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MOLECULAR CHARACTERIZATION OF MUTATION REGIONS IN HERBICIDE-RESISTANT QUINOA (*Chenopodium quinoa Willd.*) MUTANT LINES

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ABSCTRACT

Quinoa (*Chenopodium quinoa* Willd.) is a viable alternative crop due to its adaptability to unfavorable climate and soil conditions, and its seeds are nutritionally rich. However, the lack of selective herbicides for weed control in quinoa fields poses a significant challenge for cultivation. Consequently, developing herbicide-resistant quinoa lines is essential.

In this study, the Titicaca variety of quinoa was used. Sodium azide at a concentration of 1.5 mM was employed for mutagenesis. Herbicide-resistant plants were identified by applying herbicides from the imazamox to the M_3 generation. The resistant lines were designated as ET-6, ET-7, OT-11, and T-103. Among the four mutant lines obtained through seed mutagenesis, the OT-11 line exhibited a cytosine to adenine (C→A) substitution in the ALS gene, while the ET-6 line showed a thymine to guanine (T→G) substitution. These mutations in the OT-11 and ET-6 genotypes were classified as transversion-type mutations. A transition-type mutation was observed in the T-103 mutant line, involving a thymine to cytosine (T→C) substitution at nucleotide 1114.

The findings suggest that effective weed control in quinoa cultivation can be achieved by developing varieties resistant to IMI group herbicides. Continued research on herbicide resistance should focus on the ET-6, OT-11, and T-103 lines in subsequent generations.

Keywords: *Chenopodium quinoa,* Herbicide resistance, Imazamox, Mutation, Sodium azide.

INTRODUCTION

With the global population on the rise, the demand for food is also increasing. Projections indicate that the world's population will reach 9 billion by the 2050s, leading to a 70% increase in the demand for animal feed and nutritional fiber resources (Langyan et al., 2022). Urbanization and climatic conditions negatively impact agricultural fields. Additionally, improper practices in irrigation, fertilization, and land management contribute to a decline in agricultural production. To sustain soil fertility, agricultural practices must be adapted to changing climatic conditions. This adaptation includes identifying, developing and incorporating into production high-yielding and high-quality plant varieties that can thrive in adverse climatic and saline soil conditions (Gungor et al., 2022). Quinoa (*Chenopodium quinoa Willd.*), a pseudocereal originating from South America, demonstrates remarkable adaptability, growing in environments ranging from high-altitude regions to deserts and tropical climates, with temperatures between -8°C and 40°C and relative humidity around 88% (Tapia, 2015). It can grow in soils with pH levels from 4.5 to 9.06, making it suitable for sodic and alkaline soils (Jacobsen, 2003). Quinoa's adaptability positions it as a viable alternative to traditional crops under adverse climatic conditions (Sosa-Zuniga et al., 2017). The seeds of quinoa are nutrient-rich, containing an average of 12% protein with balanced amino acids and a substantial mineral content, surpassing that of wheat and rice. The seeds provide K (927 mg/100 g), Ca (149 mg/100 g), Mg (250 mg/100 g), P (384 mg/100 g), S (150–220 mg/100 g), Fe (13.2 mg/100 g), and Zn (4.4 mg/100 g) (Konisi et al., 2004). This nutritional profile enables quinoa to meet essential human dietary needs (Ocampo et al., 2023).

Quinoa cultivation has expanded to over 100 countries, with Peru and Bolivia accounting for over 90% of the global production. Global quinoa production is approximately 160.000 tonnes, with an average yield of 0.93 tonnes per hectare. The United States is the largest consumer and importer of quinoa (Patan et al., 2024; FAO, 2024). Although quinoa exhibits slow initial growth, it rapidly develops in subsequent stages, reaching harvest maturity 3 to 3.5 months after planting, depending on the variety. The plant's sufficient dry matter content makes it suitable for silage production (Carpıcı et al., 2023). Its short

