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ORIGINAL ARTICLE

Arabinogalactan proteins improve plant regeneration in barley (*Hordeum vulgare* L.) anther culture

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Abstract Androgenesis-based methods of doubled haploid (DH) production show considerable variation in efficiency in different barley genotypes. Arabinogalactan proteins (AGPs) have been shown to play a key role in several developmental processes, including embryogenesis, in different plant species. In this study we investigated the effect of exogenous AGPs from gum arabic on androgenesis and the regeneration efficiency in barley anther culture. Supplementation of the induction medium with 10 mg l^{-1} gum arabic increased the total plant regeneration rate up to 2.8 times; when exposure to GA was extended to also include the pretreatment step, the regeneration rate was up to 6.6-times higher than in control. The effect of gum arabic was reversed by the Yariv reagent, an AGPs antagonist. This suggests a direct involvement of AGPs in androgenic development from barely microspores. Addition of gum arabic reduced cell mortality, increased the frequency of mitotic divisions of microspores and the number of multicellular structures (MCSs) when compared to control. The positive effect of gum arabic also included reduction in time required for the androgenic induction and substantially improved the quality of formed embryos. Observations made in this study imply a complex role of AGPs during androgenic development and confirmed the usefulness of gum arabic in production of barley androgenic plants.

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Introduction

Haploid embryogenesis is an unusual development of male cells of the gametophytic pathway. Under the influence of exogenous stress the microspores change their pathway to sporophytic. They divide mitotically and develop into embryos capable of regenerating plants (Goralski et al. 2005). After spontaneous or induced doubling of the chromosome number, plants obtained from androgenic embryos become doubled haploids (DHs). DH generation techniques have been of great value to breeders, by speeding up cultivar development (Forster et al. 2007; Germanà 2011) as well as to researchers by creating ideal material for transformation or genetic mapping (Kumlehn et al. 2006; Poland et al. 2012). The usefulness of androgenesis as the means of DHs production depends on reproducibility and efficiency, preferably independent of genetic constitution of the donor plants stocks (Ferrie and Caswell 2011; Grauda et al. 2014).

Barley (*Hordeum vulgare* L.) is considered a model plant for androgenesis research as it offers high regeneration efficiency, at least in some cultivars such as Igri (Cistué et al. 1998; Kasha et al. 2001). In Igri, over 1000 plantlets per 100 responding anthers have been obtained, with a high fraction (88%) of green plants (GPs) (Jacquard et al. 2006). However, in most barley genotypes the efficiency of GPs production is much lower (Cistué et al. 1998; Li and Devaux 2001; Castillo et al. 2014). For a range of winter and spring barleys the average GPs regeneration rate per 100A was 6.4 and 1.3, respectively (Makowska et al. 2015). This wide disparity in androgenic response among genotypes may depend on a number of endo- and exogenous elements. One

of them is production of androgenic structures with varying regeneration abilities (Larsen et al. 1991; Pretová et al. 2006; Makowska et al. 2015). In a model course of androgenesis, microspores develop into embryos similar to the zygotic ones (Sangwan and Sangwan-Norreel 1996; Wrobel et al. 2011), however in practice, haploid cells may develop into a variety of structures. Among them are embryos with well-formed scutellum, root and shoot apical meristems and coleoptile. These convert directly into haploid or DH plants. In most cases, however, microspores develop into calli which, after transfer onto regeneration media, may convert into plants through organogenesis, undergo second embryogenesis, or in many cases may cease further development (Konieczny et al. 2007; Corral-Martínez and Seguí-Simarro 2014; Oleszczuk et al. 2014).

A factor reducing the level of plant regeneration is the duration of the in vitro culture. Culture periods longer than 18 days on the induction medium promote excess callusing with consequent low embryo formation and low conversion rate into plants (Kao et al. 1991; Sriskandarajah et al. 2015). Extended time in culture may induce variation at different levels among regenerants, promote clone formation—(sets of genetically identical plants), and increase the frequency of chromosome aberrations or the proportion of albino plants (APs) (Jähne et al. 1991; Oleszczuk et al. 2011, 2014). Each one of those reduces the overall efficiency of androgenesis. From this point of view, the most desired is direct, fast and effective DHs regeneration from embryos. One way to achieve this is by making use of a nurse culture which secretes various components to the medium to stimulate microspores to rapid divisions and embryo formation. This approach is often used in wheat (Zheng et al. 2002; Cistué et al. 2006; Broughton 2008) as well as in barley (Li and Devaux 2001; Lu et al. 2008; Lippmann et al. 2015). However, nurse culture, such as an ovary-culture, can only be used in liquid media and requires additional labor. A more convenient approach that can be used both in liquid and solid media, and in a safer way, is making use of chemical compounds known to enhance culture efficiency. One of such compounds are arabinogalactan proteins (AGPs) which are among various substances secreted from nurse cultures (Paire et al. 2003).

