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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

Some species of phytopathogenic fungi have developed strong resistance to agrochemicals used for their control, which raises concern about the negative economic and environmental consequences in global food production (1, 2). The ascomycete fungus *Alternaria alternata* (Fr.) Keissler is of particular concern, as it causes early blight in tomato and incurs basal stem lesions on seedlings, stem lesions on adult plants, and fruit rot (3, 4), resulting in significant losses in crop yields (up to 79 %) and subpar nutritional quality. Furthermore, *A. alternata* and other *Alternaria* species produce mycotoxins (5) and pose a serious health threat to humans and livestock (6–10).

In vitro tests have revealed that several isolates of *A. alternata* are resistant to pyraclostrobin, boscalid, strobilurine (11), and azoxystrobin (12). In addition, consumers are increasingly concerned about pesticide residues in food, which is why fungicides will not be the future first choice for controlling fungal diseases (13). Recent years have seen a growing interest for environmentally friendly alternatives, and boronic acids have caught the eye of the scientific community, as they can inhibit a wide range of fungi (14) and are not toxic for the environment (15). This particularly concerns boric acid (BA), a common disinfectant (16), and its phenyl derivative, phenylboronic acid (PBA), a commercially available chemical with antimicrobial (17, 18), antitumor (19), antibacterial (20, 21), and antifungal properties (22, 23) confirmed against several species of human fungi (24, 25). PBA is not toxic to the environment (18, 26–28), while boron is in fact an essential micronutrient for plants (29).

However, no research has yet investigated PBA activity against pathogenic fungi that attack agriculturally interesting plants, and the aim of this study was to address this gap by testing antifungal effects of PBA and BA on *A. alternata*.

MATERIALS AND METHODS

A. alternata isolation

Tomato (*Solanum lycopersicum* cv. Rutgers) leaves with early blight symptoms were collected in the field and the infected plant material was incubated on potato dextrose agar (PDA, Sigma-Aldrich, St. Louis, MO, USA) in a climate chamber at 25 °C for five days as detailed by Nagrale et al. (30) to stimulate fungal growth. The obtained pure culture of the isolated fungi was determined morphologically (30) and with polymerase chain reaction (PCR) (31).

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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.

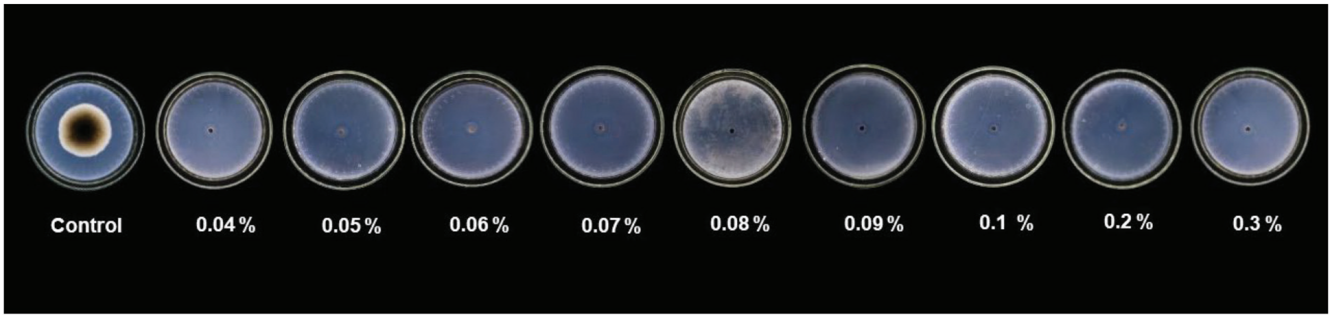

Figure 1 Effect of different doses of phenylboronic acid (volume concentration, %) on mycelial growth of pathogen *Alternaria alternata* on potato dextrose agar after 5 days of incubation at 25 °C

Table 1 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 ± 0.5^{b}	0.33 ± 0.1^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}	0^{a}

Values are presented as means ± SD. Means with the same superscript letters across columns are not significantly different (One-way ANOVA; Tukey's test, $P<0.05$)

