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Full Length Research Paper

Chemotaxis movement assay of *Eurycoma longifolia* using wild and disarmed strains of *Agrobacterium rhizogenes*

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Bacterial chemotaxis is considered the first step in the interaction between motile bacteria and plant cells. Chemotaxis initiates the process of bacterial infection towards the plant cells and thus conferring beneficial attributes to the host. In this study, 5 wild strains and 2 disarmed strains of *Agrobacterium rhizogenes* were tested for chemotaxis assay using the swarm agar plate method. As expected, strong positive chemotactic response was observed in most of the tested bacteria strains and all the tested strains of *Agrobacterium rhizogenes* showed positive chemotactic response towards the tested root and somatic embryos of the valuable medicinal plant, *Eurycoma longifolia.* Therefore, induction of hairy roots is possible in *Eurycoma longifolia.* Generating hairy roots in *Eurycoma longifolia* will be highly beneficial mainly to the pharmaceutical industry as this medicinal plant possesses the capacity to produce many secondary metabolites which is proposed to increase sexual virility properties and to have anti cancer properties.

Key words: *Eurycoma longifolia,* chemotactic movement, *Agrobacterium rhizogenes,* roots, somatic embryos.

INTRODUCTION

Eurycoma longifolia has always been regarded as one of the most important traditional remedies in countries of South-East Asia. Various parts of this plant have been and are still are being used to cure numerous diseases. The root extracts of *E. longifolia* have been scientifically proven to enhance virility and sexual prowess (Gimlette et al., 1977) when administered. In addition, Ang et al. (2002) were able to provide scientific evidence of orientation activities (anogenital sniffing, licking and mounting) in sexual behavior among middle-aged male rats after administering *Eurycoma 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* possess anti-malarial activity. Malaria causes millions of death worldwide every year. A cure for this pandemic is yet to be found due to the resistant *Plasmodium sp.* 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. Anti cancer properties of *E. longifolia* roots have been demonstrated by Kardono et al. (1991) by isolating and characterizing five cytotoxic constituents and scientifically prove that it possesses cytotoxic effects against various human cancer cell types which includes breast cancer, colon cancer, fibrosarcoma, lung cancer, melanoma and also against murine lymphocytic leukemia. Researches are currently being done to scientifically prove the medicinal value of the various parts of this plant, which are commonly used in folk medicine.

Chemical attraction of bacteria towards plant exudates

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plays a major role for the bacteria prevalence in the rhizosphere. The capacity to colonize the rhizosphere of a host plant could be favoured and even increased by several components of the root exudates, and could induce some temporary modifications in the structure of bacterial lipopolysaccharides (Begonia et al., 1999). Chemotaxis is visualized as a sharp ring of growth that forms and spreads to the edge of the plate as bacteria cells swim through the agar following the gradient of attractant created as they metabolize the attractant compound (Rebecca et al., 2002). This mechanism is activated by changes in pH, temperature, osmolarity, viscosity, chemicals and some of them are nutrients or related compounds, such as secondary metabolites (Blair, 1995). Generally, bacteria evolve to accommodate and survive in an ever-changing, often hostile environment. Due to this reason, many bacteria have flagella, usually external organelles that propel bacteria through a liquid or near-liquid medium, or sometimes over surfaces. These flagella consist of a helical structure linked to a basal body in the membrane through a hook (Macnab, 1992). Bacteria usually monitor the changing concentrations of favourable or unfavourable substances and adjust their tendency to run or tumble so that they move into a favourable region (Hedblom et al., 1980).

Agrobacterium rhizogenes carries T-DNA that induces the formation of hairy roots after its integration into the plant genome. This Ri T-DNA alters the phenotype of the transformed roots and the plants that regenerate from them (Tepfer, 1984). Hairy roots have been induced in many dicotyledonous plants by transformation with *A. rhizogenes* Ri T-DNA (Costamtino et al., 1994).

