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Unravelling impact of Na+, Cl- and their respective transporter in detrimental effects of salt stress on the African rice species (*Oryza glaberrima* Steud.) †

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Abstract: Salinity resistance of the African rice species (*Oryza glaberrima*) is poorly documented and the specific responses of the plant to Na+ and Cl- toxic ions remain unknown. Moreover, the roles of Na+/H+ antiport and cation-chloride-cotransporter in salt stresses still remain unclear. In 1st experiment, two distinct cultivars TOG5307 and TOG5949 were maintained for 15 days on iso-osmotic nutrient solutions containing 50 mM NaCl, or a combination of Cl- salts (Cl- - dominant) or Na+ salts (Na+ - dominant). Plant water status, ion accumulation, gas exchange and fluorescence related parameters; carbon (Δ13C) and nitrogen (δ15N) isotope discrimination were analyzed. But Only ion content results were displayed in this paper. TOG5307 exhibited a higher level of resistance than TOG5949 in terms of growth and photosynthesis maintenance and control of Cl- and Na+ accumulation. NaCl was the most detrimental treatment, followed by Na+ -dominant treatment while Cl- -treatment had the lowest effect. Impact of Na+ and Cl- on considered parameters are additive. In 2nd experiment, the two contrasted cultivars (TOG5307 and TOG5949) were exposed during 3 days in nutrient solutions to 75 mM NaCl containing 100 µM amiloride (inhibitor of Na+-H+ antiporters) or 200 µM bumetanide (inhibitor cation-chloride-cotransporters). Amiloride increased Na+ accumulation in roots and leaves to a higher extent in salt-resistant TOG5307 than in salt-sensitive TOG5949. Bumetanide reduced Cl- accumulation in both cultivars as well as K+ accumulation in TOG5307 and Na+ accumulation in TOG5949. Cultivars exhibiting contrasting levels of salinity resistance are available in *Oryza glaberrima* and salt-tolerant genotypes may constitute a valuable source of gene for classical rice improvement.

Keywords: Salt stress; *Oryza glaberrima*; sodium; chloride; transporter.

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1. Introduction

Soil salinization is a major environmental constraint that hampers crop production in more than 5% of agricultural land and 20% of irrigated cultivated areas [1]. Unfortunately, rice is a typical glycophyte species exhibiting a high level of salt-sensitivity at the seedling and the flowering stages [2,3]. *Oryza glaberrima* has been only marginally cultivated but it now appears as a promising source of readily available genetic diversity for rice improvement [4]. This is especially valid for resistance to numerous abiotic and biotic constraints since numerous accessions of *O. glaberrima* exhibit interesting properties for resistance to high temperatures [5], drought [4], iron toxicity [6] and several diseases [7]. As far as salinity is concerned, *O. glaberrima* received recent attention to Na+ exclusion [8] or microRNA involved in biological pathways of salinity tolerance [9]. Accessions displaying contrasting levels of salt-resistance were recently identified [10]. Salinity is a complex environmental constraint that comprises two major components: i) an osmotic component related to a decrease in the external water potential which compromises plant water absorption and ii) an ionic component related to the accumulation of toxic ions [11,12].

The aim of the present work was therefore to compare the impacts of Na+, Cl- and their simultaneous presence as NaCl on two cultivars of *O. glaberrima* differing in salinity resistance. Plant behavior was analyzed in relation to mineral nutrition, plant water status, and photosynthetic properties. In order to gain additional information regarding the involvement of ions transporters in salinity resistance in *O. glaberrima*, a pharmacological approach was performed by exposing young seedlings from the two cultivars to NaCl in the absence or in the presence of the inhibitors : amiloride, a powerfull inhibitor of Na+/H+ antiporter [13] while bumetanide is inhibiting the cation-chloride-cotransporter [14].

