Asymbiotic seed germination and *in vitro* seedling development of *Epidendrum radicans* Pav. Ex Lindl. (Orchidaceae).

María Dolores García-Suárez[1] *, Yareli Odemaris Buendía López[1], José Ángel Lechuga Corchado [2] and Héctor Serrano[3].

[1]Department of Biology, [2] Department of Biotechnology and [3] Department of Health Sciences Universidad Autónoma Metropolitana, Iztapalapa. México City, México.
Ave. Ferrocarril San Rafael Atllixco 186, Mexico City 09340, México

ABSTRACT

Epidendrum radicans is a terrestrial orchid native to México. Here we evaluated its in vitro seed germination and early protocorm development under the effects of twelve asymbiotic media combinations consisted of three basic formulations (Minimum Medium, Murashige & Skoog and Knudson C) supplemented with four naphthaleneacetic acid concentrations as plant growth regulator. Maximum seed germination percentage where an improved seed germination of 48% was achieved, was Knudson C media added with 0.1 mg/L NAA, whereas under 3.0 mg/L, no growth was observed. The best media for asymbiotic seed germination, protocorm growth and leaf development for *Epidendrum radicans* in order to contribute to its conservation, was obtained with Knudson C supplemented with 0.1 mg/L NAA or without growth regulators as well as with MS medium supplemented with 1.0 mg/L NAA or without growth regulators. SEM micrographs were taken on the five different stages observed during germination and protocorm development. It is known that each orchid species need special peculiarities for their seed germination and protocorm development, and the description of its growth and development is important as well, Epidendrum radicans is not exception, its mass propagation is in urgent need, as it is an economically important orchid, with commercial demands that need mass propagation for its conservation , also due to a continuous destruction of its natural habitat, unauthorized trade, ruthless collection, many orchid species in nature are disappearing.

Key words: Orchidaceae, *Epidendrum radicans*, seeds, *in vitro* germination.

Introduction

Orchidaceae is one of the largest families of flowering plants consisting of 27,801 species under 736 genera (The Plant List, 2014; Chase et al., 2015), the largest for monocotyledons. *Epidendrum* with around 1,435 species, is known as one of the largest genus of orchid family (Chase et al., 2003) now considered in Epidendroideae subfamily, Epidendreae clade (Chase et al., 2003; Chase et al., 2015) the species of this genera can be found in America from Florida down to Argentina. Most of its species are epiphytes or litophytic, sometimes terrestrial (Stevens et al., 2001). *Epidendrum radicans* (Pav. Ex Lidl), is a terrestrial native species from México. Several floricultural aspects made it an important orchid, either as an ornamental plant or as breeding parent, due to its striking red-orange flowers known as fire stars that bloom several times over the season (Pateli et al. 2003). In Mexico, *Epidendrum* plant population is distributed along the coast of Gulf of México from the states of San Luis Potosí down to Chiapas, including Oaxaca (García-Cruz and Sánchez, 1999; Hágsater et al. 2005). Once pollinated, orchids typically form capsules, produce 0.1-6 mm microscopic seeds, commonly known as "dust-seeds" (Arditti and Ghani 2000; Arditti, 2008; Zeng et al., 2014). Orchids have associations with mycorrhizal fungi that are considered necessary for seed germination, as wells as for their growth and development (Rasmussen, 1995), mycorrhizal fungi *Tulasnella*, *Ceratobasidium*, *and Sebacina* have been observed to promote seedling development in *Epidendrum secundum* (Pereira et al., 2014; Durán-López et al., 2019). As orchid seed germination occurs, embryo starts a general growth pattern common to all orchid species, it initiates with seed imbibition, embryo emergence from seed coat or testa, protocorm formation sometimes covered with rhizoids in its basal surface, and with a later radicle growth, leaves are at protocorm apical meristem (Veyret, 1974; Stewart et al., 2003).

Orchid seed germination is complex. As they lack endosperm, require specific mycorrhizal association for their *in-situ* seed germination in natural habits, which provide embryo with the necessary nutrition, to germinate, seedling development and establishment (Otero et al., 2002; Otero et al., 2004; Arditti, 2008; Dearnaley et al., 2013; Otero et al., 2013). In *Epidendrum secundum* symbiotic germination has been achieved showing that mycorrhizal fungi promote seedling development as well (Pereira et al., 2014; Durán-López et al., 2019). Under *in vitro* conditions, nutrients are supplied by culture media such as Knudson C and Murashige and Skoog, which have different concentrations of macro and micro nutriments, and supplemented with plant growth regulators such as cytokinins, auxins, gibberellins,

brassinoesteroids, jasmonates thidiazuron, salicylic acid and supplemented with activated charcoal, coconut water, or banana slurps, that might enhance orchid seed germination, growth and development (Arditti, 2008;
Abraham et al., 2012; Zeng et al., 2014; Teixeira et al., 2015). Each species has its own need for germination, and little is known particularly for this *Epidendru*m species. Here we propose an asymbiotic *in vitro* seed germination and seedling development protocol for *Epidendrum radicans* employing three different media as well as the effect of naphthaleneacetic acid (NAA) that might also facilitate protocorm development which will be observed under stereomicroscope and with a scanning electron microscopy (SEM). This might establish an effective *in vitro* propagation system for a large-scale propagation of *E. radicans* that might meet commercial needs and reestablish natural populations.

Material and Methods.

Seed source and sterilization. *Epidendrum radicans* seeds were obtained from mature capsules prior to dehiscence, which were generated by auto pollination from plants of a private collection. Capsules were dried and stored in paper bags in darkness at 20°C prior to use. Seeds were surface sterilized by a 5 min immersion in a 6% hypochlorite water solution, followed by three repetitive 1 min water rinses. After seed surface sterilization, seeds were rinsed twice for 1 min each in sterile distilled water.

