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Additional Information

Refining the method for eggplant microspore culture: effect of abscisic acid, epibrassinolide, polyethylene glycol, naphthaleneacetic acid, 6-benzylaminopurine and arabinogalactan proteins

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Abstract

Microspore embryogenesis is an inducible pathway interesting from basic and applied perspectives. For plant breeding, it is a powerful tool to produce doubled haploids, useful as pure lines. The most efficient way to produce them is through isolated microspore culture. In eggplant, one of the most important vegetable crops, this method is still poorly explored. So far, it is possible to produce doubled haploids, but not directly from embryos, because they are converted into calli early during their development. In this work we evaluated the effect of abscisic acid, epibrassinolide, polyethylene glycol, and arabinogalactans and arabinogalactan proteins, previously described as promoters of embryo induction and development in other species. When added individually to the standard protocol, all of them significantly increased induction of microspore embryogenesis and callus cell proliferation, producing more and larger calli. Particular combinations of them further improved the efficiency of the method. In particular, gum arabic containing arabinogalactans and arabinogalactan proteins allowed embryos to progress beyond the globular stage, constituting a significant improvement in order to achieve the desired direct induction of viable, germinating embryos. We also evaluated the effect of altering the concentration and relative ratio of naphthaleneacetic acid and 6-benzylaminopurine, used in the standard protocol. Significantly better results were obtained by reducing their concentration. Together, our results shed light on the morphogenic and regulatory roles of these substances on microspore embryogenesis, opening ways to further increase the efficiency of production of androgenic doubled haploids through microspore culture in eggplant.

Keywords: androgenesis, doubled haploid, haploid, microspore embryogenesis, *Solanum melongena*.

Introduction

The deviation of the microspore towards embryogenesis is a powerful biotechnological tool to produce doubled haploid (DH) pure lines, essential for hybrid seed production (Dunwell 2010; Germanà 2011). This technology consists on the *in vitro* deviation of microspores from their original gametophytic fate to develop a haploid or DH plant. To be triggered, this biotechnological pathway requires a source of abiotic stress (heat, cold, starvation, osmotica, etc; Shariatpanahi et al. 2006) to be applied to the microspores. After this initial inductive stage, microspores enter embryogenesis. Technically, this can be achieved by directly culturing anthers (anther culture), or through the isolation of microspores from the anthers and subsequent culture in liquid medium (isolated microspore culture). Microspores usually develop into microspore-derived embryos (MDEs) through haploid embryogenesis, but in some species they tend to proliferate as calli. From them, DH plants can be obtained through organogenesis. Examples of MDE-producing species include well-known model systems such as rapeseed, barley, tobacco or maize, among many others (Maluszynski et al. 2003). Examples of species where DHs have been obtained from callus include coffee, loquat, poplar, cereals such as rye, oat or wild barley relatives, ornamentals such as lily, narcissus, coneflower, *Anemone*, *Dianthus* or chrysanthemum, and recalcitrant solanaceae as tomato or eggplant (reviewed in Seguí-Simarro 2010).

Eggplant (*Solanum melongena* L.) is one of the most important vegetables worldwide. In eggplant, embryos can be successfully induced from microspores through anther culture (Salas et al. 2011; Salas et al. 2012). However, previous research has evidenced several limitations of this practical approach, including the occasional

appearance of somatic embryos from anther tissues, the uncontrollable secretory effect of the tapetum, which precludes from a strict control of culture conditions, and an efficiency limited to only a few embryos per anther cultivated. All of these limitations can be overcome by the direct isolation and culture of microspores. However, the number of studies published on the successful production of DH plants from isolated microspores is still very limited. Apart from the pioneering studies of Gu (1979) and the work of Miyoshi (1996), only a very recent paper dealt with this topic (Corral-Martínez and Seguí-Simarro 2012). These three studies demonstrated that DH plants can be obtained through organogenesis from the calli formed upon culturing eggplant microspores in liquid medium. However, the latter study also revealed that actually, microspores are not directly converted to calli. A detailed study of the process of microspore proliferation showed that immediately after induction, eggplant microspores enter an initial stage of embryogenesis that arrests at the globular embryo stage. Instead of experiencing the radial-to-bipolar transition typical of zygotic embryos, eggplant MDEs enter a proliferative, undifferentiated development as callus-like structures (Corral-Martínez and Seguí-Simarro 2012). Haploid and DH plants can be regenerated from these organogenic calli.

As deduced from this study, eggplant microspore cultures were characterized by a blockage of embryogenesis and by a good but still improvable efficiency. Thus our next efforts were devoted to identify factors that could help to overcome this arrest, as well as to improve the efficiency of embryogenesis induction. For this goal, we evaluated in this work the effect of different substances on the initial stages of eggplant microspore embryogenesis, i.e. the promotion of microspore induction, embryo development and conversion to callus. These substances included abscisic acid (ABA),

epibrassinolide, polyethylene glycol (PEG), and gum arabic. These compounds have been previously described in other species as promoters of microspore induction to embryogenesis, and as regulators of MDE development. For example, a number of reports have clearly shown that ABA plays a role during both zygotic and microspore-derived embryogenesis in several species including *Brassica napus* (Hays et al. 2001), barley (van Bergen et al. 1999), and tobacco (Imamura and Harada 1980; Kyo and Harada 1986). Brassinosteroids, and in particular 24-epibrassinolide (EBr) has yielded very positive results in terms of increased frequency of induction of both somatic (Azpeitia et al. 2003; Pullman et al. 2003) and microspore embryogenesis (Ferrie et al. 2005; Malik et al. 2008), and also in terms of the acquisition of a proper shoot apical meristem identity and its further development and function (Belmonte et al. 2010). Osmotic, non-metabolizable agents such as PEG have been described as effective for improving embryogenesis induction (Corral-Martínez and Seguí-Simarro 2012; Ferrie and Keller 2007; Ilic-Grubor et al. 1998; Shariatpanahi et al. 2006), due to the additional stress provided by the change in osmotic potential. Arabinogalactan proteins (AGPs) and arabinogalactans have also been attributed a decisive role during the inductive phase, as well as for MDE development (Letarte et al. 2006; Paire et al. 2003; Tang et al. 2006).

In this work we tested the effect of adding these substances, either alone or combined, to the standard protocol for eggplant microspore culture previously published by us (Corral-Martínez and Seguí-Simarro 2012). In addition, we evaluated the effect of altering the concentration and relative ratio of the auxin and cytokinin used in the mentioned protocol, naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP), respectively. The results hereby presented have the potential to be applied for the

efficient production of eggplant DHs through microspore culture. In addition, they provide new insights on the morphogenic and regulatory roles of these substances.

Material and methods

Plant materials and culture conditions

As donors of microspores, we used Bandera plants (a F1 hybrid from Seminis Vegetable Seeds Iberica, S.A., Spain). Different batches of plants were grown in 30 cm pots at COMAV greenhouses (Universitat Politècnica de València), at 20ºC under natural light at autumn, winter, and spring months during two consecutive years.

Microspore culture and plant regeneration

Flower buds at the appropriate stage of development (containing anthers with a majority of vacuolate microspores and young bicellular pollen) were selected according to Salas et al. (2012), immediately transported to the laminar flowhood under melting ice, and basically processed as previously described (Corral-Martínez and Seguí-Simarro 2012). Anthers were excised, surface sterilized with 70% ethanol (30 s) followed by a 10% solution of commercial bleach (40 g/l of NaClO) for 5 min. It must be noted that eggplant anthers have extremely thick walls, as we recently showed (Salas et al. 2012). This allows anthers to be in contact with bleach for 5 min with no damage for microspores. After sterilization, anthers were crushed under sterile distilled water. The locular content was filtrated through 40 µm nylon meshes (Millipore), spun down (850 rpm for 4 min) and washed thrice with distilled water. Microspore pellets were suspended in sterile distilled water, adjusting the concentration to 500,000 microspores per ml, plated in 6-cm petri dishes and incubated at 35ºC for 3 days. Then, microspores

were spun down and resuspended in liquid NLN culture medium supplemented with 2% sucrose, 0.5 mg/L NAA, and 0.5 mg/L BAP, pH 5.9. Dishes were incubated at 25ºC in darkness during one month, after which they were analyzed as explained below. For the analysis of the percentage of organogenic calli, one-month old induced microcalli exceeding 1 mm were isolated and individually transferred to dishes (10 calli per dish) with solid MS medium supplemented with 0.4% phytagel, 3% sucrose, 0.1 mg/L indoleacetic acid, and 2 mg/L zeatin. Dishes were incubated at 25ºC under a 16/8 h photoperiod (300 µE m^{-2} s^{-1}), subculturing every 15 days for a total of 1.5 months (2.5 months from microspore isolation), after which calli were analyzed as described below.