2. Methods

Plants material and growth conditions

Seeds of *O. glaberrima* were obtained from Africa Rice (Bouaké, Ivoiry Coast). Previous researches demonstrated that accession TOG5307 (AccNumber WAB0021855) is salt-tolerant while TOG5949 (AccNumber WAB0020144) is salt-sensitive [10]. Seeds of each cultivar were germinated in glass vessels on two layers of filter papers (Whatman 85 mm, Grade 1) moistened with 10 mL of sterile deionized water. Glass vessels were placed in a germination chamber at 25 °C under a 16 h daylight period (150 μmole m^{-2} s^{-1}). Daytime humidity was 70% and illumination was provided by Sylvania fluorescent tubes (F36W/840-T8, cool white). Ten days old seedlings (plants at the 3 leaves stage) of uniform size from the two cultivars were transferred into a phytotron and fixed on polystyrene plates floating on Yoshida's nutritive solution (YNS) [15]. 18 seedlings of each cultivar were distributed per tank containing 16 L of YNS. The temperature was maintained at 29 °C during the day and 26 °C during the night and the illumination was provided by PHILIPS metal iodide lamp (HPIT/400W) for 16 h.day-1 with a photon flux density of 300 μmoles m-2 s-1. Daytime humidity was maintained between 65% and 80%.

1st experiment: After acclimatization during two weeks, Na+, Cl- or NaCl stresses were applied as described by Tavakkoli et al. [16]. The used solutions consisted in: Control (YNS, no amendments); 50 mM of Na+-dominant salts (YNS plus 7.5 mM Na2SO4, 7.5 mM Na2HPO4, 20 mM NaNO3); 50 mM of Cl--dominant salts (YNS plus 7.5 mM CaCl2.2H2O, 7.5 mM MgCl2.6H2O, 20 mM KCl); and NaCl (YNS plus 50 mM NaCl).

Ion content

For shoots and roots of each cultivar per treatment, *c.a.* 20 mg DW were digested with 4 mL of 0.5 % of nitric acid at 80 °C. After complete evaporation, residues were dissolved with HNO_3 (68%) + HCl_{cc} (1:3, *v/v*) and incubated under gentle agitation of 80 rpm for 48 h. The solution was then filtered using a layer of filter paper (Whatman 85 mm, Grade 1). The filtrate was used to estimate the concentrations of Na^+ and K^+ by flame emission using an Atomic Absorption Spectrometer (Thermo scientific S series model AAS4, Thermo Fisher Scientific Waltham, MA, USA). Anions were extracted according to Hamrouni et al. [17]. The concentrations of Cl^- and S^{2-} were determined by liquid chromatography (HPLC-Dinex ICS2000, Dionex Corporation, Sunnyvale, California, U.S.A.) using an AS15/AG15 column/precolumn system and 20-38 mM KOH as eluent for 40 min.

2nd experiment: inhibitors were added to 20 tanks per cultivars: 10 tanks received 150 μM amiloride and 10 others received 200 μM bumetanide (both provided by Sigma-Aldrich chemical). One day after inhibitor application, NaCl was added to half of the tanks (including those which did not receive inhibitor) to reach a concentration of 75 mM which was maintained for 3 days until plant harvest. Electrical conductivity was 0.82 ± 0.07

mS.cm-1 and 7.13 ± 0.24 mS.cm-1 for control and salt-solution, respectively; the presence of inhibitor had no impact on electrical conductivity.

Statistic treatment of the data

ANOVA 2 was performed considering cultivar and treatment as main factors. Analysis were performed on 5 biological replicates. Normality of the data was preliminary checked using Shapiro-Wilk tests and data were transformed when required. Statistical analysis was performed using SAS Entreprise Guide 6.1 (SAS 9.4 system for Windows).

3. Results and Discussion

Though the African rice species *Oryza glaberrima* displays a high level of intraspecific variability [6,10], it has rarely been characterized for its behaviour in the presence of NaCl. On the one hand, the present work demonstrates that the two tested cultivars exhibited contrasting levels of salinity resistance to salt stress: in the presence of NaCl, TOG5307 accumulated lower concentrations of Na+ in shoots and roots comparatively to TOG5949 while Cl- content was lower in the shoots but higher in the roots of TOG5307 (Figure 1). On the other hand, the present research shows that Amiloride increased Na+ accumulation in roots and leaves to a higher extent in salt-resistant TOG5307 than in salt-sensitive TOG5949. Bumetanide reduced Cl- accumulation in both cultivars as well as K+ accumulation in TOG5307 and Na+ accumulation in TOG5949 (Figure 2). This suggests that efficient regulation of Na+ absorption and Cl- translocation from root to shoot may be involved in salinity resistance in *O. glaberrima*. Until recently, the physiological impact of Cl- accumulation has often been neglected in studies devoted to salt stress but some data confirm that reduced net xylem loading of Cl- contributes to salt stress resistance in cereal species [18].

