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Research article

Enhancement of downy mildew disease resistance in pearl millet by the G_app7 bioactive compound produced by *Ganoderma applanatum*

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ABSTRACT

Pearl millet (*Pennisetum glaucum*) stands sixth among the most important cereal crops grown in the semi-arid and arid regions of the world. The downy mildew disease caused by *Sclerospora graminicola*, an oomycete pathogen, has been recognized as a major biotic constraint in pearl millet production. On the other hand, basidiomycetes are known to produce a large number of antimicrobial metabolites, providing a good source of anti-oomycete agrochemicals. Here, we report the discovery and efficacy of a compound, named G_app7, purified from *Ganoderma applanatum* on inhibition of growth and development of *S. graminicola*, as well as the effects of seed treatment with G_app7 on protection of pearl millet from downy mildew. G_app7 consistently demonstrated remarkable effects against *S. graminicola* by recording significant inhibition of sporangium formation (41.4%), zoospore release (77.5%) and zoospore motility (91%). Analyses of G_app7 compound using two-dimensional nuclear magnetic resonance spectroscopy and liquid chromatography-mass spectrometry revealed its close resemblance to metominostrobin, a derivative of strobilurin group of fungicides. Furthermore, the G_app7 was shown to stably maintain the inhibitory effects at different temperatures between 25 and 80 °C. In addition, the anti-oomycete activity of G_app7 was fairly stable for a period of at least 12 months at 4 °C and was only completely lost after being autoclaved. Seed treatment with G_app7 resulted in a significant increase in disease protection (63%) under greenhouse conditions compared with water control. The identification and isolation of this novel and functional anti-oomycete compound from *G. applanatum* provide a considerable agrochemical importance for plant protection against downy mildew in an environmentally safe and economical manner.

1. Introduction

Plant pathogenic oomycetes can cause devastating effects on crops and natural ecosystems. A classical example of oomycete pathogen is the downy mildew or 'green ear' disease of pearl millet (*Pennisetum glaucum* L. R. Br.) caused by *Sclerospora graminicola* (Sacc.) Schroet., which occurs most destructively in Asia and Africa, resulting in yield loss up to >80% in case of epidemics (Sudisha et al., 2011; Thakur and Mathur, 2002). The fungus is heterothallic in nature which reproduces asexually by means of sporangia consisted of zoospores and sexually through oospores. These characteristics of the fungus make it highly variable and adaptable to diverse environmental conditions (Attard et al., 2008; Kumar et al., 2012). Oomycete plant diseases are generally managed by the application of phenylamide-based compounds, such as metalaxyl (Apron 35 SD), which specifically inhibit RNA polymerase I (Sukul and Spiteller, 2000). However, indiscriminate use of fungicides creates the risk of development of pathogenic strains that are resistant to metalaxyl. Moreover, these groups of fungicides are recognized as being hazardous to environment and human health (Parra and Ristaino, 2001; Thakur and Mathur, 2002). The application of host resistance would be a useful strategy; however,

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sources of durable host resistance are not available and the cost involved in breeding for resistant varieties is prohibitive. These limitations have opened door for such a new and innovative strategy that is affordable in terms of cost and sustainability. In addition, the public demand for environmentally safer control methods has propelled the industry to identify and isolate compounds with bioactive potential from microbial sources which can act as immunity stimulants against opportunistic plant and human pathogens (Ash, 2010; Shweta et al., 2013).

Among microbial origin, basidiomycete groups of fungi produce large number of secondary metabolites (Anke, 1989), which show antibacterial, antifungal, antiviral, cytotoxic and hallucinogenic activities as well as potential sources of plant growth regulators or flavors (Faccin et al., 2007; Gebhardt et al., 2007; Vamanu, 2012; Wang and Ng, 2006). It is only recently that basidiomycetes and other higher fungi have been re-investigated (Li et al., 2012), mainly due to the increasing difficulties and cost of isolating novel bioactive compounds from members of the order Actinomycetales, such as the slow growth rate and the low yields of products (Brizuela et al., 1998). The fact that basidiomycetes have been re-considered due to its broad range of antimicrobial activities suggests that they may be a natural new source for isolation of bioactive compounds (Anke, 1989; Anke et al., 1990; Florianowicz, 1999; Rosa et al., 2003). One such discovery was that strobilurin compounds were isolated from the wood-decaying *Strobilurus tenacellus* 21602 strain (Anke et al., 1977). Fungicides of natural origin like strobilurins have become an integral part of plant disease management programs and have been shown to possess an abroad spectrum of activities for their applications as fungicides against plant diseases caused by ascomycetes, basidiomycetes, fungi imperfecti and oomycetes (Bartlett et al., 2002). These fungicidal strobilurins have been proved to be excellent candidates for yield increase and quality of agricultural products (Margot et al., 1998; Sudisha et al., 2005, 2010).

