The Agriculturists 7(1&2): 72-81 (2009) ISSN-1729-5211
A Scientific Journal of Krishi Foundation

Factors Enhancing Virulence Response of Agrobacteria in Cucumber (*Cucumis sativus* L.)

A. K. M. Mohiuddin[1,5*], Zaliha C. Abdullah[2], M. K. U. Chowdhury[3], K. Harikrishna[4], and Suhaimi Napis[1]

[1]*Dept of Biotechnology, University Putra Malaysia, 43400 Serdang, Selangor DE, Malaysia*
[2]*CRAUN Research Sdn. Bhd., Lot 3147, Block 14, J. Sultan Tengah, 93055, Kuching, Sarawak, Malaysia*
[3]*Department of Biology, Claflin University, 400 Magnolia Street, Orangeburg, SC 29115, USA*
[4]*Sime Darby Technology Centre, Biotechnology Div., 2 Jln. Tandang 46050, PJ, Selangor D.E., Malaysia.*
[5]*Department of Biotechnology and Genetic Engineering, Mawlana Bhashani Science and Technology University, Santosh, Tangail- 1902, Bangladesh*

[*]*Corresponding author and Email: akmmohiu@yahoo.com*

Abstract

The virulence response of five wild type *Agrobacterium tumefaciens* (tumorigenic) strains and five wild type *A. rhizogenes* (oncogenic) strains was studied in cucumber (*Cucumis sativus* L.), cv. Spring Swallow. Several physical and chemical factors e.g. *Agrobacterium* strains, tissue type (stem and petiole tissues), light intensity, dark incubation, concentrations of acetosyringone (a phenolic compound) were tested. The virulent response of Agrobacteria was found to be depend on plant tissue type, *Agrobacterium* strain, tissue-*Agrobacterium* interaction and both chemical-physical factors. Specific light intensity, 2500-lux, as well as the acetosyringone concentration, 20-μM, played important role in virulence response. Among the different strains, LBA 4404 and 15834 were highly virulent to cucumber compared to the other strains tested. *A. tumefaciens* strains A348 and A281 were failed to initiate crown galls in both stem and petiole tissues, however, in the presence of acetosyringone they produced crown galls. Similarly, *A. rhizogenes* strain A4 initiated hairy roots in the presence of acetosyringone. All developed crown galls and hairy roots showed an autonomous growth on a hormone free medium containing cefatoxime. Thus, enhancement of virulence response was not only dependent on *Agrobacterium* strains, but also on particular physical and chemical factors also play vital role.

Keywords: Tumourigenic and oncogenic *Agrobacterium*, virulence response, cucumber, physical-chemical factors

1. Introduction

To develop a successful protocol for *Agrobacterium*-mediated transformation in plants it is essential to determine the virulence of strain(s) of *A. tumefaciens* and *A. rhizogenes* to the plant species concerned (Machado *et al.*, 1997). A high degree of virulence means that the plants were more susceptible to *Agrobacterium* infection and hence this improved transformation efficiency. Some *A. tumefaciens* or *A. rhizogenes* strains are more virulent on explants from certain plant species than on others (Draper *et al.*, 1988). The isolation of super virulent strains of *A. tumefaciens* and *A. rhizogenes* can facilitate the increased production of transform plants containing novel gene(s) (Mohiuddin *et al.*, 2000, Marinangeli *et al.*, 2006).

The crown gall and hairy root diseases are produced by the specific action of the bacteria *A. tumefaciens* (Smith and Townsend, 1907) and *A. rhizogenes* (Hopkins and Durbin, 1971),

respectively on plant explants. A crown gall is produced by wild-type *A. tumefaciens* in all situations where the Ti (Tumour inducing) plasmid vector has virulence (*vir*) and oncogenic (*onc*) genes and hairy roots are produced by wild type *A. rhizogenes* strains containing the Ri (root inducing) plasmid (Draper *et al.*, 1988). A part of the Ti and Ri plasmids called the T-DNA, is transferred into the plant cell during the transformation process and is integrated into chromosome and maintained in the tumour as well as in hairy root tissue in the absence of the bacterium (Chilton *et al.*, 1977).

