

# Stress treatments and in vitro culture conditions influence microspore embryogenesis and growth of callus from anther walls of sweet pepper (*Capsicum annuum* L.)

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**Abstract** Production of doubled haploids (DHs) is a convenient tool to obtain pure lines for breeding purposes. Until now, the easiest and most useful approach to obtain pepper DHs is via anther culture. However, this method has an associated possibility of producing calli from anther wall tissues that would be coexisting in the anther locule with embryos derived from microspores. Using two established protocols for anther culture, Dumas de Vault et al. (*Agronomie* 2:983–988, 1981) and Supena et al. (*Sci Hort* 107:226–232, 2006a; *Plant Cell Rep* 25:1–10, 2006b) callus and embryo development was assessed in four sweet pepper cultivars. For all genotypes tested, the protocol of Dumas de Vault et al. (*Agronomie* 2:983–988, 1981) promoted both embryo development and callus growth, whereas the protocol of Supena et al. (*Sci Hort* 107:226–232, 2006a; *Plant Cell Rep* 25:1–10, 2006b) produced no callus but only embryos. However, differences in embryo production were observed among these genotypes. In parallel, anthers were exposed to a 35 °C inductive heat shock for 4, 8, 12 and 16 days, prior to culture at 25 °C. The duration of the heat shock had significant effects in embryo production, but also in callus generation. Callus generation increased with prolonged exposures to 35 °C. Embryo and callus origin was analyzed by flow cytometry, light microscopy and molecular markers. Tests conducted demonstrated a gametophytic origin for all

of the embryos tested, and a sporophytic origin for all of the calli. Together, our results reveal that culture conditions have a significant influence on the presence of calli derived from anther walls, which could be minimized by reducing heat shock exposure and/or using a shed-microspore approach.

**Keywords** Androgenesis · *Capsicum annuum* · Heat stress · Microspore embryogenesis · In vitro culture

## Introduction

Induction of androgenesis is one of the most convenient ways to obtain haploid and doubled haploid (DH) individuals. Pepper (*Capsicum annuum*) is, together with tomato and eggplant, one of the three solanaceous crops most recalcitrant to the induction of androgenic DHs (Seguí-Simarro et al. 2011). Apart from the spontaneous occurrence of some cases of in vivo androgenesis with no practical relevance (Campos and Morgan 1958), haploids in pepper were first obtained through parthenogenesis (reviewed in Regner 1996). Soon after their discovery by Guha and Maheshwari (1964), anther cultures were explored as a way to haploidy, and since then, they have been used as a tool to produce pepper DHs for breeding programs (Jiang and Li 1984; Dumas de Vault and Pochard 1986; Hwang and Paek 1998; Arnedo Andrés et al. 2004). Despite the recent progress made on isolated microspore culture (Lantos et al. 2009; Ferrie and Caswell 2011; Kim et al. 2012; Lantos et al. 2012), anther culture is still considered the method of choice for pepper DH production due to its simplicity (Germanà 2011). However, this technique carries a number of drawbacks. Among others, these include: a limited efficiency, producing only a few embryos per cultured anther; the uncontrollable secretory effect of the tapetum, which precludes a strict control of

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culture conditions; and the undesirable presence of calli produced from anther wall tissues (Seguí-Simarro et al. 2011).

The study of callus formation in anther cultures is not a trivial issue, at least in recalcitrant solanaceous species. Whereas in some species calli may have a gametophytic origin, in many others a sporophytic origin has been demonstrated for some of the calli produced. It was recently demonstrated that, under certain experimental conditions, microspore-derived embryos in eggplant may transform into haploid or DH calli (Corral-Martínez and Seguí-Simarro 2012). However, callus generation from anther wall cells is also frequent in eggplant anther cultures, as demonstrated in 12 different eggplant accessions (Salas et al. 2011). In tomato, haploid and DH plants could be regenerated from calli produced by anthers containing meiocytes (Seguí-Simarro and Nuez 2007). Regrettably, 83 % of these calli were produced by anther wall tissues (Corral-Martínez et al. 2011). Although there are no specific studies addressing this topic, callus production has been frequently reported in the literature about pepper anther culture, with many protocols producing both calli and embryos, and few producing only calli (reviewed in Irikova et al. 2011). Interestingly, the first attempts to culture pepper anthers produced calli, from which plants were regenerated through organogenesis (George and Narayanaswamy 1973; Kuo et al. 1973; Wang et al. 1973). As deduced from the literature, it would be important to know where calli come from, to what extent they may constitute a drawback, as well as to find ways to overcome or at least minimize such a drawback.

