Pak. J. Bot., 41(4): 2043-2054, 2009.

OPTIMIZED SELECTION AND REGENERATION CONDITIONS FOR *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF CHICKPEA COTYLEDONARY NODES

M.T. ÖZ, F. EYİDOĞAN[1], M. YÜCEL AND H.A. ÖKTEM[*]

Department of Biological Sciences, Middle East Technical University, 06531, Ankara, Turkey.
[1]Başkent University, Faculty of Education, 06810, Ankara, Turkey.

Abstract

An efficient selection system and *Agrobacterium* mediated transformation for chickpea (*Cicer arietinum* L.) cotyledonary nodes were investigated. Effect of selective agents and antibiotics on multiple shoot and root induction of cotyledonary nodes and effects of mechanical injury and vacuum infiltration on transformation efficiency were evaluated. Selective agents and antibiotics were applied to explants at different concentrations for one month and numbers of regenerated shoots and roots were recorded. Kanamycin at 100 mg dm^{-3}, hygromycin at 20 mg dm^{-3}, phosphinotricin at 3 mg dm^{-3} and glyphosate at 5 mg dm^{-3} were found to be appropriate to select chickpea transformants. Lowest concentrations of all selective agents (50 mg dm^{-3} kanamycin, 10 mg dm^{-3} hygromycin, 3 mg dm^{-3} phosphinotricin, 1 mg dm^{-3} glyphosate) totally inhibited rooting of the regenerated shoots. Among the *Agrobacterium*-eliminating-antibiotics, timentin significantly increased and carbenicillin significantly decreased shoot induction after 4 weeks of culture. On the other hand, cefotaxime at all concentrations significantly decreased root induction. Sulbactam, a β-lactamase inhibitor, in combination with carbenicillin and cefotaxime displayed effective inhibition of *Agrobacterium tumefaciens* growth. Furthermore, selection procedure formulated in this study was used in chickpea transformation studies. Histochemical GUS staining was performed 4 and 16 days after transformation to analyze putative transgenics. On the other hand, efforts were exerted on transformation to increase the efficiency. Mechanical injury prior to transformation and vacuum infiltration at 200 mm Hg for 40 min., during bacterial inoculation might be employed to increase the efficiency of chickpea transformation.

Introduction

Selection and recovery of transformed cells or tissues and elimination of *Agrobacterium* from the cultures require the use of selective agents and/or antibiotics. Genes encoding antibiotic resistance and herbicide tolerance are widely employed as selective markers to identify the rare transformed explants (de Vetten *et al*., 2003; Miki & McHugh, 2004). Selective agent concentration to be used in gene transfer should be optimized prior to transformation to determine agent effective on shoot and root regeneration and to determine lethal dose for each agent.

The continued presence of *Agrobacterium* interferes with the growth, development, and rooting rates; and even it causes the necrosis of transformed explants (Tang *et al*., 2004). Moreover, elimination of *Agrobacterium* from transformants is a prerequisite in preventing the possibility of gene release when these plants are transferred to soil (Barrett *et al*., 1997). Bacterial presence on putative transgenics may also result in false positives during molecular analyses. Most commonly used antibiotics for elimination of various strains of *Agrobacterium* are carbenicillin, cefotaxime and timentin (Nauerby *et al*., 1997).

[*]E-mail: haoktem@metu.edu.tr

Legume cotyledonary nodes (CN) were found to be one of the best responding explants in tissue culture; therefore, transformation procedures using CN were developed for various legumes such as soybean (Hinchee *et al*., 1988), lentil (Mahmoudian *et al*., 2002), blackgram (Saini *et al*., 2003), and pigeonpea (Thu *et al*., 2003). To date a number of reports described genetic transformation of chickpea *via Agrobacterium* (Fontana *et al*., 1993; Kar *et al*., 1996; Krishnamurthy *et al*., 2000; Sarmah *et al*., 2004; Senthil *et al*., 2004; Polowick *et al*., 2004; Tewari-Singh *et al*., 2004; Sanyal *et al*., 2005) or *via* microprojectile bombardment (Kar *et al*., 1997; Husnain *et al*., 1997). Explants used in these reports were mostly embryo axes devoid of root meristem and shoot apex. Chickpea CN explants were used in the study of Sanyal *et al*., (2005).

Shoot regeneration from chickpea embryo axes was evaluated under various concentrations of kanamycin and phosphinotricin (PPT) (Krishnamurthy *et al*., 2000); however no comprehensive evaluation has been carried out on responses of chickpea CNs to the commonly employed selective agents. Effects of *Agrobacterium* eliminating antibiotics on different plant species (tobacco, wheat, barley, potato and apple) have been reviewed by Nauerby *et al*., (1997); however no literature data is available on chickpea.

Therefore, in this study we investigated effects of four selective agents (kanamycin, hygromycin, PPT and glyphosate) and four *Agrobacterium* eliminating antibiotics (carbenicillin, cefotaxime, timentin, and augmentin) on multiple shoot and root induction in chickpea CN. Furthermore, effect of sulbactam, a β-lactamase inhibitor, in combination with other antibiotics on chickpea regeneration and effect of these antibiotics on *A. tumefaciens* growth were evaluated. On the other hand, *Agrobacterium* mediated transformation of chickpea CN was performed and the optimized procedure of selection was employed during transformation studies. Effects of mechanical injury and vacuum infiltration on transformation were also studied.

