

# Galactoglucomannan oligosaccharides are assumed to affect tracheary element formation via interaction with auxin in *Zinnia* xylogenic cell culture

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## Abstract

**Key message** Galactoglucomannan oligosaccharides seem to interact with auxin in xylogenic cell culture, thus influencing mainly metaxylem-like tracheary element differentiation depending on timing with hormones and the process kinetics.

**Abstract** Complex mapping of *Zinnia* mesophyll cell transdifferentiation into tracheary elements with or without prior cell division was documented after palisade and spongy parenchyma cell immobilization during the first 4 days of culture. Here, we report a positive effect of galactoglucomannan oligosaccharides on cell viability and density and higher metaxylem-like tracheary element formation in xylogenic cell culture. The maximal positive effect was achieved by the simultaneous addition of the oligosaccharides and growth hormones (auxin, cytokinin) to the cell culture medium. Moreover, a large number of metaxylem-like tracheary elements were observed in a low-auxin medium supplemented with oligosaccharides, but not in a low-cytokinin medium, suggesting a close relationship between auxin and the oligosaccharides during tracheary element formation.

**Keywords** Auxin–galactoglucomannan oligosaccharides interaction · Auxin-mediated process · Cell division · Metaxylem-like tracheary elements · Xylogenic cell culture

## Introduction

Vascular plants contain many cell types that work in a coordinated way to support growth, development, reproduction, and to respond to environmental stress. The expression of intrinsic cell differentiation programs during the life cycle leads to the production of various cell types. Tracheary elements (TEs) are distinct from other cells in their ontogenic process and behavior (Kuriyama and Fukuda 2000). Their morphological features allow them to be easily distinguished within a heterogeneous cell culture, making TEs an excellent model to study plant cell differentiation.

TE induction and formation is difficult to study in intact plants. However, Fukuda and Komamine (1980a) introduced an excellent in vitro cell system by which, under certain conditions, *Zinnia* mesophyll cells, formerly adapted for photosynthesis, can transdifferentiate into TEs. TEs are easily identified based on their morphological characteristics, the hormone-initiated induction process is straightforward, and many biochemical and molecular markers have been identified. These features enable the study of TE cell differentiation at the cellular level.

In *Zinnia* cells, various signaling inputs are known to influence single steps in the differentiation process. This process could be induced by a wound signal together with light, auxin, cytokinin, ethylene, brassinosteroids, phytosulfokine (Kuriyama and Fukuda 2000), or oligosaccharins, which are relatively less well-known

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signaling molecules involved in intercellular communication (Roberts et al. 1997). One interesting group of cell wall polysaccharides, which is also a source of biologically active oligosaccharides, are mannans. Mannans are widespread among plants, where they serve as structural elements in cell walls, as carbohydrate reserves, and potentially perform other important functions. Mannan polysaccharides in the plant cell wall may fulfill a role comparable to that of xyloglucans, impacting flexibility and forming growth-constraining networks with cellulose (Whitney et al. 1998; Schröder et al. 2001, 2004). Galactoglucomannans (Sims et al. 1997; Ebringerová et al. 2005) belong to these hemicelluloses and despite their low molecular mass, they are closely associated with either cellulose microfibrils or another insoluble cell wall polymer, most likely through hydrogen bonding (Schröder et al. 2009). Galactoglucomannan oligosaccharides (GGMOs) belong to a class of oligosaccharins which possess biological activity that is closely connected to auxin/growth hormones. Auxtová-Šamajová et al. (1996) demonstrated that GGMOs inhibit 2,4-dichlorophenoxyacetic acid (2,4-D)-induced elongation growth and hypothesized that GGMOs could directly bind free auxin or an auxin receptor and thereby inactivate it. Further studies (Kollárová et al. 2005, 2010) indicated that GGMOs are potential competitive antagonists of auxins. GGMOs inhibited primary root elongation of mung bean induced by low auxin (IBA) concentration ( $10^{-8}$  M) and stimulated this elongation inhibited by high auxin (IBA) concentration ( $10^{-4}$  M). However, GGMOs also affect morphogenesis in vitro and influence the viability and regeneration of isolated protoplasts (Lišková et al. 1999; Kákoniová et al. 2010).

In xylogenic *Zinnia* cell culture, GGMOs (50  $\mu\text{g/ml}$ ) affected cell population density and secondary wall patterning (Beňová-Kákošová et al. 2006). *Zinnia* macroarrays, established with cDNA from a PX-rich library by Pesquet et al. (2005), probed with transcriptomic profiles of cells cultured 60 h (corresponding to the onset of TE secondary wall deposition) in the presence or absence of GGMOs indicated that the majority of genes (21 out of 27) were down-regulated in response to GGMOs. The fact that an auxin-induced gene, *zIAA8*, was down-regulated by GGMOs provided an argument that GGMOs may also interact with auxin-signaling pathways during in vitro TE formation.

