https://doi.org/10.32854/agrop.v13i12.1773

In vitro conservation of *Vanilla planifolia* hybrids in minimal growth conditions

Hernández-Leal, Enrique[1]; Reyes-López, Delfino[*2]; Castillo-Martínez, Carlos R.[3]; Avendaño-Arrazate, Carlos H.[4]; Corona-Torres, Tarsicio[5]; García-Zavala, J. Jesús[5]; Pascual-Ramírez, Fermín[6]

[1]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Campo Experimental La Laguna, Matamoros, Coahuila, México. [2]Benemérita Universidad Autónoma de Puebla, Facultad de Ingeniería Agrohidráulica, Teziutlán, Puebla, México, [3]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Centro Nacional de Investigación disciplinaria en Conservación y Mejoramiento de Ecosistemas Forestales, Coyoacán, Ciudad de México. [4]Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias Campo Experimental Rosario Izapa. Tuxtla Chico, Chiapas, México, [5]Colegio de Postgraduados, Campus Montecillo, Texcoco, Estado de México, México. [6]Universidad Nacional Autónoma de México. Instituto de Investigaciones en Ecosistemas y Sustentabilidad, Morelia, Michoacán, México.

***Corresponding author**: delfino.reyes@correo.buap.mx

ABSTRACT

Objective: To maintain minimal growth in *in vitro Vanilla planifolia* hybrids.

Design/Methodology/Approach: Explants of seven interspecific hybrids of *V. planifolia* with different origin parents were used. The treatments consisted of different doses of mannitol and sucrose in the culture medium which varied from 0, 5, 10, 15, 20, 25 and 30 g L^{-1}. The number of nodes, shoots and roots was recorded every 30 d for six months.

Results: 30 g L^{-1} mannitol and no sucrose in the culture medium allowed minimal growth in most of the hybrids. The higher the mannitol and lower the sucrose content, the length, number of between nodes, shoots and roots of the explants was lower ($P \leq 0.05$).

Limitations of the study/implications: There is a differential behavior between the biological material and the used culture medium, particularly in hybrids, due to their new genetic combinations. Therefore, for their conservation, the culture medium components must be adjusted.

Conclusions: 30 g L^{-1} mannitol without sucrose in in vitro culture medium significantly reduces growth during 180 d in vanilla hybrids.

Keywords: *Ex-situ* conservation, *in vitro* culture, vanilla, mannitol.

INTRODUCTION

Vanilla (*Vanilla planifolia*) is a species of the Orchidaceae family (Hagsater *et al*., 2005; Freuler, 2007). Soto (2003), Bouétard *et al*. (2010), and Gigant *et al*. (2011) reported that there are around 110 species in the Vanilla genus distributed throughout the tropical areas of the world. This number keeps increasing due

Agroproductividad: *Vol. 13, Núm. 12, diciembre. 2020. pp: 3-10.*
Recibido: *julio, 2020.* **Aceptado:** *noviembre, 2020.*

Imagen de DTelloc en Pixabay

to reports of new species, such as *V. rivasii* in Colombia (Molineros *et al*., 2014), *V. soto arenasii* M. Pignal in Costa Rica (Azofeifa *et al*., 2017) and *V. yanesha* in Peru (Damián, 2019). In Mexico, there are ten reported vanilla species (Soto, 2003). The assessment of the morphometric variation of fruits and seeds in four species of the *Vanilla* genus by Reyes *et al*. (2014) proposed the separation of three clones within *V. planifolia*. Flores *et al*. (2017) report seven species distributed throughout nine states in Mexico, taking into account georeferenced data from national and international herbaria and data from the vanilla germplasm bank; These authors mention that, in Mexico, the number of species in the Vanilla genus may increase, because not all the areas where *Vanilla* species possibly exist in the wild have been assessed, and therefore taxonomic studies are necessary to identify species and clones within species. Ramos-Castellá *et al*. (2016) separated accessions of *V. planifolia* and wild cultivars from Oaxaca and Quintana Roo, Mexico, using molecular techniques. These last three studies indicate that the vanilla diversity in Mexico may be more extensive. The cultivation of vanilla in Mexico presents technical, organizational, environmental and ecological problems that limit its production (Hipolito-Romero, 2010; López-Juárez *et al*., 2019; Santillan *et al*., 2019). *Vanilla planifolia* is the species with the largest planted area worldwide and the most demanded in the food industry for its aromatic qualities (Bory *et al*., 2008; Greule *et al*., 2010). FAO (1995) considers it as a species with a high degree of genetic erosion. In recent years in Mexico the sown area has been reduced and low yields are recorded (Luis-Rojas, 2020); In addition, it is considered a secondary economic activity with elderly producers and in a smallholding situation (Santillán-Fernández, 2019). The NOM-059-SEMARNAT-2010 standard considers it as a species subject to special protection. Faced with this situation, it is necessary to create technological strategies for both *in situ* and *ex situ* conservation that can help to stop the loss of genetic diversity of the *Vanilla* genus in Mexico and worldwide. In this regard, Azofeifa *et al*. (2014) mention that the conservation of the genus *Vanilla* must be under an integrated strategy, that sustainably allows its perpetuity, conserves the greatest possible genetic variability and significantly reduces the vulnerability of some of its species.

