Universidade Federal Rural do Semi-Árido
Pró-Reitoria de Pesquisa e Pós-Graduação
https://periodicos.ufersa.edu.br/index.php/caatinga
ISSN 1983-2125 (online)

http://dx.doi.org/10.1590/1983-21252024v3711850rc

Scientific Article

In vitro establishment of *Sideroxylon obtusifolium* (Roem. & Schult.) T. D. Penn

Estabelecimento *in vitro* de *Sideroxylon obtusifolium* (Roem. & Schult.) T. D. Penn

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ABSTRACT - *Sideroxylon obtusifolium* is a native species recognized by traditional communities for its versatility, as it is used to treat various diseases, in fence construction, furniture manufacturing, and tool handles. It is also used for both human and animal consumption. However, extractive use combined with environmental impacts such as habitat loss and wildfires can cause a significant reduction in this species. Therefore, the adoption of biotechnological techniques such as plant tissue culture has been recognized as a significant resource to support the preservation of species with economic and medicinal importance and endangered. Thus, this study aimed to determine an efficient protocol for the *in vitro* establishment of *S. obtusifolium*. To achieve this, the seeds were disinfected using different chemical agents (carbendazim, chlorine dioxide, and sodium hypochlorite) for varying lengths of time to establish an efficient disinfection method. Additionally, MS ½ and WPM media, with and without the addition of activated charcoal, were tested to determine the most efficient medium composition for germination. The results indicated that sodium hypochlorite at 2% for 25 minutes was effective for seed asepsis. For germination, the most suitable medium was WPM containing activated charcoal, resulting in healthy and uniform seedlings that can serve as plant material for *in vitro* propagation of the species.

Keywords: Micropropagation. Organogenesis. Sapotaceae. Medicinal plant.

RESUMO - *Sideroxylon obtusifolium* é uma espécie nativa, reconhecida pelas comunidades tradicionais por sua versatilidade, sendo empregada para o tratamento de diversas doenças, na construção de cercas, fabricação de móveis e cabo de ferramentas. Além disso é utilizada tanto na alimentação humana quanto animal. No entanto, o uso extrativista somado aos impactos ambientais como perda de habitat e queimadas pode ocasionar redução considerável dessa espécie. Dessa forma, a adoção de técnicas biotecnológicas, como a cultura de tecidos vegetais, tem sido reconhecida como um recurso significativo para apoiar a preservação de espécies com relevância econômica, medicinal e ameaçadas de extinção. Assim, este trabalho teve como objetivo determinar um protocolo eficiente para o estabelecimento *in vitro* de *S. obtusifolium.* Para isso, as sementes foram desinfestadas utilizando diferentes agentes químicos (carbendazim, dióxido de cloro e hipoclorito de sódio) por tempo variado visando estabelecer um método eficiente de desinfestação. Além disso, o meio MS ½ e WPM, com e sem adição de carvão ativado foram testados para determinar a composição do meio mais eficiente para germinação. Os resultados indicaram que o hipoclorito de sódio a 2% durante 25 minutos foi eficiente para a assepsia das sementes. Para a germinação o meio mais adequado foi o WPM contendo carvão ativado, resultando em plântulas sadias e uniformes e que podem servir de material vegetal para propagação *in vitro* da espécie.

Palavras-chave: Micropropagação. Organogênese. Sapotaceae. Planta medicinal.

Conflict of interest: The authors declare no conflict of interest related to the publication of this manuscript.

Received for publication in: March 18, 2023.
Accepted in: May 17, 2024.

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INTRODUCTION

Sideroxylon obtusifolium (Roem. & Schult.) T. D. Penn is a fruit tree of the Sapotaceae family, distributed in humid areas and on the banks of rivers of the arboreal Caatinga, in coastal restingas and in the Chaquenha forest of the Pantanal of Mato Grosso (ALVES-ARAÚJO, 2023). Also known in Portuguese as *quixabeira*, *quixaba*, *sapotiaba*, *sacutiaba*, *coronilha*, *coca*, *maçaranduba-da-praia*, *miri* and *rompe-gibão*, this species is widely used in folk medicine for the treatment of various infections (SILVA et al., 2021) by traditional communities (NASCIMENTO et al., 2009).

