In Vitro Conservation and Genetic Stability in *Vanilla planifolia* Jacks.

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Abstract. *Vanilla planifolia* Jacks. is a species of great economic importance, since vanillin, a compound highly valued in the food and pharmaceutical industry, is extracted from its pods. This species is in the category of special protection, so it is important to take actions for its conservation and to maintain the genetic stability of the conserved germplasm. An adequate way to achieve this is through the minimal growth in vitro conservation techniques. The present work aimed to establish an in vitro conservation protocol for vanilla germplasm that allows the genetic stability of the conserved material. For the establishment of the minimal growth in vitro conservation protocol: two concentrations of basal Murashige and Skoog (MS) medium (50% and 100%), two incubation temperatures (4 and 22 °C) and two concentrations of abscisic acid (ABA) (3 and 5 mg·L⁻¹) were evaluated. To evaluate the genetic stability of the germplasms used in this study (cultivated, wild, and *V. insignis* morphotypes) by analyzing the profiles of molecular markers SSR (simple sequence repeats) and ISSR (inter simple sequence repeats). The MS medium (100%) supplemented with 3 mg·L⁻¹ of ABA and incubated at 22 °C, was the best treatment for the in vitro conservation of *Vanilla* spp. Compared with the control treatment, it allowed us to obtain smaller shoots (1.17 × 0.17 cm), which showed high genetic stability, given by the low percentages of polymorphism detected in morphotypes cultivated and wild (SSR 0%, ISSR 2%) and *V. insignis* (SSR 0%, ISSR 0%). We conclude the usefulness of the established protocol to conserve the genetic variation of the evaluated *Vanilla* germplasm.

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Vanilla planifolia Jacks. is a species of great economic value for its pods, from which vanillin is extracted, a flavoring compound, widely used in the pharmaceutical, food, and cosmetic industries (Buccellato, 2018). In Mexico, wild as well as cultivated germplasm of *V. planifolia* make up the primary genetic heritage of the species. However, wild populations of *V. planifolia* have been severely affected by excessive and

illegal collection, to the point of bringing the species to the brink of extinction in their natural habitat (SEMARNAT, 2010; Soto-Arenas, 2006). For this reason, although *V. planifolia* is currently threatened with extinction, it is officially considered only in the category of special protection (SEMARNAT, 2010).

The area of vanilla culture is mostly composed of clones derived from the “Mansa” morphotype (Bory et al., 2007), which has low genetic variation (Minoo et al., 2007) largely due to its extensive vegetative propagation. For this reason, this crop has become vulnerable to various biotic and abiotic factors (Soto-Arenas, 1999), which has caused serious production losses in its plantations (Farreyrol et al., 2010). Therefore, conservation efforts are urgently needed.

One of the most effective strategies for the conservation of plant germplasm is based on the use of plant tissue culture techniques to help establish an in vitro germplasm bank (Kendon et al., 2017). In vitro conservation in the medium term, allows conserving aseptically, in a small area, many individuals under controlled conditions for several months, and even years (Cruz-Cruz et al., 2013). This type of in vitro conservation has been used successfully for the germplasm conservation of different plant species such as *Solanum tuberosum* L. (Gopal et al., 2005), *Manihot esculenta* Grantz. (Barrueto and Carvalho, 2008), *Acanthostachys strobilacea* (Schultes f.) Klotzsch (Carvalho et al., 2014), *Asparagus racemosus* Willd. (Thakur et al., 2015), *Heliconia champneiana* L. (Rodrigues et al., 2018), and *Dillnia indica* L. (Abd El-Kader et al., 2019) in the orchid *Epidendrum chlorocorymbos* Schltr. (Lopez-Puc, 2013). However, to establish an effective minimal growth in vitro conservation protocol, it is necessary to reduce the metabolism of plants. For this purpose, low temperatures and light intensity have been used, as well as the addition of osmotic compounds such as mannitol or sucrose, and different growth retardants in the culture medium (Rodrigues et al., 2018; Thakur et al., 2015; Trejgell et al., 2015).