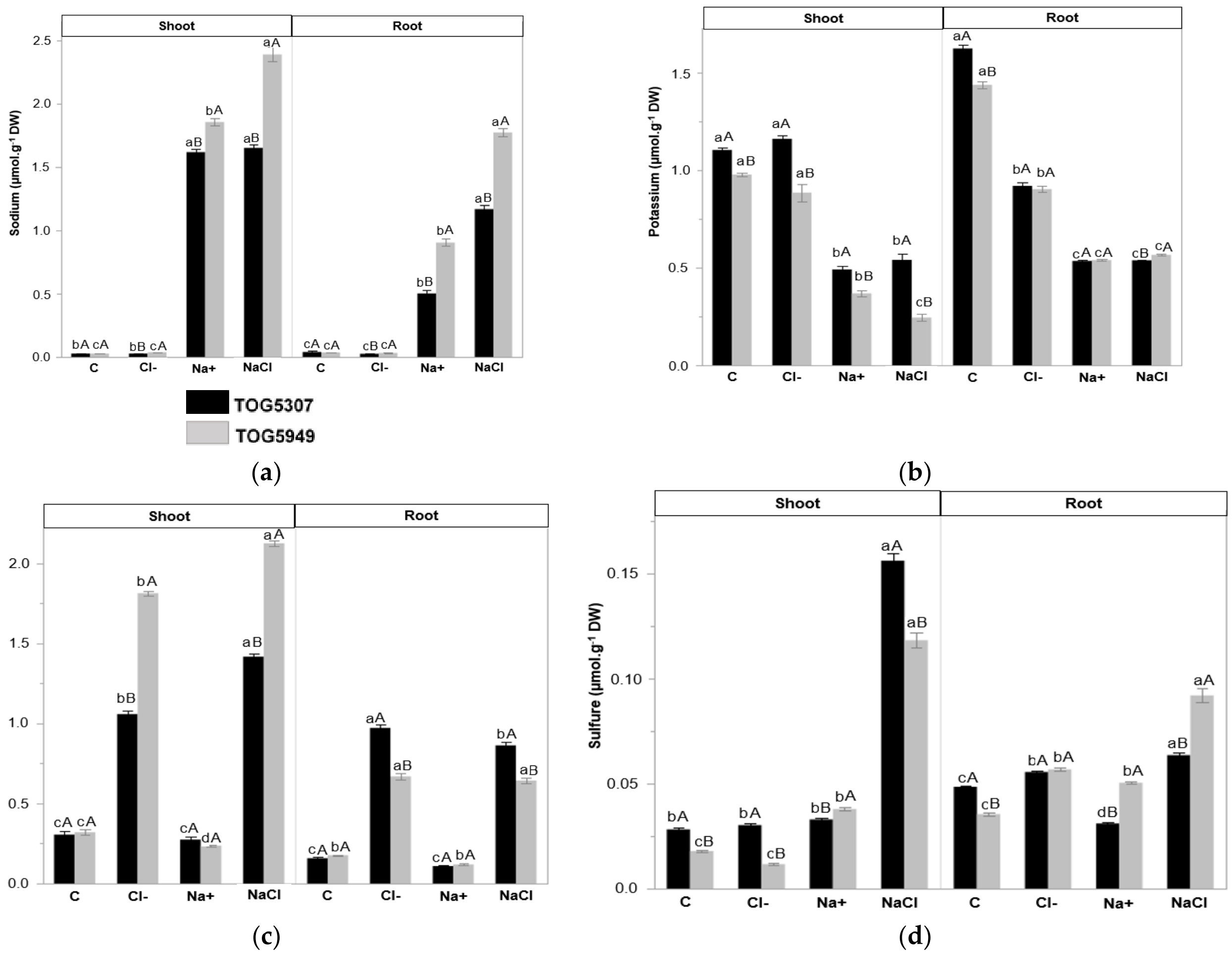
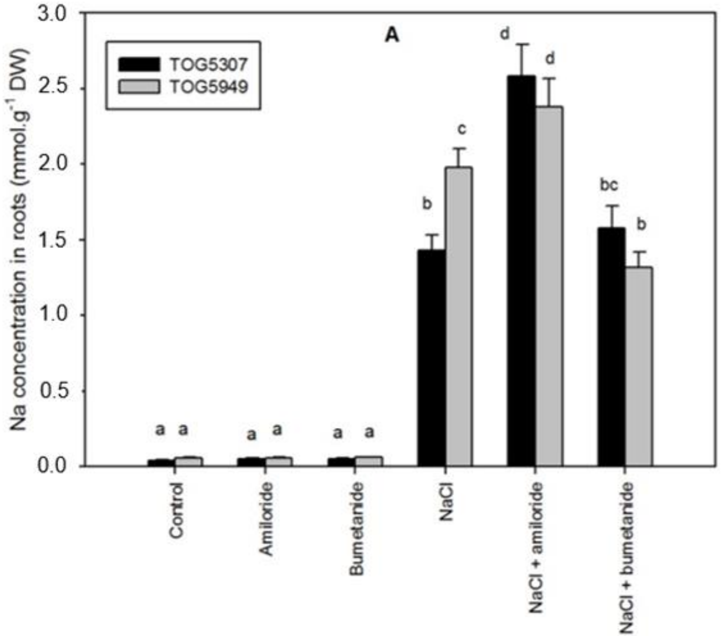
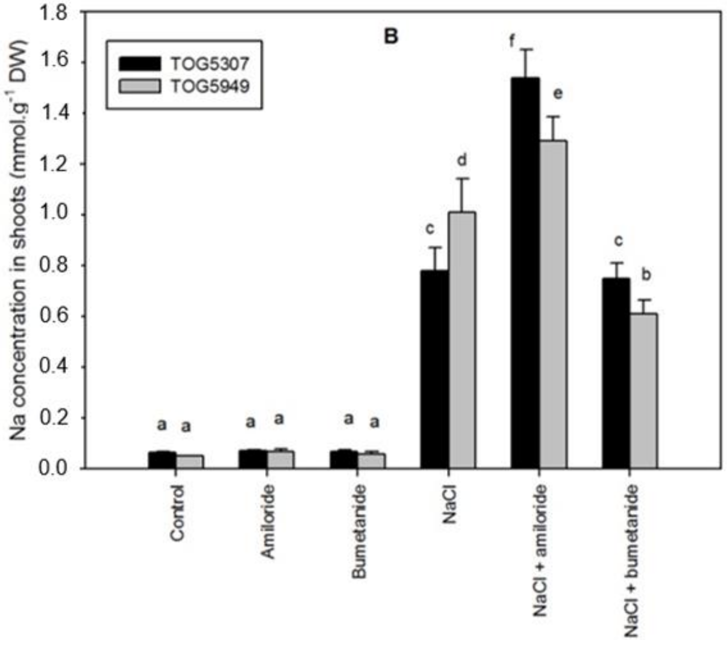


