Journal of Forestry Research (2011) 22(2): 209–215
DOI 10.1007/s11676-011-0152-1

ORIGINAL PAPER

In vitro morphogenic response of leaf sheath of *Phyllostachys bambusoides*

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Received: 2010-08-07 Accepted: 2010-09-01

Abstract: Nodal segments from secondary branches of saplings of *Phyllostachys bambusoides* were inoculated in MS medium to assess the *in vitro* morphogenic response of leaf sheath through the induction to callogenesis by Picloram (4-amino-3,5,6-trichloropicolinic acid) at different concentrations of carbohydrate under the same conditions with presence or absence of luminosity. In our experiment, secondary explants were kept in MS medium containing 8.0 mg·L^{-1} of Picloram for the callus formation. Calluses were transferred in MS medium supplemented with sucrose, fructose and glucose (control, 2%, 4% and 6%). Results show that Picloram induced the callogenesis in leaf sheath. The secondary embryogenesis was formed in yellow-globular callus. The sucrose as carbohydrate source in the absence of light was more efficient to induce rhizogenesis. Glucose was more efficiency in the presence of light. Callogenic induction and further embryogenesis evidenced the competence and determination of leaf sheath cells.

Keywords: poaceae; tissue culture; callus induction; cell competence; determination

Foundation project: This study is supported by FAPESP (São Paulo Research Foundation, Brazil) and CAPES (Coordination for the Improvement of Higher Level, Brazil).
The online version is available at http:// www.springerlink.com

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Responsible editor: Zhu Hong

Introduction

Bamboo is a plant of the Poaceae (Gramineae family), the *Bambusoideae* subfamily, and its woody stem has great agricultural potential to contribute to a sustainable development in reducing the risk of erosion. In addition, economic interests have also been focused because of its diversified use in the production of pulp and paper, in the construction sectors (furniture production, handicrafts and pharmaceutical products), as well as in the food industry (Suzaki and Nakatsubo 2001; Sombroek et al. 2004; Rother et al. 2009). While there are still large natural populations of this group, there is concern with the preservation of species populations and conservation of germplasm (Gielis et al. 2001).

Phyllostachys bambusoides Sieb. & Zucc. is a bamboo species that are difficult to be reproduced from seeds. Its blooming periods are long intervals and irregular, ranging from 12 to 120 years (John and Nadgauda 2001; Mu et al. 2003; Zhao-Hua et al. 2003). Thus, the most largely method used for its reproduction is the separation of clumps, which is costly and unfeasible (Gielis et al. 2001). Therefore, the micropropagation technique can be considered as an excellent tool to continuously obtain plants and in large scale, regardless of their blooming and seeds for the reproduction.

Currently, *in vitro* propagation of bamboo is realized through somatic embryogenesis (Lin et al. 2004) and axillary buds (Jiménez et al. 2006; Ramanayake et al. 2006). However, Alexander and Rao (1968) reported successful *in vitro* culture of zygotic embryos from bamboo. Although there are several reports related to *in vitro* culture of some species of bamboo, such as *Dendrocalamus asper* (Arya et al. 2002), *Dendrocalamus giganteus* (Ramanayake et al. 2001), *Bambusa balcooa* (Lin et al. 2004; Das and Pal 2005; Kapoor and Rao 2006), *Guadua angustifolia* (Jiménez et al. 2006), to date, there are no records of this technique for the *Phyllostachys bambusoides*. Studies on bamboo leaf sheath used as explants are also unknown.

The kind of used explants can directly affect the *in vitro* morphogenic responses during the micropropagation process. The culture medium has great effect on growth and morphogenesis of cells, tissues and organs, besides playing a role in nutrition and changes of osmotic potential (Ramarosandratana et al. 2001).

Therefore, the objectives of this study were to assess: (1) the ability of *in vitro* morphogenic response from callus induced by Picloram from leaf sheath of *P. bambusoides*; (2) the induction of indirect embryogenesis and organogenesis by the source and carbohydrate; (3) rhyzogenesis under the same conditions with presence or absence of luminosity.

