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EFFECT OF *LUGUS* SP. FEEDING AND A SAPONIN APPLICATION ON VOLATILES RELEASED BY QUINOA

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Abstract

In consequence of insect feeding and saponin application tested quinoa plants released large amounts of volatile organic compounds (VOCs) to compare to control. For cv. 'Faro' these were the following components: (Z)-3-hexenal – (Z)-3-HAL, (E)-2-hexenal – (E)-2-HAL, (Z)-3-hexen-1-ol – (Z)-3-HOL, (E)-2-hexen-1-ol – (E)-2-HOL, (Z)-3-hexen-1-yl acetate – (Z)-3-HAC, 1-hexyl acetate – 1-HAC, (Z)-ocimene - (Z)-OCI, benzyl acetate - BAC, methyl salicylate - MAT, β-caryophyllene – β-CAR, (E)-β–farnesene – (E)-β-FAR. Cv. 'Puno' released 7 VOCs and these were: (Z)-3-HAL, (Z)-3-HOL, (Z)-3-HAC, (Z)-OCI, MAT, β-CAR, and (E)– β-FAR. The fragrance bouquet of the third of variety tested (cv. 'Titicaca') consisted of 6 components: (Z)-3-HAL, (E)-2-HAL, (E)-2-HOL, (Z)-3-HAC, (Z)-OCI, and β-CAR. In general, much larger VOCs emission was observed in plants after insect feeding compared to saponin applications and especially control.

Key words: European tarnished plant bug; *Lygus rugulipennis* Popp; Quinoa; *Chenopodium quinoa* Willd.; Orientation; Behavior.

Introduction

Quinoa (*Chenopodium quinoa* Willd.) is a pseudocereal from South America where it has been cultivated since the times of the Incas. Its seeds are rich in good-quality protein with a high share of essential amino acids, especially lysine. They contain many minerals: Mg, Mn, Fe, P, Cu, vitamins (Abugoch James, 2009; Vega-Gálvez *et al.*, 2010), fat, including unsaturated fatty acids as well as antioxidants, polyphenols, phytosterols and flavonoids (Hirose *et al.*, 2010; Debski *et al.*, 2014; Lema-Rumińska *et al*., 2018). Such valuable qualities have triggered interest in quinoa in European countries. Researches supported by FAO and the European Community aim at launching quinoa cultivation as an alternative crop and the source of functional food. Quinoa synthesizes high amounts of saponins, however only at the last stages of growth and development, and mostly in seeds. Those are compounds showing antifungal effects and their availability also defends against pests, including birds and insects, during the physiological maturing in plants (Correo *et al.*, 2010). Numerous reports confirm that saponins are a toxic metabolite which occurs in fruit-seed coat in quinoa. They also act as repellents (De Geyter *et al*., 2012). An example can be seen from their application for *Plutella xylostella* L. larvae which makes their survival on the plant impossible (Badenes-Perez *et al.*, 2014). De Geyter *et al*., (2012) demonstrate that saponins show a strong cytotoxic effect on the middle intestine cells in insects.

New European conditions of the environment for quinoa have triggered new problems which need to be solved. Some of them refer to the protection of the species from diseases and pests. Due to promoted organic cultivation of that species, biological plant protection agents have been searched for as well as the defense mechanisms which developed throughout evolution need to be determined. Protecting crops against pests with environmental-friendly practices is a growing movement in Europe what led the European commission to limit the use of some pesticides, banning a number of existing pesticides that were found to be inefficient or too harmful for the environment (Skoczek *et al*., 2017).

