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Xyloglucans from *Hymenaea courbaril* var. *stilbocarpa* seeds affect *Arabidopsis thaliana* seedling growth by enhancing lateral root development

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ABSTRACT – (Xyloglucans from *Hymenaea courbaril* var. *stilbocarpa* seeds affect *Arabidopsis thaliana* seedling growth by enhancing lateral root development). The effect of crude xyloglucan (XG) preparations from *jatobá* (*Hymenaea courbaril* var. *stilbocarpa* (Hayne) Y. T. Lee & Langenh.) seeds on *Arabidopsis thaliana* (L.) Heynh. root system development was investigated. The XG extracts exerted a dual effect on root system development by slowing down root growth and improving lateral root formation. These observed morphological changes were not due to oligosaccharides that could be generated following hydrolysis of the XG polymers, since XG hydrolysate induced a drastic inhibition of the overall growth process of the *Arabidopsis thaliana* seedlings. Histochemical test of *GUS* gene expression assay performed on seven and 14-days-old transgenic *Arabidopsis thaliana* plants carrying the CycB1;1-*GUS* fusion indicated that the improvement of the lateral root development by *jatobá* XG extracts was not correlated with the expression of this cell cycle marker gene in the root system. A potential agricultural application of *jatobá* seeds XG extract is discussed.

Key words - *jatobá*, rhizogenesis, xyloglucan activity

RESUMO – (Xiloglucanas de sementes de *Hymenaea courbaril* var. *stilbocarpa* afetam o crescimento de plântulas de *Arabidopsis thaliana* aumentando o desenvolvimento de raízes laterais). O efeito de preparações de xiloglucanas (XG) de sementes de jatobá (*Hymenaea courbaril* var. *stilbocarpa* (Hayne) Y. T. Lee & Langenh.) no desenvolvimento do sistema radicular de *Arabidopsis thaliana* (L.) Heynh. foi investigado. Os extratos de XG exerceram duplo efeito no sistema radicular, diminuindo o crescimento das raízes e aumentando a formação de raízes laterais. As alterações morfológicas observadas não foram causadas pelos oligossacarídeos que poderiam ter sido produzidos a partir da hidrólise do polímero de XG, já que o hidrolisado de XG induziu uma drástica inibição do processo de crescimento das plântulas de *Arabidopsis thaliana*. A análise histoquímica de expressão do gene *GUS* realizada com plantas de *Arabidopsis thaliana* transgênicas, com sete e 14 dias de idade, carregando a fusão CycB1;1-*GUS*, indicou que o melhor desenvolvimento das raízes laterais, com os extratos da XG de jatobá, não foi correlacionado com a expressão desse gene marcador do ciclo celular no sistema radicular. Uma potencial aplicação na agricultura dos extratos de XG de sementes de jatobá é discutida.

Palavras-chave - atividade da xiloglucana, jatobá, rizogênese

Introduction

One of the major cross-linking glycans of all primary cell walls of flowering plants is xyloglucan (XG), which possesses a β-(1,4)-glucan backbone to which α-(1,6)-xylosyl residues are attached (Reid 1985, Hayashi 1989, Carpita & McCann 2000, Lima *et al.* 2004). In germinating seeds of several plant species, the secondary cell wall of the cotyledons as well as of the endosperm contain little or no cellulose consisting mainly of XG that could have several functions such as mechanical dormancy, embryo protection and/or delivery of carbohydrates that are digested during germination (Carpita & McCann 2000).

Loosening and rearrangement of the cell wall during plant cell growth and differentiation requires cleavage and molecular grafting of XG chains, a process mediated by several enzymes among which xyloglucan endotransglucosylase (XET) is shown to play a critical role (Rose *et al.* 2002). In the root system of diverse vascular plants, high XET activity was localized in the epidermic cell wall of the elongation zone and in trichoblasts in the differentiation zone (Vissenberg *et*

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al. 2003). On the other hand, it has been demonstrated that the integration of XG mediated by the action of wall-bound XET suppressed cell elongation in pea stem segments by affecting microtubule orientation (Takeda *et al.* 2002), supporting the significance of XG integration in controlling the cell growth process. In contrast, these authors showed that the integration of XG-derived oligosaccharides (XGOs) accelerates cell elongation. Some of the XG-derived oligomers with particular structural features (such as reduced forms of XXLG or XXXG) have been shown to act as signalling molecules, suggesting the occurrence of specific recognition systems for these oligosaccharides in plants (Vargas-Rechia *et al.* 1998).

