THE EVALUATION OF THE BACTERICIDE ACTIVITY USING THE GENETICALLY MODIFIED STRAIN OF THE CAUSATIVE AGENT OF BACTERIAL SPOT OF TOMATO

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Abstract. *Xanthomonas vesicatoria* strain 111 1/B, the causative agent of bacterial spot of tomato, was transformed using electroporation with the pHC60 plasmid containing the gene for green fluorescent protein (GFP). The transformed strain did not differ both in growth rate on the culture medium and the pathogenic characteristics from the initial one. The principle possibility of quick evaluation of bactericides by measuring the GFP fluorescence intensity of the transformed pathogene strain has been shown.

Key words: green fluorescence protein, GFP, bacterial spot of tomato

Bacterial spot of tomato is one of the most harmful bacterial diseases. It frequently damages tomato and pepper in Russia and the Commonwealth of Independent States. During epiphytotic years its prevalence varies from 40 to 70%. In Russia the disease is reported to occur in the North Caucasus, Krasnodar Territory, Altai Territory, Voronezh region, Chita region, Volgograd region and other regions [1]. In the European Union it is considered to be a quarantine pathogen (EPPO, list A2). The causative agent used to be classified as *Xanthomonas campestris pv. vesicatoria* [6]. Nowadays the four pathovars are proposed instead: *X. euvesicatoria, X. vesicatoria, X. perforans, X. gardneri* [6].

The main sources of the infection are seeds and plant residues. However, the peculiarities of its biology and pathogenesis such as the ability to survive in epiphytic state on the surface of nonhost plants and the duration of survival in the soil are still insufficiently known. In addition, the improvement of the technique of evaluating the matters able to limit the propagation of the pathogen is required. Such experiments can be performed using labeled strains. The most appropriate means of labeling is known to be the transformation with the gene for green fluorescence protein (GFP). GFP was isolated from jellyfish *Aequorea victoria* and exhibits green fluorescence when exposed to blue light. GFP is widely applied as a fluorescent label in cell and molecular biology [5]. The given approach has been proved efficient repeatedly on a number of phytopathogenic and symbiotic bacteria [3, 9].

The purpose of the present study was to develop the *X. vesicatoria* strains transformed with GFP. Such strains are useful in model experiments on evaluation of antibacterial activity of different bactericides.

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Materials and methods

The strain 1111/B of *X. vesicatoria* used in the experiments was provided by the State collection of phytopathogenic microorganisms (Russian Research Institute of Phytopathology (VNHF), Russian Academy of Agricultural Sciences). The pHC60 plasmid carrying the genes for GFP and resistance to tetracycline was applied for transformation [3]. The bacteria were stored in 15% glycerol at -70°C. For the cultivation of *X. vesicatoria* YDC medium was used [8].

The electro- and chemically competent cells were prepared for heat-shock transformation and electroporation, respectively. [7]. The standard procedures of heat-shock and electroporation were applied [7]. Electroporation was performed in electroporation cuvettes (BIO-RAD, Almabion) with 0.1 cm distance between electrodes using an electroporator Gene Pulser (BIO-RAD) according to the manufacturer's recommendations [4]. The selection of the transformed colonies expressing GFP was carried out on the medium with tetracycline (20 mg/1) using a stereomicroscope SteREO Lumar.V12 (Zeiss). The species of the transformed bacteria was checked by polymerase chain reaction (PCR) with the pathogen-specific primers Xnth 804/1405 in a thermocycler «Tertsik» (DNA-Technology, Moscow) using the following conditions for amplification of DNA: initial denaturation (7min at 96°C); 30 cycles including denaturation (40s at 94°C), annealing (30s at 62°C) and extension (40s at 72°C); final extension (7 min at 72°C). 10 ц1 of the amplified PCR fragments were separated on 1,5% agarose gel stained with ethidium bromide in 0.5><TBE buffer solution and visualized using UV light.

The comparison of pathogenicity between the initial and transformed strains was performed by infiltrating a bacterial suspension of 10^9 CFU/ml into leaves of tomato cv. Belyj Naliv with a syringe without needle. The isolation of the pathogen from the leaves was carried out on YDC medium containing 20 mg/1 tetracycline.