The hairy roots are able to regenerate whole viable plants with high genetic stability. Hairy roots offer a valuable source of root-derived phyto-chemicals that are useful as cosmetics, pharmaceuticals products and food additives. Transformed roots of many plant species have been widely studied for the *in vitro* production of secondary metabolites (Mukundan et al., 1998). They can be a promising source for the continuous and standardized production of secondary metabolites. Hairy roots produce secondary metabolites over several successive generations without losing genetic or biosynthetic stability. This property can be utilized by genetic engineering to enhance their biosynthetic capacity. Hairy roots would be the best choice for metabolic engineering of secondary metabolite pathways to enhance the accumulation and secretion of high value metabolites. Thus this attribute makes hairy roots a better choice compared to propagation through tissue culture method, which is a very laborious process. The hairy root system is more advantageous because the tissue culture process can only be done under sterile laboratory conditions using sterile media. However, the hairy root system can be induced and grown without the laboratory condition, which not only saves the production cost but also increases many fold the yield of the secondary metabolite production in a much simpler way. In addition, The secondary metabolites can be harvested directly with few extraction steps compared to the conventional tissue culture techniques.

In this present study, we report that it is possible to induce hairy roots in *E. longifolia* since there is evidence that both wild and disarmed *A. rhizogenes* are chemically attracted to this medicinal plant. Thus, this is the first report that focuses on the chemotaxis movement of *A. rhizogenes* towards the valuable medicinal plant, *E. longifolia.*

MATERIALS AND METHODS

Plant materials

Root explants measuring 1 cm in length were used in this work. For intact roots, the 1 cm roots was used and for wounded roots, were prepared by cutting intact roots at the length of 1 cm and slicing it to same size to obtain 4 slices. Somatic embryo explants were prepared by isolating embryos with sizes of 3-4mm. For intact embryos, the 3 to 4 mm embryos were used and for wounded embryos, they were prepared by taking the 3-4mm embryos and cutting each embryo into 4 slices. In the positive control, the root and somatic embryo explants were replaced with filter paper with the same length and dipped in either liquid Chemotaxis Media with Bacteriological Agar (CMBA) or Chemotaxis Media with Luria Bertani agar (CMLBA). In the negative control, the root inducing bacteria *A. rhizogenes* were replaced with *Escherichia coli* DH5α.

Bacteria strains

In this experimentation, 5 wild strains of *A. rhizogenes* namely MAFF106590, MAFF106591, MAFF201265, MAFF301726 and MAFF720002 (gifts from the Institute of Agrobiological Sciences, Japan) and 2 disarmed strains namely AR14 and LBA9402 (gifts from Dr. David Tepfer) were used. Prior to use in this experiment, all the bacteria were grown in Luria Bertani Agar to obtain single colonies.

Chemotaxis assays

Chemotaxis assays were carried out according to Shaw's protocol (1995) for the modified swarm agar plate method. Using a toothpick, bacteria were inoculated in the middle of a Petri dish containing chemotactic media (CM: 10 mM phosphate buffer, pH 7.0; 1 mM ammonium sulfate; 1 mM magnesium sulfate; 0.1 mM potassium-EDTA) partially solidified with 0.2%(w/v) bacteriological agar or Luria Bertani Agar. Chemotaxis was quantified after 24 h incubation at 28°C. The swarming distance from the point of bacterial inoculation toward (T) and backwards (B) from the source of tissue exudates were measured and used to obtain a ratio(R) of the bacterial movement using the following formula:

$$R = T / B$$

Thus, R values over and under 1.00 represent positive or negative chemotaxis, respectively. Each set of experiment were repeated twice in triplicates. Means in the experiment were analyzed through a two-way ANOVA and differentiated with Duncan's test.

