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RESEARCH ARTICLE OPEN ACCESS

Plant Defence Related Enzymes in Rice (*Oryzae sativa* L.,) Induced by *Pseudomonas* sp VSMKU2

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Abstract

In recent days, antibiotic producing fluorescent pseudomonads (FPs) has been used as a bioorganic tool for the control of sheath blight disease of rice. Combined application of antagonistic microorganism showed that significant bio control activity and enhances plant growth by induced systemic resistance (ISR). The present study, we carryout morphological, physiological and biochemical analysis and then identified, the selected isolate VSMKU2 is *Pseudomonas* sp. Maximum level of phenylalanine ammonia lyase (PAL) was quantified in the treatment of *Pseudomonas* sp VSMKU2 + *R. solani* on the 7th day (97.50 nmol trans-cinnamic acid/min/g). Similarly, the cell free culture filtrate of VSMKU2 challenged with *R. solani* demonstrated lower level of PAL activity on 7th day (91.76 nmol trans-cinnamic acid/min/g) compared to control. Peroxidase (PO) and polyphenoloxidase (PPO) gave higher activity in *Pseudomonas* sp VSMKU2 challenged with *R. solani* on 7th day (0.94 and 0.95 unit/min/g of proteinrespectively) but 14th and 21st day after challenged inoculation of *R. solani* had been reduced (0.92, 0.75 and 0.82, 0.65 unit/min/g of protein) compared to control. The total phenol content activity was significantly increased with *Pseudomonas* sp VSMKU2 (148.27 µg catechol/mg/g of protein) and cell free culture filtrate of VSMKU2 (137 µg catechol/mg/g of protein) treated in rice seedlings on 7th day after challenged inoculation of *R. solani* compared to control. The results obtained in the current study imply to *Pseudomonas sp* VSMKU2 was able to rise defence response, thereby contribute resistance to sheath blight disease.

Keywords: *Pseudomonas* sp VSMKU2, *R. solani*, Rice seedlings, defence related enzymes.

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INTRODUCTION

Sheath blight of rice (ShB) is a critical diseases caused by *Rhizoctonia solani***.** Rice yield was condensed up to 69% in tropical Asian countries like India and China[1]. For management of ShB of rice using chemical fungicides cause severe threat to the environment and public health. Earlier days, chemical fungicides are used for control of soil borne fungal pathogens, but these chemical fungicides persist in the agriculture ecosystem and cause toxicity to beneficial microbes and develop resistance to the plant pathogens[2]. Hence, we need to find out alternative approach for control of plant disease through bio control methods without causing any environmental problems and health hazards[3]. In recent days, biological control is one of the best choices and extensively documented as both safe and consistent clarification for sustainable agriculture. Bio control method is ecofriendly approach to minimize the risk of possible resistance under selection pressure[4]. Recent findings reported many microorganism considered as potential biocontrol agents such as *Pseudomonas aeruginosa* MML2212, *Burkholderia, Ceratobasidium, Bacillus pumilus* MTCC7615 and *Streptomyces aurantiogriseus* VSMGT1014[3,5-8]. Among different beneficial microbial population, fluorescent pseudomonads have drawn much attention worldwide, since, it has plant growth promotion efficiency and major biocontrol potential for fungal pathogens. Moreover it does not cause any environmental problems and health hazards[9]. Fluorescent pseudomonads (FPs) are reported to be a major associated bacteria[10]. FPs demonstrates has the ability to produce IAA, ACC deaminase, siderophore, hydrogen cyanide and lytic enzymes. Previous report showed that FPs strains facilitate to raise seed germination, plant growth and yield [11].

Pseudomonas spp are activates systemically in the plant system through induced systemic resistance (ISR). Recent report demonstrated that, plant growth promoting rhizobacteria (PGPR) activating defence genes encoding chitinase, POX, PPO and PAL in plants[12]. *P. fluorescens* is providing plant growth promotion against plant diseases such as sheath blight, sheath rot, blast of rice, bacterial blight of cotton, ground nut, Pythium disease of tomato and hot pepper[13-16]. The ISR induced by *Pseudomonas* sp was established in bean, carnation, rice, cucumber and raddish[13,17-20].