Normally, the extractive use of *S. obtusifolium* bark for medicinal purposes causes the death of the plants, leading to the reduction of individuals in their natural habitat (BARBOSA; LUCENA; CRUZ, 2019). In addition, it is propagated by means of seeds, whose germination may show some difficulties, such as seed coat dormancy, which requires strategies to be overcome (REBOUÇAS et al., 2012), and the dependence on abiotic factors such as favorable temperature, humidity and photoperiod to ensure reproductive success (SILVA; DANTAS, 2017). Therefore, studies seeking methods to assist in the production of seedlings for sustainable exploitation of this species are needed.

The application of biotechnological techniques, such as the culture of plant

cells and tissues, has shown promise to assist in the conservation of rare species, especially those of economic and medicinal importance and endangered. This technique allows efficient seed germination, producing seedlings faster than conventional methods; in addition, these seedlings are produced in controlled environments, ensuring uniform and pathogen-free material (CHEN et al., 2019; VICENTE; PÊGO, 2024). This technique is also used in the formation of germplasm banks, genetic improvement, and in the development of molecular biology protocols (PORFÍRIO et al., 2019).

In vitro establishment encompasses the choice of explant (OLIVEIRA; FREIRE; ALOUFA, 2019), followed by the choice of the disinfestation method, as the application of efficient protocols is essential at this stage, since the presence of microorganisms represents limitations in obtaining aseptic cultures (ESPOSITO-POLESI, 2020). Finally, the nutrient medium will provide macro and micronutrients, organic compounds such as vitamins, and water, which are necessary for seedling development (KARIMAH; YUNIATI; HANDAYANI, 2020).

Considering the lack of studies on *in vitro* propagation with *S. obtusifolium*, the objective of this study was to determine the most efficient chemical agent for seed disinfestation and the culture medium for *in vitro* establishment of this species.

MATERIAL AND METHODS

Collection site and experimental site

The seeds were collected in the municipality of Ipirá, Bahia (12° 9' 14" S and 39° 44' 42" W), and the experiments were carried out at the Plant Tissue Culture Laboratory (LCTV) of the Horto Florestal Experimental Unit (UEHF), belonging to the State University of Feira de Santana (UEFS).

Seed disinfestation with different chemical agents

After removing the seed coat, the seeds were washed in running water for 10 minutes and then immersed in 70% alcohol for 1 minute in the laminar flow chamber. Then, the seeds were subjected to different treatments: control without the addition of chemical agents; immersion in sodium hypochlorite solution at different concentrations (0.2, 0.5 and 2%), plus three drops of neutral detergent for 10 minutes; immersion in chlorine dioxide (Tcsa-clor®) at different concentrations (1, 3 and 5%) of the active ingredient for 10 minutes; and immersion in carbendazim (Bendazol®) fungicide solution at different concentrations (1, 3 and 5 mL L^{-1}) for 30 minutes, followed by immersion in sodium hypochlorite solution at 2%, plus 3 drops of neutral detergent for 10 minutes.

Next, the seeds were washed three times in distilled water and then inoculated into test tubes (25 x 150 mm) containing 10 mL of WPM (Woody Plant Medium) culture medium from Lloyd and McCown (1980), with addition of 30 g L^{-1} of sucrose and solidified with 7 g L^{-1} of agar (basic medium). The experimental design was completely randomized (CRD) with 10 treatments, five replicates and 10 plots, totaling 50 seeds per treatment. Seed contamination was evaluated after 40 days of inoculation, determined by visual analysis of the seeds and expressed as a percentage.

Disinfestation of seeds with different immersion times in sodium hypochlorite

The seeds were immersed in 2% sodium hypochlorite for 10, 15, 20 and 25 min before removal of the seed coat. Then, the seed coat was removed and the seeds were subjected to 70% alcohol for 1 min. After that, they were immersed in sodium hypochlorite for 10, 15, 20 and 25 min plus 3 drops of neutral detergent. At the end of each immersion time, the seeds were washed three times in distilled water and inoculated in a test tube containing 10 mL of the basic medium.

The experimental design was completely randomized and consisted of four treatments with 30 replicates. Seed contamination was evaluated after 30 days of inoculation, determined by visual analysis and expressed as a percentage.