Asymbiotic culture media. Three solid basal media were examined for their effectiveness in promoting germination and protocorm development of *E. radicans*: Minimum Hoagland medium and two commercial prepared media by Sigma Chemical Co. (St. Louis, MO): Murashige - Skoog (MS) medium and modified Knudson C (KC) medium (Murashige and Skoog 1962; Knudson 1946). All media were added with the proper amount of stock solution of naphthaleneacetic acid (NAA) to obtain 0.0, 0.1, 1.0 and 3.0 mg/L. making a total of 12 nutrient media combinations. All media contained 0.8% agar 3% sucrose, adjusted to pH 5.8 prior to autoclaving at 120 kPa for 20 min at 121ªC. The 12 autoclaved media were dispensed as 10 ml aliquots into a 6 multi-well dish 128 x 86 mm with a culture area of 9.6 cm^2 (Nunc, Thermo Scientific).

Seed Inoculation. In a laminar airflow chamber, at least 100 surface sterilized seeds were placed into the center of each well and evenly spread on the medium using a sterile spatula. Each media combination was tested in triplicate wells in the same plate and maintained at 30 ±2ºC in a 16/8 h photoperiod for 12 weeks.

Evaluation of germination and seedling development. Seeds were visually daily checked with a stereomicroscope (Zeiss Stemi DV4) at 20x magnification to assess seed germination and protocorm development. To evaluate progress, a modified scale done for a terrestrial orchid by Stewart and Kane (2006) of five different stages scale, was used. Three Stages for seed germination were defined and the last two were used for seedling growth: 1) Imbibition or hydration, 2) initial protocorm-like body structure or swelled embryo 3) germination, as the time where initial protocorm like bodies or embryo enlargement causes rupture of testa, 4) first leaf or foliar primordium emergence 5) leaf elongation. Germinated and non-germinated seeds were counted. In vitro seedling development were scored when emergence of first leaf, as well as for leaf elongation, stages 4 and 5. Effect of treatments were recorded after two weeks of incubation.

Scanning electron microscopy (SEM). Seed morphology, germinative and developmental characteristics were followed by scanning electron microscopy micrographs techniques according to Munien et al. (2015). Criteria for evaluation of *Epidendrum radicans* into each experimental stage was from Stewart et al., (2003), Stewart and Kane (2006), and Barthlott et al., (2014). In short, fresh material from seeds, germinated, grown protocorm, and developed leaves from *Epidendrum radicans* were fixed in 3% glutaraldehyde, washed in phosphate buffer, dehydrated with gradual increased alcohol, and kept for 8 hours in absolute alcohol. Samples were critic point dried by using a Tousimis critical point dryer (Tousumis, Rockville, MD). Orchid materials were mounted over aluminum slides using a double carbon tape and covered with a gold-palladium for 80 seconds (under a 0.5 Torr vacuum for 40 min) in a Dentron Vacumm Desk III (Dentrum, Moorestone, NJ). Samples were observed and scanning micrographs of orchid seeds, germination and their growth were obtained using a JSM-5900LV scanning electron microscope (JEOL, Tokyo, Japan).

Statistical analysis. Germination percentages were calculated by dividing the number of germinated seeds in every individual well by the total number of viable seeds in each well. Statistical data were analyzed using a two-way ANOVA and Tuckey facilities of the NCSS 2007 software. Statistical significance level was taken at $p< 0.05$.

Results.

Asymbiotic media comparison and auxin effect. *Epidendrum radicans* seeds germinated on eleven of the twelve proven media. The exception was minimum Hoagland media added with 3.0 mg/L NAA, where embryo turned white and stopped growth, as shown in Figure 1,

Seeds began swelling within a week after medium inoculation; for most tested media combinations, germination started after two weeks. Response to NAA treatment was quite similar for MS and Knudson C media since the highest NAA concentrations, the largest the time for germination to start., a 0.1 mg/L proved to be the most effective treatment since on day 16 the first germinated seeds were shown. Germination in other NAA concentrations took longer time. This behavior was also found in KC-NAA combinations where the use of 1 mg/L NAA gave the conditions for germination at day 16 whereas the other NAA additions resulted in longer germination time. The most conspicuous media was MS. In this case, germination showed an inverse dose-dependent behavior since higher NAA amounts reduced the germination starting time, as can be seen in Figure 2.

Seed germination percentages. Final *E. radicans* seed germination percentages were obtained 90 days after sowing, for maximal seedling number on the three media tested: minimum Hoagland Medium, Knudson C or MS were obtained when added with 0.01 mg/L NAA concentrations, and in all three media tested, higher concentration of NAA lowered germination percentages. Nevertheless, significant differences were obtained when comparing NAA concentrations as well as between the three different media (P's<0.0001), indicating that such differences depend on NAA concentration (P=0.000003 of interaction), as shown in Table 2.

The interaction between the growth regulator and media culture showed the best germination percentage on 0.1 mg/L NAA Knudson C media (Table 3). *In vitro* seedling development effects of media effects on all developmental data were statistically analyzed, using ANOVA.

To obtain morphological characteristics on seed germination and protocorm development in *Epidendrum radicans*, the use of scanning electron micrographs showed the most conspicuous characteristics of *E. radicans* asymbiotic development, based on a modified Stewart and Kane (2006) five stages description and as can be seen on Table 4, Stages 1 and 2 were reached in all proven treatments, Stage 3 germination, was not achieved at Minimun media with 3.0 mg/L NAA, Stage 4 leaf emergence, obtained in 8 proven media and Stage 5 leaf elongation, were reached only on three media. The scanned micrographs showed: Stage 1. Imbibition, is highly conspicuous (Figure 3a), the enlarged photo showed the rectangular or polygonal forms on the seed testa (Figure 3b). Stage 2. protocorm, except for the use of 3.0 mg/L NAA in minimal Hoaghland medium, the presence of spherical cells out of the testa identifies the protocorm like bodies structures, (Figure 3c) and show the start of the differentiation process (Figure 3d). Stage 4. The emergence of the foliar primordium can be observed on (Figure 3e) and on Stage 5. Elongation of leaves coming from the apical zone of the protocorm (Figure 3f). These last two stages were reached only on those media where the highest germination percentages were obtained. For Stage 4, the best media was minimum Hoagland medium either with 1.0 mg/L or no NAA addition and Knudson C supplemented with 3 mg/mL NAA. Embryo development was induced in Hoagland minimum medium or Knudson C but not on MS media. Stage 5 was achieved on MS media added with 0.0 y 1.0 mg/L NAA and on Knudson C media without NAA.