Study of the effect of BAP, NAA, ABA, EBr, PEG, and gum arabic

For the experiments of BAP and NAA concentration and ratio, the concentrations of BAP and NAA mentioned above were modified as described in Results. For the rest of the factors studied, experiments consisted on the addition, either individually or combined, of ABA (Duchefa Biochemie), EBr (OlChemIm Ltd), PEG 4000 (Fluka BioChemika), and gum arabic (Fluka BioChemika) at different concentrations as explained in Results. Gum arabic is a mixture of arabinogalactans and AGPs traditionally obtained from exudates of *Acacia* trees. For this factor, additional experiments adding 20µM β-D-glucosyl Yariv reagent (βGlcY) were performed. βGlcY is a synthetic phenylglycoside that specifically binds to AGPs (Yariv et al. 1967), preventing their roles during embryogenesis in a concentration-dependent manner (Tang et al. 2006). For all experiments, a minimum of five replicas of each concentration were performed, being all replicas and concentrations performed under the same experimental conditions and at the same time, all of them coming from the same pool of isolated microspores. Each experiment was repeated a minimum of three times

(sessions). In all cases, controls excluding the factor tested were included. Effects were evaluated in 1-month old cultures, unless otherwise specified in Results. For quantitative studies, the following parameters were measured per individual dish: number of total calli per dish, total callus fresh weight per dish, and number of calli larger than 1 and 2 mm per dish, as an estimation of callus size. Numbers of calli were measured by hand counting under a dissecting microscope. Callus fresh weight was measured by isolating them from the culture medium and weighing in a precision weighing scale. For the experiments of combined factors, callus weight was not measured since they were needed to evaluate the percentage of organogenic calli 1.5 months after transfer to solid medium. This percentage was calculated by counting the number of calli developing organogenic buds or shoots, divided by the total of calli per dish (10). To analyze all factors studied, a General Linear Model (GLM) of a commercially available statistics package (Statgraphics Plus, Version 4.1, STSC Inc., Rockville, MD, USA) was performed for each experiment, including as fixed effects the treatment and the session factors. Statistical significance was indicated by a P value < 0.05.

Results

Analysis of the effect of the exogenous addition of ABA

We studied the effect of adding ABA to the induction and culture medium at different concentrations, collecting data one month after application. As a preliminary trial, we first applied a wide range of ABA concentrations, including 2.10^{-6}M, 5.10^{-6}M, 10.10^{-6}M and 15.10^{-6}M (data not shown). From them, only 2.10^{-6}M produced acceptable

results, with higher numbers of calli larger than 1 and 2 mm, but with no differences in terms of number of calli. Higher concentrations consistently produced remarkably less calli than controls. According to this, we decided to perform a second test with a lower range of ABA concentrations, ranging from $0.5.10^{-6}$M to 2.10^{-6}M (Figure 1).

Microscopic observation of these experiments after 15 days in culture showed that control dishes with no ABA presented irregular, disorganized and highly proliferative callus-like structures (Figure 1A). The use of $0.5.10^{-6}$M ABA (Figure 1B) gave rise to similar structures, perhaps slightly smaller in general and more rounded. 1.10^{-6}M (Figure 1C) and 2.10^{-6}M ABA (Figure 1D) generated round, globular embryo-like structures, instead of the small, irregular, callus-like structures observed in the preliminary trial with 5.10^{-6}M (Figure 1E) or higher concentrations. From these experiments, it could be concluded that the application of ABA at concentrations ranging from 1.10^{-6}M to 2.10^{-6}M appeared to have a positive effect preventing embryo disorganization into callus. However, one month after culture initiation all the cultures presented callus-like structures, where the morphological differences observed at day 15 associated to the use of different concentrations were no longer evident (data not shown). No embryo progression beyond the globular stage was observed at any ABA concentration. Next, we performed a detailed quantitative analysis (Figures 1F-G'), and the concentration yielding best results in general was $0.5.10^{-6}$M, since it provided the highest number of calli (Figure 1F), the highest callus weight (Figure 1F') the highest number of calli larger than 1 mm (Figure 1G), and the second highest number of calli larger than 2 mm (Figure 1G'). Together, the quantitative and qualitative results obtained from ABA application showed that $0.5.10^{-6}$M ABA was the most beneficial

concentration in terms of callus number, size and weight. However, it was unable to promote embryo progression.

Analysis of the effect of the exogenous addition of EBr

In parallel, we studied the effect of the application of EBr at different concentrations from 10^{-9} M to 10^{-5} M. After one month of culture, the microspore-derived structures presented important qualitative differences. As seen in Online Resource 1, increasing concentrations of EBr had negative effects in the total amount of mass produced per plate, and also in the morphology of calli. Lower EBr concentrations produced calli with a disorganized architecture similar to those observed in control dishes, and higher EBr concentrations produced more compact, rounded and opaque calli. However, we could not identify any structure similar to late globular embryos at any of the concentrations used. Contrasting with these results, a quantitative analysis (Figure 2) revealed that EBr concentrations ranging from 10^{-9}M to 10^{-8}M had a positive effect in the number (Figure 2A) and weight (Figure 2B) of the calli produced, being 10^{-8}M the best to increase the number of callus produced. Higher concentrations had null or negative effects. With respect to callus size (Figures 2C, D), application of EBr at any concentration was clearly detrimental. In summary, the application of EBr at a concentration of 10^{-8}M increased the number of microspore-derived structures, whereas higher concentrations reduced this number but favored the appearance of smaller and more compact structures.

Analysis of the effect of the exogenous addition of gum arabic

Our third set of experiments aimed to the evaluation of the qualitative and quantitative effects of gum arabic (a source of mixed arabinogalactans and AGPs), applied to the cultures at different concentrations. In a first experiment, we tested concentrations ranging from 1 to 100 mg/L. No effect in callus morphology was observed after a month of culture at any of the concentrations of this range (data not shown). However, a positive correlation was found between concentration and callus number, size and weight (Online Resource 2), being 100 mg/L the best of the range. In order to find the optimal concentration of gum arabic, we designed a second experiment using a higher range (100 to 400 mg/L). As with the first range, we observed no changes in callus morphology. However, progressive increases in callus number and total weight were found (Online Resource 3A, B). Changes in callus size were less consistent, with no clear ascending or descending trends (Online Resource 3C, D). Then, we tested a third interval of gum arabic concentrations, ranging from 400 to 1600 mg/L. Again, a positive correlation was found between concentrations of gum arabic and number of calli and total weight (Figures 3A, B), with an optimum at 1600 mg/L. In addition, this concentration produced the highest number of calli larger than 1 mm (Figure 3C), but not than 2 mm (Figure 3D). A fourth and final experiment was performed using concentrations higher than 1600 mg/L (3000, 5000, 7500 and 10000 mg/L). However, this experiment using extremely high concentrations gave very inconsistent results, with extreme differences between repetitions, and always below or similar to controls (data not shown). Therefore, we concluded that the use of concentrations higher than 1600 mg/L was not advisable. Once established the positive effect of gum arabic in androgenesis induction, we performed an additional experiment to confirm the role of arabinogalactan and AGPs in such an effect. We cultured microspores with the standard protocol without gum arabic (control), with gum arabic at

1600 mg/L, and combining gum arabic (1600 mg/L) with βGlcY in order to precipitate the AGPs present in gum arabic. As shown in Figure 4A, addition of gum arabic was beneficial as expected, increasing the number and size of callus produced after one month of culture. However, the addition of βGlcY reduced dramatically the efficiency of the method, producing very few calli, and being only one per plate larger than 1 mm. Together, these results pointed to AGPs as the main responsible for the observed increases, suggesting a specific, positive and dose-dependent effect in the production of microspore-derived structures.

Nevertheless, the most important effect of the use of 1600 mg/L of gum arabic was observed at the morphological level. In addition to callus-like structures, we observed embryo-like structures at stages beyond the globular stage (Figure 4B). Some of them were round, whitish and with a defined, smooth surface, typical of globular embryos (arrow in Figure 4B). Others presented a clear polar embryo axis (asterisk in Figure 4B) with a clearly defined, elongated hypocotyl and in some examples, elongating roots (arrowhead in Figure 4B). When observed under the inverted microscope (Figure 4C), elongated embryos presented a differentiated vascular cylinder and a distinct radicle at the basal end of the hypocotyl. However, in all cases embryos presented absent or defective shoot apical meristems, with blunt or flat apical ends, and no traces of cotyledons. In conclusion, the use of gum arabic at 1600 mg/L not only promoted the induction of significantly more microspore-derived structures, but also prevented their conversion into calli, giving rise to embryo-like structures with defective shoot apices.

Combined effects of ABA, EBr, PEG and AGPs

Once determined the optimal concentrations of ABA, EBr and AGPs when added alone to the standard protocol, we next evaluated their combined effect when used together with AGPs and at the optimal concentration (Figure 5A). We decided to include AGPs in all combinations because this was the factor that yielded better results, in both quantitative and qualitative terms. We also considered for this experiment the use of 1% PEG, previously demonstrated to be beneficial when used together with a parallel reduction of sucrose concentration from 2% to 1% (Corral-Martínez and Seguí-Simarro 2012). First, we tested the inductive effect of combinations of two, three and the four factors. A quantitative analysis of the results revealed that despite its positive effect on androgenesis induction when applied alone, the use of ABA combined with any other factor was either not or just slightly beneficial for callus production. On the other hand, all combinations excluding ABA gave rise to callus numbers remarkably higher than controls. However it must be noted that most of the combinations, both including and excluding ABA, produced calli of larger size, as revealed by the increase in the number of calli larger than 1 and 2 mm. Nevertheless, the best overall results were obtained with ABA-excluding combinations. Among them, the best performing included the combined use of PEG+AGPs, followed by EBr+AGPs and EBR+PEG+AGPs. Thus, it could be concluded that ABA would somehow inhibit the inductive effect of the other factors. Among these other factors, the best results were always related to the use of AGPs combined with PEG, EBr or both.

