

LUDMILA NAYARA DE FREITAS CORREIA

***IN VITRO* NEOFORMATION OF ORGANS IN *Bixa orellana* L.:  
MORPHOANATOMY, CLONING AND CHARACTERIZATION OF  
PLURIPOTENCY-ASSOCIATED PUTATIVE TRANSCRIPTION FACTORS**

Thesis presented to the Universidade Federal de Viçosa, as part of the requirements of the Plant Physiology Graduate Program for obtention of the degree of *Master Scientiae*.

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## RESUMO

CORREIA, Ludmila Nayara de Freitas, M.Sc., Universidade Federal de Viçosa, Julho 2016. ***In vitro* neof ormation of organs in *Bixa orellana* L.: morphoanatomy, cloning and characterization of pluripotency-associated putative transcription factors.** Orientador: Wagner Campos Otoni. Co-Orientadores: Andréa Dias Koehler e Elyabe Monteiro de Matos

A pluripotencialidade é uma característica das células vegetais que permite uma única célula originar a maioria dos tecidos que irão compor o corpo da planta. A organogênese é uma via de regeneração que se baseia nesses princípios para induzir a neof ormation de órgãos. Os hormônios de crescimento vegetais associados aos eventos moleculares são essenciais para o sucesso dessa via morfogênica. Diante disto, os objetivos desse trabalho foram: avaliar a responsividade morfogênica *in vitro* de diferentes explantes de *Bixa orellana* submetidos a condições de indução organogênica; caracterizar as mudanças citológicas que ocorrem durante a formação de brotos e raízes, acompanhar a mobilização de reservas; e isolar e caracterizar genes expressos durante os eventos organogênicos. Para avaliar a formação de brotos hipocótilos, hipocótilos feridos, hipocótilos enraizados inoculados com a polaridade invertida, e folhas pecioladas foram inoculados em meio JADS suplementado com vitaminas B5, sacarose (3% w/v), mio-inositol (0,01% w/v), ágar (0,6% w/v) e 4,56 µM de zeatina, 2,07 ou 4,14 µM de meta-topolina. Amostras com 0, 3, 6, 9, 18, 21, 24, e 30 dias foram coletadas para microscopia de luz, microscopia eletrônica de varredura, e testes histoquímicos todas seguindo protocolos específicos. Para avaliar a formação de raízes, plântulas foram inoculadas em meio JADS em condições similares citadas acima, mas contendo 0 ou 2,46 µM de ácido indolilbutírico (AIB). Após 0, 2, 4, 6, 8, 10, 18, e 20 amostras foram coletadas e procedimento citados acima foram seguidos. A partir de material organogênico extraiu-se o RNA total e obteve-se o cDNA, o qual serviu como molde para amplificação das sequências codantes utilizando-se *primers* degenerados para os genes *BABY BOOM (BBM)* e *SHOOT MERISTEMLESS (STM)*. Observou-se que em *B. orellana* tanto a zeatina quanto a meta topolina induziram a formação de brotos, porém o ferimento do hipocótilo, bem como a inversão da polaridade em hipocótilos enraizados otimizaram o processo, quando comparados aos hipocótilos e folhas pecioladas. As primeiras divisões celulares foram observadas nas primeiras semanas de cultivo em meio de indução, principalmente em explantes cultivados em

meio acrescido de zeatina. Após 30 dias de cultivo, zeatina e meta topolina induziram a formação de estruturas caulinares e diferenciação de primórdios foliares nos explantes avaliados. Na histoquímica, reservas proteicas foram evidenciadas principalmente em hipocótilos enraizados e folhas pecioladas. Grãos de amido foram evidenciados em todos tipos de explante avaliados, e um aumento gradual na concentração desse composto de reserva foi observado ao longo do período de indução. O teste de vermelho neutro não indicou a presença de lipídios durante o processo organogênico. Explantes caulinares cultivados em meio JADS com AIB apresentaram divisões celulares após quatro dias de cultivo, que deram origem a primórdios de raízes laterais. A manutenção em meio de indução, suplementado com AIB, por 20 dias levou a formação de raízes laterais. Neste mesmo período primórdios de raízes laterais foram notados no controle. Os testes histoquímicos evidenciaram a presença de proteínas, que foram consumidas ao longo do evento organogênico, além de grãos de amido e lipídios presentes em todos os tempos avaliados. Sequências consenso obtidas a partir de primers dos degenerados de *BBM* e *STM* foram clonadas e sequenciadas. As sequências consenso obtidas foram comparadas em bancos de proteínas não redundantes do NCBI, Phytozome e TAIR, usando o algoritmo BLAST. A análise e o alinhamento das sequências geraram três contigs semelhantes a membros da família *APETALA2-like*, especialmente *BBM*, *WRINKLED (WRI)* e *AINTEGUMENTA (ANT)*, e três contigs com alta similaridade aos fatores de transcrição da Class I *KNOX*, especialmente *STM*. Estes resultados demonstram as respostas morfogênicas de diferentes tipos de explantes submetidos aos meios de indução, e os possíveis membros das famílias AP2-like e Class I *KNOX* clonados a partir das sequências analisadas, e que estão associadas com a aquisição de competências para formação de órgãos *in vitro* em *Bixa orellana*.

## ABSTRACT

CORREIA, Ludmila Nayara de Freitas, M.Sc., Universidade Federal de Viçosa, July 2016. . ***In vitro* neoformation of organs in *Bixa orellana* L.: morphoanatomy, cloning and characterization of pluripotency-associated putative transcription factors.** Advisor: Wagner Campos Otoni. Co-Advisers: Andréa Dias Koehler and Elyabe Monteiro de Matos

Pluripotency is the characteristic of plant cells that allows a single cell to originate the tissues that will compose the plant body. Organogenesis is a regeneration pathway with basis of these principles to induce organ neoformation. The plant growth hormones associated with molecular events are essential to the success of these morphogenic pathways. Thus, the objectives of this study were: to evaluate the morphogenic response *in vitro* from different explants of *Bixa orellana* submitted to organogenic induction; to characterize the cytological changes that occur during shoot and root formation, as well as to follow the mobilization of reserves, and to isolate and characterize genes expressed during the organogenic events. To assess the shoot formation, hypocotyls, injured hypocotyls, rooted hypocotyls with reversed polarity, and petiolate leaves were inoculated in JADS medium, supplemented with B5 vitamins, sucrose (3% w/v), myo-inositol (0.01% w/v), agar (0.6% w/v) and 4.56  $\mu$ M zeatin, or 2.07  $\mu$ M meta-topolin, or 4.14  $\mu$ M meta-topolin. Samples with 0, 3, 6, 9, 18, 21, 24 and 30 days were collected for light microscopy, scanning electron microscopy, and histochemical tests following specific protocols. In order to assess the root formation, plantlets were inoculated in JADS medium in similar conditions as cited above, but with 0 or 2.46  $\mu$ M indolilbutyric acid (IBA). After 0, 2, 4, 6, 8, 10, 18 and 20 days, samples were collected to perform the same analyses mentioned before. Total RNA was extracted from the organogenic material, from which the cDNA was obtained and used as a template for the amplification of coding sequences using degenerate primers for *BABY BOOM* (*BBM*) and *SHOOT MERISTEMLESS* (*STM*). It was observed that in *Bixa orellana* zeatin and meta-topolin induced shoot formation, but the injured hypocotyls as well as rooted hypocotyls with reversed polarity optimized the process when compared with hypocotyl and petiolate leaves. The first cell divisions were observed in the first weeks of culture in induction medium, especially in explants cultured in medium supplemented with zeatin. After 30 days of cultivation, zeatin and meta-topolin induced the formation of leaf primordia in the evaluated explants. Proteins were evidenced mainly in rooted

hypocotyls and petiole leaves. Starch grains were found in all types of explant evaluated, and a gradual increase in the concentration of this reserve compound was observed during the induction period. The neutral red test did not indicate the presence of lipids during the organogenic process. Plantlets inoculated in JADS with IBA have cell divisions after four days of culture, originating lateral root primordia. The maintenance in the induction medium supplemented with IBA for 20 days led to the formation of lateral roots. In the same period, lateral root primordia were observed in the control. The histochemical tests evidenced the presence of protein, which were consumed during the organogenic event, in addition to starch grains and lipids present in all evaluated days. Consensus sequence obtained from *BBM* and *STM* degenerate primers were cloned and sequenced. The consensus sequences obtained were compared to non-redundant protein databases NCBI, Phytozome TAIR, using the BLAST algorithm. The analysis and alignment of sequences yielded three contigs similar to members of *APETALA2-like* family, especially *BBM*, *WRINKLED (WRI)* and *AINTEGUMENTA (ANT)*, and three contigs with high similarity to transcription factors of Class I *KNOX*, especially *STM*. These results demonstrate the morphogenetic responses of different types of explants subjected to induction medium, and the possible members of the AP2-like and Class I KNOX families cloned from the analyzed sequences, which are associated with the acquisition of competence for *in vitro* organogenesis in *Bixa orellana*.

## INTRODUCTION

*Bixa orellana* L. (Bixaceae) is a perennial shrub or small tree, native from Central and South America and widely cultivated in African and Asian countries (Venugopalan *et al.*, 2011). Also known as ‘achiote’, annatto or ‘urucum’, this species has a high economic value due the amount of carotenoid produced, especially, in the seeds (Lorenzi & Matos, 2002; Giuliano *et al.*, 2003).

Carotenoids are yellow, orange or reds pigments, synthesized by microorganisms and plants (Walter & Strack, 2011). In plants, carotenoids are stored in the plastids of flowers, fruits and some roots (Shumskaya &Wurtzel, 2013; Cárdenas-Conejo *et al.*, 2015). In addition to ecological functions, such as attraction of pollinators, carotenoids show antioxidant activity, which plays an important role to protect against free radicals, they are also source of vitamin A in animals, and abscisic acid in plants (Ram & Mathur, 1984; Howitt & Pogson, 2006; Shumskaya &Wurtzel, 2013; Cárdenas-Conejo *et al.*, 2015). In annatto stands out the bixin, a liposoluble compound that causes red coloration in seeds, and have been widely used as natural dye in foods industry, milk products, beverages, paint, cosmetic, pharmaceutical and textile (Giuliano *et al.*, 2003; Dias *et al.*, 2011; Marcolino *et al.*, 2011; Mala *et al.*, 2015, Cárdenas-Conejo *et al.*, 2015).

Due the large application of the annatto and in order to achieve high-producing carotenoid elite plants, *in vitro* propagation techniques have been proposed. One of the characteristics of annatto that allows *in vitro* studies is its high regenerative capacity. Several studies have proven the pluripotency of the cells, regardless of the explant used, such as nodal segments and shoot (D’Souza & Sharon 2001), hypocotyl segments (Paiva Neto *et al.* 2003b; Parimalan *et al.* 2007), inverted rooted hypocotyls (Paiva Neto *et al.* 2003/04a), cotyledonary nodes (Carvalho *et al.* 2005), cotyledons (Parimalan *et al.* 2007), root segments (Cruz *et al.* 2014), petiole segments (Mohammed *et al.* 2015), and from nodal and internodal adult stem segments (Cruz *et al.* 2015).

Pluripotency is the ability of a single cell to originate most, but not all, cell types that will constitute plant (Verdeil *et al.*, 2007), process known as *de novo* organogenesis. It comprises various stages: the specification of founder cell, acquisition of competency, cell determination, and the cell differentiation that will result in organ development (Cheng *et al.*, 2013; Motte *et al.*, 2014). However, the pluripotency capacity varies in different cells types, in addition to being influenced by growth regulators, environmental conditions, and a pattern of gene expression which regulates

the formation of new organs (Verdeil *et al.*, 2007; Atta *et al.*, 2009; Cheng *et al.*, 2013). *De novo* organogenesis, allows a control of the biotic and abiotic conditions, representing an efficient system for studying the events taking place during the establishment of new organs, especially those related to hormonal cross-talk and pattern of gene expression, essential for the success of the morphogenic events.

Since the discovery of the plant growth regulators, they are widely used in tissue culture supporting the promotion of regenerative induction events (Amoo & Staden, 2013). The balance between concentrations of cytokinins and auxins regulates the cell proliferation and differentiation (Skoog and Miller, 1957). The efficiency of shoot organogenesis in annatto is achieved by the use of the cytokinins Thidiazuron (TDZ) and Zeatin (ZEA) (Paiva Neto *et al.*, 2003b; Cruz *et al.*, 2014). However, the species exhibit inherent characteristic that influence the responses to different cytokinins (Aremu *et al.*, 2012). So, it is necessary to consider other growth regulators to establish more effective protocols. The meta-topolin, and its derivatives, are naturally occurring as aromatics cytokinins in several plants species (Jones *et al.*, 1996; Strnad *et al.*, 1997; Tarkowská *et al.*, 2003), and its use have been arising in plant culture tissue (Aremu *et al.*, 2012), due to satisfactory results in important parameters such as like multiplication rate, favoring acclimatization and rooting, in assition to relieving physiological disorder and lessening hyperhydricity (Magyar-Tábori *et al.*, 2001; Bairu *et al.*, 2007, Amoo *et al.*, 2011; Aremu *et al.*, 2012; Amoo & Staden, 2013). Despite the need of an adequate application of the cytokinins, regarding type and concentration, the knowledge of meta-topolin influences upon regenerative events is still poorly explored.

The auxins have synergistic, antagonistic and additive interactions with cytokinins, influencing the regulation of physiological responses, and inducing root formation when in high concentrations (Skoog & Miller, 1957; Coenen & Lomax, 1997; Amoo & Staden, 2013). One of the earliest detectable events in founder cells specification is the establishment of local auxin maximum and activation of responses in pericycle cells, controlled by the biosynthesis of local auxin in addition with the influx and efflux from specific transportations (Dubrovsky *et al.*, 2008; Löffke *et al.*, 2013; Ruiz Rosquete *et al.*, 2012; Sauer *et al.*, 2013). Thus, plant growth regulators acts coordinating cell differentiation leading to morphogenic events.

Besides hormonal influence, the expression of keys genes is essential by checking the identity and the maintenance of new organs (Verdeil *et al.*, 2007; Duclercq *et al.*, 2011; Cheng *et al.*, 2013; Motte *et al.*, 2014). Among several genes during the

neof ormation of shoot meristems there is the expression of gene *SHOOT MERISTEMLESS* (*STM*), a homebox gene belonging to the KNOX family involved in the startup and organization of the shoot apical meristem (SAM) (Scofield & Murray, 2006), more particularly preventing stem cells to differentiate before specification of the cell fates (Gallois *et al.*, 2002; Verdeil *et al.*, 2007; Scofield *et al.*, 2013). The gene *WUSCHEL* acts together to maintain the SAM organization (Scofield & Murray, 2006; Traas & Monéger, 2010).

Many different genes are expressed during the acquisition of embryogenic competency process from somatic cells, among them family members of transcription factors *BABY BOOM* (*BBM*) e *Somatic Embryogenesis Receptor-Like Kinases* (*SERK*), first characterized in competent embryogenics cell culture of *Brassica napus* and *Daucus carota*, respectively (Schmidt *et al.* 1997; Boutilier *et al.*, 2002; Kulinska-Lukaszek *et al.*, 2012). The *BBM* gene is a transcription factor that belongs to the AP2/ERF family, whose expression occurs in microspores, in basal region of zygotic embryos, root meristems and lateral roots of *Arabidopsis thaliana* (Boutilier *et al.*, 2002; Casson *et al.*, 2005; Galinha *et al.* 2007).

The genes of *SERK* family encode a transmembrane factor, LRR-RLK II, with leucine-rich repeat region, containing a highly conserved structural organization. Besides its expression during embryogenesis events, *SERK* has also been associated the organogenic events (Pilarska *et al.*, 2016; Rocha *et al.*, 2016), in which the expression occurs in pluripotent cells, with capacity to originate shoots or roots (Nolan *et al.*, 2009; Savona *et al.*, 2012).

Previous studies have characterized the expression of these genes associating them with the pattern formation of the new organs (Schmidt *et al.*, 1997; Gallois *et al.*, 2002; Hibara *et al.*, 2006; Rocha *et al.*, 2016). Furthermore, it is important to understand the influence of different growth regulators in the formation of organs and in the patten of gene expression.

Thus, this study aims to evaluate the morphoanatomical and molecular changes associated with the cell pluripotency acquisition in different organogenic systems in *in vitro* *B. orellana*, in order to establish a more efficient regeneration protocols, which can be applied to genetic transformation, enabling the study of the regeneration pathway, as well as the carotenoid biosynthetic pathway, broadening the knowledge about these events.

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## Chapter 1

### **Influence of the type of explant and growth regulators on plant cell pluripotency in *Bixa orellana* L. cultured *in vitro***

**Abstract-** Characterization of changes that occurs at cell levels during organogenesis is essential for understanding factors associated with cell pluripotency, which determinates acquisition of competency, cell differentiation and organ formation. The establishment of efficient *in vitro* regeneration protocols is important to study the gene expression patterns involved with morphogenesis, and its application in genetic transformation purposes to run further functional gene analyses. This study aimed to describe morphoanatomical alterations associated to the acquisition of organogenic competency in different explant types in *Bixa orellana* L. Hypocotyl segments, longitudinally wounded hypocotyl, rooted hypocotyl with reverse polarity and petiolate leaves were inoculated in JADS medium supplemented with either 4,56  $\mu\text{M}$  zeatin, 2,07  $\mu\text{M}$  meta-topolin, or 4,14  $\mu\text{M}$  meta-topolin. Explants at different development stages were collected and submitted to conventional techniques in light and scanning electron microscopy. Histochemical tests were also performed to investigate the reserve mobilization during organogenesis. After nine days of culture the first cell division in explants treated with zeatin were visible. The meristemoids formation was observed along the incision in wounded hypocotyl, however restricted at the tips in the others explants. At the end of evaluation period the ongoing cell de-differentiation led the formation of shoot structures and leaf primordia in all treatments. In the later organogenesis stages the cells showed a large amount of starch grains, polysaccharides and less protein that the starting explants. These results provide detailed information on anatomical changes involved at the acquisition of organogenic competency in *Bixa orellana*.

**Key words:** *Bixa*, Annatto, adventitious organogenesis, anatomical characterization, Bixaceae, shoot apical meristem.

## Introduction

*Bixa orellana*, a species native from Central and South America, is a tree widely cultivated especially due to the presence of the bixin and norbixin pigments, found in the seeds (Scotter *et al.*, 1998; Venugopalan *et al.*, 2011; Giuliano *et al.*, 2003). These carotenoids provide red color to the seeds, and have a wide application in food industry, due restrictions in the use of artificial dyes, but also as an alternative to consume natural products (Venugopalan *et al.*, 2011; Mohammed *et al.*, 2015). Furthermore, the bixin has been used in cosmetic and pharmaceutical industries, and more recent as an alternative in hypoglycemia treatment (Giuliano *et al.*, 2003; Dias *et al.*, 2011; Marcolino *et al.*, 2011; Venugopalan *et al.*, 2011; Roehrs *et al.*, 2014; Mala *et al.*, 2015).

The propagation of annatto is mostly made via seeds and vegetative cuttings. However due to its predominantly cross-pollinated reproduction the genetic variability is high, influencing the amount of seed per capsules and the quality of bixin and norbixin produced (Paiva Neto *et al.*, 2003b; Rivera-Madrid *et al.*, 2006). The *in vitro* propagation is a viable alternative to generate high carotenoid and seeds producing elite material. Thus, it is demanded to the establishment efficient regeneration protocols. Several studies have pointed the high regenerative capacity of *Bixa orellana* from various explant types (D'Souza & Sharon 2001; Paiva Neto *et al.*, 2003/04a; Paiva Neto *et al.*, 2003b;; Carvalho *et al.*, 2005; Parimalan *et al.*, 2007; Cruz *et al.*, 2014; Mohammed *et al.*, 2015; Cruz *et al.*, 2015; ; Matos *et al.*, 2016).

