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In vitro callus induction from different explants of *Senna alata* (L.) Robx. (FABACEAE)

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ABSTRACT: *Senna alata* (Sin. *Cassia alata*), has been considered a promising species for the development of phytotherapy products, and it is necessary to supply biological material with high genetic quality for the pharmacology. Thus, the objective of the study was to evaluate the stimulating effects of plant growth regulators on callus formation of *Senna alata*. Nodal and leaf segments were inoculated in MS medium supplemented with different concentrations of 2,4-D (0; 2.21; 4.42; 6.63 and 8.84 mg L^{-1}) under the influence of LED light (white and colored) and darkness. It was also evaluated the effect of 2,4-D (1.0 mg L^{-1}) in combination with TDZ and BAP in the concentration of 1.0, 3.0 and 5.0 mg L^{-1} in the presence and absence of activated charcoal for callus induction. The percentage of responsive explants and/or callus formation was evaluated after 4 weeks of culture. The results showed that the conditions of white LED are favorable for callus induction in leaf explants (77.5%) and nodal explants (90.0%). In addition, the interaction of 2,4-D with cytokinins was also favorable for the development of callus. However, activated charcoal did not favor the process of callogenesis. This study concludes that the production of friable callus can be obtained from axenic tissue of *S. alata*.

Indução de calos *in vitro* em diferentes explantes de *Senna alata* (L.) Robx. (FABACEAE)

RESUMO: *Senna alata (*Sin. *Cassia alata*), tem sido considerada uma espécie promissora para o desenvolvimento de produtos fitoterápicos. Nesse contexto, é necessário fornecer material biológico de alta qualidade genética para a farmacologia. O objetivo do estudo foi avaliar os efeitos estimulantes de reguladores de crescimento de plantas na formação de calos de *Senna alata*. Segmentos nodais e foliares foram inoculados em meio MS suplementado com diferentes concentrações de 2,4-D (0; 2,21; 4,42; 6,63 e 8,84 mg L^{-1}) sob a influência da luz LED (branca e colorida) e escuridão. Também foi avaliado o efeito do 2,4-D (1,0 mg L^{-1}), em combinação com TDZ e BAP na concentração de 1,0; 3,0 e 5,0 mg L^{-1} na presença e ausência de carvão ativado. A porcentagem de explantes responsivos e/ou formação de calos foi avaliada após 4 semanas de cultivo. Os resultados mostraram que as condições de LED branco são favoráveis para indução de calos em explantes foliares (77,5%) e nodais (90,0%). Além disso, a interação do 2,4-D com as citocininas também favoreceu o desenvolvimento de calos. Entretanto, o carvão ativado não contribuiu no processo de calogênese. Esse estudo conclui que a produção de calos friáveis pode ser obtida a partir de tecidos axênicos de *S. alata.*

Introduction

Senna alata (Sin. *Cassia alata)* has been considered a promising species for the development of phytotherapy products due to different types of compounds such as alkaloids, reina, β-sitosterol, chrysophenol and kaempferol (Fatmawati et al. 2020). Several chemical constituents of leaf extract of *S. alata* have been showed growth inhibition for many pathogens such as fungi and bacteria (Timothy et al. 2012; Tatsimo et al. 2017). Moreover, it has antioxidant properties like as anti-inflammation (Sagnia et al. 2014), anticancer and anti-metastatic activity (Kittiwattanokhun et al. 2021; Chahardehi et al. 2021).

The importance of biological properties in this species has awakening the interest of the pharmaceutical industry in the search for homogeneous raw material on a large scale, however it is necessary to develop cultivation methods that make it possible to obtain and extract bioactive compounds. Cell suspension and callogenesis stand out among the main techniques for the production of secondary metabolites. Callogenesis consists of the differentiation of plant tissue and the formation of a mass of plant cells called callus with a high degree of cell division (Bourgard et al. 2001).

However, the success of *in vitro* propagation of plants is mainly related to the use of growth regulators, cultivation in aseptic conditions and the use of antioxidant substances (Morais et al. 2012). Among the growth regulators, the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D) is generally used in low and high concentrations, for induction of adventitious roots and for induction of callus (Silva et al. 2008). In this context, the study aimed to establish cultivation and production of *S. alata* callus.

