Aroma Compounds Are Responsible for an Herbaceous Off-Flavor in the Sweet Cherry (Prunus avium L.) cv. Regina during Fruit Development

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Abstract: An herbaceous/grassy-like flavor has been reported by Chilean producers of Regina sweet cherry. There are no previous academic reports related to this flavor occurrence. Sweet cherries from five phenological stages were collected from six orchards with high herbaceous flavor incidence spanning Chilean production zones during the 2019/2020 season. Four experienced panelists tasted the fruit to identify the off-flavor incidence and intensity from four phenological stages, and the same cherries were analyzed for volatile compounds. Thirty-nine volatiles were identified and semi-quantified using solid-phase microextraction (SPME) and GC-MS. The highest off-flavor incidence was found at the bright red (stage 3) and mahogany colors (stage 4). No single volatile explained the herbaceous flavor consistently among orchards. However, it appeared that the off-flavor was related to delayed ripening in cherries, with more C6 aldehydes and less esters. Furthermore, rainfall and the elevation of the orchard had a significant effect on the incidence of off-flavor. Preharvest practices that promote fruit ripening along with avoiding early harvests are recommended to reduce the incidence of herbaceous flavor in Regina.

Keywords: sweet cherry; Prunus avium L.; sensory analysis; herbaceous flavor; aroma compounds

1. Introduction

Sweet cherry is a fruit highly desired because of its health benefits and flavor. Its beneficial health effects have been associated to polysaccharides such as pectin, polyphenols and antioxidants in the fruit [1]. It is well-known that these constituents reduce oxidative stress and inflammation [2,3]. The cytoprotective effect of sweet cherries is a trending topic, which still requires clinical trials to fully understand the anti-cancer properties [4]. Consumer preference for sweet cherries can be related to quality characteristics such as size, color, firmness and flavor, all characteristics being valued by the sweet cherry industry [5].

Chilean sweet cherry exports reached USD 1.1 billion in the 2019 season, making Chile the main exporter of this fruit in the Southern Hemisphere [6]. Nowadays, the most exported sweet cherry cultivars are Bing, Lapins, Santina and Regina. Regina is also one of the important cultivars worldwide [5]; it showed the highest overall consumer preference among six cultivars evaluated in the U.S.A. [7]. In addition, cv. Regina is suitable for fast processing and long-distance shipping due to its rheological properties, which result
in less susceptibility to mechanical damage [8]. This mechanical property is especially important since the majority of the Chilean sweet cherry production is sent to China (94%) for which a 40-day travel under refrigerated marine containers by boat is required. However, buyers have noticed that some of the Regina fruit produced in Chile develops an undesired taste/flavor when they arrive on the market described as “herbaceous”, “unripe-like” or “grass-like” [9].

Flavor perception is the combination of different stimuli, mainly taste and aroma. Taste is produced by the interaction of non-volatile molecules with receptors located in the taste buds, which account for the five basic tastes, bitter, sour, sweet, salty and umami, while the characteristic flavor of food is attributed to the aroma volatiles, perceived by retronasal olfaction [10].

Sweet cherry cultivar Bing’s volatile profile was characterized by Mattheis et al. [11,12] during fruit development, ripening and storage. Aldehydes, alcohols, ketones and esters were the main constituents of Bing’s volatiles, with esters being associated with ripe fruit. In their study, benzaldehyde, (E)-2-hexenal and hexanal were considered to be the main contributors to cherry aroma [11], but no sensory evaluation was performed to confirm this hypothesis. Other authors have reported volatile profiles for several sweet cherry cultivars [13–17], showing commonality within the species, but the distribution and quantity of these compounds is highly cultivar-dependent and so is the final perceived flavor.

The aroma profile of the Regina sweet cherry was characterized by Papapetros et al. [18] who identified 20 volatile compounds within the main group of volatiles, including 3 aldehydes, 2 alcohols, 1 ketone and 6 terpenes. In the present research, we carried out a more complete characterization of the volatile profile of the Regina sweet cherry including changes in the volatile compounds during fruit development and differences among geographical growing areas of sweet cherry in Chile with the goal of exploring the nature of the reported off-flavor. We hypothesized that the accumulation of volatile compounds in sweet cherry Regina during fruit ripening correlates with the development of herbaceous off-flavors and that there is an environmental effect due to the climate and geographical zone. To the best of our knowledge, this is the first report on the cultivar Regina’s herbaceous off-flavor, with an attempt to understand the biological basis of this off-flavor through volatile characterization, sensorial analyses and geographical features of the fruit production zones. The ultimate goal is to provide information to growers to minimize Regina’s herbaceous off-flavor in the future.

2. Materials and Methods

2.1. Plant Material

Regina sweet cherries used in this work came from orchards located in different geographical regions where the off-flavor had been reported previously.