Previous reports showed, the seed treatment with soil application of *P. fluorescens* DABBV4 enhanced seed germination and vigour index. Further, wilt disease was considerably reduced by *P. fluorescens* treated seeds challenge with R. *solanacearum*[21]. The objectives of the current study deal with the identification of selected isolate VSMKU2. To carry out green house experiment for sheath blight of rice with treatment of VSMKU2 and their cell free culture filtrate against *R. solani*. After 1-3 weeks of *R. solani* inoculation, we examine PAL, PO, PPO activity and total phenol content.

MATERIALS AND METHODS

Antagonistic and Pathogenic culture collection and maintenance

The culture collection and maintenance of selected antagonistic isolate VSMKU2 and pathogen *R. solani* were obtained from our lab culture collection in the Department of Microbial Technology, School of Biological Sciences, Madurai Kamaraj University, which was isolated from rice rhizosphere. Sheath blight disease causing R*. solani* was used in this study. The selected antagonistic isolate VSMKU2 and *R. solani* were kept at 4°C in King's B and Potato Dextrose Agar (PDA) for regular research work. For long time storage, the isolate VSMKU2 was stored in 40% glycerol at - 80°C.

Morphology and Biochemical analysis

The selected isolate VSMKU2 was identi ed by colony morphology, cells shape, including gram staining and pigmentation. The pyocyanin pigment was observed on King's B medium. Isolate VSMKU2 was observed as rod shape under light microscope by staining with Grams reaction. Biochemical analysis was performed by Bergey's Manual of Determinative Bacteriology[22].

Green house experiment

The greenhouse experiments were performed in earthenware pots with rice seedling in complete randomized block design (CRBD) with triplicate. Wet nursery was organized in earthenware pots filled with sterilized field soil and rice seeds of IR-50 were sown as per the treatments. After 25 days, rice seedlings were transplanted to bigger pots for various treatments.

Pseudomonas sp VSMKU2 treatment and challenge inoculation with _R. solani_ in green house

Pseudomonas sp VSMKU2 was used for defence reaction against *R. solani*. The treatments included (1) Healthy control (IR-50 seeds treated with sterile distilled water) (2) Inoculation of 25g of *R. solani* hull/rice seedlings (3) *Pseudomonas* sp VSMKU2 treated in seeds + soil application (25 mL bacterial cells, 7 x 10^8 Cfu/ml) with *R. solani.* (4) A 25 ml of cell free culture filtrate of *Pseudomonas* sp VSMKU2 and *R. solani*. The treatments were duplicated for three times. In 21 days' time course of study, every one week interval; the samples were taken from all the treatments for defence related stress enzymes assay such as PAL, PO, PPO and total phenol.

Preparation of rice leaf extracts

Defence related enzyme assay was performed using rice leaf extracts. Rice leaves were collected on 7th, 14th and 21st days after challenged inoculation of *R. solani* and were stored at -80°C until extract was prepared. From all the treatments, rice leaves were collected every three rice seedlings about 10cm.

Phenylalanine ammonia lyase (PAL)

Phenylalanine ammonia lyase estimation was performed according to Dickerson *et al.* (1984)[23]. Briefly, One gram of rice leaves were taken and homogenized then followed by the above said method.

Peroxidase (PO)

Peroxidise activity was carryout at 30°C by the method of Hammerschmidt et al [24]. Briefly one gram of rice leaves were homogenized using 2ml of 0.1 M phosphate buffer (pH 7.0) at 4 °C and then followed the above said method.

Polyphenol Oxidase (PPO)

Polyphenol oxidase activity was examined by the method of Mayer *et al.* (1965)[25]. One gram of rice leaf tissues were homogenized using two ml of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 16,000 g for 15 min at 4 °C and then followed by the above said method.

Total phenol content

Total phenol content was quantified according to Mayer *et al*, (1966)[26]. One gram of fresh leaf tissues were homogenized with 10 ml of 80% methanol and then followed the above mentioned method.

