MICROBIAL BIOTECHNOLOGY
Open Access

RESEARCH ARTICLE

Methionine biosynthetic genes and methionine sulfoxide reductase A are required for *Rhizoctonia solani* AG1-IA to cause sheath blight disease in rice

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Abstract

Rhizoctonia solani is a polyphagous necrotrophic fungal pathogen that causes sheath blight disease in rice. It deploys effector molecules as well as carbohydrate-active enzymes and enhances the production of reactive oxygen species for killing host tissues. Understanding *R. solani* ability to sustain growth under an oxidative-stress-enriched environment is important for developing disease control strategies. Here, we demonstrate that *R. solani* upregulates methionine biosynthetic genes, including *Rs_MET13* during infection in rice, and double-stranded RNA-mediated silencing of these genes impairs the pathogen's ability to cause disease. Exogenous treatment with methionine restores the disease-causing ability of *Rs_MET13*-silenced *R. solani* and facilitates its growth on 10 mM H_2O_2-containing minimal-media. Notably, the *Rs_MsrA* gene that encodes methionine sulfoxide reductase A, an antioxidant enzyme involved in the repair of oxidative damage of methionine, is upregulated upon H_2O_2 treatment and also during infection in rice. *Rs_MsrA*-silenced *R. solani* is unable to cause disease, suggesting that it is important for the repair of oxidative damage in methionine during host colonization. We propose that spray-induced gene silencing of *Rs_MsrA* and designing of antagonistic molecules that block MsrA activity can be exploited as a drug target for effective control of sheath blight disease in rice.

INTRODUCTION

Rhizoctonia solani is a necrotrophic fungal pathogen that causes economically important diseases in various cereals, vegetables and ornamental plants (Francis et al., 2023; Liao et al., 2022; Molla et al., 2020; Zrenner et al., 2020). The pathogen has been broadly classified into 14 different anastomosis groups and its strains belonging to AG1-IA are known to cause sheath blight disease, one of the most devastating diseases in rice (Ghosh et al., 2014, 2017; Senapati et al., 2022; Singh et al., 2019; Zhang et al., 2024). Considering that the source of complete disease resistance against this deadly pathogen is not available in the rice germplasm (Molla et al., 2020), efforts have been made to identify various molecular determinants that play important roles during host-pathogen interactions (Dauda et al., 2023; Francis et al., 2023; Ghosh et al., 2019, 2021; Wibberg et al., 2016; Yang et al., 2023; Zheng et al., 2013). Under favourable conditions, the infection propagules of *R. solani* (i.e., sclerotia) germinate to produce infection cushions that potentially absorb sugar and nitrogen from the

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host plant to fuel its growth (Copley et al., 2017; Kanwar & Jha, 2019; Sinclair, 1970). It has been proposed that fungal pathogens like *Magnaporthe oryzae* (causal agent of rice blast disease) need to synthesize certain amino acids including methionine, that are present in scarce amounts in the leaf apoplast (Saint-Macary et al., 2015).

Methionine (Met), a sulfur-containing amino acid is necessary for several important physiological and metabolic activities, including the initiation of protein synthesis and methylation of proteins, RNA and DNA (Escaray et al., 2022; Saint-Macary et al., 2015; Scott et al., 2020; Shrivastava et al., 2021). It plays a pivotal role in regulating cell proliferation (Sutter et al., 2013; Walvekar et al., 2018), autophagy (Sutter et al., 2013) and respiration (Tripodi et al., 2018) in fungi. Methylenetetrahydrofolate reductase (MTHFR) catalyses the conversion of methylenetetrahydrofolate to methyl triglutamate tetrahydrofolate and plays a key role in methionine biosynthesis (Yan et al., 2013). Silencing of methionine biosynthesis genes compromises the growth and virulence of plant pathogenic fungi, including *Magnaporthe*, *Alternaria*, *Aspergillus* and *Fusarium* (Frandsen et al., 2010; Gai et al., 2021; Li et al., 2023; Saint-Macary et al., 2015; Scott et al., 2020; Sieńko et al., 2007). Methionine residues in various proteins are prone to oxidative damage, wherein they are oxidized to inactive forms i.e., methionine-S-sulfoxide/ methionine-R-sulfoxide (Aledo, 2019; Moskovitz & Smith, 2021). Most organisms possess two copies of methionine sulfoxide reductases (Msr), MsrA and MsrB, to repair the damage by catalysing the reduction of methionine-S-sulfoxide and methionine-R-sulfoxide residues to methionine, respectively (Moskovitz & Smith, 2021). Notably, the role of MsrA/B homologues in fungal growth and pathogenesis remains poorly understood.

In this study, we report that methionine promotes hyphal branching, and the formation of infection cushions and potentially assists in the management of oxidative stress during colonization of *R. solani* in rice. The silencing of *R. solani* methionine biosynthetic genes (*Rs_Met13*, methionine tetrahydrofolate reductase; *Rs_MET6*, 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase; and *Rs_ ArAT*, aromatic amino acid transaminase) compromise the pathogen to cause sheath blight disease in rice. We also present evidence that *R. solani* MsrA (Rs_MsrA) is required for successful infection and propose that it can be an important target for designing antifungal molecules for sheath blight disease control.

EXPERIMENTAL PROCEDURES

Growth of *R. solani*

Rhizoctonia solani (AG1-IA strain BRS1) was maintained on PDA (39 g/L; Potato Dextrose Agar; Himedia, Mumbai, India) plates at 28°C (Ghosh et al., 2021). For various treatments, *R. solani* sclerotia was grown in Czepek Dox media (a semi-synthetic minimal media, MM; Swain et al., 2017) with or without supplementation of methionine (3, 7 and 14 mM; Sigma Aldrich Co.), hydrogen peroxide (1 and 10 mM, Thermo Fisher Scientific Inc.) and methionine sulfoxide (7 mM; Tokyo Chemical Industry Co., Ltd.). The fungal growth diameter and sclerotia count were recorded at 3 and 7 dpi, respectively.

