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Stevia rebaudiana (Bert) Bertoni: influence of osmotic stress and seed priming on seed germination under laboratory conditions

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ABSTRACT. The foremost factor necessary for plant growers cultivating large acreages of *Stevia rebaudiana* (Bert) Bertoni is the production of qualitative bedding plants. The objective of this study was to evaluate the influence of osmotic-priming on the uniformity of seed germination. First, we evaluated the percentage of normal seedlings from two seed samples harvested in 2011 and 2012. The seeds harvested in 2012 produced 71.4% normal seedlings and thus they were used in the next experiments. The seeds were subjected to osmotic stress using five concentrations of polyethylene glycol (PEG-6000) at -0.2, -0.4, -0.6, -0.8, and -1.0 MPa in contrast with distilled water. Based on these first results, only -0.8 and -1.0 MPa were evaluated in the third experiment. The seeds were immersed in both concentrations of polyethylene glycol (PEG-6000) for imbibing at 20ºC for four, five, six, and seven days. Thereafter, we evaluated the time to the first normal seedling (Ti), time to the last normal seedling (Tf), percentage normal seedlings at the initial time (Pi) and percentage of normal seedlings at the end of every treatment (Pf). Osmotic priming increased the percentage of normal seedlings of the *Stevia rebaudiana* and reduced the time to the first and last germination events.

Keywords: osmotic priming, radicle protrusion, time to seed germination, sweeteners.

Stevia rebaudiana (Bert) Bertoni: influência do estresse osmótico e do "priming" na germinação das sementes em condições de laboratório

RESUMO. O fator mais importante para que os agricultores possam cultivar *Stevia rebaudiana* is a produção qualitativa de mudas. Assim, o objetivo destes experimentos foi o de avaliar a influência do "priming" na uniformidade das ocorrências germinativas. Primeiro, nós avaliamos a porcentagem de plântulas normais de duas amostras colhidas nos anos 2011 e 2012. As sementes colhidas no ano de 2012 desenvolveram 71,4% de plântulas normais, e por isso foram usadas nos demais experimentos. Depois, as sementes foram estressadas osmoticamente usando cinco concentrações de polietileno glicol (PEG-6000) -0,2; -0,4; -0,6; -0,8 e -1,0 MPa, juntamente com um controle com água destilada. Com base nestes resultados, apenas as concentrações à -0,8 e -1,0 MPa foram avaliadas no terceiro experimento. Assim, as sementes foram imersas para a embebição à 20ºC por quatro, cinco, seis e sete dias. Em seguida, nós avaliamos to tempo para a primeira ocorrência germinativa (Ti), o tempo para a última (Tf), a porcentagem inicial de plântulas normais (Pi) e a porcentagem final de cada tratamento (Pf). O priming aumentou a porcentagem de plântulas normais e reduziu o tempo para a primeira e última ocorrência germinativa.

Palavras-chave: condicionamento osmótico, protrusão de radículas, tempo de germinação, adoçantes.

Introduction

Stevia rebaudiana (Bert) Bertoni is a short-day plant that produces many foliar steviol glycosides sweeter than sucrose (Yadav, Singh, Dhyani, & Ahuja, 2011; Brandle, Starratt, & Gijzen, 1998; Carneiro, 1990). These non-caloric glycosides have been recommended for diabetic, overweight and obese patients requiring natural alternatives to artificial sweeteners, such as the industrial aspartame and saccharin (Moraes, Donega, Cantrell, Mello, & McChesney, 2013; Lemus-Mondaca, Vega-Galvez, Zura-Bravo, & Ah-Hen, 2012; Geuns, 2003). Furthermore, the industrial use of Stevia goes beyond the presence of sweeteners because glycosides, such as rubusoside, which is also partially responsible for the sweet taste, have remarkable solubilizing properties that can be used by the pharmaceutical industry (Liu et al., 2011). The leaf composition indicates financial possibilities in the food and pet industries, reflecting the richness of these plants in dietary iron, calcium, and potassium, in addition to low levels of fat. The agro-industry of

Stevia rebaudiana also offers opportunities in the cosmetic industry because of the large number of essential oils. Recently, significant quantities of inulin were detected in the stems and roots of Stevia plants. Inulin is a reserve oligosaccharide with pre-biotic effects in humans, and currently, this compound is under high demand by the food industry (Oliveira et al., 2011). These recent chemical approaches prompt a new economic discussion for cultivating *Stevia rebaudiana* because the high crop yield of the stem by-products (Moraes et al., 2013) can be a complementary and safe source of net income for Stevia growers.