Both *Agrobacterium* species are unable to transfer its T-DNA to all plant species due to its host limitation traits, but fortunately, they can transfer T-DNA to cucumber plants (De-Cleene and De-Lay, 1976; 1981), oil-seed crops (James, 2004) lentil (Hassan *et al.*, 2007) and jute (Sarker *et al.*, 2008). For the last few years work on some *Agrobacterium* infection work has been carried out on cucumber species (Matsumoto *et al.*, 1986) but sometimes they showed poor response to the recalcitrant cucumber cultivars. In this investigation, a susceptibility test was carried out through the formation of crown galls and hairy roots that improved the virulent response of different recalcitrant *Agrobacterium* strains in cucumber plants using some natural (physical) and chemical factors, which will make a prime base for cucumber transformation through genetic manipulation techniques.

2. Materials and Methods

2.1. Germination of seedlings

Seeds of the cucumber cultivar Spring Swallow (SS) used for this study were soaked in distilled water for approximately 10-20 minutes prior to manual removal of the testa. The peeled seeds (with endotesta) were treated with 70% denatured ethanol for one minute, and then surface sterilised by a solution of 20% (v/v) Clorox, a commercial bleach containing 5.25% sodium hypochlorite, with a dose of 6 drops (per liter) of Tween 20 for 15 minutes. Seeds were then washed extensively, 3-4 times, with sterile water. Healthy and unwrinkled seeds were used for germination (one seed per box) aseptically in Magenta boxes (GA 7, 110-mm height) on hormone free MS (Murashige and Skoog, 1962) full strength basal medium supplemented with 2% phytagel. The medium was adjusted to pH 5.7 before autoclaving at 121°C for 15 min at 1.05 kg/cm^{-2} pressure (15-20 psi). Magenta boxes were sealed with semi-transparent and moisture-resistant Parafilm^{R}. The seeds were then incubated in an incubation chamber at 24±1°C under 16h photoperiod of 2500-lux light intensity provided by cool-white fluorescent and incandescent lights.

2.2. Medium for agrobacterium culture

LB (Luria-Bertani) medium (broth) containing 1% Bacto-peptone, 0.5% Bacto-yeast extract and 1% NaCl (w/v) was used for *A. tumefaciens* culture. Similarly, for *A. rhizogenes* YMB medium (Yeast Mannitol Broth) containing K_2HPO_4, $MgSO_4.7H_2O$, NaCl, yeast extract and mannitol were used. Both the media were adjusted to pH 7.2 before autoclaving at 121°C for 15 min at 1.05 kg/cm^{-2} pressure (15-20 psi). For YMB medium-mixture (i) K_2HPO_4, NaCl, yeast extract and mannitol and mixture (ii) $MgSO_4.7H_2O$ were autoclaved separately. Five strains of both tumorigenic *A. tumefaciens* and oncogenic *A. rhizogenes* were cultured with the LB and YMB media, respectively and agitated at 150-175 rpm overnight at 27°C (Mihaljevic *et al.*, 1996).

2.3. Agrobacterium strains

Five tumorigenic *A. tumefaciens* strains e.g. LBA4404, A737, C58, A348 & A281 and five oncogenic *A. rhizogenes* strains e.g. 9402, 15834, A4, 8196 and A105 (Rubber Research Institute, Malaysia) were used for susceptibility test through inoculation of explants of cucumber, cv. Spring Swallow.

2.4. Explants and inoculation

Stem and petiole explants of three week-old cucumber plants (20 in each) were inoculated with each strain of both *Agrobacterium* species. Firstly, aseptically grown cucumber explants were wounded by a sterile scalpel blade and subsequently, the wound sites were inoculated

with freshly prepared *A. tumefaciens* and *A. rhizogenes* (mentioned above, OD=0.6) using a sterile needle. Each of 20 cucumber plants were inoculated with each *Agrobacterium* strain.

2.5. Incubation conditions

Inoculated plants were pre-incubated for two days in a dark chamber at 24±1°C for equal growth (Trulson and Shahin, 1986) and subsequently transferred into a culture room maintained at same temperature with 16h light (2500-lux)/8h dark regime. Two sets of same number of explants (20 in each) inoculated by each *Agrobacterium* strain were incubated separately in the same culture room with two different light intensity e.g. 3500-lux and 1500-lux. Those explants, which initiated callus from the infected sites but did not form crown galls or hairy roots were also incubated under 16 h photoperiod with 3500-lux and 1500-lux light intensity. Three weeks later, crown galls and hairy roots were separated from infected plants and cultured onto both hormone free MS medium containing 500 mg/l cefotaxime and MS medium containing 0.1 and 0.3 mg/l 2,4-D in combination with 500 mg/l cefotaxime. The cultures were incubated in the same culture room under 16 h light (2500-lux)/8 h dark regime. There were 20 replicates for each experiment.

