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RESEARCH ARTICLE

Chemotypical variation in *Vanilla planifolia* Jack. (Orchidaceae) from the Puebla-Veracruz Totonacapan region

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Abstract One of the threats in the diversity loss of the primary gene pool of *Vanilla planifolia* is the lack of information on existing level of polymorphism in cultivated germplasm, and the different expressions of this polymorphism. For this reason, it is proposed to study the chemical polymorphism of the four phytochemicals that define the vanilla aroma quality in fruits (vanillin, vanillic acid, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid) by HPLC analysis (High Performance Liquid Chromatography) of 25 collections of unknown genotype, grown in the region Totonacapan Puebla-Veracruz, Mexico. The results identified a selection process, domestication in fruit aroma of vanilla, during which increased the participation of vanillin and reduced the presence of three minor compounds (vanillic acid, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid) in the global aroma. We distinguished a total of six chemotypes of *V. planifolia* in the Totonacapan region, some chemotypes with wild aromatic characteristics (low participation of vanillin) related to the material less cultivated in the region and domesticated chemotypes with high participation of vanillin, for the most cultivated material. The results show that the diversification of the chemotypes of *V. planifolia* is not related to environmental variation. The data indicate that in the possible center of origin of vanilla, there is phytochemical polymorphism, which indirectly suggests the existence of genetic polymorphism, essential for the design of a breeding program for optimizing the use and conservation of diversity of the primary gene pool of *Vanilla planifolia*.

Keywords Chemotypical variation · Clone diversity · Primary gene pool · Vanilla aroma · *Vanilla Planifolia*

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Introduction

Vanilla planifolia G. Jack. (Orchidaceae) is one of the most important aromatic plants used in the food

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industry. It is an orchid native to the tropical forest of eastern Mexico (Soto 2003; Gonzalez-Arnao 2009, and as a genetic resource, it is one of the most important agro-biological legacies of the Mesoamerican cultures of the region (Lubinsky et al. 2008; Bory et al. 2007; Hágsater et al. 2005). Commercial production of vanilla in Mexico has been linked to the Totonaca people, who have maintained the germplasm in traditional systems of cultivation and production for at least 250 years (Hágsater et al. 2005; Bory et al. 2007). Outside of its center of origin, *V. planifolia* germplasm from the Totonacapan region, especially the "mansa"-type clone, served as the basis for establishing commercial plantations of the material denominated "Mexican" or "bourbon" vanilla, which today supplies 95% of the international demand (Ecott 2004; Bory et al. 2007; Lubinsky et al. 2008).

The aroma and flavor that characterize the vanilla beans are the result of a complex mixture of volatile compounds produced only in mature pods subjected to a curing process that lasts 3–6 months (Soto 2003; Sinha et al. 2008). During this period the aromatic constituents present in the fruits in their non-volatile conjugated forms hydrolyze through the action of the enzyme β-glucosidase and becomes volatile (Soto 2003; Ranadive 1992; Voisne et al. 1995). In *V. planifolia*, around 200 volatile compounds have been identified, including acids, ethers, alcohols, heterocyclics, esters, and phenolic and carbonylic compounds (Klimes and Lamparsky 1976; Sinha et al. 2008). Of these volatile compounds four phenols are recognized as indicators of commercial quality because of their high concentrations and important role in the aroma: (1) vanillin (4-hydroxy-3-methoxybenzaldehyde) in concentrations of 1,000–20,000 ppm, (2) *p*-hydroxybenzaldehyde (2,000 ppm), (3) vanillic acid (4-hydroxy-3-methoxybenzoic acid) (2,000 ppm) and (4) *p*-hydroxybenzoic acid (200 ppm) (Ranadive 1992; Wescott et al. 1994; Sostaric et al. 2000; Betazzi et al. 2006; Pérez-Silva et al. 2006; Sharma et al. 2006).

Commercially, two types of aromatic quality of *Vanilla planifolia* are sensorially recognized. The Mexican vanilla, produced mainly in the Totonacapan region of Mexico and bourbon vanilla, produced in the Reunión-Madagascar-Comoras triangle, although it has been shown that there are no genetically significant differences between the two germplasm sources. The variation in organoleptic quality could be due to the method of pod curing (Lubinsky et al. 2008; Bory et al. 2007). This has led to the idea of establishing, for commercial purposes, a single aroma of *V. planifolia* in Mexico with a characteristic pattern of concentrations of its four major aromatic compounds.

In a comparative study on the concentrations of vanillin, vanillic acid, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid in accessions of *V. planifolia* from different growing regions, which included material from Mexico, Ranadive (1992) observed that in controlled conditions of fruit maturity and curing process, certain accessions had variations in the concentration of vanillin and vanillic acid, while in the concentrations of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid the variation was not significant. He proposed that the geographic origin, fruit maturity and method of curing affected the concentration of vanillin and particularly that of vanillic acid, but not the contents of *p*-hydroxybenzaldehyde or *p*-hydroxybenzoic acid. The latter two could be determined by factors intrinsic to the species, such as polymorphic type genetic variations (chemical polymorphism; Gross et al. 2009). In aromatic and medicinal plants, it has been detected that within the same species or population, there are subpopulations with variations in the typical composition and concentration of the major secondary metabolites that determine their phytochemical quality (Lebot and Levesque 1996; Ruiz et al. 2007; Medina-Holgin et al. 2008). These subpopulations have been recognized as chemical polymorphisms, or chemotypes, which are defined as local phytochemical adaptations that are genetically controlled and related to the species' interaction with its habitat, although modifications in its morphology or physiology may be negligible (Gross et al. 2009).