Although *Stevia rebaudiana* acreage has been expanding worldwide to supply the needs of various agro-industrial programs (Carneiro, 1990; Brandle et al., 1998; Ramesh, Singh, & Megeji, 2006), high and cost-effective crop yields depend on the field establishment of vigorous bedding plants. Furthermore, these bedding plants require seed production with high physiological quality evaluated by the percentage of seed germination over a period of ten days, seed vigor tests, or both.

The high-quality production of uniform bedding plants depends on high and uniform percentage of seed germination, and high seedling resistance to stressful weather conditions (Carneiro, Muniz, & Guedes, 1997; Heydecker, Higgins, & Turner, 1975). Seed imbibition using priming technology is one organic strategy in which the hydric conditions initiate the mechanisms essential to seed germination, but it does not induce the protrusion of radicles. Hydric stress, otherwise, reduces both the percentage and rate of germination events (Bewley & Black, 1985). Polyethylene glycol (PEG-6000) has been used in the osmotic priming technology (Carneiro & Guedes, 1992b) because it is chemically inert and does not has toxic effects on the seedlings. Michel and Kauffmann (1973) initially proposed the use of osmotic potential to evaluate germination responses under hydric stress. Currently, this method is broadly applied to the commercial seeds of numerous species.

The objective of this study was to evaluate the influence of osmotic priming on seed germination of *Stevia rebaudiana*. Specifically, the objectives were to select the better seeds for further experimental evaluation, assess the seed germination under hydric stress conditions, and evaluate the concentration and optimal period for osmotic priming treatments.

Material and methods

Three experiments were conducted in the Seed Science and Technology Laboratory, Iguatemi Research Station 20 km apart from the central campus at 23° 21' SL, 52° 04' WL and 542 m of altitude, and in the NAPD (Núcleo de Estudos Avançados em Ciência de Plantas Daninhas) at 20°20'SL, 52°04'WL and altitude of 506 m in the central campus of the State University at Maringá, Maringá County, North Paraná State, in Brazil. In 2011 and 2012, achene fruit-like seeds were harvested under glasshouse conditions from plants cultivated in 20-L black plastic pots. The seed samples were visually hand cleaned and graded as light and heavy based on the seed length and discriminated using a stereomicroscope (model SMZ-140 N2GG, Motic, from Asia). We eliminated from the samples the presence of immature, empty, or both achene fruit-like seeds. Subsequently, the hairy pappus was removed using hand friction on the surface of germination test papers. This method improves the contact of the seed with the germination medium (Carneiro & Guedes, 1992a; Carneiro, 1996). The samples were stored at 5± 1°C and 20% relative humidity before every experiment. Thereafter, the seeds were germinated at 25°C using germi-test paper (Cel 065, JProlab, Brazil) in germination boxes (11 x 11 x 5 cm, JProlab, Brazil).

First experiment: The germination responses from two seed lots harvested in 2011 and 2012 were evaluated to discriminate the samples with better percentages of seed germination. The experimental design was completely randomized with six replications, and every germination box was an experimental unit. Three germination test papers (Cel 065, JProlab, Brazil) were wetted two and half times their weight in grams with distilled, deionized water (ISTA, 2014; Brasil, 2009). The germination temperature was 25±1°C under daylight lamps for seven days, and subsequently we counted data from normal and abnormal seedlings, and ungerminated seeds (Carneiro & Guedes, 1992a).

Second experiment: The conditions of the first experiment were improved using eight replications and modified through the application of distilled, deionized water as the control (T_1). In contrast we prepared five concentrations of polyethylene glycol (PEG-6000, Labsynth, Brazil) according to Villela, Doni Filho, and Siqueira (1991): -0.2 MPa (T_2) with 119.571 g L^{-1}, -0.4 MPa (T_3) with 178.343 g L^{-1}, -0.6 MPa (T_4) with 223.664 g L^{-1}, -0.8 MPa (T_5) with 261.948 g L^{-1} and -1.0 MPa (T_6) with 295.713 g L^{-1} of PEG-6000. These concentrations were estimated by the following equation of Michel and Kaufmann (1973): $\Psi_{os} = (1.18 \times 10^{-2}) \star C - (1.18 \times 10^{-4}) \star C^2 + (2.67 \times 10^{-4}) \star C \star T + (8.39 \times 10^{-7}) \star C^2 \star T$, in which: Ψ_{os} = osmotic potential (bar); C = concentration (g L^{-1}) of polyethylene glycol, and T = temperature (°C). All samples were treated with Thiram 200 SC® at 0.2% (p/v).