2.6. Dark incubation

Inoculated explants, which initiated callus from the infected sites but did not form crown galls or hairy roots were incubated under continuous dark condition at 24±1°C.

2.7. Acetosyringone concentrations

Both *A. tumefaciens* and *A. rhizogenes* strains were further cultured into LB and YMB media, respectively, and three different concentrations of acetosyringone e.g. 20, 40 and 100 µM were added separately to the media one hour before inoculation. The stem and petiole explants of cucumber were then inoculated by the *Agrobacterium* strains, grown in media containing acetosyringone, and consequently incubated at 24±1°C under 16 h (2500-lux)/8h light/dark regime. Each treatment had 20 replications

2.8. Statistical analysis

A completely randomised design (CRD) was used for all the experiments. Descriptive statistics such as mean and standard error were used for percentage of crown gall and hairy root induction as well as the average number of hairy roots produced from petiole and stem explants of cucumber species.

3. Results and Discussion

3.1. Effect of A. tumefaciens strains on crown-gall induction

Crown galls were produced from both stem and petiole portions of the cucumber plants at 2500-lux after inoculation by different strains of *A. tumefaciens*. Most of the *A. tumefaciens* strains were capable of inducing crown galls from cucumber plants. However, type of *A. tumefaciens* strains influenced the efficiency of crown gall induction (Table 1). Stems were found more susceptible to *A. tumefaciens* than petioles. These results suggest that the susceptibility test of *A. tumefaciens* strains in the form of crown gall production to cucumber somatic cells is strongly affected by several factors like the strains of *A. tumefaciens*, tissue (explant) type and tissue-*Agrobacterium* interaction. Similar affects have been observed in other plant species; e.g. eucalyptus (Machado *et al.*, 1997); wormwood (Nin *et al.*, 1997) and lentil (Hassan et al., 2007).

Most of the cucumber seedlings inoculated with virulent strains of *A. tumefaciens* developed callus growth at the site of infection within 7 to 8 days, and crown gall development from the callus was initiated between 2 to 3 days later. Virtually all stem explants of cucumber inoculated in the present study with LBA4404, A373 and C58 strains initiated callus, and among those that initiated callus, all developed crown galls. Only A281 and A348 strains failed to induce crown galls in cucumber plants, which died within 1 to 2 weeks of infection (Table 1). It is possible that probably these strains were not virulent to cucumber plants and died due to lack of sufficient nutrients in the medium.

Table 1. Strain-dependent variation in percent crown gall and hairy root induction on stem and petiole explants of cucumber.

Agrobacterium tumefaciens strains	% of explants produced crown galls±SE (2500-lux)		*Agrobacterium rhizogenes* strains	% of explants produced hairy roots±SE (2500-lux)	
	stem	petiole		stem	petiole
LBA4404	55±0.4	45±0.3	8196	20±0.6	10±0.5
A737	45±0.4	40±0.4	15834	20±0.6	15±0.6
C58	30±0.3	30±0.3	9402	5±0.4	10±0.5
A348	0	0	A4	0	5±0.4
A281	0	0	A105	15±0.6	10±0.4

Adventitious crown galls were formed in 30 to 55% of cucumber explants (Table 1). Among the five strains of *A. tumefaciens*, the strain LBA4404 was highly infectious to explants of cucumber. This strain was capable of inducing crown galls (Fig. 1a) from 55% and 45% of infected stem and petiole explants of cucumber, respectively (Table 1). On the other hand, the strains A737 and C58 were found less infectious to cucumber explants therefore these two strains are less suitable to cucumber for virulent study. This variation in virulence may be due to host range limitation. Analogous works on crown gall induction by various strains of *A. tumefaciens* were reported by other workers (Machado *et al.*, 1997; Unger *et al.*, 1985; Smarrelli *et al.*, 1986). Different rates of crown gall induction obtained from the same explant of cucumber after inoculation with different *A. tumefaciens* strains suggests that the rates of tumorigenicity are dependent on the strains of *Agrobacterium*. Higher rates of crown galls were induced from the stems of cucumber with different *A. tumefaciens* strains compared to petioles of cucumber (Table 1). This demonstrates that the stem of cucumber is more susceptible to *A. tumefaciens* infection, therefore, stem explant could be used for future gene transformation. Katavic *et al*. (1991) found similar host tissue differences in pumpkin by *Agrobacterium* with regards to susceptibility.

