

# The Delay of Multiplication of East Asian Passiflora Virus - Amami-O-shima Isolate (EAPV-AO) by the Pre-inoculation of EAPV - Ibusuki Isolate (EAPV-IB)

Hisashi IWAI<sup>†</sup>, Megumi TAKANO, Miwako RIKITAKE and Masayuki NAKAMURA  
(Laboratory of Plant Pathology and Entomology)

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## Summary

The effects of the pre-inoculation of a mild strain of East Asian Passiflora virus (EAPV-IB) on a severe strain of EAPV-AO were investigated. The longer a period between the pre- and challenge-inoculation (from 7 days up to 60), the stronger the multiplication of AO was suppressed. Finally, AO was detected in the leaves of the apex and lateral branches, and IB did not provide a complete attenuated virus to prevent AO. However, the delayed effects of IB pre-inoculation against an AO-challenge indicate that IB has the latent ability to be the basic material for a more effective attenuated virus.

**Key words:** East Asian Passiflora virus, attenuated virus, cross protection, potyvirus, passionfruit

## Introduction

A potyvirus isolated from the passionfruit hybrid cultivar “Ruby star” - purple passionfruit (*Passiflora edulis*) x yellow passionfruit (*P. edulis f. flavicarpa*) - collected from the Amami main island of Kagoshima Prefecture has previously been described as the *Passionfruit woodiness virus* [6] - Amami-O-shima isolate (PWV-AO) [4], and recently renamed the East Asian Passiflora virus - Amami-O-shima isolate (EAPV-AO) [5]. Subsequently, at the Ibusuki Experimental Botanical Garden of Kagoshima University we found some purple passionfruit (*Passiflora edulis*) plants showing dappled or faded fruits and mosaic symptoms on their foliage; more specifically, the surfaces of their pericarps were not accompanied by “woodiness” symptoms but were slightly malformed. We confirmed that this disease was caused by the distinguishable isolate from AO, and named it the EAPV - Ibusuki isolate (EAPV-IB) [5]. IB infected plants do not show this “woodiness”, and are expected to act as a potential attenuated virus that is a type of “vaccine” against the AO infection. IB still induces slight foliar symptoms, and also has the potential risk of strengthening the general symptoms of AO infection by mixing with of AO. Thus, we investigated whether there were neutral effects on passionfruit or whether the effects were multiplied with a challenge

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<sup>†</sup> Correspondence to: H. IWAI (Laboratory of Plant Pathology and Entomology, Kagoshima University),  
Tel & Fax: (099)285-8681;  
Email: topcrop@agri.kagoshima-u.ac.jp

inoculation of AO by the pre-inoculation of IB.

## Materials and Methods

### Plant materials

For the EAPV-AO infected plant, a hybrid cultivar of passionfruit (*P. edulis* Sims. x *P. edulis* Sims. f. *flavicarpa* Deg. "Summer queen"), previously infected by the green peach aphid (*Myzus persicae* Sulzer) [4] and preserved in a greenhouse was used. For the preparation of the EAPV-IB infected plant, "Summer queen" (called "passionfruit" hereafter) which had been inoculated with IB through a side graft of virus-infected sweet calabash (*P. malformis* Linn.) [5] was used. As the AO- and IB- source plants, twigs of passionfruit that had one node and leaf were planted in the rooting mold "Kanuma soil" on Jun. 23, 2004, transplanted to a soil mix of clay loam and compost at a ratio of 9:1 in a small clay pot (90mm-diameter) on Aug. 6, and then transplanted again into an orchid pot (200mm-diameter and eight liters-capacity) containing the same soil mix on Aug.27. Twigs of the health passionfruit for the rootstock were planted on Jun. 21, transplanted to a soil mix on Aug. 2, and transplanted again on Aug. 19. Each time graft inoculation was performed, the scion, consisting of the two nodes below the apical leaves of the newly developed shoot of the infected plant, mentioned above, was used as the source of infection.