The optical density of a bacterial suspension (OD 600) was measured by a spectrophotometer NanoDrop 1000 (Thermo Scientific, USA) at 600 nm. The concentration of bacteria (in colony forming units, CFU) was measured by serial dilutions [8].

To select the culture medium providing both the most intense growth of the transformed strain and the expression of GFP the following media were evaluated: LB, King B, modified King B with 0.1% glycerol and 0.2% peptone, modified King B with 0.1% glycerol, YD (YDC without calcium carbonate). Bacterial suspensions were plated on the media to the final concentration of 10^5 CFU/ml and incubated in a shaker at 200 rpm at 27°C. After cultivation for 4, 8, 12,16,20,24 and 48 hours samples were selected to measure the level of fluorescence, optical density at 600 nm (OD 600) and concentration (CFU/ml).

The intensity of GFP fluorescence in a bacterial suspension was measured using a fluorimeter «Dzhin» (DNA-Technology, Moscow) in arbitrary units (a.u.).

To evaluate bactericide activity antibiotic 3750 UA/mg phytobacteriomycin (Farmbiomedservice, Moscow) was tested. The antibiotic was added in different amounts to the King B liquid medium inoculated with the transformed strain. After cultivation for 4, 8, 12, 16, 20 and 24 hours samples of 600 ц1 were selected to measure the level of fluorescence.

The experiments were replicated three times. The data were statistically analyzed using the comparison of means with the Duncan's criterion using SPSS 15 software.

Results and discussion

To develop the fluorescently labeled strain of the causative agent of bacterial spot of tomato the pHC60 plasmid was applied. As a result of comparison between the transformation methods, heat shock and electroporation, the latter has turned out to be more

efficient. Unlike the heat-shock, the electroporation has produced transformed strains of the phytopathogenic bacteria expressing GFP. The transformed bacteria were plated twice on LB medium containing 20 mg/1 tetracycline. 11 isolated colonies have shown high level of fluorescence (fig. 1), their belonging to *X. vesicatoria* has been proved using PCR with the specific primers (fig. 2). The transformed strain was designated 1111/BGFP.

