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Growth and Morphological Characteristics of $Asp_{376}Glu$ Mutation in AHAS-Resistant and-Susceptible Yellow Burrhead (*Limnocharis flava* (L.) Buchenau) Populations

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Abstract

Herbicides are considered the most effective weed management approach in the world. Acetohydroxyacid synthase (AHAS, EC 2.2.1.6) is widely used due to its advantages in crop selectivity, effectiveness to control broad-spectrum weeds and low mammalian toxicity has become one of the most popular herbicides used by farmers. The overreliance on the same herbicides over the years to control the weeds led to the evolution of herbicide resistance by several weed species, including *Limnocharis flava* (L.) Buchenau is among the resistant weed species in the rice fields of Southeast Asia. This species has been reported to develop resistance to bensulfuron-methyl (AHAS inhibitor). This study aims to determine the morphological characteristics of susceptible and resistant *L. flava* populations by comparing the growth and development of the plants. Results showed that the Asp-376-Glu mutation in the AHAS gene of the *L. Flava*-resistant population has exhibited significantly stronger (dry weight) or no significantly different impacts as compared to the susceptible population in the fresh weight, height, epicuticular weight, and leaf area. The insignificant differences were observed in the leaf structure and morphology of R and S plants which appears to have no possible fitness cost in the R population. This is the confirmation of the differences between the AHAS-resistant and susceptible populations that emphasizes the morphological characteristics that is crucial for herbicide application for controlling *L. Flava* populations.

Keywords: Acetohydroxyacid synthase; Herbicide resistance; *Limnocharis flava*; Rice weed.

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1. Introduction

Limnocharis flava is a perennial species in humid tropical regions but behaves as an annual herb in shallow and transient water bodies and sites. This semi-aquatic weed species, belonging to the Limnocharitaceae/Alismataceae family is a common and competitive rice fields weed in Southeast Asia, including Malaysia [1, 2]. This species invades rice fields including irrigation systems and competes with inhabitant plants for space, nutrients, and light, it has been a control target of Acetohydroxyacid synthase (AHAS, EC 2.2.1.6) inhibitors such as bensulfuron-methyl (SU) and synthetic auxin herbicides (i.e. 2, 4-D).

Acetohydroxyacid synthase (AHAS, EC 2.2.1.6) is the first common enzyme involved in the synthesis of the branched-chain, essential amino acids valine, leucine, and isoleucine in plants. This enzyme has become the target of commercial AHAS inhibitors/herbicides: the sulfonylureas (SUs), imidazolinones (IMIs),pyrimidinyl(thio)benzoates (PTB), triazolopyrimidines (TPs), and sulfonyl-aminocarbonyl-triazolinone (SCT). The inhibition of AHAS in the plant has been reported to stop the biosynthesis of the branched-chain amino acid valine, leucine, and isoleucine, which consequently leads to plant death [3, 4].

The overreliance on a single herbicide has contributed to the development of herbicide resistance that has been reported in several weed species populations worldwide and is not exceptional for the *L. Flava* population. Based on the report made by Heap [5] in Pulau Pinang and Perak in 1998, *Limnocharis Flava* was recorded to be among the weed species with various resistances to synthetic auxin herbicides (2,4-D) and AHAS inhibitor (bensulfuron-methyl). It is a well-accepted fact that the majority of the resistance cases are due to target site mutations in the AHAS gene by encoding several amino acid substitutions [5-7].

The populations of weeds that were resistant to AHAS inhibitors were found to be substituted with the amino acid at several positions in the AHAS gene (122, 197, 205, 376, 377, 574, 653, and 654). It is also known that certain substitutions of the AHAS gene contributing to herbicide resistance confer pleiotropic effects and fitness costs in

plant growth [4, 8]. With the findings being highlighted, this article presents the morphological characteristics, growth, and development of the resistant plant in comparison with the susceptible plant.

2. Material and Methods

2.1. Plant Materials

Seed samples of confirmed Asp-376-Glu mutation in AHAS gene of resistant *Limnocharis Flava* population (hereinafter called R) in our previous work originated from the field-evolved resistant population in Malaysian Agriculture Research and Development Institute (MARDI) commercial rice fields, Bertam, Seberang Perai, Pulau Pinang, Malaysia (5_32037''N, 100_2803''E). The known AHAS-inhibitor susceptible (hereinafter called S) *L. Flava* population seeds were obtained from surrounding non-field areas with no known herbicide exposure and were included as control [9, 10].

