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ORIGINAL ARTICLE

Mutations associated with boscalid and pyraclostrobin resistance of *Botrytis cinerea* from vegetable fields in Turkey

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Abstract

Botrytis cinerea Pers. is a polyphagous fungal pathogen that can cause significant damage in the field, warehouse, and greenhouse conditions. In Turkey, fungicides with site-specific modes containing the active ingredients boscalid and pyraclostrobin are used to control *B. cinerea*. In this study, it was aimed to determine the mutations associated with resistance to boscalid and pyraclostrobin active substances in *B. cinerea* isolates obtained from tomato, pepper, eggplant, and lettuce in Turkey. In the *in vitro* bioassay, a conidial germination test was performed. It was determined that 26% of the isolates used in the bioassay test were resistant. After that, mutations associated with resistance were investigated. Mutations associated with boscalid resistance were found in 18 isolates whose *SdhB* gene was sequenced. No mutations were detected in sensitive MH51 and Ant34 isolates. It has been determined that N230I and H272R mutations were found to be frequent in *B. cinerea* populations in Turkey. P225F mutation was detected only in the B4 isolate obtained from the pepper in Antalya. The mutations associated with boscalid resistance detected in this study are reported for the first time in Turkey. The G143A mutation associated with pyraclostrobin resistance was detected in all the isolates sequenced.

Keywords *Botrytis* · Mutation · Boscalid · Pyraclostrobin · Vegetable

Introduction

Botrytis cinerea Pers. is a polyphagous pathogen that can cause significant damage in field, warehouse, and greenhouse conditions. The fungus belongs to the *Sclerotiniaceae* family of the phylum *Ascomycota*. It can grow in different phenotypic characters as sclerotial and mycelial (Kuzmanovska et al. 2012; Zhang et al. 2017; Gül and Karakaya 2020). In addition, fungicide resistance may cause variation in the phenotype of the pathogen (Lalève et al. 2014). For this reason, multigene phylogenetics is widely used to identify cryptic *Botrytis* species at the present time (Walker et al. 2011; Harper et al. 2019).

Fungicides are currently the most effective and widely used method in controlling gray mold disease caused by *B. cinerea*. However, *B. cinerea* is one of the pathogens with a fungicide resistance problem. Fungicide resistance to various active substances has been reported in vegetable fields in different geographical areas (Banno et al. 2009; Kim et al. 2014; Rodríguez et al. 2014; Konstantinou et al. 2015; Liu et al. 2016; Kanetis et al. 2017).

The most important mechanism of fungicide resistance is target-site mutations in the protein coding genes of fungi. These mutations have been observed in all site-specific fungicides (Hahn 2014). A single point mutation causing an amino acid change can quickly and effectively block fungicide binding to the target site and generally resulting in high levels of resistance (Hollomon 2015).

Pyraclostrobin belongs to the group of quinone outside inhibitor fungicides. According to Fungicide Resistance Action Committee (FRAC 2023), QoI group fungicides include the ones with a high risk of developing resistance. QoI fungicides that bind to the Qo region inhibit mitochondrial respiration and cause fungal death. (Fernández-Ortuño et al. 2012). In most cases, resistance to QoI fungicides results from a point mutation in the mitochondrial cytochrome b (*cytb*) gene (Ma and Michailides 2005). These mutations inhibit the binding of the QoI fungicides. The G143A mutation in this gene causes high resistance to QoI,

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while F129L or G137R mutations cause moderate resistance to QoI (Fernández-Ortuño et al. 2012).

The risk of developing fungicide resistance to boscalid in the succinate dehydrogenase inhibitor (SDHIs) group is medium to high (FRAC 2023). These fungicides inhibit mitochondrial respiration in fungi. The target enzyme of the fungicides in the SDHIs group is SDH, which consists of four subunits (A, B, C, D). The binding site of SDHIs consists of B, C, and D subunits. Mutations that cause resistance to these fungicides can occur in these three subunits (FRAC 2023). However, mutations mostly occur in the *SdhB* unit (Leroux et al. 2010).