Media

The chemotaxis assay was done using two different types of

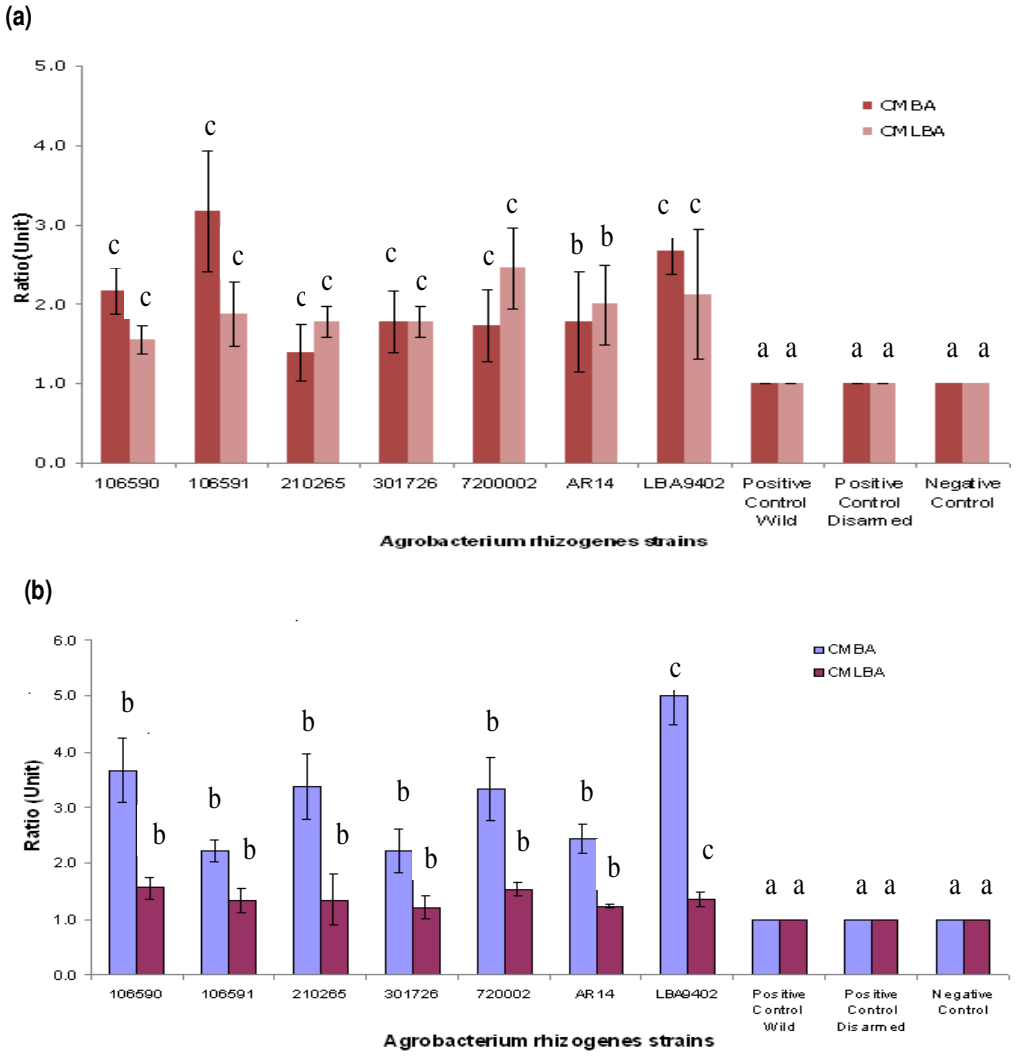

Figure 1. Chemotaxis assay on *in vitro* roots of *Eurycoma longifolia* (a) intact and (b) wounded explants. Each set of experiment were done in triplicates. Means in the experiment were analyzed through two-way ANOVA and differentiated with Duncan's test.

chemotaxis assay media. The first type of media was prepared using the method described by Shaw's protocol (1995) and the second type of media was prepared with a slight modification of the above mentioned method. The first type of media was named Chemotaxis Media with Bacteriological Agar (CMBA) and the second type of media, with a slight modification, was named as Chemotaxis Media with Luria Bertani Agar (CMLBA).

Statistical analyses

Each set of experiment was done in triplicates. Means in the experiment were analyzed through a two-way ANOVA and differentiated with Duncan's test.

