Article

Can Spectrophotometry Be Used to Quantify Zingiberene Sesquiterpenoids in Tomato Leaflet Extracts?

Mohammad Dawood[1] and John Snyder [2],*

[1] University of Kufa, College of Agriculture, Department of Soil Science and Water Resources, El-Najaf 54003, Iraq; mohammadh.dawood@uokufa.edu.iq

[2] University of Kentucky, Department of Horticulture, Lexington, KY 40503; snyder@uky.edu

* Correspondence: snyder@uky.edu; Tel.: 1-859-257-5635

Abstract: Tomato (*Solanum lycopersicum*), an important vegetable crop, is a host for numerous pests and pathogens. Consequently, tomato breeders are striving to improve pest resistance. Many accessions of the wild relative *S. habrochaites* display high resistance towards arthropod pests such as spider mites and whiteflies and the presence of the sesquiterpene hydrocarbon 7-epi zingiberene in its trichomes has been associated with arthropod resistance. Consequently, tomato breeders are attempting to introgress 7-epi zingiberene from wild to cultivated tomato, which requires the ability to quantify zingiberene. 7-Epi zingiberene has conjugated double bonds causing it to absorb uv light. In fact, de Freitas, *et al.* [1] have claimed that measurement of absorbance at 270 nm of tomato leaflet washes can be used to quantify zingiberene. However, this claim has never been critically evaluated. We initially evaluated the use of uv absorbance to estimate zingiberene content of leaflet washes from an interspecific hybrid tomato BC4F2 generation that was segregating widely for presence and abundance of zingiberene. When absorbances at 270 nm were compared with zingiberene measured by gas chromatography the resulting relationship did not obey the Beer-Lambert law. Consequently, we examined in detail aspects of the uv-absorbance of zingiberenoids by: 1) obtaining uv absorbance spectra for three isolated zingiberenoids and determining the relationship between the abundance of each isolated compound and absorbance at single wavelengths; 2) obtaining uv absorbance spectra of leaflet washes of relevant wild accessions and parents; 3) obtaining average absorbance spectra for interspecific generations of tomato that were segregating for abundance of zingiberenoids and determining their λmax values in the 250-280 nm region; 4) evaluating by regression the relationship between absorbance at a single wavelength and the concentration of zingiberene and when present, its derivatives, as measured by gas chromatography in several interspecific hybrid generations; and 5) exploring the potential use of absorbances at multiple wavelengths to improve estimates of concentrations of zingiberenoids. Results indicated that isolated zingiberenoids as well as leaflet washes of wild tomatoes that contain zingiberenoids have broad absorbance spectra in the uv region with a λmax of 264 nm. The relationship between abundance of isolated zingiberenoids and absorbance at a single wavelength of 264 nm did obey the Beer-Lambert Law. Average absorbance spectra for leaflet washes from BC3F5 generation plants sampled by two methods, as well as from a BC5F1 and a BC3F3 generation showed subtle shifts in the λmax away from 264 nm. Furthermore, the relationship between absorbance at 264 nm and zingiberenoid content of these generations did not obey the Beer-Lambert Law in that in each case regression of absorbance on zingiberenoid content of the leaflet washes resulted in non-zero intercept estimates that varied considerably among generations and sampling methods. Use of multiple wavelengths for estimation zingiberenoids in these breeding generations was explored and inclusion of additional absorbances at one or two wavelengths identified by stepwise regression always improved estimates of zingiberenoid content from absorbance data. However, the identified wavelength(s) differed among generations. Taken together the results indicate that measurement of absorbance of tomato leaflet washes at a single wavelength is not a reliable quantitative estimate of zingiberenoids in the leaflet wash. These estimates can be improved by utilizing absorbance at multiple wavelengths, but the particular wavelengths will likely vary among generations. Lastly, measurement of absorbance may be a useful tool in identifying those relatively rare individuals in a generation that is widely segregating for zingiberenoid content. However, even in this situation the determination

of the actual zingiberene content e.g., for comparison with the donor parent, would need to be back-stopped by a valid quantitative method such as gas chromatography.

1. Introduction

Tomato (*S. lycopersicum*) is a host for numerous pests and pathogens [2]. Tomato breeders have focused on increasing fruit quantity and quality with little focus on enhancing resistance to arthropods [3]. As a result, tomato plants are vulnerable to pest attacks throughout the crop cycle; thus, it is necessary to find a way to avoid damage that can reduce yield and quality. Currently, chemical sprays are the predominant method used for pest control. The use of chemical pest control can, however, cause serious damage and deleterious effects on growers, the health of consumers and can increase production costs [4-6]. The future use of arthropod resistance derived from wild relatives of cultivated tomato is expected to be a more sustainable control method of arthropod pests such as spider mites, aphids, and whiteflies [7,8].

Several classes of allelochemicals are found in glandular trichomes on the leaflet surface of wild tomatoes [9,10] and may be associated with arthropod resistance. One class is acylsugars initially found in *S. pennellii* [11,12]; another class is comprised of methyl ketones such as 2-tridecanone found in *S. habrochaites* f. *glabratum* [13-17]; and the third class is the semi-volatile mono- and sesquiterpenes, [18-21]. All classes of allelochemicals have been associated with arthropod resistance, but the focus of this research is on the terpenoid class.

According to Besser, *et al.* [22], the *S. habrochaites* species contains 8-fold more terpenoids than the cultivated *S. lycopersicum* species. Zingiberene is an example of the sesquiterpenoid class of allelochemical. It is commonly present is wild accessions of tomato, mainly *S. habrochaites* [23]. Many researchers have associated the resistance of certain accessions of *S. habrochaites* with the presence of zingiberene [10,21,24-35]. Because of the widely reported anti-insect activity of zingiberene, there is great interest in the introgression of the presence of the compound from wild to cultivated tomato. However, the breeding activity of introgression requires a method of selection for the presence and abundance of this allelochemical. A selection method, especially one that can rapidly quantify allelochemicals responsible for deterring pests would be a valuable tool for aiding the development of new arthropod-resistant cultivars.

Zingiberene in wild tomato was first identified in 1985 [36]. Zingiberene, specifically 7-epi zingiberene (Figure 1), produced by wild tomato is a diastereoisomer of that present in ginger where it was first identified [37,38]. In the wild tomato it is produced in glandular trichomes by the action of sesquiterpene synthase on 2z, 6z-farnesyl pyrophosphate [23]. 7-Epi zingiberene is a naturally occurring allelochemical that belongs to the monocyclic sesquiterpene group. It has the chemical formula $C_{15}H_{24}$ (Figure 1) having three double bonds and the two in the ring are conjugated.
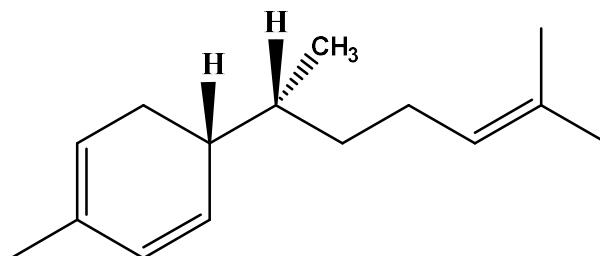
Figure 1. Structure of 7-epi zingiberene ($C_{15}H_{24}$) showing the conjugated double bonds in the ring.

There are several methods for quantifying 7-epi zingiberene. One option is gas chromatography [10,39-42]. However, this method is relatively expensive, somewhat slow and may not be accessible or affordable in plant breeding programs. Because of the presence of conjugated double bonds in 7-epi zingiberene, it may be possible to quantify it by spectrophotometry in a fashion similar to that used for β-phellandrene [43]. If so, spectrophotometric quantification of 7-epi zingiberene could be a convenient approach that might be used by plant breeders to transfer this trait to cultivated tomato, leading to tomato cultivars that are resistant to arthropods [44,45].

The idea of using spectrophotometry to assess zingiberene content of tomato leaves was initially addressed by de Freitas, Maluf, das Graças Cardoso and Benities [1] in 2000. Utilizing plants of two varieties of tomato, plants of *S. habrochaites* accession PI127826, and interspecific F1 hybrids they reported a correlation of 0.85 between absorbance at 270 nm and the quantity of zingiberene in leaves of the plants, as assessed by HPLC. Based on this correlation, the authors concluded that measurement of absorbance of leaflet washes at 270 nm can " *seja adotada na quantificação do zingibereno em plantas de tomateiro*," which translates to "be adopted in the quantification of zingiberene in plants of tomato,".

The well-known Beer-Lambert law [46] provides the basis for quantitation of compounds such as zingiberene based on their absorbance of light i.e. spectrophotometry. This fundamental law is expressed in equation form as $A = abc$, where A is the measured absorbance, a is the absorptivity of the compound, b is the path length, and c is the concentration of the compound. For a given wavelength and compound, a is constant, and generally b is held constant, often 1.0 cm. So, at a given wavelength and path length, the equation reduces to $A = c$, in which A is directly proportional to c, the concentration of the compound and when c is equal to zero, A, the absorbance must also be equal to zero. In the above equation if a, the absorptivity of the compound is known, then c, the concentration can be calculated directly. However, it is common to generate a standard curve in which absorbance is regressed on the quantity of the compound. In order to obey the Beer-Lambert Law, the resulting regression should be linear having good fit (R^2 value) and have a predicted intercept of zero indicating that a sample having an absorbance of zero would have a compound concentration of zero. A non-zero intercept could indicate the presence of compounds other than the target compound contributing to absorbance. If all samples have the same concentration(s) of non-target compound(s) that contribute to absorbance, some form of background subtraction can be performed, resulting in a standard curve that begins at the origin (has an intercept of zero). When the concentration(s) of non-target compounds vary among samples, the resulting regression will have a predicted intercept greater than zero and likely, will have reduced fit.

