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Enhanced Virulence Gene Activity of *Agrobacterium* in Muskmelon (*Cucumis melo* L.) cv. 'Birdie'

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Abstract

Muskmelon (*Cucumis melo* L.) cultivar 'Birdie', was evaluated for its response to the tumorigenic *Agrobacterium tumefaciens* and the oncogenic *A. rhizogenes* strains. Stem and petiole of three week-old *in vitro*-grown muskmelon plants were inoculated with five strains of *A. tumefaciens* and *A. rhizogenes* each and observed phenotypic expressions i.e. induction of crown galls and hairy roots. This phenotypic expression was efficaciously increased when virulence gene activity of different strains of two *Agrobacterium* species was enhanced. Intensive studies on enhancement of virulence gene activity of *Agrobacterium* found to be correlated to the appropriate light intensity (39.3 µmol m^{-2} s^{-1}) with a specific concentration of monocyclic phenolic compound, acetosyringone (20 µM). The gene activity was also influenced by several other physical factors e.g. plant tissue type, *Agrobacterium* species and their strains, and plant tissue-*Agrobacterium* interaction. Among the different *A. tumefaciens* strains, LBA4404 showed the best virulence gene activity in both stem and petiole through the formation of higher rate of crown galls. On the other hand, strain 15834 of *A. rhizogenes* showed better gene activity in stem and 8196 in petiole through the formation of higher rate of hairy roots as well as higher average number of hairy roots. Among the two different types of explants, petiole was more susceptible to both *Agrobacterium* species. Thus it was concluded that future muskmelon transformation study can efficiently be carried out with LBA4404, 15834 and 8196 strains using petiole explants by adding 20 µM of acetosyringone in the medium.

Keywords: *Agrobacterium*, Muskmelon, tumorigenic and oncogenic , virulence gene, wild type

Introduction

Both *Agrobacterium tumefaciens* and *A. rhizogenes* are soil-borne pathogenic bacteria. They are able to transfer their plasmid-encoded genes (T-DNA) and virulence (*vir*) proteins into plant cells (Hodges *et al*., 2004). The T-DNA of *Agrobacterium* is stably integrated in the plant genomes and expressed that eventually causes crown gall or hairy root diseases (Hodges *et al*., 2009) and it is maintained in the plant cells in the absence of bacteria (Chilton *et al*., 1977). This novel method is utilized as an efficient and excellent technique in plant genetic transformation study in many laboratories (Boyko *et al*., 2009).

Success in plant genetic transformation through *Agrobacterium*-mediated method depends on the virulence of strain(s) of *A. tumefaciens* and *A. rhizogenes* to a particular plant species concern (Machado *et al*., 1997). In many cases, *Agrobacterium* is found to be less virulent or less susceptible to infection of a particular plant species or explant indicating poor transformation efficiency or no transformation (Mohiuddin *et al*., 2009). A high degree of virulence means that plants are more susceptible to infection and totipotency of infectious cells improves transformation efficiency to produce transgenic plants (Boyko *et al.,* 2009). Many *A. tumefaciens* and *A. rhizogenes* strains are more virulent in transgene integration on explants from some plant species than on others (Draper *et al*., 1988). Therefore, the isolation of virulent or super virulent strains of *A. tumefaciens* and *A. rhizogenes* and their improvement of virulence gene activity study is important to facilitate the transformation in recalcitrant plant species containing biotic and abiotic stress tolerant gene(s).

The crown gall and hairy root diseases are produced by the specific action of integration and expression of the bacterial gene in plant genomes (Hodges *et al*., 2009). A crown gall is produced in plants by the wild-type *A. tumefaciens* in all situations where the transferred Ti (Tumour inducing) plasmid vector has virulence (*vir*) and oncogen-

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ic (*onc*) genes and hairy roots are produced by wild type *A. rhizogenes* strains containing the Ri (root inducing) plasmid (Drapper *et al*., 1988; Smith and Townsend, 1907; Hopkins and Durbin, 1971). Host limitation is a drawback of both *Agrobacterium* species hence unable to transfer its T-DNA to all plant species concern (Cleene and Ley, 1976, 1981); however, it can be overcome through the application of chemical compounds, results in activation of *vir* gene leading to the processing of its T-DNA from the Ti-plasmid and transfer to recipient host cells (Gelvin, 2006). For the last few years some *Agrobacterium* infection works have been carried out on muskmelon (Matsumoto *et al*., 1986) and also in many other plant species i.e. lotus (Jian *et al*., 2009), *Brassica sp.* (Kong *et al*., 2009) and garlic (Kenel *et al*., 2010). The present study was carried out to enhance the *vir* gene activity of different wild type *Agrobacterium* species through the application of chemical compounds along with physical factors and evaluate the *vir* gene activity through the phenotypic expression i.e. formation of crown galls or hairy roots on muskmelon cultivar 'Birdie'.

Materials and methods

Seedling germination

Seeds of the muskmelon (*Cucumis melo* L.) cultivar 'Birdie' (Sakata Seed Corporation, Japan) were used for this present study. Seeds were soaked in distilled water for approximately 10-20 minutes and the testa removed manually. The decoated 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 6 drops (per liter) of Tween 20 [polyoxyethylene (20) sorbitan monolaurate, Sigma, USA] for 15 minutes (Tabei *et al*., 1991). Seeds were then washed 3-4 times, with sterile distilled water.

