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# *In vitro* germination and growth of *Vriesea incurvata* Gaudich. (Bromeliaceae)

## Germinación y crecimiento *in vitro* de *Vriesea incurvata* Gaudich. (Bromeliaceae)

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## Abstract

*Vriesea incurvata* in the natural environment shows some difficulties associated to the low seed germination capacity. Aiming to provide basis for the seedlings production, the results of the seeds germination percentage and the initial growth of *V. incurvata* seedlings *in vitro* conditions in different culture media, are reported. Completely randomized design was comprised of eight treatments and eight replications. The treatments were as culture media: MS (Murashige & Skoog); MS½; KC (Knudson); KC½; MS + activated carbon (AC); MS½ + AC; KC + AC; and KC½ + AC. The germination percentage was calculated from the division between the number of seeds with primary root extrusion by the total number of sowed seeds. The initial growth was evaluated considering the values of total fresh biomass, percentage of normal and dead seedlings, number and roots length, stem length, number of leaves and percentage of chlorotic, necrotic and dead leaves, respectively. All the cultures promoted high germination percentages (> 82.8%). In MS and MS½ medium, it was evidenced the highest percentage of normal seedlings, the highest values of fresh biomass production, stem growth and number of leaves. KC and KC½ medium also promoted highest percentages of normal seedlings and low percentages of necrotic and dead leaves. The addition of activated carbon in the culture media was unfavorable to promote the growth of seedlings. This suggests that MS and MS½ are the most suitable culture media for the production of *Vriesea incurvata in vitro.*

**Keywords:** Bromeliaceae, culture media, *in vitro* propagation, native plant, seed germination.

## Resumen

*Vriesea incurvata* en el ambiente natural presenta algunas dificultades asociadas a la baja capacidad de germinación de semillas. Con el objetivo de proporcionar una base para la producción de plántulas, se reportan los resultados de la evaluación del porcentaje de germinación de semillas y crecimiento inicial de plántulas de *V. incurvata,* en condiciones *in vitro* en diferentes medios de cultivo. El diseño fue completamente al azar con ocho tratamientos y ocho repeticiones. Los tratamientos fueron los medios de cultivo: MS (Murashige & Skoog); MS½; KC (Knudson); KC½; MS + carbón activado (CA); MS½ + CA; KC + CA; y KC½ + CA. El porcentaje de germinación fue calculado a partir de la división entre el número de semillas con extrusión de la raíz primaria por el número total de semillas sembradas. El crecimiento inicial fue evaluado considerando los valores de biomasa fresca total, porcentaje de plántulas normales y muertas, número y longitud de raíces, longitud del tallo, número de hojas y porcentaje de hojas cloróticas, necróticas y muertas. Todos los medios de cultivo promovieron altos porcentajes de germinación (>82,8%). En los medios de cultivo MS y MS½ se evidenció el mayor porcentaje de plántulas normales, mayores valores de producción de biomasa fresca, crecimiento del tallo y número de hojas. Los medios de cultivo KC y KC½ también promovieron mayores porcentajes de plántulas normales y bajos porcentajes de hojas necróticas y muertas. La adición de carbón activado en los medios de cultivo fue desfavorable en la promoción del crecimiento de las plántulas. Se concluye que MS y MS½ son los medios de cultivo más adecuados para la producción *in vitro* de *Vriesea incurvata*.

**Palabras clave:** Bromeliaceae, medios de cultivo, propagación *in vitro*, planta nativa, germinación de semillas.

## Introduction

*Vriesea incurvata Gaudich.* (Bromeliads) is a native epiphytic and endemic species from the tropical rain forest - Atlantic Forest, with occurrence recorded in the southeast and south of Brazil (Fontoura, Scudeller & Costa, 2012).

Plant height is between 30-40 cm, have a compact architecture, compact architecture, green, smooth and shiny leaves and without thorns, which contrast with the red inflorescence, confers to *V. incurvata* strong ornamental appeal. This species is referenced among the ten most commercialized bromeliads in the flower segment in Paraná State (Negrelle & Anacleto, 2012).