Materials and Methods

Preparation of plant material: A *kabuli* type chickpea (*Cicer arietinum* L.) cultivar Gökce (kindly donated by Exporter Unions Seed and Research Company) was used throughout the study. Seeds of chickpea were surface sterilized in 3% (v/v) Sodium hypochloride including 0.1% (v/v) Tween-20 for 90 min. Then they were rinsed in sterile distilled water five times and blotted dry on sterile filter papers. Dried seeds were placed onto half strength MS media (Murashige & Skoog, 1962) lacking vitamins and supplemented with 1.5% (w/v) sucrose and 0.6% agar (w/v). Germination was carried out at 24±2ºC in dark for 4 days. CNs were isolated from 4 days-old etiolated chickpea seedlings. The radicle and emerging shoot primordium were removed with single cuts leaving 5-6 mm of tissue on both sides of the node. Then the cotyledons were excised from the node, leaving 2-3 mm of tissue on explant (Fig. 1a).

Bacterial strain and culture media: *A. tumefaciens* strain KYRT1 (Torisky *et al*., 1997) containing pTJK136 (Kapila *et al*., 1997) was used both to transform chickpea CNs and to determine the effect of antibiotics on bacterial growth. Yeast extract broth (YEB) containing nutrient broth (13.5 g dm^{-3}), yeast extract (1 g dm^{-3}), sucrose (5 g dm^{-3}) and magnesium sulphate (2 mM) at pH 7.2 was used to grow *A. tumefaciens* KYRT1. The medium was supplemented with filter sterilized rifampin (100 mg dm^{-3}), carbenicillin (100 mg dm^{-3}), gentamicin (40 mg dm^{-3}), streptomycin (300 mg dm^{-3}) and spectinomycin (125 mg dm^{-3}). Bacteria were grown in YEB at 200 rpm and 28±1ºC unless otherwise indicated. Binary vector pTJK136 carries an intron containing *uidA* (*gusA*) reporter gene and *nptII* selectable gene as plant selection markers.

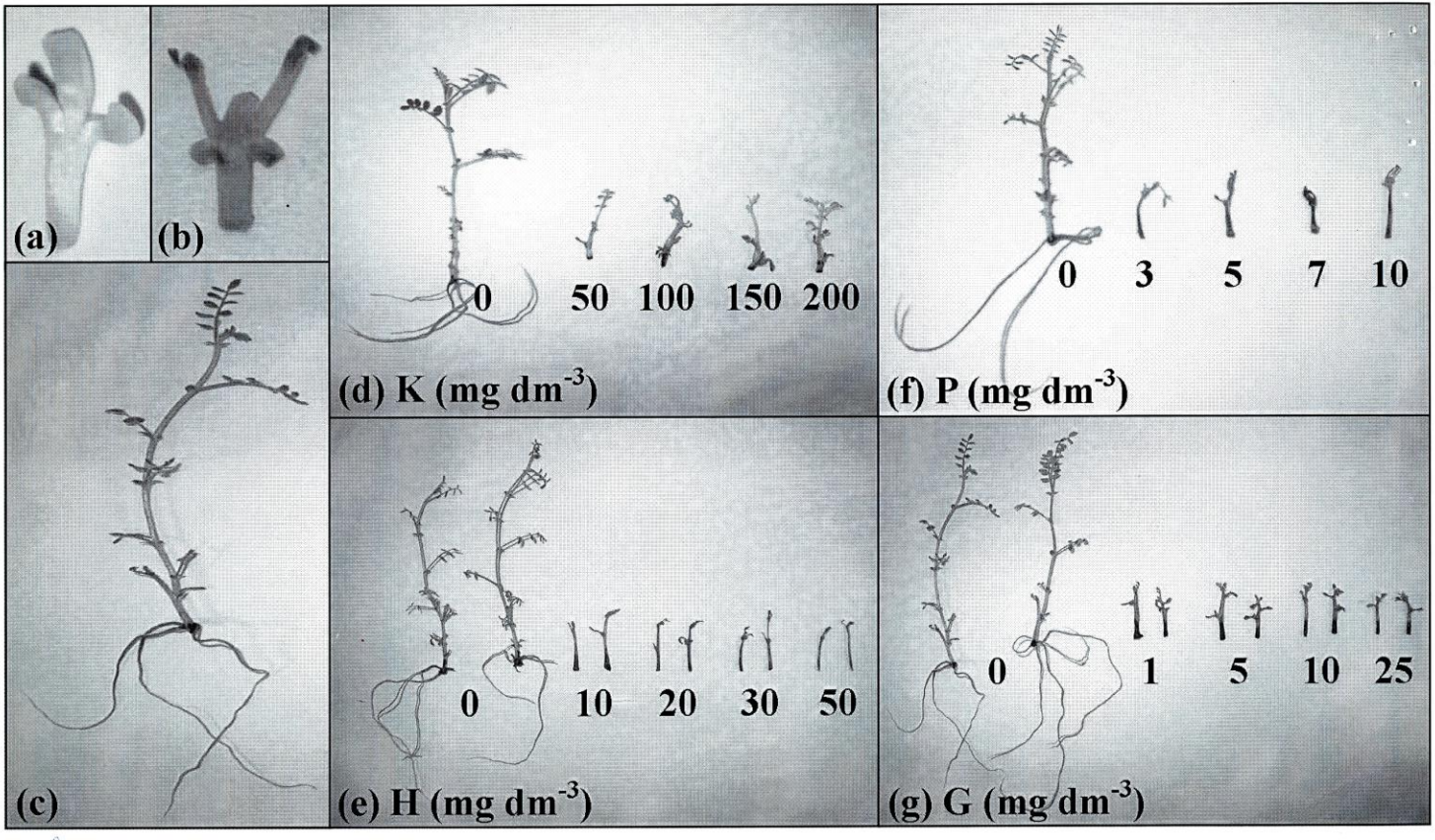

Fig. 1. (a-c) Chickpea regeneration *in vitro* via direct organogenesis and (d-g) effects of selective agents on root induction. [(a) CN explant on 4th day, (b) shoots formed on CN on 12th day, (c) a regenerated chickpea plant with roots on 28th day, (d) K: Kanamycin, (e) H: Hygromycin, (f) P: Phosphinotricin, (g) G: Glyphosate]

