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Triiodobenzoic acid affects the regenerative competence in root explants of *Adenocalymma nodosum* (Bignoniaceae)

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ABSTRACT

Adenocalymma nodosum, a native plant species from Brazilian Savanna, has a very interesting propagation mechanism by root segments, mainly, in areas with weed mechanical control or soil prepared by farming equipment such as plough that results in the cited root segments, enabling high regenerative capacity to generate adventitious shoots. This regenerative competence of root segments, without exogenous plant growth regulators, was previously showed to occur on *in vitro* conditions. Aiming to study the effect of the auxin transport inhibition on the regenerative capacity of the root segments of this species, root explants were placed in different orientations (horizontal, upright and inverted upright) on Murashige and Skoog medium supplemented or not with triiodobenzoic acid (TIBA). It was possible to verify that root explants have a polar response with shoot buds being directly differentiated from pericycle cells only at root proximal ends. Use of TIBA, an auxin transport inhibitor, inhibited organogenesis and induced callogenesis, showing that auxin transport is essential to the favorable hormonal balance to bud differentiation at the explant proximal end.

Index terms: TIBA; *de novo* organogenesis; pericycle cells; cerrado biome.

INTRODUCTION

In natural conditions of the Brazilian Savanna, locally known as Cerrado, it is not uncommon to find plants with vegetative propagation strategies by subterranean systems and some of them are clearly means of vegetative reproduction. *Adenocalymma nodosum* (Silva Manso) L. G. Lohmann (Bignoniaceae) popularly named "carobinha do campo" is a perennial shrub mainly found in the "Cerrado" (Silva, 1998). Until recent years this shrub was botanically known as *Memora nodosa* (Manso) Miers. However, Alcantara and Lohmann (2010) reclassified and renamed it to *Adenocalymma nodosum*. Oliveira et al. (2010) reported a notable capacity of this plant species to regenerate shoot buds from root fragments, mainly after a drastic pruning, a phenomenon also confirmed by these authors to occur under *in vitro* conditions when using root microcuttings. This characteristic makes it to assume a behavior of invasive plant in "Cerrado" areas, mainly those with mechanical weed control, because this kind of control results in the harmful propagation by providing the roots fragmentation and, on this condition, *A. nodosum* could be a plant easily propagated and consequently widely distributed.

Shoot buds may develop from any plant tissue for a large number of species and several researches have been studying shoot buds regeneration from root segments of many species under *in vitro* conditions. A few species can produce shoot buds from root explants under *in vitro* conditions without plant growth regulators in the induction medium, as obtained by Delgado-Paredes et al. (2016) in *Ipomoea batatas* and Picolotto et al. (2017) in *Cyrtopodium paludicolum*.

However, plant hormones play a crucial role in regulating plant development and the plastic

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and flexible shaping of the plant architecture in response to variable environmental conditions. The final developmental and physiological output of the hormonal signaling in plants is the typically resulting of combined a hormonal pathways. Several hormonal pathways are involved in the regulation of root and shoot induction on differentiated cells from various explants, with auxin and cytokinin being the principal players (Ruzicka et al., 2009).

The pioneering work has shown that a high auxin-cytokinin ratio induces root regeneration, whereas a low ratio promotes shoot induction (Skoog; Miller, 1957). This indicates that auxin and cytokinin might have a cross-talk during *in vitro* organogenesis. However, the molecular mechanism of such interaction between auxin and cytokinin in the *in vitro* meristem induction remains mostly unknown (Su et al., 2011), despite their absolute necessity in some protocols (Rodrigues et al., 2015; Abraham; Thomas, 2015).

Auxin and cytokinin play fairly important roles in many aspects of plant growth and development. Su et al. (2011) affirm that auxin polar transport is required to direct auxin flows and to form auxin gradients in plants, which are critical for developmental pattern formation.

Here we investigated the influence of the explant orientation and use of triiodobenzoic acid in the competence and regeneration polarity of root explants of *A. nosodum* to *de novo* shoot organogenesis.

