Influence of acetosyringone concentration on induction of carrot hairy root by Agrobacterium rhizogenes

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Carrot hairy root induction by two different strains of Agrobacterium rhizogenes- MTCC- 532 and MTCC- 2364 grown in yeast extract mannitol agar medium (YEMA) was studied in carrot (Daucus carota). The maximum hairy root induction was observed when carrot was simmered with 48 h old culture of A. rhizogenes MTCC-532. With different concentrations of acetosyringone (AS) tested, addition of 150 µM acetosyringone was found to enhance the transformation frequency up to 75(±2.60) and 60 (±2.08) percentage by A. rhizogenes MTCC-532 and MTCC 2364 strains, respectively. Transformation efficiency was highly dependent on the acetosyringone concentrations, type of bacterial strains and carrot genotype. Transfer of Ri Ti-DNA was confirmed by polymerase chain reaction (PCR) analysis, the detection of ags gene in transformed carrot hairy root. A. rhizogenes transformed hairy roots had the ability to form copious lateral roots as well as a negative geotropic growth habit in a shorter period of time. The Murashige and Skoog (MS) medium was found to be the best medium for hairy root mass multiplication, which induced high root biomass production and rapid root tip elongation.

Key words: Carrot, Agrobacterium rhizogenes, acetosyringone, hairy roots, Murashige and Skoog (MS) medium, arbuscular mycorrhizae.

INTRODUCTION

Abundant studies have demonstrated that arbuscular mycorrhizal (AM) fungi are obligatory symbionts which colonize the roots of approximately 80% of terrestrial plants (Lekberg and Koids, 2005) and improve the nutrient mobilization from soil, plant growth and disease tolerance (Douds and Siedel, 2012; Elsen et al., 2008). The presently available open pot culture methods for mass production of arbuscular mycorrhizae is having limitations like high cross contamination, being time consuming process; besides, only small amount of inoculum production is achieved. To overcome this problem, in vitro cultivation of AM fungi by root organ culture seems to be promising. Mosse and Hepper (1975) first proposed the use of root organ cultures with excised roots as host partner in AM symbiosis. The Agrobacterium rhizogenes is a well known tumor inducing, Gram-negative soil bacterium, which is able to induce hairy roots rapidly at the infection site (Baranski, 2008). The agrobacterium
mediated transformation frequency is based on vir gene expression (Mohiuddin et al., 2011). Transcription of high level vir gene expression is induced by acetyosyringone released by wounded plant cells and it has been reported that the compound increases agrobacterium mediated transformation frequencies in a number of plant species (Kumar et al., 2006). Genetically modified carrot (Daucus carota L.) roots, show profuse lateral branching and rapid root tip elongation within two to three weeks. Transformed hairy roots are genetically and biosynthetically stable for long periods (Sawssan et al., 2012). The negative geotropism of transformed roots facilitates contacts with hyphae of AM fungi. The success of in vitro cultivation on AM fungi depends on host partner growth. Therefore, the objective of the study was to optimize the acetyosyringone concentration, a wound response molecule known for enhanced hairy root formation for maximum transformation efficiency and also to select suitable A. rhizogenes strains for maximum hairy root induction in carrot.

MATERIALS AND METHODS

Preparation of bacterial strain

The strains of A. rhizogenes such as MTCC-532 and MTCC-2364 were used for induction of hairy root. The A. rhizogenes strains obtained from microbial type culture collection (IMTCC), Chandigarh, India, were grown in yeast extract mannitol agar medium (YEMA), which comprised (g/l) of mannitol -10, Yeast extract -0.5 g, Sodium chloride - 0.1, magnesium sulfate- 0.2, K2H PO4- 0.5 g, agar - 20, distilled water- 1000 ml, finally adjust pH-6.8.