Tissue culture studies: CNs were regenerated on MS media, supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar. The media were prepared with distilled water and the pH was adjusted to 5.6-5.8. Media were sterilized at 121ºC for 20 min. Growth regulators, selective agents, antibiotic solutions and sulbactam (Pfizer) were filter sterilized with 0.2 µm pore sized filters (Sartorius, Minisart) and added freshly to the sterilized and cooled media. Multiple shoot induction from CNs and rooting of regenerated shoots were performed with 1 mg dm^{-3} of benzylaminopurine (BA) and 0.1 mg dm^{-3} of indole butyric acid (IBA), respectively. The explants were cultured for 4 weeks at 24±2ºC under light (100 µmol m^{-2} s^{-1}) with a 16/8 h photoperiod. Number of shoots per CN, number of roots per shoot and rooting frequency (percent of shoots having roots) were recorded.

Antibiotic sensitivity testing: To determine the effects of antibiotics on growth of *A. tumefaciens* cells, a modified agar diffusion test was used. An overnight grown culture was first diluted to a final OD_{600} of 0.8. 100 µL of this bacterial culture was spread on YEB media solidified with 1.5% (w/v) agar. Surface was air dried for about 5 min. Then sterile filter paper discs loaded with different concentrations of antibiotics were placed on the agar surface. The plates were incubated at 28±1ºC for 2 days. Diameters of growth inhibition zones were recorded.

Transformation studies: The utilized transformation procedure was adopted from Mahmoudian *et al*., (2002). *A. tumefaciens* strain KYRT1 was grown overnight in liquid YEB (pH 5.6) supplemented with necessary antibiotics, 10 mM MES (2-[N-Morpholino] ethanesulfonic acid), and 20 µM acetosyringone (3',5'-Dimethoxy-4-hydroxyacetophenone), till OD_{600} reaches to 0.8. Then the bacterial culture was centrifuged at 1,500*g* for 15 min at 4ºC. The pellet was resuspended in MS (pH 5.6) lacking vitamins and containing 2% (w/v) sucrose, 10 mM MES and 200 µM acetosyringone, with OD_{600} value of 2.4-2.5. Then bacterial suspension was incubated for 60 min., at 24±2ºC under fluorescent lights. CNs were inoculated with bacteria in this suspension for 40 min., under the same conditions. Then CNs were blotted dry on sterile filter papers and placed onto MS media containing 1 mg dm^{-3} BA for co-cultivation, which was performed for 4 days at 24±2ºC under light with a 16/8 h photoperiod.

Mechanical injury of CN was performed prior to bacterial inoculation, with a fine glass needle, by poking 6 to 8 times at each axillary region of cotyledonary petiole. To determine the effect of infiltration on transformation efficiency, mechanically injured CNs were vacuum infiltrated during inoculation of CNs with bacteria. Vacuum infiltration was performed for 40 min., at evacuation pressures of 200, 400 and 600 mmHg.

Selection was performed with 100 mg dm^{-3} kanamycin and this concentration was decreased to 50 mg dm^{-3} during root induction. Elimination of bacteria was achieved by addition of 200 mg dm^{-3} carbenicillin, 400 mg dm^{-3} cefotaxime and 100 mg dm^{-3} sulbactam to the regeneration media. For the analysis of putative transgenics, GUS histochemical staining (Jefferson, 1987) was performed 4 and 16 days after transformation. Results of GUS staining on 4^{th} day, were recorded as number of shoots exhibiting GUS activity per CN and as percent of CN explants exhibiting GUS activity. On the other hand, results of GUS staining on 16^{th} day, were recorded as GUS expressing area relative to the total surface area of tissues. This value of relative GUS positive area was measured by image analysis system (Zeiss® KS300).

Statistical analysis: All of the statistical analyses were carried out by using Minitab 13.0 software. Means and standard error of means (SEM) were calculated with this software. One-way analysis of variance (ANOVA) at a 95% confidence interval (p<0.05) was used to detect variances in means and was used to investigate the relationship between response variables.

Results and Discussion

In this study, chickpea regeneration was performed *via* direct organogenesis using CN; and complete plant regeneration lasted for a total of 28 days starting from seed surface sterilization (Fig. 1a-c). First shoots emerged in 7-8 days of culture. Upon excision of previous ones, new shoots emerged from each axillary region each week. This continuous differentiation and absence of callus phase decreased the time required for regeneration and increased the number of regenerated plants. 1 mg dm^{-3} BA and 0.1 mg dm^{-3} IBA were found to be appropriate for multiple shoot induction from axillary meristems of CN and for root induction from regenerated shoots, respectively (data not shown).

Effects of selective agents on multiple shoot induction: Multiple shoot induction under 50 mg dm^{-3} kanamycin or 10 mg dm^{-3} hygromycin was significantly lower than control after 2 weeks of culture (Table 1). On the other hand, effects of inhibition were more drastic when 100 mg dm^{-3} kanamycin or 20 mg dm^{-3} hygromycin were used. Kanamycin and hygromycin are inactivated through phosphorylation. Chickpea might have non-specific phosphotransferase activity, which might explain the regeneration of minimum numbers of shoots and which might result in background activity during transformation.