Materials and methods

Plant materials

Nodal segments with 10 to 15 mm of length containing dormant buds were sectioned from secondary branches of saplings of *Phyllostachys bambusoides* Sieb. & Zucc. and kept for three months under greenhouse conditions (shade house), receiving fertilizer (N: P: K: 10: 6: 10 + micronutrients) every 15 days.

Disinfection

The nodal segments (primary explants) were superficially washed for 15 min in running water, immersed in NaOCl (sodium hypochlorite) 30% (v/v) for 10 min, and were washed four times in sterile distilled water.

Treatment and culture conditions

After disinfection, the developed nodal segments with buds after culturing of four to five weeks were inoculated in MS medium (Murashige and Skoog 1962) supplemented with 0.5 $mg \cdot L^{-1}$ BA (6-benzylaminopurine). The leaf sheaths were extracted (secondary explants) and inoculated in MS medium added to 8.0 $mg \cdot L^{-1}$ Picloram. Both explants (primary and secondary) were kept in growth room at (25±2) °C and irradiance of 42 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a photoperiod of 16 h.

The calluses obtained from the leaf sheaths were inoculated in MS medium supplemented with three carbohydrate sources (sucrose, fructose and glucose), and each source was evaluated at concentrations of 0 (control treatment), 2%, 4% and 6%, in presence and absence of luminosity for the morphogenesis induction (Table 1).

Histological analysis

The samples were fixed in FAA-50 (formaldehyde acetic ethanol), dehydrated in tertiary butyl alcohol series and embedded in paraffin. After inclusion, the material was cut with 10 to 13 mm thick, stained with safranin and fast green (Sass 1951), analyzed and photomicrographed with a adapted camera (SANSUNG) in light microscope (ZEISS – JENEMED II) with the images obtained on the same scale.

Table 1. Carbohydrate source in the MS medium to induce the morphogenesis in callus of *Phyllostachys bambusoides*

Carbohydrate source	Concentration		
	%	mM	$mg \cdot L^{-1}$
Control	0.0	0.0	0.0
Sucrose (mw*: 342,30 / $C_{12}H_{22}O_{11}$ / Nuclear®)	2.0	58.4	20.0
	4.0	116.8	40.0
	6.0	175.2	60.0
Fructose (mw: 180,16 / $C_6H_{12}O_6$ / Ecibra®)	2.0	111.0	20.0
	4.0	222.0	40.0
	6.0	333.0	60.0
Glucose (mw: 180,16 / $C_6H_{12}O_6$ / Reagen®)	2.0	111.0	20.0
	4.0	222.0	40.0
	6.0	333.0	60.0

Note: * mw = molecular weight.

Statistical analysis

A randomized design in factorial arrangement (4×3) with four replicates was used, and four concentrations (control, 2%, 4% and 6%) and three different sources carbohydrates (sucrose, fructose and glucose) were evaluated, comprising six explants per replicate. The formation of morphogenic structures, the number of roots and the presence or absence of clusters of the meristematic cells were all evaluated weekly with the aid of a stereoscopic microscope.

Results and discussion

Phyllostachys bambusoides calluses were obtained from leaf sheaths after five weeks of *in vitro* cultivation. It's noteworthy that there are no reports on the use of the leaf sheath for the induction of calluses in bamboos (Ramanayake et al. 2001; Arya et al. 2002; Lin et al. 2003; Das and Pal 2005; Jiménez et al. 2006; Kapoor and Rao 2006; Agnihotri and Nandi 2009; Mudoi and Borthakur 2009). Hassan and Deberg (1987) were succeeded in inducing embryogenic and non-embryogenic calluses from leaf primordia of *Phyllostachys viridis*. In our study, the white-friable callus and yellow-globular callus (Fig. 1A) were observed. Rout and Das (1997) and Godbole et al. (2002) obtained the same results, however only the yellow-globular callus was characterized as embryogenic calluses.

Ramanayake and Wanniarachchi (2003) studied callus formation in *Dendrocalamus giganteus* at age of 70 years, and reported the formation of globular mucilaginous callus, which yielded proembryos-like structures. However, in our study, results showed that this type of callus (mucilaginous, Fig. 1A) is not yielded *in vitro* morphogenic response even after successive subcultures.