Mycelial branching pattern in *R. solani*

Rhizoctonia solani sclerotia were grown on thin layered agar (1%) slides, with and without methionine (7 mM). The slides were incubated in a petri-dish containing moist sterile filter paper (to maintain humidity) at 28°C for 3 days. Upon staining with WGA-FITC (20 μg/mL; Sigma-Aldrich Co.) for 30 min and thorough washing with sterile MilliQ water, the slides were analysed under a GFP filter of Confocal Laser Scanning Microscope (AOBS TCS-SP5, Leica, Germany) using the 20× objective. LAS AF Version: 2.6.0 build 7266 software was used to analyse images. The experiment was performed using three biological and technical replicates.

Computational analysis

The target genes (*Rs_MET13*, *Rs_MET6*, *Rs_ArAt*, *Rs_MsrA*) were identified in the *R. solani* genomes using homologous gene sequences of *Saccharomyces cerevisiae*. Further, the protein sequences of Rs_MET13/ Rs_MsrA homologues in other fungal species were downloaded from the NCBI database and subjected to phylogenetic analysis using MEGAX, following the Neighbour-joining algorithm with 500 bootstrap values. The evolutionary distances, measured as the number of amino acid substitutions per site, were calculated using the JTT matrix-based technique. The Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) was used to align the amino acid sequences. Domain analysis was performed using InterPro (ftp.ebi.ac.uk/pub/databases/interpro/).

R. solani infection in rice

Rice (*Oryza sativa* cultivar PB1, *indica* genotype) was grown under greenhouse conditions, at 28°C temperature, 80% relative humidity and 16/8 h of day/night. The sheath of 45-day-old tillers of rice was inoculated with either buffer-treated/dsRNA-silenced/MTX-treated (Methotrexate;1 mM, TCI Chemicals) *R. solani* sclerotia (Ghosh et al., 2014) to monitor disease progression. Wherever mentioned, methionine (7 mM) was sprayed onto the infected rice tilers, daily up to 4 dpi. The vertical sheath colonization (VSC), i.e., the distance between

tips of the lowest and highest lesions in the sheath was recorded at 4 dpi, as described in (Ghosh et al., 2018). The relative vertical sheath colonization (RVSC) was calculated using the formula: VSC/sheath length × 100. The experiment was performed in at least five plants using three tillers per plant and repeated three times (biological replicates).

qRT–PCR-based expression studies

Total RNA was isolated from infected rice sheaths using an RNeasy plant mini kit (Qiagen), and cDNA was synthesized from 2 μg RNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The expression of target genes was quantified by qRT-PCR using the gene-specific primer pairs (Table S1) and PowerUp SYBR® Green Master Mix (Applied Biosystems), as per manufacturer's instructions. 18S rRNA sequence of *R. solani* was used as a reference gene to normalize gene expression (Chandan et al., 2023; Kant et al., 2019). The fold change was estimated by $2^{-\Delta\Delta Ct}$ method (Chandan et al., 2023; Livak & Schmittgen, 2001). To quantify fungal load in the infected samples, relative fold change was calculated using $2^{-\Delta Ct}$ method, wherein ΔCt is the difference between Ct values of fungal 18S rRNA and host 18S rRNA (Ghosh et al., 2021; Kant et al., 2019). In each experiment, the sheath of three different rice plants was pooled as a single biological replicate and the mean of three independent biological replicates was used for calculating standard error.

Yeast complementation assay

Homozygous diploid yeast knockout met13 strain (YGL125W; Clone Id:34492) was procured from Yeast Knockout Collection (Horizon, USA). The full-length *Rs_Met13* (~1.3 kb) gene was cloned into a pYES2 vector (Invitrogen; Thermo Fisher scientific; containing GAL1 as a galactose inducible promoter and ampicillin as a selectable bacterial marker) and transformed into met13 yeast mutant using yeast transformation kit (Yeastmaker Yeast Transformation System 2, Takara bio inc.). Positive transformant was selected on SD-Ura (MP Biochemicals, USA) plates and the growth of serially diluted (1:10) culture was analysed on SD-Met (SD minus methionine) plates.

dsRNA-mediated silencing of *R. solani* genes

The unique fragments of the target genes (*Rs_MET13*, *Rs_MET6*, *Rs_ArAt* and *Rs_MsrA*) with no off-target in *R. solani*, as well as host (rice) genome, was identified using the siFi21 software (http://labtools.ipk-gatersleben.

de/). 300–400 bp region of each of these genes was amplified from the cDNA of infected rice, using primers that contain a 5′ flanking region of T7 RNA polymerase promoter for transcription initiation. Thereafter, an in-vitro transcription reaction was set up using one μg of purified PCR product using MEGAscript T7 Transcription Kit (Invitrogen™, Thermo Fisher Scientific Inc.), as per the manufacturer's protocol (Francis et al., 2023; Ghosh et al., 2021). 30 μg of dsRNA was used to treat sclerotia for 12 h, following which the sclerotia were used for infecting rice and growth assay under laboratory conditions. The experiment was performed using at least three independent biological replicates.