PPT even at the lowest concentration (3 mg dm^{-3}) totally inhibited shoot regeneration (Table 1). The explants were totally decolorized lacking chlorophyll synthesis. This lethal effect was observed even after 1 week of culture. Senthil *et al.,* (2004) also observed shoot necrosis caused by 2.5 mg dm^{-3} PPT. Glyphosate at concentration of 5 mg dm^{-3} significantly decreased the number of shoots regenerated from chickpea CN after 2 weeks of culture (Table 1). Shoots regenerated under the stress of high concentrations (5 mg dm^{-3} or more) of glyphosate were shorter and less developed compared to ones regenerated on herbicide-free media. At such high concentrations of herbicide, shoot buds were formed but they did not develop into whole adventitious shoots. Inhibition of meristematic-tissue-elongation was also observed during selection after transformation of wheat (Hu *et al*., 2003). Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), a key enzyme of the shikimate pathway, which is required for the synthesis of aromatic amino acids and other compounds including vitamins, plant growth substances and lignin. Therefore, elongation of shoot buds might be inhibited because of depletion of such vital compounds. There are limited numbers of reports on effects of glyphosate on plant tissues in culture. Overall, these results might show that herbicides are better selective agents compared to antibiotics for chickpea transformation.

Effects of antibiotics on multiple shoot induction: High frequency transformation using *Agrobacterium* depends not only on the efficiency of plant regeneration but also on the elimination of bacteria from transformed cells. According to the number of shoots per explant, augmentin and cefotaxime up to 600 mg dm^{-3} were determined to possess no effect on chickpea CNs (Table 2). Similarly augmentin was defined as an efficient

antibiotic for elimination of *A. tumefaciens* C58C1, in selection of *Artemisia annua* L. with no significant effect on explant (Vergauwe *et al*., 1996). At all concentrations, carbenicillin significantly decreased the numbers of shoots per explant after 4 weeks of culture (Table 2). Carbenicillin is a β-lactam antibiotic and phenylacetic acid, a naturally occurring auxin, is one of the breakdown products of carbenicillin. Auxin/cytokinin ratios in the media or explant might be altered as a result of carbenicillin breakdown. This might reduce shoot induction. Timentin, which is an effective inhibitor of bacterial growth (Cheng *et al*., 1998), possessed a positive effect on multiple shoot induction (Table 2). Formation of significantly increased numbers of shoots was observed after 2 weeks at 300 mg dm^{-3}. After 4 weeks of culture all concentrations of timentin resulted in significantly high numbers of shoots compared to control. Estopa *et al*., (2001) reported that 100 mg dm^{-3} timentin increased the number of shoots regenerated from carnation leaf explants, which well correlates with our findings. Although breakdown product of timentin is also an auxin-like compound, this product does not lead to toxic levels of auxin activity. On the contrary, this auxin-like compound might be stimulating shoot induction in chickpea CN.

Antibiotics used in this study can be inactivated by β-lactamases of *Agrobacterium*. Therefore, a β-lactamase inhibitor, sulbactam (100 mg dm^{-3}), was added into media to test its effects on explants. Compared to control, a significant decrease in number of shoots under all combinations was observed after 4 weeks of culture (Table 2). This decrease probably stems from the presence of carbenicillin in all 3 combinations. However numbers of shoots regenerated from CN were not significantly different among the all 3 combinations. Therefore, it might be stated that 100 mg dm^{-3} sulbactam does not have any influence on multiple shoot induction. On the other hand, concentrations of carbenicillin and cefotaxime might be reduced when sulbactam is supplemented, since it has no antimicrobial effect of its own and also has no inhibitory effect on multiple shoot induction. Similar effect of sulbactam was previously reported on selection of an endospermous legume guar (Joersbo *et al*., 1999).

Effects of selective agents and antibiotics on root induction: All selective agents at minimum concentrations totally inhibited the root induction from regenerated shoots (Fig. 1d-g). Complete inhibition of rooting under kanamycin was consistent with the report of Estopa *et al*., (2001) in which non-transgenic carnation shoot tips were cultured with 150 mg dm^{-3} kanamycin. Polowick *et al*., (2004), indicated the importance of selective agents and increased the concentration of agent in root induction media for chickpea. Likewise Tewari-Singh *et al*., (2004) used 100 mg dm^{-3} kanamycin or 5 mg dm^{-3} PPT during chickpea rooting.

For the explants cultured on antibiotic-free media, rooting frequencies were around 85 to 90% and number of roots per shoot was around 5 (Table 2). Augmentin at all concentrations exhibited no significant change in root induction. On the other hand, cefotaxime, at all concentrations reduced the frequency of rooting and number of roots per shoot significantly (Table 2). Decreases in both parameters indicated that cefotaxime not only inhibited root growth but also inhibited formation of root primordia. Regenerated roots in the presence of cefotaxime were shorter and thicker; and shoots were less developed compared to the ones cultured in antibiotic-free media. These morphological changes are generally observed under abiotic stresses; indicating that cefotaxime might have toxic effects inducing stress response in plant tissues. Negative effects of cefotaxime on rooting efficiency of carnation shoot tips were also reported by Estopa *et al*., (2001).

Table 1. Effects of selective agents on multiple shoot induction.

