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Development of an Efficient Mechanical Inoculation Technique to Screen Barley Genotypes for Resistance to *Barley mild mosaic virus* Disease and its Comparison to Natural Infection

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Barley mild mosaic virus **(BaMMV) is a soilborne *Bymovirus* vectored by root-infecting fungus, *Polymyxa graminis*. Mechanism of cultivar's resistance to BaMMV in field tests are difficult to assess since resistance could be either due to the virus or to *P. graminis*, or both. Whereas, available mechanical inoculation methods for BaMMV and other related viruses are labor intensive, give inconsistent results and generally result in low infection rates. Inoculation method using stick with gauze (SWG) was developed for BaMMV. The improved method proved to be simple, efficient, and reliable. The infected leaf tissues were preserved by drying in a frozen state under high vaccum (freeze dried barley infected leaves) to circumvent reduction of virus infectivity during storage. Five Korean barley cultivars were mechanically inoculated with BaMMV-infected sap by the improved method. Infection rates obtained were compared with natural infection. Cultivar Naehanssalbori showed resistance to BaMMV in the field trials but was found highly susceptible in the greenhouse tests by mechanical inoculation, indicating that the field resistance may be possibly due to resistance to *P. graminis*.**

Keywords **:** *Bymorvirus*, *Hordeum vulgare*, *Polymyxa graminis*, soilborne viruses

Barley mild mosaic virus (BaMMV) is one of the most economically important virus diseases in barley (*Hordeum vulgare* L.) reported in Korea (Lee et al., 1996), Japan (Kashiwazaki, et al., 1998), China (Zheng et al., 1999) and Europe (Huth, 1991). The causative virus belongs to the *Bymovirus* genus of the *Potyviridae* family, and is transmitted by the root-infecting fungus *Polymyxa graminis* Led. Yield loss accounted for BaMMV-infection was at least 30-40% but can be rise to as high as 80% (Kashiwazaki et al., 1998). There is no control method found except for planting resistant cultivars (Kanyuka et al., 2003).

The mechanical transmissions through finger rubbing or air spray are not always reliable and are cumbersome. Production of large quantities of inoculum with high virus infectivity to provide a contiuous supply of inoculum of the same source is still a major problem. Previous studies showed that virus infectivity in infected leaves was reduced by 20% after freezing (Adams et al., 1986; Freidt, 1983). For these reasons, screening for resistance is commonly done in the field.

Field resistance tests however had some drawbacks. First, infection and symptom appearance is delayed in the field. The incubation period of virus when transmitted by *P. graminis* requires approximately 5-6 months before resistance evaluation can be assessed (Kashiwazaki et al., 1989). Secondly, field testing allows only one cycle of resistance test per growing season and that only winter season (Huth et al., 1984). Third, field resistance evaluation may vary from year to year due to changing environmental condition (Freidt, 1983). Finally, which is the most important of all is the problem of mixed infection (Kanyuka et al., 2003). Considering that BaMMV is transmitted by *P. graminis*, the resistance might not only be due to resistance to the virus but be due to resistance to the fungal vector, or both. Comparison of cultivar reactions by mechanical and natural transmissions would answer this question.

In this study, we deveoloped stick with gauze (SWG), an improved mechanical inoculation technique which was simple, efficient, inoculum-saving and reliable. We also tested the use of freeze drying of BaMMV infected leaves for inoculation. In addition, we are reporting the responses of selected Korean barley cultivars to BaMMV under mechanical and natural infection.