XG extracted from *Hymenaea courbaril* var. *stilbocarpa* (Hayne) Y. T. Lee & Langenh. (*jatobá*) seeds, a Brazilian native tree, was found to be useful as a partial substitute for agar as a gelling agent in culture media for *in vitro* plant micropropagation (Lima-Nishimura *et al.* 2003). These authors also showed that a mixture of 0.4% agar and 0.2% *jatobá* XG resulted in an improved multiplication rate, reduced callus formation and shoot hyperhydricity and particularly a better rooting performance. In the absence of auxin, the rooting rate of Marubakaido apple microcuttings reached 70.8% on a medium solidified with a mixture of agar and XG, instead of 6.7% on the agar control medium.

In the present study *Arabidopsis thaliana* (L.) Heynh. was used as a model plant in order to assess the effect of *jatobá* seeds XG on root system development.

Material and methods

Xyloglucan extracts – Seeds of *Hymenaea courbaril* (Fabaceae) were collected at two different locations in Brazil: Cuiabá (south of Mato Grosso State) and Sinop (centre of Mato Grosso State). These seeds were weighted and the tegument was removed manually after seed treatment with boiled water. The polysaccharide was extracted from the seeds with water in a blender with controlled velocity. The viscous extracts obtained were centrifuged at 7000 rpm for 40 minutes and the supernatant was treated with sodium chloride 0.1 M and 1-2 volumes of ethanol. The precipitated polysaccharide was collected and dried at 25 °C, giving XGC (xyloglucan extracted from *jatobá* seeds from Cuiabá) and XGS (xyloglucan extracted from *jatobá* seeds from Sinop). For the purification process samples of XGC in aqueous solution were passed through Millipore filter membranes with pore sizes of 3 and 0.8 μm, this product was called XGCP and through membranes with pore sizes of 3, 0.8 and 0.22 μm, this product was called XGCPP. After filtrations the polysaccharide was precipitated with 1-2 volumes of ethanol.

Xyloglucan analysis – Total XGC carbohydrate content was determined by the phenol-sulphuric method (Dubois *et al.* 1956) and the protein content of samples of XGC, XGCP and XGCPP was determined by the method of Hartree (1972). Monosaccharide contents of XGC were determined by complete acid hydrolysis with 2 M trifluoroacetic acid (TFA) at 100 °C for 8 hours (Adams 1965). The products of hydrolysis were reduced with sodium borohydride ($NaBH_4$) and acetylated with pyridine-acetic anhydride (Wolfrom & Thompson 1963). The resulting alditol acetates were analysed by GLC using an HP model 5890-2, with a DB-225 cappilary column at 220 °C, a flame ionization detector at 250 °C and nitrogen as carrier gas. Freitas *et al.* (2005) assayed the XGS by the same method.

XGC hydrolysis – XGC was hydrolysed with a cellulase from *Trichoderma reesei* (Celluclast 1.5L) (Novo Nordisk®, Bioindustrial do Brasil). The enzymatic solution was prepared with 144 mg cellulase (3.36 EGU mL^{-1}) dissolved in 0.5 M acetate buffer (pH 5.0). Cellulase solution and XGC were mixed (144 mg cellulase for 150 mg XGC) at 64.5 °C for 45 minutes. After hydrolysis, enzymatic inactivation was carried out for 20 min at 100 °C. The product of this hydrolysis was freezed, lyophilized and called XGCH.