Visualization of reactive oxygen species in *R. solani* mycelia

R. solani was grown on nitrocellulose membrane placed on PDA plates for 5 days. The mycelia growing on the membrane were placed on sterile glass slides, treated with 30 μg of gene-specific dsRNA and incubated for 12 h under moist conditions. Subsequently, upon H_2O_2 treatment (1 and 10 mM) for 3 h, the mycelia were stained with H_2DCFDA (50 μg/mL; 2′,7′-dichlorodihydrofluorescein diacetate, Thermo Fisher Scientific Inc.) and visualized under GFP filter of Confocal Laser Scanning Microscope (TCS-SP8, Leica, Germany) using 63× objective. The images were analysed using LAS AF Version: 2.6.0 build 7266 software. Fluorescence intensity (calculated across the coloured region of interest [ROI]), signifying the level of intracellular ROS accumulation in *R. solani* mycelia, was measured using ImageJ software (https://imagej.nih.gov/ij/) and represented as a line graph. The experiment was performed using three independent biological replicates and three technical replicates.

Statistical analysis

One-way analysis of variance was performed using Sigma Plot 11.0 software (SPSS, Chicago, IL, USA), using the Student–Newman–Keuls test considering $p \leq 0.001$, $p \leq 0.01$ and $p \leq 0.05$ as statistically significant. Where applicable, the significance is mentioned in the figure legend.

RESULTS

Methionine promotes *R. solani* growth, hyphal branching and infection in rice

We investigated the effect of methionine supplementation on *R. solani* [AG1-IA strain BRS1 (Francis et al., 2023)] growth under laboratory conditions and observed that it

significantly enhances hyphal branching (Figure S1A), radial growth (Figure S1B) and sclerotia formation (Figure S1C) on solid media. Methionine also promoted fungal growth in liquid media (Figure S1D). Light microscopy (Figure 1A) as well as confocal microscopy upon WGA–FITC staining (Figure 1B) further reinforced that methionine enhances hyphal branching in *R. solani*.

We also investigated the effect of methionine spray during establishment of sheath blight disease in rice. The data reflected that disease symptoms (Figure 1C), relative vertical sheath colonization (RVSC) index (Figure 1D) and pathogen load i.e abundance of *R. solani* biomass estimated by qRT–PCR (Figure 1E) were significantly higher in the methionine-treated rice, compared to the buffer-treated control.

Methionine biosynthesis is important for successful infection of *R. solani* in rice

Methylenetetrahydrofolate reductase (MTHFR) encoded by *MET12/ MET13* gene is important for methionine biosynthesis in diverse organisms (Figure S2A). Genome mining identified a single copy of *MET13* (*Rs_MET13*), while *MET12* is absent in *R. solani* AG1-IA strain BRS1 (Figure S2B). Heterologous expression of *Rs_MET13* complemented the growth defects of *Δmet13* yeast mutant on SD (synthetic defined) media lacking methionine (Figure S2C).

To investigate further, we silenced the gene using specific double-stranded RNA (dsRNA) (Francis et al., 2023; Ghosh et al., 2021) and observed that the growth of *Rs_MET13*-silenced *R. solani* was significantly compromised on minimal media (MM) plates (Figure 2A,B). It failed to produce sclerotia, however, methionine supplementation restored the fungal growth and sclerotia formation (Figure 2A–C). qRT-PCR analysis reflected

that *Rs_MET13* is upregulated during *R. solani* infection in rice (Figure 2D). Thereafter, we investigated the effect of *Rs_MET13*-silencing (Figure S3A) during disease establishment in rice. The disease symptoms (Figure 2E), RVSC index (Figure 2F) and pathogen load (Figure 2G) were significantly reduced in rice infected with *Rs_MET13*-silenced *R. solani*, compared to those infected with buffer-treated ones. Notably, methionine (7 mM) treatment restored disease severity in *Rs_MET13*-silenced *R. solani*-infected rice (Figure 2E–G).

We observed that 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (*Rs_MET6*), and aromatic amino acid transaminase (*Rs_ ArAT*), genes involved in methionine biosynthesis (Figure S2A) were also upregulated during *R. solani* infection in rice (Figure S4A). Silencing of *Rs_MET6* (Figure S3B)/ *Rs_ ArAT* (Figure S3C) also compromised growth (Figure S4B,C) as well as virulence of *R. solani* (Figure S4D–F). Considering the above, we speculate that the upregulation of methionine biosynthesis is important for sustaining *R. solani* growth during host colonization. This was supported by our observation that treatment with MTX (Methotrexate, 1 mM), a methionine biosynthesis inhibitor (Scott et al., 2020), prevents *R. solani* infection in rice (Figure 2E–G).

Methionine is important for *R. solani* to manage oxidative stress

The ability to manage an oxidative stress-enriched environment is considered to be important for *R. solani* to cause disease in rice (Hu et al., 2021; Kumar et al., 2023; Li et al., 2021; Molla et al., 2020). We observed that the pathogen is capable of growth on 1 mM, but not on 10 mM H_2O_2-containing MM plates (Figures 3A and S5). The growth defects of
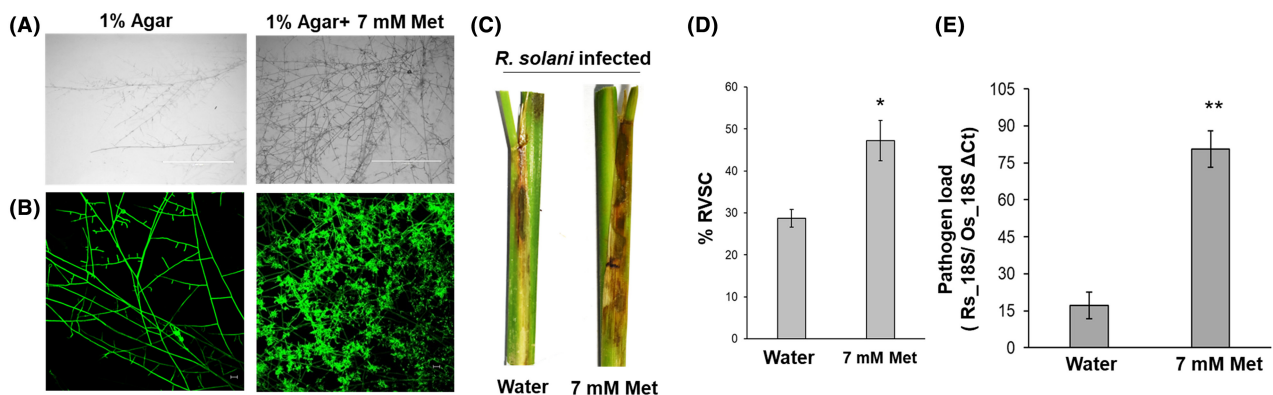

