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Combinatorial effect of mutagenesis and medium component optimization on *Bacillus amyloliquefaciens* antifungal activity and efficacy in eradicating *Botrytis cinerea*

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ABSTRACT

This work is directed towards *Bacillus amyloliquefaciens* strain BLB371 metabolite production for biocontrol of fungal phytopathogens. In order to maximise antifungal metabolite production by this strain, two approaches were combined: random mutagenesis and medium component optimization. After three rounds of mutagenesis, a hyper active mutant, named M3-7, was obtained. It produces 7 fold more antifungal metabolites (1800 AU/mL) than the wild strain in MC medium. A hybrid design was applied to optimise a new medium to enhance antifungal metabolite production by M3-7. The new optimized medium (35 g/L of peptone, 32.5 g/L of sucrose, 10.5 g/L of yeast extract, 2.4 g/L of KH_2PO_4, 1.3 g/L of $MgSO_4$ and 23 mg/L of $MnSO_4$) achieved 1.62 fold enhancement in antifungal compound production (3000 AU/mL) by this mutant, compared to that achieved in MC medium. Therefore, combinatory effect of these two approaches (mutagenesis and medium component optimization) allowed 12 fold improvement in antifungal activity (from 250 UA/mL to 3000 UA/mL). This improvement was confirmed against several phytopathogenic fungi with an increase of MIC and MFC over than 50%. More interestingly, a total eradication of gray mold was obtained on tomato fruits infected by *Botrytis cinerea* and treated by M3-7, compared to those treated by BLB371. From the practical point of view, combining random mutagenesis and medium optimization could be considered as an excellent tool for obtaining promising biological products useful against phytopathogenic fungi.

1. Introduction

Plant's diseases caused by microorganisms are one of the major issues which threats crops and plant production (Perez-García et al., 2011). The worldwide important tomato crop is attacked by various fungi, especially when mature-green fruits were stored because of their vulnerability to chilling injury at temperatures below 10 °C and to increased microbial decay (Marangoni and Stanley, 1991). Many fungi genera could attack tomato fruits such as *Fusarium* spp., *Alternaria* spp., *Aspergillus* spp., and *Botrytis* spp. (Oladiran and Iwu, 1993; Osbourn, 1996; Williamson et al., 2004; Moss, 2008). Gray mold disease which is caused by the fungus *Botrytis cinerea* is one of the most severe diseases affecting tomato. Several fungicides effectively controlled this disease at preharvest stage. However, excessive use of chemical fungicides has contributed to the biodiversity impoverishment. It has got disastrous consequences for plants, animal species and public health (Geiger et al., 2010). In the other hand, *B. cinerea* frequently acquires resistance against these fungicides (Locke and Fletcher, 1988; Elad et al., 1992; Yourman and Jeffers, 1999), which has led to the development of safe alternatives.

Biological pest control, such as the use of biocontrol organisms against phytopathogenic fungi, has been investigated as an alternative control method to manage tomato diseases (Tscharntke et al., 2005; Cuppels et al., 2013). Several microorganisms such as *Trichoderma* (Gajera and Vakharia, 2010), *Burkholderia cepacia* (Kilani-Feki et al., 2011) and *Bacillus amyloliquefaciens* (Chiou and Wu, 2003; Mezghanni et al., 2012; Ji et al., 2013) have demonstrated their efficacy in controlling different species of fungi. *B. amyloliquefaciens* is particularly promising, due to its capacity to produce a variety of antibacterial and antifungal metabolites essentially

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zwittermicin-A kanosamine and lipopeptides (surfactins, iturin, and fengycin) (Yu et al., 2002; Ji et al., 2013). Iturins and fengycins were demonstrated as the most important compounds in the biocontrol activity of *Bacillus* strains against various phytopathogenic fungi and in different plants species. Besides, *B. amyloliquefaciens* strains are able to colonize roots of the host plant and to induce its growth and defense responses (Raupach and Kloepper, 1998). It produces also resistant spores to UV light and heat which makes these microorganisms efficient in the formulation and the development of biopesticide products resistant to environmental conditions (Raaijmakers et al., 2002).

In a previous work, the antifungal metabolite production medium MC of a *B. amyloliquefaciens* strain, named BLB 371, has been optimized (Mezghanni et al., 2012). In this work, in order to develop a more economic and efficient biofungicide, a hyperactive mutant against several fungi was generated by mutagenesis using nitrous acid. By using response surface methodology, a new antifungal metabolite production medium of this mutant has been optimized and its efficacy in protecting tomato against the causal agent of gray mold disease, *B. cinerea*, was proved.

2. Materials and methods

2.1. Microorganisms and growth conditions

The *B. amyloliquefaciens* BLB371 was obtained from the bacterial strain collection of our laboratory and was isolated by Mezghanni et al. (2012) from a Tunisian soil. To produce antifungal metabolites, the *B. amyloliquefaciens* strains (mutants and wild strain) (See Mutagenesis section) were cultured at 30 °C and 200 rpm, for 48 h in production medium MC (pH 7.0), containing 25 g/L sucrose, 20 g/L peptone, 4.5 g/L yeast extract, 2 g/L KH_2PO_4, 0.6 g/L $MgSO_4$ and 6 mg/L $MnSO_4$ (Mezghanni et al., 2012). For DNA extraction and inoculums preparation, these strains were grown at 30 °C in LB medium.