Composition of culture medium on *in vitro* germination and initial growth

Initially, the seeds were immersed in sodium hypochlorite (2%) for 25 min. After this period, the seed coat was removed and the seeds were immersed in 70% alcohol for 1 min. Subsequently, they were immersed in sodium hypochlorite for 25 min plus 3 drops of neutral detergent. Then, the seeds were washed three times in distilled water and inoculated in a test tube containing 10 mL of different types of culture medium: WPM and MS (MURASHIGE; SKOOG, 1962) with half the concentration of salts (MS ½), plus different concentrations of activated charcoal (0 and 1 g L^{-1}), supplemented with 30 g L^{-1} of sucrose and solidified with 7 g L^{-1} of agar.

The experimental design was completely randomized in a 2 x 2 factorial arrangement (two types of culture medium and two concentrations of activated charcoal), totaling four treatments with 40 replicates. Seed germination was evaluated after 30 days of inoculation, determined by radicle protrusion and expressed as a percentage. After 45 days of inoculation, shoot length, root length, number of leaves and nodal segments were evaluated.

RESULTS AND DISCUSSION

Seed disinfestation with different chemical agents

For the disinfestation treatments in relation to the percentage of contamination, a significant effect ($p \leq 0.05$) was observed at 40 days after inoculation.

It was observed that *S. obtusifolium* seeds, inoculated *in vitro*, showed the presence of bacteria and fungi (Figure 1), attesting to contamination as one of the major obstacles in the *in vitro* establishment of plants (CID; TEIXEIRA, 2014).

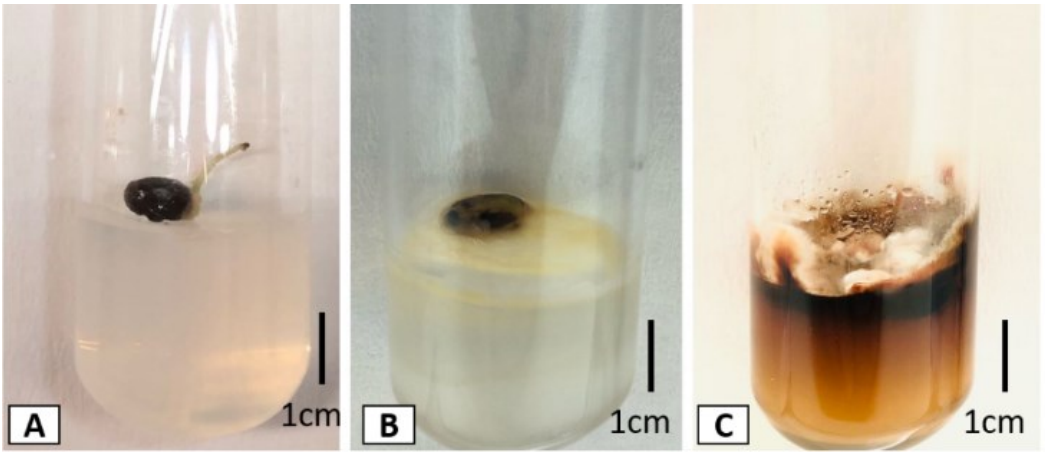

Figure 1. Appearance of *S. obtusifolium* seed 40 days after inoculation: (A) seed without contamination; (B) bacterial contamination; (C) fungal contamination.

The highest percentages of contamination were observed in the control (without disinfestation) and in the treatment with 0.2% sodium hypochlorite, 88 and 100%, respectively. On the other hand, the treatments with 3% and 5% chlorine dioxide, fungicide at a concentration of 5 mL L^{-1} and 2% sodium hypochlorite resulted in contamination levels of 2%, 6%, 10% and 18%, respectively, which are statistically lower than those found in the control and with 0.2% hypochlorite (Table 1).

The success of asepsis depends, among other factors, on the concentration, time of immersion in the solution and disinfecting agent. Thus, low concentrations associated with short immersion time may not be efficient in disinfestation (AZEVEDO et al., 2023). This fact was observed in the present study, as the 0.2% sodium hypochlorite solution resulted in a higher percentage of contamination (100%), while the highest concentration of this agent, 2%, reduced contamination to 18% (Table 1).

Table 1. Percentage of contamination in *Sideroxylon obtusifolium* seeds under the action of different chemical agents.