RESULTS AND DISCUSSION

Studies on attraction and migration of beneficial rhizosphere bacteria provide important information about ecological traits for root colonization (Macario et al., 2003). Chemotaxis assay on *in vitro* explants of *E. longifolia* were done for both intact and wounded roots and embryos. Chemotaxis assay on *in vitro* roots indicates a higher ratio of positive mobility in wounded roots compared to intact roots. In wounded roots, the strongest chemotactic mobility was observed in *A. rhizogenes* LBA9402 and followed by the *Agrobacterium* strain MAFF106590 (Figure 1b). Almost similar results in terms of mobility were obtained for *Agrobacterium* strains namely MAFF 210265 and MAFF 720002 (Figure 1b). The strongest chemotactic mobility was observed in *A. rhizogenes* MAFF 106591 and followed by the strain *Agrobacterium* LBA 9402 in intact roots (Figure 1a).

Chemotaxis assay for somatic embryogenesis were done for somatic embryos measuring 3 to 4 mm in size.

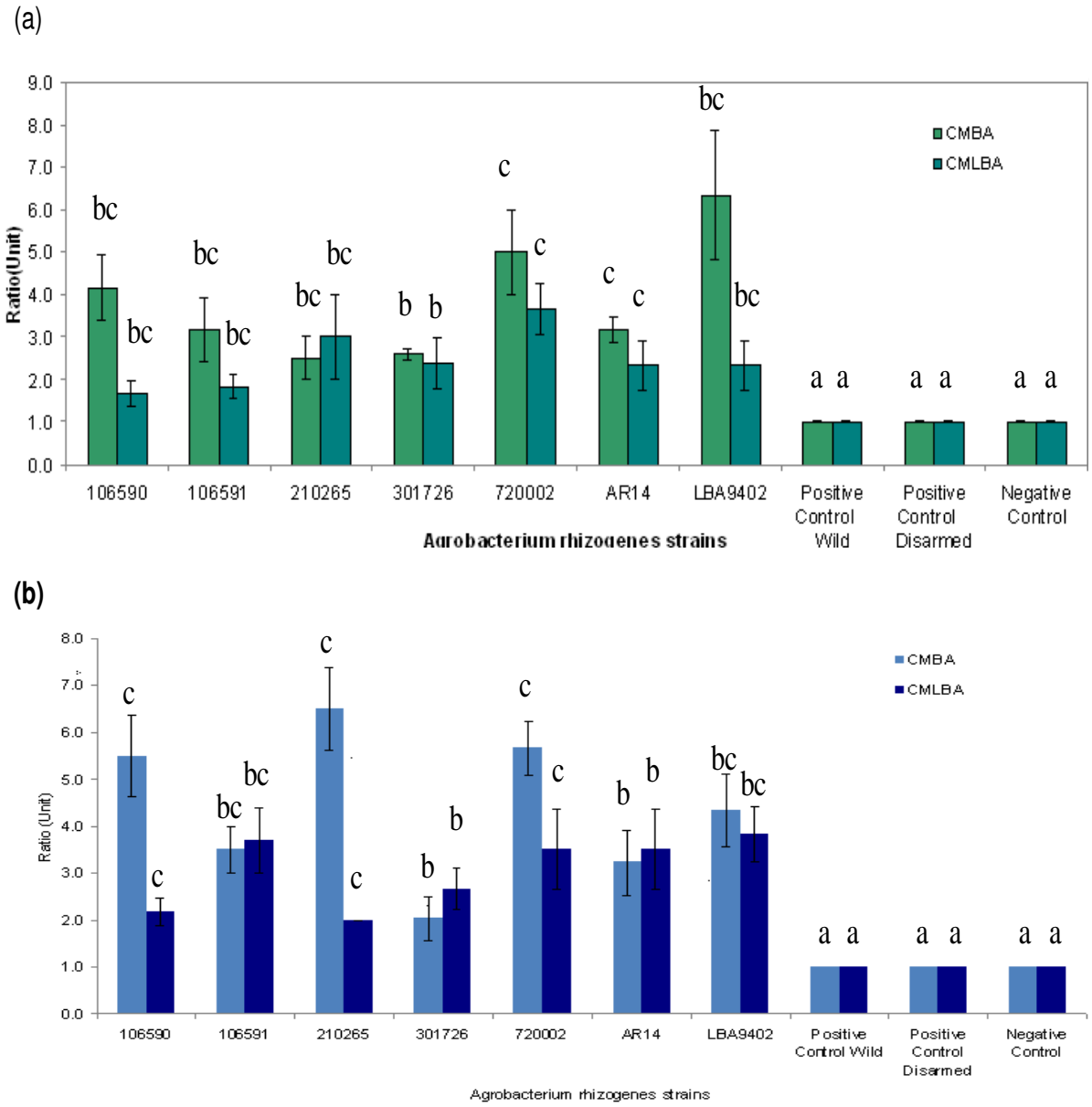

Figure 2. Chemotaxis assay on somatic embryogenesis of *Eurycoma longifolia* (a) intact and (b) wounded explants. Means in the experiment were analyzed through a two-way ANOVA and differentiated with Duncan's test.

Somatic embryos of 1 to 2 mm in size were not used in this study because they were found not suitable as they tend to dry up very fast during the study and the accurate mobility measurement could not be obtained from them. For intact somatic