Selective agent (mg dm^{-3})	# of shoots / explant (2 weeks)	# of shoots / explant (4 weeks)
Kanamycin		
0	2.13 ± 0.08a	3.13 ± 0.14a
50	1.83 ± 0.08b	2.11 ± 0.10b
100	1.48 ± 0.11c	1.66 ± 0.10c
150	1.54 ± 0.11c	1.62 ± 0.11c
200	1.50 ± 0.12c	1.57 ± 0.11c
Hygromycin		
0	2.19 ± 0.07a	3.52 ± 0.13a
10	1.71 ± 0.08b	2.22 ± 0.10b
20	1.25 ± 0.11c	1.54 ± 0.10c
30	1.15 ± 0.11c	1.43 ± 0.10cd
50	1.06 ± 0.12c	1.22 ± 0.11d
PPT		
0	2.17 ± 0.08	3.27 ± 0.14
3	0	0
5	0	0
7	0	0
10	0	0
Glyphosate		
0	2.22 ± 0.07a	3.56 ± 0.13a
1	1.86 ± 0.07b	2.74 ± 0.11b
5	1.22 ± 0.11c	1.67 ± 0.10c
10	1.12 ± 0.10c	1.37 ± 0.09d
25	1.06 ± 0.12c	1.22 ± 0.11d

Results are averages ± SEM of 4 independent experiments, which contain 15 explants for each concentration (n=60 for each concentration). Values in the same column indicated with same letter are not significantly different (p<0.05).

Carbenicillin, only at 300 mg dm^{-3}, and timentin at high concentrations (200 and 300 mg dm^{-3}) significantly reduced rooting efficiency of chickpea shoots. Effects of antibiotic and sulbactam combinations on root induction are presented in Table 2. All combinations significantly reduced the rooting frequency of chickpea shoots and number of roots per shoot. This sharp decrease was similar to one observed under cefotaxime alone; indicating that the decrease might be originating from the presence of cefotaxime. Any similar inhibitory effect of cefotaxime was not observed in multiple shoot induction. The possible reason for this might be the placement of CN onto media. CNs were inserted into media and emerging shoots were not in touch with the medium escaping from the toxic effects of the antibiotic.

Table 2. Effects of antibiotics on multiple shoot and root induction.

Antibiotic (mg dm^{-3})	# of shoots / explant (2 weeks)	# of shoots / explant (4 weeks)	# of roots / shoot	rooting frequency (%)
Augmentin				
0	2.26 ± 0.08a	3.56 ± 0.10a	4.95 ± 0.25a	87.0 ± 3.8a
200	2.18 ± 0.08a	3.61 ± 0.11a	4.89 ± 0.30a	86.4 ± 4.6a
400	2.23 ± 0.07a	3.60 ± 0.12a	4.86 ± 0.27a	91.7 ± 7.9a
600	2.25 ± 0.06a	3.62 ± 0.13a	4.94 ± 0.31a	85.7 ± 4.5a
Cefotaxime				
0	2.23 ± 0.08a	3.52 ± 0.12a	5.32 ± 0.28a	90.5 ± 2.8a
200	2.15 ± 0.08a	3.48 ± 0.13a	2.91 ± 0.25b	57.9 ± 4.2b
400	2.20 ± 0.07a	3.47 ± 0.13a	2.83 ± 0.27b	54.6 ± 4.2b
600	2.21 ± 0.07a	3.47 ± 0.13a	2.64 ± 0.28b	55.0 ± 9.6b
Carbenicillin				
0	2.24 ± 0.08a	3.52 ± 0.08a	5.67 ± 0.31a	85.7 ± 4.1a
100	2.13 ± 0.06a	3.23 ± 0.10b	5.61 ± 0.33a	81.8 ± 3.7a
200	2.10 ± 0.07a	3.15 ± 0.10b	3.59 ± 0.24b	81.0 ± 6.9a
300	2.16 ± 0.06a	3.12 ± 0.09b	3.25 ± 0.22b	57.1 ± 1.4b
Timentin				
0	2.16 ± 0.07a	3.42 ± 0.08a	5.41 ± 0.34a	89.5 ± 3.7a
100	2.23 ± 0.06a	3.81 ± 0.11b	5.32 ± 0.30a	86.4 ± 3.3a
200	2.27 ± 0.06ab	3.93 ± 0.11b	3.86 ± 0.25b	70.0 ± 4.6b
300	2.35 ± 0.07b	4.02 ± 0.10b	3.27 ± 0.24b	55.0 ± 5.0c
Combinations				
Control	2.28 ± 0.08a	3.67 ± 0.09a	5.25 ± 0.27a	90.9 ± 8.3a
200 C + 400 Cf	2.15 ± 0.06a	3.36 ± 0.09b	3.08 ± 0.23b	63.2 ± 3.3b
200 C + 400 Cf + 100 S	2.13 ± 0.06a	3.25 ± 0.09b	3.08 ± 0.21b	61.9 ± 1.8b
50 C + 100 Cf + 100 S	2.18 ± 0.06a	3.23 ± 0.09b	3.31 ± 0.26b	65.0 ± 5.0b

Results are averages ± SEM of 4 independent experiments which contain 15 explants for each concentration (n=60 for each concentration) for multiple shoot induction and of 2 independent experiments which contain 10 explants for each concentration (n=20 for each concentration) for root induction. Antibiotic-free media were used as control. Values in the same column indicated with same letter are not significantly different (p<0.05). (C: Carbenicillin; Cf: Cefotaxime; S: Sulbactam).

Table 3. Effects of mechanical injury and vacuum infiltration on multiple shoot induction and histochemical GUS staining 4 and 16 days after transformation.

