British Biotechnology Journal
1(3): 53-60, 2011

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Establishment of Regeneration and Transformation System of *Lycopersicon esculentum* MicroTom

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Research Article

Received 22nd May 2011
Accepted 11th June 2011
Online Ready 5th July 2011

ABSTRACT

In this article in order to build up an efficient regeneration system for cotyledons or hypocotyls of tomato MicroTom, two kinds of seed disinfectant (3% NaClO and 0.1% $HgCl_2$ used for 5, 10, 15, 20 min), two types of basal medium (MS: Murashige and Skoog, 1964, and B5: Gamborg et al.,1968), different ratio of Indole Butyric Acid (IBA: 0.05, 0.1, 0.2 mgL^{-1}) and 6-benzylaminopurine (6-BA: 1.0, 1.5, 2.0, 3.0 mgL^{-1}) were tested. As for gene transform system, kanamycin (Kan: 0, 25, 50, 75, 100, 150mgL^{-1}), carbenicillin, cephalosporin, cefoperazone sodium and sulbactam sodium for injection (Carb, CS, CSSS respectively) at the rate 100, 200, 300, or 500 mgL^{-1}, respectively were added in medium B5 in order to find the suitable concentration for bacteriostasis and shoot regeneration. Moreover, cell concentration of Agrobacterium EHA105 (OD600: 0.3, 0.5, or 0.8) and infection time (5, 10, 15 min) were screened and optimized in this article.

The results showed that 3% NaClO for 20 min is optimal as for the disinfect efficiency on the seed surface. The most suitable medium to induce adventitious bud is basal medium B5+0.05 mgL^{-1} IBA＋1.5 mgL^{-1} 6-BA. The cotyledons and hypocotyls were cultivated in culture medium B5+IBA @ 0.05 mgL^{-1} for 2 d, then infected by Agrobacterium EHA105 (OD600=0.5) for 10 min, then plantlets were transferred to a fresh regeneration medium which contained 50 mgL^{-1} Kan and 300 mgL^{-1} CSSS, which was proved to be the most suitable transformation system for MicroTom.

Keywords: MicroTom; Lycopersicon esculentum, regeneration; transformation; B5;

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ABBREVIATIONS

6-BA: 6-Benzylaminopurine; IBA: Indolebutyric acid; Kan: kanamycin; Carb: carbenicillin; CS: cephalosporin; CSSS: cefoperazone sodiumand sulbactam sodium for injection.

1. INTRODUCTION

The annual output of tomato was about 130 million tons in the world (Xiahua et al., 2008). As for tomato fruit containing a lot of bioactive compounds such as lycopene and β-carotene, which have the function of antioxidant, delaying ageing and preventing cancer, it became a kind of important crop. At the same time, tomato has a short life cycle, a small genome and could be easily genetic transformed. So, it was claimed as model plant for the study of flesh fruit.

MicroTom is a tomato mutant which has a shorter life cycle (70-90 days), is able to bear closely planting, transforming conveniently and its hereditary information is as same as common tomato, except for tow chief genes(dwarf gene and miniature gene). So, it is better used in studying on the gene function (Meissner et al., 1997).

There is a layer of gum out of the tomato seed, then if the disinfectant were not too strong or uneasy to wash off, the medium would get bacterial polluted or the germination might decrease very much. And for different gene type of tomato, the germination and regeneration would have different reaction to the seed disinfectant, basal medium, plant hormone and kanamycin (Xuexi et al., 2003; Yunhua et al., 2005; Luxia et al., 2008). When being transferred some gene into this plant through Agrobacterium-mediated gene transformation, the Agrobacterium type and cell concentration, infection time, the type and dosage of bacteriostatic agent would be the important factors (Nan et al., 2006; Qiu et al., 2007). But for MicroTom, those parameters are studied not too comprehensive. In this paper some factors effecting on the regeneration and infection by AgrobacteriumEHA105 were selected and discussed in order to make a foundation for gene transfer into MicroTom.

