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SOIL SCIENCE

Liming as a means of reducing copper toxicity in black oats

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ABSTRACT: *Soils which are cultivated with grapevines have high available copper (Cu) content, which can be toxic to cover crops cohabiting vineyards, such as black oats. This study aimed to assess the effect of liming in reducing Cu toxicity in black oats grown in sandy soils. Samples of a Typic Hapludalf were collected at 0-20cm, dried and subjected to the addition of Cu (0 to 50Mg kg^{-1}) and limestone (0, 1.5, and 3.0Mg ha^{-1}). The soil was placed in a rhizobox and black oats were grown for 30 days. We assessed root and shoot dry matter production, copper (Cu), calcium (Ca) and magnesium (Mg) contents in the tissues; Cu content in the root symplast and apoplast, as well as Cu, carbon and pH values in the rhizosphere and bulk soil. Liming reduced Cu toxicity in black oats. Cu was preferentially accumulated in the roots, mostly in the apoplast, which may be the result of a plant tolerance mechanism to prevent the transport of Cu to the shoots.*
Key words: *heavy metal, phytotoxicity, limestone, rhizosphere.*

Calagem como forma de redução da toxidez por cobre em aveia preta

RESUMO: *Solos cultivados com videiras possuem alto teor de cobre (Cu) disponível, que pode ser tóxico às plantas de cobertura do solo que coabitam vinhedos, como a aveia preta. O estudo objetivou avaliar o efeito da calagem na redução da toxidez por Cu em plantas de aveia preta cultivadas em solo arenoso. Amostras de um Argissolo Vermelho foram coletadas na camada de 0-20cm, secas e submetidas à adição de duas doses de Cu (0 e 50Mg kg^{-1}) e três de calcário (0, 1,5 e 3,0Mg ha^{-1}). O solo foi acondicionado em rhizobox e submetido ao cultivo de aveia preta durante 30 dias. Avaliaram-se a produção de matéria seca das raízes e da parte aérea, o teor de cobre (Cu), cálcio (Ca) e magnésio (Mg) nos tecidos; o teor de Cu no simplasto e apoplasto das raízes, e os teores de Cu, de carbono e valores de pH no solo rizosférico e não rizosférico. A aplicação de calcário reduziu a toxidez por Cu na aveia preta. O Cu foi preferencialmente acumulado nas raízes, especialmente no apoplasto, o que pode ser resultado de mecanismo de tolerância das plantas para evitar o transporte de parte do elemento para a parte aérea.*
Palavras-chave: *metal pesado, fitotoxidez, calcário, rizosfera.*

INTRODUCTION

The frequent application of copper-based (Cu) fungicides, such as Bordeaux mixture (Ca $(OH)_2$ + $CuSO_4$), for the preventive control of foliar fungal diseases in grapevines (*Vitis* sp.) causes the increase of the Cu content in vineyard soils, especially in topsoil. In the soil, Cu may be complexed by soil organic matter (SOM) and sorbed by iron (Fe), aluminum (Al) and manganese (Mn) oxides and clay minerals, through physicochemical bonds in which the binding energy of Cu depends on soil pH and on the nature of the ligand (MICHAUD et al., 2007, CASALI et al., 2008). Therefore, increased Cu in the soil may increase the amount of more labile Cu fractions, which are more accessible and may cause toxicity to plants (CASALI et al., 2008; MIOTTO et al., 2017).

In the roots, uptake and radial transport of Cu can happen via the symplast, by means of specific transporters located in the plasma membrane, or by diffusion via the apoplast until finding diffusional barriers, such as the Casparian strip in the endodermis. The apoplast has charges derived from sulfuric (R-SH$^-$) and carboxylic (R-COO$^-$) groups, as well as organic compounds present in the cell wall. The pH can alter the charges of these groups, which can adsorb metal cations such as Cu and lead to compartmentalization of Cu in the root apoplast, preventing its transport to the shoots (YRUELA, 2009). However, Cu accumulation has also been observed in the symplast due to the formation of chelates with high affinity

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binding compounds such as organic acids, amino acids and peptides, such as metallothioneins (HALL, 2002). The complexed Cu is typically transferred to the vacuole to be compartmentalized and accumulated (YRUELA, 2009). The maintenance of Cu in the roots, complexed by organic molecules and/or compartmentalized in the vacuole, prevents its transport to the shoots, where Cu concentrations between 20-100mg kg^{-1} may cause serious changes in metabolism (KABATA-PENDIAS, 2011).

