In vitro antifungal effect of phenylboronic and boric acid on Alternaria alternata

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The ascomycete fungus Alternaria alternata causes early blight, one of economically the most important tomato diseases. Due to frequent use of fungicides, A. alternata has developed resistance with negative economic and environmental consequences. Research of new ways to control fungal pathogens has turned its eye to environmentally friendly chemicals with low toxicity such as boronic acids. The aim of our study was therefore to test the antifungal effects of phenylboronic and boric acid in vitro on A. alternata. We isolated the pathogen from a symptomatic tomato plant and determined the minimum inhibitory concentration of phenylboronic and boric acid on A. alternata mycelial growth using the poisoned food technique. The antifungal effect was tested on a wide range of phenylboronic and boric acid concentrations (from 0.04 % to 0.3 %) applied separately to agar with mycelial disc of the pathogen. After five days of incubation, phenylboronic acid at low concentration (0.05 %) completely inhibited mycelial growth. Boric acid, in turn, did not significantly slow down mycelial growth but did reduce sporulation and confirmed its fungistatic effect. Our findings point to the potential use of phenylboronic acid to control phytopathogenic fungi. This is, to our knowledge, the first report on its antifungal effect on an agriculturally important pathogen in vitro. Moreover, since A. alternata is also a human pathogen, these results may have clinical ramifications.

KEY WORDS: boronic acids; early blight; minimal inhibitory concentration; mycelial growth; sporulation; tomato
Preparation of PBA and BA in concentration range

PBA (Sigma-Aldrich, CAS No. 98-80-6) and BA (Sigma-Aldrich, CAS No. 10043-35-3) were used in a wide range of concentrations (0.04 %, 0.05 %, 0.06 %, 0.07 %, 0.08 %, 0.09 %, 0.1%, 0.2 % and 0.3 %, which corresponds to 0.4 mg/mL to 3.0 mg/mL). Five hundred milligrams of PBA or BA were dissolved in 50 mL of sterile distilled water to give a 1 % PBA or BA stock solution. Based on the dilution factor, an appropriate volume of 1 % PBA or BA solution was pipetted into 50 mL of melted PDA nutrient medium, which was poured in three Petri dishes for three repetitions.

Determination of minimum inhibitory concentrations

We used the poisoned food technique (32) with slight modifications (33) to determine the minimum inhibitory concentration (MIC) of PBA and BA on mycelial growth of A. alternata. Melted PDA agar with a varying PBA or BA concentrations was poured onto three plates for each concentration. Mycelial discs of A. alternata with a diameter of 5 mm were cut with a circular cutter and placed in the centre of the solidified PDA plates. Mycelial discs were assessed under a stereomicroscope (SZ 4045, Olympus, Tokyo, Japan) at 250x magnification. Control Petri dishes did not contain PBA or BA. Petri dishes were incubated in a climate chamber at 25 °C for five days to allow A. alternata colonies to develop enough for us to quantify the antifungal effect of PBA or BA.

Grown fungal colonies of A. alternata in Petri dishes were photographed on the colony counter (Scan 100, Interscience, France) and the obtained photos processed with the ImageJ open-source software (US National Institutes of Health, Bethesda, Maryland, USA) (34) according to Guzmán et al. (35). The growth of A. alternata was quantified by measuring the surface area of the grown colony and calculating the mean of three repetitions.

Statistical analysis

Mean surface areas of fungal colonies treated with PBA or BA were compared with control using the one-way analysis of variance (ANOVA), followed by Tukey’s test to identify significant differences (P<0.05).

