

# Valuation Of Crucial Factors For Implementing Shed-Microspore Culture Of Indonesian Hot Pepper (*Capsicum Annuum* L.) Cultivars

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## Abstract

A shed-microspore culture protocol was developed in Wageningen for producing doubled haploid plants in several genotypes of Indonesian hot pepper (*Capsicum annuum* L.). For transfer of technology to Indonesia, three factors were studied that appeared crucial for successful implementation in practice. First, application in the culture medium of a combination of the antibiotics timentin and rifampicin at the concentrations of 200 and 10 mg/l, respectively, prevented bacterial contamination from the donor explants. Second, in vitro application of colchicine (100  $\mu$ M) during the first week of culture was highly effective in increasing the percentage of doubled haploid plants. Third, a comparative analysis of the ploidy level of plants regenerated from shed-microspore-derived embryos using chloroplast counts in guard cells of leaf stomata and flow cytometric measurement of leaf nuclear DNA content, revealed that the first procedure is a reliable and an easy to use method for ploidy determination with hot pepper.

**Keywords:** *Capsicum*; Contamination; Chloroplast; Colchicine; Haploid; Ploidy; Rifampicin; Timentin

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## 1. Introduction

An efficient shed-microspore culture protocol was developed for producing doubled haploid (DH) plants in various genotypes of Indonesian hot pepper (*Capsicum annuum* L.) cultivars ([Supena et al., 2005](#)). This protocol is very promising to transfer to Indonesia for enhancing local breeding programs. The protocol has partly been simplified and is adapted to less sophisticated laboratories, i.e. it is using autoclaved media instead of filtered sterile media and is using anthers as explants instead of isolated microspores that need to be carefully washed and purified by centrifugation. However, there were still some problems that hampered the implementation of the technology under the local conditions of Indonesia, namely: (i) a high chance of microbial contamination of the cultures; (ii) non-availability of an effective in vivo method for chromosome doubling; and (iii) the absence of an easy and reliable procedure for analysing the ploidy levels.

Contamination in plant tissue culture, mainly due to the difficulty to grow healthy donor plants in tropical countries, remains to be a persistent problem. Endogenous microbial contamination is known to be the most serious problem ([Kneifel and Leonhardt, 1992](#) and [Cassells, 2001](#)). Antibiotics have been widely used to eliminate bacterial contamination problems in plant tissue culture for more than 50 years ([Falkner, 1998](#)). But antibiotics (e.g. rifampicin), which are effective in preventing the growth of bacteria, usually cause phytotoxic effects on plant tissue culture ([Leifert et al., 1992](#), [Nauerby et al., 1997](#) and [Teixeira da Silva et al., 2003](#)). In contrast, a few antibiotics (e.g. timentin) have been reported that give negligible negative effects, or even a minor positive effect ([Nauerby et al., 1997](#) and [Costa et al., 2000](#)). Timentin and rifampicin have a complementary effect on bactericide activity because timentin is highly active against Gram-negative bacteria ([Nauerby et al., 1997](#)), whereas rifampicin is more active against Gram-positive bacteria ([Young et al., 1984](#)). In the present study, we studied the combined application of these two antibiotics for solving the contamination problems without causing phytotoxic effect in shed-microspore culture of Indonesian hot pepper.

Another important aspect for implementing the shed-microspore culture is the availability of an efficient procedure for chromosome doubling. The frequency of spontaneous DH plants in shed-microspore culture of hot pepper was relatively low (35%) ([Supena et al., 2005](#)). This is similar to the results obtained in anther culture of bell pepper, i.e. 35.6% by [Dumas de Vaulx et al. \(1981\)](#), or 32.6% by [Gyulai et al. \(2000\)](#). Chromosome doubling of haploid pepper plants grown in a glasshouse or in the open air is generally known to be inefficient and often troublesome (personal communication from breeding companies). Previous studies showed that in vitro colchicine treatment in pepper applied to regenerated haploid explants resulted in a 75% diploidization success rate ([Mitykó and Fári, 2001](#)). The application of colchicine during the earlier periods of in vitro culture has been reported as an efficient method to increase the

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production of DH plants in anther or microspore culture of some species, such as *Brassica napus* (Möllers et al., 1994 and Zhao et al., 1996), maize (Saisingthong et al., 1996, Antonie-Michard and Beckert, 1997 and Barnabás et al., 1999) and wheat (Hansen and Andersen, 1998 and Redha et al., 1998). In the present study, the effect of in vitro colchicine treatment during the first week of culture in shed-microspore culture of Indonesian hot pepper was investigated.