AGPs are highly glycosylated proteoglycans present in almost all plant organs. They are localized in the plasma membrane, apoplast and secretions e.g. extracellular matrix surface network that are involved in signaling during the vegetative and reproductive development (for review see Ellis et al. 2010). Specific AGPs extracted from embryogenic cultures were used to induce, maintain, and restore the embryogenic potential in several experimental systems (Kreuger and Holst 1993, 1996; Paire et al. 2003; Ben Amar et al. 2007; Wiśniewska and Majewska-Sawka 2007). More recently, a crucial role of AGPs in embryo patterning has been suggested (Yu and Zhao 2012; El-Tantawy et al. 2013). Indeed, the presence in the medium of β-D-glucosyl Yariv reagent (βGlcY), a chemical compound which specifically binds and precipitates AGPs (Yariv et al. 1962, 1967), was responsible for a reduction in the frequency of somatic embryogenesis as well as distortion of the embryo development (Thompson and Knox 1998; Chapman et al. 2000; Borderies et al. 2004; Simonovic et al. 2015). Aside from the role in signaling and molecular interactions at the cell surface, structural and protective functions of AGPs during plant differentiation have also been proposed (Šamaj et al. 1995).

In cereals, the involvement of AGPs in growth and development is poorly documented (Coskun et al. 2010, 2013). It has been reported that addition of AGPs to the culture medium increased green plant regeneration rate in wheat (Letarte et al. 2006). However, the mechanism by which AGPs affect androgenesis remains unknown.

This study was designed to test the effect of exogenous AGPs on androgenic regeneration from microspores in barley anther culture. As the source of AGPs, gum arabic was used. Gum arabic is a natural exudates from stem and branches of a tropical tree, *Acacia senegal*. It is composed of three main fractions: the arabinogalactan fraction, the AGPs fraction and the glycoprotein fraction, each with high biological activity (Menzies et al. 1996; Kong et al. 2014). To verify the involvement of AGPs in the androgenic development we have employed the Yariv reagent known to perturb the molecular function of AGPs from gum arabic (Corral-Martínez and Seguí-Simarro 2014). Quantitative studies on the total plant regeneration rate are supplemented by microscopic observations on microspore viability and the anatomy of androgenic structures maintained on media with gum arabic different content.

Materials and methods

Plant material

Two breeding lines of spring barley, NAD2 and NAD19, were kindly provided by Poznan Plant Breeders LTD—Nagradowice, Poland. Both have high androgenic potential but with a rather low level of plant regeneration and high proportion of APs (Makowska et al. 2017). Seeds were germinated on moistened filter paper in Petri dishes for 5 days at 4 °C and then for 7 days at 16 °C in the dark. Seedlings were grown in an 18-cm pots with a soil–sand mixture (3:1) in growth chamber at 16/12 °C day/night temperature, 16-h long days with light intensity of approximately 240 µE m^{-2} s^{-1} provided by sodium lamps.

Pretreatment of anthers

Tillers were collected when the majority of microspores from the middle of the spikes were at the mid- and late developmental stage. Harvested spikes were surface-sterilized in 70% ethanol for 1 min, followed by 10% sodium hypochlorite for 20 min, and rinsed five times with sterile water. Anthers were aseptically excised and placed in 55 mm diameter Petri dishes with 10 ml of the pretreatment solution, containing 62 g l^{-1} mannitol and 2.5 mg l^{-1} $CuSO_4$ *x* $5H_2O$ (Jacquard et al. 2009). In tests with prolonged supplementation of gum arabic, the pretreatment solution was supplemented with 5 or 10 mg l^{-1} gum arabic. During pretreatment, anthers were incubated at 4 °C in the dark for 5 days. The duration of the pretreatment was based on our previous experiments (data not shown).

Anther culture

Following the pretreatment, anthers from each Petri dish were aseptically transferred onto a 55 mm Petri dish with solid medium N6L containing macro- and micro-elements according to Chu (1978) with modifications (Makowska et al. 2015). Depending on the type of experiment one or both genotypes were tested. Both genotypes were used to estimate the effect of gum arabic for GPs and APs regeneration. Here the induction medium was supplemented with three different concentrations of gum arabic (5, 10, 25 mg l^{-1}). Medium without gum arabic was used as a control. The βGlcY (Bio supplies Australia Pty Ltd.) was added to confirm the effect of AGPs included in gum arabic. In this experiment, βGlcY was tested on NAD2, at concentrations of 5, 15 or 30 µM in the induction medium containing 10 mg l^{-1} gum arabic. Medium with 10 mg l^{-1} gum arabic but lacking βGlcY served as a control. The βGlcY was filter sterilized and aseptically added to previously autoclaved and cooled medium. In all experiments, cultures were grown in the dark at 26 °C. Androgenic structures (calli and embryos) at approximately 1 mm in diameter were successively transferred onto the regeneration medium K4NB (Kumlehn et al. 2006) with modifications (Makowska et al. 2015). Androgenic structures on the regeneration medium were kept under 16 h day/8 h night photoperiod at 26 °C for 2 weeks and the numbers of GPs and APs were counted. GPs were transferred to flasks with the N6I rooting medium containing macro- and micro-elements (Chu 1978) with modifications (Makowska et al. 2015). Plants with well-developed roots and shoots were potted and grown in a growth chamber. All media were prepared according to Makowska et al. (2015).