In acidic soils with high Cu content, such as those of the vineyards in southern Brazil, the use of liming as a management practice may reduce Cu toxicity in plants such as black oats and grapevines (AMBROSINI et al., 2015). Liming causes the copper hydroxide increase in the soil solution by pH increase causes Cu complexation and precipitation, and increase cation exchange capacity (CEC), enhancing heavy metals adsorption (JORIS et al., 2012). In addition, liming increases calcium (Ca) and magnesium (Mg) content in the soil, which compete with Cu for absorption pathways (KOPITTKE, et al., 2011), reducing Cu roots accumulation and shoot transport (JUANG et al., 2014). However, there are few studies in the literature demonstrating the beneficial effects of liming on black oats grown in sandy soils with high Cu content. The study aimed to assess the effect of liming in reducing Cu toxicity in black oats grown in sandy soil.

MATERIALS AND METHODS

Samples of a Typic Hapludalf (Soil Survey Staff, 2006) were collected at 0-20cm in a grassland area adjacent to vineyards in the city of Santana do Livramento, located in the region of the Campanha Gaúcha, state of Rio Grande do Sul (RS), southern Brazil. The soil was air dried, passed through a 2mm mesh sieve, and homogenized. A soil sample was subjected to chemical analysis and grain size analysis. The soil had 30g kg^{-1}, 61g$kg^{-1}$and 909g kg^{-1} of clay, silt and sand, respectively (The Pipette method - EMBRAPA, 1997); 5.1g kg^{-1} of total organic carbon (TOC) (Wet oxidation - EMBRAPA, 1997); 4.5 water pH (TEDESCO et al., 1995); 4.8mg kg^{-1} and 30.7mg kg^{-1} of available phosphorous (P) and potassium (K), respectively (extracted by Mehlich-1 - TEDESCO et al., 1995); 3.1$cmol_c$ kg^{-1}, 2.0$cmol_c$ kg^{-1} and 1.8$cmol_c$ kg^{-1} of exchangeable aluminum (Al), Ca and Mg, respectively (extracted by KCl 1 molL^{-1} - TEDESCO et al., 1995); and 2.4mg kg^{-1} of available Cu (EDTA extractor 0.01 molL^{-1} - CHAIGNON et al., 2009). The rest of the reserved soil was divided into three portions, and three doses of limestone were added: 0, 0.5g kg^{-1} and 1.0g kg^{-1} of soil –equal to 0, 1.5Mg $ha^{-1}$and 3.0Mg ha^{-1}, respectively. For more details on soil preparation see AMBROSINI et al. (2016).

The experimental design was a randomized block with five replicates in a 2x3 factorial arrangement, i.e., two doses of Cu (0 to 50mg kg^{-1}) and three doses of limestone (0, 1.5, and 3.0Mg ha^{-1}), totaling six treatments. Plants were grown for 30 days in a controlled environment at a temperature of 25±2°C and a photoperiod of 16 hours of light, with photosynthetically active radiation of 200µmol of photons m^{-2} s^{-1}. A modified HOAGLAND &ARNON (1950) solution was added throughout the cultivation to supply the nutrients, except Cu, Ca and Mg. For more details on the experimental units and cultivation see AMBROSINI et al. (2016).

At the end of the cultivation period, shoots were cut close to the soil surface and the roots were separated from the soil by hand. Afterwards, the root and shoot fresh mass (FM) was determined on a precision balance. The fresh roots were separated into two parts: one was immediately stored in a freezer (-20°C) for further analysis of Cu in the apoplast and symplast, and the other, along with the shoots of black oats, was dried in an oven with forced air at 65°C until constant mass. With this material, dry matter (DM) was quantified on a precision balance. Roots and shoots were ground and set aside for the analysis of the total contents of Cu, Ca and Mg.

In order to collect the rhizosphere and bulk soils, the acrylic plate of the rhizobox was removed and the roots were carefully separated from the soil. Roots were shaken three times and the soil that remained adhered to them was considered the rhizosphere, and it was carefully removed from the roots with a brush. The soil released from the roots during the shaking was considered bulk soil. Both were air dried, ground in a porcelain mortar and reserved.