The type of explant and growth regulators together have great influence on *in vitro* morphogenesis. Some studies address the organogenesis from hypocotyl (Paiva Neto *et al.*, 2003/04; Paiva Neto *et al.*, 2003b; Parimalan *et al.*, 2007; Cruz *et al.*, 2015) and show that it represents an excellent system of *in vitro* regeneration. The wound may optimize this process, since when an incision occurs at the plant induces cell divisions that are able to restore physiological connections and form news organs (Asahina & Satoh, 2015). However, there is lack of information about the organogenesis from of leaves and petioles in *Bixa* (Mohammed *et al.*, 2015).

Plant hormones are also essential during the development of lateral organs. The cytokinins act inducing the formation of shoots, mainly by regulating the gene expression needed for the cell acquisition of competency (Duclercq *et al.*, 2011). Among these genes the *SHOOT MERISTEMLESS (STM)*, which activates the cytokinin synthesis by expression of IPT (isopentyl transferase) gene (Jasinski *et al.*, 2005; Yanai

*et al.*, 2005; Scofield *et al.*, 2013), and has its expression controlled by cytokinins. This network, jointly with *WUSCHEL* (*WUS*) and *CLAVATA1-2-3* (*CLV-1*; *CLV2*; *CLV3*) genes, regulate and maintain the initial stem cells population (Scofield & Murray; 2006; Traas & Monéger, 2010; Scofield *et al.*, 2013).

Some studies show that when added in culture medium the cytokinins zeatin (ZEA) and thidiazuron (TDZ), induce high organogenesis efficiency in *Bixa*, being the best result, for the shoots formation, obtained with the ZEA (Paiva Neto *et al.*, 2003b; Cruz *et al.*, 2014). Other cytokinin that have had an increasing application in tissue culture is meta-topolin (Aremu *et al.*, 2012). But its effect in organogenesis of *Bixa* is still unknown. Furthermore, still there is a gap the understanding of the morphoanatomical and chemical influence of this hormone.

The foundations for the regulation of the *in vitro* morphogenetic processes at the cellular, molecular and physiological levels have been increasing (Motte *et al.*, 2014; Altamura *et al.*, 2016; Matos *et al.*, 2016; Rocha *et al.*, 2016). Considering the high regenerative capacity, linked to proven cell pluripotency in *Bixa orellana* from various types of explants, and the responses to cytokinins this study aimed to evaluate the influence of these factors in the neoformation of shoots and to characterize the chemical and morphoanatomical alterations linked to organogenic acquisition of competency.

## **Materials and Methods**

### *Plant material and in vitro seeds germination*

Mature seeds of *Bixa orellana* “Piave Vermelha” provided by Chr. Hansen Brazil (Valinhos, São Paulo, Brazil), were mechanically scarified to remove the opposite side of the embryonic axis. Seeds were then immersed in 70% ethanol (v/v) for 1 min, transferred to a 2.5% (v/v) solution from a commercial sodium hypochlorite with 0.1% (v/v) Tween 20 for 20 min, followed by washing in distilled water and autoclaved at 120°C and 108 kPa for 20 min.

Subsequently, seeds were inoculated in 25x150 mm test tubes, containing 10 mL of MS medium (Murashige and Skoog, 1962) with 50% of the basal salts concentration, supplemented with B5 vitamins (Gamborg *et al.* 1968), sucrose (1.5% w/v), myo-inositol (0.005% w/v), and agar (0.6% w/v Merck KGAA, Darmstadt, Germany). The pH of the medium was adjusted to  $5.7 \pm 0.1$ , previous to autoclaving. The cultures were kept in the dark, at  $25 \pm 1^\circ\text{C}$  for 15 days. Following cultures were transferred to growth

room under 16-h photoperiod, under  $36 \mu\text{mol m}^{-2} \text{s}^{-1}$  irradiance from two fluorescent lamps (Osram Luz do Dia, São Paulo, Brazil), at  $25 \pm 1^\circ\text{C}$  for 15 days.

#### *Induction of de novo shoot organogenesis*

Hypocotyl segments (10-20 mm of length) were excised from 30 d-old seedlings. The hypocotyl segments were wounded longitudinally with a scalpel. Explants were inoculated horizontally with wounded region facing up in 300 mL glass jars containing 30 mL of JADS medium (Correia *et al.*, 1995), supplemented with B5 vitamins, sucrose (3% w/v), myo-inositol (0.01% w/v), agar (0.6% w/v) and  $4.56 \mu\text{M}$  zeatin, or  $2.07 \mu\text{M}$  of meta-topolin, or  $4.14 \mu\text{M}$  meta-topolin. pH of the medium was adjusted to  $5.7 \pm 0.1$ . Concomitantly, other amount of seedlings (50-60 mm of length) had the hypocotyls cut 1-2 mm below the cotyledon node and were inoculated vertically, with the reverse polarity, in jars glass of 500 mL with 50 mL of JADS medium as described above. Petiolate leaves were also excised and inoculated in jars glass of 300 mL containing 30 mL of JADS medium.

The experiment was conducted in factorial design 3x 3 (cytokinin x hypocotyls type) with 7 (replicates). All data were subjected to analysis of variance. When relevant, the averages were tested by Scott & Knott test at 5% probability. The jars glasses were kept in conditions similar of light and temperature cited in the previous item.

#### *Anatomical characterization*

After 0, 3, 6, 9, 15, 18, 21, 24, and 30 days of culture in induction medium explants were collected and fixed in Karnovsk solution (Karnovsky, 1965). Fixed samples were dehydrated in an ethanol series, included in methacrylate resin (Historesin, Leica<sup>®</sup>) and embedded in plastic histomolds. Five micrometer thickness cross and longitudinal sections were obtained using an automatic rotary microtome (RM 2155, Leica) and stained using toluidine blue (O'Brien & McCully 1981). The specimens were mounted in Permount on glass slides. Photographs were taken using a microscope stereoscope Olympus SZH and microscope (Olympus AX70), U-Photo system attached.

To observation of the micromorphological characters samples were fixed in Karnovsky solution, and dehydrated in ethanol series, then dried in critical point of  $\text{CO}_2$  (Robards, 1978) (CPD 020, Bal-Tec, Balzers, Liechtenstein), fixed on aluminum support, and coated with colloidal gold (Sputter Coater, model FDU 010, Bal-Tec,

Balzers, Liechtenstein). Photography were performed in scanning electron microscope (Zeiss, LEO 1430 VP, Cambridge, England).

### *Histochemical analyses*

The histochemical tests were realized in material fixed in Karnovsky solution, included in methacrylate resin (Historesin, Leica®), and embedded in plastic histomolds. Cross and longitudinal sections were obtained from an automatic rotatory microtome (RM 2155, Leica). In the analyses were used the following reagents: lugol solution (Johansen, 1940) for starch, Schiff reagent and periodic acid (PAS) (Maia, 1979) for neutral polysaccharides, Xylidine Ponceau (Vidal, 1970) for protein, and neutral red (Kirk, 1970) for total lipids. Examinations and photography were performed in microscope stereoscope Olympus SZH and microscope Olympus AX70, with U-Photo system. The analyses with fluorochromes (neutral red) and autofluorescence were realized in the same microscope, using epifluorescence system with UV filter (WU: 340-380 nm), dichroic mirror (400nm) and barrier filter (420nm).

## **Results**

The development of adventitious shoots occurred in all treatments tested (Figure 1). There was no significant effect of the cytokinins used, neither for the interaction cytokinins x explantes types. There was significant effect only for the explants types, where wounded hypocotyl and rooted hypocotyl inoculated with reverse polarity showed more shoots numbers than hypocotyl segments and petiolate leaves (Tabela 1).

After 30 days of culture in JADS medium longitudinally wounded hypocotyl and rooted reverse polarity hypocotyls showed greater number of shoots than hypocotyl, and petiolate leaves (Table 1).

After 18 days of culture wounded hypocotyl kept in JADS medium supplemented with ZEA form meristemoids from incision (Figure 2E), whereas the explants cultivated in JADS medium supplemented with 2.07  $\mu\text{M}$  of meta-topolin, or 4.14  $\mu\text{M}$  of meta-topolin formed, mostly healing responses (Figure 2F, G), characterized by the swelling of explant. The formation of meristemoids was obvious in wounded hypocotyls kept in all cytokinins tested after 30 days (Figure 2H-J). However, direct organogenesis was observed in explants cultivated with meta-topolin, independent of the concentration.

**Table 1** - Mean number of adventitious buds developing from cultured of different explants of *Bixa orellana* L., after 30 d in culture in induction medium. Means followed by the same letter are not statistically significant by Scott & Knott's test at 5% probably.

<b>Explant type</b>	<b>Total shoot number</b>
Wounded hypocotyl	3.2381 a
Rooted hypocotyl with reverse polarity	2.9048 a
Hypocotyl segments	1.1429 b
Petiolate leaves	1.1429 b

Hypocotyls with the reverse polarity show cell divisions and formed of meristemoids after nine days of culture (Figure 3B-D). After 18 days, the treatments with ZEA formed meristemoids vascularized (Figure 3E), while the treatments with meta-topolin induced cell divisions. After 30 days all cytokinins led to the formation of adventitious shoots (Figure. 3H-J).

After nine days of cultivation, hypocotyls in JADS medium supplemented with ZEA showed greater cell division (Figure 4C). After 30 days leaf primordia were formed in all cytokinins tested, limited to the explants tips, and accompanied to callus formation (Figure 4F-K).

Petiolate leaves also showed cell division after nine days of cultured in all cytokinins tested (Figure 5B-D). After 18 days a callus tissue was observed in the petiole tip (Fig. 5E-G), which was also noted 30 days of culture (Figure 5H-J).

The histochemical test indicated the presence of storage compounds in epidermal, mesophyll, and vascular region. Starch grains, was evidenced by the lugol, and confirmed by a positive reaction to PAS uniformly distributed in the mesophyll, vascular region cells and near the sites of regeneration in wounded hypocotyl (Figure 6), rooted hypocotyl with reverse polarity (Figure 7), hypocotyl segments (Figure 8), and petiolate leaves (Figure 9). A gradual increase in starch grains was observed during the adventitious shoot organogenesis, independent of cytokinin and explant type used, especially in petiolate leaves.

Protein bodies were not observed in wounded hypocotyl (Figure 6C, F, I, L, O, R, U). Positive reaction for Xylidine Ponceau was observed in rooted hypocotyl with

reverse polarity from 0 day (Figure 7C), 18 and 30 days of culture in medium with 4.56  $\mu\text{M}$  zeatin (Figure 7F, I). Protein bodies were also detected 18 days in explants cultured in medium with 2.07  $\mu\text{M}$  and 4.14  $\mu\text{M}$  meta-topolin (Figure 7L, R), however after 30 days no protein bodies was evidenced (Figure 7O, U). In hypocotyl segments, protein bodies were evidenced only at 30 days of culture with 4.14  $\mu\text{M}$  meta-topolin (Figure 8U). Petiolate leaves showed protein bodies at 0 (figure 9C), 18 and 30 days of cultured in medium with 4.56  $\mu\text{M}$  zeatin and 2.07  $\mu\text{M}$  meta-topolin (Figure F, I, L, O). But no protein bodies were evidenced in petiolate leaves cultured in medium with 4.14  $\mu\text{M}$  meta-topolin (figure 9R, U).

Neutral red test was realized for identify lipids bodies, however no lipid bodies was identified.

## Discussion

Plant cell pluripotency is essential for the success of organogenesis (Verdeil *et al.*, 2007), but it is clear that not all cell types have the similar regenerative capacity (Atta *et al.*, 2009). When hypocotyls were wounded longitudinally or had the polarity reversed plant cells active signaling pathways required for the protection that induce the division and cell differentiation, after of the development of adventitious shoots (Asahina *et al.* 2002, 2006, 2011, 2015).

Organogenesis in *Bixa orellana* is efficiently induced by the addition of growth regulators in culture medium (Paiva Neto *et al.*, 2003; Cruz *et al.*, 2014). Also, the explant type contributes to the formation of news organs. Wounded hypocotyls longitudinally and rooted hypocotyl with the reverse polarity showed more efficiency in shoots differentiation,

In wounded hypocotyls the development of the shoots started from of the incision. When an incision interrupts the vascular system a neoformed vascular tissue can be induced. Nevertheless, molecular and physiological events that control these process are still poorly understood (Reid & Ross 2011; Asahin & Satoh, 2015).

The wound activates hormone biosynthesis, such as the ethylene and jasmonic acid (Bostock & Stermer 1989; Leon *et al.*, 2001. Sasaki *et al.*, 2002; Asahina *et al.*, 2011). These hormones together with auxin and cytokinins are involved in process of xylem and phloem differentiation (Mattsson *et al.*, 1999; Sachs, 2000; Ye, 2002; Yin *et al.*, 2012). Furthermore, previous studies suggested that the regulation of genes

responsible for regenerative process is controlled by some transcriptional factors (Kubo *et al.*, 2005; Mitsuda *et al.*, 2005; Yamaguchi *et al.*, 2008; Sena *et al.*, 2009; Asahina *et al.*, 2011; Ikeuchi *et al.*, 2013 ). Nonetheless, there is still a lack of studies that clarify the action of hormones and genes in regenerative processes from wounded tissues, since the requirements for cell division and differentiation can vary among species, as demonstrated for *Cucumis sativus*, *Solanum lycopersicum* and *Arabidopsis thaliana* (Asahin & Satoh, 2015).

The reverse of polarity in rooted hypocotyls favored the formation of adventitious shoots, corroborating with the results found by Paiva Neto *et al.* (2003) and Parimalan *et al.* (2007). Differently of the data reported by these authors for explants cultured in TDZ supplemented medium, here both ZEA and meta-topolin induced direct organogenesis and shoots elongation. For the success of the organogenic process it is necessary a crosstalk between auxin and cytokinin, which determines the cell fate (Cheng *et al.*, 2013). The responses of explants to cytokinins are influenced by the spation-temporal auxin gradient, established by the local biosynthesis and its polar transport, thus disturbances in the distribution of auxin affects shoot development (Cheng *et al.*, 2013). Based on this, it seems that the reverse of polarity do not compromise the hormone action.

In addition to the observations made by Paiva Neto *et al.* (2003) and Parimalan *et al.* (2007), this work demonstrated that hypocotyls are a excellent source for organogenesis. However this process is restricted to the explant tips, which significantly lessens the number of shoots as compared to the wounded hypocotyls. Petiolate leaves showed similar responses to hypocotyls segments, being the organogenesis also restrict to the petiole tips, and accompanied by callusing, independent of the cytokinin used. Studies on adventitious organogenesis in *Bixa* from leaves or petioles are scarce, and additional studies are necessary to better comprehensionthe pluripotency of these cells.

Previous studies evidenced the role of ZEA in the development of adventitious shoots in *Bixa* (Paiva Neto *et al.*, 2003; Cruz *et al.*, 2014), although the influence of meta-topolin was still not evaluated. Here we demonstrate the efficiency of meta-topolin in organogenesis, inducing shoot formation and elongation, especially through direct organogenesis. Although all cytocynins induced shoots development similarly, it was observed that in meta-topolin-supplemented medium organogenesis was lower.

*WUS*, *CLV*, *STM* and *CUC* genes are responsible by the maintenance of initial stem cell population and SAM development. Some studies have shown the relationship

between expression of these genes and the plant hormones, in special cytokinin and auxin, which are responsible by the cell proliferation and differentiation, and organogenic induction (Verdeil *et al.*, 2007; Duclercq *et al.*, 2011; Motte *et al.*, 2014). *WUS* gene inhibits the transcription of Type-A ARABIDOPSIS RESPONSE REGULATORS (ARRs) which negatively regulates signaling of cytokinin, in parallel *STM* gene active the synthesis of this hormone (Jasinski *et al.*, 2005; Yanai *et al.*, 2005; Scofield *et al.*, 2013). Besides these, several other genes are expressed during the plant development, a lot of them, such as *BBM* and *SERK* genes are associated with embryogenesis (Schmidt *et al.* 1997; Boutilier *et al.*, 2002; Kulinska-Lukaszek *et al.*, 2012), but because of the complex genetic and hormonal interaction network it is possible that these genes are also involved with the shoots and roots development (Nolan *et al.*, 2009; Savona *et al.*, 2012; Pilarska *et al.*, 2016; Rocha *et al.*, 2016; ), and are essential for meristem establishment. The activation of gene expression can occur via exogenous hormonal perception, but the responses are inherent the different cell types, which influences gene expression in a temporal pattern. In *Bixa* the initial responses to ZEA occurred earlier when compared the meta-topolin, and an investigation of the gene expression pattern will contribute to knowledge regarding these interactions.

The storage compounds evidenced by the histochemical tests are essential during the morphogenic events (Tozzi & Takaki, 2011; Branca *et al.*, 1994; Cangahuala-Inocente *et al.*, 2004). The great amount of starch grains observed during the organogenesis, in all explants, can be justified by the synthesis of these compounds to keep a high cell activity, once the starch is considered the first source of energy for cell elongation and proliferation (Stamp, 1987; Martin *et al.*, 2000; Pinto *et al.*, 2010). Starch grains also were observed substituting the reserve of lipids in the initial stages of the embryogenic process in *Passiflora cincinnata* (Rocha *et al.*, 2012), and during the somatic embryos formation in different species (Verdeil *et al.*, 2001; Queiroz-Figueroa *et al.*, 2002; Moura *et al.*, 2008; Pinto *et al.*, 2010). But, the presence of sucrose in culture medium also can influence starch formation during *in vitro* morphogenesis (Pinto *et al.*, 2010; Buckeridge *et al.*, 2004).

Cell expansion also is influenced by protein and lipids bodies (Jiménez, 2001), together these compounds are important for cell membranes composition, cellular reorganization and differentiation (Zienkiewicz *et al.*, 2011; Kraus *et al.*, 2012), however the small amount of the stored compounds did not coincide with the structural

alterations. This can be justified by the consumption of these compounds in the start of the formation of the meristemoids, when the cells acquired meristematic characteristics, as observed during the embryogenic process in *P. cincinnata* (Rocha *et al.*, 2012).

That work showed that zeatin and meta-topolin are efficient for morphogenesis *in vitro* in *Bixa orellana*. In addition the explant type influences in organ neoformation, being that longitudinally wound hypocotyls and rooted hypocotyls inoculated with reverse polarity are more prone to generate adventitious shoots formation. The maintenance of starch grains during the organogenesis is high, and lasts throughout the entire period of the acquisition of competency, differentiation and organ neoformation, replacing other compounds such as protein and lipids during the morphogenic events. These results provide important information for application in tissue culture *in vitro*. Studies about the pattern gene expression will give more concrete results about the hormonal action, besides this, will contribute with information about the gene expression associated with the SAM establishment and shoots development, contributing for the establishment of more efficient protocols in *Bixa*, allowing application genetic transformation.