Microspore viability

Microspore viability was tested in NAD2 at three intervals: in freshly harvested material (T0), after the pretreatment (T1) and on the 6th day of culture (T2). Pretreatment solutions were the same for all combinations. The culture media were in three combinations with 0, 5, 10 mg l^{-1} gum arabic. Three anthers from six repetitions of each combination in every analyzed phase of androgenesis were collected, incubated for 30 min with a 0.25% aqueous solution of Evans Blue, rinsed three times with mannitol and observed under a light microscope. The number of inviable (stained by Evans Blue) and viable (unstained by Evans Blue) cells from five fields of view in three randomly chosen repetitions were counted.

Histological studies

Histological studies were performed on NAD2. Anthers from spikes before and after the pretreatment step as well as the explants after 3, 5, 7, 10, 14, 21 and 28 days of culture on media with or without 10 mg l^{-1} gum arabic were fixed and embedded as described by Pilarska et al. (2013). Sections (5 µm thick) were stained with 0.1% water solution of toluidine blue (Sigma, Germany) and observed under a light microscope. Frequencies of multicellular structures (MCSs) were counted after 5, 7 and 10 days of culture on 10–15 tissue slices, from five randomly chosen anthers. The morphology and anatomy of regenerated structures was assessed after 14, 21 and 28 days of culture. In total, 15 regenerated structures from 10 different explants were assayed for each medium at each time point.

Data analysis

In tests of different concentrations of gum arabic, three independent experiments were performed. Each experiment included six Petri dishes with approximately 66 anthers per dish, for one or both genotypes. The following parameters were recorded: microspore viability, expressed as a percentage of all cells from each combination separately with approximately 1200 cells counted for each combination, the frequency of MCSs expressed as a percentage of all microspores (1752–2165 microspores per examined combination), the frequency of embryos on callus, counted for 30 randomly chosen responding anthers from six Petri dishes for control and examined combination, total plant regeneration (TP) defined as the sum of GPs and APs regenerated per 100 plated anthers (A), the green plant regeneration rate defined as the number of GPs/100A, the level of albinism expressed as the number of APs/100A, and the proportion of GPs to APs. The effects of tested variables was examined by a one-way analysis of variance (ANOVA). The statistical significance of differences was tested using the Tukey multiple

range test in Statistica 12.5 (StatSoft Inc., 2014). Differences were considered significant at $p \leq 0.01$.

Results

Gum arabic preserves microspore viability

The viability of fresh microspores (T0) was 86% (Fig. 1a). After the pretreatment phase (T1), microspore viability dropped to 29% and then dropped even lower, to 15%, after 6 days of culture on the induction media (T2) (Fig. 1b, c). However, in anthers plated on induction media supplemented with two different levels of gum arabic 10 and 5 mg l^{-1}, at T2 the microspore viability was 23 and 21%, respectively (Table 1). Addition of gum arabic clearly reduced microspore mortality.

Table 1 Effect of gum arabic added to induction medium on microspore viability at different time of experiment; T0—before anther pretreatment, T1—after pretreatment, T2—6th day of culture

Gum arabic concentration (mg l^{-1})	Viability of microspores [%]		
	Time of experiment		
	T0	T1	T2
0	86 ± 1a	29 ± 2a	15 ± 1a
5	86 ± 1a	29 ± 2a	21 ± 2ab
10	86 ± 1a	29 ± 2a	23 ± 2b

Each data represent the mean ± standard error. Values marked with the same letter do not significantly differ at $p \leq 0.01$ according to Tukey-multiple range test

Gum arabic enhances androgenic response

Histological observations confirmed the androgenic nature of regenerated structures. Regeneration from somatic cells such as anther walls was not observed. The initial stages of the sporophytic microspore development were similar in all evaluated cultures and involved an induction of symmetrical divisions of the microspores, followed by production of MCSs and the development of calli (Fig. 2a, b).

The first microspore division occurred at least 3 days earlier on media with gum arabic relative to controls. However, the onset of microspore divisions was not affected by the gum arabic concentration, and was evident after 3–5 days of culture. With continued culture, further mitotic divisions within the microspores gave rise to MCSs comprised of few thin-walled and cytoplasm-dense cells with large nuclei containing 2–5 nucleoli (Fig. 2c). The MCSs, still surrounded by the exine, were observed from 5 to 7 days of culture on media with and without gum arabic, respectively. The numbers of MCSs in a single anther kept increasing up to the 10th day of culture and were positively correlated with the gum arabic concentration in the medium (Table 2). Direct regeneration of embryos from MCSs was not observed. Instead, the MCSs proliferated and increased in size which eventually led to the rupture of the exine and the initiation of endogenous calli after 10 days of culturing (Fig. 2d).

Gum arabic improves quality of androgenic embryos

After 14 days on media supplemented with gum arabic and after 17 days in control culture, yellowish and nodular calli became visible on anthers surfaces. The frequency of callogenesis was high and similar on all media combinations tested. At this stage, calli obtained on different media had similar structure and consisted of compact meristematic tissue at the periphery and loosely attached parenchymatous cells in the inner region (Fig. 3a–c).