Healthy and unwrinkled seeds were sorted and germinated (one seed per box) aseptically in Magenta boxes (GA 7, 110-mm height, USA) on hormone free MS (Murashige and Skoog, 1962) full strength basal medium (MSO) supplemented with 2% phytagel (Sigma, USA). 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} (Sigma, USA). The seeds were incubated for one day at 26/27°C in a dark chamber to ensure uniform and rapid germination (Trulson and Shahin, 1986). The germinated seeds were subsequently transferred to an incubation room at 24±1°C for elongation under 16 h photoperiod of 39.3 µmol m^{-2} s^{-1} light intensity provided by cool-white fluorescent and incandescent lights.

Media for Agrobacterium culture

LB (Luria-Bertani) medium (broth) was prepared using 1% Bacto-peptone, 0.5% Bacto-yeast extract and 1% NaCl (w/v) for wild type *A. tumefaciens* culture. Similarly, for *A. rhizogenes* YMB medium (Yeast Mannitol Broth) containing K_2HPO_4, $2H_2O$ (0.5 g/l), $MgSO_4$, $7H_2O$ (0.2 g/l), NaCl (0.1 g/l) yeast extract (0.4 g/l) and mannitol (10.0 g/l) was prepared. The both 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 mixture (ii) $MgSO_4$, $7H_2O$ were autoclaved separately. Five tumorigenic strains of *A. tumefaciens* and five oncogenic strains of *A. rhizogenes* were cultured in tubes with freshly prepared LB and YMB media, respectively and agitated at 150-175 rpm overnight at 27°C (Mihaljevic *et al*., 1996). The optimal density (OD) of each overnight-cultured *Agrobacterium* strain was monitored by UV spectrophotometer and selected as 0.6 for inoculation.

Agrobacterium strains

Five tumorigenic *A. tumefaciens* strains e.g. LBA4404, A737, C58, A348, and A281 and five oncogenic *A. rhizogenes* strains e.g. 9402, 15834, A4, 8196 and A105, collected from Rubber Research Institute of Malaysia, were used for susceptibility test and increase their virulence (*vir*) gene activity through inoculation of seedlings of muskmelon cv. 'Birdie'.

Muskmelon tissue and inoculation

Stem and petiole of three week-old *in vitro*-grown muskmelon plants (20 in each) were inoculated with each strain of *A. tumefaciens* and *A. rhizogenes*. Stem and petiole of each plant were aseptically wounded by a sterile scalpel blade and the wound sites were inoculated with freshly prepared (tumorigenic and oncogenic) *A. tumefaciens* and *A. rhizogenes* strains, respectively, using a sterile needle. Twenty plants of muskmelon were inoculated with each *Agrobacterium* strain (OD=0.6) for each explants (stem and petiole) in each treatment. A total of 600 plants (20 plants x 10 strains x 3 treatments) were inoculated

Incubation conditions

Inoculated plants were pre-incubated for two days in a dark chamber at 24±1°C for uniform growth (Trulson and Shahin, 1986) and then 200 plants (20 plants x 10 strains) transferred into a culture room maintained at same temperature with 16 h light (39.3 µmol m^{-2} s^{-1})/8 h dark treatment. Two sets of 200 plants (20 plants x 10 strains) were incubated into the same culture room with two different light intensity e.g. 55.0 µmol m^{-2} s^{-1} and 23.6 µmol m^{-2} s^{-1} provided by cool-white fluorescent and incandescent lamps. A subset of those explants which initiated callus from the infected sites but had not formed crown galls or hairy roots at 39.3 µmol m^{-2} s^{-1}, were also incubated under 16 h photoperiod with 55.0 µmol m^{-2} s^{-1} and 23.6 µmol m^{-2} s^{-1} 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 (39.3 µmol m^{-2} s^{-1})/8 h dark treatment. Twenty replicates of crown galls and hairy roots were used for each experiment.

Dark incubation

A subset of those explants which initiated callus from the infected sites after inoculation but had not formed crown galls or hairy roots, were incubated under continuous dark condition at 24±1°C.

Acetosyringone concentrations

Both wild type *A. tumefaciens* and *A. rhizogenes* strains were further cultured into LB and YMB media, respectively, and four different concentrations of acetosyringone e.g. 10, 20, 40 and 100 µM added separately to the media one hour before inoculation. The stem and petiole of muskmelon were then inoculated by the same wild type *Agrobacterium* strains, grown in media containing acetosyringone, and incubated at 24±1°C under 16 h (39.3 µmol m^{-2} s^{-1})/ 8 h light/dark treatment. Twenty replicates were used for each experiment with particular treatment. A total of 800 plants (20 plants x 10 strains x 4 acetosyringone concentrations) were tested.

Statistical analysis

A completely randomised design (CRD) was used for all 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 muskmelon species.

