maintained at 35+2 °C and 21+2 °C, respectively. The blast of inoculated plants was confirmed using a scale of 0–5 (Bonman, 1986) 6–7 days following inoculation. Phenotyping and evaluation for agro-morphological traits were done in the BC_1F_2 population. For this, BC_1F_2 plants and the respective parents, i.e., K 343 and DHMAS, were evaluated for agronomic traits using augmented designs using K 343 and DHMAS as checks. Data were recorded on all plants found homozygous for the *PI54* for different traits mentioned in Table 1. Data analysis was done by SPAD software using the online data analysis service of the Indian Agricultural Statistical Research Institute, New Delhi (www.iasri.res.in/design)

RESULTS AND DISCUSSION

Marker validation linked to *the Pi54* gene

Of the three closely linked SSR markers considered for foreground selection of *Pi54* locus (TRS26, TRS33, and RM206), specific differential banding patterns have been observed for the RM206 primer. The amplicon size amplified using RM206 primer was 170bp for the parent K343 and 150bp for DHMAS. Therefore, the RM206 primer was extensively used for foreground selection of the *Pi54* gene in BC_1F_1 and BC_1F_2 populations. The reports regarding the suitability of marker RM206 for selecting *the Pi54* gene have been demonstrated (Sharma *et al.*, 2005).

Polymorphism study of SSR markers between parental lines K 343/DHMAS

In order to identify the polymorphic markers between the two parental genotypes, i.e., K343 and DHMAS, 450 SSR markers were utilized (Figure 1). Out of these, 50 markers (11.11%) were polymorphic concerning two parents. The resemblance in the genetic background may be due to donor and recipient parents belonging to *the indica* type (Govindaraj *et al.*, 2015; Rathour *et al.*, 2008; Sarao *et al.*, 2010; Sharma *et al.*, 2005).

Confirmation of hybridity of F_1s

Out of the 12 F_1 plants (K 343/DHMAS), six plants were confirmed as hybrids when analyzed at the molecular level (Figure 2). Confirmed F1 hybrid plants were used to develop F_2 and for backcross populations (Baliyan *et al.*, 2018; Collard *et al.*, 2005; Divya *et al.*, 2014; Hittalmani *et al.*, 2000; Ratna Madhavi *et al.*, 2016; Singh *et al.*, 2012; Vijay Kumar *et al.*, 2018).

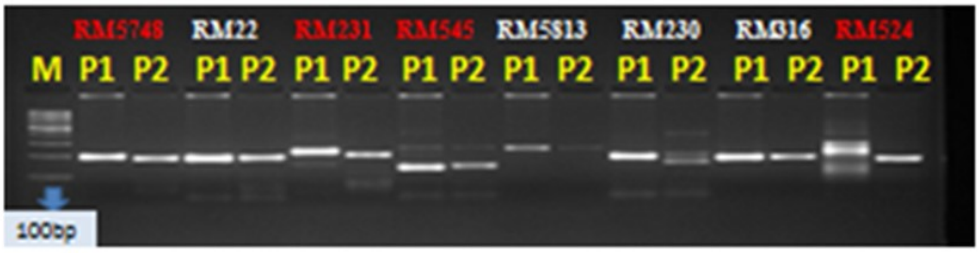

Figure 1. *The banding pattern of SSR markers concerning parents (P1=K 343, and P2=DHMAS); Markers in red color are polymorphic.*

Figure 2. *SSR banding profile of parents and F1 plants with different markers to confirm hybridity (P_1 = K343; P_2 =DHMAS, 1-6= F_1 plants). Plants marked red were confirmed as a hybrid.*

Foreground and background selection in BC_1F_1 genetic stocks

27 BC_1F_1 plants were developed and screened for the *Pi54* gene using closely linked marker, RM206. Of these, 14 plants were found positive for the *Pi54* gene (Figure 3a), which was subject to self-pollination to develop BC_1F_1 seeds. Background selection was made using SSR markers (Figure 3b).

Foreground and background selection in the BC_1F_2 population

Foreground selection of 125 individual BC_1F_2 plants and their parents was performed (Figure 4). To identify the plants possessing the resistance gene (*Pi54*), individual plants were screened with the RM206 marker. Of these, 35 plants showed heterozygosity, while 61 showed homozygosity for the *Pi54* gene. BC_1F_2 plants that showed homozygosity to *the Pi54* gene were used for revealing the genetic stocks having maximum recovery of RPG. These processes permit the direct selection of genes controlling the target phenotype and help identify plants with more significant than the average recovery of RPG, hence providing an option to surmount the shortcomings of conventional approaches (Dekkers and Hospital, 2002; Hospital, 2009; Jena and Mackill, 2008; Servin *et al.*, 2004).

