**Phenylboronic acid as a novel agent for controlling plant pathogenic bacteria**

**Running title: Antibacterial activity of phenylboronic acid**

Katarina Martinko1, Siniša Ivanković2, Edyta Đermić1, Damir Đermić3\*

1University of Zagreb Faculty of Agriculture, Division of Phytomedicine, Department of Plant Pathology, Zagreb, Croatia; 2Ruđer Bošković Institute, Division of Molecular Medicine, Zagreb, Croatia; 3Ruđer Bošković Institute, Division of Molecular Biology, Zagreb, Croatia

**Abstract**

BACKGROUND

Phenylboronic acid (PBA) is an environmentally nontoxic substance with antimicrobial activity. Due to increasing ecological limitations in phytopharmacy and considering the development of resistance of phytopathogenic bacteria to available antibacterial agents, here we explore a possible role of PBA as an antibacterial agent of choice.

RESULTS

We determined a minimal inhibitory concentration (MIC) of PBA *in vitro* on the *Pseudomonas syringae* pv*. tomato* (*Pst*) (0.5 mg/ml) and *Erwinia amylovora* (0.8 mg/ml), two of the most damaging plant pathogenic bacteria. In comparison, boric acid MIC was 2.5-6-fold higher than that of PBA, indicating enhanced antibacterial efficacy of the latter. Moreover, we determined the effect of PBA on cell growth and viability of both bacteria and have shown that PBA has bactericidal effect in concentrations >1.0 mg/ml, whereas in lower concentration it is bacteriostatic. In addition, we have shown that PBA impairs *Pst* ability to cause symptoms on tomato plants in a dose-dependent manner, whereas solely applied PBA did not affect plant morphology at bactericidal concentrations.

CONCLUSION

We report, for the first time, that PBA is a suitable agent for controlling phytopathogenic bacteria. PBA has bacteriostatic activity in lower, and bactericidal activity in higher (>1.0 mg/ml) concentrations. When applied on tomato plants, PBA managed to suppress symptoms caused by *Pst*, while having no adverse effect on plants at the bactericidal concentrations. As an additional benefit, PBA is environmentally friendly.

Key words: PBA, *Pseudomonas* *tomato*, *Erwinia amylovora*, MIC, bacterial speck.

**1 INTRODUCTION**

Phytopathogenic bacteria cause several economically and ecologically important diseases, such as bacterial speck, which is caused by the gram-negative bacterium *Pseudomonas syringae* pv*. tomato* (Okabe) Young, Dye and Wilkie. It is an economically significant disease in tomato (*Lycopersicon esculentum* Mill.) production.1, 2 Another destructive disease is fire blight, affecting rosaceous plants (such as apple and pear fruit trees), which is caused by gram-negative bacterium *Erwinia amylovora* (Burrill) Winslow et al.

Since according to Phytosanitary information system (https://fis.mps.hr/trazilicaszb) the use of antibiotics is curtailed in crop production, and the use of chemical bactericides for controlling the aforementioned diseases has led to the development of resistance of both the *P. syringae* pv*. tomato* (*Pst*) and *E. amylowora* (*Ea*) to such agents,3-5 the problem arises of a limited choice of antibacterial agents.6 One such candidate may be a phenyl derivative of boric acid (BA), phenylboronic acid (PBA), which was synthetized, and its antibacterial activity noticed more than a century ago,7, 8 and yet its antimicrobial activity is still poorly characterized. Recent studies have shown antimicrobial effect of PBA on several species of human-hosted bacteria and fungi, 9-11 as well as antitumor activity of PBA.12

Regarding PBA effect against plant pathogenic microbes, few studies described wood preservation due to PBA antifungal activity.13, 14 Recently, we have reported the prophylactic activity of PBA on early blight pathogenic fungus *Alternaria* *alternata* in tomato,15 while no study is known to us dealing with effects of PBA on phytopathogenic bacteria. It is known, however, that PBA does not cause morphological effects in mature tomato plants,16 and is generally considered nontoxic to the environment.13 In fact, boron is an essential micronutrient for plants.17

**2 MATERIALS AND METHODS**

2.1. Bacterial strains used

The antibacterial activity of PBA was tested on the AF320 strain of the *Pst* isolated from tomato with symptoms of bacterial speck, which was morphologically and molecularly determined at species level, using the published procedure and PCR primers.18 *Ea* strain used for testing of PBA and BA antibacterial activity was EaCro16K.19