Discussion.

Germination and protocorm development. Orchidaceae is one of the most endangered plant families, principally terrestrial orchids are considered as a very vulnerable group due to their difficulty to germinate in the absence of mycorrhizal fungi. (Durán-López et al., 2019). The use of *in vitro* propagation techniques is a good alternative to cultivate this type of orchids from seeds, particularly in *in vitro* asymbiotic systems, which also play an important role in its conservation (Arditti, 2008). Asymbiotic conditions *in vitro* with different media and plant growth regulators enhance the germination of the very special orchid seeds, that lack of endosperm and present a slow growth, indicating probably the need of a mycorrhizal symbiosis for their germination, as in natural conditions (Chen et al., 2019). Asymbiottic seed germination and seedling development of *Epidendrum radican*s a terrestrial orchid can be achieved as for other orchid species, with the selection of a proper medium (Arditti et al., 1979; Arditti et al., 1980; Arditti & Ghani, 2000).

Epidendrum radicans initiated germination after 16-31 culture days, depending on media composition and NAA combination employed. In Knudson C media added with 0.1 mg/L NAA, we obtained the highest (48%) germination percentage. Similar results were obtained on *Vanda dearei* where 20% seed germination was obtained in Knudson C medium and increased up to 48% when 1.0 mg/L NAA was added (Jualang et al. 2014).

It has been suggested that for a better terrestrial orchid conservation the use of mycorrhizal fungi might improve both the method as well as the physiological response of the plantlets (Zettler, 1997; Zettler et al., 2013). However, by now it is difficult to generalize the use of mycorrhizal fungi. When mycorrhizal symbionts were included in *Habeneria macroceratitis*, they inhibited protocorm development (Stewart & Kane, 2006). In general, most studies that include symbionts, have shown contradictory results for *in vitro* seed germination and plantlet development.

These results have been attributed to the use of non-specific mycorrhizal symbionts since generic fungi do not give standard results or even have deleterious results. Currently, we lack enough knowledge on specific symbionts for orchids (Zettler, 1997; Otero & Bayman, 2009).

As shown in Figure 1, supplemented media with the highest NAA concentration do not support nor induce seed germination regardless the media formulation. When germination occurred in these media, the protocorm turned white and died. This phenomenon was also observed for another terrestrial orchid *Bipinnula pennicillata* under asymbiotic conditions in half power MS medium, even though after 61 days a 49% germination percentage was obtained, their development was slower than for epiphytic species (Arditti & Ernst, 1993; Dalzotto & Lallana, 2015). Taken together, these results show that for terrestrial orchid species, one disadvantage of asymbiotic seed germination is the difficulty to find the appropriate culture medium for its appropriate asymbiotic seed germination. Other breakthrough is the susceptibility to contamination with pathogenic bacteria or fungi, that some authors indicate that can be diminished with symbiotic culture rather than by inoculation of seeds with mycorrhizal fungi (Porras & Bayman, 2007; Otero et al., 2013).

If germination has been achieved, seeds forms clumps, protocorms and plantlets giving rise to characteristic young protocorm morphology, that might be encapsulated as protocorm like bodies for future propagation programs (Saisprasad & Polisetty, 2003; Yeung, 2017). In our case, all tested media supplemented with 0.1 mg/L NAA showed seedling development. Seeds exposed to MS media added with 0.0, 1.0 mg/L NAA and Knudson C with no NAA reached stages 4 and 5 with leaf development. For other terrestrial orchid species, it has been proven that soluble sugars are required (Rasmussen, 1995).

Epidendrum seed testa morphology show that some seeds are no viable, sometimes empty without embryo, they are transparent with simple wall, ornamented with an opening at the base as for other orchid seed testa (Arditti et al., 1979; 1980; Healey et al., 1980; Gallo et al., 2016). As for many orchid seeds, those of *Epidendrum radicans* are scobiform or fusifom, (Arditti et al., 1979; Gallo et al., 2016). They are particularly considered as very large seeds (3000-6000 µm), whitish in color with a translucid reticulated as in *Laelia eyermaniana* (Nava et al., 2011), *Cattleya walkeriana* (Fernandes Galdiano et al., 2014), *Himantoglossum robertianum* (Aybeke, 2014), *Cattleya loddigesii*, *Cattley*a *tigrina*, *Handrolaelia purpurata*, *Schomburgkia gloriosa* and *Sophronitis cernua* (Gallo et al., 2016). E*pidendrum radicans* present a balloon thread seed with a prominent medial sector extending to form filiform ends. The testa is formed by elongated cells in their longitudinal axis, irregular and rouded at the end (Barthlot et al. *2*014) The anticlinal walls are straight, the transverse wall is prominently elevated (Kurzweil, 1993)

To be able to reintroduce orchid plants in the field, several aspects need to be studied. Information is needed on the ecological specificity of orchids and their associated fungi that could improve the results obtained for orchids cultured *in vitro.* Also, little is known about their seed dispersal and habitat selection; although based on the seed morphology and size, is generally assumed that wind could promote a long-distance dispersion (Van den Broeck et al., 2014), conclusive evidence has not been reported (Arditti & Ghani, 2000). Seed dispersal is essential for successful plant reproduction and adaptation, recent work has found that distance from mother plant is important to obtain the probability of the fungal component near the maternal plants for orchid seeds, so close vicinity is important (Brzosko et al., 2017).

