Expression Pattern of NHX1 and BADH2 Genes of Quinoa (*Chenopodium quinoa* Wild.) under Drought Stress and Nutrient Uptake

Mohamad Forouzandeh* (ORCID)

Department of Agronomy and Plant Breeding, Agriculture Institute, Research Institute of Zabol, Zabol, Iran

ARTICLE INFO

ABSTRACT

Quinoa is one of the plants due to its high nutritional value its cultivated area is expanding rapidly in the world. These findings on the stress tolerance of quinoa, focusing on key genes explain drought signal transduction pathways. This perception can raise the comprehension of drought tolerance in quinoa. Betaine Aldehyde Dehydrogenase (BADH) and Na^+/H^+ antiporter (NHX1) were evaluated in response to drought and fertilizer stresses in quinoa. The expression of NHX gene was highly upregulated compared with BADH gene under drought stress. The results provide a new insight into the function of NHX and BADH in plant mineral nutrition. Under fertilizer-treated conditions NHX gene was highly upregulated compared with BADH gene. Also NPK fertilizer induces a higher tolerance ability in quinoa. The evaluation of network topology showed that NHX1 gene had the maximum number of intergenic connections with SOS1, SOS3, and AVP1 genes in the network. The AVP1 gene forms complex in cooperation with SOS mutants and plants will be able to withstand higher salt stress as well as water deficit in response. Our finding reinforces the idea that the favorable NHX allele must be ongoingly selected for quinoa growth under unpredictable future climate worsening.

1. Introduction

Climate change is part of the biggest research challenges that agricultural researchers are currently facing. Future droughts due to climate change can increase without interruption and greatly until the end of this century and limit access to resources needed for photosynthesis (Sun *et al*., 2020). The plant's potential to tolerate water deficit as the main limiting agent for plant growth and development is determined by several biochemical pathways such as chloroplast protection, ion homeostasis, synthesis of osmotically active metabolites, inhibition of oxygen radicals and changes in the expression of stress-responsive genes, which maintain or facilitates water absorption (Tang *et al*., 2017).

Quinoa (*Chenopodium quinoa* Wild.) is a plant susceptible to the effects of global warming, which adapts and grows to the most severe weather conditions such as drought, high salinity, and frost (Jaikishun *et al*., 2019). Plants of this family, unlike other plants, need sodium for their growth and survival. By accumulating sodium ions in their tissues, these plants raise their internal osmotic pressure, in this way, despite the high osmotic pressure (due to salinity and dehydration), they can absorb water and store it in their cells (Gharibi *et al*., 2018). Quinoa adopts several strategies complex, numerous and various to diminish drought stress, such as high root-to-shoot ratios, morphological adaptations, osmotic adjustments, regulation of cellular homeostasis, activation of stress-related transcription factors and proteins, etc (Forouzandeh *et al*., 2023).

To create this tolerance in plants, there is a major function in the transport of cytosolic Na^+ and the decline of its toxic factors, which causes the placement of excess amounts of sodium ions in the vacuoles, which is done by the vacuolar membrane antiporter (NHX). NHX proteins are transmembrane antiporters

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* Corresponding author.
  E-mail address: Mohamad.forouzandeh@gmail.com

belonging to the cation/proton antiporter gene family and play a role in cell membrane transport processes to participate in the maintenance of calcium homeostasis and plant growth, development, and defense (Ayadi *et al*., 2020). The role of NHX family genes has also been frequently persuaded. For example, qRT-PCR analysis of shoots and roots of potatoes treated by PEG6000 corroborated that the NHX genes are affected in this plant's response to drought stress. (Yihong *et al*., 2024). NHX2 expression level was positively and significantly correlated with drought tolerance in potatoes (Wang *et al*., 2016). The expression level of NHX gene in maize shoots and roots increased under drought and salinity stress. Meanwhile, functional characterization showed that maize NHX genes were a significant duty in drought and salt endurance (Huang *et al*., 2018).