Results

Effect of A. tumefaciens strains on phenotypic expression (crown-gall induction)

Seedlings of muskmelon inoculated with different virulent strains of *A. tumefaciens* in *in vitro* condition developed callus at the site of infection within 7 to 8 days, and crown gall development from the callus was initiated after 2 to 3 days. The crown galls were observed in both stems and petioles at 16/8 h light (39.3 µmol m^{-2} s^{-1})/dark treatment. Stems of muskmelon were found less susceptible than petioles to *A. tumefaciens*. The virulence gene of *A. tumefaciens* strains, LBA4404, A737, C58 and A348 were capable of inducing crown galls in muskmelon plants. However, the strain types of *A. tumefaciens* strains influenced the efficiency of crown gall induction in stem and petiole explants of muskmelon (Fig. 1). Among the *A. tumefaciens* strains tested in this study, only A281 strain failed to induce crown galls in muskmelon plants and they died within few weeks of inoculation. The colour of the muskmelon stem-derived calluses were white (Fig. 12a).

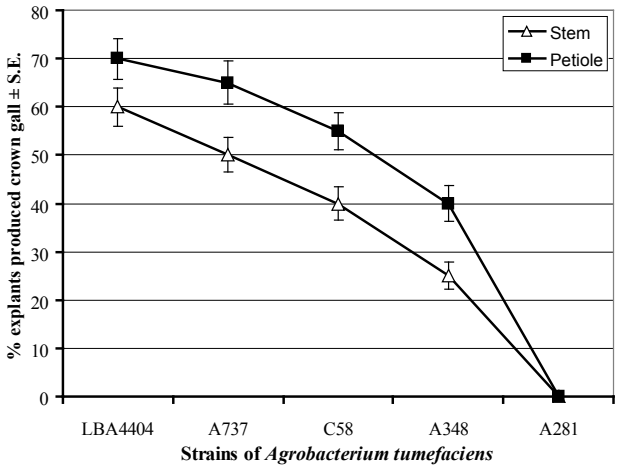

Fig. 1. Strain-dependent variation in crown gall induction on muskmelon explants at 39.3 µmol $m^{-2}s^{-1}$

The best results of crown gall induction were seen in the petiole explants, seeing that the crown galls were formed in 25 to 60% of muskmelon stem and 40 to 70% petiole explants, respectively (Fig. 1). Among the five different strains of *A. tumefaciens* tested in this study, the virulence genes of strain LBA4404 was highly infectious to both explants of muskmelon. This strain was capable of inducing crown galls from 60% and 70% of infected stem and petiole explants of muskmelon, respectively (Fig. 1). On the other hand, the virulence gene of strains A348 and C58 were less infectious to muskmelon explants compared to LBA4404 (Fig. 1).

Effect of A. rhizogenes strains on phenotypic expression (hairy root induction)

All five *A. rhizogenes* strains (8196, 15834, 9402, A4 and A105) tested in this study induced hairy roots production from the infected/inoculated sites of muskmelon (stem and petiole) at 16/8 h light (39.3 µmol m^{-2} s^{-1})/dark treatment. Gene virulence of *A. rhizogenes* strains 8196 and 15834 was more effective in the petioles, whereas 8196, 15834 and 9402 strains were more effective in the stem explants (Fig. 2). Most of the muskmelon stems and petioles inoculated with virulent strains of *A. rhizogenes* 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. The appearance of induced hairy roots from muskmelon stem is illustrated in Fig. 12b.

The virulence gene of all *A. rhizogenes* strains showed phenotypic expression in 15 to 70% of muskmelon explants in the form of adventitious hairy roots formation (Fig. 2). Among the five different strains of *A. rhizogenes* the virulence gene of strain 8196 and 15834 were highly effective to both the stem (Fig. 12c) and petiole of muskmelon. These strains were capable of inducing hairy roots from nearly 65% of stems and petioles. On the other hand, similar level of expression of virulence genes of strain 9402 was observed in stem only. Therefore, the strain 8196 will

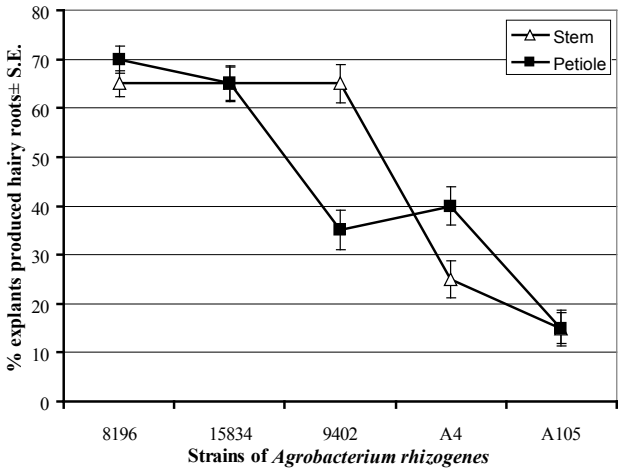

Fig. 2. Strain-dependent variation in hairy root induction on muskmelon explants at 39.3 μmol $m^{-2}s^{-1}$