embryos, the strongest chemotactic movement were observed in strain LBA 9402 and followed by *A. rhizogenes* MAFF 720002 (Figure 2a). However, in wounded embryos, the results show a higher *A. rhizogenes* mobility in *A. rhizogenes* strain MAFF 210265 (Figure 2b). The mobility ratio for strains MAFF 106591 and MAFF 720002 were quite similar (Figure 2b). The overall results for somatic embryos indicate a higher ratio of positive mobility in wounded embryos compared to the intact embryos. Therefore, results obtained for both roots and somatic embryogenesis explants indicate a higher ratio in the wounded treatment. Under natural conditions, chemotaxis of *Agrobacterium* to wounded plant cells is the first event for bacterial infection towards plant cell (Sreeramanan et al., 2009).

Chemotaxis assay

Both results shows strong positive chemotactic response

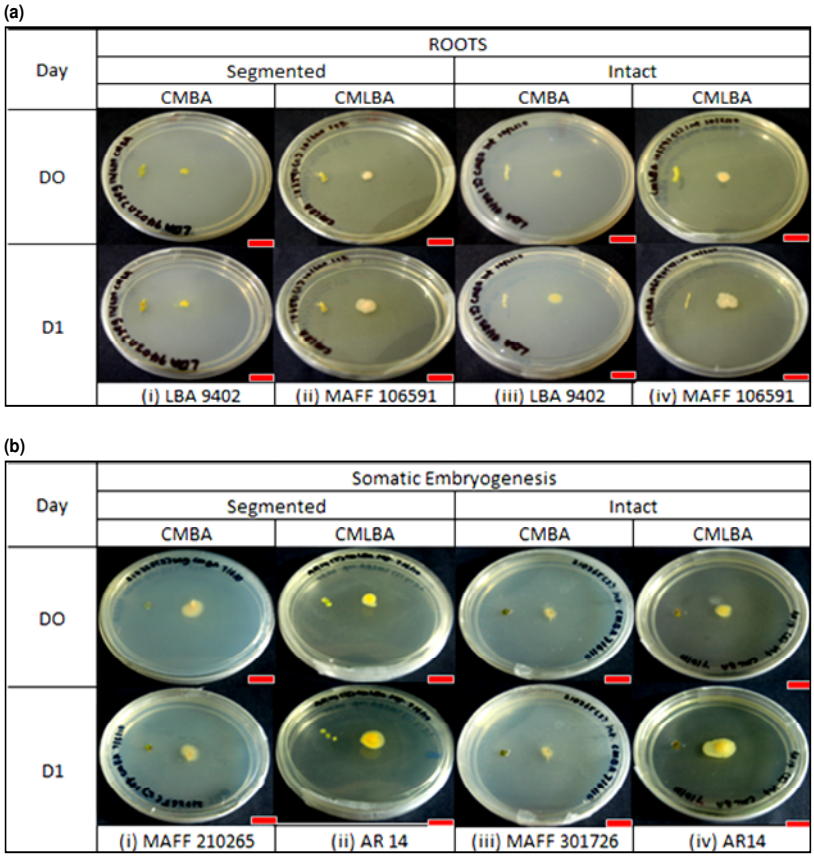

Figure 3. Chemotaxis assay for (a) roots and (b) somatic embryogenesis of *Eurycoma longifolia* using swarm agar plate method. Pictures show chemotaxis of *Agrobacterium rhizogenes* strain LBA 9402 for intact (a [i]) and wounded (a [iii]), strain MAFF 106591 for intact (a [ii]) and wounded (a[iv]), strain MAFF 210265 for intact (b[i]) and wounded (b [iii]) and strain AR14 for intact (b [iii]) and wounded (b [iv]) for roots and somatic embryogenesis of *Eurycoma longifolia* on the day of inoculation (DO) and after 24 h (D1) (bar represents 2 cm in length) in CMBA and CMLBA media.