growing period allows it to be used as a second crop in suitable climates. With proper genotype selection, quinoa can yield up to 10 tonnes per hectare under dry conditions and 20 tonnes per hectare under irrigated conditions (Tan and Temel, 2018). Despite increased cultivation fields in the past decade, quinoa yield has not proportionately increased. One of the major challenges to improving quinoa yield is weed competition, which can cause up to 34% yield losses (Basaran, 2021). Effective weed control is hindered by the lack of quinoa-specific herbicides. Herbicides that disrupt plant functions such as photosynthesis and amino acid synthesis can also harm quinoa due to the similarity of these functions between cultivated plants and weeds (Basaran, 2021). Developing herbicide-resistant quinoa through biotechnological methods, such as Clearfield® technology, can enhance yield and reduce production costs. Herbicides targeting the AHAS genes, like Sulfonylurea (SU), imidazolinone (IMI), triazolopyrimidine (TP), pyrimidinyl-thiobenzoates (PTB), and sulfonyl-aminocarbonyl-triazolinone (SCT), inhibit the synthesis of essential amino acids, leading to plant death. Clearfield® technology aims to confer herbicide resistance to plants through various biotechnological applications, thereby improving weed control and increasing crop yields (Rizwan et al., 2015).

Genetic diversity of plants and increasing this diversity is very important in plant breeding (Chuchert et al., 2022). This study aims to develop herbicide resistant quinoa lines (resistant to Imazamethabenz-methyl, Imazamox, Imazapic, Imazapyr, Imazaquin and imazethapyr) using classical breeding methods. These herbicide resistant lines can serve as genetic resources for variety development.

MATERIAL AND METHOD

Material

In this study, the early and dwarf Titicaca variety of quinoa was utilized, which has been deemed suitable for forage and seed yield in our country (Yazar and Kaya, 2014; Tan and Temel, 2018). Earliness is a desirable trait for high-altitude fields such as Erzurum, which have a short growing season. Titicaca is a Danish variety, also known as Q-52, developed from Peruvian quinoa.

Study Duration and Location

The study was conducted from 2019 to 2020 in field and greenhouse experiments at the plant production field of Atatürk University, Faculty of Agriculture. The experimental details are provided under the following sub-headings.

Seed Preparation

This phase was completed in 2018, following the method used for developing imidazolinone (IMI) herbicide-resistant wheat in the USA (Newhouse et al., 1992). Initially, 400 g of quinoa seeds were soaked in cold tap water for 24 hours. For sterilization, the seeds were treated with 1.5% sodium hypochlorite (NaOCl) for 20 minutes and rinsed three times with sterile distilled water. Subsequently, the seeds were treated with ethanol (EtOH) three times at 5-minute intervals, followed by three rinses with sterile distilled water. The rinsed seeds were then treated with 1.5 mM sodium azide at room temperature for 3 hours in closed bottles with magnetic stirrers. After treatment, the seeds were dried on filter papers at room temperature for 24 hours and prepared for use in field and greenhouse studies.

M_1 Generation

Chemically mutagen-treated quinoa seeds were planted in irrigated experimental fields of the Atatürk University Plants Production and Research Center in May 2018. Sowing was done manually in prepared rows. Based on soil analysis, fertilizer was applied at 125 kg N ha^{-1} and 80 kg P_2O_5 ha^{-1} (Geren, 2015). All phosphorus was supplied during planting; 75 kg ha^{-1} of nitrogen was applied at planting, with the remaining 50 kg ha^{-1} applied when the plants reached 30-40 cm in height. Weeds were manually removed, and the plants were irrigated as needed. A total of 166.7 mm of rainfall occurred between May and September, with an average temperature of 16.075°C during the growing period (Mgm, 2020). M1 generation seeds were planted in the fields, and Imazamox was applied to the green parts of the plants at a rate of 30 g ai ha^{-1} when they had three leaves. Harvesting occurred in August-September when the seeds matured. All plants were bulked together, and M_2 seeds were collected for herbicide tolerance screening.

Determination of Resistant Plants in M_2 and M3 Generations

M_2 seeds were obtained from plants identified as resistant at the end of the process. The bunches were sun-dried, and the seeds were manually separated from the husks. At the end of this period, 22 lines were identified for resistance. To verify the durability of resistance, M3 stage tests were conducted under greenhouse conditions. Fertilizer was applied at 125 kg N ha^{-1} and 80 kg P2O5 ha^{-1} (Geren, 2015), with all phosphorus supplied at planting and 75 kg ha^{-1} of nitrogen applied at planting and 50 kg ha^{-1} when the plants were 30-40 cm tall. Under greenhouse conditions, the plants were irrigated as needed. When the plants had three leaves, the herbicide was re-applied at a dose of 30 g ai ha^{-1}. It was observed that some lines initially resistant in the field were susceptible and died in the greenhouse test. Ultimately, four lines were identified as resistant.