Although in vitro conservation protocols have been established for *V. planifolia* (Bello-Bello et al., 2015; Divakaran et al., 2006), the genetic stability of conserved germplasm has not been evaluated yet. But genetic stability is an aspect of crucial importance to consider in the in vitro conservation protocols of germplasm because in vitro conditions may cause the appearance of physiological and biochemical alterations generated by genetic and epigenetic changes (Bednarek and Orłowska, 2019; Larkin and Scowcroft, 1981; Sánchez-Chiang and Jiménez, 2009). Some of these genetic changes have been evaluated by means of molecular markers (Sánchez-Chiang and Jiménez, 2009). The molecular markers SSR and ISSR have been some of the most used in the evaluation of the genetic stability of germplasm conserved

in vitro of different plant species, such as *Glycyrrhiza glabra* L. (Mehrotra et al., 2012) and *Tetrastigma hemsleyanum* Diels et Gilg. (Peng et al., 2015).

The objective of this study was to develop an efficient protocol for minimal growth for the conservation in vitro of *V. planifolia*, for which we evaluated different treatments: basal MS concentrations (50% and 100%), incubation temperatures (4 and 22 °C), and abscisic acid concentrations (3 and 5 mg·L⁻¹). Finally, we verify the usefulness of the best in vitro conservation treatment by evaluating the genetic stability of different *Vanilla* germplasm preserved in vitro using SSR and ISSR molecular markers.

Materials and Methods

Plant materials. We used two wild morphotypes of *V. planifolia*, “La Gran Lucha” and “Armadillo Chico,” both from Oaxaca, Mexico, as well as the cultivated morphotype “Mansa” of *V. planifolia* and germplasm of *V. insignis*, a species genetically related to *V. planifolia*, which also has outstanding characteristics (Bory et al., 2008), particularly for its greater tolerance to pathogen attack and water deficit (Soto-Arenas, 1999). Originating from the latter, from the locality of “Emiliano Zapata,” Veracruz, Mexico. All of these were previously established in vitro, in the plant tissue culture laboratory, at the Institute of Biotechnology and Applied Ecology (INBIOTECA), Universidad Veracruzana, Xalapa, Veracruz, Mexico.

In vitro conservation. For in vitro conservation by minimal growth, shoots of *V. planifolia* morphotype “Mansa” (≈0.5–0.8 cm in length) were transferred to test tubes (22 × 220 mm) containing 10 mL of MS culture medium (Murashige and Skoog, 1962), without plant growth regulators (RCVs). Phytigel (Sigma-Aldrich, St. Louis, MO) of concentration 2.2 g·L⁻¹ was used as the gelling agent. Two concentrations (50% and 100%) of the MS culture medium, as well as two concentrations (0, 3, and 5 mg·L⁻¹) of ABA (Sigma-Aldrich) were evaluated. In addition, the effect of two incubation temperatures (4 ± 2 and 22 ± 2 °C) on the in vitro conservation of *V. planifolia* was evaluated. The pH of the culture media was adjusted to 5.8 with 0.5 N NaOH and subsequently sterilized at 121 °C for 15 min in an autoclave (FE-299 Felisa, San Juan de Ocotán Zapopan, Jalisco, México) at 1.5 kg·cm⁻². All cultures were maintained under a light irradiation of 50 ± 5 μmol·m⁻²·s⁻¹, provided by fluorescent lamps and a photoperiod (16 h light/8 h dark). After 120 d of cultivation, the percentage of survival, the length of shoots, the number of leaves, and the length and number of roots were evaluated.

