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Optimised Protocols for Efficient Plant Regeneration and Gene Transfer in Pepper (*Capsicum annuum* L.)

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Abstract

An efficient *in vitro* regenaration system and an optimised *Agrobacterium* mediated transformation protocol are described, based on the use of young seedling cotyledons of *Capsicum annuum* L. Optimal regeneration efficiency can be obtained by cultivating cotyledon explants on media containing 4 mg/L benzyladenine and 0.1 mg/L indolacetic acid. The effect of antibiotics used to eliminate *Agrobacteria*, as well as the toxic level of some generally used selection agents (kanamycin, geneticin, hygromycin, phosphinotricin and methotrexate) in regenerating pepper tissues were determined. To enable the comparison of different selection markers in identical vector background, a set of binary vectors containing the marker genes for *NPTII*, *HPT*, *DHFR* and *BAR* respectively, as well as the CaMV 35S promoter/enhancer-*GUS* chimaeric gene was constructed and introduced into four different *Agrobacterium* host strains.

Introduction

Regarding its ability to *in vitro* regeneration and genetic transformation, pepper is considered to be a definitely difficult, recalcitrant object. Though doubled haploid pepper plants via *in vitro* anther culture have been efficiently produced for more than twenty years, but regeneration from somatic tissues is restricted to a few genotypes and explant types of this species. Reports on successful genetic transformation in pepper have been published recently (Yu-Xian Zhu et al., 1996; Manoharan et al., 1998), but the authors of these publications reported about a low transformation efficiency and the published regeneration and transformation protocols could not be reproduced in other laboratories. Since no reproducible and routinely working pepper transformation system is presently available, the elaboration of an efficient and reproducible regeneration and transformation system in pepper is still actual.

A highly efficient regeneration system have been developed in our laboratory, that uses the basal part of young cotyledons for *in vitro* shoot induction. Though the application of this protocol results in whole plant regeneration in a wide range of genotypes, but considerable efficiency and reproductivity could only be obtained with the pepper line No 40017 R13, that was definitely selected and bred for high *in vitro* responsiveness (Fri et al., 1992).

In this paper we report results of systematic investigations for optimising the most important factors of an efficient and reproducible *in vitro* regeneration and genetic transformation system.

Materials and Methods

Plant materials

Seeds of the pepper line No. 40017 were surface sterilised by immersing for 20 min in a solution of 10 % $CaCl_2O_2$ supplemented with a few drops of Tween 20. Thereafter they were rinsed several times in sterile distilled water. The seeds were placed for germination in 1000 mL commercial jars containing 100 mL MS basal medium (Murashige and Skoog,

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1962) with 2 % sucrose. Incubation was carried out in a culture room at 25℃ under 16 h/day photoperiode.

Culture conditions and media

Cotyledons of 16-21 days old seedlings were used as explants. Intact cotyledons with petioles were cut from the plants and transferred to shoot inducing media containing MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968) and 2 % glucose (MSB5gl) supplemented with different growth regulators. Ten explants were put into one 9 cm Petri dish. After 21 days cotyledons with adventitious shoot primordia (active explants) were transferred into 100 mL Erlenmeyer flasks containing 25 mc of MSB5E elongation medium (MS salts, B5 vitamins 1 % glucose 1 % maltose 1 % sucrose, supplemented with 1.65 mg/L BAP and 1 mg/L GA_3). The explants were transferred onto fresh elongation medium in every 3 weeks. Regenerated shoots were cut from the explants and transferred in 100 mL Erlenmeyer flasks containing 25 mL MSR rooting medium (MS medium supplemented with 0.5 mg/L IAA). Media were solidified with 7 g/L agar and pH was set to 5.8 before autoclaving. Antibiotics were added from filter sterilised stock solutions to media cooled below 55℃. In some cases 5 mg/L bromocresol purple indicator was added to the medium for monitoring changes in the pH values.

Molecular techniques and bacterial cultures

Vector construction, plasmid isolation and *E. coli* transformation were carried out by using of standard molecular techniques (Sambrook et al., 1989). *E. coli* was cultivated at 37℃ in LB medium, *Agrobacterium tumefaciens* in at 28℃ in YEB medium (Vervliet et al., 1975), or in AB minimal medium (Chilton et al., 1974) with the appropriate antibiotics (50 mg/L carbenicillin, 100 mg/L kanamycin, 100 mg/L rifampicin). Binary vectors were transferred from *E. coli* to *Agrobacteria* via triparental mating (Ditta et al., 1980) using the plasmid pRK 2013 (Figurski and Helinski, 1979) as helper.