Six different orchards were sampled during the 2019/2020 season from the central to south valley of sweet cherry commercial growing regions of Chile (from north to south ~1400 km), (orchard 1: 34°03′02.0″ S 70°40′19.3″ W 530 m (above sea level); orchard 2: 35°12′56.7″ S 71°06′37.6″ W 515 m; orchard 3: 35°06′07.9″ S 71°18′25.4″ W 230 m; orchard 4: 37°47′57.4″ S 72°38′38.6″ W 80 m; orchard 5: 40°51′21.7″ S 73°13′20.1″ W 104 m; and orchard 6: 46°32′43.7″ S 71°40′41.7″ W 216 m) dominated by temperate Mediterranean, (Orchard 1, 2, 3 and 4) and south Sub-Mediterranean (Orchard 5) climates characterized by warm to hot summers and cool, rainy winters. Orchard 6 is located in the South Austral Macro region associated with polar Tundra climate, but orchard 6 is located in a microclimate classified as sub-Mediterranean climate according to Köppen–Geiger climate classification [19]. In each orchard, the sweet cherries were hand-picked and collected from at least 30 trees (an entire row) where the herbaceous flavor was detected according to the grower.

Based on our preliminary study [9], five phenological stages (2–6) were analyzed: véraison (yellow stage), bright red (UC color chart 3.0; hue = 10–12°), mahogany (UC color
chart 3.5; hue = 7–9°) or commercial harvest, dark mahogany (UC color chart 4.0; hue = 3–6°) and black or overripe (UC color chart 5.0; hue = 2°) (Figure 1). The fruit were assessed to guarantee commercial quality parameters were met following standard protocol for fruit analyses described by Param and Zoffoli (2016) (data not shown) [8]. Sweet cherries were stored up to 6 days at 0 °C enclosed in a bag (=100 units per bag) under saturated condition to prevent desiccation and transferred to room temperature (20 ± 1 °C) at least 2 h before the sensory and volatile analyses.

2.2. Fruit Classification by Sensory Evaluation

During the first year of the research, four staff members evaluated cherries from orchard 1 and classified them as “normal” and “off-flavor” [9]. When identified with off-flavor, those cherries were cut in smaller pieces so that all tasters could agree on the quality and intensity of the off-flavor. Those tasters agreed on the quality of the off-flavor being “herbaceous”, with intensity ranging from 0 to 15 with 0 = non-herbaceous or absent, 15 = extremely herbaceous and 7.5 as the middle point. In the following year (year of the present study), 100 Regina cherries from each phenological stage (stages 3 to 6) were halved and tasted by the same staff members, occasionally repeating the previous year’s protocol to assure consensus in quality and intensity of the herbaceous flavor. Panelists tasted 25 half cherries from one orchard and one phenological stage in one sitting to recognize the presence/absence of herbaceous flavor (25 cherries each in a randomized order), as well as assigning an intensity on a 15–cm continuous line scale [20]. The procedure was repeated on different days for each orchard and phenological stage (3 to 6) with a maximum of two tests per day and a waiting period of ~4 h between tests. The first half of each cherry was eaten to classify the fruit into normal (control) or herbaceous flavor. Procedure-wise, each panelist put the second half on a plate labelled as above. These second halves of the fruit, stone removed, were processed fresh for volatile analysis.

2.3. Volatile Extraction

For volatile identification, a composite sample was prepared from randomly selected cherry halves previously classified as herbaceous or non-herbaceous in the sensory evaluation. For each composite sample representing a replication unit, 20 grams of fresh sweet cherry flesh and peel were ground for 5 min with mortar and pestle and added to a 50-mL glass flask, then 35% w/w NaCl solution was added in 1:1 ratio to stop further enzymatic activity. 1-Heptanol (0.10 mg/kg) was used as internal standard. Flasks were sealed immediately after addition of internal standard with parafilm and a screw cap with breather in order to introduce the SPME in the flask. The volatiles compounds were adsorbed on a 2-cm PDMS/DVB SPME fiber (65 μm polydimethylsiloxane/divinylbenzene coated fiber, Supelco, Steinheim, Germany) for 30 min at 40 °C while stirring. Three replications were prepared for “normal” cherries, less for off-flavored cherries if there were not enough fruit.