The aim of this work was to examine how GGMOs affect the developmental process in xylogenic cell culture at the physiological level. The impact and comparison of different GGMOs concentrations, timing of GGMO addition with respect to growth regulators, and the possible interactions between GGMOs and growth regulators were determined.

## Materials and methods

### Plant material

Seeds of zinnia (*Zinnia elegans* L. cv. Envy provided by Hem Zaden B.V., Venhuizen, the Netherlands) were grown in potting soil in controlled environmental chambers under a 16 h photoperiod, 170  $\mu\text{E/m}^2/\text{s}$  irradiance, at 27 °C.

### Xylogenic cell culture

Mesophyll cells were isolated from the first true leaves of 14-day-old seedlings and cultured in a liquid medium containing 0.1 mg/l 1-naphthaleneacetic acid (NAA) and 0.2 mg/l 6-benzyl-aminopurine (BAP) in the dark at 27 °C under continuous rotation on a revolving drum at 100 rpm (Fukuda and Komamine 1980a). The solid medium was supplemented with 0.75 % (w/v) agarose (type VII: low gelling temperature, Sigma-Aldrich, Saint Quentin Fallavier, France).

### Galactoglucomannan oligosaccharides

GGMOs (mixture of DP 4–8) were prepared by partial acid hydrolysis of galactoglucomannan from spruce (*Picea abies* L. Karst) secondary cell walls and purified by gel-permeation and paper chromatography (complete characterization in Capek et al. 2000). GGMOs backbone consists of (1  $\rightarrow$  4)-linked  $\beta$ -D-mannopyranosyl and  $\beta$ -D-glucopyranosyl residues distributed at random, having single stubs of (1  $\rightarrow$  6)-linked  $\alpha$ -D-galactopyranosyl residues attached to both mannosyl and glucosyl residues with slight preference to mannosyl residues. Oligosaccharides were filter sterilized before use and were added to the culture medium after autoclaving. Stock solutions of GGMOs were aqueous; therefore, the control was also treated with water. Hormones (NAA and BAP) and GGMOs (concentrations 0.0008, 20, 50, and 100  $\mu\text{g/ml}$ ) were added to culture media immediately at the beginning of culture, if not shown otherwise.

### Light microscopy

Cell viability was determined by staining with 0.2 % (w/v) Evans Blue. Cell density, as the number of viable cells in 1 ml of medium, was calculated after staining with 0.01 % (w/v) Calcofluor White (Fluorescent brightener 28, Sigma-Aldrich, Saint Quentin Fallavier, France) and 0.1 % (w/v) DAPI (Sigma-Aldrich, Saint Quentin Fallavier, France). For cell viability and density determination, 200 cells from each of three parallels (600 cells per treatment) were counted using inverted microscope (DMIRBE, Leica, Wetzlar, Germany) with bright field optics or epifluorescence

illumination. Photographs were made by a CCD camera (Color Coolview, Photonic Science, UK).

Annular, annular-helical, and helical secondary cell wall patterns were assigned to protoxylem-like (PX-like) TEs, helical-reticulate and reticulate to reticulate (R) TEs and scalariform, reticulate-pitted, and pitted pattern to metaxylem-like (MX-like) TEs. The percentage of PX, R, MX-like TEs was calculated by dividing the number of PX, R, MX by total TE number and multiplying by 100.

#### Statistical analysis

The results were analyzed statistically by Statgraphics 5.1. The differences among experimental groups were evaluated by analysis of variance (ANOVA) and comparisons among the mean values were made by the least significant difference (LSD) test at  $P < 0.05$  and standard deviations (SD) were calculated.

## Result and discussion

### *Zinnia* system in liquid and solid culture

Adding auxin and cytokinin to freshly isolated *Zinnia* mesophyll cells in liquid medium induced synchronous TE differentiation. Cytological studies previously described that 60 % of differentiated cells are single; they have not undergone previous division (Fukuda and Komamine 1980b). In contrast, in our liquid culture we found only 25 % of differentiated cells are single and the other differentiated cells had been divided. In addition to cell division, during dedifferentiation (the first 2 days), cell expansion, vacuolization, and loss of chloroplasts have been noted. The elongation and division of most cells began after 48 h of cultivation, although Pesquet and Tuominen (2011) pointed out that the first cell divisions already occurred after 36 h. On the contrary, Mourelatou et al. (2004) reported that only some *Zinnia* cells were elongated and DAPI and Calcofluor staining proved the first cell divisions after 72 h.

To monitor secondary cell wall thickenings in individual cells, *Zinnia* cells were immobilized in a solid culture medium. Although certain sculptured patterns characteristic of TEs appeared on the 3rd day of culture, mature TEs were observed on the 4th day. Those cells transdifferentiated into TEs with or without prior cell division (Fig. 1).