Co-culturing of cotyledon explants with Agrobacteria

Two different co-culturing procedure were applied: For liquid co-culture 10 cotyledon explants were placed into a 9 cm Petri dish containing 20 mL of liquid MSB5gl medium. *Agrobacteria* were added to an initial density of 10^7 bacteria/mL from a fresh overnight culture. In the other procedure cotyledon explants were immersed into a fresh liquid overnight *Agrobacterium* culture for 1-2 min dried briefly, on sterile tissue paper and placed onto solid MSB5gl medium. In both cases, dishes were incubated for 48 h at 24℃ under dim light. After co-culture explants were rinsed in liquid MSB5gl medium containing 500 mg/L cefotaxime or 400 mg/L augmentin. After drying on sterile tissue paper the expants were placed on solid MSB5gl medium containing 4 mg/L BAP plus 0.5 mg/L IAA and 500 mg/L cefotaxime, or 400 mg/L augmentin. If pre-incubation was applied, the cotyledon explants were cultivated on shoot inducing medium for two days before co-culture.

Results and Discussion

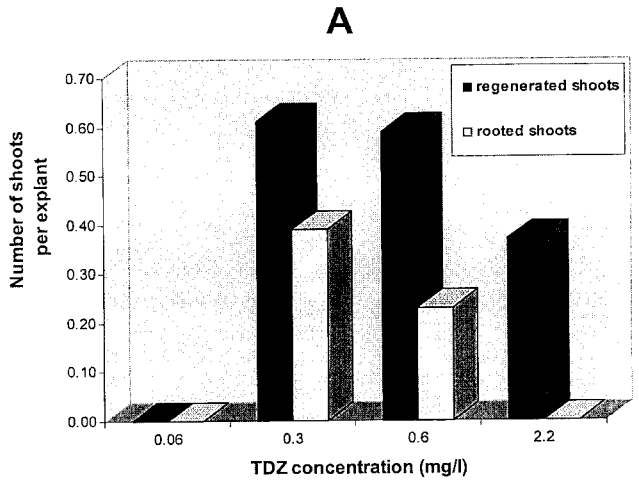
Optimisation of the factors of in vitro regeneration

The type and level of cytokinines are the most important factors for the *de novo* shoot regeneration on the *in vitro* cultivated pepper cotyledons. In an earlier work on a similar regeneration system Szász et al. (1995) reported that the application of 2.2 mg/L thidiazuron resulted in the highest number of regenerated shoots. We found that after the application of 2.2 mg/L thidiazuron intensive callus induction can be observed in the second stadium of the regeneration. The effect of different cytokinins, like 2IP, zeatin, TDZ and BAP was compared in combination and separately. In the preliminary experiments TDZ and BAP gave the best responses. Therefore we decided to refine the dosing of this selected growth regulators. I was found that the number of regenerated shoots was considerably higher when TDZ concentration was between 0.3 and 1 mg/L in contradiction to 2.2 mg/L used by Szász et al. (1995, Figure 1A).

However, the best shoot induction was obtained when the growth regulator combination of 4 mg/L BAP plus 0.1 to 0.5 mg/L IAA was applied (Figure 1B). By increasing of the IAA concentration from 0.1 mg/L to 0.5 mg/L the number of shoot primordia was slightly decreased, but since growing capacity of shoots was better at a lower density, the highest number of rooted regenerants was obtained by using of 4 mg/L BAP plus 0.5 mg/L IAA (Figure 1B).

The second stage of the original regeneration protocol included a 21 day long incubation on MSB5E medium. This often resulted in the vitrification of the regenerated shoots. We found that by reducing the incubation time on this medium to 7 days, followed by a subculture on MSB5gl medium containing 0.1 mg/L BAP almost entirely prevents the vitrification of the shoot primordia without reducing the number of the regenerated shoots.

