*Short scientific report*

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# Transient expression of chitinase gene in *Bacillus subtilis* : An endophyte of *Hevea brasiliensis*

*Keywords: Bacillus* sp., Rubber, Bio-control, chitinase, *Corynespora* leaf disease, endosymbionts

The leaf fall disease caused by the fungus *Corynespora cassiicola* is one of the major diseases affecting *Hevea*. *Corynespora* causes leaf spot and leaf fall in both immature and mature rubber trees (Situmorang *et al.*, 1996). Severity of the disease mainly depends upon factors like climate, virulence of the pathotypes and susceptibility of the host. To control *Corynespora*, Bordeaux mixture or 0.24 % Zineb (Dithane Z78) (Ramakrishnan and Pillai, 1961), carbendazim (Bavistin) spray (Rajalakshmy *et al.*, 1980), and mancozeb (Dithane/Indofil M45) (Jacob, 1997) are used. However, biological control is the most opted one as it is safer to the environment. In nature, biological control occurs at some level in all agricultural ecosystems. Biological control of fungal diseases involves degradation of chitin, the main constituent of the fungal cell wall by chitinase enzyme through hydrolysis of its β-1,4-linkage between N-acetyl glucosamine residues. *Hevea* produces chitinase as a pathogenesis related (PR) protein, against various fungal infections (Neuhaus, 1999). But most of the fungi, including *C. cassiicola* develop resistance so quickly and spread the disease in a short period. To counter this, it would be appropriate to have higher levels of chitinase expressed in the host.

Recently, endophytic bacteria such as *Bacillus subtilis*, (Philip *et al.*, 2005) with antifungal activity have been reported to be present in leaf, stem, bark, etc. of rubber trees. Endosymbionts are known to render resistance to fungal pathogens (Kloepper *et al.*, 1999; Gray and Smith, 2005; Compant *et al.*, 2005a), stress tolerance (Timmest and Wagner, 1999), inducing systemic resistance (Kloepper and Ryu, 2006) and in regulation of growth (Verma *et al.*, 2001; Pirttilla *et al.*, 2004; Wakelin *et al.*, 2004; Lee *et al.*, 2004, Compant *et al.*, 2005b). These endosymbionts, if transformed to express a foreign gene, can be a potential medium for inducing resistance to various stresses/diseases in *Hevea*. Hence, this study was initiated to develop a transformation protocol for *Bacillus subtilis* (the endosymbiont isolated from *Hevea*) and to over express chitinase gene (from *Hevea*) by using a *Bacillus* specific expression vector (pHCMC05) (Nguyen *et al.*, 2005). This vector (Fig. 1) was obtained from *Bacillus* Genetic Stock Centre (BGSC), Ohio and chitinase gene was placed in its expression cassette. Expression of chitinase in *Bacillus* cells was assessed and discussed in this report.

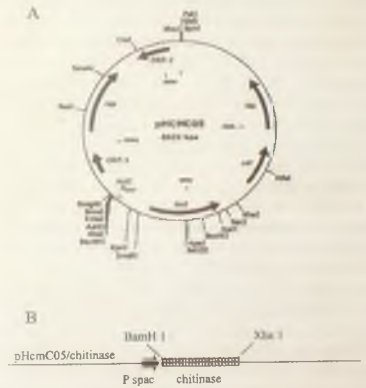


Fig. 1. A. The plasmid map of *Bacillus subtilis* transformation vector pHCMC05

B. Map of pHCMC05 inserted with chitinase in the *Bam*H I and *Xba* I restriction sites immediately after the Pspac promoter.

Total RNA was isolated from *Corynespora cassiicola* infected leaves (clone GT1) using Ambion RNA isolation kit and cDNA was synthesized using chitinase specific primers flanked with restriction sites [5' GGATCCCATGGCCAAAAGAAC CCAAGCC 3' and 5'TCTAGACCGAATAATTTCTCCCAATTCA 3') (*Bam*H I in the forward primer and *Xba* I in the reverse

primer]. PCR was performed according to standard procedure and the products were purified using PCR purification kit by Sigma, USA. Restriction digestion, ligation and transformation steps were performed using standard procedures and according to the manufacturers instructions. Restriction enzymes such as *Bam*H I and *Xba* I were purchased from Stratagene, USA. Gel elution was done using MinElute gel purification kit, Qiagen. T4 DNA Ligase (Promega, USA) was used in the ligation reactions. Competent cells of *E. coli* was purchased from GenHunter, USA.