The seeds evaluated in this experiment had the best germination responses in the first evaluation when the percentage of normal seedlings was 71.4%. The frequencies of seed germination were verified at eight-hour intervals when every seedling had roots measuring 2 mm in length. The test was finished after 384 hrs when no further germination events were observed. The abnormal seedlings were detected by the presence of one black spot in the differentiating region of the root system, and cotyledons indicating the presence of phytophagous insects. Furthermore, no germination events were observed at -0.8 and -1.0 MPa, and both concentrations were used in the third experiment in which we applied the osmotic-priming treatment.

Third experiment: The seeds were imbibed in a solution of polyethylene glycol 6000 at 20°C for four, five, six, and seven days in contrast with the control treated with distilled, deionized water. The germination paper was wetted with 20 mL of the PEG-6000 solution using a hypodermic syringe, and the germination box was sealed using polypropylene tape. After the priming period, we washed all the seeds with tap water for 1 min., followed by germination at 25°C for 196 hrs. The following response variables were recorded: time to the first normal seedling (Ti), time to the last normal seedling (Tf), percentage of normal seedlings (Pi) at the initial time (Ti), and percentage of total normal seedlings (Pf).

Data analyses

The germination data from these three experiments were reported by exploratory data analysis using box-and-whiskers plots according to Banzatto and Kronka (2008). Box-and-whiskers plots are based on sample statistics. This method uses communicative figures to show sample data when the variability is more prominent than the means (Krzywinski & Altman, 2014) as typically observed for *Stevia rebaudiana* seeds. The graphic ranges from the first (Q1) to the third quartile (Q3) of the distribution and represents the inter-quartile range (IQR). The line across the box indicates the median, and the whiskers are lines extending from Q1 and Q3 to their end points, typically defined as the most extreme data within Q1 – 1.5 × IQR and Q3 + 1.5 × IQR, respectively (Streit & Gehlenborg, 2014). Each outlier is represented as an individual mark. Alternatively, the minimum and maximum values from every data set are used as the end points for the whiskers. One benefit of the box-and-whiskers plots is the requirement of only five units for analysis, and these data are readily comparable across three or more samples. For $n < 5$, however, the best decision is to show the individual data points (Krzywinski & Altman, 2014). The control of variability during the germination and bedding plant development of *Stevia rebaudiana* produces uniform plants under crop field conditions.

Results and discussion

On average, the highest percentage (71.4%) of germination was obtained from seeds harvested in 2012 in contrast to the normal seedlings (67.0%) from seeds harvested in 2011, and both samples were stored at 5°C under 20% relative humidity. Stevia seeds are sensitive to storage conditions likely reflecting the quantitative oil contents evaluated by reddish embryos after immersion in Sudan IV for 20 minutes (Carneiro, 1990).

The average percentage of normal seedlings (71.4%) reflected the number of non-germinated seeds at 25°C, instead the level of the abnormal seedlings in the sample, despite their presence in the test. The elimination of the hairy pappus has also been motive of seedlings abnormalities. The variability was higher than in the seed sample harvested in 2011, when the reduced percentage of normal seedlings (67.0%) reflected the presence of abnormal seedlings. Based on the variability indicated by the inter-quartile ranges (IQR), both samples maintained the development stage $V_{1.7}$ (Carneiro, 2007). The selection of newly harvested seeds for use in the following experiments was based on a whisker fence value higher than 80.0% and asymmetry on the right (Figure 1a), suggesting room for DNA repair, if any, and a higher percentage of normal seedlings after the osmotic priming. This variability is typical in germination tests when the seed quality is not uniform. This non-uniformity requires further investigation to understand how to reduce variations in bedding plant production under nursery conditions.