Microscopical observations of microspore-derived structures in two-week old cultures revealed that almost all treatments produced large, disorganized callus-like structures (Figure 5B), similar to those observed for low ABA concentrations (Figures

1A-C) and for EBr (Online Resource 1). The only two exceptions were the PEG+AGPs and ABA+PEG+AGPs combinations, which produced smaller, rounded globular embryo-like structures (Figure 5C), very similar to those shown in Figure 4B (arrow). Observations in one-month old cultures revealed that all treatments, including the PEG+AGPs and ABA+PEG+AGPs combinations, gave eventually rise to callus-like structures, with no traces of embryo progression beyond the globular stage. Thus, it appeared that PEG+AGPs, with or without ABA, had a slightly beneficial effect in preserving embryo identity for longer time. This effect, however, would be insufficient to allow for embryo progression.

Then, we evaluated the effect of the initial exposure to these factor combinations for one month, in the promotion of organogenesis from the calli obtained. Upon transfer of calli to solid medium at month 1 of culture, we cultured them during 1.5 additional months and then calculated the percentage of calli undergoing organogenesis and developing organogenic buds and then shoots at their surface. As seen in Figure 5D, results for regeneration were in general consistent with those obtained for induction, but with a principal difference: the clearly positive effect on embryogenesis induction of EBr-including combinations was found moderate with respect to induction of organogenesis from calli. In turn, ABA had a positive effect on organogenesis promotion when combined with EBr and AGPs. Nevertheless, the best results (considering both embryogenesis induction from microspores and organogenesis from calli) were obtained combining PEG+AGPs, with or without EBr, as for induction.

Effects of BAP and NAA concentration and ratio

In the last experiment, we evaluated the effect of altering the concentrations and relative proportions of BAP and NAA, the two growth factors used in the standard protocol (Figure 6). We tested the effect of changing BAP and NAA concentrations to a half (0.5x), one fifth (0.2x), twice (2x) and five-fold (5x) the initial concentration of the standard protocol (1x, control). In parallel, we altered the BAP:NAA ratio by changing BAP concentration to a half (0.5 BAP) and twice (2x BAP) the initial, and also by changing NAA concentration to a half (0.5 NAA) and twice (2x NAA) the initial. Figure 6 shows that the inductive response of eggplant microspore cultures was inversely proportional to the concentration of BAP and NAA used. Moreover, we found that the 1x concentration initially used was not the best. Instead, a 0.2x reduction of both regulators yielded the best results in terms of callus number and size. The individual reduction of BAP only was found to be beneficial, whereas an individual decrease of NAA, or the individual increase of either BAP or NAA did not produce significant differences with respect to control dishes. In conclusion, a proportional reduction of both regulators to one fifth of the initial concentration was found better than any other alteration of the BAP and NAA concentration or ratio.

Discussion

ABA slightly promotes embryo induction and growth, but not embryo progression

We showed that addition of $0.5.10^{-6}$M ABA had the most markedly beneficial effect for callus production and growth. With respect to controls, more microspores were induced to divide, and they divided faster, as revealed by the increase in callus

number, weight and size. This is in accordance with previous results in tobacco or barley. In tobacco, Kyo and Harada (1986) demonstrated that ABA application stimulated embryo production, and Imamura and Harada (1980) proposed that a specific level of ABA was required for induction of androgenesis. In barley anther cultures, a peak of endogenous ABA after the first 24 h of pretreatment was proposed to prevent microspore death, increasing the amount of viable microspores (van Bergen et al. 1999). In our eggplant system, low ABA concentrations promoted MDE induction, perhaps by increasing microspore viability as suggested by van Bergen et al. (1999) or by limiting the extent of osmotic stress-induced oxidative damage. Indeed, ABA was considered a key blocker of H_2O_2 production due to osmotic stress (Ozfidan et al. 2012). According to the morphology of the structures observed (Figures 1A-E), low ABA concentrations had also a role in the maintenance of the identity of globular embryos, preventing embryo arrest and callus growth but only up to a certain extent, since all embryos eventually converted to calli. Therefore, low ABA doses would promote embryo induction but would not be able to maintain embryo identity during the transition from globular to heart-shaped embryos. In other words, low exogenous ABA doses would have a slightly positive effect during the initial stages of embryogenesis, but not at later stages. In the context of the protective role of ABA against abiotic stress, this is not surprising, since it was previously shown that exogenous ABA played a dual role in the regulation of stress-protective defense strategies, by which a beneficial role in the initial stages shifted to a detrimental one under prolonged treatments (Xiong et al. 2006).

EBr has a beneficial role limited to microspore induction towards embryogenesis

As to the effect of EBr, our results showed a positive effect on microspore induction when added at 10^{-8}M. However, these microspores soon diverted from the embryogenic pathway to become small, disorganized microcalli. As discussed for ABA, the role of EBr may be related to the protection against abiotic stresses provided by EBr. It is known that EBr has a positive impact in the acquisition of thermotolerance (Divi et al. 2010). In the context of the inductive protocol used hereby, characterized by the exposure to sublethal temperatures (35ºC) during 3 days, this protection during the initial stages appears essential to increase the survival rate. These results are consistent with the role as embryogenesis enhancers reported for several *B. napus* and *B. juncea* cultivars (Ferrie et al. 2005). EBr, however, was unable to promote embryo progression and further differentiation. Thus, EBr could possibly be considered as part of a practical protocol aimed to produce callus-derived eggplant DH plants, but not to produce them through embryogenesis.

AGPs promote microspore induction and proliferation, and are sufficient to promote the globular-to-bipolar embryo transition in eggplant MDEs

Among the different substances tested in this work, the most effective to promote microspore induction and callus growth was gum arabic. Chemically, gum arabic is a complex mixture of arabinogalactans (~90%) and AGPs (~10%), together with a residual ~2% of glycoproteins (Phillips 2009). Both arabinogalactans and AGPs have been related with beneficial effects in microspore embryogenesis. For example, Larcoll (an arabinogalactan extracted from *Larix occidentalis*) was shown to decrease mortality rate and to stimulate rapid cell division of cultured wheat microspores (Letarte et al. 2006). In maize, AGPs were found essential for the viability and development of

both MDEs and zygotic embryos (Paire et al. 2003). It is believed that AGPs are secreted to the culture medium by the growing MDE (Borderies et al. 2004; El-Tantawy et al. 2013). When applied to recalcitrant maize genotypes, the AGP-conditioned medium is able to augment their androgenic response (Borderies et al. 2004). Our results were consistent with these and other studies reporting beneficial effects of AGPs on embryogenesis induction. However, our experiments with βGlcY indicated that at least in our system, the principal responsible of the increases in callus number, size and weight was the ~10% of AGPs present in gum arabic, and not the major fraction of arabinogalactans. In addition, we could speculate that βGlcY would also be interfering with endogenous AGPs synthesized by microspores and MDEs, since addition of βGlcY produced a remarkable decrease of the induction rate, notably lower than controls with neither AGPs nor βGlcY.

Despite the fact that all the comparisons between factors were made at the same time and with the same microspore pool, the different experiments and repetitions provided different results in quantitative terms. This is evident when controls of the different charts shown in this work are compared. It is also observed in the case of AGP-including experiments, where differences can be observed in the different AGP vs control ratios of Figures 3A and 4A, for example. These differences can easily be explained as a direct consequence of the different plant batches used for each experiment, and principally by the widely accepted seasonal dependence of androgenesis. It is known that the efficiency of induction depends to a high extent on growth conditions of the donor plants (Dunwell 2010; Seguí-Simarro 2010). Thus, it is not surprising that even using the same *in vitro* procedures, microspores from greenhouse plants grown at different months yield different rates of embryogenesis

induction. This could be possibly due to changes in microspore viability or in androgenic competence. Nevertheless, what is important in this type of experiments is the consistency of the comparisons. In this context, we showed that the use of gum arabic at the optimal concentration always produced an improvement of induction efficiency.

Considering the ranges of concentration tested by us, it can be deduced that up to a limit of 1600 mg/L, increasing concentrations of AGPs are directly related to the induction of more microspores to divide (nearly 8x with 1600 mg/L), and to the production of larger calli, as revealed by the increases in number, size and weight. From 3000 mg/L and beyond, it is possible that these very high concentrations could exert some kind of deleterious effects, perhaps due to the high osmotic pressure created, or maybe due to high levels of toxic or inhibitory compounds also present in gum arabic. This would not be surprising, since it has been reported for other common components of *in vitro* culture media made from plant extracts, such as agar (Dunwell 2010; Kohlenbach and Wernicke 1978). Nevertheless, our results indicate that AGPs exert in eggplant a strong stimulatory effect over microspore viability and division of microspore-derived cells, as also reported in other species. Additionally, the use of AGPs allowed us to overcome the blockage in embryo progression initially observed at the transition between globular and heart-shaped stages (this study; Corral-Martínez and Seguí-Simarro 2012), obtaining MDEs of a morphological quality at least similar to those obtained by anther culture (Salas et al. 2011; Seguí-Simarro et al. 2011). Indeed, we showed elongating MDEs that progressed through the radial-to-bipolar transition, characterized by the unambiguous presence of a root apex and an elongated hypocotyl composed of structured cell layers surrounding a clearly distinguishable provascular

cylinder. However, they also presented many of the severe morphological abnormalities and functional problems observed in anther-derived eggplant MDEs (Salas et al. 2011; Seguí-Simarro et al. 2011), principally at the shoot apex. It is known that AGPs are necessary to modulate the developmental fate of the early embryo, in particular during the globular to heart-shaped embryo transition (this study; Tang et al. 2006). Many plant growth and differentiation events are also influenced by AGPs, including the establishment of the shoot and root apical meristems (Tang et al. 2006), the formation and vascularization of cotyledons, and embryo germination (Zhong et al. 2011). However, we showed that in eggplant, addition of AGPs was not sufficient to promote adequate shoot apical development, which suggest that other external factors would be needed to promote, in conjunction with AGPs, shoot apex establishment and proper development.