The spectrophotometric method developed by de Freitas, Maluf, das Graças Cardoso and Benities [1] was underpinned only by the estimation of fit, ($R^2=0.78$) and provided no estimates of slope or intercept. Thus, they did not provide evidence that their proposed method obeys the Beer-Lambert law. Their method has been cited at least eight times [3,31,33,45,47-51], and all of these publications seem to make the assumption that all absorbance observed at 270 nm was attributable to zingiberene, a valid assumption only if the relationship between absorbance and zingiberene concentration passes through the origin i.e. has an intercept of zero. In none of these subsequent publications was zingiberene content verified by an independent, quantitative method. Furthermore, the nature of the relationship between measured zingiberene content and absorbance for an interspecific hybrid generation of tomato that is segregating for presence and/or abundance of zingiberene has never been reported.

The presence of oxidized forms of 7-epi zingiberene in trichome secretions could be a source of interference with detection and quantitation of 7-epi zingiberene by spectrophotometry. Oxidized sesquiterpenoids, mainly carboxy acids of sesquiterpenes have been documented in *S. habrochaites*, often as predominate components of trichome

secretions [52,53]. The wild tomato accession *S. habrochaites* LA2329, the donor parent for our breeding population, has three major allelochemical components, 7-epi zingiberene, 9-hydroxy zingiberene and 9-hydroxy-10,11-epoxy zingiberene in its trichome secretions. The latter two compounds are oxidized derivatives of 7-epi zingiberene. Each of these compounds contains conjugated double bonds in their chemical structures and there are indications that oxidized forms may have greater arthropod repellency compared to the unoxidized form [21]. Because 7-epi zingiberene absorbs light in the uv region, it is likely these oxidized sesquiterpenoids will also absorb uv light and may interfere with quantitation of 7-epi zingiberene by uv absorbance.

For approximately ten years we have been pursuing the introgression of 7-epi zingiberene and its oxidized forms from the wild accession of *S. habrochaites* LA2329 into cultivated tomato. With regard to 7-epi zingiberene, this effort has been successful, and lines that breed true for the presence and abundance of zingiberene are in the process of public release [54]. In our introgression effort quantitation of zingiberene and its derivatives was accomplished by gas chromatography. As a result of the introgression effort, we had the opportunity to compare the spectrophotometric method proposed by de Freitas, Maluf, das Graças Cardoso and Benities [1] with our method based on gas chromatography. Our initial objective for the research reported herein was to conduct this comparison on an interspecific hybrid BC4F2 generation that was widely segregating for presence and abundance of zingiberene. Because results of this effort revealed that the method proposed by de Freitas, Maluf, das Graças Cardoso and Benities [1] did not obey the Beer-Lambert Law, we subsequently evaluated aspects of the uv-absorbance of zingiberenoids by: 1) obtaining uv absorbance spectra for isolated 7-epi zingiberene, and its derivatives, 9-hydroxy zingiberene, and 9-hydroxy, 10,11-epoxy zingiberene and determining the relationship between the abundance of each of these isolated compounds and absorbance at single wavelengths; 2) obtaining uv absorbance spectra of leaflet washes of wild accessions and parents relevant to our breeding population and determining their λmax values in the 250-280 nm region; 3) obtaining average absorbance spectra for interspecific generations of tomato that were segregating for abundance of zingiberenoids and determining their λmax values in the 250-280 nm region; 4) evaluating by regression the relationship between absorbance at a single wavelength and the concentration of zingiberene and when present, its derivatives, as measured by gas chromatography in several interspecific hybrid generations from our breeding population; and 5) exploring the potential use of absorbances at multiple wavelengths to improve estimates of concentrations of zingiberene and its derivatives.

2. Materials and Methods

This experiment was conducted at the Horticulture Department, University of Kentucky and at the Horticulture Research Farm, Lexington, KY. Plants of *S. habrochaites* used in this research included representatives of the accessions LA1777, LA2329, and LA2167, all obtained from the Tomato Genetics Resource Center, Davis CA. The *S. habrochaites* accession PI127826 was originally obtained from the USDA - ARS, Plant Genetic Resources Unit Geneva, NY. Two chemotypes of LA2329, LA2329-A and LA2329-B, were used in this research and their development and characterization are described in Dawood and Snyder [21]. The majority of the research reported herein relied on interspecific hybrid populations (BC3F5, BC4F2 and BC5F1) that resulted from initial crosses between *S. habrochaites* LA2329 and *S. lycopersicum* Zaofen 2, which was also the recurrent parent used in the backcross scheme. Zaofen 2 is a determinate, early, soft-fruited tomato cultivar released in the 1960's. The breeding scheme used was a modified backcross design [55]. The other generation used, a BC2F3 generation. was a complex hybrid between Zaofen 2, LA2329 and LA1777.

The plants sampled for the BC4F2 were field grown. For this generation, seeds were germinated on moist filter paper in an incubator (27°C). After radicle emergence, seeds were planted in 72-cell trays containing ProMix BX. Six weeks later, seedlings were

transplanted into the field at the Horticulture Research Farm, Lexington, KY. Cultural methods for transplant and field production followed those recommended in ID-36 [56].

Other than the BC4F2 generation, all other plants were produced in a greenhouse. Plants were grown in pots filled with ProMix BX (Premier Tech Horticulture, Quakertown, PA, USA) and auto-fertigated every day using a fertilizer solution containing Peter's Professional 5-11-26 (ICL SF USA & Canada, Dublin, OH) plus $CaNO_3$ (Viking Yara, Tampa, FL) to provide 180 ppm of nitrogen. After seedling establishment, plants were maintained by cuttings when needed. Plants were grown under normal daylength conditions, during spring, summer, and fall. Throughout this time, the average greenhouse temperature and relative humidity were 24 ± 2°C and 67% RH, respectively.

All of the analytical procedures relied on hexane leaflet washes. These were obtained by steeping leaflets or pieces of leaflets in a known amount of hexane for five minutes. When needed the area of the steeped tissue was determined after extraction by use of digital scanner and image analysis of digitized images with ImageJ [57]. Volume of hexane used, amount of tissue and the manner in which leaflet tissue was collected varied somewhat among generations and those details are provided below in the relevant methods paragraphs for each generation.

Gas chromatography with flame ionization detection (GC-FID) was used to quantify compounds of interest in this research. The column was an RTX-5 column -- 5% diphenyl 95% dimethyl polysiloxane, 15 m, 0.53-mm ID, 0.5 μm (Restek Corporation, Bellefonte, PA, USA). Temperatures were as follows: injector 250°C, detector 300°C, oven initial temperature 50°C for 1 min, then increasing at 20°C/min to 260°C. The gas chromatograph used was a Hewlett Packard 5890 Series II. To allow quantitation of compounds detected by GC-FID, a standard curve using tetradecane was constructed over the range of 0 to 100 ppm (0 to 100 ng/μL). Tetradecane was also used as an internal and external standard. Ginger oil was used as a source of α-zingiberene and was used as an external standard for retention time on the gas chromatograph.

For spectrophotometry, a Thermo Scientific Evolution 60S UV-Visible scanning spectrophotometer was employed. Absorbances of the leaflet washes from the BC4F2 population were determined at a single wavelength 270 nm; all other leaflet washes and samples were scanned from 200 to 500 nm wavelengths at a scanning rate of 1 nm·sec^{-1}. Results of each scan was stored electronically, with an absorbance value for each wavelength from 200 to 350 nm. In all cases the scans in the region between 350 and 500 nm had no absorbance relative to the blank, so only absorbance values in the 200 to 350 nm range were used.

To initially evaluate the use of the spectrophotometer for quantitation of 7-epi zingiberene a widely segregating population, an interspecific BC4F2 population, was evaluated. Leaflet washes from the entire population had previously been evaluated by GC-FID to determine concentrations of 7-epi zingiberene; oxidized forms of zingiberene were absent. We chose 38 plants from the population that represented a wide range of zingiberene concentrations. Leaflet washes were obtained by taking the middle 1/3 to 1/4 of each of three leaflets from the third or fourth leaf position of each plant, approximately ~10 cm² foliage, and placing the leaflet tissue in 4.0 ml of hexane. After vortexing and removing the leaflet tissue, absorbances of the samples at 270 nm were determined spectrophotometrically and concentrations of 7-epi zingiberene were determined by GC-FID.

7-epi zingiberene, 9-hydroxy zingiberene and 9-hydroxy-10,11-epoxy zingiberene from the leaflet wash of LA2329-A were isolated by open column chromatography on silica gel as described by Dawood and Snyder [21]. Purity and quantity of the isolated compound of interest in each fraction was determined by GC-FID and then each isolated fraction was spectrophotometrically scanned. There were 15 fractions of 7-epi-zingberene, 14 fractions of 9-hydroxy zingiberene and 10 fractions of 9-hydroxy-10,11-epoxy zingiberene.

Leaflet washes from the donor and recurrent parents, as well as from the several accessions of *S. habrochaites* were used for qualitative characterization by spectrophotometry. Leaflet washes were prepared by steeping leaflets in hexane (~2 ml per leaflet) for a few minutes, vortexing, removing leaflets, and then scanning the leaflet washes with the

spectrophotometer. Samples were diluted as needed with hexane, to provide clarity in data presentation.

To evaluate the BC3F5 generation two sampling procedures (A and B) were used. This was done to explore the accuracy of the spectrophotometric analysis for zingiberene determination, especially with regard to ratio of leaflet tissue to *hexane* in the leaflet wash and age of leaflet tissue. The same twenty-five genotypes of the BC3F5 interspecific hybrid population were used for each procedure. In procedure A, three small, young and intact leaflets were taken (average leaflet area = 28.0 cm^2, range= 21.7– 37.3 cm^2), and the leaflets of each genotype were placed in a 20 mL disposable scintillation vial containing 4 mL of hexane. With procedure B the middle parts (center 1/3 to 1/4 of the leaflet) from each of three leaflets (average = 14.4 cm^2, range= 9.5 – 20.5.3 cm^2) were taken and placed in a 20 mL disposable scintillation vial containing 4 mL of hexane. For both procedures, vials were vortexed, and then all samples were scanned with the spectrophotometer and 7-epi zingiberene concentrations were determined by GC-FID.

The BC5F1 interspecific hybrid population had low 7-epi zingiberene concentrations and produced no oxidized forms of zingiberene as determined by GC-FID. Ten cm^2 of foliage from the third and fourth leaf position of each of 10 plants was taken in duplicate (10 plants × 2 replications) by use of a leaf punch. Leaflet washes were prepared in the usual fashion with hexane and then evaluated by spectrophotometer and by GC-FID.