The occurrence of white-friable callus with granular texture and shiny appearance (Fig. 1B) was also reported in other bamboo species, such as *D. hamiltonii* (Godbole et al. 2002) and *D. giganteus* (Ramanayake and Wanniarachchi 2003). However, this type of callus also showed no morphogenic response in *P.*

bambusoides, being verified only in the increase of callogenic mass. These results corroborate the observations of Godbole et al. (2002) for *Dendrocalamus hamiltonii*. However, Rout and Das (1997) described the root formation from friable callus in *Bambusa vulgaris*.

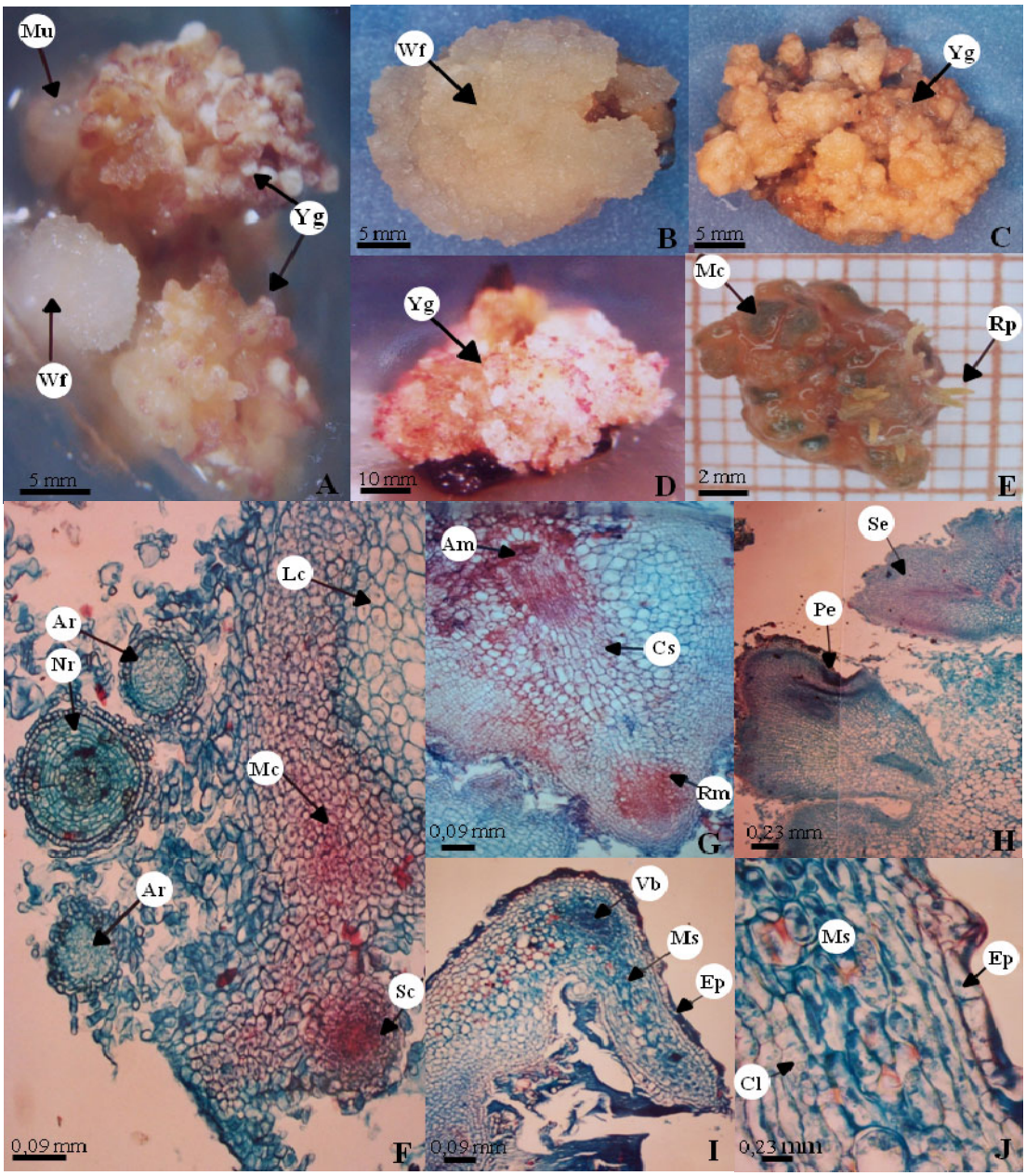

Fig. 1 Callus induction from leaf sheath of *Phyllostachys bambusoides*. **A-E** are types of calluses: **A** is Yellow-globular callus (Yg), white-friable (Wf) and mucilaginous (Mu); **B** White-friable callus (Wf); **C** Yellow-globular callus (Yg); **D** Yellow-globular callus with red-purple pigments (Yg); **E** Aspect of the yellow-globular callus with meristematic centers (Mc) and root primordia (Rp); **F-J** Photomicrographs of histological sections of yellow-globular callus showing: **F** Meristematic cells of meristematic centers (Mc); small cell with elevated NPR (nucleus plasmatic ratio) (Sc); large cell (Lc); normal root (Nr) and abnormal root (Ar); **G** Formation of embryogenic structure showing the apical meristem (Am), root meristem (Rm) and central cylinder-procambium (Cc); **H** Primary embryogenic structures (Pe) and secondary embryogenic structures (Se); **I** Leaf neoformation with flat structure dorsiventral showing: mesophyll differentiated in chlorenchyma (Ms), presence of vascular bundle (Vb) and epidermis (Ep); **J** Detail of the mesophyll showing the chlorenchyma (Ms), chloroplast (Cl) and epidermis (Ep).

The yellow-globular callus in *P. bambusoides* (Fig. 1C) showed a compact texture with some areas of red-purple pigmentation at the top of the globules (Fig. 1D). This pigmentation can be attributed to the presence of anthocyanin. According to Muralidharan and Mascarenhas (1987), there is a correlation between pigmentation and the ability for organogenic response. Cuenca et al. (2000) observed in the micropropagation process of *Fagus sylvatica* and *F. orientalis* that the regions that callus can

product differentiation buds were reddish. Nevertheless, Yoshihiro and Komamine (1981) emphasized that there are strong relationships between the anthocyanin synthesis and the somatic embryogenesis of *Daucus carota* L. cv. Kurodagosuns.