Although a number of studies have reported the isolation of various natural products for screening antimicrobial compounds, not much attention has been paid on isolating novel compounds like strobilurin group of fungicides from basidiomycetes. In our previous study, we conducted a trial using crude extracts of 17 different basidiomycete species and screen for their inhibitory action against pearl millet downy mildew pathogen *S. graminicola* (Sudisha and Shetty, 2009). On the basis of our successful detection of the bioactive activity of a *Ganoderma applanatum* strain against *S. graminicola*, in the present study we purified and characterized the functional bioactive anti-oomycete compound, named G_app7, from this *G. applanatum*. Our tests of the G_app7 compound with pearl millet under controlled greenhouse conditions have provided considerable agrochemically potential of G_app7 for eco-friendly application in agriculture against downy mildew disease.

2. Materials and methods

2.1. Chemical fungicides

Amistar 250 SC (azoxystrobin; (E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate), a derivative of strobilurin fungicides and Apron 35 SD (Metalaxyl; methyl-D-L-N-(2-[6-dimethyl phenyl)-N-(2′-methoxyacetyl-alaninate)]N-(2,6-dimethylphenyl)-N-(2-methoxyacetyl)-DL-alaninate) were procured from Syngenta and used in the study.

2.2. Preparation and extraction of G. applanatum

The fresh *G. applanatum* (accession number ABUOM-1) was collected from the upper bark of *Artocarpus hirsutus* tree of Waynad forest region, India (N11° 15′, E7570′, 2322 m altitude, sandy clay loam soil). Extraction of *G. applanatum* was carried out using chloroform ($CHCl_3$) (1:2 w/v) as previously described (Sudisha and Shetty, 2009). Subsequently, the $CHCl_3$ extract was subjected to thin-layer chromatography (TLC) for purification of the anti-oomycete G_app7 compound.

2.3. Purification of the anti-oomycete compound G_app7 using column chromatography

The TLC spots that exhibited a high activity against *S. graminicola* were re-chromatographed on C120 silica gel column (35 × 10 mm, Sisco Research Laboratories, Mumbai). Briefly, the TLC spots developed from $CHCl_3$ extract of *G. applanatum* were pooled together. One hundred mg of TLC fraction which showed high anti-oomycete activity were dissolved in the $CHCl_3$ (1:1 ratio) and loaded at the top of the column. The chromatogram was run down with a stepwise gradient system of C_6H_6, C_6H_6:$CHCl_3$ (9:1–1:9) and $CHCl_3$, and the fractions were collected at 20 min intervals. The solvents were evaporated from the collected fractions and dissolved in $CHCl_3$ and analyzed using TLC (C_6H_6:$CHCl_3$, 6:4, v/v). The spots developed on the TLC from each fraction were dissolved in $CHCl_3$, and 10 μg ml^{-1} of G_app7 solution were tested for their activity against *S. graminicola*.

2.4. Suspension culture assay of the purified G_app7 compound on sporangium formation, zoospore release and zoospore motility of S. graminicola

Infected leaves of pearl millet cv. 7042S were collected in the evening, washed with running tap water and then blot-dried. Subsequently, the washed leaves were cut into small pieces and placed on petri dishes lined with moist blotters and incubated in a humidity chamber (15–18 °C) for overnight. Sporangia produced on the leaves were harvested into sterile distilled water (SDW), and the concentration of sporangia was adjusted to 1.5×10^4 sporangia ml^{-1} using a haemocytometer (Sudisha et al., 2005). Ten ml of the 1.5×10^4 sporangia ml^{-1} were treated with 10 μg ml^{-1} of G_app7 compound in the 1:1 ratio (v/v) for 15 min under dark conditions at room temperature (22 °C ± 2 °C) (Sudisha et al., 2010). Apron 35 SD (0.015 mg ml^{-1}) and Amistar 250 SC (2 μg ml^{-1}) dissolved in SDW (Sudisha and Shetty, 2009), and $CHCl_3$ and SDW were also applied to 10 ml of 1.5×10^4 sporangia ml^{-1} in the 1:1 ratio (v/v) under the same conditions as positive and negative controls, respectively. Observations were made for zoospore release by counting the empty and intact sporangia in treated and control sets. Zoospore suspension of *S. graminicola* (5×10^4 zoospores ml^{-1}) adjusted by haemocytometer using SDW was treated with purified G_app7 in 1:1 ratio (v/v) for 15 min, and motility of the zoospores was observed under light microscope (5 different fields). The relative percentage of zoospore motility was determined using the number of zoospores showing motility in each treatment and that of control sets.

2.5. Leaf disc assay of the purified G_app7 compound on sporangium formation, zoospore release and zoospore motility of S. graminicola

In a separate experiment, infected leaves of pearl millet cv. 7042S were collected, washed and blot-dried. Subsequently, the leaves were cut into approximately 1 cm^2 in size, and then smeared with 5 ml of G_app7 compound (10 μg ml^{-1}). The treated leaf pieces were placed on petri dishes lined with moist blotters and incubated in a humidity chamber (15–18 °C) for overnight. Apron 35 SD (0.015 mg ml^{-1}), Amistar 250 SC (2 μg ml^{-1}), $CHCl_3$ and SDW (5 ml/