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Full Length Research Paper

Hairy roots induction from difficult-to-transform pharmacologically important plant *Eurycoma longifolia* using wild strains of *Agrobacterium rhizogenes*

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Wild strains of *Agrobacterium rhizogenes* were used to induce hairy roots from *Eurycoma longifolia*, a high value medicinal plant in South-East Asia. Among 5 wild strains of *A. rhizogenes* tested, 3 strains namely MAFF 210265, MAFF 301726 and MAFF 720002 exhibited the ability to induce hairy roots from *E. longifolia*. After many unsuccessful attempts to produce hairy roots from this recalcitrant plant, hairy roots were successfully initiated, through infecting the hypocotyls region of this plant. The generated hairy roots were observed to be thin, non-geotropic, hairy and brittle in appearance. Amplification of the deoxyribonucleic acid (DNA) fragments which is about 1100 bp using *rol* genes that extended between *rol C* and *D* gene sequence by Polymerase chain reaction (PCR) method confirmed the integration of the portions of transferred deoxyribonucleic acid (T-DNA) into the transformed hairy roots. Generating hairy roots in *E. longifolia* will be highly beneficial mainly to the pharmaceutical industry as through the hairy root induction increased the amount of valuable secondary metabolites which is directly linked to its root differentiation can be achieved from low biomass starting material. This is the first report on hairy root induction from the medicinally eminent plant, *E. longifolia* using wild type strains of *A. rhizogenes.*

Key words: *Eurycoma longifolia,* hairy roots, transformation, wild strains of *Agrobacterium rhizogenes.*

INTRODUCTION

Eurycoma longifolia is one of the most important traditional remedies in South-East Asia. Various parts of this plant have been and are still being used to cure numerous diseases. The root extracts of *E. longifolia* have been scientifically proven to enhance the virility and sexual prowess (Zanoli et al., 2009) when orally administered to sexually sluggish and impotent male rats. In addition, Ang et al. (2002) were able to provide scientific evidence of orientation activities (anogenital sniffing, licking and mounting) in sexual behavior among the middle-aged male rats after administering the *E. longifolia* root extracts. Therefore, *E. longifolia* is marketed as an alternative medicine to Viagra and believed to posses no side effects. In addition, *E. longifolia* possesses anti-malarial activity. Previously, Chan et al. (1986) tested the extracts of *E. longifolia* for antiplasmodial activity against a multi-drug resistant Thailand strain (K-1) of *Plasmodium falciparum* under *in vitro* conditions, which showed antimalarial activities. In addition, Kardono et al. (1991) isolated and characterized five cytotoxic constituents from *E. longifolia* roots and scientifically prove that it possesses cytotoxic effects against various human cancer cell types which includes

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breast cancer, colon cancer, fibrosarcoma, lung cancer, melanoma and also against murine lymphocytic leukemia. Currently, the most common method of propagating *E. longifolia* plant is through seeds. However, being a recalcitrant plant, the seeds have a low precentage of germination and it takes a long time to germinate due to extremely immature state of zygotic embryo at the time of dispersal (Sobri et al., 2005). The gradual dissapearance of this plant are due to the massive collection of its roots from wild drug preparations.

As a conclusion, it needs to be rapidly mass-multiplied on a commercial scale to comply with the need of the herbal and pharmaceutical industry (Sobri et al., 2005). Therefore, *in vitro* techniques can be an important alternative approach to produce useful plant chemical products from *E. longifolia.* Hairy roots application can be used extensively in areas like phytochemical and recombinant protein production (Georgiev et al., 2007; McCoy et al., 2008), phytoremediation (Doran et al., 2009), molecular breeding (Giri et al., 2000; Christensen and Müller 2009), rhizosphere physiology and biochemistry (Rios-Estepa and Lange, 2007), metabolic engineering (Mehrotra et al., 2010), bioreactor design and optimization (Srivastava and Srivastava 2007; Mishra et al., 2008). Ono et al. (2011) reported that hairy root cultures induced by *A. rhizogenes* accumulate phytochemicals to levels comparable to that of intact plants and are usually stable in their biosynthetic capacity. When optimized for liquid cultures, hairy roots can be grown in industrial-scale bioreactors providing a convenient, abundant and sustainable source of phytochemicals that are useful as cosmetics, pharmaceuticals products and food additives. Thus this attribute makes hairy roots a better choice compared to propagation through tissue culture method, which is a very laborious process (Monica et al., 2011). In addition, the secondary metabolites can be harvested directly with few extraction steps compared to the conventional tissue culture techniques (Monica et al., 2011). In this present study, we have induced hairy roots from a recalcitrant medicinal valuable plant: *E. longifolia,* and we hoped to provide new information and tools for the optimization for induction and production of *E. longifolia* hairy roots cultures from the genetic transformation technology which have not been reported previously. This information is vital as it enable us to formulate the optimum medium and conditions for the optimal production of the hairy roots. Thus, makes this as the first report of hairy root induction from *E. longifolia* using wild strains of *A. rhizogenes.*