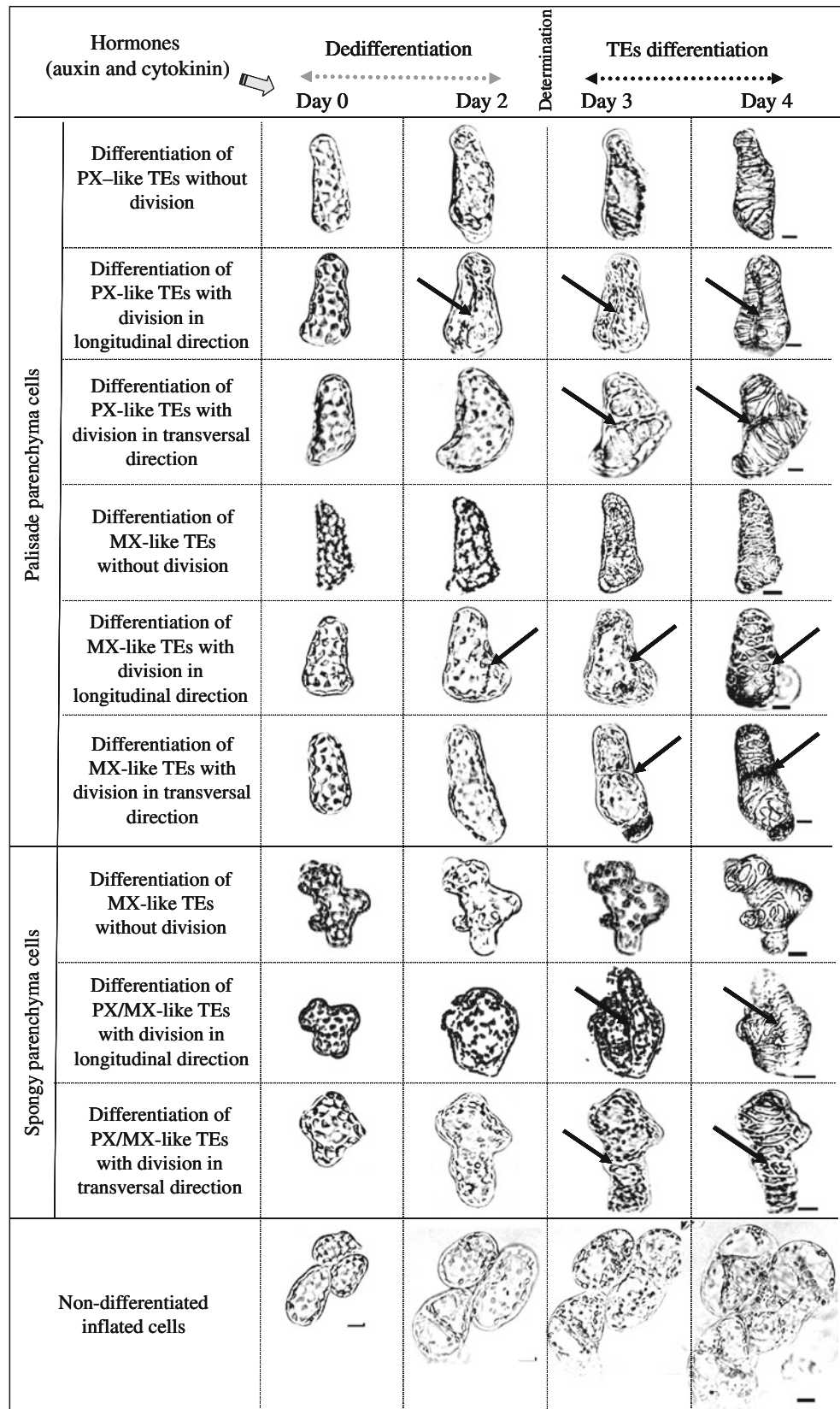
Fukuda (1996) reported TE induction from various cell types including phloem parenchyma cells, root cortex, pith parenchyma in shoots, tuber parenchyma or mesophyll and epidermal leaf cells. Likewise, although the majority of differentiated cells investigated in our experiments were derived from the palisade parenchyma, differentiation also

occurred from spongy parenchyma. The differentiation of both PX-like and MX-like TEs took place with or without prior cell division. Some palisade parenchyma cells differentiated into PX-like TEs ( $13 \pm 0.2$  %) and MX-like TEs ( $13 \pm 0.8$  %) without prior cell division. PX-like TEs originated from cells after transverse ( $20 \pm 0.8$  %) or longitudinal ( $14 \pm 1.9$  %) division. MX-like TEs represented after transversal division  $34 \pm 2.4$  %, and after longitudinal division approximately  $7 \pm 0.7$  % of the TE population. Ten to twenty percent of differentiating cells originated from spongy parenchyma cells, of which MX-like TEs represented up to 98 % of these differentiating cells, the rest being PX-like TEs. Numerous, highly inflated cells with at least two nuclei were also observed on the 4th day among living non-differentiated cells.