MATERIAL AND METHODS

Plant material and culture conditions

Seed germination and organogenesis from root explants were performed according to Oliveira et al. (2010). Seeds were pre-disinfested by immersion in hypochlorite solution (3% active chlorine) for five minutes (min) followed by manual coat removal. After that, embryos were dipped into an ethanol solution 70% (v/v) for 1 min, and afterwards in a sodium hypochlorite solution (1% active chlorine) for 5 min, and rinsed 5 fold with sterile distilled water. Seeds were subsequently transferred to 250-mL glass jars (5 seeds per jar; a total of 50 seeds) containing 40 mL MS medium (Murashige; Skoog, 1962) supplemented with myo-inositol (0.01% w/v), sucrose (3% w/v), and Agar-Agar (0.6% w/v) (*HiMedia* Laboratories Pvt. Ltd., India); the pH of the medium was adjusted to 5.8 ± 0.1 prior to autoclaving process (15 min and 121 °C). The jars were sealed with PVC film (Dispafilm do Brasil Ltda, Brazil). All jars were maintained in a temperature-controlled growth room (27 ± 2 °C) equipped with 2 fluorescent lamps (20 W; Osram Luz do Dia, São Paulo, Brazil) that provided a 16-h photoperiod (36 µmol m^{-2} s^{-1} irradiation) for 50 days.

Experiment I: influence of explant orientation on root regenerative competence

Roots from 50-day-old seedlings were segmented (15-20 mm), except for root apex (1 cm away), incubated in 250-mL glass jars containing 30 mL MS medium, and maintained at light intensity and temperature as described previously. The experiment had three different explant orientations (horizontal, upright, inverted upright), each one with 7 jars with 5 root explants in each, resulting in 21 jars. Thirty days after incubation, number of roots and shoot buds differentiated in both root explants extremities, distal and proximal, were analyzed.

Experiment II: influence of TIBA on regenerative competence of root explants

A second and similar experiment using root explants from *M. nodosa* seedlings, as described prior, was performed, but now with addition of different concentrations of sterile-filtered triiodobenzoic acid - TIBA (0.0, 0.15, and 0.30 µM) on MS culture medium, after medium autoclaving. Considering the results from Experiment I and due to reduced root explants availability, this experiment had only two different explant orientations (horizontal and upright), each one with 7 jars with 5 root explants in each. Thirty days after incubation, number of shoot bud differentiation, callus diameter in both explant extremities (distal and proximal), and total fresh weight of the explants (shoot bud removal) were analyzed.

Root anatomy

For anatomical studies to understand the regeneration process, root explants coming from experiment I were fixed in FAA 70 FAA70 (Formaldeyde - Acetic acid - Alcohol 70%) (Johansen, 1940), dehydrated in a graded ethanol series, and embedded in methacrylate (Historesin, Leica Instruments, Germany). Cross and longitudinal

sections (5-µm thick) were obtained using an automatic rotary microtome (RM 2155, Leica Microsystems Inc., USA) and stained with toluidine blue. The samples were mounted in Permount on glass slides. Photographs were taken using a light microscope (Olympus AX70TRF; Olympus Optical, Japan) equipped with a digital camera (Spot Insight Color 3.2.0; Diagnostic Instruments Inc., USA).

Statistical analyses

Data from experiments I and II were analyzed using an analysis of variance (ANOVA) followed by a Tukey's test for comparison of means at 5% probability level using the statistical program Sisvar (Ferreira, 2011), with transformed data [square root (x+1), wherein x is the number of shoot bud or roots].

RESULTS AND DISCUSSION

The regenerative capacity was observed in intact roots of *A. nodosum* seedlings maintained for a long time on *in vitro* conditions, resulting in *de novo* shoot buds differentiation throughout root zones, in a direct organogenesis process along all root extension (Figure 1A). However, in the experiment I was possible to demonstrate two things: *i*) when roots were segmented, they maintained a similar capacity to *de novo* shoot organogenesis when compared to intact roots (Figure 1A), and without addition of exogenous plant growth regulator (PGR) (Figure 1B-D); *ii*) when roots were segmented, this *de novo* shoot organogenesis occurred linked with a polarity response, regardless of the explant orientation