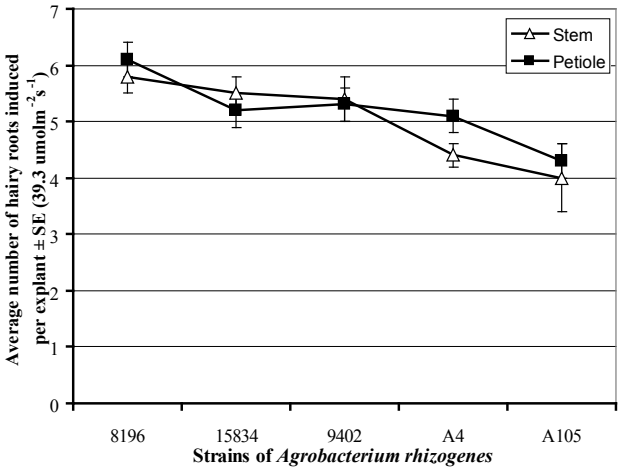

Fig. 3. Strain-dependent variation in number of hairy root induction on muskmelon explants

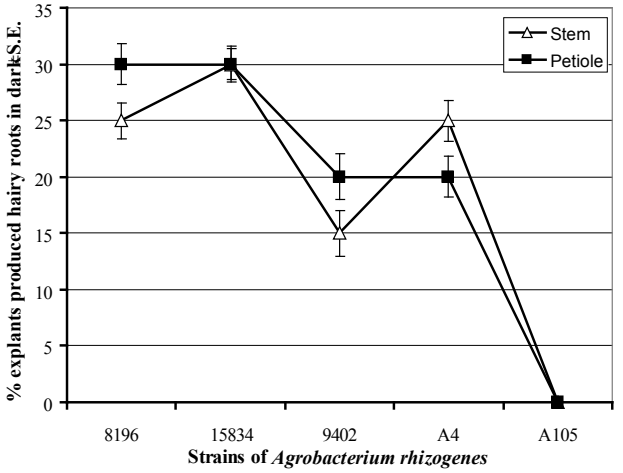

Fig. 4. Strain-dependent variation in hairy root induction in the dark on muskmelon explants

be most suitable for transformation study using petiole explant. Similarly, strain 8196, 15835 and 9402 will be also suitable for transformation study using stem explant. The A105 strain was least effective (15%) in inducing hairy roots in muskmelon stem and petiole (Fig. 2).

The types of *A. rhizogenes* strains used in this study influenced the production of numbers of hairy roots from muskmelon stem or petiole when inoculated at 39.3 μmol $m^{-2}s^{-1}$ (Fig. 3). The strain 8196 was highly virulent and induced an average of 5.8 and 6.1 hairy roots from muskmelon stem and petiole, respectively. The other strains were less virulent and induced lower numbers of hairy roots from stem and petiole explants (Fig. 3). However, the difference among the strains 8196, 15834 and 9402 was not statistically significant.

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

Among the three light intensities treatment tested (23.6 μmol $m^{-2}s^{-1}$, 39.3 μmol $m^{-2}s^{-1}$ and 55.0 μmol $m^{-2}s^{-1}$) only 39.3 μmol $m^{-2}s^{-1}$ was suitable for inducing crown gall or hairy roots from stem and petiole of muskmelon (data not shown).

Effect of dark incubation on crown-gall and hairy root induction

Under continuous dark condition, only two out of five strains of *A. tumefaciens* tested in this study (LBA 4404 and A737) were able to form crown galls in muskmelon. However, the response expressed as percentage was much lower compared to under 16 h light (39.3 μmol $m^{-2}s^{-1}$) 15% in stem and 25% in petiole (data not shown). Moreover, the galls induced under dark conditions were smaller in size in comparison to galls induced under 16 h light (39.3 μmol $m^{-2}s^{-1}$) condition. The other three strains did not produce any crown gall in muskmelon stem or petiole incubated under continuous dark.

Similarly, lower number of muskmelon explants produced hairy roots under dark conditions (Fig. 4) compared to the explants exposed under 16 h light (39.3 μmol $m^{-2}s^{-1}$) incubation (Fig. 2).

The A105 strain of *A. rhizogenes* failed to produce hairy roots when incubated under dark condition (Fig. 4, 5). Although the other four *A. rhizogenes* strains 8196, 15834, 9402 and A4 produced hairy roots in muskmelon stem and petiole, their response rate were less than half of that was observed under under 16 h light (39.3 μmol $m^{-2}s^{-1}$). Moreover, the number of hairy roots produced by the four strains in the dark condition was approximately 25% leass than that of under 16 h light (39.3 μmol $m^{-2}s^{-1}$) condition (Fig. 5).

Effect of acetosyringone on crown gall formation

Although the *A. tumefaciens* strain A281 failed to induce crown gall in stem and petiole of muskmelon (Fig. 1) the same strain was able to induce crown galls in stem and

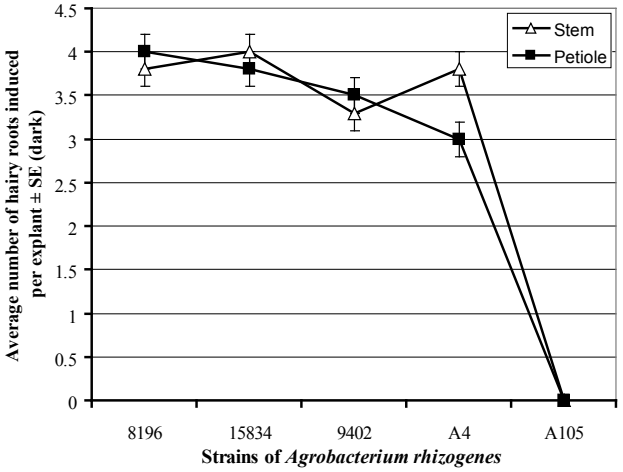

Fig. 5. Effect of dark factor on number hairy root induction in muskmelon explants