In this work, we addressed these issues. In order to shed light on the relationship between culture conditions and callus production, we compared the influence on callus and embryo production of two successfully used media. We also checked the effect of different durations of a 35 °C heat shock. Embryo and callus origin was also analyzed by flow cytometry, light microscopy and microsatellite molecular markers. Our results demonstrate that culture conditions have a significant influence not only on the production of microspore-derived embryos, but also of calli derived from anther wall tissues. We propose ways to minimize such callus presence.

## Materials and methods

### Plant material

The following four commercial F1 hybrids of pepper (*C. annuum* L.) were used: ‘Herminio’ (Lamuyo type, from Syngenta Seeds), ‘Coyote’, ‘Quito’ (California types, both from Syngenta Seeds), and ‘Vélez’ (California type, from

Enza Zaden). Plants were grown in 30 cm pots at COMAV greenhouses (Universitat Politècnica de València), at a minimum of 18 °C under natural light during 9 months from March to November.

### Anther culture

We cultured anthers of the four genotypes above mentioned. The number of anthers cultured for each genotype was as follows: for ‘Herminio’, 491 anthers (corresponding to 90 buds); for ‘Coyote’, 381 anthers (64 buds); for ‘Quito’, 378 anthers (64 buds); and for Vélez, 389 anthers (68 buds). Selection of buds was based on a sepal length being around 80 % of petal length, according to Parra-Vega et al. (2012). Anthers with purple distal tips, containing mostly vacuolate microspores and young bicellular pollen grains (Parra-Vega et al. 2012), were extracted from selected buds. After surface sterilization with 10 % commercial bleach (40 g l<sup>-1</sup>) for 5 min, anthers were plated and cultured according to two previously published, different protocols: (1) the protocol of Dumas de Vault et al. (1981) for anther culture in agar-based solid medium (hereinafter referred to as the DDV protocol), and (2) the protocol published by Supena et al. (2006a, b) for anther culture in a biphasic (solid–liquid) medium, also known as the shed-microspore method (hereinafter the SM method). Five repetitions with five dishes per repetition (6 anthers per dish) were performed at different months from March to November for each culture method. Mean and standard deviation were calculated. Additionally, anthers of the four genotypes were cultured according to the DDV method with modification of the duration of the 35 °C exposure to 4, 8, 12 and 16 days. A minimum of three repetitions with five dishes per repetition (6 anthers per dish) were performed for each combination of genotype and exposure time, and the mean and standard deviation were calculated. For all comparisons, data of the corresponding experiments were subjected to standard analysis of variance using the Sigmastat software (Systat Software, Inc. Germany) and means were separated using a Holm-Sidak test with  $p \leq 0.05$ .

### Flow cytometry

Small pieces of anther-derived calli and embryos were processed for flow cytometry as described in Abdollahi et al. (2012). Additionally, young leaf samples from donor plants were analyzed and used as standards for 2C DNA content. Briefly, samples were chopped with a razor blade and processed using the CyStain UV Precise P kit for nuclear extraction and staining (Partec GmbH, Münster, Germany) according to manufacturer’s specifications. Extracted nuclei were filtered through 30 µm CellTricks

filters (Partec GmbH, Münster, Germany) and immediately analyzed in a Partec PA-I Ploidy Analyzer.