Resistant plants surviving sulfonylurea and pyrimidinyl(thio)benzoate herbicides at the above recommended rates were maintained and isolated to prevent the ingress of foreign pollen [9]. When all individuals were flowering, individual plants were insulated in 350 × 450 mm micro-perforated plastic bags. At maturity, the seed was collected from fertilized flowers representing the sulfonylurea-selected progeny subset of the *L. Flava* population (hereinafter referred to as Progeny 1) and pyrimidinyl(thio)benzoate-selected progeny subset of the *L. Flava* population (hereinafter referred to Progeny 2).

The collected seeds from the progeny subset of the *L. Flava* R population (Progenies 1 and 2) and the herbicide-susceptible (S) populations were soaked in a 0.2% solution of potassium nitrate (KNO3) for 48 hours and placed in 9 mm Petri dishes containing wet Whatman No. 1 filter papers. Following that, healthy pre-germinated seeds from all populations with distinguishable coleoptiles were then transplanted into plant trays containing 2/3 of paddy soil for subsequent experiments [9, 10].

2.2. The Growth and Development

The pre-germinated seeds from all populations were sown into seedling plastic trays (30×10×50 cm) containing paddy soil and maintained in external conditions with average maximum temperatures range of 32/18°C for 30-35 days old. At the four to sixth true-leaf stage, the aboveground parts of the plants were trimmed by 1 cm, followed by drying in an oven at 75°C four 48 hours for the measurement of plant height, and fresh and dry weights.

The collection of epicuticular wax: The seeds from all populations were sown in seedling plastic trays into three replicates 30×10×50 cm tray (8 seedlings per tray) and maintained in the glasshouse as previous procedure. At the fourth to sixth leaf stage, the fourth leaf from the eight plants were removed from the stem, followed by the removal of epicuticular waxes, in which 20 ml of distilled chloroform at 45 °C was stirred for 20 seconds. The protocol for this process was the adaptation of the protocol in Bouzoubaâ, *et al.*'s study [11]. Then, the measurement of the area of the leaf was determined using the Li-3000 leaf area meter (Li-Cor, Inc. Lincoln, Nebraska, USA).

2.3. Stomata Counts

Three seedlings at 4–6-leaf stages were selected for stomata counts. In brief, the fourth leaf from above ground soil was analysed at the lab after being harvested. In this process, the mid-area of the adaxial and abaxial epidermis of the leaf was carefully smeared with nail varnish for around 20 minutes. An approximately 1.5x1.5 cm of the thin membranous transparent layer was cautiously removed from the leaf surface using forceps. The thin film was then placed in distilled water, followed by the preparation of Safranin solution in another watch glass. Using rigger brush (0.8×17 mm), the thin film was then transferred and stored in this solution for approximately 30 seconds until a stain was formed on the film. However, the excess stain was removed by placing it in distilled water, mounting it on a Neubauer-improved haemocytometer (Assistant, Germany), and placing a coverslip on top of it. The number of stomata per unit area (mm^2) was determined by using a compound microscope [12].

2.4. Scanning Electron Microscope (SEM)

The fourth to sixth leaf stage of plants were treated with bensulfuron-methyl (Buron 600) containing 60% of w/w ai at the recommended rate (40 g ai ha^{-1}). Adjuvant (MPC Padix, Agrow Synergy (M) Sdn. Bhd., Selangor, Malaysia) was added at 0.15% v/v. Net photosynthetic rate per unit leaf area, stomatal conductance, intercellular carbon dioxide, and transpiration rate were measured using a 0.25l chamber connected to a portable photosynthesis system (LI-6200, Li-Cor, Inc., Lincoln, NE, USA) under ambient temperature (25-27°C), and irradiance (approximately 900 µmol m^{-2} s^{-1}) were taken from 4th leaf of an aboveground soil 24 h before and after herbicide treatment. The leaves were cut to continue a process of Scanning Electron Microscope (SEM).