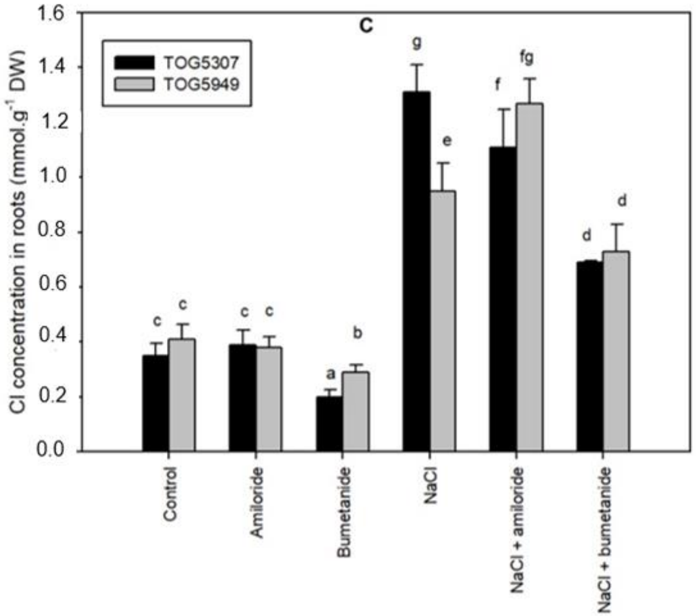
Figure 1. (A) sodium, (B) potassium, (C) chloride and (D) chloride concentration in shoots and roots of African rice seedlings (*Oryza glaberrima* Steud.) from cv. TOG5307 (black bars) and TOG5949 (grey bars) cultivated during 2 weeks in control conditions (C) or in the presence of 50 mM of either Cl- (chloride dominant), Na+ (sodium dominant) or NaCl. Each value is the mean of three replicates per treatment and vertical bars are standard errors of the mean. Treatments followed by the same lowercase latter for a particular cultivar do not differ statistically. Cultivars followed by the same uppercase latter in a particular condition do not differ statistically.