Among the different methods of *ex situ* conservation is *in vitro* conservation, which has advantages such as space and labor saving, development of healthy plants, high multiplication rates, constant supply of plants to producers and maintenance of the genetic fidelity of the material (García-Águilar, 2007; Engelmann and González, 2013; Bonilla *et al*., 2015). The minimal growth of explants in vitro has allowed successful medium and long-term conservation (Divakaran *et al*., 2006; Rayas *et al*., 2013; Bello *et al*., 2015). For this technique development, components have been used in the culture medium that retard the growth of the explants to avoid subcultures in short periods. Osmotic regulators such as mannitol and sorbitol (Montalvo *et al*., 2007; Hassan *et al*., 2014) and growth inhibitors such as abscisic acid (ABA) (Pence *et al*., 2002; Sarasan *et al*., 2006; Barrueto and Carvalho, 2008) and paclobutrazol (Ziv, 2000) have been used. Ávila and Salgado (2006) used sucrose, mannitol and sorbitol in the *in vitro* conservation of water yam (*Dioscorea alata*) and white yam (*Dioscorea rotundata*). Rayas *et al*. (2013) studied the effect of mannitol and silver nitrate for *in vitro* conservation of malaga (*Xanthosoma* spp.). Muñoz *et al*. (2019) reported that mannitol and sorbitol significantly reduce *in vitro* growth in three native Chilean potato (*Capsicum* sp.) genotypes. López-Puc (2013) developed an *in vitro* slow growth protocol for the conservation of the orchid *Epidendrum chlorocorymbos* SHLTR, where 1 % sorbitol and MS at half its ionic strength proved to be effective. In vanilla, Divakaran *et al*. (2006) used mannitol in the culture medium for minimal growth in several species of the genus *Vanilla* from India, allowing their conservation for more than seven years. In Mexico, Bello *et al*. (2015) used mannitol and poliethylenglycol, abscisic acid and paclobutrazol for *V. planifolia* conservation in slow-growth conditions, prolonging the period between subcultures every 180 days, without affecting the viability and phenotype of the plants.

The studies in Mexico for minimum growth in vanilla has focused only on *V. planifolia* and not on its interspecific hybrids. Such information is necessary for a genetic improvement program of vanilla, given that the crosses generate genetic combinations different from the parents. Also, each hybrid behavior may be different in the culture media used for the conservation of materials with high agronomic potential. Therefore, the objective of this work was to evaluate the effect of mannitol and sucrose, both individually and jointly, at different doses in a culture medium for in vitro conservation for minimal growth conditions.

MATERIALS AND METHODS

The biological material used was seven single-cross hybrids obtained from ten accessions of *V. planifolia* from

Chiapas, Puebla, Quintana Roo and Veracruz, Mexico, from the vanilla germplasm bank of the Benemérita Universidad Autónoma de Puebla. The crosses were manually carried out during March and April 2013. The fruits were harvested 60 d after pollination. The in vitro F1 seedlings were obtained in the in vitro and cryopreservation laboratory of the National Center for Genetic Resources (CNRG) in Tepatitlán, Jalisco, Mexico. *In vitro* culture was carried out in four stages: disinfection, sowing, incubation, and multiplication.

For the maintenance and multiplication of the *in vitro* plants, the culture medium proposed by Murashige and Skoog (1962) was used, supplemented with 30 g L^{-1} sucrose, 1 g L^{-1} activated carbon and 8.5 g L^{-1} agar. The pH was adjusted to 5.7 with a potentiometer (HANNA®), the medium was then sterilized in a Yamato model SM200 vertical autoclave during 15 min at 121 °C. The plants were incubated at 18 °C, in a 16 h light and 8 h darkness photoperiod, with a 68 μmol m^{-2} s^{-1} light intensity, using white fluorescent lamps. Twenty protocorms from each cross were grown to a height of 12-15 cm. This procedure lasted 2 years (2015 to 2016).

For the *in vitro* culture under minimum growth conditions, 15 plants of each cross were used, from which, 1 cm length axillary buds were cut inside a laminar flow hood. These buds were then sown in 22 × 220 mm test tubes containing 15 mL culture medium. The medium was MS with (0.22 % (w/v)) Gelrite™ (Sigma®) as a gelling agent, mannitol and sucrose were added as a carbon source at concentrations of 0, 5, 10, 15, 20, 25 and 30 g L^{-1}. The explants were incubated at 18 °C during 16 h light and 8 h dark photoperiod, with a light intensity of 68 μmol m^{-2} s^{-1}, using white fluorescent lamps. The different the treatments were the concentrations of mannitol and sucrose (Table 1), which were distributed in a completely randomized experimental design with three repetitions, and 10 explants were used per treatment. The assessed variables were the length of the explant, number of internodes, and the number of shoots and roots. Five evaluations took place every 30 days, for six months. An analysis of variance and means comparison was performed using the Tukey test ($P \leq 0.05$), a simple linear regression analysis considering the explants growth and the time of evaluation with the SAS v9 software (SAS Institute, 2002).

Table 1. Mannitol and sucrose concentration in culture media for minimal growth of *Vanilla planifolia* hybrids.

Treatment	Mannitol (g L^{-1})	Sucrose (g L^{-1})
T1	0	0
T2	0	30
T3	5	25
T4	10	20
T5	15	15
T6	20	10
T7	25	5
T8	30	0

RESULTS AND DISCUSSION

Treatment T8, with 30 g L^{-1} mannitol and without sucrose in the culture medium, allowed the shortest explants length, with 11.42 mm; although it has no statistical differences with T7, T6, T5 and T1, had no roots and allowed lower values in its between nodes and number of shoots (Table 2). The data suggest that the higher the mannitol concentration the slower the explants growth is and similar to T1. This behavior was due to the fact that in T1 the culture medium was not supplemented with sucrose and mannitol. Similar results are reported by Rukundo (2012) who found slow growth in banana seedlings when he used mannitol as a carbon source. Fortes and Scherwinski (2001), and Espinosa *et al*. (1986) reported that, in potato (*Solanum tuberosum*), mannitol reduces growth and the number of shoots per explant. Borges *et al*. (2003) reported a minimal growth effect in buds of *D. alata* when using

Table 2. Effect of mannitol and sucrose on *in vitro* growth of interspecific hybrids of *Vanilla planifolia* in minimal growth conditions.