Table 1. Physiochemical and biochemical characteristics of *Pseudomonas* sp VSMKU2

Test	Results
Biochemical test	-
Gram's Staining	
Motility	Motile
Colony	small, circular and yellow in colour
Pigment production	+
Optimum temperature for growth	37°C
Optimum pH for growth	7
Salt tolerance for growth	0.1-1M
Indole test	+
Methyl red test	-
Voges-Proskauer	-
Citrate utilization	+
Gelatin liquefaction	+
Nitrate reduction	+
TSI	acid butt, alkaline slant, H_2S production
Catalase	+
Oxidase	+
Carbohydrate utilization test	
Glucose	+
Fructose	+
Sucrose	+
Mannitol	+
Lactose	-
Maltose	-
Xylose	-
Arabinose	-
Lytic enzyme Production	
Amylase	+
Cellulase	+
Gelatinase	+
Protease	+
Chitinase	-
Pectinase	-

Note: - absence, + presence.

Statistical analysis

The pot experiments were carryout in a randomized design. The enzyme activity was presented as means ± standard deviations (S.D.) All treatments were repeated in triplicates with three plantlets per pots.

RESULTS AND DISCUSSION

Identification of VSMKU2 and Characterization

PGPR have the ability to improve plant growth promotion and indirectly control fungal

pathogens like *R. solani*, *Pythium aphanidermatum*, *Colletotrichum orbiculare*, *Fusarium oxysporum*[3, 27-29]. In the same way the current study, discover the potential antagonistic rhizobacterium enhance rice defence related stress enzymes in the inoculation and noninoculation of pathogen *R. solani*. Based on the plant growth promotion and bio control potential against fungal pathogen *R. solani* by the isolate VSMKU2 (data not shown) was selected for defence related stress enzymes in rice plants and its role for the management of sheath blight of rice.

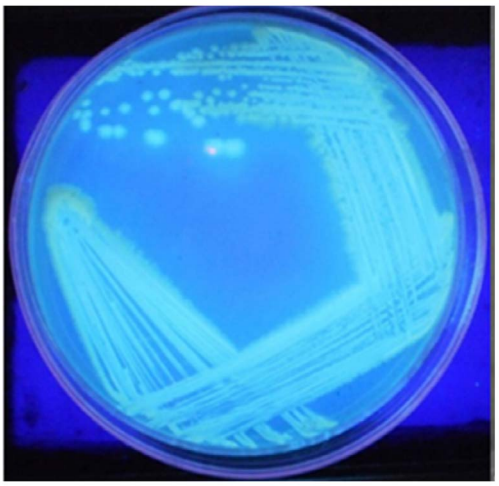

Fig. 1. Morphology of *Pseudomonas sp.* VSMKU2. Culture plate showing fluorescent pigment production under UV-transilluminator at 365 nm.

Morphology

Isolate VSMKU2 exhibited good growth on King's B agar medium with fluorescent colonies (Fig.1). It secretes a variety of pigments, including blue-green (pyocyanin) in different growth media. Light microscopic visualization revealed that the VSMKU2 is a rod-shaped bacterial cell. The light microscope images of VSMKU2 exhibited a detailed structure showing a rod shape.

Biochemical characteristics

Biochemical tests exposed that the isolate VSMKU2 is a Gram negative organism. It demonstrates positive reactions such as oxidase, catalase, citrate utilization, indole production, nitrate reduction and triple sugar iron (acid butt, alkaline slant, H_2S production) tests. It exhibited negative reactions to MR and VP tests (Table 1). The isolate VSMKU2 effectively fermented for various carbon sources like glucose, fructose, sucrose and mannitol. However, it did not ferment arabinose, lactose, maltose and xylose (Table 1). The hydrolytic enzyme assay on different substrate amended medium was exposed, the VSMKU2 isolate secrete only amylase, cellulase, gelatinase and protease, however not produced pectinase and chitinase (Table 1) compared to control. Our