Materials and Methods

Virus source and inoculum. Barley plants showing BaMMV symptoms were collected at Naju Province, Korea and were tested for BaMMV, *Barley yellow mosaic virus* (BaYMV) and soilborne *Wheat mosaic virus* infection by Enzyme linked immunosorbent assay (ELISA). Plants infected with only BaMMV were selected and used as the disease source for sap inoculation. Seedlings of a susceptible cultivar, Baegdong were mechanically inoculated and

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kept in a growth chamber (model # VS-91G09M-0, Vision Scientific Co. Ltd., Korea) with temperature maintained at 10/12°C (day and night at 12 hrs each) with a lux of nearly 2000 and <500, respectively. Then infected leaves showing clear symptoms were harvested, wrapped 5 gram in foil and lyophilized. Lyophilized infected tissues were conducted based on the protocols for other crops (Gardiner, 1998; Saha et al., 1997) with some modifications. Infected leaves were wrapped with foil, and then foils were perforated prior to loading into a lyophilizer chamber (Eyela, FDU-1100, Japan). Initial temperature was at approximately -50°C and vacuum at ≤10 microns. During the run, the vacuum was maintained at ≤100 microns and condenser temperature at ≤-50°C. Samples were dried for 48 hours and stored with desiccant in sealed plastic bags at -35°C.

Inoculum was prepared by following the combined methods of Freidt (1983) and Nomura et al. (1996) with some modifications. Five gram of lyophilized leaf tissue was placed in a desiccator's chamber for 25-30 min a prior to grinding into powder using a mortar and pestle. Tissue powder was collected in a 50 ml Falcon tube and shaken vigorously with 20 ml (1:4 dilution) chilled grinding buffer (0.04 M K_2HPO_4 + KH_2PO_4 buffer, pH 7.0, containing 0.001 M Potassium cyanide). For fresh leaves, 5 g of tissues were ground vigorously with chilled grinding buffer using pre cooled mortar and pestle. The homogenates of either lyophilized or fresh tissues were gauze-filtered, placed in 50 ml Falcon tubes, mixed with carborundum and placed in an ice bath for inoculation.

Inoculation methods. Two inoculation techniques (SWG and spraying) using lyophilized BaMMV inoculum were tested. The SWG is an improvised inoculator made of ordinary wooden chopsticks with gauze folded 3 times (4 cm wide and 15 cm long). Gauze was rolled at the end of the stick then secured with a string (Fig. 1). For inoculation, 3-4 leaves of each seedling were held flat on the plastic glove-covered palm and dusted with the inoculum in a rubbing motion starting at the base to the leaf tips (Fig. 1). To ensure uniform application, inoculum mixture was agitated every time when the SWG was immersed into the inoculum suspension. To remove excess inoculum and maximize inoculum use the immersed SWG were lifted by slid along the inner wall of the tube. The spraying method was conducted following the methods by Slykhuis (1974). Briefly an entire leaf surface of test plants was sprayed using an airbrush (Model GATX/ZECO, GSI Creos Corp. Japan) operated at an air pressure of 2.1 kg/cm while holding the nozzle approximately 3 cm above from the surface of the leaves, then the bottle was swirled frequently so that the carborundum was applied evenly and not clogging the nozzle. For healthy controls, grinding buffer without infected plant tissue was inoculated similarly. Inoculated plants were placed in the shade overnight, and then sprayed with water to remove excess carborundum. Plants were grown in the growth chamber as mentioned above. Watering was done daily.

Fig. 1. Inoculation of barley seedlings by rubbing method using stick with gauze (SWG).

SWG was compared with air spraying for their efficiency, handling and amount of inoculum required per inoculation. One month after inoculation, plants were visually scored for infection and individual leaf per plant were sampled and tested by RT-PCR. All experiments were repeated for 3 times.

Test plants. Five Korean cultivars including two malting barley such as Hopumbori and Jinyangbori and three naked barley such as Baegdong, Sessalbori and Naehanssalbori were tested in this study. Reports (Park et al., 2005; Hyun et al., 2006) on field resistance evaluation to BaMMV and BaYMV based on visual disease severity rating indicated the following cultivar reactions: Hopumbori and Sessalbori were moderately resistant; Baegdong and Jinyangbori were susceptible; and Naehanssalbori was resistant. Seeds were pre germinated and transplanted in plastic pots (16 cm diameter and 13 cm height) at a rate of 10 seedlings per pot. Seedlings were allowed to grow to 18-25C temperature controlled greenhouse until 3-4 leaf stage (approximately 3 wks) for mechanical inoculation.