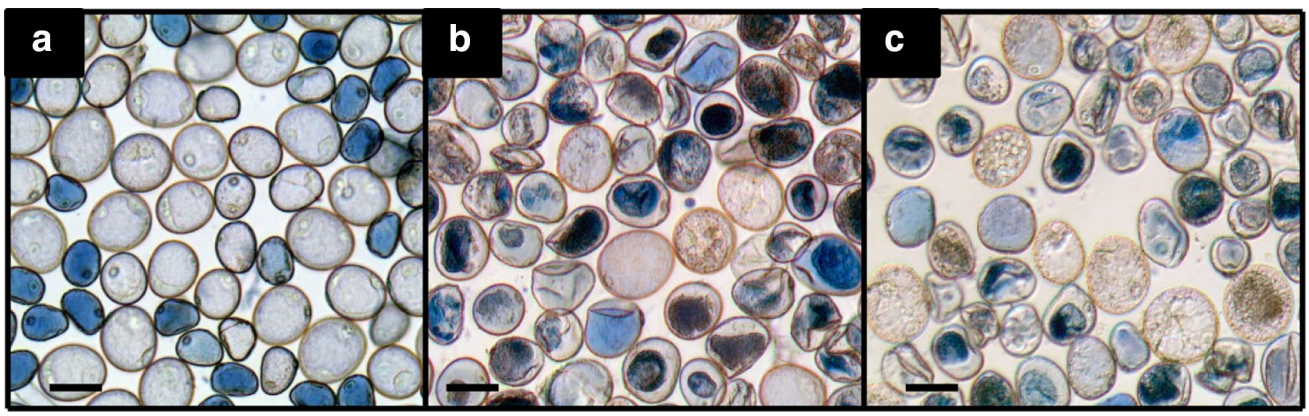

Fig. 1 Microspores viability in anther culture: **a** vacuolated microspores before the stress treatment, **b, c** microspores on 6th day of culture on induction medium (**b**) without and (**c**) with 10 mg l^{-1} gum arabic. Evans Blue staining reveals dead microspores as *blue* cells. *Bar* 25 µm (**a–c**). (Color figure online)

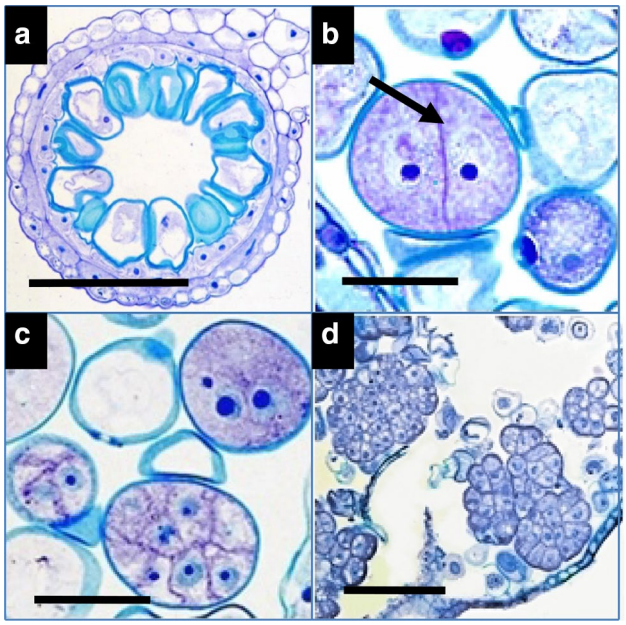

Fig. 2 Sections of anthers before culture (**a**) and maintained induction media (**b–d**)—(**a**) microspores in pollen sac before explantation, (**b**) initial androgenic symmetrical division of microspore on the 5th day of culture on medium with gum arabic, (**c**) multicellular structure enclosed within the exine on the 10th day of culture on medium with gum arabic, (**d**) callus released from exine after 17 days on medium lacking gum arabic. *Arrow* on (**b**) points cell wall formation. *Bar* 100 µm (**a, d**) and 25 µm (**b, c**)

Table 2 The frequency of MCSs in anthers maintained on media differing in gum arabic concentration

Gum arabic concentration (mg l^{-1})	MCSs (%)		
	Days of culture		
	5	7	10
0	0	1.3 ± 0.04a	2.3 ± 0.3a
5	1.6 ± 0.07a	4.5 ± 0.08b	8.7 ± 0.66b
10	8.3 ± 0.46b	13.3 ± 1.2c	16.5 ± 1.05c

Each data represent the mean ± standard error. Values marked with the same letter do not significantly differ at $p \leq 0.01$ according to Tukey-multiple range test

The onset of organized growth became evident after 21 days of culture on media supplemented with gum arabic. In control media (no gum arabic) it was observed 3 days later. This organized growth occurred regularly from meristematic cells at the periphery of callus and involved production of polarized embryos and branched outgrowths of different shapes and sizes. All formed structures were broadly attached to the mother tissue indicating their multicellular origin. The frequency of embryos on callus growing in the presence of gum arabic was 4.3 times higher than that on control media (22 ± 1 embryos per responding anther vs. 5 ± 1 embryos per responding anther on media with and without gum arabic, respectively). Conversely, the branched outgrowths were prevalent in control cultures.