Total contents of Ca, Mg and Cu in tissues were determined according to EMBRAPA (1997). The analysis of Cu in the root apoplast and symplast was carried out according to CHAIGNON & HINSINGER (2003). The Cu contents in the rhizosphere and bulk soil were determined by two different methods: 0.05mol L^{-1} Na_2-EDTA/1.0mol L^{-1}ammonium acetate, pH 6.0 (CHAIGNON et al., 2009), herein called Cu-EDTA; and 0.01mol L^{-1} $CaCl_2$ (NOVOZAMSKY et al., 1993), herein called Cu-$CaCl_2$.The determination of the pH of the rhizosphere and bulk soil was done in water, in 1:1 ratio (TEDESCO et al., 1995). The TOC content was analyzed by wet oxidation using potassium dichromate in sulfuric acid (0.2mol L^{-1}) medium, and the determination was done by titration with 0.1mol L^{-1} ammonium ferrous sulfate (EMBRAPA, 1997).

Data were subjected to analysis of variance (ANOVA) and, when the F test was significant ($p < 0.05$), means were compared by the Tukey test ($p < 0.05$).

As it is a factorial design, in cases where there was interaction between factors (Table 1), only the results of the interaction were presented and discussed; in cases where the interaction was not significant (Table 1) only the results of main effects were presented and discussed.

RESULTS AND DISCUSSION

Dry matter production and plant nutrient content

The application of 50mg kg^{-1} Cu in the soil reduced dry matter production of the roots and shoots in 2.5 and 1.9 times (on average), respectively (Table 2). With regard to the root system, the plants grown in soil with the addition of Cu alone (no limestone) did not produce sufficient dry matter to be assessed. However, there was root growth in the treatments with liming and the addition of Cu and shoot dry matter production was twice as much in comparison to the treatment without liming, regardless of the corrective dose (Table 2). The toxic effects of Cu in black oats were minimized by the addition of 1.5 and 3.0Mg ha^{-1} limestone. This possibly happened due to the increase in pH and cation exchange capacity (CEC) of the soil, reducing Cu availability to plants and, consequently, reducing the effects of toxicity, which allowed the growth of roots and shoots (AMBROSINI et al., 2015).

The average Cu content in the shoots of black oats in the treatments without addition of Cu was 23.2mg kg^{-1} (Table 2), which falls within the normal range (5 to 30mg kg^{-1}) for most cultures (KABATA-PENDIAS, 2011). However, when black oats were grown in the treatment with the addition of 50mg kg^{-1} Cu without liming, the average Cu content in the shoots was 95.7mg kg^{-1} (Table 2), which represents on average an increase of 157% in Cu content. Application of 1.5 to 3.0Mg ha^{-1} limestone reduced the Cu content by 39% and 49% in the shoots of black oats, respectively.

In the roots, the increase of the dose of limestone did not change Cu content. However, in the treatment with the addition of Cu without liming, there was no root dry matter production and Cu content could not be determined (Table 2). However, in the treatments without Cu addition, where there was root dry matter production, Cu contents were on

Table 1 - Analysis of variance for the variables determined in the shoots and roots of black oats and in the rhizosphere and bulk soil, without and with the addition of copper, combined with doses of limestone.

Variable	---Sources of variation---			CV (%)
	Cu (A)	Limestone (B)	A x B	
	---Shoots---			
Dry matter	324.74**	20.59**	1.69^{ns}	12.63
Cu content	146.59**	146.59**	38.68**	15.42
Ca content	27.89**	15.91**	8.81**	8.52
	10.52**	16.28**	9.39**	6.92
	---Roots---			
Dry matter	24.99**	1.05^{ns}	0.85^{ns}	23.45
Cu content	2782.34**	2.31^{ns}	0.57^{ns}	8.40
Ca content	13.05**	15.88**	1.26^{ns}	18.20
Mg content	30.78**	11.89**	18.17**	13.15
Apoplast Cu	755.62**	17.91**	29.83^{ns}	15.59
Symplast Cu	453.72**	0.81^{ns}	2.14^{ns}	14.67
	---Rhizosphere soil---			
pH in water (1:1)	7.81*	256.00**	0.00^{ns}	21.43
TOC	0.16^{ns}	1.96^{ns}	0.59^{ns}	14.67
Cu-$CaCl_2$	3.09^{ns}	0.57^{ns}	1.06^{ns}	11.21
Cu-EDTA	1402.91**	3.55^{ns}	10.01**	26.71
	---Bulk soil---			
pH in water (1: 1)	39.49**	414.57**	1.62^{ns}	2.24
TOC	0.07^{ns}	3.79^{ns}	1.46^{ns}	9.97
Cu-$CaCl_2$	0.80^{ns}	2.85^{ns}	0.35^{ns}	16.21
Cu-EDTA	1402.91**	3.55^{ns}	10.01**	17.49

