

## Callus induction in *Bertholletia excelsa* immature seeds

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### ABSTRACT

*Bertholletia excelsa* H.B.K., known as Brazil nut tree, belongs to the Lecythidaceae botanic family and is endemic to the Amazonian Region. Its nuts have high economic and nutritional value. The objective of this work was to establish efficient protocols for callus induction from immature seeds of *B. excelsa*, as a preliminary step to its further micropropagation. After disinfestation, fragments of immature seeds were cultivated in WPM (Wood Plant Medium) supplemented with 2,4-D (0, 1, 2, 4 and 8 mg.L<sup>-1</sup>) and TDZ (0, 1.6 and 3.2 mg.L<sup>-1</sup>) in factorial combination. Twenty-one days later the callus induction was evaluated. Cultures were kept in a growth room in the dark at 24±2°C. The highest percentage of callus induction was found with the combination of 2 mg.L<sup>-1</sup> 2,4-D with 3.2 mg.L<sup>-1</sup> TDZ.

**Keywords:** callus induction, growth regulators, Amazon Rainforest.

## Indução de calos em sementes imaturas de *Bertholletia excelsa*

### RESUMO

*Bertholletia excelsa* H.B.K., conhecida como castanheira-do-brasil, pertence à família Lecythidaceae e é uma espécie endêmica da Amazônia. Suas castanhas têm importante valor econômico e nutricional. Objetivou-se com esse trabalho determinar um protocolo eficiente para indução de calos em explantes de sementes imaturas de *B. excelsa*, primeiro passo para o estabelecimento de um protocolo de micropropagação da espécie. Após desinfestação, fragmentos de sementes imaturas foram cultivados em meio WPM (Wood Plant Medium) acrescido de 2,4-D (0, 1, 2, 4 e 8 mg.L<sup>-1</sup>) e TDZ (0, 1,6 e 3,2 mg.L<sup>-1</sup>) em combinação fatorial. Após 21 dias, foi avaliada a indução de calos. Os cultivos foram mantidos no escuro, em sala de crescimento, a 24±2°C. A condição que resulta em maior porcentagem de calogênese foi encontrada na combinação de 2 mg.L<sup>-1</sup> de 2,4-D com 3,2 mg.L<sup>-1</sup> de TDZ.

**Palavras chave:** Indução de calos, reguladores de crescimento, Floresta Amazônica.

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## INTRODUCTION

*Bertholletia excelsa* H. B. K., known as Brazil nut tree, is an endemic Amazonian species in the Lecythidaceae botanic family which has great economic and social value due to its high quality wood and edible seeds (Serra et al. 2000). Its nuts have high nutritional value and is called "vegetable meat" for its remarkable content of protein, calories, fat, and minerals, with the half the protein level and twice the caloric level as a 100 grams piece of beef; the "milk" produced from the Brazil nut has approximately as much as protein as cow's milk, and can be used as a culinary replacement; there are also high concentrations of methionine, sulfuric amino acid quite limited in the Amazon diet and often found in meat and beans (Cymerys et al. 2005). It is considered one of the major sources of selenium, which is used as an antioxidant and anticarcinogenic, and helps in the prevention of some types of viruses, Parkinson's and Alzheimer's (Fillion 2011).

The trees occur naturally in clusters; the density per hectare varies and the average is around one productive tree per hectare; the production per tree can reach 2,000 fruits with 8-24 seeds each (Shanley et al. 2010, Clement 1999). Almost all harvested Brazil nut is exported and after the decline of the rubber industry the Brazil nut became the main product for export from northern Brazil in the category of commodities (Serra et al. 2000). In 1999, Brazilian production was 19,000 tons; the state of Acre was the largest producer with 10,000 tons; in 2000, production rose to 33,000 tons and generated an income of more than US\$ 18 million (Shanley et al. 2010).

