Inhibitory effects of *Alpinia zerumbet* extract against plant virus infection in solanaceous plants

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Abstract  
Tomato mosaic virus (ToMV) and tobacco mosaic virus (TMV) are critical pathogens causing severe crop production losses of solanaceous plants. The present study was undertaken to evaluate the antiviral effects of extracts of *Alpinia* plants on ToMV and TMV infection in *Nicotiana benthamiana*. The aqueous extracts of *Alpinia zerumbet* (Pers.) B.L. Burtt and R.M. Smith and *Alpinia kumatake*, which grow widely in subtropical and tropical regions including East Asia, were effective in reducing ToMV infection when plants were treated prior to virus inoculation. We also found that the extracts of *A. zerumbet* isolated from Okinawa (Japan), locally referred to as *shima-gettou*, strongly inhibited ToMV and TMV infection. To obtain an active fraction, the aqueous extract of *A. zerumbet* isolate OG1 was separated by ethyl acetate, and the antiviral active compound was found to be present in the water layer. Based on our results, the extract of *Alpinia* plants has potential as an antiviral reagent for practical application in solanaceous crop production.

Key words: antiviral activity, *Alpinia zerumbet*, tobacco mosaic virus, tomato mosaic virus.
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et al. 2007a, 2007b; Tu and Tawata 2015; Yonaha et al. 2013; Zoghbi et al. 1999). These uses and properties make *A. zerumbet* an attractive candidate for use as a novel agricultural resource. However, its bioactive phytochemicals have not been sufficiently utilized or evaluated as agricultural materials.

In this study, we examined the antiviral activity of extract from the stem and leaves of three greenhouse grown varieties of *Alpinia* plants: two *A. zerumbet* isolates (OG1 and AG1) were collected from Nakijin-son, Okinawa, and the Amami island, respectively, and an isolate of *Alpinia kumatake* (KB1) (Sharma and Hashinaga 2004), known as *kumatakerann* in Japan, was collected from Uruma-shi, Okinawa.

Aqueous extracts were obtained by squeezing leaves and stems of *Alpinia* plants with a sugar cane squeezer (YBK-2, Yabiku, Japan). The extracts were centrifuged at 3,260×g for 10 min, and the supernatant was first filtered with Whatman no. 1 filter paper and then with a membrane filter (0.22 µm, Bottle Top Filter, TPP). To evaluate the inhibitory effect of the *Alpinia* extracts, OG1, AG1, and KB1, the extracts were applied to *N. benthamiana* plants (the third true leaf stage) as foliar sprays, and 3 days after the treatment the plants were inoculated with a plant virus as described below.

To prepare inoculum of ToMV, pTL-derived plasmids (pTLBN.G3), which contain a full length ToMV cDNA as well as a gene encoding green fluorescent protein (GFP) (Kubota et al. 2003), were linearized by restriction with *MluI* and used as a template for in vitro transcription. The AmpliCap-Max T7 High Yield Message Maker Kit (CELLSCRIPT, USA) was used for in vitro transcription according to the manufacturer’s instructions at 37°C for 40 min. The transcription mixture was then diluted by 40-fold in water. The diluted mixture mixed with abrasive carborundum (600 mesh; Nacalai Tesque, Japan) was mechanically applied to the third true leaves of *N. benthamiana* that had been treated with the *Alpinia* extract. Two other antiviral agents, i.e., l-ascorbic acid (Fujiwara et al. 2013) and Lentemin (NSK Co., Ltd., Japan), were also used as controls to evaluate antiviral activity. Control plants underwent the inoculation with only water.

Green fluorescent protein foci were used to detect virus infection, and they were observed under blue-light irradiation 3 and 11 days post-infection (dpi). The antiviral activity was assessed based on the number of GFP foci formed on the control and treated *N.
benthamiana leaves. The number of GFP foci formed on the inoculated leaves was calculated at 3 dpi.

Our results showed that treatments with the extract of A. zerumbet isolates OG1 and AG1, as well as A. kumatake isolate KB1 effectively protected N. benthamiana leaves against ToMV-GFP infection, when compared with the control (Figures 2 and 3). In addition, the application of extract from isolate OG1 showed greater antiviral activity than the application of extracts from isolates AG1 and KB1. Furthermore, we used quantitative real-time polymerase chain reaction (qPCR) analysis to confirm these findings at the RNA level. Equal amounts of total RNA were subjected to cDNA synthesis and then specific ToMV sequences were amplified. The results obtained from qPCR (Figure 3C) were not significantly different from those obtained from assays based on the number of GFP foci (Figure 3A, B). These results indicate that the number of GFP foci is a highly correlated indicator for ToMV infection.

In both N. benthamiana and tomato hosts, the extracts of A. zerumbet isolate OG1 protected both inoculated and uninoculated upper leaves against ToMV as compared with water-treated plants (Figure 4). Furthermore, A. zerumbet extract application on N. benthamiana and tomato was more effective than the application of L-ascorbic acid or Lentemin in reducing
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Viral infection. These results indicate that the *Alpinia* extracts play positive role in plant protection to ToMV.

Given the effectiveness of *A. zerumbet* OG1 extract, we also tested *N. benthamiana* plants treated with it against TMV tagged with GFP. To prepare inoculum of TMV, plasmids of pTMV-30B:GFP (Shivprasad et al. 1999) were linearized by restriction with KpnI, and used as a template for in vitro transcription using the AmpliCap-Max T7 High Yield Message Maker Kit according to the manufacturer’s instructions at 37°C for 40 min. The transcription mixture was then diluted 6-fold in water. Inoculation was performed using the same method used in the ToMV-inoculation. The results showed that the extract protected treated *N. benthamiana* plants against TMV infection as well (Figure 5).

Producing enough extract for evaluation was challenging, and if the extract were to be used in a commercial application, it would need to be produced in larger quantities. To increase the amount of active fraction obtained, the aqueous extract of *A. zerumbet*
isolate OG1 was separated by ethyl acetate (EtOAc). The EtOAc layer was dried using a rotary evaporator and stored at −80°C until use. The water layer was also freeze-dried and stored at −80°C until use. The concentration of these fractions was adjusted to be the concentration of the original solution. The application of water layer of the extraction was also able to protect N. benthamiana plants against ToMV infection (Figure 6).

Since the different Alpinia isolate extracts had varying levels of antiviral activity, we determined how closely related the isolates were by using sequences of nrDNA internal transcribed spacer (ITS), which is the spacer DNA situated between the small-subunit ribosomal RNA (rRNA) and large-subunit rRNA genes as molecular markers to distinguish the Alpinia plants. Sequences of ITS regions for all of the species examined were identical, indicating that the species and isolates are closely related to each other.

In this study, we demonstrated that the extract of Alpinia plants, especially isolate OG1, had high antiviral activity on two different viruses, ToMV and TMV, when used to treat tomato and N. benthamiana. Moreover, in comparing GFP foci and qPCR analysis, measuring GFP foci appears to be a useful indicator for measuring antiviral activity. Based on our results, the extract of Alpinia plants has potential as an antiviral reagent for practical application in crop production. A limitation on the use may be producing sufficient extract quantities; however, our results also showed that extract production protocols can be improved. Future work on identifying the active compound within the extracts with antiviral activity and characterizing the antiviral mechanism will provide further insight into the use of antiviral agents in crops.

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