AGRONOMY SCIENCE AND BIOTECHNOLOGY

Influence of Nitric oxide donor nanoencapsulation on *Dyckia excelsa* Lema (Bromeliaceae) germination

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ABSTRACT

Bromeliads are used in landscaping due to the beauty of their leaves and flowers. The use of plant regulators such as nitric oxide (NO) promotes the stimulus of germination and it has been a way out to enhance production and reduce the search for plants in nature and nanoencapsulation aims to optimize its effect. The objective of this work was to evaluate the effect of using free and nanoencapsulated NO donor on the germination of *D. excelsa*. The treatments consisted of soaking the seeds for 5 minutes with s-nitrosoglutathione (GSNO), chitosan/sodium tripolyphosphate nanoparticles containing GSNO (NP CS/TPP-GSNO) and empty (NP CS/TPP) at doses: 15 mM and 20 mM. The control consisted of imbibition in distilled water. For each treatment, 4 replications of 50 seeds were used. The following variables were evaluated: percentage of germination (GER), first germination count (FGC), germination speed index (GSI), average germination time (AGT) in addition to the length (SL) and seedling dry weight (SDW). To characterize the seeds, water content and viability were evaluated. *D. excelsa* seeds had 9.9% water content and 64% viability. For GER, treatments with GSNO ranged from 43 to 60%. The application of GSNO stimulated the germination process of *D. excelsa* and the nanoencapsulation did not cause any difference in the results compared to the free GSNO. It was concluded that the application of 15 or 20 mM of the GSNO donor is recommended for stored seeds of *D. excelsa.*

Keywords: Bromeliaceae, domestication, extractivism, floriculture, nanotechnology, plant growth regulator.

INTRODUCTION

In Brazil, several works have been done in Horticulture area (Carvalho et al., 2019; Colombo, Favetta & Faria, 2015; Frölech et al., 2019; Roberto, Mashima & Colombo, 2015; Takane et al., 2020). In relation to the plants used in this research, the frequent use of bromeliad plants in landscaping projects is due to the exuberance, shape and colours of the leaves, flowers and stem. Plant height, diameter of the flower and the presence of thorns are characteristics that define the allocation of the species, and contribute to landscape use (Zucchi, Silva, Sibov, & Pires, 2019).

Bromeliaceae is one of the largest families of floriferous plant in the neotropical region, including 95 genus e 3,568 species in literature (World Flora Online [WFO], 2022). A large sum of this group is found in Brazil, representing 56 genus and 1,386 species, some of them with a high endemic rate, comprising of 1.186 species and 24 genus (Reflora 2022).

The rise in use of this species in landscaping is resulting in high amounts of these plants being removed from its natural habitats, factor that contributes to the increased threat of extinction of some species. The southeast, northeast and south regions of Brazil have noticed extractive activities and the state of Paraná this activity has reached expressive levels, mainly in poor communities (Anacleto & Negrelle, 2019).

Bromeliads propagate sexualy and asexualy. Vegetative propagation is the choice for forming a new individual, with a rapid growth rate. Sexual reproduction involves the formation of a seed with a higher count of individuals from a single plant, however, with certain limitations regarding the maturity of seedlings and a long interval until flowering. In that context, basic information about germination is crucial to lead the plant to its full potential (Marialva, Lopes, Valente, & Chagas, 2019; Tamaki, Carvalho, Lazarini, & Nievola, 2020).

The genus *Dyckia* comprises 145 species of which 130 are recurrent in Brazil. The states of Minas Gerais, Goiás and Rio Grande do Sul register the highest specific diversity, however, this occurrence is also registered in 16 other states, distributed across all geographic regions of the country (WFO, 2022).

Dyckia excelsa Leme. had its first natural record in the cities of Corumbá and Ladário (MS), made by Paggi et al. (2015). It was described in 1993 as part of Roberto Burle Marx's particular collection. This species thrives in stony soils or rock crevices. It has an average size of 0.70 to 1.30cm, propagating through buds and seeds (Ruas, Paggi, Aguiar-Melo, Hirsch, & Bered, 2020).