A
TOG5307
TOG5949
Na concentration in roots (mmol.g-1 DW)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Control
Amiloride
Bumetanide
NaCl
NaCl + amiloride
NaCl + bumetanide

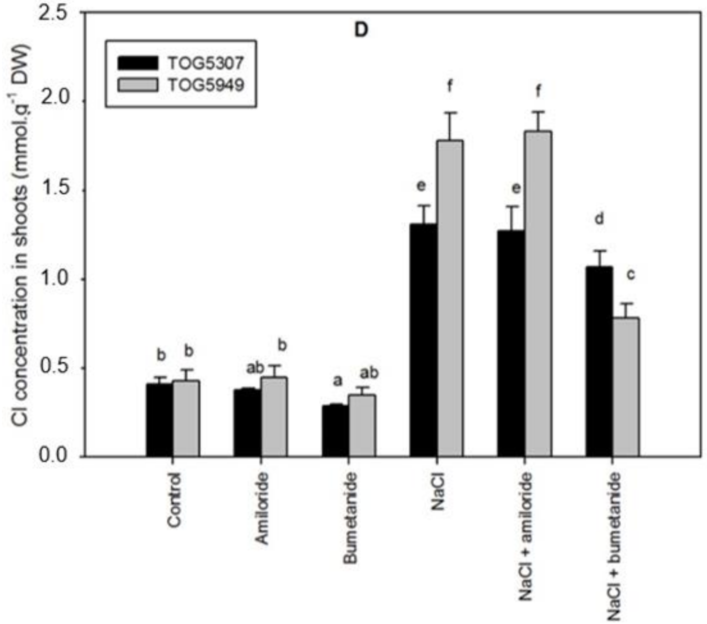
(**a**)


B
TOG5307
TOG5949
Na concentration in shoots (mmol.g-1 DW)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
Control
Amiloride
Bumetanide
NaCl
NaCl + amiloride
NaCl + bumetanide

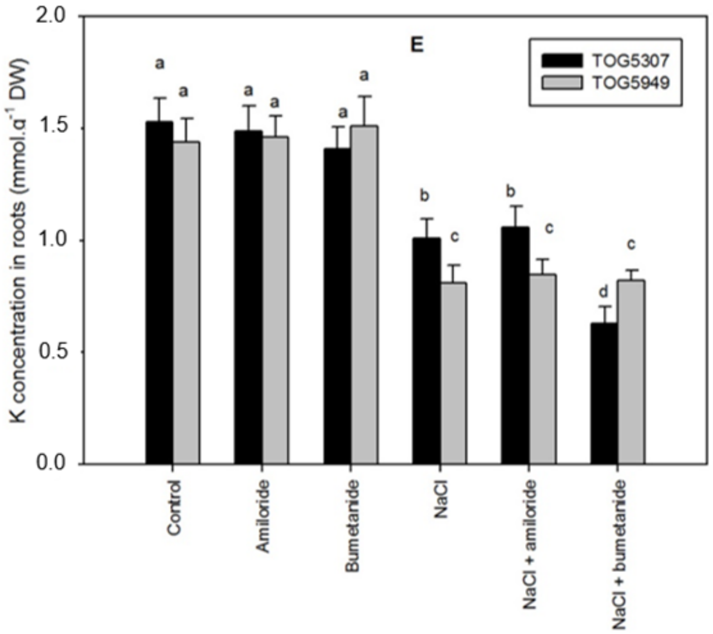
(**b**)