#### Genetic analysis with microsatellite molecular markers

Prior to their use to determine the origin of the plants obtained from anther cultures, donor plants of the commercial F1 hybrids ‘Herminio’ and ‘Vélez’ were screened using microsatellite markers (SSR). Young leaf tissue was sampled from 5 different, randomly chosen donor plants, and genomic DNA was isolated from 50 mg of tissue using the modified CTAB (hexadecyl trimethylammonium bromide) method described in Ferriol et al. (2003). Each donor plant was analyzed using the following 16 SSR markers known to be polymorphic in other pepper materials (Minamiyama et al. 2006; Portis et al. 2007): EPMS670, EPMS755, EPMS643, EPMS716, EPMS689, EPMS747, EPMS924, EPMS704, EPMS725, EPMS757, EPMS745, EPMS650, EPMS749, CAMS117, CAMS340 and CAMS806. The forward primers were labeled with different fluorescent dyes and six loci were simultaneously detected using an ABI PRISM 310 Genetic Analyzer. Heterozygous loci were consistently found for six SSR markers (CAMS117, CAMS340, EPMS650, CAMS806, EPMS670 and

EPMS643) in all ‘Herminio’ and ‘Vélez’ donor plants analyzed. These markers were used as described above to check the origin of embryo-derived plants.