In the second experiment, the time to first germination (T_i) was recorded at 64 hrs for seeds under control conditions (1). Along these levels of water stress, the seeds delayed a time to the first event of more than 150 hrs when evaluated under -0.6 MPa, except for two outliers. This finding might reflect the diversity in the investigated seed population in this study (Figure 2a). We did not find any radicle protrusion under osmotic stress conditions of -0.8 and -1.0 MPa (Figure 2b and c). The percentage of normal seedlings from the control was higher than the initial seed germination of 71.4%, suggesting significant physiological events the seed sample. Again the superior fence of the whisker was higher than 80% of

normal seedlings. The graphical responses in Figure 2 indicate asymmetry in the data distribution from the treatment with -0.6 MPa, where the highest variability in time to germination was observed. In the control, the highest variability was observed in the time to first germination and initial percentage of normal seedlings (Figure 2a and c). These responses require more investigation concerning the best methodology for applying priming methods in Stevia seeds. In summary, concentrations of -0.8 and -1.0 MPa were the most favorable to study the osmotic priming of *Stevia rebaudiana* seeds

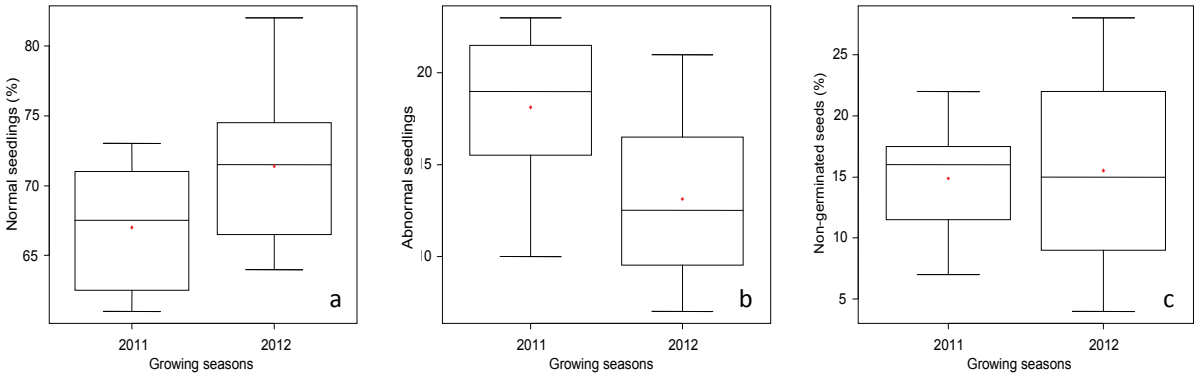

Figure 1. Box- and-whiskers plots of components from the germination test using *Stevia rebaudiana* (Bert) Bertoni seeds harvested in two growing seasons in the Iguatemi Research Station, Maringá County, Paraná State, Brazil.

