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2,4-Dichlorophenoxyacetic acid increases reserve compounds and spectaline contents in *Senna spectabilis* calli

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The aim of this study was to develop an *in vitro* culture system for *Senna spectabilis* and to quantify contents of storage compounds and spectaline in induced calli in relation to exogenous auxin. Explants (cotyledon, hypocotyl, epicotyl, and leaf) were cultured on MS medium containing different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D). The soluble carbohydrate, starch, soluble protein and spectaline contents in the induced calli were quantified. Treatment with 0.12 mg L^{-1} of 2,4-D induced callus formation and was optimal for propagation and vegetative growth, owing to the higher concentrations of reserve compounds in the calli. Hypocotyl and epicotyl produced calli on medium containing 0.5 mg L^{-1} 2,4-D. The lowest concentrations of 2,4-D induced a higher incidence of oxidation and explants showed low viability. Spectaline accumulated at low concentrations in the different callus types and 2,4-D treatments, indicating spectaline was a constitutive compound in callus. Hypocotyl with 10.0 mg L^{-1} 2,4-D for up to five days induced cell proliferation and starch accumulation, followed by treatment with 0.12 mg L^{-1} 2,4-D to increase tissue mass and accumulation of reserve compounds enables the production of friable callus suitable for the establishment of a tissue culture system and for *in vitro* spectaline production.

Key words: Alkaloid, auxin, Fabaceae, carbohydrate, tissue culture.

INTRODUCTION

Senna spectabilis (DC.) Irwin et Barn. (syn. *Cassia spectabilis* DC., Fabaceae), popularly known as 'canafístula or cassia northeast', is a deciduous, heliophytic, selective xerophytic plant native to Central and South America. The species is found mainly in the cerrado, caatinga (savannah like) and, forests areas of small rainfall of central and northeast Brazil (Braga, 1982). In traditional medicine, extracts of this species are used as an anti-inflammatory to treat human disease, it has analgesic, laxative, anesthetic, and antimicrobial properties, exhibit inhibitory activity against superoxide generation, and show promise for treatment of Alzheimer's disease (Viegas et al., 2006).

Plant alkaloids are not only toxic to herbivores and

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microorganisms, but can also be of pharmacological importance in popular medicine, and show diverse biological activities such as reduction of blood pressure, relief of pain and spasms, stimulation of blood circulation and respiration, stabilization of mental illness, and antitumor activity (Brown and Charlwood, 1986; Wink, 2008). Plant alkaloids are also utilized as food additives, as coloring agents, for provision of a specific aroma or taste, and as preservation compounds). Because of their complexity, the chemical synthesis of many biological molecules is not yet feasible, and commercial exploitation is still dependent on agricultural production of the selected species.

Recent studies indicate that piperidine alkaloids are responsible for the pharmacological properties of *Senna* species. Such alkaloids include inhibitors of acetylcholinesterase, which have potential applications in the treatment of Alzheimer's disease (Bolzani et al., 1995, Moreira et al., 2003). *S. spectabilis* is rich in piperidine alkaloids (Viegas et al., 2006, 2013), and hence has attracted much pharmaceutical interest, which has motivated research groups to develop methodologies to yield higher quantities of alkaloids than those produced by natural sources. Pivatto et al. (2005) confirmed the presence of piperidine alkaloids in ethanol extracts from flowers and fruits of *S. spectabilis*. Melo et al. (2014) described the leishmanicidal activity of the flower crude extract and Paguigan et al. (2014) reported the anti-ulcer activity in methanol extracts of leaves. Therefore, studies on alkaloid biosynthesis, and synthesizing and accumulating of these secondary compounds by *in vitro* induced callus are relevant to explore the possible development of biotechnological production systems for piperidine alkaloids.

In vitro plant tissue culture can be initiated using organized tissues such as buds or roots, from which meristematic cells usually give rise to the same organ, or fragments of organs (for example leaf, stem, root, or petal explants) plus auxin to induce proliferation of an unorganized cell mass (callus) on an injured surface. With the proper balance of growth regulators, the callus can be propagated indefinitely or induced to differentiate into new organs or a whole plant (Loyola-Vargas and Vazquez-Flota, 2006; George et al., 2008). The transport and storage of alkaloids are correlated with specialized tissues (Wink and Roberts, 1998), and these secondary compounds are synthesized in undifferentiated plant cell cultures in very low quantities or with different chemical structures when compared to those of whole plants.