2.4. Chromatographic Settings

Quantitative analyses were performed with a PerkinElmer® Clarius680 (PerkinElmer, Inc., Norwalk, CT, USA) gas chromatograph equipped with a mass detector PerkinElmer® Clarius SQ8T (PerkinElmer, Inc., Norwalk, CT, USA). Volatile compounds were separated with an Elite-WAX column (60 m, 0.25 μm film thickness, 0.32 mm ID; PerkinElmer, Inc., Norwalk, CT, USA). The transfer line and ion source temperatures were 250 °C, the injector temperature was 250 °C, set for splitless injection, and the carrier gas was helium at 1 mL/min. The temperature program was 40 °C for 5 min, ramped to 250 °C at a rate of 7 °C/min and held at 250 °C for 10 min. Mass range was recorded from m/z 50 to 250.
2.5. Volatile Identification

Tentative identification was made by comparison of the mass spectra with the National Institute for Standard and Technology (NIST) library 2014 using Turbo mass 6.0.1 software (PerkinElmer, Inc., Norwalk, CT, USA), as well as comparing retention indices from published retention indices on the same column [21–23]. A series of n-alkanes (n = 10 to n = 20) were run under the same conditions as the samples. Kovats indices were calculated for all the tentatively identified compounds following the equation KI = 100n + 100(tr (unknown) − tr (n)/tr (N) − tr (n)) [24], where n is the number of carbon atoms in the smaller alkane, N is the number of carbon atoms of the larger alkane and tr is in all cases the retention time. A semi-quantification based on the internal standard 1-heptanol was performed by extrapolating the area of the internal standard to the integrated area of every volatile compound (assuming correction factor = 1). The semi-quantification is showed as heatmaps below in the document and detailed in Supplementary Material Table S1 and Figure S1.

2.6. Statistical Analysis

A two-way ANOVA analysis was carried out with the ‘incidence’ data (angular transformation (arcsin) applied) to identify the effect of ‘orchards’ and ‘phenological stages’ on the off-flavor incidence. Furthermore, two principal component analyses (PCA) were performed with the volatile data from both affected and non-affected cherries at phenological stages 3 to 6. One PCA was performed using the relative concentration of the 10 most abundant volatiles in Regina (hexanal, (E)-2-hexenal, nonanal, decanal, benzaldehyde, 1-hexanol, (E)-2-hexen-1-ol, benzyl alcohol, hexyl acetate, (E) 2-hexenyl acetate) and the cherry phenological stages; the other PCA was built using the odor activity values (OAVs) of compounds calculated with the following equation:

\[
\text{OAV} = \frac{\text{concentration}(\mu\text{g}/\text{kg})}{\text{Odor threshold}(\mu\text{g}/\text{kg})}
\]

OAVs have been used in flavor chemistry to assess the contribution of compounds to foods. It is assumed that compounds with OAVs greater than 1.0 contribute to a food flavor [25, 26]. However, it has also been demonstrated that compounds in mixtures have a lower threshold [27, 28]. We therefore arbitrarily set a minimum OAV value of 0.5 to build the second PCA (values shown in Supplementary Material Table S2), which included the volatiles mentioned above plus eight others ((Z)-3-Hexenal, heptanal, octanal, 1-octen-3-ol, 2-nonanal, β-linalool, 2,6-nonadienal and (E)-2-decenal).

In addition, a stepwise regression test with fixed intercept = 0 was carried out, the dependent variable was the off-flavor incidence (%) in each orchard and the independent variables were the phenological stages and eight climatic variables (daily minimum air temperature, maximum air temperature, average solar radiation, accumulated solar radiation, rainfall, degree days base 10 °C, accumulated degree days and altitude), obtained from the Instituto de Investigaciones Agropecuarias (INIA) meteorological network located near orchards used in the study (Supplementary Material Table S2). The accumulated degree days for each harvest date are provided in Supplementary Material Figure S2. Another step-wise regression was performed with the concentration of the 10 most abundant volatiles in Regina (hexanal, (E)-2-hexenal, nonanal, decanal, benzaldehyde, 1-hexanol, (E)-2-hexen-1-ol, benzyl alcohol, hexyl acetate, (E) 2-hexenyl acetate) and the phenological stages values as independent variables, and the off-flavor incidence (%) as response variable, with the intercept fixed to 0 (Supplementary Material Table S2). The stepwise multiple regression test, PCAs and correlation analyses were implemented by using STATGRAPHICS Centurion XIX version 19.1.2 software (Statpoint Technologies, Warrenton, VA, USA). The ANOVA, t-test and graphs were obtained by using GraphPad Prism 8.0.1 (GraphPad Software, Inc., San Diego, CA, USA).
3. Results

3.1. Off-Flavor Occurrence by Sensory Evaluation.

Regina sweet cherries were tested during the fruit development stages (Figure 1) in order to identify at which phenological stage the off-flavor occurs. The herbaceous flavor was strongly perceived at stages 3 and 4 (commercial harvest time), while it decreased in both incidence and intensity at phenological stage 6.