Conclusions

*Epidendrum radican*s is a beautiful orchid, exploited as an ornamental plant, the best media for its germination is Knudson C media supplemented with 0.1 mg/L NAA. For an appropriate round protocorm and plantlet formation MS medium either supplemented with 1.0 mg/L NAA or without any NAA added and Knudson C medium without plant growth regulator proved to be the best.

This investigation contributes for *ex situ* conservation of this species that might fulfill the commercial demand, by using the suggested *in vitro* seed culture techniques. More information is needed to understand its growth and development requirements in the field such as its seedling stage, dormancy process, mycorrhizal needs, contribution to seed bank, as well as to understand other ecological alternatives that might be useful for their gemination under field conditions that might as well contribute to give strategies for their conservation.

ACKNOWLEDGEMENTS

The authors are grateful to Jorge García-Sánchez + for providing mature capsules of *Epidendrum radicans* from its private collection; to Drs. José Sepulveda from the Microscopy Laboratory and Claudia Barbosa Martínez, Biology Department for their technical assistance in taking SEM images and María del Rocío Zarate-Hernández, for statistical assistance at Universidad Autónoma Metropolitana Iztapalapa México.

References

Abraham, S., Augustine J., & Thomas, T. D. (2012). Asymbiotic seed germination and *in vitro* conservation of *Coelogyne nervosa* A. Rich. an endemic orchid Western Ghats. Physiology and Molecular Biology of Plants. 18(3):245-251.

Arditti, J. (2008). Micropropagation of Orchids. (2nd ed.) Blackwell, Cambridge. 756 pp.

Aditti, J., Michaud J. D., & Healey P. L. (1979). Morphometry of orchid seeds: I *Paphiopedilum* and native California and related species of *Cypripedium*. American Journal of Botany 66: 1128-1137. DOI: 10.4236/oje.2014.42005.

Arditti, J., Michaud J. D., & Healey P. L. 1980. Morphometry of orchid seeds: II Native California and realated species of *Calypso, Cephalanthera, Corallorhiza* and *Epipactis.* American Journal of Botany. 67: 347-365. DOI: 10.2307/2442345.

Arditti, J., & Ernst, R. (1993). Micropropagation of Orchids. New York, John Wiley & Sons. 640 pp.

Arditti, J., & Ghani, A. K. A. (2000). Tansley Review no 110. Numerical and physical properties of orchid seeds and their biological implications. New Phytologist. 145:367-421. DOI: 10.1046/j.1469-8137.2000.00587.x

Aybeke, M. (2014). Morphological and histochemical investigations on *Himantoglossum robertianum* (Loisel.) P. Delforge (*Orchidaceae*) seeds. Plant Sistematics and Evolution. 300: 91-97.

Barthlott, W., Große-Veldmann, B., & Korotkova, N. (2014). Orchid seed diversity A scanning electron microscopy survey Botanic Garden and Botanical Museum Berlin-Dahlem . 245pp.

Brzosko, E., Ostrowiecka, B., Kotowicz, J., Bolesta, M., Gromotowicz, A., Gromotowicz, M.G., Orzechowska, A., Orzolek J., & Wojdalska M. (2017). Seed dispersal in six species of terrestrialorchids in Biebrza National Park (NE Poland). Acta Soc Bot Pol 86(3): 3557- doi.org/10.5586/asbp3557

Chase, M.W., Cameron, K.M., Barrett, R.L., & Freudenstein, J.V. (2003). DNA data and Orchidaceae systematics: a new phylogenetic classification. In : Dixon KM, Kell SP, Barrett rl, Cribb PJ, eds. Orchid conservation. Kota Kinabalu: Natural History Publications , 69-89.

Chase, M.W., Cameron KM, Freudenstein, J.V., Pridgeon, A.M., Salazar, G., Vanden Berg, C., & A. Schuiteman. (2015). An updated classification of Orchidaceae. Botanical Journal of the Linnean Society. 177: 151-174.

Chen, L., Wang, Y.C., Qin, L.Y., He, H.Y., Yu, X.L., Yang, M.Z., & Zhang, H.B. (2019) Dynamics of fungal communities during *Gastrodia elatagrowth*. BMC Microbiol. 19*: 158*

Dalzotto, C.A., & Lallana, V.H. (2015). Efecto de la testa en la germinación *in vitro* de *Bipinnula pennicillata* (Rchb. F.) Sisternas & Salazar (Orchidaceae). Investigación Agraria. 17(2): 116-121.

Dearnaley, J. D.W., Martos, F., & Selosse, M. A. (2012). Orchid Mycorrhizas: Molecular Ecology, Physiology, Evolution and Conservation Aspects. In: Esser K., Hock B. (eds.), The mycota (A Comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research). Springer-Verlag. Berlin-Heidelberg. IX:207-230.

Durán-López, M. E., Caroca-Cáceres, R., Jahreis, K., Narváez-Vera, M., & Cazar, M. E. (2019). The mycorrhizal fungi *Ceratobasidium* sp. and *Sebacina vermifera* promote seed germination and seedling development of the terrestrial orchid *Epidendrum secundum* Jacq. SouthAfr. J. Bot. 125: 54–61.

Fernandes Galdiano R., Cibele Mantovani J., Soores Gomes E., Custodio Gasparino E. C., Vitti Moro F., & de Macedo Lemos E. G. (2014). Seed germination morphology, and *in vitro* growth of *Cattleya walkeriana* Gardner under different culture media. Comunicata Scientiae 5: 456-463.