However, easy access to the environments where naturally bromeliads grow, high density of this resource in these places, associated to lack of financial resources, land distribution problems, lack of technical and agricultural support and environmental limitation are factors that discourage the implementation of cultivation systems and reinforce the extractive standard (Negrelle & Anacleto, 2012).

Natural vegetative propagation of bromeliads is slow, due to the low number of side shoots produced by the plants after flowering (Alves, Vesco & Guerra, 2006). In addition, the reproduction of bromeliads in the natural environment shows some difficulties related to the low capacity of seed germination. Negrelle & Muraro (2006), reported *V. incurvata* with low formation of clone shoots (average = 2±0.37) per plant. The same authors characterize *V. incurvata* as a species with annual flowering, with high level of flower production, low fruit production, but relatively significant seed production. However, Muraro, Negrelle & Anacleto (2014), showed that the percentage of germination (emergence) of this species is relatively low (best result = 40%) under natural conditions.

In this context, there is a permanent demand for the search of alternatives that support the production of *V. incurvata* as for its reintroduction in environments impacted by extractive as for its implementation in cultivation systems that can adequately meet the commercial demand of ornamental flowers and plants.

Faced with this problem, the *in vitro* germination of *V. incurvata* can be considered an option. This technique involves the sowing of seed on culture media, in order to promote the seedlings germination and survival under aseptic conditions, with controlled light and temperature. Among the advantages provided by this technique are the optimization and improvement of the nutritional requirements of the cells and tissues in culture medium, and besides it is a very important technique in the commercial and ecological context. Plants produced through this way, may be used in native species reintroduction programs in areas of environmental preservation due to genetic variability generated by explant (Schneiders, Pescador, Booz & Suzuki, 2012).

However, is necessary to evaluate the response of each species to different culture media available for this technique of cultivation, as this response is not homogeneous (Chu, Tavares, Kanashiro, Giampaoli & Yokota, 2010; Zeng, Wu, Silva, Zhang, Chen, Xia & Duan, 2012). These culture media should enriched with supplements that promote the survival, growth and development of the explants.

Given these concerns, in order to support the production of *V. incurvata* seedlings, results of the assessment of the percentage of seed germination and growth and the initial development of seedlings *in vitro* conditions in different culture media, are presented.

## Material and methods

### Plant material and seed collection

Seeds of *V. incurvata* were collected in the tropical rain forest - Atlantic Forest remaining (25º 48 'S and 48º 55' W, 393 m.a.s.l., in Guaratuba - Paraná, Brazil). This region is characterized by super-humid tropical climate without dry season and free from frost, with an average temperature in the coldest month of 18°C.

In February 2015, at random traversal, was identified 20 specimens of *V. incurvata,* which showed closed and brown coloring capsules. This fruits characteristic had been previously evaluated as an indicator of seeds pre-dispersal phase. Therefore, is indicative of seeds in the final stage of maturation.

Capsules of *V. incurvata* were collected (n=30), placed in paper bags, transferred immediately to the laboratory where they were kept under refrigeration until the removal of the seeds (15 days).

### Seed preparation and sterilization

After the removal, the seeds were evaluated for recognition of the seed coat and the embryo under microscope. In this process, was also performed the manual removal of the plumose appendix. Then, the seeds were soaked in distilled water containing detergent Twen-20®, for 10 min. Subsequently, they were washed three times in distilled water. Therefore, seeds were immersed into 70% ethanol for 2 min, and washed twice in distilled water. Finally, the seeds were immersed into the solution of 15% (v/v) of commercial

sodium hypochlorite solution (2.0-2.5% active chlorine) for 10 min, under constant shaking. With the help of a micropipette and sterile tips, the hypochlorite solution was removed from the recipients and the seeds were washed four times in sterile and distilled water.