Transgenic *Arabidopsis thaliana* (C24) plants – Transgenic *Arabidopsis thaliana* (C24) plants containing the Arath;CycB1;1-*GUS* fusion (Ferreira *et al.* 1994) were grown under axenic conditions. Surface disinfected seeds were incubated for germination in Murashige & Skoog (1962) semi-solid culture medium (MS) supplemented with 0.029 M sucrose. After addition of XGs or XGCH and 2,4-dichlorophenoxyacetic acid (2,4-D), the media were autoclaved for 20 minutes at 120 °C and 1.5 atm. The seeds were incubated at 6 °C overnight and then maintained at 26 ± 2 °C under fluorescent light (Philips "white comfort") providing a photon flux density of 40 μmol m^{-2} s^{-1}, and photoperiod of 16 hours. Four-day-old seedlings were used to test the effect of XGs and XGCH. To calculate the XGC and XGS molar concentrations, the molecular masses, respectively, of 2.2×10^6 and 1.05×10^6 g mol^{-1}, as determined by Freitas *et al.* (2005), were used. To calculate the XGCH molar concentration the molar mass of the octasaccharide that appears in highest amount in the polysaccharide structure of *jatobá* seeds, the oligosaccharide XXLG was used.

Each treatment formulated consisted of two replicates with five seedlings each. The experiment was repeated twice. After seven and 14 days of treatment, the root length, the number of lateral roots and the hypocotyl length were scored. The histochemical β-glucuronidase (*GUS*) assays were performed on seven and 14-day-old seedlings as described by Jefferson *et al.* (1987). The data were analyzed statistically by one-way analysis of variance. The mean values were compared by Tukey's multiple range tests using the Michigan State University statistical package.

Results

Xyloglucan composition – The total carbohydrate content of XGC was 73.5% (w/w). The protein composition of the samples (w/w) was 8.7% for XGC, 5.6% for XGCP and 5.6% for XGCPP. The monosaccharide composition of the XGC, as alditol acetate derivative, was glucose, xylose and galactose, in the molar ratio of 3:2.6:1, respectively. The results obtained by Freitas *et al.* (2005) showed that the protein and carbohydrate levels are only slightly different. Total carbohydrate content of XGC was 86% and XGS was 85%. The protein content of the samples was 2.9% for XGC and 2.2% for XGS.

Arabidopsis thaliana seedling growth – After seven days of culture in the presence of 500 nM XGC and 1200 nM XGS extracts, the root length was reduced in comparison to the MS control cultures (figure 1A). This effect was greater after 14 days of culture, particularly for seedlings grown in the presence of XGS extract, which showed a significant root growth reduction even at a 10-fold dilution (120 nM). Interestingly, the number of lateral roots increased in seedlings cultured for 14 days in both *jatobá* XG extracts (figure 1C). Among the different treatments, the maximum number of lateral roots was observed after 14 days of culture in the medium supplemented with 1.2 or 120 nM XGS. Finally, hypocotyl length was slightly affected by the XG extracts, in the presence of 500 nM XGC after seven days of culture and in 1200 nM XGS after seven and 14 days of culture (figure 1B). In fact, in *A. thaliana* seedlings cultured in the presence of 0.1 µM 2,4-D, root length was reduced and number of lateral roots was increased, when compared to control auxin-free cultures (figure 3).

To better characterize the physiological activity observed, the effect of cellulase hydrolysed XGC (XGCH) was investigated. As shown in figure 2, the overall growth parameters of the *A. thaliana* seedlings were affected by the XGCH. Root (figure 2A) and hypocotyl (figure 2B) growth were significantly reduced in seedlings cultured for 14 days in the presence of 250 nM XGCH. Moreover, these seedlings did not form lateral roots (figure 2C). When seven-day-old seedlings were transferred to XGCH-free medium, seedling growth resumed, indicating a reversible growth-inhibiting effect. However, growth of 14-day-old treated seedlings did not resume on XGCH-free medium, indicating a toxic effect of XGCH after prolonged time of incubation (Salamoni 2004).

Change of expression in transgenic *Arabidopsis thaliana* seedlings – The effects of *jatobá* XG on the growth of *A. thaliana* seedlings were monitored by following the