In asexual or clonal crop plant populations, the chemotypes are more likely preserved and heritability is high over time due to vegetative reproduction is the fastest and most effective way for the domestication of a material through the selection of outstanding individuals to some trait of interest (Frankel et al. 1995). The chemical variations found in clonal crops may be chemical polymorphism when are influenced mainly by the parent genotype and chemical plasticity when are influenced by environmental sources (Lebot and Levesque 1996). In this sense, although it has been proposed that the production of secondary

metabolites in plants is related to plant-environment interaction systems (Lebot and Levesque 1996), in some aromatic and medicinal crop species, particularly those that reproduce by clones, it does not appear that the diversity of chemotypes within plant cultivars or varieties is the result of natural selection. Rather, it seems to be the product of a long process of human selection of outstanding individuals, in which farmer preferences have influenced the selection of utilitarian traits of the mother plant that originated the material (Lebot and Levesque 1996). Aroma has been an aspect highly valued by farmers for thousands of years and has functioned as a criterion for artificial selection, contributing to the generation of chemical variants (chemotypes) and cultivars of genetic resources such as rice, mango, kava and some spices (Fitzgerald et al. 2009; Sagar et al. 2009; Lebot and Levesque 1996).

Totonacapan region is considered the center of selection which originated the clone now being cultivated around the world (Ecott 2004; Bory et al. 2007; Lubinsky et al. 2008). Even today, the vanilla production in the region is based in traditional systems of selection of cuttings that responds to distinct cultural and sensorial appreciations of the vanilla resource (Baltazar 2010). Therefore it is possible that in the Totonacapan region exists chemical polymorphism in the aroma of the fuits of *V. planifolia*, and that it may be not related with environmental variations, but with a process of human selection which had modified the aroma of the fruits of *V. planifolia* in its wild condition, similar to other not cultivated aromatic Vanilla species (*V. pompona* Schiede and *V. insignis* Ames). For this reason, the main aim of this study was to evaluate cured pods from 25 accessions of *V. planifolia*, two accessions of *V. pompona* and two accessions of V. *insignis*, to identify chemotypical variation within the germplasm of the Puebla-Veracruz Totonacapan region in Mexico through quantitative analysis of the four phenolic compounds (vanillin, *p*-hydroxybenzaldehyde, vanillic acid, and *p*-hydroxybenzoic acid) that define the species' aromatic quality. To this end, fruit maturity at harvest, method of curing, storage conditions and time, and extraction method were controlled since these factors are considered those that most influence the concentration of the aromatic compounds in vanilla pods (Sharma et al. 2006).

Materials and methods

Reagents

HPLC grade reagents were used, including vanillin, *p*-hydroxybenzaldehyde, vanillic acid and *p*-hydroxybenzoic acid (Sigma-Aldrich Co., USA).

Fruits

Flowers from 25 accessions of *Vanilla planifolia* G. Jack and *Vanilla planifolia* G. Jack 'Rayada', two accessions of *Vanilla pompona and two accessions of Vanilla insignis,* were labeled and pollinated manually during the last week of April and the first week of May, 2007, in plantations of 22 localities of the Puebla-Veracruz Totonacapan region. The fruits were collected 28 weeks after pollination and subjected to a traditional process of curing, which lasted 14 weeks. The traditional curing process began by scalding the green pods (90°C) for 1 min to detain vegetative development. The pods were stored in a hermetically sealed box for 24 h for slow cooling. Later, the pods were subjected to 21 cycles of a process called "sunning-sweating"; during this process, the pods were exposed to the sun, reaching a temperature of approximately 45°C, for 3 or 4 h a day. They were stored in hermetically sealed boxes during the night to conserve the temperature, favoring the enzymatic activity that hydrolyzes the precursors of the aroma. After 5 or 6 "sweatings", and depending on the environmental conditions, the pods were dried. In this step, the pods were placed on wooden beds to air and prevent contamination by fungi. Finally, the pods were placed in plastic bags and stored in the dark at room temperature to complete the development of the sensorial characteristics of the vanilla aroma. It is considered that by controlling the flowering date and using the same type of curing, uniformity was achieved in fruit age at harvest, curing method, and storage time and conditions.

Extraction

Cured pods were frozen in liquid nitrogen and ground in a blender (Osterizer). Later, 36 mL of an extraction solution was added to 100 mg of tissue; this solution was composed of water, ethyl ether, and

pentane 4:16:16 v/v/v. The mixture was processed immersed in ice in a homogenizer DIAX 600–9,500 rpm. The organic phase was recovered and sodium sulfate was added to eliminate residual water. The organic phase was evaporated in a rotavapor (Heidolph) at 32°C until dry. The residue was resuspended in 1 mL of methanol (25%) H_3PO_4 10^{-2} M (75%), and filtered in 0.45 µm acrodiscs (titan2™). The extraction process was made according to Pérez-Silva et al. (2006).

HPLC analysis

The extracts were analyzed by high performance liquid chromatography (HPLC). The HPLC instrument Perkin Elmer, model Series 200, equipped with a UV detector and auto-sampler was used under the following conditions: column: PR-18 Spheri-5, injection volume: 20 µL, flow: 1.5 mL min^{-1}, isocratic mobile phase (25% methanol-75% H_3PO_4 10^{-2}M), run time (20 min), and detection at 254 nm. The process was modified based on the study by Pérez-Silva et al. (2006).

