Poster:

The effect of strain virulence on *Agrobacterium rhizogenes* transformation efficiency in *Eurycoma longifolia*

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Abstract. *Eurycoma longifolia*, known locally as 'Tongkat Ali' in Malaysia, is popularly sought out as herbal remedy in many parts of South East Asia. Consequently, this has resulted in the indiscriminate collection of the taproot from the wild, leading to fears of extinction among conservationists. *Agrobacterium rhizogenes* is a naturally occurring soil bacterium that infects injured plants and causes a massive proliferation of roots, known as hairy roots. The objective of this study is to determine the transformation efficiency of 2 different strains of *Agrobacterium rhizogenes* on somatic embryos of *Eurycoma longifolia* using transient GUS expression as an indicator of successful transformation. Somatic embryos cultured *in vitro* for 4 to 5 weeks were used as explants and were pre-cultured for 2 days in full strength MS medium containing 0.5 mg/L IBA added with 1% PVP and 2mg/L DTT. The explants were transformed using *Agrobacterium rhizogenes* strains AR12 and AR14. GUS assay was carried out 1 week after transformation and observed. Observations indicate that AR12 is more effective in transforming somatic embryos of *Eurycoma longifolia* compared to AR14. Therefore, *Agrobacterium rhizogenes* strain AR12 is a potential candidate for the successful transformation of *Eurycoma longifolia* somatic embryos, leading to the induction of sustainable hairy root cultures.
Keywords: Virulence, *Agrobacterium rhizogenes*, AR12, AR14, *Eurycoma longifolia*, somatic embryos.

Introduction

Eurycoma longifolia, a plant in the Simaroubaceae family, is native to South East Asia and is often used as folk medicine in the treatment of aches, persistent fever, tertian malaria, sexual insufficiency, glandular swelling and as health supplements (Kuo *et al*. 2004). However, the indiscriminate collection of the taproot from the wild as the raw material for the drug preparations has triggered concerns about diminishing populations, loss of genetic diversity, local extinctions and habitat degradation. Therefore, 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).

The soil-borne bacterium *Agrobacterium rhizogenes* is the causative agent of hairy root disease in dicotyledonous plants. This disease results from the transfer and integration of T-DNA of root-inducing (Ri) plasmid to the plant genome (Schmülling *et al*. 1989). The transfer of T-DNA is mediated by virulence genes, which form the *vir* region of the Ri plasmid and the bacterial *chv* genes (Giri & Narasu, 2000). Among the various factors influencing the frequency of *Agrobacterium*-mediated transformation, one of the most important is the infecting ability of the *Agrobacterium* strain, known as its virulence (Omid 2008).

Therefore, this study was conducted to determine the best condition for hairy root formation in somatic embryos of *Eurycoma longifolia* by infecting them with two strains of *A. rhizogenes*, AR12 and AR14, and using transient GUS expression as an indicator of successful transformation.

Materials and Methods

Plant material

The source of *Eurycoma longifolia* plant used in this study was kindly provided by Felda Biotechnology Centre, Kuala Lumpur, Malaysia. The *in vitro* embryogenic callus cultures provided were induced into somatic embryos and multiplied in embryo multiplication medium as specified by Sobri *et al*. (2005).

Bacterial strains

A. rhizogenes strains AR12 and AR14 (courtesy of Dr. Lene H. Madsen, Denmark), were used to determine transformation efficiency. The bacteria were maintained on Luria Bertani (LB) semi solid media containing 100mg/L rifampicin. Prior to infection the bacterial strains were grown for 24 hours in 30ml LB broth medium at 28°C on a rotary shaker at 120rpm.

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Transformation of somatic embryos

4-5 weeks old clumps of somatic embryos sized between 5-6mm were pre-cultured in full strength MS (Murashige & Skoog, 1962) medium with 0.5mg/L indole-3-butyric acid (IBA) added with 1% (v/v) polyvinyl pyrrolidone (PVP) and 2.0mg/L dithiothreitol (DTT) at 24°C in the dark for 2 days. OD_{600} of the overnight bacterial suspension was adjusted to 0.5-0.6 and added with 100µM acetosyringone. The pre-cultured embryos were then cut in half, into about 2-3mm length pieces and immersed in the bacterial suspension for 20 minutes. The explants were blotted dry on sterile filter paper to remove excess bacteria and placed back on their original culture plates. In addition, embryos were also immersed in LB broth medium containing 100mg/L rifampicin and incubated in the same conditions as control. After 3 days of co-culture at 24°C in the dark, the explants were washed in hormone-free full strength MS broth medium containing 500mg/L cefotaxime to eliminate bacteria and transferred to fresh semi solid MS medium, similar to pre-culture conditions for 4 days. The explants were washed again with 500mg/L cefotaxime before conducting GUS assay (Jefferson 1987) using a modified protocol described by Alpizar *et al*. (2006).