2. EXPERIMENTAL DETAILS

2.1 Materials and Reagents

Kan (kanamycin), Carb (carbenicillin), CS (cephalosporin) and CSSS (cefoperazone sodiumand sulbactam sodium for injection) were produced by HainNan SiDa drugs manufacture limited company. The other chemical reagents made in China are chemically pure.

2.2 Disinfection of Microtom Seed

Tomato seeds were soaked by 70% alcohol for 30 s, washed with sterile water, disinfected by 3% sodium hypochlorite (or 0.1% $HgCl_2$) for 5, 10, 15, 20 min, washed by sterile water for 5 times, absorbed the water with sterile filter-paper, planted in Ms solid medium and, cultured under 25±1 °C in darkness.

The cotyledons were cut into 0.5 cm×0.5 cm pieces and hypocotyls were cut into around 1.0 cm section for the following tests.

2.3 Comparison of the Basic Medium MS (Murashige and Skoog, 1962) and B5 (Gamborg et al., 1968)

Two basic medium for MS and B5 with 0.1 mgL^{-1} IBA, 2.0 mgL^{-1}6 - BA, 30 mgL^{-1} sucrose, 7.5 gL^{-1} AGAR, were transplanted with MicroTom cotyledons, five pieces in each bottle and, each medium in fifteen bottles. The explants regeneration was investigated 20 d after transplantation.

Callus formation rate and regeneration rate of adventitious root were calculated by the following equations:

Callus formation rate = number of explants with callus / total explants × 100%.

Regeneration rate of adventitious shoot = Number of adventitious shoot / total explants × 100%.

2.4 Screening of IBA (Indole Butyric Acid) and 6-BA (6-Benzylaminopurine) Rate for More Regeneration

IBA (0.05, 0.1, 0.2 mgL^{-1}) and 6-BA (1.0, 1.5, 2.0, 3.0 mgL^{-1}) were added in medium B5 in order to find the suitable ratio for shoot regeneration. Five pieces of cotyledons or hypocotyls were taken in each bottle, and each medium in fifteen bottles. The explants regeneration was investigated 20 d after transplantation.

2.5 Influences of Kan Concentration on Regeneration of Microtom

Kan (0, 25, 50, 75, 100, 150mgL^{-1}) were added in medium B5 in order to find the suitable concentration for antimicrobials and shoot regeneration. Five pieces were taken in each bottle and, each medium in fifteen bottles. The explants regeneration was investigated 30 d after transplantation.

2.6 Infection Concentration of Agrobacterium EHA105

MicroTom explants were infected for 5 min by Agrobacterium EHA105 (OD600 = 0.3, 0.5, or 0.8, then co-cultured for 2 d, washed 2 times with sterile water, transferred into regeneration medium B5 + IBA 0.05 mgL^{-1} + 6-BA 1.5 mgL^{-1} + Kan 50 mgL^{-1} + CSSS 300 mgL^{-1}. Ten pieces were taken in each bottle and, each medium in ten bottles. The contamination rate was investigated 7 d after subculture.

The contamination rate was calculated by the following equation:

Contamination rate (%) = Number of bottle of Agrobacterium appearing/total number of inoculated bottle ×100.

2.7 Infection Time of Agrobacterium EHA105

Agrobacterium cell concentration OD600 0.5 was chosen in liquid suspension medium B5. First, explants were infected for 5, 10, or 15min, cocultured for 2 d, then rinsed 3 times using sterile water, shifted to regeneration medium B5 + IBA 0.05 mgL^{-1} + 6-BA 1.5 mgL^{-1} + Kan 50 mgL^{-1} + CSSS 300 mgL^{-1}. The contamination rate was investigated 7 d after transplant.

2.8 Variety and Dosage of Bacteriostatic Antibiotics

After co-culture ended, explants were transferred to the different kinds of differentiated medium with different antibiotic (Carb, CS, CSSS), which respectively containing 100, 200, 300, or 500 mgL^{-1}. Each disposal has ten bottles, each bottle inoculated 10 explants. After cultivating 15d, observed the growth situation of Agrobacterium to choose the type and dosage of appropriate antibiotics.