The growing demand for native species in last few years has generated interest in many propagation methods, mainly to support the reintroduction of the species in forests, a reflection of recent environment problems. Nevertheless, all research still lack knowledge related to the physiological potential and adequated care for seed analysis (Silva et al., 2019). In this context, *Dyckia* germination studies were carried out with some species, nonetheless, there are few and each species have particularities, reinforcing the need of studies contemplating other species of the genus (Flores et al., 2018; Paula, Men, Biz, Ribeiro Júnior, & Faria, 2020).

The interaction between levels of hormones, enzymes and moisture have a fundamental role in the seed germination (Guariz et al., 2022). NO has a positive effect on several plant cellular procedures, including: Breaking seed dormancy, growth and plant defense against stress. It can even has an effect on abscisic acid expression in dormancy breaking, acting positively on germination (Silva et al., 2019; Pereira et al., 2020).

NO should be applied trough donor compounds due to its gassy formation, and its frequently used to minimize the effects of saline, hydric and heavy metal stress (Badem & Söylemez, 2022; Meng, Jing, Huang, Shen, & Zhu, 2022). One of NO donors is S-nitrosoglutathione (GSNO), a molecule found naturally in plant cells, with increased Half-life and stabilitty (Jahnová, Luhová, & Petřivalský, 2019).

However, donors have an unstable character and are highly susceptible to environmental factors. The use of nanoparticles as NO carries is an alternative capable to increase the stability and efficiency of these molecules, protecting against the excess NO release.

Due to the importance of studies on germination native species and the potential use of plant regulators through nanotechnology, this work aims to evaluate the effect of free and nanoencapsulated GSNO in *D. excelsa* seeds.

MATERIAL AND METHODS

Plant material and batch characterization

The experiment was carried out at the Phytotechnics Laboratory of the State University of Londrina. *Dyckia excelsa* Leme seeds were provided by collector Mr. Walter Miguel Kranz and collected in October 2020, in Londrina city, PR-Brazil. The collected fruits were placed in paper bags and taken to the laboratory. After being dried at room temperature for three days, protected from the sun, to complete maturation, the seeds were extracted and stored in a refrigerator in Kraft® bags at a temperature of 10 ± 2°C for 8 months.

To characterize the lot, the viability and water content of the seeds were determined following the Rules for Seed Analysis recommendations (Ministério da Agricultura, Pecuária e Abastecimento [MAPA], 2009).

In order to establish the water content, 0.2 g of seeds were placed in an oven at 105 ± 3°C for 24h00 hours and 4 replications were performed. The result was obtained by the difference in the weight of the seeds and the average of the repetitions was obtained. In carrying out the tetrazolium test, 4 repetitions of 50 seeds each were used. The seeds were placed in cryovials with a capacity of 2 mL, and filled with distilled water. After 24h00 hours at 25°C in a germination chamber, the water was removed and a 1% tetrazolium chloride solution was added and the seeds were kept in a germination chamber in the lack of light for 24h00 hours at 30°C. After this period, the percentage of viable seeds was evaluated using a magnifying glass. Seeds that did not have an embryo were not included in the calculation of viable seeds.

Obtaining free and nanoencapsulated nitric oxide

Free GSNO (S-nitrosoglutathione) and suspensions of chitosan (CS) nanoparticles (NPs) containing GSNO were provided by Prof. Dr. Amedea Barozzi Seabra from the Federal University of ABC, SP-Brazil. GSNO was synthesized and characterized according to the methodology of Silveira et al. (2016). Reduced glutathione (GSH) was dissolved in hydrochloric acid (1 mol L^{-1}) to 1.2 mol L^{-1}. An equimolar amount of sodium nitrite ($NaNO_2$) was added to the GSH solution in order to nitrosate GSH, in an ice bath for 30 minutes and magnetic stirring. Subsequently, acetone was added and this solution was filtered and washed several times with cold water, obtaining the precipitated GSNO. The obtained solid was lyophilized for 24h00 and stored at -20°C.

To obtain NPs containing GSNO, these were prepared using the ionic gelation method (Marcato et al., 2013; Pelegrino, Silva, Watashi, Haddad, & Seabra, 2017). Briefly, chitosan (CS) was dissolved in acetic acid (1%) and 26 mmol L^{-1} of GSH was added to the solution. After 90 minutes of magnetic stirring at room temperature (25 ± 2°C), a 0.6 mg mL^{-1} solution of sodium tripolyphosphate (TPP) was added dropwise to the CS/GSH solution. The final mixture was magnetically stirred for at least 90 minutes, obtaining a final concentration of GSH equal to 20 mmol L^{-1}.