### Inoculation of virus by the side graft

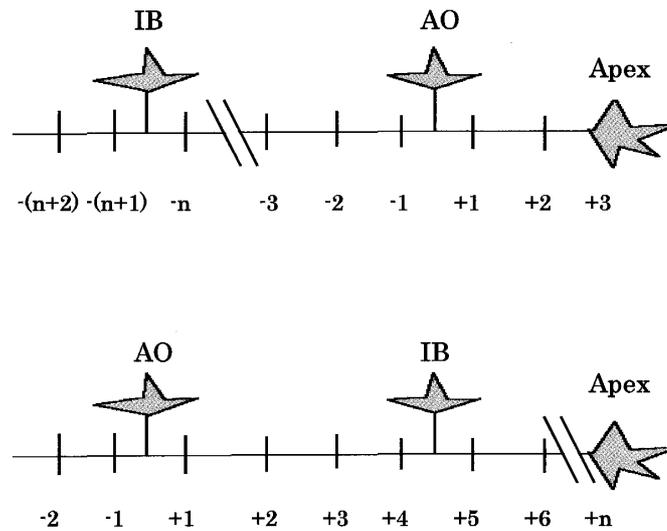
As the first inoculation, IB infected scions were grafted onto the main stem of the healthy rootstock between the 3rd and 4th nodes below the apical leaves on Sep.6. On a certain number of days after the first inoculation, the AO infected scions were grafted, as the challenge inoculation, on inter - 3rd and 4th nodes below the apical leaves of the newly developed vine or inter - 4th and 5th nodes below the IB-inoculated (IB infected scions-grafted) point. As the positive control, the infected scions of IB or AO were grafted singly. As shown in Fig. 1, the AO-inoculated (AO infected scions-grafted) point was considered "point 0", and from that point, the leaves were counted to the upper-nodes plus-integrally, and to the downward-nodes minus-integrally.

### Setting up the experimental plots based on the interval between IB- and AO-inoculation

The eight types of experimental plots were set up as follows: challenge inoculations of AO were done one week, two weeks, three weeks, 36 days, and 60 days after the first inoculation of IB. Exceptionally, two weeks after the first inoculation of IB, the challenge inoculation of AO was also done to the point between 4th and 5th nodes counted downwards from the first inoculated point, as shown in Fig.1. As the control, AO and IB were graft-inoculated singly. Every experiment was performed twice. The temperature of the greenhouse where the plants were preserved was maintained at 20-35C.

### The double antibody sandwich-enzyme-linked immuno-sorbent assay (DAS-ELISA) for detecting the PWV-AO

The production of polyclonal antiserum to PWV-AO and the ELISA procedures followed those described by Omatsu et al. [7] and Clark and Adams [1]. The ELISA values (optical density of the reacted substance at 405nm) of the AO-IgG system to the homologous-AO-antigen were 2.05 for the infected leaf sap (20 times dilution applied in this experiment) and 2.25 for the purified virus (10  $\mu$  g/ml). However, those to the heterologous-IB-antigen were 0.01 and 0.03, respectively. This



**Figure 1.** Schema of the relation between the graft-inoculation point and node number. First, the inoculation of IB between the 3rd and 4th node counted downwards from the apex was followed by the 2nd challenge inoculation of AO between the 3rd and 4th node counted downwards from the newly developed apex (Upper), and second, two weeks after the IB inoculation, between the 4th and 5th node counted downwards from the IB inoculated point (Bottom).

difference comes from the low percent homology of the amino acids sequences of the amino terminal regions of the coat protein between the two strains [5]. Therefore, we were able to use the AO-IgG system for the peculiar detection of the translocation of EAPV-AO without the cross reaction for EAPV-IB. ELISA values were graded as 0 ( $\sim 0.15$ ), 1 ( $0.15 \sim 0.5$ ), 2 ( $0.5 \sim 1.0$ ), 3 ( $1.0 \sim 2.0$ ) and 4 ( $2.0 \sim$ ).

## Results and Discussion

The passionfruit was infected with both EAPV-AO and IB by a side graft inoculation. The symptoms of the three types of experimental plots (Fig.2) were as follows: the single inoculation of AO induced severe mosaic and rugose of the upper leaves one week after the inoculation, the single inoculation of IB induced mild mottling and occasional curling of the upper leaves not accompanied with rugose two to three weeks after the inoculation, and the challenge inoculation of AO three weeks after the first inoculation of IB also showed mild mottling of the leaves without rugose an



**Figure 2.** Comparison of leaf symptoms. Six weeks after the IB single inoculation (Left), six weeks after the AO - in other words nine weeks after the IB inoculation (Middle), and six weeks after the AO single inoculation (Right). Every leaf was set at the third node down from the apex.