3.2. Effect of A. rhizogenes strains on hairy root induction

Hairy roots were produced from infected sites of both cucumber stem and petiole explants by *A. rhizogenes*. Strains 8196, 15834 and A105 of *A. rhizogenes* were found to be more infective with the stem of cucumber (Table 1). Strain A4 is only infective with cucumber petioles. These findings suggest that phenotypic expression of *A. rhizogenes* in the form of hairy root initiation is strongly affected by *A. rhizogenes* strain, explant type as well as tissue-*Agrobacterium* interaction. Similar results have also been reported in *Cucumis* and pine tree when infected with *A. rhizogenes* strains (Katavic *et al.*, 1991, Mihaljevic *et al.*, 1996; McAfee *et al.*, 1993).

Different strains of *A. rhizogenes* influenced both the efficiency of initiation and production of different number of hairy roots from cucumber explants. Most of the cucumber explants inoculated with virulent strains of *A. rhizogenes* also developed callus growth at the site of infection within 7 to 8 days, and hairy root development from the callus was initiated 3 to 4 days later. Virtually all stem explants of cucumber inoculated with strain 8196 initiated callus, and among those that initiated callus all developed hairy roots. On the other hand, strains 15834, 9402, A4 and A105 initiated callus at infection sites of stem and petiole explants and strain 8196 initiated callus at infection sites of petioles and among those that initiated callus, a few of them consequently developed hairy roots (Table 1). Some of the cucumber plants died within 2 to 3 weeks after infection with *A. rhizogenes* strains. It is possible that these strains are not virulent to cucumber plant species and died due to insufficient nutrients in the medium.

Adventitious hairy roots were formed in 5 to 20% of cucumber explants (Table 1). The strain 8196 was capable of inducing hairy roots from 20% stem and 10% of petiole explants. *A. rhizogenes* strain 15834, 9402 and A105 induced hairy roots from 20%, 5% and 15% of stem explants, respectively. Similarly, the same strains also produced hairy roots from 15%, 10% and 10% of petiole explants, respectively (Table 1). It is important to point out that stem explants of cucumber were more susceptible to some *A. rhizogenesis* strains, whereas petioles were more susceptible to the other strains. It is not clear yet why this discrepancy for hairy root induction exists, however it could be due to some form of interaction between the *A. rhizogenes* strain and the explant types.

Different rates of hairy root induction obtained from the same explant type of cucumber after inoculation with different *A. rhizogenes* strains suggest that the rates of rhizogenesis are dependent on the strains of *Agrobacteria*. Strain A4, on the other hand, did not show any response to stems of cucumber, however, this strain produced hairy roots from the lowest number of cucumber petiole (5%). This variation in virulence may be due to host range limitation. Several workers reported hairy root induction by various strains of *A. rhizogenes* (Katavic *et al.*, 1991; Smarrelli *et al*., 1986).

The time period that was needed to produce callus, crown galls and hairy roots from cucumber explants from both *A. tumefaciens* and *A. rhizogenes* infection sites was almost the same. It may be due to the fact that a specific time period is required for the transfer of T-DNA from both *A. tumefaciens* and *A. rhizogenes* to the cucumber plant cells. Matsumoto *et al.*, (1986) also reported that time period is required to form galls from the same species.

3.3. Effect of light intensity on crown-gall and hairy root induction

Crown galls as well as hairy roots were produced from both stem and petiole portions of cucumber plants at 2500-lux light intensity (Table 1). However the other two different light intensities (1500 and 3500-lux) used, were not effective to induce crown galls and hairy roots neither from stem nor from petiole explants of cucumber (data not shown). The maximum number of crown galls was initiated from the both explants at the light intensity of 2500-lux (Table 1). Similarly, promotive effect on the production of higher number of hairy roots was shown at the same light intensity (Table 2). No promotive effects of light intensity, 1500 and 3500-lux were observed on the production of hairy roots (data not shown). These findings indicated that physical factor, 2500-lux light intensity, is critical to form crown galls and hairy roots from infected spot of cucumber.