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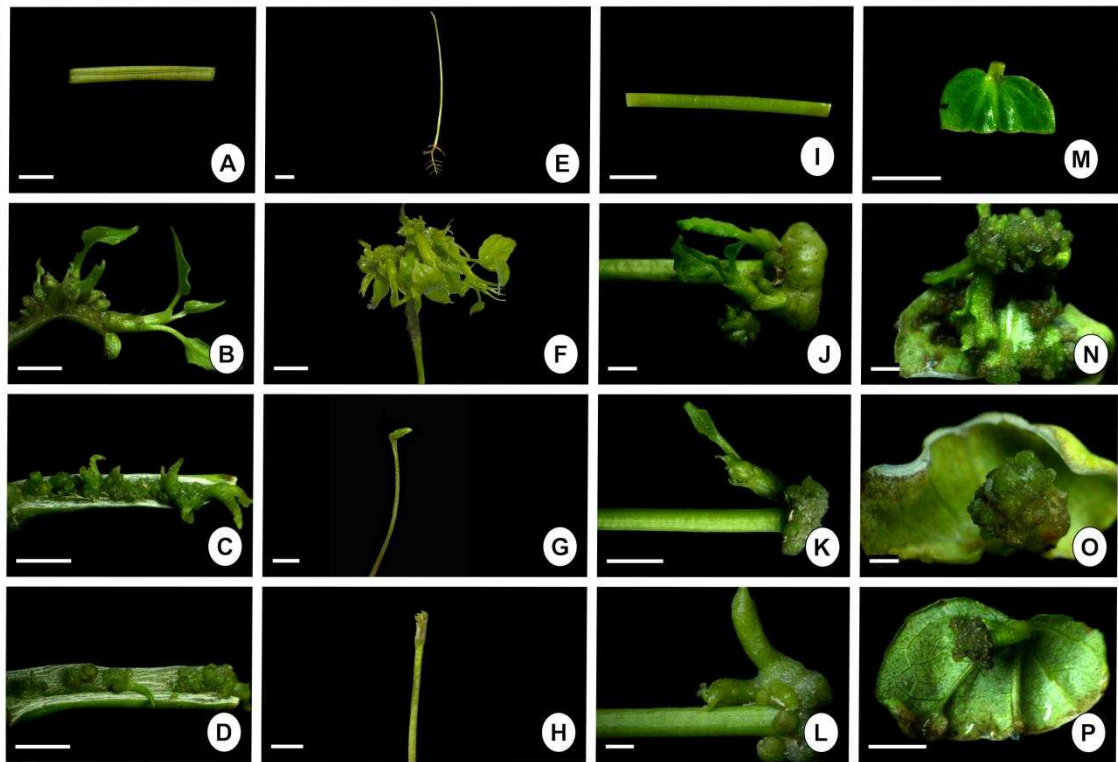
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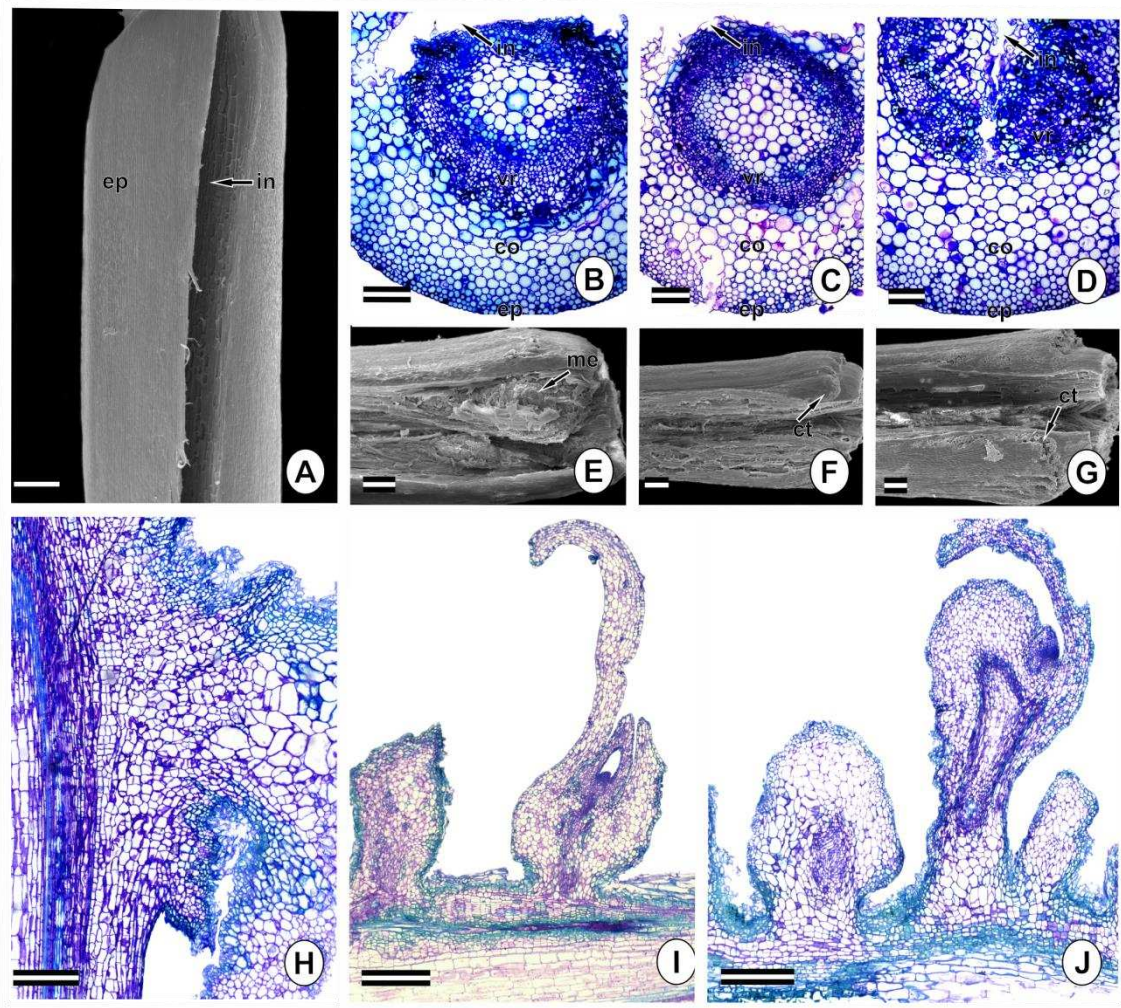
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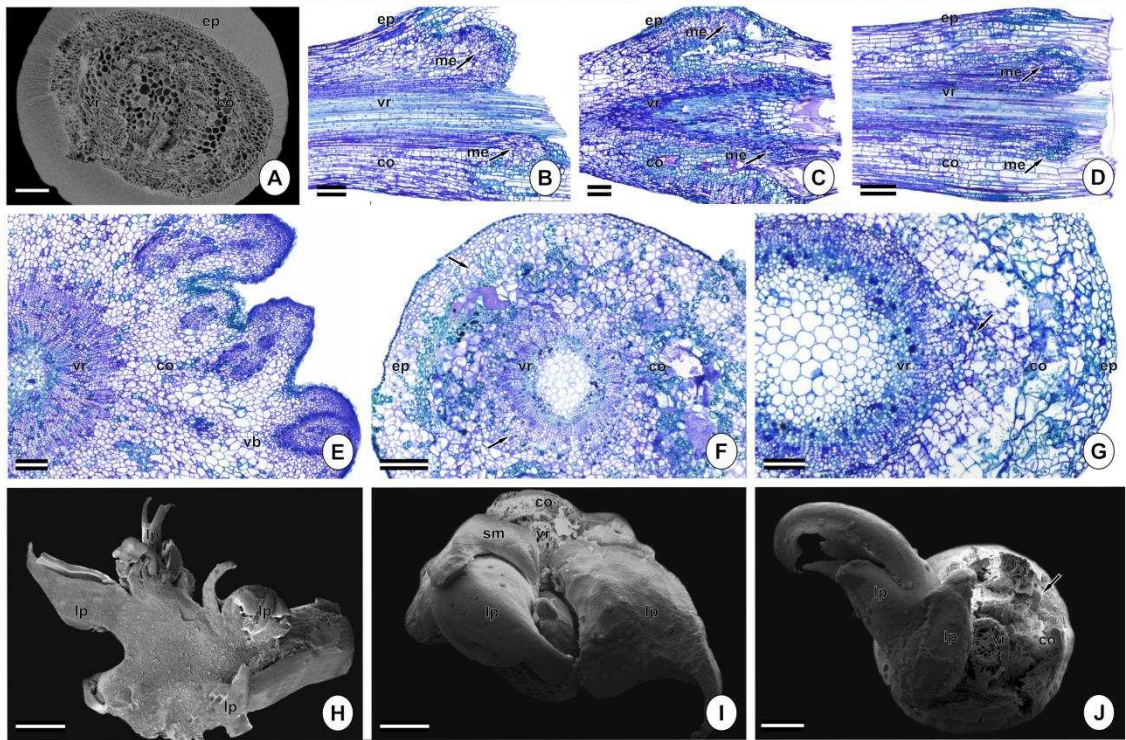
## Figures



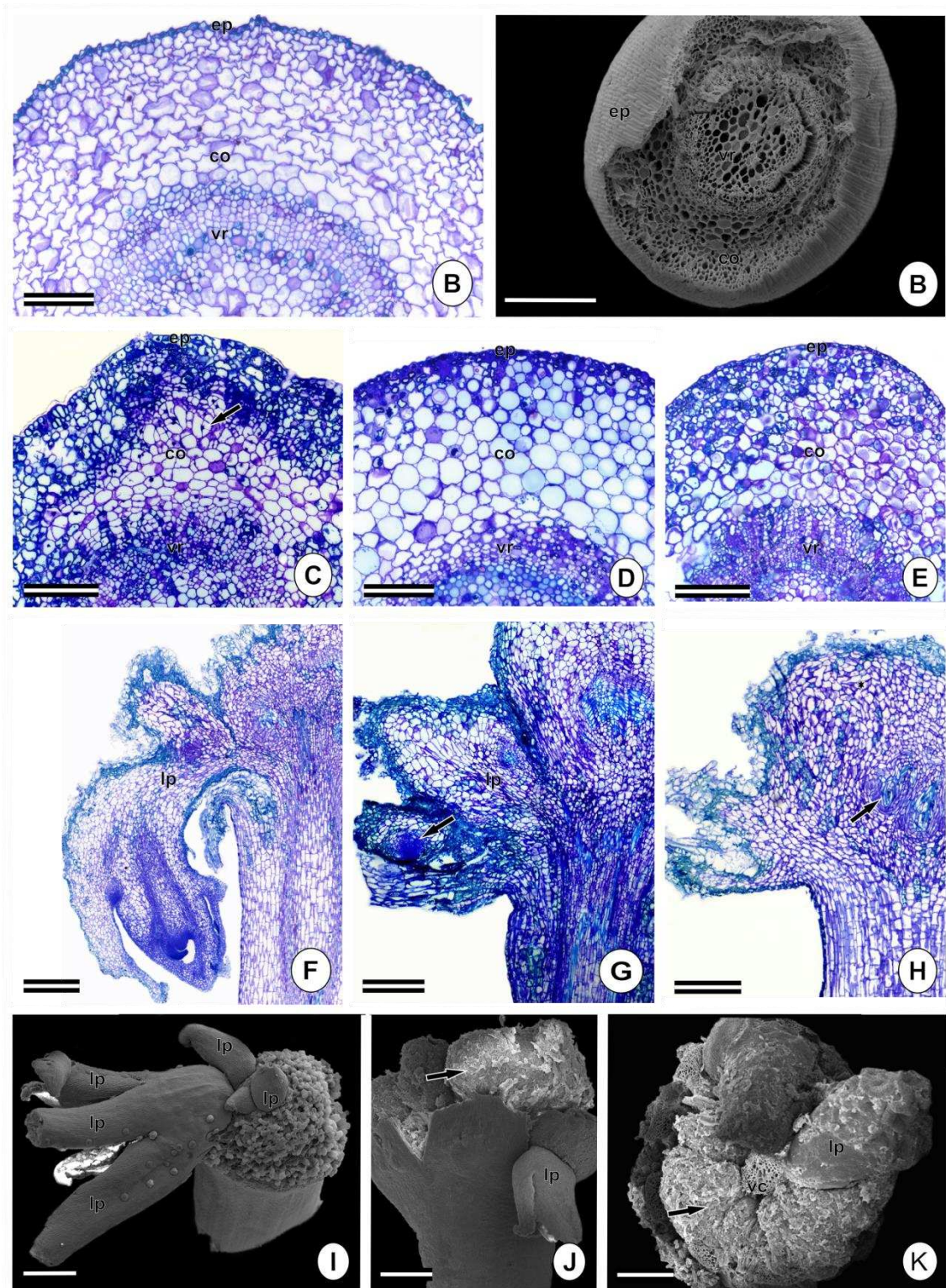
**Figure 1** - *In vitro* adventitious organogenesis of *Bixa orellana* L. from of different types of explants. Wounded hypocotyl (A-D); rooted hypocotyl with reverse polarity (E-H); hypocotyl segments (I-L); and petiolate leaves (M-P). Morphology to 0 days (A, E, I, M) and responses obtained after 30 days of culture on medium JADS supplemented with 4.56  $\mu\text{M}$  of zeatin (B, F, J, N), 2.07  $\mu\text{M}$  of meta-topolin (C, G, K, O), 4.14  $\mu\text{M}$  of meta-topolin (D, H, L, P). Bars: A- D, I, K, M, P = 25  $\mu\text{m}$ ; E = 100  $\mu\text{m}$ ; F-H = 50  $\mu\text{m}$ ; J, L, N, O = 10  $\mu\text{m}$ .



**Figure 2:** *De novo* shoot organogenesis from wounded hypocotyl explants of *Bixa orellana* L. cultured in JADS medium with cytokinin types: 4.56  $\mu\text{M}$  of zeatin (B, E, H), 2.07  $\mu\text{M}$  of meta-topolin (C, F, I), 4.14  $\mu\text{M}$  of meta-topolin (D, G, J). Scanning electron microscopy (SEM) (A, E, F, G) and light microscopy (B, C, D, H, I, J). **A:** Explants at day zero evidencing the incision. **B-D:** Cross sections showing the incision (arrow) in hypocotyl explants after 9 days of culture; note that there were no anatomical changes. **E-G:** SEM photomicrographies evidencing the wounding site in hypocotyl explants after 18 days of culture. Note the beginning of meristemoids formation at 4.56  $\mu\text{M}$  of zeatin (E), and the swelling reaction of explants cultured in medium supplemented with 2.07 (F) or 4.14  $\mu\text{M}$  of meta-topolin (G). **H-J:** Longitudinal sections evidencing the direct neoformation of shoot structures and leaf primordia at 4.56  $\mu\text{M}$  of zeatin (H), 2.07 (I) and 4.14  $\mu\text{M}$  of meta-topolin (J). Abbreviations: ep, epidermis; in, incision; co, cortex; vr, vascular region, me, meristemoid; ct, cicatrization tissue. Bars = A, E = 200 $\mu\text{m}$ ; B-D = 100  $\mu\text{m}$ ; F-J = 300 $\mu\text{m}$ .

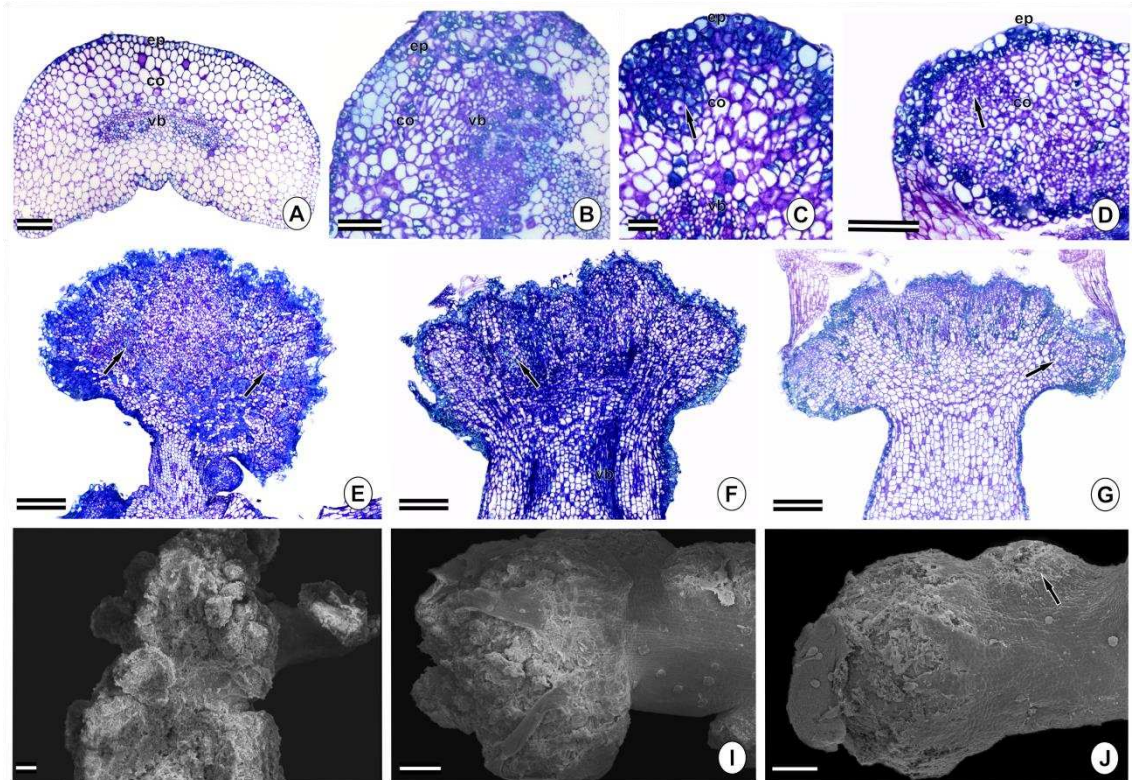


**Figure 3:** *De novo* shoot organogenesis from rooted hypocotyl explants of *Bixa orellana* L. cultured in JADS medium with cytokinin types: 4.56  $\mu\text{M}$  of zeatin (B, E, H), 2.07  $\mu\text{M}$  of meta-topolin (C, F, I), 4.14  $\mu\text{M}$  of meta-topolin (D, G, J). Scanning electron microscopy (SEM) (A, H, I, J) and light microscopy (B, C, D, E, F, G). **A:** Explants at day zero. **B-D:** Longitudinally sections showing the meristemoids formation (arrow) in rooted hypocotyl explants after 9 days of culture. **E-G:** Cross sections of explants after 18 days of culture. Note the shoots-buds formation at at 4.56  $\mu\text{M}$  of zeatin (E), and cells division in explants cultured in medium supplemented with 2.07 (F) or 4.14  $\mu\text{M}$  of meta-topolin (G). **H-J:** SEM photomicrographies evidencing the shoot formation in rooted hypocotyl after 30 days of culture cultured at 4.56  $\mu\text{M}$  of zeatin (H), 2.07 (I) or 4.14  $\mu\text{M}$  of meta-topolin (J). Abbreviations: ep, epidermis; co, cortex; vr, vascular region; me, meristemoid; lp, leaf primordial, sm, shoot meristem.. Bars = A, G = 100  $\mu\text{m}$ ; B, C, E, I, J = 300  $\mu\text{m}$ ; D, F = 200  $\mu\text{m}$ , H = 1000  $\mu\text{m}$ .