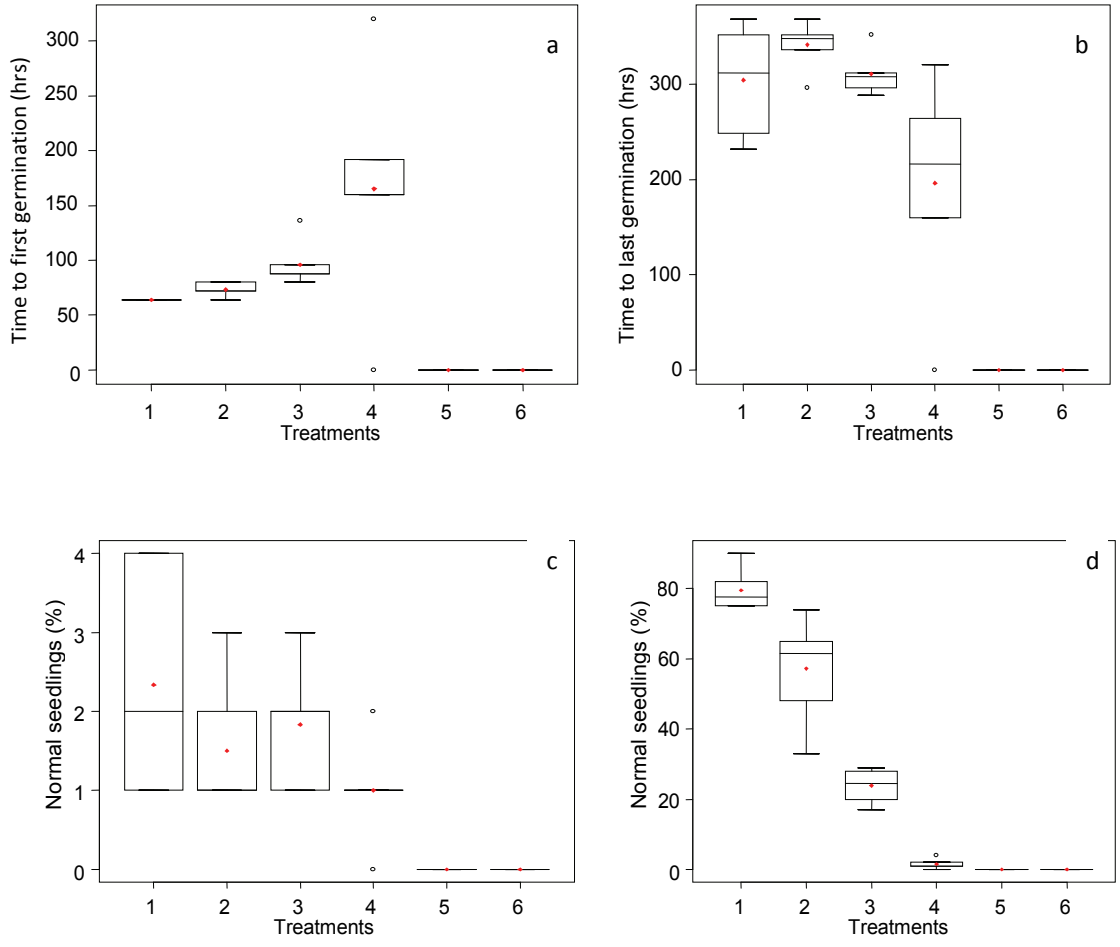

Figure 2. Performance of *Stevia rebaudiana* (Bert) Bertoni seeds under the control (1) and five osmotic priming treatments (2 = -0.2 MPa, 3 = -0.4 MPa, 4 = -0.6 MPa, 5 = -0.8 MPa, and 6 = -1.0 MPa).

In the third treatment, all the primed seeds experienced the first protrusion within 12 hrs after the beginning of the germination test (first records, data not shown), despite the number of days of priming. The average end of every treatment was different for six and seven days (Figure 3a), but the variability of these treatments was higher than expected, despite the similarity of the median and mean. The whiskers were larger on the right than in seeds primed for four and five days. Similar responses were reported for temperate carrot seeds treated with PEG-6000 at -1.0 MPa, when cumulative germination data were fit to the parameters of the Weibull distribution (Carneiro & Guedes, 1992b). The lower fence of the box-and-whiskers plots for seeds immersed in the PEG-6000 for six days was approximately 40 hrs, and the treatment for seven days delayed the last germination event to more than 160 hrs. This response contrasts with the six days of treatment, when the last observation was approximately 140 hrs (Figure 3a). In the first records, the percentage of normal seedlings reached values close to 80.0% (Figure 3b), but high variability was detected by the parameters of the box-and-whiskers plots, where five days had data skewed to the left. One explanation for skewness is the time required for all seeds to germinate.

Osmotic priming with PEG-6000 at -1.0 MPa for seven days showed a final percentage higher than 75%, and a small IQR range for 50% of normal seedlings. These higher responses suggest DNA repair by this osmotic treatment but with data still skewed to the left. Another explanation for these results is the non-uniform dimension of these seeds, particularly the seed length. Thus, the next step in seed technology research is to improve the seed grade using classification equipment based on the length or density of seeds. Osmotic treatment at -1.0 MPa increased the germination stage from $V_{1.7}$ (Figure 1a) to $V_{1.8}$ (Figure 3f), as verified using other priming treatments. However, the IQR was reduced compared with the control and treatment at -0.8 MPa. These results were similar to those of Carneiro and Guedes (1992b), when they improved the responses of primed carrot seeds at -1.0 MPa using PEG-6000. In summary, two foremost responses were observed: the reduction of IQR and the length of the whiskers, despite the stretching of the first quartile (Figures 1a and 3f). These responses also suggest the presence of cohorts during the germination period and these effects suggest further investigations.