After many years of scientific disagreement, it has been accepted that plants communicate with one another basing on chemicals. Plants to defend themselves against stressors have evolved a number of defense mechanisms (Piesik *et al.*, 2011; Wenda-*Piesik et al*., 2017). They release a spectrum of volatile organic compounds (VOCs) into the atmosphere, like terpenes, fatty acids, benzenoids and phenylpropanoids, where quantities are often affected by plant exposure to biotic and abiotic stresses. (Holopainen & Gershenzon, 2010; Clavijo McCormick *et al*., 2012; Gonzalez *et al*., 2015) VOCs have various functions including defense against pathogens and herbivores, attracting pollinators and transmitting information to neighboring plants (Rodriguez-Saona *et al.*, 2009; Piesik *et al.*, 2011; Bengtsson *et al*., 2014; Burkle & Runyon, 2017). The monoterpenes ((E)-β-ocimene and linalool), the sesquiterpenes [(E,E)-α-

farnesene and (E)-β-caryophyllene], and the fatty acid derivatives known as green leaf volatiles (GLVs) [(Z)-3-hexen-1-ol or (Z)-3-hexenyl acetate], are frequent components of volatile blends released after injury (Kigathi *et al.*, 2009; Schaub *et al*., 2010; Bruce & Pickett, 2011; Danner *et al*., 2011; Witzgall *et al*., 2012; Gantner & Najda, 2013; Piesik *et al*., 2014).

In present study the effect of insect feeding (*Lygus rugulipennis* Popp.) and saponin application on VOCs emission of three tested quinoa (*Chenopodium quinoa* Willd.) plants was studied.

Materials and Methods

Quinoa cultivation: Experiments were performed in 2015 at the Plant Growth Center (PGC) of the UTP University of Science and Technology, Bydgoszcz, Poland. In the beginning of May the seeds of three cultivars ('Faro', 'Puno', 'Titicaca') were sown and after two weeks the seedlings were transplanted to a permanent place into pots, 30 cm in diameter. Plants were grown in the glasshouse with the following temperature schedule: 22°C for 16 hours (day) and 18°C for 8 hours (night), with air circulation augmented by a cooling system, and humidity 60–70%. After 75 days of growth, at the full flowering stage, the allocated plants were transferred to separate rooms to prevent the plants receiving unwanted signals. One plant was placed into each pot containing sterilized soil and maintained with supplemental light and ambient humidity. The photoperiod was 16L: 8D, daytime temperature was 22 ± 2°C, and overnight temperature was 18 ± 2°C. Plants were watered four times weekly, and fertilized twice per week with a 20-20-20 NPK fertilizer (Peters, USA). Up to 37% soil moisture was maintained during the experiment.

Infestation by insects: Adult insects were collected from the field. They were put into the wire-cage for 24 h and kept in starve. The plants prepared for pest feeding were covered by Nalophane bags (Charles Frères-Saint Etienne-France), where two pairs of *Lygus rugulipennis* Popp. (female and male '*in copula*') were released. The plants were subjected to feeding for 48 h. The feeding insects were then removed immediately prior to VOCs collection, so VOCs were measured only from the injured plants.

Saponins application: The concentration of the saponins was arrange as 100 $g \cdot L^{-1}$. The spray was made by applying a 3 ml solution on a single plant. VOCs were measured from the plants after 24 h following the application.

Volatile collection system: Volatiles were collected from experimental plants enclosed within Nalophan (polyethylene terephtalate), odor and taste-free cooking bags (Charles Frères-Saint Etienne-France). Odours from 4 plants were collected simultaneously, and collection lasted 2 h. A volatile collector trap (Analytical Research Systems, Inc., Gainesville, Florida, USA) containing 30 mg of Super-Q adsorbent was inserted into each of 4 Tygon tubes (connection between airflow meter and collector trap). Purified, humidified air was delivered at a rate of 1.0 $L \cdot min^{-1}$ over the plants, and a vacuum pump sucked 20% less (0.8 $L \cdot min^{-1}$) to avoid collecting odors from any gap of the system. Additionally, six blanks (odors from empty Nalophan bags) were collected to verify the lack of background.