Treatment	Longitude mm	Number Internodes	Number shoots	Number roots
T1	11.60^{c}	0.16^{f}	0.93^{b}	0.03^{e}
T2	26.56^{a}	2.39^{a}	1.09^{a}	1.28^{a}
T3	21.36^{b}	1.91^{b}	$1.^{11a}$	0.94^{ab}
T4	19.05^{b}	1.78^{bc}	1.10^{a}	0.63^{bc}
T5	15.10^{c}	1.32^{cd}	1.12^{a}	0.46^{cd}
T6	14.36^{c}	1.09^{de}	1.0^{ab}	0.37^{cde}
T7	13.09^{c}	0.69^{e}	0.98^{ab}	0.11^{de}
T8	11.42^{c}	0.11^{f}	0.98^{ab}	0.0^{e}
DMS	3.71	0.46	0.14	0.40

Means with the same letter in columns are statistically equal (Tukey, 0.05).

mannitol at higher than 5.5 % concentrations. Starisky *et al*. (1985) observed that mannitol in culture medium significantly reduces the growth of shoots of *Colocasia esculenta* and *Xanthosoma brasiliense*. The mannitol concentration in the culture medium are variable, since it depends on the species or plant variety, because this compound can be naturally produced in various plant species such as olives, celery, carrot, parsley, coffee, pumpkin, bean, pea etc. (Stoop *et al*., 1996).

In relation to sucrose as a source of energy and carbon, T2 with 30 g L^{-1} allowed the highest length values, number of internodes and roots in the explants with 26.56 mm, 2.39 and 1.28, respectively; this due the added sucrose in T2 which is recommended to obtain good growth of the explants. Menchaca *et al*. (2011) used 20 g L^{-1} of sucrose in culture medium, and reported good results in for seed germination *in vitro* of a hybrid of *V. planifolia* and *V. pompona*. Others, like Gatjens *et al*. (2018), used 30 g L^{-1} sucrose in the culture medium for massive propagation and formation of vanilla protocormic callus from root tips.

The lowest values were recorded on culture medium with no sucrose. These were 11.42 mm in the explant length and 0.11, 0.98 and 0.0 number of internodes, shoots and roots, respectively. The other treatments showed intermediate growth, since when sucrose decreased and mannitol increased, the explant growth was minimal. Lemoine (2013) remarks that the sugars synthesis, transport and metabolism are important in plants that grow in the field, since they determine their growth. However, in *in vitro* culture conditions, the explants are not sufficiently autotrophic, because the conditions are not very suitable for photosynthesis, and the addition of sugars to the culture medium is essential to satisfy the energy and carbon demand for the adequate development of explants (Pierik, 1990; Ertola *et al*., 1995; Rukundo, 2012). In a research on *V. planifolia*, Divakaran *et al*. (2006) managed to conserve shoots *in vitro* in MS culture medium supplemented with 15 g L^{-1} sucrose.

The *in vitro* behavior of the interspecific vanilla hybrids in the different treatments with sucrose and mannitol were statistically different ($P \leq 0.05$). H6 allowed the shortest length, with 13.40 mm, and the lowest number of internodes, 0.66. H4 allowed the greatest length, 19.18 mm, and 1.61, 0.88, and 0.0 for the number of internodes, shoots and roots, respectively. The other hybrids displayed an intermediate behavior, presenting an interval between 15.16 to 18.34 mm in length and 0.96 to 1.61 knots on the stems (Table 3).

The H4 was the hybrid with the lowest number of shoots (0.88), while H2, registered the highest number of roots (1.03). H4 and H5 had no roots during the six conservation months. The differentiated behavior recorded in the hybrids may be due to their genetic condition as a result of the crosses with different origin parents (Figure 1). Raya *et al*. (2009) found that the *in vitro* rooting of the Freedom and Saltereck vine rootstocks varied between genotypes and also depended on the source and concentration of sugar, as well as the osmotic potential in the medium. This shows that the conditions to induce rooting are defined by each genotype and should not be generalized. In this regard, Gianni and Sottile (2015) stated that, in the *in vitro* conservation of plum germplasm under slow growth the capacity of the sprouts was closely related to the genotypes that generate new combinations in the F1 explants. Márquez (1988) remarks that when a cross is made, the hybrid vigor is taken advantage of, this phenomenon that occurs in progeny with genetic characteristics, they tend to be better than the parents.

Table 3. Behavior of *Vanilla planifolia* hybrids in minimal growth conditions.

Hybrid (Parents)	Longitude mm	Number Internodes	Number shoots	Number roots
H1 (27×27)	16.57^{ab}	0.96^{cd}	1.07^{a}	0.41^{c}
H2 (195×20)	18.34^{ab}	1.44^{ab}	1.15^{a}	1.03^{a}
H3 (111×39)	17.90^{ab}	1.34^{abc}	1.04^{a}	0.86^{ab}
H4 (111×7)	19.18^{a}	1.61^{a}	0.88^{b}	0^{d}
H5 (112×21)	15.16^{bc}	1.09^{bc}	1.05^{a}	0^{d}
H6 (124×27)	13.40^{c}	0.66^{d}	1.04^{a}	0.45^{c}
H7 (124×28)	15.90^{abc}	1.15^{bc}	1.06^{a}	0.62^{bc}
DMS	3.40	0.42	0.13	0.37

Means with the same letter in column between treatments are statistically equal (Tukey, 0.05); mm = millimeters.