Eight fungal strains, including *Aspergillus niger* (CTM1099), *Alternaria alternate* (CTM10230), *Fusarium culmurum* (ISPAVE21w), *Fusarium graminarium* (ISPAVE271), *Fusarium oxysporum* (CTM10402), *Pythium aphanidermatum* (LPAP32), *Rhizoctonia solani* (food isolate) *and B. cinerea* (food isolate), were used to evaluate the antifungal activity of the patent strain BLB371 and the obtained mutants. The indicator fungi were obtained from the local culture collections of the Centre of Biotechnology of Sfax (CBS), Tunisia. All fungi were grown on Potato Dextrose Agar (PDA) for 5–7 days at 25 °C. Spore number was determined using a Thomas counter.

2.2. Mutagenesis

Nitrous acid treatment was applied to *B. amyloliquefaciens* BLB371 cells as described by Ghribi et al. (2004) with some modifications of mutant selection. Because of the lack of a simple and rapid method for screening the mutants improved in their antifungal activity, all surviving clones were screened for their antifungal activity against *A. niger* and only mutants exhibiting higher antifungal activity than the parent strain BLB371 were selected for further studies. The mutants' stability is checked by 20 successive subculturing on MC medium and quantification of their antifungal activity on PDA (Jack et al., 1995).

The most active mutant was selected to apply on it another random of nitrous acid treatment. This method was repeated three times in order to obtain a hyperactive strain.

2.3. Antifungal activity evaluation

The antifungal activity was assessed by well diffusion method (Jack et al., 1995). Hundred μL of *A. niger* spore suspension (10^6 spores/mL) were displayed on a plate filled with PDA medium with chloramphenicol (30 μg/mL). Wells were drilled in the PDA. In each well, 100 μL of diluted culture supernatant were added. Antifungal activity was expressed as arbitrary units (AU/mL), defined as the reciprocal 1 of the dilution of supernatant giving the lowest inhibition ratios (Delgado et al., 2005).

2.4. Taxonomical study

M3-7 and BLB 371 identity (belonging to *B. amyloliquefaciens*) was confirmed by molecular approach using the 16S rDNA sequencing. The universal primers Fd1 (5′-AGAGTTTGATCCTGGCTCAG-3′) and Rd1 (5′-AAGGAGGTGATCCAGCC-3′) were used for amplification by PCR. After the genomic DNA extraction (Sambrook and Russell, 2001), an amplification of the bacterial 16S rDNA was carried out. It consisted of an initial denaturation at 94 °C for 2 min followed by 30 cycles; each one was composed of denaturation at 94 °C for 30 s, annealing at 53 °C for 1 min, and extension at 72 °C for 2 min. The PCR product was purified, sequenced in an automatic sequencer (Avant Genetic analyzer, 3100 model) and compared by Blast algorithm to find homology. Partial 16S rRNA nucleotide sequence was deposited in GenBank and an accession number (GenBank accession no. **KX579982**) was obtained.

The mutant identity was also verified using molecular approach described above and compared with parent strain.

2.5. Biomass determination

After 48 h of incubation at 30 °C, growth of the wild strain and the hyperactive mutants was monitored by counting the colony forming unit (CFU) by plating 100 μL of the appropriate cell dilutions which are treated at 80 °C for 10 min, on LB agar medium. The counting is made after incubation for 24 h at 30 °C.

2.6. Experimental design

To maximise the antifungal activity produced by *B. amyloliquefaciens* mutant M3-7 a new culture medium was optimized for this strain. Therefore, based on the medium MC of BLB371 (Mezghanni et al., 2012) six media components (sucrose, peptone, yeast extract, KH_2PO_4, $MgSO_4$ and $MnSO_4$) were chosen for this study. A hybrid design (Lewis et al., 1999) of 40 experiments was generated by Nemrod W software (Mathieu et al., 2000). It includes six centre points (run no. 28–33) and seven test points (run no. 34-40) as shown in Table 1.

The variables of the experiments were coded on the basis of the following equation (Eq. (1)):

$$X_{ij} = (U_{ij} - U_{j(0)})/\Delta U_j \quad (1)$$

Where X_{ij} is the dimensionless coded value of the independent variable U_j for the i^{th} experiment, U_{ij} is the actual value of U_j for the i^{th} experiment, $U_{j(0)}$ is the actual value of U_j at the centre point and ΔU_j is the step change value of U_j.

The relationship between the six variables and the antifungal metabolites production was determined by fitting the second order polynomial equation (Eq. (2)) to data obtained from the experiments (runs n° 1 to 33).

$$\hat{y} = b_0 + \sum_{j=1}^{6} b_j X_j + \sum_{j=1}^{6} b_{jj} X_j^2 + \sum_{j=1}^{6} \sum_{j<k}^{6} b_{jk} X_j X_k \quad (2)$$