By the use of these modified growth regulator re-



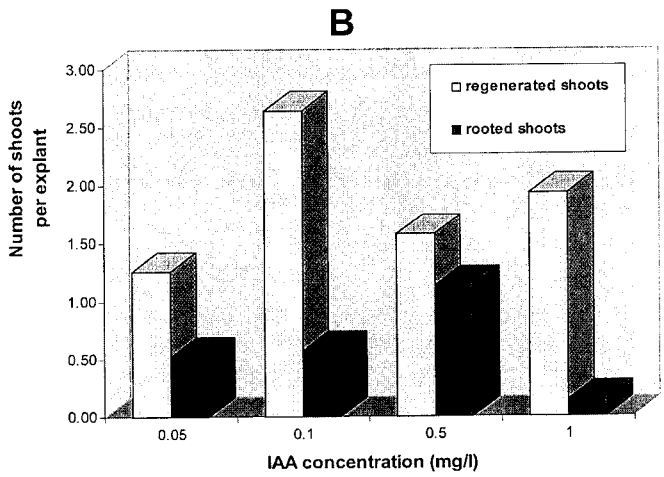


Figure 1. Effect of the increasing TDZ(A) and IAA (B) concentration on the number of the regenerated shoots

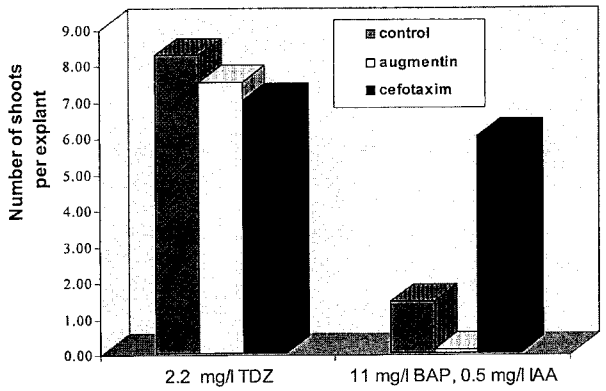


Figure 2. Effect of cefotaxime and augementin on the *in vitro* regeneration

gimes the number of regenerated shoots per explants was considerably increased and they could be rooted at nearly 100% efficiency (Figure 3).

Effect of antibiotics on the in vitro regeneration

Typically semi-synthetic penicillins, like carbenicillin, ticarcillin or the structurally related cephalosporin antibiotics (e.g. cefotaxime) are used to eliminate *Agrobacteria* after co-culture. These antibiotics are generally considered as inert and non-toxic in plant cell cultures, but in several cases it was found that the regeneration procedure might be significantly influenced by such antibiotics. The growth regulator like activity of penicillins is known for a long time (Nickell and Celmer, 1965). Mathias and Boyd (1986) found that cefotaxime stimulated callus growth and embryogenesis in wheat. Also positive effect of cefotaxime was found in an apple shoot regeneration system (Yepes and Aldwinckle, 1994). On the contrary, cefotaxime negatively influenced the adventitious bud formation in *Camellia williamsii* (Tosca et al., 1996). In a sensitive transformation system the choice of antibiotics can be critical. In the case of tomato Ling et al. (1998) found that the transformation frequency was about 40 % higher when the antibiotic betabactyl (ticarcillin/potassium clavulanate) was used instead of cefotaxime.

Obviously, the type and concentration of used antibiotics can especially critical when a low level and a narrow balance of growth regulators is required. We compared the effect of generally used antibiotics on the shoot regeneration efficiency. Cefotaxime (500 mg/L) and augmentin (amoxycillin/potassium clavulanate, 400 mg/L) were applied at two different growth regulator regimes. (11 mg/L BAP plus 0.5 mg/L IAA , vs. 2.2 mg/L TDZ). In each group 16 explants were cultured. On the 2.2 mg/L TDZ containing medium both cefotaxime and augmentin decreased the number of the regenerated shoots. In the

case the 11 mg/L BAP plus 0.5 mg/L IAA we found, that while the 400 mg/L augmentin strongly decreased the shoot regeneration efficiency, the application of 500 mg/L cefotaxime resulted in the stimulation of shoot regeneration (Figure 2).

As betalactam antibiotics and clavulanic acid did not interfere with biochemical pathways of plants, one of the possible interpretation for their influence on shoot regeneration could be their pH shifting effect. In an other experiment the effect of four antibiotics (cefotaxime, augmentin, carbenicillin and vancomycin) on the shoot regeneration efficiency was compared.

The antibiotics were added in three different concentrations, 100, 500 and 1000 mg/L respectively to shoot inducing MSB5gl medium containing 2.2 mg/L TDZ. The media were supplemented with the bromocresol purple indicator which enabled the continuous and relative precise monitoring of changing the pH values during culturing. At optimal pH (5.6 to 5.8) the indicator show a neutral, light amber colour. The antibiotics changed the pH values of the medium differently: cefotaxime and vamcomycin lowered the pH to about 3.5 - 3.8 (yellow colour), augmentin shifted the pH to about 7.5 (pink colour), while in the case of carbenicillin there was no change in colour (Table 1). After three weeks of culture the pH value was stabilised at about pH 6 in all case. The number of regenerated shoots was considerably lower in the case of all antibiotics, roughly correlating with the applied concentration. We conclude that the decrease in shoot forming capacity is due to the growth regulator effect of the antibiotics. Our finding that tobacco leaf explants frequently produce roots on growth regulator free medium containing cefotaxime or augmentin (V. Mihlka, unpublished results), also confirms this hypothesis.