The use of *in vitro* culture techniques can accelerate cultivar development, assist in conventional breeding programs and is a potential biotechnological tool for the study of metabolism, physiology, development, plant reproduction and synthesis of biological molecules of pharmacological interest. Such techniques also enable the production of large quantities of plantlets from a small number of explants (George and Debergh, 2008).

The aim of the present study was to induce callus formation with 2,4-dichlorophenoxyacetic acid (2,4-D) from different tissues of axenic plantlets of *S. spectabilis*, quantify reserve compounds (total soluble carbohydrates, starch and soluble proteins), clarify their relationship with callus type and cell multiplication cycle, and verify the *in vitro* production of the alkaloid spectaline.

MATERIALS AND METHODS

Plantlets of *S. spectabilis* were obtained by *in vitro* seed germination from mature fruits of three specimens growing in the Institute of Botany, São Paulo, Brazil. The seeds were sterilized in concentrated sulfuric acid for 45 min followed by five washes with sterile distilled water, then germinated on complete Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 3.0% (w v^{-1}) sucrose and 0.8% (w v^{-1}) agar (Bacto-agar, Difco). The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 20 min. When seedlings reached 3.0 cm in length, the cotyledons, hypocotyl, epicotyl and leaves were excised with a scalpel under aseptic conditions and cultured on MS medium supplemented with 3.0% sucrose and 0, 0.12, 0.25, 0.5, 2.5, 5, 10, 20 or 40 mg L^{-1} 2,4-D in 100 mL flask containing 30 mL of medium. Each treatment comprised 20 flasks containing one explant from each tissue type. All cultures were incubated at 26±2°C with a 12-h photoperiod under white fluorescent lights (16.2 µmol m^{-2} s^{-1}). The number, type, and fresh and dry mass (lyophilized) of callus were determined after 45 days of cultivation.

The induced calli were classified as friable, nodular, and compact or oxidated combined according to the original explant type and treatment, then subdivided into three samples (0.5-2.0 g fresh mass for each sample) and stored at -20°C.

For analysis of reserve compounds, the callus samples were lyophilized, the dry mass was determined on an analytical balance, then ground using a mortar and pestle. The samples were homogenized and extracted twice with 80% (v v^{-1}) ethanol at 80°C for 5 min. After centrifugation for 10 min at 1,250 × *g*, the ethanolic supernatants (soluble carbohydrate fraction) were combined and the volume measured. Soluble proteins were extracted twice from the residue with 0.2 M phosphate buffer (pH 5.7) at room temperature for 10 min, centrifuged for 10 min at 500 × *g* at 4°C, then the supernatants were pooled and the volume measured. The soluble protein content was determined by staining with Coomassie Blue reagent and absorbance was measured with a spectrophotometer (Biospectro SP-22) at 595 nm using bovine albumin as the standard (Bradford, 1976). Starch was extracted twice from the protein residues with 52% (v v^{-1}) perchloric acid and estimated according to the method of McCready et al. (1950). The total soluble carbohydrate and starch contents were determined colorimetrically according to the phenol-sulphuric acid method (Dubois et al., 1956; McCready et al., 1950).

For preparation of the spectaline standard, fresh *S. spectabilis* flowers (500 g) were collected and lyophilized. The dried samples were homogenized in 80% ethanol at 80°C and incubated for 5 min, then cooled and the supernatant was concentrated on a rotary evaporator (Pivatto et al., 2005). Aliquot (10 mL) of the concentrated extract was lyophilized, weighed, and then 60 mL of 5% sulfuric acid was added and incubated at 4°C for 16 h. The extract was filtered and 15 mL hexane was added for lipid extraction. The aqueous acid phase was set to 9.0 using 1 N ammonium hydroxide and dichloromethane was used for liquid–liquid separation, with the crude alkaloid fraction contained in the organic phase. The ethanolic extracts from the different treatments