### GGMOs impact of different concentrations on *Zinnia* xylogenic cell culture

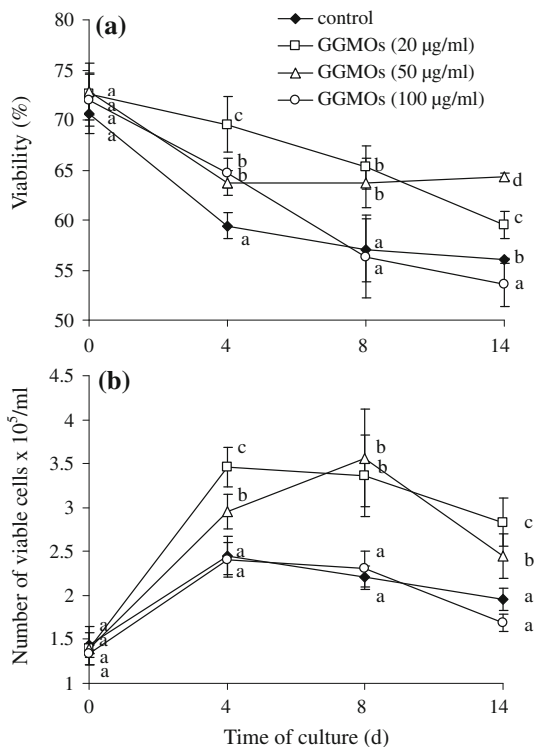
It is evident that the effect of GGMOs on cell cultures is likely to be complex. That claim is underlined by the fact that stimulatory or inhibitory effects of GGMOs on auxin-induced elongation growth are dependent on the concentration and timing of oligosaccharide action, the plant organ, and their interaction with hormones (e.g. auxins, gibberellin—Kollárová et al. 2005, 2006, 2009). Based on these experiments and inspired by Navazio et al. (2002) who tested the role of oligogalacturonides (200 µg/ml) in soybean cells, we were interested in determining the effect of various concentrations (20, 50, 100 µg/ml) of GGMOs on xylogenic *Zinnia* cultures. The impact of 50 µg/ml GGMOs was previously reported (Beňová-Kákošová et al. 2006); however, herein, we report a range of concentrations to pinpoint the possible complexities underlying GGMO activity. To characterize GGMOs as potential signaling factors in *Zinnia* cultures, we firstly monitored their effect on cell viability and density. Although the highest GGMO concentration (100 µg/ml) had no effect on either viability or density of living cells, lower GGMO concentrations (20 and 50 µg/ml) demonstrated a positive effect on both parameters from the 4th day of culture (Fig. 2a, b). GGMOs increased the viability of *Zinnia* cells as in the case of spruce protoplasts isolated from callus cultures (Kákoniová et al. 2010). The lowest (20 µg/ml) GGMO concentration appeared to be more effective for cell viability and density at the beginning of the time course, but its effectiveness decreased at later stages. Next, we monitored the effect of GGMOs on TE formation. As a result, the highest stimulation of MX-like TE formation by 20 and 100 µg/ml GGMOs was observed between the 8th and 14th day of culture (Fig. 3). Our observation also confirms the high efficiency of 50 µg/ml GGMOs on all investigated parameters as published by Beňová-Kákošová

**Fig. 1** Tracheary element differentiation from *Zinnia* mesophyll (*palisade and spongy parenchyma*) cells on solid cell culture medium with or without prior cell division. Cell divisions are indicated with *arrows*. The inflated cells originate from undifferentiated mesophyll cells. Light microscopy without staining. Bars 10 μm