2.2 Determination of minimal concentration of PBA and BA showing inhibitory effect on *Ea* and *Pst*

Poisoned food technique was applied according to the modified *in vitro* method 20 to determine the minimum inhibitory concentration (MIC) of PBA on the *Pst,* and PBA and BA on the *Ea*. We used PBA (CAS No. 98-80-6, Sigma-Aldrich) and BA (Sigma-Aldrich, USA, CAS No.10043-35-3) at a wide range of concentrations: 0.4 mg/ml, 0.5 mg/ml, 0.6 mg/ml, 0.7 mg/ml, 0.8 mg/ml, 0.9 mg/ml, 1.0 mg/ml, 2.0 mg/ml and 3 mg/ml (which is equivalent to the w/v concentrations from 0.04% to 0.3%). Based on the dilution factor, the prepared PBA solution of 10 mg/ml was pipetted into 50 mL of liquid King B nutrient medium for *Pst* or LB medium21 for *Ea* in a specific volume, such that each medium concentration variant was poured in 3 replicates (Petri plates). BA (100 mg/ml) was added to LB medium at final concentration of: 1.0 mg/ml, 1.5 mg/ml, 2.0 mg/ml, 3.0 mg/ml, 4.0 mg/ml, 5.0 mg/ml, 6.0 mg/ml and 7.0 mg/ml (corresponding to the w/v range from 0.1% to 0.7 %) (Fig.1). 100 µl of a serially diluted suspension of overnightgrown *Pst* (10-5) and *Ea* (10-6) cultures, containing 2000-3000 colony forming units (CFU)/ml, was evenly distributed on the agar surface (Fig. 1). As a (positive) control, the bacterial suspension was applied on plates containing no PBA or BA. The plates were incubated in an air chamber at 28.5 °C for 72-120 hours to allow colonies of *Pst* and *Ea* to develop, which were counted by *ImageJ* software.22

2.3 Growth kinetics measurement and viability determination of *Pst* and *Ea*

Described procedure was used.23 In summary, we grew *Pst* in a liquid NA medium and *Ea* in LB medium overnight at 28.5 °C, with aeration.24 The bacteria were diluted 12 fold into the fresh NA or LB medium, allowed to grow for 90 min at 28.5 °C, with aeration, and then were diluted 10 fold into the NA medium supplemented with PBA in following concentrations: 0.4 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 2.0 mg/ml and 3.0 mg/ml (which is equivalent to the w/v concentrations from 0.04% to 0.3%) for *Pst*, while PBA concentrations in LB medium for *Ea* included 0.7 mg/ml, 0.8 mg/ml, 1.0 mg/ml, 2.0 mg/ml and 3.0 mg/ml (which is equivalent to the w/v concentrations from 0.07% to 0.3%) (Fig.1). During the incubation of the bacteria in the PBA-enriched medium (at 28.5 °C, with aeration) the samples were periodically taken, and their optical density measured at 600 nm (OD600) by Novaspec II colorimeter (Amersham Pharmacia Biotech). In addition, the titer of viable bacteria was determined in the samples. For that, the bacterial cultures were serially diluted in 67 mM phosphate buffer and plated on King B agar plates or LB plates lacking PBA, which were then incubated at 28.5 °C for 72 hours.24 The OD600 and the titer of viable cells at the start of incubation were taken as a reference and the changes during incubation expressed in relation to them for each bacterial culture.

2.4 PBA protection of tomato plants against infection by *Pst*

The experiments under greenhouse conditions were performed in Zagreb during spring of 2021. The effect of a range of MIC concentrations of PBA on *Pst* ability to produce symptoms in inoculated plants was investigated using a modified *in vivo* method.25 To study the *in vivo* effect, preventive application of PBA in the concentration of 0.25 mg/ml (0.5 MIC), 0.5 mg/ml (1 MIC), 1.0 mg/ml (2 MIC) and 1.5 mg/ml (3 MIC) was carried out by foliar spraying of the plants (tomato cultivar Rutgers in the postembryonic tomato phenophase with developed 9th leaf) with a hand sprayer. The leaves were sprayed by suspension of *Pst* (5.6 x 106 CFU/ml) 7 days post PBA application (Fig. 1). Plants inoculated with *Pst* and distilled water (no PBA) were used as a (positive) control. Each variant was assessed in 3 repetitions with a total of 18 plants. Symptoms of bacterial speck are chlorotic and necrotic lesions on the leaves, whose area was measured by computerized image processing using *ImageJ*, 26 14th day post-inoculation.