![Figure 1. Phenological stages of cherry fruit. From left to right: (1) green or pre-<em>véraison</em>, (2) yellow or <em>véraison</em>, (3) bright red (UC color chart 3.0; hue = 10–12°), (4) mahogany (UC color chart 3.5; hue = 7–9°) or commercial harvest, (5) dark mahogany (UC color chart 4.0; hue = 3–6°) and (6) black or overripe (UC color chart 5.0; hue = 2°).](image)

When the orchard was included in the analysis, the ANOVA results showed that factors phenological stage and orchard were all significant sources of incidence variation (Table 1).

| ANOVA Table          | p Value       | % of Total Variation |
|----------------------|---------------|----------------------|
| Orchard              | p < 0.0001    | 20.9                 |
| Phenological Stage (3–6) | p < 0.0001   | 18.56                |
| Interaction          | p = 0.1796    | 17.35                |

Likewise, the geographical study revealed that the incidence of the herbaceous flavor varied among orchards and phenological stages (Figure 2). The incidence of the herbaceous flavor was the highest (17–18%) in orchards 1 and 2, located near to the center of the country (Figures 2A and 2B).

Conversely, orchards 3 and 4 showed the lowest incidence, with <5% of fruit with herbaceous flavor (Figure 2B). The off-flavor intensity of each affected cherry is shown on a 0 to 15 linear scale (Figure 2C). A higher intensity was found in orchards 5 and 6 (southern orchards), followed by orchards 1 and 2 (northern orchards). On the other hand, phenological stages 3 and 4 showed the highest herbaceous flavor intensity in all orchards.

3.2. Identification and Semi-Quantitation of the Volatile Compounds

In total, 39 compounds among aldehydes, esters, alcohols and terpenes were identified. The most important volatiles in terms of abundance and presence in all samples and throughout the developmental stages were the following: hexanal, (E)-2-hexenal, 1-hexanol, (E)-2-hexen-1-ol, nonanal, decanal, benzaldehyde and benzyl alcohol, together with hexyl acetate and 2-hexenyl acetate. These volatiles were the most represented compounds in the aroma profile of sweet cherry cv. Regina, which confirms the preliminary data obtained in the previous season [9].
The concentration of the aroma compounds varied along the phenological stages and orchards. Significant differences between the volatile profiles of herbaceous cherries compared to the non-affected ones from the same orchard were found. For instance, in orchard 1, levels of (E)-2-hexenal and (E)-2-hexenol were significantly higher in herbaceous cherries at stage 5 that had fewer herbaceous cherries but were significantly lower at stage 3. Likewise, the benzaldehyde levels were also significantly higher at stage 5. (Supplementary Material Figure S1).

Figure 2. Herbaceous flavor incidence and intensity during fruit development of sweet cherry cv. Regina in orchards 1 to 6 during the 2019/2020 season. (A) Geographical distribution of the studied sweet cherry orchards in Chile. (B) Herbaceous flavor incidence per orchard. (C) Perceived intensity of the herbaceous flavor.

The semi-quantification of the volatile data from the six evaluated orchards, located in several locations across Chile, are shown as heatmaps in Figures 3–5 and Supplementary Material Table S1.

The most abundant aldehydes in Figure 3 were hexanal, (E)-2-hexenal, benzaldehyde and to a lesser extent, nonanal. The C6 aldehydes’ concentrations increased as the sweet cherry fruit ripened and were notably higher in the southern orchards (5 and 6) compared to the northern ones. In orchards 1, 2 and 5, these aldehydes were lower in the affected cherries at stage 3 compared with the non-affected ones and were higher at stage 5. Nonanal, an aldehyde described as green, was higher in off-flavored cherries at unripe stages (2 and 3) in orchards 2, 3, 4, 5 and 6; orchard 1 was one exception with a nonanal peak at
stage 5. Moreover, in orchard 3 (one of the orchards with a lower off-flavor incidence) nonanal was present only at stage 2.

Figure 3. Volatile aldehydes concentration in pulped Regina sweet cherries during development in orchards 1 to 6. 3*, 4*, 5* and 6* indicate samples with the herbaceous flavor. White cells mean absent or not detected.
Figure 4. Volatile alcohols concentration in pulped Regina sweet cherries during development in orchards 1 to 6. 3*, 4*, 5* and 6* indicate samples with the herbaceous flavor. White cells mean absent or not detected.