**Table 1. Recovery of EAPV-AO on passionfruit after single inoculation**

Terms after the inoculation of AO	Number of leaf nodes <sup>1)</sup>												
	-4	-3	-2	-1	+1	+2	+3 <sup>2)</sup>	+4	+5	+6	+7	+8	+9
1 week	0 <sup>3)</sup>	0	0	0	0	0	4	4	4	4			
2 weeks	0	0	0	0	0	0	4	4	4	4	4	4	4

- 1) Stem nodes numbers counted from the point of AO inoculation. AO-inoculated (AO infected scion-grafted) point was between the nodes number -1 and +1.
- 2) Apical leaf when AO was inoculated.
- 3) The grades of ELISA values: 0; ~0.15, 1; 0.15~0.5, 2; 0.5~1.0, 3; 1.0~2.0, 4; 2.0~.

**Table 2. Recovery of EAPV-AO on passionfruit after the challenge inoculation of AO one week after the first inoculation of EAPV-IB**

Terms after the inoculation of AO	Number of leaf nodes <sup>1)</sup>														
	-4	-3	-2	-1	+1	+2	+3 <sup>2)</sup>	+4	+5	+6	+7	+8	+9	+10	+11
1 week	0 <sup>3)</sup>	0	0	0	0	0	0	0							
2 weeks	0	0	0	0	0	1	0	0	3	1	3	4			
3 weeks	0	0	0	0	0	0	3	4	4	4	4	4	4		
4 weeks	0	2	0	0	0	3	4	4	4	4	4	4	4		
5 weeks	0	0	0	0	0	2	3	4	4	4	4	4	4	4	4
6 weeks	0	0	0	0	0	3	4	4	4	4	4	4	4	4	4

- 1) Stem nodes numbers counted from the point of AO inoculation. IB-inoculated (IB infected scion-grafted) point was between the nodes number -4 and -3, and AO-inoculated (AO infected scion-grafted) point was between the nodes number -1 and +1.
- 2) Apical leaf when AO was inoculated.
- 3) The grades of ELISA values: 0; ~0.15, 1; 0.15~0.5, 2; 0.5~1.0, 3; 1.0~2.0, 4; 2.0~.

**Table 3. Recovery of EAPV-AO on passionfruit after the challenge inoculation of AO two weeks after the first inoculation of EAPV-IB**

Terms after the inoculation of AO	Number of leaf nodes <sup>1)</sup>									
	-4	-3	-2	-1	+1	+2	+3 <sup>2)</sup>	+4	+5	
1 week	0 <sup>3)</sup>	0	0	0	0	0	0	0		
2 weeks	0	0	0	0	0	0	0	4		
3 weeks	0	0	0	0	0	0	4	4	4	
4 weeks	0	0	1	0	0	0	4	4	4	
5 weeks	0	0	3	0	1	4	4	4	4	

- 1) Stem nodes numbers counted from the point of AO inoculation. IB-inoculated (IB infected scion-grafted) point was between the nodes number -4 and -3, and AO-inoculated (AO infected scion-grafted) point was between the nodes number -1 and +1.
- 2) Apical leaf when AO was inoculated.
- 3) The grades of ELISA values: 0; ~0.15, 1; 0.15~0.5, 2; 0.5~1.0, 3; 1.0~2.0, 4; 2.0~.

additional three weeks after the challenge inoculation. However, the mixed infection of IB and AO showed a tendency to repress the main stem development in some experimental plots (Tables 3, 4). That phenomenon might have been caused by the multiplier effect of the double infection of IB and AO, but the detailed mechanism of this growth repression is unknown at present.