**3 RESULTS AND DISCUSSION**

3.1 Minimal inhibitory concentration of PBA and BA on *Pst* and *Ea*

In the MIC determination assay, we observed a considerable antibacterial effect of PBA on the *Pst* pathogen. The effect of PBA was dose dependent, since the CFU number was significantly reduced, by 37%, on a nutrient medium containing 0.4 mg/ml PBA, compared to the untreated control (Tukey test, P**<**0.05). Conversely, no bacterial colonies developed on media containing from 0.5 mg/ml to 3.0 mg/ml PBA (Table 1). Hence, the decrease in colony growth is proportional to increase in PBA concentration in the culture medium used, and the PBA concentration of 0.5 mg/ml is the *in vitro* MIC for *Pst* (Table 1). The results are in accord with previous reports on the antibacterial effect of PBA on medicinally important species of the genus *Pseudomonas* under laboratory conditions, which showed that the use of PBA at concentrations of 0.7 mg/ml and 1.0 mg/ml inhibits the bacterial cutinase activity of *Pseudomonas putida* by 63% and 80%, respectively.27 More recent works described inhibitory *in vitro* effect of PBA on the medicinally important species of *Pseudomonas aeruginosa*.28, 29

By using the same method as here, we have recently shown that BA MIC on *Pst* is 3 mg/ml,30 which is 6-fold higher than MIC for PBA, thus indicating increased antibacterial efficacy of the latter.

We also determined that PBA MIC on *Ea* is 0.8 mg/ml (Table 1), which is somewhat higher than that of *Pst,* while being dose dependent as well. As a comparison, we measured BA MIC on *Ea* and found that the bacterium did not produce colonies on LB agar containing ≥2 mg/ml BA, which is more than double MIC of PBA. *Ea* grew on medium with lower BA concentration (n=3): 299.95 ± 32.4 and 312.5 ± 7.1 at 1.0 mg/ml and 1.5 mg/ml, respectively, which is ~ 5 % lower CFU than the untreated control (315.1 ± 19.2), and this difference is not significant (p>0.05, t-test).

3.2 PBA impairs growth kinetics and viability of *Pst* and *Ea*

Next, we determined the effect of PBA on growth kinetics and viability of bacterial cells to elucidate whether the PBA has bacteriostatic or bactericidal activity. As shown in Fig. 2A, the addition of PBA to liquid growth medium caused retardation of *Pst* growth in a dose-dependent manner. The mass-doubling time of *Pst* was ~77 min when grown in medium devoid of PBA, which increased to ~150 min and ~220 min in medium containing 0.4 mg/ml and 0. 5 mg/ml PBA, respectively. The bacterial growth was effectively stopped in medium containing 1.0 mg/ml PBA, whereas OD600 decreased in media containing 2.0 mg/ml and 3.0 mg/ml PBA (Fig. 2A). These results indicate that PBA concentration <1.0 mg/ml have bacteriostatic effect on *Pst*, whereas the higher concentrations (2.0 mg/ml and 3.0 mg/ml) are bactericidal.

Analogous results were obtained when measuring growth kinetics of *Ea*, namely PBA also caused retardation of bacterial growth (Fig. 3A). The mass doubling time of *Ea* was ~93 min in an LB medium lacking PBA, which increased more than 4-fold to ~ 400 min when grown in medium containing 0.7 mg/ml or 0.8 mg/ml PBA. In medium containing 1.0 mg/ml PBA no OD600 growth occurred, while OD600 fell in medium having 2.0 or 3.0 mg/ml PBA, in a dose-dependent way (Fig. 3A).

3.3 PBA impairs viability of *Pst* and *Ea*

We determined a viable cell count to directly measure the effect of PBA on bacterial viability. During *Pst* growth in medium lacking PBA, the titer of viable bacteria (those capable of forming a colony, CFU) doubled in less than 90 min (Fig. 2B), which is in accord with their mass doubling time of ~77 min. On the other hand, CFU count rose much slower in medium containing 0.4 mg/ml or 0.5 mg/ml PBA, with the number of viable cells still not doubled after 180 min of incubation (Fig. 2B), indicating retardation in bacterial division, *i.e.,* the moderate bacteriostatic effect. This effect was boosted in medium containing 1.0 mg/ml PBA, in which effectively no bacterial division occurred (Fig. 2B), suggesting full bacteriostatic effect. Furthermore, upon prolonged, overnight (ON) incubation in 1.0 mg/ml PBA medium, the titer of viable bacteria fell about 3-fold from initial inoculum count (Fig. 2B), indicating a moderate bactericidal effect. The drop in a viable cell count is more pronounced with increasing PBA concentration, the CFU titer dropped about 4- and 9-fold in cultures grown for 90 min and 180 min with 2.0 mg/ml PBA, respectively, while upon ON incubation it fell more than 105-fold (Fig. 2B). These results show fast, exponential kinetics of bacterial killing by PBA. The rate of killing bacteria is even higher in medium supplemented with 3 mg/ml PBA, where titer of viable bacteria fell about 8-, 40- and more than106-fold upon 90 min, 180 min and ON incubation, respectively (Fig. 2B).