Adjacent to NHXs are K^+ efflux antiporters that transport large amounts of cytosolic K^+ into the vacuole which may somewhat describe overexpression of these genes and reduction yield at low K^+ usage (Soleimani *et al*., 2008; Huertas *et al*., 2013). Ying et al. (2023) showed that expression of HtNHX2 from *Helianthus tuberosus* enhances rice tolerance to general deficiency of nutrients. There was also, the LeNHX1 gene low expression induced in the arbuscular mycorrhizal fungi plants (He and Huang, 2013). In another study was observed an increase in BADH2 gene expression of rice by growth-promoting rhizobacteria (PGPR) vs. their non-inoculated counterparts under water stress (Nikhil *et al*., 2023). According to the results, 250 ppm ZnO NPs nanopriming caused an ascent in BADH and P5CS expression of wheat, which are known as stress signaling molecules, and the production of responsive genes to drought. which function as stress signaling molecules and trigger the production of genes that are responsive to drought (Pandya *et al*., 2023). The chemical fertilizer application (NPK2 and NPK3) caused a considerable increase in BADH2 gene expression of rice varieties (Mohammed and Farhood, 2020). The goal of the current study is to determine how organic and chemical fertilizers affect the expression of drought-responsive genes of quinoa under drought stress conditions.

2. Materials and methods

Conditions and experiment location: A factorial experiment was conducted with complete randomized block design at the Agricultural Research Institute of Zabol, Iran during the 2023-2024 cropping season in the geographical location of 61 degrees and 41 minutes east longitude and 30 degrees and 54 minutes north latitude and it was done at 481 altitude meters above sea level. The soil test had a loamy sand texture. The soil chemical analysis results of the experiment site are shown in Table 1.

Table 1. Soil physical and chemical properties (Depth of 0 to 30 cm)

Soil texture	EC (dS m^{-1})	pH	O.C.	S.A.R.	T.N.V.	N	P	K	Cu	Fe	Zn	Mn
				(%)					(mg kg^{-1})			
Loamy Sand	3	7.8	0.09	25	10.7	0.11	3.4	150	1.24	5.08	0.8	6.54

The main plots were irrigation regimes at three levels: 100, 75, and 50 percent of crop water requirement (as control, mild, and severe water deficit stress, respectively). and sub-plots of fertilizer levels in the four levels of the control (Non-fertilizer), manure 25 ton. ha^{-1}, vermicompost 5 ton ha^{-1}, and 100% of the recommended basic fertilizer (Chemical fertilizer). The variety used in this study was Sadouq. The required seeds of this genotype were prepared from the National Salinity Research Center (NSRC), Yazd, Iran. Irrigation was done using a pressurized irrigation system. For this purpose, irrigation was done by spaghetti pipes and water meter. Before planting and based on the results of soil analysis to provide the primary necessities of plant, nitrogen fertilizer (as urea) an equivalent of 300 kg ha^{-1} (before planting and twice at 8 to 10 leaves and flowering stages), 225 kg ha^{-1} potash sulfate and 225 kg ha^{-1} triple superphosphate were added to each plot.

2.1. Analysis of real-time PCR

In order to extract RNA, sampling was done after applying the treatments and at the 95% physiological ripening stage, from the flag leaves of 5 plants and a homogeneous mixture was prepared from each plot. Manual RNA extraction was done using the Japelaghi protocol (Japelaghi *et al*., 2011). The quality and quantity of extracted RNA were measured by spectrophotometer and agarose gel electrophoresis. The good values were then applied for cDNA synthesis