Carrot discs preparation

The commonly cultivated carrot cultivar Ooty-1 obtained from Kavi farm, Santhur, Nilgiris, Tamil Nadu, India, was used as an experimental material. Freshly harvested carrots were washed 2 to 4 times thoroughly with tap water. Then they were surface sterilized with 0.1% HgCl2 for 10 min with continuous stirring. They were further rinsed three times (each for 5 min) with sterile distilled water and dipped in 70% ethanol for 30 s and superficially flamed and peeled out. Each carrot was sliced into 0.5 cm thick discs and was placed on 0.5% MS (half strength) (Murashige and Skoog, 1962) plates with the basal sides facing upwards (Figure 1). The sterile needle was used to prick manually for wounding on carrot surface. For pre-cultivation, the plates were incubated at 28°C in dark for 24 h.

Conformation of transformed hairy root by PCR analysis

Carrot hairy root were cut into the small pieces and the genomic DNA was isolated from transformed carrot hairy root line by CTAB methods (Doyle and Doyle, 1990). The PCR was performed to amplify T-DNA agroipine synthase (ags) transformed hairy root. The specific primer used to amplify ags genes were forward primer (5-GCGCATCCCCAGGGCAT-3) and reverse primer (5-AGGTGTGGCGATCGCAGGA-3). PCR amplification was performed with a program of initial denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min. and a final extension at 72°C for 7 min and storage at 4°C. The amplification was analyzed by agarose gel electrophoresis. Plasmid DNA from A. rhizogenes strains MTCC-532 and 2364 was used as a positive control.

Mass production of hairy roots

After two to three weeks of incubation, carrot discs showed profuse white, turbescent and non-ramified apexes hairy roots. Fresh hairy roots were cut into minimum 3 cm long and then transferred to MS medium containing plates and incubated in inverted position under dark at 27°C. Bacteria free hairy root were obtained by subsequent subculture three times in fresh MS medium containing, cefotaxime at 250 mg/l (HiMedia, Mumbai, India) to make it free of A. rhizogenes. The bacterial free hairy roots could be used for in vitro mass production of AM fungi.

Statistical analyses

All the data were subjected to statistical analysis with software, Microsoft Excel for Windows 2007 add-in with XLSTAT Version 2010.5.05 (XLSTAT, 2010). Statistically significant differences between the treatments were analyzed using analysis of variance (ANOVA) and Duncan’s multiple range test (DMRT) at a 5 % significance level.

RESULTS

Transformation

After 5 to 10 days of co-culture with A. rhizogenes, callus initiation (Figure 2.) was observed on the surface of carrot discs, followed by appearance of the transformed roots on the side wall of discs (Figure 3). Hairy root initiation continued to occur from 10 days to two to three weeks. A typical hairy root was formed quickly from

Figure 1. Pre-cultivation of carrot discs.
numerous lateral roots (Figure 4.), which grew both on the surface and penetrated into the media, exhibiting the negative geotropic growth habit (Figure 5). Some carrot discs were observed without any hairy root induction. This may be due to instability of the new genome or due to non-expression of genes involved in root induction. The carrot discs produced hairy roots up to three weeks and later on rotting was noticed. Among the four different concentrations of acetosyringone tested, 150 µM was the most effective in enhancing hairy root induction from both strains, as compared to the control. The maximum hairy root induction percentage (75±2.60), number of lateral roots (12±0.42) and number of negative geotropic roots (9±0.31) was observed from carrot discs simmered with 48 h old \textit{A. rhizogenes} MTCC-532 on MS plates supplemented with 150 µM acetosyringone (Table 1). In the \textit{A. rhizogenes} MTCC 2364 strain used for same condition only (60±2.08) percentage of hairy root initiation, (9 (0.31)) number of lateral roots and (5 (0.17)), number of negative geotropic roots was observed. The transformation frequency declined at both lower (<150 µM) and higher concentrations of acetosyringone (Table 2). These transformed hairy roots were cut around 3 cm long roots and transferred to a sterile hormone-free MS medium (Figure 6). The transformed carrot hairy root was multiplied as per the above procedure described in Materials and Methods (Figures 7 and 8). Hairy roots were maintained with regular sub culturing at three weeks interval.