In respect of the reproductive propagation, a major difficulty is the slow and uneven germination process that takes 12-18 months to occur naturally, due to endogenous seed dormancy that appears to be favorable for increasing the effective storage period of Brazil nut; during storage, embryo maturation may take place and/or a chemical inhibitor is leached from the embryos, resulting in improved germination (Kainer et al. 1999). Moreover, the seeds are recalcitrant, decreasing the ability of germination with reduction in moisture content. Because of this, a period of moist-storage over five and a half months is recommended to achieve approximately 75% germination (Kainer et al. 1999). Besides, crossed pollination generally leads to variability among progenies, what can be undesirable in terms of production (Santos et al. 2010a).

The establishment of commercial plantations depends on the use of technologies that allow the vegetative propagation of this species (Shanley et al. 2010, Müller and Freire 1979). However, in the specific case of *B. excelsa* the conventional vegetative propagation is achieved by grafting and this method depends on the establishment of the rootstock through seed germination and plantlet development (Locatelli and Souza 1990), which leads to the same problems of the reproductive propagation.

Plant tissue culture can contribute to overcome propagation problems. This comprises a set of techniques in which each explant (cell, tissue, or organ) is isolated and grown under aseptic conditions in artificial nutritive medium (Grattapaglia and Machado 1998). Through tissue culture it is possible to obtain virus-free plants, conservation and exchange of germplasm, interspecific and intergeneric hybridization, mutations induced by somaclonal variation, production of secondary metabolites, and others (Ferreira 2006). The plant tissue culture has enormous potential for use in many different areas, especially in plant breeding; and the most practical application of tissue culture is micropropagation, which has shown wide applications in agriculture and forestry science (Barbosa 2001).

Micropropagation has as main advantages the rapid increase in the number of individuals and the possibility of germplasm conservation, ensuring the maintenance of biodiversity and can occur through multiplication by indirect organogenesis, with a callus phase in which any plant tissue can be used as an explant for callus induction (Grattapaglia and Machado 1998). However, calluses are in general induced more easily in explants containing meristematic tissue or having a greater capacity to express totipotency and explants of young tissues, not lignified, are more appropriate to initiate a callus culture (Nogueira et al. 2007). The establishment of an appropriate protocol for callus induction with regenerative potential can help the future adoption of unconventional breeding techniques (Oliveira et al. 2007). Callus formation has been used for mass propagation of various species by the formation of multiple shoots or somatic embryogenesis, morphogenetic responses from which seedlings can be produced with good agronomic characteristics (Santos et al. 2010b). Thus, the objective of this work was to establish an efficient

protocol for callus induction in *B. excelsa* immature seeds, since currently there is no

## MATERIALS AND METHODS

### Plant Materials and Sterilization.

Immature seeds were collected from healthy *Bertholletia excelsa* H.B.K. stock plants from the experimental field of Embrapa Rondonia, in Porto Velho, Rondônia State. At the Plant Biotechnology Laboratory the seeds were washed with running tap water and a detergent agent for five minutes. After that, in a laminar flow cabinet, the seeds were immersed in 70% (v/v) ethanol for 1 minute and soaked in a 1% (v/v) sodium hypochlorite solution for 10 minutes, and then rinsed three times with sterile water. Explants were produced by cutting the seeds with a scalpel into pieces of approximately 1 cm<sup>3</sup> in sterile Petri dishes.

### Medium and Culture Conditions.

Test tubes were used containing 10 mL of a Wood Plant Medium (Lloyd and McCown 1981) with 3% (w/v) sucrose and 0.8% (w/v) agar. In order to achieve the callus the medium was supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D) and

efficient system of propagation, either by grafting or by seed.

Thidiazuron (TDZ). The pH of the medium was adjusted to 5.8 before addition of agar followed by autoclaving at 121°C for 20 minutes. All the cultures were incubated in a growth chamber at 24 ± 2°C under a photoperiod of 16 hours of light provided by cool white fluorescent lamps (50 mmol.m<sup>2</sup>.s<sup>2</sup>).

### Callus Induction.

Seed explants were individually transferred onto medium supplemented with a factorial combination of 2,4-D at the concentrations of 0, 1, 2, 4, and 8 mg.L<sup>-1</sup> and TDZ at the concentrations of 0, 1.6, and 3.2 mg.L<sup>-1</sup>, totaling 15 treatments.