The SEM extraction method was made with reference to the protocol established at *Microscopy Unit*, Institute of Bioscience, Universiti Putra. Malaysia (*UPM*). The leaf was cut into three rectangular pieces of 1 cm^2 per replicate. The SEM was used take image of whole leaves to examine leaf surface topography. Leaf tissue samples were fixed for 2 hours in 2.0% buffered glutaraldehyde solution followed by rinsing in 0.1 M phosphate buffer (pH 7.0) for 20 min three times. After one hour in 1% osmium tetroxide, specimens were rinsed three times in 0.1 M phosphate buffer for 20 min. Dehydration consisted of an ethanol series of 30%, 50%, 70%, 80%, 90%, 95%, and three times in 100% ethanol for 20 min each. Finally, samples were critical-point dried (bal-tec cpd 030) and mounted on 12 mm aluminum stubs with double-sided adhesive carbon tape and gold sputter coated (bal-tec scd 005) for 2 min. Images were obtained using a jsm-6400 scanning microscope (Joel) at the Institute of Bioscience, UPM Microscopy Laboratory.

2.5. Statistical Analysis

All experiments were arranged in Randomized Complete Block Design (RCBD) with three replications. Data were analysed using SAS software version 9.4 (SAS Institute Inc., North Carolina, USA). Separation of treatment means at 0.05 probability level was conducted using the LSD test, as outlined in SAS procedure.

3. Results

3.1. The Development and Morphological Characteristics of Resistant and Susceptible *L. flava* Plants

The general appearance of the progeny subset of the *L. flava* resistant population (Progenies 1 and 2) compared with the herbicide-susceptible (S) population. There were no significant differences of the Progenies 1, 2 and S. While the cotyledons of all populations were emerged at the same time and fully developed leaf with no malformation appearance of the leaves were observed.

Although no significant difference was found in the area of leaf and plant height of all populations, notable differences were observed in the dry and fresh weights of the shoot, and epicuticular weight of the plants ($P < 0.05$). Overall, the comparison between S, Progeny 1 and 2 in the aspect of fresh and dry weights, epicuticular wax weight, height, and leaf area measurement is illustrated in Table 1. Similarly, no significant difference was found between the stomatal densities of S and both R progenies at probability $P < 0.05$ due to the equal distribution of stomata on the abaxial and adaxial surfaces of the populations (Table 2). It was seen from this analysis that the Asp-376-Glu resistant mutation in R plants only led to a minor impact in the stomata appearance on the surfaces of both abaxial and adaxial leaves (Table 2).

Table-1. The comparison of the progeny subset of the *L. flava* resistant population (Progenies 1 and 2) and herbicide-susceptible (S) on fresh and dry weights, height, epicuticular weight, and leaf area at the fourth to sixth leaf stage

***L.flava* Plants**	**Fresh weight (g)**	**Dry weight (g)**	**Height (cm)**	**Epicuticular weight (ug/cm^2)**	**Leaf area (cm^2)**
Susceptible	5.13 ± 0.25^{b}	0.36 ± 0.01^{c}	22.19 ± 0.79^{a}	37.29 ± 1.22^{ab}	9.47 ± 0.29^{a}
R (Progeny 1)	4.80 ± 0.17^{b}	0.44 ± 0.01^{b}	22.29 ± 0.80^{a}	37.49 ± 1.15^{a}	9.96 ± 0.33^{a}
R (Progeny 2)	6.56 ± 0.30^{a}	0.53 ± 0.02^{a}	22.73 ± 1.57^{a}	37.1 ± 1.18^{b}	10.5 ± 0.26^{a}
LSD	0.74	0.06	3.01	0.21	1.05

Data are expressed as means ± Standard Error.
There is no significant difference between the means with a similar letter in a column at $P < 0.05$.

The results showed the comparison between the general appearances of S and R *L. flava* plants (Progenies 1 and 2), which carry Asp-376-Glu mutation (Figure 1, 2, 3), no notable difference was identified after the appearance of the plants. This finding could be illustrated from the uniformity of the S and R leaves in terms of colour and full extension, which did not show any irregularity in the leaf structure. The shapes of S and R leaves of *L. flava*, such as elongated lanceolate to predominate type, are the most common shapes [2, 13].