There are several studies on synthesis and accumulation of anthocyanins in calluses of different species (Mathur et al. 2009). After 18 months of cultivation of the leaf sheath of *P. bambusoides* we verified that morphogenic response only occurred in the areas with red-purple pigmentation on the yellow-globular calluses. The red-purple pigmentation always occurs before the organogenic induction, as a result of meristematic centers development (Fig. 1E). These results occurred independently from the type and concentration of the carbohydrate.

In these types of calluses, it is possible to distinguish the meristematic centers due to the presence of the clusters of small cells (Fig. 1F). The clustering these cells are defined as meristematic regions with intense activity of cell division. Cassells (1979) sustains that this peripheral meristematic activity, when they are replaced or complemented by the formation of meristematic centers, is essential for cellular differentiation in callus.

Considering that the embryogenic cells present typical characteristics (Durzan 1988), the histology allows to identify the multicellular origin of embryos (Fig. 1G) and also permits to observe the distal (shoot meristem) and proximal (root meristem) polarization. The origin of multicellular embryo was also reported by Saéns et al. (2006) in study with *Cocos nucifera*.

Transversal sections of yellow-globular callus of *P. bambusoides* (Fig. 1H) revealed that the primary embryogenic structures are similar to those observed by Toonen and Vries (1996) in zygotic embryos of monocots. However, the development of somatic embryos was not observed in *P. bambusoides* microplants, probably due to the occurrence of secondary somatic embryogenesis (Fig. 1H) (Li et al. 2002). According to Haccius (1978), the treatments were possibly effective in inducing embryogenesis from pro-embryonic cells. Secondary embryogenesis was also reported in study with the micropropagation of *Calamus manan* (Goh et al. 1999) and *Calamus merrillii* (Goh et al. 2001).

The addition of different carbohydrate sources in yellow-globular calluses is favored to the development of meristematic centers. The best result for inducing the meristematic centers was observed at 4% glucose (Fig. 2A), contradicting with results from Vu et al. (1993) that sucrose is the best source for cellular differentiation and microplant development.