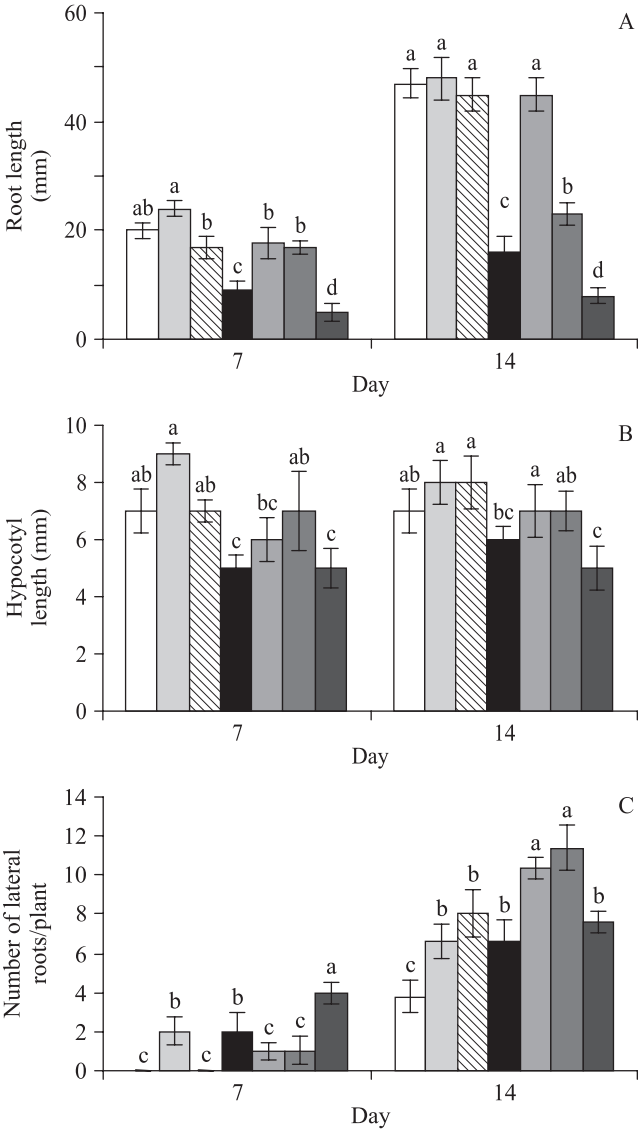

Figure 1. Effect of two xyloglucans (XGC and XGS) on root length (A), hypocotyl length (B) and number of lateral roots/plant (C) of *A. thaliana* seedlings. Each value represents the mean (± SD) of ten plantlets. (□ = MS; ▭ = XGC 0.5 nM; ▨ = XGC 50 nM; ■ = XGC 500 nM; ▭ = XGS 1.2 nM; ▭ = XGS 120 nM; ▭ = XGS 1200 nM).

changes in expression of CycB1;1 in transgenic plants which carry the CycB1;1 promoter-*GUS* fusion. The expression profile of Arath;CycB1;1 is restricted to G2 and M phases and to actively dividing cells (Ferreira *et al.* 1994, Shaul *et al.* 1996). Transgenic *A. thaliana* seedlings were cultured either in a control MS medium or in the same basal medium supplemented with 500, 1200 and 250 nM of XGC, XGS and XGCH, respectively. The combined effect of XGs and 2,4-D (0.1 µM) was also investigated (figure 3). In an auxin-free medium, *GUS* expression was not detected within root system of seven