The BC2F3 generation was chosen because it was segregating for the presence and abundance of 7-epi zingiberene, and its two oxidized forms. Based on GC-FID analysis, 31 plants were chosen for this phase of the research. Twenty-eight of the plants were producing zingiberene and its oxidized forms in their trichome secretions and three plants were producing zingiberene only. Leaflet tissue was obtained in a fashion identical to that used for the BC4F2. Leaflet tissue (average 14.5 ± 0.44, range 9.1 – 29.4 cm) was placed into 20 ml scintillation vials, 5 ml of hexane was added and then samples were vortexed. Subsequently, because of high absorbance values of undiluted samples, the samples were diluted 1:5 with hexane prior to spectrophotometric and GC-FID analysis. All samples were duplicated.

Statistical analyses were performed via the SAS 9.4 statistics package (SAS Institute Inc., 2016) and Excel (Microsoft 365, 2019). Average spectra were obtained by determining the mean absorbance at each wavelength from 200 to 350 nm for each generation or in the case of the BC3F5, for each sampling procedure.   The wavelength for the maximum absorbance value in the 250-280 nm region was identified for each genotype and replication by use of the Means Procedure in SAS. Once the wavelength of maximum absorbance was identified for each sample, the mean, maximum, minimum, and standard error values of the identified wavelengths for each generation evaluated were then calculated in the usual fashion.

Simple regression analysis (SAS 9.4) was used to investigate the association between the absorbance at a single wavelength and the concentration of 7-epi zingiberene or other compounds of interest. In some cases, multiple wavelengths were evaluated as a means to improve predictions of zingiberene from uv absorbance values. This was accomplished by use of stepwise regression with forward selection in SAS 9.4 using the GC-FID area units for the target compound(s) as the dependent variable, and the absorbance recorded for each wavelength in the 200-350 nm region of the absorbance spectrum as independent variables. Only variables significant at $P \leq 0.01$ (SLE = 0.01) were allowed entry into the regression, thus minimizing the number independent variables in the models. After these significant wavelengths were identified, their absorbance values were used as independent variables to calculate a predicted value for compounds of interest.

3. Results

3.1. Regression of   absorbance at 270 nm on 7-epi zingiberene content in a BC4F2 generation

For the leaflet washes obtained from the 38 individual BC2F4 plants there was a strong association (R²=0.92) between 7-epi zingiberene content as measured by GC-FID

and absorbance at 270 nm (Figure 2). This is the first publication of the linear estimation of the relationship between absorbance at 270 nm and zingiberene content of leaflet washes for an interspecific hybrid tomato generation that was segregating for the abundance and presence of 7-epizingiberene. Regression analysis indicated that slope and interestingly, the intercept were highly significant (Table 1). Because the predicted relationship did not pass through the origin, we examined in more detail the absorbance values for samples that had no detectable zingiberene as determined by GC-FID, i.e., those samples that displayed scatter at the origin (Figure 2). For those samples that contained no zingiberene (n=19), absorbance at 270 nm ranged from 0.158 to 0.534 and averaged 0.367 absorbance units, so even in the absence of zingiberene, there was considerable absorbance detected at 270 nm in these 19 samples. Because the predicted relationship between absorbance at 270 and zingiberene content did not pass though the origin, the relationship does not obey the Beer-Lambert law. Consequently, we decided to examine more closely the relationship between absorbance of zingiberene and its derivatives in the uv region. This included: 1) obtaining uv absorbance spectra for isolated 7-epi zingiberene, and its derivatives, 9-hydroxy zingiberene, and 9-hydroxy, 10,11-epoxy zingiberene and determining the relationship between the abundance of each of these isolated compounds and absorbance at single wavelengths; 2) obtaining uv absorbance spectra of leaflet washes of wild accessions and parents relevant to our breeding population; 3) obtaining average absorbance spectra for interspecific generations of tomato that were segregating for abundance of zingiberene and determining their λmax values in the 250 – 280 nm region; 4) evaluating by regression the relationship between absorbance at a single wavelength and the concentration of zingiberene and when present, its derivatives, as measured by gas chromatography in several interspecific hybrid generations from our breeding population and; 5) exploring the potential use of absorbances at multiple wavelengths to improve estimates of concentrations of zingiberene and its derivatives.
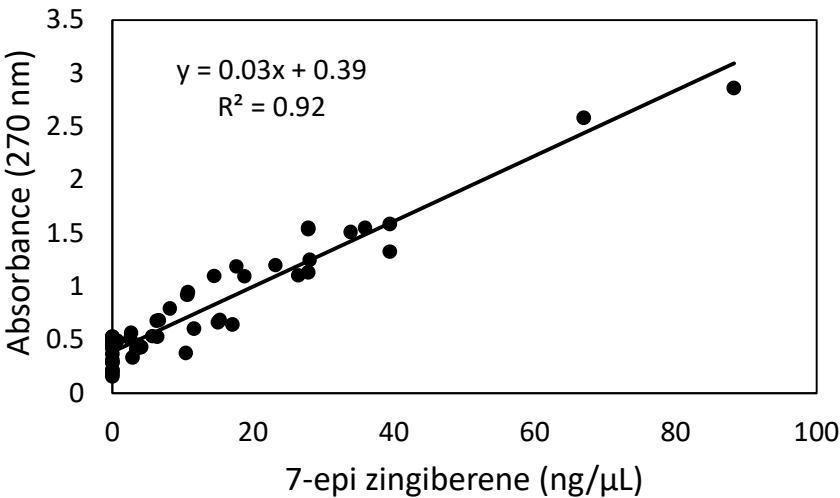

Figure 2. Association between absorbance at 270 nm and 7-epi zingiberene concentration (ng/µL) measured by GC-FID of hexane leaflet washes from BC4F2 interspecific hybrid plants, n=38.

Table 1. Estimates of intercept, slope and their respective standard errors and t-values for the regression of absorbance at 270 nm on 7-epizingiberene content measured by GC-FID for 38 BC2F2 interspecific hybrid tomato individuals.

Variable	Parameter estimate	Standard error	t-value
Intercept	0.387	0.026	14.98 **
Slope – 270 nm	0.031	0.001	25.09**

ns - not significant; ** - significant at $P < 0.01$

3.2. UV absorbance spectra of isolated tomato sesquiterpenoids

We successfully separated 7-epi-zingberene, 9-hydroxy zingiberene and 9-hydroxy-10,11-epoxy zingiberene by silica gel chromatography. The purity of the isolated zingiberene was high, about 88% as judged by GC-FID. Isolated 9-hydroxy zingiberene and 9-hydroxy-10,11-epoxy zingiberene were less pure, averaging about 49% and 43%, respectively and based on GC-FID, were each the predominate component of their respective isolated fractions. Importantly, these isolated fractions were not cross contaminated. Non-target compounds were numerous in the fractions that contained 9-hydroxy zingiberene or 9-hydroxy-10,11-epoxy zingiberene and individually the non-target compounds were less than 5% of the total detected by GC-FID in a fraction. Scans of isolated fractions containing 7-epi-zingberene, 9-hydroxy zingiberene or 9-hydroxy-10,11-epoxy zingiberene were identical qualitatively, sharing a broad absorbance peak with a λmax of 264 nm (Figure 3). The average λmax values for the isolated fractions of each compound was 264 ± 0.3.
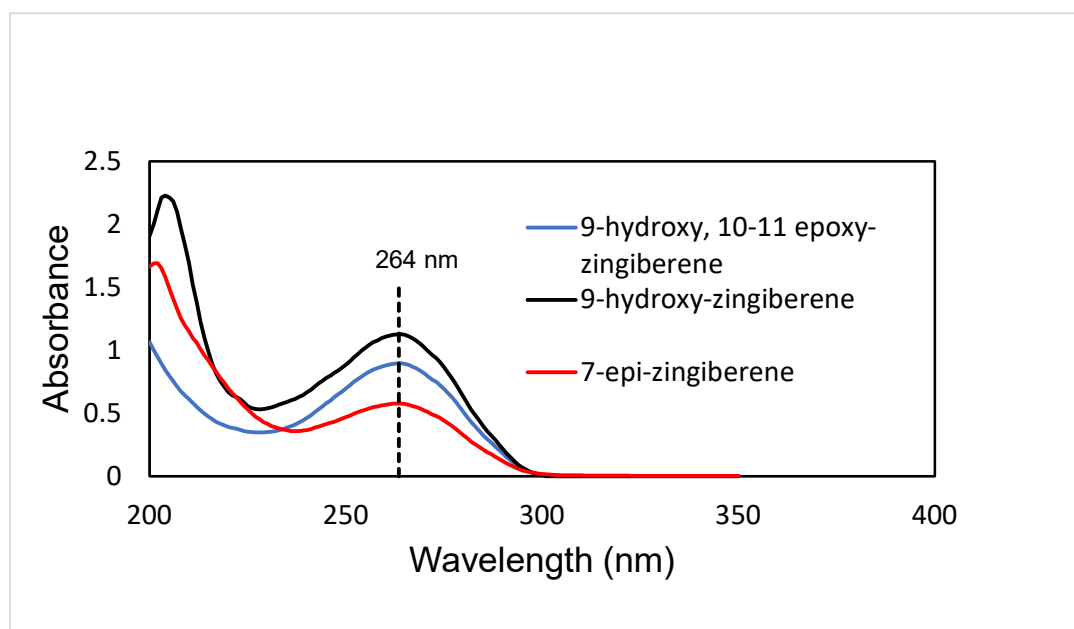

Figure 3. Example absorbance spectra for 7-epi zingiberene, 9-hydroxy zingiberene and 9-hydroxy-10,11-epoxy zingiberene isolated by silica gel chromatography. The vertical dashed line identifies 264 nm, the λmax in the 250-280 nm region for these compounds.