Statistical analysis. All experiments were run using a randomized, repeated twice design with 30 replicates each (each replicate consisted of a tube with 10 mL of culture medium), and one explant (nodal segments or individual shoots). The data obtained was processed statistically with the IBM SPSS

Statistics (version 25) software. An analysis of variance followed by a Tukey’s test ($P \leq 0.05$) was performed. Normality and variance homogeneity were checked by the Kolmogorov-Smirnov and Levene tests, respectively. The data for the variables stem length and root length required transformation to natural logarithm (LN). Shoots, previously rooted in vitro, of 8–10 cm long, were rinsed with tap water. These were subsequently planted in 50 × 30 × 5 cm trays, containing a sterile substrate consisting of a 1:1 ratio of peatmoss and Agrolite substrate. The in vitro plantlets were grown in greenhouse conditions under 50% shade, an 80% to 95% relative humidity, and a temperature between 28 and 32 °C. The in vitro plantlets were watered with tap water three times a week and fertilized once a week with the (Nitrofoska, Santiago de Querétaro, México) foliar fertilizer (25N–10P–17K). After 8 weeks of cultivation in the greenhouse, the survival rate of acclimatized in vitro plantlets was recorded.

Evaluation of genetic stability. We evaluated the genetic stability of different germplasms after 120 d of being subjected to the best treatment for conservation with ABA (*V. planifolia* wild morphotypes from “La Gran Lucha” and “Armadillo Chico,” *V. planifolia* cultivated morphotype “Mansa” and *V. insignis* Ames).

Extraction of DNA. Total DNA was extracted from 0.2 g of leaf tissue, from 10 shoots per treatment, using the protocol of Doyle and Doyle (1987). The concentration and purity of the extracted DNA was determined on a spectrophotometer (ultraviolet) (JENWAY, Staffs, UK) and its integrity was evaluated by horizontal electrophoresis (CONSORT) in 0.8% agarose gels, stained with ethidium bromide (1 mg·L⁻¹) and observed on a ultraviolet light transilluminator (UVP MultiDoc-It, Cambridge, UK).

Amplification of SSR and ISSR markers. Fourteen SSR primers (mVplCIR002, mVplCIR003, mVplCIR005, mVplCIR010, mVplCIR015, mVplCIR016, mVplCIR019, mVplCIR022, mVplCIR025, mVplCIR026, mVplCIR028, mVplCIR031, mVplCIR047, mVplCIR050) (Bory et al., 2008) and 10 ISSR primers (A04, C01, C02, C03, C07, C08, C09, C10, T05, T06) (Martínez-Castillo et al., 2008; Verma et al., 2009) were evaluated. The amplification reactions were performed in a final volume of 25 μL, containing 40 ng of genomic DNA, 2.5 μL of 10X PCR buffer, 0.2 mM of deoxynucleoside triphosphate, 0.2 μM of each pair of SSR primers or each ISSR primer examined, 2 mM magnesium chloride (MgCl₂), and 0.5 U of Taq DNA polymerase (INVITROGEN, Waltham, MA). The amplification of the DNA fragments was performed in a Thermocycler (GenAmp PCR System 9700; Applied Biosystems, Foster City, CA). The amplification program for the SSR consisted of 4 min at 94 °C for initial denaturation, 34 cycles of 30 s at 94 °C for denaturation, 45 s for annealing based on the temperatures required for each pair of primers (Table 1), 1 min extension at 72 °C, and a final extension at 72 °C for 5 min. The amplification program for the ISSR consisted of 7 min

at 94 °C, for initial denaturation, 34 cycles of 30 s at 94 °C, for denaturation, 45 s for annealing, based on the temperatures required for each primer, and an extension at 72 °C, for 1:30 min, and a final extension at 72 °C, for 10 min.

Separation, visualization, and analysis of amplified products. The amplified products (SSR and ISSR) were separated into 6% and 5% polyacrylamide gels respectively. A 1X Tris Borate EDTA (TBE) buffer solution [89 mM Tris-hydroxymethyl-aminomethane (Tris), 89 mM boric acid (H₃BO₃), and 2 mM ethylenediaminetetraacetic acid (EDTA)] was used. The gels were loaded with 5 μL of PCR product added with 4 μL of bromophenol blue.