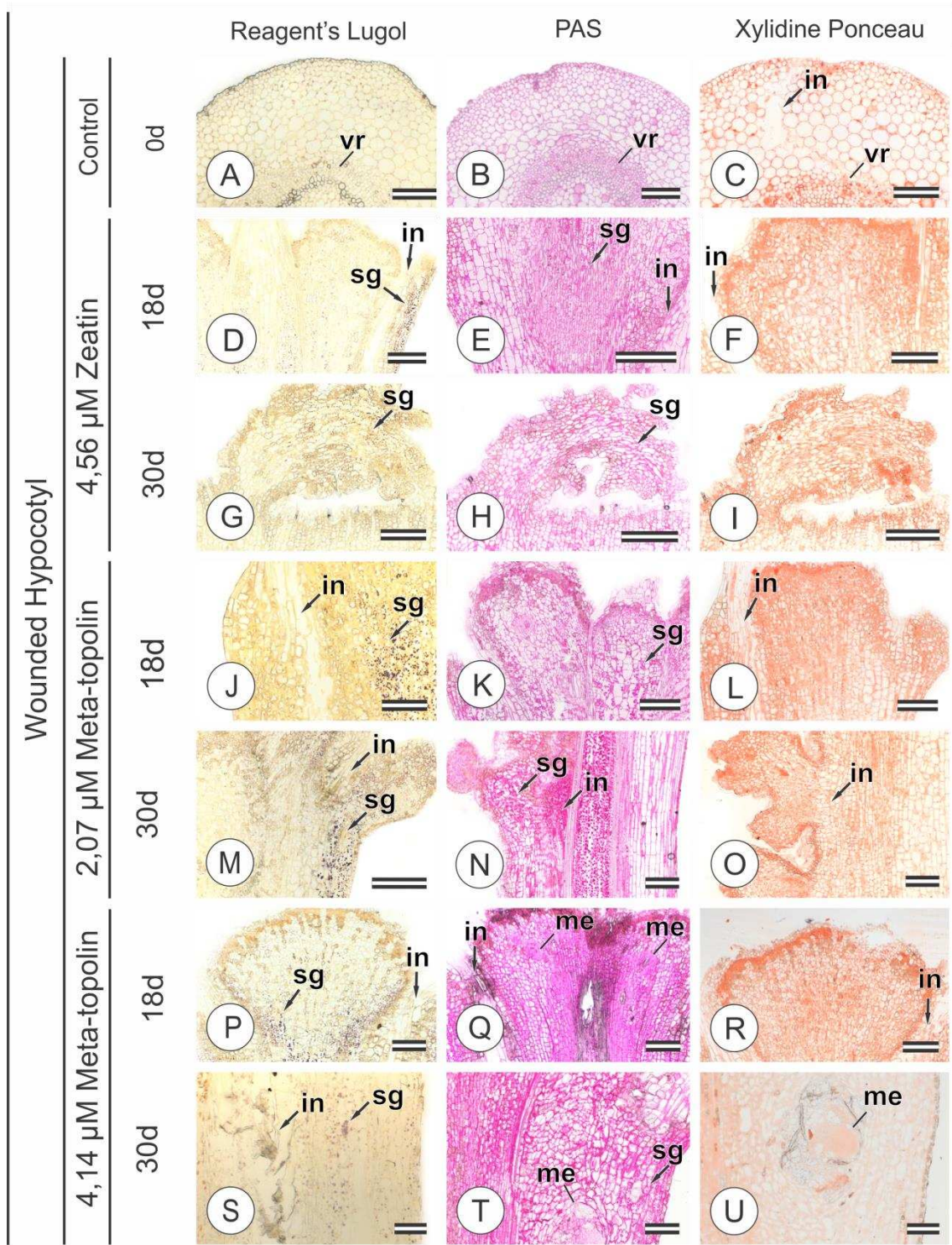
**Figure 4:** *De novo* shoot organogenesis from hypocotyl segments cultured in JADS medium supplemented with cytokinins types: 4.56  $\mu\text{M}$  of zeatin (C, F, I), 2.07  $\mu\text{M}$  of meta-topolin (D, G, J), and 4.14  $\mu\text{M}$  of meta-topolin (E, H, K). Details from scanning electron microscopy (B, I-K) and light microscopy (A, C-H). **A-B:** Explants at 0 day. **C-E:** Cross sections of explants after 9 days of culture showing cell division and

meristemoids formation (arrow) in treatments with zeatin. **F-K:** Explants after 30 days of culture, F-G longitudinal sections showing the SAM establishment and shoots-buds formation (arrow) I-K SEM photomicrographies demonstrate the SAM establishment in all treatments accompanied by callus formation (arrow). Abbreviations: ep, epidermis; co, cortex, vr, vascular region, lp, leaf primordial. Bars = A, D = 100  $\mu\text{m}$ ; B, C, E = 200  $\mu\text{m}$ ; F-I = 300  $\mu\text{m}$ ; J, K= 400  $\mu\text{m}$ .



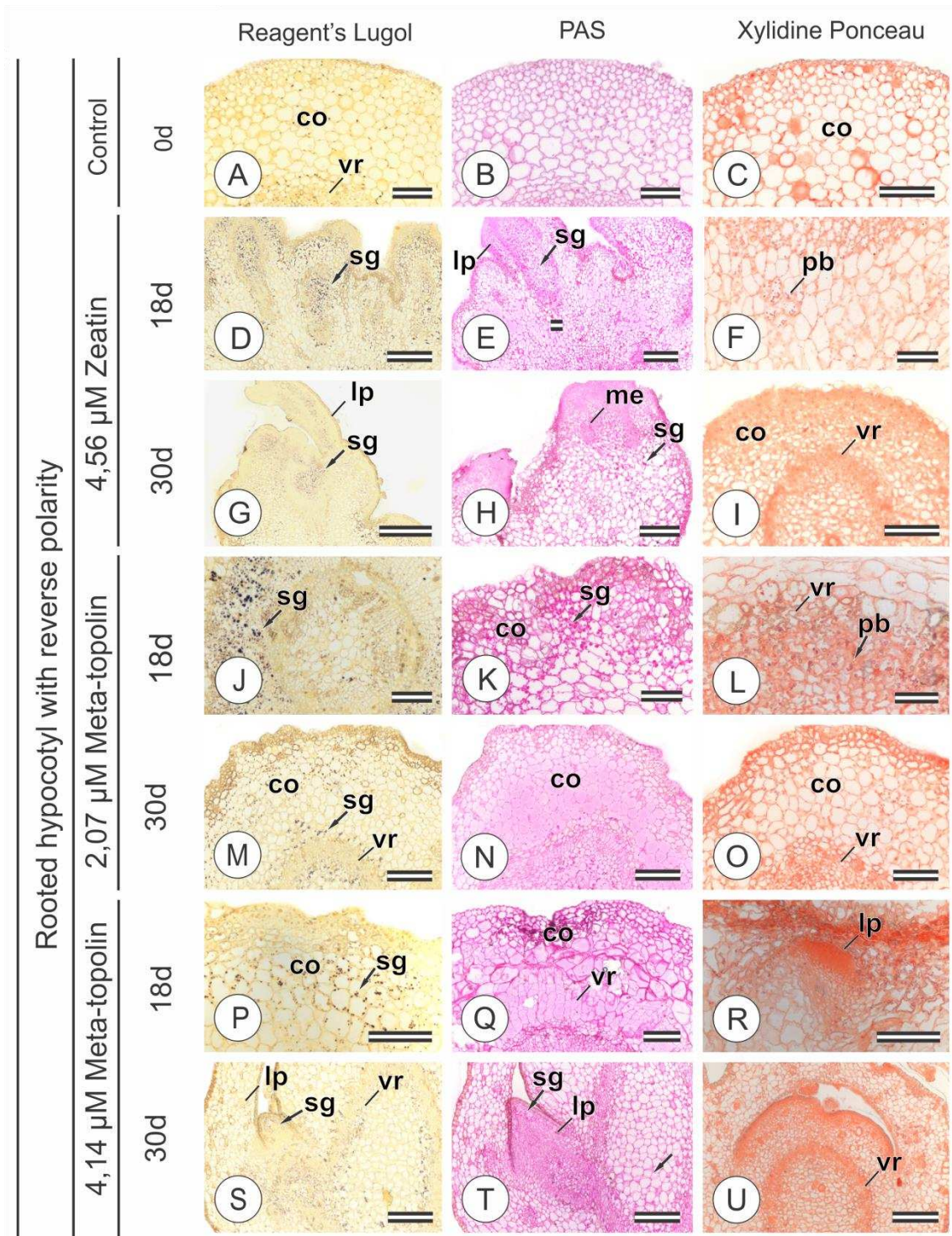
**Figure 5:** *De novo* shoot organogenesis from petiolate leaves explants of *Bixa orellana* L. cultured in JADS medium with cytokinin types: 4.56  $\mu\text{M}$  of zeatin (B, E, H), 2.07  $\mu\text{M}$  of meta-topolin (C, F, I), 4.14  $\mu\text{M}$  of meta-topolin (D, G, J). Light microscopy (A-G) and scanning electron microscopy (SEM) (H-J). **A:** Explants at day zero. **B-D:** Cross sections showing cells divisions (arrow) in petiolates leaves explants after 9 days of culture; note that there is meristemoid formation at 2.07  $\mu\text{M}$  of meta-topolin (C) and 4.14  $\mu\text{M}$  of meta-topolin (D). **E-G:** Longitudinally sections showed the meristemoid development (arrow) after 18 days of culture at 4.56  $\mu\text{M}$  of zeatin (E), and 2.07 (F) or 4.14  $\mu\text{M}$  of meta-topolin (G). **H-J:** SEM photomicrographies evidencing the callusing at 4.56  $\mu\text{M}$  of zeatin (H), 2.07  $\mu\text{M}$  of meta-topolin (I) and 4.14  $\mu\text{M}$  of meta-topolin (J).

Abbreviations: ep, epidermis; co, cortex, vb, vascular bundle. Bars = A, B = 100  $\mu\text{m}$ ; C = 50  $\mu\text{m}$ ; D, H-J = 200  $\mu\text{m}$ ; E-G = 300  $\mu\text{m}$



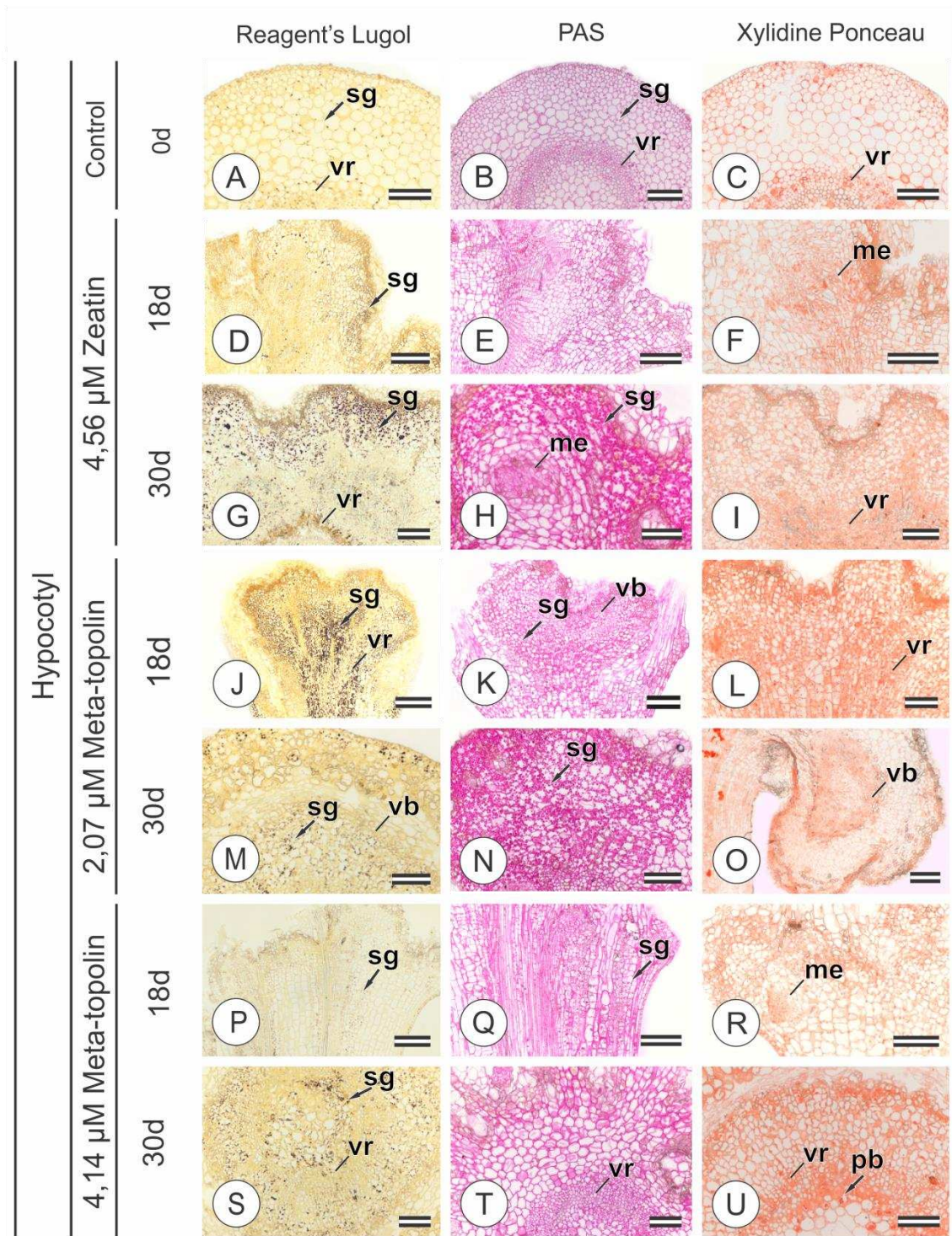
**Figure 6:** Histochemical analysis during the organogenesis from of hypocotyl wound in *Bixa orellana* with 0 (A-C), 18 and 30 days of cultivation medium with 4,56  $\mu\text{M}$  zeatin (D-I), 2,07  $\mu\text{M}$  meta-topolin (J-O) and 4,14  $\mu\text{M}$  meta-topolin (P-U). Histological

sections subjected to reagent's lugol (A, D, G, J, M, P, S), periodic acid-Schiff (PAS: B, E, H, K, N, Q, T), and Xylidine Ponceau (C, F, I, L, O, R, U). The positive reaction to lugol and PAS evidenced a high density of starch grains uniformly distribute in explant. No evidence of protein bodies. Abbreviations: vr vascular region; in, incision; sg, starch grains. Bars =A-C, R, S = 100  $\mu\text{m}$ ; F, J = 150  $\mu\text{m}$ ; K, L, N-Q, T, U = 200  $\mu\text{m}$ ; D, E, G-I, M = 300.



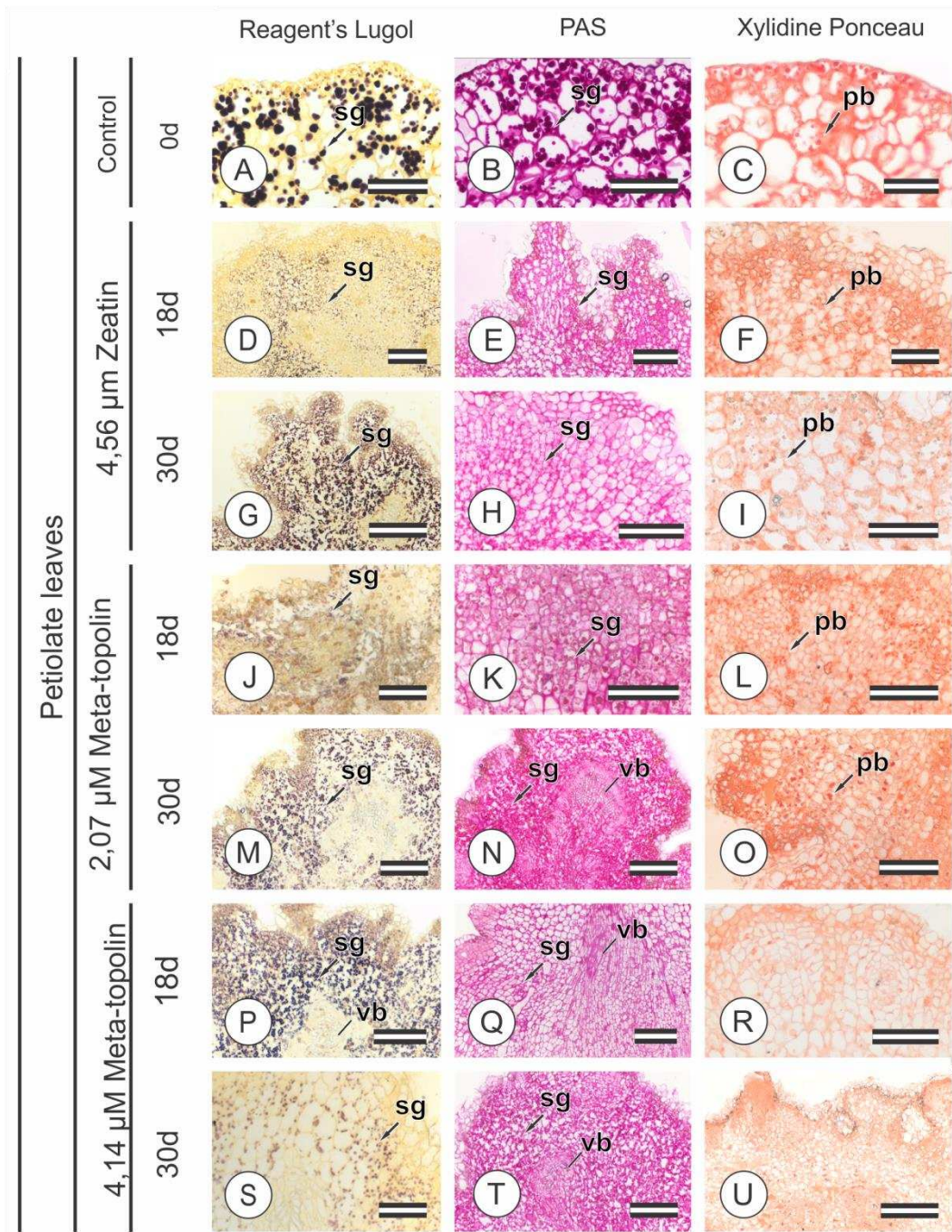
**Figure 7:** Histochemical analysis during the organogenesis from of rooted hypocotyl with reverse polarity in *Bixa orellana* with 0 (A-C), 18 and 30 days of cultivation medium with 4.56  $\mu\text{M}$  zeatin (D-I), 2.07  $\mu\text{M}$  meta-topolin (J-O) and 4.14  $\mu\text{M}$  meta-topolin (P-U). Histological sections subjected to reagent's lugol (A, D, G, J, M, P, S), periodic acid-Schiff (PAS: B, E, H, K, N, Q, T), and Xylidine Ponceau (C, F, I, L, O, R, U). A-C: The initial stages showed few reserve compounds. The positive reaction to

lugol and PAS evidenced starch grains in the explant to the 18 and 30 days in all cytokinins. To the 18 days protein bodies were evidenced by the positive reaction of Xylidine Ponceau in all treatments (F, L, R), after 30 days no evidenced protein bodies (I, O, U). Abbreviations: co, cortex; vr, vascular region; lp, leaf primordia; me, meristemoid; sg, starch grains; pb, protein bodies. Bars = L = 50  $\mu\text{m}$ ; A-C, F, I-K, P-R = 100  $\mu\text{m}$ ; M-O, S-U = 150  $\mu\text{m}$ ; H= 200  $\mu\text{m}$ ; D, E, G = 300  $\mu\text{m}$ .



**Figure 8:** Histochemical analysis during the organogenesis from of hypocotyl in *Bixa orellana* with 0 (A-C), 18 and 30 days of cultivation medium with 4,56  $\mu\text{M}$  zeatin (D-I), 2,07  $\mu\text{M}$  meta-topolin (J-O) and 4,14  $\mu\text{M}$  meta-topolin (P-U). Histological sections subjected to reagent's lugol (A, D, G, J, M, P, S), periodic acid-Schiff (PAS: B, E, H, K, N, Q, T), and Xylidine Ponceau (C, F, I, L, O, R, U). The positive reaction to lugol evidenced starch grains uniformly distribute in all treatments (A, D, G, J, M, P, S), especially near the sites of adventitious shoot formation. The negative reaction of PAS

was observed to 18 days in the treatments with 4,56  $\mu\text{M}$  zeatin (E), and the 30 days in treatments with 4,14  $\mu\text{M}$  meta-topolin (T). Protein bodies was evidenced only the 30 days in treatment with 4.14  $\mu\text{M}$  meta-topolin (U). Abbreviations: vr, vascular region; vb, vascular bundle; me, meristemoids; sg, starch grains; pb, protein bodies. Bars = A-C, H, J- M, S-U = 100  $\mu\text{m}$ ; F, R = 150  $\mu\text{m}$ ; I, N = 200  $\mu\text{m}$ ; D, E, O-Q = 300  $\mu\text{m}$ ; G= 400  $\mu\text{m}$ .