FIGURE 1 Methionine supplementation promotes hyphal growth and branching in *R. solani*. Representative images of (A) light microscopic analysis showing hyphal growth and branching pattern of *R. solani* on 1% agar, (B) confocal microscopic analysis of WGA-FITC-stained mycelia. (C) Sheath blight disease symptoms in the infected rice plants, at 4 dpi, with and without methionine (Met) treatment. (D) The % relative vertical sheath colonization (RVSC) disease index and, (E) qRT-PCR-based quantification of pathogen load in the infected plants, at 4 dpi. Pathogen load was quantified as the relative abundance of 18S rRNA of *R. solani* using 18S rRNA of rice, as endogenous control. The graph shows mean values ± standard error of three biological replicates. * and **indicate significant difference at $p \leq 0.05$ and $p \leq 0.01$, respectively.
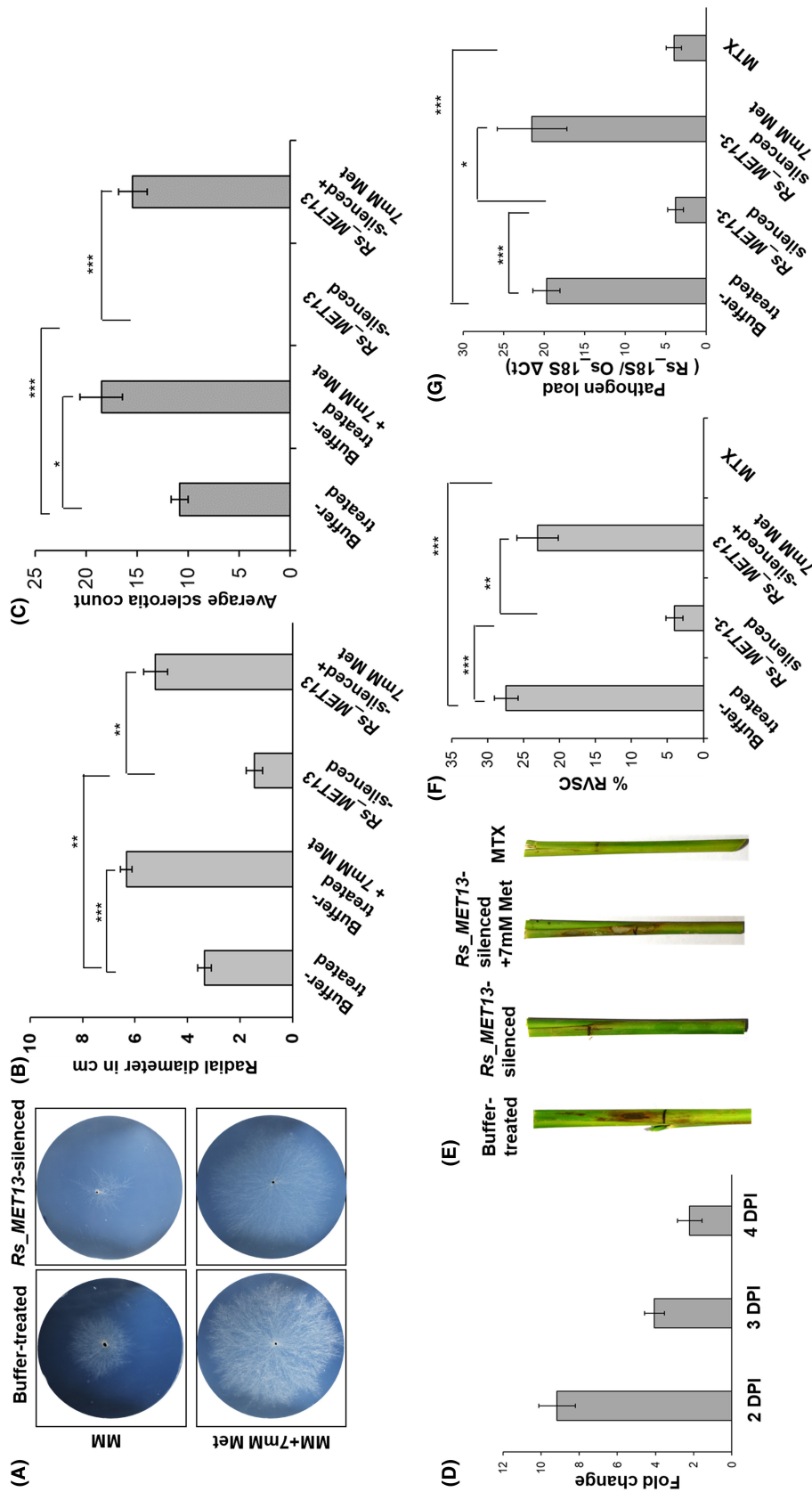
FIGURE 2 *Rs_MET13* is important for facilitating *R. solani* growth and infection in rice. (A) Representative images of buffer-treated and *Rs_MET13*-silenced *R. solani* growing on minimal media plates (MM), with and without methionine (Met) supplementation, at 3 dpi. (B) Average radial diameter reflecting mycelial growth, at 3 dpi. (C) Average sclerotia count at 7 dpi. (D) qRT-PCR based expression analysis of *Rs_MET13* during infection in rice, at different time points. The fold change in gene expression was