MATERIALS AND METHODS

Plant materials

Different parts of *E. longifolia* were used to initiate the hairy roots. The *in vivo* explants that were used are *in vivo* leaves, petiole and rachis. The *in vitro* parts used are *in vitro* leaves and root segments. In addition, three weeks germinated seedlings, embryos and somatic embryo of *E. longifolia* were used. Each of the plant parts were surface sterilized and described individually as follows:

In vivo leaves, petiole and rachis

The leaves, petioles and rachis were obtained from the Nursery School of Biological Sciences. The explants were washed under running tap water for 30 min with the addition of Tween-20 (R and M chemicals). After the initial washing, the *in vivo* explants were transferred into the laminar hood for the subsequent washing. Following the method described by Luthfi (2004) the explants were subjected to further surface sterilization. In the laminar hood, the *in vivo* explants were soaked in 13% (v/v) Clorox® Regular Bleach and continuously stirred for 15 min. Then, the *in vivo* explants were rinsed to wash away the traces of Clorox® by series of washing in the sterile distilled water for 3 times. The explants were then rinsed again for 5 min in 5% (v/v) Clorox®. Explants were rinsed to wash away the traces of Clorox® Regular Bleach by series of washing in the sterile distilled water for 3 times. Finally, the leaves explants were cut at the size of 1 cm^2 and both the rachis and petioles were cut at the length of 1 cm as to prepare them for the hairy root induction experiment.

In vitro leaves and root segments, three weeks grown seedlings, somatic embryos and embryo of *E. longifolia*

*E. longifolia s*eeds were collected from the Nursery School of Biological Sciences, Universiti Sains Malaysia. The seeds were surface sterilized using series of washing in Tween-20 (R and M chemicals) and Clorox® Regular Bleach. Seeds were transferred into the laminar hood for the subsequent washing. Then, the seeds were washed in 20% (v/v) Clorox® Regular Bleach for 20 min by continuous shaking. After the first step of washing, the seed coat and the fleshy layer were removed using sterile blades size 24. In the second washing step, the embryo with the outer layer was washed using 12% (v/v) Clorox® Regular Bleach for 15 min by continuous shaking. In the final wash, the embryo was washed using 5% (v/v) sodium hypochlorite for 5 min by continuous shaking.

The embryo was inoculated in Murashige and Skoog (MS) (1962) semi-solid media which was supplemented with 3% sucrose and 0.8% Gelrite and germinated at the 26±2°C and under 16 h photoperiod (Philips TLD, 36W) at 150 $\mu mol.m^{-2}.s^{-1}$. Medium was adjusted to pH 5.7 using pH meter (Mettler Toledo, USA) before autoclaving at 121°C for 15 min. The time of inoculation were noted till the emergence of the plumul and radical from the inoculated *E. longifolia* seeds. The leaves that emerges were cut at the size of 1 cm^2, the root were cut at the size of 1cm and the 3 weeks old seedling at its hypocotyls region were used to induce hairy roots. Embryos after the final washing step were used as explants to induce hairy roots.

Wild strains of *A. rhizogenes*

In this study, 5 wild strains of *A. rhizogenes* namely MAFF106590, 106591, 201265, 301726 and 720002 were used. Prior to be used in this experiment, all wild strains of *A. rhizogenes* were grown in Luria Bertani (LB) agar to obtain single colonies. Then, the single colony was inoculated in liquid LB medium at 60 rpm for 18 h at 26±2°C.

Hairy root Induction

In vivo* leaves, petiole, rachis and in vitro leaves, roots, embryo and somatic embryo *of E. longifolia

To induce hairy roots in the *in vivo* leaves, petiole, rachis and *in vitro* leaves, roots, embryo and somatic embryo of *E. longifolia,* the *A. rhizogenes* suspension culture (18 h of overnight culture) was drawn into a sterile fine needle syringe. After that, few drops of the *A. rhizogenes* suspension culture were injected randomly into the explants at maximum of 3 different regions. Then, the excess bacteria suspension was wiped off using sterile filter paper (Whatman # 1 lab Filter paper).