Evaluated traits

In order to characterize the phytochemical quality of the vanilla germplasm, a total of 10 variables were considered in the statistical analysis. Four variables correspond to the content of each one of the compounds that define vanilla quality: *p*-hydroxybenzoic acid (C1), vanillic acid (C2), *p*-hydroxybenzaldehyde (C3), denominated minor compounds, and vanillin (C4) (Ranadive 1992; Wescott et al. 1994; Sostaric et al. 2000; Betazzi et al. 2006; Sharma et al. 2006); the sum of the minor compounds ($\sum MC = C1 + C2 + C3$), the total of the minor compounds divided by vanillin content ($\sum MC/C4$), and the interaction of each of the minor compounds in proportion to the vanillin content (indexes C1/C4, C2/C4, C3/C4, C1 + C2/C4).

Agroecological zones

In order to analyze the effect of the environmental characteristics of the zone where *V. planifolia* grows and relating it on the content of its phenolic compounds, three agroecological zones were considered based on climate (temperature and humidity), precipitation and altitude (Table 1), because these represents the three main ecosystems and vanilla production systems of the Totonacapan region.

Statistical analysis

Two statistical designs were used to analyze the aromatic compounds of vanilla. (1) The effect of the ecological zone on the concentration of aromatic compounds of *V. planifolia* as a source of variation was analyzed. Three treatments (agroecological zones) were considered with different numbers of replications. (2) For the analysis of concentrations of aromatic compounds in the different accessions of *V. planifolia*, the collection was considered the source of variation. Twenty-five treatments with five replications, a total of 125 samples, were evaluated. In both cases, the data of each treatment were analyzed using a model equivalent to a completely randomized

Table 1 Principal characteristics of the three agroecological zones where *V. planifolia* germplasm is cultivated in the Puebla-Veracruz Totonacapan region, Mexico

Agroecological zone	Mean anual temperature	Mean anual precipitation (mm)	Altitude (m asl)	Accessions		
Z I	18–22°C	2595	301–920	Vp-1	Vp-4	Vp-7
				Vp-2	Vp-5	Vp-8
				Vp-3	Vp-6	
Z II	>22°C	1382	141–300	Vp-9	Vp-12	Vp-15
				Vp-10	Vp-13	Vp-16
				Vp-11	Vp-14	
Z III	>22°C	1582	1–140	Vp-17	Vp-20	Vp-23
				Vp-18	Vp-21	Vp-24
				Vp-19	Vp-22	Vp-25

design, unbalanced for design 1 (PROC GLM, SAS 2002) and balanced for design 2 (PROC ANOVA, SAS 2002). Means between localities were compared with the Tukey test (SAS 2002).

Numerical analysis

Two numerical analysis methods were used in the multivariate analysis of the groups of *V. planifolia*: principal components (PCA) and cluster (Sneath and Sokal 1973) with Euclidian distance and average link as the measure of distance and method of grouping, in the statistical software SAS v. 9.1 (SAS 2002). The numerical analyses used the means of each of the 10 traits evaluated of the specimens from each accession. The information was arranged in a 125 × 10 matrix, whose rows corresponded to accessions and columns to traits.

Results and discussion

HPLC revealed that the compound with the shortest retention time was *p*-hydroxybenzoic acid with an average of 6.7 min, followed by vanillic acid with a mean time of 7.6 min, *p*-hydroxybenzaldehyde with 8.4 min, and finally, vanillin with 10.1 min.

Effect of the ecological zone on concentration of *V. planifolia* aromatic compounds

The effect of the characteristics of the zone where *V. planifolia* is grown and collected, on the content of its phenolic compounds was analyzed. It was found that significant differences ($P < 0.0001$) existed only in the concentration of vanillic acid (X = 586.88 ppm). The Tukey comparison of means ($\alpha = 0.05$) indicated that the concentration of this compound was higher in humid temperate climate and high precipitation, corresponding to the agroecological zone I. The content of the other aromatic compounds and other variables were not affected by environment (Table 2).

The variation observed in the concentration of vanillic acid in cultivated *V. planifolia* coincide with data published by Ranadive (1992), who observed variations in the same compound in specimens cultivated in different geographic regions. This suggests that vanillic acid content is highly influenced by environmental characteristics of the region.

Ranadive (1992) point out that the concentration of vanillin is affected mainly by the stage of maturity and method of curing the pods, but these factors seem to have minimum or no effect on the concentrations of *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid. It was particularly notable that there were no significant differences in vanillin content among the three evaluated agroecological zones, since the conditions of the different accessions from Totonacapan region were homogeneous in terms of ripeness, method of curing, storage conditions and time and method of extraction. Thus, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid and vanillin, as traits, can contribute information on genetic variations related to chemical polymorphisms within the species because they are not affected by environment.

Table 2 Means and coefficients of variation of the 10 variables assessed in 25 accessions of *Vanilla planifolia* from three agroecological zones of the Puebla-Veracruz Totonacapan region, Mexico

	Variables	Mean (ppm[a])	Coefficient of variation
Compounds			
C1	*p*-Hydroxybenzoic acid	79.7^{NS}	25.7
C2	Vanillic acid	586.9***	20.5
C3	*p*-Hydroxybenzaldehyde	459.9^{NS}	33.4
C4	Vanillin	13701.0^{NS}	17.8
Proportion of MC/content of vanillin			
C1/C4	Hydroxybenzoic acid/vanillin	0.01^{NS}	31.4
C2/C4	Vanillic acid/vanillin	0.04^{NS}	11.8
C3/C4	*p*-Hydroxybenzaldehyde/vanillin	0.03^{NS}	35.7
(C1 + C2)/C4	(C1 + C2)/C4	0.05^{NS}	12.5
∑MC/C4	Ratio MC/vanillin	0.08^{NS}	18.3

NS not statistically significant

*** $P < 0001$

[a] mg kg^{-1} cured vanilla

Table 3 Means and coefficients of variation of the 10 variables assessed in 25 accessions of *Vanilla planifolia* from the Puebla-Veracruz Totonacapan region, Mexico