Table 2. Strain-dependent variation in numbers of hairy root induction on stem and petiole explants of cucumber.

Agrobacterium rhizogenes strains	Average number of hairy roots induced per explant±SE (2500-lux)		Average number of hairy roots induced per explant±SE (dark)	
	stem	Petiole	stem	petiole
8196	5.0±0.6	4.0±0.0	0	0
15834	4.7±0.3	4.3±0.3	3.5±0.2	3.0±0.3
9402	5.0±0.0	3.5±0.5	0	0
A4	0	4.0±0.0	0	0
A105	3.7±0.3	4.5±0.5	0	0

3.4. Effect of dark incubation

Inoculated cucumber plants when incubated under continuous dark conditions did not produce any crown galls although some callus were produced at the infection sites. Similarly, lower number of cucumber explants produced hairy roots under dark conditions (strain 15834 only) whereas, higher numbers of explants produced hairy roots under 16 h light (2500-lux)/8 h dark regime (Table 2). Cucumber plants infected by only *A. rhizogenes* strain 15834 produced hairy roots from stem and petiole explants with an average of 3.5 and 3.0 hairy roots per stem and petiole explants, respectively. The other strains did not produce hairy roots from explants under the dark conditions (Table 2). It indicated that the dark incubation plays a negative role on crown gall and hairy root production.

3.5. Effect of acetosyringone on crown gall formation

The crown gall induction rate was enhanced from inoculated stem and petiole explants when explants were infected with different strains of *A. tumefaciens*, which had been grown in LB medium containing 20 μM of acetosyringone (Table 3). Strain C58 grown in medium containing 40 μM of acetosyringone also increased gall induction rates from both stem (35%) and petiole (35%) explants of cucumber (Table 3) in comparison to the respective controls (e.g. 30% and 30%) (Table 1). It indicates that addition of specific concentration of acetosyringone has potential role in enhancement of the crown gall production.

Some *A. tumefaciens* strains, A348 and A281, which were unable to form crown galls from infected explants, were able to induce crown galls when grown with acetosyringone (Table 3). With the strain A281 only 20 μM acetosyringone induced crown galls from 10% of infected stem explants of cucumber (Table 3) but without acetosyringone it showed no gall induction (Table 1). However, the use of 40 and 100 μM of acetosyringone did not result in induction of crown galls. On the other hand, the strain A348 grown with 20 and 40 μM acetosyringone induced crown galls from 20% and 10% infected stem explants, respectively whereas, a 100 μM of acetosyringone did not induce any crown gall (Table 3). These findings indicate that specific concentration of acetosyringone induced virulent response of recalcitrant *A. tumefaciens* strain that phenotypically express crown gall production at infected sites of cucumber.

All of the *A. tumefaciens* strains grown in medium containing the three different concentrations of acetosyringone induced crown galls with petiole explants of cucumber. Among the three-acetosyringone concentrations used, 20 μM, was found to be the most effective one in enhancing crown gall induction from both the explants in comparison to the higher concentrations (40 and 100 μM) (Table 3). On the other hand, very poor response observed with 10 μM of acetosyringone compared to the three other concentrations studied (data not shown). These results clearly pointed out that 20 μM of acetosyringone is highly effective as chemical factor in inducing crown galls from cucumber explants suggesting that this concentration may promote the high infectivity of *A. tumefaciens*. Furthermore, this chemical factor would be a crucial additive in future gene transformation in cucumber. The results obtained with the highest concentrations of acetosyringone (100 μM) indicate an inhibitory action rather than stimulatory action on the induction of crown galls.

A concentration of 20 μM acetosyringone promoted crown galls production from the most stem and petioles of cucumber e.g. 65% and 60%, respectively when used in conjunction with LBA4404 (Table 3). The two other concentrations of acetosyringone reduced crown gall production from both explants. The rates of crown gall production from both explants by four other *A. tumefaciens* strains grown in LB medium containing 20, 40 and 100 μM of acetosyringone were lower in comparison to the LBA4404. It specifies that interaction between specific concentration of acetosyringone and *A. tumefaciens* strain played vital role in enhancement of virulence response.

Table 3. Effect of acetosyringone on induction of crown galls in stem and petiole explants of cucumber.