**Figure 9:** Histochemical analysis during the organogenesis from of petiolate leaves in *Bixa orellana* at 0 (A-C), 18 and 30 days of cultivation in medium with 4.56  $\mu$ M zeatin (D-I), 2.07  $\mu$ M meta-topolin (J-O) and 4.14  $\mu$ M meta-topolin (P-U). Histological sections subjected to reagent's lugol (A, D, G, J, M, P, S), periodic acid-Schiff (PAS: B, E, H, K, N, Q, T), and Xylidine Ponceau (C, F, I, L, O, R, U). The positive reaction to lugol and PAS evidenced by a high amount starch grains in all treatments in the different times evaluated. High amount of starch grains was observed in callus tissue. Protein bodies were evidenced in the treatment with 4.56  $\mu$ M zeatin (C, F) and 4.14  $\mu$ M

meta-topolin (R,U). L, O: negative reaction for protein bodies. Abbreviations: vb, vascular bundle; sg, starch grains; pb, protein bodies. Bars = B = 50  $\mu\text{m}$ ; C- F, H, L, M, N, R, U = 100  $\mu\text{m}$ ; A, G, I, O, T = 150  $\mu\text{m}$ ; J, K, P, Q = 200  $\mu\text{m}$ ; S= 300  $\mu\text{m}$ .

## Chapter 2

### Lateral root development *in vitro* in *Bixa orellana* L.: morphoanatomical and histochemical characterization

**Abstract-** The tissue culture *in vitro* is an alternative for faster propagation of elite clones. A requirement for of *in vitro* culture efficiency is the rooting of propagated plants. The induction of *in vitro* rizogenesis allows the study of changes and molecular markers associated with cell acquisition of competency. This study aimed to characterize morphoanatomical and chemical changes during lateral root development in *Bixa orellana*. Seedlings were inoculated in JADS medium with 0 or 2,46  $\mu\text{M}$  of IBA. Explants in different development stages were processed for light microscopy and scanning electron microscopy. Histochemical tests were realized for the detection of reserve compounds. After four days in induction medium the first cell divisions was observed. Lateral root primordium was observed with six days in induction medium supplemented with IBA, and with 10 days in the control. After 20 days in induction medium supplemented with IBA, the lateral root development with vascularization and root cap formation were observed. The histochemical tests evidenced the reserve compounds: protein, lipids, starch grains, and neutral polysaccharides. Protein were consumed during organogenesis, while lipids, starch grains and polysaccharides gradually increased. This data constitutes important information about the cytological changes that occurs during lateral root formation in *Bixa orellana*, and support future studies about the molecular events associated to organogenesis.

**Key-words:** *Bixa*, lateral root, root primordia, rizogenesis, anatomical characterization, histochemical characterization.

## Introduction

Currently, there is an increased demand for natural products that can replace the industrialized in the human diet. This demand also includes the dyes, which are widely used in food industry and in food preparation. One of the main dye extracted from plants used in human and animal feeding comes from annatto (*Bixa orellana* L.) (Venugopalan *et al.*, 2011). The production of this dye, consumed in the whole world, is possible due to carotenoids bixin e norbixin that confers coloration, especially in the seeds (Scotter *et al.*, 1998). Besides these properties the dye is also common in others industry lines such as t xtil, pharmaceutical and cosmetics (Giuliano *et al.*, 2003; Selvi *et al.*, 2013; Roehrs *et al.*, 2014).

Due to the dye consumption the demand for plants that produce high quality fruits, with more seeds per capsule and greater amount of carotenoids, is raising. *Bixa* is a gender represented by perennial shrubs or small trees propagated via seeds, which generates a high variation in the fruit characteristics (Falesi, 1987; Paiva Neto *et al.*, 2003; Rivera-Madrid *et al.*, 2006). The *in vitro* culture allows the production of selected seedlings, with commercially interesting features. For achieve success *in vitro* is necessary the addition of plant hormone, like auxin and cytokinin, which induce the cell division and proliferation (Skoog & Miller, 1957). Other requirement for the efficiency of *in vitro* morphogenesis is the rooting. After the root formation, plants can be acclimatized (Dabski & Parzymies, 2007).

The root formation is achieved by adding auxin in the culture medium, this hormone is an important short and long distance indicator and is responsible by cell division and elongation (Ding & Friml, 2010; Wolf *et al.*, 2012). Moreover, the auxin also can be responsible for the maintenance of initial cell population in quiescent center (Blilou *et al.*, 2005; Ding & Friml, 2010), to induce responses such as tropism (Sanchez-Rodriguez *et al.*, 2010; Depuydt & Hardtke, 2011), and activate the synthesis of ethylene, which contributes to the lateral root development (Pan *et al.*, 2002, Paiva Neto *et al.*, 2003). In addition, the temporal and spatial pattern of gene expression, act together with the hormones in order to define the root architecture (Ditengou *et al.*, 2008).

Among the genes that act during the root apical meristem (RAM) establishment are the *WUSCHEL- related homeobox 5 (WOX5)* and the *PLETHORA (PLT)* gene, that are the main regulators to the initial cell population maintenance and ensure the specification of the quiescent center (Ainda *et al.*, 2004; Vernoux & Benfey, 2005;

Galinha *et al.*, 2007; Verdeil *et al.*, 2007; Motte *et al.*, 2014; Heidstra & Sabatini, 2014). But, the interaction network that determines new organ formation is complex, and so, others genes can play elementary functions during this process.

Recent studies suggest that the *Somatic Embryogenesis Receptor-Like Kinases (SERK)* gene, expressed in cells with embryogenic competency is a possible candidate to act also during organogenic events (Rocha *et al.*, 2016; Pilarska *et al.*, 2016). Since the expression also occurs in pluripotent cells, with capacity for originate shoot or root (Nolan *et al.*, 2009; Savona *et al.*, 2012). In addition to that *BABY BOOM* gene is also, associated with the embryogenic process, can likewise be important marker to the process. Similarly, the *PLT* gene which encodes a putative transcription factor type AP2, and such expression was verified in RAM and lateral root of *Arabidopsis thaliana* (Boutillier *et al.*, 2002; Casson *et al.*, 2005; Galinha *et al.*, 2007).

Thus, the hormonal and genetic interaction network contributes with the process of lateral root development, however there is still a gap in the knowledge about these events. So there is a need of studies that consider the spatio-temporal expression patterns of these and other genes that are not clear yet. To tackle it, there is a need to understand the morphoanatomic aspects associated with the acquisition of competency and the formation of lateral roots. So, the aim of this work was to characterize the morphoanatomic and chemical alterations during the development of lateral roots in *Bixa orellana* L. cultured *in vitro*.

## **Materials and Methods**

### *Plant material and in vitro seed germination*

Mature seeds of *Bixa orellana* provided by the company Chr. Hansen Brazil (Valinhos, São Paulo, Brazil), were mechanically scarified to remove the opposite side of the embryonic axis. Seeds were then immersed in 70% ethanol (v/v) for 1 min, transferred to a 2.5% (v/v) solution from a commercial sodium hypochlorite with 0.1% (v/v) Tween 20 for 20 min, followed by washing in distilled water and autoclaved at 120°C and 108 kPa for 20 min.

Subsequently, seeds were inoculated in 25x150 mm test tubes, containing 10 mL of MS medium (Murashige e Skoog, 1962) with 50% of the basal salts concentration, supplemented with B5 vitamins (Gamborg *et al.* 1968), sucrose (1.5% w/v), myo-inositol (0.005% w/v), and agar (0.6% w/v Merck KGAA, Darmstadt, Germany). pH of the medium was adjusted to  $5.7 \pm 0.1$ . The seeds were kept in the dark for 15 days. And

the seedlings transferred to growth room under 16-h photoperiod, under  $36 \mu\text{mol m}^{-2} \text{s}^{-1}$  irradiance from two fluorescent lamps (Osram Luz do Dia, São Paulo, Brazil), at  $25 \pm 1$  for 15 days.

#### *Induction of lateral root formation in vitro*

Seedling from 30d-old had the shoot tip excised and were inoculated in 300 mL glass jars containing 30 mL of JADS medium (Correia, 1997), supplemented with B5 vitamins, sucrose (3% w/v), myo-inositol (0.01% w/v), agar (0.6% w/v ) and  $4.56 \mu\text{M}$  zeatin. pH of the medium was adjusted to  $5.7 \pm 0.1$ . The jar glasses were kept in similar conditions of light and temperature cited in the previous item, where remained for 60 days. After, the seedling were transferred for 300 mL glass jars containing 30 mL of medium JADS, in similar conditions cited above, but containing 0 or  $2.46 \mu\text{M}$  IBA.

The experiment was conducted in a completely randomized design, with four jars containing four explants for each treatment.

#### *Anatomical characterization*

After 0, 2, 4, 6, 8, 10, 18, and 20 days of culture in induction medium explants were collected and fixed in Karnovsk solution (Karnovsky, 1965). Fixed samples were dehydrated in an ethanol series, included in methacrylate resin (Historesin, Leica®) and embedded in plastic histomolds.  $5 \mu\text{m}$  of thickness cross and longitudinal sections were obtained using an automatic rotary microtome (RM 2155, Leica) and stained using toluidine blue (O'Brien & McCully 1981). The specimens were mounted in Permount on glass slides. Photographs were taken using a microscope stereoscope Olympus SZH and microscope (Olympus AX70), U-Photo system attached.

To observation of the micromorphological characters samples were fixed in Karnovsky solution and dehydrated in ethanol series, then dried in critical point of  $\text{CO}_2$  (Robards, 1978) (CPD 020, Bal-Tec, Balzers, Liechtenstein), fixed on aluminum support, and coated with colloidal gold (Sputter Coater, model FDU 010, Bal-Tec, Balzers, Liechtenstein). Photographs were performed in scanning electron microscope (Zeiss, LEO 1430 VP, Cambridge, England).

### *Histochemical test*

The histochemical tests were assessed in material fixed in Karnovsky solution, included in methacrylate resin (Historesin, Leica<sup>®</sup>), and embedded in plastic histomolds. Cross and longitudinal sections were obtained from an automatic rotatory microtome (RM 2155, Leica). In the analyses the following reagents were used: lugol solution (Johansen, 1940) for starch; Schiff reagent and periodic acid (PAS) (Maia, 1979) for neutral polysaccharides; Xylidine Ponceau (Vidal, 1970) for protein; and neutral red (Kirk, 1970) for total lipids. Examinations and photographs were performed in microscope stereoscope Olympus SZH and microscope Olympus AX70, with U-Photo system. The analyses with fluorochromes (neutral red) and autofluorescence were realized in the same microscope, using epifluorescence system with UV filter (WU: 340-380 nm), dichroic mirror (400nm) and barrier filter (420nm).

### **Results**

IBA increased the number of developed lateral roots when compared to the control. The explants used for induction of rhizogenesis are formed by uniseriate entire epidermis, cortex with parenchyma cells, cambium and evident secondary xylem and phloem, and pith with parenchyma cells (Figure 1A).

After four days in rooting induction medium (RIM), explants showed the first cell divisions (Figure 1B, C). At this stage periclinal cell divisions were observed, and cortex with isodiametric parenchyma cells.

After six days in RIM supplemented with IBA, cross and longitudinal sections evidenced lateral root primordia formation (Figure 1F, G), from parenchyma cells adjacent to vascular system. These cells have dense cytoplasm and large nuclei (Figure 1G).

At 10 days in RIM lateral root primordia was formed in the control and periclinal divisions were visualized (Figure 1H, I). Lateral root primordia observed in RIM with IBA (Figure 1J, K),

The continue cell de-differentiation of lateral root primordial observed at medium with IBA, led to the lateral root formation (Figure 1N, O). In cross section was noted the vascularization of root, cortex and epidermis with isodiametric cells, and root cap (Figure 1N). In longitudinal section it is possible to observe the RAM where the cell showed high nucleus/cytoplasm ratio (Figure 1O). In induction medium lacking auxin, after 20 days the lateral root had still not reached the outer cell layer of the

explant (Figure 1L, M). Scanning microscopy evidenced the swelling in cultured explant at control after 20 days (Figure 1P), whereas in the same period IBA-supplemented medium effectively induced the lateral root formation and its elongation (Figure 1Q).

The histochemical test indicated the presence of reserve compounds in cortex and vascular cylinder cells. Protein bodies were confirmed at initial explants (Figure 2A) and after 10 days in induction medium with IBA (Figure 2C) by the positive reaction of Xylidine Ponceau. However protein bodies were not identified after 20 days of culture (Figure 2D, E). Lipids were evidenced by the positive reaction to neutral red in cuticle of the explant at initial stages (Figure 2F). After 10 days lipids were observed in the parenchymal cell closer to region involved in the regeneration process, as well as in cuticle (Figure 2G, H). After 20 days at control it was observed lipids distributed near at lateral root primordia (Figure 2I), formed in medium with IBA (Figure 2J). Few starch grains were observed at initial stages (Figure 2K). But during the rhizogenesis process there was a gradually increase in these compounds observed at control and at IBA treatments (Figure 2L-O). Neutral polysaccharides were evidenced in the evaluated treatments (Figure 2P-T). At the end of the evaluation period starch grains and polysaccharides were plentiful and uniformly distributed.

## **Discussion**

Auxin regulates and coordinates the process of lateral root development, controlling the process of cell division, and establishing the identity of the founder cell (Ding & Friml, 2010; Lavenus *et al.*, 2013). As observed in this work, the beginning of lateral root formation is morphologically characterized by anticlinal asymmetrical cell division, followed by periclinal divisions (Dubrovsky *et al.*, 2001, Lavenus *et al.*, 2013). This cell divisions lead to the formation of an organized group of cell such as in RAM, from which will emerge a lateral root (Malamy & Benfey, 1997; Lucas *et al.*, 2013).

In some species, in special forest species, lateral root can be originate from different tissue like parenchyma cell of vascular cylinder, cambium, vascular rays and pith (Ginzburg, 1967; Baltierra *et al.*, 2004; Beyl & Trigiano, 2008). Here was noted the root development from parenchymal cell adjacent the vascular system that differentiated. These cell division events are possible due the capacity of cells to respond to high auxin level and the competency associate with them (Beeckman *et al.*,

2001; Himanen *et al.*, 2004; Dubrovsky *et al.*, 2008), which is an important mechanism to determinate when and where the lateral root will emerge (Overvoorde *et al.*, 2010).

As demonstrated in this work, when is available a source or there is overproduction of auxinin a greater number of lateral roots is formed. This happens because the auxin is essential not only for lateral roots initiations, but also for lateral root primordial development and root emergence (Ainda *et al.*, 2004; Fukaki & Tasaka 2009; Motte *et al.*, 2014). IBA is an endogenous plant compound (Epstein *et al.*, 1989; Ludwig-Muller 2000), converted in free IAA for peroxissomal enzymes (Zolman *et al.*, 2000, 2007, 2008). The conversion and transport of this hormone are important stages for determinate the root architecture (Overvoorde *et al.*, 2010). Nevertheless, is still not clear the conversion mechanism in IAA and if IBA can act such as a molecular signal.

In *A. thaliana* the exogenous auxin promoted the development of lateral root by activation of genes related to cell cycle such as cyclin and the cuclin-dependent kinases (Overvoorde *et al.*, 2010). Lateral root formation is also influenced by members of AUX/LAX family. AUX1 transports basipetal auxin to the basal region through cell layers (Swarup *et al.*, 2001; De Smet *et al.*, 2007), while LAX3 affects cortex cell auxin influx promoting the emergence of lateral roots (Swarup *et al.*, 2008).

Despite auxin is essential for the lateral roots it is still required the expression of genes responsible for organogenesis. Some works highlight genes such as *WOX* and *PLT* acting since the start of organogenesis (Aida *et al.*, 2004; Heidstra & Sabatini, 2014). The *WOX* gene is found in quiescent center cell and keeps the initial cell population of RAM (Haecker *et al.*, 2004; Sarkar *et al.*, 2007). It was observed in *A. thaliana* that *WOX5* gene is expressed few hours after the plant receive the stimulus, promoting the activity of the pericycle cells, which will originate lateral root primordial (Ditengou *et al.*, 2008). This gene has the expression controlled by auxin levels and ARF activity resulting in an initial differentiation of RAM (Wang *et al.*, 2005; Ding & Friml, 2010).

The *PLT* gene is required for specification and maintenance of RAM initial cells (Ainda, *et al.*, 2004; Vernoux & Benfey, 2005), expression also is stimulated by auxin and dependent of the transcriptions factors ARF (Ainda *et al.*, 2004; Overvoorde *et al.*, 2010). Some experimental and computational modeling data highlighted the importance of auxin distribution at genetic expression of *PLT* (Blilou *et al.*, 2005; Grieneisen *et al.*, 2007; Laskowski *et al.*, 2008), regulating the lateral root formation, but the response of this to auxin occurs in later stages when compared with primary responses genes (Ainda

*et al.*, 2004). *PLT* can also regulate also the gene expression of PIN family keeping high auxin levels and *PLT* at RAM (Blilou *et al.*, 2005; Galinha *et al.*, 2006, Heidstra & Sabatini, 2014). Furthermore, the perception of auxin is dependent, partially, of dimer formed by transcription factors *PLT* and *BBM* (Galinha *et al.*, 2007).

*BBM* belongs to the AP2/ERF family and have been associated with the embriogenic events. But, when associated the *PLT* these genes guarantee the maintenance of initial quiescent center cells such as observed in *A. thaliana*, although *PLT1* and *PLT2* are sufficient for the formation of initial cells in the root (Galinha *et al.*, 2006; Aida *et al.*, 2004). Neverthelles, the expression patter, especially of *BBM* in pluripotent cell is unclear, as well as the influence of auxin in the expression of this gene.

Another gene characterized in competent embriogenic cell culture, which had been investigated during the organogenic events is the *SERK* gene (Pilarska *et al.*, 2016; Rocha *et al.*, 2016). Studies with *Medicago truncatula* and *Cyclamen persicum* demonstrated that the expression of this gene occurs also in pluripotent cells, with the capacity to produce new organs (Nolan *et al.*, 2009; Savona *et al.*, 2012; Rocha *et al.*, 2016; Pilarska *et al.*, 2016). However there is still gap in the knowledge of the influence of the *SERK* gene during the organogenic events, and how the plant hormones can to influence the expression of this gene. So it is necessary to investigate a possible temporal and spatial expression of *SERK* and *BBM* gene during the formation of RAM and development of roots.

The histochemical tests evidenced the presence of reserve compounds such as protein, lipids, starch grains and neutral polysaccharide. The presence of protein bodies in the initial stages and the reduction of these compounds during lateral root formation support the idea that these compounds are essential to keep the cell differentiation. Similar results were founded during the somatic embryogenesis in *Passiflora edulis* and *P. cincinnata* (Rocha *et al.*, 2012; Silva *et al.*, 2015).

Lipids, starch and polysaccharides were observed during all the organogenesis induction process. These compounds are essential for the morphogenesis *in vitro* events, and some studies have showed that the mobilization occurs during the organogenic and embryogenic events (Martin *et al.*, 2000; Cangahuala-Inocente *et al.*, 2004, 2009; Pinto *et al.*, 2010). Here, was observed an increase in the amount of these compounds. Lipids together with protein are responsible by cell membrane development, while starch and polysaccharides are the main source of energy to maintain the regeneration process

(Martin *et al.*, 2000; Pinto *et al.*, 2010 Kraus *et al.*, 2012). The starch catabolism result in intermediary glycolic compounds generating the amount of ATP necessary to keep cell metabolism (Martin *et al.*, 2000), thus the increase in amount of starch grains can be justified by the energetic demand of regeneration process.

The results obtained on this work provide detailed anatomical and chemical information about the process of rizogenesis in *Bixa orellana*. These data can assist research involving the *in vitro* rizogenesis, and subsidize studies of dynamic of genetic expression involved with the organ neoformation, such as be applied in studies of genetic transformation.