Mature embryos showed typical features of zygotic embryos, with an oval shield-like scutellum attached to one side of the embryo axis (Fig. 4a). Most embryos showed well differentiated root and shoot apical meristems and a centrally located provascular strand which extended from near the shoot meristem up into the coleoptile and scutellum. The root apical meristem of the embryo was usually covered by coleorhiza whilst fully developed coleoptiles were rare, with shoot apical meristems entirely or partially exposed (Fig. 4b, c). After transferring onto the regeneration medium, most embryos developed into seedlings which rooted easily and established themselves as plants.

Unlike the embryos, the branched outgrowths never differentiated the provascular tissue and typical shoot and root apical meristems (Fig. 4d). They were mostly parenchymatous structures with few zones of compact meristematic tissue localized mainly in the upper part of branches (Fig. 4e, f). Sporadically, development of tracheary elements throughout the parenchymatous body was observed. With continued culture, these branched structures enlarged in size and produced shoots or leaf-like structures when transferred on the regeneration medium.

Improved morphology of formed structures results in a higher rate of plant regeneration

The highest average number of TP/100A was observed on the induction medium supplemented with 5 or 10 mg l^{-1} gum arabic. In NAD19 it was 2.8 times higher than in control. Concentration of 25 mg l^{-1} gum arabic generated the same plant regeneration rate as the control (Table 3). Addition of gum arabic to the induction medium did not have a significant effect on the GP regeneration in NAD2. However, in NAD19 in combinations where an increase of the TP regeneration was observed, there was a higher GP increase relative to AP (Table 3).

Addition of gum arabic to the pretreatment solution and the induction medium increases regeneration efficiency

In a search for an optimal protocol of the AGPs supplementation we have tested if prolonged exposure to gum arabic generates an additive effect. Three schemes of gum arabic supplementation were tested: to the pretreatment solution, to induction media, or to both the pretreatment solution and induction media. In all three schemes the total plant regeneration rate was improved relative to control, with 73 to 146 TP/100A in gum arabic

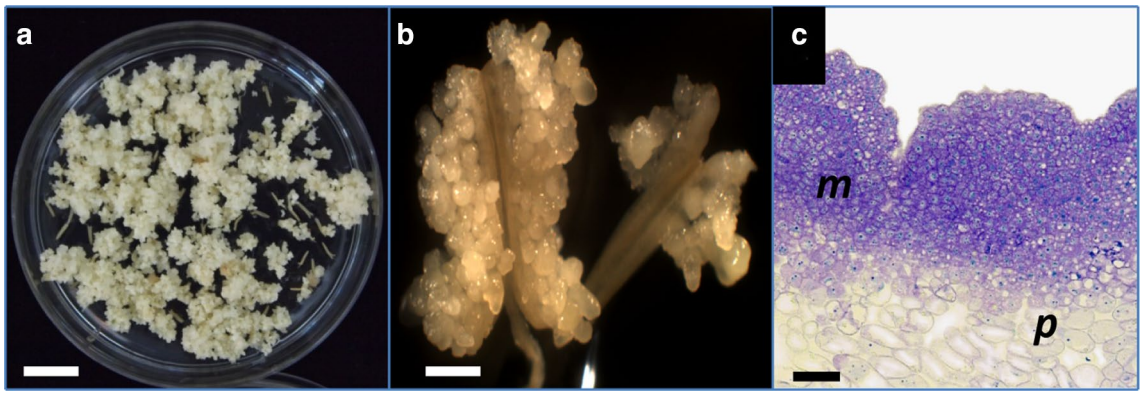

Fig. 3 Calli development on induction medium supplemented with GA—(**a, b**) macroscopic view on callogenesis on 14th (**a**) and 17th (**b**) day of culture, (**c**) histological section through callus on 21th day of culture. *m* Meristematic zone, *p* parenchyma cells. *Bar* 1 cm (**a**), 1 mm (**b**) and 100 µm (**c**)