Field test. Seeds were raised in an experimental field, Honam Agricultural Research Institute (HARI), Iksan, Korea in late October, 2005. This experimental site had a history of uniform distribution of *P. graminis* infection and where barley cultivar trials to BaMMV and BaYMV were conducted for over 10 years. For field experiment, furrow per variety was planted at a distance of 40 cm×18 cm×1 m (furrow width × seeding space × seeding length). The seed were pre treated with fungicide Bitaziram and the seeding

rate was 18 kg/10a. The field lay out was conducted in 3 replications. In March, 2006, three leaves per plant at 5 sampling points (approximately 20 cm interval between plants) per cultivar/replication (total of 15 samples per cultivar) were sampled and tested by ELISA for the presence of BaMMV and BaYMV.

RT-PCR and ELISA. RT-PCR and ELISA were used for the detection of BaMMV. For RT-PCR, RNA was extracted from approximately 100 mg of infected leaves using the RNeasy kit (Qiagen, USA) and/or using TRIzol reagent (Invitrogen, Paisley, UK) following manufacturer's protocol and finally dissolved in 50 µl RNAse free water. RNA was denatured at 70°C for 5 min and immediately placed on ice. RT-Mix and PCR-PreMix kits (AccuPower, Bioneer Corp., Korea) were used for cDNA synthesis and PCR, respectively using a MJ thermal cycler (model # PTC-200, MJ Research Inc., USA). BaMMV primers' sets used were either S15/S17 (Forward / Reverse) designed by Lee (1998) or S3/S7 designed by Kashiwazaki and Hibino (1996), to amplify BaMMV coat protein (CP) fragment. Also, field infected leaf sap was tested by ELISA using a kit manufactured by Loewe Biochemica from Germany. Absorbance values were determined using a µ-Quant Universal Microplate Spectrophotometer (KCJunior, Bio-Tek Instruments Inc., Vermont, USA).

Data analysis and scoring. In mechanical inoculation test, a month after the inoculation, plants were tested for BaMMV infection by RT-PCR. While infected plants from the field test were tested by ELISA 5 months after seeding. All data obtained were subjected to Two-way ANOVA with Dunnett's post test using GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA, www.graphpad.com. Bar graphs was performed showing the means and corresponding standard deviations.

Cultivar resistance was based on rate of infection: 0-30% = resistant; 31-50% = moderately resistant; and 51-100% = susceptible.

Results

Inocula and inoculation methods. The infectivity test of inocula prepared from lyophilized tissues and fresh tissues showed no significant differences indicating that freeze drying did not reduce virus infectivity (Fig. 2). The lyophilized tissues remained its infectivity until 1 year so far tested in this study. For the efficiency between SWG and spray inoculation methods (Fig. 3) also showed no significant differences in the infection rates between the two methods. However, cultivars Sessalbori and Jinyangbori showed lower infection rates with relatively high standard

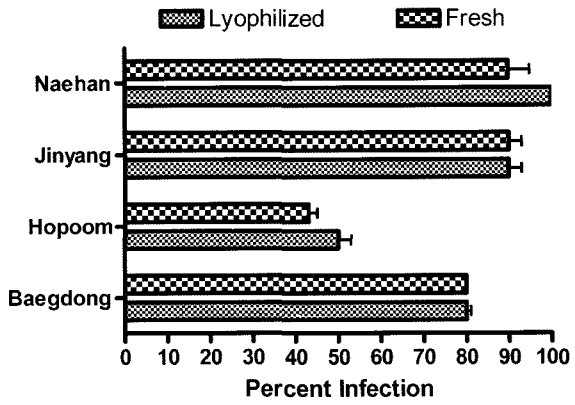

Fig. 2. Percent seedling infection with BaMMV on four cultivars inoculated with extracts of lyophilized or fresh tissues by the SWG method. Seedlings were grown in a chamber maintained at 10-12°C and tested by RT-PCR one month after the inoculation.