The next important step for the implementation of an in vitro DH production system in practice is to have a fast and accurate method of determining the ploidy level of microspore-derived plants. Chromosome counting from mitotic cells in root tips and from meiotic cells in flower buds is a suitable method, but difficult for tissue culture material because plantlets have to be first grown under in vivo conditions (Jansen, 1974 and Wang, 1998). On the other hand, flow cytometric determination of the ploidy level is very fast, simple and accurate, but it requires expensive equipment. Qin and Rotino (1995) showed that the number of chloroplasts in guard cells of leaf stomata could be used to determine the ploidy levels of haploid and diploid plantlets in bell pepper. This method was also routinely used to estimate the ploidy levels of anther-culture-derived potato plants (Singsit and Veilleux, 1991) and of regenerated cell- and tissue culture plants of tomato (Jacobs and Yoder, 1989 and Koornneef et al., 1989). In the present study, we tested the chloroplast counting for the hot pepper microspore-derived plants and compared the procedure to flow cytometry.

In the present study, antibiotic treatments to prevent bacterial contamination, chloroplast counting in guard cells of stomata of microspore-derived plants and colchicine treatment are optimized for DH plant production in Indonesian hot pepper.

## 2. Materials and methods

### 2.1. Plant material and microspore stage determination

Two Indonesian hot pepper (*C. annuum* L.) types were used: large type 'Galaxy' and 'Tombak' and curly type 'Cemeti' and 'Laris', all being open-pollinated varieties produced by Indonesian seed companies. All the plants were grown in the glasshouse in Wageningen, The Netherlands from early April to October according to the conditions described in Supena et al. (2005). We used also other Indonesian large type hot pepper varieties 'Gada', 'Prabu' and 'Marathon', which were grown in the breeding company East West Seed Indonesia (EWINDO) Purwakarta, West Java, Indonesia, for antibiotic experiments carried out under the local conditions in Bogor, Indonesia.

Flower buds of the desired size (petals slightly longer than sepals) were harvested in the morning, pre-treated at 4 °C for 1 day, and then disinfected for 10 min in 2% NaOCl with 0.05% (v/v) Tween-20 added, followed by rinsing for three times in sterile tap water. Anthers at the proper developmental stage were chosen on the basis of the morphological indicator, i.e. with faint purple tip, which contained more than 50% of microspores in the late unicellular stage, and with amounts of mid unicellular microspores and of bicellular pollen not exceeding 20 and 50%, respectively (Supena et al., 2005).

## 2.2. Shed-microspore culture and germination

The shed-microspore culture protocol developed by [Supena et al. \(2005\)](#) was used. The method is briefly outlined here. A double-layer medium was used, in which the under layer consisted of Nitsch components ([Nitsch and Nitsch, 1969](#)) with 2% maltose and 0.5 or 1.0% activated charcoal (Duchefa), and was solidified with 0.6% Plant Agar (Duchefa). The liquid upper layer medium contained the Nitsch components plus 2% maltose. Media for both layers were sterilized by autoclaving, prior to which the pH was adjusted to 5.8. Petri dishes (35 mm × 10 mm) were used with approximately 1.5 ml solid under layer and 1 ml liquid upper layer medium. After incubation of the anthers, cultures were kept at 9 °C for 1 week and then transferred to 28 °C continuously, always in darkness. After 7 weeks of culture, the normal-looking embryos developed (with one or two visible cotyledons) were germinated on medium containing half-strength MS elements ([Murashige and Skoog, 1962](#)) with addition of 2% sucrose and 0.1 μM 6-benzylaminopurine, which was solidified with 0.6% Plant Agar. Germination was performed in 6 cm Petri dishes, kept under 16 h light (25–30 μmol m<sup>-2</sup> s<sup>-1</sup>) at 25 °C. After 3–4 weeks, seedlings showing cotyledons and a first true leaf were transferred into honey jars (±8 cm diameter and 12 cm high) with a 1 cm thick layer of vermiculite wet with half-strength liquid MS medium supplemented with 1% sucrose. Seedlings that had formed four to six leaves were transferred into soil-compost medium.