Material and Methods

The study was conducted at the Laboratory of Tropical Silviculture and Digital Technologies from the National Institute for Research in the Amazon (LASTED/INPA), Manaus, Brazil. The plant material was obtained from axenic plants germinated *in vitro* for 30 days.

Culture media and conditions

Murashige and Skoog (MS) medium supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar-agar were used throughout the experiments. The pH of the culture medium was checked to 5.8 ± 0.2 with sodium hydroxide (NaOH) or hydrochloric acid (HCl) at 0.1 N before autoclaving, which was carried out at a temperature of 121 ºC and pressure of 1 atm for 15 minutes.

Effect of 2,4-dichlorophenoxyacetic (2,4-D) on callus induction in S. alata explants (leaf and nodal segments) in the presence and absence of light.

Leaf segments with an approximate size of 1 cm^2 in area and nodal segments of 1 cm in length were obtained from aseptic seedlings germinated *in vitro*. They were inoculated on MS medium supplemented with different concentrations of 2,4-D (0; 2.21; 4.42; 6.63 and 8.84 mg L^{-1}) under the influence of different light sources (Tecnal® TEC-LAMP-060 FS LED blue-red and Tachiba® LED T8-TUBE LAMP white) and dark conditions. Glass flasks of 250 ml were used with 40 ml of culture medium. The cultures were kept in a growth room at a temperature of 25 ± 2 ºC, with a photoperiod of 16 hours.

The experimental design was completely randomized, in a factorial scheme 3 (light source) x 5 (concentrations of 2,4-D) with six repetitions per treatment, each one consisting of five explants. After 30 days, it was registered the percentage of the area of explants covered by callus, and the color and texture of the callus was also evaluated.

Effect of auxin (2,4-D) in interaction with cytokinins (BAP and TDZ) in the induction of callus in foliar explants of S. alata

Seedling leaf segments germinated *in vitro* with 30 days were inoculated in MS medium supplemented with auxin (2,4-D) at 1.0 mg L^{-1} concentration, in combination with cytokinins (TDZ and BAP) at concentration of 1.0; 3.0 and 5.0 mg L^{-1}. Each treatment had 5 repetitions and each repetition with 6 explants arranged in 250 ml glass flasks.

The experimental design was completely randomized, in factorial 1 (2,4-D) x 2 (BAP x TDZ) x 3 concentrations, with 5 repetitions per treatment, each repetition composed of 6 experimental units (n = 30). At 30 days, was also evaluated, the percentage of explants with callus formation, and its characteristics (consistency, texture and color).

Effect of the interaction of auxins (2,4-D) with cytokinins (BAP and TDZ) on the induction of callus in foliar explants of S. alata in the presence of activated charcoal.

Seedling leaf segments germinated *in vitro* with 30 days of culture were inoculated with MS medium supplemented with auxin (2,4-D) at 1.0 mg L^{-1} concentration, in combination with cytokinins (TDZ and BAP) at concentration of 1.0; 3.0 and 5.0 mg L^{-1} with addition of activated charcoal (2 mg L^{-1}). Each treatment had 5 repetitions and each repetition with 6 explants was arranged in 250 ml glass flasks.

The experimental design was completely randomized, arranged in factorial 1 (2.4 D) x 2 (BAP x TDZ) x 3 concentrations, with 5 repetitions per treatment, each repetition composed of 6 experimental units (n = 30). After 30 days was also evaluated, the percentage of explants with callus

formation and its characteristics (consistency, texture and color), as well as the presence of adventitious roots.

Statistical analysis

The data were submitted to statistical analysis, by analysis of variance (ANOVA) in the means compared to the Tukey test ($p \leq 0.05$) using the Minitab® Statistical Software version 18.

Results and Discussion

Percentage of explants induced callus in the presence and absence of light

The statistical analysis of data regarding callus induction on different treatments showed significant results for the type of luminosity. The explants (nodal and leaf segments) with the highest percentage of callus covered area were obtained under white and colored LED conditions in relation to the dark conditions (Table 1).