Molecular Characterization of herbicide resistance plants

To elucidate the molecular nature of the IMI-herbicide-resistant mutant lines, the mutant region was amplified via PCR, and the type and location of the mutation were determined through sequencing. The mutant lines and the corresponding region of the non-mutated control variety were amplified by PCR and subjected to sequence analysis. The resulting DNA sequences were aligned to identify potential mutations in the gene sequence. DNA was extracted from both the mutant lines and the control quinoa

variety using a modified CTAB method (Ausubel et al., 1994).

PCR reactions were conducted in a 20 µl reaction mixture containing 50 ng DNA, 0.25 µM primers, 200 µM dNTPs, 1.5 mM MgCl2, 1x PCR buffer, and 0.5 U Taq DNA polymerase. The PCR parameters were as follows: initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 95°C for 30 seconds, 56-60°C for 30 seconds, and 72°C for 45 seconds, with a final extension at 72°C for 10 minutes. Primers specific to the AHAS gene of Chenopodium, Amaranthus, Bassia, and Salsola were used for PCR analysis (Table 1). The PCR products were analyzed in 1.5% agarose gel with 1X SB buffer at 100 V/cm for 150 min and finally were stained with ethidium bromide (0.2 µg mL-1) and visualized under UV light of Universal Hood II (Bio-Rad, Hercules, CA, USA).

Table 1. Sequences, annealing temperature of primers used

Primer Pairs	Nucleotide sequence (5′-3′)	Annealing Temperature	Genus
F1- R4	TTTTGTTTCCCGATTTAGCCC AATCAAAACAGGTCCAGGTC	58 ^{0}C	*Amaranthus* *Amaranthus*
CHALSF1 R4	GCGTCTACTTGTKCAAA AATCAAAACAGGTCCAGGTC	57 ^{0}C	*Chenopodium* *Amaranthus*
ALS1FB ALSGR1	ATCACCCCTTCTCTTCTTCAA CATCAAACCTAACCCCGAAA	58 ^{0}C	*Chenopodium* *Chenopodium*
ALSGF2 ALS1RD	TTTCGGGGTTAGGTTTGATG AGTAGTAGCAAGCAGCATGTG	58 ^{0}C	*Chenopodium* *Chenopodium*
F1 RUTH-R-3B	TTTTGTTTCCCGATTTAGCCC AACTTGTTCTTCCATCACCTTCG	58 ^{0}C	*Amaranthus* *Salsola*
CHALSF4 RUTH-R-3B	GACCTGGACCTGTTTTGATT AACTTGTTCTTCCATCACCTTCG	57 ^{0}C	*Chenopodium* *Salsola*
RUTH-F-1C RUTH-R-3B	CKGGCCGTGTKGGTGTTTG AACTTGTTCTTCCATCACCTTCG	60 ^{0}C	*S Salsola* *Salsola*

Sequence analysis of PCR products was performed using Sanger sequencing through services provided by Medsantek. The resulting DNA sequences were subjected to multiple alignment using the ClustalW module within the CLC Sequence BioEdit package (Hall et al., 1999). Following alignment, the specific nucleotides where mutations occurred and the mutation patterns were identified.

RESULTS

Molecular Characterization of herbicide resistance plants

a. DNA extraction of IMI-tolerant candidate quinoa plants

In the M_2 generation, 20,000 plants were screened under field conditions. Twenty-two plants identified as resistant were monitored until they reached harvest maturity and were harvested by cutting the clusters during the ripening period. The M_3 generation study was conducted under greenhouse conditions. At the conclusion of this stage, four of the twenty-two lines were confirmed to be resistant. These resistant lines were designated as ET-6, ET-7, OT-11, and T-103.

b. Molecular analyses of candidate mutant lines

To elucidate the molecular nature of the IMI-herbicide-resistant mutant lines, we amplified the acetolactate synthase (ALS) gene region from M3 generation mutant lines using various primer pairs. The target region, approximately 1939 base pairs in length (nucleotides 198 to 2137), was sequenced. Mutant lines OT-11, T-103, ET-6, and ET-7, along with the unmutated control quinoa cultivar, underwent PCR amplification and sequencing using ALS gene sequences obtained from a gene bank. The sequences were aligned using multi-clustalw to identify mutations in the herbicide-resistant lines.