According to the simple linear regression analysis on the length of explants over time (Table 4), T8 had the lowest regression coefficient, 0.34, followed by T1 and T7 with 0.40 and 0.75, respectively. T2 and T3 registered the highest regression coefficients, with 3.97 and 2.76, each. T4, T5 and T6 presented intermediate regression

coefficients (Figure 2) since it is observed that during the six months that the hybrids were in growth, T8 was the one that showed minimal growth compared to the other treatments.

Overall, the behavior of mannitol and sucrose together in the culture medium turned out to be effective to reduce *in vitro* growth of hybrids of *V. planifolia*. Few similar works carried out for the species, like that of Bello *et al*. (2015) where two osmotic agents (mannitol and poliethylenglycol) and two plant growth inhibitors (abscisic acid and paclobutrazol) were used for their conservation under slow-growth conditions, the length of the explants tended to be greater compared to those obtained in the present work in a similar period.

The information obtained in this study has been essential for the vanilla genetic improvement program at the Benemérita Universidad Autónoma de Puebla, since the hybrids that are currently being developed in fields with good agronomic characteristics (Figure 3), must be conserved *in vitro* under minimal growth for later use massively by producers in Mexico.

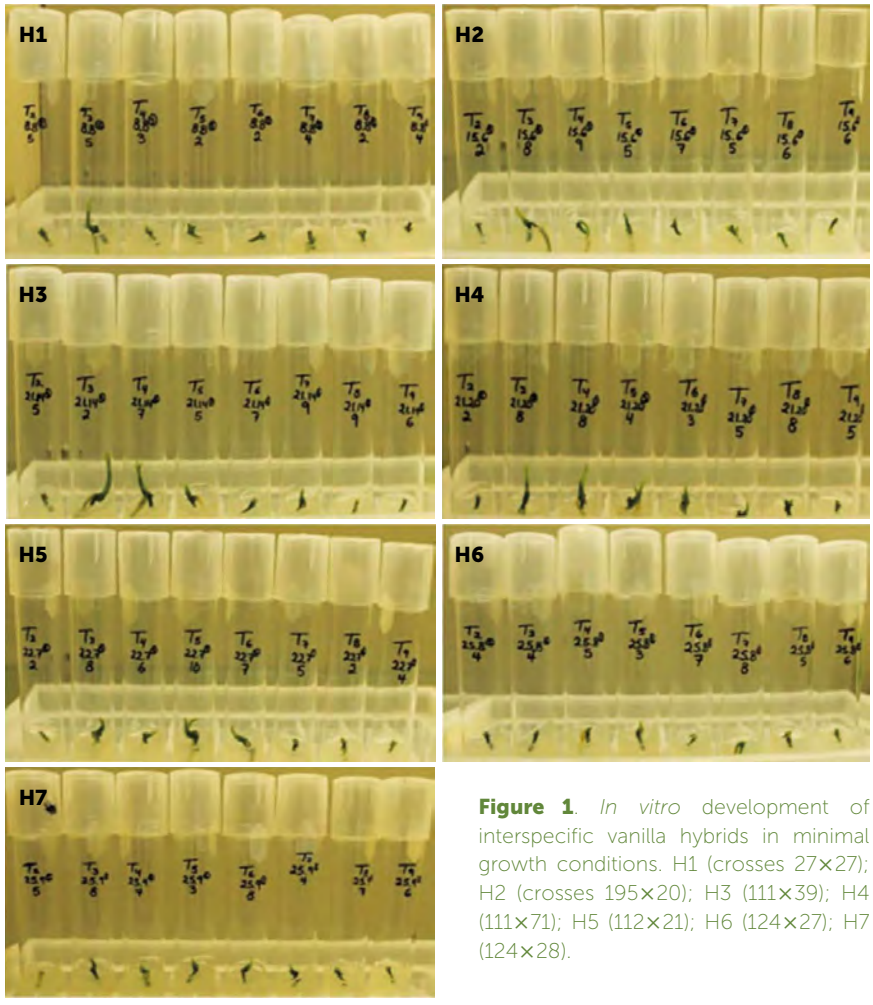

Figure 1. *In vitro* development of interspecific vanilla hybrids in minimal growth conditions. H1 (crosses 27×27); H2 (crosses 195×20); H3 (111×39); H4 (111×71); H5 (112×21); H6 (124×27); H7 (124×28).

CONCLUSIONS

Mannitol and sucrose significantly reduce the explant growth of interspecific hybrids of *V. planifolia* when developed in a culture medium with concentrations of 30 g L^{-1} mannitol without sucrose. The differences on the behavior of *V. planifolia* hybrid explants at the different concentrations of mannitol and sucrose could be due to the new genetic combinations generated when making the crosses between progenitors of different origin.

Table 4. Linear regression coefficients in eight treatments for growth of seven interspecific crosses of *Vanilla planifolia* under minimal growth conditions.

Treatment	Intersect	Regression coefficient	R^2	RMSE
1	9.58	0.394 **	0.995	0.05
2	5.06	3.97 **	0.957	1.53
3	6.95	2.76 **	0.980	0.56
4	7.99	1.99 **	0.997	0.16
5	7.6	1.78 *	0.801	1.62
6	8.89	1.08 **	0.996	0.13
7	9.16	0.75 **	0.987	0.16
8	9.64	0.34 **	0.973	0.10

REFERENCES

Ávila, D.I., & Salgado, G.R. (2006). Propagación y mantenimiento *in vitro* de orquídeas mexicanas, para colaborar en su conservación. Biológicas, 8,138-149.