A total of 61 BC_1F_2 plants, found positive and homozygous for the target locus (*Pi54*) via foreground selection, were evaluated for agro morphological traits (Table 1) as well as screened with 50 polymorphic SSR markers for selection of plants that possess maximum recovery of RPG (Figure 5). The maximum recovery of RPG was observed in PP41 (80.3%), followed by PP40 (78.6%), PP11 (76.6%), and PP32 (75.2%). It was observed that the maximum recovery of RPG was associated with chromosome numbers 1 and 2. Therefore, marker-assisted background selection is a promising way to categorize plants with more significant than the average recovery of RPG and thus speed up crop improvement in contrast to conventional backcrossing. Integration of foreground, background, and phenotypic selection to attain high recovery of RPG and phenome has been demonstrated in different investigations (Ratna Madhavi *et al.*, 2016; Singh *et al.*, 2012).

Agronomical and pathological status of maximum RPG recovery genetic stocks K 343*2/DHMAS

The genetic stocks of K 343*2/DHMAS with maximum recovery of RPG were evaluated based on agronomical as well as pathological with the recurrent parent (Table 1). The maximum recovered RPG in plant numbers PP41, PP40, PP11, and PP32 showed broader agronomical uniformity to the recurrent parent and pathologically related to the donor parent. Screening of the backcross genetic stocks with Palampur Local-1 (PLP-1) strain of *Magnaporthe oryzae* under a controlled environment showed the variable reaction of the individual plants varied from resistant to highly resistant. It signifies that the *Pi54* gene imparts strong resistance to the most prominent blast fungus isolate (PLP-1). These stocks could be exploited for further backcross breeding to increase the recovery of RPG to cultivate improved K343. In addition, they can be used as potential donors in future breeding strategies for blast resistance genes. Several studies have competently combined marker-assisted backcross breeding with phenotyping for superior plant architecture and pathotyping for blast resistance to develop rice blast-resistant lines/varieties (Hittalmani *et al.*, 2000; Kumari *et al.*, 2013; Singh *et al.*, 2012; Tang *et al.*, 2006; Ye and Smith, 2008).

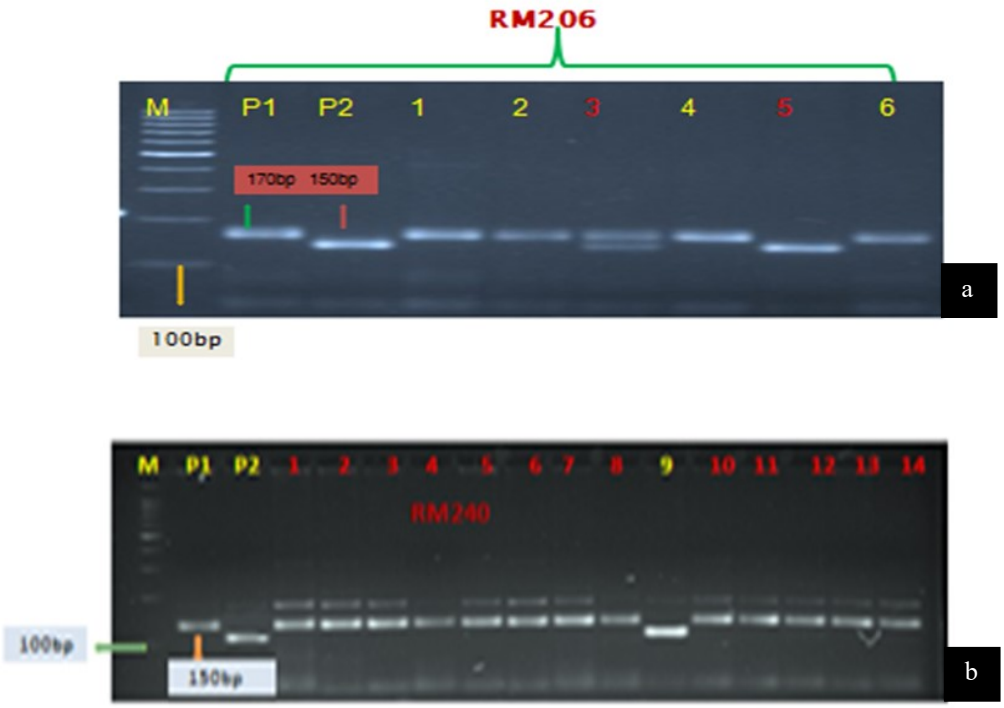

Figure 3 (a). *Foreground selection of Pi54 gene in BC_1F_1 generation using RM206 marker, where P_1 = K 343; P_2 =DHMAS; 1-27=BC_1F_1 plants.* ***(b)*** *Background selection using SSR markers, where P_1 = K 343; P_2=DHMAS; 1 to 14 = BC_1F_1 plants.*