3.3. Spectrophotometric quantitation of isolated zingiberenoids

Regressions between absorbance at 264 nm, the λmax for these compounds and GC-FID detector response were generated for the isolated fractions of 7-epi zingiberene, 9-hydroxy zingiberene and 9-hydroxy-10,11-epoxy zingiberene obtained by silica gel chromatography (Figure 4). The resulting regression equations were similar, but not identical (Table 2). R^2 values were high (>0.98) for all three compounds. Slope for 9-hydroxy zingiberene was somewhat lower that those obtained for the other two compounds. For two of the compounds, 7-epi zingiberene and 9-hydroxy-10,11-epoxy zingiberene, intercepts did not differ significantly from 0, but the intercept for 9-hydroxy zingiberene was small but significantly greater than 0 (Table 2). Regression of absorbance at 270 nm on GC-FID detector response was also accomplished for the isolated fractions of each compound. Results were very similar to those obtained for regression of absorbance at 264 nm, except that the slopes were about 10% lower (Table 2).
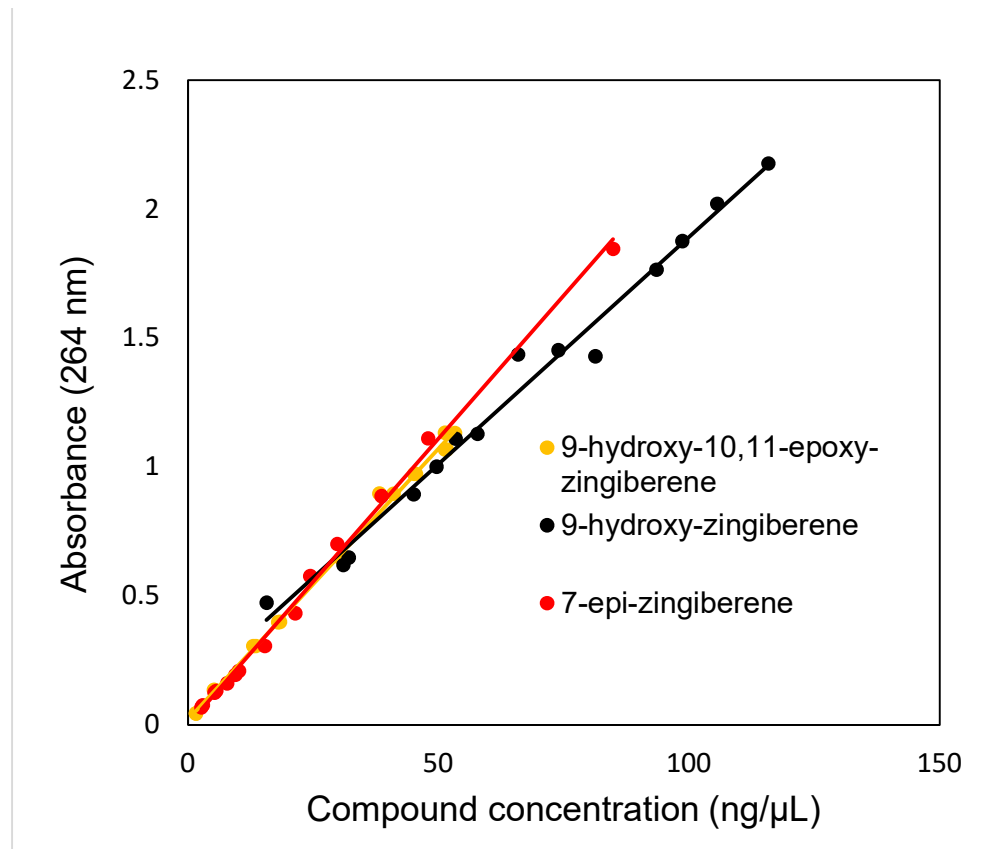
Figure 4. Linear relationships between absorbance measured at 264 nm and compound concentration (ng/µL) for fractions of 7-epi zingiberene and its two oxidized forms isolated by silica gel chromatography.

Table 2. Results of regression of absorbance at 264 nm and 270 nm on GC-FID detector response for fractions of 7-epi zingiberene, 9-hydroxy zingiberene and 9-hydroxy, 10,11 epoxy zingiberene isolated by silica gel chromatography.

Isolated compound	n	R^2	Intercept ± SE	t-value (intercept)	Slope ± SE	t-value (slope)
			264 nm			
7-epi zingiberene	14	0.997	0.000 ± 0.011	0.03^{ns}	0.022 ± 0.0003	60.06**
9-hydroxy zingiberene	14	0.986	0.130 ± 0.044	2.97*	0.018 ± 0.0006	28.98**
9-hydroxy-10,11-epoxy zingiberene	18	0.996	0.020 ± 0.011	1.83^{ns}	0.021 ± 0.0003	65.18**
			270 nm			
7-epi zingiberene	14	0.997	-0.000 ± 0.011	-0.02^{ns}	0.020 ± 0.0003	59.05**
9-hydroxy zingiberene	14	0.990	0.103 ± 0.034	3.02*	0.016 ± 0.0005	34.16**
9-hydroxy-10,11-epoxy zingiberene	18	0.995	-0.003 ± 0.011	-0.27^{ns}	0.020 ± 0.0003	58.44**

ns - not significant; * and ** significant at $P < 0.05$ and $P < 0.01$, respectively

3.4 Absorbance spectra of leaflet washes from accessions and parents

The absorbance spectra of hexane leaflet washes for the *S. habrochaites* accession that produces 7-epi zingiberene only -- LA2329-B and for those that produce 7-epi zingiberene, 9-hydroxy zingiberene and 9-hydroxy-10,11-epoxy zingiberene, LA2329-A, PI127826, and LA2167 [21] had similar broad absorbance peaks (λmax) at 264 ± 0 nm (Figure 5). Lines or accessions that do not produce these compounds, e.g., LA1777 and our recurrent parent Zaofen 2, did not have a λmax in this region of their spectra.
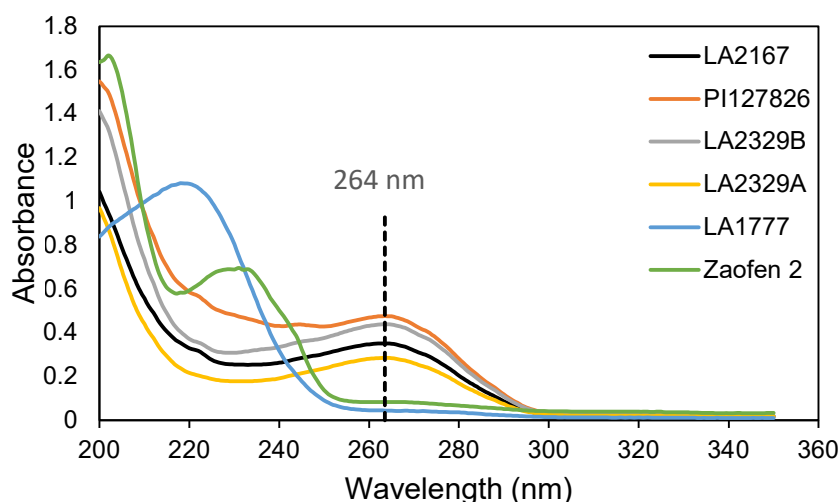

Figure 5. Examples of uv absorbance spectra for hexane leaflet washes from five accessions of *Solanum habrochaites* and one tomato (*S. lycopersicum*) variety, Zaofen 2. The vertical dashed line identifies 264 nm, the λmax in the 250-280 nm region of the spectra for accessions that produce 7-epi zingiberene and its oxidized forms.

3.5 Average absorbance spectra of leaflet washes from three interspecific hybrid generations segregating for zingiberene/zingiberenoids

3.5.1 BC3F5 generation

Average absorbance spectra in the 200-350 nm region for the A and B samples of the BC3F5 were very similar in shape (Figure 6). However, the average absorbance values were uniformly higher for the A samples, compared to the B samples. Furthermore, the λmax values in the 260 – 270 nm region were below 264 nm, the λmax for isolated zingiberene or the λmax observed for the leaflet washes obtained from wild tomato accessions that produce zingiberenoids (Table 3).
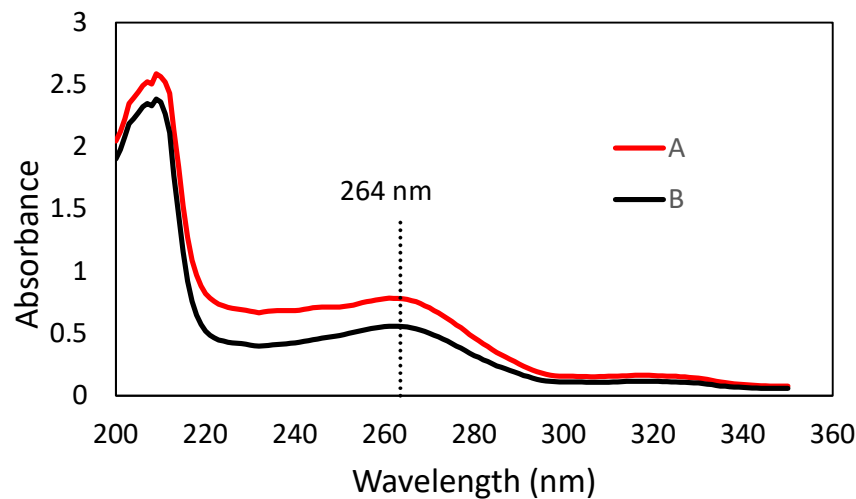
Figure 6. UV absorbance spectra for hexane leaflet washes of BC3F5 plants obtained by two sampling methods, A and B (see text). The vertical dashed line identifies 264 nm, the λmax for isolated 7-epi zingiberene in the 250-280 nm region.

Table 3. Average λmax values ± SE in the 250-280 nm region of their absorbance spectra for the BC3F5 generation sampled by two methods, A and B, and for the BC5F1 and BC3F5 generation.