	Variables	Mean (ppm[a])	Coefficient of variation
Compounds			
C1	*p*-Hydroxybenzoic acid	79.67***	13.2
C2	Vanillic acid	586.88***	7.5
C3	*p*-Hydroxybenzaldehyde	459.90***	16.9
C4	Vanillin	13700.98***	6.8
Sum of minor compounds (MC)			
C1 + C2 + C3	∑MC	1126.44***	10.2
Proportion of MC/content of vanillin			
C1/C4	Hydroxybenzoic acid/vanillin	0.006***	12.7
C2/C4	Vanillic acid/vanillin	0.043***	3.6
C3/C4	*p*-Hydroxybenzaldehyde/vanillin	0.034***	15.1
(C1 + C2)/C4	(C1 + C2)/C4	0.050***	4.2
∑MC/C4	Ratio MC/vanillin	0.080***	7.8

*** $P < 0001$

[a] mg kg^{-1} cured vanilla

Effect of the accessions on the concentration of aromatic compounds in *V. planifolia*

The analysis of the effect of the factor collection of *V. planifolia* on the content of aromatic compounds revealed highly significant differences ($P < 0.0001$) in all the variables analyzed (Table 3). The coefficients of variation had low values because the phytochemical evaluation of the specimens corresponded to infraspecific variation of material propagated vegetatively.

Through Tukey comparison test it was observed that most of the compounds analyzed, were statistically different among the accessions and were not related with environmental variations. *p*-hydroxybenzoic acid was found to be the least abundant compound of the four major aromatic compounds of *V. planifolia*, with means that ranged between 47.78 ± 2.21 and 127.67 ± 6.46 ppm (Table 4).

In vanillic acid content, there was wide variation among the accessions. The specimens having the highest content belonged to accessions Vp15 (860.93 ± 9.80 ppm), Vp7 (782.02 ± 58.85) and Vp2 (754.59 ± 19.36 ppm). Those with the lowest content (391.743 ± 22.64 ppm) were from collection Vp23 of *V. planifolia* "rayada" (Table 4). Vanillic acid was found to be the most abundant compound of the minor compounds (MC) which define the vanilla aroma. As mentioned above, the concentration of vanillic acid is affected by the environmental conditions of the Puebla-Veracruz Totonacapan region. The accessions with the highest content were located in temperate climate zones where annual precipitation is high (2,251–3,250 mm), while those with low concentrations were located in the warm to hot zones with lower precipitation (1,352 mm).

A broad range of variation in the content of *p*-hydroxybenzaldehyde among the *V. planifolia* accessions was observed, oscillating between 219 and 795 ppm of cured vanilla (Table 4). The highest concentrations were found in collection Vp3 (795 ± 57.53 ppm), followed by accessions Vp19 (733 ± 37.76 ppm) and the lowest content was recorded in accessions Vp20 (265 ± 57.53 mg kg^{-1}) (Table 4).

Vanillin ranged from 10,407 to 18,657 ppm in the different accessions (Table 4). The highest concentrations were found in accessions Vp19 (18,657 ± 638.66 ppm), followed by specimens from accessions Vp1 and Vp2 (17,599 ± 1685.47 and 17264 ± 504.29 ppm) (Table 4).

The concentration of minor compounds (MC) in the extract was independent of the concentration of vanillin; that is, those specimens with high concentrations of MC did not necessarily have high concentrations of vanillin. For this reason and with the aim of analyzing the interactions among the four major aromatic compounds of *V. planifolia*, the ratio of MC to vanillin content in the extract was calculated as ∑MC/vanillin.

Tukey test identified a total of 16 groups of means with ratio values between 7 and 13%. The values close

Table 4 Mean contents of *p*-hydroxybenzoic acid in 25 accessions of *V. planifolia* from the Puebla-Veracruz Totonacapan region, Mexico