Chemical agents	Contamination (%)
Control	88 a
Sodium hypochlorite (0.2%)	100 a
Sodium hypochlorite (0.5%)	46 b
Sodium hypochlorite (2%)	18 c
Chlorine dioxide (1%)	42 b
Chlorine dioxide (3%)	2 c
Chlorine dioxide (5%)	6 c
Carbendazim (1 mL L^{-1}) + sodium hypochlorite (2%)	44 b
Carbendazim (3 mL L^{-1}) + sodium hypochlorite (2%)	24 bc
Carbendazim (5 mL L^{-1}) + sodium hypochlorite (2%)	10 c

Means followed by equal letters in the column did not differ from each other by Tukey test ($p \leq 0.05$).

The choice of an efficient treatment for disinfestation is of great importance in *in vitro* culture, as it makes it possible to keep the explants free of microorganisms (SOUSA et al., 2018). Sodium hypochlorite is widely used for this purpose because, in addition to not being expensive, it provides satisfactory results for several species. In a study conducted by Pinheiro et al. (2016), it was found that asepsis with 2% sodium hypochlorite for 1 min was efficient for the health of seeds of the forest species *Bauhinia forficata*, *Cedrela fissilis*, *Parapiptadenia rigida* and *Senegalia bonariensis*.

As demonstrated in the analysis of the results of this experiment, the lowest means of contamination were obtained using 3 and 5% chlorine dioxide, fungicide at a concentration of 5 mL L^{-1} and 2% sodium hypochlorite. Thus, due to the efficacy and ease of acquisition of sodium hypochlorite, new tests were performed in order to evaluate different seed immersion times for greater contamination control.

Disinfestation of seeds with different times of immersion in 2% sodium hypochlorite

The results indicated a significant difference ($p \leq 0.05$) between the different times of immersion in the 2% sodium hypochlorite solution in relation to the percentage of contamination.

Increasing time of immersion in the sodium hypochlorite solution caused a significant reduction in the

presence of microorganisms in the culture medium. When the seeds were immersed in sodium hypochlorite for 10 min, the percentage of contamination was 36%, statistically superior to that found in the treatment with immersion for 25 min, which resulted in contamination of only 3% (Figure 2). Thus, it was possible to observe the efficiency of sodium hypochlorite in the asepsis of *S. obtusifolium* seeds.

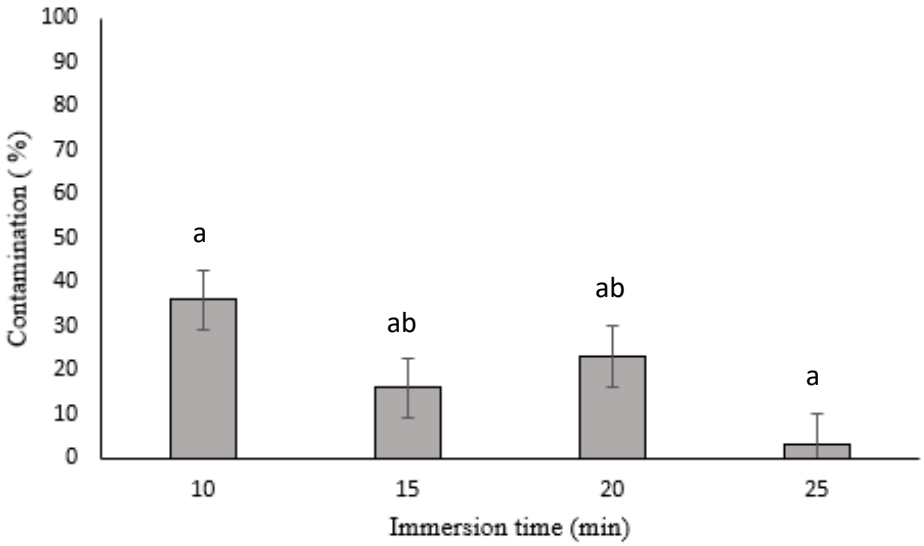

Figure 2. Contamination of *Sideroxylon obtusifolium* seeds under different times of immersion in sodium hypochlorite. Means followed by the same letter did not differ from each other by Tukey test ($p \leq 0.05$).