Azofeifa, B.J.B., Gigant, R., Nicolás, G.M., Pignal, M., Tavares, G.F.B., Hágsater. E., Salazar, Ch.G.A., Reyes, L.D., Archila, M.F.L., García, G.J.A., Denis da Silva, Allibert, A., Solano, C.F., Rodríguez, J.G. Del C., Paniagua, V.A., Besse, P., Pérez, S.A., & Grisoni, M. (2017). A new vanilla species from Costa Rica Closely related to *V. planifolia* (Orchidaceae). European Journal of Taxonomy, 284,1-26. http://dx.doi.org/10.3732/ajb.

Azofeifa-Bolaños, J.B., Paniagua-Vásquez, A., & García-García, J.A. (2014). Importancia y desafíos de la conservación de *Vanilla* spp. (Orquidaceae) en Costa Rica. Agronomía Mesoamericana, 25,189-202.

Barrueto, L.P., & Carvalho, C.B. (2008). Importance of abscisic acid (ABA) in the *in vitro* conservation of cassava (*Manihot esculentus*).

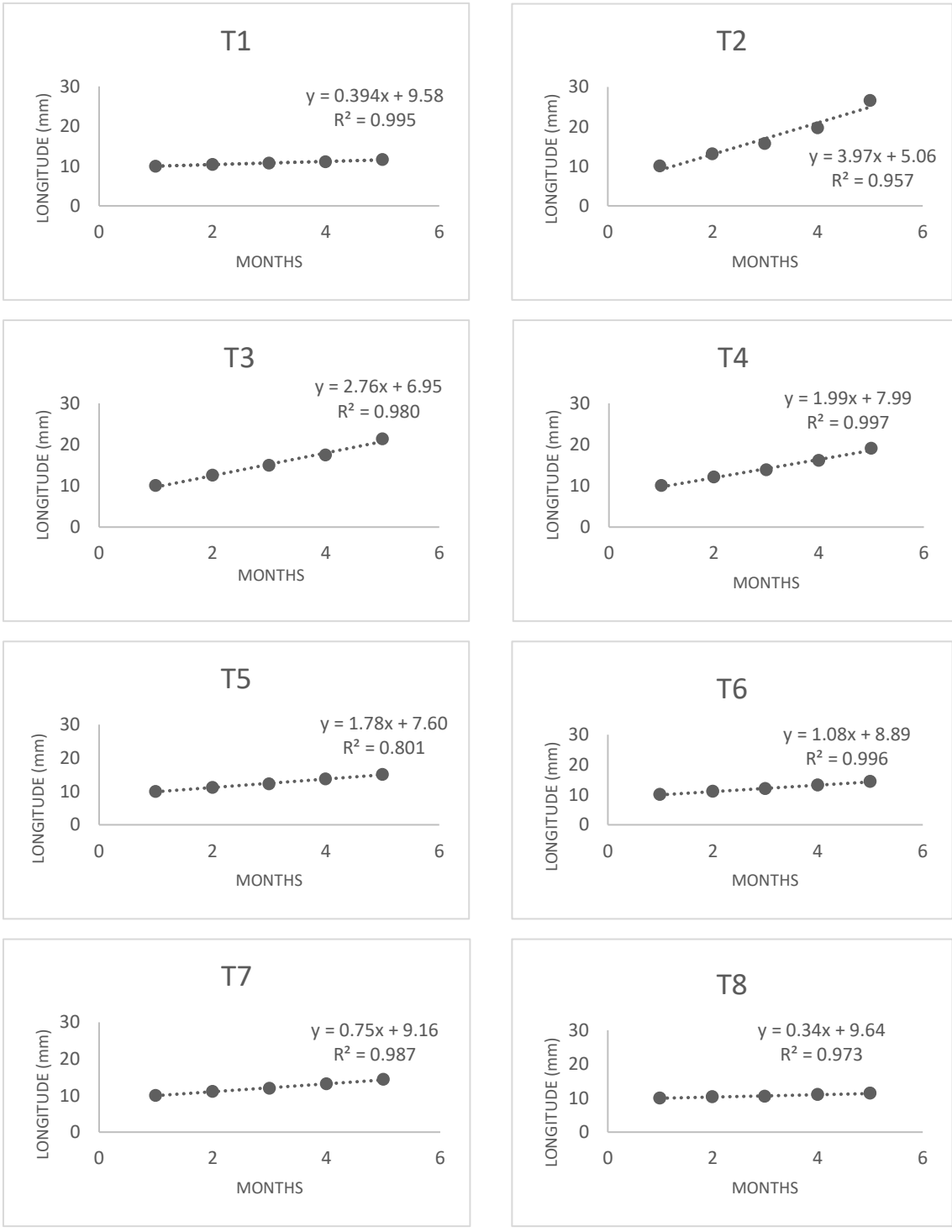

Figure 2. Growth behavior of hybrids of *V. planifolia* in different concentrations of mannitol and sucrose in *in vitro* culture medium. mm = millimeters. T1, T2, T3, T4, T5, T6, T7 and T8 = Treatments.

Chilean Journal of Agricultural Research, 68, 304-308. http://dx.doi.org/10.4067/S0718-58392008000300011

Bello, B.J.J., García, G.G.G., & Iglesias, A.L. (2015). Conservación de vainilla (*Vanilla planifolia* Jacks.) bajo condiciones de lento crecimiento *in vitro*. Revista Fitotecnia Mexicana, 38(2), 165-171. https://www.revistafitotecniamexicana.org/contacto.html

Bonilla, M.M.M., Mancipe, M.C., & Aguirre, M.A.C. (2015). Conservación *in vitro*: una perspectiva para el manejo de los recursos fitogenéticos. Revista de Investigación Agraria y Ambiental, 1(6), 67-81. https://doi.org/10.22490/21456453.1264

Borges, M., Meneses, S., Vázquez, J., & García, M. (2003). Conservación *in vitro* de Germoplasma de *Dioscorea alata* L. por crecimiento mínimo. Plant Genetic Resources Newsletter, 133, 8-12.