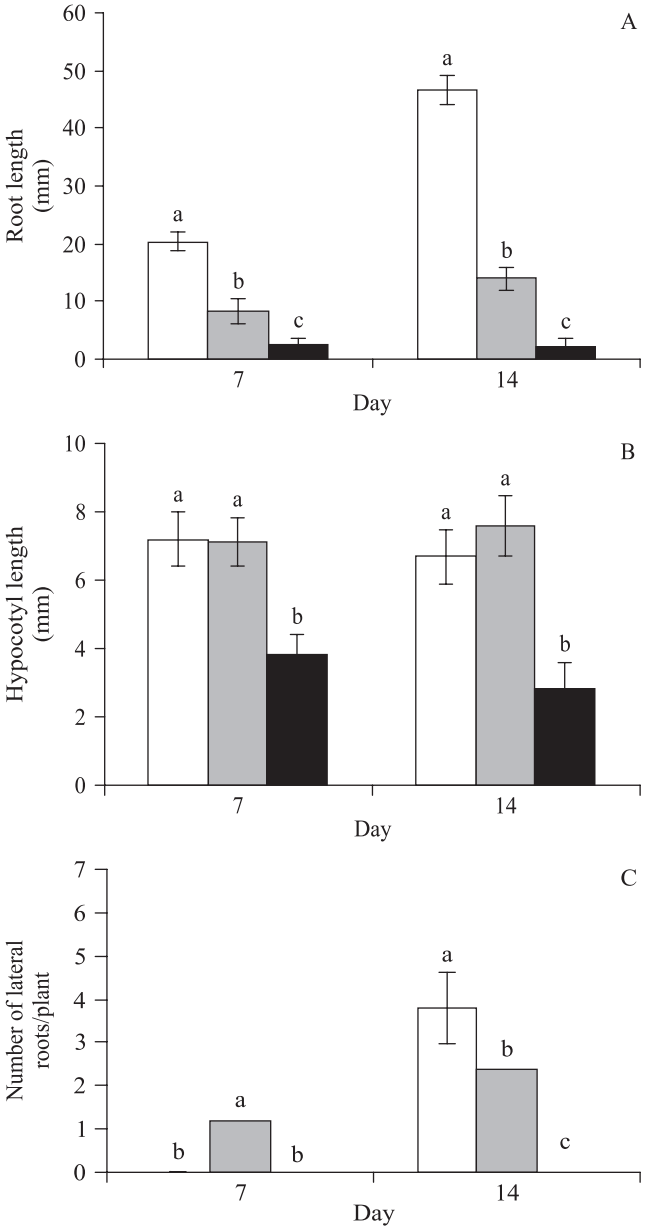

Figure 2. Effect of XGCH on root length (A), hypocotyl length (B) and number of lateral roots/plant (C) of *A. thaliana s*eedlings. Each value represents the mean (± SD) of ten plantlets. (□ = MS; ▒ = XGCH 25 nM; ■ = XGCH 250 nM).

and 14-day-old seedlings independently from XGs or XGCH supply (figure 3A-H). In 2,4-D supplemented medium, *GUS* expression within the root system was localized in lateral root meristems (figure 3I). These new root meristems further developed into lateral roots and *GUS* expression was no longer detected (figure 3J). However, when crude XG extracts (XGC or XGS) were combined with 2,4-D, *GUS* expression within the root system was maintained throughout the culture period till day 14 (figure 3K-N). Moreover, these induced root meristems did not elongate further, resulting in a short lateral root system (figure 3L and N), whereas lateral roots were well developed in XG supplemented medium only (figure 3D and F). These data suggest a synergic effect between XG and auxin. XGCH inhibited both main root growth and lateral root formation independently from the 2,4-D supply. Within the aerial part of the plantlets cultured in the presence of XGS, *GUS* expression was localised in the cotyledons and in the 2-3 first leaves (figure 3E, F, M and N). This expression was much reduced or absent in plants grown on XGC media (figure 3C, D, K and L).

Discussion

In an attempt to improve the performance of *in vitro* micropropagated plants, it has been found that in the absence of auxin, a mixture of agar-*jatobá* XG as gelling agent for culture media enhances the multiplication rate, reduces the occurrence of hyperhydric shoots and improves rooting of adventitious shoots (Lima-Nishimura *et al.* 2003). The present study was conducted in order to better characterize the effect of crude *jatobá* XG extract and XG hydrolysate on root system development.

As the difference between XGC and XGCP protein composition was low (8.7 to 5.6%), it was not necessary to purify the extract through Millipore filter membranes. Therefore, the crude XGCs characterized as glycopeptides were used.

We were able to demonstrate that crude *jatobá* XG extracts (XGC and XGS) can act by inhibiting root growth and improving lateral root formation. This dual physiological effect is dose-dependent (figure 1) and shows auxin-like effect. Furthermore, *jatobá* XG hydrolysate reversibly inhibits the root system development. The auxin-like effect of *jatobá* XGs on *A. thaliana* root development (figure 3) may be due to the presence of active oligosaccharides released by hydrolysis of XG following metabolization by the plant enzymes xyloglucan endotransglucosylases, glucanases and xylosidases. However, this effect may also be due to the hydrolysis of oligosaccharides into the monosaccharides glucose, xylose and galactose.