Agrobacterium tumefaciens strains	% of explants produced crown galls in stem±SE			% of explants produced crown galls in petiole±SE		
	20 μM	40 μM	100 μM	20 μM	40 μM	100 μM
LBA4404	65±0.5	40±0.5	25±0.4	60±0.6	40±0.4	30±0.4
A737	50±0.6	40±0.5	25±0.5	50±0.6	35±0.5	25±0.5
C58	45±0.4	35±0.4	20±0.3	40±0.5	35±0.5	30±0.4
A348	20±0.4	10±0.2	0	25±0.4	15±0.3	5±0.3
A281	10±0.2	0	0	15±0.2	10±0.2	5±0.2

3.6. Effect of acetosyringone on hairy root induction

The rates of production as well as numbers of hairy roots produced per plant were also enhanced from inoculated explants (stem and petiole) when infected with *A. rhizogenes* strains which had been grown in YMB medium containing 20 μM acetosyringone. Strains 8196, 15834, 9402 and A105 grown in medium containing 40 μM of acetosyringone also showed an increased hairy root induction rate from stems of cucumber (Table 4) compared to the respective control *A. rhizogenes* strains (Table 1). In addition, the strains 8196, 15834, 9402, A4 and A105, grown in medium containing 40 μM of acetosyringone also had increased ability on hairy root induction from petiole explants (Table 4) compared to the rates obtained from same explant infected by the above mentioned control strains (without acetosyringone) (Table 1). These findings indicate that addition of specific concentration of acetosyringone not only promoted crown gall production, but also enhanced the hairy root induction in cucumber.

A. rhizogenes strain A4 that was unable to induce hairy roots from infected stems of cucumber was able to produce hairy roots when explants were inoculated with this strain grown in the presence of acetosyringone at 20 and 40 μM (Table 4). This findings state that the recalcitrant *A. rhizogenes* strain can achieve virulence potentiality to infect cucumber in the presence of acetosyringone. All other strains grown with 100 μM of acetosyringone produced hairy roots from a lower number of inoculated cucumber explants (Table 4) show an inhibitory action on hairy root induction in cucumber.

A concentration of 20 μM acetosyringone added with strain 8196 was sufficient to induce hairy roots from the maximum numbers of inoculated sites on cucumber stem (45%). Similarly, the same concentration of acetosyringone (20 μM) added to 15834 induced hairy roots from the maximum number of inoculated sites of cucumber petiole (35%) (Table 4). The same concentration of acetosyringone added to the other strains of *A. rhizogenes* 15834, 9402, A4 and A105 also induced hairy roots from stem however at lower rates compare to 8196. Similarly, 20 μM of acetosyringone added to the strain 8196, 9402, A4 and A105 induced lower rates of hairy roots from petioles compare to 15834. On the other hand, acetosyringone at 40 and 100 μM added to the same *A. rhizogenes* strains induced lower rates of hairy roots from both stem and petiole explants. Moreover, very poor response observed in hairy root induction with 10 μM of acetosyringone compare to three other concentrations studied (data not shown). From these findings it is suggested that 20 μM acetosyringone is optimal for enhancement of virulence response of Agrobacteria to cucumber.

a **b** **c** **d**

Fig. 1. *In vitro* susceptibility of *Agrobacterium* to cucumber explants, stem and petiole. (a) Crown galls induced at the infected site. (b) Hairy roots induced from infected stem. (c) Hairy roots elongated with profuse branches on hormone free MS medium containing cefatoxime. (d) Root elongation stopped in presence of 2,4-D.

Table 4. Effect of acetosyringone on induction of hairy roots in stem and petiole explants of cucumber.

Agrobacterium rhizogenes strains	% of explants produced hairy roots in stem±SE			% of explants produced hairy roots in petiole±SE		
	20 µM	40 µM	100 µM	20 µM	40 µM	100 µM
8196	45±0.5	35±0.4	15±0.2	25±0.5	15±0.4	5±0.2
15834	40±0.4	30±0.3	15±0.2	35±0.5	20±0.4	10±0.3
9402	20±0.3	15±0.2	5±0.1	25±0.5	15±0.3	5±0.2
A4	20±0.3	10±0.2	0	15±0.4	10±0.3	5±0.2
A105	30±0.4	20±0.3	10±0.2	30±0.5	15±0.4	5±0.2

Table 5. Effect of acetosyringone on number of hairy roots induced in stem and petiole explants of cucumber.