Figure 5. Other volatiles concentration in pulped Regina sweet cherries during development in orchards 1 to 6. 3*, 4*, 5* and 6* indicate samples with the herbaceous flavor. White cells mean absent or not detected.
The most important alcohol in the sweet cherry Regina volatile profile was \((E)-2\text{-hexen}-1\text{-ol}\) followed by 1-hexanol and benzyl alcohol. The southern locations showed consistently higher levels of these volatiles than the northern ones. In addition, in orchards 1 and 2, the production of \((E)-2\text{-hexen}-1\text{-ol}\) in affected cherries with the off-flavor showed a lower concentration (lighter blue) at stage 3. The minor alcohol concentrations were under the 5 µg/kg in most cases, which is lower than the odor threshold for some of them (Table 2). Furthermore, volatiles from other chemical nature such as esters and terpenoids were present in low concentrations (Figure 5); among these compounds, \((E)\)-hexenyl acetate, hexyl acetate, d-limonene and geranyl vinyl ether were present in the majority of the samples during each phenological stage.

| Compound                        | Odor Description                  | Odor Threshold (µg/kg) | Concentration Ranges (µg/kg eq. I.S) | Reference |
|---------------------------------|-----------------------------------|------------------------|--------------------------------------|-----------|
| Ethanol                         | dry, dust                          | 100000                 | 0–1.7                                | [29]      |
| Hexanal                         | green-grassy, slightly fruity       | 4.5                    | 2.3–139.9                            | [30]      |
| \((Z)\)-3-Hexenal              | green, cut leaf                    | 0.25                   | 0–1.8                                | [30]      |
| Heptanal                        | soapy, stale, citrus, rancid       | 3                      | 0–9.1                                | [31]      |
| d-limonene                      | citrus, fruity, floral             | 200                    | 0.5–6                                | [31]      |
| \((E)\)-2-Hexenal              | green-fruity, fresh, grassy        | 17                     | 7–127.4                              | [30]      |
| Hexyl acetate                   | sweet, fruity, floral              | 2                      | 0.3–4.3                              | [32]      |
| Octanal                         | sweet, green, fruity               | 0.7                    | 0–3.2                                | [33]      |
| \((Z)\)-2-heptenal             | mushroom, soapy                    |                        | 0–4.1                                |           |
| \((E)\)-2-Hexenyl acetate      | fruity, banana peel                | 210                    | 0.8–12.7                             | [34]      |
| 6-Methyl-5-hepten-2-one         | earthy, rancid green, sweet, fruity, rubbery toasted, green, dry, resin, flower | 400 | 39.6–238.1 | [29] |
| 1-Hexanol                       | pungent/green, grassy, fresh green, fruity, herbaceous, leaf floral | 2500 | 5.4–123.2 | [35] |
| Nonanal                         | pungent, fruity, earthy sour, acidic, fruity, green, soapy | 1 | 0–7 | [35] |
| \((E)\)-2-Hexen-1-ol            | green, fruity, herbaceous, leaf floral | 400 | 39.6–238.1 | [29] |
| 2-Octanol                       | 0–1.5                               | 2                      | 0–1.6                                | [36]      |
| \((E)\)-2-Octenol              | 0–1.5                               | 1                      | 0–1.3                                | [36]      |
| 1-Octen-3-ol                    | pungent, fruity, earthy sour, acidic, fruity, green, soapy | 2 | 0–4.2 | [35] |
| Decanal                         | green, floral                       | 300                   | 0–2                                   | [36]      |
| 2-Ethyl-hexanol                 | green, floral                       |                        |                                       |           |
| Volatile          | Description                              | Concentration | Range             | Reference |
|-------------------|------------------------------------------|---------------|-------------------|-----------|
| Benzaldehyde      | sweet, almond, burnt, caramel            | 350           | 0.4–164.3         | [30]      |
| 2-Nonenal         | stale/hay, grassy, cucumber              | 0.08          | 0–3.6             | [30]      |
| β-Linalool        | fruity, floral, sweet, grape sweet-floral| 6             | 0–5.3             | [30]      |
| 1-Octanol         | fruity, citrus fresh, watermelon, cucumber| 110          | 0.4–1.6           | [35]      |
| 2,6-Nonadienal    |                                          | 0.01          | 0–1.2             | [37]      |
| Geranyl vinyl ether |                                        |               |                   |           |
| (E)-2-Decenal     | citrusy-green, soapy                     | 0.4           | 0–3.7             | [35]      |
| 1-Nonanol         | fat, green, herbageous                   | 50            | 0.5–3.1           | [35]      |
| Hexanoic acid     | sweaty, bitter, metallic                 | 3000          | 0–5.1             | [35]      |
| Geranyl acetone   | floral, rose, green sweet, floral        | 60            | 0–2.1             | [30]      |
| Benzyl alcohol    |                                          | 10,000        | 0.3–76.4          | [35]      |