Analytical methods: Volatiles were eluted from the Super-Q in each volatile collection trap with 225 µL of hexane, followed by adding 7 ng of decane as an internal standard. Individual samples (1 µL) were injected and analyzed by coupled gas chromatography-mass spectrometry (GC/MS). The GC/MS Auto System XL/Turbomass (Perkin Elmer Shelton, CT, USA) fitted with a 30 m Rtx-5MS capillary column (0.25 mm ID, 0.25 µm film thickness; Restek, USA). The temperature program increased from 40°C to 200°C at 5°C·min^{-1}. The identification of volatiles was verified with authentic standards purchased from commercial sources (Sigma-Aldrich). The emission rate (ng·hr^{-1}) of each VOC was calculated by comparing the peak area of each VOC relative to the peak area of the internal standard.

Statistical analysis

All analyses' were made separately for varieties: 'Faro', 'Puno', and 'Titicaca'. Multivariate analysis of variance (MANOVA) was performed on the basis of following model using a procedure MANOVA in GenStat 17th edition:

$$\mathbf{Y} = \mathbf{XT} + \mathbf{E},$$

where: **Y** is ($n \times p$)-dimensional matrix of observation, n is number of all observations, p is number of VOCs, **X** is ($n \times k$)-dimensional matrix of design, k is number of IS (insects and saponins), **T** is ($k \times p$)-dimensional matrix of unknown effects, **E** is ($n \times p$)-dimensional matrix of residuals. Next, one-way analyses of variances (ANOVA) were performed in order to verify the zero hypothesis on a lack of differences between insects and saponins in terms of values of observed VOCs on the basis of following model:

$$y_{ij} = \mu + \tau_i + \varepsilon_{ij},$$

where: y_{ij} is jth observation of ith IS, μ is grand mean, τ_i is effect of ith IS and ε_{ij} is an error observation.

For individual VOCs mean values and standard deviations (s.d.) were calculated. Moreover, the Fisher's least significant differences (LSDs) were also estimated at the significance level $\alpha = 0.05$.

The relationships between observed VOCs were assessed on the basis of Pearson's correlation coefficients. All the analyses were conducted using the GenStat v. 17 statistical software package.

Results and Discussion

Results of MANOVA indicate that the IS were significant (Wilk's $\lambda = 0.001441$; $F_{1,14} = 251.95$; $p<0.0001$) different for all four VOCs. Results of analysis of variance for all VOCs (except β-CAR) confirm variability of tested IS at the significance level $\alpha = 0.05$ (Table 1).

Mean values for observed VOCs for Faro were presented in Figure 1. The large mean values were observed for insects than in saponins for all VOCs. Correlation coefficients between all pairs of VOCs for Faro were presented in Table 3. β-CAR was not correlated with others VOCs. Additionally, β-FAR was not correlated with: (E)-2-HAL, (Z)-3-HOL, (Z)-OCI and MAT (Table 2).

Results of MANOVA indicate that the IS were significant (Wilk's $\lambda = 0.02049$; $F_{1,14} = 54.64$; $p<0.0001$) different for all four VOCs. Results of analysis of variance for all VOCs (except β-CAR and β-FAR) confirm variability of tested IS at the significance level $\alpha=0.001$ (Table 1). Mean values for observed VOCs for 'Puno' were presented in Figure 2.

Results of MANOVA indicate that the IS were significant (Wilk's $\lambda = 0.006355$; $F_{1,14} = 234.52$; $p<0.0001$) different for all four VOCs. Results of analysis of variance for all VOCs (except β-CAR) confirm variability of tested IS at the significance level $\alpha=0.001$ (Table 1). Mean values for observed VOCs for 'Titicaca' were presented in Figure 3.

The large mean values were observed for insects than in saponins for all VOCs. Correlation coefficients between all pairs of VOCs for Titicaca were presented in Table 4. All pairs of VOCs were correlated, except: β-CAR with (E)-3-HAL, β-CAR with (Z)-3-HOL, and β-CAR with (Z)-3-HAC (Table 4).