Generation	λmax ± SE (nm)
BC3F5 - A samples	262 ± 0.4
BC3F5 - B samples	262 ± 0.3
BC5F1	263 ± 3.0
BC3F5	266 ± 0.2

3.5.2 BC5F1 generation

There was a subtle, broad, and variable peak with a λmax = 263±3.0 nm in the 250-280 nm region of the spectra obtained for the BC5F1 generation (Table 2, Figure 7).
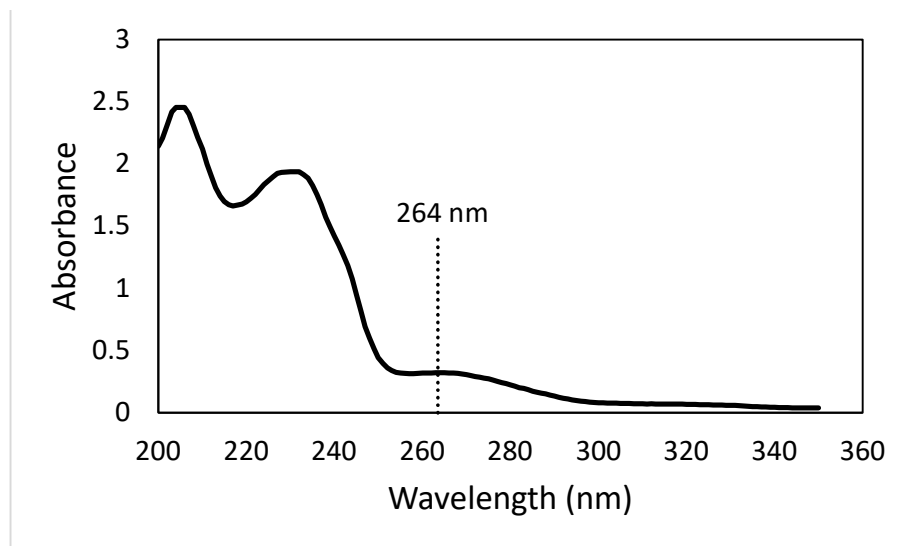
Figure 7. Average absorbance spectrum in the 200-350 nm region for the leaflet washes obtained from the BC5F1 generation. The vertical dashed line identifies 264 nm, the λmax for isolated 7-epi zingiberene.

3.5.3 BC2F3 generation

The average absorbance spectra obtained for this generation displayed a broad peak in the 250-280 nm region having a λmax of 266 ± 0.2 nm (Table 2, Figure 12).
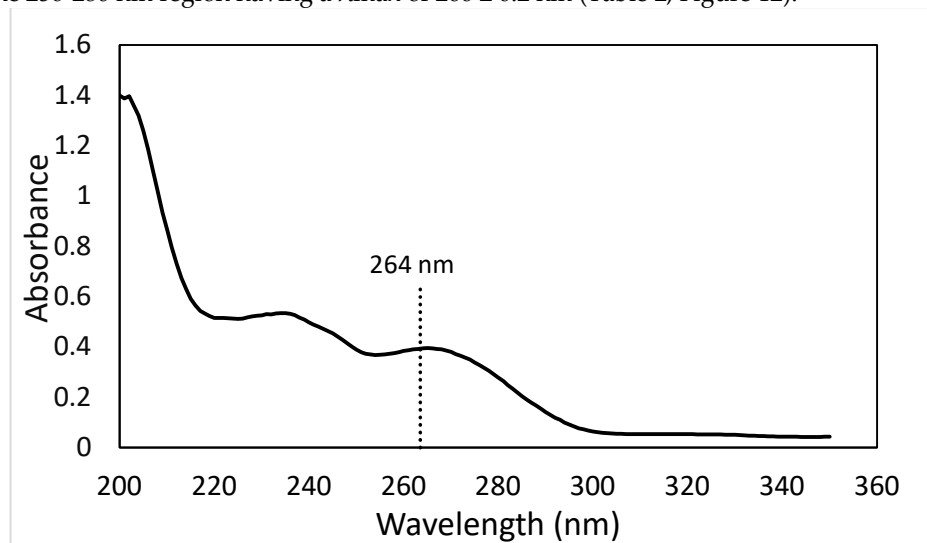

Figure 8. Average absorbance spectrum in the 200-350 nm region for leaflet washes obtained from the BC2F3 generation plants. The vertical dashed line identifies 264 nm, the λmax of isolated 7-epi zingiberene.

3.6 Simple regression of absorbance at 264 nm on zingiberene content

3.6.1 BC3F5 generation

When absorbance at 264 nm was regressed on 7-epi zingiberene content for the A and B samples there were apparent differences, mainly in the intercepts (Figure 9, Table 4). Compared to the regression for the B samples, the regression for the A samples had a considerably lower R^2 value, indicating greater scatter, and also had a higher intercept, two-fold greater.
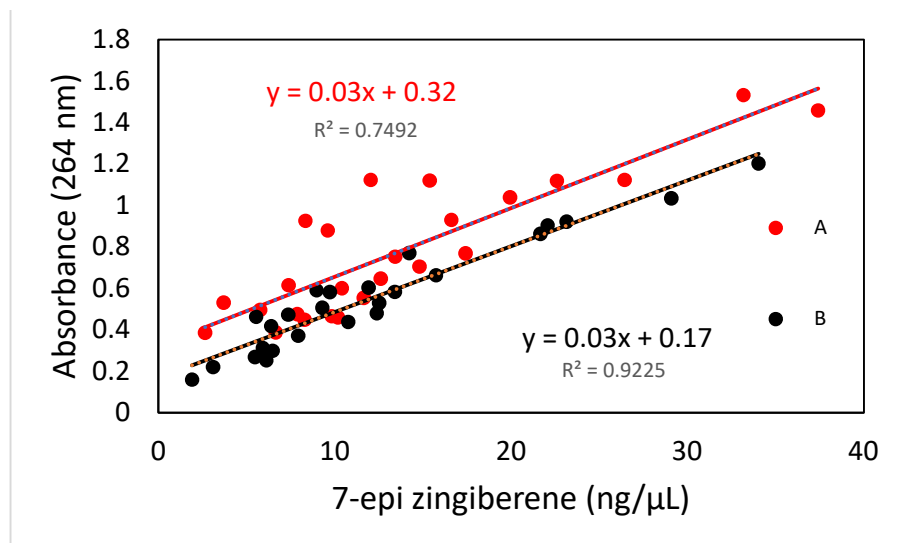
Figure 9. Relationship between absorbance at 264 nm and 7-epi zingiberene contents of BC3F5 plants sampled by two methods, A and B (see text).

Table 4. Estimates of intercepts, slopes, and their t- and R^2-values from regression of absorbance at 264 nm on 7-epizingiberene content leaflet washes, measured by GC-FID (ng/μL) for two sampling procedures of the BC3F5 generation and for the BC5F1 and BC3F3 generations of interspecific hybrid tomatoes. For the BC3F3 generation estimates are for the regression of absorbance on total zingiberenoid content (ng/μL of 7-epi zingiberene + hydroxy zingiberene + epoxy zingiberene) measured by GC-FID.

Generation	Sample	Intercept ± SE	t-value (intercept)	Slope ± SE	t-value (slope)	R^2
BC3F5	A	0.325 ± 0.065	5.03**	0.033 ± 0.0040	8.29**	0.75
	B	0.168 ±0.028	6.02**	0.032 ± 0.0019	16.55**	0.92
BC5F1	--	0.118 ± 0.031	3.8**	0.034 ± 0.0038	9.1**	0.82
BC3F3	--	0.270 ± 0.017	15.84**	0.025 ± 0.0029	8.73**	0.53

ns - not significant; ** - significant at $P < 0.01$

3.6.2 BC5F1 generation

For the regression of absorbance at 264 nm on zingiberene content of leaflet washes of the BC5F1 generation there was a significant linear relationship between the two variables and a non-zero intercept (Table 4). The degree of scatter was intermediate to those observed for the BC3F5 generation.
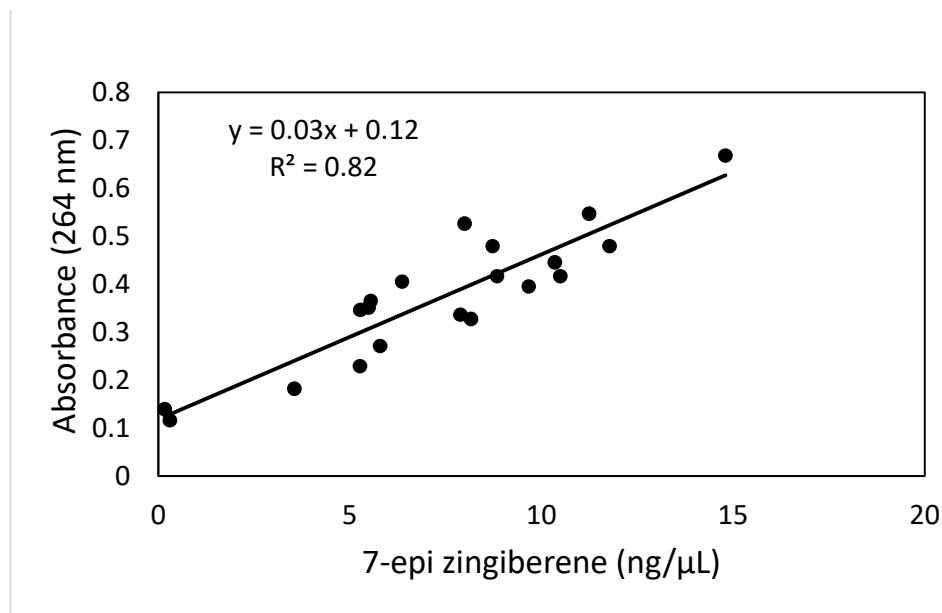
Figure 10. Relationship between absorbance at 264 nm and 7-epi zingiberene content (ng/μL) determined by GC-FID of leaflet washes obtained from BC5F1 genotypes.