Table-2. The ANOVA of the comparison between the stomatal densities of progeny subset of the *L. flava* resistant population (Progenies 1 and 2) and herbicide-susceptible (S) at the fourth to sixth leaf stage

		Mean of squares
Source of variance	Df	Stomatal density
Leaf surface	1	1.44 ns
L. flava individuals	2	90.03^{ns}
Leaf surface × *L. flava* individuals	2	2.72^{ns}
Block	2	35.26^{ns}
Error	10	26.61
Coefficient variance		6.12

Note: ^{ns} indicates "not significant"

3.2. Physiological Response of Resistant and Susceptible *L. flava* Plants Treated with Bensulfuron-Methyl

Prior to herbicide treatment both R and S plants, the stomata of both plants were open (Figure 4 a-d). However, closed stomata were observed in S plants after 24 hours of bensulfuron-methyl application, while stomata remained opened in R plants (Figure 5 a-d). The possible reason for this physiological modification was injury experienced by the plants after the herbicide application.

A statistically significant difference was found between the degrees of stomatal conductance in the R and S plants 24 hours before and after the herbicide treatment (Table 3). Nevertheless, no change was recorded in other parameters such as transpiration rate, net photosynthetic rate, and intercellular CO_2 (Table 3).

Table-3. Physiological features of herbicide-susceptible (S) and herbicide-resistant (R) *Limnocharis flava* plants at the fourth to sixth leaf stage before and after the 24-hours of bensulfuron-methyl treatment

		Intercellular CO_2			Net photosynthetic rate (µmol m^{-2} s^{-1})			Stomata conductance (mmol m^{-2} s^{-1})				Transpiration rate (mmol m^{-2} s^{-1})			
Before[1]	Without herbicide (C)	225.37	±	5.00[a]	17.46	±	1.46[a]	0.27	±	0.02	[a]	2.87	±	0.42[a]	
	With herbicide	211.44	±	9.89[a]	15.26	±	0.10[a]	0.17	±	0.02	[bc]	2.20	±	0.04[a]	
After[1]	Without herbicide (C)	247.84	±	1.14[a]	16.16	±	1.10[a]	0.26	±	0.01	[ab]	2.67	±	0.30[a]	
	With herbicide	174.53	±	4.67[a]	10.10	±	1.09[a]	0.15	±	0.06	[c]	0.96	±	0.30[a]	
Before[2]	Without herbicide (C)	223.52	±	8.11[a]	15.85	±	1.34[a]	0.17	±	0.01	[c]	2.63	±	0.19[a]	
	With herbicide	231.31	±	0.99[a]	18.00	±	1.44[a]	0.17	±	0.04	[bc]	2.54	±	0.37[a]	
After[2]	Without herbicide (C)	242.17	±	13.81[a]	17.88	±	1.57[a]	0.33	±	0.01	[a]	3.49	±	0.61[a]	
	With herbicide	239.40	±	22.55[a]	18.71	±	1.10[a]	0.13	±	0.03	[c]	3.28	±	0.43[a]	
LSD		27.7	0		3.10			0.09				0.77			

[1] Susceptible, [2] Resistant, C = Control.
Data are represented by means ± Standard Error.
There is no significant difference recorded in the LSD test on the same letter in a column for each factor at $P < 0.05$.

4. Discussion

The morphological characteristics of the plants that were associated with AHAS mutation at Asp-376-Glu in the progeny subset of the *L. flava* resistant population (Progenies 1 and 2) and herbicide- susceptible plant (S) were evaluated in this study. Although the amino acid changes in the AHAS enzyme was also found to result in harmful pleiotropic effects on the fitness traits of weed species, but however this was not observed in the resistant *L. flava* population. This result is comparable with several works of research on plant fitness that had been conducted in various resistant weed species with different AHAS mutations, such as *Lolium rigidum* [4, 14] and the dicot species *Raphanus raphanistrum* L. [15]. As a result, no significant impact was recorded in the reported mutations in these species on plant growth, competitiveness, and AHAS enzyme kinetics [4, 14].

