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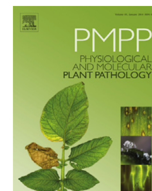

Hordenine is responsible for plant defense response through jasmonate-dependent defense pathway

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ABSTRACT

Organic agriculture does not rely on synthetic chemical fungicides. An alternative pest management strategy to chemical fungicides is the use of bioactive natural compounds. Hordenine [4-(2-dimethylaminoethyl)] is a phenethylamine alkaloid found in barley. Although hordenine has various pharmacological effects, including antibiotic activity against microorganisms, no studies have been carried out to investigate the inhibitory effects of hordenine on phytopathogenic fungal infection in host plants. Both grape downy mildew and strawberry anthracnose were suppressed by hordenine treatment. Hordenine had no effect on mycelial growth of phytopathogenic fungi, whereas plant defense response through the jasmonate-dependent defense pathway was enhanced in hordenine-treated plants. The concern over environmental pollution has led to the introduction of new pesticides, including bioactive natural compound based pesticide. Hordenine may be used in organic agriculture as an innovative elicitor of plant defense response to downy mildew and anthracnose.

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1. Introduction

The research and development of alternatives to chemical fungicides has seen an upsurge of interest. In viticulture, vine growers are facing severe problems in vineyard pest management because of the emergence of chemical fungicide resistant fungal phytopathogens. For example, *Plasmopara viticola* (Berk. & M.A. Curtis) Berl. & De Toni, which causes grape downy mildew, is a high-risk phytopathogen as regards the acquisition of chemical fungicide resistance [7]. Indeed, the resistance of certain *P. viticola* populations to quinine outside inhibitor and carboxylic acid amide was confirmed in European vineyards in 1999 [11] and in 2004 [9], respectively. The decrease in the control of *Colletotrichum gloeosporioides*, a pathogen of grape ripe rot and strawberry anthracnose, for benzimidazoles in China was attributes to the development of the resistances [15].

Two alternative pest management strategies to chemical fungicides have received great attention from plant pathologists. One is biological control. Biological control agents are microorganisms isolated from nature [4]. The microorganisms are safer than chemical fungicides. A large number of microorganisms have been identified as candidates for biological control agents, and some microorganisms, such as *Bacillus subtilis* QST-713, have been developed as a biofungicide and used to this day [6]. Another strategy is the use of bioactive natural compounds. New pesticide registration procedures, such as the Food Quality Protection Act in the United States [12], have reduced the number of synthetic chemical fungicides available in agriculture [3]. Natural compounds are being discovered and developed to replace chemical fungicides that have been banned from use in agriculture [19]. The increasing environmental concern about chemical fungicides has also heightened awareness of biofungicides and bioactive natural compounds. In addition, the adoption of alternative strategies can inhibit the emergence of resistance to chemical fungicides by reducing the frequency of application of chemical fungicides.

Bioactive natural compounds have been used indirectly to protect plants from phytopathogens by induction of systemic acquired resistance (SAR) [3]. Natural compounds elicitors may bind to specific receptors in plants, activate the salicylic acid (SA)-dependent [23] and/or jasmonate (JA)-dependent defense pathway [5] and then suppress plant disease by accumulating plant defence-

Abbreviations: DMSO, dimethyl sulfoxide; JA, jasmonate; PR protein, pathogenesis-related protein; SA, salicylic acid; SAR, systemic acquired resistance.

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related products such as pathogenesis-related (PR) proteins [24] and phytoalexins [1]. We have investigated natural compound elicitors of plant origin to grapevine using commercial chemical compound libraries representing bioactive natural compounds from plant. The preliminary experiments demonstrated that hordenine upregulated pathogenesis-related (PR) protein encoding genes in grape cell culture (Ishiai et al., unpublished data). Hordenine [4-(2-dimethylaminoethyl)] is a phenethylamine alkaloid found in barley [18], and has various pharmacological effects, including antibiotic activity against microorganisms [20]. However, no studies have been carried out to investigate the inhibitory effects of hordenine on phytopathogenic fungal infection in host plants.

The objective of this study was to clarify whether hordenine suppresses plant disease caused by phytopathogenic fungi. Here, we report the effect of hordenine on the severity and incidence of grape downy mildew and strawberry anthracnose. We also demonstrate that the JA-dependent defense pathway is involved in the defense response triggered by hordenine.

2. Materials and methods

2.1. Chemicals

Hordenine and methyl jasmonate were purchased from Tokyo Chemical Industry (Tokyo, Japan). Dimethyl sulfoxide (DMSO) was obtained from Sigma-Aldrich (Tokyo, Japan). Commercial fungicides, Ranman FL (a.i. cyazofamid, 9.4% w/w; Ishihara Sangyo, Osaka, Japan) and Topjin M (a.i. thiophanate-methyl, 70% w/w, Nippon Soda, Tokyo, Japan) were used for *in vitro* antagonistic tests.

2.2. Plant materials

Two-year-old seedlings of *Vitis vinifera* cv. Koshu were cultivated in perlite:vermiculite (1:1) soil in a growth chamber (11.8 W m^{-2} for 14 h in a day) at 27 °C. Seedlings of *Fragaria* × *ananassa* cv. Nyoho were grown in 55% peat moss, 10% perlite, 5% vermiculite, and 30% decomposed granite soil at 25 °C in a greenhouse under natural light condition.