(Fig. 1). For this purpose, unique primers for the amplification of NHX, BADH and ACTIN7 genes were designed by CLC main workbench v.7 and Primer3 plus softwares. The optimum annealing temperature for each gene was identified as the optimum annealing temperature using the temperature gradient thermocycler, Then, based on the optimal temperature, the real-time PCR reaction was performed using a Roto Gene 3000 model (Table 2; Fig. 2). Finally, the relative expression of each gene in comparison to the control sample was calculated based on CTs using GenEX 6.0 (Mangalam *et al.*, 2001) software and $\Delta\Delta^{CT}$ method (Fig. 3). To calculate fold change in real time, Δ^{CT} of the desired genes must be subtracted. The answer obtained by subtracting Δ^{CT} is called $\Delta\Delta^{CT}$. (Livak and Schmittgen, 2001). The synovial network of two genes NHX and BADH was prepared using STRING v.11 data center (Szklarczyk *et al.*, 2017) and at the end, network imaging was done by Cytoscape v.3.7.1 software (Shannon *et al.*, 2003).
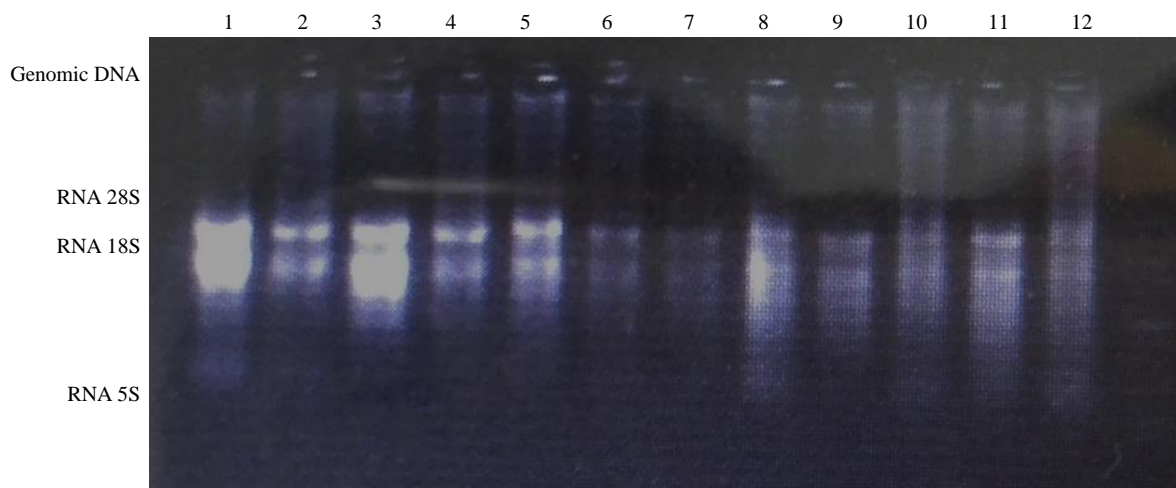

Figure 1. Rhizobia 18S and 28S bands obtained from electrophoresis of RNA samples on 7% agarose gel. 1: Control (non stress)+ Control (Non fertilizer), 2: Control (non stress)+ Manure, 3: Control (non stress)+ Vermi compost, 4: Control (non stress)+ Chemical fertilizer, 5: Mild stress+ Control (Non fertilizer), 6: Mild stress+ Manure, 7: Mild stress+ Vermi compost, 8: Mild stress+ Chemical fertilizer, 9: Severe stress+ Control (Non fertilizer), 10: Severe stress+ Manure, 11: Severe stress+ Vermi compost and 12: Severe stress+ Chemical fertilizer.