CV = coefficient of variation; ns = not significant; * and ** = significant by the F test at 5 and 1% probability, respectively.

Table 2 - Dry matter and copper, calcium and magnesium contents in the shoots and roots of black oats grown in soil without and with the addition of copper combined with limestone doses.

Cu (mg kg^{-1})	Limestone (Mg ha^{-1})			Mean
	0.0	1.5	3.0	
	Shoots			
	Dry matter (g plant^{-1})			
0	0.090	0.104	0.116	0.103A
50	0.026	0.052	0.050	0.042B
Mean	0.058b	0.078a	0.083a	
	Cu content (mg kg^{-1})			
0	21.72aB[1]	27.34Ab	20.6aB	23.23
50	95.74aA	44.42Ba	39.07bA	59.74
Mean	58.73	35.88	29.84	
	Ca content (g kg^{-1})			
0	11.03aB	9.56Ab	10.56aA	10.38
50	15.93aA	11.96bA	10.66bA	12.85
Mean	13.48	10.76	10.61	
	Mg content (g kg^{-1})			
0	4.18aB	4.02aA	3.93aA	4.05
50	5.48aA	3.98bA	4.02bA	4.49
Mean	4.83	4.01	3.97	
	Roots			
	Dry matter (g plant^{-1})			
0	0.074	0.084	0.066	0.074A
50	NA	0.042	0.038	0.040B
Mean	NA	0.063	0.052	
	Cu content (mg kg^{-1})			
0	40.88	16.37	37.83	31.69B
50	NA	445.70	454.01	449.85A
Mean	NA	231.03	245.92	
	Ca content (g kg^{-1})			
0	6.01	4.36	8.24	6.21B
50	NA	6.81	9.40	8.11A
Mean	NA	5.58 b	8.82 a	
	Mg content (g kg^{-1})			
0	3.36a	2.22bA	4.19aA	3.26A
Mean	NA	3.26 b	4.31 a	

average 31.69mg kg^{-1} (Table 2). When the black oats were subjected to the addition of 50mg kg^{-1} of Cu, the average Cu content in the roots was 449.85mg kg^{-1} (Table 2), which represents an increase of 14 times.

This increase in Cu content in the root, but not in the shoot, is due to its preferential accumulation in the root system, because of its high affinity for ligands of the root cell wall, causing low mobility within the plant (AMBROSINI et al., 2016). Accumulation in roots is an important mechanism to prevent Cu transport to the shoots, which in high concentrations causes oxidative damage (DALCORSO et al., 2014). However, exposure of the root system to high concentrations of Cu may cause damage to its structure, such as reduced cap, disorganized root apex cells and reduced root growth (AMBROSINI et al., 2015; GUIMARÃES et al., 2016).

Although, we did not carry out structural analysis on the root system in this study, root death in the treatment with the addition of Cu without liming indicates that excess of Cu presents severe damage to the formation of roots of black oats, reducing DM production, thus confirming the results obtained by GUIMARÃES et al. (2016) with the same species. This inhibitory effect of excess Cu on root growth may have reduced the uptake of water and nutrients by the plant and; consequently, caused a decrease in growth and shoot dry matter production of black oats (AMBROSINI et al., 2016).