To obtain chitosan NPs containing GSNO, an equimolar amount of sodium nitrite ($NaNO_2$) was added to the NP-CS/TPP-GSH suspension, followed by holding for 60 minutes in the dark. The final concentration of NP-CS/TPP-GSNO was 20 mM. The same procedure was performed without the presence of GSH, to obtain NPs containing CS/TPP only.

Assembly of treatments

To select the doses of the NO donor (GSNO) different concentrations of GSNO were initially tested and, finally, the doses (15 mM and 20 mM) were selected, as they respond better to the germination of this species.

Before setting up the treatments, the seeds were sterilized in a 1% sodium hypochlorite solution for 1 minute, followed by immersion in 70% alcohol for 1 minute and then washed with autoclaved water. The treatments were: control (water), GSNO (15 and 20 mM), NP-CS/TPP-GSNO (15 and 20 mM) and NP-CS/TPP (15 and 20 mM). For each treatment 200 seeds were used which, after imbibition, were separated into 4 repetitions of 50 seeds. The seeds were soaked for 5 minutes in flasks containing 5 mL of each dose (treatment).

Evaluated parameters and statistical analysis

Tests were carried out for the first and last germination count, with the first count on the 4th day and the last count on the 10th, as there is no standardization for the germination test for this species. Germination percentage (GER), first germination counting (FGC), germination speed index (GSI), average germination time (AGT), in addition to seedling length (SL) and seedling dry weight (SDW) were evaluated.

The seeds were placed to germinate on blotting paper moistened with distilled water at 2.5 times the mass of the non-hydrated paper, and placed in crystal polystyrene boxes (Gerbox®) of dimensions 11 cm x 11 cm x 3 cm. The gerbox® were kept in a growth chamber (BOD type) at a temperature of 25 ± 2°C, with a photoperiod of 8 hours, as recommended by the Seed Analysis Rules (MAPA, 2009).

The first germination count (FGC) was conducted with the germination test, counted at the time of the primary root protrusion of the first seedling and the results were expressed as percentage of normal seedlings (Krzyzanowski, França-Neto, Gomes-Junior, & Nakagawa, 2000). TThe count was performed on the 4th day.

Along with the germination test, the number of germinated seeds was counted to establish the germination speed index (GSI), obtained by Maguire's formula (1962). GSI = G1/N1 + G2/N2 + ... + Gn/Nn, where: G1, G2 and Gn = number of normal seedlings, computed in the first, second and last counts; N1, N2, Nn = number of days from sowing in the first, second and last count.

The average germination time (AGT) was determined by the criterion established by Labouriau and Valadares (1983) and performed concomitantly with the germination test, counting daily the number of seeds germinated after the test

was installed. This index represents the average time required for germination, taking daily germination as a weighting factor, calculated by the equation:

AGT= (G1T1+G2T2+........GnTnG1+G2....+Gn)/(G1+G2....+Gn).

Whereby:

AGT = is the average time, in days, required to reach maximum germination;

G1, G2 and Gn = is the number of seeds germinated and at times T1, T2 and Tn, respectively.

The percentage of total germination was determined by computing all normal seedlings, which showed potential to continue their development and give rise to normal plants when grown under favorable conditions (MAPA, 2009).

On the 10th day, the percentage of germination and abnormal seedlings was determined, and the seedling length (mm) was determined using a caliper, through the random selection of twenty seedlings. Then, from the same twenty seedlings, the dry matter was determined. The seedlings were placed in paper bags and kept in an oven with forced air circulation at 65°C, until reaching constant mass and weighed on an analytical balance (accuracy ±0.0001g) with result expressed in mg.

The experimental design adopted was completely randomized and the assumptions of normality and homogeneity of variances were tested by Shapiro-Wilk and Bartlett ($p \geq 0.05$) respectively, and subsequently submitted to analysis of variance ($p \leq 0.05$). The data were processed using the R software (R Core Team, 2022), and submitted to the Tukey test considering a significance level of 5% error probability.

RESULTS AND DISCUSSION

In the characterization of the seed batch of *D. excelsa*, they had 9.9% of water content and viability of 64%.