The sequence analysis revealed specific nucleotide substitutions in the ALS gene of the mutant lines. In OT-11, cytosine was replaced by adenine (C→A) at the 935th nucleotide. In ET-6, thymine was replaced by guanine (T→G) at the 1712th nucleotide. Both OT-11 and ET-6 mutations are classified as transversion-type mutations. In T-103, a transition-type mutation was observed, with thymine replaced by cytosine (T→C) at nucleotide 1114. No mutations were detected in ET-7 within the analyzed 1939 base pair region, suggesting that the mutation might be located in the unexamined 521 kb fragment (Table 2).

Table 2. Location of mutations in AHAS gene of quinoa lines

	910	920	930	940	950
	……..|……|	……..|……..|	……..|……..|	……..|……..|	……..|……..|
Quinoa_ALS	TCTGGTAGGC	CTGGACCTGT	TTTGATTGAT	ATTCCTAAAG	ATATTCAGCA
OT-11	TCTGGTAGGC	CTGGACCTGT	TTTGATTGAT	ATTCATAAAG	ATATTCAGCA
ET-6	TCTGGTAGGC	CTGGACCTGT	TTTGATTGAT	ATTCCTAAAG	ATATTCAGCA
Et-7	TCTGGTAGGC	CTGGACCTGT	TTTGATTGAT	ATTCCTAAAG	ATATTCAGCA
T (Control)	TCTGGTAGGC	CTGGACCTGT	TTTGATTGAT	ATTCCTAAAG	ATATTCAGCA
T-103	TCTGGTAGGC	CTGGACCTGT	TTTGATTGAT	ATTCCTAAAG	ATATTCAGCA
	1110	1120	1130	1140	1150
	……..|……|	……..|……..|	……..|……..|	……..|……..|	……..|……..|
Quinoa_ALS	GGGAGGTGGG	TGTTTGAATT	CTGGTGAGGA	ATTGAGGAAA	TTCGTCGAAT
OT-11	GGGAGGTGGG	TGTTTGAATT	CTGGTGAGGA	ATTGAGGAAA	TTCGTCGAAT
ET-6	GGGAGGTGGG	TGTTTGAATT	CTGGTGAGGA	ATTGAGGAAA	TTCGTCGAAT
Et-7	GGGAGGTGGG	TGTTTGAATT	CTGGTGAGGA	ATTGAGGAAA	TTCGTCGAAT
T (Control)	GGGAGGTGGG	TGTTTGAATT	CTGGTGAGGA	ATTGAGGAAA	TTCGTCGAAT
T-103	GGGAGGTGGG	TGTCTGAATT	CTGGTGAGGA	ATTGAGGAAA	TTCGTCGAAT
	1710	1720	1730	1740	1750
	……..|……|	……..|……..|	……..|……..|	……..|……..|	……..|……..|
Quinoa_ALS	CTCAGGTGGT	TTAGGAGCCA	TGGGGTTTGG	GCTACCAGCT	GCTATTGGAG
OT-11	CTCAGGTGGT	TTAGGAGCCA	TGGGGTTTGG	GCTACCAGCT	GCTATTGGAG
ET-6	CTCAGGTGGT	TGAGGAGCCA	TGGGGTTTGG	GCTACCAGCT	GCTATTGGAG
Et-7	CTCAGGTGGT	TTAGGAGCCA	TGGGGTTTGG	GCTACCAGCT	GCTATTGGAG
T (Control)	CTCAGGTGGT	TTAGGAGCCA	TGGGGTTTGG	GCTACCAGCT	GCTATTGGAG
T-103	CTCAGGTGGT	TTAGGAGCCA	TGGGGTTTGG	GCTACCAGCT	GCTATTGGAG

Several methods are employed to develop herbicide-resistant plants, including target gene modification, altering gene expression, inhibiting target enzyme activity, detoxifying the herbicide, and preventing herbicide uptake or transport to the target gene (Tan et al., 2005). Among these, target gene modification and herbicide detoxification are widely used (Kirkwood, 2002), especially in plants resistant to amino acid synthesis inhibitors (Duke, 2005). Commercial examples include Clearfield®, Roundup Ready®, and LibertyLink® plants, which are resistant to imidazolinone, glyphosate, and glufosinate herbicides, respectively (Tan et al., 2005).