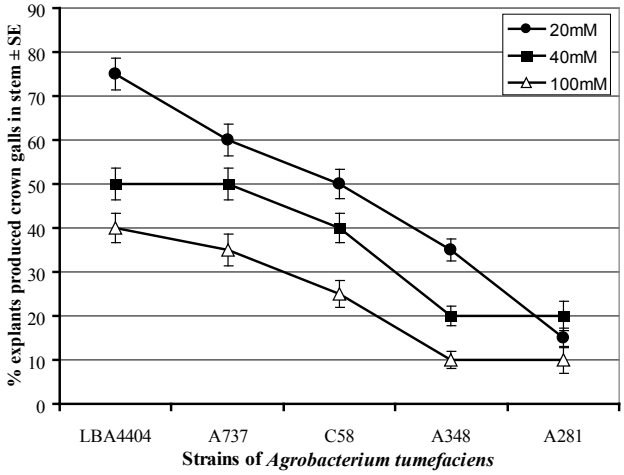

Fig. 7. Effect of acetosyringone on crown galls induction in petiole of muskmelon

petiole of muskmelon after acetosyringone was added in LB medium (Fig. 6 and 7). Among the four acetosyringone concentrations tested, 20 µM was most effective in enhancing crown gall induction from both stem and petiole of muskmelon compared to the control (Fig. 1) followed by 40 and 100 µM concentrations (Fig. 6 and 7). Similarly, A348 that had grown with 40 µM acetosyringone induced bit higher percent of crown gall (45%) (Fig. 7) than control (40%) (Fig.1). Acetosyringone at the concentration of 10 µM was also found in our study ineffective or less effective (data not shown) as 40 and 100 µM.

LBA4404 strain together with 20 µM acetosyringone promoted the greatest crown gall induction in stem and petiole of muskmelon e.g. 75% and 85%, respectively (Fig. 6 and 7). The other virulent strains of *A. tumefaciens* (A737, C58, A348 and A281) that had grown in the same concentration (20 µM) also promoted crown galls induction in stem e.g. 60%, 50%, 35% and 15%, respectively that was approximately 10% higher than the control (Fig. 1 and 6). Similar result was also observed with petiole when *A. tumefaciens* strains had grown with acetosyringone 20 µM (Fig. 1 and 7). The two other concentrations of acetosyringone that had supplemented all *A. tumefaciens* strains except A281 reduced crown gall production from both explants (Fig. 1, 6 and 7). The LBA4404 strain had a perform consistently better than the other four *A. tumefaciens* strains tested in this study for crown gall induction both from stem and petiole in the presence or absence of acetosyringone (Fig. 1, 6 and 7).

Effect of acetosyringone on hairy root induction

The percent hairy root induction per wound site was also enhanced (approximately 23% higher) when infected by *A. rhizogenes* strains grown in YMB medium containing 20 µM of acetosyringone (Fig. 8 and 9) and compared to the control (Fig. 2). However, the other two concentrations (40 µM and 100 µM) of acetosyringone tested did not show a better result (Fig. 2, 8 and 9). The strains 15834 and 8196 showed approximately 30% higher hairy roots production response in both stem and petiole of

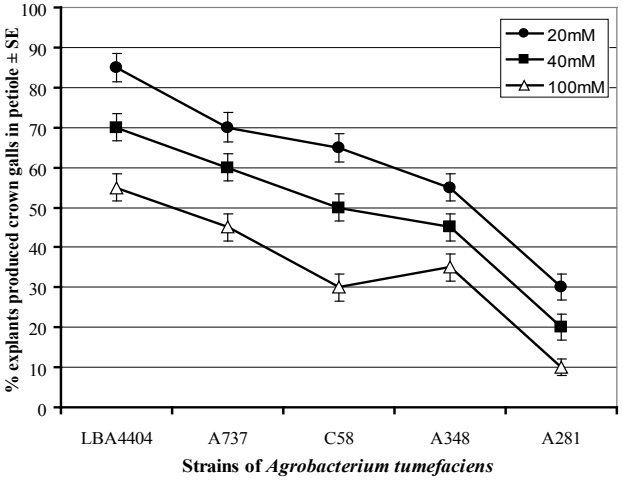

Fig. 6. Effect of acetosyringone conc. on crown gall induction in stem of muskmelon

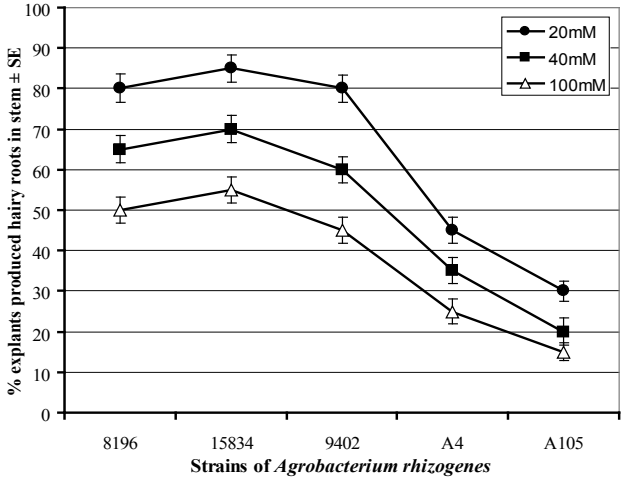

Fig. 8. Effect of acetosyringone on induction of hairy roots in stem of muskmelon