1 Concentration ranges for phenological stages 2 to 6 in Regina sweet cherries without off flavor.

The PCA (Figure 6A) shows the semi-quantitation data of nine of the most abundant volatiles in the cherry samples at four phenological stages (3 to 6), for cherries with (red dots) and without (blue dots) off-flavor. The PCA shows a clear separation of orchard 6 from the other orchards, in the upper right quadrant of the PCA (blue ellipse). Orchard 6 was characterized by higher concentrations of benzaldehyde, benzyl alcohol, the C6 aldehydes hexanal and (E)-2-hexenal and (E)-2-hexen-1-ol, showing the different compositions between the cherries from northern and the southern orchards. Taken orchard by orchard, there was little difference between the cherries with and without off-flavor. However, a trend could be observed with an increase in volatile content as fruit ripening progressed: there was a shift towards the lower right quadrant, with 1-hexanol, hexyl acetate and 2-hexenyl acetate characterizing fruit in advanced ripening stages (Stages 5 and 6). Overall, more cherry samples with off-flavor were located in middle and left quadrant, suggesting a delay in ripening of those affected fruit.
Figure 6. PCA of sweet cherry cv. Regina harvested from six orchards at four phenological stages: (A) volatiles with the highest concentration, and (B) volatiles with OAVs greater than 0.5. The points are labeled by orchard number (O1 to O6) followed by a hyphen and the phenological stage number (3 to 6), also, the tag ‘OFF’ was added for samples affected with off-flavor.

Figure 6B shows the PCA of the same cherry samples, with variables being volatiles with OAVs greater than 0.5. There was no separation between affected (off flavored) and non-affected cherries, within each orchard and phenological stage, except for cherries from orchards 4 and 5 at phenological stage 3 (for both orchards) and phenological stage 6 (orchard 4), which had higher contribution of octanal, nonanal and decanal (higher OAVs, upper right quadrant of the graph). The different composition of orchard 6 observed in Figure 6A also showed in Figure 6B, indicates that cherries from this orchard are more flavorful than cherries from orchards located further north. Indeed, the panelists commented on the flavor of cherries from orchard 6.
3.3. Climatic Factors That Influence the Herbaceous Flavor Incidence in Cultivar Regina.

The climatic variables temperature, radiation, rainfall (total amount in mm), altitude and degree days were tested for correlation with the incidence of ‘herbaceous’ flavor; however, the test showed low correlation values with single variables (data not shown). Conversely, when a stepwise multiple regression analysis of eight variables (max. temperature, min. temperature, rainfall, average radiation, accumulated radiation, altitude, degree days and the phenological stages) was carried out, the model showed that rainfall and altitude significantly contributed to the variation in the incidence of herbaceous flavor (R2: 81.13%; adjusted R2: 78.78%). The standardized partial regression coefficients, p-values and predictions of this model are included in Supplementary Table S2.

The multiple regression equation is as follows:

\[
\text{offflavor incidence} \% = 0.0282 \times \text{Rainfall} + 0.0217 \times \text{Altitude}
\]

As for the regression equation using the volatiles as independent variables, it did not show any significant result that could help explain the incidence of the herbaceous flavor.

4. Discussion

It was possible to identify the herbaceous off-flavor, which appeared in phenological stages 3, 4 and 5. The off-flavor was more frequent and intense in stages 3 and 4 (commercial harvest). These stages of fruit development were also characterized by an increase in the total volatile content, in agreement with previous studies in sweet cherry [12,13,38]. Volatile production in most non-climacteric fruit species is part of the ripening process, depending on the plant growth regulator abscisic acid (ABA). It has been demonstrated that abscisic acid increases at the beginning of maturation and accumulates during ripening [39]. Hence, future research should be addressed regarding the association of aroma production with ABA synthesis and accumulation during the ripening process.

Thirty-nine compounds were tentatively identified and included thirteen aldehydes, fourteen alcohols, three terpenes, four esters, four ketones and two organic acids. In general, the herbaceous off-flavor could not be explained by a single component apparition/omission in the affected sweet cherries when compared to the non-affected ones. However, in three instances, straight chain aldehydes, octanal, nonanal and decanal, showed higher OAVs in off-flavored cherries. Nevertheless, we hypothesize that the off-flavor may be related to a difference in the aromatic compound concentrations and the relative abundance of VOCs between affected and non-affected Regina sweet cherries. The main group of VOCs and their possible influence on the herbaceous flavor perception is discussed below.