Rolland & Sheen (2005) indicated the role of sugars as signalling molecules in plants. The flexible and reversible responses to both low and high glucose signals in plant growth promotion and inhibition, respectively, depend on cell type, developmental state, multiple nutrients status and environmental conditions. According to Léon & Sheen (2003) the transcript levels

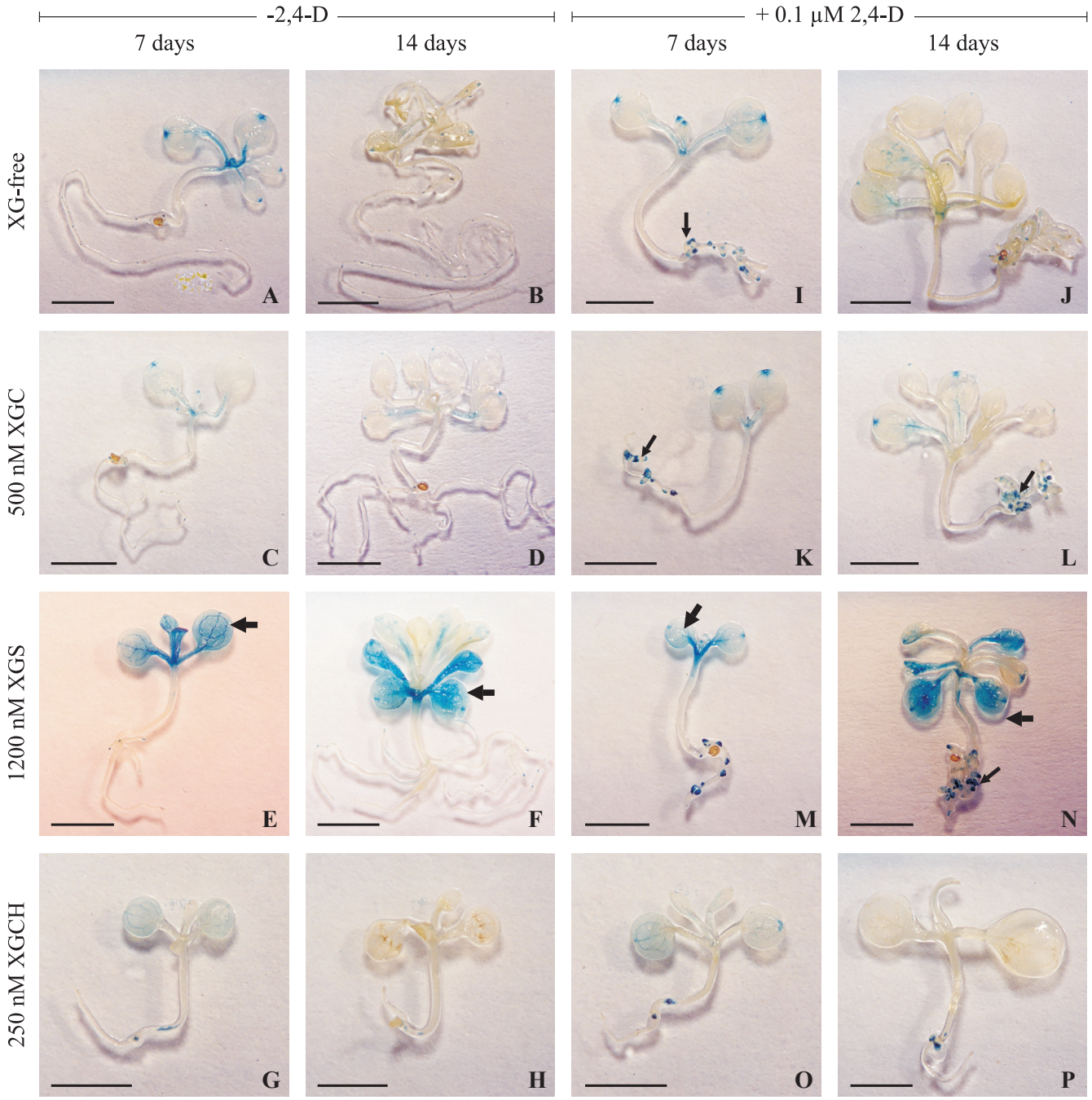

Figure 3 (A-P). *GUS* expression pattern and seedling phenotypes of 7- and 14-day-old *A. thaliana* seedlings cultured in XG-free medium or in medium supplemented with 500, 1200 or 250 nM of XGC, XGS or XGCH, respectively. The experiment was performed in auxin-free medium or in the presence of 0.1 µM of 2,4-D. Narrow arrows show the lateral root meristem. Thick arrows show the *GUS* expression in the aerial part of the plantlets. Bar = 5 mm.