Subsequently, the explants were inoculated in MS (1962) semi-solid media which was supplemented with 3% sucrose and 0.8% Gelrite and germinated at the 26±2°C and under dark condition. After 3 days, all the explants were transferred into MS media with 300 mg/L cefotaxime sodium. The explants were subsequently transferred until the excess bacteria were eliminated. For negative control the seedling were subjected to the same condition as above except that the explants were not inoculated with *A. rhizogenes* suspension culture. Each set of experiment were done in triplicates. Each set were repeated 12 times.

Three weeks old germinated seedlings of *E. longifolia*

Three weeks old germinated *E. longifolia* seeds were used in this study. The plumule region was excised using a sharp blade and the hypocotyl region was maintained. The *A. rhizogenes* suspension culture (18 h of overnight culture) was drawn into a sterile fine needle syringe and few drops of the *A. rhizogenes* suspension culture were injected randomly at 3 sites of the hypocotyl region. Then, the excess bacteria suspension was wiped off using sterile filter paper (Whatman # 1 Filter paper). The seedlings along with its root were then planted vertically in a 30 ml basal MS semi-solid media prepared in test tubes. The seedlings were placed in culture room at 24±2°C and under dark condition. Negative control seedlings were subjected to the same condition as above except that they were not inoculated with *A. rhizogenes* suspension culture. Each set of experiment were done in triplicates. Each set were repeated 12 times.

Establishment of the hairy root cultures in solid medium

The induced hairy roots were transferred to test tube containing 30 ml solid basal MS semi-solid medium supplemented with 3% sucrose and 0.8% Gelrite.

The induced root was subjected to transfer thrice (each transfer at 24 h interval) into basal MS semi-solid medium to ensure that the over growth of *Agrobacterium rhizogenes* are completely eliminated.

Genomic DNA isolation and detection of the transformed *rol* genes by PCR

The total plant genome from the transformed and control plants was extracted using Gen Elute Plant Genomic DNA miniprep kit (Sigma-Aldrich, USA). PCR was performed using set of primers that complement with the regions of *rol C* and *D* (Kiyokawa et al., 1992). 50 ng of genomic DNA, 1X PCR buffer with KCl (100 Mm Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% (v/v) Nonidet P40), 2.5 mM deoxynucleotide triphosphates (dNTPs), 0.3 µM of each primer, 3.0 mM $MgCl_2$ and 1U Taq polymerase (Fermentas) were added to make a final volume of 25 µl. The reaction mixture were subjected to initial denaturation of 94°C, 5 min followed by 30 cyles of denaturation at 95°C for 1 min, annealing at 55°C for 2 min, extension at 72°C for 2 min and a final extension at 72°C for 4 min (Seibi and Prakash, 2000) using Bio-Rad thermocycler. The PCR products were electrophoresed in 1.0% agarose gel at 100V, stained with ethidium bromide and viewed under ultraviolet (UV) illuminator.

Scanning electron microscope (SEM) analysis of the transformed hairy roots and the untransformed (control) roots

The SEM tissue preparation was done using the Hexamethyldisilazane (HMDS) method. The transformed and the control roots were fixed in the Mc Dowell-Trump fixative prepared in 0.1M phosphate buffer (pH 7.2) for 7 days. After 7 days, the samples were washed using the 0.1M phosphate buffer (pH 7.2) for 10 min for each session for three times. Then the samples were post fixed in 1% Osmium tetroxide prepared in 0.1M phosphate buffer (pH 7.2) at room temperature for 2 h. After 2 h, the samples were washed using distilled water for 10 min each session twice. Then a series a dehydration process were done to the samples first dehydration using 50% ethanol for 15 min, followed by dehydration using 75% ethanol for 15 min, 95% ethanol for 15 min (twice) and finally dehydrated in 100% ethanol twice. After that, the dehydrated tissues were immersed in 2 ml of HMDS for 10 min. The HMDS was removed from the specimen vial and the tissues were let to air dry at room temperature. The dries samples were then mounted onto a SEM specimen stub with a double sided sticky tape. The specimens were then coated with gold and viewed using SEM.

