Platinum Open Access Journal (ISSN: 2069-5837)

Flag Leaf Tolerance Study in Moroccan Barley (*Hordeum vulgare* L.) Varieties Submitted to a Severe Salt Stress

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Received: 22.04.2021; Revised: 28.05.2021; Accepted: 3.06.2021; Published: 8.08.2021

Abstract: Salt stress is the most significant abiotic stress that can severely limit crop growth and productivity. This problem gets worse in the context of climate change. The Knowledge of genetic pool behavior under such environmental constraints is imperative for growing and research. Here, we tested salt stress tolerance in six barley varieties ('Amira', 'Oussama', 'Tamellalet', 'Adrar', 'Taffa', and 'Laanaceur'). To this end, a set of biochemical parameters (chlorophylls, proline, sodium, potassium levels and K⁺/Na⁺ ratio) were measured. Salt constraint significantly reduced chlorophyll content and K⁺/Na⁺ but resulted in high records of proline and Na⁺. Our outcomes show that treatment was the main variability since it explained more than 75% in data variability followed by variety effect. Wide variabilities were found among varieties for the measured parameters. Higher proline levels and K⁺/Na⁺ were found in 'Adrar', 'Tamellalet' and 'Taffa'. These two later varieties also displayed a higher record of K⁺. Lower Na⁺ values were recorded in 'Laanaceur', 'Taffa', and 'Amira' were relatively salt-sensitive due to their higher Na⁺ and lowered K⁺/Na⁺ and proline content. Resistant varieties could represent a good background for breeding for barley salt tolerance.

Keywords: Barley; Hordeum vulgare; salt stress; biochemical parameters; salt tolerance.

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1. Introduction

As a staple food in diverse civilizations, barley (Hordeum vulgare L.) has been noted historically for health-promoting benefits [1]. This crop is among the most tolerant salt crops grown in very high salinity areas [2]. In Morocco, barley is the second important cereal after wheat in terms of production and consumption [3]. Unfortunately, according to these authors, the arable land is increasingly affected by salinity, and the saline area reached more than 500,000 ha with damaged soil. Indeed, one of the main factors of soil degradation is salinization; about 19.5% of irrigated land and 2.1% of the arid land are threatened by salinity [4]. In semi-arid and arid regions, salinity is a major adverse factor, severely reducing plant growth and crop productivity [1,5–9].

Salt stress affects metabolic activities and nutrient absorption in plants [10]. It has been shown that the salt response depends on the species, variety, salt concentration, growing conditions, and stage of development [10,11]. Salt stress is widely reported to cause a series of unfavorable biochemical, physiological, and morphological changes that damage

photosynthesis and other biochemical processes combined with plant growth, development and productivity [12,13]. Indeed, salinity is likely to disrupt the mineral nutrition of plants in interfering with the removal of some essential elements like potassium and calcium. In addition, the increase in NaCl concentration in the surrounding root area has been shown to cut down the absorption of potassium and calcium, interferes with their physiological functions [14].

Under salt stress, plants have improved their mechanisms to cope with salt constraints and to adapt to osmotic and ionic stress caused by salinity [5,8,15]. In this context, there is an association between osmoregulation and some potent osmoprotectants such as proline, soluble sugars, and potassium [16]. The change of proline concentration is generally related to the adaptation or tolerance of salt stress [17,18]. The known role of proline is membrane stabilization and osmotic adjustment, and detoxification of harmful ions in plants grown in saline environments [16]. Chlorophyll content was reported to decrease drastically in salt-sensitive plants compared to salinity-tolerant plants [1]. High salinity results in reduced growth, which may be due to reduced leaf area and hence a lower light interception [19], even with low salt concentrations, there was a decrease in chlorophyll [6,20,21].

A literature review shows that there is a scarcity of information on Moroccan barley varieties' behavior under salt stress conditions. Hence the originality of our work, which had as goals, (i) to screen some biochemical parameters in six Moroccan barley varieties submitted to severe salt stress and (ii) to compare these varieties in terms of different measured biochemical parameters in order to select the most resistant varieties.

