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Agrobacterium tumefaciens-mediated transformation of *Trichoderma harzianum* CFAM-422 using an herbicide resistance gene as selection marker

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ABSTRACT

The use of lignocellulosic materials that are the constituents of the plant cell wall have shown to be a great opportunity for sustainable industrial development. The polysaccharide part of these materials, the cellulose and the hemicellulose, can be degraded into their monomeric sugars by microorganisms, such as Trichoderma harzianum, that secrete extracellular enzymes. However, the industrial production of hydrolytic enzymes rely on the development of molecular biology tools to achieve the economical production of enzyme pools with greater hydrolytic potential. In this work the fungus T. harzianum (CFAM-422) *was transformed by Agrobacterium tumefaciens and a herbicide was used as a selective agent, making it an efficient strategy to obtain strains with higher potential for the hydrolysis of lignocellulosic materials.*

1. INTRODUCTION

The abundance of lignocellulosic biomass associated with the search for alternatives to the use of fossil fuel became crucial for the establishment of the future bio-economy based on the concept of biorefinery nevertheless the high cost of production of the enzymes is a great obstacle (Zampieri, 2015; Hamre et al., 2015). Thus, the growing interest in the hydrolysis of lignocellulosic residues has justified the prospection and the studies of the gene expression in microorganisms producing enzymes related to the degradation of this material (Zampieri, 2015). The microorganisms of the genus *Trichoderma* have a great industrial importance. Its application extends from enzyme production to pesticides production and use as biocontrol agents (Mukherjee et al., 2013; Liu, 2004). This fungus is one of the most studied for the production of cellulolytic enzymes. *T.*

harzianum has been shown as a potential producer of a well-balanced cellulolytic complex, presenting rapid kinetics for the production of endoglucanases and β-glucosidases (Castro, 2010). Our group is applying genetic engineering techniques for strain improvement in this fungus. In this way, the adaptation of genetic transformation protocols for the manipulation of genes of interest is essential. Although there are selective marker genes available for fungal transformation, their use should be evaluated for each strain to be transformed. In this context, an *Agrobacterium tumefaciens*-mediated transformation protocol was previously adapted for *T. harzianum* CFAM-422 using hygromycin as a selective agent (Shimizu et al., 2015). The aim of this work was to adapt an *Agrobacterium*-mediated transformation protocol using phosphinothricin acetyltransferase (*bar*) gene as a selection marker, which confers resistance to the herbicide glufosinate, in order to expand the genetic tools to manipulate this cellulolytic fungus.

2. METHODS

2.1. Herbicide as Selective Agent

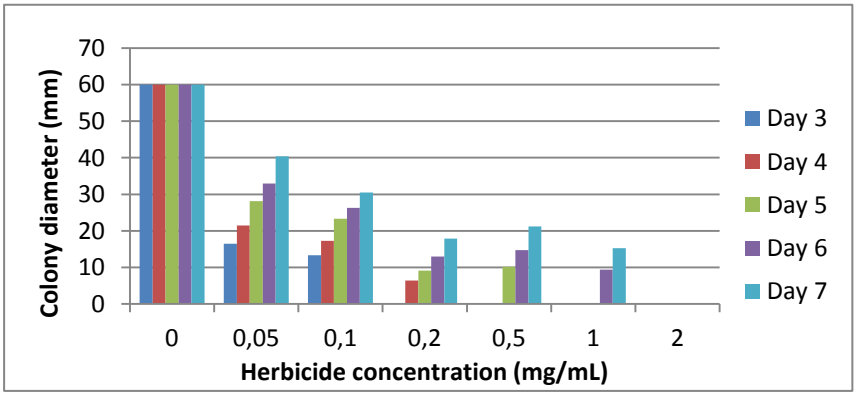
For the determination of the concentration of herbicide glufosinate as selective agent, 100 µL of spore solution of CFAM-422 strain was inoculated into Petri dishes (60x15mm) containing minimal medium with varying concentrations of herbicide (0; 0.05; 0.1; 0.2; 0.5; 1 and 2 mg/mL). Plates were incubated at 28 °C for 7 days and the growth diameter of the microorganism was monitored.

2.2. Fungal Transformation

Cells of *A. tumefaciens* EHA105 containing the binary vector p7UG-AB (with the *bar* gene of *Streptomyces hygroscopicus*, conferring resistance to the herbicide glufosinate ammonium, or phosphinothricin) was harvested from the stock in solid YEP culture medium plus spectinomycin [100 µg/mL] and rifampicin [100 µg/mL] and incubated for 2 days at 28°C. After growth, a colony was added in liquid YEP medium, added with the antibiotics and incubated at 28°C, 200 rpm until reaching OD_{600nm} 0.7. Subsequently, a volume of this pre-inoculum was added in liquid induction medium containing 200 µM acetosyringone, and incubated under the same conditions until OD_{600nm} 0.6. A suspension with $1x10^{6}$ conidia/mL of CFAM-422 in the solution was added and 100 µL was spread on the plates containing solid medium with 200 µM and 400 µM acetosyringone plus 0.1% Triton 100X containing nylon membranes. The membranes were transferred to new plates containing minimal medium plus 2 mg/mL of commercial herbicide Liberty and antibiotics after 24 and 48 hours. Transformants that were grown were counted, purified, and stored in Castellani stock.

3. RESULTS AND DISCUSSION

To determine the concentration of herbicide glufosinate to be used as a selective agent, the sensitivity of CFAM-422 strain was verified to a range of the reagent concentrations. From the third day of incubation the control colony (without herbicide addition) had already taken the entire plate. However, in the presence of increasing concentration of the herbicide the strain presented a decaying growth until the concentration of 1 mg/mL. Cell growth was fully inhibited with the concentration of 2 mg/mL. As such the herbicide glufosinate at this concentration was selected for the transforming strains in a context where only the transformants containing the plasmid that confer resistance to the herbicide would be able to grow.



Grafic 1: CFAM-422 sensitivity to herbicide glufosinate. Monitoring of the diameter growth of colonies of CFAM-422 from the third to the seventh day of incubation in the presence of several concentrations of herbicide (0; 0.05; 0.1; 0.2; 0.5; 1.0 and 2.0 mg/mL).

In the continuation of the work four different conditions, using acetosyringone at 200 µM and 400 µM after 24 and 48 hours of incubation were tested for the development of transformants in the presence of herbicide [2mg/mL] as selective agent. All tested conditions allowed the isolation of resistant colonies and a total of 72 transformants were obtained. The use of 200 µM of acetosyringone after the 24-hour of incubation generated the highest number of colonies as shown below. In the absence of acetosyringone, no transformant was recovered. This phenolic compound is required for the transfer of the T-DNA into the fungal cells.

Table 1: Number of transformants per Petri dishes after 24 and 48 hours of incubation using 200 and 400 µM of acetosyringone.

	Number of colonies				
	200 µM acetosyringone		400 µM acetosyringone		Control
Incubation time	24 hours	48 hours	24 hours	48 hours	48 hours
Plate 1	12	3	6	2	-
Plate 2	6	4	6	2	-

Plate 3	9	6	6	10	-

To confirm the transformation, the colonies obtained were grown on PDA medium containing herbicide and compared to the wild-type strain. As expected, all transformants grew while parental strain was inhibited.

4. CONCLUSION

Trichoderma harzianum (CFAM-422) that has been identified as a producer of a well-balanced cellulolytic complex was subjected to studies aiming its transformation by *Agrobacterium tumefaciens* using herbicide as a selective agent. This method that can be a major step to the future insertion of genes capable of increasing the potential for enzymatic hydrolysis.

5. REFERENCES

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