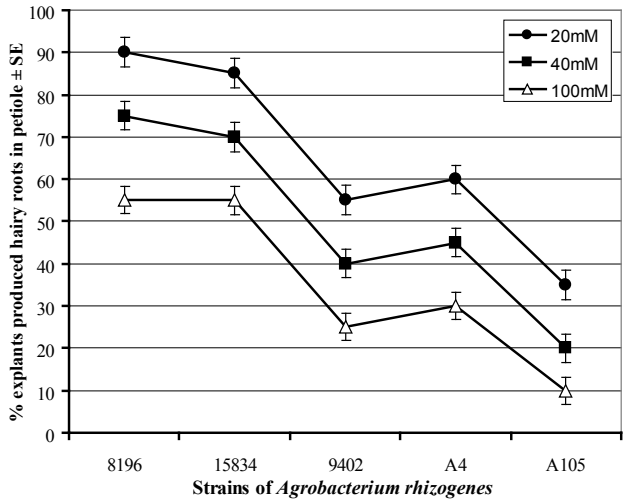

Fig. 9. Effect of acetosyringone on induction of hairy roots in petiole of muskmelon

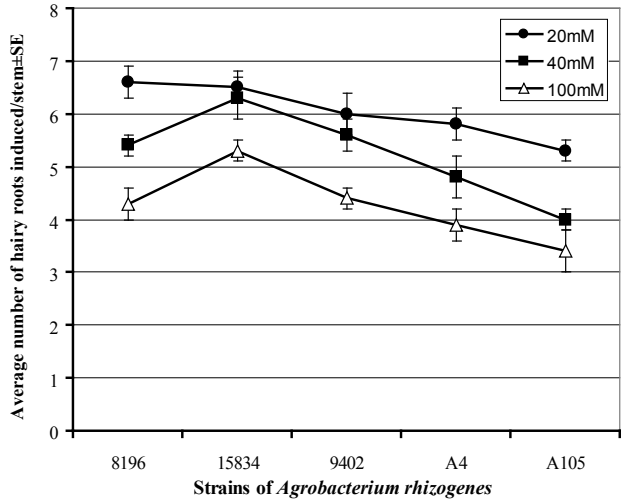

Fig. 10. Effect of acetosyringone on number of hairy roots induced in stem of muskmelon

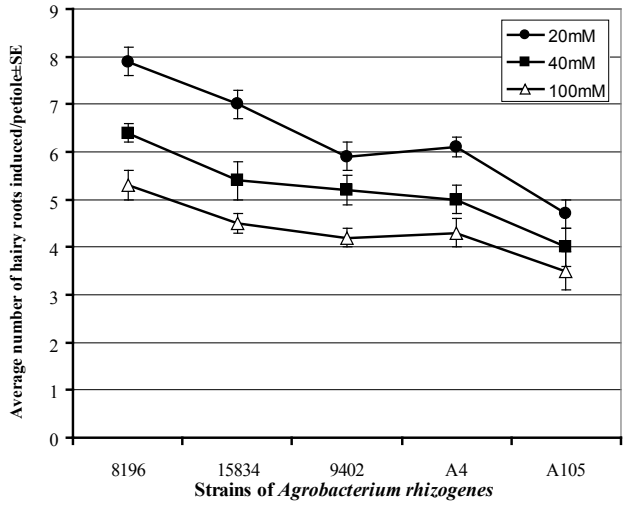

Fig. 11. Effect of acetosyringone on number of hairy roots induced in petiole of muskmelon

muskmelon with the treatment with YMB medium plus 20 µM acetosyringone when compared to the control (Fig. 2, 8 and 9). It was also observed an increase over 20% in the number of hairy roots in both stem and petiole of muskmelon when *A. rhizogenes* strains 15834 and 8196 had grown in YMB medium containing 20 µM of acetosyringone (Fig. 3, 10 and 11).

Discussion

After the inoculation a variable time period is required for transferring T-DNA from *A. tumefaciens* to the plant cells and to produce callus. Matsumoto *et al.* (1986) also reported similar results for the same phenotypic expression i.e. crown gall production. These variations may be related to some steps/factors applied and the results obtained on crown gall induction suggest that the virulence gene activity of *A. tumefaciens* in the form of crown gall production in muskmelon tissue (stem or petiole) is strongly affected by several factors as the *A. tumefaciens* strains, tissue type, plant tissue-*Agrobacterium* interaction and light intensity. Similar effects have been observed in other plant species, e.g. *Eucalyptus* (Machado *et al.*, 1997) and lentil (Hassan *et al.*, 2007). Better gall induction was found in petiole when compared to stem clearly demonstrating that petiole is more susceptible to virulent gene of *A. tumefaciens* and could be used for future gene transformation study.