Table 1. Mean squares from analysis of variance of VOCs for three cultivars.

Source of variation	d.f.	FARO										
		(Z)-3-HAL	(E)-2-HAL	(Z)-3-HOL	(E)-2-HOL	(Z)-3-HAC	1-HAC	(Z)-OCI	BAC	MAT	β-CAR	β-FAR
Insects/Saponins	1	226314***	5603***	27531***	5213***	217622***	2463***	31382***	7704***	6593***	54	2783*
Residual	14	2050	10.74	41.42	22.8	1930	5.984	328.9	14.45	215.8	932.7	594.4
Source of variation	**d.f.**	PUNO										
		(Z)-3-HAL	(Z)-3-HOL	(Z)-3-HAC	(Z)-OCI	MAT	β-CAR	β-FAR				
Insects/Saponins	1	63277***	15000***	43869***	12438***	1732.6***	443.1	1125.6				
Residual	14	929.1	59.33	1269	89.47	63.04	160.8	308.2				
Source of variation	**d.f.**	TITICACA										
		(Z)-3-HAL	(E)-2-HAL	(E)-2-HOL	(Z)-3-HAC	(Z)-OCI	β-CAR					
Insects/Saponins	1	134066***	2153***	1770***	156895***	9389.6***	610.1					
Residual	14	735.1	7.54	10.67	748.2	97.87	165.3					

* $p<0.05$; *** $p<0.001$; d.f. - degrees of freedom

Table 2. Correlation coefficients for VOCs for Faro.

VOCs	(Z)-3-HAL	(E)-2-HAL	(Z)-3-HOL	(E)-2-HOL	(Z)-3-HAC	1-HAC	(Z)-OCI	BAC	MAT	β-CAR	β-FAR
(Z)-3-HAL	1										
(E)-2-HAL	0.9204***	1									
(Z)-3-HOL	0.9169***	0.9868***	1								
(E)-2-HOL	0.9673***	0.9555***	0.9425***	1							
(Z)-3-HAC	0.9195***	0.9142***	0.9439***	0.9141***	1						
1-HAC	0.9438***	0.9538***	0.9662***	0.9577****	0.955***	1					
(Z)-OCI	0.8387***	0.9152***	0.9441***	0.8645***	0.886***	0.9485***	1				
BAC	0.9428***	0.9893***	0.9732***	0.9725***	0.9122***	0.9731***	0.9287***	1			
MAT	0.8509***	0.8379***	0.8439***	0.8275***	0.7704***	0.7882***	0.8087***	0.8395***	1		
β-CAR	0.0735	-0.0712	-0.0789	0.0472	-0.048	-0.153	-0.2856	-0.104	0.106	1	
β-FAR	0.5824*	0.4515	0.4361	0.5836*	0.5415*	0.5542*	0.4039	0.5204*	0.327	-0.094	1

* $p<0.05$, *** $p<0.001$

Table 3. Correlation coefficients for VOCs for Puno

VOCs	(Z)-3-HAL	(Z)-3-HOL	(Z)-3-HAC	(Z)-OCI	MAT	β-CAR	β-FAR
(Z)-3-HAL	1						
(Z)-3-HOL	0.8715***	1					
(Z)-3-HAC	0.8003***	0.8681***	1				
(Z)-OCI	0.8868***	0.9512***	0.9352***	1			
MAT	0.8723***	0.7804***	0.8132***	0.8795***	1		
β-CAR	0.4181	0.2721	0.1724	0.3399	0.4657	1	
β-FAR	0.4938	0.4002	0.4487	0.5233*	0.5519*	0.4589	1

* $p<0.05$, *** $p<0.001$

β-CAR was not correlated with others VOCs. Additionally, β-FAR was not correlated with: (E)-2-HAL, (Z)-3-HOL and (Z)-3-HAC (Table 2)

Table 4. Correlation coefficients for VOCs for Titicaca.