The effects of 2,4-D concentration and explant type on the callus characteristics are explained separately. There was a not significant difference among explant types for callus induction percentage. Callus induction in leaf explants started ten days after inoculation. The 2,4-D concentration at 8.84 mg L^{-1} resulted in a callus induction percentage of 75%. Callus induction in nodal explants started from the fifth day after inoculation. The explants reached a percentage of area covered by calluses of 90% at concentration of 2.21 mg L^{-1} of 2,4-D.

Concerning texture and color, in the control and in the absence of light, the explants showed appearance of swelling. In addition, the nodal segments showed greater oxidation and callus formation in the dark condition. In the white and blue-red colored LED light, the explants (nodal and leaf segments) showed a more uniform cell mass. We observed the formation of friable callus in nodal explants and the first 15 days after inoculation, in foliar explants the callus tended to compact, increasing in size and also showed greater oxidation. (Figure 1).

Table 1. Percentage of covered area by calluses in *S. alata* explants, inoculated in MS medium with the addition of different concentrations of 2,4-D under LED light and dark conditions.

2,4-D(mg L^{-1})	Leaf explants (%)			Nodal explants (%)		
	Dark	**White**	**Colored**	**Dark**	**White**	**Colored**
0	0 ^{d}	0 ^{d}	0 ^{d}	0 ^{d}	0 ^{d}	0 ^{d}
2.21	25.0 ^{b}	50.0 ^{a}	50.0 ^{a}	35.0 ^{b}	90.0 ^{a}	77.5 ^{a}
4.42	25.0 ^{b}	52.5 ^{a}	60.0 ^{a}	30.0 ^{c}	87.5 ^{a}	82.0 ^{a}
6.63	20.0 ^{c}	77.5 ^{a}	67.5 ^{a}	30.0 ^{c}	85.0 ^{a}	85.0 ^{a}
8.84	20.0 ^{c}	75.0 ^{a}	67.5 ^{a}	40.0 ^{a}	80.0 ^{a}	90.0 ^{a}

The same letter averages do not differ at the level of 5% by the Tukey test.

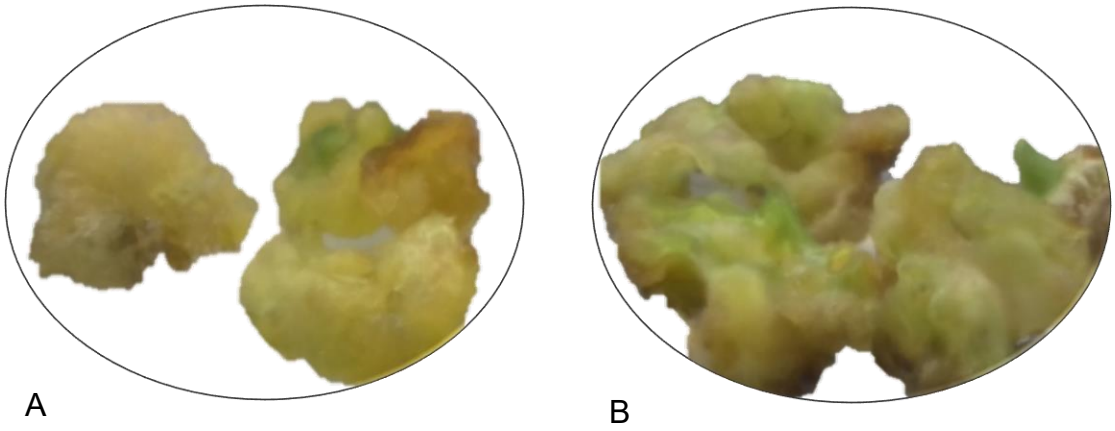

Figure 1. (A) Callus texture and coloring of *S. alata* obtained from nodal segments grown in MS medium with 2,21 mg L^{-1} of 2,4-D after 15 days of inoculation. (B) Callus texture and coloring of *S. alata* obtained from leaf segments grown in MS medium with 8,84 mg L^{-1} of 2,4-D after 30 days of inoculation Source: Corredor Lara, 2020.