It has been reported that resistance to the boscalid and pyraclostrobin used for the control of *B. cinerea* is formed in strawberry fields, stored apples and blueberries (Kim and Xiao 2010; Fernández-Ortuño et al. 2014; Saito et al. 2016). In Turkey, fungicides with site-specific modes containing the active ingredients boscalid and pyraclostrobin are used to control *B. cinerea*. This study aimed to determine the mutations associated with resistance to boscalid and pyraclostrobin active substances in *B. cinerea* isolates obtained from tomato, pepper, eggplant and lettuce in Turkey.

Materials and Methods

Botrytis cinerea isolates

In this study, 20 *B. cinerea* isolates stored in agar slants at +4°C in Ankara University, Faculty of Agriculture, Department of Plant Protection were used (Gül et al. 2023).

In vitro bioassays

In the *in vitro* bioassay, a conidial germination test was performed. A commercial fungicide with boscalid 26.7% + pyraclostrobin 6.7% active ingredients was used in Petri dish tests. Conidial germination tests were carried out in 2% water agar (WA) (CONDA) at 5 µg/ml and 100 µg/ml fungicide doses in 3 replications (Kim and Xiao 2010; Yin et al. 2011).

A stock solution at 100 mg/ml concentration of salicylhydroxamic acid 99% (SHAM) (SIGMA S607-5G) was prepared by dissolving it in methanol. To prevent alternative oxidase respiration, 1 ml of SHAM stock solution was added to 1 liter of WA medium at 100 µg/ml fungicide concentration. The same amount of solution was added to the control Petri dishes.

The *B. cinerea* isolates used in the *in vitro* bioassay were grown in a potato dextrose agar (PDA) media (Merck) for 10 days. Then, 10 ml of sterile distilled water was poured on it and the fungal cover was scraped with a sterile scalpel. After the spore suspension was passed through sterile cheesecloth, the spore concentration was calculated using a hemocytometer. The spore suspension of each isolate was prepared at a concentration of 2×10^5 conidia/ml. One hundred µl of them were transferred to WA medium and spread with a sterile Drigalski spatula.

After the Petri dishes were incubated for 12 hours at 20°C, conidial germination was evaluated under a binocular microscope. Those with no spore germination at a dose of 5 µg/ml were considered sensitive, and those with spore germination at a dose of 100 µg/ml were considered resistant (Kim and Xiao 2010).

DNA extraction and PCR amplifications

DNA extraction was performed according to Aljanabi and Martinez (1997). PCR reaction for the *SdhB* and *cytb* genes was prepared with a total volume of 25 µl. It was contained 5x PCR master mix (Solis BioDyne: 04-25-00125) 3 µl, forward primer 0.75 µl, reverse primer 0.75 µl, DNA 1.5 µl (50-100ng), and water 19 µl.

Primers IpBcBeg-F (5'-CCACTCCTCCATAATGGC TGCTCTCCGC-3') and IpBecEnd2-R (5'-CTCATCAAG CCCCCTCATTGATATC-3') were used to amplify of *SdhB* gene, and primers Qo13ext-F (5'-GGTATAACCCGACGG GGTTATAGAATAG-3') and Qo14ext-R (5'-AACCATCTC CATCCACCATACCTACAAA-3') were used to amplify of *cytb* gene (Leroux et al. 2010).

PCR conditions of the *SdhB* gene were performed as follows; first denaturation at 95°C for 12 min, at 95°C for 30 s, at 63°C for 30 s, at 72°C for 40 s, 35 cycles, and last elongation at 72°C for 10 min. PCR conditions of the *cytb* gene were performed as follows; first denaturation at 95°C for 12 min, at 95°C for 30 s, at 58°C for 30 s, at 72°C for 40 s, 33 cycles, and a final extension at 72°C for 10 min.

Five µl of PCR products were loaded onto a 1.2% agarose gel containing 3 µl (10 mg/ml) ethidium bromide. Five µl of 1 kb ladder (Thermo GeneRuler) was loaded into the gel, and a PCR mixture containing water was loaded into the last well as a negative control. Agarose gel electrophoresis was carried out at 100 V for 45 minutes using 1X TBE buffer. It was then imaged using an UV transilluminator. The remaining amounts of PCR products, from which bands were obtained in the gel image, were used for sequence analysis. In the *in vitro* bioassay, sequence analyses of 18 isolates found to be resistant and 2 isolates found to be sensitive were performed. Purification and sequencing of PCR products were performed by BMLabosis (Ankara, Turkey).