Acc.	Agroec zone	Hydroxybenzaldehyde		*p*-Hydrobenzoic acid		Vanillin		Vanillic acid		Ratio ∑MC/vai	
		Mean (ppm[a])	S.D.	Mean (ppm[a])	S.D.	Mean (ppm[a])	S.D.	Mean (ppm[a])	S.D.	Mean (%)	
Vp-3	I	794.98[a]	57.5	127.67[a]	6.5	12684.4[ghijkl]	924.6	703.76[bcd]	57.0	13	a
Vp-5	I	542.70[cde]	33.4	83.59[cdef]	6.1	11798.3[ijklm]	368.5	540.43[ghijkl]	30.2	10	bc
Vp-14	II	413.42[efghi]	90.5	111.88[ab]	31.6	11610.5[jklm]	1185.8	564.77[fghijk]	71.9	10	bcd
Vp-18	III	483.36[defgh]	45.6	96.15[bcd]	11.7	11056.5[klm]	1229.3	528.41[ghijkl]	65.0	10	b
Vp-23	III	674.59[abc]	49.8	111.40[ab]	3.5	12998.6[fghijk]	636.0	556.47[fghijk]	35.2	10	b
Vp-8	I	514.26[cdefg]	11.2	80.93[cdefg]	3.9	13118.2[fghijk]	660.4	497.34[ijklm]	24.2	9	defg
Vp-10	II	497.27[cdefgh]	148.0	66.14[efgh]	12.8	12327.4[hijklm]	1370.6	464.10[jklmn]	50.1	9	defg
Vp-11	II	515.54[cdefg]	112.6	76.71[cdefg]	13.3	11472.1[jklm]	773.5	438.58[lmn]	30.0	9	bcde
Vp-12	II	530.64[cdef]	194.7	81.26[cdefg]	20.1	14132.7[efgh]	2134.8	557.76[fghijk]	78.8	9	cdef
Vp-24	III	600.10[bcd]	48.3	89.57[bcde]	4.7	14344.1[efgh]	1154.9	579.73[fghi]	35.4	9	bcde
Vp-25	III	497.87[cdefgh]	58.0	75.85[cdefg]	11.1	10407.5 [m]	477.5	391.43[n]	22.6	9	bcd
Vp-19	III	732.98[ab]	37.8	85.69[cdef]	9.6	18657.2[a]	638.7	716.55[bc]	34.8	8	defgh
Vp-7	I	497.72[cdefgh]	61.7	99.67[bc]	3.0	17004.3[abc]	375.8	782.02[ab]	58.9	8	defghi
Vp-15	II	344.05[ghij]	18.3	84.62[cdef]	5.3	16727.3[abcd]	389.9	860.93[a]	9.8	8	efghij
Vp-1	I	497.41[cdefgh]	65.2	73.90[defg]	7.6	17598.9[ab]	1685.5	693.23[bcde]	78.9	7	fghij
Vp-2	I	325.01[hij]	55.9	80.59[cdefg]	7.0	17264.2[ab]	504.3	754.58[b]	19.4	7	hij
Vp-4	I	360.08[efghij]	78.5	67.79[efgh]	8.0	14722.6[defg]	1015.0	627.73[cdefg]	50.8	7	fghij
Vp-6	I	377.55[efghij]	21.0	68.48[efgh]	3.3	10697.7 [lm]	1028.2	410.50[mn]	10.9	7	defghij
Vp-9	II	423.63[defghi]	163.3	58.22[gh]	13.1	15624.6[bcde]	439.6	565.66[fghij]	44.8	7	ij
Vp-13	II	347.78[fghij]	65.2	70.74[efgh]	8.7	12522.4[ghijklm]	1130.5	602.63[cdefg]	55.7	8	defghi
Vp-16	II	373.20[efghij]	55.0	58.28[gh]	4.7	15028.0[cdef]	559.6	595.96[efghi]	28.1	7	ghij
Vp-17	III	346.32[fghij]	25.2	62.23[fgh]	1.3	10961.5[klm]	400.9	460.01[klmn]	27.1	7	defghij
Vp-20	III	264.65[ij]	19.7	62.35[fgh]	2.4	12288.7[hijklm]	323.7	512.24[hijklm]	17.2	7	ghij
Vp-21	III	323.74[hij]	32.0	70.17[efgh]	3.9	13574.8[efghij]	598.9	621.38[cdefg]	24.4	7	efghij
Vp-22	III	218.56[j]	22.4	47.78 [h]	2.2	13901.8[efghi]	468.5	645.79[cdef]	19.9	7	j

[a] mg kg^{-1} of cured vanilla, Different letters indicate statistical difference, Tukey ($\alpha = 0.05$), *S.D* standard deviation, *Acc* accession

to "0" indicated low presence of minor compounds and predominance of vanillin in the total aroma, while values close to "10" describe larger presence of minor compounds and a smaller proportion of vanillin, qualitatively determining sweeter, more perfumed and floral notes in the essence of the extract. Although Tukey test distinguished more groups, in Table 4 it can see that there are five groups of the ratio ∑MC/vanillin means (13, 10, 9, 8, 7%; Table 4).

Distribution of variation

Dispersion of the 25 accessions of *V. planifolia*, represented in the space determined by the first three principal components, together explained 98% of the accumulated overall variation of the 10 variables studied (Table 5). The first principal component (PC1) explained 52% of the overall variation and was more associated with attributes related to the proportion of the minor compounds relative to the vanillin content of the extract, that is, by the type of aroma (∑MC/C4), content of *p*-hydroxybenzoic acid (C1) and the proportion of *p*-hydroxybenzoic acid to vanillin content (C1/C4) (Table 5). The second PC2 explained 28% of the overall variation and was determined largely by the content of vanillic acid (C2) and vanillin (C4) (Table 5), while PC3 explained 18% of the total variation and was defined

Table 5 Eigenvalues, Eigenvectors and accumulated proportion of the variation explained by each variable in the first three dimensions of the characterization of 125 specimens of *V. planifolia*

Variable	Principal component (PC)		
	PC1	PC2	PC3
C1	**0.410**	0.107	0.032
C2	0.043	**0.596**	−0.044
C3	0.332	-0.004	**0.478**
C4	−0.102	0.525	0.311
∑MC	0.298	0.359	0.314
∑MC/C4	**0.422**	-0.145	0.012
(C1 + C2)/C4	0.319	0.159	**−0.471**
C1/C4	**0.391**	-0.208	−0.139
C2/C4	0.242	0.266	**−0.516**
C3/C4	0.360	-0.261	0.257
Eigenvalue	5.20	2.77	1.80
Proportion	0.52	0.28	0.18
Accumulated	0.52	0.80	0.98

Values in bold indicate the variables that most influence each principal component

mainly by the proportion of vanillic acid/vanillin (C2/C4), *p*-hydroxybenzaldehyde (C3), and the proportion of *p*-hydroxybenzoic acid and vanillic acid/vanillin content (C1 + C2/C4) (Table 5).