Recovery of the challenge-inoculated AO in the passionfruit was detected by DAS-ELISA. In the case of the single inoculation of AO, the uppermost leaves showed high values of multiplication

**Table 4. Recovery of EAPV-AO on passionfruit after the challenge inoculation of AO three weeks after the first inoculation of EAPV-IB**

Terms after the inoculation of AO	Number of leaf nodes <sup>1)</sup>								
	-5	-4	-3	-2	-1	+1	+2	+3 <sup>2)</sup>	+4
1 week	0 <sup>3)</sup>	0	0	0	0	0	0	0	0
2 weeks	0	0	0	0	0	0	0	0	0
3 weeks	0	0	0	0	0	0	0	0	0
4 weeks	0	0	0	0	0	0	0	1	0
5 weeks	0	0	0	0	0	1	0	2	1
7 weeks	0	0	0	0	0	3	2	3	3
9 weeks	0	0	0	0/4 <sup>4)</sup>	0	2	1	1	4

1) Stem nodes numbers counted from the point of AO inoculation. IB-inoculated (IB infected scion-grafted) point was between the nodes number -5 and -4, and AO-inoculated (AO infected scion-grafted) point was between the nodes number -1 and +1.

2) Apical leaf when AO was inoculated.

3) The grades of ELISA values: 0; ~0.15, 1; 0.15~0.5, 2; 0.5~1.0, 3; 1.0~2.0, 4; 2.0~.

4) ELISA value of main stem leaf / ELISA value of lateral branch leaf

**Table 5. Recovery of EAPV-AO on passionfruit after the challenge inoculation of AO 36 days after the first inoculation of EAPV-IB**

Terms after the inoculation of AO	Number of leaf nodes <sup>1)</sup>														
	-11	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	+1	+2	+3 <sup>2)</sup>	+4
1 week	0 <sup>3)</sup>	0	0	0	0	0	0	0	0	0	0	1	0	0	0
2 weeks	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3 weeks	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4 weeks	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5 weeks	0	- <sup>4)</sup>	0	0	0	0	0	0	0	0	0	0	0	0	4
7 weeks	0	-	0	0	0	0	0	0	1	0	0	0	0	4	-
9 weeks	2/4 <sup>5)</sup>	4	0	0	3	0	0	1	2	0	1	2	0	0	-
11 weeks	2/4	-/4	1/4	0/4	2/4	3	2	3	3	0/4	3	1/4	1	4	4

1) Stem nodes numbers counted from the point of AO inoculation. IB-inoculated (IB infected scion-grafted) point was between the nodes number -11 and -10, and AO-inoculated (AO infected scion-grafted) point was between the nodes number -1 and +1.

2) Apical leaf when AO was inoculated.

3) The grades of ELISA values: 0; ~0.15, 1; 0.15~0.5, 2; 0.5~1.0, 3; 1.0~2.0, 4; 2.0~.

4) Leaf sample was not set.

5) ELISA value of main stem leaf / ELISA value of lateral branch leaf

quickly after one week (Table 1). In the case of the inoculation of AO one and two weeks after the first inoculation of IB, accumulations of AO were suppressed up to one week after, but the high values of multiplications in the uppermost leaves occurred two weeks after (Tables 2, 3). With the inoculation of AO three weeks after the first inoculation of IB, the accumulation of AO was suppressed at least 3 weeks, but a week after, AO gradually started to multiply in the upper leaves, and particularly high-grade ELISA values were shown in the leaves of the apex and lateral branch 9 weeks after (Table 4). By the inoculation of AO 36 days after the first inoculation of IB, the accumulation of AO was suppressed for at least 4 weeks, but a week after, AO gradually started to multiply in the upper leaves, and two weeks after that, AO began to be detected in the lower leaves of the

**Table 6. Recovery of EAPV-AO on passionfruit after the challenge inoculation of AO 60 days after the first inoculation of EAPV-IB**

Terms after the inoculation of AO	Number of leaf nodes <sup>1)</sup>											
	-8	-7	-6	-5	-4	-3	-2	-1	+1	+2	+3 <sup>2)</sup>	+4
1 week	0 <sup>3)</sup>	0	0	0	0	0	0	0	0	0	0	0
2 weeks	0	0	0	0	0	0	0	0	0	0	0	0
3 weeks	0	0	0	0	0	0	0	0	0	0	0	0
4 weeks	0	0	0	0	0	0	0	0	0	0	0	0
6 weeks	- <sup>4)</sup>	0	0	0	0	0/3	0	0	0	0	0	-
8 weeks	-3 <sup>5)</sup>	0	0	0	0	0/4	0	0	0	0	0	0
9 weeks	-/3	0	0	0	0	0/3	1	0	0	0	0	4
10 weeks	-/3	0	0	0	0/2	0/3	1	1	0	0	0	3

1) Stem nodes numbers counted from the point of AO inoculation. IB-inoculated (IB infected scion-grafted) point was between the nodes number -8 and -7, and AO-inoculated (AO infected scion-grafted) point was between the nodes number -1 and +1.