3.6.2 BC3F3 generation

When absorbance at 264 nm was regressed on the quantity of 7-epi zingiberene determined by GC-FID, the R^2 value was unsurprisingly rather low, $R^2 = 0.30$, because this regression did not take into account the presence of derivatives of zingiberene, which based on our earlier results also have a λmax at 264 nm and were present in leaflet washes from most plants of this generation. When absorbance at 264 nm was regressed against the sum of zingiberene and its derivatives (zingiberenoids), the R^2 value improved from 0.31 to 0.53 (Figure 11). The predicted intercept was greater than 0 (Table 4). The relationship between the two variables displayed considerable scatter.
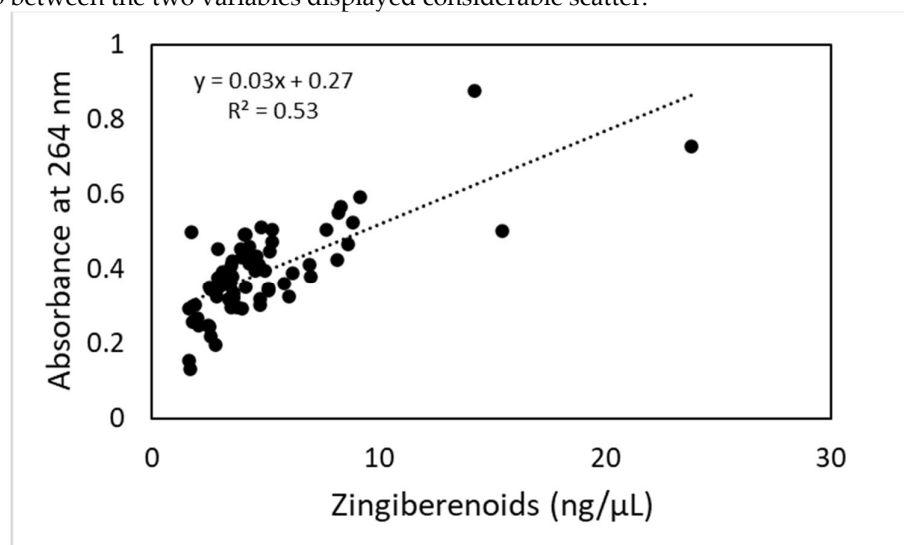

Figure 11. Relationship between absorbance at 264 nm and the zingiberenoid (7-epi zingiberene + 9-hydroxy zingiberene + 9-hydroxy, 10, 11-epoxy zingiberene) content (ng/μL) determined by GC-FID of leaflet washes obtained from BC3F3 genotypes.

3.7 Stepwise regression

For the BC3F5, BC5F1 and BC3F3 generation we used stepwise regression in an attempt to identify multiple wavelengths that could improve estimation of content of zingiberene and related compounds in these leaflet washes from plants of each generation. For the BC3F5 plants, absorbances at two wavelengths met the criterion for inclusion in the model, absorbance at 275 and 229 nm (Table 5). A similar approach using stepwise regression was taken with the BC5F1 generation (Table 6) which identified absorbances at two wavelengths, 265 and 324 nm, that met criteria for entry into the model. A similar approach was used for the BC3F3 generation, except that regression was based on zingiberenoid (7-epi zingiberene + 9-hydroxy zingiberene + 9-hydroxy, 10, 11-epoxy zingiberene) content, not just zingiberene. Absorbance at three wavelengths met the requirement for model entry, 262, 295 and 253 nm (Table 7).

Table 5. Results of stepwise regression between zingiberene content as determined by GC-FID and absorbance values from 200 to 350 nm for the BC3F5 generation.

Variable	Parameter estimate	Standard error	F-Value	Partial R^2	Model R^2
Intercept	0.99	0.59	2.81^{ns}		
275 nm	38.08	3.77	828.58**	0.79	0.79
229 nm	-35.04	2.24	244.62**	0.18	0.97

ns - not significant; ** - significant at P < 0.01

Table 6. Results of stepwise regression between zingiberene content as determined by GC-FID and absorbance values from 200 to 350 nm for the BC5F1 generation.

Variable	Parameter estimate	Standard error	F-Value	Partial R^2	Model R^2
Intercept	1.72	1.05	2.68^{ns}		
265 nm	38.08	3.77	102.03**	0.76	0.76
324 nm	-118.98	25.41	21.93**	0.16	0.92

ns - not significant; ** - significant at P < 0.01

Table 7. Results of stepwise regression between zingiberenoid content as determined by GC-FID and absorbance values from 200 to 350 nm for the BC2F3 plants.

Variable	Parameter estimate	Standard error	F-Value	Partial R^2	Model R^2
Intercept	0.34	0.68	0.25^{ns}		
262 nm	88.70	8.38	70.16**	0.52	0.52
295 nm	-77.91	9.55	57.29**	0.23	0.74
253 nm	-61.75	9.16	45.42**	0.11	0.85

ns - not significant; ** - significant at P < 0.01

4. Discussion

Our initial attempt to apply the method of de Freitas, Maluf, das Graças Cardoso and Benities [1] for quantitation of 7-epizingiberene was on a BC4F2 interspecific generation that was widely segregating for presence and abundance of zingiberene. Regression analysis of the resulting data confirmed the results of de Freitas, Maluf, das Graças Cardoso and Benities [1] that the concentration of zingiberene in leaflet washes of this generation was highly correlated with zingiberene content (R^2 = 0.92), but the regression results also refuted their claim that the method can be used to quantitate zingiberene in tomato plants

because the predicted regression equation had a non-zero intercept (Table 1) and thus does not obey the Beer-Lambert law. The most likely explanation for a non-zero intercept predicted by regression analysis is that there were compounds present in the leaflet washes that were not zingiberene but contributed to absorbance at 270 nm. Our subsequent examination of the absorbance of BC4F2 leaflet washes from individuals having no zingiberene detected by GC-FID provided information supporting this hypothesis. Absorbance of these leaflet washes ranged from 0.158 to 0.534 and was not equal to 0 for any of the individuals. Thus, the degree of interference due to non-target compounds also likely varied among individuals in this generation. Because of these results we decided to delve further into the use of spectroscopy as a tool for characterizing zingiberenoid content in tomato.

Because our initial results did not completely confirm the claims of de Freitas, Maluf, das Graças Cardoso and Benites [1], with regard to the ability to quantitate 7-epi zingiberene in tomato leaflet washes we decided to look at the relationship between uv absorbance and zingiberene concentration in more detail. This included: 1) verifying the λmax of isolated zingiberene and its derivatives; 2)` determining the relationships between absorbance at the identified λmax and concentrations of isolated zingiberenoids; 3) examining absorbance spectra of leaflet washes from parental lines, other wild accessions and three interspecific hybrid breeding generations to determine if spectral scanning could provide an indication of the presence of interfering compounds; 4) examining the relationship between absorbance at a single wavelength and zingiberenoid concentration in three generations of interspecific hybrid tomatoes; and 4) exploring the use of multiple regression, which might permit more accurate estimates of zingiberenoid content of leaflet washes by spectrophotometry.

It is clear from our results that isolated 7-epi zingiberene and its two derivatives have λmax values of 264 nm (Figure 3). Furthermore, their absorbance spectra were indistinguishable having very similar shapes. The absorbance peaks were not sharp, but rather broad. When absorbance at 264 nm was regressed on quantities of isolated zingiberene and 9-hydroxy-10,11-epoxy zingiberene, regression results predicted intercepts that were not different from 0 (Table 2). These results support the idea that when these compounds are sufficiently pure, the relationship between quantity and absorbance at 264 nm obeys the Beer-Lambert Law. The slopes estimated for the three compounds were very similar. However, the slope for 9-hydroxy zingiberene was somewhat less than those for the other two compounds, and the predicted intercept for this compound was greater than 0. It is possible that the presence of non-target compounds in the isolated fractions of the 9-hydroxy zingiberene were responsible for these differences. The similarity of slopes among the three compounds (Table 2) supports the hypothesis that the absorptivity or molar extinction coefficients for the three compounds are very similar, if not identical among the three compounds. However, additional research is needed along these lines. Lastly, when regression was conducted with absorbance at 270 nm, the wavelength suggested by de Freitas, Maluf, das Graças Cardoso and Benites [1], on quantities of isolated compounds, and results were compared to regression based on absorbance at 264 nm, the slopes were somewhat reduced (Table 2). Given the shape of the absorbance spectrum for zingiberenoids,   with its broad peak, these results were expected. Thus, when attempting to quantify 7-epi zingiberene and its derivatives, measurement of absorbance at 264 nm should provide the widest dynamic range compared to measuring absorbance at other wavelengths near the λmax of zingiberene.

Because the isolated compounds had similar absorbance spectra and λmax values of 264 nm, the presence of interfering compounds in leaflet washes might be suggested by shifts in observed λmax of leaflet washes that are expected to contain 7-epi zingiberene or its derivatives. Leaflet washes of wild tomato accessions that produce 7-epi zingiberene and its derivatives all demonstrated a λmax at 264 nm; the wild tomato accession LA1777 and the tomato cultivar Zaofen 2, neither of which produce 7-epi zingiberene or its derivatives had absorbance at 264 nm but lacked a λmax in this region of their spectra (Figure 5). Average absorbance spectra for the leaflet washes of our interspecific generations that

were spectrophotometrically scanned all demonstrated subtle shifts away from the 264 nm λmax associated with zingiberenoids (Table 3). This observation is consistent with the idea that compounds other than zingiberenoids were present in leaflet washes of plants of these three interspecific generations and were contributing to absorbance in this region of the spectra. It is likely that a shift of λmax away from 264 nm in spectral scans of tomato leaflet washes can be used as an indication of the presence of compounds that may interfere with quantitation of 7-epi zingiberene or its derivatives by measurement of absorbance at a single wavelength such as 264 nm.

Regressions between absorbance at 264 nm and zingiberenoid content in the three generations had similarities. Predicted intercepts were all greater than 0, and predicted slopes were all greater than those observed for isolated compounds (Table 4). The non-zero intercepts provide additional evidence that measurement of absorbance of these leaflet washes at 264 nm or another single wavelength does not obey the Beer-Lambert law for determination of zingiberenoid content. The predicted slopes from these regressions were greater than those obtained for isolated compounds also supporting the idea that non-target compounds contributed to absorbance at 264 nm in these leaflet washes. R^2 values ranged from 0.53 to 0.92 among generations indicating that the degree of interference by non-target compounds likely varied considerably among generations. Likewise, the extent of interference was different between the two sampling methods of the BC3F5 generation, which had distinct predicted intercepts and R^2 values, with the larger intercept and lower R^2 value associated with the A procedure compared to the B procedure.