Transmission electron microscope (TEM) sample preparation with spurr's resin

Samples were fixed in McDowell Trump fixative which was prepared using 0.1 M phosphate buffer (pH 7.2) at 4°C for 2 weeks. On the sample preparation day, the samples were washed thrice for 10 min each time using 0.1M phosphate buffer. Then the samples were post fixed in 1% Osmium tetroxide prepared in 0.1 M phosphate buffer at room temperature for 2 h. After that, the samples were washed twice for 10 min in distilled water. The samples were subjected to series of hydration, dehydration in 50% ethanol (15 min), 75% ethanol (15 min), 95% ethanol (15 min × twice), 100% ethanol (30 min × twice) and finally in 100% acetone (10 min × twice). Then, the samples were infiltrated with resin using the mixture of acetone: Spurr's resin at the ration of 1:1 in a rotator for 30 min. After that, the mixture was removed and spurr's resin were added and rotated overnight. The next day, the samples were infiltrated in a new change of spurr's resin for 5 h. The samples were embedded and cured at 60°C for 24 h. The samples pelleted were then subjected to coarse, fine and 25° angle cutting. Water boats were prepared for collecting sliced specimens. Then, thin microtome cutting with 99 nm thickness were done to obtain rainbow color on the water boat. Slicing was continued until gold color slice were obtained with continuous adjustment of the water level in the boat. Few gold slices were obtained from the water boat and stained with toluene blue and observed under light microscope. Finally, the gold slices were mounted to be viewed on TEM.

RESULTS AND DISCUSSION

Various explants of *E. longifolia* such as *in vivo* leaves, petiole, rachis and *In vitro* leaves, root segments, embryo and somatic embryo fail to transform for the hairy roots induction despite the transformation process were done

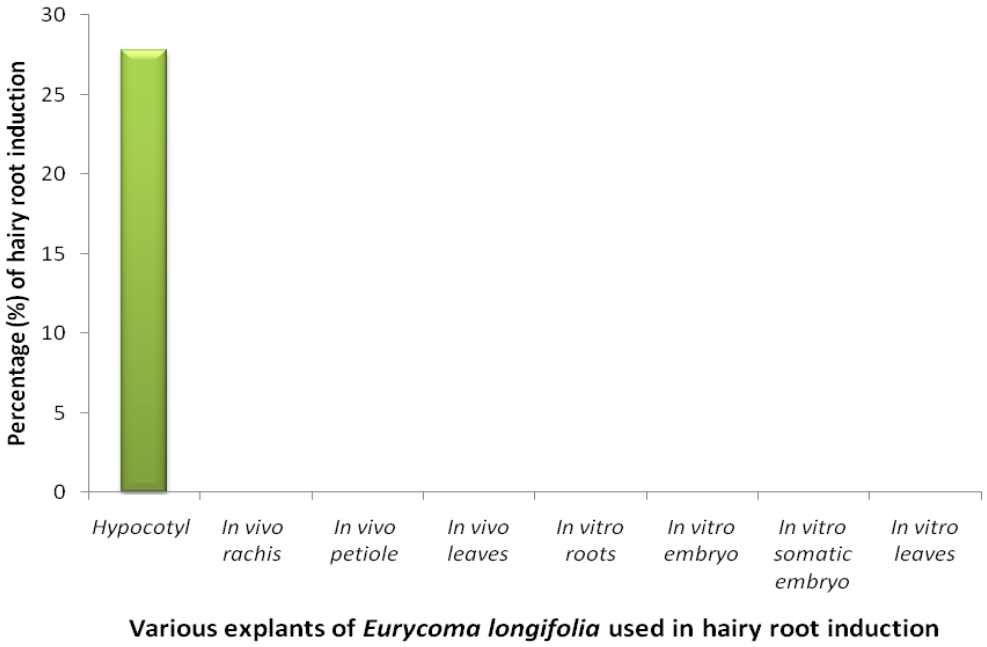

Figure 1. Percentage of hairy root induction from various explants of *E. longifolia* used to induce hairy roots. Each set of experiment were done in triplicates. Each set were repeated 12 times.