The findings reported in the present research showed that the *A. tumefasciens* LBA4404, A373 and C58 strains have relatively high virulence activities. Similar expression of C58 strain was also observed in soybean (Delzer *et al.*, 1990). Our observation confirmed that LBA4404 strain is most suitable for muskmelon transformation study. This strain found suitable for successful transformation study in many other crops such as potato with 58.4% of internode explants (Sarker *et al.*, 2009) and garlic (Kenel *et al.*, 2010). Different rates of crown gall induction obtained from the same explant, inoculated with different *A. tumefaciens* strains evidently suggest that the rates of tumorigenicity are strain-dependent. Delzer *et al.* (1990) and Katavic *et al.* (1991) found similar differences in soybean and pumpkin, respectively when different strains of *A. tumefaciens* were tested. On the other hand, it is likely that the strain A281 may be not so virulent to muskmelon plants and died in the medium due to lack of adequate nutrients.

The results in the form of hairy root induction in muskmelon tissue suggested that the virulence gene activity of *A. rhizogenes* is also dependent on different physical factors such as the *A. rhizogenes* strain, plant tissue type, tissue-*A. rhizogenes* strain interaction and light intensity. The effects of physical factors were also reported in many other crops like pumpkin (Katavic *et al.*, 1991), pine (McAfee *et al.*, 1993) and tobacco (Kumar *et al.*, 2006). The variation in virulence gene activity in stem and petiole is different. It is not clear why this discrepancy for hairy root induction

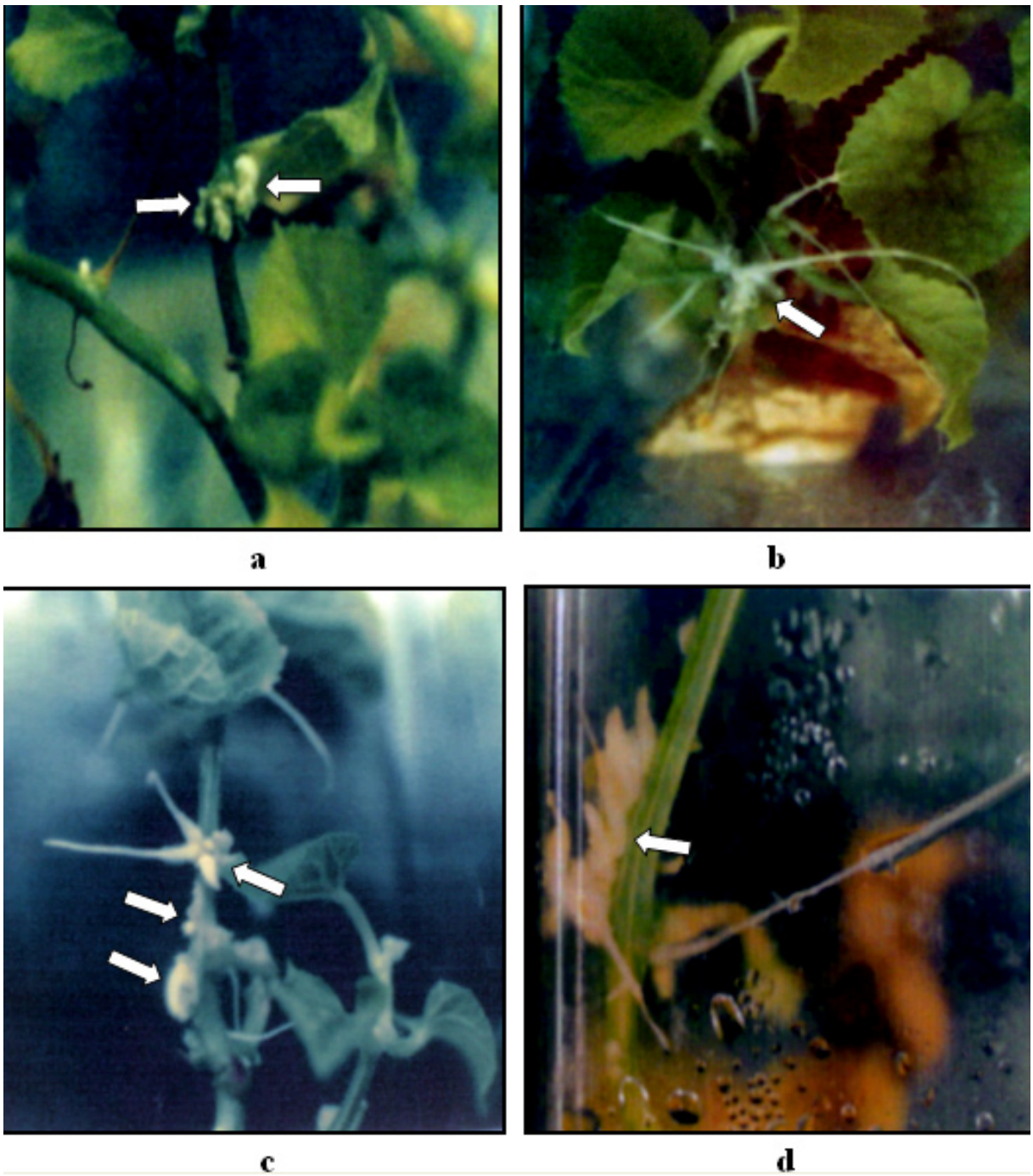

Fig. 12. Phenotypic expression of virulence gene of *Agrobacterium* in muskmelon: a) Crown galls initiated from wound site (stem) when infected with LBA 4404 strain; b) Hairy roots initiated from infected stem by *A. rhizogenes* strain A 105 at 39.3 μmol $m^{-2}s^{-1}$; c) Hairy roots initiated when infected with *A. rhizogenes* strain 8196; d) Hairy roots instigated from stem vigorously when inoculated with *A. rhizogenes* strain 8196 in addition of acetosyringone at the concentration of 20 μM