Results and Discussion

Two strains of *A. rhizogenes* were used to infect *in vitro* somatic embryo explants of *E. longifolia* and transient GUS expression was observed in explants transformed with AR12 and AR14. Blue spots indicative of positive GUS activity were not observed on any of the negative control explants. The transformation efficiency of AR12 is 84.17% and is significantly higher than AR14 at 68.33%. The lack of β-glucuronidase activity on negative control explants affirms that *E. longifolia* somatic embryos do not possess indigenous GUS genes.

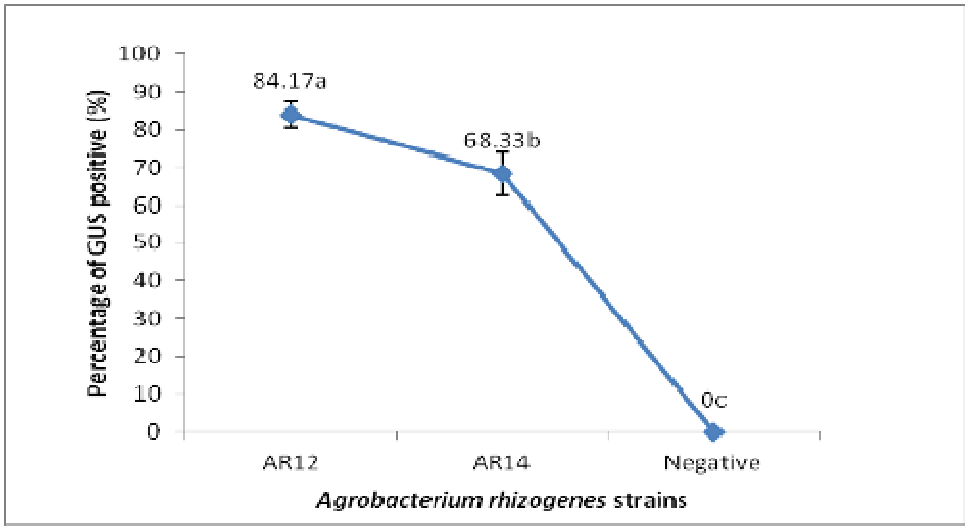

Figure 1. Frequency of transient GUS expression on *E. longifolia* somatic embryos transformed with *A. rhizogenes* strains AR12 and AR14. Mean with different alphabet on top is significantly different (Tukey's Test, α= 0.05).

Different strains of *A. rhizogenes* vary in their ability to successfully infect and transform the target plant. Morteza *et al*. (2008) reported that there was strain and species specificity in the hairy root induction of four *Hyoscyamus* species using five different strains of *A. rhizogenes*. Similarly, in the genetic transformation of *Torenia fournieri*, the strain with the highest root induction had 65% transformation frequency while the lowest stood at 28.3% (Tao & Li, 2006). When Alpizar *et al*. (2006) compared five strains of *A. rhizogenes* for their transformation efficiency on two varieties of coffee plants, they determined that A4RS, an agropine type strain, was highly virulent compared to others strains with 80% efficiency. The difference in virulence could be explained by the plasmids harboured by bacterial strains (Nguyen *et al*. 1992) and the presence of *rolA*, *B* and *C* genes were demonstrated to be sufficient for producing the hairy root phenotype (reviewed in Christey 2001).

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Conclusions

A systematic study using two different strains of *A. rhizogenes* was carried out to determine the best condition for hairy root formation in *E. longifolia*. The data presented confirm the results of previous investigations that different bacterial strains have various transformation efficiencies. A. rhizogenes strain AR12 is a potential candidate for the successful transformation of *E. longifolia* somatic embryos. However, further research is needed to evaluate hairy root induction from transformed somatic embryos.

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