VOCs	(Z)-3-HAL	(E)-2-HAL	(E)-2-HOL	(Z)-3-HAC	(Z)-OCI	β-CAR
(Z)-3-HAL	1					
(E)-2-HAL	0.9161***	1				
(E)-2-HOL	0.8996***	0.9399***	1			
(Z)-3-HAC	0.9771***	0.9459***	0.921***	1		
(Z)-OCI	0.9425***	0.9193***	0.8419***	0.9225***	1	
β-CAR	0.4291	0.5622*	0.4322	0.4308	0.5314*	1

* $p<0.05$, *** $p<0.001$

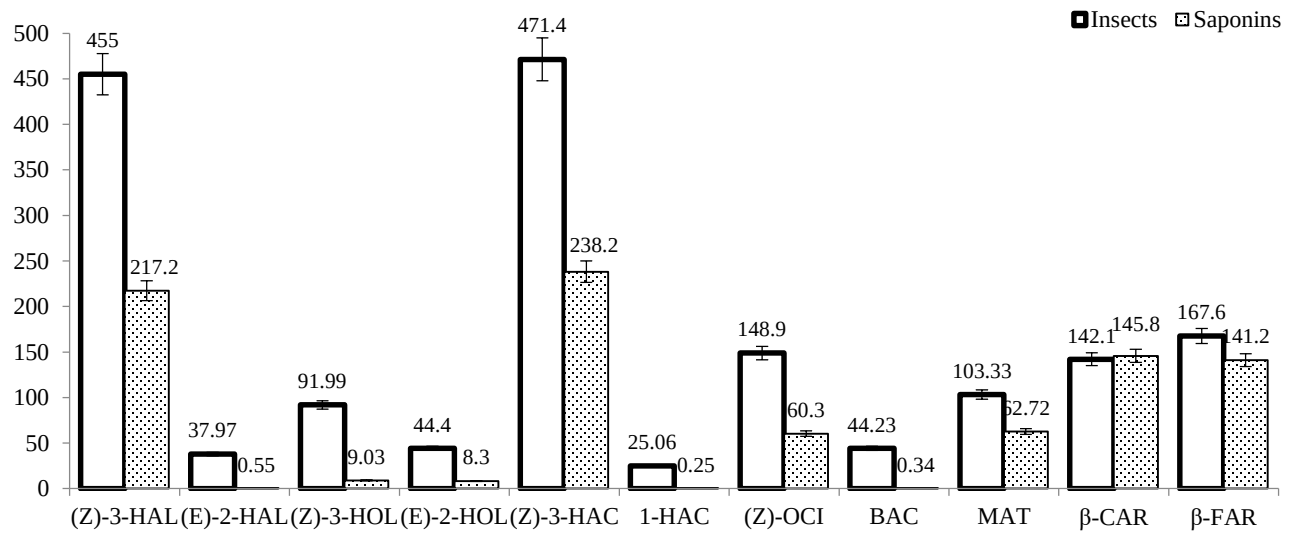

Fig. 1. Mean values and standard deviations (s.d.) of VOCs for Faro.