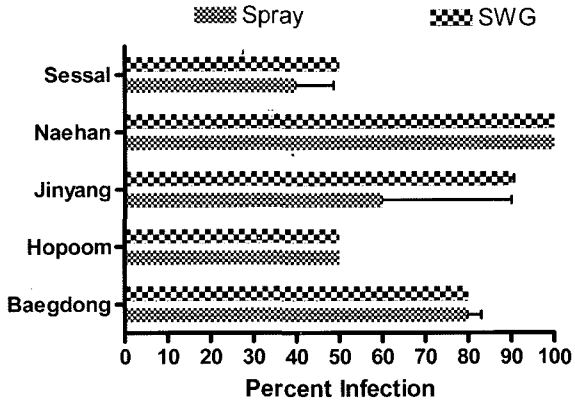

Fig. 3. Percent seedling infection with BaMMV on five cultivars inoculated by spraying and SWG rubbing method. Inoculated seedlings were grown in a chamber maintained at 10-12°C. Leaves were tested by RT-PCR one month after inoculation.

deviation in spray method than SWG indicating that SWG method was more stable than spraying method. For the inoculum volume required, a 20 ml inoculum was tested and the number of plants it could inoculate was determined. Results showed that a 20 volume of inoculum could inoculate approximately 20 plants using spray method and 40 plants for SWG indicating that SWG was more inoculum saving than spray method. The handling of the two methods in inoculating plants was also compared. The spray method could inoculate one leaf at a time due to the close distance spraying (3 cm) while SWG method having a broad size of gauze could inoculate 3-4 leaves at one time (Fig. 1), thereby making SWG inoculation became faster than either by the spray or the finger rubbing methods.

Cultivar reactions by mechanical and natural infection. Table 1 shows cultivar reactions by mechanical and natural infections under greenhouse and in field tests. In

Table 1. Reactions of five Korean barley cultivars to mechanical inoculation with BaMMV in the greenhouse and to natural infection with BaMMV and BaYMV

Cultivar	Mechanical Inoculation[a]	Natural Infection[b]			
	BaMMV	Total	Viruses detected[c]		
	Percent Infection	Percent Infection	BaMMV alone	BaYMV alone	BaMMV+BaYMV
Baegdong	85.7 (S)[d]	86.0 (S)	46.0	0	40.0
Hopumbori	47.7 (MR)	13.2 (R)	6.6	0	6.6
Jinyangbori	79.7 (S)	40.0 (MR)	0	40.0	0
Naehanssalbori	96.7 (S)	19.6 (R)	6.6	13.0	0
Sessalbori	45.0 (MR)	79.0 (S)	33.0	0	46.0

[a] Inoculated 3-4 leaf stage and maintained at growth chamber with temperature controlled at 10-12°C and tested by RT-PCR three experiments.
[b] HARI experimental field, Iksan, Korea.
[c] By ELISA and obtained in three replicates.
[d] Letters in parenthesis indicates resistance to cultivars based on rate of infection: 0-30% = R, resistant; 31-50% = MR, moderately resistant; and 51-100% = S, susceptible.

the mechanical infection, Jinyangbori, Baegdong, and Naehanssalbori had high infection rates (79.0, 86.0, and 96.0%, respectively) showing susceptible reactions with severe mosaic symptoms. Hopumbori and Sessalbori had 48.0 and 45.0% infections, respectively, indicating moderate resistance with moderate mosaic symptoms. Apparently, only cultivar Baegdong had consistent susceptible reactions under natural infection and in the mechanical infection. While cultivars Hopumbori and Jinyangbori and Sessalbori showed erratic reactions but Naehanssalbori showed extremely different reactions from mechanical and natural infection. Naehanssalbori showed resistant reaction with only below 20% total infection in the field tests, confirming previous report (Park et al., 2005). However, in mechanical infection of Naehanssalbori showed the highest infection with severe mosaic symptoms indicating that it is especially susceptible to BaMMV.