Bory, S., Grisoni, M., Duval, M.F., & Besse, P. (2008). Biodiversity and preservation of *Vanilla*: present state of knowledge. Genetic Resources and Crop Evolution, 55, 551-571. https://doi.org/10.1007/s10722-007-9260-3

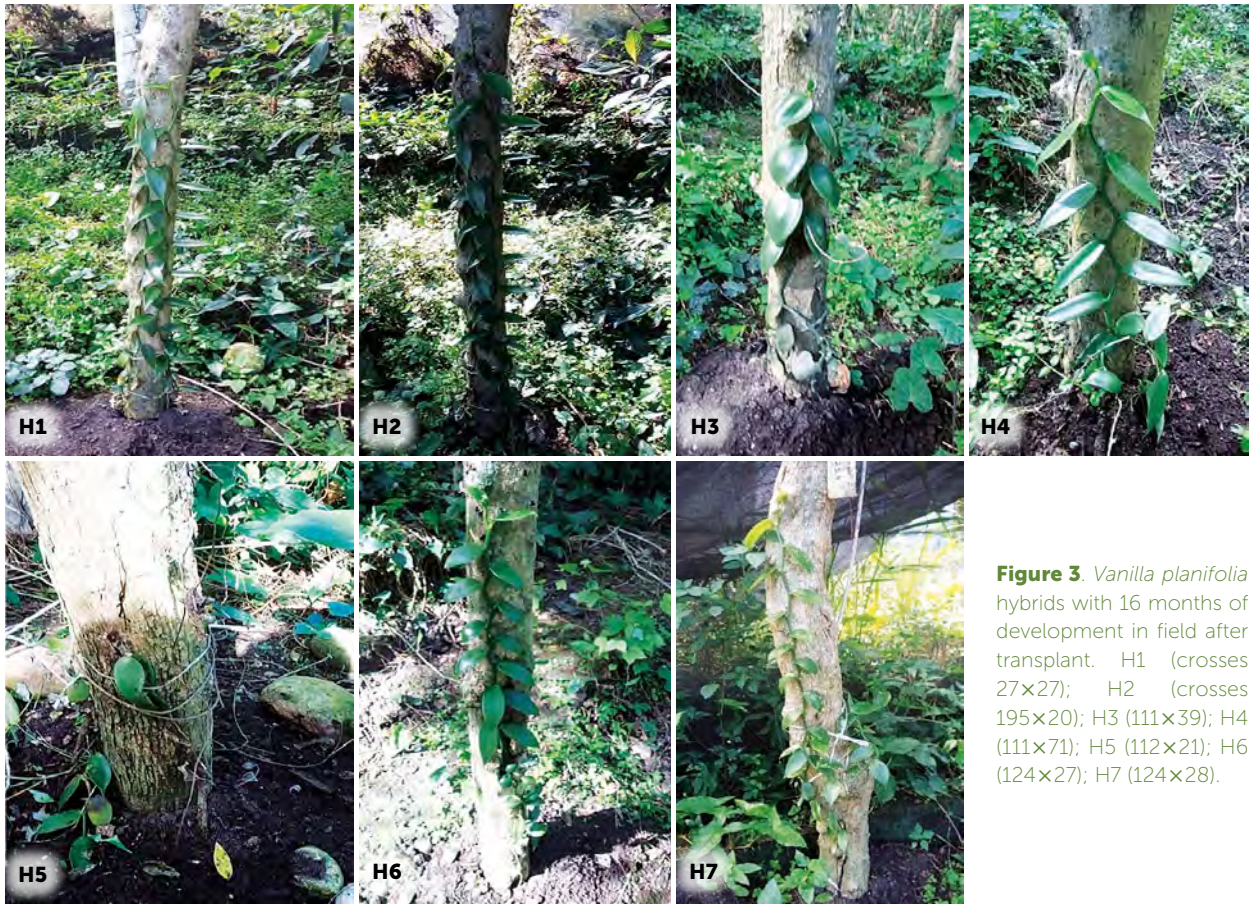

Figure 3. *Vanilla planifolia* hybrids with 16 months of development in field after transplant. H1 (crosses 27×27); H2 (crosses 195×20); H3 (111×39); H4 (111×71); H5 (112×21); H6 (124×27); H7 (124×28).

Bouétard, A., Lefeuvre, P., Gigant, R., Bory, S., Pignal, M., Besse, P., & Grisoni, M. (2010). Evidence of transoceanic dispersion of the genus *Vanilla* based on plastid DNA phylogenetic analysis. Molecular Phylogenetics and Evolution, 55, 621-630.

Damián, P.A. (2019). *Vanilla yanesha* (orchidaceae) a new species of the membranaceous-leaved group from the central rainforest of Peru. Willdenowia, 49, 5-9. https://doi.org/10.3372/wi.49.49101

Divakaran, M., Babu, K.N., & Peter, K.V. (2006). Conservation of *Vanilla* species, *in vitro*. Scientia Horticulturae, 110, 175-180. DOI: 10.1016/j.scienta.2006.07.003

Engelmann, F., & González, A.M.T. (2013). Introducción a la conservación *ex situ* de los recursos genéticos vegetales. *In*: F. Engelman, & A.M.T. González, (Eds). Crioconservación de plantas en América Latina y el Caribe. (pp. 25-35). San José, Costa Rica, IICA.