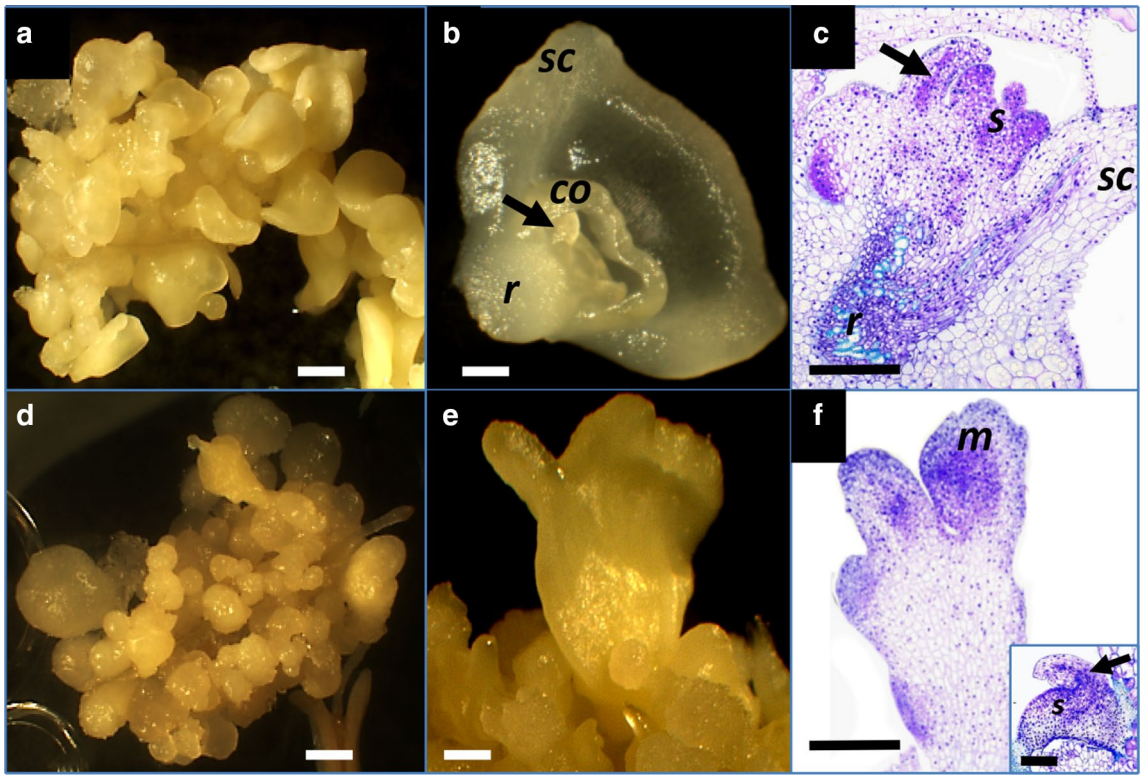

Fig. 4 Regeneration from anther-derived calli on 28th day of culture on media with gum arabic (**a–c**) and lacking this supplement (**d–f**)—(**a, b**) macroscopic view on zygotic-like embryo, (**c**) longitudinal section through zygotic-like embryo, (**d, e**) macroscopic view on regenerated branched outgrowths, (**f**) longitudinal section through typical branched outgrowth with subsequent regeneration of leaf (*inset*), *co* coleoptile, *m* meristematic zone, *r* root, *s* shoot apical meristem with leaf primordia, *sc* scutellum; *arrows* point developing leaf. *Bar* 100 µm (**a, d**), 3 µm (**b, e**) and 200 µm (**c, f**)

treatments versus 22 TP/100A of the control (Fig. 5a, b). In the best case, the increase was 6.6-times, observed for the 10–10 gum arabic combination (the third scheme: pretreatment solution—culture medium). The other two protocols produced similar results. (Table 4). Prolonged supplementation of gum arabic during culture did not reduce the AP production. In most combinations, the higher level of TP regeneration appeared to have resulted from increased regeneration of AP rather than GP (Table 4).

Table 3 Effect of different gum arabic concentration in induction medium on plant regeneration

Gum arabic concentration (mg l^{-1})	Plant regeneration							
	TPs/100A		GPs/100A		APs/100A		GPs:APs	
	Genotypes: NAD2	NAD19	NAD2	NAD19	NAD2	NAD19	NAD2	NAD19
0	57 ± 9bc	54 ± 7bc	19 ± 3bc	11 ± 3b	38 ± 7b	43 ± 6b	1:2	1:4
5	98 ± 11a	98 ± 2ab	33 ± 4a	50 ± 5a	65 ± 9ab	48 ± 7ab	1:2	1:1
10	95 ± 12ab	151 ± 30a	27 ± 4ab	52 ± 5a	68 ± 9a	99 ± 26a	1:3	1:2
25	49 ± 3c	36 ± 2c	8 ± 3c	6 ± 1b	41 ± 3ab	30 ± 2b	1:5	1:10

Each data represent a mean ± standard error. Values within a column followed by the same letter do not significantly differ at $p \leq 0.01$ according to Tukey-multiple range test

A plated anthers, *TP* total plant, *GP* green plant, *AP* albino plant

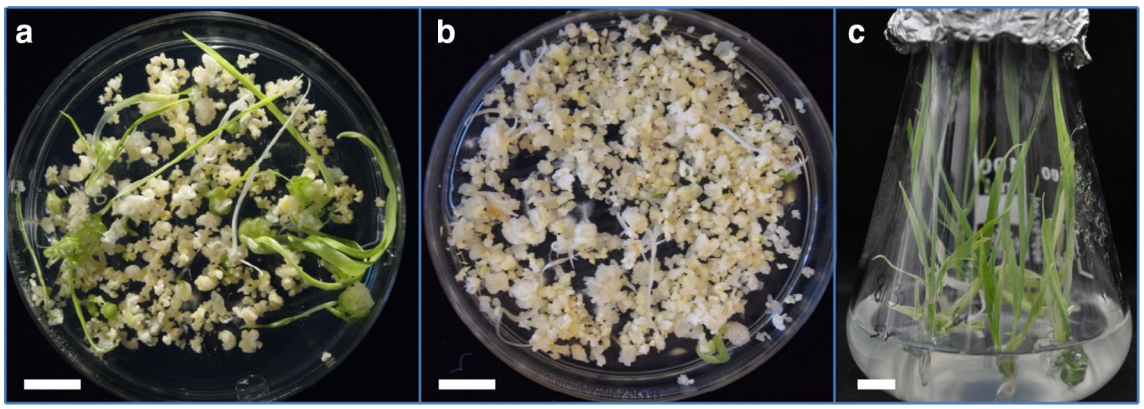

Fig. 5 Differences in efficiency of plant regeneration on tested media—(**a**) calli with predominance of zygotic-like embryos which were developed on induction medium with gum arabic, (**b**) mostly undifferentiated calli obtained on induction medium without gum arabic, (**c**) plants on regeneration medium. *Bar* 1 cm (**a**–**c**)