The size of the SSR alleles was determined using a 10-bp reference marker (INVITROGEN 1821-015). The electrophoretic separation was performed for 4 h at 320 V in a vertical electrophoresis chamber (SQ3 sequencer; Hoefer Scientific Instruments, San Francisco, CA). Similarly, the detection of the ISSR markers by electrophoresis was performed for 12 h at 320 V, using the same vertical electrophoresis chamber and a 123-bp DNA ladder (Axygen, Glendale, AZ). Consequently, each of the gels was revealed, fixed, and dried using the silver staining technique, based on the methodology of Martínez-Castillo et al. (2008).

The fragments amplified with the ISSR primers were recorded in a binary base matrix with values of 0 and 1, where 1 indicates the presence and 0 indicates the absence of bands. The fragments amplified with the SSR primers were recorded in a genotypic matrix. For this purpose, the number and frequency of alleles for each locus were recorded by coding them according to their total size in bp. The size of the SSR alleles was determined with a 10-bp DNA ladder as a reference (INVITROGEN), which was placed between samples during the electrophoresis. Only the SSR and ISSR bands that showed high resolution and reproducibility were recorded. Each band obtained was considered an independent locus. For both markers (SSR and ISSR), the percentage of polymorphic loci was calculated as well as the number and frequency of alleles for each locus based on their total size in bp. A similarity matrix was calculated using Jaccard’s coefficient and the dendrogram was generated using the unweighted pair group method (UPGMA). The clustering method was evaluated by bootstrap analysis with 1000 replicates using the PAST program (PAleontological Statistics, Version 2.17) (Hammer et al., 2001).

Results

In vitro conservation by minimal growth. Shoots of *V. planifolia* preserved in vitro at 4 ± 2 °C showed high sensitivity to low temperatures, since after 3 weeks of cultivation, at this temperature there was a 100% reduction in shoot survival, regardless of the concentrations of MS and ABA treatments examined (Fig. 1). Significant differences were observed between the treatments incubated at a temperature of 22 ± 2 °C (Table 1). The

Table 1. Effect of abscisic acid (ABA) and Murashige and Skoog (MS) concentrations on the in vitro conservation of *Vanilla planifolia*.

Treatments	Survival (%)	Length (cm)	Number of leaves	Number of roots	Length of roots (cm)
MS	100.0	2.58 ± 0.13 D	2.67 ± 0.33 AB	2.00 ± 0.15 A	1.72 ± 0.44 D
MS + 3 mg·L ⁻¹ ABA	93.3	1.10 ± 0.17 B	1 ± 0.45 A	2.00 ± 0.58 A	0.79 ± 0.20 B
MS + 5 mg·L ⁻¹ ABA	66.7	0.71 ± 0.16 A	1.17 ± 0.40 A	0.67 ± 0.33 A	0.40 ± 0.10 B
1/2 MS	100.0	3.03 ± 0.24 E	3.67 ± 0.21 B	1.00 ± 0.10 A	1.67 ± 0.43 CD
1/2 MS 3 mg·L ⁻¹ ABA	80.0	1.63 ± 0.18 C	2.67 ± 0.33 AB	2.33 ± 0.33 A	1.11 ± 0.29 C
1/2 MS 5 mg·L ⁻¹ ABA	60.0	1.08 ± 0.21 BC	1.20 ± 0.20 AB	1.00 ± 0.10 A	0.21 ± 0.05 A

The values represent the mean ± SE. Means with different letters were significantly different (Tukey, $P \leq 0.05$).

Fig. 1. Temperature effect on *Vanilla planifolia* at 4°C. Bar = 1 cm. (A) 50% MS, (B) 100% MS, (C) 100% MS + 3 mg·L⁻¹ of abscisic acid (ABA), and (D) 100% MS + 5 mg·L⁻¹ of ABA.

highest survival rates (100%) were obtained in the control, 100% MS, and 50% MS treatments. However, the development variables were unaffected by the treatment. On the other hand, the ABA concentrations used produced a reduction in the length of shoots, length, and number of roots, as well as the number of leaves. Therefore, the highest survival percentage using ABA (93.3%) was observed in MS medium added with 3 mg·L⁻¹ ABA. Shoots cultivated on this treatment showed a stem length of 1.1 cm, with 0.67 roots showing a length of 0.79 cm after 120 d of cultivation (Fig. 2). A 100% survival was observed from shoots that came from the material conserved in MS + 3 mg·L⁻¹ ABA 4 weeks after the transfer of the in vitro plantlets to greenhouse conditions.