Detection of \textit{agropine synthase (ags)} gene in transformed carrot hairy root

PCR analysis was done using a pair of gene specific primer (forward and reverse) which amplifies the T-DNA \textit{agropine synthase} gene. The total genomic
Table 1. Effect of *A. rhizogenes* (MTCC-532) strain with their ability to induce carrot hairy roots on various concentrations of acetosyringone.

<table>
<thead>
<tr>
<th>Acetosyringone (µM)</th>
<th>Total no. of carrot discs</th>
<th>No of discs inducing hairy root</th>
<th>Transformation rate (%)</th>
<th>Number of lateral roots</th>
<th>No of roots showed negative geotropism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>03</td>
<td>15 (±0.52)d</td>
<td>2 (±0.07)e</td>
<td>0 (±0.00)d</td>
</tr>
<tr>
<td>50</td>
<td>20</td>
<td>05</td>
<td>25 (±0.87)cd</td>
<td>4 (±0.14)d</td>
<td>1 (±0.03)d</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>07</td>
<td>35 (±1.21)bc</td>
<td>7 (±0.24)c</td>
<td>3 (±0.10)d</td>
</tr>
<tr>
<td>150</td>
<td>20</td>
<td>15</td>
<td>75 (±2.60)a</td>
<td>12 (±0.42)a</td>
<td>9 (±0.31)a</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
<td>08</td>
<td>40 (±1.39)b</td>
<td>10 (±0.35)b</td>
<td>6 (±0.21)d</td>
</tr>
</tbody>
</table>

Values are mean (±SE) (N=20) and values followed by the same letter in each column are not significantly different from each other as determined by DMRT (p≤0.05).

Table 2. Effect of *A. rhizogenes* (MTCC-2364) strain with their ability to induce carrot hairy roots on various concentrations of acetosyringone.

<table>
<thead>
<tr>
<th>Acetosyringone (µM)</th>
<th>Total no. of carrot discs</th>
<th>No. of discs inducing hairy root</th>
<th>Transformation rate (%)</th>
<th>Number of lateral roots</th>
<th>No. of roots showed negative geotropism</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>03</td>
<td>10 (±0.35)c</td>
<td>1 (±0.03)e</td>
<td>0 (±0.00)d</td>
</tr>
<tr>
<td>50</td>
<td>20</td>
<td>05</td>
<td>15 (±0.52)c</td>
<td>3 (±0.10)d</td>
<td>1 (±0.03)d</td>
</tr>
<tr>
<td>100</td>
<td>20</td>
<td>07</td>
<td>30 (±1.04)b</td>
<td>5 (±0.17)c</td>
<td>2 (±0.07)d</td>
</tr>
<tr>
<td>150</td>
<td>20</td>
<td>15</td>
<td>60 (±2.08)a</td>
<td>9 (±0.31)a</td>
<td>5 (±0.17)a</td>
</tr>
<tr>
<td>200</td>
<td>20</td>
<td>08</td>
<td>35 (±1.21)b</td>
<td>7 (±0.24)b</td>
<td>3 (±0.10)d</td>
</tr>
</tbody>
</table>

Values are mean (±SE) (N=20) and values followed by the same letter in each column are not significantly different from each other as determined by DMRT (p≤0.05).

DNA was extracted from the transformed carrot hairy roots to observe the presence of Ri Ti-DNA. Also, for positive control of PCR analysis, the total genomic DNA was extracted from *A. rhizogenes* and resolved in agarose gel electrophoresis. The primer showed amplification, which confirmed the successful transformation of T-DNA *agropine synthase* gene, which produced the band of size approximately 341 bp (Figure 9).
DISCUSSION

Carrot is one of the most suitable and well known model plant species for hairy root production (Bidondo et al., 2012). So far, the co-cultivation of arbuscular mycorrhizal fungi with carrot hairy root was used because of their easy propagation and fast growth rate over normal root (Yinli et al., 2004). Carrot hairy root are better adapted to low level of nutrient content and survive for long time without sub culturing than normal root (Saravanakumar et al., 2012).