Detection of mutations associated with fungicide resistance

In the agarose gel, bands of 953 bp in the *SdhB* gene (Fig. S2) and 560 bp in the *cytb* gene were obtained. The *cytb* sequences

were aligned together with sensitive reference sequences with accession numbers FJ217740.1 and FJ217742.1 in the NCBI gene bank using MEGA11 (Fig. 1). Protein-coding regions of sequences were used to detect mutations in the *SdhB* gene (Veloukas et al. 2011). In this study, mutations in the *SdhB* gene of 20 isolates were determined using the MEGA11 program together with reference sequences in the NCBI, which contain different mutations associated with boscalid resistance in the *SdhB* gene (Fig. 2).

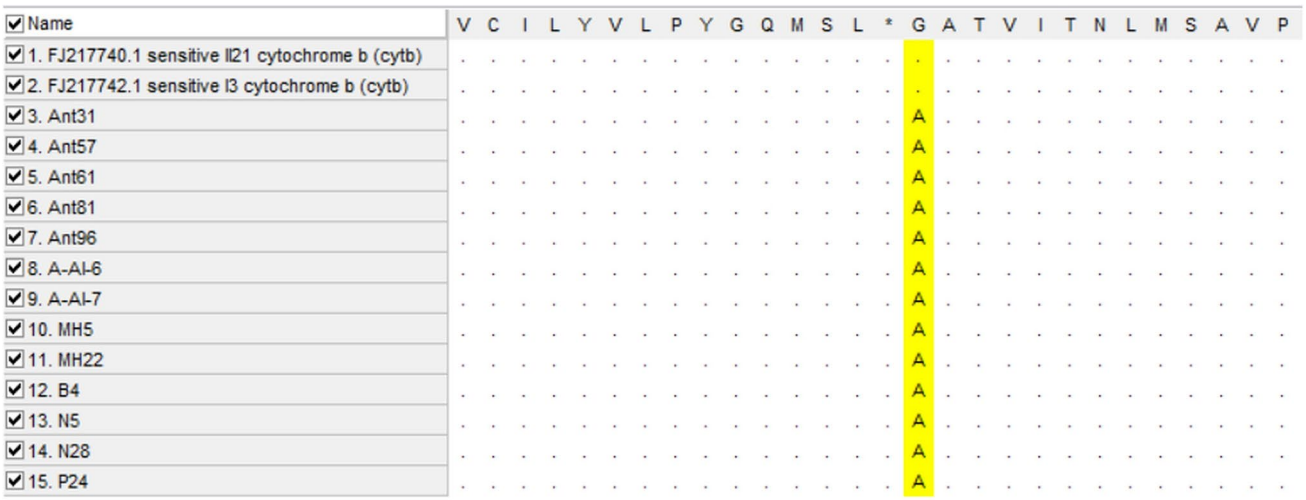


Fig. 1 Location of G143A mutation in the *cytb* gene of *Botrytis cinerea* isolates obtained from Turkey. *Numbers 1 and 2 represent reference sequences without mutations. Numbers 3-15 are the amino acid sequences of the Turkish isolates in which the mutation was detected