4.1. Aldehydes

The three most abundant aldehydes in the volatile profile were hexanal, (E)-2-hexenal and benzaldehyde; these compounds have been previously described as the principal contributors to the sweet cherry aroma in Bing cultivar [12]. Benzaldehyde, which is produced by amygdalin hydrolysis and confers the sweet almond-like aroma to the sweet cherry [13], was higher in phenological stage 3 and decreased as fruit development progressed. This is in agreement with findings in cultivar Bing [12] and is opposite to the benzaldehyde behavior in Chinese cultivar Hongdeng [13]. Thus, its production in cherries seems to be cultivar dependent. Interestingly, the concentration of benzaldehyde decreased at a slower rate and later in affected than in non-affected cherries (after stage 4), but the total content was higher at phenological stage 5 in the affected cherries. The C6 aldehydes hexanal and (E)-2-hexenal have been well described as green-herbaceous contributors to the aroma and flavor of fruits [40,41], and they are produced from fatty acids through the lipoxygenase (LOX) pathway [42,43]. There were no significant differences in the levels of these compounds between the affected and non-affected cherries within the same orchard (considering the six localities). Regardless of this fact, the concentration of
hexanal and \((E)\)-2-hexenal (<10 µg/kg eq. I.S) in orchard 3 (one of the locations with the lowest incidence) was several times lower than in the other orchards, suggesting that there could be a positive relationship of the C6 aldehydes production in sweet cherry Regina and the development of the herbaceous off-flavor in this growing area.

Regarding the aldehydes with lower concentrations such as \((Z)\)-3-hexenal, heptanal, octanal, nonanal, decanal, 2-nonenal, 2,6-nonadienal and \((E)\)-2-decenal, their relative contribution to the aroma can be predicted, at least partially, with their detection threshold values (Table 2) and their OAVs. For instance, \((Z)\)-3-hexenal, described as green/cut-leaf and with an odor threshold of 0.25 (µg/L) [44], showed a lower concentration than its threshold level in the non-affected cherries but slightly over it in the affected ones at phenological stage 5. This small increment in concentration may influence the herbaceous flavor detected by the tasters, especially when combined with other compounds. Less abundant aldehydes, \((Z)\)-3-hexenal, heptanal, octanal, nonanal, decanal, 2-nonenal, 2,6-nonadienal and \((E)\)-2-decenal, showed OAVs greater than 0.5 in the affected cherries and likely contributed to the herbaceous flavor altogether. Moreover, Figure 6B showed a clear relationship of high OAVs in octanal, nonanal and decanal with the perceived off-flavor of cherries from orchards 4 and 5, stage 3.

Considering that the aldehyde content is highly variable among sweet cherry cultivars [16], and that seven of the identified aldehydes have been described as green, grassy or herbaceous (Table 2), including hexanal and \((E)\)-2-hexenal, it is likely that these compounds are, at least in part, responsible for the herbaceous off-flavor.

4.2. Alcohols

1-hexanol and 2-hexen-1-ol were the most abundant volatiles in this group, and both compounds are produced mainly by the alcohol dehydrogenase enzyme from the corresponding aldehyde [45]. The results from orchard 1, one of the most affected orchards, showed that the content of 1-hexanol increased consistently as the fruit ripened from 6.43 (µg/kg) at stage 2 to 19.97 (µg/kg) at stage 5 in the non-affected cherries, while the concentration in the affected cherries was 2.03 (µg/kg) at stage 3 and increased to 23.21 (µg/kg) at stage 5 (Supplementary Material Figure S1). Additionally, the \((E)\)-2-hexen-1-ol levels were less variable with concentrations between 50 and 70 (µg/kg) during all developmental stages in the non-affected cherries, whereas in the affected cherries, \((E)\)-2-hexen-1-ol increased from 12.60 (µg/kg) at stage 3 to 96.72 (µg/kg) at stage 5. Overall, the LOX-derived C6 alcohol production seemed delayed in the development of the affected sweet cherries. In other words, since the volatile production is ripening-dependent, the ‘late’ peak of production of these alcohols suggests a delay in the ripening process, resulting in the detection of more green/grassy notes than fruity/sweet notes conferred by the esters.

4.3. Esters

Generally, the appearance of esters is correlated with the decrease in aldehyde levels and the development of ‘fruity’ flavors [46,47]. However, our results showed that in Regina cultivar, only \((E)\)-2-hexenyl acetate and hexyl acetate were present, and their concentrations were lower than the respective \((E)\)-2-hexanal and hexanal aldehydes through all the developmental stages. In addition, the ester concentration in the affected cherries was slightly lower than in the non-affected cherries at the equivalent harvest stages (Figures 3, 4, 5 and 6), which could also be a factor in the perceived enhancement of the herbaceous flavor. Esters are biosynthesized in the final step by acyl transferases. The genes encoding acyl transferases are often expressed in ripening-dependent manner [48]; thus, ripening variations may be also related to the perception of the herbaceous flavor.
4.4. Other Factors

Odor mixture studies have shown that the interaction among aroma components is not simply additive: synergistic or suppression phenomena occur, influencing the final odor perception [49]. It has been reported that (E)-2-hexenyl acetate had the ability to diminish or suppress the perceived intensity of other odorants [50]. This suggests that (E)-2-hexenyl acetate, as well as other esters, could have an effect by suppressing the perception of C6 aldehydes, hence decreasing the herbaceous flavor in ripe sweet cherries. The OAVs analysis showed that in most cases, the volatile contribution to the perceived flavor is similar between affected and non-affected cherries, which may indicate that non-volatiles components could also influence the off-flavor perception. Sugar content, acidity and other non-volatiles such as anthocyanins and phenolic compounds should be studied to fully understand the nature of the herbaceous flavor in Regina sweet cherry. Odor/flavor reconstitution studies including volatiles, sugars and acids would confirm the role of each compound when mixed together.