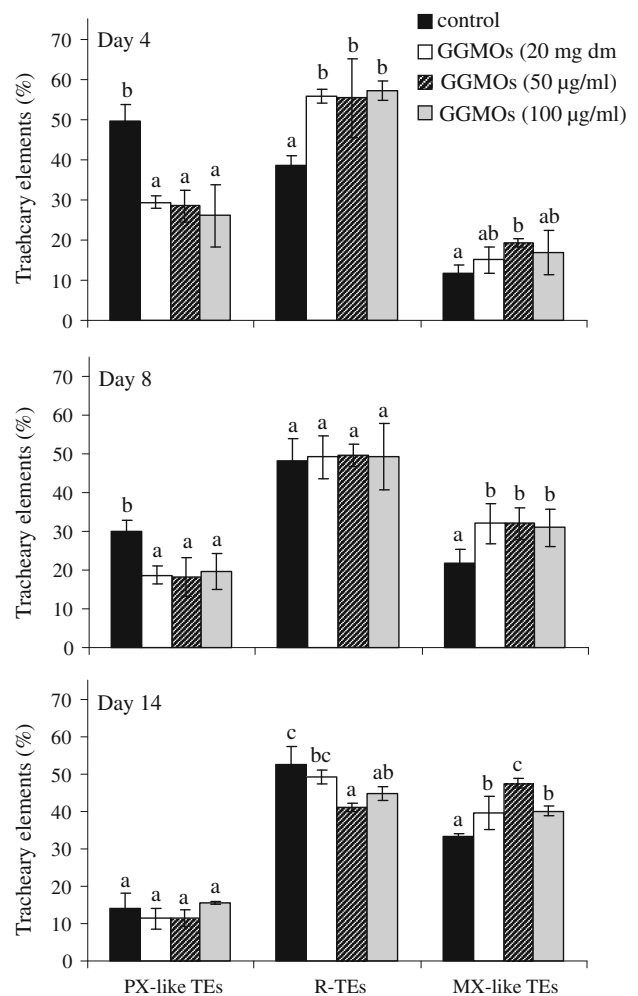


et al. (2006). From results obtained, it is evident that GGMOs in the concentration 50 µg/ml are probably reliable for the significantly highest MX-like TE formation. Positive impact of GGMOs on MX-like TE formation increased with time. This result correlates with a study by Roberts et al. (1997) who reported a naturally occurring oligosaccharide produced at about the 7th day of culture in xylogenic *Zinnia* cultures that was involved in the coordination of cell expansion, differentiation and regulation of MX-like TEs.

It is known that GGMOs have several biological activities even at very low ( $10^{-8}$ – $10^{-10}$  M) concentrations (Auxtová et al. 1995; Auxtová-Šamajová et al. 1996; Lišková et al. 1995). We therefore tested the low concentration of GGMOs (0.0008 µg/ml =  $10^{-9}$  M) previously used in auxin-induced elongation growth experiments (Auxtová-Šamajová et al. 1996) in *Zinnia* TE cultures. As expected, it was statistically indicated that this concentration of GGMOs positively affected cell viability (Fig. 4a), cell density (Fig. 4b), as well as MX-like TE formation (Fig. 5). Notably, the percentage of MX-like TEs after this GGMOs treatment was 1.5–2.5 times higher between the 6th and 12th day of culture compared with the control.



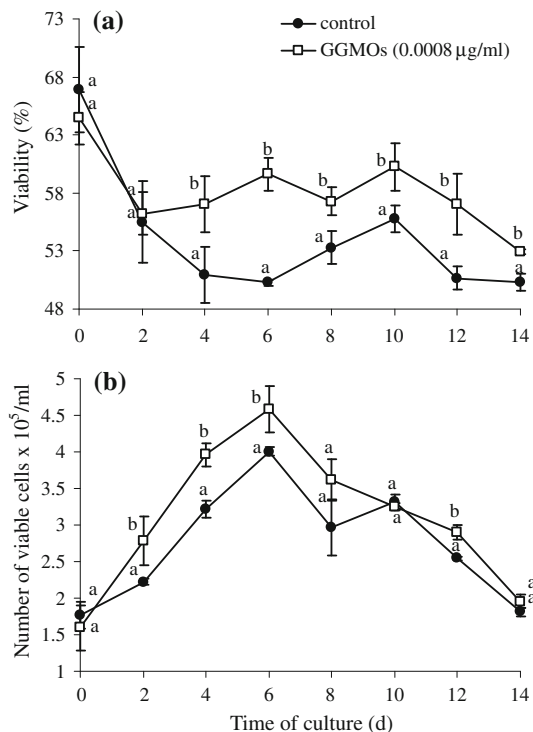
**Fig. 2** Addition of GGMOs (20 or 50 µg/ml) to *Zinnia* xylogenic cell cultures has a positive effect on cell viability (a) and cell density (b) as compared with control or 100 µg/ml GGMO cultures. Values are the means ± SD of three independent experiments. Different letters above bars indicate significant differences ( $p < 0.05$ )



**Fig. 3** Comparison of TEs in cultures supplemented with different concentrations of GGMOs (20, 50 and 100 µg/ml) on the 4th, 8th and 14th day of culture. 50 µg/ml GGMOs showed the most stimulatory effect on MX-like TE formation. Values are the means ± SD of three independent experiments. Different letters above bars indicate significant differences ( $p < 0.05$ )

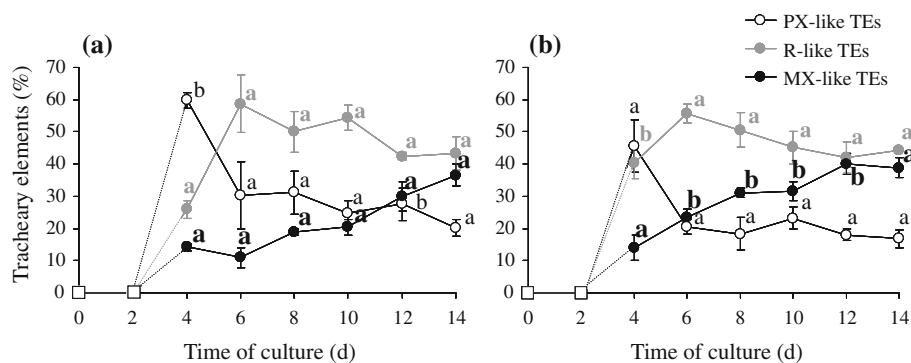
### Timing of GGMOs and hormones action in *Zinnia* xylogenic cell culture