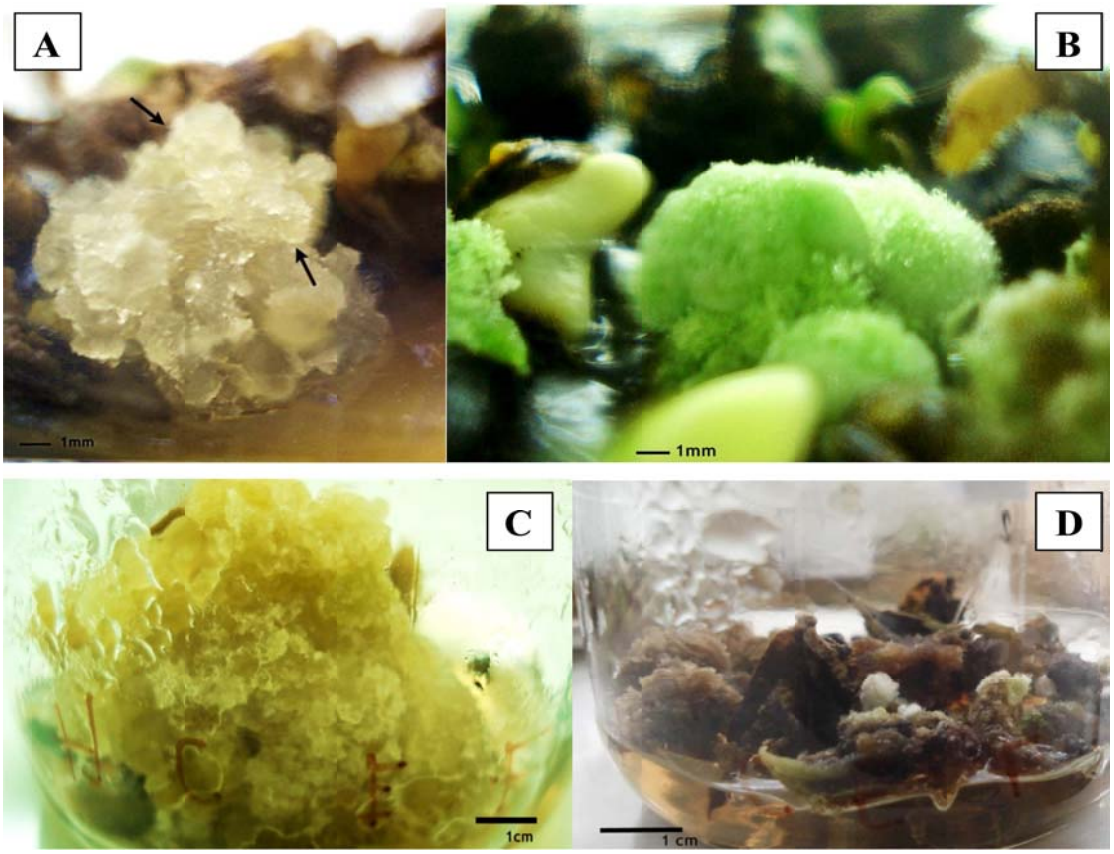


Figure 1. *Senna spectabilis* calli after cultured for 45 days on medium containing 2,4-D. **(A)** Nodular (arrows indicate callus structure). **(B)** Compact. **(C)** Friable. **(D)** Oxidized callus.

and callus types were prepared following the methodology described above, with reagents and solvents in proportional volumes. Spectaline analysis were performed with gas liquid chromatography (Silva, 2009) using a Varian Chrompack CP-3380 column and injection of 1 µL of each concentrated sample in 4.0 mg mL^{-1} ethyl acetate using the following conditions: injector 300°C; flame ionization detector 300°C; initial temperature 210°C increasing by 1°C min^{-1} to 250°C for 40 min; and 3.0 cm^3 min^{-1} helium as carrier gas.

Differences among treatments were determined by analysis of variance (ANOVA) with a post Tukey–Kramer test using Sigma Stat version 3.1 (Sigma Stat Software, Chicago, USA). Data were expressed as the mean ± standard deviation. The data were considered significant at $P < 0.05$.

RESULTS

Callus induction and growth occurred in all treatments. The calli were friable, nodular or compact (Figure 1A to C). The color of compact calli was usually green or white, not transparent and with a velvety surface, whereas the friable calli were whitish, yellowish or greyish with a spongy appearance. Nodular calli were morphologically similar (globular) to callus containing somatic embryos, suggesting a relationship between calli morphology and color.

Callus induction from hypocotyls and epicotyls was most frequent (>80%) at 0.5 mg L^{-1} 2,4-D, whereas 70% of cotyledons formed callus at 2.5, 10 and 20 mg L^{-1} 2,4-D and under 3% formed callus at 0 mg L^{-1} 2,4-D. Primary leaves showed no significant callus induction at 0 mg L^{-1} 2,4-D, whereas 80% of leaves formed callus at 2.5 mg L^{-1} 2,4-D (Table 1).