## 2.3. Antibiotic treatments, chromosome doubling and ploidy analysis

Antibiotics timentin (50, 100 and 200 mg/l) and rifampicin (10 and 20 mg/l) alone and in combination were applied to shed-microspore cultures to study their effect to prevent bacterial contamination, but also the effect on embryo production was evaluated. Embryos were counted and classified into normal-looking embryos (with one or two cotyledons) and abnormal ones (missing cotyledons and meristem).

In order to develop an efficient protocol for chromosome doubling, in vitro application of colchicine (50–800 μM) during the first week of culture at 9 °C was tested. After this treatment, liquid medium with colchicine was removed and immediately replaced by fresh liquid medium and then culture was transferred to 28 °C. Seedlings obtained from the normal-looking embryos were analysed for ploidy level, using a Coulter Epics XL-MCL (Beckman-Coulter, USA) flow cytometer at Plant Research International, Wageningen, according to the protocol described by [Lanteri et al. \(2000\)](#).

To develop an efficient method of analysing the ploidy levels in Indonesia, we studied the correlation between the data obtained by flow cytometric measurement of leaf nuclear DNA content and chloroplast number counts in guard cells of leaf stomata. The same plants were analysed in both procedures. For the chloroplast counting, the lower epidermis of the fully expanded leaf was peeled, placed onto a microscopic glass slide, and stained with a few drops of a 1% silver-nitrate solution ([Qin and Rotino, 1995](#)). The number of chloroplasts per guard cell pair was counted under phase contrast microscope from at least 10 stomata of each of three leaflet samples.

In the present study, each experiment consisted of five treatments. Per treatment, five Petri dishes were used. Each Petri dish consisted of six anthers taken randomly from six different buds. Experiments were repeated two to three times during different periods. The experimental data were analysed by standard analysis of variance using General Linear Model of SPSS 10.0 for Windows software, and least significant differences (LSD) were calculated to determine the statistical significance of treatment effects.

### 3. Results

#### 3.1. Antibiotics prevent the contamination in shed-microspore culture

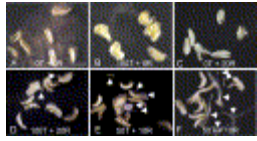
The combined treatments with the antibiotics rifampicin (10 and 20 mg/l) and timentin (100 and 200 mg/l) were successfully used for preventing the bacterial contamination (73–82% contamination-free dishes), whereas all the untreated control cultures were contaminated ([Table 1](#); [Fig. 1](#)). The application of timentin alone does not seem to be effective in preventing the contamination problem, whereas rifampicin alone appears to be partly effective in preventing the contamination, but gave rise to detrimental effect on microspore embryogenesis ([Fig. 1C](#)). Embryos were obtained in the contamination-free dishes in all the three genotypes tested, ‘Gada’, ‘Prabu’ and ‘Marathon’, especially after the combined treatment with 10 mg/l rifampicin and 50 mg/l timentin ([Fig. 1E and F](#)). Combination of the antibiotics timentin and rifampicin showed two interesting synergistic effects, i.e. (1) addition of timentin to rifampicin improved the embryogenic ability of media with rifampicin alone ([Fig. 1](#), compare C with D, E and F), while (2) rifampicin added to timentin increased the contamination preventing effect of timentin ([Fig. 1](#), compare B and D), and even less amount of the latter was sufficient and allowed higher embryo yield ([Fig. 1](#) compare D with E and F).

Table 1.

Effect of single treatments with rifampicin (Rif) and timentin (Tim) as well as the combined treatment of both antibiotics for preventing the bacterial contamination in shed-microspore culture of Indonesian hot pepper (*C. annuum* L.)