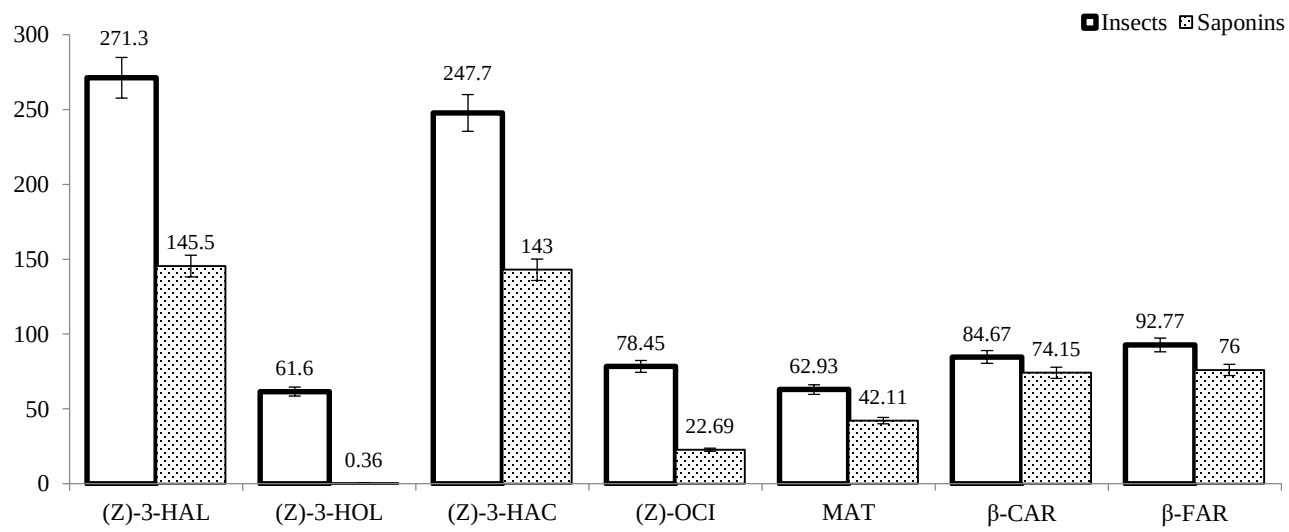

Fig. 2. Mean values and standard deviations (s.d.) of VOCs for Puno Generally, the large mean values were observed for insects than in saponins for all VOCs. Correlation coefficients between all pairs of VOCs for 'Puno' were presented in Table 3.

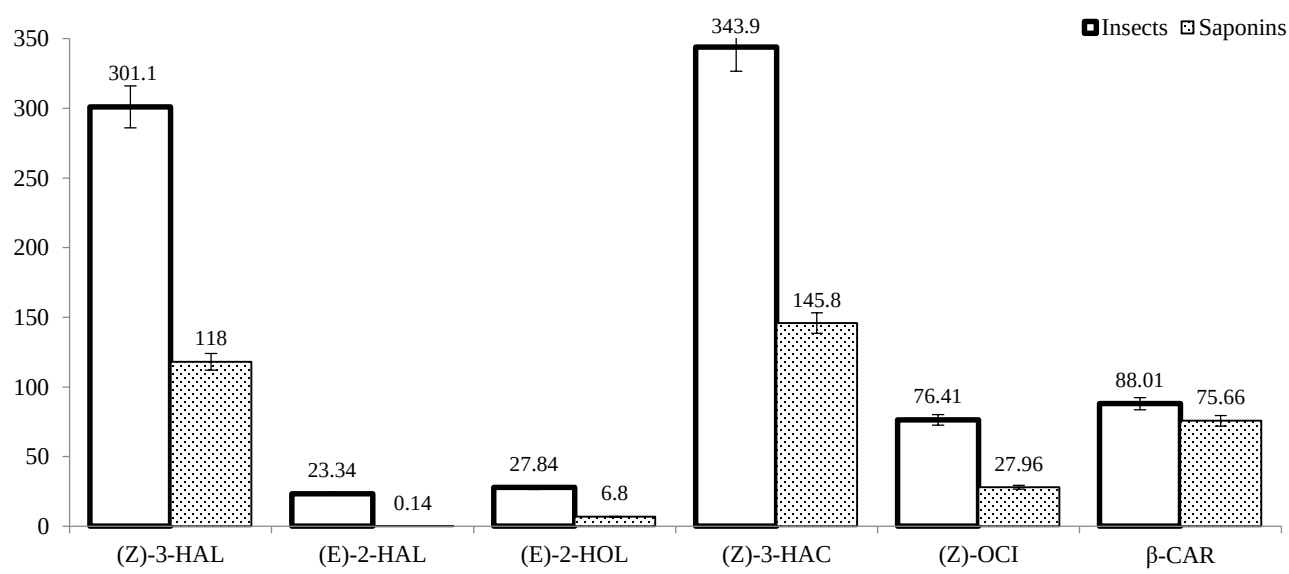

Fig. 3. Mean values and standard deviations (s.d.) of VOCs for Titicaca.