### Experimental Design and Evaluation.

The 15 treatments were arranged in a complete randomized design. There were four replications per treatment and each replication was composed by five test tubes, totaling 20 tubes per treatment and 300 explants in the whole experiment. The number of callus per explant was evaluated at the 21<sup>st</sup> day of culture and submitted to analysis of variance and quadratic regression by using the Genes statistical program.

## RESULTS AND DISCUSSION

Callus formation in immature seeds of *B. excelsa* occurred from the 12<sup>th</sup> to the 15<sup>th</sup> day after inoculation. The explants became tumid, which indicated the start of the callus induction process (Fig. 1).

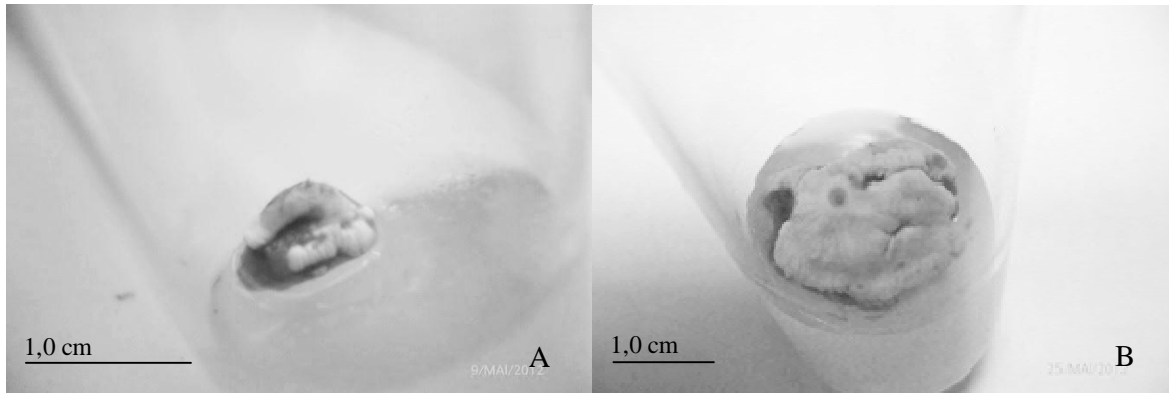
According to the analysis of variance (Table 1) there was interaction between the two growth regulators. The three concentrations of TDZ did

not differ from each other. However, the five concentrations of 2,4-D differed significantly. Thus, a comparison of these concentrations in combination with each TDZ concentration was carried out. It was possible to observe that the concentrations of 2,4-D differed significantly only when they were in combination with 3.2 mg.L<sup>-1</sup> TDZ. In this way, a regression curve was established to this later situation (Fig. 2).

**Table 1:** Calculated F values for percentages of callus induction in explants of immature seeds of *B. excelsa* in response to different concentrations of 2,4-D and TDZ in MS medium 21 days after inoculation. Porto Velho, Rondônia State, Embrapa Rondônia, 2013.

Sources of variation	f	MS	F
Between treatments	14	4,20	7,78**
Within treatments	45	0,54	
TDZ	2	0,07	0,12 <sup>ns</sup>
2,4-D	4	6,79	12,58**
TDZ x 2,4-D	8	3,94	7,30**
2,4-D (0 mg.L <sup>-1</sup> TDZ)	4	0,8	1,48 <sup>ns</sup>
2,4-D (1.6 mg.L <sup>-1</sup> TDZ)	4	0,01	0,02 <sup>ns</sup>
2,4-D (3.2 mg.L <sup>-1</sup> TDZ)	4	4,05	7,5**
CV(%)		9,71	