2. Materials and Methods

2.1. Plant material and culture conditions.

This work was carried out in a pot experiment in the growth chamber at the experimental station Polydisciplinary Faculty of of the Taza. Six barley (Hordeum vulgare L.) varieties used in this experiment were selected from several cultivars tested for sensitivity to salt stress, widely grown in Morocco. Seeds were supplied by the National Institute for Agricultural Research (INRA) and included in the official catalog of varieties (Table 1). They were grouped into two periods, namely old (released between 1980 and 1990) and intermediate (released between 1990 and 2000). Seeds were first disinfected with 5% (v/v) commercial bleach sodium hypochlorite solution (NaOCl) for 5 min and rinsed 3 times with distilled water as described in Taibi et al. (2006) and Athar et al. (2015) [22,23]. Twenty seeds of each variety were sown in 10 L plastic pots containing a mixture of soil and peat (1:1) in a completely randomized block design with three replicates.

Varieties	Origin	Year of release
Old	'Tamellalet' INRA Morocco	1984
Intermediates	'Laanaceur' INRA Morocco	1991
	'Taffa' INRA Morocco	1994
	'Oussama' INRA Morocco	1995
	'Amira' INRA Morocco	o 1996
	'Adrar' INRA Morocco	1998

Table 1. Description of the six varieties barley used in this study.

After germination, density was adjusted to three plants per pot, and the water content was adjusted at 80% f field capacity. Saline treatment was applied at the third-leaf stage. Treated plants were irrigated using saline solutions (up to 300 mM NaCl). Salinity was

monitored during the experiment period so that saline treatment was increased gradually to get an electrical conductivity in pots of 8 mS/cm. Control pots were irrigated using distilled water without salt. Plants were grown at 22 °C under artificial light with PAR of 300 μ mol photons.m⁻².s⁻¹. Fresh leaf samples used for analysis were harvested 75 days after the beginning of salt treatment.

2.2. Determination of biochemical parameters.

Proline accumulation was assessed as described in Bates *et al.* (1973) [24]. Briefly, 0.5 g of fresh leaf tissues (flag-leaf) from each treatment were added and homogenized in 10 mL of sulphosalicylic acid (3% w/v). The resulting homogenate was then filtrated. The extract was then treated with 2.5% ninhydrin solution and glacial acetic acid. The reaction mixture was kept at 100 °C for 60 min in a water bath. Toluene was added to separate chromophores. Optical density was recorded at 520 nm using a UV-VIS spectrophotometer (Jenway Model 16100, Dunmow, Essex, UK). The proline concentration was determined from a standard curve that was previously prepared using L-proline and expressed as mg of proline per g of fresh leaf weight (FW).

The chlorophyll content in flag leaves was determined using the DMSO method as described by Burnison (1980) [25]. To this end, sliced 20 mg of leaf tissue (flag leaf) was placed in a vial containing 7 mL DMSO. Mixtures were incubated in a glass tube at 65 °C with regular shaking intervals for at least 60 minutes (tissues became colorless). Absorbance was read at two wavelengths 663 and 645 nm, using a UV-VIS spectrophotometer (Jenway Model 6100, Dunmow, Essex, UK). The content of chlorophyll a (Chl a), chlorophyll b (Chl b), and total chlorophyll (Chl T) was calculated using the following equations [26]. The content of each phlorophyll fraction (Chl a, Chl b, and Chl T) was then expressed as mg per g of leaf fresh weight (FW).

Chl a (mg/L) = $(0.0127 \times A_{663}) - (0.00269 \times A_{645})$ Chl b (mg/L) = $(0.0229 \times A_{645}) - (0.00468 \times A_{663})$ Chl T (mg/L) = $(0.0202 \times A_{645}) + (0.00802 \times A_{663})$

Where,

A_{645:} Absorbance at $\lambda = 645$,

A₆₆₃: Absorbance at $\lambda = 663$.

 Na^+ and K^+ contents were determined using the flame photometer method [27]. Dry samples of 50 mg of plant material (sampled from flag leaf) were treated with a mixture of 10 mL acid nitric-perchloric (4 :1) at 120 °C for 120 min. The homogenate was diluted in distilled water 10% (v/v) and filtered through a Whatman filter paper. The extract was used to determine free inorganic ions K⁺ and Na⁺ contents by flame emission photometry (Jenway PFP7, Jenway, Australia) described by Miller (1998) and Turan *et al.* (2010) [28,29]. Na⁺ and K⁺ contents were expressed as mg per g of DW. The K⁺/Na⁺ ratio was computed as a good criterion to assess salt stress tolerance [30].