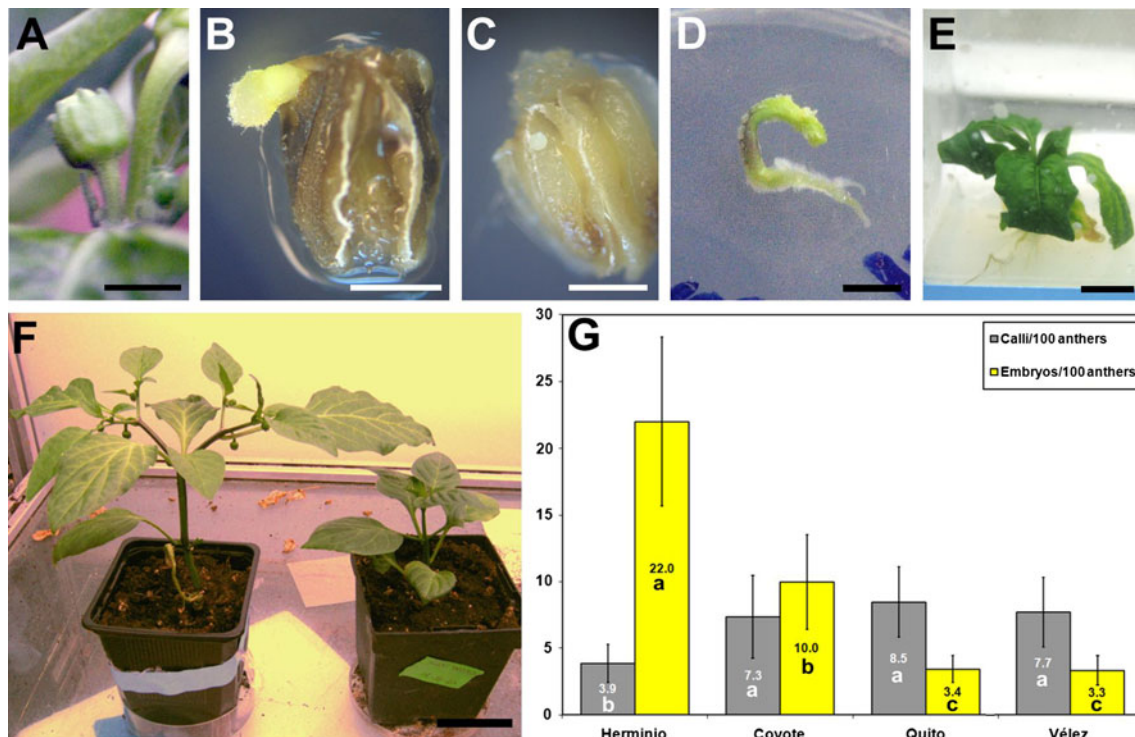
#### Light microscopy

Cultured anthers at different culture days were picked up and processed for light microscopy as described in Seguí-Simarro and Nuez (2005). Briefly, anthers were fixed in Karnovsky fixative (4 % formaldehyde + 5 % glutaraldehyde in 0.025 M cacodylate buffer, pH 7), dehydrated in ethanol series and embedded in Technovit 7100 according to manufacturer’s specifications. Thin (1.5  $\mu$ m) sections were obtained with a Leica UC6 ultramicrotome and observed under phase contrast with a Nikon Eclipse E1000 light microscope.

## Results

#### Callus and embryo production with different anther culture methods

In order to elucidate the influence of culture conditions on embryo and callus production, we cultured anthers of the



**Fig. 1** Anther culture in cv ‘Herminio’ using the DDV method. **a** Flower bud at the right stage for anther isolation. **b** Cultured anther producing a microspore-derived embryo. **c** Cultured anther producing calli. **d** Germinating microspore-derived embryo. **e** In vitro microspore-derived plantlet. **f** Ex vitro, fully acclimated microspore-derived plantlets. **g** Comparison between callus (dark bars) and

embryo production (light bars) in the four genotypes studied. Results are expressed as number of callus or embryos produced per 100 cultured anthers. Different letters indicate statistically significant ( $p < 0.05$ ) differences among genotypes. Bars: **a**, **d** 5 mm; **b**, **c** 1 mm; **e** 1 cm; **f** 5 cm

‘Herminio’, ‘Coyote’, ‘Quito’ and ‘Vélez’ genotypes using two markedly different culture conditions: the DDV and the SM protocols. In the DDV method, flower buds at the appropriate stage of development (Fig. 1a) were excised and their anthers extracted and cultured in semisolid, agar-based culture medium. Three to 4 weeks after culture initiation, the first embryos were observed emerging out of the walls of the necrosing anther (Fig. 1b). In parallel, a whitish, callus-like mass of cells emerged from the anther locule (Fig. 1c). Upon separation from the anther, individualized embryos germinated (Fig. 1d), giving rise to *in vitro* plantlets (Fig. 1e). After acclimatization, these developed into normal pepper plants (Fig. 1f). In quantitative terms (Fig. 1g), all four genotypes responded to *in vitro* induction by producing both calli and embryos. However, the embryo-to-callus ratio was remarkably different among genotypes, being embryo formation more frequent than callus production in ‘Herminio’, similarly frequent in ‘Coyote’ and less frequent in ‘Quito’ and ‘Vélez’. Production of embryos was heterogeneous as well, ranging from 3.3 embryos/100 anthers for ‘Vélez’ to 22 for ‘Herminio’. In contrast, production of calli was more homogeneous, ranging from 3.9 calli/100 anthers in ‘Herminio’ to 8.5 in ‘Quito’. Thus, it appeared that embryo production was more genotype-dependent than callus production.

In the SM method, anthers were extracted from flower buds at the appropriate stage of development and cultured in liquid medium (Fig. 2a). Upon dehiscence, open anthers released microspores to the liquid medium (Fig. 2b), sinking to the solid–liquid interphase and then transforming into microspore-derived embryos (Fig. 2c). Some embryos germinated and upon acclimatization transformed into entire plants (Fig. 2d). Quantitative analysis of these cultures (Fig. 2e) revealed a pattern of embryo and callus production