In summary, AGPs are capable to promote direct embryogenesis from eggplant isolated microspores more effectively than any other substance tested to date. Although more work needs to be done to overcome the functional limitations observed, this is an important step towards an efficient protocol for DH production through direct microspore embryogenesis, still absent.

Low BAP and NAA concentrations increase the efficiency of microspore induction

The main goal of this work was to optimize the previously existing method to obtain DHs from isolated eggplant microspores, evaluating the effect of new factors, but also revising its previous composition. Since growth regulators play an essential role in

most *in vitro* proliferation and differentiation processes, we previously checked the role of different growth regulators in the process of plant regeneration from microspore-derived calli (Corral-Martínez and Seguí-Simarro 2012). In this work, we re-evaluated the role of growth regulators, but in the process of microspore switch towards embryogenesis. We found that microspore induction could be increased by reducing the individual concentration of either BAP or NAA. However, the highest increase (four fold) was achieved by proportionally reducing both BAP and NAA to one fifth of their original concentrations. Although it is widely known that the auxin:cytokinin ratio is key to induce/inhibit most *in vitro* responses including proliferation, differentiation, rooting or shoot formation (Skoog and Miller 1957), it seems that eggplant microspores are considerably more sensitive to the absolute concentrations of BAP and NAA than to their relative proportions. This is consistent with the notion that true microspore embryogenesis should be induced regardless of growth regulators. In the model species where microspore embryogenesis is consistently induced and high amount of quality embryos are obtained, growth regulators are not used, or used in very low amounts (Seguí-Simarro 2010), suggesting that hormonal autotrophy defines true embryogenesis, either zygotic or androgenic (Aionesei et al. 2005). According to this, hormones are used in relatively recalcitrant systems where hormone-free inductive treatments are still insufficient to promote a sustained proliferation of microspore-derived cells.

The combined use of ABA, EBr, PEG, and AGPs leads to positive and negative interactions between them

Despite its positive effect on embryo induction when used alone, we found that when ABA was combined with EBr, PEG or AGPs, results were not satisfactory

enough. Although clearly positive differences with respect to control experiments were found in terms of callus size or organogenic competence, the effects in the number of induced calli were not so evident. In some cases, it seemed that the addition of ABA masked the positive effects of other factors. This was the case of the EBr+AGPs and PEG+AGPs combinations. Without ABA, the number of embryos produced was nearly twice and thrice higher than controls, respectively, whereas with ABA they were just slightly higher than controls. Somehow, ABA could be interacting with the signaling pathways or with the effects triggered by EBr, PEG and/or AGPs, thus reducing their stressing and therefore their inductive role during microspore embryogenesis. For the cases of EBr and PEG, this speculation would be reasonable, since it is known that ABA may mask or even inhibit EBr effects in plant responses against stresses such as salt and heat (Divi et al. 2010), and PEG-derived osmotic stress may be compensated by exogenous application of ABA (Ozfidan et al. 2012). This way, ABA would be preventing the stressing (inductive) effects of PEG. Considering the limited effect of ABA in the promotion of microspore embryogenesis and its null effect in MDE progression, together with its negative interaction with other, more beneficial factors, ABA should be discarded as part of an efficient protocol for androgenic eggplant DH production.

This study also demonstrated a limited effect of EBr when used alone. However, Figure 5A showed that when combined with AGPs (with or without PEG), the difference with controls is higher than when used alone. With respect to organogenic competence, when combined with AGPs and PEG, the percentage of organogenic calli was among the highest (Figure 5D). In other words, EBr seems to have positive instead of deleterious effects when combined with AGPs and PEG, adding its protective effects

to those derived from AGPs and from PEG-derived osmotic stress. It is known that EBr exerts anti-stress effects both independently as well as through interactions with other growth factors (Divi et al. 2010). In addition to the negative interaction described above for EBr and ABA, positive interactions EBR-PEG, and EBr-AGPs might well be possible. In the case of PEG, EBr would be protecting against the deleterious effects of osmotic stress while not affecting its inductive potential. With respect to the possible EBr-AGP interaction, our results suggest that this would be more than a possibility, since the combination of EBr+AGPs gave the second highest number of calli. In addition, the size of the calli produced with this combination was the highest. This observation is important, because the use of EBr alone gave rise to more calli, but significantly smaller than controls. It seems that AGPs somehow "compensate" for this, allowing for the production not only of more calli, but also of larger calli.

Refining the protocol to obtain eggplant androgenic DHs

As explained above, one of the principal conclusions of this study is that AGPs should be routinely used to increase the efficiency of microspore embryogenesis, to reduce embryo-to-callus conversion, and to increase the rate of organogenic calli. According to our combinative experiments, AGPs could be used for callus production either alone or together with EBr and/or PEG. According to their efficiency in microspore induction, the combined use of PEG+AGPs would be advised. According to the percentage of organogenic calli, however, the addition of EBr to PEG+AGPs would not have a significant impact. Therefore, considering the high price of commercial EBr, we would not recommend to include it in a practical protocol for routine production of eggplant DHs. In conclusion, according to our results such a protocol should be

optimized by reducing BAP and NAA concentrations to one fifth, by replacing 2% sucrose by 1% sucrose + 1% PEG, and by adding 1600 mg/l of gum arabic.

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Figure legends

Figure 1: Effects of the addition of ABA. A-E: Callus-like structures after 15 days in culture with different ABA concentrations. A: Control without ABA. B: $0.5.10^{-6}$M. C: 1.10^{-6}M. D: 2.10^{-6}M. E: 5.10^{-6}M. F-G': Quantitative effects expressed as total number of calli/dish (F), total callus weight (g)/dish (F'), and number of calli/dish larger than 1 mm (G), and 2 mm (G'). Error bars represent s.d. Different letters indicate statistically significant differences ($p<0.05$). Bars in A-E: 200 μm.

Figure 2: Quantitative effects of the addition of EBr, expressed as total number of calli/dish (A), total callus weight (g)/dish (B), number of calli/dish larger than 1 mm (C) and 2 mm (D). Error bars represent s.d. Different letters indicate statistically significant differences ($p<0.05$).

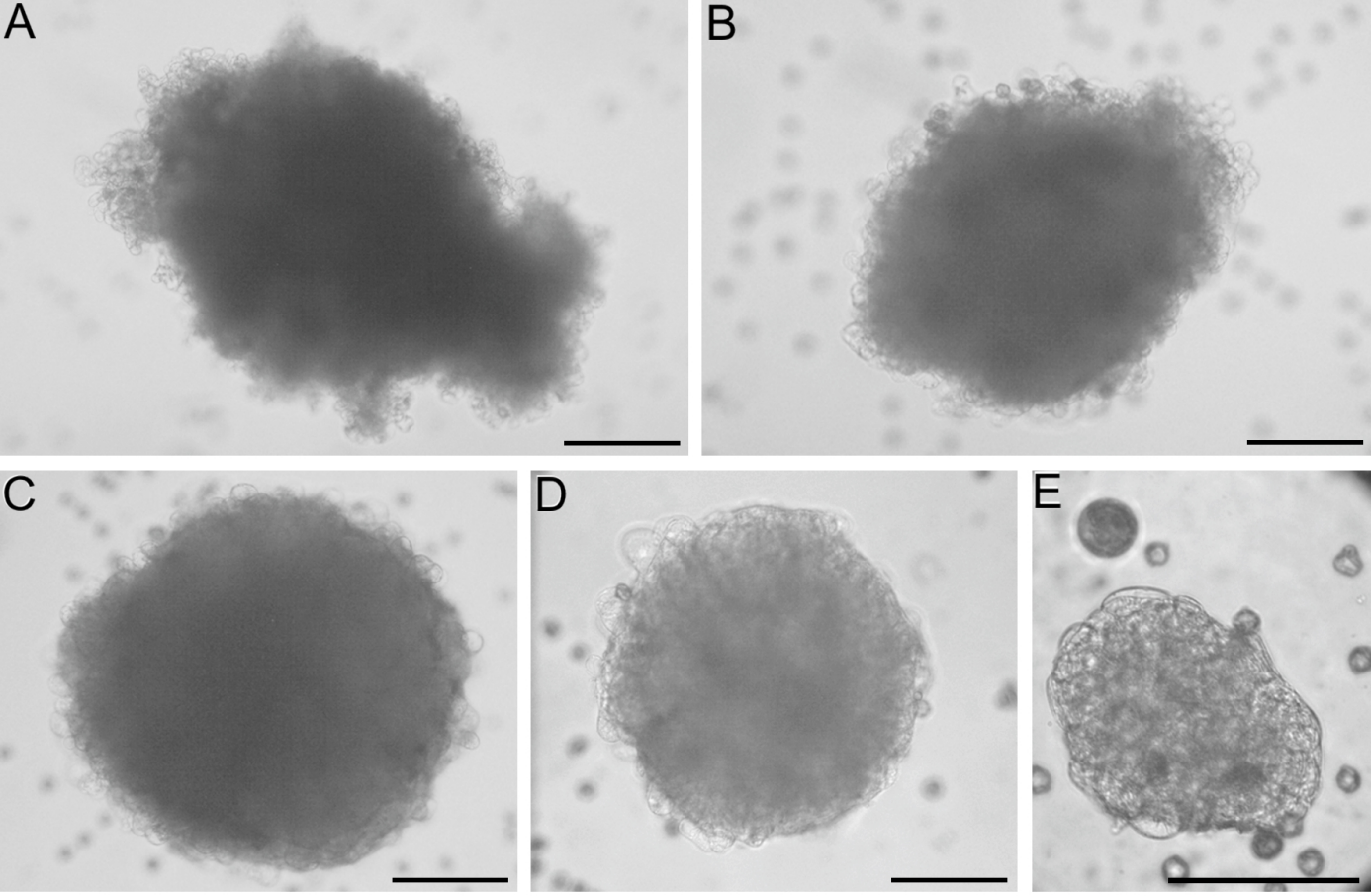
Figure 3: Quantitative effects of the addition of gum arabic at concentrations ranging from 400 to 1600 mg/L, expressed as total number of calli/dish (A), total callus weight (g)/dish (B), number of calli/dish larger than 1 mm (C) and 2 mm (D). Error bars represent s.d. Different letters indicate statistically significant differences ($p<0.05$).