According to the spatial distribution of the first three principal components, four groups of data were distinguished in *Vanilla planifolia* (Fig. 1). The distribution of the germplasm based on PC1 placed the accessions with higher proportions of MC per vanillin content (∑MC/C4) on the positive side of the axis (Groups I, II and III), while the germplasm with lower proportions were placed on the negative side (Group IV) (Fig. 1). PC2 concentrated the accessions with higher concentrations of vanillic acid (C2) and vanillin (C4) in the positive quadrant (Groups I, II and IV). According to PC3, the *V. planifolia* with higher concentrations of *p*-hydroxybenzaldehyde (C3) and lower proportions of vanillic acid/vanillin content (C2/C4) were located on the positive side of the axis (Groups II and III) (Fig. 1). In this way, the following groups of *Vanilla planifolia* germplasm from the Puebla-Veracruz Totonacapan were identified: **Group I.** Vp-3, **Group II.** Vp-19, **Group III.** Vp-5, Vp-6, Vp-18, Vp-14, Vp-23, Vp-8, Vp-12, Vp-10, Vp-24, Vp-11, Vp-25 y **Group IV.** Vp-1, Vp-2, Vp-4, Vp-9, Vp-16, Vp-17, Vp-20, Vp-13, Vp-21, Vp-22, Vp-7, Vp-15 (Fig. 1).

Variation grouping

With cluster analysis, a grouping pattern similar to that obtained with the principal components analysis can be observed. Particularly, groups I and II are maintained in both analysis, but more details were distinguished in groups III and IV. A Euclidian distance of 1.2 defined two groups of accessions by the degree of participation of minor compounds relative to the vanillin content (∑MC/C4). The first (cl1) integrated specimens with larger participation of MC in the aroma (13%), corresponding to collection Vp3, and the second (cl2) grouped the rest of the accessions with greater participation of vanillin in the aroma 7–10% (Fig. 2). At a distance of 1.0, the group with greater participation of vanillin in the aroma (cl2) separated into two blocks: (cl3) accessions with higher total content of minor compounds (∑MC) (1,290–1,535 ppm), which included accessions Vp-19, Vp-15 and Vp-7, and (cl4) accessions with content lower than 1290 ppm (Fig. 2).

Each of these blocks, in turn, subdivided into two groups at a distance of 0.9. Block cl3, with higher total MC concentration subdivided into two groups according to the content of vanillin: (cl5) high content (18,657 ppm) for collection Vp-19 and (cl6) medium high content ($\approx$17,000 ppm) for accessions Vp-7 and Vp-15, while block c14 subdivided into two groups according to the aroma (∑MC/C4): (cl7) aroma with intense notes of vanillin (7–8%) and (cl8) aroma with subtle notes of vanillin (9–10%) (Fig. 2). Finally, at a distance of 0.8, subgroups were defined within cluster cl8 that grouped accessions with subtle vanillin aroma (cl8); differences in indirect aroma (∑MC/C4) were appreciated. A group of specimens (cl10) was defined with high participation of minor compounds in the aroma ($\approx$10%), which confer sweet chocolaty notes in the aroma of accessions Vp-5, Vp-14, Vp-18 and Vp-23. Accessions (cl11) with medium high participation of the minor compounds ($\approx$9%) were detected with subtle cinnamon-like notes in the aroma (Vp-8, Vp-12, Vp-24, Vp-10, Vp-11 and Vp-25) (Fig. 2). Thus, the accessions were classified into six homogeneous groups in function of the distances inspection of the dendrogram, in which the cutting off point for the identification of groups was based, taking as a reference a distance of 0.8 units. (Fig. 2).

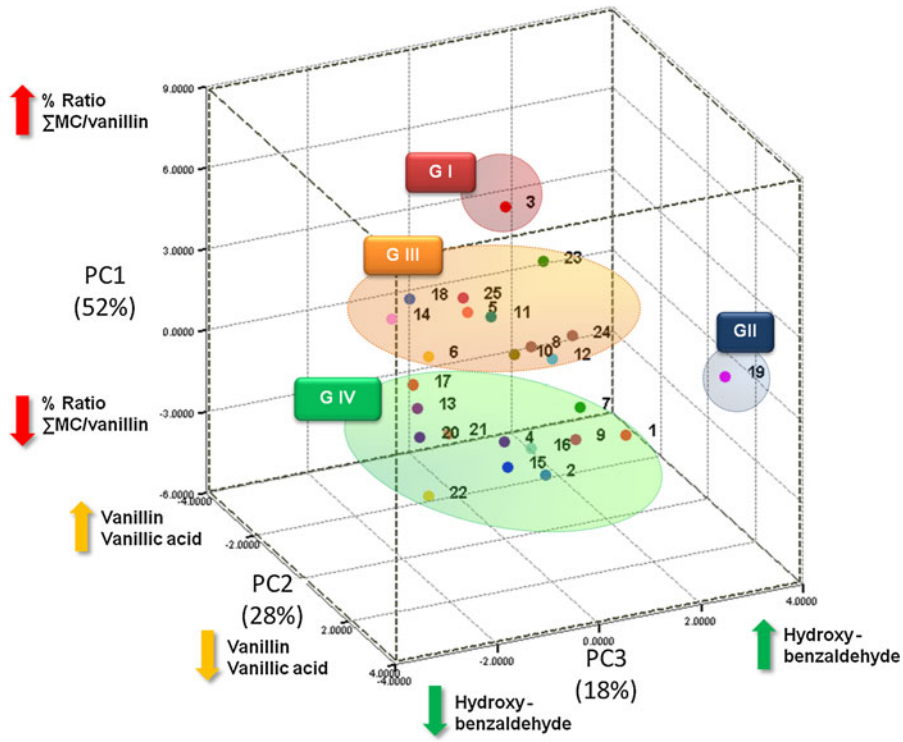

Fig. 1 Dispersion of 25 *Vanilla planifolia* accessions from the Puebla-Veracruz Totonacapan region based on the first three principal components of the analysis of 10 variables grouped by population means