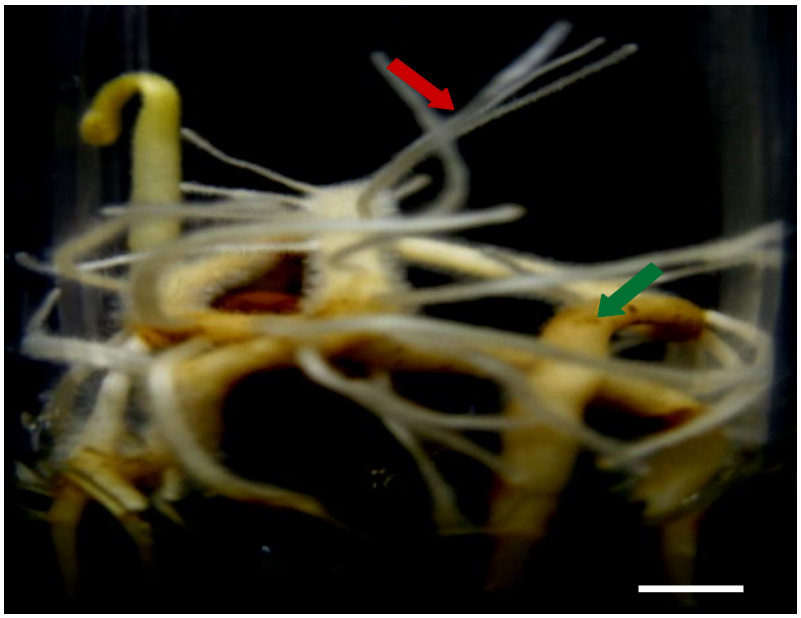

Figure 2. Hairy roots emergence from transformation using *A. rhizogenes* strain MAFF 210265 after 3 weeks (red arrow) from normal roots (green arrow) (Scale bar 1 and 1 cm).

repeatedly using all the wild strains of *A. rhizogenes.* In contrary, the inoculation of wild strains of *A. rhizogenes* at the hypocotyls region of 3 weeks germinated *E. longifolia* embryo generated the hairy roots. The percentage of hairy root induction from the hypocotyl region is about 28% (Figure 1). Of all the tested 5 strains of *A. rhizogenes*, 3 strains successfully transform the hypocotyl region of *E. longifolia*. The 3 wilds strains of the