The different findings was observed on resistant *Amaranthus powellii* populations with Trp-574-Leu AHAS resistant mutation to have a potent pleiotropic effect on plant growth [3]. The *A. powellii* plant leaves were found to have a smaller size and distorted shape compared to the susceptible leaves, including an abnormal morphological and structural pattern. Moreover, compared to the susceptible plants, the development of the resistant plants was reported to be slower, leading to the smaller leaf area and less biomass by 58% and 67%, respectively.

The distinguished epicuticular waxes did not result in a non-target site, which decreased the herbicide absorption. Therefore, the survival probability of resistant plants from the herbicides was nullified as the epicuticular wax became thicker. It was found that the water loss from plant leaves was managed by epicuticular wax controls, contributing to resistance to dry condition [11]. Furthermore, the epicuticular wax was also found to be an efficacious prevention method from herbicide absorption, as illustrated by coca (*Erythroxylum coca* var. coca (Lam.)). In this case, there was a significant increase in the absorption of glyphosate herbicide after the epicuticular wax removed using chloroform, which was followed by comparison with plants in the presence of leaf epicuticular wax [16].

The evaluation attempted to determine the effect of AHAS resistant mutation at Asp-376-Glu in R population on *L. flava* physiological reactions as compared to the S plants after bensulfuron-methyl treatment. The inhibition by AHAS on the synthesis of branched-chain amino acid resulted in insufficient amount of branched-chain amino acid in S population. Following The deficiencies of branched-chain amino acid caused by AHAS herbicides was the reduction in protein synthesis, which sequentially led to the decline in cell division rate and cell death [17, 18]. The first symptom would emerge in the meristemic tissues as a result of the biosynthesis of the amino acid, which occurred primarily in young tissues. Meanwhile, amino acids with bigger pools, which were formed in protein reserves and mature tissues, could be catabolised for amino acids. Therefore, the phytotoxic impact on the mature tissue would require a longer duration to appear [18].

The overall changes in the AHAS enzyme activity could effectively contribute to pleiotropic impacts, which would eventually lead to plant fitness reduction [19]. Furthermore, the diverse replacements of amino acid in the AHAS gene were contributed by prominent populations of plant, which resulted in various pleiotropic impacts on plant fitness [3]. Overall, it was demonstrated from these findings that resistance-endowing target site mutation did not adversely influence the physiological features of R plants in comparison to the physiological characteristics of S plants.

5. Conclusion

The present study reveals the effect of the mutation at Asp-376-Glu on morphological and physiological characteristics in R as compared to the S population. Growth and morphological characteristics of S and R showed no significant difference in terms of fresh weight, height, epicuticular weight, stomata density, and leaf area except for shoot dry weight. The results proved that the Asp-376-Glu resistant mutation on R plants produced no significant effect on the number of stomata density for both adaxial and abaxial leaf surfaces at the 4-6 leaf stage. Leaf morphology was compared for both populations and showed no observable difference between these two populations. The AHAS Asp-376-Glu substitution on the R plant showed that this mutation did not affect the physiological characteristics of the plants compared to that of S plants. Closing of stomata was observed in S plants 24 h after bensulfuron-methyl treatment as compared to R plants, possibly due to the phytotoxic effect on S plants, which indicates that the S plants could have deficiencies of branched-chain amino acid due to the application of AHAS inhibitor.

Later it decreases protein synthesis and slows down the rate of cell division, eventually causing cell death and/ or inducing a rapid accumulation of 2-AB to cause phytotoxic effects in the plant. There is also the possibility of feedback inhibition of the AHAS enzyme by branched-chain amino acids in S plants that can cause cell death [8]. However, this feedback inhibition of AHAS enzyme in *L. Flava* populations has not been discovered yet, hence requiring further investigation.

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Declaration of Interests Statement

The authors declare not conflict of interests.