calculated during infection at 2, 3, 4dpi with respect to 0dpi, using 18S rRNA of *R. solani* as endogenous control. (E) Representative images of infected rice, at 4 dpi. The effect of treatment with MTX (1 mM, methotrexate), a methionine biosynthesis inhibitor is shown. (F) Relative vertical sheath colonization (RVSC) disease index and (G) qRT-PCR-based quantification of pathogen load in infected plants, at 4 dpi. Pathogen load was quantified as the relative abundance of 18S rRNA of *R. solani* using 18S rRNA of rice, as endogenous control. The graph shows the mean value ± standard error of three biological replicates, each having four technical replicates. *, ** and ***indicate significant difference at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, respectively.
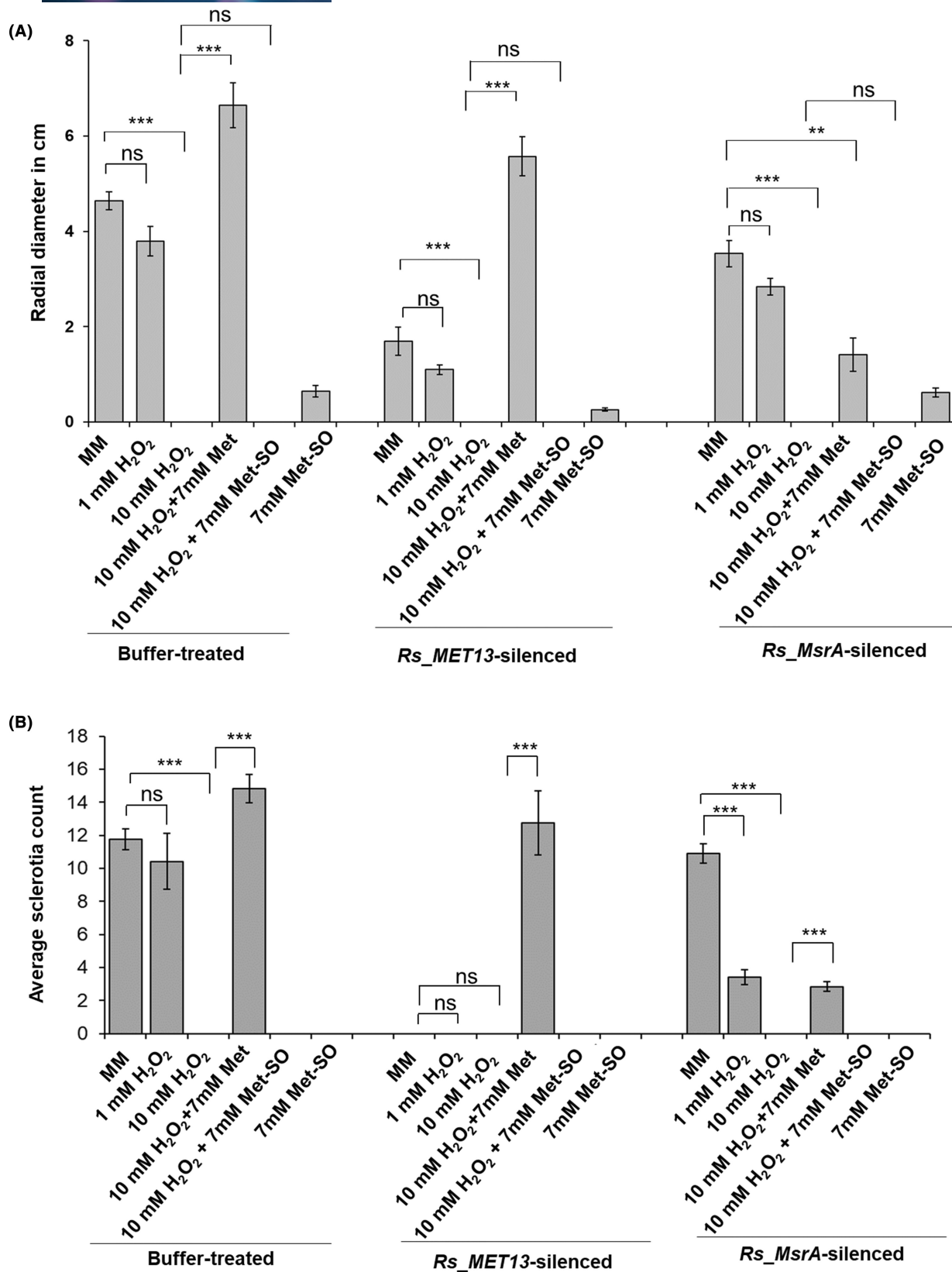
FIGURE 3 Methionine is important for the management of oxidative stress in *R. solani*. (A) Average radial diameter of mycelial growth at 3 dpi and (B) Average sclerotia count at 7 dpi, of buffer-treated and *Rs_MET13/ Rs_MsrA*-silenced *R. solani* when grown on minimal media plates (MM), with and without H_2O_2, methionine (Met) and methionine sulfoxide (Met-SO) treatment. The graph shows mean values ± standard error of three biological replicates, each having four technical replicates. ** and ***indicate significant difference at $p \leq 0.01$ and $p \leq 0.001$, respectively.