Table 2. Primers used in RT-PCR reaction on *Chenopodium quinoa*

| Gene name	Gene ID	Primers 5'-3'	Product length (bp)	Annealing temperatures (°C)
sodium/hydrogen exchanger-1 (NHX)	XM_021886762.1	Fw CAGCCTTTGTCTTCCCTCTG Rv GTAGGACAAGGCCACGGTAA	137	58
betaine aldehyde dehydrogenase (BADH)	XM_021878004.1	Fw TCCTGTTGTCAGCAAGGGAC Rv AAATGCTCAGGACGAGAGCC	105	42
Actin-7	XM_021888078.1	Fw TTCCCAAGTATTGTTGGTCGTC Rv CCAGATCTTCTCCATGTCATCC	168	55
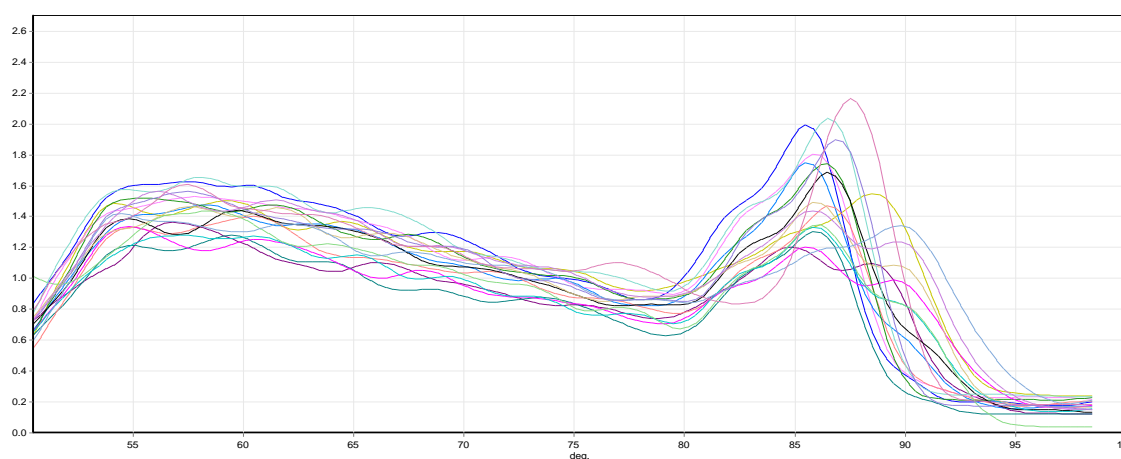
Figure 2. Melting curve of NHX1 in Real-Time PCR amplification process. Each DNA fragment that is amplified in the PCR process has a unique melting temperature based on the number of purine and pyrimidine bases
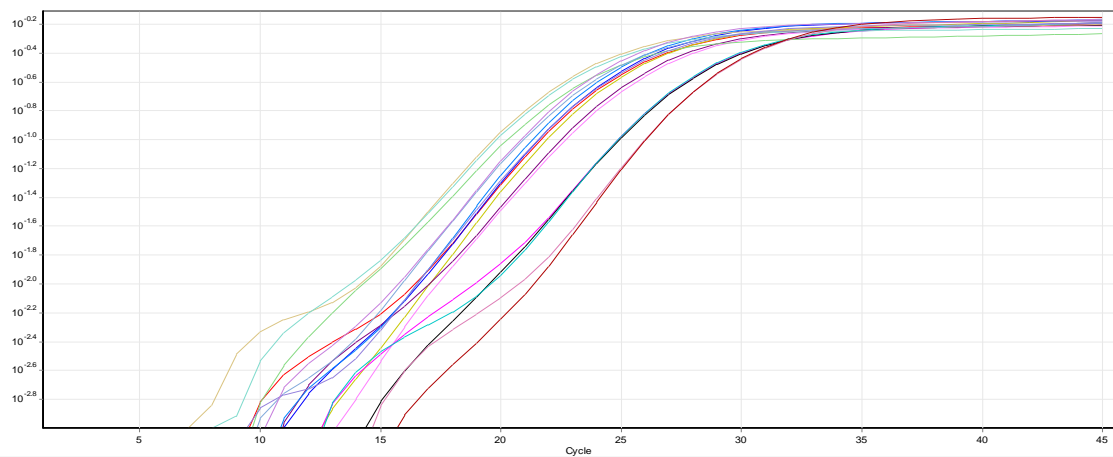
Figure 3. NHX1 CT value curve

2.2. Co-expression network drawing

Based on the co-expression and experimental parameters, the aim genes correlation trend was calculated by the STRING-db datacenter (Szklarczyk *et al*., 2017). Ultimately, the expression network was drawn using Cytoscape software (Shannon *et al*., 2003). Network topology was examined based on components closeness centrality (CC) and betweenness centrality (BC) using network analyzer software (Assenov *et al*., 2007). High levels of CC and BC for a node demonstrate the importance of this node in the co-expression network. In simpler language, the CC is the shortest distance from one node to another one and BC is the degree of centrality of a node in a complex network and is calculated based on the number of communication lines of each node (Forouzandeh *et al*., 2023).

3. Results and discussion

Fig. 4 shows that with increasing drought stress levels, NHX gene expression increases, while BADH gene expression decreases. At this level, the decrease or increase in the expression of genes compared to the control can be calculated at the level of expression of the desired gene in the zero sample. The expression of NHX gene was significantly increased in conditions of moderate stress and severe stress compared to the control. Several studies showed that NHX gene expression increased in Camellia sinensis (Paul *et al*., 2021), potato (Yihong *et al*., 2024) and rice (Khare *et al*., 2021) in response to drought stress.