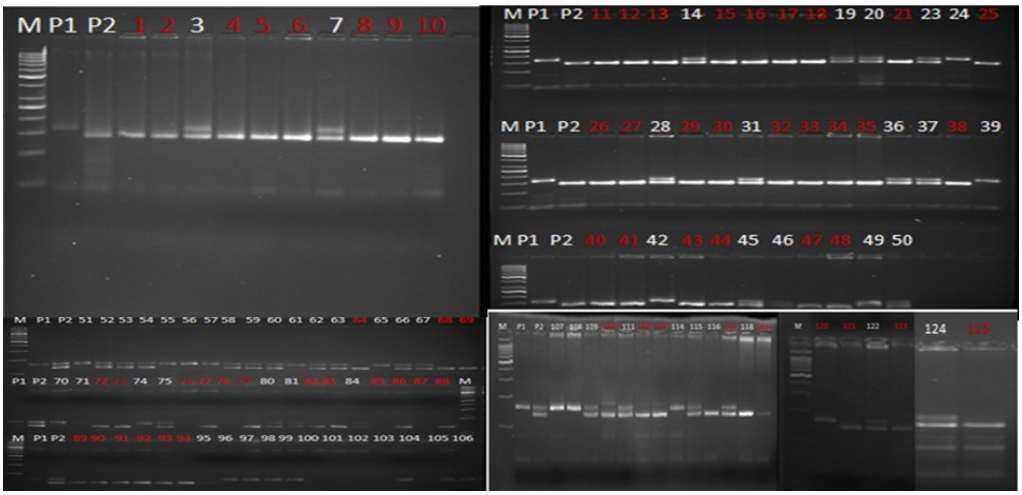

Figure 4. *Foreground selection of BC_1F_2 plants using RM206 markers, where P1=K 343 and P2=DHMAS; 1 to 125 = BC_1F_2 plants. 61 plants marked red indicates the presence of a donor allele in the homozygous state.*

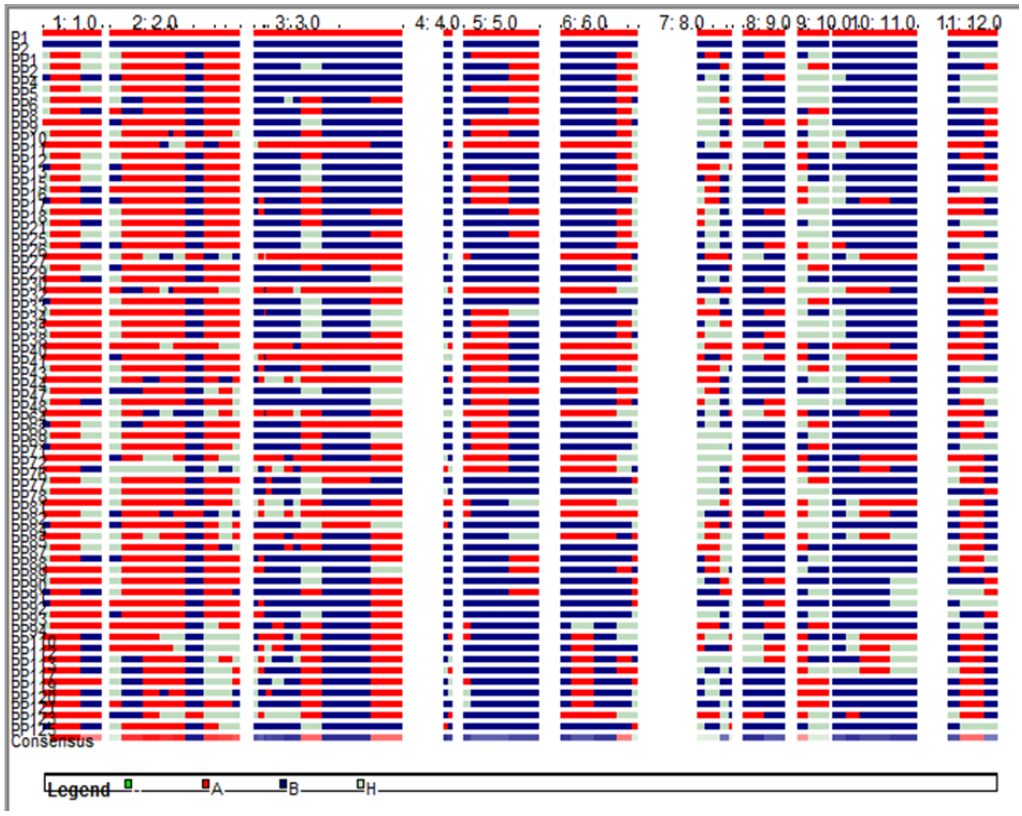

Figure 5. *RPG recovery in 61 BC_1F_2 genetic stocks.*

Conclusion

This study concluded that the RM206 primer was extensively used for foreground selection of the *Pi54* gene in BC_1F_1 and BC_1F_2 populations. The genetic stocks of K 343*2/DHMAS with maximum recovery of RPG showed broader agronomical uniformity to the recurrent parent and pathologically related to the donor parent. The backcross genetic stocks with the *Pi54* gene can be exploited for further backcross breeding to cultivate improved K343 and can be used as potential donors in future breeding strategies for blast resistance genes. The study indicated that MAS is a promising way to categorize plants with more significance than the average recovery of RPG and thus speed up crop improvement in contrast to conventional backcrossing.

ACKNOWLEDGEMENTS

This study has been undertaken through the funding of the Science and Engineering Research Board (SERB), Department of Science and Technology, Government of India, under the grant number SERB/SB/SO/PS/51/2013.

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