Table 1 shows the F values of the analysis of variance of the variables germination (GER), first germination count (FGT), germination speed index (GSI), average germination time (AGT), length (SL) and seedling dry weight (SDW) of *D. excelsa* seeds. There was a statistical difference between treatments for all variables, except for SL with a variation from 7.02 to 8.49 mm.

Table 1. F values of the variance analysis of the variables germination (GER), first germination count (FGT), germination speed index (GSI), average germination time (AGT), length (SL) and seedling dry weight (SDW) of *D. excelsa* seeds.

	GER (%)	*GSI^{t}*	*AGT (dias)*	*FGC (%)^{t}*	*SL (mm)*	*SDW (mg)*
F	5.658**	7.941**	11.299**	2.826*	1.268^{ns}	7.283**
Coefficient of variation (%)	21.15	9.93	4.98	13.95	12.09	10.99

^{*,**,ns}: Significant at 5%, 1% and not significant by the F test. ^{t} Transformed data.

For GER, treatments with NO application ranged from 43 to 60%, whether with free or nanoencapsulated GSNO donors. On the other hand, there was no difference between the control treatment (32%) and the treatments with empty nanoparticles

(NP CS/TPP), which varied between 35 and 38%. In the GSI variable, the result was similar with higher values in treatments with GSNO application, ranging from 5.86 to 8.25. The control treatment obtained GSI of 4.69 and did not differ from the treatments with free nanoparticles (NP CS/TPP), which ranged from 4.53 to 5.08 (Figure 1).

For AGT, seeds germinated faster in treatments with GSNO application, with faster germination in treatments with NP CS/TPP-GSNO, with 3.21 (10 mM) and 3.29 days (15 mM). The control treatment, for example, had a TMG of 3.95 days. For the first count, despite the difference by ANOVA, there was no difference by the Tukey test, with a variation from 9 to 16% of seeds germinated on the 4th day of the germination test (Figure 1).