2.3. Statistical analysis.

All determinations and calculations were made, at least, in triplicates. Combined analyses of variance (ANOVA) were performed over varieties and salt treatment. The least

significant difference's test (LSD) was used to compare means for varieties and treatments at 5% as a probability level [31]. Correlations matrix among studied parameters was established based on mean values. Principal component analyses (PCA) were carried out using mean values. The STATGRAPHICS package, version XVII (Stat point Technologies, Inc., Virginia, USA) was used for all statistical analyses.

3. Results and Discussion

- 3.1. Results.
- 3.1.1. Analyses of variance (ANOVA).

Table 2 shows mean squares of the combined analyses of variance for proline content, chlorophyll a, chlorophyll b, total chlorophyll, sodium (Na⁺), potassium (K⁺), and K⁺/Na⁺ ratio in leaves of six barley varieties grown under salt stress conditions. This table shows that both factors (treatment and variety) and their interaction significantly impacted most of the studied biochemical parameters. However, the main variability source was the treatment effect since it explained more than 77% of the total variance for K⁺ and about 93% of the total variability in the remaining parameters. The magnitude of genotypic effect (variety) was of a lesser extent and allowed explaining around 3% of the total variance for the investigated parameters except for K⁺ for which genotypic effect was about 17%. Treatment by variety interaction was lower since it explained around 2% of the total variance in almost studied parameters.

Table 2. Mean squares of the combined analyses of variance for proline content (Proc), chlorophyll a (Chl a), Chlorophyll b (Chl b), Total Chlorophyll (Chl T), Sodium (Na⁺), Potassium (K⁺), and ratio (K⁺/Na⁺) in leaves of six barley varieties grown under salt stress conditions during the 2017-2018 crop season. Df = degree of freedom * ** and *** indicate significance at 0.05, 0.01, and 0.001 levels of probability, respectively.

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Source of variation	Df	ProC	Chl a	Chl b	Chl T	Na ⁺	K ⁺	K ⁺ /Na ⁺
Treatment (Trt)	1	331.30***	1.985***	0.569***	4.738***	382.67***	3.15***	19.34***
Varieties (Var)	5	7.26***	0.118***	0.049**	0.213**	8.56***	0.71***	0.33
Replicate (Trt)	4	0.04	0.006	0.004	0.015	0.21	0.01	0.15
$Trt \times Var$	5	10.08***	0.056*	0.012	0.069	8.34***	0.15**	0.45*
Residual	20	0.31	0.015	0.008	0.035	0.06	0.037	0.15
Total (corrected)	35							

3.1.2. Treatment effects.

Mean values of both salt treatment and control are summarized in Table 3. As evidenced in this table, there were significant variations between the salt treatment and control. Salt treatment displayed the greatest values of proline content (8.22 mg/g FW) and sodium ion content (8.37 mg/g DW). However, the lowest values of was recorded in chlorophyll a (1.31 mg/g FW), chlorophyll b (0.89 mg/g FW), total chlorophyll (2.30 mg/g FW), sodium ion content (1.85 mg/g DW), and K⁺/Na⁺ ratio.

Table 3. Mean values of varieties and treatments for proline content (Proc), Chlorophyll a (Chl a), Chlorophyll b (Chl b), Total Chlorophyll (Chl T), Sodium (Na⁺), Potassium (K⁺), and ratio (K⁺/Na⁺) in leaves of six barley varieties grown under salt stress conditions during the 2017-2018 crop season. In each column, values followed by the same letter are not significantly different at 5% as a probability level.

