Efficacy of a biological control agent *Rhizobium vitis* ARK-1 on grapevine crown gall against higher cell density of tumorigenic isolates, and investigation on the optimal application timing

Mizuho Nita[1], Abdullah Nahiyan[1], and Akira Kawaguchi[2]

[1] Alson H. Smith Jr. Agricultural Research and Extension Station, School of Plant and Environmental Sciences, Virginia Polytechnic Institute and State University, Winchester, Virginia, USA

[2] Western Region Agricultural Research Center (WARC), National Agricultural and Food Research Organization (NARO) Fukuyama, Hiroshima, Japan

Abstract

Crown gall of grapevine, caused by the bacterium *Rhizobium vitis*, is an economically significant disease worldwide. The objectives of this study are to determine the efficacy of a biological control agent *R. vitis* ARK-1 against a higher cell number of tumorigenic inoculum mixture (Ti-mix) collected from vineyards in Virginia, USA, and the best timing of ARK-1 application using *in planta* assays in grapevines (*Vitis vinifera*, 'Chardonnay 'and 'Merlot') and tomato (*Solanum lycopersicum*, 'Beefsteak'). Based on the optical density (OD_{600} = 0.1, ~ 5 x 10^7 CFU/mL), a range of concentrations of ARK-1 and Ti-mix were prepared as the treatments of 1:1, 1:2, 1:3, 1:4, and 1:5 ratio of ARK-1 to Ti-mix. In a separate experiment, ARK-1 was applied 48, 24, 6, 3, and 1 hour before or after the inoculation of the Ti-mix. Treatments were applied as a cell suspension to artificial wounds in lignified cane tissue. ARK-1 significantly ($P \leq 0.05$) reduced both gall incidence and gall diameter up to a 1:4 ratio. ARK-1 significantly reduced the gall formation than any other treatment when it was applied at 24 hours or earlier than the Ti-mix application. ARK-1 was also able to significantly reduce the gall formation compared with a positive control when it was applied within 24 hours of application of the Ti-mix.

Introduction

Rhizobium vitis causes crown gall in grapevines (*Vitis* spp.), which is considered an economically significant disease in the grape-growing regions worldwide. The formation of galls (Fig. 1) can block the vascular system, which results in a decline of the infected vines. Removal of diseased vines for replanting is commonly recommended due to the lack of effective management options.

Figure 1. Typical grapevine crown gall symptom caused by *Rhizobium vitis,* on *Vitis vinifera* 'Petit Verdot'.

A biological control agent *R. vitis* ARK-1 is an antagonistic, endophytic, and non-tumorigenic strain. ARK-1 has been proven effective in reducing gall formation *in planta* against tumorigenic isolates in Japan and the US (1,2). Given the success of the past studies, the objectives of this study are 1) to determine the threshold of ARK-1 against higher cell number of tumorigenic *R. vitis* strains, and 2) to examine the best application timing for ARK-1.

References

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Materials and Methods

Preparation of inoculums: A mixture of four tumorigenic *R. vitis* isolates (Ti-mix) from different geographic regions in Virginia, USA was used for the ratio and timing experiments. Bacterial suspension of each isolate was grown in yeast extract mannitol (YEM) broth for 48 hours at 28°C and 135 rpm on a shaker before the start of the experiments. Cell density was estimated based on the optical density (OD_{600} = 0.1 = 5 x 10^7cells/mL) value. Then the four isolates were mixed to create the Ti-mix.

Cell ratio experiment: Ratio treatments of 1:1, 1:2, 1:3, 1:4, and 1:5 of ARK-1 to Ti-mix were prepared. The estimated cell number of ARK-1 was 1 x 10^8 cells/mL. Five artificial wounds were made in the hardwood stem of grapevine cutting (*V. vinifera*, 'Merlot') and tomato stem (*Solanum lycopersicum*, 'Beefsteak') with a 3.25 mm drill bit and sterile needle, respectively. Fifty microliters (10 µl in tomato) of inoculum were injected into each wound with a sterile micropipette. For all experiments, two plants were used per experimental run, and three independent runs were conducted.

Application timing experiment: Wounds were made in grapevine (*V. vinifera*, 'Chardonnay') and tomato (*S. lycopersicum*, 'Beefsteak') as above. Fifty microliters (5 µl in tomato) of ARK-1 were applied 48, 24, 6, 3, and 1 hour before or after, and 5 and 30 minutes after inoculation of equal volume and concentration of the Ti-mix in each wound (**Fig. 2**).

Disease assessment and data analysis: Following 12 weeks (6 weeks in tomato) of inoculation, gall diameter in mm and gall incidence data were recorded. The effect of treatment on gall diameter and incidence was analyzed with the linear mixed model assuming the normal distribution and generalized regression assuming the binomial distribution and penalized with adaptive Lasso, respectively. The Tukey-Kramer test was used as a mean separation method. Statistical analyses were conducted with JMP Pro (ver. 15, SAS Institute, Cary, NC).