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Figure-1. The susceptible (S) Limnocharis flava leaves. The fourth leaves were collected at the fourth and fifth leaf stages. Bars: 1 cm

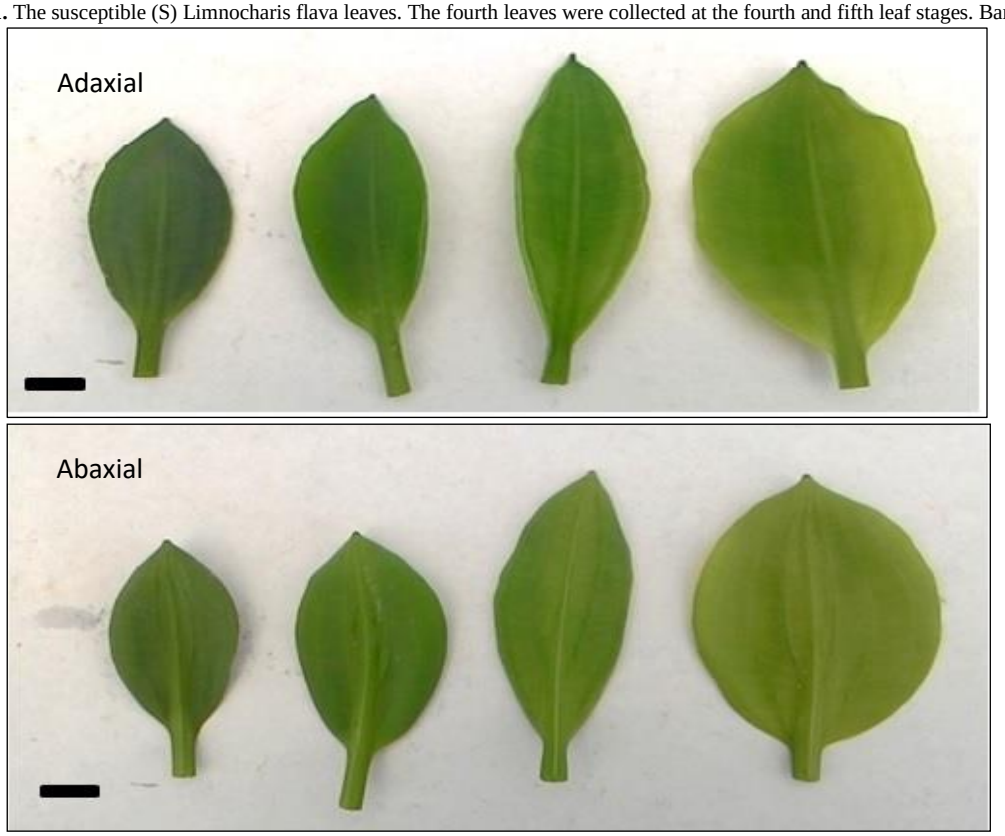

Figure-2. The herbicide-resistant (R) Progeny 1 *Limnocharis flava* leaves. The fourth leaves were collected at the fourth and fifth leaf stage. Bars: 1 cm