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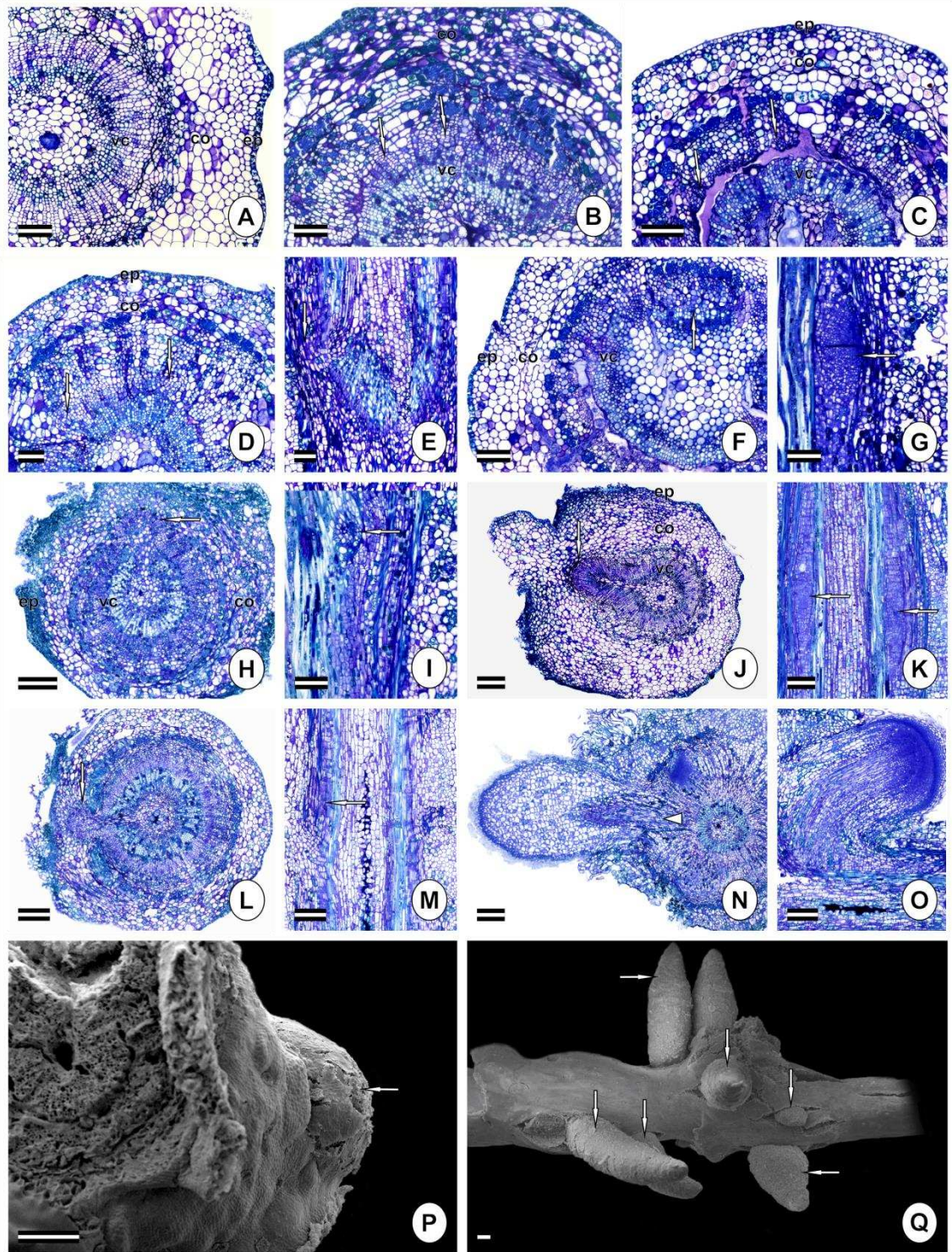
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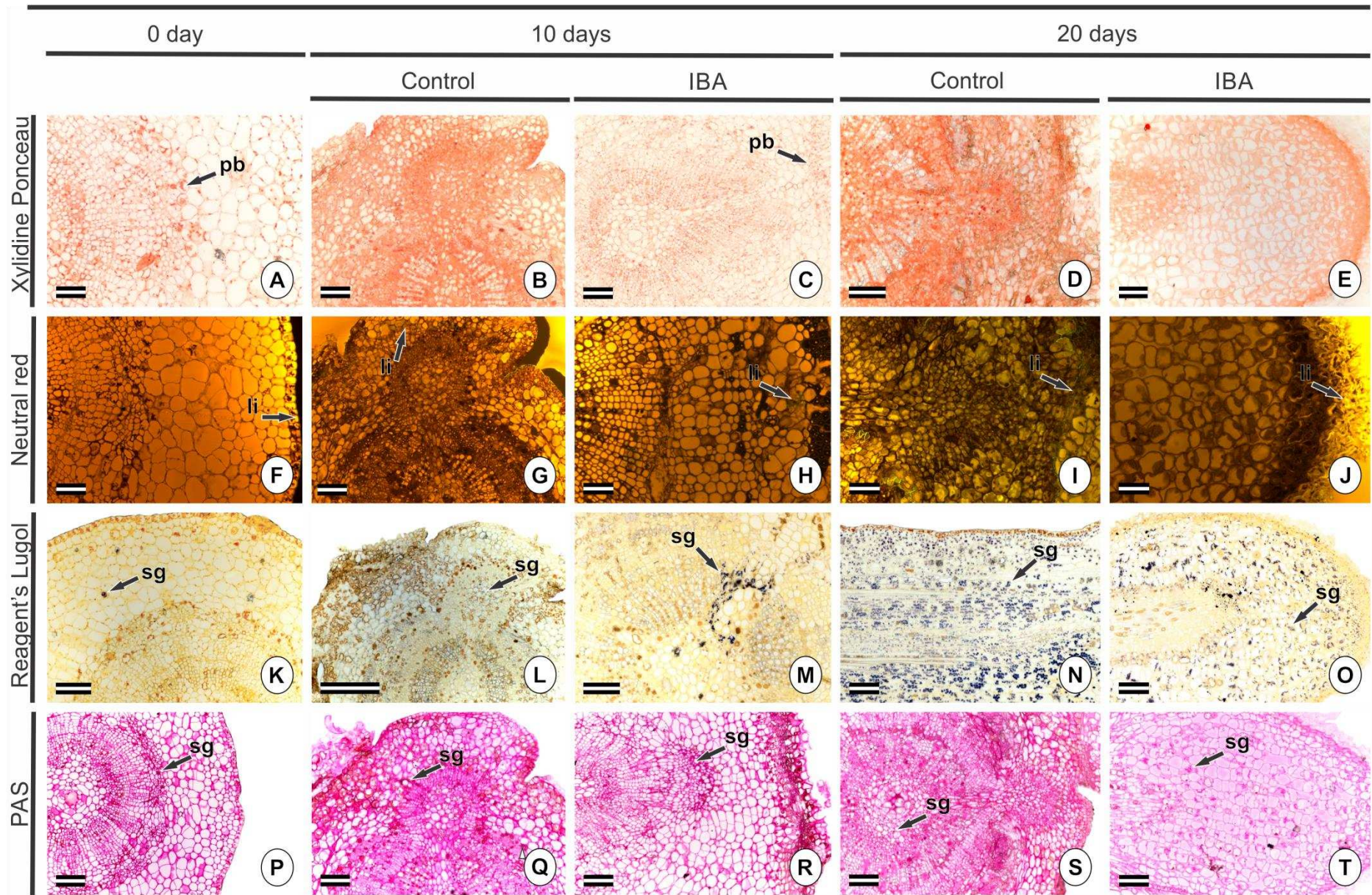
## Legend for Figures

**Figure 1:** Rhizogenic responses of *Bixa orellana* L. shoot cultures maintained in JADS medium with 0 (B, D, E, H, I, L, M, P) or 2.46  $\mu$ M de IBA (C, F, G, J, K, N, O, Q). Light microscopy (A-O) and Scanning electron microscopy (SEM) (P-Q). **A:** Cross section of initial stages of differentiations showed secondary growth. **B-C:** After 4 days of culture, cross sections evidencing periclinal divisions (arrow). **D, F:** cross sections, and **E, G:** longitudinal sections of explants after 6 days. Note the periclinal divisions (arrow) at control, and lateral root primordial formation (arrow) at medium with IBA, from parenchymal cell adjacent to vascular system. **H-K:** After 10 days cross and longitudinal sections evidenced initial formation of lateral root at control and at medium with IBA. **N,O:** After 20 days of culture observed lateral root with vascular connection (arrowhead) in segments cultured at medium with IBA. **P, Q:** SEM evidenced the swelling at explant (P) and lateral root (Q). Abbreviations: ep, epidermis; co, cortex, vc, vascular cylinder. Bars = A- G = 100  $\mu$ m; H-P = 200  $\mu$ m; Q = 1000  $\mu$ m.

**Figure 2:** Histochemical Analysis during lateral root development in *Bixa orellana* L. from hypocotyl segments with 0, 10 and 20 days of cultivation in induction medium supplemented with 0 (control) or 2.46  $\mu$ M de IBA. Histological sections subjected to Xylidine Ponceau (XP; A-E), Neutra red (F-J), Lugol (K-O), and periodic acid-Schiff (PAS; P-T). Positive reaction to XP evidenced protein bodies (arrowhead) in explants with 0 day (A), and 10 days at medium with IBA (C). After 20 days was not observed protein bodies in the treatments (D, E). Lipids were evidenced in cuticle at initial stages (F). After 10 and 20 days (G-J) was observed lipids composing the cuticle and near to lateral root primordial (arrowhead). Few starch grain were evidenced at initial stages (K) (arrowhead). After 10 days (L, M) observed an increase in these compounds distributed in the vascular cylinder, near to lateral root primordial. After 20 days (N, O) there was high amount of starch grains distributed in the cortex, and in the vascular cylinder at control treatments, and by all lateral root formed at IBA treatments. Polysaccharides were evidenced in all treatments by the PAS test (arrowhead). Note that at control, 10 and 20 days (Q, S) there is a small amount of polysaccharide compared with the explants cultured with IBA (R, T). In lateral root the polysaccharides were uniformly distributed in the organ (T). Bars = A, F, H-J = 50  $\mu$ m; B-D, G, L-T = 100  $\mu$ m; E, K= 200  $\mu$ m.

# Figures





### Chapter 3

#### **Cloning and characterization of putative Class I *KNOX* and *AP2-LIKE* transcription factors expressed during organ neof ormation in *Bixa orellana* L.**

**Abstract-** The organ neof ormation is influenced by growth regulators, and by genes which are expressed during the acquisition competence events. The spatio temporal gene expression patterns are essential to ensure the organization and maintenance of the shoot apical and root apical meristems, as well as the development of adventitious organs. However, still there are gaps in knowledge on the molecular events that occur during *in vitro* organogenesis. This study aimed to cloning and characterizing members of Class 1 *KNOX* and *APETALA2-like* family during organogenesis in *Bixa orellana* (annatto). Aiming at pursuing hypocotyl segments were inoculated in JADS medium supplemented with 4.56  $\mu$ M of zeatin, and collected in different development stages for RNA extraction. Single cDNAs were used as template for amplification of the coding sequences using degenerate primers for *BBM* and *STM*. The amplification products were cloned and sequenced. The consensus sequences obtained were compared to non-redundant proteins of NCBI, Phytozome and TAIR, using the BLAST algorithms. After analysis and alignment of the sequences three contigs similar to members *APETALA2-like* family were generated. The BoContig1 was isolated from proembryogenic mass cDNA in annatto, whereas BoContig2 and BoContig3 were amplified from cDNA generated from material organogenic. BoContig1 showed greater similarity with the *BABY BOOM* transcription factors. BoContig2 showed similarity with *WRINKLED* members, while BoContig3 was more similar with *AINTEGUMENTA*. Sequence analysis of the products amplified with degenerated primers for *STM* led to formation of three distinct contigs. All the contigs showed high similarity to Class I *KNOX* transcription factor, especially with *SHOOT MERISTEMLESS (STM)* gene. The multiple alignment sequence revealed the presence of characteristic structural domains of the *KNOX* family. These results suggest that analyzed sequences are possible members and *AP2-like* family of Class I *KNOX*, potentially associated with pluripotency and *in vitro* organ neof ormation in annatto.

**Key-words:** *BABY BOOM*, *Bixa*, organogenesis events, pluripotency, *SHOOT MERISTEMLESS*, transcription factorS.

## Introduction

The *in vitro* regeneration is based on the capacity of plant cells to acquire competency for reprogramming themselves to follow new developmental paths culminating in the neoformation of organs. Based on that, the tissue culture has effectively contributed to the contemporary plant biotechnology, permitting that the basic mechanisms regulating morphogenesis and the changes that occur during the process are studied in detail, using molecular tools (Duclercq *et al.*, 2011; Rai & Shekhawat, 2013; Us-Camas *et al.*, 2014; Harada *et al.*, 2015).

Somatic embryogenesis and organogenesis are the main morphogenetic regeneration pathways, and efficient propagation protocols using these techniques have been successfully established for many species (Mohan Jain & Hägmann, 2007; Mohan Jain & Ochatt, 2010; Germanà & Lambardi, 2016). However, the organogenic pathway is preferentially used in methods of biotechnological improvement because of the efficiency of the process (Duclercq *et al.*, 2011; Cheng *et al.*, 2013; Motte *et al.*, 2012). The organogenesis is the *in vitro* formation of a monopolar structure, shoot or roots, in function of explant type, as well of the environmental conditions and chemical factors (Skoog & Miller, 1957; Duclercq *et al.*, 2011; Cheng *et al.*, 2013; Rocha *et al.*, 2014; Motte *et al.*, 2014). Besides this, the organogenesis also is dependent of the pluripotency of plant cell (Duclercq *et al.*, 2011), which refers to capacity of stem cells give rise to the cell and tissue at shoot apical meristem (SAM) and root apical meristem (RAM), nevertheless do not have ability to form an embryo (Verdeil *et al.*, 2007).

The maintaining of population of initial cells, the organization and the differentiation are essential to ensure the establishment of the SAM and RAM (Heidstra & Sabatini, 2014). These events are mediated by the plant growth regulators auxin and cytokinin, and by express genes in all stages of development, giving identity to the new organ, as well as maintenance (Verdeil *et al.*, 2007; Duclercq *et al.*, 2011; Cheng *et al.*, 2013; Motte *et al.*, 2014). Some studies characterized the expression of several genes involved in the organogenic events, and demonstrated the functions in the determination the organ architecture (Hibara *et al.*, 2006; Scofield *et al.*, 2013).

Among the expressed genes the *KNOX* (*KNOTTED1-like* homeobox) encodes a small group homeobox transcriptions factors that are essentials on formation and maintenance of the shoot apical meristem (SAM), and leaf development (Gao *et al.*, 2015; Hay & Tisians, 2006). They belong to TALE superfamily homeoboxes genes,

and are part of a large homeodomain protein family, being distinguished by presence of four characteristic domains: N-terminal *KNOX* domain (with *KNOX1* and *KNOX2* subdomains), ELK, followed by the HD homeobox at the C-terminus (Tsuda & Hake, 2016). Studies based on sequence similarity, intron position, expression pattern has been separated *KNOX* genes in two classes: Class I (*KNOX I*) and Class II (*KNOX II*). In angiosperms, class I *KNOX* gene have been intensively studied and shown to play indispensable roles in the establishment and maintenance of the SAM. Therefore *KNOX* genes are used as markers for the undifferentiated or meristematic state in various species (Tsuda & Hake, 2016). Functional studies in *Arabidopsis thaliana* has highlighted the importance of four genes class I *KNOX*: *STM*, *KNAT1*, *KNAT2* and *KNAT6*. *KNAT1* and *KNAT6* contribute to function of SAM and inflorescence development. *KNAT2* by its turn promotes pattern formation during development of the inflorescence.

The *STM* (*SHOOT MERISTEMLESS*) gene, in addition to the *WUSCHEL* gene is required as a meristem-promoting factor, preventing stem cell differentiation (Gallois *et al.*, 2002; Verdeil *et al.*, 2007; Scofield *et al.*, 2013 Heidstra & Sabatini, 2014). *STM* inhibits the activity of the differentiation factor *ASI* in the meristem center, and activates the synthesis of cytokinins through of the expression of gene *IPT* (*Isopentenyl transferase*), which regulates the cell division (Jasinski *et al.*, 2005; Yanai *et al.*, 2005; Scofield *et al.*, 2013). Mutations in the *STM* gene resulted in the lack of a SAM, demonstrating that this gene is essential for the organization of apical meristem (Daimon *et al.*, 2003).

Members of the APETALA-2 family, such as *BABY BOOM* (*BBM*), *PLETHORA* (*PLT*) and *AINTEGUMENTA* (*ANT*) are responsible by diverse transcriptional networks and developmental process during the morphogenic events (Ouakfaoui *et al.*, 2010). These genes are defined by the presence of two AP2 domains separated by a conserved linker region which together constitute the DNA binding domain (Nole-Wilson & Krizek, 2000; Krizek, 2003). *BBM* gene was postulated to be involved in the acquisition embryogenic competence by somatic cells (Boutilier *et al.*, 2002; Gaj *et al.*, 2005; Deng *et al.*, 2009; Heidmann *et al.*, 2011; Flores *et al.*, 2015), activating a complex network of development pathways (Passarinho *et al.*, 2008). *PLT* regulates of root growth promoting cell proliferation (Aida *et al.*, 2004; Galinha *et al.*, 2007), and guarantee the specification of the quiescent center and maintenance of the initial cells (Aida, *et al.*, 2004; Blilou *et al.*, 2005; Vernoux & Benfey, 2005), *ANT* is

responsible by the development of floral organ and auxiliary in maintenance of meristems (Nole-Wilson *et al.*, 2005; Dash & Malladi, 2012)

It is widely accepted that its necessary an efficient regeneration system to support studies of the molecular events which occurs during the morphogenesis *in vitro*. In that sense, *Bixa orellana* L. shows high regenerative capacity associated to cell pluripotency (D'Souza & Sharon 2001; Carvalho *et al.*, 2005; Paiva Neto *et al.*, 2003/4a; Paiva Neto *et al.*, 2003b; Parimalan *et al.*, 2007; Cruz *et al.*, 2014; Mohammed *et al.*, 2015; Cruz *et al.*, 2015). So it is a species prone for studies of the regenerative pathways and of the molecular mechanisms that regulates *in vitro* morphogenesis. Besides this, *Bixa* has large economic value associated to the carotenoids bixin and norbixin found in seeds coat, which are used in food, cosmetic and pharmaceutical industries (Giuliano *et al.*, 2003; Dias *et al.*, 2011; Marcolino *et al.*, 2011; Mala *et al.*, 2015). Thus genetic improvement for generation of plants with increase in the number of seeds, and increase pigment accumulation on seed coats is necessary.

The study of the molecular events responsible by the organogenesis in annatto will contribute with relevant information about the morphogenesis, including the organization, maintenance, and development of SAM and RAM. The goal this study was cloning and characterize members *APETALA2-like* family and *KNOX* genes, expressed during the *in vitro* organ neoformation in *Bixa orellana* L.

## **Materials and Methods**

### *Plant material and in vitro seeds germination*

Mature seeds of *Bixa orellana* “Piave Vermelha” provided by Chr. Hansen Brazil (Valinhos, São Paulo, Brazil) were mechanically scarified to remove the opposite side of the embryonic axis. Seeds were then immersed in 70% ethanol (v/v) for 1 min, transferred to a 2.5% (v/v) solution from a commercial sodium hypochlorite with 0.1% (v/v) Tween 20 for 20 min, followed by washing in distilled water and autoclaved at 120°C and 108 kPa for 20 min.

Subsequently, seeds were inoculated in 25x150 mm test tubes, containing 10 mL of MS medium (Murashige and Skoog, 1962) with 50% of the basal salts concentration, supplemented with B5 vitamins (Gamborg *et al.*, 1968), sucrose (1.5% w/v), myo-inositol (0.005% w/v), and agar (0.6% w/v Merck KGAA, Darmstadt, Germany). The pH of the medium was adjusted to  $5.7 \pm 0.1$ . The seeds were kept in the dark for at  $25 \pm 1^\circ\text{C}$  15 days. And the seedlings transferred to growth room under 16-h photoperiod,

under  $36 \mu\text{mol m}^{-2} \text{s}^{-1}$  irradiance from two fluorescent lamps (Osram Luz do Dia, São Paulo, Brazil), at  $25 \pm 1^\circ\text{C}$  for 15 days.