The Clearfield production system combines herbicide-resistant plants with IMI group herbicides for effective weed control. Artificial mutagenesis and the selection of desirable traits in mutant plants are key strategies for developing herbicide resistance. Chemical mutagens like NaN3 are preferred for their efficacy at inducing mutations, typically resulting in AT→GC base changes and subsequent amino acid and phenotype alterations (Al-Qurainy and Khan, 2009).

IMI group herbicides effectively control broad-spectrum weeds that other herbicides cannot, such as red rice (Oryza sativa L.), a major global rice cultivation problem. Herbicide-resistant rice varieties developed using Clearfield technology have solved this issue (Webster and Masson, 2001). Similarly, herbicide-resistant quinoa varieties can control weeds like Chenopodium album and Amaranthus retroflexus without harming the crop, improving seed quality and yield.

Clearfield technology has significantly impacted agriculture by enabling effective weed control and minimizing damage from other agricultural practices. In Kenya, for instance, the harvest index increased by 17 % in maize cultivated in Orobanche-infected soils using Clearfield technology. This technology is also effective against broomrape in sunflower cultivation.

Mutation breeding differs from GMO technology as it does not involve transferring foreign genes into the plant genome. Instead, it induces small changes within the plant's own genome, minimizing potential negative effects. This method is extensively used in corn and canola farming. In 2002, 15% of the 4.9 million tons of corn produced in the USA were from Clearfield technology seeds, and in 2000-2001, 20% of the canola grown in Canada was derived from Clearfield seeds (Tan et al., 2005; Beckie, 2004). Additionally, Clearfield technology has resolved issues caused by organophosphate insecticides in corn, which affect the ALS gene. This demonstrates that Clearfield technology not only addresses weed problems but also reduces the adverse effects of other agricultural inputs, gaining wider acceptance among producers and consumers wary of GMO plants.

CONCLUSIONS

Weed control is a major challenge in agricultural production, and chemical control remains the most effective method. However, the development of herbicide-resistant weeds and the emergence of new weed species necessitate the creation of new herbicides. Although quinoa has been cultivated since ancient times, large-scale

cultivation has only become feasible in recent decades. The presence of morphologically similar plants and the lack of plant-specific herbicides complicate weed management in large fields. Effective weed control is crucial for high-yield quinoa cultivation. Given the lengthy process of developing plant-specific herbicides, focusing on herbicide-resistant plants is more practical. IMI group herbicides are preferred for their efficacy at low doses and lower toxicity to organisms.

This study aimed to induce resistance to IMI group herbicides in the quinoa cultivar Titicaca through mutagenesis. Prior to mutagenesis, preliminary studies determined the 50 % lethal dose and application time for quinoa seeds. Based on these results, a protocol involving 1.5 mM sodium azide mutagen at room temperature was established. The durability of the resulting lines was assessed by comparing their ALS gene base sequences with those of the resistant lines, revealing amino acid changes typically observed in mutation studies.

Developing quinoa varieties resistant to IMI group herbicides can enhance weed control in agriculture. Additionally, quinoa's high forage yield and nutrient-rich seeds can contribute to animal feed. Herbicide resistance studies should continue in lines ET-6, OT-11, and T-103, advancing through several generations. Artificial mutation applications can expedite the generation of wide genetic variation, a critical and challenging aspect of breeding. Resistant quinoa varieties could be integrated into crop rotations with herbicide-resistant corn, soybean, and cotton.

In regions with high soil salinity and elevated groundwater levels, such as the 3.6 million hectares in our country affected by these conditions (Kanber et al., 2005) quinoa's known salt tolerance could be beneficial. Resolving weed issues could make these otherwise unusable lands productive through quinoa cultivation.

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