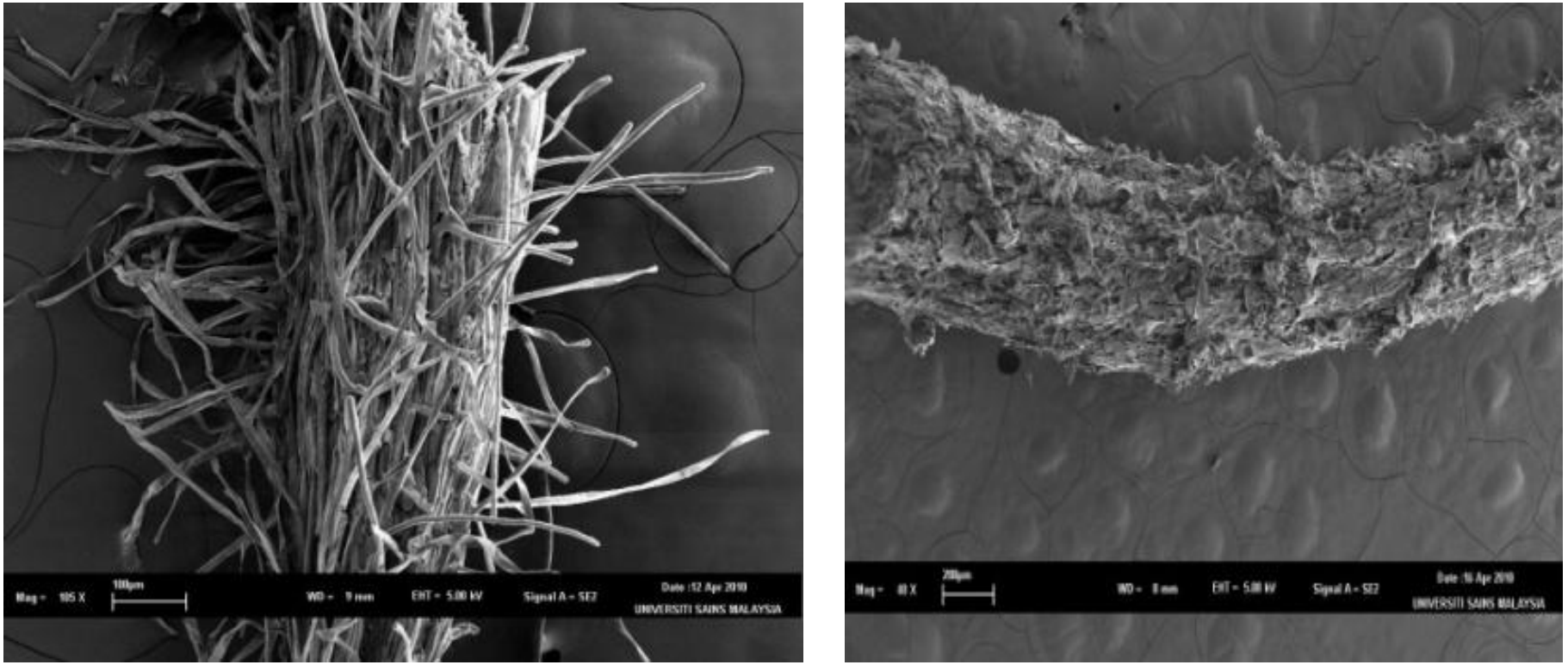

Figure 3. SEM analysis on (A) hairy roots transformed using *Agrobacterium rhizogenes* strains MAFF 210265 and (B) Untransformed root.

A. rhizogenes that were able to transform the hypocotyl region of *E. longifolia* are strains MAFF 210265 (Figure 2), 301726 and 720002. The reason that only the hypocotyl region is transformed may due to the reason that root inducing genes (*rol* genes) have similar tissue specific expression pattern and are mainly confined to root meristems and the phloem (Ove et al., 1997). In addition, detailed analysis showed that whereas the *rolB* promoter seems to be generally expressed in phloem, phloem parenchyma (Altamura et al., 1991; Nilsson et al., 1997) and ray cells (Nilsson et al., 1997), the *rolC* promoter is specifically expressed in phloem companion cells (Guivarch et al., 1996; Nilsson et al., 1996).

The SEM analysis was done to analyse the surface of the untransformed roots and the transformed roots using wild strains of *A. rhizogenes* MAFF 201265 (Figure 3). The result shows that the hairy roots that were generated after the transformation have numerous tiny hair roots like structure (Figure 3A) compared to untransformed (control) roots (Figure 3B). The

Figure 4. *A. rhizogenes* MAFF 201265 attachment photos on hypocotyl region in which the polar binding was observed that aids the transformation process of the bacteria to the plant surface.

control roots (Figure 3B) were essentially hairless. In addition, the control roots were observed to be much thicker with less or no tiny hairs than the generated hairy roots (Figure 2). In addition, another interesting features that was observed using SEM is the aggregation of the *A. rhizogenes* onto the surface of *E. longifolia* (Figure 4). The polar binding that aids the transformation process is clearly seen in Figure 4. The polar binding process aids the T-DNA transfer from the Ri (root inducing) plasmid of the *A. rhizogenes* to be integrated in the genome of *E. longifolia*. Weak attachment to the plant cell is first achieved through synthesis of acetylated polysaccharides, followed by strong binding through the