#### *Induction of de novo shoot organogenesis*

Hypocotyl segments (10-20 mm of length) were excised from 30d-old seedlings and inoculated horizontally in 300 mL glass jars containing 30 mL of JADS medium (Correia *et al.*, 1995), supplemented with B5 vitamins, sucrose (3% w/v), myo-inositol (0.01% w/v), agar (0.6% w/v) and  $4.56 \mu\text{M}$  zeatin. The pH of the medium was adjusted to  $5.7 \pm 0.1$ . Following cultures were transferred to growth room under 16-h photoperiod, under  $36 \mu\text{mol m}^{-2} \text{s}^{-1}$  irradiance from two fluorescent lamps (Osram Luz do Dia, São Paulo, Brazil), at  $25 \pm 1^\circ\text{C}$  for 30 days.

The experiment was conducted in a completely randomized design, with three glass jars by treatment and seven explants by jars. The jars glasses were kept in conditions similar of light and temperature as previously mentioned.

#### *RNA extraction and Synthesis of cDNA*

After 0, 3, 6, 9, 15, 18, 21, 24, and 30 days of culture in induction medium explants were collected in Eppendorf® RNase free tubes, immediately frozen in liquid nitrogen and stored to  $-80^\circ\text{C}$ .

The total RNA was extracted used the Kit RNA Aqueous (Applied Biosystems™), according to the manufacturer's recommendations. The quantitation of RNA was realized used NanoDrop ND-1000 (NanoDrop Technologies™), with absorbance measurements at 260 nm, and the integrity of RNA sample was verified by agarose gel electrophoresis at 1.2% (RNase free)

The cDNAs single strands were synthesized from of  $3.0 \mu\text{g}$  of total RNA obtained from of a pool of RNA of sample with 9, 15 and 30 days. Was used the kit *Super Script™ II, First- Strand Synthesis System* (Invitrogen™), according to the manufacturer's recommendations.

#### *Amplification of coding sequence of the genes linked to organogenesis*

The cDNA obtained from total RNA of organogenic and embriogenic samples were used as template for the amplification of coding sequence of *BBM* genes and *STM*. A different combination of degenerated primers was used, designed from conserved domains of each gene, using sequences of different species available at National Center

for Biotechnology Information (NCBI) and at The Arabidopsis Information Resource (TAIR).

The PCR reaction was conducted in the final volume of 50  $\mu$ L containing: 2.0  $\mu$ L of cDNA, 5  $\mu$ L of buffer 10x, dNTPs (0.2 mM each), 2.0 mM of MgSO<sub>4</sub>, 200 nM of each primer and 0.2  $\mu$ L of Taq DNA polymerase Platinum High Fidelity® (Invitrogen). The reaction was realized in thermocycler C1000™ Touch Thermal Cycle (Bio-Rad) with the program: denaturation at 94 °C (5 min), followed of 40 cycles at 94°C (1 min), 50-60 °C (1 min) as each primer pair used, 68 °C (1 min), and final extension step at 68 °C for 5 min.

The amplification fragments were cut out of the gel and purified with the Kit Wizard® SV Gel and PCR clean up (Promega®), according to the manufacturer's recommendations. The fragments were linked at pGEM®-T Easy Vector Systems (Promega®) vector, in proportion of 3:1 (insert:vector), with the T4 DNA ligase (Promega®) enzyme, according to the manufacturer's recommendations.

Recombinant plasmids obtained were introduced into *Escherichia coli* (DH5-alpha) by thermal shock. For transformation were used 5  $\mu$ L of insert and 100  $\mu$ L of competent cells (1x 10<sup>8</sup>). After incubation at 37°C by 1 h the cell were locked in selective solid medium LB (Sambrook & Russel, 2001), containing 100  $\mu$ g/mL of ampicillin, 20  $\mu$ g/mL of 5-bromo-4-chloro-3-indolyl- $\beta$ -d-galactopiranoside (X-Gal) and 0.1 mM of isopropyl-beta-D-thiogalactopiranoside (IPTG) and then incubated at 37 °C overnight.

For confirmation of presence of the insert in the vector, the plasmid DNA of the colonies which showed white colors were cleaved with the *Eco*RI restriction enzyme, and the fragments were separated by agarose gel electrophoresis at 1.0%

#### *Sequence analysis and phylogenetic inference*

Ten positive clones were chosen for sequencing using universal T7 and SP6 primers. The obtained sequences were processed using the PHRED (Ewing *et al.*, 1998) and the CAP3 (Huang & Madan, 1999). Contigs sequences were compared to public databases at NCBI, Phytozome and TAIR using the BLAST algorithm (Altschul *et al.*, 1997). The identification of Open reading frame and protein prediction were made using the ORF Finder (<http://www.ncbi.nlm.nih.gov/projects/gorf/>), and the comparison among the sequences were made using the app *bl2seq* (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The presence of conserved motifs protein in the

deduced protein sequences from the obtained sequenced, was observed using CDD Conserved Domain database (NCBI) (Marchler-Bauer *et al.*, 2015). Multiple sequence alignments obtained from of possible counterparts of *Arabidopsis* and others species available at databases were performed using CLUSTALW (Thompson *et al.*, 1994) at Bioedit. Phylogenetic tree were constructed using the neighbor-joining (Saitou & Nei, 1987) with MEGA 7 (<http://www.megasoftware.net>). Bootstrap values were calculated from 1000 replicates.

#### *Protein Structure analysis*

The analysis of putatives KNOX protein the conservation score for Homeodomain was determined using the CONSURF webserver (Ashkenazy *et al.*, 2010; Glaser *et al.*, 2005). The analysis was performed from alignment with 21 sequences identified as KNOX protein, and using AtSTM and BoContig2 as query sequence. The cristal structure used as template was the homeodomain of *Drosophyla melanogaster* (PDB id: 1enh). The molecular graphics and analysis were performed with PyMOL Viewer version 0.99rc6.

## **Results and Discussion**

### *Isolation of three putative AP2-Like family transcription factors associated to in vitro shoot development*

The combinations used of the degenerated primers for the *BBM* genes (Table 1) amplified of predict size fragments. Three putative members of the AP2-domain were isolated from amplifications of degenerated primer pairs BBM F1; BBM R and BBM F2; BBM R. The amplified fragments showed approximate size of 289 and 293 pb, respectively. Ten clones of all combinations of the degenerate primers were sequenced. After the analysis of the sequence three contigs were generate: BoContig1 (397 pb), BoContig2 (289 pb), BoContig3 (289 pb). The BoContig1 was assembly from clones of amplified products of cDNA of embryogenic material, whereas BoContig2 and BoContig3 were isolated from cDNA from organogenic samples.

The nucleotide sequences of the contigs generated were compared to the nucleotide sequences deposited in the non-redundant database NCBI, using the Blastn tools. All the sequences analyzed by the Blastn showed identity with the ones described as members APETA2-like family for other species available in the GenBank. The BoContig1 showed high identity (between 77 and 98%) with sequences descibed as

*BBM1* and *BBM2* of other species such as *Vitis vinifera*, *Arabidopsis thaliana* and *Zea mays*. The BoContig2 showed identity between 80 and 88% with AP2-like ethylene responsive transcription factor family of other species. The BoContig3 showed identity between 85 and 86% with sequence annotated as *AINTEGUMENTA (ANT)* of other species.

The three contigs showed Open reading frames (ORFs) of different sizes, as follows: BoContig1 (132 amino acids); BoContig2 (84 amino acids); and BoContig3 (65 amino acids). The comparison of the sequences with the database of non-redundant protein NCBI, by the Blastp and Blastx showed similarity of contigs with predicted proteins of the AP2-like family. BoContig1 showed similarity between 88 and 97% with *BBM* and *PLT*. BoContig2 was similar between 79 and 95% with described proteins as members of AP2-like family. BoContig3 showed similarity between 91 and 97% with *ANT*.

The multiple alignments of the deduced amino acids sequences were generated using sequences of other species such as *Theobroma cacao*, *Arabidopsis thaliana*, *Vitis vinifera* and *Carica papaya* found in databases. This alignment confirmed the conservation of AP2-like domain characteristic of members of this family (Figure 1). Was noted high similarity of BoContig1 with the *BBM* transcription factor, of the BoContig2 with *WRINKLED (WRI)* transcription factor, and of the BoContig3 with *ANT*.

The phylogenetic inferences of the BoContigs1, 2 and 3 considered the sequences included in the AP2-Like conserved region of *BBM*, *ANT*, *PLT*, and *WRI*. From of the phylogenetic analysis was noted the composition of three different groups (Figure 2). BoContig1 grouped with the *BBM* transcription factors of various species, being more close to *T. cacao*, *A. thaliana* and *Brassica rapa* (Figure 2). BoContig2 formed a second group with *WRI* transcription factor of *A. thaliana* (Figure 2). BoContig3 formed a group with *ANT* describe for *A. thaliana* (Figure 2).

Three partial sequences of members of the AP2-like were isolated from cDNA derived from embryogenic and organogenic competent tissue in *Bixa orellana*. The analysis of the sequences showed high similarity of these contigs with members of the AP2-like family annotated as *BBM*, *PLT*, *ANT* and *WRI* of other species available in Non-Redundant Protein Sequence database of NCBI

BoContig1 grouped together to the cluster corresponding to *BBM* sequences in databases. In species like *Brassica napus* (Boutilier *et al.*, 2002) *T. cacao* (Florez *et al.*,

2015), and *A. thaliana* (Casson *et al.*, 2005) *BBM* gene is involved with embryo development, and also relates to the developmental competence of the seedling to keep cells in a relatively undifferentiated or juvenile state (Passarinho *et al.*, 2008). When *BBM* gene was overexpressed in *A. thaliana* and *Glycine max* somatic embryos regenerated without hormone application (Boutilier *et al.*, 2002; Ouakfaoui *et al.*, 2010), however in *T. cacao* the ability of *BBM* gene to activate somatic embryogenesis pathways was dependent of other factors (Passarinho *et al.*, 2008). therefore, *BBM* genes can be involved with other processes during the morphogenetic events, since in tobacco heterologous *BBM* expression induces shoot and callus formation (Srinivasan *et al.*, 2007), and in *A. thaliana* *BBM* gene was involved with vascular tissue development and differentiation, and is also expressed in seedling roots (Galinha *et al.*, 2007; Passarinho *et al.*, 2008).

*BBM* gene can also interact with *PLETHORA (PLT)* gene, transcription factors of APETALA2-domain, forming a dimer which accumulates along gradients of auxin (Pettersson *et al.*, 2009; Galinha *et al.*, 2007). The *PLT* gene is first expressed at the basal embryo region, and later in the root meristem stem cell niche ensuring the stem cell specification and maintenance in the RAM (Aida *et al.*, 2004; Galinha *et al.*, 2007) Kareem *et al.*, 2015). Recently, it was verified the expression of *PLT* gene in competent cells for shoot formation, this was associated to capacity this gene keep the pluripotent cell population and to active the gene expression involved with the shoot formation (Kareem *et al.*, 2015).

The BoConting2 expressed during organogenic events in *Bixa* showed identity with annotated homologs as *WRINKLED (WRI)* transcriptions factors of *A. thaliana*. This transcription factor might be conserved within eudicots and monocots (Shen *et al.*, 2010), and has been associated with the carbohydrate metabolism, fatty acid synthesis in maturing *A. thaliana* seed, and upregulation genes involved in fatty acid production during the seed maturation phase, binding to promoter sequences of late glycolytic and fatty acid biosynthetic genes (Cernac & Benning, 2004; Baud *et al.*, 2007; Baud *et al.*, 2009). However, the transcriptional machinery that regulates the fatty acid biosynthetic pathway is not influenced only by the *WRI* transcriptional factors, but probably involves multiple regulatory proteins (To *et al.*, 2012).

In *Ricinus communis* *WRI* was expressed specifically in developing seed (Tajima *et al.*, 2013), however *WRI1*, *WRI2*, *WRI3* and *WRI4* were differentially expressed during the morphogenic events and have not identical functions in plants (To *et al.*,

2012). *WRI1*, *WRI3* and *WRI4* promoted yeast growth on selection medium, and are involved in the activation of fatty acid biosynthesis in developing petals and sepals, but only *WRI1* enhances the transcription of fatty acid biosynthetic genes in maturing seeds (To *et al.*, 2012). The functions of *WRI2* are not clear, and further studies are necessary to elucidate the role of this transcription factor.

The BoContig3 also was expressed in sample with organogenic characteristics cultured in induction medium, and was grouped with annotated sequences as *AINTEGUMENTA (ANT)* in the databases for *A. thaliana*. *ANT* is required for ovule development, floral organ growth and regulates the number and the differentiation of stem cell in the meristems (Elliott *et al.*, 1996; Klucher *et al.*, 1996; Mizukami & Fischer, 2000; Nole-Wilson *et al.*, 2005). In apple the expression of *ANT* was associated with the cell proliferation and with the regulation of others genes involved in regulating organ growth (Dash & Malladi, 2012).

With the multiple alignment of the sequences it was possible to observe substitutions of amino acids and deletions or insertions represented by gaps. The absence of gaps in conserved region do AP2-domain and the sequences describes in databases suggests that in *Bixa orellana* members of AP2-like family were cloned. The phylogenetic analysis confirms these results, by means of three distinct groups formed by the *BBM*, *WRI* and *ANT* transfactors. However, it was not possible to name the contigs, because partial sequences are involved. Thus, future studies are necessary for infer more details about the amplified products.

#### *Characterization of three putatives class I KNOX genes associated to in vitro shoot development*

Using combinations of degenerated primers a fragment of around 1,2 kb was amplified. The amplification products were cloned and ten positive clones were sequenced. Sequences analysis resulted in the generation of three distinct contigs: Contigs 1 and 2, both with 1019 pb, and a third Contig with 1233 pb. Analysis of BLASTX and BLASTP against the Non-Redundant Protein Sequence database of NCBI, Phytozome and TAIR database revealed high similarity of the all contigs with proteins from KNOX I class.

The predict ORF for each contig was 382 amino acids for Contig1, 352 amino acids for Contig2 and 338 amino acids for Contig3. The BLAST results against the Non-Redundante protein sequences database (NCBI) and Phytozome demonstrated

between 81 and 88% of identity (e-value 0,0) for all contigs with predict homeobox proteins of species as *Theobroma cacao*, *Grossypium hirsutum* and *Manihot esculenta*. When compared against the *Arabidopsis* database (TAIR) the complete sequences showed among 72 and 73% (identical and positives amino acids) of similarity with the class I STM protein. Lower similarity percentages and score values were observed in relation to other members of KNOX I class, as KNAT1 and KNAT6.

The analysis of the deduced sequence amino acids of the contigs in the CDD Conserved Domain database (NCBI) by BLASTP revealed the presence of the four characteristic structural domains of KNOX I class protein: KNOX1, KNOX2, ELK and Homeodomain (Figure 3). The multiple sequence alignment with the predict amino acid sequences of the contigs with KNOX I class representatives of *A. thaliana*, *Theobroma cacao* and *Zea mays*, revealed high degree of conservation of structural domains (Figure 4). Furthermore, in relation to contigs of *B. orellana* was observed a greater identity with the Class I STM protein of *A. thaliana*.

Analysis of BLASTP also was performed from of functional domains of each contig using the TAIR datase. The comparison made in the region comprised between KNOX1 and KNOX2 domains revealed between 82 and 89% of similarity with AtSTM protein, and the contig2 showed greater similarity. When compared the sequence comprised between ELK and Homeodomain was observed 98% of similarity was observed for all contigs.

The phylogenetic tree construted based from amino acid alignment sequences of representatives homologs of class I and class 2 KNOX and other homeodomain proteins of species available in databases, showed the formation of distincts groups supported by bootstrap value above 50%. The contigs of *B. orellana*, named as BoSTM, were grouped a single clade, being observed a well near relationship with *KNOX* I class protein of other species (Figure 5).

Analysis of CONSURF program was performed aimed to determine the conservation score of the Homeodomain DNA-binding domains for 21 sequences identified as *KNOX* protein, and using AtSTM and BoContig2 as query sequence. The cristal structure used as template was the homeodomain of *Drosophyla* (PDB id: 1enh). The results were showed on ribbon diagram of the structure, where cyan represent to low score and red-violet to higher conservation score (Figure 6). The corresponding residues of homedomains, are defined by 60 amino acids forming a structure containing three alpha-helices, of which the second and third form a helix-turn-helix motif

(Gehring *et al.*, 1994). The structure of BoContig2 showed important conservation degree of residues, reinforcing the assumption that this isolated sequence is a homeodomain transcription factor associated with *in vitro* development in this species.

This preliminary results suggested that the three partial contigs are possible orthologs for the class I *KNOX STM* transcription factors. The coding sequences are complete at 5' portion, being a region well variable between members of the family and among species. For the 3' region, based on alignment with AtSTM (382 amino acid), supposedly are missing around 22 amino acids. Currently, 5' and 3' RACE experiments are being conducted aiming to get the full-length mRNA sequences with untranslated region (UTRs).

The proximity observed among the amplified products (contigs) suggested that are possible possible duplication events (paralogs), transcript variant or isoforms that may be redundant or have different functions in this species. For this nature of, studies the use of genomic functional tool and a more detailed phylogenetic approach are necessary in order to unveil the function for this genes in *annatto*.

In this work were cloned and sequenced six partial sequences corresponding to putative transcription factors of *AP2*-like family and *KNOX* I class, potentially associated with pluripotency and the *in vitro* organ neorformation in *Bixa orellana*, considered a plant prone for *in vitro* regeneration studies. Further 5' and 3' RACE experiments are necessary to obtain the complete sequences for these genes. The function of these genes will be investigated using functional genomic tools, aiming to characterize temporal and spacial conservation patterns of gene expression or even identifying new expression patterns. The present study will contribute for the better understanding of the transactors regulating the morphogenesis in this species.