Because all of our results pointed to the contribution of non-target compounds to absorbance at 264 nm in our hybrid generations, we explored the use of multiple regression to as a way to improve estimates of zingiberenoids by measurement of absorbance at multiple wavelengths. *A priori*, the expected result of multiple regression would be identification of a wavelength near the λmax of zingiberenoids -- 264 nm associated with a positive slope between absorbance and abundance, and identification of one or more wavelengths where absorbance is negatively associated with abundance, reflecting the interference associated with non-target compounds. In all cases examined stepwise regression provided highly satisfactory models, with intercepts that did not differ significantly from 0, and highly significant R^2 values ranging from 0.85 to 0.97 among the three generations evaluated (Tables 5-7). In each case adding absorbance at one additional wavelength considerably improved the regression. The first wavelength chosen for entry into these models always had a positive slope and in two of the three cases examined, the chosen wavelength was very near the 264 nm λmax for zingiberenoids. Negative slopes were consistently associated with additional   wavelengths entered into the models likely associated with abundance of non-target compounds. However, the second wavelengths chosen for model entry were very diverse, supporting the idea that the presence and/or abundance of the non-target compounds that contributed to absorbance at 264 nm also varied among the samples and generations.

The method as proposed by de Freitas, Maluf, das Graças Cardoso and Benities [1] holds great attraction for plant breeders mainly in its low cost, minimal equipment needed and speed of determination. However, we have clearly demonstrated their method does not obey the Beer-Lambert law, and thus is inadequate for quantitation of 7-epi zingiberene. That said, based on the general correlation of 7-epi zingiberene content of leaflet washes to single wavelength absorbance at a wavelength near the λmax of 7-epi zingiberene, i.e. the method of de Freitas, Maluf, das Graças Cardoso and Benities [1], it may be possible to identify those relatively rare individuals in a breeding generation that is widely segregating for presence and abundance of 7-epi zingiberene such as the BC4F2 examined in this work. The frequency of these rare individuals in this generation was ~1:16. However, even in this circumstance we assert that the actual level of 7-epi zingiberene in these segregating populations should be assessed by a quantitative method so that the zingiberenoid concentration in the chosen individuals can be compared, such as to the level present in the donor parent, or to the results of other breeding efforts. Only

with a quantitative method does breeder know whether the trait has been fully introgressed.

Accurate quantitation is also important in development of pure lines that produce known levels of 7-epi zingiberene for subsequent breeding. Accurate quantitation is also needed to identify genotypes for testing in bioassays with target pests, etc. For the latter, genotypes having high, intermediate, low and zero levels of zingiberenoids would be highly desirable and based on our results, would be difficult to reliably achieve with zingiberenoid levels estimated by single wavelength absorbance alone. Furthermore, since it appears that there are non-target compounds present in leaflet washes that contribute to absorbance in the 264 nm region, selection based solely on single wavelength absorbance could inadvertently result in increased concentration of these non-target compounds. Results of selection by single wavelength needs to be verified by a valid quantitative method.

Scanning of leaflet washes, especially with the ability to store the scan digitally, provides an additional avenue for the use of spectrophotometry as a component of selection for 7-epi zingiberene. From these spectra, shifts of λmax values away from 264 nm in spectra of tomato leaflet washes likely indicate the presence of non-target compounds that contribute to absorbance at this wavelength. Also, we have demonstrated that use of multiple wavelength uv-absorbance data and stepwise regression for identification of wavelengths that can be used to improve quantitation of 7-epi zingiberene by spectrophotometry. Unfortunately, it appears that the wavelengths are not at all uniform among generations that we evaluated, so even this approach needs to be backstopped by a reliable quantitative method such as GC-FID that would need to be utilized for each generation evaluated.

Use of measurement of absorbance can probably play a role in breeding for presence and abundance of 7-epi zingiberene in tomato. However, our evidence indicates that such use needs verification by a method that can accurately quantify 7-epi zingiberene and its derivatives. This could take place by validation of a few individuals selected by single wavelength absorbance, or by use of a training population for developing multiple wavelength model for each generation evaluated. Finally, it is abundantly clear from our results that attributing any absorbance detected at 270 or 264 in the manner of de Sena Fernandes, Fernandes, Silva, da Silva, Pinto and Ramos [45] should not be interpreted as indicative of the presence of zingberene.

References

1. de Freitas, J.A.; Maluf, W.R.; das Graças Cardoso, M.; Benities, F.R.G. Métodos para quantificação do zingibereno em tomateiro, visando à seleção indireta de plantas resistentes aos artrópodes-praga. *Acta Scientiarum. Agronomy* **2000**, *22*, 943-949.
2. Lange, W.H.; Bronson, L. Insect pests of tomatoes. *Annual Review of Entomology* **1981**, *26*, 345-371.
3. Zeist, A.R.; da Silva, A.A.; de Resende, J.T.V.; Maluf, W.R.; Gabriel, A.; Zanin, D.S.; Guerra, E.P. Tomato breeding for insect-pest resistance. In *Recent Advances in Tomato Breeding and Production*, IntechOpen: 2018; pp. 1-20.

4. Ansari, M.S.; Moraiet, M.A.; Ahmad, S. Insecticides: impact on the environment and human health. In *Environmental Deterioration and Human Health*, Springer: 2014; pp. 99-123.

5. Weaver, R.D.; Evans, D.J.; Luloff, A. Pesticide use in tomato production: Consumer concerns and willingness-to-pay. *Agribusiness* **1992**, *8*, 131-142.

6. Racke, K.D. Degradation of organophosphorus insecticides in environmental matrices. In *Organophosphates Chemistry, Fate, and Effects*, Chambers, J.E., Levi, P.E., Eds. Academic Press: San Diego, 1992; pp. 47-52.

7. Rick, C.M. Germplasm resources in the wild tomato species. *Acta Horticulturae* **1984**, *190*, 39-47.

8. Rick, C.; Chetelat, R. Utilization of related wild species for tomato improvement. *Acta Horticulturae* **1995**, *412*, 21-38.

9. Van Haren, R.; Steenhuis, M.; Sabelis, M.; De Ponti, O. Tomato stem trichomes and dispersal success of *Phytoseiulus persimilis* relative to its prey *Tetranychus urticae*. *Experimental & Applied Acarology* **1987**, *3*, 115-121.

10. Carter, C.D.; Gianfagna, T.J.; Sacalis, J.N. Sesquiterpenes in glandular trichomes of a wild tomato species and toxicity to the Colorado potato beetle. *Journal of Agricultural and Food Chemistry* **1989**, *37*, 1425-1428.

11. Liedl, B.E.; Lawson, D.M.; White, K.K.; Shapiro, J.A.; Cohen, D.E.; Carson, W.G.; Trumble, J.T.; Mutschler, M.A. Acylsugars of wild tomato *Lycopersicon pennellii* alters settling and reduces oviposition of *Bemisia argentifolii* (Homoptera: Aleyrodidae). *Journal of Economic Entomology* **1995**, *88*, 742-748.

12. Walters, D.S.; Steffens, J.C. Branched chain amino acid metabolism in the biosynthesis of *Lycopersicon pennellii* glucose esters. *Plant Physiology* **1990**, *93*, 1544-1551.

13. Farrar, R.; Kennedy, G. Inhibition of *Telenomus sphingis* an egg parasitoid of *Manduca* spp. by trichome/2-tridecanone-based host plant resistance in tomato. *Entomologia experimentalis et applicata* **1991**, *60*, 157-166.

14. Kauffman, W.; Kennedy, G. Toxicity of allelochemicals from wild insect-resistant tomato *Lycopersicon hirsutum* f. *glabratum* to *Campoletis sonorensis*, a parasitoid of *Heliothis zea*. *Journal of Chemical Ecology* **1989**, *15*, 2051-2060.

15. Gonçalves, M.; Maluf, W.; Gomes, L.; Barbosa, L. Variation of 2-tridecanone level in tomato plant leaflets and resistance to two mite species (*Tetranychus* sp.). *Euphytica* **1998**, *104*, 33-38.

16. Weston, P.; Johnson, D.; Burton, H.; Snyder, J. Trichome secretion composition, trichome densities, and spider mite resistance of ten accessions of *Lycopersicon hirsutum*. *Journal of the American Society for Horticultural Science* **1989**, *114*, 492-498.

17. Maluf, W.; Barbosa, L.; Santa-Cécília, L.C. 2-Tridecanone-mediated mechanisms of resistance to the South American tomato pinworm *Scrobipalpuloides absoluta* (Meyrick, 1917)(Lepidoptera-Gelechiidae) in *Lycopersicon* spp. *Euphytica* **1997**, *93*, 189-194.

18. Bleeker, P.M.; Mirabella, R.; Diergaarde, P.J.; VanDoorn, A.; Tissier, A.; Kant, M.R.; Prins, M.; de Vos, M.; Haring, M.A.; Schuurink, R.C. Improved herbivore resistance in cultivated tomato with the sesquiterpene biosynthetic pathway from a wild relative. *Proceedings of the National Academy of Sciences* **2012**, *109*, 20124-20129.

19. Kang, J.-H.; Liu, G.; Shi, F.; Jones, A.D.; Beaudry, R.M.; Howe, G.A. The tomato odorless-2 mutant is defective in trichome-based production of diverse specialized metabolites and broad-spectrum resistance to insect herbivores. *Plant Physiology* **2010**, *154*, 262-272.

20. Ekanayaka, E.P.; Li, C.; Jones, A.D. Sesquiterpenoid glycosides from glandular trichomes of the wild tomato relative *Solanum habrochaites*. *Phytochemistry* **2014**, *98*, 223-231.

21. Dawood, M.H.; Snyder, J.C. The alcohol and epoxy alcohol of zingiberene, produced in trichomes of wild tomato, are more repellent to spider mites than zingiberene. *Frontiers in Plant Science* **2020**, *11*, 35.