As far as we know, there are not enough reports on the effect of light and dark on callus induction and plant regeneration with respect to physiological and morphological development. The effect of light and dark on callus induction can vary from one species to another. In this way, in some plants, callus occurs in the dark, in the case of wheat (Islam 2010), barley (Haque and Islam 2014), *Vicia faba* (Almaghrabi 2014), corn (Pathi et al. 2013; Morshed et al. 2014), and taro (Paul et al. 2014); while in other plants the callus develops better in light conditions like as in tomato (Sherkar and Chavan 2014) and tobacco (Siddique and Islam 2015). For their part, our findings are consistent with the previous report that light positively affected callus induction and growth. In most cases, corns can form well in adequate light intensity, but may have lower metabolite content (Siddique and Islam 2015).

Another factor that influences the development of callus is the concentration of auxin, 2,4-D. Reports on the optimal concentration vary from species to species. Castro et al. (2009), Vasconcelos et al. (2012), reported that low concentrations of 2,4-D in the culture provided greater areas per explant covered by callus in *Stryphnodendron adstringens* and *Myracrodruon urundeuva*, respectively. Therefore, Costa et al. (2008) report that despite 2,4-D being a synthetic auxin, most commonly used for callus induction, in high concentrations (2.5 and 5.0 mg L^{-1}), it causes phytotoxicity in explants of *Piper hispidinervum*.

The callus is formed at different concentrations of 2,4-D and show heterogeneous color and appearance, in addition may present different metabolite contents. Sado (2009) showed that 0.12 mg L^{-1} of 2,4-D induced friable callus with higher soluble carbohydrate content in *Senna spectabilis*. A concentration of 0.5 mg L^{-1} of 2,4-D induced friable callus with higher protein values, and a concentration of 10 mg L^{-1} of 2,4-D induced nodular callus with higher starch content.

Observations from other authors indicate that the type of culture medium and kind of explant are important factors in quantity and quality of callus. Some authors reported that internodal segments are responsive to callogenesis and allow the highest percentage of friable callus formation (Kumlay and Ercisli 2015; Keshvari et al. 2018; Rodriguez et al. 2020).

Effect of the interaction of auxin 2,4-D with cytokinins BAP and TDZ in leaf explants.

There was a significant effect on callus formation depending on the cytokinin concentration. Except for the control treatment, callus formation was observed in all other treatments. We observed a greater formation of callus in treatments with the same concentration of growth regulators as 73.3% in the 2,4-D + TDZ treatment (1.0 + 1.0 mg L^{-1}) and 70.7% in the 2,4-D + BAP treatment (1.0 + 1.0 mg L^{-1}) (Table 2). However, the percentage of callus decreased when the cytokine concentration was higher. Regarding texture and color, we observed that the addition of cytokinins induced callus with nodular or globular structures, with a color ranging from brown to greener coloring (Figure 2A).

Table 2. Callus formation in response of *S. alata* leaf explants to the interaction of auxin (2,4-D) with cytokinins (BAP and TDZ)

Trat[0]	Dose Growth regulator (mg L^{-1})		Callus (%)
T1	Control	0	0 [c]
T2	2,4-D + BAP	1.0 + 1.0	70.7 [a]
T3	2,4-D + TDZ	1.0 + 1.0	73.3 [a]
T4	2,4-D + BAP	1.0 + 3.0	61.6 [a]
T5	2,4-D + TDZ	1.0 + 3.0	63.3 [a]
T6	2,4-D + BAP	1.0 + 5.0	34.9 [b]
T7	2,4-D + TDZ	1.0 + 5.0	33.3 b

The same letter averages do not differ at the level of 5 % by the Tukey test

Effect of the interaction of auxin 2,4-D with cytokinins BAP and TDZ in the presence of activated charcoal in leaf explant.