Standard method for *Bacillus* transformation was followed. 50 µl of inoculum from overnight grown *Bacillus subtilis* culture was subcultured into 50 ml fresh LB medium and grown overnight in an incubation shaker at 37°C with 200 rpm. 100 µl of this culture was inoculated into 100 ml of freshly prepared GM1 medium followed by overnight incubation at 27°C without shaking. Next morning, the culture was shaken at 37°C with 200 rpm until it reached the stationary phase. The cell growth was monitored every hour using the 'Nanodrop' spectrophotometer (at 560nm). One and a half hour after reaching stationary phase, 1.0 ml of culture was transferred into 9.0 ml of GM2 medium and incubated at 37°C for 60 min. with 200 rpm shaking. 900 µl from this culture was incubated with 100 µl of plasmid DNA [20ng/µl] at 37°C in a shaker at 200 rpm for 60 min. After an hour of incubation, the culture was spun at 5000 rpm for 2 to 3 min. and the pellet was resuspended in 400 µl of GM2. The mixture was plated on suitable selection medium and incubated at 37°C overnight. To measure the chitinase activity, LB medium (50 ml) was inoculated with *Bacillus subtilis* cells and incubated in a shaker at 37° C with 200 rpm till mid log phase at which IPTG was added to a final concentration of 1mM. 1ml of sample was collected at hourly intervals till seven hours and the collected samples were centrifuged at 8000 rpm for 5 min. The pellets after resuspending in minimal volume (200 µl) of medium and lysozyme (250 µg/ml) were sonicated (12W; 6×15 with 15 second intervals) and centrifuged at low rpm (430 x g) for 10 minutes. Chitinase acitivity was measured in the aliquoted supernatant according to Boller and Mauch (1988). To test the colonizing ability of the transformed endosymbionts in *Hevea* leaves, the leaves applied with transformed *Bacillus subtilis* were collected after 72 hours of application, surface disinfected, ground, briefly spun and supernatant spread on Lauria Bertini agar plates with suitable antibiotic and kept for overnight incubation at 37°C. The numbers of colonies formed per unit leaf (cfu) area were recorded.

A 978 bp coding region of chitinase gene from leaves of *Hevea* was PCR amplified (Fig. 2) and cloned into pGEM-T Easy vector. This clone when sequenced showed 99 % similarity with the chitinase gene

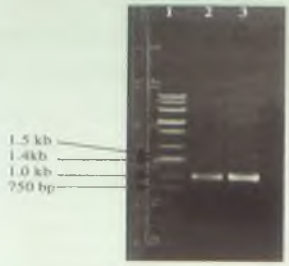


**Fig. 2. PCR amplification of chitinase gene from cDNA synthesized from leaf samples of *Hevea brasiliensis***

(ACCESSION DQ873889) available in the GenBank database. Later, chitinase gene in pGEM-T Easy vector was restriction digested with restriction enzymes *Bam*H I and *Xba* I and the released fragment was gel eluted after running on agarose gel electrophoresis. After gel purification, this fragment was ligated into the corresponding sites in the pHCMC05 to obtain the pHCMC05/chitinase construct. This ligated mixture was used to transform *E. coli* cells and the transformed colonies were selected for further plasmid DNA preparation. Subsequently, this plasmid DNA (pHCMC05/chitinase) was used to transform *Bacillus subtilis* using protocol standardized in our laboratory and the transformed cells were selected on Luria Bertani agar medium with chloramphenicol. Transformation was confirmed by slot lysis and PCR of the isolated plasmid (Fig. 3). Expression of chitinase gene in *Bacillus* cells was confirmed by estimating the chitinase activity. The cells had very high chitinase activity when compared to the negative control (pHCMC05 vector alone in *Bacillus* cells) (Fig. 4).

The experiment conducted to assess the colonizing ability of the transformed endosymbionts in *Hevea* leaves, yielded quite a good number of colony forming units indicating the colonizing ability of the transformed *Bacillus subtilis* in the intercellular regions of *Hevea* leaves. The colonies were further confirmed by plasmid DNA isolation and PCR amplification of chitinase gene.

These results have proved beyond doubt that *Bacillus subtilis*, an endosymbiont of *Hevea* could be employed as a viable medium for facilitating over-


Lane 1: Wide range DNA marker (Sigma)
Lane 2-4: pGEM-T/chitinase construct in *E. coli* sample 1-3
Lane 5-7: pHCMC05/chitinase construct in *Bacillus* sample 1-3

**Fig. 3. Confirmation of presence of chitinase gene in Bacillus cells after transformation by chitinase specific PCR amplification**

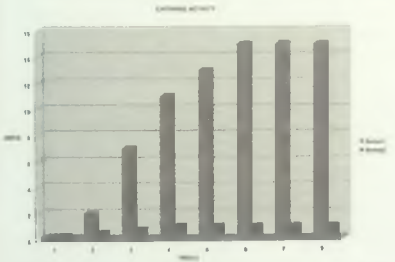

**Fig. 4. Chitinase activity in units per glucosamine produced $min^{-1}$ $ml^{-1}$ culture**

**X axis -Time in hrs after IPTG induction, 1unit = 1hr**
**Z axis - Chitinase activity as absorbance at 585 nm, 1unit = 0.02 OD value**
**Red: Bacillus subtilis with pHCMC only (Negative control)**
**Violet: Bacillus subtilis with pHCMC/chitinase (Test sample)**

expression of foreign genes in *Hevea*. The difficulties being experienced in tissue culture and genetic transformation experiments in a tree crop like *Hevea* makes the use of endosymbionts more attractive as they are easier for manipulation and needs a shorter time for development.

Stability of this particular vector in transformed cells had been reported to be apparent between forty to sixty generations after which loss of plasmid DNA has been noticed (Nguyen *et al*., 2007). This property of the vector is an added advantage that the desired gene product intended for a shorter period can be successfully expressed and hence long time expression is not warranted. We can safely employ the endosymbionts transformed with this vector as and when required. Hence, this system of over-expression of gene of interest can be successfully tried in *Hevea* for rendering stress/ disease resistance.

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