### Culture media and conditions for seed germination and initial growth

Experimental design was completely randomized with eight treatments and eight replicates, each one represented by a flask (300 ml) with 50 ml of culture medium.

Seeds were sowed into eight formulations of culture media: (1) MS (Murashige & Skoog, 1962); (2) MS½; (3) KC (Knudson, 1946); (4) KC½; (5) MS + activated carbon (AC); (6) MS½ + AC; (7) KC + AC; and (8) KC½ + AC. Activated carbon (3.0 g $L^{-1}$) was used. All culture media were supplemented with sucrose (30 g $L^{-1}$) and agar (4.5 g $L^{-1}$). The pH was adjusted to 5.6 with NaOH and HCl before autoclaving at 121 °C for 15 min. During the sowing of seeds, it was also added 1 mL of distilled water in each flask. After the seeding, the flasks containing the seeds were kept in a growth room under sterile conditions with a temperature of 25±3°C, irradiance of 48 µmol $m^{-2}$ $s^{-1}$ and photoperiod of 16 h.

Percentage of seed germination was evaluated 20 days after date of sowing and calculated by dividing the number of seeds with the primary root extrusion per the total number of sowed seeds in each flask. Initial growth was recorded 190 days after sowing. Subsequently was evaluated the traits as follows: a) fresh biomass of each seedling; b) percentage of normal seedlings (considered those alive seedlings that had stem portion and primary root without damage such as chlorosis or necrosis) and percentage of dead seedlings; c) size of medium stem (considered the measure of stem base until the end of the larger leaf); d) average number of leaves; e) percentage of chlorotic, necrotic and dead leaves; f) average number of formed roots and g) average length of larger root.

## Statistical analysis

Data were statistically analyzed with R version 3.3.0, using analysis of variance (ANOVA) to detect significant differences among treatments. Means differing significantly were compared by Tukey test at $p \leq 0.05$. Data of percentage of normal seedlings, percentage of dead seedlings, percentage of chlorotic, necrotic and dead leaves were √(X+1) transformed before subjecting it to ANOVA.

## Results

Figure 1, shows the percentage germination of *Vriesea incurvata* seeds, which performed high rates of seed germination and was higher than 82.8% and was observed for all culture media.

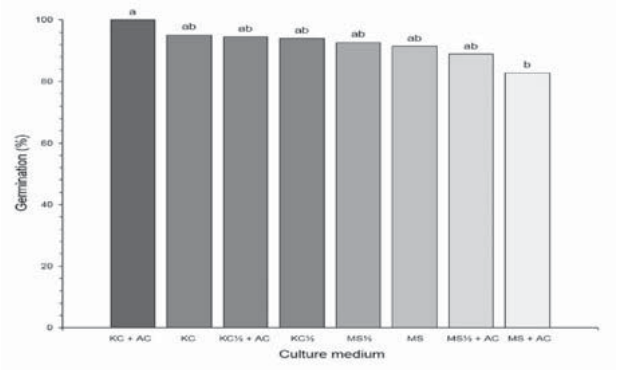


**Figure 1.** Percentage germination of *Vriesea incurvata* seeds cultured *in vitro* in different culture media.

Means followed by the same letter do not differ at 5% by Tukey test. MS: Murashige and Skoog. KC: Knudson C. AC: Activated carbon.

Percentage of seeds germination with KC medium + AC increased in 17.2% when compared to MS medium + AC (Figure 1). The results obtained with *V. incurvata* also confirm the positive response already observed for other species of *Vriesea*, concerning to the use of KC medium in the *in vitro* germination.

Total fresh biomass of the seedling was significantly different in the culture media. MS medium had minimum increment of 52.94% of total fresh biomass, when compared to KC½ medium + AC, KC½ medium, KC medium and KC medium + AC, respectively (Table 1).