Ca contents in the shoots and roots increased by 31 and 35% with Cu addition, respectively, except for the roots of the treatment with the highest dose of limestone. (Table 2); while, the Mg content in the shoots increased by 31% (Table 2). Increase of Ca and Mg contents by adding Cu was probably caused by concentration of these nutrients in the plant, since there was reduced dry matter production in these treatments (Table 2). The excessive increase of a cation in the soil, in this case Cu, due to mass effect, makes it more competitive compared to other cations, such as Ca and Mg, by the adsorption by the ligands of the root cell wall, which favors its uptake by the plant (KOPITTKE et al., 2011).

Copper content in the root apoplast and symplast

Cu content in the apoplast and symplast, and total Cu in the roots were higher in plants grown in soil with the addition of Cu, with average increases of 36 and 14 times, respectively (Figure 1A and 1B). However, the application of 3.0Mg ha^{-1} limestone reduced the Cu content in the apoplast by 36% (Figure 1A), while it did not change the Cu content in the symplast (Figure 1B).

The plant cell wall has -COOH, -OH, and -SH groups that have affinity for divalent and trivalent cations at the pH commonly reported in the apoplast. These interactions occur mainly with polysaccharides rich in carboxyl groups, such as homogalacturonans (HGAs), which are part of pectins (KRZESŁOWSKA, 2010). With the increase in pH derived from liming, these groups are expected to exhibit lower binding capacity and; therefore, less Cu will be adsorbed to the wall. In addition, Ca^{2+} is a ligand commonly reported in pectins and; therefore, liming may result in occupying the binding sites, preventing Cu adsorption (KRZESŁOWSKA, 2010).The use of liming clearly decreased the Cu content in the apoplast (Figure 1A),

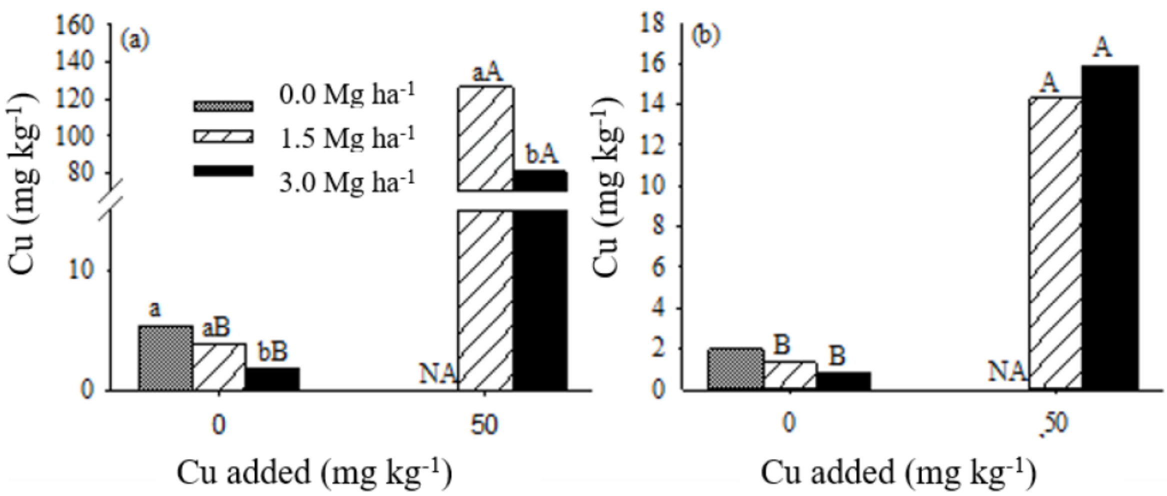

Figure 1 - Copper (Cu) content in the root apoplast (a) and symplast (b) of black oats grown in soil with and without the addition of Cu, combined with limestone doses. Means followed by the same capital letter (Cu factor) and lowercase letter (liming factor) do not differ by the Tukey test (P <0.05); NA = not analyzed.

but no change was observed in the Cu content in the symplast (Figure 1B).Conversely, the increase in the dose of Cu significantly increased the Cu content in both apoplast and symplast.

Soil attributes

For the soil attributes, only pH values in water and Cu extracted by EDTA (Cu-EDTA) were influenced by treatments (Table 3). TOC and Cu extracted by $CaCl_2$ (Cu-$CaCl_2$) were neither affected by the addition of Cu nor by liming. Of these attributes, Cu-$CaCl_2$ was possibly not altered because of its low values (from 0.02mg $kg^{-1}$to 0.15mg kg^{-1}), which made detection difficult.