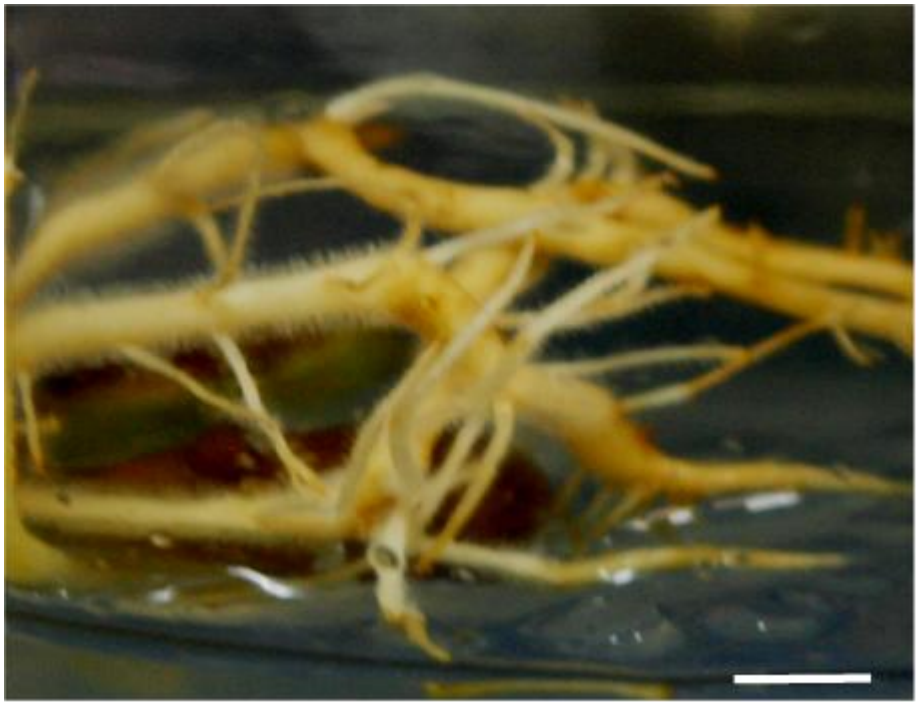

Figure 5. Establishment of the hairy root cultures in solid medium of transformed roots using *A. rhizogenes* strain MAFF 210265 (Scale bar 1and 1.5 cm).

extrusion of cellulose fibrils (Gelvin, 2000; Daniel et al., 2011). Furthermore, the TEM analysis was done to analyse the internal structure of the transformed roots. From TEM analysis *A. rhizogenes* MAFF 210265 integration into the genome of *E. longifolia* observed in which the *A. rhizogenes* MAFF 210265 structures have been deeply integrated into the genome of *E. longifolia* (Figure 6).

The establishment of the hairy root cultures in semi-solid medium was done by transferring the induced hairy root which is still attached to its cotyledon to a MS semi-solid media. During the initial time period the growth of the hairy roots were recorded slow as the emergence of new roots were observed after 30 days of subculture (Figure 5). Although, the proliferations of the hairy roots were not rapid, but new hairy roots were produced. The phenotype of the generated hairy roots was whitish, thin, non-geotropic, hairy and brittle. In contrary, the normal roots which were either a single tap roots or with less branching continued to grow at a very slow rate on MS semi-solid media compared to hairy roots (Seibi and Prakash, 2000). Different *A. rhizogenes* metabolizes different types of opine as a carbon source. Isogai et al. (1990) indicated that *A. rhizogenes* strain MAFF 03-01724 utilizes mikimopine as its carbon source and possess plasmid pRi1724 (Seibi and Prakash, 2000). Kiyokawa et al. (1994) elucidated the nucleotide sequence of plasmid pRi1724 and proposed two primers namely 1724C and 1724D for MAFF

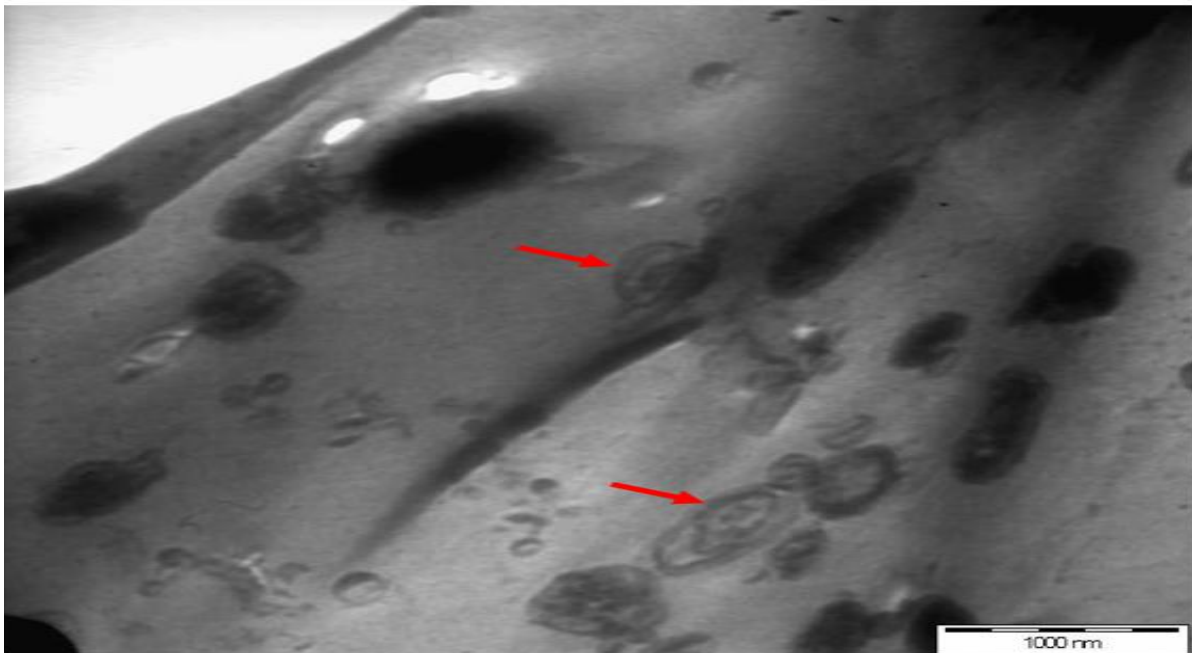

Figure 6. *A. rhizogenes* MAFF 210265 integration into the genome of *E. longifolia* observed using transmission electron microscope (TEM). The red arrows indicate the *A. rhizogenes* MAFF 210265 structures that have deeply integrated its root inducing genes (*rol* genes) into the genome of *E. longifolia.*