Agrobacterium rhizogenes strains	Average number of hairy roots induced per stem explant±SE			Average number of hairy roots induced per petiole explant±SE		
	20 µM	40 µM	100 µM	20 µM	40 µM	100 µM
8196	6.4±0.3	4.8±0.2	3.2±0.3	5.2±0.3	4.3±0.2	3.0±0.0
15834	6.1±0.3	4.3±0.4	3.3±0.2	5.6±0.3	4.8±0.4	3.4±0.0
9402	5.5±0.4	4.3±0.3	3.9±0.2	4.7±0.3	3.6±0.3	3.0±0.3
A4	4.0±0.3	3.5±0.4	0	5.2±0.2	4.4±0.3	3.0±0.0
A105	4.6±0.2	3.5±0.2	2.3±0.4	5.2±0.3	4.6±0.4	4.0±0.0

The maximum number of hairy roots 8.0 (average 6.4), was initiated from stem explants of cucumber (Fig. 1b) by inoculation with *A. rhizogenes* strain 8196 grown with 20 μM of acetosyringone (Table 5). However, the *A. rhizogenes* strain 15834 grown with 20 μM acetosyringone produced the maximum average numbers of hairy roots (5.6) from petiole explants of cucumber (Table 5). The above number of hairy roots was higher than control (acetosyringone not added to *A. rhizogenes* culture medium) (Table 1). The other strains grown with 40 and 100 μM of acetosyringone produced lower number of hairy roots from stem and petiole explants (Table 5). On average, the lowest numbers of hairy roots were produced from stem and petiole explants of cucumber inoculated with A4 and 9402 strains, respectively grown with any of the three different concentrations of acetosyringone (Table 5). These results clearly point out that 20 μM of acetosyringone is highly effective at inducing hairy roots from cucumber explants suggesting that this concentration may promote the high infectivity of *A. rhizogenes*. However, a high concentration of acetosyringone (40 μM) also promoted high virulence of A4 to cucumber stems only. The results obtained with the highest concentrations of acetosyringone (100 μM) indicate an inhibitory action rather than stimulatory action on the induction of hairy roots.

The hairy roots induced from both explants of cucumber elongated with profuse branches on hormone free MS medium containing cefatoxime (Fig. 1c) however, on MS medium with 2,4-D the roots initiated callus, which did not grow further (Fig. 1d). Similar result was also shown in crown gall. Crown gall and hairy roots enlarged on hormone free MS medium whereas, enlargement stopped on MS medium containing 2,4-D which confirm that these crown gall and hairy root tissue have been transformed by T-DNA and can synthesise hormones that play an accessory role in root and gall induction. The hormone synthesis genes are found within the T-DNA of agrobacteria (White *et al.*, 1985) and any external hormone(s) may be deleterious to this process.

4. Conclusions

The present study confirmed that physical and chemical factors particularly specific light intensity, 2500-lux, as well as the acetosyringone concentration, 20-μM, played important role in enhancement of virulence response in agrobacteria. Application of these factors in future gene transformation study will facilitate the production of transformed plants with novel genes.

5. Acknowledgement

The authors express their sincere thanks to the Ministry of Science, Technology and the Environment of Malaysia for financial support in the form of Graduate Assistantship (IRPA grant No UPM-51267) to accomplish this study.

References

Chilton, M.-D., Drummond, H. J., Merlo, D. J., Sciaky, D., Montoya, A. L., Gordon, M. P. and Nester, E. W. 1977. Stable incorporation of plasmid DNA into higher plant cells: The molecular basis of crown gall tumourgenesis. *Cell*, 11: 263-271.

De Cleene, M. and De Ley, J. 1976. The host range of crown galls. *Botanical Review*, 42: 389-466.

De Cleene, M. and De Ley, J. 1981. The host range of infectious hairy root. *Botanical Review*, 47: 147-194.

Draper, J., Scott, R. and Hamil, J. 1988. Transformation of dicotyledonous plant cells using the Ti plasmid of *Agrobacterium tumefaciens* and the Ri plasmid of *A. rhizogenes*. In: *Plant genetic transformation and gene expression*, (Eds.) J. Draper, R. Scott, and P. Armitage. Blackwell Scientific Publications, Oxford, UK, 69-160 pp.