To elucidate possible GGMO–hormone interactions on TE development, we added GGMOs (50 µg/ml) and hormones at different time points with respect to each other (sequentially or together) to *Zinnia* cell culture. The treatments were as follows: (1) hormones and GGMOs were added together at the beginning of the experiment, (2) GGMOs added 24 h after preincubation with hormones, (3) hormones and GGMOs added together after 24 h of preincubation of cells in hormone-free medium, and (4) GGMOs added at the beginning of the culture and hormones added 24 h later. The second treatment, addition of GGMOs 24 h after preincubation with hormones, showed a positive effect on all of the parameters investigated (viability, density, MX-like TEs) on



**Fig. 4** Effect of very low GGMO concentration on *Zinnia* culture fitness. 0.0008 µg/ml GGMOs increased both cell viability (a) and density (b). Values are the means ± SD of three independent experiments. Different letters above bars indicate significant differences ( $p < 0.05$ )

the 4th day of culture, but the effect on the 8th day was weaker than that in the first treatment in which GGMOs were added simultaneously with hormones at the beginning of the culture (Figs. 6a, b and 7a). Beňová-Kákošová et al. (2006) found that GGMOs down-regulated an auxin-induced gene, *zIAA8*. From this result, it was assumed that GGMOs could be involved in MX-like TE formation through an auxin-signaling pathway; however, the final type of the TE formation could be dependent on appropriate timing.



**Fig. 5** Effect of very low GGMO concentration on *Zinnia* TE formation. Comparison of *Zinnia* TE formation in control cell cultures (a) and in cell cultures treated with 0.0008 µg/ml GGMOs (b). GGMOs increased the proportion of MX-like TEs from the 6th to the

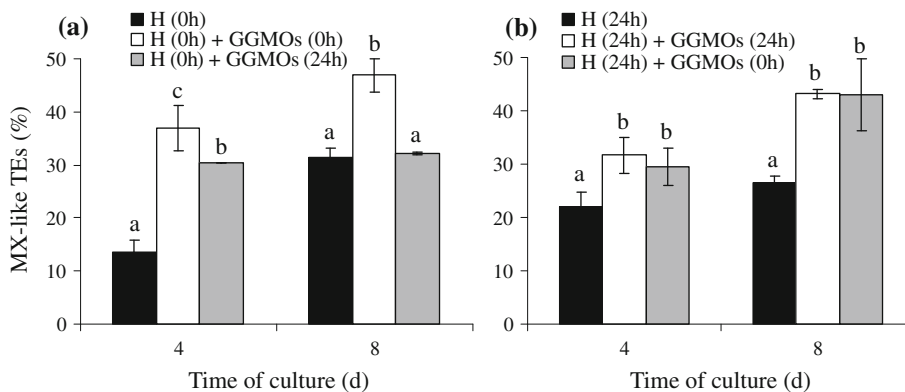
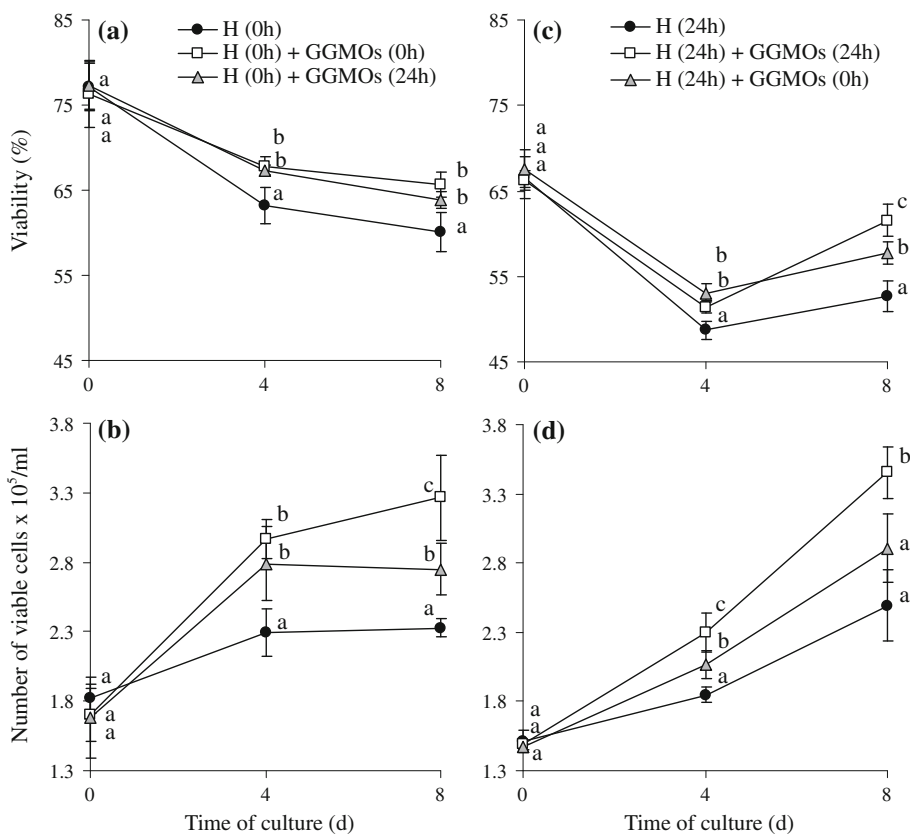
12th day. Statistical comparison was performed between (a) and (b). Values are the means ± SD of three independent experiments. Different letters above bars indicate significant differences ( $p < 0.05$ )