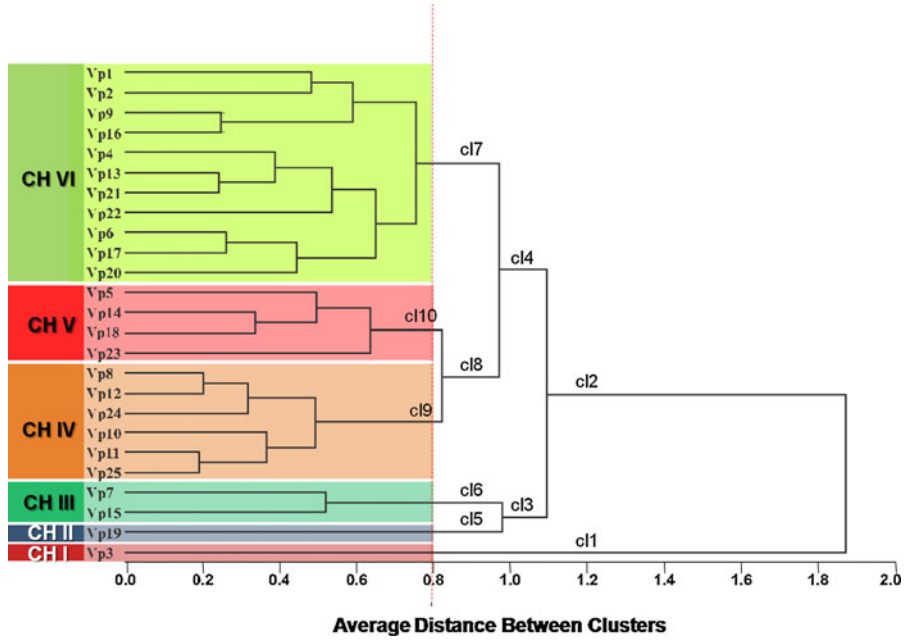

Fig. 2 Dendrogram of 25 accessions of *Vanilla planifolia* in the Puebla-Veracruz Totonacapan region, based on averages of 10 variables and grouping by similarity distances. Chemotypes CH I-CH VI

Chemotype I, (CH I: HA)

This type is represented by Vp-3 germplasm, characterized by having the highest concentration of *p*-hydroxybenzoic acid (127 ppm), high concentrations of *p*-hydroxybenzaldehyde (794 ppm) and vanillic acid (703 ppm), as well as a low content of vanillin (12,684 ppm). It has the highest proportion of minor

Table 6 Principal aromatic characteristics of *V. planifolia* chemotypes in the Puebla-Veracruz Totonacapan Region. C1: *p*-hydroxybenzoic acid, C2: vanillic acid, C3: *p*-hydroxybenzaldehyde, C4: vanillin, ∑MC/C4: proportion of minor compounds relative to vanillin content

Chemotype	C1 (ppm)	C2	C3	C4	∑MC/C4 (%)
V. planifolia CH I	127	794	703	12,684	13
V. planifolia CH II	86	716	733	18,657	8
V. planifolia CH III	85–100	782–861	344–498	16,727–17,004	8
V. planifolia CH IV	66–90	391–580	497–600	10,407–14,344	9
V. planifolia CH V	84–112	528–565	413–675	11,056–12,998	10
V. planifolia CH VI	58–81	411–755	219–497	10,698–17599	7

compounds in the aroma of the extract relative to the content of vanillin (13%), giving it sweet, floral notes to the overall aroma (Table 6). It is distributed in zones with a mean annual rainfall of 1,751 and a hot humid climate type with mean annual temperatures above 22°C and above 18°C in the coldest month of the year.

Chemotype II, (CH II: VAI)

This chemotype corresponds to collection Vp-19. It is distinguished by having a medium content of *p*-hydroxybenzoic acid (86 ppm), high concentration of vanillic acid (716 ppm) and *p*-hydroxybenzaldehyde (733 ppm) and the highest content of vanillin (18,657 ppm). Vanillin predominates in its aroma and there is a medium participation of minor compounds (8%) (Table 6). It is distributed in the zone with hot humid climate, mean annual temperature above 22°C, temperature of the coldest month of 18°C, and mean annual rainfall of 1,351 mm.

Chemotype III, (CH III: VA)

This type comprises accessions Vp-15 and Vp-7. It is identified by a medium high content of *p*-hydroxybenzoic acid (85–100 ppm), high concentrations of vanillic acid (782–861 ppm), medium low contents of *p*-hydroxybenzaldehyde (344–498 ppm) and high content of vanillin (16,727–17,004 ppm). There is medium participation of the minor compounds (8%), slightly predominating notes of vanillin (Table 6). It is distributed in the zone with hot and warm humid climates with mean annual temperatures above 18°C and temperature of the coldest month below 18°C and mean annual precipitation between 1,351 and 1,751 mm.

Chemotype IV, (CH IV: H-VA$^-$)

This group comprises the accessions Vp-8, Vp-12, Vp-10, Vp-24, Vp-11 and Vp-25. It is characterized by a medium–low content of *p*-hydroxybenzoic acid (66–90 ppm), low concentrations of vanillic acid (8,381–580 ppm), medium high concentrations of *p*-hydroxybenzaldehyde (497–600 ppm) and medium–low content of vanillin (10,407–14,344 ppm). In its aroma there is a medium–high participation of minor compounds ($\approx$9%) that gives subtle cinnamon-like notes (Table 6). It is distributed in the zone with hot humid climate with mean annual temperature above 22°C and temperature of the coldest month above 18°C. Mean annual precipitation is in the range of 1,000–2,751 mm.