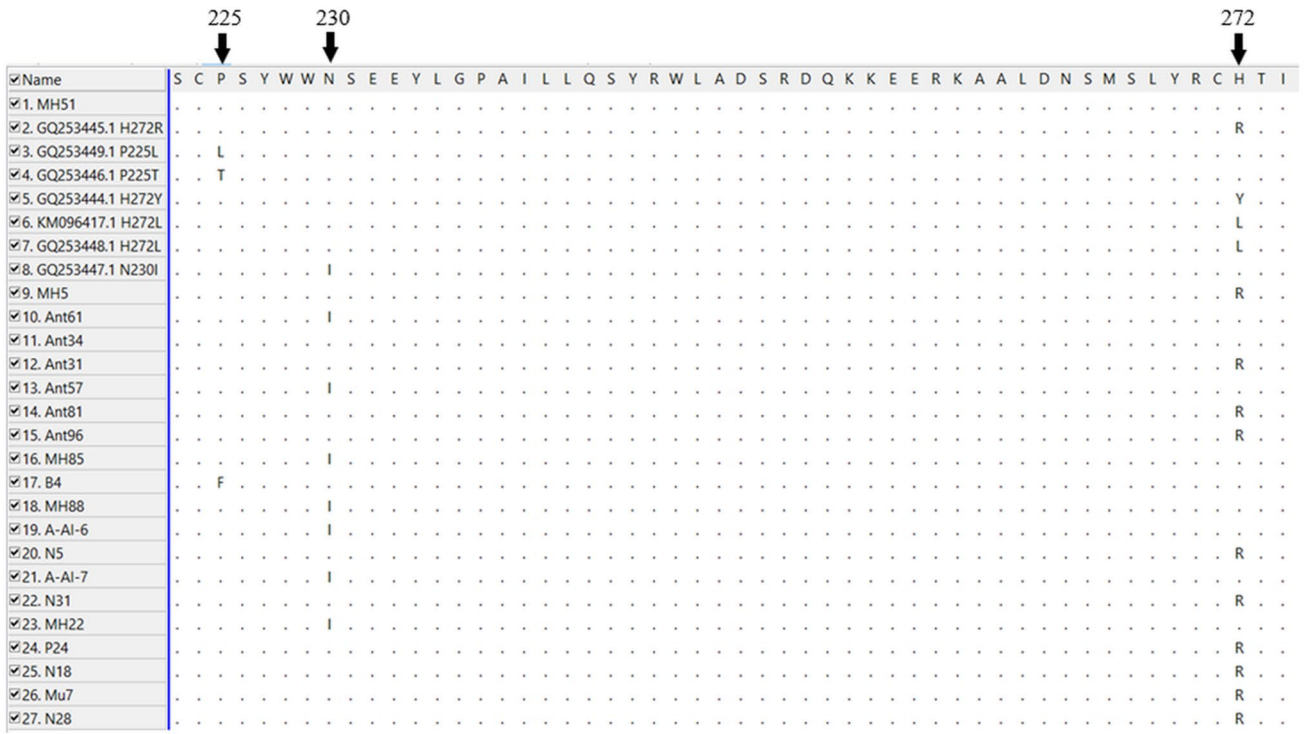


Fig. 2 Locations of P225F, N230I, and H272R mutations detected in the amino acid sequences obtained through alignment of the protein-coding regions of the *SdhB* gene of 12 *Botrytis cinerea* isolates (B4, Ant31, Ant57, Ant61, Ant81, Ant96, A-Al-6, A-Al-7, MH5, MH22, N5, N28, P24, MH85, MH88, N18, N31, Mu7). * Accession numbers GQ253445.1, GQ253449.1, GQ253446.1, GQ253444.1, KM096417.1, GQ253448.1, and GQ253447.1, located in rows 2-8, are reference sequences of H272R, P225L, P225T, H272Y, H272L and N230I mutations

Results

It was determined that 26% of the isolates used in the bioassay test were resistant. Mutations associated with boscalid resistance were found in 18 isolates whose *SdhB* gene was sequenced. No mutations were detected in sensitive MH51 and Ant34 isolates. H272R mutation was detected in MH5 isolate obtained from eggplant in Mersin province of Turkey. This mutation was also detected in all isolates obtained from lettuce in Ankara, Turkey. N230I mutation was detected in the A-Al-6 isolate obtained from eggplant in Antalya province and isolates obtained from tomatoes in Mersin province. P225F mutation was detected only in the B4 isolate obtained from the pepper in Antalya. It has been determined that N230I and H272R mutations were found to be common in the *SdhB* gene of *B. cinerea* populations in Turkey. H272R mutation was detected in 10 isolates, and N230I mutation was detected in 7 isolates (Table 1) (Fig. 3). P225H, P225L, P225T, H272L, H272Y, and I274V mutations reported in previous studies (Stammler et al. 2008; Leroux et al. 2010; Malandrakis et al. 2022) were not detected in this study. With this current study, mutations associated with boscalid resistance are reported for the first time in Turkey.