3. RESULTS AND DISCUSSION

3.1 Sterilizing Effects of NaClO and $HgCl_2$

From Table 1, it can be observed that the sterilizing effects of 0.1% $HgCl_2$ is better than 3% NaClO, but the germination rate decreased obviously. It also shows that $HgCl_2$ have pernicious effects on seed. So, 3% NaClO for 15~20 min is suitable for the surface disinfection of MicroTom seeds, which could get low pollution (less than 5%) and high germination percentage (more than 80%). So for tomato seed disinfection, NaClO is better than $HgCl_2$ (Luxia et al., 2008).

Table 1. Effects of two types of disinfectors on seed germination (GR) and contamination ratio (CR)

Disinfector	5 min		10 min		15 min		20 min	
	GR%	CR%	GR%	CR%	GR%	CR%	GR%	CR%
NaClO (3%)	96	60	95	30	90	5	80	0
$HgCl_2$ (0.1%)	35	10	25	0	0	0	0	0

3.2 Comparison of Basic Medium MS and B5

The kind of basal culture medium could lead to different regeneration rate for different gene type of tomato (Yunhua et al., 2005). From table 2, it is clear that the regeneration rate of shoot form MicroTom in medium B5 is obviously higher than that in medium MS (+ 12.0%).

Table 2. Effects of base media on bud regeneration from MicroTom cotyledon

Base media	No. of explants	Differentiation rate of bud
MS	100	66%
B5	100	74%

3.3 Influence of IBA and 6-BA Ratio on Differentiation

From Table 3, it can be seen that in medium B5, the regeneration rate of MicroTom decreased with IBA concentration rising, and the shoot regeneration rates from cotyledons and hypocotyls were highest when the concentration of 6-BA reached 1.5 mgL^{-1}, and it reached 95.8% and 60.0%, respectively.

Table 3. Effect of IBA and 6-BA on bud induction from MicroTom explants

6-BA concentration (mgL-1)		IBA concentration ($mg \cdot L^{-1}$)		
		0.05	0.1	0.2
Cotyledons	1.0	83.3%	75.0%	65.0%
	1.5	95.8%	83.3%	62.5%
	2.0	87.5%	70.8%	66.7%
	3.0	75.0%	66.7%	50.0%
Hypocotyls	1.0	50.0%	47.8%	36.7%
	1.5	60.0%	43.3%	33.3%
	2.0	43.3%	33.3%	43.3%
	3.0	23.3%	16.7%	14.8%

When cotyledons and hypocotyls of MicroTom as the explants, Caixia et al. (2007) found that the appropriate medium of shoot differentiation induced is MS + 6-BA 2.0 mgL^{-1} + IAA 0.2 mgL^{-1}, which of the bud differentiation rate can be as high as 92%. The study of BO(2005) showed that the medium MS + ZT/IAA (9:1) is suitable for shoots differentiation of MicroTom cotyledons, the rate of bud differentiation reached to 93.3%. In this paper the regeneration rate of shoot obtained as high as 95.8%, it is probable that which has particular relation with basic medium B5.

3.4 Influence of Kan Concentration on Regeneration

The gene type of tomato has different sensibility to Kanamycin, for T79 and T151 it is 50-100 mgL^{-1} (Sulan et al., 2003), but for Zhongshu 6-10 mgL^{-1} of kanamycin is advisable (Xuexi et al., 2003).

Table 4. Effect of Kan concentration on MicroTom regeneration

Kan concentration (mgL^{-1})	No. of explants inoculated	Differentiation rate of callus (%)	Differentiation rate of buds (%)
0	50	96	90
25	50	80	40
50	50	40	10
75	50	10	0
100	50	6	0
150	50	0	0

From the Table 4, it is clear that Callus induction rate decreased obviously when concentration of Kan at 50 mgL^{-1}, bud differentiation rate was very low (10%). When

concentration of Kan is more than 75 mgL^{-1}, the capacity of bud differentiation lost. So 50 mgL^{-1} Kan is the appropriate selection-pressure.