GGMOs added 24 h before the hormones induced a similar effect on the cell viability, density, and MX-like TE formation as in the culture with GGMOs and hormones added simultaneously after 24 h of preincubation in the medium without auxin and cytokinin (Figs. 6c, d, and 7b). We can assume that the GGMOs and auxin interaction is dependent on timing of their action. The most effective was their simultaneous application (either at the beginning of culture or 24 h after cells preincubation in the hormone-free medium) which is contrary to probable interaction of GGMOs and growth hormones in the process of elongation growth, where the highest values of growth inhibition induced by GGMOs were observed in case when the plant material was 24 h preincubated with GGMOs and auxin was added after this time. It could indicate a different mechanism of GGMO action with growth hormones in the differentiation process compared with elongation growth (Kollárová et al. 2006, 2010). The results suggest a certain degree of specificity in terms of oligosaccharide action in distinct developmental processes, in cells with different determination of ontogenesis, and the subsequent processes which they undergo, probably species specificity, their concentration in certain physiological process, and their interaction with different types and concentrations of auxin (Kollárová et al. 2005, 2006, 2010).

#### GGMOs versus auxin and/or cytokinin interaction in *Zinnia* xylogenetic cell culture

Auxin and cytokinin evidently play pivotal roles in TE formation. Transcriptional profiling in an *Arabidopsis* or *Zinnia* vascular system revealed that an auxin response factor, *MP/ARF5*, (Badescu and Napier 2006) and a functional cytokinin receptor, *WOL/CRE1/ATHK4*, (Mähönen et al. 2000) are expressed in procambium, suggesting that auxin and cytokinin are key factors controlling procambium maintenance and/or development. Demura et al.

**Fig. 6** Timing of GGMO and hormone action in *Zinnia* cell cultures. GGMOs (50 µg/ml) were added simultaneously with hormones at the beginning of the experiment, 24 h after hormone addition (a, b), 24 h before hormone addition, or simultaneously with hormones after 24 h of incubation in basal medium (c, d). GGMOs enhanced cell viability (a, c) and density (b, d) when added 24 h before or after hormone addition. The most pronounced effect of GGMOs was observed when GGMOs were added simultaneously with hormones. Values are the means ± SD of three independent experiments. Different letters above bars indicate significant differences ( $p < 0.05$ )



**Fig. 7** Timing of GGMO and hormone action on MX-like TE formation. GGMOs (50 µg/ml) were added simultaneously with hormones at the beginning of culture, 24 h after hormone addition (a), 24 h before hormone addition, or simultaneously with hormones after 24 h of incubation in basal medium (b). Simultaneous GGMO and

hormone addition had the most pronounced effect on MX-like TE formation. Values are the means ± SD of three independent experiments. Different letters above bars indicate significant differences ( $p < 0.05$ )

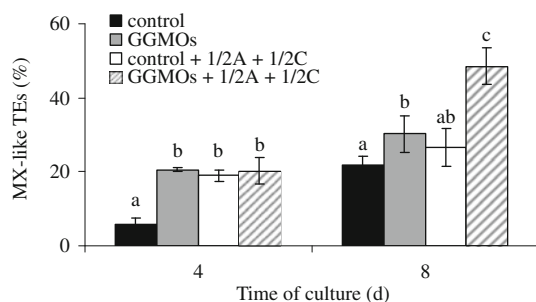
(2002) reported that in the presence of both auxin and cytokinin, the expression of genes associated with auxin signal transduction occurs during the stage corresponding to the process of differentiation from procambial initials into precursors of TEs (24–48 h from the beginning of *Zinnia* cell culture), which in turn regulates the xylem differentiation-dependent program. Therefore, crosstalk between auxin and cytokinin occurring in procambium or procambial initials influences transcription of genes

responsible for xylem development. Based on these data and because the fact that as signaling molecules GGMOs stimulate TE differentiation in *Zinnia* xylogenic cell cultures, we were interested in how the reduction of auxin and cytokinin concentration in the medium with GGMOs would influence TE differentiation.