Gallo, F.R., Souza, L. A., Milaneze, G. M. A., & Almeida O. J. G. (2016). Seed structure and *in vitro* seedling development of certain Laeliinae species (*Orchidaceae*). Revista Mexicana de Biodiversidad. 87: 68-73.

García-Cruz, J., & Sánchez L. (1999). *Orchidaceae* II. *Epidendrum*. Flora de Veracruz. Fascículo 112. Instituto de Ecología, A. C. México. 1-9.

Hágsater, E., Soto, M., Salazar, G. A., Jiménez, R., López, M., & Dressler R. L. (2005). Las orquídeas de México. Instituto Chinoín, México. 302 pp.

Healey, P. L., Michaud J. D., Arditti J. (1980). Morphometry of orchid seeds. III. Native California and related species of *Goodyera, Piperia, Platanthera* and *Spiranthes*. American Journal of Botany. 67: 508-518.

Jualang, A. G., Devina, D., Hartinie, M., Sharon, J. S., & Roslina, J. (2014). Asymbiotic seed germination and seedling development of *Vanda dearei*. Malaysian Applied Biology. 43: 25-33.

Knudson, L. (1946). A new nutrient solution for germination of orchid seed. American Orchid Society Bulletin 15: 214-217.

Kurzweil, H. (1993). Seed morphology in Southern African Orchidoideae (*Orchidacea*e). Plant Systematics and Evolution. 185: 229-247.

Munien, P., Naidoo Y., & Naidoo, G. (2015). Micromorphology, histochemmistry and ultastructure of the foliar trichomes of *Withania somnifera* (L.) Dunal (Solanaceae). Planta. 242: 1107-1122.

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

Nava, J. J. F., Jiménez, A. A. R., De Jesús, S. A., Arenas, O. M. L., Ventura, Z. E., & Evangelista L. S. (2011). Estudio de la morfología y aclimatación de plantas *de Laelia eyermaniana* RCHB. F. generadas *in vitro*. Polibotánica. 32: 107-117.

Otero, J. T., Ackerman J. D., & Bayman P. (2002). Diversity and host specificity of *Rhizoctonia*-like fungi from tropical orchids. American Journal of Botany. 89:1852- 1858.

Otero, J. T., Ackerman J. D., & Bayman P. (2004). Differences in mycorrhizal preferences between two tropical orchids. Molecular Ecology. 13:2393-2404

Otero, J.T., & Bayman, P. (2009). Symbiotic vs. asymbiotic seed germination in epiphytic orchids. Germinación simbiótica y asimbiótica en semillas de orquídeas epifitas. Acta Agronómica 58(4): 270-276.

Otero, J. T., Mosquera A. T., & Flanagan N. S. (2013). Tropical orchid mycorrhizae: potential applications in orchid conservation, commercialization, and beyond. Lankesteriana 13:57-63.

Pateli, P.; Papafotiou, M., & Chronopoulos, J. (2003).Influence of *in vitro* culture medium on Epidendrum radicans seed germination, seedinggrowth and ex vitro establishment. Acta Hortic. 616: 189–192.

Pereira, M. C., Coelho, I. S., Valadares, R. B. S., Oliveira, S. F., Bocayuva, M., Pereira, O. L., Araújo, E. F., & Kasuya, M. C. M. (2014). Morphologicaland molecular characterization of Tulasnella spp. fungi isolated from the roots of Epidendrum secundum, a widespread Brazilianorchid. Symbiosis 62: 111–121

Porras, A. & Bayman, P. (2007). Mycorrhizal fungi of *Vanilla:* diversity, specificity, and effects on seed germination and plant growth. Mycol. 99:510 - 525.

Rasmussen, H. N. (1995). Terrestrial orchids from seed to mycotrophic plant. Cambridge, Cambridge University Press. Cambridge Massachussets.

Saiprasad, G., & Polisetty, R. (2003). Propagation of three orchid genera using encapsulated protocorm-like bodies. *In Vitro Cellular and Developmental Biology-Plant*, 39, 42-48.

Stevens, W. D. C., Ulloa U., Pool, A., & Montiel, O.M. (eds.). (2001). Flora de Nicaragua. Vol. 85, tomos I, II y III. Missouri Botanical Garden Press. St. Louis,Missouri.

Stewart, S. L., Zettler, L. W., Minso, J., & Brown, P.M. (2003) Symbiotic germination and reintroduction of *Spiranthes brevilabris* Lindley, an endangered orchid native to Florida. Selbyana 24:64–70

Stewart, S. L., & Kane, M. E. (2006) Asymbiotic seed germination and in vitro seedling development of *Habenaria macroceratitis* (Orchidaceae) a rare Florida terrestrial orchid. Plant cell Tiss. Organ Cult. 86: 147-158.

Teixeira da Silva, J. A., Tsavkelova, E. A., Bun, N. T., Parthibhan, S., Dobránszki, J., Cardoso J. C., Rao, M. V., & Zeng S. (2015). Asymbiotic *in vitro* seed propagation of *Dendrobium*. Plant Cell Reports. 34: 1685-1706.

The Plant List. (2014 april). http://www.theplantlist.org/

Vanden Broeck, A., Van Landuyt, W., Cox, K., de Bruyn, L., Gyselings, R., Oostermeijer, G., Vakebtubm. B., Bozic, G., Dolinar, B., Illyés, Z., & Mergeay, J. (2014). High levels of effective long-distance dispersal may blur ecotypic divergence in a rare terrestrial orchid. BMC Ecology 14:20. doi.org/10.1186/1472-6785-14-20

Veyret, Y. (1974). Development of embryo and young seedling stages of orchids. In: Withner C. L. (eds.). The orchids: Scientific studies. John Wiley and Sons Ltd. New York. 223-265.