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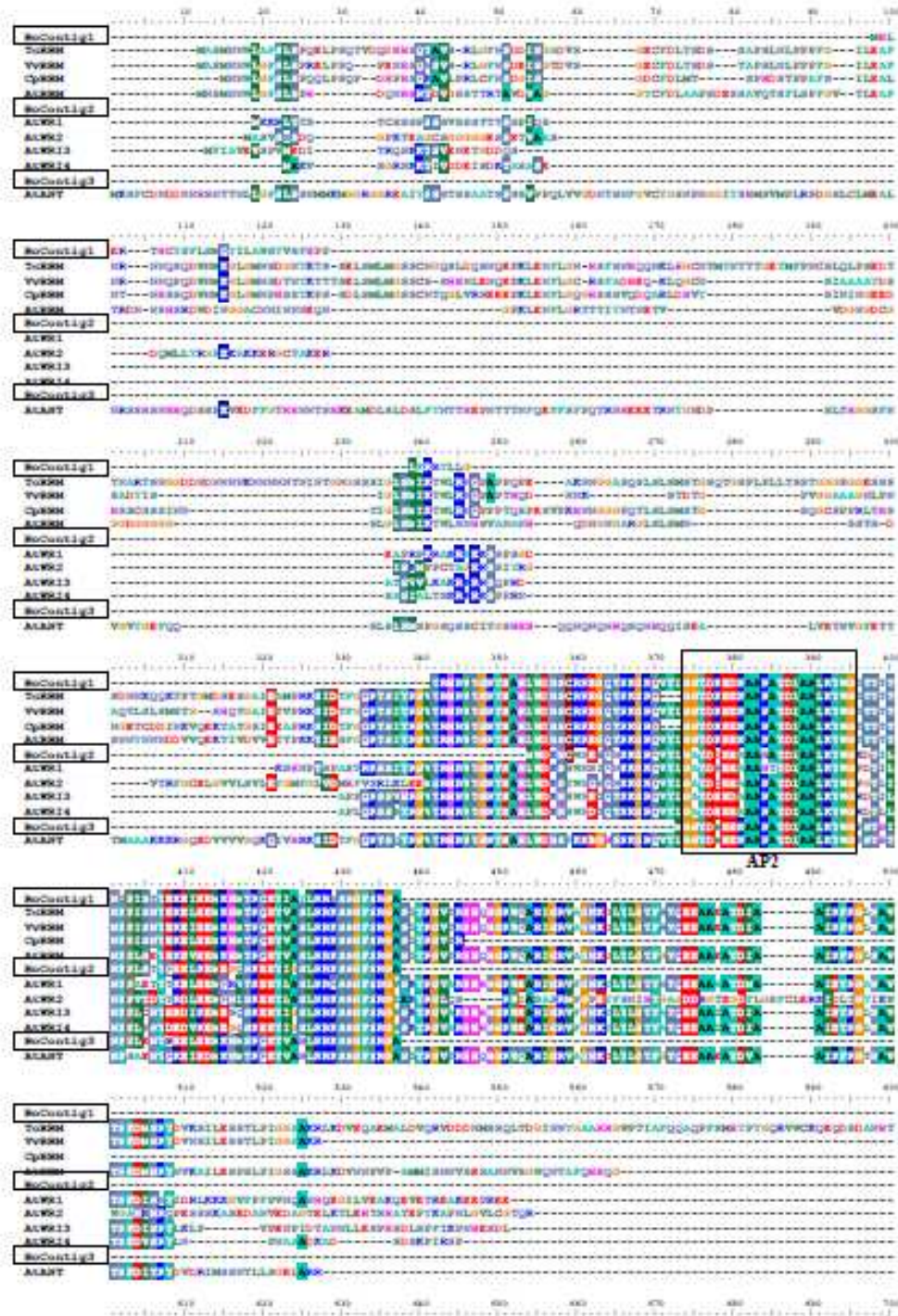
## Figures and Legends

**Table 1-** Degenerated primers sequences used to amplify the coding sequence of *BBM* and *STM* genes from cDNA of *Bixa orellana* L.

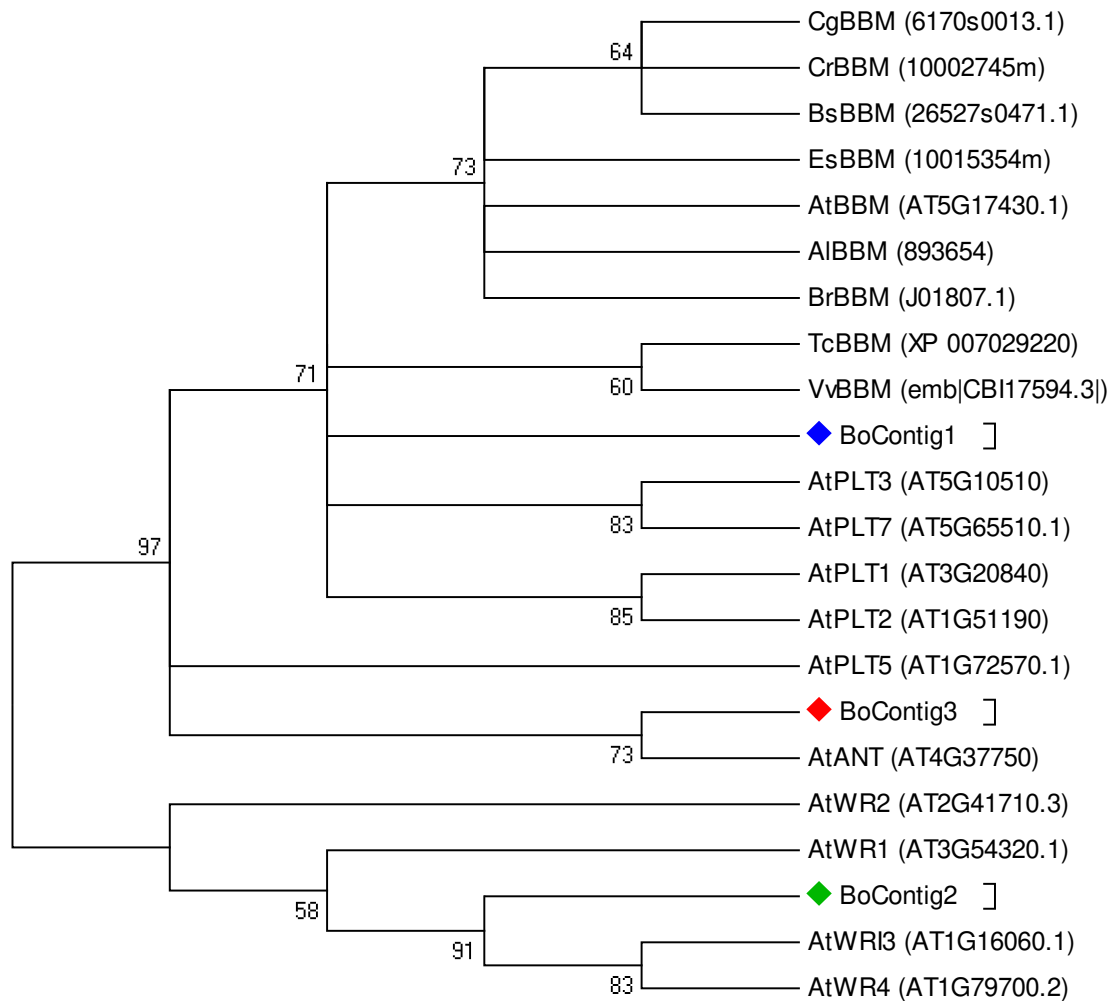
Primers	Sequence	Structural Domain
<b><i>BBM</i></b>		
BBMF1	5'ACAAGGCATMGRTGGACDG3'	AP2
BBMF2	5'TGTHACAAGGCATMGRTGGACDG3'	AP2
BBMR	5'ATGCACDCGWDGARAAMCCAC3'	AP2
<b><i>STM</i></b>		
STMF1	5'-ATGGAIGGTGGTGCTAATAGCA-3'	Região N-terminal
STMR1	5'-TGGTGAGGATGTGTTGCGTC-3'	Região C-terminal

W = A or T; R = A or G; M = A or C; Y = C or T; H = A or C or T; S = C or G; D = A, G or T. F:

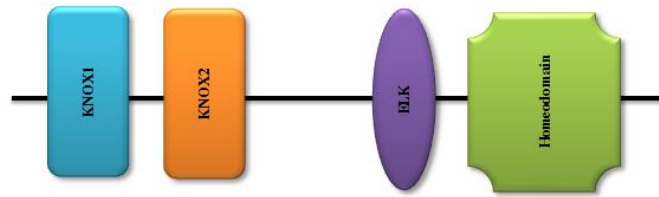
Forward, R: Reverse.



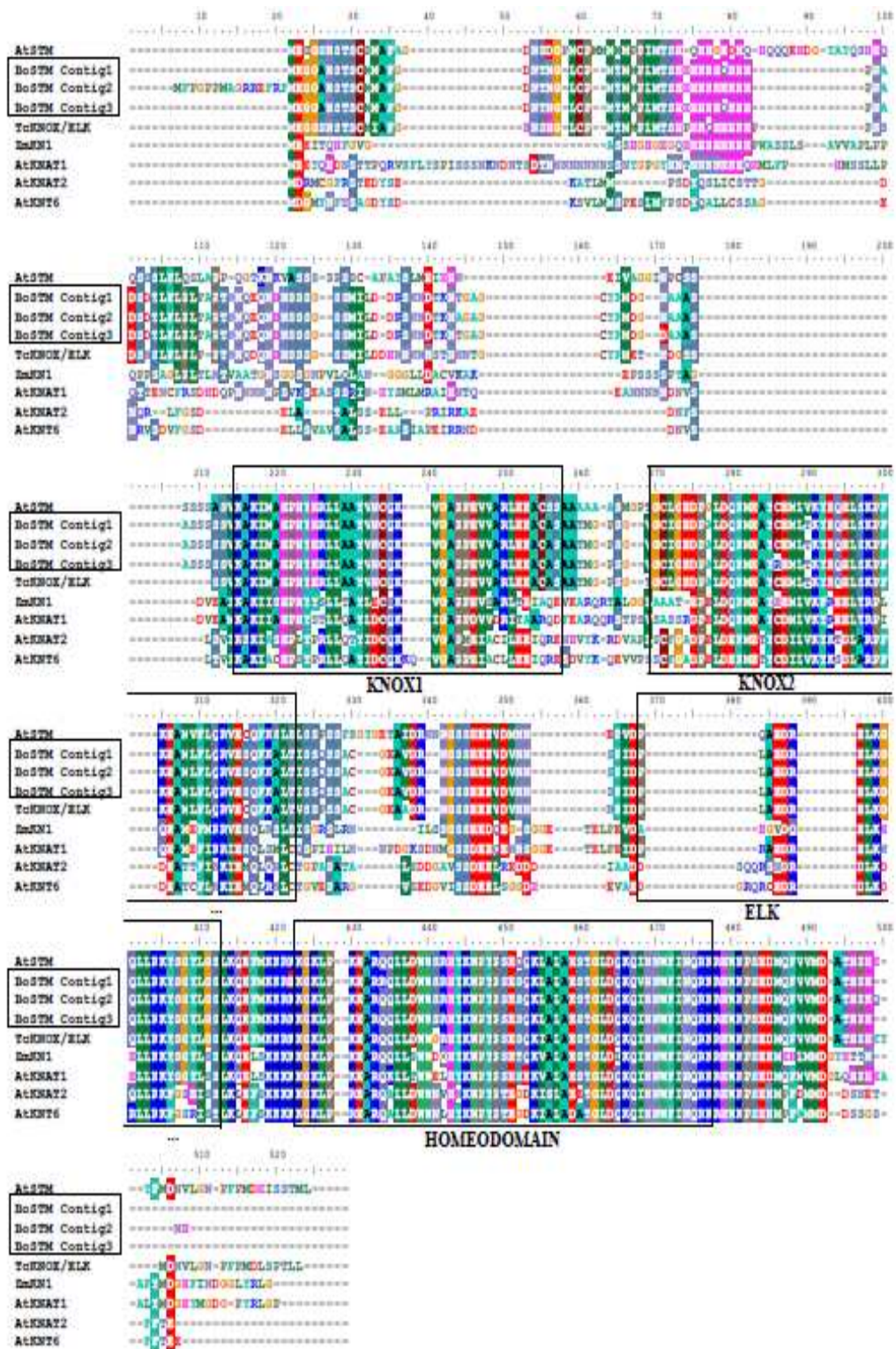
**Figure 1** – Alignment of deduced amino acid sequences of the BoContigs1, 2 and 3 with APETALA2-like transcription factors of *Theobroma cacao*, *Vitis vinifera*, *Carica papaya* and *A. thaliana*. The accession number of the sequences are available in phylogenetic tree (figure 3). The conserved domain AP2 are featured in the black boxes



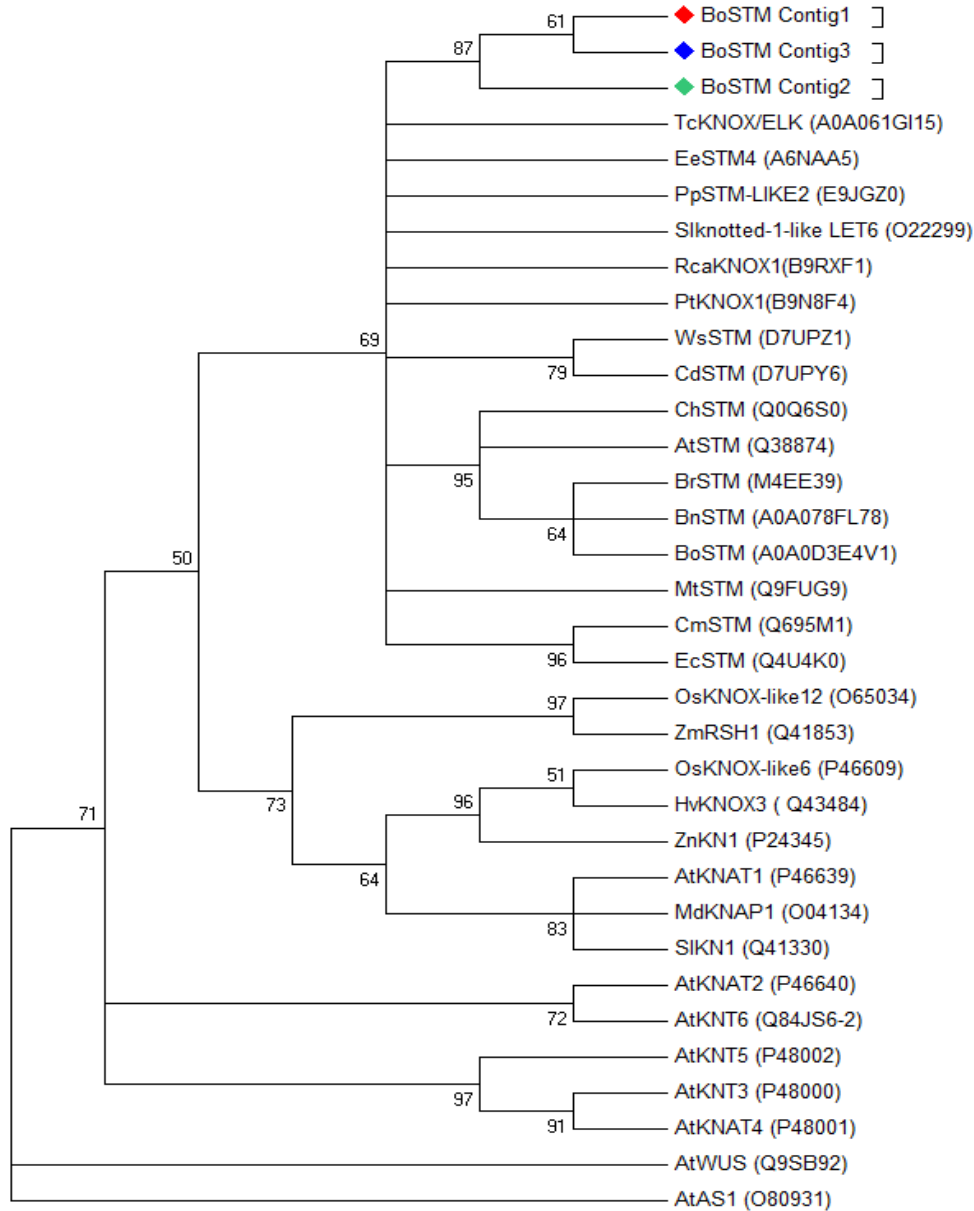
**Figure 2:** Unrooted phylogenetic tree constructed from deduced amino acid sequences from Bocontigs1, 2 and 3 and APETALA2-like family homologs available on databases. The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 9.14849522 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The evolutionary distances were computed using the JTT matrix-based method [3] and are in the units of the number of amino acid substitutions per site. The analysis involved 34 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 140 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [4].



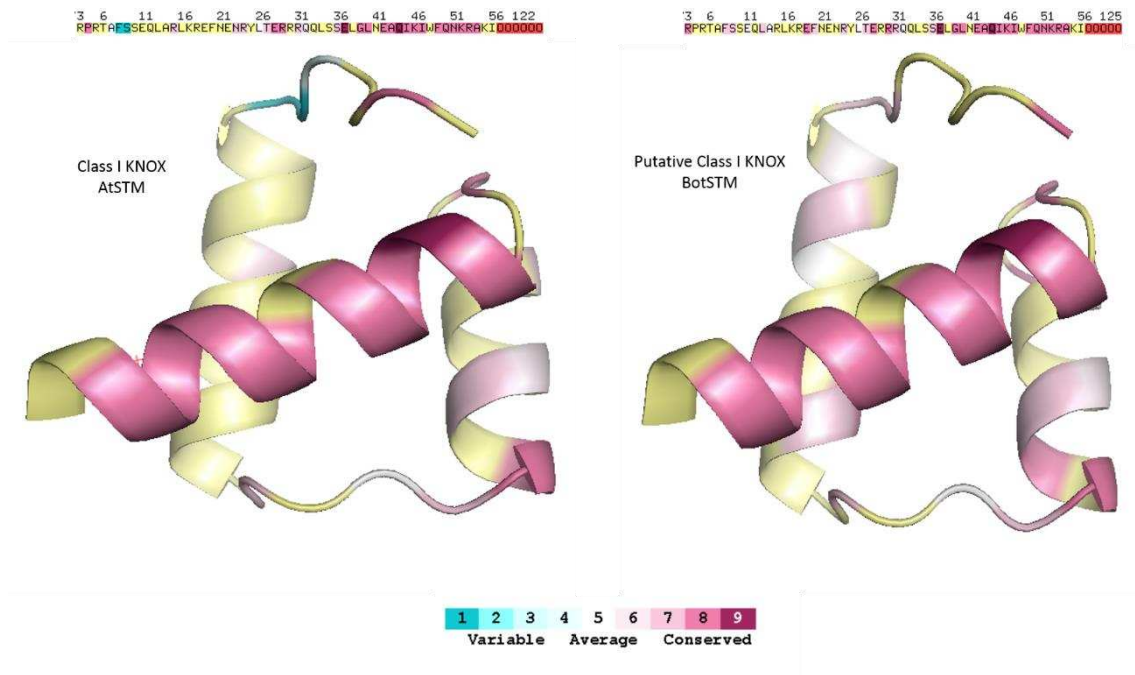
**Figure 3** – General scheme of BoSTM Contigs showing the conserved Class I KNOX domain protein, identified by CDD Conserved Domain database (NCBI).



**Figure 4** – Alignment of deduced amino acid sequences of the BoSTMContigs1, 2 and 3 with class I KNOX protein of *A. thaliana*, *Theobroma cacao* and *Zea mays*. The accession number of the sequences are available in phylogenetic tree (figure 5). The conserved domains KNOX1, KNOX2, ELK and homeodomain are featured in the black boxes



**Figure 5** - Unrooted phylogenetic tree constructed from deduced amino acid sequences from BocontigsSTM and Class I KNOX homologs available on databases. The evolutionary history was inferred using the Neighbor-Joining method [1]. The optimal tree with the sum of branch length = 9.14849522 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches [2]. The evolutionary distances were computed using the JTT matrix-based method [3] and are in the units of the number of amino acid substitutions per site. The analysis involved 34 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 140 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 [4].



**Figure 6** – Conservation in the homeodomain DNA-binding domain for AtSTM and a Putative Class I KNOX BoSTM. Score conservation for each deduced amino acid residue was determined by Consurf Algorithm, using as template the crystal structure of *Drosophila melanogaster* homeodomain (PDB id: 1enh). The input was the MSA of 21 plant Class I KNOX protein.

## GENERAL CONCLUSIONS

*In vitro* organogenesis in *Bixa orellana* is orchestrated by the cytokinins zeatin and meta-topolin, regardless of the explant type used. However, the wound and polarity inversion of the hypocotyl optimize the neoformation responses. The low accumulation of proteins and the absence of lipids as revealed by the histochemical tests did not coincide with structural changes. However, the high amount of starch grains observed indicates that this compound is an important reserve compound during the organogenic events in *Bixa orellana* L.

Adventitious rizogenesis process in *Bixa orellana* revealed that the formation of the lateral root occurred from of anticlinal and periclinal divisions of the parenchymatic cells adjacent to vascular system. The storage compounds like lipids and starch grains observed during the formation of lateral root confirm the essentiality of these compounds during the *in vitro* morphogenesis.

The amplification of cDNA with different degenerated primers allowed the isolation of partial sequences of transcription factors related with the organogenesis in *Bixa orellana*. The phylogenetic analyses suggest that the sequences analyzed are possible members of the *AP2-like* and Class I *KNOX* family, associated with the acquisition of competence, essential for expression of the marked pluripotency of annatto explant cells.