Corroborating this result, Azevedo et al. (2023) found that the most efficient treatment for asepsis of African mahogany (*Khaya grandifoliola*) seeds was exposure for 20 min to 2.5% sodium hypochlorite solution. The use of sodium hypochlorite has also been shown to be effective for the asepsis of safflower (*Carthamus tinctorius* L.) seeds, reducing the incidence of phytopathogens and promoting an increase in germination (MENEGAES et al., 2021). These authors found that the immersion of safflower seeds in 2.5% sodium hypochlorite resulted in contamination of 17%, while the immersion in a 5% active ingredient for 30 min led to 12% of seeds infested by microorganisms.

Souza et al. (2014) used systemic fungicide for asepsis of *S. obtusifolium* seeds, which were kept for 1 hour under 80 rpm, followed by immersion in 2% sodium hypochlorite for 20 min, and obtained 96% of plants without contamination. However, after 20 and 30 days, they found the presence of bacteria in the culture medium, which demonstrated the ineffectiveness of this protocol. On the other hand, in the present study, treatment with 2% sodium hypochlorite for 25 min proved to be an efficient protocol for disinfesting *S. obtusifolium* seeds and obtaining pathogen-free *in vitro* plants.

Composition of culture medium on *in vitro* germination and initial growth

For the variables percentage of germination, seedling formation and number of segments in the different treatments (WPM medium and MS ½ combined or not with activated charcoal), there were significant differences ($p \leq 0.05$). On the other hand, the variables shoot length, number of leaves and root length were significantly affected ($p \leq 0.05$) by the addition of activated charcoal to the culture medium.

Although no significant difference was found between the treatments, MS ½ medium resulted in 100% seed germination without the addition of charcoal and 97% germination in the presence of activated charcoal. In both MS ½ media, 57% of seedlings were formed (Figures 3A and 3B). Cultivation in WPM medium with the addition of charcoal led to 95% of germinated seeds, while cultivation in WPM medium without the addition of charcoal led to 97% germination. However, the presence of activated charcoal in the medium resulted, on average, in 73% of seedlings, with good growth and development, and it was possible to collect explants for *in vitro* multiplication (Figure 3C) compared to the charcoal-free medium, 65% (Figure 3D).

The composition of the medium for *in vitro* cultivation influences several metabolic processes, as it must provide all the necessary components for plant development. Thus, the use of media with lower concentration of salts has led to better results for germination and development of seedlings of woody species, such as baru (*Dipteryx alata*) (PINHAL et al., 2017). These authors obtained a higher percentage of complete seedlings using MS medium with reduction in salt concentration. On the other hand, for the *in vitro* germination of *Hippeastrum hybridum* seeds, WPM medium was the most effective, promoting 100% germination (RODRIGUES; CARVALHO; AYUB, 2020).

Seeds of barbatimão (*Stryphnodendron adstringens* (Mart.) Coville), a medicinal species from the Cerrado, were cultivated in MS and WPM medium and resulted in a high percentage of germination, regardless of the culture medium (CASTRO et al., 2007). This result corroborates those found in the present study, in which high germination of *S. obtusifolium* was obtained for both culture media (MS ½ and WPM).