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	ProC (mg/gFW)	Chl a (mg/gFW)	Chl b (mg/gFW)	Chl T (mg/gFW)	Na ⁺ (mg/gDW)	K ⁺ (mg/g D W)	K ⁺ /Na ⁺	
Varieties								
'Amira'	3.48 e	0.92 cd	0.70 cd	1.72 b	5.19 b	2.89 ab	0.94 ab	
'Laanaceur'	4.64 d	1.26 a	0.82 ab	2.18 a	3.60 d	2.73 b	1.23 a	
'Oussama'	4.88 cd	1.14 ab	0.64 d	1.88 b	6.50 a	2.75 b	0.69 b	

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	ProC	Chl a	Chl b	Chl T	Na ⁺	\mathbf{K}^+	K ⁺ /Na ⁺
	(mg/gFW)	(mg/gFW)	(mg/gFW)	(mg/gFW)	(mg/g D W)	$(mg/g\mathbf{D}W)$	
'Adrar'	5.53 bc	1.07 bc	0.74 bcd	1.90 b	6.53 a	2.04 c	1.35 a
'Taffa'	6.09 ab	0.91 d	0.78 abc	1.79 b	4.47 c	3.04 a	0.95 ab
'Tamellalet'	6.53 a	1.18 ab	0.89 a	2.14 a	4.37 c	2.67 b	1.12 ab
Treatment							
Control	2.16 b	1.31 a	0.89 a	2.30 a	1.85 b	2.98 a	1.78 a
NaCl	8.22 a	0.84 b	0.64 b	1.57 b	8.37 a	2.39 b	0.31 b

3.1.3. Genotypic effects.

Table 3 presents the mean values of varieties of the investigated biochemical parameters. From these outcomes, there were significant variations among most of the six studied barley varieties. 'Tamallelet' was found to have the highest scores of proline (6.53 mg/g FW) and chlorophyll b (0.89 mg/g FW) contents. 'Taffa' displayed the smallest chlorophyll content (0.91 mg/g FW) but the greatest value of K⁺ (3.04 mg/g DW). 'Adrar' was found to have the greatest values of Na⁺ (6.53 mg/g DW), K⁺ (2.04 mg/g DW) and K⁺/Na⁺ ratio (1.35). The lowest values of chlorophyll b (0.64 mg/g DW), and K⁺/Na⁺ ratio (0.69 mg/g DW) contents were recorded in 'Oussama', while 'Amira' was characterized by the lowest level of proline content (3.48 mg/g FW) and total chlorophyll (2.18 mg/g FW). 'Laanaceur' presented the greatest scores of chlorophyll a (1.26 mg/g FW), total chlorophyll (2.18 mg/g FW), this variety showed the lowest value of sodium ion content (3.66 mg/g DW).

3.1.4. Correlations among the studied parameters.

The correlations matrix among the studied parameters are shown in Table 4. According to our results, important positive and negative correlations were highlighted among the investigated biochemical parameters. In this regard, proline content was positively linked to sodium ion content and negatively associated with chlorophyll a, chlorophyll b, and total chlorophyll, potassium ion content, and the K⁺/Na⁺ ratio. Moreover, chlorophyll a, b, and total chlorophyll values were positively and significantly associated with each other and with potassium ion content, but they were negatively associated with sodium ion content (Table 4). Potassium ion content was negatively linked to sodium ion content and positively associated with the K⁺/Na⁺ ratio.

 Table 4. Correlation coefficients among the studied parameters. Matrix correlation was carried out on mean values for each parameter. *, **, and *** indicate significance at 0.05, 0.01, and 0.001 levels of probability, respectively.

respectively.										
	ProC	Chl a	Chl b	Chl T	Na ⁺	K^+	K ⁺ /Na ⁺			
ProC		-0.641***	-0.544 **	-0.6278**	0.671***	-0.800***	-0.684***			
Chl a			0.762***	0.941***	-0.734***	0.479**	0.730***			
Chl b				0.911***	-0.832***	0.569**	0.826***			
Chl T					-0.812***	0.518*	0.797***			
Na ⁺						-0.618**	-0.986***			
K+							0.689***			
K+/Na+										

3.1.5. Principal Component Analysis.

PCA was used as a multivariate statistical approach to discriminate between treatments and varieties. The two first PCs were retained since they allowed explaining over 89% of the total variability in our results. PC1 and PC2 accounted for 79% and 10%, respectively. Points plotted on the surface delimited by axis 1 and 2 (Figure 1) are related to treatments, which seem to be distributed along PC1. Towards the positive direction of this component, NaCl treatment

interacted with higher values of proline and sodium ion contents. Control interacted, on the positive side of PC1, with higher scores of chlorophyll a, b, and total chlorophyll, potassium ion content, and the K^+/Na^+ ratio.