Ertola, R.J., Giulietti, A.M., & Castillo, F.J. (1995). Design, Formulation, and optimization of media. In J.A. Asenjo, & J.C. Merchuk. (Eds). Bioreactor System Design (pp. 89-137). Marcel Dekker, New York. USA.

Espinosa, N., Estrada, R., Tovar, P., Bryan, J., & Dodds, J.H. (1986). Tissue culture micropropagation, conservation and export of potato germplasm. Specialized Technology Document 1. International potato Center, Lima, Peru.

FAO, Food Agricultural Organization. (1995). Conservación y utilización sostenible de los recursos fitogenéticos de América Central y México. Conferencia Técnica Internacional sobre los Recursos fitogenéticos. San José, Costa Rica.

Flores, J.A., Reyes, L.D., Jimenez, G.D., Romero, A.O., Rivera, T.J.A., Huerta, L.M., & Pérez, S.A. (2017). Diversidad de *Vanilla* spp. (Orchidaceae) y sus perfiles bioclimáticos en México. Revista Biologia Tropical 65(3), 975-987. http://dx.doi.org/10.15517/rbt.v65i3.29438.

Fortes, G.R., & Scherwinski, P.J.E. (2001). *In vitro* storage of potato under acetyl salicylic acid and two carbohydrate sources. Pesquisa Agropecuaria Brasileira. 36, 1261-1264. http://dx.doi.org/10.1590/S0100-204X2001001000007.

Freuler, M.J. (2007). Orquídeas. Argentina. Albatros.

García-Águilar, L., De Feria, M., & Acosta, K. (2007). Aspectos básicos de la conservación *in vitro* de germoplasma vegetal. Biotecnología Vegetal, 2(7), 67-79.

Gätjens, B.O., Acuña, M.C.L., Montero, C.W., Díaz, C., & Torres, S. (2018). Propagación masiva y formación de callos protocórmicos de vainilla a partir de ápices radicales. Polibotánica, 45, 157-180. http://dx.doi.org/10.18387/polibotanica.45.12.

Gianní, S., & Sottile, F. (2015). *In vitro* storage of plum germplasm by slow growth. Horticultural Sciences, 42, 61-69. doi: 10.17221/186/2014-HORTSCI

Gigant, R., Bory, S., Grisoni, M., & Besse, P. (2011). Biodiversity and evolution in the *Vanilla* genus. *In*: O. Grillo, & G. Venora, (Eds), The dynamical processes of biodiversity - case studies of evolution and spatial distribution. (pp.1-26). Francia: InTech. DOI: 10.5772/24567

Greule, M., Tumino, L., Kronewald, T., Hener, U., Schleucher, J., Mosandl, A., & Keppler, F. (2010). Improved rapid authentication of vanillin using 813C and 82H values. European Food Research and Technology, 231, 933-941.

Hassan, N.A., Stino, R.G., Gomaa, A.H., & Al-Mousa, R.M. (2014). *In vitro* medium-term germplasm conservation and genetic stability of grape (*Vitis vinifera* L.). Journal of Horticultural Science & Ornamental Plants, 6(1), 9-17. DOI: 10.5829/idosi.jhsop.2014.6.1.1133

Hágsater, E., Soto, A.M.A., Salazar, CH.G.A., Jimenez, M., López, R.M.A., & Dressler, R. L. (2005). Las Orquídeas de México. Instituto Chinoín, México D.F.

Hipólito-Romero, E. (2010). Modelo de intervención con enfoque ecosistémico para el desarrollo empresarial rural de pequeños productores: Estudio de caso en la región Totonaca del Estado de Veracruz, México. Tesis de posgrado en Ecología Tropical. Centro de Investigaciones Tropicales (CITRO), Universidad Veracruzana. Xalapa de Enríquez, Veracruz. México. 211 pp.

Lemoine, R.S., La Camera, S., Atanassova, R., Dédaldéchamp, F., Allario, T., Pourtau, N., Bonnemain, J.L., Laloi, M., Coutos, T.P., Maurousset, M.L., Faucher, M., Girousse, C., Lemonnier, P., Parrilla, J., & Durand, M. (2013). Source-to-sink transport of sugar and regulation by environmental factors. Frontiers in Plant Science, 4, 1-21. DOI:10.3389/fpls.2013.00272

López-Juárez, S.A., Hipólito-Romero, E., Cerdán-Cabrera, C.R., Ortiz-Ceballos, G.C. & Reyes-López, D. (2019). Asociación entre cultivos de cacao (*Theobroma cacao* l.) y vainilla (*Vanilla planifolia* Jacks. ex Andrews) en un sistema agroforestal en COMALCALCO, TABASCO. Tropical and Subtropical Agroecosystems, 22, 613-629.

López-Puc, G. (2013). An effective *in vitro* slow growth protocol for conservation of the orchid *Epidendrum chlorocorymbos* SCHLTR. Tropical and Subtropical Agroecosystems, 16, 61- 68.

Luis-Rojas, S., Ramírez-Valverde, B., Díaz-Bautista, M., Pizano-Calderón, J., & Rodríguez-López, C. (2020). La producción de vainilla (*Vanilla planifolia*) en México: análisis y pronóstico. Revista Mexicana de Ciencias Agrícolas. 1:175-187. DOI: https://doi.org/10.29312/remexca.v11i1.2065

Márquez, S. F. (1988). Genotecnia vegetal métodos, Teoria, resultados. Tomo II, AGT EDITOR, S. A. México, D. F.