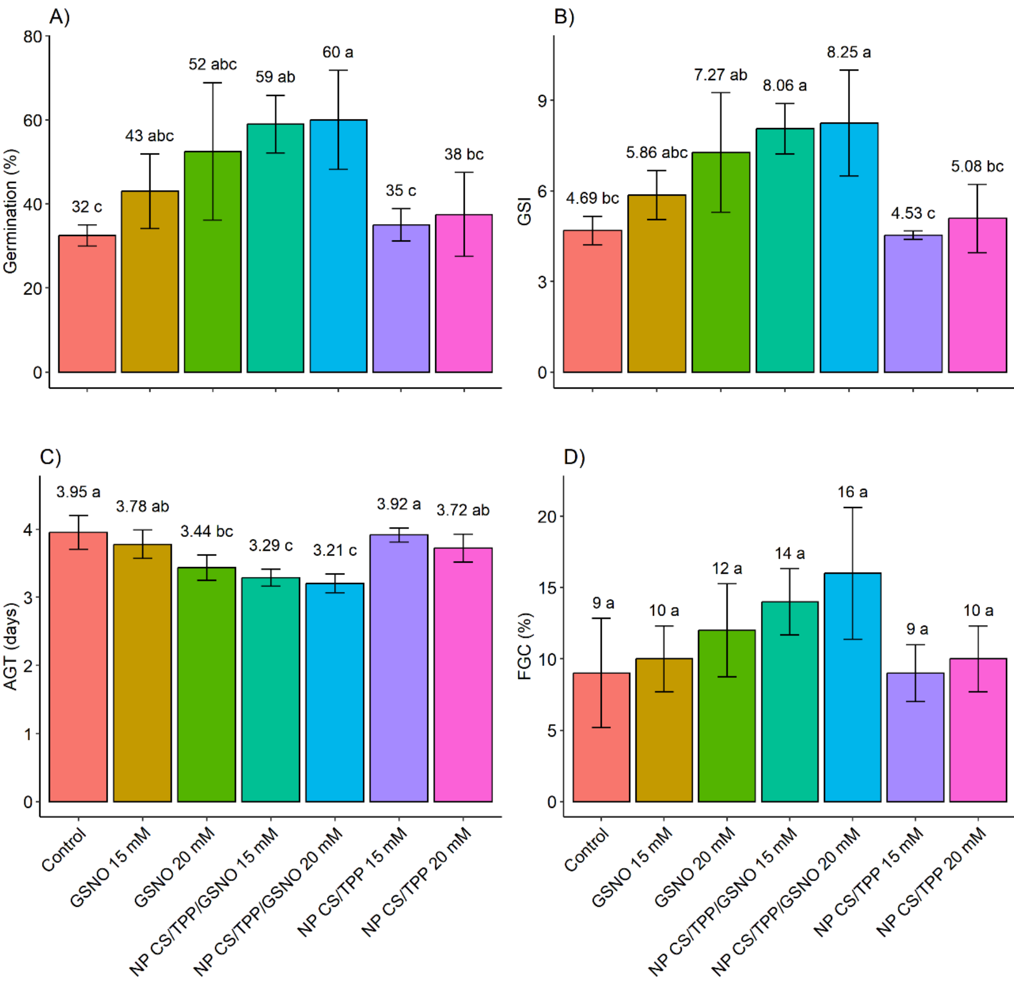

Figure 1. Germination (A), germination speed index (B), average germination time (C) & first germination counting (D). Average followed by the same lowercase letter in the columns do not differ by Tukey's test at 5% significance.

*GSNO (S-nitrosoglutathione), NP (nanoparticles), CS (chitosan), TPP (sodium tripolyphosphate).

For SDW, the application of GSNO stimulated a greater mass of seedlings, with better responses in treatments with NO, ranging from 7.20 to 8.18 mg (Figure 2). The lowest values of SDW were obtained in the control treatment (5.67 mg) and for the empty NP (NP CS/TPP) ranging from 6.03 to 6.35 mg.

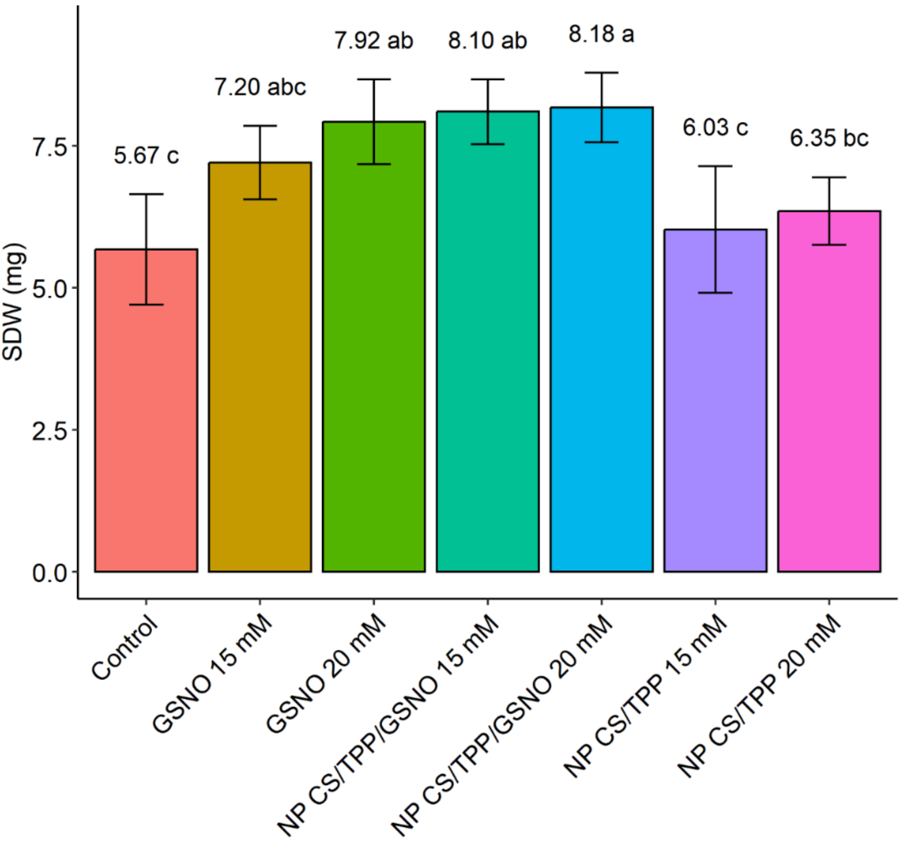

Figure 2. Seedling dry weight (SDW). Average followed by the same lowercase letter in the columns do not differ by Tukey's test at 5% significance.
*GSNO (S-nitrosoglutathione), NP (nanoparticles), CS (chitosan), TPP (sodium tripolyphosphate).