Chemotype V, (CH V: H-VA)

This group is integrated by accessions Vp-5, Vp-18, Vp-14 and Vp-23. It is characterized by its medium–high content of *p*-hydroxybenzoic acid (84–112 ppm), similar proportions of vanillic acid (528–565 ppm) and *p*-hydroxybenzaldehyde (413–675 ppm) and medium–low content of vanillin (11,056–12,998 ppm). In its aroma the high participation of minor compounds ($\approx$10%) gives it sweet chocolaty notes (Table 6). It is distributed in the hot to warm humid climate zone with mean annual temperature of 18°C and the temperature of the coldest month is below 18°C. Mean annual precipitation is between 1,351 and 3,501 mm.

Chemotype VI, (CH VI: H$^-$-HA$^-$)

This group comprises accessionsVp-4, Vp-6, Vp-17, Vp-20, Vp-13, Vp-21, Vp-22, Vp-1, Vp-2, Vp-9 y

Vp-16. It is distinguished by its lower contents of *p*-hydroxybenzoic acid (411–755 ppm) and variable content of vanillin, low to high (10,698–17,599 pm). The minor compounds have a medium–low participation (≈7%) in the aroma, predominating the intense vanillin notes (Table 6). It is distributed in hot humid climate zones with mean annual temperature above 18°C and temperature of the coldest month is below 18°C, but with a wide range of mean annual participation, 1,000–4,000 mm. This group seems to be the most affected by environment, since it has a wide variation in vanillic acid concentration, which is the compound that is most sensitive to environmental factors, and coincides with the broad margins of mean annual precipitation in the zone of distribution.

In the case of *V. planifolia* from the Totonacapan region, explanation of the distribution pattern of chemotypical variation observed in PCA and cluster, was not related to geographic or climatic factors since all the specimens were from cultivated material. For this reason, the socio-cultural context in which these plants develop, particularly the differences in how the aroma is appreciated, could provide more explanation of chemotypical variation within the region.

The results show that the vanillin content, which to a great extent defines the dynamics of commercial market quality, has not been the trait that has influenced the diversification of *V. planifolia* genetic resources in the Totonacapan region. Rather, the proportion of minor compounds relative to the content of vanillin (∑MC/C4), that is, aroma, is the trait that has received the greatest selection pressure by the Totonaca culture of Puebla and Veracruz, Mexico. A gradient in the participation of the minor compounds (MC) was found in the aroma of the vanilla germplasm from the Totonacapan region (Table 7). The species *V. pompona*, *V. insignis* and *V. planifolia* from Oaxaca, considered wild or little cultivated, had the highest proportions of minor compounds in the aroma (23, 16 and 13%, respectively). In the material cultivated in the Totonacapan region, it can be observed that the participation of minor compounds in the aroma declines from materials with "wild" characteristics, such as chemotype I (13% MC), to highly modified material, such as chemotype VI (7%) (Table 7). This suggests that through the human selection process based on aroma and clonal reproduction of the species, the Totonaca farmers of the Puebla-Veracruz Totonacapan region have preserved chemotypical variation in *V. planifolia* germplasm.

In the case of vanilla, like that of other species, aroma has been a determining aspect in its development as a phytogenetic resource. Especially at the local level, it has been observed that aroma, not only of vanilla but of other resources such as rice, mango, kava and some spices, has been a highly valued aspect used in selection by the cultures for thousands of years and has contributed to the generation of plant varieties and cultivars (Lebot and Levesque 1996; Fitzgerald et al. 2009; Sagar et al. 2009). Thus, identification of chemical typical variation in *V. planifolia* germplasm of the Totonacapan region,

Table 7 Main aromatic characteristics of *V. planifolia* chemotypes in the Puebla-Veracruz Totonacapan region and complementary accessions. C1: *p*-hydroxybenzoic acid, C2: vanillic acid, C3: *p*-hydroxybenzaldehyde, C4: vanillin, ∑MC/C4: proportion of minor compounds relative to vanillin content

Chemotype	C1 (ppm)	C2	C3	C4	∑MC/C4 (%)
V. pompona	63	83	104	1,115	23
V. insignis	48	43	84	866	16
V. planifolia (OAX)[a]	255	1315	873	19,118	13
V. planifolia CH I	127	794	703	12,684	13
V. planifolia CH V	84–112	528–565	413–675	11,056–12,998	10
V. planifolia CH IV	66–90	391–580	497–600	10,407–14,344	9
V. planifolia CH II	86	716	733	18,657	8
V. planifolia CH III	85–100	782–861	344–498	16,727–17,004	8
V. planifolia CH VI	58–81	411–755	219–497	10,698–17,599	7

[a] Pérez-Silva et al. 2006

although it has meant important progress, is still a complex challenge for conservation in its center of origin and diversity. Under the international schemes of commercialization of vanilla, more importance is given to maximization and uniformity in vanillin contents. This can lead to depletion of materials with low vanillin content and the loss of variation and aromas in response to biotic and abiotic factors. Furthermore, clones cultivated both regionally and worldwide are highly vulnerable to extinction because of factors such as genetic erosion, phytosanitary problems, and destruction of habitat by human and climatic phenomena. The adequate use and conservation of chemotypical variation in *V. planifolia* requires in depth analyses of the human systems of valuation and selection that have configured variation in an aroma as complex and exquisite as that of *V. planifolia* in the Totonacapan region.

Conclusions

Chemotypical variation exists among the cultivated specimens of *V. planifolia.* They were grouped into six chemotypes, which indirectly suggests the existence of genetic polymorphism in the Totonacapan region. The study of chemotypical variation of *V. planifolia* in the Totonacapan region revealed a presumably process of selection-domestication by Totonaca groups. During which the concentration of the three minor compounds, *p*-hydroxybenzoic acid, vanillic acid and *p*-hydroxybenzaldehyde, decreased and the content of vanillin, which is the most abundant compound making up the aroma of *V. planifolia*, increased.

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