Media containing 0 and 0.25 mg L^{-1} 2,4-D did not induce nodular callus. Treatments 0.12, 0.25 and 0.5 mg L^{-1} 2,4-D had oxidized callus (Figure 1D) and cells undergoing senescence (Table 1). The fresh weight was higher ($P < 0.05$) in friable callus cultured on high concentrations of 2,4-D (Figure 2A). The dry weight was significantly higher in compact callus compared to that of friable callus ($P < 0.01$) (Figure 2B). Treatment with 0.12 mg L^{-1} 2,4-D induced compact callus with the highest dry weight compared to the other treatments. The friable callus induced by 20 and 40.0 mg L^{-1} 2,4-D showed the lowest dry weight.

Table 1. *Senna spectabilis* callus induction (%) by treatment with different concentrations of 2,4-D in the culture medium.

2,4-D (mg L^{-1})	Calli induction (%)				Type of calli (%)		
	Hypocotyl	Epycotyl	Cotiledon	Leaf	Friable	Nodular	Compact
0.0	25.0	21.9	3.1	1.6	25.0	0.0	75.0
0.12	65.8	71.0	61.8	52.6	51.1	2.0	46.9
0.25	82.0	65.3	66.7	68.1	81.8	0.0	18.2
0.5	86.7	83.3	45.0	78.3	41.9	45.2	12.9
2.5	76.6	78.1	73.4	79.7	48.8	39.5	11.6
5.0	70.0	61.7	55.0	50.0	52.6	38.6	10.5
10.0	71.7	73.3	73.3	58.3	57.9	38.6	5.3
20.0	60.0	70.0	72.5	65.0	51.5	45.5	3.0
40.0	11.7	11.7	45.0	21.7	40.0	40.0	20.0