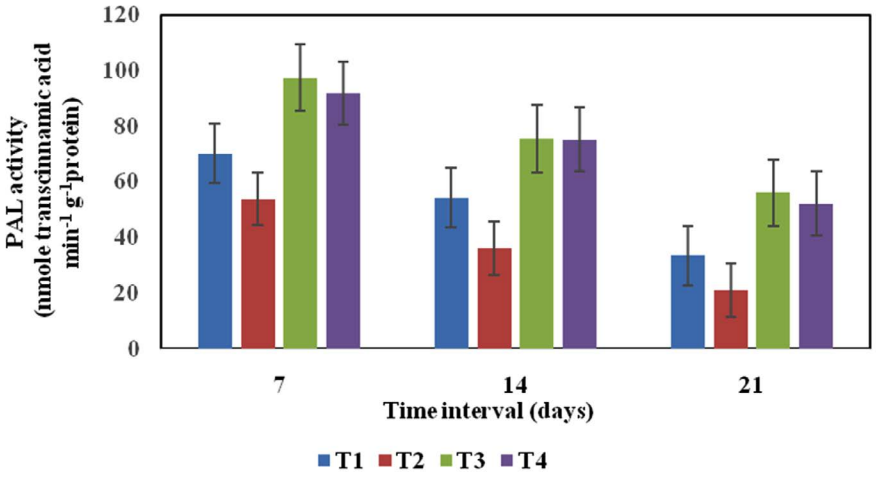

Fig. 2. Phenylalanine ammonia lyase (PAL) activity profile of rice leaves variety IR-50 in different treatments. T1- Healthy control (rice variety IR-50 treated with sterile distilled water); T2- Disease control (rice seed inoculated with *R. solani*); T3- *P. aeruginosa* VSMKU2 culture + *R. solani*; T4- *P. aeruginosa* VSMKU2 culture filtrate + *R. solani* at 7th, 14th and 21st day. The data represent the mean values based on three replicates in each treatment, Vertical bar indicate standard error.

present findings coherence with previous results [30-32].

Induction of defence related stress enzymes

Isolate VSMKU2 belongs to genus *Pseudomonas* sp were involved for the control of fungal and bacterial plant pathogens through antagonism, competition and by developing straight communications with host plants through Induced systemic resistance (ISR). In this study, we discuss the following defence related stress enzymes such as PAL, PO, PPO and total phenol.

Phenylalanine ammonia lyase (PAL)

The seed treatment and soil application of *Pseudomonas* sp VSMKU2 significantly induced maximum level of PAL activity (97.50 nmol trans-cinnamic acid/min/g) on 7th day inoculation of *R. solani,* whereas 14th and 21st day after inoculation of *R. solani*, the PAL activity reduced compared

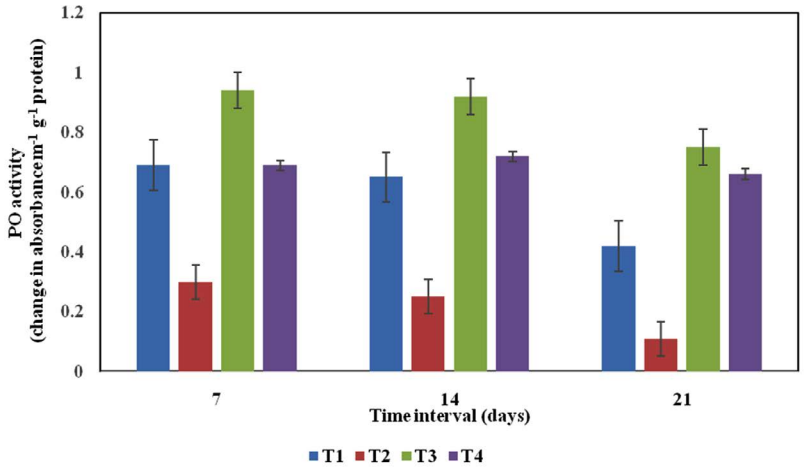

Fig. 3. Phenol oxidase (PO) activity profile of rice leaves variety IR-50 in different treatments. T1- Healthy control (rice variety IR-50 treated with sterile distilled water); T2- Disease control (rice seed inoculated with *R. solani*); T3- *P. aeruginosa* VSMKU2 culture + *R. solani*; T4- *P. aeruginosa* VSMKU2 culture filtrate + *R. solani* at 7th, 14th and 21st day. The data represent the mean values based on three replicates in each treatment, Vertical bar indicate standard error.