Tolerance of pepper tissues against commonly used selection agents

It is a generally accepted statement that kanamycin resistance is not an optimal selection marker for genetic transformation of pepper, because this species is highly tolerant against this selection agent. This can lead to the appearance of kanamycin tolerant 'escaped' plants in the transformation experiments (Fri et al.,1992; Sankhla et al., 1996).

In our experiments non-transformed pepper cotyledons could tolerate a kanamycin concentration as high as 150 mg/L. On the other hand, the kanamycin tolerance of the different explant types showed a high variance and it was very difficult to determine a level of kanamycin concentration that is suitable for the selection of transformed pepper tissues. Consequently, there is a need of searching for alternative selection agents and marker genes that give better results in the pepper transformation. Since no systematic studies are known on determining the sensitivity of pepper tissues against the commonly used selection agents such as G-418, hygromycin, phosphinotricin and methotrexate, we decided to carry out such investigations. The results of these investigations show that except kanamycin the minimal lethal doses of the most selection agents in pepper are similar to that found in other dicotyledonous species: 25 mg/L for geneticin (G 418), 25 mg/L for hygromycin, 0.05 mg/L for methotrexate, and 1 mg/L for phosphinotricin (Figure 4). All tested alternative drugs proved to be better selection agents than kanamycin.

Construction of a new set of vectors with different marker genes

The comparison of different marker gene can be properly made by when the used *Agrobacterium* host strains, helper plasmid and vector sequences (except the marker gene sequences) are identical.

The members of binary vector set pGPTV (Becker

Table 1. Effect of different antibiotics on the regeneration

Anti-biotics	Conc. (mg/L)	pH of the medium	Number of explants	Number of regenerated shoots	Number of shoots per explants
Control		5	20	88	4.4
Cefotaxime	100	5	20	9	0.45
	500	3.5	20	3	0.15
	1000	3.5	20	0	0
Augmentin	100	7.5	20	77	3.85
	500	7.5	20	55	2.75
	1000	7.5	20	36	1.8
Vancomycin	100	5	20	61	3.05
	500	5	20	74	3.7
	1000	3.5	20	1	0.05
Carbenicillin	100	5	20	61	3.05
	500	5	20	53	2.65
	1000	5	20	15	0.75

et al., 1992) carry the selection marker genes *npt*II (kanamycin/geneticin resistance), *hpt* (hygromycin resistance), *dhfr* (methotrexate resistance) and *bar* (phosphinotricin resistance) respectively, under the transcription control of the nopalin synthase (*nos*) promoter. These vectors also contain a promoterless gus (β-glucuronidase) gene. To fulfil the requirements of histochemical localisation in transformed cells, we added regulatory sequences to the gus gene: the Cauliflower Mosaic Virus (CaMV) 35S promoter/enhancer region from the plasmid pFF19 (Timmermans et al., 1990) was inserted in front of the gus coding sequence (Figure 5). The members of the new plasmid set-named RGG-were mobilised into different *Agrobacterium* hosts as listed on Table 2.

The integrity of the modified plasmids and the

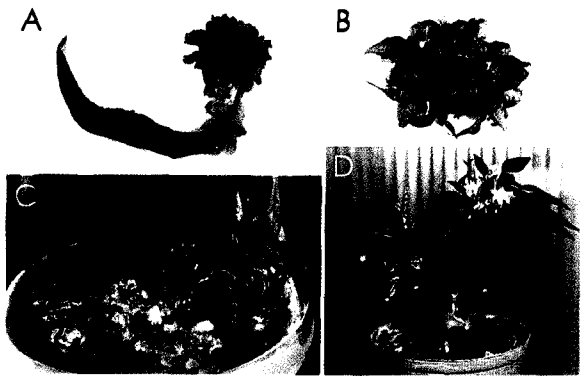


Figure 3. Steps of organogenesis in the pepper genotype No. 40017 R13
A: Cotyledons with adventitious shoot primordia on 4 mg/l BAP and 0.5 mg/l IAA containing MSB5gl medium
B: Adventitious shoot primordia on MSB5 medium
C: Regenerated shoots on MSB5gl medium containing 0.1 mg/l BAP
D: Regenerated greenhouse plants No 40017 R13