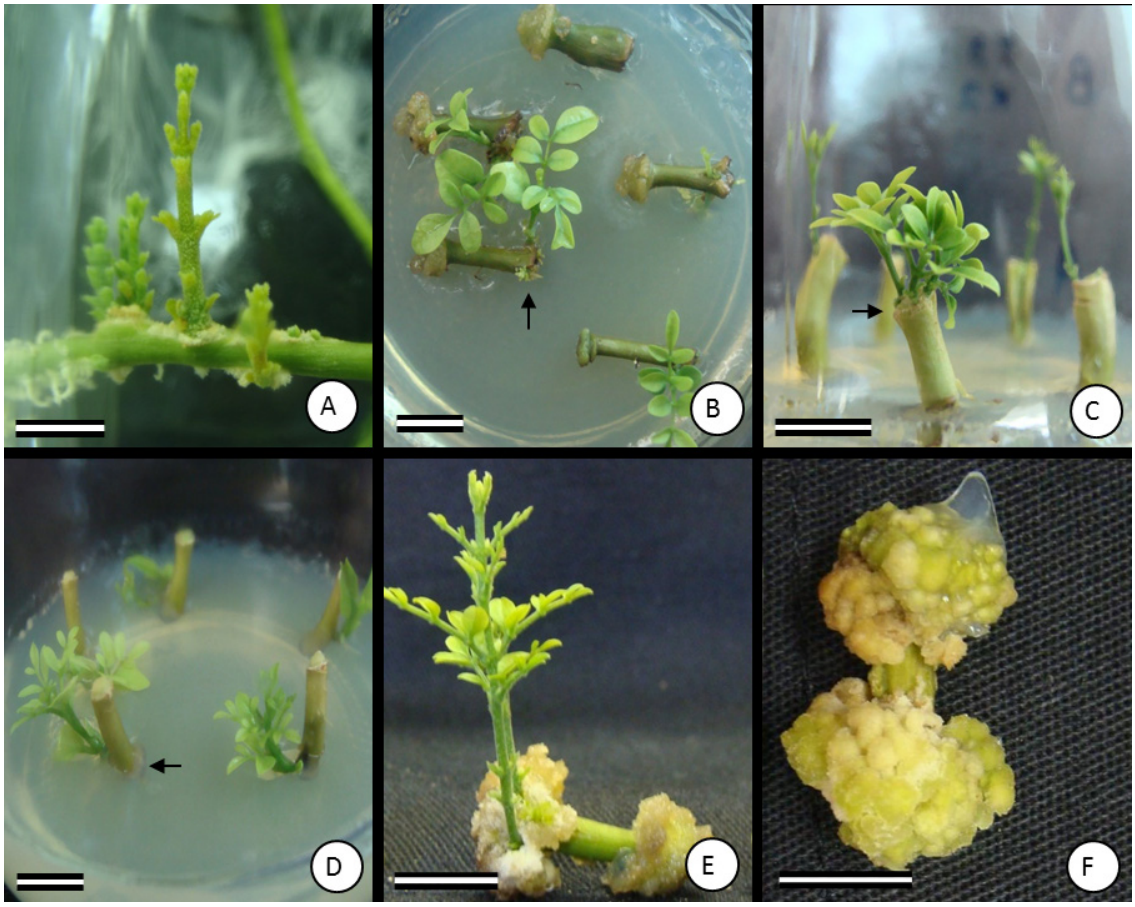

Figure 1: Organogenic responses of intact root (A) and segmented root explants (B-F) of *Adenocalymma nodosum* inoculated in different orientations on Murashige and Skoog (MS) nutritive medium supplementend or not with acid 2,3,5-triiodobenzoic (TIBA). A: shoot buds in intact roots of seedling cultivated for more than 60 days on *in vitro* conditions; B: horizontal explants; C: inverted upright explant orientation; D: upright explant orientation; E: horizontal explant with 15µM TIBA; F: horizontal explant with 30µM TIBA. Bar = 1cm; arrows = proximal end.

during induction on PGR-free medium (Figure 1B-D). The shoot buds were visible 12 to 15 days after initial explants incubation. This species presented a well-marked morphogenic polarity when root is segmented, notably forming 1.5 shoot buds per explant only at proximal end (basal end) of the root explants (data not shown). This pattern of polarized response was maintained for differentiation of root primordial, and in this case, only cells of the distal (apical end) of root explants showed that competence, with mean number of 1.0 root per explant (data not shown). Statistical analyses showed that explant orientation did not modify responses for both number of shoot buds and number of roots, signalizing that auxin polar transport can be involved in this behavior.

The alteration of the *in vitro* regeneration pattern showed by *A. nodosum* segmented roots may be related with changes in endogenous hormonal balance in intact roots when compared with segmented roots. Probably, this morphogenic competence with well-defined polarity is conditioned by endogenous hormonal balance and it could be related with high levels of cytokinins and acropetal transport of auxin in the root explants. These factors probably generate an accumulation of auxin at the distal end of the explants while cytokinin becomes more concentrated at the proximal end, making such regions with favorable hormonal balance to make their respective competent cells to differentiate into root and shoot primordial, respectively. This relationship between auxin and cytokinin in modulating morphogenic responses, including morphogenic polarity, was reported by Bishopp et al. (2011) and Sharma (2012).

The negative effect of cytokinin on auxin signaling and transport might be of general significance in explaining the antagonistic interaction of cytokinin-auxin (Bishopp et al., 2011). The two hormones are known to have opposite effects on *de novo* auxin induced organogenesis (Pernisová et al., 2009). It is likely that the response found here is the result of this pattern of antagonistic relationship between these two hormonal classes.