buffer-treated and *Rs_MET13*-silenced *R. solani* on 10 mM H_2O_2 plates were fully restored upon methionine, but not with methionine sulfoxide (MetSO, an oxidized form of methionine) supplementation (Figures 3A and S5). Considering that organisms utilize methionine sulfoxide reductases (Msr) to repair oxidized methionine (Hazra et al., 2022; Moskovitz & Smith, 2021), we explored whether *R. solani* utilizes Msr to manage oxidative stress during infection in rice.

Methionine sulfoxide reductase A is important for *R. solani* to manage oxidative stress

Most organisms possess two methionine sulfoxide reductases, A and B (MsrA/B) that are specific to the S- and R-diastereomers of MetSO, respectively (Hage et al., 2021; Tarrago et al., 2022). We observed that *R. solani* strains, including our laboratory strain (AG1-IA strain BRS1), encode MsrA (Rs_MsrA; Figure S6A) but not MsrB. Rs_MsrA harbours Met_Sox_Rdtase_MsrA domain (peptide methionine sulfoxide reductase A domain, InterPro: IPR002569) (Figure S6B) and conserved catalytic (Cys-47) as well as recycling (Cys-197) amino acid residues that are important for its enzymatic activity (Kim et al., 2009) (Figure S6C). Expression analysis reflected *Rs_MsrA* to be upregulated upon 3 h of H_2O_2 treatment during growth in MM broth (Figure 4A). Also, the gene was upregulated during 3 and 4 dpi of infection in rice (Figure 5A), which coincides with the necrotrophic phase of *R. solani* infection (Ghosh et al., 2017). In the presence of 1 mM H_2O_2, although the hyphal growth of *Rs_MsrA*-silenced *R. solani* was comparable to that on MM plates (without H_2O_2) (Figures 3A and S5), its sclerotia formation ability was severely compromised (Figure 3B). The growth of *Rs_MsrA*-silenced *R. solani* was completely prevented on 10 mM H_2O_2-containing plates, and methionine (but not MetSO) supplementation could only partially restore the growth as well as sclerotia formation ability (Figures 3A,B and S5). Upon 1 mM H_2O_2 treatment, *Rs_MsrA*-silenced *R. solani* had enhanced intracellular ROS level (detected by confocal microscopic analysis of H_2DCFDA stained mycelia), compared to buffer-treated control (Figure 4B,C). On the other hand, upon 10 mM H_2O_2 treatment, both control as well as *Rs_MsrA*-silenced *R. solani* exhibited enhanced ROS accumulation (Figure 4B,C). This suggested that *Rs_MsrA* is required for *R. solani* to manage oxidative stress. We observed that upon *Rs_MsrA* silencing (Figure S3D), disease symptoms (Figure 5B), RVSC index (Figure 5C) and pathogen load (Figure 5D) were significantly reduced, compared to rice infected with buffer-treated *R. solani*. Moreover, exogenous methionine spray failed to

rescue *Rs_MsrA*-silenced *R. solani* to establish disease (Figure 5B–D). These analyses emphasize that *Rs_MsrA* is involved in management of oxidative stress during infection in rice.

DISCUSSION

Enhanced production of reactive oxygen species (ROS), leading to cell death of the infected tissues has been one of the prime strategies of various necrotrophic pathogens, including *Rhizoctonia solani*, the causal organism of sheath blight disease, which is a challenge for sustainable rice cultivation (Kant et al., 2019; Kumar et al., 2023; Molla et al., 2020). An ability to grow under an oxidative stress-enriched environment has been advantageous for such pathogens to survive under hostile environment and to establish disease. However, molecular players that facilitate *R. solani* to manage oxidative stress remains poorly understood. In this study, we present evidence that *R. solani* upregulates methionine biosynthesis and utilizes exogenous methionine (potentially from the host) to sustain growth under oxidative stress-enriched environment that is encountered during infection in plants.