exists, however it could be due to some form of interaction between the *A. rhizogenes* strain and the tissue type of the explant. Future studies could reveal the reason behind it. For hairy root initiation also, a few days is critical. Matsumoto *et al.* (1986) reported that a time period is crucial to release DNA from *Agrobacterium* to plant cells as well as its phenotypic expression.

The strains 8196, 15834, and 9402 showed comparatively high virulence activity; therefore the competent cells of stem and petiole visibly expressed and formed hairy roots. Kumar *et al*. (2006) observed similar result with A4 strain when infected tobacco leaf tissue. The variation in the percentage of hairy root induction in same explant (stem or petiole) inoculated with different strains evident-

ly suggests that the rate of oncogenecity are dependent on the strains of *A. rhizogenes*. The findings based on induction of higher number of hairy roots confirm that 8196 is most suitable compared to others. On the other hand, the strain A105 induced lowest number of hairy roots in both stem and petiole indicates that this strain is less virulent.

The root induction response difference due to different light intensity indicates that the light intensity plays an important role for activation of virulence gene or for the synthesis of some essential compound important for the gene activity. This may be due to the alteration of the rooting environment due to hormonal or other secretions during the incubation of infected explants at the optimal light intensity. The importance of appropriate light intensity is also evident from the negative role of continuous darkness in the production of crown galls and hairy roots in muskmelon.

The findings pointed out to, in addition to the importance of the specific concentration of acetosyringone (20 μM) that this has played a role in enhancement of virulence gene activity of all *A. tumefaciens* strains resulting in an increase in crown gall induction in both stem and petiole explants. Hess *et al*. (1991) reported that this phenolic compound must be constant for both biological activities of *vir* gene expression as well as for the expression maintenance of *vir* gene. Many other reports also pointed out that the *vir* genes are inducible in response to the monocyclic phenolic compound like acetosyringone (Parke *et al.*, 1987; Ashby *et al.*, 1988; Shaw *et al.*, 1988). The results obtained with the higher and lowest concentrations of acetosyringone (10, 40, and 100 μM) indicated an inhibitory action rather than stimulatory. Similar result was also observed by Kumar *et al.* (2006) in tobacco with high concentration of acetosyringone. The importance of acetosyringone in *vir* gene induction is further confirmed from the response of *A. tumefaciens* strains A281, which failed to form crown galls in stem or in petiole but was able to induce crown galls when grown with acetosyringone at three different concentrations e.g. 20, 40 and 100 μM. The findings of different *A. tumefaciens* strains on crown gall induction suggest that LBA4404 strain is most virulent for both stem and petiole of muskmelon. It also indicates that interaction between specific concentration of acetosyringone and *A. tumefaciens* strain played very important role in enhancement of virulence gene activity.

The crucial role of specific concentration of acetosyringone in enhancement of virulence gene activity was also evident from the increased qualitative and quantitative response of all *A. rhizogenes* strains in hairy root production in both stem and petiole of muskmelon. Higher percent hairy root induction by 15834 strain in stem and by 8196 strain in petiole indicated that the interaction between specific concentration of acetosyringone (20 μM) and *A. rhizogenes* strain played crucial role in enhancement of virulence gene activity. The above finding suggested that these two strains could be tested in future transformation study with other Cucurbitaceae species.

The hairy roots induced from both explants of muskmelon elongated with profuse branches on hormone free MS medium containing cefatoxime however, on MS medium with 2, 4-D the roots initiated callus, which did not grow further. Similar result was also shown in crown gall. These findings confirmed that these crown gall and hairy root tissues had been transformed by T-DNA containing virulence gene and could synthesise hormones that played an accessory role in root and gall induction. Profuse branching of hairy roots in hormone free medium confirmed the subsequent selection of transformed roots (Christey and Braun, 2005; Srivastava and Srivastava, 2007) that justified the present results. The genes for hormone synthesis are found within the T-DNA of agrobacteria (White *et al*., 1985) and any external hormone(s) may be deleterious to this process.

Conclusions

The present study confirmed that both physical and chemical factors, mainly accurate light intensity (39.3 μmol m^{-2} s^{-1}) and the appropriate acetosyringone concentarion (20 μM), together with the specific strains of *Agrobacterium tumefaciens* (LBA4404) and *A. rhizogenes* (8196 and 15834) played vital role in the enhancement of virulence gene (*vir*) activity in both stem and petiole of muskmelon, cv. 'Birdie'. This finding will provide valuable information in future transformation study with recalcitrant species and also a valuable contribution for a betterment of breeding program.

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