RESULTS AND DISCUSSION

Antifungal activity of PBA against A. alternata

The antifungal effect of PBA on A. alternata mycelial growth is shown in Table 1 and Figure 1. After five days of incubation at 25 °C, no pathogen growth was observed at concentrations ranging from 0.05 % to 0.3 %, whereas 0.04 % PBA reduced fungal growth by 98 % compared to control. Mycelial discs showed no hyphal growth. Fungal growth reduction was statistically significant at all tested PBA concentrations compared to control (Tukey’s test, P<0.05), which points to highly effective antifungal activity against A. alternata. These results support earlier in vitro findings reported by Liu et al. (23), in which the application of 0.3 % PBA completely inhibited the growth and development of basidiomycete fungi that cause decay of Japanese cedar wood. In fact, in our study A. alternata has shown much higher sensitivity, as its growth was completely inhibited at much lower PBA concentrations, starting with 0.05 %.

Antifungal activity of BA against A. alternata

Unlike PBA, BA did not completely inhibit the mycelial growth of A. alternata after five days of incubation (Table 2 and Figure 2). Highest inhibition was achieved with mid-range concentrations, and the non-linear relationship between BA concentrations and mycelial growth may point to experimental variation.

![Figure 1](image-url) Effect of different doses of phenylboronic acid (volume concentration, %) on mycelial growth of pathogen Alternaria alternata on potato dextrose agar after 5 days of incubation at 25 °C.

![Table 1](table-url) Mycelial-growth area of Alternaria alternata after five days of incubation on potato dextrose with different phenylboronic acid volume concentrations (%) at 25 °C.

| PBA concentration (%) | 0   | 0.04 | 0.05 | 0.06 | 0.07 | 0.08 | 0.09 | 0.1  | 0.2  | 0.3  |
|-----------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Mean of colony area ± SD | 17.7±0.5b | 0.3±0.1a | 0a | 0a | 0a | 0a | 0a | 0a | 0a | 0a |

Values are presented as means ± SD. Means with the same superscript letters across columns are not significantly different (One-way ANOVA; Tukey’s test, P<0.05)
Pathogen, this study has potential pharmaceutical ramifications, especially as PBA is well tolerated by mammals (19, 27). Another advantage of PBA is that it is environmentally friendly.

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Conflicts of interest

None to declare.

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Askomicetna gljiva Alternaria alternata uzročnik je koncentrične pajgavosti, jedne od ekonomski najvažnijih bolesti rajčice. Zbog česte primjene fungicida, ta je gljiva razvila otpornost na agrokemikalije koje se koriste u njezinu suzbijanju, s negativnim ekonomskim i ekološkim posljedicama. Novi načini suzbijanja gljivičnih patogena uključuju upotrebu ekološki prihvatljivih i manje toksičnih spojeva, među koje potencijalno spadaju boronske kiseline. Pokusom in vitro istražen je antimikotički učinak fenilboronske i borne kiseline na gljivu Alternaria alternata.

Nakon izolacije patogena iz rajčice, određena je minimalna inhibitorna koncentracija fenilboronske i borne kiseline za rast micelija primjenom tehnike poisoned food. Antimikotički učinak testiran je na širokom rasponu koncentracija fenilboronske i borne kiseline (od 0,04 % do 0,3 %), pojedinačno umiješanih u hranjivu podlogu na kojima je tijekom petodnevnog rasta rast kulture patogena.

Fenilboronska je kiselina pri niskoj koncentraciji (0,05 %) potpuno inhibirala rast micelija. Primjena borne kiseline u različitom rasponu koncentracija nije značajno umanjila rast micelija, ali je primijećen smanjenje sporulacije patogena, čime se potvrđuje fungistatski učinak borne kiseline. Prema našoj spoznaji, ovo je prva studija koja opisuje in-vitro antimikotički učinak fenilboronske kiseline na patogena koji je važan u poljoprivredi. Štoviše, s obzirom na to da je A. alternata i patogen ljudi, studija ima i potencijalni medicinski značaj.

**KLJUČNE RIJEČI:** boronske kiseline; koncentrična pajgavost; minimalna inhibitorna koncentracija rajčica; sporulacija

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**In vitro antimikotički učinak fenilboronske kiseline i borne kiseline na patogenu gljivu Alternaria alternata**

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