Parameter	# of shoots / explant (4th day)	# of shoots exhibiting GUS activity / explant (4th day)	% of explants exhibiting GUS activity (4th day)	% of GUS expressing area (16th day)
Mechanical injury				
Control	1.71 ± 0.05a	0a	0a	0a
Not injured	1.58 ± 0.05ab	0.49 ± 0.06b	42.6 ± 4.1b	2.1 ± 0.4b
Injured	1.47 ± 0.02b	1.21 ± 0.03c	78.6 ± 2.6c	14.9 ± 1.7c
Vacuum infiltration				
Control	1.76 ± 0.06a	0a	0a	0a
0 mm Hg	1.46 ± 0.03b	1.06 ± 0.04b	74.5 ± 3.6b	16.1 ± 2.4b
200 mm Hg	1.41 ± 0.03b	1.16 ± 0.04b	79.1 ± 2.8b	16.3 ± 3.4b
400 mm Hg	0.73 ± 0.02c	0.33 ± 0.02c	58.6 ± 2.8c	19.2 ± 4.8b
600 mm Hg	0.34 ± 0.03d	0.10 ± 0.02d	47.7 ± 2.4d	20.2 ± 3.3b

Results are averages ± SEM of 7 independent experiments which contain a minimum of 25 explants for control groups and 50-75 explants for treatments. In control groups, explants were not inoculated with bacteria. Values in the same column indicated with same letter are not significantly different (p<0.05).

Effects of antibiotics on *A. tumefaciens*: Efficiency of various antibiotics in controlling growth of *Agrobacterium* was tested using agar diffusion assay. According to the diameters of inhibition zones, it was found that *A. tumefaciens* was resistant to all concentrations of carbenicillin (Fig. 2) since it contains carbenicillin resistance gene (Torisky *et al*., 1997). Inhibition zones formed around the discs of augmentin, cefotaxime and timentin increased in a dose dependent manner. Among the three antibiotics; augmentin produced the narrower zones (8-15 mm) whereas cefotaxime produced the wider zones (32-42 mm) at all concentrations. Although the concentrations of both are equal (200, 400 and 600 µg) and augmentin contains clavulanic acid (a β-lactamase inhibitor), cefotaxime was found to be superior over augmentin in controlling *A. tumefaciens* (Fig. 2). A similar observation in inhibition of *A. tumefaciens* strain AGL1 using disc diffusion assay was also reported by de Mayolo *et al*., (2003). Cefotaxime is a cephalosporin antibiotic where augmentin and timentin contains penicillin G group antibiotics. The methoxyimino moiety of cefotaxime confers stability to β-lactamases.

Sulbactam presence exhibited a significant increase in effects of other antibiotics. Sulbactam (100 µg), when applied together with 200 µg carbenicillin and 400 µg cefotaxime, displayed a zone with a diameter of 47 mm (Fig. 2). Also a decrement in concentrations of carbenicillin to 50 µg and cefotaxime to 100 µg in the presence of sulbactam resulted in an effective inhibition. Effects of antibiotic combinations together with 100 µg kanamycin were also investigated since kanamycin and antibiotics are used together during selection of transgenics. Kanamycin (100 µg), carbenicillin (200 µg), cefotaxime (400 µg) and sulbactam (100 µg), when applied together, effectively controlled bacterial growth (Fig. 2); therefore, this combination might be employed after chickpea transformation.

Transformation and analysis of putative transgenics: The optimized system of selection and bacteria removal was used after transformation of chickpea CNs with *A. tumefaciens* KYRT1. Results of histochemical GUS staining on 4^{th} and 16^{th} day are displayed in Table 3. Mechanical injury significantly increased the transformation efficiency. Injury of axillary region of CN increased the number of cells infected at this specific region. However, the relative GUS expressing regions were still smaller in area compared to total surface area of shoots. Although regenerated shoot number slightly decreased upon mechanical injury, number of GUS positive shoots per explant increased 3 fold and frequency of GUS positive explants increased nearly 2 fold (Table 3). Injury also increased the surface area of tissues exhibiting GUS activity by 7 fold (Table 3). On the other hand, vacuum infiltration increased the efficiency of transformation only at 200 mm Hg. Higher values of pressure (400 or 600 mm Hg) significantly decreased the number of regenerated shoots, number of GUS positive shoots and frequency of GUS positive explants (Table 3). These sharp reductions probably stem from the detrimental effects of vacuum or bacterial penetration derived by vacuum. Although GUS-positive-surface-area of tissues increased slightly under 400 and 600 mm Hg, these increases were not significant compared to application of 200 mm Hg (Table 3). Overall, it might be concluded that mechanical injury prior to transformation and vacuum infiltration at 200 mm Hg for 40 min., during bacterial inoculation might be employed to increase the efficiency of chickpea transformation.