Figure 1. Principal component analysis (PCA) projections on PC1 and PC2. The eigenvalues are symbolized as blue segments representing traits that most affect each principal component. The 12 points are the treatment mean values of each studied parameter of six barley varieties grown under NaCl treatment during the 2017-2018 crop season. Proc: proline content, Chl a: Chlorophyll a, Chl b: Chlorophyll b, Chl T: Total Chlorophyll, Na⁺: Sodium, K⁺: Potassium, and K⁺/Na⁺: ratio of K⁺ out of Na⁺.

Similar to Figure 1, points plotted on the surface delimited by axis 1 and 2 (Figure 2) are related to varieties. PC2 appears to discriminate between both 'Tamellalet' and 'Adrar' towards the negative side of PC2 with higher proline content, sodium ion, chlorophyll a, b, and total chlorophyll contents, and K⁺/Na⁺ ratio. However, varieties 'Taffa', 'Amira', and 'Oussama' were distributed on the positive side of PC2 with higher scores of potassium ion content. Points related to 'Oussama' were close to 0 with lower scores of most studied parameters. PCA outcomes confirmed the results obtained in mean comparisons highlighted in Table 3.



Figure 2. Principal component analysis (PCA) projections on PC1 and PC2. The eigenvalues are symbolized as blue segments representing traits that most affect each principal component. The 12 points are the mean accession values of each studied parameter of six barley varieties grown under NaCl treatment during the 2017-2018 crop season. Proc: proline content, Chl a: Chlorophyll a, Chl b: Chlorophyll b, Chl T: Total Chlorophyll, Na⁺: Sodium, K⁺: Potassium, and K⁺/Na⁺: ratio of K⁺ out of Na⁺.

3.2. Discussion.

It is well known that salinity is a major abiotic stress that can severely reduce plant growth and crop productivity [32]. In this context, a pressing need is faced with overcoming such environmental constraints through technological means. Improving salinity tolerance requires a good knowledge of the physiological mechanisms linked to plant response to salt stress. In this work, we reported some biochemical responses of six Moroccan barley varieties under salt stress conditions. As highlighted in the results section, different biochemical parameters were mainly under treatment effects, while genotypic effects were lesser. Such results were in agreement with other previously reported works in barley and other crops [1,10,12,33]. It has been demonstrated that salt stress tolerance is highly inherited in barley [34–38]. It is controlled by the action of several genes that are highly influenced by the environment and genotype-by-environment interaction. Under salt constraint, [36] found higher values of heritability of leaf chlorophyll content (0.86) and Na⁺ and K⁺ contents (0.80).

Mean comparison between treatments (salt treatment and control) showed that applying salt stress reduced chlorophyll contents (a, b, and the total), potassium ion content (K⁺), and K⁺/Na⁺ ratio on the one hand and increased the proline and sodium ion contents on the other hand. Our findings were in line with previously published works [1,10,12,39–41]. Under salt treatment, our varieties experienced a reduction exceeding 66% in the case of chlorophyll a and the K⁺/Na⁺ ratio, about 30% in total chlorophyll, and less than 20% in chlorophyll b and potassium ion content compared to their respective controls. A differential reduction in different chlorophyll fractions has been reported by Athar *et al.* (2015) [23]. This chlorophyll content decline could be attributed to the chlorophyll synthesis inhibition, along with the activation of its enzymatic degradation via the chlorophyllase enzyme [21,42].

With regard to ions leakage, under salt stress, there was a significant increase in Na⁺, while K^+ and K^+/Na^+ increased. These results were in agreement with published literature [1,38,43,44]. Under salt stress conditions, ions content and transport are altered as reviewed by Arzani and Ashraf (2006) [34]. Different strategies are used by plants to cope with the toxicity caused by Na⁺. In this context, some plants transport Na⁺ to leaves and accumulate it in vacuoles, while others accumulate this ion at the roots level [45]. Increasing K⁺ uptake is also a known strategy to counteract the entry of Na⁺ [46]. Such decline in K⁺ content at higher salinity levels could be ascribed to decreased competitive absorption with Na⁺ [46]. This suggests that a strong ability of K⁺ retention is thought to be one of the mechanisms behind their higher salt tolerance, as explained by Sun et al. (2015) [48]. Along with ionic leakage, the synthesis and accumulation of compatible solutes (like proline) are required to balance the osmotic potential of the vacuolar Na⁺ [43]. Our results found a strong increase in proline levels under salt treatment compared to the control. This confirmed previously published works [12,23,40,43]. Proline synthesis, under salt conditions, is deemed to be one of the osmolytes widely reported to accumulate in plants under salt stress conditions. In fact, proline protects plants against salinity stress mostly via maintaining osmotic adjustment, ROS scavenging, and regulating antioxidant metabolites, but also modulating major enzymatic components involved in antioxidant defense system as reviewed in [49].