On the other hand, Hopumbori showed moderately resistant reaction from mechanical inoculation but showed resistant reaction from the field test. ELISA test revealed that from those collected leaves samples from cultivar Hopumbori plants showed a total percent infection of 13.2% and no BaYMV alone infection detected. However, in cultivar Jinyangbori had 40% infection and virus detection revealed only BaYMV infection. Sessalbori showed relatively high total percent infection (79%) with both viruses present in infected plants under natural infection but showed moderate BaMMV resistance in the mechanical inoculation (Table 1).

Discussion

Existing mechanical inoculation methods were inefficient and cumbersome. Thus, in this study, the improved mechanical inoculation method using SWG was demonstrated. The SWG method was very easy in applying. The wooden stick served as a handle for easy application allowing less chance of uneven application unlike the spraying method. Based on the results in Fig. 3, the SWG rubbing method was more stable and efficient than spraying method. The low infection rate of Jinyangbori and Sessalbori was demonstrated by relatively high standard deviation and this might be due to human error such as careless spraying since the distance of nozzle to the leaf surface is critical. The distance of nozzle to the leaf surface should be 3 cm away from leaf area. In this case, the distance might be ignored resulted in lower infection rate. The SWG method gave stable infections and offers speedy application without jeopardizing results. In fact, the Freidt's (1983) finger rubbing method required two inoculations at 7 day intervals for increased infection. In the improved method, one time application was enough to generate a complete infection in susceptible barley plants. The spray method required more inoculum compared with the improved method. Consequently, the SWG is efficient method, its application is easy and uses low levels of inoculum compared to other methods.

A number of papers have reported preservation of virus infectivity in infected leaves by freeze drying (Gardiner, 1998; Saha et al., 1997). In this study, virus infectivity in BaMMV-infected tissues was well preserved up to a year of storage (so far tested in this study) after being subjected to freeze drying. Thus, frequent virus propagation is unnecessary and, more importantly, working repeatedly with the same inoculum sources is now possible. Another advantage of preserving inoculum by freeze drying is that grinding of the preserved leaves into powder is much easier and not necessarily to be on ice while grinding.

Here, five selected barley cultivars were evaluated under mechanical and natural means of infection. Our results showed that cultivar reactions between mechanical and natural infection varied from each other except from

susceptible check cultivar, Baegdong. Also, in this study, our results further proved the occurrence of mixed infections in the field that explained the discrepancy in the resistance evaluation for specific virus. Hopumbori showed moderately resistant reaction to BaMMV in mechanical inoculation but resistant in the field test. Hopumbori was derived from a cross between cultivars Misato Golden and Sacheon 6 which are resistant and susceptible to BaYMV, respectively. Misato Golden possessed *rym5* gene for resistance to BaYMV (Hyun et al., 2006)). Results further confirmed that the resistance to BaYMV in Hopumbori is due to *rym5* gene. However, it is not determined whether *rym5* gene also involves in the moderate resistance to BaMMV. Sessalbori showed moderate resistance in the mechanical inoculation and susceptible reaction in the field test (Table 1). The difference is probably attributed to the differences in virus strain present in Iksan field, and the virus isolate (collected at Naju) used in the inoculation. Jinyangbori showed moderately resistant in BaMMV in mechanical inoculation but was found infected only with BaYMV in the field test. This may also be due to characteristic features of BaMMV strain in the experimental field. In the case of Naehanssalbori, it showed a level of resistance to BaMMV and BaYMV in the field test but was highly susceptible to BaMMV in the mechanical infection. This possibly indicates that the field resistance to BaMMV in Naehanssalbori is due to its resistance to the fungal vector. However, further research is required.

In Korea, screening of barley genotypes to BaMMV and also to BaYMV has been done largely in fields with a history of natural infection. Greenhouse screening by mechanical inoculation has been limited due to the difficulty and required care when inoculating plants. Using the improved mechanical inoculation method with freeze-dried leaf tissues as the inoculum source and the SWG for inoculation, the screening was improved substantially. Further, by testing genotypes in the greenhouse and also in the field, cultivars with resistance to the viruses can be differentiated from cultivars with resistance to their vector *P. graminis*. However, additional experiments are needed to further confirm evidence of *P. graminis* resistance.

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