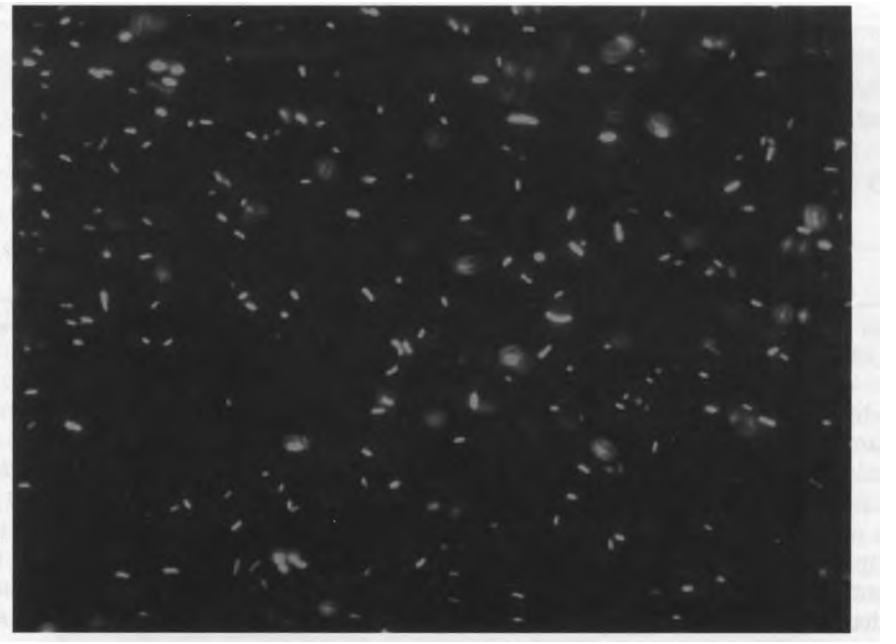

Fig. 1. The GFP fluorescence of the 1111/BGFP cells

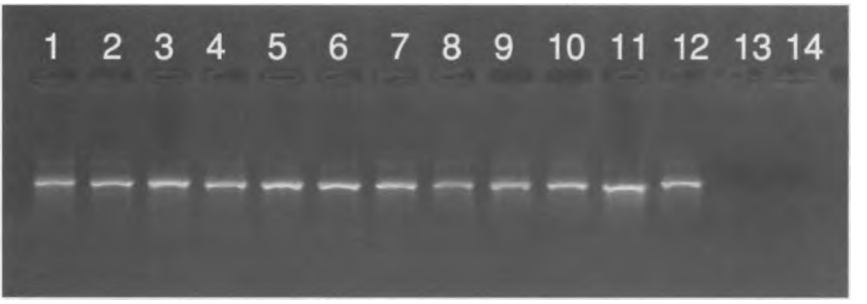

Fig. 2. The electrophoregram of the PCR products amplified with Xnth 804/1405.
Lanes: 1-11 —the transformed colonies of X. *vesicatoria* 1111/B,
12 — 1111/B (positive control), 13 — *E. coli* DH10B (negative control),
14 — water (negative control)

The next step was to select the culture medium providing both rapid growth of 1111/BGFP and the highest level of GFP fluorescence. The results has shown that among 8 evaluated media the standard King B medium provides both the highest growth rate of

1111/BGFP culture and the highest level of fluorescence (table 1). The differences were statistically significant.

Table 1

Growth and fluorescence of 1111/BGFP in different liquid culture media

Culture medium	OD 600	Intensity of fluorescence, a.u.
Mineral base + 1% glucose	0,017 a *	1,00 a
Mineral base + 1% glycerol	0,018 a	1,23 a
Mineral base + 1% sucrose	0,019 a	1,25 a
King B (0,1% glycerol)	0,280 b	26,22 b
King B (0,1% glycerol + 0,2% peptone)	0,450 c	30,53 c
LB	0,520 d	32,97 d
YD	0,750 e	36,72 e
King B	0,800 f	43,16 f

Note. In the tables 1, 2 and 3 between the variants labeled with the same letters there are no statistically significant differences at 95% probability level.

Additionally, the comparison of the characteristics between the initial and transformed *X. vesicatoria* strains was performed. After cultivation for 24 hours in LB liquid medium using shaker the OD 600 of the initial strain was 0.147 and that of 1111/BGFP was 0.140. No significant differences have been found between these data. That indicates that the presence of the pHC60 plasmid does not affect the bacteria growth rate on culture medium. The comparison of the pathogenic characteristics by leaves inoculation has not revealed significant differences between the initial and transformed strains. The given results have shown that the fluorescently labeled strain developed by us can be used as an adequate model to study various aspects of pathogenesis of plant bacterioses.

The study of the 1111 /BGFP growth kinetics by measuring OD 600, CFU concentration and fluorescence intensity was performed on the selected medium King B.

The optical density of the bacterial suspension significantly increased from 12 h and continuously increased during all the period of sampling (table 2). The CFU concentration reached the maximum after cultivation for 24 h and did not increased during the next 24 h of cultivation. The fluorescence intensity significantly increased after cultivation for 8 h; in addition, the growth was observed in the period of 24-48 h despite the CFU concentration reached the plateau. It is the evidence for the stability of the fluorescent protein and, consequently, possible participation of GFP of dead cells in the total fluorescence.

Table 2

Growth dynamics and fluorescence intensity of 1111/BGFP in King B liquid medium

Time of cultivation, hours	OD 600	*Ln* CFU/ml	Fluorescence intensity, a.u.
0	0,018 a	14,78 a	6,29 a
4	0,020 a	15,41 ab	8,47 a
8	0,120 a	17,99 bc	13,80 b
12	0,217 b	19,25 c	34,01 c
16	0,300 c	20,28 d	35,64 c
24	0,480 d	22,59 d	39,58 d
48	0,750 e	20,39 cd	64,08 f

The comparison of the growth rate of 1111/BGFP in the presence and absence of a selective factor (tetracycline) has shown that on the medium without tetracycline propagation of the bacteria is more rapid. However, the fluorescence intensity of the two variants has shown no significant difference (table 3). Therefore, we assume that a part of the bacteria can lose the pHC60 plasmid during the propagation on the medium without the selective factor (tetracycline).