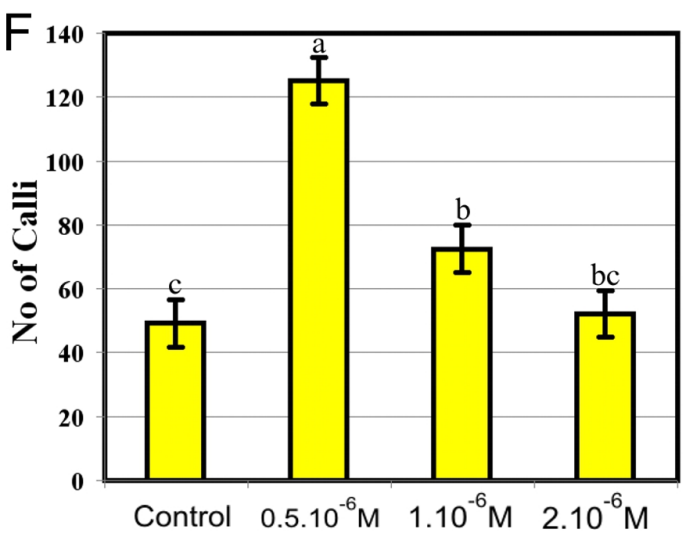
Figure 4: Effects of the addition of gum arabic. A: Quantitative effects of adding gum arabic alone (AGPs) and together with βGlcY (AGPs+ βGlcY), expressed as total number of calli/dish (light bars), number of calli/dish larger than 1 mm (grey bars) and 2 mm (dark bars). Error bars represent s.d. Different letters indicate statistically significant differences ($p<0.05$). B: MDEs and calli obtained with 1600 mg/L gum arabic. Arrow points to a globular embryo, asterisk marks an elongating embryo, and

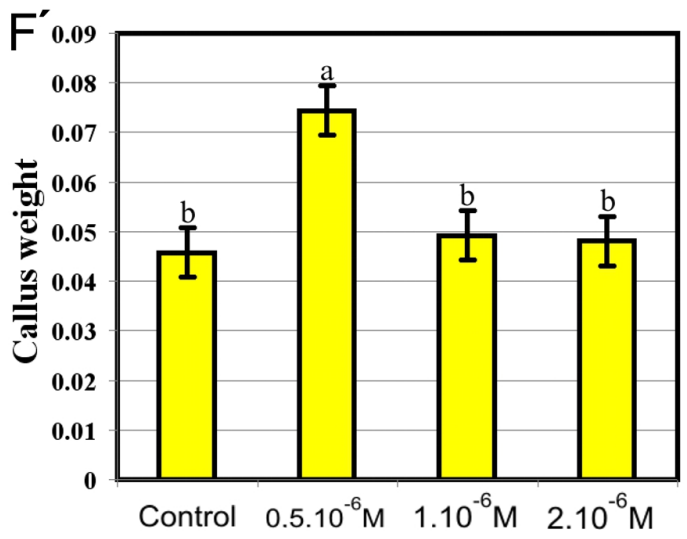
arrowhead points to an embryo with a germinated root. C: Eggplant MDE showing a normal, zygotic-like hypocotyl and root apex, and a defective shoot apex. Bars: B: 500 µm; C: 200 µm.

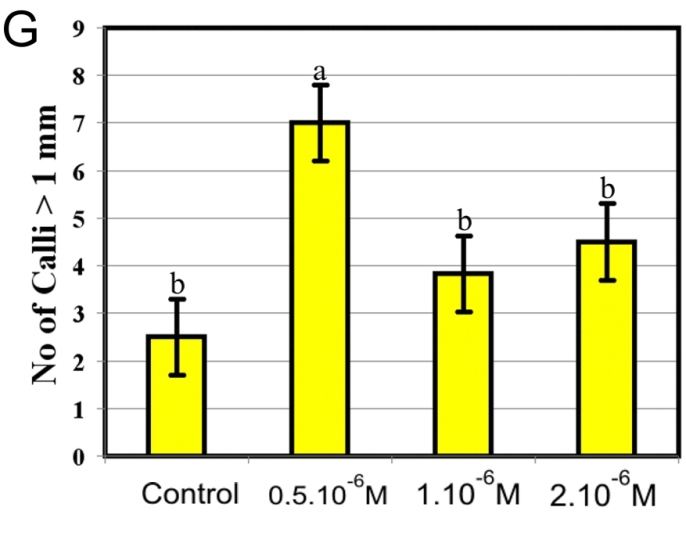
Figure 5: Effects of the combined addition of AGPs, ABA, EBr and PEG. A: Quantitative effects on microspore induction, expressed as total number of calli/dish (light bars), number of calli/dish larger than 1 mm (grey bars) and 2 mm (dark bars). B: Examples of structures obtained with all factor combinations except for PEG+AGPs amd ABA+PEG+AGPs, which are illustrated in C. Bars: 50 µm. D: Quantitative effects on regeneration from microspore-derived calli, expressed as percentage of organogenic calli from total number of calli per plate. Error bars represent s.d. Different letters indicate statistically significant differences ($p<0.05$).

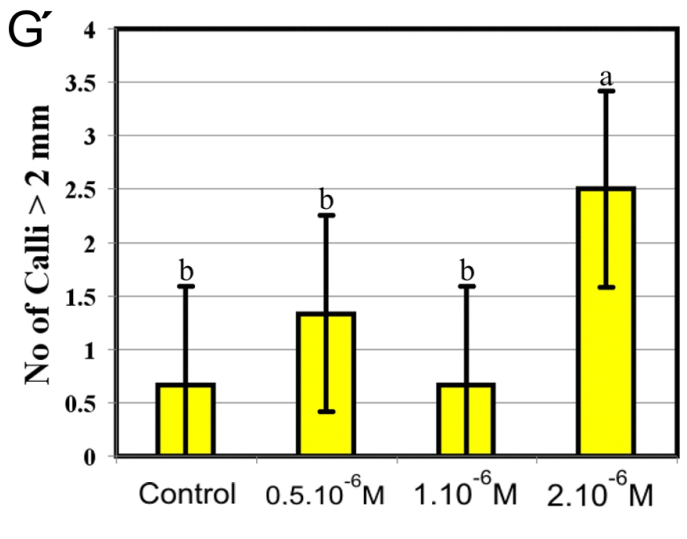
Figure 6: Quantitative effects of altering concentration and ratio of BAP and NAA, expressed as total number of calli/dish (light bars), number of calli/dish larger than 1 mm (grey bars) and 2 mm (dark bars). Error bars represent s.d. Different letters indicate statistically significant differences ($p<0.05$).