In the present study, the investigation of the NHX1 gene expression in response to fertilizer type helped understand whether fertilizer application affected

quinoa drought tolerance ability. The expression pattern of the NHX and BADH genes, under fertilizer, and treatment can be seen in Fig. 5. BADH gene as a drought stress resistance gene is reduced in all treatments, but vermicompost treatment with NPK fertilizer has the least reduction effect and vermicompost treatment has the most reduction effect. However, vermicompost had no significant effect on NHX gene expression. Also, the NHX gene expression increased the most under the influence of NPK treatment, and the use of manure ranked the next level. This implies that exposure to NPK fertilizer induces a higher tolerance ability in quinoa.
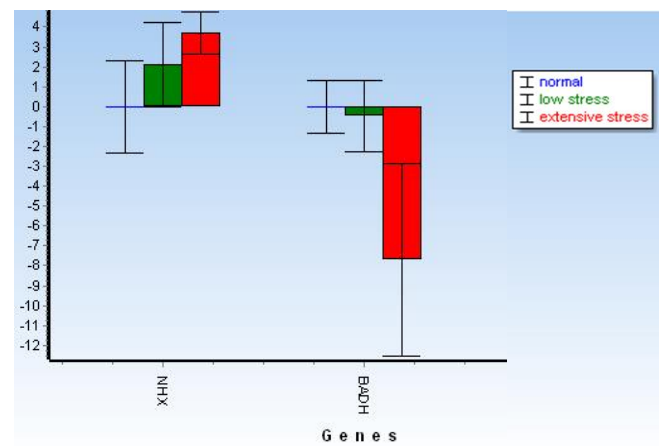

Figure 4. Expression of NHX and BADH genes in three levels of drought stress

In this study, to know how to function and genes were reconstructed in the co-expression network. The co-expression network of two genes has a common connection. The evaluation of network topology (Fig. 6) showed that NHX1 gene with 4 links and SOS1, SOS3, AVP1, and HKT1 genes with 3 links each had

the maximum number of intergenic connections in this network. Further, the maximum BC values were observed for NHX1 and BADH2 respectively. Four genes, SOS1, SOS3, AVP1, and HKT1 had the highest BC values at the next level. More review of network topology specified that CMO, BADH1, and FSD1 ALDH7B4 genes had the one connection with BADH2 gene was the highest and identical CC in the gene network. Also, these four genes had joint BC.
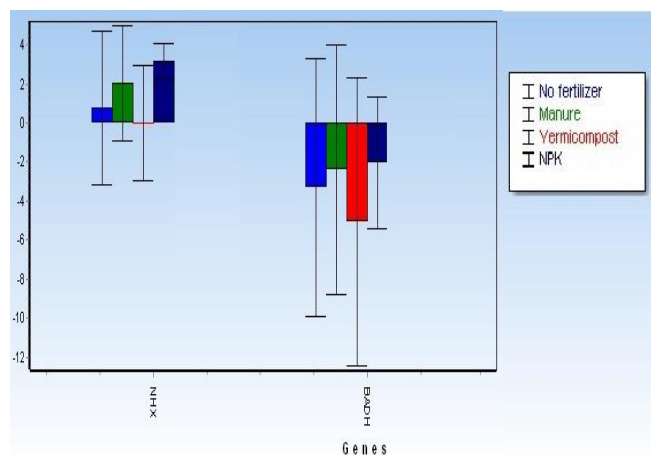

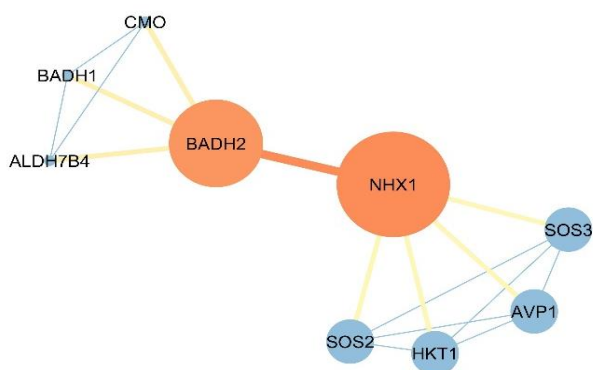**Figure 5. Expression of NHX and BADH genes in fertilizer application**

Figure 6. The co-expression network of NHX1 and BADH2. Color lines from orange (high CC) to blue (low CC) and difference node size for BC inclusive big size for bigger values and on the contrary.

Stressed plants regulate salt accumulation in the vacuole by reducing the osmotic potential to partially provide the necessary conditions for the continued absorption of water from soil and reducing the absorption of toxic ions. Also, plants can use different ways to deal with the negative effects of salinity stress, among others, control the absorption and transfer of ions to the aerial organs, the output of ions, the specific replacement of ions in the cell, the synthesis of osmolytes and genomic alterations (Rostami, 2018).