Table 1 The mutations in the *SdhB* and *cytb* genes of *Botrytis cinerea* isolates obtained from Turkey

	Genes and mutations			
	SdhB			*cytb*
Isolates	P225F	N230I	H272R	G143A
B4	+	-	-	+
Ant31	-	-	+	+
Ant57	-	+	-	+
Ant61	-	+	-	+
Ant81	-	-	+	+
Ant96	-	-	+	+
A-Al-6	-	+	-	+
A-Al-7	-	+	-	+
MH5	-	-	+	+
MH22	-	+	-	+
N5	-	-	+	+
N28	-	-	+	+
P24	-	-	+	+
MH85	-	+	-	*
MH88	-	+	-	*
N18	-	-	+	*
N31	-	-	+	*
Mu7	-	-	+	*
MH51	-	-	-	*
Ant34	-	-	-	*

- no mutation + there is a mutation * unsequenced isolates for *cytb*

G143A mutation associated with pyraclostrobin resistance was detected in 13 isolates (Fig. 1). The geographical distribution of the mutations is shown in Fig. S1 and the mutations detected in the isolates are listed in Table 1.

Discussion

There are many studies that show that the fungus gains resistance to boscalid and pyraclostrobin active substances and that there are some mutations associated with fungicide resistance (Leroux et al. 2010; Malandrakis et al. 2022). The occurrence of modifications in the target site where fungicides bind leads to the emergence of fungicide resistance.

In our previous *in vitro* study, it was determined that some *B. cinerea* isolates obtained from greenhouse tomatoes in Antalya province had resistance to boscalid and pyraclostrobin active substances (Gül et al. 2021). Therefore, this study aimed to identify mutations associated with resistance to these active substances in addition to *in vitro* tests.

In previous studies carried out in Turkey, in the *in vitro* fungicide tests of *B. cinerea* isolates obtained from lettuce in Bilecik in the Marmara region, it was reported that there was a decrease in the sensitivity level against boscalid+pyraclostrobin fungicide (Kavak 2022). Moreover, the G143A mutation associated with QoI fungicide resistance has been reported on *B. cinerea*. G143A mutation was detected in all *B. cinerea* isolates obtained from vineyards in the Aegean region (Selvi et al. 2016), and in 42% of *B. cinerea* isolates obtained from greenhouses in the Mediterranean and Aegean regions (Aghdam 2017). Similarly, in this current study, G143A mutation was detected in all isolates obtained from lettuce in the Central Anatolia region, and in all isolates obtained from pepper, tomato and eggplant in the Mediterranean region (Fig. S1). G143A mutation has been reported to cause high resistance to QoI (Fernández-Ortuño et al. 2012). Based on these results, it appears that *B. cinerea* populations in Turkey have a high level of resistance to QoI fungicides. Resistance-related F129L and G137R mutations were not detected in this study.

According to FRAC 2023, boscalid active substance, which is in the SDHIs group, is indicated in the medium-high risk of resistance. This group of fungicides inhibits mitochondrial respiration. Energy production in the cell is inhibited and death occurs in the fungus. The occurrence of modifications in the target site where fungicides bind leads to the emergence of fungicide resistance. The target enzyme of the fungicides in this group is SDH, which consists of four subunits (A, B, C, D). Mutations may develop in these three subunits (FRAC 2023), but mostly occur in the *SdhB* (Leroux et al. 2010).

The boscalid active ingredient is one of the most widely used fungicides in the control against *B. cinerea*. Fungicide