4.5. Environmental Factors

The geographical study showed differences in the herbaceous flavor incidence among the six orchards, which were grouped for the analysis as follows: orchards 1 and 2 with high incidence (>15%); orchards 5 and 6 with intermediate incidence (10–15%); and orchards 3 and 4 with low incidence (<5%). It is to be noted that orchards 2 and 3 were separated by only 22 km with different results in terms of incidence, suggesting that the off-flavor could be the result of soil, microclimate, sun exposure, as well as preharvest practices altering the rate of ripening and is less strongly influenced by the geographical location [51]. In addition, it is important to mention that color index for fruit harvest may not represent the same physiological stage through the different geographical areas, making the comparison among orchards unequal.

A stepwise regression revealed that the incidence of herbaceous flavor in cherries was related to the altitude and rainfall of each orchard. Rainfall has been reported to promote or to inhibit the production of certain volatiles. For example, Vallat et al. [52] related the influence of rainfall in the volatile emissions from apple fruit, leaves and twigs. In their findings, Vallat et al. [52] reported that the C-6 volatile compounds (E)-2-hexenal and (Z)-3-hexen-1-ol were negatively related with rainfall, whereas benzaldehyde and nonanal were positively correlated. It is well known by the wine industry that rainfall affects the aromatic qualities in their wines, for instance, a rainfall before veraison negatively affected the production of terpenes and positively affects the C-6 compounds and alcohols of grapes cv. Muscat [53]. On the other hand, in Glera grapes grown at different altitudes, a difference in the ripening process and aromatic profile were reported [54]. These researchers associated altitude with temperature, where the differences in the aroma profile were determined by the minimum air temperatures at night. In another study, the rainfall and altitude affected the production of 2-acetyl-1-pyrroline (2AP), an important aroma constituent of a fragrant colored rice [55]. In their study with 29 samples of rice under different rain regimes and altitude ranges (26.26 to 1033.41 m above sea level), 2AP decreased with increasing rainfall, and those samples harvested from higher altitudes presented higher 2AP content than those from lower altitudes. Likely, the effect of rainfall on sweet cherry aromas is not simply due to the amount of rainfall but mostly the physiological period when the rainfall happens, similar to what has been reported in wine grapes [53]. The precipitation pattern in Chile increases from northern to southern areas. For instance, the rainfall range in the northern orchard was 3.6–6.6 mm, and it was 42.2–218.9 mm in the southern orchard, which explains the higher volatile production in Orchard 6.
5. Conclusions

Our findings show that the herbaceous flavor in sweet cherries cv. Regina is present during phenological stages 3 to 6, with up to 15% incidence at the stage of the commercial harvest. The most southern orchard (orchard 6) had a different volatile profile compared to orchards 1–5, indicating effect of climate and soil. The off-flavor appeared to be related with a delay in ripening, affecting the secondary metabolism with slower accumulation of total volatiles, and the growing area has an influence on the overall aroma profile of the cherries. It was not possible to explain the herbaceous flavor with one single volatile compound, therefore, we conclude that off-flavor is the result of a combination of environmental, developmental and volatile perception factors. The regression equation with environmental factors may serve as a tool for growers, helping them to make better decisions about cultivation zones. For instance, growers in high elevations should plant cultivars other than Regina to avoid producing cherries that have a potential of being rejected due to off-flavor. Likewise, the fact that the volatile profile is being delayed in its developmental process makes this work valuable for cherry producers, since they could avoid early harvests when the aroma biology affects the fruit quality and sensory characteristics perceived by the consumer. Considering the complexity of flavor perception, the contribution of non-volatiles compounds should be studied together with volatiles to have a complete picture of the nature of the herbaceous flavor in Regina sweet cherry.

Supplementary Materials: The following are available online at www.mdpi.com/article/10.3390/agronomy11102020/s1: Figure S1: Concentration of the main aroma compounds of sweet cherry cv. Regina from orchard 1 at phenological stages 2 to 6 in 2019/2020 season, Figure S2: Harvest date and accumulated degree days base 10°C at each phenological stage for the six cultivation zones in the study, Table S1: Concentration of the tentatively identified volatile compounds by orchard and phenological stages, Table S2: Volatiles, OAVs and climatic data used for PCAs and stepwise regressions.

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