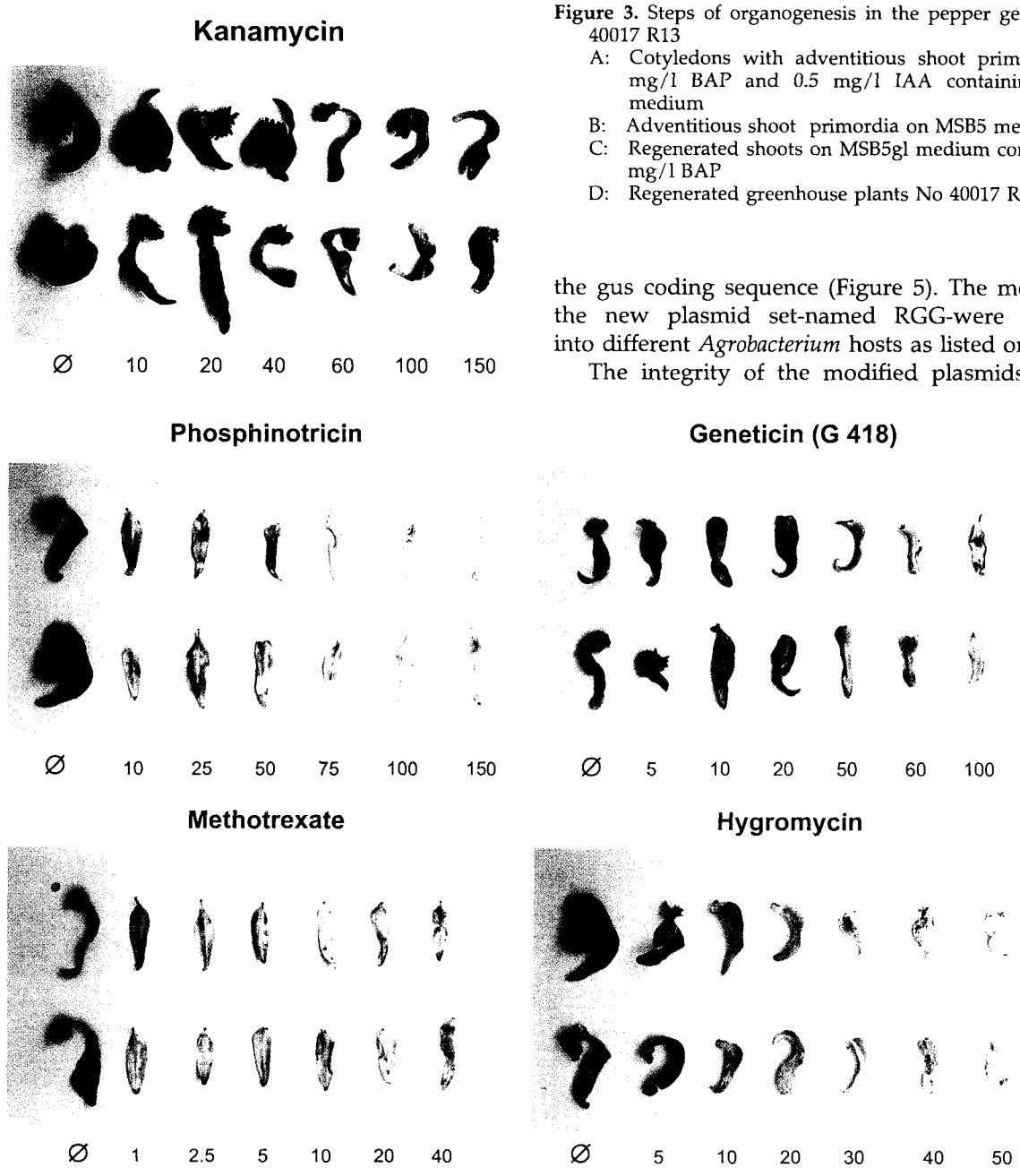


Figure 4. Effect of commonly used selection agents on the *in vitro* regeneration from pepper cotyledons

Table 2. List of *Agrobacterium* strains used as recipients of the pRGG plasmid set