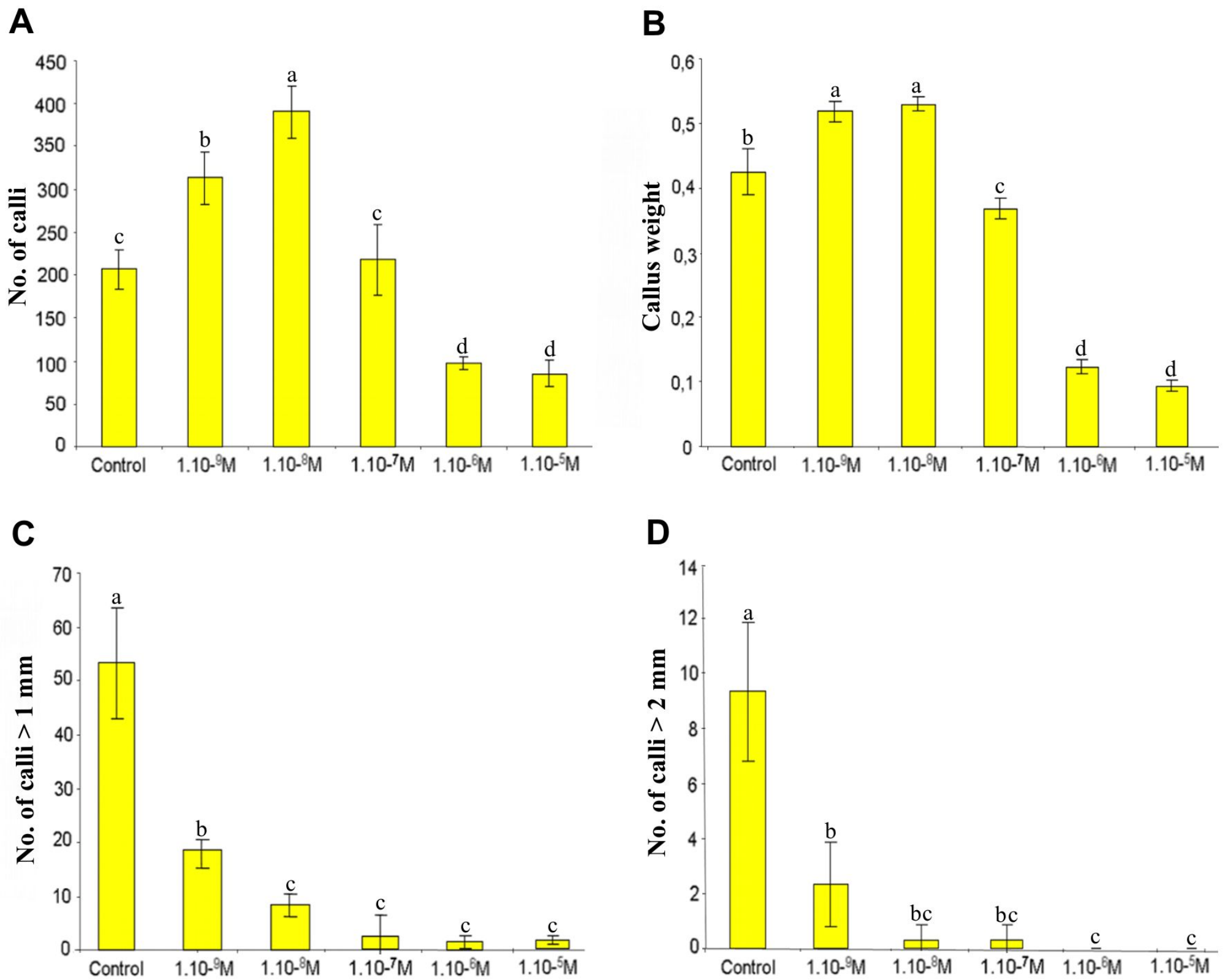

A
B
C
D
E

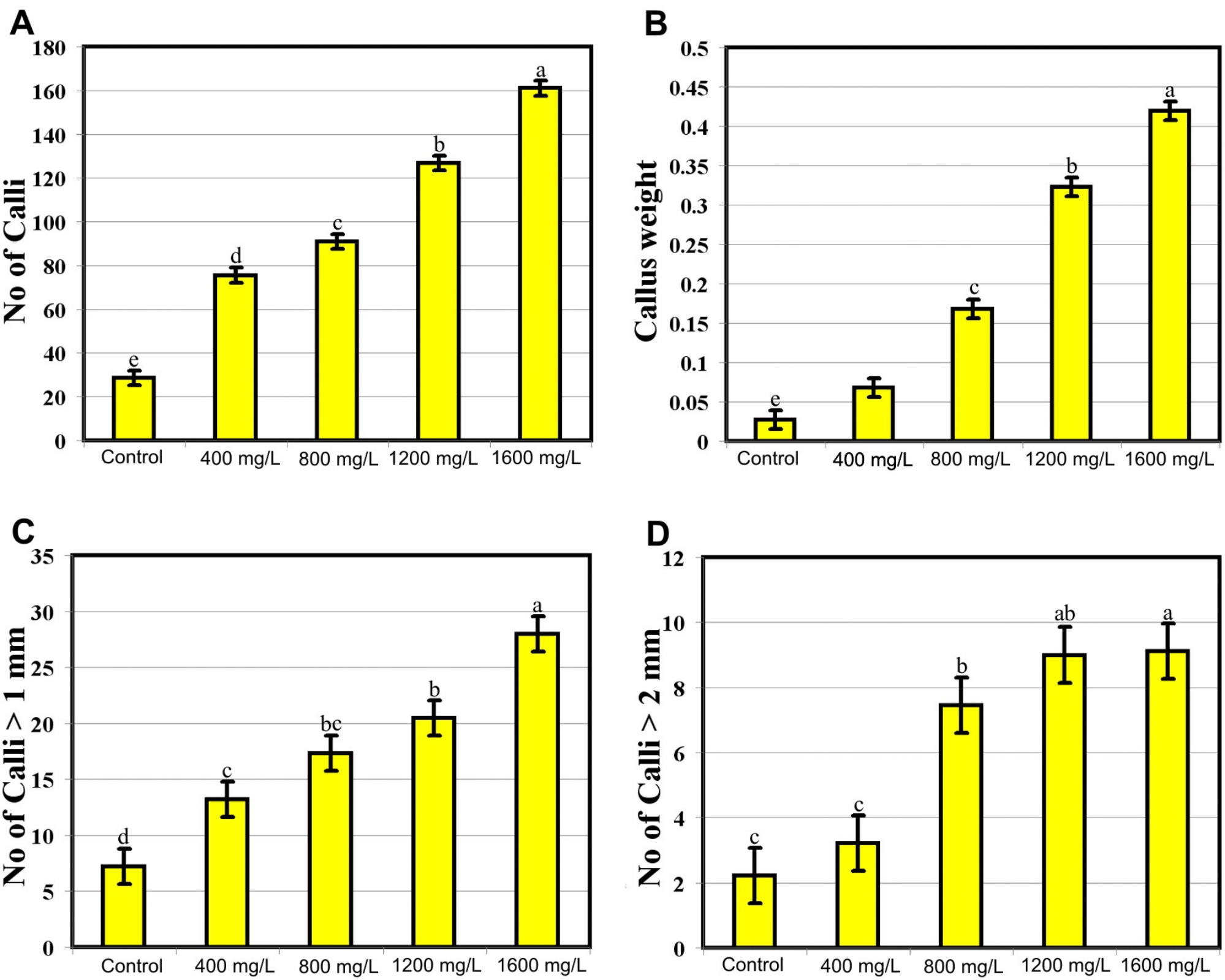

F
No of Calli
140
120
100
80
60
40
20
0
a
b
bc
c
Control
0.5.10-6M
1.10-6M
2.10-6M

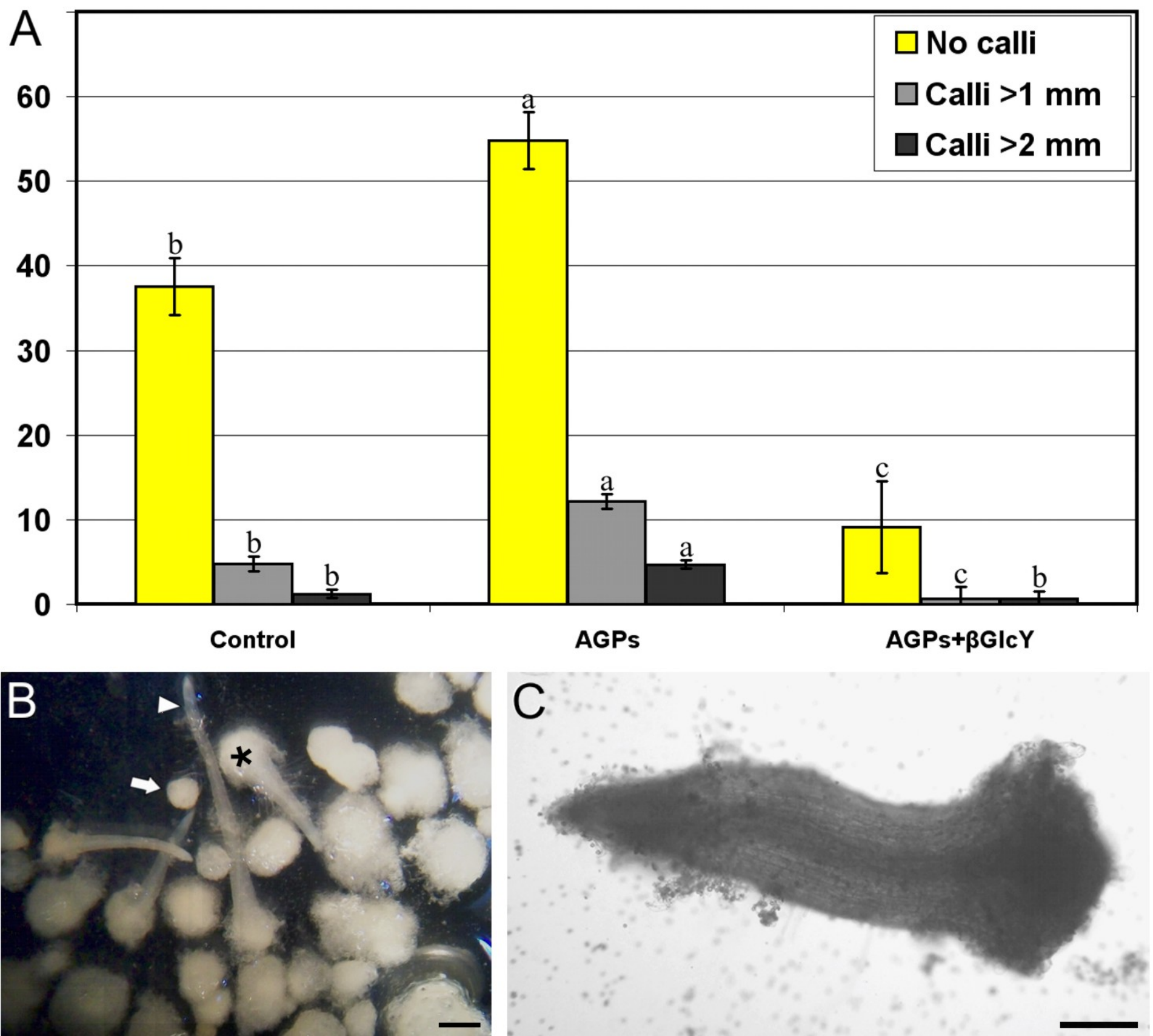

F´
Callus weight
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
a
b
b
b
Control
0.5.10-6M
1.10-6M
2.10-6M