**Table 1.** Total fresh biomass of seedlings (FBM), percentage of normal and dead seedlings, number (NR) and length of roots (LR) of *Vriesea incurvata* cultured *in vitro* in different culture media.

| Culture medium | FBM | Seedlings (%) | | NR | LR |
|---|---|---|---|---|---|
| | (g seedling$^{-1}$) | Normal | Dead | | (mm) |
| MS | 0.034 a | 89.49 abc | 10.51 abc | 3.67 $^{n.s.}$ | 5.38 b |
| MS½ | 0.026 abc | 93.88 abc | 6.12 abc | 4.00 | 9.63 b |
| KC | 0.014 cd | 97.78 ab | 2.22 ab | 3.79 | 9.63 b |
| KC½ | 0.014 cd | 100.0 a | 0.00 a | 3.71 | 12.04 ab |
| MS + AC | 0.026 abc | 82.12 c | 17.88 c | 3.42 | 5.67 b |
| MS½ + AC | 0.027 abc | 85.97 bc | 14.03 bc | 4.13 | 9.67 b |
| KC + AC | 0.010 d | 100.0 a | 0.00 a | 3.54 | 10.92 ab |
| KC½ + AC | 0.016 bcd | 94.50 abc | 5.50 abc | 3.42 | 18.00 a |

Means followed by different letters within a column are significantly different at $p \leq 0.05$ by Tukey test. Data of percentage seedlings were √(X+1) transformed before subjecting it to ANOVA. $^{n.s.}$ not significant. MS: Murashige and Skoog. KC: Knudson C. AC: Activated carbon.

High percentage of normal seedlings (> 82%) was recorded for all culture media. The highest values were obtained in KC½ medium and KC medium + AC. The lowest values were observed in MS medium + AC and MS½ medium + AC (Table 1). The achievement of high percentage values of normal seedlings (> 82%) can be considered a favorable indicator for the employment of *in vitro* cultivation in the development of *V. incurvata* propagation and reintroduction programs.

Length of roots of *V. incurvata* seedlings, was significantly affected by the composition of the culture media. The highest values were observed in KC½ medium + AC (18 mm), KC½ medium (12.04 mm) and KC medium + AC (10.92 mm) (Table 1). However, roots production was not significantly affected by the culture media (Table 1). Number and length of roots are important variables in the initial growth of bromeliads due to the fact the roots become the principal plant organ to uptake the nutrients provided by the culture medium under *in vitro* conditions.

MS½ medium and MS medium, were also highlighted as the best response in relation to the stem length and leaf production. Shoot length was significantly different among the culture media tested, especially as regards to MS½ medium (25.29 mm) when compared to KC medium + AC (15.50 mm) (Table 2). Production of leaves, was significantly affected by the culture media. The greatest amount of leaves was observed in MS medium (9 leaves in total) and the lowest amount was recorded in KC medium + AC (6.3 leaves in total) (Table 2).

Percentage of necrotic and dead leaves of *V. incurvata* seedlings, was significantly different among the culture media tested. However, for the percentage of chlorotic leaves was not significantly different in relation to the culture media (Table 2). The lowest percentages of necrotic leaves were recorded in KC½ medium (0.48%), KC medium (1.74%) and MS½ medium (2.56%) and the highest percentages were in MS medium + AC (11.78%) (Table 2).

**Table 2.** Stem length (SL), number of leaves (NL), percentage of chlorotic, necrotic and dead leaves of *Vriesea incurvata* cultured *in vitro* in different culture media.