The pH in water increased with the increasing doses of limestone both in the rhizosphere and bulk soil (Table 3), which was expected. Moreover, in both, pH in water were lower in soil with the addition of 50mg kg^{-1} Cu compared to the soil without Cu addition (Table 3), which may have been caused by

Table 3 - Values pH in water, total organic carbon (TOC), copper extracted by EDTA (Cu-EDTA) and copper extracted by $CaCl_2$ (Cu-$CaCl_2$) in soil without and with the addition of this metal, combined with doses of limestone.

Copper (mg kg^{-1})	------Limestone (Mg ha^{-1})------			Mean
	0	1.5	3.0	
	------Rhizosphere soil------			
	------pH in water (1:1)------			
0	5.08	6.04	6.85	6.44 A[(1)(2)]
50	NA	5.74	6.56	6.15 B[(2)]
Mean	NA	5.89 b	6.70 a	
	------TOC (g kg^{-1})------			
0	4.70	5.10	5.03	5.06[(2)]
50	NA	5.66	4.96	5.31[(2)]
Mean	NA	5.37	4.98	
	------Cu-$CaCl_2$ (mg kg^{-1})------			
0	0.02	0.00	0.00	0.00[(2)]
50	NA	0.15	0.04	0.10[(2)]
Mean	NA	0.08	0.02	
	------Cu-EDTA (mg kg^{-1})------			
0	1.89 b	3.05aB	2.47aB	2.76B[(2)]
50	NA	32.68aA	38.20aA	35.44A[(2)]
Mean	NA	17.86	20.33	
	------Bulk soil------			
	------pH in water (1:1)------			
0	4.96	5.94	6.59	5.83 A
50	4.71	5.53	6.37	5.53 B
Mean	4.84 c	5.73 b	6.48 a	
	------TOC (g kg^{-1})------			
0	5.33	5.53	4.66	5.17
50	5.83	5.03	4.86	5.24
Mean	5.58	5.28	4.70	
	------Cu-$CaCl_2$ (mg kg^{-1})------			
0	0.04	0.14	0.10	0.09
50	0.02	0.08	0.10	0.06
Mean	0.03	0.11	0.10	
	------Cu-EDTA (mg kg^{-1})------			
0	2.03aB	2.32aB	2.61aB	2.32B
50	35.73aA	36.89aA	39.36aA	37.33A
Mean	18.88	19.61	20.98	

[(1)]Means followed by the same capital letter in the column and by the same lowercase letter in the row do not differ by the Tukey test ($P < 0.05$); NA = not analyzed;[(2)]Means of the doses of 1.5 and 3.0 Mg ha^{-1} limestone, as there is no rhizosphere soil in the treatment without liming and with 50 mg kg^{-1}Cu.

the adsorption of Cu in functional groups of organic and inorganic reactive particles, and desorption of H^+ ions to the soil solution (DUPLAY et al., 2014).

Rhizosphere and bulk soils with the addition of 50mg Cu kg^{-1}soil exhibited the highest Cu contents extracted by EDTA compared to treatments without the addition of Cu, while the different doses of limestone did not change the Cu contents extracted by EDTA (Table 3).The absence of reduction in Cu contents in soil by the increase in limestone doses can be explained by the high correlation of the Cu extracted by EDTA and total Cu in the soil, as observed by BRUN et al. (1998), because the Cu extracted by EDTA is only slightly influenced by pH and soil CEC. Furthermore, according to these authors, the Cu extracted by EDTA is not always a good indicator of the amount of Cu uptake by plants.

Although, the soil Cu contents extracted by EDTA and $CaCl_2$ did not decrease with increasing doses of limestone, the effects of liming in reducing Cu toxicity in black oats are clear, since the increase in doses of limestone promoted increased plant growth, resulting in more dry matter production (Table 2).

CONCLUSION

Liming reduced Cu toxicity in black oats and can be recommended to reduce the negative effects on sandy soils with low organic matter content. Liming with a dose of 1.5Mg ha^{-1} limestone capable of raising soil to pH 5.5, reduce the phytotoxic effects of Cu in vineyard soils with a history of copper-based fungicide application.

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DECLARATION OF CONFLICT OF INTEREST

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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