Yeung, E.C. (2017). A perspective on orchid seed and protocorm development. Bot Stud. 58: 33 -47. DOI 101186/s40529-017-0188-4

Zettler, L. W. (1997) Terrestrial Orchid Conservation by symbiotic seed germination: techniques and perspectives. Selbyana 18 (2): 188-194.

Zettler, L. W., Corey, L.L., Jacks, A.L., Gruender L.T.& López A.M. (2013). *Tulasnella irregularis* (basidiomycota: Tullasnellaceae) from rotos of *Encyclia tampensis* in south Florida, and confirmation of its mycorrhizal significance through symbiotic seed germination. Lankesteriana 13(1–2): 119—128.

Zeng, S., Zhang, Y., Teixeira da Silva, J. A., Wu, K., Zhang, J., & Duan, J. (2014). Seed biology and *in vitro* seed germination of *Cypripedium* Crit Rev Biotechnol, 2014; 34(4): 358–371 DOI: 10.3109/07388551.2013.841117

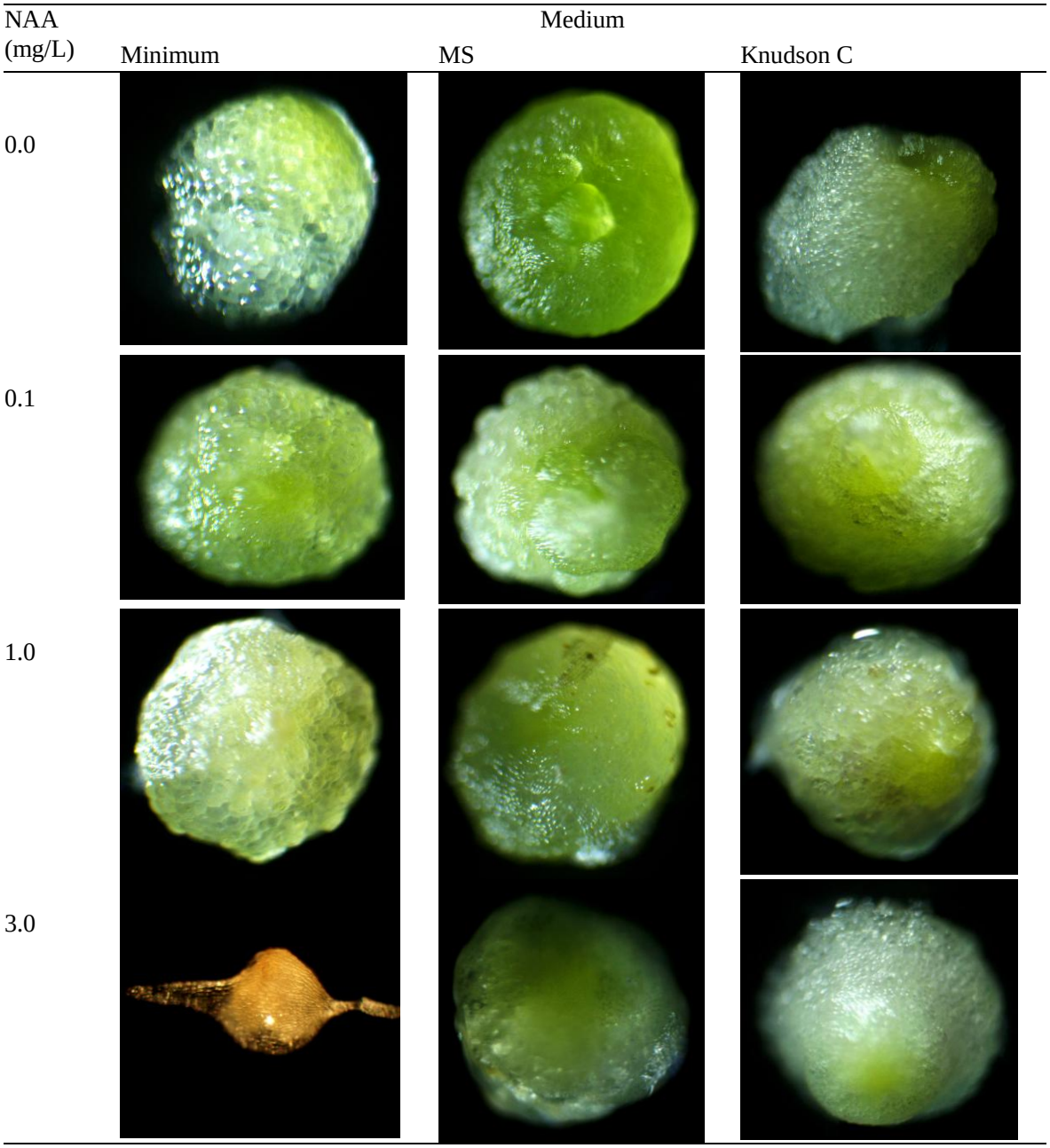

Figure 1. ***Epidendrum radicans*** **germinated seeds under different NAA (mg/L) concentrations and media. Micrographs obtained with optic microscope camara Omax A35100U at 40 diam.**