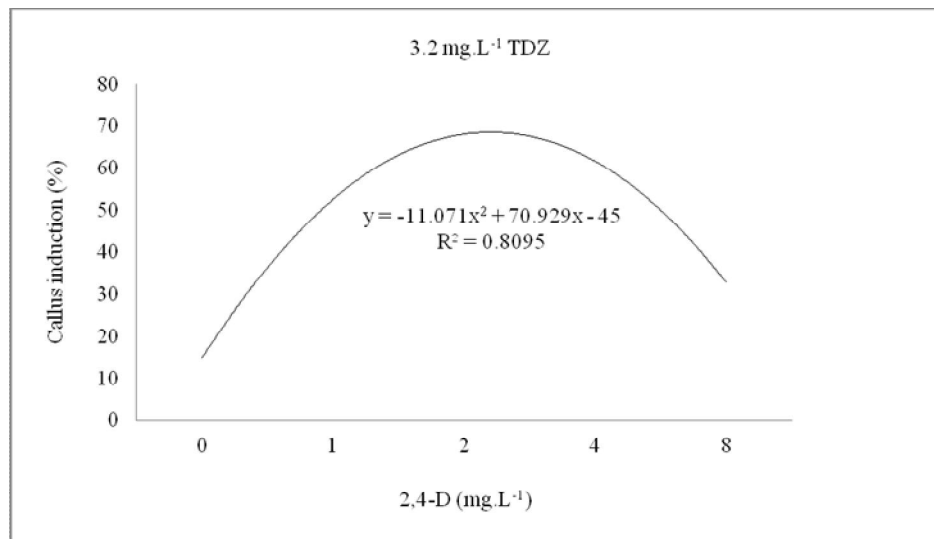
<sup>ns</sup> not significant; \*\* significant at 1% of probability.



**Figure 1:** Response of explants of *B. excelsa* immature seeds to supplementation of 2.0 mg.L<sup>-1</sup> 2,4-D and 3.2 mg.L<sup>-1</sup> TDZ in MS medium. (A) 15 days after inoculation, in the beginning of callus induction; and (B) 21 days after inoculation, with the entire surface covered by callus structure. Porto Velho, Rondônia State, Embrapa Rondônia, 2013.

Serra et al. (2000) also observed callus formation at the 15<sup>th</sup> day of culture in leaf explants of *B. excelsa* by supplementing the medium with 0.5 mg.L<sup>-1</sup> 2,4-D and 2.0 mg.L<sup>-1</sup> 6-benzylaminopurine (BA). The authors found an exponential phase

between the 30<sup>th</sup> and the 53<sup>rd</sup> days, when major growth occurred (74%) and the deceleration phase between the 60<sup>th</sup> and the 67<sup>th</sup> days, when the minor growth occurred. The general growth of the callus is slow, probably due to a long cellular cycle.



**Figure 2:** Regression curve of callus induction in explants of immature seeds of *B. excelsa* in response to different concentrations of 2,4-D in combination with 3.2 mg.L<sup>-1</sup> TDZ in MS medium 21 days after inoculation. Porto Velho-RO, Embrapa Rondônia, 2013.

Little is known so far about the molecular mechanisms of auxin-cytokinin interactions. It is believed that a possible interaction could be found in the metabolism of both hormones, one hormone

class could influence the activity of enzymes involved in the biosynthesis or inactivation of the other (Kerbaui 2004).

Callus induction is dependent on a balance of auxin and cytokinin, which acts in cell cycle regulation, and probably the supply of endogenous cytokinins plus cytokinins present in the nutrient medium would result in a high cytokinin concentration in relation to auxin, causing a decrease in callus formation (Kerbauy 2004). The induction of callus in seeds of tree species in general involves a combination of cytokinins and auxins and has been studied by several authors: Khan et al. (2001) obtained 63.8% callus induction from *Bixa orellana* entire seeds inoculated in MS medium supplemented with 0.93 mg.L<sup>-1</sup> NAA and 0.56 mg.L<sup>-1</sup> BA. Tang et al. (1998) achieved the maximum percentage of 35.7% callus induction from mature zygotic embryos of *Pinus taeda* L. by using 10.0 mg.L<sup>-1</sup> NAA and 4.0 mg.L<sup>-1</sup> BA in TE medium. Bonneau et al. (1994) also utilized mature zygotic embryos for callus induction in *Euonymus europaeus* L. and obtained 16.7% with 1.0 mg.L<sup>-1</sup> NAA and 0.1 mg.L<sup>-1</sup> BA in MS medium.