Antibiotic treatments (mg/l)	Contamination-free cultures (%)
Control (without antibiotic)	0
Rif 20	64
Tim 100	27
Rif 10 + Tim 50	73
Rif 20 + Tim 100	82

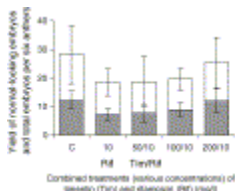
Data are the means of total 11 Petri dishes per treatment of ‘Gada’, ‘Prabu’ and ‘Marathon’ genotypes; each dish contained six anthers from six different buds. Donor-plants for the anthers were grown in Indonesia.



[Full-size image](#) (94K)

Fig. 1. Shed-microspore culture of Indonesian hot pepper (*C. annuum* L.) ‘Gada’ after single and combination treatments of antibiotics. (A) Control without antibiotic treatment (0T + 0R) showing contaminated culture; (B) culture treated with 100 mg/l timentin (100T + 0R) showing contamination; (C) culture treated with 20 mg/l rifampicin (0T + 20R) showing no contamination, but without undergoing embryogenesis; (D) culture treated with 100 mg/l timentin + 20 mg/l rifampicin (100T + 20R) showing no contamination, where some embryos can be seen (arrows); (E and F) culture treated with 50 mg/l timentin + 10 mg/l rifampicin (50T + 10R) showing no contamination, where more embryos can be seen (arrows). The culture was with 0.5% activated charcoal in solid medium. Donor-plants for the anthers were grown in Indonesia. (A–E) After 5 weeks of culture; (F) after 6 weeks of culture. Bar = 4 mm.

To enhance the positive effect of timentin (i.e. for reducing the negative effect of rifampicin) on embryo yield, we studied the effect of various concentrations of timentin (50, 100 and 200 mg/l) in combination with rifampicin (10 mg/l) in shed-microspore culture of ‘Tombak’ in Wageningen (Fig. 2). The results showed the highly beneficial effect of the combination treatment with 200 mg/l timentin and 10 mg/l rifampicin, where timentin almost completely neutralised the negative effect of rifampicin on embryogenic ability (Fig. 2 and Fig. 3).



[Full-size image](#) (23K)

Fig. 2. The effect of various timentin concentrations (50, 100 and 200 mg/l) in combination with 10 mg/l rifampicin on shed-microspore culture of hot pepper (*C. annuum* L.) ‘Tombak’. The yield of total embryos (□) and normal-looking embryos (■) was determined after 7 weeks of culture. The donor-plants were grown in Wageningen. The culture was with 1% activated

charcoal in solid medium. Data are the means ( $\pm$  standard errors) of three replicated experiments. C: control; Tim/Rif: combined treatment of timentin and rifampicin.

[Full-size image](#) (43K)

Fig. 3. High embryogenic ability in shed-microspore culture of hot pepper (*C. annuum* L.) ‘Tombak’ after combined treatment of 200 mg/l timentin and 10 mg/l rifampicin in liquid medium. The solid medium contained 1.0% activated charcoal. The photograph was taken after 7 weeks of culture. Bar = 2.5 mm.

### 3.2. In vitro application of colchicine increases DH plant production

A protocol for producing DH plants in vitro is considered as efficient when it increases not only the frequency of plants produced, but also the frequency of spontaneous diploidization in the system. In order to develop such a protocol for chromosome doubling, in vitro application of colchicine at various concentrations, i.e. 50–800  $\mu$ M was tested in cv. ‘Galaxy’ ([Fig. 4](#)). We found that the treatment with 100  $\mu$ M colchicine during the first week of culture in vitro was effective in increasing DH plant production. The percentage of DH plants increased by almost two-fold (i.e. from 33 to 61%) without severely decreasing the embryo yield, especially the production of normal-looking embryos. The application of colchicine at a lower concentration (50  $\mu$ M) did not significantly increase the percentage of DH plants, whereas the colchicine treatment applied at higher concentration ( $\geq$ 200  $\mu$ M) caused detrimental effect on the yield of normal-looking embryos and total embryos. Observations of diploid plants obtained after treatment with colchicine (100  $\mu$ M) in culture revealed normal fertility, without any indication of mutations, which is similar to that observed in DH plants regenerated from shed-microspore culture not treated with colchicine (data not shown).