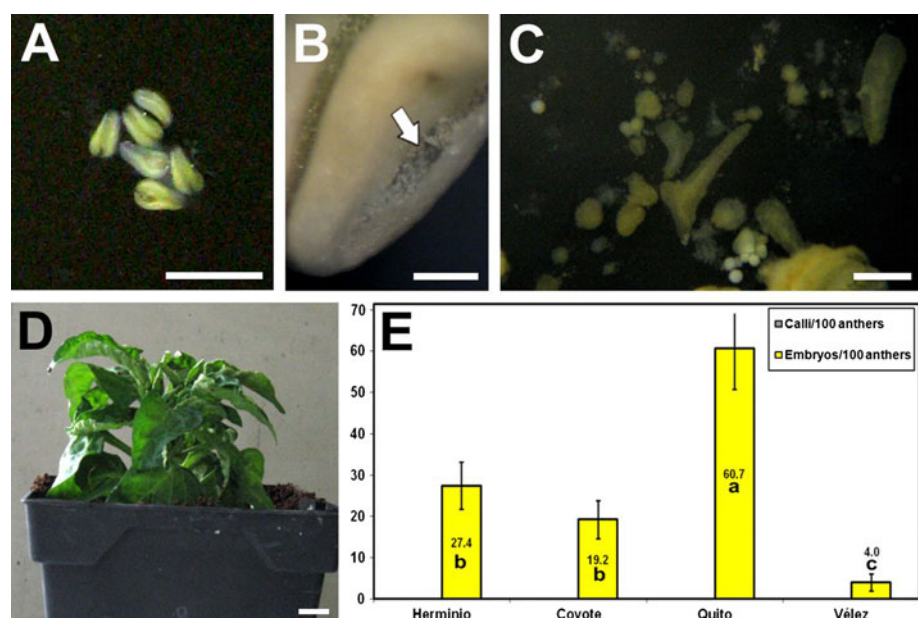
markedly different from the DDV method. Although embryo production was similar using both methods for the ‘Herminio’ (22.0 vs 27.4 embryos/100 anthers) and ‘Vélez’ genotypes (3.3 vs 4.0 embryos/100 anthers), ‘Coyote’ and principally ‘Quito’ showed a dramatically increased response, nearly doubled for ‘Coyote’ (10.0 vs 19.2 embryos/100 anthers) and 17 times higher for ‘Quito’ (3.4 vs 60.7 embryos/100 anthers). Production of calli was not observed in any case. Thus, as observed with the DDV method, with the SM method callus production was less influenced by the genotype than embryo production.

#### Callus and embryo response under different pretreatment durations

Anthers of the ‘Herminio’, ‘Coyote’, ‘Quito’ and ‘Vélez’ genotypes were cultured according to the DDV method but modifying the duration of the 35 °C exposure to 4, 8, 12 and 16 days. The embryogenic response was highly dependent of the genotype for the four durations assayed. ‘Herminio’ (Fig. 3a) showed the highest levels of embryo production, followed by ‘Coyote’ (Fig. 3b), ‘Quito’ (Fig. 3c) and ‘Vélez’ (Fig. 3d). Quantitative differences in terms of embryo production, probably due to seasonal effects, were observed. Nevertheless, the pattern of differences observed among genotypes when cultured for 8 days at 35 °C (as in the original DDV protocol) was consistent with that shown in Fig. 1g. Equally consistent was the observation that callus formation was less dependent on the genotype, with differences smaller than for embryo production.

The effect of prolonged 35 °C treatments on embryo production was found to be detrimental in all cases, with a

**Fig. 2** Anther culture in cv ‘Herminio’ using the SM method. **a** Anthers floating on the liquid upper phase. **b** Anther opening at the dehiscence line (arrow), releasing microspores to the liquid medium. **c** Microspore-derived embryos originated from induced microspores. **d** Ex vitro, fully acclimated microspore-derived plantlet. **e** Comparison between callus and embryo production in the four genotypes studied. Results are expressed as number of callus or embryos produced per 100 cultured anthers. Different letters indicate statistically significant ( $p \leq 0.05$ ) differences among genotypes. Bars: **a** 5 mm; **b** 500  $\mu$ m; **c** 200  $\mu$ m; **d**: 1 cm