in all the *A. rhizogenes* strains tested, as there is no negative value in the results obtained. All the values for the ratio had more than one values. Results obtained from the two way ANOVA indicates that there were significant differences ($P<0.05$) in all the strains' ability to respond to the chemical stimulus. This is because the O-Specific Polysaccharide (OPS) is responsible for the antigenic property (Christina et al., 2008) of LPSs (lipopolysaccaharides) which is the main component of the bacteria cell membrane. The composition or the size of the OPS modulates the virulence potential of the bacterium. The reason for the structural variability observed here is mainly due to the different sugar substituent(s) and attachment, which are often constituted by phosphate groups (Christina et al., 2008). Therefore, different strains of *A. rhizogenes* used in this study may differ in their pathogenicity and virulence.

In addition, the type of media significantly influences the chemotactic ability of the *A. rhizogenes* strains in its mobility towards its stimulus. It has been revealed that the mobility of *A. rhizogenes* strains in CMBA media is better than in the CMLBA media. The *A. rhizogenes* strain LPSs plays a major role in the bacteria–plant interaction. The different media composition influences chemotactic movement. The CMLBA media which is the *in situ* nutrition rich media influences less mobility of bacteria towards the plant exudates due to the fact that since it is a nutrition rich media, the bacteria were not so attracted to the explants as the nutrients to grow were available *in situ*. However in CMBA media, since there is no *in situ* nutrient available for the bacteria, *Agrobacterium rhizogenes* were chemotactically attracted to the explants. Comparatively, more exudates were released from wounded explants compared to intact explants. Therefore, there was a higher ratio of mobility of *A. rhizogenes* towards plant exudates in CMBA compared to CMLBA media for both type of treatment and explants (Figure 3).

In bacteria complex gene expression, systems have been developed to turn on or off certain groups of genes in response to specific environmental conditions (Liam et al., 1995). To transfer its t-DNA into the plant cell, the bacterium has to be adsorbed on the wounded area modulated by the components of the external membrane of the bacterium; both the proteins and the LPSs (Pueppke et al., 1984).

Currently, the most common method of propagating *E. longifolia* is through its seeds. However, being a recalcitrant plant, the seeds have a low germination precentage and it takes a long time to germinate due to its extremely immature state of zygotic embryo at the time of dispersal. The gradual dissapearance of this plant is attributed mainly to the indiscriminate collection of its tap root as the raw material for the preparation of drugs. Thus, it needs to be rapidly mass-multiplied on a commercial scale to meet the needs of the herbal as well as pharmaceutical industries (Sobri et al., 2005). Therefore, *in vitro* techniques like induction of hairy root can be an important alternative approach to produce useful plant chemical products. Hairy roots offer numerous advantages, due primarily to their fast growth rate in the phytohormone-free medium. The greatest advantage of hairy roots is that its cultures often exhibit some or greater biosynthetic capacity for secondary metabolite production as compared to their mother plants. Many valuable secondary metabolites are synthesized in roots *in vivo* and often, synthesis is linked to root differentiation. Therefore, chemotaxis of *A. rhizogenes* towards plant root cells is the first event required for bacterial infection and disease development, a process of primary importance for an opportunistic pathogen which plays a major role in the induction of hairy roots.

In conclusion, all the tested strains of *A. rhizogenes* showed positive chemotactic response towards the tested root and somatic embryogenesis of the recalcitrant valuable medicinal plant, *E. longifolia.* This study may contribute to the development of more efficient *E. longifolia* production systems due to the colossal market for *E. longifolia* derived products.

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