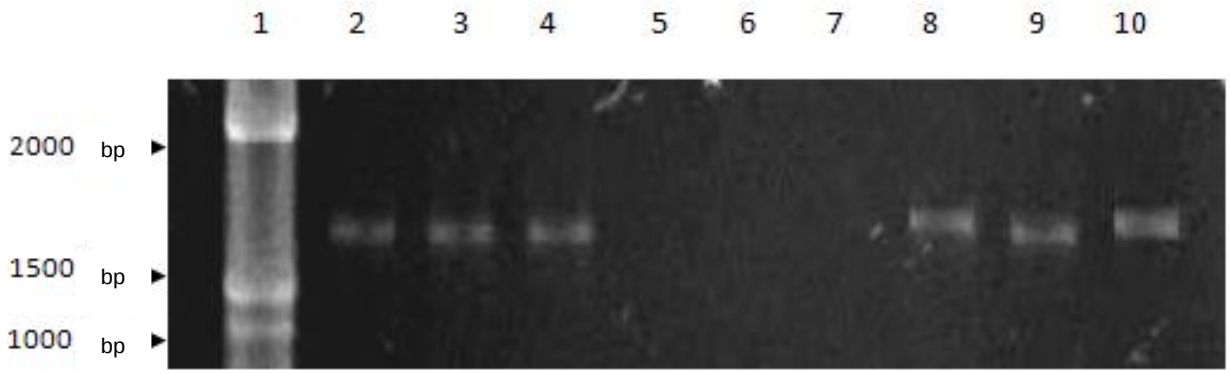

Figure 7. Detection of the amplified DNA fragment (≈1100 bp) using 100 bp DNA ladder; Lane1: DNA marker (Amersco® 100 bp DNA ladder). Lane 2, 3 and 4: Amplified DNA fragment from hairy roots induced from *A. rhizogenes* strains MAFF 210265, MAFF 301726 and MAFF 720002 (lane 2, 3 and 4). Lane 5, 6 and 7: Amplified DNA fragment from control roots from *A. rhizogenes* strains MAFF 210265, MAFF 301726 and MAFF 720002 (lane 5, 6 and 7). Lane 8, 9 and 10: Amplified DNA fragment from *A. rhizogenes* strains MAFF 210265, MAFF 301726 and MAFF 720002 (lane 8, 9 and10).

A. rhizogenes strain. These primers can amplify the *rol C* through the open reading frame13 (*rol* D), thus gives an amplified region of *rol* genes at the size of 1132 base pair (bp) (Kiyokawa et al., 1992). All the wild type of *Agrobacterium rhizogenes* strains were obtained from the same source which is from Institute of Agrobiological Sciences, Japan, therefore, it were expected to have the same sequence of rol genes and hence, the same primers 1724C and 1724D were used to test the all the transformed plants.

The gel electrophoresis results for hairy roots and for the *A. rhizogenes* MAFF 210265, MAFF 301726 and MAFF 720002 for the amplified DNA product exhibited the expected product size of 1100 bp as observed in Lanes 2, 3, 4, 8, 9 and 10 (Figure 7). No band or amplified DNA products were observed at Lanes 5, 6

and 7 (Figure 7), that electropherosised with DNA product of the normal roots. As to determine whether, the amplified DNA product is not due to bacterial contamination but rather due to the integration into the plant genome, method described by Seibi and Prakash (2000) were followed. Prior to PCR experiment, hairy root segments were cultured in both MS liquid and LB broth over a period of 30 days and no bacterial contamination were detected in both the media. Therefore, the PCR products obtained were from the integration of the *rol* genes from the T-DNA of bacterial plasmid into the plant genome and not due to bacterial contamination.

As conclusion, this study showed that hairy roots can be induced from the valuable medicinal plant *E. longifolia*. More studies can be conducted to further enhance the proliferation of the hairy roots.

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