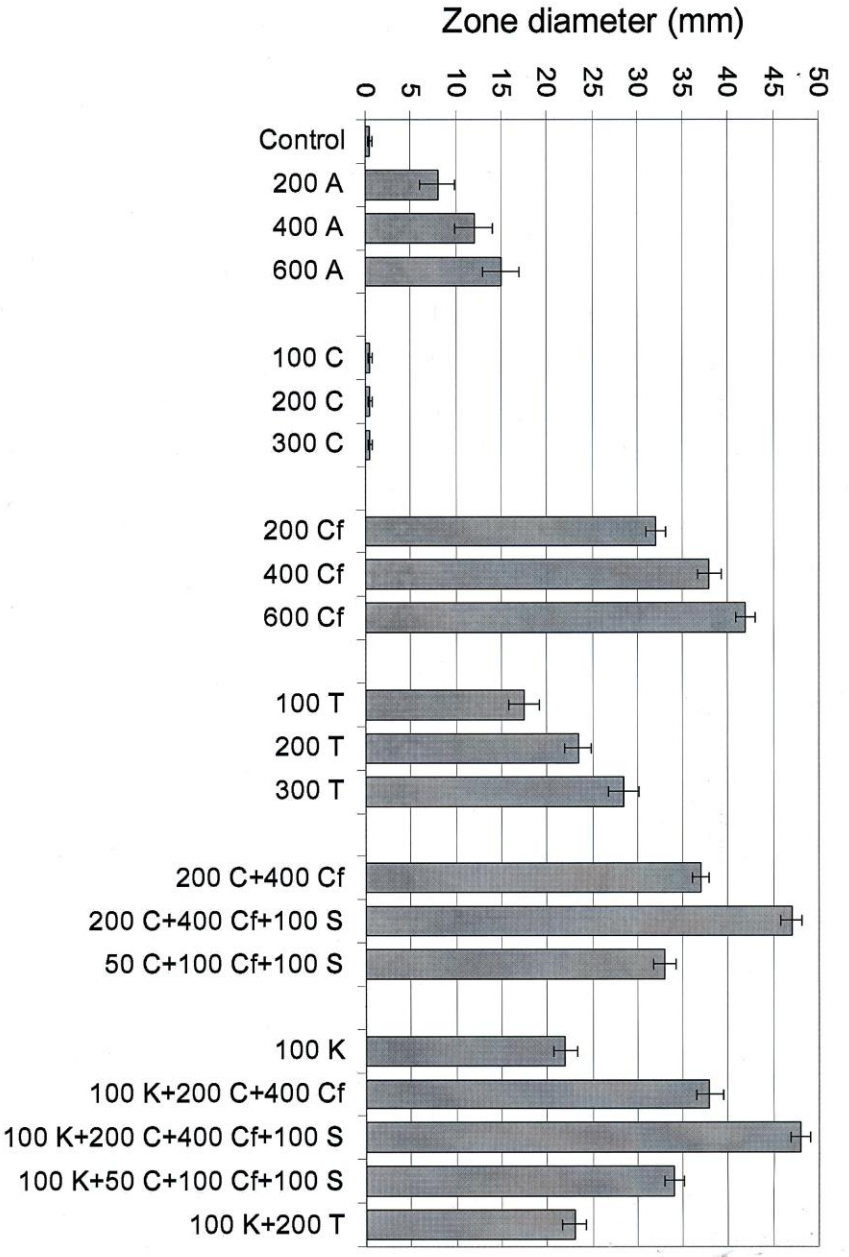

Fig. 2. Antibiotic sensitivity testing for *A. tumefaciens* strain KYRT1. The effects of various concentrations (µg) and combinations of Augmentin (A), Carbenicillin (C), Cefotaxime (Cf), Timentin (T), Kanamycin (K) and Sulbactam (S) on bacterial growth are represented as diameter of growth inhibition zone in millimeters (mm).

Acknowledgments

This work was supported by the funds; BAP-08-11-DPT2002K120510 and BAP-2005(R)-08-11-03, which are gratefully acknowledged.

References

Barrett, C., E. Cobb, R. McNicol and G. Lyon. 1997. A risk assessment study of plant genetic transformation using *Agrobacterium* and implications for analysis of transgenic plants. *Plant Cell Tiss. Org. Cult.,* 47: 135-144.

Cheng, Z.M., J.A. Schnurr and J.A. Kapaun. 1998. Timentin as an alternative antibiotic for suppression of *Agrobacterium tumefaciens* in genetic transformation. *Plant Cell Rep.,* 17: 646-649.

de Mayolo, G.A., S.N. Maximova, S. Pishak and M.J. Guiltinan. 2003. Moxalactam as a counter-selection antibiotic for *Agrobacterium* mediated transformation and its positive effects on *Theobroma cacao* somatic embryogenesis. *Plant Sci*., 164: 607-615.

de Vetten, N., A.M. Wolters, K. Raemakers, I. van der Meer, R. ter Stege, E. Heeres, P. Heeres and R. Visser. 2003. A transformation method for obtaining marker-free plants of a cross-pollinating and vegetatively propagated crop. *Nat. Biotechnol.,* 21: 439-442.

Estopa, M., V. Marfa, E. Mele and J. Messeguer. 2001. Study of different antibiotic combinations for use in the elimination of *Agrobacterium* with kanamycin selection in carnation. *Plant Cell Tiss. Org. Cult.,* 65: 211-220.

Fontana, G.S., L. Santini, S. Caretto, G. Frugis and D. Mariotti. 1993. Genetic transformation in the grain legume *Cicer arietinum* L. (chickpea). *Plant Cell Rep.,* 12: 194-198.

Hinchee, M.A.W., D.V. Connor-Ward, C.A. Newell, R.E. McDonnell, S.J. Sato, C.S. Gasser, D.A. Fischhoff, D.B. Re, R.T. Fraley and R.B. Horsch. 1988. Production of transgenic soybean plants using *Agrobacterium*-mediated DNA transfer. *Biotechnology,* 6: 915-922.

Hu, T., S. Metz, C. Chay, H.P. Zhou, N. Biest, G. Chen, M. Cheng, X. Feng, M. Radionenko, F. Lu and J. Fry. 2003. *Agrobacterium*-mediated large-scale transformation of wheat (*Triticum aestivum* L.) using glyphosate selection. *Plant Cell Rep.,* 21: 1010-1019.