Histological analyses of the root structure of *A. nodosum* from experiment I, showed an uniseriate epidermis, a cortical parenchyma with several cell layers of varying sizes, a polyarch vascular cylinder, and a central region filled with uncommon pith tissue with thin walls and cells of varying sizes (Figure 2). Additionally, it was possible to show that shoot buds were formed directly from the pericycle cells of the *A. nodosum* root explants (Figure 2B-C). The shoot buds development resulted in the disruption of the cortex and root epidermis (Figure 2B-C). Shoot buds arose endogenously with the vascular system connected to the main vascular tissue of the initial explant (Figure 2B), confirming the organogenic regeneration pattern. Organogenic process in *A. nodosum* root explants was asynchronous, resulting in the presence of shoot buds in different stages of development in the same explant (Figures 1B-D, 2B-C).

The pericycle has been proposed to be an extended meristem in the plant body (Smet et al., 2006). A number of studies have reported the involvement of pericycle in the regeneration of *de novo* shoot buds from root explants (Rocha; Vieura; Tanaka, 2012; Chatfield et al., 2013), which is consistent with the current knowledge of the molecular mechanisms involved in the induction of shoot meristems in roots. Atta et al. (2009) demonstrated the ability of pericycle cells to rapidly re-enter the cell cycle upon induction with cytokinin. Pericycle cells are lateral root initiation sites (Smith; Smet, 2012), and exposed to proper conditions, they may also be involved in the formation of shoot meristems (Rocha et al., 2012). Molecular studies have shown that the competence to form shoot buds is acquired at sites where spontaneous formation of lateral roots occurs (Atta et al., 2009; Motte et al., 2011).

The use of root explants for *in vitro* regeneration is limited to a few plant species and the success of shoot differentiation is, in general, dependent on culture media supplementation with growth regulators. Conversely, *A. nodosum* enable an *in vitro* system in which root explants generate shoot-buds without the need of exogenous hormones, providing a relevant and interesting system to study certain physiological aspects of *de novo* shoot bud induction, since organogenic capacity occurs without exogenous plant growth regulators addition, showing an endogenous hormonal balance adequate to both shoot and root *de novo* organogenesis. Additionally, this organogenic process could be a promising model to investigate cytokinin and auxin transport in root cuttings.