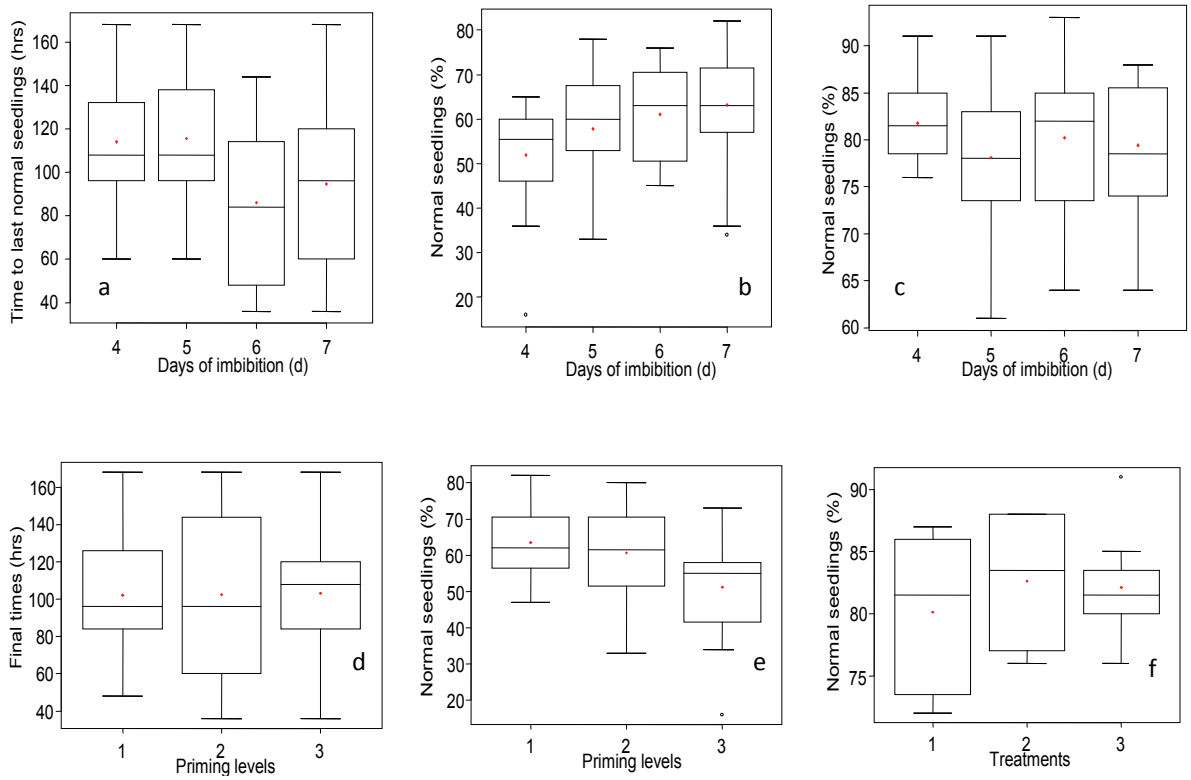

Figure 3. Performance of *Stevia rebaudiana* (Bert) Bertoni seeds germinated after treatments at 25ºC for four, five, six, and seven days (a, b, and c) in the control (1), -0.8 (2), and -1.0 MPa (3) (d, e, and f) under laboratory conditions.

Conclusion

Osmotic priming increased the final percentage of normal *Stevia rebaudiana* seedlings and reduced the time to the first and last germination events.

References

Banzatto, D. A., & Kronka, S. N. (2008). *Experimentação Agrícola* (4a. ed.). Jaboticabal, SP: Funep.

Bewley, J. D., & Black, M. (1985) *Seeds*: physiology of development and germination. New York, US: Plenum Press.

Brandle, J. E., Starratt, A. N., & Gijzen, M. (1998). *Stevia rebaudiana*: Its agricultural, biological, and chemical properties. *Canadian Journal of Plant Science*, *78*(4), 527-536.

Brasil. (2009). Ministério da Agricultura, Pecuária e Abastecimento. *Regras para análise de sementes*. Brasília, DF: Mapa/ACS.

Carneiro, J. W. P. (1990). *Stevia rebaudiana* (Bert) *Bertoni: produção de sementes*. Maringá, PR: Imprensa Universitária.