| Culture Medium | SL (mm) | NL | Leaves (%) | | |
|---|---|---|---|---|---|
| | | | Chlorotic | Necrotic | Dead |
| MS | 21.61 ab | 9.0 a | 0.00 $^{n.s.}$ | 6.12 abc | 18.23 c |
| MS½ | 25.29 a | 8.5 ab | 0.54 | 2.56 ab | 4.10 ab |
| KC | 20.75 ab | 7.3 ab | 1.16 | 1.74 ab | 0.66 a |
| KC½ | 20.71 ab | 7.5 ab | 0.00 | 0.48 a | 0.00 a |
| MS + AC | 20.58 ab | 7.2 ab | 0.00 | 11.78 c | 11.79 bc |
| MS½ + AC | 21.52 ab | 7.9 ab | 0.00 | 8.81 bc | 5.54 ab |
| KC + AC | 15.50 b | 6.3 b | 0.68 | 3.98 abc | 5.99 ab |
| KC½ + AC | 17.54 ab | 6.8 ab | 0.56 | 4.92 abc | 1.26 ab |

Means followed by different letters within a column are significantly different at $p \leq 0.05$ by Tukey test. Data of percentage leaves were √(X+1) transformed before subjecting it to ANOVA. $^{n.s.}$ not significant. MS: Murashige and Skoog. KC: Knudson C. AC: Activated carbon.

Additionally, for the percentage of necrotic leaves, were observed differences between KC½ medium when compared to MS½ medium + AC and MS medium +AC. (Table 2). For the percentage of dead leaves, KC½ medium (0%) and KC medium (0.66%) showed the lowest percentages in relation to MS medium + AC (11.79%) and MS medium (18.23%) (Table 2).

## Discussion

The choice of the culture medium, nutritional supplements, physiological germination conditions, and the conditions of the seeds as the origin and maturity of the capsule influence significantly the *in vitro* seed germination (Zeng, Zhang, Silva, Wu, Zhang & Duan, 2013).

The highest percentage of seeds germination, was obtained with the KC medium + AC (100%) and the lowest (82.8%) was obtained in MS medium + AC (Figure 1). When compared to the results of the *ex vitro* germination (40% according to Muraro, Negrelle & Anacleto (2014), who was showed that the *in vitro* condition can optimize in an expressive way the production of *V. incurvata* seedlings, doubling the percentage of germination. The production of seedlings of this species is therefore, favored by this seed germination technique, which provides the nutritional requirements appropriate for the emergence, survival and development of the seedlings under aseptic conditions, with controlled temperature and light (Schneiders, Pescador, Booz & Suzuki, 2012; Zeng, Wu, Silva, Zhang, Chen, Xia & Duan, 2012, among others).

High percentages of germination using KC medium, were also observed by Droste, Silva, Matos & Almeida (2005), in *V. gigantea* and *V. philippocoburgii* species with 99% and 89%, respectively. Mercier & Kerbauy (1994), reported up to 90% of seeds germination in *V. hieroglyphica* using KC½ medium.

However, despite the KC medium, has been reported as more effective for the growth of ornamental species such as Orchidaceae (Soares, Araújo, Pasqual, Rodrigues & Assis, 2009); this medium was not suitable to promote the initial growth of *V. incurvata*. This result could be related to the low concentration of potassium and nitrogen (Knudson, 1946).

MS medium had achieved a higher concentration of nutrients than the KC medium, is likely due to the high concentration of nutrients in MS medium, which favored the increasing of the total fresh biomass of *V. incurvata* seedlings. This result was also seen in *Nidularium minutum* (Bromeliaceae) when cultivated in MS medium and MS½ medium (Carvalho, Hayashi, Braga & Nievol, 2013).

Root systems with long and numerous roots are important, because they ensure the proper storage of carbohydrate reserves for the growth under *in vitro* conditions (Chu, Tavares, Kanashiro, Giampaoli & Yokota, 2010). Already in advanced stages of the plant, the bromeliad roots have as the main function the nutrients fixing, due to plant nutrition is carried out by leaf trichomes (Vanhoutte, Ceusters & Proft, 2016).