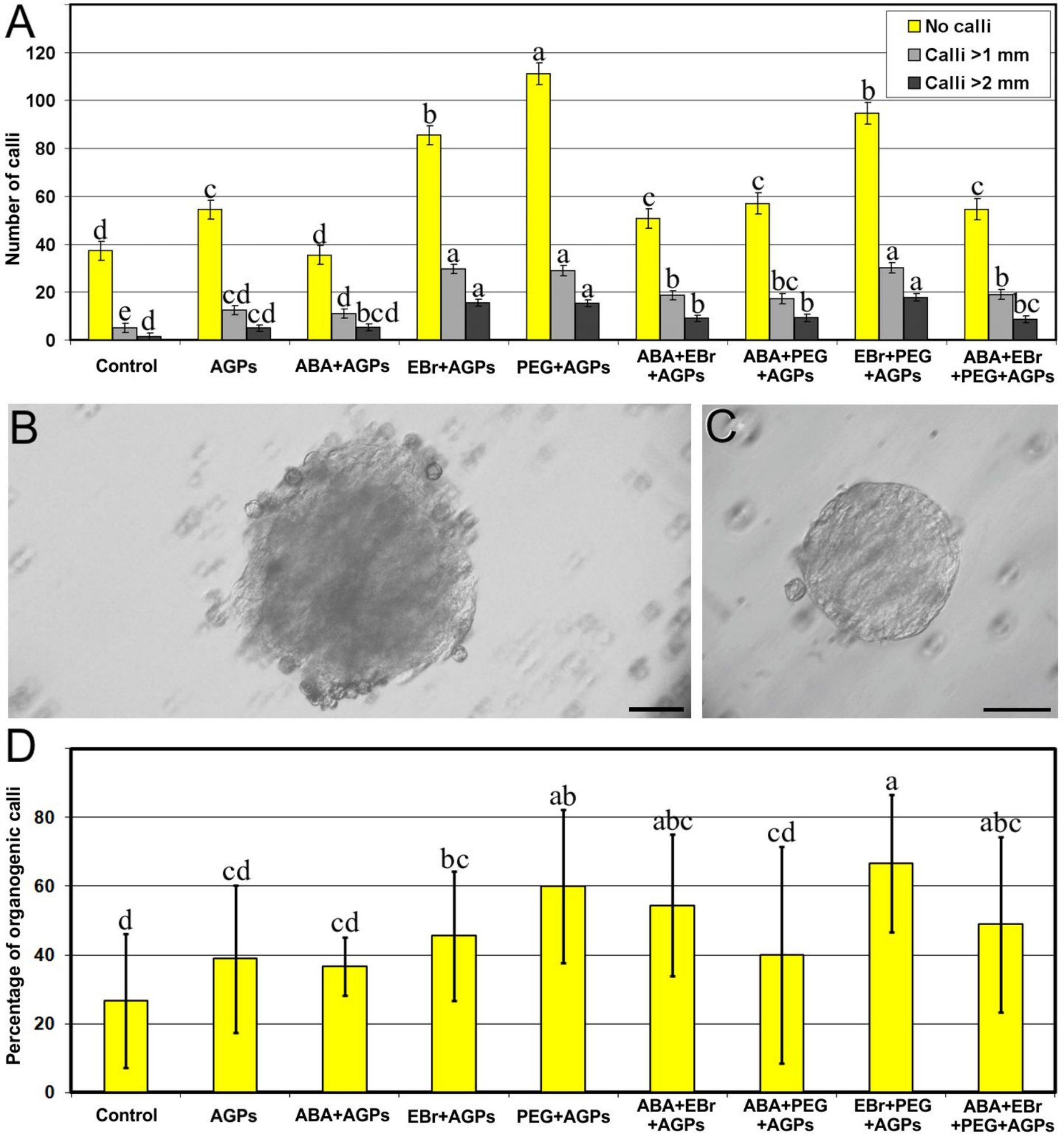

G
No of Calli > 1 mm
9
8
7
6
5
4
3
2
1
0
a
b
b
b
Control
0.5.10-6M
1.10-6M
2.10-6M

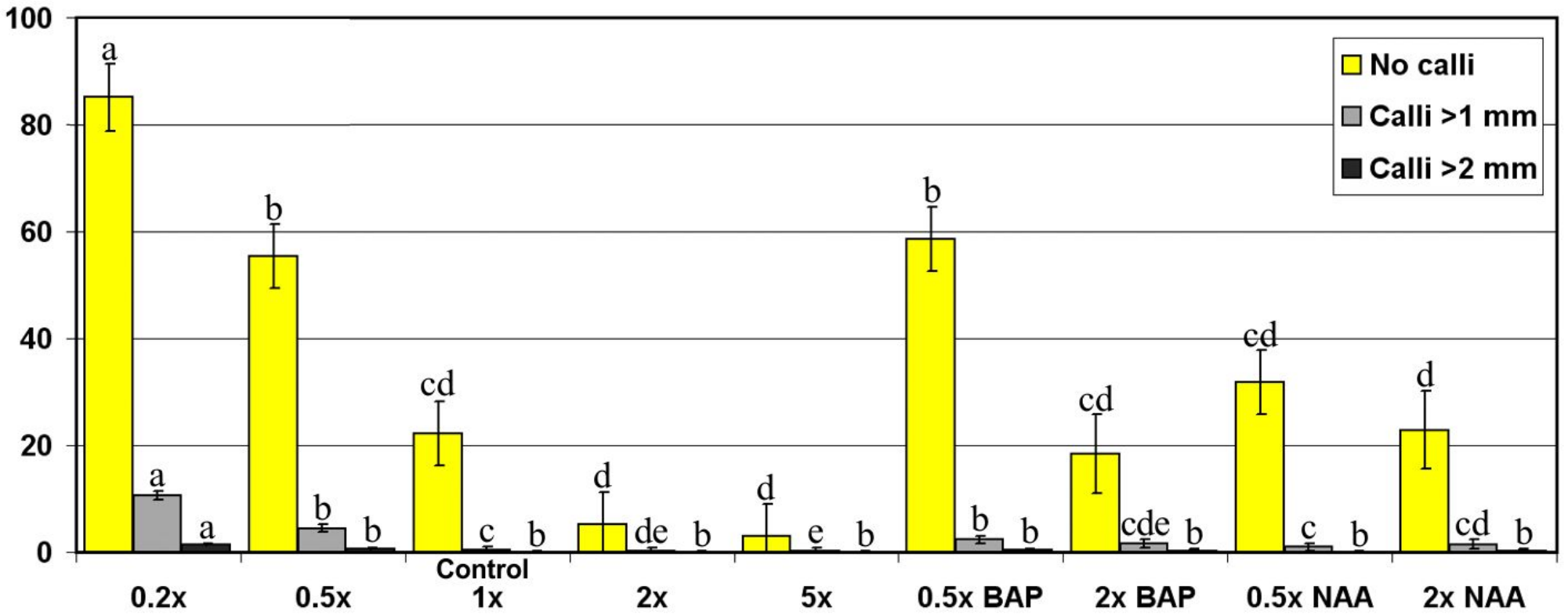

G´
No of Calli > 2 mm
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
b
b
Control
0.5.10-6M
1.10-6M
2.10-6M