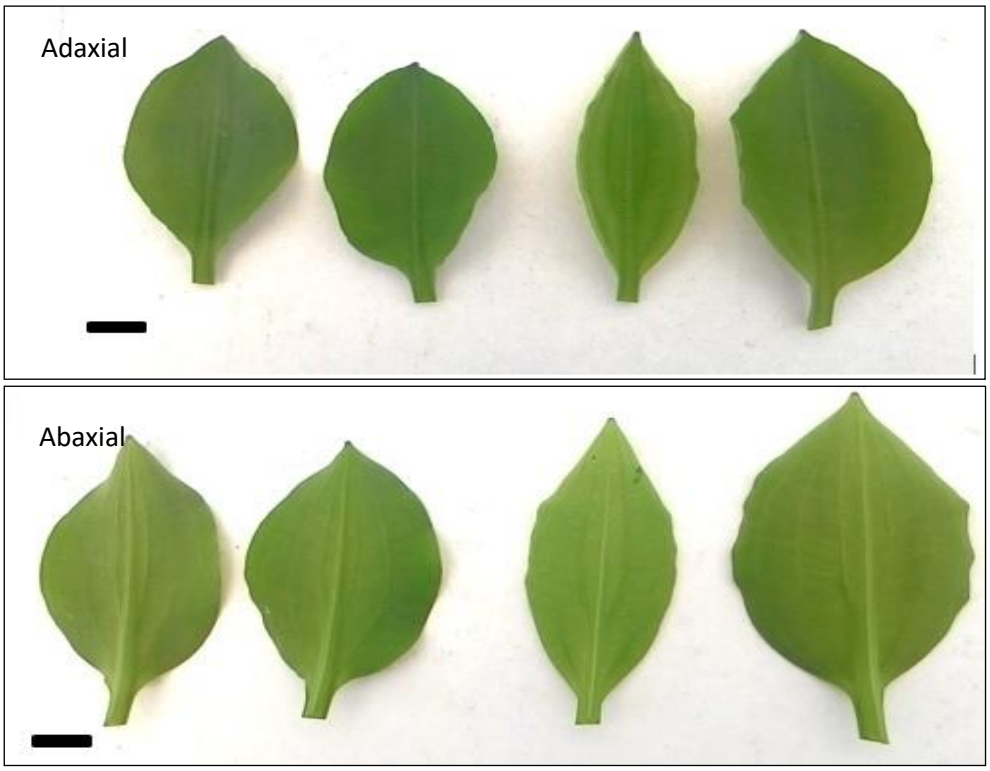

Figure-3. The herbicide-resistant (R) Progeny 2 *Limnocharis flava* leaves. The fourth leaves were collected at the fourth and fifth leaf stages. Bars: 1 cm

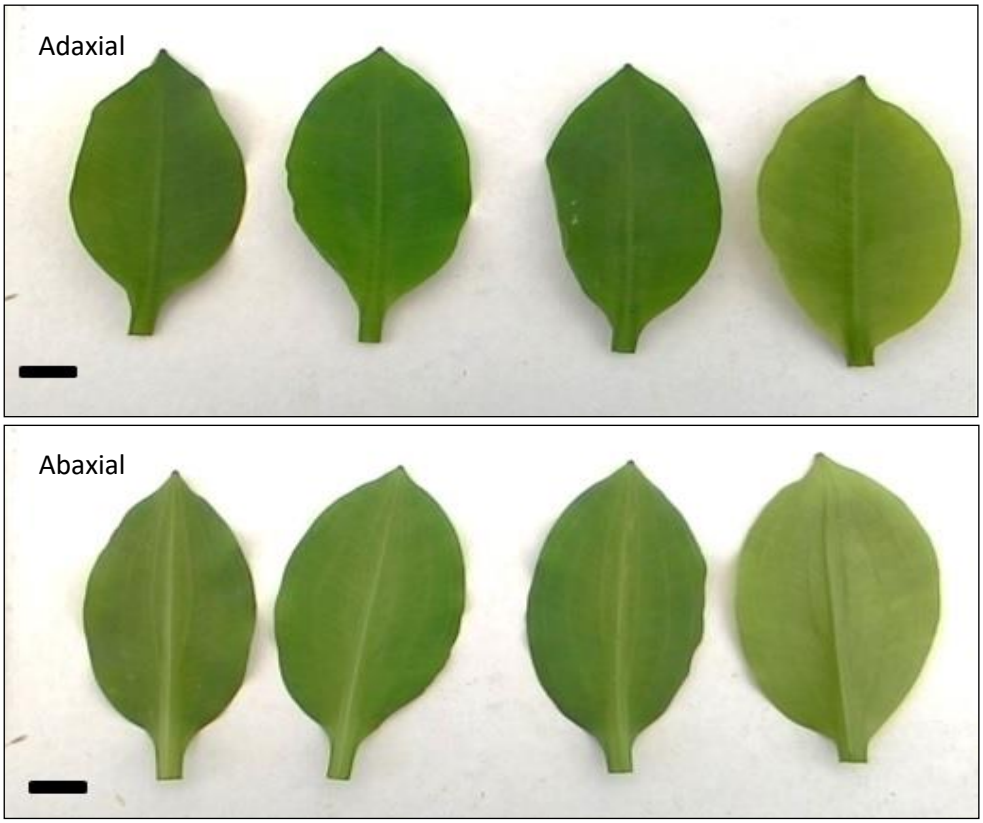

Figure-4. Stomatal weight and form in the epidermis of the fourth leaf of susceptible (S) (a; b) and resistant (R) (c; d) *Limnocharis flava* plants without treatment. In this case, the opening of stomata was observed with no notable difference between both populations in the aspect of stomata weight and form in the absence of bensulfuron-methyl treatment. The structure of the stomata comprises kidney-shaped epidermal cells, each containing pore, which refers to an opening in the centre of the cell