Table 4 Effect of gum arabic supplementation in different step of androgenesis on regeneration efficiency

Gum arabic concentration (pretreatment solution-induction medium) (mg l^{-1})	Plant regeneration			
	TPs/100A	GPs/100A	APs/100A	GPs:APs
0	22 ± 2c	6 ± 2b	16 ± 3b	1:3
0–5	85 ± 12a	14 ± 3b	71 ± 10c	1:5
0–10	85 ± 3a	15 ± 3b	70 ± 3c	1:5
5–0	73 ± 7a	14 ± 3b	59 ± 4c	1:4
10–0	107 ± 8ab	34 ± 5a	73 ± 5c	1:2
5–5	100 ± 7a	17 ± 4b	83 ± 6bc	1:5
10–10	146 ± 17b	11 ± 2b	135 ± 18a	1:12

Each data represent a mean ± standard error. Values within a column followed by the same letter do not significantly differ at $p \leq 0.01$ according to Tukey-multiple range test

A plated anthers, *TP* total plant, *GP* green plant, *AP* albino plant

Yariv reagent counters the effect of gum arabic on plant regeneration rate

Addition of 5 and 15 µM βGlcY did not cause any significant change in plant regeneration compared to the 10 mg l^{-1} of gum arabic-only variant. A reduction in plant regeneration was observed for the 30 µM concentration of βGlcY. The highest concentration of the Yariv reagent inhibited the total plant regeneration, which was 4.6 times lower compared to control. Addition of the Yariv reagent to the induction medium did not have a significant effect on the rate of regeneration of GP and AP (Table 5).

Table 5 Effect of βGlcY added to the medium with gum arabic at 10 mg l^{-1} on total plant regeneration

Yariv concentration (μM)	Plant regeneration			
	TPs/100A	GPs/100A	APs/100A	GPs:APs
0	51 ± 5a	20 ± 2a	31 ± 4a	1:2
5	46 ± 8a	15 ± 3a	31 ± 5a	1:2
15	35 ± 7ab	12 ± 1a	23 ± 6a	1:2
30	7 ± 2b	4 ± 1b	3 ± 1b	1:1

Each number is a mean ± standard error. Values within a column followed by the same letter do not significantly differ at $p \leq 0.01$ according to Tukey-multiple range test

A plated anthers, *TP* total plant, *GP* green plant, *AP* albino plant

Discussion

After years of trying there is still no simple and reproducible method of anther culture in barley that would guarantee high efficiency of DH production independent of the genotype used. Here we demonstrate an impressive effect of a source of AGPs—gum arabic capable of improving the plant regeneration rate from barley anthers by 6.6-times relative to standard protocols. It appears that this improvement is a consequence of several events in the course of the androgenic process.

Addition of gum arabic reduced microspore mortality in the first days of culture—the crucial moment in the induction of androgenesis, when microspores switch their development from the gametophytic to sporophytic pathway. It has been shown that in barley culture the typical mortality in this step reaches 74% (Ritala et al. 2001) and our control treatments were very much in the same range. Further reduction of microspore viability over the first days of culture was less dramatic with gum arabic than in controls. Here, we were able to limit cell mortality from 85% in control conditions to 77% with gum arabic, a ca. 53% increase in the proportion of viable cells. A comparable effect was observed in wheat microspore culture where Larcoll was used (Letarte et al. 2006). Larcoll is a source of arabinogalactans but does not have the protein fraction. The protection effect of Larcoll was higher when it used together with the ovary culture. This could suggest that the ovary culture offered some additional protection against cell death. It can be hypothesized that both the polysaccharide and the protein fractions of gum arabic are involved in maintaining cell viability. This is supported by reports that AGPs are involved in the control of the Programmed Cell Death (Gao and Showalter 1999) which takes part at least two levels: during induction of androgenesis and transition multicellular structure to proembryos (Maraschin et al. 2005; Rodríguez-Serrano et al. 2012). A recent report suggests that arabinogalactan proteins may play a role in early microspore wall development, potentially maintaining cell viability (Li et al. 2017).