Dyckia seeds are considered orthodox, namely they tolerate drying, remain alive with water content around 5% to 10%, withstand temperatures below zero and can remain viable for many years. Thus, the storage conditions of the seeds of the species under study are in ideal conditions, however, it is noteworthy that this content is not applicable to all species of the genus, as each has a tolerable limit regarding desiccation (Rajanaidu & Ainul, 2013).

The aim of study was to understand how NO can regulate germination, emphasizing the importance of understanding the germination process. Germination can be defined as the sequence of molecular and physiological events initiated by imbibition of the non-dormant seed and leading to radicle protrusion that marks the end of germination.

In the soaking process, the dry seed passes successively through three main stages of water absorption. The first stage consists of a rapid imbibition that leads to the progressive resumption of metabolic activity, gene expression (transcription), protein synthesis and processing, and DNA repair. During the second stage, the water content increases slightly while important metabolic changes occur within the seeds. At the end of this second stage, if the mechanisms to proceed with germination occur, the embryo's growth potential overcomes the mechanical restrictions imposed by the surrounding layers, leading to the successive rupture of the testa and

the endosperm. The protrusion of the radicle through the tegument is therefore obtained as a result of cell elongation (Sano et al., 2012).

NO is known to release dormancy and providing a stimulus for seed germination in several species (Arc, Galland, Godin, Cueff, & Rajjou 2013). A study by Liu et al. (2009) found a rapid accumulation of NO in Arabidopsis seeds, possibly in the endosperm layer, causing abscisic acid (ABA) catabolism and breaking dormancy.

It is known that NO is responsible for the degradation of reserves present in the seeds endosperm through the mobilization of the main enzymes that carry out this degradation, such as amylases. Silva et al. (2019), who worked with *Leucaena leucocephala* (Lam.) de Wit, confirmed this information. Seeds also prepared in KNO_3, and through the release of NO, confirmed the significant role in the mobilization of the main enzymes during the germination of the seeds of this species.

NO is also able to reduce the effects of seed aging and increase tolerance to various abiotic adversities, such as water, saline and heavy metal stress (Pires, Souza, Cardoso, Dias, & Borges, 2016). Many of the deleterious processes suffered by plants subjected to adverse conditions are mediated by reactive oxygen species (ROS). However, at low concentrations, ROS act as signalers for the activation of the stress defense system (Kohli et al., 2019).

These products in excess cause harmful chain reactions for plants, targeting macromolecules and altering their functionality. Excessive ROS production causes chlorophyll destruction, DNA fragmentation, protein damage, lipid peroxidation and ion leakage, causing cell death. The plant's defense mechanism includes enzymatic activity as ascorbate peroxidase (APX), catalase (CAT), guaiacol peroxidase (POD) and superoxide dismutase (SOD). NO has the ability to activate this antioxidant defense complex, thus having the ability to contain the cellular damage caused by ROS, aiding in their detoxification (Kohli et al., 2019).

NO can also react with tyrosines by the nitration process or with thiol residues by oxidation. These complexes may be related to the regulatory effects of NO transcription factors and certain enzymes (Silva, Santos Dias, Borges, Ribeiro, & Silva 2015). NO also acts on signaling with other plant hormones such as auxins, a fact that also explains its effect on seedling growth variables, since auxin, at optimal levels, plays a relevant role in the development of the apical meristem (Salmi, Clark, & Roux, 2013).

The evaluation of free nanoparticles aims to avoid a false positive result based on the plant growth regulator, in this case the GSNO. This is because chitosan, a polymer present in the formulation of nanoparticles is widely applied in agriculture for the preservation of fruits, vegetables and seeds against deterioration by microorganisms, to stimulate the plant's immune system, protecting the plant against the attack of pathogens and favoring its growth, consequently increasing plant production. Thus, it participates in inducing the synthesis and production of some enzymes and proteins, which may interfere with germination results (Malerba & Cerana, 2016). The results make clear the similarity of the empty NPs with the control, leading to the observation that the GSNO actually promoted the germination stimulus, the SDW and the reduction of the AGT.

CONCLUSIONS

The application of GSNO stimulates the germination process of *D. excelsa*, however, the nanoencapsulation does not cause difference in the results. It is concluded that the application of 15 or 20 mm of the GSNO donor is recommended for stored seeds of this species.

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