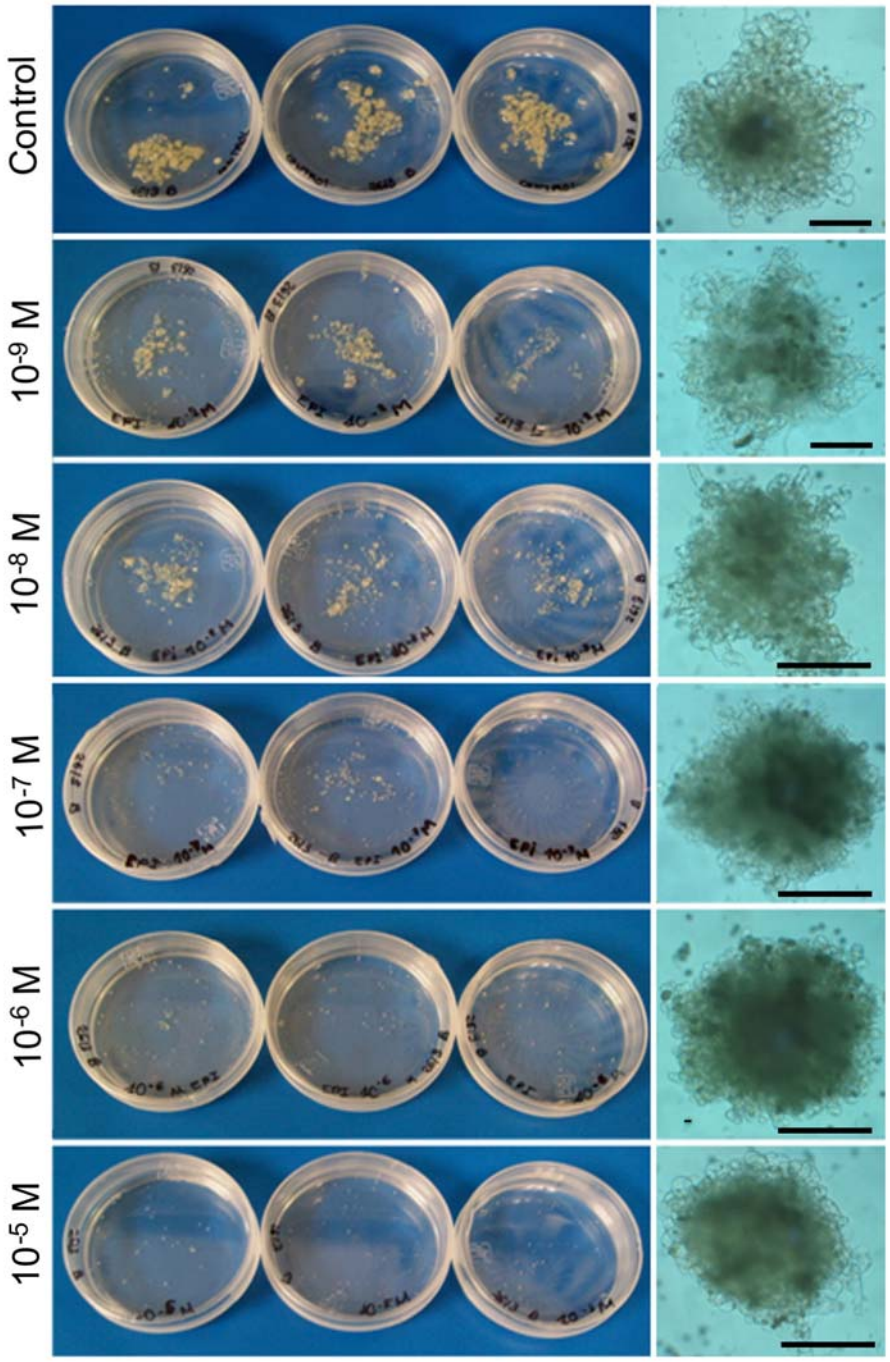

A
No. of calli
450
400
350
300
250
200
150
100
50
0
a
b
c
c
d
d
Control
1.10-9M
1.10-8M
1.10-7M
1.10-6M
1.10-5M
B
Callus weight
0,6
0,5
0,4
0,3
0,2
0,1
0
a
a
b
c
d
d
Control
1.10-9M
1.10-8M
1.10-7M
1.10-6M
1.10-5M
C
No. of calli > 1 mm
70
60
50
40
30
20
10
0
a
b
c
c
c
c
Control
1.10-9M
1.10-8M
1.10-7M
1.10-6M
1.10-5M
D
No. of calli > 2 mm
14
12
10
8
6
4
2
0
a
b
bc
bc
c
c
Control
1.10-9M
1.10-8M
1.10-7M
1.10-6M
1.10-5M


A
No of Calli
180
160
140
120
100
80
60
40
20
0
a
b
c
d
e
Control
400 mg/L
800 mg/L
1200 mg/L
1600 mg/L
B
Callus weight
0.5
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
b
c
e
Control
400 mg/L
800 mg/L
1200 mg/L
1600 mg/L
C
No of Calli > 1 mm
35
30
25
20
15
10
5
0
a
b
bc
c
d
Control
400 mg/L
800 mg/L
1200 mg/L
1600 mg/L
D
No of Calli > 2 mm
12
10
8
6
4
2
0
a
ab
b
c
c
Control
400 mg/L
800 mg/L
1200 mg/L
1600 mg/L

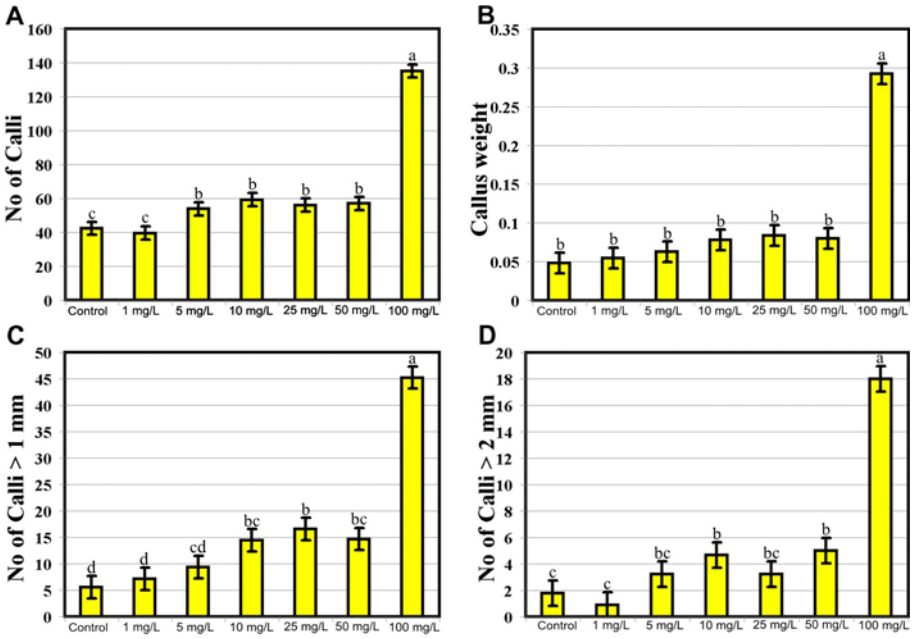

A
No calli
Calli >1 mm
Calli >2 mm
60
50
40
30
20
10
0
b
b
b
a
a
a
c
c
b
Control
AGPs
AGPs+βGlcY
B
C


A
No calli
Calli >1 mm
Calli >2 mm
Number of calli
120
100
80
60
40
20
0
d
e
d
c
cd
cd
d
d
bcd
b
a
a
a
a
a
c
b
b
c
bc
b
b
a
a
c
b
bc
Control
AGPs
ABA+AGPs
EBr+AGPs
PEG+AGPs
ABA+EBr +AGPs
ABA+PEG +AGPs
EBr+PEG +AGPs
ABA+EBr +PEG+AGPs
B
C
D
Percentage of organogenic calli
80
60
40
20
0
d
cd
cd
bc
ab
abc
cd
a
abc
Control
AGPs
ABA+AGPs
EBr+AGPs
PEG+AGPs
ABA+EBr +AGPs
ABA+PEG +AGPs
EBr+PEG +AGPs
ABA+EBr +PEG+AGPs

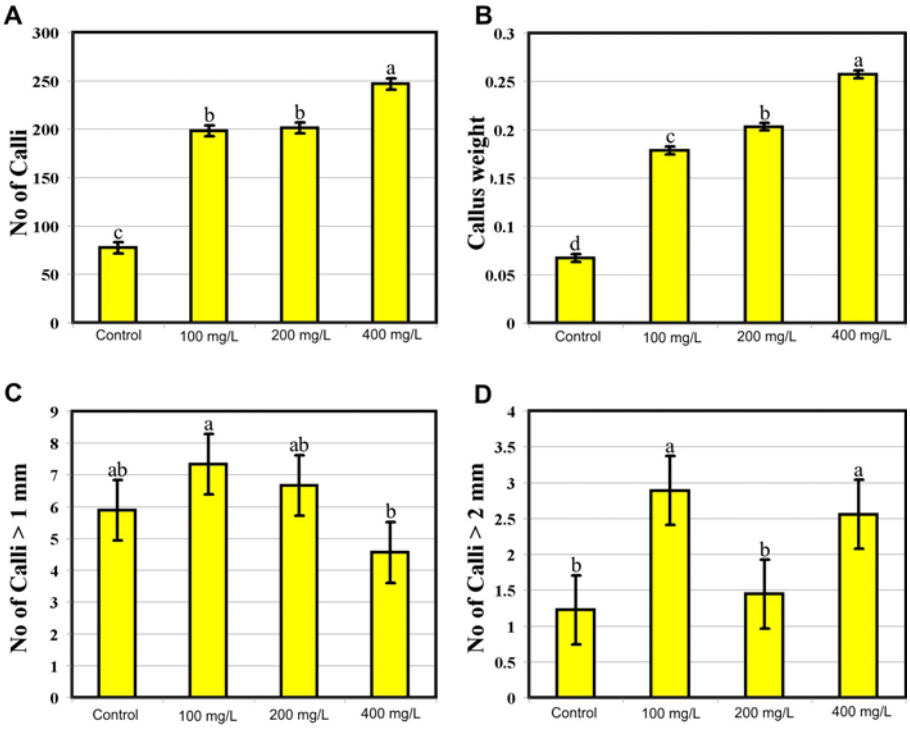

100
80
60
40
20
0
No calli
Calli >1 mm
Calli >2 mm
a
a
a
b
b
b
cd
c
b
d
de
b
d
e
b
b
b
b
cd
cde
b
cd
c
b
d
cd
b
0.2x
0.5x
Control
1x
2x
5x
0.5x BAP
2x BAP
0.5x NAA
2x NAA



Online Resource 1: Callus-like structures after 30 days in culture with different EBr concentrations. Left column shows three replicas of each concentration, where differences in callus number and size can be observed. The B column shows microscopic images of the calli produced at each concentration. Bars: 200 µm.



Online Resource 2: Quantitative effects of the addition of gum arabic at concentrations ranging from 1 to 100 mg/L, expressed as total number of calli/bud (A), total callus weight (B), number of calli/bud larger than 1 mm (C) and 2 mm (D). Different letters indicate statistically significant differences ($p \leq 0.05$).



Online Resource 3: Quantitative effects of the addition of gum arabic at concentrations ranging from 100 to 400 mg/L, expressed as total number of calli/bud (A), total callus weight (B), number of calli/bud larger than 1 mm (C) and 2 mm (D). Different letters indicate statistically significant differences ($p \leq 0.05$).