Similar effects were observed in the *Ea* viability assay (Fig. 3B). Namely, an exponential increase in CFUs was observed in cultures lacking PBA, which greatly slowed down at concentrations close to MIC (0.7 mg/ml and 0.8 mg/ml), with effectively arrested increase at 1.0 mg/ml PBA (Fig. 3B). Hence, we can conclude that PBA concentrations ≤1.0 mg/ml have bacteriostatic effects on *Ea* grown in liquid LB medium. Conversely, increased PBA concentrations, namely 2.0 mg/ml and 3.0 mg/ml, acted bactericidally on *Ea* cultures, which was especially noticeable with 3.0 mg/ml PBA, where viable cell titer decreased sharply: more than 50-fold after 90 min incubation with PBA and more than 104- and 106-fold after 180 min and ON incubation, respectively (Fig. 3B).

Therefore, we may conclude that antibacterial activity of PBA against both *Pst* and *Ea* is dose dependent, namely PBA has the bacteriostatic activity in lower concentrations (≤1.0 mg/ml), while showing the strong bactericidal activity when present at concentrations of 2.0 mg/ml or 3.0 mg/ml. Since we observedlower MIC and less adverse effect of 1.0 mg/ml and 2.0 mg/ml PBA on *Pst* than *Ea* cellular viability, we infer that *Pst* is somewhat more sensitive than *Ea* at lower PBA concentrations. Conversely, 3.0 mg/ml PBA concentration has more adverse effects on *Ea* than on *Pst*, since *Ea* cells were killed faster (compare Fig. 2B and Fig. 3B).

The different inhibiting PBA concentrations that we observed in the two *in vitro* assays (0.5 mg/ml and 0.8 mg/ml in the MIC assay and 1.0 mg/ml in the viability assay for *Pst* and *Ea* respectively) are likely due to different exposure durations in the two tests, namely bacteria were exposed to the PBA for 72 h in the MIC assay and 24 h, at most, in the viability assay.

3.4 PBA reduces symptoms of *Pst* infection of tomato plants

Testing of the PBA activity on the *Pst* pathogen under *in vivo* conditions was carried out in the postembryonic tomato phenophase with developed 9th leaf. We first sprayed the leaves with PBA, which were 7 days later inoculated with bacteria. Reduction in the symptoms of bacterial speck was measured in all tested samples 14 days after application of bacteria. Foliar application of PBA at a concentration of 0.5 MIC reduced the incidence of bacterial speck symptoms by 85% compared to the control group of plants (Table 2), which is significant compared to the control and the other test treatments (P**<**0.05, Tukey test). Analogous results were obtained when plants were treated with 1 MIC, which caused significant reduction, 88%, of bacterial speck symptoms, compared to control plants (P**<**0.05, Tukey test) (Table 2). The decrease of the symptoms severity on tomato leaves was 95% and 66% when PBA was applied at a concentration of 2 and 3 MIC, respectively (Table 2), which is significant compared to the other test treatments and the control (P**<**0.05, Tukey test). Notably, tomato plants treated solely with PBA (no bacterial inoculation) did not develop chlorotic symptoms, whereas leaf blades of control plants on which pathogen suspension was applied instead of PBA did show chlorotic symptoms.

The results show a positive correlation of the antibacterial effect of PBA in the concentration range of 0.25 mg/ml (0.5 MIC), 0.5 mg/ml (1 MIC) and 1.0 mg/ml (2 MIC), which is in accord with the *in vitro* test (Table 1). The effect of 3 MIC concentration was smaller than that of the lower concentrations, suggesting that higher PBA concentrations affect plants' physiology in such a way that the plants show more severe symptoms of bacterial infection.

On the other hand, no serious symptoms appeared on the host plants after treatment with 3 MIC (1.5 mg/ml) in a control without bacteria, suggesting that PBA does not have adverse effect on tomato plants even in quite high, bactericidal concentrations. Hence, our results further expand on the range of PBA concentrations that can be safely applied on tomato plants, since the highest concentration is about 5-fold higher than that in the previous studythat also showed no morphological effect of PBA (300 ppm, 0.3 mg/ml) on mature tomato plants.16

Briefly, in this study we have shown that PBA has a strong antibacterial effect on economically important bacterial species *Pst* and *Ea* at a low concentration of 0.5 mg/ml and 0.8 mg/ml in the *in vitro* MIC test. In comparison, BA has 2.5- (this study) and 6-fold higher MIC for *Ea* and *Pst*,30 respectively, thus indicating its lower antibacterial efficacy.