2) Apical leaf when AO was inoculated.

3) The grades of ELISA values: 0; ~0.15, 1; 0.15~0.5, 2; 0.5~1.0, 3; 1.0~2.0, 4; 2.0~.

4) Leaf sample was not set.

5) ELISA value of main stem leaf / ELISA value of lateral branch leaf

**Table 7. Recovery of EAPV-AO on passionfruit after the challenge inoculation of AO was done at the point downwards from the EAPV-IB-inoculated point, two weeks after the first inoculation of IB**

Terms after the inoculation of AO	Number of leaf nodes <sup>1)</sup>													
	-2	-1	+1	+2	+3	+4	+5	+6	+7	+8	+9	+10 <sup>2)</sup>	+11	+12
1 week	0 <sup>3)</sup>	0	0	0	0	0	0	0	0	0	0	0	0	0
2 weeks	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3 weeks	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4 weeks	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5 weeks	0	0	0	0	0	0	0	0	0	0	0	0	- <sup>4)</sup>	0
6 weeks	0	0	0	0	0	0	0	0	0	0	0	1	3	-
8 weeks	0	0	0	0	0	0	0	0	0	0	0	0	2	3
10 weeks	-/4 <sup>5)</sup>	0/4	3	0/2	0	0	0/2	0	0	0	0/4	2	4	4

1) Stem nodes numbers counted from the point of AO inoculation. IB-inoculated (IB infected scion-grafted) point was between the nodes number +4 and +5, and AO-inoculated (AO infected scion-grafted) point was between the nodes number -1 and +1.

2) Apical leaf when AO was inoculated.

3) The grades of ELISA values: 0; ~0.15, 1; 0.15~0.5, 2; 0.5~1.0, 3; 1.0~2.0, 4; 2.0~.

4) Leaf sample was not set.

5) ELISA value of main stem leaf / ELISA value of lateral branch leaf

main stem. In this plot, AO was often detected in the leaves of the lateral branches, without being detected from the leaves of the main stems originating in the same nodes (Table 5). By the inoculation of AO 60 days after the first inoculation of IB, the accumulation of AO in the main stem leaves was suppressed for at least 8 weeks, but a week after that, AO started to multiply in the uppermost leaves, and was independently detected from the leaves of the lateral branches starting at 6 weeks after the inoculation of AO (Table 6). When the challenge inoculation was done at the point below the first IB-inoculated point, the accumulation of AO was much more delayed than when the

challenge inoculation was done at the point above the first-inoculated point (Table 7).

As shown in these results, the longer a period of time between the first and challenge inoculation, the stronger the multiplication of AO was suppressed. However, AO was finally detected in every experimental plot from the young leaves of the apex and lateral branches, and in the latter periods, from the fully expanded old leaves attached to the main stem. Thus, IB was not shown to be a complete attenuated virus that can protect against severe AO infection. The deduced amino acids sequences of the coat protein of IB share only 83% sequence identity with AO. The resistance or attenuation of symptoms were observed in host plants cross-protected with an attenuated isolate when the challenging isolates shared over 93% sequence identity with the attenuated isolates [3, 8, 9]. Thus, based also on the phylogenetic relation, IB by itself might not be the appropriate mild strain for restraining the AO multiplication. Nevertheless, there were still delay effects of pre-inoculated IB against a challenging AO. We expect IB to be used as one of the basic materials for the real attenuated virus by reverse genetics [2] in plant viruses.

This experiment was started on Jun. 21, 2004 (planting twigs) and finished on Dec. 31 (final detection in Table 6). This half-year period was not long enough for the plant to bear fruits. When raising passionfruit from a twig in greenhouses, it takes plants at least one and half years to bear fruits. Because of this, we could not assess whether IB helped prevent the deformed fruits caused by AO. To better assess IB for practical use, experiments conducted for a longer period of time under the various controlled and natural conditions need to be performed.

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