C
TOG5307
TOG5949
Cl concentration in roots (mmol.g-1 DW)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
Control
Amiloride
Bumetanide
NaCl
NaCl + amiloride
NaCl + bumetanide

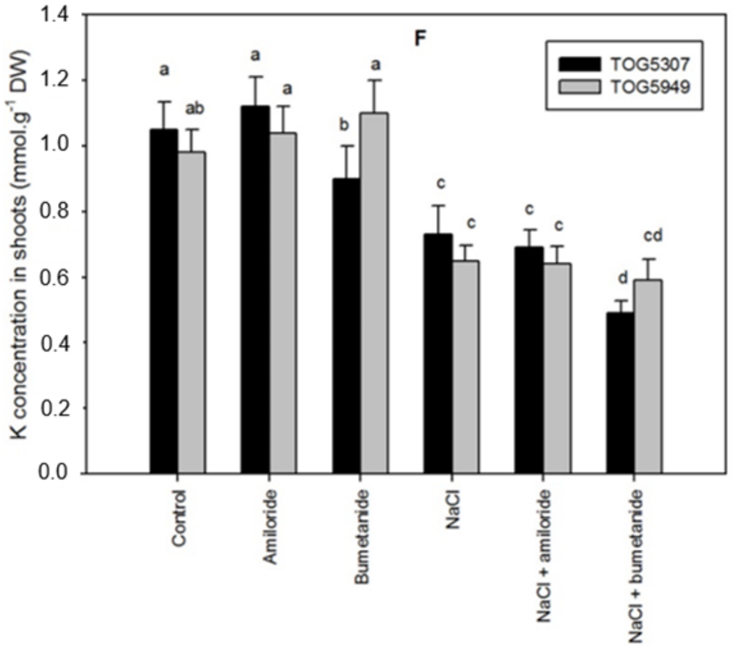
(**c**)


D
TOG5307
TOG5949
Cl concentration in shoots (mmol.g-1 DW)
0.0
0.5
1.0
1.5
2.0
2.5
Control
Amiloride
Bumetanide
NaCl
NaCl + amiloride
NaCl + bumetanide

(**d**)



(**e**)



(**f**)

Figure 2. Concentrations of Na+ (A;B), Cl- (C;D) and K+ (E;F) in roots (A, C and E) and in shoots (B, D and F) of two cultivars of the African rice specis *Oryza glaberrima* (TOG5307 : Scheme 5949. salt-sensitive). Plants were exposed during 72h to 0 (control) or 75 mM NaCl in the presence or absence of amiloride (100 μM) or bumetanide (200 μM). Each value is the mean of 5 biological replicates and bars represent standard errors. Values with different letters are significantly different at P = 0.05.

Salinity is a complex environmental constraint that exhibits a water stress component resulting from a decrease in the external osmotic potential. The ionic component of salt stress is itself a complex environmental constraint since it is related to Na+ and Cl- accumulation. In order to distinguish the Na+ and the Cl- effects, the present study compares the impact of NaCl solution with the impact of Na+ or Cl--enriched solution. Synergistic interaction between Na+ and Cl- ions was observed as reported in previous studies [16,19-21].

4. Conclusions

It is concluded that the differential impact of NaCl on two African rice (*Oryza glaberrima*) cultivars is mainly related to the ionic rather than to the osmotic component of salinity. Sodium appeared more toxic than Cl- on a wide range of parameters. In most cases, Na+ and Cl- acted in an additive way. Inhibition of Na+-H+ antiporters in roots induced an increase in Na+ concentration in all organs and a higher impact was recorded in the salt-resistant genotype suggesting that antiporter such as SOS1 play a major role in salinity resistance of African rice *Oryza glaberrima*. The CCC transporter is directly involved in Cl- accumulation but the counterion involved in the symport was preferentially K+ in the salt-resistant cultivar and Na+ in the salt-sensitive one.

Author Contributions: HP performed the experiment and analyzed the data; WI helps with the statistical treatment, GL performed the isotope discrimination analysis; SL and CG conceived the experiment and managed the research; SL and HP wrote the first draft of the manuscript; All the authors prepared the final version and approved submission.

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Conflicts of Interest: The authors declare no conflict of interest.

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