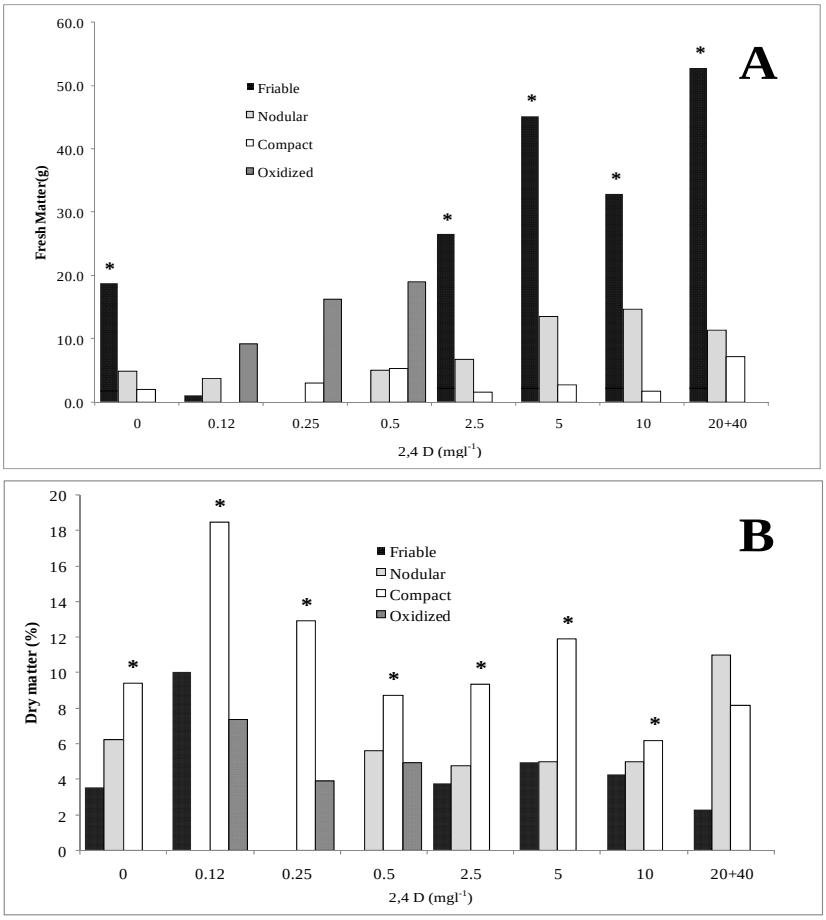


Figure 2. A) Fresh weight of *S. spectabilis* calli. *A significant difference (P < 0.05) existed between nodular, compact and oxidized calli for treatments 0, 0.5, 2.5, 5, 10 and 20+40 mg L^{-1} 2.4-D. **B)** Dry mass (%) of different calli types (friable, nodular, compact and oxidized) cultured with different 2,4-D concentrations. *A significant difference (p < 0.05) existed for friable callus at all concentrations of 2,4-D except 20+40 mg L^{-1} 2,4-D. **C)** Soluble carbohydrate content (mg g^{-1} dry matter). **D)** Soluble protein content (mg g^{-1} dry matter). **E)** Starch content (mg g^{-1} dry matter). **F)** Spectaline content (µg g^{-1} dry matter). **G)** Total content of reserve compounds (mg g^{-1} dry matter) in the different calli. Lowercase letters above bars in the same graph indicate a significant difference (C–E).

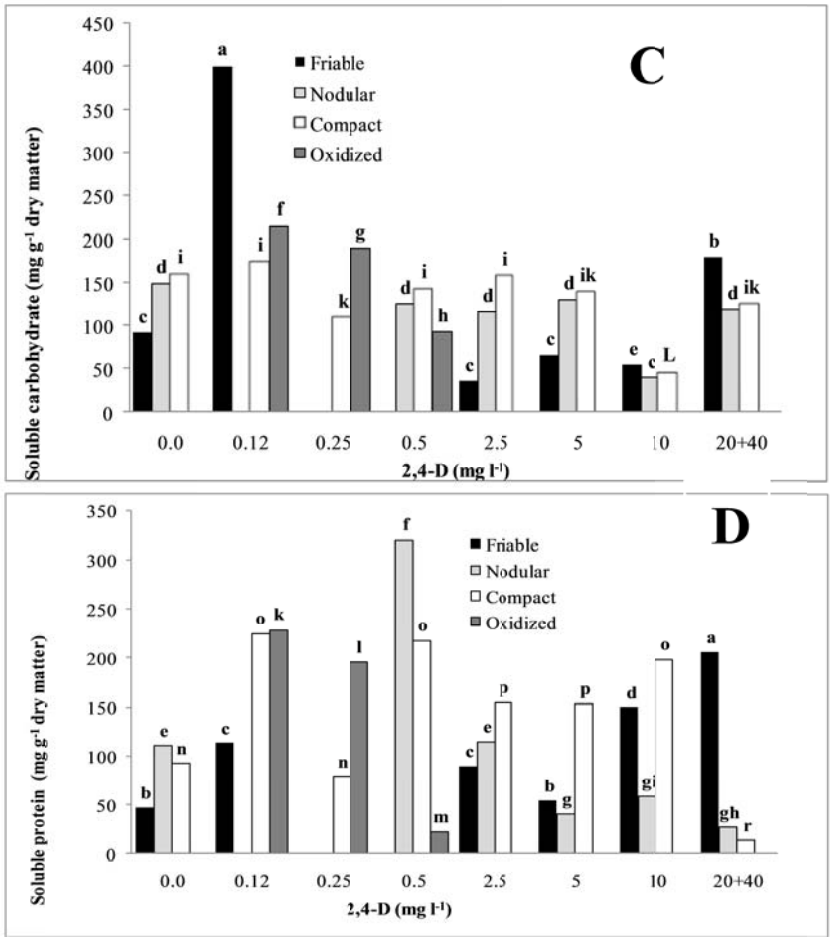


Figure 2. Contd.