In the absence of both regulators there was 30% of callus induction probably due to the physical injury to which the explants were submitted, when cut by scalpel. Callogenesis is often associated with presence of auxins in the medium, but is not necessarily auxin-dependent (Borges et al. 2006). During the *in vitro* establishment of explants growth regulators act supplementing the possible lack or low level of endogenous hormones, as the explant is isolated from the production areas of the plant (Machado et al. 2011).

Thurrow et al. (2009) achieved high callus induction in leaf explants of *Prunus salicina* Lindl. with 1 mg.L<sup>-1</sup> 2,4-D. Ferreira et al. (2007) also observed callogenesis in leaf explants of *Ficus carica* L. with 4 mg.L<sup>-1</sup> 2,4-D. Silva et al. (2009) induced callus in anthers of *Coffea arabica* L. with 2 mg.L<sup>-1</sup> 2,4-D.

Auxins are capable of initiating cellular division and controlling growth processes and cellular elongation. 2,4-D acts in the RNA metabolism inducing the RNA messengers capable of decoding proteins involved with plant growth and disordered proliferation (Nogueira et al. 2007).

Growth regulators do not influence plant responses exclusively by changing their concentrations, as the regulation can also be exerted by changes in the sensibility of responsive cells. In this way,

responses to a determined growth regulator can be altered by changes in a) the number and affinity of receptors, and b) the level of other endogenous substances (Guerra et al. 1998).

The highest callus induction corresponded to 75% of the explants and occurred with the combination of 2 mg.L<sup>-1</sup> 2,4-D with 3.2 mg.L<sup>-1</sup> TDZ. This cytokinin is a potent inducer of multiplication, as it can promote the biological activity of endogenous cytokinins (Ribeiro et al. 2010). The percentage of 75% is satisfactory, as the callus can grow and be multiplied several times according to its final utilization – organogenesis or embryogenesis.

Higher concentrations of 2,4-D resulted in lower levels of callogenesis, as an outcome of the phytotoxicity of this growth regulator at high concentrations. The shape of the curve observed in Figure 2 is typical for callus induction and often represents an increase on the percentage of explants with callus cells until reach a maximum; followed by a decrease that means saturation and maybe toxic effect of high concentrations. The same was obtained by Santos et al. (2012) with leaf explants of *Bactris gasipes* H.B.K. submitted to different concentrations of 2,4-D and BA.

Deus et al. (2007) induced calluses in seeds of *Eucalyptus urophylla* S.T. Blake by using alpha-naphthaleneacetic acid (NAA) combined with TDZ. It was found that 0.5 mg.L<sup>-1</sup> TDZ was unable to induce calluses, but its combination with 0.1 mg.L<sup>-1</sup> NAA resulted in callus induction in 100% of the explants.

Costa et al. (2008) tested different auxins (NAA, IBA, IAA, and 2,4-D) at the concentrations of 0, 2.5, and 5.0 mg.L<sup>-1</sup> in leaf and internodal explants of *Piper hispidinervum* C. DC. 2,4-D caused the highest levels of cellular death. On the other hand, Werner et al. (2009) achieved friable callus in leaf explants of *Caesalpinia echinata* Lam. at concentrations from 5.0 to 20.0 mg.L<sup>-1</sup> 2,4-D, which were considered phytotoxic in the study of Costa et al. (2008). Correia (2010) induced callus in shoot tips of *Bactris gasipaes* Kunth. by combining 10.0 mg.L<sup>-1</sup> 2,4-D with 3.0 mg.L<sup>-1</sup> BA. The growth regulators 2,4-D and TDZ used in this study were effective for callus induction in fragments of immature seeds of *B. excelsa*. The obtained percentages were satisfactory and the induced callus will be used to continue the process of *in vitro* culture aiming to regenerate plantlets from these calluses.

## CONCLUSION

The highest callus induction levels in immature seeds of *B. excelsa* can be achieved by supplementing WPM culture medium with a combination of 2.0 mg.L<sup>-1</sup> 2,4-D and 3.2 mg.L<sup>-1</sup> TDZ in MS medium.

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