Assessment of genetic stability. Of the 10 ISSR primers tested, only 6 of them (C03, A04, T05, T06, C07, C09) produced clear and reproducible bands in a molecular weight

range between 347 and 2,214 bp. Although a total of 51 bands were obtained, only 4 of them were found to be polymorphic, representing 2% of the genetic polymorphism detected in the in vitro plantlets of “La Gran Lucha,” “Armadillo Chico,” and the morphotype “Mansa” conserved by minimum growth, which were evaluated in this way. However, for *V. insignis*, a total of 49 bands were detected, whose molecular weight varied between 394 and 2460 bp, but all were monomorphic (Table 2).

Of the 14 SSR primers evaluated, only 8 of them (mVpICIR002, mVpICIR010, mVpICIR015, mVpICIR016, mVpICIR019, mVpICIR025, mVpICIR031, mVpICIR047) produced distinctive and reproducible bands. These primers threw a total of 12 bands for “La Gran Lucha,” “Armadillo Chico,” and the morphotype “Mansa”. Regarding *V. insignis*, a total of 9 bands were obtained. The mVpICIR016 primer was discarded because it

did not produce good-quality amplification. The molecular weight range of the bands obtained was 215–346 bp (Table 2). It was of interest to observe the presence of a great monomorphism in the SSR profiles of 100% obtained in the preserved *V. planifolia* seedlings, which apparently evidences their genetic stability. The results of the dendrograms obtained from the UPGMA clusters analysis based on Jaccard similarity coefficients revealed high genetic similarity between clustering patterns of SSR and ISSR (Fig. 3).

Discussion

Various osmotic and growth-regulating agents have been evaluated for the preservation of *Vanilla* spp. (Bello-Bello et al., 2015; Divakaran et al., 2006). However, the effects of low temperatures, high doses of growth inhibitors, and the genetic stability of the preserved material have not yet been evaluated.

Some authors (Divakaran et al., 2006; Engelmann 2011) have indicated that the use of low temperatures of 0 to 10 °C is an effective way to reduce the growth rate in in vitro conservation work. However, it has been indicated (Lambardi et al., 2006) that the most appropriate incubation temperature to use in in vitro conservation work for minimum growth depends on each species under study. It is possible that the sensitivity to low temperatures shown by *V. planifolia* shoots incubated at low temperatures is because, as this species usually grows in tropical and subtropical climates, at temperatures between 20 and 30 °C (Ranadive, 2005), it is not able to show adequate tolerance to low temperatures.

A beneficial effect on the reduction of mineral element content in the culture medium was not observed, as expected, considering the results obtained by Lopez-Puc (2013), who found that 1/2 MS was effective for the conservation of the orchid *Epidendrum chlorocorymbos* Schltr. Other authors, such as Ramirez-Mosqueda et al. (2019), found that the use of 3/4 MS was equally effective for the conservation of the orchid *Laelia anceps* Lindl.

ABA is a plant growth inhibitor that has been widely used in in vitro conservation programs for different crops of agri-food, pharmaceutical, and ornamental interest. It was used for this purpose in some species, such as *Garcinia mangostana* L. and *Lansium domesticum* Corr. (Keatmetha et al., 2011), *Saccharum* spp. (Bello-Bello et al., 2014), and *Dillnia indica* L. (Abd El-Kader et al., 2019). ABA has different physiological effects on plants, among which are the inhibition of plant elongation and cell

Fig. 2. Effect of the concentration of MS medium and abscisic acid (ABA) on the in vitro conservation of *Vanilla planifolia* Jacks. MS (A) 5, (B) 3, and (C) 0 mg·L⁻¹ of ABA from left to right and 50% MS (D) 5, (E) 3, and (F) 0 mg·L⁻¹ of ABA from left to right. Bar = 1 cm.