Based on the results of the previous test, activated charcoal was added to the culture medium to eliminate or reduce undesirable compounds that favor the oxidation of explants. Even so, it was observed that in the presence of activated charcoal, the percentage of callus formation decreases considerably, resulting in 23.3% and 19.9% in the treatments 2,4-D + TDZ (1.0 + 1.0 mg L^{-1}) and 2, 4-D + BAP (1.0 + 1.0 mg L^{-1}), respectively. This fact showed that the presence of this antioxidant compound does not help in the callogenesis process. At the same time, there was a greater induction of roots that resulted in 86.7% in the treatment with 2,4-D + TDZ (1.0 + 5.0 mg L^{-1}) (Table 3). In the presence of activated charcoal, the texture of the callus was friable, but the area of the explant covered by the callus showed adventitial root formation. (Figure 2 B).

Table 3. Callus and root formation in *S. alata* leaf explants in the interaction of auxin (2,4-D) with cytokinins (BAP and TDZ) in the presence of activated charcoal.

Trat[0]	Dose Growth regulator (mg L^{-1})		Callus (%)	Root (%)
T1	Control	0	0 [c]	0 [d]
T2	2,4-D + BAP	1.0 + 1.0	70.7 [a]	73.2 [a]
T3	2,4-D + TDZ	1.0 + 1.0	73.3 [a]	70.0 [a]
T4	2,4-D + BAP	1.0 + 3.0	61.6 [a]	65.7 [a]
T5	2,4-D + TDZ	1.0 + 3.0	63.3 [a]	83.3 [a]
T6	2,4-D + BAP	1.0 + 5.0	34.9 [b]	54.9 [b]
T7	2,4-D + TDZ	1.0 + 5.0	33.3 b	86.7 [a]

The same letter averages do not differ at the level of 5% by the Tukey test

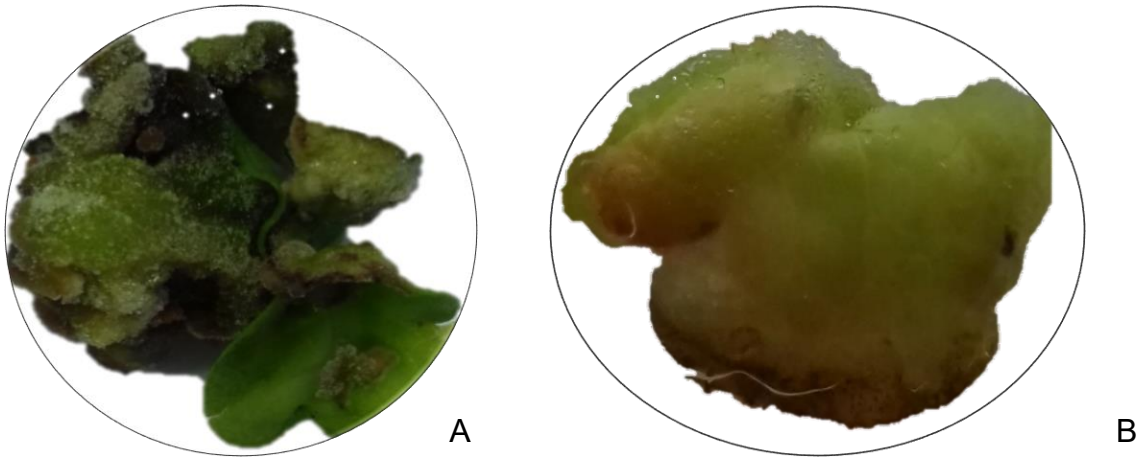

Figure 2.A) Leaf explants with 2,4 D + TDZ (1.0 + 3.0 mg L^{-1}) after 30 days of inoculation. B) Leaf explants with 2,4 D + TDZ (1.0 + 3.0 mg L^{-1} supplemented with activated charcoal) after 30 days of inoculation. Source: Corredor Lara, 2020.

In theory, the equivalent amount of auxin and cytokinin promotes callus formation, but in practice it differs with each species, largely due to variation in the endogenous level of phytohormones. In the present study, the most effective culture medium for callus induction was found in the combination of 2,4-D (1.0 mg L^{-1}) with TDZ (1.0 mg L^{-1}). We observed that with the addition of cytokinins, callus proliferation generally improves in terms of size and appearance.