[Full-size image](#) (24K)

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Fig. 4. The effect of colchicine treatment applied during the first week of shed-microspore culture of hot pepper (*C. annuum* L.) ‘Galaxy’. The yield of total embryos (□) and normal-looking embryos (■) was determined after 6–8 weeks of culture. The culture was with 0.5% activated charcoal in solid medium. Data are the means ( $\pm$  standard errors) of three replicated experiments. The ploidy levels of at least 15 seedlings derived from normal-looking embryos in each treatment were analysed by flow cytometry and the percentage of DH plants (—◆—◆—) was calculated.

### 3.3. Rapid analysis of ploidy levels of plants

In order to have a more practical method of analysing the ploidy levels, we have studied two methods, i.e. (1) flow cytometric measurement of leaf nuclear DNA content, and (2) chloroplast counts in guard cells of leaf stomata. The comparison of results presented in [Table 2](#) and [Fig. 5](#) showed a 100% correlation between the chloroplast number counts and the ploidy levels determined by flow cytometry. The chloroplast number of DH plants had almost double the number of chloroplasts in haploid plants. Thus, the haploid plants can be easily distinguished from the DH ones. Also, the number of chloroplasts per guard cell pair in DH plants and the diploid plants grown from seeds was similar. The chloroplast number was also similar ( $p = 0.05$ ) among varieties with the same ploidy level (i.e. large type and curly type). It was easy to observe that the haploids showed smaller and narrower leaves than those of DH plants regenerated from shed-microspore-derived embryos of the same genotype ([Fig. 5G](#)).

Table 2.

Chloroplast number in guard cells of stomata in four varieties of Indonesian hot pepper (*C. annuum* L.)

Hot pepper varieties	Chloroplast number per guard cell pair <sup>a</sup>		
	Haploids	Doubled haploids	Diploids
Large type			
Galaxy	9.0 a <sup>b</sup>	16.3 b	16.7 b
Tombak	8.9 a	15.8 b	16.5 b
Curly type			
Cemeti	9.2 a	17.1 b	17.3 b



Hot pepper varieties	Chloroplast number per guard cell pair <sup>a</sup>		
	Haploids	Doubled haploids	Diploids
Laris	9.6 a	17.4 b	17.3 b

Haploid and doubled haploid plants were regenerated from shed-microspore-culture-derived embryos and the ploidy levels were determined by flow cytometry analysis prior to the chloroplast counting. Correlation between both ploidy analysis methods was 100%. Diploid plants for control were grown from seeds.

<sup>a</sup> Chloroplast number per guard cell pair was based on the analysis of 30 stomata from three leaflets.

<sup>b</sup> Means of chloroplast numbers followed by the same letters were not significantly different at  $p = 0.05$ .

[Full-size image](#) (93K)

Fig. 5. Analysis of ploidy level of plants by flow cytometric determination of leaf nuclear DNA contents (A, C and E) and by chloroplast counts in guard cells of leaf stomata (B, D and F). (A and B) Diploid plant derived from a seed of ‘Cemeti’; (C and D) microspore-derived haploid plant of ‘Cemeti’; (E and F) microspore-derived DH plant of ‘Cemeti’; (G) phenotype of microspore-derived haploid (left) and DH (right) plants of ‘Galaxy’. 1C and 2C DNA contents refer to the G1 and G2 phases in microspore-derived haploid plants; 2C and 4C DNA contents refer to the G1 and G2 phases in diploid plants obtained from seeds and DH plants from microspore-derived embryos. Spike refers to the DNA content in seeds (G1 phase) of *B. napus*, which was used as a standard (about 2.3 pg/nucleus). The DNA content of 2C (G1 phase) in diploid plants of ‘Cemeti’ obtained from seeds is about 8.1 pg/nucleus. Bar = 7  $\mu$ m for B, D and F.

#### 4. Discussion

The results obtained in the present study show that the combination treatment of timentin and rifampicin was highly effective in preventing the bacterial contamination in local conditions of

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Bogor, Indonesia. After applying this treatment, embryos were produced from contamination-free cultures. One possible reason for overcoming the bacterial contamination problem in shed-microspore culture is that timentin and rifampicin have a complementary effect on bactericide activity against both Gram-negative and Gram-positive bacteria ([Nauerby et al., 1997](#) and [Young et al., 1984](#)). This antibiotic combination gave a highly beneficial effect in reducing the negative effect of in particular rifampicin alone on the embryogenic capacity in culture.