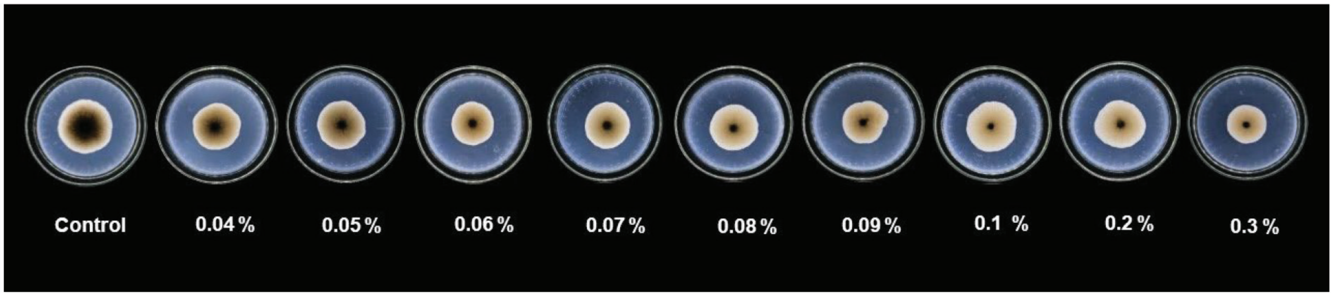

Figure 2 Effect of different doses of boric acid (volume concentration, %) on mycelial growth of pathogen *Alternaria alternata* on potato dextrose agar after 5 days of incubation at 25 °C

Table 2 Mycelial-growth area of *Alternaria alternata* after five days of incubation on potato dextrose agar with different boric acid concentrations at 25 °C

BA 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 ± 0.5^{c}	12.9 ± 1.1^{ab}	11.2 ± 0.7^{ab}	9.7 ± 0.4^{a}	11.8 ± 1^{abc}	12.2 ± 0.5^{ab}	11.2 ± 1.3^{ab}	14 ± 0.1^{bc}	14.9 ± 1^{abc}	16.2 ± 0.6^{bc}
Inhibition (%)	0	27.1	36.7	45.2	33.3	31.1	36.7	20.9	15.8	8.5

Values are presented as means ± SD. Means with the same superscript letters across columns are not significantly different (one-way ANOVA; Tukey's test, $P<0.05$)

Morphological characteristics of the mycelia grown on media supplemented with BA differed from control. The mycelium was less coloured and less branched than the dark brown mycelium that grew on control substrate. This could be because BA at mid-range concentrations reduced sporulation without inhibiting significantly mycelial growth. Considering that *A. alternata* is a foliar fungus whose pathogenicity depends on sporulation and the way its conidia spread, this effect on sporulation is important for arresting or inhibiting pathogenesis.

Our findings are in line with one early report of *A. alternata* being tolerant to BA (36), whereas one study reported diminishing inhibitory effects on the germination of spores in *Alternaria* spp. from 44 % to 36 % over 48 h as BA concentrations rose from 0.2 % to 1 % (37). In contrast, another *in vitro* study (38) reported rising growth inhibition in *A. solani* with BA concentration over seven days of incubation, namely 90.16 % with 1 % and 95.22 % with 2 %

In an extension of this study, we investigated *in vivo* activity of BA and PBA against *A. alternata* infection of tomato plants in the same concentration ranges, which confirmed stronger prophylactic activity of PBA, in controlling early blight symptoms in test plants (28).

CONCLUSION

This study has established that PBA completely inhibits the growth of *A. alternata* at quite low doses, while BA inhibits sporulation. To our knowledge, this is the first report about *in vitro* antifungal activity of PBA against this agriculturally important pathogen and mycotoxin producer. Since *A. alternata* is also a human 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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In vitro antimikotički učinak fenilboronske i borne kiseline na patogenu gljivu *Alternaria alternata*

Askomicetna gljiva *Alternaria alternata* uzročnik je koncentrične pjegavosti, 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 *A. 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 petodnevne inkubacije uzgajan micelarni disk 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 primijećeno 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 patogen 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 pjegavost; minimalna inhibitorna koncentracija rajčica; sporulacija