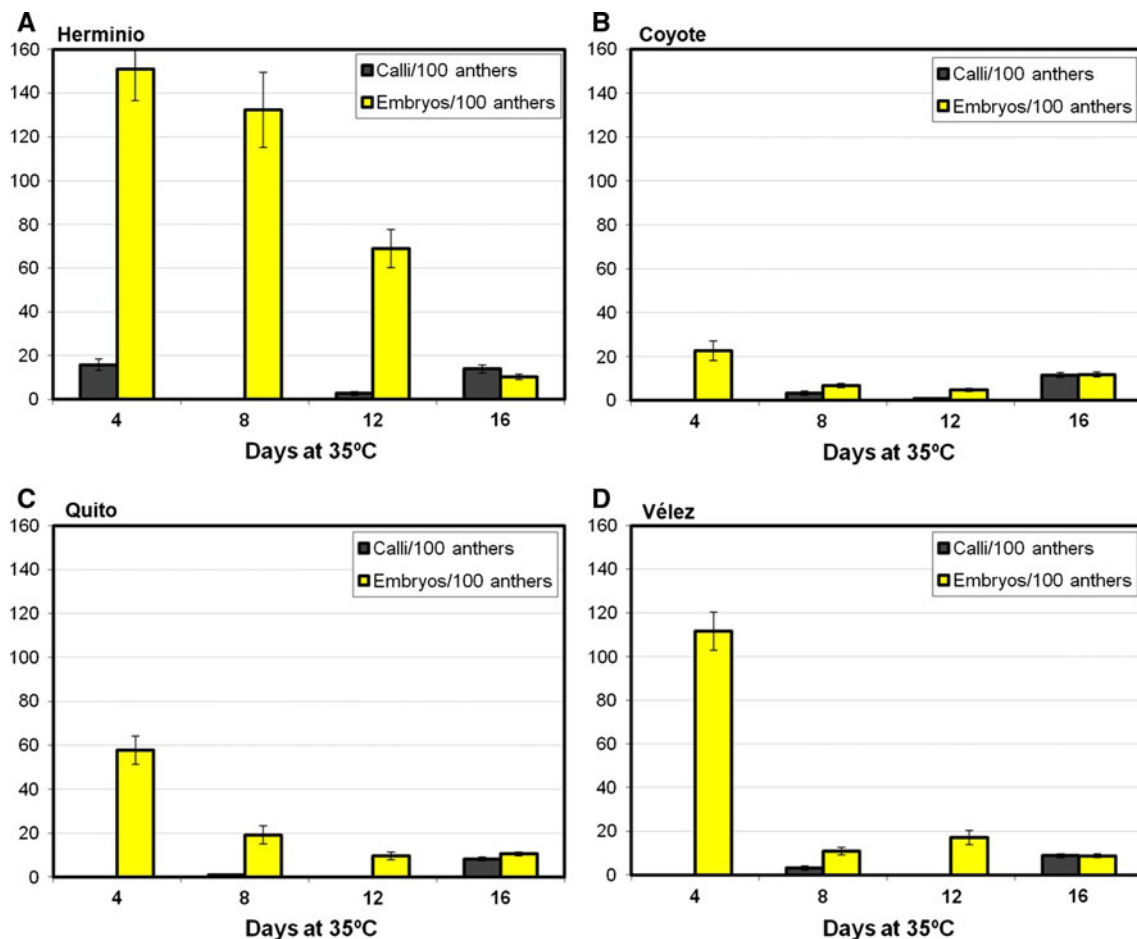
general trend pointing to a reduced embryo production with increased durations. Conversely, the shortest duration (4 days) was found to be the most effective in all four genotypes studied. However, it must be noted that in the case of ‘Herminio’ the difference between 4 and 8 days was not significant. In terms of callus production it was difficult to extract any defined trend for 4, 8 and 12-day treatments, since some genotypes responded with a rather discrete or even null callus production rate (Figs. 3a–d). Interestingly, all four genotypes responded to 16-day treatments with a similar rate of callus production, ranging between 8 and 14 calli/100 anthers, and similar to the rate of embryos/100 anthers. It appeared that prolonged exposures to 35 °C increased the rate of callus production in a genotype-independent manner.