of several abscisic acid (ABA) biosynthesis genes are raised by glucose, suggesting a direct, specific glucose modulation of ABA biosynthesis genes and ABA accumulation. The effect of excess glucose during the seedling establishment stage results in an arrest of seedling growth and differentiation that seems to be mediated partly by the increase in ABA biosynthesis and by the activation of same ABA signaling genes. It will be interesting to carry out more-detailed analyses of the expression patterns of other ABA biosynthesis genes and their responses to sugars as well as their roles in sugar signaling. While sucrose is the most important transport sugar in plants, most regulatory effects can be ascribed to glucose (Ramon *et al*. 2008).

The observed effects cannot be attributed to a possible modification of the physical properties of the solid medium following addition of XG extract, since rheologic measurements indicated that the consistency of these media was identical to that of medium containing agar only (Lima-Nishimura *et al.* 2003). Moreover, experiments conducted with *A. thaliana* plantlets cultured in liquid medium supplemented with either XGs or XGCH, confirmed the effects on rooting that we observed in solid medium (Salamoni 2004). Differences in *GUS* expression in *A. thaliana* seedlings grown in medium supplemented with XGC or XGS indicated a difference in the effect of both *jatobá* XG extracts, probably related with their fine chemical composition which may differ in function of their geographical origin.

Freitas *et al.* (2005) related differences in the relative proportion of XXXG, XXLG, XLLG + XXXXG and XXLXG oligosaccharides between both xyloglucan polymers. The concentration of the mixture XLLG+XXXXG was 30.3% for XGC and 25.8% for XGS. So, as firstly observed by Buckeridge *et al.* (1997) for *jatobá* from São Paulo, the XGC probably presents more oligosaccharides of the new series. Moreover, there was little difference in the total content of carbohydrates and proteins for *jatobá* seeds collected in different regions of Brazil.

Mechanical changes underlying primary cell wall re-modelling and consequently the growth process has been shown to be mediated by the XETs (Chanliaud *et al.* 2004). Beside their function as a catalyst of cell wall loosening, XETs are responsible for the incorporation of new XG molecules synthesized during plant growth (Sulová *et al.* 2003). The suppression of root elongation observed in the presence of 500 nM XGC (figure 1A) could be explained by the integration of XG into the cell wall by the plant XET activity. A similar effect has been described for pea stem segments where incorporation of XG suppressed cell wall extension and, consequently, cell elongation (Takeda *et al.* 2002). These authors showed that integration of XG into the primary cell wall induces rearrangement of cortical microtubules from a transverse to a longitudinal position resulting in the suppression of cell elongation.

Seedlings cultured for 14 days in the presence of XG extracts (figure 3D and F) or 2,4-D (figure 3J) developed a similar number of lateral roots but for the latter the root length was reduced, indicating a different mode of action between XG and auxin at least during the root elongation process. Further molecular analyses based on differential gene expression between XG and 2,4-D treated plants will certainly contribute to the identification of molecular markers associated with root meristem formation and/or root elongation.

It was also shown that morphological changes induced on *A. thaliana* root system cultured in the presence of XG were not due to oligosaccharides that could be generated following hydrolysis of the XG polymers because XGCH induced a drastic inhibition effect on the overall growth process of the *A. thaliana* seedlings, indicating a possible action of the XGCH component(s) on fundamental growth processes in plant. Nevertheless, up to 7 days post-treatment, this inhibiting effect is reversible, as seedling growth resumed after transfer into an XGCH-free medium. New studies need to be carried out with isolated XGOs in order to understand the role of each of them on root growth and lateral root formation.

From the results presented in this study, a potential agricultural and/or horticultural application may be considered for *jatobá* XGs. Root system implementation is a crucial developmental process especially during the first stage of plant growth. A well-developed root system results in an increased root surface allowing efficient nutrient assimilation by the plant either directly from the soil or through rhizosphere-associated plant growth promoting rhizobacteria and fungi. Seeds pre-treated with *jatobá* XG extract may therefore represent an interesting approach for the improvement of plant growth efficiency, by providing seedlings with a well-developed root system. Moreover, *jatobá* XG can be used as a substitute for auxin during the rooting stage of *in vitro* micropropagated plantlets.

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