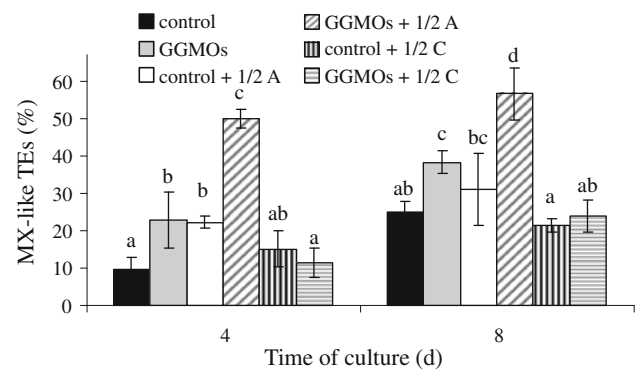
We first compared TE cultures in medium containing standard hormone concentrations (NAA 0.1 µg/ml and BAP 0.2 µg/ml) in the presence or absence of 50 µg/ml

GGMOs with those cultivated in medium supplemented with half of the standard hormone concentrations (0.05  $\mu\text{g/ml}$  NAA and 0.1  $\mu\text{g/ml}$  BAP) in the presence or absence of GGMOs. As shown in Figs. 7a and 8, on the 4th day of culture, the number of MX-like TEs increased more than twofold in cultures with standard hormone concentrations supplemented with GGMOs as compared with control cultures. Interestingly, the same level of increase was also observed in cells cultured in medium containing half the standard hormone concentration with or without GGMOs. In addition, MX-like TE formation remains two times higher in the culture with GGMOs and half concentration of both auxin and cytokinin compared with the control on the 8th day of culture.

We then investigated which of the two hormones could be associated with the action of GGMOs during TE differentiation. Toward this aim, we performed experiments with a standard cytokinin concentration (0.2  $\mu\text{g/ml}$ ) and half the standard auxin concentration (0.05  $\mu\text{g/ml}$ ), or the opposite; that is half standard cytokinin (0.1  $\mu\text{g/ml}$ ) and standard auxin concentrations (0.1  $\mu\text{g/ml}$ ) in the presence or absence of 50  $\mu\text{g/ml}$  GGMOs. As a control, the standard medium described by Fukuda and Komamine (1980a) (auxin 0.1  $\mu\text{g/ml}$  and cytokinin 0.2  $\mu\text{g/ml}$ ) in the presence or absence of GGMOs was used. In cultures with reduced auxin, a relatively higher proportion of MX-like TEs was observed compared with the cultures with standard medium on the 4th day of culture (Fig. 9). MX-like TEs occurred in higher amounts not only in cultures with GGMOs, but also in cultures with half of the normal auxin concentration in the absence of GGMOs. The presence of MX-like TEs increased almost two times in cell cultures



**Fig. 8** The effect of GGMOs on MX-like TE formation in *Zinnia* cultures grown in medium containing half of the standard concentration of both auxin (1/2A) and cytokinin (1/2C). 50  $\mu\text{g/ml}$  GGMOs in both standard medium and in medium containing a decreased hormone concentration stimulated MX-like TE formation compared with the control on the 4th and 8th day of culture. MX-like TE formation was also higher in the culture with half-hormone concentrations, even in the absence of GGMOs as compared with the control but only on the 4th day of culture. Values are the means  $\pm$  SD of three independent experiments. Different letters above bars indicate significant differences ( $p < 0.05$ )



**Fig. 9** The effect of GGMOs on MX-like TE formation in *Zinnia* cultures grown in medium containing either half of the standard auxin or cytokinin concentration (1/2A and 1/2C, respectively). 50  $\mu\text{g/ml}$  GGMOs added to control and half-auxin medium stimulate MX-like TE formation compared with controls on the 4th and 8th day of culture. MX-like TE formation was also higher in half-auxin cultures in the absence of GGMOs compared with controls on the 4th day of culture. Half-cytokinin concentrations had no effect on TE formation in the presence or absence of GGMOs. Values are the means  $\pm$  SD of three independent experiments. Different letters above bars indicate significant differences ( $p < 0.05$ )

grown on media with reduced auxin concentration and five times with the addition of GGMOs compared with the cultures without GGMOs and standard hormone content. MX-like TE number was significantly higher in the medium with GGMOs also on the 8th day while the percentage of these TEs was 20 % higher in the culture with GGMOs and half-auxin compared to the culture with GGMOs and standard hormone concentrations.

It is known that auxin concentration influences the development of individual TE types (Leyser 2002). For example, higher auxin content causes a decrease in TE diameter, whereas on the contrary, lower auxin concentration leads to TEs with a larger diameter (Klee et al. 1987). Esau (1977) suggested that MX vessels have a larger diameter and they differentiate after the cessation of organ expansion in discord with PX. Our observations in xylogenic *Zinnia* cultures with reduced auxin are comparable with the above-mentioned results in which lower auxin concentration caused TE formation with larger diameter.

Although Mähönen et al. (2006) reported that cytokinins can negatively control protoxylem vessel differentiation, in our case, cytokinin concentration did not significantly affect TE formation (Fig. 9).

In conclusion, it is likely that GGMOs act as regulatory factors in TE differentiation, mainly of MX-like TEs and their interaction with auxin may be an essential feature in the cytodifferentiation process.

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