#### Analysis of callus and embryo origin

Calli from ‘Herminio’ and ‘Vélez’ obtained using the DDV protocol were analyzed in order to clarify their cellular

origin. For this, we analyzed by flow cytometry 20 randomly selected calli. All of them presented a diploid DNA content, identical to the diploid parental plants used as controls. Not a single haploid signal was observed in any histogram (data not shown). During processing for flow cytometry, all of the calli had to be mechanically detached from the anther, indicating that they were physically connected to the anther tissue. Light microscopy sections of fixed and embedded anthers (Fig. 4) revealed that these calli originated by proliferation of the connective tissues of the anther. Proliferating calli first invaded and collapsed the anther locule (Fig. 4a) and then ruptured the anther walls, emerging out of the anther (Fig. 4b). These observations, together with the absence of haploid cells demonstrated by flow cytometry, indicated that calli were originated from tissue layers of the anther walls.

We also analyzed by flow cytometry 20 plants produced from anther-derived MDEs of ‘Herminio’ hybrids’. From them, 12 (60 %) showed a 1C DNA content, equivalent to a haploid genome, 7 (35 %) showed a 2C DNA content,



**Fig. 3** Comparison between callus and embryo production in anthers of ‘Herminio’ (a), ‘Coyote’ (b), ‘Quito’ (c) and ‘Vélez’ (d), cultured according to the DDV method but exposed to 35 °C during 4, 8, 12

and 16 days. Results are expressed as number of callus or embryos produced per 100 cultured anthers

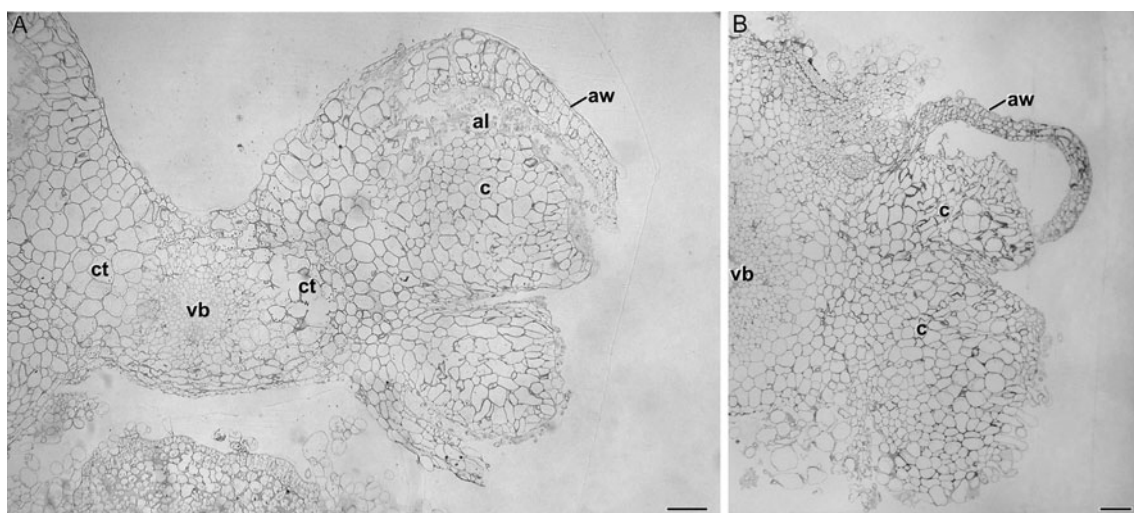
equivalent to diploid controls (donor plants) and 1 (5 %) showed a 3C, triploid DNA content. According to this, a gametophytic origin could be unambiguously assigned to the 12 haploid plants. The other 8 were analyzed using microsatellite (SSR) molecular markers. All 8 plantlets were homozygous for the six SRR markers found heterozygous for their corresponding donor plants. These data, together with those coming from flow cytometry indicated that all the embryos produced had a microspore origin, i.e. they were true androgenic embryos.