We dissected the methionine biosynthetic pathway and observed that *R. solani* encodes MTHFR (*Rs_MET13*), a critical regulator in methionine biosynthesis (Yan et al., 2013); 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase (*Rs_MET6*), a cobalamin-independent methionine synthase (Shrivastava et al., 2021) and 4-Methylthio-2-oxobutyrate transaminase (*Rs_ArAT*), involved in regenerating methionine by salvage pathway (Brault & Labbé, 2020; Pirkov et al., 2008). Each of these genes was upregulated during infection and silencing of them compromised the pathogen to cause sheath blight disease in rice. The exogenous spray of a methionine biosynthesis inhibitor, MTX (Scott et al., 2020) prevented disease, while methionine treatment enhanced disease severity. Although it remains to be investigated whether methionine can influence plant-associated microbial populations, that otherwise have a profuse impact on plant health (Azar et al., 2023; Das et al., 2021; Fitzpatrick et al., 2020; Noel et al., 2023; Singh, Agrawal, & Bednarek, 2023; Singh, Vaishnav, et al., 2023). However, we observed that under axenic conditions (during growth in minimal media) methionine treatment facilitated intricate and dense hyphal branching that resemble infection cushions, characteristic structures formed during the necrotrophic phase of *R. solani* infection in rice (Ghosh et al., 2017; Singh et al., 2003). On the contrary, defects in methionine biosynthesis upon *Rs_MET13*/ *Rs_MET6*/ *Rs_ArAT* silencing compromised the growth in minimal media. Overall, this suggests that methionine is required for supporting *R. solani* growth and it also acts as a
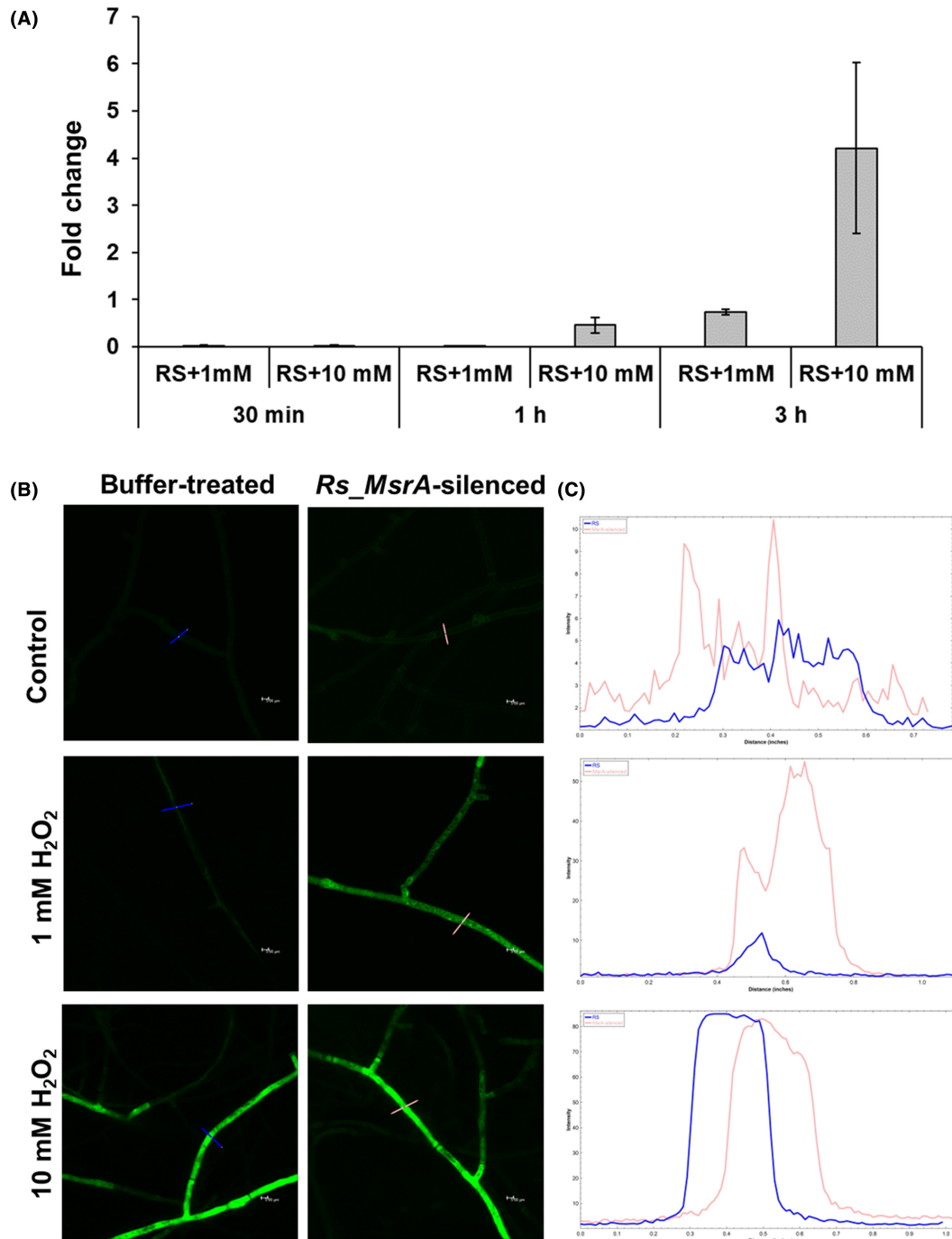
FIGURE 4 *Rs_MsrA* is required for the management of oxidative stress in *R. solani*. (A) qRT-PCR based expression analysis of *Rs_MsrA* upon H_2O_2 treatment, during growth of *R. solani* in minimal media broth (MM), at different time points. The fold change was calculated upon H_2O_2 treatment, with respect to the expression in MM, using 18S rRNA of *R. solani* as endogenous control. The graph shows mean values ± standard error of three biological replicates. (B) Representative confocal micrographs of H$_2$DCFDA (a reactive oxygen species indicator)-stained *Rs_MsrA*-silenced and control (buffer-treated) mycelia, upon H_2O_2 treatment. (C) Fluorescence intensity (calculated across the coloured region of interest [ROI]) quantifying ROS accumulation in the mycelia is drawn as a line graph, wherein blue and pink represent control (buffer-treated) and *Rs_MsrA*-silenced-*R. solani*, respectively.