22. Besser, K.; Harper, A.; Welsby, N.; Schauvinhold, I.; Slocombe, S.; Li, Y.; Dixon, R.A.; Broun, P. Divergent regulation of terpenoid metabolism in the trichomes of wild and cultivated tomato species. *Plant Physiology* **2009**, *149*, 499-514.

23. Falara, V.; Akhtar, T.A.; Nguyen, T.T.; Spyropoulou, E.A.; Bleeker, P.M.; Schauvinhold, I.; Matsuba, Y.; Bonini, M.E.; Schilmiller, A.L.; Last, R.L. The tomato terpene synthase gene family. *Plant Physiology* **2011**, *157*, 770-789.

24. Bleeker, P.M.; Diergaarde, P.J.; Ament, K.; Guerra, J.; Weidner, M.; Schutz, S.; de Both, M.T.; Haring, M.A.; Schuurink, R.C. The role of specific tomato volatiles in tomato-whitefly interaction. *Plant Physiology* **2009**, *151*, 925-935.

25. Bleeker, P.M.; Diergaarde, P.J.; Ament, K.; Schütz, S.; Johne, B.; Dijkink, J.; Hiemstra, H.; de Gelder, R.; de Both, M.T.; Sabelis, M.W. Tomato-produced 7-epizingiberene and R-curcumene act as repellents to whiteflies. *Phytochemistry* **2011**, *72*, 68-73.

26. Carter, C.D.; Sacalis, J.N.; Gianfagna, T.J. Zingiberene and resistance to Colorado potato beetle in *Lycopersicon hirsutum* f. *hirsutum*. *Journal of Agricultural and Food Chemistry* **1989**, *37*, 206-210.

27. de Azevedo, S.M.; Faria, M.V.; Maluf, W.R.; de Oliveira, A.C.B.; de Freitas, J.A. Zingiberene-mediated resistance to the South American tomato pinworm derived from *Lycopersicon hirsutum* var. *hirsutum*. *Euphytica* **2003**, *134*, 347-351.

28. Eigenbrode, S.D.; Trumble, J.T.; Millar, J.G.; White, K.K. Topical toxicity of tomato sesquiterpenes to the beet armyworm and the role of these compounds in resistance derived from an accession of *Lycopersicon hirsutum* f. *typicum*. *Journal of Agricultural and Food Chemistry* **1994**, *42*, 807-810.

29. Freitas, J.A.; Maluf, W.R.; das Graças Cardoso, M.; Gomes, L.A.; Bearzotti, E. Inheritance of foliar zingiberene contents and their relationship to trichome densities and whitefly resistance in tomatoes. *Euphytica* **2002**, *127*, 275-287.

30. Júnior, J.; Resende, J.; da Silva, A.; Gabriel, A.; Zeist, A.; Favaro, R.; Nascimento, D.; Zeist, R.; Camargo, C. Resistance to *Helicoverpa armigera* mediated by zingiberene and glandular trichomes in tomatoes for industrial processing. *Genetics and Molecular Research* **2018**, *17*.

31. Lima, I.; Resende, J.; Oliveira, J.; Faria, M.; Resende, N.; Lima-Filho, R. Indirect selection of industrial tomato genotypes rich in zingiberene and resistant to *Tuta absoluta* Meyrick. *Genetics and Molecular Research* **2015**, *14*, 15081-15089.

32. Maluf, W.R.; de Fátima Silva, V.; das Graças Cardoso, M.; Gomes, L.A.A.; Neto, Á.C.G.; Maciel, G.M.; Nízio, D.A.C. Resistance to the South American tomato pinworm *Tuta absoluta* in high acylsugar and/or high zingiberene tomato genotypes. *Euphytica* **2010**, *176*, 113-123.

33. Oliveira, C.M.d.; Andrade Júnior, V.C.d.; Maluf, W.R.; Neiva, I.P.; Maciel, G.M. Resistance of tomato strains to the moth *Tuta absoluta* imparted by allelochemicals and trichome density. *Ciência e Agrotecnologia* **2012**, *36*, 45-52.

34. Oliveira, J.R.F.d.; Resende, J.T.V.d.; Roberto, S.R.; Silva, P.R.d.; Rech, C.; Nardi, C. Tomato breeding for sustainable crop systems: High levels of zingiberene providing resistance to multiple arthropods. *Horticulturae* **2020**, *6*, 34.

35. Zanin, D.S.; de Resende, J.T.V.; Zeist, A.R.; de Lima Filho, R.B.; Gabriel, A.; Diniz, F.C.P.; Perrud, A.C.; Morales, R.G.F. Selection of F 2 BC 1 tomato genotypes for processing containing high levels of zingiberene and resistant to tomato pinworms. *Phytoparasitica* **2021**, *49*, 265-274.

36. Lundgren, L.; Norelius, C.; Stenhagen, G. Leaf volatiles from some wild tomato species. *Nordic Journal of Botany* **1985**, *5*, 315-320.
37. Breeden, D.C.; Coates, R.M. 7-Epizingiberene, A novel bisabolane sesquiterpene from wild tomato leaves. *Tetrahedron* **1994**, *50*, 11123-11132.
38. Eschenmoser, A.; Schinz, H. Zur Kenntnis der Sesquiterpene und Azulene. 91. Mitteilung. Zur Konstitution des Zingiberens. *Helvetica Chimica Acta* **1950**, *33*, 171-177.
39. Lin, S.Y.; Trumble, J.T.; Kumamoto, J. Activity of volatile compounds in glandular trichomes of *Lycopersicon* species against two insect herbivores. *Journal of Chemical Ecology* **1987**, *13*, 837-850.
40. Chatzivasileiadis, E.A.; Boon, J.J.; Sabelis, M.W. Accumulation and turnover of 2-tridecanone in *Tetranychus urticae* and its consequences for resistance of wild and cultivated tomatoes. *Experimental and Applied Acarology* **1999**, *23*, 1011-1021, doi:10.1023/a:1006394109643.
41. Antonious, G.F.; Kochhar, T.S. Zingiberene and curcumene in wild tomato. *Journal of Environmental Science and Health, Part B* **2003**, *38*, 489-500.
42. Snyder, J.C.; Thacker, R.R.; Zhang, X. Genetic transfer of a twospotted spider mite (Acari: *Tetranychidae*) repellent in tomato hybrids. *Journal of Economic Entomology* **2005**, *98*, 1710-1716.
43. Formighieri, C.; Melis, A. Regulation of β-phellandrene synthase gene expression, recombinant protein accumulation, and monoterpene hydrocarbons production in Synechocystis transformants. *Planta* **2014**, *240*, 309-324.
44. de Freitas, J.; Maluf, W.; Cardoso, M.d.G.; de Oliveira, A. Selection of tomato plant resistant to arthropod pests mediated by zingiberene. *Acta Scientiarum* **2000**, *22*, 919-923.
45. de Sena Fernandes, M.E.; Fernandes, F.L.; Silva, N.O.; da Silva, D.J.H.; Pinto, F.G.; Ramos, R.S. Selection of tomato hybrids with zingiberene concentration for breeding programs to pest resistance. *Journal of Agricultural Science* **2014**, *6*, 148.
46. Swinehart, D.F. The Beer-Lambert law. *Journal of Chemical Education* **1962**, *39*, 333.
47. Fernandes, M.E.d.S. Capacidade combinatória entre fontes de resistência de *Solanum* sp. à *Tuta absoluta* (Meyrick)(Lepidoptera: Gelechiidae). Dissertation, Universidade Federal de Vicosa, Minas Gerais, Brazil, 2011.
48. Gonçalves, L.D.; Maluf, W.R.; Cardoso, M.d.G.; Resende, J.T.V.d.; Castro, E.M.d.; Santos, N.M.; Nascimento, I.R.d.; Faria, M.V. Relação entre zingibereno, tricomas foliares e repelência de tomateiros a *Tetranychus evansi*. *Pesquisa Agropecuária Brasileira* **2006**, *41*, 267-273, doi:10.1590/s0100-204x2006000200011.
49. Leite, G.L.D. Resistência de tomates a pragas. *Unimontes Científica* **2008**, *6*, 129-140.
50. Lima, I.P.; Resende, J.T.; Oliveira, J.R.; Faria, M.V.; Dias, D.M.; Resende, N.C. Selection of tomato genotypes for processing with high zingiberene content, resistant to pests. *Horticultura Brasileira* **2016**, *34*, 387-391.
51. Silva, A.A.d.; Andrade, M.C.; Maluf, W.R.; Moraes, J.C.; Rezende, J.F. Resistance of tomato plant genotypes with high foliar allelochemical contents to the leafminer *Liriomyza trifolii*. *Arquivos do Instituto Biológico* **2017**, *84*.
52. Coates, R.M.; Denissen, J.F.; Juvik, J.A.; Babka, B.A. Identification of α-santalenoic and endo β-bergamotenoic acids as moth oviposition stimulants from wild tomato leaves. *The Journal of Organic Chemistry* **1988**, *53*, 2186-2192.
53. Snyder, J.C.; Guo, Z.; Thacker, R.; Goodman, J.P.; Pyrek, J.S. 2,3-Dihydrofarnesoic acid, a unique terpene from trichomes of *Lycopersicon hirsutum*, repels spider mites. *Journal of Chemical Ecology* **1993**, *19*, 2981-2997, doi:10.1007/bf00980597.
54. Snyder, C.J.; Dawood, M.H.S.A.; Al-Bayati, A.S. Introgression of zingiberene and type IV trichome density from *Solanum habrochaites* LA2329 into *S. lycopersicum* – Progress Report Available online: http://tgc.ifas.ufl.edu/TBRT%202018/TBRT_Program_Book_Final.pdf (accessed on June 2).
55. Rick, C.M. Fruit and pedicel characters derived from Galápagos tomatoes. *Economic Botany* **1967**, *21*, 171-184.
56. Saha, S.; Bessin, R.; Strang, J. Vegetable Production Guide for Commercial Growers. Available online: http://www2.ca.uky.edu/agcomm/pubs/ID36/ID36.pdf (accessed on February 15. 2017).
57. Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **2012**, *9*, 671-675.