PBA shows bacteriostatic effect in concentrations at about 1.0 mg/ml, while the higher PBA concentrations (tested up to 3.0 mg/ml) have pronounced bactericidal effects. Such a dose-dependent antibacterial activity is notably reflected in the *in vivo* assay of *Pst* infection oftomato plants. At the same time, no adverse effect of PBA on host plants was observed at bactericidal (1.5 mg/ml) PBA concentration in the absence of bacteria. Hence, these results give us an orientation on how to apply PBA in effective prophylactic doses that sufficiently impair the *Pst*, while having no notable effect on plant health. Certainly, one or two MIC of PBA (0.5 mg/ml -1.0 mg/ml ) are especially interesting in that regard.

This is, to our knowledge, the first report on PBA application on plant pathogenic bacteria. It demonstrates that PBA is effective in suppressing symptoms of the disease, while having no adverse effect on host plants even when applied in bactericidal concentrations. This effectively qualifies PBA as a suitable antibacterial agent of choice, especially since it is not harmful for the environment and, importantly, bacteria apparently lack intrinsic resistance to it (as it is not a naturally- occurring chemical, but a human-made one, which is currently not used against pathogenic bacteria).

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**CONFLICT OF INTEREST**

The authors declare no conflicting interests.

**AUTHOR CONTRIBUTIONS**

Đermić, D., Ivanković, S., Đermić, E. and K. Martinko conceptualized the design of the study. Martinko, K. and D. Đermić performed the laboratory analyses. Martinko, K. and D. Đermić collected and analysed the data, and interpreted it together with Đermić, E. and S. Ivanković. Martinko, K. and D. Đermić drafted the article, and together with Đermić, E. and S. Ivanković critically revised the manuscript and gave the final approval of the article version to be published.

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 Table 1. *In vitro* antibacterial effect of PBA on pathogen *Pseudomonas syringae* pv*. tomato* (top row)and *Erwinia amylovora* (bottom row) (Poisoned Food Technique after 72 h incubation at 28 °C).

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| PBA concentration (mg/ml)  | 0 | 0.4 | 0.5 | 0.6 | 0.7 | 0.8 | 0.9 | 1.0 | 2.0 | 3.0 |
| Average number of colonies ± SD (n=3) | 187.3c± 5.6 | 119.0b± 2.5 | 0a | 0a | 0a | 0a | 0a | 0a | 0a | 0a |
| 225.7b ± 12.7  | 224.0b ± 25.3 | 213.7b ± 18.6 | 220.3b ± 21.2 | 212.7b ± 25.5 | 0a | 0a | 0a | 0a | 0a |

Note: Different letters mark statistically significant difference according to Tukey test at the level P**<**0.05.

 Table 2. *In vivo* effect of PBA on symptoms caused by pathogen *Pseudomonas syringae* pv*. tomato* 14 days postinfection.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| PBA concentration (mg/ml) | Control(0) | 1/2 MIC (0.25) | 1 MIC (0.5) | 2 MIC (1.0) | 3 MIC (1.5) |
| Average percentage of symptoms (%) ± SD (n=3) | 40.4c ±2.6 | 6.11ab ±5.4 | 4.8ab±3.6  | 2.1a±3.1 | 13.6b±5.5  |

Note: PBA was applied on tomato leaves 7 days before infection. Different letters mark statistically significant difference according to Tukey test at the level P**<**0.05.

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**Figure 1.** Experimental setting. Phenylboronic acid was applied either on solid or liquid nutrient medium, or on tomato leaves*. Pseudomonas syringae* (*Pst*) or *Erwinia amylowora* (*Ea*) were then added and their growth, viability and the ability to cause symptoms on tomato plants were assessed.

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**Figure 2.** Kinetics of bacterial growth (A) and titer of viable cells, CFU (B) in a liquid culture of *Pseudomonas syringae* pv*. tomato* incubated for 90 min, 180 min and overnight (ON) in the presence of PBA, at 28.5 °C. Each value is a mean of three independent experiments, with error bars representing SD.



**Figure 3.** Kinetics of bacterial growth (A) and titer of viable cells, CFU (B) in a liquid culture of *Erwinia amylovora* incubated for 90 min, 180 min and overnight (ON) in LB medium supplemented with PBA, at 28.5 °C. Each value is a mean of three independent experiments, with error bars representing SD.