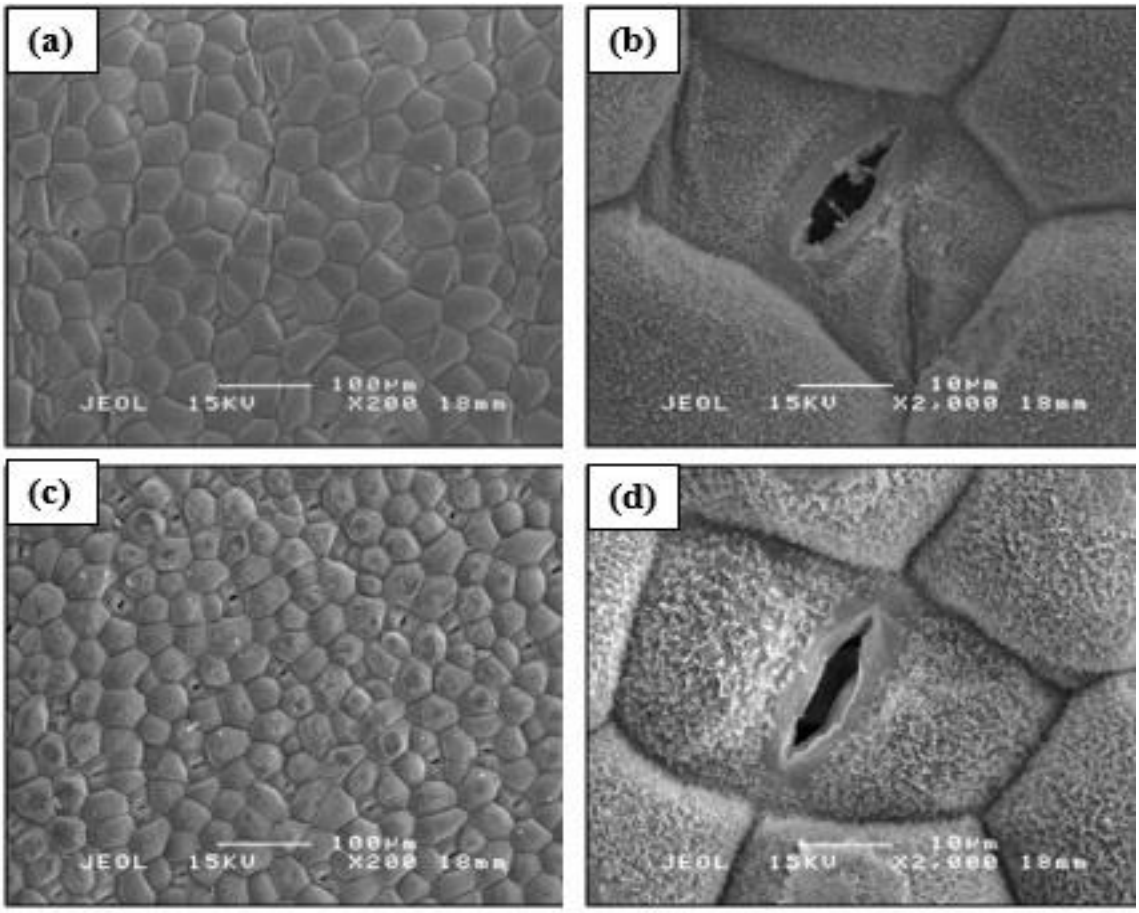

Figure-5. Stomatal weight and form of susceptible (S) (a; b) and resistant (R) (c; d) *Limnocharis flava* plants after being treated by bensulfuron-methyl for 24 hours, leading to the opening of R plant stomata (d) The closing of S plant stomata after 24 hours of bensulfuron-methyl treatment (b). This notable finding was attributed to herbicide application, in which no impact was observed on R plants, while the early affected-symptom from the herbicide was present in S plants