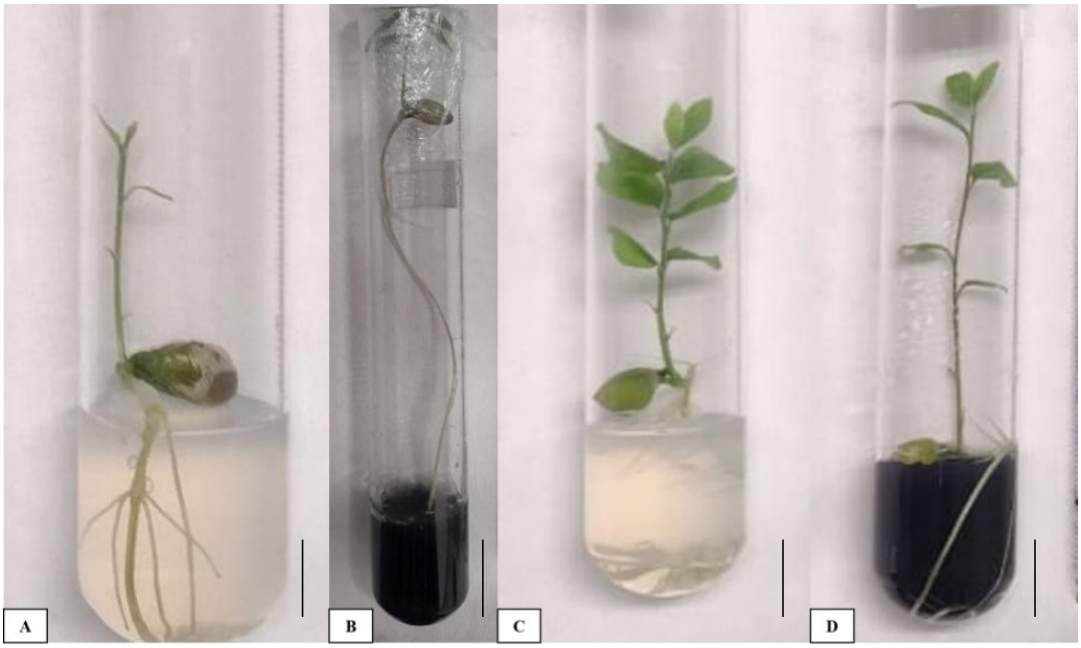

Figure 3. *Sideroxylon obtusifolium* seedling at 30 days of cultivation: (A) Seedling in MS ½ medium without charcoal; (B) Seedling in MS ½ medium with charcoal; (C) Seedling in WPM medium without charcoal; (D) Seedling in WPM medium with charcoal. Bar: 1cm.

Several formulations of basic media have been used in *in vitro* cultivation, not only providing nutrients, but also influencing cell growth and morphogenesis (PIERINE; GIANINI; MORAES, 2019). For woody species, however, the MS medium is not satisfactory because it has a higher osmotic potential, hindering seed germination, as observed in the germination of *Handroanthus chrysotrichus* seeds, so more diluted compositions in terms of macronutrients have better performance in the germination of the species (BARBOSA et al., 2020).

Addition of charcoal to the culture medium led to better results in epicotyl length, number of leaves and root length compared to the absence of activated charcoal (Table 2).

Table 2. Effect of activated charcoal on epicotyl length (EL), number of leaves (NL) and root length (RL) of *Sideroxylon obtusifolium*, after 45 days of *in vitro* cultivation.

Charcoal (g L^{-1})	EL (cm)	NL	RL (cm)
0	4.19 b	8.53 b	11.13 b
1	5.06 a	9.50 a	13.78 a

*Means followed by equal letters in the column did not differ from each other by Tukey test ($p \leq 0.05$).

Activated charcoal has been widely used in the medium for *in vitro* cultivation, as it helps in seedling development and germination, removing the toxicity released by seeds, and because it performs activities related to pH, controlling the acidity of the medium (PAULINO et al. 2021). In the present study, addition of charcoal to the medium resulted in seedlings with greater epicotyl length (5.06 cm) compared to the medium without charcoal. An opposite effect was observed in the cultivation of pequi (*Caryocar brasiliense*), as the authors found a significant difference in shoot length in the absence of charcoal (LONDE et al., 2022).

After 45 days, *S. obtusifolium* plants cultivated in the presence of activated charcoal showed higher root growth (13.78 cm) when compared to seedlings grown in the absence of charcoal (11.13 cm). In the *in vitro* cultivation of ipê-roxo (*Handroanthus impetiginosus*), Máximo et al. (2020) observed the highest value of main root length in the medium with addition of 2.0 g L^{-1} of activated charcoal. This may be associated with the fact that charcoal stimulates dark conditions, under which the roots develop better due to the reduction of light incidence in the growth zone of the root system, besides adsorbing toxic substances that can affect the development of the explant (MÁXIMO et al., 2020).

In short, this study is a pioneer in the *in vitro* cultivation of *S. obtusifolium*, since it was successful in the *in vitro* establishment and obtaining of parent plants to be used in *in vitro* propagation. In addition, the results obtained are promising and open new possibilities for the large-scale production of seedlings of this species. Thus, we expect that this study can contribute significantly to the preservation of the species and its sustainable use.

CONCLUSION

The use of 2% sodium hypochlorite for 25 min is efficient for asepsis of *S. obtusifolium* seeds, and WPM medium with the addition of activated charcoal is indicated for the *in vitro* establishment of this species.

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