Carrot hairy roots have been used to initiate monoxenic culture of AM fungi since 1988. Suitable methods for their maximum hairy root induction in shorter period of time and mass multiplication has not been described yet. In this present study, it was observed that A. rhizogenes strain MTCC-532 showed higher transformation efficiency and higher lateral branching, more number of negative geotrophic roots as compared to MTCC-2364 strain, so that transformation efficiency is highly dependent on the type of bacterial strain used. This result was supported by Ahlawat et al. (2012), who used different A. rhizogenes strains like LBA 9402, LBA 920, LBA 301, MTCC 532, NRRL B193, A4 for induction of Artemisia annua L. hairy root. Among the strains, LBA 9402 showed 100% transformation frequency within five to six days of hairy root induction. Inoculation of carrot discs with 48 h old culture and incubation in darkness at 28°C provided a suitable condition for bacterial strains to insert their maximum copies of Ri t-DNA.

The present findings is in line with those reported by Ridgway et al. (2004), who used 24 h old culture of A. rhizogenes (A4T) strain and achieved 53% of hairy root induction when freshly harvested carrot was used as explants. Among the different concentration of acetylsyringone used in our study, specific concentration (150 µM), which played a role in enhancement of virulence vir gene activity of two different A. rhizogenes strains, resulted in enhanced transformation frequency of carrot explants. These findings are supported by Mohiuddin et al. (2011), who reported that acetylsyringone is an amino acid derivative and a phenolic compound, which must be constant for biological activity and maintenance of vir gene expression in Muskelon explants. Many other reports also pointed out that the vir genes are inducible in response to the monocylic phenolic compound like acetylsyringone (Shaw et al., 1988, Ridgway et al., 2004). The results obtained from the higher (200 µM) and lower (100 µM) concentration of acetylsyringone indicated an inhibitory action rather than stimulatory.

Similar result was also observed by Kumar et al. (2006) in Nicotiana tabacum with high concentration (>200 µM) of acetylsyringone to reduces the transformation efficiency. Freshly harvested carrots are invariably better in initiating hairy roots overall. This may be due to the active nature or less dormant nature of the carrots. However, hairy root induction also depends on the carrot genotype and origin of plant (Danesh et al., 2006). Molecular analysis of carrot hairy root through PCR was done using a pair of primer, which amplified the T DNA agropine synthase gene and confirmed the transformation at 341 bp segment. Similar primer was also used by Garland et al. (2001) and Rajkumar and Murugesan (2014) in Ulmus procera, Psoralea corylifolia hairy root respectively to confirm the transformation. The confirmation of rolC gene transfer from A. rhizogenes R1000 by PCR detection at 557 bp was also reported in Withania somnifera L. (Saravanakumar et al., 2012). The MS medium has been used for growing transformed roots, because it allows significantly better growth and rapid root tip elongation.

The same trends was observed by Pratap Chandran and Potty (2010), where in different media like modified whites’ medium, MSR medium, MS medium were used for hairy root multiplication. Among the media, MS salts with B5 vitamins were used for successful mass production of carrot hairy root. Similar result also observed by Diop (2003) pointed out that MS medium is the most suitable medium used for mass production of hairy root for in vitro culture of AM fungi.
Conclusion

This study demonstrates the ability of *A. rhizogenes* (MTCC-532) strain and 150 µM concentration of acetosyringone combination showed maximum hairy root induction and growth under *in vitro* conditions. The potential role of *Agrobacterium* strain and host explant genotype in hairy root induction is of great scientific interest, which may allow the rational manipulation of hairy root biomass production on large scale to develop monoxenic culture of AM Fungi.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES