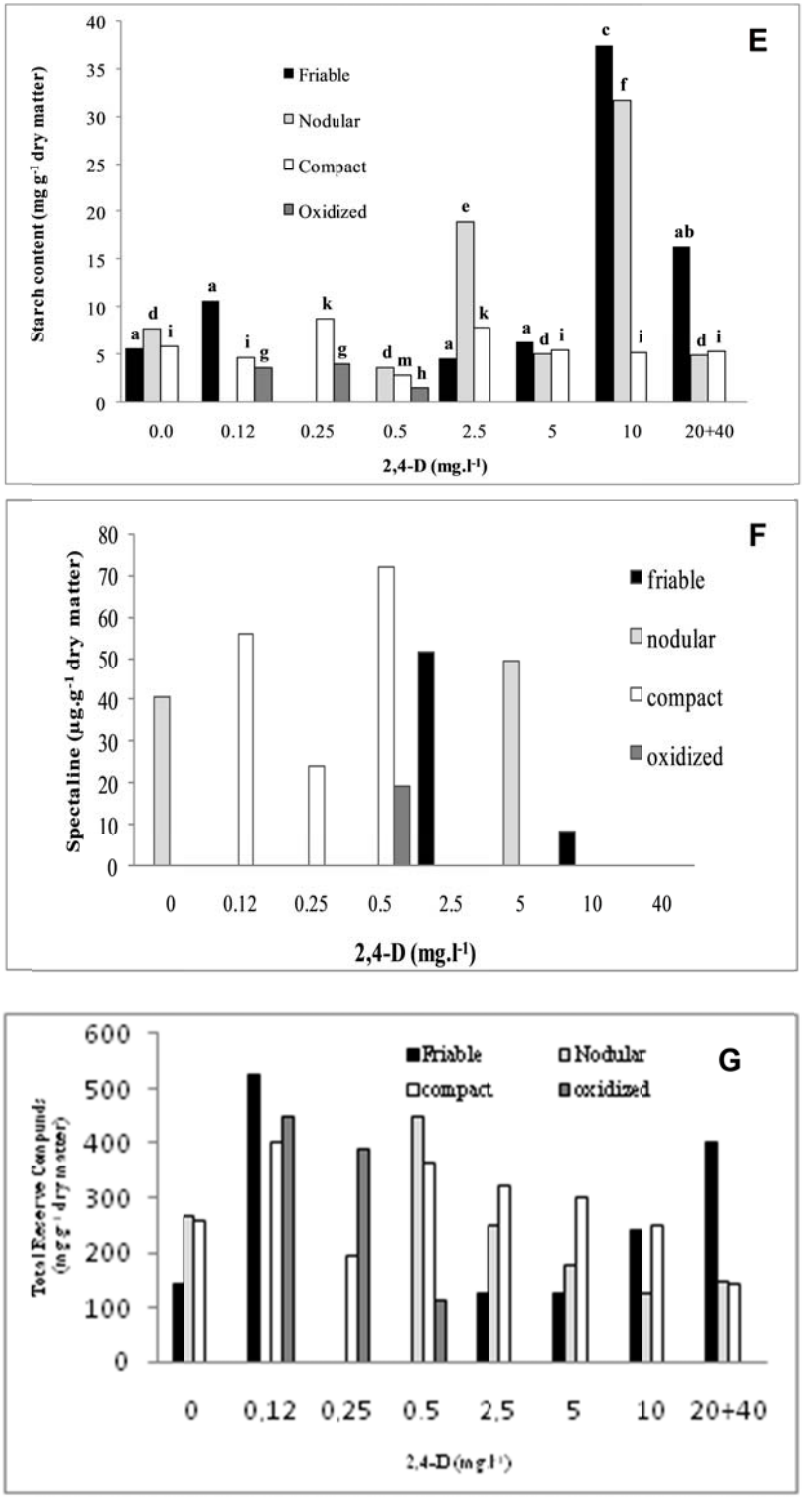
The soluble carbohydrates content was significantly higher in friable callus induced by 0.12 mg L^{-1} 2,4-D (P < 0.001) and 40.0 mg L^{-1} 2,4-D (P < 0.001) than at 0, 2.5, 5.0 and 10.0 mg L^{-1} 2,4-D (Figure 2C). Soluble carbohydrates in friable callus at 0.25 mg L^{-1} 2,4-D was lower compared to that at 0, 0.12, 0.5, 2.55 and 10.0 mg L^{-1} 2,4-D (P < 0.005). Nodular callus induced by 10.0 mg L^{-1} 2,4-D showed lower accumulation of soluble carbohydrates (P < 0.001) compared with that induced by all other 2,4-D treatments. Friable callus induced by 0.12 mg L^{-1} 2,4-D accumulated higher soluble carbohydrate contents than at all other 2,4-D concentrations (P < 0.05).

The 10.0 and 40.0 mg L^{-1} 2,4-D treatments induced friable callus with higher protein contents (P < 0.05) compared to that of the other treatments (Figure 2D). The nodular callus induced by 0.5 mg L^{-1} 2,4-D contained higher levels of soluble protein compared to that of all other treatments (P < 0.05). Soluble protein content in compact callus was significantly higher at 0.12, 0.5, 2.5, 5.0 and 10.0 mg L^{-1} 2,4-D (P < 0.01) compared to the 0.0, 0.25 and 40.0 mg L^{-1} 2,4-D treatments. Oxidized calli showed higher levels of soluble proteins in the 0.12, 0.25 and 0.5 mg L^{-1} 2,4-D treatments.

Friable and nodular callus induced by 10.0 mg L^{-1} 2,4-D contained the highest concentrations of starch (P < 0.001) compared to that of the other treatments (Figure 2E). However, the starch content of nodular callus induced at 0.25 and 2.5 mg L^{-1} 2.4-D was also significantly higher than the other treatments and callus types (P < 0.05).

Spectaline was detected in nodular callus induced by 0 and 5.0 mg L^{-1} 2,4-D (Figure 2F, Table 2), compact callus at 0.12, 0.25 and 0.5 mg L^{-1} 2,4-D, oxidized callus at 0.5 mg L^{-1} 2,4-D, and friable callus at 2.5 and 10 mg L^{-1} 2,4-D.

All types of calli induced by 0.12 mg L^{-1} 2,4-D showed higher contents of reserve compounds compared to that of the other treatments (Figure 2G). At 0.5 mg L^{-1} 2,4-D, nodular callus with large amounts of reserve compounds developed, but friable callus induced by the same treatment showed a low content of reserve compounds.



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Treatment with 5.0 mg L^{-1} 2,4-D induced compact callus with higher concentrations of reserve compounds compared to those of nodular and friable callus in the same treatment.

Table 2. Spectaline content (μg g^{-1} dry mass) detected by GC-FID in different callus types cultured on medium containing different 2,4-D concentrations (* not detected).

2,4-D (mg L^{-1})	Callus type			
	Friable	Nodular	Compact	Oxidized
0.0	0*	41.0	0*	0*
0.12	0*	0*	56.0	0*
0.25	0*	0*	24.0	0*
0.5	0*	0*	72.0	19.0
2.5	51.0	0*	0*	0*
5.0	0*	49.0	0*	0*
10	8.0	0*	0*	0*
20+40	0*	0*	0*	0*
Standard (flowers)	6,820.0			

DISCUSSION

The presence of spectaline in cultured calli occurred randomly, except for the 40 mg L^{-1} 2,4-D treatment, in which spectaline was not detected, showing an herbicide toxic effect in the calli growth. The accumulation of spectaline in different callus types indicates their potential for *in vitro* biosynthesis of the alkaloid. According to Shrivastava et al. (2006) and Karuppusamy (2009), the chemical composition of the culture medium, presence of growth regulators, physical components of the axenic culture, basic knowledge of the biosynthetic routes, elicitor compounds or treatments (abiotic or biotic) triggering the formation of secondary metabolites, genetic manipulation and metabolic engineering may improve the accumulation of compounds.

Trees species of Fabaceae are considered recalcitrant on tissue culture, and do not show tissue or organ induction in lower concentrations of growth regulators as recommended on protocols for cell multiplication (Gharyal and Maheshwari, 1990; Machuka et al., 2002). In the present study, all *S. spectabilis* explants have competent cells that were receptive to stimulation, induction, dedifferentiation and proliferation by auxin in the culture medium. Diverse organs from dicot tissues are easily induced, moreover, callus proliferation is more easily established from certain organs than others due some cells have the ability to perceive, translate and respond to a signal, otherwise they are not suitable targets for plant growth regulators action (Osbourne and McManus, 2005). Callus appearance is considered an important factor for successful induction of somatic embryogenesis. Pal et al. (2006), in a study of regeneration through organogenesis from hypocotyl and cotyledon fragments of *Curcubita pepo* L., considered friable and nodular callus with a milky aspect to be potentially organogenic, and discarded callus that was overly moist and spongy, too compact or brown, and the presence of green dots indicated the beginning of the somatic embryos maturation. The nodules observed on the callus surface of *S. spectabilis* could be associated with globular embryo-genic protuberances or with the globular stage of somatic embryo (Rodriguez and Wetzstein, 1998; Sharma et al., 2014).