morphogen that induces hyphal branching leading to the formation of infection cushions. Generally, rice apoplast contains traces of various amino acids, including methionine (Saint-Macary et al., 2015; Wang et al., 2022) which may not be adequate to sustain pathogen growth. Therefore, an ability to upregulate its own methionine biosynthetic machineries serves as an important strategy for *R. solani* to colonize rice. Moreover, extensive usage of various fertilizers that contain methionine to enhance rice productivity, can indirectly be responsible for promoting the occurrence of sheath blight disease.
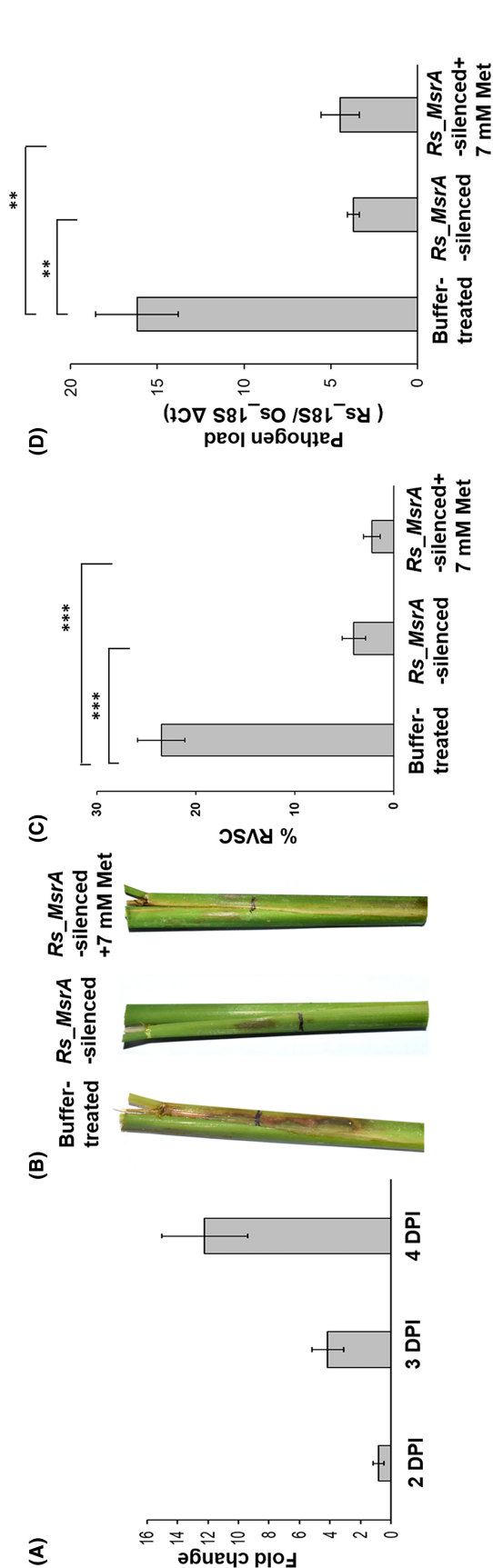
FIGURE 5   *Rs_MsrA* is important for *R. solani* infection in rice. (A) qRT-PCR based expression analysis of *Rs_MsrA* during infection in rice. The fold change was calculated during 2, 3, and 4 dpi, with respect to 0 dpi, using 18S rRNA of *R. solani* as endogenous control. (B) Representative images of disease symptoms in rice infected with buffer-treated and *Rs_MsrA*-silenced *R. solani*, with or without methionine (Met) spray, at 4 dpi. (C) % RVSC (relative vertical sheath colonization) disease index in rice, at 4 dpi. (D) qRT-PCR-based quantification of pathogen load in infected plants. Pathogen load was quantified as the relative abundance of 18S rRNA of *R. solani* using 18S rRNA of rice, as endogenous control. The graph shows mean values ± standard error of three biological replicates. ** and *** Indicate a significant difference at *p*≤0.01 and *p* ≤ 0.001, respectively.

The role of methionine in modulating fungal growth and virulence has been reported in other pathogens. For example, silencing of *Met13* in *Magnaporthe oryzae* (rice blast pathogen) reduced hyphal growth and virulence (Yan et al., 2013), while knock-down of methionine synthase (*MetH*) caused fungalstasis in *Aspergillus* sp. (Frandsen et al., 2010; Scott et al., 2020). The mutant of *MetR*, a transcriptional regulator of methionine metabolism in *Alternaria alternata* was hypersensitive to ROS and defective in conidia formation (Gai et al., 2021). Moreover, additional methionine biosynthetic genes were also involved in providing protection against oxidative burst and promoting *A. alternata* infection in citrus plants (Gai et al., 2021). In corroboration with these studies, we have observed that *Rs_Met13*-silenced *R. solani* was compromised in growth under oxidative stress, which was fully restored by external supplementation of methionine, but not with MetSO (methionine sulfoxide). It is known that oxidative stress causes the oxidation of methionine residues to MetSO in various proteins (including antioxidative enzymes and regulatory proteins), leading to their inactivation. An antioxidant enzyme, methionine sulfoxide reductase (Msr) is used by plants to repair MetSO, back to methionine (Hazra et al., 2022; Lim et al., 2011). We observed that *MsrA* homologue in *R. solani* (*Rs_MsrA*) gets upregulated upon H_2O_2 treatment and also during the necrotrophic phase of infection in rice. *Rs_MsrA*-silenced *R. solani* was severely defective in causing disease and even methionine supplementation failed to restore virulence. As *Rs_MsrA*-silenced *R. solani* exhibited enhanced ROS accumulation upon H_2O_2 treatment, we anticipated that Rs_MsrA-mediated repair of oxidative damage may be important for *R. solani* to establish disease in rice. It will be important to identify *R. solani* proteins that are subject to MetSO accumulation during oxidative stress and investigate Rs_MsrA-mediated repair of such damage. We anticipate that similar to plants (Hazra et al., 2022), various antioxidant enzymes (such as catalase and ascorbate peroxidase) of *R. solani* may be prone to MetSO-accumulation under oxidative stress and Rs_MsrA-mediated repair of these enzymes may be important for causing disease. In recent years, the spray-induced gene silencing (SIGS) approach has gained impetus to control plant diseases (McRae et al., 2023; Mitter et al., 2017; Qiao et al., 2021; Wang & Jin, 2017) and we emphasize that a suitable formulation for dsRNA-mediated silencing of *Rs_MsrA* will be an effective approach to control sheath blight disease in rice. In addition, Rs_MsrA can be explored as a drug target for developing novel anti-sheath blight molecules.

AUTHOR CONTRIBUTIONS
Joyati Das: Data curation; formal analysis; investigation; methodology; validation; visualization; writing – original draft; writing – review and editing. **Srayan Ghosh:** Data curation; formal analysis; investigation; methodology; visualization; writing – original draft. **Kriti Tyagi:** Data curation; formal analysis; investigation; validation. **Debashis Sahoo:** Data curation; formal analysis. **Gopaljee Jha:** Conceptualization; funding acquisition; project administration; resources; supervision; writing – original draft; writing – review and editing.

CONFLICT OF INTEREST STATEMENT
The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.