Table 2. Analysis of the genetic stability of *Vanilla* germplasm conserved in vitro by minimum growth evaluated with inter simple sequence repeat (ISSR) and simple sequence repeat (SSR) markers.

ISSR markers					
Accessions	Total number of loci	Monomorphic number of loci	Polymorphic number of loci	Rate of Polymorphism	Molecular weight range
La Gran Lucha	51	46	4	2%	347–2214
Armadillo Chico	51	46	4	2%	347–2214
Emiliano Zapata	51	49	2	2%	347–2214
<i>V. insignis</i>	49	49	0	0	394–2460

SSR markers					
Accessions	Total number of loci	Monomorphic number of loci	Polymorphic number of loci	Rate of Polymorphism	Molecular weight range
La Gran Lucha	12	12	0	0%	215–346
Armadillo Chico	12	12	0	0%	215–346
Emiliano Zapata	12	12	0	0%	215–346
<i>V. insignis</i>	9	9	0	0%	215–346

Fig. 3. Dendrogram of *Vanilla* spp. based on the Jaccard's coefficient of (A) inter simple sequence repeat and (B) simple sequence repeat markers.

division, stomatic occlusion, induction of seed dormancy, and prevention of premature germination (Li et al., 2015; Selvakumar et al., 2016).

In our study, ABA, at a concentration of 3 mg·L⁻¹, produced a reduction in the in vitro growth of *V. planifolia*. These results coincide with those obtained by Bello-Bello et al. (2015), which highlighted the beneficial effect of the addition of ABA on the conservation of minimal growth of *V. planifolia*. However, these authors did not evaluate if the proposed protocol affected the genetic stability of the germplasm conserved in vitro.

The analysis of the genetic stability of the materials preserved in vitro is essential because during the conservation process, somaclonal variants may arise that cause alterations in the genetic homogeneity of the conserved germplasm (Tyagi et al., 2010). In *V. planifolia*, there are few studies on the analysis of somaclonal variation in micropropagated plants. In this regard, the presence of 71.66% of genetic polymorphism in in vitro plantlets of this species regenerated by indirect organogenesis has been reported (Ramírez-Mosqueda and Iglesias-Andreu, 2015) and more than 15% of polymorphism from the sixth subculture in micropropagation works in *V. planifolia* (Pastelín-Solano et al., 2019). Studies of genetic stability in cryopreservation using ISSR primers have revealed that the composition of the medium and the number of

subcultures influence genetic stability (Isidro-Adolfo et al., 2019). However, studies on the genetic stability of the germplasm of these species conserved in vitro are limited.

Previous studies carried out by Borbolla-Pérez et al. (2016) to detect the variation in the SSR profiles of two *V. planifolia* plantations from “Papantla,” Veracruz, Mexico, showed the presence of 18.18% of genetic polymorphism, even though the plants analyzed came from the clonal propagation of the “Mansa” morphotype. This evidence the genetic variability of some *V. planifolia* plants used as donors of explants in this study.

The integral analysis of the results obtained with both molecular markers allow us to conclude that genetic stability was maintained not only in commonly cultivated *V. planifolia* (Mansa), but also in its wild germplasm and in *V. insignis* germplasm, by minimal growth. This result could be useful for increasing the subculture period from 45 to 120 d without any genetic alterations. However, it is recommended to carry out future studies to evaluate, with other molecular markers, such as methylation-sensitive-Amplified Fragment Length Polymorphism, the possible epigenetic variations that could have arisen in the germplasm of *Vanilla* preserved by the procedure of minimum growth in vitro established in this work.

In conclusion, MS media containing 3 mg·L⁻¹ of ABA allows to obtain survival of 93.3%, lower height and length of the roots,

so it can be used for in vitro conservation by minimum growth of germplasm of *V. planifolia*. Under these conservation conditions, minimal variation genetic was detected by ISSR and SSR markers in different germplasms of *Vanilla* spp. Therefore, the use of the in vitro conservation protocol established to conserve, in the medium term, this valuable genetic resource with adequate genetic stability is recommended.

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