A. tumefciens Strain	Helper plasmid	Important characterisitics
C58C1Rif^R	pGV 2260	Disarmed strain, Rif^R, Cb^R
LBA 4404	pAL 4404	Disarmed strain, Rif^R
EHA 101	pEHA 101	Disarmed strain, Nal^R
A 281	pTi Bo542	Wild type, supervirulent strain, Km^R

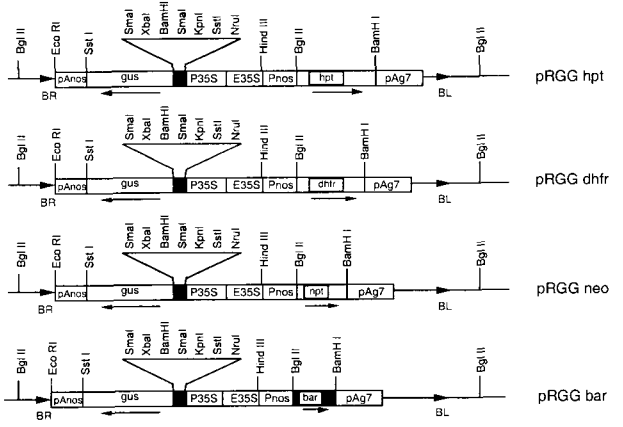


Figure 5. T-DNA structure of the pRGG plasmids

funcionality of all vectors and chimaeric genes was tested by restriction mapping, specific PCR amplifications and by tobacco leaf disk transformations (data not shown). By the combination of these vectors and host strains now it is possible to compare the effects of different selection markers using identical host and vector background, as well as to compare different *Agrobacterium* strains with identical vector sequences.

Optimisation of the factors of co-culturing and transformation

The co-cultivation with the *Agrobacterium* is carried out either on solid or in liquid medium. In case of some plant species the cultivation in liquid medium results in a decrease of regeneration efficiency and in an increase of callus formation. In some regeneration experiments a pre-incubation on induction medium before co-culturing with *Agrobacteria* proved to be advantageous. In our experiment cotyledon explants were co-cultivated with *Agrobacteria* either in liquid or in solid medium with or without 48 h pre-incubation on shoot inducing medium. In each group 80 cotyledon explants were

Table 3. Effect of different factors of co-culture on the shoot regeneration

Medium	Pre-culture	Number of explants	Number of active Explants	Number of regenerated shoots	Number of shoots per explants
Liquid	+	80	74	25	0.31
Liquid	-	80	64	12	0.15
Solid	+	80	71	28	0.35
Solid	-	80	49	7	0.09

cultivated and the numbers of shoots regenerated under non-selective conditions were scored. The number of regenerated shoots were at least two times higher when pre-incubation was applied (Table 3). The co-culture itself considerably lowered the shoot regeneration efficiency: after co-culture 0.1-0.4 shoots per explants were obtained on average, while untreated explants normally produced 1 to 4 shoots. In contrary to our expectations the co-culturing in liquid medium did not lowered the regeneration efficiency.

At a low transformation efficiency the choice of the proper *Agrobacterium* strain can be critical. Liu et al. (1990) found that pepper genotypes show a consistent wide difference in response to the different *Agrobacterium* strains. It is very difficult to make exact quantitative comparisons of different host strains before a relatively large number of stabily transformed plants are produced. However, based on the early transformation events we can conclude that in pepper the largest number of primary transformation events can be obtained when the wild type strain A281 is used as host. Under the disarmed strains the performance of C581C1 Rif^R and EHA 101 are roughly the same, whereas a considerably lower number of primary transformant cells was obtained with the host strain LBA 4404.

Conclusions

Using young seedling cotyledons as explants, in the case of the pepper genotype No. 40017 R13 effective shoot regeneration can be obtained at relatively high level of cytokinines. The highest number of plants could be regenerated when MSB5gl medium supplemented with 4 mg/L BAP plus 0.1 mg/L IAA was used. Because of the relatively high tolerance of pepper tissues against kanamycin, the use of geneticin as selection agent seems to be advantageous, when the vectors contain *npt*II as selective marker gene. Alternative selection markers like

hygromycin-, phosphinotricin-, and methotrexate resistance can be used efficiently as well. Because of the growth regulator effect of penicillin derivatives and cephalosporins the right choice of antibiotic to eliminate *Agrobacteria* can be critical. In our experiments augmentin proved to be less injurious for shoot induction than cefotaxime, vancomycin or carbenicillin. The response of pepper tissues to different *Agrobacterium* host strain show essential differences. The disarmed host strains C58C1Rif^R and EHA 101 seem to be more efficient for pepper transformation than LBA 4404.

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