To demonstrate that SA and/or JA-dependent defense pathways are involved in defense response triggered by hordenine, *Arabidopsis thaliana* wild-type (Col-0), SA-insensitive mutant *npr1-5* (CS3724) [25] and JA-insensitive mutant *atmyc2* (SALK_039235) [16] were used. Seeds of each *Arabidopsis* were sown on rockwool blocks (2.5 × 2.5 × 3.8 cm) and incubated at 22 °C in an incubator (11.8 W m^{-2} for 16 h in a day).

2.3. Bioassay for severity of downy mildew in grape leaf disks

An *in vivo* bioassay using grape leaf disks as described previously [8] was performed to evaluate whether hordenine suppresses grape downy mildew. Briefly, the third to fifth leaves, counted from the shoot tip, were collected from grapevine seedlings. Leaf disks (15 mm diameter) were punched out from the leaves and placed upside down on moistened filter paper in Petri dishes. Three disks were sprayed with 1.2 mL of 1 mM hordenine using an atomizer (30 mL size, AsOne, Tokyo, Japan). Water or DMSO was used in control experiments. The disks were dried in a flow cabinet overnight. To prepare spore suspension, spores of *P. viticola* were washed off with sterile water from downy mildew infected grapevine leaves of seedlings in the laboratory and adjusted to a concentration of 5000 spores/mL. Each pretreated disk was inoculated with 20 μL of the spore suspension. The inoculated disks were placed in a plastic box containing moistened paper towel to achieve approximately 100% humidity, and the box was incubated at 20 °C under a constant light condition (11.8 W m^{-2}). Downy mildew symptoms on each disk were assessed 18 days after inoculation and scored according to the symptom index of 0–4, as shown in Fig. 1. Means of the symptom indexes from two independent experiments with three leaf disks were calculated as disease severity. Disease incidence was calculated using the following formula.

Disease incidence (%) = (the number of leaves infected with *P. viticola*/the number of total leaves tested) × 100.

2.4. Bioassay for severity of anthracnose in strawberry leaves

An *in vivo* bioassay using strawberry leaves as described previously [1] was performed to evaluate whether hordenine suppresses strawberry anthracnose caused by *C. gloeosporioides*. Leaves were detached from strawberry seedlings. Three detached leaflets were sprayed with 600 μL of 1 mM hordenine 24 h prior to the experiment. Water or DMSO was used in control experiments. The center of each leaflet was punctured with a sterile needle of 1 mm diameter. *C. gloeosporioides* inoculum was incubated on a potato dextrose agar plate (PDA, Becton and Dickinson, MA) at 25 °C for 10 days. *C. gloeosporioides* agar plugs (4 mm diameter) were prepared by using a cork borer, and one plug was placed on the wound of the leaflet that was turned upside down. The leaflets were incubated at 25 °C in a plastic box containing moistened paper towel under light/dark condition (11.8 W m^{-2} for 14 h in a day). On the 7th days post incubation, lesion diameter on each leaf was measured to determine anthracnose severity. Means of the lesion diameters from two independent experiments with three leaflets were calculated.

2.5. Effect of hordenine on mycelial growth of phytopathogenic fungi on agar media

As *P. viticola* is an obligate biotrophic oomycete, it is impossible to perform *in vitro* experiments with it. Accordingly, to investigate the direct antagonistic effect of hordenine on phytopathogenic fungal growth, the *in vitro* antagonistic activity of hordenine on *C. gloeosporioides* and oomycete phytopathogens was examined. *Pythium aphanidermatum*, which causes *Pythium* stalk rot, and *Pythium ultimum*, which causes damping-off and root rot, were used as oomycete phytopathogens instead of *P. viticola*. These fungi were incubated on a PDA plate at 25 °C for 3 days. Mycelial agar plugs (4 mm diameter) were cut out of the plate and placed at the center of new PDA plates. The plates were incubated at 25 °C for one day. Sterilized paper disks (diameter 10 mm, Advantech Toyo, Tokyo, Japan) were placed on the plates and then inoculated with 50 μL of 100 mM hordenine, sterilized water, DMSO, or fungicide. Cyazofamid (100 ppm) or thiophanate-methyl (700 ppm) was dissolved in water and used as fungicide against *Pythium* or *Colletotrichum*, respectively. The plates were incubated at 25 °C for one day for *Pythium* or 3 days for *Colletotrichum*, respectively. The antagonistic effect of hordenine on fungal growth was visually evaluated by examining the growth inhibition zones formed on the plates in five independent experiments.

2.6. Treatment of arabidopsis seedlings with hordenine

Each of the 25-day-old *Arabidopsis* seedlings was sprayed with 1 mL of 1 mM hordenine using an atomizer. DMSO was used in a control experiment. The seedlings were incubated at 22 °C for 24 h in an incubator (11.8 W m^{-2} for 16 h in a day). For the analysis of gene expression in *Arabidopsis*, all rosette leaves (approximately 15 rosette leaves) of a seedling were used in an experiment and six independent experiments were performed.

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