Carneiro, J. W. P. (1996). Determinação do número de sementes para avaliar o desempenho germinativo de Stevia (*Stevia rebaudiana* (Bert) Bertoni). *Revista Brasileira de Sementes*, *18*(1), 1-5.

Carneiro, J. W. P. (2007). *Stevia rebaudiana* (Bert) Bertoni: Stages of plant development. *Canadian Journal of Plant Science*, *87*(4), 861-865.

Carneiro, J. W. P., & Guedes, T. A. (1992a). Influencia do contato das sementes de Stevia (*Stevia rebaudiana* (Bert) Bertoni) no substrato, avaliada pela função de distribuição de Weibull. *Revista Brasileira de Sementes*, *14*(1), 65-68.

Carneiro, J. W. P., & Guedes, T. A. (1992b). Influência da temperatura no desempenho germinativo de sementes de cenoura (*Daucus carota* L.) avaliada pela função de distribuição de Weibull. *Revista Brasileira de Sementes*, *14*(2), 207-213.

Carneiro, J. W. P., Muniz, A. S., & Guedes, T. A. (1997). Greenhouse bedding plant production of *Stevia rebaudiana* (Bert) Bertoni. *Canadian Journal of Plant Science*, 77(3), 473-474.

Geuns, J. M. C. (2003). Molecules of interest: Stevioside. *Phytochemistry*, *64*(5), 913-921.

Heydecker, W., Higgins, J., & Turner, Y. J. (1975). Invigoration of seeds. *Seed Science and Technology*, *3*(3/4), 881-888.

International Seed Testing Association [ISTA]. (2014). *International rules for seed testing*. Bassersdorf, SW: ISTA.

Krzywinski, M., & Altman, N. (2014). Visualizing samples with box-plots. *Nature Methods*, *11*(2), 119-120.

Lemus-Mondaca, R., Vega-Galvez, A., Zura-Bravo, L., & Ah-Hen, K. (2012). *Stevia rebaudiana* Bertoni, source of a high potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. *Food Chemistry*, *132*(3), 1121-1132.

Liu, Z., Koh, G. Y., Zhang, F., Jeansonne, D., Stout, R., Dong, L., & Enright, F. (2011). Medicinal plants and cancer: solubility-enhanced reformulation of paclitaxel, an old drug. *Louisiana Agriculture, Winter*, *54*(1), 6-7.

Michel, B. E., & Kaufmann, M. R. (1973). The osmotic potential of polyethylene glycol 6000. *Plant Physiology*, *51*(6), 914-916.

Moraes, R. M., Donega, M. A., Cantrell, C. L., Mello, S. C., & McChesney, J. D. (2013). Effect of harvest timing on leaf production and yield of diterpene glycosides in *Stevia rebaudiana* Bert: A specialty perennial crop for Mississippi. *Industrial Crops and Products*, *51*(11), 385-389.

Oliveira, A. J. B., Gonçalves, R. A. C., Chierrito, T. P. C., Santos, M. M., Souza, L. M., Gorin, P. A. J., Sassaki, G. L., & Iacomini, M. (2011). Structure and degree of polymerisation of fructo-oligosaccharides present in roots and leaves of *Stevia rebaudiana* (Bert) Bertoni. *Food Chemistry*, *129*(2), 305-311.

Ramesh, K., Singh, V., & Megeji, N. W. (2006). Cultivation of Stevia (*Stevia rebaudiana* (Bert) Bertoni): A comprehensive review. *Advances in Agronomy*, *89*(3), 138-179.

Streit, M., & Gehlenborg, N. (2014). Bar charts and box plots. *Nature Methods*, *11*(2), 117.

Takahashi, L., Melges, E., & Carneiro, J. W. P. (1996). Desempenho germinativo de sementes de *Stevia rebaudiana* (Bert) Bertoni sob diferentes temperaturas. *Revista Brasileira de Sementes*, *18*(1), 1-5.

Villela, F. A., Doni Filho, L., & Siqueira, E. L. (1991). Tabela de potencial osmótico em função da concentração de polietileno glicol 6000 e da temperatura. *Pesquisa Agropecuária Brasileira*, *26*(11/12), 1957-1968.

Yadav, A. K., Singh, S., Dhyani, D., & Ahuja, P. S. (2011). A review on the improvement of Stevia [*Stevia rebaudiana* (Bertoni)]. *Canadian Journal of Plant Science*, *91*(1), 1-27.

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