However, the values related to the length of roots detected in *V. incurvata* were much lower than those reported for other species of bromeliads. The representatives of *Nidularium minutum* (Bromeliaceae) held for six months under *in vitro* conditions reached maximum roots length of 6 cm using MS medium and MS½ medium (Carvalho, Hayashi, Braga & Nievol, 2013). On the other hand, Carvalho, Santos & Nievol (2014), using MS medium in *Acanthostachys strobilacea* (Bromeliaceae) observed that seedlings *in vitro* with 90 days maintained at temperatures of 20 to 25°C, reached the root length of at least 7.6 and 8 cm, respectively. Additional studies on roots production and length in representatives of *V. incurvata* produced in natural environment, through sexual propagation, should support the better understanding of this *in vitro* response.

MS medium shows as its main characteristic high nitrogen concentration, essential macro element in the production of proteins and in the physiological processes that occur in plants as the growth (Murashige & Skoog, 1962).

These results support that the addition of activated carbon was unfavorable to promote this growth. Activated carbon is widely used in *in vitro* propagation of several plants because it contributes in the root formation process and in the absorption of toxic substances present in the culture medium (Thomas, 2008). However, the addition of activated carbon can also determine inhibitory responses (Nicoloso, Erig, Martins & Russowski, 2001). This seems to be the case of *V. incurvata*, which determined in some culture media at least the lowest accumulation of total fresh biomass, leaf production and higher percentages of necrotic and dead leaves. Additionally, these results may be associated to the relatively high concentration of phosphate in the KC medium (1.84 mmol $l^{-1}$) in relation to MS medium (1.25 mmol $l^{-1}$) (Knudson, 1946; Murashige & Skoog, 1962). According by Kerbauy (2012), the phosphorus deficiency in plants can lead to premature senescence of leaves, reduction of leaf expansion and retrace in the formation of the reproductive organs.

## Conclusion

*In vitro* cultivation is suitable to promote high rates of *V. incurvata* germination.

MS and MS½ are the best culture media to promote the achievement of a greater number of normal seedlings, higher production rates of total fresh biomass, more leaves and better performance of stem growth. MS½ medium contains half of nutrients concentration related to standard medium (MS), showing a major advantage in terms of cost/benefit.

KC and KC½ medium, also promote highest percentages of normal seedlings and low percentages of necrotic and dead leaves of *V. incurvata*. Conversely, the addition of activated carbon in the culture media is unfavorable to promote the growth of *V. incurvata*.

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## References

Alves, G. M., Vesco, L. L. D., & Guerra, M. P. (2006). Micropropagation of the Brazilian endemic bromeliad *Vriesea reitzii* through nodule clusters culture. *Scientia Hortic*, 110 (2), 204–207. *https://doi.org/10.1016/j.scienta.2006.06.014*

Carvalho, C. P., Hayashi, A. H., Braga, M. R. & Nievol, C. C. (2013). Biochemical and anatomical responses related to the *in vitro* survival of the tropical bromeliad *Nidularium minutum* to low temperatures. *Plant Physiol Bioch*, 71, 144-154. *https://doi.org/10.1016/j.plaphy.2013.07.005*

Carvalho, V., Santos, D. S. & Nievol, C. C. (2014). *In vitro* storage under slow growth and *ex vitro* acclimatization of the ornamental bromeliad *Acanthostachys strobilacea*. *S Afr J Bot*, 92, 39-43. *https://doi.org/10.1016/j.sajb.2014.01.011*

Chu, E. P., Tavares, A.R., Kanashiro, S., Giampaoli, P. & Yokota, E. S. (2010). Effects of auxins on soluble carbohydrates, starch and soluble protein content in *Aechmea blanchetiana* (Bromeliaceae) cultured *in vitro*. *Sci Hortic*, 125(3), 451-455. *https://doi.org/10.1016/j.scienta.2010.04.021*

Droste, A., Silva, A. M., Matos, A. V. & Almeida, J. W. (2005). *In vitro* culture of *Vriesea gigantea* and *Vriesea philippocoburgii*: two vulnerable bromeliads native to Southern Brazil. *Braz Arch Biol Technol*, 48 (5), 717-722. *http://dx.doi.org/10.1590/S1516-89132005000600006*

Fontoura, T., Scudeller, V. V. & Costa, A. F. (2012). Floristics and environmental factors determining the geographic distribution of epiphytic bromeliads in the Brazilian Atlantic Rain Forest. *Flora - Morphology, Distribution, Functional Ecology of Plants*, 207(9), 662-672. *https://doi.org/10.1016/j.flora.2012.05.003*

Kerbauy, G.B. (2012). *Fisiologia Vegetal*. 2nd Edition. Guanabara Koogan. 446 p.