Vast differences were highlighted between all varieties studied here in terms of different biochemical parameters. These outcomes were in accordance with several studies that examined barley's biochemical and physiological behavior under salt stress conditions [1,12,23,38,43]. As outlined by other authors, such genotypic variations were assigned to a

differentiated expression of genes that encode for these traits [12,48,51]. Moreover, several authors reported a positive correlation between free proline content, K^+ content, the K^+/Na^+ ratio on the one hand, and salt tolerance, on the other hand, suggesting the use of these parameters as indices to screen salt tolerance potentials among genotypes [1,43,52–54]. In our results, 'Tamellalet' and 'Taffa' were characterized by relatively higher proline, K^+ , and ratio K^+/Na^+ but lower values of Na⁺. This suggests their salt stress resistance as compared to the remaining varieties. In contrast, 'Oussama' and 'Amira' were relatively salt-sensitive because of lower levels of proline, Na⁺, and K⁺/Na⁺ ratio but a higher Na⁺ content. Suppose we consider the K⁺/Na⁺ ratio as a criterion to assess salt stress tolerance, as suggested by Widodo *et al.* (2009), Somasundaram *et al.* (2019), and Vasilakoglou *et al.*(2021) [38,43,55]. Both 'Laanacer' and 'Adrar' could be considered the most resistant varieties because of their relatively higher recorded values in the K⁺/Na⁺ ratio. Correlations among different ions (Na⁺, K⁺, and K⁺/Na⁺ ratio) and among these ions and other induced salt stress metabolites like amino acids (including proline) were investigated previously. Such associations could be ascribed to genes linkage or pleiotropic effects between genes that encode these traits [36,50,56–58].

PCA was proved to be an efficient discriminative tool [59–65]. It was used as a multivariate statistical approach to discriminate among treatments and varieties. Several authors have used this tool for the same purpose in barley and other crops [11,38,42,66–81]. Our data variability was explained mainly by the two first components, as we indicated in the results' section. The first component was environmental, allowing a better separation of treatments (salt stress and control). In contrast, the second component is separated among varieties (genotypic component).

4. Conclusions

We evaluated biochemical responses of six Moroccan barley varieties grown under salt stress conditions. Data variability was mainly associated with the treatment effects, while genotypic effects were significant for different measured biochemical parameters. Salt treatment-induced proline accumulation and significant Na⁺ content increase, while there were significant declines in terms of different chlorophyll fractions, K⁺, and K⁺/Na⁺ ratio. Wide genotypic variations were revealed among all studied varieties. From these results, 'Tamellalet' and 'Taffa' had relatively higher proline, K⁺ contents, and higher K⁺/Na⁺ ratio, but these varieties showed lower Na⁺ values, which could be linked to their salt stress resistance. In contrast, 'Oussama' and 'Amira' were relatively salt-sensitive because of lower proline levels, Na⁺ contents, and K⁺/Na⁺ ratio, coupled with higher Na⁺ content. Considering the K⁺/Na⁺ ratio as a criterion to assess salt stress tolerance, both 'Laanaceur' and 'Adrar' could be considered the most resistant varieties because of their relatively higher K⁺/Na⁺. Further investigations are needed to investigate the effects of salt constraint on phenology, yield, and grain quality in these varieties.

Funding

This research was funded by ARIMNet2 –Coordination of Agricultural Research in the Mediterranean, FP7-ERANET-2013 RTD –KBBE.2013.1.4-0.3, grant number 618127.

Acknowledgments

This research has no acknowledgment. https://biointerfaceresearch.com/

Conflicts of Interest

The authors declare no conflict of interest.

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