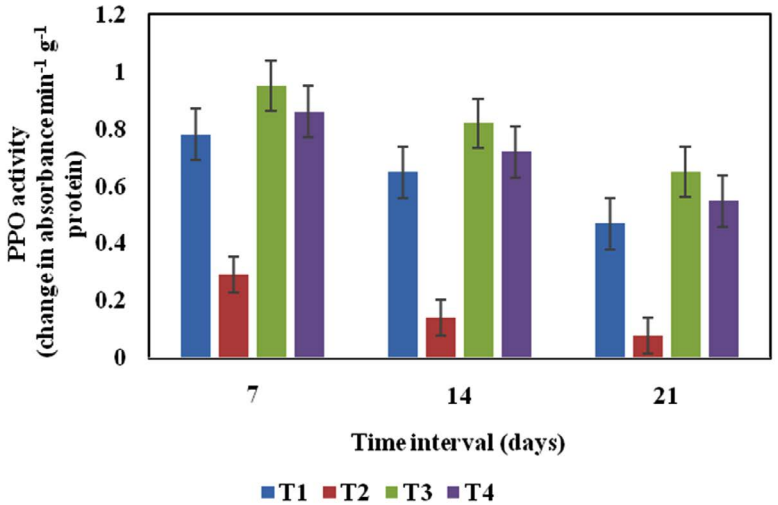

Fig. 4. Polyphenol oxidase (PPO) activity profile of rice leaves variety IR-50 in different treatments. T1- Healthy control (rice variety IR-50 treated with sterile distilled water); T2- Disease control (rice seed inoculated with *R. solani*); T3- *P. aeruginosa* VSMKU2 culture + *R. solani*; T4- *P. aeruginosa* VSMKU2 culture filtrate + *R. solani* at 7th, 14th and 21st day. The data represent the mean values based on three replicates in each treatment, Vertical bar indicate standard error.

to control (Fig. 2). But, the cell free treatment of *Pseudomonas* sp VSMKU2 about 20 to 30% reduction of PAL activity was observed for all three consequent days of evaluation compared to pathogen and untreated control. Similar result was reported by Reshma *et al.* (2018)[33] , showed that seed treatment and root dipping of *Pseudomonas* sp maximum in PAL activity on 7th day of challenge inoculation of *R. solani* in rice plants. Numerous fluorescent pseudomonads were showed to induce ISR[33]. Similarly, ISR condensed infection and enhanced plant growth promotion was reported in several crops[34]. Since, PAL is the primary enzyme in phenylpropanoid metabolism and phenolics and phytoalexins which reduced the development of pathogen35. The present study showed increased PAL activity due to *Pseudomonas* sp VSMKU2 action, which has the capacity to prevent the establishment of *R. solani* in rice roots and leaves. PAL has been involved a significant task in phenylpropanoid pathway, since lignin is a major product. Lignin accumulation is a provoke defence mechanism and strengthening against infection development. Superior PAL activity by *Pseudomonas* spp was reported in tomato [36], pearl millet[37], cucumber[12], tomato[16, 20] and mulberry[38].

Peroxidase (PO)

Peroxidase is the principal enzyme during biosynthesis of lignin. Due to production of PO, it gives strengthening to plant tissues and avoids pathogen entry in to the plants. PO could afford fortification from oxidative stress, through which lipid peroxidation ensuing in damage to the macromolecules, thereby inhibiting photosynthesis and other enzyme activities. In our study, PO activity has been increased on 7th and 14th days of challenged inoculation of *R. solani* with *Pseudomonas* sp VSMKU2 as seed and soil treatment in rice seedlings compared to control. Whereas, in the cell free culture filtrate treatment showed significant activity of PO on 14th days after challenge inoculation of *R. solani* in comparison to other two (7th and 21st) consequent days (Fig. 3). In concurrence with our result, Podile and Lakshmi (1998) [39] reported that PO activity was increased in pea plants treated by *Bacillus subtilis* against *Fusarium udum* after 7 day of inoculation. On the other hand, PO level has been improved after immunization of pathogen and accomplish its greatest at 9th hours after *Ralstonia solancearum* inoculation in tomato plants. Similarly, PAL and PO lower activity was observed in tomato seedlings treated with *R.*