Similarly to several monocot and dicot species (Konieczny et al. 2003; Daghma et al. 2014), the onset of the androgenic development in barley anthers manifests itself by an induction of a symmetrical division of the microspore. The time required for the first androgenic cell division appears to depend on the media used, and it is significantly shorter on the medium containing gum arabic. This may well be the first observation that the gum arabic accelerates the first androgenic cell division in barley. A similar effect was observed when barley microspores were co-cultured with ovaries (Lu et al. 2008). In that study, the authors also reported an increase in the frequency of the MCSs formation. In our experiments, the frequency of the MCSs formation was about 8-times higher on media with gum arabic than without it. Moreover, the number of MCSs formed appeared to be related to the concentration of gum arabic in the medium. Thus, gum arabic appears to mimic the effect of ovary co-culture in barley; however the pathways by which this stimulates the androgenic switch need not be the same. Tang et al. (2006) reported that AGPs which bind to βGlcY are involved in the initiation and maintenance of *Brassica oleracea* microspore androgenesis, whilst recent studies of Li et al. (2017) revealed a role of AGPs in development and patterning of the microspore wall. Cell wall composition is tightly controlled by the protoplast, but it also has some reciprocal functions as the mechanical and chemical properties of the cell wall affect cell fate (Rodakowska et al. 2009). Taking it into account the possible function of exogenously applied gum arabic in the patterning of the microspore wall and/or in the initiation of the cell wall signaling pathway that leads to the androgenic switch cannot be ruled out. However, it should also be kept in mind that AGPs from gum arabic were suggested to have a role in would healing (Schowalter 2001); this also allows us also to speculate on the possible involvement of gum arabic in nutrition of cultured anthers and/or in reducing the stress associated with the initiation and maintenance of tissue culture.

Histological observations in this study have shown that plant regeneration occurred via calli. Supplementation of gum arabic into the induction medium caused more than a fourfold increase in the number of typical zygotic-like embryos on callus surface relative to the control medium. The mechanism by which AGPs affects embryo development remains unclear. However, specific localization of some AGPs epitopes on embryogenic cells (Chapman et al. 2000) as well as on embryo structures (Pilarska et al. 2013) suggest the involvement of these proteins in the induction and maintenance of cell polarity. Indeed, a majority of barley embryos obtained in the presence of gum arabic were bipolar and displayed all structures typical for zygotic embryos, such as scutellum, coleoptile and coleorhiza. Conversely,

branched and mostly parenchymatous outgrowths were prevalent in the control culture. Recently, we have observed that aberrant embryo morphology may be the prime cue for low conversion rate or even total inability to develop into plants after subculture on the regeneration media (Konieczny et al. 2012). Interestingly, the anthers-derived calli of barley cultured in the presence of gum arabic showed a significantly higher ability to regenerate plants relative to calli maintained without gum arabic. The positive effect of gum arabic can be reversed by the AGPs antagonist—βGlcY, which is in line with observations in the eggplant microspore culture (Corral-Martinez and Segui-Simarro 2014). Tang et al. (2006) have shown that in *Brassica napus* βGlcY seriously disturbs both the radial and axial patterning of developing androgenic embryos. All these results point to the AGPs fraction of gum arabic as the factor affecting the embryo conversion in barley, possibly by its effect on embryo patterning.

When added to the induction medium gum arabic increased plant regeneration from barley anthers in a dose-dependent manner. Concentrations of 5 and 10 mg l^{-1}gum arabic were the most effective for both genotypes tested, and brought about a twofold higher frequency of the total plant regeneration relative to control. Similar concentrations of gum arabic increased plant recovery from barley mature zygotic embryos (Coskun et al. 2010) as well as in androgenic cultures of wheat (Letarte et al. 2006) and white cabbage (Yuan et al. 2012). Supplementation of the induction medium with 25 mg l^{-1} gum arabic was apparently too high for the tested genotypes and reduced the effectiveness of plant regeneration. Interestingly, in the eggplant microspore culture a much higher concentration (1600 mg l^{-1}) was suggested to improve the regeneration protocol (Corral-Martinez and Segui-Simarro 2014). It appears, therefore, that the gum arabic concentration must be individually tested for each species.

Taking into account the role of AGPs in preventing cell death and in acquisition of the embryogenic potential we tested gum arabic not only in the induction medium but also in the pretreatment solution. This improved the regeneration rate by about 6.6 times compared to control. Gum arabic applied only during the pretreatment stage produced a similar effect as gum arabic applied only in the induction medium. It was the combination of the two that produced the best effect indicating that AGP should be supplemented from the earliest steps of androgenesis and provided continuously during embryo formation.

While AGPs present in gum arabic improved the regeneration potential of androgenic structures, they did not reduce the number of APs. These results are in line with those presented by Letarte et al. (2006) in wheat, where a treatment with gum arabic and Larcoll increased the absolute number of APs compared to control. APs are a perennial problem in barley androgenesis. In some genotypes almost all regenerated plants are albino (Caredda 2000, 2004). AGPs have not been noted as involved in plastid development so the absence of an effect on albinism was not unexpected. This also provides further evidence that the development of embryos converting into the plants and chloroplast biogenesis during androgenesis are two independent processes (Chaudhary et al. 2003; Makowska et al. 2017).

We have demonstrated that AGPs from gum arabic have a significant role in increasing the regeneration rate from barley anthers. Gum arabic apparently mimics the effect of ovary co-culture in isolated microspore culture (Lu et al. 2008) and represents a simple approach to improve some of the critical stages which contribute to the overall effectiveness of DH production in barley. Checking the effect of ovary co-culture and AGPs together in both anther and isolated microspore culture within the same cultivar would be helpful in searching an optimal protocol for DH production in barley.

Author contributions KM, RK and JZ conceived and designed the research. KM and RK drafted the manuscript. KM, SO performed the experiments in tissue culture. MK performed the histological analyses. AC helped during the data analysis. KM and RK designed the figures. SO, JZ revised the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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