However, plants develop disorganized cell masses such as calluses and tumors in response to various biotic and abiotic stimuli. Wound-induced callus formation has long been observed in nature, the phenomenon is used in various tree debarking contexts for horticultural use (Chen et al. 2013). Tumors induced by pathogens gall is a plant disease caused by gram-negative bacteria *Agrobacterium tumefaciens* and occurs in plant species by introducing their T-DNA into the genome. These bacteria enter plants through wounds and promote the tumor growth of a disorganized cell mass (Gohlke and Deeken 2014). The expression of bacterial genes encoding biosynthetic enzymes of auxin and cytokinin forces infected plants to produce galls.

The production of tumors in plants can also be induced by other pathways. Viral infection is another source of tumorization. Wound tumor viruses (WTVs), also called large vein clover virus, belong to the family of Group III viruses with the double-stranded RNA genome and induces gall formation in the plant host. Genetic tumors induced by interspecific hybrids refer to the disorganized overproliferation of cells that occurs as a result of interspecific crosses (Ikeuchi et al. 2013).

Furthermore, since the historical discovery that the combination of two growth-promoting hormones, auxin and cytokinin, induces calluses in plant explants *in vitro*, there has also been an attempt to identify the mechanisms of cell reprogramming produced by the lesion that activate cell proliferation and the generation of this undifferentiated tissue, but the underlying molecular mechanisms remain largely unknown yet the inheritance activates multiples regulators of development. (Ikeuchi, et al., 2017).

The presence of secondary metabolites is related to the processes of cell differentiation and, therefore, not all species of medicinal plants used in the conventional production of secondary metabolites produce these metabolites in callus cultures or in suspension cell cultures. Such observations were found in *Caesalpinia ferrea* with callus induction (90%) obtained from nodal segment explants cultured in MS medium supplemented with 1.0 mg L^{-1} of 2,4-D and 5.0 mg L^{-1} of TDZ (Silva et al. 2018; Silva et al. 2020).

Other studies of callus induction for the genus *Cassia* reported high concentrations of cytokinin and low concentration of auxin. Vats and Ramal (2014) found that addition of 5.0 mg L^{-1} 2,4-D and 0.02 mg L^{-1} KIN resulted in 87.2% of calogenesis in *Cassia occidentalis*. Yang et al. (2020) found that adding 2.0 mg L^{-1} 6-BA and 0.5 mg L^{-1} ANA could effectively promote callus induction from *Cassia mimosoides* leaf explants, and the induction rate was 100%. Santos et al. (2014) and Oliveira et al. (2018) reported that the addition of cytokinins improved the proliferation of calluses in terms of size and appearance in general.

Pervin et al., (2013) and Ikeuchi et al. (2013) reported that to obtain embryogenic calluses are taken into account multiple factors that contribute to tissue dedifferentiation as culture medium, additives, and culture conditions. Silva et al. (2020) observed that MS and B5 media supplemented with 2.21 and 4.42 mg L^{-1} of 2,4-D induced 100% formation of friable callus of *Libidibia ferrea* cultivated under

red-blue LED, demonstrating that the light quality significantly influenced callogenesis.

The use of activated charcoal in culture medium does not have a positive effect on callogenesis, however, it controls phenolic oxidation and stimulates the formation of adventitious roots. Activated charcoal and other oxidation control agents have been used in the culture medium to overcome this problem, they can inhibit callus growth and delay callus formation (Werner et al. 2009; Yang et al. 2020). The results obtained in this study showed that *Senna alata* tissues are competent for callus differentiation. In addition to reinforcing the need for more studies related to the control of phenol synthesis with the aim of expression of the determination pathway, somatic organogenesis or embryogenesis and species conservation.

Conclusions

Leaf explants and nodal segments cultivated in the MS medium supplemented with 2,4-D and maintained under white and colored Led conditions promoted the callus formation of *S. alata*.

The interaction 2,4-D with cytokinins BAP and TDZ had a positive effect on the induction of friable calluses in leaf explants in the absence of activated charcoal. Activated charcoal did not favor the formation of calluses in *S. alata* explants.

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