Hassan, F., Hoque, M. I., Kiesecker, H. and Jacobsen, H.-J. 2007. Transient GUS expression in decapitated lentil embryos. *Plant Tissue Culture and Biotechnology*, 17: 97-102.

Hopkins, D. L. and Durbin, R. D. 1971. Induction of adventitious roots by culture filtrates of the hairy root bacterium, *Agrobacterium rhizogenes*. *Canadian Journal Microbiology*, 17: 1409-1412.

James, C. 2004. Global status of commercialized transgenic crops. *ISAAA*, Metro Manila, Philippines.

Katavic, V., Jelaska, S., Bakran-Petricioli, T. and David, C. 1991. Host-tissue differences in transformation of pumpkin (*Cucurbita pepo* L.) by *Agrobacterium rhizogenes*. *Plant Cell Tissue Organ Culture*, 24:35-42.

Machado, L. de O. R., Andrade, de G. M., Cid, L. P. B., Penchel, R. M. and Brasileiro, A. C. M. 1997. *Agrobacterium* strain specificity and shoot tumour formation in eucalypt (*Eucalyptus grandis* x *E. urophylla*). *Plant Cell Reports*, 16: 299-303.

Marinangeli, P., Deluchi, B., Rois, R., Franzone, P., Galmarini, C, Rossello, F. and Carvetto, N. 2006. Towards genetic transformation of local onion varieties. *Electronic Journal of Biotechnology*, 9(3):327-339.

Matsumoto, S., Machida, Y. and Takebe, I. 1986. Rapid method for assaying tumorigenicity of *Agrobacterium tumefaciens*. *Plant Molecular Biology Reports*, 4(1): 42-47.

McAfee, B. J., White, E. E., Pelcher, L. E. and Lapp, M. S. 1993. Root induction in pine (*Pinus*) and Larch (*Larix*) spp. Using *Agrobacterium rhizogenes*. *Plant Cell Tissue Organ Culture*, 34:53-62.

Mihaljevic, S., Stipkovic, S., and Jelaska, S. 1996. Increase of root induction in *Pinus nigra* explants using agrobacteria. *Plant Cell Reports*, 15: 610-614.

Mohiuddin, A. K. M., Harikrishna, K., Chowdhury, M. K. U., Abdullah, Z. C. and Napis, S. 2000. Influence of acetosyringone on transformation of cucumber mediated by *Agrobacterium tumeficiens*. *Plant Tissue Culture*, 10(2): 167-174.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologium Plantarum*, 15: 473-497.

Nin, S., Bennici, A., Roselli, G. D., Schiff, M. S. and Magherini, R. 1997. *Agrobacterium*-mediated transformation on *Artemisia absinthium* L. (wormwood) and production of secondary metaborlites. *Plant Cell Reports*, 16: 725-730.

Sarker, R. H., Al-Amin, G. M., Hasan, F. and Hoque M. I. 2008. *Agrobacterium*-mediated genetic transformation of two varieties of jute (*Corchorus capsularies* L.). *Plant Tissue Culture and Biotechnology*, 18: 7-16.

Smarrelli, J. J., Watters, M. T. and Diba, L. H. 1986. Response of various cucurbits to infection by Plasmid-harbouring strains of *Agrobacterium*. *Plant Physiology*, 82: 622-624.

Smith, E. F. and Townsend, C. O. 1907. A plant tumour of bacterial origin. *Science*, 25: 671-673.

Trulson, A. J. and Shahin, E. A. 1986. *In vitro* plant regeneration in the genus Cucumis. *Plant Science*, 47: 35-43.

Unger, L., Ziegler, S. F., Huffman, G. A., Knauf, V. C., Peet, R., Moore, L. W., Gordon, M. P. and Nester, E. W. 1985. New class of limited-host-range *Agrobacterium* mega-tumor-inducing plasmids lacking homology to the transferred DNA of a wide-host-range, tumour inducing plasmid. *Journal of Bacteriology*, 164: 723-730.

White, F. F., Taylor, B. H., Huffman, G. A., Gordon, M. P. and Nester, E. W. 1985. Molecular and genetic analysis of the transferred DNA region of the root-inducing plasmid of *Agrobacterium rhizogenes*. *Journal of Bacteriology*, 164:33-44.