## Discussion

It is widely accepted that anther culture promotes the formation of microspore-derived embryos, and that the genotype has a remarkable influence in the percentage of microspores deviated towards embryogenesis and effectively transformed into embryos (Seguí-Simarro and Nuez 2008; Dunwell 2010; Seguí-Simarro 2010; Irikova et al. 2011). It is also known that in addition to embryos, calli may also be formed in anther cultures. The results presented in this work, using four different sweet pepper cultivars subjected to two different anther culture methods, are consistent with these notions. However, we also showed that under the same conditions and for the same four cultivars, callus induction from sporophytic anther tissues appeared less genotype-dependent. In the case of the DDV method, all four cultivars produced calli, in similar amounts. In the case of the SM method, no calli were produced in any of the four sweet pepper cultivars we tested. Furthermore, no callus (just embryo) presence was reported by Supena et al. neither in their original publications of the shed-microspore method

(Supena et al. 2006a, b) nor in a further refinement of the method (Supena and Custers 2011). An additional confirmation to this notion was provided by the experiment where different durations of the 35 °C treatment led to different rates of callus production, ranging from zero to 15 calli/100 anthers. Thus, we could speculate that at least in pepper, callus induction seems more dependent upon culture conditions than upon the genotype. Similar results have been described in other solanaceous species such as tomato and eggplant. In tomato, anthers of 6 out of 8 cultivars produced callus when cultured using a defined protocol but not with other protocols (Seguí-Simarro and Nuez 2007). The other two cultivars did not produce callus under any circumstances. Later on, it was demonstrated that 83 % of the calli produced originated from anther wall tissues (Corral-Martínez et al. 2011), as we showed hereby for pepper. In eggplant, anthers of 11 out of 12 genotypes cultured using the Dumas de Vaulx and Chambonnet (1982) method produced anther-derived callus, whereas only 5 of them produced embryos under the same experimental conditions (Salas et al. 2011).

Our flow cytometry and light microscopy analysis demonstrated that in pepper, calli are produced by proliferation of cells from anther wall tissues. It might be argued that these calli could potentially be a problem in terms of producing callus-derived embryos (for example, through secondary embryogenesis). These calli might give rise to non-DH plants. This, in turn, would imply the consumption of time and resources to identify and dispose of the useless individuals. However, in our genotypes this possibility seemed unlikely since for all of the embryos analyzed, a haploid origin was clearly assigned. Notwithstanding this, the occurrence of calli from anther wall tissues should be



**Fig. 4** Light microscopy analysis of callus growth in cultured ‘Herminio’ anthers. **a** Anther with a callus (c) derived from connective tissues (ct) invading the anther locule (al). **b** Anther with

developing calli that tear off the anther wall (aw) and emerge out of the anther. vb vascular bundle. Bars 100 μm

minimized in order to maximize the availability of resources for microspore growth and conversion to embryos.

A straightforward way to achieve this would be to use a method that minimizes or excludes callus formation. For example, the shed-microspore method produced no callus. However, we showed that this method may not work efficiently in some genotypes. It is likely that in other cultivars, not tested by us, this method may not work at all. Thus, a previous validation of the method should be required. A second suggestion would be to use a method with a wider range of application, such as the DDV, but modifying particular conditions in order to minimize callus production. For example, the duration of the heat treatment could be optimized. We showed that prolonged periods at 35 °C stimulate callus formation. It is likely that other conditions would promote similar effects. For example, in *C. annuum* var. Grossum Sendt, it was shown that when cultured in solid MS medium, 25 °C promoted callus growth but 35 °C prevented it (Mythili and Thomas 1995). In the same study it was also shown that anthers cultured under continuous darkness produced more calli than those cultured under a 16/8 photoperiod. Thus, photoperiod could well be another candidate factor.

In conclusion, we showed that culture conditions have a notable influence, even higher than the genotype, on the formation of callus from sweet pepper anther walls. This influence could be minimized in general by reducing heat shock exposure to a minimum when cultured in solid medium, or by using the shed-microspore approach in particularly sensitive genotypes.

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