The swelling and expansion observed in the explants has been observed on several previous studies in genus *Cassia*. Shrivastava et al. (2006) noticed swelling and increased size of explants of *Cassia senna* L. three weeks after inoculation. A similar result was observed by Agrawal and Sardar (2006) during induction of embryogenesis and organogenesis of *Cassia angustifolia* M. Vahl. Auxin stimulates acidification of the cell wall allowing extension or stretching, and during this process the cell absorbs large amounts of water (Raven et al., 2001, Silveira et al., 2004). These factors explain the amount of water absorbed by the cells, as indicated by comparison of the fresh and percentage dry mass of friable callus and low dry mass compared to that of compact callus, which suggests friable callus absorbed more water than did the compact callus.

In the present study notable concentrations of soluble proteins were present in the different callus types. In contrast, the highest soluble proteins content recorded by Silveira et al. (2004) for cell suspension cultures of *Pinus taeda* L. was 0.73 mg g^{-1} fresh weight (estimated 10% of this value for dry weight). These authors suggested that the increase in the concentration of soluble proteins might be related to mitotic activity during the exponential growth phase, when specific proteins are synthesized at the beginning of the cell cycle and which lead to biochemical and morphological changes throughout the mitotic cycle. The presence of these specific proteins in embryogenic cells might be associated with formation of proembryogenic groups (Cangahuala-Inocente et al., 2004). Total soluble protein in roots and in the photo-

synthetic aerial parts of *Phalaenopsis amabilis* were related to enzymes and their synthesis, coenzymes, nucleic acids, chlorophyll and primary plant metabolism (Ori et al., 2014). The authors suggest that these proteins are possibly not for reserve as found in potato tubers and soybean seeds. This rise expresses that the synthesis of proteins formed from the stage of degradation of carbohydrates, which is necessary for production of energy which will be used on the exponential growth phase, when the rate of cell division increases.

Reserves compounds are crucial for *in vitro* morphogenesis (Branca et al., 1994) and some studies correlated reserve consumption patterns with the development of organogenesis and also to somatic embryogenesis (Mangat et al., 1990; Martin et al., 2000). Pinto et al. (2010) pointed that starch begins to accumulate early during induction of *Eucalyptus globulus* somatic embryos with 3 mg L^{-1} α-naphthalene acetic acid, probably due to the sucrose present in the culture medium and in meristematic regions a lower abundance of starch were presented due consumption in mitotically active tissues that was reported earlier for other species (Barciela and Vieitez, 1993; Canhotoa and Cruz, 1996).

Induction of cell proliferation caused by the synthetic auxin 2,4-D also leads the cells to accumulate reserve compounds, because the cell division process has a great demand for energy and basic compounds for DNA duplication, specific protein synthesis, multiplication of organelles and production of new cell walls. Thus, treatment with 0.12 and 0.5 mg L^{-1} 2,4-D, which induced higher rates of reserve compound accumulation, is inferred to represent the optimal concentrations of auxin to induce cell proliferation in *S. spectabilis* callus. However, treatment with 10 mg L^{-1} 2,4-D represented the best option to induce friable and nodular callus with higher accumulation of starch. *Carya illinoiensis* (Wangenh.) K. Koch embryogenic clump formation is preceded by accumulation of starch grains, which are rapidly consumed during the induction of the embryogenic regions, but starch is absent in the embryo maturation stages (heart and torpedo stages) (Rodriguez and Wetzstein, 1998).

The possibility that the available sucrose, auxin and cytokinin acted as a checkpoint in the control of cell division in the plant cycle is another interesting aspect to highlight. Although the levels of reserve compounds present in the calli favored cell growth and division, the presence of high concentrations of auxin and sucrose in the culture medium was insufficient to induce differentiation of somatic embryos, buds or roots, which indicated that other factors were absent or present at inadequate concentrations to stimulate differentiation in the G2 phase checkpoint during the experiment as observed by Chu et al. (2008) for *C. echinata*.

However, data obtained in the present study could serve as the foundation for new protocols for *in vitro* propagation and differentiation of *S. spectabilis*. Our results indicate that hypocotyl fragments with an auxin shock (10 mg L^{-1} 2,4-D) for a short period (up to five days) to induce cell proliferation and starch accumulation, followed by culture with 0.12 mg L^{-1} 2,4-D to increase in tissue mass and accumulation of other reserve compounds (soluble carbohydrates and proteins), induces the production of friable callus suitable for establishment of an *in vitro* tissue culture system.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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