Knudson, L. (1946). A new nutrient solution for the germination of orchid seed. *American Orquid Society Bulletim*, 14, 214-217.

Mercier, H. & Kerbauy, G. B. (1994). *In vitro* culture of *Vriesea hieroglyphica*, an endangered bromeliad from the Brazilian Atlantic forest. *Journal Bromeliad*, 44, 120-124.

Muraro, D., Negrelle, R. R. B. & Anacleto, A. (2014). Germination and survival of *Vriesea incurvata* Gaudich. under forest canopy on different substrates. *Scientia Agraria Paranaensis*, 13 (3), 251-258. *http://dx.doi.org/10.1818/sap.v13i3.8448*

Murashige, T. & Skoog, F. (1962). A revised method for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15 (3), 473-497. *http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x*

Nicoloso, F. T., Erig, A. C., Martins, C. F. & Russowski, D. (2001). Micro propagation of Brazilian ginseng [*Pfaffia glomerata* (Spreng.) Pedersen]. *Rev Bras Pl Med*, 3 (2), 11-18.

Negrelle, R. R. B. & Anacleto, A. (2012). Bromeliads wild harvesting in State of Paraná. *Ciênc Rural*, 42 (6), 981-986. *http://dx.doi.org/10.1590/S0103-84782012000600005*

Negrelle, R. R. B. & Muraro, D. (2006). Phenological and reproductive aspects of *Vriesea incurvata* Gaudich (Bromeliaceae). *Acta Sci Biol Sci*, 28 (2), 95-102. *http://dx.doi.org/10.4025/actascibiolsci.v28i2.1011*

Schneiders, D., Pescador, R., Booz, M. R. & Suzuki, R. M. (2012). Germination, growth and development in vitro of orchids of the genus *Cattleya*. *Rev Ceres*, 59 (2), 85-191. *http://dx.doi.org/10.1590/S0034-737X2012000200006*

Soares, J. D. R., Araújo, A. G., Pasqual, M., Rodrigues, F. A. & Assis, F. A. (2009). Concentrações de sais do meio Knudson C e de ácido giberélico no crescimento in vitro de plântulas de orquídea. *Ciênc Rural*, 39 (3), 772-777. *http://dx.doi.org/10.1590/S0103-84782009000300021*

Thomas, T. D. (2008). The role of activated carbon in plant tissue culture. *Biotechnology Advances*, 26 (6), 618–631. *https://doi.org/10.1016/j.biotechadv.2008.08.003*

Vanhoutte, B., Ceusters, J. & Proft, M. P. (2016). The 'tubing' phenomenon in commercial cultivation of Guzmania: morphology, physiology and anatomy. *Scientia Horticulturae*, 205, 112–118. *https://doi.org/10.1016/j.scienta.2016.04.008*

Zeng, S., Wu, K. L., Silva, J., Zhang, J. X., Chen, Z. L., Xia, N. H. & Duan, J. (2012). Asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii* Sumerh., an endangered terrestrial orchid. *Sci Hortic*, 138, 198–209. *https://doi.org/10.1016/j.scienta.2012.02.026*

Zeng, S., Zhang, Y., Silva, J. A. T., Wu, K. L., Zhang, J. & Duan, J. (2013). Seed biology and *in vitro* seed germination of Cypripedium. *Critical Reviews in Biotechnology*, 34, 358-371. *https://doi.org/10.3109/07388551.2013.841117*