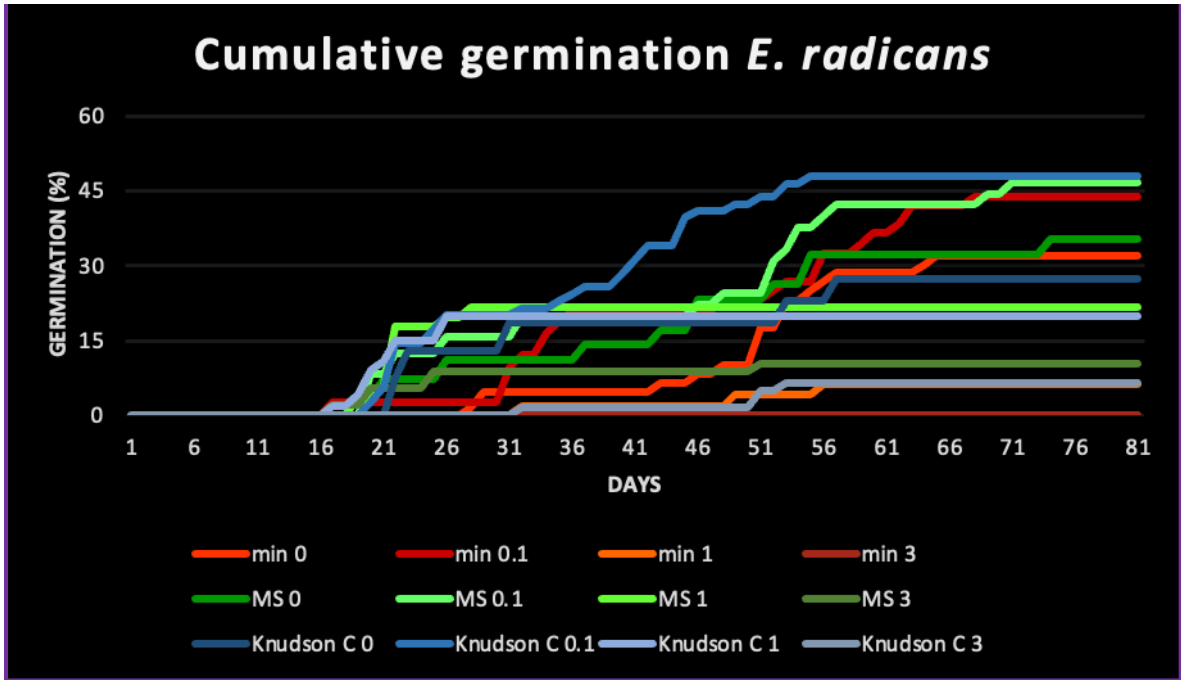

Figure 2. *Epidendrum radicans*, seed germination Effect of culture media: Minimum, MS and Knudson C added with different NAA concentrations. Data show cumulated seed germination through time with 12 proven media.

Table 1. Mean percentage of Seed germination respect four NAA concentrations proven in *Epidendrum radicans.*

NAA (mg/L)	Mean % of seed germination
0.0	17.29 [a*]
0.1	**26.24** [b]
1.0	11.79 [c]
3.0	3.64 [d]

* Mean values within a column followed by a letter are significative different by Tukey test (P<0.05),

.

Table 2. Mean percentage of seed germination respect three proven media on *Epidendrum radicans.*

Culture Media	Seed germination mean%
Minimum	10.22 [a*]
MS	**17.31** [bc]
Knudson C	16.70 [bc]

* Mean values within a column followed by different letter are significative different by Tukey test (P<0.05),

Table 3. *Epidendrum radicans* seed germination mean percentage respect the interaction between NAA concentration and culture media with 12 treatments.

NAA concentration and Media	Mean % seed germination
0.0, Minimum	15.05
0.0, MS	19.21
0.0, Knudson C	17.60
0.1, Minimum	**22.78**
0.1, MS	**25.74**
0.1, Knudson C	**30.20**
1.0, Minimum	3.05
1.0, MS	16.85
1.0, Knudson C	15.47
3.0, Minimum	0
3.0, MS	7.41
3.0, Knudson C	3.52