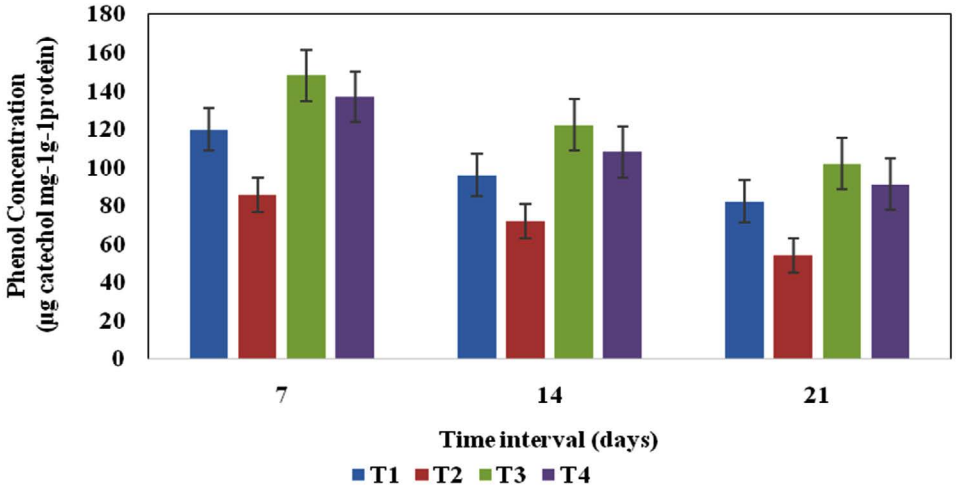

Fig. 5. Profiling of total phenol content in rice leaves variety IR-50 in different treatments. T1- Healthy control (rice variety IR-50 treated with sterile distilled water); T2- Disease control (rice seed inoculated with *R. solani*); T3- *P. aeruginosa* VSMKU2 culture + *R. solani*; T4- *P. aeruginosa* VSMKU2 culture filtrate + *R. solani* at 7th, 14th and 21st day. The data represent the mean values based on three replicates in each treatment, Vertical bar indicate standard error.

solancearum but superior activity was noticed in plants treated with *Pseudomonas fluorescens* and control seedlings[40]. The enhancement of PO activity obtains by *P. fluorescens* in various seedlings such as cucumber[12], rice[13], tomato[16] and mulberry[38].

Polyphenol oxidase (PPO)

High level of PPO activity was recorded in seed treatment, soil application and culture filtrate of *Pseudomonas* sp VSMKU2 on 7th day of *R. solani* inoculation in rice seedlings compared to treated and untreated control (Fig. 4). Similar to other enzyme activity PPO level reduced on 14th and 21st day after pathogen inoculation. PPO catalyses, the oxidation of phenolic compounds to more toxic quinones are a key role in plant disease resistance. In tomato seedlings treated with *P. fluorescens*, the ISR induced PPO activities were observed. PPO activity was maximum in plant pre-treated with *P. fluorescens* and inoculation with *R. solanacearum*. In contrary, *R. solanacaerum* treated plants were showed PPO activity was less (Chen *et al*)[12]. Also they reported that many rhizobacteria and *P. aphanidermatum* induce PPO activity in cucumber plants. PPO level was increased in *P. fluorescens* treated banana plants[41], tomato[20] and mulberry[38].

Total Phenol content

Phenol content increased in seed treatment and soil application and cell free culture filtrate of *Pseudomonas* sp VSMKU2 on 7th day after the pathogen *R. solani* inoculation in rice seedlings compared to treated and untreated control (Fig. 5). Whereas, 14th and 21st day up on the pathogen inoculation, the activity of total phenol content almost same in both seed and soil application and culture filtrate of *Pseudomonas* sp VSMKU2 compared to control. Our result in coherence with the reports of Anita and Samiyappan (2012)[42], the induced defence mechanism exposed more amount of phenol present in bacterized rice roots treated with *Meloidogyne graminicola*. However, the accretion of phenol was reported after seven days of inoculation, where as maximum level of phenol was observed in bacterized seedlings on 14th day after nematode inoculation. Phenolic compounds are known to be a key role for plant defence mechanism against various pathogens. *P. fluorescens* liberate lytic enzymes to build up of phenolic compounds[15] and secretion of indole acetic acids are involved in the phenol metabolism in plants[43].

CONCLUSION

The induction of ISR by beneficial rhizobacteurium can put forth for protective mechanism against soil borne pathogens. Hence, our present findings concluded that *Pseudomonas* sp VSMKU2 could be used as a bioinoculant for the management of sheath blight of rice.

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CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

KN, contributed the data and drafted the manuscript. VS interpretation supervised and reviewed the manuscript. NB helped for preparation of figures, interpretation and draft improvisation. KN, VS and NB read and approved the manuscript.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable.

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