The co-expression network results of quinoa showed that tolerance to stress at the molecular level has a

complex messenger network that contains several message transmission pathways so that the plant can respond quickly and appropriately. In this study, the regulatory mechanisms responsive to drought stress belong to several family differences. Members of three SOS, HKT1 and NHX1 gene families have roles in mediating Na^+ and K^+ transport, translocation, and intracellular compartmentation (Calzone *et al.*, 2021). NHX gene functions to transport cations such as Na^+ ion from the cytoplasm to the vacuole or outside the cell by two proton pumps for cell volume regulation and cytoplasmic detoxification (Yokoi *et al.*, 2002) The reports proved that HtNHX1 and HtNHX2 increased rice tolerance to soil acidity via various methods by cell wall mechanism and the altering the K^+ and H^+ fluxes (Li *et al.*, 2020). The results of numerous studies indicate the potential of plants containing the SOS gene to deal with abiotic stresses (Haroon *et al.*, 2021; Huang *et al.*, 2024). Molecular analysis of SOS mutants led to the identification of three proteins involved in this system, SOS1, SOS2, and SOS3, which connects a calcium message induced by ion stress to ion homeostasis, and the high sensitivity of salt is neutralized by the SOS1, SOS2, and SOS3 mutations (Chai *et al.*, 2020). Our results show that the AVP1 gene forms a complex in cooperation with SOS mutants. The expression of AVP1 transcripts in transgenic lines of sugarcane showed a longer and more fertile root system in transgenic plants compared to control plants (Kumar *et al.*, 2014). Therefore, plants will be able to withstand higher salt stress as well as water deficit in response to stress conditions simultaneously thus showing tolerance to salinity and drought stresses. The aldehyde dehydrogenase (ALDH) gene has been activated in the BADH gene cascade. Transcript analysis of ALDH7B4 shows that stress responses are regulated by the phytohormone abscisic acid and other pathways that affect sugar metabolism and fatty acid composition of lipid membranes (Kirch *et al.*, 2005).

Up-expression of the drought stress-responsive gene (NHX) in severe stress levels significantly enhanced drought tolerance in quinoa. The important role of NHX in significant increase of proline content, SOD, photosynthesis activities and reduction of MAD content were found and alleviated the physiological damage caused by drought stress in sweet potato (Wang *et al.*, 2016). The expression changing trend of

BADH gene in wheat was the same as that of our study and severe drought stress resulted in the decrease of BADH gene expression (Demirkol, 2020). BADH when expressed at a high level can increase the content of betaine (Abbas *et al*., 2023). Our study indicates that the expression level of BADH declines under drought stress. This might be due to not effect of increased betaine content in these conditions but, it can be chosen in the face of worsening climate in the future.

A four-year field experiment showed lower BADH activity was recorded in urea, superphosphate, and potassium chloride and organic-inorganic compound treatments than no fertilizer application (Luo *et al*., 2021). Results showed that quinoa Na^+ homeostasis and drought tolerance ability were also affected by fertilizer exposure and exhibited type fertilizers response. It has also been reported that expression of NHX could more promote K^+ uptake, and salt tolerance and increase rice yield at sufficient fertilizer application conditions in both hydroponic culture and paddy field (Wang *et al*., 2023).

4. Conclusion

To achieve a high yield of agricultural products, it is inevitable to use all kinds of fertilizers as well as the high cost of these inputs. Knowing the genetic characteristics and its K^+, NO_3^- and $H_2PO_4^-$ absorption mechanisms can significantly reduce fertilizer consumption and production costs. The results obtained from measuring NHX and BADH gene expression showed that in quinoa under fertilizer application and drought stress conditions, the expression of NHX and BADH genes increased and decreased, respectively.

Abbreviation

BADH: betaine aldehyde dehydrogenase; NHX1: Na^+/H^+ antiporter; PGPR: growth-promoting rhizobacteria; BC: betweenness centrality; CC: closeness centrality.

Ethics approval and consent to participate

No humans or animals were used in the present research. The authors have adhered to ethical standards, including avoiding plagiarism, data fabrication, and double publication.

Consent for publications

Hereby give my consent for the to be published in Agrotechniques in Industrial Crops.

Availability of data and material

The datasets analyzed in the current study are available from the corresponding author upon reasonable request.

Funding/Support

Acknowledgement

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