Results and Discussion

Cell ratio experiment*:* In grape, the mean gall incidence and gall diameter varies from 0 to 100% and 0 to 8.2 mm, respectively (**Fig. 3**). ARK-1 treatment significantly reduced mean gall incidence (X^2 = 5,402.4, $P < 0.01$) and mean gall diameter (F = 60.35, $P < 0.01$). In tomato, the mean gall incidence and gall diameter varied from 0 to 100% and 0 to 6.4 mm, respectively. ARK-1 treatment significantly reduced mean gall incidence (X^2 = 87.78, $P < 0.01$) and mean gall diameter (F = 62.67, $P < 0.01$) *Since the results of grape and tomato were similar, only grape results are shown in this presentation.*

In grape, ARK-1 treatment significantly reduced the gall formation ($P \leq 0.05$) up to 1:5 ratio of ARK-1 to Ti-mix comparing with the ARK-1 untreated controls, and the level of reduction level reduced with the increasing Ti-mix concentration (**Fig. 3**). ARK-1 was able to provide a practical reduction in gall formation up to 4X concentration of the Ti-mix (~67% reduction in gall incidence).

Application timing experiment: In grape, the mean gall incidence and diameter ranged from 0% to 100%, and 0.0 to 8.9 mm, respectively (**Fig. 4**). Preventative application of ARK-1 significantly reduced gall incidence (X^2 = 3,239.4, $P < 0.01$) and diameter (F = 34.9, $P < 0.01$). In tomato, the mean gall incidence and diameter ranged from 0% to 100%, and 0.0 to 9.2 mm, respectively. Preventative application of ARK-1 significantly reduced gall incidence (X^2 = 7,741.8, $P < 0.01$) and diameter (F = 40.96, $P < 0.01$) in tomato.

When ARK-1 was applied prior to the inoculation of the Ti-mix, all treatments resulted in the mean gall size that was not significantly different from the sole ARK-1 (negative) control ($P \leq 0.05$) (**Fig. 4**). With the mean gall incidence, although all pre-application of ARK-1 resulted in significantly lower gall incidence than the positive control, ARK-1 applied 48 hours prior to the Ti-mix resulted in the mean that was not significantly different from the sole ARK-1 control which resulted in no gall formation.

When ARK-1 was applied after the inoculation of the Ti-mix, all, but post 48 hours application resulted in a significant reduction in gall diameter and incidence ($P \leq 0.05$) (**Fig. 4**). Interestingly, ARK-1 applied at 3 hours after the inoculation of the Ti-mix resulted in a significantly lower gall incidence than other post-inoculation application timings.

Figure 2. Application timing experiment shown as a pictorial diagram. ARK-1 was applied to artificial wounds at various time points before and after inoculation of the Ti-mix to the same wound. A representative picture from each treatment.

Figure 3. Effect of ARK-1 treatment against a mixture of four tumorigenic strains (Ti-mix) up to 5 times higher in cell density (i.e., 1 x 10^8 vs. 5 x 10^8 cell/ml) treatments were applied to artificial wounds on grape cane (*V. vinifera* 'Merlot') grown in the greenhouse in 2020. Blue and orange bar shows mean gall diameter and incidence, respectively, per treatment.

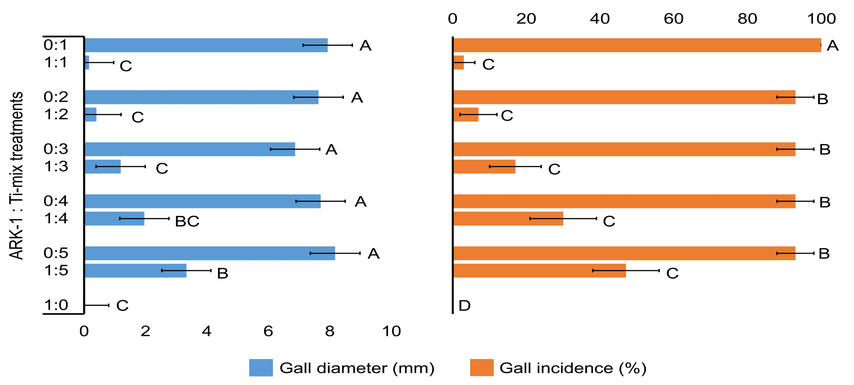

Figure 4. Effect of timing of ARK-1 application against a mixture of four tumorigenic strains (Ti-mix) applied at the same cell density (~5 x 10^7 cell/ml). Treatments were applied to artificial wounds on grape cane (*V. vinifera* 'Chardonnay') grown in the greenhouse in 2020. Blue and orange bar shows mean gall diameter and incidence, respectively, per treatment.