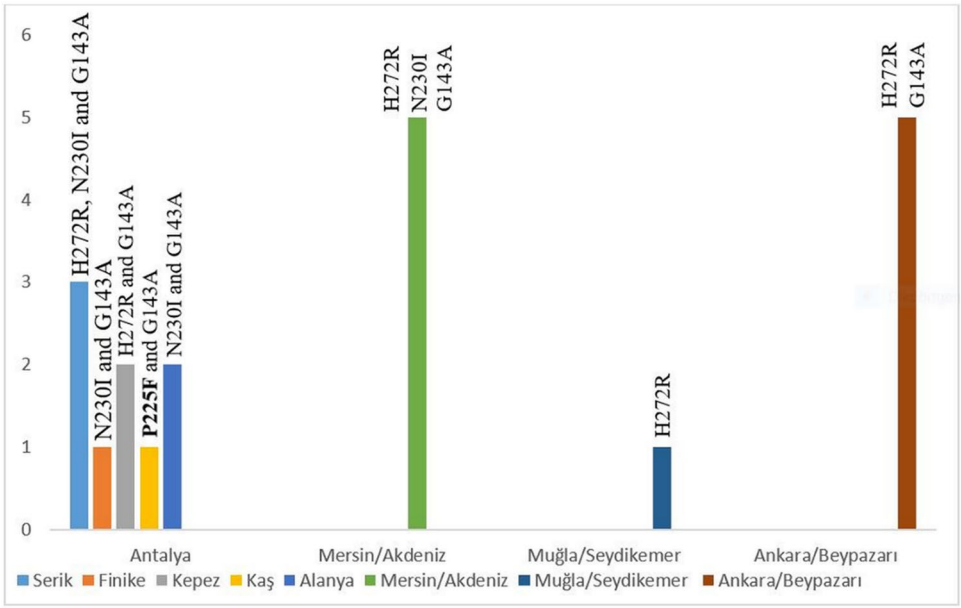


Fig. 3 Distribution of mutations detected in the *SdhB* and *cytb* genes of *Botrytis cinerea* isolates according to Turkish provinces and districts

resistance to this active ingredient has been reported in numerous countries (Veloukas et al. 2011; Leroch et al. 2011, 2013; Konstantinou et al. 2015; Kanetis et al. 2017). In Turkey, it has been stated that isolates show a high risk of resistance against the active substances pyrimethanil, boscalid, iprodione and fenhexamide, which are frequently used in the control of *B. cinerea* in vineyards in the Aegean region in Turkey (Selvi et al. 2016).

In the study conducted with isolates obtained from tomato and strawberry in Greece, it was determined that the H272R mutation, one of the *SdhB* mutations, was dominant (Konstantinou et al. 2015). This mutation was also found to be dominant in this study and detected in isolates obtained from tomatoes in Antalya and Muğla, eggplant in Mersin, and lettuce in Ankara. N230I mutation was the other commonly detected mutation. This mutation was detected in isolates obtained from tomato and eggplant in Antalya province and from tomato in Mersin province in Turkey (Fig. S1, Tables S1 and S2).

ROS (Reactive Oxygen Species) are small molecules generally produced as byproducts of metabolic processes in organisms (Zhang et al. 2020). At the beginning of pathogen infection, plant cells can rapidly accumulate abundant ROS (Wojtaszek 1997). ROS play an important role on fungi and plant interaction (Shetty et al. 2008). ROS produced by fungal pathogens accumulate in hyphal tips during the infection process (Heller and Tudzynski 2011). Moreover, the level of ROS affects the virulence of the fungus (Zhang et al. 2020). The possible consequences of *SdhB* mutations on ROS production in *B. cinerea* were investigated by Lalève et al. 2014. They found that the mutant containing H272R produced significantly less ROS than the wild type. When the effect on pathogenicity was examined, it was reported that the H272R mutation highly affected pathogenicity. It has been stated that the decrease in ROS production may be the main reason for the decrease in pathogenicity. It has been stated that mutations in the *SdhB* gene region of *Botrytis* isolates may also have effects on mycelial development and sclerotia formation (Lalève et al. 2014). These data show that fungicide resistance may lead to increased phenotypic variation in the fungus.

There is a study on the absence of the *Botrytis* S group in the population in which phenotypes resistant to boscalid are dominant. It has been stated that *B. cinerea* is more prone to boscalid resistance than *Botrytis* S group and therefore *B. cinerea* species may be more dominant than S group in the population (Konstantinou et al. 2015). *Botrytis* S group has not been detected before in Turkey and it has been reported that the dominant species in tomato greenhouses is *B. cinerea* (Gül et al. 2023). *Botrytis* S group may not have been detected in the Turkish population due to the intensive use of fungicides containing the active ingredient boscalid.

In conclusion, in this study, it is reported for the first time that P225F, N230I and H272R mutations are associated with boscalid resistance in *B. cinerea* in Turkey. G143A mutation associated with pyraclostrobin resistance was detected in all isolates sequenced in Turkey.

The fungicide resistance makes controlling the *B. cinerea* difficult and increases crop losses caused by the pathogen. Therefore, developing effective control programs are necessary. More extensive studies are needed to reveal the fungicide resistance profiles in *B. cinerea* populations. Thus, control programs that can be used for the pathogen can be prepared.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s40858-024-00646-4.

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Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of Interest The authors declare that they have no conflict of interest.

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