Further, the present data revealed that when the microspore cultures were not treated with antibiotics, they were all contaminated. Growing healthy donor plants in tropical environment is very difficult, and moreover, the endogenous microbial contamination is a serious problem. Although the combined treatment of timentin and rifampicin was highly effective in preventing the contamination problem, it should be noted here that the application of antibiotics in tissue culture has a bacteriostatic function. The repeated use of antibiotics in plant tissue culture may cause resistance development of the bacteria ([Kneifel and Leonhardt, 1992](#)). The best way in plant tissue culture is to use healthy donor plant material. Fully controlled conditions, such as a phytotron, are highly desirable for obtaining healthy donor material as well as for providing excellent flower buds to start shed-microspore culture in tropical conditions. Our preliminary studies in Wageningen indicated that the hot pepper plants of cv. 'Galaxy' and other pepper type grew well in phytotron conditions, and served as a better material for shed-microspore culture, resulting in high embryo yield (data not published). However, although it would be the best solution for Indonesia, buying a phytotron mostly is too expensive.

The application of 100  $\mu$ M colchicine treatment during the first week of culture at 9 °C in darkness was highly efficient in increasing the DH plant production in shed-microspore culture. As earlier found, during this period about 70% of the microspores go through the first mitotic division ([Supena et al., 2005](#)). The efficiency of colchicine treatment appears to be not only due to the level of colchicine concentration, but also due to the optimal developmental stages and culture conditions (e.g. temperature). The optimal chromosome doubling is also associated with the duration of colchicine treatment and the culture temperatures ([Saisingtong et al., 1996](#)). The most effective period for the application of colchicine in anther or microspore culture is usually during the induction phase, i.e. the first microspore mitosis ([Barnabás et al., 1991](#), [Möllers et al., 1994](#), [Saisingtong et al., 1996](#), [Zhao et al., 1996](#) and [Antonie-Michard and Beckert, 1997](#)). It is known that colchicine efficiently arrests mitosis through microtubule depolymerization, resulting in homozygous doubled-haploid microspores ([Barnabás et al., 1999](#)). Thus, in vitro colchicine application has obvious advantages compared to the conventional in vivo application: (1) the procedure is simple and rapid; (2) it results in the occurrence of a high frequency of diploidization; and (3) low concentration of the chemical can be used, avoiding toxic effects. Hence, we suggest here that the application of 100  $\mu$ M colchicine during the first week of culture should become an integral part of the refined shed-microspore culture protocol of hot pepper.

The chloroplast number in guard cells of leaf stomata can be used to clearly distinguish between haploid and doubled haploid plants in hot pepper. Therefore, the counting of chloroplast number in guard cells of stomata can be considered as an effective method for the analysis of ploidy levels in hot pepper plants of both large and curly types. Similarly, the chloroplast number had been reported as an effective ploidy analysis in bell pepper types of in vitro grown anther-

derived plantlets ([Qin and Rotino, 1995](#)), and some other diploid and tetraploid pepper plants ([Srivalli et al., 1995](#)). However, it should be noted that different growth conditions, such as light intensity and temperature ([Zachleder and Cepák, 1987](#) and [Zhang et al., 2003](#)), and developmental stages, such as the age and leaf position ([Possingham and Saurer, 1969](#), [Asahi and Toyama, 1982](#) and [Olszewska et al., 1982](#)), may influence the number of chloroplasts in guard cells. Therefore, it is highly desirable to use the plants grown under the same conditions and the leaves at the same developmental stage of growth.

In conclusion, the results obtained in this study show that the combined treatment of antibiotics could solve the severe bacterial contamination problems for shed-microspore culture of hot pepper under the tropical conditions of Indonesia. In vitro colchicine treatment could be integrated in the doubled haploid production procedure for the crop. The chloroplast counting in guard cells of stomata is an effective practical method to determine the ploidy level of regenerated plants in Indonesian hot pepper.

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