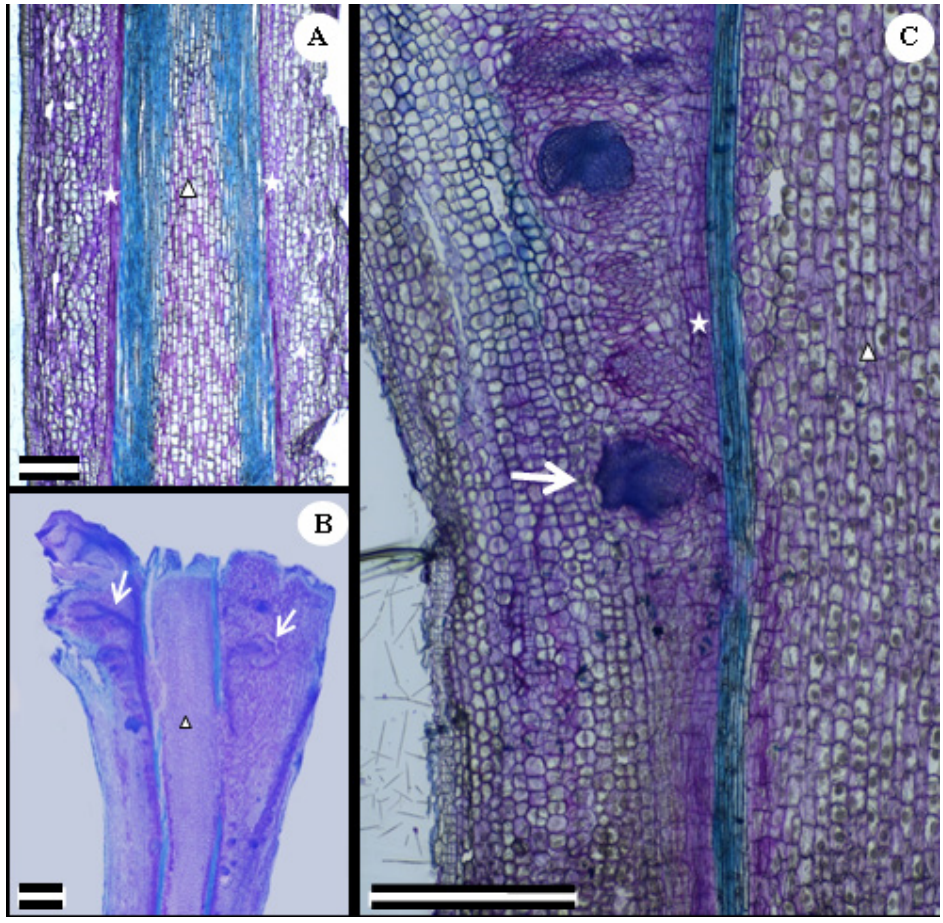

Figure 2: Photomicrographs of longitudinal sections of root explants of *Adenocalymma nodosum* grown *in vitro* on Murashige and Skoog (MS) nutrient medium. A: longitudinal section of the initial explant, B and C: longitudinal section showing the pericycle cells division in early and later organogenesis and shoot buds (arrows) at different stages derived from pericycle cells.

As shown in the experiment II, the role of auxin in the regeneration of root cuttings of *A. nodosum* cultivated *in vitro* indicates that the root explant position with regard to gravity does not fundamentally affect the inherent tendency of these cuttings to manifest *de novo* shoot organogenesis in proximal end, while the distal end of root explants proved to be incompetent for the purpose, as commented before. Interference in the auxin polar transport with addition of crescent TIBA doses (0.15 or 0.30 mM) caused failure in the bilateral symmetry organogenesis, in which number of differentiated shoots at the proximal end was reduced (Table 1) while root differentiation at distal end was abolished (data not shown). On the other hand, the presence of callusing and consequently increase in the explant fresh weight were incremented with increasing in TIBA (Table 1). Additionally, it was observed that, when explant was horizontally positioned on culture medium, in the presence of TIBA, its response pattern was modified when compared to upright position, mainly showing reduced shoot number and enhanced callusing at proximal end and higher explants fresh weight (Table 1, Figure 1E-F). These differences in relation to explant orientation can be attributed to higher absorption capacity of the horizontal explant due to its higher contact surface with culture medium, as obtained by Al-Ramamneh et al. (2017).

Table 1: Variance analysis and Tukey's test of the mean shoot number (SN), callus diameter at proximal (CDP) and basal (CDB) ends and fresh weight (FW) of the *A. nodosum* root explants cultivated on MS medium added with triiodobenzoic acid (TIBA) and different orientation during cultivation.

Source of variation	F value			
	SN*	CDP (cm)*	CDB (cm)*	FW (g)*
TIBA doses (T)	24.47**	4.40**	4.75**	4.67**
Explant orientation (O)	5.36**	33.76**	0.015^{ns}	4.84**
F (T x O)	2.38^{ns}	0.18^{ns}	1.482^{ns}	2.01^{ns}
TIBA (µM) (T)	SN*	CDP (cm)*	CDB (cm)*	FW (g)*
0.0	1.31a	3.93 b	5.30 b	0.23 b
0.15	0.51b	4.71 ab	6.91 ab	0.28 ab
0.30	0.25b	5.15 a	8.29 a	0.38 a
Explant orientation (O)	SN*	CDP (cm)*	CDB (cm)*	FW (g)*
Horizontal	0.54 B	5.58 A	6.79 A	0.34 A
Upright***	0.84 A	3.61 B	6.88 A	0.25 B

* Original data; ** Significant according to ANOVA ($p \leq 0.05$). ^{ns} Not significant according to ANOVA ($p \leq 0.05$); *** Explant with basal end inside medium.

Means followed by the same lowercase letter on TIBA doses (column) and uppercase letter on different explant orientation (column) are not significantly different according to Tukey test ($p \leq 0.05$).

Further it can also be deduced that endogenous auxin in both explant extremities plays an important role in the induction of organogenesis and that the root and shoot differentiation in root explants is also related to the polar transport of endogenous auxin, which alters the auxin-cytokinin balance, as also presented by Warmke and Warmke (1950) with *Taraxacum* and *Cichorium* root explants, and with *Mesembryanthemum crystallinum* hypocotyls explants.

CONCLUSIONS

The shoot bud organogenic competence was restricted to proximal end of *A. nosodum* root segments, independently of explant orientation, and this competence was affected by TIBA, an auxin transport inhibitor. Anatomical images exposed that pericycle cells were responsible for shoot bud differentiation. These are basic information for future experiments that will allow us to understand the regenerative capacity of *A. nodosum*, enabling the development of strategies to inhibit its vegetative propagation.

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