Looking at tendency of VOCs emission in 'Faro' (Z)-3-HAL and (Z)-3-HAC were released in twice higher amounts after insect feeding to compare to saponin application (455.0 and 471.4 $ng \cdot h^{-1}$, respectively). For (Z)-OCI the induction of plants was even 2.5 times stronger. The largest differences concerned (E)-2-HAL, 1-HAC and BAC, where 63, 83 and 147 times greater emission by plants attacked by insects compared to saponin application was observed. It is interesting that the releasing of β-CAR and (E)-β-FAR for both tested factors was similar. The control plants emitted only traces of VOCs. The production and emissions of VOCs is reported to be the key or the only means of contact between plants and the environment (Das *et al*., 2013). The compounds are released at low amounts by healthy plants, but their emissions may increase strongly during the feeding of herbivores. It was reported that even mechanically damaged cotton released only β-pinene, myrcene, (Z)-3-hexen-1yl acetate, (E)-β-farnesene (Röse & Tumlinson, 2005).

Cv. 'Puno' was less active in the secretion of VOCs in both quantitative and qualitative terms. The emission of (Z)-3-HAL and (Z)-3-HAC was almost twice higher after insect feeding to compare to saponin application (271.3 and 247.7 $ng \cdot h^{-1}$, respectively). The largest difference concerned (Z)-3-HOL 152 times greater emission by plants attacked by insects compared to saponin application was observed. Plant volatiles provide host recognition cues to insects that use them to determine not only whether they are approaching the correct host plant species, but also to judge the nutritional quality of the host (Bruce & Pickett, 2011). VOCs can be involved in direct and indirect plant defenses against herbivores (War *et al*., 2012).

The emission activity of the cv. 'Titicaca' was similar to the 'Puno' variety after insect feeding, although the quantity bouquet was different. The largest difference in the VOCs emission was observed for (E)-2-HAL. It has been noticed 233 times higher emission after the biotic stress (23.3 $ng \cdot h^{-1}$). It is worth noting that the tested varieties differed in the number of volatile compounds emitted. It was due to genetic differences. Quinoa is characterized by a large variety of forms and varieties (Benlhabib *et al*., 2016; Kowalski *et al*., 2016; Aluwi *et al*., 2016; Maliro *et al*., 2017) and their adaptation to new environmental conditions (Bazile *et al*., 2016a; Bazile *et al*., 2016b). Its plasticity is very wide and also applies to volatile plant ingredients. The analyzed varieties differed in the length of the growing season and the final growth of plants. European varieties (Puno and Titicaca) are characterized by a shorter growing season and smaller growth than Faro and have fewer compounds. The American variety Faro, on the other hand, emits a wider range of volatile compounds and its plants are higher and later bloom.

VOCs induction effects on plants will need further investigation to explore the role of these active substances for ecology (Kessler & Heil, 2011).

Conclusion

Looking at tendency of VOCs emission in 'Faro' (Z)-3-HAL and (Z)-3-HAC were released in twice higher amounts after insect feeding to compare to saponin application. It is interesting that the releasing of β-CAR and (E)-β-FAR for both tested factors was similar. Cv. 'Puno' was less active in the secretion of VOCs in both quantitative and qualitative terms. The emission activity of the cv. 'Titicaca' was similar to the 'Puno' variety after insect feeding, although the quantity bouquet was different. The control plants emitted only traces of VOCs.

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