Husnain, T., T. Malik, S. Riazuddin and M. Gordon. 1997. Studies on the expression of marker genes in chickpea. *Plant Cell Tiss. Org. Cult.,* 49: 7-16.

Jefferson, R.A. 1987. Assaying chimeric genes in plants: The GUS gene fusion system. *Plant Mol. Biol. Rep.,* 5: 387-405.

Joersbo, M., J. Brunstedt, J. Marcussen and F.T. Okkels. 1999. Transformation of the endospermous legume guar (*Cyamopsis tetragonoloba* L.) and analysis of transgene transmission. *Mol. Breeding,* 5: 521-529.

Kapila, J., R. DeRycke, M. van Montagu and G. Angenon. 1997. An *Agrobacterium* mediated transient gene expression system for intact leaves. *Plant Sci.,* 122: 101-108.

Kar, S., D. Basu, S. Das, N.A. Ramkrishnan, P. Mukherjee, P. Nayak and S.K. Sen. 1997. Expression of *cryIA(c)* gene of *Bacillus thuringiensis* in transgenic plants inhibits development of pod-borer (*Heliothis armigera*) larvae. *Transgenic Res.,* 6: 177-185.

Kar, S., T.M. Johnson, P. Nayak and S.K. Sen. 1996. Efficient transgenic plant regeneration through *Agrobacterium*-mediated transformation of chickpea (*Cicer arietinum* L.). *Plant Cell Rep.,* 16: 32-37.

Krishnamurthy, K.V., K. Suhasini, A.P. Sagare, M. Meixner, A. de Kathen, T. Pickardt and O. Schieder. 2000. *Agrobacterium* mediated transformation of chickpea (*Cicer arietinum* L.) embryo axes. *Plant Cell Rep.,* 19: 235-240.

Mahmoudian, M., M. Yücel and H.A. Öktem. 2002. Transformation of lentil (*Lens culinaris* M.) cotyledonary nodes via vacuum infiltration of *Agrobacterium tumefaciens*. *Plant Mol. Biol. Rep.,* 20: 251-257.

Miki, B. and S. McHugh. 2004. Selectable marker genes in transgenic plants: applications, alternatives and biosafety. *J. Biotechnol.,* 107: 193-232.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 15: 473-479.

Nauerby, B., K. Billing and R. Wyndaele. 1997. Influence of the antibiotic timentin on plant regeneration compared to carbenicillin and cefotaxime in concentrations suitable for elimination of *Agrobacterium tumefaciens*. *Plant Sci.*, 123: 169-177.

Polowick, P.L., D.S. Baliski and J.D. Mahon. 2004. *Agrobacterium tumefaciens*-mediated transformation of chickpea (*Cicer arietinum* L.): gene integration, expression and inheritance. *Plant Cell Rep.*, 23: 485-491.

Saini, R., S. Jaiwal and P.K. Jaiwal. 2003. Stable genetic transformation of *Vigna mungo* L. Hepper via *Agrobacterium tumefaciens*. *Plant Cell Rep.*, 21: 851-859.

Sanyal, I., A.K. Singh, M. Kaushik and D.V. Amla. 2005. *Agrobacterium*-mediated transformation of chickpea (*Cicer arietinum* L.) with *Bacillus thuringiensis* cry1Ac gene for resistance against pod borer insect *Helicoverpa armigera*. *Plant Sci.*, 168(4): 1135-1146.

Sarmah, B.K., A. Moore, W. Tate, L. Molvig, R.L. Morton, D.P. Rees, P. Chiaiese, M.J. Chrispeels, L.M. Tabe and T.J.V. Higgins. 2004. Transgenic chickpea seeds expressing high levels of a bean α-amylase inhibitor. *Mol. Breeding*, 14: 73-82.

Senthil, G., B. Williamson, R.D. Dinkins and G. Ramsay. 2004. An efficient transformation system for chickpea (*Cicer arietinum* L.). *Plant Cell Rep.*, 23: 297-303.

Tang, W., H. Luo and R.J. Newton. 2004. Effects of antibiotics on the elimination of *Agrobacterium tumefaciens* from loblolly pine (*Pinus taeda*) zygotic embryo explants and on transgenic plant regeneration. *Plant Cell Tiss. Org. Cult.*, 70: 71-81.

Tewari-Singh, N., J. Sen, H. Kiesecker, V.S. Reddy, H.J. Jacobsen and S. Guha-Mukherjee. 2004. Use of a herbicide or lysine plus threonine for non-antibiotic selection of transgenic chickpea. *Plant Cell Rep.*, 22: 576-583.

Thu, T.T., T.T.X. Mai, E. Dewaele, S. Farsi, Y. Tadesse, G. Angenon and M. Jacobs. 2003. *In vitro* regeneration and transformation of pigeonpea [*Cajanus cajan* (L.) Millsp]. *Mol. Breeding*, 11: 159-168.

Torisky, R.S., L. Kovacs, S. Avdiushko, J.D. Newman, A.G. Hunt and G.B. Collins. 1997. Development of a binary vector system for plant transformation based on the supervirulent *Agrobacterium tumefaciens* strain Chry5. *Plant Cell Rep.*, 17: 102-108.

Vergauwe, A., E.V. Geldre, D. Inze, M. van Montagu and E. van den Eeckhout. 1996. The use of amoxicillin and ticarcillin in combination with a β-lactamase inhibitor as decontaminating agents in the *Agrobacterium tumefaciens*-mediated transformation of *Artemisia annua* L. *J. Biotechnol.*, 52: 89-95.

(Received for publication 6 December 2007)