Regarding specifically the role of sugars in organogenesis, they can act in the regulation of gene expression through specific signaling, especially sucrose, during transcription and translation of mRNA (Messenger ribonucleic acid) in plants, including also the induction of genes, protein production, expression and repression of genes (Farrar et al. 2000; Coruzzi and Zhou 2001; Koch 2004; Şener et al. 2008; Smet et al. 2009).

Concerning rhyzogenesis observed for *P. bambusoides*, the concentration of carbohydrate added to the culture medium in all treatments was always higher, compared to the control. According to Paiva and Otoni (2003), variations of carbohydrates concentration in the culture medium (e.g. sucrose, fructose or glucose) may influence the organogenic processes with callus formation by altering the osmotic potential.

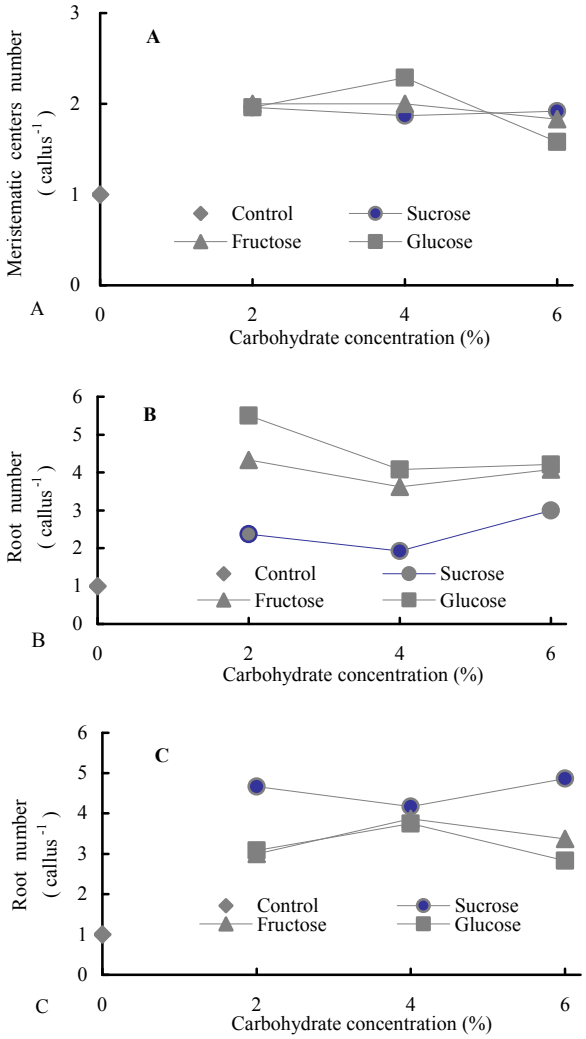

Fig. 2 Graphic representation of average number for meristematic centers and roots induced in yellow-globular callus of *Phyllostachys bambusoides* in the presence of sucrose, fructose and glucose at 56 days of *in vitro* culture. A is Average number for the meristematic centers; **B** Value for the roots induced under a photoperiod of 16 hours at 56 days of *in vitro* culture; **C** Average roots inducted without luminosity.

Assessments of rhizogenesis of *P. bambusoides*, in the presence of luminosity with sucrose, corroborate with the results presented by Jo et al. (2009) and Romano et al. (1995), who affirm the importance of the preconditioning explants at high concentrations of carbohydrates in inducing adventitious roots. However, our best result for rooting was obtained when the leaf sheathes were cultivated at 2% glucose (5.5 roots per callus) or 2% fructose (4.3 roots per callus) on average (Fig. 2B), agreeing with Lane (1978) that reported a proportional relation between reduction of carbohydrate (sucrose) and root induction. Coruzzi and Zhou (2001) described that there is strong interaction between the levels of carbohydrate and endogenous hormone, which directly affects the morphogenic process.

Relevant influence to *in vitro* morphogenic processes is attributed to luminosity. Ferreira and Handro (1988) found in *Stevia rebaudiana* that the organogenesis of callus tissue occurred preferentially in the presence of light. However, Ferreira and Handro (1987) also observed the neoformation of buds in absence of light. To *P. bambusoides* in the absence of light (Fig. 2C), the number of roots had average values of 4.7 (2% sucrose) and 4.9 (6% sucrose) per callus in media. It is shown that the sucrose had the best results, compared to the other carbohydrate sources.