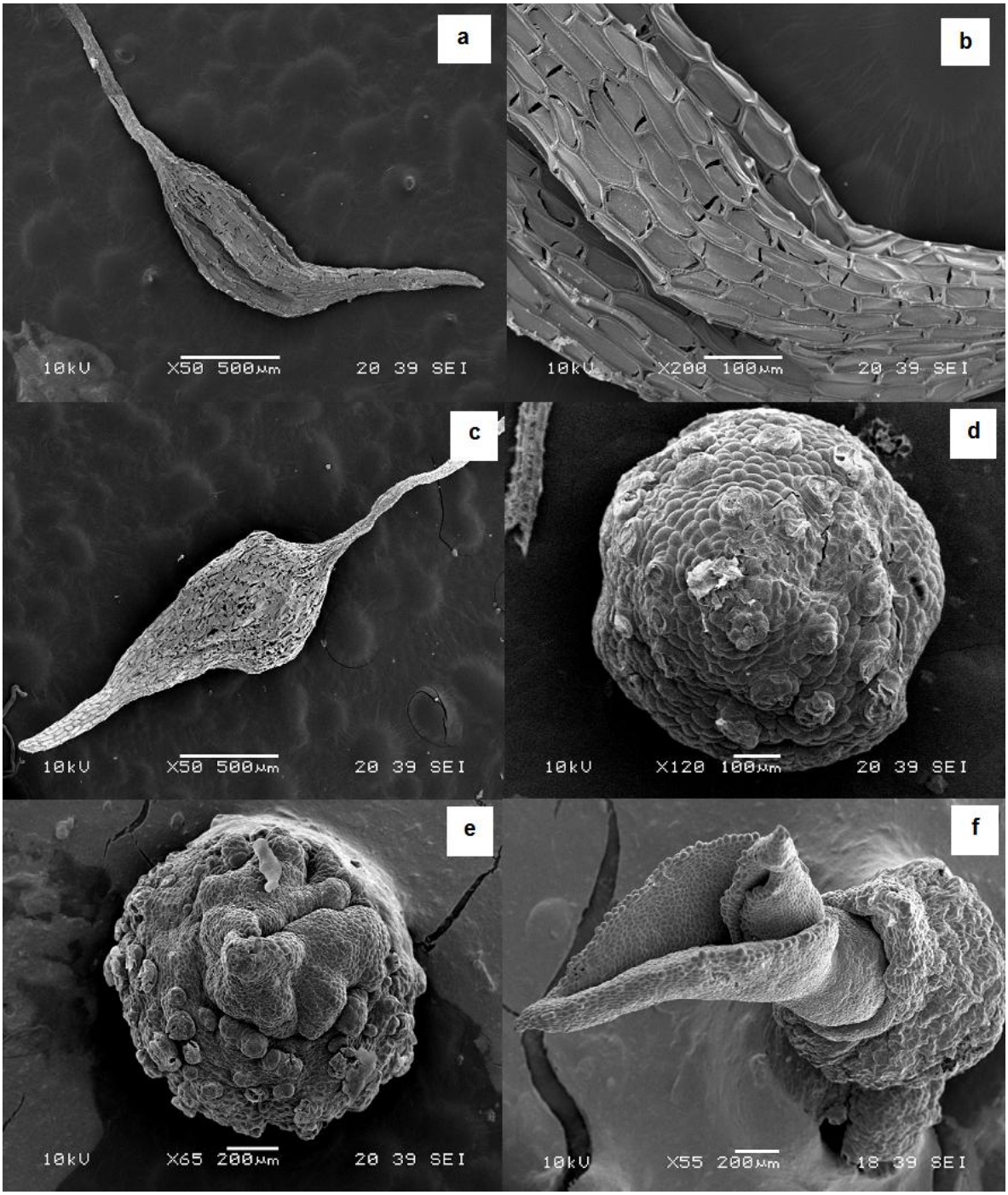

Figure 3. *Epidendrum radicans*. a) seed stage 1, b) enlarged seed, c) seed stage 2, d) protocorm stage 3, e) protocorm stage 4, f) protocorm stage 5. Micrographs taken with a SEM microscope JEOL model JSM-5900LV.

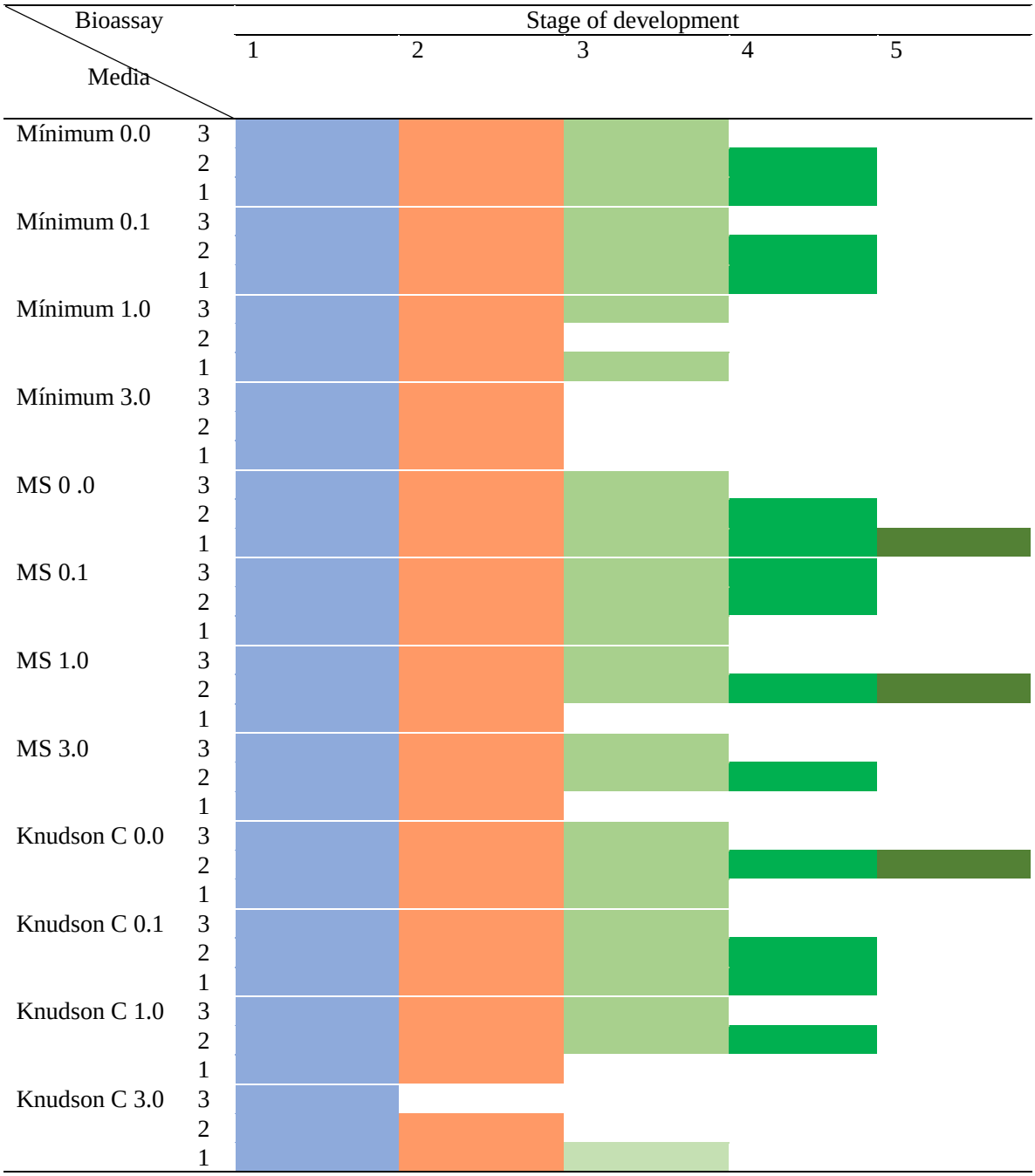

Table 4. Stages of seed germination and protocorm development of *Epidendrum radicans*, after three different bioassays: Stage: 1. Imbibition, 2. Protocorm like body, 3. Germination, 4. Leaf emergence, 5. Leaf elongation