3.5 Microbial Cell Concentration for Infection

As Agrobacterium has overgrown, it is difficult to restrain, and the excessive concentrations of bacterium fluid has adverse impaction on growth and differentiation of the explants; the concentrations is too low to get enough Agrobacterium attached on the explants incision and the transformation frequency reduced greatly (Guixia, 1999; Nan et al., 2006; Qiu et al., 2007).

When Agrobacterium cell concentration was around OD600 0.8 in this paper, the Agrobacterium colonies became obvious after coculture for 2 d, eventually all explants brown and died. When it was OD600 0.5, there were some contamination, but the progress was slow (Table 5). Thus it could be figured out that Agrobacterium cell concentration around OD600 0.5 was the suitable concentration for Agrobacterium EHA105.

Nan et al.（2006）found that for Agrobacterium LBA4404, OD600 0.6-0.8 of bacterium cell is the most appropriate range for infection of MicroTom. The antibiotic dosages increased can control the proliferation of Agrobacterium, but also it seriously inhibited the differentiation of shoot. But Caixia et al.（2007）proved that the concentration OD600 0.14 of Agrobacterium (Agl1) was the best concentration for MicroTom. This showed that different Agrobacterium strains have different toxicity to MicroTom.

Table 5. Effect of bacterial concentration for incubation on rate of contamination

Bacterial concentration for incubation(OD600)	Rate of contamination co-culturing for 2 d (%)	Rate of contamination co-culturing for 7 d (%)
0.3	0	0
0.5	18	30
0.8	60	100

3.6 Selection of Infection Time

Table 6. Effect of incubation time on the rate of contamination (7 d after infection)

Incubation time (min)	5	10	15
Rate of contamination (%) after co-culturing 7 days	15	46	67

The contamination rate on medium with 300 mgL^{-1} Carb increased greatly when the time of infection prolonged, whereas, the contamination rate was low (15%) when infected for 5 min, but the contamination rate was quit high (67%) when infected for 15 min (Table 6). Considering the efficiency of transformation, it was wise to choose 10 min of infection and change the type and dosage of bacteriostatic agent. Xiaoling et al. (2004), Quanhua et al. (2007) gave the alike suggestion.

3.7 Selection of Antibiotic

From table 7, it can be seen that when the Carb concentration was 500 mgL^{-1}, ideal bacteriostatic effect achieved, and relatively lower dosage of CSSS and CS could get the same bacteriostatic affection, which suggested that Agrobacterium EHA105 may be more sensitive to cephalosporins penicillin. But CS had obvious browning effect on MicroTom explants, therefore, CSSS from 300 to 500 mgL^{-1} is the most appropriate choice to restrain Agrobacterium EHA105 for MicroTom explants.

Table 7. Restrain effect of antibiotic for EHA105

Antibiotic	Concentration ($mg \cdot L^{-1}$)			
	100	200	300	500
Carb	+++	++	+	±
CS	++	+*	±*	-*
CSSS	++	+	±	-

Note : "+++" infected extremely serious and all browning explants died.
"++" infected serious and part of browning explants died.
"+" less pollution and bit of explants brown.
"±" agrobacterium colonies occasionally appeared around explants incision.
"-" Agrobacterium colonies hard to see on culture medium.
"" medium turned light yellow and part of explants browning.*

4. CONCLUSION

- If immersed in 3% sodium hypochlorite for 20 min, MicroTom seed could be disinfected efficiently without any restrain to germination.
- The most suitable medium for MicroTom to induce adventitious bud is B5+IBA0.05 mgL^{-1}＋6-BA 1.5 mgL^{-1}.
- The cotyledons or hypocotyls were cultivated in culture medium B5+IBA0.05 $mg \cdot L^{-1}$ for 2 d, infected by agrobacterium EHA105 (OD600=0.5) for 10min, then transferred to a fresh regeneration medium which contained 50 mgL^{-1} Kan and 300 mgL^{-1} Sulbactam, which is the most suitable transformation system for MicroTom.

ACKNOWLEDGEMENTS

Authors are thankful to Professor Zhihui Cheng in Northwest Agriculture & Forestry University for providing MicroTom (miniature *Lycopersicon esculentum* Mill.) and Agrobacterium EHA105.

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