Zimmerman (1984) affirmed that the absence of light is favorable for the rooting of many species. Nevertheless, Rugini et al. (1988) reported that the absence of light in the early days of culture, promotes rooting by reducing the action of IAA-oxidase (Indoleacetic acid oxidase), which underscores that there is the strong relationship between the carbohydrate source and plant hormone in proportion (Coruzzi and Zhou 2001). Therefore, it is believed that the embryo induction, followed by the death of the epicotyls (shoot apexes), facilitated the roots development, explaining why the somatic embryos do not develop themselves in plants of *P. bambusoides* in all treatments.

Most likely, rhizogenesis in callus of *P. bambusoides* occurred mainly due to the permanence of the callus in rhizogenesis induction medium. According to Dettmer et al. (2009), the *in vitro* formation of shoots and roots is controlled by the ratio of auxin to cytokinin, therefore, the higher concentration of auxin promoted the induction of roots. This effect can also be linked to the carbohydrate source (Coruzzi and Zhou 2001).

Histological analysis showed that the roots formed from callus of *P. bambusoides* presented distinct protoderm (i.e. typical roots), nevertheless, there was also the formation of atypical roots with noted structural disorganization (Fig. 1F). According to Christianson and Warnick (1988), the process of *in vitro* organogenesis can be divided into six stages: dedifferentiation; competence acquisition; induction; determination; differentiation and organ formation, thus, it appears that the structural disorganization in these roots, probably occurred in the stage of competence acquisition, which is related to the ability of a cell or tissue to respond to a stimulus. The failure of competence of a tissue may reflect in the lack of receptors for the plant growth regulator that will induce the organogenesis (MacArthur et al. 2009; Smet et al. 2009; Papp and Plath 2011; Smet and Beeckman 2011). Moreover, Coruzzi and Zhou (2001) reported that deactivation of the plant hormone may occur due to the hydrolysis of sucrose, glucose and fructose by altering the balance of active molecules of auxin and cytokinin.

The cellular division with a specification, arrangement and organization correct of the cells, are needed for normal plant development. These processes are controlled mainly by phytohormones in transcriptional networks, which act in feedforward loops for the growth and development of tissues (MacArthur et al. 2009). Currently, physiological and genetic analyses have proven that the position of the cell (cell-niche) in the tissue is the importance for their differentiation and subsequent development (Smet et al. 2009). This concept, also called habituation, is already known and is reported by Meins Jr. (1989) and Gaspar et al. (2002).

The loss of genetic capacity during cultivation is a limitation that occurs during successive subcultures. The causes for the loss of regenerative ability are still little studied and these losses vary with the circumstances of cultivation and species (Gaspar et al. 2002). In the case of induction by cytokines, it is believed that habituation would be maintained by a positive feedback loop in which cytokinins would induce their own synthesis or inhibit their own degradation (Meins Jr. 1989). In this sense, the ability of cell habituation is influenced by the developmental and physiological states of the cell.

The competence for habituation in plants emerges and persists when the cells are cultured *in vitro* (Meins Jr. 1989). Corroborating with the observations from Smet et al. (2009), the position of the cell into the tissue can influence the differentiation of some cells formed in calluses in *P. bambusoides* (Fig. 1I-J). The transdetermination in this case could be attributed to the change in the determination of the pericycle cells in the production of radicels and leaves.

In general, it can be inferred that Picloram showed ability to induce calluses in *P. bambusoides*. Although the primary and secondary somatic embryo induction was observed from yellow-globular callus, the plant development is not occurred. As for the rhizogenesis in the presence of luminosity, the glucose as a carbohydrate source was more effective. However, in the absence of light, the sucrose had better effect.

Histological sections of roots from yellow-globular calluses evidenced the possible occurring of transdetermination in pericycle cells, emphasizing the morphogenic competence of the pericycle cells.

The cellular competence and determination evidenced by leaf sheath cells allowed induction of callus, with subsequent organogenesis and somatic embryogenesis. However, future studies should be performed in order to maximize somatic embryogenesis and induce regeneration of plants.

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