Menchaca, G.R.A., Ramos, P.J.M., Moreno, M.D., Luna, R.M., Mata, R.M., Vázquez, G.L.M., & Lozano, R.M.A. (2011). Germinación *in vitro* de híbridos de *Vanilla planifolia* y *V. pompona*. Revista Colombiana de Biotecnología, 13(1), 80-84. DOI: 10.15446/rev.colomb.biote

Molineros, H.F., González, M.R.T., Flana, G.N., & Tupac, O.J. (2014). *Vanilla rivasii* (Orchidaceae), A new species from the Colombian pacific region. LANKESTERIANA. 13(3), 353-357. DOI 10.15517/lank.v13i3.14423.

Montalvo, P.M.C., Iglesias, A.L.G., Mijangos, C.J.O., Nahuat, D.S.L., Barahona, P.F., Canto, F. A., & Santana, B.N. (2007). *In vitro* germplasm conservation of habanero pepper (*Capsicum chinense* Jacq.). Hortscience, 42, 1247-1252. DOI:https://doi.org/10.21273/HORTSCI.42.5.124

Muñoz, M., Díaz, O., Reinún, W., Winkler, A., & Quevedo, R. (2019). Slow growth *in vitro* culture for conservation of *Chilotanum* potato germplasm. Chilean Journal of Agricultural Research, 79(1), 26-35. http://dx.doi.org/10.4067/S0718-58392019000100026.

Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture, Physiologia Plantarum, 15, 473-497.

Pence, V.C., Sandoval, J.A., Villalobos, V.M., & Engelmann, F. (Eds.). (2002). *In vitro* collecting techniques for germplasm conservation. IPGRI Technical Bulletin No. 7. International Plant Genetic Resources Institute, Rome, Italy.

Pierik, R.L.M. (1990). Cultivo *in vitro* de plantas superiores. Departamento de Horticultura y Agricultura, Universidad de Wageninigen, Edit. Mundi-Prensa.

Ramos-Castellá, A., Iglesias-Andreu, L., Martínez-Castillo, J., Ortíz-García, M., Andueza-Noh, R., Octavio-Aguilar, P., & Luna-Rodriguez, M. (2016). Evaluation of molecular variability in germplasm of vanilla (*Vanilla planifolia* G. Jackson in Andrews) in Southeast Mexico: Implications for genetic improvement and conservation. Plant Genetic Resources, 15(4), 310-320. DOI: 10.1017/S1479262115000660

Raya, M.Y.A., Villegas, M.A., & Arellano O.G. (2009). Cinética de enraizamiento *in vitro* de portainjertos de vid en respuesta a la fuente y concentración de azúcar. Revista Fitotecnia Mexicana, 32(2), 111-117.

Rayas, A., Cabrera, M., Santos, A., Basail, M., López, J., Medero, V. & Beovides, Y. (2013). Efecto del manitol y el nitrato de plata en la conservación *in vitro* de la malanga (*Xanthosoma* spp.). Revista Colombiana de Biotecnología, 15(1), 167-171. Disponible en: <https://revistas.unal.edu.co/index.php/biotecnologia/article/view/28620/42015>.

Reyes, L.D., Flores, J.A., Huerta, L.M., Kelso, B.H.A., Avendaño, A.C.H., Lobato, O.R., Aragón, G.A., & López, O.J.F. (2014). Variación morfométrica de fruto y semilla en cuatro especies del género Vanilla. Ecosistemas y Recursos Agropecuarios, 1(3), 205-218.

Rukundo, P. (2012). Comparative study of effects of table sugar, Laboratory grade sucrose and manitol on growthof banana plantlets under *in vitro* condition. Rwanda Journal, 76(28), 76-83. DOI: 10.4314/rj.v28i1.6

Santillán-Fernández, A., Trejo, C.M., Martínez, S.A., Martínez, A.L., Vásquez, B.N., Luis, M.S. (2019). Potencial productivo de Vanilla planifolia Jacks en el Totonacapan, México, mediante técnicas geográficas. Revista Mexicana de Ciencias Agrícolas, 4(10), 789-802.

SAS Institute. (2002). User´s Guide of SAS (Statistical Analysis System). SAS Institute Inc. Cary, N. C. USA.

Sarasan, V., Cripps, R., Ramsay, M.M.C., Atherton, M.M., Prendergast, G., & Rowntree, J.K. (2006). Conservation *in vitro* of threatened plants-progress in the past decade. *In Vitro* Cellular and Developmental Biology-Plant, 42, 206-214. https://doi.org/10.1079/IVP2006769

Soto, A.M.A. (2003). Vanilla. In A.M. Pridgeon, P.J. Cribb, M.W. Chase, & F.M. Rasmussen. (Eds.). Genera Orchidacearum. Orchidoideae (Part two), Vanilloideae. (pp. 321-334). Oxford University Press.

Staritsky, G., Dekkers, A.J., Louwaars, N.P., & Zandvoort, E.A. (1985). *In vitro* conservation of aroid germplasm at reduced temperatures and under osmotic stress. In L.A. Withers, P.G. Alderson, (Eds.). Plant Tissue Culture and its Agricultural Applications. Butterworths, London, UK.

Stoop, J., Williamson, J., & Pharr, D.M. (1996). Mannitol metabolism in plants: a method for coping with stress. Trends Plant Science. 1:139-144. https://doi.org/10.1016/S1360-1385(96)80048-3

Ziv, M. (2000). Bioreactor technology for plant micropropagation. In, J. Janick, (Ed.). Horticultural Reviews, (Vol. 24). (pp. 1-30). John Wiley & Sons, Inc. New York.