Table 3

Optical density and fluorescence intensity of 1111/BGFP bacterial suspension after 24 h of cultivation in King B liquid medium

Medium	OD 600	Fluorescence intensity, a.u.
King B	0,420 a	19,36 a
King B + tetracycline 20 mg/l	0,380 b	21,38 a

No differences between the initial and transformed strains allowed us to use the latter as a model to evaluate the efficiency of bactericides. Phytobacteriomycin was chosen for the test as an antibiotic that is able to suppress most of phytopathogenic bacteria [2]. The antibiotic was added to King B liquid medium and subsequently the strain was cultivated in it. As a result, the propagation and fluorescence of the tested object are significantly suppressed at the antibiotic concentration of l mg/ml and totally suppressed at more than 5 mg/l (fig. 3). The differences of the fluorescence level between the experiment variants and control (without the antibiotic) were significant after 4 h of cultivation already.

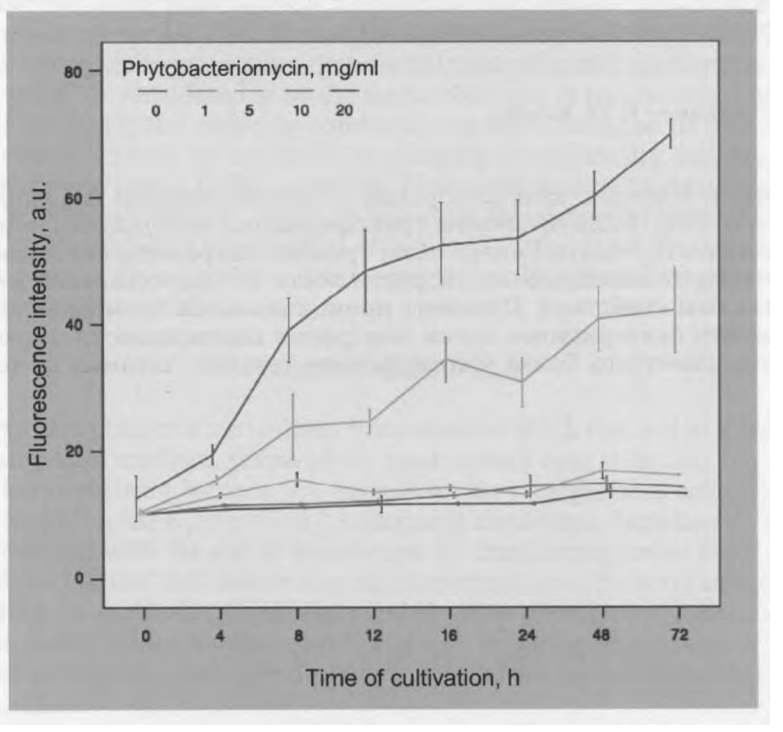

Fig. 3. GFP fluorescence intensity during the cultivation of 1111/BGFP on King B medium at different concentration of phytobacteriomycin

Thus, we have shown the possibility of quick evaluation of the bactericide activity by measuring fluorescence intensity of the transformed strain of the phytopathogen. The method can be useful in laboratory screening of means of plant protection from bacterial diseases.

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***Translation into english* — P. Yu. Krupin**

Аннотация. Методом электропорации с использованием плазмиды pHCбO, содержащей ген GFP, была проведена трансформация возбудителя черной бактериальной пятнистости томата. Генетически трансформированный штамм X. vesicatoria не отличался от исходного по скорости роста на искусственной питательной среде и патогенным свойствам. Показана принципиальная возможность экспресс-оценки активности бактерицидов путем измерения интенсивности флуоресценции зеленого флуоресцентного белка у трансформированного штамма фитопатогена.